1 **The impact of life-long strength versus endurance training on muscle fiber morphology**

2 **and phenotype composition in older men**

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19 **Running head:** Myofiber alterations with age

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27 **NEW & NOTEWORTHY**

28 Aging is associated with loss of fast-twitch type II myofibers, motor unit remodeling, and
29 grouping of myofibers. This study reveals, for the first time, that strength training preserves
30 neural innervation of type II fibers, resulting in similar myofiber type distribution and
31 grouping in life-long strength trained master athletes as young moderately active adults. In
32 contrast, life-long endurance trained master athletes and recreationally active old adults
33 demonstrated higher proportion of type I fibers accompanied by more marked grouping of
34 type I myofibers, and more atrophic fibers compared to strength trained master athletes and
35 young individuals. Thus, strength training should be utilized as a training modality for
36 preservation of fast-twitch musculature, maximal muscle strength, and rapid force capacity
37 (RFD) with advancing age.

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ABSTRACT

Aging is typically associated with decreased muscle strength and rate of force development (RFD), partly explained by motor unit remodeling due to denervation, and subsequent loss of fast-twitch type II myofibers. Exercise is commonly advocated to counteract this detrimental loss. However, it is unclear how life-long strength- versus endurance-training may differentially affect markers of denervation and reinnervation of skeletal myofibers and, in turn, affect the proportion and morphology of fast-twitch type II musculature. Thus, we compared fiber type distribution, fiber type grouping, and the prevalence of atrophic myofibers ($\leq 1494\mu m^2$) in strength-trained (OS) versus endurance-trained (OE) master athletes and compared the results to recreationally active older adults (all >70yr, OC) and young habitually active references (<30yr, YC). Immunofluorescent stainings were performed on biopsy samples from vastus lateralis, along with leg press maximal strength and RFD measurements. OS demonstrated similar type II fiber distribution (OS:52.0±16.4%; YC:51.1±14.4%), fiber type grouping, maximal strength (OS:170.0±18.9kg, YC:151.0±24.4kg), and RFD (OS:3993±894N·s^{-1}, YC:3470±1394N·s^{-1}) as young, and absence of atrophic myofibers (OS:0.2±0.7%; YC: 0.1±0.4%). In contrast, OE and OC exhibited more atrophic fibers (OE:1.2±1.0%; OC: 1.1±1.4%), more grouped fibers, and smaller proportion of type II fibers (OE:39.3±11.9%; OC: 35.0±12.4%) than OS and YC (all p<0.05). In conclusion, strength-trained master athletes were characterized by similar muscle morphology as young, which was not the case for recreationally active or endurance-trained old. These results indicate that strength training may preserve type II fibers with advancing age in older men, likely as a result of chronic use of high contractile force generation.

Key words: fast-twitch type II fibers, denervation, neural cell adhesion molecule (NCAM), nuclear clumps, older adults

INTRODUCTION

The maximal strength and rapid force capacity (rate of force development; RFD) of human skeletal muscle typically decline with age (9, 42, 51, 59). One of the main contributors to this decline is the peripheral denervation, and subsequent loss of myofibers (19, 22, 23) accompanied by a preferential atrophy of fast-twitch type II fibers (9, 20, 28). Reinnervation and remodeling of motor units may occur as a compensatory response to age-related denervation (31, 41), potentially causing fiber type changes and leading to signs of fiber type grouping (14). There appears to be more pronounced grouping of type I fibers (27, 41), due to age-related denervation of type II fibers followed by reinnervation of part of the denervated myofiber pool by low-threshold motor units that originally innervate type I fibers resulting in a higher proportion of type I fibers and grouping of these fibers (17) .

Although the decline in maximal muscle strength with age appears to be inevitable, it may be decelerated by physical exercise (6). As a testament to successful aging, older female master athletes, consisting of both endurance- and strength-trained athletes, have been characterized by signs of attenuated neurogenic atrophy and superior reinnervating capacity compared to their frail age-matched counterparts (41). This was manifested as fewer nuclear clumps, a smaller decline in fiber type IIa/I size ratio, and less variable inter-subject accumulation of neural cell adhesion molecule (NCAM)-positive fibers and atrophic fibers in master athletes compared to frail controls (41). NCAM is recognized as a marker of denervation (38, 39), but may also be upregulated during re-innervation (38), whereas the presence of atrophic fibers (i.e., very small fibers) and nuclear clumps may reflect neurogenic atrophy (5, 16, 37) with the latter detectable several years after complete upper motor neuron injury (5). There are also indications that life-long training may prevent the loss of motor units, as track and field master athletes had a higher number of estimated motor units than older untrained participants (33, 34). However, this has not been a universal finding, as similar motor unit numbers have been observed in endurance and strength trained master athletes compared to untrained, older controls (30, 31). This discrepancy may be explained by methodological differences regarding the methods used to estimate motor units (electrophysiological estimates) (1). Moreover, investigations of life-long training in master athletes typically do not distinguish between training modes, thus it remains elusive whether specific exercise modalities (i.e., endurance versus strength training) may provide more protective effects to prevent the age-related denervation of myofibers.

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116   We have previously documented that maximal muscle strength and RFD was higher in 70

117   year-old life-long strength trained master athletes compared with moderately active young

118   (20-30 years) individuals (51). Moreover, the high muscle strength levels observed in strength

119   trained master athletes is accompanied by a superior descending neural drive (elevated evoked

120   V-wave responses) to maximally contracting myofibers, when compared to sedentary and

121   recreationally active age-matched individuals (49), indicating that strength training may be

122   particularly beneficial for maintaining recruitment and in turn innervation of fast-twitch type

123   II fibers. In contrast, endurance trained master athletes appear to be characterized by different

124   neural pathways, dissociated from the activation of type II fibers and the ability to perform

125   very strong muscle contractions (45). In line with this notion, life-long strength trained master

126   athletes have been documented to have significantly larger type IIa and IIx fiber areas,

127   respectively, compared to life-long endurance trained master athletes as well as sedentary age-

128   matched older adults (58), further suggesting that long-term strength training may offer

129   significant benefits compared to other exercise modalities, for the preservation of type II fiber

130   morphology with increasing age.

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132   There is mounting evidence that strength-training may be imperative for the maintenance of

133   maximal force generating capacity with advancing age. However, markers of denervation and

134   fiber type grouping have not previously been investigated along with the assessment of

135   myofiber size and phenotype composition in chronically strength trained versus endurance

136   trained master athletes. Thus, the aim of the present study was to investigate if life-long

137   trained master athletes exhibit different patterns of myofiber distribution, fiber type grouping,

138   and myofiber atrophy, or differential signs of denervation and reinnervation depending on

139   their chronic training background (strength vs. endurance), and to make similar comparisons

140   to young and older habitually active adults. Specifically, it was hypothesized that master

141   athletes engaged in life-long strength training would demonstrate maintenance of type II fibre

142   distribution and attenuated or absent signs of myocellular denervation (muscle fiber grouping,

143   atrophic fibers, and number of nuclei clumps) and muscle regeneration (NCAM-positive

144   fibers), respectively, compared to age-matched life-long endurance trained master athletes,

145   and recreationally active older individuals. Further, we hypothesized that young habitually

146   active adults would exhibit fewer signs of myofiber denervation and regeneration compared to

147   aged adults irrespective of their training status.

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MATERIALS & METHODS

Participant characteristics

A total of forty-two men were included in the study. Participants (> 65 years) were recruited based on their physical training status, and are also part of another study (45). Strength trained master athletes (OS, 73 ± 4 years, n = 10) were recruited from local powerlifting and weightlifting clubs, while endurance trained master athletes were recruited from local Track and Field clubs (OE, 72 ± 6 years, n = 8) limited to athletes participating in endurance events of long duration ($\geq$ 3000 meters). Participants were considered athletes if they had been actively training and competing over the past two years in their respective sport. Master athletes had trained systematically most of their lives, although they reported some sporadic breaks throughout their career. As such, they are considered life-long trained athletes. No female strength trained master athletes could be identified in the age groups we aimed to recruit, despite our best efforts to find female master athletes engaged in strength sports by searching in data bases and results from past competitions at regional and national levels. Half of the endurance-trained master athletes reported performing some general strength exercises <1 session per week. This was typically performed as circuit training with a focus on core muscles. In contrast, the strength-trained master athletes reported mostly heavy strength training performed with few repetitions (typically 5 reps or less) using heavy exercise loads (>80 % of one repetition maximum; 1RM) in squat, deadlift, snatch, clean and jerk, and bench press. Strength-trained master athletes also reported some sporadic endurance-based activities, such as swimming, cycling, and going for walks. Recreationally active control participants, engaging in activities like e.g., hiking and skiing (OC, 75 ± 6 years, n = 13) were recruited from senior societies, and finally moderately active young references (YC, 25 ± 4 years, n = 11) were recruited among local University students. The activity level of recreationally active older and young was confirmed using the International Physical Activity Questionnaire (IPAQ). Exclusion criteria included: documented history of neurological, musculoskeletal, cardiovascular disease, and/or pulmonary disease. The study was approved by the Regional Ethics Committee (REC number: 2018/1207) and conducted in accordance with the Declaration of Helsinki. All subjects gave their written informed consent prior to inclusion in the study. Subject characteristics are summarized in Table 1.

