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Native whey- and milk-protein supplementation combined with resistance exercise, induces similar anabolic signaling-responses downstream of mTOR in elderly.

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Abstract

Background: Reduced muscle mass seen with age, often referred to as age-related atrophy or sarcopenia, has negative effects on physical function. There are frequent reports of a blunted anabolic response in elderly to both resistance exercise (RE) and protein intake, and this is referred to as anabolic resistance. Translation initiation and elongation signaling responses after a single exposure of RE and protein intake is often used as indexes of anabolic responsiveness.

Aim: To examine whether habitual intake of un-denatured and specially filtrated native whey (NW) with a faster absorption-rate and higher leucine content than milk, would lead to greater hypertrophy and 1RM strength-gains after 11 weeks of resistance training, mirrored by increased anabolic signaling.

Methods: Two groups of elderly (total of 9 men and 7 women), mean age 73 years completed a 10RM whole-body workout combined with intake of 20 g NW- or milk-protein before (acute 1) and after (acute 2) 11 weeks of strength training. Muscle biopsies were collected from m. *vastus lateralis* pre- and 2 hour post-exercise. Tissue samples were analyzed for total protein- and phosphorylated levels of p706SK, 4E-BP1 and eEF2.

Results: The ratio of phosphorylated/total p706SK increased significantly on acute 2, and with a strong tendency on acute 1. Ratio of phosphorylated/total 4E-BP1 and eEF2 remained unchanged from baseline in both groups on acute 1 and 2. There was no differences ($P>0.05$) in any of the studied signaling proteins within each group, or between groups on either acute day. Both groups experienced similar increases in m. *vastus lateralis* thickness (+6-7%), lean body mass (2.1-2.3 kg) and 1RM strength (+20-40%). Phosphorylation patterns of our studied signaling proteins 2 hours post-RE and -protein intake were not correlated to any long-term outcome measure on an individual level.

Discussion and conclusion: The difference in leucine-content between NW and milk might have been too small to detect differences between our groups. Supplementation with equal amounts of protein from NW and milk resulted in similar signaling for muscle hypertrophy and similar gains in *m. vastus lateralis* muscle thickness, muscle mass and muscle strength. Our findings emphasize that acute measures of intracellular anabolic signaling responses downstream of mTORC1 should be interpreted with caution if used as indexes of responsiveness to repeated RE and protein intake.

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Preface

This thesis is the result of a year of hard work, completing a study much more comprehensive than I had imagined it to be. It has been a great experience with a vast increase of my own knowledge in this field of research. It has been a long process that has given me first-hand experience into how to plan and conduct a study of this size. Through countless hours of trial and error, I have learned the necessary precision and skill needed in lab-work at this level. Being a large study iam proud to have been a part of, there are several people involved that deserve to be mentioned.

I want to thank Truls Raastad and Håvard Hamarsland for guidance along the way and an almost annoyingly positive outlook on everything, even in times when everything seemed impossible. A special thank you goes to Håvard, being the head organizer of this study. All those times we had to unexpectedly reschedule testing and training, it was always nice to be able to reply our participants with "send an e-mail to Håvard, he knows how we can organize this for you".

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Background

Maintaining an adequate amount of skeletal muscle is crucial for human locomotion, sports performance and several health parameters such as avoiding obesity, type 2 diabetes and age-related atrophy in the elderly. In this context it is of the greatest importance to develop efficient strategies to enhance or maintain muscle mass. Interventions combining resistance exercise (RE) and protein-intake show that these external stimuli are potent regulators of muscle mass and strength gains (Cermak et al, 2012). Understanding how these external stimuli affects internal signaling cascades related to hypertrophy/atrophy will increase the efficacy of recommendations with regards to resistance training and protein intake.

There are frequent reports of a blunted response in elderly to both exercise and protein intake (Cuthbertson et al, 2005; Katsanos et al, 2005; Kumar et al, 2009; Paddon-Jones et al, 2004), and this is referred to as anabolic resistance (Cuthbertson et al, 2005). Data is unequivocal in regards to diminishing muscle mass seen with age, often referred to as age-related atrophy or sarcopenia (Janssen & Ross, 2005). This loss of muscle mass could be a consequence of anabolic resistance or a more sedentary lifestyle as seen with aging. Anabolic resistance could lead to a more sedentary lifestyle, as well as a sedentary lifestyle itself could lead to anabolic resistance. Nonetheless, it becomes apparent that in the aging population, we need efficient countermeasures to overcome the well-documented anabolic resistance and reductioins in muscle mass seen with age.

Of different exercise-modes, RE is by far the most efficient countermeasure to atrophy. There is wealth of evidence that progressive resistance training causes hypertrophy in healthy individuals, regardless of gender and age (Peterson et al, 2010; Peterson et al, 2011; Cermak et al, 2012). Protein intake has been examined with regards to dosing, timing and types of protein used. These factors are often mentioned to be main contributors to the varying degrees of subsequent anabolic signaling and hypertrophy in relation to protein intake. While it is well established that the most potent anabolic effects of protein intake require sufficient dosing and an amino acid profile containing a high amount of essential amino acids (especially leucine), there is limited data showing protein timing to be of significant importance,

as long as sufficient amount of protein is consumed. Of the most frequent protein sources examined, whey, soy and casein are all proven to enhance muscle protein synthesis (MPS), but of different magnitude. Whey has been shown to be superior to soy and casein in creating a fast and robust MPS-response, often explained by its faster digestion-rate and higher leucine-content (Tang et al, 2009; Reidy et al, 2014).

In general, measuring functionally relevant changes in muscle mass over time is done with different methods of a non-invasive character (Ultrasound, DXA-scan and MRI). Acute changes are measured by more invasive methods (tracer infusion and biopsies). Methods of a non-invasive character are generally used to quantitatively assess changes in muscle mass and size, fat mass, bone mass etc. Combining such outcome-measures with more invasive methods can also give insight into the mechanisms behind these changes. With tracer infusion of labelled amino acids one can calculate and analyze the temporal MPS accumulation in the acute post-workout phase. Muscle tissue from biopsies is frequently used together with immunohistochemical and Western blot analysis to reveal changes in the phosphorylation patterns of intramuscular signaling proteins related to MPS (Cuthbertson et al, 2005; Reitelseder et al, 2011; Churchward-Venne et al, 2012).

Theoretically, acute anabolic responses to exercise and protein intake would accumulate and translate into hypertrophy after a prolonged intervention period. Acute studies are often with participants in the fasted state, and in highly controlled laboratory settings, not necessarily reflecting everyday life. Combining both non-invasive and invasive methods within the same study group over a substantial timeframe could give further insight to how we can relate acute intramuscular changes with long-term achieved hypertrophy and strength gains. Before exercise and nutritional strategies to slow or reverse the progression of sarcopenia can be maximized, there are several areas of research that still require further investigation.

Translation initiation and elongation signaling responses after a single exposure of RE and protein intake is often used as indexes of anabolic responsiveness. By collecting biopsies and through subsequent Western-blot analysis we wanted to examine whether hypertrophy and strength-gains are mirrored by anabolic intracellular signaling responses. To make data from this study applicable to a larger population

we have included both men and women. This study could provide an improved understanding of the cellular mechanisms that contribute to the protein synthetic response in skeletal muscle, thus provide a basis to develop more promising exercise and protein-intake strategies.

We have conducted a randomized controlled longitudinal study in an elderly population combining measures of long-term hypertrophy, strength gains and body composition changes with acute intracellular responses. By this study-design we wanted to examine whether habitual intake native whey with a faster absorption-rate and higher leucine content than milk (Laahne J.A, 2013; Aas, S, 2014), would lead to greater hypertrophy and 1RM strength gains after 11 weeks of resistance training. Furthermore, we wanted to examine whether greater hypertrophy and strength gains with native whey were mirrored by increased anabolic intramuscular signaling, thought to give an anabolic advantage.

1.1. Hypothesis

Supplementation with native whey immediately after resistance exercise will lead to greater activation of key signaling proteins downstream of mTORC1 than consuming the same amount of protein through milk, resulting in greater hypertrophy and 1RM strength gains in elderly men and women after 11 weeks of strength training. Furthermore, we expect 11 weeks of strength training and protein supplementation to be efficient countermeasures to the anabolic resistance in elderly, and expect enhanced anabolic signaling post-intervention compared to pre-intervention.

2. Theory

2.1. The dynamic equilibrium of human skeletal muscle and net protein balance

Human skeletal muscle exist in a dynamic equilibrium which constantly responds to the load of training and energy intake to determine the rate of muscle protein synthesis (MPS) and breakdown (MPB) (Adams & Bamman, 2012). The dynamic state of MPS and MPB (MPS-MPB = net protein balance) will over time determine subsequent hypertrophy/atrophy/equilibrium. In healthy individuals, net protein balance is determined and constantly altered, mainly by external stimuli such as mechanical stimuli (e.g. RE) and nutrients (protein intake) (Tang et al, 2009; Tipton et al, 1999).

MPB exceeds MPS in the post absorptive (fasted) state and MPS exceeds MPB in the postprandial (fed) state. Post-exercise, both MPS and MPB remain elevated, and MPS exceeds MPB only when amino acids are available (Biolo et al, 1995; Tipton et al, 1999). To understand how net protein balance determines subsequent hypertrophy/atrophy we have to map out how external stimuli (e.g. RE and protein intake) translates into intracellular signaling cascades and subsequent positive net protein balance. At the most fundamental level it is necessary to up-regulate the translational machinery to sustain a net increase in protein balance and achieve hypertrophy. MPS is individually up regulated by both RE (Phillips et al, 1997; Burd et al, 2010) and protein intake (Biolo et al, 1997; Moore et al 2009). The combination of the two stimuli has an additive effect on MPS, causing a more potent positive effect on net protein balance than either stimulus by itself (Dreyer et al, 2008; Moore et al, 2009; Burd et al, 2010). RE and protein intake in relation to MPS will be further elucidated in sections below.

MPB is less documented and understood than MPS *in vivo*, because of more difficult and less developed study-techniques of MPB after exercise and/or protein intake (Glynn et al, 2010). There are multiple processes involved in MPB including the ubiquitin-proteasome system (UPS), lysosomal and calcium-activated systems, and caspases (Fry et al, 2013). Within the UPS, there are two E3 ubiquitin ligases called MuRF1 (muscle specific RING Finger 1) and MAFbx (muscle atrophy F-box, also

known as atrogin-1). Both these ligases are regulated through the Forkhead box (FOX) family of transcription factors (FOXO1, FOXO3a, FOXO4), inhibited by Akt through phosphorylation (Glynn et al, 2010; Fry et al, 2013). High levels of insulin activate Akt, so even though carbohydrate ingestion itself has no effect on MPS (Koopman et al, 2007), it has been shown to reduce MPB through Akt, when provided in sufficient amounts (>40 g) after exercise (Borsheim et al, 2004). Thus, the role of insulin in net protein balance is limited to minor anti-catabolic effect (reduced MPB) (Glynn et al, 2010, Fry et al, 2013). Furthermore, there appears to be no difference in MPB post-exercise between young and elderly (Fry et al, 2013), making MPS the main target for strategies to induce hypertrophy/maintaining muscle mass. At the same time a certain level of MPB is necessary to remove proteins that are no longer functional, thus completely removing MPB remains disadvantageous.

2.2. Resistance Exercise and MPS

Independent of contraction-type, RE has been shown to increase MPS for up to 48 hours post-exercise in both genders in an untrained group (Phillips, et al, 1997), whereas in a trained group it was elevated for 24 hours (Tang et al, 2008). Exercise-induced MPS is prolonged and of greater magnitude in the contractile elements than the sarcoplasmic protein synthesis (Moore et al, 2009). The same study (Moore et al, 2009) showed that the sarcoplasmic protein synthesis was exclusively stimulated by protein intake. Intensity and volume has been shown to determine the MPS-response to an RE bout, with increased volume and intensity augmenting and prolonging the MPS-response post-exercise (Kumar et al, 2009; Burd et al, 2010). Both young and old showed no significant increase in MPS at intensities <40% of 1RM whereas at intensities >60% of 1RM, MPS can increase 2- to 3-fold (Kumar et al, 2009). However, this does not infer that RE at lower intensities is incapable of inducing an MPS-response, if volume is increased (Burd et al, 2010). At 30% of 1RM, the magnitude of MPS-response is comparable to 90% of 1RM, if performed to failure and volume of work is not matched (Burd et al, 2010). Furthermore, RE stimulates MPS by increasing amino acid transport into active skeletal muscles (Biolo et al, 1995) an effect shown to be independent of age and gender (Drummond et al, 2011). RE up regulates both MPS and MPB, thus making post-exercise protein-intake necessary to support a lasting MPS-response. If one remains in the post absorptive

state, MPS is depressed and MPB stays elevated, causing a negative net protein balance (Biolo et al, 1995; Phillips et al, 1999; Tipton et al, 1999).

2.3. Protein intake and MPS

Increased availability of circulating amino acids leads to a more positive net protein balance at rest (Biolo et al, 1997; Smith et al, 1998) and after resistance-exercise (Biolo et al, 1997; Phillips et al, 1997). The anabolic effects of feeding are shown to be entirely contributed by the essential amino acid (EEA) content (Cuthbertson et al, 2005; Volpi et al, 2003). Adding additional carbohydrate post-exercise does not enhance the MPS-response (Koopman et al, 2007). Cuthbertson et al (2005) showed that EEA stimulates MPS independently of insulin, and that a basal rate of MPS is not decreased in the elderly compared to the young. Furthermore, a decreased sensitivity and delayed responsiveness to EEA intake in the elderly has been shown (Drummond et al, 2008; Paddon-Jones et al, 2004).

The anabolic effect of EEA in the young is dose-dependent from 2.5 g and saturates at amounts equivalent of ~10 g EEA, ~2.1 g leucine (Cuthbertson et al, 2005) or ~20 g of high quality protein (Moore et al, 2009). The MPS response peaks ~2 hours after protein intake, and at this time point skeletal muscle becomes refractory to further stimulation, despite continuous infusion of amino acids (Bohé et al, 2001; Atherton et al, 2010). This EEA saturation is termed the "muscle full" concept (Atherton et al, 2010). The importance of dosing rather than delivery-profile of protein intake has been reported by two recent studies (Kim et al, 2015; Mitchell et al, 2015). Kim et al (2015) showed an increase in MPS when doubling the recommended dietary allowance (RDA) in elderly, but no effect of how this protein amount was spread out during the day. Furthermore, Mitchell et al (2015) showed that equal protein doses gave the same MPS response and muscle-full effect regardless of whether protein intake was ingested as a bolus or spread out in smaller portions. In summary, the anabolic effects of feeding are entirely contributed by EEA-content, which according to the muscle full concept has a saturating response at around 20 g high quality protein, ~10 g EEA and ~2 g leucine

2.4. Additive effect of resistance exercise and protein intake

The combination of RE and protein intake has an additive effect on net protein balance that exceeds either of the stimuli alone (Biolo et al, 1995; Biolo et al, 1997; Dreyer et al, 2008; Moore et al, 2009; Burd et al, 2010). The time aspect of these anabolic stimuli is varied, a result of whether they are combined or not, and on the age and training status of the population examined (Phillips et al, 1997; Tang et al, 2008).

Protein intake results in an increased MPS-response which diminishes after ~2 hours (Bohe et al, 2001; Atherton et al, 2010). With prior RE and subsequent protein intake, MPS can be elevated for up to 48 hours in an untrained population (Phillips et al, 1997). Provision of sufficient EEA is critical to the induction of a positive net protein balance by exercise, with an EEA-deficiency abolishing this effect (Biolo et al, 1997). After unilateral knee-extension was performed for 8 weeks with the untrained leg acting as a control, MPS returned to baseline 28 hours after an acute bout of RE in the trained leg whereas MPS was significantly elevated in the untrained leg after 28 hours (Tang et al, 2008). In the same study (Tang et al, 2008) MPS in the trained leg was significantly elevated above the untrained leg at 4 hours post-exercise, showing a faster rise and decline in MPS. Because of a sustained MPS >28 hours, the untrained leg showed a greater total MPS-response to acute RE than the trained leg (Tang et al, 2008).

RE improves the hypertrophic response to protein intake by widening the previously described ”muscle full” concept. This widening allows for more amino acids to be incorporated into muscle protein during the postprandial period in both young and old (Pennings et al, 2011) but the effects are dependent on protein source (Yang et al, 2012a; Yang et al, 2012b). Elderly men aged 70, showed an incremental MPS-response to whey intakes ranging from 20 g to 40 g after RE, but a saturated MPS-response at just 20 g of whey, at rest (Yang et al, 2012b). When soy was used as a protein source, a more modest effect on MPS was shown with similar dosing (Yang et al, 2012a). In terms of the anabolic effect of EEA’s, the branched-chain amino acid (BCAA) leucine has received special attention. More on different protein sources will be further elucidated in the protein source section.

2.5. Anabolic resistance

The dynamic interplay between MPS and MPB governs changes in skeletal muscle size. Current methodologies suggest that relative to MPB, the rate of MPS is more dynamic and responsive to potent anabolic stimulus, such as the aforementioned RE and protein intake (Fry et al, 2013; Dreyer et al, 2008; Moore et al, 2009; Burd et al, 2010). Theoretically when repeated regularly, acute anabolic responses to these stimuli will accumulate over time and lead to gains in muscle size and strength. With increasing age, there is a decline in muscle mass with concomitant loss of muscle strength, referred to as sarcopenia (Janssen & Ross, 2005). This age-related loss of muscle mass is suggested to be a result of blunted responses to anabolic stimuli. Compared to young, elderly has been shown to have a blunted and delayed response to smaller boluses of protein intake (~10 g EEA) and RE (Moore et al, 2015; Cuthbertson et al, 2005; Katsanos et al, 2005; Kumar et al, 2009; Paddon-Jones et al, 2004) . These blunted responses to protein intake and RE has been termed ”anabolic resistance” (Cuthbertson et al, 2005).

Before protein intake is able to stimulate MPS, digestion, absorption, basal rates of MPS and amino acid transport into muscle fibers are possible limiting factors for the subsequent anabolic response. Early work by Boirie et al (1997) suggested that reductions in amino acid absorption and digestion in elderly could explain the blunted response to protein intake. Increased first-pass hepatic extraction is shown in elderly compared to young, which translates into a larger proportion of amino acids directed towards use in internal organs (Boirie et al, 1997; Volpi et al, 1999). In contrast, a more recent study (Koopman et al, 2009) showed no difference in first-pass hepatic extraction in elderly compared to young. The discrepancy between these studies could be related to small repeated feedings (Volpi et al, 1999) in contrast to a single large protein intake (Koopman et al, 2009). In spite of these differences, both studies (Volpi et al, 1999; Koopman et al, 2009) found no differences in MPS-response to protein intake between young an old. Furthermore, basal rates of MPS and MPB do not seem to differ between young and old (Cuthbertson et al, 2005; Fry et al, 2013; Katsanos et al, 2006), and is independent of gender (Fujita et al, 2007a).

2.6. Muscle uptake of dietary amino acids

As RE and increased amino acid availability increases amino acid transport (Biolo et al, 1995; Drummond et al, 2010), the reported blunted MPS responses in elderly (Cuthbertson et al, 2005; Drummond et al, 2008; Kumar et al, 2009; Fry et al, 2011) could infer reduced amino acid transport into muscle fibers in elderly. In this context there are studies that report a more rapid decline in plasma-leucine concentrations in younger compared to elderly after protein intake, which may be linked to reduced amino acid transport (Drummond et al, 2008; Koopman et al, 2009).

