The role of denervation in skeletal muscle of pre-frail/frail elderly women

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Abstract

The aging process is associated with progressive decline in skeletal muscle mass and function contributing to impaired physical function and increased risk of frailty. Investigations on underlying mechanisms contributing to age-related muscle atrophy have revealed an important role of mitochondrial dysfunction and motor unit (MU) remodeling and loss. We have previously shown the presence of denervation and its modulating role on mitochondrial function in octogenarian men. However, limited data exists in elderly women, who are at a higher risk than men of becoming physically frail and who live longer with functional dependence. The aims of this Dissertation are to assess (i) mitochondrial function changes, and the potential modulating role of denervation on mitochondrial function in prefrail/frail elderly women, and (ii) the significance of denervation to muscle atrophy in pre-frail/frail elderly women. For comparison, we assessed the degree of muscle denervation in world class octogenarian master athletes (MA), who better retain muscle mass and physical function in advanced age. Here we showed that frail elderly women (FE, 77.9 ± 1.5y) compared to young inactive (YI, 24.0 ± 1.0) controls, have reduced *vastus lateralis muscle* respiratory capacity, which seemed to be due to reduced mitochondrial content rather than intrinsic dysfunction of mitochondria because the reduction in respiration was proportional to a reduction in the mitochondrial outer membrane protein, voltage dependent anion channel (VDAC). On the other hand, FE women displayed increased reactive oxygen species (ROS) per unit of VDAC that was normalized by blocking the denervation-responsive ROS signal through pharmacological inhibition of cytoplasmic phospholipase A_2 ($cPLA_2$). As such, our results suggest that denervation modulates mitochondrial function in FE similar to our previous observations in octogenarian men. Consistent with these findings, we also demonstrated the presence of denervation-induced morphological changes in the FE group compared to YI controls, including grouping of myofibers of the same type, changes in myofiber type (myosin heavy chain (MHC) protein

isoform) characterized as increased abundance of fibers co-expressing multiple MHC isoforms, and an accumulation of small fibers that frequently have angular shape. Compared to YI group, FE women also displayed a higher abundance of neural cell adhesion molecule (NCAM) positive fibers and the presence of pyknotic nuclei, which are well-established marker of denervation. Next, we demonstrated that a population that maintains high physical function in advanced age, world class octogenarian track and field athletes (MA, 80.7 ± 2.5y), compared to FE group, had lower abundance of fibers expressing NCAM and greater slow fiber type grouping with increased fiber number per group. This suggests that retention of muscle mass and function in high functioning elderly is associated with a greater capacity for reinnervating denervated muscle fibers such that over time, fewer fibers are lost. All together these data demonstrate that in FE women, denervation is an important modulator of mitochondrial function and contributes to age-related muscle atrophy and physical impairment as indicated by the presence of denervation-induced morphological changes and biomarkers in aging muscle. In contrast, highly functioning elderly adults exhibit features consistent with a greater reinnervation capacity, which likely plays a key role in their lesser degree of muscle degeneration and greater retention of physical function.

Résumé

Le processus de vieillissement est associé à un déclin progressif de la masse musculaire squelettique et de sa function, entraînant une diminution de la fonction physique et un risque accru de fragilité. Les recherches sur les mécanismes contribuant à l'atrophie musculaire liée à l'âge ont révélé un rôle important dans le remodelage et la perte de la fonction mitochondriale altérée et de l'unité motrice (MU). Nous avons précédemment montré la présence de dénervation et son rôle modulateur sur la fonction mitochondriale chez les hommes octogénaires. Cependant, il existe peu de données sur les femmes âgées, qui risquent davantage de devenir physiquement fragiles et vivent plus longtemps avec une dépendance fonctionnelle. Le but de cette thèse est d'évaluer les modifications de la fonction mitochondriale, la présence d'une dénervation musculaire persistante et son rôle modulateur sur la fonction mitochondriale chez les femmes âgées pré-fragiles / fragiles, ainsi que l'importance de la dénervation pour l'atrophie musculaire chez les femmes âgées pré-fragiles / fragiles. À titre de comparaison, nous évaluons l'ampleur de dénervation musculaire chez les athletes maîtres (MA), qui conservent leur masse musculaire et leur fonction physique à un âge avancé. Nous montrons ici que les femmes âgées fragiles (AF, 77,9 ± 1,5 ans) comparées aux jeunes contrôles inactifs (JI, 24,0 ± 1,0), ont la capacité respiratoire mitochondriale du muscle latéral lateral réduite, ce qui semble être dû à une réduction du contenu mitochondrial plutôt qu'à un dysfonctionnement intrinsèque de mitochondries. D'autre part, les femmes AF ont présenté un taux élevé de dérivés réactifs de l'oxygène (DRO) augmentées par unité de mitochondrie normalisée en inhibant le signal ROS sensible à la dénervation en inhibant la phospholipase A_2 cytoplasmique, suggérant que la dénervation module la fonction mitochondriale dans AF de la même manière que nos précédentes observations chez les hommes octogénaires. Conformément à ces résultats, nous avons démontré la présence de changements morphologiques induits par la dénervation dans le groupe AF, notamment le groupement de myofibres du même type, des changements dans le type de myofibre (isoforme de la chaîne lourde de la myosine (MHC) avec une abondance accrue de fibres co-exprimant différentes protéines isoformes du MHC et accumulation de petites fibres angulaires. Les femmes AF ont également présenté une plus grande abondance de fibres positives à la molécule d'adhésion des cellules neurales (NCAM) et la présence de

noyaux pycnotiques, considérés comme des marqueurs associés à la dénervation. Ensuite, nous avons démontré qu'une population qui maintient une fonction physique élevée, les maîtres en athlétisme octogénaires de classe mondiale (MA, 80,7 ± 2,5 ans), par rapport au groupe FE, avait une plus faible abondance de fibres exprimant la NCAM et un plus de regroupement de fibres lentes avec une quantité accrues de fibres par groupe. Cela suggère que la rétention de la masse musculaire et de la fonction musculaire chez les personnes âgées dont le fonctionnement est optimal est associée à une plus grande capacité de réinnervation. Toutes ces données démontrent que, chez les femmes AF, la dénervation a le potentiel de moduler la fonction mitochondriale et contribue à l'atrophie musculaire et au handicap physique liés à l'âge, comme l'indique la présence de changements morphologiques et de biomarqueurs induits par la dénervation dans le muscle vieillissant. En revanche, les personnes âgées très fonctionnelles présentent des caractéristiques compatibles avec une plus grande capacité de réinnervation, compatibles avec l'idée que la dégénérescence musculaire et l'érosion résultante de la fonction physique avec le vieillissement sont en partie liées à l'ampleur de la dénervation persistante.

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Se posebej bi se rada zahvalila svoji mami, ocetu in sestri. Ne morem si predstavljati kako bi lahko dosegla to kar sem brez vasih nasvetov, vase spodbude, podpore in poterpezljivost. Rada vas imam!

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PREFACE AND CONTRIBUTIONS OF AUTHORS

This dissertation is prepared in accordance with the guidelines of the McGill University Office of Graduate and Postdoctoral Studies for the preparation of a manuscript-based thesis. The second manuscript in this Dissertation was published by the Journal of Gerontology: Biological Sciences ([1]; doi.org/10.1093/gerona/glz066). Below is a statement required by the McGill University Office of Graduate and Postdoctoral Studies about the contributions of authors to the manuscripts that compose parts of this Dissertation.

Chapter 2

Title: Fidelity of Reinnervation Modulates Susceptibility to Aging Muscle Impact and Frailty in Elderly Women

Publication status: In preparation

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Contributions of authors: VS and RTH planned the experimental design; VS and CS recruitment, screening and clinical testing of young participants; KJ and SC recruitment, screening and clinical testing of prefrail/frail elderly participants; TT recruitment of Master athletes; SS processed the samples from Master athletes; JM performed muscle biopsies, supervised participant's recruitment and provided input into the design, VS mounting and sectioning of muscle tissue for histology; VS performed immunohistological labeling of muscle tissue sections; VS and KJ imaging of immunolabeled muscle sections; VS performed qPCR; MRZ analyzed muscle sections for the presence of pyknotic nuclei; VSand RTH analyzed and interpret the data; VS and RTH wrote the manuscript; RTH supervised the project.

Chapter 3

Title: Reduced Mitochondrial Content, Elevated ROS, and Modulation by Denervation in Skeletal Muscle of Pre-frail/Frail Elderly Women

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Contributions of authors: VS, SS JM and RTH planned the experimental design; VS and CS recruitment of young participants; KJ recruitment of prefrail/frail elderly participants, JM performed muscle biopsies, supervised participant's recruitment and provided input into the design ; VS and AP tissue collection and mounting of muscle tissue for histology; VS and CS sectioning of muscle tissue for histology; VS and MV muscle fiber separation for mitochondrial function measurements; VS, SS, AP, KJ, KMand FCB performed mitochondrial function measurements; VS performed western blots; VS, SS and RTH analyzed and interpret the data; VS and RTH wrote the manuscript; RTH supervised the project.

Other contributions

In addition to the two manuscripts that represent the main part of this Dissertation, I have contributed to other published original pieces during this PhD.

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ORIGINALITY OF THE PRESENT DISSERTATION

As required by the Office of Graduate and Postgraduate Studies, the following statements contain elements of the thesis that are considered original scholarship and distinct contributions to knowledge.

Chapter 2: This study is the first to perform a comprehensive assessment of the presence of denervation-induced morphological changes in skeletal muscle of prefrail/frail elderly women, including severe myofiber atrophy, accumulation of MHC co-expressing fibers, fiber type grouping, expression of NCAM within muscle fibers and the presence of pyknotic nuclei. Furthermore, this was the first study to evaluate denervation-induced transcriptional (MuSK, AChRα, AChRγ, AChRε) changes in skeletal muscle of prefrail/frail elderly women.

Chapter 3: This study is the first to obtain detailed assessment of mitochondrial respiratory capacity and ROS production in saponin-permeabilized muscle fibers of the *vastus lateralis* muscle in prefrail/frail elderly women. It is also the first study to evaluate denervation as an important modulator of mitochondrial function in pre-frail/frail elderly women.

INTRODUCTION

Skeletal muscle aging is characterized by progressive loss of muscle mass and function leading to a decline in physical function that often precipitates physical frailty [2]. Frailty is recognized as a biological syndrome that is due to the impairment of various physiological systems, and is often described as a pre-disability condition [3]. The incidence of frailty is higher in women, which is partly due to their higher life expectancy [4]. As a result, women live longer with functional dependence [5]. Degeneration of skeletal muscle with aging is an important component of frailty. Although the underlying mechanisms leading to age-related muscle atrophy and weakness remain under study, loss of skeletal muscle innervation and mitochondrial functional impairment are amongst the most studied physiological factors contributing to the complex etiology of muscle atrophy. This is because both factors are important for normal cellular function. Mitochondria regulate energy production, apoptotic signaling and reactive oxygen species generation [6, 7]. Similarly, intact innervation enables efficient neuromuscular transmission to permit muscle activation/contraction and movement. Alterations in mitochondrial function and muscle innervation have been linked to impaired muscle fatigue and reduced muscle strength in aging muscle [8-12], which are associated with low physical function in elderly individuals [13].

Moreover, impaired muscle innervation leads to recurring cycles of denervation-reinnervation that cause motor unit remodeling (e.g., fiber type shift and type-grouping) that eventually precipitate accelerated muscle atrophy when reinnervation capacity is exhausted [6]. Interestingly, although experimental models of denervation demonstrate altered muscle mitochondrial function [14-16], the potential for denervation to modulate mitochondrial function in aging muscle has only recently been considered in aging men [17]. The latter study not only showed that accumulation of small angular fibers (characteristic of persistent denervation) is accompanied by changes in denervation-responsive transcripts, but also that

denervation has a modulating role on mitochondrial function [17]. This suggests that denervation is amongst the factors contributing to age-related muscle atrophy in very old men (>75y of age) and that muscle mitochondrial function alterations appear at least partially secondary to denervation [17]; however, if this is also true in elderly women has not been previously assessed. Moreover, there is limited data on denervation-induced muscle changes in elderly frail women, who are characterized by a greater functional impairment and may perhaps exhibit a greater burden of muscle denervation. Further to this point, how skeletal muscle mitochondrial function is altered and whether mitochondrial function is also modulated by denervation in frail elderly women is unknown.

On the other side of the aging spectrum, high functioning octogenarian individuals (e.g., master athletes), who maintain a high degree of physical function in advanced age, may exhibit less denervation and/or enhanced reinnervation and thus less muscle dysfunction, as has been suggested previously [18]. Therefore, the comparison of pre-frail/frail elderly women to octogenarian athletes will give us further insight into differences in muscle function between these low and very high functioning individuals.

On the basis stated above, the purpose of the present dissertation was to assess the presence of age-related motor unit remodeling and mitochondrial impairment, and their relationship and impact on muscle structure and function in prefrail/frail elderly women. Specifically, we tested the following hypotheses:

- **Hypothesis 1A:** Skeletal muscle denervation contributes to age-related muscle atrophy in FE elderly women.
- **Hypothesis 1B:** We further hypothesized that world class octogenarian master athletes exhibit better re-innervation process of muscle fibers that contributes to better preservation of muscle in advanced age.

- **Hypothesis 2:** Mitochondrial function is altered in the muscle of prefrail/frail elderly women and modulated by denervation.

LIST OF ABREVIATIONS

AACOCF3: arachidonyl trifluoromethyl ketone

AChR; Acetylcholine receptor

ACR: Acceptor control ratio

ADP: Adenosine diphosphate

AIF: Apoptosis inducing factor

ANT: Adenine nucleotide exchanger

ATP: Adenosine triphosphate

ATPase: Mitochondrial ATP synthase

CAMP: compound muscle activation potential

$cPLA_2$: calcium-dependent cytosolic phospholipase A_2

CSA: Cross-section area

Cyp D: cyclophilin D

Dok-7: Docking protein 7

Drp1: Dynamin related protein 1

ECM: Extracellular matrix

EMG: electromyography

EndoG: Endonuclease G

ETC: Electron transport chain

FA-OOH: Free fatty acid hydroperoxide

FE: Frail Elderly

FGFBP1: fibroblast growth factor-binding protein 1

FGF: Fibroblast grow factor

Fis1: Fission 1 protein

FoxO: Forkhead box O

$iPLA_2$: calcium-independent PLA_2 isoform

Lrp4: low-density lipoprotein receptor-related protein 4

MA: Master Athletes

MAFbx: Muscle atrophy F-box

MFN2: Mitofusin 2

MHC: Myosin heavy chain

mPTP: Mitochondrial permeability transition pore

mtDNA: Mitochondrial DNA

MU: Motor unit

MuSK: Muscle specific kinase

MuRF1: Muscle RING Finger-1

MUNE: Motor unit number estimate

MUP: Motor unit potential

Nav: Voltage gated sodium channel

NCAM: Neural Neuro Cell Adhesion Molecule

Nedd4: Neuronal cell-expressed developmentally downregulated protein 4

NMJ: Neuromuscular junction

NRF1: Nuclear respiratory factor 1

OPA1: Optic atrophy gene 1

OXPHOS: Oxidative phosphorylation

PCr: Phosphocreatine

PKA-R1: protein kinase A R1

MRS: Magnetic resonance spectroscopy

ROS: Reactive oxygen species

SERCA1: sarco(endo)plasmic reticulum calcium ATPase 1

CHAPTER 1: LITERATURE REVIEW

1.1. Physical frailty

Projections from the year 2015 predict that by the year 2030, almost 29% of Canadians will be 60 years of age or older [19]. While life expectancy is rapidly increasing, quality of life is often compromised. Moreover, the rapid growth of the elderly population and their age-related health issues have a substantial socio-economic impact and place additional strain on available resources. One of the most prominent physiological changes that occur with aging is the progressive decline of physical mobility [5]. By 80 years of age, a large portion of the elderly population is partially or completely physically dependent with only a small proportion being capable to perform vigorous everyday life activities independently [20, 21]. Generally speaking, impaired mobility represents the starting point of a progressive deterioration of physical function that, when severe, can lead to physical frailty [5]. Frailty is recognized as a biological syndrome due to the impairment of various physiological systems, where frail individuals exhibit a low ability to respond adequately to physical and psychosocial stressors. It can be described as a pre-disability condition, which can eventually lead to the development of disabilities and hospitalization [2, 22]. Although generally the risk of frailty increases with age, frailty is not universally present in elderly individuals [23]. Incidence of frailty ranges from 7% to 16.3% in adults aged ≥65 years and rise up to ~ 40% in adults aged ≥80 years [24, 25]. It should also be noted that the number of people >80 years of age increased by almost 77% from 71 million in 2000 to 125 million in 2015 and that number is estimated to increase even more in years to come [19]. Women represent a greater proportion of the population most at risk of physical frailty, partly due to their higher life expectancy [19]. As a result, not only do women have a greater risk of developing frailty than men, they also live longer with functional dependence.

1.2. Age-related skeletal muscle atrophy

The biggest component of physical frailty is progressive skeletal muscle weakness and atrophy that occurs with advancing age. Loss of muscle mass and strength, and the presence of muscle weakness are important components of the Fried criteria for frailty, which is predominantly used in the clinic to assess the Frail Phenotype [2]. Age-related skeletal muscle atrophy is associated with (i) reduced quality of life, (ii) higher rates of falls, fractures, and hospitalization, and (iii) eventual loss of independence with need for long-term care in older people [26]. Indirect effects of in-home health care and nursing homes costs were estimated as $132 billion per year [27]. In the United States, the health care cost estimates associated directly with age-related muscle atrophy and weakness were $18.5 billion in 2000 [28]. Although it is clear that age-related muscle atrophy represents a great health problem in the elderly population, and despite years of research into the functional, clinical and societal consequences of age-related muscle atrophy, there is still a lack of consensus on the underlying mechanisms.

Clinical and experimental evidence demonstrate that the aging process promotes structural and functional remodeling of the skeletal muscle [29-32]. Skeletal muscle mass starts gradually to decline after the age of 45 and by the age of 80y 30-60% of muscle mass loss is evident [29, 33]. More specifically, elderly women display a 25-30% lower cross-sectional area of the quadriceps muscle when compared to younger controls [34-37]. Nevertheless, after the age of 75 years muscle mass loss appears to accelerate [29, 38], which is in line with the higher prevalence of age-related muscle atrophy within the elderly population [39, 40]. However, the prevalence and impact of age-related muscle atrophy appears to differ between men and women [33]. Longitudinal studies reported that around 75 years of age, the rate of muscle mass loss of the legs in men is 0.80 to 0.98% per year and 0.64 to 0.70% per year in women [39]. Although both men and women demonstrate a decrease in muscle mass with increasing age (especially in the muscles of the lower extremities), men demonstrated greater muscle mass loss throughout

the aging process, whereas women above 60 years of age had a higher prevalence of age-related muscle atrophy, which may be due in part to the occurrence of menopause [33, 39, 41, 42]. Indeed, around 70% of women above 80 years of age showed presence of muscle mass loss, whereas in men 50% were characterized with age-related muscle atrophy [33, 39]. A potential reason for this finding is that the initial muscle mass in women is smaller than in men and therefore women have less functional reserves, which may lead to a greater risk of functional impairment and disability with advancing age [33, 39, 43]. Additionally, the rate of muscle strength loss throughout the aging process has been reported to precede and to be greater than muscle mass loss [39, 44, 45]. Studies reported a 30% reduction in leg muscle strength between 37 and 61 years of age [45], with an additional 20-30% of strength loss by the 8^{th} decade of life [38, 45]. After around 75 years of age the rate of strength loss accelerates to 3.0-4.0% per year in men and 2.5-3.0% per year in women [32, 39, 44]. Age-related reduction in muscle force production and contraction velocity can result in impaired balance, increased risks of falls and having movement difficulties that require sit-to-stand motion [46]. Hence, muscle strength loss may represent a better risk factor for disability than loss of muscle mass [39]. Although the prevalence of age-related muscle atrophy and weakness is higher in women than men, and despite the knowledge that women are at a greater risk of becoming frail than men, studies assessing age-related skeletal muscle changes have mainly been conducted in men leaving women significantly understudied.

1.3. Age-related changes in myofiber morphology

1.3.1. Myofiber atrophy

Various studies [29, 37, 38, 47] reported that the age-related reduction in skeletal muscle mass is due to both myofiber loss and atrophy [37, 48, 49]. Human cadaveric studies showed an almost 39% reduction in the total myofiber number between 25 and 80 years of age, with no preferential loss of either type I or IIa fibers within the whole *vastus lateralis* muscle [29, 50]. Furthermore, muscle samples obtained from the *vastus lateralis* of 70-80 years old subjects observed increased accumulation of small, angular fibers (individually or in groups), dispersed between normally sized and shaped fibers and even some larger fibers [29, 47, 51]. Nevertheless, the majority of studies agree that there is a selective reduction of type IIa fiber size with age [29, 30, 34, 49, 52-55], whereas some studies suggest a decrease in type I fiber size as well [51, 56, 57]. A study in pre-frail elderly men reported a 47% reduction of type IIa fiber size and a 17% reduction of type I fiber size compared to young controls [56]. Another study in a frail group (consisting of 75% elderly frail women) reported a 57% decrease of type IIa and 25% decrease of type I fiber cross-section area when compared to young controls [51]. Moreover, type IIa fiber size was reported to be significantly (35%) smaller in low-functioning compared to high-functioning elderly individuals [58].

It has been also reported that age-related morphological differences of muscle fibers exist between men and women [45]. Type IIa muscle fiber size in elderly women (~72y of age) was found to be significantly smaller compared to young women and elderly men (~72y of age)[45]. Interestingly, there was no significant age-related change in type IIa or type I fiber size in men, although type I fibers in elderly men (~72y of age) were around 15% bigger compared to young controls [45]. However, these findings contradict other studies which reported the presence of type IIa fiber atrophy in elderly men (75-81y of age) [48, 53, 59, 60]. As discussed in the next section, these contradictory findings may reflect differences in age

between individuals in elderly groups, which can vary from 65-80y of age, and the method used to assess the presence of different fiber types. Nevertheless, it appears that age-related muscle mass loss in women is mainly due to type IIa fiber atrophy [45], whereas in men it is mainly due to reduced number of muscle fibers [32, 38, 45]. Another interesting finding was that the muscle from elderly women showed greater fiber size heterogeneity compared to muscle of elderly men [45]. Indeed, the heterogeneity in fiber cross-sectional area (CSA) increases with advancing age and varies between subjects and within fiber types [48, 51]. Additionally, in the very old muscle (>75y of age) increased accumulation of small angular fibers was noticed [61]. It has been further suggested that non-atrophic myofibers compensate for the high degree of myofiber atrophy by compensatory hypertrophy [29, 32, 61, 62], which could mask the actual degree of atrophic fibers and potentially contribute to the notion that type I fibers are less susceptible to atrophy. It should be noted that the sex-dependent differences in myofiber morphology may additionally contribute to discrepancy found between studies as most of studies used a mix cohort of elderly men and women to assess age-related muscle changes. Nevertheless, because type IIa fibers are more powerful than type I, they are more important for initiation of fast movements that help to prevent falls [45, 63]. Hence, atrophy of type IIa fibers in frail elderly women could contribute to weaker and less powerful muscles, which may result in a greater incidence of falls and consequent mobility impairment [45, 63, 64].

