

DISSERTATION FROM THE
NORWEGIAN SCHOOL OF
SPORT SCIENCES
2019

Sigve Nyvik Aas

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Table of contents

Summary	III
Sammendrag (summary in Norwegian).....	V
Preface and acknowledgements.....	VII
List of papers	IX
Abbreviations	X
1. Introduction	1
1.1. Frailty	2
1.2. Impact of aging	2
1.3.1. <i>Atrophy</i>	2
1.3.2. <i>Muscle activation</i>	3
1.3.3. <i>Intramuscular fat</i>	4
1.3.4. <i>Single fiber specific tension</i>	6
1.3.5. <i>Muscle protein turnover</i>	8
1.4. Impact of strength training	12
1.4.1. <i>Hypertrophy</i>	12
1.4.2. <i>Muscle activation</i>	12
1.4.3. <i>Intramuscular fat</i>	13
1.4.4. <i>Single fiber specific tension</i>	15
1.4.5. <i>Muscle protein turnover</i>	17
2. Research aims and hypotheses.....	20
3. Methods.....	22
3.1. Subjects	22
3.2. Study design and intervention adherence.....	23
3.3. Training program and exercise protocol	25
3.4. Supplements and diet	25
3.5. Measures of muscle mass, size and density	26
3.6. Muscle strength, activation level and functional tests.....	27
3.7. Specific strength.....	29
3.8. Biopsy analyses.....	30
3.8.1. <i>Western Blot</i>	30
3.8.2. <i>Immunohistochemistry</i>	32

3.8.3.	<i>Single fiber measurements</i>	33
3.8.4.	<i>Reverse transcription polymerase chain reaction (RT-PCR)</i>	34
3.9.	Statistical analyses	34
3.9.1.	<i>Article I</i>	35
3.9.2.	<i>Article II</i>	35
3.9.3.	<i>Article III</i>	35
3.9.4.	<i>Article IV</i>	35
4.	Results and discussion	37
4.3.	Relationships between muscle mass, strength and function	37
4.4.	Cross-sectional comparison	39
4.4.1.	<i>Impact of age and frailty on autophagy markers</i>	40
4.4.2.	<i>Impact of age and frailty on heat shock proteins</i>	42
4.4.3.	<i>Autophagy and specific strength</i>	45
4.4.4.	<i>Differences between men and women</i>	45
4.5.	Effects of strength training in pre-frail and frail elderly	46
4.5.1.	<i>Muscle mass, muscle strength and functional capacity</i>	46
4.5.2.	<i>Muscle activation</i>	49
4.5.3.	<i>Muscle cross-sectional area, density and intermuscular adipose tissue</i>	51
4.5.4.	<i>Intramyocellular and extramyocellular lipids</i>	53
4.5.5.	<i>Single fiber properties</i>	54
4.6.	Autophagy - acute and long-term effects of strength training	57
4.6.1.	<i>Anabolic signaling</i>	58
4.6.2.	<i>Ubiquitin-proteasome pathway</i>	58
4.6.3.	<i>Autophagy and mitophagy</i>	59
4.6.4.	<i>Heat shock proteins</i>	61
4.7.	What determines the effect of training in pre-frail and frail elderly?	61
4.8.	Methodological considerations	63
4.9.	Ethical considerations	64
5.	Conclusion	66
6.	Perspectives	67
7.	References	68
	Article I, II, III, IV and Appendix I, II	80

Summary

With aging, the loss of muscle strength exceeds the loss of muscle mass. Specific strength, defined as strength normalized to the cross-sectional area of a muscle or muscle group, declines accordingly. A reduced voluntary activation level may explain some of the decline in specific strength, but also decreased muscle quality appears to be involved. Autophagy is responsible for the degradation of dysfunctional proteins and organelles in muscle cells, and studies in rodents suggest that autophagy is impaired with aging. Whether or not autophagy is impaired with aging also in humans, and whether a possible decline in autophagic activity contributes to the reduction in muscle quality, remains to be investigated. Contrary to aging, specific strength increases following a period of strength training, and this is particularly clear in frail elderly. However, the relative contribution from changes in activation level and muscle quality is not known. Moreover, whether or not changes in protein quality control systems contribute to improved muscle quality has not been investigated. The main goal of this thesis was to investigate the effect of heavy load strength training on a broad number of factors related to specific strength in pre-frail and frail elderly. In addition, a cross-sectional study was conducted to assess if autophagy is different in elderly and frail compared to healthy and young humans.

In total, 93 men and women (17 young, 76 old) were included in the studies. In the cross-sectional study, the following four groups were compared: Young, old non-frail, old pre-frail and old frail. Specific strength was measured as maximal isometric knee extension torque (MVC) normalized to leg lean mass assessed by DXA. Muscle biopsies were obtained from the vastus lateralis muscle, and analyzed for proteins related to muscle protein synthesis (p70S6K, 4E-BP1, eEF2), autophagy (p62, LC3-I, LC3-II) and cellular stress (heat shock proteins; Hsp70, α B-crystallin) by western blot. In the main intervention study, pre-frail and frail elderly underwent heavy load strength training twice weekly for ten weeks. Specific strength was measured as knee extension MVC normalized to cross-sectional area (CSA) of the quadriceps femoris muscles, assessed by computed tomography (CT). Voluntary activation level was assessed by the twitch interpolation technique, intermuscular adipose tissue (IMAT) and muscle density by CT, and intramyocellular lipids (IMCL) by Oil Red O staining on muscle biopsy sections. In addition, single fiber specific tension was investigated, and the biopsies were also analyzed for proteins related to muscle protein synthesis, autophagy and cellular stress. Functional capacity was assessed by five times chair rise time and gait velocity.

In the cross-sectional study, specific strength was lowest in old frail subjects. No between-group differences were observed for p70S6K, 4E-BP1, eEF2 or p62, whereas the levels of LC3-I, LC3-II and certain heat shock proteins (HSPs) were higher in the elderly subjects. Higher levels of LC3-I and LC3-II might reflect attenuated fusion of autophagosomes and lysosomes, and higher levels of HSPs might indicate increased levels of denatured and dysfunctional proteins. In the intervention study, a

strong correlation was observed between quadriceps femoris CSA and knee extension MVC at baseline ($r = 0.89$, $P < 0.01$). The training intervention did not elicit changes in activation level, IMAT, IMCL or single fiber specific tension. An increase in muscle density was observed, but increased density was not correlated with changes in IMCL content or single fiber specific tension. The intervention had limited effects on autophagy proteins and HSPs. In contrast to our hypothesis, the increase in quadriceps femoris CSA (7%) corresponded well with the improvements in knee extension MVC (7%). The training intervention improved habitual and maximal gait velocity, but not five times chair rise time.

In contrast to several previous studies, our findings suggest that even in pre-frail and frail elderly, most of the variation in muscle strength is explained by differences in muscle cross-sectional area. Moreover, the training-induced increase in muscle strength was primarily due to increased muscle quantity, not quality in our study. Nevertheless, we found indications of impaired autophagy and higher cellular stress in elderly and frail individuals compared to young and healthy individuals. Furthermore, specific strength was lowest in the elderly and frail, and within the elderly there was a correlation between markers of autophagy and specific strength, supporting a possible relation between impaired protein quality control and reduced muscle quality. Altogether, impaired autophagy may play a role in the age-associated decline in specific strength, but muscle mass seems to be the most important determinant of muscle strength and function even in old and frail individuals. It is too early to conclude that strength training does not affect protein quality control systems in this population, and it is possible that a higher frequency or longer duration of training is required to elicit greater changes.

Sammendrag (summary in Norwegian)

Med aldring er tapet av muskelstyrke større enn reduksjonen i muskeltverrsnitt skulle tilsi, og spesifikk styrke (styrke/muskeltverrsnitt) er derfor redusert hos eldre. Redusert aktiveringsgrad ser ut til å forklare noe av reduksjonen, men det ser også ut til at muskelkvaliteten er redusert, spesielt hos eldre med veldig lavt funksjonsnivå. Autofagi er et av systemene som bryter ned dysfunksjonelle proteiner og organeller i muskelcellen. I dyrestudier har man sett at autofagi reduseres med økende alder, men hvorvidt dette er tilfellet hos mennesker er uvisst. Det er også usikkert om en eventuell reduksjon i autofagi bidrar til den aldersrelaterte reduksjonen i spesifikk styrke. Styrketrening fører til økt spesifikk styrke, og dette er spesielt tydelig hos eldre personer med lavt funksjonsnivå. Man vet likevel lite om det relative bidraget fra eventuelle endringer i aktiveringsgrad og muskelkvalitet, og hvorvidt endringer i muskelcellenes nedbrytningssystemer er involvert. Målet med denne doktorgraden var derfor å undersøke effekten av tung styrketrening på ulike faktorer relatert til spesifikk styrke hos eldre med lavt funksjonsnivå. I tillegg ble det gjennomført en tverrsnittstudie for å undersøke om autofagi er redusert hos eldre, og om en eventuell reduksjon er mer fremtredende hos eldre med lavt funksjonsnivå.

Totalt deltok 93 menn og kvinner (17 yngre, 76 eldre) i studiene. I tverrsnittstudien ble forsøkspersonene delt i fire grupper basert på alder og funksjonsnivå. Spesifikk styrke ble målt som maksimal isometrisk kneekstensjon normalisert til fett- og beinfri masse i beina målt med DXA. Muskelbiopsi ble tatt fra vastus lateralis, og analysert for proteiner relatert til muskelproteinsyntese (p70S6K, 4E-BP1, eEF2), autofagi (p62, LC3-I, LC3-II), og cellulært stress (Hsp70, α B-crystallin) med western blot. I intervensjonsstudien gjennomførte eldre med lavt funksjonsnivå styrketrening to ganger per uke i ti uker. Spesifikk styrke ble målt som maksimal isometrisk kneekstensjonsstyrke normalisert til tverrsnittsareal av quadriceps femoris målt med CT. Aktiveringsgrad ble undersøkt med interpolert twitch-teknikk (ITT), intermuskulært fettinnhold og muskeltetthet med CT, og intramyocellulært fett med Oil Red O-merking på biopsisnitt. I tillegg ble spesifikk kraft for enkeltfibre undersøkt, og biopsiene ble analysert for proteiner relatert til muskelproteinsyntese, autofagi og cellulært stress. Funksjonsnivå ble testet ved å måle normal og maksimal ganghastighet, samt ved gjennomføring av en stoltest.

I tverrsnittstudien var spesifikk styrke lavest i gruppen med høyest alder og lavest funksjonsnivå. Vi fant ingen forskjeller mellom gruppene for proteiner relatert til muskelproteinsyntese, mens proteinnivå av LC3-I, LC3-II og enkelte heat shock proteiner var høyere hos de eldre. Høyere nivå av LC3-I og LC3-II kan bety redusert fusjon av autofagosomer og lysosomer, mens høyere nivå av heat shock proteiner kan indikere økt mengde denaturerte og dysfunksjonelle proteiner. I intervensjonsstudien fant vi en sterk korrelasjon mellom tverrsnittsareal av quadriceps femoris og isometrisk kneekstensjon ved baseline ($r = 0.89$, $P < 0.01$). Treningsintervensjonen førte ikke til

endringer i aktiveringsgrad, fettinnhold eller spesifikk styrke på fibernivå. En økning i muskeltetthet ble observert, men økningen muskeltetthet korrelerte ikke med endringer i intramyocellulære lipider eller spesifikk styrke på fibernivå. Intervensjonen hadde minimal effekt på autofagimarkører og heat shock proteiner, og i motsetning til vår hypotese, stemte økningen i muskeltverrsnitt (7%) godt overens med økningen i isometrisk muskelstyrke (7%). Intervensjonen førte til en økning i normal og maksimal ganghastighet, men ikke signifikant forbedring på stoltesten.

I tverrsnittstudien fant vi forskjeller i autofagi, samt indikasjoner på høyere cellulært stress hos eldre med lavt funksjonsnivå sammenlignet med yngre. Spesifikk styrke var også lavest hos gruppen med lavest funksjonsnivå, og blant de eldre deltakerne fant vi korrelasjoner mellom autofagimarkører og spesifikk styrke. Disse funnene støtter en potensiell sammenheng mellom forstyrrelser i nedbrytningsystemer og redusert muskelkvalitet. Det ser likevel ut til at muskelmassen er den viktigste faktoren for muskelstyrke og funksjon, selv hos denne gruppen individer. På nåværende tidspunkt er det for tidlig å konkludere med at styrketrening ikke påvirker autofagi og cellulært stress hos eldre, og det er mulig at en høyere treningsfrekvens, eventuelt en lengre treningsperiode er nødvendig for å påvirke disse systemene i større grad.

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Trude, my wife. The last four years would have been much more challenging without your support, understanding and love. You are amazing!

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I. **Aas SN**, Seynnes O, Benestad HB, Raastad T. Strength training and protein supplementation improve muscle mass, strength, and function in old pre-frail individuals. Submitted
- II. **Aas SN**, Hamarsland H, Cumming TC, Rognlien SH, Aase OJ, Nordseth M, Karsrud S, Godager S, Tømmerbakke D, Handegard V, Raastad T. The impact of age and frailty on skeletal muscle autophagy markers and specific strength: A cross-sectional comparison. Manuscript
- III. **Aas SN**, Breit M, Karsrud S, Aase OJ, Rognlien SH, Cumming KT, Reggiani C, Seynnes O, Rossi AP, Toniolo L, Raastad T. Effects of 10 weeks of heavy load strength training on muscle activation level, muscle quality and single fiber function in frail elderly men and women. Manuscript
- IV. **Aas SN**, Tømmerbakke D, Godager S, Nordseth M, Armani A, Sandri M, Benestad HB, Raastad T. Effects of acute and long-term strength training on skeletal muscle autophagy in frail elderly men and women. Manuscript

Abbreviations

ANOVA	analysis of variance
AMPK	AMP-activated protein kinase
CSA	cross-sectional area
CT	computed tomography
4E-BP1	4E binding protein 1
DXA	dual-energy X-ray absorptiometry
eEF2	eukaryotic elongation factor 2
EMCL	extramyocellular lipids
HSP	heat shock protein
HU	Hounsfield units
IMAT	intermuscular adipose tissue
IMCL	intramyocellular lipids
KE	knee extension
LC3	microtubule-associated protein 1A/1B-light chain 3
MPB	muscle protein breakdown
MPS	muscle protein synthesis
MVC	maximal voluntary contraction
mTORC1	mechanistic target of rapamycin complex 1
p62	sequestosome 1
p70S6K	ribosomal protein S6 kinase beta-1
PA	phosphatic acid
UPP	ubiquitin proteasome pathway
1RM	one repetition maximum

1. Introduction

Over the next decades, projections foresee a growing number and proportion of elderly in the world's population, with a particularly rapid increase in the number of very old individuals. In Norway, predictions propose that the number of persons aged 70 years or more will double within three decades – from 600 000 today to nearly 1.2 million (Tønnessen, Leknes, & Syse, 2016). For those aged 80 or more, the numbers will double in just two decades – from 220 000 today to 440 000. The increased number and proportion of elderly individuals are primarily due to the assumption of a steadily increasing life expectancy. In addition, the large birth cohorts from the post-war era will gradually become the eldest in society (Tønnessen et al., 2016). While it is positive that life expectancy continues to rise, it is not so clear that additional years of life are welcome if characterized by a range of medical problems and disabilities. The number of *healthy life years* combine information on mortality with data on health status, and provide an indication of the number of years that a person can expect to live free from any form of disability (Beltran-Sanchez, Soneji, & Crimmins, 2015). Although the two measures are associated with one another, they do not always go hand in hand. For example, although the life expectancy of women exceed that of men by 4-5 years in European countries, the number of healthy life years is not different between genders (European Commission, 2015). Hence, although we can expect that future increases in life expectancy will be accompanied by increases also in the number of healthy life years, it seems reasonable that the focus should be to increase the latter rather than the former. Indeed, increasing the proportion of healthy life years will likely reduce the impact on our health care system in the coming years, and increase quality of life at the individual level.

Functional capacity predict future loss of mobility (Vasunilashorn et al., 2009), whereas low gait velocity increases the risk of both institutionalization and death (Jung et al., 2018; Studenski et al., 2011). Consequently, it seems reasonable to conclude that the number of healthy life years depend on the extent to which functional performance is maintained during aging. A better understanding of the mechanisms causing the functional decline will therefore improve the basis for developing effective countermeasures. The purpose of this introduction is to give a brief overview of how aging affects different aspects of muscle function, and how strength training might counteract some of these changes. Particular emphasis will be given to the notion that the strength of a muscle does not always correlate with its size.

1.1. Frailty

A hallmark characteristic of aging is the gradual loss of skeletal muscle mass and strength, which begins as early as the fifth decade of life (Hughes et al., 2001). The age-related loss of muscle mass and strength may eventually lead to frailty, characterized by diminished strength, endurance and function. Frailty increases the risk of poor health outcomes, such as falls, disability, hospitalization, and mortality (Bandeem-Roche et al., 2006; Ensrud et al., 2009; Fried et al., 2001; Keevil & Romero-Ortuno, 2015). The Fried Frailty Criteria (FFC) are commonly used to assess frailty (Fried et al., 2001), and consist of the five criteria weight loss, weakness, exhaustion, slowness and low activity level. An individual fulfilling one or two of the criteria is categorized as *pre-frail*, whereas the term *frail* is used to describe an individual fulfilling three or more criteria. A study conducted in the UK found that the prevalence of frailty in individuals aged 60 or more was 14 % (Gale, Cooper, & Sayer, 2015). The authors also observed rising prevalence with increasing age, from 6.5 % in those aged 60-69 years to 65 % in those aged 90 or over (Gale et al., 2015).

The causes of frailty are likely to be multi-factorial, but decreased skeletal muscle function seems to play a pivotal role (Batista et al., 2012). Hence, more knowledge about the mechanisms behind the age-related declines in muscle mass and strength is needed. Over the next pages some of the factors considered to be involved are covered, beginning with the quantitative alterations that occur, followed by changes at the qualitative level.

1.2. Impact of aging

1.3.1. Atrophy

In human autopsy studies, aging has been characterized by a reduced number and diameter of spinal motor neurons (Mittal & Logmani, 1987), leading to denervation of muscle fibers (Aagaard, Suetta, Caserotti, Magnusson, & Kjaer, 2010). Some, but likely not all of the denervated muscle fibers are reinnervated through collateral sprouting of nearby surviving motor axons or motor end plates (Aagaard et al., 2010). Muscle fibers that are not reinnervated, are thought to undergo the process of apoptosis and necrosis (Cheema, Herbst, McKenzie, & Aiken, 2015). Although some controversy exist on this topic (Nilwik et al., 2013), it seems that the total number of muscle fibers is reduced with aging (Lexell, Taylor, & Sjoström, 1988; McPhee et al., 2018), thus contributing to the overall loss of muscle mass.

In addition, atrophy of remaining muscle fibers considerably contribute to muscle mass declines. The atrophy is particularly clear in type II fibers, which are approximately 25-40 % smaller in healthy 70 year-olds compared to 20-year-olds (Verdijk et al., 2012; Verdijk et al., 2014). Cross-sectional area of type I fibers has also been observed to undergo some atrophy, but to a lesser extent, with atrophy of 3-15 % (Verdijk et al., 2012; Verdijk et al., 2014). Type II muscle fibers have a faster cross-bridge cycle than type I fibers, and they are very important when we need to develop force rapidly (i.e. power production). As muscle power seems to be a better predictor of functional performance than muscle strength (Bean et al., 2002), it is clear that the predominant atrophy of type II fibers is disadvantageous for elderly individuals. The atrophy of type II fibers is also considered to increase the risk of falls (Kramer et al., 2017), as muscle power is imperative to quickly correct unexpected perturbations that might occur during different daily situations, such as walking. The greater atrophy of type II fibers might relate to a greater degree of disuse compared to the type I fibers. In addition, an age-related reduction in satellite cell content in type II fibers have been observed (Verdijk et al., 2012), perhaps contributing to their specific atrophy.

The results of a 5-year longitudinal study demonstrated that after the age of seventy, the annual reduction in thigh muscle cross-sectional area was approximately 1% and 0.7% in men and women, respectively (Delmonico et al., 2009). However, from several studies it is evident that the loss of muscle strength exceeds the reductions in muscle size, with annual declines of approximately 2-5% in 70-year-olds (Delmonico et al., 2009; Goodpaster et al., 2006; Hughes et al., 2001). Specific strength, defined as the ratio between the strength and size of a muscle or muscle group, declines accordingly (Goodpaster et al., 2006). Identifying the factors involved are important, and some of the potential contributors to the age-related reduction in specific strength will be discussed next.

1.3.2. Muscle activation

Central muscle activation can be assessed by electrical muscle stimulation superimposed onto a maximal voluntary contraction (MVC). When the force output is increased by these superimposed electrical stimuli, the subject's voluntary activation is considered sub-maximal. Studies consistently report a close to maximum activation level (92-95 %) of the knee extensors in young individuals (Billot, Duclay, Simoneau-Buessinger, Ballay, & Martin, 2014; Roos, Rice, Connelly, & Vandervoort, 1999; Suetta et al., 2009; Venturelli et al., 2015). Although not a consistent finding (Cannon, Kay, Tarpinning, & Marino, 2007; Roos et al.,

1999), voluntary activation appear to be slightly reduced in healthy elderly, with activation levels of 85-89% (Billot et al., 2014; Suetta et al., 2009). These studies suggest that some of the decline in specific strength with healthy aging may be explained by differences in activation level, but its contribution appears rather limited. Few studies have investigated activation level in very old and frail elderly. However, Venturelli and colleagues (2015) observed a lower activation level in very old wheelchair bound individuals (78%), compared to both non-frail elderly (85%) and young subjects (94%). An activation level of ~80% in pre-frail and frail elderly have also been observed by others (Harridge, Kryger, & Stensgaard, 1999; Hvid et al., 2016), and it therefore seems likely that a reduced activation level contribute to the very low muscle strength observed in this population.

1.3.3. Intramuscular fat

Infiltration of adipose tissue in the musculature is another potential factor influencing specific strength. Before discussing the impact of aging, a brief introduction to some of the methods used to assess skeletal muscle lipid content will be given, one of which is computed tomography (CT). A CT image of the thigh represents a two-dimensional map of pixels corresponding to a three-dimensional section. The numerical value of each pixel corresponds to the level of gray in the image (attenuation), and is referred to as *Hounsfield Units* (HU). Based on the attenuation of the pixels, CT images can therefore differentiate adipose tissue (-190 to -30 HU), muscle (0 to 100 HU), and bone (700 to 3000 HU) in vivo (Goodpaster, Thaete, & Kelley, 2000). In the literature, different terms are used to describe adipose tissue within and between muscles. The terms used in this thesis are presented in **figure 1.1**. Intermuscular adipose tissue (IMAT) translates to adipose tissue *between* muscles. However, when CT is used to investigate adipose tissue, IMAT refers to adipose tissue both between adjacent muscle groups, and also larger fat depots *within* a muscle. If other methods are used, depots of fat within the muscle may be referred to as *intramuscular* or *extracellular* fat.

In addition to the differentiation of fat and muscle tissue, CT images are used to evaluate the proportion of normal-density (31-100 HU) and low-density muscle (0-30 HU). Moreover, it is common to calculate the mean attenuation value for all pixels within the 0-100 HU range, where a lower value is considered to reflect skeletal muscle with higher levels of intramyocellular lipids (IMCL). In this thesis, the term *muscle density* is used instead of muscle attenuation. However, to measure IMCL content directly, histological sections of muscle biopsies can be stained with Oil Red O (Mehlem, Hagberg, Muhl, Eriksson, &

Falkevall, 2013). This method stains neutral lipids such as triglycerides as well as lipid droplets (Franklin & Kanaley, 2009). An inverse relationship was observed between IMCL content assessed by ORO and muscle density in 45 young individuals ($r = -0.43$) (Goodpaster, Kelley, Thaete, He, & Ross, 2000), supporting the idea that low-density and normal-density muscle reflect muscle with higher and lower levels of IMCL, respectively.

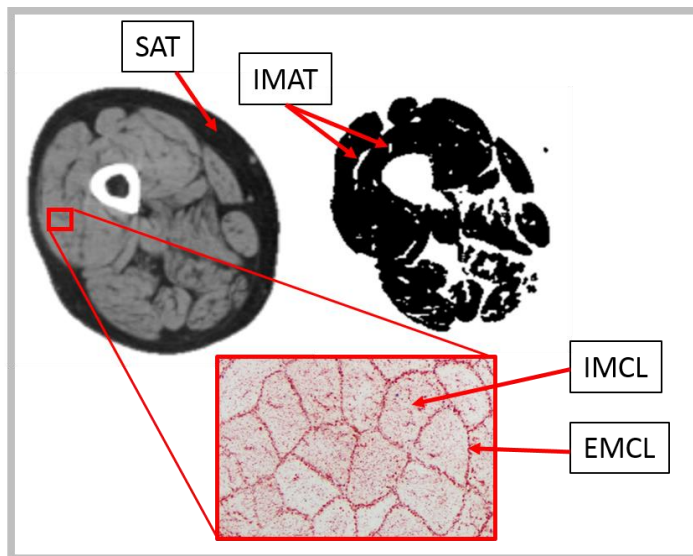


Figure 1.1 Subcutaneous adipose tissue (SAT) is fat located directly underneath the skin. Intermuscular adipose tissue (IMAT) is larger depots of fat located mostly between, but also within muscles. SAT and IMAT can be measured by CT. Intramyocellular lipids (IMCL) are fat stored within the muscle cells, whereas extramyocellular lipids (EMCL) refers to lipids between the muscle fibers. IMCL and EMCL can be stained by Oil Red O on histological sections from muscle biopsies.

Aging increases the area of IMAT in the locomotor muscles (Delmonico et al., 2009; Song et al., 2004), and is associated with muscle weakness, decreased functional capacity, and increased risk of future mobility limitation (Addison, Marcus, Lastayo, & Ryan, 2014). One study suggests that this may be partly due to a negative effect of IMAT on voluntary activation level (Yoshida, Marcus, & Lastayo, 2012). In addition, due to its close proximity to muscle bundles, it is possible that increased levels of IMAT directly impede the muscles ability to contract effectively.

Muscle density (mean HU) is also decreased with aging (Goodpaster, Carlson, et al., 2001). Although not very strong ($r = 0.20$), a correlation was observed between muscle density and specific strength in 2627 elderly individuals (Goodpaster, Carlson, et al., 2001). Interestingly,

a higher muscle density was observed for quadriceps femoris (41 HU) than for hamstrings (29 HU) in the same study, perhaps indicating that IMCL content is higher in the latter muscle group. Despite the decrease in muscle density with aging, direct evidence of an age-related increase in IMCL from analyses on muscle biopsies is scarce. Gueugneau and colleagues (2015) assessed lipid content by Oil Red O, and reported higher lipid content in old compared to young men (Gueugneau et al., 2015). Another study investigated IMCL content by electron microscopy and observed similar numbers of lipid droplets in young men, young women, old men and old women. However, the lipid droplets in old men were twice as large as in the other groups (Crane, Devries, Safdar, Hamadeh, & Tarnopolsky, 2010). Nevertheless, several studies suggest that IMCL content is closer associated with obesity (Choi et al., 2016; Goodpaster, Theriault, Watkins, & Kelley, 2000) and insulin sensitivity (Goodpaster, He, Watkins, & Kelley, 2001), than aging *per se* (Krssak et al., 1999; St-Jean-Pelletier et al., 2016). Based on the latter, and considering the rather weak correlation between IMCL and muscle density (Goodpaster, Kelley, et al., 2000), it seems reasonable to conclude that increased IMCL content is not the only explanation for the age-related decrease in muscle density assessed by CT.

Nevertheless, it seems evident that increased levels of fat within the muscle, whether an effect of aging, obesity or inactivity, have a negative impact on specific strength. Interestingly, a recent study observed an inverse relationship between single fiber specific tension and IMCL content in overweight elderly individuals (Choi et al., 2016), suggesting that the potential link between lipid content and whole muscle specific strength might be related to function at the single fiber level.

1.3.4. Single fiber specific tension

By isolating single muscle fibers under the microscope, and subsequently mounting these fibers to an experimental apparatus where the fibers are subjected to calcium, the force-generating capacity of the fibers can be investigated. To obtain a measure of specific force at the single fiber level, maximum calcium-activated force is normalized to the cross-sectional area of the fiber. This measurement is referred to as *single fiber specific tension* in this thesis. Multiple cross-sectional studies have examined the impact of age on this variable. Some studies suggest lower specific tension in elderly (D'Antona et al., 2003; Frontera et al., 2000; Larsson, Li, & Frontera, 1997; Ochala, Frontera, Dorer, Van Hoecke, & Krivickas, 2007; Power et al., 2015; Yu, Hedstrom, Cristea, Dalen, & Larsson, 2007), whereas others observe

no difference (Frontera et al., 2003; Hvid et al., 2017; Korhonen et al., 2006; Murgia et al., 2017; Reid et al., 2012; Trappe et al., 2003; Venturelli et al., 2015). These studies included primarily healthy elderly individuals, and data on single fiber specific tension in frail elderly is scarce. However, Venturelli and colleagues (2015) observed similar single fiber specific tension in young subjects and wheelchair-bound elderly. This contrasts observations that single fiber specific tension is decreased following both short-term and long-term disuse in elderly (D'Antona et al., 2003; Hvid et al., 2017; Hvid, Ortenblad, Aagaard, Kjaer, & Suetta, 2011). Clearly, more research is required to better elucidate the impact of factors such as age, disuse and frailty on single fiber function. Despite the controversy with regards to the effect of aging, a consistent observation is that specific tension is 10-40% higher in type II compared to type I fibers (Frontera et al., 2000; Ochala et al., 2007; Trappe et al., 2003; Venturelli et al., 2015). This may partly be due to a higher IMCL content in the type I fibers (Choi et al., 2016; St-Jean-Pelletier et al., 2016). Thus, regardless of whether age affects single fiber specific tension or not, specific strength at the whole muscle level will likely be reduced by the primary atrophy of type II fibers (Verdijk et al., 2014).

An important point to keep in mind is that the analysis of single fiber function is very time-consuming and therefore often includes few subjects, and only twenty or so fibers from each biopsy. Thus, it is possible that some of the controversy in this field can be explained by statistical type II errors. Since studies either show reduced or unchanged specific tension, it is reasonable to assume that specific tension is *most likely* reduced with aging. A reduction in single fiber specific tension could rely on several factors. As stated previously, increased IMCL content could be one of the underlying mechanisms (Choi et al., 2016; St-Jean-Pelletier et al., 2016). Reduced myosin content is another potential contributor (Hvid et al., 2017). In addition, evidence suggests that skeletal muscles of older individuals display increased levels of heat shock proteins (Yamaguchi et al., 2007), mitochondrial protein carbonylation (Beltran Valls et al., 2015), and lipofuscin aggregation (Orlander, Kiessling, Larsson, Karlsson, & Aniansson, 1978), all indicative of deficits in mechanisms related to protein quality control. Thus, inefficient degradation of proteins and organelles might be involved in the age-related reduction in specific strength. Some of the processes regulating muscle protein turnover will be introduced next.

1.3.5. Muscle protein turnover

The turnover rate of skeletal muscle protein is approximately 1-2% each day (Volpi, Sheffield-Moore, Rasmussen, & Wolfe, 2001), and the continuous degradation of dysfunctional proteins and simultaneous synthesis of new proteins allow the muscle to maintain its function and adapt to a variety of stimuli. **Figure 1.2** gives an overview of the main factors regulating muscle protein synthesis and muscle protein breakdown in skeletal muscle, and also introduce some of the stimuli known to affect these signaling pathways. Age-related *anabolic resistance* is manifested by a reduced stimulation of muscle protein synthesis following protein intake (Cuthbertson et al., 2005) and muscular activity (Kumar et al., 2009), presumably due to reduced activation of mTORC1 and its downstream targets p70S6K and 4E-BP1.

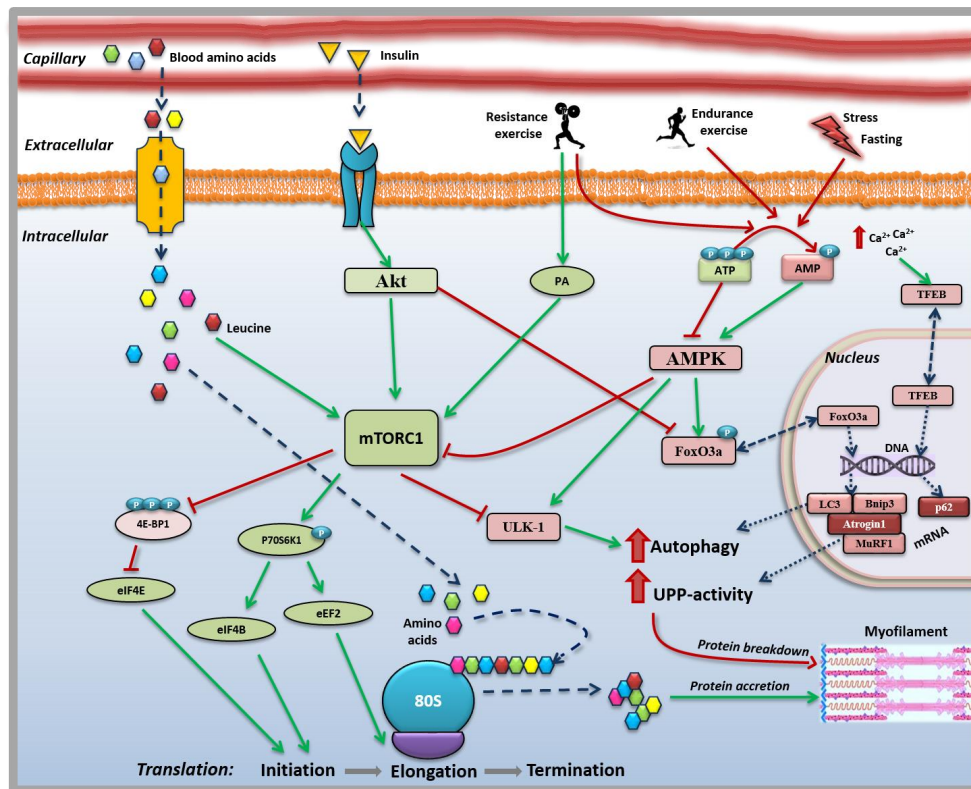


Figure 1.2 mTORC1 and AMPK holds central roles in the regulation of muscle protein synthesis and breakdown in muscle cells. mTORC1 is activated by growth factors, insulin, certain amino acids and tension (resistance exercise), and promote protein accretion through its effect on 4E-BP1 and p70S6K. AMPK is activated by exercise and fasting, due to increases in the AMP/ATP ratio. AMPK promote muscle protein breakdown by activating autophagy and the ubiquitin-proteasome pathway (UPP).

Age-related anabolic resistance likely contribute to the age-related loss of muscle mass and strength. At rest, however, evidence suggest that mTORC1 is hyperphosphorylated in older individuals (Markofski et al., 2015). Although this does not appear to impact the basal protein synthesis rate (Markofski et al., 2015; Volpi et al., 2001), it may affect pathways related to protein degradation, due to the substantial cross-talk between MPS and MPB signaling (**figure 1.2**).

Protein degradation in skeletal muscle is mainly controlled by the ubiquitin proteasome pathway (UPP) and the autophagosome-lysosome system. UPP is largely responsible for the degradation of myofibrillar proteins (Solomon & Goldberg, 1996), and is regulated by ubiquitin ligases such as muscle ring finger-1 (MuRF-1), atrogin-1 (Fbox32/MAFbx) and MUSA-1 (Fbox30). These ligases transfer ubiquitin to a target protein, promoting its degradation by the 26S proteasome (Bodine & Baehr, 2014). Gene and protein expression data indicate either unchanged (Fry et al., 2013; Whitman, Wacker, Richmond, & Godard, 2005) or increased UPP-activity with aging (Dalbo, Roberts, Hassell, Brown, & Kerksick, 2011; Raue, Slivka, Jemiolo, Hollon, & Trappe, 2007).

The autophagosome-lysosome system can degrade intracellular proteins, but also macromolecules and organelles (Tanida, Ueno, & Kominami, 2008). The autophagic process is shown in **figure 1.3** (next page). First, a membrane structure called a phagophore is formed; thereafter, the phagophore expands, a process requiring the conjugation of microtubule-associated protein 1 light chain (LC3-I) to PE (phosphatidylethanolamine) to form its lipidated form, LC3-II. Cargo receptors such p62 recruit proteins and organelles to the growing autophagosome which eventually will fuse with lysosomes to form an autolysosome, where the content is degraded (Tanida et al., 2008). Dysfunctional mitochondria normally undergo fission, before being removed by *mitophagy*, a selective form of autophagy. Autophagy also degrade lipid droplets, a process termed *lipophagy*. Studies in rodents suggest that autophagy is reduced with aging (Kim, Kim, Oh, Kim, & Song, 2013; Wohlgemuth, Seo, Marzetti, Lees, & Leeuwenburgh, 2010; Zhou et al., 2017), but information on how autophagy is affected by aging in human skeletal muscle is scarce. So far, observations indicate that sedentary behavior (Carnio et al., 2014) and overweight (Potes et al., 2017) might suppress autophagy more than aging *per se*. However, to our knowledge, no study has investigated autophagy in very old and frail individuals, in whom specific strength is reduced the most (Metter et al., 1999; Moore et al., 2014).

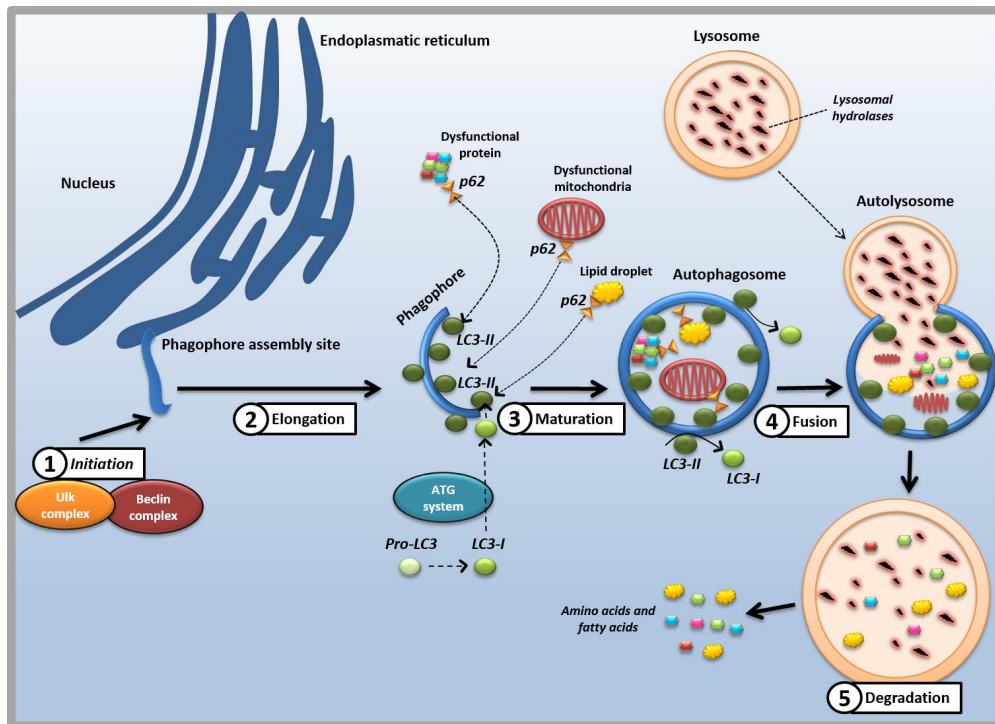


Figure 1.3 Schematic presentation of the steps involved in autophagy. Initiation of autophagy involves the Ulk- and Beclin-complexes (**step 1**). Elongation and maturation of the phagophore (**step 2-3**) requires the lipidation of LC3-I to LC3-II, governed by the ATG system. LC3-II is a receptor for p62, which binds to ubiquitinated proteins, mitochondria and lipid droplets. These substrates are recruited to the maturing phagophore, and eventually enclosed within the autophagosome. LC3-II on the outside of the autophagosomal membrane is recycled back to cytosolic LC3-I. The autophagosome then fuses with a lysosome to form an autolysosome (**step 4**). Finally, all components trapped within the autolysosome are degraded by lysosomal hydrolases, including LC3-II and p62 (**step 5**). The end products, including amino acids and fatty acids, can be reused by the cell.

In theory, attenuated autophagy can have a negative impact on single fiber specific tension through several routes. For example, autophagy is critical for the selective removal of damaged mitochondria (Sandri, 2013). Insufficient removal of damaged mitochondria may therefore increase production of reactive oxygen species (ROS), which can subsequently lead to increased carbonylation of proteins (Beltran Valls et al., 2015). Evidence suggests that carbonylated proteins can escape degradation and form high-molecular-weight aggregates that accumulate with age (Nystrom, 2005). Considering that autophagy is also required to degrade these aggregates, they may not be removed effectively, and consequently the muscle's force-generating capacity may be affected in a negative manner (Beltran Valls et al., 2015). A second route by which attenuated autophagy may negatively affect single fiber specific force

is related to the degradation of lipid droplets. A decline in lipophagy could lead to intracellular accumulation of lipid droplets (Lam et al., 2016; Singh & Cuervo, 2012), thus reducing the density of contractile proteins, negatively affecting the strength of muscle fibers (Choi et al., 2016).

Interestingly, attenuated autophagy might not only have an impact on muscle quality, but could also negatively affect muscle strength through atrophy and denervation. Muscle-specific knockout of the Atg7 gene (autophagy initiator) in aged mice led to muscle atrophy, in addition to a significant increase in the proportion of denervated fibers (Carnio et al., 2014). It has been hypothesized that damaged mitochondria that are not removed efficiently in aging, due to suppressed mitophagy, release mitochondrial contents to the cytosol of the cell, inducing an apoptotic cascade ending with DNA fragmentation and removal of nuclei (Alway, Mohamed, & Myers, 2017). Loss of nuclei in muscle cells may then lead to death and removal of the entire muscle cell. In support of this hypothesis, increased apoptosis signaling has been observed both in aged rodents (Chabi et al., 2008; Dirks & Leeuwenburgh, 2002; Marzetti et al., 2008; Wohlgemuth et al., 2010) and humans (Gousspillou et al., 2014; Whitman et al., 2005). Interestingly, a reduced number of nuclei per fiber has also been observed in type II fibers in old compared to young individuals (Verdijk et al., 2012). Collectively, these studies suggest that a suppression of autophagy with aging might be detrimental for skeletal muscle function in different ways, contributing both to the loss of muscle mass and muscle quality.

Increased levels of heat shock proteins in aging cells supports the idea that a reduced capacity for protein degradation may lead to aggregates of misfolded proteins and increased cellular stress. Proteins such as Hsp70 and α B-crystallin demonstrate a protective role through repair and refolding of misfolded peptides (Dubinska-Magiera et al., 2014). An age-related increase of both proteins have been observed previously (Thalacker-Mercer, Dell'Italia, Cui, Cross, & Bamman, 2010; Yamaguchi et al., 2007), perhaps as a compensatory mechanism against increased levels of misfolded and dysfunctional proteins. It has also been suggested that heat shock proteins may have regulatory control over the autophagic process (Dokladny et al., 2013), but how these two systems interact is not well elucidated at this point.

Undoubtedly, several factors are involved in the age-related reduction in muscle functioning. The reduction in muscle size is likely due to a loss of muscle fibers and atrophy of remaining fibers, whereas the reductions in specific strength might be due to a combination of reduced activation level, increased lipid content, and reduced single fiber specific tension. Whether or

not suppressed autophagy is involved in the age-related reduction in specific strength is currently unknown. Interestingly, both quantitative and qualitative adaptation seems to take place during strength training, even in frail elderly individuals (Fiatarone et al., 1994; Seynnes et al., 2004; Weening-Dijksterhuis, de Greef, Scherder, Slaets, & van der Schans, 2011). Factors underlying the training-induced improvements in muscle size and quality will be discussed next.

1.4. Impact of strength training

1.4.1. Hypertrophy

In general, the adaptation to strength training in elderly appears to be similar to that of young individuals, with substantial increases both in muscle size and strength (Cannon et al., 2007). Several studies have demonstrated similar hypertrophic responses in young and old subjects (Cannon et al., 2007; Ivey et al., 2000; Knight & Kamen, 2001; Mayhew, Kim, Cross, Ferrando, & Bamman, 2009; Roth et al., 2001), whereas other studies suggest greater adaptation in younger cohorts (Kosek, Kim, Petrella, Cross, & Bamman, 2006; Welle, Totterman, & Thornton, 1996). Although it is established that training-induced hypertrophy can be achieved also in very old (Bechshoft et al., 2017) and frail persons (Fiatarone et al., 1994), the gains in muscle mass are typically less prominent (Raue, Slivka, Minchev, & Trappe, 2009; Stewart, Saunders, & Greig, 2014). Importantly, the improvements in strength are similar in old and young individuals (Peterson, Rhea, Sen, & Gordon, 2010), and perhaps even greater in very old and frail subjects (Bechshoft et al., 2017; Stewart et al., 2014), indicating a substantial increase in specific strength in this population. Therefore, studies investigating the effect on strength training on activation level, muscle adipose tissue and single fiber specific force will be addressed next.

1.4.2. Muscle activation

In healthy elderly individuals, minimal or relatively small improvements in activation level are observed after strength training (Cannon et al., 2007; Knight & Kamen, 2001; Reeves, Narici, & Maganaris, 2004), indicating that increased activation level does not contribute greatly to the large improvement in knee extensor strength (Knight & Kamen, 2001). Few studies have measured activation level in very old or frail individuals. Harridge and colleagues (1999) observed a non-significant improvement in activation level from 81% at

baseline to 85% after ten weeks of heavy load strength training in very old and frail subjects. Moreover, Hvid and colleagues (2016) observed a six percent increase in activation level following high load power training in pre-frail elderly (Hvid et al., 2016). These findings suggest that increased voluntary activation may contribute more to strength gains in this population, compared to non-frail elderly. Indeed, a meta analysis concluded that greater gains were achieved in subjects with lower voluntary activation level at baseline (Arnold & Bautmans, 2014). Hvid et al (2016) observed a correlation between improvements in activation level and improvements in gait velocity, demonstrating that the improvement in activation level and thus strength is also important from a functional point of view. Studies investigating the effect of strength training on knee extensor activation level are summarized in **table 1.1**.

Table 1.1 Effect of resistance training on knee extensor activation level in elderly individuals.

	Age	N	% W	Intervention	Method	Activation (%)	
<u>Healthy elderly</u>							
Cannon et al., 2007	70 ± 7	8	100	KE: 3 x 10 reps at 75% of 1RM. 3 sessions/week for 10 weeks	IT-ratio	PRE 96 ± 2	POST 98 ± 2
Knight et al., 2001	67-81	7	14	KE: 3 x 10 reps at 75% of 1RM X. 3 sessions/week for 10 weeks.	CAR	PRE 97 ± 6	POST 98 ± 4
Reeves et al., 2004	74 ± 4	9	56	LP and KE: 2 x 10 reps at 80% of 5RM. 3 sessions/week for 14 weeks.	CAR	PRE 90 ± 12	POST 95 ± 6*
<u>Pre-frail/frail elderly</u>							
Hvid et al., 2016	82 ± 1	16	56	LP: 3 x 8-10 reps at 70-80% of 1RM. Concentric phase with maximum velocity. 2 sessions/week for 12 weeks.	IT-ratio	PRE 79 ± 4	POST 85 ± 2*
Harridge et al., 1999	85-97	11	73	KE: 3 x 10 reps at 50-80% of 1RM. 3 sessions/week for 12 weeks.	IT-ratio	PRE 81 ± 7	POST 85 ± 10

W: women, KE: knee extension, 1RM, one repetition maximum, LP: leg press, IT-ratio: interpolation twitch ratio, CAR: central activation ratio. *significantly different from baseline. Some of the values are estimations based on figures.

1.4.3. Intramuscular fat

Few studies have investigated the effect of strength training on intermuscular adipose tissue (IMAT) and intramyocellular lipids (IMCL). Goodpaster et al. (Goodpaster et al., 2008) demonstrated that a 12-month, multimodal exercise program prevented the increase in IMAT observed in the control group. In addition, decreases in IMAT have been observed following

six months of multimodal exercise combined with dietary restriction (Manini et al., 2014; Santanasto et al., 2011). However, in one of the latter studies (Santanasto et al., 2011), the exercise program alone did not significantly reduce IMAT, suggesting that the greater weight loss in the exercise and diet group was the main factor behind the change. Sipila and colleagues (1995) reported that the proportion of IMAT in the thigh decreased after 18 weeks of strength training in 76-78 year-old women (Sipila & Suominen, 1995), but the change was primarily due to an increase in lean tissue, and not due changes in IMAT *per se*. Moreover, IMAT did not change following 24 weeks of detraining and 12 weeks of retraining in elderly men and women, although a tendency for a decrease was observed following retraining (Taaffe et al., 2009). Thus, training does not appear to have a huge impact on IMAT area. Nonetheless, Marcus et al (2013) investigated changes in specific strength (force / lean tissue area) following multimodal training (walking, strength, flexibility) in 78 elderly subjects with either low, moderate or high levels of IMAT at baseline. Interestingly, specific strength changed by 11, 8, and -3 %, respectively, in the three groups. The authors speculate that the mechanism behind this blunted response in the high IMAT group may involve the potential impact of IMAT on inflammatory pathways in the muscle cells (Marcus, Addison, & LaStayo, 2013). In conclusion, strength training performed alone or in combination with other types of training does not appear to have a huge impact on IMAT in elderly individuals, although gains in lean tissue might reduce its proportion of the total cross-sectional area. However, the potential negative role of IMAT in blunting training responses should be taken into account when investigating training-induced changes in specific strength.

Some of the studies referred to in the paragraph above also reported results for muscle density (mean attenuation [HU]). Because IMAT is excluded from the calculation of muscle density, lower values are considered to reflect increased levels of lipids within (IMCL) or between (EMCL) individual muscle fibers (Aubrey et al., 2014; Goodpaster, Kelley, et al., 2000). In two of these studies, no changes in muscle density were observed (Goodpaster et al., 2008; Santanasto et al., 2011), whereas in the study by Sipila and colleagues (1995), increased muscle density was observed for the lower leg, but not quadriceps femoris and the hamstring muscles. Taaffe and colleagues (2009) observed decreased and increased quadriceps femoris density following detraining and retraining, respectively. However, since muscle biopsies were not obtained in these studies, we do not know if the changes in muscle density were due to changes in IMCL content, as suggested by the authors (Taaffe et al., 2009). In the only study so far to investigate the effect of strength training on IMCL content by Oil Red O, no

changes were observed in the deltoid muscle following fourteen weeks of upper-body strength training in active elderly men (Ngo, Denis, Saafi, Feasson, & Verney, 2012). Clearly, more research is required to fully elucidate the role of skeletal muscle adipose tissue with regards to training adaptation in the elderly. Interestingly, no study has investigated the effect of strength training on these aspects in very old and frail individuals.

1.4.4. Single fiber specific tension

As covered previously, several studies have observed lower single fiber specific tension in old compared to young individuals, and it therefore seems reasonable that increased single fiber specific force may contribute to the training-induced increases in muscle strength. However, in most of the studies conducted so far, strength training did not affect single fiber specific tension (**table 1.2**).

Table 1.2 Effect of resistance training on vastus lateralis single fiber specific tension in elderly individuals.

Authors	Subjects				Intervention	Type I ST % change	Type II ST % change
	Age	N	%W	Status			
Frontera et al., 2003	74 ± 3	7	100	UT	KE: 4 x 8 reps at 80% of 1RM 3 sessions/week for 12 weeks	+4 #	-
Godard et al., 2002	73 ± 2	6	100	UT	KE: 2 x 10 reps at 80% of 1RM + 1 set to failure. 3 sessions/week for 12 weeks	-5	+12
Miller et al., 2017	70 ± 2	17	59	OA	LP and KE: 2 x 8 reps at 60% of 1RM. 3 sessions/week for 12 weeks.	0%	+2
Parente et al., 2008	78 ± 4	4	100	UT	LP: 1 x 12 reps at 60% of 1RM twice a week, in addition to 1 home session with 20 repetitions of LP using elastic bands. 3 sessions/week for 1 year	+24 *	+37 *
Raue et al., 2009	85 ± 1	6	100	UT	KE: 3 x 10 reps at 70-75% of 1RM 3 sessions/week for 12 weeks	+2	-1
Slivka et al., 2008	82 ± 1	6	0	UT	KE: 3 x 10 reps at 70% of 1RM 3 sessions/week for 12 weeks	+4	-4
Trappe et al., 2000	74 ± 2	7	0	UT	KE: 2 x 10 reps at 80% of 1RM + 1 set to failure. 3 sessions/week for 12 weeks	+7	0
Trappe et al., 2001	74 ± 2	7	100	UT	KE: 2 x 10 reps at 80% of 1RM + 1 set to failure. 3 sessions/week for 12 weeks	-18	+5
Wang et al., 2018	65-80	16	39	OW	LP and KE: 3 x 10 reps at 70% of 1RM. 3 sessions/week for 22 weeks	+27 *	+18 *

W: women, ST: specific tension, LP: leg press, KE: knee extension, 1RM: one repetition maximum, UT: untrained, OA: osteo-arthritis (knee), OW: overweight. *significantly different from baseline, # significantly different from change in control group. Some of the values are estimations based on figures.

