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Autophagy regulation. Impact of age, resistance exercise and protein supplementation

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Summary

Background: Aging is associated with a decline in skeletal muscle mass, strength, and quality a condition known as sarcopenia, which further relates to the loss of functional independence. The pathways regulating protein degradation via autophagy have displayed age-related reductions, reflecting insufficient clearance of damaged proteins and organelles, compromising the function of the cell and ultimately contributing to the development of sarcopenia. The purpose of this thesis was therefore to examine markers of protein degradation via the autophagy-lysosome system in young, healthy elderly and frail elderly and investigate how they respond to acute resistance exercise and protein supplementation.

Methods: Twenty-nine men and women were divided into three different age groups; Young (n = 7, 20-43 years old), elderly (n = 10, 70-82 years old) and frail elderly (n = 12, 67-96 years old). The groups performed either a whole-body resistance exercise (young and elderly) or lower body resistance exercise bout (frail elderly), followed by protein supplementation. Muscle biopsies were obtained from *m. vastus lateralis* before and after the resistance exercise bout and protein intake. Muscle tissue samples were fractionated into cytosol and membrane fractions and analyzed for LC3, p62 and Foxo3a.

Results: No age-related differences were shown in the basal LC3-II/I ratio in the cytosolic or membrane fraction ($p>0.05$). The LC3-II/I ratio had a tendency towards, or was significantly reduced in young, elderly and frail elderly groups ($p<0.10$, $p<0.05$, $p<0.05$, respectively), which was mainly driven by a decline in LC3-II. We observed a significant decrease in the cytosolic FoxO3a fraction in both young and elderly individuals. Merging all groups revealed an increased p62 and LC3-I at the membrane fraction level ($p<0.05$).

Conclusion: The present study found no age-related differences for any of the autophagy-related markers measured. Furthermore, no differences were demonstrated between protein supplementation and the combination of protein supplementation and resistance exercise on autophagy-related markers in the frail elderly individuals.

Contents

Preface and acknowledgments

This thesis presents data based on the results from two larger studies; Amarone (2014-2015) and STAS (2016-2017) projects, at the Department of Physical Performance at the Norwegian School of Sport Sciences (NSSS).

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"Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough."

Richard Feynman

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1. Introduction

Skeletal muscle is the largest organ in the body, constituting ~40% of total body mass and plays vital roles in maintaining skeletal structure, locomotion permitting essential daily activities and overall metabolism (Reid & Fielding, 2012). Therefore, the conservation of skeletal muscle throughout our lifespan confers protection from several metabolic morbidities and preserves physical independence (Rizzoli et al. 2013). However, it is widely recognized that aging is associated with a progressive decline in skeletal muscle mass, strength and quality, a condition known as sarcopenia and dynapenia (Candow & Chilibeck, 2005; Roubenoff, 2000). Sarcopenia has been estimated to be prevalent in 5 – 13 % of older adults aged 60 – 70 years, and 11 – 50 % for those aged 80 years or above (von Haehling, Morley, & Anker, 2010). The development of sarcopenia may be a consequence of a multi-factorial process that is related to hormone imbalances (Lapauw et al., 2008), chronic inflammation (Brinkley et al., 2009) neurodegeneration (McNeil, Doherty, Stashuk, & Rice, 2005), abnormal fat deposition (Goodpaster et al., 2001), reduced satellite cell functionality (Kadi, Charifi, Denis, & Lexell, 2004), attenuated anabolic response to nutrition and exercise (Cuthbertson et al., 2005; Kumar et al., 2009) and genetic factors (B. E. Phillips et al., 2013). Moreover, certain lifestyle factors such as age-related sedentary behavior (Kortebein et al., 2008), nutritional deficiencies (Beasley, Shikany, & Thomson, 2013) and acute bouts of hospitalization (Ali et al., 2008) are likely to enhance the course of sarcopenia. Studies have shown an age-related reduction in skeletal muscle mass of approximately 3-8 % per decade after the age of 30 years, and after the age of 60 years these rates accelerates. Interestingly, the rate of decline in muscle strength is much more rapid than the concomitant loss of muscle mass (Fragala, Kenny, & Kuchel, 2015). As a consequence, muscle quality defined as muscle strength relative to muscle quantity correspondingly declines with age which further results in the loss of functional independence among the elderly (Irving, Robinson, & Nair, 2012). The pathways regulating protein breakdown via autophagy have been shown to decline with age in higher organisms (Cuervo et al., 2005). Reduced capacity in protein degrading systems may reflect insufficient clearance of damaged proteins and organelles, compromising the function of the cell, and ultimately contributing to the development of sarcopenia (Irving et al., 2012).

One of the primary methods of measuring protein degradation via autophagy activity is by monitoring the LC3 proteins turnover (Mizushima, Yoshimori, & Levine, 2010), which can be challenging to interpret by itself (Klionsky et al., 2016). This ubiquitin-like LC3 orchestrates autophagosome initiation, biogenesis, and functions as an adaptor protein for select autophagy(Y.-K. Lee & Lee, 2016). Measuring additional proteins could help us get a better picture of the autophagic flux. The p62/SQSTM1 is a multifunctional protein involved in signal transduction, and the degradation of proteins and organelles can serve as a link between LC3 and ubiquitinated substrates and could thus function as a protein marker of autophagy (Taniguchi, Yamachika, He, & Karin, 2016). Together with their upstream regulator, FoxO3a, that appears to partially govern the autophagy-lysosome degradation pathway (Mammucari et al., 2007; Mizushima & Yoshimori, 2007; Zhao et al., 2007).

The purpose of this thesis was to examine markers of protein degradation via the autophagy-lysosome system in young, healthy elderly and frail elderly individuals. More specifically, we measured autophagy-related markers, LC3, p62 and FoxO3a, and investigated how they respond to resistance exercise and protein supplementation. We hypothesized that protein supplementation and resistance exercise would result in an age-dependent difference in response with suppressed ratio of LC3-II/I but elevated p62, suggesting autophagy interruption with an attenuated autophagic flux in the frail elderly. We also hypothesized that protein supplementation would have a greater effect on the inhibition of the autophagy-lysosome system than the combination of protein intake and resistance exercise in the frail elderly individuals.

2. Theory

2.1 Skeletal muscle protein turnover

Skeletal muscle tissue displays an outstanding plasticity and is continuously changing and remodeling through the simultaneous processes of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Mitchell, Churchward-Venne, Cameron-Smith, & Phillips, 2015). During the period in-between meals, skeletal muscle proteins are broken down, and amino acids are released as gluconeogenic substrates and building blocks for protein synthesis in muscles and other tissues (Greenhaff et al., 2008; Owen et al., 1998). These basal and fasted rates of MPB ranges from 0.08 – 0.11% h^{-1} (S. M. Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997; S. M. Phillips, Tipton, Ferrando, & Wolfe, 1999) and exceed those of MPS (0.03 - 0.07%· h^{-1}) (Kumar et al., 2009; Mittendorfer et al., 2005; S. M. Phillips et al., 1997; Welle, Thornton, & Statt, 1995), thus creating a net negative protein balance and hence a loss of muscle amino acids. However, upon the consumption of dietary protein, these amino acids are replenished through the stimulation of MPS (Bennet et al., 1989; Rand et al., 2003), so that on a daily basis the net protein balance is neutral (Brook, Wilkinson, Smith, & Atherton, 2016). This balance between MPS and MPB is often characterized as protein turnover, and it is the changes in net protein balance that over time regulates muscle mass (Deutz & Wolfe, 2013; S. M. Phillips, 2004; Rennie, Wackerhage, Spangenburg, & Booth, 2004).

This constant oscillation between anabolic and catabolic conditions provides a mechanism for protein maintenance, and the capability to replace dysfunctional proteins (Rennie, 2007). In healthy people at rest, the rate of the renewal and remodeling of muscle protein is approximately 1-2% per day (G. I. Smith & Mittendorfer, 2016). This flux through protein synthesis and degradation is affected by a number of variables including ageing, disuse, resistance exercise, disease and diet (Areta et al., 2014; Cermak, Res, de Groot, Saris, & van Loon, 2012; S. M. Phillips, 2009; S. M. Phillips et al., 1997; Rudrappa et al., 2016). Whereas MPS appears to be the more dynamic, MPB plays an important role in the modulation of muscle mass. However, due to methodological challenges MPB can be problematic to study in vivo (Brook et al., 2016).

2.1.1 The effect of resistance exercise on muscle protein synthesis

Given the substantial role skeletal muscle plays in the development and maintenance of human health, the literature on how exercise affects skeletal muscle protein turnover is progressing rapidly (McGlory, Devries, & Phillips, 2017; Wolfe, 2006). Resistance exercise has been shown to be a potent stimulator of MPS (Biolo, Maggi, Williams, Tipton, & Wolfe, 1995; S. M. Phillips et al., 1997; Yarasheski, Zachwieja, & Bier, 1993), with a single bout shown to induce a two- to threefold increase in MPS (Holm et al., 2010; Kumar et al., 2009; S. M. Phillips et al., 1997). In the fasted state, MPS peaks ~1-2 h after a resistance exercise bout and myofibrillar MPS returns to baseline approximately 4 h later (Kumar et al., 2009), although the duration is highly dependent on the exercise intensity and volume (Burd, Holwerda, et al., 2010; Burd, West, et al., 2010; Kumar et al., 2009). With adequate nutrition in conjunction with resistance exercise, this increased MPS can be sustained for over 24 h (Cuthbertson et al., 2005; MacDougall et al., 1995; Miller et al., 2005; S. M. Phillips et al., 1997), and could persist for eight days (D. J. Wilkinson et al., 2014). Nonetheless, without nutritional support, a single bout of resistance exercise executed in the fasted state increases MPB above MPS and is thus catabolic (Biolo et al., 1995). This negative protein balance is due to increased MPB which occurs to replace damaged tissue or promote tissue remodeling (Biolo, Tipton, Klein, & Wolfe, 1997; Chesley, MacDougall, Tarnopolsky, Atkinson, & Smith, 1992; M. Louis et al., 2003; Witard et al., 2014).

Table 2.1: *Examples from the literature of rates of muscle protein synthesis captured 2-4 h following resistance exercise*

Fractional synthetic rate (%/h)	Rest	RE 2-4 h
Mixed	0.05-0.07	0.07-0.12
Myofibrillar	0.02-0.05	0.07-0.9
Mitochondrial	0.05-0.10	0.10-0.15
Sarcoplasmic	0.04-0.06	0.08-0.10

Resistance exercise (RE), Mixed and myofibrillar values obtained from Reidy and Rasmussen (2016), sarcoplasmic values from Burd, West, et al. (2010) and mitochondrial protein turnover rates were obtained from S. B. Wilkinson et al. (2008).

Resistance exercise-induced stimulation of MPS follows a sigmoidal dose-dependent relationship for training load, reaching an upper limit between ~60 – 90 % of one-repetition maximum (1-RM) when matching the external load (Kumar et al., 2009). Nonetheless, higher mechanical loads do not always equal greater MPS. Burd, Holwerda, et al. (2010) demonstrated that resistance exercise at 30 % of 1 RM to fatigue

increased mixed MPS equal to that at 90 % of 1 RM (Burd, Holwerda, et al., 2010). Hence, suggesting the importance of maximizing muscle fiber recruitment for MPS stimulation.

Some researchers have described elevated resting MPS rates in the trained compared to untrained state (P. L. Kim, Staron, & Phillips, 2005; S. M. Phillips et al., 2002), while others found no such difference (S. M. Phillips et al., 1999; Tang, Perco, Moore, Wilkinson, & Phillips, 2008). Interestingly, S. M. Phillips et al. (2002) observed an increased basal MPB in the trained stated, which could suggest that trained individuals have an increased protein turnover (S. M. Phillips et al., 2002; Reidy et al., 2017). The initial increase in mixed MPS in the untrained state is less prominent and peaks later but lives longer than in the trained state (Damas, Phillips, Vechin, & Ugrinowitsch, 2015). Due to limited data, estimating the precise time course of myofibrillar protein synthesis in different training states is problematic (P. L. Kim et al., 2005; S. B. Wilkinson et al., 2008). However, the few available studies may collectively indicate greater myofibrillar protein synthesis in the untrained compared to trained state after resistance exercise, which could be reflective of increased muscle damage (Damas et al., 2016; P. L. Kim et al., 2005; S. B. Wilkinson et al., 2008).

2.1.2 The effect of amino acids on muscle protein synthesis

Amino acids yield a significant stimulatory effect on the MPS. From the rested state, rates of MPS can almost double after food consumption (Babraj et al., 2005; Cuthbertson et al., 2005; Moore et al., 2009). This anabolic response peaks at 1.5 – 2 h after oral intake, and persists for 2 – 3 h before declining towards basal values (Atherton et al., 2010; Bohe, Low, Wolfe, & Rennie, 2001; Churchward-Venne, Murphy, Longland, & Phillips, 2013; Cuthbertson et al., 2005). In addition to being anabolic, protein supplementation (and carbohydrates) has shown to have pancreatic beta-cell secretagogue properties, thus increasing plasma insulin (Atherton et al., 2010; Juntunen et al., 2002; Staples et al., 2011). This hyperinsulinemia can suppress MPB (Koopman et al., 2007; Staples et al., 2011; Wilkes et al., 2009), which elevated plasma concentrations of amino acids cannot do unaided (Greenhaff et al., 2008). Once the dietary protein is ingested, it is the amino acids themselves that are driving the process of MPS (Biolo et al., 1997; Moore et al., 2009; Trommelen, Groen, Hamer, de Groot, & van Loon, 2015). It is also quite clear that the potent increase in MPS arises almost

entirely from the essential amino acids (EAAs)(K. Smith, Barua, Watt, Scrimgeour, & Rennie, 1992), and leucine seems to be decisive to these effects (K. Smith, Reynolds, Downie, Patel, & Rennie, 1998), with little to no role for the nonessential amino acids (Tipton, Gurkin, Matin, & Wolfe, 1999). Dietary protein with higher leucine content stimulates MPS to a greater extent than proteins with lower leucine content (Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe, 2006).

The ingestion of 20-25 g of high-quality protein seems sufficient to maximize post-exercise MPS rates (Moore et al., 2009; Witard et al., 2014), with the consumption of 40 g eliciting no further increase in MPS but instead, stimulating amino acid oxidation (Moore et al., 2009). A significant limitation of these reports is that the exercise protocols were limited to lower body exercises. Recently, Macnaughton et al. (2016) showed that when performing whole-body resistance exercise, the ingestion of 40 g of protein resulted in a 16% greater stimulation of MPS as compared with 20 g protein in healthy resistance-trained young men (Macnaughton et al., 2016), suggesting that the ingestion of ~20 g protein is only sufficient to maximize post-exercise MPS rate with lower body resistance exercise (Areta et al., 2013; Moore et al., 2009; Witard et al., 2014). In contrast, following whole-body resistance exercise the required dose might exceed 20 g of protein (Macnaughton et al., 2016). Nevertheless, this is currently the only study demonstrating a higher optimal protein dose with whole-body resistance exercise. As such, despite the potential benefits of higher protein doses to maximize MPS, it cannot be readily confirmed. It therefore remains to be determined by future research.

2.1.3 The influence of the aging process

The underlying causes of age-related loss of muscle and function are multifactorial, but the decline in muscle mass (primarily as a reduction in type II fiber size) suggests that an alteration in the balance between MPS and MPB occurs in older age (Nilwik et al., 2013; Verdijk et al., 2007). Initially, it was suggested this was due to a significant reduction in post-absorptive rates of MPS (Balagopal, Rooyackers, Adey, Ades, & Nair, 1997; Welle, Thornton, Jozefowicz, & Statt, 1993; Welle et al., 1995; Yarasheski et al., 1993). However, this notion has been questioned as most studies have revealed equivalent basal rates of post-absorptive MPS and MPB between young and older adult males (Cuthbertson et al., 2005; Drummond et al., 2008; Markofski et al., 2015;

Paddon-Jones et al., 2004; Symons, Sheffield-Moore, Wolfe, & Paddon-Jones, 2009; Volpi, Mittendorfer, Wolf, & Wolfe, 1999). Some studies have pointed towards an age-related sexual dimorphism in the basal MPS rate, with greater rates observed in older women compared to older men (G. I. Smith et al., 2012; G. I. Smith et al., 2014). However, older women also appear to have a blunted anabolic response to mixed meal ingestion and exercise training (G. I. Smith et al., 2008; G. I. Smith et al., 2012). Nevertheless, the inability of skeletal muscles to support an adequate MPS response to resistance exercise (Kumar et al., 2009) and protein feeding (Katsanos et al., 2006), led to the theory of anabolic resistance. With postprandial MPS increases revealed to be attenuated in older compared to younger adults (Cuthbertson et al., 2005; Guillet et al., 2004; G. I. Smith et al., 2012; Volpi, Mittendorfer, Rasmussen, & Wolfe, 2000). Older adults also appear to have a blunted MPS response to resistance exercise across a range of exercise intensities (Fry et al., 2011; Kumar et al., 2012; Kumar et al., 2009; Sheffield-Moore et al., 2005).

