Skeletal Muscle Mass & Function in Older Women
Skeletal Muscle Mass & Function in Older Women
Health-Enhancing Influences of Combined Resistance Exercise & Diet
Abstract


Ageing is accompanied by a progressive decline in skeletal muscle mass and strength which may lead to impaired ability to perform activities of daily living in older adults. Although the exact cause of the gradual decline in muscle mass is unknown, identifying efficient strategies aiming to prevent age-related loss of muscle mass and strength is important in order to promote healthy ageing. The overall aim of this thesis was to explore the effects of resistance training alone or combined with a healthy diet on skeletal muscle mass and function of healthy recreationally active older women and to determine mechanisms by which elevated systemic inflammation may contribute to the age-related decline of muscle mass in older adults. The combination of resistance training and a healthy diet induced gains in leg lean mass as well as greater gains in dynamic explosive force than resistance training alone in healthy recreationally active older women. The observed gains in leg lean mass were accompanied by increases in the size of type IIA muscle fibres together with down-regulation in gene expression of a pro-inflammatory factor (IL-1β) and upregulation in gene expression of a regulator of cellular growth (mTOR) in skeletal muscle of older women. Additionally, reduced muscle protein synthesis and size of muscle cells may mediate the detrimental effects of elevated circulating markers of inflammation on muscle mass in older adults. In conclusion, the present thesis depicts mechanistic links between elevated systemic marker of inflammation and muscle mass and provides new information on the effects of combined resistance training and healthy diet on muscle mass and strength in a group of healthy recreationally active older women. This knowledge is instrumental for development of strategies aiming to prevent age-related loss of muscle mass and function.

Keywords: Healthy ageing, Chronic inflammation, C-reactive protein, Omega-3 fatty acids, Resistance training, Physical function

Emelie Strandberg, School of Health Sciences, Örebro University, SE-701 82, Sweden, emelie.strandberg@oru.se
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<th>Description</th>
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<tbody>
<tr>
<td>1RM</td>
<td>One-repetition maximum</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>4E-binding protein 1</td>
</tr>
<tr>
<td>5-STS</td>
<td>Five sit-to-stand</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>BIA</td>
<td>Bioelectrical impedance analysis</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CSA</td>
<td>Cross-sectional area</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterium oxide</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual-energy x-ray absorptiometry</td>
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<tr>
<td>E%</td>
<td>Energy percent</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>eIF4G</td>
<td>Eukaryotic translation initiation factor gamma 1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EPAQ2</td>
<td>EPIC-Norfolk physical activity questionnaire</td>
</tr>
<tr>
<td>FoxO</td>
<td>Forkhead box</td>
</tr>
<tr>
<td>FoxO1</td>
<td>Forkhead box 1</td>
</tr>
<tr>
<td>FSR</td>
<td>Fractional synthetic rate</td>
</tr>
<tr>
<td>GABARAP</td>
<td>GABA(A) receptor associated protein</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>ICCs</td>
<td>Intraclass correlation coefficients</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IsoRFD</td>
<td>Rate of force development during isometric contraction</td>
</tr>
<tr>
<td>LC3</td>
<td>Light chain 3</td>
</tr>
<tr>
<td>MAFbx</td>
<td>Muscle atrophy f-box/atrogin-1</td>
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</table>
MPB          Muscle protein breakdown
MPS          Muscle protein synthesis
mTOR         Mechanistic target of rapamycin
MSTN         Myostatin
MuRF1        Muscle RING finger 1/TRIM 63
MVPA         Moderate-to-vigorous physical activity
n-3          Omega-3
n-6          Omega-6
NF-κB        Nuclear factor kappa-light-chain-enhancer of activated B cells
NFκB1        Nuclear factor NF-kappa-B p105 subunit
NSAID        Non-steroidal anti-inflammatory drug
PI3K         Phosphatidylinositol-3-kinase
PL           Phospholipids
PUFAs        Poly unsaturated fatty acids
RCT          Randomized controlled trial
RelA         Nuclear factor NF-kappa-B p65 subunit
RFD          Rate of force development
S6K1         Ribosomal protein S6 kinase beta-1
SC           Satellite cell
sIL-6R       Soluble interleukin 6 receptor
SJ           Squat jump
sjMAX        Maximal ground reaction force during squat jump
sjRFD        Rate of force development during squat jump
SLS          Single-leg-stance
Smad2        SMAD family member 2
Smad3        SMAD family member 3
sTNF-RI      Soluble tumour necrosis factor alpha receptor 1
sTNF-RII     Soluble tumour necrosis factor alpha receptor 2
TGF β        Transforming growth factor beta
TLC          Thin-layer chromatography
TLR1         Toll-like receptor 1
TNF-α        Tumour necrosis factor alpha
TUG          Time-up-and-go
Introduction

By the year 2050 the proportion of the world’s population aged over 60 years is expected to double from 11% to 22% (WHO, 2014). The same trend is described for Europe where the proportion of people over 60 years will represent 34% (WHO, 2016). These demographic changes are driven by an increase in life expectancy, together with a decline in birth rate and infant mortality (Harper, 2014). Increased life expectancy is accompanied by increased disease risk including cancer, cardiovascular diseases, metabolic, musculoskeletal and neurological disorders (Niccoli and Partridge, 2012). This will have profound implications at the level of the healthcare systems (Beaudart et al., 2014). Therefore, an important challenge for modern societies is to develop strategies to delay onset of disease.

Ageing

Ageing is a complex, multifactorial process characterised by several biologically significant changes. An age-related functional decline occurs at the level of many organs and systems including musculoskeletal, cardio-respiratory, metabolic, hormonal, immune and endocrine. Another important aspect highlighted in older adults is an age-related altered ability of various physiological functions to adapt to external stimuli. This might contribute to tissue/organ dysfunction and systemic illness. Factors such as oxidative stress (Finkel and Holbrook, 2000), mitochondrial dysfunction (Lin and Beal, 2006), cellular senescence (Campisi and d'Adda di Fagagna, 2007) and telomere attrition (Benetos et al., 2001) are often addressed for their role in ageing. Low-grade chronic systemic inflammation has gained interest among the scientific community as several studies reported slight increases in levels of circulating inflammatory markers in older adults (Ferrucci et al., 2005). Chronic systemic inflammation has been linked to increased risk of disease such as cardiovascular diseases, type 2 diabetes mellitus, Alzheimer and Parkinson’s disease (Libby, 2006, Duncan et al., 2003, Holmes et al., 2009). Low-grade chronic inflammation has also been associated to loss of muscle mass and function with age (Visser et al., 2002, Schaap et al., 2009, Schaap et al., 2006).
Healthspan

The concept of healthspan was recently introduced in the field of ageing research (Seals and Melov, 2014, Kirkland and Peterson, 2009). As proposed by Seals et al. (2016), ageing can be regarded as a two-phase process, a phase of relative disease-free healthy ageing (healthspan) followed by a phase characterised by the occurrence of diseases and disabilities. Hence, the extension of healthspan is an important public health concern (Niccoli and Partridge, 2012, Seals and Melov, 2014). In this respect, regular physical activity has been identified as an important lifestyle factor associated with reduced risk of developing cardiovascular diseases, type 2 diabetes mellitus, osteoporosis, obesity, colon cancer, breast cancer, anxiety and depression (Nelson et al., 2007). Interestingly, despite the beneficial effects of regular physical activity, significant functional decline also occurs in apparently healthy and physically active older adults after the age of 50 years (Hughes et al., 2002). Therefore, there is a need to understand mechanisms underlying the age-related functional decline and to design non-pharmacological strategies able to delay loss of function.

Decline of skeletal muscle mass and function with ageing

Depending on gender and age, skeletal muscle mass represents 30 to 40% of the total mass of the human body (Janssen et al., 2000). Ageing is accompanied by a progressive loss in muscle mass and strength. It is generally accepted that muscle mass and strength are maintained throughout adulthood. However, from the middle of the 5th decade of life, annual rates of loss of approximately 1% of muscle mass and 3% of muscle strength occur (Janssen et al., 2000, Goodpaster et al., 2006). The accumulated loss of muscle mass and strength over several years may lead to sarcopenia (Rosenberg, 1997), which is defined as reduced muscle mass and strength and/or physical function (Cruz-Jentoft et al., 2010, Muscaritoli et al., 2010, Fielding et al., 2011, Studenski et al., 2014, Baumgartner et al., 1998). Interestingly, the loss of muscle mass has been shown to be greater in lower-limb muscle groups compared to upper-limb muscles (Janssen et al., 2000), indicating the need to specifically target lower limbs muscles in resistance training interventions. Previous epidemiological studies addressing the prevalence of sarcopenia reported no gender differences, higher prevalence in women or higher prevalence in men (reviewed in (Morley et al., 2014). For instance, low lean mass was present in 33 % of women and 10 % of males
in a cohort from Barcelona (Masanes et al., 2012) and the prevalence of sarcopenia has been recently shown to be higher in older women in comparison to older men (Dam et al., 2014). Although the relative loss of muscle mass and strength has been reported to be similar for men and women (Lindle et al., 1997) women’s susceptibility to develop sarcopenia at old age could been linked to lower skeletal muscle mass and strength compared to men throughout adulthood (Janssen et al., 2000, Skelton et al., 1994). As women can be at higher risk of developing sarcopenia, it is important to design specific strategies able to counteract the decline of muscle mass and function in older women.

The age-related annual loss in muscle strength exceeds the annual loss in muscle mass (Goodpaster et al., 2006) and is referred to as dynapenia (Clark and Manini, 2012). It was previously believed that the age-related atrophy of skeletal muscle fully accounted for poor muscle strength (Clark and Manini, 2012). However, it is currently acknowledged that muscle atrophy cannot fully account for the loss of muscle strength (Clark et al., 2006b, Clark et al., 2006a, Delmonico et al., 2009). Indeed, detrimental changes in nervous system including higher rate of denervation (Hepple and Rice, 2016), impaired motor nerve function (Ward et al., 2015) and loss of spinal motor neurons (Aagaard et al., 2010) have been suggested to contribute to loss of muscle strength.

The ability to rapidly generate muscle force (i.e. explosive capacity), is considered as a strong predictor of functional status in older adults (Foldvari et al., 2000). Indeed, several reports have highlighted a greater age-related decline in lower limb explosive capacity compared to maximal muscle strength (Hakkinen et al., 1996, Skelton et al., 1994, Izquierdo et al., 1999, Bassey et al., 1992). An age-related decline in lower-limb explosive capacity in older adults has been associated with increased fall risk and impaired ability to perform activities of daily living such as climbing stairs and rising from a chair (Skelton et al., 2002, Skelton et al., 1994).