An interesting observation from **table 1.2** is that increased specific tension was only observed following the two training interventions with the longest duration (5 and 12 months) (Parente et al., 2008; Wang et al., 2018), suggesting that 12 weeks of strength training might not be sufficient to affect the mechanisms underlying this qualitative adaptation. Moreover, the subjects in the study by Wang and colleagues (2018) were overweight and obese, and the researchers reported that increased specific tension of type II fibers correlated with reductions in thigh fat volume (SAT+IMAT) assessed by CT. IMCL content was not measured, but it is interesting to speculate that the reduction in thigh fat volume was accompanied by reduced IMCL content, and that this contributed to the increase in single fiber specific tension. The latter suggests that the potential for improvement might be less in non-obese subjects with lower levels of IMAT and IMCL.

All these studies included non-frail elderly individuals (Frontera et al., 2003; Godard, Gallagher, Raue, & Trappe, 2002; Miller et al., 2017; Parente et al., 2008; Raue et al., 2009; Slivka, Raue, Hollon, Minchev, & Trappe, 2008; Trappe et al., 2001; Trappe et al., 2000; Wang et al., 2018). It can be assumed that activity levels are lower in frail elderly individuals using aids such as walkers in their everyday life, and we know that disuse negatively affect specific tension of both type I and type II fibers (D'Antona et al., 2003; Hvid et al., 2017). Hence, although one study suggests otherwise (Venturelli et al., 2015), specific tension might in fact be reduced in frail elderly, and strength training could represent an effective counter-measure. However, the effect of strength training on single fiber specific tension has to date not been investigated in this population, despite evidence that strength gains are substantial, and greatly exceed the gains in muscle size (Bechshoft et al., 2017; Cadore et al., 2014; Fiatarone et al., 1994).

So far, we have seen that improved voluntary activation level may explain part of the training-induced improvements in strength in elderly individuals, and perhaps slightly more in frail elderly. However, from the literature it is clear that a substantial part of the training-induced improvements in strength is due to alterations at the level of the muscle cells, primarily due to increased fiber size, but perhaps also due to increases in fiber quality. Changes at the muscle fiber level are related to altered signaling in the pathways governing muscle protein synthesis and muscle protein breakdown. Therefore, in the last chapter of this introduction, the acute and long-term effect of strength training on pathways related to MPS and MPB will be covered, with emphasis on the process of autophagy.

1.4.5. Muscle protein turnover

As discussed earlier, aging is associated with a reduced muscle protein synthetic response to protein intake and muscular activity, termed anabolic resistance (Cuthbertson et al., 2005; Fry et al., 2011; Kumar et al., 2009), which may ultimately contribute to the development of sarcopenia and frailty. Luckily, in both young and healthy elderly, it has been demonstrated that resistance exercise performed prior to protein intake "sensitize" the muscle cells, resulting in a greater MPS response compared to protein intake alone (Yang et al., 2012). This appears to be due to an enhanced activation of mTORC1 and its downstream targets, such as p70S6K (Francaux et al., 2016) (**figure 1.2**, page 8). However, to what extent an acute session of strength training potentiates the muscles of very old and frail individuals, and whether this depend on training status, remains to be investigated. Based on studies showing limited training-induced hypertrophy in this population (Bechshoft et al., 2017; Chale et al., 2013; Fiatarone et al., 1994), it is necessary that we improve our understanding of how muscle protein synthesis is regulated in response to strength training and protein intake in these individuals.

In addition to stimulating MPS, strength training also leads to acute increases in muscle protein breakdown (Biolo, Maggi, Williams, Tipton, & Wolfe, 1995; Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997), which is likely important in terms of facilitating degradation of dysfunctional proteins and organelles. AMPK appears to be central to the increase in MPB, by dephosphorylating FoxO3, thus promoting its translocation to the nucleus. In the nucleus, FoxO3 increases the gene expression of UPP-related genes MuRF1 and atrogin-1, and autophagy-related genes LC3 and Bnip3 (Mammucari et al., 2007). In addition, AMPK may increase ULK1 activity and thus autophagy, through suppression of mTORC1 (**figure 1.2**, page 8). Moreover, it appears that exercise-induced increases in calcium promote translocation of TFEB from cytosol to the nucleus (Mansueto et al., 2017), where TFEB increases the gene expression of p62 as well as genes involved in lysosomal biogenesis (Settembre et al., 2011). Hence, it is clear that the strength training-induced increase in MPB may involve altered regulation in several pathways of muscle protein degradation.

Studies consistently report increased gene expression of MuRF-1 the first hours after strength training in both young and healthy older individuals (Fry et al., 2013; Raue et al., 2007; Stefanetti, Lamon, et al., 2014; Stefanetti, Zacharewicz, et al., 2014), suggesting that increased activity of the ubiquitin-proteasome pathway (UPP) is central to the training-induced increase in protein breakdown. However, the UPP response to strength training has

never been investigated in frail elderly individuals. Moreover, if the acute response depends on training status in this population is currently unknown.

Autophagy and mitophagy have received less focus in relation to strength training compared to the UPP. Recent observations of a reduced LC3-II/LC3-I ratio the first hours after strength training suggest acute inhibition of autophagy (Dickinson et al., 2017; Fry et al., 2013).

However, gene expression data suggest a later stimulation of autophagy, because increased mRNA levels of both LC3 and p62 have been observed (Dickinson et al., 2017; Ogborn et al., 2015). Moreover, long-term strength training has been shown to increase several autophagic markers, both in rodents (Luo et al., 2013) and humans (Hentila et al., 2018; Tanner et al., 2015). Hence, the autophagic response to strength training warrants further investigation, and acute responses should be interpreted in light of long-term effects, to better elucidate the role of autophagy in strength training adaptation.

There are several ways in which increased muscle protein breakdown could contribute to improvements in muscle function. Increased UPP-activity might simply have a positive impact through clearance of dysfunctional myofibrillar proteins, and at the same time provide building blocks for the synthesis of new proteins. A functional heat shock protein system can rescue proteins from aggregation through its ability to refold denatured proteins, but also facilitate degradation of proteins damaged beyond repair. Increased autophagic activity may improve cellular function in various ways. First, protein aggregates formed by denatured proteins that are not refolded, are degraded by autophagy (Lamark & Johansen, 2012). Second, clearance of damaged and dysfunctional mitochondria by mitophagy may reduce apoptosis signaling and thus prevent fiber atrophy and loss (Alway et al., 2017). Clearance of damaged mitochondria may also reduce the production of reactive oxygen species (ROS), and thus prevent carbonylation of other cellular proteins which could otherwise compromise contractile function (Barreiro & Hussain, 2010). Third, in individuals with high levels of intramyocellular lipids (St-Jean-Pelletier et al., 2016), and large lipid droplets (Crane et al., 2010), increased lipophagy may be another benefit of training-induced increases in autophagy. Hence, it is clear that muscle protein breakdown should not be neglected when the goal is to elucidate the mechanisms behind adaptation to strength training.

Much progress has been made in this field of research over the last decades, and our understanding of how skeletal muscle respond to aging and strength training has expanded substantially. Nevertheless, there are still many questions, several of which stems from the relative lack of data on frail elderly individuals, who suffer most from the declines in skeletal

muscle function. Frail elderly are also the individuals in whom the development of effective counter-measures are needed the most.

2. Research aims and hypotheses

The overall aim of this thesis was to investigate the effect of heavy load strength training on a broad number of factors related to specific strength in pre-frail and frail elderly, and to assess their contribution to improvements in absolute strength and functional capacity. Another objective was to investigate the impact of age, frailty and strength training on the process of autophagy, and to explore if this aspect of protein quality control relates to specific strength.

The aim of the pilot study (*article I*) was, in addition to test the feasibility of our training protocol, to investigate the effect of heavy load strength training and protein supplementation on muscle mass, muscle thickness, strength and function in pre-frail elderly individuals. Our hypotheses were:

- Heavy load strength and protein supplementation will increase muscle thickness, muscle strength and functional capacity.
- Isometric and dynamic knee extension strength will exceed the increase in quadriceps femoris muscle thickness.
- Increases in muscle strength will correlate with improvements in functional capacity.

The aim of the cross-sectional study (*article II*) was to compare levels of autophagic markers and heat shock proteins in groups known to differ in specific strength. Our hypotheses were:

- Autophagic markers LC3-I, LC3-II, and p62 will show evidence for attenuated autophagy in non-frail elderly compared to young individuals, and an even stronger attenuation in frail elderly individuals.
- The level of heat shock proteins Hsp70 and α B-crystallin will be higher in non-frail old compared to young subjects, and even higher in frail elderly.
- Markers of increased autophagy will correlate positively with specific strength.

The aim of the main intervention study (*article III*) was to investigate how different aspects of specific strength change in response to heavy load strength training in pre-frail and frail elderly individuals. Our hypotheses were:

- Strength training will increase specific strength (knee extension isometric torque / quadriceps femoris muscle cross-sectional area).

- Strength training will increase activation level, muscle density and single fiber specific tension.
- Increases in muscle density will correlate with changes in skeletal muscle lipid content and single fiber specific tension.

As a part of the main intervention study, an acute experiment was conducted before and after the intervention period. The aim of the acute study (*article IV*) was to investigate acute and long-term effects of strength training on markers of muscle protein synthesis and breakdown in pre-frail and frail elderly participants, with emphasis on autophagy. Our hypotheses were:

- The acute stimulation of regulators of protein synthesis (p70S6K, 4E-BP1, eEF2) will be more pronounced after resistance exercise and protein ingestion than after protein ingestion alone.
- The acute upregulation of mRNA related to UPP and autophagy will be more pronounced after resistance exercise and protein supplementation than after protein supplementation alone.
- 10 weeks of heavy load strength training will increase markers of autophagy activity at rest, manifested by reduced levels of p62 and LC3-I, and increased levels of LC3-II.
- 10 weeks of heavy load strength training will reduce resting levels of heat shock proteins Hsp70 and α B-crystallin.

3. Methods

This thesis presents data from four studies, referred to as A, B, C and D. Study A was carried out in the autumn 2014, whereas study B was conducted in the spring of 2015. These two studies were part of a previous PhD thesis (Hamarsland, 2017), and only baseline data from these projects are included here (in *article II*).

Our research group had limited experience with training of pre-frail and frail elderly individuals, and a pilot study (study C) was conducted prior to the main intervention study (study D). The pilot study was conducted in the autumn of 2015, whereas the main intervention study ran during the autumn of 2016, with an additional round of participants in 2017.

3.1. Subjects

A total of 93 participants gave their written, informed consent to participate in the four studies (**table 3.1**). Study A, B and D were approved by the Regional Ethics Committee (REC) for Medical and Health Research of South-East Norway. Study C was by REC evaluated to be outside the remit of the Act on Medical and Health Research (2008), and therefore implemented without their approval (*letter of exemption, appendix I*). The projects were registered at clinicaltrials.gov as NCT03033953 (A and B), NCT03723902 (C), and NCT03326648 (D), and performed in accordance with the *Declaration of Helsinki (World Medical, 2013)*.

Table 3.1 Subject characteristics. Note that study A and B were part of a previous PhD thesis, and that these subjects are only included in the cross-sectional study (article II)

Study	N (♂/♀)	Age (yr.)	Body mass (kg)	Body fat (%)	Lean mass (kg)	Leg lean mass (kg)
A	10/7	29 ± 7	81 ± 14	30 ± 7	54 ± 10	19 ± 4
B	12/8	74 ± 3	78 ± 15	32 ± 8	50 ± 10	17 ± 4
C	7/15	85 ± 6	73 ± 11	38 ± 9	43 ± 5	15 ± 2
D	18/16	86 ± 7	69 ± 14	34 ± 7	44 ± 9	15 ± 4

Inn all studies only untrained participants were included, and participants were excluded if they had severe cognitive impairment, unstable chronic illness, cardiac arrhythmia, fracture of

a lower extremity during the last year, or other musculoskeletal problems making testing and training impossible.

In study C, participants were screened using the Short Physical Performance Battery (SPPB) (**appendix II**). The SPPB consists of timed standing balance, gait speed, and timed chair-rise assessments (Guralnik, Ferrucci, Simonsick, Salive, & Wallace, 1995). Performance for each of these tasks is scored between 0 and 4, with a summary score of 0–12. Participants with an SPPB score ≤ 10 were included. In addition, individuals using aids such as walkers, canes or Nordic walking poles in their daily life were included, regardless of SPPB score.

In study D, subjects fulfilling three of the five Fried Frailty Criteria (FFC; **appendix II**) were included (Fried et al., 2001). In addition, subjects fulfilling two FFC criteria amongst "slowness", "weakness", "low activity level" were included. Furthermore, individuals with a score of $\leq 6/12$ on the Short Physical Performance Battery (SPPB) were included, regardless of categorization based on the FFC.

3.2. Study design and intervention adherence

Since only baseline data from study A and B are included in this thesis, methodological aspects extending beyond what is relevant for the presented results will not be covered.

Study C (*article I*) was carried out as a randomized controlled trial to investigate the effect of strength training in combination with protein supplementation on muscle hypertrophy, muscle strength and functional performance in pre-frail elderly individuals. Another goal of this study was to test the feasibility of our training protocol. Subjects were randomized into a group performing heavy load strength training three times weekly for 10 weeks with daily protein supplementation (ST), or a control group (CON). All subjects completed the intervention, but one subject was excluded from the results, because the participant began exercising despite being a part of the control group (**figure 3.1, Study C**).

Study D (*article III and IV*) was designed as a randomized controlled trial to investigate the effect of strength training on different aspects of specific strength/muscle quality in pre-frail and frail elderly individuals. Subjects were randomized to a group performing heavy load strength training twice a week for 10 weeks with daily protein supplementation (ST), or a non-training control group (CON), only receiving the protein supplement. Due to a high drop-out rate in the training group ($n = 7$), seven subjects allocated to CON agreed to perform the ST intervention after the initial intervention period, and were then retested. Hence, the original

design (RCT) was abandoned to increase statistical power. Three subjects withdrew after randomization, and six subjects dropped out during the intervention, due to death ($n = 1$), lack of motivation ($n = 2$), pain during training ($n = 2$), and health issues unrelated to the study ($n = 1$) (**figure 3.1, Study D**). Moreover, several of the subjects in study D were excluded from specific tests. More details are provided in *article III*.

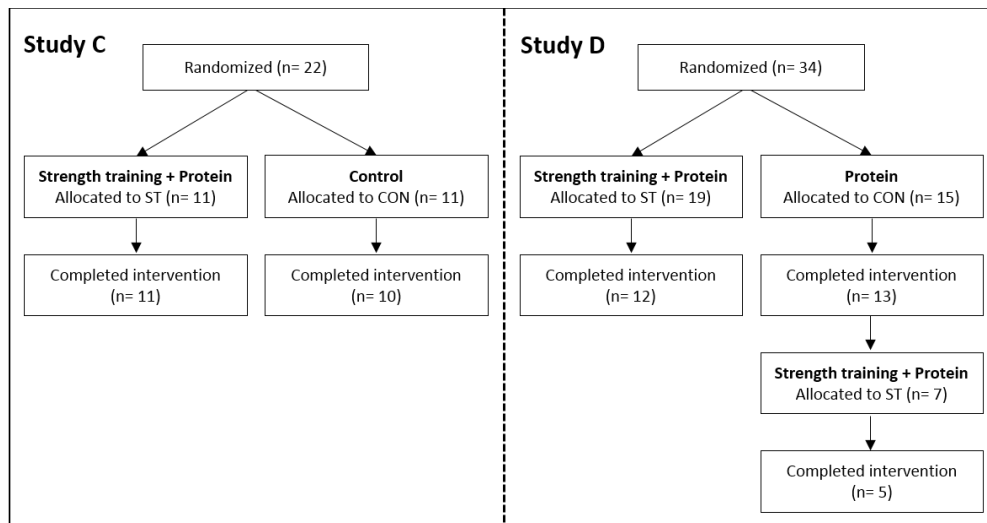


Figure 3.1 Simplified flow charts for the pilot study (study C) and the main intervention study (study D).

An acute experiment (*article IV*) was conducted before and after the ten-week intervention period in study D, to investigate the acute response to strength training followed by protein intake in the untrained and trained state. Both groups performed the acute experiment, but in CON the training session was replaced by a rest period (**figure 3.2**).

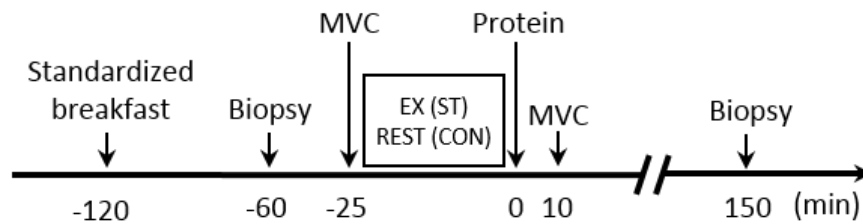


Figure 3.2 Acute experiment conducted before and after the intervention in study IV. MVC: maximal voluntary contraction, EX: strength training session, REST: rest period.

The cross-sectional study (*article II*) included young subjects from study A, old non-frail subjects from study B, and pre-frail and frail subjects from study D. The division into pre-frail and frail was based on the SPPB score. Participants with an SPPB score ≤ 6 were assigned to old frail, whereas subjects with a score ≥ 7 were assigned to old pre-frail. Subjects from study C was not included in the cross-sectional study, because muscle biopsies were not obtained in this project.

3.3. Training program and exercise protocol

Participants performed a lower body strength training program with three (study C) or two training sessions per week (study D). Both training programs included leg press and knee extension. In addition, adapted one leg squat was performed in study C. Participants performed 1-2 sets of 12 repetitions at submaximal loads the first week, and from week 2-10 subjects gradually progressed from 3 sets of 12 repetitions to failure (12RM) to 3-4 sets of 6 repetitions to failure (6RM). Each set was followed by approximately 2-min rest periods. ST sessions were preceded by a lower extremity warm up exercise, using a step platform. In addition, specific warm up was performed with one submaximal set in leg press and knee extension. In study C, one of three weekly sessions was a submaximal work-out, with loads corresponding to 80-90 % of RM loads, whereas for the other two sessions RM-loads were used. In study D, RM-loads were used in both weekly work-outs. The training programs are presented in the end of this thesis (**appendix II**). Experienced instructors supervised the participants at all training sessions.

During the acute experiment (**figure 3.2**) conducted before and after the intervention in study D, subjects in the training group performed a training protocol consisting of 6 sets of 8 repetitions to failure (8RM sets) of knee extension, with a new set starting every 3rd min.

3.4. Supplements and diet

The same supplement was used in study C and D. Supplements were produced by Tine ASA (Oslo, Norway). Each supplement contained 17 g of milk protein, 18 g of carbohydrate, and 1 g fat (149 kcal [627 kJ]). In study C, the training group ingested two supplements each day. On days with no training, supplements were consumed in the morning and in the evening. On training days, one of the servings was consumed within two hours after the training session. In study D, subjects in both groups ingested one supplement each day. The supplement was

consumed in the evening, except on training days (ST), when the supplement was consumed within two hours after training. In both studies, participants were encouraged to continue their habitual diet in addition to the supplements. 24-hour diet recall interviews were conducted in study C (for details, see *article D*).

In study A, B and D, the participants received a standardized breakfast following an overnight fast on the test days with muscle biopsies. The standardized breakfast contained oatmeal, water, sugar and butter, constituting 30 kJ (study A), 25 kJ (study B) and 20 kJ (study D) per kg body mass. The protein content of the breakfast was 0.14, 0.11 and 0.09 g per kg body mass in study A, B and D, respectively. Water was provided ad libitum in all experiments.

3.5. Measures of muscle mass, size and density

In all studies, body composition was assessed by dual-energy X-ray absorptiometry (Lunar iDXA GE Healthcare, Madison, Wisconsin, USA). Participants were scanned from head to toe in a supine position, providing values for total lean tissue and fat mass. In addition, regional analyses were performed to measure leg lean mass. The coefficient of variation (CV) for the assessment of leg lean mass is low in our lab (<1.7%).

In study C, ultrasound was used to measure muscle thickness in vastus lateralis, rectus femoris and vastus intermedius of the dominant leg, with a 50-mm linear probe (5–12 MHz) connected to a Philips HD11 XE ultrasound apparatus (Royal Philips Electronics, Amsterdam, Netherlands). Scans of vastus lateralis were obtained at 40% of the distance between the knee joint and the greater trochanter, and halfway between the iliac crest and the myotendinous junction for rectus femoris. Vastus intermedius was measured on the same image as vastus lateralis, where the latter was thickest in the medio-lateral direction. Probe position was recorded for each measurement on transparent, acetate paper positioned over the thigh. Several images were obtained for each muscle at each registration time point, and the three best images were chosen for analysis, which was performed using OsiriX v5.5.1 (Bernex, Switzerland). The shortest distance between the upper and lower aponeurosis was measured at 25, 50 and 75% of the width of the field of view. Muscle thickness was calculated as the average of these measurements across images.

In study D, changes in quadriceps femoris muscle cross-sectional area was measured by Computed Tomography (CT) before and after the intervention. CT scans (8 mm thick) were obtained from both legs at 50% of the distance between the knee joint and the greater

trochanter (Toshiba Aquilion Prime 80, Canon Medical Systems, Otawara, Japan), and saved as DICOM images. Analyses were performed in a blinded manner using Fiji software (ImageJ, U.S. National Institutes of Health, Maryland, USA), and only results for quadriceps femoris muscles are included in the present investigation. Other muscles, bone and subcutaneous adipose tissue were excluded manually by drawing a line along the muscle fascia (**figure 3.3 A-B**). According to previously defined ranges (Goodpaster, Kelley, Wing, Meier, & Thaete, 1999), scans were analyzed to quantify the cross-sectional areas of total muscle (0 to 100 Hounsfield units [HU]), low-density muscle (0 to 30 HU), normal-density muscle (31 to 100 HU), and adipose tissue (-190 to -30 HU) within the defined area (**figure 3.3 C-D**). Pixels between these ranges (-29 to 0 HU) are thought to reflect a mix of adipose tissue and muscle (Goodpaster, Kelley, et al., 2000), and constituted a small area in all subjects (~1-2 cm²). This area was excluded from the analyses. Muscle attenuation (referred to as *density* in this thesis), was measured as the mean attenuation value from all pixels within the range of 0 to 100 HU in quadriceps femoris (Goodpaster, Thaete, Simoneau, & Kelley, 1997).

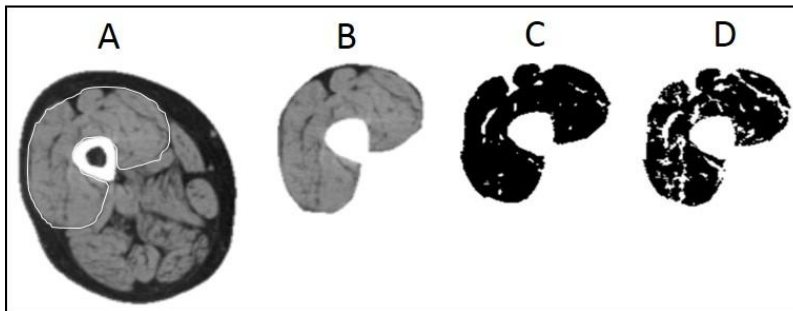


Figure 3.3 Analysis of muscle cross-sectional area, intermuscular adipose tissue (IMAT) and low- and normal-density muscle area from the right leg of an 84-year-old male subject. **A:** cross-sectional area of the mid-portion of the right thigh. **B:** Manual isolation of quadriceps femoris, excluding subcutaneous adipose tissue, bone and other muscles. **C:** Tissue characterized as muscle (0 to 100 HU) is depicted in black, whereas white areas show intermuscular adipose tissue (IMAT). **D:** Normal-density muscle (31 to 100 HU) is depicted in black, whereas white areas represent low-density muscle (0 to 30 HU) and IMAT (-190 to -30 HU).

3.6. Muscle strength, activation level and functional tests

In the intervention studies (C and D), maximal strength in knee extension was measured by 1 repetition maximum (1RM) before and after the intervention. Testing was preceded by a specific warm-up procedure with 10, 6, 3 and 1 repetition at 50, 70, 80 and 90% of expected 1RM, respectively. 2-3 min of rest was given between attempts, and range of motion was strictly controlled.

In all studies, unilateral maximal knee extension was assessed by isometric maximal voluntary contraction (MVC). The test was performed in a custom-made knee-extension apparatus (Gym2000, Geithus, Norway), in which participants were seated with a belt fixing their hip. Joint angle in hips and knees were 90°. Three attempts of 3-4 s were given for each leg to reach MVC. Force was measured with a force transducer (HMB U2AC2, Darmstadt, Germany).

In study D, isometric strength testing was combined with the interpolated twitch technique to evaluate voluntary muscle activation. In short, participants were seated in a Humac NORM dynamometer (CSMi, Stoughton, MA, USA) with a four-point belt fixing the chest and hips, and with a knee joint angle of 70° (0° corresponding to full extension). Two percutaneous surface stimulation electrodes (Veinoplus, Ad Rem Technology, 8 x 13 cm, Paris, France) were placed over the distal and proximal parts of the quadriceps muscle belly. Subjects were instructed to contract as forcefully as possible, and maintain maximal force exertion for 3-4 s, to assess isometric maximal voluntary contraction (MVC). Following a 3-min break, subjects performed a second MVC to assess activation capacity. To this end, paired (doublet) stimuli were evoked (10 ms interval) when the plateau of maximal torque exertion was reached (**figure 3.4**). In addition, a doublet was delivered approximately two seconds after the end of the voluntary contraction, when the muscle was still potentiated. Two more MVC trials were performed, with and without stimulation, interspersed by 3-min breaks. Voluntary muscle activation was calculated using the equation: $\text{activation (\%)} = 100 - ((D * (T_{\text{Stim}} / T_{\text{MVC}}) / T_{\text{StimRest}}) * 100)$ where D is the difference between torque at stimulation (T_{Stim}) and total torque (voluntary + electrically evoked force response), T_{MVC} is the highest voluntary torque, and T_{StimRest} is torque achieved due to stimulation in the rested muscle.

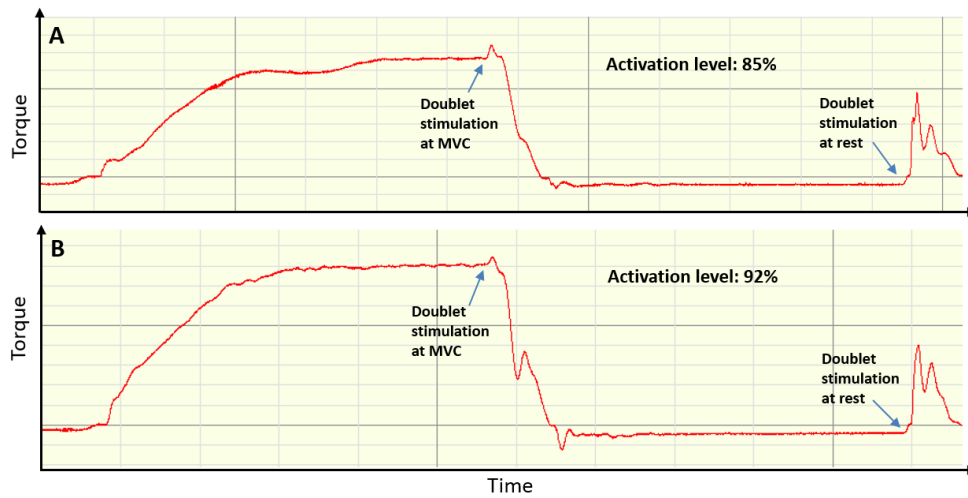


Figure 3.4 Assessment of knee extensor voluntary muscle activation using the interpolated twitch technique. Examples from a 98-year-old female (A), and an 84 year old male participant (B).

In all studies including elderly participants (study B, C and D), various tests were performed to assess functional performance. In all three studies, *five times chair rise* was performed. Participants were instructed to rise from a chair to a full stand five times as fast as possible. If they were not able to stand up without using their hands, limited use of hands was allowed both before and after the intervention period. Time was measured using a compression based sensor (TC-Start Pod and Touch Pad, Brower Timing Systems, Utah, USA) in study B and C, and a stop-watch in study D. In study C, participants also performed a *stair climb test*, in which participants were instructed to walk a set of stairs as fast as possible without transitioning into running. Risers were 18 cm high and 30 long (horizontal length). The total height was 3.6 m and the total length was 6 m. Time was assessed by photo cells (Speedtrap 2, Brower Timing Systems, Utah, USA). In study C and D, also *gait velocity* was measured. Participants were asked to walk 10 meters at their habitual gait velocity, and photo cells (Brower Timing Systems, USA) were placed to measure the time from 2 to 8 meters. In study D, also maximal gait velocity was assessed.

3.7. Specific strength

Specific strength was calculated as knee extension MVC normalized to leg lean mass in the cross-sectional study (*article II*), because quadriceps femoris muscle CSA was not measured in the young (study A) and non-frail elderly (study B) individuals.

In *article III* (on data from study D), specific strength was calculated as knee extension MVC normalized to quadriceps femoris muscle CSA (0 to 100 HU). In addition, the term *dynamic* specific strength is used, representing knee extension 1RM normalized to quadriceps femoris muscle CSA (0 to 100 HU).

3.8. Biopsy analyses

Muscle biopsies were obtained from the mid portion of vastus lateralis in study A, B and D. The procedure was conducted under local anesthesia (Xylocaine with adrenaline, 10 mg*ml⁻¹ + 5 ug*mg ml⁻¹, AstraZenica London, UK), and approximately 200 mg of tissue was obtained using a modified Bergström technique with suction. Blood, fat and connective tissue was quickly removed, and the biopsy sample was rinsed in physiological saline. Thereafter, the sample was divided in pieces for further analyses. Samples intended for immunoblot analyses were weighed and quickly frozen in liquid nitrogen (protein phosphorylation) or isopentane cooled on dry ice (cellular sub-fractions). Tissue to be analyzed by immunohistochemistry (IHC) was mounted in Tissue-Tek (Sakura Finetek, Torrance, CA, USA) and rapidly frozen in isopentane cooled on dry ice. All muscle samples to be analyzed by western blot and IHC were stored at -80°C for later treatment and analysis. Muscle tissue intended for RT-PCR was immersed in RNAlater® solution (Ambion, TX, USA), stored for 24-48 hours at 4 °C, and thereafter stored at -20 °C for later treatment and analysis. The samples intended for single fiber measurements were put in a tube containing skinning solution (K propionate 150 mM, Mg acetate 5 mM, Na ATP 5 mM, EGTA 5 mM and KH₂PO₄ 5 mM with protease inhibitors: E64 10 µM and leupeptine 40 µM), and stored at 4°C. The skinning solution was changed every four hours for 24 hours, before the sample was put in a tube containing 50% skinning solution and 50% glycerol. These samples were stored at -20°C until analysis (within two weeks).

3.8.1. Western Blot

Muscle samples intended for analyses on soluble proteins (50 mg) were homogenized in 1 ml T-PER (Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA), 20 µl Halt Protease and Inhibitor Cocktail (Thermo Scientific) and 20 µl EDTA (Thermo Scientific). Muscle samples intended for analyses of the cytosolic, membrane, nuclear and cytoskeletal fractions were homogenized using a subcellular extraction kit according to the supplier's instructions (ProteoExtract Subcellular Proteome Extraction Kit, Calbiochem®,

Darmstadt, Germany). The different fractions were obtained by stepwise extraction using different buffers and centrifugation. Protein concentration was measured by using a commercial kit (BioRad DC protein micro plate assay, BioRad, Hercules, CA, USA), a filter photometer (Expert 96, ASYS Hitech, Cambridge, UK) and the provided software (Kim, ver. 5.45.0.1). Protein measurements were performed in triplicates.

Equal amounts of protein were loaded in each well within a gel (5-30 µg) and separated by 4-20% gradient Mini-PROTEAN TGX Stain-Free Precast protein gels (4568093, Bio-Rad Laboratories). Electrophoresis was performed under denatured conditions for 30-45 min at 200 volts in cold Tris/Glycine/SDS running buffer (161-0732, Bio-Rad Laboratories). After gel-electrophoresis, proteins were transferred onto a PVDF-membrane at 100 volts for 60 min (Criterion™ Blotter; Tris/Glycine buffer 161-0734, Bio-Rad). Membranes were blocked at room temperature for 2 hours in a 5% fat free skimmed milk TBS solution added 0.1% Tween 20 (TBS, 170-6435, Bio-Rad; Tween-20, 437082Q, VWR International, Radnor, PA, USA; Skim milk, 1.15363.0500, Merck), and incubated overnight at 4 °C with primary antibodies (table 3.2).

Table 3.2 Antibodies used in article II and IV.

Antibody	Dilution	Cat. no	Manufacturer
LC3B (LC3-I and LC3-II)	1:1000	2775	Cell Signaling
p62 / SQSTM1	1:2000	56416	Abcam
αB-crystallin	1:4000	222	Enzo Life Sciences
Hsp70	1:4000	812	Enzo Life Sciences
P70S6K1	1:1000	2708	Cell Signaling
Phospho-P70S6K ^{Thr389}	1:1000	9234	Cell Signaling
eEF2	1:5000	2332	Cell Signaling
Phospho-eEF2 ^{Thr56}	1:5000	2331	Cell Signaling
4E-BP1	1:1000	9644	Cell Signaling
Phospho-4EBP-1 ^{Thr37/46}	1:1000	2855	Cell Signaling
Secondary anti-rabbit	1:3000	7074	Cell Signaling
Secondary anti-goat	1:30000	31430	Thermo Scientific

The next day, membranes were washed and incubated at room temperature for 1 hour with a secondary antibody. Protein bands were visualized using a HRP-detection system (Super Signal West Dura Extended Duration Substrate, 34076, Thermo Fisher Scientific, Rockford,

IL, USA), and chemiluminescence was measured using a ChemiDoc MP System (Bio-Rad Laboratories). Band intensities were calculated with Image Lab (Bio-Rad Laboratories). For some of the analyses (phospho-proteins), membranes were stripped of primary and secondary antibodies using Restore Western Blot Stripping Buffer (21059, Thermo Fisher Scientific), blocked for 2 hours at room temperature, and incubated at 4 °C overnight with antibodies against total protein. All antibodies were diluted in a 1% fat free skimmed milk TBS solution added 0.1% Tween-20. Between stages, membranes were washed in TBS added 0.1% Tween 20. All samples were analyzed in duplicates. Duplicates of a control sample was added to each gel to allow comparisons between gels.

3.8.2. Immunohistochemistry

The immunohistochemistry analyses are explained in detail in *article II*. In short, samples were cut at 8 and 10 µm thickness at –20°C using a cryostat (CM1860 UV, Leica Microsystems GmbH, Nussloch, Germany), mounted on microscope slides (SuperFrost Plus, Thermo Scientific), and air-dried before being stored at –80°C. Sections for fiber type composition (8 µm thickness) were blocked before overnight incubation at 4°C with primary antibodies against dystrophin (Ab15277, Abcam, Cambridge, UK; dil. 1:500) and myosin heavy chain I (BA-D5, DSHB, Iowa, IA, USA). After incubation and washing, sections were incubated for 60 min at room temperature with secondary antibodies (Alexa Fluor 488, A11001 and Alexa Fluor 594, A11012, Invitrogen Molecular Probes, Carlsbad, CA, USA). Subsequently, sections were washed and mounted with ProLong Gold Antifade Reagent (P36935, Invitrogen Molecular Probes) and covered with a coverslip. Sections intended for intramyocellular lipid analyses (10 µm thickness) were stained as described by Mehlem and co-workers (Mehlem et al., 2013). Briefly, sections were incubated for 10 min in an Oil Red O (ORO) solution followed by washing of excess solution in running tap water for 30 min. Subsequently, the sections were mounted with Aquatex mounting agent (108562, Merck, Darmstad, Germany) and covered with a coverslip. Sections stained with ORO were imaged within 24 hours after staining.

All muscle sections were visualized and micrographed using a high-resolution camera (DP72, Olympus Corp., Tokyo Japan) mounted on a light microscope (BX61, Olympus Corp.). For analyses with Oil Red O staining, two separate analyses were performed. First, a representative area of the stained section (> 50 fibers) were analyzed, including staining within (intramyocellular lipids; IMCL) and between (extramyocellular lipids; EMCL) fibers. This analysis is hereafter referred to as total staining intensity. Next, staining within type I and type

II fibers were measured. Range of interest (ROI) within the fibers was set to include close to the whole fiber area excluding the cell membrane. At least 40 randomly selected muscle fibers per sample was included in this analysis and related to their respective fiber type using a neighboring section (**figure 3.5**).

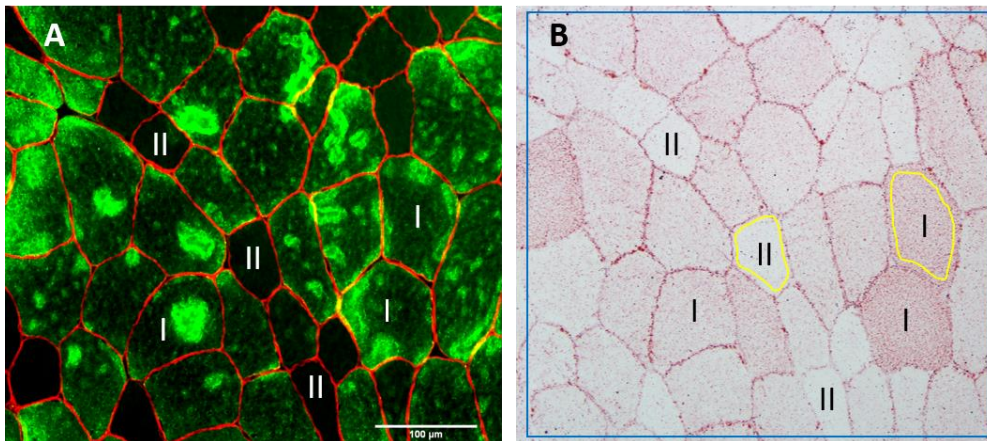


Figure 3.5 Antibody against myosin heavy-chain I was used to differentiate between type I (green) and type II fibers (no staining) (**A**). Oil Red O staining was performed on a neighbor section (**B**). Range of interest (ROI) within each fiber was set to include close to the whole fiber area excluding the cell membrane (area within yellow lines). In addition, total staining intensity was measured for the whole area, including staining within and between fibers (area within blue line).

3.8.3. Single fiber measurements

The single fiber measurements are explained in detail in *article IV*. In short, single muscle fiber segments were dissected and short segments (approximate length 1 mm) were equipped with two aluminum clips. The segments were then transferred to the set up and mounted in a drop (70 μ l) of relaxing solution between the force transducer (AME-801 SensorOne, Sausalito, California) and the electromagnetic puller (SI, Heidelberg, Germany) equipped with a displacement transducer. A movable aluminum plate hosted three drops (70 μ l volume) of relaxing, pre-activating, and activating solution, respectively. Under each solution drop, an opening through the aluminum plate made the fiber segment visible via the objective piece of the inverted microscope which was connected to a digital camera (Optikam B5, OPTIKA) to collect images at 300x magnification. A stereomicroscope (Konus Diamond, KONUS) placed above the inverted microscope allowed a careful manipulation of the fiber segments. For the functional test, fiber segments were transferred from the relaxing solution into the pre-activating solution for at least 1 min and then maximally activated by immersion in the activating solution (pCa 4.6). During maximal activation isometric force (F_0) was measured

with a quick shortening-relengthening maneuver (duration 5 ms). The cycle activation-relaxation was repeated at least three times and the average force value was used for further analysis. Cross sectional area (CSA) was calculated from the measurements of three fiber diameters, assuming a circular shape of the fiber section. Furthermore, specific force ($P_o = F_o \cdot CSA^{-1}$) was also calculated. At the end of the functional experiment, the fiber segments were removed from the set-up and immersed in 10 μ l of Laemmli solution, for subsequent determination of the composition in MyHC isoforms. To this end, protein separation was performed on 8% polyacrylamide slab gels after denaturation in SDS (SDS-PAGE) as described previously (Doria et al., 2011). Gels were silver stained and three bands were separated in the region of 200 kD, corresponding to MyHC-1, MyHC-2A and MyHC-2X, in order of migration from the fastest to the slowest.

3.8.4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from biopsies by the use of a Trizol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, each muscle sample was rinsed from RNAlater® solution in cold sterile PBS and immediately homogenized with Trizol by mean of a metal bead and Tissue Lyser apparatus (Qiagen, Venlo, Netherlands). Subsequent RNA extraction was carried out following Trizol manufacturer manual. Total RNA was resuspended in RNase/DNase free water (GIBCO) and quantified using RNA BR assay test for Qubit fluorometer (Invitrogen). 400 ng of total RNA were retrotranscribed using SuperScript IV kit (Invitrogen) according to the supplier's instructions. Complementary DNA (cDNA) was finally resuspended in 50 μ l with RNase/DNase free water and 1 μ l of diluted cDNA was used as a template for RT-qPCR analysis. qPCR analysis and Ct detection were carried out with QuantStudio5 machine (Applied Biosystems, CA, USA) using PoweUP SYBR green technology (Applied Biosystems). mRNA expression level of the following genes was detected: actin, MuRF-1, Atrogin1, MUSA1, ATG7, LC3, p62, BNIP3, BNIP3L, OPA1 and DRP1. Actin mRNA expression appear to be stable following acute resistance exercise (Mahoney et al., 2004), and was therefore chosen as house-keeping gene for the mRNA analyses. Expression level was expressed as fold change normalized to actin expression.

3.9. Statistical analyses

All statistical analyses were performed using GraphPad Prism6 software (GraphPad Software, Inc., La Jolla, CA). If not otherwise stated, all data in figures and tables are presented as mean

\pm standard deviation. To assess whether or not data sets were normally distributed, the D'Agostino-Pearson omnibus normality test was performed. Non-normally distributed data were log-transformed prior to statistical analyses. Between-group differences at baseline in the intervention studies were investigated by unpaired Student's *t*-tests, and associations between variables were investigated using Pearson's correlation coefficient. Correlations were considered weak if $r < 0.4$, moderate if $r = 0.4-0.6$, strong if $r = 0.6-0.8$, and very strong if $r = > 0.8$. Statistical significance was set at $P < 0.05$.

3.9.1. Article I

A two-way ANOVA and Sidak's multiple comparison test was used to evaluate the effect of time and group for variables measured both before and after the intervention period. Differences in response between vastus lateralis, rectus femoris and vastus intermedius were analyzed with one-way ANOVA, and Tukey's multiple comparisons test. Power calculations performed a priori indicated that 22 participants would be needed to identify a difference of 0.5 kg between the ST and CON in lean mass, with alpha set at 0.05 and beta at 0.20.

3.9.2. Article II

Statistical comparisons between groups were performed using a one-way ANOVA followed by a Tukey's multiple comparison test (absolute values). Sex differences between men and women were investigated using the Student's *t*-test.

3.9.3. Article III

Differences between fiber types for the single fiber measurements (I, I-IIA, IIA, IIA-IIX and IIX) were investigated with one-way ANOVA with Tukey's multiple comparisons test. A two-way ANOVA with Bonferroni's multiple comparison test was used to evaluate the effect of time and group for variables measured both before and after the intervention period (absolute values). Power calculations performed a priori indicated that 16 participants in each group would be needed to identify a difference of 10% between the ST and CON in specific strength, with alpha set at 0.05 and beta at 0.20.

3.9.4. Article IV

All gene expression data were log-transformed prior to statistical analyses. A two-way ANOVA with Bonferroni's multiple comparison test was used to evaluate the effect of time

and group (absolute values). In addition, paired *t*-tests were performed to investigate changes from baseline within the groups.

4. Results and discussion

This chapter will give an overview of the main findings from the four studies, and the findings will be discussed continuously. Detailed results for each specific study is presented in the manuscripts in the end of this thesis.

4.3. Relationships between muscle mass, strength and function

The thesis includes data from a total of 76 elderly individuals, covering non-frail elderly (study B), pre-frail elderly (study C and D) and frail elderly individuals (study D). Several tests were performed in all three projects. Combining results from the different studies allow for correlation analyses at baseline, providing information about the association between different parameters of muscle functioning.

The age of the elderly subjects was negatively correlated with leg lean mass for men ($r = -0.55$, $P < 0.001$), and a tendency for the same was observed in women ($r = -0.31$, $P = 0.056$; **figure 4.1 A**). Moreover, an inverse relationship was observed between age and knee extension strength, both in men ($r = -0.60$, $P < 0.001$), and women ($r = -0.49$, $P < 0.01$; **figure 4.1 B**).

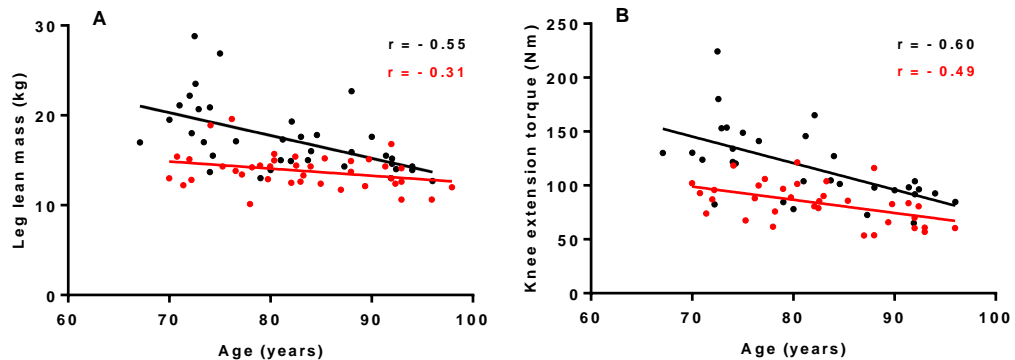


Figure 4.1 Correlation between age and leg lean mass (A), and knee extension isometric torque (B), for men (●) and women (●) included in study B, C and D.

In line with previous reports (Delmonico et al., 2009; Hughes et al., 2001), these data show that the decline in muscle strength is rapid after the age of 70. The notion that the age-related reduction in muscle mass seems to be greater in men than in women is supported by others

(Delmonico et al., 2009; Janssen, Heymsfield, Wang, & Ross, 2000). Interestingly, both our results and others show only small differences in leg lean mass between men and women after the age of 85, and minimal differences after the age of 90 (Janssen et al., 2000).

The age-related reduction in muscle mass and strength is worrying, because muscle strength is closely related to functional capacity (Hayashida, Tanimoto, Takahashi, Kusabiraki, & Tamaki, 2014). This is also evident from our results, based on the correlation between relative muscle strength and habitual gait velocity, both in men ($r = 0.62$, $P = 0.003$) and in women ($r = 0.60$, $P < 0.001$) (**figure 4.2**).

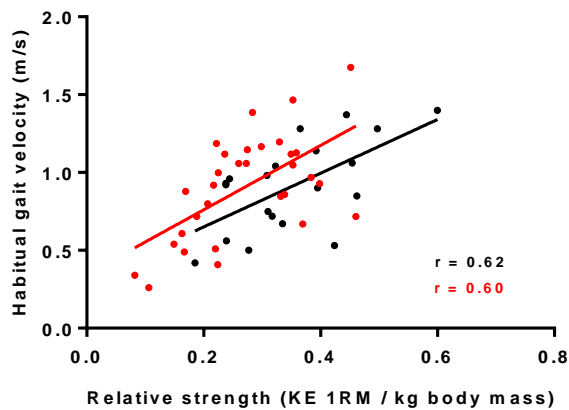


Figure 4.2 Correlation between knee extension (KE) 1RM normalized to body weight and habitual gait velocity for men (●) and women (●) included in study C and D. Values for relative 1RM strength are the mean of the right and left leg.

Gait speed predicts both institutionalization and survival in elderly individuals (Jung et al., 2018; Studenski et al., 2011), and these data therefore support the notion that maintaining a high muscle strength throughout life should be a sought-after goal (Mayer et al., 2011).

Although the correlations presented in **figure 4.1** indicate that much of the decline in muscle strength between the age of 70 and 95 is explained by loss of muscle mass, we know that other factors are involved as well. This is evident both from previous studies (Delmonico et al., 2009), and from our cross-sectional study. Results from the latter will be presented and discussed next.

4.4. Cross-sectional comparison

Combining baseline data from study A, B and D allowed for cross-sectional comparison of young, old non-frail, pre-frail and frail individuals (*article II*). From this study, it was evident that specific strength, defined as unilateral knee extension MVC torque normalized to single leg lean mass, was lower in all elderly groups compared to the young subjects. In addition, a significant difference was observed between non-frail and frail elderly individuals (**figure 4.3**).

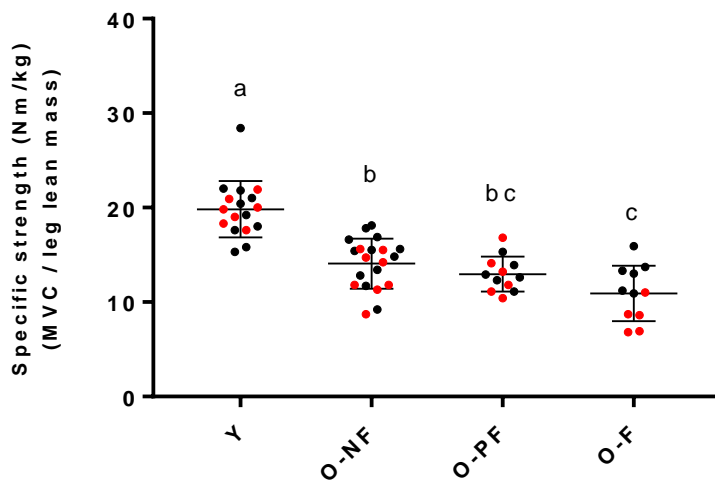


Figure 4.3 Specific strength in young (Y; 29 ± 7 yr.), old non-frail (O-NF; 74 ± 3 yr.), old pre-frail (O-PF; 84 ± 8 yr.) and old frail (O-F; 88 ± 6 yr.) men (●) and women (●). Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

As discussed in the introduction of this thesis, the lower specific strength is likely a result of multiple factors, and may include reductions in activation level (Billot et al., 2014), increased fat infiltration (Goodpaster, Carlson, et al., 2001), and reduced single fiber specific tension (Frontera et al., 2000). However, the young and non-frail elderly subjects were part of previously conducted studies where neither of these measures were included.

Fiber area was measured in a subset of subjects in each group, and in line with previous reports (Verdijk et al., 2012; Verdijk et al., 2014), our results support specific atrophy of type II fibers, although also type I fibers tended to be smaller in the older groups (**figure 4.4**).

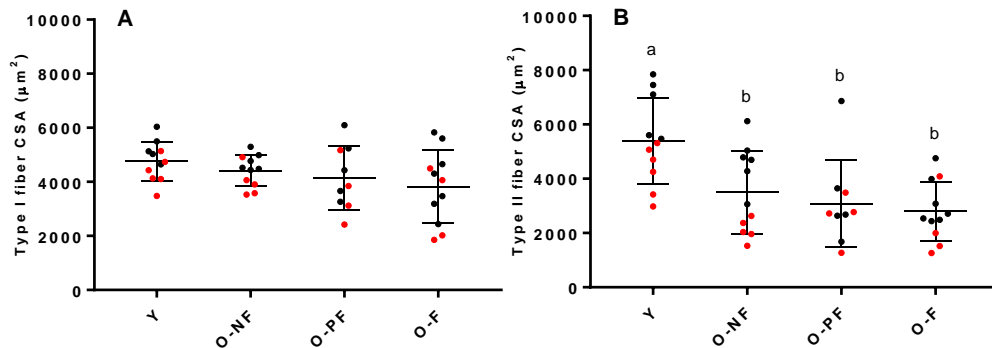


Figure 4.4 Type I (A) and type II (B) muscle fiber area in young (Y), old non-frail (O-NF), pre-frail (O-PF) and frail (O-F) men (●) and women (●). Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

The proportion of type I fibers was not different in Y ($46 \pm 14\%$), O-NF ($47 \pm 18\%$), O-PF ($46 \pm 19\%$) and O-F ($48 \pm 13\%$) individuals, and it was therefore expected that the percentage area occupied by type I muscle fibers would be higher in the older subjects. Although no significant differences between groups were observed, we did see a trend for this, as the percentage area covered by type I fibers was 54-55% in the older groups, compared to 44% in the young group. Thus, because specific tension is higher in type II fibers (Trappe et al., 2003), it is likely that the predominant atrophy of type II fibers contributed to the between-group difference observed for specific strength. Nevertheless, the main aim of the cross-sectional study was to test the hypothesis that autophagy is attenuated in frail elderly individuals, and that markers of autophagy correlate with specific strength. Moreover, our objective was to investigate if the level of heat shock proteins Hsp70 and αB -crystallin were different between groups.