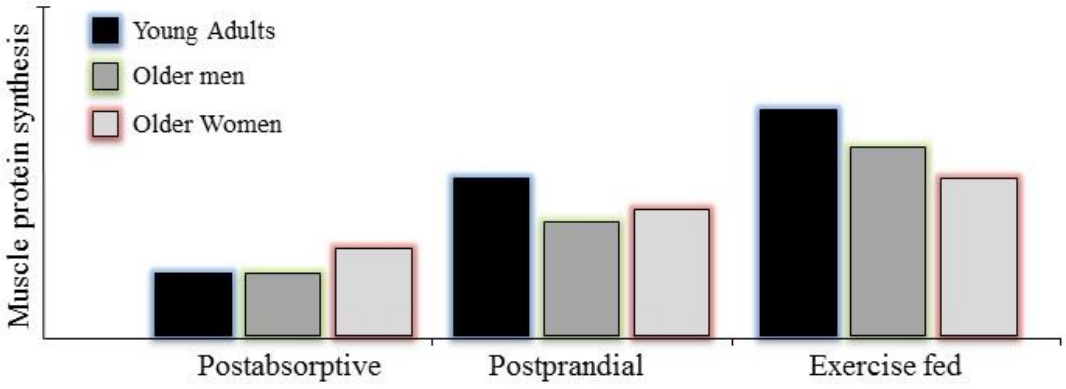

Figure 2.1: *Schematic presentation of the age-related changes in muscle protein synthesis. Aging is associated with an increase in the basal rate of MPS in women. Both men and women have a diminished MPS response to amino acids and exercise. However, the reduction is greater in women than in men. The figure is redrawn from G. I. Smith and Mittendorfer (2016).*

Anabolic resistance results in a net negative protein balance that may over time lead to a loss of skeletal muscle mass. Aging per se may play a secondary role, as anabolic resistance could be a consequence of increased sedentary time, muscle disuse and a reduction in daily steps by the older population (Breen et al., 2013; Burd, Gorissen, & van Loon, 2013; Drummond et al., 2012). After just five days of muscle disuse, young adults were observed to have a diminished ability to utilize amino acids for MPS in skeletal muscle (Wall et al., 2016). In contrast, resistance exercise can sensitize young

and older adults skeletal muscle tissue to the anabolic properties of protein-based nutrition (Pennings et al., 2011).

The physiological mechanism underlying the reduced anabolic sensitivity in aging muscle remains unknown. It has been suggested to be related to diminished skeletal muscle translational capacity in older adults (Chaillou, Kirby, & McCarthy, 2014; Kirby et al., 2015), thus lacking the ability to achieve a potent MPS stimulus with the same aminoacidemia. However, this fails to explain why additional amounts of protein consumption seem to restore protein synthetic rates (S. M. Phillips, 2015). Moreover, impaired transport of amino acids into muscle (Dickinson, Drummond, Coben, Volpi, & Rasmussen, 2013; Dickinson et al., 2014), lipid-induced muscle insulin resistance (Stephens et al., 2015), attenuated protein digestion and absorption (Boirie, Gachon, & Beaufrere, 1997), and dysregulation of nutritive blood flow to skeletal muscle (Fujita, Glynn, Timmerman, Rasmussen, & Volpi, 2009; Meneilly, Elliot, Bryer-Ash, & Floras, 1995; Rasmussen et al., 2006), have also been suggested to cause anabolic resistance.

On the other hand, due to different biopsy time points blunted anabolic signaling is not always observed (Drummond et al., 2008; Mayhew, Kim, Cross, Ferrando, & Bamman, 2009). Also, many research groups did not compare younger and older adults directly (Churchward-Venne, Cotie, et al., 2014; Dickinson et al., 2014; Dreyer et al., 2008; Witard et al., 2014; Y. Yang et al., 2012). Those studies that did compare different age-groups primarily measured mixed MPS, which may not reflect synthesis of contractile proteins, and may explain why no age-related differences were shown. Importantly, when myofibrillar protein synthesis components were measured anabolic blunting was often revealed (Kumar et al., 2009; Moore et al., 2015).

Becoming gradually more evident is that frail elderly consume less protein than recommended (Fulgoni, 2008), coming from low-leucine sources (Tieland, Borgonjen-Van den Berg, van Loon, & de Groot, 2012), and we are more sedentary with advancing age (Harvey, Chastin, & Skelton, 2015; Martin et al., 2014), all factors related to a reduced muscle anabolic response. Nevertheless, due to the significant variability in experimental methodology as to the presence or absence of age-related anabolic resistance, the findings are inconsistent (Shad, Thompson, & Breen, 2016).

2.2 Intracellular Signaling Pathways

2.2.1 PI-3K/Akt/mTOR pathway

Several studies have shown that the phosphatidylinositol-3-kinase (PI3-K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway promote protein synthesis (Glass, 2010; Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). Activated by PI3-K, the serine/threonine kinase Akt is considered an important upstream regulator of MPS, due to its capability to phosphorylate and modify the activity of several signaling molecules, including mTOR (Bodine, 2006). Among the downstream targets of Akt are growth regulatory molecules such as, glycogen synthase kinase 3β (GSK3β) (Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995), proline-rich Akt substrate of 40 kDa (PRAS40) (Kovacina et al., 2003), tuberous sclerosis complex 2 (TSC2) (Inoki, Li, Zhu, Wu, & Guan, 2002) and forkhead box class O (FoxO) transcription factors (Tran, Brunet, Griffith, & Greenberg, 2003). It is through the phosphorylation and the inactivation of TSC2 that mTOR activation is facilitated by Akt (Y. Li, Corradetti, Inoki, & Guan, 2004). However, more recent studies have demonstrated loading-induced mTORC1 activation is independent of both PI3-K (Miyazaki, McCarthy, Fedele, & Esser, 2011) and Akt (Deldicque et al., 2008a; Miyazaki et al., 2011; Vissing et al., 2013).

mTOR exists in two different complexes, mTORC1 is recognized by the regulatory-associated protein of mTOR (RAPTOR)(D. H. Kim et al., 2002), while mTORC2 binds rapamycin-insensitive companion of mTOR (RICTOR) (Sarbassov, Guertin, Ali, & Sabatini, 2005). Following activation, mTORC1 propagates downstream signaling through the phosphorylation and activation of the 70-kDa ribosomal protein S6 kinase (P70S6K), and the inhibition of 4E-binding protein-1 (4E-BP1) (Dickinson et al., 2011; Gingras et al., 1999). Correspondingly, P70S6K and 4E-BP1 are important signaling molecules that regulate the initiation of protein translation and are frequently used to signify mTORC1 activation. The inhibition of 4E-BP1 by mTORC1, reduces its affinity for eukaryotic initiation factor 4E (eIF4E), thus enabling eIF4E to bind with eIF4G and eIF4A, forming the eIF4F complex to begin translation initiation (Egerman & Glass, 2014). Furthermore, mTORC1 phosphorylates P70S6K1which in addition to upregulating translation initiation (Ma, Yoon, Richardson, Julich, & Blenis, 2008) serves to enhance translation elongation through eukaryotic elongation factor-2 kinase

(eEF2K) (Wang et al., 2001). Independent of its activation of mTORC1 (Cross et al., 1995), Akt also phosphorylates and inactivates GSK-3β thereby commencing the activity of translation initiation factor eIF-2B (Egerman & Glass, 2014).

In skeletal muscle, mTORC1 signaling is activated in response to a variety of stimuli, including mechanical loading, feeding and growth factors (Bond, 2016; Drummond et al., 2009; Gan, Yoo, & Guan, 2006; Rommel et al., 2001), and also regulates several other anabolic processes, as well as catabolic processes (Zoncu, Efeyan, & Sabatini, 2011).

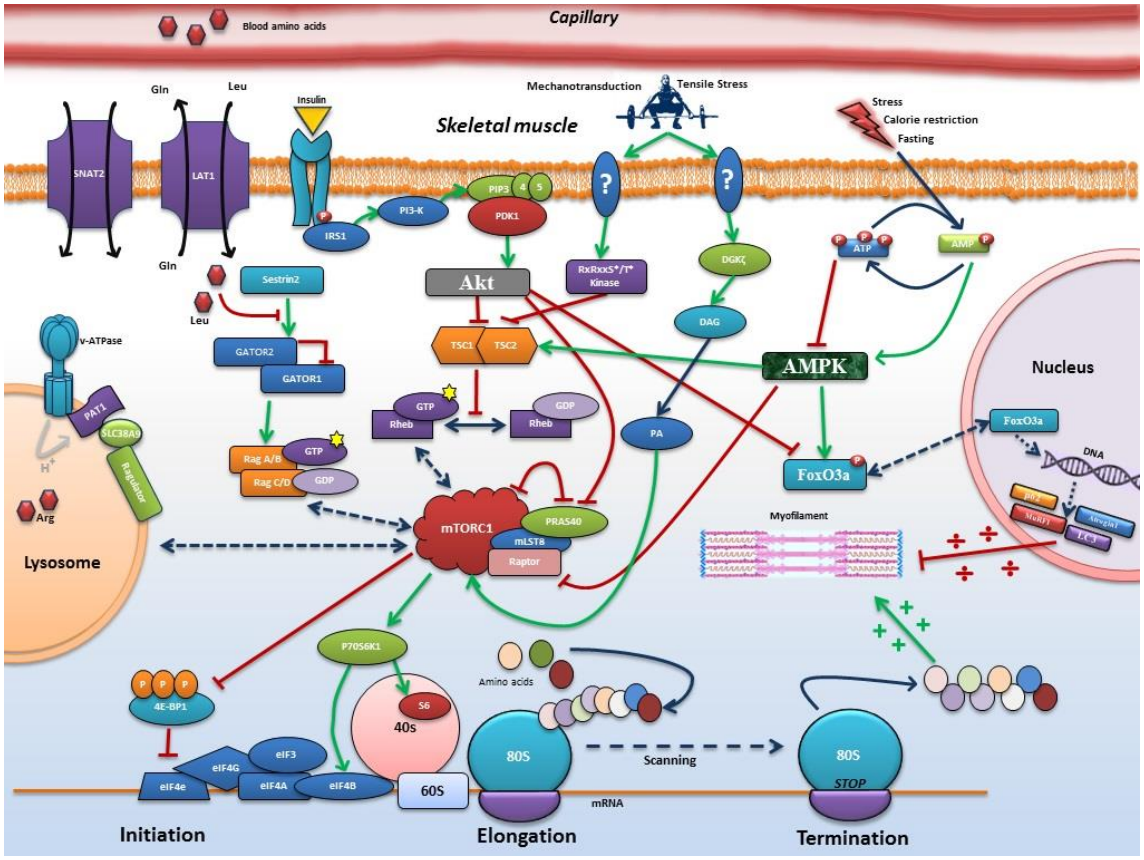

Figure 2.2: *A Schematic overview of the cellular signaling responses regulating muscle protein synthesis. The figure is based on the work of Egerman and Glass (2014), McGlory and Phillips (2016), Moro, Ebert, Adams, and Rasmussen (2016), Marcotte, West, and Baar (2015), Rindom and Vissing (2016) and Reidy and Rasmussen (2016).*

The downstream intracellular signaling targets in protein synthesis have been described to be diminished with age, which includes reductions in the activity of PI3K, Akt, mTOR, P70S6K1, 4E-BP1 and eIF2B (Cuthbertson et al., 2005; Leger, Derave, De Bock, Hespel, & Russell, 2008; Pallafacchina, Calabria, Serrano, Kalhovde, &

Schiaffino, 2002; Terada et al., 1994; Welsh et al., 1997). These observations of reduced Akt activity match with the reduced differentiation and hypertrophy of myotubes (Deane et al., 2013; Sharples et al., 2012), although human skeletal muscle cells also detect these phenotypes (Pietrangelo et al., 2009).

Despite an apparent decrease in Akt activity, the level of phosphorylated P70S6K, eIF4E amount, and eIF2B activity increased between 12 and 27 months of age in rats (Kimball, O'Malley, Anthony, Crozier, & Jefferson, 2004). In humans, no differences in the protein levels of Akt, mTOR, raptor, S6K1, 4E-BP1, and PRAS40 were shown when young adults were compared to old sedentary and old active adults (Sandri et al., 2013). In a recent study, when comparing old with younger participants, a significant higher basal mTORC1 and S6K1 phosphorylation were observed (Markofski et al., 2015).

A large body of animal research proposes that inhibiting mTOR, and thus blunting protein synthesis, is related to increased lifespan and could be promoted as a longevity treatment (Kapahi et al., 2010; Passtoors et al., 2013; Slagboom et al., 2011). Paradoxically, despite inhibition of mTOR increases lifespan and could improve numerous age-related pathologies; mTOR also plays a critical role in maintaining skeletal muscle mass and anabolism (Sharples et al., 2015). This raises the dilemma of improving lifespan or improving healthspan by maintaining muscle mass, strength and function through an active life.

2.2.2 Summary

Aging is associated with a progressive decline in skeletal muscle mass, strength, and quality, a condition known as sarcopenia and dynapenia. The regulation of muscle mass is dictated by the changes in muscle protein balance over time. This constant oscillation between MPS and MPB provides a mechanism for protein maintenance, and its flux is affected by many variables including aging, disuse, resistance exercise, disease, and diet. Resistance exercise has been shown to be a potent stimulator of MPS. However, without nutritional support, a single bout of resistance exercise is catabolic. The consumption of protein induces both an increase in MPS and suppression in MPB, and the amino acid leucine seems to be decisive to these effects. The ingestion of 20 g high-

quality protein can maximize post-exercise MPS rate with lower body resistance exercise, but whole-body resistance exercise may require more than a 20 g protein dose. Reduced anabolic sensitivity with age has led to the theory of anabolic resistance, which relates to skeletal muscles inability to support an adequate MPS response to resistance exercise and protein feeding. However, greater amounts of stimulus could be required to maximize the anabolic response in aging muscle. The mammalian target of rapamycin (mTOR) signaling pathway is essential for stimulating protein synthesis and is activated by mechanical loading, feeding, and growth factors. Nevertheless, PI3-K/Akt/mTOR levels seem to decrease with age and studies using sarcopenic muscles have produced inconsistent results. The cellular pathways regulating protein breakdown via autophagy have also been shown to decline with age in higher organisms. These reductions may reflect insufficient clearance of dysfunctional proteins and organelles, compromising the function of the cell, and eventually contributing to the development of sarcopenia.

2.3 Cellular mechanisms of protein breakdown

2.3.1 Autophagy-Lysosome System

The term autophagy, derived from Greek meaning “self-eating,” describes a catabolic process that through a lysosomal pathway degrades and recycles cytoplasmic components (Neel, Lin, & Pessin, 2013; Sandri, 2010a, 2011). This evolutionarily conserved process occurs in all eukaryotic cells from yeast to humans and is active in nearly all tissues. Autophagy possibly exists as an efficient mechanism for regulation of metabolism, removal of defective organelles, protein quality control, and pathogen removal (Vainshtein, Grumati, Sandri, & Bonaldo, 2014). Initially, it was described to be activated during catabolic conditions in muscle cells (Bechet, Tassa, Taillandier, Combaret, & Attaix, 2005; Deval et al., 2001; Tassa, Roux, Attaix, & Bechet, 2003). Presently, however, autophagy has been found to be modulated in numerous muscle conditions, including cancer (Penna et al., 2013), ageing (Penna et al., 2013; Wohlgemuth, Seo, Marzetti, Lees, & Leeuwenburgh, 2010), fasting (Mammucari et al., 2007), chemotherapy (Smuder, Kavazis, Min, & Powers, 2011), disuse (Brocca et al., 2012) and denervation (O'Leary, Vainshtein, Carter, Zhang, & Hood, 2012; Zhao et al., 2007).

Three different types of autophagy have been recognized; microautophagy, chaperone-mediated autophagy, and macroautophagy, which mainly differs in their mode of substrate delivery and the type of cargo delivered to the lysosome. Microautophagy degrades substrates in the surrounding area of the lysosome through lysosomal membrane invaginations (W. W. Li, Li, & Bao, 2012). Chaperone-mediated autophagy is a targeted degradation mechanism through which chaperones recognize designated substrates and deliver them to lysosomes for degradation (Kaushik & Cuervo, 2012). The process of macroautophagy is an intracellular homeostatic mechanism used for the degradation and recycling of long-lived proteins and whole organelles (Mizushima & Komatsu, 2011). Furthermore, the engulfment of materials leads to the development of a nascent double-bilayer enclosed vesicle recognized as an autophagosome. The development of the autophagosome signifies the early stage of macroautophagy, whereas the later maturation phase entails the transport and subsequent fusion with lysosomes to form autolysosomes. This formation of autolysosomes marks the transition to the degradative phase of autophagy. Here the cytoplasmic cargo, the proteins responsible for substrate recognition and delivery and the autophagosomes themselves, are degraded and recycled for the synthesis of new proteins (Gallagher, Williamson, & Chan, 2016; Vainshtein & Hood, 2016). Macroautophagy (hereafter referred to as autophagy) is the most studied of the three and will be the focus of this thesis.

An increasing number of specific cargo receptors have been identified, p62/SQSTM1, Nbr1, Bnip, Nix (Bnip3L), and optineurin (Shaid, Brandts, Serve, & Dikic, 2013). These cargo receptors are critical for the selectivity of the autophagy process and have the capability to recognize and bind directly to proteins and organelles tagged for degradation through specific molecules or post-translational modifications. Also, the cargo receptors possess an LC3-interacting region domain, capable of recruiting and binding essential autophagosome membrane proteins (McEwan & Dikic, 2011; Sakuma, Aoi, & Yamaguchi, 2017).

At least three molecular complexes control de novo formation of autophagosomes, ultimately leading to the conversion of LC3 to LC3-II (Mizushima & Komatsu, 2011). During the initial step of autophagy, LC3 exists free in the cytosol in its inactive form with an amino acid tail, that is cleaved off by Atg4 giving rise to LC3-I (Kabeya et al., 2000). The Atg12-Atg5-Atg16L complex, along with Atg7, plays an essential role in the

conjugation of phosphatidylethanolamine (PE) to LC3-I, forming LC3-II (Kirisako et al., 2000; Yorimitsu & Klionsky, 2005). The amount of LC3-II increases as the LC3-II protein is attached to both the inner and outer side of the expanding phagophore membrane and is required for the elongation and closure of the autophagosome membrane (Lippai & Szatmari, 2017; Mizushima et al., 2001). Due to the LC3-II protein localization on the autophagosomal membrane, it serves as a widely used marker for autophagosomes (Kabeya et al., 2000; Mizushima, 2004). The amount of LC3-II protein has been shown to correlate with the number of autophagosomes (Kabeya et al., 2000) and could, therefore, be an indication of enhanced autophagy activity (Martinez-Lopez, Athonvarangkul, & Singh, 2015; Tam & Siu, 2014). After autophagosome closure, the LC3-II located on the cytosolic surface of the membrane is recycled back to LC3-I through delipidation by Atg4 (Satoo et al., 2009; Tanida et al., 2004). The closed autophagosomes ultimately fuse with lysosomes, thereby creating autolysosomes and acquiring lysosomal enzymes and membrane proteins required for degradation. The LC3-II protein bound to the intra-autophagosomal lumen and cytoplasmic cargo is degraded by lysosomal hydrolases (Tanida, 2011).