**Mechanisms behind loss of muscle mass and function**

**Low-grade chronic systemic inflammation**
Low-grade chronic systemic inflammation, defined as elevated basal levels of acute-phase proteins and cytokines (Bruunsgaard and Pedersen, 2003), is currently considered as an important factor contributing to the development...
of chronic diseases. C-reactive protein (CRP), Interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) are common circulating inflammatory markers studied in older adults. CRP is an acute phase protein synthesised by hepatocytes, which main function is to recognize pathogens and damaged cells (Gabay and Kushner, 1999). IL-6 is a pleiotropic cytokine produced by many cell types, for example T-cells and macrophages, and exerts its pro- and anti-inflammatory properties through specific IL-6 receptors (Eklund, 2009). TNF-α exerts its pro-inflammatory action through two types of receptors, TNF-RI and TNF-RII and is produced predominantly by immune cells like macrophages and lymphocytes and also by endothelial and epithelial cells (Eklund, 2009). Associations between increases in circulating markers of inflammation and many age-related disorders and functional impairments have been reported (Tracy et al., 1997, Ridker, 2001, Ridker et al., 1998, Lindmark et al., 2001, Bruunsgaard et al., 2000, Cohen et al., 1997).

Elevations of pro-inflammatory cytokines are frequently reported in ageing populations (Singh and Newman, 2011, Ferrucci et al., 2005) and associations between chronic inflammation and reduced muscle mass and function have been elucidated in several studies (Visser et al., 2002, Schaap et al., 2009, Schaap et al., 2006). Very few studies have addressed the link between chronic systemic inflammation and muscle protein synthesis (MPS) and they yielded conflicting results. While systemic inflammation was associated with reduced MPS in two studies (Balage et al., 2010, Toth et al., 2005), there was no association between CRP level and protein metabolism in a third study (Buffiere et al., 2015). The influence of the systemic environment on muscle regenerative capacity has also been investigated. Using a parabiotic model in mice, the regenerative capacity of injured old skeletal muscle was improved when exposed to a youthful systemic environment (Conboy et al., 2005). Furthermore, reduced proliferative rate was observed in myogenic cells exposed to sera from older women with elevated CRP levels (Wahlin-Larsson et al., 2014). It has also been shown that the exposure of myogenic cells to sera from young can rejuvenate cell proliferative capacity (Conboy et al., 2005, Carlson et al., 2009, Barberi et al., 2013). These experiments suggest that changes in the systemic environment may have an influence on skeletal muscle function. It can be hypothesized that promoting an anti-inflammatory environment by pharmacological or non-pharmacological approaches might improve muscle function. Interestingly,
administration of non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to restore muscle protein synthesis in old rats (Rieu et al., 2009). Nevertheless, daily administration of anti-inflammatory medications might be questionable due to potential side effects. Therefore, future studies should focus on non-pharmacological strategies aiming to induce systemic changes and examine their impact on muscle function in older adults.

**Cellular alterations in skeletal muscle**

Human skeletal muscle is made of slow and fast muscle fibres. Slow twitch type I fibres are characterised by a high oxidative enzyme content, a dense capillary network and a high resistance to fatigue. Fast twitch fibres are subdivided into IIA and IIX fibres. Type IIX fibres are rich in glycolytic enzymes and are characterised by high and rapid force development. Type IIA fibres correspond to the fast oxidative glycolytic fibres with intermediate metabolic and mechanical properties between type I and IIX fibres (Schiaffino and Reggiani, 2011, Pette and Staron, 2000). Skeletal muscle fibres are multinucleated cells containing hundreds of myonuclei. It is commonly accepted that each myonucleus controls gene expression and protein synthesis over a limited area of cytoplasm, a concept named myonuclear domain (Cheek, 1985). At cellular level, though reported by very few studies, the loss of muscle fibres is suggested to contribute to age-related decline in muscle mass (Lexell et al., 1988). In contrast, a fibre-type specific decrease in cross-sectional area (CSA) of type II fibres is a more commonly reported finding in older adults (Nilwik et al., 2013). Currently, there is very little support in favour of an age-related loss of myonuclei (Gundersen and Bruusgaard, 2008, Kadi et al., 2004a). However, it is hypothesized that increased rate of apoptosis may drive the onset of age-related muscle loss (Marzetti and Leeuwenburgh, 2006).

**Apoptosis**

Programmed cell death or apoptosis is an important mechanism to maintain the integrity of proliferative tissues (Elmore, 2007). When cell death is triggered, different caspases are activated leading to cellular degradation and DNA fragmentation. Apoptosis is regulated by the balance between pro- and anti-apoptotic factors. The pro-apoptotic factors Bax (Bcl-2 associated X protein) and Bad (Bcl-2-associated death promoter) initiate the caspase cascade, whereas the anti-apoptotic factor Bcl-2 (B-cell lymphoma 2) pro-
motes cell survival (Zimmermann et al., 2001). In skeletal muscle, a multi-nucleated post-mitotic tissue, the role of apoptosis is less defined (Dupont-Versteegden, 2006). Accelerated rate of apoptosis has been mostly reported in animal models of muscular dystrophy (Sandri et al., 1995) and in extreme catabolic conditions (Marzetti et al., 2012). The role of apoptosis in age-related decline in muscle mass remains to be elucidated.

**Impaired regenerative capacity of skeletal muscle**

Satellite cells (SC) are mononucleated cells located between the sarcolemma and the basal lamina of muscle fibres and were first described by Mauro (Mauro, 1961). The activation and proliferation of SC give rise to daughter cells which can either generate new muscle fibres, provide new myonuclei to the parent fibre or return to a quiescent state (Hawke and Garry, 2001, Kadi and Thornell, 2000). Decreases in SC content predominantly in type II muscle fibres have been reported in older adults (Verdijk et al., 2007, Kadi et al., 2004a). More importantly, impaired activation and proliferation capacity of myogenic cells was reported in cells isolated from older compared to younger donors (Barani et al., 2003, Barberi et al., 2013, Conboy et al., 2003, Shefer et al., 2006, Brack and Rando, 2007) Moreover, it has been shown that myogenic cells isolated from older donors had a reduced capacity to repair myofibres and re-populate the myogenic cell reservoir compared to cells isolated from young donors (Cosgrove et al., 2014). Currently, it remains to determine whether the occurrence of age-related changes in intrinsic properties of myogenic cells may contribute to reduced regenerative potential (Brack and Rando, 2007).

**Impaired balance in muscle protein synthesis and breakdown**

Muscle mass is maintained through tightly regulated major processes; muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Phillips et al., 2012a). MPS and MPB are controlled by coordinated signalling pathways which activity is under control of hormonal and cytokine signals. A slight chronic imbalance between MPS and MPB may be responsible for gradual decline in muscle mass (Mosoni et al., 1999).

**Muscle protein synthesis**

Previous studies suggested that reduced basal protein synthesis rate in older adults may promote loss of muscle mass (Short et al., 2004, Welle et al., 1993, Welle et al., 1995). However, other studies failed to report reduced
basal levels of MPS rates (Cuthbertson et al., 2005, Volpi et al., 2001, Markofski et al., 2015). In fact, it is proposed that MPS response to anabolic stimuli such as exercise (Kumar et al., 2009, Koopman and van Loon, 2009) or protein ingestion (Wall et al., 2015) may be impaired in older adults. Currently, the occurrence of anabolic resistance, a condition describing impaired response to anabolic stimuli is suggested to cause loss of muscle mass in older adults (Burd et al., 2013).

MPS is known to be regulated by the PI3K (phosphatidylinositol-3-kinase) /Akt (protein kinase B) /mTOR (mechanistic target of rapamycin) signalling pathway (Bodine et al., 2001b), which can be activated in response to resistance exercise or amino acid ingestion (Dreyer et al., 2006, Atherton et al., 2010). mTOR is the central regulator of cellular growth and it is suggested that the mTOR cascade may play an important role in the adaptive response to anabolic stimuli, coordination of metabolism, protein synthesis, cell growth and proliferation (Soliman, 2013). Activation of mTOR promotes MPS through its downstream targets S6K1 (Ribosomal protein S6 kinase beta-1) and 4E-BP1 (4E-binding protein 1) (Schiaffino et al., 2013). Activation of S6K1 promotes the synthesis of the components of the translational machinery (ribosomal proteins, translation initiation, and elongation factors) and activation of 4E-BP1 initiates the first steps of protein translation (Bodine et al., 2001b). Once phosphorylated, 4E-BP1 is dissociated from eIF4E (Eukaryotic translation initiation factor 4E) and thereby permitting the binding of eIF4E to eIF4G (Eukaryotic translation initiation factor 4 gamma 1) promoting the translation initiation (Bodine et al., 2001b). The importance of this major cellular growth control pathway in aged human skeletal muscle remains unclear. Decreased basal protein levels of mTOR, S6K1 and 4E-BP1 (Cuthbertson et al., 2005) and reduced levels of mTOR and 4E-BP1 in their active conformation, i.e. phosphorylated state (Li et al., 2012) have been described in older adults.

**Muscle protein degradation**

MPB is mediated by two main degradation systems, the ubiquitin-proteasomal pathway and the autophagic/lysosomal pathway (Lecker et al., 1999). Muscle specific ubiquitin-ligases, and E3 enzymes in particular, play a major role in protein degradation process. The E3 ligases MAFbx (muscle atrophy f-box/atrogin-1) and MuRF1 (muscle RING finger 1/TRIM63), have been shown to be up-regulated in atrophy models including ageing (Bodine and Baehr, 2014). The expression of both MAFbx and MuRF1 is
under the control of FoxO (Forkhead box) transcription factors. To promote their action, FoxO transcription factors have to translocate to the nucleus and this process is inhibited by Akt (Manning and Cantley, 2007, Sandri et al., 2004). FoxO1 (Forkhead box 1) activation was shown to be necessary, however not sufficient, to increase MuRF1 and MAFbx gene expression in cultured myotubes (Stitt et al., 2004). Indeed, MuRF1 and MAFbx transcriptional activities have been shown to be up-regulated by the activation of another pathway, myostatin-Smad2/3 (SMAD family member 2/3) (Sartori et al., 2009, Trendelenburg et al., 2009). Myostatin, a member of the transforming growth factor beta (TGFβ) superfamily, is an important endogenous inducer of muscle atrophy (Schiaffino et al., 2013). Myostatin gene and protein expressions have been shown to be up-regulated in older adults (Leger et al., 2008) and myostatin down-regulation has been shown to induce skeletal muscle hypertrophy (McPherron et al., 1997) indicating a potential role in the control of age-related decline in muscle mass. Myostatin signalling is mediated by activin type II receptors and acts through the receptor-associated proteins Smad2 and Smad3, which translocate into the nucleus to activate the transcription of target genes such as MuRF1 and MAFbx (Carnac et al., 2007). The myostatin-mediated negative regulation of muscle mass also rely on the suppression of Akt signalling (Rodriguez et al., 2014, Trendelenburg et al., 2009). The implication of these pathways in decline in muscle mass in humans remains poorly understood.