4.4.1. Impact of age and frailty on autophagy markers

The most commonly used markers to assess autophagic activity is LC3-I, LC3-II, and p62. An increased ratio of LC3-II/LC3-I is considered to reflect increased autophagosome formation, because the lipidation of LC3-I to LC3-II is crucial for phagophore elongation (Mizushima & Yoshimori, 2007). Moreover, the level of LC3-II has been observed to reflect the abundance of autophagosomes (Kabeya et al., 2000), and is therefore often reported independent of LC3-I. Because it is unclear whether LC3-I is converted to LC3-II prior to, or after its binding to the phagophore membrane, LC3-I was measured both in the cytosolic and membrane fraction

in the present study. Also p62 was measured both in the cytosolic and membrane fraction, since p62 first targets polyubiquitinated organelles and proteins, and thereafter recruit these substrates to the phagophore membrane where p62 interacts with LC3-II (Bitto et al., 2014). Increased cytosolic p62 levels are interpreted as reduced autophagic flux, because cytosolic p62 accumulates when autophagy is suppressed (Kuma, Komatsu, & Mizushima, 2017).

In the cross-sectional study, no between-group differences were observed neither for cytosolic nor membrane-bound p62. However, old non-frail, pre-frail and frail subjects displayed 48, 63 and 100% higher levels of cytosolic LC3-I compared to young individuals, respectively (**figure 4.5 A**), although only the difference between Y and O-F reached statistical significance ($P < 0.01$). Moreover, the level of LC3-II was 43, 51, and 63% higher in O-NF, O-PF and O-F, compared with Y, but the difference did not reach statistical significance for any of the groups (**figure 4.5 B**). However, if all subjects from the old groups were pooled, the difference between young and old participants was statistically significant for LC3-II ($P = 0.021$). In addition to separate analyses of LC3-I and LC3-II, the ratio between the two is commonly calculated as a measure of autophagosome formation. The ratio, calculated as $(LC3-II_{\text{membrane}}) / (LC3-I_{\text{cytosol}} + LC3-I_{\text{membrane}})$ in the present investigation, was not different between Y (1.90 ± 0.69), O-NF (1.76 ± 0.91), O-PF (2.29 ± 1.46) and O-F (1.80 ± 1.47).

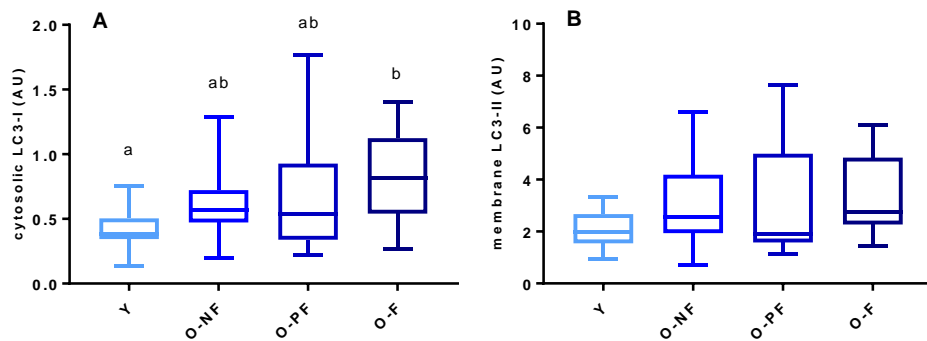


Figure 4.5 Cytosolic LC3-I (**A**) and membrane LC3-II (**B**) young (Y), old non-frail (O-NF), pre-frail (O-PF), and frail (O-F) subjects. Data are presented as box plots with median, upper and lower quartile and lowest and highest value. Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

The consensus that autophagy declines with aging stems primarily from studies performed in rodents (Kim et al., 2013; Wohlgemuth et al., 2010; Zhou et al., 2017). In addition to changes in LC3-I and LC3-II, an age-related decrease in LAMP-2A protein expression was observed in two of these studies (Kim et al., 2013; Zhou et al., 2017). LAMP-2A is involved in the

autophagosome-lysosome fusion (Hubert et al., 2016), but whether or not this process is affected by aging in human skeletal muscle is currently unknown. Interestingly, and in line with our findings, a trend for higher levels of both LC3-I and LC3-II was recently observed in both untrained and trained elderly (mean age: 62 years), compared to young subjects (Dethlefsen et al., 2018). It is important to acknowledge that the possible increase in autophagosome number with aging, as suggested by increased LC3-II levels in the present study and the study by Dethlefsen and colleagues (2018), does not equal increased autophagic flux. The functionality and effectiveness of autophagy also directly depends on the capacity of the lysosome to degrade the delivered cargo (Klionsky et al., 2012). Hence, increased levels of both LC3-I and LC3-II would also be expected in a situation where autophagosome formation is blunted, but where the fusion of autophagosomes and lysosomes is blunted even more. Interestingly, Potes and colleagues (2017) observed higher levels of p62 in overweight compared to normal-weight elderly (Potes et al., 2017). Accumulation of p62 was accompanied by increased levels of both LC3-I and LC3-II, together with decreased gene expression of LAMP-2A. Their findings imply that several of the autophagic steps may be affected in overweight elderly individuals. Our findings expand on these observations, by suggesting that autophagy is also altered in frail, normal-weight elderly individuals. However, because cytosolic p62 was not different between groups in our study, we cannot exclude the possibility that higher levels of LC3-I and LC3-II in fact reflected increased autophagy.

4.4.2. Impact of age and frailty on heat shock proteins

A possible impairment in autophagy may lead to accumulation of dysfunctional proteins and protein aggregates. Since heat shock proteins have the ability to repair and refold denatured and misfolded proteins, their abundance is of interest, and may provide information about the degree of cellular stress. Therefore, analyses of α B-crystallin and Hsp70 were performed in the cross-sectional study. If all subjects from the old groups were pooled, the cytosolic Hsp70 was higher in old compared to young subjects ($P < 0.01$). In addition, higher levels of cytoskeletal Hsp70 were observed in old pre-frail, compared to young and old non-frail subjects. The level of cytosolic α B-crystallin was higher in both pre-frail and frail elderly, compared to the young group (**figure 4.6**).

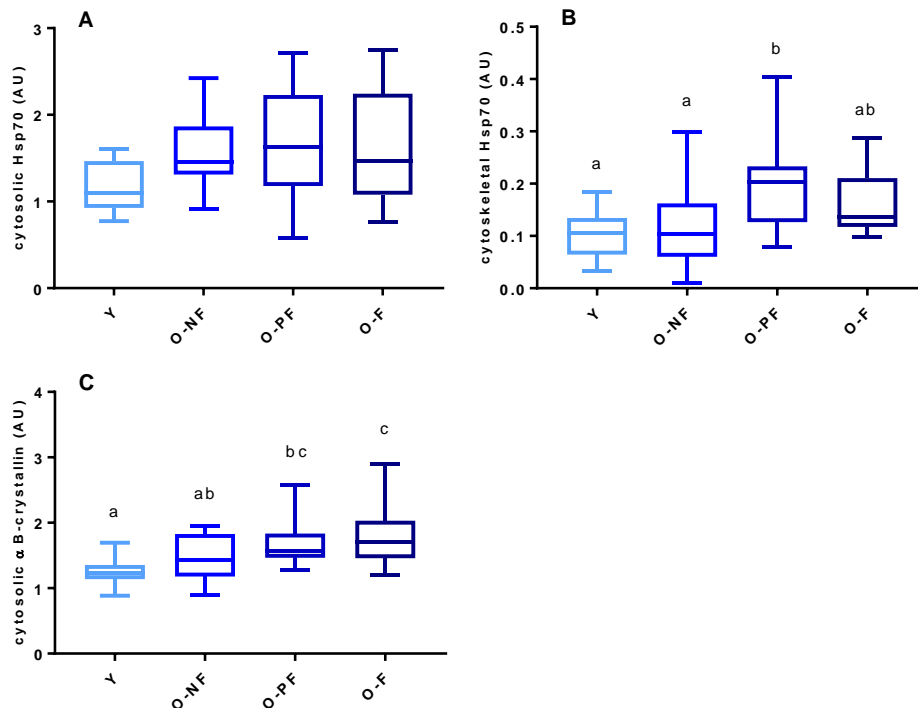


Figure 4.6 Hsp70 in the cytosolic (A) and cytoskeletal fraction (B), and cytosolic α B-crystallin (C) in young (Y), old non-frail (O-NF), pre-frail (O-PF) and frail (O-F) subjects. Data are presented as box plots with median, upper and lower quartile and lowest and highest value. Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

An age-related increase in α B-crystallin has been observed previously, both in rodent (Doran, Gannon, O'Connell, & Ohlendieck, 2007) and human skeletal muscle (Yamaguchi et al., 2007). In contrast, Beltran Valls and colleagues did not observe differences in α B-crystallin protein expression between young, old, and old sarcopenic subjects, neither in the sarcoplasmic nor myofibrillar fraction (Beltran Valls et al., 2015). However, a trend for higher levels of Hsp70 in the old sarcopenic subjects were observed (Beltran Valls et al., 2015). Furthermore, Joseph and colleagues (2012) observed higher levels of Hsp70 in low-functioning compared to high-functioning elderly individuals (Joseph et al., 2012), suggesting that physical function might be more important than aging per se. The latter might explain why higher levels of cytoskeletal Hsp70 and cytosolic α B-crystallin were observed in pre-frail and frail, but not non-frail, elderly individuals. The up-regulation of certain heat shock proteins might be an auto-protective mechanism against increased levels of misfolded proteins, possibly due to inefficient

degradation. One possible interpretation of the results for the autophagic markers and heat shock proteins is presented in **figure 4.7**.

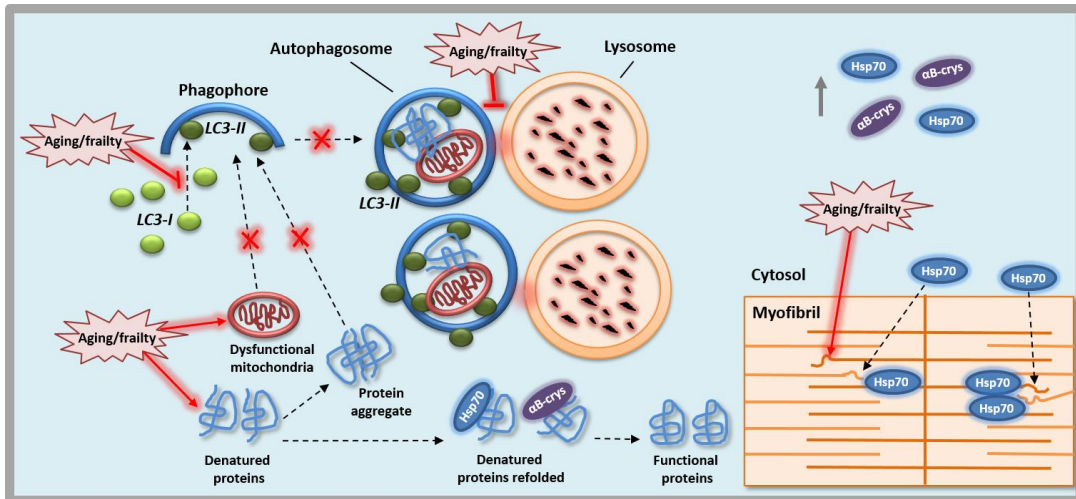


Figure 4.7 Reduced lipidation of LC3-I to LC3-II may impede phagophore elongation and thus autophagosome formation, and lead to higher levels of LC3-I. The increase in membrane LC3-II may be due to a simultaneous but stronger suppression of autophagosome-lysosome fusion, leading to accumulation of autophagosomes. The increase in cytosolic α B-crystallin and Hsp70, as well as cytoskeletal Hsp70, may represent a compensatory mechanism against increased levels of denatured and dysfunctional proteins.

An interesting observation both from the results for the autophagic markers (**figure 4.5**) and the heat shock proteins (**figure 4.6**), is that the within-group variation was much higher in the elderly subjects, compared to the young subjects. Hence, the impact of aging and frailty on the systems of protein quality control does not seem to be a uniform one, and it is possible that autophagy is increased in some individuals and suppressed in others. Another possible explanation is that there are between-subject differences in which step of the autophagic process that is affected. In conclusion, our results suggest that aging is accompanied by altered regulation of autophagy. The results for p62, LC3-I and LC3-II are consistent with both an increase and decrease in autophagic activity. However, we propose that the simultaneous increase in LC3-I and LC3-II reflect suppressed formation of autophagosomes, accompanied by an even greater suppression of autophagosome-lysosome fusion.

4.4.3. Autophagy and specific strength

Among the elderly subjects, specific strength was negatively correlated with cytosolic LC3-I ($r = -0.34$, $P = 0.028$) and positively correlated with the ratio of membrane-bound to cytosolic p62 ($r = 0.41$, $P = 0.006$). Although speculative, the membrane-to-cytosolic ratio of p62 might represent the rate of substrate delivery to the maturing phagophore, whereas increased levels of LC3-I might represent attenuated lipidation to LC3-II, and thus attenuated autophagosome formation. Accordingly, both correlations indicate a positive relationship between autophagy and specific strength.

4.4.4. Differences between men and women

Women have a greater life expectancy than men (Johansson, 1989), but face an increased probability of encountering functional disability (Janssen, Heymsfield, & Ross, 2002), a greater likelihood of experiencing frailty (Mitnitski et al., 2005), and a greater risk of developing metabolic disease (Ravaglia et al., 2006). Yet, many gaps exist in the literature regarding underlying causes. Therefore, we pooled the three elderly groups in the cross-sectional study to investigate differences between elderly men and women. This comparison represented 26 men (80 ± 8 yr.) and 19 women (82 ± 9 yr.). Interestingly, specific strength was lower in women (11.7 ± 2.9 Nm/kg) compared to men (13.9 ± 2.9 Nm/kg; $P = 0.01$; **figure 4.8 A**). In addition, the level of membrane LC3-II was lower ($P = 0.049$), whereas the level of LC3-I tended to be higher ($P = 0.076$), in women. Thus, also the LC3-II/LC3-I ratio was lower in women ($P = 0.033$; **figure 4.8 B**), indicative of reduced autophagic flux.

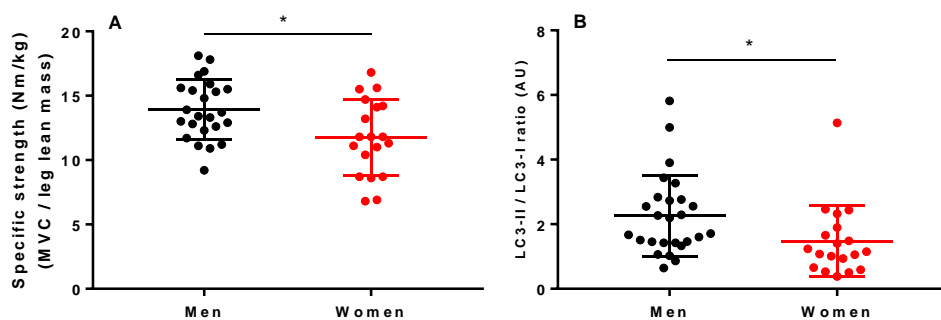


Figure 4.8 Specific strength (A) and LC3-II/LC3-I ratio (B) in elderly men (●) and women (●).

In addition to the differences observed for the autophagic markers, the level of cytosolic Hsp70 was 22% higher in women than in men ($P = 0.041$), and a similar tendency was

observed in the cytoskeletal fraction ($P = 0.074$). No sex differences were observed for proteins involved in the regulation of muscle protein synthesis. Potential explanations for the difference in specific strength will be discussed next. Here, also some baseline data from *article III* will be included. It is interesting to speculate that that the difference in autophagy between men and women represents a suppression of protein quality control in women, and that this contributes to the difference observed in specific strength. For example, it could be hypothesized that attenuated lipophagy in women results in accumulation of intramyocellular lipids, negatively affecting the fibers force-generating capacity. Indeed, both we (*article III*) and others have observed an inverse relationship between IMCL content and single fiber specific tension (Choi et al., 2016). However, if this was the underlying mechanism, differences between men and women should be evident with respect to both single fiber specific tension and IMCL content. A previous study did in fact observe lower specific tension in type I fibers of elderly women compared to elderly men (Miller et al., 2017). However, looking at the baseline data in study D, single fiber specific tension was not different between men (92 ± 26 mN/mm²) and women (96 ± 21 mN/mm², $P = 0.15$). Moreover, IMCL content was not different, neither in type I (men: 48 ± 8 AU, women: 45 ± 8 AU, $P = 0.42$), nor in type II fibers (men: 44 ± 8 AU, women: 39 ± 7 AU, $P = 0.13$). If anything, IMCL content was higher in men. Thus, at least in pre-frail and frail elderly, decreased lipophagy and thereby increased IMCL levels does not appear to explain the difference between men and women in specific strength.

The baseline data in study D revealed that also muscle density was similar in women (44.8 ± 5.9 HU) and men (men: 45.2 ± 4.4 HU). In contrast, although not significant ($P = 0.15$), mean voluntary activation level was 3.6% lower in women ($85.2 \pm 6.4\%$) compared to men ($88.8 \pm 5.0\%$). Because this comparison represented a relatively low number of subjects (men = 12, women = 13), we cannot exclude the possibility that a difference in activation level exist between men and women, and that this is part of the explanation for the sex-specific difference we observe in specific strength.

4.5. Effects of strength training in pre-frail and frail elderly

4.5.1. Muscle mass, muscle strength and functional capacity

Results from the two intervention studies (pilot and main intervention study) will be presented and discussed next. The results from the pilot study are summarized in **table 4.1**. In short, the

pilot study demonstrated that a weekly training volume of only ninety minutes (3 x 30 min) in combination with protein supplementation, increased leg lean mass, as well as muscle thickness of vastus lateralis, rectus femoris, and vastus intermedius in pre-frail elderly individuals. In relative terms, these three muscles contributed similarly to the knee extensor hypertrophy. The gains in muscle mass were accompanied by increased strength and improved functional performance. No changes were observed in CON.

Table 4.1 Body composition, muscle thickness, muscle strength and functional performance before and after the ten-week intervention in ST (strength training three times per week + protein supplementation) and CON (no training or supplementation) in study C.

	ST			CON			ANOVA <i>P</i> -value
	Pre (kg)	Post (kg)	% change	Pre (kg)	Post (kg)	% change	
Body mass (kg)	73.6 ± 12.7	74.3 ± 12.9	1 ± 2	71.5 ± 9.5	72.7 ± 10.4	2 ± 2	0.54
Leg lean mass (kg)	15.0 ± 2.3	15.7 ± 2.4	4 ± 2	14.2 ± 1.5	14.3 ± 1.7	1 ± 3	<0.01
VL thickness (mm)	16.5 ± 3.9	17.2 ± 4.2	4 ± 3	15.4 ± 4.6	15.5 ± 4.9	0 ± 1	<0.01
RF thickness (mm)	18.1 ± 2.8	19.2 ± 2.4	7 ± 5	16.3 ± 4.3	15.8 ± 3.9	2 ± 3	<0.01
VI thickness (mm)	11.8 ± 3.9	12.5 ± 4.0	6 ± 6	11.1 ± 3.8	10.6 ± 3.6	0 ± 1	<0.01
KE 1RM (kg)	28.2 ± 8.4	34.0 ± 8.6	23 ± 15	23.8 ± 5.2	24.3 ± 4.2	3 ± 7	<0.01
KE MVC 90° (Nm)	98 ± 28	115 ± 24	20 ± 11	81 ± 14	80 ± 12	-1 ± 7	<0.01
Gait velocity (m/s)	1.16 ± 0.21	1.23 ± 0.16	7 ± 10	1.17 ± 0.22	1.11 ± 0.31	-6 ± 13	0.03
Chair rise x 5 (s)	9.7 ± 3.1	8.5 ± 1.9	-10 ± 13	9.9 ± 5.5	9.5 ± 3.2	1 ± 17	0.13
Stair climbing (s)	11.9 ± 3.1	10.6 ± 2.9	-11 ± 12	11.9 ± 3.4	13.4 ± 7.4	8 ± 24	0.02

VL: vastus lateralis, RF: rectus femoris, VI: vastus intermedius, KE: knee extension, 1RM: one repetition maximum, MVC: maximal voluntary contraction. Knee extension 1RM and MVC show the mean of the right and left leg. Data are presented as mean ± SD. ANOVA *p*-value show group x time interaction.

Importantly, all subjects randomized to the training group completed the ten-week training intervention, suggesting that heavy load strength training is feasible in this population. Although quadriceps femoris muscle CSA was not measured, it has been shown previously that a 7.5% increase in vastus lateralis thickness corresponded to a 5.2% increase in cross-sectional area (CSA) (Franchi et al., 2018). Consequently, the improvements in knee extension strength seem to have exceeded the increase in cross-sectional area of the knee extensors, reflecting an increase in specific strength.

The results from the main intervention study (study D) will be presented and discussed next. Because the subjects recruited for this study had a lower functional capacity compared to study C, and because previous reports have demonstrated substantial gains in strength from only two weekly training sessions in this population (Cadore et al., 2014; Hvid et al., 2016), two instead of three weekly training sessions were conducted. The aim of this study was to investigate the

effect of strength training on a broad number of factors related to specific strength in frail elderly.

Thirty-four pre-frail and frail elderly individuals were included in study D. Not all participants performed all tests, hence the number of subjects varies for the different measurements (for details, see *article III*). Very strong correlations were observed between quadriceps femoris muscle CSA and both knee extension 1RM and MVC at baseline, suggesting that most of the between-subject variation in muscle strength was in fact explained by differences in muscle size (**table 4.2**). This contrast a previous study (Goodpaster, Carlson, et al., 2001), where muscle CSA only explained ~25% of the variance in muscle strength in healthy 70-year-olds. Nevertheless, muscle activation and muscle density also explained some of the variation in both absolute and specific strength (**table 4.2**).

Table 4.2 Correlations between knee extensor strength and various measures of muscle size and composition at baseline in study D.

Measure	Absolute strength		Specific strength	
	KE 1RM (kg)	KE MVC (Nm)	KE 1RM / QF CSA	KE MVC / QF CSA
QF CSA (cm ²)	.87* (33)	.89* (30)	-	-
QF muscle activation (%)	.34 (22)	.53* (22)	.22 (21)	.54* (21)
QF IMAT (cm ²)	.03 (33)	-.01 (30)	-.04 (33)	-.14 (30)
QF muscle density (HU)	.43* (33)	.40* (30)	.30 (33)	.19 (30)
VL total ORO staining intensity (AU)	.38 (24)	.39 (23)	.08 (24)	.08 (23)
VL single fiber specific tension (mN·mm ⁻²)	-.09 (18)	-.19 (17)	.07 (18)	.02 (17)

QF CSA: Quadriceps femoris cross-sectional area, IMAT: intermuscular adipose tissue, HU: Hounsfield units, VL: vastus lateralis, ORO: Oil red O, AU: arbitrary units, KE: knee extension, 1RM: one repetition maximum, MVC: isometric maximal voluntary contraction. Numbers in parenthesis denote the number of XY pairs for the given correlation. * $P < 0.05$.

There were no significant effects of the intervention on body mass, total lean mass or fat mass. Surprisingly, changes in leg lean mass only showed a tendency for a group x time interaction ($P = 0.076$) (**table 4.3**). Knee extension 1RM improved from baseline in ST ($P < 0.001$), but the interaction with CON did not reach statistical significance ($P = 0.067$). In addition, isometric MVC improved from baseline in ST ($P = 0.025$), but again only a tendency for a group x time interaction was observed ($P = 0.088$). No group x time interactions were observed for specific strength (MVC / quadriceps femoris muscle CSA). The change in five times chair-rise showed only a tendency for a group x time interaction ($P = 0.08$), whereas significant interaction effects were observed for both habitual ($P < 0.01$) and maximal gait velocity ($P = 0.015$; **table 4.3**).

Table 4.3 Body composition, strength, specific strength and functional performance before and after the ten-week intervention in ST (strength training twice weekly + protein supplementation) and CON (only protein supplementation) in study D.

	ST			CON			ANOVA
	Pre (kg)	Post (kg)	% change	Pre (kg)	Post (kg)	% change	P-value
Body mass (kg)	68.3 ± 12.5	69.3 ± 13.0	1 ± 2	74.2 ± 11.7	74.2 ± 11.6	0 ± 4	0.27
Leg lean mass (kg)	14.6 ± 3.0	14.9 ± 3.1	2 ± 4	15.3 ± 3.9	15.1 ± 3.7	0 ± 1	0.08
KE 1RM (kg)	20.2 ± 8.9	23.1 ± 9.1	17 ± 16***	22.0 ± 8.9	22.5 ± 8.3	4 ± 13	0.07
KE MVC 70° (Nm)	102 ± 31	108 ± 33	7 ± 10*	115 ± 34	115 ± 35	0 ± 6	0.09
SS (MVC / CSA)	2.43 ± 0.55	2.39 ± 0.57	-1 ± 9	2.51 ± 0.28	2.54 ± 0.36	1 ± 7	0.42
Habitual gait (m/s)	0.69 ± 0.24	0.75 ± 0.25	12 ± 27	0.82 ± 0.25	0.74 ± 0.25	-10 ± 10**	<0.01
Maximal gait (m/s)	1.14 ± 0.49	1.21 ± 0.51	10 ± 28	1.23 ± 0.41	1.13 ± 0.39	-7 ± 8*	0.02
Chair rise x 5 (s)	19.6 ± 19.5	14.9 ± 8.8	-13 ± 20*	13.0 ± 4.5	12.8 ± 3.9	1 ± 19	0.08

KE: knee extension, 1RM: one repetition maximum, MVC: maximal voluntary contraction, SS: specific strength, CSA: quadriceps femoris cross-sectional area. Knee extension 1RM and MVC show the mean of the right and left leg. Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ different from baseline within group. ANOVA p-value show group x time interaction.

It is clear that the overall improvements were greater in study C (**table 4.1**) compared to study D (**table 4.3**). For example, leg lean mass increased by 4% in the pilot study, whereas a 2% non-significant increase was observed in the main training intervention. Moreover, knee extension 1RM changed by 23 and 17%, in study C and D, respectively. Although changes in isometric strength in the two studies should be compared with caution (different machines and tested at 90° and 70°), the improvement in the pilot study of 20% greatly exceeded the 7% improvement in the main intervention. Because the training sessions were very similar in the two studies, it is likely that the differences in training effects were mainly due to the differences in training frequency (2 vs. 3 sessions per week). In addition, subjects in study C received twice protein supplements each day, compared to one in study D. A more in depth discussion around determinants of training adaptation in the two studies will be given later (chapter 4.7). Next, results for activation level, muscle density, lipid content and single fiber specific tension will be presented and discussed.

4.5.2. Muscle activation

Ten (ST) and nine (CON) subjects performed the interpolated twitch technique both before and after the intervention. Muscle activation did not change from baseline in ST (from 89.7 ± 4.7 to 86.7 ± 5.7%) or CON (from 87.9 ± 5.1 to 89.0 ± 6.1%), and no group x time interaction was observed (**figure 4.9**).

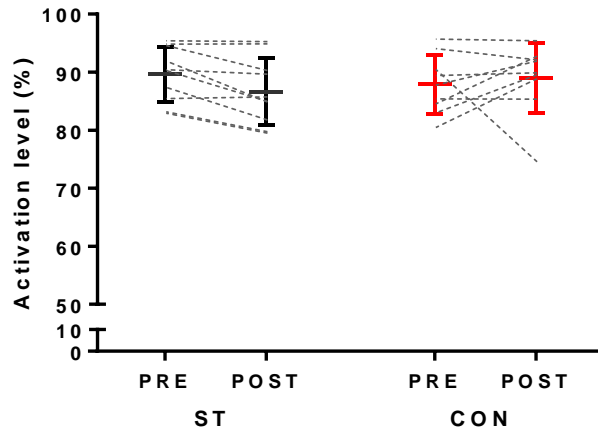


Figure 4.9 Voluntary activation level during an isometric knee extension MVC, before and after the intervention period in ST (strength training twice weekly + protein supplementation) and CON (only protein supplementation). The figure displays individual values, as well as mean \pm SD before and after the intervention.

A correlation between activation level and specific strength was observed at baseline (**table 4.2**), suggesting that some of the between-subject variation in specific strength was indeed due to differences in voluntary activation level. Contrary to our hypothesis, activation level did not improve following the ten-week training intervention. To our knowledge, only two previous studies have investigated the effect of heavy load strength training on knee extensor activation level in pre-frail or frail elderly individuals. Hvid and colleagues (2016) observed an improvement in activation level from 79 to 85% following 12 weeks of heavy load power training performed twice weekly in pre-frail elderly (Hvid et al., 2016), whereas Harridge and colleagues (1999) observed a non-significant improvement from 81 to 85% after 12 weeks of heavy load strength training performed three times per week in very old frail individuals. The most noticeable difference between these studies and ours is the difference in activation level at baseline, as the subjects in our training group displayed an activation level of 90%. Accordingly, the potential for improvements is reduced (Arnold & Bautmans, 2014). It is difficult to unravel why our subjects displayed a much higher activation level at baseline, but it is interesting to note that several of the subjects in the previous two reports displayed activation levels between 50 and 70%. In our experience, such low activation levels are only observed in subjects experiencing pain during the contraction (e.g. from knee arthritis). Indeed, one subject was excluded from the results in the present investigation due to knee pain during the test, and this subject had an activation level of 62%. In summary, our results suggest that the voluntary

activation level of quadriceps femoris is close to 90% even in frail elderly individuals. Moreover, changes in activation level did not contribute to strength gains following a ten-week intervention with two weekly strength training sessions.

4.5.3. Muscle cross-sectional area, density and intermuscular adipose tissue

Pre and post data represent fifteen (ST) and thirteen (CON) subjects for the CT analyses. The area of IMAT in quadriceps femoris was small at baseline, and did not change in ST (from 1.3 ± 1.1 to 1.5 ± 1.1 cm²) or CON (from 1.6 ± 0.9 to 1.7 ± 1.0 cm²). In contrast to a previous study (Yoshida et al., 2012), IMAT area was not correlated with activation level ($r = -0.28$, $P = 0.21$). Moreover, IMAT did not correlate with absolute strength or specific strength (**table 4.2**), nor functional performance (results not shown). In contrast to the study by Marcus and colleagues (2013), the area of IMAT at baseline did not predict training-induced changes in specific strength. Thus, our results suggest a limited negative impact of IMAT on quadriceps femoris function in frail elderly individuals. Although not analyzed in the present investigation, it is apparent from **figure 3.3 A** (page 27 in *Methods*) that IMAT constituted a much larger area in the posterior part of the thigh. How IMAT impact hamstring strength in this population is therefore interesting and warrants further investigation.

There was a significant group x time interaction between ST ($6.8 \pm 7.4\%$) and CON ($1.2 \pm 5.1\%$) for the change in quadriceps femoris muscle CSA ($P = 0.002$) (**figure 4.10 A**, next page). The increase in muscle CSA was exclusively attributable to an increase in area of normal-density muscle ($10.1 \pm 9.0\%$), which did not change in CON (-1.5 ± 4.9) (group x time interaction, $P < 0.001$) (**figure 4.10 B**). In contrast, low-density muscle did not change in either ST (-1.4 ± 17.1) or CON (-1.5 ± 10.2) (**figure 4.10 C**). Hence, a group x time interaction was observed for muscle density (measured as mean HU), with changes of $1.5 (\pm 1.6)$ and $-0.2 (\pm 0.9)$ HU, in ST and CON, respectively ($P < 0.001$; **figure 4.10 D**).

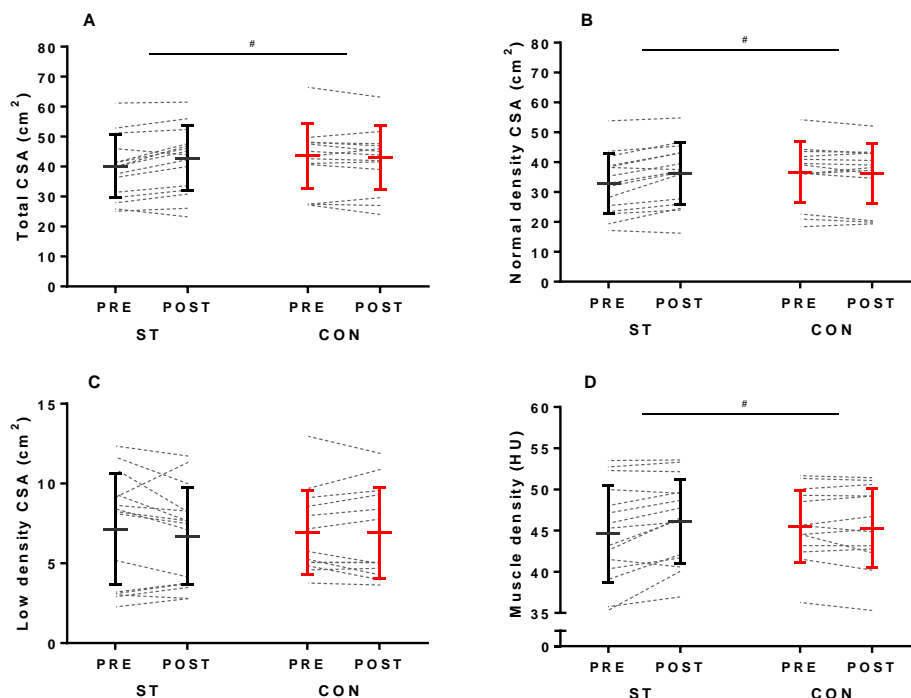


Figure 4.10 Quadriceps femoris muscle total cross-sectional area (A), normal-density muscle area (B), low-density muscle area (C), and muscle density expressed as mean HU (D) in ST (strength training twice weekly + protein supplementation) and CON (only protein supplementation). The figure displays individual values, as well as mean \pm SD before and after the intervention. # $P < 0.05$ (group x time interaction).

As covered in the introduction of this thesis, aging is associated with reduced mid-thigh density values, and a negative correlation has been observed between muscle density and specific strength of quadriceps femoris (Goodpaster, Carlson, et al., 2001). Because IMAT is excluded from the calculation of muscle density, lower values are considered to reflect increased amounts of lipid droplets within (IMCL), and between (EMCL) muscle fibers (Aubrey et al., 2014; Goodpaster, Kelley, et al., 2000). Studies investigating the effect of resistance training on muscle density in elderly individuals report either increases in muscle density (Cadore et al., 2014; Taaffe et al., 2009), or no change (Goodpaster et al., 2008; Santanasto et al., 2011). However, because muscle biopsies were not obtained in these studies, we do not know if the changes in muscle density were in fact due to changes in IMCL content, as proposed by the authors (Cadore et al., 2014; Taaffe et al., 2009). Therefore, histological sections from vastus lateralis biopsies were analyzed by Oil Red O in the present investigation.

4.5.4. Intramyocellular and extramyocellular lipids

For pre and post data twelve (ST) and eight (CON) subjects are included in the analyses of intra- and extramyocellular lipids. Total staining intensity, thought to reflect both IMCL and EMCL, showed correlations with BMI ($r = 0.75$, $P = 0.001$) and body fat percentage ($r = 0.51$, $P = 0.011$) at baseline. However, total staining intensity did not correlate with quadriceps femoris muscle density ($r = 0.01$, $P = 0.95$), or IMAT ($r = 0.32$, $P = 0.13$) assessed by CT. Total staining intensity did not change in ST (from 49.6 ± 7.8 to 51.3 ± 10.2 AU) or CON (from 51.0 ± 5.8 to 49.8 ± 8.3 AU) following the intervention (group x time interaction, $P = 0.38$; **figure 4.11**).

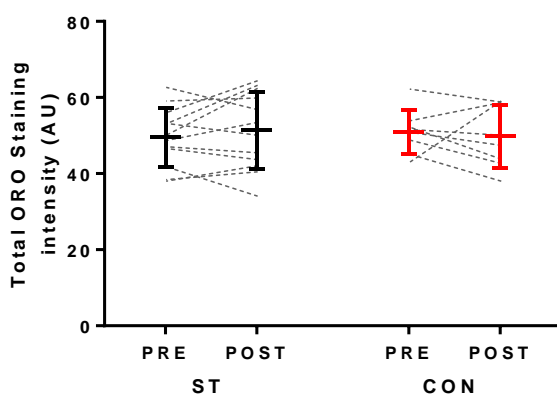


Figure 4.11 Total Oil Red O (ORO) staining intensity in ST (strength training twice weekly + protein supplementation) and CON (only protein supplementation). An area including at least 50 fibers were analyzed per biopsy, including staining within (IMCL) and between (EMCL) muscle fibers. The figure displays individual values, as well as mean \pm SD before and after the intervention.

Staining intensity within the cell membrane of type I fibers, considered to reflect IMCL content, did not change in ST (from 49.6 ± 9.2 to 52.0 ± 11.1 AU) or CON (49.8 ± 5.1 to 48.2 ± 9.9 AU) (group x time interaction, $P = 0.447$). Moreover, no group x time interaction was observed for staining within type II fibers, with no changes in ST (from 43.8 ± 5.2 to 45.9 ± 9.3 AU) or CON (from 47.8 ± 6.9 to 44.5 ± 8.0 AU) (group x time interaction, $P = 0.143$). If the two groups were pooled at baseline, IMCL content was higher in type I compared to type II fibers ($P = 0.026$).

The lack of correlations between muscle density and skeletal muscle lipid content at baseline was surprising in light of a previous study observing a correlation between the two (Goodpaster, Kelley, et al., 2000). However, Goodpaster investigated a very heterogeneous group of young individuals (BMI between 18.5 and 35.6), and this could partly explain the discrepancy. Interestingly, we observed increased muscle density in absence of changes in IMCL and EMCL,

and our results therefore suggest that the training-induced increase in muscle density was not due to changes in lipid content.

It is important to acknowledge that increased muscle density does not necessarily require qualitative changes at the single fiber level, or changes in lipid content *per se*. Because of the somewhat limited resolution of CT (0.78 x 0.78 mm in our study), depots of extracellular adipose tissue smaller than the pixel's resolution would not necessarily be categorized as adipose tissue (Goodpaster, Kelley, et al., 2000), but could instead contribute to low-density muscle area. Moreover, in the boundary between muscle tissue and larger areas of IMAT, it might be that some pixels are categorized as low-density muscle if the pixels contain most muscle, but also a small portion of adipose tissue. In support of this, we did observe a positive correlation between IMAT and low-density muscle ($r = 0.46$, $P = 0.007$) at baseline. Along this line, muscle density would be expected to increase following muscle hypertrophy, even when IMAT and IMCL content does not change, and the latter could explain why we observed an increase in normal-density muscle, without changes in low-density muscle. It is also important to be aware that lipid staining by Oil Red O on histological muscle sections is prone to much variation (Watt, Heigenhauser, & Spriet, 2002), hence this method might not be sensitive enough to detect small changes in IMCL content.

Both from our results and previous observations (Gueugneau et al., 2015; He, Goodpaster, & Kelley, 2004; St-Jean-Pelletier et al., 2016), it is evident that lipid content is higher in type I compared to type II fibers. Because training-induced hypertrophy mainly occurs in type II fibers (Kryger & Andersen, 2007; Leenders et al., 2013), an increase in the percentage area covered by fast-twitch fibers would also be expected to contribute to the increase in muscle density. Interestingly, the latter could also contribute to the age-related reduction in muscle density (Goodpaster, Carlson, et al., 2001), considering that age-related atrophy primarily affect type II-fibers (Verdijk et al., 2014). In fact, evidence that the level of IMCL is increased with aging is scarce. IMCL content appear to be more closely associated with overweight and obesity, than aging *per se* (Goodpaster, He, et al., 2001; Goodpaster, Theriault, et al., 2000; St-Jean-Pelletier et al., 2016). The correlations we observed between skeletal muscle lipid content and both BMI and fat percentage support this notion.

4.5.5. Single fiber properties

To investigate differences between fiber types at baseline, all fibers from twenty-two baseline biopsies were pooled. Type IIA and IIX fibers were 15 ($P = 0.082$) and 33 % ($P = 0.012$) smaller

than type I fibers. Specific tension was 44% ($P = 0.001$) higher in type IIX, compared to type I fibers. The difference of 17 % between type I and IIA fibers did not reach statistical significance ($P = 0.108$). Our findings are therefore in line with previous reports of higher specific tension in fast-twitch fibers (Frontera et al., 2000; Ochala et al., 2007; Trappe et al., 2003; Venturelli et al., 2015). Interestingly, we observed an inverse correlation between single fiber specific tension and IMCL content in type I fibers ($r = -0.53$, $P = 0.044$), suggesting that the lower specific tension of type I fibers might be related to increased IMCL content.

Pre and post results represent eight (ST) and five (CON) subjects. Due to the low number of hybrid fibers and pure IIX-fibers, two separate analyses were performed. First, all fibers from each subject were pooled, regardless of fiber type. Fiber cross-sectional area did not change in ST ($4310 \pm 861 \mu\text{m}^2$ to $4809 \pm 861 \mu\text{m}^2$) or CON ($5346 \pm 2068 \mu\text{m}^2$ to $4807 \pm 1550 \mu\text{m}^2$) (group x time interaction: $P = 0.38$). No group x time interaction was observed for maximal isometric force (ST: 0.36 ± 0.16 to 0.50 ± 0.41 mN, CON: 0.51 ± 0.20 to 0.48 ± 0.15 mN, $P = 0.43$). Moreover, specific tension, defined as maximal isometric force normalized to fiber area, did not change in ST (85 ± 30 to $99 \pm 30 \text{ mN} \cdot \text{mm}^{-2}$) or CON (101 ± 8 to $100 \pm 14 \text{ mN} \cdot \text{mm}^{-2}$) (group x time interaction: $P = 0.51$) (**figure 4.12**). Subsequently, type I-fibers were analyzed separately, whereas IIA, hybrid IIA-X, and IIX-fibers were pooled and analyzed separately. Regardless of categorization, no interaction effects were observed for fiber area, maximal isometric tension or single fiber specific force (results not shown).

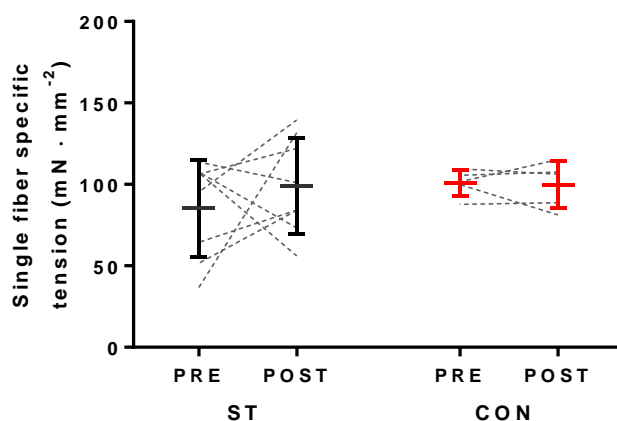


Figure 4.12 Single fiber specific tension before and after the intervention in ST (strength training twice weekly + protein supplementation) and CON (only protein supplementation). All fibers from each biopsy are included, regardless of myosin heavy chain expression. The figure displays individual values, as well as mean \pm SD before and after the intervention.

Hence, contrary to our hypothesis, single fiber specific tension did not improve in the strength-training group. Increases in specific tension has been observed following both short-term (9 weeks) (Erskine et al., 2011) and long-term (1 year) (Pansarasa et al., 2009) strength training in young individuals. However, as is evident from **table 1.2** (page 15 in *Introduction*), the only two studies demonstrating clear effects of strength training on specific tension in elderly, were of longer duration (5-12 months) (Parente et al., 2008; Wang et al., 2018). Frontera and colleagues (2003) also observed an intervention effect following 12 weeks of strength training, but this was primarily due to a decrease in the control group. A closer look at **table 1.2** also reveals that the two studies including the oldest subjects (+80 years) did not observe any changes in specific tension. Collectively, these data therefore indicate limited "qualitative" adaptation at the single-muscle fiber level among the very old, at least following 10-12 weeks of training. Whether a longer duration of training elicit improvements in single fiber specific tension in this population remains to be investigated. Nevertheless, our study confirms that there is a negative correlation between IMCL content and specific tension in type I fibers, and it is therefore interesting to speculate whether the strength training-induced increase in specific tension observed in overweight elderly subjects (Wang et al., 2018) were related to alterations in lipid content.

Our muscle biopsy analyses are subject to some limitations. First, the low number of subjects with both pre and post measurements for single fiber function (ST = 8, CON = 5) increase the risk of statistical type II-errors. Second, a recurring challenge with analyses on human muscle biopsies is to which extent a single biopsy is representative of the whole muscle. This point might be even more applicable in very old and frail individuals, because aging and frailty seem to increase the variation between different areas in the muscle, due to denervation and fiber type grouping (Andersen, 2003; Kelly et al., 2018). The large variation we observed in single fiber specific tension could be related to this phenomenon.

The muscle biopsies were also analyzed by western blot, to investigate the acute and long-term effects of strength training and protein supplementation on mRNAs and proteins related to muscle protein synthesis and degradation. Detailed results can be found in *article IV*, but the main findings are presented and discussed next.

4.6. Autophagy – acute and long-term effects of strength training

From the cross-sectional comparison it was evident that elderly, and particularly frail elderly, displayed higher levels of certain autophagic markers and heat shock proteins compared to young subjects. As previously discussed, we propose that these differences reflect alterations in protein quality control. The aim of the acute study was to assess how different protein quality control systems responded to acute and long-term strength training in pre-frail and frail elderly individuals. In addition, proteins related to muscle protein synthesis were included, as well as genes involved in autophagy, mitophagy and the ubiquitin-proteasome system. The results from the acute study are summarized in **table 4.4**. Note that the two groups (ST and CON) are referred to as ST+PRO and PRO below.

Table 4.4 Summary of gene and protein expression data at Test Day 1, Test Day 2 and following the intervention (basal levels) in ST+PRO (strength training and protein supplementation) and PRO (protein supplementation only).

	Phosphorylation status	Test Day 1		Test Day 2		Intervention	
		ST+PRO	PRO	ST+PRO	PRO	ST+PRO	PRO
Muscle protein synthesis	Phospho-p70 S6K / total	↑	↗	↑	↗	-	-
	Phospho 4E-BP1 / total	↗	-	↑	↗	↗	-
	Phospho eEF2 / total	-	-	-	-	-	-
Macro-autophagy	Gene expression						
	LC3	↑	-	-	-	-	-
	P62	↑	-	-	-	-	-
	ATG7	↑	-	-	-	-	-
	Protein expression						
	Cytosolic LC3-I	↑	-	-	-	-	-
	Membrane LC3-I	-	-	-	-	↘	-
	Membrane LC3-II	↓	↓	↓	↓	-	-
	Cytosolic p62	-	-	-	-	-	-
Membrane p62	-	-	-	-	↘ #	↗	
Mitochondrial quality control	Gene expression						
	BNIP3	-	-	-	-	-	-
	BNIP3L	↗	-	-	-	-	-
	OPA1	↗	-	-	-	-	-
	DRP1	↑	-	-	-	-	-
Ubiquitin proteasome system	Gene expression						
	MuRF-1	↑	-	-	↘	-	-
	Atrogin-1	-	-	↓	↓	-	-
	MUSA1 / Fbox30	↑	-	-	-	-	-
Heat shock proteins	Protein expression						
	Cytosolic Hsp70	-	-	-	-	-	-
	Cytoskeletal Hsp70	-	-	-	-	-	-
	Cytosolic αB-crystallin	↗	-	↑	-	-	-

↑ $P < 0.05$, different from baseline within group. ↗ $P < 0.10$, different from baseline within group. # $P < 0.05$, group x time interaction.

4.6.1. Anabolic signaling

The phosphorylation ratio of p70S6K increased from baseline in ST+PRO both after the first and last training session. In addition, the phosphorylation ratio of 4E-BP1 increased by 34 and 37% at test day 1 and test day 2, respectively, although only the change at test day 2 reached statistical significance. Both p70S6K and 4E-BP1 are under mTORC1 control, and promote translation initiation when phosphorylated and hyper-phosphorylated, respectively (Wang & Proud, 2006). In addition, p70S6K promote translation elongation, through dephosphorylation, and thus activation of eEF2 (Wang & Proud, 2006). The change in phosphorylation of p70S6K and 4E-BP1 have been observed to correlate with the change in fractional synthetic rate after a strength training session (Burd et al., 2012; Burd et al., 2010). Thus, our results suggest that a session of heavy load strength training followed by protein intake upregulates muscle protein synthesis in frail elderly individuals, and that this response occurs regardless of training status. In PRO, only ingesting the protein supplement, phosphorylation of p70S6K tended to increase at both test days, whereas 4E-BP1 phosphorylation tended to increase at test day 2. These results suggest that ingestion of 17 grams of milk protein also increase muscle protein synthesis in frail elderly individuals, albeit perhaps to a lesser extent than when protein intake is preceded by exercise. Hence, our results correspond well with previous observations in young and non-frail elderly individuals (Francaux et al., 2016). Basal MPS-signaling did not change following the 10-week training intervention, although the phosphorylation ratio of 4E-BP1 tended to increase from baseline in ST+PRO.

4.6.2. Ubiquitin-proteasome pathway

No group x time interactions were observed for the UPP genes investigated, and the changes from baseline within the groups should therefore be interpreted with caution. Regardless, the 2.9-fold increase in MuRF1 expression 2.5 hours after the first training session is in line with previous reports (Dickinson et al., 2017; Fry et al., 2013; Raue et al., 2007; Stefanetti, Zacharewicz, et al., 2014). In addition, increased expression from baseline was observed for the ubiquitin ligase MUSA1. No changes were observed for MuRF1 or MUSA1 2.5 hours after the last training session, suggesting limited activation of UPP at this stage of training, when the participants were well accustomed to the exercise protocol. Thus, our results imply that UPP is activated following a session of heavy-load strength training in the untrained state, but that this response decline with improved fitness. This corresponds well with the

findings by Stefanetti and colleagues (Stefanetti, Lamon, et al., 2014), observing limited UPP-activation following a bout of strength training after 10 weeks of strength training in young men. Also in line with our findings, Phillips and colleagues (1999) observed greater increases in fractional breakdown rate following a strength training session in untrained, compared to trained subjects (same relative training load) (Phillips, Tipton, Ferrando, & Wolfe, 1999). Basal gene expression did not change after the ten-week intervention in the present study for any of the UPP-genes. Because we did not measure protein levels, we cannot exclude the possibility that increased mRNA expression following the first training sessions led to increased protein levels of ubiquitin ligases or other components of the UPP-system, potentially increasing UPP activity at rest.

4.6.3. Autophagy and mitophagy

In response to the first training session, increased gene expression of ATG7, LC3 and p62 were observed in ST+PRO. Ogborn and colleagues (2015) observed increased gene expression of LC3 and p62, but not ATG7, 3 hours after a strength training session in untrained young and old individuals (Ogborn et al., 2015). Moreover, the increase in gene expression was accompanied by increased protein levels of both LC3-I and p62 48 hours later (Ogborn et al., 2015). In contrast, Fry and colleagues (Fry et al., 2013) did not observe changes in either LC3 gene expression or LC3-I protein expression 3, 6 and 24 hours after strength training in physically active young and elderly. In the present investigation, the increases in gene expression were accompanied by increased cytosolic LC3-I 2.5 hours after the training session, suggesting that the increase in LC3-I protein could be due to an increased *de novo* synthesis. However, at this time point, the level of LC3-II was significantly reduced, and a positive correlation was observed between the change in p70S6K-phosphorylation and the change in cytosolic LC3-I ($r = 0.56$, $P = 0.037$). Hence, it seems more likely that enhanced mTORC1 activity suppressed lipidation of LC3-I to LC3-II, and that this was the reason for the increased LC3-I levels in ST+PRO. The observation that changes in autophagic proteins did not significantly differ between ST+PRO and PRO, supports the interpretation that autophagy was suppressed 2.5 hours after both strength training and protein ingestion. However, based on the gene expression data, it is possible that a later increase in autophagy occurred. The training session performed after the training period did not elicit changes in mRNA levels of ATG7, LC3 or p62 mRNA, whereas autophagy proteins, in essence, changed in a similar manner as in response to the first training session. Thus, also strength training

followed by protein intake in the trained state appear to acutely suppress autophagy in frail elderly individuals.

BNIP3L mRNA tended to increase following the first training session in ST+PRO ($P = 0.058$), accompanied by a significant increase in DRP1 mRNA. BNIP3L mediates mitophagy (Yuan et al., 2017), whereas DRP1 regulates mitochondrial fission (Frank et al., 2001). Evidence suggests that mitophagy might be attenuated with aging (Joseph et al., 2013; Sebastian et al., 2016), and it has been hypothesized that this may contribute to the development of sarcopenia (Alway et al., 2017). Interestingly, alterations in mitochondrial dynamics may be involved in the age-related reduction in mitochondrial function, based on observations of higher abundance of small, fragmented mitochondria (Iqbal, Ostojic, Singh, Joseph, & Hood, 2013), as well as very large mitochondria (Leduc-Gaudet et al., 2015) in muscles from old rodents. Indeed, both the mRNA and protein levels of important fusion and fission proteins have been reported to be lower with aging in both rodents (Ibebunjo et al., 2013) and humans (Joseph et al., 2012; Tezze et al., 2017). Interestingly, reduced DRP1 expression has been observed in inactive frail, compared to active elderly women in a previous study (Drummond et al., 2014), indicating that mitochondrial dynamics may be more affected in individuals with low functional capacity. The results in the present study are therefore interesting, and suggest that unaccustomed resistance exercise might activate mitophagy in frail elderly, and that increased mitochondrial fission might be involved. No changes were observed in response to the last training session, but whether this was due to increased basal levels of mitophagy proteins is uncertain.

