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Long term high-level exercise promotes muscle reinnervation with age

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ABSTRACT

Histology of aging muscle suggests that denervation contributes to atrophy and that immobility accelerates the process, while routine exercise protects against loss of muscle tissue and motor units. Here, we compare muscle biopsies from active and sedentary seniors (71.8+/-3.5 and 68.3+/-4.0 [mean+/- SD] years, respectively, $p > 0.05$) and discover that the seniors with a long history of high-level recreational activity, continuing up to muscle biopsy, present with: 1. lower loss of muscle strength (relative to young men), 32% knee contraction strength loss in sportsmen vs 51% in sedentary seniors; 2. fewer small-size angular (denervated) myofibers than sedentary seniors, 1.8+/-3.9% vs 6.5+/-3.8%; 3. a considerably higher percentage of fiber-type groupings (reinnervated muscle fibers), almost exclusively of the slow type, 7.9+/-7.4% vs 0.5+/-0.6%; and 4. sparse presence of regular-size muscle fibers co-expressing fast and slow myosin heavy chains (MHCs), 0.6+/-0.6% vs 1.8+/-1.7%, an observation that is not compatible with an exercise-driven muscle type transformation. The biopsies from the senior sportsmen fluctuate from those with sparse fiber-type groupings to almost fully transformed muscle, going through a process in which isolated co-expressing fibers appear to fill gaps. Altogether, the data show that long term increased physical activity promotes reinnervation of muscle fibers, suggesting that decades of high level exercise allows the body to adapt to age-related denervation by saving otherwise lost muscle fibers through recruitment to slow motor units. These effects on axon terminals maintain size, structure and function of myofibers, delaying the functional decline and loss of independence that are often seen in late aging; these results suggest that long term high level activity may also counteract muscle atrophy in other neuromuscular and metabolic diseases.
Trial Registration: ClinicalTrials.gov: NCT01679977

Key words (up to 7): aging; human skeletal muscle; recreational sport activity; denervation and re-innervation; fiber type grouping; co-expression of fast and slow myosin heavy chains.

INTRODUCTION

Aging is characterized by a gradual decline that impairs cell homeostasis and functional reserves. Degeneration, apoptosis and death of all cell types, accompanied by loss of regenerative capacity, progressively accumulate and ultimately lead to organism death (1-4). Histological studies of muscle have shown that denervation is among the numerous mechanisms (5,6) which contribute to tissue atrophy and degeneration in aging. The term "disseminated neurogenic atrophy" was coined to describe the progressive accumulation and clustering of small, angular fibers (7-10) and supporting evidence of a progressive loss of α-motoneurons has been described (11,12). Corroborating electrophysiological studies have confirmed that there is a decrease in the number of motor units with a concomitant increase in their size with age. These results suggest that some reinnervation events follow muscle fiber denervation (13). Further evidence supporting the occurrence of rounds of denervation and reinnervation includes the increased clustering of myofiber types in the motor units of rodents and other mammals as they age (11,14). In adult humans, also, fiber types appear randomly distributed across the muscle and become increasingly grouped together with age (15). Therefore, it has been proposed that, beyond axonal disorders, apoptosis of α-motoneurons in the spinal cord, with subsequent incomplete reinnervation of fibers by surviving motor neurons, may contribute to the loss of muscle strength and mass as people grow older (16). These rearrangement processes are generally accompanied by a progressive increase in the proportion of slow muscle fibers, although the literature does provide some evidence to the contrary (for review see 17). Some of the discrepancies have been dispelled by comparisons of muscle from normally active and immobile older patients which show that muscle wasting in "normally active" seniors is accompanied by a shift toward a slow twitch phenotype, whereas the inactive senior demonstrates a shift toward fast twitch isoform expression. This latter case is common in "unloaded" muscle undergoing atrophy, e.g., during limb suspension, immobilization, paralysis and spaceflight (18-22). To further complicate the situation, conflicting results regarding fast to slow myosin transition arise in endurance training studies using animal models and in clinical trials of humans involving either voluntary exercise or electrical stimulation (both directly to denervated muscle and indirectly to muscle through nerve stimulation) (19,21,23-27). Whether the aging-related shifts are under neural control or the result of the direct influence of use/disuse on myogenic processes remains to be clarified.

In the present study, we analyzed muscle biopsies harvested from the *Vastus lateralis* of sedentary and active seniors (65 to 79 years). The active senior subjects routinely practiced sport activities usually more than three times a week up to the time of biopsy. In agreement with previous studies of master athletes (28-30), we show that long term high-level physical activity considerably increases the percentage of slow-type myofibers and the number of muscle fiber-type groupings. The latter provides direct evidence that long term cycles of denervation/reinnervation occurred. In recent interim reports (31-33), we showed, and here confirm, that muscle properties of these senior recreational sportsmen are more similar to those of active young men than to those of sedentary seniors. To our knowledge, we are the first to show that these events occur with recreational activity levels by analyzing co-expression of fast and slow isomyosins in the muscle biopsies. Our studies support the concept that long term high-level exercise has beneficial effects on reinnervation of the muscle fibers, resulting in preservation of muscle size, structure, ultrastructure (34) and function, thereby delaying the functional decline and loss of independence that are commonly seen in aging.

MATERIALS AND METHODS

Study Subjects. Approval from the national committee for medical ethics was obtained before study onset (EK08-102-0608). With the exception of two female subjects in the sedentary group, recruited subjects were male volunteers. All subjects received detailed information on the study and gave informed consent. Three groups were enrolled: Young men (n=5; aged 22-33 years, 10 *Vastus l.* biopsies); seniors with a sedentary life style (n=6; aged 67-74 years, 10 *Vastus l.* biopsies); and seniors with a long history of high-level recreational sport activities (n=7; aged 65 to 79 years, 10 *Vastus l.* biopsies) (Supplemental Table 1). All subjects were healthy and declared not to have any specific mobility impairment or disease. Further, all the subjects declared they had no prescriptions for anti-inflammatory therapy related to neuropathies or myopathies. The seniors were not outpatients of any rehabilitation clinic, but were located by newspaper advertisements which stressed that only healthy subjects without mobility impairment would be enrolled. Sedentary seniors were enrolled on the basis of their declaration that they had not performed any routine physical activity/training during the previous 10 years. Upon enrollment in the study, needle muscle biopsies were harvested from the *Vastus lateralis* muscles through a small skin incision (6 mm) and then frozen for light microscopy or fixed for electron microscopy as described (21,34).

