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MUSCULAR AGING AND ITS RELATION TO PHYSICAL ACTIVITY AND FUNCTION - LONGITUDINAL AND CROSS-SECTIONAL ANALYSES

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Muscular aging and its relation to physical activity and function - longitudinal and cross-sectional analyses

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To Emelie and Nora

POPULAR SCIENCE SUMMARY OF THE THESIS

The human body has more than 600 muscles that are essential for movement. As we age, muscle mass is lost and physical function and strength decline. This decline begins as early as the 3rd to 5th decade of life and accelerates in older people. This decline occurs even in healthy and active people, although regular exercise seems to slow the deterioration. There is some conflicting data on the amount of muscle mass that is lost with age, and measurements of muscle mass need to be refined and validated. There are many factors that contribute to the loss of muscle function and mass with age, and there is an imbalance between muscle gain and muscle loss. Older people do not respond as well to exercise as younger people and older people require more protein to build muscle. The reasons for this reduced response are only partially known and need further research. In this thesis, different mechanisms of muscular aging were studied in muscle biopsies (very small pieces of muscle), both longitudinally (the same individuals were studied at 70 and 88-90 years of age) and cross-sectionally (at one point in time), by studying genes involved in muscle protein degradation and muscle differentiation, and by studying muscle morphology (viewing muscles under the microscope using different techniques to stain different structures in the muscle, Study I). The morphology of muscle biopsies from very old people with a history of endurance exercise was compared with very old people who had either high or low physical function (study II). In addition, a semi-automated measurement of muscle mass and muscle fat content in eight lower body muscles was compared to a manual method of circling each muscle (study III). In addition, muscular adaptations were analyzed in terms of changes in muscle area and fat content in a 6-month physical activity intervention in which participants received either a nutritional supplement or a placebo drink (study IV).

The most important finding of this work was a reduced adaptability of the muscles in the oldest age groups. This was evident in both the gene expression analysis and the physical activity intervention. The muscles of the endurance athletes had characteristics beneficial for oxygen delivery (more blood vessels, capillaries) and oxygen utilization (more slow fibers and better preserved mitochondrial enzyme). The two groups who differed in physical function did not differ in morphological assessment, suggesting that physical function may not be related to muscle characteristics only. The semi-automatic method of analyzing muscle area and fat infiltration was accurate for most muscle groups, although the threshold used in this work may not be optimal for muscles with high fat content. The physical activity intervention significantly increased physical function, although only some of the eight lower body muscles studied showed an increase in area and a decrease in fat infiltration. The changes in muscle area and fat infiltration were relatively small and unrelated to improvements in physical function.

ABSTRACT

With age, muscle mass decreases and muscle function and strength decline, which is associated with loss of independence, hospitalization, morbidity and mortality. The aim of this thesis was to investigate the mechanisms involved in the age-related loss of muscle mass, physical function and strength by 1) studying gene expression and muscle morphology in muscle biopsies of vastus lateralis longitudinally (Study I) and muscle morphology cross-sectionally (Study II) in a cohort of older men, i.e. the Uppsala Longitudinal Study of Adult Men (ULSAM, Study I and II), and in men with a lifelong history of endurance training (Study II), 2) evaluation of a semi-automated and manual method to analyze the cross-sectional area and radiological attenuation (RA) of eight lower extremity muscle groups in mobility-limited individuals aged over 70 years (Study III), 3) evaluation of the effect of a 6-month physical activity intervention on muscle size and density of the same locomotor muscles, with or without an oral nutritional supplement containing protein and vitamin D (study IV). In study I, muscle morphology including fiber types, fiber area, and satellite cells (SC) and 14 genes involved in muscle remodeling were analyzed at ages 70 and 88-90 years. Study II examined muscle morphology in seven elite endurance athletes aged 82-92 and 19 ULSAM men aged 87-91 years, who were divided into high and low function groups based on tests for physical function and strength. In Study III and IV, measurements were done in a cohort of mobility limited individuals over 70 years (the Vitality, Independence, and Vigor in the Elderly 2 Study, VIVE2). Eight different locomotor muscles were measured using both the semi-automated and manual method and two independent observers performed the manual measurements (Study III) and manual measurement only (Study IV).

In Study I, type II fibers decreased with age, whereas fibers co-expressing myosin heavy chain type I+IIA increased. Expression of genes involved in muscle remodeling were higher at age 70 compared to healthy adult men. Some of these genes were also expressed at higher levels at age 70 than at age 88-90 and in those who survived beyond age 82 compared to those who died before that age. The higher expression of genes involved in remodeling at age 70 in survivors were considered beneficial since muscle mass was relatively stable between 82 and 88 years. Lifelong endurance training was associated with a better oxidative profile, with more type I fibers, more capillaries and fewer COX negative fibers even after 80 years of age (Study II). Manual and semi-automated measurements of area and RA correlated well between methods, especially in normal-density muscles, as shown in study III. A 6-month physical activity intervention increased area and RA in some but not all muscle groups and these changes were not related to the more pronounced changes observed in physical function (study IV). In conclusion, muscle plasticity decreases in very old men, as evidenced by altered gene expression profile. Morphological characteristics are to some extent unrelated to physical function, while lifelong endurance training is associated with some beneficial morphological features even at very advanced ages. There is also a reduced muscular adaptive response to physical activity in old men and women and these changes are at least partially unrelated to physical function.

LIST OF SCIENTIFIC PAPERS

I. **Elisabeth Skoglund***, Max Grönholdt-Klein*, Eric Rullman*, Lars-Eric Thornell, Anna Strömberg, Anu Hedman, Tommy Cederholm, Brun Ulfhake and Thomas Gustafsson. Longitudinal Muscle and Myocellular Changes in Community-Dwelling Men Over Two Decades of Successful Aging—The ULSAM Cohort Revisited *J Gerontol A Biol Sci Med Sci, 2020, 75, 654-663*

II. **Elisabeth Skoglund**, Per Stål, Tommy R Lundberg, Eric Rullman, Thomas Gustafsson, Per A Tesch, Lars-Eric Thornell. Skeletal muscle morphology, satellite cells and oxidative profile in relation to physical function and lifelong endurance training in very old men-*manuscript*

III. Hans E Berg, Daniel Truong, **Elisabeth Skoglund**, Thomas Gustafsson, Tommy R Lundberg. Threshold-automated CT measurements of muscle size and radiological attenuation in multiple lower-extremity muscles of older individuals *Clin Physiol Funct Imaging, 2020, 40, 165-172*

IV. **Elisabeth Skoglund**, Tommy R Lundberg, Eric Rullman, Roger A. Fielding, Dylan R Kirn, Davis A Englund, Åsa von Berens, Afsaneh Koochek, Tommy Cederholm, Hans E Berg, Thomas Gustafsson. Functional improvements to 6 months of physical activity are not related to changes in size or density of multiple lower-extremity muscles in mobility-limited older individuals-*In revision*

*Shared first authorship.

This thesis is the result of close collaborations between the Department of Laboratory Medicine at Karolinska Institutet, Stockholm, the Department of Public Health and Caring Sciences at Uppsala University, and the Department of Integrative Medical Biology at Umeå University.

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LIST OF ABBREVIATIONS

ADL	Activities of Daily Life
CFPE	Capillary-to-Fiber Perimeter Exchange
COX	Cytochrome C Oxidases
CSA	Cross-Sectional Area
CT	Computed Tomography
DXA	Dual energy X-ray Absorptiometry
EWGSOP	European Working Group on Sarcopenia in Older People
FDR	False Discovery Rate
HU	Hounsfield Unit
LTPA	Leisure Time Physical Activity
MRI	Magnetic Resonance Imaging
MRF	Myogenic Regulatory Factors
mtDNA	mithocondrial DNA
MyHC	Myosin Heavy Chain
OPLS	Orthogonal Partial Least Squares
PCA	Principal Component Analysis
RA	Radiological Attenuation
RFD	Rate of Force Development
SDH	Succinate Dehydrogenase
SC	Satellite Cells
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TUG	Time Up and Go
ULSAM	Uppsala Longitudinal Study of Adult Men
UPS	Ubiquitin Proteasome System
VIVE2	Vitality, Independence, and Vigor in the Elderly 2 Study
VIP	Variable Important for Projection

1 INTRODUCTION

The elderly population is steadily increasing worldwide and as we age, we lose muscle strength and physical function as well as muscle mass. This loss is part of the aging process and when it is pronounced it is called sarcopenia. It is associated with functional impairment, increased number of falls, hospitalizations, morbidity and mortality [1-10].

The mechanism behind sarcopenia is multifactorial, with an imbalance between muscle protein synthesis and breakdown and include both extra-cellular components such as alterations in hormone levels, increased inflammation, decreased physical activity, nutritional status and neurological factors and effects on intra-myocelluar components such as reduction in muscle fiber size, decreased number of satellite cells (SC), SC dysfunction and mitochondrial dysfunction [11, 12]. It is believed that some of the age-related changes or their consequences in the muscles can be reduced or compensated for by physical activity and, to some extent, by nutritional supplements [6, 13-17].

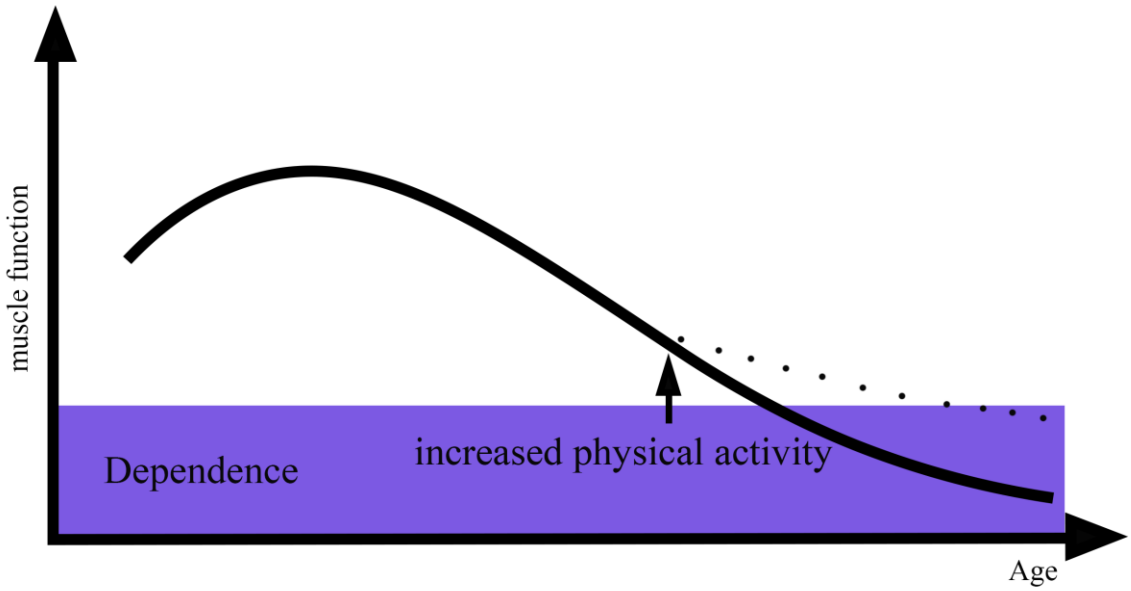

Figure 1. Simplified schematic illustration of muscle function in relation to age and physical activity. Blue shading represents when muscle function is too poor to cope with activities of daily living and arrow indicates the start of a regular physical activity program.

The decline in muscle mass can begin as early as the third to fifth decade of life and accelerates in older age [18-21]. Loss of muscle mass and function is also observed in active individuals, although the decline is slower [22, 23]. Cross-sectional studies tend to report less decline in muscle mass compared to longitudinal studies and results also vary between studies, while the extent of loss of muscle function and strength appears to be larger and more uniform between studies compared to loss of muscle mass [21, 24-30]. In addition, there is evidence that muscle mass and muscle function follow different trajectories [31-33]. Different muscle groups appear to respond differently to aging and inactivity as well as exercise, suggesting that some muscles are more vulnerable to aging [29, 30, 34, 35]. The

mechanism behind the age-related decline in muscle function and mass as well as the difference between muscle groups, the greater loss in function compared to mass and the protective effects of physical activity are insufficiently explored and it is important with standardized and uniform measurement of muscle quantity and density. Furthermore, longitudinal studies are sparse, and the time span studied is often much shorter compared to cross-sectional studies, seldom more than 15 years [24-30, 36-38].

2 LITERATURE REVIEW

The human body relies on skeletal muscle for locomotion and movement. In humans there are more than 640 muscles with different tasks [39]. Muscles contain from several hundred to approximately a million of muscle fibers (myofibers), and the length of the muscle can vary from about 2–600 mm [39]. Skeletal muscle is striated and the force generators in each muscle fiber responsible for contraction are called myofibrils, which contain sarcomeres (the smallest contractile units). The sarcomere consists of thick filaments and thin filaments, with the thick filament containing mainly myosin, while the thin filament consists of actin, tropomyosin and troponin. The myosin filaments are located in the center of the sarcomere and their length remains unchanged during contraction. When the muscle is activated, calcium is released from the sarcoplasmic reticulum which initiates the binding of the myosin head to actin. Subsequent movements of the myosin heads relative to actin (the cross bridge cycle) mediate muscle contraction [40]. The contraction can be isometric (muscle length remains constant), isotonic (muscle shortens with constant load) or isokinetic (a contraction at constant velocity). The energy for the cross-bridge cycle comes from the breakdown of ATP (ATP→ADP+P). Myosin contains heavy and light chains, with the myosin head consisting of two heavy chains. The property of the myosin heavy chain (MyHC) as well as resting calcium levels are some of the factors determining the speed of the cross-bridge cycle and thus the fiber type (fast or slow) [40]. There are three main types of fibers in human muscle, type I (slow, fatigue resistant), type IIA (fast, fatigue resistant) and type IIX (very fast, and fast fatiguing). In addition, there are hybrid fibers that contain different MyHC and developmental MyHC that are mainly expressed during development [41, 42].

Muscle fibers are post-mitotic, multinucleated and highly adaptable to external stimuli such as increased or decreased load. Adaptations vary depending on the type of stimuli, intensity, and volume. In general, endurance training (repetitive bouts) results in changes that increase the muscles' ability to extract and use oxygen and produce greater amount of energy (ATP) over a longer period of time. For example, regular endurance training increases cardiac output, capillaries in muscle, and enzymes in the citric acid cycle [43-47]. Strength training, on the other hand, results in increased strength and power, mediated by larger muscle fibers (hypertrophy) and neural adaptations [48, 49]. For muscle hypertrophy, muscle protein synthesis must outweigh muscle protein breakdown over time [50]. In addition, there are adaptations of connective tissue and tendons in which strength training increases stiffness, which is favorable for force transfer from muscle to skeletal bone [51, 52]. The changes in the muscle due to regular exercise are very plastic and many of the adaptations to training disappear after a period of disuse [48, 53].

Sarcopenia

The term sarcopenia was proposed in 1988 and at that time referred to the loss of muscle mass as the term means “loss of flesh” [54]. Over the first years after the introduction the

clinical uptake of the concept was sparse. In 2010 The European Working Group of Sarcopenia in Older People (EWGSOP) published its first clinical definition of sarcopenia, which included gait speed, handgrip, and muscle mass [4]. This step marked an emerging interest to integrate the concept of sarcopenia into clinical practice. A similar approach was performed in the Asian context [55, 56]. Recently, an updated version of the sarcopenia definition has been published that places more emphasis on muscle function, while grouping together low muscle mass or quality and finally adding physical function (e.g. gait speed, time up and go (TUG), short physical performance battery (SPPB) or 400 m walk) as an indicator of disease severity [5]. Depending on the definition used, the prevalence of sarcopenia can vary widely in the same cohort [57, 58]. A meta-analysis using 3 different definitions of sarcopenia found an overall prevalence of 10% in both men and women over 60 years of age [59]. Sarcopenia is now a clinical diagnosis, with ICD-10 code M62.84 [60]. Differences in reported rates of muscle loss differ between studies due to variation in study design (cross-sectional or longitudinal) and different methods used to analyze muscle mass [21].

Quantitative and qualitative changes of skeletal muscle

The World Health Organization has recently introduced "the decade of healthy aging" to raise awareness about the importance of functional abilities in the elderly population [61]. Finding individuals at risk of developing sarcopenia is of great clinical value. Thus, accurate measurements of muscle mass, strength and function are important. There are several methods for quantifying muscle mass and size. Dual energy X-ray absorptiometry (DXA) is commonly used to determine lean mass, and correlate well with computed tomography (CT) and magnetic resonance imaging (MRI) measurements of muscle mass. DXA is relatively fast, and radiation is low, while it cannot measure specific muscles or muscle fat infiltration [62]. For assessing muscle area and density of specific muscles or muscle groups, MRI provides high resolution but is expensive and time consuming. CT is cheaper and the examination is faster than MRI, easily accessible but time-consuming to analyze the images and includes radiation [62]. When using CT, muscle density is measured using Hounsfield units (HU) and decreased density is usually seen with increased fat infiltration [63, 64]. The muscles or muscle groups can be manually encircled or a threshold setting can be used to identify muscle mass and fat infiltration based on HU cut offs. Unfortunately, there is no single threshold for defining skeletal muscle, often -29 or 0 is used as the lower reference value and +100-150 HU defines the upper limit, while fat is usually defined as -30 to -190 HU [63, 65]. There is some evidence that low density muscle is overlooked when 0 HU is used as the lower reference point for defining skeletal muscle mass [63]. Ultrasound is simple and fast, can measure both muscle quantity as well as density and is reliable for measuring muscle size [5, 66]. It can be used to estimate but not to measure muscle mass [66]. Thus, standardizing the measurements used would make it easier to compare studies and thereby understand the underlying mechanisms and identify potential treatments [67]. In addition, D_3-

creatine dilution is another measurement of muscle mass that involves oral ingestion of deuterium-labeled creatine followed by measurement in the urine [68]. The correlation between muscle mass loss with the D_3-creatine dilution method and DXA measurement of total body lean mass is moderate [69]. When a muscle biopsy is obtained, the area of individual muscle fibers can be measured, although this is a small part of the muscle and it is known that there is variation in, for example, fiber types depending on the depth of the muscle, although this variation may be smaller for old individuals [70, 71]. In addition, the random variation in the percentage of fiber types can be substantial between biopsy sites. Differences of up to 47% in type II fibers have been observed [71]. The biopsy procedure is invasive, sometimes associated with pain, but generally well tolerated and complications are rare [72].