**Autophagy**

The autophagic/lysosomal pathway ensures removal of misfolded or aggregated proteins and damaged organelles (Glick et al., 2010). GABARAP (Gamma-aminobutyric acid receptor-associated protein) and LC3 (Light chain 3) carry out important functions in autophagosome formation leading to subsequent degradation after fusion with lysosomes. GABARAP and LC3 ensure cross-talk between ubiquitination and autophagy processes (Shaid et al., 2013). Although very few studies have investigated autophagy in aged skeletal muscle, it has been hypothesized that an observed age-related chronic imbalance of the autophagic process may significantly contribute to age-related muscle loss (Bonaldor and Sandri, 2013). Increased autophagy has been shown to be promoted by activation of AMPK (AMP-activated protein kinase), a key energy sensor and regulator of cellular metabolism (Kim et al., 2011). Currently, the implications of the autophagic/lysosomal
Inflammatory factors in skeletal muscle
The NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) pathway is considered as a major pro-inflammatory signalling pathway (Lawrence, 2009) able to activate important pro-inflammatory factors such as IL-1 and TNF-α (Lawrence, 2009). Increases in protein levels of two members of the NF-κB family, the nuclear factor NF-kappa-B p65 subunit (RelA) and the nuclear factor NF-kappa-B p105 subunit (NFKB1) have been reported in older men and women (Buford et al., 2010, Thalacker-Mercer et al., 2010). Additionally, NF-κB has also been shown to be an important factor involved in skeletal muscle atrophy (Li et al., 2008). Elevated IL-1β (Interleukin 1 beta) gene expression level in skeletal muscle has also been reported and it is suggested that this change may limit muscle hypertrophy in older adults (Przybyla et al., 2006). In animal models, increased IL-1β gene expression in skeletal muscle was associated with reduced muscle mass (Schulze et al., 2003). Additionally, in cultured C2C12 muscle cells, exposure to IL-1β reduces myofibrillar protein content and myotube diameter (Li et al., 2009). These studies highlight the possible implication of local inflammatory processes in age-related loss of muscle mass.

Interventions to preserve muscle mass during ageing
Several types of interventions aiming to prevent the onset of muscle loss in older adults have been proposed including hormonal, nutritional and exercise interventions. However, long-term prescription of drugs is currently not feasible. Non-pharmacological strategies, such as exercise and nutritional approaches can be integrated in habitual lifestyle and have the potential to delay age-related functional decline (Peterson et al., 2011, Peterson et al., 2010, Candow et al., 2012).

Resistance training interventions
Among strategies used to delay the decline in muscle function, resistance training is currently considered as the most important non-pharmacological preventive measure (Peterson et al., 2011, Peterson et al., 2010). For example, 12 weeks of resistance training in older adults induced gains in muscle strength by 117% in knee extensor and gains in quadriceps area by 9-12%
Another study showed that prolonged resistance training resulted in gains in muscle strength (+26%) and mass (+5%) in lower limbs of healthy older men (Verdijk et al., 2009a). Interestingly, a 24 week intervention induced similar strength (+30%) and muscle mass (+9%) gains in older men and women (Leenders et al., 2013a), indicating that men and women could benefit equally from resistance training. However, there is an increasing number of studies that failed to report gains in muscle mass. For example, Vincent et al. (2002) and Kosek et al. (2006) did not observe significant training-induced gains in muscle mass in older men and women. Furthermore, Hanson et al. (2009) observed gains in muscle mass in older men but not in older women.

The ability of resistance training to improve explosive capacity in elderly has previously been investigated, yielding conflicting findings (Ferri et al., 2003, Hakkinen et al., 1998a, Hakkinen et al., 1998b, Hakkinen et al., 2001, Holviala et al., 2014, Jozsi et al., 2000, Frontera et al., 1988, Skelton et al., 1995). Thus, improvements of the explosive capacity have been less documented than gains in maximal muscle strength in aged populations.

Performance in functional tests such as five sit-to-stand (5-STS), single-leg-stance balance (SLS) and timed-up-and-go (TUG) may predict risk of falls and loss of independency (Guralnik et al., 1995, Podsiadlo and Richardson, 1991, Springer et al., 2007). In this respect, resistance training not only improves muscle mass and strength but also induces improvements in walking speed, chair rising and stair climbing (Fiatarone et al., 1990, Capodaglio et al., 2007, Skelton et al., 1995), reflecting improved ability to perform activities of daily living.

At a cellular level, important gains in CSA of type II fibres have been observed in response to resistance training in older adults (Nilwik et al., 2013, Verdijk et al., 2009a, Leenders et al., 2013a). When hypertrophy of muscle fibres reaches a given threshold (~+26% of hypertrophy), enlargement of muscle fibres is accompanied by increases in myonuclear content (Kadi and Thornell, 2000, Petrella et al., 2006, Leenders et al., 2013a). Below that threshold, existing myonuclei can support enlargement of muscle fibres (Kadi et al., 2004b, Mackey et al., 2007, Verdijk et al., 2009a, Petrella et al., 2006). Thus, increases in myonuclear number is not prerequisite for the occurrence of fibre hypertrophy (Hikida et al., 2000, Petrella et al., 2006, Verdijk et al., 2009a, Kadi et al., 2004b). Resistance training may also promote increases in SC number in older adults (Verdijk et al., 2014,
Verdijk et al., 2009a, Mackey et al., 2007, Kadi et al., 2004b, Verney et al., 2008). The meaning of increases in SC number measured in muscle cross-sections is unclear as it does not provide information regarding the proliferative capacity of SC and the regenerative potential of muscle tissue.

Resistance training may also influence low-grade systemic inflammation. However, there are discrepancies among the few studies addressing the efficiency of long-term resistance training in reducing the systemic inflammatory level. Small decreases in CRP levels were reported after 1 year resistance training in young and middle-aged overweight sedentary women with elevated baseline CRP levels (Olson et al., 2007). However, in this study, CRP levels were still elevated at the end of the intervention. Another study reported reduced CRP and TNF-α levels in obese older women after 12 weeks of resistance training (Phillips et al., 2012b). In contrast, IL-6 and TNF-α were not altered by 12-weeks resistance training program in frail older men and women (Bruunsgaard et al., 2004).

**Nutritional interventions**

The anabolic effect of nutrition is principally driven by the transfer and incorporation of amino acids captured from dietary protein sources into skeletal muscle proteins (Atherton and Smith, 2012). Therefore, a main focus of nutritional interventions in aged population has been to increase protein intake, either through whole-diet approaches or by using supplementation with specific protein or amino acid mixtures.

Improvements in muscle mass or function in response to protein supplementation have been reported in aged populations in several reports. However, there is a considerable number of studies that failed to report any significant improvement in muscle mass, strength or function after protein supplementation in healthy older adults (Fiatarone et al., 1994, Rosendahl et al., 2006, Carlsson et al., 2011). Improvements in muscle mass and/or function in response to protein supplementation commonly occur in older frail adults and patients with chronic diseases. Protein supplementation improved muscle mass and power in frail older adults (Bonnefoy et al., 2003) and induced gains in muscle strength but not muscle mass in older adults with metabolic disturbances (Borsheim et al., 2008). Moreover, Tieland et al. (2012b) did not observe any improvements in either muscle mass or strength after 24 weeks of protein supplementation in frail older men and women.
In recent years, omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have gained interest owing to their putative anabolic properties. Supplementation with n-3 PUFAs has been shown to attenuate age-related decline in muscle mass and function (Smith, 2016) and inhibit protein degradation (Wang et al., 2013). Moreover, supplementation with n-3 PUFAs has also been shown to improve muscle strength, muscle mass volume and muscle protein synthesis in older adults (Smith et al., 2011, Smith et al., 2015) possibly through enhanced activation of the mTOR pathway (Yoshino et al., 2016). In contrast, another study failed to report improvements in body composition, muscle strength or physical performance in older subjects supplemented with n-3 PUFAs (Krzyminska-Siemaszko et al., 2015).

n-3 PUFAs have also been suggested to have anti-inflammatory properties (Calder, 2006, Ferrucci et al., 2006). Data from clinical trials show that n-3 PUFAs may decrease levels of pro-inflammatory cytokines in patients with rheumatoid arthritis and inflammatory bowel disease (Wall et al., 2010). Moreover, a significant decrease in the level of the pro-inflammatory precursor arachidonic acid (AA) has been reported in healthy middle-aged subjects supplemented with the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Fisher et al., 1990, Kelley et al., 1999). These data are of clinical relevance as higher concentrations of AA are associated with smaller muscle size (Reinders et al., 2015). Moreover, AA has been shown to be involved in the induction of muscle protein degradation through increased expression and activity of the ubiquitin–proteasome pathway (Whitehouse et al., 2003). Thus, supplementation with n-3 PUFAs may be considered as an approach to delay age-related deteriorations in skeletal muscle.

**Combined resistance training and dietary approaches**

Different dietary approaches have been used in combination with resistance training in order to optimize training effects in older adults. Combined protein supplementation and resistance training induces greater gains in muscle mass than resistance training alone in older adults (Cermak et al., 2012, Tieland et al., 2012a). However, other studies failed to report similar findings (Leenders et al., 2013b, Verdijk et al., 2009b, Thomas et al., 2016). Noteworthy, no additional benefits are reported when resistance training is combined with protein supplementation in subjects with already adequate
protein intake (Iglay et al., 2007). Older adults with inadequate protein intake may benefit from the combination of resistance training and protein supplementation as protein availability is important for resistance training-induced growth of muscle mass.

There are only a few studies addressing the effects of combined resistance training with n-3 PUFAs intake on skeletal muscle. Compared to eighteen weeks of resistance training alone, resistance training combined with n-3 PUFAs supplementation is associated with greater gains in maximal isometric torque in older women but not in men (Da Boit et al., 2017). Additionally, 90 days of combined resistance training and n-3 PUFAs induced greater improvements in muscle strength than resistance training alone in older women (Rodacki et al., 2012).

It is important to note that many previous studies examining the benefits of exercise in older populations have included participants with low habitual physical activity level, chronic diseases and signs of frailty. Therefore, findings from these studies may not apply to healthier older with an active lifestyle and adequate nutritional habits. Currently, there is a need to explore the beneficial effects of combining exercise and dietary changes in already healthy and recreationally active older adults. Exercise and dietary changes should be compatible with long-term adoption and implementation. This would delay age-related chronic diseases and loss of function.
Aim

The overall aim of this thesis was to explore the effects of resistance training alone or combined with a healthy diet on skeletal muscle mass and function of healthy recreationally active older women and to determine mechanisms by which elevated systemic inflammation may contribute to the age-related decline of muscle mass in older adults.

The specific aims were:

To examine the effects of 24-weeks resistance training combined with a healthy diet on skeletal muscle mass and strength and systemic markers of inflammation in healthy recreationally active older women (study I)

To explore the effects of 24-weeks resistance training combined with a healthy diet on maximal strength, explosive force capacity and physical function of healthy recreationally active older women (study II)

To determine mechanisms underlying the effects of 24-weeks resistance training combined with a healthy diet on skeletal muscle mass in healthy recreationally active older women (study III)

To identify mechanistic links underlying the detrimental effects of elevated systemic inflammation on muscle mass in older adults (study IV)
Methods

Subjects
In studies I, II and III, elderly women were recruited through advertisement in a local newspaper. A medical history and electrocardiograms were assessed by a physician. Exclusion criteria were (1) living in a nursing home, (2) self-reported inability to walk, (3) cardiovascular, pulmonary, metabolic, rheumatologic and psychiatric disease, (4) musculoskeletal problems, (5) using medication, (6) food allergy and (7) unexplained weight loss, (≥ 5% of body weight during the preceding 12 months). In order to be included in the study elderly women had to be aged between 65 and 70 years and with a BMI < 30, fasting glucose < 6 mmol/L, fasting cholesterol < 8 mmol/L, systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg. Additionally, subjects had to be recreationally physically active. The subjects physical activity behaviours were assessed by a previously validated questionnaire (EPAQ2, (EPIC-Norfolk physical activity questionnaire)) (Wareham et al., 2002). All subjects included in the study participated in various recreational physical activities such as walking, Nordic walking, jogging, cycling, swimming and skiing, none of the subjects had previously participated in structured resistance training. A three-armed randomized controlled trial (RCT) was performed. Randomization was done by block-design to ensure equal numbers of elderly women assigned to a control group (CON, n=21; 68 ± 1 years), a resistance-training group (RT, n=21; 68 ± 2 years) and a resistance-training plus healthy diet group (RT-HD, n=21; 67 ± 1 years). In order to ensure that the comparison groups were as similar as possible with regard to baseline inflammatory level, the randomization was stratified by serum CRP level.