The ten-week training intervention did not change protein levels of cytosolic LC3-I, membrane LC3-I or membrane LC3-II. Consequently, also the LC3-II/LC3-I ratio was unaffected by training and protein supplementation. Interestingly, although cytosolic p62 did not change following the ten-week intervention, a group x time interaction was observed for p62 in the membrane fraction, due to a 15% reduction in ST+PRO and a 34% increase in PRO. The reduction in membrane p62 was accompanied by non-significant reductions in membrane LC3-I (-20%) and membrane LC3-II (-8%), perhaps representing a slight reduction in autophagosome abundance. A potential training-induced reduction in autophagosome abundance is interesting, considering that we observed increased LC3-II levels in elderly subjects in the cross-sectional study (*article II*). Hence, the potential long-term effect of strength training on autophagic markers warrants further investigation, and it is possible that a

greater training frequency (>2 sessions/week) and/or longer duration of training (>10 weeks) is needed to elicit greater changes.

4.6.4. Heat shock proteins

The strength training session elicited an acute increase in the ratio of cytoskeletal to cytosolic α B-crystallin in ST+PRO, both before and after the training intervention, although the increase at test day 1 did not reach statistical significance ($P = 0.067$). However, in contrast to our hypothesis, the ten-week training intervention did not reduce resting levels of Hsp70 or α B-crystallin, neither in the cytosolic nor cytoskeletal fraction. Moreover, we did not observe any correlations between autophagy markers and heat shock proteins at baseline, and the acute training-induced heat shock response observed for α B-crystallin did not correlate with changes in any of the autophagic markers.

The main finding in the acute study was that unaccustomed strength training increased the expression of genes related to the ubiquitin proteasome pathway, autophagy and mitochondrial fission in pre-frail and frail elderly, but that this response was absent in the trained state. Nevertheless, the observed changes in autophagic proteins indicated that autophagy was suppressed 2.5 hours after strength training and protein ingestion both before and after the training intervention. An intervention effect was observed for p62 in the membrane fraction, but not for any of the other autophagic proteins. Moreover, ten weeks of strength training had no effects on anabolic signaling proteins, heat shock proteins, and basal expression of genes and proteins related to the ubiquitin-proteasome pathway and autophagy.

4.7. What determines the effect of training in pre-frail and frail elderly?

In both training interventions (Study C and D) large within-group variation in training effects were observed for muscle mass, strength and functional capacity. Thus, to explore potential determinants of training adaptation, correlation analyses were performed both within each study, and by combining the results from the two studies.

In study D, weekly training frequency (range: 1.6-2.0) correlated significantly with the change in quadriceps femoris muscle CSA ($r = 0.55$, $P = 0.032$). Leg lean mass was measured in both studies, and if combined, a correlation was also observed between training frequency (range: 1.6-3.0) and change in leg lean mass ($r = 0.39$, $P = 0.04$). In contrast, training frequency did

not correlate with changes in knee extension 1RM ($r = 0.19$, $P = 0.32$), chair rise time ($r = 0.08$, $P = 0.7$) or habitual gait velocity ($r = -0.05$, $P = 0.8$). Previous studies have shown that one session per week can be equally effective as two or three sessions a week with regards to improvements in muscle strength in healthy elderly individuals (DiFrancisco-Donoghue, Werner, & Douris, 2007; Taaffe, Duret, Wheeler, & Marcus, 1999), although superior effects of two or three compared to one session have also been reported (Farinatti et al., 2013). Our findings suggest that at least in pre-frail and frail elderly individuals, a higher training frequency should be pursued to optimize gains in muscle mass. However, since the protein supplementation was not equal in study C and D, strong conclusions cannot be drawn based on these correlations.

There are some reports suggesting that the hypertrophic response to strength training is partly blunted in very old individuals (Greig et al., 2011; Raue et al., 2009; Stewart et al., 2014). Subjects completing the training intervention in the two training studies ranged from 74 to 94 years old, allowing us to investigate if age of the subjects influenced the adaptation to training. Interestingly, no correlations were observed between age at baseline and change in leg lean mass, quadriceps femoris muscle CSA, 1RM strength or any of the functional tests in neither of the two interventions.

Both men and women were included in the two studies. A reduced capacity for hypertrophy in response to resistance exercise in older women compared to men has been observed (Bamman et al., 2003; Brose, Parise, & Tarnopolsky, 2003; Kosek et al., 2006), and it has been suggested that a reduced capacity and responsiveness in anabolic signaling might be the reason for these differences. In contrast, we did not observe any differences between men and women for changes in muscle size, strength or functional capacity, suggesting that at least among pre-frail and frail elderly individuals, gender does not appear to be decisive for the training adaptation.

As we have seen, neither training frequency, age nor gender could explain changes in muscle strength and functional performance. Instead, correlation analyses in study C revealed that performance at baseline was inversely correlated with improvements in 1RM (-0.53 , $P = 0.09$), chair rise time (-0.66 , $P = 0.028$), and habitual gait velocity ($r = -0.75$, $P = 0.008$), demonstrating that subjects with lower strength and function at baseline experienced the greatest improvements. The same pattern was evident in the main intervention study, for 1RM ($r = -0.30$, $P = 0.25$), chair rise time ($r = -0.62$, $P = 0.007$) and habitual gait velocity ($r = -0.66$, $P = 0.011$).

In summary, these results indicate that a training frequency of approximately 3 sessions per week might be required to optimize gains in muscle mass and size in pre-frail and frail elderly individuals. Based on the observation of a fairly low protein intake at baseline in many of the subjects in study C (*article I*), the inclusion of a protein supplement in addition to strength training seems reasonable. Moreover, our findings imply that similar improvements in muscle mass and strength can be expected in 70-, 80-, and 90-year-olds, and that women can expect similar relative improvements as men. Lastly, the notion that subjects with a low functional performance at baseline experienced the greatest improvements, underscore that heavy load strength training can be beneficial regardless of functional status.

However, nine out of 26 subjects allocated to training in study D did not complete the 10-week intervention, which equals a drop-out rate of 35%. This starkly contrasted study C, where all eleven subjects randomized to the training group completed the intervention. Geirsdottir and colleagues (2017) investigated predictors of drop-out during a 12-week heavy load strength training intervention (3 sessions per week). The overall drop-out rate in 236 elderly individuals with a mean age of 74 years was 12%. However, in individuals above 80 years ($n = 32$), the drop-out rate was almost 30%. Moreover, low gait speed and low physical activity were the strongest predictors of drop-out (Geirsdottir et al., 2017). Collectively, these findings emphasize that even though heavy load strength training *can* be very beneficial regardless of age and functional capacity, earlier initiation will increase the likelihood of long-term commitment.

4.8. Methodological considerations

There are some methodological considerations to our studies that should be taken into account when interpreting the results. An important difference between this cross-sectional study and the majority of the previous studies in this field is that our subjects received a standardized breakfast 1 hour prior to the muscle biopsy (after an overnight fast). Although the breakfast contained only small amounts of protein, we cannot exclude the possibility that some of the between-group differences we observe for autophagic proteins are at least partly due to differences in breakfast amount, digestion, uptake, transport, or cellular signaling responses to the nutrients consumed. However, no between-group differences were observed for the anabolic proteins investigated (p70S6K, 4E-BP1 and eEF2), suggesting that the mTOR pathway, an important negative regulator of autophagy, was equally affected by the standardized breakfast across groups. Additionally, our autophagy analyses (*article II and IV*) are limited by the lack

of information about the stage of autophagosome-lysosome fusion, as we were unable to obtain antibodies with satisfactory specificity for the proteins of interest.

With only two groups in study C, where the intervention group both performed strength training *and* received protein supplementation, we are not able to discern effects from training and effects from supplementation. However, the aim of the pilot study was to investigate the effects of an optimized strategy, combining heavy-load strength training and protein supplementation. Study D was initially designed as a randomized controlled trial, but we were forced to abandon this design in order to increase statistical power. However, due to several drop-outs in combination with some analytical challenges, the results for some variables still represent a quite low number of subjects, increasing the risk of type II errors. The number of subjects were especially low for the biopsy analyses, mainly because we were unable to obtain a muscle biopsy from several subjects due to a small muscle belly and much subcutaneous adipose tissue. The latter is an important methodological consideration for future studies where muscle biopsies are planned for this population. Another important consideration is that western blot analysis on muscle biopsies only provide a snapshot of the state of the muscle at the time of sampling, and does not provide information about the rate of MPS or MPB. In study D, we planned to include measurements of muscle protein breakdown rate over the last 14 days of the intervention, to see if regular training was associated with increased MPB rates. This was to be done by oral administration of deuterium oxide (D₂O) approximately 70 days before the measurement period. Although it has been reported that mild vertigo can be experienced the first hours after intake (Holm et al., 2013), dizziness and nausea were more prevalent and lasted longer than expected in the frail elderly, and we therefore decided to discontinue these measurements.

4.9. Ethical considerations

There are several ethical considerations in studies such as the ones presented in this thesis, where heavy load strength training and invasive tests are performed in frail elderly. Much of the ethical considerations revolve around the aspect of benefit and burden. The *Declaration of Helsinki* states that "medical research involving human subjects may only be conducted if the importance of the objective outweighs the risk and burdens of the research subjects" (World Medical, 2013). Several measures were taken to limit the burdens imposed on the participants in these studies.

Any medical procedure that makes an incision in the skin (such as muscle biopsies) carries some risk of infection. In addition, a muscle biopsy can be painful (Dengler et al., 2014). By using the Bergström technique, only a small incision in the skin was made (1-2 cm), and the whole procedure was carried out with the use of sterile equipment. On the acute test days with two biopsies, we also collected the biopsies from the same incision, to reduce discomfort. Moreover, participants withdrawing from the biopsy were still allowed to be included in the study without biopsies. Electrical stimulation by the interpolated twitch technique can also be painful, and the threshold for termination of the test was therefore low.

There will always be a small risk of injury during exercise. Liu and Latham (2010) found that adverse events were reported more often in trials that recruited participants with certain functional limitations or a sedentary life style, and in trials that applied high training intensity. The most commonly reported events were joint pain or muscle strains (Liu & Latham, 2010). Therefore, all training sessions were supervised by experienced instructors, and the training load was gradually increased in both training interventions. If subjects experienced pain or discomfort during training, modifications of technique or other adjustments were performed. In some occasions, training sessions were terminated.

An ethical issue relevant to many studies in the field of exercise physiology is the inclusion of control groups. Both in study C and study D, the control group underwent the same tests as the intervention group, without receiving the benefits of training adaptation. To compensate for this, these subjects were offered 10 weeks of training guidance after the initial intervention period.

5. Conclusion

The overall aim of this thesis was to investigate the effect of heavy load strength training on a broad number of factors related to specific strength in frail elderly, and to assess their contribution to improvements in absolute strength. Another objective was to investigate the impact of age, frailty and strength training on the process of autophagy, and to explore if this aspect of protein quality control relates to specific strength. The main findings are summarized below.

Cross-sectional comparison of young, non-frail, pre-frail and frail elderly

- Specific strength was lower in frail elderly, compared to both young and non-frail elderly.
- Levels of cytosolic LC3-I were higher in old frail subjects, compared to the young group. If the elderly groups were pooled, membrane LC3-II levels were also higher in old, compared to young subjects. These results may indicate suppressed autophagosome formation, accompanied by impaired autophagosome-lysosome fusion.
- If the elderly groups were pooled, levels of cytosolic Hsp70 were higher in old compared to young participants. Cytosolic α B-crystallin was higher in old pre-frail and frail subjects, compared to the young group, whereas cytoskeletal Hsp70 levels were higher in frail elderly.
- Levels of cytosolic LC3-I were inversely correlated with specific strength among the elderly participants, indicating a positive relationship between autophagy and muscle quality.

Acute and long-term effects of strength training on autophagy in pre-frail and frail elderly

- Decreased LC3-II levels indicated suppressed autophagy 2.5 hours after a strength training session followed by protein intake, regardless of training status.
- Increased gene expression of LC3, p62 and ATG7 after a bout of unaccustomed strength training indicated a later stimulation of autophagy.
- Ten weeks of strength training had minimal effects on proteins related to autophagy (p62, LC3-I, LC3-II), muscle protein synthesis (p70S6K, 4E-BP1, eEF2), and cellular stress (Hsp70, α B-crystallin).

Effect of strength training on determinants of specific strength in pre-frail and frail elderly

- Strength training twice weekly for ten weeks did not change voluntary activation level, intramyocellular lipid content or single fiber specific tension.
- Strength training twice weekly for ten weeks increased muscle density, but the increase in muscle density did not correlate with changes in IMCL content or single fiber specific tension.
- Strength training twice weekly for ten weeks increased quadriceps femoris CSA and the increase in muscle CSA corresponded well with improvements in isometric strength.

6. Perspectives

The age-related loss of muscle strength can decline to a level where it has implications for the ability to perform various tasks of daily living. The consequence is a vicious circle where inactivity caused by reduced functional capacity accelerates the loss of muscle mass and strength. The rapid functional decline in pre-frail and frail elderly was also evident in our studies, as gait velocity was reduced over ten weeks in controls. In contrast, strength training elicited improvements in functional capacity, underscoring the potential of strength training to combat the *circle of loss* (**figure 6.1**). Another interesting observation was that similar improvements were achieved regardless of age, and that low functional capacity at baseline in fact was associated with greater improvements. Nevertheless, the many dropouts in the main training intervention suggest that the likelihood of long-term commitment is reduced in individuals with very low physical function. The latter observation emphasize that strength training should be applied as early as possible, with a goal of *preventing* rather than *treating* frailty.

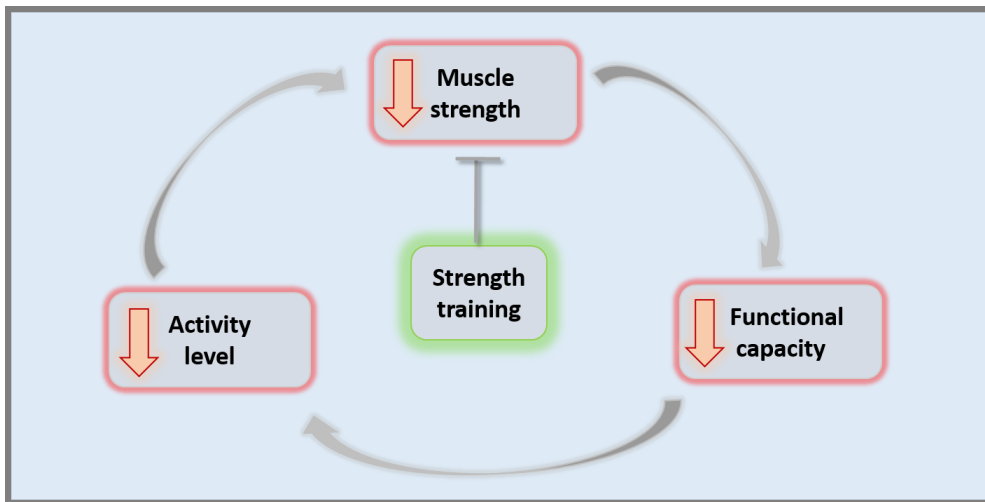


Figure 6.1. The *circle of loss* and the potential effect of strength training.

7. References

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ARTICLE I

**Strength training and protein supplementation improve muscle mass,
strength, and function in old pre-frail individuals**

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ABSTRACT

Aim: The aim of this study was to investigate the effects of a lower body strength training regime, combined with protein supplementation, on muscle mass and thickness, strength, and functional capacity in pre-frail elderly individuals. In addition, the relationship between changes in the different variables were investigated.

Method: Twenty-two elderly men and women (73-92 years) were randomized to either a group performing thirty minutes of heavy-load strength training three times a week, with daily protein supplementation, for ten weeks (ST), or a control group (CON). Body composition was assessed with dual-energy X-ray absorptiometry (DXA), and muscle thickness with ultrasound imaging. Muscle strength was measured as knee extension one-repetition maximum (1RM) and isometric maximal voluntary contraction (IMVC). Functional capacity was assessed by five times chair rise, gait velocity and stair climbing performance.

Results: Leg lean mass increased from baseline in ST (0.7 ± 0.3 kg). Moreover, increased muscle thickness of vastus lateralis ($4.4 \pm 3.2\%$), rectus femoris ($6.7 \pm 5.1\%$), and vastus intermedius ($5.8 \pm 5.9\%$) were observed. The hypertrophy was accompanied by increased strength (20-23%) and improved functional performance (7-11%). No changes were observed in CON. In ST, neither the change in leg lean mass nor muscle thickness correlated with changes in muscle strength. However, correlations between changes in muscle strength and functional performance were observed.

Conclusions: A weekly training volume of only ninety minutes was sufficient to elicit gains in muscle mass, as well as improvements in strength and functional capacity in pre-frail elderly individuals. In relative terms, vastus lateralis, rectus femoris and vastus intermedius contributed equally to the quadriceps femoris hypertrophy. The correlations observed between changes in strength and function suggests that deficits in strength should be addressed when the goal is to improve functional performance.

Keywords: resistance exercise, frailty, sarcopenia, hypertrophy, mobility-limited, aging, skeletal muscle

INTRODUCTION

Aging is accompanied by loss of muscle mass, which reaches approximately 1% each year after the age of 70 (Goodpaster et al., 2006). Moreover, the annual reduction in muscle strength is even greater (Delmonico et al., 2009). In elderly individuals, muscle strength is associated with functional performance (Hayashida, Tanimoto, Takahashi, Kusabiraki, & Tamaki, 2014), and accordingly, various tasks of daily living are hampered by the decline in strength. The consequence is a vicious circle, where inactivity caused by reduced functional capacity accelerates the loss of muscle mass, strength and physical function.

The Short Physical Performance Battery (SPPB) is commonly used to assess functional capacity, where individuals with a score of 10 or less out of maximum 12 may be categorized as pre-frail. These individuals represent a group of particular interest, as small-to-moderate limitations in functional status assessed by SPPB is associated with higher odds of losing the ability to walk 400 m three years later (Vasunilashorn et al., 2009). Therefore, strategies to prevent the loss of – or even better increase – functional capacity are important in this population.

It is established that heavy-load strength training, alone or in combination with protein supplementation, can improve muscle mass, strength and function in elderly individuals (Cermak, Res, de Groot, Saris, & van Loon, 2012; Pinto et al., 2014; Verdijk, Jonkers, et al., 2009). Although most studies have focused on healthy older adults, heavy-load strength training also seems like an effective strategy in very old pre-frail and frail individuals (Chale et al., 2013; Fiatarone et al., 1994; Seynnes et al., 2004). However, the extent to which training-induced gains in muscle mass and size are related to improvements in strength and functional capacity is still poorly understood, because few intervention studies in this population have quantified hypertrophy precisely. Moreover, to improve the basis for investigating such relationships, it might be beneficial to include a protein supplement to optimize the gains in muscle mass (Tieland et al., 2012).

Therefore, the aim of this study was to investigate the effects of a lower-body strength training regime combined with protein supplementation in pre-frail elderly individuals. The endpoints were changes in body composition, the relative changes in different compartments of the quadriceps femoris muscles, and the relationships between changes in muscle mass, muscle thickness, strength and functional capacity.

METHOD AND DESIGN

This study was conducted from August to December 2015 in Oslo, Norway. Twenty-two elderly men and women aged between 73 and 92 [85 ± 6 years; mean \pm standard deviation (SD)] were recruited through advertisement in the local community and visits at a local senior activity center. Upon inclusion, subjects were screened using the Short Physical Performance Battery (SPPB). The SPPB consists of timed standing balance, gait speed, and timed chair-rise assessments (Guralnik, Ferrucci, Simonsick, Salive, & Wallace, 1995). Performance for each of these tasks is scored between 0 and 4, with a summary score of 0–12. Participants with an SPPB score ≤ 10 were included. In addition, individuals using aids such as walkers, canes or Nordic walking sticks in their daily life were included, regardless of SPPB score.

The study complied with the standards set by the Declaration of Helsinki. The nature and goals of the study were thoroughly explained, and all participants provided a written informed consent. The study data storage methods were in accordance with The Norwegian Centre for Research Data. The study was retrospectively registered at clinicaltrials.gov as NCT03723902. After baseline assessment, stratified randomization was performed on ranked baseline knee extensor strength (normalized to body weight), five times chair-rise and stair climbing performance. Randomization was performed using an online random allocation software, and participants were assigned in a 1:1 ratio to a group performing heavy load strength training three times per week and receiving daily protein supplementation, both for ten weeks (ST), or a control group continuing their normal daily activities and dietary habits (CON).

Training

Training of participants in ST was carried out at a local senior activity center. The strength training protocol was a supervised progressive program, performed three times per week for ten weeks. The training was performed in groups of two to three participants, and each session took approximately thirty minutes to complete, including warm-up. Sessions were usually performed Mondays, Wednesdays and Fridays, where Mondays and Fridays were repetition maximum (RM) sessions. On Wednesdays, the load was adjusted to approximately 80-90% of RM loads. Each session included leg press, knee extension, and adapted one-leg squat. In the latter exercise, subjects stood sideways toward a wall bar for support (one hand), with the opposite leg on a step platform (initial height of 20 cm). From this position, subjects extended their knee and hip, before returning to the starting position. After a given number of repetitions, the participant turned around 180 degrees, to perform the exercise for the other leg. Progression was achieved by gradually increasing the height of the step platform, and by adding weight (dumbbell in the arm not used for support). For all three

Article I

exercises, subjects were instructed to use 1-2 seconds both on the concentric and eccentric phase. The participants performed 1-3 sets of 12 repetitions at submaximal loads the first week, and from week 2-10 subjects gradually progressed from 3 sets of 12RM to 3-4 sets of 6RM (**Table 1**). Each set was followed by approximately 2-min rest periods. Strength training sessions were preceded by a lower extremity 3-min warm-up exercise, using a step platform.

Supplementation

Participants in ST received 34 g of milk protein each day, divided in two equal servings (each contained 17 g of protein, 18 g of carbohydrate, 1 g fat; giving 149 kcal [627 kJ]). Participants consumed one supplement in the morning and one in the evening. On training days, subjects were instructed to consume one of the servings within two hours after training. Participants were encouraged to continue their regular food routines in addition to the supplements.

Tests and measurements

All tests were monitored by the same investigator before and after the intervention period. Neither participants nor test personnel were blinded. All testing took place at Norwegian School of Sport Sciences.

Diet assessment

24-hour diet recall interviews were performed to evaluate whether the supplement had changed the participants' diet. Two interviews were conducted before, and two toward the end of the intervention period.

Body composition

Dual energy x-ray absorptiometry. Fat and lean mass was assessed with a DXA scan after an overnight fast. In addition, regional analyses were performed to measure leg lean mass. The coefficient of variation (CV) for the assessment of leg lean mass is low in our lab (<1.7%).

Quadriceps thickness

Muscle thickness was measured in vastus lateralis, rectus femoris, and vastus intermedius muscles of the dominant leg, with a 50-mm linear probe (5–12 MHz) connected to a Philips HD11 XE ultrasound apparatus (Royal Philips Electronics, Amsterdam, Netherlands). Scans of vastus lateralis were obtained at 40% of the distance between the knee joint and the greater trochanter, and halfway between the iliac crest and the myotendinous junction for rectus femoris. Vastus intermedius was measured on the same image as vastus lateralis, where the latter was thickest in the medio-lateral

Article I

direction. Probe position was recorded for each measurement on transparent, acetate paper positioned over the thigh.

Several images were obtained for each muscle at each registration time point, and the three best images were chosen for analysis, which was performed using OsiriX v5.5.1 (Bernex, Switzerland). The shortest distance between the upper and lower aponeurosis was measured at 25, 50 and 75% of the width of the field of view. Muscle thickness was calculated as the average of these measurements across images.

Muscle strength

Maximal strength was assessed with a one-repetition maximum (1RM) in knee extension. After a general warm-up (5 min bicycling), a specific warm-up/preparation procedure was performed using loads corresponding to 50%, 70%, 80% and 90% of expected 1RM – conducting 10, 6, 3, and 1 repetitions, respectively. Optimally, 2–5 attempts were used to establish 1RM. The loads could be adjusted in steps as low as 0.5 kg. The left and right leg was tested interchangeably (unilateral), and each leg rested for approximately three minutes between attempts. The range of motion was strictly controlled.

Isometric maximal voluntary contraction (IMVC) was also tested for the knee extensors. The participants were securely strapped to the testing chair, with an angle of 90° in the hip and knee joints (0° corresponding to full extension). The lever arm of the knee-extension device with an attached strength gauge (HBM U2AC2, Darmstadt, Germany) was adjusted so that the contact point with the participant's calf was just proximal to the line of the medial malleolus. The participants performed three 5-second attempts and were instructed to reach maximum torque (Newton meter - Nm) as rapidly as possible. This was done to obtain a measure of the rate of torque development (RTD), both in relative terms ($RTD_{max}/\text{maximal torque}$) and absolute terms (torque at 100 ms). The left and right leg was tested interchangeably, with sixty seconds of rest between each attempt. The best result of three trials per leg was used in the analyses.

Functional performance

Sit to stand x 5

Participants were asked to rise from a chair to a full stand five times as fast as possible. Subjects were instructed to keep their hands crossed on their chest. If they were not able to stand up without using their hands, the use of hands was allowed, and performed the same way at both registration time points. Time was measured with a pressure sensor (TC-Start Pod and Touch Pad, Brower Timing Systems, Draper, USA). The best time of two trials was used in the analyses.

Article I

Stair climbing test

Participants were asked to climb a stair with twenty steps as fast as possible, without running. Time was assessed by photocells (Brower Timing Systems, Draper, USA). Steps were 18 cm high and 30 long (horizontal length). The total height was 3.6 m and the total length was 6 m. The participants were encouraged to climb the stairs without using the handrail, but were allowed to do so if they felt it was necessary. If so, hands were used at both registration time points. The best time of two trials was used in the analyses.

Habitual gait velocity

Participants were asked to walk 10 meters at their habitual gait velocity. Photo cells (Brower Timing Systems, Draper, USA) were placed to measure the time between 2 to 8 meters. The mean time of three trials was used in the analyses.

Statistical analyses

Power calculations performed a priori indicated that 22 participants would be needed to identify a difference of 0.5 kg between the ST and CON in lean mass, with alpha set at 0.05 and beta at 0.20. Non-normally distributed data (D'Agostino and Pearson omnibus normality test) were log-transformed prior to statistical analysis. Differences at baseline were assessed with an unpaired Student's t test. A two-way ANOVA and Sidak's multiple comparison test was used to evaluate the effect of time and group for variables measured both before and after the intervention period. Differences in response between vastus lateralis, rectus femoris and vastus intermedius were analyzed with one-way ANOVA, and Tukey's multiple comparisons test. Associations between variables were investigated using Pearson's correlation coefficient. Correlations were considered weak if $r < 0.4$, moderate if $r = 0.4-0.6$, and strong if $r > 0.6$. Statistical analyses were performed using GraphPad Prism6 software (GraphPad Software, Inc., La Jolla, CA). Two-sided statistical significance was set at $P < 0.05$.

RESULTS

There were no significant differences between ST and CON in physical characteristics or protein intake at baseline, although ST tended to be older than CON (**Table 2**). All 22 subjects completed the initial part of the study. However, one subject in CON was excluded from the evaluation of results, as the subject started strength training during the intervention period. Thus, 11 subject in ST and 10 subjects in CON remained for the per-protocol analyses. Moreover, one additional subject in CON did not perform the ultrasound measurement after the intervention. Thus, for these

Article I

analyses the number of subjects in ST and CON is 11 and 9, respectively. No adverse events occurred in any of the groups.

Baseline correlations

A strong correlation was observed between leg lean mass and knee extension 1RM at baseline ($r = 0.80$, $P < 0.001$). In addition, 1RM strength normalized to body weight showed moderate correlations with habitual gait velocity ($r = 0.46$, $P < 0.05$) and stair climbing time ($r = -0.50$, $P < 0.05$), but not with five times chair-rise ($r = -0.25$, $P = 0.26$) (**Table 3**).

Adherence

All subjects in ST performed at least 90% of the training sessions in the ten-week period (95.8 ± 2.6). There were no correlations between adherence and training effect on any variable.

Protein intake

ST and CON had a protein intake of $0.91 (\pm 0.17)$ and $0.83 (\pm 0.29)$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ at baseline, respectively. As a consequence of the protein supplement, ST increased their daily protein intake to $1.22 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ($P = 0.002$). No significant change was observed in CON, and the group x time interaction only tended to be significant ($P = 0.058$) (**Figure 1**).

Body composition

Body mass tended to increase from baseline in CON (1.1 ± 1.5 kg, $P = 0.056$), but not in ST (0.7 ± 1.5 kg, $P = 0.24$). No group x time interaction was observed ($P = 0.54$). However, a group x time interaction was observed for leg lean mass ($P = 0.001$), due to an increase from baseline in ST (0.7 ± 0.3 kg, $P < 0.001$), and no change in CON (0.1 ± 0.3 kg, $P = 0.69$) (**Figure 2A**). For fat mass, a tendency towards an increase was observed in CON (0.6 ± 0.7 kg; $P = 0.052$), whereas a non-significant reduction was observed in ST (-0.5 ± 0.9 kg, $P = 0.15$). Thus, for fat mass, a group x time interaction was observed ($P = 0.007$) (**Figure 2B**).

Muscle thickness

In ST, increased thickness of vastus lateralis ($4.4 \pm 3.2\%$), rectus femoris ($6.7 \pm 5.1\%$), and vastus intermedius ($5.8 \pm 5.9\%$) was observed ($P < 0.001$ for all muscles) (**Figure 3**). Muscle thickness did not change in CON, and a group x time interaction was observed for all muscles investigated ($P < 0.01$). There were no differences between the relative change in vastus lateralis, rectus femoris and vastus intermedius in ST ($P = 0.38$). A strong correlation was observed between change in vastus lateralis thickness and the change in leg lean mass in the training group ($r = 0.67$, $P < 0.05$),

and the correlation was slightly stronger if the mean of the change in vastus lateralis, rectus femoris and vastus intermedius was used ($r = 0.69$, $P < 0.05$).

Muscle strength

ST improved KE 1RM and isometric MVC by $23 \pm 15\%$ and $20 \pm 11\%$, respectively, and a group x time interaction was observed for both tests ($P < 0.001$) (**Figure 4**). The change in knee extension 1RM was not correlated with the change in leg lean mass, nor with the change in thickness of vastus lateralis, rectus femoris or vastus intermedius. Force at 100 milliseconds during the MVC improved from baseline only in ST ($22 \pm 22\%$), and a group x time interaction was observed ($P = 0.039$).

Functional performance

ST participants used less time on five times chair-rise and stair climbing after the intervention, with improvements of 10 ± 13 ($P = 0.05$) and $11 \pm 12\%$ ($P = 0.034$) from baseline, respectively (**Figure 5**). A group x time interaction was observed for stair climbing ($P = 0.019$), but not five times chair-rise ($P = 0.13$). In addition, a group x time interaction was observed for habitual gait velocity ($P = 0.028$), due to a non-significant improvement in ST ($7 \pm 10\%$; $P = 0.17$), along with a non-significant decline in CON ($-5 \pm 14\%$, $P = 0.24$) (**Figure 6**). No significant correlations were observed between training-induced changes in knee extension 1RM and changes in five times chair-rise time ($r = -0.46$, $P = 0.15$) or stair climbing time ($r = 0.04$, $P = 0.89$). However, the change in knee extension 1RM tended to correlate with the change in habitual gait velocity ($r = 0.54$, $P = 0.09$). Furthermore, a strong correlation was observed between the change in isometric MVC and the change in habitual gait velocity in the training group ($r = 0.70$, $P = 0.015$). No significant correlations were observed between the improvement in force at 100ms during MVC, and changes in functional performance.

DISCUSSION

Thirty minutes of heavy load strength training performed three times per week in combination with protein supplementation increased leg lean mass, as well as strength and functional capacity, in pre-frail elderly individuals. Concerning hypertrophy of vastus lateralis, rectus femoris and vastus intermedius we found no significant differences between the muscles. Importantly, the increases in muscle mass and strength were translated into improved physical function. Although we did not observe any correlations between the changes in muscle mass/thickness and changes in muscle strength in the training group, we did observe correlations between changes in strength and changes in some measures of functional capacity.

Body composition

Leg lean mass increased by 0.7 kg in the training group in the present study. A meta-analysis of 49 studies, representing a total of 1328 participants, examined the effect of whole-body strength training on lean body mass (LBM) in individuals > 50 years. The mean intervention duration was 20.5 weeks, and the weighted pooled estimate of mean LBM change was 1.1 kg (Peterson, Sen, & Gordon, 2011). Interestingly, the meta-analysis also found a negative association between age and change in LBM. With a mean age of 86.6 years, the subjects in our experimental group can be categorized as *very old*, and thus an increase of 0.7 kg in leg lean mass after only ten weeks of leg exercise is higher than expected. Nevertheless, despite a possible negative correlation between age and LBM change (Peterson et al., 2011), it is established that also very old individuals can experience hypertrophy after a period of strength training (Bechshoft et al., 2017; Fiatarone et al., 1990), and our findings further strengthen this observation.

The literature is equivocal with regard to the importance of protein supplementation during a period of strength training in elderly individuals (Cermak et al., 2012; Chale et al., 2013; Fiatarone et al., 1994; Finger et al., 2015; Thomas, Quinn, Saunders, & Greig, 2016; Tieland et al., 2012). Due to the design of our study, we are not able to make any conclusions regarding the contribution that the protein supplement may have had on the observed effects. According to the diet assessments, the experimental group had a protein intake of 0.91 g·kg⁻¹·day⁻¹ at baseline. Although this is above the Recommended Dietary Allowance (RDA) of 0.8 g·kg⁻¹·day⁻¹ (Volpi et al., 2013), it is below the Nordic recommendations of 1.2 g·kg⁻¹·day⁻¹ for elderly (*Nordic Nutrition Recommendations*, 2012). Indeed, recent consensus statements have argued that protein intakes between 1.0 and 1.5 g·kg⁻¹·day⁻¹ may be necessary to slow down or counteract sarcopenia in old individuals (Bauer et al., 2013; Deutz et al., 2014; Paddon-Jones et al., 2015). At baseline, the variability in protein intake in the training group was quite high (range: 0.6-1.2 g·kg⁻¹·day⁻¹). It is therefore possible that the protein supplement had additive effects at least in the individuals with a low protein intake at baseline. Regardless, protein supplementation was feasible and did not interfere with the normal diet of the participants. Therefore, inclusion of a protein supplement might be a good strategy to ensure optimal effects of strength training in very old individuals, in which protein intake is highly variable (Mendonca et al., 2017).

Muscle thickness

In a systematic review, Stewart and coworkers investigated the hypertrophic response to strength training in elderly individuals aged > 75 years (Stewart, Saunders, & Greig, 2014). Three of four studies observed increased cross-sectional area (CSA) of quadriceps femoris, showing

Article I

improvements of 2-9%. Recently, a 3.4% increase in quadriceps femoris CSA was observed in very old individuals (range: 83-94) following twelve weeks of heavy-load strength training and protein supplementation (Bechshoft et al., 2017). As we measured muscle thickness and not cross-sectional area, we cannot directly compare our results with the above findings. However, a 7.5% increase in vastus lateralis thickness assessed by ultrasound corresponded to a 5.2% increase in CSA assessed by MRI in young individuals, following twelve weeks of resistance training (Franchi et al., 2018). Accordingly, our observation of 4-7% increases in muscle thickness corresponds well with previous observations (Bechshoft et al., 2017; Chale et al., 2013; Fiatarone et al., 1994; Sipila & Suominen, 1995).

No significant differences between vastus lateralis, rectus femoris and vastus intermedius were observed in the present investigation, with improvements of 4.4, 6.7, and 5.8% respectively. To our knowledge, no study has investigated the relative hypertrophy of different compartments of quadriceps femoris following strength training in this population previously. However, in somewhat younger individuals (67 ± 5 years), increases of 6% and 16% were observed for vastus lateralis and rectus femoris, respectively, after 12 weeks of strength training (Bjornsen et al., 2016). Also in young individuals greater increases have been observed for rectus femoris (~18%), compared to both vastus lateralis (~8%) and vastus intermedius (~9%) (Ema, Wakahara, Miyamoto, Kanehisa, & Kawakami, 2013). Although direct comparisons across studies should be performed with care, the overall greater improvements in muscle thickness in young (Ema et al., 2013), as well as in individuals in their late sixties (Bjornsen et al., 2016), imply that the hypertrophic response to strength training might be reduced at very high age. This is indeed supported by others (Greig et al., 2011; Peterson et al., 2011; Raue, Slivka, Minchev, & Trappe, 2009). Additionally, our results suggest that vastus lateralis, rectus femoris and vastus intermedius in relative terms contribute equally to the strength training induced increase in quadriceps femoris in pre-frail elderly individuals.

Muscle strength

The observed increase in maximal knee extension strength in ST by 23% was in the expected range. In general, elderly individuals show similar relative improvements in muscle strength as young individuals after strength training (Hakkinen, Kraemer, Newton, & Alen, 2001; Moritani & deVries, 1980). In a meta-analysis combining data from 28 studies, and subjects with a mean age of 67 years, an average improvement of 33% was observed for knee extension strength (Peterson, Rhea, Sen, & Gordon, 2010). With a mean intervention duration of 17.6 weeks and an average training frequency of 2.7 days/week, the increase of 33% corresponded to an improvement of approximately 0.7% per training session (Peterson et al., 2010). Our trained subjects increased their maximal knee extension

Article I

strength by 23%, following an average of 28.7 training sessions, corresponding to an 0.8% increase per session. These very rough evaluations emphasize that heavy load strength training is effective in improving muscle strength even in very old pre-frail individuals.

In the present study, the change in knee extension 1RM was not significantly correlated with the change in leg lean mass or quadriceps femoris thickness, suggesting that factors other than increased cross-sectional area contributed to the improvements in strength. In this regard, increased activation level, muscle density, muscle length and single fiber specific force may all be involved (Arnold & Bautmans, 2014; Frontera et al., 2003; Sipila & Suominen, 1995). Although not significant, we did observe associations between the change in knee extension 1RM and change in habitual gait velocity ($r = 0.54$, $P = 0.09$) and time used during five times chair-rise ($r = -0.46$, $P = 0.15$). Additionally, the change in isometric MVC was significantly correlated with the change in habitual gait velocity ($r = 0.70$, $P < 0.05$). Moreover, we observed significant correlations between knee extension 1RM and performance on two of the three functional tests at baseline. Collectively, these findings support addressing deficits in strength to improve functional capacity.

Rate of torque development

The improvements of knee extension 1RM and peak IMVC were paralleled by improvement of torque at 100 ms during MVC. Torque at 100 ms represents the capacity of the muscle as a whole to produce force rapidly, and is therefore an absolute measure of rate of torque development (RTD). However, if torque at 100 ms were normalized to maximal torque, no effect of training was observed (results not shown). Moreover, normalized RTD_{max} , given as the rise in torque development relative to maximal torque, did not improve (results not shown). Hence, both these relative measures of RTD suggest that the intrinsic properties of the knee extensors were not affected by strength training and protein supplementation in the present study. In theory, it would not be surprising if we had observed increased relative rate of torque development, due to the expected preferential hypertrophy of type II-fibers, especially in elderly individuals (Kosek, Kim, Petrella, Cross, & Bamman, 2006; Kryger & Andersen, 2007; Verdijk, Gleeson, et al., 2009). Regardless, increasing muscle strength alone will allow the muscle to produce more force during a short time interval, and thus improve the muscle's ability to produce power. This is important, since functional performance correlates even better with muscle power than muscle strength (Bean et al., 2002; Reid & Fielding, 2012). In addition, improved ability to produce force rapidly might prevent falls, due to an increased ability to correct unexpected imbalances that might easily occur during daily life (e.g. walking) in the elderly (Pijnappels, Reeves, Maganaris, & van Dieen, 2008).

Functional performance

The improvement in habitual gait velocity of 7% in the trained subjects is in accordance with two meta-analyses showing improvements of approximately 5-10% following strength training in elderly individuals (Hortobagyi et al., 2015; Van Abbema et al., 2015). The importance of addressing this aspect of functional decline is demonstrated by the observed relationship between gait velocity and risk of mortality (Studenski et al., 2011; White et al., 2013). Interestingly, the control subjects in the present study displayed a non-significant reduction (5%) in gait velocity over the 10-week period, indicating that the functional decline in this population is rapid. The latter observation emphasize the importance of countermeasures. Training also improved five times chair-rise performance by 10% from baseline. Data concerning the effect of strength training on chair-rise performance are contradictory. Some have observed improved performance (Chale et al., 2013; Dias et al., 2015; Pinto et al., 2014; Schot, Knutzen, Poole, & Mrotek, 2003), whereas others have not (Judge, Whipple, & Wolfson, 1994; Schlicht, Camaione, & Owen, 2001). In addition to gait velocity and chair-rise, stair-climbing performance is often tested to assess the functional status of elderly individuals. Strength training seems to improve performance by 8-18% (Chale et al., 2013; Dias et al., 2015; Seynnes et al., 2004), again in accordance with our findings. It has been suggested that high-speed power training has some advantages over traditional strength training with regards to improvements in function (Sayers & Gibson, 2014). Nevertheless, our findings demonstrate that traditional heavy-load strength training effectively improves functional performance in pre-frail elderly individuals.

Based on the findings in this and previous studies, it seems evident that increasing muscle strength through heavy-load strength training in combination with protein supplementation is an effective, as well as feasible, strategy to improve functional capacity in pre-frail elderly individuals. Importantly, no adverse event occurred and adherence to the training was high, demonstrating that such training can be safely implemented. The lack of correlations between muscle hypertrophy and strength, as well as the mismatch between increases in muscle thickness (4-7%) and maximal isometric torque (20%), supports the notion that factors other than gains in muscle mass contribute to the training induced improvements in strength. However, the association between muscle strength and functional capacity, both at baseline and for changes in the training group, imply that one should aim to address deficits in muscle strength when the goal is to improve functional capacity. In this regard, thirty minutes of heavy load strength training performed three times per week in combination with protein supplementation represented an effective strategy.

Article I

Limitations

With only two groups, where the intervention group both performed strength training *and* received protein supplementation, we are not able to discern effects from training and effects from supplementation. Including a protein only and training only group would have provided this information. However, the aim of the present study was to investigate the effects of an optimized strategy, combining heavy-load strength training and protein supplementation.

Perspectives

Among pre-frail elderly individuals living at home, many are at the verge of being institutionalized. Increasing functional capacity through strength training may allow these individuals to become less dependent on assistance in performing various daily activities, to an extent where their activity level can be better maintained, and further loss of muscle mass and strength can be blunted or even prevented. Such strategies are important both from a socioeconomic and individual perspective. Future studies should address the mechanisms underlying the often-observed mismatch between improvements in muscle strength and size. A deeper understanding of such factors will strengthen the basis for the development of optimal countermeasures.

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Article I

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Article I

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Article I

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TABLES

Table 1. Strength training program performed in ST.

	Week 1			Week 2-4			Week 5-7			Week 8-10		
	WO 1	WO 2	WO 3	WO 1	WO 2	WO 3	WO 1	WO 2	WO 3	WO 1	WO 2	WO 3
Leg press	2 x 12 (<RM)	2 x 12 (<RM)	2 x 12 (<RM)	3 x 12 (RM)	3 x 10 (<RM)	3 x 10 (RM)	3 x 10 (RM)	3 x 10 (<RM)	3 x 8 (RM)	3 x 8 (RM)	3 x 10 (<RM)	4 x 6 (RM)
Knee extension	3 x 12 (<RM)	3 x 12 (<RM)	3 x 12 (<RM)	3 x 12 (RM)	3 x 10 (<RM)	3 x 10 (RM)	3 x 10 (RM)	2 x 10 (<RM)	3 x 8 (RM)	4 x 8 (RM)	4 x 10 (<RM)	4 x 6 (RM)
Adapted one-leg squat	1 x 12 (<RM)	1 x 12 (<RM)	1 x 12 (<RM)	1 x 12 (RM)	1 x 10 (<RM)	1 x 10 (RM)	1 x 10 (RM)	2 x 10 (<RM)	2 x 8 (RM)	2 x 8 (RM)	2 x 10 (<RM)	2 x 6 (RM)

WO, work-out; RM, repetition maximum

Table 2. Baseline participant characteristics.

	ST (n = 11) N or Mean \pm SD	CON (n = 11) N or Mean \pm SD	P value
Men / women (n)	4 / 7	3 / 8	
Age	86.6 \pm 6.0	82.6 \pm 4.5	0.09
Height (m)	1.68 \pm 0.07	1.65 \pm 0.10	0.50
Weight (kg)	73.6 \pm 12.7	71.5 \pm 9.5	0.67
Lean mass (kg)	44.8 \pm 5.6	41.5 \pm 4.5	0.14
BMI (body mass/height ²)	26.1 \pm 4.2	26.3 \pm 3.8	0.94
Protein intake (g/kg/day)	0.91 \pm 0.17	0.83 \pm 0.29	0.47
Short Physical Performance Battery (SPPB)	9.0 \pm 1.4	8.9 \pm 2.2	0.91

N, number; BMI, body mass index; SD, standard deviation

Table 3. Correlations at baseline for all participants (n: 22). Values for leg lean mass, vastus lateralis thickness, and knee extension 1RM were normalized to body weight prior to correlation analyses.

Measure	1	2	3	4	5	6
1. Leg lean mass (kg)	—					
2. Vastus lateralis thickness (mm)	.29	—				
3. Knee extension 1RM (kg)	.80***	.22	—			
4. Habitual gait velocity (m/s)	.25	.13	.46*	—		
5. Five times chair rise (s)	-.02	.02	-.25	-.54**	—	
6. Stair climbing (s)	-.40	.01	-.50*	-.69***	.53*	—

1RM, one repetition maximum. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

FIGURES

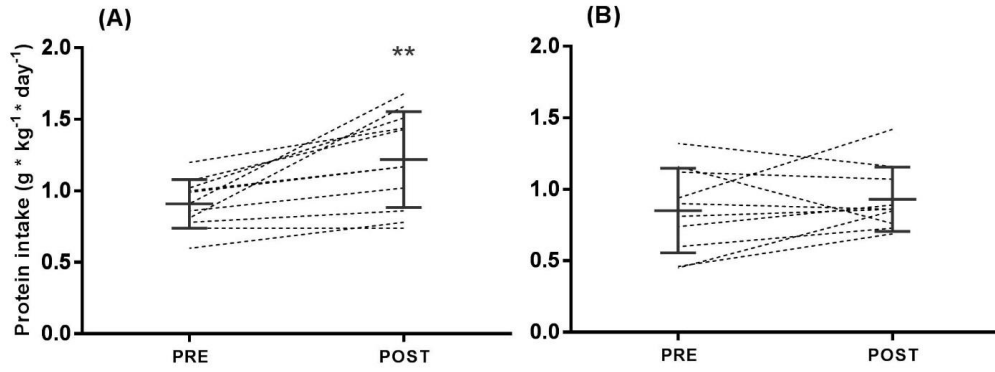


Figure 1. Change in daily protein intake in ST (A) and CON (B). ** $P < 0.01$, compared to baseline within group.

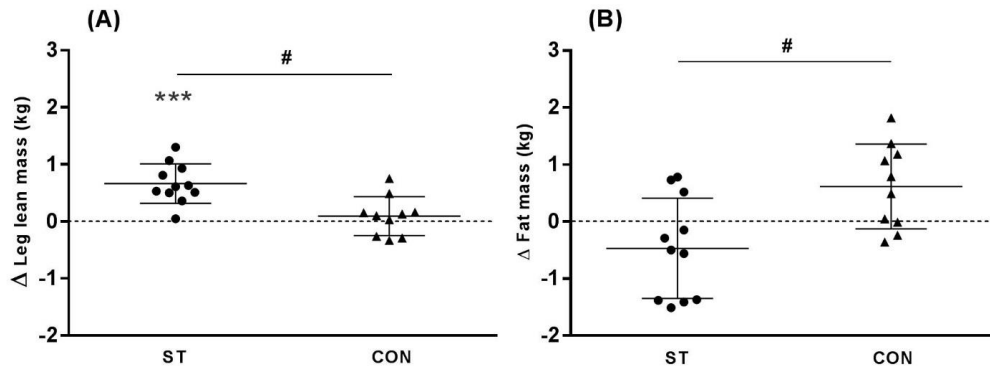


Figure 2. Change in leg lean mass (A) and total body fat mass (B), in ST (●), CON (▲). # $P < 0.05$, group x time interaction. *** $P < 0.001$, compared to baseline within group. Results are presented as individual values and with mean and standard deviation (SD) for each group.

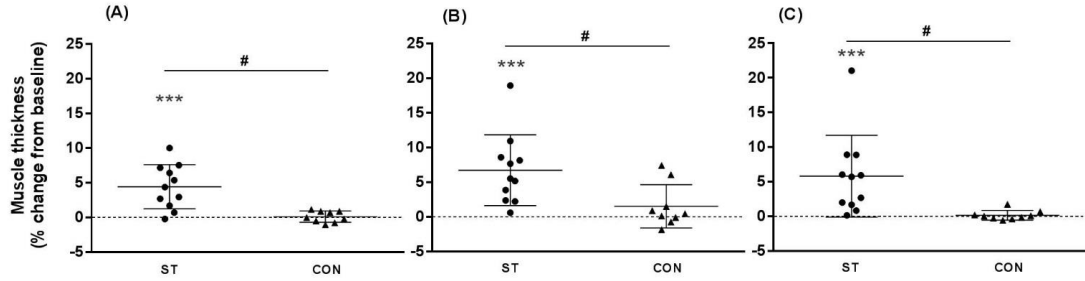


Figure 3. Percentage changes in ST (●) and CON (▲), measured in vastus lateralis (A), rectus femoris (B), and vastus intermedius (C). # $P < 0.01$, group x time interaction. *** $P < 0.001$, compared to baseline within group. Results are presented as individual values and with mean and standard deviation (SD) for each group.

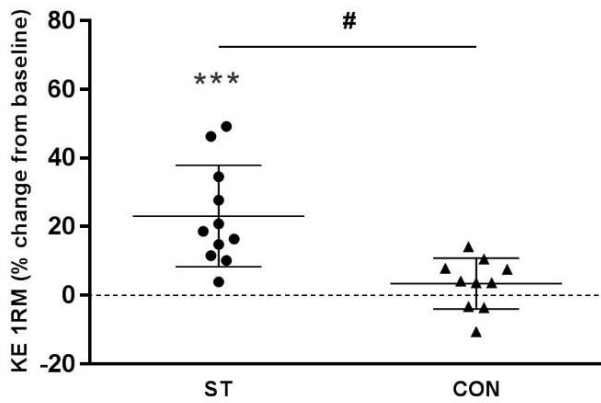


Figure 4. Percentage change in knee extension 1RM in ST (●) and CON (▲). # $P < 0.001$, group x time interaction. *** $P < 0.001$, compared to baseline within group. Results are presented as individual values and with mean and standard deviation (SD) for each group.

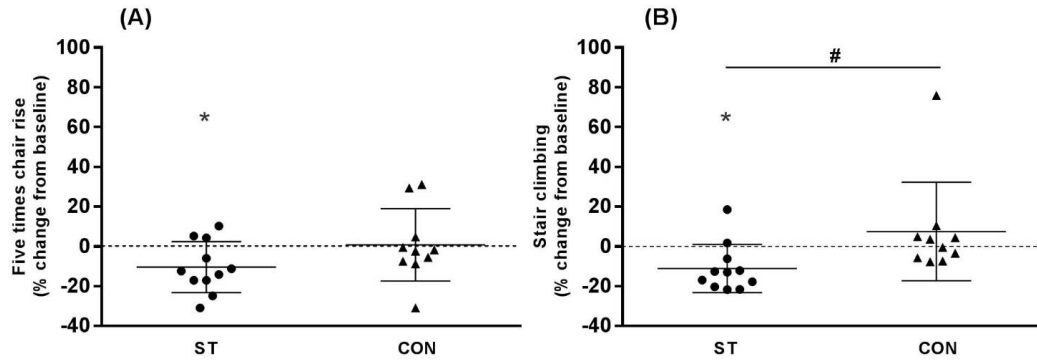


Figure 5. Percentage change in time used during five times chair rise (A) and stair climbing (B) in ST (●) and CON (▲). # $P < 0.05$, group x time interaction. * $P < 0.05$, compared to baseline within group. Results are presented as individual values and with mean and standard deviation (SD) for each group.

