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Lack of muscle fibre hypertrophy, myonuclear addition, and satellite cell pool expansion with resistance training in 83-94-year-old men and women

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Abstract

Aims: To examine satellite cell and myonuclear content in very old (≥83 years) individuals, and the response to heavy resistance training.

Methods: A group of very old men and women (Old, 83-94 years, n = 29) was randomized to 12 weeks of heavy resistance training or untrained controls. A group of young men who did not resistance train (Young, 19-27 years, n = 9) were included for comparison.

Results: Compared to young men, prior to training the old men had smaller type II fibres (−38%, $P < 0.001$), lower satellite cell content (−52%, $P < 0.001$), smaller myonuclear domain (−30%, $P < 0.001$), and a trend for lower myonuclear content (−13%, $P = 0.09$). Old women were significantly different from old men for these parameters, except for satellite cell content. Resistance training had no effect on these parameters in these old men and women. Fibre-size specific analysis showed strong correlations between fibre size and myonuclei per fibre and between fibre size and myonuclear domain for both fibre types ($r = 0.94$-0.99, $P < 0.0001$). In contrast, muscle fibre perimeter per myonucleus seemed to be constant across the range in fibre size, particularly in type I fibres ($r = -0.31$, $P = 0.17$).

Conclusions: The present data demonstrate that type II fibre size, satellite cell content and myonuclear domain is significantly smaller in very old men compared to young men, while myonuclear content is less affected. These parameters were not improved with heavy resistance training at the most advanced stage of ageing.

KEYWORDS
ageing, heavy resistance training, hypertrophy, myonuclear domain, myonuclei, satellite cells

1 | INTRODUCTION

Ageing is associated with a gradual loss of muscle mass and strength, also termed sarcopenia, which is related to an increased risk of functional impairment, loss of independency and a reduced quality of life.[1-4] The definition of the "oldest-old" or "very old" individuals varies in the literature, in the present study we refer to the United Nations definition (≥80 years).[5] Heavy resistance training has proven effective to increase muscle mass and strength in elderly individuals,[6] but the effect may be blunted with increasing age.[7] The balance between protein synthesis and proteolysis ultimately determines muscle fibre size, with myonuclei playing a key role

in protein synthesis.[8] Each myonucleus in the multinucleated skeletal muscle fibre support a finite volume of the fibre, the myonuclear domain,[9] defined as muscle fibre cross-sectional area per myonucleus in 2-dimensional (2D) muscle biopsy cross-sections. Myonuclei are post mitotic and therefore addition of new myonuclei relies on the myogenic precursor cells, the satellite cells (SC).[10] A lower number of SCs per fibre with ageing have been reported specifically in the type II fibres from the 7th[11,12] and 8th[13-18] decade of life, while information in very old individuals (≥80 years) is sparse. There is also evidence to suggest that ageing is associated with a lower number of myonuclei[14,15,18-20] and smaller myonuclear domains[15,21,22] in type II fibres. However, the age-related loss of myonuclei is less consistently reported, but may be more pronounced later in life. This is an abbreviation and I believe the correct form is: i.e. in very old individuals (≥80 years).

While not yet investigated in very old individuals in the 9th decade of life, heavy resistance training has resulted in an increase in the number of SCs and fibre size in type II fibres in old individuals.[15,16,18,19,23] However, the SC response to a single bout of exercise may be blunted or delayed with ageing,[24,25] and it is not known if SCs respond to resistance exercise in very old individuals. A blunted SC response with advancing age may be a limiting factor for muscle fibre hypertrophy, as the response to resistance training seems to be related to SC content and/or responsiveness.[15,20,21,26,27] Being the source of new myonuclei, SCs play a key role during hypertrophy if myonuclear addition is required.[21,26] However, whether, and if so when, the addition of new myonuclei is required for muscle hypertrophy is not fully clarified at this point.[27,28] Interestingly, a recent meta-analysis concluded that myonuclear addition occurs during small (<10%) increases in fibre size,[33] while it has previously been suggested that there is no increase in myonuclear content before a 15%-26% increase in fibre size has occurred.[21,34] It has also been suggested that the myonuclear domain can only increase to an upper limit or "ceiling" of approximately 2000-2250 μm^2 during muscle fibre hypertrophy,[21,26] from where further hypertrophy requires addition of new myonuclei from SCs. Accordingly, small myonuclear domains could represent a large potential for muscle fibre hypertrophy without myonuclear addition, until the myonuclear domain reaches the "ceiling" and myonuclear addition is necessary. To get more detailed insight into the relationship between fibre size and myonuclei, we previously applied a detailed cluster analysis, grouping fibres of similar size.[35] This approach revealed that myonuclear domain is not constant across the range in fibre size in muscle biopsies, and is markedly smaller in the smallest fibres (fibres <3000 μm^2).[35] In contrast, myonuclear content seemed to be strictly regulated with respect to fibre size, as we observed a strong linear correlation between these parameters.[35] Together these observations suggest that small myonuclear domains per se, do not reflect a potential for hypertrophy without myonuclear addition.[33,35]

There were two aims of the present study: Part (1) To examine in a cross-sectional comparison the differences in muscle fibre size, SCs, myonuclear content and the myonuclear domain in young and very old (≥83 years) individuals; and: Part (2) To examine in a training study the changes in these parameters in very old individuals after 12 weeks of heavy resistance training.

In the cross-sectional part of the study (part 1) it was hypothesized that advanced ageing would be associated with a substantial lower number of SCs in the type II fibres compared to previous observations at earlier stages of ageing. It was furthermore hypothesized that ageing would be associated with a lower number of myonuclei and a smaller myonuclear domain in type II fibres. A detailed analysis of the relationship between fibre size and myonuclei was applied to gain further insight into the myonuclei to fibre size relationship at this advanced stage of ageing.

In the training part of the study (part 2), including only the very old men and women, it was hypothesized that heavy resistance training would partially or fully restore the number of SCs in the type II fibres. In addition, it was hypothesized that both the number of myonuclei and the size of the myonuclear domain would increase in response to heavy resistance training. To further evaluate the effects at the cellular level of heavy resistance training at this stage of aging, gene expression levels in a broad selection of genes were measured in the Old subjects before and after the intervention.

2 | RESULTS

2.1 | Part 1: Cross-sectional comparison—Young vs Old, biopsy means

Age-related differences were compared between Young men and Old men for fibre size, SCs, myonuclear content and myonuclear domain (Figure 1A-D). Compared to type II fibres in Young men, Type II fibres in Old men were significantly different with respect to fibre size (−38%, $P < 0.001$, Cohen's D 1.84, Figure 1A), SCs per fibre (−52%, $P < 0.001$, Cohen's D 2.71, Figure 1B) and myonuclear domain (−30%, $P < 0.001$, Cohen's D 1.59, Figure 1D). In addition, the Old men had significantly lower number of myonuclei per type II fibre compared to their own type I fibres (−14%, Cohen's D 0.85, $P < 0.001$), and there was a trend for a significant lower number of myonuclei in type II fibres in Old men vs type II fibres in Young men (−13%, $P = 0.09$, Cohen's D 0.69, Figure 1C).

For these parameters the sex-related differences between the Old men and the Old women are presented in Figure 1E-H. Main effects of fibre type and sex were observed between Old men and Old women for all parameters ($P < 0.001$, Figure 1E,G,H), except for the number of SCs per type II fibre (no main effect of sex, $P = 0.74$, Figure

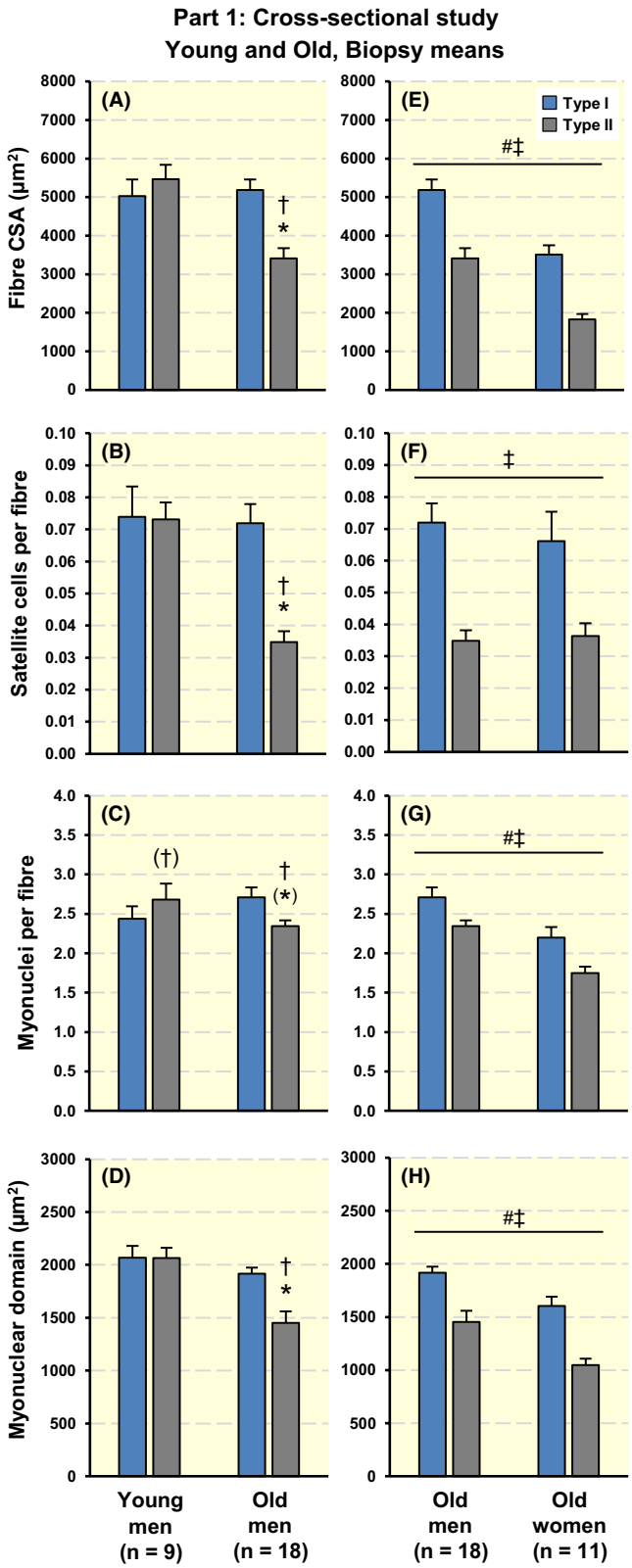

FIGURE 1 Cross-sectional data (part 1) from biopsy analysis of type I fibres (blue bars) and type II fibres (grey bars). The effect of age is compared between a group of Young men (n = 9) and Old men (n = 18) in the left panel. Sex-differences between Old men (n = 18) and Old Women (n = 11) are compared in the right panel. The data are fibre CSA (cross-sectional area, A and E), the number of satellite cells (B and F) and myonuclei (C and G) per fibre, and the myonuclear domain (fibre CSA per myonucleus, D and H). ( ) around symbols illustrate a trend for significant effect ($P = 0.05$-0.1), $^{*}P < 0.05$ vs type II fibres in Young men, †$P < 0.05$ vs type I fibres within group, #$P < 0.001$ main effect of sex, ‡$P < 0.001$ main effect of fibre type. Data are means ± SEM

1F). Type II fibre size distribution in the Young men, Old men and Old women can be seen in Figure 2. The figure illustrates the higher prevalence of smaller type II fibres in Old men vs Young men, and in Old women vs Old men and Young men.