Muscle strength and physical function

Muscle strength can be measured with dynamometers that measure isometric, isotonic and isokinetic strength. Measurements may include one joint (single joint) or more than one joint (multiple joints) and this can affect to what extent the muscle is activated during an exercise [73-76]. Dynamic strength can be measured with one repetition maximum of an exercise. In older people, there is evidence of a reduction in explosive strength (strength developed in relation to the speed of contraction), which can be measured, for example, by rate of force development (RFD) [77]. These strength measurements quantifies the torque that a muscle/muscle group can produce, whereas functional measurements are more complex and includes coordination/balance. Functional measurements assess physical performance, which often involves a task that uses multiple muscle groups, but other factors such as balance and endurance components can also be included [5]. Examples of functional measures include gait speed, TUG, and SPPB [5].

The current definition of sarcopenia from EWSOP, uses simple tests that are applicable in a clinical setting. These include handgrip strength, as well as functional measures such as chair stand and gait speed [5]. Low handgrip strength and gait speed are risk factors for disability and mortality [78, 79], while chair stand has been suggested as a proxy for quadriceps strength [5]. Other functional measures that are easy to use in a clinical setting include TUG, stair raise and SPPB. TUG has high validity and can be used for quantifying functional mobility [80]. The SPPB is a composite muscle function measure that includes balance, gait, and chair stand test [81]. It has high validity and has been shown to be predictive of adverse outcomes such as mortality [4, 82]. Clinically meaningful changes have been suggested for both gait speed (0.04-0.06 m/s) and SPPB (0.54 points) [83].

Age-associated changes of skeletal muscle

Muscle fiber composition

Muscle aging has been extensively studied, and morphological changes with fiber type grouping and atrophy may be seen in some muscles as early as the second decade of life and increase thereafter [84]. By 65-80 years of age, there is evidence of de- and re-innervation manifested in muscle as type II fiber atrophy, fiber size variability and fiber type grouping [70, 85-99]. There are indications of alteration in the fiber type composition, both a decrease in type I fibers [29] and an increase with age [88, 100] have been reported. However, most studies report no difference [37, 38, 70, 90, 96, 101-107]. Hybrid fibers co-expressing MyHC are thought to increase due to denervation [108] and increase during aging [109, 110], although some studies indicate consistent levels at older ages [111, 112]. These conflicting results regarding age and hybrid type I+IIA fibers needs clarification, especially at the most advanced ages (over the age of 80 years).

Satellite cells

SC are muscle progenitor cells that were localized between the basal lamina and the sarcolemma in 1961 [113]. They are important for muscle regeneration and are activated to proliferate and fuse with existing muscle fibers during repair and hypertrophy [114-116]. SC are often quantified by immunofluorescence, although there are methodological problems with this quantification as there are no specific markers for SC. Instead, several different markers are used in combination with location (between the basal lamina and the sarcolemma) to identify SC cells. The multiple marker method has suggested a combination of two markers, NCAM and Pax7, in combination with laminin and DAPI for more accurate quantification [117]. The SC pool decreases with age in most studies, especially the SC in type II fibers are lost with aging [87, 88, 93-95, 118-122]. It has been suggested that the SC are not too few but rather that they are not activated properly [123, 124]. In mouse models, depletion of SC resulted in increased extracellular matrix, suggesting that loss of SC may increase fibrosis in muscle [125]. SC has a mutation accumulation rate of 13 somatic mutations per genome/year that target myoblast activity [126]. These mutations appear to spread into muscle where they may potentially impair muscle remodeling capacity. The role of SC during aging is under debate and it is uncertain whether the decrease in number affects the regenerative capacity of the muscles [114, 116, 119], and data on individuals between 80 and 90 years old are sparse.

Vascular supply

The microvascular network is also thought to play a role in the age-related loss of muscle fibers and function, possibly by contributing to the anabolic resistance or by the cross-talk with endothelial cells and SC [11, 127]. The number of capillaries per fiber appear to decrease with age, whereas capillaries in relation to muscle cross sectional area (capillary density, CD) seems to be rather constant, likely due to a concomitant decrease in fiber area

[11]. Capillaries can also be measured as the number of capillaries adjacent to fiber (CAF) also called capillary contacts (CC) [47, 128]. In addition, capillaries adjacent to fiber in relation to the fiber area (CAFA) takes into account the fiber area while CD may take area of the whole section into account, including extra cellular space [47]. Furthermore, physical function has been shown to be related to capillary density at early aging (mean age 71 years) [129]. The number of capillaries in relation to physical function and endurance training in the oldest ages needs further attention as most studies are on earlier aging (<80 years).

Oxidative capacity and mitochondria

Mitochondria are cellular organelles that contain their own DNA, mitochondrial DNA (mtDNA). They are important for many cellular processes such as energy production, calcium homeostasis, production of reactive oxygen species (ROS), and regulation of apoptosis [130]. During aging there is evidence of decreased mitochondrial function such as a decrease in TCA enzyme activity, increased ROS production, and mtDNA deletion mutation [130-132] In a recent genome-wide RNA sequencing study of muscle biopsies from both sarcopenic and non-sarcopenic elderly, genes involved in oxidative phosphorylation, TCA cycle regulation and respiratory chain were suppressed in sarcopenic individuals [133]. Cytochrome C oxidases (COX) are mitochondrial enzymes. During aging, there is an increase in muscle fibers that lack COX activity, and these fibers appear to co-locate with mtDNA deletion mutation [131, 134]. The enzyme succinate dehydrogenase (SDH), complex II of the electron transport chain, is encoded by nuclear DNA, while COX, complex IV in the electron transport chain, is coded by mtDNA and COX-SDH staining can be used to quantify COX deficiency in tissue sections [135]. Furthermore, decreased mitochondrial function during aging is also thought to be associated with increased inflammation [130].

Intra-muscular fat

During aging, there is an increase in intramuscular fat, both between muscle fibers and within muscle fibers (intra myocellular fat) [63, 64, 136]. This intramuscular fat is associated with reduced muscle strength, insulin resistance and hospitalization, and is thought to be important for increased low-grade chronic inflammation during aging [3, 64, 137-139]. The association between intramuscular fat and reduced strength is independent of muscle area and may explain more of the variance in physical function than lean mass [64, 140]. Inflammatory markers such as CRP, Il-6 and TNF-α may increase during aging [141] and muscle gene expression of Il-6, but not TNF-α, has been associated with both intramuscular fat and physical function, suggesting a link between intramuscular fat, inflammation and physical function [142].

Suggested mechanisms of muscular aging

A simplified description states that aging is the accumulation of un-degraded waste and damage with a decline in cellular ATP production and depletion of capacity to replicate, inducing a state of non-responsiveness, called senescence. Whether the origin of sarcopenia is primarily neurogenic or myogenic is under debate and at advanced stages there is a variety of changes in the peripheral innervation as well as in the muscle, making the determination of cause and effect quite difficult [12].

Muscle innervation

Muscle fibers are innervated by an α-motor neuron, groups of fibers are innervated by the same α-motor neuron, and this is called a motor unit (MU). Fatigue-resistant MU innervates type I fibers, and each unit may include 30-120 fibers. Fast fatigable MU innervates type II fibers and hundreds to thousands of fibers may be included in a fast MU [12]. During aging, denervation and subsequent reinnervation of neighboring motor neurons occurs through collateral sprouting [11, 92], with initial enlargement of surviving MU and eventual loss of MU, especially the large fast-fatigable ones [11, 12, 143]. In humans, the loss of motor neurons in the lumbosacral region begins around the age of 60 years, and beyond that there are large inter-individual variations. It has been reported that up to 50% of the motor neurons may be lost across the life-span [144].

Skeletal muscle protein synthesis

At older ages, there appears to be an imbalance between muscle protein synthesis and breakdown [10] and protein intake is important for muscle protein synthesis and muscle mass in the elderly [145-148]. More protein is required to elicit an anabolic response, termed anabolic resistance [149, 150]. It may therefore be of importance to achieve a certain amount of protein intake at each meal to stimulate muscle protein synthesis [151]. Whey protein is rich in the specific amino acids leucine, which can trigger anabolic signaling in part through the mTOR pathway and increase muscle protein fractional synthetic rate [152, 153]. The amount of protein is likely to be most important and older people require higher doses compared to young healthy individuals, although the timing in relation to strength exercise of protein intake may also enhance the hypertrophic response [151, 154-157].

Markers of skeletal muscle remodeling

Developmental Myosins and NCAM

Developmental MyHC are normally expressed during the neonatal/fetal period and most transition to type I and type IIA fibers in the third trimester of gestation while the transition to type IIX occurs in the first week of life [41]. In animal models, developmental MyHC, such as embryonic and fetal MyHC, seem to reappear in denervated muscle [158]. The expression of these developmental MyHC differs depending on the muscle group studied [159] and in

vastus lateralis there appears to be an increase with age [87, 109], but individual variation is quite large [160]. Moreover, fetal MyHC and Neural Cell Adhesion Molecule (NCAM) expression is also considered to be markers of regeneration and NCAM of neuromuscular instability. Therefore, the increase in developmental myosins and/or NCAM in vastus lateralis during aging is thought to be both due to regeneration and the denervation process [41, 87, 160-163]. How physical function and endurance training affect these markers after 80 years of age is unknown, especially in combination with patho-morphological evaluation.

Activation of ubiquitin ligase system

Animal studies of muscle aging have shown that the ubiquitin ligase system containing the muscle-specific genes MuRF-1 and Atrogin-1/ MAFbx is differently regulated at older ages [164]. Murf-1 and Atrogin-1 are E3 ubiquitin ligases downstream of myostatin/TGF-β, probably via Smad2/3, and are responsible for poly-ubiquitination and proteolysis via proteasome 26S and are important for muscle protein degradation during atrophy [165-168]. Mice deficient in ether MuRF-1 or Atrogin-1 were more resistant to atrophy after denervation than wild-types [167]. Trim 32 is another ubiquitin ligase, and studies in mice have shown that MuRF-1 ubiquitylates thick filaments, whereas Trim 32 ubiquitylates thin filaments [169, 170]. Studies in aged rats have shown evidence of activation of Ubiquitin Proteasome System (UPS) during early aging and also changes in innervation [164, 171-173], possibly due to increased remodeling. In the oldest muscle, there are signs of dysregulation of UPS [164] and a decreased ability to reinnervate myofibers [174]. Maintenance of proteolytic activity during aging is important to maintain sarcomere protein quality control and remodeling [175], and it has been suggested that degradation of dysfunctional proteins is altered in old rats [176]. Taken together, the role of the ubiquitin ligase system during advanced aging and its relation to denervation is important but unclear.

Myogenic regulatory factors

Other genes that may be important for muscle remodeling in humans are the myogenic regulatory factors (MRFs) (MyoD, myogenin, MRF4 and Myf5). In postnatal myogenesis, these factors are mainly thought to regulate the resident stem cell in skeletal muscle – the SC (see above). In this context, MYF5, MyoD and MRF4 act as myogenic determinants and myogenin as a differentiation factor [177]. In mice, myogenin may also be involved in regulating atrophy during denervation by regulating MuRF-1 and Atrogin-1 [178], while Atrogin-1 regulates MyoD [179]. In mature skeletal muscle fibers, MRFs appear to be downregulated, with the exception of MRF4. Moreover, in rats, MRF4 regulates skeletal muscle mass by repressing another family of transcription factors MEF2 [180]. Myogenin and MyoD have been shown to have a higher expression in older adults than in young [181, 182], and immunoblotting has also shown higher myogenin protein levels in old compared to young, with no differences in MyoD protein levels [183]. Thus, many studies regarding myogenic differentiation factors originate from animal studies and studies of early aging, while longitudinal data on very old humans are lacking.

Suggested factors influencing muscle aging

Physical activity

Regular exercise affect almost all organ systems of the body [184] and can partially prevent or possibly reverse to some degree parts of the changes seen during aging [6, 101, 185]. For example, changes in mitochondria following exercise are opposite to those induced by aging [13, 186], although molecular age-related changes are slightly different from those induced by exercise [187].

Strength training in elderly increases physical function and strength substantially, while muscle mass may not increase as much [14, 188]. In addition, there is evidence that muscle hypertrophy is more pronounced in some muscle groups after exercise [14, 189]. There appears to be a reduction in strength that is not only related to fiber loss/atrophy, as there is also a reduction power per single fiber, even when adjusted for CSA, possibly due to changes in the contractile elements in the fiber [190, 191].

After strength training, the area of type II fiber increases, although this response may be less pronounced in old compared to young people [14, 192, 193]. It has been suggested that regular endurance or strength training may enhance the reinnervation process [101, 112], although neither endurance nor strength training appear to protect against increases in developmental MyHC, or co-expression of type I and IIA MyHC [111, 112]. In addition, there is evidence of an increase in capillary supply following both endurance and strength training at early aging (<80) [43-47, 194-196]. As for SC, most types of exercise training appears to increase the number of SC, and the increase seems to be greater in young people indicating a reduced SC capacity at older ages [88, 197, 198].

Some of the genes involved in muscle differentiation and ubiquitin ligase system seem to be up-regulated during aging and after a single bout of exercise. The up-regulation after exercise may be dependent of training status of the individuals, although the literature is conflicting [101, 181, 199-206].

Based on the findings described above and population-based data, a reduction in physical activity has been highlighted as a key environmental factor responsible for the loss of muscle function and mass during aging, and it is thought that increased physical activity levels may counteract this decline [6]. Thus, when locomotor muscles are used to study aging, inactivity is often a potential confounder. An approach to reduce this bias is to study master athletes [207]. Most studies on master athletes suggest that regular strength or endurance training reduces but does not eliminate age-related muscular changes [112, 208-211]. Lifelong endurance training is associated with higher capillarization and oxidative enzyme activity in vastus lateralis and higher $V0_2$max compared to healthy older controls [208, 212]. In the gastrocnemius, mitochondrial enzymes succinate dehydrogenase and beta-hydroxyacyl-CoA dehydrogenase might even be higher in master runners than in young runners, indicating a sustained mitochondrial response to exercise in the early aging [213]. Furthermore,

individuals who have participated in endurance training throughout their lives have a higher number of SC per fiber area, which may indicate that exercise protects against this loss [87]. Taken together, there are indications that lifelong training is associated with better muscle health, although genetic traits may also play a role (see below). Physical activity also appears to influence age-related neurological changes, which may be partly responsible for the observed effects on skeletal muscle function [48, 49].

Nutritional supplements

Nutritional status is important with increasing age, and several factors may influence food intake and appetite [214]. The use of dietary supplements to reduce age-related muscle loss has been tested with mixed results. Protein intake is known to be important for maintaining muscle mass [145-148] and low vitamin D levels have been associated with intramuscular fat accumulation and impaired gait speed and balance [215]. It has been controversial whether vitamin-D receptors are present in skeletal muscle. They have not been found in some studies [216] and it has been suggested that vitamin D has ligand-independent effects on skeletal muscle [217]. However, it has also been suggested that the vitamin D receptors are present at low levels and possibly only in the SC of muscle [218].

Several studies have examined the effect of vitamin D supplementation on muscle mass and function, but with conflicting results. The PROVIDE study examined the effects of vitamin D and leucine on muscle mass and function in sarcopenic elderly. This study showed better response on chair stand test and muscle mass in the supplemented group compared to placebo, although there were no differences between groups in the SPPB test and hand grip [219]. Beaudart et al. conducted a meta-analysis showing effects on muscle strength but not muscle mass or power following vitamin D (+/- calcium) supplementation, not including complex oral nutritional supplement. There appeared to be a better response in older individuals and in those with low vitamin D levels at baseline (<30nmol/L) [220]. Several other studies examining the effects of vitamin D on muscle mass and function also involve proteins or specific amino acids, which complicates conclusions regarding vitamin D alone, as protein is known to be important for muscle protein synthesis and muscle mass maintenance in the elderly individuals [145-148]. However, it is possible that vitamin D mainly affects fat infiltration [221], while protein may be more anabolic, or that they have synergistic effects [222]. It is therefore important to study them together as well.

Genetic factors

In addition to these studies supporting the theory that regular exercise and nutrition may reduce some phenotypic changes during muscle aging, there is also evidence that genes are responsible for physical function and performance [223]. It is thought that up to 50% of muscle strength in adults is due to heredity, although this may be lower in older ages where environmental factors can have a greater influence [21, 224]. However, it is possible that people with certain genetic predispositions remain physically active at higher rates. Heritability for physical activity may be close to 50% [225, 226]. When studying aged elite

athletes, it is reasonable to assume that these individuals have inherited some traits that enhance their performance. In fact, 60% of baseline VO_2 max and 50% of training-induced changes in VO_2 max are estimated to be heritable [223]. Thus, genes and environmental factors are closely linked and may influence both performance and the desire to exercise.

Summary

Overall, many changes in skeletal muscle during aging are known, while the underlying mechanisms are yet to be deciphered. Many studies are cross-sectional in nature with individuals from small birth cohorts, and therefore results are sometimes inconsistent. Many mechanistic studies are on animals or in humans during early aging. Quantification of muscle changes are performed using different methods and it is important to include measures of fat infiltration since it appears to be of great clinical importance [3]. There are several lines of evidence that exercise and some dietary components such as proteins can reverse or slow many of these age-related changes in skeletal muscle, including muscle mass and fat infiltration. However, the response to exercise and/or protein is not as strong as in young people and once muscle mass is lost, it is more difficult to regain [53]. The mechanisms behind this attenuated response, and the differences between muscle groups, are to a large extent still unknown. Furthermore, morphological changes during aging are extensively studied up to an age of approximately 80 years (referred to as early aging in this thesis), while again studies of older age (80+) are rare. It is therefore unclear whether the muscular changes are similar or exacerbated at very old ages.

3 RESEARCH AIMS

The overall aim of this thesis was to investigate the mechanisms involved in age-related loss of muscle mass and function in advanced aging. More specifically, this thesis 1) examined gene expression and muscle morphology using muscle biopsies from vastus lateralis in the longitudinal ULSAM study, (Study I) and cross-sectionally in ULSAM participants and old endurance athletes (Study II), 2) analyzed the cross-sectional area and radiological attenuation of multiple lower extremity muscle groups in mobility-limited individuals over 70 years of age using a semi-automated and manual method (Study III), and finally, 3) evaluated the effect of a physical activity intervention on multiple locomotor muscles in mobility-impaired individuals over 70 years of age and the effect of an oral nutritional supplement containing protein and vitamin D (study IV).

4 MATERIALS AND METHODS

Overview of study participants

ULSAM (Study I, II)

The Uppsala Longitudinal Study of Adult Men (ULSAM) is a cohort of all men born between 1920 and 1924 and living in Uppsala County, Sweden, at age 50 [227]. Studies on the ULSAM cohort are still ongoing. To date, individuals aged 50, 60, 70, 77, 82, 88, and 93 have been studied. Muscle biopsies from a subset of the cohort aged 70 and new biopsies at age 88-90 years were used. DXA was performed at 82 and 88 years of age. Measurements of muscle function were performed at age 88 (Figure 2). In study II, they were divided into a high function group (HF) and a low function group (LF) based on TUG, gait speed, chair stand test and handgrip strength. Baseline characteristics at age 70 were obtained from the ULSAM database.