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182 *Study outline*

183 Participants performed all physical testing procedures on a single day. Upon arrival in the lab,
184 subjects were weighed, and anthropometric measurements were obtained. Subjects proceeded
185 to physical testing, where familiarization and warm-up procedures were combined and
186 instructions on how to perform each individual test were provided. 1RM muscle strength and
187 RFD were measured in an instrumented leg press apparatus (44, 49) followed by
188 measurement of maximal oxygen uptake ($\dot{V}O_{2max}$) during walking or running on a treadmill.
189 Subjects were also asked to answer a questionnaire about current or previous steroid and
190 growth hormone usage. Biopsies were obtained from the vastus lateralis (VL) 10-14 days
191 after the physical tests.

192

193 *Anthropometry*

194 Thigh muscle volume was estimated using skinfold caliper recordings to the nearest 1 mm
195 (Baty International) using following equation, previously described, and validated against
196 magnetic resonance imaging (MRI) (18):

197 $\quad Vol_{anthropo} = (L/12\pi) \cdot (C1^2 + C2^2 + C3^2) - [(S - 0.4) / 2] \cdot L \cdot [(C1 + C2 + C3) / 3]$ $\quad$ (1)

198 where L is thigh length, S is skinfold thickness, and C is circumference. All measurements
199 were conducted three times at each site (C1, C2, C3), and the average value was used for
200 analysis. Thigh length was measured from the greater trochanter of the femoral head to the
201 lateral femoral epicondyle. Thigh circumference was measured at the mid-point of the thigh
202 length (C2) and 10 cm proximal (C1) and distal (C3) to the mid-point. Skinfold measurements
203 were performed at the mid-point laterally, medially, and anteriorly. Subsequently, muscle
204 volume was estimated using the following equation (18):

205 $\quad Thigh\ muscle\ volume\ (cm^3) = 0.866 \cdot Vol_{anthropo} - 1750$ $\qquad\qquad$ (2)

206

207 *Maximal muscle strength and rate of force development*

208 1RM strength of the leg extensors was measured using a horizontal leg press apparatus
209 instrumented with a force plate and movable weight (Technogym silver line, Gambettola,
210 Italy), as previously described (44). The subjects performed three warm-up sets of 8 down to

211   2 repetitions with increasing loads up to ~60 % of expected 1RM, prior to commencing the

212   1RM test. Instructions were given to the participants to lower the weight slowly (starting from

213   near 180° knee flexion), have a short pause (~1 second) at the bottom of the movement (at

214   ~90° knee flexion) before maximally contracting their lower limb muscles to move the load as

215   forcefully as possible back to its starting position. These instructions were given already

216   during warm-up and repeated for each attempt throughout the 1RM strength test. The load

217   was gradually increased by 5-20 kg if the participant was able to lift the load successfully, and

218   1RM typically was reached within 5 attempts. Each attempt was separated by ~3 minutes of

219   rest. The highest weight successfully lifted according to the instructions was recorded as 1RM

220   strength.

221   A piezo-electric force plate was used to record dynamic RFD in the same leg extensor

222   apparatus, where the orthogonal reaction force under the feet was digitally sampled at 2 kHz

223   (model 9286AA; Kistler, Winterthur, Switzerland). The test load was set to the closest 10 kg

224   level matching or above the subject's body weight in accordance with previous procedures

225   (49). Instructions during the RFD measurements were similar to those given during 1RM

226   testing, emphasizing a slow, controlled eccentric movement followed by a marked stop at the

227   bottom of the movement (~1 second), before initiating a concentric movement at maximal

228   intentional speed. The highest RFD out of three trials was selected for analysis. RFD ($\Delta F/\Delta t$)

229   was calculated as the rise in force (ΔF) divided by the duration of the time interval (Δt)

230   between 10 and 90 % peak concentric force (47).

231

232   *Maximal oxygen uptake*

233   Participants walked at 4.5 km·h^{-1} at 5 % inclination for 5 minutes to warm up before the

234   treadmill (Woodway, Wisconsin, USA) speed and/or inclination was gradually increased

235   every 3 minutes to reach $\dot{V}O_{2max}$ (54). Pulmonary gas exchange variables were obtained every

236   10 s (Metamax II, Cortex Biophysik GmbH, Leipzig, Germany). The highest 30-s average of

237    $\dot{V}O_2$ was reported as $\dot{V}O_{2max}$. $\dot{V}O_{2max}$ was accepted when no further increase in $\dot{V}O_2$ was

238   observed despite increased speed/inclination and a respiratory exchange ratio ≥ 1.05 was

239   present.

240

241 *Muscle biopsy sampling*

242 About 10-14 days following physical testing in all groups, muscle biopsies were obtained

243 from a depth of ~3.5 cm from the middle portion of the vastus lateralis, slightly distal to the

244 ventral midline of the muscle (2, 36). Participants were asked to refrain from alcohol and

245 strenuous exercise within 48 hours leading up to the biopsy. A 6-mm Bergström needle

246 attached to a suction syringe was used after injecting local xylocaine (1 %) anesthesia at the

247 superficial level of the muscle fascia. Following removal, the tissue samples were aligned

248 and mounted in Tissue-Tek (4583, Sakura Finetek, AV Alphen aan den Rijn, The

249 Netherlands) and subsequently frozen in isopentane pre-cooled in liquid nitrogen and stored

250 in a -80 °C freezer until further analyses. Before analysis, transverse serial sections (8 μm) of

251 the embedded muscle biopsy specimen were cut at -22 °C using a cryostat (HM560; Microm,

252 Walldorf, Germany) and were mounted on glass slides.

253

254 *Immunofluorescence staining procedure and analysis*

255 To visualize NCAM-positive fibers, cryosections were mounted in a 4 % formaldehyde

256 fixation solution (Triton X-100 (10%), formaldehyde (37%) and phosphate buffered saline

257 (PBS, x10)) for 10 minutes, whereas sections for visualization of fiber types (I, IIa and IIx)

258 and the myofiber perimeter was not. Subsequently, for all sections a wash procedure (3 x 3

259 minutes in PBS, x10) was completed prior to blocking (Vector SP6000) for 10 minutes. Next,

260 NCAM staining was performed using a two-step protocol consisting of anti-NCAM (DAKO,

261 1:100, M7304) and anti-Laminin (DAKO, 1:1000, Z0097) incubation followed by MHC-slow

262 (BA-F8, 1:50, Developmental Studies Hybridoma Bank (DSHB)) using the following

263 secondary antibodies (A21424, A21206and A21140, ThermoFisher). For visualization of

264 fiber types (I, IIa and IIx) and myofiber membrane perimeter, sections were stained anti-MHC

265 slow, IIa and IIx and anti-laminin (BA-F8 1:50, SC-71 1:250, 6H1 1:25; DSHB and Z0097

266 1:1000; DAKO), respectively, followed by a secondary antibody step (A21240, A21140,

267 AB_2535711 and A21206; ThermoFisher). All primary and secondary antibody steps were

268 incubated for 60 minutes at room temperature, separated by wash procedures. Lastly, all

269 stained sections were mounted with mounting medium (H5501; Vector). Immunofluorescence

270 images were captured using a microscope (Carl Zeiss Axio Imager M1, Germany) and a high-

271 resolution AxioCam (Carl Zeiss). All images were obtained using the x10 objective and

272 between image standardized exposures.