2.2 | Part 1: Cross-sectional comparison—Young vs Old, cluster analysis

The relationship between fibre size, myonuclei and age was further examined for fibres of similar size grouped in 2000 µm^2 fibre size-clusters. There was no significant difference between the clusters in the Old men and women, with the exception of the 500-2500 µm^2 cluster in the type II fibres (see Figure S1), but this was to some extend affected by smaller fibres in the old women in that cluster. Data from Old men and Old women were pooled before comparison with data from the Young men, and the Young men did not have enough of these small type II fibres to create the smallest cluster. Overall, the 2000 µm^2 analysis revealed that the number of myonuclei increased with increasing fibre size in both Young and Old ($P < 0.05$-0.001, Figure 3A-B). Within each cluster Old had numerically higher number of myonuclei per fibre compared to Young, which was significantly higher for the type I fibres in the 2500-4500 µm^2 cluster ($P < 0.01$, Figure 3A).

The detailed 100-fibres per cluster analysis in Old revealed a strong positive linear relationship between fibre size and myonuclei per fibre in the type I fibres ($r = 0.98$, $P < 0.0001$, Figure 4A) and the type II fibres ($r = 0.97$, $P < 0.0001$, Figure 4B). In addition, the myonuclear domain increased with fibre size in both type I fibres ($r = 0.94$, $P < 0.0001$, Figure 4C) and type II fibres ($r = 0.99$, $P < 0.0001$, Figure 4D). The muscle fibre perimeter per myonucleus was not significantly correlated with fibre size in the type I fibres ($r = -0.31$, $P = 0.17$, Figure 4E), while there was a modest positive correlation in the type II fibres ($r = 0.67$, $P < 0.001$, Figure 4F).

2.3 | Part 1: Baseline MHCn/e positive fibres and central myonuclei

The prevalence of fibres positive for neonatal or embryonic myosin (MHCn/e) in Young subjects was very low (only present in one subject). A total of 171 normal sized strongly stained (MHCn/e-ST: 0.10% [0.02%:0.54%]) and

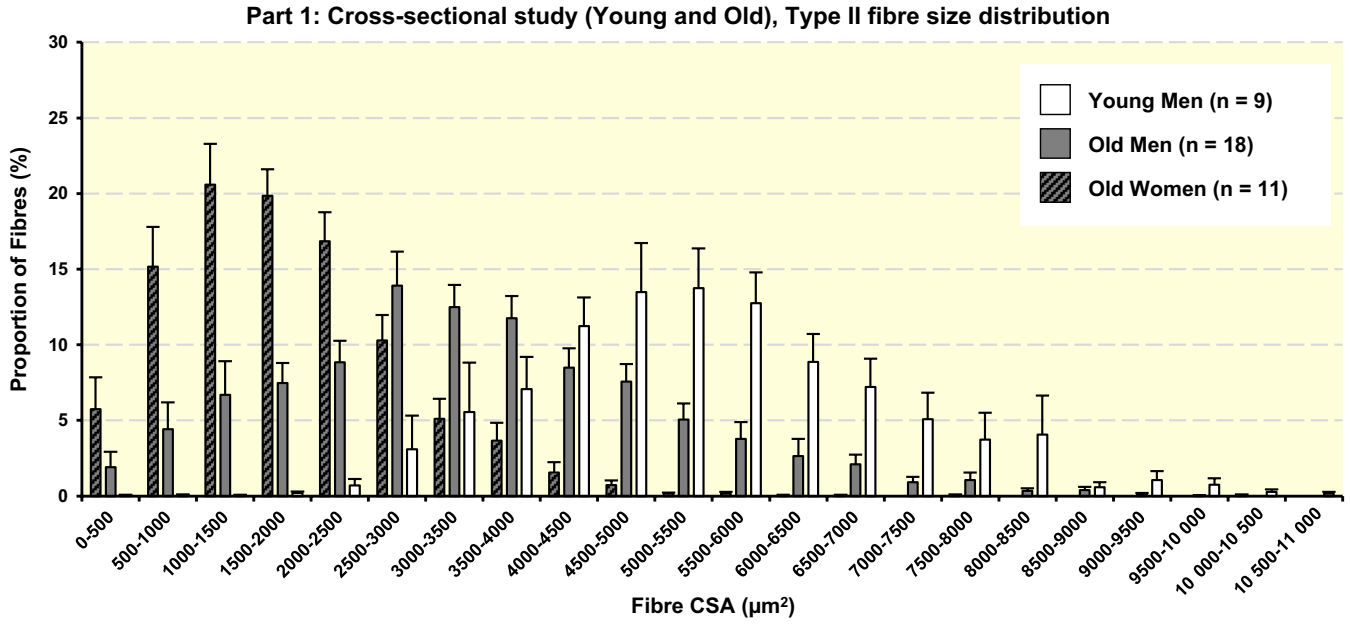

FIGURE 2 Distribution of type II fibre size (part 1, CSA, cross-sectional area) in Young men (white bars), Old men (grey bars) and Old women (hatched grey bars). Data are means ± SEM

188 normal sized moderately stained (MHCn/e-MOD: 0.18% [0.05%:0.58%]) MHCn/e positive fibres were found in 26 of the 29 baseline biopsies from the Old subjects (median [IQR; 25%:75%]). In comparison, a total of 259 (<50 μm^2) and 79 (50-200 μm^2) very small MHCn/e positively stained fibres were observed in 23 (<50 μm^2) and 26 (50-200 μm^2) of the 29 baseline biopsies from the Old subjects. Central nuclei were observed in all biopsies except one Young subject, with no significant difference in the proportion of fibres with centrally located nuclei between Young (0.72% [0.21%:1.36%]) and Old (1.03% [0.52%:1.52%], $P = 0.41$).

2.4 | Part 2: Intervention—Old, RT vs CON

A histogram of sex-separated fibre size distribution before and after the heavy resistance training intervention in the Old subjects is available in Figure S2. The resistance training group (RT) and the control group (CON) had similar, but non-significant, increases in type II fibre size (RT: +6.8%, Cohen's $D = 0.16$, CON: +7.1%, Cohen's $D = 0.21$, Figure 5A), as there was no significant time × treatment interaction for type I fibre size ($P = 0.59$) or type II fibre size ($P = 0.89$). There was furthermore no main effect of time for type I fibre size ($P = 0.64$) and type II fibre size ($P = 0.34$), but a main effect of treatment for type II fibre size was observed ($P < 0.05$). There were no significant time × treatment interaction for the number of SCs per fibre (type I: $P = 0.39$, Cohen's D: RT = 0.22, CON = 0.19, type II: $P = 0.62$, Cohen's D: RT = 0.35, CON = 0.11, Figure 5B), myonuclei per fibre (type I: $P = 0.37$, Cohen's D: RT = 0.40, CON = 0.05, type II: $P = 0.57$, Cohen's D: RT = 0.25, CON = 0.01, Figure 5C) or myonuclear domain (type I: $P = 0.10$, Cohen's D: RT = 0.44, CON = 0.25, type II: $P = 0.56$, Cohen's D: RT = 0.34, CON = 0.27, Figure 5D), but a main effect of treatment was observed for myonuclear domain in the type II fibres ($P < 0.05$, Figure 5D).

2.5 | Part 2: Intervention—Old, RT vs CON—MHCn/e positive fibres and central myonuclei

Individual data from the RT-intervention in the Old subjects are presented in Figure 6C-H. A significant decline in the proportion of MHCn/e-ST fibres was observed in the RT group from Pre to Post ($P < 0.05$, Figure 6C), while MHCn/e-MOD fibres where unchanged after 12 weeks of RT ($P = 0.29$, Figure 6D). There were no significant changes in the proportion of these fibres in the CON group from Pre to Post (MHCn/e-ST: $P = 0.50$, Figure 6F; MHCn/e-MOD: $P = 0.10$, Figure 6G). There was a significant increase in the proportion of fibres with central nuclei from Pre (1.2% [0.6%:2.0%]) to Post (1.6% [0.8%:2.8%], $P < 0.05$) in the RT group (Figure 6E), while no changes was observed in the CON group ($P = 0.17$, Figure 6H).

2.6 | Part 2: Intervention—Old, RT vs CON—mRNA

To obtain a general overview of the potential changes taking place in the muscle over time during the RT-intervention, a selection of mRNA markers were tested. Many of the measured genes were significantly elevated as an effect of time,

with no time x treatment interactions (Figure 7). Time x treatment interactions were found for Collagen IV ($P < 0.01$), TNFα ($P < 0.05$) and MHC IIX ($P < 0.05$). The post hoc test revealed significant differences between RT and CON in the changes in gene expression from Pre to Post (Collagen IV: $P < 0.001$; TNFα: $P < 0.01$; MHC IIX: $P < 0.01$). The post hoc test furthermore revealed a significant upregulation from Pre to Post in the RT group of Collagen IV ($P < 0.001$), and a significant upregulation of TNFα from pre to post in the CON group ($P < 0.05$), while MHC IIX showed a trend for an upregulation in the CON group ($P = 0.097$) from pre to post.