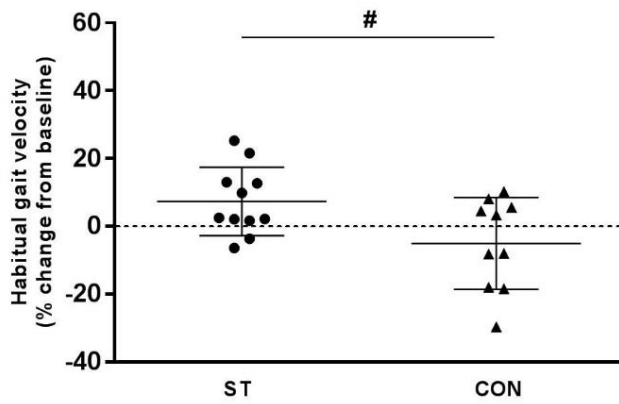


Figure 6. Percentage change in habitual gait velocity in ST (●) and CON (▲). # $P < 0.05$, group x time interaction. Results are presented as individual values and with mean and standard deviation (SD) for each group.

ARTICLE II

The impact of age and frailty on skeletal muscle autophagy markers and specific strength: A cross-sectional comparison

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ABSTRACT

Background: Aging is associated with reduced specific strength, defined as strength normalized to the cross-sectional area of a given muscle or muscle group. Dysregulated autophagy, impairing removal of dysfunctional proteins and organelles, is suggested as one of the underlying mechanisms. Therefore, the aim of this study was to investigate levels of autophagic markers in skeletal muscle in groups known to differ in specific strength. We hypothesized that autophagy would be attenuated in elderly, and even more so in pre-frail and frail elderly individuals.

Methods: Sixty-two volunteers were assigned to the following study groups: young (Y, 7 women/10 men, 29 ± 7 years), old non-frail (O-NF, 8 women/12 men, 74 ± 3 years), old pre-frail (O-PF, 6 women/7 men, 84 ± 8 years), and old frail individuals (O-F, 5 women/7 men, 88 ± 6 years). Body composition was assessed with dual-energy X-ray absorptiometry (DXA), quadriceps muscle strength by isometric maximal voluntary contraction (IMVC), and functional capacity by the short physical performance battery (SPPB), five times chair rise, and habitual gait velocity. The abundance of autophagic proteins within skeletal muscle cytosolic and membrane sub-fractions were determined by western blotting. In addition, the level of heat shock proteins and proteins involved in the regulation of protein synthesis were measured.

Results: Significant between-group differences were observed for maximal strength, relative strength and specific strength (strength per unit muscle mass). The abundance of LC3-I was higher in O-F compared to Y. If the three elderly groups were pooled, the level of LC3-II was higher in old compared to young subjects. No between-group differences were observed for p62, LC3-II/LC3-I ratio, or any of the anabolic signaling molecules. The level of cytosolic α B-crystallin was higher in old pre-frail and frail subjects compared to the young group. For cytoskeletal Hsp70, a significant difference was observed between O-PF and Y. A negative correlation was observed between cytosolic levels of LC3-I and specific strength.

Conclusions: Higher levels of LC3-I in the frail elderly individuals might represent attenuated autophagosome formation. However, higher LC3-II levels indicate an increased abundance of autophagosomes. Thus, our results could imply that both the process of autophagosome formation and autophagosome-lysosome fusion are affected in frail elderly. The higher levels of heat shock proteins might represent an auto-protective mechanism against increased levels of misfolded proteins, possibly due to inefficient degradation. In conclusion, the reduction in specific strength with aging and frailty may partly be caused by alterations in muscle protein quality control.

Keywords: Aging, protein degradation, heat shock protein, sarcopenia, muscle quality

INTRODUCTION

Aging is associated with reduced specific strength, defined as strength normalized to the cross-sectional area of a given muscle or muscle group^{1, 2}. Identifying the mechanisms behind this phenomenon is highly relevant for the prevention of functional impairment with aging. The continuous degradation of dysfunctional proteins and organelles, and the simultaneous synthesis of new proteins, allow the muscle to maintain its function and adapt to a variety of stimuli. It is generally accepted that the loss of muscle mass with aging to a great extent is caused by reduced activity and anabolic resistance, manifested by an attenuated response to anabolic stimuli such as protein intake³ and resistance training⁴. However, the less studied mechanisms of protein breakdown likely also play a part, perhaps especially in relation to the age-related reduction in specific strength⁵.

Macroautophagy, hereafter referred to as autophagy, is one of several pathways by which cellular content can be degraded. It involves the formation of a double-membrane vesicle called an autophagosome, and inclusion of cytosolic substrate within the autophagosome. Subsequently, the autophagosome fuses with a lysosome to form an autolysosome, where the engulfed organelles and proteins are degraded⁶. It is well established that autophagy is critical for the selective removal of damaged mitochondria⁷, and autophagy-deficient mice mimic several age-related characteristics such as loss of muscle mass and quality⁸, suggesting that autophagy is a key mechanism for protecting against cellular impairments during aging. Studies in rodents suggest that autophagy is indeed reduced with aging⁹⁻¹¹, but information on how autophagy is affected by aging in human skeletal muscle is scarce. Observations indicate that sedentary behavior⁵ and overweight¹² might be as important as aging *per se*. However, to our knowledge, no study has investigated autophagy in very old and frail individuals, in whom specific strength is reduced the most^{1, 13}.

Heat shock proteins (HSPs) are a family of proteins that are upregulated by cells in response to stressful conditions. These proteins demonstrate a protective role through repair, refolding of misfolded peptides and are involved in the controlled degradation of irreparable proteins^{14, 15}. Insufficient removal of damaged mitochondria by autophagy might increase the production of reactive oxygen species (ROS), thus increasing protein carbonylation and damage¹⁶. In this regard, heat shock proteins might play a crucial compensating role. Although not a consistent observation¹⁶, the level of certain heat shock proteins has been observed to increase with aging^{17, 18} and frailty¹⁹. Measuring the level of heat shock proteins in addition to markers of autophagy will thus provide a more comprehensive picture of the overall status of the cell. Anabolic and catabolic signaling is closely linked²⁰. Therefore, investigation of proteins involved in the regulation of protein synthesis will assist in the interpretation of the autophagic markers. The basis for interpreting the results will

also be improved by performing analyses on cellular sub-fractions, rather than whole cell lysate, because many of the proteins involved in muscle protein quality control alter their cellular location in response to various stimuli^{21,22}.

Hence, the aim of this study was to compare markers of autophagy, heat shock proteins and anabolic signaling molecules in young, old non-frail, pre-frail and frail individuals. We hypothesized that autophagic proteins would indicate impaired autophagy in old compared to young subjects, and that this would be even more apparent in pre-frail and frail elderly. In addition, we hypothesized that the level of heat shock proteins would be higher in frail elderly compared to young.

MATERIALS AND METHODS

Study design and participants

Baseline data from two different clinical trials performed in the period 2014-2017 was included in this study. Both studies were approved by the Regional Ethics Committee for Medical and Health Research of South-East Norway (2016/895/REK sør-øst C and 2014/834/REK sør-øst C) and performed in accordance with the Declaration of Helsinki. The nature and goals of the study were thoroughly explained, and all participants provided a written informed consent prior to inclusion. The trials were registered at clinicaltrials.gov as NCT03326648 and NCT03033953. Twenty-six women, and thirty-six men, aged from 20 to 96 years (yr.) old were assigned to the four following groups: young (Y; 29 ± 7 yr.; N= 17), old non-frail (O-NF; 74 ± 3 yr.; N= 20), old pre-frail (O-PF; 84 ± 8 yr.; N= 13) and old frail (O-F; 88 ± 6 yr.; N= 12). All subjects in Y and O-NF were healthy, independently living and recreationally active. Among the old pre-frail (O-PF) and old frail (O-F) subjects, 15 and 42% lived in a nursing home, respectively. Several of the subjects in these groups were dependent of aids, such as Nordic walking poles, canes, walkers, or wheelchairs. All subjects included in O-PF and O-F fulfilled at least two of the five Fried Frailty Criteria²³. The division into pre-frail and frail was performed using the Short Physical Performance Battery (SPPB). The SPPB consists of timed standing balance, gait speed, and timed chair-rise assessments^{24, 25}. Performance for each of these tasks is scored between 0 and 4, with a summary score of 0–12. Participants with an SPPB score ≤ 6 were assigned to O-F, whereas subjects with a score ≥ 7 were assigned to O-PF. **Table 1** provides an overview of the criteria used for inclusion. Subject characteristics are presented in **Table 2**.

Data included in this cross-sectional study was collected on three separate test days. Muscle strength and functional capacity were tested on two occasions, separated by a minimum of three days. Body composition was measured on one of these occasions. The biopsy procedure was performed on a

separate day of testing, approximately one week later. Subjects were instructed to refrain from strenuous physical activity for three days before each day of testing.

Body composition

Body composition was assessed by dual-energy X-ray absorptiometry (Lunar iDXA GE Healthcare, Madison, Wisconsin, USA). Participants were scanned from head to toe in a supine position, providing values for total lean tissue and fat mass. In addition, regional analyses were performed to measure leg lean mass. The coefficient of variation for the assessment of leg lean mass is low in our lab (CV <1.7%).

Muscle strength

Isometric maximal voluntary contraction torque (IMVC) was tested unilaterally for the knee-extensors. The participants were securely strapped with a four-point belt to the testing chair, with an angle of 90° in the hip and knee joints (0° corresponding to full extension). A strength gauge (HBM U2AC2, Darmstadt, Germany) was used to measure knee extension force. The participants performed three maximal 5-second attempts. Sixty seconds of rest was given between each attempt. The highest value from the three trials was used in the analyses. To calculate specific strength, leg IMVC was normalized to leg lean mass of the same leg (from regional DXA).

Functional capacity

Five times chair rise was performed in all three groups of elderly individuals. Participants were asked to rise from a chair to a full stand five times as fast as possible. Subjects were instructed to keep their hands crossed on their chest and lift their feet from the ground between every repetition. If they were not able to stand up without using their hands, limited use of hands was allowed. Time was measured using a compression based sensor (TC-Start Pod and Touch Pad, Brower Timing Systems, Draper, USA) in the old non-frail participants (O-NF), and a stopwatch in the old pre-frail (O-PF) and frail (O-F) participants. The fastest time of two trials was used in the analyses.

Habitual gait velocity was assessed in O-PF and O-F. Participants were asked to walk 10 meters at their normal gait velocity. Walkers, canes or Nordic walking poles were allowed only if subjects did not feel safe walking without these devices. Photocells (Brower Timing Systems, Draper, USA) were placed to measure the time between 2 and 8 meters. The mean time of three trials was used in the analyses.

Biopsy protocol

Subjects refrained from strenuous physical activity for 3 days before the muscle biopsy. On the day of the biopsy procedure, subjects arrived at the laboratory by car or public transportation after an overnight fast, and received a standardized breakfast containing oatmeal, water, sugar and butter.

Article II

The breakfast constituted 30 kJ (young), 25 kJ (old non-frail) and 20 kJ (old pre-frail and frail) per kg body mass. The protein content of the breakfast was 0.14, 0.11 and 0.09 g per kg body mass in Y, O-NF and O-PF/O-F, respectively. Water was provided ad libitum. One hour after the breakfast, a percutaneous needle biopsy was obtained from the vastus lateralis muscle from the right leg (Y and O-NF), or the left leg (O-PF and O-F). The procedure was conducted under local anesthesia (Xylocaine with adrenaline, 10 mg/ml lidocaine + 5 µg/ml adrenaline, AstraZeneca, London, UK), and approximately 200 mg ($2-3 \times 50-150$ mg) of muscle tissue was obtained using a modified Bergström technique. Tissue intended for immunoblot analyses was quickly rinsed in physiological saline, before fat, connective tissue and blood were removed and discarded. Subsequently, the sample was weighed and quickly frozen in isopentane cooled on dry ice (immunoblot analyses on cellular sub-fractions), or liquid nitrogen (immunoblot analyses of protein phosphorylation). Samples intended for immunohistochemical analyses were embedded in OCT (Cellpath O.C.T embedding matrix, Newtown, UK) before being frozen in isopentane cooled down on liquid nitrogen. All muscle samples were stored at -80°C for later analyses.

Pre-analytical analyses

Approximately 50 mg of muscle tissue was homogenized in 1 ml T-PER (Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA), 20 µl Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and 20 µl EDTA (Thermo Scientific). Another 50 mg of muscle sample was homogenized and fractionated into cytosolic, membrane, cytoskeletal, and nuclear fractions using a commercial fractionation kit (ProteoExtract Subcellular Proteome Extraction Kit, Cat#539790, Calbiochem, EMD Biosciences, Germany) according to the manufacturer's procedures. Protein concentration was measured using a commercial kit (BioRad DC protein microplate assay, Cat#0113, Cat#0114, Cat#0115, Bio-Rad Laboratories, Hercules, CA, USA), a filter photometer (Expert 96, ASYS Hitech, UK) and the software provided (Kim, ver. 5.45.0.1, Daniel Kittrich, Prague, Czech Republic).

Immunoblot analyses

Equal amounts of protein were loaded per well (5-30 µg) and separated by 4-20% gradient Mini-PROTEAN TGX Stain-Free Precast protein gels (4568093, Bio-Rad Laboratories). Electrophoresis was performed under denaturized conditions for 30-35 min at 200 volts in Tris/Glycine/SDS running buffer (161-0732, Bio-Rad Laboratories). After gel-electrophoresis, proteins were transferred onto a PVDF-membrane at 100 volts for 60 min (CriterionTM Blotter; Tris/Glycine buffer 161-0734, Bio-Rad Laboratories). Membranes were blocked at room temperature for 2 hours in a TBS solution with 5% fat-free skimmed milk and 0.1% Tween 20 (TBS, 170-6435, Bio-Rad Laboratories; Tween-20, 437082Q, VWR International, Radnor, PA, USA; Skim milk,

Article II

1.15363.0500, Merck, Darmstadt, Germany). Blocked membranes were incubated overnight at 4°C with primary antibodies (see below) followed by incubation with appropriate secondary antibodies for 1 h at room temperature (see below). Between stages, membranes were washed in 0.1% TBS-t. Protein stripping was conducted for phospho-proteins using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL, USA). All samples were analyzed in duplicates, and bands were visualized using an HRP-detection system (Super Signal West Dura Extended Duration Substrate, 34076, Thermo Fisher Scientific). Chemiluminescence was measured using a ChemiDoc MP System (Bio-Rad Laboratories), and band intensities were measured with Image Lab (Bio-Rad Laboratories). All intensities were normalized to a control sample that was applied in each gel (in duplicates), to allow comparisons between gels.

Primary and secondary antibodies were diluted in a 1% fat-free skimmed milk and 0.1% TBS-t solution. Antibodies against p70S6K (2708), phospho-p70S6K Thr389 (9234), 4E-BP1 (9644), phospho-4EBP1 Thr37/46 (2855), eEF2 (2332), phospho-eEF2 Thr56 (2331) and LC3-I/II (2775) were obtained from Cell Signaling Technology (Beverly, MA, USA), whereas the antibody against p62 (ab56416) was obtained from Abcam (Cambridge, UK). Antibodies against α B-crystallin (ADI-SPA-222) and Hsp70 (ADI-SPA-812) were purchased from Enzo Life Sciences (Farmingdale, NY, USA), and secondary antibodies from Cell Signaling Technology (goat anti-rabbit IgG, 7074) and Thermo Scientific (goat anti-mouse, 31430). Representative Western blots are presented in **Figure 1**.

Immunohistochemical analyses

Immunohistochemical analyses were performed in a subset of forty-two subjects. Samples were cut at an 8- μ m thickness at -21°C using a cryostat (CM1860 UV, Leica Microsystems GmbH, Nussloch, Germany), mounted on microscope slides (SuperFrost Plus, Thermo Scientific), and air-dried before being stored at -80°C. Muscle sections were blocked in 1% BSA (bovine serum albumin) in PBS containing 0.05% Tween-20 (PBS-t) before overnight incubation at 4°C with primary antibodies against dystrophin (Ab15277, Abcam, Cambridge, UK) and myosin heavy chain I (BA-D5, developed by Schiaffino, S., obtained by DSHB, Iowa, IA, USA) diluted in the blocking buffer. Thereafter, sections were washed 3 \times 10 min in PBS-t solution, followed by incubation for 60 min at room temperature with appropriate secondary antibodies (Alexa Fluor 488, A11001 or Alexa Fluor 594, A11012, Invitrogen Molecular Probes, Carlsbad, CA, USA) diluted in the blocking buffer. After incubation, the sections were again washed 3 \times 10 min before they were covered with a coverslip and mounted with ProLong Gold Antifade Reagent (P36935, Invitrogen Molecular Probes). Sections were left to dry overnight at room temperature before viewed and imaged under the microscope. Muscle sections were visualized and micrographed using a 4x 0.13

NA air objective (UplanFL N, Olympus Corp., Tokyo, Japan) using a high-resolution camera (DP72, Olympus Corp.) mounted on a light microscope (BX61, Olympus Corp.) with a fluorescence light source (X-Cite, 120PCQ, EXFO Photonic Solutions Inc., Mississauga, ON, Canada). Fiber specific cross-sectional area and fiber type distribution were analyzed using TEMA software (CheckVision, Hadsund, Denmark). The mean number of analyzed fibers per subject was 423 (Range: 40-858).

Statistical analyses

Data in tables are presented as mean \pm standard deviation. Figures display individual values with mean and standard deviation, or box plots with median, upper and lower quartile and lowest and highest value. To assess whether or not data sets were normally distributed, the D'Agostino-Pearson omnibus normality test was performed. Non-normally distributed data were log-transformed prior to the statistical analyses. Statistical comparisons between groups were performed using a one-way ANOVA followed by a Tukey's multiple comparison test. Differences between men and women were investigated using the Student's *t*-test. Pearson correlation coefficients were calculated to investigate the relationships between variables. Statistical significance was set at $P < 0.05$. Statistical analyses and preparation of figures were performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

The O-PF and O-F groups were significantly older compared with the O-NF group (**Table 2**). Furthermore, differences between the groups were observed for lean body mass, leg lean mass, muscle strength and five times chair rise, whereas BMI and body fat percentage were similar between groups. As per study design, the O-F group had lower SPPB score and habitual gait velocity compared to the O-PF group.

Specific strength

Specific strength was significantly higher in young subjects (19.8 ± 3.0 Nm/kg), compared to non-frail (14.1 ± 2.7 Nm/kg), pre-frail (12.9 ± 1.9 Nm/kg) and frail elderly individuals (10.9 ± 2.9 Nm/kg) (all $P < 0.001$) (**Figure 2**). Furthermore, a significant difference in specific strength was observed between old non-frail and old frail subjects ($P = 0.014$).

Muscle fiber area and fiber type distribution

Type I muscle fiber area was not significantly different between young (4760 ± 715 μm^2), old non-frail (4406 ± 581 μm^2), pre-frail (4138 ± 1186 μm^2) or frail (3811 ± 1350 μm^2) individuals ($P = 0.169$; **Figure 3A**). If all subjects from the old groups were pooled, type I fiber area tended to be lower compared to the young group ($P = 0.074$). Type II fiber area was significantly larger in Y

($5384 \pm 1576 \mu\text{m}^2$), compared to O-NF ($3500 \pm 1535 \mu\text{m}^2$, $P = 0.045$), O-PF ($3084 \pm 1604 \mu\text{m}^2$, $P = 0.01$) and O-F ($2806 \pm 1090 \mu\text{m}^2$, $P = 0.002$; **Figure 3B**). No significant differences were observed between the groups of older subjects. The proportion of type I fibers was not different in Y ($46 \pm 14\%$), O-NF ($47 \pm 18\%$), O-PF ($46 \pm 19\%$) and O-F ($48 \pm 13\%$) ($P = 0.986$). Furthermore, the percentage area occupied by type I muscle fibers was not significantly different between the four groups, with $44 \pm 15\%$, $54 \pm 18\%$, $54 \pm 19\%$, and $55 \pm 15\%$ in Y, O-NF, O-PF and O-F, respectively ($P = 0.391$).

Autophagy

P62 (also known as SQSTM1) was measured both in the cytosolic and membrane fraction, since p62 first targets polyubiquitinated organelles and proteins, and thereafter recruit these substrates to the phagophore membrane (immature autophagosome) where p62 interacts with LC3-II. Thereafter, both p62 and the substrates are degraded²⁶. In the present study, no between-group differences were observed neither for cytosolic nor membrane-bound p62 (**Figure 4A/B**).

LC3-I was also measured both in the cytosolic and membrane fraction, because it is unclear whether LC3-I is converted to LC3-II prior to, or after its binding to the phagophore membrane. Old non-frail, pre-frail and frail subjects displayed 48, 63 and 100% higher levels of cytosolic LC3-I compared to young individuals, respectively (**Figure 4C**). However, a significant difference was only observed between Y and O-F ($P = 0.005$). Furthermore, the level of LC3-I in the membrane fraction was higher in both old non-frail and old frail subjects compared to the young group (Y vs. O-NF; $P = 0.016$, Y vs. O-F; $P = 0.004$) (**Figure 4D**). Interestingly, pre-frail elderly did not differ from the young group.

LC3-I is subsequently converted to its lipidated form, LC3-II²⁷, and this conversion represents a crucial step for the formation of autophagosomes. Indeed, the amount of LC3-II is closely correlated with the number of autophagosomes²⁸. The level of LC3-II was 43, 51, and 63% higher in O-NF, O-PF and O-F, compared with Y, but the difference did not reach statistical significance for any of the groups (**Figure 4E**). However, if all subjects from the old groups were pooled, the difference between young and old participants was statistically significant for LC3-II ($P = 0.021$). In addition to separate analyses of LC3-I and LC3-II, the ratio between the two is commonly used to estimate the rate of autophagosome formation. The ratio, calculated as $(\text{LC3-II}_{\text{membrane}}) / (\text{LC3-I}_{\text{cytosol}} + \text{LC3-I}_{\text{membrane}})$ in the present investigation, was not different between Y (1.90 ± 0.69), O-NF (1.76 ± 0.91), O-PF (2.29 ± 1.46) and O-F (1.80 ± 1.47) (**Figure 4F**).

Heat shock proteins

Hsp70 measured in the cytosolic fraction was 32, 47 and 37% higher in O-NF, O-PF and O-F, respectively, compared with the young subjects, but neither comparison reached statistical significance (Y vs. O-NF: $P = 0.081$, Y vs. O-PF: $P = 0.052$, Y vs. O-F: $P = 0.159$) (**Figure 5A**). If all subjects from the old groups were pooled, the difference between young and old participants was statistically significant ($P = 0.003$). In the cytoskeletal fraction, higher levels of Hsp70 were observed in old pre-frail, compared to young individuals ($P = 0.008$), whereas the difference between young and old frail individuals did not reach statistical significance ($P = 0.125$) (**Figure 5B**).

In the present study, α B-crystallin was not detected in the cytoskeletal fraction, thus only results for the cytosolic fraction is presented. Old pre-frail (O-PF) and frail (O-F) subjects displayed 37% ($P = 0.008$) and 60% ($P < 0.001$) higher levels of cytosolic α B-crystallin compared to the young subjects, respectively (**Figure 5C**). The old non-frail individuals (O-NF) did not significantly differ from Y, and displayed lower levels than O-F ($P = 0.001$).

Anabolic proteins

No differences between the four groups were observed for phosphorylated or total p70S6K, 4E-BP1 or eEF2 (**Figure 6**). Furthermore, no between-group differences were observed when investigating the ratio of phosphorylated to total amount of these proteins (results not shown).

Sex differences

The three elderly groups were pooled to investigate differences in protein levels between elderly men and women. Thus, this comparison represented 26 men (80 ± 8 yr.) and 19 women (82 ± 9 yr.). The level of membrane LC3-II was lower ($P = 0.049$), whereas the level of LC3-I tended to be higher ($P = 0.076$), in women compared to men. Hence, also the LC3-II/LC3-I ratio was lower in women ($P = 0.033$), indicative of reduced autophagic flux. In addition, the level of cytosolic Hsp70 was 22% higher in women than in men ($P = 0.041$), and a similar tendency was observed in the cytoskeletal fraction ($P = 0.074$). No sex differences were observed for the other proteins investigated. Interestingly, specific strength was also lower in women (11.7 ± 2.9 Nm/kg) than in men (13.9 ± 2.9 Nm/kg; $P = 0.01$).

Relationships between biopsy variables and muscle function

Correlations between biopsy analyses and measurements of muscle function and functional capacity were investigated across the groups of elderly subjects (O-NF, O-PF and O-F) (**Table 3**). Absolute strength was positively correlated with muscle fiber cross-sectional area ($r = 0.49$, $P = 0.007$), and negatively correlated with cytosolic LC3-I ($r = -0.38$, $P = 0.012$). Cytosolic LC3-I was also

negatively correlated with specific strength ($r = -0.34$, $P = 0.028$). Furthermore, a positive correlation was observed between specific strength and the ratio of membrane-bound to cytosolic p62 ($r = 0.41$, $P = 0.006$). In addition, a positive correlation was observed between fat percentage and cytosolic p62 ($r = 0.36$, $P = 0.016$). No significant correlations were observed between any of the autophagic markers and functional capacity assessed by five times chair rise and habitual gait velocity (**Table 3**).

DISCUSSION

To the best of our knowledge, this is the first study to investigate the abundance of autophagic markers and heat shock proteins, in multiple cellular fractions, in groups vastly different in age and functional capacity. The results for body composition and muscle strength demonstrate that frailty is characterized by a very low muscle mass and specific strength. Moreover, we observed between-group difference for autophagy markers and certain heat shock-proteins. Collectively, our findings indicate altered muscle protein quality control in older individuals, and that these effects are more pronounced in pre-frail and frail elderly.

The influence of age and frailty on muscle fiber cross-sectional area and fiber type distribution

Although not significant, the type I fibers from non-frail, pre-frail and frail elderly individuals were 7, 19, and 20% smaller than those of the young individuals, corresponding well with previous observations²⁹⁻³¹. The size of the type II fibers, which were 35% (O-NF), 44% (O-PF) and 48% (O-F) smaller than those of the young group, are also in line with other studies.²⁹⁻³¹ Hence, although type I fibers tend to undergo some atrophy with aging, our findings support the notion that the age-related atrophy mainly affects type II fibers. An often debated issue is whether or not fiber type composition of skeletal muscle change with aging. Despite some evidence of altered proportion³², most studies do not observe differences between young and old individuals^{29-31, 33}. Our results are in support of the latter, demonstrated by an almost identical type I fiber proportion in the four groups (46-48%), despite vast differences in age.

The influence of age and frailty on autophagy

The level of LC3-I was higher in old frail compared to young individuals, both in the cytosolic and membrane sub-fraction. Old non-frail individuals also displayed higher levels of LC3-I in the membrane fraction, compared to young subjects. Increased levels of LC3-I may represent increased processing from proLC3, reduced lipidation to LC3-II, or increased delipidation of LC3-II. Therefore, the ratio of LC3-II to LC3-I is commonly assessed to estimate the rate of autophagosome formation³⁴. In the present study, the LC3-II/LC3-I ratio did not differ between groups, because the higher levels of LC3-I in old frail subjects coincided with a tendency towards higher LC3-II levels.

Article II

Interestingly, a trend for higher LC3-I and LC3-II levels was recently observed in both untrained and trained elderly (mean age: 62 years), compared to young individuals³⁵. Moreover, a similar tendency for LC3-I has been observed previously in 70-year-olds³⁶. The consensus that autophagy declines with aging stems primarily from studies performed in rodents⁹⁻¹¹. In addition to changes in LC3-I and LC3-II, an age-related decrease in LAMP-2A protein expression was observed in two of these studies^{9, 10}. LAMP-2A is involved in the autophagosome-lysosome fusion³⁷, but whether or not this process is affected by aging in human skeletal muscle is unknown. In this regard, it is important to acknowledge that the possible increase in autophagosome number in the elderly individuals, as suggested by the tendency of higher LC3-II levels, does not equal increased autophagic flux, since the functionality and effectiveness of autophagy also directly depends on the capacity of the lysosome to degrade the delivered cargo³⁴. Hence, higher levels of both LC3-I and LC3-II would also be expected in a situation where autophagosome formation is blunted, but where the fusion of autophagosomes and lysosomes is blunted even more.

We did not observe between-group differences for p62, neither in the cytosolic nor membrane sub-fraction. To the best of our knowledge, the level of p62 in different cellular sub-fractions has not been investigated in human skeletal muscle previously. Sakuma and colleagues³⁸ observed higher levels of p62 in the cytosolic, but not membrane fraction, in quadriceps muscle of old mice. Accumulation of p62 has been observed together with reductions in the LC3-II/LC3-I ratio⁹, and thus interpreted as attenuated autophagic flux. Hence, our observation of similar p62 levels across groups seems to contrast the observed accumulation of LC3-I. However, the degree of p62 accumulation will likely depend on which step of the autophagy process that is blunted. It is plausible that a reduction in autophagy initiation results in more cytosolic p62 accumulation than a reduction in autophagosome-lysosome fusion. Moreover, because p62 regulate a range of metabolic processes^{39, 40}, it should be interpreted carefully. A positive correlation was observed between cytosolic p62 and body fat percentage in our elderly subjects, in line with a study observing higher levels of p62 in old overweight, compared with normal-weight individuals¹². Hence, the lack of p62 accumulation in our elderly groups could be related to the fact that BMI and body fat percentage was similar in the four groups (**Table 2**). Interestingly, in the study by Potes and colleagues¹², the accumulation of p62 was accompanied by higher levels of both LC3-I and LC3-II, together with lower gene expression of LAMP-2A. Their findings therefore imply that several of the autophagic steps may be affected in overweight elderly individuals. Our findings expand on these observations, by demonstrating differences in autophagy also between young and frail normal-weight elderly individuals, although perhaps to a lesser extent given the lack of p62 accumulation.

Article II

A previous study observed higher phosphorylation of mTORC1 and p70S6K in old compared to young individuals at rest⁴¹. Based on the substantial cross-talk between mTORC1 signaling and autophagy⁴², it could be hypothesized that the between-group differences we observed for autophagic proteins were related to hyperphosphorylation of the mTORC1 pathway in the elderly. However, no between-group differences were observed for mTORC1 targets p70S6K, 4E-BP1 and eEF2 in the present study, suggesting that this was not the case.

Autophagy and specific strength

Inhibition of autophagy leads to reduced single fiber specific force in mice⁵, presumably due to insufficient degradation of damaged and dysfunctional peptides and proteins. Given the age-related decline in single fiber specific force⁴³, along with observations of attenuated autophagy⁵, it is possible that dysregulated autophagy might be one of the factors contributing to the reduction in muscle quality in humans. Thus, we investigated the relationship between autophagy markers and specific strength in the present investigation. Specific strength was negatively correlated with cytosolic LC3-I and positively correlated with the ratio of membrane-bound to cytosolic p62. Although speculative, the membrane-to-cytosolic ratio of p62 might represent the rate of substrate delivery to the maturing autophagosome, whereas higher levels of LC3-I might represent attenuated lipidation to LC3-II, and thus attenuated formation of autophagosomes. Accordingly, both of these correlations indicate a positive relationship between autophagy and specific strength. Interestingly, our findings also suggest that the autophagic flux is higher in elderly men than in women, and this corresponded with the observation that also specific strength was higher in elderly men in the present study. The possible sex-specific difference in skeletal muscle autophagy warrants further investigation.

The influence of age and frailty on heat shock proteins

Higher levels of cytoskeletal Hsp70 were observed in old pre-frail, compared to young and old non-frail subjects, whereas the level of cytosolic α B-crystallin was higher in both pre-frail and frail elderly, compared to the young group. An age-related increase in α B-crystallin has been observed previously, both in rodent⁴⁴ and human skeletal muscle¹⁷. In contrast, Beltran Valls and colleagues did not observe differences in α B-crystallin protein expression between young, old, and old sarcopenic subjects, neither in the sarcoplasmic nor myofibrillar fraction¹⁶. However, the level of Hsp70 tended to be higher in the old sarcopenic subjects¹⁶. Furthermore, Joseph and colleagues (2012) observed higher levels of Hsp70 in low-functioning compared to high-functioning elderly individuals¹⁹, suggesting that physical function might be more important than aging per se. The latter might explain why higher levels of cytoskeletal Hsp70 and cytosolic α B-crystallin were observed in pre-frail and frail, but not non-frail elderly. We propose that the differences observed for the heat shock proteins were due to increased cellular stress in elderly, and particularly in pre-

frail and frail elderly subjects. The possible up-regulation might be an auto-protective mechanism against increased levels of misfolded proteins, perhaps due to inefficient degradation. Interestingly, protein levels of Hsp70 were reduced after resistance-type training in rats⁴⁵, suggesting that the possible increase in cellular stress with aging and frailty might be reversible.

Limitations

The cross-sectional nature of the study design cannot discern causation of the observed associations. Second, our index of specific strength was suboptimal, since the strength of the quadriceps femoris muscle was normalized to the lean mass of the whole leg. Another limitation is the lack of information about the stage of autophagosome-lysosome fusion, because we were unable to obtain antibodies with satisfactory specificity for the proteins of interest. Furthermore, an important difference between the present study and a majority of the previous studies in this field is that our subjects received a standardized breakfast 1 hour prior to the muscle biopsy (after an overnight fast). Thus, our study provides information about the post-prandial rate of autophagy after a high-carbohydrate meal. Although the breakfast contained only small amounts of protein in all groups, we cannot exclude the possibility that some of the between-group differences we observe for autophagic proteins are at least partly due to differences in meal amount, or differences in digestion, uptake, transport, or cellular signaling responses to the nutrients consumed. However, no between-group differences were observed for the anabolic proteins investigated (p70S6K, 4E-BP1 and eEF2), suggesting that the mTOR pathway, an important negative regulator of autophagy, was equally affected by the standardized breakfast across groups.

Perspectives and conclusions

The annual decline in muscle strength can be up to three times greater than the reduction in muscle cross-sectional area in elderly individuals². Given the close relationship between muscle strength and functional capacity⁴⁶, and the observation that small-to-moderate limitations in functional capacity is indicative of future mobility loss⁴⁷ and even death⁴⁸, it is imperative to gain knowledge about the mechanisms underlying the decline in specific strength. In the present study, we observed between-group differences for certain autophagic markers and heat shock proteins, suggesting that these two processes of muscle protein quality control are affected in old non-frail individuals, and that these effects are even more prominent in frail elderly. Furthermore, correlations between specific strength and some markers of autophagy were observed, suggesting that the two factors may be linked. Future investigations should aim to gain knowledge about how aging and frailty affects the different stages of the autophagic process, and how interventions like exercise, nutrition and medication could influence autophagic processes and specific strength.

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TABLES

Table 1. Inclusion criteria in the four groups.

Group	Inclusion criteria
Young (Y)	Age < 45 No strength training past 6 months
Old Non-Frail (O-NF)	Age > 70 No strength training past 6 months
Old Pre-Frail (O-PF)	Age > 70 No strength training past 6 months Fried Frailty Criteria $\geq 2/5$
Old Frail (O-F)	Age > 70 No strength training past 6 months Fried Frailty Criteria $\geq 2/5$ SPPB ≤ 6

SPPB, Short Physical Performance Battery

Table 2. Subject characteristics.

Characteristics	Young	Old Non-Frail	Old Pre-Frail	Old Frail
N (♂/♀)	(10/7)	(12/8)	(7/6)	(7/5)
Age (years)	29 ± 7 ^a	74 ± 3 ^b	84 ± 8 ^c	88 ± 6 ^c
Body mass (kg)	80.5 ± 14.4 ^a	76.7 ± 15.0 ^{ab}	69.8 ± 17.8 ^{ab}	64.0 ± 11.1 ^b
Lean body mass (kg)	54.3 ± 9.5 ^a	50.1 ± 9.7 ^{ab}	45.8 ± 11.3 ^{ab}	41.8 ± 5.6 ^b
Leg lean mass (kg)	19.3 ± 3.8 ^a	17.3 ± 4.3 ^{ab}	15.3 ± 4.8 ^b	14.2 ± 2.4 ^b
Body fat (%)	29.9 ± 7.4	31.8 ± 7.6	32.1 ± 8.8	31.9 ± 5.6
BMI (kg/m ²)	25.3 ± 3.7	25.5 ± 4.2	24.7 ± 4.9	23.6 ± 3.4
KE IMVC (Nm)	191 ± 46 ^a	121 ± 38 ^b	95 ± 27 ^{bc}	76 ± 26 ^c
Relative strength (MVC/BM)	2.38 ± 0.41 ^a	1.59 ± 0.36 ^b	1.41 ± 0.28 ^{bc}	1.22 ± 0.29 ^c
Chair rise x 5 (sec)	-	7 ± 2 ^a	12 ± 3 ^a	29 ± 29 ^b
Gait velocity (m/s)	-	-	0.85 ± 0.15 ^a	0.55 ± 0.21 ^b
SPPB score*	-	-	7.8 ± 1.7 ^a	3.4 ± 1.3 ^b

Data are presented as mean ± standard deviation. BMI, body mass index; BM, body mass; SPPB, Short Physical Performance Battery; KE, knee extension; MVC, maximal voluntary contraction. Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

Table 3. Correlations between absolute strength, specific strength, functional capacity and markers of autophagy in the elderly subjects (O-NF, O-PF, and O-F).

Measure	KE IMVC (Nm)	SS (Nm/LLM)	Five times chair rise (s)	Gait velocity (m/s)
Mean muscle fiber area	.49** (29)	.19 (29)	.26 (31)	.11 (20)
P62 cytosol	-.11 (43)	-.24 (43)	.27 (45)	.08 (25)
P62 membrane	.15 (43)	.27 (43)	.00 (45)	.12 (25)
P62 ratio (membrane/cytosol)	.26 (43)	.41** (43)	-.17 (45)	.13 (25)
LC3-I cytosol	-.38* (43)	-.34* (43)	.13 (45)	-.20 (25)
LC3-I membrane	-.25 (43)	-.14 (43)	-.07 (45)	-.32 (25)
LC3-II membrane	.10 (43)	.03 (43)	.24 (45)	.06 (25)
LC3-II/LC3-I ratio	.29 (43)	.20 (43)	.15 (45)	.30 (25)

Data are presented as correlation coefficients (r), and numbers in parenthesis denote the number of XY pairs for each correlation. KE: knee extension, IMVC: isometric maximal voluntary contraction. SS: specific strength measured as maximal strength normalized to single leg lean mass (LLM, kg). * $P < 0.05$, ** $P < 0.01$. Gait velocity was only tested in O-PF and O-F.

FIGURES

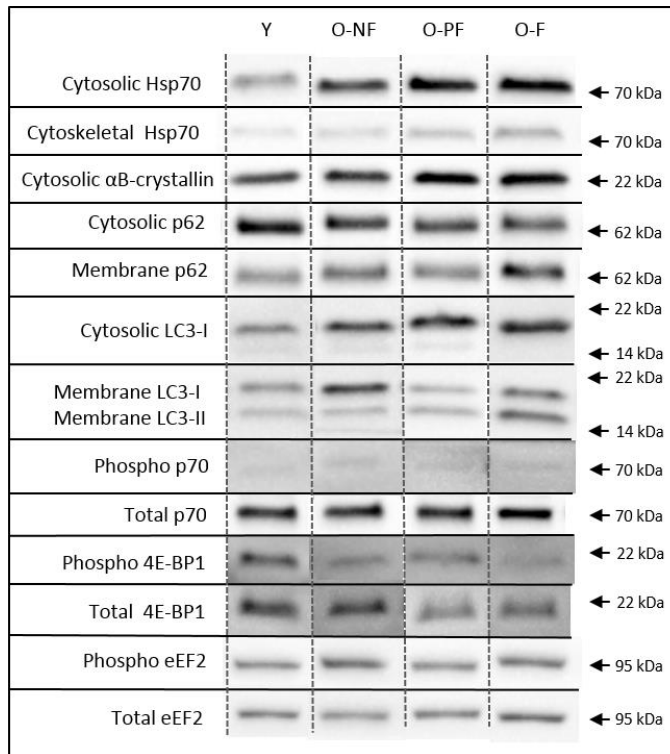


Figure 1. Representative Western blots for all measured proteins, in young (Y), old non-frail (O-NF), pre-frail (O-PF) and frail (O-F) individuals. kDa, kilodaltons.

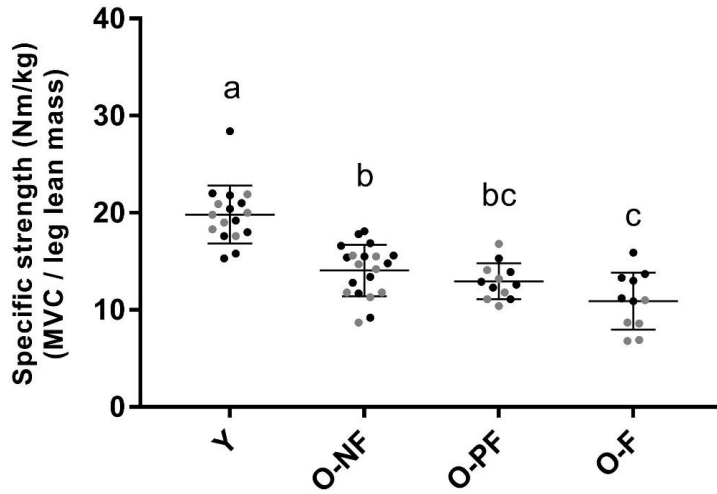


Figure 2. Specific strength (knee extension maximal isometric voluntary contraction normalized to single leg lean mass) in young (Y), old non-frail (O-NF), pre-frail (O-PF), and frail (O-F) subjects. Data presented are individual values with mean \pm SD. Women (grey), men (black). Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

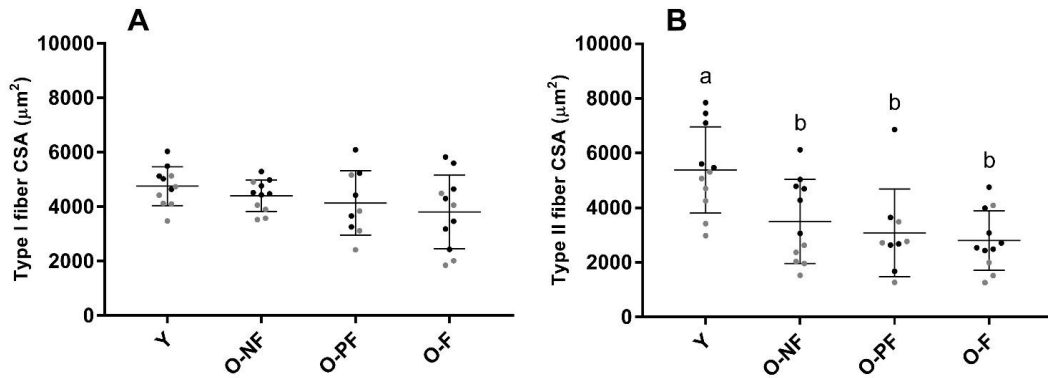


Figure 3. Muscle fiber cross-sectional area (CSA; μm^2) in type I (panel A) and type II fibers (panel B) in young (Y), old non-frail (O-NF), old pre-frail (O-PF), and old frail (O-F) subjects. Data are presented as individual values with mean \pm SD for women (grey) and men (black). Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

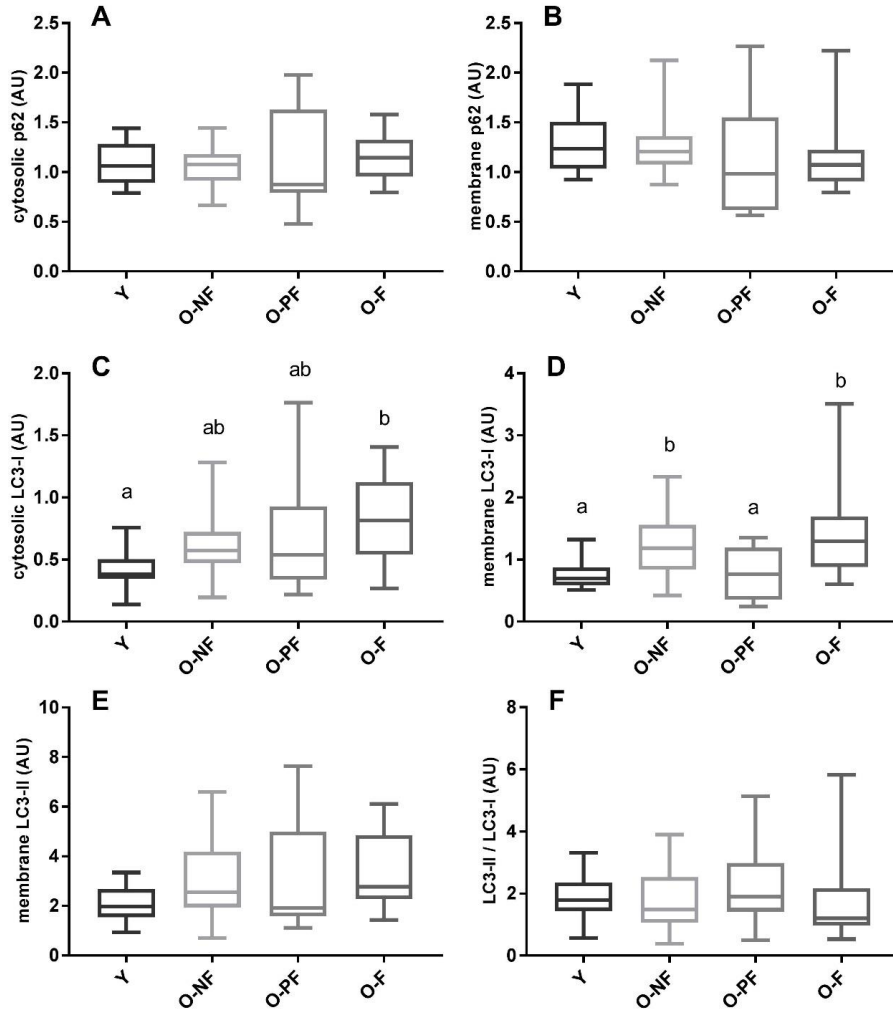


Figure 4. Autophagy proteins young (Y), old non-frail (O-NF), pre-frail (O-PF), and frail (O-F) subjects. Presented are results for cytosolic p62 (Panel A), membrane p62 (Panel B), cytosolic LC3-I (Panel C), membrane LC3-I (Panel D), membrane LC3-II (Panel E), and LC3II/LC3-I ratio (Panel F). Data are presented as box plots with median, upper and lower quartile and lowest and highest value. Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

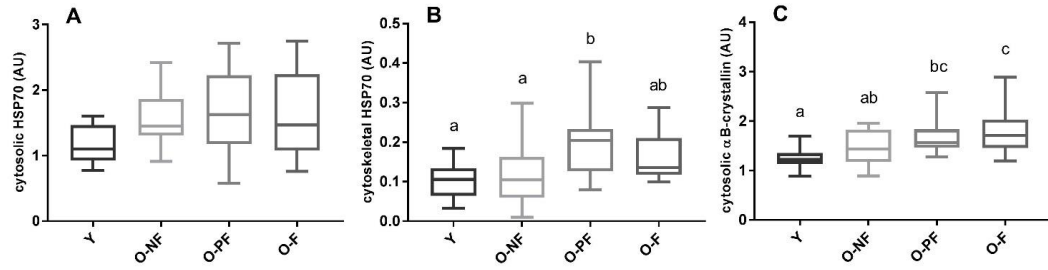


Figure 5. Hsp70 in the cytosolic (panel A) and cytoskeletal fraction (panel B), and cytosolic α B-crystallin (panel C) in young (Y), old non-frail (O-NF), pre-frail (O-PF) and frail (O-F) subjects. Data are presented as box plots with median, upper and lower quartile and lowest and highest value. Groups not sharing the same letter are significantly different from each other ($P < 0.05$).

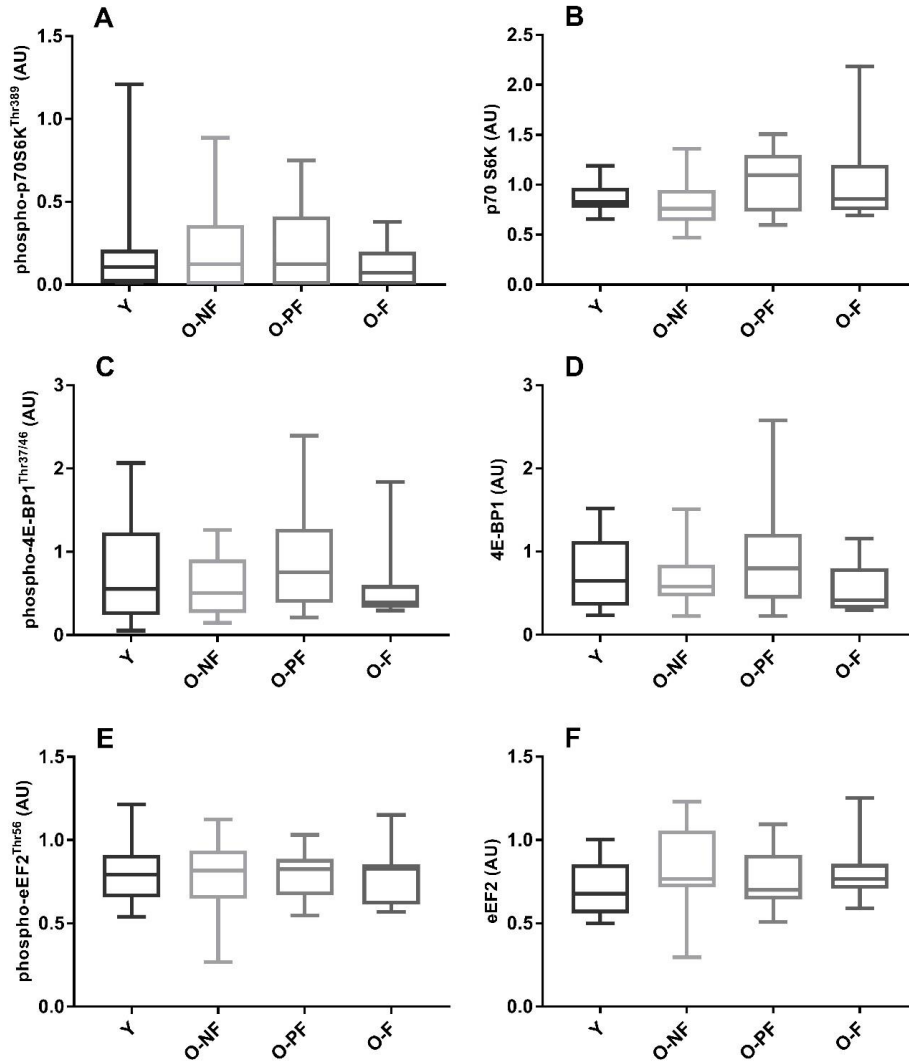


Figure 6. Phosphorylated and total level of anabolic signalling molecules in young (Y), old non-frail (O-NF), pre-frail (O-PF) and frail (O-F) subjects. Presented are results for phosphorylated p70S6K^{Thr389} (Panel A), total p70S6K (Panel B), phosphorylated 4E-BP1^{Thr37/46} (Panel C), total 4E-BP1 (Panel D), phosphorylated eEF2^{Thr56} (Panel E), and total eEF2 (Panel F). Data are presented as box plots with median, upper and lower quartile and lowest and highest value.

ARTICLE III

Effects of 10 weeks of heavy load strength training on muscle activation level, muscle quality and single fiber function in frail elderly men and women

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ABSTRACT

The improvement in muscle strength generally exceeds the increase in muscle size following strength training in frail elderly, highlighting the complex etiology of strength deficit in aging. The aim of this study was to investigate the effect of heavy load strength training on a broad number of factors related to specific strength in frail elderly. Thirty-four frail elderly men ($n = 18$) and women ($n = 16$) aged 67 to 98 (86 ± 7 years) were randomized to either a group performing strength training twice a week for ten weeks (ST), or a non-exercising control group (CON). Knee extension muscle strength was tested as one repetition maximum (1RM) and isometric maximal voluntary contraction torque (MVC). Muscle activation was assessed by the interpolated twitch technique, and muscle density (mean Hounsfield units [HU]) and intermuscular adipose tissue (IMAT) by computed tomography scans of quadriceps femoris. Muscle biopsies from vastus lateralis were obtained to investigate changes in intramyocellular lipids and single fiber specific tension. In ST, knee extension 1RM and MVC improved by 17 and 7%, respectively. Muscle cross-sectional area of quadriceps femoris increased by 7%, accompanied by increased muscle density (+ 1.5 HU). No changes in IMAT, voluntary activation level, single fiber specific tension or lipid content were observed. The latter suggests that the training-induced increase in muscle density was not due to changes in skeletal muscle lipid content, but may instead reflect an increased ratio of muscle tissue relative to adipose tissue, due to the gains in muscle mass. These findings indicate that the increases in muscle strength was mainly due to an increase in muscle quantity, not quality.

Keywords: Resistance exercise, sarcopenia, specific strength, muscle attenuation, lipid content

INTRODUCTION

A hallmark characteristic of aging is the gradual loss of skeletal muscle mass that begins as early as the fifth decade of life (Hughes et al., 2001). After the age of seventy, the annual reduction in thigh muscle cross-sectional area is approximately 1 and 0.7 % in men and women, respectively (Delmonico et al., 2009). However, the loss of muscle strength greatly exceeds the reductions in muscle size, with annual declines of 3-4% (Delmonico et al., 2009). Consequently, specific strength, defined as the ratio between the strength and cross-sectional area of a muscle or muscle group, declines accordingly. Strength training is a well-established counter-measure against the declines in muscle mass, strength and functional (Hortobagyi et al., 2015; Peterson, Rhea, Sen, & Gordon, 2010; Peterson, Sen, & Gordon, 2011). Interestingly, the improvement in muscle strength greatly exceeds the increase in muscle size (Reeves, Narici, & Maganaris, 2004), and this is particularly evident in individuals of very high age or with reduced functional capacity (Bechshoft et al., 2017; Chale et al., 2013). Both the age-related reduction and the training-induced increase in specific strength is likely multi-factorial, and both neural and muscular factors are thought to play a role.