273   All analyses were performed using a digital analysis program (Carl Zeiss, AxioVision

274   4.6). Type I (blue), type IIa (red), and type IIx (green) myofibers were identified and analyzed

275   for fiber number and cross-sectional area. Myofiber distribution was expressed as proportion

276   of total fiber number and proportion of mean fiber area (area weighted fiber distribution). The

277   method of Jennekens, Tomlinson and Walton (15) was used to quantify fiber type grouping

278   of type I fibers in given cross-sections. An 'enclosed fiber' was defined as a type I muscle

279   fiber surrounded by only type I fibers. A 'fiber type group' was defined as a group of fibers

280   with at least one enclosed fiber (26). The number of enclosed fibers was counted manually,

281   along with number of enclosing fibers (a fiber that surrounded an enclosed fiber) and

282   remaining fibers (fibers that were neither enclosed or enclosing fibers, but contiguous with the

283   enclosing fibers and not separated by a visible fascicle (17); Figure 1). The number of fiber

284   type groups was expressed per 1000 fibers. The number of enclosed and enclosing fibers were

285   presented as a percentage of type I fibers, and the total number of grouped fibers (enclosed +

286   enclosing + remaining fibers) was presented as percentage of type I fibers and percentage of

287   whole fiber type count. Group size was presented as number of fibers per group. Atrophied

288   fibers were identified as the mean fiber area represented by the lowest 1st percentile in YC

289   following analysis of all fibers in this group, as previously used (41, 60), which corresponded

290   to fibers with a size $\leq$ 1494 μm^2. Previous findings suggest that fibers of this size are

291   atrophied, as >90 % expressed $Nav_{1.5}$, a marker of denervation (37). On average 867 $\pm$ 471

292   myofibers (mean $\pm$ SD) were analyzed per biopsy for the assessment fiber type distribution,

293   611 $\pm$ 348 for grouping, and 160 $\pm$ 111 for fiber area.

294   NCAM-positive myofibers were identified as fibers with a NCAM expression

295   exceeding 2 SD above the mean expression of two clearly negative fibers. NCAM-positive

296   fibers were reported per 1000 fibers. Moreover, the percentage of NCAM-positive fibers co-

297   expressing MHC I was calculated while the mean fiber area of NCAM-positive fibers was

298   determined and compared to the average area of type I and type II fibers identified in the

299   analyses of all fibers. Only subjects with NCAM-positive fibers were included in the latter

300   analyses. An average 667 $\pm$ 197 myofibers were analyzed per biopsy sample for NCAM.

301   All fiber type and grouping analyses were performed by the same assessor, who was

302   blinded to subject ID and group adherence.

303

304   *Histochemistry staining procedure and analysis*

305   Haematoxylin and Eosin (H&E) were used to stain myofiber cross-sections to assess nuclear

306   clumps. The presence of nuclear clumps (nuclear bags) were used as a marker of long-term

307   neurogenic atrophy (16, 41).

308   H&E images were captured using a microscope (Nikon TI Eclipse Widefield) and a

309   high-resolution camera (sCMOS, Oxford Instruments). All analysis was performed using

310   AxioVision. Nuclear clumps were counted by an assessor who was blinded in terms of

311   subject-ID and group. On average 335 ± 74 fibers were analyzed per biopsy sample.

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313   *Statistical analysis*

314   Normality (Gaussian distribution) was assessed using the Kolmogorov-Smirnov test and by

315   visual inspection of Q-Q-plots after removal of outliers. Outliers were identified by examining

316   box plots and histograms and removed if outside the 3rd quartile + 1.5*interquartile range and

317   1st quartile - 1.5*interquartile range (8). 1RM, RFD, $\dot{V}O_{2max}$, fiber type data, and NCAM-

318   positive fibers co-expressing MHC I were found to follow a normal distribution. As expected,

319   grouping variables in YC did not follow normal distribution, as there were many individuals

320   without grouping. However, since they were used as a young reference group relative to the

321   older subject groups (all of whom were normally distributed), a parametric test was used. As

322   such, a one-way ANOVA was used to detect between-group interactions, followed by

323   Fisher's LSD to investigate differences between groups. The number of nuclear clumps,

324   NCAM-positive fibers, and atrophic fibers did not follow normal distribution. Consequently,

325   the Kruskal-Wallis test was used to detect between-group differences, followed by Mann-U-

326   Whitney testing between groups (24). The area of NCAM-positive fibers was compared to the

327   average area of all fibers (type I and type II combined) using paired samples t-testing, as these

328   data adhered to a normal distribution. Significance level was $p \leq 0.05$ (2-tailed), and

329   borderline effects were presented when $p \leq 0.10$.

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RESULTS

None of the participants reported any previous or current intake of growth hormones, testosterone, or related anabolic substances. One outlier in YC was identified in all grouping variables except group size. Similarly, one outlier in OS was identified in all grouping variables except group size and number of grouped fibers (% type I fibers). Further, one outlier from OC was removed from the analysis of number of groups per 1000 fibers and one outlier from OE was removed from the group size analysis. Two outliers were removed from the analysis of NCAM-positive fibers in OC and one from OE, while four outliers were removed from the analysis of atrophied myofibers (one outlier in each group). Nuclear clumps content was not analyzed in one participant in OS as well as one participant in OE due to ice-crystal artefacts in the biopsy material.

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Maximal strength and rate of force development

Leg press 1RM strength differed between subject groups (main effect $p < 0.001$, Figure 2A), where OS demonstrated higher 1RM than OC and OE (both $p < 0.001$) while tending to be higher than YC ($p = 0.080$). OE tended to have higher 1RM strength than OC ($p = 0.083$), while YC showed higher 1RM than OC ($p < 0.001$) and tended to show higher 1RM than OE ($p = 0.064$).

A main effect for RFD also was observed ($p < 0.001$, Figure 2B), revealing OS to have higher leg press RFD than OC ($p < 0.001$) and tended to have higher RFD than OE ($p = 0.072$). There was no difference detected between YC and OS ($p = 0.329$). OE had a higher RFD than OC ($p = 0.049$). Finally, there was no difference between YC and OE ($p = 0.334$), whereas OC demonstrated lower RFD than YC ($p = 0.002$).

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Maximal oxygen uptake

Both relative (expresed relative to body mass) and absolute $\dot{V}O_{2max}$ differed between subject groups (main effect $p < 0.001$; Table 1). Specifically, OE showed higher relative $\dot{V}O_{2max}$ than OS and OC (both $p < 0.001$). Likewise, YC demonstrated higher $\dot{V}O_{2max}$ than all older groups

366 (all p < 0.001), whereas there was no difference between OS and OC (p = 0.469). YC also had

367 higher absolute $\dot{V}O_{2max}$ than all older groups (p < 0.001), whereas there was no difference in

368 absolute $\dot{V}O_{2max}$ between any of the older groups (p = 0.135-0.664).

369

370 *Muscle fiber distribution*

371 Type II fiber distribution (% number of fibers) was higher in OS (52.0 ± 16.4 %; main effect

372 p = 0.012) compared to OC (35.0 ± 12.4%; p = 0.006) while tending to be higher than OE

373 (39.3 ± 11.9 %; p = 0.063). No difference in type II fiber distribution could be detected

374 between OS and YC (51.1 ± 14.4 %; p = 0.895). YC had higher type II fiber distribution than

375 OC (p = 0.007) and borderline higher than OE (p = 0.075). Type I fiber distribution (%

376 number of fibers) showed inverse trends compared to type II fibers (OS: 48.1 ± 16.4 %, OE:

377 60.7 ± 11.9 %, OC: 65.0 ± 12.4 %, YC: 48.9 ± 14.4 %).

378   Type IIa fiber distribution (% number of fibers) was higher in OS (41.4 ± 9.2 %; main

379 effect p = 0.008) compared to OC (29.7 ± 11.0 %; p = 0.006) and borderline higher than OE

380 (33.5 ± 6.5 %; p = 0.088). No difference could be detected between OS and YC (41.9 ± 9.8

381 %; p = 0.918). YC showed higher type IIa distribution (% number of fibers) than OC (p =

382 0.004) while tending to be higher than OE (p = 0.067). There was no main effect for the

383 distribution of type IIx fibers across subject groups (OS: 10.5 ± 12.9 %, OE: 5.9 ± 5.8 %, OC:

384 5.3 ± 7.1 %, YC: 9.3 ± 8.9 %; p = 0.480).

385   Area weighted type II fiber distribution (% fiber area; main effect p = 0.010; Figure 3)

386 was higher in OS compared to OE (p = 0.016) and OC (p = 0.006). There was no difference

387 detected between OS and YC (p = 0.652). YC had higher area weighted type II fiber

388 distribution than OC (p = 0.017) and OE (p = 0.039).

389   There was no main effect for mean cross-sectional area of type I fibers (p = 0.590),

390 type II fibers (p = 0.140), type IIa fibers (p = 0.157), or type IIx fibers (p = 0.250; Table 2).