In study IV, one hundred and eighteen elderly women (age: 67 ± 2 years) were included. They were non-smokers, had no disability regarding mobility and were free of metabolic and inflammatory diseases, diagnosed coronary heart disease or diabetes mellitus. None of the subjects had a serum CRP higher than 6 mg/L. Muscle biopsies from 7 older women (66.9 ± 1.2 years) were obtained for the in-vitro model.
Resistance training (Study I, II, III)
Supervised progressive resistance training was performed twice a week during 24 weeks. Subjects performed 3 sets per exercise with 2 min rest between sets and 3 min rest between exercises. The initial workload corresponded to 50% of the one-repetition maximum (1RM) during the first two weeks where the subjects performed 12-15 repetitions per set. A workload of 75-85% 1RM (8-12 reps/set) was set for the rest of the intervention. Training load was adjusted throughout the intervention. The following exercises were performed: squat, leg-extension, leg-press, seated-row and pull-down. Additionally, 5 minutes of core stability exercises and 7 squat jumps were included. Training sessions ended with 5 minutes stretching exercises.

Healthy diet intervention (Study I, II, III)
The subjects in RT-HD group attended a dietary consultation and were given a diet plan. Details of the prescribed diet are summarized in table 1. Briefly, the dietary plan was based on an intake of 44 E% (energy percent) of carbohydrates (fibre intake >25 g/day), 36 E% of fat (mainly monounsaturated and polyunsaturated fatty acids) and 20 E% of protein with the following major adjustment: the n-6/n-3 (omega-6/omega-3) ratio <2. Subjects in CON and RT were instructed to maintain their habitual dietary intake throughout the study. The dietary intake was monitored by using a food record over a period of 6 days at baseline, week 12, and week 24.
Table 1. Nutrient goal and prescribed key food items for the dietary intervention

<table>
<thead>
<tr>
<th>Nutrient goals</th>
<th>Dietary Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate, E%</td>
<td>44</td>
</tr>
<tr>
<td>Fibre, g/day</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Protein, E%</td>
<td>20</td>
</tr>
<tr>
<td>Total fat, E%</td>
<td>36</td>
</tr>
<tr>
<td>Saturated fat, E%</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Unsaturated fat, E%</td>
<td>≥2/3 of total fat</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Prescribed foods</td>
<td></td>
</tr>
<tr>
<td>Cereal products</td>
<td>High whole-grain (rye, oat, barley)</td>
</tr>
<tr>
<td>Vegetables/fruits/berries</td>
<td>≥600 g/day</td>
</tr>
<tr>
<td>Fish and seafood</td>
<td>≥500 g/wk</td>
</tr>
<tr>
<td>Dietary fats</td>
<td>Rape seed oil, olive oil, nuts and seeds</td>
</tr>
<tr>
<td>Meat products</td>
<td>Lean meat</td>
</tr>
<tr>
<td>Dairy products</td>
<td>≤0.5 l/day (low fat)</td>
</tr>
<tr>
<td>Soft drinks/ juice</td>
<td>Be avoided/&lt;1.5 dl juice/day</td>
</tr>
</tbody>
</table>

E%, energy percent; n-6, omega-6; n-3, omega-3

Assessment of physical activity level (Study I, II, III)

Recreational physical activity was assessed by accelerometry (Actigraph model GT3x, Pensacola, FL, USA) at baseline, week 12 and week 24. This assessment was performed in order to monitor physical activity level outside the intervention. The accelerometer was worn with an elastic belt on the right hip for six consecutive days. Participants were informed to wear the monitor all waking hours except for bathing or swimming. The monitors were initialized and downloaded using the manufacturer software ActiLife 6. None-wear time was defined as periods of at least 60 consecutive minutes of zero values. The total number of counts per minutes per day (cnts·min⁻¹·day⁻¹) and the average number of minutes spent in moderate-to-vigorous physical activity (MVPA) per day were derived. MVPA was defined as all activities with an intensity ≥760 cnts·min⁻¹, a threshold frequently previously used in elderly women (Orsini et al., 2008). The resistance training sessions for the RT and RT-HD groups at week 12 and 24 were excluded from the analysis.
Assessment of body composition

**Dual-energy x-ray absorptiometry (Study I & II)**
Regional- and whole-body composition was estimated by using Dual-energy x-ray absorptiometry (DXA) (LUNAR Prodigy, GE Medical systems) performed in the fasted state. The system software (Hologic Apex version 2.3) provided the mass of lean mass, fat mass and bone mineral content.

**Bioelectrical impedance analysis (Study IV)**
Muscle mass was assessed using bioelectrical impedance analysis (BIA) (TANITA BC-420MA, Tanita Corporation, Japan). Skeletal muscle mass was calculated using the equation of Janssen et al. (2002): Skeletal muscle mass (kg) = [(height^2/BIA-resistance x 0.401) + (gender x 3.825) + (age x - 0.071)] + 5.102. A skeletal muscle mass index in percentage (SMI %) is calculated as follows: skeletal muscle mass/body mass x 100. According to Janssen et al. (2002) low muscle mass is defined as an SMI below 28%.

**Assessment of muscle strength (Study I & II)**

**One repetition maximum**
Maximum strength was assessed using the 1RM test. During a familiarization session, the proper lifting technique was practiced and maximum strength was estimated using a multiple repetition test procedure. The 1RM was determined for each exercise during the second visit. Following a warm-up period, the load was set at 90-95% of the estimated 1RM and increased with approximately 2.5-5% after each successful lift until failure. A 3-min resting period was allowed between consecutive attempts and the 1RM was obtained within five attempts.

**Maximal dynamic leg power and time to peak power**
Test were performed using a knee-extension machine with the subject firmly strapped while seated with a 90° hip angle and 80° knee angle. The axis of the adjustable lever arm was aligned with the centre of the knee joint, and an adjustable padded crossbar was applied against the tibia at a point above the malleoli corresponding to 1/3 of the distance between the lateral femoral epicondyle and lateral malleolus. A force sensor (K. TOYO 333A) and a linear encoder (Muscle Laboratory; Ergotest Technology, Oslo, Norway) were used to monitor force and vertical displacement generated during the
concentric phase of knee extension exercise. Subjects were instructed to perform a maximal bilateral knee extension as forcefully and quickly as possible. Verbal encouragements were provided during each trial. All tests were performed against a load corresponding to 70% of 1RM assessed at baseline. Each subject performed three trials, and trials with an initial countermovement (identified by a visible drop in the force signal) were excluded and a new trial was performed. Onset of muscle contraction was defined as the time point where the knee extension power exceeded 1.0 W above the baseline level. Time to peak power was calculated from the trial with the highest peak power output. Intraclass correlation coefficients (ICCs) of 0.98 and 0.91 were obtained for knee extension peak power and time to peak power, respectively.

**Maximal isometric leg strength and rate of force development during isometric contractions**

Tests were performed in seated position using an adjustable chair with 90° angle of the hip and knee joints and with restraining straps crossing the torso and the tested leg. A force sensor (K. TOYO 333A) was attached above the malleoli at one-third of the distance between the lateral femoral epicondyle and lateral malleolus. Subjects were instructed to exert maximal muscle force as “fast and forcefully” as possible and to maintain it for 3-5 s. Verbal encouragement were provided during each trial. All isometric measurements were performed on the dominant leg, and each subject performed three knee extensions at maximal voluntary effort separated by a rest period of 2.5 min. Trials with an initial countermovement (identified by a visible drop in the force signal) were excluded and a new trial was performed. Onset of muscle contraction was defined as the time point where the knee extension force exceeded 2.0 N above the baseline level, which corresponded to ~1% of maximal peak force (Aagaard et al., 2002). The trial with the highest isometric force was selected for further analysis of the rate of force development during isometric contraction (isoRFD). IsoRFD was determined as the greatest increases in force in a given 50-ms time period (Hakkinen et al., 2001). ICCs of 0.93 and 0.88 were obtained for trial-to-trial reliability regarding isometric peak force and RFD (rate of force development), respectively.
Assessment of physical function (Study II)
Five sit-to-stand (5-STS), single-leg-stance (SLS), time-up-and-go (TUG), and squat jumps (SJ) were used to assess physical function. During the 5-STS, subjects were instructed to stand fully upright from a chair and sit down and repeat this sequence for five times (Guralnik et al., 1995). For the SLS balance test, subjects were instructed to stand barefoot on one leg (the dominant leg) with arms crossed and eyes closed (Springer et al., 2007). For the TUG, the subjects were instructed to stand up from a chair, walk at a “comfortable speed” to the 3-m mark, turn around, walk back, and sit down (Podsiadlo and Richardson, 1991). To obtain a measure of physical function in relation to muscle power, a SJ was performed on a force platform (Kistler 9281 B; Kistler Nordic). The SJ started from a static position with knee bent in a 90° angle. The hands were kept on the hip during the whole jump. Subjects performed familiarization trials with gradually increased effort. Each subject performed three maximal trials separated by 1.5 min of rest. Trial with an initial countermovement (identified by a visible drop in the force signal) were excluded and a new trial was performed. During each squat jump, maximal ground reaction force (sjMAX) and rate of force development (sjRFD) were calculated from the concentric phase of the squat jump. The two parameters (sjMAX and sjRFD) are directly correlated to jump performance and are not influenced by technical aspects such as landing technique. ICCs of 0.97 and 0.88 were obtained for trial-to-trial reliability regarding sjMAX and sjRFD, respectively.

Determination of plasma CRP, IL-6, fibrinogen, sIL-6R and sTNF-RII (Study I & III)
Blood samples were obtained between 7.00 and 9.00 am after an overnight fast in 54 participants (CON, n=17; RT, n=17 and RT-HD, n=20). CRP level was measured using a high-sensitivity C-reactive protein (Hs-CRP) kit by a fully automated immunoturbidimetric assay (Advia 1800, Chemistry System, Siemens, Germany). Plasma fibrinogen was quantified using an automated immunoassay method with a polyclonal rabbit anti-human antibody (Dako, Glostrup, Denmark). Commercially available enzyme linked immunosorbent assay (ELISA) kit was used (Quantikine HS, R&D Systems) for the assessment of IL-6 levels. Plasma fibrinogen was quantified using an automated immunoassay method with a polyclonal rabbit anti-human an-
tibody (Dako, Glostrup, Denmark). Human soluble cytokine receptors interleukin-6 receptor (sIL-6R) and tumour necrosis factor receptor II (sTNF-RII) (Millipore, Germany) were measured in a multiplex assay according to the manufacturer’s recommendations and measurements were made using the Luminex xMAP technology (Luminex Corporation, Austin, TX, U.S.A). Data analysis was performed using the xPONENT software (Luminex). All samples were run in duplicates and the average of two values was used for data analysis.

