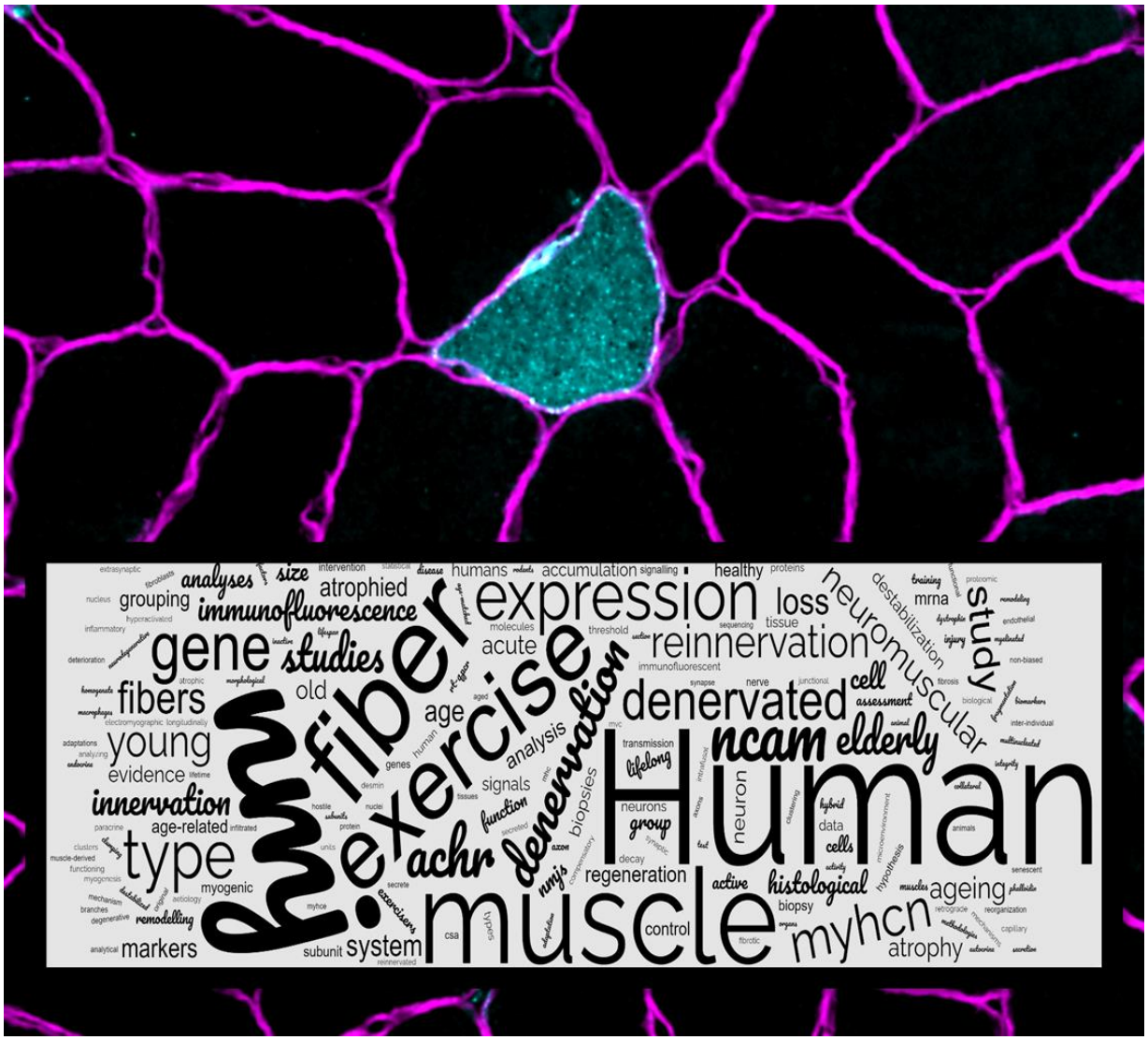
UNIVERSITY OF COPENHAGEN
Faculty of Health and Medical Sciences

PhD Thesis

Casper Søndenbroe

Maintaining Muscle Innervation Through Exercise



Supervisors: Abigail L. Mackey-Sennels & Jesper L. Andersen

This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen, 25th of February 2022

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Submitted on: 25th of February 2022

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Preface and acknowledgements

The studies included in this thesis cover the work done during my 4 years at the Institute of Sports Medicine Copenhagen (ISMC), the last 3 of these as a PhD student. It's a bit odd to imagine that a failed gymnastics exam, was the decisive element that led me to ISMC. Jesper L. Andersen was the first person with whom I was in contact with, and he (apparently) believed in me enough to co-supervise me during both my master's and PhD studies. I thank you for that, as well as for always trying to understand my way of thinking. To solidly place my foot inside the door of ISMC has, without a doubt, been the right decision, as I found myself in a supportive environment with freedom within limits and a sense of unity, which is realized by the leadership of Michael Kjær. In terms of supervision and mentoring, no one has been more influential to me than Abigail Mackey. You offer not only scientific creativity and sound explanations, but also an atmosphere where it is okay to launch stupid ideas and news on failed experiments. I also want to thank Peter Schjerling for often putting me on the spot to justify arguments and decisions with your laser-sharp questions and comments.

Although it is only my name that is highlighted on the front page, several people have contributed to this thesis. There are too many to mention here, but a big thanks goes to Anders Karlsen, Rene Svensson, the secretary office (Maria, Else and Charlotte) and Vibeke Pless (Dpt. of Dermatology). I also want to extent my gratitude to the study participants, without whom, scientific progress would not be possible. Last, but not least, I warmly acknowledge the contributions made by the lab technicians, Camilla Sørensen, Anja Jokipii-Utzon and Ann-Christina Reimann. Although your work does not always receive the recognition that it deserves, the extent of your contributions is only matched by the pleasure of your company.

I have initiated several collaborations during my PhD, and I believe that there are interesting discoveries waiting down the line. For that, I'm thankful towards Jens Hannibal, Claire Meehan, and Julien Ochala. I also had the privilege of visiting the lab of Benedicte Chazaud. While this was a scientifically successful endeavour, nothing tops repeatedly defeating her senior researcher (Rémi) in badminton. The stay was cut short due to external circumstances, but it had the effect of showing me that going abroad is not so bad after all.

A quote to end: "the days have been long, but time have gone by quickly". What this means is that I have spent a lot of time working on my project, which have taken time away from being at home. It is therefore fitting to say that my wife, and soon to be mother of my child, Laura Søndenbroe, has showed exceptional understanding and consideration on an almost daily basis. **Thank you and thank you all.**

List of papers

Paper 1

Soendenbroe C, Heisterberg MF, Schjerling P, Karlsen A, Kjaer M, Andersen JL & Mackey AL (2019). Molecular indicators of denervation in ageing human skeletal muscle. Muscle & Nerve 60, 453–463. *Published.*

Paper 2

Soendenbroe C, Bechshøft CJL, Heisterberg MF, Jensen SM, Bomme E, Schjerling P, Karlsen A, Kjaer M, Andersen JL & Mackey AL (2020). Key Components of Human Myofibre Denervation and Neuromuscular Junction Stability are Modulated by Age and Exercise. Cells 9, E893. *Published.*

Paper 3

Soendenbroe C, Heisterberg MF, Schjerling P, Kjaer M, Andersen JL & Mackey AL. The influence of 16 weeks of heavy resistance exercise on gene expression and protein markers of denervation in elderly human skeletal muscle. *Under revision.*

Paper 4

Soendenbroe C, Dahl C, Meulengracht C, Tamas M, Svensson RB, Schjerling P, Kjaer M, Andersen JL & Mackey AL (2022). Preserved stem cell content and innervation profile of elderly human skeletal muscle with lifelong recreational exercise. J Physiol. *Accepted 14/2-22.*

Abbreviations

AChR: Acetylcholine receptor

BMI: Body mass index

BrdU: 5-bromo-2-deoxyuridine

BSA: Bovine serum albumin

CRP: C-reactive protein

CSA: Cross sectional area

DEXA: Dual energy X-ray absorptiometry

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GPS: L-Glutamine-Penicillin-Streptomycin solution

HbA1c: Haemoglobin A1c

IgG: Immunoglobulin G

LBM: Lean body mass

LLEX: Lifelong exercise

mRNA: Messenger Ribonucleic Acid

MTJ: Myotendinous junction

MuSK: Muscle-specific-kinase

MVC: Maximal voluntary contraction

MyHC: Myosin heavy chain

MyHCe: Embryonic myosin heavy chain.

MyHCn: Neonatal myosin heavy chain

Nm: Newton meter

NMJ: Neuromuscular junction

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

RNA: Ribonucleic Acid

ROI: Region of interest

RPLP0: Ribosomal Protein Lateral Stalk Subunit P0

RT qPCR: Reverse transcription quantitative polymerase chain reaction

SED: Sedentary

TBS: Tris-buffered saline

Summary

Ageing leads to a loss of muscle mass and function, which is associated with frailty and loss of independence. This loss is driven by detrimental changes in the neuromuscular system. The neuromuscular system is composed of motor neurons, axons, neuromuscular junctions (NMJ) and muscle fibres. The loss of connection between a motor neuron and the muscle fibres it previously innervated is called denervation. Denervation upregulates the expression of several genes and proteins in the entire muscle fibre, including neural-cell-adhesion-molecule (NCAM), neonatal myosin heavy chain (MyHCn) and acetylcholine receptors (AChR). However, this knowledge is mostly derived from animal studies, and the number of human studies that assess denervation is sparse. Furthermore, there is evidence from animal studies and from human studies using electromyography, that exercise might protect against denervation, either through protective effects on motor neurons or through a stabilizing effect on NMJs. The overall aim of this thesis was to investigate muscle fibre denervation in muscle tissue obtained from young and elderly individuals, at rest and in response to acute, long-term, and lifelong exercise. This was done across four studies.

The aim of **study 1** was to investigate muscle fibre denervation in muscle tissue samples collected from a large (n=70) cohort of elderly men (65-94 years old) using three different immunofluorescent markers and AChR gene expression. It was hypothesized that indices of denervation would be positively correlated with age and that there would be a high level of agreement between markers. Two out of three immunofluorescent markers (NCAM and MyHCn) were observed in higher levels in the elderly participants compared to values previously reported for young individuals. Also, the gamma AChR subunit showed, as the only marker, a correlation with age (negative). Several questions arose from this study which necessitate a young reference group. Therefore, in **study 2**, 11 elderly females, 12 young females and 25 elderly males, were studied. The purpose was to compare markers of denervation directly between young and old, to investigate the effect of acute resistance exercise on AChR gene expression and finally to evaluate the ability of satellite cells *in vitro* to transcribe genes related to NMJ maintenance. A higher number of denervated fibres and a higher AChR gamma subunit gene expression was observed in muscle of elderly women compared to young. Furthermore, age-related differences in expression of genes related to NMJ maintenance were observed in cultured satellite cells. Lastly, AChR gene expression was affected by acute resistance exercise in a subunit and time dependent manner. This finding suggested that repeated bouts of exercise

(long-term training) might positively impact the neuromuscular system, which then became the objective of **study 3**. The hypothesis was that a long-term intervention of resistance exercise would reduce the number of denervated muscle fibres and alter AChR gene expression positively. 38 healthy elderly men underwent 16 weeks of heavy resistance exercise, while 20 individuals served as sedentary controls. Tests were performed before, midway through (8 weeks) and after (16 weeks) the exercise period. The training intervention led to hypertrophy and increased muscle strength but did not reduce the number of denervated fibres on the protein level. However, two out of five AChR subunits showed a favourable response after 8 weeks, suggesting positive adaptations at the level of the NMJs. Based on these findings it was speculated that an even longer timeframe was necessary to observe exercise induced alterations in denervation on the protein level. As such, in **study 4**, the purpose was to investigate denervation, muscle morphology and satellite cell quantity and function in lifelong recreational exercisers compared to sedentary age-matched and young individuals. It was hypothesized that lifelong recreational exercise would partially protect against age-related changes, including denervation. In accordance with the hypothesis, lifelong training was associated with improved muscle function during an exercise challenge, a higher number of type II muscle fibre associated satellite cells and a remarkable resemblance in AChR gene expression profile between lifelong exercisers and young counterparts.

The studies conducted for this thesis have provided evidence that muscle fibre denervation is an integral part of human ageing, with ensuing muscle atrophy and muscle weakness. Exercise appears to offer some protection against denervation, although differences between active and sedentary individuals likely become clearer at late stages of ageing. Future studies should attempt to uncover the mechanisms associated with the mitigating effect of exercise on age-related changes in the neuromuscular system.

Resumé (dansk)

Aldring er forbundet med tab af muskelmasse og -funktion, hvilket leder til forringet funktionsevne, øget skrøbelighed, og tab af uafhængighed. Dette aldersrelaterede henfald kan i vidt omfang tilskrives ændringer i det neuromuskulære system. Det neuromuskulære system består af motor neuroner, nervefibre, neuromuskulære synapser og muskelfibre, og aldersrelaterede ændringer kan observeres i alle disse komponenter. Konsekvensen af disse ændringer er hæmmet eller fuldstændigt tab af forbindelse mellem et motorneuron og dets muskelfibre; dette kaldes denervering. Denervering leder til opregulering af en række gener og proteiner i hele muskelfiberen, herunder *neural-cell-adhesion-molecule* (NCAM), *neonatal myosin heavy chain* (MyHCn) og acetylkolinreceptoren (AChR). Denervering leder også til et øget antal muskel stamceller (kaldet satellitceller). Dyreforsøg har vist at denervering kan detekteres i musklerne på både gen- og proteinniveau, men dette er utilstrækkeligt undersøgt i humant væv. Derudover, findes der, dels fra dyreforsøg og dels fra humane forsøg med anvendelse af elektromyografi, evidens for at fysisk aktivitet kan modvirke denervering, formodentlig via en beskyttende effekt på motorneuroner eller ved at bevare neuromuskulære synapser. Det overordnede formål med denne afhandling var at evaluere brugen af molekylære markører for denervering på gen- og proteinniveau i muskelvæv udtaget fra unge og ældre forsøgspersoner, i hvilende tilstand samt i respons på akut eller længerevarende og livslang træning. Dette formål blev undersøgt hen over 4 studier.

Formålet med **studie 1** var at evaluere brugen af tre forskellige immunfluorescerende markører for denervering samt at undersøge AChR genekspressionen i en stor kohorte af ældre mænd. Hypotesen var, at indenfor et aldersspænd på 65-94 år, ville denerveringsmarkørerne være positivt associeret med alder, samt at de enkelte markører I vidt omfang ville markere de samme muskelfibre. To ud af tre immunfluorescerende markører (NCAM og MyHCn) viste højere niveauer i muskler fra ældre forsøgspersoner, sammenlignet med hvad der tidligere er rapporteret for unge forsøgspersoner. Derudover, så var AChR gamma underenheden, som den eneste markør, associeret med alder (negativt). En række nye spørgsmål opstod som følge af dette projekt, hvilket nødvendiggjorde sammenligning med en ung referencegruppe, samt en klarlæggelse af betydningen af fysisk aktivitet for disse markører. Dette ledte til **studie 2**, hvor formålet var 1) at sammenligne muskulære denerveringsmarkører mellem unge og ældre forsøgspersoner, 2) at undersøge effekten af et akut styrketræningspas på genekspressionen af de fem AChR underenheder, samt 3) at undersøge satellitcellers evne til at transskribere gener af betydning for vedligeholdelse af neuromuskulære synapser *in vitro*. Hypotesen var at tydelige

aldersrelaterede forskelle ville blive observeret, samt at et akut styrketræningsarbejde ville påvirke AChR genekspressionen. Dette blev undersøgt i to studier bestående af 11 ældre og 12 unge kvindelige forsøgspersoner, samt af 25 ældre mandlige forsøgspersoner. Der blev observeret flere denerverede muskelfibre i ældre sammenlignet med unge, mens AChR gamma underenheden var markant højere udtrykt i den ældre muskel. Derudover var satellitcellers evne (*in vitro*), til at opregulere ekspression af gener relateret til neuromuskulære synapser, påvirket af aldring. Slutteligt, så blev AChR genekspressionen påvirket af et akut styrketræningspas i et AChR underenheds- og tidsspecifikt mønster. Som følge heraf gav det mening at undersøge effekten af længerevarende træning på det neuromuskulære system. Formålet med **studie 3**, var derfor at undersøge effekten af længerevarende tung styrketræning på både gen- og proteinniveau denerveringsmarkører. Hypotesen var at træningsinterventionen ville reducere antallet af denerverede muskelfibre samt påvirke AChR genekspressionen positivt. 38 ældre mænd gennemgik 16 ugers tung styrketræning, mens 20 ældre mænd fungerede som inaktive kontroller. Test af muskelstyrke samt udtagelse af muskelbiopsier blev foretaget før, midtvejs (8 uger) samt efter (16 uger). Træningsinterventionen afstedkom markante forøgelser af muskelstørrelse og -styrke, men ledte ikke til en reduktion af denerverede muskelfibre, evalueret med immunfluorescens. Omvendt, så var to AChR underenheder nedreguleret efter 8 uger, hvilket indikerer at træningen havde en positiv indvirkning på stabiliteten af neuromuskulære synapser. Disse fund antyder at en længere tidshorisont kunne være nødvendig for at effekten af fysisk aktivitet på forekomsten af denerverede muskelfibre på proteinniveau kunne detekteres. Dette ledte til **studie 4**, hvor formålet var at undersøge denervering, muskelmorfologi samt satellitcelle antal og funktion hos ældre personer som enten havde været rekreativt fysisk aktive eller sedentære hele livet. Hypotesen var at livslang fysisk aktivitet ville lede til delvis beskyttelse mod aldersrelateret fald i muskelfunktion, bl.a. via beskyttelse mod denervering. Foruden 16 fysisk aktive ældre og 15 sedentære ældre, så blev også 15 unge sedentære forsøgspersoner rekrutteret. I overensstemmelse med hypotesen, så medførte livslang træning en optimeret muskelfunktion under et akut styrketræningspas, et højere antal type II muskelfibre associerede satellitceller samt stor lighed i AChR genekspressionen sammenlignet med den unge referencegruppe.

Overordnet, så har denne afhandling påvist at aldring leder til muskelfibre denervering samt ændringer i AChR genekspression. Det neuromuskulære system undergår drastiske ændringer ved aldring, hvor denervering er en drivende kraft bag tabet af muskelfunktion. En række positive fund blev gjort i relation til fysisk aktivitet og dets beskyttende effekt mod denervering, ikke desto mindre er flere studier er påkrævet for at verificere disse fund.

Introduction

Age-related changes in the neuromuscular system

The skeletal muscles, and the motor neurons residing in the spinal cord make up key components of the neuromuscular system and is, by virtue of input received from the brain, responsible for the generation of voluntary movements (Stifani, 2014). Motor neurons are irreplaceable post-mitotic cells that convey signals from the brain by sending action potentials along the axon until reaching the synapse connecting to the muscle fibres; the neuromuscular junction (NMJ). Acetylcholine released from the pre-synaptic compartment cross the synaptic cleft and bind to Acetylcholine receptors (AChR) clustered at the post-synaptic compartment causing depolarization and muscle contraction. A simplified overview of the neuromuscular system is shown in figure 1. Notice that an axon from one motor neuron branch and innervate multiple muscle fibres; these are collectively called a motor unit.

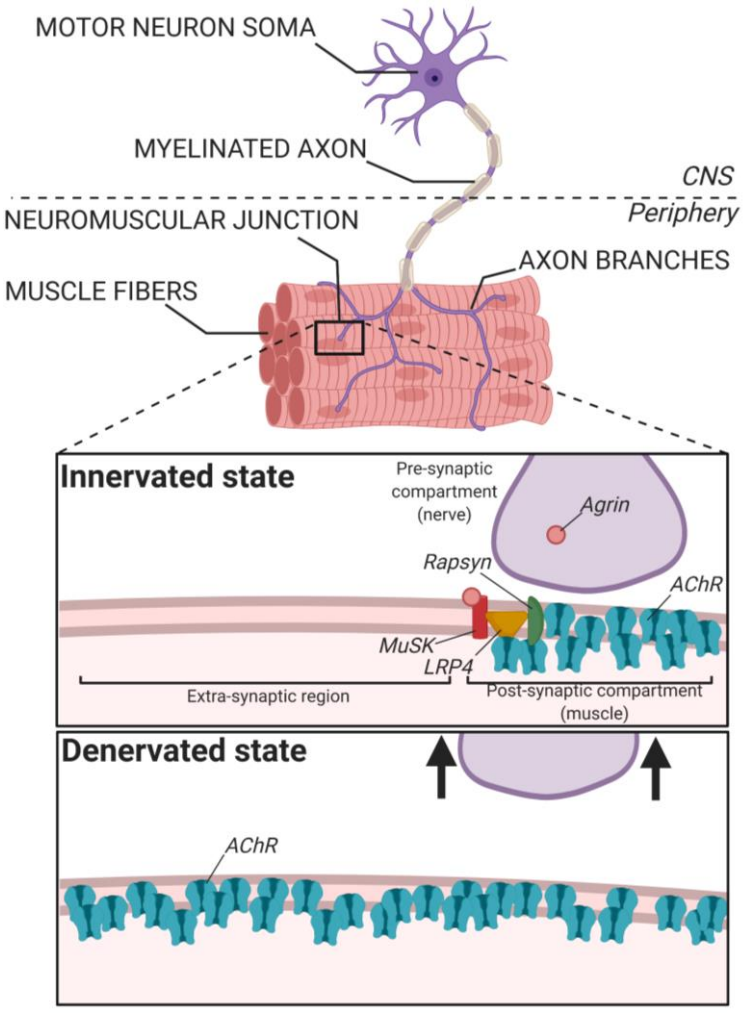

Figure 1: Motor neurons, that reside in the spinal cord, send out myelinated axons that innervated all skeletal muscles in the body. A single motor neuron and all the individual muscle fibres that it innervates is called a motor unit. The intersection between axon and muscle fibre is collectively called the neuromuscular junction, which consists of a pre-synaptic compartment (nerve side) and a post-synaptic compartment (muscle side). In the innervated state, acetylcholine released from the nerve terminal bind to AChRs imbedded in the muscle fibre membrane and cause muscle contraction through a series of sequential steps. Clustering of AChRs on the muscle fibre membrane opposite of the nerve terminal is organized by a signalling cascade involving the release of Agrin and a subsequent interaction between MuSK, LRP4 and rapsyn. In the denervated state, AChRs are no longer clustered in the synaptic region, but are expressed homogenously in the muscle. Other elements central for functioning NMJs have been omitted from the drawing for simplicity, but include Schwann cells, voltage-gated channels, axon potentials and acetylcholinesterase. This illustration was created using BioRender.

Profound changes in the neuromuscular system takes place during ageing, which over time causes defect in synaptic transmission and ultimately muscle fibre denervation. In the neuronal part of the neuromuscular system, a loss of motor neurons have been documented in rodents (Ishihara *et al.*, 1987; Rowan *et al.*, 2012; Fogarty *et al.*, 2018) and in humans (Kawamura *et al.*, 1977; Tomlinson & Irving, 1977). Furthermore, a reduction in the number of myelinated axons have also been documented in rodents (van Steenis & Kroes, 1971; Samorajski, 1974; Hashizume *et al.*, 1988; Ansved & Larsson, 1990) and in humans (Corbin & Gardner, 1937; Gardner, 1940; Swallow, 1966; Tohgi *et al.*, 1977; Mittal & Logmani, 1987). It should be pointed out that age-related loss of motor neurons is not a consistent finding in rodents (Chai *et al.*, 2011; Bütikofer *et al.*, 2011) and that reports in humans are sparse. The decay of motor neurons is problematic as there are no stem cells that can replace lost motor neurons, which therefore can lead to muscle fibre denervation, as a signal is no longer transported across the synapses to the muscle fibres. Given that a single motor neuron can control from tens to thousands of muscle fibres, depending on the muscle, the loss of a single motor neuron will jeopardize the survival of many muscle fibres.

In addition to a loss of motor neurons, degenerative alterations in the morphology and functionality of NMJs occur during ageing in rodents (Courtney & Steinbach, 1981; Smith & Rosenheimer, 1982; Fahim & Robbins, 1982; Rosenheimer, 1990; Valdez *et al.*, 2010; Chai *et al.*, 2011; Bütikofer *et al.*, 2011; Cheng *et al.*, 2013; Chung *et al.*, 2017; Liu *et al.*, 2017; Gillon *et al.*, 2018) and in humans (Arizono *et al.*, 1984; Oda, 1984; Wokke *et al.*, 1990). The morphological changes include increased fragmentation and degeneration of junctional folds in the post-synaptic compartment, while the pre-synaptic compartment show distorted branching and a decrease in the abundance of nerve terminals (Iyer *et al.*, 2021). There is some ambiguity in these findings and the number of studies that assess NMJ morphology in humans is limited (Arizono *et al.*, 1984; Oda, 1984; Wokke *et al.*, 1990; Jones *et al.*, 2017; Boehm *et al.*, 2020*b*; Aubertin-Leheudre *et al.*, 2020; Boehm *et al.*, 2020*a*; Alhindi *et al.*, 2021). Also, the importance of morphological changes has been questioned, as synaptic efficiency has been shown to be preserved in old mice despite substantial changes in morphology (Willadt *et al.*, 2016). However, that fundamental idea remains that age-related changes of the NMJ lead to a state of destabilization which hampers synaptic transmission resulting in muscle fibre denervation. The underlying reason for the degenerative alterations at the NMJ is not clear, although it likely involves disturbances of the Agrin-MuSK-LRP4-Rapsyn signalling cascade (see figure 1).

Briefly explained, a neural specific type of Agrin is synthesized in the motor neuron cell body, transported along the axon, and released from nerve terminals into the synaptic cleft, where it binds to the lamina of the juxtaposed muscle fibre. This leads to activation of a transmembrane receptor called Muscle-Specific-Kinase (MuSK), which, through an interaction with LDL-receptor-related-protein 4 (LRP4), recruits the cytoplastic protein rapsyn to the synaptic area where it, in collaboration with other proteins, stabilize and cluster AChRs (Sanes & Lichtman, 2001; Hepple & Rice, 2016).

The histological assessment of motor unit numbers and NMJ morphology have been corroborated by electrophysiological studies in humans that indirectly estimate the number of motor units in specific muscles. The methodologies vary slightly between studies, but have been used to show a lower number of motor neurons in elderly compared to young individuals (Brown, 1972; Campbell *et al.*, 1973; Vandervoort & McComas, 1986; Brown *et al.*, 1988; Doherty *et al.*, 1993; Doherty & Brown, 1993; Galea, 1996; McNeil *et al.*, 2005; Power *et al.*, 2010, 2012; Piasecki *et al.*, 2016*b*; Gilmore *et al.*, 2018). Another feature of the electrophysiological studies is that they can be used for studying the stability of synaptic transmission (Stålberg & Sonoo, 1994), by analysing the variability in the action potential. Using this approach, increased variability, indicative of decreased NMJ efficiency have been reported in aged muscle (Power *et al.*, 2016; Gilmore *et al.*, 2018), which is in support of the histological findings previously mentioned. Additionally, ageing has been associated with reduced motor unit firing frequency (Connelly *et al.*, 1999; Kamen & Knight, 2004) and nerve conduction velocity (Walsh *et al.*, 2015), as well as, during maximum contractions, a reduced voluntary activation (Klass *et al.*, 2007) and an increased antagonist activation (Häkkinen *et al.*, 2000; Macaluso *et al.*, 2002).

The neuronal changes outlined above translate into severe consequences for the skeletal muscles. It has been shown in both rodents (Caccia *et al.*, 1979; Ishihara *et al.*, 1987; Ansved & Larsson, 1990; Bütikofer *et al.*, 2011) and humans (Inokuchi *et al.*, 1975; Lexell *et al.*, 1988) that the number of muscle fibres declines with age. Muscle fibre numbers can also be estimated based on individual fibre cross sectional area (CSA) relative to total muscle CSA and using this approach at least two studies have reported a loss of fibres (Frontera *et al.*, 2000; McPhee *et al.*, 2018). The loss of fibres is believed to be a direct consequence of denervation (Hepple & Rice, 2016). In addition to the loss of fibres, it is well known that the CSA of each muscle fibre declines with age (Lexell *et al.*, 1988). The is especially true for type II muscle fibres, where it is not

uncommon to observe fibres in very old individuals that are less than half the size of those in young individuals (Andersen, 2003). The cumulative effect of a loss of muscle fibres and atrophy at the single fibre level is a marked reduction in muscle mass and muscle function. In confirmation of this, several large-scale studies containing numerous subjects have provided convincing evidence for an age-related reduction in lean body mass (LBM) (Kostka, 2005; Goodpaster *et al.*, 2006; Jackson *et al.*, 2012; Suetta *et al.*, 2019), quadriceps CSA (Janssen *et al.*, 2000), muscle strength (Goodpaster *et al.*, 2006; Suetta *et al.*, 2019) and muscle power (Kostka, 2005; Alcazar *et al.*, 2020). Low muscle mass and muscle function is associated with functional impairment in elderly individuals (Bean *et al.*, 2002; Janssen *et al.*, 2002; Reid *et al.*, 2008), which highlight the direct connection between detrimental changes in the neuromuscular system and the clinical diagnosis of sarcopenia.

Compensatory reinnervation

Muscle fibre denervation stems from a loss of motor neurons or a weakening of transmission efficiency at the NMJ, and it is believed to be a relatively frequent phenomenon that all muscle fibres occasionally encounter (Hepple & Rice, 2016). If a mechanism that could subdue the negative impact of denervation did not exist, then the age-related decline in muscle function would be markedly steeper. The reason that not all denervated fibres perish when denervated, is that they become reinnervated by sprouts from nearby axons or by the old axon, if it remains intact. This is a highly dynamic process, where fibres are thought to become transiently disconnected multiple times throughout a lifespan. Compelling evidence for the existence of the reinnervation process comes from animal models using nerve damage or other kinds of partial or temporary denervation, which serves to imitate denervation at the fibre level (Morris, 1953; Barker *et al.*, 1966; Brown & Ironton, 1978). Here it was observed that, within 4 days following partial denervation, there was an increase in the number of nerve fibre branches from intramuscular axons (Morris, 1953), suggesting a highly dynamic response to nerve insult. These early studies also suggested a substantial capacity for enlargement of motor units, as remaining units were able to innervate and control more than 5 times the initial pool of muscle fibres (Brown & Ironton, 1978).

The reorganization of the neuromuscular system with fewer motor neurons that each control a larger sum of muscle fibres have also been shown in aged human muscle using electrophysiological measurements (Campbell *et al.*, 1973; Power *et al.*, 2010). Additionally,

using muscle histology, one of the derived effects of denervation and subsequent reinnervation is muscle fibre type grouping (Lexell & Downham, 1991). Fibre type grouping, or the closely associated number of enclosed fibres (Jennekens *et al.*, 1971), has been studied in the aged muscle (Lexell & Downham, 1991; Andersen, 2003; Aare *et al.*, 2016; Kelly *et al.*, 2018; Sonjak *et al.*, 2019; Messa *et al.*, 2020). A high degree of fibre type grouping is usually interpreted as a sign of previously denervated fibres that have become successfully reinnervated (Mosole *et al.*, 2014). It can however also be interpreted as a preferential loss of one fibre type over another, which then leads to grouping of the remaining fibre type. The former explanation is often used as backing for the argument that repeated cycles of denervation and reinnervation occurs during ageing (Hepple & Rice, 2016), while the latter explanation relies on the assumption that denervation, due to either loss of motor neurons or destabilization of NMJs, affect type II muscle fibres more and/or at an earlier time point (Power *et al.*, 2012). The general belief is that the compensatory mechanism of axon sprouting plays a vital role in maintaining muscle fibre innervation in ageing. Eventually, compensatory mechanisms are unable to cope with extensive denervation in highly aged muscle, which leads to accumulation of denervated fibres and eventual loss of fibres (Power *et al.*, 2013). The presence of denervated muscle fibres, which is a sign that denervation is starting to outpace reinnervation, will be investigated in this thesis.

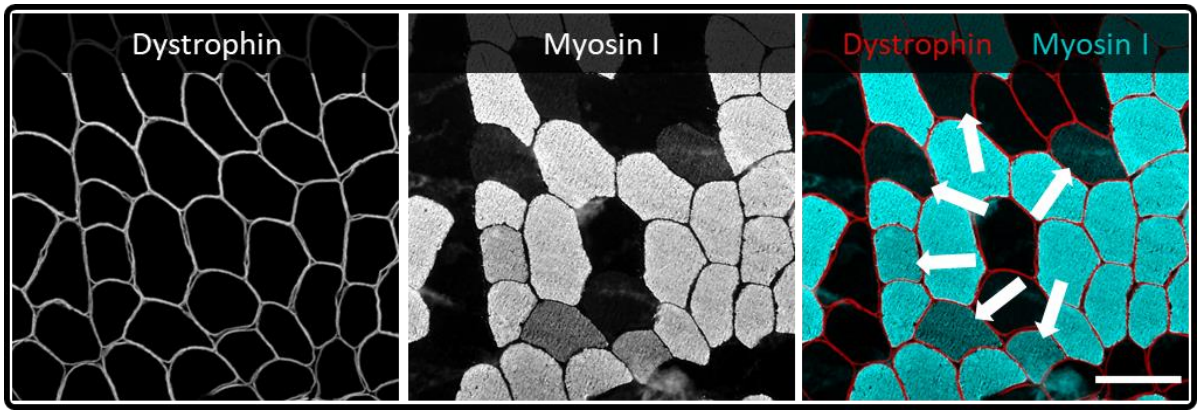

Figure 2: Example of cross-section of human muscle biopsy obtained from old individual, and immunofluorescently stained with dystrophin and MyHC I. Image is shown as split channels (left and middle) and composite image (right). White arrows point to fibres with low amounts of MyHC I (type I/II hybrid fibres). Scalebar is 100 µm.

Tentative markers of muscle fibre denervation

Several histological hallmarks of recurring cycles of denervation and reinnervation have been suggested. In a properly functioning motor unit, there is complete consistency between the

intrinsic properties of the motor neuron and all the muscle fibres that it innervates (Gardiner & Kernell, 1990). However, at later stages of ageing there is a substantial increase in fibres that co-express multiple myosin heavy chain (MyHC) isoforms, usually referred to as hybrid or mismatched fibres (Andersen *et al.*, 1999; Rowan *et al.*, 2012). An example is provided in figure 2. Type IIa/IIx hybrids are common in young muscle, while there is an emergence of I/IIa, I/IIx and I/IIa/IIx hybrids in elderly muscle (Andersen *et al.*, 1999). The presence of these fibres in aged muscle suggests either that multiple MyHC isoforms are simultaneously transcribed, meaning that a motor neuron is no longer able to efficiently dictate the contractile properties of the fibres within its control. Or it could suggest that some muscle fibres have previously been denervated and then reinnervated by a motor neuron of another type, thereby giving rise to the multiple MyHC isoforms. Other histological hallmarks of denervation and reinnervation are the presence of atrophic fibres and clumps of nuclei (Oertel, 1986; Viguie *et al.*, 1997; Rowan *et al.*, 2012; Sonjak *et al.*, 2019). The presence of atrophic fibres reflects, that during a solitary period where no muscle contraction takes place atrophy ensues. Similarly, clumps of nuclei represent the relatively larger loss of myofibrillar content compared to myonuclei that occur following denervation (Viguie *et al.*, 1997).

Most studies that investigate muscle fibre denervation take advantage of the fact that loss of neural input represents a severe insult to a muscle fibre, which subsequently leads to drastic changes at the mRNA and protein level (Lang *et al.*, 2017). It is thus conceivable that expression of specific genes and proteins not observed in innervated muscle fibres can be used to assess innervation status. On the protein level, the markers of denervation include the sodium channel Na_v 1.5 (Kallen *et al.*, 1990; Rowan *et al.*, 2012; Gillon & Sheard, 2015), nestin (Vaittinen *et al.*, 1999, 2001), cardiac troponin T (Saggin *et al.*, 1990; Xu *et al.*, 2017), neural-cell-adhesion-molecule (NCAM) (Covault & Sanes, 1985) and neonatal myosin heavy chain (MyHCn) (Schiaffino *et al.*, 1988; Sieck & Zhan, 2000). Presence of embryonic myosin heavy chain $(MyHCe)^+$ and $MyHCn^+$ fibres are observed during muscle development (Butler-Browne & Whalen, 1984), in intrafusal fibres (Walro & Kucera, 1999; Schiaffino *et al.*, 2015), in certain facial muscles (Sartore *et al.*, 1987; Butler-Browne *et al.*, 1988; Stål *et al.*, 1994), during regeneration (Schiaffino *et al.*, 1988; Mackey & Kjaer, 2017) and in relation to various neuromuscular diseases (Fitzsimons & Hoh, 1981; Winter & Bornemann, 1999; Gosztonyi *et al.*, 2001; Doppler *et al.*, 2008). NCAM is observed in fibres devoid of innervation, such as those observed during muscle development (Moore & Walsh, 1985; Fidziańska & Kamińska, 1995), regeneration (Irintchev *et al.*, 1994; Mackey & Kjaer, 2017) and neuromuscular disease (Walsh

& Moore, 1985). Importantly, both MyHCn and NCAM have been shown to increase following denervation or disturbance of synaptic transmission (Butler-Browne *et al.*, 1982; Covault & Sanes, 1985; Covault *et al.*, 1986; Schiaffino *et al.*, 1988; Olsen *et al.*, 1995; Sieck & Zhan, 2000; Xing *et al.*, 2015; Lin *et al.*, 2021). A small number of studies have evaluated the age-related presence of NCAM and MyHCn in healthy muscle from rodents (Olsen *et al.*, 1995; Deschenes & Wilson, 2003; Snow *et al.*, 2005; Gillon & Sheard, 2015; Hendrickse *et al.*, 2018; Burke *et al.*, 2021) and from humans (Monemi *et al.*, 1996; D'Antona *et al.*, 2003; Mackey *et al.*, 2014; Mosole *et al.*, 2014; Sonjak *et al.*, 2019). It is generally observed in these studies that a higher number of $NCAM^+$ and $MyHCn^+$ fibres is observed in aged muscle compared to young. This thesis will explore the presence of NCAM and MyHCn in human muscle biopsies obtained from young and old individuals.

On the gene expression level several potential denervation markers also exists, including AChR subunits (Theroux *et al.*, 2002; Zhu *et al.*, 2007; Gigliotti *et al.*, 2015), histone deacetylases (Daou *et al.*, 2020), Runt-related transcription factor 1 (Baehr *et al.*, 2016; Messi *et al.*, 2016; Hughes *et al.*, 2017) and MuSK (Glass *et al.*, 1996; Castets *et al.*, 2019). Innervated muscle fibres are characterized by clustering of AChRs at the NMJ (Axelsson & Thesleff, 1959; Fambrough, 1979; Merlie & Sanes, 1985; Gundersen *et al.*, 1995), due to transcriptional specialization of synaptic myonuclei (Merlie & Sanes, 1985; Schaeffer *et al.*, 2001) and electric activity, enabled through the NMJ, which suppresses AChR gene expression in the non-synaptic nuclei (Frank *et al.*, 1976; Schaeffer *et al.*, 2001). Loss of innervation disperses this specialization (Pestronk & Drachman, 1978; Goldman & Staple, 1989; Witzemann *et al.*, 1991; Gundersen *et al.*, 1995; Missias *et al.*, 1996), due to lifting of the repressive electrical activity (Sanes & Lichtman, 2001), with changes in gene expression throughout the muscle fibre observed within 12 hours following denervation (Tsay & Schmidt, 1989). This is illustrated as the "denervated state" in figure 1). Thus, the gene expression of AChR subunits, of which there are five in skeletal muscle (alpha1, beta1, gamma, delta and epsilon) have been reported to increase following nerve signal blockade (Mishina *et al.*, 1986; Goldman & Staple, 1989; Witzemann *et al.*, 1991; Carlson *et al.*, 2002; Apel *et al.*, 2009). Especially the gamma subunit, which is supposedly a foetal subunit, (Mishina *et al.*, 1986; Gu & Hall, 1988), have received attention as a marker of denervation. It has been described that the gamma subunit is replaced by the adult epsilon subunit during maturation and that it is reinstated following denervation (Mishina *et al.*, 1986; Gu & Hall, 1988). However, the number of studies that measure AChR subunit gene expression in humans is limited, and most studies focus on specific diseases

(Whiting *et al.*, 1986; Hesselmans *et al.*, 1993; MacLennan *et al.*, 1997; Croxen *et al.*, 2001; Theroux *et al.*, 2002; Gattenlöhner *et al.*, 2002; Zhu *et al.*, 2007; Gigliotti *et al.*, 2015; Kelly *et al.*, 2018; Karlsen *et al.*, 2019, 2020; Daou *et al.*, 2020).

Crucially, there is no established gold standard for the assessment of ongoing denervation at either the mRNA or the protein level, and many of the tentative markers have only been investigated in animal muscle, thus creating a need for their assessment in human ageing. This thesis will focus on NCAM and MyHCn as protein markers of denervation and AChR and MuSK as markers on the gene level.

Models of exercise

Muscle mass, assessed as LBM or muscle CSA, and muscle function, assessed as muscle strength, has been shown to be protected by lifelong physical activity during ageing (Klitgaard *et al.*, 1990; Grassi *et al.*, 1991; Pearson *et al.*, 2002; Ojanen *et al.*, 2007; Aagaard *et al.*, 2007; Lanza *et al.*, 2008; Safdar *et al.*, 2010; Wroblewski *et al.*, 2011; Mikkelsen *et al.*, 2013; Mosole *et al.*, 2014; Zampieri *et al.*, 2015; Drey *et al.*, 2016; Power *et al.*, 2016; Unhjem *et al.*, 2016; Piasecki *et al.*, 2016*a*, 2019; St-Jean-Pelletier *et al.*, 2017; Sonjak *et al.*, 2019; McKendry *et al.*, 2020; Grosicki *et al.*, 2021). However, the type of activity and the level of mastery obtained within the activity are both of paramount importance for the degree of protection. Exercise, especially resistance exercise, also remains a potent stimulus for increased muscle function, even among very old individuals (Kryger & Andersen, 2007; Bechshøft *et al.*, 2017). Surprisingly, it has not been sufficiently investigated whether exercise can directly augment muscle innervation status during ageing. The rationale for an effect of exercise to ameliorate the aged phenotype could be the secretion of molecules by muscle that exert local and/or systemic effects on motor neurons (Farrash *et al.*, 2021). Accordingly, it has been shown that exercise is beneficial for the brain, where it increases neurogenesis and neurotransmission (Vecchio *et al.*, 2018).

Some findings based on either electrophysiological measurements or animal models have indicated a positive effect of exercise on innervation status during ageing. For example, Power et al., showed that lifelong runners had preserve their motor units in their active leg muscles compared sedentary peers (Power *et al.*, 2010), although this protective effect did not cover the upper arms of the runners (Power *et al.*, 2012), indicating local effects of exercise. Similarly, improved NMJ stability was observed in a group of track & field athletes (Power *et al.*, 2016).

However, these positive findings are not unopposed, as another group have failed to observe a difference between active and sedentary elderly individuals using similar electrophysiological measurements (Piasecki *et al.*, 2016*a*, 2019). One of the major limitations of this kind of cross sectional studies, is that the influence of genetics is not taken into account. Animal models generally accommodate this by virtue of their nearly identical genetic background. There are several studies using various animal models and various exercise modalities that assess the influence of exercise on innervation. The findings of these studies are mixed, although leaning towards a positive effect of exercise in relation to age-related denervation (Nishimune *et al.*, 2014). The observed effects include favourable morphological changes at the NMJ (Valdez *et al.*, 2010; Cheng *et al.*, 2013; Gillon *et al.*, 2018), optimized NMJ transmission (Chugh *et al.*, 2021) and higher MUNE (Giorgetti *et al.*, 2019).

The study of innervation status and how it is influenced by exercise is inherently difficult to study in humans. There are multiple reasons for this; firstly, there is a lack of reliably markers that can be used. Secondly, there is limited access to NMJs. Thirdly, the magnitude of any change induced by exercise must overcome the inherent change caused by ageing itself in order to be detectable. There are only three studies in humans using histochemical methodologies that have specifically investigated the influence of exercise habits on muscle innervation status during ageing (Mosole *et al.*, 2014; Sonjak *et al.*, 2019; Messa *et al.*, 2020). These studies each take very different approaches to this question, but overall, the findings indicate that exercise can diminish muscle fibre denervation and improve reinnervation. Acute studies, typically including a single bout of exercise, long-term training studies with multiple training sessions and studies of lifelong exercisers and sedentary peers are three of the most commonly used exercise models. In this thesis all these models will be used to investigate the influence of exercise on innervation status in elderly human individuals.

Aims

The main aim was to assess molecular markers of muscle denervation on the mRNA and protein level in young and elderly muscle at rest and in response to acute, long-term, and lifelong exercise. This aim was assessed across the 4 original research publications included in the thesis.

The objective of **study 1** was to evaluate three immunofluorescent markers of denervation and assess AChR gene expression in a large cohort of healthy elderly men (Soendenbroe *et al.*, 2019). It was hypothesized, that within the age-range of 65-94 years, the molecular indicators of denervation would be associated with age and that the individual markers would generally be co-expressed.

The aim of **study 2** was three-fold (Soendenbroe *et al.*, 2020). Firstly, muscle fibre denervation was compared between young and elderly individuals. Secondly, the effect of acute resistance exercise on AChR gene expression was evaluated. Thirdly, the capacity of cultured satellite cells to transcribe genes related to NMJ formation, in relation to both age and acute exercise, were measured. It was hypothesized that clear age-related effects would be observed, and that acute exercise would alter the AChR mRNA levels.

In **study 3**, the influence of a 16-week long program of heavy resistance exercise, which led to hypertrophy and increases in muscle strength, on markers of muscle fibre denervation was assessed. It was hypothesized that the intervention would reduce the number of denervated fibres and alter AChR mRNA levels positively.

In **study 4**, the goal was to comprehensively study the influence of lifelong recreational exercise on indices of muscle fibre denervation, muscle morphology and satellite cell quantity and function (Soendenbroe *et al.*, 2022). This was accomplished by recruiting lifelong recreational exercisers and age and body mass index (BMI) matched sedentary controls, and a young reference group. It was hypothesized that recreational physical activity would partially protect against the age-related decline in muscle function and provide some improvement in indices of muscle fibre innervation.

Participants and study design

The data for this thesis comes from four projects, three of which were previously conducted at the department, and the fourth which was a newly conducted project. Given that the previously conducted projects have already been described in detail in several peer-reviewed articles (Heisterberg *et al.*, 2018*a*, 2018*b*; Bechshøft *et al.*, 2019; Jensen *et al.*, 2020), less detailed descriptions will be provided of these projects in the thesis. In terms of terminology, study 1 is referred to as "aged baseline", study 2 as "acute-exercise", study 3 as "16-week training" and study 4 as "lifelong exercise".

Participants

All projects were carried out in accordance with the Declaration of Helsinki and were assessed and authorized by the Committees on Health Research Ethics for the Capital Region of Denmark (H-3-2012-081, H-15005761, H-15017223 and H-19000881). Recruitment for all projects was done using online and/or newspaper advertisement. All participants were screened by telephone and in-person meeting and signed an informed consent. A table showing basic characteristics of all subjects included in the thesis is shown below.

	Age (yr)	Height (cm)	Weight (kg)	BMI (kg/m²)
Study 1 (aged baseline) (n=70)	72 ± 6 [65-94]	178 ± 7 [161-191]	84 ± 11 [57-110]	26 ± 3 [19-33]
Study 2 (acute exercise)				
Young women (n=12)	23 ± 3 [20-28]	168 ± 7 [157-177]	64 ± 8 [53-75]	23 ± 2 [19-26]
Old women (n=11)	74 ± 3 [71-78]	166 ± 3 [162-169]	69 ± 10 [57-84]	25 ± 4 [20-30]
Old men (n=25)	70 ± 7 [64-90]	180 ± 5 [172-189]	82 ± 10 [67-98]	26 ± 3 [21-31]
Study 3 (16-week training)				
EX (n=38)	72 ± 5 [65-83]	178 ± 7 [162-191]	85 ± 11 [57-108]	27 ± 3 [19-33]
SED (n=20)	72 ± 6 [66-85]	179 ± 7 [162-190]	83 ± 11 [62-102]	26 ± 3 [21-32]
Study 4 (lifelong exercise)				
Young (n=15)	26 ± 5 [20-36]	183 ± 7 [169-193]	82 ± 13 [62-105]	24 ± 3 [20-30]
LLEX (n=16)	73 ± 4 [68-82]	176 ± 6 [166-185]	76 ± 9 [63-94]	24 ± 3 [21-31]
SED (n=15)	73 ± 4 [68-82]	178 ± 8 [161-195]	82 ± 11 [65-109]	26 ± 3 [22-32]

Tabel 1: Age, height, weight, and BMI of all participants across studies given as average ± SD [range]. Abbreviations: EX, exercise; SED, sedentary, LLEX, lifelong exercise

Study 1 (aged baseline) and 3 (16-week training)

Study 1 and 3 is based on the same project, with the former focusing on the baseline samples and the latter on the change over time following the 16-week training intervention. The aim of the original study was to examine the effect of taking the blood pressure lowering medication Losartan on the response to 16 weeks of heavy resistance exercise in healthy elderly muscle (Heisterberg *et al.*, 2018*b*). To participate, individuals had to be male, ≥65 years old, non-smoking, normotensive and with a BMI between 19-34 kg/m^2. Exclusion criteria were known diseases, anticoagulation medication and engagement in resistance exercise, or other types of physical activity on a moderate to high level. Recruited participants were allocated in a double-blinded fashion into one of three groups: Losartan supplementation and resistance exercise, Losartan supplementation and sedentary and placebo supplementation and resistance exercise. In study 1, the baseline samples, which were obtained before supplementation with Losartan was initiated, were analysed of all participants from the three groups. Importantly, in study 3 the two training groups were collapsed into one, as no effect of drug treatment was observed in the original study. Also, only participants that completed the study and were evaluated at all time points are included. The groups used in study 3 are called exercise (EX) and sedentary (SED). Statistical tests for the influence of drug treatment were also conducted and showed no effect of receiving the drug (supplemental material of study 3).

Study 2 (acute-exercise)

Study 2 is based on two projects. In one part elderly men were allocated in a double-blinded manner into one of two groups, either receiving Losartan or placebo for 18 days, with multiple muscle biopsies obtained during the study. The aim was to study the effect of Losartan supplementation on the acute response to heavy resistance exercise in healthy elderly muscle (Heisterberg *et al.*, 2018*a*). Several muscle and connective tissue related genes were analysed in the original study and Losartan supplementation only affected one gene, myostatin. It was therefore decided to collapse the two groups into one for the present study. However, statistical tests for the influence of drug treatment were also conducted in the present study and showed no effect of receiving the drug (supplemental material of study 2). To participate, individuals had to be male, ≥64 years old, non-smoking, normotensive, normal weight (BMI between 20–31 kg/m^2), free of any self-reported diseases and not using anticoagulation medication.
In the other part both young and elderly females were recruited as pairs, that underwent all experimental procedures, including an acute resistance exercise bout, in parallel. The aim of the

original study was to investigate the effect of age and acute exercise on myogenesis *in vitro* (Bechshøft *et al.*, 2019). To participate, individuals had to be female, ≥65 or ≤30 years of age, no more than mildly physically active and normal weight (BMI between 20–31 kg/m^2). Exclusion criteria were smoking, anticoagulation medication, high alcohol consumption, more than one previous biopsy from vastus lateralis, knee pain and knee/hip arthroplasty.

Study 4 (lifelong exercise)

223 individuals responded to advertisement and were subsequently screened. Inclusion criteria were male gender, age between 18-39 or ≥68, BMI between 20-32 kg/m^2. Exclusion criteria were smoking, >14 alcoholic drinks per week, >1 prior vastus lateralis biopsy, self-reported disease, knee pain, and anticoagulation medication. Potential participants were also carefully screened on their prior and current patterns of physical activity. 56 men were included into one of three groups: young sedentary (Young), elderly lifelong exercise (LLEX) and elderly sedentary (SED).

The participants in Young and SED were generally in good health, but did not take part in structured physical activity, like racket ball sports or rowing. They also did not do extensive walking or cycling for transportation. This overall description covered at least 10 and 30 years prior to participation, for Young and SED respectively. In contrast, the participants in LLEX had been recreationally active for at least 30 years prior to participation. To be included, they had to have at least partially performed an activity which was assumed to lead to type II muscle fibre recruitment. The subjects were physically active at a level where they met the recommendations for physical activity set by the World Health Organization but did not desire to compete. This places them in tier 1 in the classification framework by McKay et al. (McKay *et al.*, 2022). The specific activities reported are very broad and were not necessarily performed by the individual for their whole adult life. The number of participants performing a specific activity were: Resistance exercise (10), ball games (5), racket sports (5), cycling (5), rowing (4), running (4), gymnastics (3), athletics (2), martial arts (1), and swimming (1). The number of participants performing a specific activity as their main activity were: Resistance exercise (3), ball games (3), racket sports (3), cycling (3), rowing (1), running (1), gymnastics (1) and athletics (1). Main activity was defined as “many years” of participation and/or the primarily performed activity.

Three LLEX subjects that were included in the early phase of the project were later deemed too unfit to go into the LLEX group as they trained too little or too lightly. Additionally, 7 participants dropped out of the study either due to lack of interest (1), injury unrelated to project (1), exercise related knee pain (1), biopsy could not be obtained from both legs (3) or unknown (1). In total, 46 (15 young, 16 LLEX and 15 SED) participants completed the study with data availability for all tests and conditions.

Study design

Study 1 (aged baseline) and 3 (16-week training)

The participants underwent a 16-week periodized program of heavy resistance exercise, consisting of Leg Extensions, Leg Press and Leg Curl performed at 15-6 repetition maximum for 3-5 sets per exercise. Maximal voluntary contraction (MVC) was assessed, and muscle biopsies obtained before (pre) after 8 weeks (mid) and after the intervention (post). See figure 3 for an overview. The pre and post biopsies were obtained from the same leg, separated by 4 centimetres, and the mid biopsy was obtained from the contralateral leg. The time of day for biopsy procedures were standardized for each participant. Two days separated the most recent training session and the mid and post biopsies. The drug treatment (Losartan or placebo) was initiated after the pre-test had been performed. Biopsies were frozen and analysed by immunofluorescence and RT-qPCR.

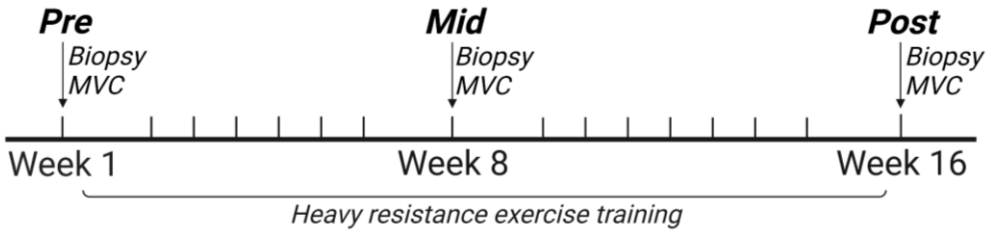

Figure 3: Study design in study 1 and 3. Test rounds are indicated by arrows and contained a single muscle biopsy and determination of MVC. Pre test was performed before the exercise intervention was commenced, and mid and post tests took place two days after the latest training session. Study 1 is based on the pre test only, whereas study 3 used all three timepoints. Abbreviations: MVC, maximal voluntary contraction.

Study 2 (acute-exercise)

The male participants underwent a single bout of unilateral heavy resistance exercise and had muscle biopsies obtained before (at rest), 4.5 hour after exercise and on day 1, 4 and 7 (see figure 4). Drug administration (Losartan or placebo) was initiated 10 days before the exercise bout. Only gene expression analysis was conducted on these samples.

In the project with the female subjects two muscle biopsies were taken in immediate succession from each leg, 5 days after a single bout of unilateral heavy resistance exercise. The leg that underwent the exercise bout is the "exercised leg", and the leg that did not exercise is the "control leg". The participants were not allowed to partake in physical activity during the period of the study. Part of the biopsies were frozen and analysed by immunofluorescence and another part was used for cell culture experiments.

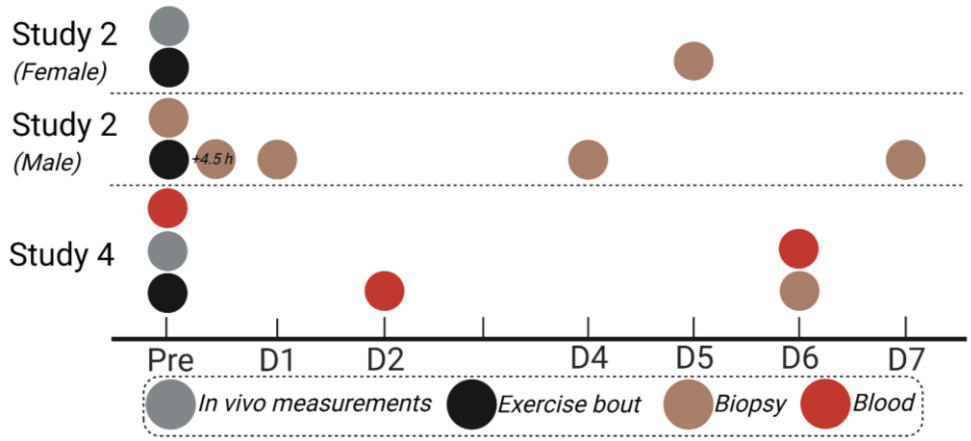

Figure 4: Study design in study 2 (both female and male part) and 4. Acute resistance exercise (black), blood samples (red), muscle biopsies (brown) and *in vivo* measurements (grey) are indicated by coloured circles (see legend) and the relative timing is indicated by the timeline. *In vivo* measurements in study 2 was determination of 1-repition maximum, and in study 4 it was DEXA and MVC.

Study 4 (lifelong exercise)

Participants reported three times to the research facility over a week (see figure 4). First, a dual energy X-ray absorptiometry (DEXA) scan was performed, and a blood sample was acquired. MVC was assessed, and participants then underwent a single bout of unilateral heavy resistance exercise. Another blood sample was obtained two days later. On day 6, two muscle biopsies were taken in immediate succession from each leg. Like in study 2, the terminology of "exercised leg" and "control/rested leg" is used to describe effects of the acute exercise bout.

Participants were instructed to consume a handed-out protein shake (Bodylab ShakeUp!, 330 mL, 26 g protein, 284 kcal) at home instead of their regular breakfast on the days of the exercise bout and the biopsies, approximately 2 hours before start of the experiment. The participants were also instructed not to perform physical activity during course of the study. Part of the biopsies were frozen and analysed by immunofluorescence and RT-qPCR and another part was used for cell culture experiments.