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Muscle fiber grouping

The number of enclosed fibers (% of type I fibers; main effect p = 0.004; Figure 4A) was lower in OS than OC (p = 0.005), but there was no difference between OS and OE (p = 0.265). No difference was detected between OS and YC (p = 0.595), OE and OC (p = 0.103) or OE and YC (p = 0.102), whereas OC had more enclosed fibers than YC (p < 0.001). Figure 5 displays representative images of fiber type distribution and myofiber grouping in each group.

There were fewer enclosing fibers (% of type I fibers; main effect p < 0.001) in OS (7.9 ± 5.2 %) than OC (19.7 ± 11.5 %; p = 0.004) and OE (17.2 ± 11.5 %; p = 0.036), whereas no difference was noted between OE and OC (p = 0.526). No differences were observed between OS and YC (2.1 ± 2.8 %; p = 0.168), whereas OE and OC both showed more enclosing fibers than YC (p < 0.001).

Likewise, the number of grouped fibers (% of type I fibers; main effect p < 0.001; Figure 4B) was lower in OS than OC (p = 0.008) and OE (p = 0.039), whereas there was no difference between OE and OC (p = 0.703). There was a tendency for a difference was detected between OS and YC (p = 0.057), while OE and OC showed more grouped fibers than YC (p < 0.001).

Fewer grouped fibers (% of total fiber type count; main effect p < 0.001) were observed in OS (12.3 ± 9.3%) than OC (35.9 ± 23.4 %; p = 0.004) and OE (31.4 ± 21.9 %; p = 0.031), whereas there was no difference between OE and OC (p = 0.566). Likewise. no difference was detected between OS and YC (4.5 ± 6.3 %; p = 0.335), whereas OE (p = 0.003) and OC (p < 0.001) had more grouped fibers than YC.

Group size (main effect p = 0.005; Figure 4C) was smaller in OS compared to OC (p = 0.023) but not OE (p = 0.240). No difference was detected between OC and OE (p = 0.440) or between YC and OS (p = 0.248), whereas OE (p = 0.032) and OC (p < 0.001) had larger group size than YC.

424   Number of fiber type groups per 1000 fibers (main effect $p < 0.001$) was lower in OS

425   (5.6 ± 3.8) compared to OC (10.1 ± 5.2; $p = 0.033$) while tending to be lower than OE ($9.7 \pm$

426   6.0; $p = 0.073$), but not different than YC (2.1 ± 2.7; $p = 0.107$). OC and OE had higher

427   number of fiber groups than YC ($p < 0.001$) with no differences between OC and OE ($p =$

428   0.865).

429

430   *Atrophic fibers*

431   Atrophic fibers (fiber cross-sectional area < 1494 μm^2) were detected in a total of 19 subjects

432   (two in OS, two in YC, seven in OE and eight in OC). There was a main effect ($p = 0.006$),

433   revealing that OC ($p = 0.034$) and OE ($p = 0.009$) had higher percentage atrophic fibers than

434   OS (Figure 4D). A higher abundance of atrophic fibers also was observed in OC ($p = 0.024$)

435   and OE ($p = 0.005$) compared to YC. In contrast, atrophic fiber content did not differ between

436   OS and YC ($p = 0.878$) or OC and OE ($p = 0.606$).

437

438   *Denervation markers*

439   Nuclear clumps were detected in a total of eight subjects, three in OS and five in OC. A main

440   effect was evident for nuclear clumps ($p = 0.046$; Table 2), where OS ($p = 0.044$) and OC ($p =$

441   0.025) showed higher content than YC, whereas there was no difference between YC and OE

442   ($p = 1.000$). Also, there was no difference between OS and OC ($p = 0.727$), OS and OE ($p =$

443   0.103), however a tendency emerged for higher count of nuclear clumps in OC than OE ($p =$

444   0.068).

445   NCAM-positive fibers were detected in a total of 27 subjects: six in YC and OS, four

446   in OE, and 11 in OC. There was no main effect across subject groups in the number of

447   NCAM-positive fibers ($p = 0.097$; Table 2).

448   A higher percentage of NCAM-positive fibers co-expressing MHC I (main effect $p =$

449   0.038) was observed in OC (Figure 6C) compared to OS ($p = 0.047$), OE ($p = 0.039$), and YC

450   ($p = 0.015$). No difference was detected between OS, OE and YC ($p = 0.646$-0.927).

451   NCAM-positive fibers were smaller than the mean fiber size in OC (3340 ± 1923 vs.

452   4655 ± 1464 μm; $p = 0.020$). No difference was detected in OE (2888 ± 1449 vs. 4147 ± 247

453 µm; p = 0.216), OS (3561 ± 2665 vs. 4542 ± 848 µm; p = 0.417), or YC (2699 ± 1792 vs.
454 4218 ± 1068 µm; p = 0.132).

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462 **DISCUSSION**

463 The main findings of the present study were that strength-trained master athletes had a type II

464 fibre distribution similar to young individuals, and a higher proportion of type II fibers

465 compared to endurance-trained master athletes and recreationally active older adults.

466 Moreover, the strength-trained master athletes also displayed fewer signs of myocellular

467 denervation, reflected by less prevalent myofiber grouping and fewer atrophic fibers

468 compared to endurance-trained and recreationally active older adults, and again not being

469 different from young. The present data imply that chronic strength training is an effective

470 training modality for the maintenance of neuromuscular properties related to the recruitment

471 of the fastest and largest motor units, in turn preserving type II fibers integrity, maximal

472 muscle strength and rapid force capacity at increasing age.

473

474 *Training modality and fast twitch type II fiber properties*

475 Fiber type distribution and grouping can be seen as the converging consequence of successive

476 fiber denervation with or without subsequent reinnervation (14). A higher area-weighted

477 proportion of type II fibers was observed in strength-trained master athletes compared to

478 endurance-trained master athletes and recreationally active old. This appears to be explained

479 by a combination of higher proportion of type IIa fibers and larger type II fiber area. Although

480 evidence from studies investigating the effects of life-long strength- compared to endurance-

481 training on type II fiber maintenance is scarce, our results are in accordance with previous

482 results (58). Strikingly, type II fiber proportions and area-weighted distributions were highly

483 similar between life-long strength trained older adults (~75 years) and the young reference

484 population (~25 years) recruited for the present study, both expressing a ~50% type II fiber

485 distribution typically expected in young (23).

486

Training modality and fiber type grouping

Strength-trained master athletes were not different from young adults for any variable used to assess fiber type grouping. In contrast, older life-long endurance-trained master athletes and recreationally active older adults showed signs of type I fiber grouping, including larger fiber grouping size, higher fraction of grouped fibers, higher number of fiber type groups, and more atrophic fibers than young adults. Previous studies have failed to observe any differences in fiber type grouping between master athletes and older controls, which may be related to these studies collapsing endurance- and strength-trained master athletes into one group (26, 41). Thus, to our best knowledge, this is the first study to compare fiber type grouping in endurance- and strength-trained master athletes, which appears to be an important distinction. Different approaches to quantify fiber type grouping may also cause inconsistent results, as grouping may be overestimated even in young populations (26) where signs of fiber type grouping would not be expected (17, 21). Moreover, studies applying indirect neurophysiological estimates of motor unit numbers and size, an indicator of fiber reinnervation have left inconclusive evidence. Some observations suggest pronounced motor unit remodeling (increased motor unit size and/or low number of motor units) may take place in both strength- and endurance-trained master athletes compared to young (29, 30), while other studies have shown no motor unit remodeling in endurance-trained master athletes compared to recreationally active old (33, 34), suggesting a neuroprotective effect from endurance training. The current fiber grouping data derived from muscle biopsy analysis strengthen the notion that habitual recreational activity as well as high (life-long) endurance training volumes may offer similar benefits for fiber reinnervation with aging, but that they are of a different magnitude than strength training.