**Determination of fatty acid composition (Study I)**
Serum lipids were extracted with chloroform and phospholipids (PL) were separated from other lipids by thin-layer chromatography (TLC) and transmethylated with methanol and sulfuric acid (Boberg et al., 1985). The percentage composition of methylated fatty acids was determined by gas chromatography (GC) with flame ionization detection. GC used for the analysis consisted of 30-m capillary column coated with Thermo TR-FAME (Thermo Electron Corporation, USA) and Agilent Technologies system (GC 6890N, Autosampler 7683, and Agilent ChemStation). The temperature used was between 150°C-260°C. The fatty acids were identified by comparing the retention time of each peak with the methyl ester standard (Nu Check Prep, Elysian, MN, USA).

**Muscle Biopsy**
Muscle biopsies were taken from *m.vastus lateralis* under local anaesthesia (2 ml lidocaine-adrenaline, 1 %) using the Weil-Blakesley’s conchotome technique at baseline and after the intervention (four days after the last training session in RT and RT-HD). Muscle samples were dissected free of visible blood, fat and connective tissue and frozen in liquid nitrogen before storage at -80°C until analysed.

**Assessment of muscle fibre composition, fibre size, myonuclear and satellite cell content (Study III)**
Morphometric analyses were performed in skeletal muscle of 34 participants (CON, n=11; RT, n=12 and RT-HD, n=11) using methods previously described (Mackey et al., 2009). Serial 6-μm thick cross-sections were cut at -20°C using a cryostat microtome (Leica CM 1850), mounted on glass slides and air dried at room temperature. All sections were fixed for 8 min
with a 5% formaldehyde solution (Histolab, Gothenburg, Sweden). Blocking of non-specific binding sites was performed by incubation for 60 min with a blocking buffer containing 0.01% Triton, 1% bovine serum albumin (BSA), 1% skimmed milk powder. A two-step procedure based on the use of two serial cross-sections was used to assess the size of muscle fibres and fibre-type specific myonuclear and SC content. The first section was first labelled with the monoclonal antibody (mAb) against Pax7 (Pax7, Developmental Studies Hybridoma Bank) followed by a second incubation in a mixture of two mAb against slow myosin (A4.840, Developmental Studies Hybridoma Bank) and laminin (2E8, Developmental Studies Hybridoma Bank). Visualization of Pax7+SCs was performed by successive incubations with a biotinylated horse-anti-mouse secondary antibody (Vector BA-9200, Burlingame, CA, USA), Vectastain ABC (PK6100, Vector Laboratories, USA) reagent and DAB (Diaminobenzidine) substrate kit (SK-4100, Vector Laboratories, USA). Pax7+SCs were stained brown and visualized using light microscopy. Visualization of the antibody mixture containing A4.840 and 2E8 was achieved by incubation with Alexa Fluor 488 goat-anti-mouse (Invitrogen, Thermo Fisher Scientific) yielding a green-fluorescent staining. Nuclei are stained blue using molecular probes prolong gold antifade reagent containing 4’,6 Diamidino-2-phenylindole (DAPI), (Invitrogen, Thermo Fisher Scientific). This first step allowed the visualization on the same section of brown-stained Pax7+SCs using light microscopy, green fluorescent basal lamina and type I muscle fibres and blue fluorescent myonuclei located beneath the basal lamina using fluorescent microscopy. Type II fibres are unstained. The second step of the procedure allows to further distinguish between type IIA and IIX muscle fibres by incubating the second section with the mAb N2.261 (Developmental Studies Hybridoma Bank) and with the Alexa Fluor 488 goat-anti-mouse secondary antibody (Invitrogen, Thermo Fisher Scientific). Fibre type composition was performed on the whole cross-section. Type I and type IIA fibres were the predominant fibre types. Type IIX fibres were very rare and were not included in the analysis. The cross-sectional area (CSA) of type I and type IIA muscle fibres was measured using Image J software (U. S. National Institutes of Health, Bethesda, MD). Myonuclear and Pax7+SCs contents were assessed in type I and type IIA fibres. As a measure of fibre circularity, form factors were calculated using the following formula: \((4\pi^{*}\text{CSA})/(\text{perimeter})^{2}\) as previously described (Charifi et al., 2003).
**Total RNA extraction and reverse transcription**

Total RNA was extracted from biopsy samples of 31 subjects (CON, n=11; RT, n=10 and RT-HD, n=10) using Nucleospin RNA extraction kit (Machery-Nagel, Düren, Germany) and RNeasy clean-up and concentration kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA concentration and purity were determined using a spectrophotometer (NanoDrop 2000, Thermo Scientific). The use of Heat&Run gDNA removal kit (ArticZymes, Tromsø, Norway) prior to reverse transcription ensured complete removal of any contaminating DNA. RNA was reverse transcribed to give complementary DNA (cDNA) according to the manufacturer’s instructions (RT² First strand kit: Qiagen, Hilden, Germany).

**Real-time quantitative PCR**

Quantitative real-time PCR was performed on Rotor-Gene Q (Qiagen, Hilden, Germany) using RT² SYBR® Green ROX Fast Master mix (Qiagen, Hilden, Germany) and gene specific primers (muscle mass regulation: IGF-I, mTOR, myostatin, FoxO1, Inflammation: IL-1β, NFKB1, RelA, TLR1, Apoptosis: Bcl-2, Bad and Bax, Autophagy: GABARAP) according to the manufacturer’s instructions. The thermocycler parameters were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30s. Each run was followed by a melting curve analysis in order to confirm the presence of only one single PCR product. The quantification of the PCR results was generated using comparative quantification (CQ). The CQ method allows for the calculation for individual PCR reaction efficiencies, obviating the need to generate standard curves estimate efficiencies (McCurdy et al., 2008). This method is used to calculate the point at which the exponential phase of amplification begins. This point, so-called Take Off Point (TOP), is used in the equivalent manner as the Ct value as described by (Livak and Schmittgen, 2001). McCurdy et al. (2008) demonstrate a strong correlation between these two methods which further validates CQ as an effective quantification method. As no significant changes in TOP values were observed over time (before intervention period: CON: 15.6 ± 0.1; RT: 15.8 ± 0.2; RT-HD: 15.9 ± 0.2 and after intervention period: CON: 15.5 ± 0.2; RT: 15.7 ± 0.2; RT-HD: 15.8 ± 0.2), the use of GAPDH as a reference gene was validated.
Cell culture experiments (Study III & IV)
Small muscle pieces were scissor-minced and explants were trapped inside a thin layer of 6 mg/ml Matrigel (Matrigel Matrix, BD Biosciences, Le Pont de Claix, France) in 35 mm collagen-coated Petri dishes with growth media (Dulbecco’s Modified Eagle Medium, DMEM, Sigma) supplemented with 20% fetal bovine serum (HyClone Perbio), 2% Ultroser G (Pall Corporation, France), 10 mM Hepes (H0887, Sigma Aldrich) and 50 μg/ml Gentamicin (G1397, Sigma Aldrich). After 6 to 8 days, cells migrated out of the explants. Migrating cells were enzymatically harvested using dispase (BD Biosciences) and subcultured in growth medium. Harvested cells were purified by immunomagnetic cell sorting using magnetic activated cell sorter (MACS) microbeads coupled to an antibody against CD56 (Miltenyi Biotec, Paris, France) (Wahlin-Larsson et al., 2014). The immunomagnetic cell sorting procedure yields highly purified myoblasts from m. vastus lateralis biopsies as evidenced by staining with the muscle cell-specific marker desmin, showing that more than 99% of the cells were desmin+myoblasts. At cell isolation, all myoblasts were considered to be at 1 population doubling (PD) and all experiments were performed at 4 PD.

Assessment of proliferative rate of human myoblasts
On the basis of DXA-derived measures of leg lean mass (Strandberg et al., 2015), we sought to examine the in-vitro proliferative capacity of myoblasts isolated from a subset of participants exhibiting muscle hypertrophy in response to intervention (n=8) and in participants exhibiting no muscle hypertrophy (n=5). Myoblasts were seeded at 3×10^4 cells/dish onto 35-mm collagen-coated dishes. At 96h, the cells were counted using a hemocytometer (Bürker). Myoblast doubling time (DT) was calculated as follows: DT=96/(ln (y/x)/ln2), where x is the number of cells counted at 0 h and y the number of cells counted at 96h (Wahlin-Larsson et al., 2014).

Effects of exogenous CRP on the size of human myotubes (Study IV)
Myoblasts obtained from 7 older women were seeded at 1×10^6 cells/dish onto 35 mm collagen-coated dishes and cultured in growth medium. When the cell monolayers reached confluence, they were differentiated into myotubes in differentiation medium. Differentiated myotubes were kept in differentiation medium without (Control) and with CRP (50µg/ml, C1617 Sigma Aldrich) for 72h. Cells were then fixed in 2% formaldehyde, treated with 0.25% Triton and immunostained for 2h at room temperature using
the primary antibodies against myogenin (1:100; mouse monoclonal, Santa Cruz) and troponin T (1:100; rabbit polyclonal, Sigma Aldrich). Secondary antibodies Alexa Fluor 488 (goat anti-mouse, Invitrogen, Thermo Fisher Scientific) and Alexa Fluor 568 (goat anti-rabbit, Invitrogen, Thermo Fisher Scientific) were used. Nuclei are stained blue with DAPI. Myotube diameter was quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, MD) on 6 randomly chosen fields yielding approximately 140 myotubes per cell culture. The average diameter of one myotube was calculated as the mean of 5 measurements performed along the length of that myotube.