Additionally, p62/SQSTM1 could also be used as a protein marker for autophagy activity under certain settings (Germain et al., 2011; Mizushima & Yoshimori, 2007). p62 is a multifunctional protein involved in multiple cellular functions and plays a role in signal transduction, in the degradation of proteins and organelles, and can serve as a link between LC3 and ubiquitinated substrates (Bjorkoy et al., 2005; Johansen & Lamark, 2011; Nezis & Stenmark, 2012). The p62 protein and the polyubiquitinated proteins bound to it can merge with, and be degraded by the autophagosomes, thus serving as an index of autophagic degradation. In mammals and Drosophila, impaired autophagy correlates with increased levels of p62 (Ichimura, Kominami, Tanaka, & Komatsu, 2008; Komatsu et al., 2007). On the contrary, reduced p62 levels are associated with autophagy activation (Klionsky et al., 2016; Mizushima et al., 2010). This signifies that the steady-state p62 protein level is reflective of autophagic status (Bartlett et al., 2011; Cui et al., 2012; Klionsky et al., 2016; Masiero et al., 2009).

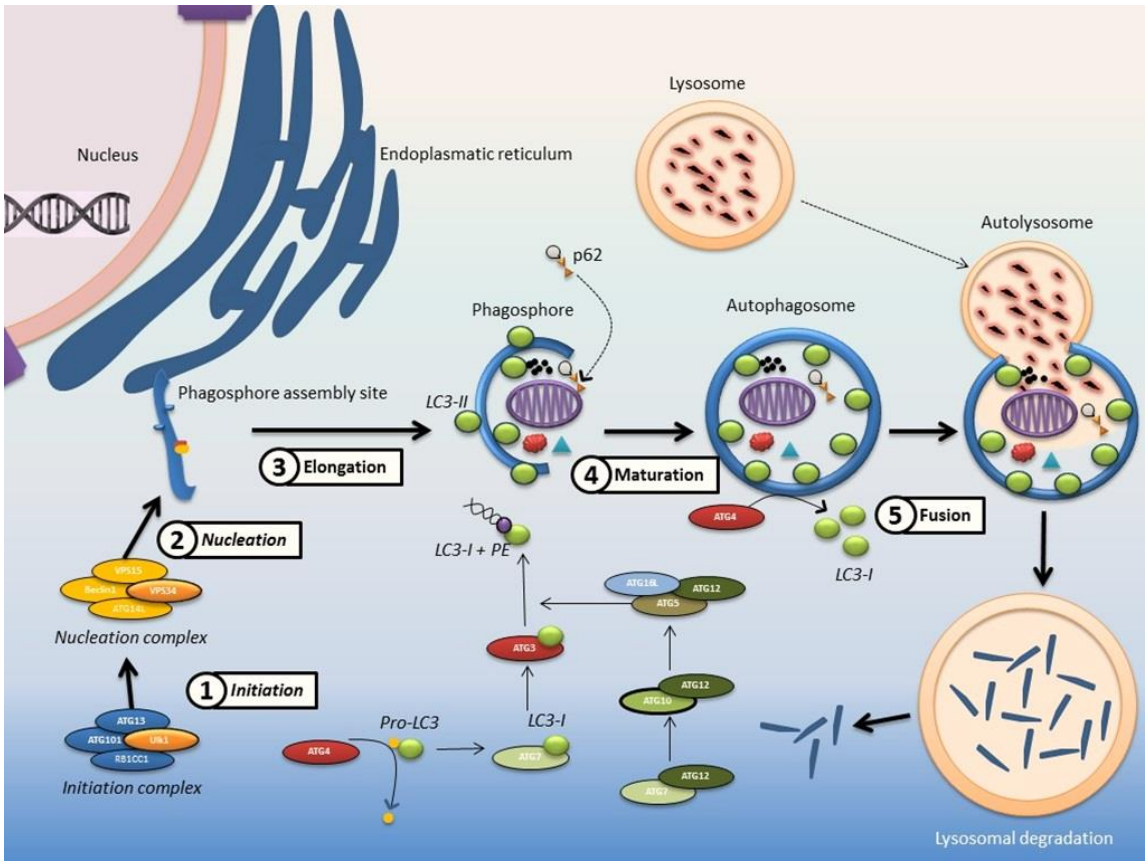

Figure 2.3: *A Schematic overview of the autophagy-lysosome system. The figure is based on the work of Lippai and Szatmari (2017), Rubinsztein, Marino, and Kroemer (2011) and Tanida (2011).*

The autophagy-lysosome degradation pathway seems to be regulated by transcription factor Forkhead box O3a (FoxO3a) (Mammucari et al., 2007; Zhao et al., 2007), which has shown to upregulate several autophagy-related genes, including LC3, p62/SQSTM1, GABARAPL1, Atg12, BNIP and Nix (BNIP3L) (Milan et al., 2015; van der Vos et al., 2012; Zhao et al., 2007; Zhao, Brault, Schild, & Goldberg, 2008). Also, upstream Akt-mediated phosphorylation promotes nuclear export of FoxO to the cytoplasm, thus suppressing FoxO-dependent transcription of target genes. Conversely, the absence of growth factor signaling or cellular stress transfers FoxO into the nucleus, thus activating FoxO-dependent gene expression (Martins, Lithgow, & Link, 2016). This activation of FoxO upregulates 4E-BP1 (Demontis & Perrimon, 2010) and suppresses mTOR (J. H. Lee et al., 2010), hence FoxO3 reduces total protein synthesis in adult muscle (Reed, Sandesara, Senf, & Judge, 2012).

2.3.2 The effects of age on Autophagy-lysosome system

Numerous studies have reported age-related reductions in the autophagy-lysosome system (Cuervo, 2008; Cuervo et al., 2005; Del Roso et al., 2003; Drummond et al., 2014; Gaugler et al., 2011; Rubinsztein et al., 2011; Sakuma et al., 2016; Wohlgemuth et al., 2010). Moreover, tissue-specific knockouts of Atg7 or Atg5 have shown that younger knockout mice mimicked age-associated manifestations observed during sarcopenia such as neurodegeneration (Komatsu et al., 2006), damaged and defective mitochondria (I. Kim, Rodriguez-Enriquez, & Lemasters, 2007), accumulation of lipid droplets (Dong & Czaja, 2011) increased protein oxidation (Nezis & Stenmark, 2012) and decreased muscle mass (Masiero et al., 2009). These findings suggest that defective autophagy plays a role in sarcopenia, although more investigation is needed (Martinez-Lopez et al., 2015; Sakuma et al., 2017).

One of the primary methods to investigate autophagic flux is the monitoring of LC3 turnover, which is established by the observation that LC3-II is degraded in autolysosomes (Mizushima et al., 2010). The most common approach is to perform analyses on whole homogenate, and the studies referred to in the following paragraphs were carried out on whole homogenate if not otherwise stated. Whereas the ratio of cytosolic LC3-II to LC3-I is related to the extent of autophagy-lysosomal activity (Kadowaki & Karim, 2009; Karim et al., 2007), its age-related changes have produced mixed results. Wohlgemuth et al. (2010) detected significantly increased protein expression levels of both LC3-I and LC3-II in aged rats. This LC3-II/I protein ratio was only slightly but not significantly decreased in the aged compared to younger animals. Nevertheless, aging did not impact the amount of Atg7 or Atg9 proteins (Wohlgemuth et al., 2010). In humans, an age-dependent reduction in the ratio of LC3-II to LC3-I proteins and the amount of Atg7 protein were demonstrated using young and older volunteers (Carnio et al., 2014). Interestingly, Iida, Kanko, Suga, Morito, and Yamane (2011) showed that the adaptation of the LC3-I and LC3-II proteins differed among several muscles. Using a type of short-lived mouse model displaying various ageing-related phenotypes, they revealed noticeable increases of the LC3-II to LC3-I ratio in masseter and tongue muscles, but not in the gastrocnemius muscles (Iida et al., 2011) . In recent research, Sakuma et al. (2016) did not detect any upregulation of LC3-I and

LC3-II protein in the quadriceps muscle of older mice. However, they did detect a marked age-related increase in p62/SQSTM1 (Sakuma et al., 2016).

The myofiber atrophy in 24-month-old mice is accompanied by accumulation of p62 in the cytosolic cell fraction (Sakuma et al., 2016), similar to the p62 accumulation observed in aged Drosophila muscles (Demontis & Perrimon, 2010). Also, myofibers that are p62-positive appears to be smaller than nearby myofibers with lower p62 levels (Sakuma et al., 2016), suggesting that the inhibition of autophagy leads to myofiber atrophy and muscle mass loss in the aged. Nezis and Stenmark (2012) demonstrated that artificially increasing p62/SQSTM1 expression may have similar effects to mTORC1 inhibition for longevity, with perhaps the advantage of maintaining cell growth and proliferation (Nezis & Stenmark, 2012). This overexpression of p62/SQSTM1 could have some damaging consequences, specifically when autophagy is impaired (Komatsu et al., 2010). On the contrary, the expression of p62/SQSTM1 has also been shown to decrease with age in mice (J. Kwon et al., 2012). The loss of p62/SQSTM1 has proved to reduce lifespan, and a reduced p62/SQSTM1 level correlates with age-related pathologies in human tissues (Bitto et al., 2014; Lerner et al., 2013). These observations suggest a role for p62/SQSTM1 in the processes that prevent aging.

The mechanism underlying attenuated autophagy with aging remains unclear, and the possible mechanisms contributing to autophagy inhibition are complex and multifactorial (Martinez-Lopez et al., 2015). This age-related decline in autophagy may be due to reduced autophagy-related gene expression (Drummond et al., 2014; Joseph et al., 2013), or lower protein levels of autophagy central components and mitophagy regulators (Russ, Boyd, McCoy, & McCorkle, 2015; Sebastian et al., 2016). The formation and clearance of autophagosome could be reduced, either by an inability of autophagosome to fuse with lysosomes or by a decreased lysosomal activity (Hohn et al., 2017). Interestingly, mTOR signaling might contribute to the suppression of autophagy with age (Ravikumar et al., 2004; Settembre et al., 2012), as hyperphosphorylation of mTOR have been found in both advancing age and obesity (Cornu, Albert, & Hall, 2013; Johnson, Rabinovitch, & Kaeberlein, 2013; Z. Yang & Ming, 2012). There is considerable evidence indicating that AMP-activated kinase (AMPK) signaling can inhibit mTOR and thus suppresses protein synthesis (Bolster,

Crozier, Kimball, & Jefferson, 2002; Gwinn et al., 2008; Mihaylova & Shaw, 2011). AMPK is an evolutionarily conserved energy sensor and is activated by ATP depletion prompted by energy deficiency. This, in turn, stimulates catabolic processes to maintain energy homeostasis and consequently inhibits energy consuming reactions such as protein synthesis. AMPK can inhibit mTORC1 function by phosphorylating TSC2 or Raptor, while also stimulating the activity of FoxO3 (Salminen, Kaarniranta, & Kauppinen, 2016). Although FoxO3a stimulates the expression of several autophagy-related genes, it can also inhibit nuclear factor kappa B (NF-κB) signaling, thus preventing age-related inflammatory responses (Lin, Hron, & Peng, 2004). Also, the activation of 4E-BP1 in mice muscles regulates autophagy by increasing FoxO activity (Tsai et al., 2015), signifying that FoxO/4E-BP1 signaling could prevent skeletal muscle aging through enhanced autophagy in mice as shown earlier in Drosophila (Demontis & Perrimon, 2010).

Autophagy and FoxO3 signaling have been shown to be crucial longevity factors (Salminen et al., 2016), and how autophagy may facilitate longevity is not entirely clarified. It could be due to its role in facilitating the disposal of harmful proteins and organelles, thus preventing their accumulation and supporting cellular renewal (Vainshtein & Hood, 2016).

2.3.3 The effects of exercise on Autophagy-lysosome system

The first study connecting the autophagy pathway with exercise dates back to 1984 (Salminen & Vihko, 1984). Until recently, the molecular evidence and functional significance of exercise-induced autophagy in skeletal muscle have been widely neglected (Grumati et al., 2011; He et al., 2012). Like nutrient deficiency, muscle contraction creates a form of energetic stress which leads to changes in molecular messengers, as soon as energy demand is greater than energy supply; the AMP-to-ATP ratio increases and activates AMPK. The activation of AMPK with endurance exercise is widely recognized, and recent evidence suggests that it is associated with autophagy activation during and after exercise (He et al., 2012; Moller et al., 2015).

In response to endurance exercise, several studies have noted increases in autophagy markers in both rodents and humans (Grumati et al., 2011; Jamart et al., 2012; Y. A. Kim, Kim, Oh, Kim, & Song, 2013). However, a single bout of high-intensity exercise

appears to be more efficient in inducing autophagy flux than prolonged exercise with moderate intensity when measured by an increase in the LC3-II/LC3-I ratio and decrease of p62 levels (Fritzen et al., 2016; Pagano, Py, Bernardi, Candau, & Sanchez, 2014). In humans, high-intensity but not low-intensity exercise activates the autophagy flux and the AMPK pathway, thus increasing the mRNA level of LC3B, p62/SQSTM1, GABARAP, and Cathepsin L (Schwalm et al., 2015). Therefore, exercise intensity, rather than duration, seems to govern the level of autophagy initiation. Even though the influence of endurance exercise on autophagy is widely documented, only a few rodent (I. Kwon, Jang, Cho, Jang, & Lee, 2017; Luo et al., 2013; Monico-Neto et al., 2015) and human (Fritzen et al., 2016; Fry et al., 2013; Glynn et al., 2010; Mejias-Pena et al., 2017; Tanner et al., 2015; Ulbricht et al., 2015) studies have investigated the impact of resistance exercise on autophagy markers.

In a recent study in young rats, LC3-II/I ratio was reduced following 8 weeks resistance training, due to substantial upregulation of LC3-I. Moreover, significantly elevated p62 levels were also observed (I. Kwon et al., 2017). Luo et al. (2013), using older rats, also noted that 9 weeks resistance training resulted in a decrease in the LC3-II/LC3-I ratio, but contrastingly, they demonstrated a significant decline in the autophagy substrate protein p62/SQSTM1. Nonetheless, upregulation in the expression of total AMPK, phosphorylated AMPK and FoxO3a, were also seen (Luo et al., 2013). In human studies, following a bout of resistance exercise Fry et al. (2013) observed an acute decrease in mRNA expression of GABARAP and LC3-II/LC3-I protein ratio in human skeletal muscle, with no age-related differences between younger and older adults. Furthermore, age-related increases in basal protein levels of Atg7 and Beclin1 were identified (Fry et al., 2013). On closer inspection of this study, the older participants possessed quite similar lean mass and muscle strength as the younger participants, and thus the regulation of the autophagy-lysosome system may respond differently in frail elderly. Fritzen et al. (2016) also observed the LC3-II/LC3-I ratio decreased in response to 3 weeks of one-legged exercise training, and this change was primarily driven by an increase in LC3-I content (Fritzen et al., 2016). A study by Tanner et al. (2015) observed an 80 % lower LC3-II/I protein ratio levels in older compared to younger adults at baseline. However, following 5 days bedrest the LC3-II/LC3-I ratio increased in older adults and interestingly further increased following 8 weeks of high-intensity

resistance exercise, reaching an absolute expression level that was comparable to young adults (Tanner et al., 2015). This suggests that a dysfunctional autophagy-lysosomal system in aging muscle cells could be reversible. Furthermore, recent work in agreement with previous human studies observed that acute resistance exercise reduced LC3-II/I ratio which was driven primarily by a reduction in LC3-II in elderly participants. However, higher ingestion of leucine appeared to facilitate a greater suppression of basal autophagosome degradation 24 h postexercise (Dickinson et al., 2017). Additionally, this increased reduction through higher leucine ingestion would be supported by greater intracellular leucine availability, which has been shown to reduce autophagic activity (Glynn et al., 2010; Yan et al., 2012).

2.3.4 Summary

The key proteolytic systems of the cell, ubiquitin-proteasome and autophagy-lysosome system have a crucial role in the removal of proteins and dysfunctional organelles. The ATP-dependent ubiquitin-proteasome system is essential for regulating and removing proteins upon alterations in muscle activity. The Autophagy-lysosome system is an efficient mechanism for regulation of metabolism, removal of defective organelles, protein quality control, and pathogen removal, and engulfs materials in a vesicle called an autophagosome. The autophagy-lysosome degradation pathway seems to be regulated by transcription factor Forkhead box O3a (FoxO3a) which has shown to upregulate several autophagy-related genes, including LC3 & p62/SQSTM1, which might relate to autophagic status. Numerous studies have reported age-related changes in the autophagy-lysosome system. In response to acute exercise, several studies have noted decreases in total LC3-II/I protein levels, whereas long term training seems to increase some autophagy markers in both rodents and humans. A limited amount research has investigated autophagy in humans, and consequently, we know little about how the aging process affects this system. To our knowledge, no previous studies have examined autophagy in frail elderly humans. This research would be of considerable interest due to the significant declines in muscle quality previously observed in this population, which further relates to their loss of functional independence.

In this randomized controlled trial, we investigated the acute response of several autophagy-related markers, LC3, p62, and FoxO3a, to a resistance exercise bout and

protein supplementation. We compared three different age groups (young, elderly and frail elderly), to gain insight into how this stimulus affects the autophagy-lysosome system and if this response is age-dependent.

3. Methods

This thesis presents data based on the results of two larger studies at the Norwegian School of Sport Sciences (NSSS); STAS and Amarone projects. The studies were approved by the South-East Regional Ethical Committee of Norway and carried out in agreement with the Declaration of Helsinki. All participants were informed about potential risks related to the experiments and gave written informed consent before entering the studies. The following description of methods focuses on procedures relevant for the purpose of the present thesis.