One likely explanation for the present signs of type I area-weighted dominance and fiber type grouping in endurance-trained master athletes and recreationally active older adults may be that the type II fibers are rarely used during repetitive contractions with low force requirement (13), which is typically the case in endurance exercise settings. When large, high-threshold motor units, critical for forceful and fast muscle contractions, become more inactive with age, their motor neurons may degrade. The resultant denervated myofibers, typically type II, may either be reinnervated by axon sprouting from adjacent motor neurons leading to myofiber type changes and myofiber grouping, or slowly waste away resulting in neurogenic atrophy, ultimately resulting in a loss of myofibers (14, 56). Although the low (absent) prevalence of

520 fiber grouping in strength-trained master athletes could also potentially reflect denervation of
521 fibers without subsequent reinnervation, this does not seem a plausible scenario due to these
522 master athletes' superior maximal strength, lack of fiber atrophy, high proportion of type II
523 fibers, and enlarged thigh muscle volume, as also previously documented (45). Thus,
524 although, we cannot exclude that the strength-trained master athletes had an inherent
525 advantage compared to their aged-matched counterparts, our data are in line with observations
526 in older adults performing high-load strength training, where increased type II muscle fiber
527 area and distribution is observed (28, 52, 53). Interestingly, the differences observed between
528 life-long strength-trained master athletes vs. endurance-trained master athletes and untrained
529 older adults in muscle morphology and fiber type grouping appear to perfectly mirror
530 previous experimental observations on the neural side of the spectrum; namely that strength-
531 trained master athletes demonstrate increased descending motor drive to maximally
532 contracting skeletal muscle compared to endurance-trained, recreationally active and
533 sedentary older adults (45, 49). Moreover, increased descending neural drive following short-
534 term strength training has been documented in older adults (46, 48, 50). Collectively, these
535 data suggest that strength training preserve neuromuscular activation to recruit the largest
536 motor units innervating type II fibers, potentially due to performing recurrent high-force
537 explosive-type motor activities dictated by their training protocols. This could be a key factor
538 in maintaining the integrity of large-sized motor axons and for retaining type II fiber
539 morphology at increasing age.

540

541 The present data may suggest that neither habitual physical activity nor high levels of long-
542 term endurance training may be sufficient to maintain type II fiber properties and innervation
543 with aging. Hence, despite higher volume of physical activity in the endurance trained master
544 athletes no differences in muscle fiber composition, grouping, or atrophic fibers were detected
545 compared with recreationally active older adults, suggesting that the specific training
546 modality and its pattern of muscle loading are of greater importance than training volume *per
547 se* for type II fiber maintenance. However, it should be noted that the fiber type distribution
548 patterns observed in recreationally active- and endurance trained older adults could also
549 reflect a training adaptation facilitating the high oxidative capacity associated with type I
550 fibers. Yet, such oxidative myocellular characteristics would be expected to be more
551 pronounced in the endurance-trained master athletes, which does not seem to be the case.
552 Consequently, these patterns may instead reflect an aged phenotype.

553

554 *Training modality and markers of denervation and reinnervation*

555 In line with the present fiber distribution and grouping data, life-long strength-trained master

556 athletes demonstrated a lower density of atrophic fibers compared to endurance-trained master

557 athletes and recreationally active old, with no difference compared to young controls. An

558 abundance of atrophic fibers has previously been interpreted as poor reinnervation status as a

559 consequence of progressive denervation (37, 60). Alternatively, an abundance of atrophic

560 fibers could also reflect a lack of maximal voluntary motor unit recruitment, which similarly

561 to denervation would result in a negative net protein balance in the affected fibers that would

562 over time result in myofiber atrophy.

563

564 Somewhat surprisingly, a higher accumulation of muscle fibers with nuclear clumps was

565 observed in strength-trained and recreationally active older compared to young controls in the

566 present study, suggesting the presence of more fibers with severe neurogenic atrophy in these

567 aging groups (16). These data may suggest some level of complete myofiber denervation in

568 strength-trained athletes and recreationally active aged, which may in turn suggest superior

569 reinnervation in the endurance-trained. However, fibers with nuclear clumps were detected

570 only in few individuals (8/42), and in a very low number of affected fibers (mean: 2-4 ‰)

571 making it difficult to draw any firm conclusion about the functional relevance of these

572 findings. It should be noted that previous studies have reported fibers with nuclear clumps to

573 be observed only in frail older adults (41), and of higher magnitude (~2 %) than observed in

574 the present study, suggesting that the groups examined in the present study were

575 representative of healthy, active aging without severe atrophy.

576

577 NCAM-positive fibers did not differ between age groups or training modes in the present

578 study. Elevated numbers of NCAM-positive fibers have previously been reported in older

579 adults compared to young, even in life-long trained athletes (39, 40). Similar to the present

580 data Sonjak, Jacob, Morais, Rivera-Zengotita, Spendiff, Spake, Taivassalo, Chevalier and

581 Hepple (41), did not observe differences between frail older adults, age-matched master

582 athletes, and young controls. Notably, NCAM has been shown to be present during both

583 denervation and reinnervation (38) and seem not to be constantly expressed in denervated

584 muscle (12), which in combination with the inclusion of healthy individuals may explain the

585 low proportion of NCAM-positive fibers (1.0-7.4 ‰) and thus lack of age-related differences

586 in NCAM-positive fibers. Furthermore, NCAM-positive fibers may also be observed in

587 conditions not related to fiber denervation/reinnervation, such as exercise-induced

588 regeneration and myopathy (3, 55), which challenges the use of NCAM as a 1:1 marker of
589 denervation.

590

591 As all markers are not continuously expressed during muscle fiber denervation, we used a
592 combination of markers of myofiber grouping to indicate long-term denervation with
593 subsequent reinnervation (26, 27), NCAM positive fibers to indicate acute denervation (39,
594 40), and pyknotic nuclei bags and atrophic fibers (very small fibers) to indicate long-term
595 denervation without subsequent reinnervation (16, 37, 41, 60). These markers were chosen to
596 represent the denervation status in a population that was regarded as healthy, where
597 denervation had likely not progressed to a very high level. A combination of different markers
598 such as that used in the present study has also previously been utilized (41).

599

600

601 *Influence of training modality on muscle strength and rate of force development*
602 From a functional perspective, it is of interest that the strength-trained master athletes
603 demonstrated ~30-294 % higher maximal strength and RFD than their age-matched
604 counterparts. This observation is in line with previous reports from our labs comparing life-
605 long strength-trained master athletes to life-long endurance-trained master athletes and
606 untrained/recreationally active older adults (45, 49, 58). The present endurance trained master
607 athletes also demonstrated higher levels of maximal lower limb muscle strength and RFD
608 compared to recreationally active old, suggesting a degree of preserved neuromuscular
609 function in the endurance trained group. The ability to produce force rapidly, i.e., as much
610 force as possible in a short time frame, is particularly important in e.g. fall prevention, given
611 that during tripping or loss of postural balance there is only limited time to regain balance
612 (32). As it typically takes more than 300 ms to reach maximum muscle force (43, 57), RFD is
613 considered to be more functionally relevant than maximal muscle strength (25), especially at
614 older age (35). The higher RFD demonstrated in strength-trained master athletes may be
615 related, at least in part, to the previously documented higher descending drive (45) and higher
616 proportion of type II fibers, both of which have been associated to greater RFD (4, 7, 10, 11,
617 25). Although, the relationship between higher intrinsic single fibre RFD and joint-level RFD
618 has not been firmly established (7). Moreover, other factors not examined in the presented
619 study also influence RFD, such as tendon stiffness and moment arm/lever length (25).

620

621 *Methodological considerations*

622   Although the present data suggest a protective effect of life-long strength training in
623   preserving neuromuscular function related to strong and rapid muscle contractions, it is not
624   possible to exclude a role of potential confounding factors including genetics, nutritional
625   intake, and exposure/absence to various stressors. Moreover, the cross-sectional study design
626   also impedes firm conclusions regarding the evolution of fibre type composition. Further,
627   fiber grouping in the present study was defined as phenotype-specific groups of fibers with
628   one or more enclosed fibers. Of notice, the likelihood of observing enclosed fibers is higher if
629   the individual has a dominance of one fiber type over the other. To account for this, we
630   presented these variables expressed both as a percentage of total fiber count as well as
631   percentage of type I fibers. The low number of participants in each group challenges
632   definitive conclusions and the present findings should therefore be interpreted with caution,
633   although the relatively small groups are explained by the low prevalence of master athletes in
634   the general population. It should also be noted that these results may not extend to other forms
635   of endurance training, such as cycling, rowing, and skiing. Finally, while we would also
636   preferably have included females in the present study, this was not possible as we were unable
637   to identify female strength master athletes in the local area, thus limiting the participant cohort
638   to males.

639

640   *Conclusions*

641 Life-long strength-trained master athletes demonstrate neuromuscular properties related to
642   fast-twitch type II fiber force production that are of comparable magnitude to young
643   recreationally active individuals. Similarly, life-long endurance-trained master athletes and
644   recreationally active old demonstrate similar neuromuscular properties, with lower proportion
645   of type II fibers and more markers of grouping. Consequently, strength training appears to
646   counteract age-related denervation processes and concurrent atrophy of type II fibers in older
647   men to thereby promote maintenance of maximal strength and RFD, which is critically
648   important for retaining functional capacity with increasing age.