Amino acid transporters (AAT) affect net protein balance in their ability to activate the mTORC1-signaling pathway (Drummond et al, 2010; Drummond et al, 2011; Dickinson et al, 2014). Especially system-L (LAT1) and system-A (SNAT2) ATT's have received special attention because of their ability to sense changes in EEA availability (Dickinson et al, 2014; Drummond et al, 2011). Reduced amino acid transport into skeletal muscle could translate into reduced intracellular anabolic signaling. A diminished response to RE and protein intake has been reported as dampened mTOR signaling in elderly compared to young (Cuthbertson et al, 2005; Drummond et al, 2008; Kumar et al, 2009).

In contrast, others have shown no age-related reduction in intracellular signaling after RE and protein intake (Farnfield et al, 2012; Reidy et al, 2014). Drummond et al (2011) reported that increased EEA availability up-regulated mTORC1-signaling, MPS and AAT expression in both young and old, of both genders. Thus, alterations in AAT expression and function may be essential for regulating MPS after anabolic stimuli such as RE and protein intake. After RE and protein intake there is shown an up-regulation of LAT1 and SNAT2 in both young and elderly (Drummond et al, 2010; Drummond et al, 2011; Reidy et al, 2014; Dickinson et al, 2014). Furthermore, this up regulation of system L and A transporters is shown to coincide with increased MPS and phosphorylation of p706SK and 4E-BP1 (Reidy et al, 2014; Dickinson et al, 2014). Furthermore, Drummond et al (2011) reported increased AAT expression after RE to be equal in both young and elderly. In light of these studies (Drummond et al, 2011; Farnfield et al, 2012; Dickinson et al, 2014) it seems that elderly remain an efficient amino acid transport into skeletal muscle, and that participant's state of being

untrained/trained is as an important factor for downstream mTORC1-signalling as age.

2.7. Reduced anabolic response to protein intake and resistance exercise

If basal MPS in the post absorptive state is unrelated to age, the anabolic resistance in elderly compared to the young must be of postprandial character and/or related to the RE-response. It is a common observation that elderly is less sensitive than their younger counterparts to protein intake (Cuthbertson et al, 2005; Katsanos et al, 2005&2006; Moore et al, 2015) and RE (Kumar et al, 2009; Fry et al, 2011). In support of this, a meta-analysis consisting of 49 studies and 1328 participants (Peterson et al, 2011) identified a negative association between age and gains in lean body mass (LBM) after resistance training.

After resistance training without subsequent protein intake, it has been shown a greater MPS-response in young compared to the elderly (Kumar et al, 2009; Fry et al, 2011). Furthermore, protein intake under resting conditions show a need for a larger protein intake in the elderly of >20g protein, >15g EEA to maximally stimulate MPS (Cuthbertson et al, 2005; Paddon-Jones et al, 2004; Drummond et al, 2008; Yang et al, 2012b). A recent study (Moore et al, 2015) examined graded protein intakes (0-40 g) at rest, in elderly and young, showing an increased protein intake to be necessary to induce a maximal MPS-response in the elderly (0.6 g per kg lean body mass). Another recent study (Kim et al, 2015) divided elderly into two groups, one consuming the RDA of 0.8 g protein per day (1RDA) and the other consuming double the amount (2RDA). After 4 days of diet habituation, the 2RDA-group had close to a doubled net protein balance in the fed state, compared to the 1RDA-group.

When RE precedes protein intake there seems to be a higher saturation threshold in elderly compared to young, with elderly showing maximal MPS-response at 40 g of whey and the young having a saturated response at 20 g of whey (Yang et al, 2012a&b). Furthermore, Kim et al (2015) showed no effect of how protein quantities were spread out during the day, emphasizing the superior importance of protein quantity over intake pattern. This has been confirmed in young people were the

muscle full effect was dose dependent and not dependent on feeding pattern (bolus vs spread) (Mitchell et al (2015). The blunted response in elderly to smaller boluses (7 g) of EEA (Katsanos et al, 2005) is restored when EEA is doubled (15 g) (Paddon-Jones et al, 2004; Drummond et al, 2008). Drummond et al (2008) showed an equal MPS-response after RE and 20 g of EEA between young and old over a 6 hour period, with the young having a more pronounced MPS-response the first 3 hours, and the elderly showing a delayed but increased response between 3-6 hours. Combining resistance training and ingestion of 20 g casein (~7 g EEA) was shown to induce equal MPS-responses in young and old (Pennings et al, 2011). At rest, a moderate-protein meal of lean beef (30 g protein) was shown to induce a similar MPS-response in young and old (Symons et al 2009). Based on the above studies, elderly seem to respond to EEA, albeit in a delayed fashion, and may require larger doses than their younger counterparts.

The findings in the above mentioned studies (Katsanos et al, 2005; Paddon-Jones et al, 2004; Symons et al, 2009; Moore et al, 2015; Kim et al, 2015; Yang et al, 2012a&b; Mitchell et al, 2015) is further elucidated in several recent reviews (Deer&Volpi, 2015; Wall et al, 2014; Deutz et al, 2014), and it seems beneficial for the elderly to consume ~30 g of protein per meal and about 1.2-1.6 g/kg of protein per day.

2.8. Protein source

In addition to the demand for increased protein quantities in elderly, different sources of protein has been shown to affect the MPS-response of various magnitudes. Whey has been proven superior to casein and the plant-based soy-protein (Tang et al, 2009; Yang et al, 2012a&b; Burd et al, 2012). Following RE and matched intakes of 10 g EEA through either whey, soy or casein, digestion rates and MPS-response of the different protein sources proved to be whey>soy>casein in young men (Tang et al, 2009). Despite the fact that both whey and soy were deemed ”fast”-proteins in this study (Tang et al, 2009), whey had a superior MPS-response, possibly due to higher leucine content than in soy.

Whey protein is usually derived from cheese production, but newer technology allows extraction of whey from milk through filtration techniques, resulting in a higher leucine content. Native whey used in this study is produced in this manner, leaving the whey un-denatured as opposed to traditional whey supplements. Ingestion of native whey has been shown to be more readily absorbed, with significantly raised blood leucine concentration compared to milk and other whey supplements (Laahne, J.A, 2013; Aas, S, 2014).

2.9. Leucine

In addition to the effect of absorption-rate (Tang et al, 2009), leucine content is shown to be an important factor for subsequent MPS-response (Burd et al, 2012; Yang et al, 2012a&b) and is not dependent on any of the other amino acids (Katsanos et al, 2005; Rieu et al, 2006; Casperson et al, 2012). Other amino acids seem in this context to be only permissive for a longer lasting MPS-response (Tipton et al, 1999). Whey protein (2.8 g leucine) induced a greater MPS-response than casein (1.6 g leucine) when given as 20 g doses, both at rest and post-exercise in elderly (Burd et al, 2012). Corroborating these data, Yang et al (2012a&b) supplemented with 20 g and 40 g of either whey or soy protein, given at rest and post-exercise in elderly. Combining data from these studies (Yang et al, 2012a&b), whey induced a superior MPS-response compared to soy at both 20 g and 40 g dosing, both at rest and post-exercise. Whey and soy had a leucine-content of 2 g and 1.6 g respectively at 20 g supplementation. Tang et al (2009) also contributes some of the increased MPS-response in their study to increased leucine content in whey (2.3 g) compared to soy and casein (both being 1.8 g).

The attenuated MPS-response to protein intake in elderly is diminished by increasing leucine content (Katsanos et al, 2006; Rieu, 2006; Casperson et al, 2012; Dickinson et al, 2014). When given 6.7 g of EEA post-exercise, Katsanos et al (2006) showed that increasing leucine content from 26% to 41% restored the MPS-response in the elderly, reaching similar MPS as seen in the young. Furthermore, Churchward-Venne et al (2012) supplemented a sub-optimal dose of 6.25 g whey with 3 g leucine and saw a similar MPS-response as with 25 g of whey, both at rest and after RE. Rieu et al (2006) reported improved MPS when supplementing with 4 g of leucine after

ensuring a habitual intake of 0.8 g protein/day for 4 consecutive days before testing. Elderly men (mean age 69 years) went through an 8 hour isocaloric infusion protocol and the effect of leucine supplementation on MPS were entirely attributed to leucine content, as the only difference between groups was plasma leucine concentrations (Rieu et al, 2006).

Casperson et al (2012) reported increased MPS in elderly in both the post absorptive and postprandial state after 2 weeks of leucine supplementation. Leucine was not given on day 1 and 15 (testing days) but given to one participant-group as 4 g doses on top of their normal meals 3 times per day. These findings were also mirrored by similar increases in p706SK- and 4E-BP1-phosphorylation (Casperson et al, 2012) Another study (Wall et al, 2013) corroborated the above observations, and extended these findings by showing that a larger proportion of total ingested protein were directed towards MPS with leucine supplementation. However, when examining the prolonged effects of leucine supplementation, no extra effect has been shown (Verhoeven et al, 2009). Verhoeven et al (2009) supplemented each main meal with 2.5 g of leucine, three times per day for 3 months with no effect on muscle mass or strength. Based on the above-mentioned studies it appears to be a leucine ”trigger” at >2 g of leucine, causing a potent MPS-response in the first hours after ingestion. The prolonged effect of leucine stimulation in muscle is, however, less clear.

2.10. Gender differences

In the post absorptive state there appears to be no difference in MPS between young men and women (Smith et al, 2012; West et al, 2012). This also appears to be the case after isolated protein intake (Smith et al, 2009), RE (Dreyer et al, 2010), and when RE and protein intake are combined (West et al, 2012). Chevalier et al (2012) reported no differences in MPS between young and elderly women in the post absorptive or postprandial state. There also appears to be no difference in MPS between young and elderly men in the post absorptive state (Cuthbertson et al, 2005). In contrast to Chevalier et al (2012) findings, Smith et al (2012) reported increased MPS in elderly women compared to both elderly men and young women in the post absorptive state. Furthermore, Smith et al (2012) reported a blunted response to nutritional stimuli similar in both old women and men compared to their younger counterparts. The

reason for discrepancies between these studies (Chevalier et al, 2012; Smith et al, 2012) could be attributed to the leucine plasma concentration being raised 250% above basal values in Chevalier et al (2012), while in Smith et al (2012) it was only raised 40% above basal values. If these discrepancies could be attributed to protein intake, it seems that elderly women might need larger protein boluses (>30 g) to robustly stimulate MPS.

Elderly men has been shown to not benefit from larger protein doses compared to their younger counterparts (Cuthbertson et al, 2005), while other show a dose-response relationship with whey-intake ranging from 20-40 g (Yang et al, 2012a&b). Furthermore AAT expression after RE seem to be unrelated to both age and gender (Drummond et al, 2011). In summary there appears to be no obvious gender-differences in elderly men and women, but elderly women is reported to exhibit slightly increased post absorptive MPS and a slightly reduced response to protein intake compared to elderly men.

2.11. Summary

In summary, absorption kinetics, digestion, amino acid transport and basal MPS seems to be similar in both the young and old (Koopman et al, 2009; Drummond et al, 2011; Reidy et al, 2014; Dickinson et al, 2014; Moore et al, 2015). Elderly seem to maintain the ability to respond to both exercise and protein intake, but special attention to increased protein intake (>20 g protein >15 g EEA>2 g leucine) and protein source seems important to maximize MPS (Cuthbertson et al, 2005; Katsanos et al, 2006; Tang et al, 2009; Yang et al, 2012a&b; Moore et al, 2015). Timing of intake seems to be of less importance (Kim et al, 2015). The reduced anabolic effect of lower doses of protein (< 20 g) can be restored by increasing leucine content combined with rapidly digested protein sources (e.g. whey) (Katsanos et al, 2006, Yang et al, 2012a&b).

Furthermore, if RE precedes protein intake, there is a widening of the muscle full concept (Atherton et al, 2010) with an additive effect of the two stimuli in elderly, allowing for more dietary protein to be incorporated into skeletal muscle (Pennings et al, 2011; Yang et al, 2012a&b). Allowing more dietary protein to be incorporated into

skeletal muscle before MPS-response becomes refractory seems to be dose-dependent and not relying on delivery-profile, e.g. bolus vs spread feeding (Mitchell et al, 2015). As elucidated, greater training volumes of RE, increasing protein intake and select choice of protein source, independent of gender, seems to be promising strategies to overcome the anabolic resistance reported in the elderly (Cuthbertson et al, 2005; Katsanos et al, 2005; Kumar et al, 2009; Fry et al, 2011). Our study is highly relevant as it is of interest to examine whether full-body RE combined with habitual supplementation of native whey will induce greater hypertrophy and strength-gains compared to milk after 11 weeks. Furthermore, it is of interest to examine whether our exercise protocol and supplements could be efficient countermeasures to anabolic resistance.

3. Intracellular signaling

For external stimuli such as RE and protein intake to have anabolic effects, it must somehow be sensed and translated into anabolic signaling, increasing MPS for compensatory hypertrophy. The central mediator of external anabolic stimuli into anabolic internal signaling cascades is the serine/threonine kinase, mammalian target of rapamycin (mTOR) (Baar&Esser, 1999; Terziz et al, 2008; Fujita et al, 2007b). On its own, mTOR has a limited kinase activity, but form complexes with other proteins, dictating its location, activity and downstream effectors (Hara et al, 2002). Two main complexes of mTOR are formed, with either the rapamycin-sensitive raptor (mTORC1) or rapamycin-insensitive rictor (mTORC2) (Loewith et al, 2004).

3.1. mTORC1

It is widely accepted that in skeletal muscle, the rapamycin-sensitive mTORC1-complex (from now on referred to as mTOR) is the main mediator of anabolic stimuli such as mechanical-loading (exercise) (Hornberger et al, 2007; Drummond et al, 2009) and nutrient signaling (amino acids) (Atherton et al, 2010; Dickinson et al, 2011). When administering the mTOR-inhibitor rapamycin, hypertrophy was abolished in skeletal muscle, and genetic activation of mTOR in a PI3K/Akt-dependent manner, caused hypertrophy in vivo (Bodine et al, 2001). This was the first report of this matter, and led to the conclusion that mTOR-activation is both necessary and sufficient to induce hypertrophy (Bodine et al, 2001). Since growth

factors such as IGF-1 can activate PI3K/Akt, and Akt could affect a variety of mTOR-independent growth regulatory molecules, it has been proposed that mTOR may be only permissive to induce hypertrophy (Goodman et al, 2010). Goodman et al (2010) shed light on this by overexpressing Rheb (mTOR-activator), activating mTOR in an PI3K/Akt-independent manner, showing mTOR to be both necessary and sufficient to induce hypertrophy in skeletal muscle.

Furthermore, Hornberger et al (2007) used PI3K-inhibitor wortmannin, and showed mTOR-activation from mechanical loading to be PI3K/Akt-independent and rapamycin-sensitive. This was also confirmed in another study were Drummond et al (2009) used rapamycin, which blocked contraction-induced activation of mTOR, p70S6K and eEF2. Rapamycin-administration completely blocked the MPS-response, p70- and 4E-BP1 phosphorylation after ingestion of 10 g of EEA, showing the essential role of mTOR in mediating the anabolic stimuli of EEA (Dickinson et al, 2011).

A key PI3K-dependant upstream regulator of mTOR is a heterodimer consisting of TSC1 and TSC2 (Sancak et al, 2010). Growth factors and insulin signals through PI3K/Akt where TSC1/2 subsequently functions as a GTPase-activating binding protein for Rheb. GTP-bound Rheb interacts with mTOR and strongly stimulates its kinase activity. TSC1/2 negatively regulates mTOR by converting Rheb into it inactive GDP-bound form (Sancak et al, 2010). As RE and protein intake act in a PI3K/Akt-independent manner (Hornberger et al, 2007; Deldicque et al, 2008; Atherton et al, 2010; Dickinson et al, 2011) these anabolic stimuli activate mTOR by different mechanisms than through the growth factor/insulin-receptor PI3K. Growth factor/insulin-activation of mTOR ends with removing TSC1/2 from Rheb, a final step that is shared by mechanical loading (Jacobs et al, 2013).

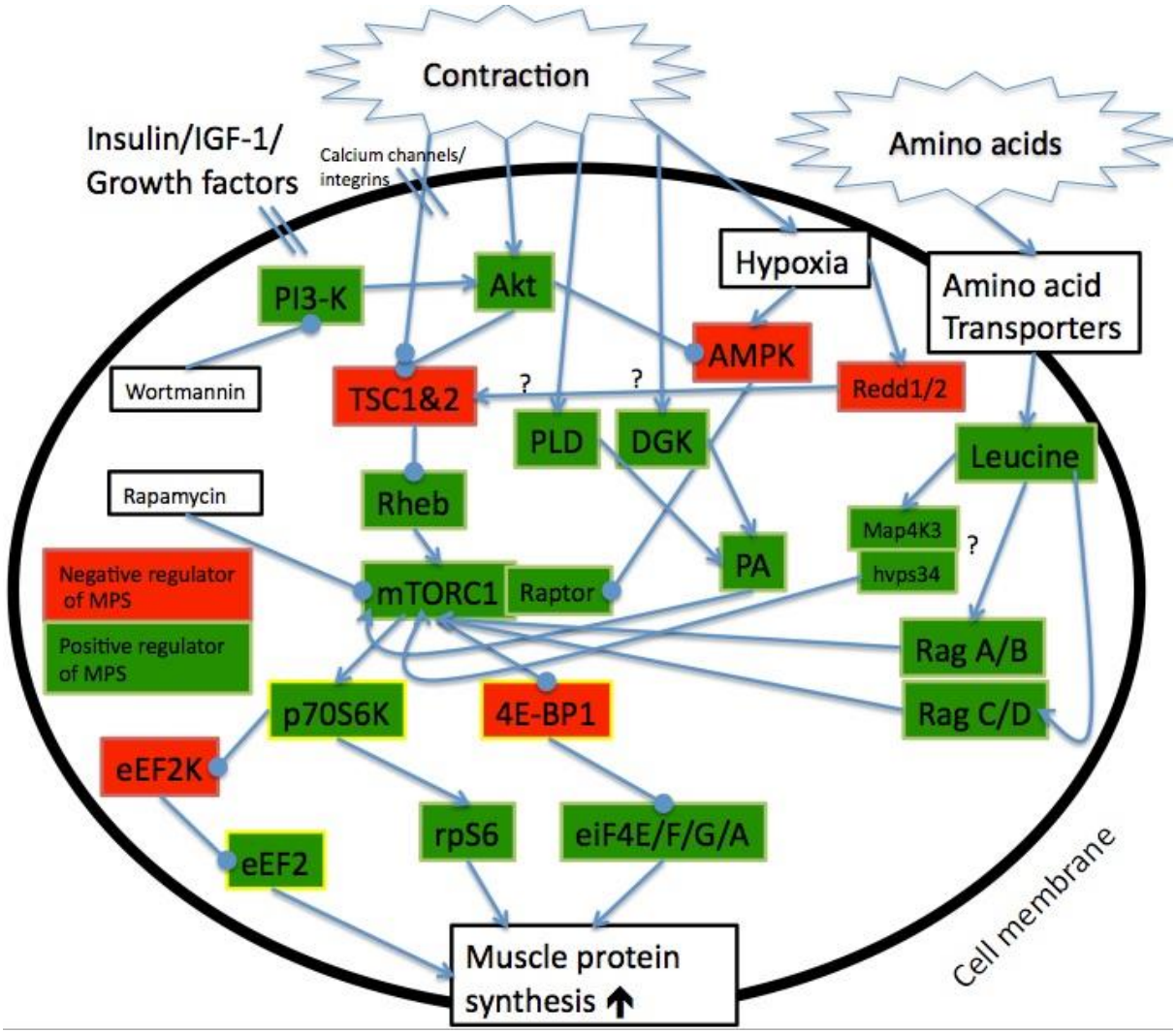

Figure 3.1: *Simplified overview of suggested signaling pathways regulating MPS in skeletal muscle. Studied signaling proteins p70S6K, 4E-BP1 and eEF2 are highlighted with a yellow line. Green boxes indicate positive regulators of MPS and red boxes indicate negative regulators of MPS.*

3.2. Resistance exercise regulating mTOR

The exact mechanism of mechanical-load transduction into increased mTOR-activation remains elusive, but increased Rheb-activity (Jacobs et al, 2013) and increased [phosphatic acid] (PA) are possible explanations (Hornberger et al, 2007; You et al, 2014). Even though growth factor/insulin- and RE-induced-activation of mTOR share the final step (Sancak et al, 2010; Jacobs et al, 2013), RE seem to activate mTOR independently of PI3K/Akt (Hornberger et al, 2007; Deldicque et al, 2008; Hulmi et al, 2009). Hornberger et al (2007) proved that mechanical stretch activated mTOR concomitant with PI3K/Akt inhibition by wortmannin. Furthermore,

RE is shown to activate mTOR while Akt-activation decreases (Deldicque et al, 2008; Dreyer et al, 2006; Coffey et al, 2005; Hulmi et al, 2009). Thus, it seems while both growth factors/insulin and RE activate mTOR by removing TSC1/2 from Rheb, they do so from different upstream regulators. A distinct upstream regulator, sensing mechanical stimuli is yet to be identified, but some possible explanations are reported (Hornberger et al, 2007; You et al, 2014).