3 | DISCUSSION

3.1 | Main findings

In this study we present novel cross-sectional data (part 1) on SC and myonuclear content in very old individuals (83-94 years), as well as novel data on the changes in SCs and myonuclear content in these very old individuals after 12 weeks of heavy resistance training (part 2). The cross-sectional comparison between Young men and Old men in part 1 of the study revealed substantial differences in type II fibre characteristics, particularly with respect to type II fibre size (−38%), SC content (−52%) and the myonuclear domain (−30%), together with a trend for a lower (−13%) myonuclear content. In the first part of the study we furthermore found that Old women had significantly smaller fibres, myonuclear content and myonuclear domains compared to the Old men, while the number of SCs was unaffected by sex. Part 2 of the study revealed that despite small myonuclear domains in the type II fibres in the RT-group (1389 ± 516 μm^2), 12 weeks of heavy resistance training did not result in significant type II fibre hypertrophy in these very old individuals, and only gene expression of collagen IV changed as an effect of RT. Furthermore, no changes were observed for SC or myonuclear content in response to RT, which could indicate a poor responsiveness of very old SCs.

To gain more insight into the relationship between muscle fibre size and myonuclear content, cross-sectional data from part 1 of the study were compared for clusters of fibres of similar size. The results of these cluster analyses showed that even at this very late stage of life, the number of myonuclei per fibre was not lower in Old compared to Young muscle fibres. In addition, there is a lower number of myonuclei per fibre and a smaller myonuclear domain in smaller fibres. Therefore, biopsy means-based reports of

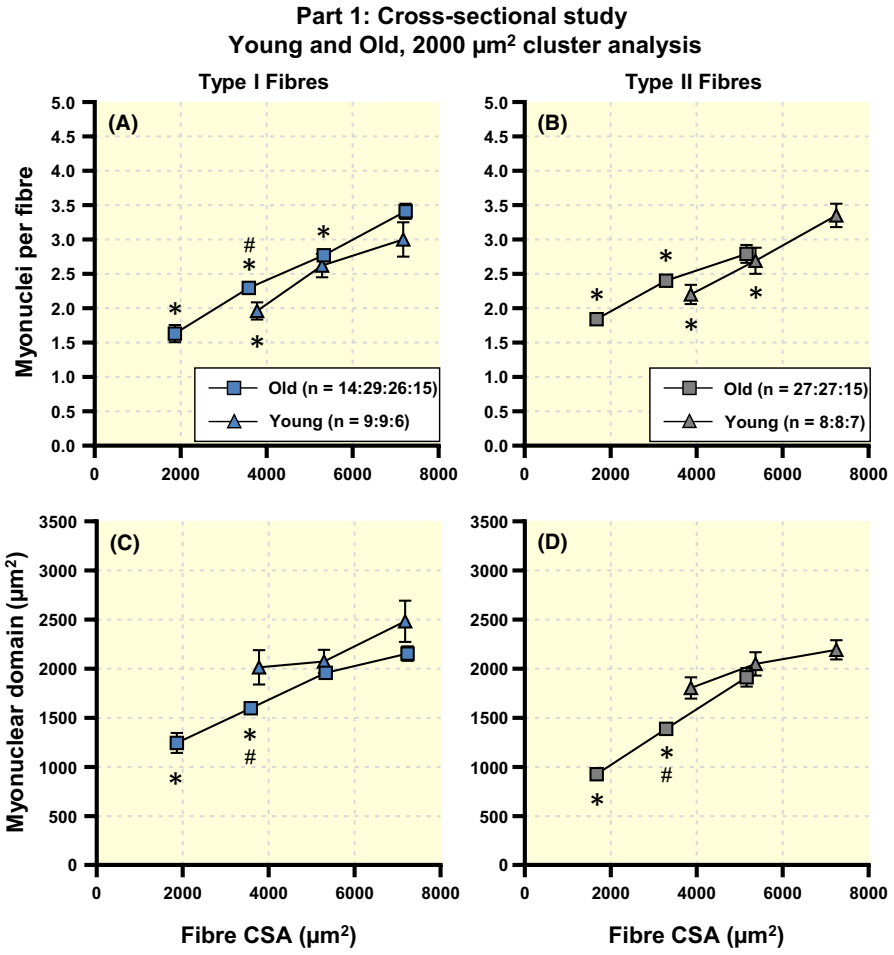

FIGURE 3 2000 μm^2 fibre size (part 1, CSA, cross-sectional area) cluster analysis in type I fibres (blue symbols, left panel) and type II fibres (grey symbols, right panel) of myonuclei per fibre (A and B) and myonuclear domain (fibre CSA per myonucleus, C and D) in clusters of fibres of similar size in Old (squares, men and women combined, n = 29) and Young men (triangles, n = 9). *$P < 0.05$ vs all larger clusters within the group, #$P < 0.05$ vs Young within that cluster. Data are means ± SEM

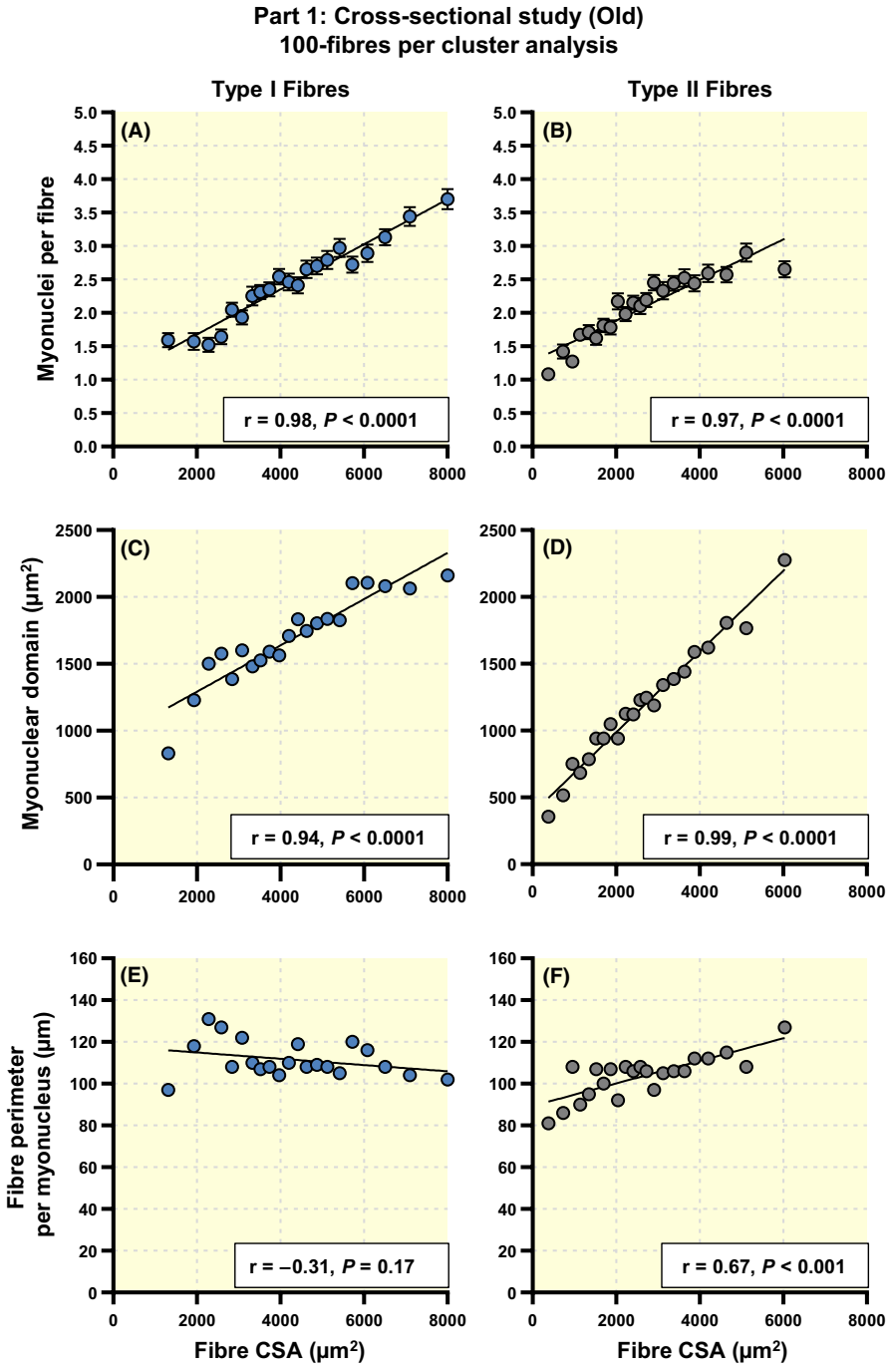

FIGURE 4 Detailed 100 fibres per cluster analysis (part 1) in type I muscle fibres (blue symbols, left panel) and type II muscle fibres (grey symbols, right panel) from 29 Old men (n = 18) and women (n = 11) illustrating the relationship between muscle fibre CSA (cross-sectional area, *x*-axis) and the myonuclear content (A and B), myonuclear domain (fibre CSA per myonucleus, C and D) and fibre perimeter per myonucleus (E and F). Each point represents a cluster of 100 fibres. Error bars are mean ± SEM

lower myonuclear content and smaller myonuclear domains in old individuals are most likely affected by the increasing proportion of small fibres with ageing. The detailed 100-fibres per cluster analysis of the Old muscle fibres lead to a very interesting observation, because, in contrast to the myonuclear domain (fibre CSA per myonucleus), the muscle fibre perimeter per myonucleus was more constant, particularly in the type I fibres, throughout a large range in fibre size. This could indicate that myonuclear content is regulated with respect to fibre circumference (or surface area in a 3D model), and not with respect to the size of the myonuclear domain. Although further confirmation is needed, this could mean that myonuclear addition is required for hypertrophy even in fibres with myonuclear domains well below the theoretical "ceiling". We therefore speculate that the lack of increase in SC and myonuclear content in the present study was a limiting factor for hypertrophy in these very old individuals.