**Effects of exogenous CRP on myotube mixed protein synthesis (MPS) (Study IV)**

Differentiated myotubes were kept in differentiation medium without (Control, CON) and with CRP (50µg/ml, C1617 Sigma Aldrich) for 24h. All samples were measured in at least duplicates. Chronic measures of protein synthesis rates were performed using the stable isotope tracer deuterium oxide (D₂O). 5% D₂O was added in both control and CRP-treated cell cultures. Following incubations, 1ml of media was sampled from each well and cells were washed twice with ice cold PBS and harvested into 200µl of ice cold homogenisation buffer (50 mM Tris-HCl (pH 7.4), 50 mM NaF, 10 mM β-Glycerophosphate disodium salt, 1 mM EDTA, 1 mM EGTA, 1 mM activated Na₃VO₄ (all Sigma Aldrich, Poole, UK) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK)). Following homogenisation, samples were centrifuged at 10000g for 10mins at 4°C to separate the sarcoplasmic proteins and 50µl of the supernatant was transferred to a fresh eppendorf and frozen for western blotting analyses. To the remaining supernatant and pellet an equal volume of 1M PCA was added to precipitate any remaining sarcoplasmic proteins. The sample was incubated on ice for 30 mins and centrifuged at 10000g for 10 mins at 4°C. The PCA supernatant (containing free amino acids) was transferred to a fresh Eppendorf and frozen, whilst the pellet was washed sequentially with PCA: 70% ethanol to remove any remaining free amino acids. The mixed muscle pellet was then hydrolysed overnight at 110°C in 1ml of 0.1M HCl and 1ml +H dowex resin. Hydrolysed amino acids were then eluted from the dowex resin the following day into 2M NH₄OH and evaporated to dryness. Deuterium labelling of protein bound alanine was then determined using GC-MS/MS.
(Trace 1310-TSQ 8000, Thermo Scientific, Hemel Hempstead, UK) following conversion to its n-methoxycarbonyl methyl ester derivative and single reaction monitoring (SRM). The media sampled from each well was measured for D2O enrichment using a modification of the acetone exchange method (Yang et al., 1998). Briefly, 2µl of 10N NaOH was added to 100µl of media, following a 15s vortex mix 1µl of acetone was added. This was incubated for 24h to allow full hydrogen/deuterium exchange. The acetone was then extracted into 200µl of n-heptane, the n-heptane layer was transferred to an autosampler vial and 0.5µl injected into the GC-MS (Trace 1300-ISQ, Thermo Scientific, Hemel Hempstead, UK). D2O enrichment was determined via SIM of m/z 58 and 59 with reference to a standard curve of known D2O enrichments. FSR (fractional synthetic rate) was calculated using the following equation:

\[
\text{FSR} \ (\%/h) = \frac{[\text{MPE}_{\text{Ala}}]}{3.7 \times (\text{MPE}_{\text{MW}}) \times t} \times 100
\]

Where MPEAla represents protein bound alanine enrichment, MPEMW represents media water enrichment and t represents time in hours.

**Western Blot analysis (Study IV)**

The sarcoplasmic fraction was used for immunoblot analysis. Sarcoplasmic protein concentrations were determined using a NanoDrop Lite spectrophotometer (Thermo Scientific, Hemel Hempstead, UK) and adjusted to 0.3µg.µl⁻¹ in 3× laemmlli buffer. Each sample was loaded onto pre-cast 12% Bis-Tris Criterion XT gels (BioRad, Hemel Hempstead, UK) at 4.5 µg per lane and separated electrophoretically at 200 V for 1h. Proteins were then wet-transferred at 100 V for 1h onto polyvinylidene difluoride (PVDF) membranes (0.22 µm pore size), blocked for 1h in 2.5% skimmed milk in 1× Tris-buffered saline/Tween-20 (TBS-T), and then incubated in primary antibodies (1:2000 dilution in 2.5% BSA in TBS-T); phospho-serine/threonine kinase Akt (p-AktSer473), phospho-ribosomal protein S6 (p-RPS6Ser235/236), phospho-AMP-activated protein kinase (p-AMPKαThr172), phospho-raptor (p-RaptorSer792), phospho-acetyl-CoA carboxylase (p-ACC-βSer79) (New England Biolabs, Hertfordshire, UK), rocking overnight at 4°C. The next day membranes were subsequently washed 3×5 min in TBS-T, incubated in HRP-conjugated secondary antibody (New England Biolabs, Hertfordshire, UK; 1:2000 in 2.5% BSA in TBS-T) at room temperature for 1h, before the last 3×5 min washes in TBS-T. Membranes were exposed to Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA-
US) for 5 min and bands quantified by Chemidoc XRS (BioRad, Hertfordshire, UK). Software measures were taken to prevent pixel saturation and protein loading were corrected to Coomassie staining.

**Statistical analysis**

Statistical analyses were performed using SPSS version 20.0 or 24.0 (SPSS, Chicago, IL) (Study I, III & IV) and SigmaStat software (SYSTAT Software Version 12) (Study II). All variables were tested for normality by using the Shapiro-Wilk normality test and skewed data were log transformed.

**Study I**: One-way ANOVA was used to examine between-group differences at baseline. The effects of the intervention were analysed using a repeated-measures ANOVA with one within-subject (time) and one between-subject (group) factor followed by Holm-Sidak post hoc procedure. An ANCOVA was performed to control for possible confounding effects of total energy intake or relative protein intake on changes in muscle mass. P values ≤0.05 were considered significant.

**Study II**: The effects of the intervention were analysed using a repeated-measures ANOVA with one within-subject (time) and one between-subject (group) factor followed by Holm-Sidak post hoc procedure. When significant changes were captured using the repeated measures ANOVA, differences in amplitude of changes between RT and RT-HD were analysed using one-way ANOVA. Associations between variables were assessed using Pearson’s correlation coefficient. P values <0.05 were considered significant.

**Study III**: Between-group comparison was performed for all data collected at baseline using one-way analysis of variance (ANOVA). The effects of the RCT were analysed using a repeated-measures ANOVA with one within-subject factor (time point) and one between-subject factor (group) followed by Holm-Sidak post hoc procedure. For gene expression data, between-group comparison in $2\Delta\Delta^{\text{TOP}}$ of selected genes was performed using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn’s post-hoc procedure. Changes in proliferative capacity of myoblasts were analysed using a repeated-measures ANOVA with one within-subject factor (time-point) and one between-subject factor (group, hypertrophy vs no hypertrophy). Associations between changes in gene expression and muscle mass were assessed using Spearman’s correlation coefficient. P values <0.05 were considered significant.
Study IV: *In-vitro* data was analysed using a paired samples t-tests with the alpha level of significance set at p<0.05. Unpaired t-test was used to assess the differences between elderly with low (SMI below 28%) and normal (SMI over 28%) muscle mass. Linear regression was used to examine association between CRP z-score and SMI z-score with adjustments for BMI and age.

**Ethical considerations**
This study has been conducted in accordance to the declaration of Helsinki and was approved by the regional ethical review board of Uppsala, DNR: 2011/033. Participants gave written informed consent and were informed about potential risks of injury associated with resistance training. All training sessions were supervised to ensure proper lifting technique. Precautions were also taken during the testing procedures to prevent occurrence of injuries. The biopsy procedure is invasive and can cause pain and discomfort. A detailed description of the procedure was provided and all biopsies were performed by a trained physician. Data storage process ensures that only authorized persons have access to them. Personal data are treated confidentially and data analysis is conducted anonymously.
Results

Study I.
The effects of 24 weeks progressive resistance training in combination to a healthy diet on muscle strength and mass and markers of chronic systemic inflammation are examined. Resistance training alone or in combination to a healthy diet improved both dynamic (RT: +20.4%, RT-HD: +20.2%) and isometric lower-limb muscle strength (RT: +19.0%, RT-HD: +15.3%). Gains in muscle mass were only observed when resistance training was combined to the healthy diet (Fig. 1). Interestingly, the observed gains in muscle mass were accompanied by a reduction in the pro-inflammatory precursor arachidonic acid (-5.3%) and an increase in the n-3 DHA (+8.3%). Circulating markers of inflammation CRP and IL-6 remained unchanged in response to the intervention.

Figure 1. Relative changes in leg lean mass in healthy recreationally active older women following 24-weeks intervention. Data are presented as means ± SEM.* p<0.05 vs. PRE
Study II.
The effects of 24 weeks progressive resistance training in combination to a healthy diet on dynamic and isometric muscle characteristics as well as physical function are studied. The explosive capacity in dynamic movements increased as evidenced by the significant changes in knee extension peak power in both RT (+15.7%) and RT-HD (+24.6%). Interestingly, changes in knee extension peak power were significantly larger in RT-HD compared with RT (Fig. 2A). Time to reach peak power also improved (i.e. shorter time) in both RT (-11.0%) and RT-HD (-20.3%), and these changes were significantly larger in RT-HD compared with RT (Fig. 2B). At baseline, there were no group differences in 5-STS, SLS and TUG performance. By the end of the 24-weeks intervention, both RT and RT-HD significantly improved their performance in 5-STS (i.e., shorter time) (-24.3% and -24.4%, respectively) and SLS (+66.7% and +50.8%, respectively). There were no significant group differences in sjMAX or sjRFD at baseline (Fig. 3). By the end of the intervention, sjMAX and sjRFD increased in both RT (+35.7% and +105.4%, respectively) and RT-HD (+58.5% and +185.4%, respectively). The increases sjMAX and sjRFD were significantly larger in RT-HD compared with RT (Fig. 3). Additionally, significant gains in whole body lean mass was observed in RT-HD only (+1.5%).

Figure 2. Knee extension peak power (A) and time to peak power (B) before (open bars) and after (closed bars) 24-weeks intervention *p<0.05, significant difference vs. before; *p<0.05, significant difference in amplitude of changes between resistance training (RT) and resistance training and healthy diet (RT-HD). Data are presented as means ± SEM.
Figure 3. Maximal ground reaction force (A) and rate of force development (RFD; B) during the concentric phase of squat jump test in CON, RT and RT-HD before (open bars) and after (closed bars) 24-weeks intervention. *p<0.05, significant difference vs. before; #p<0.05, significant difference in amplitude of changes between RT and RT-HD. Data are presented as means ± SEM. In C, a representative force-time curve during maximal squat jump showing changes (before: solid line; after: dotted line) in maximal ground reaction force and RFD in 1 subject.
**Study III.**

The mechanisms underlying the effects of 24-weeks resistance training combined with a healthy diet on skeletal muscle mass are examined. Analysis of biopsy samples of all participants collected at baseline revealed that CSA type IIA fibres is significantly smaller than CSA type I fibres. Interestingly, a significant increase in CSA type IIA occurred in RT-HD only (+23%, Fig. 4) and there were no significant changes in CSA type I fibres in any of the study groups (Fig 4). At baseline, myonuclear content was significantly lower in type IIA compared to type I fibres in participants from the 3 groups. While there were no significant changes in myonuclear content of type I and type IIA fibres in response to the intervention, the difference in myonuclear content between the two fibre types at baseline was significantly attenuated in RT-HD only, supporting the occurrence of a slight elevation of the myonuclear number in type IIA fibres in 9 out of 11 participants belonging to this group. At baseline, SC number was significantly lower in type IIA compared to type I fibres in participants from the 3 groups and no changes in SC number were detected in response to the intervention period in any of the study groups. An additional experiment was performed to determine whether increases in muscle mass (*in-vivo* hypertrophy phenotype) influence *in-vitro* proliferative capacity of myogenic cells. A significant reduction of myoblast doubling time indicating improvement of the proliferative capacity of myogenic cells occurred in participants exhibiting muscle hypertrophy and not in participants without hypertrophy (Fig. 5). We also sought to determine the chronic effects of the RCT on gene expression of specific inflammatory markers in skeletal muscle. A significant down-regulation in gene expression of the pro-inflammatory marker IL-1β occurred only in RT-HD (Fig. 6). No significant changes in the expression of NFKB1, RelA or TLR1 (toll-like receptor 1) were observed. Additionally, there was a significant upregulation of mTOR gene expression in RT-HD only (Fig. 6), while gene expressions of IGF-I (insulin-like growth factor 1), FoxO1, MSTN (myostatin), GABARAP, Bax, Bad and Bcl-2 remained unchanged.
Figure 4. Relative changes in cross-sectional area of type I and type IIA fibres in skeletal muscle of older women in response to the 24-week intervention. Data are presented as means ± SEM. *p<0.05 vs. PRE

Figure 5. Myoblast proliferative capacity isolated from participants with (HYP+) and without muscle hypertrophy (HYP-). Data are presented as means ± SEM. *p<0.05 vs participants exhibiting no muscle hypertrophy.
Study IV.