The ability to fully activate the muscles has been observed to be reduced both with aging and degree of frailty (Venturelli et al., 2015). In healthy elderly, minimal or relatively small improvements in activation level are observed after strength training (Cannon, Kay, Tarpinning, & Marino, 2007; Knight & Kamen, 2001; Reeves et al., 2004). Very few have investigated the effect of exercise in pre-frail and frail elderly, but so far evidence suggest that improvements in activation level contribute slightly more to training-induced strength gains in this population (Harridge, Kryger, & Stensgaard, 1999; Hvid et al., 2016). Nevertheless, more data is required to fully elucidate the extent to which improvements in activation level contribute to the training-induced improvements in specific strength.

Altered muscle composition has also been proposed as a contributor to both age-related and training-induced changes in specific strength. Muscle attenuation in Hounsfield Units (HU) obtained by Computed Tomography (CT) is a non-invasive measure of muscle density, and has been shown to correlate with skeletal muscle lipid content (Goodpaster, Kelley, Thaete, He, & Ross, 2000; Larson-Meyer et al., 2006). Aging is associated with reduced mid-thigh muscle density (Goodpaster, Carlson, et al., 2001), whereas strength training has been shown to have the opposite effect in both young (Poehlman, Dvorak, DeNino, Brochu, & Ades, 2000), and old individuals (Taaffe et al., 2009). If similar adaptations occur in frail elderly, has to our knowledge not been investigated. Moreover, whether altered muscle density following strength training represents changes in lipids within (intramyocellular lipids [IMCL]) or between (extramyocellular lipids [EMCL]) skeletal muscle fibers, remain to be elucidated.

Article III

Although not a consistent observation (Frontera et al., 2008; Venturelli et al., 2015), the contractile properties of single muscle fibers have also been observed to change with aging. Muscle fibers become weaker not only due to atrophy, but specific tension (force normalized to fiber area) also seems to decrease (D'Antona et al., 2003; Ochala, Frontera, Dorer, Van Hoecke, & Krivickas, 2007). In contrast, specific tension has been shown to improve following strength training in some (Parente et al., 2008; Wang et al., 2018), but not all (Erskine et al., 2011; Slivka, Raue, Hollon, Minchev, & Trappe, 2008) investigations. Importantly, the effect of strength training on single fiber specific tension has never been investigated in frail elderly, in whom training-induced improvements in specific strength appear to be substantial (Cadore et al., 2014). Another interesting observation is that the contractile properties of single fibers appear to be related to IMCL content (Choi et al., 2016), and it has been shown that IMCL content is reduced immediately following a session of heavy load strength training in young men (Koopman et al., 2006). Whether long-term strength training affects lipid content in frail elderly, and if this relates to improvements in specific tension, remains to be investigated.

The objective of this study was to examine the effects of ten weeks of heavy load strength training on voluntary muscle activation and muscle density in frail elderly individuals, to assess their relative contribution to improvements in specific strength. In addition, the aim was to explore if muscle density was related to skeletal muscle lipid content and single fiber specific tension. Based on previous observations of substantial improvements in specific strength in this population, we hypothesized that strength training would elicit improvements in both activation level and muscle density. Besides, we hypothesized that the improvements in muscle density would be related to changes in skeletal muscle lipid content and single fiber specific tension.

METHODS

Thirty-four frail elderly men and women aged between 67 and 98 (86 ± 7 years; mean \pm standard deviation [SD]) were recruited from nursing homes or day-care facilities for elderly and gave their written informed consent to participate in this experiment. The study was approved by the Regional Ethics Committee for Medical and Health Research of South-East Norway (2016/895/REK sør-øst C) and performed in accordance with the Declaration of Helsinki. The trial was registered at clinicaltrials.gov as NCT03326648.

Inclusion and randomization

Subjects fulfilling three of the five Fried Frailty Criteria (FFC) were included (Fried et al., 2001). In addition, subjects fulfilling two FFC criteria among "slowness", "weakness", and "low activity level" were included. Furthermore, individuals with a score of $\leq 6/12$ on the Short Physical Performance Battery (SPPB) were included, regardless of categorization based on the FFC. The

Article III

SPPB consists of timed standing balance, gait speed, and timed chair-rise assessment (Guralnik, Ferrucci, Simonsick, Salive, & Wallace, 1995). After baseline measurements, stratified randomization was performed based on knee extensor strength (normalized to body weight) and preferred walking speed. Participants were randomly assigned to either a group performing heavy load strength training two times per week for ten weeks (ST, n = 19) or a control group (CON, n = 15) in a 4:3 allocation ratio, due to the expected higher drop-out rate in ST. Moreover, seven subjects initially part of the control group agreed to participate to the training program after the first intervention period. After ten weeks of training, five of these subjects were re-tested, and are thus included in both ST and CON (**Figure 1**). Subject characteristics at baseline are presented in **Table 1**.

Training

The training protocol consisted in progressive resistance training, performed two times per week for ten weeks. Trained exercise instructors supervised all sessions. The exercise program included leg press and knee extension, and participants performed 2-3 sets of 12 repetitions at submaximal loads the first week, and from week 2-10 subjects gradually progressed from 3 sets of 12RM to 4 sets of 6RM. Each exercise set was followed by approximately 2-min rest periods. Training sessions were preceded by a low-intensity 3-min warm up exercise, using a step platform.

Supplementation

Participants in both groups received one dietary supplement each day throughout the intervention period, containing 17 g of milk protein, 18 g of carbohydrate, and 1 g fat (149 kcal [627 kJ]). The supplement was consumed in the evening, except on training days (ST), when the supplement was consumed within two hours after training. Participants were encouraged to continue their habitual diet in addition to the supplements. Supplements were provided to avoid a very low protein intake in some participants, based on evidence that protein intake is highly variable in this population (Mendonca et al., 2017).

Tests and measurements

All tests were monitored by the same investigator before and after the intervention period. Analyses of CT images and muscle biopsies were performed in a blinded manner. Testing of muscle strength, activation level and functional capacity were performed twice before, and once after the intervention.

Body composition

Body composition was assessed by dual energy X-ray absorptiometry (Lunar iDXA GE Healthcare, Madison, Wisconsin, USA). Participants were scanned from head to toe in a supine position, providing values for total lean tissue and fat mass. In addition, regional analyses were performed to

measure leg lean mass. The coefficient of variation (CV) for the assessment of leg lean mass is low in our lab (<1.7%).

Quadriceps femoris size and composition

Computed tomography (CT) scans (8 mm thick) were obtained from both legs at 50% of the distance between the knee joint and the greater trochanter (Toshiba Aquilion Prime 80, Canon Medical Systems, Otawara, Japan), and saved as DICOM images for further analysis. Analyses were performed in a blinded manner using Fiji software (ImageJ, U.S. National Institutes of Health, Maryland, USA), and only results for quadriceps femoris are included in the present investigation. Other muscles, bone and subcutaneous adipose tissue were excluded manually by drawing a line along the muscle fascia (**Figure 2A-B**). According to previously defined ranges (Goodpaster, Kelley, Wing, Meier, & Thaete, 1999), scans were analyzed to quantify the cross-sectional areas of total muscle (0 to 100 Hounsfield units [HU]), low-density muscle (0 to 30 HU), normal-density muscle (31 to 100 HU), and adipose tissue (-190 to -30 HU) within the defined area (**Figure 2 C-D**). Pixels between these ranges (-29 to 0 HU) are considered to reflect a mix of adipose tissue and muscle (Goodpaster, Kelley, et al., 2000), and constituted a small area in all subjects (~1-2 cm²). This area was excluded from the analyses. Muscle attenuation (referred to as *density* in the present study), was measured as the mean attenuation value from all pixels within the range of 0 to 100 HU in quadriceps femoris.

Dynamic strength

After a general warm-up (5 min bicycling), maximal strength was assessed by one-repetition maximum (1RM) in knee extension in a custom-made apparatus (Gym2000, Geithus, Norway). The participants were securely strapped to the testing chair with a belt fixing the hips, and a specific warm-up procedure was performed using loads corresponding to 50%, 70%, 80% and 90% of expected 1RM – conducting 10, 6, 3, and 1 repetition(s), respectively. Optimally, 2–5 attempts were needed to establish 1RM. The loads could be adjusted in steps as low as 0.25 kg. The left and right leg was tested interchangeably (unilateral), and each leg rested for three minutes between attempts. The pre-intervention test with the best result was used in the analysis. Knee extension 1RM was normalized to quadriceps femoris muscle CSA (0 to 100 HU), to obtain a measure of dynamic specific strength.

Maximal isometric strength and muscle activation level

Maximal isometric strength was assessed using a Humac NORM dynamometer (CSMi, Stoughton, MA, USA), and combined with the interpolated twitch technique (ITT) to evaluate voluntary muscle activation. Participants were seated in the Humac chair with a four-point belt fixing the chest and hips, with a knee joint angle of 70° (0° corresponding to full extension). Two percutaneous surface

Article III

stimulation electrodes (Veinoplus, Ad Rem Technology, 8 x 13 cm, Paris, France) were placed over the distal and proximal parts of the quadriceps muscle belly. After an isokinetic and isometric warm-up procedure, the muscle was stimulated in a resting state to identify the intensity necessary to elicit peak torque. Stimulation intensity was gradually increased until no further increase in twitch torque was observed (intensity test). Next, subjects were instructed to contract as forcefully as possible, and maintain maximal force exertion for 3-4 s, to assess isometric maximal voluntary contraction (MVC). Following a 3-min break, subjects performed a second MVC to assess activation capacity. To this end, paired (doublet) stimuli were evoked (10 ms interval) when the plateau of maximal torque exertion was reached. In addition, a doublet was delivered approximately two seconds after the end of the voluntary contraction, when the muscle was still potentiated. Two more MVC trials were performed, either with or without stimulation, interspersed by 3-min breaks. Voluntary muscle activation was calculated using the equation: $\text{activation (\%)} = 100 - ((D * (T_{\text{Stim}} / T_{\text{MVC}}) / T_{\text{StimRest}}) * 100)$ where D is the difference between torque at stimulation (T_{Stim}) and total torque (voluntary + electrically evoked force response), T_{MVC} is the highest voluntary torque, and T_{StimRest} is torque achieved due to stimulation in the rested muscle. The trial with the highest level of voluntary muscle activation was used in the analysis of activation level, whereas peak torque was used for MVC results. Knee extension MVC was normalized to quadriceps femoris muscle CSA (0 to 100 HU), to obtain a measure of isometric specific strength.

Functional performance

Five times chair-rise. Participants were asked to rise from a chair to a full stand five times as fast as possible. Subjects were instructed to keep their hands crossed on their chest. If subjects were not able to stand up without using their hands, limited use of hands was allowed, and performed the same way at both registration time points. Time was measured using a stop-watch. The best time of two trials was used in the analysis.

Gait velocity. Participants were instructed to walk 10 meters at their habitual gait velocity. Photo cells (Brower Timing Systems, Draper, USA) were placed to measure the time from 2 to 8 meters. The mean time of three trials was used in the analyses. Following measurement of habitual gait velocity, subjects were instructed to walk as fast as possible. The best time of three trials was used in the analyses, to provide a measure of maximal gait velocity. Walkers, canes or walking poles were allowed only if subjects did not feel safe walking without these devices. For these subjects, the same aids were used at both registration time points.

Biopsy protocol

Subjects refrained from strenuous physical activity for 3 days before testing. On the day of the biopsy procedure, subjects arrived at the laboratory by car or public transportation after an overnight

Article III

fast, and received a standardized breakfast containing oatmeal, water, sugar and butter (20 kJ and 0.09 g protein per kg body mass). One hour following the breakfast, a percutaneous needle biopsy was obtained from the vastus lateralis muscle from the left leg. The procedure was conducted under local anesthesia (Xylocaine with adrenaline, 10 mg/ml lidocaine + 5 µg/ml adrenaline, AstraZeneca, London, UK), and approximately 200 mg (2–3 × 50–150 mg) of muscle tissue was obtained using a modified Bergström technique with suction (Tarnopolsky, Pearce, Smith, & Lach, 2011). Samples intended for immunohistochemical analyses were embedded in OCT (Cellpath O.C.T embedding matrix, Newtown, UK) before being frozen in isopentane cooled down on liquid nitrogen. The samples were stored at -80°C for later analyses. The samples intended for single fiber measurements were put in a tube containing skinning solution (K propionate 150 mM, Mg acetate 5 mM, Na ATP 5 mM, EGTA 5 mM and KH₂PO₄ 5 mM with protease inhibitors: E64 10 µM and leupeptine 40 µM), and stored at 4°C. The skinning solution was changed every four hours for 24 hours, before the sample was put in a tube containing 50% skinning solution and 50% glycerol. Samples were stored at -20°C until analysis (within 2 weeks).

Immunohistochemical analyses

Samples for immunohistochemical analysis were cut at 8 and 10 µm thickness at -20°C using a cryostat (CM1860 UV, Leica Microsystems GmbH, Nussloch, Germany), mounted on microscope slides (SuperFrost Plus, Thermo Scientific), and air-dried before being stored at -80°C. Sections intended for fiber type composition (8 µm thickness) were blocked in 1% BSA (bovine serum albumin) in PBS containing 0.05% Tween-20 (PBS-t) before overnight incubation at 4°C with primary antibodies against dystrophin (Ab15277, Abcam, Cambridge, UK; dil. 1:500) and myosin heavy chain I (BA-D5, developed by Schiaffino, S., obtained by DSHB, Iowa, IA, USA; dil. 1:500) diluted in the blocking buffer. After incubation, sections were washed 3 × 10 min in the PBS-t solution with gentle agitation, followed by incubation for 60 min at room temperature with appropriate secondary antibodies (Alexa Fluor 488, A11001 and Alexa Fluor 594, A11012, Invitrogen Molecular Probes, Carlsbad, CA, USA; dil. 1:200) diluted in the blocking buffer. Subsequently, sections were again washed 3 × 10 min before they were mounted with ProLong Gold Antifade Reagent (P36935, Invitrogen Molecular Probes) and covered with a coverslip. Sections were left to dry overnight at room temperature before viewed and imaged under the microscope (**Figure 3A**).

Sections intended for intramyocellular lipid analyses (10 µm thickness) were stained as described by Mehlem and co-workers (Mehlem, Hagberg, Muhl, Eriksson, & Falkevall, 2013). Briefly, sections were incubated for 10 min in an Oil Red O (ORO) solution followed by washing of excess solution in running tap water for 30 min. Sections were then mounted with Aquatex mounting agent

Article III

(108562, Merck, Darmstadt, Germany) and covered with a coverslip. Sections stained with ORO were imaged within 24 hours after staining. All muscle sections were visualized and micrographed using a high-resolution camera (DP72, Olympus Corp., Tokyo Japan) mounted on a light microscope (BX61, Olympus Corp.). Sections intended for fiber type distribution were visualized with a fluorescence light source (X-Cite, 120PCQ, EXFO Photonic Solutions Inc., Mississauga, ON, Canada) and imaged through a 4x 0,13 NA air objective (UPlanFL N, Olympus Corp., Tokyo, Japan). Sections stained with ORO were imaged through a 20x 0,55 NA air objective (UPlanFL N, Olympus Corp.) with all settings on the microscope and camera fixed and standardized for all samples. 8-bit images were analyzed using FIJI (Schindelin et al., 2012) for ORO staining intensity. Two separate analyses were performed. First, a representative area of the stained section (> 50 fibers) were analyzed, including staining within (intramyocellular lipids; IMCL) and between (extramyocellular lipids; EMCL) fibers. This analysis is hereafter referred to as *total staining intensity*. Next, measurements of the staining within type I and type II fibers were performed. Region of interest (ROI) within the fibers was set to include close to the whole fiber area excluding the cell membrane (**Figure 3B**). At least 40 randomly selected muscle fibers per sample was included in this analysis and related to their respective fiber type using a neighboring section.

Single fiber thickness and contractility

On the day of the experiment, a fragment of the biopsy sample stored in 50% skinning and 50% glycerol solution (see above) was transferred to a Petri dish containing skinning solution and, after repeated washing to remove glycerol, single muscle fiber segments were dissected and short segments (approximate length 1 mm) equipped with two aluminum clips. The segments were then transferred to the set up and mounted in a drop (70 μ l) of relaxing solution between the force transducer (AME-801 SensorOne, Sausalito, California) and the electromagnetic puller (SI, Heidelberg, Germany) equipped with a displacement transducer. The signals from the force and displacement transducers were fed and stored in a personal computer after A/D conversion (interface CED 1401 plus, Cambridge, UK). For data storage, recall and analysis the software Spike 2 (CED, Cambridge, UK) was used. The force transducer and the electromagnetic puller were placed on the stage of an inverted microscope (ZEISS, Axiovert 25) and their relative position could be finely adjusted with micromanipulators. A movable aluminum plate hosted three drops (70 μ l volume) of relaxing, pre-activating, and activating solution, respectively. The drops were kept between two coverslips, the upper one being connected with a movable arm. Under each solution drop, an opening through the aluminum plate made the fiber segment visible via the objective piece of the inverted microscope which was connected with a digital camera (Optikam B5, OPTIKA) to collect images at 300x magnification. A stereomicroscope (Konus Diamond, KONUS) placed above the inverted microscope allowed a careful manipulation of the fiber segments. The

Article III

composition of the solutions in mM was as follows: relaxing solution: KCl 100, imidazole 20, MgCl₂ 5, Na ATP 5 and EGTA 5; preactivating solution had a similar composition except that EGTA concentration was reduced to 0.5 mM, and 25 mM creatine phosphate (CP) and 300 U/ml creatine phosphokinase (CPK) were added; activating solution (pCa 4.6) was similar to relaxing solution with the addition of 5 mM CaCl₂, 25 mM CP and 300 U/ml CPK. The pH of all solutions was set at 7.0 at the temperature at which experiments were performed (12 °C). Protease inhibitors (E64 10 μM and leupeptine 40 μM) were added to all solutions.

For the functional test, fiber segments were transferred from the relaxing solution into the pre-activating solution for at least 1 min and then maximally activated by immersion in the activating solution (pCa 4.6). During maximal activation isometric force (Fo) was measured with a quick shortening-relengthening maneuver (duration 5 ms). The cycle activation-relaxation was repeated at least 3 times and the average force value was used for further analysis. Cross Sectional Area (CSA) was calculated from the measurements of three fiber diameters, assuming a circular shape of the fiber section. Furthermore, specific force $Po = Fo \cdot CSA^{-1}$ was also calculated. At the end of the functional experiment, the fiber segments were removed from the set-up and immersed in 10 μl of Laemmli solution (Laemmli, 1970), for subsequent determination of the composition in MyHC isoforms. To this end, protein separation was performed on 8% polyacrylamide slab gels after denaturation in SDS (SDS-PAGE) as described previously (Doria et al., 2011). Gels were silver stained and three bands were separated in the region of 200 kD, corresponding to MyHC-1, MyHC-2A and MyHC-2X, in order of migration from the fastest to the slowest.

A total of 413 muscle fibers were analyzed from 22 baseline biopsies, corresponding to 18.8 (± 3.4) fibers per biopsy. All fibers were analyzed for cross-sectional area. However, force measurements could not be performed in 18 fibers (breakage), and force was therefore measured in 395 muscle fibers. After the intervention, 251 muscle fibers were analyzed from 13 biopsies, corresponding to 19.3 (± 2.3) fibers per biopsy. Twenty-three fibers broke during force measurements, and thus force data were available for 228 muscle fibers.

Statistics

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Statistical significance was set at $P < 0.05$. Non-normally distributed data (D'Agostino and Pearson omnibus normality test) were log-transformed prior to statistical analysis. For single fiber variables, the ROUT method (with Q set at 1%) was used to detect and exclude outliers (individual muscle fibers). Between-group differences at baseline were investigated using the Student's t-test. Fiber-type specific differences at baseline were assessed with the Student's t-test (IMCL analyses) or one-way ANOVA with Tukey's multiple comparisons test (single fiber

measurements). A two-way ANOVA with Bonferroni's multiple comparison test was used to evaluate the effect of time and group for variables measured both before and after the intervention period (absolute values). Associations between variables were investigated using Pearson's correlation coefficient. Correlations were considered weak if $r < 0.4$, moderate if $r = 0.4-0.6$, and strong if $r > 0.6$. Power calculations performed *a priori* indicated that 16 participants in each group would be needed to identify a difference of 10% between the ST and CON in specific strength, with alpha set at 0.05 and beta at 0.20. Data are presented as individual values and mean \pm standard deviation, if not otherwise stated, and all data are illustrated in original form (absolute values).

RESULTS

Baseline characteristics of participants

Even though the groups were not significantly different in terms of age, muscle strength or functional capacity, CON had a higher body fat percentage ($P = 0.042$), and body mass index (BMI) ($P = 0.039$) than ST (**Table 1**).

Adherence to the intervention

Eleven subjects did not complete the trial, thus 13 subjects in CON and 17 subjects in ST (including five subjects initially in CON) completed and remained for the per-protocol analyses (**Figure 1**). The dropouts in ST did not significantly differ from the completers for any of the variables measured. The completers in ST performed on average 18.9 (± 1.1) training sessions in 10.5 (± 0.5) weeks. Average training frequency was 1.8 (± 0.1) sessions per week.

Adherence to testing

Body composition, muscle strength and functional performance were tested in all completers. However, three subjects had previously suffered from a stroke (>5 years ago), and therefore results for gait velocity and chair rise performance were omitted. For other measurements, these subjects were included, and the unaffected limb was always tested. Cross-sectional area, muscle density and intermuscular adipose tissue (IMAT) were assessed in all but two completers (logistical challenges). Five completers were excluded from the assessment of muscle activation due to pacemakers. In addition, the test was terminated for three subjects due to discomfort, and the results for three subjects were omitted because of suboptimal measurements (*e.g.* due to joint pain). With regards to the muscle biopsy, one of the completers was excluded due to anti-coagulant medication that could not be discontinued. Another participant withdrew during the initiation of the muscle biopsy procedure due to discomfort. In addition, we were not able to obtain a biopsy from four completers either at baseline or after the intervention due to an excessive layer of subcutaneous fat and a very thin muscle belly. In four subjects, the histological sections were of low quality or showed evidence of freeze damage, and were therefore excluded from the analysis of IMCL and EMCL. Furthermore,

Article III

measurements of single fibers were only performed in a subset of 22 baseline biopsies, of whom only thirteen remained for the per-protocol analysis. Due to the reasons above, the number of subjects is different for the various tests.

Correlations at baseline

Strong correlations were observed between quadriceps femoris muscle CSA and both knee extension 1RM ($r = 0.87$, $P < 0.001$) and MVC ($r = 0.89$, $P < 0.001$) at baseline (**Table 2**). In addition, significant correlations were observed between voluntary activation level and both maximal and specific isometric strength. No other correlations were observed between specific strength and aspects of muscle quality (muscle density, IMCL, single fiber specific tension). Quadriceps femoris muscle density (mean HU) showed stronger correlations with maximal than specific strength. Both maximal and specific strength showed moderate correlations with functional performance, and dynamic strength (1RM) generally showed stronger correlations with functional performance than isometric strength. In addition, correlations between muscle strength and functional performance were slightly stronger when strength were normalized to body mass (results not shown).

Body composition (DXA)

There were no significant effects of the training program on body mass, lean mass or fat mass, and changes in leg lean mass showed only a tendency for a group x time interaction ($P = 0.076$) (**Table 3**).

Maximal strength, specific strength and functional capacity

Knee extension 1RM improved from baseline in ST ($P < 0.001$), but the interaction with CON did not reach statistical significance ($P = 0.067$; **Table 3**). In addition, isometric MVC improved from baseline in ST ($P = 0.025$), but again only a tendency for a group x time interaction was observed ($P = 0.088$). No group x time interactions were observed for isometric specific strength (MVC / quadriceps femoris muscle CSA) or dynamic specific strength (KE 1RM / quadriceps femoris muscle CSA), although the latter improved from baseline in ST ($P = 0.024$). The change in five times chair-rise showed only a tendency for a group x time interaction ($P = 0.08$), whereas significant interaction effects were observed for both habitual ($P = 0.003$) and maximal gait velocity ($P = 0.015$).

Muscle activation

Ten (ST) and nine (CON) subjects completed interpolated twitch technique both before and after the intervention. Muscle activation did not change from baseline in ST (from 89.7 ± 4.7 to $86.7 \pm$

5.7%) or CON (from 87.9 ± 5.1 to $89.0 \pm 6.1\%$), and no group x time interaction was observed (**Figure 4**).

Muscle cross-sectional area, muscle attenuation and intermuscular adipose tissue

Pre and post data represent fifteen (ST) and thirteen (CON) subjects. There was a significant group x time interaction between ST ($6.8 \pm 7.4\%$) and CON ($1.2 \pm 5.1\%$) for the change in quadriceps femoris muscle CSA ($P = 0.002$) (**Figure 5A**). The increase in muscle CSA in ST was exclusively attributable to an increase in area of normal-density muscle ($10.1 \pm 9.0\%$), which did not change in CON (-1.5 ± 4.9) (group x time interaction, $P < 0.001$) (**Figure 5B**). Low-density muscle did not change in either ST (-1.4 ± 17.1) or CON (-1.5 ± 10.2) (**Figure 5C**). Hence, a group x time interaction was observed for muscle density (measured as mean HU), with changes of $1.5 (\pm 1.6)$ and $-0.2 (\pm 0.9)$ HU, in ST and CON, respectively ($P < 0.001$; **Figure 5D**). The area of IMAT in quadriceps femoris was small at baseline, and did not change in ST (from 1.3 ± 1.1 to $1.5 \pm 1.1 \text{ cm}^2$) or CON (from 1.6 ± 0.9 to $1.7 \pm 1.0 \text{ cm}^2$).

Intramyocellular and extramyocellular lipids

For pre and post data twelve (ST) and eight (CON) subjects are included in the analyses of intra- and extramyocellular lipids. Total ORO staining intensity at baseline, thought to reflect both IMCL and EMCL, showed correlations with BMI ($r = 0.75$, $P = 0.001$) and body fat percentage ($r = 0.51$, $P = 0.011$). However, total staining intensity was not correlated with quadriceps femoris muscle density ($r = 0.01$, $P = 0.95$), or IMAT ($r = 0.32$, $P = 0.13$) assessed by CT. Total staining intensity did not change in ST (from 49.6 ± 7.8 to 51.3 ± 10.2 AU) or CON (from 51.0 ± 5.8 to 49.8 ± 8.3 AU) following the intervention (group x time interaction, $P = 0.378$; **Figure 6**).

ORO staining intensity within the cell membrane of type I fibers, considered to reflect IMCL content, did not change in ST (from 49.6 ± 9.2 to 52.0 ± 11.1 AU) or CON (49.8 ± 5.1 to 48.2 ± 9.9 AU) (group x time interaction, $P = 0.447$). Moreover, no group x time interaction was observed for staining within type II fibers, with no changes in ST (from 43.8 ± 5.2 to 45.9 ± 9.3 AU) or CON (from 47.8 ± 6.9 to 44.5 ± 8.0 AU) (group x time interaction, $P = 0.143$). If the two groups were pooled at baseline, IMCL content was higher in type I compared to type II fibers ($P = 0.026$).

Single fiber properties

To investigate differences between fiber types at baseline, all fibers from twenty-two baseline biopsies were pooled (**Table 4**). Type IIA and IIX fibers were 15 ($P = 0.082$) and 33% ($P = 0.012$) smaller than type I fibers. No differences between fiber types were observed for maximum force, but due to the differences in size, specific tension was 44% ($P = 0.001$) higher in IIX fibers, compared to type I fibers. The difference of 17% between type I and IIA fibers did not reach

Article III

statistical significance ($P = 0.108$). An inverse correlation was observed between single fiber specific tension and IMCL content in type I ($r = -0.53$, $P = 0.044$), but not for type II fibers ($r = -0.09$, $P = 0.75$).

Pre and post results represent eight (ST) and five (CON) subjects. Due to the low number of hybrid fibers and pure IIX-fibers, two separate analyses were performed. First, all fibers from each subject were pooled, regardless of fiber type. Fiber cross-sectional area did not change in ST ($4310 \pm 861 \mu\text{m}^2$ to $4809 \pm 861 \mu\text{m}^2$) or CON ($5346 \pm 2068 \mu\text{m}^2$ to $4807 \pm 1550 \mu\text{m}^2$) (group x time interaction: $P = 0.38$), but the change in ST tended to correlate with the change in quadriceps femoris muscle CSA measured by CT ($r = 0.79$, $P = 0.059$). No group x time interaction was observed for single fiber maximal isometric force (ST: 0.36 ± 0.16 to 0.50 ± 0.41 mN, CON: 0.51 ± 0.20 to 0.48 ± 0.15 mN, $P = 0.43$). Moreover, specific tension, defined as maximal isometric force normalized to fiber area, did not change in ST (85 ± 30 to 99 ± 30 mN \cdot mm⁻²) or CON (101 ± 8 to 100 ± 14 mN \cdot mm⁻²) (group x time interaction: $P = 0.51$) (**Figure 7**).

Subsequently, type I-fibers were analyzed separately, whereas IIA, hybrid IIA-X, and IIX-fibers were pooled and analyzed separately. Regardless of categorization, no interaction effects were observed for fiber area, maximal isometric tension or single fiber specific force (results not shown).

Correlations in the training group

Weekly training frequency in ST (range: 1.6-2.0) was correlated with the change in quadriceps femoris muscle CSA ($r = 0.55$, $P = 0.032$). However, the change in muscle CSA did not correlate with changes in 1RM ($r = 0.26$, $P = 0.35$) or MVC ($r = 0.26$, $P = 0.41$), and no correlations were observed between changes in muscle strength and changes in functional performance. Moreover, no significant correlations were observed between changes in isometric specific strength and changes in activation level ($r = 0.34$, $P = 0.33$), muscle density ($r = 0.43$, $P = 0.13$), IMAT ($r = 0.30$, $P = 0.30$), total ORO staining intensity ($r = -0.24$, $P = 0.51$) or single fiber specific tension ($r = 0.04$, $P = 0.95$).

DISCUSSION

In the present study, our main findings were that muscle density, assessed by CT, was the only qualitative variable that changed after ten weeks of strength training in frail elderly individuals. In contrast to previous reports, the increase in isometric strength (6.5%) and quadriceps femoris muscle CSA (6.8%) were in good agreement, suggesting that the increase in absolute strength was mainly due to increased muscle quantity, not quality.

Effect of strength training on muscle activation

A correlation between activation level and specific strength was observed at baseline, suggesting that some of the between-subject variation in specific strength was indeed due to differences in voluntary activation level. However, activation level did not change following the ten-week training intervention. In general, increased activation level following strength training is more pronounced in the plantar flexors (Arnold & Bautmans, 2014), and improvements for the knee extensors are typically in the area of 2-7% in healthy 70-year-olds (Cannon et al., 2007; Knight & Kamen, 2001; Reeves et al., 2004). However, based on the findings by Venturelli and colleagues (2015), observing an activation level of quadriceps femoris of 95, 85, and 78%, in young, very old active and very old wheelchair-bound individuals, respectively (Venturelli et al., 2015), we hypothesized that our subjects would have a low activation level at baseline, and that strength training would elicit marked improvements. Indeed, Hvid and colleagues (Hvid et al., 2016) observed an improvement in activation level from 79 to 85% following 12 weeks of power training performed twice weekly in pre-frail elderly (Hvid et al., 2016). The discrepancy between the findings of the latter study and the present one could be related to the type of training. Although a training load of 70-80% of 1RM was utilized in both studies, the concentric phase of the exercises were performed as rapid as possible in Hvid et al. (2016). However, a more likely explanation for the discrepancy is that our subjects displayed a much higher activation level at baseline (89%), indicative of a limited potential for improvements. Indeed, a meta analysis concluded that greater gains were achieved in subjects with lower voluntary activation level at baseline (Arnold & Bautmans, 2014). The reason for the difference at baseline is difficult to unravel, but pain is known to affect voluntary muscle activation (Graven-Nielsen, Lund, Arendt-Nielsen, Danneskiold-Samsoe, & Bliddal, 2002). In the present study, one subject experienced knee pain during the contraction, and was excluded from the results (activation level of 62%). If some of the subjects in the previous studies experienced pain during the test (Harridge et al., 1999; Hvid et al., 2016), and whether this could explain the very low activation level observed in some participants (50-70%) is not known.

Effect of strength training on muscle composition

The area of intermuscular adipose tissue (IMAT) in quadriceps femoris was small at baseline (~1.5 cm²), and did not change following training. Moreover, the hypertrophy observed in the training group was exclusively due to an increased area of normal-density muscle, whereas low-density muscle area did not change. Consequently, mean muscle density also increased. Aging is associated with reduced mid-thigh density values, negatively affecting specific strength (Goodpaster, Carlson, et al., 2001). Because IMAT is excluded from the calculation of muscle density, lower attenuation values are considered to reflect increased levels of lipids within (IMCL) or surrounding (EMCL) individual muscle fibers (Aubrey et al., 2014). In support of this, Goodpaster and colleagues (2000)

Article III

observed a negative correlation between mid-thigh muscle density and vastus lateralis IMCL content in young individuals (Goodpaster, Kelley, et al., 2000). Surprisingly, no correlation between muscle density and IMCL content was observed in the present study, for neither type I nor type II fibers. Between-subject variation in muscle density was neither explained by total ORO staining intensity, reflecting both IMCL and EMCL. It has been demonstrated previously that detraining results in decreased muscle density, and that retraining has the opposite effect in healthy elderly men and women (Taaffe et al., 2009). Muscle biopsies were not obtained in the latter study, but the authors speculate that the changes in muscle density could reflect changes in lipid content. In the present study, increased muscle density was observed in the absence of changes in IMCL and EMCL, and our results therefore suggest that the training-induced increase in muscle density was not related to changes in lipid content. Previously, it has been seen that myosin content change in response to disuse and retraining in elderly individuals (Hvid et al., 2017), and this could potentially explain the increase in muscle density in our training group. However, in the study by Hvid and colleagues (2017), increased myosin content was associated with increased single fiber specific tension, which was not observed in the present investigation.

It is important to acknowledge that increased muscle density does not necessarily require qualitative changes at the single fiber level, or changes in lipid content *per se*. Because of the somewhat limited resolution of CT, depots of extracellular adipose tissue smaller than the pixels will not necessarily be categorized as adipose tissue (Goodpaster, Kelley, et al., 2000), but may instead contribute to low-density muscle area. Moreover, in the boundary between muscle tissue and larger areas of IMAT, it might be that some pixels are categorized as low-density muscle if the pixels contain most muscle, but also a small portion of adipose tissue. In support of this, we did observe a positive correlation between IMAT and low-density muscle ($r = 0.46$, $P = 0.007$). Along this line, muscle density would be expected to increase following muscle hypertrophy, even when IMAT and IMCL content does not change, because the proportion of muscle "not in contact" with IMAT would increase. The latter could explain why we observed an increase in normal-density muscle, without changes in low-density muscle or IMCL. In addition, both from our results and from previous observations, it is evident that lipid content is higher in type I compared to type II fibers (He, Goodpaster, & Kelley, 2004; St-Jean-Pelletier et al., 2016). Because strength training-induced hypertrophy mainly occurs in type II fibers (Leenders et al., 2013), an increased percentage area covered by fast-twitch fibers would also be expected to contribute to the increase in muscle density. Interestingly, the latter could also contribute to the age-related reduction in muscle density (Goodpaster, Carlson, et al., 2001), considering that age-related atrophy primarily affect type II-fibers (Verdijk et al., 2014). Indeed, evidence that the level of IMCL is increased with aging is scarce (Gueugneau et al., 2015), and IMCL content appear to be more closely associated with

Article III

obesity than aging *per se* (Goodpaster, He, Watkins, & Kelley, 2001; Goodpaster, Theriault, Watkins, & Kelley, 2000; St-Jean-Pelletier et al., 2016). The correlations we observed between skeletal muscle lipid content and both BMI and fat percentage support this notion.

Effect of strength training on single fiber function

To the best of our knowledge, this is the first study to investigate the effect of strength training on single fiber specific tension in frail elderly individuals. Contrary to our hypothesis, no effects were observed. It is difficult to unravel why some studies observe training-induced improvements in specific tension in elderly individuals (Frontera et al., 2003; Parente et al., 2008; Wang et al., 2018), whereas others do not (Godard, Gallagher, Raue, & Trappe, 2002; Raue, Slivka, Minchev, & Trappe, 2009; Slivka et al., 2008; Trappe et al., 2001). However, an interesting notion is that two of the interventions eliciting improvements were of relatively long duration (5 and 12 months) (Parente et al., 2008; Wang et al., 2018), suggesting that the ten-week intervention employed in the present study might have been of insufficient duration. Moreover, the subjects in the study by Wang and colleagues (2018) were overweight/obese, and the authors reported that increased specific tension of type II fibers correlated with reductions in thigh fat volume assessed by CT. The latter study, along with the observation of an inverse correlation between IMCL content and single fiber specific power in obese elderly (Choi et al., 2016), indicate that the potential for improvements in specific tension might be greater in individuals with higher IMCL content. In the present study, an inverse relationship was observed between single fiber specific tension and ORO staining intensity in type I, but not type II fibers. Collectively, these findings imply that intramyocellular lipids do have a negative impact single fiber specific tension. However, from our results it seems that neither IMCL content or single fiber specific tension change following ten weeks of heavy load strength training in frail elderly individuals.

Our muscle biopsy analyses are subject to some limitations. First, the low number of subjects with both pre and post measurements for single fiber function (ST = 8, CON = 5) increase the risk of statistical type II-errors. Second, a recurring challenge with analyses on human muscle biopsies is to which extent a single biopsy is representative of the whole muscle. This point might be even more applicable in very old and frail individuals, because aging and frailty seem to increase the variation between different areas in the muscle, due to denervation and fiber type grouping (Andersen, 2003; Kelly et al., 2018). The large variation we observed for single fiber specific tension could be related to this phenomenon. Nevertheless, we did observe a non-significant correlation between the change in CSA assessed by CT, and change in single fiber area ($r = 0.79$, $P = 0.059$, $n=6$), suggesting that the twenty or so fibers chosen for analysis per biopsy at least were partly representative of changes in muscle *size*.

Effect of strength training on muscle strength and functional capacity

The improvements in muscle strength of 7% (MVC) and 17% (1RM) in the training group were somewhat lower than expected. Recently, improvements of 13% (MVC) and 91% (1RM) were observed in a similar group of individuals (age: 83-94) after 12 weeks of heavy load strength training (Bechshoft et al., 2017). The discrepancies could be related to the difference in training frequency (2 vs. 3 sessions/week) and total duration (10 vs. 12 weeks). Regardless, the 7% increase we observed in quadriceps femoris muscle CSA is on the high end of previous reports in the same population (Bechshoft et al., 2017; Chale et al., 2013; Fiatarone et al., 1994). In most previous and similar studies, the change in both isometric and dynamic muscle strength exceeded the improvements in muscle size (Bechshoft et al., 2017; Cadore et al., 2014; Harridge et al., 1999), suggesting that several factors other than increases in muscle mass contribute to the improvements in muscle strength. However, from our findings it seems that at least following a 10-week intervention with two weekly strength training sessions, the degree of muscle hypertrophy corresponds well with the improvement in isometric strength. Nevertheless, dynamic specific strength (1RM / CSA) improved from baseline in the training group (10%). This increase could not be explained by changes in IMCL/EMCL content or single fiber specific tension. Although activation level was not improved in MVC, a more optimal recruitment strategy cannot be excluded as a contributing factor for the improvements in knee extension 1RM. Another potential explanation is training-induced changes in muscle fascicle length (Reeves et al., 2004), possibly resulting in a more optimal overlap of actin and myosin filaments at "sticking point" (the position in the range of motion where the weight is experienced heaviest, and where 1RM tests typically fail).

An interesting observation in the present study was that habitual gait velocity was significantly reduced from baseline in controls (-10%), and a similar tendency was observed for maximal gait velocity (-7%). Hence, although the functional improvements in the training group were modest (10-12%), they starkly contrasted the apparently rapid decline in controls. Thus, although two weekly sessions of heavy load strength training had limited effects on specific strength and its determining factors, it proved effective in eliciting gains in muscle size and gait velocity.

Adherence to heavy load strength training

Nine out of 26 subjects allocated to training did not complete the 10-week intervention, which equals to a drop-out rate of 35%. Geirsdottir and colleagues (2017) investigated predictors of drop-out during a 12-week heavy load strength training intervention (3 sessions per week). The overall drop-out rate in 236 elderly individuals with a mean age of 74 years was 12%. However, in individuals above 80 years (n = 32), the drop-out rate was almost 30%. Moreover, low gait speed and physical activity were the strongest predictors of drop-out (Geirsdottir et al., 2017). Collectively, these findings emphasize that even though heavy load strength training *can* be very

Article III

beneficial regardless of age and functional capacity, earlier initiation might increase the likelihood of long-term commitment.

Strengths and limitations

Few previous studies have investigated the effects of heavy load strength training in frail elderly, and in a majority of the earlier investigations the end points have been limited to measures of muscle size, strength and functional capacity. Obtaining muscle biopsies is very rarely done in this population. Thus, our study provides novel data on how lipid content and the properties of single muscle fibers respond to strength training in frail elderly. There are also some limitations to our study. Even though this study was initially designed as a randomized controlled trial, we were forced to abandon this design in order to increase the statistical power. Furthermore, due to several drop-outs in combination with some analytical challenges, the results for some variables still represent a quite low number of subjects, increasing the risk of statistical type II errors.

Conclusions

The purpose of the present study was to investigate how different aspects of specific strength change in response to heavy load strength training in frail elderly individuals. Specifically, we aimed to investigate if the often-observed mismatch between gains in muscle strength and size were related to changes in muscle activation, muscle density, lipid content or single fiber specific tension. In contrast to our hypothesis, the improvements in isometric strength corresponded well to the increase in quadriceps femoris muscle CSA, suggesting that the increase in strength was mainly due to increased muscle quantity, not quality. Interestingly, the training-induced increase in muscle density did not reflect changes in lipid content *per se*, and might instead reflect an increased ratio of muscle tissue relative to IMAT.

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Article III

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Article III

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TABLES

Table 1. Subject characteristics at baseline in the strength training group (ST), and control group (CON).

Characteristics	ST	CON	P-value
N (men/women)	(13/13)	(8/7)	
Age (years)	86.6 ± 6.9	84.5 ± 7.2	0.341
Body mass (kg)	66.4 ± 12.5	74.3 ± 13.7	0.071
Lean body mass (kg)	42.6 ± 7.3	44.6 ± 9.5	0.469
Leg lean mass (kg)	14.2 ± 2.8	15.5 ± 4.1	0.245
Body fat (%)	33.2 ± 7.6	38.0 ± 5.9	0.042*
Body Mass Index (kg/m ²)	24.3 ± 3.7	26.8 ± 3.3	0.039*
Unilateral knee extension 1RM (kg)	19 ± 8	23 ± 11	0.236
Five times chair rise (sec)	20 ± 20	19 ± 20	0.824
Habitual gait velocity (m/s)	0.70 ± 0.25	0.76 ± 0.26	0.499
SPPB score	5.8 ± 2.9	5.7 ± 3.0	0.881

1RM: one repetition maximum, SPPB: Short Physical Performance Battery. Data are presented as mean ± SD. *sig. different between groups

Table 2. Correlations between knee extensor strength and various measures of muscle size, composition and functional performance.

Measure	Absolute strength		Specific strength	
	KE 1RM (kg)	KE MVC (Nm)	KE 1RM / QF m. CSA	KE MVC / QF m. CSA
QF muscle CSA (cm ²)	.87* (33)	.89* (30)	-	-
QF muscle activation (%)	.34 (22)	.53* (22)	.22 (21)	.54* (21)
QF IMAT (cm ²)	.03 (33)	-.01 (30)	-.04 (33)	-.14 (30)
QF muscle density (mean HU)	.43* (33)	.40* (30)	.30 (33)	.19 (30)
VL total ORO staining intensity (AU)	.38 (24)	.39 (23)	.08 (24)	.08 (23)
VL single fiber specific tension (mN·mm ⁻²)	-.09 (18)	-.19 (17)	.07 (18)	.02 (17)
Habitual gait velocity (m/s)	.51* (30)	.38* (28)	.43* (29)	.19 (27)
Five times chair rise (s)	-.39* (30)	-.30 (28)	-.47* (29)	-.41* (27)

QF: quadriceps femoris, CSA: cross-sectional area, m: muscle, IMAT: intermuscular adipose tissue, HU: Hounsfield units, VL: vastus lateralis, ORO: Oil Red O, AU: arbitrary units, KE: knee extension, 1RM: one repetition maximum, MVC: isometric maximal voluntary contraction. Numbers in parenthesis denote the number of XY pairs for the given correlation. * $P < 0.05$.

Article III

Table 3. Body composition, maximal strength, specific strength and functional capacity before and after the 10-week intervention.

	ST			CON			ANOVA <i>P</i> -value
	Pre	Post	% change	Pre	Post	% change	
Body mass (kg)	68.3 ± 12.5	69.3 ± 13.0	1.3 ± 2.4	74.2 ± 11.7	74.2 ± 11.6	0.2 ± 3.5	0.273
Lean mass (kg)	43.4 ± 8.0	43.6 ± 8.0	0.6 ± 2.6	44.3 ± 8.9	44.1 ± 9.0	-0.5 ± 3.0	0.287
Leg lean mass (kg)	14.6 ± 3.0	14.9 ± 3.1	2.3 ± 3.8	15.3 ± 3.9	15.1 ± 3.7	-0.1 ± 0.7	0.076
Fat mass (kg)	22.7 ± 6.9	23.4 ± 7.0	3.2 ± 6.4	27.6 ± 5.6	27.9 ± 5.4	1.5 ± 7.4	0.514
Knee extension 1RM (kg)	20.2 ± 8.9	23.1 ± 9.1	17.4 ± 16.3***	22.0 ± 8.9	22.5 ± 8.3	4.2 ± 13.0	0.067
Knee extension MVC (Nm)	102.3 ± 30.9	108.4 ± 32.6	6.5 ± 9.5*	115.3 ± 34.0	114.9 ± 34.5	0.1 ± 5.5	0.088
Specific force (1RM / CSA)	0.46 ± 0.15	0.49 ± 0.12	10.2 ± 15.7*	0.51 ± 0.11	0.53 ± 0.09	4.8 ± 11.5	0.814
Specific force (MVC / CSA)	2.43 ± 0.55	2.39 ± 0.57	-1.4 ± 9.1	2.51 ± 0.28	2.54 ± 0.36	1.0 ± 6.5	0.417
Gait velocity - habitual (m/s)	0.69 ± 0.24	0.75 ± 0.25	11.6 ± 27.2	0.82 ± 0.25	0.74 ± 0.25	-10.4 ± 9.8**	0.003
Gait velocity - maximal (m/s)	1.14 ± 0.49	1.21 ± 0.51	9.7 ± 27.8	1.23 ± 0.41	1.13 ± 0.39	-7.2 ± 8.2*	0.015
Five times chair rise (s)	19.6 ± 19.5	14.9 ± 8.8	-12.5 ± 19.9*	13.0 ± 4.5	12.8 ± 3.9	1.0 ± 18.9	0.080

1RM: one repetition maximum, MVC: maximal voluntary contraction, CSA: cross-sectional area. Data are presented as mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 different from baseline within group. ANOVA *P*-value show group x time interaction.

Table 4. Single fiber measurements for all baseline biopsies.

	Fiber type					ANOVA <i>P</i> -value
	I	I-2A	2A	2A-2X	2X	
Fibers (n)	164	31	104	49	25	-
Fibers (%)	44	8	28	13	7	-
fCSA (μm ²)	5220 ± 2942 ^a	4354 ± 1725 ^{ab}	4429 ± 2035 ^{ab}	4577 ± 2281 ^{ab}	3501 ± 1804 ^b	0.003
Force (mN)	0.42 ± 0.26	0.43 ± 0.21	0.45 ± 0.27	0.38 ± 0.18	0.44 ± 0.23	0.627
Force/CSA (mN/mm ²)	89 ± 45 ^a	103 ± 43 ^{ab}	104 ± 59 ^{ab}	95 ± 44 ^{ab}	128 ± 54 ^b	0.002

fCSA: fiber cross-sectional area. ANOVA: analysis of variance. Data are presented as mean ± SD. Values not sharing the same letter are significantly different from each other (*P* < 0.05).

FIGURES

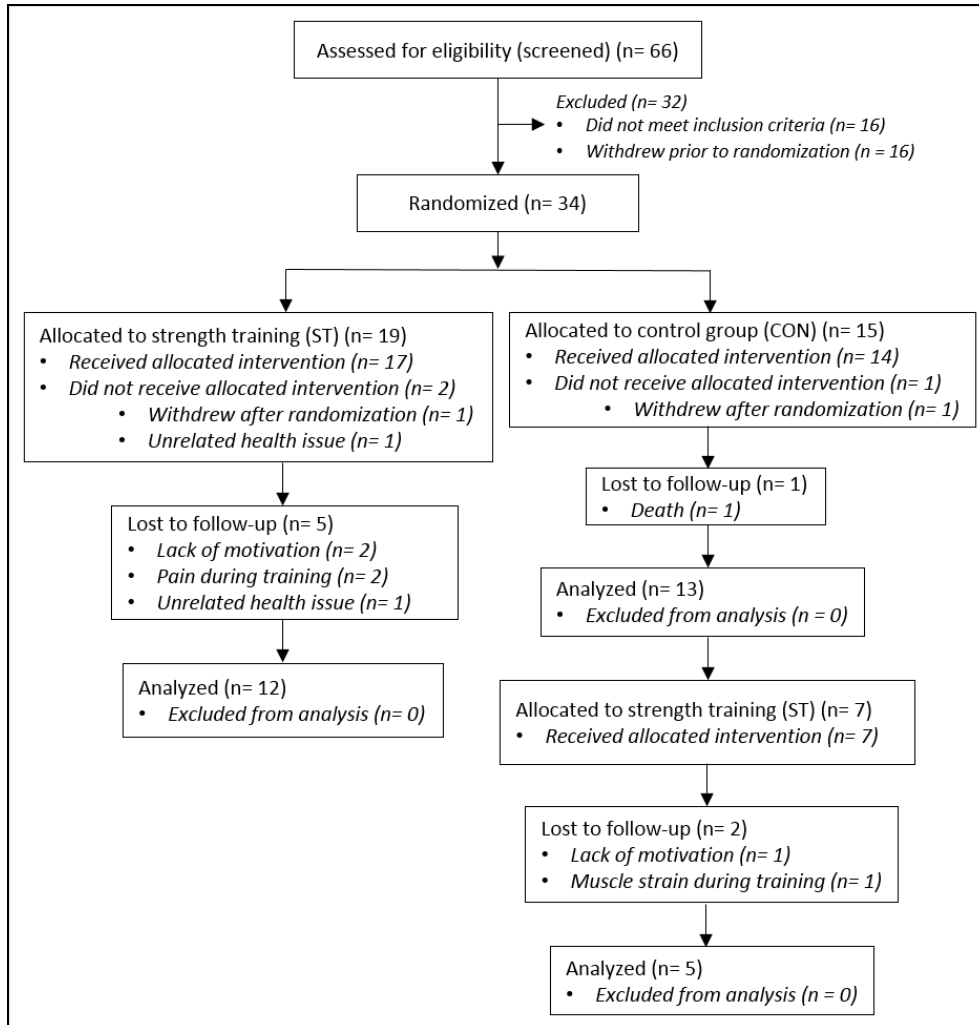


Figure 1. Participant flow diagram. The number of subjects included in the analyses varied for the different tests, and the numbers above holds true only for body composition and 1RM strength testing.

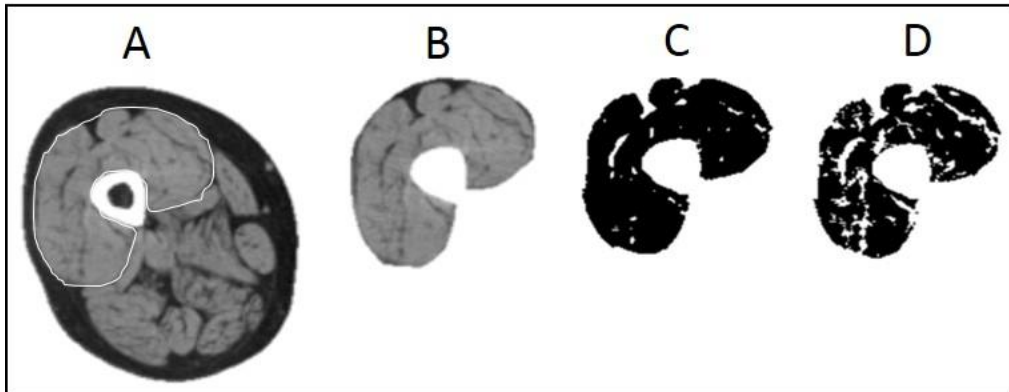


Figure 2. Analysis of muscle cross-sectional area, intermuscular adipose tissue (IMAT) and low- and normal-density muscle (right leg). **A:** cross-sectional area of the mid-portion of the right thigh. **B:** Manual isolation of quadriceps femoris, excluding subcutaneous adipose tissue, bone and other muscles. **C:** Tissue characterized as muscle (0 to 100 HU) is depicted in black, whereas white areas show intermuscular adipose tissue (IMAT). **D:** Normal-density muscle (31 to 100 HU) is depicted in black, whereas white areas represent low-density muscle (0 to 30 HU) and IMAT (-190 to -30 HU).