3.2 | Part 1: Satellite cells at the latest stage of ageing

In line with our hypothesis for the cross-sectional part of the study (part 1) we observed smaller type II fibres and a lower

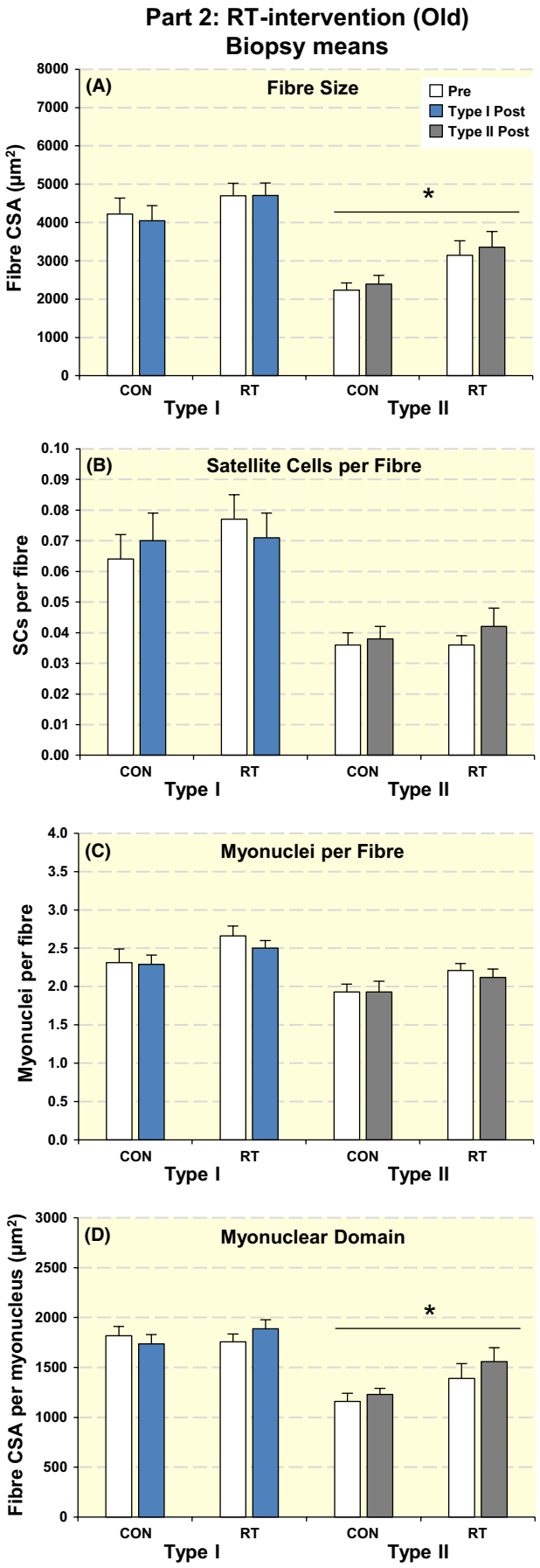

FIGURE 5 Part 2: Biopsy analysis in two mixed groups of Old men and Old women before (Pre, white bars) and after (Post, type I and II fibres in blue and grey bars respectively) 12 wk of heavy resistance training (RT, n = 12, 8 men/4 women) or in a control group (CON, n = 13, 7 men/6 women). The data are fibre CSA (cross-sectional area, A), the number of satellite cells (B) and myonuclei (C) per fibre, and the myonuclear domain (fibre CSA per myonucleus, D). *$P < 0.05$ main effect of treatment. Data are means ± SEM

SC content in the type II fibres of Old men vs Young men. Smaller type II fibres have consistently been reported in old individuals at earlier stages of ageing, while type I fibres are not affected to the same extent, and the present results extends these observations to very old individuals.[15,36] The 52% lower SC content in the type II fibres in the Old men compared to type II fibres in Young men, and compared to type I fibres within the Old men (−51%) is substantially larger than some previous reports in individuals ~10-30 year younger.[11,12,15,18,20,23,25,37,38] However, somewhat comparable results have also been reported in ~10 year younger individuals,[13-16,18] which could indicate that the SC-pool had reached a lower level or "floor" at this late stage of ageing. This seems possible as the cross-sectional data furthermore revealed that the SC content in type II fibres did not differ between the Old men and Old women (Figure 1B), despite 46% smaller type II fibres in the Old women (1831 ± 451 µm^2, n = 11) compared to the Old men (3414 ± 1107 µm^2, n = 18, Figure 1A-B).

3.3 | Part 1: Myonuclei at the latest stage of ageing

The smaller type II fibres, lower number of myonuclei per fibre and smaller myonuclear domains in the Old women compared to the Old men (Figure 1) are in line with observations in slightly younger (~70 years) men and women.[18] The cross-sectional data revealed that the Old men had significantly lower myonuclear content in their type II fibres vs their own type I fibres (−14%). However, there was only a trend for a ~13% lower number of myonuclei per type II fibre in the Old men compared to type II fibres in Young men, despite substantially smaller (~38%) type II fibres in the Old men (Figure 1). In contrast to the present results, some studies have reported a significantly lower (~14%-40%) number of myonuclei in the type II fibres at an earlier stage of ageing.[14,15,38] The discrepancies between previous reports and the present results may to some extend be explained by the challenges in the quantification of myonuclei, relying on a subjective evaluation of the positioning of nuclei inside or outside the muscle fibre membrane. The use of confocal microscopy in the present study is a clear advantage compared to widefield microscopy. Interestingly, a myonuclei specific antibody, PCM1 (pericentriolar material 1), labelling the nuclear envelope, has recently been validated in young humans and rodents, which

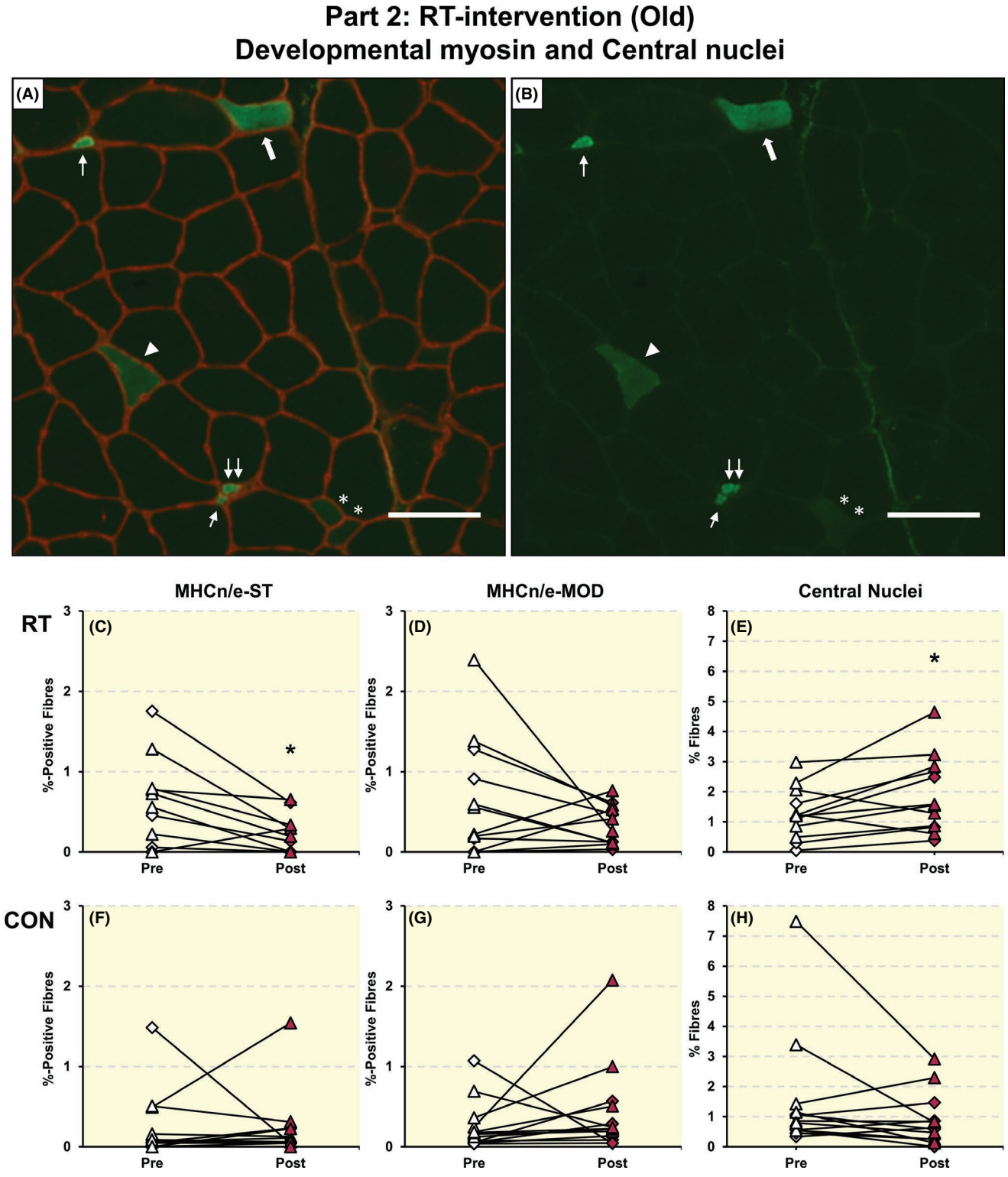

FIGURE 6 Immunohistochemical staining of (A) basement membrane (laminin, red) and neonatal/embryonic myosin (MHCn/e, green), and (B) the MHCn/e staining alone (green). The images illustrate the variation in staining intensity and size of MHCn/e positive fibres. The thick white arrow points at a normally sized strongly stained fibre (MHCn/e-ST). The white triangle is placed next to a normally sized moderately stained fibre (MHCn/e-MOD). Two asterisks are placed next to two normally sized weakly stained fibres not included for analysis. The thin white arrows point to very small strongly stained fibres not included for analysis. Scale bars 100 μm. (C-H) Individual data from part 2 of the study, illustrating the proportion of fibres strongly stained (MHCn/e-ST, C and F) and moderately stained (MHCn/e-MOD, D and G) for neonatal and embryonic myosin, and the proportion of fibres with a centrally located nucleus (E and H) in 83-94 y old men (triangles) and women (diamonds) from before (Pre, white symbols) to after (Post, red symbols) 12 wk of heavy resistance training (RT, upper panel, n = 12) and in a control group performing no training (CON, lower panel, n = 13). *$P < 0.05$ from Pre