One kinase that could be responsible for loading-induced mTOR-activation is diacylglyserol kinase zeta, an isoform of DGK (You et al, 2014). DGK-zeta can synthesize PA, which again is suggested to activate mTOR by direct binding to the FKBP12-rapamycin-sensitive site on mTOR, possibly competing with inhibiting effectors (You et al, 2014). On that note, this was only proved in vitro, in muscles from mice where DGK-knockout mice did not synthesize as much PA as wild-type mice, and increasing DGK-content combined with mechanical stretch further activated mTOR (You et al, 2014). Since absolute activation of mTOR remained unchanged compared to wild-type mice, the role of DGK can only be said to be modulating and not necessary for loading-induced activation of mTOR (You et al, 2014). Another suggested synthesizer of PA is PLD, which has been shown in vitro to be activated by mechanical loading, thus increasing PA (Hornberger et al, 2007).

3.3. Amino acid regulation of mTOR

As previously elucidated, RE and protein intake has an additive effect on MPS. Allowing such an additive effect when combining these stimuli, EEA (especially leucine) regulate mTOR by different mechanisms than growth factor/insulin and RE (Kim et al, 2008; Sancak et al, 2008; Sancak et al, 2010). In absence of amino acids, mTOR is dispersed throughout the cytoplasm (Sancak et al, 2008). However, when amino acids are added, mTOR and Rheb relocalizes to the lysosomal membrane (Sancak et al, 2010). The importance of this lysosomal localization of mTOR and Rheb seems to be attributed to the additional regulators called Ragulator and the Rag-family of small proteins (Sancak et al, 2008; Sancak et al, 2010).

These amino acid specific regulators of the mTOR-pathway (Rag-family) exist as heterodimers with GTP-bound Rag A or B bound to GDP-bound Rag C or D, with

A/B and C/D being highly similar to each other, and functionally redundant (Sancak et al, 2008). Amino acids GTP-load RagA/B which binds to mTORs regulatory complex Raptor, and subsequently translocates mTOR to the lysosomal membrane, where Rheb is situated (Sancak et al, 2008). This regulatory role has been proven by Rag-knockdown and by overexpressing negative Rags (GDP-loaded), which suppresses amino acids effect on TOR-activity (in Drosophila cells) (Kim et al, 2008). When localized at the lysosomal membrane mTOR interacts with the Ragulator complex, which then recruits GTP-activated Rags, creating proximity to Rheb, thus activating mTOR (Sancak et al, 2010). Amino acids seem to have an essential role in enhancing growth-factor/insulin and resistance-exercise-induced stimuli because of this relocalization of mTOR to the lysosomal membrane, makin mTOR more readily activated (Sancak et al, 2008; Sancak et al, 2010).

3.4. Translation initiation and elongation

A central step in MPS is translation initiation (Bodine et al, 2001; Wang&Proud, 2008). Translation initiation consists of a series of events for ribosomal complex assembly and binding of mRNA. Multiple initiation factors and signaling molecules regulate the formation of the translational apparatus and provide a highly dynamic and tightly regulated control of protein synthesis (Bodine et al, 2001; Wang&Proud, 2008). This tight and dynamic regulation is evident as exercise and protein intake has shown signaling molecules and initiation factors to respond within minutes (Bolster et al, 2003). RE and protein intake induce translation initiation through phosphorylation of mTOR and sequential phosphorylation of key downstream-effectors p706SK and 4E-BP1 (Cuthbertson et al, 2005; Kumar et al, 2009; Drummond et al, 2008; Dickinson et al, 2011; Farnfield et al, 2012). Further downstream, the sequential phosphorylation of mTOR-p706SK dephosphorylates (activates) elongation factor 2 (eEF2) and enhances translation elongation (Fujita et al, 2007b).
Downstream-effectors p70S6K and 4E-BP1 is phosphorylated by mTOR and influence translation initiation through distinct classes of mRNA (Wang&Proud, 2008). Phosphorylated p70S6K subsequently phosphorylates ribosomal protein S6 (rpS6), allowing efficient translation of mRNAs containing oligopyrimidine tracts in their 5' untranslated regions (Burnett et al, 1998). In its dephosphorylated state, eIF-4E binding protein (4E-BP1) binds to eIF4E, blocking its ability to form functional

interactions with eIF4G, which further interacts with eIF4A (Burnett et al, 1998). When 4E-BP1 is phosphorylated it releases from eIF-4E, allowing the eIF-4E/G/A-complex to assemble and initiate cap-dependent initiation (Burnett et al, 1998). The activity of eukaryotic elongation factor 2 (eEF2) is regulated through reversible phosphorylation at the Thr56-residue by eEF2 kinase (eEF2K) (Wang et al, 2001). To increase both translation initiation and elongation, thus increasing translation efficiency, p706SK phosphorylates eEF2K (inhibiting its kinase activity) which subsequently leads to dephosphorylating (activating) of eEF2 (Wang et al, 2001). Activated eEF2 binds to the ribosomes and promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome (Wang et al, 2001).

3.5. p706SK

Activation of mTOR is often evaluated by measuring changes in the phosphorylation of the Thr389 residue of the 70Kda-isoform ribosomal S6 kinase (from now on referred to as p70, and activated p70 is phosphorylated at Thr389, unless stated otherwise) (Burnett et al, 1998; Hornberger et al, 2007; Sancak et al, 2008). Burnett et al (1998) showed phosphorylation of the Thr389 residue to be crucial for p70-activation *in vivo*. Hornberger et al (2007) reported mechanical activation of p70 to be PI3K-independent and rapamycin sensitive in mice skeletal muscle cells. Furthermore, Baar&Esser (1999) reported that an initial post-exercise measurement of p70-phosphorylation correlated with subsequent hypertrophy in rats, and this is also reported after 14 weeks of resistance training in young men (Terziz et al, 2008). The degree of p70-phosphorylation after the initial exercise bout were highly correlated to the percentage increase in whole-body- and leg fat-free mass (FFM), 1RM squat and mean muscle cross sectional area (CSA) (Terziz et al, 2008).

Fry et al (2011) reported that 0, 3, 6 and 24 hours after RE there were no significant changes in p70 and 4E-BP1 phosphorylation in elderly, with increased phosphorylation seen in their younger counterparts. The lack of significant changes in p70- and 4E-BP1-phosphorylation in elderly was mirrored by a blunted MPS-response (Fry et al, 2011). This age-related blunted MPS-response, p70- and 4E-BP1-phosphorylation was also confirmed in elderly by Kumar et al (2009) 0, 1, 2 and 4

hours post-exercise at 60-90% of 1RM, in the post absorptive state. In their younger counterparts, p70-phosphorylation 1 hour post-exercise was highly correlated with MPS-response within the same timeframe (1-2 hours).

In contrast to these findings (Fry et al, 2011, Kumar et al, 2009), others did not find any age-related blunting of MPS- or intracellular signaling-responses (Farnfield et al, 2012). When combining RE and protein intake in untrained participants, Farnfield et al (2012) reported a greater p70-phosporylation in elderly compared to young. Comparing the latter studies, RE without subsequent protein intake had a blunted response in elderly (Fry et al, 2011; Kumar et al, 2009), but when exercise was followed by protein intake this blunted response was diminished (Farnfield et al, 2012).

Protein intake with leucine in particular, is shown to be a potent regulator of p70 as supplementing a sub-optimal dose of 6.25 g whey with 3 g leucine showed similar activation of p70 as 25 g of whey; both under rested conditions and 1-3 hours post-exercise (Churchward-Venne et al, 2012). Isolated leucine-intake has a potent effect, as 2 weeks of supplementing elderly with leucine (4 g/3 meals per day) improved both post absorptive and postprandial p70-phosphorylation, mirrored by a similar increase in MPS (Casperson et al, 2012). It should also be noted that Casperson et al (2012) did not supplement leucine on testing-days pre- and post-intervention that could confound these findings.

The importance of post-exercise protein intake is emphasized as resistance training alone is shown not to phosphorylate p70 (Karlsson et al, 2004; Reitelseder et al, 2011). When ingesting 0.1 g/kg body weight of branched-chained amino acids (BCAA) (45% leucine, 30% valine, and 25% isoleucine) p70 was profoundly phosphorylated 2 hours post-exercise (Karlsson et al, 2004). In contrast to the above findings (Karlsson et al, 2004; Reitelseder et al, 2011), Terziz et al (2010) reported significant p70-phosphorylation after RE without subsequent protein intake. This has also been confirmed by Dreyer et al (2006) who reported increased p70-phosphorylation 0, 1 and 2 hours after RE, with participants remaining in the post absorptive state the whole time period. Furthermore, the degree of p70 phosphorylation in relation to exercise alone seems to depend upon training volume

with increasing phosphorylation at higher training volumes (Terziz et al, 2010). When examining different exercise intensities it seems that in the range of 60-90% of 1RM, the MPS-response and intracellular signaling-responses reaches a plateau in both young and old (Kumar et al, 2009).

In addition to the potent effect of protein intake and leucine-content (Tang et al, 2009; Churchward-Venne et al, 2012; Karlsson et al, 2004), feeding pattern seems to affect intracellular signaling (West et al, 2011). To mimic a slower digested protein (e.g. casein) and to avoid protein source and leucine-content as confounding variables, West et al (2011) compared bolus vs spread intake (mimicking casein) of 25 g whey, and reported superior MPS- and intracellular signaling-responses to bolus-feeding. Both in the early (1-3 hours) and late (3-5 hours) postprandial period, bolus intake showed superior MPS-response, in concert with increasing p70-phospohyralation (West et al, 2011). Furthermore, spread intake increased MPS by 42% and 121% from baseline in the early and late postprandial period respectively, but no effect was seen on p70-phosphorylation in either period (West et al, 2011).

Training status seem to influence acute p70-phosphorylation as Coffey et al (2005) compared endurance-trained vs strength-trained men, with only the endurance-trained increasing acute p70-phosphorylation post RE, in contrast to the data reported in resistance-trained in another study (Terziz et al, 2008). Furthermore, Farnfield et al (2012) found training status to be predictive of p70-phosphorylation. A bout of RE with subsequent intake of 26.6 g of whey (3.6 g leucine) or placebo was performed before and after an intervention of 12 weeks of resistance training in both young and elderly participants (Farnfield et al, 2012). In the untrained state (pre-intervention) both young and old increased p70-phosphorylation with whey compared to placebo 2 hours post-exercise, but a greater phosphorylation was seen in the elderly. However, in the trained state (post-intervention) only the young participants maintained this p70-phosphorylation response to the exercise-bout and subsequent protein intake, with the elderly showing a blunted response in p70-phosphorylation. Regardless of p70-phosphorylation responses, both young and elderly had similar increases in 1RM strength after 12 weeks of RE (Farnfield et al, 2012).

Table 3.1: *Overview of studies examining the effect of resistance exercise (RE) and/or protein intake on phosphorylation of p70, 4E-BP1 and eEF2. +* indicate significant increase. -* indicate significant decrease. = indicate no change. (+) or (-) indidcate a tendency of change. All biopsies were collected from m. vastus lateralis*

Authors	Design	Participants	Intervention	Signaling proteins	Outcome measures
Terziz et al, 2008	RCT	12 young	RE-bout before 14 weeks of resistance training. Leg press, 6X6RM, 2 min rest between sets. Baseline biopsy in postprandial state.	p-p70	p70-phosphorylation was significantly increased 30 minutes after initial RE-bout. The magnitude of p70-phosphorylation were highly correlated with subsequent increases in 1RM strength, whole body fat free mass and leg fat free mass after 14 weeks of resistance training.
Farnfield et al, 2012	RCT	16 young 18 old	Acute day pre/post 12 week RE. 26,6 g of whey-protein (3,6g leucine)/placebo after acute RE-bout. Biopsy 2 hours after RE-bout	p-p70 p-4E-BP1 p-p70 p-4E-BP1	**Young** **Pre** **Whey** / **Placebo** (+) / = (+) / = **Post** (+) / = (+) **Old** **Pre** **Whey** / **Placebo** +* / = +* / + **Post** (+) / = +* / =
Moore et al, 2011	CT	7 young	RE in post absorptive state. Unilateral leg-press and knee-extension. 25 g of whey immediately post-RE	p-p70 p-4E-BP1 p-eEF2	**Whey** **1h** / **3h** / **5h** **+*** / **=** / **=** **+*** / **+*** / **+*** **=** / **=** / **=** **RE + whey** **1h** / **3h** / **5h** **+*** / **+*** / **+*** **+*** / **+*** / **+*** **=** / **=** / **=**
Fujita et al, 2007b	RCT	14 young men Control (n = 7) EEA (n = 7)	Fasted baseline biopsy. 20 g of EEA with 7 g leucine at rest. Control group not consuming nutrients.	p-p70 p-4E-BP1 p-eEF2	**EEA** **1 h** **+*** **+*** **-*** **Control** **1 h** **=** **=** **=**
Hulmi et al, 2009	RCT	18 young men. Protein (n = 9) Placebo (n= 9)	15 g of whey /placebo before/after RE-bout. Total of 30 g whey (3.4 g leucine)	p-p70 p-4E-BP1 p-eEF2	**Whey** **1 h** / **48 h** +* / = = / = = / = **Placebo** **1 h** / **48 h** (+) / = (-) / = = / =
Kumar et al, 2009	RCT	25 young 25 old	Post absorptive state. Unilateral knee-extension and –flexion. 60-90% of 1RM data presented.	p-p70 p-4E-BP1 p-eEF2	**Young** **10 min** / **1h** / **2h** / **4h** **=** / **+*** / **=** / **=** **(-)** / **+*** / **=** / **=** **(-)** / **+*** **Old** **10 min** / **1h** / **2h** / **4h** **=** / **=** / **=** / **=** **(-)** / **(-)** / **=** / **=** **(-)** / **+***
Drummond et al, 2008	RCT	7 young men, 6 old men	Post absorptive knee-extension RE. 20 g of EEA (7g leucine) immediatley post RE	p-p70 p-4E-BP1 p-eEF2	**Young** **1 h** / **3 h** / **6 h** +* / +* / (+) (+) / +* / +* (-) / -* / -* **Old** **1 h** / **3 h** / **6 h** +* / +* / +* (+) / +* / (+) (-) / -* / -*
Dreyer et al, 2006	RCT	11 participants, both genders	RE in fasted state. Biopsies before, 0, 1 and 2 hours after RE	p-p70 p-4E-BP1 p-eEF2	**0 hour** / **1 hour** / **2 hours** (+) / (+) / +* -* / (-) / = (+) / -* / -*
Dreyer et al, 2008	Cross-over	16 young men Control (n = 8) EEA (n = 8)	RE-bout. EEA intake 1 h after RE. Control group did not consume nutrients.	p-p70 p-4E-BP1 p-eEF2 p-p70 p-4E-BP1 p-eEF2	**0 hour Control** / **1 hour Control** / **2 hours Control** +* / +* / +* -* / = / = (+) / -* / -* **EEA** / **EEA** / **EEA** +* / +* / +* -* / = / +* (+) / -* / -*
Dreyer et al, 2010	RCT	17 young, 9 men, 8 women	Fasted RE. 10X10 knee-extension. Intensity was ~67 % of 1RM. biopsy 1 and 2 hours post-RE	p-p70 p-4E-BP1 p-eEF-2	**1 h** **Men** / **Women** +* / +* = / = -* / -* **2 h** **Men** / **Women** +* / +* = / = -* / -*

The anabolic effect of combining RE and protein intake has been shown to translate into increased intracellular signaling (Hulmi et al, 2009; Moore et al, 2011). Moore et al (2011) had their participants perform unilateral leg-press and knee-extension until failure and 25 g of whey protein was ingested immediately post-exercise, with the rested leg acting as a control. At 1, 3 and 5 hours post-exercise, both MPS and p70-phosphorylation were increased and highly associated in the exercised leg, whereas in the rested leg, MPS and p70-phosphorylation only increased 1 hour post-workout, returning to baseline values after 3 and 5 hours (Moore et al, 2011). Hulmi et al (2009) reported that only when a total of 30 g whey protein were ingested in combination with RE there was a potent effect on p70-phosphorylation compared with exercise and non-caloric placebo.

In contrast to studies showing p70-phosphorylation in concert with both hypertrophy (Baar&Esser, 1999; Terziz et al, 2008) and MPS-response (Moore et al, 2011; West et al, 2011), there appears to be a dissociation between single point-in-time changes in signaling molecule phosphorylation and the dynamic changes in MPS (Atherton et al, 2010; Mitchell et al, 2015; Greenhaff et al, 2008; West et al, 2011). Atherton et al (2010) reported that both p70- and 4E-BP1-phosphorylation (baseline after 2.5 hours) outlasted the MPS-response (baseline after 1.5 hours). When bolus vs spread protein intake at rest were examined, there were no differences in MPS-response, but p70-phosphorylation was greater with bolus feeding, explained by a more rapid aminoacidemia and leucinemia (Mitchell et al, 2015). A similar study (West et al, 2011) had a greater MPS-response with bolus feeding compared to spread, but in contrast to Mitchell et al (2015), West et al (2011) reported increased p70-phosphorylation and MPS to be highly associated after bolus feeding, but dissociated after spread feeding, as MPS increased concomitant with unchanged p70-phosphorylation. This dissociation has also been confirmed by Greenhaff et al (2008) who saw a dose-response relationship between amino acid availability, p70-phosphorylation and eEF2-dephosphorylation (activation), without concurrent changes in MPS. Churchward-Venne et al (2012) also reported a discrepancy between MPS and intracellular signaling as p70-phosphorylation was associated with leucine-intake, but not MPS.