Light microscopy and quantitative histological analyses. Serial cryosections (8 μm of thickness) from frozen muscle biopsies were mounted on polysine™ glass slides, air-dried and stained with Hematoxylin and Eosin (H&E) (35).

Immunofluorescence analyses. Serial cryosections (8 μm) from frozen muscle biopsies were mounted onto polysine™ glass slides and air-dried. Muscle sections were labeled for either fast or slow myosin heavy chain (MHC) or neural cell adhesion molecule (N-CAM) as described below.

Immunohistochemistry of fast and slow Myosin Heavy Chains (MHC). Sections were washed with PBS and permeabilizated with 0.1% Triton (Sigma-Aldrich, St. Louis, MO) in PBS for 15 min. After a PBS wash, non-specific protein interactions were blocked by incubation with 10% Fetal Bovine Serum in PBS for 30 min at room temperature (RT). The sections were then incubated for an hour (RT) in primary mouse monoclonal anti-MHC fast or anti-MHC slow antibody (Novocastra; Milano, Italy) diluted 1:10 in PBS. The sections were subsequently washed in PBS and incubated with anti-Mouse-Cy3 secondary antibody (1:100; Sigma-Aldrich) for MHC slow and with anti-Mouse-FITC (1:100; Sigma-Aldrich) for MHC fast for 1 hour. The sections were washed again in PBS and coverslips were mounted onto the glass slides using ProLong Gold antifade reagent with DAPI (Life Technologies; Carlsbad, CA). Images were acquired using a Zeis microscope connected to a Leica DC 300F camera.

Detection of N-CAM expressing myofibers. Sections were fixed in methanol for 15 min at 20°C and then labeled for 1 hour (RT) using rabbit polyclonal antibody directed against N-CAM (Chemicon, Millipore, Milan, Italy) diluted 1:200 in PBS (Zampieri S 2010). Sections were rinsed 3x5 min in PBS, and then incubated for 1 hour (RT) with Cy3 labeled conjugate directed against rabbit IgG (Chemicon, Millipore) diluted 1:200 in 10% goat serum in PBS. Negative controls were performed by omitting the primary antibodies from sample incubations. After washes, nuclei were counterstained for 5 min (RT) with Hoechst 33258 (Sigma-Aldrich), sections were coverslipped using mounting medium (Dako, Glostrup, Denmark) and observed under a Zeiss microscope connected to a Leica DC 300F camera.

Co-immunolocalizaton of fast and slow MHC on single sections. The sections were washed, permeabilized, washed again and incubated with blocking solution as described above. The sections were then incubated for an hour (RT) with mouse anti-MHC slow primary monoclonal antibody (Sigma-Aldrich) diluted 1:10 in PBS and secondarily with rabbit anti-laminin (Sigma-Aldrich) diluted 1:100 in PBS. Next, the sections were washed in PBS and incubated with the anti-mouse-Alexa 594 (1:200; Life Technologies) secondary antibody and the anti-rabbit FITC antibody (1:200;

Sigma-Aldrich) for 1 hour. After a PBS wash, the sections were incubated for an hour with an anti-MHC fast primary monoclonal antibody produced in mouse (1:10; Novocastra; Buffalo Grove, IL). The sections were then washed with PBS and incubated for another hour with an anti-mouse-Alexa 488 secondary antibody (1:200; Life Technologies). After another wash with PBS, coverslips were mounted onto the glass slides using ProLong Gold antifade reagent with DAPI (Life Technologies).

Morphometric analyses. Morphometric analyses of the fiber diameter and of the fiber type distribution were performed on cryosections using Scion Image for Windows version Beta 4.0.2 (2000 Scion Corporation) as previously described (35-38).

Statistical analysis. ANOVA tests were performed with statistical algorithms of Origin™ (OriginLab Corporation, USA). The level of statistical significance was set at $p<0.05$.

RESULTS

Demography and clinical characteristics show that the enrolled subjects are healthy and mobile.

Detailed demographic and clinical characteristics of the enrolled subjects are described in Supplemental Table 1. All subjects (young men and seniors, either sedentary or sportsmen) were healthy and declared not to have any specific mobility impairment or disease. Nonetheless, clinical and functional evaluations, in addition to electromyographic analyses, were performed in a few senior sportsmen, resulting in detection of some neuropathic or myopathic evidence (Supplemental Table 1).

Amount of weekly physical activity and knee extension strength are significantly higher in co-aged recreational sportsmen compared to sedentary seniors.

Amounts and types of physical exercise performed by the active subjects are detailed in Supplemental Table 2. Four of the five young men performed strength training; the fifth did endurance activity. They declared to have exercised 4.0-7.5 hours per week during the previous 5 years. The senior sportsmen trained 4.5-24.0 hours per week. Obviously, a few seniors spent more time training than the young men; however, the apparent large difference between the mean training times (11.7+/-7.3 vs 5.3+/-1.4 hours per week+/-SD, respectively) is not statistically significant. Sedentary seniors had not performed any exercise above normal everyday living activities throughout the previous 20 years.

Quadriceps force was measured to allow comparison of specific muscle strength among the groups (Supplemental Table 2). The mean (+/-SD) knee contraction strength in young men was found to be 3.21+/-0.55 Nm/Kg while it was 2.17+/-0.42 in senior sportsmen (a decrease of 32% relative to the young men) and 1.57+/-0.39 in sedentary seniors (a decrease of 51%). The performances in a battery of functional mobility tests of the senior sportsmen were more similar to those of young men than to those of the sedentary co-aged group (Kern et al, manuscript in revision). This is sound evidence that the enrolled senior sportsmen are a highly active group and are likely comparable to master athletes (28-30).

Small angular muscle fibers in both the young men and seniors (sedentary and sportsmen) have the size and morphology of denervated muscle fibers.