Methods

In vivo measurements

MVC

MVC was assessed in a dynamometer (model 500-11; Kinetic Communicator) (Farrell & Richards, 1986). Following 5 minutes of cycling on an ergometer, the participants were instructed in, and familiarized to the dynamometer. Multiple warmup attempts were provided, gradually increasing in force output. Isokinetic MVC was assessed from a 90 to 10° knee angle, at 60°/s (study 1+3) and 30°/s (study 4) angular velocity, with ~30 seconds between attempts. At least 4 attempts were given, continuing until two consecutive attempts were lower that the previously best. The attempt with the highest peak force was used as test outcome. Isometric MVC was assessed at a 70° of knee angle with ~60 seconds between attempts. Participants were instructed to contract as hard and fast as possible. After warmup attempts, three maximal attempts were conducted. If an attempt had any excessive backward movement the attempt was erased and replace by a new one. The attempt with the highest peak force was used as test outcome, except RFD values that were derived from the attempt with the highest impulse moment at 200 ms. Verbal encouragement and visual feedback were provided for both isokinetic and isometric tests.

Maximal knee extension strength

Following warmup and familiarization, the one repetition maximum (1RM) was determined in a Knee Extension machine (M452; TechnoGym, Cesena, Italy), by gradually increasing the load until an unsuccessful attempt.

Acute exercise bout

In study 2, a unilateral bout of heavy resistance exercise was performed in a Leg Extension machine (M452; TechnoGym, Cesena, Italy). The exercise bout consisted of multiple sets of concentric contractions at 70 % of 1RM and eccentric contractions at 110 % of 1RM, with ~2 min breaks between sets.

In study 4, a similar unilateral bout of heavy resistance exercise was performed, but in a dynamometer (model 500-11; Kinetic Communicator). The exercise bout consisted of two rounds, separated by a ~10 min break, each consisting of 4x10 concentric contractions (30°/s) at >70% of MVC and 4x5 eccentric contractions (30°/s) at >100% of MVC, with ~2 min breaks between sets. Force output was acquired from the 1st, 5th, and 10th concentric contraction and the 1st, 3^{rd,} and 5th eccentric contraction. In both types of exercise bout verbal encouragement and visual feedback were provided.

DEXA

The DEXA scan was performed using a Lunar DPX-IQ (GE-Healthcare). The participants drank 0.5 L of water 30 minutes before the scan and emptied their bladder immediately before. After being placed on the scanner the participants rested for 10 minutes. LBM, total bone mineral content, fat percentage and android fat mass were the designated outcome measures.

Blood sample

Blood samples were drawn from an antecubital vein and sent to the Department of Clinical Biochemistry for analysis of general health factors and creatine kinase (indirect measure of muscle damage).

Muscle biopsies

Biopsy procedure

Muscle biopsies were obtained from the mid-portion of the vastus lateralis muscle. Local anaesthetics (1% lidocaine) were applied, and the skin was then shaved, disinfected, and opened using a scalpel. The biopsy was extracted through the incision using either 5 or 6 mm Bergström

needles (Bergstrom, 1975). If biopsies were to be obtained from both legs, the location of the incision sites were aligned between the legs. In case multiple biopsies were obtained from the same leg over time, the incision sites were at least 2 cm apart to avoid potential scar tissue from prior biopsies. If two biopsies were obtained from the same incision site in one session, the biopsies were taken in proximal and distal direction, relative to the thigh. Pieces of tissue deemed appropriate for histology were aligned on syringe pistons, covered in Tissue-Tek (Sakura Finetek), and frozen in liquid nitrogen chilled isopentane (JT Baker). Tissue was stored at -80°C. In study 2 and 4, fresh tissue was used for cell culture experiments. Surplus tissue was frozen directly in liquid nitrogen.

Cell culture

Cell culture studies were performed in study 2 (Bechshøft *et al.*, 2019) and study 4. The cell culture protocol was largely based on a previous study (Agley *et al.*, 2013). Freshly harvested muscle biopsy tissue was placed inside a sterile 50 mL Falcon tube containing 25 mL phosphate-buffered saline (PBS) with 1 % penicillin-streptomycin and transferred to a laminar airflow bench. The tissue was washed in PBS, minced into small fragments, digested using 2 mg/ml collagenase B (11088815001; Roche) and 2 mg/ml dispase II (D4693; Sigma-Aldrich) diluted in basal medium (C-23260; PromoCell) and incubated at 37°C and 5% CO_2 for 60 minutes with trituration every 15th minute. It was then passed through a 40 µm cell strainer (352340; BD Falcon) and added to either a 25 or 75 µm^2 cell culture flask (690170/658170; Cellstar). Cells were grown in culture medium (C-23060; PromoCell) supplied with supplement mix (C-39365; PromoCell) and containing 15 % foetal bovine serum (ALB-S1810; Biowest) and 1 % L-Glutamine-Penicillin-Streptomycin solution (GPS, G6784; Sigma), for an average ± SD of 6.3 ± 1.4 days corresponding to ~ 80% confluency. Medium was replaced after 3 days, and the old medium was centrifuged, the cell pellet resuspended and returned to the flask. From there the medium was replaced on the 5th and 7th (if needed) day, but without returning unattached cells. Study 2 deviated slightly from this in that, cultures were always expanded for 7 days. Cells were separated from the flask using a 0.25 % Trypsin-EDTA (25200056; Gibco), which was diluted in PBS to 0.125%. Detached cells were incubated with 35 µl of CD56 magnetic beads (130-050-401; Miltenyi Biotec) and 170 ul of MACS running buffer (130-091-221; Miltenyi Biotec) for 15 minutes. The cells were then passed through a Pre-Separation filter (130-041-407; Miltenyi Biotec) mounted on a Large Cell column (130-042-202; Miltenyi Biotec), which were attached to a MultiStand magnet (130-090-312; Miltenyi Biotec), thereby separating into a CD56$^-$ fraction

(mostly fibroblasts) and a $CD56^+$ fraction (myogenic). The fibroblasts were not used for experiments included in this thesis.

The protocol for the cell culture of study 2 has been described in detail before (Bechshøft *et al.*, 2019) and was almost identical to the protocol in study 4. In study 4, ~12.000 and ~20.000 cells were plated on 18 mm. Ø glass coverslips (0111580; Marienfeld), placed in 12-well plates (353503; Corning) for proliferation and differentiation experiments, respectively. Three plates were used for each experiment and duplicates were plated in each well. Thus, 3 replicates were used for immunostaining and 3 were used for RNA extraction. Furthermore, as biopsies were obtained from both the exercised and control leg then these cells were cultured on the same plates. For the proliferation experiment, cultures were initially run for 3 days in culture medium, followed by the addition of 10 μM of 5-bromo-2-deoxyuridine (BrdU) for a 5-hour period. For the differentiation experiment, cultures were run for 3 days in culture medium, and then for 4 days in basal medium supplemented with 1 % GPS. The medium was changed after 2 of the 4 days. After the designated periods, cells were either processed for RNA extraction or fixed using Histofix (Histolab) for immunostaining.

Immunofluorescence, microscopy and image analyses

From muscle biopsies, 10 μm cross section were cut at −20°C in a cryostat and placed on glass slides (Thermo Scientific). Primary and secondary antibodies used in each study are shown in table 2. The general protocol for immunofluorescent staining of muscle cross sections was as follows. Slides were removed from the freezer, allowed to dry, and then encircled using a hydrophobic pen. Slides were washed once in tris-buffered saline (TBS) for 5 minutes. Primary antibodies were then diluted in 1% bovine serum albumin (BSA) in TBS, and applied to the sections for overnight incubation at 5°C. The next day, sections were washed twice in TBS (5 minutes), and then incubated with secondary antibodies diluted in 1% BSA in TBS for 45 minutes at room temperature. Following two 5 minutes washes in TBS, sections were fixed in Histofix (Histolab) for 12 minutes. The only exception is the Laminin, Pax7 and myosin I staining, where fixation was done before incubation with primary antibodies. The reasoning behind fixing after incubation with secondary antibodies is that the signal from some antibodies, MyHCe and MyHCn in particular, will then be stronger (A.L.M., personal communication). Ultimately, drops of Prolong-Gold-Antifade (P36931; Thermo Fisher Scientific) mounting medium containing 4',6-diamidino-2-phenylindole (DAPI), were applied to the sections, and

they were mounted using cover glasses. Samples were stored in dark conditions for ~2 days before being moved to a −20°C freezer.

For staining of cell cultures in study 2 and 4, cells were washed once in TBS for 5 minutes, then tritonized (9002-93-1; Sigma-Aldrich) for 8 minutes, and then washed twice in TBS for 5 minutes each. Cells were incubated with primary antibodies (desmin and BrdU or desmin and myogenin) diluted in 1% BSA in TBS overnight at 5°C. Then, cells were washed twice in TBS for 5 minutes each and incubated with secondary antibodies diluted in 1% BSA in TBS for 45 minutes at room temperature. The coverslips containing the cells were then removed from the wells, and invertedly placed on a glass slide containing a drop of Prolong-Gold-Antifade mounting medium with DAPI. Samples were stored in dark conditions for ~2 days before being moved to a −20°C freezer before imaging.

Host	Primary antibody	Company	Cat. no.	Concentration	Study used
Rabbit	Desmin, IgG	Abcam	AB32362	1:500-1:1000	2, 4
Rabbit	Laminin	Dako	Z0097	1:500	4
Mouse	CD56 (NCAM), IgG1	Becton Dickinson	347740	1:50	1, 2, 3, 4
Mouse	Myosin 1, IgG1	DSHB	A4.951	1:200	2, 4
Mouse	Pax 7, IgG1	DSHB	PAX7	1:100	4
Mouse	Myosin 1, IgG2b	DSHB	BA.D5	1:100	4
Mouse	Myogenin, IgG1	DSHB	F5D-s	1:50	2, 4
Mouse	MyHCe	DSHB	F1.652	1:100	1, 2, 3
Mouse	Merosin Laminin α2	Leica	MEROSIN-CE	1:200	4
Mouse	MyHCn, IgG1	Novocastra	NCL-MHCn	1:100	1, 2, 3, 4
Mouse	Dystrophin, IgG2b	Sigma-Aldrich	D8168	1:500	1, 2, 3, 4
Guinea pig	Collagen 22	*	KG36	1:5000	4
	Phalloidin 680	Invitrogen	A22286	1:40	4
Host	**Secondary antibody**	**Company**	**Cat. no.**	**Concentration**	**Study used**
Goat	Anti-Mouse 488, IgG	Invitrogen	A-11029	1:500	2, 4
Goat	Anti-Mouse 568, IgG	Invitrogen	A-11031	1:200	4
Goat	Anti-Rabbit 488, IgG	Invitrogen	A-11034	1:200	4
Goat	Anti-Rabbit 568, IgG	Invitrogen	A-11036	1:500	2, 4
Goat	Anti-Mouse 488, IgG1	Invitrogen	A-21121	1:500	1, 2, 3, 4
Goat	Anti-Mouse 568, IgG2b	Invitrogen	A-21144	1:200	1, 2, 3, 4

Tabel 2: Antibodies and concentrations used for immunofluorescent analyses. "Study used" refer to specific studies where a given antibody was used.

A 10×/0.30NA objective and a 0.5× camera (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany) mounted on a BX51 Olympus microscope, using the Olympus cellSens software (v.1.14) was used for microscopy. The exceptions to this were cell culture

immunofluorescence and the merosin, desmin and phalloidin staining in study 4 (see details in specific sections). All image analyses were done under blinded conditions (group, control/exercised leg, timepoint but not study), and the same investigator analysed all samples in a given analysis. For each immunofluorescent analysis, the number of muscle fibres included is provided as averages ± SD [range] per group, timepoint or fibre type (table 3, 4 and 5).

Number of fibres analysed (denervated fibres)			
Study 1 (aged baseline)	Average	SD	Range
Old men (n=70)	1144	489	355–2452
Study 2 (acute exercise)			
Young women (n=12)	1042	306	472–1623
Old women (n=11)	1170	285	805-1662
Study 3 (16-week training)			
EX (n=38)			
Pre	1162	494	355-2452
Post	1262	539	269-2733
SED (n=20)			
Pre	1142	482	507-2053
Post	1491	732	456-3027
Study 4 (lifelong exercise)			
Young (n=15)	978	369	423-1513
LLEX (n=16)	894	340	361-1751
SED (n=15)	1099	378	402-1688

Tabel 3: The number of fibres per biopsy cross section included in the analyses of denervated fibres in study 1, 2, 3 and 4, given as average ± SD [range]. Abbreviations: EX, exercise; SED, sedentary; LLEX, lifelong exercise.

Denervated fibres

The identification of denervated fibres based on NCAM, MyHCn and MyHCe is a key method for this thesis. The number of muscle fibres included in the analysis of each study is given in table 3. The method has been optimized across the four studies that it was used in. In general, overlapping images of whole section staining with dystrophin and NCAM were obtained, whereas only areas with positive staining for MyHCn and MyHCe were imaged. Overlapping images were subsequently stitched into one image using the "Stitch Directory with Images (unknown configuration)" plugin (Preibisch *et al.*, 2009) in Fiji (U.S. National Institutes of Health, Bethesda, MD, USA) (Schindelin *et al.*, 2012). See an overview image in figure 5.

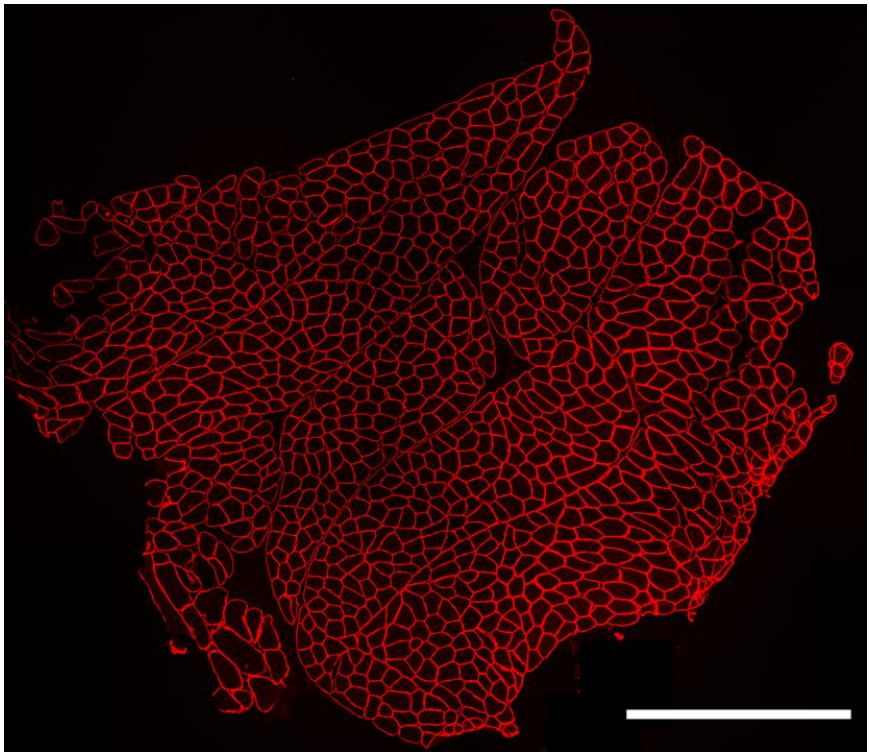

Figure 5: Overview image of an entire human muscle biopsy immunofluorescently stained with dystrophin (red). Biopsies of at least 500 muscle fibres are necessary to obtain reliably quantifications of denervated muscle fibres. Scalebar is 1000 µm.

Dystrophin, a marker of the sarcolemma, was used to quantify muscle fibres. The order of analysis was NCAM, MyHCn and MyHCe. Counted fibres were expressed relative to the total number of fibres in the given section, which was counted on the NCAM section (see table 3).

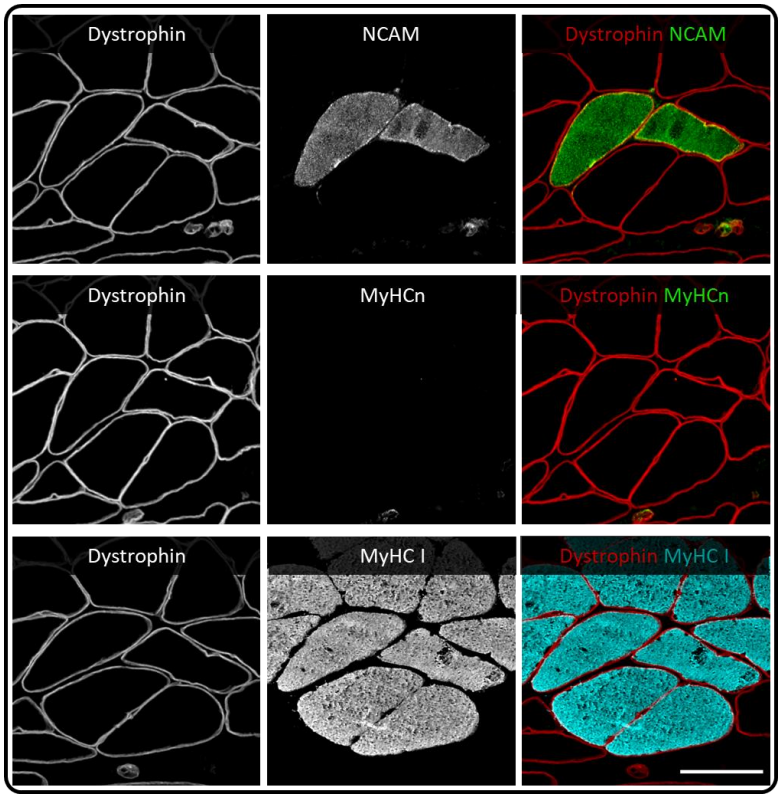

Figure 6: Example of two large NCAM$^+$ fibres (green) from a human muscle biopsy that are positive for MyHC I (cyan) but negative for MyHCn. Images are shown as split channels (left and middle column) and composite image (right column). Scalebar is 100 µm.

An example of an NCAM$^+$ fibre is shown in figure 6. The analyses of study 1 and 3 were performed simultaneously as these were the same subjects. 2040x1536 pixel images were obtained of sections stained with NCAM, MyHCn or MyHCe. Centralized nuclei were also assessed from the NCAM section. The number of fibres co-expressing MyHCn and NCAM was also assessed.

In study 2, 2040x1536 pixel images were obtained of sections stained with NCAM, MyHCn or MyHCe. MyHCe$^+$ fibres were extremely rare and were thus not analysed. In this analysis, the CSA of all transversely cut fibres was determined.

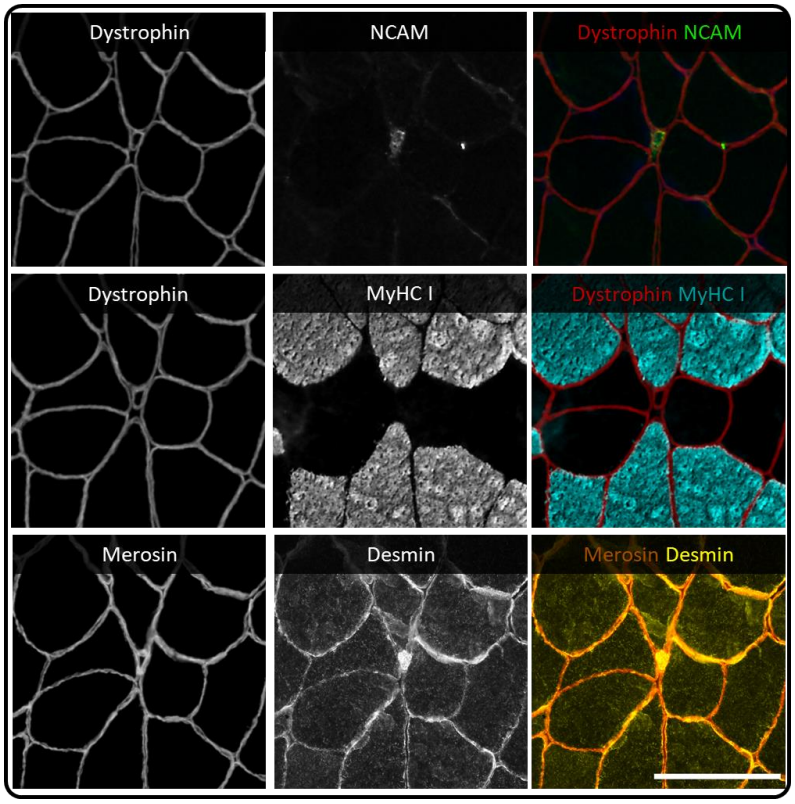

Figure 7: Example of a very small NCAM$^+$ fibre (green) from a human muscle biopsy that is negative for MyHC I (cyan) but strongly positive for merosin (orange) and desmin (yellow). Images are shown as split channels (left and middle column) and composite image (right column). Scalebar is 100 µm.

The analysis conducted in study 4 was the most comprehensive yet undertaken (see example in figure 7). Slide 1 was stained MyHCn, slide 2 for myosin I, slide 3 for NCAM and slide 4 for merosin, desmin and phalloidin. Presence of myotendinous junction (MTJ) was suspected in a small group of samples, and a fifth consecutive slide from these samples were stained with NCAM and collagen 22 (Koch *et al.*, 2004). 4080x3072 pixel images were obtained, and a x20/0.50NA objective was used for slide 4. The purpose of the merosin, desmin and phalloidin staining was to increase the likelihood that fibres that were included based on a combination of dystrophin$^+$ and MyHCn$^+$ or NCAM$^+$ staining were in fact actual muscle fibres, and not hyperactivated satellite cells or fibre branches (Schmalbruch, 1976). The number of MyHCn$^+$ and NCAM$^+$ fibres were assessed manually on composite images using the ObjectJ plugin in Fiji. The CSA of all included fibres was also determined. Furthermore, all NCAM$^+$ fibres were evaluated for co-expression of MyHCn and MyHC I. Fibres that could not be located on the next serial section were marked as “lost”.

Number of fibres analysed (fibre CSA)			
Study 2 (acute exercise)	Average	SD	Range
Young women (n=12)			
Type I	212	68	129–352
Type II	151	67	68–247
Old women (n=11)			
Type I	205	137	85-604
Type II	150	120	45–487
Study 4 (lifelong exercise)			
Young (n=15)			
Type I	227	147	39-604
Type II	222	71	72-314
LLEX (n=16)			
Type I	253	118	79-485
Type II	157	89	73-380
SED (n=15)			
Type I	248	110	124-456
Type II	261	127	69-539

Tabel 4: The number of fibres per biopsy cross section included in the analyses of muscle fibre CSA in study 2 and 4, given as average ± SD [range]. Abbreviations: SED, sedentary; LLEX, lifelong exercise.

Fibre type specific CSA and type distribution

Fibre type specific CSA and type distribution were assessed in study 2 and 4 (see figure 8 for example). In both studies, 4080×3072 pixel images were obtained without overlap, and these were analysed using a semi-automated macro [30], run in Fiji (v.1.51), as previously described (Karlsen *et al.*, 2019). Briefly, fibres included by the macro were manually assessed and excluded if longitudinally oriented, located at the edge of biopsy or around wholes in biopsy or had an incomplete dystrophin staining. Fibre type was automatically determined and manually checked based on median light intensity. Classifications were type I fibres (myosin type I^+), type II fibres (myosin type I^-) and hybrid fibres (weakly myosin type I^+). Hybrid fibres were excluded (131 fibres in study 2 and 328 fibres in study 4). The average number of fibres analysed per fibre type in each study is provided in table 4. In study 3, ATPase staining was used to analyse fibre type specific CSA in study 3, as described in detail (Heisterberg *et al.*, 2018*b*). ATPase was chosen as this allows for accurate determination of type IIx fibres.

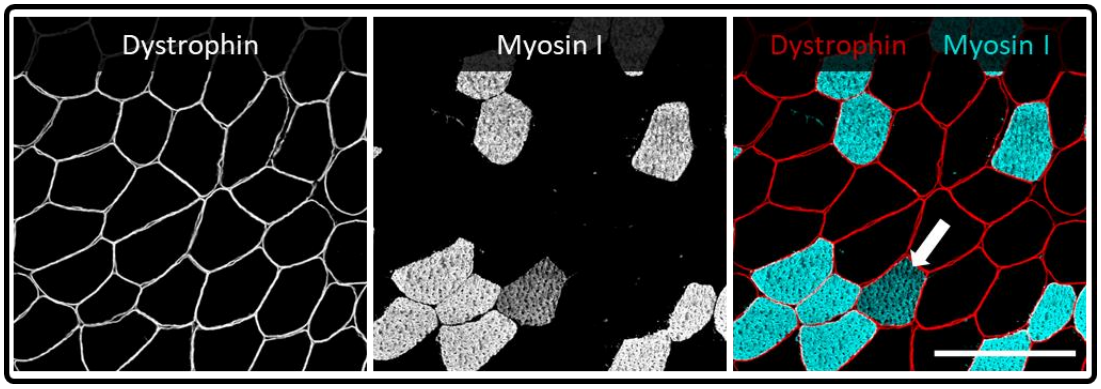

Figure 8: Example of cross-section of human muscle biopsy obtained from young individual immunofluorescently stained with dystrophin and MyHC I to determine muscle fibre CSA. Image is shown as split channels (left and middle) and composite image (right). White arrow point to a hybrid fibre. Scalebar is 200 µm.

Satellite cells

Satellite cell quantity was assessed in study 4. 4080x3072 pixel images were obtained without overlap, and manually assessed for satellite cells on composite images (laminin, Pax7, myosin I, DAPI) using the ObjectJ plugin in Fiji (see figure 9 for examples). $Pax7^+$ and $DAPI^+$ cells were defined as satellite cells and were allocated to type I (myosin type I^+) or II (myosin type I^-) fibres. 14 out of the total of 4614 satellite cells counted could not be allocated to a given fibre type and were then shared between fibre types.

Number of fibres analysed (satellite cells)

Study 4 (lifelong exercise)	Average	SD	Range
Young (n=15)			
Type I (control leg)	245	128	125-625
Type I (exercised leg)	253	131	110-621
Type II (control leg)	276	94	110-504
Type II (exercised leg)	291	108	145-512
LLEX (n=16)			
Type I (control leg)	366	185	129-837
Type I (exercised leg)	331	173	97-587
Type II (control leg)	224	94	80-387
Type II (exercised leg)	364	189	76-712
SED (n=15)			
Type I (control leg)	338	124	122-587
Type I (exercised leg)	391	142	131-636
Type II (control leg)	400	176	159-763
Type II (exercised leg)	270	107	101-442

Tabel 5: The number of type I and type II fibres per biopsy cross section included in the analyses of satellite cells in study 4, given as average ± SD [range]. Abbreviations: SED, sedentary; LLEX, lifelong exercise.

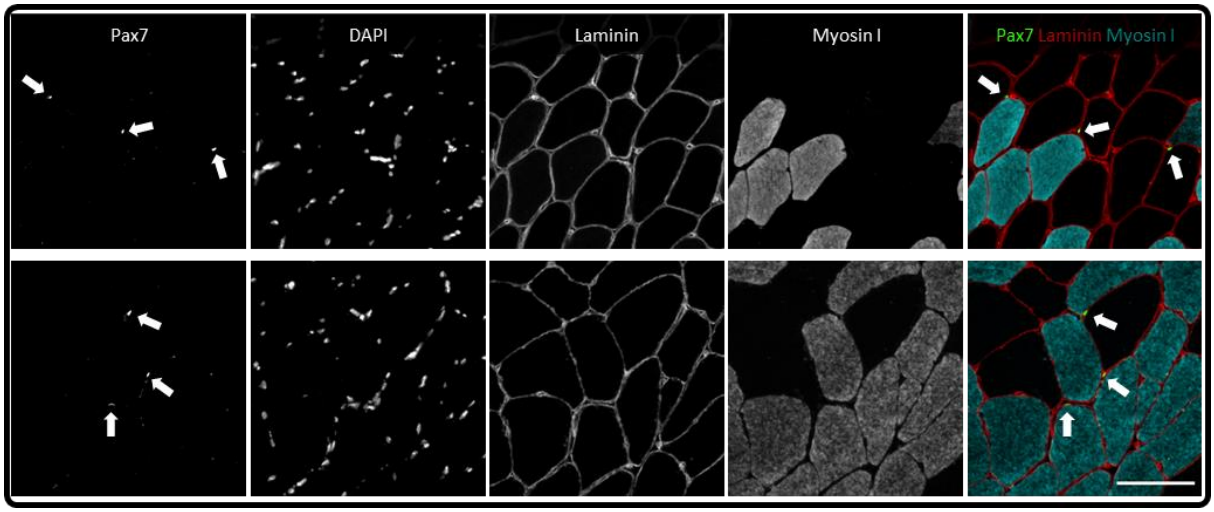

Figure 9: Examples of cross-sections from two human muscle biopsy obtained from an elderly (top row) and a young (bottom row) individual, and immunofluorescently stained with Pax7, laminin and MyHC I to determine fibre type specific satellite cell numbers. Images are shown as split channels (column 1-4) and composite image (column 5). White arrows points towards satellite cells. Scalebar is 100 µm and both panels are shown at the same magnification.

Fibre type specific satellite cell count was expressed relative to the number of fibres included in the analysis (see table 5). Two samples were excluded from the type II satellite cell quantification due to a low number of available fibres (SED control leg, n=14 and LLEX exercised leg, n=15).

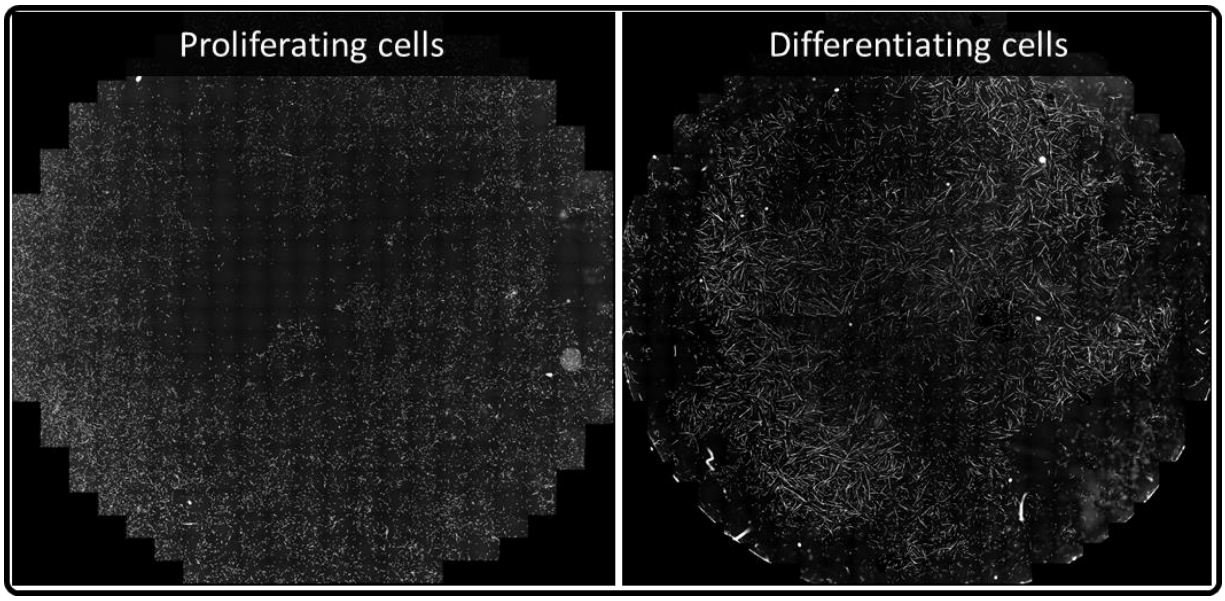

Figure 10: Overview images of proliferating and differentiating myogenic cells *in vitro* isolated from human muscle biopsies, immunofluorescently stained with desmin and imaged as many small sequential images that were stitched into one seamless image. Notice the large heterogeneity in cell localization and staining intensity, which underlines the importance of objectively determining the optimal ROI for analysis.

Cell culture

An AxioScan.Z1 (Carl Zeiss) slide scanner was used to image a pre-defined ROI covering ~90 % of the entire coverslip, containing cells stained with myogenin and desmin (see figure 10). The cells stained with BrdU and desmin were not imaged, as the BrdU staining failed. Areas with air bubbles or damage due to handling were removed from the ROI. For imaging, a plan-apochromat 0.45 NA / 10x objective with a MultiBand filter cube DAPI/FITC/TexasRed) using excitation wavelengths of 353, 493 and 577 nm (LED light source), was used. Each channel was imaged sequentially and individually with an AxioCam MR R3, using both coarse and fine focusing steps, and a 10% image overlap. Images were automatically merged and stitched in ZEN blue software (Carl Zeiss).

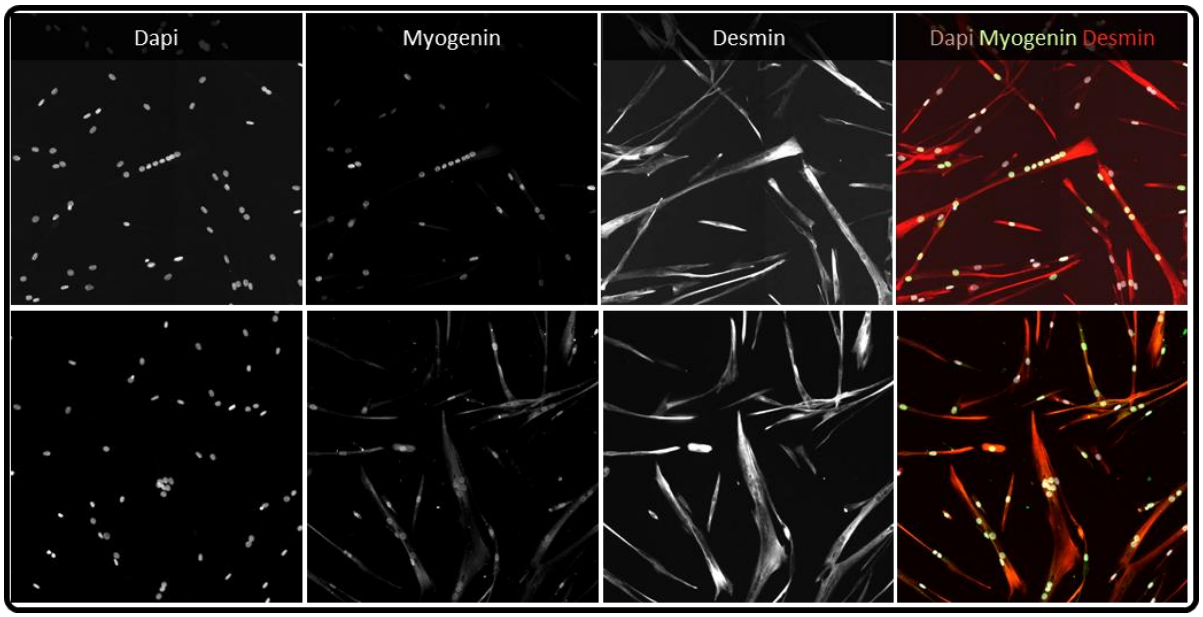

Figure 11: Examples of differentiating myogenic cells in vitro isolated from human muscle biopsies, and immunofluorescently stained with myogenin (green) and desmin (red) to determine differentiating and fusion indexes. Images are shown as split channels (column 1-3) and composite image (column 4).

This stitched image was then separated into smaller regions (2.26 x 1.80 mm ~ 3510 x 2790 pixels). Cells appeared to be centrally localized, and a central rectangular ROI was therefore defined. An estimated cell count across each region was defined using automated thresholding (DAPI), and the region with a nuclei count closest to the median for that specific coverslip was selected. Representative examples of images used in the analysis is shown in figure 11. This was done for up to three technical replicates (depending on availability) cultured on separate plates for both the exercised and control leg. Pairs of legs cultured on the same plate were analysed

together. Then, a manual correction of errors of the automated definition of nuclei was performed within the chosen ROI. This means separating clumped nuclei or melding a single nucleus. This adjusted count was then overlaid on the desmin channel, and all nuclei located within myotubes that had ≥3 nuclei were manually selected. Bleed-through of desmin into the myogenin channel was observed, so the myogenin signal was corrected by fitting the myogenin intensity against the desmin intensity outside of nuclei (which had no true myogenin signal) and then subtracting this from the intensity of the myogenin image. Furthermore, homogeneity between samples was improved by a contrast enhancement on the desmin and myogenic channels. Data lists with intensities from all channels per nucleus were extracted from Fiji and a in-house MATLAB script (MATLAB R2019a, The MathWorks Inc.) was used to collect the data and determine desmin$^+$ and myogenin$^+$ cells by a set threshold. Desmin$^+$ area was automatically measured. The ratio of fused desmin$^+$ nuclei to desmin$^+$ nuclei was set as the fusion index. The ratio of myogenin$^+$ nuclei to desmin$^+$ nuclei was set as the differentiation index. The ratio of desmin$^+$ nuclei to all nuclei was set as cell purity. A cell purity < 90% was set in place, which led to exclusion of 12 out of 92 samples (5 / 7 control / exercised leg and 1 / 7 / 4 young / SED / LLEX).

RNA extraction and purification

All studies followed the same general protocol for extraction and purification of RNA and Real-Time RT qPCR. Extraction of RNA from cells was done by moving coverslips with attached cells to an empty well in a new plate, followed by addition of 1 mL of TriReagent (TR118; Molecular Research Inc.) and mixing. The TriReagent, now containing the RNA, was moved to a BioSpec tube (5225; Bio Spec Products Inc.) and stored at -80°C. For the muscle biopsies, 100 sections of 10 µm thickness was cut and placed in BioSpec tube. The tissue was liquefied using 1 mL of TriReagent and subsequent mixing with 5 steel beads (2.3 mm, BioSpec) for 15 seconds in a FastPrep homogenizer (MP Biomedicals) and was then stored at -80°C.
100 µl of bromo-chloropropane was added to the samples for 15 minutes at room temperature. Then, the samples were centrifuged for 15 minutes at 12.000 g to separate into an organic and liquid (with the RNA) phase. Then, 80 µg of glycogen (cat. no. 10814-010; Invitrogen) and 350 µl of Isopropanol were mixed into the sample, left for 10 min, and then centrifuged for 8 minutes at 12.000 g. For the tissue samples no glycogen was added. After washing of the RNA pellet in 1 mL of 75 % ethanol, it was centrifuged two times for 5 minutes at 7.500 g, with removal of the supernatant afterwards. The final pellet was then dissolved in 100 µl of RNase-free water.

Reprecipitation was done with 200 µl 99 % ethanol and 10 µl 3 M sodium acetate (pH 5.5) washing as described above. Finally, the pellet was dissolved 10 µl of RNase-free water. Total RNA purity and concentration were assessed by spectroscopy at 260, 280, and 240 nm, and RNA integrity was confirmed by gel electrophoresis.

Real-Time RT-qPCR

RNA was converted to cDNA with OmniScript reverse transcriptase (Qiagen) and poly-dT (Qiagen), and subsequently amplified in a 25-µl SYBR green polymerase chain reaction containing 1×Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (table 6). Amplification was monitored using MX3005P real-time PCR machine (StrataGene) and a standard curve was created using known concentrations of DNA oligonucleotides (Ultramer oligos, Integrated DNA Technologies). Ct values were related to the standard curve and melting curve analysis following amplification was used to confirm the specificity of the PCR products. RPLP0 mRNA was chosen as an internal control and GAPDH was measured as another unrelated control (normalized to RPLP0). In study 2, the exercise response was expressed relative to own control sample (rested leg or baseline sample). In study 3, mid and post was expressed relative to baseline sample. In study 4, LLEX and young was expressed relative to SED (control leg) and for the exercise response data was expressed relative to own control sample (rested leg).

mRNA	Gene name	Genbank	Sense	Antisense
AChR α1	CHRNA1	NM_000079.3	GCAGAGACCATGAAGTCAGACCAGGAG	CCGATGATGCAAACAAGCATGAA
AChR β1	CHRNB1	NM_000747.2	TTCATCCGGAAGCCGCCAAG	CCGCAGATCAGGGGCAGACA
AChR γ	CHRNG	NM_005199.4	GCCTGCAACCTCATTGCCTGT	ACTCGGCCCACCAGGAACCAC
AChR δ	CHRND	NM_000751.2	CAGCTGTGGATGGGGCAAAC	GCCACTCGGTTCCAGCTGTCTT
AChR ε	CHRNE	NM_000080.4	TGGCAGAACTGTTCGCTTATTTTCC	TTGATGGTCTTGCCGTCGTTGT
COL1A1	COL1A1	NM_000088.3	GGCAACAGCCGCTTCACCTAC	GCGGGAGGTCTTGGTGGTTTT
GAPDH	GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
Ki67	MKI67	NM_002417.4	CGGAAGAGCTGAACAGCAACGA	GCGTCTGGAGCGCAGGGATA
MUSK	MUSK	NM_005592.3	TCATGGCAGAATTTGACAACCCTAAC	GGCTTCCCGACAGCACACAC
MyHCe	MYH3	NM_002470.3	CGGATATCGCAGAATCTCAAGTCAA	CTCCAGAAGGGCTGGCTCACTC
MyHCn	MYH8	NM_002472.2	CGGAAACATGAGCGACGAGTAAAA	CAGCCTGAGAACATTCTTGCGATCTT
Myogenin	MYOG	NM_002479.5	CTGCAGTCCAGAGTGGGGCAGT	CTGTAGGGTCAGCCGTGAGCAG
p16	CDKN2A	NM_000077.4	GGGGGCACCAGAGGCAGTAA	TTCTCAGAGCCTCTCTGGTTCTTTCA
RPLP0	RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG

Tabel 6: Primers used for PCR. Abbreviations: RPLP0, Ribosomal Protein Lateral Stalk Subunit P0; AChR, acetylcholine receptor; COL1A1, collagen type I alpha 1 chain; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; MuSK, muscle-specific-kinase; MyHCe, embryonic myosin heavy chain; MyHCn, neonatal myosin heavy chain.

Statistical analyses

Figures and tables were prepared using GraphPad Prism (GraphPad Software, La Jolla, USA) and Microsoft Excel (Microsoft Corporation, Redmond, USA), respectively. Statistical analyses were mostly performed using SigmaPlot (Systat Software Inc, San Jose, USA); correlation analyses were conducted using GraphPad Prism and Microsoft Excel was used for some t-tests. P values <0.05 were considered significant, and trends of $p<0.1$ are mentioned. In all studies, participant characteristics is shown as average ± standard deviation [range]. mRNA data were normalized to RPLP0 and log-transformed before statistical analysis. The specific summary data used are specified within each figure.

In study 1, data with a skewed distribution are shown as individual values with a median line. Pearson's correlation was used for age with *in vivo* measurements and gene expression data, while Spearman's correlation was used for age with immunofluorescent data.
In study 2, mRNA data from the male participants were analysed using a one-way repeated measures ANOVA, with Dunnett's post hoc test. For the female participants, unpaired two-tailed t-tests were used to test for differences between old and young in participants characteristics, mRNA data and fibre CSA. Paired two-tailed t-test were used to evaluate the exercise response (exercised versus control leg). Bonferroni (x3) corrections were used on mRNA data due to multiple t-tests. Mann–Whitney Rank Sum Test were used to compare young and old for the presence of $MyHCn^+$ and $NCAM^+$ fibres.
In study 3, differences between EX and SED in participant characteristics were analysed using unpaired two-tailed t-tests. The change from pre to post for MyHCn and NCAM were analysed using the Wilcoxon signed rank test while the changes in mRNA, MVC and fibre CSA were evaluated using two-way Repeated Measures ANOVA (time x group). Another two-way Repeated Measures ANOVA (time x drug) was used to test for the influence of receiving Losartan supplementation. If a significant interaction was observed, Dunnett's post hoc test was used.
In study 4, three types of comparisons were made. First, LLEX and SED were directly compared. Second, young were compared with old (LLEX combined with SED). Third, the exercised leg was compared to the control leg. Non-parametric statistical analyses (Mann-Whitney Rank Sum Test and Wilcoxon Signed Rank Test) were used for MyHCn/NCAM and cell culture data. Unpaired and paired two-tailed t-tests were used for all remaining data (mRNA data following log-transformation), except force values for the exercise bout, that were analysed

using a two-way ANOVA (group x round), with Holm-Sidak post hoc test. Notice that values from individual sets were average into rounds before being applied to statistical testing.

Results and Discussion

Muscle fibre denervation in the ageing population (study 1)

Age-related loss of muscle mass and function has been comprehensively described in several studies containing numerous subjects (Lexell *et al.*, 1988; Frontera *et al.*, 1991; Baumgartner, 2000; Suetta *et al.*, 2019). Yet, the relative contribution of muscle fibre denervation to this decline is unknown, given the difficulties in its assessment. The aim of study 1 was to provide a qualitative and quantitative analysis of the presence of three potential markers of denervated muscle fibres. The characteristics of the 70 participants is provided in table 7. These were well-functioning, healthy elderly men, who did not have hypertension or receive blood pressure lowering medication.

Participant characteristics *(n=70)*		
Age (yr)	72 ± 6	*65-94*
Height (cm)	178 ± 7	*161-191*
Weight (kg)	84 ± 11	*57-110*
BMI (kg/m²)	26 ± 3	*19-33*
LBM (kg)[a]	56 ± 6	*44-71*
Isometric force (Nm)	196 ± 46	*75-298*

Tabel 7: Age, height, weight, BMI, LBM, and isometric force for all participants given as average ± SD [range]. Abbreviations: LBM, lean body mass; BMI, body mass index; Nm, newton meter. [a] N = 69

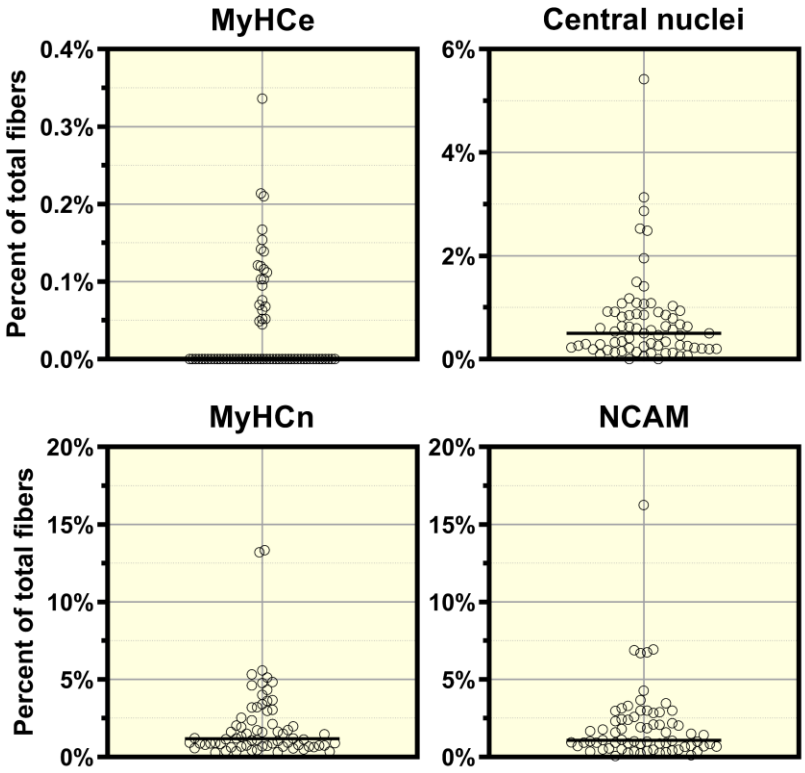

Figure 12: Percentage of muscle fibres positive for MyHCe, MyHCn and NCAM or with central nuclei determined using immunofluorescent microscopy on human muscle biopsies from in 70 healthy men (72±6 years of age). Data are shown as individual values with median (horizontal line). Notice that the y-axis is different between graphs. Abbreviations: MyHCe, embryonic myosin heavy chain; MyHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule.

The number of MyHCe$^+$, MyHCn$^+$ and NCAM$^+$ muscle fibres as well as central nuclei were assessed by immunofluorescence on muscle biopsy cross sections (figure 12). MyHCe$^+$ fibres were only observed in 22 out of 70 samples, based on a total of 33 positive fibres. In stark contrast, MyHCn, another MyHC associated with embryogenesis (Whalen *et al.*, 1981; Draeger *et al.*, 1987; Barbet *et al.*, 1991) and muscle regeneration (Sartore *et al.*, 1982; Whalen *et al.*, 1990), was observed in all samples, based on a total of 1542 positive fibres, giving a median (range) of 1.2 (0.3-13.3) %. Somewhat similarly, NCAM$^+$ fibres were observed in all samples, with 1509 positive fibres and a median (range) of 1.1 (0.1-16.2) %. Lastly, muscle fibres with centralized nuclei were observed in 68 out of 70 samples, equalling a median (range) of 0.5 (0-5.4) %. Centralized nuclei, MyHCn and NCAM are not normally found in healthy adult muscle (Fitzsimons & Hoh, 1981; Butler-Browne *et al.*, 1982; Schiaffino *et al.*, 1986; Illa *et al.*, 1992; Monemi *et al.*, 1996), except in intrafusal muscle fibres (Walro & Kucera, 1999), specific fascial muscles (Sartore *et al.*, 1987; Butler-Browne *et al.*, 1988; Stål *et al.*, 1994), at the NMJ and the MTJ for NCAM (Daniloff *et al.*, 1989; Jakobsen *et al.*, 2018) and in various neuromuscular

diseases (Winter & Bornemann, 1999; Doppler *et al.*, 2008), as reviewed here (Schiaffino *et al.*, 2015). Importantly, denervation by both nerve transection and blocking of nerve transmission using a neurotoxin lead to increases in embryonic myosins (Butler-Browne *et al.*, 1982; Schiaffino *et al.*, 1988; Xing *et al.*, 2015) and NCAM (Covault & Sanes, 1985; Covault *et al.*, 1986; Olsen *et al.*, 1995; Lin *et al.*, 2021). Previous studies that have quantified the presence of MyHCn and NCAM in young muscle using either immunofluorescence or western blot have found no or very low levels of these proteins (Kadi & Thornell, 1999; D'Antona *et al.*, 2003; Mackey *et al.*, 2011, 2014, 2016). As such, the major finding of study 1 is the surprisingly high prevalence of fibres positive for NCAM and MyHCn. This is in support of the theory that muscle fibre denervation and failed reinnervation leads to accumulation of orphaned fibres in aged muscle (Hepple & Rice, 2016).

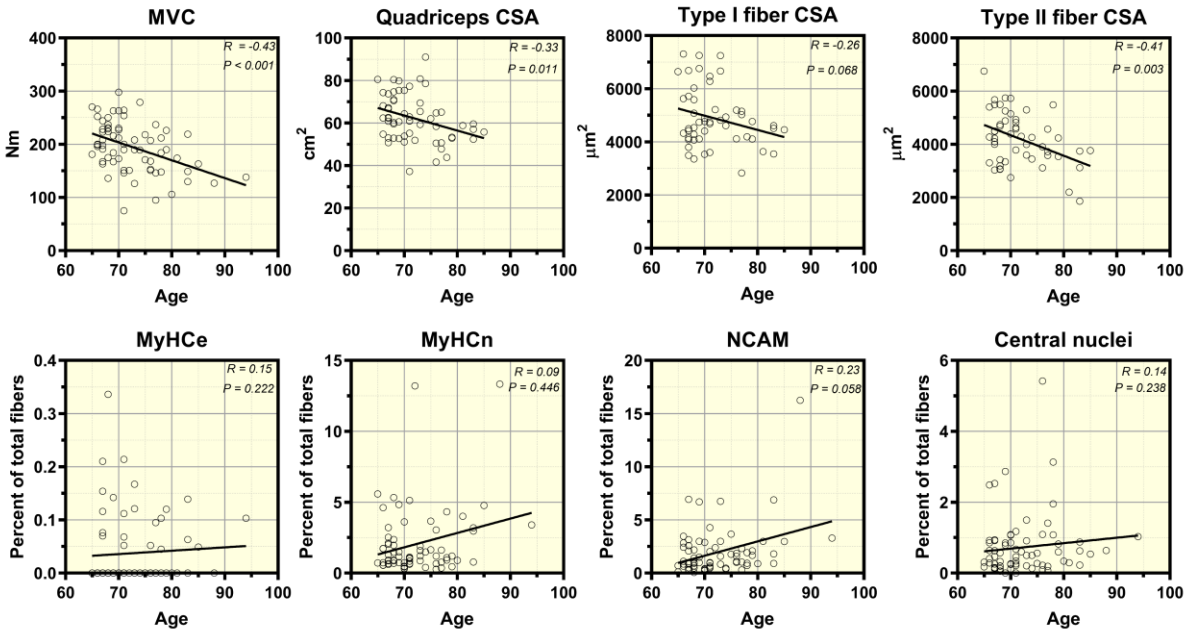

Figure 13: Correlations between age and muscle strength (n=69), quadriceps CSA (n=58), fibre CSA (n=51) and immunofluorescence-derived values (n=70). Pearson's correlation was used for top row and Spearman's correlation for bottom row. R and P values are written within figures. Abbreviations: CSA, cross sectional area; MyHCe, embryonic myosin heavy chain; MyHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule.

As shown in figure 13, there was a significant negative correlation between age and vastus lateralis CSA, MVC and type I (tendency) and II average muscle fibre CSA, among the cohort of healthy elderly men, covering an age-span of 65-94 years of age. This illustrates the gradually worsening condition of aged muscle, even among otherwise healthy individuals. Despite this,

there were no significant correlations between age and the percentage of NCAM$^+$ and MyHCn$^+$ fibres (figure 13). At least two possible explanations for this needs to be mentioned. Firstly, it might simply be that too few individuals were in the higher range of age-span for the correlation analysis to be effective. Specifically, there were only 3 and 8 subjects ≥ 85 and 80 years of age, respectively, while there were 28 at the age between 65 to 69. Secondly, the loss of innervation leaves the fibre incapable of activation and contraction, with ensuing disruption and atrophy (Viguie *et al.*, 1997; Winter & Bornemann, 1999). At one point the fibre ceases to exist or is at least at a stage where it cannot be convincingly identified using microscopy. In confirmation of this, a lower number of muscle fibres in old compared to young muscle have been reported for rodents (Ishihara *et al.*, 1987; Bütikofer *et al.*, 2011), and in the two only studies performed in humans (Inokuchi *et al.*, 1975; Lexell *et al.*, 1988). The failed reinnervation, which presumably becomes a feature at lager stages of ageing (Aagaard *et al.*, 2010; Hepple & Rice, 2016), leaves some fibres permanently denervated until they are no more.

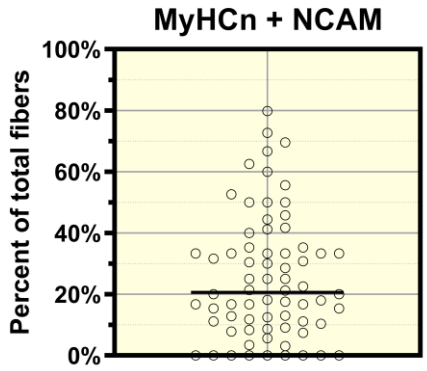

Figure 14: Percentage of MyHCn$^+$ muscle fibres that co-expressed NCAM, determined using immunofluorescent microscopy on human muscle biopsies from in 70 healthy men (72±6 years of age). Data are shown as individual values with median (horizontal line). Abbreviations: MyHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule.

Qualitative assessment and co-expression of markers

Slightly over 1 % of fibres in the aged muscle expressed NCAM and MyHCn, respectively. However, only 23.6 % of MyHCn$^+$ fibres also expressed NCAM (figure 14). Several factors could explain this discrepancy. Firstly, the lack of agreement could indicate temporal variation, as one marker might be more rapidly expressed than the other, or perhaps be expressed for a longer time. When reinnervation is prevented from occurring by complete transection of the nerve, the muscle fibres of a rat continuously express NCAM for at least 300 days (Covault &

Sanes, 1985). Similarly, NCAM is also present in muscle at late stages of various neuromuscular diseases (Winter & Bornemann, 1999). These findings indicate that NCAM can remain consistently expressed in skeletal muscle. On the other hand, expression of MyHCn is upregulated during embryogenesis (Whalen *et al.*, 1981; Draeger *et al.*, 1987; Barbet *et al.*, 1991) and regeneration (Sartore *et al.*, 1982; Whalen *et al.*, 1990) but gradually dissipates. This could perhaps indicate that MyHCn would be downregulated at some point, which could then explain some of the discrepancy. However, MyHCn is continuously present in the muscle of patients suffering from various neuromuscular diseases where denervation is prominent (Schiaffino *et al.*, 1986; Winter & Bornemann, 1999; Doppler *et al.*, 2008), suggesting that this is not the main reason. Secondly, it is also possible, although yet unknown, that there are different pools of fibres that are more amendable to express one marker of the other. This could for instance be fibre type specific, as has been suggested by Schiaffino et al., 1988 relating to MyHCn (Schiaffino *et al.*, 1988). It has also been suggested that the ability to upregulate NCAM might be diminished with ageing (Olsen *et al.*, 1995; Gillon & Sheard, 2015) although other findings seem to firmly refute this (Hendrickse *et al.*, 2018). If it was true however, then it could be theorized that dysfunctional signalling mechanism could prevent NCAM expression to increase, while the reversion to a foetal MyHC configuration might still occur. Thirdly, $MyHCn^+$ fibres, when observed longitudinally, often present in a striated pattern (Schiaffino *et al.*, 1988; Soendenbroe *et al.*, 2021), whereas $NCAM^+$ appears to be more consistently expressed. This means, that when fibres are analysed in consecutive cross sections, then a $NCAM^+$ and $MyHCn^+$ fibre can occasionally appear to only express NCAM, if the MyHCn signal has faded in the given section. Overall, none of these three factors are satisfactorily explanations on their own, but a combination of them seems likely.