3.6. 4E-BP1

Activating eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) *in vivo* is reported to occur in a two-step mechanism (Gingras et al, 1999). The two phosphorylation-sites Thr37&46 are phosphorylated by mTOR, but do not disrupt the binding of 4E-BP1 to eIF4E, thus not allow for cap-dependent translation to initiate (Gingras et al, 1999). Thr37&46-phosphorylation serves as a prerequisite for subsequent phosphorylation of Thr70 to completely disrupt the 4E-BP1-eIF4E-interaction, allowing the formation of initiation-complex eIF4E/G/A (Gingras et al, 1999).

RE is reported to decrease 4E-BP1-phosphorylation when protein is not supplemented (Deldicque et al, 2008; Dreyer et al, 2006; Hulmi et al, 2009). After an exhausting 10 rep x 10 set leg-extension bout in the post absorptive state, biopsies were withdrawn 30 seconds post-workout, with significantly decreased 4E-BP1 Thr37/46-phosphorylation, whereas p70-phosphorylation was increased 20-fold (Deldicque et al, 2008). Hulmi et al (2009) reported decreased 4E-BP1 Thr37/46-phosphorylation 1 hour after RE without subsequent protein intake. When 15 g of whey protein was ingested pre- and post-exercise this decrease was avoided and remained at baseline values, with authors (Hulmi et al, 2009) concluding this probably allowed for an increase in translation initiation. Dreyer et al (2006) reported similar findings after RE in the post absorptive state. 4E-BP1 Thr37/46-phosphorylation was decreased immediately after exercise slowly returning to baseline within the next 2 hours (biopsies withdrawn at 1 and 2 hours post-exercise) (Dreyer et al, 2006). In elderly, 4E-BP1 remained unchanged 3 and 6 hours after RE in the post absorptive state (Fry et al, 2011). When RE was followed by 25 g of whey protein, 4E-BP1 Thr37/46-phosphorylation was elevated 1, 3 and 5 hours post-exercise, but with no difference between the exercised and rested leg (Moore et al, 2011, further details in p70-section above).

Examining the effect of training status, an intake of 26.6 g of whey (3.6 g leucine) or placebo was performed post-exercise, in both the untrained state (pre-intervention) and trained state (post-intervention) (Farnfield et al, 2012, for more details see description above in the p70-section). Only the elderly increased 4E-BP1 Thr37/46-

phosphorylation with whey compared to placebo 2 hours post-exercise in the untrained state, and a greater phosphorylation was seen in the elderly in the trained state (Farnfield et al, 2012). Regardless of 4E-BP1-phosphorylation responses, both young and elderly had similar increases in 1RM strength after 12 weeks of resistance training (Farnfield et al, 2012).

The potent effect of leucine was reported by Casperson et al (2012) with improved MPS both in the post absorptive and postprandial state, mirrored by increased 4E-BP1 Thr 37/46 phosphorylation following 2 weeks of leucine-supplementation of 4 g/3 meals per day. When a group of elderly ingested 10g of EEA post-exercise with either 1.85 g (control-group) or 3.5 g of leucine (leucine-group), only the leucine group increased 4E-BP1 Thr37/46-phosphorylation 2 hours post-exercise (Dickinson et al, 2014), emphasizing the potent effect of leucine-content.

When examining the effect of different feeding patterns at rest, recent reports are contradicting (West et al, 2011; Mitchell et al, 2015). West et al (2011) reported increased 4E-BP1 Thr37/46 phosphorylation with no differences in bolus vs spread feeding of 25 g of whey, both in the early (1-3 hours) and late postprandial phase (3-5 hours), despite increased MPS-response with bolus feeding. In contrast, Mitchell et al (2015) reported equal MPS with bolus vs spread feeding, but a greater 4E-BP1 phosphorylation after bolus feeding. Examining different protein sources, Reitelseder et al (2011) reported a greater increase in 4E-BP1 Thr37/46-phosphorylation 1-6 hours post-exercise with casein than whey, despite similar MPS-responses. These findings (Reitelseder et al, 2011; West et al, 2011; Mitchell et al, 2015) underline the fact that phosphorylation-pattern of key anabolic signaling proteins does not necessarily reflect MPS-response, also reported by others (Greenhaff et al, 2008; Churchward-Venne et al, 2012).

3.7. eEF2

Not being part of the initiation process, dephosphorylated eEF2 at residue Thr 56 (activated) increases translation efficiency by enhancing translation elongation, thus contributing to an increased MPS (Fujita et al, 2007b). One hour after intake of ~20 g of EEA (~7 g of leucine) at rest, eEF2 was significantly dephosphorylated, with no

change seen in the control group not consuming nutrients (Fujita et al, 2007b). Furthermore there were significant increases in p70- and 4E-BP1 Thr37/46-phosphorylation, and authors (Fujita et al, 2007b) concluded that EEA promoted MPS through translation elongation concurrently with translation initiation. Although Fujita et al (2007b) did not measure eEF2 kinase-activity directly, their data strongly suggest that the extraordinary large increase in p70-phosphorylation caused a subsequent dephosphorylation of eEF2. In contrast to these findings, Atherton et al (2010) reported that eEF2-phosphorylation remained unchanged for 6 hours following a large saturating bolus ingestion of 48 g whey protein. Also, after ingestion of 15 g of EEA, eEF2-phosphorylation was reported to be unchanged from fasting levels with both bolus and a spread feeding pattern (Mitchell et al, 2015). Furthermore, another study comparing bolus vs spread intake of protein (West et al, 2011) reported reduced eEF2-phosphorylation after spread-, but remained unchanged after bolus feeding.

After RE, eEF2 is reported to be 20% less phosphorylated immediately post-workout, before rebounding to 120% of baseline values 1 hour post-workout, in both young and old in the post absorptive state (Kumar et al, 2009). Along with the previous study (Kumar et al, 2009), Moore et al (2011) also reported contradicting phosphorylation patterns of eEF2. A reduction in eEF2-phosphorylation was seen in the exercise and fed condition compared to rested and fed condition, but surprisingly with no change compared to rested and fasted condition (Moore et al, 2011).

In contrast to the previous studies (Kumar et al, 2009; Moore et al, 2011), Dreyer et al (2006) reported decreased eEF2- and increased p70- and 4E-BP1-activation to be highly associated with MPS-response 1-2 hours post-exercise in the post absorptive state. Furthermore, after RE with a subsequent intake of 20 g of EEA (7 g leucine) both young and old had an equal MPS-response over 1-6 hours, with the elderly showing a delayed response (Drummond et al, 2008). Despite the delayed MPS-response, elderly showed a similar tendency of reduced eEF2-phosphorylation as the young 1 hour post RE, and both age-groups showed a significant reduction 2 and 6 hours post EEA-intake (Drummond et al, 2008). Others (Hulmi et al, 2009) reported unchanged eEF2-phosphorylation 1 hour after RE in combination with a total intake of 30 g of whey protein. In summary, studies show contradicting eEF2-

phosphorylation patterns after RE and protein intake, with reports of decreased, increased and unchanged phosphorylation.

3.8. Summary, intracellular signaling

Theoretically, increased p70- and 4E-BP1-phosphorylation together with decreased eEF2-phosphorylation initiate translation initiation and elongation. After RE and protein intake, the phosphorylation-patterns of these signaling proteins are reported to be highly varied and greatly affected by time of biopsy-collection (table 3.1). Furthermore, the phosphorylation responses to the anabolic stimuli of RE and protein intake are highly influenced of whether these stimuli are combined or isolated, with the combined effect generally shown to be of greater effect. The magnitude in phosphorylation of the different signaling proteins seems to work in a dose-response relationship to RE-volume, and leucine-content and absorption rate of given protein sources.

In general, p70-phosphorylation is reported to increase 0-6 hours after RE and protein intake, with elderly reported to have a delayed and/or blunted response compared to young. 4E-BP1-phosphorylation seem to be reduced 0-2 hours after RE, but is restored to/increased above baseline values after 2-5 hours when RE is combined with protein intake of >3.5 g leucine. Phosphorylation of eEF2 0-6 hours after RE and protein intake is reported to be greatly varied. The contradicting reports in eEF2-phosphorylation response to RE and protein intake, has led to several authors question the physiological importance of the phosphorylation status of eEF2.

This study is highly relevant, as it is of interest to examine whether native whey with increased leucine content and absorption-rate compared to milk-protein, enhance the phosphorylation responses of these signaling molecules. Furthermore, this study could further elucidate whether such an intracellular anabolic advantage would lead to increased hypertrophy and strength after 11 weeks of RE and native whey vs milk supplementation.

4. Method

4.1. Overall study design

This thesis is based from the results of a larger study. The study was conducted as a randomized double-blinded controlled intervention. The intervention was eleven weeks of strength training combined with protein supplementation of 40 g per day as native whey or milk protein in an elderly population above 70 years of age. The main goal of the study was to compare acute and long-term effects of native whey vs milk supplementation combined with resistance exercise (RE) on intracellular anabolic signaling and changes in muscle mass and strength. The study was ethically approved by the South-East Regional Ethical Committee of Norway. The Norwegian Research Council and Tine SA were joint funders of this study. None of the contributors to the completion of this study had any conflicting interests or relation to the funders of the study.

Our participants went through extensive pre- and post-testing of both invasive and non-invasive manner. This has produced data on body composition, bone mineral density (BMD), upper- and lower-body 1RM strength, performance in daily living, recovery and intracellular responses. All resistance training and data collection were done at the Norwegian School of Sport Sciences (NSSS). The focus of this master thesis is on the acute intracellular hypertrophic responses downstream of mTOR, after RE combined with native whey vs milk supplementation. This RE-bout was completed before and after 11 weeks of RE, with the same group of participants (see acute study section), going from an untrained to a more trained state. Through biopsy-collection pre- and post RE and protein intake, completed before and after the 11 week intervention, I have been able to link acute phosphorylation patterns of key signaling proteins to long-term hypertrophy and strength gains. I have personally been involved in the entire process of this study, from recruitment, pre-testing, training, post-testing and subsequent analysis. Western blot analysis was performed at the laboratory of NSSS. The following method section will mainly be focused towards the acute part of the study, and methods most relevant to my analysis and personal involvement.

4.2. Recruitment and randomization of study subjects

We recruited 30 participants above 70 years of age from both genders, unfamiliar with previous strength training. Participants were recruited through flyers, dialogue with several centers for elderly and advertisement in a local newspaper (appendix 1). To participate in the study we had pre-determined inclusion and exclusion criteria (table 4.1). Upon contact, all participants received detailed information of potential risks from participating in the study.

4.3. Screening

After initial contact, all potential participants were called in for an interview and screening. This process revealed whether they were eligible for the study or not, based on the pre-determined criteria (table 4.1). The screening process also consisted of a preliminary testing day (further details are listed below). DXA-scan was performed to reveal bone mineral density (BMD) and body composition, a blood draw determined cholesterol levels, fasting glucose– and lipid-levels and measurement of blood pressure. After completing a yes/no questionnaire, participants adhering to all inclusion/exclusion criteria could begin the intervention. When in doubt we also got a doctor's declaration of the given criteria to ensure eligible participants. All participants gave informed written consent before participating in the study (appendix 2).

Table 4.1: *Overview of inclusion/exclusion criteria. All participants adhered to these criteria to participate in this study.*

Inclusion criteria	**Exclusion criteria**
- Men and women older than 70 years. - Able to attend training at NIH 3 days a week. - Able to read and speak Norwegian. - Not regularly participating in strength training.	- Use of specified medicines and supplements. - Lactose intolerance and/or milk allergy. - Allergic to local anaesthesia. - Use of corticosteroids last 6 months. - Bone mineral density (BMD) below –0.84 g/cm2 in L2-L4. - Uncontrolled hypertension. - Heart attack last 6 months.

4.4. Randomization

30 participants met our inclusion/exclusion criteria and were divided into two study groups that were supplemented with either native whey or milk (for supplement details, see nutrition section). The division into two groups was randomized, but stratified based on pre-intervention lean mass and gender (table 4.2). An individual not part of the physical testing nor any type of analysis was responsible for the randomization, stratification, and for concealing the group identity of each participant throughout the study. 4 participants withdrew from the study within the first 3 weeks, with the remaining 26 participants completing the entire intervention (completion of all tests and above 80% completion of all RE). 19 participants volunteered to be in the acute part of the study, 16 of these completed the study, making up the participant pool this thesis is based on (figure 4.1). Because of withdrawal from the study by 3 participants the groups ended up slightly skewed based on gender and number of participants.

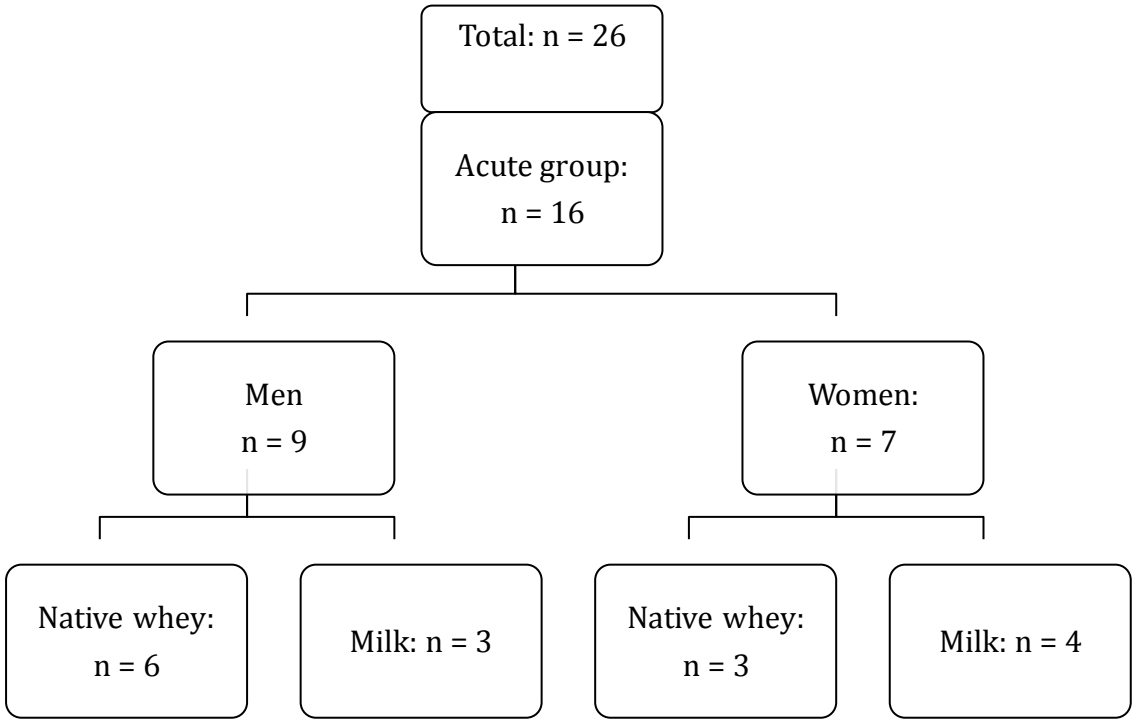

Figure 4.1: *Overview of the randomization process. Of the 26 participants completing this study, 16 took part in the acute part of the study. NW group consisted of 9 participants (6 men and 3 women) and Milk group of 7 participants (3 men and 4 women).*

4.5. Preliminary testing

For preliminary testing, all participants met in the fasted state (>8 hours) between 8 AM and 10 AM at NSSS. All preliminary testing procedures were repeated at the end of the intervention to produce post-measurements in all participants. The project manager met up with each participant before a DXA-scan was performed and blood samples were withdrawn. After fasted blood samples, blood pressure and DXA-scan, participants could eat their first meal of the day (food choices was not restricted, and each participant brought their own food).

After a 24 h recall nutrition interview and ultrasound of the m. *vastus lateralis* thickness, each participant was escorted to the school gym to perform 1RM-testing in leg press and chest press. 1RM-testing started with a 10 minute warm-up on either treadmill or stationary bike. Subsequently, several sub-maximal lifts with incremental loads were completed before 1RM were reached (further details in 1RM-testing sections below).

DXA and ultrasound were used to measure changes in body composition, bone mineral density and muscle size. 1RM-testing in leg press and chest press reflected changes in maximal strength. Functional tests were chair raise and stair climb and served as measures of performance in activities of daily living. To measure our participant's rate of force development (RFD), maximal voluntary contraction (MVC) were performed in a leg extension machine with a power gauge. MVC was conducted pre- and two times post-workout on acute days, and an additional time the following day. In this way MVC-testing served as a mean to reflect recovery after an exhausting 10RM workout on acute days. For an overview of the preliminary testing day, see figure 4.2.

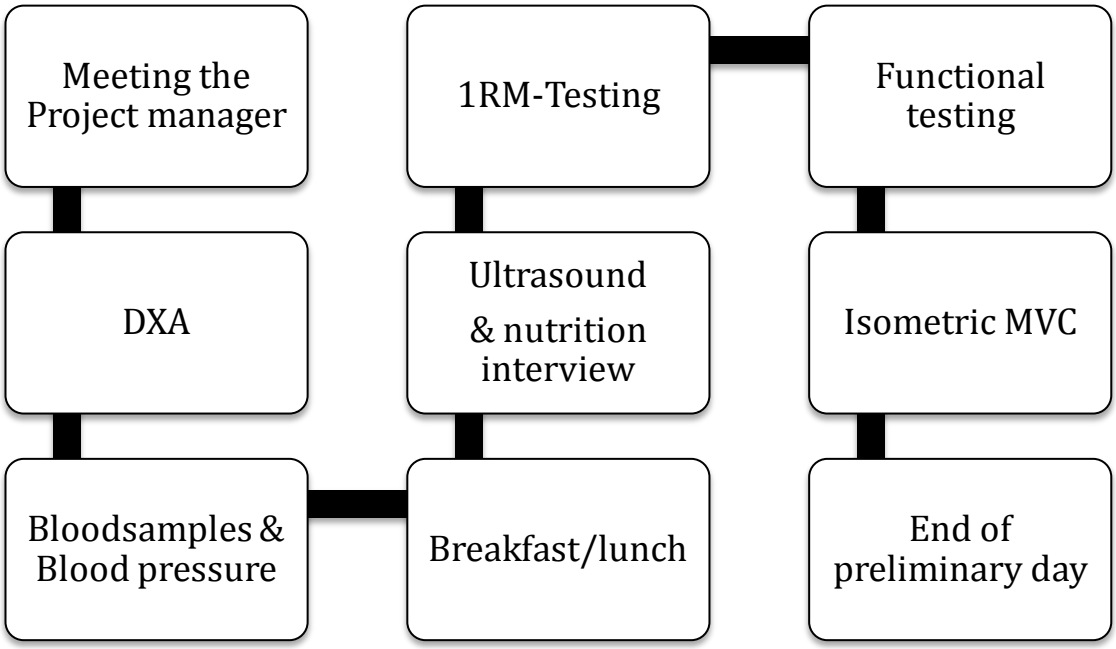

Figure 4.2: *Overview of the preliminary testing day, starting with each participant meeting up with the project manager before subsequent testing procedures were completed.*

4.5.1. Ultrasound and nutrition interview

Participants underwent ultrasound-scans of the thickness of *m. vastus lateralis*, with calculation of thickness based on the 3 best images. The coefficient of variation (CV) between the preliminary testing days for the subjects that underwent both ultrasounds scans included in the study was 2.23 %, out of 82 images. A Msc. student in clinical nutrition performed all 24 h recall interviews, and gave nutritional advice to participants. Participants were not allowed to have a total protein intake below 1.2 g/kg body weight/day during the intervention period (protein content of given supplements included).