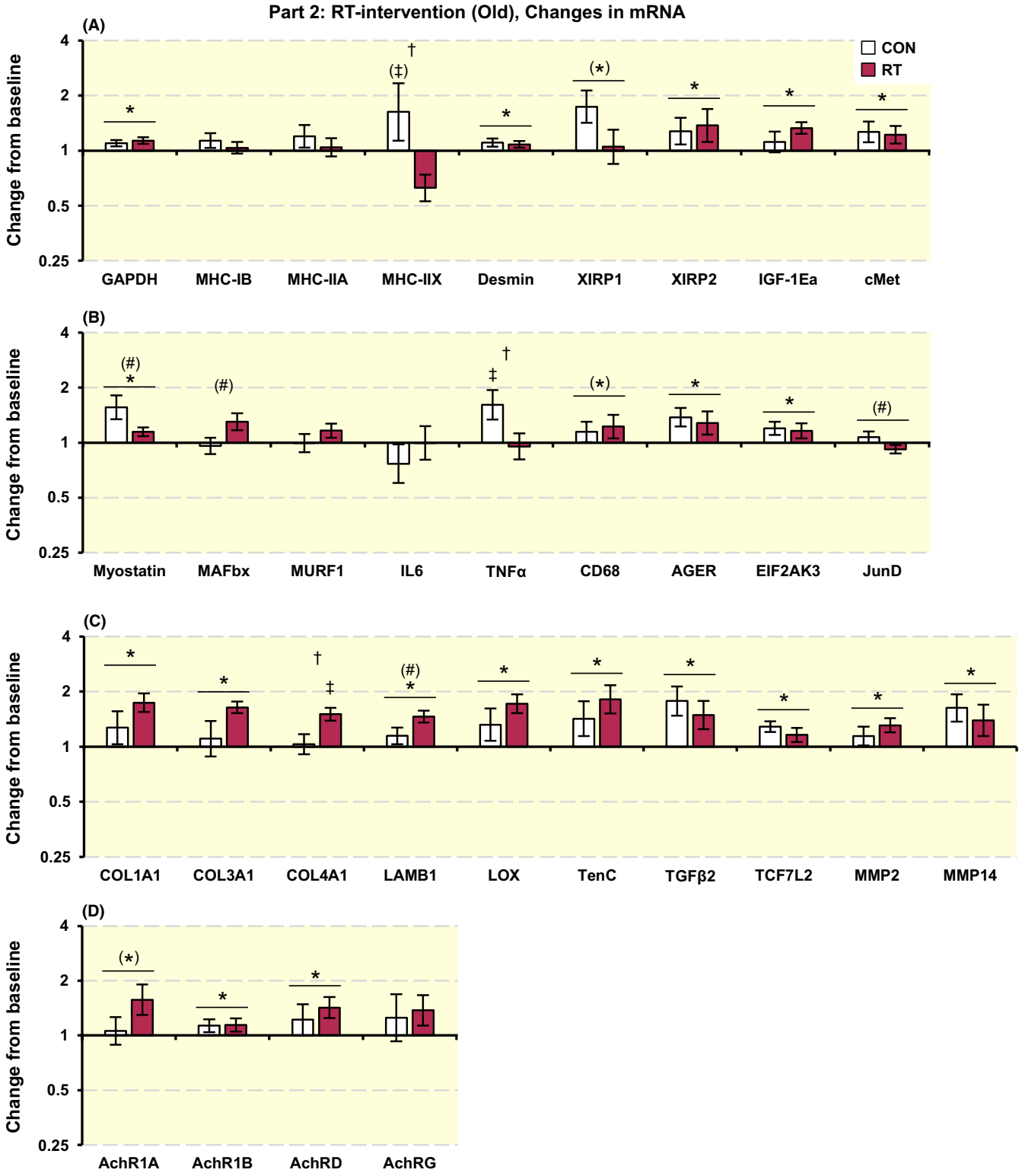

FIGURE 7 Changes in gene expression in part 2 of the study in muscle biopsies collected in 83-94 y old men and women before (Pre) and after (Post) 12 wk of heavy resistance training and protein supplementation twice a day (RT, red bars, n = 12) or 12 wk of protein supplementation only (CON, white bars). Data are normalized to RPLP0 and log2 transformed before analysis. Post intervention mRNA data are expressed relative to the individual Pre intervention mRNA data. Results are displayed as geometric means ± backtransformed SEM on a logarithmic (log2) scale *y*-axis. (*) trend for significant effect of time ($P = 0.05$-0.1), * effect of time $P < 0.05$, (#) trend for effect of treatment and for a time x treatment interaction ($P = 0.05$-0.1), † post hoc test significant difference between groups at Post $P < 0.05$, ‡ post hoc test significantly different from Pre within group $P < 0.05$

could be a very important improvement to myonuclei analysis particularly in studies where widefield microscopy is the only option.[42] The cross-sectional data in part 1 of the present study reveals only a modest effect of ageing on the number of myonuclei in Old type II fibres, indicating that muscle fibre atrophy and loss of myonuclei does not occur in a 1:1 ratio. This observation seems to be in agreement with a recent study showing no correlation between fibre size and myonuclear content in frail old women with very small type II fibres, in contrast to younger and more healthy individuals.[43]

3.4 | Part 1: Detailed cluster analysis of myonuclei and fibre size

The most important information from the 2000 μm^2 analysis was that ageing did not seem to result in a lower number of myonuclei per fibre for fibres of similar size (Figure 3A-B). In the 2000 μm^2 as well as the 100-fibres per cluster analysis it was furthermore clear that there was a relationship between fibre size and myonuclear content, as well as between fibre size and myonuclear domain. Overall the present results are fully in line with our previous observations using a similar cluster analysis in younger men.

3.4.1 | Fibre perimeter per myonucleus

It is important to emphasize that although muscle fibre perimeter and muscle fibre CSA are related, similar to a circle, the muscle fibre cross-sectional area increase approximately fourfold for every twofold increase in muscle fibre perimeter (own observations). Therefore, it matters whether myonuclear content is expressed relative to fibre CSA or fibre perimeter. It should furthermore be noted that compared to the very old type II fibres, the very old type I fibres probably better reflect a homeostatic system with respect to fibre size, SCs and myonuclear content, as the type I fibres seemed to be unaffected by ageing in these parameters. Interestingly, the 100-fibres per cluster analysis revealed that while the myonuclear domain (CSA per myonucleus) increased with increasing fibre size, the muscle fibre perimeter per myonucleus seemed to be relatively constant regardless of fibre size (a more or less horizontal regression line, no correlation in the type I fibres and only a weak correlation in the type II fibres), particularly in the type I fibres (Figure 4E). This is a very important observation, as it could indicate that myonuclear content is regulated with respect to the fibre perimeter (or surface area of the fibre in a 3D model). A similar observation was reported in single fibres from mice, as the surface area per myonucleus was relatively constant in fibres of different size.[44] These authors speculated that each nucleus was "serving" a cell surface area rather than a volume.[44] In support of this, human single fibre analysis (3D) demonstrated that the distance to the nearest neighbouring myonucleus on the surface of the myofibre is kept relatively constant, although greater variability was observed in old type I muscle fibres.[45] A constant distance between the positioning of myonuclei on the surface of the myofibre may indicate that for example signalling between myonuclei on the surface of the fibre, and not myonuclear capacity, is involved in the regulation of myonuclear content. Ultimately, it is important to emphasize that myonuclear addition is required during fibre hypertrophy, in order to maintain a constant fibre perimeter to myonucleus ratio (in 2D), regardless of the size of the myonuclear domain. Whether this means that myonuclear addition is required for muscle fibre hypertrophy at a much earlier point than previously assumed is not fully clear at this point. However, it does seem to be a possible scenario, as a recent meta-analysis concluded that myonuclear addition occurs even during low levels of muscle fibre hypertrophy (<10%),[33] but this needs to be further investigated, preferably in younger subjects with a substantial hypertrophy response.

3.5 | Part 2: The effect of heavy resistance training on fibre size, satellite cells, and myonuclei at the latest stage of ageing

The second part of the study included only muscle biopsies from the Old men and women in the RT and CON group who completed the intervention. Interestingly, the 12-week RT-intervention did not result in significant type II fibre hypertrophy in the present study, despite small myonuclear domains in these old individuals. In addition, the RT-intervention did not have an effect on SC content in the type II fibres, which was in contrast to our hypothesis and previous reports with similar interventions at earlier stages of ageing.[15,18,19,23] Ageing may be associated with a blunted SC response to exercise, when examined in acute trials with subjects decades younger.[24,25] Several factors such as SC senescence,[46] capillarization,[12,23] circulating factors[47] or the SC-niche[48] may contribute to a reduced SC-responsiveness with ageing. There is a lack of studies on SCs and changes in response to an exercise stimulus in very old individuals. The present data could indicate that it becomes very difficult to mount an SC response at the most advanced stage of ageing, but it is also possible that the lack of SC response reflect that there was no requirement for new myonuclei because there was no increase in fibre size. It should be noted that the muscle biopsies were collected 2 days after the last training session in the RT-group, a time-point where an acute increase in the SC-pool has been observed in participants accustomed to resistance training.[49] This does not seem to have affected the SC-analysis in the present study, because the SC content was unchanged from Pre to Post in the RT-group. In good agreement with the lack of increase in SC content, but in contrast to our hypothesis, the RT-intervention did not result in an increase in myonuclear content. Therefore, the small non-significant increase in

type II fibre size was paralleled by only a small but non-significant increase in the size of the myonuclear domain (Figure 5A,D). Most importantly, the myonuclear domain remained well below the theoretical ceiling even after the RT-intervention (Figure 5D). These results are interesting because they could indicate that small myonuclear domains in very old type II fibres do not indicate a large potential for muscle fibre hypertrophy without myonuclear addition. The explanation could be that myonuclear content is not regulated with respect to the size of the myonuclear domain, but is more closely related to the fibre perimeter per myonucleus (in a 2D model). The SCs may therefore play an important role during muscle fibre hypertrophy in very old individuals, although they have small myonuclear domains. More studies are therefore needed to further examine and improve the SC activation and myonuclear addition at this last stage of ageing.