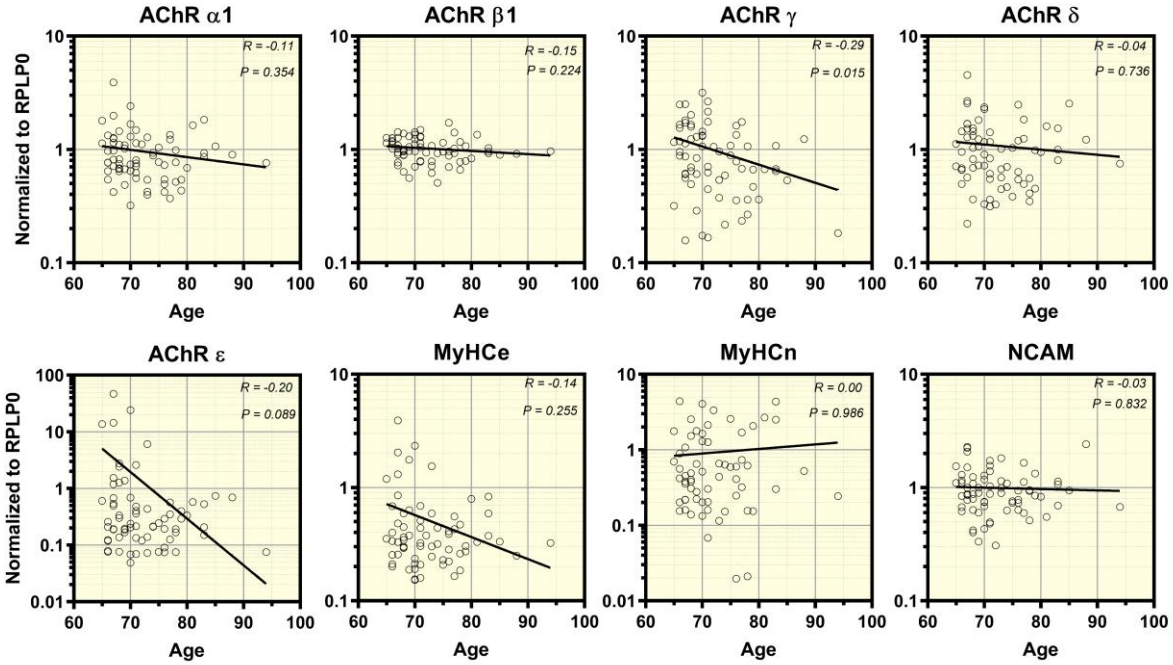

Figure 15: Correlations between age and gene expression data (n=70). Pearson's correlation was used, and R and P values are written within figures. Abbreviations: AChR, acetylcholine receptor; MyHCe, embryonic myosin heavy chain; MyHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule.

AChR gene expression in ageing muscle

Innervated muscle fibres are characterized by clustering of AChRs at the NMJ (Axelsson & Thesleff, 1959; Fambrough, 1979; Merlie & Sanes, 1985; Gundersen *et al.*, 1995), due to transcriptional specialization of synaptic myonuclei (Merlie & Sanes, 1985; Schaeffer *et al.*, 2001) and electric activity, enabled through the NMJ, which suppresses AChR gene expression in the non-synaptic nuclei (Frank *et al.*, 1976; Schaeffer *et al.*, 2001). Loss of innervation diminishes this specialization (Pestronk & Drachman, 1978; Goldman & Staple, 1989; Witzemann *et al.*, 1991; Missias *et al.*, 1996), due to lifting of the repressive electrical activity (Sanes & Lichtman, 2001), with changes in gene expression observed within 12 hours following denervation (Tsay & Schmidt, 1989). Thus, the gene expression of most AChR subunits, including gamma, have been reported to increase following nerve signal blockade (Mishina *et al.*, 1986; Goldman & Staple, 1989; Witzemann *et al.*, 1991; Carlson *et al.*, 2002; Apel *et al.*, 2009). This led to the idea that within a large cohort of men spanning an age range of ~20 years, that gene expression of AChR subunits measured in muscle biopsies would be positively associated with age, as previously shown in animals (Carlson *et al.*, 2002). However, in contrast

to this hypothesis, 4 out 5 subunits showed no significant relationship with age, whilst the gamma subunit showed a weak negative association with age (figure 15). Also, the individual data points serve to illustrate the large interindividual variation that exists for some subunits and can be upwards of 100-1000 fold between similarly aged individuals. It is interesting that it is only the gamma subunit, which is a foetal specific subunit (Mishina *et al.*, 1986; Gu & Hall, 1988; Missias *et al.*, 1996), that shows any kind of relationship with age. Why the relationship is negative is unclear, but as previously discussed in relation to the lack of correlation between age and the percentage of $MyHCn^+$ and $NCAM^+$ fibres, denervated fibres gradually atrophy and loose both structure and function (Viguie *et al.*, 1997; Gosztonyi *et al.*, 2001). The long-term denervated fibre will eventually disappear, and for a prior period contribute very little to the gene expression of whole muscle homogenate due to its already shrunken stage. Lastly, it should be pointed out that the status of the gamma subunit as a foetal subunit is primarily based on animal studies, hence making it possible that species-specific differences could explain the observation (Cetin *et al.*, 2020). Only a single study has, as far as the author is aware, documented the gamma to epsilon switch during maturation in humans (Hesselmans *et al.*, 1993).

Overall, the conclusions from study 1 are that more $NCAM^+$ and $MyHCn^+$ fibres are found in aged muscle than what has been reported in young muscle, that these markers are only coexpressed in a small number of fibres, and that the AChR gamma subunit was negatively associated with age among a large group of elderly men. These findings raised several questions that necessitated a young control group and opened up a venue to study the influence of exercise on muscle innervation.

Muscle fibre denervation and acute exercise (study 2)

Muscle fibre denervation in young and elderly individuals

Study 2 was based on participants from two previously conducted projects. The participant characteristics is provided in table 8. No direct comparison between the female and male participants is attempted given that they were involved in different studies.

Table 8 Participant characteristics

	Young women *(n=12)*		Old women *(n=11)*		Old men *(n=25)*	
Age (yr) *	23 ± 3	*20-28*	74 ± 3	*71-78*	70 ± 7	*64-90*
Height (cm)	168 ± 7	*157-177*	166 ± 3	*162-169*	180 ± 5	*172-189*
Weight (kg)	64 ± 8	*53-75*	69 ± 10	*57-84*	82 ± 10	*67-98*
BMI (kg/m²) (*)	23 ± 2	*19-26*	25 ± 4	*20-30*	26 ± 3	*21-31*
Knee extension 1RM (kg) *	39 ± 8	*30-50*	23 ± 5	*12-28*	56 ± 14	*23-82*

Tabel 8: Age, height, weight, BMI, and 1 repetition maximum for all participants given as average ± SD [range]. Abbreviations: 1RM, 1 repetition maximum; BMI, body mass index. Young and old women were compared using unpaired t-tests. * *p*<0.05 young versus old. (*) p<0.1 young versus old.

The muscle of the elderly group was characterised by significantly smaller type II fibres compared to both their own type I and to type II fibres in young, and the elderly women also had a lower 1RM knee extension compared to young (table 8 and figure 16). The fibre size distribution graph shows that the type II fibres of the elderly appear to have shifted left-ward, indicating more atrophied fibres.

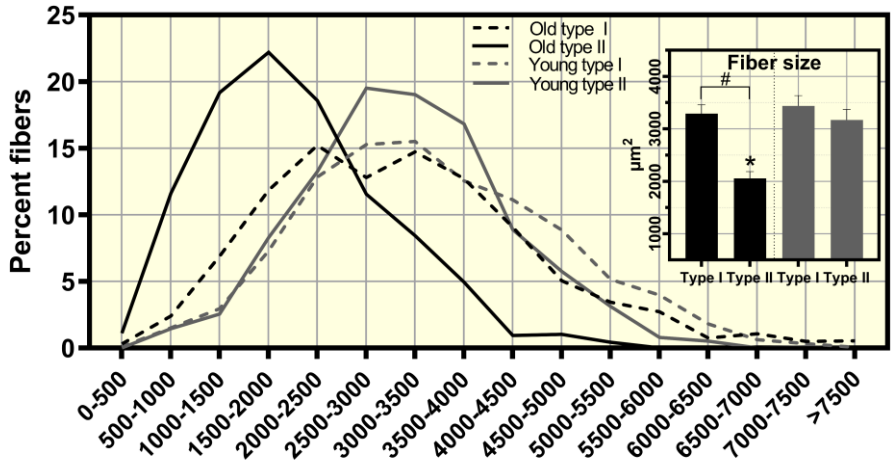

Figure 16: Muscle fibre size was assessed on cross section of biopsies from old (black) and young (grey) female participants using immunofluorescence. Histograms of type I (dotted line) and II (solid line) size distribution is provided shown and Fibre type specific fibre size is given as average ± SEM is shown in the insert. Differences within group between fibre types and within fibre type between groups were evaluated using paired and unpaired t-tests, respectively. # p <0.05 versus type I. * p<0.05 vs young type II.

Using a similar methodological approach as in study 1, the number of denervated fibres, analysed as $NCAM^+$ and $MyHCn^+$ fibres, were assessed in both young and old women, which allowed for a direct comparison between the groups. In accordance with the hypothesis,

significantly more denervated fibres were found in old compared to young (figure 17). MyHCn and NCAM were observed in 10 out of 11 samples from old women and based on 134 and 41 MyHCn$^+$ and NCAM$^+$ fibres, respectively, a median (range) of 0.8 (0.0-3.0) % and 0.2 (0.0-0.9) % were found. In young muscle, MyHCn and NCAM were observed in 3 and 4 out of 12 samples for MyHCn and NCAM, respectively. Based on a total of 15 and 24 MyHCn$^+$ and NCAM$^+$ fibres, respectively, a median (range) of 0.0 (0.0-0.8) % and 0.0 (0.0-2.0) % were found. Although no direct comparison can be made to study 1, it is notable that while the percentage of MyHCn$^+$ fibres is similar (1.2 % in study 1 versus 0.8 % in study 2), there is a marked difference in the percentage of NCAM$^+$ fibres (1.1 % in study 1 versus 0.2 % in study 2), despite the average age being similar (72 ± 6 in study 1 versus 74 ± 3 in study 2). The reason behind this potential divergence is not clear, especially given that the same antibodies, staining procedures, microscopy settings and image analyses approach were used.

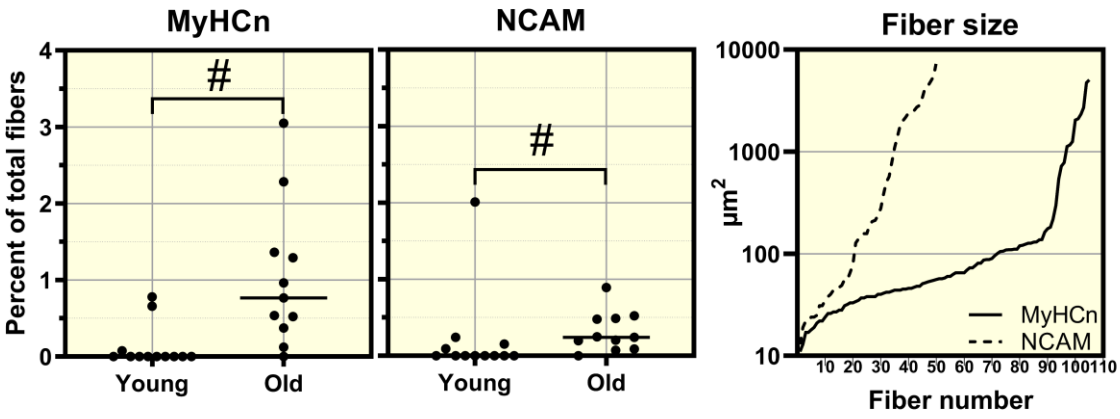

Figure 17: Percentage of muscle fibres positive for MyHCn and NCAM determined using immunofluorescent microscopy on human muscle biopsies from in 11 young and 12 old females. Data are shown as individual values with median (horizontal line). Differences between groups were evaluated using the Mann–Whitney Rank Sum Test. # p <0.05 young versus old. The fibre sizes of all MyHCn$^+$ and NCAM$^+$ fibres across groups are also shown. Abbreviations: MyHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule.

This was also the first study where the CSA area of all transversally cut NCAM$^+$ and MyHCn$^+$ fibres was measured. As seen in figure 17, most MyHCn$^+$ and NCAM$^+$ were severely atrophied, with some fibres as small as 40-50 µm2. At this size, it becomes increasingly important to discriminate NCAM signal coming from muscle fibres and from satellite cells, that also express NCAM in both the quiescent and committed state (Snijders et al., 2015; Nederveen et al., 2020).

For this purpose, Dystrophin serves as prerequisite, as it is a muscle fibre specific sarcolemma protein, that is not present in the satellite cell membrane (Marti *et al.*, 2013).

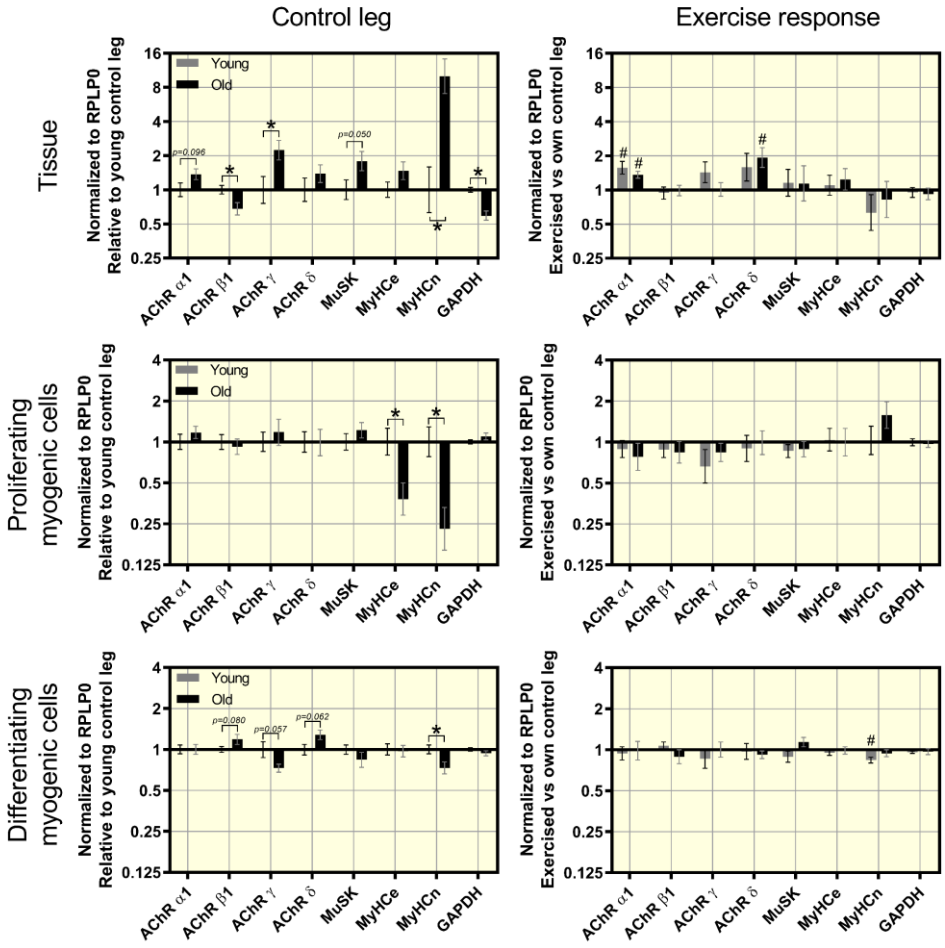

Figure 18: Gene expression in muscle biopsies (top row), proliferating (middle row) and differentiating (bottom row) myogenic cells from rested (left column) and exercised leg (right column) of young and old female participants. mRNA data are expressed relative to RPLP0 and are shown as geo mean ± back-transformed SEM. For the control leg, data are expressed relative to young group and were investigated using unpaired t-tests. For exercise response, the exercised leg is expressed relative to own control leg and were investigated using paired t-tests. Bonferroni (x3) was used due to multiple t-tests. * p<0.05 young versus old. # p<0.05 versus control leg. Tendencies are written. Abbreviations: AChR, acetylcholine receptor; MuSK, muscle-specific-kinase; MyHCe, embryonic myosin heavy chain; MyHCn, neonatal myosin heavy chain; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; RPLP0, Ribosomal Protein Lateral Stalk Subunit P0.

In relation to AChR gene expression, a higher gamma gene expression was observed in old compared to young (figure 18). This aligns with the supposition that this subunit in particular can be used to assess innervation status (Carlson *et al.*, 2002; Apel *et al.*, 2009), due to it being a foetal subunit. Furthermore, the alpha1 AChR subunit and MuSK, which is related to NMJ

maintenance (Glass *et al.*, 1996), both tended to be increased in old compared to young muscle, while the beta1 subunit was downregulated. In another study from our department, Karlsen et al., 2020 reported higher gene expression levels of alpha1 and delta subunits in old compared to young muscle at baseline (Karlsen *et al.*, 2020). Overall, these results suggest that age-related differences in AChR gene expression are often observed, appears to be subunit specific and are not easily interpreted as differences can go both ways.

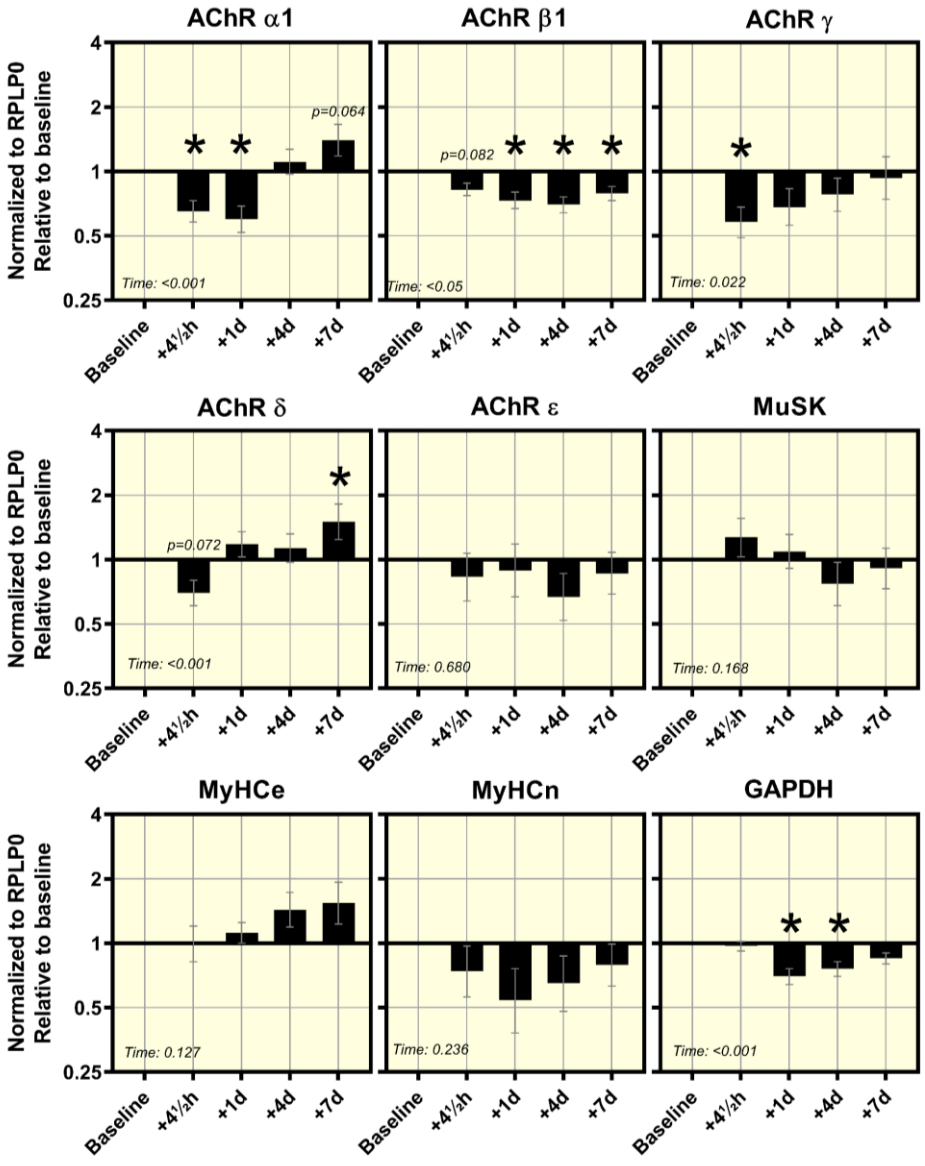

Figure 19: Gene expression in muscle biopsies at baseline and 4½ hour and 1, 4 and 7 days following acute exercise bout in healthy elderly men. mRNA data are expressed relative to RPLP0 and are shown as geo mean ± back-transformed SEM. Data are shown relative to pre. Changes in gene expression were evaluated using a one-way repeated measures ANOVA with Dunnett's post hoc test. * p<0.05 versus baseline. Tendencies are written. Abbreviations: AChR, acetylcholine receptor; MuSK, muscle-specific-kinase; MyHCe, embryonic myosin heavy chain; MyHCn, neonatal myosin heavy chain; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; RPLP0, Ribosomal Protein Lateral Stalk Subunit P0.

Effect of acute resistance exercise on AChR gene expression

This was the first study of the acute response of exercise on AChR gene expression in humans, which was studied in two ways. Firstly, young, and elderly female individuals performed a unilateral bout of heavy resistance exercise, and then had muscle biopsies obtained from both legs five days later, allowing for direct comparison between the exercised and control leg. Secondly, elderly men performed a unilateral bout of heavy resistance exercise and then had muscle biopsies obtained after 4.5 hours, and 1, 4 and 7 days. In the first part of the study, it was observed that the response to exercise in general appeared similar between young and old subjects (figure 18). The alpha1 subunit was significantly upregulated in the exercised leg of both groups, while upregulation of the delta subunit only reached significance in the exercised leg of the old subjects. No other differences between the exercised and control leg were found in gene expression of muscle homogenate. These findings show that acute exercise influences AChR gene expression, perhaps indicating a mechanism through which muscle innervation can be manipulated (Baehr *et al.*, 2016; Hughes *et al.*, 2017). Furthermore, the findings indicate that the muscle of elderly healthy individuals retain the ability to increase transcription of AChRs in the face of an exercise challenge. In the second part of the study, transient changes in the gene expression of 4 out of 5 AChRs subunits follow a bout of heavy resistance exercise was found (figure 19). Interestingly, some subunits showed a rapid downregulation (alpha1, delta and gamma) which was either normalized or switched to an increase at later time points. In contrast, beta1 is immediately downregulated (tendency for 4.5 hours) and remains so for all time points. These findings highlight that AChR gene expression is strongly responsive to exercise, and that changes are both subunit specific and time-dependant. However, it cannot be stated based on the data whether the changes are positive or negative in terms of denervation, or whether repeated bouts of exercise would lead to favourable adaptions as has been reported in animals (Valdez *et al.*, 2010).

Transcription of NMJ related genes in cultured satellite cells

mRNA of the alpha1, beta1, delta and gamma AChR subunits were also detected in both proliferating and differentiating myogenic cell cultures (figure 18). This is consistent with the idea that new receptors must be synthesized for a functional NMJ to be established (Mis *et al.*, 2017), and also indicates that myogenic cells *in vitro* must be readily available and able to transcribe NMJ related genes (Liu *et al.*, 2015, 2017). 3 out of 4 detected subunits tended to be

differently expressed between differentiating cells derived from young and old individuals. This was observed despite the use of the conservative Bonferroni correction. Notably, the observed differences were not in the same direction and are therefore unlikely to be attributable to general cell culture differences. Instead, the differences could indicate the ability of the cells to transcribe key genes related to formation and maintenance of the NMJ is affected by ageing. However, more delicate cell culture studies using cells of both myogenic and neurogenic origin are needed to test this hypothesis appropriately. It should also be pointed out that the fusion index was higher in the cells of the young subjects (Bechshøft *et al.*, 2019), which indicates a higher degree of development. There was a significantly positive correlation between fusion index and AChR gamma mRNA in differentiation myogenic cells of the elderly ($R=0.74$, $p=0.02$). This correlation could therefore explain the downregulation of the gamma subunit in old compared to young, although no relationship was observed with the other AChR subunits (see supplemental material of manuscript 2).

In agreement with the hypothesis, more denervated fibres were observed in elderly muscle compared to young. Furthermore, the study also indicated that AChR gamma subunit gene expression in muscle homogenate can be used to assess innervation status in humans and that cultured satellite cells transcribe AChR genes, possibly in a manner affected by age. As the study also showed that AChR gene expression was highly affected by acute exercise, it was prudent to investigate the long-term consequences of exercise more thoroughly.

Muscle fibre denervation and long-term training (study 3)

In study 3, the effect of a 16-week long heavy resistance exercise program on indices of muscle fibre denervation was investigated. The 58 subjects were analysed before, midway through and after the 16 weeks, and were also part of the larger cohort in study 1. The characteristics of EX and SED is provided in table 9. It should be noted that there were three and not two groups in the original study (Heisterberg *et al.*, 2018*b*), as the effect of Losartan alone was also assessed. No effect of drug treatment was observed in the original study or in the present study (see supplemental material of manuscript 3), and it was concluded that the two training groups could be collapsed in this study.

Participant characteristics				
	Exercise *(n=38)*		Sedentary *(n=20)*	
Age (yr)	72 ± 5	*65-83*	72 ± 6	*66-85*
Height (cm)	178 ± 7	*162-191*	179 ± 7	*162-190*
Weight (kg)	85 ± 11	*57-108*	83 ± 11	*62-102*
BMI (kg/m²)	27 ± 3	*19-33*	26 ± 3	*21-32*
LBM (kg)	57 ± 6	*45-71*	56 ± 5	*47-66*

Tabel 9: Age, height, weight, BMI and LBM for EX and SED given as average ± SD [range]. Exercise and sedentary group were compared using unpaired t-tests. Abbreviations: LBM, lean body mass; BMI, body mass index.

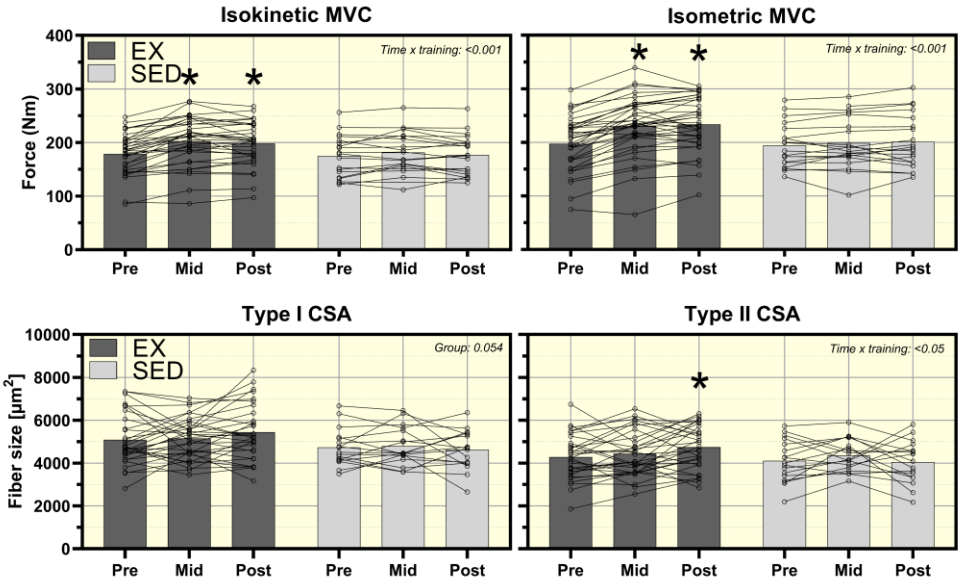

Figure 20: MVC and fibre type specific CSA determined at baseline (pre), after 8 (mid) and 16 (post) weeks in EX and SED. Data are shown as individual values and averages and were analysed by two-way Repeated Measures ANOVA (time x group) with Dunnett's post hoc test. Interactions or main effects are written within figures. * p<0.05 versus pre. Abbreviations: MVC, maximal voluntary contraction; CSA, cross sectional area; EX, exercise; SED, sedentary.

The training intervention was effective, as increases in quadriceps CSA (Heisterberg *et al.*, 2018*b*), MVC and type II muscle fibre CSA were observed (figure 20). However, the percentage of muscle fibres positive for NCAM and MyHCn or with centralized nuclei, did not change following the 16-week intervention (figure 21). The change from pre to post was the primary focus to allow for as long as possible for a change on the protein level to occur and at the same time benefit from the biopsies being obtained from the same leg. Furthermore, it could also be speculated that if muscle injury would be induced by the training of the elderly men, which have been reported before in other training studies (Singh *et al.*, 1999), then mid timepoint after only 8

weeks would be more affected by this. However, the mid time point was also not different (see supplemental material of manuscript 3).

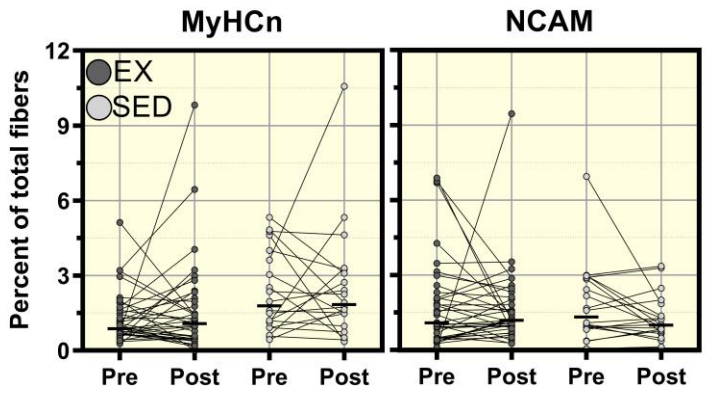

Figure 21: Percentage of muscle fibres positive for MyHCn and NCAM determined using immunofluorescent microscopy on human muscle biopsies at pre and post. Data are shown as individual values with median (horizontal line). Differences between groups were evaluated using the Mann–Whitney Rank Sum Test. Mid time point was analysed and is shown in supplemental material of study 3. Abbreviations: MyHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule.

The lack of change in the number of denervated fibres indicates that 16 weeks is insufficient time for these changes to occur. However, in opposition of this view is what is known from the nerve crush model in animals, which allows for the minute study of reinnervation. Using this model it has been shown that NCAM is first upregulated and then downregulated all within 14-18 days in rodents (Covault & Sanes, 1985; Moore & Walsh, 1986). This rapid regulation implies that, although it is a very extreme model, substantial changes in the innervation status can be achieved in short time. There is no data on the time course of reinnervation available in humans, except it is known that regenerating fibres express NCAM at least 30 days following muscle injury (Mackey *et al.*, 2011; Mackey & Kjaer, 2017). Four studies have previously assessed the number of $MyHCe^+$, $MyHCn^+$ or $NCAM^+$ fibres before and after an resistance exercise intervention (Singh *et al.*, 1999; Messi *et al.*, 2016; Karlsen *et al.*, 2019; Voigt *et al.*, 2019). Singh et al., 1999 reported a substantial amount of MyHCe and MyHCn stained area at baseline in a mixed group of nursing home residents and a further increase following 10 weeks of resistance training (Singh *et al.*, 1999). The authors suggested that the training intervention caused muscle fibre injury and subsequent regeneration. In another study, by Messi et al., 2016, a 5-month resistance exercise in overweight, elderly subjects led to a decline in the $NCAM^+$ area,

which was subsequently viewed as an adaptation favouring NMJ remodelling (Messi *et al.*, 2016). Common for the studies of Singh et al., and Messi et al., is that the quantification of either MyHCe and MyHCn or NCAM, were performed per area and not per fibre. The major concern here is what influence unspecific staining and staining of nerves or other structures might have on the area quantifications. Furthermore, most $MyHCn^+$ and $NCAM^+$ fibres will be greatly atrophied and thus take up a small area relative to the innervated (negative) fibres, creating the risk of underreporting. The percentage of $NCAM^+$ fibres were assessed by Voigt et al., 2019 before and after 14 weeks of resistance exercise in advanced stage osteoarthritis patients (Voigt *et al.*, 2019). Despite significant type II muscle fibre hypertrophy, the intervention did not change the number of $NCAM^+$ fibres, which the authors take as indicative of no or limited effect of the intervention of muscle innervation status. The combination of only 7 subjects and a potential influence of the disease means that these findings are not to be generalized to healthy individuals. In a study by Karlsen et al., 2019, the percentage of intensely stained $MyHCn^+$ combined with $MyHCe^+$ fibres were reduced following 12 weeks of heavy resistance exercise in 12 subjects aged between 83 and 94 years (Karlsen *et al.*, 2019). This result would indicate improved innervation status and happened despite the intervention not causing significant hypertrophy at the muscle fibre level. Lastly, in addition to the 4 studies focusing on resistance exercise, Brightwell et al., 2019 saw no change in the percentage of $NCAM^+$ fibres following 24 weeks of aerobic exercise (Brightwell *et al.*, 2019). It should be noted that this was only tested on a subset of samples, from the already limited 12 subjects that underwent training, which limits the extent to which these findings can be used. Taking these 5 studies and the present study together indicates, that no consensus can be made on whether the proportion of $MyHCn^+$ or $NCAM^+$ fibres can be expected increase, decrease or remain unaffected in response to a prolonged exercise intervention. If the duration of the training period is important for the presence of $MyHCn^+$ and $NCAM^+$ fibres, then lifelong exercisers should be investigated.

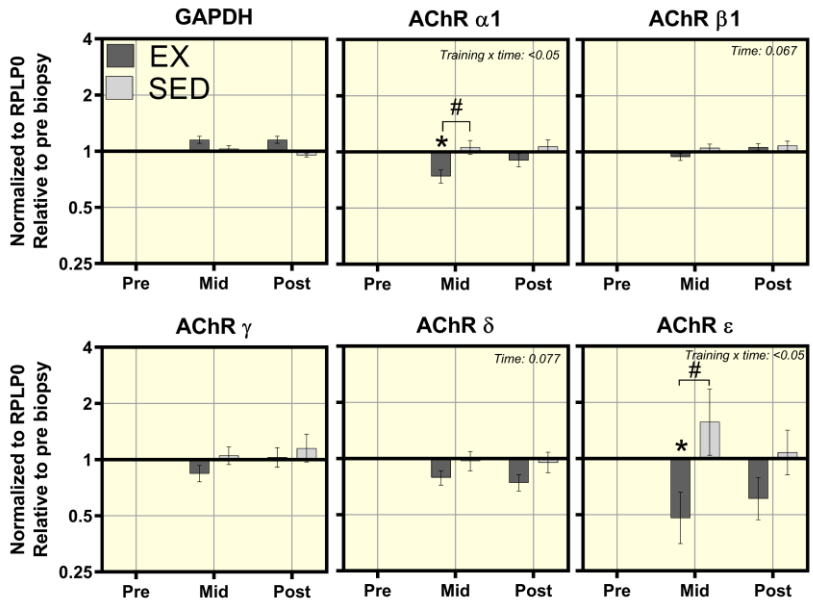

Figure 22: Gene expression in muscle biopsies at pre, mid and post in EX (dark grey, n=38) and SED (light grey, n=20). mRNA data are expressed relative to RPLP0 and are shown as geo mean ± back-transformed SEM. Data are shown relative to pre. Changes in gene expression were analysed by two-way Repeated Measures ANOVA (time x group) with Dunnett's post hoc test. Interactions or main effects are written within figures. * p<0.05 versus pre. # p<0.05 EX versus SED. Abbreviations: AChR, acetylcholine receptor; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; RPLP0, Ribosomal Protein Lateral Stalk Subunit P0.

Effect of long-term resistance exercise on AChR gene expression

AChR gene expression was again measured as a token of NMJ remodelling, and it was speculated that heavy resistance exercise, that lead to both hypertrophy and increases in muscle strength, would positively influence innervation status of the muscle. In support of this hypothesis, two subunits, alpha1 and epsilon, were significantly downregulated midway through the intervention (figure 22). Extrasynaptic presence of AChRs gene expression is indicative of muscle fibre denervation (Pestronk & Drachman, 1978; Goldman & Staple, 1989; Witzemann *et al.*, 1991; Missias *et al.*, 1996). Downregulation relative to baseline therefore implies *less* denervation (improved innervation). The altered AChR profile at 8 weeks coincides with both MVC and whole muscle CSA being significantly increased at this time point (Heisterberg *et al.*, 2018*b*), suggesting an association with the degree of tissue remodelling. In support of this, Karlsen et al., 2020 saw no change in AChR gene expression following 13 weeks of a very similar training program, where it could be speculated that the point with the highest degree of tissue remodelling had occurred earlier. Although theoretical, it could be that fibres with a destabilized NMJ, which caused the extra-synaptic presence of AChRs, were positively affected

by the intervention, thus representing a mechanism of exercise induced NMJ fortification. It should be noted that the biopsies at both mid and post were sampled 2 days after the most recent exercise bout. Given that no significant differences between SED and EX or within group relative to baseline, were observed at post, it appears more likely that the changes observed at mid reflects a training response rather than an acute response.

Study 3 is the first study to investigate muscle innervation in large group of healthy men before and after a hypertrophy-inducing resistance exercise intervention. Surprisingly, no change in the number of denervated fibres were observed, while AChR gene expression was decreased in two subunits during the intervention. Based on these results it could be speculated that an even longer time frame would be necessary for differences in the prevalence of denervated fibres to become visible. As such, study 4 was established with the main goal of investigating muscle innervation in lifelong exercisers.

Muscle fibre denervation and lifelong exercise (study 4)

To study the influence of lifelong recreational exercise on indices of muscle function, fibre denervation, muscle morphology and satellite cells, physically active elderly men and sedentary controls were recruited. The characteristics are provided in table 10. The young men were taller, had lower fat percentage and lower C-reactive protein and Haemoglobin A1c levels in the blood, compared to old.

Participant characteristics

	Young *(n=15)*		LLEX *(n=16)*		SED *(n=15)*	
Age (yr) *	26 ± 5	*20-36*	73 ± 4	*68-82*	73 ± 4	*68-82*
Height (cm) *	183 ± 7	*169-193*	176 ± 6	*166-185*	178 ± 8	*161-195*
Weight (kg)	82 ± 13	*62-105*	76 ± 9	*63-94*	82 ± 11	*65-109*
BMI (kg/m²)	24 ± 3	*20-30*	24 ± 3	*21-31*	26 ± 3	*22-32*
Fat percentage $	24.7 ± 6.6	*10-33*	23.6 ± 6.4	*12.2-33.6*	29.9 ± 5.4	*15-38*
LBM (kg) *	61.6 ± 7.2	*48.8-71.7*	58.1 ± 4.5	*51.6-67.1*	57.1 ± 6.1	*46.1-71.6*
MVC/BW (Nm/kg) * ($)	3.9 ± 0.7	*2.9-5.2*	2.7 ± 0.6	*1.8-3.5*	2.3 ± 0.6	*1.5-3.6*
RFDpeak (Nm/s) *	2585 ± 857	*1194-4048*	1425 ± 507	*692-2351*	1380 ± 479	*759-2746*
CRP (mg/L) *	1.3 ± 0.8	*1-4*	2.4 ± 2.1	*1-9*	3.0 ± 3.2	*1-13*
HbA1c (mmol/L) *	5.5 ± 0.4	*4.9-6.2*	5.9 ± 0.3	*5.5-6.8*	5.9 ± 0.5	*5.0-6.5*
Type I percentage (*) $	47.3 ± 13.5	*29.8-79.1*	60.5 ± 17.4	*23.3-84.4*	49.9 ± 15.4	*23.4-67.7*
Type I fibre size (um2) (*)	4912 ± 1147	*3283-7314*	4385 ± 727	*2891-5449*	4154 ± 1384	*2566-8031*
Type II fibre size (um2) *	5380 ± 845	*4067-7142*	3138 ± 892	*1510-5073*	3107 ± 1045	*2033-5614*

Tabel 10: Age, height, weight, BMI, blood sample parameters, DEXA derived measurements, MVC, RFDpeak and fibre type CSA for young, LLEX and SED given as average ± SD [range]. Abbreviations: BW, bodyweight; LBM, lean body mass; MVC, maximal voluntary contraction; RFD, rate of force development; BMI, body mass index; Nm, newton meter; CRP, C-reactive protein; HbA1c, haemoglobin type A1c. Young were compared to old groups combined, and LLEX was compared to SED, both using unpaired t-tests. * p<0.05 young versus old. (*) p<0.1 young versus old. $ p<0.05 LLEX versus SED. ($) p<0.1 LLEX versus SED.

Muscle mass and function *in vivo*

Declining levels of muscle mass and muscle strength with ageing have been linked to reduced functional capability (Tinetti & Williams, 1997) and increased risk of various lifestyle related diseases (Wolfe, 2006), such as impaired glucose tolerance (Rowe *et al.*, 1983; Dela *et al.*, 1996) and obesity (Michalakis *et al.*, 2013). Increases in muscle strength (Häkkinen *et al.*, 2000; Snijders *et al.*, 2019) and muscle size (Kryger & Andersen, 2007; Karlsen *et al.*, 2020) as well as reductions in fat mass (Nordby *et al.*, 2012; Gylling *et al.*, 2020) have been reported following intense, supervised interventions. However, whether these anthropometric measures are modulated by self-organized recreational physical activity is less clear. Thus, in the following section, studies comparing long-term physically active and sedentary individuals will be the central reference point.

The old had a smaller type I (tendency) and type II muscle fibre CSA compared to young, as has been shown before (Klitgaard *et al.*, 1990; Zampieri *et al.*, 2015; St-Jean-Pelletier *et al.*, 2017; Sonjak *et al.*, 2019; Karlsen *et al.*, 2020). Studies have also reported larger fibre CSA in trained elderly individuals compared to sedentary (Klitgaard *et al.*, 1990; Zampieri *et al.*, 2015), but that was not observed in the present study. Sonjak et al., 2019 reported no difference in fibre size between 17 pre-frail/frail elderly women and 7 track and field master athletes, although they did see differences in other parameters, such as accumulation of atrophic fibres (Sonjak *et al.*, 2019). The reason behind the lack of difference in the present study is likely related to the types of activity that the participants in LLEX performed. Many different activities were reported, but only 3 subjects performed resistance exercise as their primary sport. Most individuals had therefore not been exposed to a sufficiently large hypertrophic stimulus for fibre size to increase. As expected, the old groups had lower LBM, muscle strength and RFD than young (table 24). However, we saw no difference between the two old groups on these measures. It is not clear from the literature whether recreational physical activity is sufficient to increase and/or preserve LBM during ageing. Master athletes or individuals engaging in sports requiring either explosiveness or high force have a larger LBM compared to sedentary individuals (Ojanen *et al.*,

2007; Unhjem *et al.*, 2016; Piasecki *et al.*, 2016*a*; Sonjak *et al.*, 2019) and compared to individuals performing endurance exercise (Klitgaard *et al.*, 1990; Drey *et al.*, 2016). However, others have found that recreationally active individuals do not have an increased LBM compared to sedentary controls (Pearson *et al.*, 2002; Lanza *et al.*, 2008; St-Jean-Pelletier *et al.*, 2017), unless resistance exercise were performed (Grassi *et al.*, 1991; McKendry *et al.*, 2020), although some exceptions do exists (Safdar *et al.*, 2010; Mikkelsen *et al.*, 2013; Grosicki *et al.*, 2021). Regarding differences in muscle force due to age and patterns of physical activity, the literature is more clear, as most studies find that physically active elderly individuals have a higher muscle peak force compared to age-matched physically inactive peers (Pearson *et al.*, 2002; Ojanen *et al.*, 2007; Aagaard *et al.*, 2007; Safdar *et al.*, 2010; Mosole *et al.*, 2014; Power *et al.*, 2016; Unhjem *et al.*, 2016; Piasecki *et al.*, 2019; Grosicki *et al.*, 2021). However, sports requiring high force still appears more effective for maintaining muscle function (Klitgaard *et al.*, 1990; Unhjem *et al.*, 2016; Piasecki *et al.*, 2019).

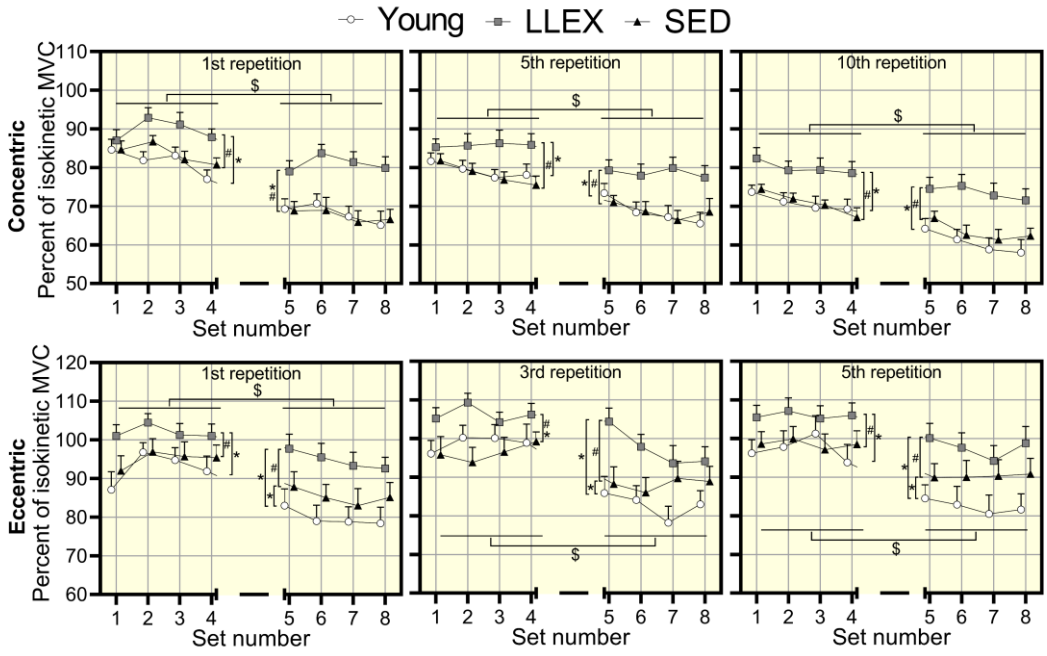

Figure 23: Force output during acute exercise bout. The first, fifth and tenth concentric and first, third and fifth eccentric repetition of each set was collected and expressed relative to isokinetic MVC (average ± SEM). For statistical analyses, set 1 to 4 and set 5 to 8 were averaged into round 1 and 2, respectively, and then subjected to two-way ANOVA (group x time), using the Holm-Sidak post hoc test. $ $p < 0.05$ between rounds. # $p < 0.05$ SED versus LLEX. * $p < 0.05$ old combined versus young. Abbreviations: MVC, maximal voluntary contraction; LLEX, lifelong exercise; SED, sedentary.

An acute exercise bout was used in the present study to test muscle function under challenged conditions (figure 23). Interestingly, LLEX consistently produced force at a higher percent of MVC across all repetitions and round compared to both SED and young, which proves that this group was exercise habituated. This also indicates that LBM and MVC might not be suitable to detect subtle differences between recreationally active individuals and their sedentary peers. Varesco et al., 2021 similarly reported that daily steps in elderly women showed a positive correlation with total work during an exercise test, but not MVC (Varesco *et al.*, 2022).

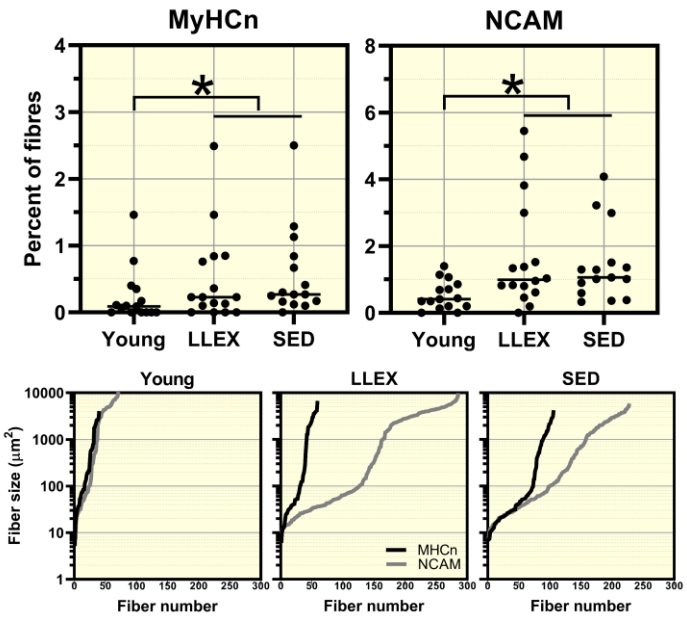

Figure 24: Percentage of muscle fibres positive for MyHCn and NCAM determined using immunofluorescent microscopy on human muscle biopsies. Data are shown as individual values with median (horizontal line). Statistically, young were compared to old combined and LLEX was compared to SED, both using the Mann–Whitney Rank Sum Test. * p <0.05 young versus old. The fibre sizes of all MyHCn$^+$ and NCAM$^+$ fibres within each group are also shown. Abbreviations: MyHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule

Muscle fibre denervation

The number of MyHCn$^+$ and NCAM$^+$ muscle fibres were assessed by immunofluorescence on muscle biopsy cross sections from all subjects (figure 24). MyHCe was not assessed due to the low abundance of fibres positive for this marker previously determined in study 1. NCAM$^+$ and MyHCn$^+$ fibres were detected in 43 and 35 out of 46 samples respectively, with a total of 563 and 190 positive fibres. As a further advancement of the method compared to study 1-3, we decided to stain for phalloidin and the muscle specific proteins merosin and desmin, on a

subsequent cross-section. Fibres that were not merosin$^+$ and desmin$^+$ or merosin$^+$ and phalloidin$^+$ were removed. This led to removal of some of the very small MyHCn$^+$ or NCAM$^+$ fibres. Additionally, 14 out of 37 NCAM$^+$ fibres in one sample were also Col22$^+$ and were subsequently removed from the analysis (MTJ tissue). As expected, young had a lower percentage of fibres positive for both NCAM and MyHCn compared to old. However, there was no difference between the two old groups, indicating that their prior pattern of lifelong physical activity did not affect the accumulation of denervated fibres.

As previously noted, the positive fibres are very heterogenous in terms of shape, size, staining intensity and whether the co-express other markers (figure 24 and study 4 in appendix). It was observed that 20-30 % of NCAM$^+$ fibres also expressed MyHCn$^+$, which was similar to what was found in study 1 (see manuscript 4). Furthermore, NCAM$^+$ fibres were found to be both positive and negative for MyHC I (see manuscript 4). In terms of size of the positive fibres, the CSA ranges from 30 to ~10000 μm^2, with most positive fibres being <1000 μm^2.

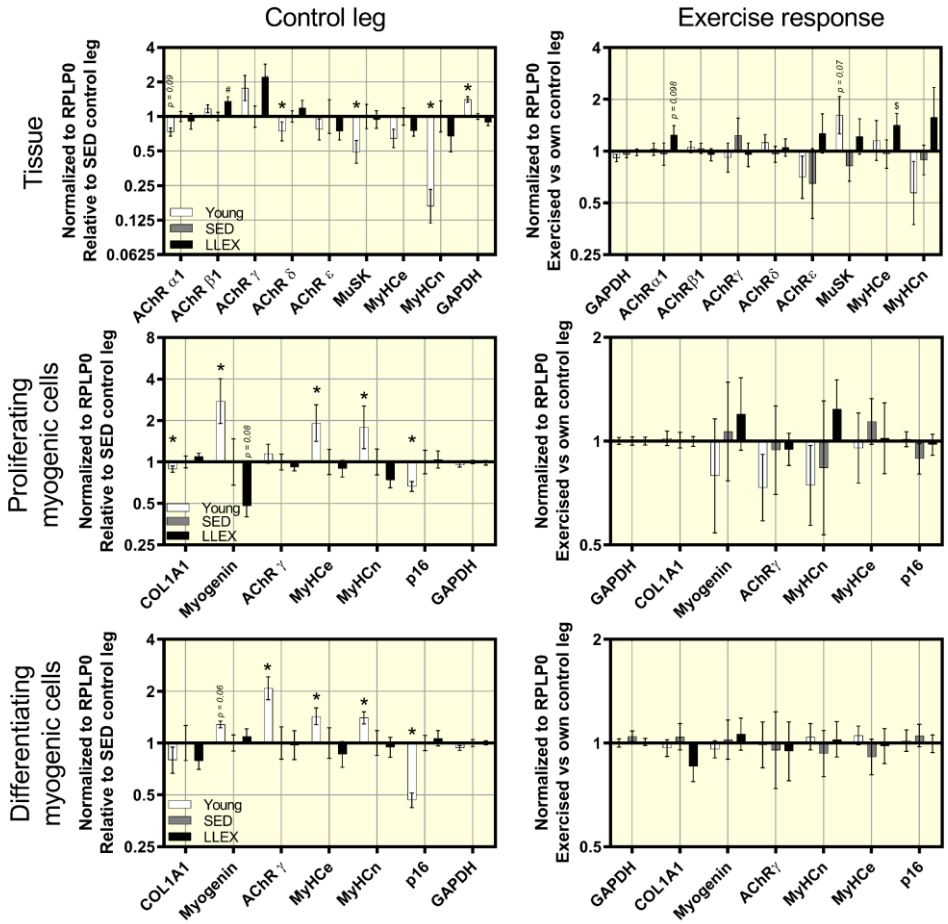

Figure 25: Gene expression in muscle biopsies (top row), proliferating (middle row) and differentiating (bottom row) myogenic cells from rested (left column) and exercised leg (right column). mRNA data are expressed relative to RPLP0 and are shown as geo mean ± back-transformed SEM. For the control leg, data are expressed relative to SED and were investigated using unpaired t-tests. For exercise response, the exercised leg is expressed relative to own control leg and were investigated using paired t-tests. * p<0.05 young versus old. # p<0.05 LLEX versus SED. $ p<0.05 versus control leg. Tendencies are written. Abbreviations: AChR, acetylcholine receptor; MuSK, muscle-specific-kinase; MyHCe, embryonic myosin heavy chain; MyHCn, neonatal myosin heavy chain; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; COL1A1, Collagen type I alpha 1 chain; RPLP0, Ribosomal Protein Lateral Stalk Subunit P0.

The mRNA levels of the AChR subunits were also assessed in muscle homogenate (figure 25). The basal level of RPLP0 was either decreased or GAPDH was increased in young compared to old. The latter seemed more likely as this would indicate a lower metabolic activity, and RPLP0 was chosen as normalizer. Old had higher levels of alpha1 (tendency) and delta compared to young, whereas both beta1 and gamma was higher in LLEX compared to SED. However, there was a striking resemblance between young and LLEX, which was not reflected in the chosen statistical analysis. Therefore, it could be theorized that the young subjects were more physically active during everyday life, which could increase their similarity to LLEX.

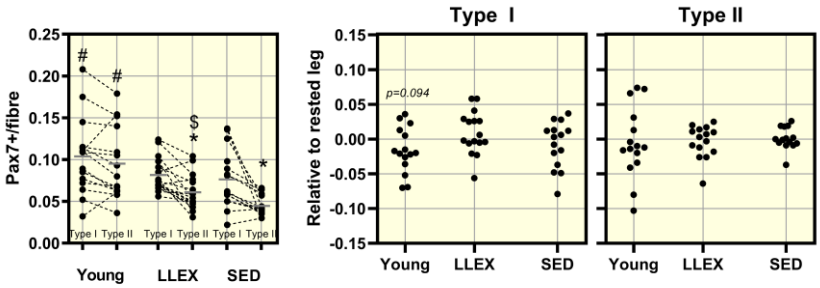

Figure 26: Satellite cell quantity was assessed on cross section of biopsies using immunofluorescence. Satellite cells per fibre for type I and II fibres, shown as individual values and average (horizontal line) and connected between fibre types by dotted line. The middle and right graph shown the exercise response (exercised leg relative to control leg). Differences in the control leg between groups within fibre type were evaluated using unpaired t-tests and differences between fibre types within groups were evaluated using paired t-tests. Differences between exercised and rested leg were evaluated using paired t-tests. * p<0.05 type I versus type II. # p<0.05 young versus old combined. $ p<0.05 LLEX versus SED. Tendencies are written. Abbreviations: LLEX, lifelong exercise; SED, sedentary.

Satellite cell quantity and function

The quantity and function of satellite cells were studied in parallel with the assessment of muscle innervation status (see figure 26), as satellite cell numbers decline with age (Verdijk *et al.*, 2014), and novel roles for satellite cells in relation to NMJ maintenance (Liu *et al.*, 2015, 2017) and compensatory myogenesis following denervation (Borisov *et al.*, 2001, 2005) have been shown in rodents. Interestingly, a 22 % higher number of type II muscle fibre associated satellite cells were observed in LLEX compared to SED, while no difference was observed for type I fibres. It has been shown that type II muscle fibre associated satellite cells are more severely affected by ageing than type I (Verdijk *et al.*, 2014; Karlsen *et al.*, 2019, 2020). However, it is also possible that this decline could be attributed to a reduced activation of type II muscle fibres, as has been shown before (Englund *et al.*, 2021). Then, these results suggests that lifelong recreational exercise can partly alleviated this decline. The larger pool of dormant satellite cells could play a role in the event of denervation or injury, as they then initiate a myogenic response (Shefer *et al.*, 2006).

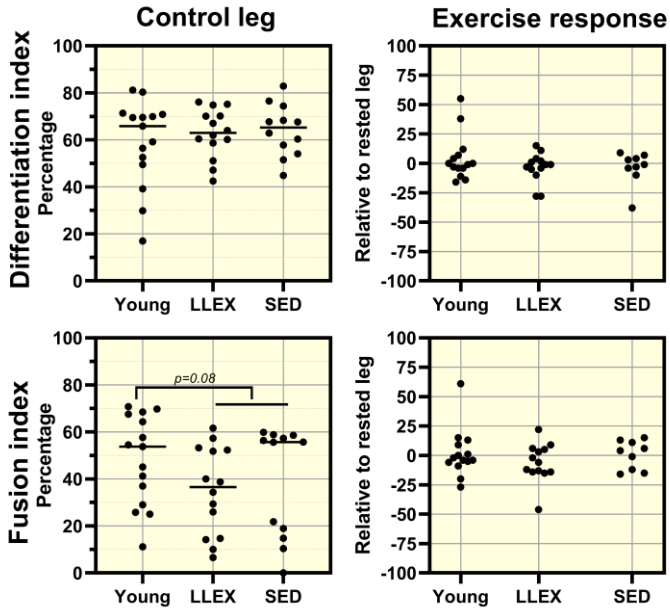

Figure 27: Satellite cell function was assessed *in vitro* using primary human cells. Differentiation (top row) and fusion (bottom row) index in control (left column) and exercise (right column) leg, shown as individual values with median (horizontal line, only control leg). Differences between groups in the control leg were evaluated using the Mann-Whitney Rank Sum test. Differences between exercised and rested leg were evaluated using the Wilcoxon Signed Rank test. Tendencies are written. Abbreviations: LLEX, lifelong exercise; SED, sedentary.