3.1 Participants

Twenty-nine men and women from three different age groups were recruited from nursing homes, sheltered housing and through written communications. Participants from the Amarone project included seven females and ten males (n = 7, 20-43 years old; n = 10, 70-82 years old), and were separated into a young (Y) and elderly (E) group, respectively. Whereas, the Frail Elderly (FE) consisted of five females and seven males (n = 12, 67-96 years old). See Table 1 for a complete overview of the participant characteristics.

Table 3.1: *Baseline characteristics of the participants included in this study.*

	YOUNG (n = 7)	ELDERY (n = 10)	FRAIL ELDERLY (n = 12)
Sex Distribution	♀= 3 ♂ = 4	♀ = 4 ♂= 6	♀ = 5 ♂ = 7
Age (years)	28.3 ± 6.9	74.8 ± 3.4	85.3 ± 8.6
Height (m)	1.77 ± 0.09	1.72 ± 0.09	1.65 ± 0.08
Body Mass (kg)	84.3 ± 16.6	79.3 ± 16.8	63.2 ± 12.6
BMI (Body mass/m^2)	26.8 ± 4.4	26.6 ± 4.8	23.1 ± 3.5
Body fat %	32.9 ± 8.8	32.1 ± 5.8	32.1 ± 6.5
Lean Body Mass (kg)	55.0 ± 9.4	51.8 ± 10.6	41.4 ± 6.6
Lean Mass Legs (kg)	19.9 ± 3.8	18.2 ± 4.8	13.9 ± 2.6
Isometric maximal voluntary contraction force (IMVC) (N)	477 ± 88	316 ± 109	209 ± 81
Relative Strength (N/Body Mass)	5.7 ± 0.8	4.0 ± 1.0	3.4 ± 1.0

3.1.1 Inclusion and Exclusion Criteria

Participants were screened and filled out questionnaires to assess eligibility for participation. Those who met the pre-determined criteria (Table 2) got included in the study. Those with musculoskeletal injuries, health conditions, used anticoagulant

medication that could not be discontinued for the biopsy procedure, or were lactose intolerant/had milk allergies, were excluded.

Table 3.2: *Summary of inclusion and exclusion criteria*

	INCLUSION CRITERIA	EXCLUSION CRITERIA
Young	Men and women between 18-45 years old.	Musculoskeletal injury or health condition Use of supplements Use of corticosteroids last six months Lactose intolerant/Milk allergies
Elderly	Men and women over 65 years old. Healthy and active.	Musculoskeletal injury or heath condition Use of supplements Blood pressure >140/90 BMD <0,84 g/cm^2 in L2-L4 Fasted glucose >6mmol Lactose intolerant/Milk allergies
Frail Elderly	Men and women over 70 years old. Fried Frailty Criteria score ≥3 (attachment 1) Short Physical Performance Battery (SPPB) score ≤6 (attachment 2)	Musculoskeletal injury or health condition Use of anticoagulant medication that cannot discontinue Allergies to local anesthesia Uncontrolled hypertension Mini Mental State Examination <22 out of 30 (attachment 3) Lactose intolerant/Milk allergies

3.2 Overview of Experimental Design

At the completion of pre-intervention assessments, the frail elderly (STAS participants) got randomized into two groups. One group performed resistance exercise and received a protein supplement (RE+PRO), whereas the other group received the same protein supplement, but did not perform resistance exercise (PRO) (Table 3). Randomization was computerized and carried out by a researcher at NSSS. The results chapter presents RE+PRO, and PRO combined for baseline values, and RE+PRO separately when comparing the acute response to exercise to young and elderly participants. Lastly, some figures will also only compare RE+PRO and PRO.

Table 3.3: *Baseline characteristics of the two frail elderly groups; RE+PRO and PRO. *Significantly different from RE+PRO*

	FRAIL ELDERLY	
	RE+PRO (n = 8)	PRO (n = 4)
Sex distribution	♀= 4 ♂ = 4	♀= 1 ♂ = 3
Age (years)	84.5 ± 9.8	87 ± 6.5
Height (m)	1.65 ± 0.1	1.64 ± 0.1
Body Mass (kg)	60.5 ± 12.4	68.4 ± 13.1
BMI (Body mass/m^2)	22 ± 3.3	25.4 ± 2.9
Body fat %	29 ± 6	38 ± 2*
Lean Body Mass (kg)	41.2 ± 6.9	41.6 ± 6.9
Lean Body Mass Legs (kg)	13.8 ± 2.6	14.3 ± 3.1
Isometric maximal voluntary contraction force (IMVC) (N)	207 ± 93	215 ± 46
Relative Strength (N/Body Mass)	3.4 ± 1.1	3.4 ± 0.4
Sit-to-Stand (sec)	23.8 ± 27.9	32.4 ± 36

3.2.1 Experimental Trial

Participants were requested to meet fasted at NSSS and received a standardized low-protein breakfast consisting of oatmeal and water. One hour following breakfast, a muscle biopsy was obtained from *m. vastus lateralis* (described below). Forty minutes later, the participants underwent a unilateral isometric maximal voluntary contraction (IMVC) test, followed by a resistance exercise bout lasting twenty minutes, and another unilateral IMVC test. Participants from the young and elderly groups conducted a full body resistance exercise bout. The leg exercises consisted of three sets of hammer squat, three sets of leg press, three sets of knee extension and the upper body exercises were three sets of chest press, three sets of seated row and three sets of shoulder press. The frail elderly conducted six sets of knee extensions. Each set for all groups started every third minute and completed at a load equivalent of ten repetition maximum (10 RM) for the young and elderly group and eight repetition max (8 RM) for the frail elderly. Immediately after the resistance exercise bout, the participants consumed a protein supplement at the same time point. Two hours (for young and elderly) and two hours and thirty minutes later (for frail elderly), another muscle biopsy was obtained from the same incision.

3.2.2 Isometric Maximal Voluntary Contraction (IMVC)

IMVC was tested unilaterally in a knee extension apparatus (GYM 2000 Gym Equipment, Geithus, Norway), seated with a knee angle 90°, and the ankle pressing against a force cell (HBM U2AC2, Darmstadt, Germany)connected to Labview 8.2 analysis software (National instr., Austin, Texas). Before the test, the participants did a

warm-up of 5 minutes on a stationary bicycle, followed by a specific warm-up in the knee extension apparatus. The specific warm-up consisted of three submaximal 5-second isometric contractions with both legs, at 25, 50 and 75% of maximal torque, separated by approximately 10 seconds of rest. During the IMVC, the participants were instructed to perform three maximum effort trials on each leg and were encouraged to produce maximal force as quickly as possible. Each maximum effort was held for three seconds, with one-minute rest between trials. The unilateral IMVC test has demonstrated a high coefficient of variation (CV) value (CV = 6.1%), in elderly subjects (n=18) supporting this test is reliable.

3.2.3 Protein Supplements

Directly after the resistance exercise bout, the participants consumed a supplement consisting of protein, carbohydrates, and fat. Although the protein content between groups was comparable, there were some differences in the nutritional content between the young and elderly (18,9 g protein, 6,9 g fat, 35,6 g carbohydrate) and the frail elderly (16,8 g protein, 0,7 g fat, 18,5 g carbohydrate), whom received low-fat milk and Tine Styrk, respectively.

3.2.4 Muscle Biopsies

The participant lay in a supine position, and after injection of local anesthesia (Xylocaine with adrenaline, 10 $mg \cdot ml^{-1}$ + 5 $\mu l \cdot ml^{-1}$, AstraZeneca, London, UK), an 1.5-2.0 cm incision was made in the skin and muscle fascia. Using a 6mm Bergström biopsy needle modified for manual suction, 1-3 small muscle samples (approx. 50 mg apiece) were obtained from *m. vastus lateralis.* The two biopsies were taken from the same incision but in opposite directions. The incision was closed with strips, and muscle tissue samples were preserved in an ultra-freezer at -80°C until further analyses.

3.3 Analyses

3.3.1 Homogenization

One piece (50 mg) from muscle tissue sample was homogenized using 1 ml of a commercial homogenization buffer, TPER® (TissueProtein Extraction Reagent, cat#78510, Thermo Scientific Rockford, IL, USA), 20 µl Halt™ protease and

phosphatase inhibitor cocktail(cat#1861281, Thermo Scientific) and 20 µl EDTA (Cat#1861274, Thermo Scientific, Rockford, IL, USA).

Another piece (50 mg) from the muscle tissue samples were fractionated into cytosol-, membrane-, nuclear-, and cytoskeletal fractions, using ProteoExtract® Subcellular Proteosome Extraction kit(Cat#539790, Calbiochem, EMD Biosciences, Schwalbach, Germany). Samples were portioned in 25 µl aliquots, then stored in an -80°C ultra-freezer.

3.3.2 Protein Measurement

Protein concentration was measured using RC/DC Protein assay kit (Bio-Rad, cat#5000121, Herkules, CA, USA). Bovine γ-globulin standard set ranging 0,125; 0,25; 0,5; 1; 1,5 $\mu g \cdot ml^{-1}$, were used as protein standards. The fractionated samples were diluted with distilled water (dH_2O) to retain protein concentrations within the defined standard protein range. Triplicates á 5µl of every sample were pipetted into a 96-well microtiter plate (Greiner Bio-One International AG, Kremsmünster, Germany). After adding the samples, 25 µl of reagent A+S (cat#500-0113 and #500-0115, Bio-Rad Laboratories Inc., USA), and 200 µl reagent B (cat#500-0114, Bio-Rad Laboratories Inc., USA) were added to each well. After 15 minutes incubation, the microtiter plates were quantified using ASYS Expert 96 (Biochrom, Cambridge, UK) at 690 nm. Protein concentrations were calculated with KIM Immunochemical Processing Software 32, and samples with CV <10% were used in subsequent Western Blot assays.

3.3.3 Western Blot

Electrophoresis and Western blot analyses were performed on fractionated samples. Analyses of proteins involved in muscle protein degradation were performed at NSSS, by investigating level and location of proteins involved in the ubiquitin-proteasome system and the autophagic pathway.

Fractionated samples were added sample buffer 1/25 5M DTT (dithiothreitol, cat#161-0610, Bio-Rad Laboratories Inc., USA), 24/25 Laemmli (4x Laemmli Sample Buffer, cat#161-0747, Bio-Rad Laboratories Inc., USA) and distilled deionized water (dH_2O). Samples were placed on a heating block for 10minutes at 70°C, before 30 µl was applied as duplicates for each biopsy time point to Stain-free gels (Mini-PROTEAN®

TGX Stain-Free™Gels, cat#456-8094, Bio-Rad Laboratories Inc., USA), with 5 µl of molecular weight marker (Protein Ladder PS 11, cat#310005, GeneOn., Germany) in the first and last well. All subcellular fractions were loaded onto the same gel to enable comparisons. Electrophoresis was performed at 200 volts for 25-45 minutes (Mini-PROTEAN® Tetra Cell, Bio-Rad) or until the 10,5kDa-marker wandered of the gel, with room tempered running buffer.

After the electrophoresis, pictures were taken of the gels with Bio-Rad ChemiDoc™ MP System (#170-8280, Bio-Rad Laboratories, Hercules, CA, USA) to control for loading errors. The gels tryptophan content were activated, and protein distribution was visualized by 2,5 minutes of UV-light exposure, PVDF-membranes were activated for 30 seconds in methanol (EMD Millipore Corporation, Billerica, MA, USA), 30 seconds in dH_2O, 2 new minutes in fresh dH_2O followed by 15 minutes in transfer buffer.

Proteins were transferred to activated PVDF membranes (cat#162-0177, Bio-Rad, CA, USA) in a Criterion™ Blotter (BioRad, CA, USA), at 50 volts for 60 minutes in cold transfer buffer. After blotting, pictures were again taken of the gels and membranes to ensure proper protein transfer.

Membranes were blocked at room temperature for 2 hours in a 5% fat-free skimmed milk powder (EMD Millipore Corporation, Billerica, MA, USA) and TBS-t solution (10X Tris Buffered Saline, cat#170-6435, Bio-Rad, CA, USA & 0,1% Tween® 20, cat#P7949-100 ml, Sigma Aldrich). Blocked membranes, were cut based on the proteins of interest; FOX03a (82-97 kDa), p62 (62 kDa) and LC3B (14 and 16 kDa), and were incubated with primary antibodies (Table 4) overnight at 4°C on a rocker-machine.

The following day, membranes were incubated with secondary antibody diluted 1:3000 in a 1% fat-free skimmed milk and TBS-t solution, for 1 hour. Between stages, the membranes were washed 15 min in TBS-t, and then 3 x 5 min in TBS. The membranes were incubated with Chemiluminescent Substrate SuperSignal WestDura® (Extended Duration Substrate, Thermo Scientific, cat#34076, Rockford, IL, USA) for 5 min before pictures were taken with ChemiDocTM MP Imaging System and analyzed with Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA, USA).

Table 3.4: *Overview of primary and secondary antibodies.*

	Antibody	Producer	Host	Dilution	Cat.no.
Primary antibody	Fox03a (75D8)	Cell Signaling	Rabbit	1:1000	#6888
	SQSTM/p62	Cell Signaling	Rabbit	1:2000	#5114
	LC3B (LC3I and LC3II)	Cell Signaling	Rabbit	1:1000	#2775
Secondary antibody	Anti-rabbit IgG HRP-linked AB	Cell Signaling	Goat	1:3000	#7074

3.4 Statistics

Data are presented as the mean ± standard deviation (±SD). Statistical analysis was conducted in Microsoft® Excel 2010 and Prism® (GraphPad Software Inc., San Diego, CA, USA). Normality of the data was assessed by visual examination of normality plots, as well as Shapiro-Wilk test and the D'Agostino-Pearson omnibus normality test. Data that passed the normality test were assessed by one-way analysis of variance (ANOVA) for between-group differences (i.e. Y, E & FE) for anthropometric and Western Blot data. In the occasion of significant effects, the Turkey *post hoc* test was used to determine specific differences within an ANOVA. The within-group differences (changes from pre to post resistance exercise bout) was assessed with a paired t-test. Between-group differences for RE+PRO vs. PRO were assessed with unpaired t-test. Non-normally distributed data were evaluated by a nonparametric Kruskal-Wallis test for between-group differences and Mann-Whitney U (for RE+PRO vs. PRO), whereas Wilcoxon signed-rank test was used for within-group differences. $P<0.05$ was chosen as a two-tail level of significance.

4. Results

4.1 Baseline Characteristics

There were no differences in weight between young and elderly, while frail elderly weighed significantly less than both groups ($p<0.05$). BMI and body fat % did not differ between young, elderly, and frail elderly. However, frail elderly had significantly lower total lean body mass compared to young and elderly (41.4±6.6 kg vs., 55.0±9.4 kg and 51.8±10.6 kg, respectively) and leg lean mass ($p<0.05$). No significant differences were observed between young and elderly group in any of the body composition variables. The young group had a significantly higher IMVC compared to frail elderly (477±88 N vs. 209±81 N, $p=0.0002$), while no significant differences were seen between young and elderly, or elderly and frail elderly groups. Both the elderly and frail elderly had lower relative strength compared to the young ($p=0.002$ & $p<0.0001$) (Table 1).

4.2 Intracellular Signaling

4.2.1 LC3-II/I Ratio

No differences in the LC3-II/I ratio in cytosolic or membrane fraction were observed between the young, elderly or frail elderly groups at baseline (Figure 4.4 A & B).

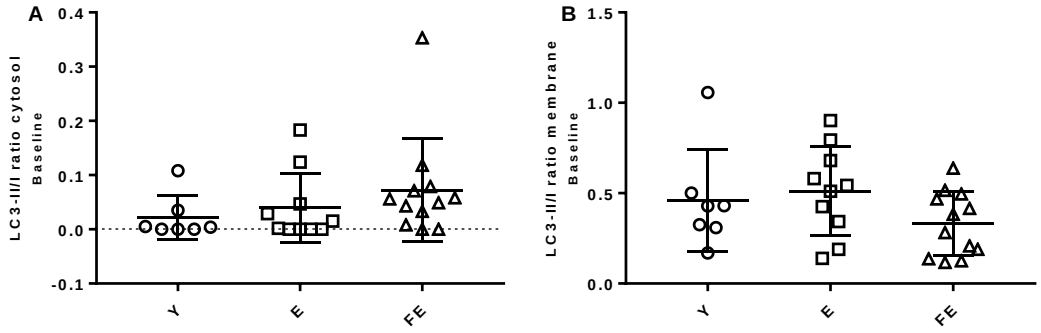

Figure 4.1: *LC3-II/I ratio at baseline in cytosol fraction (A), and membrane fraction (B). Baseline levels are illustrated with a dotted line. Y: Young, E: Elderly, FE: Frail elderly. #: difference between groups (P<0.05);*: different from pre (P<0.05).*

In response to resistance exercise and protein intake, the LC3-II/I ratio in the cytosolic (figure 4.5 A) and membrane (figure 4.5 B) fractions were significantly reduced from baseline for both elderly and frail elderly groups ($p<0.05$). Additionally, a tendency towards change was observed in the LC3-II/I ratio in the cytosolic and membrane

fraction from pre to 2.5 h post resistance exercise and protein intake for young (p=0.063 & p=0.078, respectively). However, no between-group differences were observed.