1.3.2. Mechanism of muscle fiber atrophy

The maintenance of skeletal muscle mass is greatly dependent on the balance between protein synthesis and breakdown [65]. Numerous studies have showed an important role of the ubiquitin-proteasome (UPS) pathway in muscle atrophy [66, 67]. The UPS system is regulated through the transcription factor forkhead box (FoxO3a)[67, 68] and it is based on protein ubiquitination which involves a cascade of enzymatic activity including ubiquitin activating

enzyme (E1), ubiquitin conjugating enzymes (E2) and the ubiquitin ligase enzyme (E3). E3 binds to the targeted (substrate) protein, catalyzes the transfer of the ubiquitin from E2 to the targeted protein and brings it to the 26S proteasome for degradation (Sandri 2013, Larsson 2019). The two mainly upregulated E3s in atrophic muscle are muscle ring-finger 1 (MuRF1) and muscle atrophy F-box (MAFbx or Atrogin-1) [69, 70]. Although the E3s ligases are upregulated in muscle atrophy, their target proteins are not well characterized [71]. Nevertheless, MuRF1 has been suggested to induce myofibrillar protein degradation and Atrogin-1 to regulate protein synthesis as well [72]. Despite evidence that the aging muscle has a higher proteolytic rate [73], in advanced age (>80y of age) the ubiquitin-proteasome mediated gene expression appears to be down regulated [35, 74]. This could occur due to alterations in translational capacity (e.g., ribosomal quantity and quality) or due to alterations in post-translational modifications that affect protein structure and function. A study in octogenarian women showed that elderly inactive women have an increased expression of FOXO3A and MuRF1, but with no change in Atrogin-1 when compared to young controls [35]. Another study in frail octogenarian women noted that, although there was no difference in Foxo3A protein expression when compared to active age-matched controls, the FOXO3A gene expression was significantly lower in frail elderly [74]. The same study found lower gene expression of MURF1 and Atrogin-1 in the frail group compared to controls [74]. These findings suggest that in octogenarian frail women the proteolytic system may be impaired leading to an accumulation of damaged/ not functional proteins [35, 74].

Another important pathway involved in muscle atrophy that is regulated by FoXO is the autophagy-lysosome system. This system is based on the selective degradation of damaged cellular organelles and macromolecules, which are engulfed by the autophagosome and are degraded after the fusion of autophagosome with lysosome [72].

The lysomial system is based on the selective degradation and recycling of damaged cellular organelles and macromolecules, which are engulfed by the autophagosome and degraded after the fusion of autophasome with lysosome [72]. It has been suggested that in the aging muscle the autophagy-lysosome system is impaired leading to the accumulation of protein aggregates and dysfunctional mitochondria resulting in increased oxidative stress [75-77]. Indeed, muscle of sarcopenic individuals had reduced levels of Atg7, an enzyme involved in autophagosome formation, and LC3II, an autophagosomal membrane-associated form postulated to be responsible for the reduction of age-related autophagy [76]. Another study in frail elderly women compared healthy age-matched controls showed lower gene expression of Atg7 and Beclin1 (Atg6), an autophagy initiation protein [74]. Additionally, the same study found a correlation between reduced Beclin1 levels and less distance covered in 6-minute walk test and a correlation between low Atg7 expression and low muscle mass as well as a correlation between low Atg7 expression and muscle mass [74]. The latter findings postulate that frail elderly individuals have decreased activity of autophagy [74]. Studies in old rodents showed an accumulation of LC3II and p62, an adaptor protein and reporter of autophagy activity, in the aging muscle [75, 78, 79] suggesting impaired autophagosome clearance. Other studies further postulated the presence of impaired formation of autophagosomes as indicated by reduced levels of LC3II, no change or accumulation of p62 and reduction of beclin1 [76, 80, 81]. Interestingly, a study in very old rats (35months) showed that although advanced age resulted in increased protein expression of Atg7 and transcript levels of p62, aged muscle after surgical denervation was unable to further induce autophagy markers compared to young controls [82]. Although, this suggests that aged muscle has a low capacity to increase autophagy in respond to surgical denervation, it should be noted that the increased expression of autophagy markers in muscle of old rats at baseline condition may be

secondary to high burden of denervated myofibers seen at this age [83]. From these studies, it appears that autophagy-induced signaling is highly heterogenous between fibers in old muscle. Particularly, the upregulation of autophagy markers in aging muscle could be occurring specifically in persistently denervated muscle fibers [82, 84] rather than being a general feature of all muscle fibers.

1.3.3. Myofiber type proportion

Age-related myofiber atrophy and loss have been proposed to affect fiber type distribution in aging muscle [34]. However, the age-related changes in muscle fiber type composition are still inconclusive and a subject of debate. Although some studies support the idea that there is an age-related shift towards a higher type I fiber abundance [49, 85, 86], the majority of longitudinal [30, 32, 60], cross-sectional [37, 45, 51] and cadaver *vastus lateralis* muscle [29, 50] studies have not noticed any differences in fiber type proportion with advancing age. Interestingly, a sex-dependent difference in fiber type I distribution has been noted, with women having a 5% higher abundance of type I fibers compare to men [45, 87].

Nevertheless, most studies in the past classified fibers as either type IIa or type I and were perhaps unaware of the age-related increase in fibers co-expressing different MHC isoforms or the available methods were unable to accurately detect these co-expressing fibers [29, 47, 49]. Studies in rodent models of aging suggest that the direction of fiber type change with aging is not uniform [88]. The most common fiber phenotypes present in the aging muscle are fibers co-expressing MHC I/IIa and MHC IIa/IIx isoforms, where MHC I/IIa co-expressing fibers are dominated by MHC I expression and majority of MHCIIa/IIx fibers have an equal amount of both MHC isoforms [51, 89, 90]. Other possible, although rare, MHC co-expressing fibers are MHC I/IIx and MHC I/IIa/IIx [51, 85, 90]. Even though some studies reported an age-related increase in the proportion of MHC co-expressing fibers [85, 89], the proportion of type I and IIa fibers has not been shown to change [51, 53]. Underscoring the magnitude of

MHC co-expression in aging muscle, studies using gel-electrophoresis for single myofiber analysis reported that the abundance of MHC co-expressing fibers in healthy elderly individuals (<75y of age) accounted for 35% of total fibers [91], whereas in older frail individuals (~88y of age) the abundance of MHC co-expressing fibers was almost 52% of total fibers [89], suggesting that with advanced age the abundance of co-expressing fibers increases. The muscle from frail individuals have been reported to display a high abundance of MHC I/IIa (28%) and MHC IIa/IIx (22%) co-expressing fibers [89]. MHC I/IIa were mainly dominated by MHC I isoform [89], suggesting a fiber type shift from type I to type IIa [90], whereas majority of MHC IIa/IIx fibers had similar amounts of both isoforms [89]. Although the proportion of co-expressing fibers (e.g., I/IIa and IIa/IIx) in aged muscle varies between studies, it is still higher compared to young muscle [51, 85, 89]. Moreover, other studies reported that type I fibers in old muscle express proteins specific to type IIa fibers, suggesting they were former type IIa fibers [92], suggesting that fiber type shift is bidirectional and may not result in a complete transition between fiber types [89, 92]. Interestingly, frail elderly [89] and young sedentary subjects [93, 94] have been shown to have a similar abundance of MHC IIa/IIx co-expressing fibers, suggesting that muscle disuse at a young age may lead to fiber type remodeling [93] and could potentially accelerate the deterioration of muscle [57]. Hence, young sedentary individuals could face mobility impairment earlier in adulthood. However, the cross-sectional area (CSA) of MHC IIa/IIx co-expressing fibers in pre-frail individuals has been reported to be significantly smaller [56]. Furthermore, despite the notion that type IIa fibers are more susceptible to atrophy, type I fibers are more likely to co-express MHC IIa isoform, and the co-expressing MHC fibers have been shown to display atrophy to a similar extend as type IIa muscle fiber [89, 90, 95]. Furthermore, as type I/IIa fibers are likely to be mischaracterized as type II fibers by the classical myofibrillar ATPase histochemical technique used in many prior studies of aging muscle, their atrophy behavior would thus be ascribed to type II fibers and this

likely contributes to the dominant view of preferential type IIa fiber atrophy in aging muscle seen in the literature [90]. Therefore, the degree of atrophy of type I fibers may be much greater than previously suggested [96].

A potential reason for the conflicting results is the suggested variability in fiber type proportion between and within *vastus lateralis* muscle, hence the fiber composition of the small muscle sample can vary due to biopsy location and depth [29, 48, 97]. Another confounding factor is the use of different methods to assess fiber type proportion in the aging muscle. Studies using ATPase histochemistry alluded to above [38, 51], immunohistochemistry [17, 56] or gel-electrophoresis for single myofibers analysis [85, 89] each provide different findings, which makes the interpretation of the data challenging. Especially in case of ATPase histochemistry, where the identification of MHc co-expressing fibers is very difficult and not accurate as co-expressing fibers can be easily misclassified as pure type I or type IIa fibers [90]. Furthermore, changes of MHC isoforms appear to be more complex as they can be also influenced by physical activity levels in both young and old individuals, hence not controlling for physical activity can additionally contribute to discrepancies present in the literature [56, 57, 85, 91]. Nevertheless, changes in fiber type, including incomplete shift between muscle MHC isoforms and increased abundance of MHC co-expressing fibers with advanced age can potentially contribute to altered muscle contraction speed and velocity in the elderly individuals and consequently diminished physical function [88].

1.4. Mechanisms contributing to age-related muscle atrophy and weakness

Despite years of research, the underlying mechanism(s) contributing to the complex etiology of age-related skeletal muscle atrophy and weakness has not yet been elucidated. Although there are numerous mechanisms identified to contribute to age-related muscle atrophy, the morphological and excitation-contraction coupling alterations observed in old muscle have

frequently been linked to age-related motor neuron loss and reduction of motor unit number [98, 99]. Indeed, it has been reported that structural alterations of the neuromuscular junction (NMJ) appear before muscle atrophy is evident [100]. Mitochondrial functional impairment has been also associated with age-related muscle atrophy as it is the main site of energy production and substrate metabolism, which is essential for proper muscle function [6]. Muscle mass loss, muscle fatigue and reduced muscle strength are key components of the frailty syndrome, hence both denervation [56, 89] and mitochondrial functional impairment [58, 101, 102] have been implicated in the frailty syndrome. Furthermore, it has been recently reported that skeletal muscle mitochondrial function alterations in octogenarian men are at least partly secondary to myofiber denervation [17]. Hence, a discussion of aging from the perspective of each factor and their relationship follows.

1.4.1. Age-related motor neuron loss and motor unit remodeling

1.4.1.1. Motor unit and myofiber type

The motor unit (MU) is composed of the α-motor neuron and all the myofibers it innervates, and the innervation of the muscle fibers within a MU is a key factor regulating myofiber contraction and function. Importantly, the MU's function is defined by its size and recruitment threshold [98, 103]. Smaller motor neurons innervate fewer myofibers, require lower depolarization stimuli for activation, and produce smaller forces. In contrast, larger motor neurons innervate more myofibers and form bigger, more powerful MU's with higher recruitment thresholds [98, 104]. The recruitment order of MUs starts with smaller MU and as the requirement of a stronger contraction increases, larger MUs are recruited [105]. Myofibers that belong to different MUs have different contractile and metabolic properties [106]. Myofibers within a small MU with low activation thresholds are identified as slow-twitch oxidative (type I) myofibers, while myofibers within a larger MU with high activation thresholds are fast-twitch oxidative glycolytic (type IIa) and fast-twitch glycolytic (IIx)

myofibers [104, 106]. Based on their recruitment pattern, type I fibers are slow twitching, fatigue resistant fibers and belong to MUs with a low threshold. As these fibers are recruited they have high oxidative capacity to promote fatigue resistance. Less frequently recruited are the more powerful fast twitch type IIa fibers that belong to intermediate sized MUs with a higher recruitment threshold. Type IIa fibers fatigue faster than type I fibers, and they rely on non-aerobic and aerobic processes for ATP production. Type IIx fibers belong to bigger MUs than type IIa and have the highest recruitment threshold. Type IIx fibers are fast contracting, fatigue quickly and mainly rely on glycolytic metabolism [106]. Different muscle fibers with their specific contractile and metabolic capacities enable the muscle to perform various tasks such as postural joint stabilization (low-intensity activity, type I fibers), continuous and long-lasting submaximal actions like walking (type I fibers) and fast and strong actions like jumping and kicking (usually type I and type IIa fibers) [106].

1.4.1.2. Age-related motor neuron loss

The neuromuscular system involves the brain and the spinal cord that interacts with the muscle through motor neurons at the neuromuscular junction. Initiation of voluntary movement requires activation of upper motor neurons whose cell bodies are located in the cortex and brain stem centers [107]. These motor neurons transmit action potential to the lower motor neuron cell bodies located in the ventral horn of the spinal cord and in the motor nuclei of the cranial nerves in the brainstem [107]. The lower motor neuron, also called the α-motor neuron, transmits the action potential via its axon to the neuromuscular junction where it innervates muscle fibers [107]. Throughout the aging process, different parts of the neuromuscular system can become altered leading to loss of muscle mass and function. Studies showed that the volume and weight of the brain decreases with advanced age and correlates with the need of walking assistance [108]. However, it has been suggested that reduction in the grey matter may be due to reduced neuronal size, decreased synaptic density and degeneration of the nerve cell

dendrites [109] as well as abnormalities of the myelin structure around axons [110, 111]. These factors may negatively affect axonal transport [112] and contribute to impaired electrical transmission and motor neuron degeneration. However, the contribution of the latter events to muscle denervation and consequently MU remodeling remains to be determined.

Post-mortem studies reported that the number of limb motoneurons in the lumbosacral (L1-S3) region of the spinal cord remained somewhat constant until 60 years of age and then progressively decreased with advancing age [99]. Tomlinson et al. 1977 [99] demonstrated that the loss of L1-S3 motor neurons between the ages of 20 to 60 is around 10%, whereas after 60 and up to 95 years of age the loss of L1-S3 motor neurons reached 22%, with some individuals exhibiting more that 30% loss in motor neuron counts [99]. Studies also showed an age-related decline in number and size of motor axons in the ventral nerve roots and reduced number of cell bodies in the spinal cord [113, 114]. Moreover, the progressive loss of human spinal cord motor neuron cell bodies in advanced age has been noticed to display extensive interindividual variation, indicating that some individuals have a greater degree of age-related motor neuron loss than others [99]. Importantly, the loss of motor neurons within a given MU leads to denervation of all the muscle fibers it innervates.

In vivo studies using electromyographic (EMG) techniques consistently show an age-related reduction in MU number estimates (MUNE)[98]. Likewise, it has been continuously shown that the MU potential (MUP) – an index of MU size - is higher in the aged muscle [98, 115, 116]. Moreover, muscle specific force in elderly subjects was 17% lower compared to younger controls [37]. Thus, the attenuated neuromuscular activation present in the aging muscle may have an impact on force and muscle power development, which are essential for dynamic movements and the prevention of falls.

1.4.2. Age-related skeletal muscle denervation and motor unit remodeling

The impact of age-related MU remodeling and loss can be also studied at the myocellular level. Skeletal muscle undergoes recurring cycles of denervation and reinnervation throughout adult life [6]. In this process, the connection between the myofiber and the motor axon terminal is temporarily disrupted, resulting in myofiber denervation [6]. Most of the denervation events are followed by successful re-innervation by the intact original motor axon or by collateral sprouting by a neighboring surviving motor axon for much of adult life. Due to age-related MU loss, the latter process increases the number of myofibers per MU, which leads to an increase in MU size with aging [40, 117]. However, the marked accumulation of small angular fibers in very old age [17] supports the presence of impaired reinnervation capacity due to axonal degeneration or loss of an entire MU, resulting in accelerated myofiber atrophy in very old muscle [17, 83, 95, 99, 118]. Indeed, a recent study by Piasecki et al. 2018 [117] reported that the difference between old individuals who develop muscle atrophy and those who do not lies in the capacity for successful reinnervation of denervated muscle fibers by the remaining MUs. It seems that in some individuals the remaining MUs in atrophic muscle lose their ability to expand, resulting in impaired reinnervation capacity of denervated muscle fibers, which may eventually contribute to accelerated myofiber atrophy and loss [117].

Age-related MU remodeling and persistent denervation influence fiber type distribution in muscle. Moreover, it appears that in advanced age, when myofiber denervation is most at play, muscle displays less fibers expressing a singular MHC isoform and a higher abundance of myofibers co-expressing different MHC isoforms [10, 17, 48, 51]. Indeed, this has been discovered in transgenic animal models [119, 120], experimental denervation models [121-123], and very old human muscle [10, 17, 51, 61].

Another important hallmark of aging muscle is fiber type grouping, which is displayed as clusters of fibers of the same MHC isoforms [34, 48, 124]. Fiber type grouping reflects the

recurring cycle of denervation-reinnervation during which the denervated fiber is reinnervated through collateral sprouting of a neighboring motor axon of a different type, which is displayed as grouping of myofibers with the same MHC isoform [92]. The increased fiber type grouping in aging muscle can stem from a larger group size (increased number of fibers per group) and/or from an increased number of groups [92]. Because fast or slow MUs can innervate a denervated myofiber, Kelly et al. 2018 [92] showed that some of the grouped type I myofibers expressed sarco(endo)plasmic reticulum calcium ATPase 1 (SERCA1), the isoform typically associated with type IIa fibers, indicating that they were once pure type IIa fibers and are now transitioning to type I fibers [92]. The same study also reported greater fiber type I grouping in elderly individuals compared to younger controls [92]. Another study in elderly men showed that sedentary individuals have a higher degree of type IIa fiber grouping than type I grouping, whereas active elderly individuals displayed the opposite [18]. However, in this study, [18] did not discriminate between pure type IIa fibers and fibers co-expressing MHC I/IIa or IIa/IIx isoforms. Thus, the contribution of co-expressing fibers to the higher abundance of type IIa fiber grouping cannot be excluded [18]. Relatively greater fiber type grouping in aged compared to young muscle has been associated with larger MUs [6, 92, 117, 125]. As described previously, larger MUs have a better capacity for reinnervation of denervated fibers [117], which may result as an increase in myofiber group size and muscle mass maintenance with age [92]. However, smaller MUs with impaired reinnervation capacity commonly seen in old individuals with muscle atrophy [117], could potentially lead to smaller fiber group sizes and accelerated myofiber atrophy and loss.

Interestingly, a study by Roberts et al. 2018 [45] reported that, although healthy elderly men and women had significantly higher proportion of fiber type grouping compared to young controls, old female muscle displayed 13% greater fiber type I grouping and had almost 5 times more fibers per group compared to old male muscle. However, as mentioned previously, elderly

women have a higher degree of type IIa fiber atrophy, which may potentially also contribute to greater fiber type I grouping. Recent evidence shows that a higher abundance of type I fiber grouping requires greater MU activation during sit-to-stand task test [125]. Further, it has been postulated that alterations in MU recruitment would diminish the chance of successful task completion and increase the risk of falls in elderly people [125, 126]. Although MU remodeling in aging muscle may be a compensatory response for age-related MU loss and myofiber preservation, it may not be functionally advantageous [45, 125]. Hence, the exact functional consequence of fiber grouping remains to be determined.

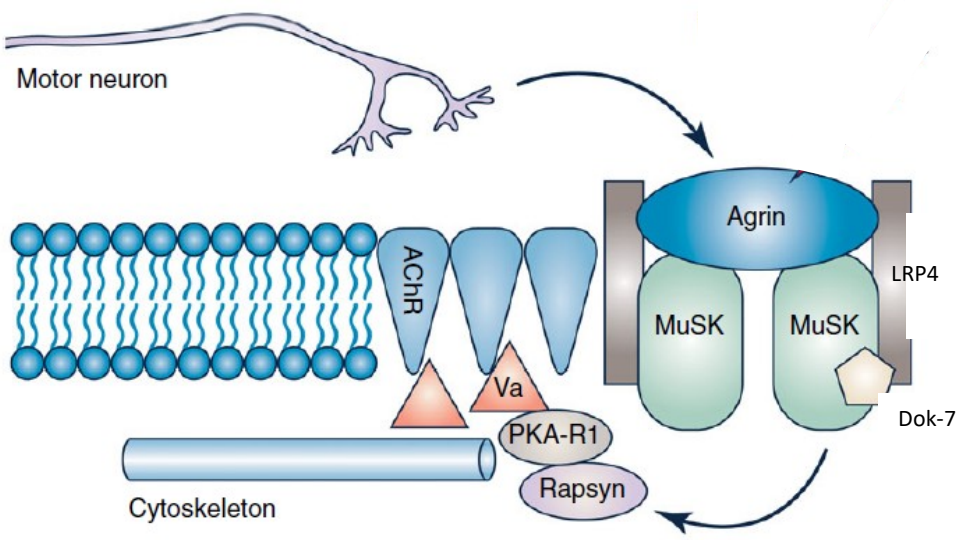
1.4.2.1. Mechanisms regulating neuromuscular junction structure and function

Motor neuron and skeletal muscle communicate through a chemical synapse called the neuromuscular junction (NMJ). The NMJ controls muscle contraction where the presynaptic axon terminal accurately aligns with the postsynaptic endplate [120, 127, 128]. To attain an efficient synaptic transmission, the acetylcholine receptors (AChRs) on the postsynaptic endplate must be densely clustered in a complex pretzel-like shape [119]. The proper function and structure of the NMJ depends on various proteins that enable nerve transmission and maintain post-synaptic structure, and consequently NMJ integrity [129] (Figure 1).

During the aging process, the motor endplate undergoes repeated modifications that changes NMJ structure and leads to the loss of the precise alignment of the pre- and post-synaptic elements as the muscle fiber becomes morphologically denervated [120, 130]. This results in impaired signal transduction, reduced excitation-contraction coupling and eventually in the accumulation of denervated muscle fibers [115, 119, 120, 131]. One of the pathways identified as being important in regulating NMJ integrity is the agrin - muscle specific kinase (MuSK) signaling axis [132] (Figure 1). Old rodent muscle and surgically denervated muscle (dissection of the sciatic nerve or individuals muscle nerve) showed increased protein expression of MuSK (128) and both, AChR ε and AChR γ isoforms (118). Moreover, AChR γ

isoform has been suggested to be involved in restoring neuromuscular function after denervation, whereas AChR ε has been reported to be upregulated in response to agrin signaling during re-innervation of muscle [122, 123]. Another study in very old (VO) rats (35-36 months) observed higher phospho-MuSK protein expression at the NMJ of VO muscle as well as higher MuSK transcript levels compared to young controls [118]. The increased expression of phosphor-MuSK in the aged muscle was also associated with increased protein expression of rapsyn [118]. Additionally, in the latter study transcript levels of AChRα, AChR γ and AChR ε were reported to be increased in VO rat muscle. These findings suggest that although reinnervation-promoting signals are induced, the reinnervation process does not occur leading to the accumulation of persistently denervated myofibers [83, 118]. Consistent with aging rodent studies, a study in elderly men showed that transcript levels of MuSK and AChR ε in muscle were significantly higher in subjects >75y of age compared to subjects <75y of age, supporting the presence of sporadic denervation in very old age [17]. Another study in 66 year old men further reported increased muscle protein levels of MuSK [125]. Other studies in sarcopenic individuals found increased C-terminal agrin fragments in the blood serum, which may suggest the presence of muscle denervation [133, 134]. However, to better understand mechanisms involved, researchers developed specific rodent models with specific impairments in key proteins involved in NMJ structure and stability.

Figure 1: Agrin-MuSK signaling pathway at the NMJ.



The NMJ is a synapse between the motor neuron and the muscle important for initiation of muscle contraction. Agrin (z-agrin) is released from the terminal axon into the synaptic basal lamina and binds to low-density lipoprotein receptor-related protein 4 (LRP4) co-receptor of MuSK present at the postsynaptic membrane. Dok-7, a muscle-specific adaptor protein, interact with MuSK in the cytoplasm and additionally stabilizes/enhances MuSK phosphorylation [135]. However, Dok-7 requires LRP4 to adequately activate MuSK [135]. The activated MuSK recruits rapsyn to the subsynaptic site where it acts in conjunction with protein kinase A R1 (PKAR1) and myosin Va (Va) to promote anchoring of the AChR to the underlying actin cytoskeleton and eventually leads to AChR clustering (pretzel like shape), which is essential for NMJ formation. The cluster formation of AChRs permits the terminal axon to correctly target the postsynaptic endplate to maintain a functional NMJ. AChRs and voltage gated sodium channels (Na_v) are present on the post-synaptic membrane and bind ACh released from the presynaptic terminal of the motor neuron to transmit the action potential throughout the myofiber. Both channels have different forms in developmental and adult stage. The adult isoform of AChR has 2α/β/δ/ε subunits and the Na_v is of the $Na_v1.4$ isoform. During the developmental stage or in case of muscle denervation the AChR contains γ subunit instead of ε subunit (2α/β/δ/γ) and the Na_v present is the $Na_v1.5$ isoform [136](Image adapted from Hepple&Rice 2016 [6]).