4.5.2. 1RM-tests and functional testing

1RM-testing started with a 10 minute general warm-up of each participant's preference, either on a stationary bike (Technogym, Italy) or treadmill (Technogym, Italy, Woodway). Before maximal lifts were attempted, every participant would undergo an exercise-specific warm-up, with series of 10, 6 and 3 repetitions of progressively increasing weights lifted.

4.5.3. Leg press

1RM leg press was performed in a leg press machine (Technogym, Italy). Participants were instructed to place both feet centered and to not allow knees falling medially inwards. Without added weight, the weight-sled was lowered to a 90 degree knee-joint angle and an individually corresponding wooden pole (ranging from 18 to 36 cm) was put underneath. This was to ensure correct individualized sled position and repeatability for all warm-up sets and 1RM-attempts. 1RM-attempts started from a completely extended knee-joint position. The weight was lowered in a controlled manner all the way down to the wooden pole (depth marker) assigned to each participant. Upon contact, participants pressed the weight-sled back to starting position (fully extended knee-joints). After a successful/unsuccessful lift, weight was added/removed or remained the same, and a new attempt was performed after 2 minutes of rest. This process was repeated until 1RM was reached as a consequence of failure, or test supervisor deemed the participant's technique to be of unsatisfactory manner (e.g. knees falling medially inwards).

4.5.4. Chest Press

1RM chest press was performed in a chest press machine (Technogym, Italy). Seat height and grip width (determined as narrow/medium/wide) was individualized. These settings were used at every test session to ensure repeatability. Participants were instructed to maintain contact with the back support under the entire lift and with their feet placed on the ground. All lifts started in a flexed elbow-joint position and a successful lift was when both arms reached a fully extended elbow-joint position. As with the leg press, weights were gradually increased until 1RM was reached as a consequence of failure or unsatisfactory technique.

4.5.5. Functional tests

1RM-testing was followed by functional tests. Participants performed a laser-timed stair-climb without and with added weight in the form of a 10 kg weight-west and two 5 kg manuals in each hand. Each participant completed 3 tries (1 set) without added weight, before a subsequent set with a 10 kg weight-west followed by a last set with additional 5 kg manuals in each hand. The best try in each set was used in subsequent analysis. Afterwards, each participant went through a sit-to-stand test (five times up and down) measured with a pressure sensor with the best of 3 tries considered as their

final score. This was followed by an isometric MVC (MVC-details in acute study section).

4.5.6. Baseline values

All pre-tests of a physical character (MVC, 1RM and functional tests) were repeated after a few days of rest. The best result achieved over these two days was set as baseline values before the intervention started. This was to ensure test familiarization and thus baseline values that best reflected their baseline condition at the start of the study. There were no significant (P>0.05) differences between groups (table 4.2).

Table 4.2: *Baseline values of the NW and Milk group. There were no significant differences between groups (P>0.05). Values are given as a mean ± (SD)*

	Native whey N = 9	Milk N = 7
Gender	♂ = 6 / ♀ = 3	♂ = 3 / ♀ = 4
Age	72.2 (1.3)	74.7 (2.7)
Body mass (kg)	79.6 (19.1)	74.5 (12.8)
Lean mass (kg)	51.2 (12.3)	49.7 (6.5)
1RM leg press (kg)	174.4 (63.3)	160.4 (35.5)
1RM chest press (kg)	48.1 (20.0)	39.3 (12.0)
Functional tests	No significant differences (P>0.05)	

4.6. Eleven week resistance training protocol

RE was performed with participants divided into groups of 3 individuals, and each training session was led by a personal trainer. This was to ensure appropriate weight and form was conducted throughout the workout. The training protocol consisted of whole-body workouts with a daily undulating periodization scheme (complete overview of training program, appendix 3). All exercises were performed in machines (Technogym, Italy) except for shoulder press, which was executed with free-weights. The choice of not using free-weights was based upon difficulties in learning appropriate form and technique, thus confounding strength gains with improvements in technique. Furthermore, the use of machines enhanced standardization of both training and testing, thus allowing weekly increases in weights lifted reflecting actual improvements in strength, and not technique.

The training program was divided in 3 week mesocycles with a gradual increase in intensity and total training volume to avoid early over-training, and a continuous progression throughout the total 11 weeks of training. Participants exercised

Mondays, Wednesdays and Fridays ensuring proper recovery and rest between workouts.

4.7. Acute part of the study

16 participants completed an acute day, pre- and post-intervention. This group underwent two biopsies (pre and post 10RM-workout) on both acute days (four biopsies in total) to investigate changes in intracellular signaling molecule phosphorylation patterns in the acute phase after an exhausting strength workout. This was done before the intervention started, and then repeated at the end of the intervention. On acute days we supervised their nutrition, training and recovery to ensure standardized test-circumstances. For a complete overview of acute days, see figure 4.3.

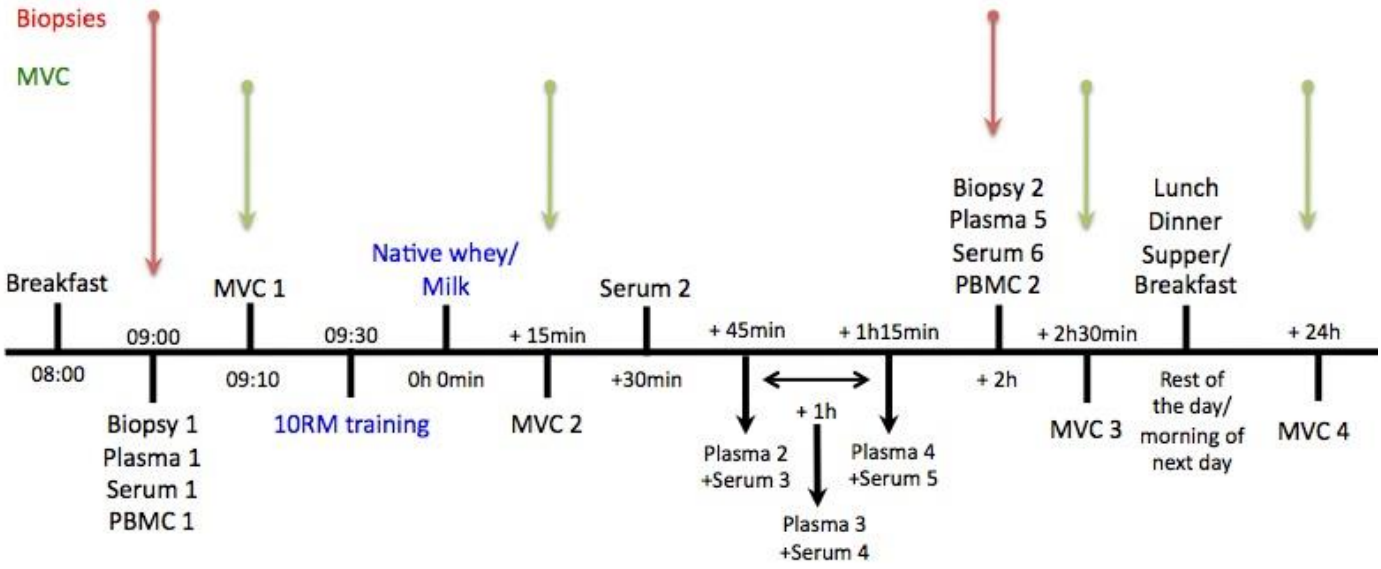

Figure 4.3: *An example timeline showing an acute day with a participant consuming a standardized breakfast at NSSS 08:00. The specific time-points for biopsies and MVC are highlighted with red and green arrows, respectively. 10RM training bout and subsequent protein intake are highlighted in blue writing.*

4.7.1. Biopsies

On acute days, two biopsies were obtained from m. *vastus lateralis*. The second biopsy was withdrawn with the biopsy-needle inclined at a different angle than the

first biopsy, to ensure the second withdrawal was ~5 cm apart from the first. Biopsies were withdrawn under sterile conditions and with l% lidocaine local anesthesia. Professor Truls Raastad was responsible for all biopsies taken. The first biopsy took place 30 minutes before the 10RM workout started and the second biopsy was taken 2 hours after the 10RM workout ended. The choice of these time points was based on previous studies, and was chosen to best reflect phosphorylation status of the studied signaling proteins. Muscle biopsies were obtained with a 6mm Bergstrom needle and vacuum pump, allowing suction to pull out maximum amount of muscle tissue (Evans WJ, Phinney SD, Young VR, 1982). About 150 mg of muscle tissue were collected at every biopsy. The tissue was divided to serve different subsequent analysis purposes, before frozen in liquid nitrogen and stored in an ultra-freezer (-80°C). 50 mg of muscle tissue was used for analysis of phosphorylation status of p706SK, 4E-BP1 and eEF2.

4.7.2. Nutrition

Nutrition on acute days was logged, and portion sizes given to each participant were based on gender, age and previously measured lean body mass. Participants met at NSSS fasted and were fed a standardized breakfast of oatmeal, water, oil, sugar and cinnamon. Immediately post-workout, a shake of native whey or milk (Prolacta®, Lactalis, France) protein was given and consumed by each participant (nutritional content and amino acid profiles, table 4.3).

This shake was consumed two times per day throughout the entire intervention period by each participant. Shakes were consumed immediately post-workout and in the evening. On days without training, each participant was instructed to consume one shake with breakfast and the other in the evening. Native whey and milk sachets were isocaloric, and only amino-acid profile differed between supplements. Lunch on acute days was provided as God Morgen yoghurt with muesli after all tests were completed. Furthermore, each participant was provided with food for the remaining day (Fjordland, ready-made meals) and another oatmeal-breakfast to be consumed the following day at home, before returning for a follow-up MVC-test at NSSS 24 hours after the end of the workout.

Table 4.3: *Table showing the amino acid profile, fat and carbohydrate content in native whey and milk. Doses were given as an individual sachet after the 10RM workout and two times per day throughout the 11 weeks of training. Essential amino acids are labeled with a *.*

Amino acids (g/100 g)	Native whey		Milk	
	g/100g	g per sachet (69 g)	g/100g	g per sachet (73 g)
Alanine	1.3	0.9	0.9	0.7
Arginine	0.8	0.6	0.9	0.6
Aspartic acid	3.1	2.1	2.2	1.6
Cysteine	0.7	0.5	0.2	0.2
Phenylalanine*	1.2	0.8	1.3	1.0
Glutamic acid	5.3	3.7	5.9	4.3
Glycine	0.5	0.4	0.5	0.4
Histidine*	0.7	0.5	0.8	0.6
Isoleucine*	1.6	1.1	1.4	1.0
Leucine*	3.4	2.3	2.7	2.0
Lysine*	2.8	2.0	2.3	1.7
Methionine*	0.7	0.5	0.7	0.5
Proline	1.8	1.2	2.7	2.0
Serine	1.4	1.0	1.5	1.1
Threonine*	1.4	1.0	1.2	0.9
Tyrosine	1.0	0.7	1.2	0.9
Valine*	1.6	1.1	1.8	1.3
Tryptophan*	0.6	0.4	0.4	0.3
Total protein	27.7	19.1	27.3	20.3
*** Total EEA**	14.0	9.7	12.6	9.3
Total NEA	13.7	9.4	14.7	11.0
Fat	10.3	7.1	10.0	7.3
Carbohydrate	57.1	39.4	51.6	37.7
Total calories	430.6	297.1	407.2	297.3

4.7.3. Recovery

MVC in m. *Quadriceps femoris* with right and left leg was measured individually 20 minutes before the start of the workout and served as a baseline MVC value. This was repeated 15, 150 minutes and 24 hours after completion of 10RM workout. MVC was performed in a GYM 2000 knee-extension machine connected to a power gauge (HBM U2AC2, Darmstadt, Germany). The power gauge was connected to a PC and MVC was analyzed with Labview 8.2 (National instruments, Austin, Texas, USA). Individual back-, seat- and leg-settings were used for each participant at every MVC.

Participants performed two warm-up contractions with both legs, and were instructed to use approximately 25% and 75% of their maximum effort and contract for 5-10 seconds. After warm-up, three maximum effort MVC on each leg was performed with every participant instructed to hold the contraction for 2-3 seconds, and as quickly as possible reach maximum power. Each leg had 1 minute rest between trials.

4.7.4. 10RM workout

30 minutes after the first biopsy was taken, each participant was individually led through a standardized 10RM workout with 6 exercises of 3 sets of each exercise (table 4.4), all completed to exhaustion. There were 3 lower body- and 3 upper-body exercises. Starting weight was based on previous 1RM results in leg-press and chest-press. If starting weight was set too low in a given exercise, weight was put on to achieve 10RM. A new set was started every third minute which made all workouts last approximately 51 minutes.

Table 4.4: *Table showing all 6 exercises performed on acute days. Training load was 10RM, and load was added for each set if sets were not exhausting.*

Exercise	Sets	Reps	Load
Hammer squat	3	10	RM
Leg press	3	10	RM
Knee extension	3	10	RM
Chest press	3	10	RM
Seated row	3	10	RM
Shoulder press	3	10	RM

4.8. Analysis

4.8.1. Homogenization

Muscle tissue from biopsies was contained in ultra-freezers at – 80°C until homogenization. Approximately 50 µg muscle tissue were homogenized with 1 ml T-PER ® (Tissue Protein Extraction Reagent, Thermo scientific, Rockford, IL, USA), 20 µl Halt^{TM} Protease and Phosphatase Inhibitor Cocktail (Thermo scientific, Rockford, IL, USA) and 20 µl EDTA (Thermo scientific, Rockford, IL, USA). Homogenization was performed in pulses of 3-5 seconds until tissue was completely dissolved. After homogenization, samples were put at 4°C for 30 minutes. Subsequently, samples were centrifuged at 10 000 G for 10 minutes at 4°C. The supernatant from every sample were then transferred to 1.5 ml tubes before being centrifuged for another 10 minutes at 10 000 G. Afterwards, the supernatant were

aliquoted in labeled 0.2 ml tubes, each containing 25 µl, before being put in an ultra-freezer at -80°C.

4.8.2. Total protein measuring procedure

To establish total protein content, RC/DC Protein Assay kit from BioRad was used (cat. no. 500-0121, Herkules, CA, USA). Standard proteins were bovine y-globulin, ranging from 0.125 to 1.5 µg/ml. Samples were diluted 1:4 to ensure that protein concentration were within the range of standard proteins used. Triplicates of 5 µl from each sample were pipetted into a 96-well microtiter plate (Greiner Bio-one International AG, Kremsmünster, Germany). After pipetting, 25 µl reagens A*S and 200 µl reagens B were added in all wells. The wells were then sealed to avoid contamination and put in a dark closet for a minimum of 15 minutes. After 15 minutes the microtiter plate was analyzed in ASYS Expert 96 (Biochrom, Cambridge, UK). Only total protein measurements, with a CV<10% were used for subsequent Western Blot analysis, calculated with KIM Immunochemical Processing Software 32.

4.8.3. Western Blot

Resting levels and acute changes in the proteins of interest (eEF2, p706SK and 4E-BP1) was determined with Western Blot analysis at the NSSS laboratory. These analyses derived from muscle tissue obtained with biopsies from the m. *vastus lateralis*. For my own complete step-by-step procedure, see appendix 4.

4.8.4. Sample preparation

0.2 ml tubes were used to mix buffer (5MDT and Laemmli sample buffer, BioRad, CA, USA), distilled deionized water (dH_2O) and muscle tissue samples, resulting in a total volume of 65 µl in each tube. 5MDT and Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) (diluted 1:14) were added in a total of 16 µl to each 0.2ml tube with the remaining volume made up of dH_2O and tissue sample (calculations based on previous total protein measurements). Samples were then put on a heat block at 70°C for 10 minutes, before being applied on Criterion™ TGX (Tris-Glycine eXtended) Stain-Free™ precast 12-well gels for PAGE (BioRad, CA, USA). Each well was applied with 30 µl sample, containing 45 µg protein content. Samples from two study subjects were applied on each gel. In the first and last well, 5 µl of Protein Marker PS11 (GeneOn, GmbH,

Germany) was applied to serve as a protein marker, ranging from 10-175 kDA (figure 4.4). Each run included duplicates of each participant on a second gel.

4.8.5. Electrophoresis

Electrophoresis was done in a Mini-PROTEAN® Tetra Cell (BioRad, CA, USA), at 200 V with a HC High-Current Power Supply (BioRad, CA, USA). Running buffer consisted of 10% Tris/Glycine/SDS #161-0732 (BioRad, CA, USA) and 90% dH_2O. Electrophoresis continued until the GeneON-marker showed satisfactory separation of the 95 and 70 kDa bands (for separating eEF2 at ~90 kDa and p70 at ~70 kDa). Satisfactory separation was consistently achieved after 44-45 minutes. After electrophoresis, gels were put in a ChemiDoc™ MP Imaging System from BioRad (Hercules, CA, USA). This step UV-activated (2.5 minutes) the gels and visualized protein distribution (figure 4.4).

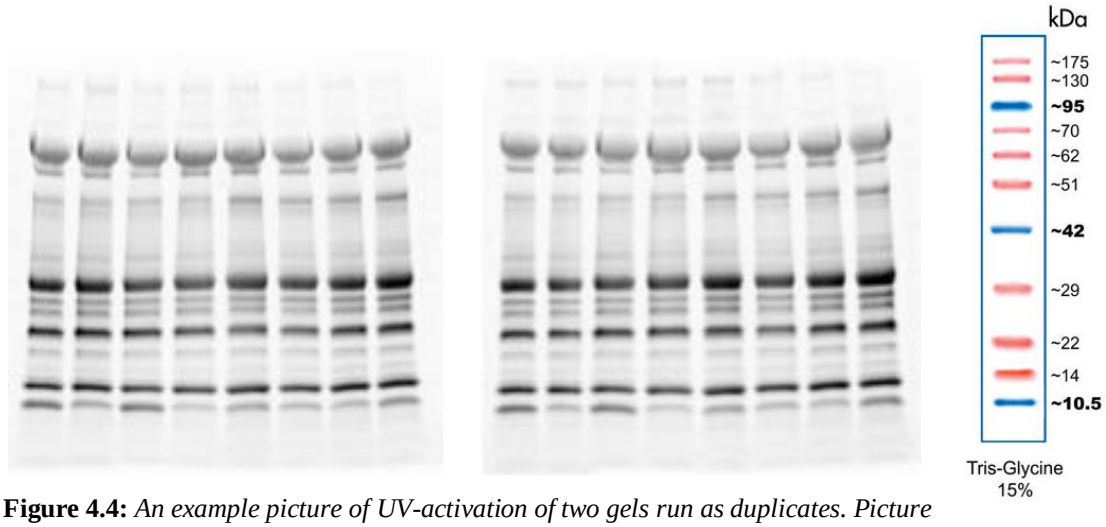

Figure 4.4: *An example picture of UV-activation of two gels run as duplicates. Picture shows protein distribution after electrophoresis. The Gene-On-marker used for later cutting of membranes is shown to the right.*

4.8.6. Blotting

Proteins were transferred to a PVDF-membrane **(**Cat#162-0177, Bio-Rad, CA, USA) in a Criterion™ Blotter (BioRad, CA, USA), with transfer buffer consisting of 10% methanol, 10% Tris/Glycine (Cat# 161-0743, BioRad, CA, USA) and 80% dH_2O. Blotting was run at 100V for 30 minutes with a magnet and ice pack inside the blotter, to ensure circulating transfer buffer, and avoid overheating.