Based on our experience with muscle biopsies from spinal cord injured paraplegics presenting with either disuse atrophy resulting from lesions of the central motoneuron or extreme atrophy due to lack of innervation secondary to complete peripheral motoneuron lesions, we identify muscle fibers with a diameter less than 30 μm as denervated (20,21,36–38). Our interpretation of these myofibers as denervated is strengthened by the facts that half of these small fibers actually have diameters less than 25 μm and that several have angular aspects. In the present study, serial sections of muscle biopsies from the young men reveal that myofibers having a diameter less than 30 μm are infrequent (0.4%, Table 1) and that those with a diameter less than 25 μm are even less abundant (0.2%, Table 1); the vast majority of the muscle fibers in these sections are basically round (Figure 1, Panels A and B, respectively). However, the muscle sections from the seniors (Figure 1, Panels C and D, sedentary and Panel E, sportsmen; Figure 2, sedentary seniors; and Figure 3, Panels A and B, sedentary seniors) reveal more abundant muscle fibers having diameters less than 30 and 25 μm (Table 1) and some of these have a peculiar angular aspect (Figure 1, white arrowheads). The biopsies taken from the sedentary seniors contain the highest percentage of denervated muscle fibers having a diameter less than 30 μm (6.5%) and of those having a diameter less than 25 μm (2.6%). These percentages are significantly higher than in the other two groups; however, the percentages of denervated fibers are not significantly different between the young men and senior sportsmen. Additionally, in sedentary seniors, the small angular myofibers show appreciable expression of N-CAM, an accepted marker of denervation (39,40) (Figure 2, Panels A-C); the N-CAM staining is less abundant in fibers from the

senior sportsmen and even less so in sections from the young men (data not shown). These data show that the sedentary seniors have more denervated fibers.

Percentages of fast and slow myofibers in senior sportsmen show a significant shift toward slow fibers relative to the young men and the sedentary seniors.

Immunofluorescence reveals both fast and slow MHC proteins in serial sections of muscle biopsies from the young men (Figure 1, Panels A and B, respectively) with the fast fibers being slightly more abundant than the slow fibers in these *Vastus lateralis* sections (Figure 1, Panels A and B; Table 2). Interestingly, this pattern of fiber type distribution is not statistically different from that observed in the matched muscle of sedentary seniors, although the latter are slightly shifted toward the slow type relative to the young men (Figure 1, Panels C and D; Table 2). However, in senior sportsmen (Figure 1, Panels E and F) the slow fibers prevail (68.5%) with the increase being statistically significant relative to both the young men (42% slow fibers) and the sedentary seniors (46% slow fibers) (Table 2).

Fiber-type groupings are almost absent in young men; however, these groupings increase in the senior subjects with senior sportsmen having the greatest number, most of which are of the slow type, while those in sedentary seniors are mainly of the fast type.

Fiber-type groupings are identified on the basis that at least one muscle fiber is completely surrounded by fibers of the same phenotype. Percentages of fiber type-groupings are determined by counting the number of muscle fibers in the biopsy that are surrounded by fibers of the same type and then dividing this number by the total number of fibers. To avoid problems related to the existence of many different fast MHC isoforms, we used an anti-fast MHC antibody that does not discriminate among the fast isoforms; therefore, we will describe and discuss fiber type clusters as either "slow" or "fast" only. Muscle sections from the young men have few fiber type groupings with those that are detected being mainly of the fast type (1%; Figure 1 and Table 3). Biopsies taken from sedentary seniors show that, although both fast and slow type groupings are present, the fast type (3.0%) are more numerous than the slow (0.5%; Table 3). Most notable is the fact that biopsies taken from senior sportsmen have the highest percentage of slow type fiber groupings with a mean of 7.9%, reaching almost 25% in the extreme cases where 93% of total myofibers are of the slow type (Table 3). It is important to note that the senior sportsmen with the highest amount of EMG neuropathic or myopathic signs are not the subjects presenting with the higher content of slow type fibers and slow fiber type-groupings (compare Supplemental Tables 1 and 3).

Muscle fibers co-expressing fast and slow MHC are sparsely present in analyzed biopsies of all study subjects, with the observed differences among groups of subjects not being statistically significant.

Muscle fibers co-expressing fast and slow MHCs are seldom observed in any of the analyzed biopsies and there are no significant differences among these groups in terms of this parameter (Table 4). However, the serial sections from sedentary seniors which do show MHC co-expression are often small-sized, angular (denervated) muscle fibers (Figure 1, Panels C and D, white arrows); therefore, we suggest that these are slow myofibers co-expressing fast isoforms of MHC by default myogenic programs. In contrast, the muscle fibers from senior sportsmen which are positive for both fast and slow MHC proteins (Figure 4, Panel C, orange fibers circumscribed by white interrupted circles) are similar in size to the pure fast (green) or pure slow (red) myofibers. In Panel C, it is interesting to note that, although some fibers are green and others are red, not all of the fibers have the same intensity of color. This could indicate that these fibers contain some variable combinations of fast and slow MHCs, but not enough of both to produce the orange color.

There is a high correlation between the slow fiber percentages and slow fiber groupings, but not between the percentages of slow fibers and the type of training performed by the senior sportsmen.

The correlation between the slow fiber percentages and slow fiber type groupings in the senior sportsmen is high ($R^2 = 0.82$; Figure 5). However, there is no correlation between the percentages of slow fibers and the prevalent kinds of training undertaken by the sportsmen (Figure 6). Indeed, the marks indicating whether the subjects had performed mainly strength training, endurance training or a mixture of both strength and endurance trainings (mixed training) are randomly distributed both among scarcely or highly transformed muscle biopsies.

DISCUSSION

In aging muscle, it has long been recognized that denervation of muscle fibers contributes to atrophy (7-17) and that disuse accelerates the deterioration process (22) while increased exercise, sustained for decades (e.g., training as performed by track and field master athletes), protects against the age-related loss of motor units (44-46) and, thereby, of lean muscle mass (47). However, the degree to which denervation causes loss of myofibers is an open issue because reinnervation events may compensate for motor neuron loss during aging as well as with spinal cord injury and/or axonal abnormalities of peripheral nerves (13-15,48-50). In the present study, we compared muscle parameters from a group of young men (training by weight lifting) with those from two groups of healthy, freely mobile seniors (Supplemental Table 1): one composed of people leading a sedentary life style and a second made up of recreational sportsmen who had trained 4.5 to 24.5 hours per week during the previous two (or more) decades and were still training at the time the muscle biopsies were taken. The data reveal that muscle from the sportsmen more closely resembles that of the younger men in terms of force generation and fiber size than it does the muscle of the sedentary older people, showing that long term exercise aids in preservation of muscle health. More interestingly, the study reveals some unique characteristics of the sportsmen muscle in terms of fiber type and fiber type groupings that suggest denervation/reinnervation plays a role in the maintenance of muscle health.