1.4.2.2. Experimental models of muscle denervation

To better understand the mechanisms behind neurological impairment in old muscle, researchers have used various experimental models to reproduce functional (e.g., muscle weakness, gait abnormalities) and myocellular (fiber atrophy and loss, MHC co-expression and NMJ instability) changes seen in aging muscle. Experimental models that are often used are surgical denervation (which is characterized by manual dissection of the sciatic nerve or individuals muscle nerve) and transgenic mice. Some of these transgenic mouse models specifically exhibit an alteration in the agrin-MuSK signaling pathway, with a deletion of the nerve-derived organizer of postsynaptic differentiation – agrin [137] or elevated cleavage of agrin by over-expressing neurotrypsin (also known as Sarco mice) [17, 118, 120] and animal models with muscle specific Lrp4 knock-out [138]. Other models used to evaluate mechanisms of NMJ alterations with aging include the superoxide dismutase 1 knock-out mouse ($SOD1^{-/-}$)[119], which exhibits increased oxidative stress and mitochondrial impairment and a mouse model with reduced expression of tyrosine kinase B receptor ($TrkB^{-/+}$) that exhibits impaired neurotrophin signaling [127]. All models mentioned display fragmentation and disruption of the pre- and postsynaptic NMJ regions, resulting in morphological changes similar to what is seen in aging muscle. These changes include NMJ AChR cluster fragmentation, fiber loss, changes in fiber size, fiber type grouping and increased abundance of MHC co-expressing fibers. Furthermore, the motor neuron number in spinal cord of these models is not affected, suggesting that early alterations in the denervation/reinnervation cycle may start at the NMJ and later proceed to the motor neurons in the spinal cord [120, 127, 137]. Additionally, loss of motor neurons may occur in the very late stage of age-related muscle atrophy when fiber atrophy is accelerated and results in the profound muscle mass loss seen at a very advanced age [29, 54, 83, 130]. Because multiple models produce similar phenotypic changes through different mechanisms, it makes it difficult to discriminate which of these mechanisms account

for the impairment in the aging motor unit [6]. It may also be the case that multiple mechanisms are at work in aging muscle. Although deterioration of the NMJ has been widely reported in various animal models stated above, the limited number of studies in human cadaveric muscle have largely observed similar NMJ fragmentation patterns with aging as seen in rodent studies [139, 140].

1.4.2.3. Identification of denervated muscle fibers in aging muscle

Beside denervation-induced alterations in the expression of proteins and their transcripts involved in NMJ structural stabilization and function (Figure 1), myofibers also secrete and express other factors that appear to facilitate to the maintenance of the presynaptic region of the NMJ. These specific markers are expressed or secreted by denervated myofibers to facilitate reinnervation and include fibroblast growth factor-binding protein 1 (FGFBP1), Nav1.5 and Neural Cell Adhesion Molecule (NCAM) [141, 142]. As such these proteins are often used to morphologically assess denervation fibers in a muscle cross-section.

1.4.2.3.1. Role of FGFBP1 in muscle denervation

FGFBP1 is one of the factors that has been suggested to promote reinnervation and contribute to the functional maintenance of the NMJ. FGFBP1 is another key protein that promotes NMJ development, repair and maintains NMJ function in skeletal muscle [143, 144]. FGFBP1 is expressed and secreted at the NMJ region of skeletal muscle fibers where it acts through the binding of muscle and nerve derived FGF ligands [143]. FGFBP1 increases the bioavailability of FGF ligands to bind to FGF receptor (FGFR) present at the presynaptic membrane and promotes the clustering of synaptic vesicles as well as stimulation of neuronal formation [145]. FGFBP1 has been also suggested to contribute to AChR aggregation at the postsynaptic region [143]. A study using old mice and mice mimicking ALS reported significant reduction of FGFBP1 transcript levels in muscle of both animal models. Moreover, aging muscle displayed reduced FGFBP1 expression in the synaptic region of the muscle [143]. Compared to wild type

mice, FGFBP1$^{-/-}$ mice have been reported to display fragmentation of the NMJ and exaggerated transcript levels of AChR ε and γ subunits, suggesting that FGFBP1 contributes to the maintenance of the NMJ structure and function [143]. Although the exact role of FGFBP1 in promoting NMJ repair and neuronal growth remains to be determined, it has been hypothesized that FGF signaling in aging muscle completely relies on binding to FGFBP1 to reach and bind to the FGFR1 [145]. This is because the wider synaptic cleft of the adult NMJ and increased ECM protein deposition negatively affect FGF signaling [145]. Although, FGFBP1 has been demonstrated to promote NMJ structural integrity, its expression with aging appears to be suppressed by one of the transforming growth factor-β (TGF-β) effectors – TGF-β2 [143]. Interestingly, TGF-β has been suggested to accumulate at the NMJ region of deteriorated muscle and inhibit FGFBP1 activity [145].

1.4.2.3.2. NCAM

NCAM has long been recognized to facilitate reinnervation of denervated myofibers and the formation of stable muscle-motor neuron contacts [146-149]. Although NCAM is mainly expressed during myogenesis, its expression becomes restricted to the NMJ with muscle fiber maturation [147]. However, after surgical denervation and nerve crush, NCAM expression increases intracellularly through the muscle fiber and at the postsynaptic region of the myofiber to restore neuronal input [147, 150]. Human [18, 151] and animal [141, 146, 148, 152] studies reported greater proportion of NCAM positive fibers in old muscle, supporting the presence of denervation. Additionally, NCAM positive fibers were often smaller in size [146, 153]. Cross-sectional studies in old humans [18, 151] found similar findings to those in animals. Although NCAM expression has not been found to be specifically associated with either type I or type IIa fiber type [147, 151], denervated fibers display different patterns of NCAM expression, with some fibers showing positive staining at the membrane and others in the cytoplasm [151]. The time course of junctional and cytoplasmic NCAM expression after surgical denervation in old

rats was reported to increase within 1 week of denervation [148]. Interestingly, after 4 weeks of denervation, the junctional NCAM returned to control levels, whereas NCAM expression persisted in the cytoplasm [148]. Nevertheless, NCAM expression levels have been shown to reduce after successful reinnervation [147, 154].

NCAM can be found in three distinct isoforms (120, 140 and 180kDA), with these isoforms differently expressed throughout the maturation of the synaptic cleft [155]. The 180 and 140 kDa isoforms alone or together are important for the formation of stable contacts and functional transmission in the early stage of NMJ development [155], whereas the 120kDA isoform becomes more prevalent in myofibers later in development [156]. Indeed, a study assessing surgically denervated muscle reported an increased content of the 140kDa NCAM isoform in denervated muscle [147]. For successful formation of stable synapses and NMJ development, both the myofiber and the motor axon need to express NCAM [155]. Studies have found that NCAM present in neurons is important for sprouting of the motor axon to reinnervate denervated muscle [157, 158], whereas postsynaptic NCAM facilitates the formation of stable myofiber-motor axon contacts [155]. The exact mechanism through which NCAM stabilizes and enables synaptic connection is not well known. It has been recently reported that NCAM expressed at the postsynaptic region somehow interacts with Lrp4 [155], a molecule important for the activation of agrin-MuSK signaling pathway and consequent aggregation of AChR and NMJ formation [136]. It has been suggested that Lrp4 can facilitate synapse formation independent of agrin-MusK signaling activation. The presence of NCAM appears to influence the independent function of Lrp4, which is important in the early stages of synapse formation as it reduces neuron cone growth motility and induces formation of stable adhesion contacts [155]. Although the possible mechanisms through which NCAM affects Lrp4 are currently not known, it has been postulated that NCAM could potentially bind to Lrp4 directly or indirectly, increasing its levels and facilitating its stabilization at the postsynaptic region [155].

Interestingly, it has been reported that NCAM can interact with MuSK [159] and potentially other molecules important for NMJ formation. Although it is not known how NCAM affects molecules at the postsynaptic region, it appears that it has an important role in contributing to NMJ formation and its structural maintenance.

1.4.2.3.3. Nav1.5

Sodium channel Nav1.5 is highly expressed in skeletal muscle fibers in the first few days postpartum and declines thereafter as it is gradually replaced by the adult Nav1.4 isoform [160]. However, the Nav1.5 isoform has been shown to reappear in adult skeletal muscle following muscle fiber denervation [160, 161] and is again reduced after successful fiber reinnervation [161, 162]. As such, the expression of Nav1.5 seems to be regulated by neural input, which is lost in denervated fibers [163]. Increased expression of Nav1.5 has been demonstrated in aged mouse forelimb muscle [164] and in small angular fibers in association with a high degree of MHC co-expression in very old rat muscle [83]. Furthermore, octogenarian men display a reduction of Nav1.4 isoform transcript, which is also associated with muscle denervation [161]. However, a study that assessed the time course of Nav1.5 expression reported that Nav1.5 in denervated muscle is increased only transiently, suggesting that this may not be the most suitable marker of muscle denervation and should be accompanied by other markers of denervation [148].

1.4.2.4. Potential mechanisms contributing to age-related motoneuron loss

As mentioned previously post-mortem studies reported that the number of limb motoneurons in the lumbosacral (L1-S3) region of the spinal cord progressively deceases with advancing age [99]. Tomlinson et al. 1977 [99] demonstrated that the loss of L1-S3 motor neurons after 60 and up to 95 years of age reaches 22%, with some individuals exhibiting more that 30% loss in motor neuron counts [99]. Studies also showed an age-related decline in number and size of motor axons in the ventral nerve roots and reduced number of cell bodies in the spinal cord

[113, 114]. Based on studies using various rodent models (section 1.4.2.2.) it appears that multiple mechanisms may be involved in the denervation process of aged muscle [6]. It has been also postulated that alterations in mitochondrial dynamics and function importantly contribute to motoneurodegeneration [131, 165]. Impaired mitochondrial dynamics in the axon and cell bodies of neurons may lead to reduce energy production, which may contribute to neuronal dysfunction [166]. Garcia et al 2013 [165] showed that mitochondria in the axonal terminals at the NMJ in old rat muscle (24 months) were swollen and had severely damaged cristae indicating the opening of permeability transition pore, which is consistent with the detection of cytochrome c in the cytoplasm of aged motoneuron terminals [165]. Interestingly, the same study did not find mitochondrial structural alterations in the soma or motoneurons in the spinal cord, which suggests that mitochondrial degeneration starts at the NMJ and progress to motoneurons in the spinal cord (also known as the dying-back phenomenon)[165]. Nevertheless, it appears that the accumulation of impaired mitochondria with increased sensitivity to permeability transition in motoneuron terminals importantly contributes to mechanisms of age-related muscle denervation. Other studies in aged muscle also showed that there is a failure of reinnervation of denervated myofibers leading to the accumulation of persistently denervated myofibers [83, 118]. A study in VO rats (35mo) showed that despite the marked presence of myofiber denervation, VO rat muscle was unable to upregulate transcript levels of neurotrophins which are important for successful reinnervation of denervated myofiber [118]. The same study further showed increased levels of specific microRNAs that suppress neurotrophins in aged muscle leading to failed reinnervation [118]. As mentioned previously (section 1.4.2.3.1.) another important innervation promoting cytokine - FGFBP1, has been shown to be significantly reduced in aged mice muscle [143]. The expression of FGFBP1 in aged muscle appears to be suppressed by one of the transforming growth factor-β (TGF-β) effectors – TGF-β2, which expression is increased in aged muscle

[143]. TGF-β2 has been suggested to inhibit FGFBP1 by suppressing microRNA-206 which modulates FGFBP1 expression in the muscle [144, 145]. Although there are various potential mechanisms contributing to muscle denervation, the exact mechanisms involved still need to be elucidated.

1.4.2.5. Role of denervation in muscle fiber atrophy

Human [17, 61, 99] and rodent [83, 95] studies support the idea that myofiber denervation contributes to age-related muscle atrophy. From the data in rodent models, it is also evident that accumulation of denervated muscle fibers is a major factor causing an acceleration of muscle atrophy in the very old age [83, 95]. Denervation has been associated with the induction of similar proteolytic pathways as those found in other causes of muscle atrophy [167]. Surgically denervated mice lacking MuRF1 and MAFbx have been shown to be partially protected from the development of muscle atrophy [167]. Neuronal cell-expressed developmentally downregulated protein 4 (Nedd4), another E3 ubiquitin ligase, has also been found to be expressed in persistently denervated muscle [168-170]. Interestingly, the expression of Nedd4 has been mainly associated with muscle denervation and disuse [169, 170]. The Nedd4 protein has been found to be localized at the muscle fiber sarcolemma and although the exact trigger leading to Nedd4 upregulation is not known, it has been suggested to be related to diminished muscle tension [169]. Nevertheless, studies in aging muscle further showed that MAFbx and MuRF1 were differentially expressed by muscle fibers [83], where MAFbx was predominantly expressed by pure type II fibers and MHC slow-fast co-expressing fibers [83, 96]. Regarding the latter finding and the notion that MHC co-expressing fibers originate from MHC slow fibers, the observed atrophy of MHC slow fibers in aging muscle may be partially induced via MAFbx expression [83, 96]. Interestingly, MuRF1 is highly expressed in long-term denervated fibers characterized by an increased expression of sodium channel isoform Nav1.5 and independently of fiber type [83], and it has been further suggested to be involved in the

regulation of AChR lifetime at the NMJ [128]. Nevertheless, MAFbx is proposed to be expressed prior to the detection of muscle loss [171], whereas MuRF1 is expressed at a later stage when muscle loss is accelerated [70, 83]. From these studies, it appears that atrophy-induced signaling is highly heterogenous between fibers in old muscle. Particularly, the upregulation of the E3 ubiquitin ligases seen in aging muscle could be occurring specifically in persistently denervated muscle fibers rather than being a general feature of all muscle fibers. Hence, measurement of muscle atrophy at the whole tissue level, which includes denervated and normal/intact myofibers, could potentially obscure atrophy-induced signals occurring in a subset of fibers, particularly those that are denervated.

1.5. Mitochondrial function in aging muscle

Muscle denervation certainly contributes to age-related muscle atrophy; however, it was recently reported that sporadic denervation occurring with normal aging has a modulating role on mitochondrial function in skeletal muscle of octogenarian men [17]. Mitochondria are important sources of cellular including energy transduction, activation of apoptotic signaling, and regulation of reactive oxygen species (ROS) production [7, 172], and through these processes can modulate a wide variety of cell signaling pathways, including those associated with muscle atrophy [173]. With aging, these components of mitochondrial function get altered, which leads to reduced oxidative phosphorylation (OXPHOS) [119], increased recruitment of mitochondrial-mediated apoptotic pathways [17], and increased ROS production [119]. The involvement of mitochondrial impairment and oxidative stress has been linked to reduced muscle mass, strength, impaired aerobic capacity and physical function [8, 9, 12, 56, 174]. Inasmuch as poor muscle strength, muscle fatigue and atrophy are the main components of the frailty syndrome, mitochondria have been considered to be involved in the frailty syndrome [74, 101, 102]. However, most of studies are conducted in men, even though women are at greater risk for developing frailty and live longer with functional dependence. Additionally, not only is there limited information about mitochondrial alterations in skeletal muscle of elderly women, but also there is a lack of studies evaluating mitochondrial function in very old age (> 80 years of age) in either sex, when the clinical impact of muscle aging is more likely to become functionally relevant. For the purposes of this thesis, only studies on mitochondrial function in elderly women, pre-frail and frail subjects will be discussed.

1.5.1. Mitochondrial function in aging muscle

Most studies in elderly women have assessed mitochondrial function indirectly by measuring the activity of numerous mitochondrial enzymes. These studies found that elderly women have reduced citrate synthase activity [101, 175, 176] in addition to reduced succinate dehydrogenase

and β-hydroxyacyl-CoA dehydrogenase activities [175]. There is only one study that measured mitochondrial function in pre-frail elderly subjects and found lower phosphocreatine recovery (PCr) time assessed by magnetic resonance spectroscopy (^{31}P-MRS), indicating diminished capacity to generate ATP [177]. These findings were associated with reduced protein abundance of complex I, IV and V, and reduced activity of complexes I, II and IV in pre-frail subjects [177]. Another study in old women with mobility impairment reported the presence of diminished oxidative capacity indicated by longer PCr recovery rate when compared to sedentary age matched controls [178]. Similar findings were observed in elderly hospitalized individuals (>80y of age) of which 69% were women [179]. Only one previous study directly measured mitochondrial function in saponin-permeabilized myofibers in low functioning elderly individuals, and this was a mixed cohort consisting of 45% elderly women [58]. The latter study reported lower ADP-stimulated respiration (state 3) in low functioning elderly individuals, which supports the presence of diminished mitochondrial respiratory capacity measured *in vivo* by ^{31}P-MRS [177]. Interestingly, researchers have also assessed mitochondrial DNA (mtDNA) in blood samples of elderly individuals and found (i) an age-related reduction in mtDNA copy number and (ii) that elderly women with lower compared to higher mtDNA copy number were more likely to be frail [102]. The same group of researchers identified a specific mtDNA variant, which is located in the region that controls mtDNA replication and contributes to alter mtDNA copy number in the frail population [180]. Although the investigators were unable to assess the cause and effect relationship, these findings support the potential mechanistic role of impaired mitochondrial function in physical frailty [102, 180]. Nevertheless, another study that assessed mitochondrial function in muscle of three generations of women, reported that age-related decline in mtDNA copy number was accompanied by reduced abundance of respiratory chain complexes [101]. The same study assessed the presence of mtDNA mutations and found that mtDNA deletions were largely present in the mtDNA

control region, which is a non-coding region of the mtDNA [101]. This suggests that mtDNA deletions found in old muscle may not be the main contributor to age-related mitochondrial dysfunction [101]. Indeed, it has been reported that the abundance of mtDNA mutations in aging muscle is less than 5%, which makes mtDNA mutations an unlikely determinant of mitochondrial dysfunction and age-related muscle atrophy [181, 182].

The current literature is rich with studies assessing changes in mitochondrial function with aging. However, the data provided is still inconclusive as some studies support [58, 183] and others dispute [184-186] a decline of mitochondrial oxidative phosphorylation with aging. Discrepancies present in the literature may be due to different factors. Firstly, experimental design and methods used differ between studies, where numerous studies use mechanically isolated mitochondria to assess mitochondrial functional indices. However, this approach has been shown to amplify mitochondrial impairments with aging compared to studies using permeabilized muscle fibers, which preserves the mitochondrial network [7]. Secondly, most of the studies consider 'old age' as a homogeneous group combining subjects aged 65-80 years, rather than considering aging across a continuum from old age (<75y) to very old age (>75 y). Thirdly, age-related alterations in mitochondrial function appear to be due, at least in part, to age-related decreases in physical activity [17, 187-190]. However, most studies did not sufficiently control for physical activity levels of their participants [186, 191]. Lastly, most studies used a mixed population of men and women and did not consider possible differences between sexes. Indeed, recent research suggested that there are sex-dependent differences in mitochondrial physiology (as discussed below) [102, 192, 193].

1.5.2. Mitochondrial ROS production and aging muscle

Based on the free radical theory of aging [194], increased mitochondrial ROS generation is mechanistically linked to mitochondrial impairment and age-related muscle atrophy. Reports on changes in muscle mitochondrial ROS production with aging are inconsistent. While

numerous studies have shown that mitochondrial ROS production increases in various conditions involving muscle atrophy [14, 195-197], only some studies have reported that mitochondrial ROS levels are elevated in aging atrophied muscle [183] others have shown no increase despite muscle atrophy [198, 199] or a highly variable response between different muscles that is independent of atrophy severity [196]. It has been previously shown that mitochondrial ROS generation is highly heterogenous between individual muscle fibers in aged muscle [196]; thus, increases in mitochondrial ROS in a subset of muscle fibers (e.g., such as those that are denervated [14]) could be obscured by the ROS signal in the surrounding muscle fibers. Because mitochondria are considered as major contributors to cellular ROS production, mitochondrial impairment has been suggested to play a role in physical frailty. Indeed, elderly individuals with low physical function have been reported to be more susceptible to oxidative stress [58]. Interestingly, another study in elderly women with mobility impairments reported that elevated serum levels of protein carbonyls (a marker of oxidative stress) are strongly associated with weak handgrip strength, suggesting that oxidative stress may contribute, at least in part, to age-related decline in skeletal muscle function [200]. Moreover, a longitudinal study in elderly women found that serum protein carbonyls were associated with severe walking disability [201]. Numerous other studies reported an association between circulating biomarkers of oxidative stress and physical frailty [202-205]. These studies showed that frail individuals have increased levels of oxidative stress indicated by high levels of protein carbonyls, hydroperoxides, oxidative DNA damage and isoprostanes as well as reduced levels of total thiol and increased glutathione oxidation. Another study reported increased levels of superoxide anions in white blood cells of frail individuals and that increased ROS are associated with slower gait speed [206]. To the best of our knowledge, we are not aware of any studies that assessed oxidative stress in skeletal muscle of frail individuals.

1.5.3. Sex-dependent differences in mitochondrial physiology

As mentioned earlier, although the current literature is abundant with studies assessing age-related changes in mitochondrial function, most studies used a mixed population of men and women and did not consider possible difference between sexes. However, recent research suggests that there are distinct sex-related differences in mitochondrial physiology [102, 192, 193]. The majority of human studies assessing these differences have been conducted in young and middle-aged individuals wherein age-related changes in mitochondrial function may not yet be apparent. Although there were no sex-dependent differences found in mitochondrial OXPHOS in permeabilized muscle fibers of *vastus lateralis* [207] and *gastrocnemius* muscle [208], women displayed higher mitochondrial volume density [209], greater fatty acid oxidation [207, 209], and lower sensitivity to ADP [207]. In addition, other factors like post-transcriptional modifications and allosteric enzyme regulations could contribute to sex differences in mitochondrial physiology. Another study using isolated mitochondria from *vastus lateralis* muscle showed that young active women have higher mitochondrial OXPHOS compared to men, although there was no difference in mitochondrial content [210]. Furthermore, women displayed similar mitochondrial OXPHOS as endurance-trained men, suggesting that greater mitochondrial OXPHOS in women vs. men may be the result of a physiological adaptation to exercise [210].

1.5.4. Mitochondrial permeability transition pore in aging muscle

The activation of apoptosis is another mitochondrial dependent mechanism thought to contribute to age-related muscle atrophy and loss of muscle function [211, 212]. A key player in this process is the mitochondrial permeability transition pore (mPTP) located at the inner mitochondrial membrane (Panel 2018). Pro-apoptotic stimuli, like oxidative stress and accumulation of unfolded proteins, have been suggested to increase the sensitivity of mPTP to calcium causing its opening, which leads to matrix swelling, loss of mitochondrial membrane

potential, and the release of proapoptotic factors, such as cytochrome c, apoptosis inducing factor (AIF), and endonuclease G (EndoG), into the cytoplasm [16, 185, 195, 213]. Data obtained from elderly subjects showed an increased sensitivity of mPTP opening in skeletal muscle of physically active septuagenarian men compared to young adult physically active controls [185], although there were no changes in abundance of proteins regulating mPTP activity (voltage dependent anion channel (VDAC), cyclophilin D (CypD) and adenine nucleotide translocator (ANT)) [185], which is in keeping with the results of other studies [213, 214]. Interestingly, the degree of sensitivity to mPTP opening did not differ between old and very old men (>80y of age) and it was independent of physical activity status, suggesting that increased sensitivity of mPTP is a primary organelle defect of the aging process [17]. In support of this interpretation, aging human muscle has been reported to have an increased fraction of muscle fiber nuclei that are positive for EndoG, coincident with a reduced Parkin protein level relative to VDAC [185]. Lower gene expression of Parkin was also found in frail elderly women [74]. The presence of sensitized mPTP [17, 185] and reduced expression of key regulators of skeletal muscle mitophagy (e.g. Parkin, BCL2/adenovirus E1B interacting protein 3 (BNIP3)) [74, 185] in aging human muscle suggest an accumulation of pro-apoptotic mitochondria due to impaired mitophagy. It is not yet well understood which are the exact underlying factors that contribute to increased sensitivity to mPTP opening in aging muscle. However, since denervation is also known to induce sensitization of mitochondria to permeability transition [16, 17], accounting for the impact of denervation on aging muscle mitochondrial function is critical in understanding the causes of these mitochondrial function alterations.