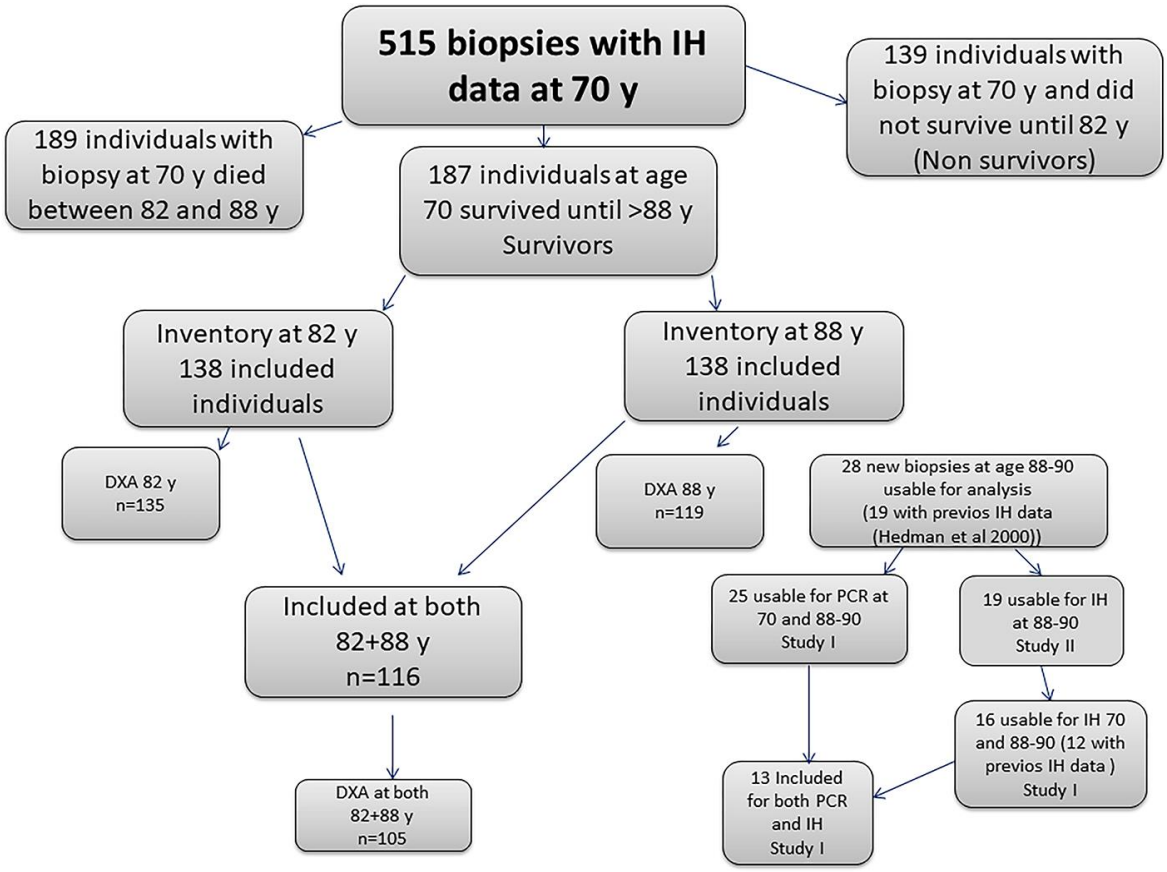

Figure 2. Flow chart of participants from the ULSAM cohort in Study I and II. Modified from supporting information paper I.

Endurance athletes (Study II)

Seven older, male elite endurance athletes, aged 82-92 years (mean 85), participated in Study II. The participants were recruited from Sweden national list of master cross country skiers. Inclusion criteria included 50 years of continued endurance exercise with maximum 6 months without training, the last 50 years. Participants were screened for their athletic history by completing an extensive questionnaire and an in-depth personal interview. All participants had performed intense endurance training 4-6 times per week over the past 50 years and participated in endurance competitions [208]. These elite skiers were compared to ULSAM participants aged 87-91 (mean 89).

VIVE2 Study participants (Study III, IV)

The Vitality, Independence, and Vigor in the Elderly 2 Study (VIVE2), was a multi-center physical activity intervention study including USA and Sweden. All participants performed a physical activity intervention and were randomized to a nutritional supplement or a placebo drink [228] Sixty-six men and women aged 70 + were recruited to the Swedish arm of the VIVE2 study. The inclusion criteria were BMI of ≤35, a serum 25(OH)D level of 22.5-60 nmol/L (9-24 ng/ml), a SPPB score of ≤9 (0-12) a Mini-Mental State Examination (MMSE) score of ≥24 (0-30), could walk 400-meter unassisted within 15 minutes, and participated in no more than 30 minutes structural physical activity per week. Participants were randomly assigned to receive either a nutritional drink or a placebo. Of the 66 individuals, 55 had complete CT image sets, 25 in the intervention group and 30 in the placebo group. Six individuals (2 from the intervention group and 4 from the control group) did not adhere to the physical activity program, attending less than 60% of the sessions. These 49 individuals (24 women, 10 in the intervention group and 14 in the control group) were included in study IV. In study III, 40 (19 women and 21 men) individuals had available images at the time of the study, 36 for hips and 39 for thigh and calf.

Overview of the methods used in the thesis

Tests of physical function and muscle strength (Study I, II and IV)

At age 88 years (Study I and II), participants in the ULSAM cohort had their physical function measured by TUG where the participants started in a seated position, stood up and walked across a mark at 3 meters distance, turned, walked back and sat down. Time was measured from when they stood until they sat down again. Gait speed was measured at a comfortable speed and “as fast as possible”. Participants walked a distance of 10 meters, and the middle 6 meters were timed. In addition, chair stand test was measured, and it included 5 raises from a chair without using the arms as support. Furthermore, hand grip strength was measured using a Baseline hydraulic hand dynamometer, (Fabrication Enterprises, White Plains NY, USA), using both hands. Each hand was measured 3 times with 10 seconds pause in between. TUG, gait speed, chair stand and hand grip were ranked from best to worst

performance, and all scores were averaged and the ULSAM group was divided into HF and LF groups based on these scores (in study II). The endurance athletes completed a standardized ramped VO_{2max} until fatigue (Study II).

In study IV, function was measured by a 400 m walk at average speed (10 laps of 20 meters), SPPB (4 meters walk, five time chair rises and a simple balance test with three different foot positions, held for 10 seconds) [81], and stair climb (10 sets of stairs as fast as possible, repeated twice with 60 seconds rest). In addition, maximal isometric torque for the knee flexors and extensors was measured at baseline and after 6 months using a Biodex System 3 Isokinetic Dynamometer (Biodex Medical Systems, Shirley, NY). During the measurements, participants alternated between flexion and extension with 30 seconds of rest in between. They were asked to apply as much force as possible for 5 seconds. Peak torque was determined using three measurements. Hand grip strength of the dominant hand was also measured in study IV at baseline and after 6 months (Jamar Handheld Dynamometer, Patterson Medical, Warrenville, IL). Two measurements were performed with 10 seconds rest in between, with the highest value being recorded [228].

Leisure time physical activity (LTPA) (Study I and II)

The assessment of leisure time physical activity among the ULSAM participants has been described previously [229]. Four questions were asked (yes or no). If the answer was yes, subsequent questions were answered until participants answered no. Then no more questions were asked, so there was only one category per person. The questions were as follows "1) Do you spend most of your time reading, watching TV, going to the cinema, or engaging in other, mostly sedentary, activities? 2) Do you often go walking or cycling for pleasure? 3) Do you engage in any active sport or heavy gardening for at least 3 h every week? (4) Do you regularly engage in hard physical training or competitive sport?" [229]. Based on these answers, participants were divided into four groups; sedentary, moderate, regular, or athletic [229]. The endurance athletes were assigned to the athletic group because they had been vigorously exercising 4-6 timer per week and competing in endurance events for 50 years [208].

Dual-energy X-ray absorptiometry (DXA) (Study I and II)

In the ULSAM cohort lean mass, fat mass and bone mineral density (BMD, g/cm^2) and bone area (cm^2) were measured on the whole body, femoral neck region of the hip, whole proximal femur, whole legs and arms, skull and lumbar spine (vertebrae L2-L4) using DXA (DPX Prodigy, Lunar Corp., Madison, WI, USA). In the endurance athletes, body composition was determined using X-ray absorptiometry (Lunar Prodigy full-body scanner, Madison, WI). EnCore 2008 software from GE Health Care were used to analyze the data [208].

CT Scans (Study III and IV)

CT scans of the hips, thighs and calves were performed with a slice thickness of 5 mm, with supine rest for 30-60 minutes before the measurements to avoid fluid shifts to influence the

measurements [230]. CSA and RA (HU), were determined using NIH ImageJ (Bethesda, MD) for different muscle groups by manually circling the muscles (Study III and IV) as previously described [231] and by semi-automated measurements for comparison (Study III). A threshold of -29 HU to + 150 HU was chosen to exclude non-muscular tissue, but to include low-density muscle in the semi-automated measurements. In study III, the time taken for the different measurements was determined. In the hip section, gluteus minimus + medius, gluteus maximus, and iliopsoas were measured. In the thigh section the knee flexors (biceps femoris, semimembranosus, semitendinosus), knee extensors (rectus femoris, vastus lateralis, vastus medialis and vastus intermedius) and the adductors (gracilis, adductor magnus, aductor longus) were measured. In the calf section, the anterior muscles (tibialis anterior, extensor hallucis longus, extensor digitorium longus, peroneus longus and peroneus brevis) and the posterior muscles (gastrocnemius, soleus, tibialais posterior, flexor hallucis longus and flexor digitorium longus) were measured.

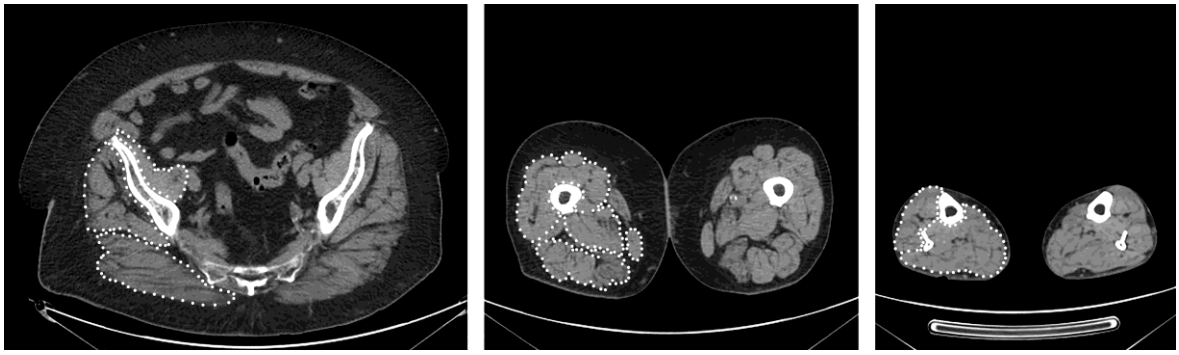

Figure 3. Example pictures from the hip, thigh and calves relevant to study III and IV. The 8 different muscle groups examined are outlined with white dots.

Physical activity intervention (Study IV)

The intervention included approximately 60 min group-based, flexibility, balance, lower extremity strength and brisk walking 3 times per week. Twenty minutes was aerobic exercise (brisk walking) and 20 minutes was lower body strength using ankle weights. Flexibility and balance were included in the warm-up and cool-down. Exercises were rated based on perceived exertion (6-20 Borg RPE scale, [232]), for aerobic exercise, the target effort was 13 (somewhat hard) according to current physical activity guidelines [233, 234]. Lower extremity strength included two sets with 10 repetitions in each set including lateral hip raise, chair rise, knee extension, knee flexion and calf raise. The target intensity was 15-16 (Borg Scale). In addition, participants were encouraged to be physically active outside of the group-based exercises to meet the current recommendations of 2.5 hours of moderate intensity physical activity per week.

Nutritional supplement (Study IV)

Participants received either a daily nutritional drink containing 150 kcal, 800 IU of vitamin D, 20 g of whey protein, 350 mg of calcium, and other vitamins and minerals, or a daily

placebo drink containing 30 kcal. Both drinks contained 119 ml and were provided by Nestle Health Science, Vevey, Switzerland. Sixty percent of the drinks had to be consumed for the participants to be considered adherent, assessed by the participants using paper logs.

Muscle biopsies (Study I and II)

Muscle biopsy data from a subset of the ULSAM cohort aged 70 years (515 individuals) were obtained from the ULSAM repository (Figure 2) [229, 235, 236]. Earlier analysis included fiber type, fiber area and capillary density and was performed using enzyme histochemistry (ATPase) for fiber types and amylase-Periodic acid-Schiff for capillaries as previously described by Hedman et al [235]. At 88-90 years of age, new biopsies were obtained from a subset of the original biopsy cohort. Of these biopsies, 28 were large enough to be used for either morphological or PCR analysis. Twenty-five biopsies were used for longitudinal gene expression analysis at age 70 to 88-90 years (Study I). Nineteen biopsies could be used for immunohistochemistry and immunofluorescence (Study II) and 16 of them for longitudinal analysis of fiber types (12 of these had previous data from Hedman et al [235]) and five for longitudinal analysis of SC and nuclei (study I). Twenty-four biopsies from ULSAM individuals aged 70 who did not survive until age 82 (non-survivors) were also included in the qPCR analysis and some morphological data were obtained from Hedman et al (Study I) [235]. Individuals that survived until the age of 88 are called survivors at age 70 and were compared to non-survivors at age 70 both morphologically (n=12 for survivors and n=24 for non-survivors) and using gene expression (n=25 for survivors and n=24 for non-survivors). See flowchart of included individuals from the ULSAM cohort in figure 2. Muscle biopsies from eight healthy men (25-35 years old) were used as reference biopsies representing young individuals (Study I). All muscle biopsies were obtained from the vastus lateralis muscle using Bergström percutaneous needle biopsy technique, with suction [237].

Histology, morphology and immunohistochemistry (Study I and II)

Hematoxylin-eosin (Htx-eosin) was used to assess general morphology. Fiber types were identified using peroxidase-antiperoxidase (PAP) staining [238]. A primary antibody, A4.951, with high affinity for myosin heavy chain I (MyHC I) was used to visualize type I fibers and a primary antibody, N2.261, with low affinity for MyHC I and high affinity for myosin heavy chain II (MyHC II) was used to identify type II fibers. To assess mixed fiber types, a combination of A4.951 and N2.261 were used (Figure 4-5). The 4C7 antibody targeting the laminin α 5 chain, which strongly stains capillaries and weakly stains basal lamina of the muscle fiber, and Merosin targeting the laminin α 2 chain, which stains muscle basal lamina strong, were used to identify the cell boundaries of muscle fibers. Fiber types, areas and capillaries were assessed using the Leica Qwin plus program. In study II, staining with antibodies targeting Fetal MyHC and NCAM was performed, and visualized using PAP [238]. COX-SDH was stained using the DAB method for COX [239] and Nitro Blue Tetrazolium for SDH. SC was identified using the multiple marker method [117]. NCAM and/or Pax7 in combination with correct location (just beneath the basal lamina) and staining

for myonuclei (DAPI) was required to be counted as SC (Figure 6). For antibodies see table 1.

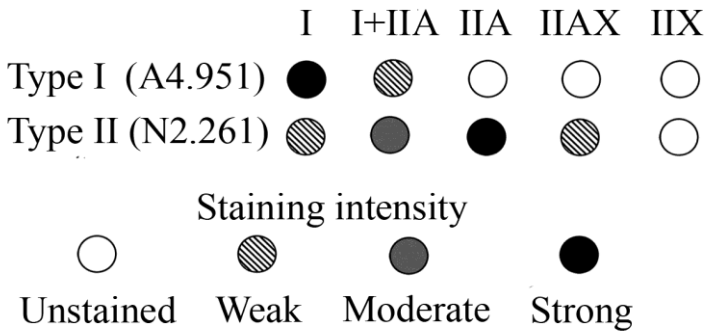

Figure 4. Schematic figure over fiber types using the antibodies A4.951 and N2.261. A4.951 har strong affinity for MyHC I, whereas N2.261 has weak affinity for MyHC I and stong affinity for MyHC II. Type IIX is identified by absence of staining from any of the antibodies. Modified and published with permission by Professor Per Stål, Umeå University.

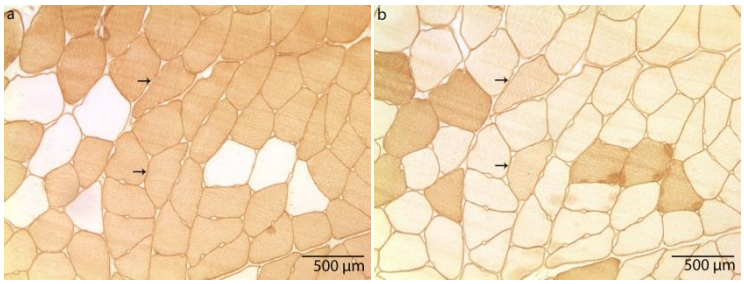

Figure 5. Staining for fiber types in cross section, Type I fibers stained with the A4.951 antibody (a) and type I fibers weakly and type IIA strongly with N2.261 antibody (b). Arrows indicate mixed fibers (type I+IIA) stained with both A4.951 and N2.261.

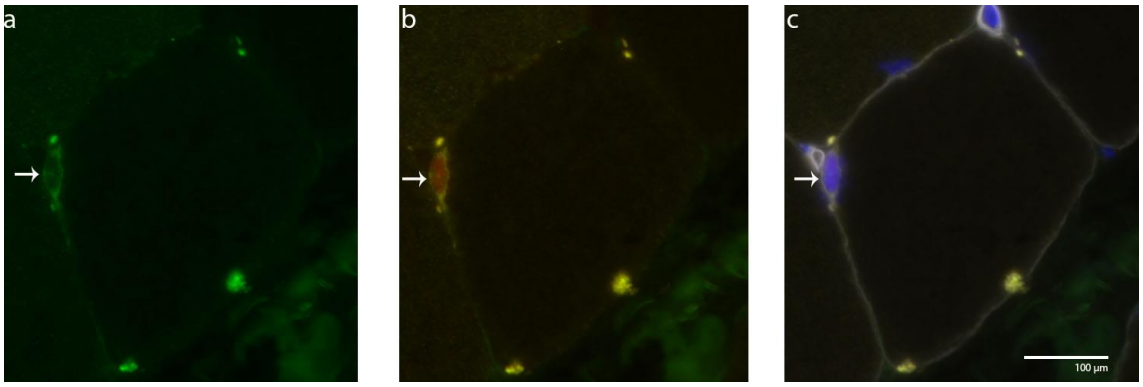

Figure 6. Cross-section of a muscle biopsy from an endurance athletes. SC are visualized using antibodies against NCAM, Pax7, laminin, and with DAPI, arrow in a, b and c. The cell border is stained for NCAM (green, a), the nucleus is stained with Pax7 (red, b). The nucleus is purple (c) due to counterstaining of Pax7 and DAPI. The NCAM staining appears yellow due to double staining with secondary antibodies (b). The satellite cell is beneath the basal lamina (white, c). Note the auto fluorescent lipofuscin granules. Modified from manuscript II.