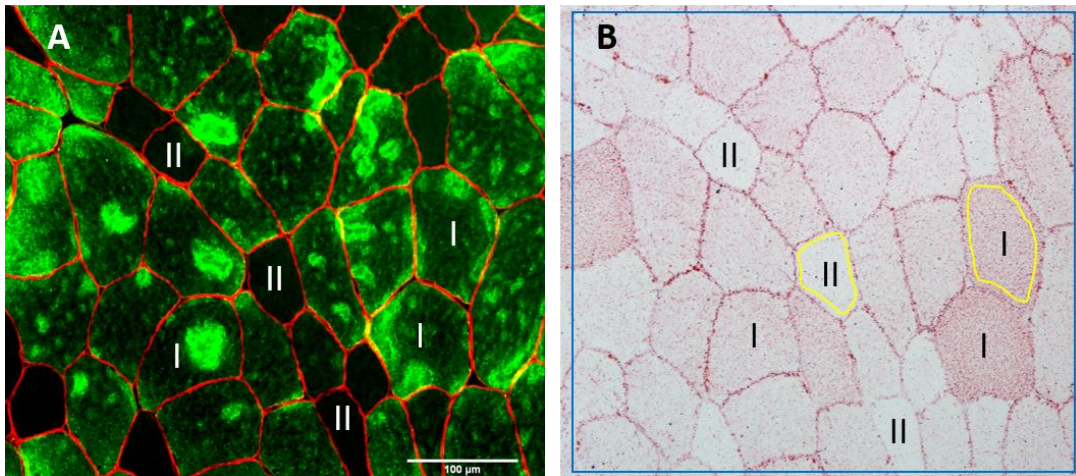


Figure 3. Antibody against myosin heavy-chain I was used to differentiate between type I (green) and type II fibers (no staining) (**A**). A neighbor section was stained with Oil Red O (**B**). Range of interest (ROI) within each fiber was set to include close to the whole fiber area excluding the cell membrane (area within yellow lines). In addition, total staining intensity was measured for the whole area, including staining within and between fibers (area within blue line).

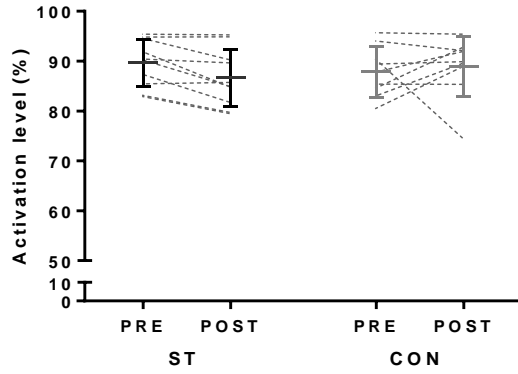


Figure 4. Voluntary activation level during an isometric knee extension MVC, before and after the ten-week intervention period in the strength training group (ST) and control group (CON). The figure displays individual values, as well as mean \pm SD before and after the intervention.

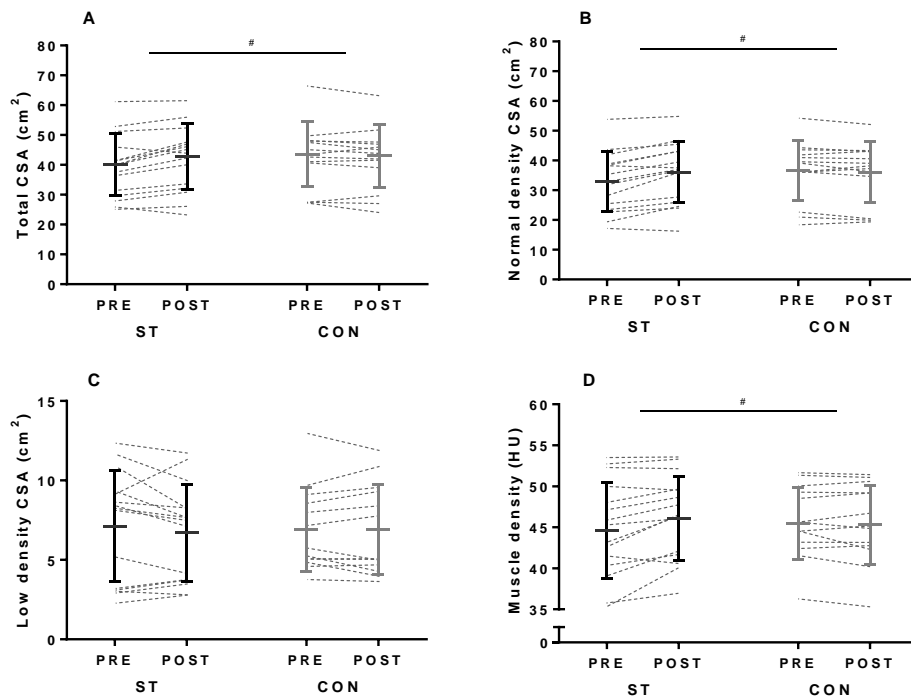


Figure 5. Quadriceps femoris total muscle cross-sectional area (panel A), normal-density muscle area (panel B), low-density muscle area (panel C), and muscle density expressed as mean HU (panel D) before and after the intervention in the strength training group (ST) and control group (CON). The figure displays individual values, as well as mean \pm SD before and after the intervention. # $P < 0.05$ (group x time interaction).

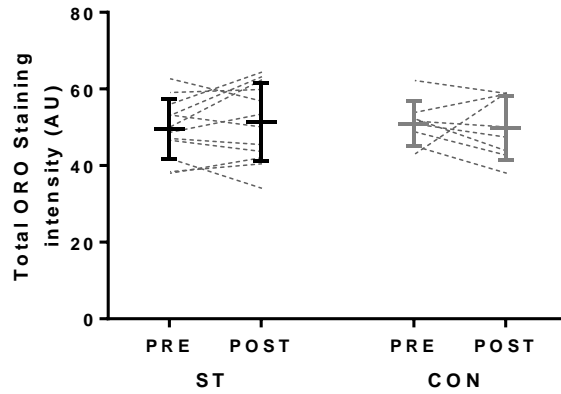


Figure 6. Total Oil Red O (ORO) staining intensity before and after the ten-week intervention period in the strength training group (ST) and control group (CON). An area including at least 50 fibers were analyzed per biopsy, including staining within fibers (IMCL) and between (EMCL) muscle fibers. The figure displays individual values, as well as mean \pm SD before and after the intervention.

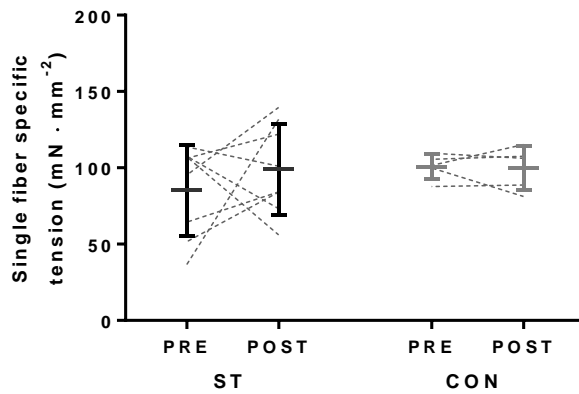


Figure 7. Single fiber specific tension (maximal isometric tension normalized to fiber cross sectional area) before and after the ten-week intervention period in the strength training group (ST) and control group (CON). All fibers from each biopsy are included, regardless of myosin heavy chain expression. The figure displays individual values, as well as mean \pm SD before and after the intervention.

ARTICLE IV

Effects of acute and long-term strength training on skeletal muscle autophagy in frail elderly men and women

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ABSTRACT

Strength training with protein supplementation has been reported to improve muscle mass and quality in frail elderly individuals. However, underlying mechanisms are not well elucidated in this population. To address this issue, we investigated acute and long-term effects of strength training with protein supplementation (ST+PRO) compared with protein supplementation alone (PRO), on protein synthesis and degradation markers in skeletal muscle biopsies (*m. vastus lateralis*) from frail elderly individuals (86 ± 7 yr.). Phosphorylation of p70S6K and 4E-BP1, indicators of protein synthesis rate, were significantly increased 2.5 hours after protein intake only if preceded by strength training (ST+PRO), and this effect was evident both before and after ten weeks of heavy load strength training. Acute increases in mRNA expression of genes related to the ubiquitin proteasome system (MuRF-1, MUSA1), autophagy (ATG7, LC3, p62), and mitochondrial fission (DRP1) were observed after the first, but not after the last training session in ST+PRO. Despite the increases in gene expression, changes in protein levels (LC3-I, LC3-II, p62) indicated an acute suppression of autophagy in both groups. The ten-week training intervention had minimal effects on resting levels of anabolic proteins, heat shock proteins, markers of the ubiquitin proteasome system and autophagy. These results indicate that resistance exercise with protein ingestion is a potent stimulus to muscle protein synthesis in frail elderly, regardless of training status. Moreover, the results indicate that strength improvements were not directly related to changes in proteins involved in cellular quality control.

Keywords: Resistance exercise, aging, sarcopenia, anabolic resistance, degradation

INTRODUCTION

Aging is associated with a reduced muscle protein synthetic (MPS) response to protein intake and muscular activity, termed "anabolic resistance" (Cuthbertson et al., 2005; Fry et al., 2011; Kumar et al., 2009), which may ultimately contribute to the development of sarcopenia and frailty. Nevertheless, strength training with protein supplementation elicit gains in muscle mass and strength in both young and healthy elderly (Phillips, Hartman, & Wilkinson, 2005; Verdijk et al., 2009). Muscle hypertrophy can be achieved also in *very* old and frail individuals (Bechshoft et al., 2017; Fiatarone et al., 1994), but evidence suggests that the hypertrophic response is partly blunted (Greig et al., 2011; Raue, Slivka, Minchev, & Trappe, 2009; Stewart, Saunders, & Greig, 2014). Muscle biopsies are rarely performed in this population; therefore underlying mechanisms are not well understood. In both young and healthy elderly, it has been demonstrated that resistance exercise performed prior to protein intake results in a greater MPS response compared to protein intake alone (Yang et al., 2012). This appears to be due to an enhanced activation of mTORC1 and its downstream targets, such as p70S6K (Francaux et al., 2016). However, whether an acute session of strength training elicits the same effect in frail elderly, and if long-term strength training influences resting levels of enzymes involved in MPS regulation, remain to be investigated.

Although the loss of muscle mass with aging is believed to mainly involve changes in MPS, the less studied mechanisms of muscle protein breakdown (MPB) probably also play a part, perhaps especially in relation to the age-related reduction in muscle quality (D'Antona et al., 2003; Frontera et al., 2000). Evidence suggests that skeletal muscles of older individuals display increased levels of heat shock proteins (Yamaguchi et al., 2007), mitochondrial protein carbonylation (Beltran Valls et al., 2015), intramyocellular lipids (Gueugneau et al., 2015) and lipofuscin aggregation (Orlander, Kiessling, Larsson, Karlsson, & Aniansson, 1978), indicating deficits in mechanisms related to muscle quality control. In this regard, strength training might represent an effective countermeasure, by facilitating degradation of dysfunctional proteins and organelles (Biolo, Maggi, Williams, Tipton, & Wolfe, 1995; Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997). Protein degradation in skeletal muscle is mainly controlled by the ubiquitin-proteasome pathway (UPP) and the autophagosome-lysosome system. UPP is largely responsible for the degradation of myofibrillar proteins (Solomon & Goldberg, 1996), and is regulated by ubiquitin ligases such as muscle ring finger-1 (MuRF-1), atrogin-1 (Fbx32/MAFbx) and MUSA-1 (Fbx30). Studies have consistently reported increased gene expression of MuRF-1 the first hours after strength training in both young and healthy elderly (Fry et al., 2013; Raue, Slivka, Jemiolo, Hollon, & Trappe, 2007; Stefanetti, Zacharewicz, et al., 2014). However, to our knowledge, the UPP response to strength training has

Article IV

never been investigated in frail elderly individuals. Moreover, whether the acute response depends on training status in this population is currently unknown.

The autophagosome-lysosome system can degrade intracellular protein aggregates, but also macromolecules and organelles (Tanida, Ueno, & Kominami, 2008). First, a membrane structure called a phagophore is formed; thereafter, the phagophore expands, a process requiring the conjugation of microtubule-associated protein 1 light chain (LC3-I) to PE (phosphatidylethanolamine) to form its lipidated form, LC3-II. Cargo receptors such as p62 recruit proteins and organelles to the growing autophagosome which eventually will fuse with lysosomes to form an autolysosome, where the content is degraded (Tanida et al., 2008). Dysfunctional mitochondria normally undergo fission, and are thereafter removed by mitophagy, a selective form of autophagy (Youle & van der Bliek, 2012). Autophagy and mitophagy have received less focus in relation to strength training compared to the UPP. Recent observations of a reduced LC3-II/LC3-I ratio the first hours after strength training suggest acute inhibition of autophagy (Dickinson et al., 2017; Fry et al., 2013). However, gene expression data suggest a later stimulation of autophagy, because increased mRNA levels of both LC3 and p62 have been observed (Dickinson et al., 2017; Ogborn et al., 2015). Moreover, long-term strength training has been shown to increase several autophagic markers, both in rodents (Luo et al., 2013) and humans (Hentila et al., 2018; Tanner et al., 2015). Hence, the autophagic response to strength training warrants further investigation, and acute responses should be interpreted in light of long-term effects, to better elucidate the role of autophagy in strength training adaptation.

Heat shock proteins (HSPs) are a family of proteins that are upregulated by cells in response to stress, and demonstrate a protective role through repair and refolding of misfolded peptides (Dubinska-Magiera et al., 2014). An age-related increase in the expression of heat shock proteins Hsp70 and α B-crystallin has been observed previously (Thalacker-Mercer, Dell'Italia, Cui, Cross, & Bamman, 2010; Yamaguchi et al., 2007), potentially as a compensatory mechanism against increased levels of misfolded and dysfunctional proteins. Interestingly, it seems that heat shock proteins may also play a part in regulation of autophagy (Dokladny et al., 2013). Hence, to gain more information about acute and long-term effects of strength training on heat shock proteins in frail elderly individuals, and the potential interaction between HSPs and autophagy, measurements of Hsp70 and α B-crystallin were included in the present investigation.

The overall aim of this study was to investigate acute and long-term effects of strength training and protein supplementation on markers of muscle protein synthesis and muscle protein breakdown in frail elderly individuals, with emphasis on autophagy. Since much uncertainty still exists with regards to the interpretation of several autophagic markers (Mizushima & Yoshimori, 2007), the

Article IV

strength training group was compared to a non-training control group only ingesting a protein supplement, a stimulus known to acutely reduce overall muscle protein breakdown (Glynn et al., 2010). We hypothesized that 1) the acute stimulation of regulators of protein synthesis would be more pronounced after resistance exercise and protein supplementation than after protein supplementation alone, 2) the acute upregulation of mRNA related to UPP and autophagy would be more pronounced after resistance exercise and protein supplementation than after protein supplementation alone, 3) that autophagy-related proteins would indicate increased autophagic activity at rest following ten weeks of strength training and protein supplementation.

METHOD AND DESIGN

Participants and ethical approval

This investigation includes a subset of participants from a previous study (Aas et al., 2019, [article III]) in which thirty-four frail elderly men and women were recruited. Only subjects from which a muscle biopsy were obtained are included in the present investigation (16 men/12 women, 86 ± 7 years). The study design, purpose and possible risks were explained to each subject before inclusion, and subjects gave their written consent to participate. The study was approved by the Regional Ethics Committee for Medical and Health Research of South-East Norway (2016/895/REK sør-øst C), and performed in accordance with the Declaration of Helsinki (World Medical, 2013). The study was registered at clinicaltrials.gov as NCT03326648.

Inclusion criteria

Subjects fulfilling three of the five Fried Frailty Criteria (FFC) were included (Fried et al., 2001). In addition, subjects fulfilling two FFC criteria among "slowness", "weakness", "low activity level" were included. Furthermore, individuals with a score of $\leq 6/12$ on the Short Physical Performance Battery (SPPB) were included, regardless of categorization based on the FFC. The SPPB consists of timed standing balance, gait speed, and timed chair-rise assessment (Guralnik, Ferrucci, Simonsick, Salive, & Wallace, 1995).

Study design

Eighteen subjects were allocated to ten weeks of strength training and protein supplementation (ST+PRO), whereas ten subjects constituted a non-training control group, only receiving protein supplementation (PRO) (**Table 1**). An acute experiment (described later) was performed before (at Test Day 1; TD1) and after (at Test Day 2; TD2) the ten-week intervention period.

Training and supplementation

The strength training protocol performed in ST+PRO was a supervised progressive program, performed two times per week for 10 weeks. The program included leg press and knee extension,

Article IV

and participants performed 2-3 sets of 12 repetitions at submaximal loads the first week, and from week 2-10 subjects gradually progressed from 3 sets of 12 repetitions maximum (RM) to 4 sets of 6RM. Each set was followed by approximately 2-min rest periods. Strength training sessions were preceded by a 3-min lower extremity warm-up exercise, using a step platform.

Participants in both groups received one dietary supplement each day throughout the intervention period, containing 17 g of milk protein, 18 g of carbohydrate, and 1 g fat (149 kcal [627 kJ]). The supplement was consumed in the evening, except on training days (ST+PRO), when the supplement was consumed within two hours after training. Participants were encouraged to continue their regular food routines in addition to the supplements.

Acute experiment

An acute experiment was conducted before and after the ten-week intervention period (**Figure 1**), to investigate the acute response to strength training followed by protein intake (ST+PRO) in the untrained (TD1) and trained state (TD2). In short, subjects refrained from strenuous physical activity for 3 days, and arrived at the laboratory by car or public transportation after an overnight fast. Participants received a standardized breakfast containing oatmeal, water, sugar and butter (20 kJ and 0.09 g protein per kg body mass). One hour after the breakfast, a percutaneous needle biopsy was obtained from the vastus lateralis muscle. Thirty minutes later, both groups performed a 5-min warm-up on an exercise bike, followed by an assessment of maximal voluntary contraction (MVC). The MVC-test was followed by a leg resistance exercise session in ST+PRO, and a rest period in PRO.

The exercise session in ST+PRO began with 10 warm-up repetitions in knee extension at 65% of 8RM load. Subsequently, subjects conducted 6 sets of 8RM in knee extension, with a new set starting every 3rd min. The exercise protocol had a duration of 19 min. Both groups received the protein supplement one hour after the baseline muscle biopsy. For ST+PRO, this coincided with the moment they had completed the training session. A second MVC-test was performed in both groups 10 minutes later. 150 minutes after consuming the supplement, a second muscle biopsy from vastus lateralis was obtained.

Maximal voluntary contraction (MVC)

Unilateral maximal knee extension strength was assessed to obtain information about the extent of fatigue elicited by the exercise session. The test was performed in a custom-made knee-extension apparatus (Gym2000, Geithus, Norway). Participants were seated in a chair with a belt fixing the hips, with 90° in the hip and knee joints. Three attempts of 3-4 seconds per leg with 1 min rest between were given to reach MVC force. Force was measured with a force transducer (HMB U2

Article IV

AC2, Darmstadt, Germany). To avoid potentially erroneous results due to biopsy-related pain, MVC data from the non-biopsied leg were used in the analysis.

Biopsy protocol

The biopsy procedure was conducted under local anesthesia (Xylocaine with adrenaline, 10 mg/ml lidocaine + 5 µg/ml adrenaline, AstraZeneca, London, UK), and approximately 200 mg (2–3 × 50–150 mg) of muscle tissue was obtained using a modified Bergström technique with suction. Tissue intended for immunoblot analyses was quickly rinsed in physiological saline, before fat, connective tissue and blood were removed and discarded. Subsequently, the sample was weighed and quickly frozen in isopentane cooled on dry ice (immunoblot analyses on cellular sub-fractions), or liquid nitrogen (immunoblot analyses of protein phosphorylation), before being transferred and stored at –80°C for later analyses. Samples intended for gene expression analyses were immersed into RNeasy® solution (Ambion, TX, USA), stored for 24-48 hours at 4 °C, and thereafter stored at –20 °C for later treatment and analysis.

Analyses

Pre-analytical analyses

Approximately 50 mg of muscle tissue was homogenized in 1 ml T-PER (Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA), 20 µl Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and 20 µl EDTA (Thermo Scientific). Another 50 mg of muscle sample was homogenized and fractionated into cytosolic, membrane, cytoskeletal, and nuclear fractions using a commercial fractionation kit (ProteoExtract Subcellular Proteome Extraction Kit, Cat#539790, Calbiochem, EMD Biosciences, Germany) according to the manufacturer's procedures. Protein concentration was measured using a commercial kit (BioRad DC protein microplate assay, Cat#0113, Cat#0114, Cat#0115, Bio-Rad Laboratories, Hercules, CA, USA), a filter photometer (Expert 96, ASYS Hitech, UK) and the software provided (Kim, ver. 5.45.0.1, Daniel Kittrich, Prague, Czech Republic).

Western blot

Equal amounts of protein were loaded within a gel (5-30 µg) and separated by 4-20% gradient Mini-PROTEAN TGX Stain-Free Precast protein gels (4568093, Bio-Rad Laboratories). Electrophoresis was performed under denaturized conditions for 30-35 min at 200 volts in Tris/Glycine/SDS running buffer (161-0732, Bio-Rad Laboratories). After gel-electrophoresis, proteins were transferred onto a PVDF-membrane at 100 volts for 60 min (Criterion™ Blotter; Tris/Glycine buffer 161-0734, Bio-Rad Laboratories). Membranes were blocked at room temperature for 2 hours in a TBS solution with 5% fat-free skimmed milk and 0.1% Tween 20 (TBS, 170-6435, Bio-Rad Laboratories; Tween-20, 437082Q, VWR International, Radnor, PA, USA; Skim milk,

Article IV

1.15363.0500, Merck, Darmstadt, Germany). Blocked membranes were incubated overnight at 4°C with primary antibodies (see below) followed by incubation with appropriate secondary antibodies for 1 h at room temperature (see below). Between stages, membranes were washed in 0.1% TBS-t. Protein stripping was conducted for phospho-proteins using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL, USA). All samples were analyzed in duplicates, and bands were visualized using an HRP-detection system (Super Signal West Dura Extended Duration Substrate, 34076, Thermo Fisher Scientific). Chemiluminescence was measured using a ChemiDoc MP System (Bio-Rad Laboratories), and band intensities were measured with Image Lab (Bio-Rad Laboratories). All intensities were normalized to a control sample that was applied in each gel (in duplicates), to allow comparisons between gels.

Primary and secondary antibodies were diluted in a 1% fat-free skimmed milk and 0.1% TBS-t solution. Antibodies against p70S6K (2708), phospho-p70S6K Thr389 (9234), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (9644), phospho-4EBP1 Thr37/46 (2855), eukaryotic elongation factor 2 (eEF2) (2332), phospho-eEF2 Thr56 (2331) and LC3-I/II (2775) were obtained from Cell Signaling Technology (Beverly, MA, USA), whereas the antibody against p62 (ab56416) was obtained from Abcam (Cambridge, UK). Antibodies against α B-crystallin (ADI-SPA-222) and Hsp70 (ADI-SPA-812) were purchased from Enzo Life Sciences (Farmingdale, NY, USA), and secondary antibodies from Cell Signaling Technology (goat anti-rabbit IgG, 7074) and Thermo Scientific (goat anti-mouse, 31430).

RNA extraction and RT-PCR

Total RNA was extracted from biopsies dedicated to gene expression analysis by mean of Trizol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, each muscle sample was rinsed from RNAlater® solution in cold sterile PBS and immediately homogenized with Trizol by mean of a metal bead and Tissue Lyser apparatus (Qiagen, Venlo, Netherlands). Subsequent RNA extraction was carried out following Trizol manufacturer manual. Total RNA was resuspended in RNase/DNase free water (GIBCO) and quantified using RNA BR assay test for Qubit fluorometer (Invitrogen). 400 ng of total RNA were retrotranscribed using SuperScript IV kit (Invitrogen) following the manufacturer's guide. cDNA was finally resuspended in 50 μ l with RNase/DNase free water and 1 μ l of diluted cDNA was used as a template for RT-qPCR analysis. qPCR analysis and Ct detection were carried out with QuantStudio5 machine (Applied Biosystems, CA, USA) using PoweUP SYBR green technology (Applied Biosystems). mRNA expression level of the following genes was detected: actin, MuRF-1, Atrogin1, MUSA1, ATG7, LC3, p62, BNIP3, BNIP3L, OPA1 and DRP1. Primer sequences are listed in **Table 2**. Expression level is presented as fold change normalized to actin expression.

Article IV

Statistics

Statistical analyses were performed using GraphPad Prism6 software (GraphPad Software, Inc., La Jolla, CA). Statistical significance was set at $P < 0.05$. Gene expression data were log-transformed prior to statistical analyses. All other non-normally distributed data (D'Agostino and Pearson omnibus normality test) were also log-transformed prior to statistical analyses. Between-group differences at baseline were investigated by unpaired Student's t-tests. A two-way ANOVA with Bonferroni's multiple comparison test was used to evaluate the effect of time and group (absolute values). In addition, paired t-tests were performed to investigate changes from baseline within the groups. Associations between variables were investigated using Pearson's correlation analysis. All data are presented as mean \pm standard deviation.

RESULTS

Due to several drop-outs in ST+PRO ($n = 7$), two subjects initially allocated to PRO performed ten weeks of training after the first intervention period, and thereafter performed TD2 in the trained state. These two subjects are therefore included in both groups for comparisons at TD2. In addition, for some biopsies we were not able to obtain enough muscle tissue for all analyses. Hence, the number of subjects included for the different analyses varies.

Subjects

The two groups were similar in terms of age, lean mass and functional capacity, but subjects in PRO had a higher body fat percentage, and tended to have a higher body mass than the subjects in ST+PRO (**Table 1**).

Muscle hypertrophy and muscle strength

Changes in muscle size and strength following the ten-week training intervention have been described in detail previously (Aas et al., 2019, [article III]). In short, ST+PRO increased quadriceps femoris muscle cross-sectional area by 7%, knee extension MVC by 7% and knee extension 1RM by 17%.

Training volume and fatigue

Eight subjects in ST+PRO performed both TD1 and TD2. In these subjects, 8RM during the training session was higher at TD2 (37.2 ± 9.7 kg) than TD1 (32.6 ± 9.5 kg; $P = 0.002$). Thus, total weight lifted was also higher at TD2 (1733 ± 452 kg vs 1486 ± 448 kg; $P = 0.002$). A reduced force-generating capacity measured as MVC force was evident in the training group 10 minutes after the training session both at TD1 ($9 \pm 10\%$ reduction) and TD2 ($11 \pm 8\%$ reduction) (**Figure 2**).

Article IV

Gene expression

Test day 1 (Table 3). No group x time interactions were observed for any of the genes investigated. However, in ST+PRO, increased expression from baseline were observed for LC3 ($P = 0.002$), p62 ($P = 0.005$), MuRF-1 ($P = 0.037$), ATG7 ($P = 0.05$), MUSA ($P = 0.031$), and DRP1 ($P = 0.017$). No significant changes were observed in PRO.

Test day 2 (Table 3). No group x time interactions were observed for any of the genes investigated. However, Atrogin-1 expression decreased from baseline both in ST+PRO ($P < 0.001$) and PRO ($P = 0.019$).

Intervention (Table 3). Basal gene expression from before to after the intervention period did not change in ST+PRO or PRO for any of the genes investigated.

Protein expression

Representative western blots are presented in **Figure 3** and **Figure 4**.

Anabolic signaling proteins

Test day 1 (Figure 5). No group x time interactions were observed for p70S6K, 4E-BP1 or eEF2. However, phosphorylation ratio of p70S6K increased from baseline in ST+PRO ($P < 0.001$), and tended to increase from baseline in PRO ($P = 0.08$). Moreover, a 49% non-significant increase in the phosphorylation ratio of 4E-BP1 was observed in ST+PRO ($P = 0.054$). Phosphorylation of eEF2 did not change in ST+PRO or PRO.

Test day 2 (Figure 5). No group x time interactions were observed for p70S6K, 4E-BP1 or eEF2. Phosphorylation of p70S6K increased from baseline in ST+PRO ($P = 0.023$), and tended to increase from baseline in PRO ($P = 0.10$). The phosphorylation ratio of 4E-BP1 increased by 38% from baseline in ST+PRO ($P = 0.011$), whereas a 31% non-significant increase was observed in PRO ($P = 0.07$). Phosphorylation of eEF2 did not change in ST+PRO or PRO.

Intervention. Phosphorylation ratio or total level of p70S6K, 4E-BP1 and eEF2 did not change following the intervention in either group, and no group x time interactions were observed (results not shown).

Autophagic markers

Test day 1 (Figure 6). No group x time interactions were observed for any of the proteins measured, but the level of cytosolic LC3-I increased by 31% from baseline only in ST+PRO ($P = 0.005$). Moreover, LC3-II in the membrane fraction was reduced to 33% of baseline values, both in ST+PRO ($P < 0.001$) and PRO ($P = 0.008$). Hence, also the LC3-II/LC3-I ratio (calculated as membrane LC3-II / cytosolic + membrane LC3-I) was reduced from baseline in both groups ($P <$

Article IV

0.05). No changes were observed for cytosolic or membrane p62. In ST+PRO, a correlation was observed between the change in p70S6K phosphorylation and the change in cytosolic LC3-I ($r = 0.56, P = 0.037$). In addition, a tendency for an inverse correlation was observed between the change in p70S6K phosphorylation and change in LC3-II ($r = -0.48, P = 0.08$).

Test day 2 (Figure 6). No group x time interactions were observed for any of the proteins measured, but LC3-II in the membrane fraction was reduced to 39 and 31% of baseline values, in ST+PRO ($P = 0.032$) and PRO ($P = 0.002$), respectively. Thus, also the LC3-II/LC3-I ratio was reduced in both groups ($P < 0.01$). No changes were observed for p62, neither in the cytosolic or membrane fraction.

Intervention. No group x time interactions were observed for LC3-I, LC3-II, or the ratio of LC3-II/LC3-I. However, a group x time interaction was observed for p62 in the membrane fraction ($P = 0.01$), due to a 15% reduction in ST+PRO and a 34% increase in PRO.

Heat shock proteins

Test day 1 (Figure 7). A tendency for a group x time interaction for the ratio of cytoskeletal to cytosolic Hsp70 was observed ($P = 0.081$), due to a 20% increase and 10% decrease, in ST+PRO and PRO, respectively. No group x time interaction was observed for the ratio of cytoskeletal to cytosolic α B-crystallin, but the ratio tended to increase from baseline in ST+PRO ($P = 0.07$).

Test day 2 (Figure 7). No group x time interaction was observed for Hsp70, but a tendency was observed for the ratio of cytoskeletal to cytosolic α B-crystallin (group x time interaction: $P = 0.071$), due to a significant increase from baseline in ST+PRO ($P = 0.003$).

Intervention. No effects of the ten-week intervention period were observed for the level of heat shock proteins in the cytosolic or cytoskeletal fraction (results not shown).

Sex differences

In ST+PRO, nine men (87 ± 9 years) and eight women (88 ± 6 years) performed the first acute day of testing (TD1), allowing for investigation of possible sex-specific differences. The level of LC3-II tended to be higher in men at rest ($P < 0.051$). Moreover, the reduction in LC3-II following strength training and protein intake tended to be greater in men compared to women ($P = 0.098$). No differences were observed for any other gene or protein investigated.

Protein measurements and muscle function

The training-induced gains in muscle mass and strength did not correlate with acute increases in anabolic signaling (p70S6K, 4E-BP1, eEF2), training-induced changes in autophagic markers (LC3-I, LC3-II, p62) or Hsp70 and α B-crystallin (results not shown).

DISCUSSION

The main finding in the present study was that unaccustomed resistance exercise increased expression of genes related to the ubiquitin-proteasome pathway, autophagy and mitochondrial fission in frail elderly individuals, but that this response was absent in the trained state. Nevertheless, the autophagic proteins indicated suppressed autophagy 2.5 hours after strength training and protein ingestion, both before and after the training intervention. Ten weeks of heavy-load strength training had limited effects on resting levels of anabolic signaling proteins, heat shock proteins, and basal expression of genes and proteins related to the UPP and autophagy.

Signaling related to muscle protein synthesis

The phosphorylation ratio of p70S6K increased from baseline in ST+PRO both after the first and last training session. In addition, the phosphorylation ratio of 4E-BP1 increased by 34 and 37% at test day 1 and test day 2, respectively, although only the change at test day 2 reached statistical significance. Both p70S6K and 4E-BP1 are under mTORC1 control (Yoon, 2017), and promote translation initiation when phosphorylated and hyper-phosphorylated, respectively. In addition, p70S6K promote translation elongation, through dephosphorylation, and thus activation of eEF2 (Wang & Proud, 2006). The change in phosphorylation of p70S6K and 4E-BP1 have been observed to correlate with the change in fractional synthetic rate after a strength training session (Burd et al., 2012; Burd et al., 2010). Thus, our results suggest that a session of heavy load strength training followed by protein intake upregulates muscle protein synthesis in frail elderly individuals, and that this response occurs regardless of training status. In the group only ingesting a protein supplement, phosphorylation of p70S6K tended to increase at both test days, whereas 4E-BP1 phosphorylation tended to increase at test day 2. These results suggest that ingestion of 17 grams of milk protein also increase muscle protein synthesis in frail elderly individuals, albeit perhaps to a lesser extent than when protein intake is preceded by exercise. Hence, our results correspond well with previous observations in young and non-frail elderly individuals (Francaux et al., 2016).

Phosphorylation of eEF2 did not change in either group. Results for eEF2 after strength training and protein intake are contradictory, with some observations of increased activation (Dreyer et al., 2008; Dreyer et al., 2010; Drummond et al., 2008), and some observations of no change (Areta et al., 2013; Hamarsland et al., 2017; Hulmi et al., 2009; Moore, Atherton, Rennie, Tarnopolsky, & Phillips, 2011). These discrepancies do not appear to be related to population investigated, timing of biopsies or antibodies utilized. In summary, although no group x time interactions were observed for neither p70S6K, 4E-BP1 or eEF2, it seems that also skeletal muscle of frail elderly individuals is "sensitized" by a session of heavy-load strength training, increasing the anabolic response to protein ingestion.

Article IV

Gene expression of ubiquitin ligases

No group x time interactions were observed for the UPP genes investigated, and the changes from baseline within the groups should therefore be interpreted with caution. Regardless, the 2.9-fold increase in MuRF1 expression 2.5 hours after the first training session is in line with previous reports (Dickinson et al., 2017; Fry et al., 2013; Raue et al., 2007; Stefanetti, Zacharewicz, et al., 2014). Moreover, increased expression from baseline was observed for the ubiquitin ligase MUSA1, whereas atrogen-1 did not change. Increased expression of atrogen-1 is observed in some (Raue et al., 2007; Stefanetti, Zacharewicz, et al., 2014), but not all studies (Dickinson et al., 2017; Fry et al., 2013). The less consistent results for atrogen-1 compared to MuRF-1 might be due to the role of atrogen-1 in regulating the half-life of MyoD and eIF3f (Bonardo & Sandri, 2013), factors involved in the MPS response. Hence, it is logical that increases in atrogen-1 is observed more consistently in situations of increased MPB and attenuated MPS, such as the first days of immobilization/disuse (Chen et al., 2007; Suetta et al., 2012; Wall et al., 2014) and muscle denervation (Bongers et al., 2013). The observation of decreased atrogen-1 expression after the last training session might also be related to this. Moreover, no changes were observed for MuRF1 and MUSA1 2.5 hours after the last training session, perhaps indicating a lower activation of UPP at this stage of training. This corresponds well with the findings by Stefanetti and colleagues (Stefanetti, Lamon, et al., 2014), observing limited UPP-activation following a bout of strength training after 10 weeks of strength training in young men. Also in line with our findings, Phillips and colleagues (1999) observed greater increases in fractional breakdown rate following a strength training session in untrained, compared to trained subjects (same relative training load) (Phillips, Tipton, Ferrando, & Wolfe, 1999).

Basal gene expression did not change after the ten-week intervention in the present study for any of the UPP genes. Because we did not measure protein levels, we cannot exclude the possibility that increased mRNA expression following the first training sessions led to increased protein levels of ubiquitin ligases or other components of the UPP-system, potentially increasing UPP activity at rest. In line with this, Phillips and colleagues (Phillips et al., 2002) observed increased fractional breakdown rate (FBR) at rest following 8 weeks of resistance training in young men. Hence, it is interesting to speculate whether long-term strength training increases UPP-activity at rest, diminishing the necessity of an acute training-induced upregulation. Nevertheless, Stefanetti and colleagues (Stefanetti, Lamon, et al., 2014) did not observe any changes in protein levels of MuRF1 or other UPP-related proteins after 10 weeks of resistance training in young men, opposing this interpretation.

Article IV

Autophagy and mitophagy

In response to the first training session, increased gene expression of ATG7, LC3 and p62 were observed in ST+PRO. Ogborn and colleagues (2015) observed increased gene expression of LC3 and p62, but not ATG7, 3 hours after a strength training session in untrained young and old individuals (Ogborn et al., 2015). Moreover, the increase in gene expression was accompanied by increased protein levels of both LC3-I and p62 48 hours later (Ogborn et al., 2015). In contrast, Fry and colleagues (Fry et al., 2013) did not observe changes in either LC3 gene expression or LC3-I protein expression 3, 6 and 24 hours after strength training in physically active young and elderly subjects. In the present investigation, the increases in gene expression were accompanied by increased cytosolic LC3-I 2.5 hours after the training session, potentially reflecting increased *de novo* synthesis. Indeed, the reduction in LC3-II at this time point could indicate increased autophagic flux, with enhanced lysosomal degradation of autophagosomes. In such a situation, increased synthesis of LC3-I would be an expected response in order to replenish the cytosolic pool of LC3. However, a positive correlation was observed between the change in p70S6K-phosphorylation and the change in cytosolic LC3-I. Hence, it seems more likely that enhanced mTORC1 activity suppressed lipidation of LC3-I to LC3-II, and that this was the reason for the increased LC3-I levels in ST+PRO. The observation that changes in autophagic proteins did not significantly differ between ST+PRO and PRO, support the interpretation that autophagy was suppressed 2.5 hours after strength training and protein ingestion. Nevertheless, since only one muscle biopsy was obtained, we do not know if the increases in gene expression of ATG7, LC3 and p62 caused a later increase in autophagic proteins, as observed previously (Ogborn et al., 2015). It can neither be dismissed that autophagic activity was enhanced *during* the training session, but that the protein levels were "normalized" 2.5 hours later due to a potent stimulation of the mTORC1 pathway.

BNIP3L mRNA tended to increase following the first training session in ST+PRO ($P = 0.058$), accompanied by a significant increase in DRP1 mRNA. BNIP3L mediates mitophagy (Yuan et al., 2017), whereas DRP1 regulates mitochondrial fission (Frank et al., 2001). Evidence suggests that mitophagy is attenuated with aging (Joseph et al., 2013; Palikaras, Lionaki, & Tavernarakis, 2015), and it has been hypothesized that this may contribute to the development of sarcopenia (Alway, Mohamed, & Myers, 2017). Interestingly, alterations in mitochondrial dynamics may be involved in the age-related reduction in mitochondrial function, based on observations of increased abundance of small, fragmented mitochondria (Iqbal, Ostojic, Singh, Joseph, & Hood, 2013), as well as very large mitochondria (Leduc-Gaudet et al., 2015) in muscles from old rodents. Indeed, both the mRNA and protein levels of important fusion and fission proteins have been reported to be lower with aging in both rodents (Ibebunjo et al., 2013) and humans (Joseph et al., 2012; Tezze

Article IV

et al., 2017). Interestingly, lower DRP1 expression was observed in frail, compared to non-frail elderly women (Drummond et al., 2014), indicating that mitochondrial dynamics may be more affected in individuals with low functional capacity. The results in the present study are therefore interesting, and suggest that unaccustomed resistance exercise might activate mitophagy in frail elderly, and that increased mitochondrial fission might be involved. No changes were observed in response to the last training session, but whether this was due to increased basal levels of mitophagy proteins is not known. Hence, the effect of strength training on mitophagy and mitochondrial dynamics warrants further investigation.

The ten-week training intervention did not change protein levels of cytosolic LC3-I, membrane LC3-I or membrane LC3-II. Consequently, also the LC3-II/LC3-I ratio was unaffected by training. Interestingly, although cytosolic p62 did not change, a group x time interaction was observed for p62 in the membrane fraction, due to a 15% reduction in ST+PRO and a 34% increase in PRO. The reduction in membrane p62 was accompanied by non-significant reductions in membrane LC3-I (-20%) and membrane LC3-II (-8%), perhaps representing a slight reduction in autophagosome abundance following the ten-week training intervention. A potential training-induced reduction in autophagosome abundance is interesting, considering that we recently observed higher LC3-II levels in old compared to young individuals (Aas et al., 2019, [article III]). Moreover, a tendency for an age-related increase in LC3-II (+~45%) has also been observed recently by others (Dethlefsen et al., 2018). The potential long-term effect of strength training on autophagic markers therefore warrants further investigation, and it is possible that a greater training frequency (>2 sessions/week) and/or longer duration of training (>10 weeks) is needed to elicit greater changes.

Heat shock proteins

Hsp70 and α B-crystallin were measured both in the cytosolic and cytoskeletal compartment of the cell, because exercise appears to elicit a transient translocation from the cytosolic pool to the stressed myofibrils after exercise (Cumming, Paulsen, Wernbom, Ugelstad, & Raastad, 2014). Acutely, the ratio of cytoskeletal to cytosolic α B-crystallin tended to increase after the first ($P = 0.067$), and increased significantly after the last training session in ST+PRO. Thus, our data indicate that strength training elicits translocation of small, cytosolic HSPs to denatured and damaged proteins in the cytoskeleton, and that this occurs regardless of training status. No acute changes were observed for Hsp70, although a tendency for a group x time interaction was observed at test day 1. The more prominent effect on α B-crystallin compared to Hsp70 is in line with previous reports, demonstrating that the myofibrillar translocation of small HSPs, such as α B-crystallin, is much more rapid (0.5-1 hour) compared to larger heat shock proteins, such as Hsp70 (>24 hours) (Cumming et al., 2014). We did not observe any correlations between autophagy markers and heat

Article IV

shock proteins at baseline, and the acute training-induced heat shock response observed for α B-crystallin did not correlate with changes in any of the autophagic proteins. Contrary to observations in rodents (Molanouri Shamsi, Mahdavi, Quinn, Gharakhanlou, & Isanegad, 2016), the ten-week strength training intervention did not alter resting levels of Hsp70 or α B-crystallin, neither in the cytosolic nor cytoskeletal fraction.

Conclusions

This study shed light on acute and long-term effects of strength training and protein supplementation on markers of muscle protein synthesis and muscle protein breakdown in frail elderly individuals, a population in which muscle biopsies are rarely obtained. Our results showed that phosphorylation of enzymes governing muscle protein synthesis tended to increase following protein intake alone, and that phosphorylation of the same enzymes increased significantly when protein intake was preceded by a session of heavy load strength training. The autophagic proteins indicated suppressed autophagy 2.5 hours after strength training and protein intake, both in the untrained and trained state. However, gene expression data indicated activation of both the ubiquitin proteasome system and autophagy after unaccustomed strength training, perhaps leading to increased protein levels and activity in these pathways at a later time point. Lastly, strength training performed twice weekly for ten weeks had limited effects on autophagic markers and heat shock proteins. Further research should investigate if a greater training frequency or perhaps a stronger metabolic stimulus (15-20 RM) is required to elicit changes in basal autophagy in frail elderly individuals.

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TABLES

Table 1. Baseline characteristics.

Characteristic	ST+PRO	PRO	<i>P</i> -value
N (men/women)	10/8	6/4	-
Age (years)	87 ± 7	84 ± 6	0.18
Body mass (kg)	64.4 ± 13.3	74.7 ± 13.4	0.06
Lean body mass (kg)	43.0 ± 8.0	45.7 ± 9.8	0.44
Lean leg mass (kg)	14.2 ± 3.0	15.6 ± 4.4	0.32
Body fat (%)	30.4 ± 7.1	37.0 ± 6.0	0.02
SPPB score	5.7 ± 2.8	6.5 ± 2.8	0.46

N, Number; SPPB, Short Physical Performance Battery. Data shown as mean ± SD. *P*-values give the statistical significance of differences between test ST+PRO and PRO.

Table 2. Primer sequences employed for reverse transcription-polymerase chain reaction.

Gene	Forward	Reverse
Actb	GGGAAATCGTGCGTGACA	GGACTCCATGCCCAGGA
Atrogin-1	GCAGCTGAACAACATTCAGATCAC	CAGCCTCTGCATGATGTTTCAGT
MuRF1	CTTCCCTTCTGTGGACTCTTCCT	CCTGAGAGGCATTGACTTTGG
MUSA1	GGGAAAAGGAAGTATCCAGA	GCCATGCTTAGGATGTCAGC
OPA1	GGCTCCTGACACAAAGGAAA	TCCTTCCATGAGGGTCCATT
DRP1	GGCGCTAATTCCTGTCATAA	CAGGCTTCTAGCACTGAGC
BNIP3	GGGATGCAGGAGGAGAGC	ACTCCGTCCAGACTCATGC
BNIP3L	CATGAATCAGGACAGAGTAGTTCC	CCTTCTCTCCTTCTACAACTTCTTC
ATG7	AGGAGATTCAACCAGAGACGG	TGGTGAGGCACAAGCCCAA
P62	GCTTCCAGGCGCACTACC	CATCCTTCACGTAGGACATGG
LC3	TCGAGAGCAGCATCCAAC	GCTGTGTCCGTTACCA

Article IV

Table 3. mRNA analyses of regulators of proteasomal degradation, autophagy/mitophagy, and mitochondrial fusion and fission after strength training with protein supplementation (ST+PRO), or protein supplementation alone (PRO). Test day 1 was conducted before, and test day 2 after the ten-week intervention.

mRNA	Test Day 1		Test Day 2		Intervention	
	<i>(fold change from baseline)</i>		<i>(fold change from baseline)</i>		<i>(fold change from baseline)</i>	
	ST+PRO	PRO	ST+PRO	PRO	ST+PRO	PRO
<i>Positive regulators of proteasomal degradation</i>						
MuRF-1	2.85 ± 2.53*	1.26 ± 1.14	1.15 ± 0.68	0.66 ± 0.44	0.97 ± 0.58	1.49 ± 0.91
Atrogin-1	1.11 ± 0.71	0.71 ± 0.27	0.62 ± 0.27*	0.72 ± 0.24*	1.34 ± 0.53	1.08 ± 0.55
MUSA1	1.51 ± 0.76*	1.24 ± 0.60	1.14 ± 0.55	1.34 ± 1.61	1.18 ± 0.49	1.63 ± 0.70
<i>Positive regulators of autophagy / mitophagy</i>						
ATG7	1.41 ± 0.69*	1.07 ± 0.33	1.03 ± 0.37	1.84 ± 1.72	0.93 ± 0.34	1.10 ± 0.58
LC3	2.90 ± 3.77*	1.30 ± 0.54	1.05 ± 0.35	2.41 ± 2.81	1.18 ± 0.64	1.53 ± 0.80
p62	1.89 ± 1.90*	1.27 ± 0.28	1.14 ± 0.25	1.49 ± 1.78	1.01 ± 0.52	2.77 ± 4.06
BNIP3	2.35 ± 4.83	1.25 ± 1.06	1.31 ± 0.61	1.30 ± 1.64	1.09 ± 0.94	3.76 ± 7.37
BNIP3L	3.71 ± 9.04	1.05 ± 0.27	1.24 ± 0.70	1.87 ± 2.38	2.73 ± 3.81	1.45 ± 0.97
<i>Regulators of mitochondrial fusion and fission</i>						
OPA1	1.68 ± 1.63	1.07 ± 0.22	0.88 ± 0.24	1.41 ± 1.07	1.29 ± 0.74	1.44 ± 0.87
DRP1	3.18 ± 3.15*	1.40 ± 0.28	1.16 ± 0.36	1.40 ± 1.20	1.73 ± 1.76	1.51 ± 0.55

All data are presented as means ± SD, and display the fold change from baseline at Test Day 1, Test Day 2, and after the ten-week intervention. MuRF1, muscle RING-finger protein-1; Atrogin-1, muscle atrophy F-Box protein 32, MUSA1, F-Box only protein 30; ATG7, autophagy related 7; LC3, microtubule-associated protein 1 light chain 3; p62, Sequestosome 1; BNIP3, BCL2/adenovirus E1B 19-kDa interacting protein 3; BNIP3L, BCL2/adenovirus E1B 19-kDa interacting protein 3 Like; OPA1, mitochondrial dynamin-like GTPase; DRP1, dynamin-related protein 1. * $P < 0.05$ vs. baseline within group.

FIGURES

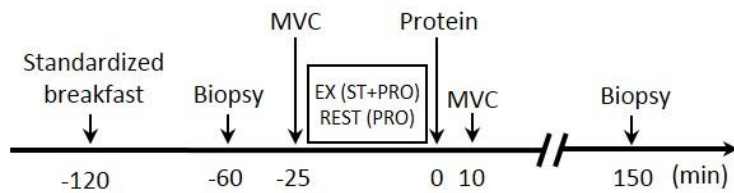


Figure 1. Experimental design for Test Day 1 (TD1) performed before the ten-week intervention, and Test Day 2 (TD2), performed after the ten-week intervention. MVC: maximal voluntary contraction, EX: strength training session, REST: rest period.

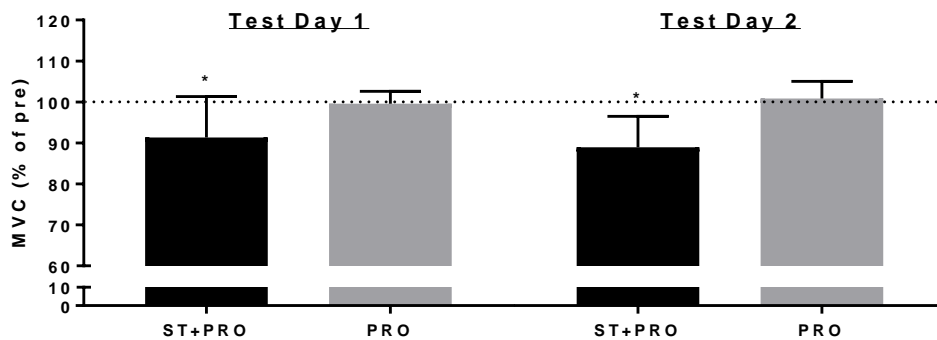


Figure 2. Isometric knee extensor force-generating capacity (MVC) relative to resting values 10 minutes after a 19-min bout of heavy load strength training (ST+PRO) or following rest (PRO), at Test Day 1 (before intervention) and Test Day 2 (after intervention). Values are mean \pm SD. * $P < 0.05$ different from baseline within group.