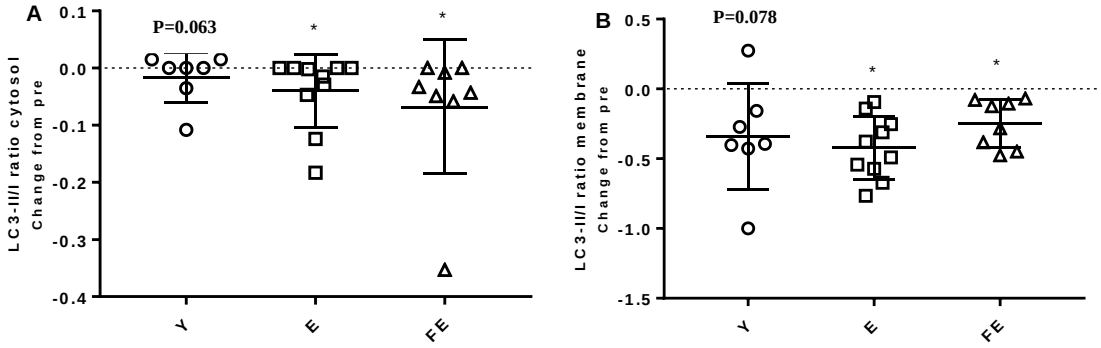

Figure 4.2: *Change in LC3-II/I ratio from baseline in cytosol fraction (A), and membrane fraction (B). Baseline levels are illustrated with a dotted line. Y: Young, E: elderly, FE: Frail elderly. #: difference between groups (P<0.05);*: different from pre (P<0.05).*

4.2.2 LC3-II

After the resistance exercise bout and protein supplementation, the elderly group experienced a statistically significant reduction in the level of the LC3-II in the cytosolic fraction, corresponding to a 100 ± 0 % decrease (p<0.05). Neither the frail elderly nor the young group observed any changes in the LC3-II cytosolic fraction from pre to post resistance exercise. However, the LC3-II cytosolic fraction immunoblots detected only 4 participants in the young group. A tendency towards a significant decrease was demonstrated in the frail elderly group (p=0.063), (figure 4.3 A). The level of LC3-II membrane fraction decreased significantly from baseline in both the elderly and frail elderly group (-80±11%, p<0.0001 & -74±39%, p<0.05, respectively), whereas the young group had one participant who saw a marked increase, the group remained unchanged (p>0.05). Nevertheless, no group differences were observed for either fraction (figure 4.3 B).

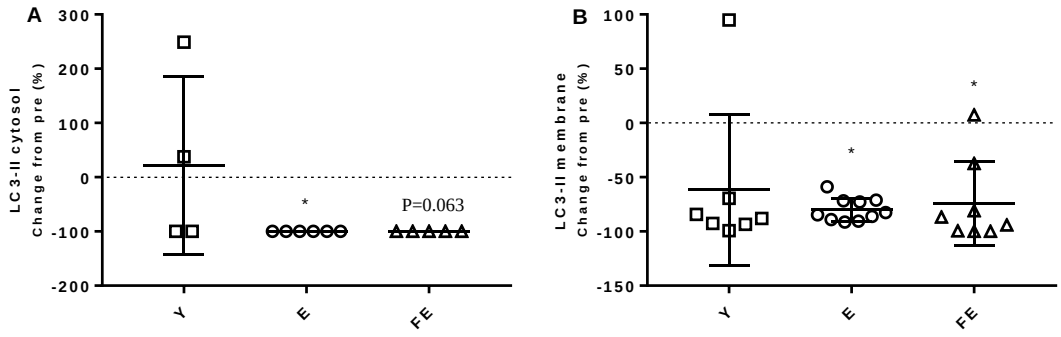

Figure 4.3: *Percent change in LC3-II from baseline in cytosol fraction (A), and membrane fraction (B). Baseline levels are illustrated with a dotted line. Y: Young, E: Elderly, FE: Frail elderly. #: difference between groups (P<0.05);*: different from pre (P<0.05).*

4.2.3 LC3-I

The level of LC3-I in the cytosolic and membrane fraction remained unchanged from baseline to after the resistance exercise and protein intake for the young and elderly groups (p>0.05). In contrast, the frail elderly group observed a suggestive but not quite significant increase in LC3-I cytosolic fraction (p=0.081), and in the membrane fraction (p=0.101) (Figure 4.2 A & B). Combining all the groups together showed a significant increase in the LC3-I membrane fraction (p<0.02). Also, when differences between the young, elderly and frail elderly groups from pre to post resistance exercise and protein intake were analyzed, no differences were shown.

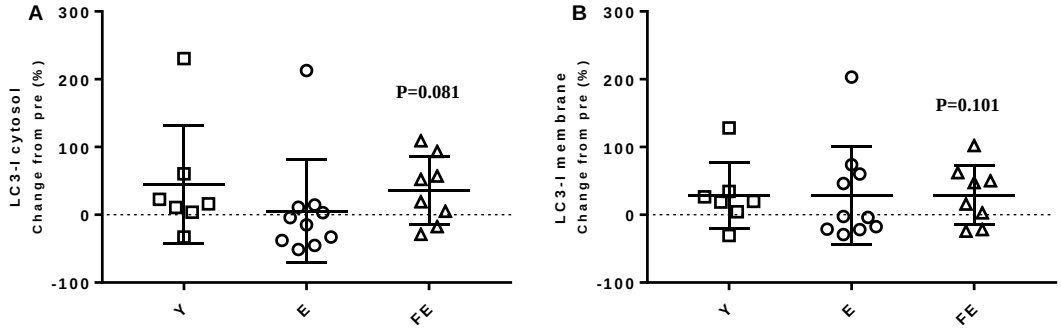

Figure 4.4*: Percent change in LC3-I from baseline in cytosol fraction (A), and membrane fraction (B). Baseline levels are illustrated with a dotted line. Y: Young, E: Elderly, FE: Frail elderly. #: difference between groups (P<0.05);*: different from pre (P<0.05).*

4.2.4 p62

The level of p62 did not change from baseline to after the resistance exercise and protein intake in any of the groups in the cytosolic fraction (figure 4.1 A). However, there was a favorable trend toward a significant increase for the elderly and frail elderly in the p62 membrane fraction (p=0.091 & p=0.065, respectively), with no change for the young group (p>0.05) (figure 4.1 B). The membrane p62 protein fraction did demonstrate a significant increase after resistance exercise and protein intake when all the groups were merged together (p<0.008). Furthermore, no group differences were observed for p62 in either of the fractions.

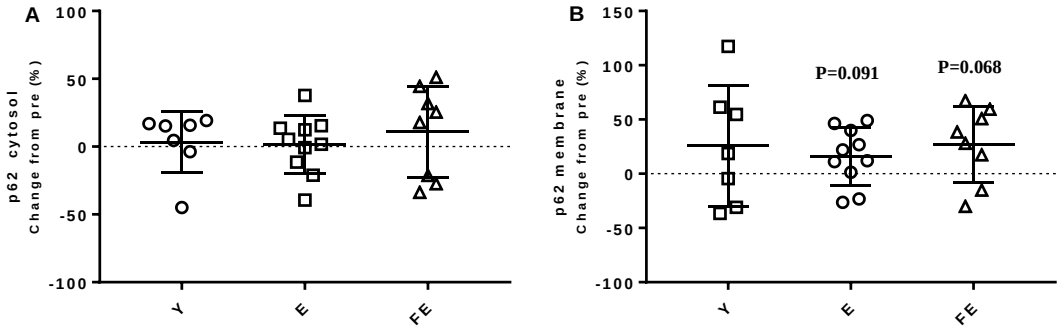

Figure 4.5: *Percent change in p62 from baseline in cytosol fraction (A), and membrane fraction (B). Baseline levels are illustrated with a dotted line. Y: Young, E: Elderly, FE: Frail elderly. #: difference between groups (P<0.05);*: different from pre (P<0.05).*

4.2.5 Fox03a

The level of FoxO3a in the cytosolic fraction was significantly reduced in the young (52 ± 49%, p<0.05) and frail elderly group (-52±53%, p<0.05) 2.5 hours following resistance exercise and protein supplementation. However, no significant change was observed in the elderly group (-45±68%, p>0.05). No group differences were found (figure 4.6).

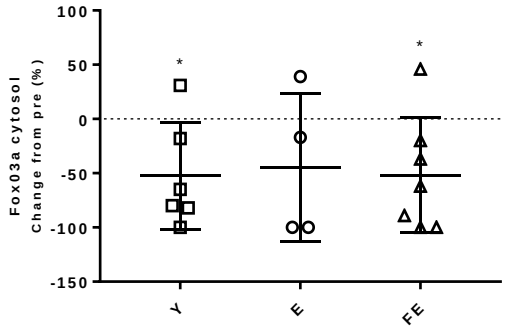

Figure 4.6: *Percent change in Fox03a from baseline in cytosol fraction. Baseline levels are illustrated with a dotted line. Y: Young, E: Elderly, FE: Frail elderly. #: difference between groups (P<0.05);*: different from pre (P<0.05).*

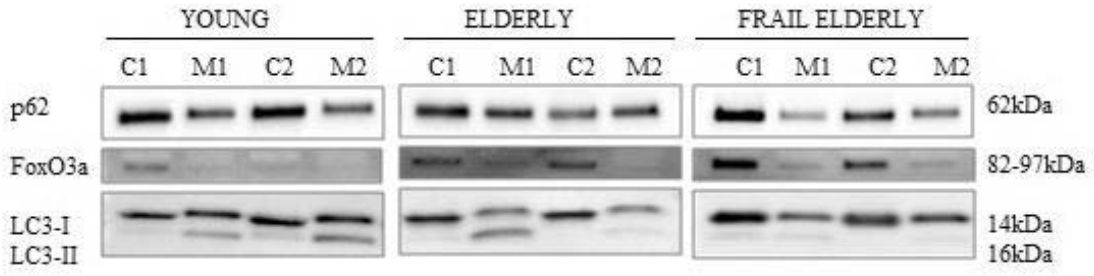

Figure 4.7: *Representative Western blot protein images for basal (C1 & M1) and 2.5h (C2 & M) following the combination of resistance exercise and postexercise protein supplementation. C1: Basal cytosolic, C2: Postexercise cytosolic, M1: Basal membrane, M2: Postexercise membrane.*

4.3 Intracellular signaling in response to exercise and protein vs. only protein ingestion in frail Elderly

4.4 Baseline characteristics frail elderly

When comparing the two groups of frail participants (RE+PRO and PRO), no between-group differences were observed, except for body fat % which was significantly higher in PRO (29±6 % and 38±2 %, p<0.03, respectively) (Table 3).

4.5 Intracellular Signaling

4.5.1 LC3-II/I Ratio

A significant reduction in the LC3-II/I cytosolic fraction ratio was observed post resistance exercise bout and protein intake for RE+PRO and after protein intake only for PRO (p<0.05 & p=0.011, respectively) (figure 4.9 A). On the other hand, only the

RE+PRO group observed a statistically significant reduction in LC3-II/I membrane fraction ratio ($p<0.05$).However, the PRO group barely fell short of significance ($p=0.051$). When we compare groups, a slight difference toward significant was observed in the LC3-II/I cytosolic fraction ($p<0.07$). However, there were no observed differences between groups in the membrane fraction. (Figure 4.9 B).

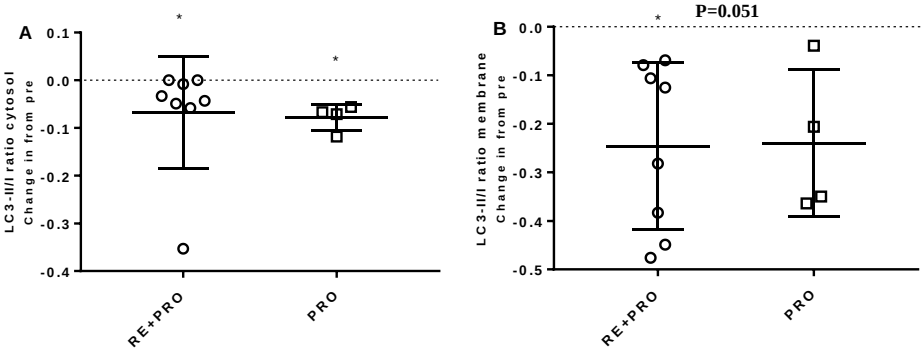

Figure 4.8: *Change in LC3-II/I ratio from baseline in cytosol fraction (A) and membrane fraction (B). Baseline levels are illustrated with a dotted line. RE+PRO: resistance exercise and protein supplement, PRO: protein supplement only, #: difference between groups (P<0.05);*: different from pre (P<0.05).*

4.5.2 LC3-II

The level of LC3-II cytosolic fraction remained unchanged from pre to post protein intake in the PRO group ($p>0.05$). However, a strong trend towards significance decrease was demonstrated in the RE+PRO group after resistance exercise and protein supplementation ($p<0.07$). Both RE+PRO and PRO experienced a statistical significant change from baseline in the level of LC3-II membrane fraction (-74±39 %, $p=0.016$; -73±33 %, $p=0.02$, respectively) (figure 4.8). No between-group differences were evident for any fraction.

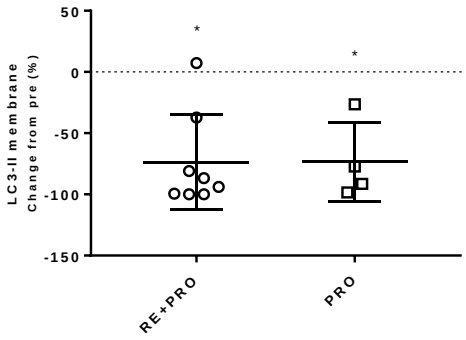

Figure 4.9: *Percent change in LC3-II from baseline in the membrane fraction. Baseline levels are illustrated with a dotted line. RE+PRO: resistance exercise and protein supplement, PRO: protein supplement only, #: difference between groups (P<0.05);*: different from pre (P<0.05).*

4.5.3 LC3-I

The RE+PRO group had a non-significant increase 2.5 h post resistance exercise in the LC3-I cytosolic fraction (p<.0.09), whereas no changes were observed in the PRO group post protein intake (p>0.05). For the LC3-I membrane fraction, neither group experienced any change from baseline. However, the RE+PRO had a weak trend towards significance (p=0.101). There were no observable differences between groups for either the cytosolic or membrane fraction.

4.5.4 p62

The level of p62 cytosolic fraction did not changed in either RE+PRO or PRO groups from baseline (p<0.05). Moreover, the RE+PRO group demonstrated a non-significant increase in the level of p62 membrane fraction post resistance exercise and protein intake (p<0.07), whereas PRO group remained unchanged after protein intake only (p>0.05). Furthermore, no differences were observed between the groups for neither cytosolic or membrane fractions (results not shown).

4.5.5 FoxO3a

The RE+PRO group showed a significant decrease in FoxO3a cytosolic fraction after resistance exercise bout and protein supplementation compared to baseline levels (-52±53 %, p<0.05), whereas the PRO group demonstrated a non-significant decrease post protein intake (p<0.10). There were no significant differences between RE+PRO and PRO groups for Fox03a in the cytosolic fraction (Figure 4.10).

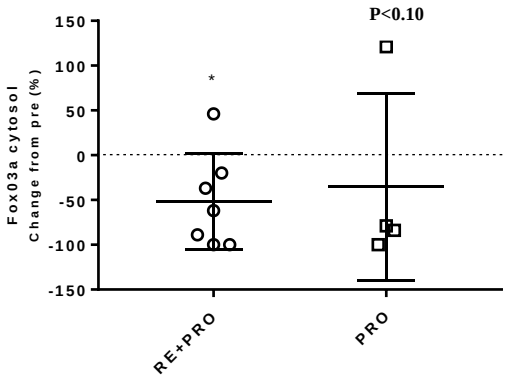

Figure 4.10: *Percent change in Fox03a from baseline in cytosol fraction. Baseline levels are illustrated with a dotted line. RE+PRO: resistance exercise and protein supplement, PRO: protein supplement only, #: difference between groups (P<0.05);*: different from pre (P<0.05).*

4.6 Summary

Table 4.1: *Overview of the protein results for the different groups. ↔=No change. ↑=Significant increase (p<0.05). ↓=Significant decrease (p<0.05). ↗=Nonsignificant increase (p<0.10). ↘=Nonsignificant decrease (p<0.10).*

				FRAIL ELDERLY	
	YOUNG	ELDERLY	FRAIL ELDERLY	RE+PRO	PRO
P62 CYTO	↔	↔	↔	↔	↔
P62 MEM	↔	↗	↗	↗	↔
LC3-I CYTO	↔	↔	↗	↗	↔
LC3-I MEM	↔	↔	↗	↗	↔
LC3-II CYTO	↔	↓	↘	↘	↔
LC3-II MEM	↔	↓	↓	↓	↓
LC3-II/I CYTO	↘	↓	↓	↓	↘
LC3-II/I MEM	↘	↓	↓	↓	↘
FOXO3A CYTO	↓	↘	↓	↓	↘

5. Discussion

In the present study, we examined markers of autophagy following the combination of an acute resistance exercise bout and protein supplementation in young, elderly and frail elderly adults. The primary and novel findings were; acute resistance exercise and protein supplementation led to a reduction in LC3-II/I ratio in cytosol and membrane fractions driven mainly by a decreased LC3-II level in the elderly and frail elderly subjects. Merging all groups revealed increased p62 and LC3-I in the membrane fraction. Furthermore, we did not demonstrate any age-related differences in the response of the autophagy-lysosome systems.

5.1 Intracellular Signaling

5.1.1 LC3

In response to an acute resistance exercise bout, we observed a significant reduction in LC3-II/I ratio in both the cytosolic and membrane fractions for the frail elderly and elderly groups, with a trend towards a decrease in the young group. This reduction in LC3-II/I ratio is in agreement with some human (Dickinson et al., 2017; Fritzen et al., 2016; Fry et al., 2013), and rodent studies (I. Kwon et al., 2017; Luo et al., 2013), but not all (Tanner et al., 2015). Previous research has demonstrated that the decline in the total LC3-II/I ratio 2 h postexercise is due in large part to a reduction in LC3-II (Dickinson et al., 2017; Fry et al., 2013; Luo et al., 2013). Other studies have contrastingly noted that an accumulation of the LC3-I protein was the primary driver behind the reduced LC3-II/I ratio (Fritzen et al., 2016; I. Kwon et al., 2017). According to Kadowaki and Karim (2009), the increased cytosolic fraction LC3-II/I ratio appears to correlate with changes in autophagy and may provide an easy quantitative method for monitoring the regulation of autophagy (Kadowaki & Karim, 2009; Karim et al., 2007). However, the change in the LC3-II/LC3-I ratio alone can be challenging to interpret (Klionsky et al., 2016), as it can reflect either a reduction or an increased autophagic flux.