1.6. The relationship between mitochondrial function and denervation

Animal models of sporadic denervation, such as $SOD1^{-/-}$ mice [119] and neurotrypsin-overexpressing mice [118, 120], as well as models of complete surgical denervation [14, 15], not only show altered muscle fiber morphology and NMJ degeneration, but also an elevated

ROS production, sensitization to permeability transition [14, 16, 118, 215], and reduced respiratory capacity [14, 118, 215]. Furthermore, the fiber type shift towards fast glycolytic fibers seen in denervated muscle [121] is associated with impaired mitochondrial function [216]. Interestingly, although the impact of denervation on mitochondrial function in these models occurs independently of aging, the mitochondrial function alterations are similar to what is seen in aging muscle [17, 56]. However, the relationship between mitochondrial function and muscle denervation, and specifically whether there may be circumstances when mitochondrial impairment occurs secondary to muscle denervation in aging muscle, has only recently been considered in aging. The first study [17] to assess mitochondrial function through the spectrum of aging, separating old and very old individuals, also determined the impact of denervation on mitochondrial function in aging human skeletal muscle. The results of this research suggested that changes in mitochondrial function can emerge before the presence of persistent denervation can be detected, providing evidence that some changes in mitochondrial function precede the accumulation of persistently denervated myofibers in aging muscle [17].

Although models of surgical denervation display high oxidative damage to lipids and proteins [15, 217-219] and exhibit higher ROS production with longer periods of denervation [14, 217], superoxide produced by the ETC in surgically denervated mouse muscle was not increased when compared to controls, suggesting that the ETC may not be the main source of ROS in denervated muscle [15].

A study conducted by Bhattacharya et al. 2009 [15] showed that the primary increase in ROS production seen in surgically denervated and $SOD1^{-/-}$ mice muscle is due to the release of fatty acid hydroperoxides (FA-OOH), which are a product of mitochondrial membrane oxidative modifications and are cleaved from the mitochondrial outer membrane by the calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$). Denervation has been shown to induce the activation of $cPLA_2$ leading to an increased release of mitochondrial-derived FA-OOH [15].

They also demonstrated a reduction in ROS signal after specific inhibition of $cPLA_2$ by $AACOCF_3$ from surgically denervated muscle and $SOD^{-/-}$ mice, where the latter model is known to exhibit substantial denervation [141]. Furthermore, the involvement of $cPLA_2$ was further confirmed with higher $cPLA_2$ protein [15] and transcript [217] levels in denervated. So far, only one study isolated the impact of denervation on mitochondrial function by using AACOCF3 to pharmacologically inhibit the activity of $cPLA_2$ in very old human muscle [17]. The investigators showed that only very old men (> 75 years of age) demonstrated a reduction in ROS emission after $cPLA_2$ inhibition, whereas no change was seen in men <75 years of age. In the same study, the presence of denervation in very old age was further supported by upregulation of denervation responsive transcripts and an accumulation of very small fibers [17]. Interestingly, the latter study showed that under basal conditions (vehicle control) in the $cPLA_2$ assay, ROS emission was not increased. One potential reason for this finding could be that mitochondrial ROS signaling in denervated fibers is elevated but it still cannot be detected at a whole muscle level, because mitochondria from denervated myofibers represent only a small fraction of the total mitochondria in the bundle examined. It is also possible that elevated ROS in denervated muscle fibers may cause a compensatory decrease in mitochondrial ROS in neighboring innervated muscle fibers. The different aspects pertaining to mitochondria, denervation and the interaction between them was incorporated in to our studies to better delineate the changes occurring with frail and physical active aging in an attempt to tease out the effect of intrinsic aging from that of physical inactivity.

1.7. Master Athletes – a model of superior aging

On the other side of the aging spectrum are octogenarian master athletes (MA) who are exceptionally gifted individuals who have kept high physical function throughout life [220], and represent the best model of superior aging. MA are characterized by greater muscle strength [221] and preserved muscle mass compared to non-active controls [222, 223]. Thus, they are an excellent cohort to include in the assessment of aging parameters to capture a wider breadth of physiological ranges with aging. Therefore, any differences seen in MA compared to young controls are more likely to be representative of true changes with aging, as long as the relevant changes are also seen in old nonathletic individuals.

PREFACE TO CHAPTER 2

As detailed in Chapter 1, skeletal muscle atrophy and weakness are key components contributing to mobility impairment in aging and physical frailty. Skeletal muscle denervation is one of the factors that has recently been linked to reduced muscle strength [10, 11] and muscle structural remodeling [17, 31, 38, 61, 83] in aging muscle. Indeed, aging muscle displays similar morphological changes as seen in animal models of denervation including myofiber loss, reduced fiber size, increased fiber type grouping and increased abundance of MHC co-expressing fibers [17, 51, 61]. Moreover, in a recent study in octogenarian men the accumulation of small angular fibers (characteristic of persistent denervation) was accompanied by changes in denervation-responsive transcripts, strongly indicating that denervation is among the factors contributing to age-related muscle atrophy in very old men [17]. However, there are no studies assessing the presence of denervation in elderly frail women, who are more functionally impaired. Understanding whether their greater muscle impact is due in part to a greater burden of denervation is important to direct development of therapeutic approaches to limit frailty. On this basis, we determined denervation-induced morphological and transcriptional alterations in *vastus lateralis* muscle of prefrail/frail elderly women in comparison with world class octogenarian MA, a population that maintains a high level of physical function in advanced age. This work will give us a better understanding of whether denervation contributes to muscle atrophy and impaired muscle function in physically frail individuals, providing key insights to therapeutic approaches aiming to reduce/prevent the development of physical frailty.

Chapter 2: Fidelity of Muscle Fiber Reinnervation Modulates Aging Muscle Impact in Elderly Women

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2.1. ABSTRACT

Background: Aging muscle degeneration is a key contributor to physical frailty; however, the factors responsible for exacerbated versus muted aging muscle impact are largely unknown. Based upon evidence that susceptibility to neurogenic impact is an important determinant of the severity of aging muscle degeneration, we examined indices of muscle denervation in pre-frail/frail elderly women compared to a population with high function in advanced age, world class octogenarian track and field athletes. We hypothesized that pre-frail/frail elderly women would exhibit marked features of denervation compared to young physically inactive females, whereas age-matched world class female masters athletes would exhibit attenuated indices of denervation compared to their age-matched pre-frail/frail counterparts.

Methods: Muscle biopsies were obtained from *vastus lateralis* muscle from pre-frail/frail elderly women (FE, n=17, 77.9±1.5y), world class masters track and field athletes (MA, n=7, 80.9±2.2y; world ranking in top 5 of their discipline) and young physically inactive (YI, n=12, 24.0±1.0y) controls, to assess denervation-related morphological and transcriptional markers.

Results: The FE group displayed marked grouping of slow fibers, accumulation of very small myofibers, a severe reduction in type IIa to type I size ratio, highly variable inter-subject accumulation of neural cell adhesion molecule (NCAM)-positive myofibers, and an accumulation of pyknotic nuclei, indicative of recurring cycles of denervation/reinnervation and a sporadic occurrence of failed reinnervation in pre-frail/frail subjects. The MA group exhibited a smaller decline in type IIa/I size ratio and fewer pyknotic nuclei, accompanied by a higher degree of type I fiber grouping and larger fiber group size, suggesting a greater reinnervation of denervated fibers in MA. In seeking to identify possible contributing factors to the difference in neurogenic impact between FE and MA, MA had higher mRNA levels of the reinnervation-promoting cytokine Fibroblast Growth Factor Binding Protein 1 (FGFBP1) than

FE, but there was no difference in a negative regulator of FGFBP1 (transforming growth factor β2) between groups.

Conclusions: Consistent with our hypothesis, our results indicate that the muscle of pre-frail/frail elderly women has significant neurogenic atrophy, whereas MA exhibit evidence of superior reinnervation capacity and this was associated with transcriptional up-regulation of the reinnervation-promoting cytokine, FGFBP1. Thus, our data show that a distinguishing feature of muscle impact with aging between low and high functioning elderly women is the robustness of the response to denervation of muscle fibers.

Keywords: Frailty, muscle atrophy, sarcopenia, denervation, motor unit

2.2. INTRODUCTION

Aging is characterized by a progressive decline in mobility and physical ability, where aging-related alterations in skeletal muscle play a pivotal role [21]. Furthermore, individuals who exhibit more severe muscle impairment with aging generally have poorer health outcomes, including a greater risk of physical frailty and death [8, 10]. There can be substantial inter-individual variability in muscle impact with aging, and whilst some individuals have greater muscle impairment that contributes to physical frailty [7], others are more resistant and maintain higher function in advanced age [22, 44]. Understanding the processes responsible for exacerbated versus diminished muscle decline can play a key role in identifying potential mechanistic targets for therapeutic intervention.

Amongst the most important processes contributing to aging muscle impairment is a loss of motor units, where a motor unit is the smallest functional unit of muscle, consisting of a motoneuron and the muscle fibers it innervates [19]. Acknowledging some disparity on this issue [13, 28], a study in human cadaver specimens [49] and some studies in rodent models [17, 43] show a reduction in the number of motoneuron cell bodies in the spinal cord with aging, and the consequences of this can be detected clinically by electromyographic techniques at the whole muscle level as a reduction in motor unit number estimates with aging [15, 29]. With respect to the aforementioned inter-individual variability in aging muscle impact, recent evidence finds that susceptibility to aging muscle atrophy relates in part to the ability to expand the remaining motor units through collateral sprouting of surviving motoneurons to reinnervate muscle fibers that lose their innervation [18]. Specifically, Piasecki et al. [36] observed in both tibialis anterior and vastus lateralis muscles that elderly individuals with low muscle mass had fewer motor units and the remaining motor units were unchanged in size relative to young adults, whereas individuals who maintained muscle mass had a smaller reduction in motor units

(vastus lateralis only) and larger remaining motor unit size, consistent with a greater capacity to expand the surviving motor units in the latter group. Further to this point, the reinnervation process can also be detected in muscle biopsies as a grouping of fibers of the same type, and Mosole and colleagues [31] reported a greater degree of fiber type grouping in high functioning 65-79 y olds with a long history of high volume physical activity, consistent with a greater ability to expand the surviving motor units in these physically active subjects. These findings support observations in both animal models and humans that suggest the severity of muscle impact with aging relates to the degree of neurological impact [34, 46], and are consistent with studies showing that an acceleration of aging muscle atrophy is associated with an accumulation of persistently denervated muscle fibers [42, 43] and failed reinnervation in advanced age [1].

On the basis of the above information, the purpose of our study was to gain novel insights into the factors responsible for variation in the severity of aging muscle impact in advanced age by comparing muscle from pre-frail/frail elderly women to world class age-matched female competitive masters track and field athletes (world ranked within the top 5 of their event). In view of the importance of denervation to aging muscle impact [19], we hypothesized that pre-frail/frail elderly women would exhibit marked features of denervation compared to young physically inactive females, whereas age-matched world class female masters athletes would exhibit attenuated indices of denervation compared to their age-matched pre-frail/frail counterparts.

2.3. METHODS

2.3.1. Subject characteristics

17 pre-frail/frail elderly women (FE; 77.9 ± 1.5y) were recruited from the local Montreal area through local press (Senior Times) and by referral from the geriatric clinics of the McGill University Health Centre. The frailty syndrome was defined based on Fried criteria [16], where a participant was defined as frail if she presented three or more of the following criteria: (i) unintentional weight loss or low muscle mass, (ii) physical inactivity, (ii) weakness, (iv) slow walking speed and (v) self-reported exhaustion. A pre-frail status was defined as having one or two of the above-mentioned criteria. 12 young physically inactive female controls (YI; 24.0 ± 1y) were recruited from the McGill University community. Subjects were screened for eligibility for physical inactivity with the Godin-Shephard leisure-time physical activity questionnaire [2] and one-week measurement of physical activity level with Actigraph accelerometer. Participants were excluded if they reported any heart, lung and/or central nervous system diseases; had an eating disorder; were diagnosed with dementia, depression, or diabetes; had abnormal complete blood count test; took blood thinning or anti-coagulant medications; or had walking aids. Additional exclusion criteria for the young participants was being physically active (≥150 min per week of structured activity) or were <20 or >30 years of age. There was no attempt to restrict the study measures to a particular phase of the menstrual cycle in YI. To provide information on skeletal muscle characteristics in individuals who maintain high physical function in advanced age, we also studied 7 female masters athletes (MA; 80.9 ± 2.2y) for whom we have previously reported on motor unit number estimates [37]. Individuals in the MA group were world class octogenarian women competing in track and field events who were ranked in the top 5 for an event in their respective age category at an international competition in the last 5 years. The MA group was composed of 4 sprint/power athletes and 3 endurance athletes, each training 3-5 times a week. Ethical approval was obtained

from McGill University Health Center (MUHC) Human Research Ethics Board (BMC-06-015; 13-211-BMB; A08-M66-12B). All participants agreed to and signed the informed consent and the study has thus been conducted in compliance with the ethical standards required by the 1964 Declaration of Helsinki and its subsequent amendments.

2.3.2. Skeletal muscle biopsy

Muscle tissue samples were collected by percutaneous skeletal muscle biopsy of the *vastus lateralis* muscle using the Bergstrøm needle technique with suction [45]. Approximately 50 mg of the sample was used for mitochondrial function measures (data not reported here). Approximately 20-30 mg of sample was used for histology and mounted with triganth gum, frozen in isopentane pre-cooled in liquid nitrogen and stored at -80°C. Approximately 5-10 mg of the sample was snap frozen in liquid nitrogen for transcript analysis.

Due to limited muscle tissue from a few participants, some measurements were only performed on a subset of muscle biopsy specimens. The sample size used for each measurement is indicated in the results section of each technique used in this study.

2.3.3. Histochemical and Immunofluorescent staining

Skeletal muscle tissue blocks were cut into 10-µmk thick sections on a cryostat (HM505E; Microm, Walldorf, Germany) at -21°C and mounted on glass slides (Double frosted microscope slides; Fisher Scientific), air dried for 2 h and consequently stored at -80°C. Muscle cross-sections were stained with Hematoxylin and Eosin to assess pyknotic nuclei, and probed using primary antibodies against the basal lamina, myosin heavy chain (MHC) isoform proteins, and Polysialylated (PSA) Neural cell adhesion molecule (NCAM).

For MHC and laminin labeling, sections were hydrated with 1x phosphate buffered saline (PBS) and blocked with 10% normal goat serum in 1xPBS for 30 min. Sections were then incubated for 1h at room temperature with the following primary antibodies: polyclonal rabbit anti-laminin IgG (L9393, 1:700; Sigma-Aldrich), monoclonal mouse anti-MHCI IgG2b

(BA-F8, 1:25), monoclonal mouse anti-MHCIIa IgG1 (Sc71, 1:200) and monoclonal mouse anti-MHCIIx IgM (6H1, 1:25). All MHC primary antibodies were purchased from Developmental Studies Hybridoma Bank (University of Iowa, USA). For NCAM labeling, slides were fixed in 4% paraformaldehyde, washed with 1x PBS and permeabilized with 0.1% Triton-X. After blocking, slides were incubated in rabbit anti-PSA-NCAM IgG antibody (AB5032; 1:100; Millipore Sigma) and mouse anti-MHCI IgG2b (to label type I MHC) overnight at 4°C. After incubation with primary antibodies, tissue sections underwent a series of 1xPBS washes and incubation with fluorescently labeled secondary antibodies for 1h at room temperature. For MHC labeling, Alexa Fluor 488 goat anti-rabbit IgG (A11008, 1:500), Alexa Fluor 350 goat anti-mouse IgG2b (A21140, 1:500), Alexa Fluor 594 goat anti-mouse IgG (A21125, 1:100), and Alexa Fluor 488 goat anti-mouse IgM (A21042, 1:500) secondary antibodies were used, whereas for NCAM Alexa Fluor 594 goat anti-rabbit (A11037, 1:200) was used. All secondary antibodies were obtained from ThermoFisher Scientific. NCAM slides were co-stained with DAPI prior to being mounted with cover slips using Prolong Gold mounting medium (Invitrogen).

2.3.4. Image acquisition and analysis

Images of the stained tissue sections were acquired with a Zeiss fluorescence microscope (Axio Imager M2, Carl Zeiss, Oberkochen, Germany) and analyzed with ImageJ (National Institute of Health, Bethesda, MD, USA). Depending on the size of the tissue section, a minimum of 100 fibers were sampled per muscle section and analyzed for their shape and cross-sectional area. Fiber type grouping was assessed as previously described [24]. Briefly, two adjacent “core” myofibers of the same type were identified by calculating a mean number and standard deviation of myofibers of the same type touching a given myofiber. Once two core myofibers were identified, all subsequent adjacent myofibers of the same type were counted as a part of the group. As we have done previously [1], atrophied fibers were defined as the size represented

by the first percentile in YI controls following the calculation of muscle fiber size distribution of all fibers for all YI subjects. This corresponded to fibers with a size ≤ 1441μm^2. As the ratio of type IIa to type I fiber cross-section area (CSA) is reduced with aging [41], we used this measure as an index of the degree to which a given individual had been impacted by aging (i.e., a greater reduction in type IIa/I size ratio was interpreted as a greater aging impact). For the assessment of NCAM expression, fibers that expressed NCAM in the cytoplasm and/or >50% of the sarcolemmal region were characterized as NCAM positive, excluding fibers only having NCAM in <50% of the sarcolemmal region (as occurs at endplates [40]). We used the presence of pyknotic nuclear clumps (also known as nuclear bags) as an indicator of long-term neurogenic atrophy [23], and this was assessed by an experienced pathologist who identified and counted pyknotic nuclei as deeply basophilic nuclear clumps.

2.3.5. Transcript analysis

Total RNA was extracted from *Vastus lateralis* muscle samples using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). RNA concertation and purity (A260/A280 ratios >1.8) were assessed using a NanoDrop-2000 spectrophotometer (Thermo Scientific). RNA (1μg) was reverse transcribed to cDNA using a qScriptTM cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA). To quantify gene expression of ACh receptors (AchRα,γ,ε), muscle specific kinase (MuSK), FGFBP1, and TGF-β2, primers were designed using Primer3Plus (http://primer3plus.com) (Table 1). TATA box binding protein (TBP) was used as endogenous control. The cDNA was amplified at 95°C for 10 min followed by 40 cycles of 95°C for 15s and 56°C for 20s with an extension step at 72°C for 20 s, using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), with samples run in triplicate along with a melt curve analysis to assess primer-dimer formation or contamination. The comparative threshold cycle (CT) method was used to calculate fold changes in expression. Relative fold changes in gene expression were presented as 2-ΔΔCT and normalized to control subjects.

2.3.6. Statistical analysis

The sample size varies due to limited sample availability of the muscle biopsies, where sample size for each group is indicated below corresponding graphs. Differences in myofiber morphology, myofiber type, myofiber grouping and gene expression between the three groups were assessed by 1-way analysis of variance. In the case of a significant main effect, Fishers's *post-hoc* test was applied for normally distributed data and Kruskal-Wallis with Dunn's *post-hoc* test for non-normally distributed data. The Pearson correlation test was used to assess the association between variables that were normally distributed, whereas in cases of non-normally distributed data a Spearman correlation test was used. The presence of outliers was tested with the ROUT method in GraphPad Prism 7 (GraphPad Software, Inc. CA, USA). In the FE group several points were recognized as outliers according to this test; however, when outliers did not affect the significance of the data, these outliers were included in the analysis to reflect the increase in inter-individual variability with aging [20]. The inclusion/exclusion of outliers is specified in figure legends where applicable. Data are presented as means and standard error of the mean (SEM). For all tests, $p \leq 0.05$ was considered statistically significant.

2.4. RESULTS

2.4.1. Clinical characteristics and physical function measurements

Subject characteristics and physical function data are indicated in Table 2. The FE group had significantly less FFM compared to YI group ($p=0.047$) and showed a trend towards reduced FFM compared to MA group ($p=0.067$). The MA group had a greater FFMI than FE group, and a greater AAMI than YI group. In addition, the MA group had significantly lower body mass compared to YI ($p=0.036$) and showed a trend towards reduced body mass when compared to FE ($p=0.059$). The % body fat content between FE and YI group did not differ, but both groups had significantly more fat compared to MA group (YI vs MA, $p=0.003$; FE vs MA, $p<0.001$). There was no difference in the remainder of the clinical parameters between groups.

We found a significant difference in performance of standard clinical physical function tests between the three groups. The FE group performed significantly fewer chair stands (FE vs YI, $p<0.001$) and was slower at the 4 m gait speed test (FE vs YI, $p=0.019$; FE vs MA, $p<0.001$) and timed up-and go (TUG; FE vs YI, $p=0.004$; FE vs MA, $p=0.008$) test compared to YI and MA group. None of these physical function tests were different between MA and YI women.

2.4.2. Fiber type and size

2.4.2.1. Fiber morphology and size

Figure 1A shows representative images of MHC labeled skeletal muscle cross-sections. We found high heterogeneity of fiber sizes and shapes when assessing the fiber pool of the complete muscle cross-section of FE participants (Fig.1A). Both aged groups (FE, MA) had smaller muscle fibers compared to young controls (Fig.1B; FE vs YI, $p<0.001$; MA vs YI, $p=0.013$). The FE group displayed a pronounced accumulation of very small muscle fibers ($\leq 1441 \mu m^2$, indicated by a vertical dashed line in Fig.1C), whereas the MA group showed a tendency to a lesser shift towards smaller fibers (Fig.1C and 1D) (FE vs YI, $p=0.002$; MA vs YI, $p=0.021$). There were no differences in fiber type proportions between groups (Fig. 2A). The type I fiber

size in MA group was significantly smaller compared to YI group (Fig. 2B; p=0.036). Fibers expressing MHC IIa and IIa/IIx were significantly smaller in both FE and MA groups compared to YI group (Fig. 2B; FE and MA vs YI, p<0.001). The type IIx fibers in FE group were trending to a smaller fiber size compared to YI group (Fig. 2A; p=0.057). Whereas type I and type IIa fibers were of similar size in YI individuals, in both older groups fiber size was significantly smaller in type IIa vs type I fibers such that the type IIa to I size ratio was significantly lower in both FE and MA group compared to YI group (Fig. 2C; FE, p<0.001; MA, p=0.002), and this was lower in FE compared to MA (p=0.02).

2.4.2. Fiber type grouping

Representative images of type I and IIa fiber grouping in muscle cross-sections are depicted in Fig. 3A. The muscle of FE and MA groups showed extensive type I fiber grouping compared to the YI group (Fig. 3B; FE vs YI, p=0.008; MA vs YI, p<0.001). Additionally, we observed a trend towards more type I fiber grouping in MA compared to FE (Fig. 3B; p=0.071). Type I fiber grouping in FE was manifest as an increased number of myofiber groups (Fig. 3C; FE vs YF, p=0.001) rather than a larger size of groups where no difference was observed compared to YI group (Fig.3D). In MA group we observed that both myofiber group size (p=0.006) and group number (p=0.035) were significantly higher compared to YI group. Additionally, the MA group exhibited significantly larger group size compared to FE group (p=0.028). There were no significant differences observed in type IIa fiber grouping, group size or group number between groups (Fig.3B). We found that the degree of type I fiber grouping negatively correlated with type IIa to I fiber size ratio, such that individuals with more severe type IIa fiber atrophy had a greater degree of type I fiber grouping (Fig. 3E; p=0.001).

2.4.3. Morphological and transcriptional markers of denervation

2.4.3.1. NCAM

We performed immunolabeling for NCAM, which is a cytokine that increases in muscle following denervation [32] and which on this basis is frequently used as a marker of muscle fiber denervation [30, 31]. Figure 4A depicts representative NCAM images. NCAM positive muscle fibers exhibited labeling of the cytoplasm and/or the muscle fiber membrane. It also appeared to affect both type I and type II fibers. As presented in Fig. 4B, the abundance of NCAM positive fibers in the FE group was remarkably heterogenous compared to YI individuals and tended to be higher ($p=0.077$). There was less heterogeneity in the MA group and in contrast to the FE group where 5 individuals had more than 10% NCAM positive fibers, no subject in the MA group had more than 8% NCAM positive fibers. We found that the abundance of NCAM+ fibers was negatively associated with type IIa to I fiber size ratio (Fig. 4C; $p=0.009$, $r= -0.557$), suggesting that denervation is related to the reduction in type IIa/I size ratio with aging.