First, to insure that the older sportsmen had maintained a significant degree of muscle function, we tested knee contraction strength in all groups. Indeed, the knee contraction strength in the older sportsmen was significantly greater than that of the sedentary seniors and not significantly different from that of the younger men (Supplemental Table 2). Further, although the senior sportsmen did generate 32% less force than the young men upon knee contraction (despite the fact that the groups had dedicated similar time to training), this latter finding is not surprising because it is well documented within the world sporting records of master athletes that young competitors out-perform older ones (28-30,51). To explore the mechanisms that delayed deterioration in the muscle of the senior sportsmen, we analyzed immunolabeled muscle biopsies taken from our groups and compared their relative amounts of: 1. small angular myofibers (i.e., denervated muscle fibers), 2. molecular markers of fast and slow muscle fiber types (a measure of residual muscle plasticity), and 3. fiber-type groupings (representing denervated/reinnervated muscle fibers). The main results are: 1. biopsies from young men seldom contain denervated, reinnervated or grouped muscle fibers; 2. biopsies from sedentary seniors contain both denervated and a few reinnervated clustered myofibers of the fast type; and 3. senior sportsmen present with a larger percentage of healthy slow myofibers, up to 90%, that appear mainly clustered in slow fiber-type groupings. The finding that senior sportsmen have a significantly higher percentage of slow type fibers and slow type fiber groupings is supported by our previous work which used histochemical myosin ATPase staining in muscle biopsies harvested from senior sportsmen (n=15; only two of those subjects also participated in this study) to show that 27 out of 28 biopsies presented with slow type groupings [31]. The increased slow type fiber content and percent slow-type fiber groupings are most interesting and specular to the accepted fact that immobilization drives muscle fibers toward atrophy and fast type transformation. The fact that these parameters are significantly higher in the sportsmen relative to the sedentary seniors makes it obvious that this is not simply a function of age. In further support of this, we show that the ages of the seniors (Supplemental Table 1) are *not* correlated with percentages of slow fibers and slow fiber-type groupings in either group of seniors: 1) Sedentary seniors, age vs % slow fibers, R^2= 0.12; age vs % slow type groupings, R^2= 0.02; and 2) Senior sportsmen, age vs % slow fibers, R^2= 0.25; age vs % slow type groupings, R^2= 0.27. There is no correlation even when the three groups are pooled: age vs % slow fibers, R^2= 0.15; age vs % slow type, R^2= 0.56. Further, the lack of correlation between the kind of training and the percentages of slow type fibers demonstrates that the type of activity is not relevant.

Interestingly, muscle fibers co-expressing fast and slow MCH proteins are seldom detected in the biopsies of any of our groups and no statistical differences were found among the groups with respect

to this parameter. It is possible that the observed co-expression is scanty either because it is actually a rare event or because the denervation that occurs is quickly followed by reinnervation so that obvious co-expression of the MHCs is short-lived. To our knowledge, this is the first evidence that fiber transformation cannot be the direct consequence of decades of high level activity. It also further supports the hypothesis that denervation events are not detectable with standard clinical electromyography (EMG). However, when these co-expressing fibers are found in the sedentary senior muscles, they are small (<30 μm) and often have the peculiar angular aspect noted after experimental or clinical denervation; also some are positive to the anti-NCAM antibody, an accepted marker of denervation (39,40). This is in stark contrast to fibers from the sections of young and senior athletes in which NCAM staining is nearly absent (approximately 1 in 25 fibers was positive in the muscle of the young men). his type of myofiber is common in unloaded muscle [i.e., resulting from spaceflight, limb suspension or immobilization] and with spinal cord injury and peripheral denervation (18-24,41-43). Thus, we consider these to be denervated muscle fibers. Further, because it is well known that slow type muscle fibers revert to the fast isotype when denervated during development and adulthood (18,27,41,42), we suggest that these fibers are denervated slow type myofibers re-expressing fast MHC through default myogenic programs. In contrast, when myofibers co-expressing fast and slow MHCs are detected in the muscles of senior sportsmen, they are similar in size and shape to the pure type fibers in the section and, therefore, cannot be lacking innervation. Further, their low density in these muscle sections is not in agreement with the concept that they belong to a motor unit that is undergoing exercise-driven, slow-type transformation of myosin heavy chains - a mechanism that is well known to occur in cross-reinnervation models (19,43), but is more presumed than demonstrated in humans performing voluntary exercise (17). It is likely that the transforming myofibers (i.e., those co-expressing fast and slow MHC proteins) contribute to the increase in slow fiber-type groupings (Figure 4); this is supported by the positive correlation between the increasing percentages of slow type fiber number and slow-type fiber groupings in sections from the sportsmen. Thus, we suggest that these few normal-sized co-expressing fibers very likely are previously denervated fast-type fibers, which have been quickly reinnervated by sprouts from slow axons, and are only temporarily co-expressing fast and slow MHC isoforms before they will finally exclusively express slow MHC. This is supported by the law of "recruitment order" which dictates that slow motor units (and thus muscle fibers) are activated more frequently than the fast motor units. In fact, the most active of the α-motoneurons are the slow-type and it is likely that this higher-lever of activity is what maintains motoneurons, muscle fibers and their MHC content. Therefore, the increase in slow type fiber groupings is evidence that some muscle plasticity still exists in both sedentary and (especially) recreational sportsmen. Additionally, the reinnervation process may be more extensive than is obvious here because if the temporarily denervated myofibers were of the slow type, they would continue their current gene expression when reinnervated by slow type α-motoneuron axon terminals and, thereby, escape our detection. Further, in our opinion, lack of fast type-groupings in senior sportsmen, is direct evidence of selective re-innervation of denervated myofibers from slow-type α-motoneurons. In summary, our working hypothesis is that muscle fibers co-expressing fast and slow MHCs are either denervated slow myofibers also expressing fast MHC isoforms by the default myogenic program (Figure 3, panels A and B) (22,24,27) or denervated fast fibers reinnervated by axons sprouting from slow motor neurons (19,42,43). This speculation certainly needs further study, in particular *in situ* MHC expression analyses on more numerous subjects and different muscle types.