Table 1. Antibodies used in paper I and II, concentrations used and company they were obtained from. Modified from manuscript II.

Antibody	Concentration	Company
4C7- Laminin A	1:200	Dako, Glostrup Denmark
NCL-Merosin-Laminin M	1:2000	Novocastra, Leica Biosystems, Newcastle, UK
A4.951	1:500	Developmental studies Hybridoma Bank, University of Iowa, Iowa City, IA, US
N2.261	1:400	Developmental studies Hybridoma Bank, University of Iowa, Iowa City, IA, US
Fetal NCL-MHCneo	1:200	Novocastra Laboratories Ltd, Newcastle, UK
Leu 19, NCAM/CD56 347740	1:10	Becton Dickinson Immunocytochemistry Systems, San Jose, CA, US
Pax7	1:10	Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, US
Laminin poly sheep, PC128	1:15000	The Binding Site Ltd, Birmingham, UK

Gene expression (Study I)

RNA was prepared by the Trizol method (Invitrogen, Life Technologies) with zircona beads using a bullet blender. RNA was then quantified spectrophotometrically by absorbance at 260 nm. To measure RNA integrity (RNA Quality Index, RQI 1-10) the Experion™ RNA StdSens & HighSens Kit (Bio-Rad Laboratories, Inc.) was used (the latter for small RNA samples). PicoDrop (Picopet01, Picodrop Ltd., Cambridge UK) was used to re-measure RNA-concentrations. Applied Biosystems High-Capacity cDNA Reverse Transcription Kit was used for reverse transcription. NCBI Primer-BLAST software was used for design primers. Agarose gel electrophoresis with ethidium bromide as stain and melt curve were used to control the length of amplicons and number of products by qPCR. Evaluation of three different genes as endogenous controls (GAPDH, β-actin and GPx1) was performed due to substantial variation in RQI between samples and because gene expression of some of the genes correlated with RNA integrity. These tests gave similar results. GPx1 was then chosen to normalize the CT values (ΔCT) because it has been used by our group in studies of small rodents and normalization with GPx1 abolished most of the correlation between RNA integrity and CT values in the samples with low RQI.

Statistical analysis

In the longitudinal ULSAM study (Study I) paired two-tailed t-test were used to compare individuals over time (longitudinal analysis from 70 to 88-90 and unpaired t-test were used to compare differences between various groups (survivors, non-survivors and healthy adult men). Principal component analysis (PCA) was used to examine the correlation of anthropomorphic data at ages 70, 82 and 88 and DXA at age 82 and 88, to find clusters of co-varying genes at age 70 and 88-90 and explore correlation of gene expression and histology at age 70. False discovery rate (FDR) was calculated due to multiple hypothesis testing.

In study II, one-way ANOVA was used to compare the different groups and unpaired T-tests were performed to follow up significant effects. Paired T-test were calculated to compare type I and IIA areas within the groups. FDR were used to correct for multiple comparisons.

In study III, Pearson's linear regression was used to analyze the relationship between manual and semi-automated measurements of muscle area and density. Bland-Altman plots were used to examine the systematic and random error (mean bias and 95% limits of agreement). The difference between the two legs and the systematic bias (difference in means between two methods) was examined for each muscle group using a Two-way ANOVA. Inter-rater reliability was assessed using a spreadsheet provided on sportsci.org (sportsci.org/2015/ValidRely.htm) with each leg considered an independent observation.

In study IV, non-adherent individuals were removed from the analysis and an unpaired T-test were performed to compare baseline characteristics between the two groups. To assess the effects of the physical activity intervention and nutritional supplement on muscle area and density of the different muscle groups, a three-way ANOVA with CSA and RA as dependent variables, time and muscle group as independent within variables, and group (nutrition or placebo) as the independent between variable were used. A paired two-tailed T-test was used to follow up significant interactions and for muscle torque and physical function. FDR were used to compensate for multiple comparison. Orthogonal Partial Least Squares (OPLS) were used to explore the effects of the physical activity intervention on muscle area, density and function. To quantify the importance of the different variables Variable Important for Projection (VIP) was used. VIP more than 1 suggests that the variable is important for the projection while values less than 0.5 suggests that it is not important [240]. An alpha level of 5% ($P < 0.05$) was considered significant in all studies.

Ethical considerations

All studies were approved by the regional ethical review boards in Uppsala, Umeå, or Stockholm. All participants gave informed consent. For the survey at age 70, the study was approved by the regional ethical review board in Uppsala Dnr 251/90, at age 82 Dnr: 02- 605, at age 88 Dnr: 2007/338 and at age 88-90 (muscle biopsies) Dnr: 2010/400. Below, I will address some of the ethical issues that need to be considered further in these studies. For the ULSAM study, the DXA measurement could be a potential issue due to its radiation and muscle biopsies, as it is an invasive method. However, since the DXA measurements are being performed for the first time at age 82 and the radiation from the DXA is low, it should not be harmful for the participants. The muscle biopsies might be associated with some pain. Complications are extremely rare and include minor bleeding and local infection [72]. Participation in the ULSAM cohort might even have health benefits for participants, as they were screened for cardiovascular risk factors and offered treatment and follow-up if needed.

For the athletes (study II) and the young male controls in Paper I, the main ethical concerns would be the muscle biopsies, which, as described above, are extremely rare to lead to complications although could be associated with some pain. Study II was approved by the regional ethical review board in Umeå (09-154M) and Uppsala (2010/400) and for the young control participants in Study I, Stockholm DNR 2006/1232-31/1 and DNR 2010/786-31/3.

In the VIVE2 study (Study III and IV), participants were exposed to some radiation during the CT-scans. At age 70+ this is not considered to be harmful to the participant if done only a few times. The health benefits of the physical activity intervention, both mental and physical, and the screening could have a positive impact on overall health. The study was approved by the Regional Ethical Committee in Uppsala, Sweden (Dnr 2012/154).

5 RESULTS

Study I

In study I, muscle mass, gene expression and morphology were analyzed longitudinally using the ULSAM cohort from age 70 to 88-90 and cross sectional in the ULSAM cohort comparing survivors and non-survivors at age 70 and also survivors compared to healthy adult men.

Muscle mass and histology

Muscle mass was assessed by DXA at ages 82 and 88. Appendicular lean mass/height^2 decreased with 0.1% per year and total lean mass decreased 0.3-0.4% per year in survivors with biopsy investigated here and all survivors, respectively. The proportion of type II fiber (including type IIA, IIA+IIX and IIX) declined, whereas the hybrid fiber type I+IIA increased from age 70 to 88-90 years. Type II fibers were smaller than type I fibers at both ages 70 and 88-90 years, and this difference seemed to increase over these years (data from Hedman et al included, Figure 7). There were no differences in fiber area or fiber frequencies at age 70 years when survivors and non-survivors were compared (using data from Hedman et al [235].

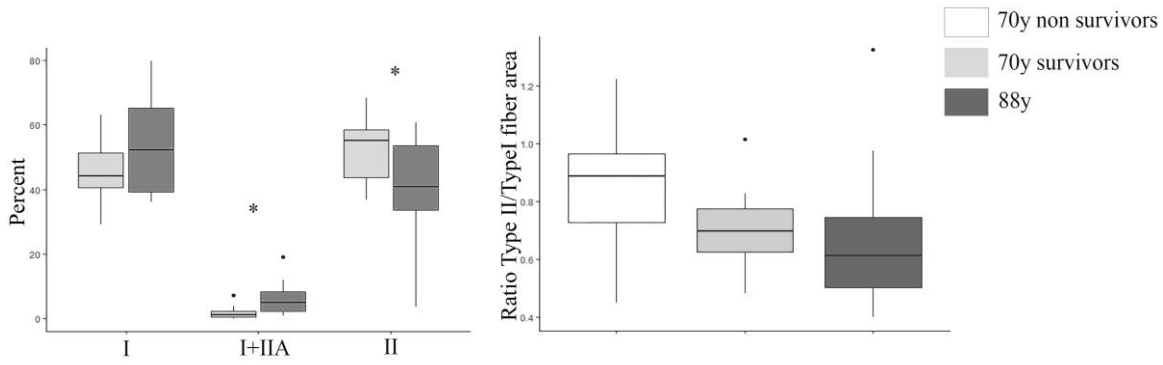

Figure 7. Boxplot of fiber type composition using immunohistochemistry at age 70 and 88-90 for the fiber types I, I+IIA and II (II includes IIA, IIAX and IIX) fibers, n=16. The fiber area ratio type II/type I using data from Hedman et al. (ATPase) for non-survivors (NS, n=24) and survivors at age 70 (S, n=12) [235] and also new data using immunohistochemistry at age 88-90 (n=12). Whiskers indicate highest and lowest value within 1.5 IQR. Singular point indicates more extreme values. *p<0.05, modified from Paper I.

SC, nuclei and internal nuclei was assessable longitudinally from 70 to 88-90 only in 5 individuals. The number of SC decreased in 3 out of 5 individuals, while nuclei and internal nuclei decreased in 4 out of 5 individuals from 70 to 88-90 (Figure 8).

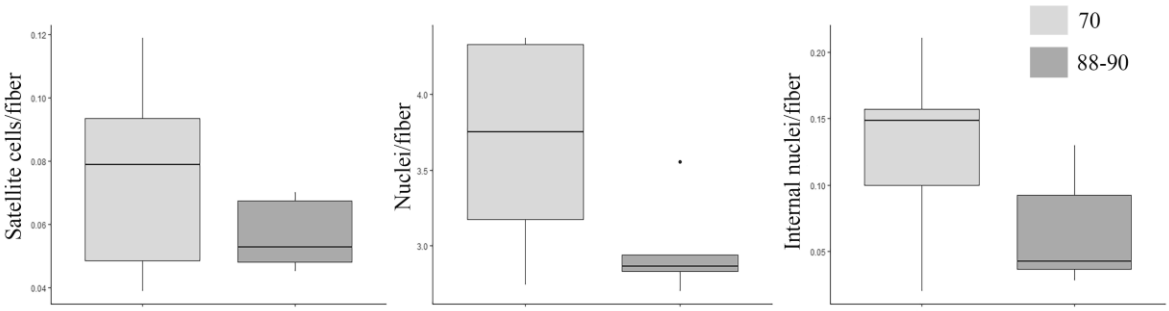

Figure 8. SC, nuclei and internal nuclei per fiber in ULSAM individuals at age 70 and 88-90 (n=5). Whiskers indicates highest and lowest value within 1.5 IQR. Singular point indicates more extreme values. Modified from supporting information Paper I.

Gene expression

Myogenin, Myo D, eMyHC, NCAM and Smad2 were higher at 70 than at age 88-90. The mechano-splice variant of IGF-1 (IGF-1Ec) was undetectable at age 88-90 years, while β-Catenin increased from 70 to 88-90. Survivors had higher levels of Myogenin, MyoD, eMyHC, NCAM, Smad2, Trim32, MuRF1 at age 70, while Smad3 and IGF-1Ec were lower compared to healthy adult men. Those who survived to age 88 had higher levels of eMyHC, Smad2, and MuRF1 at age 70 than those who died before age 82 years. Individuals with lower function (hand grip <30 kg and/or gait speed ≤0.8 m/s) at age 88-90 years had lower expression of Myogenin, Smad2 and TRIM32.

Study II

This study examined muscle morphology in a group of endurance athletes (EA) aged 82-92 years (mean 85) and contrasted them to the ULSAM cohort aged 87-91 years (mean 89), who were divided into a high-function (HF) and low-function group (LF) on the basis of gait speed, chair stand time, TUG and hand grip strength. The endurance athletes had more type I fibers, less variation in fiber size, more capillaries per fiber and capillaries in relation fiber area and fewer COX deficient fibers compared to the two ULSAM groups. There was an indication of greater type II fiber area in both EA (27%) and HF (33%) groups compared with LF (P=0.06), and type I fiber CSA were larger than type IIA in HF and LF. There were no differences in the mixed fiber type I+IIA between groups, although the individual variation was large. Only a few percent of fibers were positive for fetal MyHC (mean in the three groups 0.7-1.4%) or NCAM (mean in the two ULSAM groups 1.7-2.6%). There were no differences between the three groups in fetal MyHC expression or between the two ULSAM groups in NCAM positive fibers. Of the fibers positive for Fetal MyHC, 20.5% also expressed NCAM, and of the NCAM positive fibers only 9.2% also expressed fetal MyHC (only ULSAM groups included). There were no differences between groups in terms of nuclei per fiber, internal nuclei per fiber, SC per fiber or SC per nuclei.

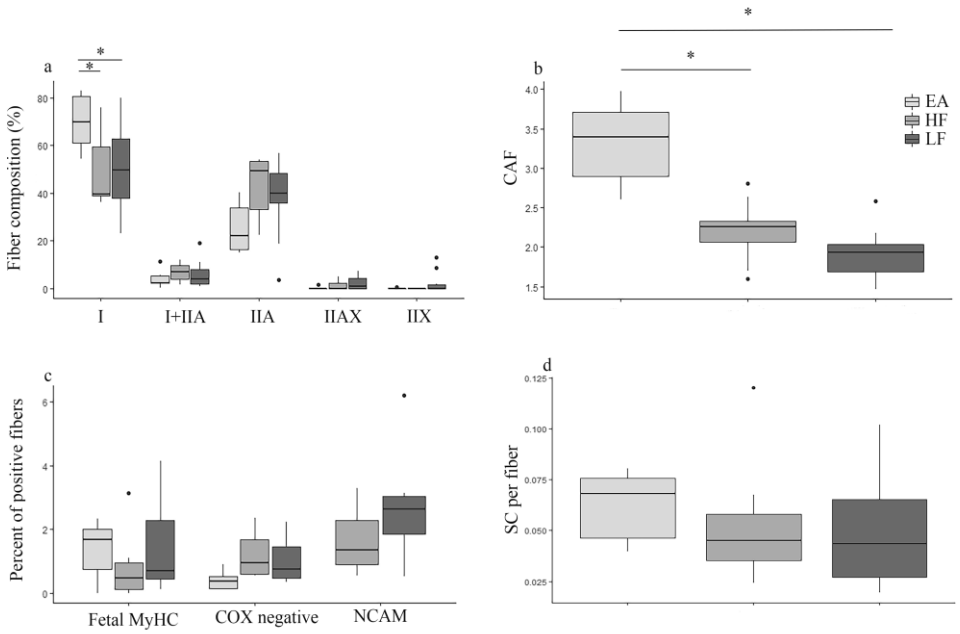

Figure 9. Fiber type composition (a), capillaries adjacent to fibers (CAF, b), percent of Fetal MyHC positive, COX negative and NCAM positive fibers (c) and SC per fiber (d) in endurance athletes (EA), ULSAM high (HF) and low function (LF) groups. Whiskers indicates highest and lowest value within 1.5 IQR. Singular point indicates more extreme values. *p<0.05

Study III

In study III, a semi-automated and a manual method were evaluated measuring area and density of 8 locomotor muscle groups. Semi-automated and manual measurements generally correlated very well, $r \geq 0.9$ for all measurements except for CSA in gluteus maximus (r=0.88) and gluteus medius+minumus (r=0.79). There was a significant bias in CSA for half of the muscle groups. In two of eight muscle groups, the bias was more than 5%, in the iliopsoas muscle it was 15.1% and, in the ankle dorsiflexors 5.3%. For RA, the plantar- and dorsiflexors showed no systematic bias between methods, whereas this was the case for the other muscle groups. For the gluteus medius + minimus muscles, it was 8.8 HU in all other muscle groups below 5 HU (Table 2.). The semi-automated method was 3.2 times faster than the manual method (p<0.01).

Table 2. Cross-sectional area (mm^2) and radiological attenuation (Hounsfield units, HU) of muscle groups examined (right and left) with manual or thresholded method. Bias in % or HU and their p-values. Modified from Paper III.

		Cross-sectional area				Radiological attenuation			
		Method data (mm^2)		Method	Method bias	Method data (HU)		Method	Method bias
Muscle group		Manual	Thresholded	bias Δ%	P-value	Manual	Thresholded	bias ΔHU	P-value
Knee extensors	*Right*	4433 (1057)	4461 (1050)	0.8	0.065	47.8 (7.5)	49.8 (6.5)	2.0	<0.0001
	Left	4426 (996)	4472 (1048)			48.0 (7.4)	49.6 (6.4)		
Knee flexors	*Right*	2726 (718)	2742 (699)	0.6	0.400	35.2 (10.5)	36.2 (8.4)	1.0	0.009
	Left	2725 (646)	2743 (638)			36.1 (12.2)	37.4 (9.6)		
Hip adductors	*Right*	2027 (675)	2005 (658)	-1.7	0.014	36.6 (9.2)	38.9 (7.0)	2.3	<0.0001
	Left	2036 (703)	1986 (694)			37.9 (9.4)	40.1 (7.7)		
Gluteus min/medius	*Right*	4130 (704)	3927 (714)	-3.8	0.034	25.2 (15.8)	34.0 (8.6)	8.8	<0.0001
	Left	4123 (633)	4011 (713)			27.6 (15.4)	35.2 (8.7)		
Gluteus maximus	*Right*	3186 (770)	3232 (732)	1.7	0.317	19.8 (13.3)	21.7 (10.0)	1.9	0.021
	Left	3207 (656)	3269 (633)			23.3 (14.2)	24.5 (11.1)		
Iliopsoas	*Right*	1294 (369)	1491 (392)	15.1	<0.0001	53.5 (6.0)	52.9 (4.9)	-0.6	0.006
	Left	1287 (364)	1481 (368)			54.8 (6.0)	53.3 (5.2)		
Ankle dorsiflexors	*Right*	1705 (335)	1792 (383)	5.3	<0.0001	45.2 (12.1)	46.7 (9.9)	1.5	0.055
	Left	1645 (306)	1736 (328)			45.6 (12.8)	46.3 (9.9)		
Ankle plantar flexors	*Right*	4146 (1088)	4150 (1014)	0.4	0.824	43.5 (13.7)	45.1 (9.5)	1.6	0.084
	Left	4151 (1144)	4179 (1173)			45.0 (14.6)	46.0 (10.5)		

The inter operator correlation was r >0.9 in all muscle measurements except CSA for gluteus medius + minimus (r=0.76) and for RA in the Iliopsoas muscle (r=0.85). The bias was more than 5% for knee extensors (5.7%), hip adductors (6.0%), gluteus maximus (5.3%) and ankle dorsiflexors (5.8%). For RA, none of the muscle groups had a bias over 5 HU (Table 3).