After blotting, both gels and membranes were put in ChemiDoc^{TM} MP Imaging System to reveal transfer of proteins on duplicates, serving as a loading control (figure 4.5). This method of loading control has been proved superior to traditional methods using reference-proteins such as α-tubulin, ß-actin and GADPH (Vigelsø A, Dybboe R, Hansen CN, Dela F, Helge JW and Grau AG. 2015).

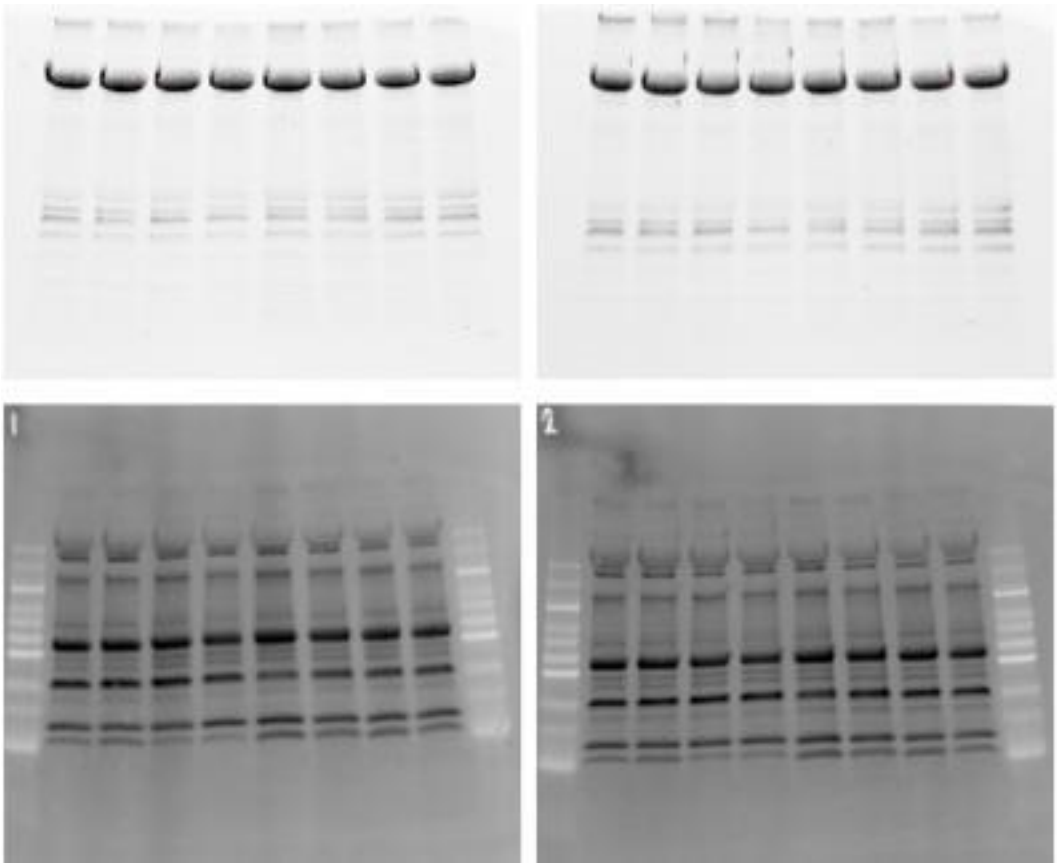

Figure 4.5: *Picture of the same duplicate gels (top) as in figure 4.4, and membranes (bottom) after blotting. Comparing figure 4.4 with the top two gels in fidure 4.5 one can clearly see how proteins has been transferred to their respective membranes (bottom) for further handling and analysis.*

4.8.7. Blocking and antibodies

After blotting and imaging of protein transfer and total protein loading, the membranes were incubated (blocked) for 2 hours in a 5% skim milk solution. After blocking, each membrane was cut to separate eEF2, p70 and 4E-BP1 into strips (with the help of GeneOn protein marker, see figure 4.4). The different membrane strips

were then incubated in their respective primary antibodies for phosphorylated protein (overview of antibody specifics and chosen dilutions in table 4.5).

Incubation was at 4°C over-night on a rocker-machine. The next day, membranes were washed for 15 minutes in TBS-T and 3 x 5 minutes in TBS (10X Tris Buffered Saline, Cat#170-6435, BioRad, CA, USA). TBS were 10-fold diluted giving 20 mM Tris, 500 mM NaCl, pH 7.4. TBS-T was the same formula with the adding of 1ml Tween 20 per litre of TBS-solution. After washing, membranes were incubated in secondary Anti-rabbit IgG HRP-linked antibody for 1 hour at room temperature, followed by another round of washing in TBS and TBS-T.

Membranes were incubated in Cheliminescent Substrate SuperSignal® WestDura (Thermo Scientific, Rockford, IL, USA) for 5 minutes before imaging in ChemiDoc™ MP Imaging. After imaging, membranes were stripped for 10 minutes in Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA) before another round of washing. After washing, membranes were blocked for 2 hours in 5% skim milk, followed by subsequent incubation over-night at 4°C in primary antibodies for total protein content. The following day was similar to the day after the first over-night incubation and ended with imaging of total protein content.

Table 4.5: *Table showing all primary and secondary antibodies used, with their respective dilutions. Primary antibodies were phospho- and total protein-specific. Secondary antibody used was Anti-rabbit IgG HRP-linked antibody*

Antibody	**Manufacturer**	**Host**	**Dilution**	**Cat.nr**
P70S6 kinase	Cell Signaling	Rabbit	1:1000	#9202S
Phospho p70S6 kinase (Thr 389)	Cell Signaling	Rabbit	1:1000	#9234S
eEF2	Cell Signaling	Rabbit	1:5000	#2332S
Phospho eEF2 (Thr 56)	Cell Signaling	Rabbit	1:5000	#2331S
4E-BP1	Cell Signaling	Rabbit	1:1000	#9452S
Phospho 4E-BP1 (Thr70)	Cell Signaling	Rabbit	1:1000	#9455S
Anti-rabbit IgG HRP-linked antibody	Cell Signaling	Goat	1:3000	#7074S

4.9. Statistics

All data are presented as means with standard deviation (±SD). In all figures in the following results-chapter, ±SD are indicated with error-bars. Pearson's r product moment correlation coefficient was used to explore the relationship between different variables. Student's t-test for paired/unpaired observations was used to evaluate differences within/between groups in phosphorylation-status of p70S6k, 4E-BP1 and eEF2 pre- and post-workout. A one-way factorial ANOVA with repeated measures was employed to evaluate physiological, anthropometrical and performance changes before and after the intervention within the NW and Milk groups. $P < 0.05$ was chosen as a two-tail level of significance. A two-way factorial ANOVA with repeated measures was employed to evaluate physiological, anthropometrical and performance changes before and after the intervention between NW and Milk groups. Calculations were done in Microsoft® Excel 2011 and Prism® 6 (GraphPad Software Inc., San Diego, CA, USA).

5. Results

5.1. Resistance training volume

There were no significant (P>0.05) differences between groups in resistance training (RT) volume (repetitions x series x intensity) on either of the two acute days (figure 5.1). RT-volume was significantly increased (P<0.0001) on acute day 2 compared to acute day 1 in both groups. RT-volume increased by 69% ± 22 and 63% ± 21 for the native whey (NW) and milk group respectively. The increase in RT-volume on acute day 2 was due to a higher training load as repetitions were set at 10 in each set. The greater albeit non-significant training volume (P>0.05) in the NW group on both acute day was mirrored by a greater (P=0.089) 1RM leg-press and chest-press pre and post-intervention (table 5.1).

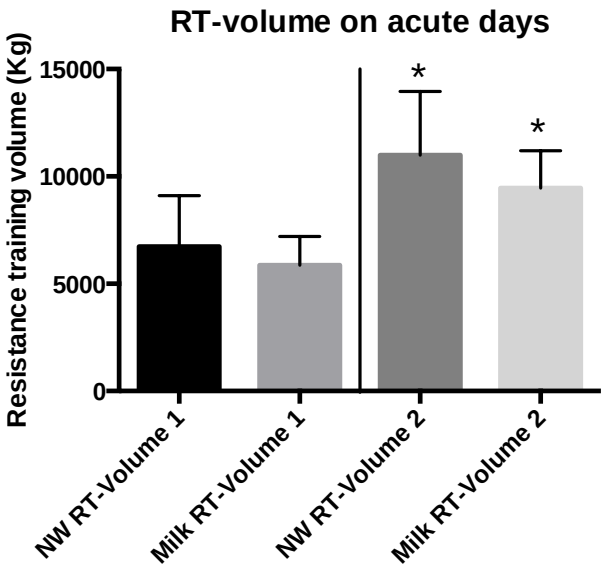

Figure 5.1: *RT-volume on acute days1 (left side) and 2 (right side) for NW and Milk groups. Y-axis shows training volume in kg (repetitions x series x intensity). * indicates significant change (P<0.0001) from acute day 1*

5.2. p70S6K

Ratio of phosphorylated p70S6K (Thr 389) to total p70S6K was significantly increased (P<0.05) from baseline (pre-biopsy) in the NW and Milk group on acute day 2 (figure 5.2). On acute day 1 ratio of phosphorylated p70S6K (Thr 389) to total p70S6K increased in the Milk and NW groups but failed to reach significance (P>0.05) with (P = 0.0529) and (P = 0.0713), respectively. There was no significant (P>0.05) differences between groups on either of the acute days. No significant differences was found within either group when the response on acute day 1 was compared with acute day 2, although a tendency of greater p70-phosphorylation was found in the NW group on day 2 compared with day 1 (P = 0.0740). When groups

were pooled by gender, comparison of acute day 1 and 2 showed no differences (P>0.05).

5.3. 4E-BP1

Ratio of phosphorylated 4E-BP1 (Thr 70) to total 4E-BP1 did not change significantly (P>0.05) from baseline (pre-biopsy) in either group on acute day 1 and 2 (figure 5.2). There was no significant (P>0.05) differences between groups on either of the acute days. No significant differences (P>0.05) was found within either group when acute day 1 was compared with day 2. When groups were pooled by gender, comparison of acute day 1 and 2 showed no differences (P>0.05).

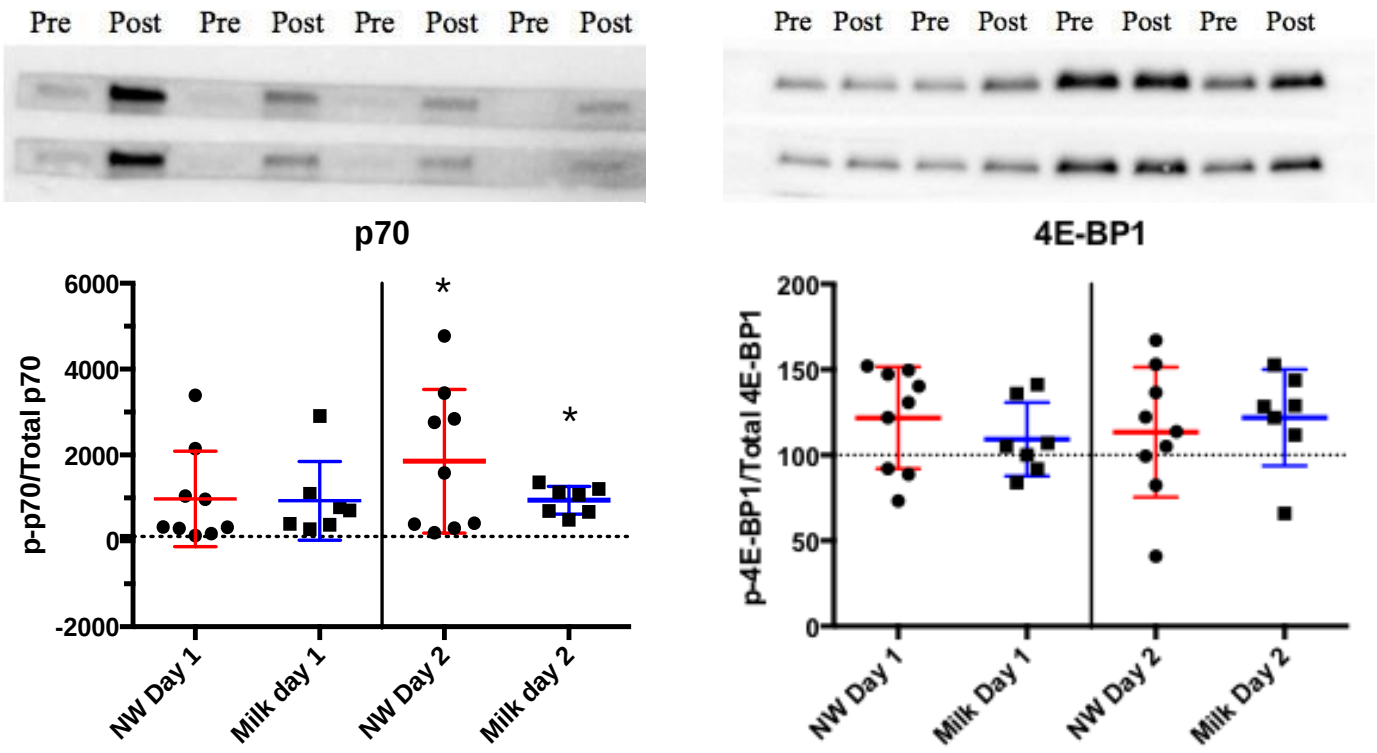

Figure 5.2: *The ratio of phosphorylated to total protein content of p706SK (left) and 4E-BP1 (right). Red colour indicates NW group and blue colour indicates Milk group. Horizontal dotted lines in both figures indicate baseline values (pre-biopsy). * indicates significant change (P<0.05) from baseline. Above p706SK and 4E-BP1 are examples of representative western blots of the respective signaling proteins*

5.4. eEF2

Ratio of phosphorylated eEF2 (Thr56) to total eEF2 did not change significantly (P>0.05) from baseline (pre-biopsy) in either group on acute days 1 and 2 (figure 5.3). There was no significant differences (P>0.05) between groups on either of the acute days. No significant differences (P>0.05) was found within either group when acute day 1 was compared with day 2. When groups were pooled by gender, comparison of acute day 1 and 2 showed no differences (P>0.05).

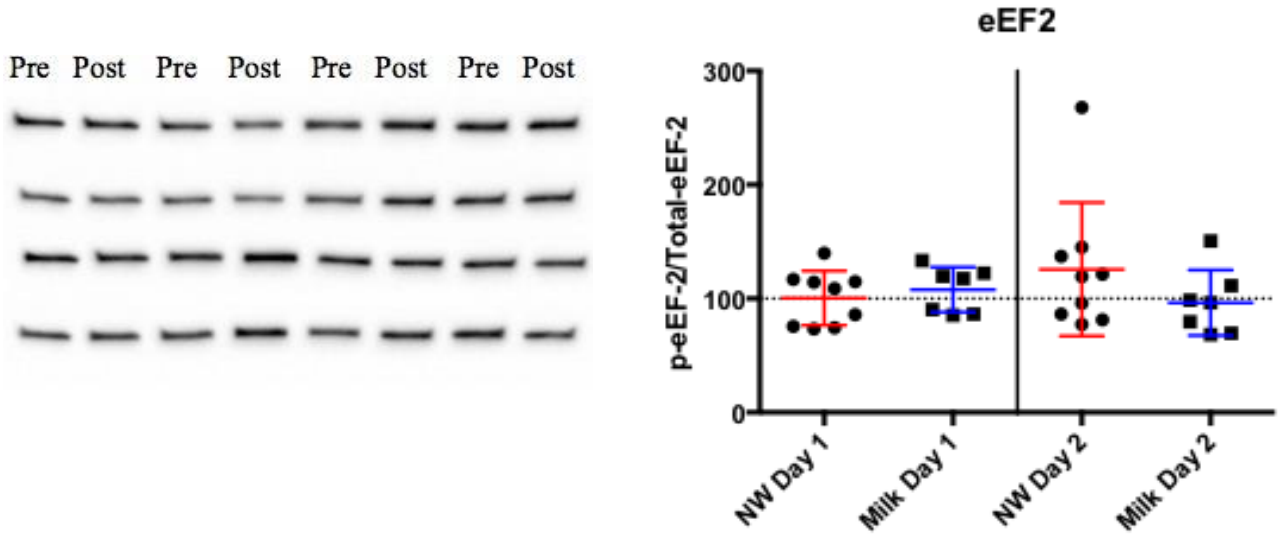

Figure 5.3: *The ratio of phosphorylated to total protein content of eEF2. Red colour indicates NW group and blue colour indicates Milk group. Horizontal dotted line indicates baseline values (pre-biopsy). To the left are examples of representative duplicate western blots of eEF2.*

5.5. Correlations

When correlations were done on group level, pooled by gender, or participants individually, between the ratio of phosphorylated/total protein content of p706SK, 4E-BP1, eEF2 and the outcome measures 1RM leg press, m. *vastus lateralis* thickness, lean body mass and lean mass in legs, no significant (P>0.05) correlations were found. There were also no correlations (P>0.05) found between the ratio of phosphorylated/total protein of p706SK, 4E-BP1 and eEF2 on either of the acute days or between acute days, on both group and individual level, or when pooled by gender.

5.6. MVC acute day 1

On acute day 1 both groups experienced a similar loss of MVC with no significant difference (P>0.05) between groups at both 15 minutes and 150 minutes after ended 10RM workout (figure 5.4). At both these time points both groups had significantly (P<0.05) reduced MVC compared to the baseline value. 24 hours after the workout, only the NW group returned to the baseline value, with the Milk group still being significantly reduced (P<0.05) compared to baseline. When comparing groups, there were no differences (P>0.05) at all-time points.