2.4.3.2. Pyknotic nuclei

The assessment of pyknotic nuclei is routinely performed in the investigation of the presence of long-term neurogenic atrophy [23]. Figure 4D illustrates pyknotic nuclei within a fiber, and as shown in figure 4E, the FE group had significantly higher accumulation of pyknotic nuclei compared to YI group ($p=0.009$), whereas the MA group was not different from YI.

2.4.4. Denervation responsive transcripts

The analysis of denervation responsive transcripts, MuSK, AChRα, AChRε, AChRγ, did not show any differences between groups (Fig.5). However, the gene expression of the reinnervation-promoting FGFBP1 was significantly upregulated in the MA group compared to YI and FE group (MA vs YI, $p=0.004$; MA vs FE, $p=0.004$), suggesting a greater reinnervation capacity was driving the greater size of the grouped fibers seen in MA.

Table 1: mRNA primers to assess denervation-induced transcripts.

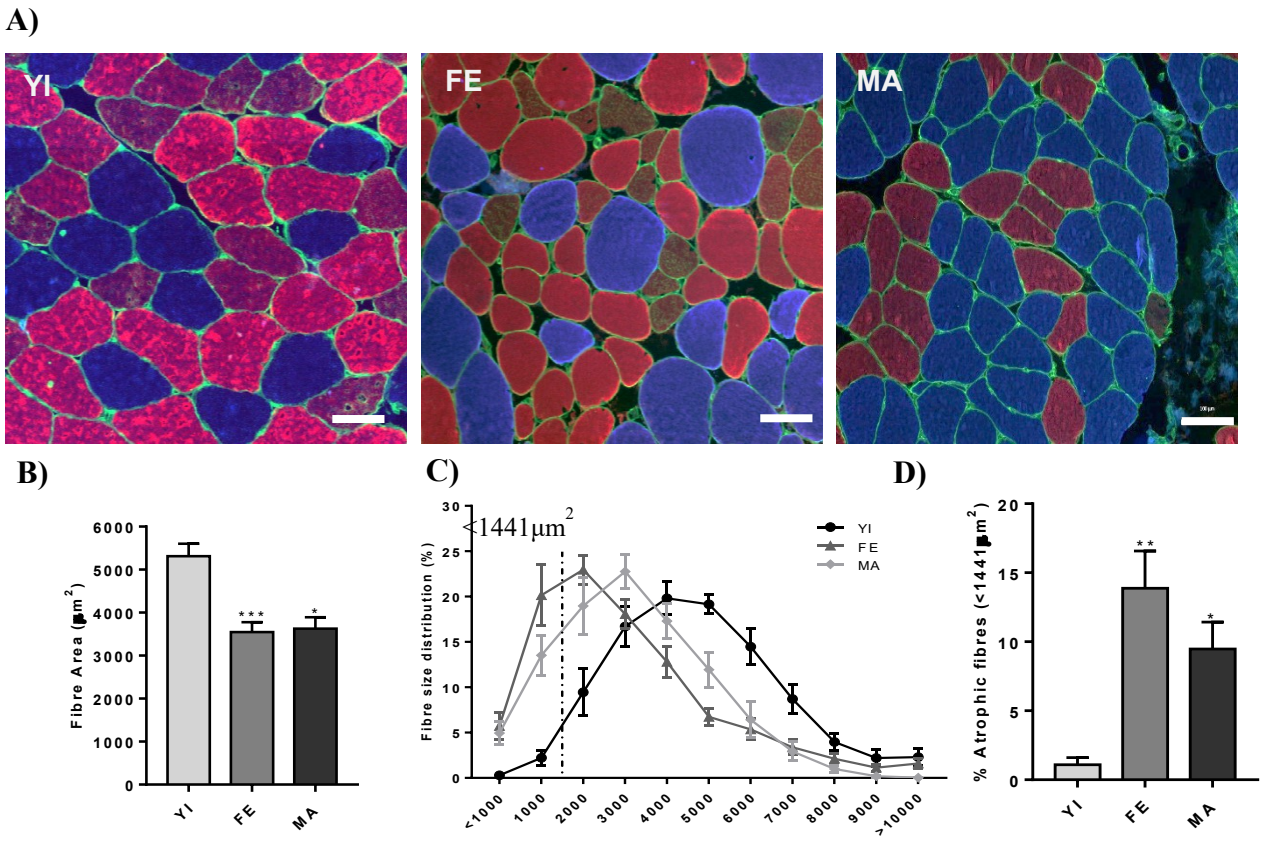
Gene name	Sequence	NCBI reference number
AChRα	F 5'-TGACTATGGCGGTGTGAAAA-3' R 5'-TCAAAGGGAAAGTGGGTGAC-3'	NM 000079.3
AChRγ	F 5'-CCACCAGAAGGTGGTGTTCT-3' R 5'-GATGGCGACGGTACACTTCT-3'	NM 005199.4
AChRε	F 5'-ATACTGAGAACGGCGAGTGG-3' R 5'-GATGGAGACCGTGCATTTCT-3'	NM 000080.3
MuSK	F 5'-GCCTTCAGCGGAACTGAG AAA-3' R 5'-GGCTGGGGGTAGGATTCCA-3'	PMID: 17192614
FGFBP1	F 5'-CCTCAGCATAGTGCAGGACA-3' R 5'-GCAGGAAACAGCCTCTGAAC-3'	NM 005130.4
TGF-β2	F 5'-CCCAAAAGCCAGAGTGCCTGAA-3' R 5'-ATGTAGCGCTGGGTTGGAGATG-3'	PMID:22107086
TBP	F 5'-TATAATCCCAAGCGGTTTGC-3' R 5'-GCTGGAAAACCCAACTTCTG-3'	NM 001172085.1

Table 2: Clinical characteristics of participants.

Characteristics	YI (n=12)	FE (n=17)	MA (n=7)
Age (years)	24 .0 ± 1.0	77.9 ± 1.5†	80.7 ± 2.5†
Hight (cm)	162.5 ± 1.8	157.7 ± 1.7	157.1 ± 2.2
Body mass (kg)	62.9 ± 3.5	61.5 ± 2.0	53.3 ± 1.6†,‡
BMI (kg/m^2)	23.5 ± 1.3	24.8 ± 0.9	21.6 ± 0.8
FFMI (kg/m^2)	15.3 ± 0.4	15.1 ± 0.3	16.5 ± 0.3‡
FFM (kg)	40.7 ± 1.5	37.4 ± 1.0†	40.8 ± 0.9
AMMI (kg/m^2)	6.5 ± 0.3	6.7 ± 0.2	7.3 ± 0.1†
AMM (kg)	17.1 ± 0.9	16.7 ± 0.5	18.1 ± 0.5
Body fat (%)	34.8 ± 2.6	37.8 ± 1.6	23.3 ± 2.2†,‡
Chair stands (n/30s)	14.0 ± 0.4	11.5 ± 0.4†	-
4m Gait speed (m/s)	1.2 ± 0.05	1.0 ± 0.03†	1.6 ± 0.07‡
TUG (sec)	8.8 ± 0.2	10.5 ± 0.4†	8.6 ± 0.4‡

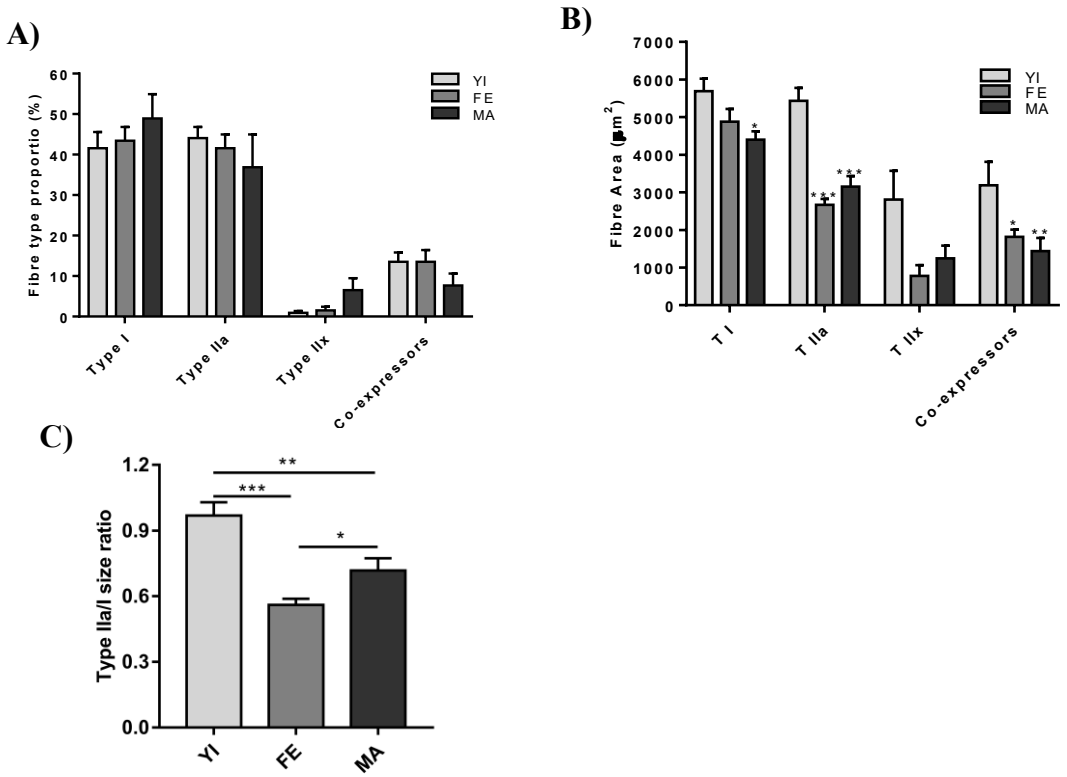
BMI = body mass index; FFMI = Fat free mass index; FFM = fat free mass; AMMI = appendicular muscle mass index; AMM = appendicular muscle mass; TUG = Timed up-and-go. Values are means ± standard error; †$p<0.05$ versus YI group, ‡$p<0.05$ versus FE group.

Figure 2: Skeletal muscle fiber morphology of YI, FE and MA.



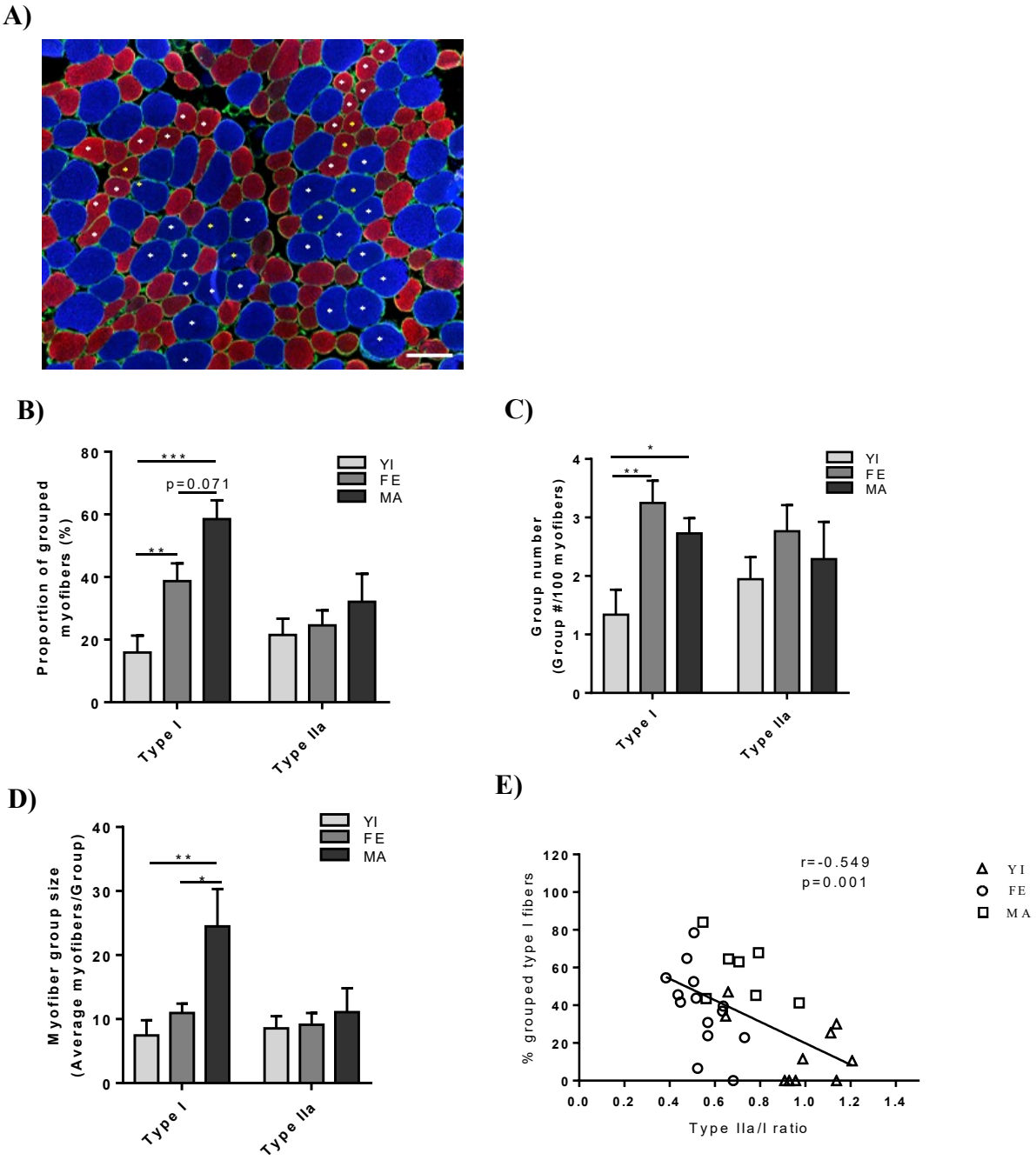
A) Representative cross-sections of a muscle samples from YI, FE and MA. MHC I (Blue); MHC IIa (Red); MHC IIx (Green); MHC IIa/IIx (Red/green); Laminin (Green). Scale bars = 100μm. **B)** Mean fiber cross-section area (YI, n=10; FE, n=17; MA, n=7). **C)** Fiber size distribution. Fibers below the dashed vertical line (<1441μm^2) are identified as atrophic (YI, n=10; FE, n=17; MA, n=7). **D)** Proportion of atrophic fibers in each group (YI, n=10; FE, n=17; MA, n=7). Graphs show mean ± SE. Scale bars = 100 μm. *versus YI; **versus YI; ***versus YI.

Figure 3: Muscle fiber size by type and type proportions in YI, FE and MA vastus lateralis muscle.



A) Fiber type proportion (YI, n=10; FE, n=17; MA, n=7). **B)** CSA of different fiber types (YI, n=10; FE, n=17; MA, n=7). **C)** Type IIa to I fiber size ratio (YI, n=10; FE, n=17; MA, n=7. Graphs show mean ± SE. Scale bars = 100 μm. *versus YI; **versus YI; ***versus YI.

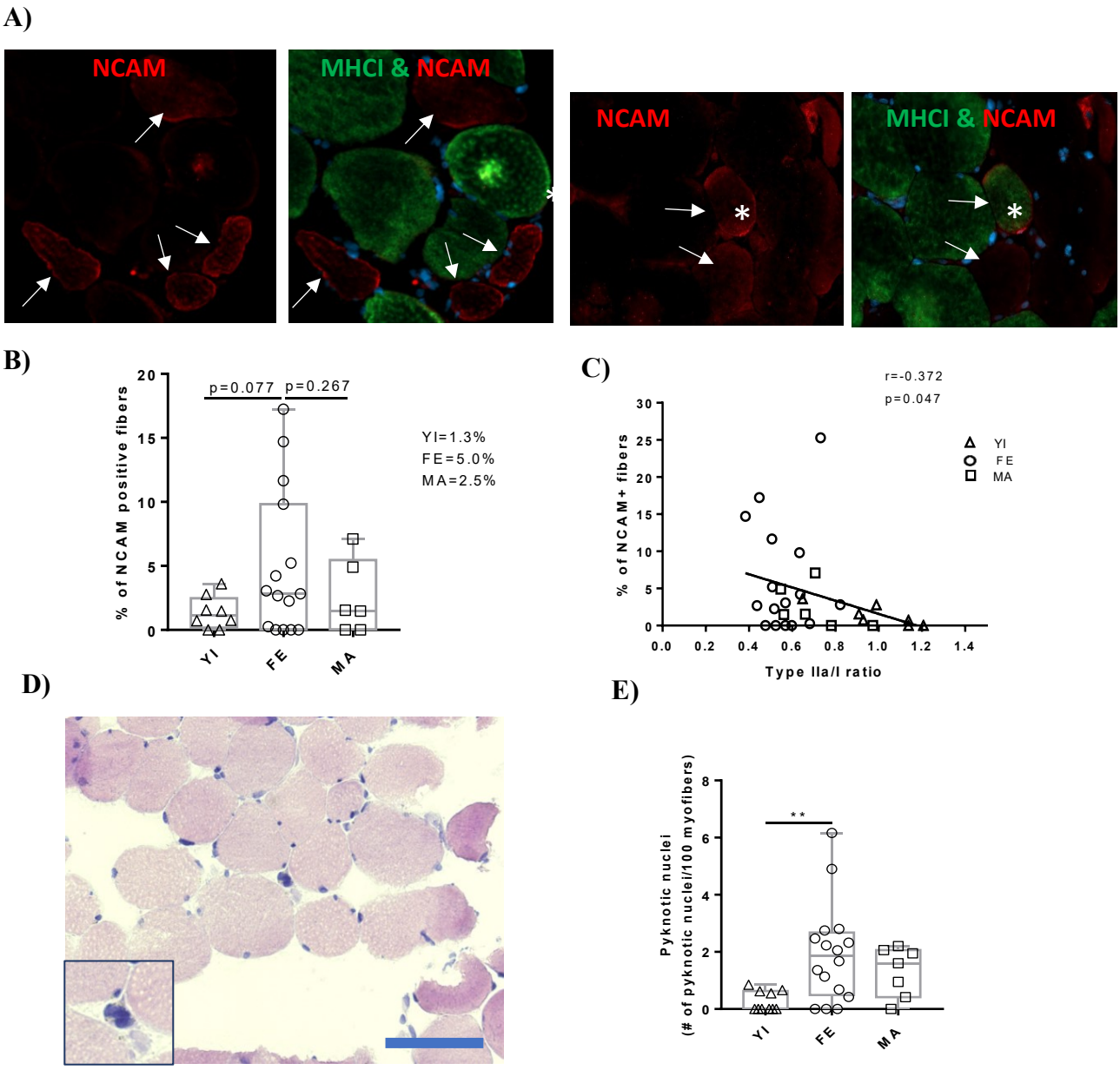
Figure 4: Presence of fiber grouping in FE and MA group.



A) Representative immunofluorescence image indicating pronounced grouped type I and type IIa myofibers in FE group (indicated by *). MHC I (Blue); MHC IIa (Red); MHC IIx (Green); MHC IIa/IIx (Red/green)(Scale bars = 100µm). **B)** Increased proportion of grouped muscle fibers in FE and MA driven by **(C)** increased number of myofiber per group only in MA and **(D)** increased number of myofibers in FE as well as MA group.

E) The relationship between fiber type I grouping and type IIa muscle fiber atrophy (YI, n=10; FE, n=14; MA, n=7; in FE group one outlier removed and two samples with insufficient material). Graphs show mean ± SE. *versus YI; **versus YI; ***versus YI.

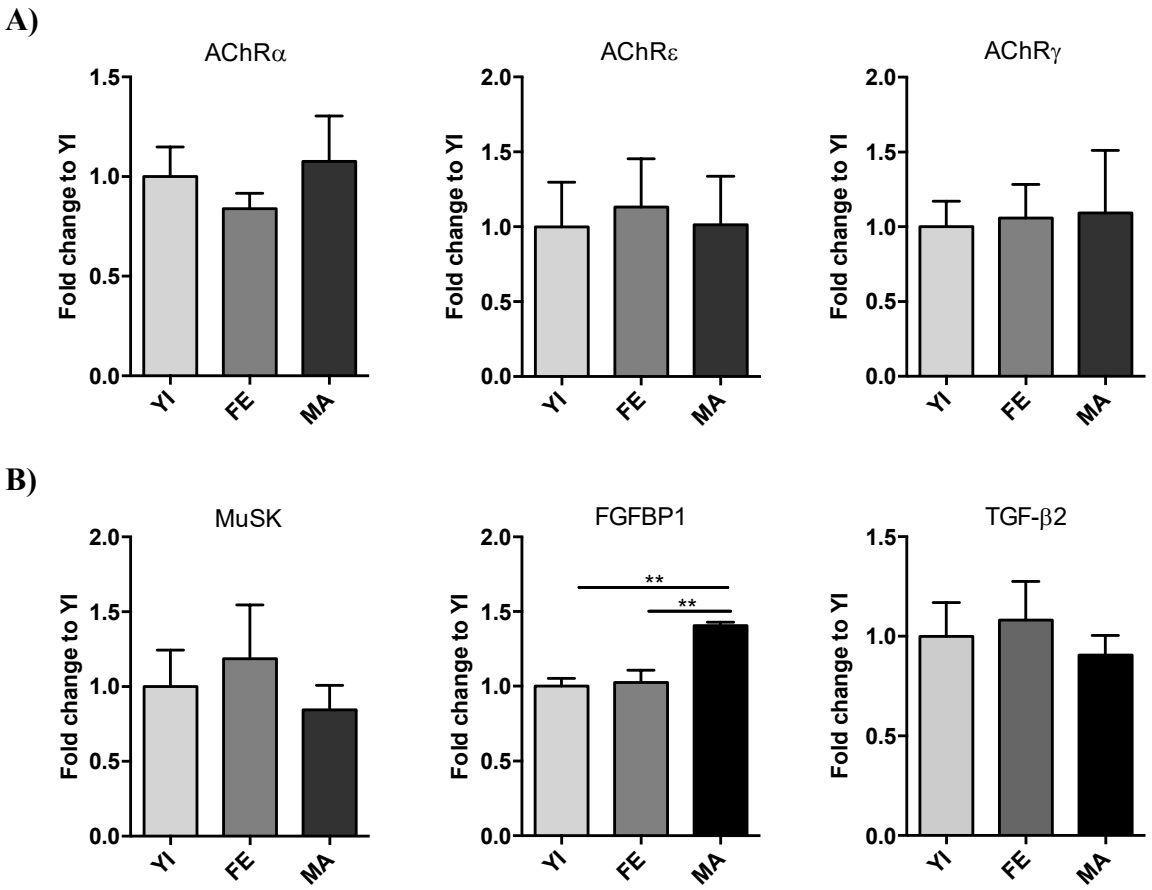
Figure 5: Presence of denervation-inducted markers in the vastus lateralis muscle of FE**.**



A) Representative immunofluorescence images of NCAM in FE muscle (NCAM = red; DAPI = Blue, MHC I = Green). NCAM positive fibers are indicated by arrow and an NCAM positive type I fiber is indicated by *. **B)** Abundance of $NCAM^{+}$ fibers in YI (n=8, two samples with insufficient material), FE (n=15, one outlier removed) and MA (n=6, n=15, one outlier removed and one sample with insufficient material) group. **C)** Negative association between the abundance of $NCAM^{+}$ fibers and type IIa to I fiber size ratio **D)** Histochemical H&E staining shows the presence of dense nuclear clumps with a rim cytoplasm = pyknotic nuclear clump

(arrow). **E)** Increased accumulation of pyknotic nuclei in FE (n=16) vs YI (n=10). Scale bars = 100µm, Graphs show mean ± SE. **versus YI.

Figure 6: Denervation-responsive transcripts.



A) Transcripts of AChR subunits. **B)** Transcripts affecting reinnervation. Graphs show mean ± SE. **versus YI.

(AChRα: YI, n=9; FE, n=9; MA, n=6; AChRε, YI, n=10; FE, n=12; MA, n=4; AChR γ, YI, n=10; FE, n=11; MA, n=5; MuSK, YI, n=10; FE, n=12; MA, n=5; FGFBP1, YI, n=10; FE, n=13; MA, n=5; TGF-β2, YI, n=10; FE, n=12; MA, n=4).