Indeed, our study has many potential confounding factors such as: 1.1 the use of the fiber type-heterogenous *Vastus lateralis* muscle, 1.2 the small size of the specimens due to the sampling method (needle biopsy) and low number of study subjects, 1.3 the inherently variable genetic backgrounds of the individual subjects; and 1.4 the differences in the kind and extent of physical activities of the senior sportsmen; and 2) the fact that muscle biopsies harvested from senior sportsmen range from those with scarce fiber-type transformation and groupings to those with almost fully transformed

muscles, in which some isolated fibers co-expressing fast and slow MHCs fill in gaps (Figure 4). Despite these limitations, the clinical significance of our observations is confirmed by the fact that the muscle properties of the senior sportsmen group are more similar to those of the active young men than to those of sedentary seniors. Specifically, relative to their sedentary cohorts, senior sportsmen have greater muscle maximal isometric force (Supplemental Table 1) along with better preserved muscle morphology and mobility as we have reported at recent meetings (32,33). Taken together, our results suggest that, beyond the direct effects of aging on the structure and function of muscle fibers (Kern et al., manuscript under revision), changes occurring in the muscle tissue of the sedentary group appear to be in part a result of sparse incremental denervation. In senior sportsmen, the increase in the percentage of "slow fiber groupings" is the result of the positive effect of long term physical activity on the motoneuron pool, which, conceivably, has mainly spared the slow motoneurons from age related lesion/death, increasing the chance that peripheral reinnervation occurs due to sprouting of slow axons. Certainly, numerous mechanisms contribute to long term muscle health or deterioration, yet our study suggests that long term exercise will allow the body to adapt to the consequences of age-related denervation (Table 2 and Figures 1, 2, 3) and to preserve muscle structure and function by saving otherwise lost muscle fibers through recruitment of muscle fibers to different, mainly slow, motor units. We further speculate that high-level activity, by either voluntary exercise or functional electrical stimulation, may be applied at any age to save neurons from disorders secondary to inactivity (52-53) and to counteract muscle atrophy in other neuromuscular or metabolic diseases (54). We conclude that, although we did not work with master athletes, the intensity of recreational training reported here is something that the general population may achieve, especially if properly motivated by specialists in the field. We show that recreational levels of activity are very effective in driving seniors toward improved functional performance and rearrangement of muscle fiber type. In particular, these levels of exercise seem to have beneficial effects on reinnervation of the muscle fibers, resulting in preservation of muscle size, structure and function, thereby delaying the functional decline and loss of independence that are commonly seen in elderly people.

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Supplemental Materials

Supplemental **Table 1. Demography and clinical characteristics of the enrolled subjects.**

	Demography			Clinical characteristics	
	Biopsy's leg (n=10)	**Sex**	**Age (yrs)**	**Electrophysiology**	**Other diseases**
Young men					
1	L/R	M	22	N.D.	no
2	L/R	M	24	N.D.	no
3	L/R	M	25	N.D.	no
4	L/R	M	27	N.D.	no
5	L/R	M	33	N.D.	no
Mean +/-SD			26.2 [a] 4.0		
Sedentary Seniors					
6	L/R	M	77	N.D.	no
7	L	F	74	N.D.	no
8	L/R	F	67	N.D.	no
9	L/R	F	71	N.D.	no
10	L/R	M	72	N.D.	no
11	L	M	70	N.D.	no
Mean +/-SD			71.8 [b] 3.5		
Senior Sportsmen					
12	L/R	M	68	NCV: N; EMG: N	no
13	R	M	69	NCV: M; EMG: N	no
14	R	M	67	N.D.	no
15	L/R	M	68	N.D.	no
16 myop/neur	L/R	M	65	NCV: N; EMG: no	
17 sl.myop.	L	M	79	NCV: M; EMG: no	
18	L	M	66	NCV: D; EMG: N	no

Mean	68.3 [c]
+/-SD	4.0

L= left, R= right; NCV= nerve conduction velocity; N.D.= not determined;

EMG= electromyography; N= normal; M= moderate; D= distinct; myop/neur, both myopathic and neurogenic changes, overall less marked; sl.myop, slight myopathic.

Statistical significance: a, $p<0.05$ vs. sedentary and sportsmen seniors; b, $p<0.05$ vs. young, but not vs. senior sportsmen; c, $p<0.05$ vs. young, but not vs. sedentary seniors.

Supplemental Table 2. Detailed characteristics of physical activity and muscle strength of the enrolled subjects at biopsy.

	Training			Force (Knee Contraction)	
	Prevalent Type	**Amount (hrs/week)**	**Years**	**(Nm/kg)**	**Decrease (Δ %)**
Young men					
1	Force	4.0	>5	2.95	
2	Force	4.5	>5	2.93	
3	Endurance	4.5	>5	2.69	
4	Force	7.5	>10	4.10	
5	Force	6.0	>5	3.36	
Mean		5.3 [a]		3.21 [b]	
+/-SD		1.4		0.55	
Sedentary Seniors					
6	-	-	-	1.46	
7	-	-	-	1.76	
8	-	-	-	1.51	
9	-	-	-	1.15	
10	-	-	-	1.29	
11	-	-	-	2.24	
Mean	-	-	-	1.57 [c]	51
+/-SD				0.39	
Senior Sportsmen					
12	Force	4.5	>20	1.98	
13	Mixed	6.9	>20	1.35	
14	Mixed	16.0	>20	2.09	
15	Endurance	16.0	>20	2.49	
16	Endurance	8.0	>20	2.33	
17	Endurance	6.0	>30	2.48	
18	Force	24.5	>20	2.49	
Mean		11.7 [a]		2.17 [d]	32
+/-SD		7.3		0.42	

Recreational activity for each subject : **1, 2, 4 and 5** : weight lifting ; **3** : interval running ; **4** : sprinting running; **12**: weight lifting; **13**: aerobic cycling and strength-endurance training of upper body and legs; **14**: tennis and soccer, skiing (winter time) and cycling (summer time); **15**: cycling, swimming and weight lifting, skiing (winter time); **16**: inline skating, swimming, cycling (summer time), skiing (winter time); **17**: 100-800 meter track running; **18**: decathlon, ice hockey, alpine skiing and ice gliding (winter time), swimming (summer time).

Statistical significance: a, not significant vs. senior sportsmen, $p>0.05$; b, $p<0.05$ vs. sedentary seniors, but not vs. senior sportmen; c, $p<0.05$ vs. young men and senior sportsmen; d, $p<0.05$ vs. sedentary seniors, but not vs. young men.

Supplemental Table 3. Overall results from immunolabeling of muscle biopsies harvested from young men and elders, either sedentary or sportsmen, with anti-fast and anti-slow myosin heavy chain (MHC) antibodies.