It should be noted that several factors other than SCs and myonuclear addition could explain the lack of muscle fibre hypertrophy, for example proper nutritional support (15 g of protein, twice daily) combined with training was necessary to increase muscle mass in frail old individuals.[20] In the present study the subjects received a supplemental drink containing 20 g of milk proteins two times a day, and the RT-group consumed one of these drinks immediately after the training sessions. However, we cannot rule out that higher doses of protein would have been beneficial, as an acute dose- response study found that 40 g of protein resulted in the highest increase in myofibrillar protein synthesis after a heavy resistance training bout in old men.[50] Furthermore, although no changes were observed in muscle fibre size, SCs and myonuclear content, the RT-group did improve isometric (+13%) and isokinetic (+11%) knee extensor peak torque.[51] Whole body lean mass and leg lean mass was unchanged in the RT-group, but a small increase (~3%) in cross-sectional area of the quadriceps femoris (magnetic resonance imaging) was observed.[51] Overall, these changes indicate that there was no negative effects of the RT-intervention, but the improvements were too small to be detected at the muscle fibre level with immunohistochemistry. Only a few studies have investigated the effect of heavy resistance training on hypertrophy in very old individuals. Some of these studies have reported a similar small effect on quadriceps cross-sectional area[52,53] or a non-significant increase in muscle fibre size,[52,54] as seen in the present study. However, significant increase in muscle fibre size has also been reported in studies including both men and women, with a 22% increase in type IIA fibre size after 12 weeks RT in 85-97 year old subjects,[55] and a 10% increase in type II fibre size in 84 ± 1 year old subjects after 10 weeks of RT.[56] Interestingly, both of these studies used a relatively simple training protocol, with a low volume and relatively high intensity (3 sets × 8 repetitions), including only a single exercise for the knee extensors in the study showing the largest response.[55] In the present study the protocol included up to 5 sets in two exercises for the knee-extensors, resulting in an up to 3 times larger training volume. At this point no study has investigated the dose-response to RT in very old individuals, but the present study seems to indicate that a high volume training protocol with 3-5 sets and 2 exercises for the knee extensors, three times per week, may not be optimal at this advanced age.

3.6 | Part 2: Changes in gene expression with heavy resistance training

The changes in gene expression levels with the RT-intervention in the Old subjects were in line with the overall lack of response to the RT intervention at the myofibre level. However, there were some exceptions, including MHC-IIX, Collagen IV and TNF-α, where differences were seen between the RT and CON groups. A downregulation in MHC-IIX mRNA in the RT group would have been in good agreement with the commonly observed fibre type shift from type IIX to type IIA with heavy resistance training, and with the trend for a decline in the proportion of type IIX fibres in the RT-group previously reported in these subjects,[51] but this was not significant in the present study ($P = 0.107$, Figure 7A). With regard to interpretation of altered transcription in the post training biopsies, it should be noted that it is not possible to distinguish between a new baseline level as a result of the 12 weeks of training and an acute response to the last training session, 48 hours prior to tissue sampling. This may be especially pertinent in relation to myosin IIX, which at the fibre protein level is down regulated with months of RT, but it has also been reported that MHC-IIX mRNA begins to decline 24 hours after a single bout of heavy load muscle contractions and remains suppressed for at least 4 days.[57] Several genes were elevated in both the RT and CON-group (effect of time), possibly indicating some effect of the nutritional supplementation at the gene expression levels in both groups. Other genes affected by the RT intervention included Collagen IV which was significantly upregulated in the RT group only, together with a similar trend in Laminin (Figure 7C), indicating training induced adaptations in the muscle fibre basement membrane and extracellular matrix.

3.7 | Developmental myosin and central nuclei

A staining for MHCn/e was included to exclude regenerating fibres from the SC and myonuclei analysis. Little is known regarding the presence of these fibres in uninjured skeletal muscles, but there are some indications in the literature that this is an age-related phenomenon.[14,22] In line with this, MHCn/e positive fibres were found in almost all biopsies from the Old subjects, while only present in one biopsy from the Young subjects. The prevalence of these fibres varied greatly within the Old subjects, with a median proportion of MHCn/e positive fibres somewhat comparable to previous

TABLE 1 Subject characteristics

	Young men (n = 9)	Old men (n = 18)	Old women (n = 11)	RT (old) (n = 12)	CON (old) (n = 13)
Age [y]	21 ± 2*	87 ± 3	87 ± 3	88 ± 4	86 ± 3
Height [cm]	180 ± 9*	174 ± 8	159 ± 8*	171 ± 11	167 ± 12
Weight [kg]	73 ± 9	78 ± 9	58 ± 10*	70 ± 14	67 ± 13
BMI [kg m^{-2}]	22 ± 2*	26 ± 3	23 ± 3*	24 ± 3	24 ± 2
SMI [kg m^{-2}][a]	N/A	7.46 ± 0.57	6.13 ± 0.92*	6.93 ± 0.86	6.82 ± 1.02
SMI < cut-off (n)	N/A	8	3	5	6
DEMMI [0-100]	N/A	82 ± 10	64 ± 21*	72 ± 20	74 ± 15
30-s CST [reps]	N/A	13 ± 5	8 ± 9	10 ± 7	13 ± 7

30-s CST, 30 second chair stand test; CON, control group; DEMMI, DeMorton Mobility Index; RT, heavy resistance training group; SMI, Skeletal Muscle Index.
SMI < cut-off level for sarcopenia: <7.26 kg m^{-2} for men and <5.45 kg m^{-2} for women.
[a]n = 17 and n = 10 for Old men and Old women respectively.
*$P < 0.05$ vs Old Men. Data are means ± SD.

reports in old individuals.[14,22] While it is well recognized that the presence of neonatal and embryonic myosin in adult muscle are indicative of regeneration after injury, it is possible it reflects muscle fibre damage from daily wear and tear,[58] or even denervated/re-innervated muscle fibres.[59] It should be noted that at this point it is not clear why there was a difference in the staining intensity between the MHCn/e positive fibres, but in the present study RT leads to a decline in the proportion of MHCn/e-ST fibres. Although speculative, this could signify a positive effect of RT on denervation/re-innervation (Figure 6C), but this was not supported by gene expression levels of acetylcholine receptors, a marker of neuromuscular junction instability/denervation,[60] where no changes were observed (Figure 7D).

4 | LIMITATIONS

The inclusion of women in the RT-intervention could potentially have affected the hypertrophy response, however, a recent study did not observe any sex-difference in the response to RT in 71 ± 1 year old men and women.[18] In the present study the four Old women in the RT group actually seemed to have a good response to the training intervention, illustrated by a clear rightward shift in type II fibre size distribution (Figure S2). While there are only 4 women in this group, and their data thus should be considered with caution, these findings indicate at the very least that the old women were not the cause for the blunted hypertrophy response in the RT group as a whole.

5 | CONCLUSION

The present study revealed a substantial lower (~50%) SC content of type II fibres in 83-94 year Old men compared to Young men, with no effect of 12 weeks of heavy resistance training on SCs in a mixed group of Old men and women. Myonuclear content seemed to be more robust to age-related changes, with only a trend for a ~13% lower number of myonuclei in Old men vs Young men. Significantly smaller myonuclear domains were therefore observed in the type II fibres in these Old individuals, but this did not seem to be beneficial for the muscle fibre hypertrophy response to heavy resistance training. The observation of smaller myonuclear domains in smaller fibres, and a constant fibre perimeter to myonucleus ratio in the type I fibres in the Old subjects, indicate that myonuclear content is not regulated with respect to the size of the myonuclear domain. Without substantial muscle fibre hypertrophy, it is not possible to conclude whether the fibre perimeter per myonucleus ratio is kept constant during fibre hypertrophy, but the present data could indicate that myonuclear addition is required for muscle fibre hypertrophy, even in muscle fibres with small myonuclear domains.

6 | MATERIALS AND METHODS

6.1 | Study design and participants

A total of 30 "Old" men and women (age 83-94 years) were randomized into two groups: (a) a heavy resistance training group (RT, n = 15, 10 men and 5 women); or (b) a control group (CON, n = 15, 9 men and 6 women). The participants completed a test-battery including functional tests and strength tests at the beginning and at the end of the intervention as described in details elsewhere, including details on recruitment, inclusion criteria, randomization and subjects who dropped out of the study.[51] All subjects consumed a 1260 kJ nutrient supplement drink (Fresubin® Protein Energy Drink, Fresenius Kabi, Bad Homburg, Germany) at breakfast and

lunch (after training for the RT group on training days) containing 20 g of milk protein, 25 g of carbohydrate and 13 g of fat.

In addition, baseline biopsies from nine young recreationally active, but otherwise untrained men (age 19-27 years), who were randomized to a Placebo group as part of another study in our lab[61] were included as a "Young" control group for cross-sectional comparison between Young men and Old men.

Based on the available biopsy material, biopsies from 29 Old subjects (n = 18 men and n = 11 women) were included for baseline analysis and biopsies from 25 Old subjects (RT: n = 12, 8 men, 4 women; CON: n = 13, 7 men, 6 women, Table 1) were included in the analysis of the RT-intervention. Two biopsies from Young subjects were excluded from the SC analysis due to poor quality of the Pax7 staining (n = 1) and insufficient number of fibres to analyse (n = 1), but included for all other analyses. For further description of the Old subjects, baseline measures of skeletal muscle index[62] (SMI = appendicular lean mass × height-2) and functional test-scores (DeMorton Mobility Index, DEMMI[63] and 30 second chair stand test, 30-s CST[64]) are included in Table 1.

All subjects gave written informed consent before entering the study that was approved by the Danish Regional Ethical Committees of the Capital Region (H-4-2013-068 and HD-2008-074) and conformed to the standards of the latest Declaration of Helsinki.

6.2 | Heavy resistance training intervention

After 2 weeks of familiarization, the RT group performed 12 weeks of heavy resistance training 3 times a week. The training started at 3 × 12 repetitions at 70% of 1RM (repetition maximum), progressing towards 5 × 6 repetitions in week 10, followed by tapering in week 11-12 (3 × 6 and 2 × 6 repetitions). The 1RM was calculated from a 3-6 RM test as described elsewhere.[51] Exercises for the legs included leg press, knee extension and leg curl (weight plate machines, MED Line, Technogym, Gambettola, Italy). Training was supervised by a physiotherapist and the 3-6 RM test was only performed at the beginning of the training intervention, where after the training load was increased in the following session whenever a subject was able to complete more repetitions than prescribed in the final set. Detailed description of the progression in training load is available in Table S1.

6.3 | Muscle biopsies

Biopsies were taken and handled following the same procedures for Young and Old. Biopsies from the Young and Old at baseline were taken before any physical tests, and 2 days after the final training session in the Old RT group. Muscle biopsies were collected from the midbelly of the vastus lateralis under local anaesthesia (1% lidocaine). Biopsies were taken 3-5 cm apart with a 5-mm biopsy needle by means of the percutaneous needle biopsy technique as described by Bergström,[65] using manual suction. Immediately after extraction, the specimen was aligned, embedded in Tissue-Tek, and frozen in isopentane precooled by liquid nitrogen, and stored at −80°C until analysis. Sections (10 µm) were cut from frozen biopsies in a cryostat (−20°C), placed on SuperFrost Plus glass slides (Menzel-Gläser, Braunsshweig, Germany), and stored at −80°C. Pre and Post samples from Old were mounted together with the samples from Young on the same slides.