Article IV

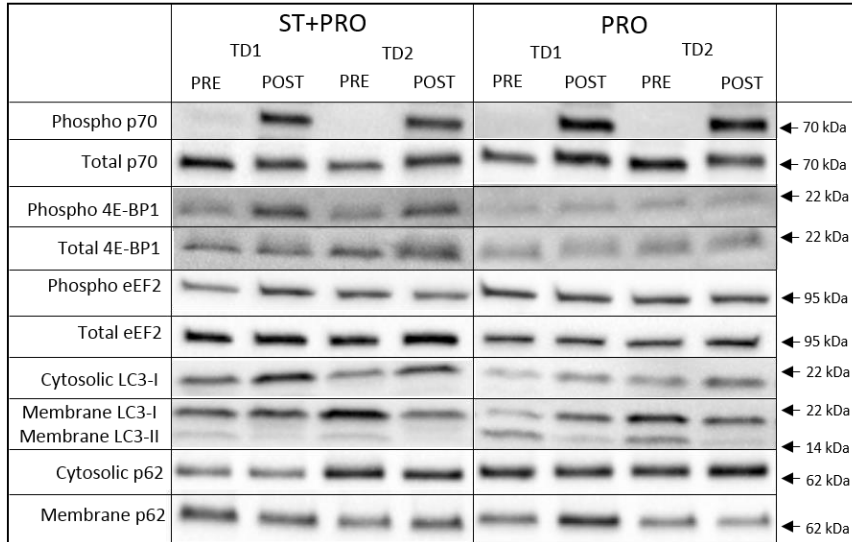


Figure 3. Representative western blots for anabolic signaling proteins and autophagic markers. TD1: test day 1, TD2: test day 2

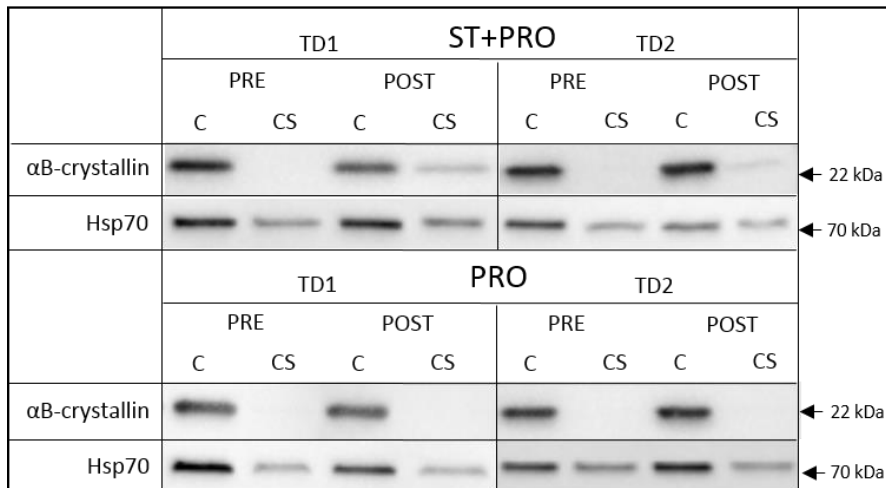


Figure 4. Representative western blots for the heat shock proteins. TD1: test day 1, TD2: test day 2, C: cytosolic fraction, CS: cytoskeletal fraction.

Article IV

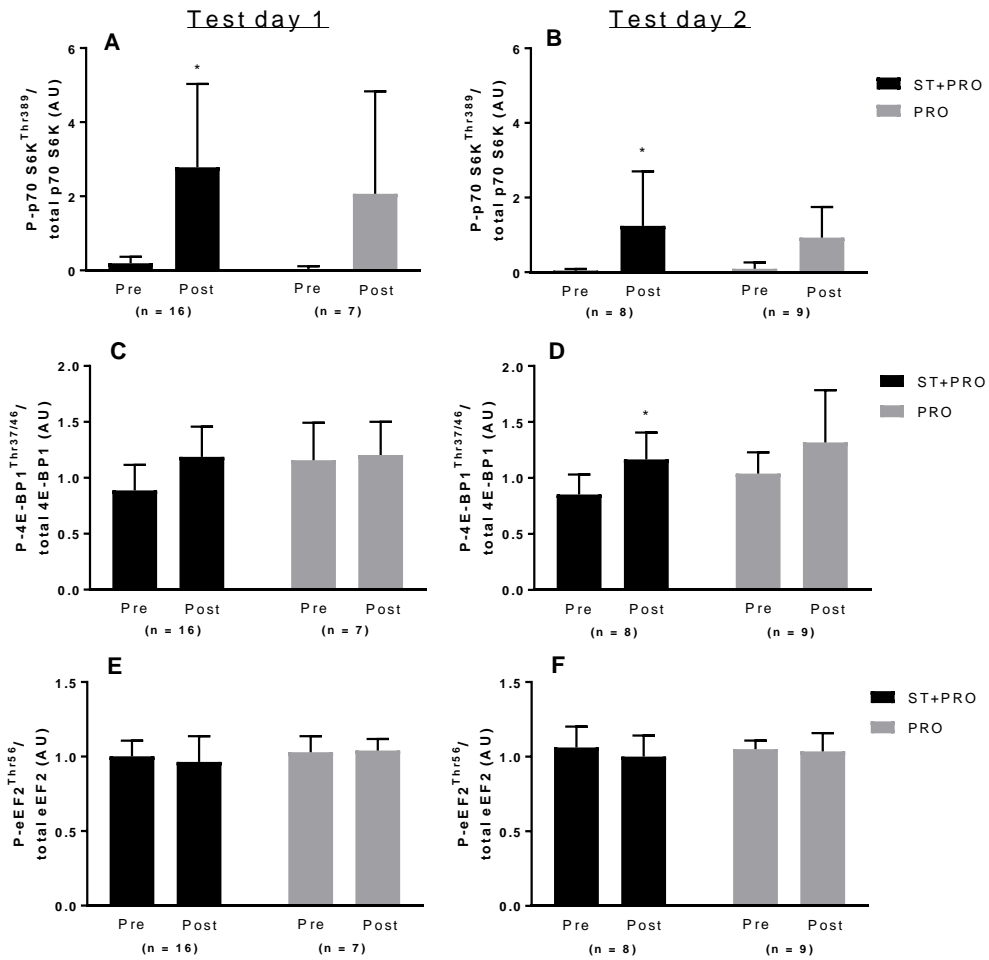


Figure 5. Change in phosphorylation ratio of proteins involved in the regulation of muscle protein synthesis at Test Day 1 (before intervention) and Test Day 2 (after intervention) in ST+PRO and PRO. Results show the ratio of phosphorylated to total p70S6K (panel A-B), 4E-BP1 (panel C-D), and eEF2 (panel E-F). Values are mean \pm SD. AU, arbitrary units. * $P < 0.05$, different from baseline within group.

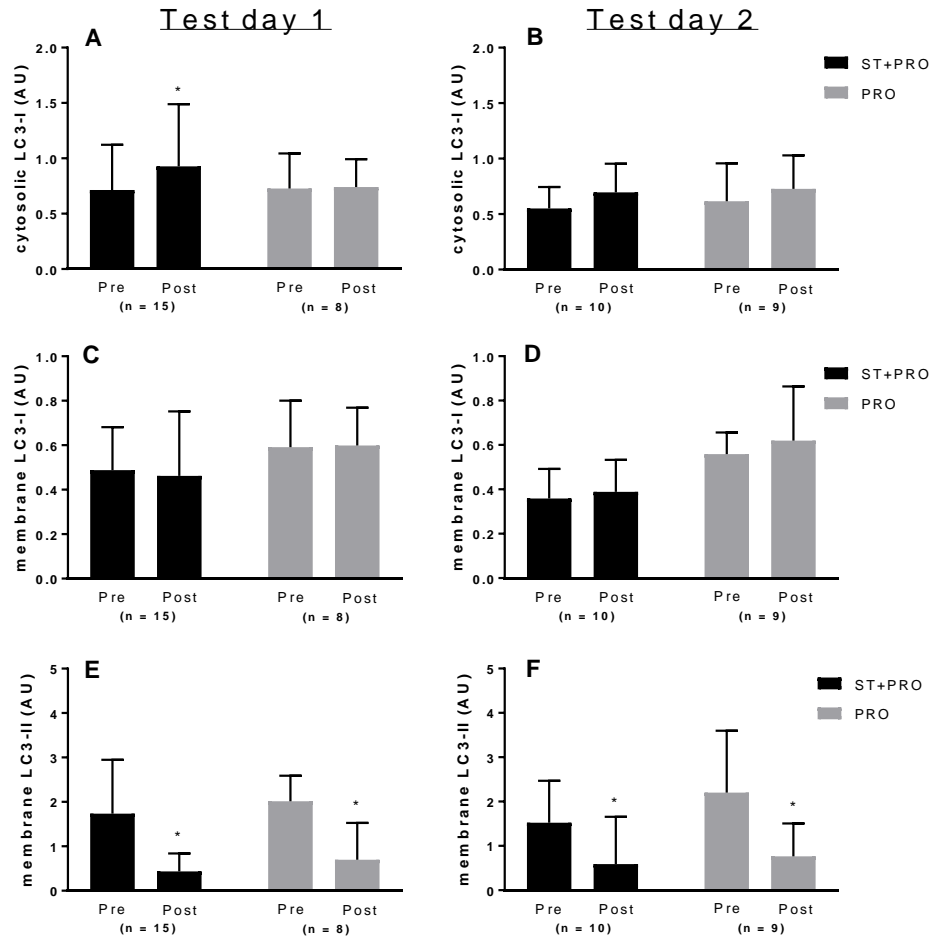


Figure 6. Change in protein levels of LC3-I and LC3-II at Test Day 1 (before intervention) and Test Day 2 (after the intervention), in ST+PRO and PRO. Results show cytosolic LC3-I (panel A-B), membrane LC3-I (panel C-D) and membrane LC3-II (panel E-F). Values are mean \pm SD. AU, arbitrary units. * $P < 0.05$, different from baseline within group.

Article IV

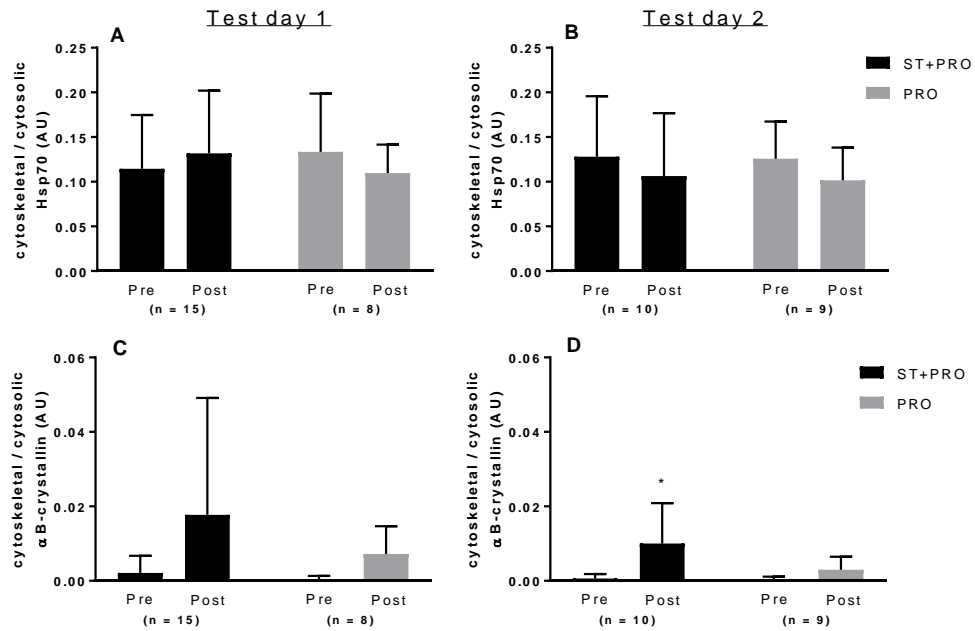


Figure 7. Change in heat shock proteins at Test Day 1 (before intervention) and Test Day 2 (after intervention), in ST+PRO and PRO. Results show the ratio of cytoskeletal to cytosolic Hsp70 (panel A-B) and α B-crystallin (panel C-D). Values are mean \pm SD. AU, arbitrary units. * $P < 0.05$, different from baseline within group.

APPENDIX I

Letter of exemption from Regional Committee for Medical and Health Research Ethics (study C)

Approval from the Regional Committee for Medical and Health Research Ethics (study D)

Participant information and consent form (study C)

Participant information and consent form (study D)

**Regional Committee for Medical
& Health Research Ethics**

South East Norway, Section C

Postbox 1130 Blindern

NO-0318 Oslo

Norway

Phone: + 47 22 84 55 98

E-mail: t.t.mosling@medisin.uio.no

Webportal: <http://helseforskning.etikkom.no>

Our ref.: 2015/995c
IRB00001870 REK Sør-Øst C

Date: 20th November 2018

To whom it may concern,

Re: REC Letter of Exemption

I am writing in reference to a request from Truls Raastad via e-mail dated 31th of October 2018, regarding a Letter of Exemption in English.

Review

The Regional Committee for Medical & Health Research Ethics, Section C, South East Norway, reviewed the Research Project *Norwegian title: Muskelstyrke og funksjon hos eldre med lav muskelmasse og muskelstyrke* at its Committee Review Meeting on the 11th of June 2015. The Project Manager is Truls Raastad and the Institution Responsible for Research is Norway's sports academy.

The application was assessed accordance with the Norwegian Research Ethics Act (2006) and Act on Medical and Health Research (2008).

The Committee's Decision

The Regional Committee for Medical & Health Research Ethics, Section C, South East Norway, found the Research Project to be outside the remit of the Act on Medical and Health Research (2008) and therefore can be implemented without its approval.

Ethics Committee System

The Ethics Committee System in Norway consists of seven Independent Regional Committees with authority to either approve or disapprove Medical Research Studies conducted within Norway, or by Norwegian Institutions, in accordance with the Act on Medical and Health Research (2008).

Please do not hesitate to contact the Regional Committee for Medical and Health Research Ethics Section South East C (REK Sør-Øst C) if further information is required, as we are happy to be of assistance.

Yours faithfully,

Britt - Ingerd Nesheim
Chair of the Regional Committee for Medical
& Health Research Ethics of South East Norway,
Section C

Tone Transeth Mosling
Senior Executive Officer

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Claus Henning Thorsen	22845515	12.09.2016	2016/895/REK sør-øst C
			Deres dato:	Deres referanse:
			10.08.2016	

Vår referanse må oppgis ved alle henvendelser

Truls Raastad
Norges Idrettshøgskole
Sognsveien 220
0806 Oslo

Forskningsansvarlig: Norges idrettshøgskole
Prosjektleder: Truls Raastad

Prosjektomtale (original):

Med økende alder reduseres muskelkvaliteten (ratio muskelstyrke/ muskelstørrelse) og dette bidrar sterkt til redusert funksjonsnivå hos eldre. Styrketrening har motsatt effekt, selv hos eldre med et lavt funksjonsnivå. Studier gjort på mus har vist at autofagi, et system som har i oppgave å bryte ned ødelagte proteiner i muskelcellene, fungerer dårligere med økende alder. Det er mulig at dette bidrar til den aldersrelaterte nedgangen i muskelkvalitet. Trening ser ut til å øke autofagi-aktiviteten i mus, men hvorvidt det samme er tilfellet hos mennesker er uvisst. Hovedformålet med denne studien er derfor å undersøke hvordan styrketrening påvirker muskelkvalitet og hvordan underliggende mekanismer endres og bidrar til økt muskelkvalitet med trening. Deltagerne trekkes til en trenings- eller en kontrollgruppe (randomisert kontrollert studie). Begge grupper vil få et proteintilskudd, men bare treningsgruppen vil gjennomføre styrketrening 2 ganger per uke i 10 uker.

Vi viser til klage mottatt 10.08.2016 for ovennevnte prosjekt. Klagen ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK sør-øst C) i møtet 18.08.2016. Vurderingen er gjort med hjemmel i helseforskningsloven § 10, jf. forskningsetikklovens § 4.

Komiteens vurdering

Komiteen mener dette er en gjennomarbeidet, oppklarende og godt begrunnet klage. Klagen synliggjør den erfaringen som finnes i forskningsgruppen, og de erfaringene man har fra gjennomføringen av lignende studier.

Det er gitt en bedre beskrivelse av studiens rasjonale. Det er etablert kunnskap at styrketrening har positiv effekt, men i dette prosjektet fremsettes muskelrelaterte hypoteser relatert til sarkopeni. Nødvendigheten av biopsiene er godt redegjort for, og det er gitt en realistisk beskrivelse av ulempene ved biopsitaking, herunder hvordan man ivaretar og forholder seg til deltagerne under denne prosedyren.

Komiteen mener det i klagen er gitt en langt bedre begrunnelse for nytten av prosjektet, samtidig som ulempene ved deltagelse er nedtonet. Komiteen innser at et viktig etisk moment er at man bør være varsom med å frata denne sårbare gruppen retten til selv å bestemme om de vil være med på et prosjekt som de oppfatter som meningsfylt, og som vil kunne være et velkomment avbrett i hverdagslivets rutiner.

Komiteen finner etter en samlet vurdering å kunne ta klagen til følge og omgjør sitt vedtak av 28.06.2016, jf. forvaltningsloven § 33 annet ledd.

Komiteen oppfatter samtykkekompetanse som en hovedutfordring i prosjektet. Screening og vurdering av samtykkekompetanse er tydelig beskrevet, men den angitte test for å vurdere inklusjon er etter komiteens oppfatning ikke avgjørende for samtykkekompetansen. Dette må vurderes nærmere, og komiteen forutsetter

at det etableres gode rutiner for samtykkevurdering. Sykehjemslegen forutsettes videre å vurdere den enkelte beboers medikasjon i relasjon til deltakelse i prosjektet.

Komiteen forutsetter videre at informasjon om studien gis i god tid, slik at deltakerne får mulighet til å tenke grundig igjennom hvorvidt de ønsker å være med, samt at det kommuniseres tydelig om muligheten til å trekke seg fra studien underveis.

Biobank

Det søkes om opprettelse av en spesifikk forskningsbiobank med navn *STAS* i prosjektet.

Ansvarshavende for forskningsbiobanken er Truls Raastad.

Forskningsansvarlig er Norges idrettshøgskole.

Forskningsbiobanken vil bestå av blodprøver og vev fra muskelbiopsier.

Komiteen setter en tidsavgrensing for forskningsbiobanken tilsvarende oppbevaringstiden for prosjektdata, det vil si til 17.09.2022. Deretter skal materialet behandles i henhold til helseforskningsloven § 30.

Komiteen godkjenner at humant biologisk materiale overføres til Danmark og Italia for analyse, jf. helseforskningsloven § 29.

Vedtak

Komiteen omgjør sitt vedtak av 28.08.2016, og prosjektet godkjennes, jf. helseforskningsloven §§ 9 og 33.

Tillatelsen er gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i søknaden og protokollen, og de bestemmelser som følger av helseforskningsloven med forskrifter.

Komiteen godkjenner opprettelse av forskningsbiobanken *STAS* i tråd med det som er angitt i prosjektsøknaden. Biobankregisteret vil bli underrettet ved kopi av dette brev.

Tillatelsen gjelder til 17.09.2017. Av dokumentasjons- og oppfølgingshensyn skal opplysningene likevel bevares inntil 17.09.2022. Opplysningene skal lagres avidentifisert, dvs. atskilt i en nøkkel- og en opplysningsfil. Opplysningene skal deretter slettes eller anonymiseres, senest innen et halvt år fra denne dato.

Komiteens avgjørelse var enstemmig.

Sluttmelding og søknad om prosjektendring

Dersom det skal gjøres endringer i prosjektet i forhold til de opplysninger som er gitt i søknaden, må prosjektleder sende endringsmelding til REK. Prosjektet skal sende sluttmelding på eget skjema, se helseforskningsloven § 12, senest et halvt år etter prosjektslutt.

Klageadgang

Komiteens vedtak kan påklages til Den nasjonale forskningsetiske komité for medisin og helsefag, jf. helseforskningsloven § 10, tredje ledd og forvaltningsloven § 28. En eventuell klage sendes til REK sør-øst C. Klagefristen er tre uker fra mottak av dette brevet, jf. forvaltningsloven § 29.

Med vennlig hilsen

Britt-Ingjerd Nesheim
prof.dr.med.
leder REK sør-øst C

Claus Henning Thorsen
Rådgiver

Kopi til: turid.sjostedt@nih.no; postmottak@nih.no; biobankregisteret@fhi.no



Forespørsel om deltakelse som forsøksperson

Hvordan bedre muskelstyrke og funksjon i daglige gjøremål hos eldre med lav muskelmasse og muskelstyrke?

Dette skrevet er til alle potensielle forsøkspersoner. Vi ber om din deltakelse i prosjektet, så fremt du oppfyller kriteriene: Du må være 75 år eller eldre og kunne gjennomføre tilpasset styrketrening (under tett oppfølging). Du skal ikke ha drevet regelmessig med tung styrketrening det siste året (>1 gang per uke). Du kan ikke ha laktoseintoleranse eller melkeallergi. Du kan heller ikke bruke noen form for kosttilskudd (proteinpulver, vitaminer, kreatin eller lignende); hvis du gjør det kan du likevel delta som forsøksperson ved at du slutter med tilskuddet senest en uke før prosjektstart.

Bakgrunn og hensikt med forsøket

Sarkopeni (aldersrelatert muskelsvinn) har de siste årene fått mye oppmerksomhet da det i tillegg til å redusere funksjon og livskvalitet i hverdagen også disponerer for flere livsstilssykdommer (blant annet type II diabetes og osteoporose). Lav muskelstyrke øker også risikoen for fall blant eldre. Styrketrening og et økt inntak av proteiner har vist seg å kunne motvirke nedgangen i muskelmasse og muskelstyrke med økende alder. Styrketrening etterfulgt av proteininntak har en umiddelbar muskeloppbyggende effekt ved at proteinsyntesen øker, og over tid vil dette kunne resultere i økt muskelmasse og muskelstyrke.

I denne studien ønsker vi å undersøke effekten av et forholdsvis enkelt styrketreningsopplegg sammen med proteinsupplementering på eldre med lav muskelmasse og muskelstyrke.

Hovedhensikten med studien er å utvikle en strategi som effektivt kan motvirke sarkopeni (aldersrelatert muskelsvinn) hos eldre.

Gjennomføringen av forsøket

Dette er et randomisert kontrollert studie, og deltakere vil trekkes tilfeldig til en av to grupper.

Dersom man blir trukket (randomisert) til treningsgruppen, skal man gjennomføre styrketrening tre ganger i uken i 10 uker. I tillegg skal man innta to enheter på 0,33 l TINE Styrk daglig. Selve treningen vil finne sted på eldresenteret du benytter deg av. Dersom du blir trukket til kontrollgruppen, skal du

verken gjennomføre styrketrening eller innta TINE Styrk, men leve som vanlig. Før og etter intervensjonsperioden vil det gjennomføres ulike tester ved Norges Idrettshøgskole, uavhengig av om man er i treningsgruppen eller kontrollgruppen.

Tester på dagsenteret/seniorsenteret

For å vurdere hvorvidt du kan inkluderes som forsøksperson i denne studien, vil vi gjennomføre noen tester ved ditt dagsenter. Vi vil blant annet gjennomføre noen funksjonelle tester, hvor vi tester balanse, ganghastighet, og hvor raskt du kan reise deg opp fra en stol (Short Physical Performance Battery; SPPB). Før du inkluderes som deltaker vil du også måtte besvare et spørreskjema omhandlende hjerteproblematikk, medisinbruk med mer. På bakgrunn av dine svar her vil vi vurdere hvorvidt en legeundersøkelse skal gjennomføres før du eventuelt inkluderes i studien.

Både de funksjonelle testene og en eventuell legesjekk vil avgjøre hvorvidt du kan inkluderes i studien eller ikke.

Tester på Norges Idrettshøgskole

Dersom du blir inkludert i prosjektet skal du møte på Norges Idrettshøgskole en gang før treningsperioden og en gang etter treningsperioden. Vi vil bistå med transport, dersom det er nødvendig. Hvert oppmøte vil vare i ca. 4 timer, og man skal møte fastende. Etter DXA (se nedenfor) og blodtrykk/fingerstikk vil du kunne spise frokost. Tidspunkter for de ulike oppmøtene avtales individuelt. Nedenfor følger en oversikt over tester som vil bli gjennomført på Norges Idrettshøgskole, både før og etter 10-ukersperioden.

- *DXA*: En DXA-analyse vil gjennomføres for å måle kroppssammensetningen. Denne testen innebærer at man ligger stille i ca. 10 minutter. Tas fastende.
- *Blodtrykk/fingerstikk*: For å måle kolesterol, glukose og triglyserider. Tas fastende.
- *Kostholdsintervju*: For å få informasjon om kostholdet ditt (ca. 15 min).
- *Ultralyd*: For å måle tverrsnitt av *vastus lateralis*, en muskel på fremsiden/yttersiden av låret.
- *Muskelfunksjonstest*: Gir et mål på styrke/eksplosivitet i musklene som strekker kneleddet. Kneleddet er låst ved 90° i denne testen.
- *1RM*: Maksimal styrke i øvelsen kneekstensjon.
- *Funksjonelle tester*: For å teste hvor raskt man kan reise seg fra en stol fem ganger på rad, samt hvor raskt man kan gå opp en trapp. Gir informasjon om funksjon i hverdagen og mobilitet. I tillegg måler vi ganghastighet.

I de to siste dagene før tester må du avstå fra all krevende fysisk aktivitet (trening)

Etter den første testdagen vil deltakere bli randomisert (trukket tilfeldig) til treningsgruppen eller kontrollgruppen. Dersom man blir trukket til kontrollgruppen vil man få tilbud om treningsoppfølging våren 2016 (én gang per uke).

Treningsperioden

Treningsperioden starter når man har gjennomført alle testene, og den varer i 10 uker. Dersom man blir trukket til treningsgruppen vil man i disse 10 ukene trene styrke tre ganger i uken i grupper på to/tre deltakere med oppfølging av en instruktør på alle økter. Hver enkelt økt vil ha en varighet på 20-40 minutter, og den vil gjennomføres ved det dagsenteret du benytter. Treningsprogrammet som skal gjennomføres inneholder trappegang (med eller uten vektvest), beinpress, kneekstensjon («knestrek») og ettbeins knebøy («halve knebøy», med støtte tilpasset den enkeltes funksjonsnivå). Treningsøvelsene som er valgt belaster muskler som innehar en viktig rolle i mange daglige gjøremål. Etter treningsperioden gjennomføres alle testene på nytt for å måle endringer.

Eventuelle ulemper ved å delta

Deltakelse i prosjektet vil kreve en del tid og oppmerksomhet. Det blir ett oppmøte på Norges Idrettshøgskole før treningsperioden, og ett oppmøte etter endt 10-ukersperiode. Dette uavhengig av om man blir trukket til treningsgruppen eller kontrollgruppen.

Treningen skal gjennomføres med forholdsvis stor belastning, og vil medføre en viss risiko for skade og følelse av sårhet/stølheth i muskulaturen.

Personvern

Vi vil kun lagre informasjon om deg under ditt forsøkspersonnummer. Undervis i forsøket vil vi oppbevare en kodeliste med navn og forsøkspersonnummer. Denne kodelisten vil fysisk være låst inne, slik at det er kun forskerne tilknyttet studien som har adgang til den. Alle som får innsyn i informasjon om deg har taushetsplikt. Innsamlet data vil bli anonymisert etter 15 år (kodelisten destrueres). Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

Innsynsrett og oppbevaring av materiale

Hvis du sier ja til å delta i studien, har du rett til å få innsyn i hvilke opplysninger som er registrert om deg. Du har videre rett til å få korrigerert eventuelle feil i de opplysningene vi har registrert. Dersom du trekker deg fra studien, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre

opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner.

Informasjon om utfallet av studien

Etter at data er samlet inn og analysert vil vi avholde et møte for alle forsøkspersonene der vi presenterer resultatene fra studien.

Forsikring

Deltakere i prosjektet er forsikret dersom det skulle oppstå skade eller komplikasjoner som følge av deltakelse i forskningsprosjektet. NIH er en statlig institusjon og er således selvassurandør. Dette innebærer at det er NIH som dekker en eventuell erstatning og ikke et forsikringsselskap.

Finansiering

Prosjektet er finansiert av Norges Idrettshøgskole

Publisering

Resultatene fra studien vil offentliggjøres i internasjonale, fagfelleverderte, tidsskrift. Du vil få tilsendt artiklene hvis du ønsker det.

Samtykke

Hvis du har lest informasjonsskrivet og ønsker å være med som forsøksperson i prosjektet, ber vi deg undertegne "Samtykke om deltakelse" og returnere dette til en av personene oppgitt nedenfor. Du bekrefter samtidig at du har fått kopi av og lest denne informasjonen.

Det er frivillig å delta og du kan når som helst trekke deg fra prosjektet uten videre begrunnelse. Alle data vil, som nevnt ovenfor, bli avidentifisert før de blir lagt inn i en database, og senere anonymisert.

Dersom du ønsker flere opplysninger kan du ta kontakt med Sigve Nyvik Aas på tlf: 414 99 074 eller Truls Raastad på tlf: 23 26 23 28 el. 913 68 896

Vennlig hilsen

Sigve Nyvik Aas (Stipendiat)

Truls Raastad (Professor)

Samtykke til deltakelse i studien

Jeg er villig til å delta i studien

(Signert av prosjektdeltaker, dato)

Jeg bekrefter å ha gitt informasjon om studien

(Signert, rolle i studien, dato)



NORGES IDRETTSHØGSKOLE

FORESPØRSEL OM DELTAKELSE I FORSKNINGSPROSJEKTET

STYRKETRENING FOR ELDRE MED LAV MUSKELSTYRKE

Dette er et spørsmål til deg om å delta i et forskningsprosjekt hvor vi ønsker å undersøke effekten av et enkelt og tidseffektivt styrketreningsopplegg sammen med proteinsupplementering på muskelmasse, muskelstyrke, muskelkvalitet og fysisk prestasjonsevne hos eldre med lavt funksjonsnivå.

Med økende alder ser man en gradvis reduksjon i både muskelmasse og muskelstyrke, men tapet av muskelstyrke er større enn tapet av muskelmasse. Som et resultat reduseres muskelkvaliteten med økende alder (definert som muskelstyrke/muskeltverrsnitt). Ved styrketrening er utviklingen den motsatte; muskelstyrken øker vesentlig mer enn muskelmassen, og muskelkvaliteten økes. Dette er spesielt tydelig hos eldre personer som i utgangspunktet har lav muskelstyrke. Vi vet likevel lite om det relative bidraget fra de ulike faktorene som kan tenkes å påvirke muskelkvaliteten ved styrketrening. Vi ønsker derfor å rekruttere eldre med lav muskelstyrke til en studie hvor vi undersøker endringer i muskelkvalitet som følge av styrketrening og proteinsupplementering. Norges idrettshøgskole er ansvarlig for gjennomføring av prosjektet, og de fleste tester vil gjennomføres her. All styrketrening gjennomføres på ditt sykehjem/dagsenter eller i nærheten av der du bor.

HVA INNEBÆRER PROSJEKTET?

Dette er en randomisert kontrollert studie. Det betyr at du trekkes tilfeldig til en av to grupper. Den ene gruppen skal gjennomføre styrketrening to ganger per uke i 10 uker, og innta en kartong Tine Styrk (0.33 l) daglig gjennom perioden. Den andre gruppen skal innta samme mengde Tine Styrk, men ikke gjennomføre styrketrening. På denne måten kan vi sammenligne effekten av økt proteininntak alene og økt proteininntak i kombinasjon med styrketrening. Dersom du trekkes til treningsgruppen, vil all trening finne sted på ditt sykehjem, dagsenter, eller like i nærheten av der du bor. Før og etter intervensjonsperioden vil det gjennomføres ulike tester ved Norges idrettshøgskole.

Tester på sykehjemmet/dagsenteret/seniorsenteret

For å vurdere hvorvidt du kan inkluderes som forsøksperson i denne studien, vil vi gjennomføre noen

tester der du holder til. Vi kommer til å måle høyde og vekt, blodtrykk og blodprofil (fingerstikk). I tillegg kommer vi til å gjennomføre ulike funksjonelle tester, hvor vi måler balanse, ganghastighet, og hvor raskt du kan reise deg opp fra en stol. Vi vil også gjennomføre en enkel test for å måle grepstyrke. Før du inkluderes som deltaker vil du også måtte besvare et spørreskjema omhandlende hjerteproblematikk, medisinbruk med mer. På bakgrunn av dine svar her vil vi vurdere hvorvidt en legeundersøkelse skal gjennomføres før du eventuelt inkluderes i studien. Vi vil også gjennomføre en test som evaluerer kognitiv funksjon (enkle tester på forståelse av ulike oppgaver). Både funksjonelle tester, kognitiv test, og en eventuell legesjekk vil avgjøre hvorvidt du kan inkluderes i studien eller ikke.

Tester på Norges idrettshøgskole

Dersom du blir inkludert i prosjektet skal du møte på Norges idrettshøgskole tre ganger før treningsperioden og to ganger etter treningsperioden. Vi vil bistå med transport. Hvert oppmøte vil vare i 2-5 timer, og en av disse dagene skal du møte fastende (ikke spise frokost før du ankommer). Tidspunkter for de ulike testdagene avtales individuelt. Felles for alle testdager er at du må avstå fra fysisk trening de siste to dagene før testing.

Testdag 1 gjennomføres den første gangen du kommer til Norges idrettshøgskole. Denne testdagen tar omtrent 3 timer å gjennomføre. Vi vil bistå med transport til og fra Norges idrettshøgskole.

- *DXA*: En DXA-analyse vil gjennomføres for å måle kroppssammensetningen din. Denne testen innebærer at man ligger stille i ca. 10 minutter.
- *Muskelfunksjonstest*: Gir et mål på styrke og eksplosivitet i musklene som strekker kneleddet.
- *Grad av muskelaktivering*: For å undersøke i hvor stor grad du greier å aktivere muskulaturen når du tar i alt du kan.
- *1RM*: Maksimal styrke i øvelsen kneekstensjon.

Testdag 2 gjennomføres andre gang du møter på Norges idrettshøgskole. Denne dagen skal du gjennomføre de samme testene som du gjennomførte testdag 1, med unntak av DXA. I tillegg skal vi gjennomføre en ultralydundersøkelse av låret ditt denne dagen. Årsaken til at mange av testene gjennomføres to ganger er at noen av testene krever litt tilvenning/trening, og ved å gjennomføre disse to ganger er det større sannsynlighet for at resultatene blir riktige. Testdag 2 vil ta omtrent 3 timer å gjennomføre.

Testdag 3 gjennomføres også på Norges idrettshøgskole. Denne dagen skal du ta muskelbiopsier og blodprøver før og etter en styrketreningsøkt (dersom du trekkes til treningsgruppen). Dersom du trekkes til gruppen som bare får proteinsupplementering, skal du gjennomføre alle testene som er oppført nedenfor, med unntak av treningsøkten. Denne dagen skal du møte fastende, men i likhet med testdag 1 vil du få frokost etter å ha gjennomført de første testene. Nedenfor følger en oversikt over denne testdagen, som tar 4-5 timer å gjennomføre. Det vil bli gode pauser mellom de ulike testene, hvor det er mulighet for å hvile. Vi vil bistå med transport til og fra Norges idrettshøgskole.

- Blodprøve (fastende)
- Standardisert frokost (havregrøt)
- Muskelbiopsi fra ytre lårmuskel
- Styrketreningsøkt med øvelsen kneekstensjon (gjelder bare treningsgruppen)
- Inntak av 0,33 ml Tine Styrk
- Muskelbiopsi fra ytre lårmuskel

Du skal totalt ta to muskelbiopsier denne dagen, men begge biopsiene vil bli tatt fra det samme snittet i huden. Muskelbiopsiene tas ut på følgende måte:

- Huden og bindevevet lokalbedøves der vevsprøven skal tas.
- Et snitt på ca. 1-2 cm gjøres gjennom hud og bindevevet over muskelen.
- En nål med diameter på 6 mm føres inn (2-3 cm) og 1-3 små biter av muskulaturen tas ut (total 200-300 mg muskelvev).
- Snittet lukkes med tape (strips).

CT på Aleris røntgen

I tillegg til testdagene på Norges idrettshøgskole, skal du gjennomføre en CT-undersøkelse ved Aleris røntgen (Oslo sentrum) både før og etter intervensjonsperioden. Hensikten med denne undersøkelsen er å måle tverrsnittet av lårmusklene dine. CT-bildene gir oss i tillegg muligheten til å undersøke grad av fettinfiltrering i muskulaturen. Denne undersøkelsen tar omtrent en halv time. Vi vil bistå med transport.

Muskelproteinnedbrytning

Vi ønsker å måle muskelproteinnedbrytning hos et utvalg av forsøkspersonene. Disse målingene gjøres ved hjelp av deuteriumoksid (D₂O), og forutsetter en ekstra muskelbiopsi mot slutten av intervensjonsperioden. Tre uker før intervensjonsperioden starter, skal du drikke en bestemt mengde deuteriumoksid (ca. 2 dl) utblandet i vanlig vann (ca. 2 dl). På denne måten vil muskelproteinene merkes, og vi vil i neste steg kunne måle nedbrytningshastigheten for muskelproteinene omtrent 80

dager senere. Bruken av dobbeltmerket vann er utbredt i forbindelse med forskning og diagnostikk.

Treningsperioden

Dersom du trekkes til treningsgruppen, skal du gjennomføre styrketrening i 10 uker.

Treningsperioden starter når du har gjennomført alle testene. Du skal gjennomføre styrketrening to ganger i uken i grupper på to/tre deltakere. Hver enkelt økt vil ha en varighet på 20-40 minutter, og den vil gjennomføres der du bor (sykehjem, dagsenter, i tilknytning omsorgsbolig). Alle treningsøkter gjennomføres med oppfølging av en instruktør. Treningsprogrammet som skal gjennomføres består av beinpress, kneekstensjon (kneestrek) og to øvelser der du går opp på en kasse. Alle øvelser vil tilpasses den enkeltes funksjonsnivå. Treningsøvelsene som er valgt belaster muskler som innehar en viktig rolle i mange daglige gjøremål. Etter treningsperioden gjennomføres testdag 1 og testdag 3 og CT på Aleris røntgen på nytt for å måle endringer.

Vi vil kun lagre informasjon om deg under ditt forsøkspersonnummer. Undervis i forsøket vil vi oppbevare en kodeliste med navn og forsøkspersonnummer. Denne kodelisten vil fysisk være låst inne, slik at det er kun forskerne tilknyttet studien som har adgang til den. Alle som får innsyn i informasjon om deg har taushetsplikt. Innsamlet data vil bli anonymisert etter 15 år (kodelisten destrueres). Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

MULIGE FORDELER OG ULEMPER

Tidligere studier har vist at styrketrening har meget god effekt på muskelstyrke og fysisk funksjonsevne, spesielt for eldre som i utgangspunktet har et lavt funksjonsnivå. Forsøkspersoner som trekkes til treningsgruppen vil derfor med stor sannsynlighet oppleve god fremgang i styrke og funksjonsnivå, og potensielt erfare at mange daglige oppgaver vil gå lettere etter treningsperioden. I tillegg vil du som deltaker få god innsikt i hvordan treningen drives slik at du vil være i stand til å fortsette slik trening etter avsluttet prosjekt. Dersom du trekkes til gruppen som bare skal innta protein, vil du få tilbud om treningsoppfølging etter at den første intervensjonsperioden er gjennomført. Du vil med andre ord få treningsoppfølging uansett hvilken gruppe du trekkes til, men du må vente 3-4 måneder hvis du trekkes til kontrollgruppen.

Deltakelse i prosjektet vil kreve en del tid og oppmerksomhet. Det blir tre oppmøter på Norges idrettshøgskole før treningsperioden, og to oppmøter etter endt 10-ukersperiode. I tillegg skal du gjennomføre en CT-undersøkelse ved Currato Røntgen i Oslo sentrum både før og etter treningsperioden. Som tidligere nevnt vil vi bistå med transport i forbindelse med all testing dersom det er nødvendig, og for å begrense belastningen for hver enkelt forsøksperson vil en del av testene

bare gjennomføres for et utvalg av forsøkspersonene. Dette vil riktignok ikke redusere antall oppmøter, men vil redusere antall tester per oppmøte.

Vevsprøvetakninger (biopsier) medfører en liten infeksjonsfare (minimal), og litt ubehag/smerter kan oppleves under inngrepet. Du kan også oppleve lette til moderate smerter i 1-2 døgn etter inngrepet. Du vil få et lite arr etter snittet i huden; arret vil sakte bli mindre tydelig. Enkelte personer vil kunne få en fortykning av huden i arrområdet.

Voluntær muskelaktivering som gjennomføres under testdag 1 og testdag 2 kan oppleves litt ubehagelig, da lårmusklene ved denne testen aktiveres ved hjelp av strøm-elektroder. Denne testen er ikke invasiv, og elektrodene er "lapper" som festes på huden.

Noen opplever å bli litt svimmel av å drikke deuteriumoksid, og i tillegg er det noen som opplever å bli litt kvalme. For å redusere sjansen for at dette skjer, skal du ikke drikke alt vannet på en gang, men i mange små doser over en 4-timersperiode.

CT-undersøkelsen medfører at forsøkspersonene utsettes for stråling. For å begrense strålemengden, undersøkes bare det ene låret på tre steder.

Selve treningen skal gjennomføres med forholdsvis stor belastning, og vil medføre en viss risiko for skade og følelse av sårhet/stølhethet i muskulaturen.

FRIVILLIG DELTAKELSE OG MULIGHET FOR Å TREKKE SITT SAMTYKKE

Det er frivillig å delta i prosjektet. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke. Dersom du trekker deg fra prosjektet, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner. Dersom du senere ønsker å trekke deg eller har spørsmål til prosjektet, kan du kontakte Sigve Nyvik Aas, tlf: 41499074, epost: s.n.aas@nih.no

HVA SKJER MED INFORMASJONEN OM DEG?

Informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Du har rett til innsyn i hvilke opplysninger som er registrert om deg og rett til å få korrigert eventuelle feil i de opplysningene som er registrert. Alle opplysningene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger gjennom en navneliste.

Prosjektleder har ansvar for den daglige driften av forskningsprosjektet og at opplysninger om deg blir behandlet på en sikker måte. Informasjon om deg vil bli anonymisert eller slettet senest femten år etter prosjektslutt.

HVA SKJER MED PRØVER SOM BLIR TATT AV DEG?

Biopsiene og blodprøvene som tas av deg vil bli oppbevart i en forskningsbiobank uten kommersielle interesser (vurdert av Regional Etisk Komite). Hvis du sier ja til å delta i studien, gir du også samtykke til at det biologiske materialet og analyseresultater inngår i biobanken. Prøvene vil bli lagret til år 2031. Ansvarlig for biobanken er Dr. Truls Raastad ved Seksjon for fysisk prestasjonsevne ved NIH. Det biologiske materialet kan bare brukes etter godkjenning fra Regional komité for medisinsk og helsefaglig forskningsetikk (REK). Hvis du sier ja til å delta i studien, gir du også ditt samtykke til at prøver og aidentifiserte opplysninger utleveres til universitetet i Padova (Italia) og København (Danmark).

FORSIKRING

Deltakere i prosjektet er forsikret dersom det skulle oppstå skade eller komplikasjoner som følge av deltakelse i forskningsprosjektet. NIH er en statlig institusjon og er således selvassurandør. Dette innebærer at det er NIH som dekker en eventuell erstatning og ikke et forsikringsselskap.

UTLEVERING AV OPPLYSNINGER TIL ANDRE

Ved å delta i prosjektet, samtykker du også til at vevsprøver (muskelbiopsier og blodprøver) kan utleveres til utlandet. Koden som knytter deg til dine personidentifiserende opplysninger vil ikke bli utlevert.

OPPFØLGINGSPROSJEKT

Det kan være aktuelt med et oppfølgingsprosjekt innen fem år etter at dette prosjektet er gjennomført. Dersom du signerer samtykkeskjemaet, kan det derfor være at vi tar kontakt med deg innen fem år etter gjennomføring av dette prosjektet. Du vil naturligvis stå helt fritt til å avstå fra deltakelse i et eventuelt oppfølgingsprosjekt.

GODKJENNING

Prosjektet er godkjent av Regional komite for medisinsk og helsefaglig forskningsetikk, REK (2016/895).

SAMTYKKE TIL DELTAKELSE I PROSJEKTET

Hvis du har lest informasjonsskrivet og ønsker å være med som forsøksperson i prosjektet, ber vi deg undertegne nedenfor, og returnere skjemaet til en av personene oppgitt nedenfor. Du bekrefter samtidig at du har fått kopi av og lest denne informasjonen.

Det er frivillig å delta og du kan når som helst trekke deg fra prosjektet uten videre begrunnelse. Alle data vil, som nevnt ovenfor, bli aidentifisert før de blir lagt inn i en database, og senere anonymisert.

Med vennlig hilsen,

Sigve Nyvik Aas, Stipendiat (tlf: 414 99 074)

Truls Raastad, Professor (tlf: 23 26 23 28 / 91 36 88 96)

JEG ER VILLIG TIL Å DELTA I PROSJEKTET

Sted og dato

Deltakers signatur

Deltakers navn med trykte bokstaver

Jeg bekrefter å ha gitt informasjon om prosjektet

Sted og dato

Signatur

Rolle i prosjektet

APPENDIX II

Short Physical Performance Battery (SPPB)

Fried Frailty Criteria (FFC)

Training programs in study C and D

Registreringsark

dd/mnd/år:

<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
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ID/navn:

<input type="text"/>

1. Balansetest

1.Samlede føtter 10 sekunder



1. sek



2.Semi-tandem 10 sekunder



2. sek



3.Tandem 10 sekunder



3. sek



Gå til gangtest

2. Gangtest



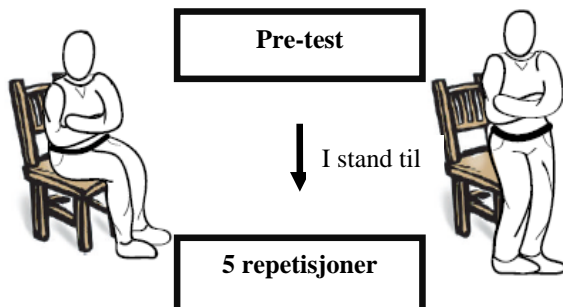
Ganghjelpemidler ved test (kryss av):

- uten
- krykke/stokk (er)
- rollator
- Annet (spesifiser) _____

Tid test 1: sek

Tid test 2: sek

3. Reise/ sette seg



Ikke i stand til → Avslutt

Setehøyde cm

Tid 5 repetisjoner uten armbruk: sek

Tester:

SCORING SPPB:

dd/mnd/år:

ID/navn:

1. Score statisk balanse

Hvis deltageren ikke har forsøkt eller mislyktes, kryss av hvorfor:

- Forsøkte, men ikke i stand til(0p)
- Deltageren kunne ikke holde stillingen uten hjelp(0p)
- Ikke forsøkt, tester følte det utrygg(0p)
- Ikke forsøkt, deltager følte seg utrygg(0p)
- Deltager tar ikke instruksjon(missing)
- Annet (spesifiser) _____
- Deltager nektet(missing)



Samlede føtter =10 sek = 1 p
<10 sek = 0 p



Semi-tandem =10 sek = 1 p
<10 sek = 0 p



Tandem =10 sek = 2 p
3 - 9.99 sek = 1 p
< 3 sek = 0 p

Sum poeng balanse:

2. Score 4m gangtest

Hvis deltageren ikke har forsøkt eller mislyktes, kryss av hvorfor:

- Forsøkte, men ikke i stand til(0p)
- Deltageren kunne ikke gå uten assistanse(0p)
- Ikke forsøkt, tester følte det utrygg(0p)
- Ikke forsøkt, deltager følte seg utrygg(0p)
- Deltager tar ikke instruksjon(missing)
- Annet (spesifiser) _____
- Deltager nektet(missing)



Deltager var ikke i stand til: = 0 poeng
Hvis tiden var > 8.7 = 1 poeng
Hvis tiden var 6.21 - 8.70 = 2 poeng
Hvis tiden var 4.82 - 6.20 = 3 poeng
Hvis tiden var < 4.82 = 4 poeng

Poeng ganghastighet (beste av to forsøk):

3. Score reise/sette seg x5

Hvis deltageren ikke har forsøkt eller mislyktes, kryss av hvorfor:

- Forsøkte, men ikke i stand til(0p)
- Deltageren kunne ikke reise seg uten hjelp(0p)
- Ikke forsøkt, tester følte det utrygg(0p)
- Ikke forsøkt, deltager følte seg utrygg(0p)
- Deltager tar ikke instruksjon(missing)
- Annet (spesifiser) _____
- Deltager nektet(missing)

Deltager var ikke istand til/brukte >60 sek = 0 poeng
Hvis tiden var ≥ 16.7 sek = 1 poeng
Hvis tiden var 13.7 - 16.69 sek = 2 poeng
Hvis tiden var 11.20 - 13.69 sek = 3 poeng
Hvis tiden var ≤ 11.19 sek = 4 poeng



Poeng reise/sette seg x5:

tester:

TOTAL SCORE SPPB 1.+2.+3.:

Fried Frailty Criteria

Kriterium 1: Matlyst <u>JA</u> <u>NEI</u>	Se neste side Spørsmål 1) Alternativ: Spørsmål 2) Alternativ: Personer som oppgir alternativ 3 på begge spørsmål, oppfyller kriteriet.				
Kriterium 2: Håndgripsstyrke <u>JA</u> <u>NEI</u>	Håndgripsstyrke for dominant hånd (gjennomsnitt av tre målinger)				
	Resultat:	BMI/mann	Cut-off (kg)	BMI/kvinne	Cut-off (kg)
	#1:	≤24	≤29	≤23	≤17
	#2:	24-26	≤30	23-26	≤17.3
	#3:	26-28	≤30	26-29	≤18
	Gj.snitt:	>28	≤32	>29	≤21
Kriterium 3: Utmattelse <u>JA</u> <u>NEI</u>	Se neste side Påstand 1) Alternativ: Påstand 2) Alternativ: Personer som oppgir alternativ 3 eller 4 på ett eller begge spørsmålene, oppfyller kriteriet "utmattelse".				
Kriterium 4: Ganghastighet <u>JA</u> <u>NEI</u>	Hentes fra SPPB. Cut-off tid på å gå 4 meter (statisk start)				
	Resultat:	Mann (cm)	Cut-offs (s)	Dame (cm)	Cut-offs (s)
	#1:	≤173	≥6.15 (0.65 m/s)	≤159	≥6.15 (0.65 m/s)
	#2:	>173	≥5.25 (0.76 m/s)	>159	≥5.25 (0.76 m/s)
	Gj.snitt:				
Kriterium 5: Aktivitetsnivå <u>JA</u> <u>NEI</u>	Se neste side Spørsmål 1) Alternativ: Personer som oppgir alternativ 3 eller 4 oppfyller kriteriet "lavt aktivitetsnivå".				

Personer som oppfyller tre av kriteriene kan inkluderes.

Personer som oppfyller to av kriteriene kan inkluderes, gitt at det er to av følgende kriterier som er oppfylt: 2, 4, 5.

I tillegg kan personer med SPPB score ≤ 6 inkluderes, uavhengig av FFC-kriterier.

Kriterium 1

Spørsmål 1: Hvordan har matlysten din vært i det siste?

- 1) God
- 2) Middels
- 3) Dårlig

Spørsmål 2: Hvor mye spiser du nå sammenlignet med for ett år siden?

- 1) Mer
- 2) Like mye
- 3) Mindre

Kriterium 3

Under finner du to påstander om hvordan du kan ha følt deg i det siste. Kryss av for hvor ofte du har følt det på denne måten i løpet av den siste uka.

Påstand 1: "Jeg følte at alt jeg gjorde var et ork"

- 1) Aldri eller nesten aldri (< 1 dag)
- 2) Litt av tiden (1 – 2 dager)
- 3) En del av tiden (3-4 dager)
- 4) Hele eller nesten hele tiden (5-7 dager)

Påstand 2: "Jeg var initiativløs "

- 1) Aldri eller nesten aldri (< 1 dag)
- 2) Litt av tiden (1 – 2 dager)
- 3) En del av tiden (3-4 dager)
- 4) Hele eller nesten hele tiden (5-7 dager)

Kriterium 5

Spørsmål 1: Hvor ofte deltar du i fysisk aktivitet med lav eller moderat intensitet?
(hagearbeid, støvsuge, gå tur)

- 1) Flere ganger i uka
- 2) 4-5 ganger per måned
- 3) 1-3 ganger per måned
- 4) Nesten aldri/aldri

Training program (Study C)

		Session 1			Session 2			Session 3		
Week	Exercise	Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
1	Leg press	2	12	<RM	2	12	<RM	2	12	<RM
	Knee extension	3	12	<RM	3	12	<RM	3	12	<RM
	One-leg squat	1	12	<RM	1	12	<RM	1	12	<RM
2-4		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
	Leg press	3	12	RM	3	10	90% of 12 RM	3	10	RM
	Knee extension	3	12	RM	3	10	90% of 12 RM	3	10	RM
5-7		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
	Leg press	3	10	RM	2	10	90% of 10 RM	3	8	RM
	Knee extension	3	10	RM	3	10	90% of 10 RM	3	8	RM
8-10		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
	Leg press	3	8	RM	3	10	80% of 8 RM	4	6	RM
	Knee extension	4	8	RM	4	10	80% of 8 RM	4	6	RM
8-10		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
	One-leg squat	1	8	RM	1	10	80% of 8 RM	1	6	RM

Training program (Study D)

Week	Exercise	Session 1			Session 2		
		Sets	Reps	Load	Sets	Reps	Load
1	Leg press	3	12	<RM	3	12	<RM
	Knee extension	3	12	<RM	3	12	<RM
2-4		Sets	Reps	Load	Sets	Reps	Load
	Leg press	3	12	RM	3	10	RM
5-7		Sets	Reps	Load	Sets	Reps	Load
	Knee extension	3	12	RM	3	10	RM
8-10		Sets	Reps	Load	Sets	Reps	Load
	Leg press	3	10	RM	3	8	RM
8-10		Sets	Reps	Load	Sets	Reps	Load
	Knee extension	4	10	RM	4	8	RM
8-10		Sets	Reps	Load	Sets	Reps	Load
	Leg press	3	8	RM	4	6	RM
8-10		Sets	Reps	Load	Sets	Reps	Load
	Knee extension	4	8	RM	4	6	RM