Young Men

Muscle Fibers			Fiber-Type Groupings	
Coexpressive (%)	**Fast (%)**	**Slow(%)**	**Fast (%)**	**Slow(%)**
0.0	56.3	43.8	0.0	0.0
1.4	54.8	45.2	0.0	0.0
1.4	52.5	47.5	0.0	0.0
0.0	64.8	35.2	6.7	0.0
0.7	60.1	39.9	0.8	0.3
0.4	64.0	36.0	0.6	0.0
0.0	53.6	46.4	0.0	0.0
0.9	54.4	45.6	0.5	0.0
0.0	59.7	40.3	0.4	0.0
0.2	60.0	40.0	0.7	0.0

Sedentary Seniors

Muscle Fibers			Fiber-Type Groupings	
Coexpressive (%)	**Fast (%)**	**Slow(%)**	**Fast (%)**	**Slow(%)**
0.0	56.6	43.4	0.5	0.0
3.3	53.1	46.9	1.8	0.9
4.2	51.6	48.4	2.2	0.9
0.0	80.3	19.7	15.8	0.0
3.5	59.5	40.5	2.1	0.0
1.9	59.7	40.3	1.9	0.0
3.5	59.1	40.9	4.7	0.0
0.0	48.4	51.6	0.0	0.9
0.0	32.5	67.5	0.0	1.5
1.5	37.3	62.7	1.0	0.4

Senior Sportsmen

Muscle Fibers			Fiber-Type Groupings	
Coexpressive (%)	**Fast (%)**	**Slow(%)**	**Fast (%)**	**Slow(%)**
2.2	6.9	93.1	0.0	25.2
0.4	46.5	53.5	0.3	1.1
0.1	25.9	74.1	0.2	12.6
0.8	33.5	66.5	0.0	5.1
1.0	23,5	76,5	0.0	9,9
0.2	20,7	79,3	0.0	10,9
0.1	35.4	64.6	0.1	2.8

0.3	38.1	61.9	0.2	2.1
0.2	57.4	42.6	0.2	0.4
0.6	26.7	73.3	0.0	8.4

Statistical significance of differences among groups are described in Tables 1 to 4.

Table 1. Denervated fibers in young men and in seniors, either sedentary or sportsmen.

		Myofiber diameter			
		< 30 μm		< 25 μm	
Subjects	**(Biopsy, #)**	**%**	**ANOVA**	**%**	**ANOVA**
Young men	(10)	0.4 +/- 0.5[a]	YES	0.2 +/- 0.5[a]	YES (vs Sedentary)
Seniors					
Sedentary	(10)	6.5 +/- 3.8[b]	YES	2.6 +/- 1.9[b]	YES (vs Sportsmen)
Sportsmen	(10)	1.8 +/- 3.9[a]	NO	0.4 +/- 1.1[a]	NO (vs Young men)

Occurrences of small fibers are expressed as percentages in respect to the total number of muscle fibers. Parentheses apply to both columns.

Within columns, groups with different lower case letter superscripts are statistically different, $p<0.05$. YES or NO refer to statistically significant differences by ANOVA tests.

Table 2. Percentages of fast and slow myofibers in young men and seniors, either sedentary or sportsmen.

	MHC Positive Muscle Fibers (%)			
Subjects	(Biopsy, #)	Fast	Slow	ANOVA
Young men	(10)	58.1 +/-4.3[a]	42.0 +/- 4.3[a]	NO (vs. Sedentary)
Seniors				
Sedentary	(10)	53.8 +/- 13.2[a]	46.2 +/- 13.2[a]	YES (vs Sportsmen)
Sportsmen	(10)	31.5 +/- 14.1[b]	68.5 +/- 14.1[b]	YES (vs. Young)

Content of fast and slow fibers are expressed as their percentage in respect to the total number of fibers in each muscle biopsy.

Within columns, groups with different lower case letter superscripts are statistically different, $p<0.05$. YES or NO refer to statistically significant differences by ANOVA tests.

Table 3. Percentages of fast and slow fiber-type groupings in young men andseniors, either sedentary or sportsmen.

		Fiber-Type Groupings (as % of central fibers in clustered areas vs total fibers)			
Subjects	**(Biopsy, #)**	**Fast**	ANOVA	**Slow**	ANOVA
Young men	(10)	1.0 +/- 2.0[a]	NO	< 0.1 +/- 0.1[a]	NO (vs Sedentary)
Seniors					
Sedentary	(10)	3.0 +/- 4.7[a]	NO	0.5+/- 0.6[a]	YES (vs Sportsmen)
Sportsmen	(10)	0.1 +/- 0.1[a]	NO	7.9 +/- 7.4[b]	YES (vs Young)

Content of reinnervated muscle fibers are expressed as the percentage of central muscle fibers surrounded by muscle fibers of the same phenotype relative to the total number of muscle fibers in each biopsy.

Within columns, groups with different lower case letter superscripts are statistically different, $p<0.05$. YES or NO refer to statistically significant differences by ANOVA tests.

Table 4. Percentages of myofibers co-expressing fast and slow MHCs in young men and inseniors, either sedentary or sportsmen

		Myofibers co-expressing fast and slow MHCs	
Subjects	**(Biopsy, #)**	**%**	**ANOVA**
Young men	(10)	0.5 +/- 0.6[a]	NO (vs Sedentary)
Seniors			
Sedentary	(10)	1.8 +/- 1.7[a]	NO (vs Sportsmen)
Sportsmen	(10)	0.6 +/- 0.6[a]	NO (vs Young)

Content of muscle fibers co-expressing fast and slow muscle fibers are expressed as their percentage relative to the total number of muscle fibers.

Groups with different lower case letter superscripts are statistically different, $p<0.05$. YES or NO refer to statistically significant differences by ANOVA tests.

LEGENDS OF FIGURES

Figure 1. *Immunofluorescence revealing either fast or slow MHC proteins in serial sections of biopsies from young subjects (A, B), sedentary seniors (C, D) and seniors sportsmen (E, F).* White arrows point to small and angular fibers, while white circles show fiber type groupings. Senior sportsmen present a lower percentage of small fibers, and a higher percentage of slow fiber-type clusters than sedentary seniors. Fiber size in senior sportsmen biopsies are comparable with those of the young men.

Figure 2. *N-CAM, one of the classical markers of denervation and neural regeneration, is expressed in myofibers of biopsies from seniors*.
Small, angulated N-CAM positive myofibers (red stained) were detected in muscle biopsies from sedentary seniors. Based on their peculiar morphological aspects, size and N-CAM expression, these myofibers (A, B, C) are indeed denervated. Myonuclei are counterstained in blue; magnification bar = 100 μm.