The mechanistic links underlying the detrimental effects of elevated systemic inflammation on muscle mass in older adults are studied. An inverse relationship between serum CRP and skeletal muscle mass were detected (Fig. 7). In order to address the mechanism underlying the relationship between muscle mass and serum CRP, we adopted an in-vitro model of exposing human muscle cells to CRP. The size of human myotubes exposed to CRP for 72h was significantly lower (p<0.05) than that of control myotubes (Fig. 8). We hypothesized that the CRP-induced reduction of the size of muscle cells is partly mediated through a reduction in muscle protein synthesis. Muscle protein FSR of human myotubes exposed to CRP for 24h was measured using the stable isotope tracer deuterium oxide (D₂O). Our findings revealed that muscle protein FSR was significantly lower (p<0.05) in CRP-treated myotubes compared to control myotubes (Fig. 9).

Figure 6. Changes in mTOR and IL-1β gene expression after 24-wk intervention in healthy recreationally active older women. Changes are expressed as Log2 Fold-Change (Post/Pre), positive values indicating up-regulation and negative values indicating down-regulation. *p<0.05 vs CON.
Figure 7. Relationship between serum CRP level and skeletal muscle mass in elderly women, ($\beta = -0.646$ (95% CI: $-0.888$, $-0.405$) $p < 0.05$).

Figure 8. Size of human myotubes exposed to CRP for 72h. Myotubes are stained with antibodies against troponin T (green), myogenin (red) and the nuclear marker DAPI (blue). *$p<0.05$ vs. CON
Figure 9. Muscle protein fractional synthetic rate (FSR) in human myotubes exposed to CRP for 24h. *p<0.05 vs. CON.
General discussion

I. Combined resistance training and healthy diet in healthy recreationally active older women

a. Muscle mass and strength

Our findings showed that resistance training alone did not induce significant gains in muscle mass in healthy recreationally active older women. In fact, previous studies have also reported absence of gains in muscle mass in response to training in older men and women (Vincent et al., 2002, Kosek et al., 2006, Mackey et al., 2007, Hanson et al., 2009). Combining resistance training to a healthy diet high in n-3 PUFAs is a strategy that optimizes gains in muscle mass in healthy recreationally active older women.

Increases in whole-body lean mass (+1.5%) and leg lean mass (+1.8%, Fig 1) in response to the combined training and healthy diet intervention are relatively modest. In previous studies, the magnitude of changes in muscle mass generally ranges between +2% and +6% (Sillanpaa et al., 2009, Geirsdottir et al., 2012, Roth et al., 2001, Hunter et al., 2001, Bamman et al., 2003, Hartman et al., 2007, Leenders et al., 2013a). For example, Sillanpaa et al. (2009) reported a 2% increase in leg lean mass following 21 weeks resistance training in elderly women and Leenders et al. (2013a) reported a 2.9% increase in leg lean mass in healthy elderly women following 6 months resistance training. Increases in fat free mass averaging 2.1% were reported after 6 months of resistance training in participants who underwent a period of 8 month detraining prior to the start of the intervention (Roth et al., 2001). Inter-individual variations in the anabolic response to a similar physiological stimulus and inclusion of older adults with different levels of habitual physical activity level may explain differences in the magnitude of changes of muscle mass. It has been proposed that anabolic resistance is an important condition accounting for the weak anabolic response to resistance training in older adults (Kumar et al., 2009). Noteworthy, the annual rate of loss of muscle mass in elderly women over the age of 65 years approximates 1% (Goodpaster et al., 2006), which is in line with the observed loss of whole-body lean mass (-0.4%, during 24 weeks) which occurred in older women belonging to our control group. Therefore, the modest increases in whole-body lean mass (+1.5%) and leg lean mass (+1.8%) observed in response to 24 weeks of resistance training and healthy
diet is of clinical relevance as it corresponds to approximately twice the annual rate of loss of muscle mass in older women (Goodpaster et al., 2006).

Resistance training in healthy recreationally active older women induces improvements in several indicators of muscle strength, indicating training-induced beneficial effects even in older individuals regularly involved in recreational activities. However, combined resistance training and healthy diet further enhanced improvements in indicators of dynamic muscle power observed in response to resistance training alone (Fig. 2). These additional gains may prove important as muscle power is particularly useful during usual activities of daily living (Hakkinen et al., 1998a). Additionally, the combined intervention further optimized gains in peak force and rate of force development during the take of phase of squat jump exercises (Fig. 3). Squat jump is a functional test that requires rapid increases in muscle force in the lower limbs under weight-bearing multi-joint conditions. Thus, an improvement in squat jump performance in older women reflects improved capacity to rapidly generate force during a complex and coordinated motor task (Caserotti et al., 2008).

In line with the observed gains in muscle mass, significant improvements in the cross-sectional area of fast type IIA fibres were observed (Fig. 4). Enlargement of type IIA muscle fibres occurred without addition of new myonuclei, indicating that existing myonuclei were able to support the ~23% hypertrophy of this specific fibre type. Findings from previous studies suggest that increases in myonuclear number is not prerequisite for the process of hypertrophy (Hikida et al., 2000, Petrella et al., 2006, Verdijk et al., 2009a) and it has been suggested that myonuclear accretion in humans only occurs when the size of muscle fibres reaches an upper limit beyond which existing myonuclei are not able to sustain cellular growth (Kadi et al., 2004b).

The occurrence of type IIA-specific muscle fibre hypertrophy in RT-HD only, was accompanied by an increase in the n-3 PUFA DHA, which is in line with data suggesting that n-3 PUFAs may promote muscle hypertrophy (Smith et al., 2011, Smith et al., 2015, Yoshino et al., 2016) through enhanced activation of the mTOR growth pathway (Yoshino et al., 2016). In the present work we report an up-regulation of mTOR gene expression in response to chronic resistance training combined to diet rich in n-3 PUFAs in older women (Fig. 6). Although phosphorylation rate rather than fluctuation of basal transcriptional activity is the hallmark of exercise-mediated
impact on mTOR-signalling in skeletal muscle, it is suggested that changes in transcriptional activity may lead to increased mTOR protein pool available for phosphorylation in muscle from older women.

b. Systemic markers of inflammation and gene expression of inflammatory factors in skeletal muscle

Our results clearly show resistance training alone or combined to healthy diet has no impact on several inflammatory markers in healthy recreationally older women with relatively low baseline systemic inflammatory status. However, in this group of older women, a reduction in circulating arachidonic acid (AA) levels as well as a down-regulation of skeletal muscle IL-1β gene expression (Fig. 6) occurred. Previous studies showed that n-3 PUFAs plasma levels are independently associated with lower levels of pro-inflammatory markers and higher levels of anti-inflammatory markers (Ferrucci et al., 2006) and that n-3 PUFAs inhibit IL-1β synthesis in PBMC (peripheral blood mononuclear cell) (Caughey et al., 1996). Reduced circulating AA may have a positive impact on muscle mass as higher concentrations of AA have been linked to reduced size of skeletal muscle (Reinders et al., 2015). Additionally, it is suggested that AA is involved in the induction of muscle protein degradation through increased expression and activity of the ubiquitin–proteasome pathway (Whitehouse et al., 2003). Moreover, the observed down-regulation of IL-1β gene expression may have facilitated muscle hypertrophy in older women. In fact, increases in IL-1β mRNA levels in humans have previously been reported and it is suggested that this age-related dysregulation may limit muscle hypertrophy in older adults (Przybyla et al., 2006). Indeed, up-regulation of IL-1β gene expression has been linked to muscle atrophy in animal models (Schulze et al., 2003) and chronic exposure to IL-1β has been shown to reduce myofibrillar protein content and myotube diameter in cultured C2C12 cells (Li et al., 2009). This effect might be mediated through enhanced expression of the atrophy related genes, atrogin1/MAFbx and MuRF1 in rodent skeletal muscle (Bodine et al., 2001a). Altogether, our data suggest that combining resistance training to healthy diet rich n-3 PUFAs may have subtle anti-inflammatory effects that facilitated increases in muscle mass.

c. Proliferative capacity of myogenic cells

The regenerative capacity of skeletal muscle relies on the proliferative capacity of satellite cells, which represent a pool of myogenic cells able to enter
a phase of proliferation to generate new muscle tissue. It is suggested that the proliferative capacity of satellite cells is attenuated with advanced age (Barani et al., 2003). Traditionally, the regenerative capacity of skeletal muscle has been indirectly explored through evaluation of satellite cell number in muscle cross-sections and there is paucity of studies directly assessing the proliferative rate of myogenic cells in human biopsies. In the present work, evaluation of satellite cell number in muscle cross-sections showed no significant changes in the number of satellite cells in skeletal muscle from older women in response to the intervention. It has been suggested that myogenic cells isolated from human biopsies may retain the in-vivo phenotype of the muscle. For example, impaired in-vitro glucose metabolism is observed in myogenic cells isolated from patients with disturbed glucose metabolism (Gaster et al., 2002, Henry et al., 1996). In our work, we sought to examine the in-vitro proliferative rate of myoblasts harvested from a subset of older women with or without hypertrophy. We report for the first time the occurrence of significant improvements of the proliferative capacity of myoblasts from older women when skeletal muscle hypertrophy occurs (Fig.5), indicating that in-vivo enlargement of skeletal muscle alters in-vitro behaviour of myogenic precursor cells. Only a few studies have previously investigated the effects of exercise in humans on in-vitro properties of myogenic cells. In one study, 8 weeks of endurance training improved in-vitro glucose metabolism of myogenic cells isolated subjects with improved in-vivo glucose metabolism (Bourlier et al., 2013). More recently, 12 weeks of combined endurance and resistance training improved in-vitro lipid metabolism of myogenic cells isolated from overweight men (Lund et al., 2017). It is suggested that an age-related decline in the proliferative capacity of muscle stem cells (Barani et al., 2003, Schultz and Lipton, 1982) may have adverse effects on the regenerative capacity of skeletal muscle (Conboy et al., 2003, Shefer et al., 2006) and contributes to the age-related muscle atrophy. Altogether, our data suggest that the occurrence of hypertrophy at the whole muscle level and at the level of individual muscle fibres has an influence on the physiological properties of muscle stem cells. The mechanisms responsible for cellular communication between the stem cell niche and its environment in skeletal muscle remain to be elucidated.
II. Mechanisms of loss of muscle mass

The exact mechanism by which ageing reduces muscle mass remains unclear. In recent years, the possible negative influence the systemic environment on muscle mass has gained interest in the scientific community. For example, the exposure of myogenic cells from old donors to sera from young may rejuvenate cell proliferative capacity (Conboy et al., 2005, Carlson et al., 2009, Barberi et al., 2013).