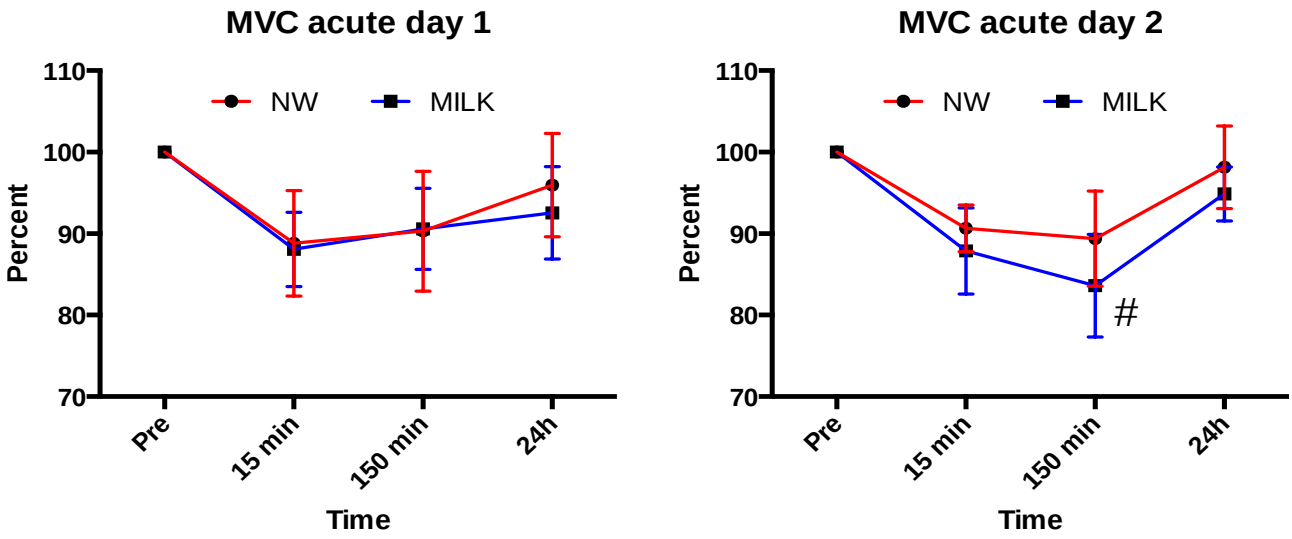

Figure 5.4: *Pre-workout MVC represent 100 percent (x-axis), and y-axis represent time after completed 10RM workout. NW and Milk groups are indicated with a red and blue line, respectively. On both acute day 1 and 2 only the NW group returned to baseline levels 24h after completed 10RM workout. # indicates significant (P<0.05) difference between groups.*

5.7. MVC acute day 2

On acute day 2 both groups experienced a similar loss (P>0.05) of MVC 15 minutes after ended 10RM workout (figure 5.4). At 150 minutes both groups still had significantly reduced (P<0.05) MVC compared to baseline. 24 hours after ended 10RM workout, only the NW group returned to baseline, with the Milk group still being significantly reduced (P<0.05) from baseline. When comparing groups, the Milk group were significantly (P<0.05) reduced compared to NW 2.5 hours after ended 10RM workout without significant differences at any other time point.

5.8. Anthropometric and performance measurements

There was no significant differences (P>0.05) between groups pre and post intervention (table 5.1), as was the case for all functional tests (data not shown). There was a tendency towards a difference (P=0.089) between groups in 1RM leg-press pre and post, with an increase of 41% ± 12 and 29% ± 14 for NW and Milk respectively.

Table 5.1: *Overview of long-term outcome measures after 11 weeks of strength training in NW and Milk groups. Pre and post intervention variables are included, along with % change from pre to post. Values of m. vastus lateralisis thickness in cm ±(SD) . Remaining values are in kg ±(SD)*

	Native Whey (n = 9) ♂ = 6 / ♀ = 3			Milk (n = 7) ♂ = 3 / ♀ = 4		
Variable (±SD)	**Pre**	**Post**	**% Change**	**Pre**	**Post**	**% Change**
Body mass	79.6 (19.1)	81.7 (19.0)	2.8 (1.5)	74.5 (12.8)	76.7 (12.5)	3.1 (2.6)
Lean mass	51.2 (12.3)	52.8 (12.4)	3.3 (2.5)	47.2 (6.6)	49.7 (6.5)	5.5 (3.2)
Lean legs	17.8 (5.6)	18.4 (5.5)	4.2 (3.4)	16.4 (13.3)	17.5 (3.2)	7.0 (4.4)
1RM Leg press	174.4 (63.3)	240 (76.0)	40.7 (12.2)	160.4 (35.5)	204.6 (36.2)	28.7 (13.6)
1RM Chest press	48.1 (20.0)	56.4 (21.9)	19.1 (7.4)	39.3 (12.0)	46.8 (10.2)	19.1 (7.4)
m. vastus lateralis	2.2 (0.5)	2.4 (0.6)	6.2 (4.7)	1.9 (0.3)	2.1 (0.4)	7.1 (7.0)

6. Discussion

The main findings in this study were: 1) After an exhausting 10RM-workout with subsequent intake of either native whey or milk-protein, p70-phosphorylation was increased 2 hours post-exercise, whereas 4E-BP1- and eEF2-phosphorylation remained unchanged. 2) There were no differences in intramuscular signaling responses between native whey and milk protein supplementation. 3) 11 weeks of strength-training produced significant increases in lean body mass- and 1RM strength. 4) Lean body mass increases, m. *vastus lateralis* thickness and 1RM outcome-measures were not different between groups, nor did they show any correlations with acute individual intramuscular signaling responses.

6.1. p70S6K

Phosphorylation of p70 increased significantly 2 hours post-workout for Milk and NW groups on acute day 2, while showing a strong tendency for Milk and NW on day 1. This is in agreement with previous studies reporting increased p70-phosphorylation when combining resistance exercise (RE) with subsequent protein intake (Farnfield et al, 2012; Karlsson et al, 2004; Moore et al, 2011; Reitelseder et al, 2011). Moore et al (2011) reported increased p70-phosphorylation in the trained leg 1, 3 and 5 hours after unilateral leg-press and knee-extension combined with intake of 25 g of whey protein, and phosphorylation was highly associated with a concomitant increase in MPS.

Our participants consumed 20 g in the form of NW or Milk-protein post-exercise, corresponding to 0.4 g of protein/kg lean body mass (LBM). The NW and milk supplements had a leucine content of 2.3 g and 2.0 g, respectively. Reitelseder et al (2011) reported an increase in p70-phosphorylation in concert with MPS-increases over 1-6 hours when consuming 0.3 g/kg LBM of protein, averaging as either 17.5 g of whey (2 g leucine) or casein (1.5 g leucine) post-exercise, with no differences between whey and casein. As no differences were seen between a fast (whey) and slow (casein) protein source (Reitelseder et al, 2011) with less protein and leucine content than the NW (fast) and Milk (slow)-supplements used in this study, it could suggest that both our supplements contained sufficient amounts of leucine for inducing maximal p70-responses after RE and protein intake.

In contrast to our findings, some studies report no significant changes in p70-phosphorylation after RE in elderly (Fry et al, 2011; Kumar et al, 2009. Both these studies (Fry et al, 2011; Kumar et al, 2009) were without post-exercise protein intake, in contrary to our study. As we report significantly increased p70-phosphorylation in elderly after RE in combination with protein intake, their lack of protein intake could explain their reported blunted p70-response in elderly (Kumar et al, 2009; Fry et al, 2011).

We report no difference in p70-phosphorylation between groups on either acute day, mirrored by a similar training volume between groups. As both exercise-volume (Terziz et al, 2010) and –intensity (Kumar et al, 2009) is shown to affect signaling responses, an unequal training-volume between groups could confound the results. Kumar et al (2009) reported in both young and old, a plateau in MPS-response and p70-phosphorylation when load is in the range of 60-90% of 1RM. Pre and post 1RM results confirm that all of our participants were within this 60-90% range of 1RM on both acute days. Furthermore, MVC-tests performed 15 minutes post-exercise shows an equal drop in MVC for both groups, indicating a similar degree of fatigue. This fatigue was present as <20% reductions in 1RM after heavy RE indicate considerable fatigue, without muscle damage (Paulsen et al, 2012). Training volume, intensity and MVC-data on both acute days all indicate similar degrees of fatigue post-exercise, thus both groups presumably had equal premises of induced p70-response.

Groups were slightly skewed by gender, with one third and two thirds being female in the NW and Milk group respectively. One study in elderly (Smith et al, 2008) reported that only men increased their MPS in the postprandial state, while p70-phosphorylation was similar between genders in both the post absorptive and postprandial state. Other studies reports similar increases in p70-phosphorylation between genders 2 hours after RE (Dreyer et al, 2010) and 1 and 4 hours after protein infusion (Smith et al, 2009). In line with the previous studies (Dreyer et al, 2010; Smith et al; 2008; Smith et al, 2009) when pooled by gender, we found no differences in p70-phosphorylation. Thus, it is unlikely that gender-differences between groups have affected the findings in this study.

Training status is reported to predict the degree of p70-phosphorylation (Farnfield et al, 2012). In the untrained state, elderly (mean age 68) significantly increased p70-phosphorylation 2 hours after RE and 27 g of whey protein intake, a response not reproduced in the trained state (Farnfield et al, 2012). All our participants were in an untrained state and unfamiliar with RE on acute day 1. After 11 weeks of RE our participants completed acute day 2 in a more trained state, as both groups had a significant ($P<0.05$) ~65% increase in training volume concomitant with significant pre- to post-intervention strength-gains. Similar to our study, Farnfield et al (2012) participants went through 12 weeks of resistance training, but in contrast to their findings we do not report a blunted response in the trained state, as p70-phosphorylation were significantly ($P<0.05$) increased on acute day 2.

Furthermore, we do not report significant differences between groups on either acute day. Within each group, comparing acute day 1 with acute day 2, the NW group had a tendency ($P= 0.0740$) of a greater p70-phosphorylation on acute day 2. If our participants were in a state of anabolic resistance pre-intervention, we expected anabolic signaling to be greater in the trained state. As both groups only showed significant increases in p70-phosphorylation on acute day 2, and the NW group showing a tendency of greater phosphorylation on acute day 2 compared to acute day 1, this is in agreement with our expectations of RE and protein intake as efficient countermeasures of anabolic resistance. A few differences in their (Farnfield et al, 2012) study-design compared to ours stand out. Their exercise protocol was not a full-body program, but single-legged knee-extension. The young participants in this study (Farnfield et al, 2012) significantly increased p70-phosphorylation in the trained state as well as the untrained state. As training volume affects p70-signalling (Terziz et al, 2010), elderly in the more trained state might need a greater exercise-volume than performed in Farnfield et al (2012). Our exercise protocol was of much greater volume than theirs (Farnfield et al, 2012), which probably is the reason for our divergent reports of p70-phosphorylation in the trained state.

In our study and Farnfield et al (2012) post-exercise biopsies were collected after 2 hours. Our baseline biopsy was obtained in the postprandial state, whereas theirs were obtained in the post absorptive state. Protein intake alone is shown to increase p70-phosphorylation (Dickinson et al, 2011; Fujita et al, 2007a) with leucine-content

proven to be of particular importance (Churchward-Venne et al, 2012). As Farnfield et al (2012) baseline p70-measurement in the post absorptive state presumably would be lower compared to ours in the postprandial state, participants being of similar age (~68 years old) and the fact that their supplement both had a higher protein- (27 g vs ~20 g) and leucine-content (3.7 g vs ~2 g), there is no obvious reason to why Farnfield et al (2012) in contrast to our findings, reported a blunted p70-response in the trained state.

We report no significant (P>0.05) differences between groups in p70-phosphorylation, m. *vastus lateralis* thickness, LBM increases and any other anthropometric measurement. Correlations were subsequently both pooled by gender and on an individual level, but no correlations between any outcome measure and the magnitude of p70-phosphorylation were found, in contrast to previous reports (Baar & Esser, 1999; Terziz et al, 2008). Phosphorylation of p70 post-exercise has been reported to be highly correlated to the degree of subsequent hypertrophy in rats (Baar & Esser, 1999). In young men, hypertrophy, increases in whole-body fat free mass (FFM), leg fat free mass (LFFM), 1RM squat and mean muscle CSA after 14 weeks of resistance training were highly correlated with p70-phosphorylation after an pre-intervention exercise bout (Terziz et al, 2008). In line with our study, Terziz et al (2008) had their participants consuming a standardized breakfast before RE and biopsies were collected from m. *vastus lateralis*. However, there are several methodic differences between our study and Terziz et al (2008) that could explain why we do not find similar correlations. Terziz et al (2008) measured p70-phosphorylation 30 minutes post RE, whereas we had a 2 hour post RE measurement. Phosphorylation states are transient and highly dynamic, making the time-points of biopsy collections a determining factor of measured phosphorylation (Drummond et al, 2008). Furthermore, our study was in an older population whereas Terziz et al (2008) had younger participants (~21 years). As previously shown (Fry et al, 2011; Kumar et al, 2009), this age difference could lead to differences in the magnitude of p70-phosphorylation. Terziz et al (2008) exercise protocol was completed in 12 minutes consisting of 6 RM leg-press with 6 sets, a lower training volume than our participants, with different RE volume shown to induce varying p70-phosphorylation (Kumar et al, 2009). Taken together, the methodological differences and different outcomes in our study and Terziz et al (2008) highlight the need for future studies

linking acute phosphorylation states of anabolic signaling proteins with longitudinal outcome measures.

In summary, exercise-volume and -intensity, MVC-data and gender is seemingly not confounding variables of our reported p70-phosphorylation between groups. Both NW- and Milk-groups significantly increased p70-phosphorylation 2 hours post-RE and protein intake on acute day 2, only showing a strong tendency on acute day 1. This suggest that our participants could have been in a greater state of anabolic resistance pre- than post-intervention. Furthermore, phosphorylation-state of p70 2 hours post RE and protein intake is not predictive of subsequent LBM and strength gains in elderly men and women after 11 weeks of resistance training. We acknowledge that our results are based on biopsies collected 2 hours post RE, and that biopsies collected at other time-points could produce different results.

6.2. 4E-BP1

In line with previous studies (Hulmi et al, 2009; Dreyer et al, 2006; Fry et al, 2011) we report non-significant increases in 4E-BP1-phosphorylation 2 hours after RE and protein intake. As our participants consumed ~20 g of either NW or Milk-protein our findings is coherent with Hulmi et al (2009) comparing 15 g of whey protein to non-caloric placebo-intake pre- and post-workout (total of 30 g whey), reporting non-significant increases in 4E-BP1-phosphorylation 1 hour post-workout with whey, but a significant decline with placebo. The above-mentioned studies, and all studies subsequently discussed measure 4E-BP1-phosphorylation at Thr 37/46, in contrast to our data, reflecting 4E-BP1 phosphorylated at Thr70. As reported by Gingras et al (1999), phosphorylation at Thr 37/46 do not disrupt the binding to eIF4E, but serve as a prerequisite for subsequent phosphorylation at Thr70 before 4E-BP1 disrupt from eIF4E, allowing cap-dependent translation. Whether the methodic differences in choice of phosphorylation sites between our study and studies in comparison, would lead to greatly divergent results is of uncertainty. Gingras et al (1999) propose that phosphorylation of Thr37/46 is necessary as a priming event for subsequent phosphorylation of Thr70, thus these methodic differences probably still allow for comparison between studies.

Without post-exercise protein intake, others report a significant reduction in 4E-BP1-phosphorylation immediately after (Deldique et al, 2008; Dreyer et al, 2008) and 1 hour post-exercise (Drummond et al, 2008), returning to baseline 2 hours post-exercise (Dreyer et al, 2006). These studies (Deldique et al, 2008; Drummond et al, 2008; Dreyer et al, 2006) report in agreement with our findings an increase in p70-phosphorylation concomitant with reduced/unchanged 4E-BP1-phosphorylation. In the post absorptive state, Dreyer et al (2008) reported in line with one of their previous studies (Dreyer et al, 2006) a significant immediate reduction in 4E-BP1-phosphorylation, returning to baseline 1 hour post-exercise. When ingesting ~20 g of EEA with ~7 g leucine 1 hour post-exercise, 4E-BP1 was significantly increased 1 and 2 hours after protein intake (Dreyer et al, 2008; Fujita et al, 2007b). In agreement with our findings, these studies (Dreyer et al, 2008; Fujita et al, 2007b) reported significant increases in p70-phosphorylation 2 hours post-exercise, but in contrast we do not report concomitant significant increases in 4E-BP1-phosphorylation.

The reported increase in p70-phosphorylation concomitant with a decline in 4E-BP1-phosphorylation in the early recovery phase (Deldicque et al, 2008; Drummond et al, 2008; Dreyer et al, 2006; Dreyer et al, 2008) seem to mirror our p70-phosphorylation pattern. Thus, the reported early 4E-BP1-decline reported by others might have preceded our 4E-BP1-measurement 2 hours post-exercise. Dreyer et al (2006) speculate that the immediate RE-induced reduction in 4E-BP1-phosphorylation is associated with increased AMPK-activity, inhibiting ATP-consuming anabolic processes such as MPS and anabolic signaling responses. Furthermore, Dreyer et al (2006) challenge the generally accepted role of mTOR directly controlling subsequent 4E-BP1-phosphorylation by reporting a disconnect between the degree of phosphorylated mTOR and 4E-BP1. It has been shown that p70-phosphorylation is rapamycin-sensitive, but 4E-BP1-phosphorylation is rapamycin-insensitive (Wang et al, 2005). Hulmi et al (2009) reported in agreement with our findings, significantly increased p70-phosphorylation concomitant with unchanged 4E-BP1-phosphorylation. The reason to why our increased p70-response is not matched by an increase in 4E-BP1-phosphorylation, might be that mTOR regulate p70- and 4E-BP1-phosphorylation via different downstream-effectors (Wang et al, 2005). However, we measured p70 and 4E-BP1 at the same time-point, and the lack of concomitant phosphorylation of both signaling proteins, may be a result of these signaling proteins

having a different time-course of increased phosphorylation after RE and protein intake

As previously described, equal training-volume on acute days 1 and 2 together with our MVC-data 15 minutes post-workout infer similar RE-induced stimuli between our groups to phosphorylate 4E-BP1. 4E-BP1-phosphorylation is reported by others to be similar between elderly men and women in basal 4E-BP1-phosphorylation, 2 hours post-exercise, and after protein infusion of 0.1 g protein/kg fat free mass (FFM) per hour for 7 hours (Dreyer et al, 2010; Smith et al, 2009). A previous study (Smith et al, 2008) showed that only elderly men increased 4E-BP1-phosphorylation and MPS in the postprandial state. When we pooled our groups by gender we found no differences in 4E-BP1-phosphorylation on acute day 1 (untrained state) and acute day 2 (more trained state). This is in agreement with the above studies (Dreyer et al, 2010; Smith et al, 2009). As we show similar RE-induced stimuli between groups and that gender does not relate to 4E-BP1-response, group differences in 4E-BP1-phosphorylation should reflect differences in supplements given.

Similar to our findings, Dickinson et al (2014) reported unchanged 4E-BP1-phosphorylation 2 hours post-exercise in elderly when participants ingested 10 g of EEA with 1.85 g of leucine. The potent effect of leucine content was elucidated when Dickinson et al (2014) supplemented 10 g of EEA with 3.5 g leucine, significantly increasing 4E-BP1-phosphorylation 2 hours post-exercise. As we found no difference between acute day 1 (untrained) and 2 (trained) within each group or when pooled by gender, we report training status not to affect 4E-BP1-phosphorylation 2 hours post RE and protein intake. Farnfield et al (2012) examined 4E-BP1-phosphorylation in elderly compared to young 2 hours post-exercise combined with a protein intake of 26.6 g of whey protein (leucine content, 3.6 g). Elderly significantly increased 4E-BP1-phosphorylation in both the untrained and trained state (after 12 weeks of RE), while their younger counterparts had unchanged 4E-BP1-phosphorylation (Farnfield et al, 2012). In agreement with our findings, neither elderly nor young showed different 4E-BP1-phosphorylation comparing pre- and post-intervention measurements.