Satellite cell function was tested using immunofluorescent and RT qPCR analysis of cell cultures (figure 27). Alterations in cellular function have been shown in relation to both exercise and disease (Sharples *et al.*, 2016), and these *in vivo* traits have also been shown to be translated into an altered behaviour and changes in gene expression of cultivated satellite cells (Teng & Huang, 2019). Here, a comprehensive semi-automated immunofluorescent analysis was undertaken to quantify the satellite cell's ability to differentiate and fuse, as well as gene expression analysis of genes related to myogenesis, NMJ remodelling and cellular senescence. Surprisingly, no differences were observed for differentiation index, while only a tendency for fusion index to be higher in young compared to old was observed. This latter finding might also be explained by differences in the cell count (see manuscript 4). These findings contrast a recent study from our group, where a clear difference in fusion index between cells from young and old individuals was observed (Bechshøft *et al.*, 2019). In contrast, when the ability of the cultured satellite cells to transcribe genes related to myogenesis, NMJ remodelling and cellular senescence were assessed, several age-related differences were observed. However, age-related differences in the function of satellite cells are not a universal finding, as some (Bechshøft *et al.*, 2019; Balan *et al.*, 2020) but not all (Alsharidah *et al.*, 2013; Chaillou *et al.*, 2020) find differences. The

underlying reasons for the lack of consistency is not clear, but the use of immortalized cell lines or primary cells, and how the analyses were carried out is likely to be involved. There was no difference between LLEX and SED in differentiation or fusion index, or in gene expression, in the present study. Several studies have observed an influence of exercise on cultured satellite cells (Green et al., 2013; Cisterna et al., 2016; Lund et al., 2017, 2018).

General discussion

Muscle fibres are giant multinucleated cells that shrink and grow throughout a lifetime in response to various stimuli. When a muscle fibre loses its connection to the nervous system it will gradually atrophy until it is eventually lost, unless it is reinnervated by an axon. Studies in both rodents (Ishihara *et al.*, 1987; Bütikofer *et al.*, 2011; Nielsen *et al.*, 2018) and humans (Inokuchi *et al.*, 1975; Lexell *et al.*, 1988) have provided evidence of loss of muscle fibres. The microenvironment surrounding the muscle fibres becomes increasingly infiltrated by fibrotic tissue with ageing (Borisov *et al.*, 2005; Nederveen *et al.*, 2020), while clumping of nuclei (Viguie *et al.*, 1997) and loss of fibre type uniformity (Rowan *et al.*, 2012; Purves-Smith *et al.*, 2012; Carraro *et al.*, 2017; Medler, 2019) are commonly observed in aged muscle. The ultimate consequences of fibre denervation, failed reinnervation and loss of fibres are muscle atrophy and loss of muscle function (Baumgartner, 2000; Janssen *et al.*, 2000; Suetta *et al.*, 2019), which was also observed in study 2 and 4, where the old participants were weaker, had lower LBM (study 4) and smaller muscle fibre CSA than their young counterparts.

The skeletal muscle system is normally capable of remarkable adaptation and regeneration. This is evidenced by substantial neuromuscular adaptations observed in response to heavy loading (Aagaard, 2003) and the coordinated regeneration of muscle tissue following severe muscle injury (Mackey *et al.*, 2016). It must then be wondered why a challenged innervation status is not in the same way responded to efficiently by the muscle fibres. It has been shown that muscle fibre denervation cause remodelling of the capillary network, increased fibrosis and a strong activation of satellite cells (Viguie *et al.*, 1997; Borisov *et al.*, 2001, 2005), where the latter can be seen as an attempt of what is termed compensatory myogenesis (I.E. a failed attempt to recover). However, the rescue of a muscle fibre by reinnervation from a nearby axon requires a change in its gene expression that reflects the denervated state and which serves to attract a nerve (Walsh *et al.*, 2000). The studies conducted as part of this thesis has provided evidence for the accumulation of denervated fibres in elderly healthy muscle. This accumulation indicates that the

underlying mechanisms responsibly for the timely and effective reinnervation of muscle fibres is somehow insufficient at advanced age.

Evidence of muscle fibre denervation

Key changes in the neuromuscular system with ageing include a decay of motor neurons located in the spinal cord (Kawamura *et al.*, 1977; Tomlinson & Irving, 1977; Ishihara *et al.*, 1987; Rowan *et al.*, 2012; Fogarty *et al.*, 2018) and fewer myelinated axons (Corbin & Gardner, 1937; Gardner, 1940; Swallow, 1966; van Steenis & Kroes, 1971; Samorajski, 1974; Tohgi *et al.*, 1977; Mittal & Logmani, 1987; Hashizume *et al.*, 1988; Ansved & Larsson, 1990) as well as degenerative changes at the NMJ (Courtney & Steinbach, 1981; Smith & Rosenheimer, 1982; Fahim & Robbins, 1982; Arizono *et al.*, 1984; Oda, 1984; Rosenheimer, 1990; Wokke *et al.*, 1990; Balice-Gordon, 1997; Valdez *et al.*, 2010; Chai *et al.*, 2011; Bütikofer *et al.*, 2011; Cheng *et al.*, 2013; Chung *et al.*, 2017; Liu *et al.*, 2017; Gillon *et al.*, 2018). This age-related deterioration of the neuromuscular system hampers synaptic transmission resulting in muscle fibre denervation. There are multiple histological indicators of denervation that each represent distinct stages of this process. The studies presented in this thesis predominantly focused on the presence of "denervation responsive" proteins and genes using immunofluorescence and RT qPCR, respectively.

Throughout the studies the method for analysing NCAM and MyHCn was refined and optimized, but in all studies a very large number of muscle fibres were included, which is important in order to obtain reliable quantifications. In study 1 and 3, which are from the same analysis, dystrophin was used as the sole prerequisite for a fibre to be counted. In contrast, both study 2 and 4 used other muscle specific markers on serial sections to improve the certainty of the analysis. The use of serial sections can be harsh on this type of analysis, as serial sections of muscle biopsies are not identical, and certain fibres or areas might disappear. However, the major advantage of the method is the spatial and morphological insight it provides and that multiple immunofluorescent markers can be combined. But there are also certain considerations that must be taken into account when interpreting the findings. First, it must be realized that taking muscle biopsies using the Bergstrom technique will, even when standardized, carry a low risk of sampling both MTJs and NMJs. In fact, among the hundreds of biopsies that were investigated in this thesis, in a few of them, some $NCAM^+$ and $Col22^+$ fibres (MTJ tissue) were observed (Vaittinen *et al.*, 1999; Koch *et al.*, 2004). This issue likely arises from the actual

sampling site within the muscle being too close to the aponeurosis that surrounds the muscle. I was not aware of the risk of obtaining MTJ in study 1 and 3, which might have led to inclusion of $NCAM^+$ fibres that were not denervated, but instead close to the MTJ. Furthermore, these is also a risk that NMJs could have been sampled by chance. NMJs are quite distinctive on cross sections and would have been noticed during the image analysis. However, in relation to the RT-qPCR analyses on muscle homogenate, this might be an actual issue, as ~100 cross-sections were blindly sectioned from the muscle biopsies.

The second consideration is that both NCAM and MyHCn are expressed in other conditions, such as regeneration (Sartore *et al.*, 1982; Irintchev *et al.*, 1994; Mackey & Kjaer, 2017) and neuromuscular disease (Fitzsimons & Hoh, 1981; Walsh & Moore, 1985) and in intrafusal fibres (MyHCn only) (Walro & Kucera, 1999; Schiaffino *et al.*, 2015). Intrafusal fibres are rare, and neuromuscular disease usually present with very distinctive histological characteristics, so it is unlikely that these conditions can explain the ~1 % positive fibres in elderly muscle. Muscle regeneration is a vital muscle remodelling process that contains both inflammatory and satellite cell mediated stages (Ciciliot & Schiaffino, 2010) where NCAM and MyHCn expression is removed as it progresses. Theoretically, a certain threshold for an insult to cause muscle injury must exists, and it can then be speculated that this threshold might be lower in old compared to young. As such, it would no longer, as is the case in young healthy muscle, require a substantial effort by the individual, to cause muscle injury (Crameri *et al.*, 2007). It might simply be that unaccustomed activities of any kind, such as stairclimbing or gardening, could cause a small-scale increase in expression of NCAM and/or MyHCn. This would then, in the snapshot of the muscle biopsy, appear as single positive muscle fibres intermingled among many negative. Nevertheless, the most likely explanation for the presence of NCAM and MyHCn in healthy muscle remains a disturbance in the transmission of nerve signals to the muscle which subsequently increases the transcription of certain denervation responsive genes. Yet, with the currently available markers -immunofluorescence and RT-qPCR – it is not possible to completely rule out that muscle regeneration also interferes.

The third and final consideration is that there is substantial inter and intra-individual variation in these measurements (both immunofluorescence and RT-qPCR). This is clearly shown in immunofluorescent data of the sedentary control group of study 3 (figure 21), where values from the same individual varies over time despite no exercise being performed. This consequently

means that large groups of subjects and/or large differences between groups are needed for a significant difference to be observed.

To summarize the histological findings on muscle fibre denervation from the studies of this thesis and the works of others, an illustration has been made (figure 28). Ageing is associated with a gradually accelerating decline in muscle function driven to a large extent by neuromuscular alterations. Healthy young muscle is characterized by random scattering of fibre types and hybrid fibres are rare (except type IIa/IIx). The NMJs show no signs of morphological changes, and there is no motor neuron death. The neuromuscular system is functioning optimally at this age. During the early stages of ageing, denervated muscle fibres, type II muscle fibre atrophy, hybrid fibres and type I fibre grouping (Rowan *et al.*, 2012; Mosole *et al.*, 2014; Sonjak *et al.*, 2019) is observed. These indices suggest that fibre denervation is a frequent event, but that it is mostly responded to by reinnervation, to the extent that overall muscle atrophy is attenuated. Muscle fibre denervation is mostly affecting type II fibres and it occurs due to both early morphological changes at the NMJ and a limited loss of MNs. At later stages of ageing reinnervation is failing which leads to pronounced accumulation of denervated fibres and accelerated loss of muscle mass. Whereas type II fibres had previously been mostly exposed to denervation, now type I fibres are also affected. There is also substantial loss of motor neurons at this stage, which severely impacts on muscle function, as each motor unit is very large (more fibres lost per motor neuron). It can be speculated whether severely atrophied fibres are able to be reinnervated or whether they are deemed expendable.

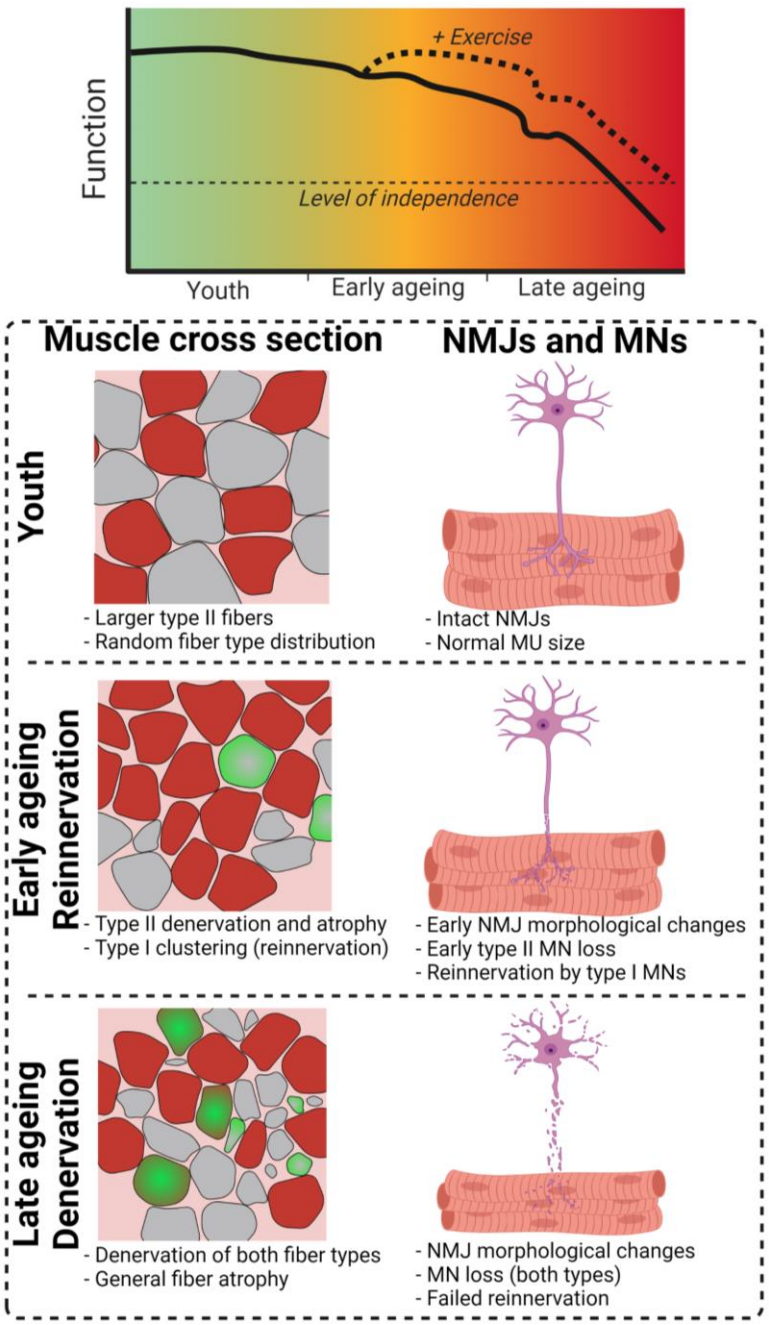

Figure 28: Alterations in the neuromuscular system drives the gradually accelerating loss of muscle function during ageing which eventually might lead to a loss of independence. Repeated sequences of muscle fibre denervation and subsequent reinnervation characterizes the early stage of ageing. There is a limited decay of motor neurons, while remodelling of the NMJs, which is detectable on the gene expression level, is frequent. These changes lead to increased presence of mostly large-sized denervated fibres, enlarged motor units, type I fibre type grouping and hybrid fibres. Exercise-induced changes in AChR gene expression indicates a sustained adaptability in aged muscle at this stage, perhaps reflecting a mechanism by which the age-related loss of muscle function can be ameliorated. At later stages of ageing, MN death and NMJ remodelling are both widespread, which, concomitant with failed reinnervation, leads to increased presence of atrophied denervated fibres, hybrid fibres and loss of fibres in general. Highly atrophied and deteriorated muscle fibres might be incapable of rescue by exercise at this stage. Abbreviations: NMJ, neuromuscular junction; MU, motor unit; MN, motor neuron. This illustration was created using BioRender.

NMJ remodelling and AChR gene expression

In addition to the histological assessment of denervation, there are several denervation responsive genes that can be studied in relation to ageing and exercise (Glass *et al.*, 1996; Baehr *et al.*, 2016; Daou *et al.*, 2020), while recent studies using RNA sequencing have provided a number of new target genes (Petrany *et al.*, 2020; Solovyeva *et al.*, 2021). Several novel findings on AChR gene expression were reported in the studies of this thesis. These include age-related differences, changes following both acute and long-term exercise and different profiles in

lifelong exercisers compared to sedentary individuals. Taking the findings together it is noticeably how often age or exercise induced differences is observed among the mere 5 gene targets. There is also a remarkable consistency in the findings between the studies. For example, alpha1 tended to be higher in old compared to young in both study 2 and 4, and both alpha1 and delta were increased 5-7 days after an acute exercise bout in the two groups of subjects in study 2. However, discrepancies still exist between studies, and these are further aggravated when taking other studies that measure the same targets into account (Messi *et al.*, 2016; Sonjak *et al.*, 2019; Karlsen *et al.*, 2019, 2020). Overall, the interpretation of these differences in AChR gene expression and how it relates to NMJ remodelling is complex. Very few studies of AChR subunit-specific gene expression in humans are available, with most knowledge on the regulation in ageing and diseases being derived from studies on rodents.

The relative contribution of NMJ destabilization and motor neuron decay to the age-related decline in muscle mass and function is unknown. AChR clusters are maintained through the Agrin-MuSK-LRP4-Rapsyn signalling cascade, where possibly defects might interfere with the stability of the NMJ. MuSK gene expression was measured in muscle homogenate in study 2 and 4. In both studies old had higher level compared to young ($p=0.05$ in study 2), whereas no effect of acute exercise was observed. While concrete interpretations of these data are difficult, the findings at least support the view that some sort of age-related disturbance of the signalling cascade is in effect. In that regard, there is a vast literature that describes age-related structural and/or functional changes in the animal NMJ (Balice-Gordon, 1997; Valdez *et al.*, 2010; Chai *et al.*, 2011; Bütikofer *et al.*, 2011; Cheng *et al.*, 2013; Chung *et al.*, 2017; Liu *et al.*, 2017; Gillon *et al.*, 2018), while the number of studies in humans is limited (Arizono *et al.*, 1984; Oda, 1984; Wokke *et al.*, 1990; Jones *et al.*, 2017; Alhindi *et al.*, 2021). The specific changes observed have been reviewed elsewhere (Iyer *et al.*, 2021), but include increased synapse fragmentation and loss of junctional foldings. The relatively low number of studies on human NMJs likely relates to its inaccessibility, as it's primarily obtained from post-mortem samples, from specific surgical procedures or alternatively using a specific biopsy technique (Aubertin-Leheudre *et al.*, 2020).

Given this difficulty in obtaining human NMJs, a prudent alternative is to study changes in extrasynaptic signals, such as AChR and NCAM. Thus, the altered AChR and MuSK gene expression profiles in elderly muscle observed in this thesis and the rapid regulation of NMJ structure and function (Mansilla *et al.*, 2018), together points toward NMJ destabilization representing a temporary phase of reduced synaptic transmission. This explanation reconciles

very well with the heterogeneity in size of $NCAM^+$ fibres observed in study 2 and 4, as some of the positive fibres were of similar size as to the $NCAM^-$ fibres from the same biopsy. NMJ destabilized fibres are the large $NCAM^+$ fibres during early ageing represented in figure 28. It can then be speculated that the synapse in some cases is reorganized or improved, while it other cases is replaced by a new one. The aetiology of muscle fibre denervation is therefore a combination of multiple processes, with highly atrophied fibres representing long-term denervation and regular-sized fibres representing NMJ destabilization. It can be further speculated that the accumulated effect of many rounds of NMJ destabilization and subsequent remodelling causes gradual atrophy of fibres, and perhaps the emergence of hybrid fibres and MyHCn expression. Even more detailed immunofluorescent analyses, taking fibre type and overall fibre size into account, would have to be conducted to answer these questions.

The influence of physical activity on muscle innervation

Muscles secrete a plethora of molecules in response to contractions (Roca-Rivada *et al.*, 2012) that can exert autocrine, paracrine or even endocrine effects (Delezie & Handschin, 2018) on multiple organs and tissues (Florin *et al.*, 2020). It has also been shown that muscle-derived signals are taken up at the NMJ and transported back along the axon to the motor neuron in a process called retrograde signalling (Chakkalakal *et al.*, 2010). A number of studies have provided compelling evidence for this biological process (Chakkalakal *et al.*, 2010; Zahavi *et al.*, 2015; Ionescu *et al.*, 2016; Maimon *et al.*, 2018, 2021; Mills *et al.*, 2018; Cantor *et al.*, 2018; Stoklund Dittlau *et al.*, 2021; Correia *et al.*, 2021), and that secreted signals can be both positive and supportive, and negative and hostile to motor neuron survival (Zahavi *et al.*, 2015). Based on this it has been speculated that exercise can facilitate reinnervation and/or NMJ maintenance and perhaps modulate the lifespan of motor neurons, by orchestrating a selective secretion of positive molecules and/or reduce negative ones (Soendenbroe *et al.*, 2021). In this thesis, innervation status in relation to exercise was investigated using, a bout of heavy resistance exercise (study 2 and 4), long-term training interventions (study 3) and lifelong exercise (study 4).

Taking the studies using the acute model together it is clear that the response to exercise in AChR mRNA levels is subunit specific and time dependent. While concrete interpretations between the studies are difficult to perform, it is clear that the stability of NMJ is modulated by acute exercise in both young and old.

In study 3 it was observed that the proportion of denervated muscle fibres assessed by immunofluorescence did not change, while there were indications on the gene expression level for an improvement in innervation after 8 weeks of training. The underlying reason for this discrepancy is unclear. It is possible that there are different pools of fibres that generate the signals, meaning that the fibres that were assessed by immunofluorescence were not the same that contributed the most in the gene expression analysis. The favourable changes in AChR gene expression thus likely reflect a strengthening of existing NMJs, whereas the majority of the $MyHCn^+$ or $NCAM^+$ fibres were severely atrophied. It can be speculated that this group of atrophied fibres might be incapable of reinnervation at that stage. This statement ties into the findings using electromyographic methods, where motor units, across various upper and lower limb muscles are lost but also increased in size (Campbell *et al.*, 1973; McNeil *et al.*, 2005; Power *et al.*, 2010; Drey *et al.*, 2014; Piasecki *et al.*, 2016*b*). This reorganization of the neuromuscular system with fewer but larger motor units is evidence that reinnervation by collateral sprouting can compensate for the decay of motor neurons. Eventually, this compensatory mechanism is unable to cope with the extensive denervation in highly aged muscle, which leads to accumulation of atrophied denervated fibres and eventual loss of fibres (Power *et al.*, 2013). These are the $NCAM^+$ and $MyHCn^+$ fibres observed using immunofluorescence, and it might be that there is no leftover capacity in the neuromuscular system to facilitate their reinnervation and/or that the muscle fibres are no longer able to attract a nerve.

Innervation status in relation to exercise was also investigated in study 4, where subjects with entirely different exercise profiles were studied, with the idea molecules secreted by active muscles would favour muscle innervation. It has been shown, both *in vivo* and *in vitro*, that cells from older animals express a senescent phenotype, and secrete negative factors (Baker *et al.*, 2011; Sousa-Victor *et al.*, 2014; Xu *et al.*, 2018), thereby sowing the seeds for an exercise related improvement to exist. There were also diverging findings in this study. The number of $NCAM^+$ and $MyHCn^+$ fibres were not different between the active and sedentary elderly men, but there was a marked similarity in AChR gene expression between the active elderly men and a young control group. This latter finding would suggest that a favourable adaptation in lifelong exercisers serves to maintain NMJ integrity.

Limitations, conclusions, and perspectives

Limitations

Several issues relating to the used methodologies have already been carefully describe in the thesis and will only briefly be mentioned here. These include the risk of sampling NMJ and MTJ in biopsy material and the challenging assessment of the very small denervated fibres. It is also worth pointing out again that when measuring gene expression of muscle biopsies, the result reflects a sum of all the cell types present and their relative proportion. Other cell types found in muscle tissue include macrophages, fibroblasts, endothelial cells and others (Giordani *et al.*, 2019). The issue being that if the presence of a specific cell type or the magnitude of its presence influences gene expression in one sample but not another, this could lead to false conclusions. Some control can be done by selecting gene targets that reflect a specific cell type, such as the use of COL1A1 as a proxy for the presence of fibroblasts in primary cell cultures. However, the crude approach is in stark contrast to the more advanced single cell or nucleus transcriptomics approaches that are becoming more widely used.

An issue might also arise from the fact that 3 out of 4 projects in this thesis were originally designed for testing other hypotheses. Study 1, 3 and part of study 2 were originally designed to evaluate the influence of Losartan supplementation on the myogenic response to resistance exercise. The data from the original studies indicated that the supplementation had limited effect, and additional statistical analyses to assess the influence of supplementation were also performed in studies of this thesis. However, despite a positive outcome from these statistical analyses, it cannot be completely ruled out that supplementation could have had some influence on the results. And if the studies had been designed de novo to test the hypothesis of this thesis, various parameters, such as time points and choices of test, might have been different.

Conclusions

The aim of this thesis was to investigate muscle denervation on the protein and mRNA levels in humans of different ages, both at rest and in response to different exercise modalities.

In **study 1** it was observed, within a large group of elderly healthy men, that muscle fibre expressing NCAM or MyHCn were frequent, which was taken as evidence of accumulation of denervated muscle fibres. However, in contrast to the hypothesis, a positive correlation with age was not observed. Furthermore, gene expression analysis revealed a substantial inter-individual

variation in AChR subunit mRNA levels, and the gamma subunit were negatively associated with age. In **study 2**, which expanded upon study 1 by inclusion of a young control group and by investigating the influence of acute exercise, denervated muscle fibres were found to be more frequent in elderly muscle, while mRNA levels of AChR subunits also differed in relation to age, which likely indicates NMJ destabilization. Furthermore, an acute bout of resistance exercise, which was conducted in both male and female participants, revealed that AChR gene expression is modifiable in a subunit and time-dependent manner, which suggests that exercise can influence innervation status. To assess the influence of repeated bouts of exercise, the 16-week training intervention in **study 3** was used to investigate muscle fibre denervation and NMJ stability in elderly individuals. Contrary to the hypothesis, the intervention did not reduce the amount of denervated muscle fibres when determined by immunofluorescence. However, the intervention did lead to reduced mRNA levels of two AChR subunits (alpha 1 and epsilon) at 8 weeks, which happened concomitantly with a high degree of tissue turnover (increased MVC and fibre CSA). This indicates that heavy resistance exercise can influence NMJ stability in elderly individuals. Lastly, in **study 4,** lifelong exercisers were compared to age-matched sedentary peers and a young control group, and while old had more denervated fibres determined by immunofluorescence than young, there was no difference between the active and inactive elderly. However, there were a remarkable similarity in the overall profile of AChR between young and the lifelong exercisers, which could indicate a preserved innervation status in the active elderly.

Overall, the studies of this thesis have shown that muscle fibre denervation, atrophied fibres and altered AChR gene expression profile is characteristic of the aged muscle. These findings suggest that the neuromuscular system undergoes drastic changes during ageing, leading to denervation, failed reinnervation and a loss of muscle function. Importantly, there were encouraging findings on to the influence of exercise and its ability to positively influence innervation status, even in old muscle.

Perspectives

The writing of this thesis has made it clear that some questions were left unanswered, while it has also led to the creation of new ideas to pursue in the future. The method for identifying denervated muscle fibres on cross sections using immunofluorescence was gradually expanded. Initially, only dystrophin was used as a myogenic marker (study 1 and 3) together with the

proteins of interest (NCAM, MyHCn and MyHCe). In study 2, the size of the $NCAM^+$ and $MyHCn^+$ fibres were assessed, and it became clear that a large part of the denervated fibres were severely atrophied. This increased the chance that some of the smallest fibres could simply be either fibre branches (Schmalbruch, 1976; Partridge, 2021) or hyperactivated satellite cells (Borisov *et al.*, 2001). To guard against this misclassification of fibres extra layers of security were added in study 4, where MyHC configuration and two other myogenic markers (desmin and merosin) were assessed on subsequent cross sections, which resulted in removal of some of the very small fibres. It could be speculated that denervated fibres might terminate mid-muscle, whereby the added layers of security would have excluded denervated fibres that should have been left in, and thus resulted in a loss of information. If this question is to be answered, very detailed analyses of long series of cross sections using quality microscopy are needed.

The classic histological assessments, such as measurement of fibre CSA, fibre clumps and fibre type grouping have been refined throughout countless studies over the last century and the findings of this thesis have added to the established arsenal of histological markers. However, there is a need for new and more specific markers of denervation, as both NCAM and MyHCn are associated with other conditions (regeneration, neuromuscular disease) and tissues (MTJ and NMJ), which challenges the interpretation. The increased ease of access, the non-biased approach and the wealth of data generated by proteomic analyses and RNA sequencing might aid in the discovery of such novel markers (Lin *et al.*, 2021). Furthermore, the influx of potential biomarkers associated with neurodegenerative changes (Kalinkovich & Livshits, 2015; Calvani *et al.*, 2017) and ageing in general (Wilkinson *et al.*, 2020) might increase the temporal resolution that innervation status can be monitored at, while simultaneously alleviate participant discomfort and lower analytical burden, relatively to histological assessments.

Finally, within a broader context, the findings of this thesis represent the currently most detailed investigation of muscle fibre denervation in human muscle and the method will be useful for further research in not only sarcopenia, but also cachexia and neuromuscular diseases. In order to truly impact the lives of people it is not enough to simply characterize inherent changes that occurs during ageing. Instead, what is needed is effective interventions that mitigate the consequences associated with ageing, which could be both exercise (Allen *et al.*, 2021) or drug treatment (Guo *et al.*, 2021). The mechanisms that underline the loss of innervation and subsequent decline in muscle function are largely unexplored in humans. One avenue for further research is to investigate the interaction between muscle cells and neurons *in vitro*, where signals

arising from one cell type and how it affects the other can be meticulously studied. Within such a design lies the possibility of identifying key molecules associated with neuron survival and axon growth, which in turn might represent potential drug targets down the line.

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Appendix

Study 1

Molecular indicators of denervation in aging human skeletal muscle.

Status: *Published*

Received: 24 January 2019 | Revised: 10 July 2019 | Accepted: 13 July 2019

DOI: 10.1002/mus.26638

BASIC SCIENCE RESEARCH

MUSCLE&NERVE WILEY

Molecular indicators of denervation in aging human skeletal muscle

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Funding information
Bispebjerg Hospital Research Funding; Nordea Foundation (Healthy Aging grant); The A.P. Møller Foundation for the Advancement of Medical Science; The Danish Agency for Culture , Grant/Award Number: FPK.2018-0036; The Lundbeck Foundation

Abstract

Introduction: Muscle fiber denervation increases with age, yet studies at the tissue level are sparse due to the challenging nature of establishing the relative role of regeneration and denervation.

Methods: Muscle biopsies were obtained from the vastus lateralis of 70 healthy men (aged 72 ± 6 years; range, 65–94). Messenger RNA (mRNA) levels of acetylcholine receptors (AchR) were measured, and sections were stained for embryonic myosin, neonatal myosin (MHC_n), and neural cell adhesion molecule (NCAM).

Results: Embryonic myosin$^+$ fibers were rare, while MHC_n^+ and $NCAM^+$ fibers were observed in all samples. Age (range, 65–94 years) was negatively associated with AchRγ mRNA.

Discussion: Muscle from healthy older individuals expressed developmental myosins to varying degrees but more than has been previously reported for young individuals. Along with the AchR correlations, we propose that these findings support the presence of neuromuscular junction destabilization, denervation, and reinnervation in aging human skeletal muscle.

KEYWORDS
acetylcholine receptor, denervation, human muscle, neonatal myosin, sarcopenia

1 | INTRODUCTION

Adult human skeletal muscle is primarily composed of three major fiber types based on their expression of one of three myosin heavy chains (MHC 1, 2a, 2x).[1] During the fetal stage of human development, almost all muscle fibers initially express two additional MHC types, embryonic (MHC_e) and neonatal (MHC_n).[2,3] Embryonic and neonatal MHC ($MHC_{e/n}$) are gradually replaced by adult MHC, and, at birth, only a minor proportion of muscle fibers express $MHC_{e/n}$.[2] Most reports describe a complete absence, or very low levels (<0.5%), of $MHC_{e/n}^+$ fibers in muscle tissue from young human adults,[4-11] with only a few known exceptions.[4,5,10,12,13] Muscle injury in adult animals and humans also leads to a process of regeneration in which $MHC_{e/n}$ become transiently reexpressed.[9,14] Because such fibers are rarely observed in young individuals, it can be assumed that these immature myosins are gradually removed in the following months. Two studies have directly compared the prevalence of MHC_n^+ fibers in young and nonfrail older individuals and have found significantly higher levels in the older individuals.[8,15] The existence of a spectrum for the prevalence of $MHC_{e/n}^+$ fibers was further supported in two studies in which more developmental MHC^+ fibers were found in older than in younger

Abbreviations: AchR, acetylcholine receptor; BMI, body mass index; CSA, cross-sectional area; DAPI, 4′,6-diamidino-2-phenylindole; DYST, dystrophin; Ig, immunoglobulin; IHC, immunohistochemical; LBM, lean body mass; MHC, myosin heavy chain; MHC_e, embryonic myosin heavy chain; MHC_n, neonatal myosin heavy chain; mRNA, messenger RNA; NCAM, neural cell adhesion molecule; NMJ, neuromuscular junction.

rodents.[16,17] The significance of the presence of these classical markers of muscle regeneration with aging is unclear. It is interesting that animal models have shown that denervation of muscles also leads to reexpression of $MHC_{e/n}$.[14,18] Given the loss of motor neurons in humans with age,[19] it is likely that the number of denervated muscle fibers increases with age, which could explain the observed reexpression of developmental myosins in older muscle. In addition, when a myofiber becomes denervated, it also expresses neural cell adhesion molecule (NCAM),[20,21] which is thought to be involved in the development and plasticity of the neuromuscular junction.[21,22] It is believed that the existence $NCAM^+$ fibers in adult, nondiseased human muscle is virtually nonexistent.[21,23]

Other potential markers of denervation are the acetylcholine receptors (AchR); it has previously been reported that the density of AchR is increased in the extrasynaptic portion of denervated muscle fibers in mice rather than being confined to the postsynaptic region as in the innervated state.[24] The five skeletal muscle subunits of the AchR are differently affected by denervation; it has been shown that the messenger RNA (mRNA) levels of the α1-, β1-, γ-, and δ-subunits but not ε-subunit are greatly overexpressed upon denervation of the extensor digitorum longus muscle in mice.[24] Divergent responses in the mRNA levels of AchR subunits have been reported with age; it has been suggested that such differences reflect neuromuscular junction (NMJ) instability in older animals.[25,26] In humans, much less is known. Acetylcholine receptor α1 mRNA has been shown to be more abundantly expressed in old adults compared with young adults, with none of the other subunits showing any age-related tendencies.[27] On the protein level, a tendency for a lower amount of AchRβ1 was found in old adults compared with young adults.[27] Otherwise, data on these receptors in aging human muscle are lacking.

It is currently unknown to what extent healthy aging human muscle contains muscle fibers positive for $MHC_{e/n}$ and NCAM. In addition, a systematic comparison between these different markers in a large group of older individuals is lacking. Therefore, the objective of the present study was to determine the prevalence of $MHC_{e/n}^+$ and $NCAM^+$ fibers, in addition to AchR gene expression, in a large group of nonfrail older persons. This question has biological relevance in contributing to understanding the age-related reduction in muscle fiber number[28] and in adding insight into markers to distinguish between denervation and regeneration.

2 | MATERIALS AND METHODS

This study arose from a study previously conducted at the Institute of Sports Medicine, Copenhagen,[29] which was approved by The Committees on Health Research Ethics for the Capital Region of Denmark (Reference: H-15005761) and conformed to the standards set by the Declaration of Helsinki. Volunteers gave written informed consent before inclusion. Exclusion criteria were smoking, body mass index (BMI) >34 or < 19 kg/m^2, more than moderate regular physical activity, hypotension, hypertension, anticoagulation medication, and participation in strength training. Cholesterol medicine and vitamin supplements were allowed. Muscle fiber type and size have previously been reported for most participants.[29] After inclusion the participants underwent the following three tests, described in detail elsewhere[29]: (a) MRI of the thighs with a Philips Ingenia 3.0 T scanner to asses quadriceps cross-sectional area (CSA), (b) whole-body dual energy x-ray absorptiometry (Lunar iDXA; GE Medical Systems, Pewaukee, Wisconsin) with lean body mass (LBM) as the primary output, (c) measurement of the 70° knee-angle quadriceps maximal isometric torque with a Kin-Com dynamometer (Kinetic 359 Communicator, model 500–11; Chattecx, Chattanooga, Tennessee).

2.1 | Muscle biopsy

A muscle biopsy was obtained from the vastus lateralis of a randomly chosen leg from each participant. Local anesthetic (10 mg/mL lidocaine, Lidokain "Mylan"; Mylan Hospital, Oslo, Norway) was applied, and an incision in the skin was made. A 5-mm Bergström needle with manual suction was used to obtain the tissue sample.[30] Extracted muscle tissue deemed appropriate for histology was aligned in Tissue-Tek (Sakura Finetek, Europe, AJ Alphen aan den Rijn, The Netherlands), frozen in liquid nitrogen cooled isopentane (2-methylbutan; JT Baker, A Vantor Performance Materials BV, Deventer, The Netherlands), and stored in a cryotube (1.8 mL; Thermo Scientific, Nunc A/S, Roskilde, Denmark) at −80°C until further analysis.

2.2 | Immunohistochemistry

Consecutive cross-sections (10 μm) of each muscle biopsy were cut at −20°C in a cryostat and placed on glass slides (Thermo Scientific). Four primary antibodies were used in the analysis: MHC_e (mouse immunoglobulin [Ig] G1, F1.652; Developmental Studies Hybridoma Bank), MHC_n (mouse IgG1, NCL-MHC_n; Novocastra, Newcastle upon Tyne, United Kingdom), NCAM (mouse IgG1, CD56, 347740; Becton Dickinson, San Jose, California), and dystrophin (DYST; mouse IgG2b, MANDYS8, D8168; Sigma). Three consecutive slides were stained, unfixed, with primary antibody combinations (a) DYST + MHC_e, (b) DYST + MHC_n, or (c) DYST + NCAM and incubated overnight at 5°C (20–24 hours). Then, the sections were incubated for 45 minutes with two secondary antibodies, Alexa-Fluor 568 goat anti-mouse IgG2b (Cat. No. A-11036; Molecular Probes/Invitrogen A/S, Taastrup, Denmark) and Alexa-Fluor 488 goat anti-mouse IgG1 (Cat. No. A-11029; Molecular Probes/Invitrogen A/S). Afterward, the sections were fixed in 5% formaldehyde (Histofix; Histolab, Gothenburg, Sweden) for 12 minutes and mounted with Prolong-Gold-Antifade (Cat. No. P36931; Molecular Probes/Invitrogen) containing 4′,6-diamidino-2-phenylindole (DAPI). Between all steps, slides were washed in two changes of 0.05 M Tris-buffered saline. Slides were stored in a dark at room temperature for 24 to 48 hours and moved to −20°C until analysis.

2.3 | Serial sections

To follow specific fibers longitudinally, ~40 consecutive 10-μm-thick cross-sections were cut from two biopsy samples with many MHC_n^+

fibers. Two serial sections were placed on each slide, resulting in 20 slides, covering a fiber distance of at least 400 μm. Sections that were lost in this process were noted so that the actual distance between sections could be determined. Approximately the same number of longitudinal sections were cut by using the same method. This set of samples was stained in the order MHC_e, MHC_n, and NCAM by skipping every fourth slide and using the same protocols previously described.

2.4 | Microscopy and image analysis

Grayscale 2040 × 1536 images were captured of the blue (nuclei), green ($MHC_{e/n}$/NCAM), and red (DYST) channels using a fixed exposure time with a DP71 Olympus camera mounted on a BX51 Olympus microscope (×10/0.30NA objective) in the OLYMPUS cellSens software (v.1.14). For the staining of $MHC_{e/n}$, only areas with fibers positive for these markers were imaged, whereas, for the NCAM staining, the whole section was imaged. Images were subsequently stitched together to reconstruct the entire cross-section in ImageJ (v.1.51; National Institutes of Health, Bethesda, Maryland). In general, areas where the fibers were longitudinally oriented or had been damaged during sectioning were excluded from analysis.

All analyses were conducted by the same person. The brightness and contrast levels were kept constant. The following sequence of analysis was used: total number of fibers in the section, fibers with central nuclei, and $MHC_{e/n}^+$ and $NCAM^+$ fibers. Muscle fibers positive for MHC_e, MHC_n, or NCAM were counted, distinguishing subjectively between small and large fibers, which later were determined to be less than or greater than ~750 μm^2, respectively, as shown in Figure S1. Fiber size was determined by manually delineating fibers in ImageJ on approximately 10 small and 10 large representative fibers. Care was taken to confirm that small fibers had their own DYST staining around the entire fiber to classify it as a muscle fiber. In addition, only fibers deemed to have sufficient staining signal were included. Three weakly stained fibers are shown in Figure S1 as examples of fibers not included. The prevalence of fibers positive for two or more markers (MHC_n + NCAM, MHC_e + MHC_n, MHC_e + NCAM, or MHC_e + MHC_n + NCAM) was also assessed across the three serial sections. Values are expressed as a percentage of the total number of fibers counted per section.

2.5 | Gene expression

Messenger RNA levels for the different AchR, $MHC_{e/n}$, and NCAM were measured by using real-time reverse transcription-quantative polymerase chain reaction as previously described.[29] The specific primers are given in Table S1.

2.6 | Statistical analysis

All figures and statistical tests were completed in GraphPad Prism (v.7.04; GraphPad Software, La Jolla, California). Participant characteristics are presented as mean ± SD (range), and immunohistochemical (IHC) data are presented as median (range). $P < .05$ was considered significant. Pearson's correlation analysis was performed for all variables except for the IHC data, which were evaluated by using Spearman's correlation. All mRNA data were log transformed before analysis.

3 | RESULTS

3.1 | Participant characteristics

In total, 71 men aged 65 or older were included. One participant was excluded from the analysis because of too few acceptable fibers in the biopsy section, leaving a total of 70 participants represented in the IHC analysis. Age, height, weight, BMI, LBM, and maximal isometric knee extensor torque of the participants are provided in Table 1. Eight participants were ≥ 80 years of age, and 28 were < 70 years of age.

3.2 | MHC_e^+, MHC_n^+, and $NCAM^+$ fibers

The prevalence of MHC_e^+, MHC_n^+, and $NCAM^+$ fibers as well as fibers with one or more central nuclei is presented in Figure 1. On average, 1144 ± 489 [range 355–2452] fibers per biopsy were included. For NCAM and $MHC_{e/n}$, values are presented for small positive and large positive fibers separately as well as for the total number of positive fibers (small + large). The median for MHC_e^+ fibers was 0% (0%–0.34%), with a total of 33 positive fibers observed in 22 of 70 participants; these were mostly small fibers. In contrast, MHC_n^+ fibers were found in all samples, and the median was 1.17% (0.28%–13.33%). The median for $NCAM^+$ fibers was 1.07% (0.06%–16.24%). Finally, the median of fibers with one or more central nuclei was 0.50% (0%–5.42%). Figure 2 shows the same area of a biopsy, with many small fibers being detectable along the entire length.

3.3 | Costainings

The prevalence of costained MHC_n^+/$NCAM^+$ fibers, expressed both as a percentage of all MHC_n^+ fibers (median 20.6% [0%–79.8%]) and as a percentage of the total number of fibers in the section (median 0.3% [0%–10.6%]), is presented in Figure 3. In absolute terms, 61 of 70 participants had at least one costained MHC_n^+/$NCAM^+$ fiber, with a total of 427 fibers observed among all participants. The median of the other costainings—MHC_e + NCAM,

TABLE 1 Participant characteristics[a]

Characteristics	Mean	±SD	Range
Age, y	72	6	65–94
Height, cm	178	7	161–191
Weight, kg	84	11	57–110
BMI, kg/m^2	26	3	19–33
LBM, kg[b]	56	6	44–71
Isometric torque, Nm	196	46	75–298

Abbreviations: *BMI*, body mass index; LBM, lean body mass; Nm, newton meter.
[a]n = 70.
[b]n = 69.

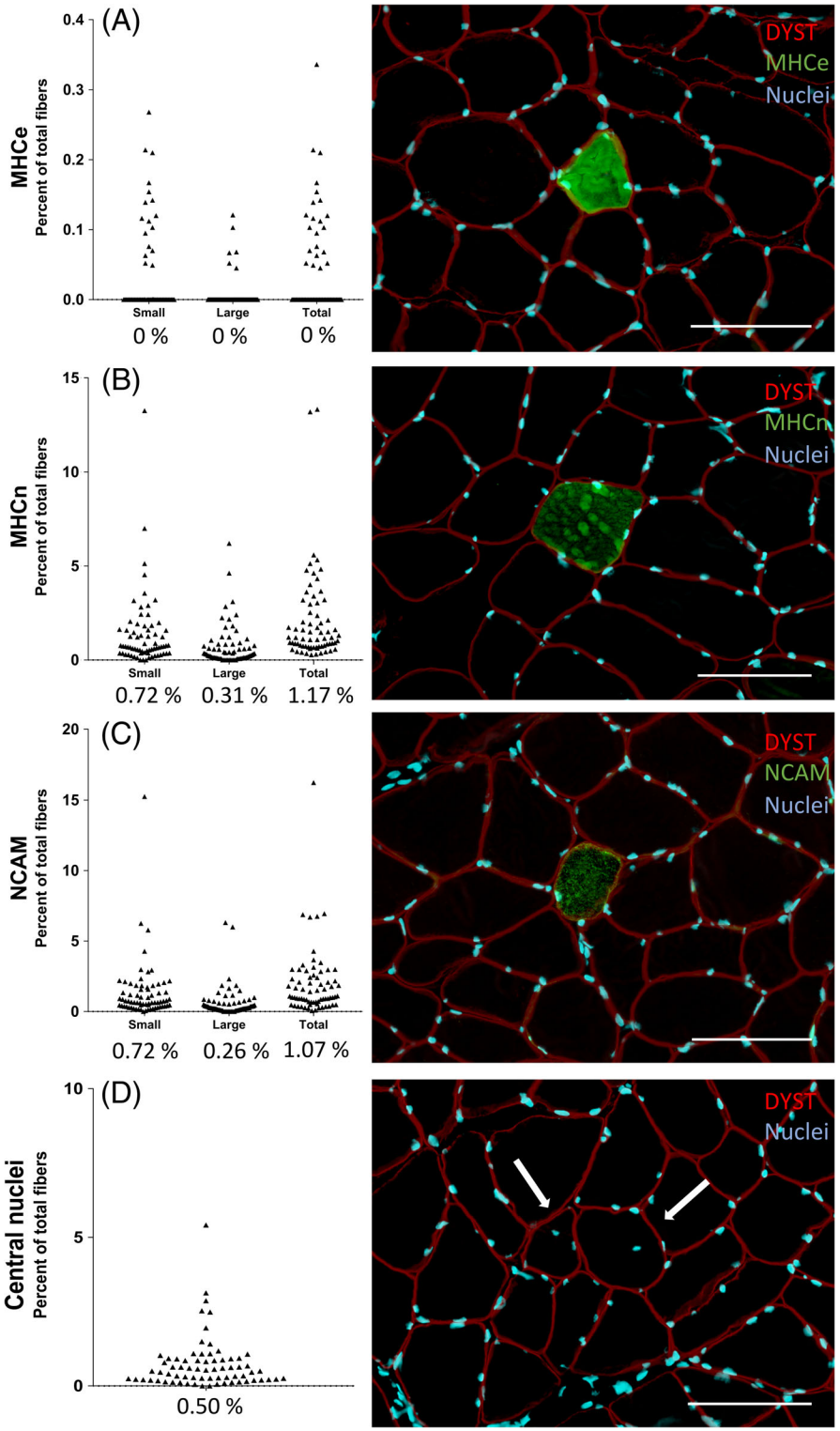

FIGURE 1 Prevalence of markers of regeneration and denervation in muscle biopsies from 70 older (aged 72 ± 6 years) participants. The graphs illustrate the prevalence of fibers positive for $MHC_{e/n}$ and NCAM (separated into small and large fibers) and centralized nuclei in percentage of total fiber count. Median is given below the X-axis. The panels to the right of the graphs are representative merged images of dystrophin (red), nuclei (blue), and MHC_e/MHC_n/NCAM (green). Images are taken with equal magnification. Arrows indicates central nuclei. Scale bars = 100 μm. DYST, dystrophin; MHC_e, embryonic myosin heavy chain; MHC_n, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule

MHC_e + MHC_n, and MHC_e + MHC_n + NCAM—was 0, with the total numbers of costained fibers being 22, 21 and 19, respectively.

3.4 | Correlations

The relationships between age and data for function, IHC, and gene expression were tested by correlations. Significant negative correlations were found between age and maximal isometric torque, type 2 fiber CSA, and quadriceps CSA (Figure 4). No significant correlations were found between age and MHC_e^+ fibers, MHC_n, or central nuclei. For the mRNA data, a significant, negative correlation was observed between age and AchRγ. Removing the 94-year-old participant from the age/AchRγ correlation resulted in *R* and *P* values of –0.21 and .085, respectively. When we compared the IHC and the mRNA data, a

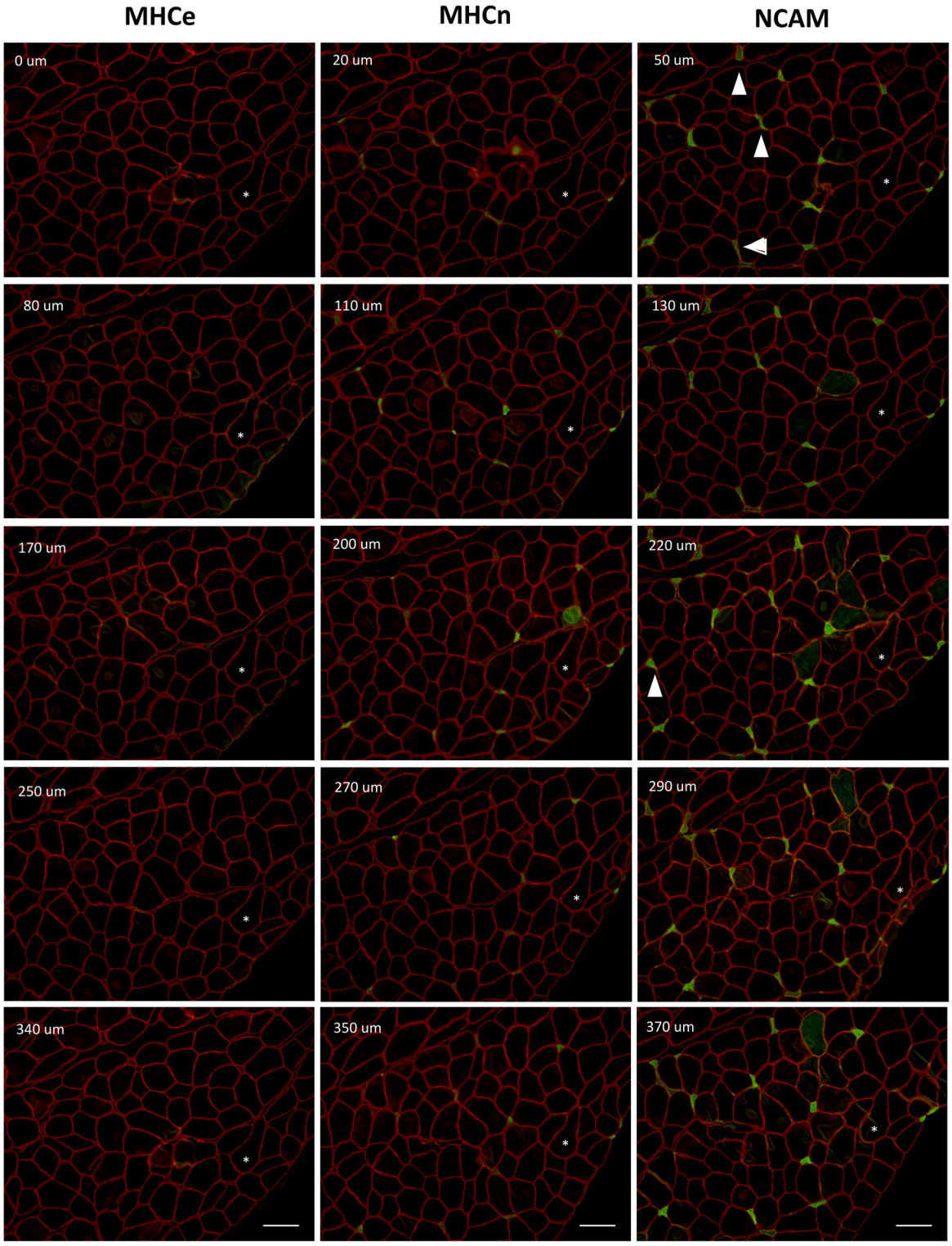

FIGURE 2 Small $MHC_n^+/NCAM^+$ muscle fibers. Approximately 40 consecutive sections were cut from this biopsy and stained with MHC_e, MHC_n, or NCAM. Arrowheads indicate $NCAM^+$ fibers that are completely or partially MHC_n^-. Asterisks indicate the same large, nonstained fiber on all sections. Varying staining intensity is clearly shown to be a feature of MHC_n^+ fibers, whereas $NCAM^+$ fibers appear more stable in staining intensity. Note that, on the MHC_e staining, small fibers frequently change size and/or shape. Red is dystrophin. Images are taken with equal magnification and brightness/contrast levels. Scale bars = 100 μm. MHC_e, embryonic myosin heavy chain; MHC_n, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule

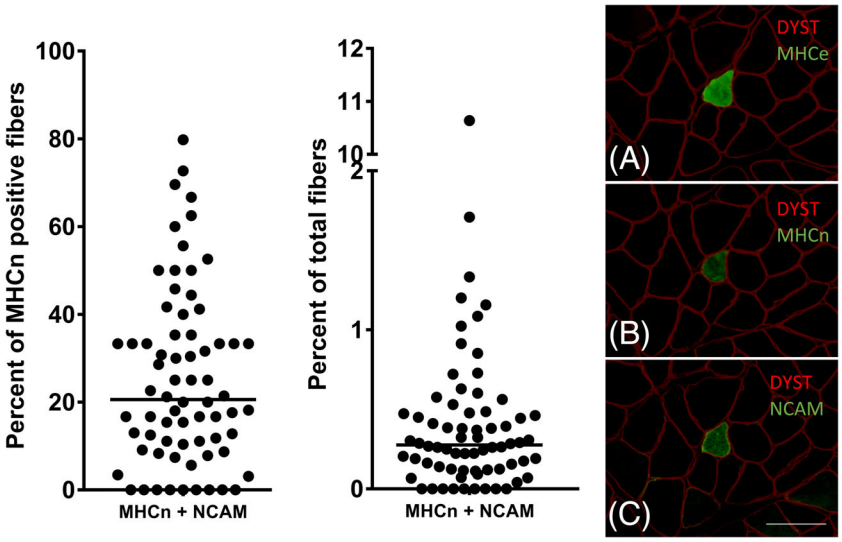

FIGURE 3 Costained fibers. Graphs illustrate the prevalence of fibers that are positive for both MHC_n and NCAM, expressed relative to the number of MHC_n^+ fibers (left graph) or the total number of fibers in the section (right graph). Individual values and median are shown; it can be seen that approximately 20% of all MHC_n^+ fibers were also $NCAM^+$ but that this population of $MHCn^+/NCAM^+$ fibers makes up less than 1% of the total number of fibers on the cross-section. The y-axis in the graph at right is hatched for clarity. A-C, images illustrate these costained fibers. Images are taken with equal magnification and brightness/contrast levels. Scale bar = 100 μm. DYST, dystrophin; MHC_e, embryonic myosin heavy chain; MHC_n, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule

significant relationship between the proportion of MHC_n^+ fibers and MHC_n mRNA was found (Figure S2). No relationship was found for NCAM or MHC_e.

3.5 | Longitudinal sections

Figure 5A-D shows longitudinal sections of a thin $MHC_n^+/NCAM^+$ muscle fiber, and Figure 5E,F shows a striated MHC_n^+ muscle fiber that is $NCAM^-$.

4 | DISCUSSION

The main finding of this study is that alleged, frequently used markers of regeneration and denervation were present to a greater extent in muscle tissue from healthy older individuals compared with what has previously been described in studies in young individuals.[4,8,9,11,15,31] In addition, mRNA levels of the AchR γ-subunit were negatively associated with age within the ~20-year age span represented by our cohort.

With increasing age there is a well-established decrease in muscle fibers and fiber CSA, the latter being more pronounced in the type 2 fibers.[29] This was confirmed in the present study as there were significant, negative correlations between age and maximal isometric torque, type 2 muscle fiber CSA, and quadriceps CSA, with no significant relationship for type 1 muscle fiber CSA. Because the participants ages ranged over a span of ~20 years, it was expected that the muscle expression of AchRs and fibers positive for $MHC_{e/n}$ and NCAM would differ according to age. This expectation is based on observations that muscle fiber denervation is reported to accelerate with advancing age,[29] leading to an increased likelihood of observing muscle fibers stained with markers of denervation as a person ages. In line with this, we detected higher levels of MHC_n^+ fibers than had been previously reported for younger individuals. Within our cohort, however, we were unable to detect any significant correlation between age and the prevalence of $MHC_{e/n}^+$ or $NCAM^+$ fibers. One possible explanation for this is that it is basically unknown whether the expression of these markers gradually accumulates with increasing age or whether they follow a different trajectory. Because muscle fiber denervation has been observed to increase severely in the ninth decade of life,[29] it could be that it is only at such a high age, above the age range of most of our participants, that these markers increase beyond the ~1.0% that we observed.

4.1 | Acetylcholine Receptors

With regard to AchR, while gene expression levels of the α1-, β1-, δ-, and ε-subunits did not correlate with age, the γ-subunit was negatively associated with age. This was contrary to our hypothesis, in that the so-called "fetal γ-subunit" has been shown to increase after denervation in rats.[32,33] During embryonic development, AchRs are evenly distributed along the entire muscle fiber.[34] In healthy adult muscle, in contrast, AchRs are clustered at high density in the synaptic region of the muscle fiber, with little transcription of AchR genes in the extrasynaptic region,[24,35] where most biopsies are taken. This tight regulation of AchR expression observed in the adult stage is reversed with muscle denervation, leading to increased transcription in the extrasynaptic region of all AchR subunit genes, except for the ε-subunit.[24,33,36,37] Furthermore, it has been suggested that the γ-subunit and ε-subunit are reciprocally regulated,[33] although we did not observe any relationship between mRNA levels of γ and ε when correlated directly (Figure S2). In addition, the large interindividual variation in mRNA levels of the AchR subunits between similarly aged participants is noteworthy. This was most clearly shown for the ε-subunit, in which some individuals had levels almost 1000-fold higher than others. The reason for this large interindividual variation is currently unclear. Finally, it should be noted that only normotensive participants were included, meaning that the participants were quite selected, and additional work is required to establish whether our observations are characteristic of the general population.