Table 3. Inter-rator bias, typical error, r-value and intra-class correlation (ICC). Modified from Paper III

	Cross-sectional area				Radiological attenuation			
	Mean bias	Typical			Mean bias	Typical		
Muscle group	(%)	error (mm^2)	r-value	ICC	(HU)	error (HU)	r-value	ICC
Knee extensors	5.7	110	0.99	0.99	3.8	0.2	0.98	0.98
Knee flexors	3.9	183	0.93	0.92	4.0	1.8	0.98	0.98
Hip adductors	6.0	211	0.91	0.91	0.8	1.5	0.97	0.97
Gluteus min/medius	4.0	371	0.76	0.75	2.6	2.1	0.98	0.98
Gluteus maximus	5.3	188	0.94	0.94	3.4	1.2	0.99	0.99
Iliopsoas	4.3	106	0.92	0.92	3.6	2.3	0.85	0.86
Ankle dorsiflexors	5.8	95	0.93	0.92	1.7	4.0	0.91	0.90
Ankle plantar flexors	2.7	120	0.99	0.99	3.3	1.2	0.99	0.99

Study IV

In study IV, muscle area and density in 8 different locomotor muscle groups, and physical function were evaluated before and after a 6 month physical activity intervention where the participants were randomized to a nutritional supplement or a placebo drink. The physical activity program preformed 3 times a week increased the CSA of the knee extensors (1.9%) and hip adductors (2.8%) but no changes in area were seen in the other six muscle groups studied. The RA increased in the hip flexors (1.1 HU), hip adductors (0.9 HU), knee extensors (1.2 HU) and ankle dorsiflexors (0.8 HU) with no changes in the other 4 muscle group studied. There was an increase in the knee flexor torque (22 %; $p<0.0001$) and improvements in walking speed (13%, $p<0.001$), stair climbing time (6%, $p<0.01$) and in SPPB (38%, $p<0.001$). Multivariate analysis revealed that SPPB, walking speed and knee flexor torque contributed significantly to the VIP model while area and muscle RA did not. The improvements in physical function did not correlate with increases in CSA or RA. The nutritional supplement did not enhance the training effects.

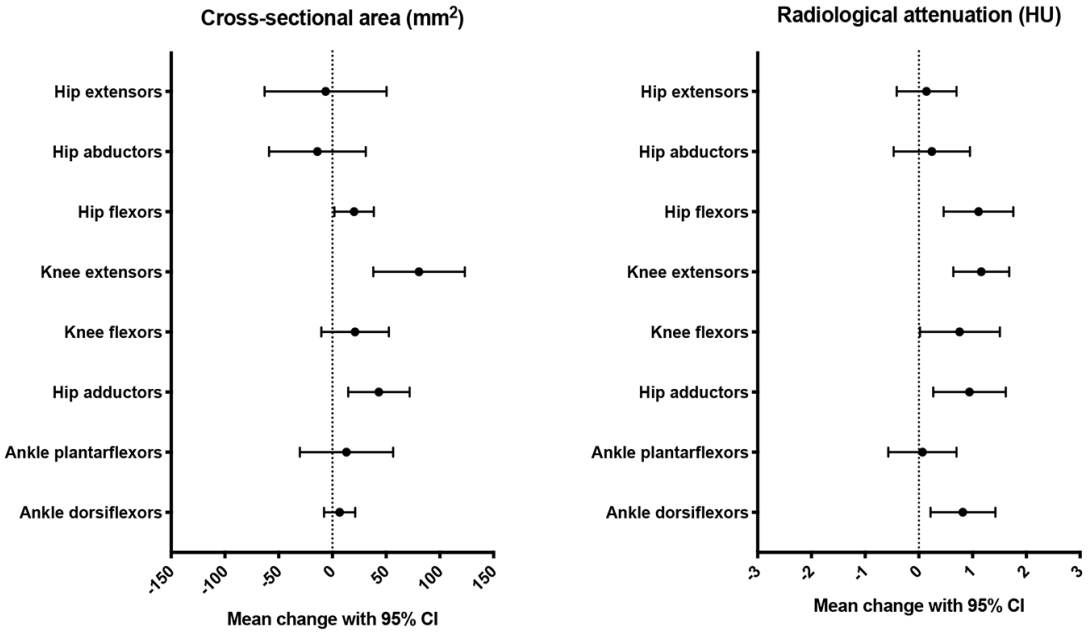

Figure 10. Mean changes, with 95% confidence interval, in cross-sectional area and radiological attenuation in the 8 different muscle groups after 6 months of physical activity intervention. From manuscript IV.

6 DISCUSSION

In this thesis, age-related muscular changes were examined both longitudinally (Study I and IV) and cross-sectionally (Study II), at gene expression (Study I), morphological (study I and II) and whole-muscle level (Study IV). The muscular changes were related to physical function (Study I, II and IV) and exercise (Study II and IV). In addition, a semi-automated method for analyzing muscle cross-sectional area and radiological density in 8 lower-extremity muscle groups were evaluated (Study III). The main findings of this thesis were that 1) muscle plasticity is decreased with advanced age, as indicated by an attenuated gene expression profile at age 88-90 years compared to 70 years. 2) Muscle mass can be relatively well preserved even at very advanced ages in a cohort of successfully aged male seniors, and some changes in muscle morphology occur regardless of endurance training background. However, lifelong endurance training appears to preserve oxidative capacity, as indicated by more type I fibers, more capillaries and fewer COX deficient muscle fibers. 3) Semi-automated measurement of muscle cross-sectional area in CT scans is time-saving and accurate, although caution is warranted when using pre-determined thresholds to determine fat infiltration in muscles with high fat content. 4) A physical activity program in the elderly with limited mobility had minor effects on muscle size and fat infiltration, and these effects varied between muscle groups and were not related to improvements in physical function.

Characteristics of the aging muscle

In the longitudinal ULSAM study (study I), muscle characteristics were quite stable during the two decades of aging (between 70 and 88-90 years of age). Other longitudinal studies have shown somewhat greater changes in muscle mass, although most studies include younger individuals compared to our cohort. A more consistent finding in both longitudinal and cross-sectional studies is decreased muscular strength and function [21, 24-30]. The relatively small loss of muscle mass may be explained by the fact that these participants were among the 28% still alive at the time for the second muscle biopsy. It could arguably be claimed that this reflects successful aging, and that our results support the importance of muscle mass maintenance for healthy aging.

With regards to the fiber size measurements, these were measured using different methods at age 70 compared to 88-90. Because of this, a ratio between the type II and type I fibers were calculated, without any statistical testing. The ratio decreased over time indicating type II fiber atrophy, with preserved type I fiber size, even at this advanced age. This is consistent with the general viewpoint of many cross-sectional studies of earlier aging that age mainly affects the area of type II fibers, while type I fibers are spared or even hypertrophied [70, 86-89, 94, 95, 99, 102]. When endurance athletes and ULSAM participants were compared in study II, there was a trend for larger type IIA fibers in the athletes and the ULSAM high function group, suggesting that both better function and lifelong endurance training may be associated with larger type IIA fibers. In support of this, the type IIA fiber area was relatively

well preserved in participants with a history of lifelong endurance training. The effect of endurance training on type II fiber area has been investigated in lifelong runners aged 64 years, and no difference was found compared to the untrained control group [87]. The most striking difference between our study and Mackey's is the age difference of approximately 20 years. It is possible that endurance training has a small positive effect on both type I and type II fiber area [241], although the relative increase in type II fibers may be influenced by age, especially compared to a sedentary control group, as the difference in physical activity between the active and inactive may be different at advanced ages.

There was a decrease in the frequency of type II fibers (including IIA, IIAX and IIX) combined with an increase in the type I+IIA hybrid fiber from age 70 to 88-90 years (Study I). Most studies report no changes in the proportions of fiber types during aging, although many include only type I and type II fibers, but no hybrid fibers [38, 70, 96, 103, 105, 107]. At age 88-90 years, type IIAX appeared to be more common in the ULSAM groups compared to athletes and type IIX appeared to be less common in the ULSAM high-function group and athletes compared to the ULSAM low-function group, although the fibers were few and therefore no statistical analysis was performed. As these two fiber types (IIAX and IIX) were expressed at very low levels, this may indicate that our participants were still physically active, as type IIX increases during immobilization and inactivity [109, 242, 243] and decreases with training. Hedman et al. reported approximately 20% type IIb (ATPase staining) fibers at age 70 in the ULSAM cohort (n=515) [235]. However, this apparent contradiction can partly be explained methodological factors, as some type IIb fibers are classified as type IIAX by the immunohistochemical method [244]. Since pure type IIX are most likely rare in healthy human muscles, the low levels in our ULSAM cohort and in endurance athletes could be considered indicative of healthy muscles [245].

Type I fibers predominated in the endurance athletes which was expected from previous literature and is most likely due to a combination of their genetic traits and the endurance training performed [42, 223, 246-248]. Furthermore, the increase in fibers co-expressing MyHC I+IIA may suggest increased denervation during these 20 years of aging, as the increase in fibers co-expressing MyHC I+IIA has been suggested to be associated with the denervation process [108]. In humans, there are conflicting results regarding the number of fibers co-expressing different MyHC. Most studies show low levels in young individuals, while data differ in older individuals, possibly due to different methods, i.e., sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), ATPase staining and immunohistochemistry, and different age groups and population sizes [109-112, 191, 249-251]. Larsson et al. used SDS-PAGE for MyHC distribution and found low levels of fibers co-expressing MyHC in young and old people (73-81 years old), with the physically active elderly possible having more fibers co-expressing MyHC I+IIA [191]. This may be due to a transition state induced by training [42]. However, using immunocytochemistry, Klitgaard found few fibers co-expressing MyHC I and II in vastus lateralis in young (1%) and in old (2%), while old swimmers had 0% and old runners and strength trained individuals had 1% [106]. On the other hand, in a study by Andersen et al., also using SDS-PAGE, type I + IIA

MyHC co-expression was found in 28.5% of the fibers from men and women aged 85-98 years and they concluded that there is an increase during aging. The participants in that study were older compared to previous studies, but no young control group was included [110]. Even though the co-expression of MyHC I+IIA increased from 70 to 88-90 in the present work (study I), it was only a few percent even at the age of 88-90. In addition, we investigated whether lifelong endurance training influences the co-expression of MyHC I+IIA. When ULSAM individuals were divided into high and low function groups and compared to endurance athletes, there were no differences between the groups (Study II). This is consistent with previous research indicating that lifelong training is not associated with changes in the frequency of co-expression MyHC I+IIA fibers [111, 112]. Thus, it is possible that the denervation process is unaffected by lifelong endurance activity. Taken together, our longitudinal and cross-sectional data suggests a small but significant relative increase in type I+IIA fibers during aging, and levels at very high ages are not related to endurance training or differences in physical function.

Fetal MyHC and NCAM are considered indirect markers of denervation [161-163]. As additional support for similar denervation between groups, there were no differences in either Fetal MyHC (between all three groups) or NCAM (between the two ULSAM groups). Interestingly, about one fifth of the fibers expressing fetal MyHC were also NCAM positive (only ULSAM individuals included), which is consistent with previous work from Soendenbroe et al. [160].This may suggest that they are expressed at different time points during the denervation process as seen in rat [252], or possibly due to the segmental staining of fetal MyHC [162]. Furthermore, because these markers are also expressed during regeneration and there can be regeneration without denervation (and vice versa), it is possible that some of the Fetal MyHC and/or NCAM positive fibers reflect a regeneration process [162, 163]. Additional support for the suggestion that denervation does not depend on physical activity level was provided by recent work showing no differences in NCAM positive fibers or mRNA expression of markers of denervation (acetylcholine receptor alpha, epsilon, gamma, and MuSK), whereas fibroblast growth factor binding protein 1 gene expression was upregulated in master athletes compared to young and frail elderly [111]. Taken together, these results suggest a similar denervation but a better reinnervation in old active compared to inactive individuals [111, 112]. It is noteworthy that there seems to be large individual differences and this discrepancy between individuals appeared to be smaller in master athletes, highlighting the need for further research in this area [111].

In study I, SC decreased in three out of five individuals between age 70 to 88-90, which is consistent with previous reports showing decreased SC content with aging [87, 88, 93-95, 118-122]. In study II, there were no differences in the number of SC between the three groups examined, suggesting that SC content is not strongly related to physical function or lifelong endurance training. However, the endurance athletes had slightly more internal nuclei than the other groups, which may indicate a history of muscle regeneration [11]. Although the difference was not significant and from morphological perspective the athletes' muscles were

well preserved, there was no evidence of large areas of ongoing foci of necrosis. In addition, there was no evidence that this lifelong endurance training had damaged the muscles.

Overall, the role of SC in aging-induced muscle mass loss is still controversial. In support of the theory that SC are still able to maintain healthy muscles, regenerative capacity following electrical stimulation was preserved in muscles from older subjects (age 67 years), with similar hypertrophy between young and old individuals in response to stimulation. The SC response was also similar between groups, possibly even a stronger response in SC in type I fibers in the older participants [253]. Even though electrical stimulation does not represent a real-life occurring phenomenon in aging muscle, these results support the idea that SCs are able to sustain muscle regeneration at earlier aging. In contrast, at more advanced age (87 years), the elderly did not increase their SC pool or fiber area in response to resistance training, suggesting decreased plasticity at this advanced age [93, 254].

Another factor that could contribute to the reduction of SC and aging atrophy of the type II fibers are the capillaries. In a 24-week resistance training study, Snijders et al. examined hypertrophy and SC changes based on capillary-to-fiber perimeter exchange (CFPE) at baseline. Participants were divided into two groups based on their CFPE count, and only participants with higher levels of CFPE at baseline increased type II fiber CSA and SC count [127]. In our comparison between the ULSAM groups and the older athletes, the athletes had more capillaries around each fiber and in relation to fiber area. This could be of clinical importance when exercise is recommended to older people as endurance training can increase both capillary per fiber and capillary density of muscles [43-47, 194]. Strength training, on the other hand, may increase capillary contacts while capillaries in relation to fiber area is more constant due to increases in fiber area [195, 196]. It is possible that endurance training is important to increases the number of capillaries in the muscle so that they can later respond to the strength training. In the ULSAM cohort, Hedman et al. found a significant positive correlation between insulin-induced leg blood flow and capillary density at age 70 [255]. This provides further support for the thesis that lower capillary density at advanced ages contributes to anabolic resistance.

As mentioned earlier, there is evidence for a reduced plasticity of human muscles in very old age [93, 254]. The cross-sectional data in the present thesis suggest an increase in muscle remodeling with up-regulation of genes involved in protein degradation and muscle differentiation (Smad2, Trim32, MuRF1, Myogenin, MyoD, eMyHC), and in denervation/neuromuscular instability (NCAM) at age 70 years compared to young healthy men, and decreased expression of Myogenin, Myo D, eMyHC, NCAM and Smad2 at age 88-90 compared to age 70. A similar pattern was seen when survivors were compared with non-survivors, with higher levels of eMyHC, Smad2 and MuRF1 in survivors. There are conflicting results on how the genes mentioned above behave during aging, inactivity, and exercise. Some studies found no differences between young and old, active and inactive individuals in the expression of MuRF-1 and Atrogin [101, 199, 203]. Others have reported lower expression of MuRF-1 and Atrogin in young compared to elderly [204] and in inactive

frail elderly women compared to active healthy elderly women [201]. Raue et al. reported higher expression of MyoD, MRF4, Myf5, MuRF-1, and FOXO3A in old women at rest and that MuRF-1, MyoD, and MRF4 were up-regulated by one bout of resistance exercise in both young and elderly women [181, 200]. Furthermore, no effect of the 10-week strength training intervention on basal levels of MuRF-1 and Atrogin-1 was detected [206].

The longitudinal study between 70 and 88-90 suggests that plasticity may be preserved at age 70 in those who survived, whereas it decreases at older ages (88-90) and that it is lower in those who did not survive to follow-up at age 82 compared to survivors at age 70. It is possible that as people age, more mutations accumulate in the SC and spread to the muscles [126] and that this process increases the number of misfolded proteins that needs to be degraded. In support, increased mRNA expression of Hsp1a (marker of misfolded protein pathway) in older non-sarcopenic individuals and an even greater increase in older sarcopenic individuals compared to middle-aged individuals has been reported [256]. In a healthy muscle, up-regulation of e.g., the ubiquitin ligases may maintain muscle fiber function at this stage. In more advanced ages, this plasticity may be lost, and atrophy or accumulation of misfolded proteins occur and impair muscle function. The ULSAM individuals with lower function in Study I (hand grip <30 kg and/or gait speed ≤0.8 m/s) showed decreased expression of Myogenin, Smad2 and TRIM32. The remarkable stable muscle mass gives additional support for the hypothesis that this expression pattern is beneficial and strengthens the idea that maintained remodeling capacity is important for healthy muscle during aging. The reduction in gene expression observed at 88-90 years may reflect a diminished response to denervation, as shown in old mice who failed to upregulate NCAM after denervation [257]. This is consistent with data from humans suggesting that muscles are not reinnervated in sarcopenic elderly [143]. In summary, the gene expression profile in ULSAM individuals suggests an initial response to intrinsic or extrinsic changes that are lost at the most advanced ages. This response during early aging may be physiological and a mechanism to maintain healthy muscle.

Assessing muscle size and density

When measuring muscle mass and density, it is important to have reliable methods. However, time (spent for assessment of the analyses) is also an important factor especially when larger studies are conducted or when assessments should be implemented at a wider scale in clinical settings. While muscle mass is generally agreed to be of great importance to muscle health, increased attention is given to the quality of the muscle. One such parameter is skeletal muscle fat infiltration, which has been associated with reduced strength and hospitalization [3, 64]. However, accurate assessment of both muscle size and fat infiltration currently requires MRI or CT scans, which can be costly and time-consuming as images most often are analyzed manually and image by image. Therefore, a semi-automated threshold method was compared to a manual method in Study III, covering 8 muscle groups of the thigh, hip and calves. The threshold for the semi-automated measurements was set to -29 to + 150 HU to

account for low-density muscles [63]. For the knee extensor and flexor muscles, which are commonly examined in training studies in both young and aged individuals, the correlations between the methods were high and the systematic bias was less than 1%. As anticipated, however, the bias of the different methods increased when semi-automated measurements were applied on muscles with a high fat content. For gluteus medius and minimus, the difference was quite large, i.e. CSA was overestimated and the fat content underestimated compared with the manual method. In some individuals, there was some intermuscular fat between the gluteus medius and maximus muscles included as muscle in the manual measurement, making CSA larger and RA lower. Therefore, in some cases, the semi-automated measurement may have been more correct and the bias less if the muscles would have been measured separately. Furthermore, the mismatch observed in muscles with high fat content might be corrected by lowering the threshold cut-off values, although this approach increase the risk to include intermuscular fat [63]. The rather large difference in the CSA of iliopsoas muscle (reported in study III) could be due the iliopsoas muscle being adjacent to the intestines, which are similar in mean density [258]. In addition, the small size of the muscle makes it more sensitive to measurement errors.