A significant decline in the LC3-II protein was observed in both cytosolic and membrane fraction in the elderly group and frail elderly group. However, no changes were shown in the young group. The decrease in LC3-II in the elderly and frail elderly

could imply an enhanced lysosomal activity, as the LC3-II protein is bound to both the inner and outer side of the expanding phagosphore membrane (Lippai & Szatmari, 2017; Mizushima et al., 2001). Due to localization on the autophagosomal membrane, the LC3-II protein in the intra-autophagosomal lumen is degraded by lysosomal hydrolases when the closed autophagosomes ultimately fuse with lysosomes, resulting in the LC3-II levels decreasing (Tanida, 2011). In support of this, several studies have demonstrated that increased amount of LC3-II with the absence or reduced activity of lysosome enzymes coincides with an impaired autophagic degradation and autophagosomal accumulation (Maruzs et al., 2015; Tatti et al., 2012). Since the LC3-II protein is degraded through autophagy, it is seen as a good indicator of autophagic flux. However, paradoxically, the amount of LC3-II increases rapidly upon autophagy induction, and decreases after longer periods of activation (Mizushima et al., 2010). Thus the key issue is to distinguish between the transient decline of autophagosomes due to attenuated induction, and their reduction due to efficient clearance. Although our study only had one biopsy time point, 2-2.5 h post resistance exercise and protein intake, more than 2 hr of starvation has shown to activate autophagy with subsequent reductions in the total LC3-II (Mizushima & Yoshimori, 2007). Therefore a reduced LC3-II does not exclude autophagy activation.

In addition to a significant reduction in LC3-II, the frail elderly group demonstrated a nonsignificant upregulation of LC3-I following resistance exercise. However, the sensitivity of LC3-I detection by anti-LC3 antibody has shown to be much lower than that of LC3-II in most cases (Kabeya et al., 2004; Mizushima & Yoshimori, 2007). In an effort to keep our sample size sufficiently large, we pooled all groups and observed a significant increase of LC3-I in the membrane fraction. This increase in LC3-I protein levels could reflect an enhanced autophagic flux because the LC3-II attached to the autophagosomal membrane facing the cytosol can be recycled back to LC3-I by Atg4 (Mizushima & Komatsu, 2011) and thus could accumulate in proportion to an increase in autophagy. However, there is an imprecise precursor-product relationship between LC3-I and LC3-II because the conversion of the former to the latter is cell type-specific and dependent on the kind of stress to which the cells are subjected. Karim et al. (2007) investigated the response of the cytosolic LC3-II/I ratio to amino acids. They demonstrated that the amino acid leucine, which is a strong regulator of autophagy,

reduced the concentration of LC3-II and concomitantly increased the concentration of LC3-I (Karim et al., 2007). Glynn et al. (2010) did not observe any changes in either total LC3-I or LC3-II 1 h after resistance exercise in humans. However, LC3-II was significantly reduced following the ingestion of 0.35 g/kg/LBM essential amino acids with no change in LC3-I (Glynn et al., 2010). In a more recent study, Dickinson et al., (2017) showed that old adults who consumed leucine post resistance exercise noted a reduction in total LC3-II and no change in LC3-I (Dickinson et al., 2017). Interestingly, Karim et al. (2007) speculate that leucine could effectively suppress the transformation of LC3-I to LC3-II, which could reflect what we see in our results (Karim et al., 2007). Fritzen et al. (2016) observed that insulin stimulation led to the phosphorylation of Unc-51-like protein kinase 1 (ULK1) together with a reduction in the LC3-II/I ratio (Fritzen et al., 2016). Insulin has been shown to be a potent inhibitor of autophagy, which might decrease LC3 lipidation through the activation of mTORC1 (Vendelbo et al., 2014). We could speculate that an increased intracellular leucine availability and insulin response from the post resistance exercise protein supplementation would reduce the LC3-I to LC3-II conversion, leading to the observed pooled significant increase in LC3-I in the membrane fraction and thus attenuate autophagic activity (Glynn et al., 2010; Yan et al., 2012).

More recently it was demonstrated that resistance exercise stimulates mTORC1 activation through a different pathway than amino acids (Marcotte et al., 2015; Rindom & Vissing, 2016). As mTORC1 activation also induces the phosphorylation and inhibition of ULK1^{Ser757} (Egan et al., 2011; J. Kim, Kundu, Viollet, & Guan, 2011; Schneider & Cuervo, 2014), it plays a central role in the attenuation of autophagy initiation. The phosphorylation of ULK1^{Ser757} by mTORC1 could inhibit the ability of AMPK to activate ULK1^{Ser555} which in turn inhibits the initiation autophagosome formation and thus prevents the transient increase of the LC3-II amount upon autophagy induction (Jung, Ro, Cao, Otto, & Kim, 2010; J. Kim et al., 2011; Shang et al., 2011). After 8 weeks of resistance training, Mejias-Pena et al. (2017) observed a significant decrease in the phosphorylation of ULK1^{Ser757} in healthy older adults (Mejias-Pena et al., 2017). I. Kwon et al. (2017) noted a resistance exercise-induced AMPK suppression 24 h after last the bout of 8 weeks training, but neither Ulk1^{Ser555} nor Ulk1^{757} levels significantly changed (I. Kwon et al., 2017). Therefore based on recent research

demonstrating regulation of autophagy is independent of mTOR (Hiebel, Kromm, Stark, & Behl, 2014; Y. Lee et al., 2016), it could also be conceivable that resistance exercise affects autophagy in an AMPK or mTOR-independent way. However, the inhibition of mTORC1 leads to an up-regulation of autophagy, seen by LC3-I conversion to LC3-II through lipidation (Klionsky et al., 2016). Vendelbo et al. (2014) observed a reduced mTORC1 activity during fasting and found that LC3-II content was significantly increased, suggesting autophagosome appearance (Vendelbo et al., 2014). Although we did not measure mTORC1 in the present study, several others have demonstrated resistance exercise combined with protein supplementation is a potent mTORC1 activator (Apro & Blomstrand, 2010; Areta et al., 2013; Churchward-Venne, Breen, et al., 2014). Thus, in the case of the significantly decreased LC3-II level in the membrane fraction in the elderly and frail elderly, and a significant increase in the pooled LC3-I, we could speculate that a reduced conversion of LC3-I to LC3-II occurred due to mTORC1 activation. Given this scenario, a greater mTORC1 activation might explain why the frail elderly participants who performed resistance exercise, the RE+PRO and not the PRO group, demonstrated this nonsignificant increase in LC3-I. However, due to a small sample size in RE+PRO (n=8) and PRO (n=4), it is possible our null finding is attributable to our study being underpowered, resulting in a type-II error. Nevertheless, even though LC3 is degraded by autophagy, the total levels of LC3 do not essentially change in a predictable way. There may be increases in the conversion of LC3-I to LC3-II, for instance upon induction of autophagy. If the lysosomal turnover is particularly rapid, the LC3-II/I ratio might decrease despite an increased LC3-I to LC3-II conversion (Klionsky et al., 2016). However, given that the LC3-I protein pointed towards an increase and the production of new LC3-I proteins takes longer than 2 hr, this is unlikely what we observe in our data.

The LC3 protein can be degraded by the 20S proteasome, but it can also convert to LC3-T which is equivalent in size to LC3-II on SDS-PAGE (Gao et al., 2010). This similarity could also create an unreliable indicator of the amount of LC3-II in our analyses, as our LC3-antibody may have some cross-reactivity with other LC3 isoforms. As the LC3 undertakes considerable transcriptional and posttranscriptional modifications and to ensure a precise interpretation of LC3 protein levels; it is also necessary to monitor the mRNA levels. The LC3-II protein will be degraded and thus

decrease upon the autophagosomal-lysosomal fusion. Therefore an increase in autophagy would require transcriptional control of LC3 to be replenished (Sandri, 2010c). Similar to the elderly group in our study, recent research showed reduced LC3-II without changes in LC3-I in humans vastus lateralis (Fry et al., 2013) and rat gastrocnemius muscles (Luo et al., 2013) suggesting attenuated autophagic flux after resistance exercise. Other studies on endurance exercise had concluded with an increased autophagy flux when the LC3-II level increased, and no significant changes in p62 were demonstrated (Jamart, Naslain, Gilson, & Francaux, 2013; Y. A. Kim, Kim, & Song, 2012). As the LC3-II assays may often not give complete information, and since some facets of LC3-II metabolism might be incompletely understood, it is a necessity to complement and evaluate other autophagy markers to get a better picture of the autophagic flux (Rubinsztein et al., 2009).

5.1.2 p62

In addition to LC3, to confirm whether the autophagy flux increased or decreased in response to resistance exercise and protein supplementation, we assessed the p62 protein levels since it can be used as a marker for autophagic flux under various conditions (Germain et al., 2011; Kovacs, Rez, Palfia, & Kovacs, 2000; I. Kwon, Lee, Cosio-Lima, Cho, & Yeom, 2015; Lira et al., 2013). In this present study, the level of p62 in the membrane fraction had a trend towards an increase in both elderly and frail elderly, with no change in the cytosolic fraction 2.5 h post resistance exercise and protein ingestion. However, when pooling all the groups, p62 membrane fraction was significantly increased. An impaired autophagy has shown to correlate with increased levels of p62 (Ichimura et al., 2008; Komatsu et al., 2007). Although temporary increases of p62 could might also reflect an increased autophagic flux in some specific situations (Klionsky et al., 2016). This increase or a trend towards an increase in p62 protein level has been shown by some (Fritzen et al., 2016; I. Kwon et al., 2017; Ulbricht et al., 2015), but not all earlier studies (Luo et al., 2013; Mejias-Pena et al., 2017).

Amino acid consumption has been shown to modulate mTORC1 activity (Jewell & Guan, 2013; Proud, 2007; Stipanuk, 2007). A recent study noted that amino acid-induced activation of mTOR is dependent on p62 (Duran et al., 2011). The p62 interacts

with Raptor, a subunit of the mTORC1 and the Rag GTPases, as a result promoting the formation and translocation of a molecular complex to the lysosomal membrane that relays the signal from amino acids to the mTORC1 pathway (Duran et al., 2011). It is conceivable that the p62-mTORC1 interaction could respond to amino acids to prevent excessive autophagy. The p62 levels might also function as a positive feedback for both autophagy and cell growth. We could speculate that low p62 levels would result in reduced mTORC1 activity, which would increase autophagy and thereby depress the p62 protein levels even further due to its breakdown during autophagic degradation processes (Komatsu, Kageyama, & Ichimura, 2012). On the other hand, high p62 levels would augment mTORC1 activity and reduce autophagy. This would result in a further increase in p62 levels, thereby sustaining cell growth (Moscat & Diaz-Meco, 2011). The latter could explain the pooled significant increase demonstrated in the p62 membrane fraction, which would indicate a reduced autophagic flux. However, it is unclear whether this elevation in p62 persists the hours following exercise. To date is not known how p62 levels correlate with autophagy initiation in vivo (Mizushima et al., 2010). Given that p62 is incorporated into the autophagosomes through direct binding to LC3 and that the autophagosomes accumulate upon autophagy induction (Mizushima et al., 2010), it may be plausible that p62 correspondingly increases with autophagosome formation. As it appears the measurement of p62 correlates well with other parameters of autophagic flux (Mizushima et al., 2010), it is thinkable that a later biopsy time point than 2.5 h postexercise would reveal a decline in p62 and due to its degradation through autophagy (Bjorkoy et al., 2005). However, during 2h of starvation decreases the level of p62 in wild-type mouse embryonic fibroblasts (MEFs) (Kuma et al., 2004). This inevitably begs the question if the autophagic induction time frame is the similar in adult humans. Although proper timeframe studies in humans are lacking, autophagic activity in rat cardiac muscle after endurance exercise peak within 2 h postexercise and start to decline after 3-4 h post exercise, with no changes in the level of p62 post exercise (Ogura et al., 2011). However, it is important to remember that the expression level of p62 can also change independent of autophagy (Kuusisto, Suuronen, & Salminen, 2001; Nakaso et al., 2004), and thus requires evaluation with additional proteins.

Luo et al. (2013) using tail-weighed resistance exercise over 9 weeks showed both reduced LC3-II/I ratio and p62 protein amounts in aged rats, measured 48 h after the last training session (Luo et al., 2013). Unlike hypertrophy signaling responses, the autophagic response may have a much shorter duration (Ogura et al., 2011), thus tissue sampling 48 h postexercise might overlook possible earlier changes. Mejias-Pena et al. (2017) also suggested that 8 weeks resistance training in healthy older adults induced a significant decrease in LC3-II/I ratio and reduced expression of p62 in older human subjects, which they indicated as an increase in autophagy activity (Mejias-Pena et al., 2017). Nevertheless, there are some major limitations surrounding this study. First, they did not collect muscle biopsies but rather analyzed venus blood samples and second, the samples were collected 5-6 days before and after the training period. However, autophagic activity is not always inversely related to the p62 content (Moller et al., 2015), and a snapshot of the intricate processes of autophagy at different time points and exercise durations, acute resistance exercise bout vs 8-9 weeks of training, and method of measurement, could explain some of the differences. Contrastingly, a study by Fritzen et al. (2016) observed a decline in LC3-II/I ratio with an elevated p62 4h postexercise after a 3 weeks training period, which also was supported by changes in gene expression (Fritzen et al., 2016). The decrease they noted in LC3-II/I ratio with training was primarily induced by an increased LC3-I protein abundance. Although the LC3-I was significantly increased when we pooled all the groups, it was mainly the significant LC3-II reduction that drove our results. Therefore it could be conceivable that resistance exercise upregulates the autophagic flux over the course of several weeks of training. Fritzen et al. (2016) also noticed LC3-I and p62 protein content increased in both legs after unilateral training, implying the release of a systemic factor during exercise training might be the underlying signal.

Luo et al. (2013) found that mTOR^{Ser2448} phosphorylation was significantly reduced after 9 weeks of resistance exercise, signifying that the inhibition of mTOR signaling may lead the activation of autophagic pathway (Luo et al., 2013). Performing resistance exercise even in the absence of protein consumption can also result in the cellular uptake of amino acids (Biolo et al., 1995). Accordingly, this could contribute to a greater positive feedback between the p62-mTORC1 interaction in the RE+PRO and not the PRO group for p62 membrane fraction, thus preventing autophagy further. These

results must be interpreted with caution since the sample was relatively small (encompassing 8 subjects for RE+PRO and 4 subjects for PRO), affording poor statistical power with a high chance or type-II errors. Nevertheless, the level of cystolic p62 remained unchanged 2-2.5 h after resistance exercise and protein supplementation in every group. Importantly, several studies argue that p62 protein content is inadequate as a marker of lysosomal degradation of autophagosomes (Barth, Glick, & Macleod, 2010; Mizushima & Yoshimori, 2007; Rubinsztein et al., 2009). Thus far it is not clear whether p62 is degraded solely through autophagy or partially through the ubiquitin-proteasome pathway(Mizushima et al., 2010). We do not exclude the possibility we might overlook probable changes that occur in a time dependent manner in the autophagy-inductive processes. For this reason, it is recommended that future studies examining resistance exercise-induced autophagy adaptation should consider a proper time frame that precisely displays autophagic responses. Moreover, since p62 can also be transcriptionally regulated during autophagy (He & Klionsky, 2009; Nakaso et al., 2004) it may complicate the interpretation of p62. Taken together, this signifies that to enable interpretation of the autophagy response, p62 requires evaluation with additional proteins and data at the mRNA level.

5.1.3 FoxO3a

Autophagy can be controlled at the posttranslational level, but transcriptional mechanisms also contributes to the regulation of autophagy (Sandri, 2010c). Akt and AMPK play key roles as antagonist regulators in the transcriptional modulation of autophagy of the downstream substrate, FoxO3a (Mammucari et al., 2007; Sanchez et al., 2012). FoxO has been shown to regulate both of the major protein breakdown systems in skeletal muscle, the ubiquitin-proteasome and the autophagy-lysosome pathways (Mammucari et al., 2007; Sandri et al., 2004; Stitt et al., 2004). Our results demonstrated that the level of FoxO3a in the cytosolic fraction was significantly reduced in young and frail elderly groups in response to resistance exercise and protein supplementation. Due to the small amount of participants, we observed no change in the E group (n = 4), which could entail a type-II error.

In the absence of growth factor signaling or upon cellular stress, FoxO translocates into the nucleus and activates FoxO-dependent gene expression. Additionally, FoxO3 has

been shown to suppress Raptor to lower mTORC1 activity (Chen et al., 2010; Martins et al., 2016; Morris, 2005). Following resistance exercise, a reduction in the phosphorylation of FoxO3a has been observed in both younger and older participants (Fry et al., 2013; Williamson, Raue, Slivka, & Trappe, 2010). Furthermore, the decrease of cytosolic FoxO3a observed in our study could indicate increased nuclear translocation (Williamson et al., 2010), and possibly reflect the induction of transcriptional upregulation of several autophagy-related genes including LC3, p62/SQSTM1 & Atg4 (Mammucari et al., 2007; Zhao et al., 2007). Williamson et al. (2010) noted that older women were unable to activate nuclear FoxO3a to the same degree as the young women after resistance exercise. Also, older subjects had a lower cytosolic FoxO3 phosphorylation and a higher total nuclear FoxO3 level compared to younger subjects at baseline (Williamson et al., 2010). Collectively our observation of reduced cytosolic FoxO3a might reflect increased nuclear translocation, potentially upregulating atrophy-related gene expression (Sandri et al., 2004; Stitt et al., 2004). Although without measuring the protein level of FoxO3a in the nuclear fraction or mRNA data, this remains just mere speculation, as a potential nuclear FoxO3 increase does not necessarily lead to increased gene expression (Williamson et al., 2010).