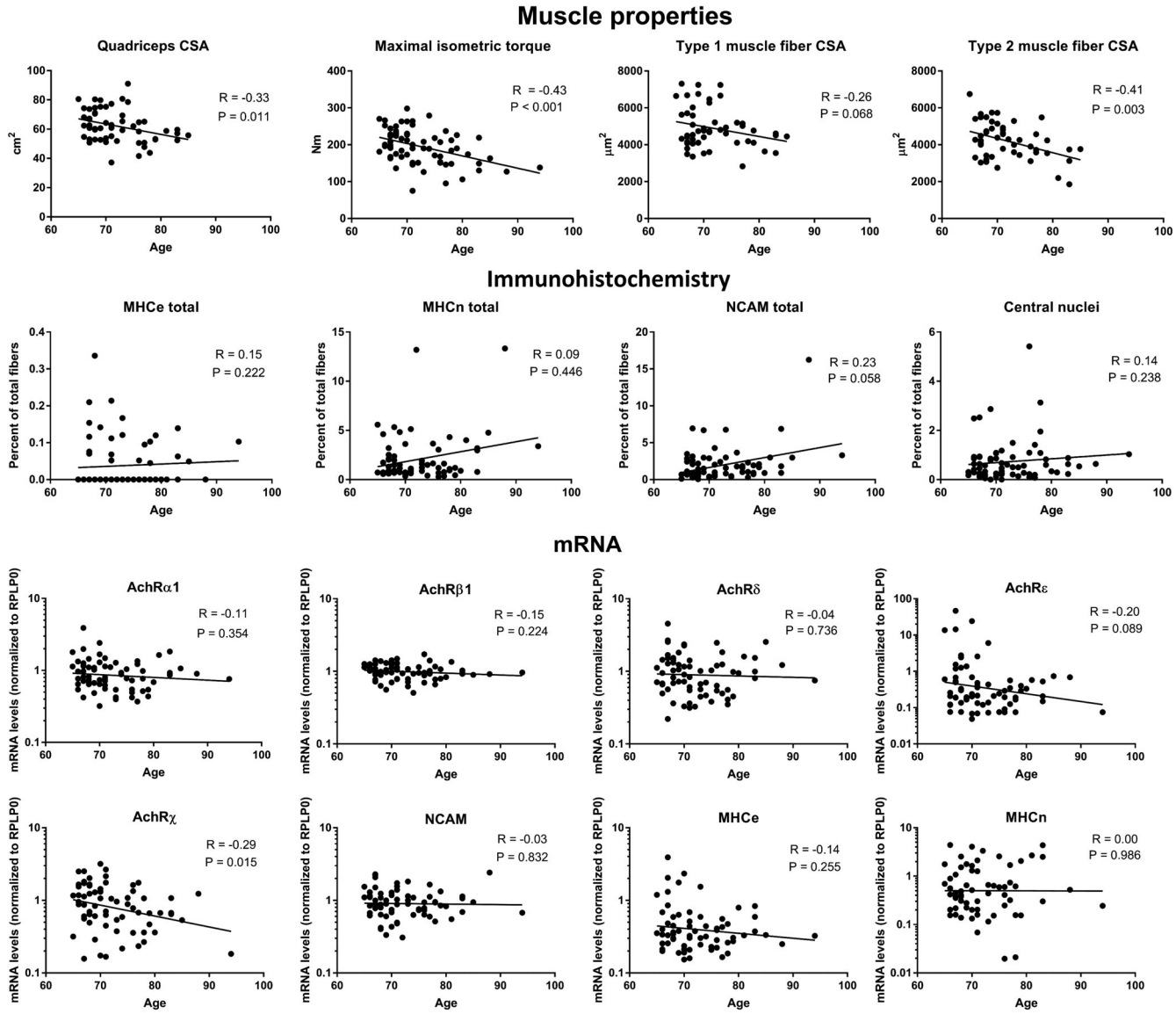

FIGURE 4 Correlations. The number of participants included in analyses varied between 51 and 70, depending on data availability. Specifically, maximal isometric torque (n = 69), type 2 and type 1 muscle fiber CSA (n = 51), quadriceps CSA measured by MRI (n = 58), and all IHC and mRNA data (n = 70). Linear correlation line and *R* and *P*-values were inserted. All mRNA data were log transformed and then subjected to Pearson's correlation with *P*-values inserted. Immunohistochemical data were subjected to Spearman's correlation with a semi-log correlation line inserted. AchR, acetylcholine receptor; CSA, cross-sectional area; IHC, immunohistochemical; MHC_e, embryonic myosin heavy chain; MHC_n, neonatal myosin heavy chain; MR, magnetic resonance imaging; mRNA, messenger RNA; NCAM, neural cell adhesion molecule

4.2 | Age-related differences in the prevalence of neonatal MHC

Neonatal myosin heavy chain is considered a classic marker of adult muscle regeneration.[6,38] Previous studies investigating the prevalence of MHC_n in vastus lateralis muscle from young untrained participants have found either no evidence of such fibers[4,9,15,31] or only very few fibers.[8,9,11] Among our group of healthy, older individuals, there was a median (range) of 1.17% (0.28%–13.33%) MHC_n^+ fibers, which, although relatively low, reflects a substantial number of such fibers. While there was no correlation between MHC_n gene expression and age within the age range of our participants, we found that the proportion of MHC_n^+ fibers determined by IHC was significantly correlated with MHC_n mRNA (Figure S2). Thus, our results, together with previous findings, indicate a clear age-related increase in the pattern of MHC_n expression.

This raises the question regarding the significance of MHC_n^+ fibers in uninjured healthy skeletal muscle. Induction of muscle injury leads to reexpression of $MHC_{e/n}$ in both rodent[39] and human muscle[9], although it should be noted that several of the methods used in earlier studies to induce muscle injury are severe, leading to necrosis and requiring de novo formation of muscle fibers through the process of adult regenerative myogenesis.[40] As such, it is highly unlikely that the MHC_n^+ fibers we detected in the basal state in our cohort of older individuals resulted from this type of muscle regeneration. However, we cannot exclude the

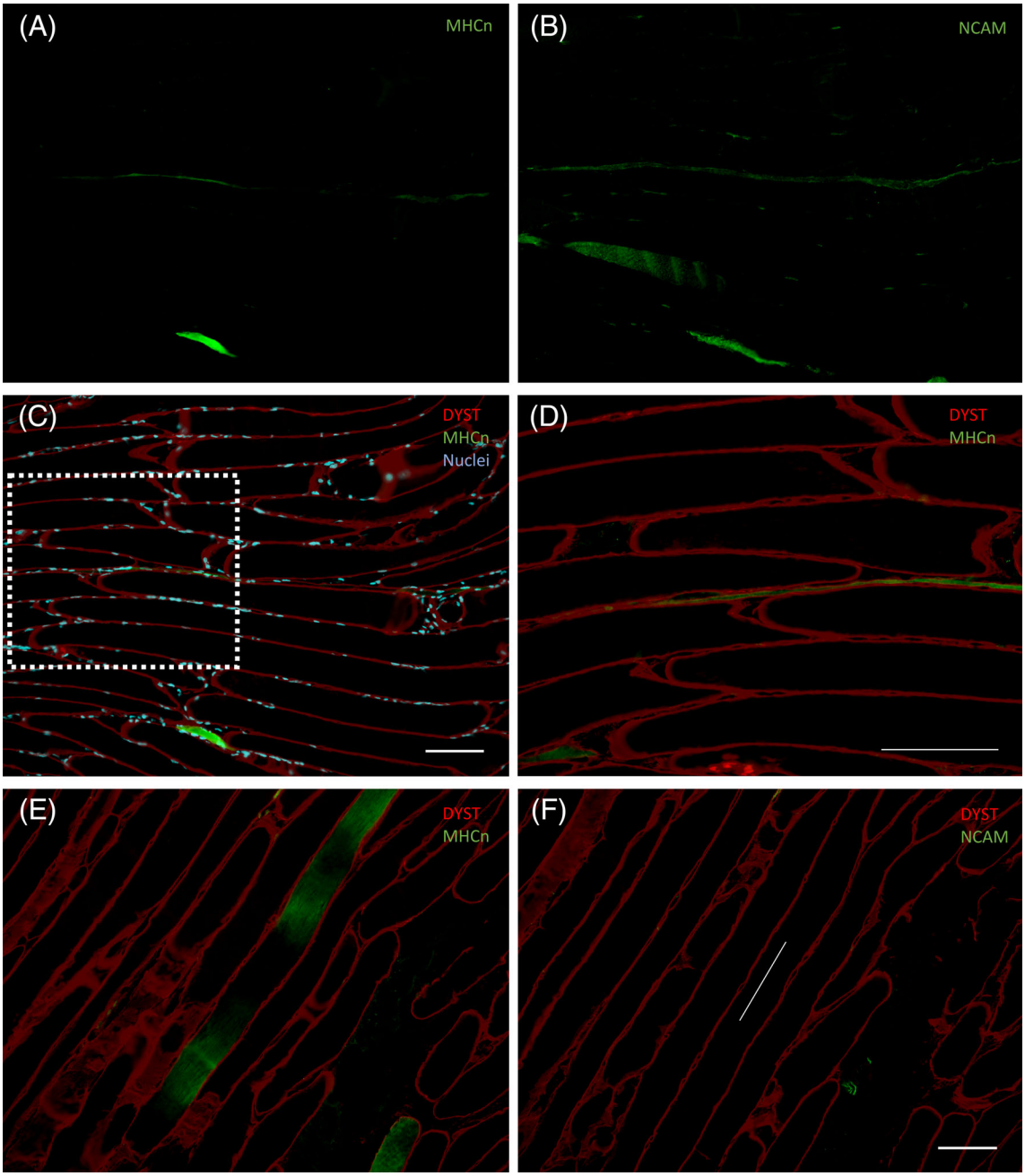

FIGURE 5 Longitudinal sections. A-D, the same thin MHC_n^+/$NCAM^+$ fiber on two consecutive sections, which spans approximal 1000 um. Notice the uneven staining pattern for the MHC_n. A-C are equal magnification, while D is a high-magnification image of the area within the dashed square in C. Notice that the DYST staining is clearly visible along the length, showing that this is truly a distinct muscle fiber. E, a muscle fiber with area specific staining of MHC_n, resulting in a striated staining pattern which has previously been described by Schiaffino et al.[14] This image clearly shows that the change from MHC_n positive to negative is gradual, which could explain why some fibers are less intensely stained than other in sections. F, the same fiber as in E (white line) on the following section where it is clearly $NCAM^-$. Scale bars = 100 μm. DYST, dystrophin; MHC_n, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule

possibility that the threshold at which muscle injury occurs is lower in old individuals, meaning that even ordinary daily activities involving eccentric contractions, such as walking down stairs, might induce muscle injury and, subsequently, expression of MHC_n. A more likely explanation, however, for the prevalence of MHC_n^+ fibers in our old individuals is that at least some of the MHC_n^+ fibers are in fact denervated muscle fibers, in line with reports in animal models of reexpression of MHC_n on muscle denervation.[14,19]

4.3 | Identifying denervated muscle fibers

In addition to a surprisingly high level of MHC_n^+ fibers, we also observed $NCAM^+$ fibers in most biopsies. In response to denervation, NCAM is expressed in the extrasynaptic region of the muscle fiber where it is not normally present in the adult, and it is believed to facilitate the process of reinnervation.[22] Denervation occurs either due to

the decay of α-motoneurons at the spinal cord[20] or due to a disturbance in the NMJ at the level of the muscle fiber itself. Previous reports on the prevalence of NCAM$^+$ muscle fibers in young healthy participants are conflicting, ranging from zero[10,41] to <0.5%[42] to 2.4%.[43] In a direct comparison between young and old participants, it was reported that older participants had more NCAM$^+$ fibers compared with young participants.[42] On the basis of these studies and the 1.07% (0.06%–16.24%%) NCAM$^+$ fibers among our group of healthy, older individuals, it remains unclear whether the prevalence of NCAM is an actual age-related phenomenon, although most studies that have examined the issue seem to suggest that this is the case; this could reflect that, with increasing age, some muscle fibers are fully denervated or going through a disturbance or period of NMJ instability. If a working synapse is reestablished, either from the same or another type of motor neuron, NCAM expression will gradually disappear,[22] and, in the latter case, clustering of fiber types could arise. However, if innervation is not reestablished, the muscle fiber will deteriorate and eventually die[44] but, in that process, continually express NCAM,[45] which has been shown in rat muscles that have been kept denervated for up to 300 days.[22] This means that truly denervated muscle fibers are likely to be smaller than normal muscle fibers, which is in line with our data showing that about two of three of the NCAM$^+$ fibers were categorized as small fibers. A limitation of our study is the lack of in vivo measurements of muscle activation, which could potentially have shown changes in the participants with the largest amount of long-term denervated muscle fibers and should, therefore, be considered in future investigations on this topic.

To investigate these potentially denervated fibers further, the prevalence of fibers positive for both NCAM and MHC$_n$ was determined, and we observed that approximately 20% of the MHC$_n^+$ fibers were also NCAM$^+$. In addition, it was generally observed during the analysis that it was mostly the small fibers that were costained. One possible interpretation of this is that there are multiple processes proceeding simultaneously, and small MHC$_n$ and NCAM costained fibers mostly represent long-term denervated fibers that have atrophied greatly over time and reverted to an immature MHC configuration. In support of this, muscle biopsies from patients with amyotrophic lateral sclerosis show a high proportion of small fibers that are positive for both MHC$_n$ and NCAM and are thought to be denervated fibers.[41] It is possible that the large NCAM$^+$ fibers are at an earlier stage of denervation when there is an instability of the NMJ, generating expression of NCAM in the extrasynaptic region to promote axon growth and establish a new NMJ [45] or stabilize the current NMJ.

It was occasionally observed that some muscle fibers were only partially stained with MHC$_n$ and/or NCAM. This was seen in the longitudinal direction as a striated pattern (Figure 5) or as distinct areas within a muscle fiber cross-section. This phenomenon has previously been described in denervated rat muscle.[14] This is seen in the consecutive cross-sections of Figure 2, in which many of the NCAM$^+$ fibers are MHC$_n^+$, but there are several that are completely or partially MHC$_n^-$. It would also appear that NCAM seems more consistently expressed along the length of a fiber. Furthermore, these small fibers were found to span at least 370 μm in length, indicating that they are likely to be independent muscle fibers and not fiber branches, as has been shown in regenerating muscle.[46] We also observed that small fibers occasionally showed a large degree of tortuosity and/or change in fiber size and shape, which highlights the importance of following fibers on consecutive sections.

These findings provide evidence that the coordination of gene transcription between all the nuclei of a myofiber is lost or that some sort of regional remodeling of the muscle fiber is ongoing.[47] The regulation of gene transcription by all nuclei in a muscle fiber is normally strictly coordinated, with the exception of the NMJ domain, such that the MHC gene expression is uniform throughout.[47] It is possible that the MHC$_n$ striated staining pattern in particular indicates that this coordination is weakened with aging.

In conclusion, the IHC analysis showed that muscle fibers from healthy older individuals express developmental myosins to varying degrees but more than what has been reported in young individuals, while the pattern for NCAM was less clear. We have also demonstrated that the mRNA levels of the AchR subunits do not appear to increase with increasing age in humans within the ~20-year age span represented by our cohort. The results of the immunohistochemical analyses provide evidence to support the theory of greater NMJ destabilization, denervation, and reinnervation, while additional studies are required to determine the significance of AchR gene expression in human skeletal muscle across a wider age range.

ACKNOWLEDGMENTS

The authors thank lab technicians Camilla Brink Sørensen and Anja Jokipii-Utzon for excellent technical assistance with preparation of the muscle biopsies and the mRNA analysis. Funding from Nordea Foundation (Healthy Aging grant), The Danish Agency for Culture (FPK.2018-0036), The Lundbeck Foundation, The AP Møller Foundation for the Advancement of Medical Science, and Bispebjerg Hospital Research Funding is gratefully acknowledged.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to disclose.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Soendenbroe C, Heisterberg MF, Schjerling P, et al. Molecular indicators of denervation in aging human skeletal muscle. *Muscle Nerve*. 2019;1–11. https://doi.org/10.1002/mus.26638

Study 2

Key Components of Human Myofibre Denervation and Neuromuscular Junction Stability are Modulated by Age and Exercise.

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Key Components of Human Myofibre Denervation and Neuromuscular Junction Stability are Modulated by Age and Exercise

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Received: 28 February 2020; Accepted: 3 April 2020; Published: 6 April 2020

Abstract: The decline in muscle mass and function with age is partly caused by a loss of muscle fibres through denervation. The purpose of this study was to investigate the potential of exercise to influence molecular targets involved in neuromuscular junction (NMJ) stability in healthy elderly individuals. Participants from two studies (one group of 12 young and 12 elderly females and another group of 25 elderly males) performed a unilateral bout of resistance exercise. Muscle biopsies were collected at 4.5 h and up to 7 days post exercise for tissue analysis and cell culture. Molecular targets related to denervation and NMJ stability were analysed by immunohistochemistry and real-time reverse transcription polymerase chain reaction. In addition to a greater presence of denervated fibres, the muscle samples and cultured myotubes from the elderly individuals displayed altered gene expression levels of acetylcholine receptor (AChR) subunits. A single bout of exercise induced general changes in AChR subunit gene expression within the biopsy sampling timeframe, suggesting a sustained plasticity of the NMJ in elderly individuals. These data support the role of exercise in maintaining NMJ stability, even in elderly inactive individuals. Furthermore, the cell culture findings suggest that the transcriptional capacity of satellite cells for AChR subunit genes is negatively affected by ageing.

Keywords: sarcopenia; denervation; neuromuscular junction; heavy resistance exercise; acetylcholine receptor; cell culture; myogenesis; neonatal myosin; neural cell adhesion molecule

1. Introduction

The rate of loss of muscle mass increases with advancing age [1], and ultimately leads to impaired physical function in elderly individuals [2–4]. This age-dependent decline in muscle mass is partly due to a loss of individual muscle fibres [5] as a result of muscle fibre denervation [6–8]. While physical exercise is recognized as a strong countermeasure against the loss of muscle mass and has consistently been shown to maintain physical function and health in the last ten years of life [9,10], it is currently unclear whether denervation can be ameliorated or reversed by exercise.

It has been shown in animals that exercise causes positive adaptations to the neuromuscular junction (NMJ) that to some extent can attenuate the age-related degeneration of the NMJ [11]. Changes in expression of acetylcholine receptors (AChRs) with acute exercise have been suggested to indicate NMJ remodelling in animals [12,13] and represent a potential target for studying this in humans [14]. AChR are present in abundance at the NMJ [15] and are almost non-existent in the extra-synaptic region of the muscle fibre [16]. Upon experimental denervation, however, the α1, β1, γ, and δ subunits increase extra synaptically [16–19], raising the possibility that these AChR subunits can be used as indicators of denervation associated with ageing. We recently observed a correlation between age and gene expression levels of the foetal γ AChR subunit in a large group (n = 70) of healthy elderly men ranging in age from 65 to 94 years, in conjunction with tissue markers of muscle fibre denervation, neural cell adhesion molecule (NCAM) and neonatal myosin (MHCn), at the protein level [20]. Direct comparisons with a younger cohort as well as the potential for exercise to influence AChR expression patterns are however lacking.

One of the challenges for ageing skeletal muscle is related to the decline in satellite cell function with age. Not only is satellite cell function important for tissue repair and maintenance, but it also has potential implications for maintenance of the NMJ, where myonuclei at this site must be capable of carrying out the specialization necessary to complete the formation of the NMJ. This includes producing a high concentration of AChRs at the membrane and a clustering of myonuclei, which become transcriptionally specialized and distinct from adjacent extra-synaptic myonuclei [21,22]. Whether this capacity declines with age is currently unknown. Satellite cells have been shown to play a vital role in maintaining the post-synaptic region in mice, both in terms of myonuclear clusters of AChRs and re-innervation of the regenerating NMJ [23,24]. In this context it is interesting that we have recently observed a poorer fusion capacity of satellite cells derived from old women compared to young women, accompanied by a distinctly different molecular profile throughout the myogenic program [25]. It remains unknown, however, to what extent this dysfunction in human satellite cells has implications for NMJ maintenance with increasing age.

Based on the above, the main purpose of this study was to investigate the influence of age and exercise on molecular markers of NMJ stability and muscle fibre denervation in healthy elderly individuals. An additional focus was to determine how ageing would alter the capacity of myonuclei in cell culture to produce key transcriptional elements for NMJ formation.

2. Materials and Methods

2.1. Experimental Design

This study is based on muscle biopsies collected from two studies, on 12 young and 12 elderly women [25], and on 25 elderly men [26], respectively. Both studies were approved by The Committees on Health Research Ethics for The Capital Region of Denmark (Ref: H-15017223, H-3-2012-081). All procedures conformed to the Declaration of Helsinki of 1975, revised in 2013, and the subjects gave written informed consent before participation. All participants were healthy, non-smokers, non-obese, and did not perform strenuous physical exercise on a regular basis. The men were part of a randomized controlled trial investigating the effect of the blood pressure-lowering medication losartan on the muscle response to exercise, where half of the participants received losartan and the other half placebo. Given the general lack of drug effect, the two groups were merged in the present study (separate group data are also provided for reference in online Supplementary Figure S1).

All participants performed a maximal strength test in a Leg Extension machine (M52, TechnoGym, Cesena, Italy) to determine the one-repetition maximum (1 RM), which was used to determine the load lifted during the subsequent bout of heavy resistance exercise. The Leg Extension exercise protocols consisted of both concentric and eccentric contractions. First, 4–5 sets of 12 concentric contractions at 70% of 1 RM were performed, followed by four sets of 4–6 eccentric contractions at 110% of 1 RM, as

previously described [25,26]. The exercise was performed with one leg only, leaving the contralateral leg as a control. No other exercise was allowed during the study period.

2.2. Muscle Biopsies

For all participants, muscle biopsies were obtained from the vastus lateralis muscle, under local anaesthetic (1% lidocaine), using the percutaneous needle biopsy technique of Bergström [27], with five 6-mm needles and manual suction. Pieces of muscle tissue were aligned, embedded in Tissue-Tek, and then frozen in isopentane, pre-cooled in liquid nitrogen, and stored at −80 °C. The men had six muscle biopsies taken over 17 days, at the following time points: −10 and −3 days before exercise from the control, non-exercised leg, and from the exercised leg at +4.5 h and on days +1, +4, and +7 post exercise. The day −3 sample was excluded from the current study since its purpose was to investigate a potential effect of losartan in the rested state and is therefore superfluous in the current context. The young and elderly women had muscle biopsies collected from each leg five days after exercise, from which a part was embedded as described above and a part was used for cell culture, where myoblasts were plated in 12-well plates for three days of proliferation (12,000 cells per well), or three days of proliferation followed by four days of differentiation (20,000 cells per well), as previously described in detail [25].

2.3. RNA Extraction

100 cryo sections, 10 µm thick, 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 four for 15 s. Cell culture cells were dissolved directly in the Trireagent. 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.

2.4. Real-Time RT-PCR

mRNA targets related to innervation were analysed for the current study. The specific primers are given in Table 1. Total RNA (500 ng for muscle and 150 ng for cell culture) was converted into cDNA in 20 µL using OmniScript reverse transcriptase (Qiagen, Redwood City, CA, USA) and 1 µM poly-dT (Invitrogen, Naerum, Denmark) according to the manufacturer's protocol (Qiagen). The same pool of cDNA used previously for the cells in culture [25] and the male muscle tissue [26] was used here. For each target mRNA, 0.25 µL cDNA were 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 1). The amplification was monitored real time using the MX3005P Real-time PCR machine (Stratagene, San Diego, 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. Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) mRNA was chosen as internal control. To validate this use, another unrelated "constitutive" mRNA, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), was measured and normalized with RPLP0. In the cell culture experiment GAPDH mRNA normalized to RPLP0 mRNA was constant, indicating that RPLP0 (and GAPDH) was indeed constant and suitable for normalization. However, in tissue the GAPDH/RPLP0 ratio was lower in the elderly female subjects and one and four days after exercise in the males, showing either a GAPDH decrease or a RPLP0 increase. However, the decrease in GAPDH was not reflected

in the general pattern of the other mRNA when normalized to RPLP0, arguing against a general normalization error. We therefore chose to use retain RPLP0 for normalization. The GAPDH mRNA data from cell culture of the females and tissue of the males have been used as internal control in already published papers [25,26].

Table 1. Primers used for PCR. RPLP0: Ribosomal Protein Lateral Stalk Subunit P0; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; AChR: acetylcholine receptor; MuSK: muscle-specific-kinase; MHCn: neonatal myosin; MHCe: embryonic myosin heavy chain.

mRNA	Genbank	Sense	Antisense
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
AchRα1	NM_000079.3	GCAGAGACCATGAAGTCAGACCAGGAG	CCGATGATGCAAACAAGCATGAA
AchRβ1	NM_000747.2	TTCATCCGGAAGCCGCCAAG	CCGCAGATCAGGGGCAGACA
AchRδ	NM_000751.2	CAGCTGTGGATGGGGCAAAC	GCCACTCGGTTCCAGCTGTCTT
AchRε	NM_000080.4	TGGCAGAACTGTTCGCTTATTTTCC	TTGATGGTCTTGCCGTCGTTGT
AchRγ	NM_005199.4	GCCTGCAACCTCATTGCCTGT	ACTCGGCCCACCAGGAACCAC
MuSK	NM_005592.3	TCATGGCAGAATTTGACAACCCTAAC	GGCTTCCCGACAGCACACAC
MHCe	NM_002470.3	CGGATATCGCAGAATCTCAAGTCAA	CTCCAGAAGGGCTGGCTCACTC
MHCn	NM_002472.2	CGGAAACATGAGCGACGAGTAAAA	CAGCCTGAGAACATTCTTGCGATCTT
GAPDH	NM_002046.6	GAGGGGCCATCCACAGTCTTCT	GACATGCCCAAGACCCAGAAGGA

2.5. Immunohistochemistry

For the female participants, cross sections (10 µm) from the biopsies of the exercised and control legs were cut at −20 °C in a cryostat. Sections from both legs of one individual were placed on the same glass slide (Thermo Scientific, Waltham, MA, USA) and stored at −80 °C until staining. For staining, two primary antibodies were diluted in 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) and applied to the sections (see Table 2), and then incubated in the refrigerator overnight. Afterwards two secondary antibodies (see Table 2) diluted in 1% BSA in TBS were applied for 45 min. At this point, the sections were fixed in 5% formaldehyde (Histofix, Histolab, Gothenburg, Sweden) for 12 min and then mounted with Prolong-Gold-Antifade (Invitrogen, Molecular Probes, OR, USA, catalogue #P36931), containing 4′,6-Di-amidino-2-phenylindole (DAPI). Slides were washed with TBS twice between all steps. Slides were kept in darkness at room temperature for 48 h and then moved to a −20 °C freezer. Two sections were also stained with NCAM and collagen XXII (made by Manuel Koch) [28], as previously described [29], since it was suspected that the NCAM staining in these sections was due to the presence of myotendinous junction and not denervated muscle fibres.

Table 2. Antibodies used for immunohistochemistry and immunocytochemistry. MHCn: neonatal myosin; MHCe: embryonic myosin heavy chain; NCAM: neural cell adhesion molecule.

Host	Antibody	Primary Antibody Company	Cat. no.	Concentration
Mouse	Dystrophin, IgG2b	Sigma-Aldrich	D8168	1:500
Mouse	Myosin 1, IgG1	Hybridoma Bank	A4.951	1:200
Mouse	MHCe, IgG1	Hybridoma Bank	F1.652	1:100
Mouse	MHCn, IgG1	Novocastra	NCL-MHCn	1:100
Mouse	NCAM, IgG1	Becton Dickinson	347740	1:50
Rabbit	Desmin, IgG	Abcam	AB32362	1:1000
Mouse	Myogenin, IgG1	Hybridoma Bank	F5D-s	1:50
Host	**Antibody**	**Secondary Antibody Company**	**Cat. no.**	**Concentration**
Goat	488, green, IgG1	Invitrogen	A-21121	1:500
Goat	568, red, IgG2b	Invitrogen	A-21144	1:200
Goat	568, red, IgG	Invitrogen	A-11036	1:500
Goat	488, green, IgG	Invitrogen	A-11029	1:500

2.6. Microscopy

All imaging was performed with a ×10/0.30NA objective and a 0.5× camera (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany) mounted on a BX51 Olympus microscope, using the Olympus cellSens software (v.1.14). For all analyses, 1.7 × 1.3 mm greyscale images were captured.

Muscle fibre size and muscle fibre type composition analysis was only performed on the control leg. Non-overlapping images of high resolution (4080 × 3072 pixels) were captured to accommodate a semi-automated macro [30], run in ImageJ (v.1.51, U.S. National Institutes of Health, Bethesda, MD, USA). All analyses were conducted by the same person blinded to the age group. All included muscle fibres were manually checked, and fibres were excluded if the dystrophin staining was incomplete or if an area of the biopsy was longitudinally oriented. Fibres at the edge and around holes and folds in the biopsies were always excluded. After delineation of the muscle fibre cross-sectional area (CSA), fibre type was determined based upon the median light intensity. Fibres were classified as type I (positive for myosin type I staining) or type II (negative for myosin type I staining). Hybrid muscle fibres (low levels of type I myosin staining) were excluded from the analysis (a total of 131 fibres from all sections).

For the analysis of embryonic myosin heavy chain (MHCe)-, MHCn-, and NCAM-positive fibres, images at a resolution of 2040 × 1536 pixels were captured. For MHCe, only areas with positive staining were imaged, while for MHCn and NCAM the entire biopsy section was imaged (due to the relatively higher prevalence of positive fibres). Positively stained muscle fibres were determined as fibres with a complete dystrophin staining and a clear staining of one of the three markers. We extended the method used in our previous study [20] by also measuring the CSA of all transversely cut positive muscle fibres in the present study. All analyses were conducted by the same person, blinded to age group and leg of the sample. All values are expressed relative to the total number of fibres in the section. In a sub-analysis, four consecutive sections from two elderly subjects (both the exercised and the control leg) were additionally analysed for MHCn-positive fibres to determine whether small fibres could be found on consecutive sections. Overview images of the sections were initially used to identify areas of the biopsy that were present on all four consecutive sections. Peripherally positioned (at edges or holes) muscle fibres were not included. In total, 31 MHCn-positive muscle fibres were included across the two subjects and followed through the four consecutive sections (see online Supplementary Figure S2 for images).

2.7. Immunocytochemistry

For the cells cultured to differentiate, the fusion index was determined as reported earlier [25]. Briefly, coverslips were stained with the primary antibodies desmin and myogenin (see Table 2 for details) followed by the secondary antibodies goat anti-rabbit 568 (catalogue #A11036) and goat anti-mouse 488 (catalogue # A11029), and mounted with Prolong-Gold-Antifade containing DAPI (catalogue #P36931, Invitrogen), as described [25]. Fusion index was calculated as the percentage of desmin-positive nuclei within myotubes (containing three or more nuclei) divided by the total number of desmin-positive nuclei.

2.8. Statistics

All figures were prepared in GraphPad Prism (v.7.04, GraphPad Software, Inc., La Jolla, CA, USA) and all statistical analyses were conducted in SigmaPlot (v. 13.0, Systat Software Inc, San Jose, CA, USA), except subject characteristics and gene expression of the female subjects, which were analysed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, Washington). p-Values below 0.05 were considered significant, and trends of $p < 0.1$ are also reported. mRNA data were normalized to RPLP0 and log-transformed before statistical analysis. For the female participants, unpaired t-tests (two-tailed) were performed between young and old for subject characteristics, fibre size, fibre type composition, and mRNA data. Paired t-tests (two-tailed) were conducted for the analysis of the

exercise response (exercised leg vs. control leg). The Bonferroni correction was applied (multiplying the p-values ×3) to the t-test analyses on the mRNA data to correct for multiple testing. For correlation analyses, mRNA data were log-transformed and then subjected to Pearson's correlation. The number of MHCe-, MHCn-, and NCAM-positive fibres, which was not normally distributed, was subjected to the Mann–Whitney Rank Sum Test and Wilcoxon Signed Rank Test to compare differences between young and old subjects, and control versus exercised leg, respectively. For the male participants, data were analysed by one-way repeated measures analysis of variance, using Dunnett's method for multiple comparisons to compare each time point with baseline, where an overall main effect of time was found. The subject characteristics are presented as means with standard deviation and range, while muscle fibre size and composition are shown as individual values. MHCn- and NCAM-positive muscle fibres are presented as median and individual values.

3. Results

3.1. Subject Characteristics

Age, height, weight, BMI, and Leg Extension 1 RM for all subjects included in the analyses are provided in Table 3. The control muscle biopsy from one elderly woman was found to show irregularities (one fascicle filled with unusually large and small muscle fibres positive for NCAM, MHCn, and MHCe), and this subject was therefore excluded from all analyses.

Table 3. Subject characteristics. Average and standard deviations with ranges (superscript). Abbreviations: BMI, body mass index; 1 RM, one-repetition maximum; yr: years, kg: kilogram.

	Young Women			Old Women			Old Men		
	$n = 12$			$n = 11$			$n = 25$		
Age (yr)	23	± 3	20–28	74	± 3	71–78	70	± 7	64–90
Height (cm)	168	± 7	157–177	166	± 3	162–169	180	± 5	172–189
Weight (kg)	64	± 8	53–75	69	± 10	57–84	82	± 10	67–98
BMI (kg/m^2)	23	± 2	19–26	25	± 4	20–30	26	± 3	21–31
Knee extension 1RM (kg)	39	± 8	30–50	23	± 5	12–28	56	± 14	23–82

3.2. Tissue Immunohistochemistry at Baseline—Young and Elderly Women

On average, the numbers of fibres included in the fibre type and size analysis were 212 (129–352) for type I and 151 (68–247) for type II fibres in the young participants. The corresponding values for the elderly were 169 (85–267) type I and 143 (45–487) type II fibres. The type I fibre percentage was 59 ± 11% (35%–74%) for the young and 58 ± 15% (22%–75%) for the elderly, with no difference between them. As seen in Figure 1, the elderly had significantly smaller type II fibres compared to both their own type I fibres (−38%, $p < 0.001$) and the type II fibres in the young (−36%, $p < 0.001$).

On average, the number of fibres included in the immunohistochemical analysis of denervated fibres was 1080 [401–2270]. MHCe-positive fibres were only found in the excluded subject and are therefore not presented. The elderly had significantly more MHCn- and NCAM-positive fibres compared to the young (Figure 2).

No significant differences between the previously exercised and the control leg were found in either the young or the elderly for MHCn or NCAM (online Supplementary Figure S3). We evaluated the fibre size of all transversely cut MHCn- and NCAM-positive fibres from the control leg. A clear majority of the MHCn- and NCAM-positive muscle fibres were smaller than 150 μm^2 (online Supplementary Figure S3).

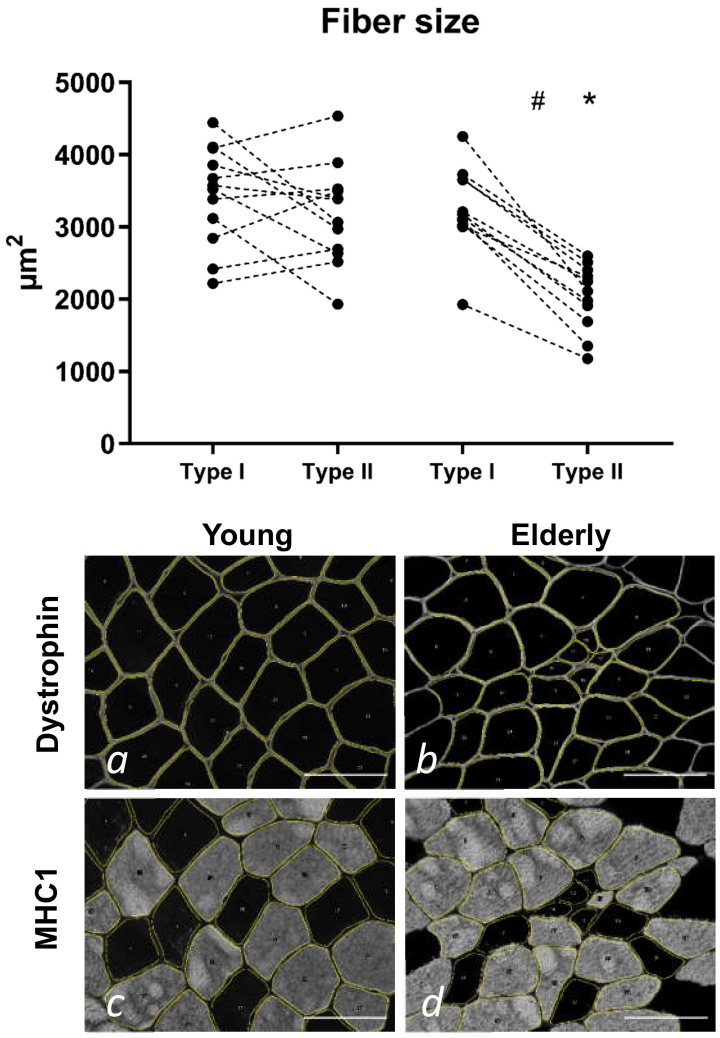

Figure 1. Muscle fibre size analysis in biopsy cross-sections from the control legs of 12 young and 11 elderly women. Individual values are displayed and with type I and II values for an individual connected by a dashed line. The type II fibres of the elderly individuals were significantly smaller than their own type I fibres and the type II fibres of the younger individuals. * $p < 0.001$ vs. young type II, # $p < 0.001$ between fibre types in elderly. Images (**a**–**d**) illustrate the analysis process. (b,d) show representative images of the same area, which has been delineated by the macro in ImageJ. This is an elderly subject with a mean fibre size of 3025 µm^2 and 1688 µm^2 for type I and II fibres, respectively. Similarly, a and c show representative images of the same area in a young subject with a mean fibre size of 3574 µm^2 and 3378 µm^2 for type I and II fibres, respectively. MHC1, myosin heavy chain 1. Scale bars = 100 µm.

One biopsy from the exercised leg of a young subject showed 13 NCAM-positive fibres (1.3% of total fibre count) all located adjacent to a thick band of connective tissue, reminiscent of the myotendinous junction (MTJ). Collagen XXII staining confirmed that this was in fact MTJ, so these fibres were not included in the analysis of this biopsy (see online Supplementary Figure S4 for image). One young subject had 13 (1.45% of total fibre count) NCAM-positive fibres, all of which were located at the edge of the biopsy. This area was not stained by collagen XXII and remained NCAM-positive on additional sections and was therefore not excluded from the analysis. In all other samples MHCn- and NCAM-positive fibres were randomly scattered in between normal muscle fibres.

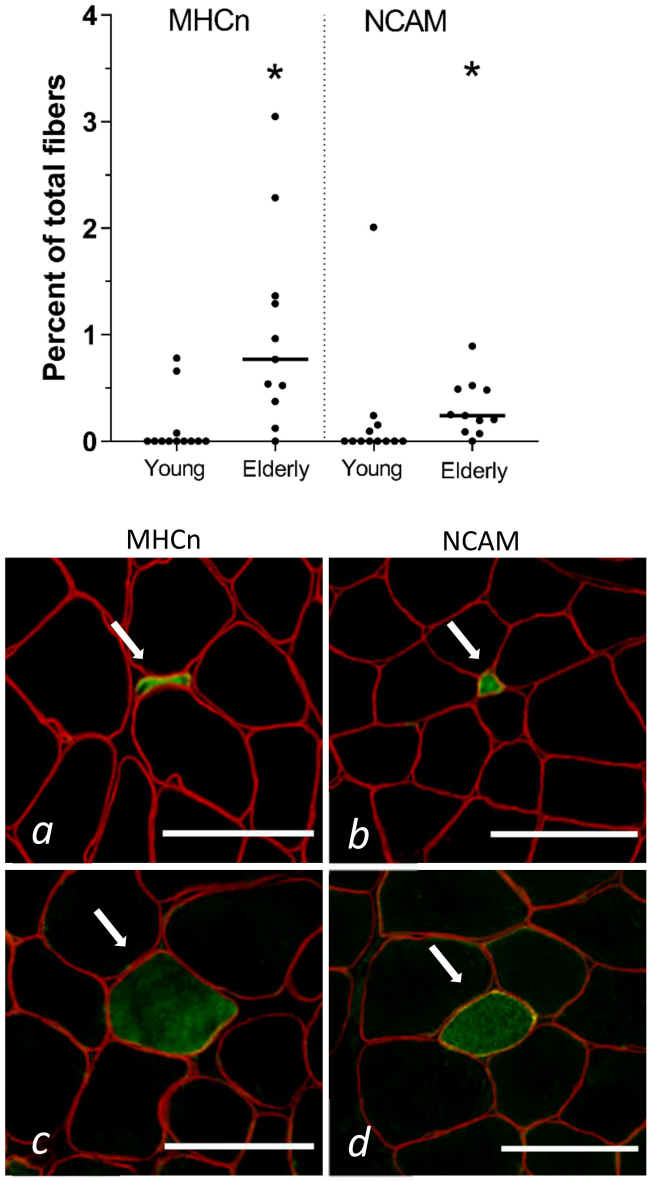

Figure 2. Muscle fibres positive for MHCn or NCAM in biopsy cross-sections from 12 young and 11 elderly women. Only the control leg is shown. Individual values are presented with the median (horizontal line). Panels show examples of small MHCn (**a**) and NCAM (**b**), and large MHCn (**c**) and NCAM (**d**) fibres (arrows). Positive fibres are green, dystrophin, red. * $p < 0.05$ vs. young. MHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule. Scale bars = 100 μm.

3.3. Tissue mRNA at Baseline and in Response to Exercise—Young and Elderly Women

The muscle tissue of the elderly women had significantly lower levels of AChR β1 mRNA compared to the young women, whereas levels of both AChR γ and MHCn mRNA were higher in the elderly compared to the young (Figure 3). Tendencies for differences were seen for gene expression levels of AChR α1 and muscle-specific-kinase (MuSK).

Both the elderly and the young women had a significant upregulation of AChR α1 mRNA in the previously exercised leg compared to the control leg (Figure 3). The exercise response of AChR δ mRNA only reached statistical significance in the elderly. AChR ε mRNA were detected in less than half of the samples at levels very close to detection limit of one molecule and with no preference for any group (data not shown).

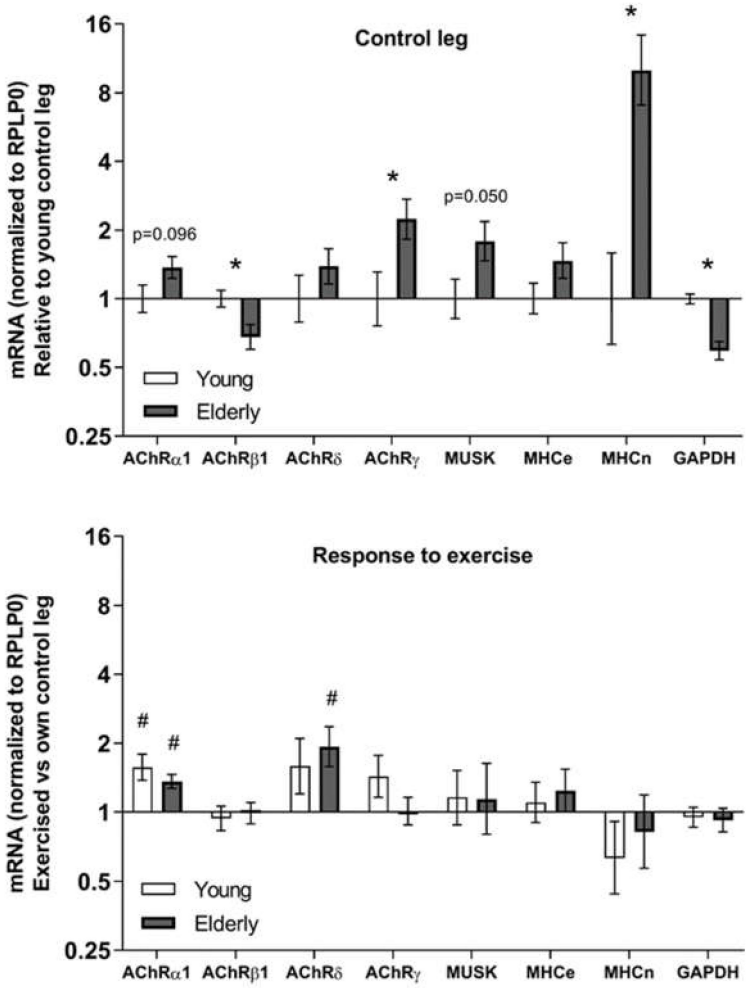

Figure 3. Gene expression in muscle biopsies of healthy young (n = 12) and elderly (n = 11) women, at rest (control) and five days after a single bout of one-legged exercise. mRNA data were normalized to RPLP0 and are shown as geometric means ± back-transformed SEM, relative to young control legs (control leg) and own control leg (response to exercise). * $p < 0.05$ elderly vs. young. # $p < 0.05$ vs. control leg. Tendencies are written. AChR: acetylcholine receptor; MuSK: muscle-specific-kinase; MHCe: embryonic myosin heavy chain; MHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; RPLP0: Ribosomal Protein Lateral Stalk Subunit P0.

3.4. Cell Culture at Baseline and in Response to Exercise—Young and Elderly Women

The fusion index of the cell cultures from the rested and exercised legs of the elderly women was 36.3 ± 4.2% and 36.1 ± 5.0%, respectively, with the corresponding values for the young group being 52.2 ± 1.8% and 49.8 ± 2.2%, respectively (main effect of age, two-way repeated measures ANOVA).

All gene expression targets were more strongly expressed in differentiating compared to proliferating cells (see online Supplementary Figure S5). In the proliferating condition, the cells from the elderly had lower gene expression levels of MHCe and MHCn compared to young (Figure 4). Similarly, we also found a significantly lower level of MHCn gene expression in the differentiating cells in the control leg in the elderly compared to the young. AChR β1, δ, and γ all showed age-related tendencies.

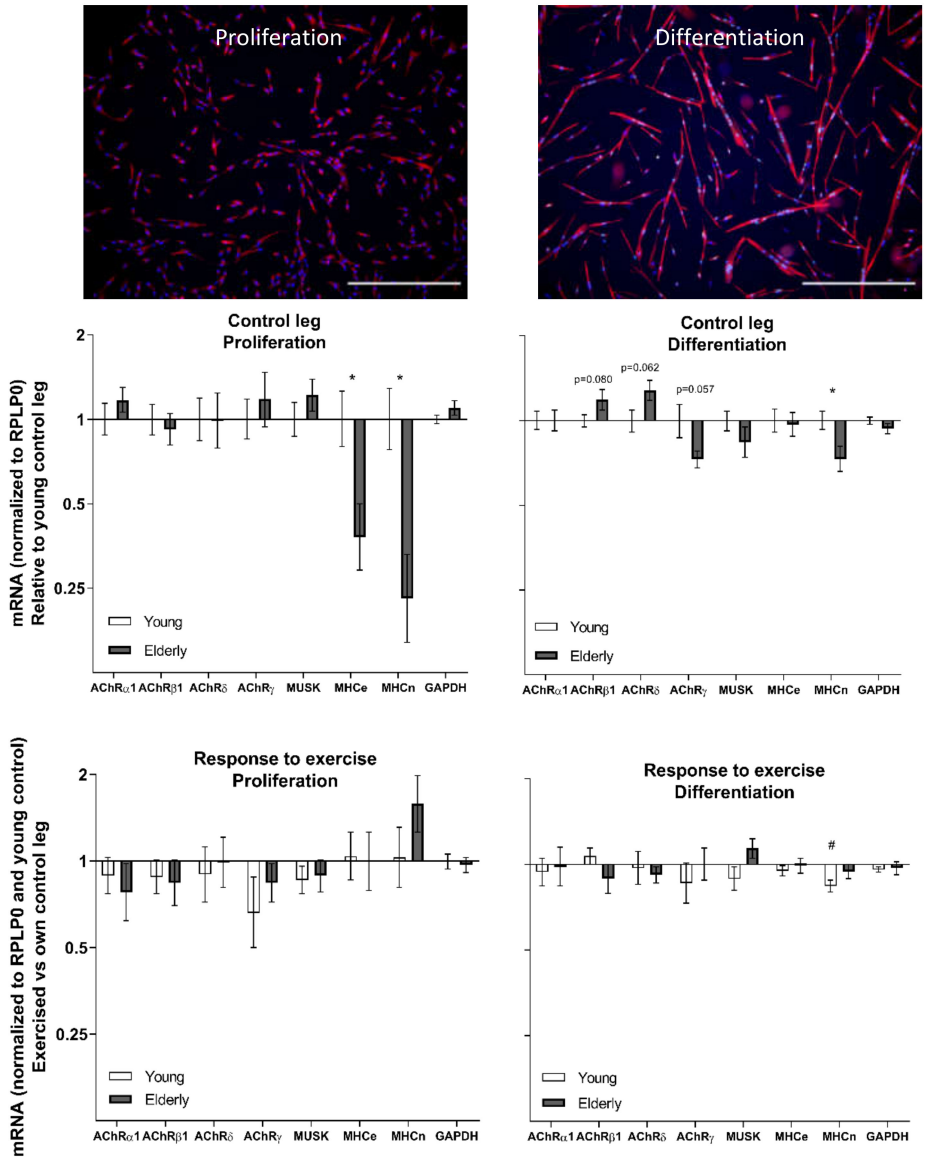

Figure 4. Images display cells in the proliferation condition (Desmin, red, and DAPI, blue) and in the differentiation condition (Desmin, red, Myogenin, green, and DAPI, blue), scale bars = 500 μm. Gene expression in cell cultures from the control and exercised legs of healthy young (n = 12) and elderly (n = 11) women. mRNA data were normalized to RPLP0 and are shown as geometric means ± back-transformed SEM, relative to young control leg (control leg) and own control leg (response to exercise). * $p < 0.05$ elderly vs. young. # $p < 0.05$ vs. control leg. Tendencies are written. AChR: acetylcholine receptor; MuSK: muscle-specific-kinase; MHCe: embryonic myosin heavy chain; MHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; RPLP0: Ribosomal Protein Lateral Stalk Subunit P0.

Differentiating cells from the previously exercised leg from the young subjects demonstrated a lower gene expression of MHCn versus the control leg (Figure 4).

3.5. Tissue mRNA in Response to Exercise—Elderly Men

In general, gene expression in four out of the five AChR measured demonstrated a response to exercise. AChR α1 mRNA was downregulated 4.5 h and one day after the exercise and returned to baseline in four days (Figure 5). AChR β1 mRNA was downregulated at 1, 4, and 7 days. AChR δ mRNA showed a tendency for a decline 4.5 h after exercise and was upregulated seven days after

the exercise. AChR γ mRNA decreased 4.5 h after the exercise bout. No significant exercise-induced changes in gene expression of the AChR ε subunit, MuSK, MHCe, or MHCn were observed.

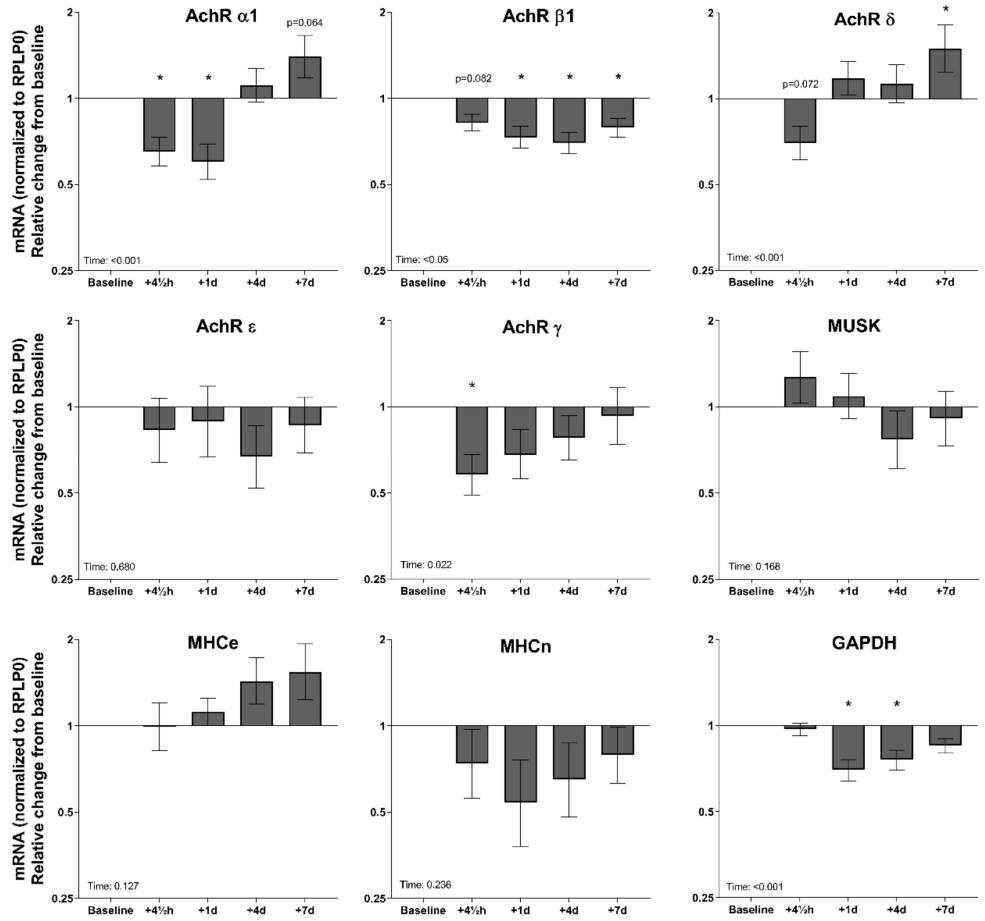

Figure 5. Gene expression in muscle biopsies of 25 healthy elderly men ten days before (baseline) and 4.5 h, one day, four days, and seven days after a single bout of exercise. mRNA data were normalized to RPLP0 and are shown as geometric means ± back-transformed SEM, relative to baseline. * $p < 0.05$ vs. baseline. Tendencies are written. AChR: acetylcholine receptor; MuSK: muscle-specific-kinase; MHCe: embryonic myosin heavy chain; MHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; RPLP0: Ribosomal Protein Lateral Stalk Subunit P0.

4. Discussions

The most notable findings of the present study was that the skeletal muscle of elderly individuals, with morphological signs of ageing as demonstrated by a reduced type II muscle fibre CSA and a heightened number of denervated muscle fibres, has a significantly elevated gene expression level of the denervation-responsive AChR γ subunit and MHCn as compared to young healthy individuals. Our data also suggest an age effect on the capacity of satellite cell-derived myotubes to transcribe AChR genes, which is fundamental for NMJ maintenance. Furthermore, we provide novel insight into the transient changes in gene expression of all five muscle AChR subunits following heavy resistance exercise in healthy elderly human skeletal muscle. Together these data support the role of exercise in stimulating the stability of the NMJ, but also indicate age-related changes, even in healthy elderly individuals.

4.1. Muscle Fibre Denervation in Elderly Humans

Healthy elderly women with clear signs of ageing (lower type II muscle fibre CSA and lower muscle strength), also show a significantly heightened number of denervated muscle fibres compared to young healthy women, as evidenced by a greater proportion of fibres positive for NCAM and MHCn. When a muscle fibre loses its neural input, the plasticity of the peripheral nervous systems allows for adjacent nerve sprouts to attempt to re-innervate denervated muscle fibres through nerve sprouting [31]. It is believed that the increased synthesis of NCAM in denervated muscle fibres facilitates this innervation process [32,33]. Denervated muscle fibres will also revert into an immature myosin heavy chain configuration, as we found more MHCn-positive fibres in old subjects compared to the young. Furthermore, we also observed a substantial 10-fold higher gene expression level of MHCn in the muscle tissue of the elderly compared to the young females, reflecting a persisting synthesis of this distinct myosin isoform. Importantly, it should be noted that there is not a complete overlap between MHCn- and NCAM-positive stained fibres, which suggests that the rate at which these proteins aggregate in the muscle fibres following denervation might differ. In terms of denervated muscle fibre morphology, we observed a persisting MHCn and NCAM protein presence in even the smallest of muscle fibres (<75 μm^2). These miniature fibres are easily missed during regular biopsy assessments and could represent long-term denervated fibres that had atrophied over time [34] and undergone deterioration of muscle proteins [35], but maintained an increased and long-lasting cytoplasmic expression of MHCn [36] and NCAM [32]. The length of these miniature muscle fibres is a matter of uncertainty. We have previously been able to follow such fibres through 400 µm of consecutive biopsy sections in a selected subject [20]. In a sub analysis in the present study, we searched for MHCn-positive fibres through four consecutive sections and found that 13, 32, and 39% of the fibres had disappeared after 1, 2, and 3 sections, respectively. This implies a substantial number of miniature fibres ends, which could indicate that long-term denervated fibres are gradually degraded both transversally and longitudinally.

4.2. Ageing and Exercise Alter Acetylcholine Receptor Gene Expression

One of our most marked findings is that the gene expression of the AChR γ subunit is robustly elevated in the skeletal muscle of elderly compared to young females. This coincides with this subunit being a functionally distinct foetal subunit [37–39], which is increasingly expressed following both denervation [39,40] and neurotransmitter blocking [41]. Interestingly, in our group of male participants the muscle homogenate gene expression of the γ subunit was acutely downregulated after the exercise bout but had already returned to baseline after one day. We were also able to detect this subunit, as well as α1, β1, and δ subunits, in both proliferating and differentiating cell cultures that were devoid of neural presence, meaning that satellite cell derived myonuclei can upregulate AChR gene expression without the presence of a nerve. As satellite cells have been shown to be crucial for maintaining the specialized post-synaptic region of the muscle fibre [23], it is interesting that we observed trends for an age effect in three out of the four subunits. It is worth noting that this is the case even with the conservative Bonferroni correction, but given that the age difference was not always in the same direction, it is possible that this is a real effect of age (and not an effect of general cell culture conditions), potentially reflecting an age-related satellite cell dysfunction that could negatively impact the maintenance of the NMJ. However, it should be noted that the cell cultures derived from satellite cells of the young subjects showed a significantly higher fusion index compared to the old subjects [25], indicative of a higher level of myotube maturity. Furthermore, we also observed a positive correlation between cell fusion index and gene expression levels of AChRγ (R = 0.74), MuSK (R = 0.75), and MHCe (R = 0.66) in the old group (Supplementary Figure S6). This would suggest that AChRγ gene expression in aneural cell cultures is increased concordant with myotube maturity and raises the possibility that the molecular differences we observed between the cell cultures from young and elderly muscle are determined by the extent of fusion. However we cannot rule out the opposite, i.e., that the lower gene expression levels contribute to the lower fusion index values.

Generally, our data show age and exercise effects on AChR γ subunit gene expression, in line with its suggested use in evaluating muscle fibre denervation in healthy individuals. In our previous study, we found a negative correlation between age and the AChR γ subunit in a large group of elderly men [20], which initially might seem to contradict our finding in the present study. However, it is important to acknowledge that a denervated muscle fibre is not in a "stable state", meaning that without the neural input the proteins will be degraded and the structure is gradually lost [34,35]. Ultimately, the muscle fibre completely disappears or is only present as a fraction of its former size and as such its contribution to the whole muscle gene expression profile will also decline.