2.5. DISCUSSION

Individuals who experience greater deterioration of skeletal muscle with aging have elevated risk of physical frailty and other poor health outcomes, including increased mortality [8, 10]. Unfortunately, the processes that lead to exacerbated muscle impact with aging are largely undetermined. Further to this point, whilst previous work has underscored the importance of neurogenic impact as a key determinant of muscle deterioration with aging [34, 43], the specific role of denervation as a determinant of the severity of muscle aging between populations with low versus high physical function has not been addressed. To this end, in this study we proposed to evaluate the hypothesis that pre-frail/frail elderly women would exhibit marked features of denervation compared to young physically inactive females, whereas age-matched world class female masters athletes would exhibit attenuated indices of denervation compared to their age-matched pre-frail/frail counterparts. Consistent with this hypothesis, skeletal muscle from pre-frail/frail elderly women demonstrated marked fiber type grouping (suggesting recurring cycles of denervation-reinnervation), remarkable inter-individual heterogeneity in accumulation of NCAM-positive muscle fibers and a significant accumulation of pyknotic nuclei (evidence of denervation), coincident with a large accumulation of very small fibers (characteristic of sporadic persistent denervation). In contrast, skeletal muscle from world class octogenarian athletes demonstrated a greater degree of fiber type grouping compared to pre-frail/frail elderly women and a greater number of fibers per type group (suggesting greater reinnervation of denervated muscle fibers), coupled with elevated transcript levels of the reinnervation promoting cytokine FGFBP1. Thus, we conclude that denervation plays an important role in the exacerbated muscle impact seen in low functioning elderly women, and that superior reinnervation capacity plays an important role in attenuating aging muscle impact in high functioning elderly women.

2.5.1 Changes in muscle fiber size and proportion with advanced age

Skeletal muscle atrophy is a well-known consequence of the aging process and is the result of both a reduction in muscle fiber number and selective atrophy of the remaining muscle fibers [26]. It is often noted that different muscle fiber types are affected by the atrophy process to different degrees, with many studies concluding that type II fibers atrophy more than do type I fibers with aging [27, 33, 47]. One of the complicating factors in understanding atrophy behavior of type I fibers, however, is that in aging muscle the degree of type I/slow fiber atrophy can be masked by co-expression of type II/fast MHC isoforms [39], which is further complicated by the potential for misclassification of MHC co-expressing fibers [38]. Keeping this caveat in mind, a study in pre-frail elderly men reported a 47% reduction of type IIa fiber size and 17% reduction of type I fiber size compared to young controls [47]. Another study examining a cohort of frail individuals, consisting of 75% frail elderly women, reported a 57% decrease of type IIa and 25% decrease of type I fiber CSA when compared to young controls [3]. Thus, caveats acknowledged, aging muscle is characterized by a relatively greater atrophy of type IIa fibers.

Consistent with this notion, in the present study we also observed a relatively greater difference in type IIa fibers size of FE and MA versus YI subjects, and this was reflected in a reduced type IIa/I size ratio in both groups. On the other hand, MA subjects had a higher type IIa/I size ratio than FE, suggesting the aging impact was smaller in these highly functioning individuals. Interestingly, although both of the aged groups (FE, MA) exhibited a lower mean fiber size versus YI, only the FE group had a lower lean muscle mass. Thus, our data suggest that the greater lean muscle mass in MA relative to FE is due primarily to a better maintenance of muscle fiber number rather than fiber size.

Apart from obvious changes in myofiber size with advancing age, changes in fiber type composition are still a subject of debate. There are only a few studies that have assessed sex-

dependent changes in fiber type portion and found that elderly women (63-72y of age) have 6-10% greater abundance of type I fibers and 8-14% lower abundance of type IIx fibers compared to men [9, 35, 41], and Roberts et al. have previously commented that in human muscle from individuals below the age of 75 y, the abundance of MHC co-expressing fibers is typically low [41]. To the best of our knowledge, there is only one study in very old frail individuals (75% were women), and interestingly, when MHC composition was assessed in single fibers by SDS PAGE almost 52% of all muscle fibers were seen to be co-expressing multiple MHC isoforms, with the most abundant fibers co-expressing MHC I/IIa (28%) and MHCIIa/IIx (22%) [5]. Our cohort of FE subjects lie in between these extremes and are consistent with previous data showing that the proportion of MHC IIa/IIx co-expressing fibers between frail elderly [5] and young sedentary subjects [25] are similar. On the other hand, the size of MHC IIa/IIx co-expressing fibers has been reported to be 36% smaller in pre-frail elderly individuals compared to young controls [47], and this, too, is consistent with our observations. Based on available data, the origins of MHC co-expressing fibers differ between young sedentary versus aged individuals, with physical inactivity being the largest contributor in young and <75 y old subjects [4, 52], and denervation playing a more important role in advanced age [43]. This difference likely contributes to the smaller size of MHC co-expressing fibers in advanced age. Interestingly, high functioning individuals (e.g., MA) have been reported to retain more pure MHC isoforms and less co-expressing fibers [31]. In the present study, we did not observe any significant difference in fiber type proportions between groups, although MA tended to have lower abundance of MHC co-expressing fibers than FE and YI.

2.5.2. Denervation and fiber type grouping

It has been long recognized that the number of motor units declines with advanced age and contributes to changes in muscle fiber type, accumulation of atrophic fibers, and accumulation of myofibers that co-express multiple MHC isoforms [19]. Indeed, there are

numerous reports in aging muscle of fibers expressing proteins indicative of denervation, including the denervation-responsive cytokine NCAM [6, 30, 31] and the denervation-responsive sodium channel isoform $Nav_{1.5}$ [43, 50]. Furthermore, in old human muscle some MHC co-expressing fibers were found to express NCAM [31], consistent with our previous report in very old rat muscle showing more than 70% of MHC co-expressing fibers expressed the denervation-responsive $Nav_{1.5}$ [43]. In the present study we found an elevated proportion of atrophic muscle fibers in FE compared to YI, and a remarkably variable accumulation of NCAM positive fibers in the FE group compared to YI, with several FE subjects having >10% of their fibers being NCAM positive. In addition, we also found significant accumulation of pyknotic nuclei in the FE group, which is indicative of prolonged persistent denervation of muscle fibers [23]. Specifically, pyknotic nuclei occur because denervation causes displacement of myonuclei that, as atrophy progresses, results in a gathering of nuclei in the middle of what remains of the severely atrophic denervated muscle fiber [12]. As such, these findings support the presence of neurogenic atrophy and a relatively high burden of denervation in FE individuals. In contrast, MA tended to have a lower abundance of NCAM positive fibers and did not have an elevated abundance of pyknotic nuclei compared to YI, suggesting a milder impact of persistent denervation with aging in these highly functioning individuals. This is consistent with our previous analysis, showing that world class octogenarian MA men and women (the same women studied here in the MA group) exhibit a greater retention of motor unit number estimates in their tibialis anterior muscle than healthy non-athlete octogenarian controls [37].

During cycles of denervation-reinnervation in aging muscle, denervated fibers may be reinnervated by the original axon if present, or by collateral axon sprouting of a viable, neighboring motor neuron. When the latter occurs, this results in an increase in the number of myofibers per motor unit, which is manifest histologically as grouping of myofibers with the

same MHC isoform [19]. Interestingly, Kelly et al. 2018 reported that grouped type I fibers in elderly individuals express proteins that are normally specific to type IIa fibers, suggesting that at some point previously they had been type IIa fibers that were subsequently denervated and then reinnervated by a slow motor neuron, leading to an incomplete transition to type I fibers [24]. Two recent studies that included healthy older women [24, 41] reported that the age-related increase in fiber type grouping is driven by a higher number of myofibers per group (myofiber group size). This has been suggested to be indicative of a larger motor unit, and one which retains the capacity of successful reinnervation to promote maintenance of muscle mass [36]. In the present study, we found that although both FE and MA groups had significantly higher type I fiber grouping compared to YI controls, the MA group showed a trend to even greater type I fiber grouping compared to FE (P=0.07). Moreover, compared to YI group, FE and MA displayed an increased number of myofiber groups, where MA also showed significantly higher number of myofibers per group compared to the FE group. On this basis, we postulate that a limited reinnervation response contributes to smaller myofiber group size in FE versus MA, whereas MA have a more robust reinnervation response that is reflected in the greater number of myofibers per group and group number found in MA versus FE, as has been suggested previously in a slightly younger cohort of highly physically active elderly [31].

2.5.3. Reinnervation of Denervated Muscle Fibers

In seeking to explain the contrasting reinnervation responses between FE and MA, previous studies in transgenic mice showed that neuromuscular junction degeneration in a model of familial amyotrophic lateral sclerosis was associated with reduced levels of the reinnervation promoting cytokine FGFBP1 [51], and knockout of FGFBP1 exacerbates neuromuscular junction fragmentation of aging muscle [48]. Thus, the higher transcript levels of FGFBP1 in MA than FE suggests an inadequate response to denervation in FE and a robust response to denervation in MA. Although microRNA-206 [51] and transforming growth factor

ß2 (TGF- ß2) [48] have a positive and negative impact on muscle FGFBP1 levels, respectively, we have previously observed an up-regulation of miR-206 in very old and severely atrophied rat muscle [1], which in theory should promote higher muscle FGFBP1 production. As such, differential miR-206 levels seem an unlikely candidate to explain the contrasting FGFBP1 response between MA and FE. Further to this, Taetzsch et al. observed an increase in TGF-$ß_2$ in aged mouse muscle, which was linked to a reduction in FGFBP1 level at the neuromuscular junction of aged mice [48]. However, we did not observe any differences in the muscle transcript levels of TGF-$ß_2$ between FE and MA. On the other hand, this does not rule out differences in the circulating levels of TGF-$ß_2$ between groups and this, and other circulating factors that could affect muscle reinnervation, should be considered in future studies.

2.6. CONCLUSION

Our study shows that denervation is an important factor contributing to muscle alterations in prefrail/frail individuals, as demonstrated by the presence of severely atrophic myofibers that correlated with a reduced type IIa/I fiber size ratio, combined with fiber type grouping and abundant pyknotic nuclei. In contrast, highly functioning octogenarian master athletes had a lesser reduction in type IIa/I size ratio, which was accompanied by more fiber type grouping (more grouped fibers and larger group size) and higher expression of the reinnervation promoting FGFBP1. Therefore, the difference in age-related muscle impact between low and high functioning individuals is closely related to the degree of muscle denervation and reinnervation capacity. Hence, our results highlight the important contribution of denervation to age-related muscle deterioration and physical frailty, and suggest therapeutic approaches aimed at preventing physical frailty should focus on strategies for reducing denervation and/or encouraging successful reinnervation.

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Conflict of Interest. The authors have no conflicts of interest to declare.

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PREFACE TO CHAPTER 3

In Chapter 2 we showed that denervation is an important factor contributing to muscle alterations in prefrail/frail individuals and that the difference in age-related muscle impact between low and high functioning individuals is closely related to the degree of muscle denervation and reinnervation capacity. Another factor recognized to contribute to age-related muscle atrophy is mitochondrial functional impairment. Although experimental denervation can alter mitochondrial function, including increasing ROS in the form of fatty acid hydroperoxides [15], whether denervation is affecting mitochondrial function in aging skeletal muscle has only recently been considered [17]. A study in octogenarian men showed that muscle mitochondrial function alterations are at least partially secondary to denervation [17]. Whether this is also true in women has not been previously examined. In general, far less is known about mitochondrial function changes in skeletal muscle of aging women compared to men. To address this oversight in the literature, we first determined mitochondrial respiration capacity and ROS production in saponin-permeabilized myofibers of prefrail/frail elderly women. Next, with the purpose of investigating a modulating role of denervation on mitochondrial function, we pharmacologically assessed the denervation-induced mitochondrial ROS response in prefrail/frail elderly women. The significance of addressing the impact of denervation as a modulating force on mitochondrial function is to identify if denervation rather than mitochondria could be a potential therapeutic target to combat age-related muscle atrophy and/or impaired muscle function in elderly individuals.

Chapter 3: Reduced Mitochondrial Content, Elevated ROS, and Modulation by Denervation in Skeletal Muscle of Pre-frail/Frail Elderly Women

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3.1. ABSTRACT

Denervation and mitochondrial impairment are implicated in age-related skeletal muscle atrophy, and may play a role in physical frailty. We recently showed that denervation modulates muscle mitochondrial function in octogenarian men, but this has not been examined in elderly women. On this basis we tested the hypothesis that denervation plays a modulating role in mitochondrial impairment in skeletal muscle from prefrail/frail elderly (FE) women. Mitochondrial respiratory capacity and reactive oxygen species (ROS) emission were examined in permeabilized myofibers obtained from *Vastus lateralis* muscle biopsies from FE and young inactive (YI) women. Muscle respiratory capacity was reduced in proportion to a reduction in a mitochondrial marker protein in FE, and mitochondrial ROS emission was elevated in FE versus YI group. Consistent with a significant accumulation of neural cell adhesion molecule positive muscle fibers in FE (indicative of denervation), a 50% reduction in ROS production after pharmacologically inhibiting the denervation-mediated ROS response in FE women suggests a significant modulation of mitochondrial function by denervation. In conclusion, our data support the hypothesis that denervation plays a modulating role on skeletal muscle mitochondrial function in FE women, suggesting therapeutic strategies in advanced age should focus on the causes and treatment of denervation.

Key words: Mitochondrial function, myofiber size, sarcopenia.

3.2. INTRODUCTION

One of the most prominent physiological changes occurring with aging is the progressive decline of physical capacity (1) which is frequently a precursor to a progressive deterioration of physical function that when severe can lead to physical frailty (2). Frailty can be described as a pre-disability condition that is associated with low ability to respond to physical as well as psychosocial stressors, which can eventually lead to the development of disabilities and loss of autonomy (3). Women have a higher rate of physical frailty (4), which is partly due to their higher life expectancy (UN 2015). Amongst the most important contributors to physical frailty are alterations in skeletal muscle that precipitate progressive weakness with advancing age (2). Although mitochondrial changes are frequently implicated in muscle alterations with aging (5), and some studies have reported greater mitochondrial impairment in elderly with low physical function (6, 7), little is known about mitochondrial function changes specifically in prefrail/frail elderly women.

Skeletal muscle denervation (8) and mitochondrial impairment (9) have long been considered important factors involved in age-related skeletal muscle atrophy. However, the relationship between these factors, and specifically whether there may be circumstances when mitochondrial impairment occurs secondary to muscle denervation in aging muscle, has only recently been considered in aging men (10). The rationale for considering a central role for denervation in causing mitochondrial impairment in aging muscle is based upon two parallel bodies of knowledge. Firstly, in very advanced age there is a large accumulation of small angular fibers (10), the majority of which express a marker of denervation in a rodent model of aging (11), and wherein denervated fibers exhibit up-regulation of ubiquitin ligases associated with muscle atrophy (11). Secondly, surgical denervation of muscle leads to elevated mitochondrial reactive oxygen species (ROS) production (12), sensitization to mitochondrial permeability transition (13), and reduced respiratory capacity (14), and these are changes

commonly seen in aging skeletal muscle (15). Consistent with this rationale, we have recently shown very similar alterations in these different indices of mitochondrial function in a mouse model of sporadic denervation independent of aging (10), and that in octogenarian men muscle mitochondrial function alterations are at least partially secondary to denervation (10). Generally speaking, there is far less known about mitochondrial function changes in skeletal muscle of women with aging. Further to this point, how skeletal muscle mitochondrial function is altered and whether mitochondrial function is also modulated by denervation in prefrail/frail elderly women is unknown, but it is important to establish in the context of identifying appropriate treatment strategies as there is evidence for sex-specific differences in skeletal muscle mitochondria (16). To address this gap, we obtained biopsies from the vastus lateralis muscle of healthy young physically inactive women and prefrail/frail elderly women and measured mitochondrial respiratory capacity and ROS production in saponin-permeabilized myofibers. We hypothesized that there would be alterations in mitochondrial function in muscle of pre-frail/frail elderly women and that denervation would play a modulating role in driving the changes in mitochondrial function. To our knowledge this is the first study assessing skeletal muscle mitochondrial function in prefrail/frail elderly women.

3.3. METHODS

3.3.1. Study participants

22 elderly pre-frail and frail elderly women (FE; 78± 5y) were recruited from the local Montreal area through local press (Senior Times) and by referral from the geriatric clinics of the McGill University Health Centre. The frailty syndrome was defined based on Fried criteria (17). A participant was defined frail if she presented three or more of the following criteria: (i) unintentional weight loss or low muscle mass, (ii) physical inactivity, (ii) weakness, (iv) slow walking speed and (v) self-reported exhaustion (Supplementary Table 1). A pre-frail status was defined as having one or two of the above-mentioned criteria. 12 young physically inactive female controls (YI; 23± 3y) were recruited from the McGill University community. They were screened for eligibility for physical inactivity with the Godin-Shephard leisure-time physical activity questionnaire (18) and one week measurement of physical activity level with Actigraph accelerometer. Participants were excluded if they reported any heart, lung and/or central nervous system diseases; had an eating disorder; were diagnosed with dementia, depression, or diabetes; had abnormal complete blood count test; took blood thinning or anti-coagulant medications; or had walking aids. Additional exclusion criteria for the young participants was being physically active (≥150min/week of structured activity) or were <20 or >30 years of age. There was no attempt to restrict the study measures to a particular phase of the menstrual cycle in YI. This study was approved by the Research Ethics Board (REB) of McGill University Health Centre (Montreal, QC, Canada)(BMC-06-015; 13-211-BMB) and all participants provided written informed consent.

3.3.2. Skeletal muscle biopsy

Percutaneous skeletal muscle biopsy of the *vastus lateralis* muscle was performed after an overnight fast using the modified Bergstrøm needle technique with suction (19). Approximately 20-30mg of sample was used for histology and mounted with triganth gum,

frozen in isopentane pre-cooled in liquid nitrogen and stored at -80°C for muscle histology (see below). 60-70mg of sample was used to assess mitochondrial function on the day of collection (see below).

Due to limited muscle tissue from a few participants, some measurements were only performed on a subset of muscle biopsy specimens. The sample size used for each measurement is indicated in the results section of each technique used in this study.

3.3.3. Muscle immunofluorescent labelling

Myosin Heavy Chains. Skeletal muscle tissue blocks were cut to 10-µm tissue sections on a cryostat (HM505E; Microm, Walldorf, Germany) at -22°C and mounted on glass slides (Double frosted microscope slides; Fisher Scientific), air dried for 2h and stored at -80°C. Muscle cross-sections were probed using primary antibodies against the basal lamina and myosin heavy chain (MHC) isoform proteins. Slides were first removed from -80°C and allowed to air dry for 1h at room temperature. Sections were hydrated with 1xphosphate buffered saline (PBS) and blocked with 10% normal goat serum in 1xPBS for 30min. Sections were next incubated for 1h at room temperature with the following primary antibodies: polyclonal rabbit anti-laminin IgG (L9393, 1:700; Sigma-Aldrich), monoclonal mouse anti-MHCI IgG2b (BA-F8, 1:25), monoclonal mouse anti-MHCIIa IgG1 (Sc71, 1:200) and monoclonal mouse anti-MHCIIx IgM (6H1, 1:25). All MHC primary antibodies were purchased from Developmental Studies Hybridoma Bank (University of Iowa, USA). After incubation, tissue sections underwent a series of 1xPBS washes and incubation with fluorescently labeled secondary antibodies for 1h at room temperature: Alexa Fluor 488 goat anti-rabbit IgG (A11008, 1:500), Alexa Fluor 350 goat anti-mouse IgG2b (A21140, 1:500), Alexa Fluor 594 goat anti-mouse IgG (A21125, 1:100), and Alexa Fluor 488 goat anti-mouse IgM (A21042, 1:500). All secondary antibodies were obtained from ThermoFisher Scientific. After incubation muscle cross-sections were washed 3x5min in 1xPBS and mounted with cover slips using Prolong-Gold mounting medium (Invitrogen,

ThermoFisher Scientific). Images of the stained tissue sections were acquired with a Zeiss fluorescence microscope (Axio Imager M2, Carl Zeiss, Oberkochen, Germany) and analyzed with ImageJ (National Institute of Health, Bethesda, MD, USA). A minimum of 100 fibers were sampled per muscle section and analyzed for their shape and cross-sectional area. Atrophied fibers were determined based on the first percentile in YI controls. The first percentile was determined following the calculation of muscle fiber size distribution of all fibers for all YI subjects, where the first percentile size corresponded to fibers with a size ≤1441 μm^2.

Neural Cell Adhesion Molecule (NCAM). In serial muscle cross-sections we labeled with a primary antibody against NCAM (AB5032; 1:100; Millipore Sigma) to provide an indication of the degree of denervation in YI versus FE muscle. Tissue slides were fixed in 4% paraformaldehyde, washed with 1xPBS and permeabilized with 0.1% Triton-X. After blocking, slides were incubated with anti-NCAM antibody and anti-MHCI antibody overnight at 4°C. The next day tissue sections were washed with 1xPBS and incubated with fluorescence labeled secondary antibodies for 1h at room temperature. For NCAM Alexa Flour 594 (1:200) and MHCI Alexa Flour 350 (1:500) were used. A serial tissue section was stained for laminin as described above.

3.3.4. Mitochondrial function measurement

Mitochondrial function measures were done as previously described (10). Briefly, muscle tissue was immediately placed in ice-cold buffer A (Supplementary Table 2) for manual dissection under a stereomicroscope. Muscle tissue was manually dissected and separated into small bundles (2-6mg) of muscle fibers. Bundles of muscle fibers were subsequently placed in ice-cold buffer A with 0.05mg/ml of saponin to allow chemical permeabilization for 30min at low rocking speed.

3.3.5. High-resolution respirometry

Following 30min saponin permeabilization, myofibers were washed 3x10min in buffer B (Supplementary Table 2). Oxygen consumption was subsequently measured using a high-resolution respirometer (Oxygraph-2k; Oroboros, Innsbruck, Austria). Polarographic oxygen sensors were calibrated by a two-point calibration for air saturation and zero oxygen concentration. 3-5mg of wet weight permeabilized myofibers were added to 2ml of buffer B in the respirometer and equilibrated at 37°C with constant stirring. Respiration was conducted under hyperoxygenated conditions to limit oxygen diffusion limitation. Oxygen flux was assessed by the titration of substrates, where each addition was followed by a short period of stabilization. To obtain state 2 respiration glutamate (10mM) and malate (5mM) were simultaneously added to the chamber. Next, with the titration of ADP (2mM), ATP synthesis was stimulated and maximal state 3 respiration driven by complex I substrates was achieved. To assess state 3 respiration with both complex I and complex II substrates, the complex II substrate succinate (10mM) was added to the chamber. To check the integrity of the outer mitochondrial membrane cytochrome c (5mM) was added to the substrate mix, wherein an increase in respiration following addition of exogenous cytochrome c signifies damage to the outer mitochondrial membrane. Next we added antimycin A (10μM) to inhibit complex III, and with the titration of ascorbate (10mM) and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (1mM) we assessed complex IV respiratory capacity independent of the upstream complexes. Values obtained by TMPD-stimulated respiration were corrected for auto-oxidation by TMPD, as done previously (10). After these measurements, the degree of mitochondrial respiration coupling was assessed by calculating the acceptor control ratio (state III respiration with GM substrates/state II respiration with GM substrates). After respiration was completed, muscle bundles were frozen in liquid nitrogen and stored at -80°C for protein measurement.

3.3.6. Measurement of mitochondrial ROS emission

To measure reactive oxygen species emission, myofibers were washed 3x10min in buffer Z (Supplementary Table 2). ROS production was detected by recording the rate of generation of the fluorescent compound resorufin from Amplex Red. Resorufin is formed by the reaction of Amplex Red and hydrogen peroxide as well as fatty acid hydroperoxides (FA-OOH) released from the mitochondria, and catalyzed by horseradish peroxidase. A fluorescence spectrophotometer (Hitachi F2500, Tokyo, Japan) was used to measure the fluorescence signal at an excitation/emission wavelength of 563/587nm. The fluorescent signal was measured with the FL Solution Software (Hitachi High Technologies Corporation, Tokyo, Japan). Approximately 3-6mg of muscle fiber bundles were added to 600µl of solution Z, with 5.5µM Amplex red and 1U/ml HRP in a glass cuvette at 37°C and with a magnetic stirrer. After a short period of baseline auto-fluorescence, muscle fibers were added into the cuvette and the following substrates were titrated: Glutamate (10mM) + Malate (2mM), Succinate (10mM), ADP (10µM), ADP (100µM) and Antimycin A (10µM).