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FIGURE LEGENDS

Figure 1 showing a schematic visualization of fiber type grouping-variables. Deep red represents type I fibers, and light red represents type II fibers. X indicates enclosed type I fibers, · indicates enclosing type I fibers, * indicates remaining (i.e., contiguous with the enclosing fibers, not separated by a fascicle) type I fibers. All enclosed, enclosing, and remaining fibers were counted as one group. The number of fibers type groups was expressed per 1000 fibers. The number of enclosed and enclosing fibers were presented as a percentage of type I fibers. The total number of grouped fibers (total number of enclosed + enclosing + remaining fibers for each subject) was presented as percentage of type I fibers and percentage of whole fiber type count. Group size was presented as number of fibers per group. Created in Biorender.com.

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Figure 2 One repetition maximum (A) and dynamic leg press rate of force development (B) in young recreationally active (n = 11), older strength athletes (n = 10), older endurance athletes (n = 8) and older recreationally active control participants (n = 13). Data are presented as mean ± SE and individual responses. a = different from older endurance athletes and older control participants, b = different from older control participants. One, two and

687 three symbols indicate significance level of $p \leq 0.05$, 0.01, and 0.001, respectively. The
688 statistical test used was one-way ANOVA.

689

690 **Figure 3** Area weighted fiber distribution (% of mean fiber area) of type II (A) and type I
691 fibers (B) in young recreationally active (n = 11), older strength athletes (n = 10), older
692 endurance athletes (n = 8) and older recreationally active control participants (n = 13). Data
693 are presented as mean ± SE and individual responses. MHC; myosin heavy chain. a =
694 different from older endurance athletes and older control participants. One symbol indicates a
695 significant level of $p \leq 0.05$. The statistical test used was one-way ANOVA.

696

697 **Figure 4** Enclosed fibers (percentage of type I fibers; A), grouped fibers (percentage of type I
698 fibers; B), group size (number of fibers per group; C), and percentage of atrophic fibers
699 (fibers < 1494 μm^2; D) in young recreationally active (n = 10 in panel A, B, D; n = 11 in
700 panel C), older strength athletes (n = 9 in panel A and D; n = 10 in panel B and C), older
701 endurance athletes (n = 7 in panel C and D; n = 8 in panel A and B) and older recreationally
702 active control participants (n = 12 in panel D; n = 13 in panel A-C). Data are presented as
703 mean ± SE and individual responses. a = different from older endurance athletes and older
704 control participants; b = different from older control participants. One, two and three symbols
705 indicate significance level of $p \leq 0.05$, 0.01, and 0.001, respectively. One-way ANOVA was
706 used in panels A-C and Kruskal-Wallis followed by Mann-U-Whitney test in panel D.

707

708 **Figure 5** Representative images of fiber type distribution and type I grouping in young
709 recreationally active, older strength athletes, older endurance athletes, and older recreationally
710 active control participants. Blue color represents myosin heavy chain type I, red color
711 represents myosin heavy chain type II and orange represents laminin.

712

713 **Figure 6** A representative image of nuclear clumps (indicated by arrow) from hematoxylin
714 and eosin-stain from older recreationally active participant (A), a representative image of
715 neural cell adhesion molecules (NCAM)-positive fiber (indicated by arrow) from one older
716 recreationally active participant (B), and percentage of NCAM-positive fibers co-expressing
717 myosin heavy chain (MHC) type I (C) in young control (n = 6), older strength athletes (n = 6),

718   older endurance athletes (n = 4) and older control participants (n = 11). Blue colour represents
719   myosin heavy chain type I, green represents laminin and orange represents NCAM-positive. b
720   = different from older control participants. One symbol indicates significance level of p ≤
721   0.05. One-way ANOVA was used.

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922 **DATA AVAILABILITY STATEMENT**

923 Data is available upon reasonable request to the corresponding author.

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925 **COMPETING INTEREST**

926 The authors declare no conflict of interest.

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The impact of life-long strength versus endurance training on muscle fiber morphology and phenotype composition in older men

METHODS

	Master athletes		Control	
	Strength	Endurance	Old	Young
1RM (kg)	170±19	131±26	113±23	152±24
VO_{2max} (ml·kg^{-1}·min^{-1})	33±7	48±8	35±4	63±7
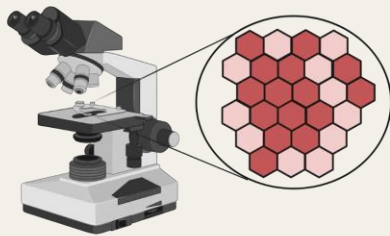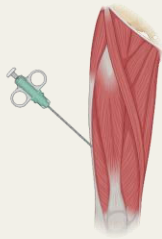
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OUTCOME

Grouped fibers (% of type I fibers)

Young — aaa
Strength — a
Endurance
Control

Old
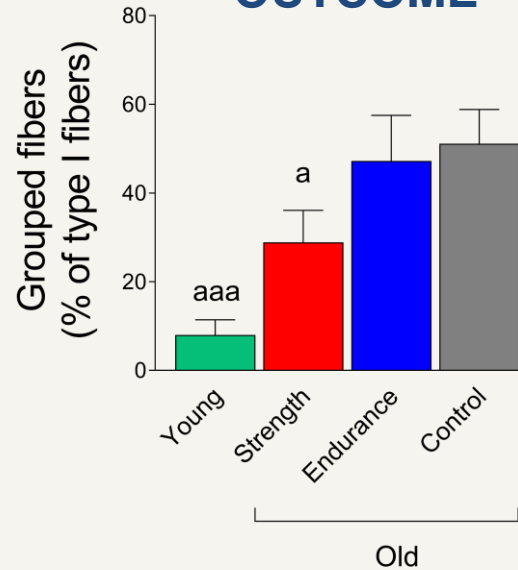
a = compared to endurance trained and old control
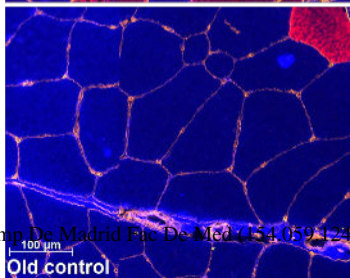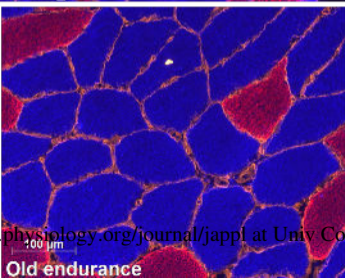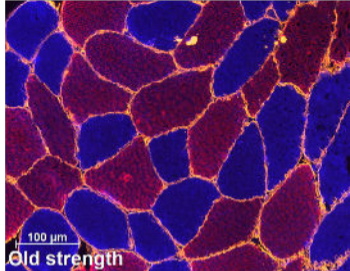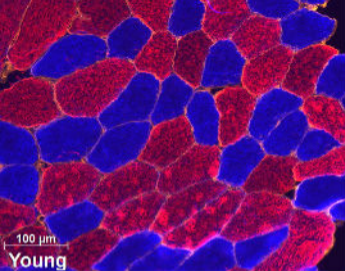
Young
Old strength
Old endurance
Old control

100 µm
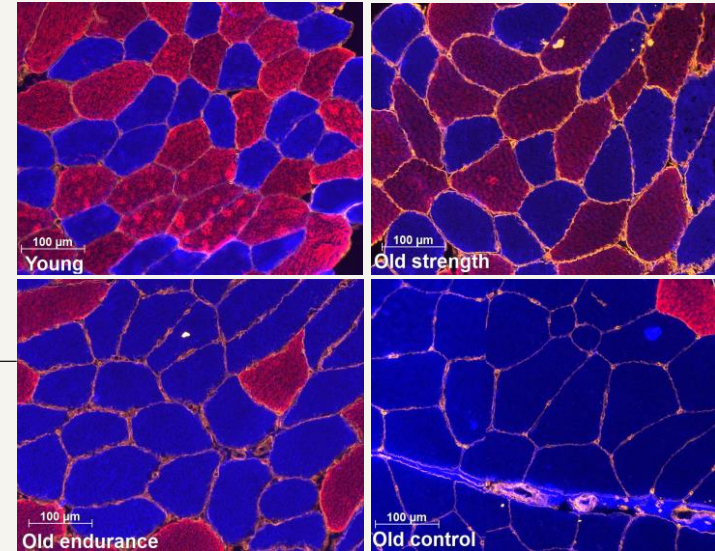
Blue represents type I fibers, red fibers represents type II fibers