Only a few studies have studied FoxOs role in exercise models (Dickinson et al., 2017; Fry et al., 2013; Glynn et al., 2010; E. Louis, Raue, Yang, Jemiolo, & Trappe, 2007; Raue, Slivka, Jemiolo, Hollon, & Trappe, 2007; Stefanetti et al., 2014; Williamson et al., 2010). Glynn et al. (2010) noted that the phosphorylation of FoxO3 was unaltered at 1 h following resistance exercise (Glynn et al., 2010). Dickinson et al. (2017), however, observed a significant increase in cytosolic FoxO3a in 2 h postexercise with 3.5 g leucine supplementation in elderly participants, the opposite of our results (Dickinson et al., 2017). The capability of protein to stimulate MPS also appears to be blunted in older adults (Moore et al., 2015). Likewise, to maximally stimulate MPS in older adults required the ingestion of 35 and 40 g of protein at rest (Pennings et al., 2012) and after resistance exercise (Y. Yang et al., 2012), compared with only 20 g in young individuals (Witard et al., 2014). However, our participants received a supplement consisting of either 18.9 g protein (young & elderly groups) or 16.8 g protein (frail elderly group). Since the young and elderly groups performed a whole-body resistance exercise bout, which potentially requires additional protein to maximally stimulate MPS

(Macnaughton et al., 2016), the protein dose our participants consumed may not have been sufficient to stimulate Akt enough to suppress FoxO3a translocation into to the nucleus. Luo et al. (2013) demonstrated FoxO3a activation through resistance exercise training, which might contribute to the activation of autophagy (Luo et al., 2013). Moreover, Fry et al. (2013) noted a significant increase in the phosphorylation of Akt with a significant decrease in FoxO3a phosphorylation. Thus, indicating that the postexercise activation of Akt was inadequate to induce phosphorylation in the downstream effector FoxO3a (Fry et al., 2013). This is also in agreement with Phillips et al. (1997) who demonstrated a 31 % increase in the fractional breakdown rate of skeletal muscle proteins 3 h postexercise, with both MPS and MPB being elevated hours and days after resistance exercise (S. M. Phillips et al., 1997). Several studies have reported elevation of MuRF1 mRNA in both younger and older participants 3-6 h post resistance exercise (Fry et al., 2013; Raue et al., 2007; Stefanetti et al., 2014) and Atrogin-1 mRNA in younger (Deldicque et al., 2008c; Stefanetti et al., 2015) and older subjects (Raue et al., 2007), which are under FoxO control (Sandri et al., 2004; Waddell et al., 2008). Glynn et al. (2010) found that MuRF1 mRNA and protein levels were significantly elevated after resistance exercise and remained elevated following protein intake (Glynn et al., 2010), suggesting resistance exercise is the main driver behind the reduction in cytosolic FoxO3a shown in our results. However, our study did not detect any differences between RE+PRO and PRO group, which could entail a type-II error due to the sample being underpowered.

5.2 Baseline Characteristics

The frail elderly group in our study weighed significantly less and had significantly lower total lean body mass and leg lean body mass compared to young and elderly, whereas no between-group differences in BMI and body fat % were shown. After the age of 60, the loss of skeletal muscle mass might escalate to a rate of 15% per decade, (Coker & Wolfe, 2012; Fielding et al., 2011; Malafarina, Uriz-Otano, Iniesta, & Gil-Guerrero, 2013), resulting in most individuals 70-80 years old only possess 60-80 % of the muscle mass they had at ~30 years old, decreasing to <50% after 80 years of age (Witard, McGlory, Hamilton, & Phillips, 2016). The frail elderly group appears to almost fit this pattern. Nevertheless, our results diverge from this as no differences were detected between young and elderly group for either total body mass or lean leg mass.

The elderly group was healthy and physically active which might explain why no differences in muscle mass were apparent between these groups. However, a 3kg difference in lean body mass between the young and elderly was observed, implying our statistical power was too low and not sensitive enough to detect small differences. Disturbingly, the trajectory of strength loss is even faster, with declines of 3-4% in men and 2.5-3% in women, annually (Goodpaster et al., 2006). We noted that the young participants had a significantly higher IMVC compared to frail elderly. This decreased strength cannot solely be attributed to the significantly reduced lean mass of the frail elderly participants (Hughes et al., 2001). Because both elderly and frail elderly also demonstrated a significantly lower relative strength compared to the young. Several researchers have noted changes in muscle fiber quality, force corrected for size, with age (Delbono, 2011; Frontera, Hughes, et al., 2000; Frontera, Suh, et al., 2000; Kostek & Delmonico, 2011). As older men have more fat in the muscle compartments of the thigh compared to their younger counterpart (Overend, Cunningham, Paterson, & Lefcoe, 1992). This excessive lipid infiltration in skeletal muscles is associated with low muscle strength and poor physical performance (Hilton, Tuttle, Bohnert, Mueller, & Sinacore, 2008; Visser et al., 2005), which hinders the contractile ability of the muscle (Lauretani et al., 2006; Schrager et al., 2007). The DXA measurements performed in the present study lacks the sensitivity to identify muscle composition, and therefore cannot detect fat that infiltrates the muscle (Fuller et al., 1999). This would explain why we did not detect any differences in fat % between groups. Consequently, the lower relative muscle strength in the elderly and frail elderly groups, would possibly to some extent be explained by reductions in intrinsic force-generating capacity of skeletal muscle fibers (Ochala, Frontera, Dorer, Van Hoecke, & Krivickas, 2007; Russ, Grandy, Toma, & Ward, 2011; Yu, Hedstrom, Cristea, Dalen, & Larsson, 2007).

5.3 Limitations

To our knowledge, this is the first study to examine autophagy markers in skeletal muscle of frail elderly humans. The investigation of resistance exercise and protein supplementation on acute changes in autophagy-related markers and comparison of three different age groups could help us gain insight into how this stimulus affects the autophagy-lysosome system and if this response is age-dependent. However, the present study exhibits several limitations. First, while we did not observe any age-related

differences in our immunoblot results, the study did not include a sufficient number of participants. Some of our analyses included only four subjects, giving it a low statistical power, which could entail a high chance for type-II errors. Another limitation is the absence of several key upstream and downstream autophagy markers and mRNA data; this could give us a clearer picture of the autophagy response to resistance exercise and protein supplementation and unravel some of the underlying regulatory signals. Furthermore, the single post exercise biopsy provides only a limited snapshot 2.5 hours after the bout had ended. By adding additional post exercise biopsies, one preferably straight after exercise and another one a couple of hours later gives greater insight into the time course of change. A final major limitation is that the present study neglect to match the resistance exercise bout, protein intake, and time from protein supplementation to the muscle biopsy between all groups. As such, it's not possible to ascertain whether our outcomes were influenced by resistance exercise and protein intake in general, or simply by the differences in the protocol.

5.4 Conclusion

The purpose of this thesis was to examine markers of protein degradation via the autophagy-lysosome system in young, healthy elderly and frail elderly. More specifically, we measured autophagy-related markers, LC3, p62 and FoxO3a, and investigated how they respond to resistance exercise and protein supplementation. The LC3-II/I ratio had a tendency towards, or was significantly reduced in all groups, which was mainly driven by a decline in LC3-II. We observed a significant decrease in the cytosolic FoxO3a fraction in both young and elderly which might reflect an increased activation of skeletal muscle protein degradation systems. Merging all groups revealed an increased p62 and LC3-I at the membrane fraction level, indicating a reduced autophagosome formation and degradation.

We hypothesized that protein supplementation and resistance exercise would result in an age-dependent difference in response with suppressed ratio of LC3-II/I but elevated p62, suggesting autophagy interruption with an attenuated autophagic flux in the frail elderly. The present study found no between-group differences for any of the proteins measured.

We also predicted that protein supplementation would inhibit the autophagy-lysosome system to a greater extent than resistance exercise and protein supplementation combined in the frail elderly. No differences were demonstrated in any autophagy-related marker between groups 2.5 hours after protein ingestion, although resistance exercise preceded protein ingestion in one of the groups.

More research is needed to elucidate the molecular mechanisms by which resistance exercise, nutrition, and aging interact to affect skeletal muscle breakdown and autophagy, and how these interactions play out in the long term and their functional outcomes.

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Overview of tables

Overview of figures

Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E binding protein 1
Akt/PKB	Protein kinase B
AMPK	Adenosine monophosphat-activated protein kinase
Atg	Autophagy-related gene or protein
Atrogin-1	muscle atrophy F-box
BMI	Body mass index
DXA	Dual X-ray absorptiometry
EEA	Essential amino acids
eEF2K	Eukaryotic elongation factor 2 kinase
eEF2B	Eukaryotic initiation factor 2B
eIF4A	Eukaryotic initiation factor 4A
eIF4E	Eukaryotic initiation factor 4E
eIF4F	Eukaryotic initiation factor 4F
eIF4G	Eukaryotic initiation factor 4G
FoxO3	Forkhead box O transcription factor 3
IMVC	Isometric maximum voluntary contraction
LBM	Lean body mass
LC3	Microtubule-associated protein light chain 3
LLM	Lean leg mass
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribo nuclei acid
mTOR	Mammalian/mechanistic target of rapamycin
MuRF1	Muscle specific RING finger 1
NFκB	Nuclear factor kappa B

P62/SQSTM1	Sequestosome 1
P70S6k	70-kD S6 protein kinase
PI3K	Phosphatidylinositol-3-kinase
RM	Repetition maximum
TSC1/2	Tuberous sclerosis 1/2
UPS	Ubiquitin-proteasome system
ULK1	Unc-51 like Autophagy Activating Kinase 1

Appendix 1

NORGES IDRETTSHØGSKOLE

- FORESPØRSEL OM DELTAKELSE I FORSKNINGSPROSJEKTET

STYRKETRENING FOR ELDRE MED LAVT FUNKSJONSNIVÅ

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 din omsorgsbolig.

- 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 to enheter Tine Styrk (0.33 ml) 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/omsorgsboligen

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 du skal 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, og du skal møte fastende. 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. Tas fastende.
    - *Frokost*
- *Kostholdsintervju*
- *Ultralyd:* For å måle tverrsnitt og arkitektur av *m. quadriceps femoris*, en muskelgruppe på fremsiden av låret.
- *Muskelfunksjonstest:* Gir et mål på styrke og eksplosivitet i musklene som strekker kneleddet.
- *Voluntær muskelaktivering:* For å undersøke i hvor stor grad du greier å aktivere muskulaturen når du tar i alt du kan.
- *1RM:* Maksimal styrke i øvelsene beinpress og kneekstensjon.
- *Funksjonelle tester:* For å teste hvor raskt du kan reise seg fra en stol fem ganger på rad, samt hvor raskt du kan gå opp en trapp. Gir informasjon om funksjon i hverdagen og mobilitet. I tillegg måler vi vanlig og maksimal ganghastighet.

Testdag 2 gjennomføres andre gang du møter på Norges idrettshøgskole, og denne gangen trenger du ikke møte fastende. Denne dagen skal du gjennomføre de samme testene som du gjennomførte testdag 1, unntatt DXA. 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 2,5 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. Også 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 testdagen, som tar 4-5 timer å gjennomføre. Vi vil bistå med transport til og fra Norges idrettshøgskole.

- Blodprøve (fastende)
- Standardisert frokost (havregrøt)
- Muskelbiopsi fra ytre lårmuskel
- Styrketreningsøkt med øvelsene beinpress og 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 Currato røntgen

I tillegg til testdagene på Norges idrettshøgskole, skal du gjennomføre en CT-undersøkelse ved Currato 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 dobbeltmerket vann (^2H_2O), og forutsetter en ekstra muskelbiopsi mot slutten av intervensjonsperioden. Tre uker før intervensjonsperioden starter, skal du drikke en bestemt mengde dobbeltmerket vann (ca. 250 mL) utblandet i vanlig vann (ca. 100 mL). 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 (knestrekk) 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 Currato 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. Denne treningen vil foregå i perioden januar-april i 2017. Du vil med andre ord få treningsoppfølging uansett hvilken gruppe du trekkes til, men du må vente til januar 2017 hvis du trekkes inn i 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, og 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 noe 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.

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

Selve treningen skal gjennomføres med forholdsvis stor belastning, og vil medføre en viss risiko for skade og følelse av sårhet/stølhet 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.a.nyvik@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 avidentifiserte 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, [sett inn saksnr. hos REK (20xx/yyy)].

• 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 avidentifisert 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 2

Aldring og helse
Nasjonal kompetansetjeneste

Helsedirektoratet

NORSK REVIDERT MINI MENTAL STATUS EVALUERING (MMSE-NR2)

Carsten Strobel & Knut Engedal, 2014

Pasient (PAS): _____ Fødselsdato/alder: _____

Nasjonalitet/morsmål/tolk: _____ Høyre-/venstrehendt: _____

Utdanning: _____ Antall år: ____ Yrke: _____

Hørsel/høreapparat: _____ Syn/briller: _____ Geriatrisk leseprøve: _____

Testleder (TL): _____ Dato: _____ Klokken: _____

Teststed/hjemmebesøk: _____ Er PAS testet med MMSE-NR samme sted tidligere? Ja ☐ Nei ☐

Hvis ja, når? _____ Når/hvor ble PAS sist testet med MMSE-NR (oppgavesett)? _____

MMSE-NR er ikke en demenstest, kun et grovt kognitivt funksjonsmål som supplerer annen utredning som somatisk undersøkelse (inkl. medikamentgjennomgang) og komparentintervju (inkl. forløp/varighet av kognitiv svikt og endret ADL-funksjon). Alle som administrerer MMSE-NR bør ha opplæring og god kjennskap til manual (lastes ned fra www.aldringoghelse.no). Følg standardisert instruksjon, ikke gi ledetråder, se retningslinjer for administrasjon, oppfølgende spørsmål og skåring på skjema og i manual. Ved lav norskspråklig kompetanse og annet morsmål enn norsk bruk fagutdannet tolk, ikke slektninger/bekjente. For oppgave 16 og 18, bruk standardiserte oversettelser og stimuliark der disse foreligger.

Instruksjon

Utfør testing en-til-en, uten pårørende til stede. Unngå at PAS ser skjema og skåring, bruk f.eks. skriveunderlag med klemme. Les fet skrift (**bold**) høyt, tydelig og langsomt. Pause (markert: [*pause*]) skal vare 1 sekund. Samtlige spørsmål skal stilles, også om PAS har besvart oppgaveledd under tidligere stilte spørsmål. Instruksjon kan gjentas, unntatt på oppgave 12 og 17 hvor det er svært viktig at instruksjon kun gis én gang. Skriv ordrett ned svar på hvert spørsmål. PAS kan korrigere svar underveis, gi derfor ikke tilbakemelding om svar er rett eller galt.

Ved retesting skift alltid oppgavesett som angitt på oppgave 11,12 og 13 for å redusere læringseffekt. Sett kryss i ruten for «0» ved feil svar og i ruten for «1» ved rett svar, gi aldri ½ poeng. Totalskåre regnes alltid fra 30 poeng: Er PAS ikke testbar på en oppgave pga. ikke-kognitive handikapp, angi hvorfor og sett ring rundt ruten for «0». Gir PAS utrykk for ikke å klare en oppgave, oppfordre likevel til å gjøre et forsøk. Er du usikker på hvordan et svar skåres etter å ha sjekket manual, rådfør deg med en erfaren kollega. Lavere alder og høyere utdanning gir ofte bedre skåre. Likeså testing på hjemmebesøk/vante omgivelser pga. stedsorienteringsledd. Lav motivasjon, dårlig dagsform, trettbarhet, afasi, lese- og skrivevansker, redusert syn og hørsel, depresjon, testangst, legemiddeleffekter (bivirkninger/interaksjoner), akutt somatisk sykdom, lav norskspråklig kompetanse, stress og liten testledererfaring kan påvirke resultat negativt. Totalskåre sier lite om spesifikke kognitive sviktområder som kan være diagnostisk og klinisk relevante, presisér derfor alltid utfall. Skåringsprofil og kvalitativ vurdering av utførelse kan også gi informasjon om kognitive restressurser og kompenserende mestringsstrategier som kan gi innspill til hvordan tilrettelegge aktivitet og samhandling. Bemerk påfallende forhold som lang tidsbruk, usikkerhet, mange korrigeringer, behov for gjentakelse av instruksjon, årsaker til testavbrudd e.l.

Skåring MMSE-NR2. Oppgavesett (ordsett/starttall oppgave 11, 12 og 13) administrert i dag: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐

		KOMMENTARER TIL SPESIFIKKE OPPGAVELEDD:	
Tidsorientering	*(oppgave 1–5)*		**/5**
Stedsorientering	*(oppgave 6–10)*		**/5**
Umiddelbar gjenkalling	*(oppgave 11)*		**/3**
Hoderegning	*(oppgave 12)*		**/5**
Utsatt gjenkalling	*(oppgave 13)*		**/3**
Språk og praksis	*(oppgave 14–19)*		**/8**
Figurkopiering	*(oppgave 20)*		**/1**
Total poengskåre			**/30**

Versjon 2, 18.05.2014

Vurderer du som testleder (TL) at samarbeid/motivasjon/testinnsats var uten anmerkning? Ja ☐ Nei ☐ Usikker ☐

Vurderer du at oppmerksomhet/bevissthetsnivå/våkenhet var uten anmerkning? Ja ☐ Nei ☐ Usikker ☐

Vurderes ikke resultat som valid/gyldig, angi årsak(er): _____

Spesielt å bemerke (henvisningsgrunn, medikamenter som kan påvirke kognitiv funksjon, atferd, dagsform, stemningsleie, smerter, afasi, ikke-kognitive handikapp, bruk av ikke-dominant hånd f.eks. ved lammelse, tidsbruk, vansker på distraksjonsbetingelsen, glemt briller/høreapparat e.l.):

_____

_____

_____

Basert på: Folstein, M.F., Folstein, S.E., & McHugh, P.R. (1975). "Mini-Mental State": A practical method for grading the cognitive state of patients for the clinician. Journal of Psychiatric Research, 12, 189-198.
Engedal, K., Haugen, PK., Gilje, K., & Laake, P. (1988). Efficacy of short mental tests in the detection of mental impairment in old age. Compr Gerontol A, 2, 87-93.
Strobel, C., & Engedal, K. (2008). MMSE-NR. Norsk Revidert Mini Mental Status Evaluering. Revidert og utvidet manual. Oslo. Nasjonalt kompetansesenter for aldring og helse.
Palmquist, S., Terzis, B., Strobel, C., & Wallin, A. (2012). Mini Mental State Examination, Svensk Revidering (MMSE-SR). Svensk Förening för Kognitiva sjukdomar.