Since our study includes one data set from males and the other from females, it is worth considering similarities in the pattern of the exercise response between the elderly male and female subjects, given that the day five timepoint of the females can be compared with the four- and seven-day timepoints of the male. In this way it seems that the α, δ, and γ subunits follow a similar pattern between the genders, with the first two subunits being upregulated in both male (α only a tendency) and female subjects after seven and five days, respectively, and the γ subunit being unaffected in both male and female subjects at these timepoints. The β subunit is consistently downregulated in the male subjects whereas this subunit is not affected in the elderly women five days after exercise. Whether this represents a true gender difference and what the functional significance might be however is unknown.

This study is to our knowledge the first to outline the gene expression time course for all AChR subunits following acute heavy resistance exercise and the first to analyse the expression of four out of five AChR subunits in both young and elderly individuals at rest and following acute exercise. The NMJ of humans is challenging to study molecularly since it is difficult to obtain actual human NMJs. Hence, we rely on extra-synaptic expression of various genes that are related to the NMJ. With this approach we observed that most subunits were found to be responsive to exercise, which would suggest that despite having reached an advanced age, there is a sustained tissue plasticity in terms of synthesizing new AChRs following an exercise stimulus. The subunit-specific responses also appear to be time-dependent, as some subunits were acutely reduced after exercise followed by a recovery phase, whereas others were downregulated for longer periods. The root of these widely diverging AChR subunit time courses is puzzling and, given evidence from animal studies that long-term exercise increases the size of the NMJ [11], it would be of interest to investigate the potential of lifelong exercise on NMJ adaptations in humans.

5. Conclusions

Taken together, these data support the concept that the loss of neural signal reverts certain muscle fibre proteins to an embryonic configuration (NCAM/MHCn/AChR γ) and that these markers are useful in evaluating the effectiveness of interventions to counteract the denervation-induced loss of muscle fibres in humans. Gene expression levels of the AChR γ subunit in particular repeatedly demonstrated sensitivity to age and exercise. The trends for age-related differences in the gene expression of AChR subunits in myotubes in cell culture were related to myogenic fusion index and potentially suggest a loss of satellite cell function in relation to the capacity to transcribe key molecules for NMJ stability. Finally, it can be speculated that the temporal manner of the AChR subunit gene expression response following exercise represents a beneficial stimulus for muscle mass preservation through strengthening of the NMJ.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/4/893/s1, Figure S1: Gene expression in muscle biopsies of elderly men receiving losartan (n = 13) or placebo (n = 12). mRNA data were normalized to RPLP0, log-transformed, and are shown as geometric means ± back-transformed SEM, relative to baseline (–10d). Data were analysed with a two-way repeated measures ANOVA (treatment/time). * $p < 0.05$ vs. baseline. Tendencies are written; Figure S2: Panel showing four consecutive biopsy sections of an area with small MHCn-positive muscle fibres (a–d). The dotted squares (b,d) highlight the areas of the inserts (b_{1+2} and d_{1+2}), in which a distinct dystrophin membrane is visible around the small fibre. Note that one of the positive muscle fibres is no longer visible in (c) and (d). MHCn-positive fibres are red, dystrophin, green, and nuclei, blue. MHCn, neonatal myosin heavy chain. Scale bars = 20 μm; Figure S3: Muscle fibre size of MHCn- and NCAM-positive fibres in biopsy cross-sections from the control leg in 12 young and 11 elderly women, pooled

and plotted on a logarithmic scale (a). The majority of the positive fibres were <100 μm^2.Muscle fibres positive for MHCn and NCAM for young and elderly women in control and exercised legs (b). No differences between control and exercised leg was observed for any variable. MHCn, neonatal myosin heavy chain; NCAM, neural cell adhesion molecule; Figure S4: Cross section of a muscle biopsy from one subject stained with NCAM (green) and collagen XXII (red). Nuclei are blue. NCAM-positive fibres are found in close proximity of a tendon-like structure and collagen XXII positivity confirms this is a myotendinous junction. These fibres were excluded from the analysis. Scalebar is 500 μm; Figure S5: Differentiating cells relative to proliferating cells in control leg of young women. mRNA data were normalized to RPLP0 and are shown as geometric means ± SEM. * $p < 0.05$ vs. proliferation; Figure S6: Myogenic fusion index correlates with cell culture mRNA levels of AChRγ, MuSK, and MHCe in rested leg of elderly ($n = 10$) but not young ($n = 11$) subjects. All mRNA data were log transformed and analysed with Pearson's correlation, with R and P values displayed.

Author Contributions: A.L.M., P.S., C.S., J.L.A., C.J.L.B., M.F.H., and M.K. contributed to the design of the project, while A.L.M., P.S., C.S., J.L.A., C.J.L.B., M.F.H., and M.K., S.M.J., E.B., A.K. acquired, analysed or interpreted the data of the project. C.S. and A.L.M. drafted the manuscript, and all authors gave intellectual feedback to the draft. All authors approve the final version of the manuscript to be published in *Cells* and are to be held accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and agreed to the published version of the manuscript.

Funding: Funding is gratefully acknowledged from The Nordea Foundation (Healthy Aging grant), The Danish Agency for Culture (FPK.2018-0036, FPK.2015-0020), The Lundbeck Foundation, The A.P. Møller Foundation for the Advancement of Medical Science, and Bispebjerg Hospital Research Funding.

Acknowledgments: The authors thank Anja Jokipii-Utzon and Camilla Brink Sørensen for excellent technical assistance with preparation of the muscle biopsies and the mRNA analysis. The monoclonal antibodies F1.652 (developmental MHC) and A4.951 (myosin heavy chain, human slow fibres), developed by Blau, H.M., were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH, and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

MHCe	Embryonic myosin heavy chain
MHCn	Neonatal myosin heavy chain
NCAM	Neural cell adhesion molecule
MHC1	Myosin heavy chain 1
DYST	Dystrophin
AChR	Acetylcholine receptor
DAPI	4′,6-Di-amidino-2-phenylindole
TBS	Tris-buffered saline
BSA	Bovine serum albumin
mTOR	Mammalian target of rapamycin

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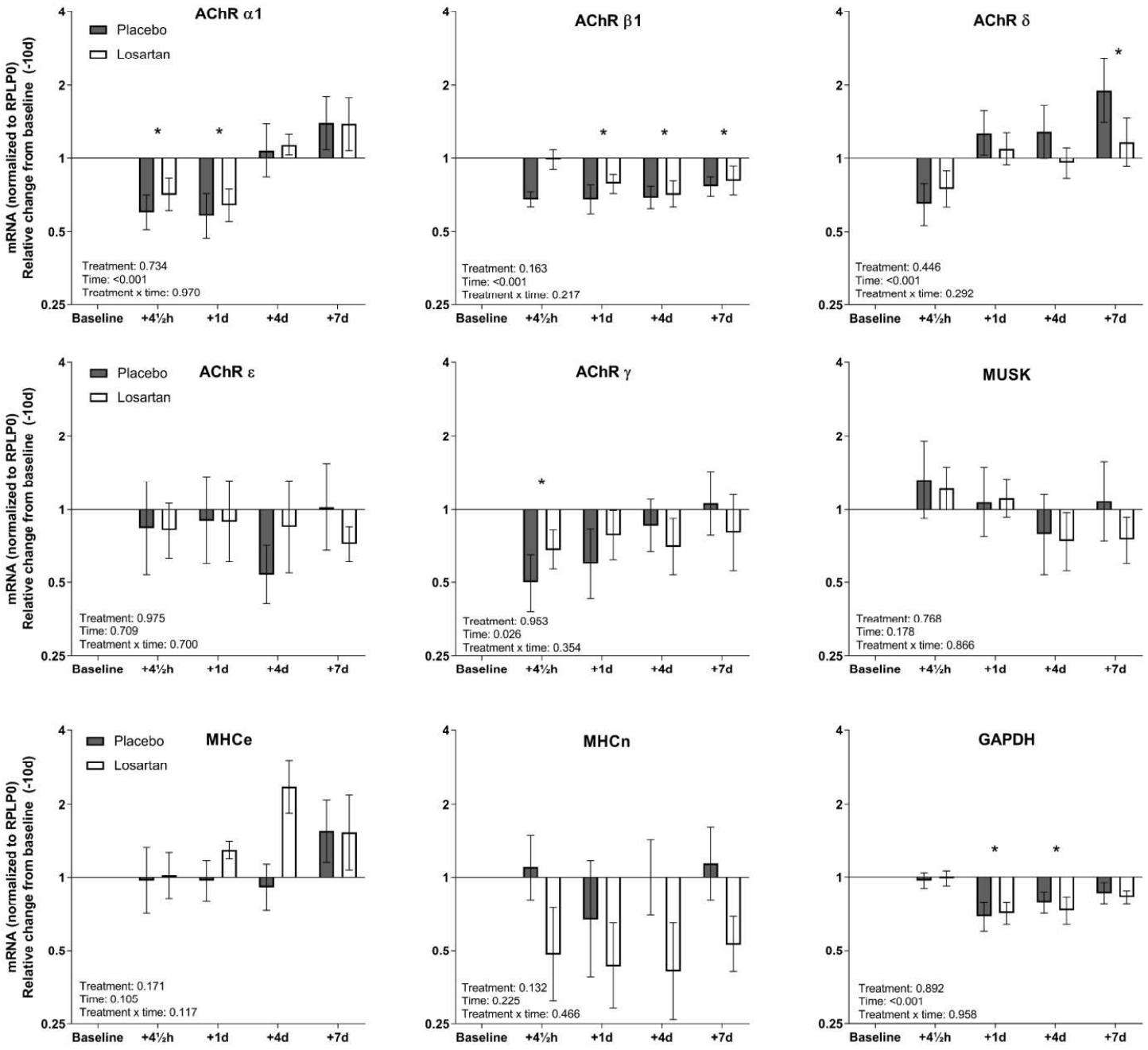
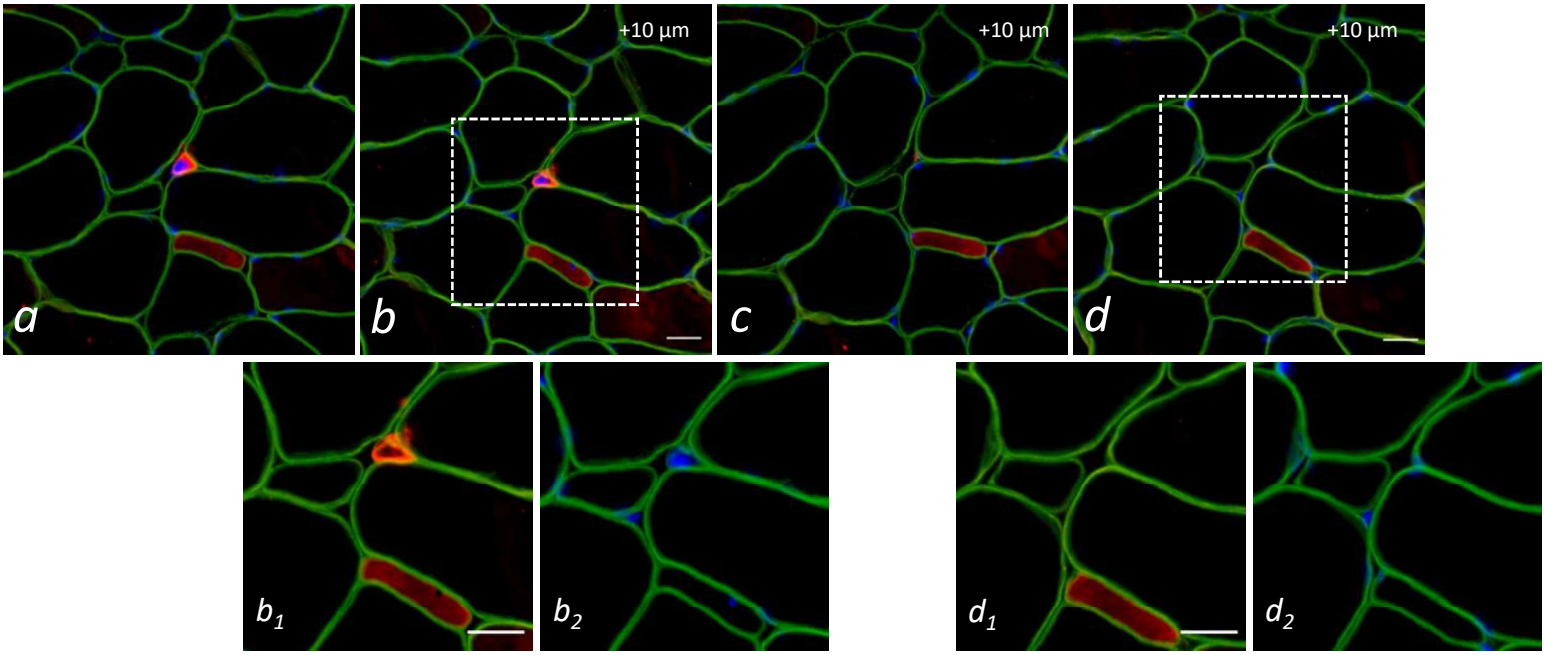
Supplemental Figure 1

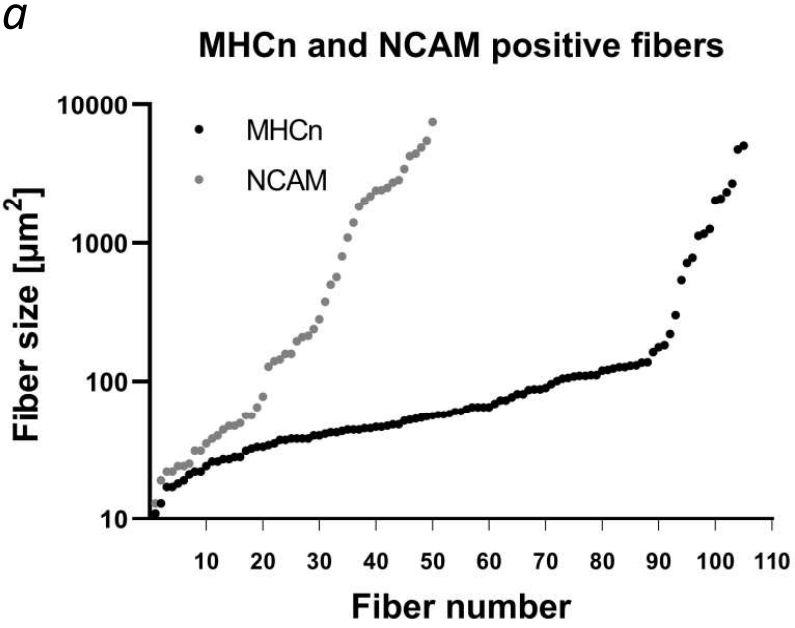




Supplemental Figure 2

Supplemental Figure 3

a



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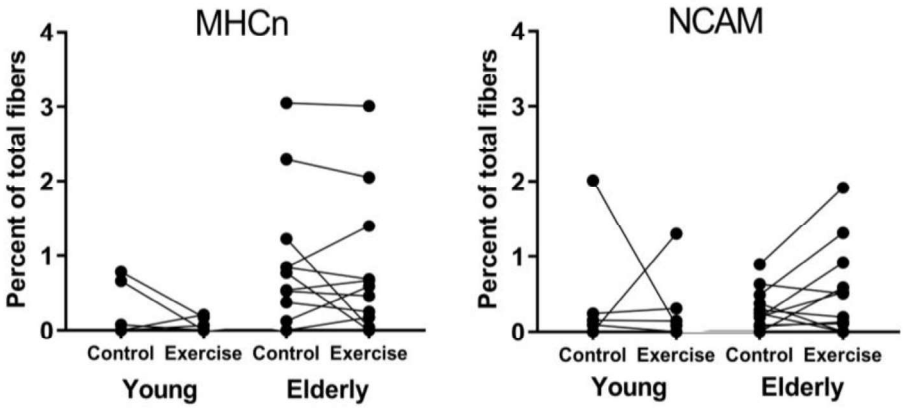
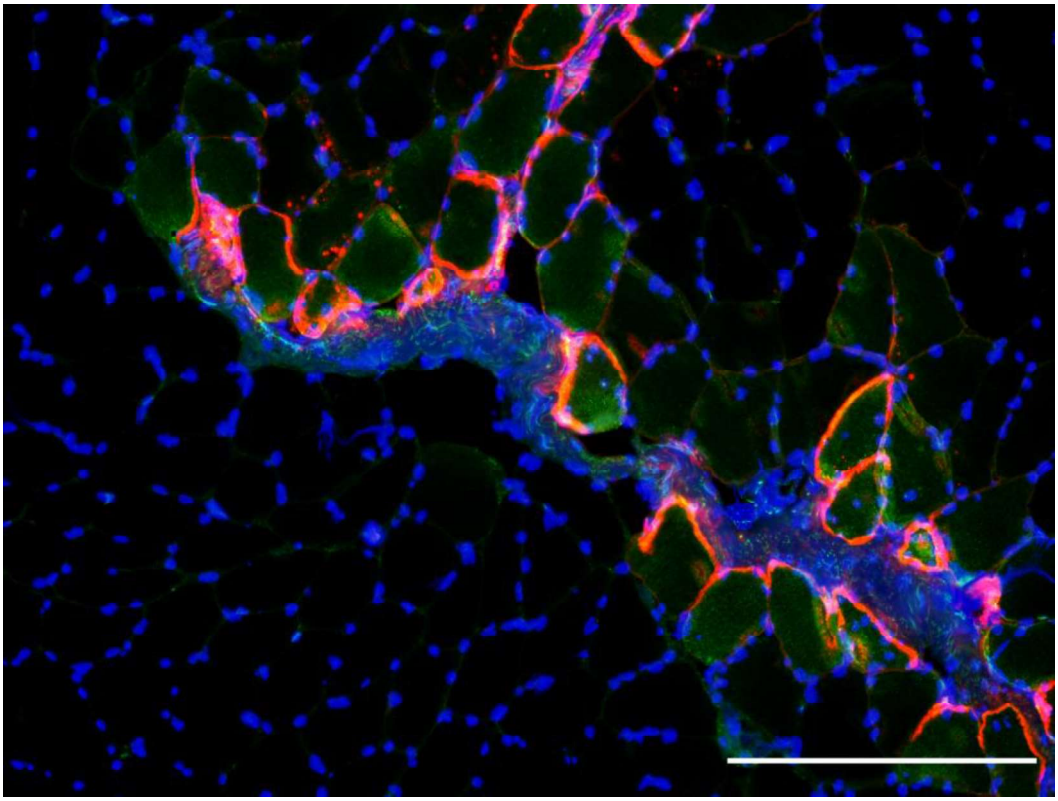
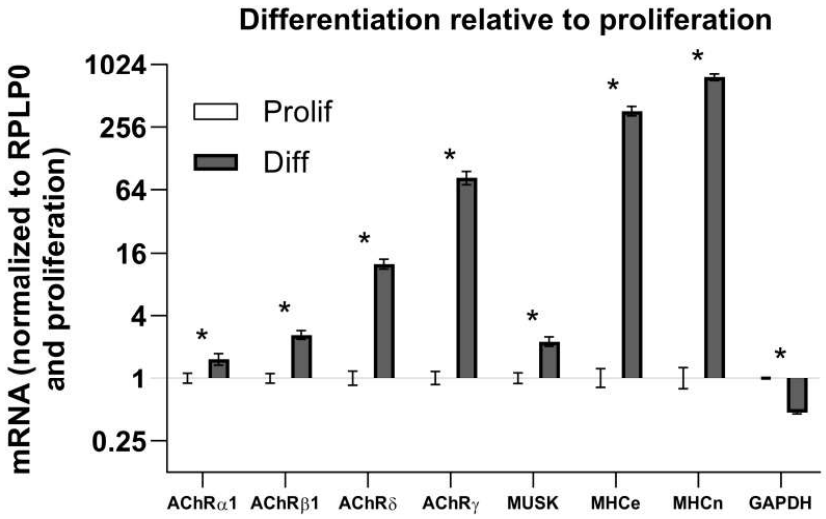


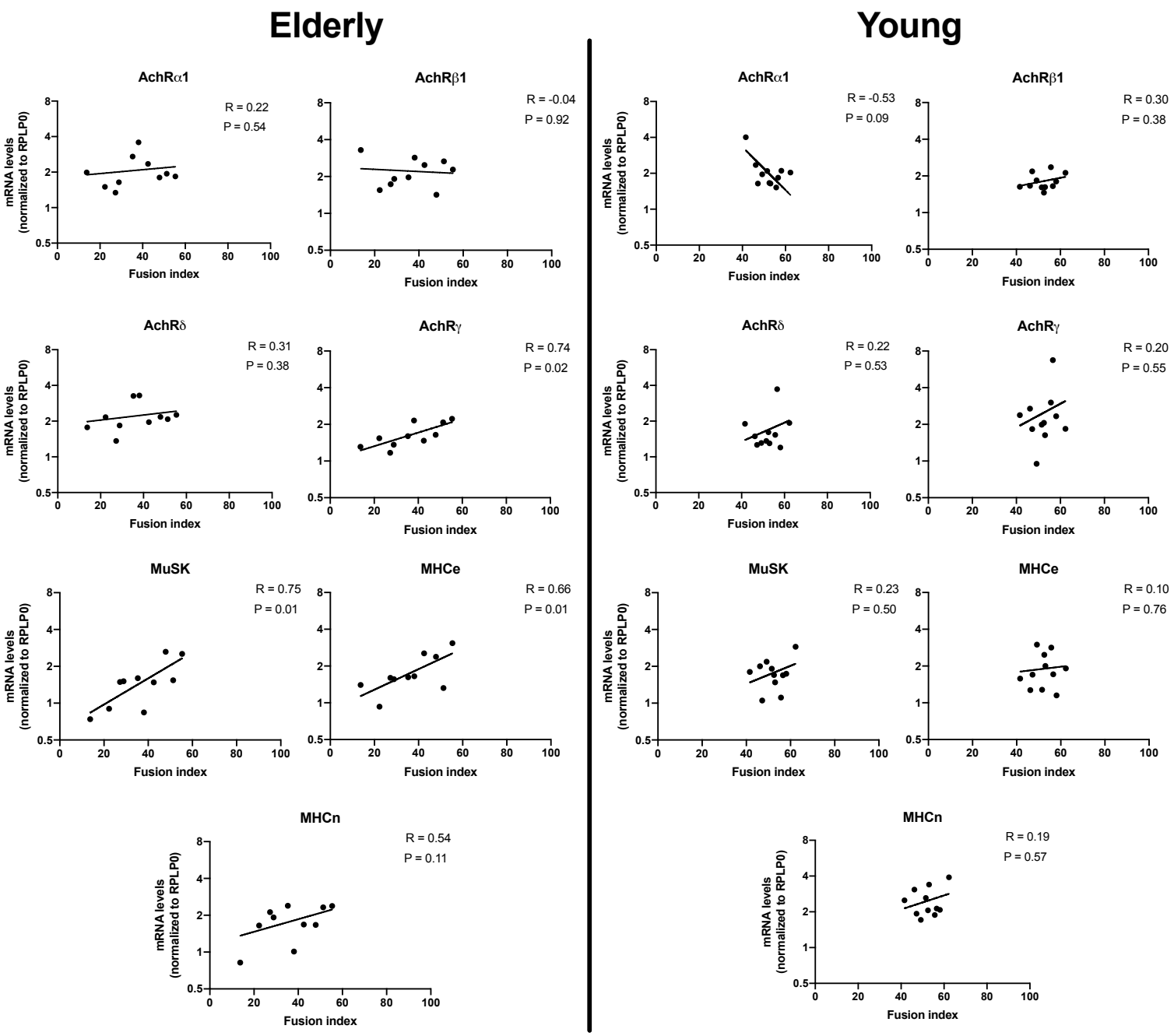
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



Study 3

The influence of 16 weeks of heavy resistance exercise on gene expression and protein markers of denervation in elderly human skeletal muscle.

Status: *Under revision*

The influence of 16 weeks of heavy resistance exercise on gene expression and protein markers of denervation in elderly human skeletal muscle

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Keywords: Heavy resistance exercise, neuromuscular denervation, acetylcholine receptor, healthy aging

Running title: Resistance exercise and muscle fiber denervation

ABSTRACT

Muscle fiber denervation is a major contributor to the decline in muscle mass and function during aging. Heavy resistance exercise is an effective tool for increasing muscle mass and strength, but whether it can rescue denervated muscle fibers remains unclear. Therefore, the purpose of this study was to investigate the potential of heavy resistance exercise to modify indices of denervation in healthy elderly individuals. 38 healthy elderly men (72±5 years) underwent 16 weeks of heavy resistance exercise while 20 healthy elderly men (72±6 years) served as non-exercising sedentary controls. Muscle biopsies were obtained pre and post training, and midway at eight weeks. Biopsies were analysed by immunofluorescence for the prevalence of myofibers expressing neonatal myosin (MHCn) and neural cell adhesion molecule (NCAM), and by RT-qPCR for gene expression levels of acetylcholine receptor (AChR) subunits, MHCn, embryonic MHC, p16 and Ki67. In addition to increases in strength and type II fiber hypertrophy, heavy resistance exercise training lead to a decrease in AChR α1 and ε subunit mRNA (at eight weeks). The prevalence of MHCn and NCAM positive fibers was not affected by exercise. Changes in gene expression levels of the α1 and ε AChR subunits with eight weeks of heavy resistance exercise supports the role of this type of exercise in targeting stability of the neuromuscular junction. Protein markers of denervation were not affected, suggesting that a longer timeframe is needed for adaptations to manifest at the protein level.

INTRODUCTION

Age-related loss of muscle mass, leading to muscle weakness and frailty, is partly caused by a loss of muscle fibers through denervation (1, 2). While heavy resistance exercise currently represents the most effective non-pharmacological countermeasure against functional decline (3), its role in ameliorating denervation specifically remains largely unexplored, particularly in humans. This is partly due to the challenges in obtaining neuromuscular junctions (NMJ) from human skeletal muscle biopsies to the extent where meaningful quantitative measurements can be made. While structural studies of the NMJ can provide direct evidence of synaptic integrity (4–6), an alternative approach is to look for signals expressed by the muscle fiber extra-synaptically. For example the entire muscle fiber in the denervated state displays immunoreactivity to neonatal myosin heavy chain (MHCn) and neural cell adhesion molecule (NCAM) (7–9), as well as increased presence of the α1, β1, γ and δ (but not ε) acetylcholine receptors (AChR) subunits (10, 11). Using these markers in muscle biopsies collected from older individuals, we have previously observed a greater prevalence of denervated muscle fibers and a modified AChR subunit profile, compared to young individuals (12, 13), supporting the usefulness of such measurements in muscle tissue samples where NMJ capture is rare. Furthermore, our findings of altered AChR subunit gene expression by a single bout of heavy resistance exercise (12, 13), and during muscle regeneration (14), demonstrates a high degree of neural plasticity at the human NMJ in healthy young and elderly individuals. Changes in AChR gene expression levels are believed to reflect stabilization of NMJs as well as re-innervation of denervated muscle fibers. The purpose of at least some of the proteins expressed by denervated fibers, e.g. NCAM, is to attract a nerve to form a NMJ with the fiber, and while re-innervation can occur in the absence of exercise, it is possible that exercise enhances this process, as discussed elsewhere (15). Furthermore, it is likely that exercise can stabilize and fortify NMJs at a stage before the muscle fiber becomes denervated. Whether resistance training can contribute to the rescue of denervated muscle fibers in elderly individuals is, however, unclear.

In the context of prolonged periods of resistance training, four human studies have investigated the prevalence of denervated muscle fibers and/or alterations in AChR gene expression in elderly individuals (14, 16–18). In a study by Messi et al., 2016, eight overweight older subjects underwent a 5-month resistance exercise training program, where the most intriguing findings in the muscle biopsy specimens were a decline in NCAM positive area and AChR γ gene expression, which was interpreted as a beneficial effect of resistance exercise on the innervation status of the muscle (16). Interestingly, while the subjects did increase their strength, no increase in muscle fiber cross-sectional area (CSA) was observed. Similarly, Karlsen et al., 2019, found a decline in the percentage of fibers strongly stained for developmental myosins (neonatal and embryonic) following 12 weeks of heavy resistance exercise in 12 elderly individuals (18). This finding was not reflected by mRNA levels of AChR subunits, as these remained unchanged at 12 weeks compared to baseline. Importantly, these subjects were very old (83-94 years), and the exercise intervention did not result in significant hypertrophy at the muscle fiber level. However, even when hypertrophy does occur, we have failed to detect changes in AChR gene expression (14), raising the possibility that changes in AChR transcription occur at an earlier stage of the training, in line with our observations following a single bout of exercise. Finally, seven older individuals with advanced stage osteoarthritis performed resistance exercise for 14 weeks, which led to an increase in type II muscle fiber diameter but did not alter the amount of NCAM staining (17). The low number of subjects and the potential influence of advanced stage osteoarthritis limits the extent to which these results can be generalized to a broader population. In addition to the four studies investigating the impact of resistance exercise on denervation, a single study has investigated endurance exercise,

and found no change in the number of NCAM positive fibers after 24 weeks of moderate intensity exercise in old individuals, despite improved peak oxygen consumption and muscle strength (19).

Taken together, very little is known about the impact of resistance exercise on muscle denervation in humans, and a combination of immunofluorescence staining and AChR gene expression analysis of muscle biopsies collected during and after the training period might provide useful insight into the potential for resistance exercise to stimulate innervation pathways. We hypothesized that a 16-week heavy resistance exercise training program leading to significant hypertrophy and increases in muscle strength in a group of healthy elderly men would cause extra-synaptic innervation adaptations, manifested as a decline in the prevalence of denervated muscle fibers and an altered AChR gene expression profile.

METHODS

Subjects and study design

This study originates from an experiment previously conducted at our department to investigate the effect of an angiotensin II type I receptor blocker (losartan) on the hypertrophic response of elderly human skeletal muscle to heavy resistance exercise (20). The study was approved by The Committees on Health Research Ethics for the Capital Region of Denmark (Reference: H-15005761) and conformed to the standards set by the Declaration of Helsinki. A total of 58 normotensive, healthy elderly men completed the study. The participants were allocated in a double-blinded manner into one of three groups; Losartan with exercise (LOS-EX), placebo with exercise (PLA-EX) and Losartan with continuation of sedentary behavior (SED, N=20). Generally, no effect of the drug was found, and the two exercise groups were therefore pooled in the present study; exercise (EX, N=38). Data from the separate exercise groups (LOS-EX and PLA-EX) are presented as a supplementary file.

Body composition was assessed by DEXA before the intervention, and maximal isokinetic and isometric voluntary force production was measured at pre, mid (eight weeks), and post (16 weeks), as described in detail (21). Muscle biopsies were obtained at pre, mid, and post, with sampling time of day standardized for each participant and the mid and post biopsies obtained 2 days after the latest training session. The pre and 16-week biopsies were taken from the same leg, and the 8-week biopsy was taken from the contralateral leg. Fiber type specific CSA and satellite cell numbers was evaluated using ATPase staining and immunofluorescence, respectively (20).

Briefly, the exercise intervention consisted of a heavy resistance exercise training program performed, under supervision, three times a week for 16 weeks. Three mandatory lower-body exercises (Leg Press, Leg Extension and Leg Curl) and two optional upper-body exercises were performed, and the loading was continuously adjusted in order to accommodate a high level of exertion in every training set.

Muscle biopsy analyses

Muscle biopsies were collected with a five mm Bergström needle, with manual suction, from the vastus lateralis muscle (22). Muscle fiber fascicles were carefully aligned and mounted in Tissue-Tek (Sakura Finetek, Europe, AJ Alphen aan den Rijn, The Netherlands), frozen in isopentane (2-Methylbutan; J.T.Baker, AVantor Performance Materials B.V., Deventer, The Netherlands) precooled by liquid nitrogen, and stored at -80°C.

Denervation immunofluorescence and microscopy
10 μm thick cross-sections from the pre, mid and post muscle biopsies of each participant were placed on the same glass slide (Thermo Scientific). For one subject in EX, the post biopsy was lost during sectioning, reducing the number of subjects to 37. As described (12), sections were stained with primary antibodies against Dystrophin (mouse IgG2b, MANDYS8, D8168; Sigma) in combination with 1) embryonic myosin heavy chain (MHCe) (mouse Ig G1, F1.652; Developmental Studies Hybridoma Bank), 2) MHCn (mouse IgG1, NCL-MHCn; Novocastra, Newcastle upon Tyne, United Kingdom), or 3) NCAM (mouse IgG1, CD56, 347740; Becton Dickinson, San Jose, California). Following incubation with secondary antibodies (Alexa Fluor 568, A-21144, Goat Anti-Mouse IgG2b and Alexa Fluor 488, A-21121, Goat Anti-Mouse IgG1), sections were mounted with Prolong-Gold-Antifade (Cat. No. P36931; Molecular Probes/Invitrogen) containing 4´,6-diamidino-2-phenylindole (DAPI).

2040x1536 pixel images were taken with a 10x/30NA objective of areas with positive staining of MHCe and MHCn, and of the whole cross-section of staining with NCAM. Overlapping images were subsequently stitched together using ImageJ (v. 1.51; National Institutes of Health, Bethesda, Maryland, USA). The number of MHCe, MHCn and NCAM positive fibers was then manually counted in a blinded fashion by the same person and expressed relative to the total number of muscle fibers on the NCAM cross-section. A distinct dystrophin staining, in addition to positive staining for MHCe, MHCn or NCAM, was required for inclusion of a fiber. The number of fibers with one or more centrally positioned nuclei was also recorded from the NCAM section and expressed as a percentage of the total number of fibers assessed. To allow as long as possible for the MHCe, MHCn and NCAM proteins to disappear from any potentially reinnervated fibers during the training period, the pre and post (16-week) biopsies were compared as the main statistical outcome relating to the immunofluorescence data. The baseline data from both EX and PLA have been published before, where they were part of a larger group (12).

Real-time reverse transcription qPCR
Real-time reverse transcription qPCR (RT-qPCR) was used to measure Messenger RNA (mRNA) levels of all five muscle AChR subunits (α1, β1, γ, δ and ε) and markers cell proliferation (Ki67), cell senescence (p16) and immature myosins (MHCe and MHCn). The primers are provided in table 1. These mRNA were measured using the same cDNA and real-time PCR procedure as previously described for the baseline samples of these subjects (20). Given that changes in AChR gene expression are likely to occur before the end of the 16-week training period, we included the 8-week time point in the statistical analysis of the gene expression data.

Statistics
T-tests were performed using Excel (Office 365, Microsoft Corporation, Redmond, WA, USA), Pearson's correlation in GraphPad Prism (v. 8.4, GraphPad Software, Inc., La Jolla, CA, USA) and all other statistical tests were performed in SigmaPlot (v. 13.0, Systat Software Inc, San Jose, California, USA). Figures were designed in GraphPad Prism and tables were prepared using Excel (Office 365, Microsoft Corporation). Subject characteristics (SED versus EX) were compared with an unpaired two-tailed t-test. RT-qPCR data were normalized to RPLP0 and log-transformed before statistical analysis and are presented as geometric means ± back-transformed SEM. The Wilcoxon signed rank test was used to compare pre and 16-week post time points for the microscopy data (NCAM, MHCn, central nuclei). The 8-week timepoint is shown in supplemental figure 2. A two-way Repeated Measures ANOVA (time x group) was used to test for changes in mRNA, strength tests, fiber type specific CSA. As the study was originally composed of three groups instead of two,

we also performed a two-way Repeated Measures ANOVA (time x drug) between the exercise groups (LOS-EX and PLA-EX) on the new mRNA targets presented in the present study. In case of significant interactions, Dunnett's post hoc test was performed. Where significant, interactions are included in the figures, and main effects when there is no interaction. Statistical significance was set at $P < 0.05$, and trends ($P < 0.1$) are also reported. Subject characteristics is presented as mean ± SD, and figures show mean or median with individual values.

RESULTS

Subject characteristics

Age, height, weight, BMI and lean body mass (LBM) are shown in table 2. No difference between SED and EX was found for any variable.

Muscle strength and hypertrophy

As reported earlier for this cohort, resistance training induced increases in muscle strength and size (20). We confirmed this was still the case in the new groups studied in the present study (figure 1). Maximal isokinetic voluntary force production in EX increased significantly by 14.6% from 177.6 ± 6.1 Nm to 203.7 ± 7.3 Nm by week eight and remained elevated at 198.3 ± 6.6 Nm by week 16. Similarly, maximal isometric voluntary force production in EX increased significantly by 17% from 195.9 ± 7.9 Nm to 230 ± 9.0 Nm by week eight and remained elevated at 230.3 ± 8.2 Nm by week 16. No changes were seen in SED during the intervention. Type II muscle fiber CSA in EX increased significantly by 11 % from 4292 ± 175 μm^2 to 4771 ± 182 μm^2 by week 16. A tendency for a main effect of group was observed for type I muscle fiber CSA ($p = 0.054$).

Denervation immunofluorescence and microscopy

MHCe positive fibers were generally rare and therefore not presented; 26 out of 38, and 13 out of 20, subjects from EX and SED respectively, did not have any MHCe positive fibers at baseline. Of the 19 subjects that had at least one MHCe positive fiber, 17 had one or two, while one subject had three and another had five positive fibers.

MHCn and NCAM positive fibers were present in almost all samples. No change in the prevalence of muscle fibers positive for MHCn, NCAM, or containing central nuclei, was observed in SED or EX over the 16-week period (figure 2). Examples of each staining are shown in figure 2. White arrows point to included fibers. NCAM positive fibers were generally smaller than NCAM negative fibers. MHCn positive fibers varied in size, with some positive fibers being equivalent to MHCn negative fibers and others severely atrophied. Central nuclei were sometimes but not always associated with NCAM positive fibers and in rare cases several central nuclei were observed within the same fiber cross-section.

RT-qPCR

Significant time x group interactions were found for mRNA levels of AChR α1 and ε, with reduced levels detected at eight weeks versus baseline for EX. A tendency for AChR α1 to be lower in EX versus SED at 16 weeks was also found. A main effect of time was observed for Ki67, and a trend for a main effect of time for AChR β1 and δ (figure 3).

Significant correlations were found between baseline and delta values of type II associated satellite cells and delta values of AChR γ gene expression (figure 4).

DISCUSSION

The main findings of the study were changes in the AChR subunit gene expression profile, reflecting NMJ remodeling, in the skeletal muscle of a large group (n=38) of healthy elderly individuals midway through 16 weeks of heavy resistance exercise training. These adaptations were accompanied by a substantial increase in muscle strength and type II muscle fiber hypertrophy, confirming the effectiveness of the training program. However, no change in the prevalence of denervated muscle fibers was observed, indicating that the NMJ remodeling initiated by the exercise may require a longer timeframe to be evident at the protein level. Alternatively, it is possible that the outcome of the AChR gene expression and immunofluorescence methods employed represent different pools of muscle fibers. Overall, our findings support the inclusion of NMJ remodeling as one of the benefits of heavy resistance exercise in healthy elderly individuals.

A novel finding in the present study is that the α1 and the ε AChR subunits, both adult isoforms (23–26), were significantly downregulated midway through the heavy resistance exercise training program, at 8 weeks. The γ AChR subunit was unaffected, while main effects of time only for β1 and δ complicate interpretation of the outcome of these subunits. The implication of the α1 and the ε AChR subunit downregulation is not entirely clear. We have previously reported that a single bout of heavy resistance exercise, or neuromuscular electrically stimulated eccentric contractions, alters AChR gene expression in the skeletal muscle of healthy young and older males and females, where the AChR α1 and δ subunits appear to be consistently upregulated 5-9 days after exercise (13, 14). Interestingly, we observed a clear trend for downregulation of four out of the five subunits 4½ hours after the exercise bout (13), together indicating a dynamic response of the AChR subunits to exercise in human skeletal muscle. The presence of AChR gene expression in the extra-synaptic region of the muscle fiber indicates muscle fiber denervation (10). Therefore, one potential explanation for our finding is that the decline in gene expression of the α1 and the ε subunits with heavy resistance exercise reflects a strengthening of the NMJ in fibers where a destabilized NMJ led to a rise in extra-synaptic AChR expression levels, potentially representing a mechanism by which resistance exercise can preserve muscle function and size with ageing. It is worth noting in the present study that the α1 and the ε subunits were downregulated at eight weeks, and not at 16 weeks, indicating that the adaptations occur temporally in parallel with the onset of strength gains and hypertrophy, when the muscle tissue is clearly remodelling at a high rate. It is unlikely that the observed changes in gene expression represent acute responses, since both mid and post biopsies were obtained 2 days after the latest training session and no changes were observed at post. Thus, our findings suggest that neural activity, either stabilization of existing NMJs or establishment of new NMJ through reinnervation, is also heightened in the earlier phase of tissue adaptation. It is possible that this can explain the lack of influence of 12 or 13 weeks of resistance training on the AChR gene expression in two earlier studies (14, 18), and that a muscle biopsy collected midway would have reflected the NMJ remodeling detected in the 8-week samples collected in the present study.

Based on the AChR gene expression changes detected at 8 weeks we had expected that this would translate into a reduced prevalence of denervated muscle fibers at 16 weeks. However, we saw no change in the number of MHCn or NCAM positive fibers following the 16-week intervention, suggesting an unchanged number of denervated fibers with resistance training. The prevalence of MHCn and NCAM positive fibers at baseline was similar to previous analyses from our laboratory of different groups of older individuals (13, 18, 27). We have also previously shown that the majority of MHCn and NCAM positive fibers found in the skeletal muscle of elderly individuals are greatly atrophied (13), typically with a CSA below <500 μm^2, and often angularly shaped, both being indices of persistent denervation (28–30). The proportion of denervated fibers in pre and post

(16-week) biopsies was compared to allow as long as possible for the denervation proteins to be removed from the fibers. From animal studies it is known that NCAM is removed from all fibers within 14 days following the onset of reinnervation secondary to nerve crush (9, 31). In humans on the other hand, no data are available on the time course of the removal of NCAM following denervation, aside from the observation that NCAM is present in regenerating fibers 30 days after a bout of electrically induced eccentric contractions (32, 33). A further strength of the direct comparison between the pre and post biopsies is that these biopsies were obtained from the same leg. However, the 8-week biopsy, from the contralateral leg, was also analyzed, and group level data did not deviate from the other time points. Taking the gene expression and staining data together, it appears that changes in gene expression occur before reinnervation can be observed by immunofluorescence at the protein level.

It is possible that the microscopy and AChR gene expression data sets reflect two different pools of muscle fibers, or at least that AChR transcription is more dynamically regulated than the presence of protein markers of denervation such as NCAM and MHCn. While extensive morphological analyses of NMJs from different species (5) and in humans of varying age (34) and health status (35) have been performed, it is also apparent that the NMJ demonstrates remarkable plasticity as it undergoes rhythmic cycles of morphological changes (36, 37). The rapidness of these changes serves to illustrate the adaptability of the NMJ. In light of this, it is possible that the onset of exercise in our untrained participants, with a gradual increase in recruitment of motor units during the first few weeks, led to a stabilization of some NMJs that were close to being lost, and that this is reflected in the AChR transcription changes detected in the muscle biopsies at 8 weeks. In accordance with this idea, structural changes in AChRs have previously been observed following short-term training interventions in ageing mice (6). Furthermore, it must be recognized that other, currently unknown, markers of muscle fiber denervation could provide more sensitive indication of the stage of denervation or reinnervation at the individual fiber level, so we cannot rule out that a reduced prevalence of denervated muscle fibers might have been detected after a longer interval than the 16 weeks in our study.

The heavy resistance exercise performed targeted the type II fibers in particular, evidenced by significant hypertrophy. Type II fibers are those that suffer most with ageing in terms of size (38, 39), possibly due to infrequent recruitment, but are also potentially lost through denervation at a greater rate than type I fibers. Interestingly, we observed positive correlations between the number of type II muscle fiber associated satellite cells at baseline and the change in AChR γ gene expression at both mid (8 weeks) and post (16 weeks). Furthermore, we observed negative correlations between the change in type II muscle fiber associated satellite cells and the change in AChR γ gene expression at both mid and post. While a concrete interpretation of this is difficult, it is interesting that it is only the γ subunit, which is supposedly a fetal subunit (23), and only type II satellite cells, which are lost to a greater extent with ageing (40), that turn out significant.

Taken together, these results demonstrate for the first time that heavy resistance exercise has the capacity to perturb expression of genes related to NMJ remodeling in healthy human skeletal muscle, although no change in the number of denervated muscle fibers was observed. Importantly, these findings are reported in conjunction with favorable adaptations in muscle strength and muscle fiber size, thus possibly representing a positive adaptation in the muscle compartment of the NMJ. It remains to be determined whether longer term training could continue to strengthen weak NMJs and lead to a reduction in the number of denervated fibers, or even protect against denervation if training activity is maintained throughout life.

ABBREVIATIONS

AChR: Acetylcholine Receptor
BMI: Body mass index
CSA: Cross-sectional area
DAPI: 4´,6-diamidino-2-phenylindole.
LBM: Lean body mass
MHCe: Embryonic myosin heavy chain
MHCn: Neonatal myosin heavy chain
mRNA: Messenger RNA
NCAM: Neural Cell Adhesion Molecule
NMJ: Neuromuscular junction
RPLP0: Ribosomal protein lateral stalk subunit P0

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors conceived the project, performed experiments and analysed data. CS and ALM drafted the manuscript and all authors contributed to, and approved, the manuscript.

ACKNOWLEDGEMENTS

Lab technicians Camilla Brink Sørensen and Anja Jokipii-Utzon provided excellent technical assistance with preparation of the muscle biopsies and the mRNA analysis and for that the authors are very thankful.
The monoclonal antibody F1.652 (Developmental MHC), developed by Blau, H.M., was obtained from the Developmental studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.
Funding from Nordea Foundation (Healthy Aging grant), The Danish Agency for Culture (FPK.2018-0036), The Lundbeck Foundation (R344-2020-254), The AP Møller Foundation for the Advancement of Medical Science, and Copenhagen University Hospital - Bispebjerg and Frederiksberg, is gratefully acknowledged.

FIGURE LEGENDS

Table 1. Primers used for real-time RT-qPCR.
RPLP0, ribosomal protein lateral stalk subunit P0; AChR, Acetylcholine Receptor; MHCe, Embryonic myosin heavy chain; MHCn, Neonatal myosin heavy chain; p16, CDKN2A.

Table 2. Subject characteristics.
Subject characteristics for the exercise group and sedentary group, shown as mean ± standard deviation, and range in italics. No difference was found between groups for any variable when tested with an unpaired two-tailed t-test. Abbreviations: BMI: Body mass index; LBM: Lean body mass.

Figure 1. Muscle strength and muscle fiber CSA
Isokinetic and isometric MVC and type I and II muscle fiber CSA measured at pre (week 0), mid (week 8) and post (week 16) for a group of healthy elderly individuals performing 16 weeks of heavy resistance exercise (EX) and a sedentary group (SED). Mean and individual values are

reported. The number of subjects in the MVC data was 36 and 18 for EX and SED respectively, and 32 and 17 for EX and SED respectively in fiber CSA analysis. Significant, or tendencies for, interactions or main effects are provided. * $p<0.05$ from pre. Abbreviations: MVC: Maximal voluntary contraction; CSA: Cross-sectional area.

Figure 2. Immunofluorescence
Immunofluorescence analyses of muscle fibers positive for NCAM (A-B), MHCn (C-D) and central nuclei (E-F) at pre (week 0) and post (week 16) for the exercise (EX) and sedentary (SED) groups. The mid (week 8) timepoint can be found in supplemental figure 2. Values are reported as median (red line) and connected individual values. The number of subjects was 37 EX and 20 SED for all analyses. Images B, D and F show examples of fibers included (white arrows). Scalebar is 100 μm. Abbreviations: MHCn, Neonatal myosin heavy chain; NCAM, Neural Cell Adhesion Molecule.

Figure 3. Gene expression
Gene expression measured by real-time RT-qPCR in muscle biopsies obtained at pre (week 0), mid (week 8) and post (week 16) for the exercise group (EX) and sedentary group (SED). mRNA levels are normalized to RPLP0 and presented relative to baseline as geometric means ± back-transformed SEM on a logarithmic scale (y-axis). The number of subjects was 38 EX and 20 SED. Significant, or tendencies for, interactions or main effects are written within figures. * $p<0.05$ from pre, # $p<0.05$ between groups. Abbreviations: RPLP0, ribosomal protein lateral stalk subunit P0; AChR, Acetylcholine Receptor; NCAM, Neural Cell Adhesion Molecule; MHCe, Embryonic myosin heavy chain; MHCn, Neonatal myosin heavy chain; p16, CDKN2A.

Figure 4. Correlations
Pearson's correlation analysis between number of type II associated satellite cells and AChR γ gene expression measured by real-time RT-qPCR for the exercise group. A) Correlation between type II associated satellite cells at baseline and delta AChR γ gene expression at mid and post. B) Correlation between delta type II associated satellite cells at mid and post and delta AChR γ gene expression at mid and post. The number of subjects was 38. mRNA data were log transformed and then subjected to Pearson's correlation with R^2 and P-values inserted. Abbreviations: RPLP0, ribosomal protein lateral stalk subunit P0; AChR, Acetylcholine Receptor.

Supplemental figure 1
Gene expression measured by real-time RT-qPCR in muscle biopsies obtained at pre (week 0), mid (week 8) and post (week 16) for Losartan+exercise group (LOS-EX), placebo+exercise group (PLA-EX) and Losartan+sedentary group (SED). mRNA levels are normalized to RPLP0 and presented relative to baseline as geometric means ± back-transformed SEM on a logarithmic scale (y-axis). Notice that the y-axis for AChR ε and Ki67 are different from other targets. The number of subjects was 38 EX and 20 SED. Main effects are written within figures. Abbreviations: RPLP0, ribosomal protein lateral stalk subunit P0; AChR, Acetylcholine Receptor; NCAM, Neural Cell Adhesion Molecule; MHCe, Embryonic myosin heavy chain; MHCn, Neonatal myosin heavy chain; p16, CDKN2A

Supplemental figure 2
Immunohistochemical analyses of muscle fibers positive for MHCn, NCAM, and central nuclei at pre (week 0), mid (week 8) and post (week 16) for exercise group (EX) and sedentary group (SED). Values are reported as median (line) and connected individual values. The number of subjects was

37 EX and 20 SED for all analyses. Abbreviations: MHCn, Neonatal myosin heavy chain; NCAM, Neural Cell Adhesion Molecule.