CONCLUSION Strength trained master athletes have similar myofiber distribution as young adults, and less signs of neuromuscular denervation compared to endurance trained master athletes and recreationally active older adults.
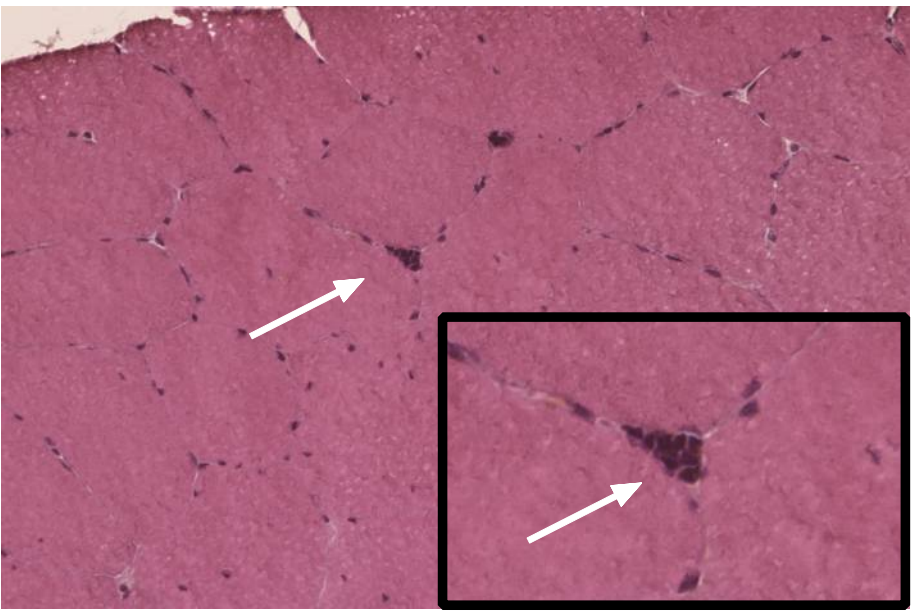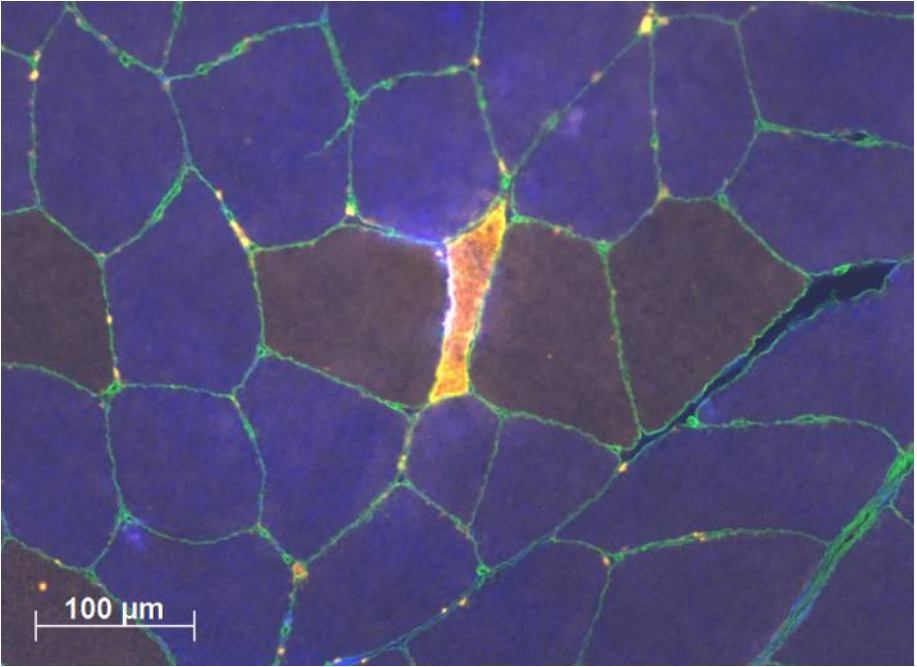
A