Figure 3. *Muscle biopsies harvested from sedentary seniors (A, B) and senior sportsmen (C, D)*. White arrows point to small angular muscle fibers, while white circles surround the central fibers that delineate fiber type groupings. Note that the clustered fibers in the sedentary biopsies are of the fast type, while those of the senior sportsmen are of the slow type.

Figure 4. *Co-immunofluorescence of fast and slow MHC proteins in a single section of Vastus lateralis from senior sportsmen reveals sparse MHC co-expressing myofibers*. Fast fiber MHC proteins and laminin are labeled in green (Panel A) while slow fiber MHC proteins are labeled in red (Panel B). Muscle fibers co-expressing both fast and slow MHCs are labeled with various levels of yellow/orange (within white circles of Panel C) in proportion to the predominance of either fast or slow myosin heavy chains, respectively. The amount of co-expressing muscle fibers is far below the large number of muscle fibers (in the hundreds) belonging to one motor unit.

Figure 5. *The percentages of slow muscle fibers and slow fiber-type groupings in muscle biopsies of senior sportsmen are strongly correlated* ($R^2 = 0.82$).

Figure 6. *The percentage of slow fibers in muscle biopsies harvested from senior sportsmen are independent of the prevalent kind of training (E = endurance, S = strength or M = mixed) undertaken by the sportsman.* This is evidenced by the fact that data from each form of exercise is randomly distributed within the normal distribution produced when the percentage of slow muscle fibers are plotted against the probability that each datum falls within the area under the curve (AUC).
Excel equation DISTRIB.NORM(X;Mean;Dev_standard; Cumulative):

$$f(x) = \frac{1}{\sqrt{2\pi\sigma^2}} e^{\frac{(x-\mu)^2}{2\sigma^2}}$$

Figure 1.

Figure 2.

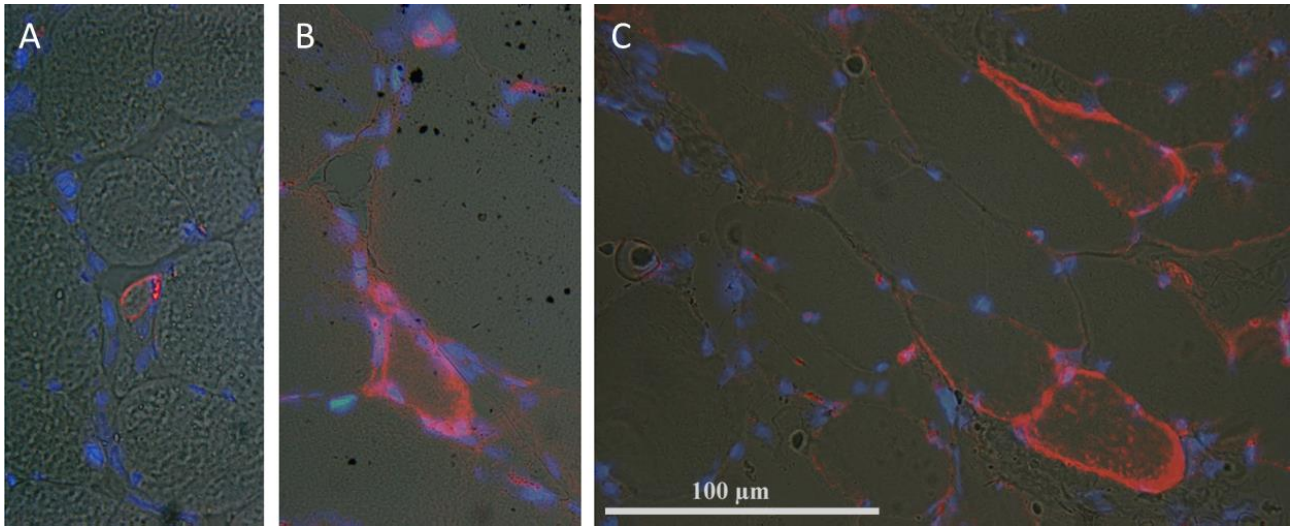

Figure 3.