Altogether, study III suggest that semi-automated measurements are time-saving and accurate when applied to thigh muscles with normal density, even in mobility limited elderly over 70 years of age. However, low-density muscles may need different thresholds, and it is important that the assessor understands the strength and limitations of each method. This is particularly important because intramuscular fat increases with age, which could affect measurement if individuals are even older or frailer than our VIVE2 cohort [64].

Counteracting age-induced muscle deterioration

Elderly individuals are recommended to engage in at least 150 min moderate aerobic exercise per week, muscle strengthening exercises twice a week and also include balance training [233, 234, 259]. The physical activity intervention in study IV aimed to explore the effects of a physical activity intervention that was feasible for individuals with limited mobility and at risk for dependency [260]. The results showed that 6 months of physical activity resulted in only small changes in some but not all muscle groups of the lower extremities, while physical function was markedly improved. There was no correlation between improvement in muscle size and density and gain in function. The reason for this is likely that neurological adaptations occurred, which might be even more important in the elderly than in the young [48, 49]. This is interesting because the morphological changes observed during aging are partly due to inactivity and the de- and reinnervation processes (as described above). The discrepancy between strength and size may in part be due to that denervated fibers maintain their contractile machinery during a period of time before they either get reinnervated or atrophy [162]. If this reinnervation is enhanced by exercise, the size will be constant while the strength increases due to an increased number of innervated fibers. In addition, there are also intra myocellular changes that may be relevant to the reduction in strength during aging,

such as Ca^{2+} handling [261]. This can be improved without muscle mass gain or fat reduction, but was not measured in this thesis. It is important to consider these aspects when designing exercise programs for the elderly, as function is essential for managing activities of daily life, while muscle mass may be important for other functions such as thermoregulation and insulin sensitivity, and also acts as an endocrine organ secreting various myokines thought to be important for e.g. immune function and regulation of energy balance [137].

In Study IV, there were no effects of the nutritional supplement containing protein and vitamin D. It is well known that protein intake is important to promote muscle protein synthesis and maintain muscle mass in the elderly [145-147]. The amount of whey protein administered should have been sufficient to stimulate muscle protein synthesis [155], although an intake of up to 0.4 g/kg body weight may be necessary to achieve maximal effect in the elderly [151, 156], as the anabolic response to amino acids/proteins is lower in elderly [149, 150]. It should be noted that although the participants displayed mobility limitations, they were not malnourished. In addition, Von Berens et al. reported that the Swedish participants joined the study in part to lose weight [262]. Thus, it is possible that the participants in the supplement group compensated by lowering their food intake. Interestingly, the small weight gains were similar in both groups even though the drinks were not isocaloric. This is consistent with the view that most people self-regulate energy homeostasis over time [263]. The nutritional drink also contained vitamin D and the effects of this vitamin on muscle mass are still debated. A robust meta-analysis found a significant effect of vitamin D supplementation on muscle strength, but not on muscle mass or muscle power [220], while two other meta-analysis found no effect on strength or muscle function if vitamin D was above 25nmol/L [264, 265]. There are several possible explanations for the slightly different results between study IV and the results reported from the thigh of the entire cohort (both Swedish and US participants), where low density muscles decreased and normal density muscles increased [65]. Importantly, Englund et al. used a different approach to analyze the muscle density data compared to Study IV. Firstly, they included the whole thigh, secondly, they only included areas with a radiological attenuation of 0-100 HU and thirdly they divided the muscles into low (0-34 HU) and normal (35-100) density muscles [65]. There is no single cut-off for defining muscle by HU, although low-density muscles are considered to be as low as -29 HU [63]. In the current thesis, muscles were manually encircled and intermuscular fat between the circled groups was excluded. Taken together, if there was an effect from nutritional supplementation, it was arguably small in this well-nourished population.

7 CONCLUSIONS

In this thesis, it is shown that muscle plasticity decreases with age and the response to physical activity intervention is less than expected from a young population. Several factors are important for this reduced plasticity. In study I, it is shown that up-regulation of genes involved in protein degradation and muscle differentiation could contribute to maintain muscle health during successful aging. However, it appears that significant functional improvements can occur without major increases in muscle size or reductions in intramuscular fat infiltration. This suggests that increases in physical function in older people with reduced mobility is due to factors beyond muscle mass. Re-innervation of denervated muscle fibers may be one. A multicomponent physical activity program will not affect all muscle groups equally. Such programs should be tailored to muscle groups at risk. In addition, lifelong endurance training is associated with greater microvascular network and oxidative profile of the muscle. It is plausible that these factors are important to counterbalance anabolic resistance, and may reduce the risk of disability.

8 POINTS OF PERSPECTIVE

The results of this thesis indicate indirectly that nerves may play important roles in muscle atrophy and the development of sarcopenia with increasing age. There has been a major focus on muscle mass during the years while physical function has gained attention the last decade. The anabolic resistance and the reduced plasticity may be important for muscle mass while it is shown in this thesis that physical function can be maintained and increase substantially even at advanced ages. Furthermore, muscle mass and morphological characteristics may be unrelated to function. Thus, when physical activity interventions are designed, it is important to have in mind that different stimuli may be needed to gain mass and physical function and for the old individuals this is important since the metabolic effects from gaining muscle mass may be more important at earlier aging while maintaining muscle function is essential to manage the task of daily living regardless of age.

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10 REFERENCES

1. Baumgartner, R.N., et al., *Epidemiology of sarcopenia among the elderly in New Mexico.* Am J Epidemiol, 1998. **147**(8): p. 755-63.
2. Janssen, I., S.B. Heymsfield, and R. Ross, *Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability.* J Am Geriatr Soc, 2002. **50**(5): p. 889-96.
3. Cawthon, P.M., et al., *Do muscle mass, muscle density, strength, and physical function similarly influence risk of hospitalization in older adults?* J Am Geriatr Soc, 2009. **57**(8): p. 1411-9.
4. Cruz-Jentoft, A.J., et al., *Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People.* Age Ageing, 2010. **39**(4): p. 412-23.
5. Cruz-Jentoft, A.J., et al., *Sarcopenia: revised European consensus on definition and diagnosis.* Age Ageing, 2019. **48**(1): p. 16-31.
6. Cruz-Jentoft, A.J., et al., *Prevalence of and interventions for sarcopenia in ageing adults: a systematic review. Report of the International Sarcopenia Initiative (EWGSOP and IWGS).* Age Ageing, 2014. **43**(6): p. 748-59.
7. Liu, P., et al., *Sarcopenia as a predictor of all-cause mortality among community-dwelling older people: A systematic review and meta-analysis.* Maturitas, 2017. **103**: p. 16-22.
8. Beaudart, C., et al., *Health Outcomes of Sarcopenia: A Systematic Review and Meta-Analysis.* PLoS ONE, 2017. **12**(1): p. e0169548.
9. Bhasin, S., et al., *Sarcopenia Definition: The Position Statements of the Sarcopenia Definition and Outcomes Consortium.* J Am Geriatr Soc, 2020. **68**(7): p. 1410-1418.
10. Cruz-Jentoft, A.J. and A.A. Sayer, *Sarcopenia.* Lancet, 2019. **393**(10191): p. 2636-2646.
11. Larsson, L., et al., *Sarcopenia: Aging-Related Loss of Muscle Mass and Function.* Physiol Rev, 2019. **99**(1): p. 427-511.
12. Gustafsson, T. and B. Ulfhake, *Sarcopenia: What Is the Origin of This Aging-Induced Disorder?* Front Genet, 2021. **12**: p. 688526.
13. Seo, D.Y., et al., *Age-related changes in skeletal muscle mitochondria: the role of exercise.* Integr Med Res, 2016. **5**(3): p. 182-186.
14. Frontera, W.R., et al., *Strength conditioning in older men: skeletal muscle hypertrophy and improved function.* J Appl Physiol (1985), 1988. **64**(3): p. 1038-44.
15. Bauer, J., et al., *Sarcopenia: A Time for Action. An SCWD Position Paper.* J Cachexia Sarcopenia Muscle, 2019. **10**(5): p. 956-961.
16. Lai, C.C., et al., *Effects of resistance training, endurance training and whole-body vibration on lean body mass, muscle strength and physical performance in older people: a systematic review and network meta-analysis.* Age Ageing, 2018. **47**(3): p. 367-373.

17. Liu, C.J. and N.K. Latham, *Progressive resistance strength training for improving physical function in older adults.* Cochrane Database Syst Rev, 2009(3): p. CD002759.

18. Tzankoff, S.P. and A.H. Norris, *Effect of muscle mass decrease on age-related BMR changes.* J Appl Physiol Respir Environ Exerc Physiol, 1977. **43**(6): p. 1001-6.

19. Kyle, U.G., et al., *Total body mass, fat mass, fat-free mass, and skeletal muscle in older people: cross-sectional differences in 60-year-old persons.* J Am Geriatr Soc, 2001. **49**(12): p. 1633-40.

20. Janssen, I., et al., *Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr.* J Appl Physiol (1985), 2000. **89**(1): p. 81-8.

21. Mitchell, W.K., et al., *Sarcopenia, dynapenia, and the impact of advancing age on human skeletal muscle size and strength; a quantitative review.* Front Physiol, 2012. **3**: p. 260.

22. Wroblewski, A.P., et al., *Chronic exercise preserves lean muscle mass in masters athletes.* Phys Sportsmed, 2011. **39**(3): p. 172-8.

23. Mikkelsen, U.R., et al., *Life-long endurance exercise in humans: circulating levels of inflammatory markers and leg muscle size.* Mech Ageing Dev, 2013. **134**(11-12): p. 531-40.

24. Hughes, V.A., et al., *Longitudinal muscle strength changes in older adults: influence of muscle mass, physical activity, and health.* J Gerontol A Biol Sci Med Sci, 2001. **56**(5): p. B209-17.

25. Dey, D.K., et al., *Changes in body composition and its relation to muscle strength in 75-year-old men and women: a 5-year prospective follow-up study of the NORA cohort in Goteborg, Sweden.* Nutrition, 2009. **25**(6): p. 613-9.

26. Koster, A., et al., *Does the amount of fat mass predict age-related loss of lean mass, muscle strength, and muscle quality in older adults?* J Gerontol A Biol Sci Med Sci, 2011. **66**(8): p. 888-95.

27. Shimokata, H., et al., *Age-related changes in skeletal muscle mass among community-dwelling Japanese: a 12-year longitudinal study.* Geriatr Gerontol Int, 2014. **14 Suppl 1**: p. 85-92.

28. Reid, K.F., et al., *Longitudinal decline of lower extremity muscle power in healthy and mobility-limited older adults: influence of muscle mass, strength, composition, neuromuscular activation and single fiber contractile properties.* Eur J Appl Physiol, 2014. **114**(1): p. 29-39.

29. Frontera, W.R., et al., *Aging of skeletal muscle: a 12-yr longitudinal study.* J Appl Physiol (1985), 2000. **88**(4): p. 1321-6.

30. Frontera, W.R., et al., *Muscle fiber size and function in elderly humans: a longitudinal study.* J Appl Physiol (1985), 2008. **105**(2): p. 637-42.

31. Clark, B.C. and T.M. Manini, *What is dynapenia?* Nutrition, 2012. **28**(5): p. 495-503.

32. Loenneke, J.P., et al., *Exercise-Induced Changes in Muscle Size do not Contribute to Exercise-Induced Changes in Muscle Strength.* Sports Med, 2019. **49**(7): p. 987-991.

33. Aas, S.N., et al., *Strength training and protein supplementation improve muscle mass, strength, and function in mobility-limited older adults: a randomized controlled trial.* Aging Clin Exp Res, 2020. **32**(4): p. 605-616.

34. Lee, H., et al., *Exercise training increases skeletal muscle strength independent of hypertrophy in older adults aged 75 years and older.* Geriatr Gerontol Int, 2019. **19**(3): p. 265-270.

35. Ogawa, M., T. Yasuda, and T. Abe, *Component characteristics of thigh muscle volume in young and older healthy men.* Clin Physiol Funct Imaging, 2012. **32**(2): p. 89-93.

36. Trombetti, A., et al., *Age-associated declines in muscle mass, strength, power, and physical performance: impact on fear of falling and quality of life.* Osteoporos Int, 2016. **27**(2): p. 463-71.

37. Aniansson, A., G. Grimby, and M. Hedberg, *Compensatory muscle fiber hypertrophy in elderly men.* J Appl Physiol (1985), 1992. **73**(3): p. 812-6.

38. Aniansson, A., et al., *Muscle morphology, enzymatic activity, and muscle strength in elderly men: a follow-up study.* Muscle Nerve, 1986. **9**(7): p. 585-91.

39. Grounds, M.D. and T. Shavlakadze, *Growing muscle has different sarcolemmal properties from adult muscle: a proposal with scientific and clinical implications: reasons to reassess skeletal muscle molecular dynamics, cellular responses and suitability of experimental models of muscle disorders.* Bioessays, 2011. **33**(6): p. 458-68.

40. Kuo, I.Y. and B.E. Ehrlich, *Signaling in muscle contraction.* Cold Spring Harb Perspect Biol, 2015. **7**(2): p. a006023.

41. Schiaffino, S., et al., *Developmental myosins: expression patterns and functional significance.* Skelet Muscle, 2015. **5**: p. 22.

42. Moreillon, M., et al., *Hybrid fiber alterations in exercising seniors suggest contribution to fast-to-slow muscle fiber shift.* J Cachexia Sarcopenia Muscle, 2019. **10**(3): p. 687-695.

43. Coggan, A.R., et al., *Skeletal muscle adaptations to endurance training in 60- to 70-yr-old men and women.* J Appl Physiol (1985), 1992. **72**(5): p. 1780-6.

44. Gavin, T.P., et al., *No difference in the skeletal muscle angiogenic response to aerobic exercise training between young and aged men.* J Physiol, 2007. **585**(Pt 1): p. 231-9.

45. Freyssenet, D., et al., *Effect of a 6-week endurance training programme and branched-chain amino acid supplementation on histomorphometric characteristics of aged human muscle.* Arch Physiol Biochem, 1996. **104**(2): p. 157-62.

46. Denis, C., et al., *Effects of endurance training on capillary supply of human skeletal muscle on two age groups (20 and 60 years).* J Physiol (Paris), 1986. **81**(5): p. 379-83.

47. Charifi, N., et al., *Enhancement of microvessel tortuosity in the vastus lateralis muscle of old men in response to endurance training.* J Physiol, 2004. **554**(Pt 2): p. 559-69.

48. Hakkinen, K., et al., *Neuromuscular adaptation during prolonged strength training, detraining and re-strength-training in middle-aged and elderly people.* Eur J Appl Physiol, 2000. **83**(1): p. 51-62.

49. Aagaard, P., et al., *Role of the nervous system in sarcopenia and muscle atrophy with aging: strength training as a countermeasure.* Scand J Med Sci Sports, 2010. **20**(1): p. 49-64.

50. Schiaffino, S., et al., *Mechanisms regulating skeletal muscle growth and atrophy.* FEBS J, 2013. **280**(17): p. 4294-314.

51. Baar, K., *Minimizing Injury and Maximizing Return to Play: Lessons from Engineered Ligaments.* Sports Med, 2017. **47**(Suppl 1): p. 5-11.

52. Hughes, D.C., M.A. Wallace, and K. Baar, *Effects of aging, exercise, and disease on force transfer in skeletal muscle.* Am J Physiol Endocrinol Metab, 2015. **309**(1): p. E1-E10.

53. Suetta, C., et al., *Effects of aging on human skeletal muscle after immobilization and retraining.* J Appl Physiol (1985), 2009. **107**(4): p. 1172-80.

54. Rosenberg, I.H., *Sarcopenia: origins and clinical relevance.* J Nutr, 1997. **127**(5 Suppl): p. 990S-991S.

55. Chen, L.K., et al., *Sarcopenia in Asia: consensus report of the Asian Working Group for Sarcopenia.* J Am Med Dir Assoc, 2014. **15**(2): p. 95-101.

56. Chen, L.K., et al., *Asian Working Group for Sarcopenia: 2019 Consensus Update on Sarcopenia Diagnosis and Treatment.* J Am Med Dir Assoc, 2020. **21**(3): p. 300-307 e2.

57. Batsis, J.A., et al., *Variation in the prevalence of sarcopenia and sarcopenic obesity in older adults associated with different research definitions: dual-energy X-ray absorptiometry data from the National Health and Nutrition Examination Survey 1999-2004.* J Am Geriatr Soc, 2013. **61**(6): p. 974-80.

58. Sobestiansky, S., K. Michaelsson, and T. Cederholm, *Sarcopenia prevalence and associations with mortality and hospitalisation by various sarcopenia definitions in 85-89 year old community-dwelling men: a report from the ULSAM study.* BMC Geriatr, 2019. **19**(1): p. 318.

59. Shafiee, G., et al., *Prevalence of sarcopenia in the world: a systematic review and meta- analysis of general population studies.* J Diabetes Metab Disord, 2017. **16**: p. 21.

60. Anker, S.D., J.E. Morley, and S. von Haehling, *Welcome to the ICD-10 code for sarcopenia.* J Cachexia Sarcopenia Muscle, 2016. **7**(5): p. 512-514.

61. WHO, *Decade of healthy ageing: baseline report.* 2020.

62. Buckinx, F., et al., *Pitfalls in the measurement of muscle mass: a need for a reference standard.* J Cachexia Sarcopenia Muscle, 2018. **9**(2): p. 269-278.

63. Aubrey, J., et al., *Measurement of skeletal muscle radiation attenuation and basis of its biological variation.* Acta Physiol (Oxf), 2014. **210**(3): p. 489-97.

64. Goodpaster, B.H., et al., *Attenuation of skeletal muscle and strength in the elderly: The Health ABC Study.* J Appl Physiol (1985), 2001. **90**(6): p. 2157-65.

65. Englund, D.A., et al., *Nutritional Supplementation With Physical Activity Improves Muscle Composition in Mobility-Limited Older Adults, The VIVE2 Study: A Randomized, Double-Blind, Placebo-Controlled Trial.* J Gerontol A Biol Sci Med Sci, 2017. **73**(1): p. 95-101.

66. Nijholt, W., et al., *The reliability and validity of ultrasound to quantify muscles in older adults: a systematic review.* J Cachexia Sarcopenia Muscle, 2017. **8**(5): p. 702-712.