B

Young | Strength | Endurance | Control

Old

A

B

Old

Young

Old strength

Old endurance

Old control

100 µm
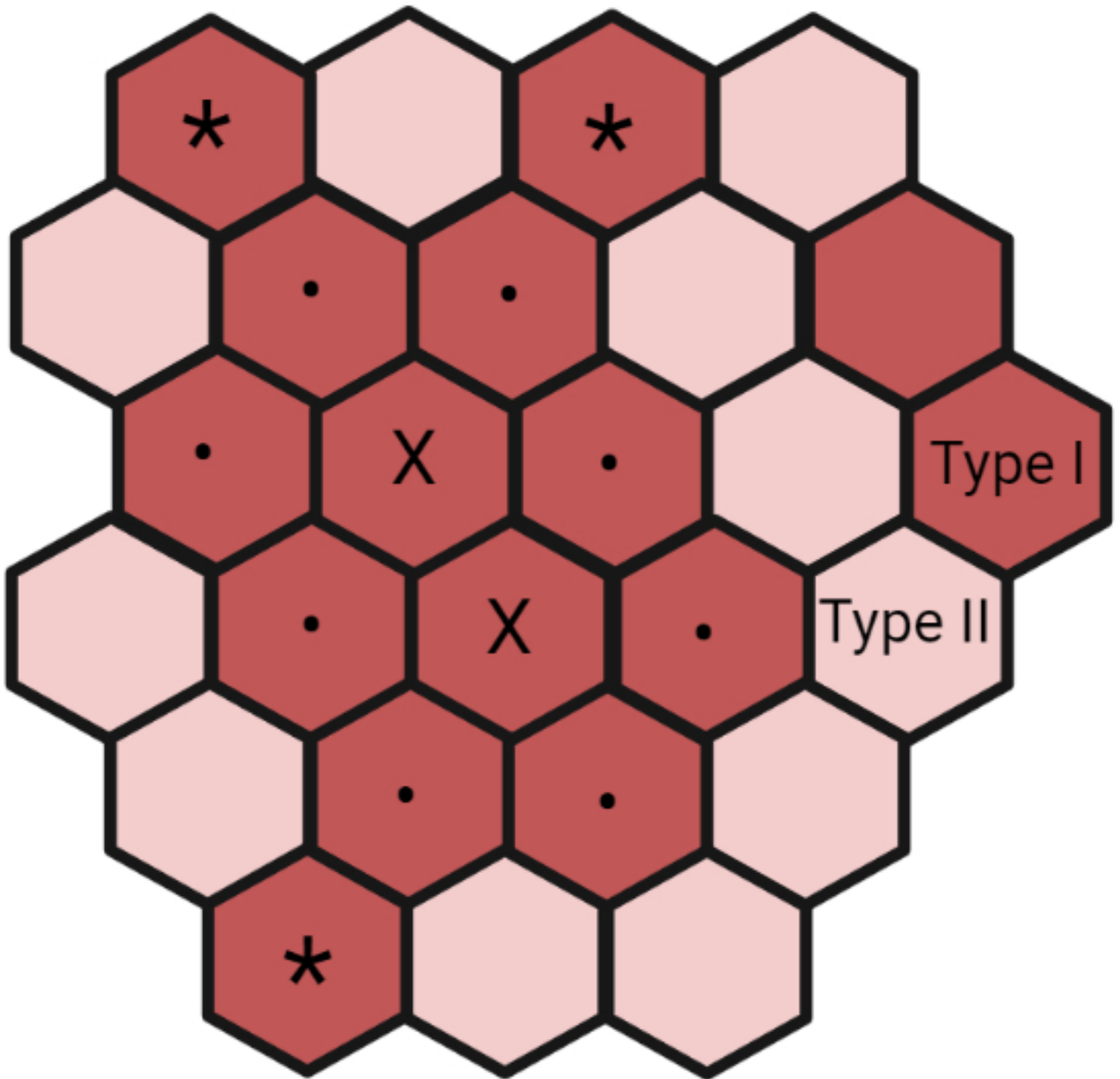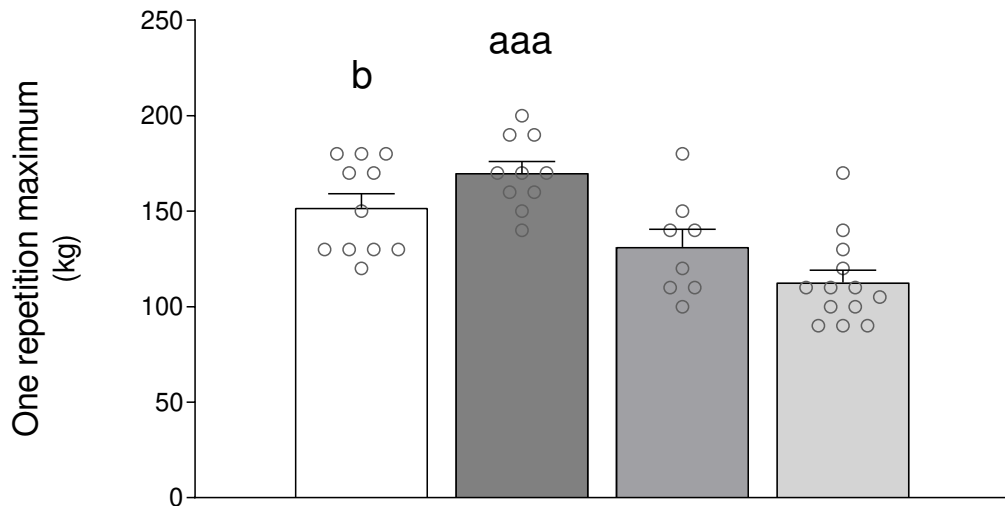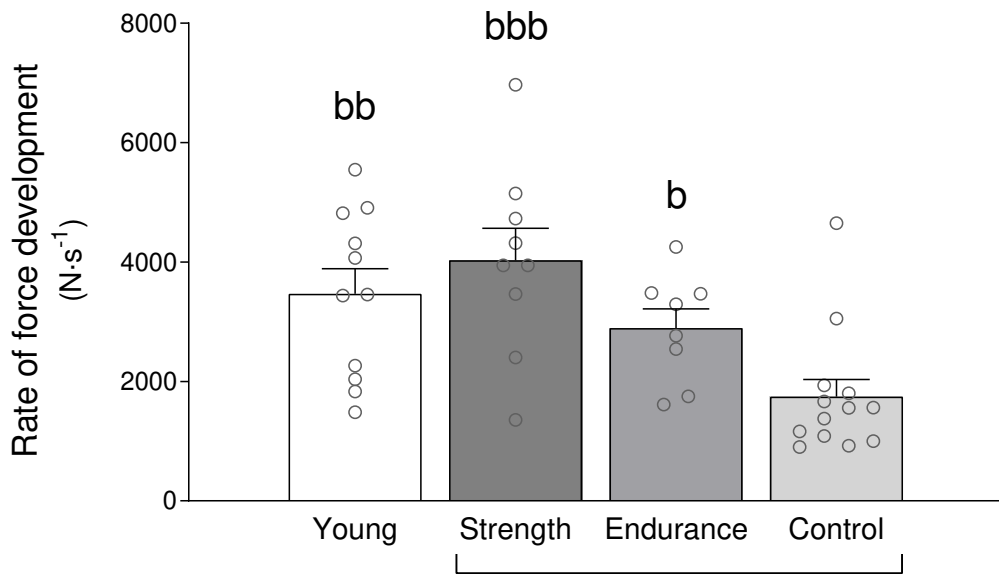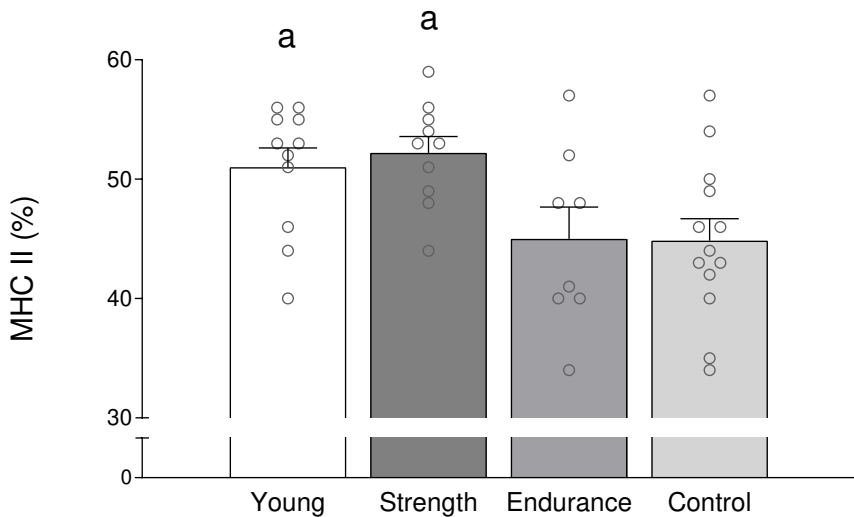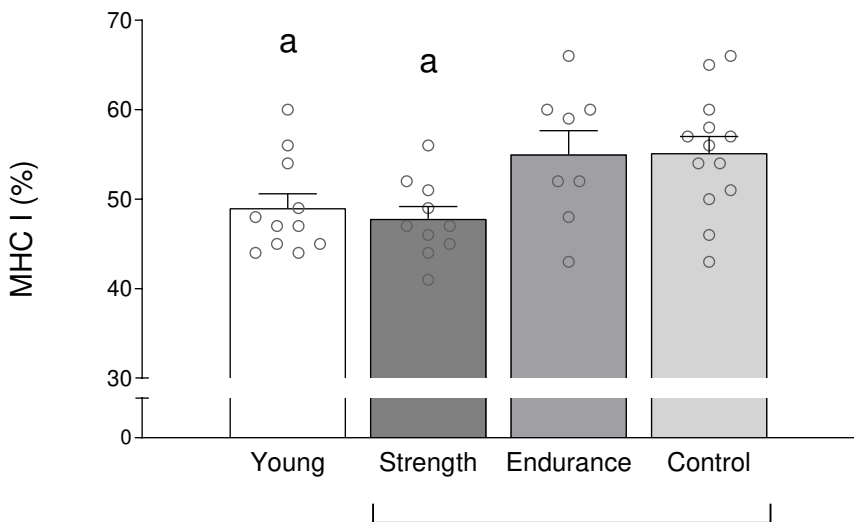
A

B

C

Table 1. Subject characteristics

		Old		
	Young (n = 11)	Strength (n = 10)	Endurance (n = 8)	Control (n = 13)
Body mass, kg	76.0 ± 8.9^{e}	85.9 ± 12.1	69.0 ± 8.1^{d}	83.5 ± 9.6
Body height, cm	180 ± 6	174 ± 6	175 ± 8	179 ± 7
Thigh muscle volume, cm^3	5259 ± 950	5199 ± 1418	4657 ± 860	4863 ± 942
$\dot{V}O_{2max}$, $mL \cdot kg^{-1} \cdot min^{-1}$	63.1 ± 6.6^{ccc}	33.2 ± 6.8	47.5 ± 8.1^{ddd}	35.2 ± 3.8
$\dot{V}O_{2max}$, $L \cdot min^{-1}$	4.79 ± 0.65^{ccc}	2.84 ± 0.61	3.24 ± 0.48	2.94 ± 0.46

Group mean $\pm$ SD. $\dot{V}O_{2max}$; maximal oxygen uptake. [c] = different from all older adults, [d] = different from older strength athletes and older controls, [e] = different from older strength athletes. One and three symbols indicate significance level of $p \leq 0.05$ and 0.001, respectively.

Table 2. Mean cross-sectional area of type I, II, IIa and IIx myofibers from vastus lateralis, and number of nuclear clumps and neural cell adhesion molecule (NCAM)-positive fibers per 1000 fibers.

		Old		
	Young	Strength	Endurance	Control
Type I, μm^2	4439 ± 1135	4932 ±1019	5059 ± 848	5039 ± 1478
Type II, μm^2	4644 ± 1204	5353 ± 1233	4176 ± 1148	4146 ± 1485
Type IIa, μm^2	5162 ± 1335	5602 ± 1289	4633 ± 1253	4427 ± 1257
Type Iix, μm^2	4127 ± 1127	5159 ± 1478	3853 ± 1197	3837 ± 1934
Nuclear clumps, per 1000 fibers	0.0 ± 0.0[d]	1.9 ± 3.7	0.0 ± 0.0	3.6 ± 6.3
NCAM-positive, per 1000 fibers	2.2 ± 2.3	7.3 ± 9.8	1.0 ± 1.4	7.4 ± 8.5

Group mean ± SD. d = different from older strength athletes and older control subjects; $p < 0.05$.