3.3.7. Measurement of denervation induced mitochondrial ROS emission

It has been established previously that there is an increase calcium-dependent phospholipase A_2 ($cPLA_2$) activity in denervated muscle that causes the release of ROS from mitochondria in the form of FA-OOH and that these FA-OOH are detected with Amplex Red (20). As the denervation-induced ROS response detected by Amplex Red is specific to $cPLA_2$ activity rather than ROS emission in general, it can be used as a means of identifying the influence of denervation on mitochondrial function, as shown in mouse models of sporadic denervation such as the SOD1 knockout and SODG93A amyotrophic lateral sclerosis models (20), and the neurotrypsin over-expressing mouse model (10). Thus, to determine whether denervation is modulating mitochondrial function we inhibited the activity of $cPLA_2$ with the application of arachidonyl trifluoromethyl ketone ($AACOCF_3$), as previously described by

Bhattacharya *et al.* (20) and used in our recent study of elderly men (10). To confirm the specificity of the ROS response to the cPLA2 isoform, and thus, to the influence of denervation, we also applied bromoenol lactone (BEL), a specific inhibitor of the calcium-independent PLA_2 isoform ($iPLA_2$) that is associated with muscle ROS production in the context of disuse muscle atrophy (21), to a subset of samples. A reduction in Amplex Red signal (resorufin) with $cPLA_2$ inhibition but not $iPLA_2$ inhibition is interpreted as evidence for modulation of mitochondrial function by denervation (20). Saponin permeabilized muscle fibers were washed 3x10 min in buffer Z with $AACOCF_3$ (20µM) and buffer Z with ethanol (EtOH; vehicle control). The Amplex Red signal was measured as mentioned previously in the presence or absence (EtOH vehicle only) of $AACOCF_3$, to assess the contribution of FA-OOH to the endogenous ROS production.

After mitochondrial function measurements were completed, muscle bundles were frozen in liquid nitrogen and stored at -80°C for protein measurement. All mitochondrial function experiments were analyzed using an in-house analysis program created using Igor Pro Software (Wavemetrics; https://www.wavemetrics.com).

3.3.8. Mitochondrial content estimation

Skeletal muscle fibers that were used in the mitochondrial function assays were subsequently probed for protein levels of voltage-dependent anion channel (VDAC) by Western blotting, where the content of the VDAC (an outer membrane protein) was used as a general indicator of mitochondrial content. Between 6 to 35mg of muscle was homogenized 2x45s with a robot homogenizer (Minibead-beater, Biospec Products, USA) with a 10x volume of RIPA extraction buffer (Supplementary Table 2). For homogenization, 1.4mm ceramic beads were used. After 2h incubation at 4°C the samples were centrifuged at 12,000g for 20min at 4°C. The supernatant was removed and used to measure protein content with the Bradford assay. Immunoblotting was performed with 15µg of tissue protein diluted in 4x Laemli buffer and

extraction buffer, and boiled at 95°C for 5min. 24µl of sample was then loaded onto a 12% acrylamide gel, electrophoresed by SDS-PAGE and transferred at 4°C to polyvinylidene fluoride membranes (Amersham Hybond ECL, GE Healthcare Life Sciences). The membranes were blocked in 5% semi-skimmed milk for 1h at room temperature and incubated over night at 4°C with a primary mouse monoclonal anti-VDAC antibody (ab14734, 1:1000; Abcam) diluted in 5% BSA. Because β-actin, GAPDH and α-tubulin tend to decrease with age, total protein loading was used to normalize protein levels (22). Membranes were incubated with rabbit anti-mouse HRP-conjugated secondary antibody diluted in 5% milk (ab6728, 1:2000; Abcam) for 1h at room temperature. Protein bands were detected with SuperSignal™ West Pico Chemiluminescent Substrate (Fisher Thermo Scientific, Waltham, MA, USA) and imaged with a BIO-RAD image system (BIO-RAD ChemiDoc™MP Imaging System). Identification of VDAC was performed using Image Lab™ Software, where both bands at the approximate molecular mass of VDAC in a given blot were summed to obtain an index of the quantity of VDAC protein (22).

3.3.9. Statistical analysis

In all cases except ROS emission for the denervation-induced ROS estimation, the comparison between YI and FE group was performed with an unpaired two-tailed Students t-test. The presence of outliers was tested with the ROUT method in GraphPad Prism 7. Results are expressed as mean ± standard deviation (SD). Differences between groups were considered statistically significant at $P<0.05$. Differences in the Amplex Red signal during the denervation-induced ROS estimation between EtOH controls and either AACOCF3 or BEL treatment were analyzed by paired t-test. Data was statistically analyzed using GraphPad Prism 7 for Microsoft Windows.

3.4. RESULTS

3.4.1. Study Participants

Based on the Fried criteria of frailty (17), 8 participants were characterized as pre-frail and 14 as frail. The main physical characteristics of the participants are summarized in Table 1. Two participants in the pre-frail group had low physical activity (PA) as the only parameter meeting the prefrail criteria (data not shown). The measurement of PA is not well standardized (3), and it has been suggested, therefore, that low PA alone is not a good indicator of the prefrail status. However, since their age and other data (described below) fits with other subjects in the prefrail/frail group, the inclusion of these 2 participants does not affect the conclusions about the FE group. Both age groups were similar in weight, height and body mass index (BMI)($p>0,05$). Within the FE group, there were no differences between prefrail and frail subjects in terms of myofiber size, mitochondrial respiration capacity and ROS production, hence prefrail and frail subject were combined into FE group.

3.4.2. Muscle fiber size and size distribution

Figure 1 shows representative micrographs of muscle cross-sections from YI and FE (A), revealing a reduced mean fiber size (B), and a marked abundance of very small muscle fibers ($\leq$1441 μm^2) in limb muscle of the FE group (C).

3.4.3. Mitochondrial content and respiration

VDAC was used as a marker for mitochondrial content. Representative western blots are depicted in Fig. 2A. The abundance of the VDAC protein was significantly lower in the FE (n=21) compared to the YI (n=12) group (Fig. 2B; p=0,004). Whereas State 3 respiratory capacity of complex I (GM+ADP) did not reach statistical difference between the groups (p=0,07), state 3 respiration driven by complex I and II substrates (GM+ADP+Succinate) was significantly lower in the FE group (Fig. 3A; p=0,019). Additionally, we found that the respiratory capacity of complex IV was significantly lower in FE versus YI (Fig. 3A; p=0,003),

as assessed using the artificial electron donor TMPD following inhibition of complex III using antimycin A.

Next, we evaluated mitochondrial respiratory capacity after normalization to mitochondrial content (VDAC protein), and found this abolished the differences in respiratory capacity between groups (Fig. 3B), suggesting that the reduced muscle respiratory capacity in FE was a function of lower mitochondrial content. We then assessed the oxidative phosphorylation coupling, by the respiratory acceptor control ratio, and found a significantly lower ACR in the older group (Fig. 3C; p=0,028). Note that based upon a >10% increase in respiration with the addition of exogenous cytochrome c following the succinate step, the integrity of the outer mitochondrial membrane was judged to be interrupted in 5 cases (FE, n=3; YI, n=2). These samples were excluded from the mitochondrial respiration capacity data set reported above.

3.4.4. Mitochondrial ROS production

ROS emission under state 2 and state 3 respiration when normalized to wet weight did not differ between groups (Fig. 4A; FE, n=20; YI, n=12; G+M; +Succinate; p>0,05). After mitochondrial ROS emission rates were expressed relative to mitochondrial content (VDAC protein), the FE group demonstrated a significantly greater ROS emission under state 1 respiration as well as under state 2 and state 3 respiration compared to the YI group (Fig. 4B; FE, n=20; YI, n=12; Fibers, p=0.02; G+M, p=0,001; +Succinate, p=0,002; ADP 0,1M, p=0,009; ADP 1M, p=0,008), revealing a greater intrinsic mitochondrial ROS emission in FE.

3.4.5. Impact of muscle denervation on mitochondrial function

To establish the extent of denervation in our sample, we labeled muscle cross-sections with an antibody against the denervation-responsive cytokine, NCAM. As shown in Fig 5A & B, there was a pronounced increase in the abundance of muscle fibers that were positive for NCAM labeling in the FE group (p=0.039). Following-up on this, to determine if muscle

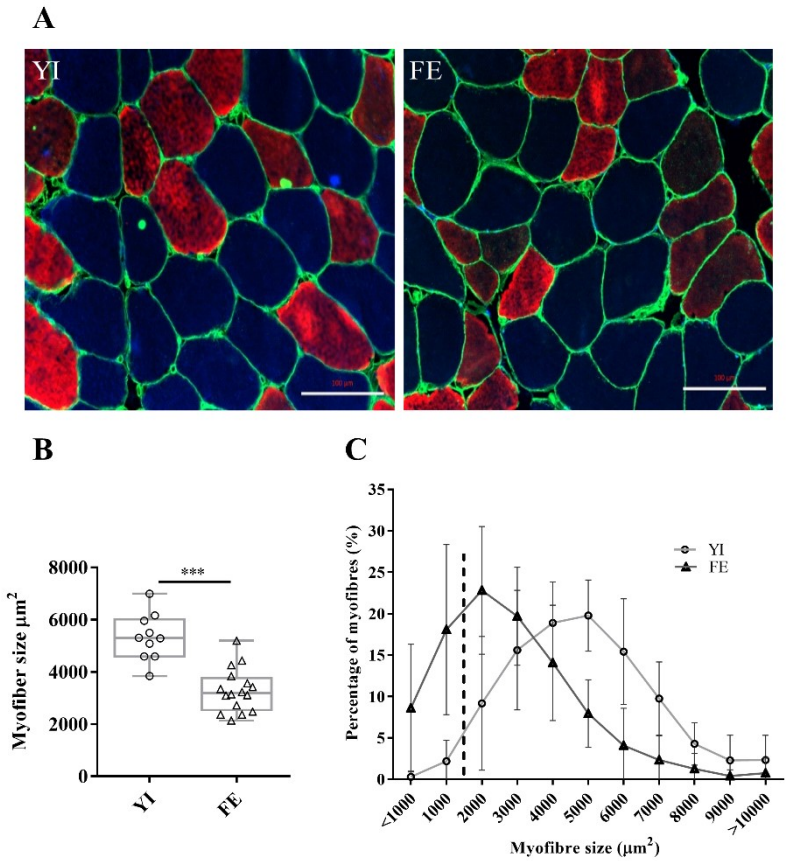
denervation was influencing mitochondrial function in our measures we used a pharmacological approach with the application of the $cPLA_2$ inhibitor $AACOCF_3$ to inhibit the denervation-induced mitochondrial ROS response (20), as we have done previously in octogenarian men and a mouse model of sporadic denervation (10). $cPLA_2$ inhibition significantly reduced ROS production compared to EtOH vehicle in FE (n=21) (Figure 5C; p<0,001), but had no effect on ROS production in the YI group (n=12) (Fig. 5C; p=0,403). In contrast to what was observed with $cPLA_2$ inhibition in FE, use of the iPLA2 inhibitor BEL had no effect on ROS production in a subset of FE (Fig. 5D), showing that a reduction in ROS emission in FE was specific to the contribution of ROS from the denervation-induced $cPLA_2$ isoform (20).

Table 3: Descriptive characteristics of YI and FE participants.

Characteristics	**YI (n=12)**	**FE (n=22)**
Age (years)	24.0 ± 3.5	78.0 ± 5.9***
Height (cm)	162.5 ± 0.1	156.9 ± 7.7*
Weight (kg)	61.8 ± 11.9	64.1 ± 10.0
BMI (kg/m^2)	23.5 ± 4.6	26.2 ± 4.7

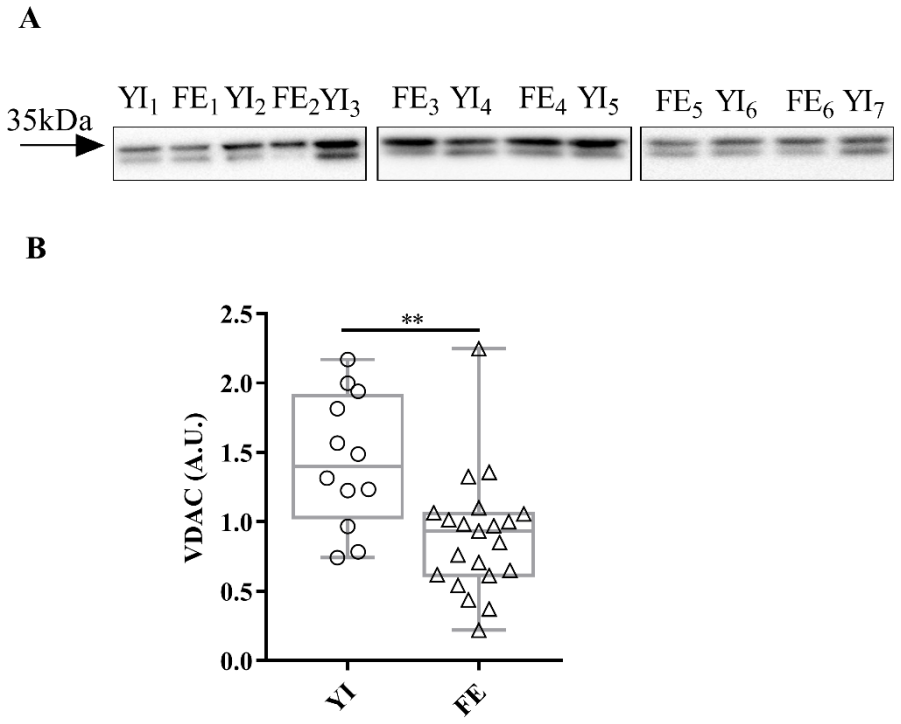
Notes: FE=pre-frail/frail elderly, YI=young inactive, BMI=body mass index
*p<0.05, ***p<0.001
Values presented as mean ± SD

Figure 7: Skeletal muscle fiber morphology in vastus lateralis muscle of YI and FE.



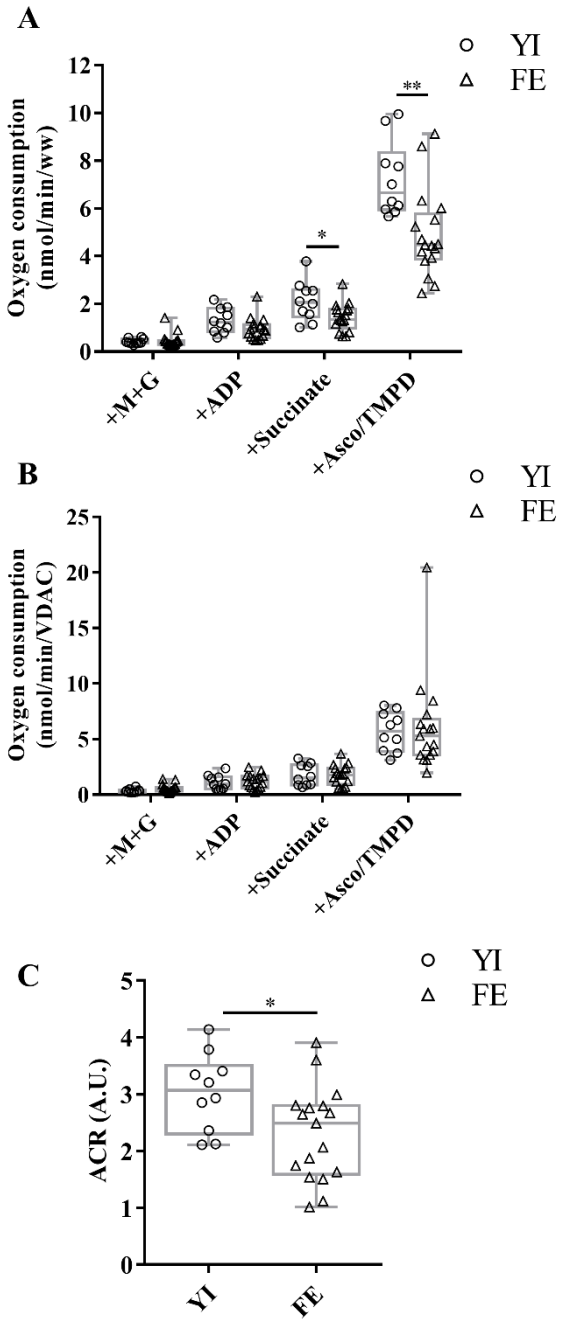
A) Skeletal muscle cross-sections immunolabeled for laminin (cell membrane, green), type I (blue), type IIa (red) and type IIx (green) myosin heavy chain isoforms. **B)** Mean fiber cross-sectional area (YI, n=10; FE, n=16). **C)** Fiber size distribution. The dashed vertical line represents fibers that were atrophied (YI 1.3%; FE 15.2%) determined based on the first percentile in YI controls. Graphs show mean ± SD. Scale bars = 100µm. ***p<0,001.

Figure 8: Changes in mitochondrial content in human muscle.



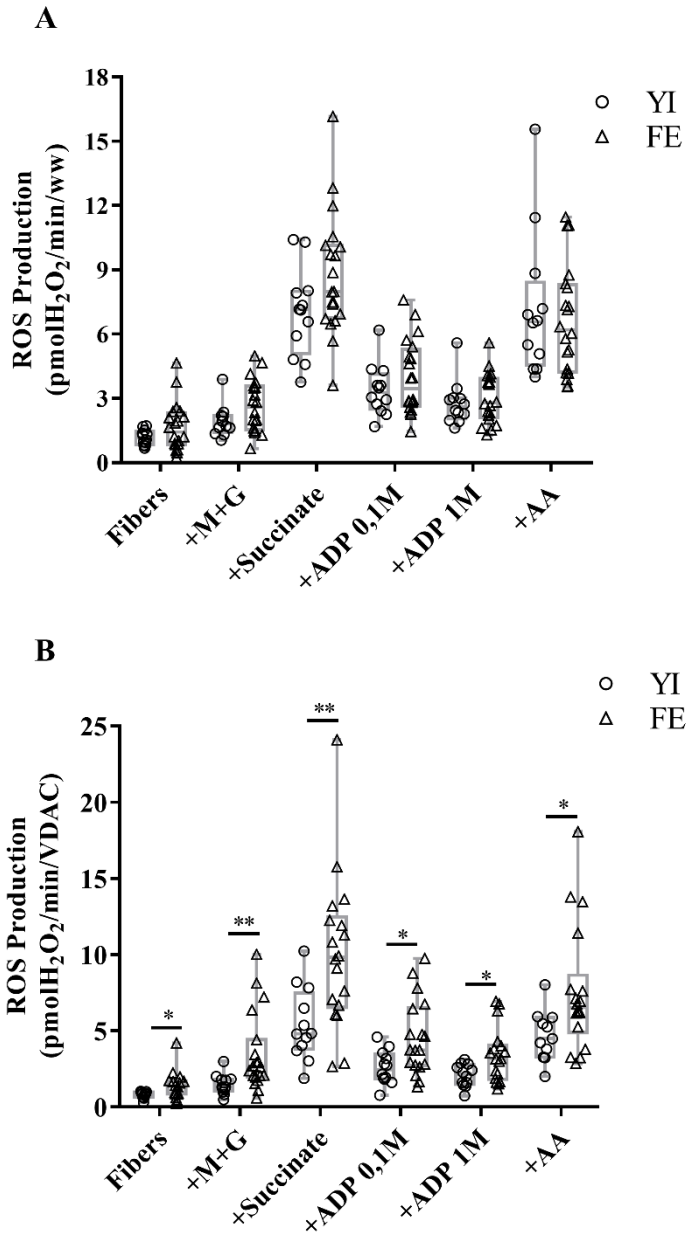
A) Representative sections from three separate western blots showing VDAC protein. Each section from a separate western blot is specifically outlined. **B)** Abundance of VDAC protein in *Vastus lateralis* muscle of YI and FE participants with corresponding western blot (YI, n = 12; FE, n = 21). Both bands at the approximate molecular weight of VDAC were summed to represent VDAC content. Graphs show mean ± SD. **p<0,01.

Figure 9: Mitochondrial respiratory capacity in permeabilized muscle fiber bundles.



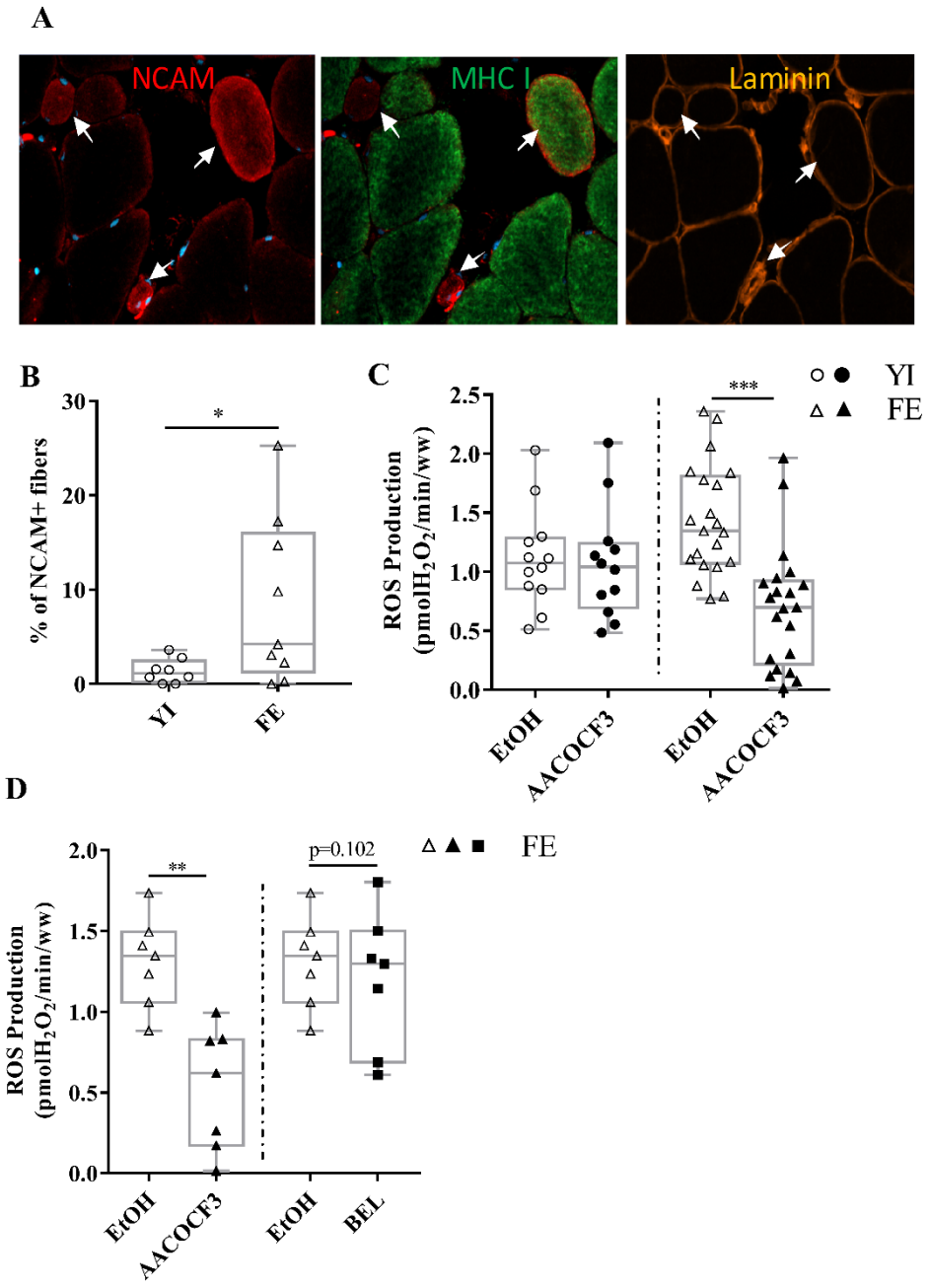
A) Oxygen consumption measured in YI (n=10) and FE (n=17) group normalized to fiber wet weight. **B)** Oxygen consumption normalized to the abundance of VDAC protein. **C)** Differences in mitochondrial oxidative phosphorylation coupling between YI and FE group. Graphs show mean ± SD. *p<0,05; **p<0,01.