67. Perkisas, S., et al., *Application of ultrasound for muscle assessment in sarcopenia: 2020 SARCUS update.* Eur Geriatr Med, 2021. **12**(1): p. 45-59.

68. Shankaran, M., et al., *Dilution of oral D3 -Creatine to measure creatine pool size and estimate skeletal muscle mass: development of a correction algorithm.* J Cachexia Sarcopenia Muscle, 2018. **9**(3): p. 540-546.

69. Duchowny, K.A., et al., *Association of change in muscle mass assessed by D3 -creatine dilution with changes in grip strength and walking speed.* J Cachexia Sarcopenia Muscle, 2020. **11**(1): p. 55-61.

70. Lexell, J., C.C. Taylor, and M. Sjostrom, *What is the cause of the ageing atrophy? Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men.* J Neurol Sci, 1988. **84**(2-3): p. 275-94.

71. Horwath, O., et al., *Variability in vastus lateralis fiber type distribution, fiber size, and myonuclear content along and between the legs.* J Appl Physiol (1985), 2021. **131**(1): p. 158-173.

72. Tarnopolsky, M.A., et al., *Suction-modified Bergstrom muscle biopsy technique: experience with 13,500 procedures.* Muscle Nerve, 2011. **43**(5): p. 717-25.

73. Wilk, K.E., et al., *A comparison of tibiofemoral joint forces and electromyographic activity during open and closed kinetic chain exercises.* Am J Sports Med, 1996. **24**(4): p. 518-27.

74. Alkner, B.A., P.A. Tesch, and H.E. Berg, *Quadriceps EMG/force relationship in knee extension and leg press.* Med Sci Sports Exerc, 2000. **32**(2): p. 459-63.

75. Stien, N., A.H. Saeterbakken, and V. Andersen, *Electromyographic Comparison of Five Lower-Limb Muscles between Single- and Multi-Joint Exercises among Trained Men.* J Sports Sci Med, 2021. **20**(1): p. 56-61.

76. Ema, R., et al., *Unique activation of the quadriceps femoris during single- and multi-joint exercises.* Eur J Appl Physiol, 2016. **116**(5): p. 1031-41.

77. Izquierdo, M., et al., *Maximal and explosive force production capacity and balance performance in men of different ages.* Eur J Appl Physiol Occup Physiol, 1999. **79**(3): p. 260-7.

78. Abellan van Kan, G., et al., *Gait speed at usual pace as a predictor of adverse outcomes in community-dwelling older people an International Academy on Nutrition and Aging (IANA) Task Force.* J Nutr Health Aging, 2009. **13**(10): p. 881-9.

79. Malhotra, R., et al., *Association of baseline hand grip strength and annual change in hand grip strength with mortality among older people.* Arch Gerontol Geriatr, 2020. **86**: p. 103961.

80. Podsiadlo, D. and S. Richardson, *The timed "Up & Go": a test of basic functional mobility for frail elderly persons.* J Am Geriatr Soc, 1991. **39**(2): p. 142-8.

81. Guralnik, J.M., et al., *A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission.* J Gerontol, 1994. **49**(2): p. M85-94.

82. Working Group on Functional Outcome Measures for Clinical, T., *Functional outcomes for clinical trials in frail older persons: time to be moving.* J Gerontol A Biol Sci Med Sci, 2008. **63**(2): p. 160-4.

83. Perera, S., et al., *Meaningful change and responsiveness in common physical performance measures in older adults.* J Am Geriatr Soc, 2006. **54**(5): p. 743-9.

84. Jennekens, F.G., B.E. Tomlinson, and J.N. Walton, *The extensor digitorum brevis: histological and histochemical aspects.* J Neurol Neurosurg Psychiatry, 1972. **35**(1): p. 124-32.

85. Jennekens, F.G., B.E. Tomlinson, and J.N. Walton, *Histochemical aspects of five limb muscles in old age. An autopsy study.* J Neurol Sci, 1971. **14**(3): p. 259-76.

86. Scelsi, R., C. Marchetti, and P. Poggi, *Histochemical and ultrastructural aspects of m. vastus lateralis in sedentary old people (age 65--89 years).* Acta Neuropathol, 1980. **51**(2): p. 99-105.

87. Mackey, A.L., et al., *Differential satellite cell density of type I and II fibres with lifelong endurance running in old men.* Acta Physiol (Oxf), 2014. **210**(3): p. 612-27.

88. Dreyer, H.C., et al., *Satellite cell numbers in young and older men 24 hours after eccentric exercise.* Muscle Nerve, 2006. **33**(2): p. 242-53.

89. Lexell, J., *Human aging, muscle mass, and fiber type composition.* J Gerontol A Biol Sci Med Sci, 1995. **50 Spec No**: p. 11-6.

90. Lexell, J., D. Downham, and M. Sjostrom, *Distribution of different fibre types in human skeletal muscles. Fibre type arrangement in m. vastus lateralis from three groups of healthy men between 15 and 83 years.* J Neurol Sci, 1986. **72**(2-3): p. 211-22.

91. Lexell, J. and D.Y. Downham, *The occurrence of fibre-type grouping in healthy human muscle: a quantitative study of cross-sections of whole vastus lateralis from men between 15 and 83 years.* Acta Neuropathol, 1991. **81**(4): p. 377-81.

92. Campbell, M.J., A.J. McComas, and F. Petito, *Physiological changes in ageing muscles.* J Neurol Neurosurg Psychiatry, 1973. **36**(2): p. 174-82.

93. Karlsen, A., et al., *Lack of muscle fibre hypertrophy, myonuclear addition, and satellite cell pool expansion with resistance training in 83-94-year-old men and women.* Acta Physiol (Oxf), 2019. **227**(1): p. e13271.

94. Verdijk, L.B., et al., *Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly.* Am J Physiol Endocrinol Metab, 2007. **292**(1): p. E151-7.

95. Verdijk, L.B., et al., *Satellite cells in human skeletal muscle; from birth to old age.* Age (Dordr), 2014. **36**(2): p. 545-7.

96. Grimby, G., et al., *Is there a change in relative muscle fibre composition with age?* Clin Physiol, 1984. **4**(2): p. 189-94.

97. Hakkinen, K., et al., *Changes in muscle morphology, electromyographic activity, and force production characteristics during progressive strength training in young and older men.* J Gerontol A Biol Sci Med Sci, 1998. **53**(6): p. B415-23.

98. Tomonaga, M., *Histochemical and ultrastructural changes in senile human skeletal muscle.* J Am Geriatr Soc, 1977. **25**(3): p. 125-31.

99. Lexell, J. and C.C. Taylor, *Variability in muscle fibre areas in whole human quadriceps muscle: effects of increasing age.* J Anat, 1991. **174**: p. 239-49.

100. Larsson, L., B. Sjodin, and J. Karlsson, *Histochemical and biochemical changes in human skeletal muscle with age in sedentary males, age 22--65 years.* Acta Physiol Scand, 1978. **103**(1): p. 31-9.

101. Zampieri, S., et al., *Lifelong physical exercise delays age-associated skeletal muscle decline.* J Gerontol A Biol Sci Med Sci, 2015. **70**(2): p. 163-73.

102. Korhonen, M.T., et al., *Aging, muscle fiber type, and contractile function in sprint-trained athletes.* J Appl Physiol (1985), 2006. **101**(3): p. 906-17.

103. Essen-Gustavsson, B. and O. Borges, *Histochemical and metabolic characteristics of human skeletal muscle in relation to age.* Acta Physiol Scand, 1986. **126**(1): p. 107-14.

104. Coggan, A.R., et al., *Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women.* J Gerontol, 1992. **47**(3): p. B71-6.

105. Houmard, J.A., et al., *Fiber type and citrate synthase activity in the human gastrocnemius and vastus lateralis with aging.* J Appl Physiol (1985), 1998. **85**(4): p. 1337-41.

106. Klitgaard, H., et al., *Function, morphology and protein expression of ageing skeletal muscle: a cross-sectional study of elderly men with different training backgrounds.* Acta Physiol Scand, 1990. **140**(1): p. 41-54.

107. Lexell, J., et al., *Distribution of different fiber types in human skeletal muscles: effects of aging studied in whole muscle cross sections.* Muscle Nerve, 1983. **6**(8): p. 588-95.

108. Rowan, S.L., et al., *Denervation causes fiber atrophy and myosin heavy chain co-expression in senescent skeletal muscle.* PLoS ONE, 2012. **7**(1): p. e29082.

109. D'Antona, G., et al., *The effect of ageing and immobilization on structure and function of human skeletal muscle fibres.* J Physiol, 2003. **552**(Pt 2): p. 499-511.

110. Andersen, J.L., G. Terzis, and A. Kryger, *Increase in the degree of coexpression of myosin heavy chain isoforms in skeletal muscle fibers of the very old.* Muscle Nerve, 1999. **22**(4): p. 449-54.

111. Sonjak, V., et al., *Fidelity of muscle fibre reinnervation modulates ageing muscle impact in elderly women.* J Physiol, 2019. **597**(19): p. 5009-5023.

112. Mosole, S., et al., *Long-term high-level exercise promotes muscle reinnervation with age.* J Neuropathol Exp Neurol, 2014. **73**(4): p. 284-94.

113. Mauro, A., *Satellite cell of skeletal muscle fibers.* J Biophys Biochem Cytol, 1961. **9**: p. 493-5.

114. Thornell, L.E., et al., *Satellite cells and training in the elderly.* Scand J Med Sci Sports, 2003. **13**(1): p. 48-55.

115. Bischoff, R. and C. Heintz, *Enhancement of skeletal muscle regeneration.* Dev Dyn, 1994. **201**(1): p. 41-54.

116. Franco, I., et al., *Healthy skeletal muscle aging: The role of satellite cells, somatic mutations and exercise.* Int Rev Cell Mol Biol, 2019. **346**: p. 157-200.

117. Lindström, M. and L.E. Thornell, *New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men.* Histochemistry and cell biology, 2009. **132**(2): p. 141-157.

118. Kadi, F., et al., *Satellite cells and myonuclei in young and elderly women and men.* Muscle Nerve, 2004. **29**(1): p. 120-7.

119. Renault, V., et al., *Regenerative potential of human skeletal muscle during aging.* Aging Cell, 2002. **1**(2): p. 132-9.

120. Roth, S.M., et al., *Skeletal muscle satellite cell populations in healthy young and older men and women.* Anat Rec, 2000. **260**(4): p. 351-8.

121. Sajko, S., et al., *Frequency of M-Cadherin-stained Satellite Cells Declines in Human Muscles During Aging.* Journal of Histochemistry & Cytochemistry, 2004. **52**(2): p. 179-185.

122. Petrella, J.K., et al., *Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women.* Am J Physiol Endocrinol Metab, 2006. **291**(5): p. E937-46.

123. Carlson, M.E., et al., *Molecular aging and rejuvenation of human muscle stem cells.* EMBO Mol Med, 2009. **1**(8-9): p. 381-91.

124. Thornell, L.E., *Sarcopenic obesity: satellite cells in the aging muscle.* Curr Opin Clin Nutr Metab Care, 2011. **14**(1): p. 22-7.

125. Fry, C.S., et al., *Inducible depletion of satellite cells in adult, sedentary mice impairs muscle regenerative capacity without affecting sarcopenia.* Nat Med, 2015. **21**(1): p. 76-80.

126. Franco, I., et al., *Somatic mutagenesis in satellite cells associates with human skeletal muscle aging.* Nat Commun, 2018. **9**(1): p. 800.

127. Snijders, T., et al., *Muscle fibre capillarization is a critical factor in muscle fibre hypertrophy during resistance exercise training in older men.* J Cachexia Sarcopenia Muscle, 2017. **8**(2): p. 267-276.

128. Nederveen, J.P., et al., *Skeletal muscle satellite cells are located at a closer proximity to capillaries in healthy young compared with older men.* J Cachexia Sarcopenia Muscle, 2016. **7**(5): p. 547-554.

129. Nicklas, B.J., et al., *Relationship of physical function to vastus lateralis capillary density and metabolic enzyme activity in elderly men and women.* Aging Clin Exp Res, 2008. **20**(4): p. 302-9.

130. Daussin, F.N., E. Boulanger, and S. Lancel, *From mitochondria to sarcopenia: Role of inflammaging and RAGE-ligand axis implication.* Exp Gerontol, 2021. **146**: p. 111247.

131. Bua, E., et al., *Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers.* Am J Hum Genet, 2006. **79**(3): p. 469-80.

132. Trounce, I., E. Byrne, and S. Marzuki, *Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing.* Lancet, 1989. **1**(8639): p. 637-9.

133. Migliavacca, E., et al., *Mitochondrial oxidative capacity and NAD(+) biosynthesis are reduced in human sarcopenia across ethnicities.* Nat Commun, 2019. **10**(1): p. 5808.

134. Muller-Hocker, J., *Cytochrome c oxidase deficient fibres in the limb muscle and diaphragm of man without muscular disease: an age-related alteration.* J Neurol Sci, 1990. **100**(1-2): p. 14-21.

135. Ross, J.M., *Visualization of mitochondrial respiratory function using cytochrome c oxidase/succinate dehydrogenase (COX/SDH) double-labeling histochemistry.* J Vis Exp, 2011(57): p. e3266.

136. Borkan, G.A., et al., *Age changes in body composition revealed by computed tomography.* J Gerontol, 1983. **38**(6): p. 673-7.

137. Biolo, G., T. Cederholm, and M. Muscaritoli, *Muscle contractile and metabolic dysfunction is a common feature of sarcopenia of aging and chronic diseases: from sarcopenic obesity to cachexia.* Clin Nutr, 2014. **33**(5): p. 737-48.

138. Goodpaster, B.H. and N.F. Brown, *Skeletal muscle lipid and its association with insulin resistance: what is the role for exercise?* Exerc Sport Sci Rev, 2005. **33**(3): p. 150-4.

139. Addison, O., et al., *Intermuscular fat: a review of the consequences and causes.* Int J Endocrinol, 2014. **2014**: p. 309570.

140. Marcus, R.L., et al., *Intramuscular adipose tissue, sarcopenia, and mobility function in older individuals.* J Aging Res, 2012. **2012**: p. 629637.

141. Dalle, S., L. Rossmeislova, and K. Koppo, *The Role of Inflammation in Age-Related Sarcopenia.* Front Physiol, 2017. **8**: p. 1045.

142. Addison, O., et al., *Intramuscular fat and inflammation differ in older adults: the impact of frailty and inactivity.* J Nutr Health Aging, 2014. **18**(5): p. 532-8.

143. Piasecki, M., et al., *Failure to expand the motor unit size to compensate for declining motor unit numbers distinguishes sarcopenic from non-sarcopenic older men.* J Physiol, 2018. **596**(9): p. 1627-1637.

144. Tomlinson, B.E. and D. Irving, *The numbers of limb motor neurons in the human lumbosacral cord throughout life.* J Neurol Sci, 1977. **34**(2): p. 213-9.

145. Lancha, A.H., Jr., et al., *Dietary protein supplementation in the elderly for limiting muscle mass loss.* Amino Acids, 2017. **49**(1): p. 33-47.

146. Wolfe, R.R., *The role of dietary protein in optimizing muscle mass, function and health outcomes in older individuals.* Br J Nutr, 2012. **108 Suppl 2**: p. S88-93.

147. Paddon-Jones, D. and B.B. Rasmussen, *Dietary protein recommendations and the prevention of sarcopenia.* Curr Opin Clin Nutr Metab Care, 2009. **12**(1): p. 86-90.

148. Deutz, N.E., et al., *Protein intake and exercise for optimal muscle function with aging: recommendations from the ESPEN Expert Group.* Clin Nutr, 2014. **33**(6): p. 929-36.

149. Cuthbertson, D., et al., *Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle.* FASEB J, 2005. **19**(3): p. 422-4.

150. Morton, R.W., et al., *A systematic review, meta-analysis and meta-regression of the effect of protein supplementation on resistance training-induced gains in muscle mass and strength in healthy adults.* Br J Sports Med, 2018. **52**(6): p. 376-384.

151. Moore, D.R., et al., *Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men.* J Gerontol A Biol Sci Med Sci, 2015. **70**(1): p. 57-62.

152. Kimball, S.R. and L.S. Jefferson, *Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis.* J Nutr, 2006. **136**(1 Suppl): p. 227S-31S.

153. Xu, Z.R., et al., *The effectiveness of leucine on muscle protein synthesis, lean body mass and leg lean mass accretion in older people: a systematic review and meta-analysis.* Br J Nutr, 2015. **113**(1): p. 25-34.

154. Esmarck, B., et al., *Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans.* J Physiol, 2001. **535**(Pt 1): p. 301-11.

155. Yang, Y., et al., *Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men.* Br J Nutr, 2012. **108**(10): p. 1780-8.

156. Churchward-Venne, T.A., et al., *What is the Optimal Amount of Protein to Support Post-Exercise Skeletal Muscle Reconditioning in the Older Adult?* Sports Med, 2016. **46**(9): p. 1205-12.

157. Candow, D.G. and P.D. Chilibeck, *Timing of creatine or protein supplementation and resistance training in the elderly.* Appl Physiol Nutr Metab, 2008. **33**(1): p. 184-90.

158. Schiaffino, S., et al., *Embryonic and neonatal myosin heavy chain in denervated and paralyzed rat skeletal muscle.* Dev Biol, 1988. **127**(1): p. 1-11.

159. Monemi, M., et al., *Fetal myosin heavy chain increases in human masseter muscle during aging.* FEBS Lett, 1996. **386**(1): p. 87-90.

160. Soendenbroe, C., et al., *Molecular indicators of denervation in aging human skeletal muscle.* Muscle Nerve, 2019. **60**(4): p. 453-463.

161. Illa, I., M. Leon-Monzon, and M.C. Dalakas, *Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells.* Ann Neurol, 1992. **31**(1): p. 46-52.

162. Soendenbroe, C., J.L. Andersen, and A.L. Mackey, *Muscle-nerve communication and the molecular assessment of human skeletal muscle denervation with aging.* Am J Physiol Cell Physiol, 2021. **321**(2): p. C317-C329.

163. Cashman, N.R., et al., *Neural cell adhesion molecule in normal, denervated, and myopathic human muscle.* Ann Neurol, 1987. **21**(5): p. 481-9.

164. Altun, M., et al., *Muscle wasting in aged, sarcopenic rats is associated with enhanced activity of the ubiquitin proteasome pathway.* J Biol Chem, 2010. **285**(51): p. 39597-608.

165. Gumucio, J.P. and C.L. Mendias, *Atrogin-1, MuRF-1, and sarcopenia.* Endocrine, 2013. **43**(1): p. 12-21.

166. Solomon, V. and A.L. Goldberg, *Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts.* J Biol Chem, 1996. **271**(43): p. 26690-7.

167. Bodine, S.C., et al., *Identification of ubiquitin ligases required for skeletal muscle atrophy.* Science, 2001. **294**(5547): p. 1704-8.