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Table 1

mRNA	Gene name	Genbank	Sense	Antisense
RPLP0	RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
AChR α1	CHRNA1	NM_000079.3	GCAGAGACCATGAAGTCAGACCAGGAG	CCGATGATGCAAACAAGCATGAA
AChR β1	CHRNB1	NM_000747.2	TTCATCCGGAAGCCGCCAAG	CCGCAGATCAGGGGCAGACA
AChR γ	CHRNG	NM_005199.4	GCCTGCAACCTCATTGCCTGT	ACTCGGCCCACCAGGAACCAC
AChR δ	CHRND	NM_000751.2	CAGCTGTGGATGGGGCAAAC	GCCACTCGGTTCCAGCTGTCTT
AChR ε	CHRNE	NM_000080.4	TGGCAGAACTGTTCGCTTATTTTCC	TTGATGGTCTTGCCGTCGTTGT
Ki67	MKI67	NM_002417.4	CGGAAGAGCTGAACAGCAACGA	GCGTCTGGAGCGCAGGGATA
p16	CDKN2A	NM_000077.4	GGGGGCACCAGAGGCAGTAA	TTCTCAGAGCCTCTCTGGTTCTTTCA
MHCe	MYH3	NM_002470.3	CGGATATCGCAGAATCTCAAGTCAA	CTCCAGAAGGGCTGGCTCACTC
MHCn	MYH8	NM_002472.2	CGGAAACATGAGCGACGAGTAAAA	CAGCCTGAGAACATTCTTGCGATCTT

Table 2 Subject characteristics

	Exercise *(n=38)*	Sedentary *(n=20)*
Age (yr)	72 ± 5 *65-83*	72 ± 6 *66-85*
Height (cm)	178 ± 7 *162-191*	179 ± 7 *162-190*
Weight (kg)	85 ± 11 *57-108*	83 ± 11 *62-102*
BMI (kg/m²)	27 ± 3 *19-33*	26 ± 3 *21-32*
LBM (kg)	57 ± 6 *45-71*	56 ± 5 *47-66*

Figure 1

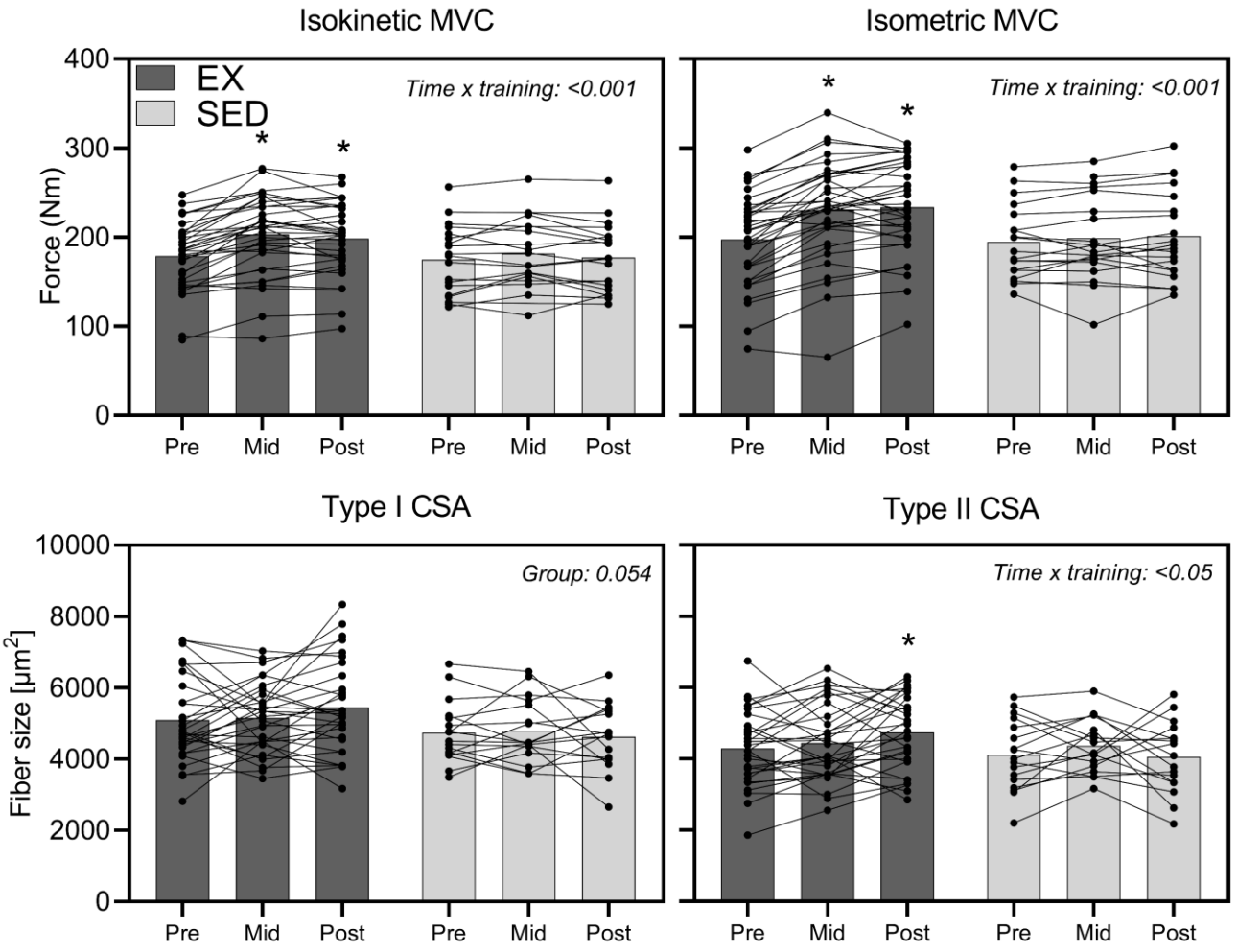

Isokinetic MVC
EX
SED
Time x training: <0.001
Force (Nm)
400
300
200
100
0
Pre
Mid
Post
Isometric MVC
Time x training: <0.001
Type I CSA
Group: 0.054
Fiber size [µm²]
10000
8000
6000
4000
2000
0
Type II CSA
Time x training: <0.05

Figure 2

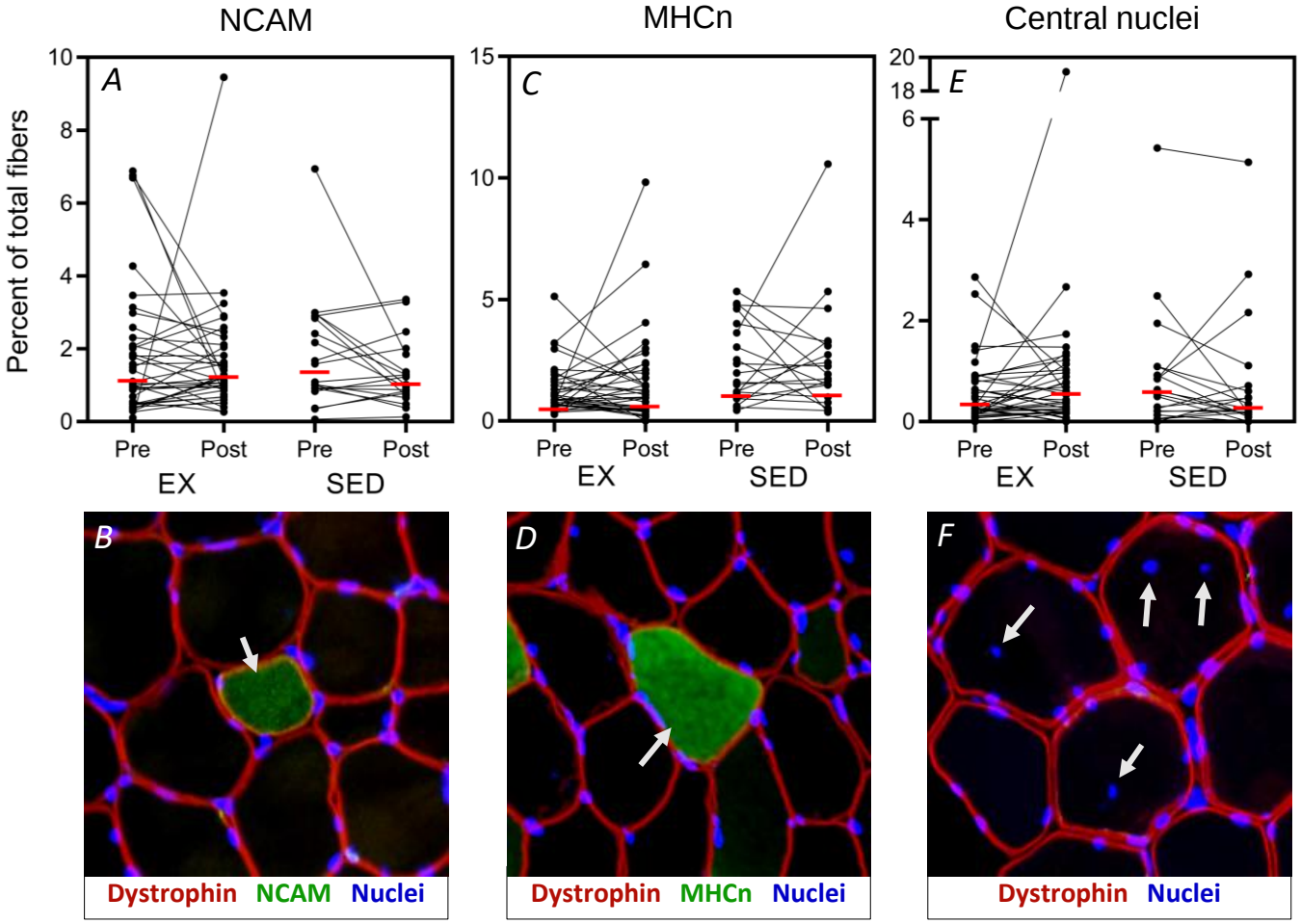

NCAM
MHCn
Central nuclei
A
C
E
Percent of total fibers
Pre
Post
EX
SED
B
D
F
Dystrophin NCAM Nuclei
Dystrophin MHCn Nuclei
Dystrophin Nuclei

Figure 3

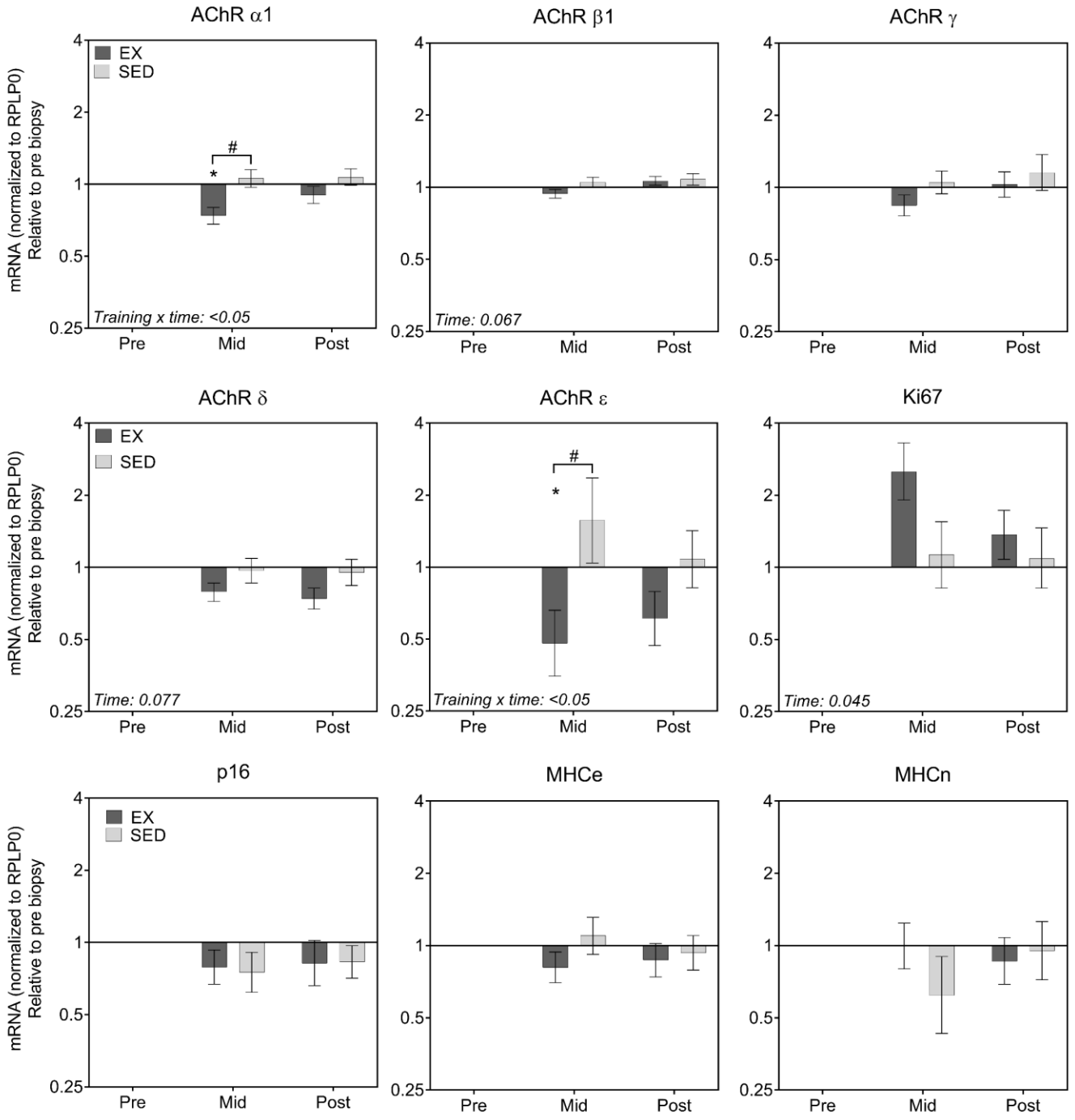
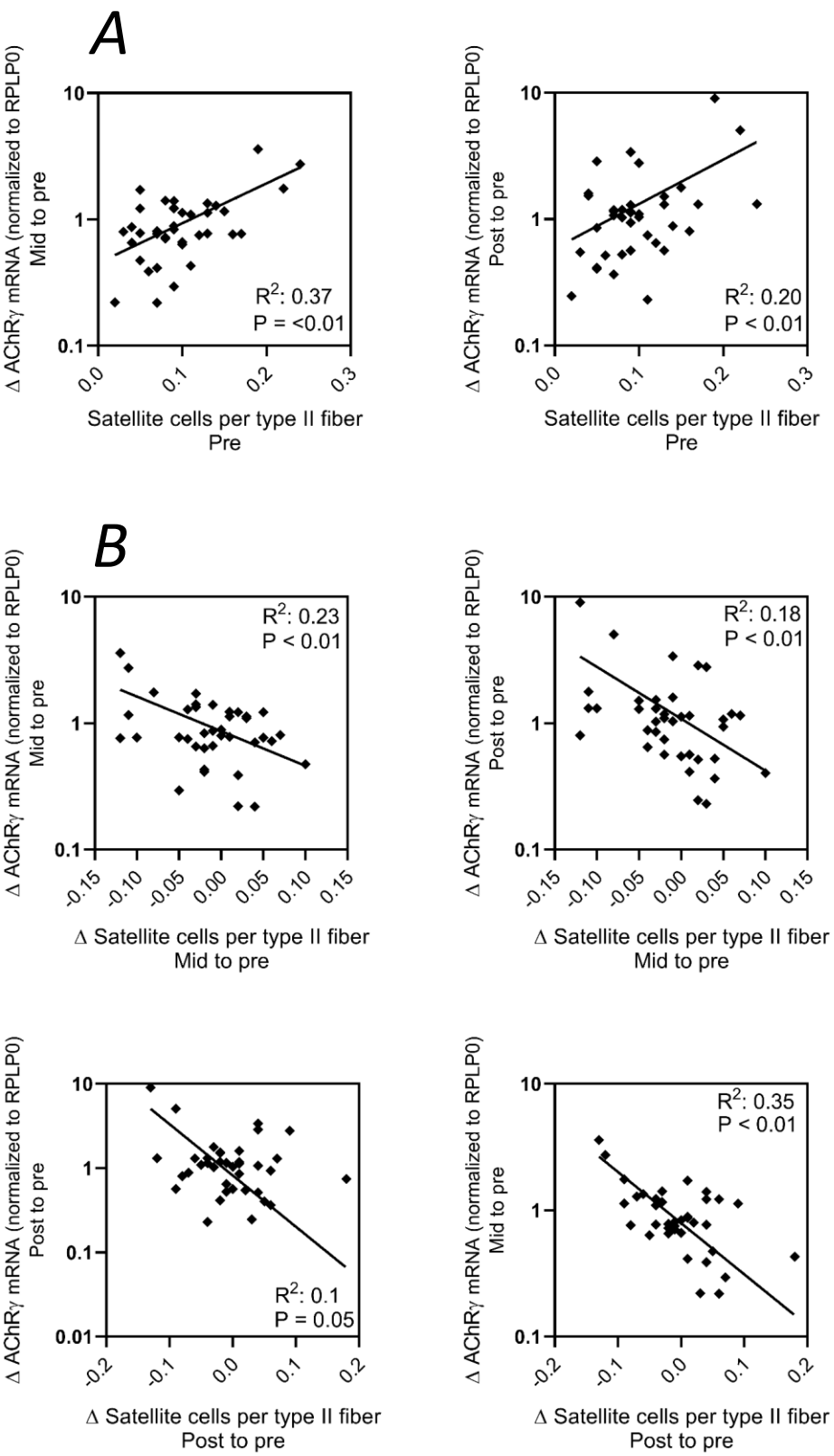
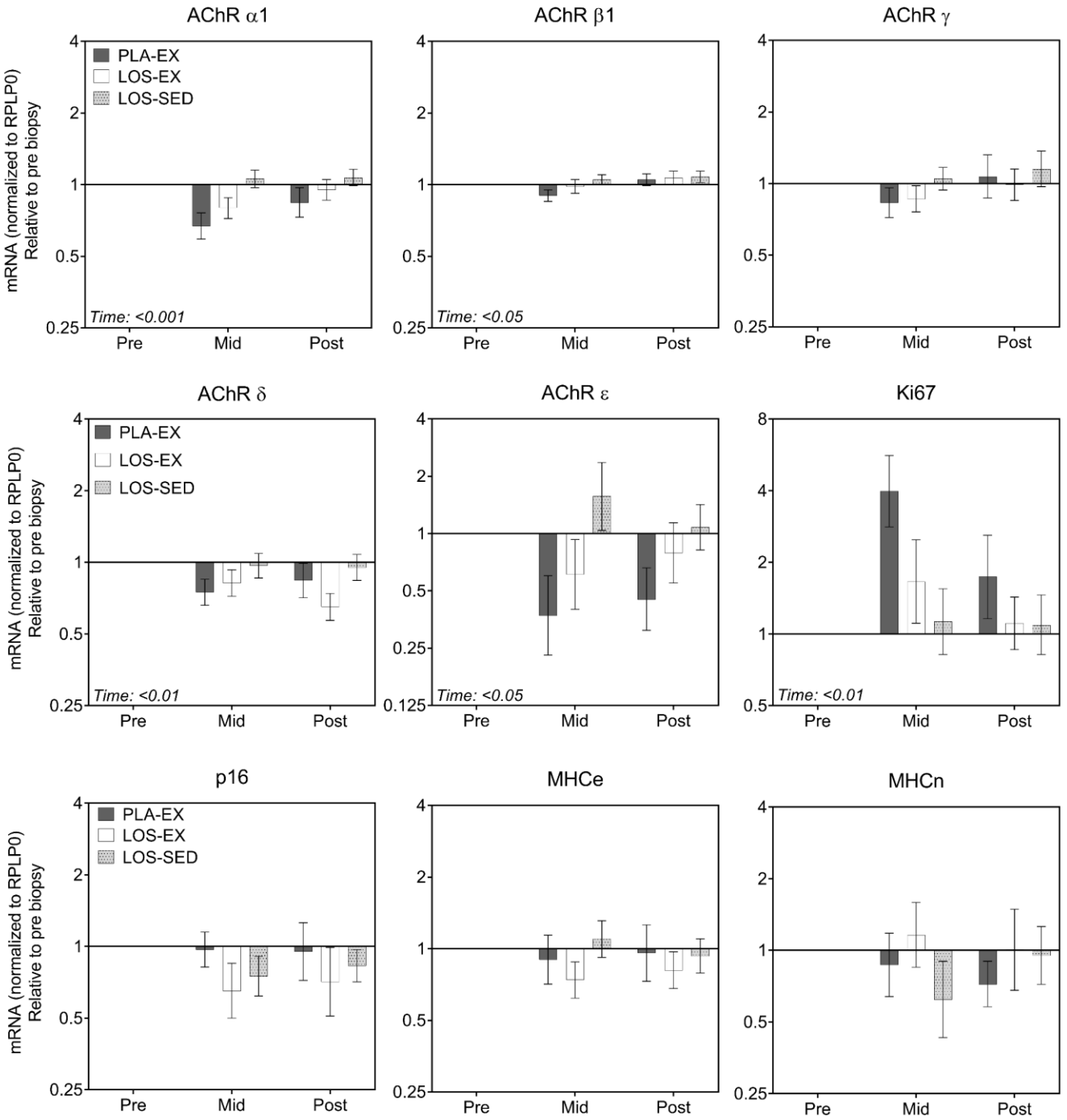

AChR α1
AChR β1
AChR γ
AChR δ
AChR ε
Ki67
p16
MHCe
MHCn
EX
SED
mRNA (normalized to RPLP0)
Relative to pre biopsy
Training x time: <0.05
Time: 0.067
Time: 0.077
Training x time: <0.05
Time: 0.045
Pre
Mid
Post
0.25
0.5
1
2
4
*
#

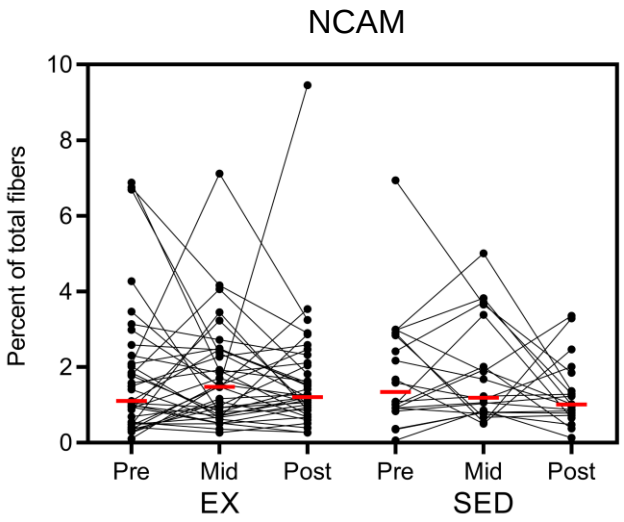
Figure 4

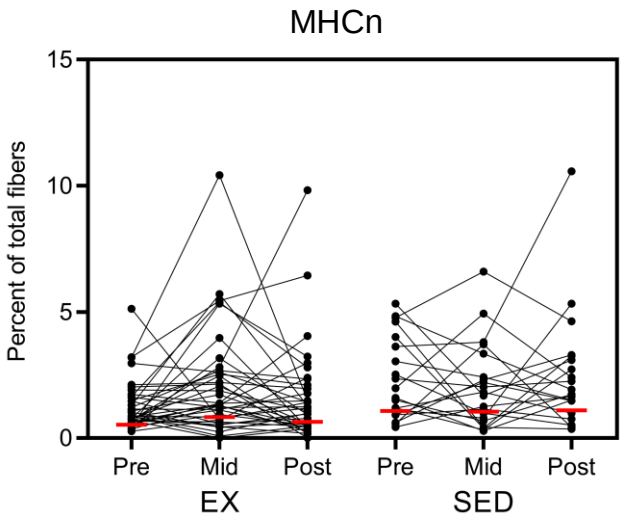

A
Δ AChRγ mRNA (normalized to RPLP0)
Mid to pre
R2: 0.37
P = <0.01
Satellite cells per type II fiber
Pre
Δ AChRγ mRNA (normalized to RPLP0)
Post to pre
R2: 0.20
P < 0.01
Satellite cells per type II fiber
Pre
B
Δ AChRγ mRNA (normalized to RPLP0)
Mid to pre
R2: 0.23
P < 0.01
Δ Satellite cells per type II fiber
Mid to pre
Δ AChRγ mRNA (normalized to RPLP0)
Post to pre
R2: 0.18
P < 0.01
Δ Satellite cells per type II fiber
Mid to pre
Δ AChRγ mRNA (normalized to RPLP0)
Post to pre
R2: 0.1
P = 0.05
Δ Satellite cells per type II fiber
Post to pre
Δ AChRγ mRNA (normalized to RPLP0)
Mid to pre
R2: 0.35
P < 0.01
Δ Satellite cells per type II fiber
Post to pre

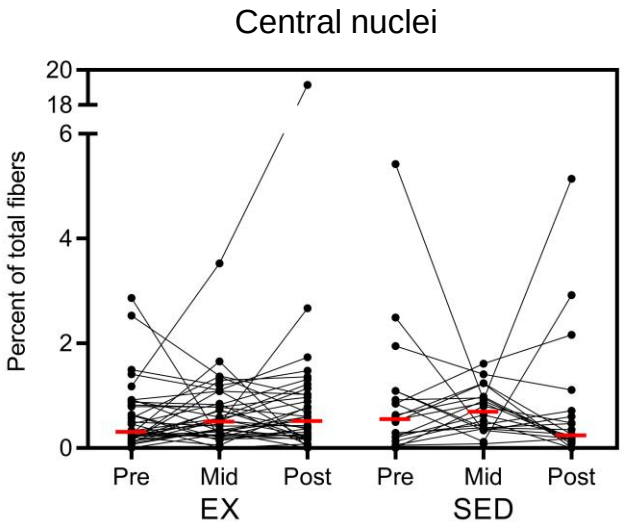
Supplemental figure 1



Supplemental figure 2







Study 4

Preserved stem cell content and innervation profile of elderly human skeletal muscle with lifelong recreational exercise.

Status: *Accepted*

Title: Preserved stem cell content and innervation profile of elderly human skeletal muscle with lifelong recreational exercise

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Keywords: sarcopenia; lifelong exercise; human skeletal muscle; denervation; satellite cells; acetylcholine receptor

Running title: Skeletal muscle of elderly lifelong recreational exercisers

Key points

- The detrimental effects of ageing can be partially offset by lifelong self-organized recreational exercise, as evidence by preserved type II myofibre associated satellite cells, a beneficial muscle innervation status and greater fatigue resistance under challenged conditions
- Satellite cell function (in vitro), muscle fibre size and muscle fibre denervation determined by immunofluorescence were not affected by recreational exercise
- Individuals that are recreationally active are far more abundant that master athletes, which sharply increases the translational perspective of the present study. Future studies should investigate recreational activity in relation to muscle health further, while also including female participants

Abstract

Background: Muscle fibre denervation and declining numbers of muscle stem (satellite) cells are defining characteristics of ageing skeletal muscle. The aim of this study was to investigate the potential for lifelong recreational exercise to offset muscle fibre denervation and compromised satellite cell content and function, both at rest and under challenged conditions.
Methods: 16 elderly lifelong recreational exercises (LLEX) were studied alongside groups of age-matched sedentary (SED) and young subjects. Lean body mass and maximal voluntary contraction were assessed, and a strength training bout was performed. From muscle biopsies, tissue and primary myogenic cell cultures were analysed by immunofluorescence and RT-qPCR to assess myofibre denervation and satellite cell quantity and function.
Results: LLEX demonstrated superior muscle function under challenged conditions. When compared to SED, the muscle of LLEX was found to contain a greater content of satellite cells associated with type II myofibres specifically, along with higher mRNA levels of the beta and gamma acetylcholine receptors (AChR). No difference was observed between LLEX and SED for the proportion of denervated fibres or satellite cell function, as assessed in vitro by myogenic cell differentiation and fusion index assays.

Conclusion: When compared to inactive counterparts, the skeletal muscle of lifelong exercisers is characterised by greater fatigue resistance under challenged conditions in vivo, together with a more youthful tissue satellite cell and AChR profile. Our data suggest a little recreational level exercise goes a long way in protecting against the emergence of classic phenotypic traits associated with the aged muscle.

Introduction

Age-related loss of muscle mass and function is often unnoticeable and negligible during mid-life, but gradually accelerates, causing most individuals entering their 8th decade of life to have a greatly diminished muscle function (Janssen *et al.*, 2000; Kostka, 2005; Suetta *et al.*, 2019). Among the myriad of changes associated with the ageing muscle, myofibre denervation and a decline in the number (Verdijk *et al.*, 2014) and function (Pietrangelo *et al.*, 2009) of muscle stem (satellite) cells are clear features. Myofibre denervation occurs following decay of α-motoneurons in the spinal cord (Campbell *et al.*, 1973; Tomlinson & Irving, 1977; Mittal & Logmani, 1987; Power *et al.*, 2010; Piasecki *et al.*, 2016) or destabilization of neuromuscular junctions (NMJ) (Bütikofer *et al.*, 2011). Loss of myofibre innervation removes the transcriptional specialization normally confined to the small synaptic area, and alters gene expression in the extra-synaptic area of the myofibre (Covault & Sanes, 1985). For example, a strong upregulation of the acetylcholine receptors (AChR), normally confined to the NMJ, is evident along the length of the myofibre upon denervation (Merlie *et al.*, 1984). We (Soendenbroe *et al.*, 2019, 2020; Karlsen *et al.*, 2019, 2020) and others (Gigliotti *et al.*, 2015; Baehr *et al.*, 2016; Kelly *et al.*, 2018; Sonjak *et al.*, 2019; Daou *et al.*, 2020; Skoglund *et al.*, 2020; Monti *et al.*, 2021; Lagerwaard *et al.*, 2021) have availed of this to indirectly investigate myofibre innervation status in human muscle tissue.

Satellite cells are indispensable during embryonic myogenesis and for muscle regeneration during adulthood (Engquist & Zammit, 2021), due to their ability to proliferate, fuse and form myotubes. Given their role as the sole source of myonuclei, satellite cells are also involved in the hypertrophic response to exercise (Murach *et al.*, 2021*a*). Studies using satellite cell depleted mice have shown that some hypertrophy can be achieved without satellite cells, but in order to maximize the response to long-term training, satellite cells are required (Englund *et al.*, 2020) . It is now also clear that satellite cells interact directly with muscle fibres (Murach *et al.*, 2021*b*) and with other cell types located in the microenvironment surrounding the muscle fibre, including fibroblasts (Mackey *et al.*, 2017; Fry *et al.*, 2017) and endothelial cells (Nederveen *et al.*, 2021). Maladaptation of the muscle is evident during persistent overload in the absence of satellite cells, such as increased ECM and fibroblast number, indicating a regulatory role for satellite cells in ameliorating unfavourable remodelling of the muscle environment (Murach *et al.*, 2018). In relation to the NMJ, it has been shown that a subgroup of satellite cells generate and maintain the specialized myonuclei at the NMJ (Liu *et al.*, 2017; Larouche *et al.*, 2021) and that depletion of satellite cells dampens the regeneration of NMJs following nerve damage (Liu *et al.*, 2015). Although not completely depleted, the aged human muscle has been shown to have fewer satellite cells, especially associated with type II fibres (Verdijk *et al.*, 2007, 2014; Karlsen *et al.*, 2019, 2020). Furthermore, a link between denervation and satellite cells has been shown, where satellite cells exit the quiescent state following denervation and mount an attempt at compensatory myogenesis (Borisov *et al.*, 2001). Long-term denervated fibres also possess viable satellite cells with preserved renewal capability (Wong *et al.*, 2021).

A key tool in improving muscle function is increasing levels of physical activity (Pahor *et al.*, 2020). Numerous studies have documented beneficial effects of intense, supervised, and short-term interventions (<1 year) on muscle mass (Gylling *et al.*, 2020), strength (Erskine *et al.*, 2011), and other parameters of health (Nordby *et al.*, 2012). However, while short-term interventions of increased physical activity undoubtedly remain an effective countermeasure against age-related loss of muscle function, the effects of self-organized physical activity are less clear. Most studies on aged exercising and sedentary individuals, focus on aged master athletes, meaning the best functioning individuals within their age group (Harridge &

Lazarus, 2017), which is a highly selected group that constitute a minor proportion of the general population (Ng & Popkin, 2012). Less than <20% of men and women aged ≥60 performed ≥20 minutes of vigorous intensity physical activity on 3 or more days per week (Hallal *et al.*, 2012). In contrast, the group of recreationally active individuals constituted around 60%. From the master athlete studies we know that high levels of physical activity, maintained over many years, preserve muscle mass, strength and power (Klitgaard *et al.*, 1990; Grassi *et al.*, 1991; Mikkelsen *et al.*, 2013; Mosole *et al.*, 2014). Furthermore, electrophysiological (Power *et al.*, 2010) and muscle biopsy (Mosole *et al.*, 2014; Sonjak *et al.*, 2019) studies indicate that exercise influences the neuromuscular system, possibly by facilitating myofibre reinnervation. However, there exists a paucity of knowledge on recreationally active individuals, especially in relation to myofibre morphology, satellite cell numbers and function, and how these relate to indices of muscle denervation.

The potential of exercise to influence the neuromuscular system is substantial, however there are discrepancies in outcomes between experimental and self-organized exercise interventions, as well as limited data on recreationally active individuals compared to master athletes. We therefore designed the present study to investigate muscle morphology, satellite cells and myofibre denervation in two well-matched groups of elderly individuals different only in their physical activity history. We hypothesized that physically active individuals would possess a higher lean body mass and better muscle function compared to sedentary individuals, although an inherent decline in muscle morphology and function due to ageing would still exist (relative to young control group). Furthermore, we hypothesized that positive effects of lifelong recreational physical activity would be evident for indices of myofibre denervation, myofibre size, type II myofibre associated satellite cells, and satellite cell function in cell culture in comparison to a sedentary lifestyle.

Methods

Ethical approval and participants

Experimental procedures were approved by The Committees on Health Research Ethics for The Capital Region of Denmark (Ref: H-19000881) and were conducted according to the standards set by the Declaration of Helsinki, except for registration in a database. Participants signed an informed consent agreement. 223 men responded to either newspaper or online advertisement and were screened by telephone including wide-ranging questions on their physical activity pattern. Exclusion criteria were age between 40-67, obesity (BMI >32 kg/m^2), smoking, >14 alcoholic beverages per week, prior muscle biopsies (vastus lateralis), knee pain, current disease and use of anticoagulant medication.
56 men were included into three groups: young, elderly lifelong exercise (LLEX) and elderly sedentary (SED). 7 individuals did not complete the study: injury not related to study (1), loss of interest (1), knee pain during exercise protocol (1), muscle biopsy only obtained from one leg (3) or no info (1). Subjects in LLEX corresponds to Tier 1 in the participant classification framework by McKay et al. (McKay *et al.*, 2022). These individuals meet the recommendations for physical activity set by the World Health Organization, often through a combination of different activities, and without a specific aim at competing. Three additional LLEX subjects were excluded, as they ultimately proved markedly less trained in comparison with the rest of the group. The final number of participants included was 46 (15 young, 16 LLEX and 15 SED).
Young and SED were healthy and had not performed structured physical activity, such as regular football or resistance exercise, or any physical activity during everyday life (e.g., cycling or walking for transportation) for at least 10 (young) 30 (SED) years prior to enrolment. LLEX had performed multiple sports throughout their adult life. We sought to include participants who had at least partially performed sports which would lead to recruitment of type II myofibres in the lower extremities (high force or high speed). Specific activities reported were as follows (individuals performing each activity; Individuals performing each activity as their primary activity): Strength training (10;3), ball games (5;3), racket sports (5;3), cycling (5;3), rowing (4;1), running (4;1), gymnastics (3;1), athletics (2;1), martial arts (1;0), and swimming (1;0).

Study design

The study was comprised of 3 visits to the research facility, taking place between 08.00 and 13.00 (figure 1.A). The participants were instructed to refrain from physical activity from two days before visit 1 and for the entire course of study, and they were asked to transport themselves to the institute by car or public transportation. On visit 1 and 3 they were instructed to drink a provided protein shake (Bodylab ShakeUp!, 330 mL, 26 g protein, 284 kcal) at home 2 hours before the experiment started instead of their normal breakfast.

Visit 1 consisted of a dual energy x-ray absorptiometry (DEXA) scan, blood sampling, maximal strength testing and a bout of unilateral heavy resistance exercise. Visit 2 consisted of a blood sample. On visit 3, another blood sample was taken, followed by bilateral muscle biopsies.

The leg that was subjected to the exercise bout was block-randomized for dominant/non-dominant, resulting in 8/7 (young), 8/8 (PA) and 6/9 (SED). The SED group ended up being unbalanced, as two participants dropped out after being allocated to a group.

DEXA scan

30 minutes before the scan, the participants drank 0.5 L of water, and they emptied their bladder immediately before lying down in the scanner (Lunar DPX-IQ, GE-Healthcare). The participants were carefully positioned and lay supine for 10 minutes before the scan. Lean body mass (LBM), total bone mineral content, fat percentage and android fat mass were chosen as the outcomes.

Blood samples

Blood samples were obtained from an antecubital vein. General health parameters were analysed on visit 1, and creatine kinase was analysed on all visits, following standard methods at the Department of Clinical Biochemistry.

Maximal voluntary contraction

Participants had their assigned leg tested for maximal voluntary contraction (MVC) in a dynamometer (KinCom, model 500-11; Kinetic Communicator). The protocol was similar to the one used in our previous study (Karlsen *et al.*, 2020), except angular velocity was 30°/s (2.67 seconds per repetition). The isometric test was repeated after the exercise bout.

Acute resistance exercise bout

Participants underwent a bout of unilateral heavy resistance exercise in the KinCom using the same leg as for the MVC. The exercise protocol is illustrated in figure 1.B. Two rounds were performed separated by a 5-10 minute break. Each round consisted of 4 sets of 10 concentric contractions (30°/s) at >70% of MVC. This was followed by 4 sets of 5 eccentric contractions (30°/s) at >100% of MVC. Torque was sampled from the first, middle and last repetition from each set. Verbal encouragement and visual feedback were provided. The participants rested for 1½-2½ minutes between sets.

Muscle biopsy

Muscle biopsies were obtained from the mid portion of the vastus lateralis muscle from both legs. Biopsies were taken under local anaesthetic (1% lidocaine), using the percutaneous needle biopsy technique (Bergstrom, 1975) with manual suction. Care was taken to align the incision sites between the legs. Two biopsies were taken from each leg in immediate succession, through the same incision, with the biopsy needle angled proximally and distally from the incision. Pieces of muscle appropriate for histology were carefully aligned in Tissue-Tek (Sakura Finetek), frozen in liquid nitrogen cooled isopentane (JT Baker), and stored at –80°C. The remaining tissue was immediately processed for cell culture.

Cell culture

The cell culture protocol has previously been described in detail (Agley *et al.*, 2017; Bechshøft *et al.*, 2019). Briefly, tissue was digested using collagenase B (11088815001; Roche) and dispase II (D4693; Sigma-Aldrich) for one hour in a humidified incubator (37°C and 5% CO_2), then filtered through a cell strainer (352340; BD Falcon) and transferred to a cell culture flask (690170/658170; Cellstar) and grown in culture medium (C-23060; PromoCell) until ~80% confluency (mean 6.3 ± 1.4 SD days). The medium was changed after 3 days and old medium was spun down, and unattached cells were returned to the flask. Afterwards, the medium was changed every second day. Cells were detached using diluted Trypsin-EDTA (25200-056; Gibco) and then incubated with MACS running buffer (130-091-221; Miltenyi Biotec) and CD56 magnetic beads (130-050-401; Miltenyi Biotec). Cells were passed through a Pre-Separation filter (130-041-407; Miltenyi Biotec) and a Large Cell column (130-042-202; Miltenyi Biotec) attached to a MultiStand magnet (130-090-312; Miltenyi Biotec), capturing the $CD56^+$ (myogenic) fraction. ~3000 and ~5000 $CD56^+$ cells/cm^2 were plated on glass coverslips (0111580; Marienfeld) in 12-well plates (353503; Corning), for proliferation (PRO) and differentiation (DIF) experiments. Three 12-well plates were used for PRO and DIF each, and cells were plated in duplicate (IHC or RNA) on each plate, providing 3 replicates for each analysis. Control leg and exercised leg for each participant were cultured on the same plates. Cells were cultured for 3 days for PRO and 3+4 days for DIF. After 3 days in CM, PRO cells were exposed to 10 µM of 5-bromo-2-deoxyuridine (BrdU) for 5 hours. For DIF, the cells were also cultured in CM for 3 days, after which the medium was changed to differentiation medium (C-23260; PromoCell). The medium was changed again after 2 days, and the experiment was stopped after further 2 days. At the end of PRO and DIF, the cells were either fixed using Histofix (Histolab) for immunostaining or processed for RNA extraction.

RNA extraction

Coverslips containing cells were moved to an empty well in a new plate. 1 mL of TriReagent (TR118; Molecular Research Inc.) was added and, after pipetting several times, the mixture were moved to a 2 mL BioSpec tube (5225; Bio Spec Products Inc.) and stored in a -80°C freezer. At the end of the experiment, all samples were thawed, and RNA purified with added glycogen as previously described (Bechshøft *et al.*, 2019).

For the tissue samples, 100 sections (10 µm each) from the frozen biopsies were transferred to the 2 mL BioSpec tubes and dissolved in 1 ml TriReagent by shaking with 5 steel beads (2.3 mm, BioSpec) for 15 seconds in a FastPrep homogenizer (MP Biomedicals). The RNA was purified as for the cell culture, except no glycogen was added.

Real-time RT-qPCR

50 ng (cell culture) or 400 ng (tissue) total RNA per sample was converted to cDNA using OmniScript reverse transcriptase (Qiagen) and poly-dT (Qiagen) as previously described (Bechshøft *et al.*, 2019). 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 for every target mRNA (table 1). An MX3005P real-time PCR machine (StrataGene) was used for monitoring the amplification, and a standard curve was made with known concentrations of DNA oligonucleotides (Ultramer oligos, Integrated DNA Technologies) corresponding to the expected PCR product. The Ct values were related to the standard curve. Melting curve analysis after amplification was used to confirm the specificity of the PCR products, and RPLP0 mRNA was originally chosen as the internal control for normalization. To support the use of RPLP0, another unrelated "constitutive" mRNA, GAPDH, was measured (normalized to RPLP0) and showed no change in response to exercise (shown together with the rest of the mRNA). But the basal level is higher in the young group showing that either GAPDH mRNA decrease by age or that RPLP0 mRNA increase by age. As the first would suggests lower metabolic activity in aged muscle and the later more protein synthesis, we find the first more likely and therefore used RPLP0 as normalizer for all the mRNA. The data are expressed relative

to the SED group (control leg) or for the exercised leg relative to the individual control leg (exercise response).

Immunofluorescence

Biopsies of both legs from each participant were sectioned (10 μm) using a cryostat, placed in duplicate on the same glass slide, and stored at –80°C. Four serial sections were used (table 2): Slide 1, dystrophin+MyHCn; Slide 2, dystrophin+myosin I (A4.951); Slide 3, dystrophin+CD56 (NCAM); Slide 4, merosin+phalloidin+desmin (figure 2). Additionally, a fifth consecutive slide from selected samples suspected to contain myotendinous junction (MTJ), were stained for collagen 22 (Koch *et al.*, 2004). Satellite cells were stained with Pax7, laminin and myosin I (BA.D5). The slides for Pax7 staining were fixed using Histofix before incubation with the primary antibodies. All other stainings were fixed after incubation with secondary antibodies. Sections were incubated overnight at 5°C with primary antibodies diluted in blocking buffer consisting of 1% BSA and 0.1% sodium azide in TBS. Then, slides were incubated for 45 minutes at room temperature with secondary antibodies diluted in blocking buffer. Slides were washed in TBS between each step. Sections were finally mounted with cover glasses using Prolong-Gold-Antifade (P36931; Thermo Fisher Scientific) containing 4',6-diamidino-2-phenylindole (DAPI).

The immunofluorescence staining protocol for the cultured cells has been described before (Bechshøft *et al.*, 2019). Briefly, cells were tritonized (9002-93-1; Sigma-Aldrich) for 8 minutes and incubated overnight with primary antibodies (desmin and myogenin for DIF and desmin and BrdU for PRO) diluted in blocking buffer (1% BSA and 0.1% sodium azide in TBS). Cells were incubated for 1 hour at room temperature with secondary antibodies diluted in blocking buffer. Coverslips containing the cells were mounted on glass slides using Prolong-Gold-Antifade containing DAPI.

Microscopy

Tissue biopsy sections were imaged using a 20x/0.50 NA (slide 4) or a 10x/0.30 NA objective and a 0.5x camera (DP71, Olympus) mounted on a BX51 Olympus microscope. Greyscale 4080x3072 or 2040x1513 pixel images were obtained, and sections stained with MyHCn or NCAM were stitched into one seamless image using Fiji (ImageJ, v.1.51).

BrdU staining of the proliferating cells was not strong enough to analyse in a reliable manner so only mRNA data are provided for PRO. Differentiating cells, stained with desmin and myogenin, were imaged with an AxioScan.Z1 slide scanner (Carl Zeiss). A standardized region of interest (ROI), which covered approximately 90% of the coverslip was defined (figure 3.A). Damaged areas (due to handling of the coverslips) or large air bubbles were removed from the ROI before imaging. Images were captured using a plan-apochromat 10x/0.45 NA objective and a MultiBand filter cube (DAPI/FITC/TexasRed) using excitation wavelengths of 353, 493 and 577 nm (LED light source) and both coarse and fine focusing steps. Each channel was imaged separately and sequentially with an AxioCam MR R3 and a 10% overlap between images. Merged images were stitched using ZEN blue software (Carl Zeiss).

Image analyses

The same person, blinded to group and leg, analysed all samples. The number of fibres included in each analysis is provided in table 3.

Myofibre size and type

Myofibre cross-sectional area, type composition and type area percentage, were analysed on composite images (dystrophin/myosin/DAPI) using a semi-automated macro, run in Fiji, as described (Karlsen *et al.*, 2019). Transversally cut myofibres were delineated and classified as type I, type II or hybrid based on median staining intensity. Hybrid fibres were detected in all three groups (0.9 [0-7.8] % in young, 0.5 [0-2.7] % in LLEX and 1.2 [0-5.6] % in SED) and were removed from the analysis. Myofibre type composition was

also manually assessed on the same composite images by counting all visible type I, type II or hybrid myofibres using the ObjectJ plugin in Fiji. Myofibre type composition obtained by manual counting and using the semi-automated macro were strongly correlated (R^2 = 0.971). Fibre type area percentage was determined as a function of fibre type percentage and fibre CSA.

Satellite cells

Satellite cells were manually quantified on composite images (laminin, Pax7, myosin I, DAPI) using the ObjectJ plugin in Fiji. $Pax7^+$ cells, also $DAPI^+$, were classified as satellite cells, and were allocated to type I or II fibres. If the "parent" fibre could not be clearly identified, the respective satellite cell was marked separately, and later shared between fibre types. This occurred for 14 out of a total of 4614 satellite cells counted. Satellite cell number was expressed relative to the number of fibres included in the analysis. Two samples were excluded from type II analysis due to a low number of fibres (SED control leg, n=14 and LLEX exercised leg, n=15).

Denervated fibres

The presence of $MyHCn^+$ and $NCAM^+$ fibres was manually assessed on composite images (dystrophin, NCAM/MyHCn, DAPI) using the ObjectJ plugin in Fiji. The CSA of all $NCAM^+$ fibres was measured, and checked for co-expression of MyHCn and MyHC I. Then, the CSA of all $MyHCn^+$ fibres was measured. Lastly, we removed all $NCAM^+$ or $MyHCn^+$ fibres that were not $merosin^+$ and $desmin^+$, or $merosin^+$ and $phalloidin^+$, as further confirmation that included cells were of myogenic origin. Fibres that had disappeared on a subsequent section or could not be convincingly located were marked separately as "lost".

Cell culture

The stitched images were separated into regions (2.26x1.80 mm, 3510x2790 pixels) equal to 3x3 of the original image tiles of the slide scanner. As it was observed that cells were more densely located toward the centre of the coverslip, only regions within a central rectangular ROI on the coverslip were used. Automated thresholding of the DAPI channel was used to determine the approximate number of nuclei within each region and the region with a nuclei count closest to the median for that coverslip was selected for further analysis. As we had three technical replicates placed on separate plates, we analysed cells of the exercised and control leg that were cultured on the same plate. The next step included a manual correction of any mistakes made by the macro in delineating nuclei, e.g., fusing a single nucleus that had been split or separating several nuclei that were clumped together. Then the corrected nuclei were superimposed on the desmin channel, and nuclei that were located within myotubes with 3 nuclei or more were manually selected. Due to a small amount of bleed-through of desmin signal in the myogenin channel, the myogenin signal in each image was corrected by fitting the myogenin intensity vs. desmin intensity outside of nuclei (containing no true myogenin signal) and subtracting this fit from the intensity of the entire myogenin image. To improve homogeneity between samples with differing staining intensity, a contrast enhancement was performed on the desmin and myogenin channels. Data lists containing intensities in all channels for each nucleus were exported from Fiji and a custom MATLAB script (MATLAB R2019a, The MathWorks Inc.) was used for aggregating the data and determining $desmin^+$ and $myogenin^+$ cells by a threshold in the intensity of the respective channels within each nucleus. Area covered by myogenic cells (area of $desmin^+$ signal) was automatically measured. Fusion index was determined as the ratio of fused nuclei to $desmin^+$ nuclei, and differentiation index was determined as the ratio of $myogenin^+$ nuclei to $desmin^+$ nuclei. Samples with a cell purity, determined as percentage $desmin^+$ cells, below 90% were removed from all data sets. 12/92 samples (5/7 control/exercised leg and 1/7/4 young/SED/LLEX) were removed (figure 3.B).

Statistical analyses

Data are presented as mean ± SD or individual values with median unless stated otherwise in figure legend. A significance level of $P < 0.05$ was chosen, with tendencies ($P < 0.1$) provided. Figures and tables were designed using Prism (v.8, GraphPad Software) and Excel 2016 (Microsoft), respectively. SigmaPlot (v. 13.0,

Systat Software) were used for statistical analyses. LLEX and SED were directly compared, and young was compared to the old groups combined. Within-group differences between rested and exercised leg was also compared. Cell culture data and NCAM/MyHCn analyses were not normally distributed, so non-parametric statistics were used (Mann-Whitney Rank Sum Test and Wilcoxon Signed Rank Test). All remaining data appeared normally distributed (mRNA data after log-transformation), prompting the use of unpaired and paired t-tests. Isometric strength tests performed before and after the exercise bout and log-transformed creatine kinase values were evaluated with one-way repeated measures ANOVA (Tukey post hoc) for each group. Data from the exercise bout were averaged into rounds and analysed using a two-way ANOVA (group x round) with the Holm-Sidak post hoc analysis.

Results

Participant characteristics and heavy resistance exercise

LLEX and SED did not differ in age, height, weight or BMI (p=0.679, 0.482, 0.124 and 0.277, table 4). Young had lower levels of CRP and HbA1c compared with old (p=0.052 and 0.001, table 4). Young were stronger and had a higher LBM than old (p<0.0001 and p=0.053), while LLEX had a lower fat percentage than SED (p=0.006, figure 4 and table 4). Relative strength tended to be higher in LLEX compared to SED (p=0.087, figure 4.B).

Force produced, expressed relative to MVC, was lower in round 2 compared to round 1 in all groups, and LLEX produced force at a higher relative level across all sampled repetitions than both young and SED (figure 5.A). There was a decline in MVC immediately following the exercise bout, and creatine kinase increased at day 2 in all groups (p<0.0001, figure 5.B-C).

Myofibre size and denervation

LLEX had a larger proportion of type I fibres compared to SED (p=0.033), while there was a tendency for young to have a lower proportion of type I fibres than old (p=0.060, figure 6.B). Fibres that were only weakly stained with MyHC I (hybrid fibres) were detected in low numbers in all three groups (0.9 [0 - 7.8] % in young, 0.5 [0 - 2.7] % in LLEX and 1.2 [0 - 5.6] % in SED). Given that hybrid fibres are common in aged muscle, and are composed of two or three distinctive MyHCs (Andersen *et al.*, 1999), we removed these fibres from our analysis as our myosin I staining provided insufficient insight into the myosin composition. Fibre type area followed a similar pattern to fibre type distribution. Young had larger type II fibres than old (p<0.0001), while their type I fibres tended to be larger (p=0.072, figure 6.C). Both old groups had smaller type II fibres compared to their own type I fibres (LLEX, p=0.003, SED, p=0.015). Myofibre morphology is illustrated in histograms, where the type II fibres of the old participants have shifted leftwards (figure 6.A).

The percentage of $NCAM^+$ and $MyHCn^+$ fibres was larger in old compared to young (p=0.003 and 0.034), while no difference was observed between LLEX and SED (p=0.984 and 0.352, figure 7.A-B). $NCAM^+$ fibres were classified as pure type I or II myofibres, or hybrids, with almost even numbers of type I and II. Furthermore, between 10-30 % of $NCAM^+$ fibres co-expressed MyHCn (figure 7.D). A large part of the $NCAM^+$ and $MyHCn^+$ fibres were <500 μm^2 (figure 7.C). We observed an area in a sample that was reminiscent of MTJ, similar to what we have previously described (Soendenbroe *et al.*, 2020). Control stainings with COL22 revealed that 14 out of 37 $NCAM^+$ fibres from that biopsy were related to the MTJ and were removed. A median (range) of 4.5 ± 4.6 and 1.8 (0-9) ± 2.1 fibres initially included in the NCAM and MyHCn counts respectively, were removed following assessment for merosin, desmin and phalloidin (26 and 28% reduction in $NCAM^+$ and $MyHCn^+$ fibres, respectively). It was predominantly the very small myofibres that could not be detected on serial sections.

Satellite cells and cell culture

In the control leg, LLEX had a greater number of type II myofibre associated satellite cells than SED ($p=0.016$), while no difference was observed for type I fibres ($p=0.609$, figure 8.A). Young had more satellite cells associated with both type I and II fibres, compared to old ($p=0.035$ and $p<0.0001$, figure 8.A). LLEX and SED had less type II associated satellite cells than type I ($p<0.0001$ and $p=0.006$, figure 8.A). No difference in differentiation index was observed ($p=0.695$), while a tendency for a higher fusion index in young compared to old was found ($p=0.091$, figure 9.A). Young had a higher cell count compared to old ($p=0.002$), and a tendency for an increased desmin area in young compared to old was also observed ($p=0.081$, figure 9.A) We observed no effect of acute exercise on satellite cell number, differentiation index or fusion index, cell count or desmin area (p values ranged from 0.094 to 0.922, figure 8.B and 9.B).

Gene expression

At the tissue level AChR δ, α1 (tendency) MuSK and MyHCn mRNA was lower in young compared to old ($p=0.047$, 0.086, 0.014 and $p<0.0001$, figure 10.A). AChR β1 and γ was higher in LLEX compared to SED ($p=0.022$ and 0.026, figure 10.A). MyHCe gene expression was upregulated in the exercised leg of LLEX ($p=0.035$), and AChR α1 and MuSK tended to be expressed higher in the exercised leg of LLEX and young, respectively ($p=0.098$ and 0.074, figure 10.A).

In proliferating myoblasts COL1a1 and p16 were lower, and myogenin, MyHCn and MyHCe higher in young compared to old ($p=0.047$, 0.016, 0.001, 0.018 and 0.013, figure 10.B). Myogenin tended to be lower in LLEX compared to SED ($p=0.078$, figure 10.B. In differentiating myotubes, p16 was lower, and AChR γ, MyHCn and MyHCe were higher in young compared to old ($p=0.0001$, 0.002, 0.024 and 0.035, figure 10.C). No differences between LLEX and SED were observed (figure 10.C). Similarly, no effects of acute exercise were in proliferating or differentiating cells for either group (figure 10.B-C).

Discussion

Skeletal muscle of lifelong recreationally active elderly individuals retains a higher number of type II fibre associated satellite cells, possesses a beneficial innervation status when assessed by RT-qPCR, and performs substantially better during acute resistance exercise, compared to sedentary individuals. These findings indicate that lifelong recreational activity can partially offset the emergence of classic phenotypic traits associated with the aged muscle.

In vivo measure of muscle function

The acute exercise bout caused a pronounced decline in force output both within sets and between sets for all groups. Strikingly, LLEX outperformed both SED and the young group, confirming their status as exercise habituated individuals. Despite this, no differences were observed in LBM and MVC between the old groups, indicating that these standard assessments may not allow for detecting subtle differences. Studies investigating the impact of a recreationally active lifestyle on LBM and MVC are inconclusive. When heavy resistance exercise is performed, or the participants are at the pinnacle of sporting performance within their age group in a strength or explosive type of event, then both muscle mass and function will have increased accordingly (Klitgaard *et al.*, 1990; Ojanen *et al.*, 2007; Unhjem *et al.*, 2016; Sonjak *et al.*, 2019). On the other hand, several studies investigating recreationally active individuals have seen limited effects on LBM and MVC (Klitgaard *et al.*, 1990; Lanza *et al.*, 2008; Unhjem *et al.*, 2016; St-Jean-Pelletier *et al.*, 2017), suggesting that these measures are unable to discriminate between recreationally active and sedentary individuals of similar age. Accordingly, it is only under challenged conditions that functional differences become apparent between recreationally active and inactive elderly individuals. In support of this notion, a recent study found no correlation between daily steps and in vivo measurements of muscle function, except during challenged conditions in elderly men and women (Varesco *et al.*, 2022).

Satellite cell quantity and function

One of the main novel findings of the present study is the difference in type II myofibre associated satellite cells between the physically active and inactive elderly men. Satellite cells are the sole source of new myonuclei and are important not only for long-term muscle growth by facilitating accretion of myonuclei (Kadi *et al.*, 2004; Fry *et al.*, 2014) but also for inter-cell communication (Murach *et al.*, 2021*b*) and NMJ maintenance (Liu *et al.*, 2017). Satellite cell quantity is reduced with ageing (Karlsen *et al.*, 2020), disease (Verdijk *et al.*, 2012) and inactivity (Arentson-Lantz *et al.*, 2016) and increased with acute (Heisterberg *et al.*, 2018) and long-term (Kadi *et al.*, 2004) exercise and during muscle regeneration (Karlsen *et al.*, 2020). Type II myofibre associated satellite cells are more severely affected by ageing than type I (Verdijk *et al.*, 2014; Karlsen *et al.*, 2019, 2020), but this decline could also be attributed to a reduced type II myofibre activation with ageing. The larger type II fibre satellite cell pool in LLEX thus provides a larger capacity to mount a myogenic response in the event of injury or denervation (Shefer *et al.*, 2006), while simultaneously secreting signals taken up by muscle fibres and single-nucleated cells in or around the satellite cell niche (Murach *et al.*, 2021*b*). Surprisingly, the exercise bout did not lead to an increase in satellite cell content, which might be related to the exclusive use of slow contractions, timing of biopsy sampling or insufficient stimulus (Hyldahl & Hubal, 2014; Snijders *et al.*, 2015). To explore the function of the satellite cells, we performed cell culture studies and compared the capacity of satellite cells to differentiate and fuse, in addition to measuring mRNA levels of genes related to myogenesis and muscle innervation. Importantly, cultivated myogenic satellite cells have been shown to retain intrinsic capabilities reminiscent of their former in vivo environment (Teng & Huang, 2019). Contrary to our hypothesis, the two primary measures of cell function, differentiation and fusion index, were similar in LLEX and SED, while only a tendency for an age-related difference for fusion index was observed, which might be explained by a higher cell number. Satellite cell proliferation could not be assessed due to problems relating to the staining protocol, so we cannot rule out potential differences between groups in myoblast proliferation. The literature on whether ageing affects satellite cell function in culture is mixed, as some studies indicate phenotypic differences (Bechshøft *et al.*, 2019; Balan *et al.*, 2020) while others do not (Alsharidah *et al.*, 2013; Chaillou *et al.*, 2020). For example, we recently showed that the fusion capabilities were reduced in old compared to young subjects (Bechshøft *et al.*, 2019), while Chaillou et al., 2020 found no difference in fusion index or myotube diameter between young and old (Chaillou *et al.*, 2020). The cause of these discrepancies between studies is unclear but may at least partly be due to differences in the employed cell culture models (cell lines or primary cells) or the immunofluorescence and image analyses. As such, a strength of the present study is that entire coverslips were imaged, which allowed for analysing areas with the most representative cell presence, and that technical replicates were used. In line with our earlier studies, several age-related differences in gene expression of proliferating and differentiating satellite cells were observed (AChR γ subunit, myogenin, COL1A1, MyHCn, MyHCe and p16) (Bechshøft *et al.*, 2019; Soendenbroe *et al.*, 2020). No significant differences were observed between LLEX and SED. Overall, the satellite cell data are supportive of age-related differences in both satellite cell quantity in vivo, measured by immunofluorescence microscopy, and function in vitro, as evidenced by differences in gene expression of several genes related to myogenesis and muscle innervation. However, neither differentiation nor fusion index, the primary measures of cell function, were affected by age, although the influence on proliferation remains to be determined. Lifelong recreational exercise affected satellite cell numbers positively, while no change in satellite cell function was observed. Next, we wanted to know if these differences amounted into differences in muscle innervation status and myofibre morphology.

Muscle innervation status

Innervation status was assessed by immunofluorescence microscopy and RT-qPCR analyses. Both methods were used as they might represent myofibres at different stages of denervation or differ in how they are regulated. NCAM and MyHCn were used as IHC markers for denervated myofibre, as we (Soendenbroe *et al.*, 2019, 2020) and others (Mosole *et al.*, 2014; Sonjak *et al.*, 2019; Daou *et al.*, 2020; Monti *et al.*, 2021; Burke *et al.*, 2021) have previously done. It should be noted that NCAM and MyHCn are also associated

with other physiological processes and structures within muscle, which can challenge the interpretation. NCAM is found at the NMJ and MTJ (Moore & Walsh, 1985; Jakobsen *et al.*, 2018), during muscle regeneration (Irintchev *et al.*, 1994; Mackey & Kjaer, 2017) and in neuromuscular disease (Walsh & Moore, 1985). MyHCn is found during muscle regeneration (Sartore *et al.*, 1982; Mackey & Kjaer, 2017), in neuromuscular disease (Fitzsimons & Hoh, 1981) and in intrafusal fibres (Walro & Kucera, 1999). However, in healthy vastus lateralis muscle tissue, MTJ and NMJ structures are easily recognized, intrafusal fibres are rare, and muscle regeneration is unlikely to be present. Furthermore, experimentally-induced muscle denervation leads to a large upregulation in the expression of NCAM and MyHCn (Covault & Sanes, 1985; Schiaffino *et al.*, 1988), together making muscle fibre denervation the most likely explanation for the observation of $NCAM^+$ and $MyHCn^+$ fibres in our study. In accordance with our prior findings (Soendenbroe *et al.*, 2020), old subjects had a higher number of $NCAM^+$ and $MyHCn^+$ fibres compared to young. However, in contrast to our hypothesis, we did not see indications of favourable innervation status in LLEX group using our immunofluorescent approach. Importantly, several novel findings relating to exercise status were observed in the gene expression data. LLEX had significantly higher mRNA levels of both AChR β1 and γ subunits compared to SED, and young had lower AChR δ and α1 (tendency) compared to old. AChR gene expression have been reported to be affected by disease (Kapchinsky *et al.*, 2018; Kelly *et al.*, 2018), injury (Gigliotti *et al.*, 2015; Karlsen *et al.*, 2020), ageing (Spendiff *et al.*, 2016; Soendenbroe *et al.*, 2020), inactivity (Monti *et al.*, 2021) and acute exercise (Soendenbroe *et al.*, 2020). Given the remarkable similarity of the AChR gene expression profile between LLEX and the young group, it could be speculated that the young group might have been habitually more active than SED, which would push them in the direction of LLEX. Activity levels are well known to change with ageing (Hallal *et al.*, 2012). As previously mentioned, very few human studies examine human AChRs, and this is the first study to report data for all muscle specific AChR subunits in life long recreationally active elderly men. Overall, it appears that the analysis of AChR gene expression is more sensitive than the currently available immunofluorescent markers of denervation. But the use of immunofluorescent markers in the present study has added important details on the morphology of the denervated fibres. Most denervated fibres are very small, often with a CSA of less than a tenth of the mean normal fibre size of the elderly groups. Furthermore, the rigorous assessment required the presence of several myogenic markers such as a dystrophin (sarcolemma), merosin (basal lamina), MyHC, and desmin, as well as containing general cell actin. Approximately similar proportions of the denervated fibres in LLEX and SED are type I, II and hybrid fibres. Interestingly, the overlap between the used markers (NCAM and MyHCn) was limited, which might be due to temporal variation in protein expression of denervated fibres, or that subgroups of denervated fibres exist. Also, MyHCn positive fibres have a segmented staining profile, which due to the cross-sectional approach could also explain at least a portion of the discrepancy (Schiaffino *et al.*, 1988; Soendenbroe *et al.*, 2019, 2021).

Lastly, myofibre morphology was comprehensively studied as it ties closely with both innervation status and satellite cell numbers. We found, as expected, that the young group had larger type II and type I (tendency) fibres than the old groups combined, which has been shown before (Klitgaard *et al.*, 1990; Zampieri *et al.*, 2015; St-Jean-Pelletier *et al.*, 2017; Sonjak *et al.*, 2019; Karlsen *et al.*, 2019). In contrast to our hypothesis however, no difference in fibre size was observed between LLEX and SED. The reason for the lack of difference in average fibre size is unclear, but it is possible that the activities performed by the individuals of the LLEX group did not possess a large enough hypertrophic stimulus for the fibres to increase in size. In general, heavy loading has been shown to be crucial for type II myofibre hypertrophy (Klitgaard *et al.*, 1990), and endurance exercise has limited effect on type II fibre CSA (McKendry *et al.*, 2020). 10 of the subjects in the present study reported performing resistance exercise, although some of these only did it once a week, some only during the off season of their primary activity and some with light loads. Only 3 subjects reported resistance exercise as their primary activity. Since muscle loading, volume and training frequency are all major determinants of hypertrophy, it is likely that the activities performed has not forced an adaptation in myofibre size. It is also noteworthy that while the amount and type of activity performed by LLEX did not appear to preserve type II myofibre size, it was associated with a preservation of the

number of type II myofibre associated satellite cells, suggesting that these two entities are not tightly regulated in healthy elderly muscle. The study of master athletes remains a suitable model to study ageing disentangled from physical inactivity (Harridge & Lazarus, 2017). However, the number of individuals performing exercise at a level where they can be considered master athletes is low (Hallal *et al.*, 2012), which coincidingly makes the study of recreationally active individuals more relevant. All individuals in the present study were independent and well-functioning, meaning that a decline in muscle function would be expected in years ahead. It has been shown that the muscle of very old individuals remain amendable to improvement (Kryger & Andersen, 2007), indicating that although few differences between the groups were observed, the recreationally active individuals might be on a different trajectory, which could benefit them later in life when phenotypic traits of the aged muscle are more pronounced.