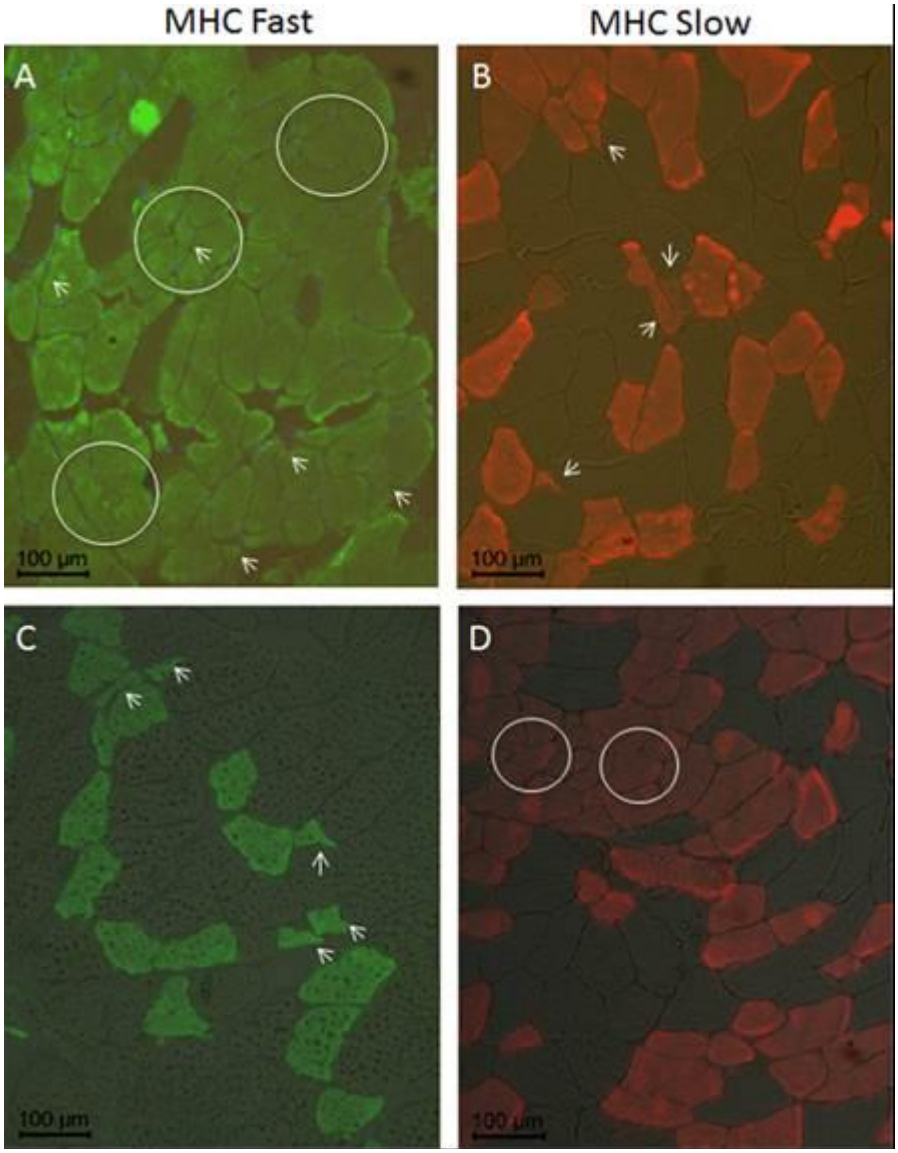

Figure 4.

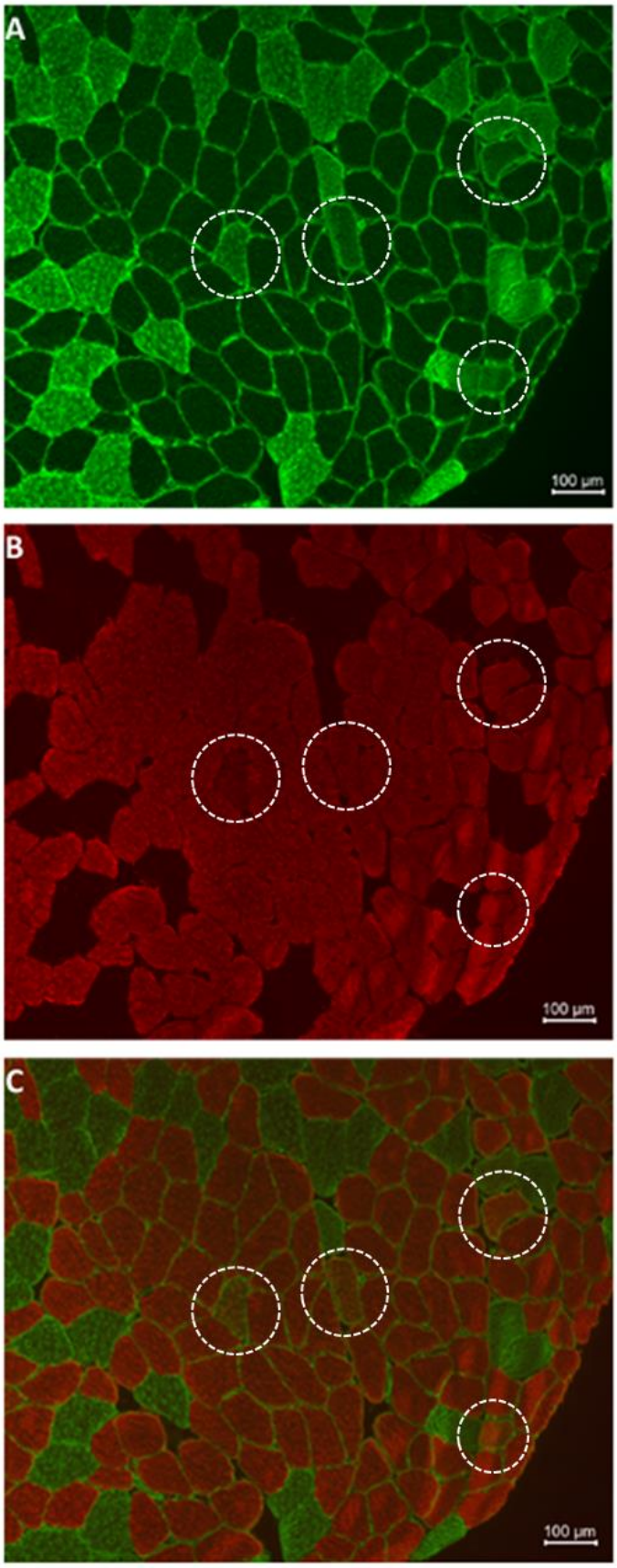

Figure 5.

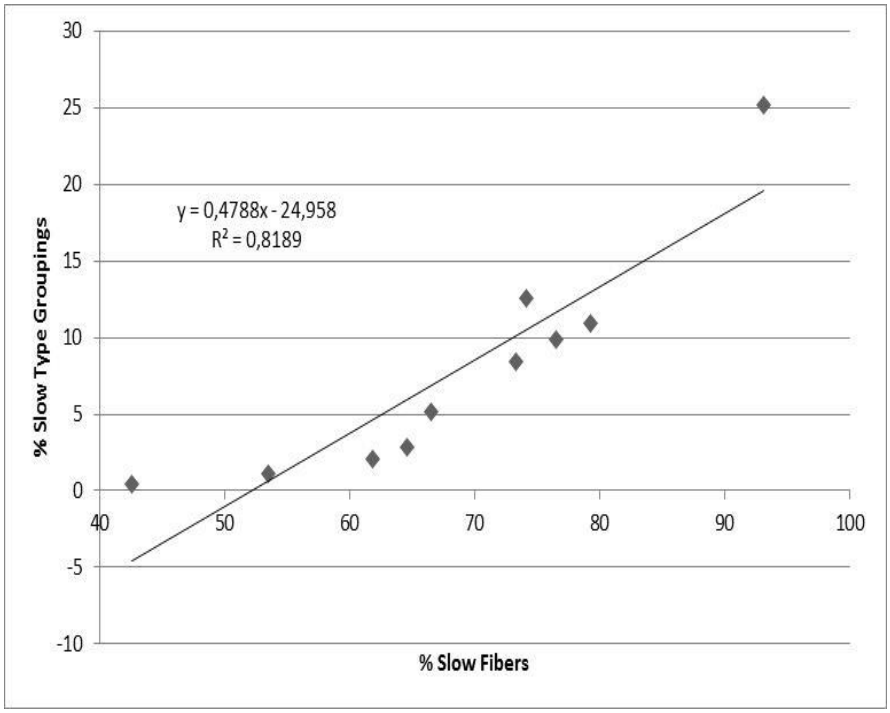

Figure 6.