Our work highlight the negative influence of circulating CRP on muscle mass. This was illustrated by the negative relationship between CRP level and muscle mass in older women (Fig. 7). In order to address the link between serum CRP and muscle mass, we adopted an in-vitro model where human myogenic cells were exposed to CRP. We identified a CRP-mediated reduction in the size of human myotubes (Fig. 8) through the reduction of muscle protein synthesis (Fig. 9). These findings are in line with the reduced proliferative capacity of myogenic cells exposed to sera from elderly women with elevated CRP levels (Wahlin-Larsson et al., 2014). Given that myofibrillar proteins represent ~85% of muscle fibre volume (Hoppeler, 1986) any factor able to influence the balance of myofibrillar protein metabolism may contribute to muscle atrophy. Therefore, our in-vitro data provide a mechanistic model supporting epidemiological links between elevated chronic inflammation and reduced muscle mass and suggest a direct action of CRP on the size of muscle cells. It is suggested that the influence of CRP on muscle protein synthesis may be mediated via alterations in two pathways playing an important role in muscle protein synthesis, Akt/mTOR growth signalling pathway and AMPK, a master regulator of cellular energy homeostasis. Nevertheless, several other signalling pathways may have mediated the CRP-induced reduction in myotube size (Schiaffino et al., 2013). The delineation of the exact pathways responsible for age-related decline in muscle is an important future challenge.

Methodological considerations and limitations

Several previous investigations on the effects of training and diet in older adults were conducted over relatively shorter periods (8-12 weeks) than our RCT (24 weeks). Our study duration was selected in order to address the effects of long term lifestyle changes. A major challenge in our work as in any other long term intervention is the retention of participants over the course of the study.
The nutritional intervention used in this work was delivered as a whole-diet approach instead of using dietary supplements. This dietary design is less rigorous than controlled-nutrient diets. However, this approach reduces participant burden and is compatible with long-term lifestyle changes. Measurement of the n-3 fatty acid DHA serum level further confirmed dietary compliance. The present work did not address potential influence of dietary changes alone on muscle function in older women. In fact, several previous studies suggest that diet alone may not trigger myotrophic effects in elderly (Fiatarone et al., 1994, Leenders et al., 2011, Verhoeven et al., 2009). Nevertheless, a few reports indicate that n-3 PUFAs supplementation may improve muscle mass and strength (Smith et al., 2015, Smith et al., 2011). The question of whether dietary changes alone may influence muscle function in healthy recreationally active older adults remains unanswered.

Participation in regular exercise training programs might lead to the adoption of new physical activity behaviours. This might influence the physiological response to the training intervention. In this work, we have assessed physical activity level of participants before, during and at the end of the training intervention and found no changes in physical activity level. Several previous investigations addressing the impact of exercise on chronic systemic inflammation included individuals with elevated inflammatory markers and study groups were not stratified by systemic inflammatory status. To ensure that comparison groups were as similar as possible with regard to inflammatory level, the randomization was stratified by CRP level.

Measurement of muscle mass can be conducted using several methods. Computed tomography (CT) and Magnetic resonance imaging (MRI) methods are more accurate than Dual-energy x-ray absorptiometry (DXA) and Bioelectrical impedance analysis (BIA) measurements. DXA used in our intervention, as in other studies, has previously been considered as a reasonable alternative to CT and MRI for assessment of body composition (Lee et al., 2001) and previous studies have reported good between-method agreement in the determination of muscle size (Fuller et al., 1999, Maden-Wilkinson et al., 2013, Visser et al., 1999). In studies reporting on associations between body composition and other parameters in large populations, BIA is considered as an accurate measurement of functioning muscle mass. In standardized conditions (body position, previous exercise, hydration status and dietary intake) and using equation cross-validated against MRI, BIA method is currently considered as an accurate measurement of functioning
muscle mass in clinical settings and epidemiological studies. The advantages of BIA (no exposure to radiation, easy to use, relatively low cost and safety) make it appropriate for large-scale studies.

The effects of resistance training on muscle strength are mediated through neuromuscular changes. In this work, the occurrence of electrophysiological changes in response to the intervention have not been addressed. However, our data clearly suggest that gains strength performance in older women are not entirely explained by changes in muscle mass and that neural factors contributed to changes in muscle performance.

The analysis of cellular changes in muscle biopsies is based on a relatively low number of muscle biopsies. However, the significant increase in the area of type IIA fibres in RT-HD and not in other groups is in line with the significant increase in leg lean mass and explosive dynamic muscle strength that occurred only in RT-HD. The data from our *in-vitro* models were also based on a relatively low number of subjects. However the use of cells from several donors allowed for biological variability.

Our work examined the impact of training and diet in healthy recreationally active older women but not in older men. In general, women might be at higher risk of developing sarcopenia, which highlights the importance of designing specific strategies able to counteract the decline of muscle mass and function in older women. Thus, our conclusions of the present intervention may not apply to a larger sample of older adults with poorer functional status and older men.

**Implications**

The practice of resistance training is recommended by major health organisations including American College of Sports (2009) (ACSM). Current recommendations state that resistance training should be conducted two or more non-consecutive days per week. In the present work, the resistance training program was based on the recommendations from ACSM, targeting muscle hypertrophy (i.e. a workload of 75-85% 1RM (8-12 reps/set)). The fact that resistance training alone did not induce significant gains in muscle mass in healthy recreationally active older women suggests that exercise recommendations in this population should take into consideration the potential additive benefits of diet-related adjustments.
Future perspectives

Our data clearly highlight the benefits of combining resistance training with nutritional changes to optimize gains in muscle mass and function in recreationally active older women. Preventing age-related functional decline would reduce risk of disability, loss of independence and hospitalization at very old ages, thus extending healthspan. Our work has a preventive dimension and it remains to examine to which extent a similar intervention can reverse (rejuvenate) age-related loss of muscle mass and function in older men and women with deteriorated function.

A healthy diet rich in n-3 PUFAs can be beneficial in term of potentiating the adaptation of skeletal muscle to resistance training in older adults. It remains to determine whether the timing of the dietary intake rich in n-3 PUFAs in combination to resistance training is a determinant factor for optimization of training adaptations.

The weak impact of resistance training alone on muscle mass in recreationally active older women raises several aspects: (1) which factors reduce the impact of training on muscle mass? (2) are recommendations for resistance training in older adults optimal? Future work is needed to determine adequate population-specific resistance training doses. It is also important to consider examining the acute effect of such interventions in order to gain knowledge on early signalling pathways involved in the muscle adaptations. In particular, the cross-talk between muscle inflammatory reactions and anabolic responses merits further attention.

Our work also shows that chronic systemic inflammation has a negative impact of skeletal muscle mass. A future major challenge is to identify the origin of the chronic elevation in systemic markers of inflammation in older adults. The effects of CRP on muscle cell size may be mediated through cross-talk between AMPK and Akt/mTOR pathway. Future investigations should provide a detailed characterisation of upstream and downstream effectors, which mediate the effects of CRP.
Conclusions

The present thesis depicts mechanistic links between elevated systemic marker of inflammation and muscle mass and provides new information on the effects of combined resistance training and healthy diet on muscle mass and strength in a group of healthy recreationally active older women. This knowledge is instrumental for development of strategies aiming to prevent age-related loss of muscle mass and function. It is concluded that

- Combined resistance training and a healthy diet rich in n-3 PUFAs induces greater gains in dynamic explosive force than resistance training alone in healthy recreationally active older women.
- Combined resistance training and a healthy diet rich in n-3 PUFAs rather than resistance training alone induces gains in leg lean mass of healthy recreationally active older women.
- Gains in leg lean mass in response to combined resistance training and a healthy diet rich in n-3 PUFAs are accompanied by increases in the size of type IIA muscle fibres together with down-regulation in gene expression of a pro-inflammatory factor (IL-1β) and up-regulation in gene expression of a regulator of cellular growth (mTOR) in skeletal muscle of older women.
- Reduced muscle protein synthesis and size of muscle cells may mediate the detrimental effects of elevated circulating markers of inflammation on muscle mass in older adults.
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Svensk sammanfattning

Åldersrelaterad förlust av muskelmassa och styrka kan ha en negativ inverkan på äldres vardag. Att identifiera nya effektiva strategier för att motverka denna åldersrelaterade förlust blir då viktig för ett hälsosamt åldrande.

I denna avhandling undersöktes effekterna av styrketräning med eller utan kombinationen av en hälsosam diet på muskelmassa och styrka hos friska, fysiskt aktiva äldre kvinnor. Vidare så undersöktes även hur ökade inflammationsnivåer kan bidra till den åldersrelaterade förlusten av muskelmassa.


Delarbete I undersökte effekterna av interventionen på muskelstyrka, muskelmassa och inflammation hos friska och fysiskt aktiva äldre kvinnor. Resultat från denna studie visar att muskelstyrka ökar oavsett inkluderingen av en hälsosam diet, men att kombinationen av styrketräning och den hälsosamma dieten behövs för att muskelmassan i benen ska öka. Vidare så observerades inga signifikanta förändringar i de inflammatoriska markörerna CRP och IL-6. I delarbete II undersökte effekterna av interventionen på dynamisk och isometrisk styrka, explosiv muskelstyrka samt fysisk funktion. Resultat från denna studie visar att explosiv muskelstyrka ökade i båda grupperna som deltog i styrketräningen. Ökningen i explosiv muskelstyrka var större i gruppen som kombinerade styrketräningen med den hälsosamma dieten. I de funktionella testerna var förbättringarna likartad mellan de två grupperna som deltog i styrketräningen. Delarbete III undersökte cellulära och molekylära förändringar i muskulaturen efter den 24 veckor långa interventionen. I denna studie observerades signifikanta ökningar i typ IIA muskelfiberstorlek endast hos gruppen som kombinerade styrketräning med den hälsosamma dieten (+23%). I samma grupp identifierades även en nedreglering i genuttryck av en pro-inflammatorisk faktor (IL-1β) och uppreglering i genuttryck av en faktor involverad i cellulär tillväxt (mTOR). I delarbete IV undersökes sambandet mellan inflammation

Denna avhandling påvisar länken mellan ökad inflammation och minskad muskelmassa. Dessutom bidrar den med ny kunskap om effekter av styrketräning i kombination med en hälsosam diet på muskelmassa och styrka bland friska, fysiskt aktiva äldre kvinnor. Resultaten från denna avhandling kan bidra till utveckling av strategier som syftar till att förhindra åldersrelaterad förlust i muskelmassa och styrka. Denna avhandling konkluderar:

- kombinationen av styrketräning och hälsosam diet, med ökat omega-3 intag, resulterar i större ökningar i explosiv muskelkraft än endast styrketräning hos friska, fysiskt aktiva äldre kvinnor.

- kombinationen av styrketräning och hälsosam diet, med ökat omega-3 intag, resulterar iökning av muskelmassa i benen hos friska, fysiskt aktiva äldre kvinnor.

- ökningen av muskelmassa i benen, som observerades när styrketräning kombinerades med den hälsosamma dieten, åtföljdes av en ökning i typ IIA muskelfiberstorlek tillsammans med en nedreglering i genuttryck av en pro-inflammatorisk faktor (IL-1β) och uppreglering i genuttryck av en faktor involverad i cellulär tillväxt (mTOR).

- reducering i muskelproteinsyntesen och minskning av muskelcellstorlek kan förmedla effekterna av ökade inflammationsnivåer på muskelmassa hos äldre.
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