6.4 | Immunohistochemistry

Sections for analysis of fibre cross-sectional area (CSA), fibre type, SCs and myonuclei were fixed in 4% PFA for

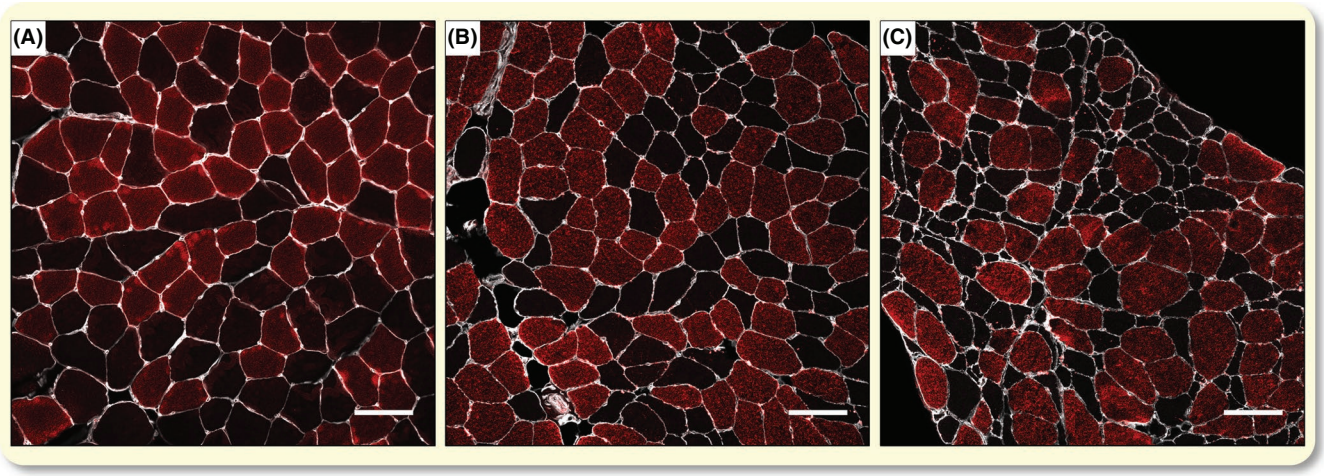

FIGURE 8 Confocal microscopy images of biopsy cross-sections immunohistochemically stained for laminin (white, pseudo color) and myosin heavy chain I (red) from (A) a 20-year-old Young man, (B) an 88-year-old man, (C) a 94-year-old woman. Scale bars 100 µm

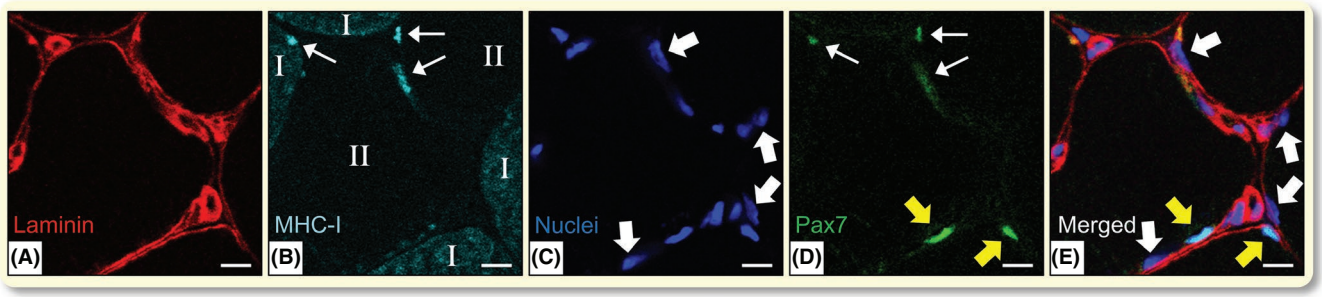

FIGURE 9 Confocal microscopy images of an immunohistochemically stained cross-section from one of the participants. The laminin (far-red channel) and MHC-I (red channel) colors are pseudo colors. The images show staining for (A) the basement membrane (laminin), (B) myosin heavy chain type I fibres (MHC-I), (C) nuclei (Hoechst dye), (D) satellite cells (Pax7), (E) merged image of laminin, nuclei and Pax7. Thin white arrows indicate autoflourescent lipofuscin, thick arrows indicate those cells considered to be myonuclei (thick white arrows) and satellite cells (thick yellow arrow). Scale bars 10 μm

10 minutes and incubated overnight at 4°C with primary antibodies for basement membrane (rabbit anti-laminin IgG; 1:1000; Z0097; DAKO), SCs (mouse anti-Pax7 IgG1; 1:20; Pax7, DSHB) and myosin heavy chain I (mouse anti-MHC-I IgG2b; 1:200; BA.D5; DHSB). The following day slides were incubated for 45 minutes in secondary antibodies for laminin (far-red, Alexa Fluor 680 goat anti-rabbit IgG; 1:500; A-21076), Pax7 (green, Alexa Fluor 488 goat anti-mouse IgG1; 1:200; A-21121) and MHC-I (red, Alexa Fluor 568 goat anti-mouse IgG2b; 1:500; A-21144), followed by incubation for 5 minutes with Hoechst dye (Hoechst 33342; 2.5μg mL^{-1}; H1399; Invitrogen) diluted in TBS for visualization of nuclei (blue), and mounted with cover glasses in mounting medium (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36930). Representative images from a young man, old man and old woman are presented in Figure 8.

To ensure that small type II fibres (MHC-I negative fibres) were not regenerating fibres, it was decided to include a staining for neonatal and/or embryonic myosin (MHCn/e) in a second set of biopsies. The sections were incubated on day 1 with primary antibodies for basement membrane (rabbit anti-laminin IgG; 1:500; Z0097; DAKO), neonatal myosin (mouse anti-MHCn IgG1; 1:100; NCL-MHCn; Novocastra) and embryonic myosin (mouse anti-MHCe IgG1; 1:100; F1.652; Hybridoma Bank). The following day secondary antibodies were applied for laminin (red, Alexa Fluor 568 goat anti-rabbit IgG; 1:500; A-11036) and MHCn/e (green, Alexa Fluor 488 goat anti-mouse IgG1; 1:500; A-21121). Slides were then fixed for 12 minutes in Histofix (Histolab, Gothenburg, Sweden) and mounted with cover glasses in mounting medium with DAPI (blue, Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36931). All primary and secondary antibodies were diluted in 1% BSA in TBS (tris-base 50 mM, NaCl 154mM, pH 7.4-7.6), sections were washed 2 × 5 min in TBS between each protocol step.

6.5 | Image acquisition and analysis

Details on image acquisition and analysis can be found in the Supporting Information. Briefly, images of the sections stained for fibre size/fibre type/SCs/myonuclei were digitally captured using a confocal laser scanning microscope with a 20×/0.8NA objective and pinhole set to 3 μm (section thickness). All image analyses were performed in ImageJ (version 1.51n; National Institute of Health; USA) by the same investigator (AK) who was blinded to subject ID, age, treatment and time point. Fibre size was analysed with a semi-automatic macro written for ImageJ in 220 ± 113 type I fibres (MHC-I positive fibres) and 186 ± 100 type II fibres (MHC-I negative fibres, mean ± SD, see Supporting Information and Figure S3 for details on the macro and procedures). Regions with longitudinally cut fibres and fibres on the edges of the biopsy were not included for analysis. Myonuclei were analysed in 75 ± 9 type I fibres and 77 ± 16 type II fibres randomly selected from the fibres in the fibre size analysis (see Supporting Information). Myonuclei were manually identified by visual inspection and were defined as all Pax7-negative nuclei with approximately more than half of the nucleus inside the laminin defined edge of the fibre (Figure 9C-E and Figure S4). Satellite cells (Pax7 positive nuclei within the laminin defined edge of the fibre, Figure 9D-E and Figure S4) and centrally located nuclei were analysed in 441 ± 218 type I fibres and 403 ± 267 type II fibres. It should be noted that autofluorescent lipofuscin was frequently observed in close proximity to nuclei in biopsies from Old subjects. Lipofuscin was observed in the green, red and far-red channel (Figure 9B,D,E), but not in the blue channel, and additional steps were included to prevent false positive SC counts (see Supporting Information).

Images of the sections stained for MHCn/e were captured using a widefield microscope with a 4×/0.10NA objective (see Supporting Information). MHCn/e positive fibres were quantified from all fibres in the biopsy section (1752 ± 890

fibres per biopsy cross-section) and expressed as a percentage of positive fibres of the total number of fibres within a biopsy. All fibres in the biopsy were counted in the laminin channel. At this magnification level, fibres <200 μm^2 were so small that they were often not included as fibres during the fibre count. However, the following inspection of the MHCn/e channel revealed that some of these small fibres were MHCn/e positive. To give an impression of the abundance of these small fibres at baseline it was decided to report the total count of these fibres together with the total count of normal sized MHCn/e positive fibres (>200 μm^2). These very small fibres were not included in the analysis of MHCn/e positive fibres. Because the MHCn/e staining intensity differed between fibres, these were categorized as either strongly stained (MHCn/e-ST) or moderately stained (MHCn/e-MOD). Fibres with a weak MHCn/e staining intensity, close to the background staining intensity, were not included as they could not be quantified in a reliable manner. See the Supporting Information File and Figure S5 for more details. Briefly, when the images were visually inspected it was clear that some fibres stained stronger than others. It is unclear what the difference is between a strong and moderately stained fibre, but because of the clear difference it was decided to distinguish between these fibres in the analysis. After an initial subjective evaluation, a cut-off value for the median pixel intensity was chosen (only the strongly stained fibres were visible above the cut-off level). The cut-off was a pixel value of 30 above the median background pixel value (described in detail in the Supporting Information and Figure S5). Fibres positive for MHCn/e were excluded from the fibre size/SC/myonuclei analysis.