Conclusion

Recreational physical activity preserves type II myofibre associated satellite cells during ageing, and leads to a more beneficial muscle innervation status. These data strongly suggest that detrimental effects of ageing can be partially offset by lifelong self-organized recreational exercise. Furthermore, this is the first attempt in humans to investigate satellite cells and myofibre denervation in parallel, and how they are each influenced by exercise. The study is limited by the lack of objective measures of levels of physical activity and the inclusion of only male participants. In our earlier study on young and elderly females, similar findings on myofiber denervation were reported (Soendenbroe *et al.*, 2020). Clearly, studies of lifelong exercise in females are needed. The translational perspective of the present study is heightened due to the focus on recreationally active individuals rather than master athletes, as the former constitute a far larger part of the general population aged 60 and above.

Abbreviation

AChR: Acetylcholine receptor
BrdU: Bromodeoxyuridine
BSA: Bovine serum albumin
COL1a1: Collagen type I alpha 1 chain
CSA: Cross-sectional area
DAPI: 4',6-diamidino-2-phenylindole
DEXA: Dual energy x-ray absorptiometry
MyHC: Myosin heavy chain
MyHC I: Myosin heavy chain 1
MyHCe: Embryonic myosin heavy chain
MyHCn: Neonatal myosin heavy chain
mRNA: Messenger ribonucleic acid
MTJ: Myotendinous junction
MVC: Maximal voluntary contraction
NCAM: Neural cell adhesion molecule
NMJ: Neuromuscular junction
PBS: Phosphate-buffered saline
RT-qPCR: Reverse transcription quantitative polymerase chain reaction
TBS: Tris-buffered saline

Data availability statement

mRNA data from tissue and cells can be found in online supplemental material. Additional original data can be provided, in an anonymized manner, to interested parties. Contact C.S. and A.L.M. and describe the specific data that is needed and the intended use of the data.

Author contributions

A.L.M. and C.S. contributed to the first hypothesis generation and A.L.M. provided resources. C.S., P.S., M.K., J.L.A., and A.L.M. contributed to conceptual design and A.L.M. and J.L.A. supervised this work. C.S., M.T., R.B.S., P.S., J.L.A. and A.L.M. developed the methodology. C.S., C.M. and M.T. performed the experiments and C.S., C.L.D., R.B.S. and P.S. performed data analysis and visualization. C.S., R.B.S., P.S., M.K., J.L.A., and A.L.M. performed analysis and data interpretation and C.S. and A.L.M. wrote the manuscript. All authors edited and reviewed the manuscript.

Acknowledgments

We acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen, where the AxioScan.Z1 slide scanner images were obtained. The Department of Clinical Biochemistry, Bispebjerg Frederiksberg Hospital, University of Copenhagen, Copenhagen, Denmark is acknowledged for analysing the blood samples.

The monoclonal antibodies A4.951 (myosin heavy chain, human slow fibres), BA-D5 (myosin heavy chain, human slow fibres), PAX7 and F5D (myogenin), developed by Blau, H.M., Schiaffino, S., Kawakami, A., and Wright, W.E., respectively, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH, and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. The Collagen 22 antibody was kindly provided by Manuel Koch.

The authors thank Anja Jokipii-Utzon and Ann-Christina Ronnié Reimann for excellent technical assistance with preparation of the muscle biopsies and the mRNA analysis.

Conflicts of interests

The authors declare that they have no competing interests

Funding

We gratefully acknowledge grants from The Lundbeck Foundation (R344-2020-254), the Nordea Foundation (Center for Healthy Aging), the Danish Agency for Culture (FPK.2018-0036), the AP Møller Foundation for the Advancement of Medical Science and Copenhagen University Hospital - Bispebjerg and Frederiksberg.

Figure legends

Abstract figure

Lifelong exercisers were studied alongside age-matched sedentary individuals and young subjects. Muscle biopsies were obtained from all subjects and used for immunofluorescent analyses and cell culture experiments. *In vivo* measurements of muscle mass and function were also performed. Lifelong exercise was associated with a preserved number of type II myofibre associated satellite cells, an improved innervation status that was similar to the young control group, and better muscle function under challenged conditions. The findings suggests that even low amounts of physical activity over many years have a positive impact on muscle health and innervation status. Figure was created using BioRender. Publication licence has been obtained.

Table 1 Primers used for PCR

RPLP0: Ribosomal Protein Large P0; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; AChR: acetylcholine receptor; MuSK: muscle-specific-kinase; MyHCn: neonatal myosin; MyHCe: embryonic myosin heavy chain

Table 2 Primary and secondary antibodies used for immunofluorescence microscopy

Host, antibody name, company, category number and dilution are provided. * Antibody provided by Manuel Koch. MyHCn: neonatal myosin heavy chain

Table 3 Number of fibres included in each image analysis
Values are given as average with standard deviations and ranges . Abbreviations: CSA, cross section area

Table 4 Participant characteristics
Values are given as averages with standard deviations and ranges. Data were analysed using unpaired t-tests. Specific p values are provided in the table. Abbreviations: LBM, lean body mass; BMC, bone mineral content; BMI, body mass index, CRP, C-reactive protein; DEXA, dual energy x-ray absorptiometry; RFD, rate of force development

Figure 1 Study design and exercise protocol
A) 3 visits spread over 7 days, with timing of exercise, blood samples and biopsies indicated.
B) Unilateral bout of heavy resistance exercise performed on visit 1. 2 rounds, separated by a 5-10 minute break, each consisting of 4 sets of concentric and 4 eccentric isokinetic contractions. The 1^{st}, 5^{th}, and 10^{th} concentric repetitions and the 1^{st}, 3^{rd}, and 5^{th} eccentric repetition from each set was sampled. MVCs was performed before and immediately after the exercise bout and after a 5 min break. Abbreviations: DEXA, dual energy x-ray absorptiometry; MVC, maximal voluntary contraction

Figure 2 Cross-sectional profiles of denervated fibres
Split channel view of 4 serial sections from two vastus lateralis biopsies obtained from healthy elderly individuals. Sections have been stained with dystrophin + MyHCn (slide 1), dystrophin + MyHC I (slide 2), dystrophin + NCAM (slide 3) and Merosin + Desmin (slide 4). Notice in A the denervated fibre is positive for NCAM and more strongly positive for desmin than neighbouring fibres. Notice in B the NCAM signal in the upper right corner (arrows) which is not a myofibre (based on lack of staining for dystrophin, desmin, and merosin). Asterisk in the merged image indicates the same denervated fibre on serial sections. Scalebars are 100 µm

Figure 3 Cell culture image analysis
A) An example of an overview widefield microscope image, a stitched image covering entire coverslips with the rectangular ROI, a separation of regions each equal to 3x3 of the original image tiles of the slide scanner and a chosen region used for analyses. B) Cell purity data from both control and exercise leg determined semi-automatically from DAPI and desmin stain. A threshold was set at 90 % purity; samples below were excluded from all analyses. C) Correlation between total cell count and total RNA for each sample (n = 90).

Figure 4 Muscle mass and strength
A) Lean body mass, B) relative strength and C) rate of force development is provided for each group as averages with standard deviations. n = 15 (young), 16 (LLEX) and 15/14 (SED). Data were analysed using unpaired t-tests. * $p<0.05$ versus Young. Tendencies are written

Figure 5 Acute bout of heavy resistance exercise
A) The 1^{st}, 5^{th}, and 10^{th} concentric repetitions and the 1^{st}, 3^{rd}, and 5^{th} eccentric repetition from each set was sampled during the exercise bout. Maximum torque values are expressed relative to concentric isokinetic MVC and is shown as averages with standard deviations. n = 15 (young), 16 (LLEX) and 15 (SED). Set 1-4 (round 1) and 5-8 (round 2) average values were statistically evaluated using two-way ANOVA (group x round) with the Holm-Sidak post hoc analysis. # $p < 0.05$ versus SED, * $p < 0.05$ versus young, $ $p < 0.05$ round 2 versus round 1. B) Isometric MVC, shown as individual values, before, immediately after the exercise bout and following a 5 min rest period. n = 15 (young), 16 (LLEX) and 9 (SED). Data were analysed using one-way RM ANOVA with the Tukey post hoc analysis* $p < 0.05$ versus Before. C) Creatine kinase was

694 measured on day 0, 2 and 6 and is shown as geometric mean with 95% CI. n = 15 (young), 15 (LLEX) and 14
695 (SED). Data were analysed using one-way RM ANOVA with the Tukey post hoc analysis. * $p < 0.05$ versus
696 before/day 0. Abbreviations: MVC, maximal voluntary contraction
697
698 **Figure 6** Muscle morphology
699 A) Type I (open circle, dotted line) and II (filled circle, stippled line) fibre size distribution shown as averages
700 with standard deviations. B) Fibre type distribution and fibre type area shown as averages with standard
701 deviations. C) Fibre size of type I and II shown as connected individual values and averages. n = 15 (young),
702 16 (LLEX) and 15 (SED). * Significantly different from type I within group, # = Significantly different from old,
703 (#) = tendency for a difference from old, $ = Significantly different from SED, ($) = tendency for a difference
704 from SED
705
706 **Figure 7** Muscle innervation
707 A) Percentage of fibres expressing NCAM shown as individual values and median. n = 15 (young), 16 (LLEX)
708 and 15 (SED). Data were analysed using a Mann-Whitney Rank Sum test. * Significantly different from
709 young. B) Percentage of fibres expressing MyHCn shown as individual values and median. C) Fibre size given
710 in μm^2 of all $NCAM^+$ and $MyHCn^+$ positive fibres for each group (y-axis is logarithmic). D) Coexpression of
711 NCAM fibres. Left shows the percentage of $NCAM^+$ fibres than are type I, II, hybrid or not found (lost). Right
712 shows the percentage of $NCAM^+$ fibres that are $MyHCn^+$, $MyHCn^-$ or not found (lost). Number in each bar is
713 the absolute number of fibres within that category.
714
715 **Figure 8** Satellite cell quantity
716 A) Satellite cells per fibre in control leg given as individual values for both type I and II fibres (connected by
717 dashed line) with average value (horizontal line). Data were analysed using unpaired t-tests. B) Exercise
718 response in type I and II satellite cells shown as individual values and averages (horizontal line). Data were
719 analysed using paired t-tests. n = 15, 16 and 15 for young, LLEX and SED. * = Significantly different from
720 type I within group, # = Significantly different from old, $ = Significantly different from SED. Tendencies are
721 written. C) Example of type I (black arrow) and type II (white arrow) myofibre associated satellite cells in
722 young (left), LLEX (middle) and SED (right). Scalebar is 100 µm.
723
724 **Figure 9** Satellite cell function
725 A) Differentiation index, fusion index, cell count and desmin area of human myogenic cells cultured for 7
726 days shown as individual values with median line (n = 15, 14 and 12 for young, LLEX and SED). Data were
727 analysed using Mann-Whitney Rank Sum test. B) Exercise response in differentiation index, fusion index,
728 cell count and desmin area shown as individual values with median line (n = 14, 13 and 9 for young, LLEX
729 and SED. Data were analysed using Wilcoxon Signed Rank test. * Significantly different from old. Tendencies
730 are written. C) Representative example of cell culture; arrows and arrowheads points to fused and non-
731 fused nuclei, respectively.
732
733 **Figure 10** Gene expression in biopsies and cells
734 (A) Gene expression in muscle biopsies (n = 15, 16 and 15 for young, LLEX and SED). (B) Proliferating
735 myoblasts (n = 14/13, 15/14 and 12/8 for control/exercise leg of young, LLEX and SED). (C) Differentiating
736 myotubes (n = 15/14, 14/13 and 14/11 for control/exercise leg of young, LLEX and SED). Control (left) and
737 exercised (right) leg. mRNA data were normalized to RPLP0 and are shown as geometric means with 95 %
738 confidence intervals. Control leg is shown relative to SED control leg and exercise response is shown
739 relative own control leg. Baseline differences were analysed using unpaired t-tests, and exercise responses
740 were analysed using paired t-tests. * $p<0.05$ young versus old. (*) $p<0.1$ young versus old. # $p<0.05$ LLEX
741 versus SED. (#) $p<0.1$ LLEX versus SED. $ $p<0.05$ exercised versus control leg. ($) $p<0.1$ exercised versus
742 control leg.
743

Tables

Table 1

mRNA	Gene name	Genbank	Sense	Antisense
RPLP0	RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
AchRα1	CHRNA1	NM_000079.3	GCAGAGACCATGAAGTCAGACCAGGAG	CCGATGATGCAAACAAGCATGAA
AchRβ1	CHRNB1	NM_000747.2	TTCATCCGGAAGCCGCCAAG	CCGCAGATCAGGGGCAGACA
AchRδ	CHRND	NM_000751.2	CAGCTGTGGATGGGGCAAAC	GCCACTCGGTTCCAGCTGTCTT
AchRε	CHRNE	NM_000080.4	TGGCAGAACTGTTCGCTTATTTTCC	TTGATGGTCTTGCCGTCGTTGT
AchRγ	CHRNG	NM_005199.4	GCCTGCAACCTCATTGCCTGT	ACTCGGCCCACCAGGAACCAC
MUSK	MUSK	NM_005592.3	TCATGGCAGAATTTGACAACCCTAAC	GGCTTCCCGACAGCACACAC
MyHCe	MYH3	NM_002470.3	CGGATATCGCAGAATCTCAAGTCAA	CTCCAGAAGGGCTGGCTCACTC
MyHCn	MYH8	NM_002472.2	CGGAAACATGAGCGACGAGTAAAA	CAGCCTGAGAACATTCTTGCGATCTT
COL1A1	COL1A1	NM_000088.3	GGCAACAGCCGCTTCACCTAC	GCGGGAGGTCTTGGTGGTTTT
Myogenin	MYOG	NM_002479.5	CTGCAGTCCAGAGTGGGGCAGT	CTGTAGGGTCAGCCGTGAGCAG
p16	CDKN2A	NM_000077.4	GGGGGCACCAGAGGCAGTAA	TTCTCAGAGCCTCTCTGGTTCTTTCA

Table 2

Primary antibody				
Host	Antibody	Company	Cat. no.	Concentration
Rabbit	Laminin	Dako	Z0097	1:500
Rabbit	Desmin, IgG	Abcam	AB32362	1:500-1:1000
Mouse	Dystrophin, IgG2b	Sigma-Aldrich	D8168	1:500
Mouse	Myosin 1, IgG1	DSHB	A4.951	1:200
Mouse	Pax 7, IgG1	DSHB	PAX7	1:100
Mouse	Myosin 1, IgG2b	DSHB	BA.D5	1:100
Mouse	Merosin Laminin α2	Leica	MEROSIN-CE	1:200
Mouse	MyHCn, IgG1	Novocastra	NCL-MHCn	1:100
Mouse	CD56 (NCAM), IgG1	Becton Dickinson	347740	1:50
Mouse	Myogenin, IgG1	DSHB	F5D-s	1:50
Guinea pig	Collagen 22	*	KG36	1:5000
	Phalloidin 680	Invitrogen	A22286	1:40
Secondary antibody				
Host	Antibody	Company	Cat. no.	Concentration
Goat	Anti-Mouse 488, IgG	Invitrogen	A-11029	1:500
Goat	Anti-Mouse 568, IgG	Invitrogen	A-11031	1:200
Goat	Anti-Rabbit 488, IgG	Invitrogen	A-11034	1:200
Goat	Anti-Rabbit 568, IgG	Invitrogen	A-11036	1:500
Goat	Anti-Mouse 488, IgG1	Invitrogen	A-21121	1:500
Goat	Anti-Mouse 568, IgG2b	Invitrogen	A-21144	1:200

Table 3

	Young		LLEX		SED	
Myofibre CSA						
Type I	227 ± 147	*39 - 604*	253 ± 118	*79 - 485*	248 ± 110	*124 - 456*
Type II	222 ± 71	*72 - 314*	157 ± 89	*73 - 380*	261 ± 127	*69 - 539*
Myofibre type composition						
Type I	436 ± 272	*303 - 1108*	549 ± 253	*170 - 1091*	533 ± 219	*303 - 1108*
Type II	451 ± 173	*169 - 721*	324 ± 172	*61 - 743*	588 ± 275	*233 - 1009*
Satellite cells						
Type I, control leg	245 ± 128	*125 - 625*	366 ± 185	*129 - 837*	338 ± 124	*122 - 587*
Type I, exercised leg	253 ± 131	*110 - 621*	391 ± 142	*131 - 636*	331 ± 173	*97 - 587*
Type II, control leg	276 ± 94	*110 - 504*	224 ± 94	*80 - 387*	400 ± 176	*159 - 763*
Type II, exercised leg	291 ± 108	*145 - 521*	270 ± 107	*101 - 442*	364 ± 189	*76 - 712*
Denervated fibres						

control leg	978 ± 369 *423 - 1513*	894 ± 340 *361 - 1751*	1099 ± 378 *402 - 1688*

Table 4

	Young vs old	LLEX vs SED	Young n=15	LLEX n=16	SED n=15
Anthropometric					
Age (yr)	*<0.0001*	*0.679*	26 ± 5 *20 - 36*	73 ± 4 *68 - 82*	73 ± 4 *68 - 82*
Height (cm)	*0.016*	*0.482*	183 ± 7 *169 - 193*	176 ± 6 *166 - 185*	178 ± 8 *161 - 195*
Weight (kg)	*0.365*	*0.124*	82 ± 13 *62 - 105*	76 ± 9 *63 - 94*	82 ± 11 *65 - 109*
BMI (kg/m^2)	*0.507*	*0.277*	24 ± 3 *20 - 30*	24 ± 3 *21 - 31*	26 ± 3 *22 - 32*
Blood sample					
CRP (mg/L)	*0.052*	*0.525*	1.3 ± 0.8 *1.0 - 4.0*	2.4 ± 2.1 *1.0 - 9.0*	3.0 ± 3.2 *1.0 - 13.0*
HbA1c (mmol/L)	*0.001*	*0.989*	5.5 ± 0.4 *4.9 - 6.2*	5.9 ± 0.3 *5.5 - 6.8*	5.9 ± 0.5 *5.0 - 6.5*
DEXA					
Leg LBM (kg)	*0.004*	*0.455*	22.7 ± 2.9 *18.3 - 27.4*	20.6 ± 2.1 *17.6 - 24.6*	20.0 ± 2.4 *16.7 - 25.8*
Total BMC (kg)	*0.792*	*0.881*	3.1 ± 0.5 2.4 - 4.0	3.1 ± 0.3 2.6 - 3.8	3.1 ± 0.4 2.4 - 3.9
Fat percentage	*0.356*	*0.006*	24.7 ± 6.6 *10.0 - 33.0*	23.6 ± 6.4 *12.2 - 33.6*	29.9 ± 5.4 *15.0 - 38.0*
Android fat mass (kg)	*0.151*	*0.016*	1.7 ± 0.8 0.2 - 3.5	1.7 ± 1.0 0.5 - 3.5	2.6 ± 0.9 0.8 - 4.3
KinCom					
RFD30ms (Nm/s)	*<0.0001*	*0.702*	2145 ± 921 *742 - 3841*	924 ± 572 *269 - 1990*	851 ± 432 *346 - 2094*
RFD200ms (Nm/s)	*<0.0001*	*0.729*	1232 ± 372 *688 - 1836*	784 ± 204 *409 - 1062*	759 ± 175 *555 - 1272*

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Figure 1

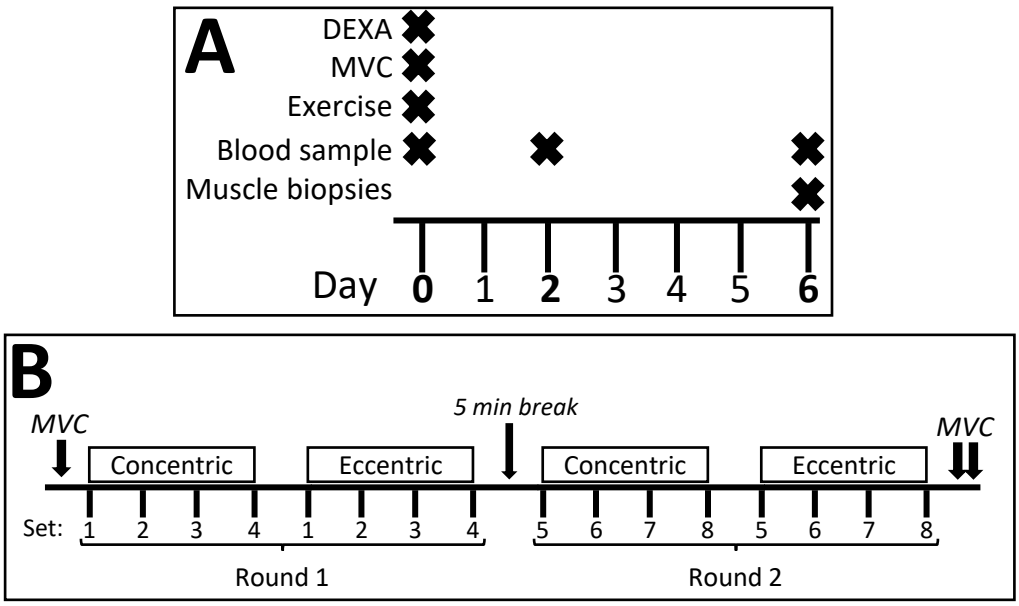

A
DEXA
MVC
Exercise
Blood sample
Muscle biopsies
Day 0 1 2 3 4 5 6
B
MVC
5 min break
MVC
Concentric
Eccentric
Concentric
Eccentric
Set: 1 2 3 4 1 2 3 4 5 6 7 8 5 6 7 8
Round 1
Round 2

Figure 2

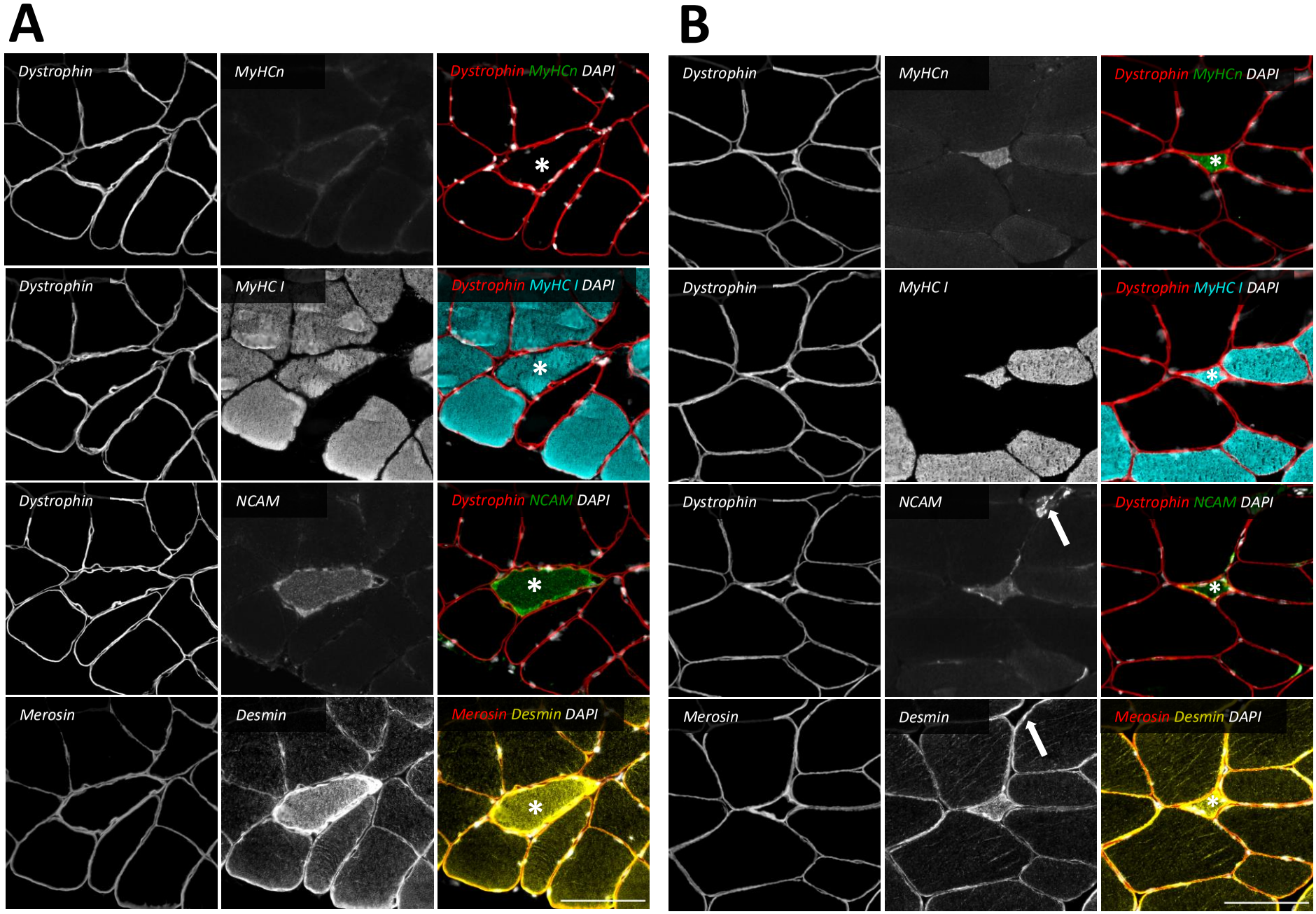
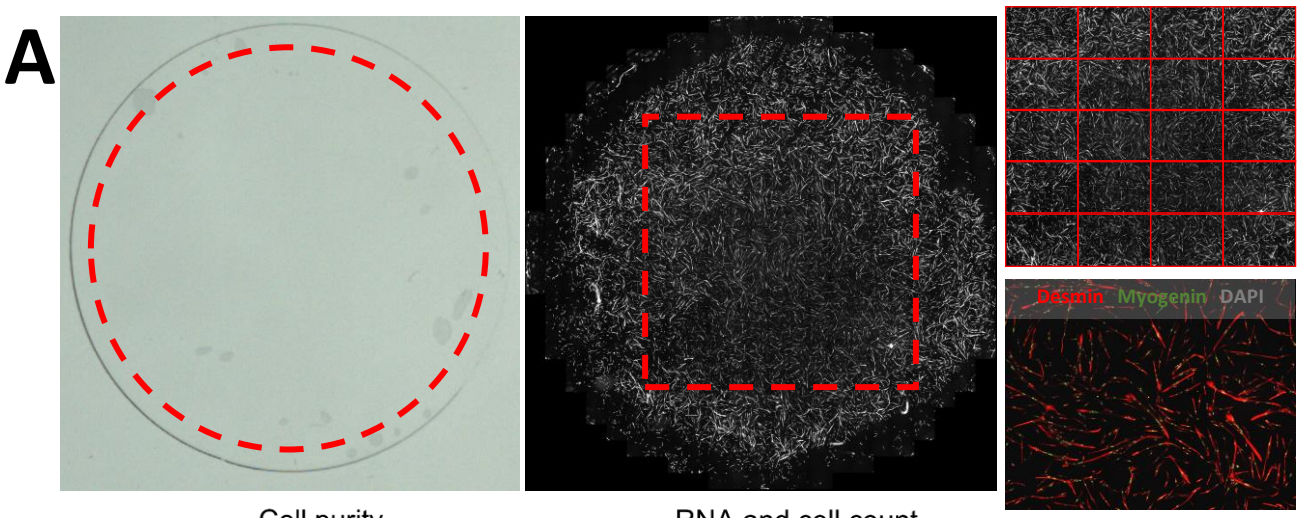
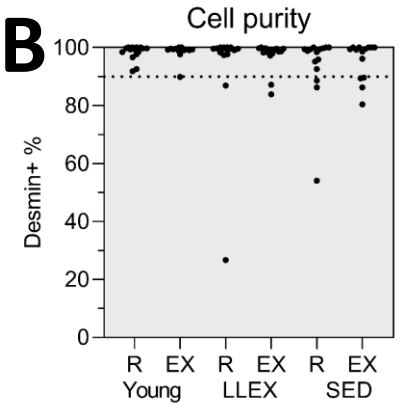

A
B
(Slide 1)
+20 µm (Slide 2)
+40 µm (Slide 3)
+60 µm (Slide 4)
Dystrophin
MyHCn
Dystrophin MyHCn DAPI
MyHC I
Dystrophin MyHC I DAPI
NCAM
Dystrophin NCAM DAPI
Merosin
Desmin
Merosin Desmin DAPI

Figure 3


A
Desmin Myogenin DAPI


B
Cell purity
Desmin+ %
100
80
60
40
20
0
R EX R EX R EX
Young LLEX SED

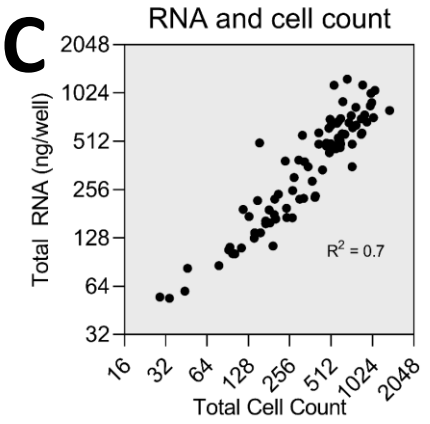

C
RNA and cell count
Total RNA (ng/well)
2048
1024
512
256
128
64
32
R² = 0.7
16 32 64 128 256 512 1024 2048
Total Cell Count

Figure 4

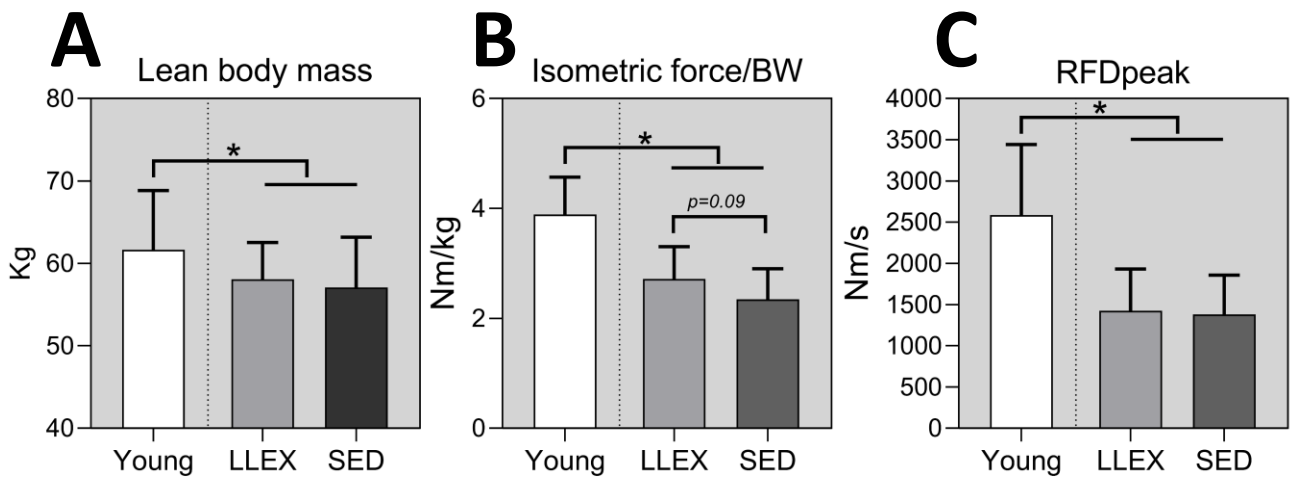
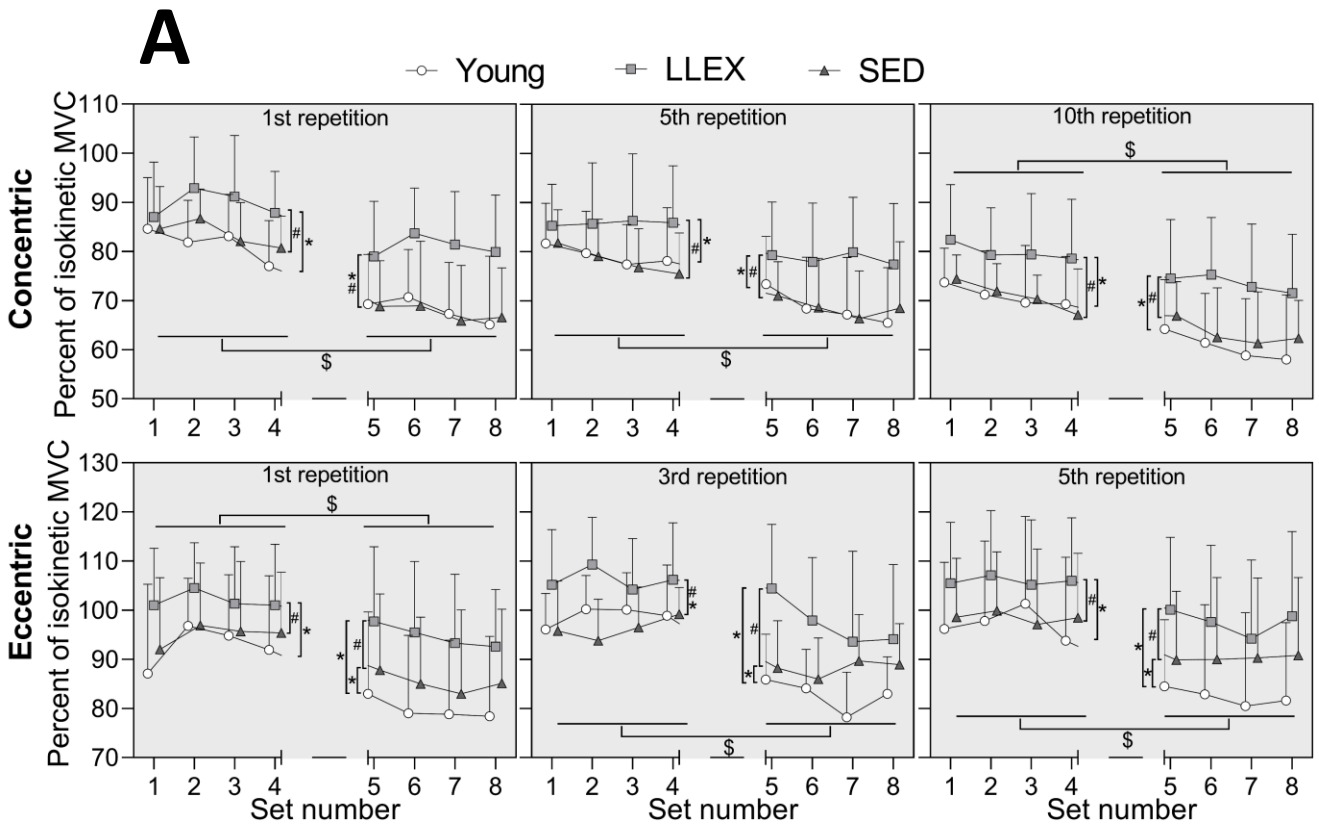
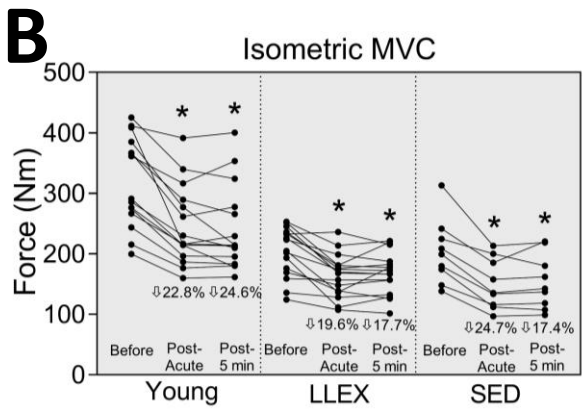

A
Lean body mass
Kg
80
70
60
50
40
*
Young
LLEX
SED
B
Isometric force/BW
Nm/kg
6
4
2
0
*
p=0.09
Young
LLEX
SED
C
RFDpeak
Nm/s
4000
3500
3000
2500
2000
1500
1000
500
0
*
Young
LLEX
SED

Figure 5


A
Young
LLEX
SED
Concentric
Eccentric
Percent of isokinetic MVC
1st repetition
5th repetition
10th repetition
3rd repetition
Set number


B
Isometric MVC
Force (Nm)
⇩22.8% ⇩24.6%
⇩19.6% ⇩17.7%
⇩24.7% ⇩17.4%
Before
Post-Acute
Post-5 min
Young
LLEX
SED

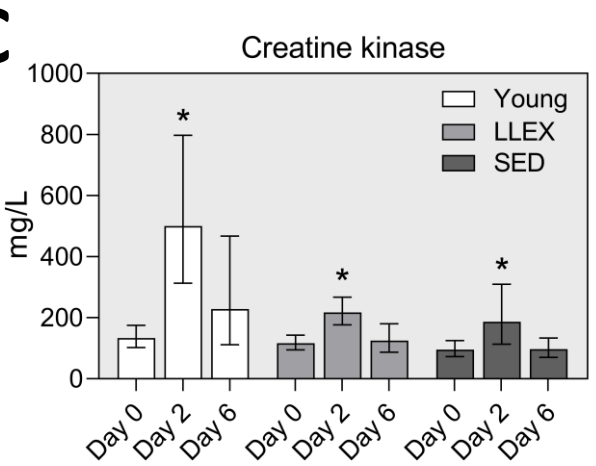

C
Creatine kinase
mg/L
Young
LLEX
SED
Day 0
Day 2
Day 6

Figure 6

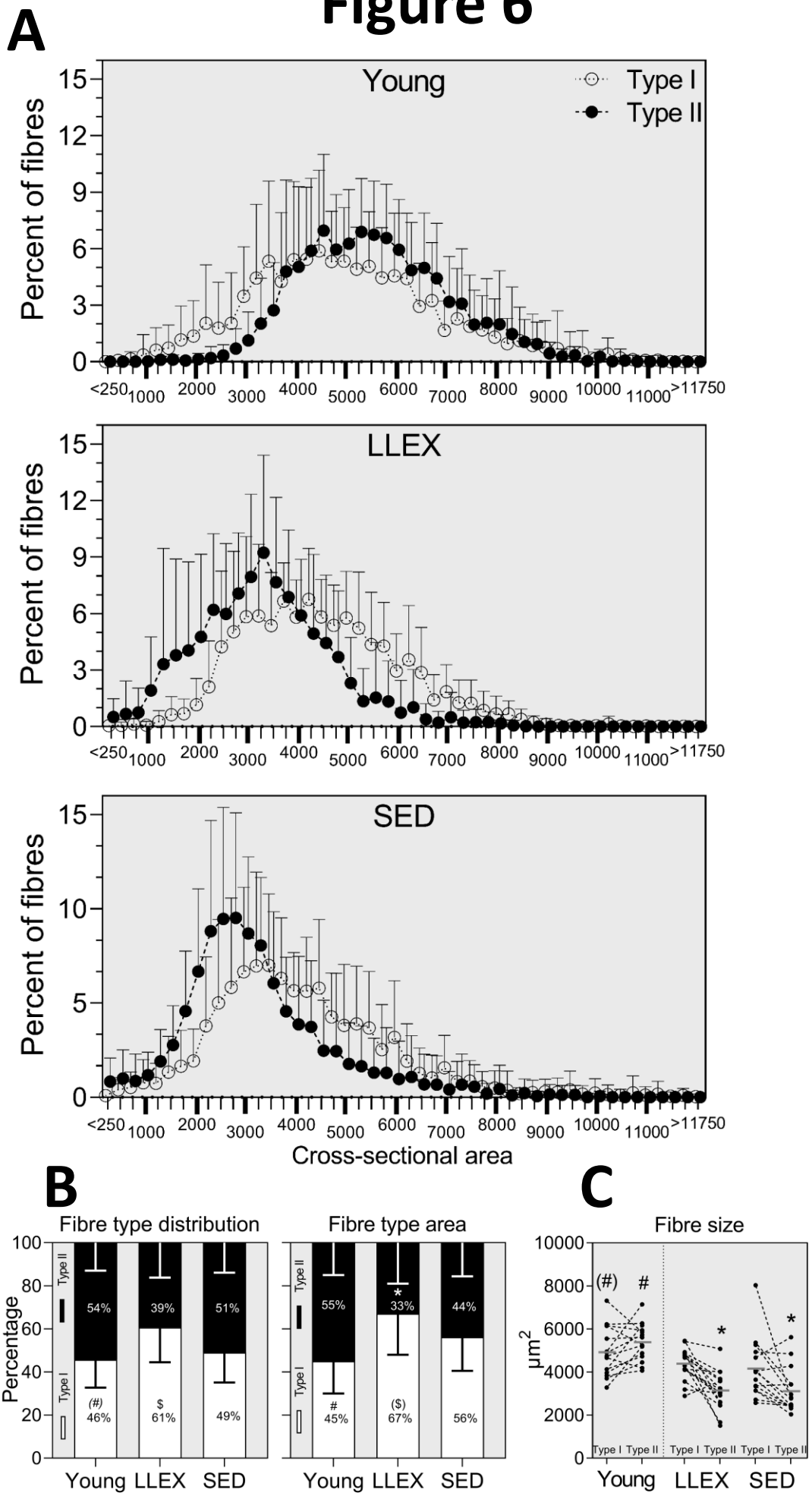

A
Young
Type I
Type II
Percent of fibres
LLEX
SED
<250
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
>11750
Cross-sectional area
B
Fibre type distribution
Fibre type area
Percentage
54%
39%
51%
55%
33%
44%
(#) 46%
$ 61%
49%
45%
($) 67%
56%
Young LLEX SED
C
Fibre size
µm2
(#)
#
*
Type I Type II Type I Type II Type I Type II
Young LLEX SED

Figure 7

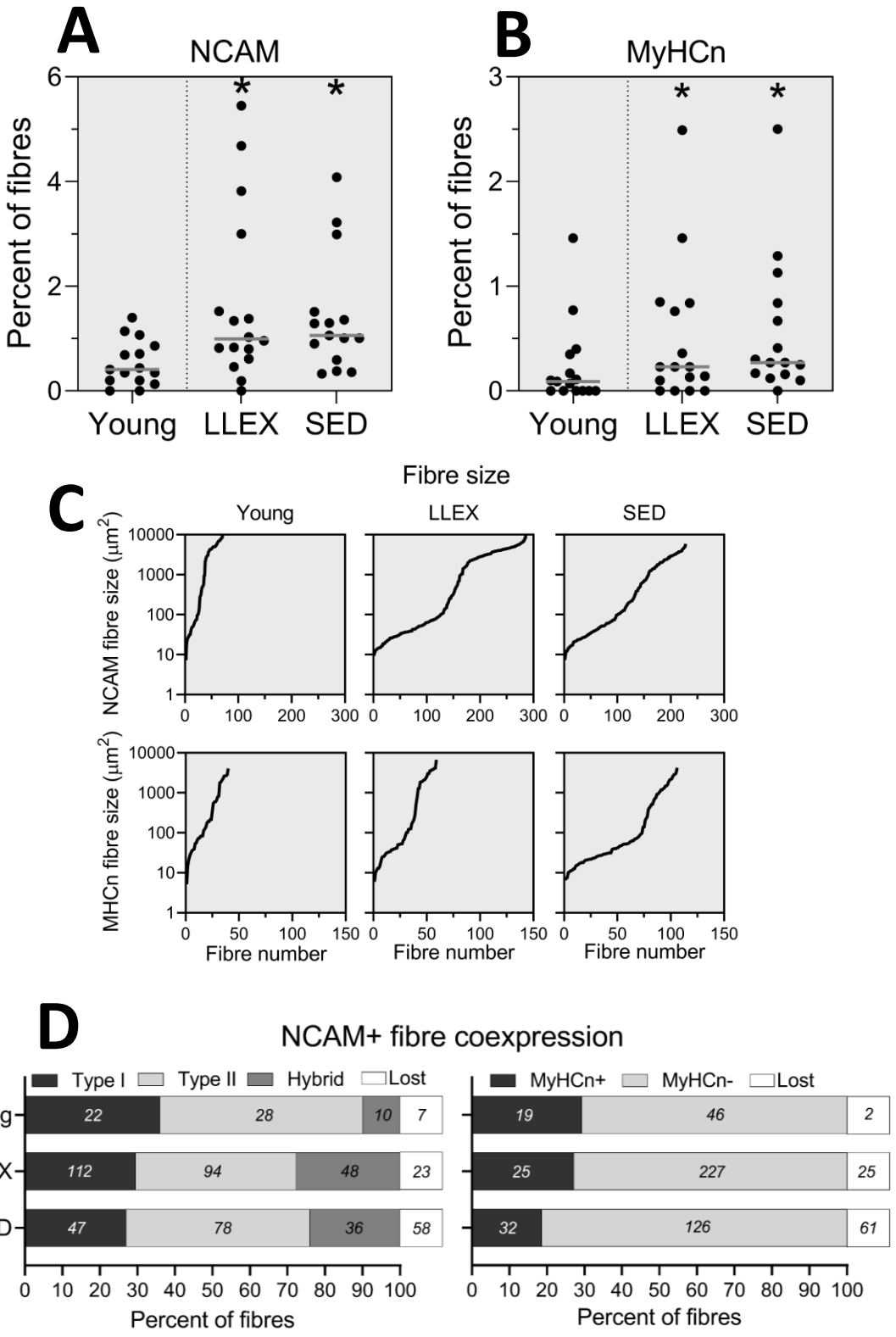
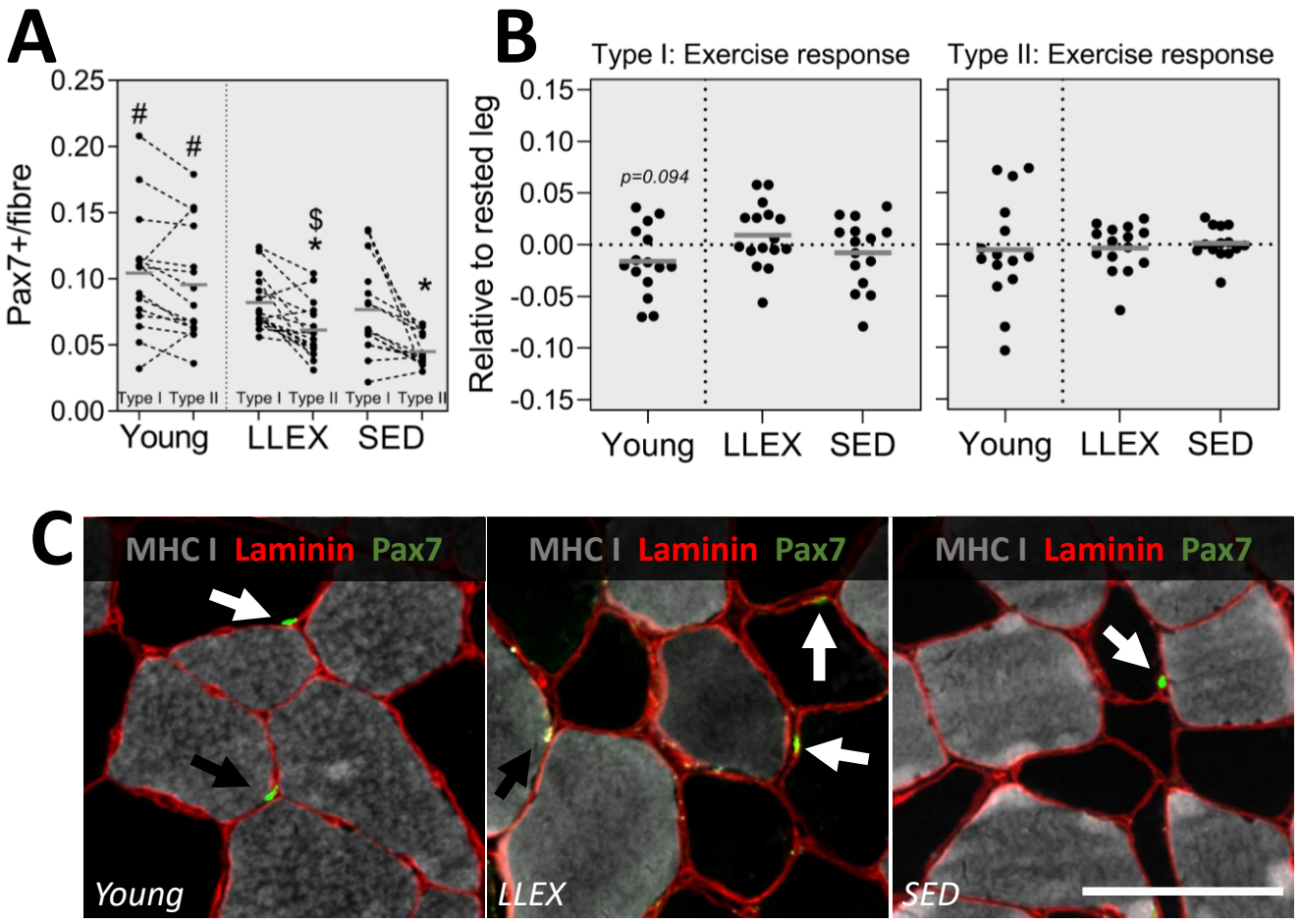

A
NCAM
Percent of fibres
Young
LLEX
SED
B
MyHCn
Percent of fibres
Young
LLEX
SED
C
Fibre size
Young
LLEX
SED
NCAM fibre size (μm²)
MHCn fibre size (μm²)
Fibre number
D
NCAM+ fibre coexpression
Type I
Type II
Hybrid
Lost
MyHCn+
MyHCn-
Lost
Young
LLEX
SED
22
28
10
7
112
94
48
23
47
78
36
58
19
46
2
25
227
25
32
126
61
Percent of fibres
Percent of fibres

Figure 8


A
Pax7+/fibre
0.25
0.20
0.15
0.10
0.05
0.00
#
#
* $
*
Type I Type II Type I Type II Type I Type II
Young
LLEX
SED
B
Type I: Exercise response
Type II: Exercise response
Relative to rested leg
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
p=0.094
Young
LLEX
SED
Young
LLEX
SED

C
MHC I Laminin Pax7
MHC I Laminin Pax7
MHC I Laminin Pax7
Young
LLEX
SED

Figure 9

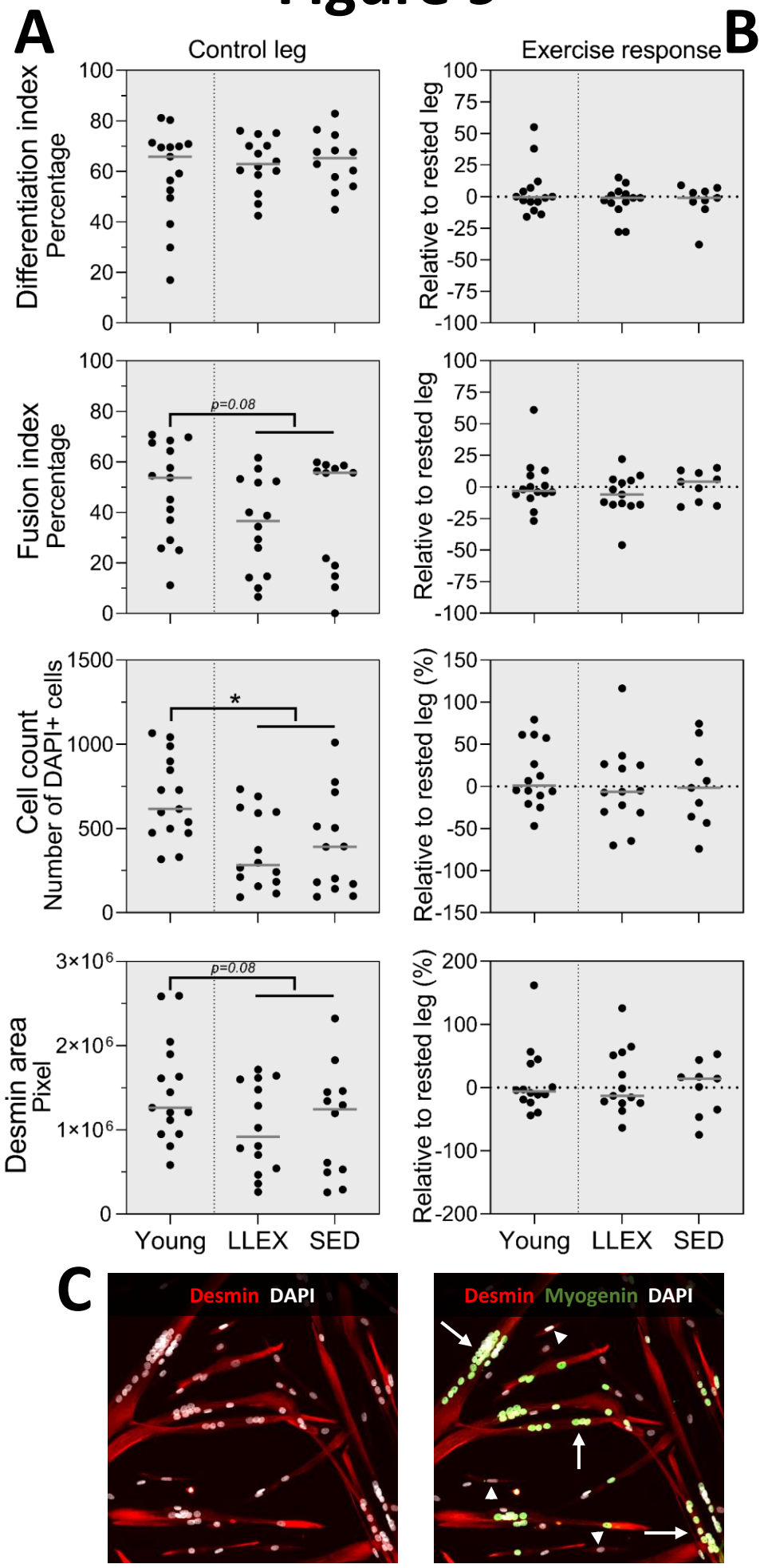
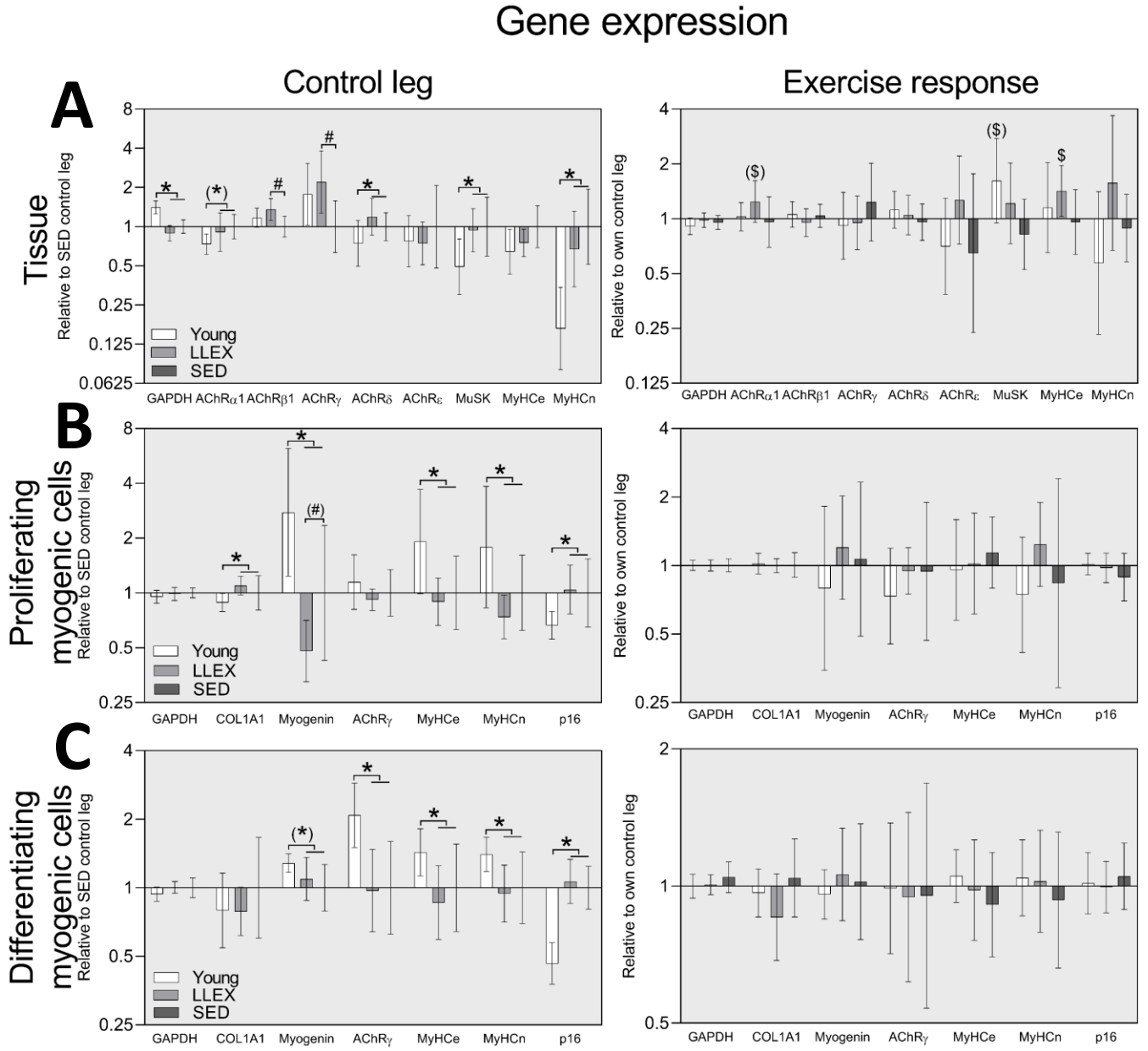

A
Control leg
B
Exercise response
Differentiation index
Percentage
Relative to rested leg
Fusion index
Percentage
p=0.08
Cell count
Number of DAPI+ cells
*
Relative to rested leg (%)
Desmin area
Pixel
p=0.08
Young
LLEX
SED
C
Desmin DAPI
Desmin Myogenin DAPI

Figure 10


Gene expression
Control leg
Exercise response
A
Tissue
Relative to SED control leg
Relative to own control leg
Young
LLEX
SED
GAPDH
AChRα1
AChRβ1
AChRγ
AChRδ
AChRε
MuSK
MyHCe
MyHCn
B
Proliferating myogenic cells
Relative to SED control leg
Relative to own control leg
COL1A1
Myogenin
p16
C
Differentiating myogenic cells
Relative to SED control leg
Relative to own control leg