Start med introduksjonsspørsmålet: **Synes du hukommelsen har blitt dårligere siste år?** Ja ☐ Nei ☐ Usikker ☐
Jeg skal nå stille deg noen spørsmål vi bruker bl.a. for å undersøke hukommelsen. Svar så godt du kan.

TIDSORIENTERING

Det er TL sitt ansvar å forhindre at PAS kan ta i bruk ledetråder: Se ut av vindu (årstid, måned), bruke kalender, avis, innkallingsbrev (årstall, måned, ukedag, dato), sjekke dato på klokke, mobil e.l.

1. **Hvilket årstall har vi nå?** (Kun fullt årstall med 4 sifre gir poeng) _____ 0☐ 1☐
2. **Hvilken årstid har vi nå?** (Ta hensyn til vær og geografiske forhold) _____ 0☐ 1☐
3. **Hvilken måned har vi nå?** (Kun rett navn på måned gir poeng, ikke nummer på måned) _____ 0☐ 1☐
4. **Hvilken dag har vi i dag?** (Kun rett navn på ukedag gir poeng) _____ 0☐ 1☐
5. **Hvilken dato har vi i dag?** (Unngå følgefeil: Kun dagsledd må være rett, måned/år kan være feil) _____ 0☐ 1☐

STEDSORIENTERING

Bruk best egnet stedsord og spørsmålsstilling, sett ring rundt valgt alternativ. Landsdel* skal kun benyttes ved testing i Oslo.

6. **Hvilket land er vi i nå?** _____ 0☐ 1☐
7. **Hvilket (fylke/landsdel*) er vi i nå?** (For landsdel gi poeng for Østlandet og Sør-Norge) _____ 0☐ 1☐
8. **Hvilken (by/tettsted/kommune) er vi i nå?** _____ 0☐ 1☐
9. **Hva heter dette (stedet/sykehuset/sykehjemmet/legekontoret e.l.)?** Eller **Hvor er vi nå?** _____ 0☐ 1☐
10. **I hvilken etasje er vi nå?** (Still spørsmål selv der bygg kun har én etasje. Ta hensyn til språk/kultur) _____ 0☐ 1☐

Unngå at PAS kan se ut av vindu (sted, etasje). Avhengig av inngang vil bygg i skrånende terreng kunne oppfattes å ha ulik etasje-angivelse for samme etasje. Gi poeng om PAS i tråd med språk/kultur benevner norsk 1. etasje som grunnplan (f.eks. Erdgeschoss, ground floor, stuen) og norsk 2. etasje som 1. etasje (1. Stock/Etage, first floor, 1. sal). Ved testing på hjemmebesøk, se manual.

UMIDDELBAR GJENKALLING

Bruk alltid nytt ordsett som angitt ved retesting for å hindre læringseffekt fra tidligere administrasjon. Sett ring rundt dagens ordsett. Ved 1. adm. bruk oppgavesett 1, ved 2. adm. bruk sett 2 osv., ved 6. adm. bruk sett 1, ved 7. adm. bruk sett 2 osv.

11. **Hør godt etter. Jeg vil si 3 ord som du skal gjenta etter meg. Disse skal du også prøve å huske, for jeg kommer til å spørre deg om dem litt senere. Er du klar?**
Nå kommer ordene: ..... [*pause*], **.....** [*pause*], **.....** [*pause*]**. Nå kan du gjenta disse ordene.**
Gjentar ikke PAS alle 3 ord, repeteres hele ordsettet inntil alle 3 ord gjengis i samme forsøk, opptil 3 presentasjoner. Gi *kun* poeng for riktige ord etter 1. presentasjon, rekkefølge PAS sier ordene er uten betydning. Antall presentasjoner: _____ stk.

Oppgavesett:	1	2	3	4	5		
Nå kommer ordene: ...	**HUS**	**STOL**	**SAFT**	**KATT**	**FLY**	_____	0☐ 1☐
	KANIN	**BANAN**	**LAMPE**	**AVIS**	**EPLE**	_____	0☐ 1☐
	TOG	**NÅL**	**BÅT**	**LØK**	**SKO**	_____	0☐ 1☐

Etter 3 gjenkalte ord eller 3 presentasjoner, si: **Husk disse ordene, for jeg vil spørre deg om hvilke de er litt senere.**

HODEREGNING (Bruk alltid obligatorisk distraksjonsbetingelse i tillegg)

Bruk alltid nytt starttall som angitt ved retesting. Ved 6. adm. bruk oppgavesett 1 osv. Sett ring rundt dagens starttall, skriv ned tallsvar. Unngå følgefeil: Gi poeng når svar er minus 7 fra forrige tall, uavhengig av om forrige tallsvar var rett eller galt.

12. **Nå vil jeg at du trekker 7 fra .....** [*Gir ikke PAS tallsvar, si:* **Hva er ..... minus 7?**] [*Rett etter tallsvar, si*]: **Og så fortsetter du å trekke 7 fra tallet du kom frem til, helt til jeg sier stopp.** [*Instruksjon gis kun én gang. Ikke informer underveis om subtraksjonstall eller hvor langt PAS har kommet*]. Ved færre enn 5 tallsvar, gå til distraksjonsbetingelsen.

Oppgavesett:	1	2	3	4	5		
Starttall: **Nå vil jeg at du trekker 7 fra ...**	**80**	**50**	**90**	**40**	**60**		
Og så fortsetter du å trekke 7 fra tallet du kom frem til, helt til jeg sier stopp ⟶	73	43	83	33	53	_____	0☐ 1☐
	66	36	76	26	46	_____	0☐ 1☐
Ved behov si: **Og så videre**	59	29	69	19	39	_____	0☐ 1☐
Ved behov si: **Og så videre**	52	22	62	12	32	_____	0☐ 1☐
Ved behov si: **Og så videre**	45	15	55	5	25	_____	0☐ 1☐

Etter 5 subtraksjoner, si: **Fint, det holder** [Gå til distraksjonsbetingelsen].

Obligatorisk distraksjonsbetingelse – OBS, er ikke poenggivende!
Bruk alltid distraksjonsbetingelsen for å sikre tilstrekkelig tidsopphold med distraksjon. Dette for å fremme reell kartlegging av langtidshukommelse fremfor arbeidshukommelse på oppgave 13. Be PAS telle baklengs fra 100 ca. 30 sekunder med følgende instruksjon: **Nå vil jeg at du teller baklengs fra 100 på denne måten: 99, 98, 97..., helt til jeg sier stopp. Vær så god!** [*Etter ca. 30 sek. si:*] **Fint, det holder.**

UTSATT GJENKALLING

13. **Hvilke 3 ord var det jeg ba deg om å huske?** [*Ikke gi ledetråder/stikkordshjelp, sett ring rundt dagens ordsett*]

Oppgavesett:	1	2	3	4	5		
	HUS	STOL	SAFT	KATT	FLY	_____	0☐ 1☐
	KANIN	BANAN	LAMPE	AVIS	EPLE	_____	0☐ 1☐
	TOG	NÅL	BÅT	LØK	SKO	_____	0☐ 1☐

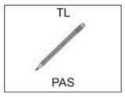
Nevnes mer enn 3 ord, må PAS velge hvilke 3 ord som skal være svaret, rekkefølge er uten betydning. Gi *kun* poeng for dagens ordsett og eksakt gjengivelse, dvs. bolighus, hytte, kaninen, kaniner, hare, togbane, lokomotiv e.l. gir ikke poeng.

BENEVNING

14. **Hva heter dette?** [*Vis stimuliarket riktig vei og pek på blyanten*] _____ 0☐ 1☐

15. **Hva heter dette?** [*Vis stimuliarket riktig vei og pek på armbåndsuret*] _____ 0☐ 1☐

Alternative poenggivende svar: Penn, gråblyant, fargeblyant, ur, klokke, klokkerem e.l. Bruk kun stimuliarket i farger med blyant og armbåndsur, ikke andre objekter, gjelder også retesting. Eneste unntak er testing av sterkt synshemmete eller blinde, hvor stimuliobjektene blyant og armbåndsur kan presenteres taktilt med konkreter.



FRASEREPETISJON

16. **Gjenta *ordrett* det jeg sier. Er du klar?** [*Si tydelig*]: **«ALDRI ANNET ENN OM OG MEN».**

Gi poeng når hele frasen gjengis korrekt med alle 6 ord i riktig rekkefølge. Dialektvarianter godtas.

TL kan si frasen 3 ganger, men gi *kun* poeng etter 1. presentasjon. Antall presentasjoner: _____ stk.

ALDRI ANNET ENN OM OG MEN _____ 0☐ 1☐

3-LEDDET KOMMANDO

Legg et ubrukt A4-ark på bordet midt foran PAS, kortsiden mot PAS. For å unngå at PAS starter før hele instruksjonen er gitt, legg egen hånd på arket til all instruksjon er gitt. Gi poeng for hver korrekt utførte delhandling.

17. **Hør godt etter, for jeg skal be deg gjøre 3 ting i en bestemt rekkefølge. Er du klar?**
Ta arket med én hånd [*pause*]**, brett arket på midten kun én gang, med en eller begge hender** [*pause*]**, og gi arket til meg** [*pause*]**. Vær så god!** [*Instruksjon gis kun én gang, enkeltledd kan ikke repeteres*]

TAR ARKET MED KUN *EN* HÅND _____ 0☐ 1☐

BRETTER ARKET PÅ MIDTEN KUN *EN* GANG _____ 0☐ 1☐

GIR ARKET TIL TL (gi også poeng om arket legges på bordet tydelig foran TL) _____ 0☐ 1☐

LESNING

18. **Nå vil jeg at du gjør det som står på arket** [*Vis PAS teksten*]. PAS må lukke øynene for poeng. Lukker ikke PAS øynene, kan instruksjon gjentas 2 ganger til. Hver presentasjon gir mulighet for poeng. Antall presentasjoner: _____ stk.

LUKK ØYNENE DINE _____ 0☐ 1☐

SETNINGSGENERERING

Legg nedre del av neste side MMSE-NR skjema med kortsiden foran PAS, og gi vedkommende en blyant.

19. **Skriv en meningsfull setning her.** [*Pek på øvre del av neste side*] _____ 0☐ 1☐

Skrives imperativsetning med kun ett ord, f.eks. «Spis», si: **Skriv en lengre setning.** Skrives intet eller tidligere gitt setning/frase, f.eks.«Lukk øynene dine» eller «En meningsfull setning», si: **Skriv en setning du lager selv.**

Skriver ikke PAS noe nå heller, si: **Skriv om været.**

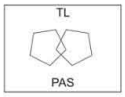
Setningen må gi mening, men trenger ikke ha objekt og tidvis heller ikke subjekt eller verb, se manualeksempler. Ignorer stave- og grammatikalske feil. Gi poeng ved rett utførelse etter supplerende instruksjon og for spørresetning, om kriterier ellers er innfridd.

FIGURKOPIERING

Legg figurarket som vist med figurspissene mot PAS over øvre del av neste side (over setningen PAS skrev), viskelær ved siden av (skal ikke brukes som linjal). PAS får ikke rotere eller flytte på figurarket som TL må sørge for at blir liggende til PAS er ferdig.

20. **Kopier figuren så nøyaktig du kan her.** [*Pek på nedre del av neste side*] _____ 0☐ 1☐
Du kan bruke viskelær. Ta deg god tid. Si fra når du er ferdig.

Er PAS misfornøyd med utførelse, oppfordre til å korrigere/tegne figuren på nytt, maks. 3 poenggivende forsøk. Gi poeng når to 5-kantede figurer former en 4-sidet figur der 5-kantene overlapper: 5-4-5. Rotert utførelse, størrelsesforskjell mellom 5-kantene eller hvor de overlapper er ikke avgjørende for skåring om kriterier ellers er innfridd, se skåringseksempler i manual. Er TL i tvil om utførelse er korrekt, be PAS tegne figuren på nytt.



Appendix 3

SCORING SPPB:

dd/mnd/år: ☐☐ ☐☐ ☐☐☐☐

ID/navn:

1. Score statisk balanse

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

1. ☐ Forsøkte, men ikke i stand til(0p)
2. ☐ Deltageren kunne ikke holde stillingen uten hjelp(0p)
3. ☐ Ikke forsøkt, tester følte det utrygg(0p)
4. ☐ Ikke forsøkt, deltager følte seg utrygg(0p)
5. ☐ Deltager tar ikke instruksjon(missing)
6. ☐ Annet (spesifiser)_____
7. ☐ 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 ☐

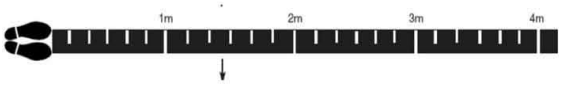
=

Sum poeng balanse: ☐

2. Score 4m gangtest

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

1. ☐ Forsøkte, men ikke i stand til(0p)
2. ☐ Deltageren kunne ikke gå uten assistanse(0p)
3. ☐ Ikke forsøkt, tester følte det utrygg(0p)
4. ☐ Ikke forsøkt, deltager følte seg utrygg(0p)
5. ☐ Deltager tar ikke instruksjon(missing)
6. ☐ Annet (spesifiser)_____
7. ☐ 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:

1. ☐ Forsøkte, men ikke i stand til(0p)
2. ☐ Deltageren kunne ikke reise seg uten hjelp(0p)
3. ☐ Ikke forsøkt, tester følte det utrygg(0p)
4. ☐ Ikke forsøkt, deltager følte seg utrygg(0p)
5. ☐ Deltager tar ikke instruksjon(missing)
6. ☐ Annet (spesifiser)_____
7. ☐ 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.: ☐

Registreringsark dd/mnd/år: ☐☐ ☐☐ ☐☐☐☐

ID/navn:

1. Balansetest

1.Samlede føtter 10 sekunder

↓

2.Semi-tandem 10 sekunder

↓

3.Tandem 10 sekunder

↓

Gå til gangtest

1. ☐☐.☐☐ sek

2. ☐☐.☐☐ sek

3. ☐☐.☐☐ sek

---

2. Gangtest

Ganghjelpemidler ved test (kryss av):

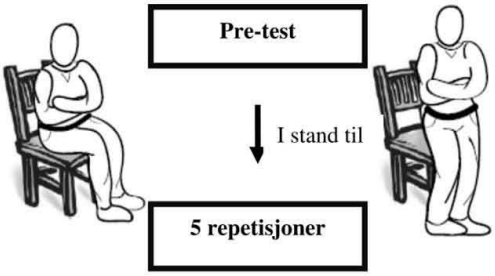
1. ☐ uten
2. ☐ krykke/stokk (er)
3. ☐ rollator
4. ☐ Annet (spesifiser)_____

Tid test 1: ☐☐.☐☐ sek

Tid test 2: ☐☐.☐☐ sek

---

3. Reise/ sette seg



Pre-test

↓ I stand til

5 repetisjoner

→ Avslutt

Ikke i stand til

Setehøyde ☐ cm

Tid 5 repetisjoner uten armbruk: ☐☐.☐☐ sek

Tester:

Appendix 4

Fried Frailty Criteria, modified

<table>
<tr><td>Shrinking,
i.e. weight loss</td><td colspan="4">Unintentional loss of at least 5% of the previous year's body weight</td></tr>
<tr><td rowspan="5">Weakness, i.e. low handgrip strength</td><td colspan="4">Grip strength of the dominant hand (mean of three measurements)</td></tr>
<tr><td>BMI/male</td><td>Cutoff (kg)</td><td>BMI/female</td><td>Cutoff (kg)</td></tr>
<tr><td>≤24</td><td>≤29</td><td>≤23</td><td>≤17</td></tr>
<tr><td>24-26</td><td>≤30</td><td>23-26</td><td>≤17.3</td></tr>
<tr><td>26-28
>28</td><td>≤30
≤32</td><td>26-29
>29</td><td>≤18
≤21</td></tr>
<tr><td>Poor endurance, i.e. self-reported exhaustion</td><td colspan="4">Evaluation of two statements of the CES-D scale:
a) I felt that everything I did was an effort
b) I could not get going
Criteria fulfilled if at least one condition is present for 3 days or more during the last week.</td></tr>
<tr><td rowspan="4">Slowness, i.e. low gait speed</td><td colspan="4">Cutoff for time to walk 4 meters (static start)</td></tr>
<tr><td>Height/male (cm)</td><td>Cutoffs (s)</td><td>Height/female (cm)</td><td>Cutoffs (s)</td></tr>
<tr><td>≤173</td><td>≥6.15 (0.65 m/s)</td><td>≤159</td><td>≥6.15 (0.65 m/s)</td></tr>
<tr><td>>173</td><td>≥5.25 (0.76 m/s)</td><td>>159</td><td>≥5.25 (0.76 m/s)</td></tr>
<tr><td>Low activity, i.e. reduced energy consumption</td><td colspan="4">Physical activity will be assessed during an interview. According to the level of leisure physical activity performed daily during the last year, participants will be assigned to one of the following categories.

1) completely inactive or performing light-intensity physical activity (i.e., walking, light housework) less than 1 hour per week;
2) light physical activity: light-intensity physical activity 2–4 hours per week;
3) moderate–high physical activity: light physical activity at least 5 hours per week or moderate physical activity (i.e., gymnastics, playing soccer, gardening) at least 1–2 hours per week

The low activity-criteria is fulfilled only for participants in category 1.</td></tr>
</table>