6.6 | Detailed cluster analysis of the relationship between myonuclei and fibre size

In order to compare myonuclear content and myonuclear domain in fibres of similar size between Young and Old, clusters of fibres with a 2000 μm^2 range in size were created and analysed as previously described.[35] For details of the method see Supporting Information. The large number of fibres analysed for myonuclei in the biopsies from the 29 Old subjects (2172 type I and 2241 type II fibres) were included for a further detailed 100-fibres per cluster analysis of the relationship between fibre size and: (a) myonuclei per fibre; (b) myonuclear domain; (c) fibre perimeter per myonucleus. Fibres were ordered by size and a cluster was created for every 100 fibres throughout the full fibre-size range. This analysis was a modified version of a previously published 500 μm^2 cluster analysis.[35]

6.7 | mRNA

Biopsies from the Old subjects in the RT and CON group were included for analysis of mRNA. The particular targets were chosen in order to assess a range of possible adaptations, in myofibres (e.g. myosin isoforms, myostatin), extracellular matrix (e.g. collagens, TenC) and inflammatory markers (e.g. TNF-α, CD68). In addition, gene expression of the acetylcholine receptor subunits was included based on the findings of fibres expressing developmental myosin.

6.7.1 | RNA extraction

100 cryo sections of 10 μm (2-5 mg tissue) from the embedded muscle tissue were homogenized in 1 mL of TriReagent (Molecular Research Center, Cincinnati, OH, USA) containing five stainless steel balls of 2.3 mm in diameter (BioSpec Products, Bartlesville, OK, USA), and one silicon-carbide sharp particle of 1 mm (BioSpec Products), by shaking in a FastPrep®-24 instrument (MP Biomedicals, Illkirch, France) at speed level 4 for 15 seconds. Following homogenization, bromo-chloropropane was added in order to separate the samples into an aqueous and an organic phase. Following isolation of the aqueous phase, RNA was precipitated using isopropanol. The RNA pellet was then washed in ethanol and subsequently dissolved in 20 μL RNAse-free water. Total RNA concentrations and purity were determined by spectroscopy at 260, 280 and 240 nm. Good RNA integrity was ensured by gel electrophoresis.

6.7.2 | Real-time RT-PCR

500 ng total RNA was converted into cDNA in 20 μL using the OmniScript reverse transcriptase (Qiagen, CA, USA) and 1 μM poly-dT (Invitrogen, Naerum, Denmark) according to the manufacture's protocol (Qiagen). For each target mRNA, 0.25 μL cDNA was amplified in a 25 μL SYBR Green polymerase chain reaction (PCR) containing 1× Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (Table 2). The amplification was monitored real time using the MX3005P Real-time PCR machine (Stratagene, CA, USA). The Ct values were related to a standard curve made with known concentrations of cloned PCR products or DNA oligonucleotides (Ultramer™ oligos, Integrated DNA Technologies, Inc, Leuven, Belgium) with a DNA sequence corresponding to the sequence of the expected PCR product. The specificity of the PCR products was confirmed by melting curve analysis after amplification. RPLP0 mRNA was chosen as internal control. To validate this use, another unrelated "constitutive" mRNA, GAPDH, was measured and normalized with RPLP0. This showed a small but significant increase over time, indicating that either GAPDH increased or RPLP0 decreased due to the protein supplementation. As it is unlikely that a ribosomal protein, RPLP0, would decrease due to protein supplementation, it is considered more likely that GAPDH increased. Hence, RPLP0 was used for normalization.

TABLE 2 Primers for real-time RT-PCR

Target	Primer name	Sense	Anti-sense
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
MHC-IB	NM_000257.3	GCCGAGTCCCAGGTCAACAAG	TGAGCAGATCAAGATGTGGCAAAG
MHC-IIA	NM_017534.5	TTGCTGAGTCCCAGGTGAACAA	TTTGTGCCTGTCTTCAGTCATTCC
MHC-IIX	NM_005963.3	CTGAGGGTGAAGAGCAGGGAGGT	TTTTCACATTTTGTGCATTTCTTTGG
Desmin	NM_001927.3	AAGATGGCCCTGGATGTGGAG	TTGAGGGCAGAGTAGGTCTGGATG
XIRP1	NM_194293.3	AAACACCCCATCAGCCAAGAGA	GAGAAGAAGGGGCAGAGATGAGGA
XIRP2	NM_001079810.3	CAAGGTGAAGAAACAATTTGAGGACGA	GTGCCAACCTGGCTGCTATGAA
IGF-1Ea	NM_000618.3	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC
cMet	NM_001127500.2	AACCCGAATACTGCCCAGACCC	TGATATCCGGGACACCAGTTCAG
Myostatin	NM_005259.2	TGCTGTAACCTTCCCAGGACCA	GCTCATCACAGTCAAGACCAAAATCC
Atrogin1/MAFbx	NM_058229.3	TGTTACCCAAGGAAAGAGCAGTATGGA	ACGGAGCAGCTCTCTGGGTTATTG
MURF1	NM_032588.3	TGGGGGAGCCACCTTCCTCT	ATGTTCTCAAAGCCCTGCTCTGTCT
IL6	NM_000600.4	GAGGCACTGGCAGAAAACAACC	CCTCAAACTCCAAAAGACCAGTGATG
TNFα	NM_000594.3	TTCCCCAGGGACCTCTCTCTAATC	GAGGGTTTGCTACAACATGGGCTAC
CD68	NM_001251.2	CAGCTTTGGATTCATGCAGGACC	CTCTGCCCCAGGGGTGCTTG
AGER	NM_001136.4	TGCCTCTGAACTCACGGCTGGT	TTCCCATCCAAGTGCCAGCTAA
EIF2AK3	NM_004836.6	TCAGCACTCAGATGGAGAGAGTCAGG	CTTGAACCATCACGTACTCACAAGGA
JunD	NM_005354.5	CGAGTCCACATTCCTGTTTGTAATCCT	GAAAACAGAAAACCGGGCGAAC
COL1A1	NM_000088.3	GGCAACAGCCGCTTCACCTAC	GCGGGAGGACTTGGTGGTTTT
COL3A1	NM_000090.3	CACGGAAACACTGGTGGACAGATT	ATGCCAGCTGCACATCAAGGAC
COL4A1	NM_001845.5	TCGCTGTGGATCGGCTACTCTT	CGATGAATGGCGCACTTCTAAAC
LAMB1	NM_002291.2	GGACAAGAGCAATGAGGAGCTGAGA	AGCAACTGCTTCAATGCTGTCCAA
LOX	NM_002317.6	CGCTGTGACATTCGCTACACAGGAC	CATTGGGAGTTTTGCTTTGCCTTCT
TenC	NM_002160.3	CAGCCAAGATCCAGGCACTCAA	GTCCTTGGGGAAGGGGTACAGG
TGFβ2	NM_003238.4	CCCAAAAGCCAGAGTGCCTGAA	ATGTAGCGCTGGGTTGGAGATG
TCF7L2	NM_001146274.1	CGGAAGGAGCGACAGCTTCAT	GTCTCTCCCGGCTGCTTGTCC
MMP2	NM_004530.5	CCGCCTTTAACTGGAGCAAAAACA	TTGGGGAAGCCAGGATCCATTT
MMP14	NM_004995.3	CCTACCGACAAGATTGATGCTGCT	TCCACTGCCCTGAGCTCTTCGT
AchR1A(CHRNA1)	NM_000079.3	GCAGAGACCATGAAGTCAGACCAGGAG	CCGATGATGCAAACAAGCATGAA
AchR1B(CHRNB1)	NM_000747.2	TTCATCCGGAAGCCGCCAAG	CCGCAGATCAGGGGCAGACA
AchRD(CHRND)	NM_000751.2	CAGCTGTGGATGGGGCAAAC	GCCACTCGGTTCCAGCTGTCTT
AchRE(CHRNE)[a]	NM_000080.4	TGGCAGAACTGTTCGCTTATTTTCC	TTGATGGTCTTGCCGTCGTTGT
AchRG(CHRNG)	NM_005199.4	GCCTGCAACCTCATTGCCTGT	ACTCGGCCCACCAGGAACCAC

[a]Number of molecules too low for analysis.

6.8 | Statistics

The change in SC- and myonuclear-content in the RT-group was the primary outcome in the present study. For this age-group there were no data available on these outcomes for a power calculation. A retrospective power calculation revealed that we could detect a change of 0.015 SCs per type II fibre and 0.27 myonuclei per type II fibre (with 12 subjects in the RT-group, setting a power of 0.8, a significance level at 0.05, an SD of 0.019 and 0.33 for the change in type II fibre SC-content and myonuclear content respectively). Anthropometric data for Young and Old were analysed with a 2-tailed unpaired *t*-test. Cross-sectional comparison of fibre size, SCs per fibre, myonuclei per fibre and myonuclear domain between Young men and Old men, and between Old men and Old women was performed with a mixed 2-way ANOVA with one group factor and one repeated factor for age and fibre type (age [Young men vs Old men, group factor] × fibre type [type I vs type II, repeated factor]), and for sex and fibre type (sex [Old men vs Old women, group

factor] × fibre type [type I vs type II, repeated factor]). The effect of the RT-intervention in Old subjects on these parameters was analysed for each fibre type with a mixed 2-way ANOVA with one group factor and one repeated factor: (time [Pre vs Post, repeated factor] × treatment [RT vs CON, group factor]). Linear correlation was evaluated with Spearman's rho (r) for the relationship between myonuclear parameters in the 100-fibres per cluster analysis. Between groups differences within a 2000 μm^2 cluster were analysed with a 2-tailed unpaired t-test, and differences between 2000 μm^2 clusters within groups were tested with a one-way ANOVA. Data for MHCn/e and central myonuclei were non-normally distributed, and were analysed with the Mann-Whitney Rank Sum test (Young vs Old), and with the Wilcoxon Signed rank test for changes in Old from Pre to Post within RT and CON. mRNA data were log2 transformed before statistical analysis. Individual Post intervention mRNA data were expressed relative to the individual Pre mRNA data, before being analysed with a mixed 2-way ANOVA with one group factor and one repeated factor (time [Pre vs Post, repeated factor] x treatment [RT vs CON, group factor]). Cohen's D was calculated to provide effect sizes on parametric data. Post hoc testing was performed with the Holm-Sidak method. Data are means ± SD, unless otherwise stated. A P-value <0.05 was considered statistically significant. All statistical analyses were performed with SigmaPlot vs 13.0 (Systat Software Inc, San Jose, CA).

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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