Figure 10: Mitochondrial ROS production in permeabilized muscle fiber bundles.



A) Differences in ROS signal between YI (n=12) and FE (n=20) group when normalized to wet weight. **B)** Differences in ROS signal between groups (YI, n=12; FE, n=20) when normalized to the abundance of VDAC protein. Graphs show mean ± SD. *p<0,05; **p<0,01.

Figure 11: Evidence of denervation and its modulating role on mitochondrial function.



A) Representative images of a section labeled with NCAM, MHC I, and laminin from a FE subject. **B)** Plot of the abundance of NCAM positive muscle fibers in YI (n=8) and FE (n=9) groups. **C)** Changes in ROS signal compared between vehicle control (EtOH) and the addition of the cytoplasmic phospholipase A_2 ($cPLA_2$) inhibitor, $AACOCF_3$ in the older group (YI, n=12; FE, n= 21).

D) Changes in ROS signal amongst FE (n=7) with the addition of the $cPLA_2$ inhibitor AACOCF3 compared to the addition of the calcium-independent phospholipase A2 ($iPLA_2$) inhibitor bromoenol lactone (BEL). Graphs show mean ± SD. **p<0,01; ***p<0,001.

3.5. DISCUSSION

Skeletal muscle is abundant in mitochondria that help to provide the energy required for normal muscle function. Furthermore, mitochondria are also involved in homeostasis (e.g., pH, Ca^{2+}), apoptotic signaling, and are an important source of ROS generation in the cell (9). Alterations in these processes occur in association with age-related skeletal muscle atrophy (15). Muscle mass loss, exacerbated muscle fatigue and reduced muscle strength are key components of the frailty syndrome, hence mitochondria have also been implicated in the frailty syndrome (7, 23, 24). Nevertheless, although women are at a greater risk of becoming frail, there is limited information about mitochondrial alterations in skeletal muscle of elderly women. Furthermore, it has been recently shown that skeletal muscle mitochondrial function alterations in octogenarian men are at least partly secondary to myofiber denervation (10) and this has not yet been addressed in pre-frail/frail elderly women. As such, the purpose of the present study was to assess mitochondrial function changes in skeletal muscle, and the potential modulating impact of denervation, in pre-frail/frail elderly women.

3.5.1. Mitochondrial function in aging muscle

Several studies have shown declines in the activity of various mitochondrial enzymes in older women (25-27), but only one has assessed this issue in frail elderly women, finding a lower phosphocreatine recovery time by magnetic resonance spectroscopy and lower mitochondrial respiratory complex activities (28). Interestingly, another study found that elderly women with lower mitochondrial DNA (mtDNA) copy number assessed in blood samples were more likely to be frail (24). This group have also identified a specific mtDNA variant which is associated with the control region of mtDNA replication that is suggested to alter mtDNA copy number in the frail population (24, 29). Although the cause and effect relationship could not be assessed, these findings also support the potential role of mitochondria in the frailty syndrome. Another study in frail women found reduced levels of nuclear respiratory factor 1 (NRF1) transcript in

skeletal muscle tissue when compared to healthy active elderly women (23), which may indicate that frail women have altered mitochondrial biogenesis secondary to reduced physical activity. A study that assessed various aspects of mitochondria in skeletal muscle of three generations of women, found that elderly women had lower citrate synthase activity and mtDNA copy number, whereas indices of mitochondrial biogenesis were not affected (26). The same study found that mtDNA mutations may not be the main contributor to age-related mitochondrial dysfunction, based upon the deletions being present in the noncoding regions of the mtDNA (26). Elderly women also appear to have reduced mitochondrial size, as assessed by electron microscopy, suggesting a degree of mitochondrial fragmentation (30). Whereas one study in very old (mean age of 83y) physically inactive women observed a tendency to reduced transcript levels of mitofusin 2 (mfn2) and dynamin related protein 1 (Drp1), suggesting impaired mitochondrial fission and fusion signaling (23), another study examining a cohort consisting of 45% elderly women reported no changes in protein abundance of mitofusion 1, Drp1 or fission 1 protein (Fis1), but a significant decline in optic atrophy gene 1 (OPA1) (7). In the only previous study to examine mitochondrial function directly, Joseph and colleagues reported lower state 3 respiratory capacity in saponin-permeabilized myofibers obtained from a mixed cohort of low functioning elderly men and women (45% women) (7). Our results are generally consistent with this finding, wherein we observed a reduced mitochondrial respiratory capacity in FE women and, based upon a lower VDAC protein level, this appears to be due to reduced mitochondrial content rather than intrinsic mitochondrial respiratory capacity impairment. We acknowledge, however, that a single mitochondrial protein may not represent the dynamics of the hundreds of proteins that comprise a mitochondrion and that the gold standard measure here is mitochondrial volume density. In this respect, in the only study to have specifically made this measure in older women only a trend to a reduction in intermyofibrillar mitochondrial volume density was seen in moderately physically active

women <70 of age compared to activity-matched young women (30). Since our FE subjects were approximately a decade older and quite sedentary by comparison to the older women in this previous study (30), a reduced muscle mitochondrial content in FE women as suggested by our VDAC measures is not unexpected. We also note, that in other experiments we did not observe a difference in the relative abundance of type I versus type II fibers between groups (data not shown), so fiber type does not appear to relate to the differences in mitochondrial abundance in the YI versus FE groups. The FE group also displayed lower ACR, suggesting a reduced mitochondrial oxidative phosphorylation (OXPHOS) coupling efficiency, which may be an adaptive response to limit age-related increased generation of mitochondrial ROS (31). Thus, our findings add to the previous studies noted herein and support the notion that mitochondrial alterations are an important component of frailty in elderly women, and muscle alterations in aging women in general.

3.5.2. Mitochondrial ROS production

Although, numerous studies have shown that mitochondrial ROS increases in various conditions involving muscle atrophy (12, 32), only some studies have reported that mitochondrial ROS is elevated in aging atrophied muscle (33, 34), whereas others have shown no increase despite muscle atrophy (35, 36) or a highly variable response independent of atrophy (37). On the other hand, we have previously shown that oxidative damage is highly heterogenous between individual muscle fibers in aged muscle (38), and thus increases in mitochondrial ROS in a subset of muscle fibers (e.g., such as those that are denervated (12)) could be obscured by the ROS signal in the surrounding muscle fibers. Notwithstanding this point, because mitochondria are a main site of ROS production in the cell, mitochondrial impairment has been suggested to play a role in frailty. Consistent with this notion, it has been shown that skeletal muscle from elderly individuals with reduced physical function is more susceptible to oxidative damage (7). Various studies have also presented an association between

oxidative stress and frailty by measuring circulating biomarkers in plasma samples (39-41), wherein frail individuals presented increased protein and glutathione oxidation, reduced levels of total thiol, elevated levels of isoprostanes as well as oxidative DNA damage. Another study assessed the presence of oxidative stress more directly, observing an increased production of superoxide anions in white blood cells from frail individuals (42). Interestingly, to the best of our knowledge none of the studies in frail individuals measured ROS in skeletal muscle. Thus, our study shows for the first time that ROS emission in vastus lateralis muscle is markedly higher in the FE group after normalization to mitochondrial content, suggesting that mitochondria in limb muscle of very old pre-frail/frail women have features that potentiate ROS production.

3.5.3. Sex-specific changes in mitochondrial function with aging

The current literature is rich with studies assessing changes in mitochondrial function with aging; however, most of these studies do not account for possible difference between sexes. Recent research suggests that there is significant between-sex dimorphism in mitochondrial physiology in healthy adults (16). Indeed, studies have found that, compared to men, women had reduced sensitivity to ADP (43), higher mitochondrial volume density (44) and increased lipid oxidation (43, 44). However, there were no sex-dependent differences found in mitochondrial maximal OXPHOS of vastus lateralis (43) and gastrocnemius muscle (45), suggesting other factors may contribute to sex-dependent differences in mitochondrial function, such as post-transcriptional modifications or allosteric enzyme regulation. It should be noted that participants in the studies noted above were young or middle-aged individuals, and thus mitochondrial functional differences with aging may not yet be apparent. Hence, our study could provide a foundation for a better understanding of sex-specific age-related changes in mitochondrial function by making some comparisons of the FE women studied here to our previous study of octogenarian men (10). In this respect, it is noteworthy that whereas our

current study finds proportional changes in muscle respiratory capacity and VDAC protein in FE vs YI women, our prior study (10) found no significant reduction in muscle respiratory capacity or VDAC protein with aging in octogenarian men. This suggests women but not men exhibit a decline in mitochondrial content in advanced age. In contrast, our prior study (10) found no increase in mitochondria-specific ROS emission in octogenarian men, whereas our current results show an increase in mitochondria-specific ROS emission in FE women. This latter result suggests women but not men exhibit a mitochondria-specific exacerbation of ROS generation in advanced age. Interestingly, these differences occurred despite the evidence that denervation was modulating mitochondrial function in both octogenarian men (10) and the FE women studied here (see next section). In addition, we cannot rule out the possibility that the frailty status of the current cohort of FE women was more severe than that of the octogenarian men reported previously. Thus, the biological basis for these sex-dependent differences cannot be revealed by our analysis and additional study of sex-specific variation in skeletal muscle mitochondrial function and its impact on skeletal muscle mass and function is clearly warranted.

3.5.4. Muscle denervation

Frail individuals appear to have exacerbated alterations in the central nervous system indicated by increased prevalence of dementia (46), a neurological disorder that affects neuronal connectivity of the brain (reviewed in (47). Hence, it is possible that they may also exhibit exacerbated motor neuron degeneration, which is known to contribute to age-related muscle atrophy (10, 11). Consistent with this notion, we observed a marked increase in the abundance of muscle fibers positive for the denervation-responsive cytokine, NCAM, in FE muscle.

Muscle fibers undergo repeated cycles of denervation and reinnervation for much of adult life; however, in very old age (>75 years of age in humans) persistently denervated fibers start to accumulate and often occur as grouped fiber atrophy, implicating loss of an entire motor unit or a large axon innervating multiple fibers (8). Studies in animal models suggest that failed

reinnervation of muscle fibers is responsible for the accumulation of persistently denervated fibers in advanced age (11, 48) and that this is a key contributor to an acceleration of atrophy in very old age (49). Interestingly, Bhattacharya et al. (20) have previously shown that denervation increases mitochondrial ROS emission in the form of FA-OOH cleaved from the outer mitochondrial membrane by the enzyme $cPLA_2$ and that the denervation-induced ROS signal can be ablated by inhibiting $cPLA_2$. We have also shown in both a mouse model of sporadic denervation and octogenarian men, whose muscle exhibited morphological and transcriptional changes indicative of denervation, that $cPLA_2$ inhibition reduces ROS emission (10), showing that this method can be used to uncloak the impact of denervation on mitochondrial function. As mentioned in the preceding section, these latter observations in octogenarian men are consistent with the current study where we found a significant reduction in mitochondrial ROS emission following $cPLA_2$ inhibition in FE but not YI women. We further showed that the reduction in ROS emission occurred only with $cPLA_2$ inhibition and not with $iPLA_2$ inhibition in fibers from FE, which based upon Bhattacharya et al.'s previous results showing that the denervation-induced ROS response is specific to $cPLA_2$ action (20), indicates that denervation is modulating the ROS response in FE. Finally, similar to our previous study in octogenarian men (10), the modulation of mitochondrial function by denervation in prefrail/frail elderly women was accompanied by a significant accumulation of severely atrophied muscle fibers, as is typical of persistent denervation in aging muscle (11, 48). Thus, our results support our hypothesis that denervation contributes to mitochondrial impact in limb muscle of prefrail/frail elderly women. Although this could suggest that exacerbated muscle denervation may be a risk factor for frailty, it is not possible with the current study design to discern causality in this relationship. Thus, a more comprehensive study in elderly women including very old non-frail participants is needed.

3.6. CONCLUSIONS

Loss of muscle mass and function are major contributors to physical frailty, and therefore, intervening with these processes could be effective in reducing physical frailty. In this respect, our results show that denervation is an important modulator of mitochondrial function in pre-frail/frail elderly women as demonstrated by the reduction in mitochondrial ROS emission after $cPLA_2$ inhibition. This is consistent with the histological presence of very small fibers typical of persistent denervation in skeletal muscle cross-sections from the elderly women. Additionally, it would be valuable to determine whether the reduction in mitochondrial content suggested by lower VDAC that we observed in FE group could be prevented through physical activity, as suggested by other studies. As such our results add to the existing data in octogenarian men and confirm that denervation is present in both elderly men and women, suggesting that when aging muscle atrophy becomes clinically relevant denervation rather than the mitochondrion may be a more effective therapeutic target.

Conflict of interests: None

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CHAPTER 4: LIMITATIONS, CONCLUSIONS AND FUTURE DIRECTIONS

4.1. Limitations

Skeletal muscle sampling in studies of human muscle is usually performed by percutaneous needle biopsy, which limits the analysis to only a few hundred myofibers, which may not accurately represent the whole *vastus lateralis* muscle. In our particular studies, the small amount of tissue obtained (around 150mg) was separated into four pieces for four different assays (transcript and protein expression, mitochondrial function and histology). Because of this separation, the abundance of denervated fibers may vary between them (e.g., muscle bundle used for protein analysis had less denervated myofibers than the bundle used for histology or mitochondrial function). Moreover, during the separation process of the muscle bundles allocated for mitochondrial assay some of the more fragile fibers are lost, which could skew results to the more healthy fibers and thus, dilute the real impact of aging.

Because of the small tissue sample obtained from some participants (specifically in those where muscle yield was <100mg), we did not have enough tissue to carry out all of the analyses planned. Hence, the sample size in some measurements, especially for gene expression, was very low. Additionally, in some cases the size of tissue used for gene expression was less than 10mg, which may not accurately represent the whole muscle (see additional discussion bellow) and could have additionally contribute to the variation in the data.

With advanced age the heterogeneity in fiber size and type also increases and can vary between individuals, which makes it even more difficult to obtain representative information from a biopsy sample, hence a bigger sample size would give us greater power to detect any differences. Furthermore, because of the fiber type grouping evident in aging muscle, upon subdividing the muscle biopsy into smaller pieces it is possible that one may get a sample that is skewed towards one fiber type.

Another factor potentially contributing to higher variability within the elderly group might have been the inclusion of both pre-frail and frail individuals instead of frail individuals only. Additionally, the very low physical activity levels of the young control group, may not have matched the sedentariness of the physically prefrail/frail group. Although we did our best to recruit the most inactive young women, their activity levels still might have been higher compared to those of prefrail/frail elderly women. Beside physical activity levels, most elderly individuals have been suggested to consume insufficient amounts of dietary protein, which may contribute to age-related muscle atrophy and eventually development of frailty [224, 225]. However, in the present study prefrail/frail elderly women were taking the current recommended dietary allowance for protein (0.8g/kg/day). We do recognize that this amount of protein intake may not be enough as elderly individuals seem to require greater amount of daily protein consumption (1.0–1.2g/kg/d) for optimal stimulation of muscle protein synthesis [226, 227].

4.2. Conclusions

One of the most prominent physiological changes occurring with aging is the progressive decline of physical mobility, which represents a starting point of a progressive deterioration of physical function that, when severe, can lead to physical frailty [5]. Elderly women are at a greater risk of physical frailty, wherein age-related degeneration of skeletal muscle plays a primary role. Important physiological factors implicated in age-related muscle degeneration are impaired mitochondrial function and denervation of muscle fibers. Throughout this dissertation, with the use of histological, biochemical and molecular methods, we demonstrated the important contribution of muscle denervation to age-related muscle atrophy and mitochondrial function in prefrail/frail elderly women. We showed for the first time that prefrail/frail elderly women exhibit persistent muscle denervation, demonstrated by the presence of severely atrophic fibers, fiber type grouping, greater expression of NCAM and abundant pyknotic nuclei. These findings contrasted with observations made in skeletal muscle from world class octogenarian track and field MA, a population with remarkably high function in advanced age. Specifically, MA exhibited a substantially smaller accumulation of small fibers and less NCAM expression, accompanied by greater fiber type grouping and an elevated expression of the reinnervation promoting cytokine FGFBP1. Thus, the difference in muscle impact between FE and MA related closely to differences in the vulnerability to denervation and failed reinnervation. As such, our results underscore the importance of denervation as a critical factor determining the relative impact of aging on skeletal muscle, and suggest therapeutic approaches aimed at preventing physical frailty should focus on strategies for reducing denervation and/or encouraging successful reinnervation.

In addition to the above, our findings illustrate that denervation is an important modulator of mitochondrial function in pre-frail/frail elderly women as demonstrated by the reduction in mitochondrial ROS emission after $cPLA_2$ inhibition. Prefrail/frail women also

displayed lower mitochondrial content compared to young controls, which may be secondary to low physical activity. As such our results add to the existing data in octogenarian men and confirm that denervation is present in both elderly men and women, suggesting that when aging muscle atrophy becomes clinically relevant denervation, rather than the muscle mitochondrion, may be a more effective therapeutic target. Our work presents a starting point for future studies to develop appropriate therapeutic strategies tailored to elderly women to improve muscle function and perhaps reverse the frailty state.

4.2. Future directions

The work conducted in this dissertation is the first to show the important impact of denervation on age-related muscle atrophy and physical function, and its modulating role on mitochondrial function prefrail/frail elderly women. However, more work is needed to improve our understanding on the underlining mechanisms that contribute and may potentially accelerate muscled denervation and alter reinnervation capacity of aging muscle. This may be especially important in women, who are understudied relative to men and have a higher degree of muscle atrophy and are at greater risk of becoming physically frail.

Firstly, a more comprehensive study in elderly women including very old non-frail participants is needed as this would give us a better understanding if the impact of denervation occurs at the same age as seen in men [17]. This is important because women have generally less muscle mass than men [33, 39] and consequently less functional reserves. Hence, the presence of denervation in elderly women, could make the muscle more vulnerable to the aging process and accelerate functional impairment. A longitudinal study design would further help to decipher the presence and degree of denervation load on muscle with advancing age and to better evaluate the relationship between the degree of denervation and alterations in muscle function over time. This type of study design would also enable to assess potential time-dependent differences in the expression of denervation markers (e.g., NCAM, $Nav_{1.5}$ etc.) – e.g., to see if the expression of denervation induced proteins declines after a certain period of persistent denervation. Another approach to identify differences in protein and gene expression between denervated and normal myofibers would be by using the Laser capture microdissection (LCM) technique. This technique would enable the investigator to dissect denervated fibers from a muscle tissue section, which can be then used to analyses their proteome and transcriptome and compare the results with normal innervated myofibers.

Moreover, there are various neurotrophic factors (e.g., brain-derived neurotrophic factor, neurotrophin-4) known to contribute to the innervation capacity of the motor neurons and to maintain NMJ function [228]. Hence, it would be of great interest to further investigate if levels of these factors are associated with the degree of muscle denervation in elderly individuals. We showed that MA have increased expression of FGFBP1, which may indicate the presence of better reinnervation capacity. FGFBP1 is positively regulated by miRNA-206 and negatively by TGF- ß$_2$, which is increased in aged muscle [143]. Although we have previously observed an up-regulation of miR-206 in very old and severely atrophied rat muscle [1], we did not see any increase in FGFBP1 gene expression in the muscle of prefrail/frail individuals or an increase in the muscle transcript levels of TGF-ß$_2$. It would be valuable to assess protein levels of FGFBP1 and TGF- ß$_2$ as it would give us a clearer picture of their role in muscle denervation process. Additionally, it would be interesting to see if blocking TGF- ß$_2$ in old rodents or using animal models overexpressing FGFBP1 would improve FGFBP1 levels and consequently innervation capacity of aging muscle. These approaches would help to better understand mechanisms involved in muscle denervation. Furthermore, circulating levels of miRNA-206 have been found to be increased in individuals with ALS [229]. Hence measuring miRNA-206 levels in blood of elderly individuals could represent a biomarker for tracking muscle denervation progression.

We showed that MA, who maintain their muscle function into old age, have superior innervation capacity, which may be partially due to their active lifestyle. It would be valuable to determine if exercise intervention initiated in advanced age has the capacity to improve/facilitate myofiber reinnervation through upregulation of neurotrophins, which contribute to the maintenance of NMJ structure and promote reinnervation. However, there should also be investigation to determine at which age exercise intervention may be most efficient. This is because with advanced age, there is a reduction in MU number [99, 117], that

cannot be recovered. As such, exercise intervention could place a higher burden on the remaining MU and contribute to accelerate muscle denervation. This notion is consistent with a longitudinal study of endurance exercise training performed in aging rats where the exercise trained group demonstrated exacerbated muscle loss in advanced age that was associated with a marked accumulation of small fibers with high levels of oxidative damage [230]. Interestingly, another study in mice suggested that starting with physical activity in middle-age can impede age-associated loss of nerve terminal [231]. Caloric restriction is another intervention, beside exercise, proposed to delay the deleterious effect of aging on muscle innervation. A study in rodents showed that the addition of resveratrol, a caloric restriction mimetic, to regular chow diet reduces postsynaptic fragmentation of NMJ [232].

Future research should focus on assessing men and women as separate groups and account for possible sex-dependent differences in biological mechanisms (e.g., mitochondrial function) contributing to muscle mass and function loss. For example, our results here with pre-frail/frail elderly women demonstrated a reduction in muscle mitochondrial content, whereas a previous study from our group showed that a similar aged group of healthy elderly men tended to have elevated not reduced levels of mitochondrial proteins [17]. Additionally, future studies measuring mitochondrial function *ex vivo* in permeabilized muscle fibers could consider applying physiological levels of ADP, which better mimic every day muscle contractions and may provide additional resolution of the nature of mitochondrial function perturbation in aging muscle. Moreover, it would be valuable to examine if exercise training can increase mitochondrial content in very old individuals, as data from a rat model of treadmill exercise training finds a lack of induction of the master regulator of mitochondrial biogenesis PGC-1a, accompanied by a lack of increase in mitochondrial marker enzymes [233].

CHAPTER 5: APPENDIX

Physical Frailty criteria:

Frailty criteria	Method of measurement	Cut-off values for women
Low muscle mass	DEXA Self-reported	SMI: < 6kg/m^2 > 10lbs(4.5kg) in past year
Physical inactivity	CHAMPS Physical Activity Questionnaire	<125 points
Weakness	Hand grip	≤ 20kg
Slowness	4-meter gait speed	≤ 1m/s
Self-reported exhaustion	Based on CES-D depression scale - two questions are asked: How often do you feel like a) Everything you did was an effort? b) You could not get going?	If answered "most of the time" or "moderate amount of the time"

DEXA, Dual-energy X-ray absorptiometry
SMI, Skeletal muscle mass index
CHAMPS, Community Healthy Activities Model Program for Seniors
CES-D, Center for Epidemiologic Studies Depression Scale

Physical function measurement

Administration of Chair Stand Test

1. Use a chair without arms.
2. Ask the participant to…
    a. Sit on the chair, back straight
    b. Feet shoulder width apart, flat on floor, one foot slightly in front of other (for balance)
    c. Fold their arms across their chest. They should not push off of their thighs to stand.

3. At the signal “Go!” the participant rises to full stand and returns to initial sitting position.
4. Participant is encouraged to complete as many full stands as possible within 30 seconds.
5. Instruct participant to sit FULLY between each stand
6. Count how many stands are completely in 30 seconds, and how many seconds are required to complete 10 stands. Do not count incorrectly executed stands (i.e. using hands to stand)

Administration of Timed up and go (TUG)

1. Have subject sit in chair with arms and feet flat on floor.
2. Arms should rest on the arms of the chair.
3. On the “Go!” the subject will be timed on how long it takes them to stand up from the chair (they can push off the arm chair if needed), and walk to the 3-meter mark, turn around and sit back down in the chair.
4. Time subjects form when you say “GO!” to when they are fully seated (bottom touches the sear of the chair).

   Normal time (young adults): <10 seconds

   Normal time (older adults): <12 seconds

Administration of Gait Speed (4-meter)

1. Mark both ends of 4-meter line on the floor.
2. Explain to subject they will be timed on how long it takes them to walk from one mark to the other at their usual pace.
3. Start timer when subject starts their first step and stop timer when subject crosses the 4- meter mark.
4. Calculate gait speed as distance/time.

CHAPTER 6: REFERENCES

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