168. Gomes, M.D., et al., *Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy.* Proc Natl Acad Sci U S A, 2001. **98**(25): p. 14440-5.

169. Cohen, S., et al., *During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation.* J Cell Biol, 2009. **185**(6): p. 1083-95.

170. Cohen, S., et al., *Ubiquitylation by Trim32 causes coupled loss of desmin, Z-bands, and thin filaments in muscle atrophy.* J Cell Biol, 2012. **198**(4): p. 575-89.

171. Hepple, R.T. and C.L. Rice, *Innervation and neuromuscular control in ageing skeletal muscle.* J Physiol, 2016. **594**(8): p. 1965-78.

172. Tintignac, L.A., H.R. Brenner, and M.A. Ruegg, *Mechanisms Regulating Neuromuscular Junction Development and Function and Causes of Muscle Wasting.* Physiol Rev, 2015. **95**(3): p. 809-52.

173. Egerman, M.A. and D.J. Glass, *Signaling pathways controlling skeletal muscle mass.* Crit Rev Biochem Mol Biol, 2014. **49**(1): p. 59-68.

174. Aare, S., et al., *Failed reinnervation in aging skeletal muscle.* Skelet Muscle, 2016. **6**(1): p. 29.

175. Sandri, M., et al., *Signalling pathways regulating muscle mass in ageing skeletal muscle: the role of the IGF1-Akt-mTOR-FoxO pathway.* Biogerontology, 2013. **14**(3): p. 303-23.

176. Baehr, L.M., et al., *Age-related deficits in skeletal muscle recovery following disuse are associated with neuromuscular junction instability and ER stress, not impaired protein synthesis.* Aging (Albany NY), 2016. **8**(1): p. 127-46.

177. Schiaffino, S., K.A. Dyar, and E. Calabria, *Skeletal muscle mass is controlled by the MRF4-MEF2 axis.* Curr Opin Clin Nutr Metab Care, 2018. **21**(3): p. 164-167.

178. Moresi, V., et al., *Myogenin and class II HDACs control neurogenic muscle atrophy by inducing E3 ubiquitin ligases.* Cell, 2010. **143**(1): p. 35-45.

179. Tintignac, L.A., et al., *Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase.* J Biol Chem, 2005. **280**(4): p. 2847-56.

180. Moretti, I., et al., *MRF4 negatively regulates adult skeletal muscle growth by repressing MEF2 activity.* Nat Commun, 2016. **7**: p. 12397.

181. Raue, U., et al., *Myogenic gene expression at rest and after a bout of resistance exercise in young (18-30 yr) and old (80-89 yr) women.* J Appl Physiol (1985), 2006. **101**(1): p. 53-9.

182. Welle, S., et al., *Skeletal muscle gene expression profiles in 20-29 year old and 65-71 year old women.* Exp Gerontol, 2004. **39**(3): p. 369-77.

183. Bamman, M.M., et al., *Myogenic protein expression before and after resistance loading in 26- and 64-yr-old men and women.* J Appl Physiol (1985), 2004. **97**(4): p. 1329-37.

184. Hawley, J.A., et al., *Integrative biology of exercise.* Cell, 2014. **159**(4): p. 738-49.

185. Aagaard, P., et al., *Mechanical muscle function, morphology, and fiber type in lifelong trained elderly.* Med Sci Sports Exerc, 2007. **39**(11): p. 1989-96.

186. Hood, D.A., et al., *Maintenance of Skeletal Muscle Mitochondria in Health, Exercise, and Aging.* Annu Rev Physiol, 2019. **81**: p. 19-41.

187. Phillips, B.E., et al., *Molecular networks of human muscle adaptation to exercise and age.* PLoS Genet, 2013. **9**(3): p. e1003389.

188. Slivka, D., et al., *Single muscle fiber adaptations to resistance training in old (>80 yr) men: evidence for limited skeletal muscle plasticity.* Am J Physiol Regul Integr Comp Physiol, 2008. **295**(1): p. R273-80.

189. Abe, T., et al., *Time course for strength and muscle thickness changes following upper and lower body resistance training in men and women.* Eur J Appl Physiol, 2000. **81**(3): p. 174-80.

190. Frontera, W.R., et al., *Skeletal muscle fiber quality in older men and women.* Am J Physiol Cell Physiol, 2000. **279**(3): p. C611-8.

191. Larsson, L., X. Li, and W.R. Frontera, *Effects of aging on shortening velocity and myosin isoform composition in single human skeletal muscle cells.* Am J Physiol, 1997. **272**(2 Pt 1): p. C638-49.

192. Kosek, D.J., et al., *Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults.* J Appl Physiol (1985), 2006. **101**(2): p. 531-44.

193. Hunter, G.R., J.P. McCarthy, and M.M. Bamman, *Effects of resistance training on older adults.* Sports Med, 2004. **34**(5): p. 329-48.

194. Hepple, R.T., et al., *Resistance and aerobic training in older men: effects on VO2peak and the capillary supply to skeletal muscle.* J Appl Physiol (1985), 1997. **82**(4): p. 1305-10.

195. Hepple, R.T., et al., *Quantitating the capillary supply and the response to resistance training in older men.* Pflugers Arch, 1997. **433**(3): p. 238-44.

196. Harris, B.A., *The influence of endurance and resistance exercise on muscle capillarization in the elderly: a review.* Acta Physiol Scand, 2005. **185**(2): p. 89-97.

197. Charifi, N., et al., *Effects of endurance training on satellite cell frequency in skeletal muscle of old men.* Muscle Nerve, 2003. **28**(1): p. 87-92.

198. Kadi, F. and L.E. Thornell, *Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training.* Histochem Cell Biol, 2000. **113**(2): p. 99-103.

199. Whitman, S.A., et al., *Contributions of the ubiquitin-proteasome pathway and apoptosis to human skeletal muscle wasting with age.* Pflugers Arch, 2005. **450**(6): p. 437-46.

200. Raue, U., et al., *Proteolytic gene expression differs at rest and after resistance exercise between young and old women.* J Gerontol A Biol Sci Med Sci, 2007. **62**(12): p. 1407-12.

201. Drummond, M.J., et al., *Downregulation of E3 ubiquitin ligases and mitophagy-related genes in skeletal muscle of physically inactive, frail older women: a cross-sectional comparison.* J Gerontol A Biol Sci Med Sci, 2014. **69**(8): p. 1040-8.

202. Fry, C.S., et al., *Skeletal muscle autophagy and protein breakdown following resistance exercise are similar in younger and older adults.* J Gerontol A Biol Sci Med Sci, 2013. **68**(5): p. 599-607.

203. Stefanetti, R.J., et al., *Ageing has no effect on the regulation of the ubiquitin proteasome-related genes and proteins following resistance exercise.* Front Physiol, 2014. **5**: p. 30.

204. Tanner, R.E., et al., *Age-related differences in lean mass, protein synthesis and skeletal muscle markers of proteolysis after bed rest and exercise rehabilitation.* J Physiol, 2015. **593**(18): p. 4259-73.

205. Dickinson, J.M., et al., *The impact of postexercise essential amino acid ingestion on the ubiquitin proteasome and autophagosomal-lysosomal systems in skeletal muscle of older men.* J Appl Physiol (1985), 2017. **122**(3): p. 620-630.

206. Aas, S.N., et al., *Effects of acute and chronic strength training on skeletal muscle autophagy in frail elderly men and women.* Exp Gerontol, 2020. **142**: p. 111122.

207. Lazarus, N.R. and S.D. Harridge, *Inherent ageing in humans: the case for studying master athletes.* Scand J Med Sci Sports, 2007. **17**(5): p. 461-3.

208. Trappe, S., et al., *New records in aerobic power among octogenarian lifelong endurance athletes.* J Appl Physiol (1985), 2013. **114**(1): p. 3-10.

209. McKendry, J., et al., *Muscle morphology and performance in master athletes: A systematic review and meta-analyses.* Ageing Res Rev, 2018. **45**: p. 62-82.

210. Willy, R.W. and M.R. Paquette, *The Physiology and Biomechanics of the Master Runner.* Sports Med Arthrosc Rev, 2019. **27**(1): p. 15-21.

211. Drey, M., et al., *Relation between muscle mass, motor units and type of training in master athletes.* Clin Physiol Funct Imaging, 2016. **36**(1): p. 70-6.

212. Gries, K.J., et al., *Cardiovascular and skeletal muscle health with lifelong exercise.* J Appl Physiol (1985), 2018. **125**(5): p. 1636-1645.

213. Coggan, A.R., et al., *Histochemical and enzymatic characteristics of skeletal muscle in master athletes.* J Appl Physiol (1985), 1990. **68**(5): p. 1896-901.

214. Malafarina, V., et al., *The anorexia of ageing: physiopathology, prevalence, associated comorbidity and mortality. A systematic review.* Maturitas, 2013. **74**(4): p. 293-302.

215. Tagliafico, A.S., et al., *Relationship between fatty degeneration of thigh muscles and vitamin D status in the elderly: a preliminary MRI study.* AJR Am J Roentgenol, 2010. **194**(3): p. 728-34.

216. Wang, Y. and H.F. DeLuca, *Is the vitamin d receptor found in muscle?* Endocrinology, 2011. **152**(2): p. 354-63.

217. Girgis, C.M., *Vitamin D and Skeletal Muscle: Emerging Roles in Development, Anabolism and Repair.* Calcif Tissue Int, 2019. **106**(1): p. 47-57.

218. Olsson, K., et al., *Evidence for Vitamin D Receptor Expression and Direct Effects of 1alpha,25(OH)2D3 in Human Skeletal Muscle Precursor Cells.* Endocrinology, 2016. **157**(1): p. 98-111.

219. Bauer, J.M., et al., *Effects of a vitamin D and leucine-enriched whey protein nutritional supplement on measures of sarcopenia in older adults, the PROVIDE study: a randomized, double-blind, placebo-controlled trial.* J Am Med Dir Assoc, 2015. **16**(9): p. 740-7.

220. Beaudart, C., et al., *The effects of vitamin D on skeletal muscle strength, muscle mass, and muscle power: a systematic review and meta-analysis of randomized controlled trials.* J Clin Endocrinol Metab, 2014. **99**(11): p. 4336-45.

221. Gilsanz, V., et al., *Vitamin D status and its relation to muscle mass and muscle fat in young women.* J Clin Endocrinol Metab, 2010. **95**(4): p. 1595-601.

222. Salles, J., et al., *1,25(OH)2-vitamin D3 enhances the stimulating effect of leucine and insulin on protein synthesis rate through Akt/PKB and mTOR mediated pathways in murine C2C12 skeletal myotubes.* Mol Nutr Food Res, 2013. **57**(12): p. 2137-46.

223. Kim, D.S., M.T. Wheeler, and E.A. Ashley, *The genetics of human performance.* Nat Rev Genet, 2021.

224. Zempo, H., et al., *Heritability estimates of muscle strength-related phenotypes: A systematic review and meta-analysis.* Scand J Med Sci Sports, 2017. **27**(12): p. 1537-1546.

225. den Hoed, M., et al., *Heritability of objectively assessed daily physical activity and sedentary behavior.* Am J Clin Nutr, 2013. **98**(5): p. 1317-25.

226. Schutte, N.M., et al., *Heritability of objectively assessed and self-reported sedentary behavior.* Scand J Med Sci Sports, 2020. **30**(7): p. 1237-1247.

227. Hedstrand, H., *A study of middle-aged men with particular reference to risk factors for cardiovascular disease.* Ups J Med Sci Suppl, 1975. **19**: p. 1-61.

228. Kirn, D.R., et al., *The Vitality, Independence, and Vigor in the Elderly 2 Study (VIVE2): Design and methods.* Contemp Clin Trials, 2015. **43**: p. 164-71.

229. Hedman, A., et al., *Muscle morphology, self-reported physical activity and insulin resistance syndrome.* Acta Physiol Scand, 2002. **175**(4): p. 325-32.

230. Berg, H.E., B. Tedner, and P.A. Tesch, *Changes in lower limb muscle cross-sectional area and tissue fluid volume after transition from standing to supine.* Acta Physiol Scand, 1993. **148**(4): p. 379-85.

231. Rasch, A., et al., *Reduced muscle radiological density, cross-sectional area, and strength of major hip and knee muscles in 22 patients with hip osteoarthritis.* Acta Orthop, 2007. **78**(4): p. 505-10.

232. Borg, G., *Perceived exertion as an indicator of somatic stress.* Scand J Rehabil Med, 1970. **2**(2): p. 92-8.

233. Piercy, K.L., et al., *The Physical Activity Guidelines for Americans.* JAMA, 2018. **320**(19): p. 2020-2028.

234. Bull, F.C., et al., *World Health Organization 2020 guidelines on physical activity and sedentary behaviour.* Br J Sports Med, 2020. **54**(24): p. 1451-1462.

235. Hedman, A., et al., *Relationships between muscle morphology and insulin sensitivity are improved after adjustment for intra-individual variability in 70-year-old men.* Acta Physiol Scand, 2000. **169**(2): p. 125-32.

236. Hedman, A., R. Reneland, and H.O. Lithell, *Alterations in skeletal muscle morphology in glucose-tolerant elderly hypertensive men: relationship to development of hypertension and heart rate.* J Hypertens, 2000. **18**(5): p. 559-65.

237. Bergstrom, J., *Percutaneous needle biopsy of skeletal muscle in physiological and clinical research.* Scand J Clin Lab Invest, 1975. **35**(7): p. 609-16.

238. Sternberger, L.A., *The unlabeled antibody (PAP) method, introduction.* Journal of Histochemistry & Cytochemistry, 1979. **27**(12): p. 1657-1657.

239. Seligman, A.M., et al., *Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB).* J Cell Biol, 1968. **38**(1): p. 1-14.

240. Trygg, J.W., S., *Orthogonal projections to latent structures (O-PLS).* Journal of Chemometrics 2002. **16**(3): p. 119-128.

241. Konopka, A.R. and M.P. Harber, *Skeletal muscle hypertrophy after aerobic exercise training.* Exerc Sport Sci Rev, 2014. **42**(2): p. 53-61.

242. Scott, W., J. Stevens, and S.A. Binder-Macleod, *Human skeletal muscle fiber type classifications.* Phys Ther, 2001. **81**(11): p. 1810-6.

243. Vikne, H., et al., *Human skeletal muscle fiber type percentage and area after reduced muscle use: A systematic review and meta-analysis.* Scand J Med Sci Sports, 2020. **30**(8): p. 1298-1317.

244. Monemi, M., et al., *Opposite changes in myosin heavy chain composition of human masseter and biceps brachii muscles during aging.* J Muscle Res Cell Motil, 1999. **20**(4): p. 351-61.

245. Murach, K.A., et al., *Fiber typing human skeletal muscle with fluorescent immunohistochemistry.* J Appl Physiol (1985), 2019. **127**(6): p. 1632-1639.

246. Simoneau, J.A. and C. Bouchard, *Genetic determinism of fiber type proportion in human skeletal muscle.* FASEB J, 1995. **9**(11): p. 1091-5.

247. Proctor, D.N., et al., *Oxidative capacity of human muscle fiber types: effects of age and training status.* J Appl Physiol (1985), 1995. **78**(6): p. 2033-8.

248. Trappe, S.W., et al., *Skeletal muscle characteristics among distance runners: a 20-yr follow-up study.* J Appl Physiol (1985), 1995. **78**(3): p. 823-9.

249. Purves-Smith, F.M., N. Sgarioto, and R.T. Hepple, *Fiber typing in aging muscle.* Exerc Sport Sci Rev, 2014. **42**(2): p. 45-52.

250. Klitgaard, H., et al., *Ageing alters the myosin heavy chain composition of single fibres from human skeletal muscle.* Acta Physiol Scand, 1990. **140**(1): p. 55-62.

251. Staron, R.S., et al., *Fiber type composition of the vastus lateralis muscle of young men and women.* J Histochem Cytochem, 2000. **48**(5): p. 623-9.

252. Hendrickse, P., et al., *An evaluation of common markers of muscle denervation in denervated young-adult and old rat gastrocnemius muscle.* Exp Gerontol, 2018. **106**: p. 159-164.

253. Karlsen, A., et al., *Preserved capacity for satellite cell proliferation, regeneration, and hypertrophy in the skeletal muscle of healthy elderly men.* FASEB J, 2020. **34**(5): p. 6418-6436.

254. Lundberg, T.R. and T. Gustafsson, *Fibre hypertrophy, satellite cell and myonuclear adaptations to resistance training: Have very old individuals reached the ceiling for muscle fibre plasticity?* Acta Physiol (Oxf), 2019. **227**(1): p. e13287.

255. Hedman, A., et al., *Insulin-mediated changes in leg blood flow are coupled to capillary density in skeletal muscle in healthy 70-year-old men.* Metabolism, 2001. **50**(9): p. 1078-82.

256. Brzeszczynska, J., et al., *Alterations in the in vitro and in vivo regulation of muscle regeneration in healthy ageing and the influence of sarcopenia.* J Cachexia Sarcopenia Muscle, 2018. **9**(1): p. 93-105.

257. Gillon, A. and P. Sheard, *Elderly mouse skeletal muscle fibres have a diminished capacity to upregulate NCAM production in response to denervation.* Biogerontology, 2015. **16**(6): p. 811-23.

258. Baker, M.E., et al., *Mural attenuation in normal small bowel and active inflammatory Crohn's disease on CT enterography: location, absolute attenuation, relative attenuation, and the effect of wall thickness.* AJR Am J Roentgenol, 2009. **192**(2): p. 417-23.

259. WHO, in *WHO Guidelines on Physical Activity and Sedentary Behaviour*. 2020: Geneva.

260. Guralnik, J.M., et al., *Lower-extremity function in persons over the age of 70 years as a predictor of subsequent disability.* N Engl J Med, 1995. **332**(9): p. 556-61.

261. Tieland, M., I. Trouwborst, and B.C. Clark, *Skeletal muscle performance and ageing.* J Cachexia Sarcopenia Muscle, 2018. **9**(1): p. 3-19.

262. von Berens, A., et al., *"Feeling More Self-Confident, Cheerful and Safe". Experiences from a Health-Promoting Intervention in Community Dwelling Older Adults - A Qualitative Study.* J Nutr Health Aging, 2018. **22**(4): p. 541-548.

263. Woods, S.C., et al., *Food intake and the regulation of body weight.* Annu Rev Psychol, 2000. **51**: p. 255-77.

264. Rosendahl-Riise, H., et al., *Vitamin D supplementation and its influence on muscle strength and mobility in community-dwelling older persons: a systematic review and meta-analysis.* J Hum Nutr Diet, 2017. **30**(1): p. 3-15.

265. Stockton, K.A., et al., *Effect of vitamin D supplementation on muscle strength: a systematic review and meta-analysis.* Osteoporos Int, 2011. **22**(3): p. 859-71.