We found no difference (P>0.05) in 4E-BP1-phosphorylation between groups on acute day 1 or 2. Our supplementation with NW and Milk containing 2.3 g and 2.0 g leucine respectively might be inadequate to give a potent 4E-BP1-response in elderly, as 3.4-7 g of leucine has been proven sufficient to significantly increase 4E-BP1-phosphorylation, with training status not affecting the degree of phosphorylation (Dreyer et al, 2008; Dickinson et al, 2014; Drummond et al, 2008; Fujita et al, 2007b; Farnfield et al, 2012). Similar to our findings, supplementing with a leucine-content in the range of 1.5-2.3 seem to be insufficient to potently increase 4E-BP1-phosphorylation (Dickinson et al, 2014; Reitelseder et al, 2011).

Hulmi et al (2009) supplemented a total of 3.4 g leucine, but did not find increased 4E-BP1-phosphorylation, in contrast to the previously mentioned studies (Dreyer et al, 2008; Dickinson et al, 2014; Drummond et al, 2008; Fujita et al, 2007b; Farnfield et al, 2012). The reason for this discrepancy could be explained by the early post RE-measurement (1 hour) and a spread feeding pattern (Hulmi et al, 2009), shown to give inferior 4E-BP1-phosphorylation (Mitchell et al, 2015). As both our study and Hulmi et al (2009) measured 4E-BP1-phosphorylation to remain at baseline values 2 hour post RE, this might be a consequence of counterbalancing the previously mentioned 4E-BP1 decline in the early recovery phase (Deldicque et al, 2008; Drummond et al, 2008; Dreyer et al, 2006; Dreyer et al, 2008). As Hulmi et al (2009) report a strong tendency of 4E-BP1-phosphorylation to decrease in their placebo group, it seems that protein supplementation in both ours and their study is sufficient to counterbalance the RE-induced 4E-BP1 decline. Furthermore, it seems that protein intake is the main determinator of 4E-BP1-phosphorylation status as Moore et al (2011) showed equal 4E-BP1-phosphorylation in the rested vs exercised leg 1, 3 and 5 hours post unilateral knee-extension combined with protein intake.

If we had additional biopsies withdrawn at later time points we might have seen increases in 4E-BP1-phosphorylation, as anabolic signaling is shown to be delayed with aging (Drummond et al, 2008). In line with our findings, Drummond et al (2008) reported unchanged 4E-BP1-phosphorylation in the early recovery phase (0-2 hours after RE and protein intake, but it was significantly increased 3 hours post-exercise in elderly. Drummond et al (2008) supplemented with 20 g of EEA, highly enriched with leucine (7 g of leucine) 1 hour post exercise, while our study supplemented

protein immediately post exercise, thus making their 3 hour post-workout 4E-BP1-measurement comparable with our measurement 2 hours post RE and protein-intake. Furthermore, Drummond et al (2008) reported 4E-BP1-phosphorylation returning to baseline values after 6 hours. Considering the different time-points of 4E-BP1 measurements in light of the aforementioned studies, our 2 hour post RE and protein intake seem to reflect a 4E-BP1-phosphorylation state counterbalancing the RE-induced immediate decline, and before subsequent peak phosphorylation and decline to baseline values at later time points (3-6 hours) (Drummond et al, 2008).

Feeding pattern and source of protein is reported to affect both anabolic signaling responses and MPS (West et al, 2011; Mitchell et al, 2015; Reitelseder et al, 2011). We supplemented NW and Milk-protein as a bolus dose of 20 g immediately post-exercise, proven to give a superior 4E-BP1-response compared to spread feeding (Mitchell et al, 2015). In contrast, West et al (2011) report no difference between bolus and spread feeding on 4E-BP1-phosphorylation. However, West et al (2011) report increased MPS-response to bolus feeding, while Mitchell et al (2015) report unchanged MPS-response in association with feeding patterns. Based on these studies (Mitchell et al, 2015; West et al, 2011) the choice of giving our participants post-exercise protein intake as a single bolus should not induce a reduced response in 4E-BP1- and p70-phosphorylation compared to a spread feeding pattern.

Reitelseder et al (2011) (for further details, see p70-section) compared post-exercise whey vs casein intake, and reported that 4E-BP1 was phosphorylated in favour ($P<0.05$) of casein at 1, 3.5 and 6 hours after protein intake. Regardless of different 4E-BP1-responses between whey and casein, MPS remained equal for both supplements 1-6 hours (Reitelseder et al, 2011). Furthermore, neither whey nor casein was able to significantly increase 4E-BP1-phosphorylation at any time point over 1-6 hours, with whey showing a tendency of decreased 4E-BP1-phosphorylation (Reitelseder et al, 2011).

Thus, based on our data and studies in comparison (Reitelseder et al, 2011; Mitchell et al, 2015; West et al, 2011) it appears to be a disconnect between single point-in-time changes in signaling molecule phosphorylation, the dynamic changes in MPS, subsequent hypertrophy and strength gains. This disconnect is also reported by

Atherton et al (2010) after isolated bolus feeding of 48 g whey protein (saturating protein-dose, according to previously described ”muscle-full” concept) with 4E-BP1- and p70-phosphorylation (baseline after 2.5 hours) outlasting the associated MPS-response (baseline after 1.5 hours).

The apparent RE-induced immediate reduction (Deldique et al, 2008) in 4E-BP1-phosphorylation is restored by protein intake (Hulmi et al, 2009; Dreyer et al, 2008; Farnfield et al, 2012) and in the recovery period (1-6 hours) (Dreyer et al, 2006; Drummond et al, 2008; Fry et al, 2011). We found no correlations between the degree of individual 4E-BP1-phosphorylation and any of the other outcome measures, also when pooled by gender. Isolated, the effect of RE seems to dephosphorylate 4E-BP1 (Hulmi et al, 2009; Dreyer et al, 2006; Deldicque et al, 2008) and according to our data and others (Hulmi et al, 2009; Farnfield et al, 2012; Mitchell et al, 2015; Atherton et al, 2010) subsequent protein intake phosphorylates and restores this RE-induced dephosphorylation, with no strong association to the concomitant MPS-response (Reitelseder et al, 2011; West et al, 2011; Mitchell et al, 2015).

As our participants experienced significant increases in lean body mass and strength gains, we have to conclude in agreement with Hulmi et al (2009) that unchanged acute 4E-BP1-phosphorylation (not dephosphorylated) seems sufficient for the eIF4E/G/A complex to form and contribute to translation initiation, allowing accumulating hypertrophy and strength gains after 11 weeks of RE. In line with this conclusion Farnfield et al (2012) reported increased 4E-BP1-phosphorylation in elderly while being unchanged in young participants in both the untrained and trained state, but with both age-groups having similar increases in 1RM strength after 12 weeks of RE. We conclude that when measured 2 hours post RE and protein intake, the degree of 4E-BP1-phosphorylation is not predictive or associated with the magnitude of individual hypertrophy or strength gains in elderly after 11 weeks of RE.

6.3. eEF2

We report eEF2-phosphorylation to remain unchanged 2 hours post-exercise and protein intake, with no differences between groups on either of the acute days. This is

in agreement with Moore et al (2011) who reported eEF2-phosphorylation to be unchanged in both the rested and exercised leg 1, 3 and 5 hours after bilateral knee-extension combined with 25 g of whey intake. Furthermore, Hulmi et al (2009), reported unchanged eEF2-phosphorylation 1 hour post exercise in combination with ingestion of 30 g of whey protein. Fujita et al, (2007b) reported eEF2-phosphorylation to be significantly reduced (activated) after ingestion of 20 g EEA with a higher leucine content (7 g) than our supplements at rest, and after RE combined with the same amount of EEA and leucine (Dreyer et al, 2008; Drummond et al, 2008). Whereas two of these studies were conducted in young participants (Dreyer et al, 2008; Fujita et al, 2007b), Drummond et al (2008) shows a delayed response in intracellular signaling in elderly after RE and protein intake. We report unchanged eEF2-phosphorylation 2 hours post RE and protein intake, with Drummond et al (2008) reporting unchanged eEF2-phosphorylation 1 hour post RE, but a significant reduction in eEF2-phosphorylation after 3 and 6 hours. Thus, if we had measured eEF2-phosphorylation at a later time-point we might have seen a significant reduction. On that note, Drummond et al (2008) had their participants ingesting 20 g of EEA (7 g leucine) 1 hour post RE making their 3 hour post RE-measurement (2 hour post protein intake) comparable with our 2 hour post RE and protein intake-measurement.

The leucine-content used in studies with similar doses of whey (Hulmi et al, 2009; Moore et al, 2011) as our NW- and Milk-supplements were far below the highly enriched leucine-content (7 g) used in other studies (Dreyer et al, 2008; Drummond et al, 2008; Fujita et al, 2007b) and may explain the discrepancy in our findings. Furthermore, Fujita et al (2007b) reported unchanged eEF2-phosphorylation in their control group not consuming nutrients, suggesting leucine-content in our supplements to be insufficient and comparable to not ingesting nutrients. Our reported increase in p70-phosphorylation was unexpectedly not mirrored by reduced eEF2-phosphorylation, as phosphorylated p70 activates eEF2 through phosphorylation of eEF2 kinase (Wang et al, 2001). Fujita et al (2007b) point to their robust increase in p70-phosphorylation as a probable explanation for their reported decrease in eEF2-phosphorylation. Thus, our findings are in contrast to several studies showing reduced eEF2-phosphorylation concomitant with increasing p70-phosphorylation (Fujita et al, 2007b; Dreyer et al, 2008; Dreyer et al, 2010; Drummond et al, 2008).

In addition to no group differences in eEF2-phosphorylation we found no differences within groups in eEF2-phosphorylation when comparing acute day 1 and 2. Similar to our measurements of 4E-BP1-phosphorylation, we find no association between eEF2-phosphorylation and training status. In elderly as previously elucidated, training status has been shown to have a blunting effect on p70-phosphorylation and no effect on 4E-BP1-phosphorylation (Farnfield et al, 2012). However, Farnfield et al (2012) did not include eEF2 in their study for further comparisons.

Our groups were as previously described, slightly skewed by gender. As we found no group differences, and training status to be unrelated to eEF2-phosphorylation, we pooled participants by gender to examine whether this skewness could confound group measurements. In elderly men and women, it is shown a similar decrease in eEF2-phosphorylation 2 hours post-exercise, and after isolated protein intake (Dreyer et al, 2010; Smith et al, 2009). In agreement with these studies (Dreyer et al, 2010; Smith et al, 2009) we found no differences in eEF2-phosphorylation between genders, but in contrast we found eEF2-phosphorylation to remain unchanged after RE and protein intake.

We show equal RE-induced stimuli between groups and that gender or training status does not relate to eEF2-response, thus potential group differences in eEF2-phosphorylation should reflect differences in our given supplements. As we found no group differences, we report no supplemental effect on eEF2-phosphorylation, and previously mentioned studies (Dreyer et al, 2008; Drummond et al, 2008; Fujita et al, 2007b) suggest leucine content in both our supplements might be insufficient to robustly decrease eEF2-phosphorylation. It should be noted that in these studies (Dreyer et al, 2008; Drummond et al, 2008; Fujita et al, 2007b) baseline biopsies were collected in the post absorptive state, thus subsequent biopsies collected post RE and protein intake would presumably reveal greater changes in phosphorylation-state compared to our biopsies collected in the fed state. These methodic differences has been shown to affect eEF2-phosphorylation after RE and protein intake (Deldicque et al, 2010). Deldicque et al (2010) had their participants either fasting or fed a carbohydrate-rich breakfast pre-exercise, with post-exercise biopsies collected 1 and 4 hour after the workout ended, and greater reductions in phosphorylated eEF2 was seen in the group not consuming breakfast pre-exercise (Deldicque et al, 2010).

Our contradicting findings of increased p70-phosphorylation not being mirrored by reduced eEF2-phosphorylation are in line with studies questioning the importance of acute eEF2-phosphorylation. The phosphorylation pattern of eEF2 in response to isolated RE and protein intake, and the two stimuli combined seem to be contradicting. When consuming a large bolus of 48 g of whey, eEF2-phosphorylation remained unchanged for 6 hours (Atherton et al, 2010), in contrast to reduced eEF2-phosphorylation in other studies with 20 g of EEA highly enriched leucine content (Dreyer et al; 2006; Drummond et al, 2008; Fujita et al, 2007b).

West et al (2011) compared bolus vs spread feeding pattern, with spread feeding inducing a significantly greater reduction in eEF2-phosphorylation compared to bolus feeding. However, the greater reduction in eEF2-phosphorylation after spread feeding was concomitant with reduced MPS compared to bolus feeding 1, 3 and 5 hours post protein intake (West et al, 2011). In another study there were no differences between bolus and spread feeding in regards to MPS, with eEF2 remaining unchanged from baseline with both feeding patterns (Mitchell et al, 2015). Kumar et al (2009) reported in both young and old that eEF2 was 20% less phosphorylated immediately after RE before rebounding to 120% of baseline values 1 hour after (authors stated this as a highly inactivated state), in great contrast to similar studies (Dreyer et al, 2006; Fujita et al, 2007b). Furthermore, Moore et al (2011) reported a reduction in eEF2-phosphorylation in the exercise and fed condition compared to rested and condition, but unexpectedly unchanged compared to rested and fasted condition. In line with these contradicting findings, Kumar et al (2009) stated acute eEF2-phosphorylation to be of little biological relevance and agrees with our reports of eEF2-phosphorylation 2 hours post RE and protein intake not being individually correlated to any outcome measure after 11 weeks of resistance training in elderly men and women.

6.4. Hypertrophy and strength

The most obvious finding from our study was the great improvements in the physical state of our participants, emphasizing the potent effect of progressive resistance training combined with protein supplementation. As elucidated in a comprehensive meta-analysis (Cermak et al, 2012), it is likely that our protein supplementation, ensuring a daily intake of >1.2 g/kg/body mass during the 11 weeks of resistance

exercise was a contributor to our participants' increases in 1RM strength and hypertrophy. Our participants experienced a large increase in 1RM strength and lean body mass (LBM) compared with meta-analysis in both young and old (Cermak et al, 2012) and old >65 years (Peterson et al, 2010, Peterson et al, 2011). Similar to our study, mean number of exercises performed was ~8, with ~10 repetitions in each exercise, and training frequency was ~3 times per week (Cermak et al, 2012; Peterson et al, 2011; Peterson et al, 2010). Mean duration of the training interventions was ~17-20 weeks (Peterson et al, 2010&2011) and ~12 weeks (Cermak et al, 2012). When examining studies in older populations of both genders (Peterson et al, 2011), mean gains in whole body fat free mass was ~1.1 kg after 20 weeks of training, with our participants showing a mean increase of ~2 kg in 11 weeks. Increases in 1RM leg press and chest press from baseline after ~17 weeks of resistance training have been reported to be ~32 kg and ~10 kg respectively (Peterson et al, 2010), with our participants showing a mean increase in leg press and chest press of ~55 kg and ~8 kg respectively, in only 11 weeks. Thus, our full-body progressive resistance training program combined with protein supplementation seems to be efficient countermeasures to the reported age-related atrophy and anabolic resistance (Janssen & Ross, 2005; Cuthbertson et al, 2005, Katsanos et al, 2005&2006; Kumar et al, 2009; Paddon-Jones et al, 2004; Yang et al, 2012a&b).

6.5. Recovery

When examining MVC data, the Milk group had a significantly reduced ($P<0.05$) MVC 2.5 hours after RE and protein intake on acute day 2 compared to the NW group. The reduction in MVC was, however, not associated with the magnitude of phosphorylation of p70, 4E-BP1 and eEF2. As we report similar training volume and MVC 15 minutes post RE and protein intake, group differences 2.5 hours after exercise could reflect differences in recovery from metabolic fatigue and partial recovery of structural damages (Paulsen et al, 2012). Etheridge et al (2008) showed that immediate post RE protein intake resulted in MVC returning faster to baseline (24h) after an exhausting downhill-run, inducing delayed onset muscle soreness (DOMS), while the placebo-group were significantly reduced compared to baseline (Etheridge et al, 2008). Since NW is more readily absorbed and digested than milk, group differences in recovery 2.5 hours after exercise might be contributing to a more

rapid and enhanced recovery with NW. Furthermore, on both acute days, only the NW group returned to baseline-values 24 h after RE and protein intake, although differences between groups at 24 h failed to reach significance. Since our participants only exercised 3 times per week, >48 h recovery between exercise-bouts might be sufficient for equal recovery between groups. These findings make it interesting to examine whether our studied supplements would reveal different longitudinal outcome measures when given to participants adhering to a more frequent exercise-regime. If resistance exercise was performed on a daily basis, rapid recovery would be of greater importance, not reflected in our participants with >48 h recovery between exercise bouts.

6.6. Linking acute phosphorylation to long-term hypertrophy and strength gains

None of our studied signaling proteins were phosphorylated differently between or within groups, and groups were similar in all long-term outcome measures. We expected intracellular signaling to be enhanced on acute day 2, due to chronic RE and protein supplementation overcoming the assumed anabolic resistance in the untrained state. The only indication of enhanced signaling in the trained state was p70-phosphorylation being significantly increased on acute day 2 in both groups, while only showing a strong tendency of increased phosphorylation in both groups on acute day 1. However, only the NW group showed a tendency of greater p70-phosphorylation on acute day 2, compared to acute day 1.

The difference in leucine content between the NW (2.3 g) and milk supplements (2.0 g) might have been too small to detect differences in the current study design. However it is surprising we did not find supplemental differences as the faster NW absorption-rate has been reported to significantly increase [blood leucine] 0-5 hours post RE and protein intake (Aas, S, 2014). We acknowledge that we do not have blood data to confirm whether NW and milk induced different [blood leucine] in our study. Furthermore, unchanged phosphorylation of 4E-BP1 and eEF2 might have been due to both supplements not being sufficiently above the leucine trigger at >2 g, as several studies with >3.5 g leucine show increased and decreased phosphorylation of 4E-BP1 and eEF2, respectively. Yang et al (2012b) reported a saturated MPS-

response with 20 g of whey at rest in elderly, and an incremental response from 20-40 g of whey after RE. We gave our participants equal doses of native whey/milk both at rest and after RE, thus doubling our given doses after RE might have revealed supplemental differences we could not detect in our current study-design. We saw a tendency of increased 1RM leg-press in the NW group compared to the Milk-group. In light of this, future studies should examine whether increasing our given doses after RE could reveal supplemental differences between native whey and milk.

As individual signaling responses were highly varied between participants, the group mean itself do not identify potential responders/non-responders. Thus, in addition to examining correlations on a group level, this was also done in participants individually and pooled by gender. When examined individually there was no pattern to be seen in terms of a high/low-responder in e.g. p70-phosphorylation simultaneously being a high/low-responder in 4E-BP1- and eEF2-phosphorylation. Furthermore, we wanted to examine whether our participants' physiological outcome-measures were mirrored by phosphorylation-states of key anabolic signaling proteins after an acute exercise bout. We conclude that outcome-measures were not related to the individual phosphorylation-states of p70, 4E-BP1 and eEF2, 2 hours after an initial exposure to an exhausting 10RM-bout combined with protein intake of either native whey or milk. As there were no group differences in phosphorylation and outcome-measures, our reported phosphorylation pattern, with an increase in p70-phosphorylation together with unchanged 4E-BP1- and eEF2-phosphorylation 2 hours after RE and protein intake seem to be permissive for subsequent hypertrophy and strength gains on a group level.

In contrast to our study, Mayhew et al (2009) has shown that acute p70- and 4E-BP1-phosphorylation is related to subsequent gains in LBM after 16 weeks of RE in both elderly and young. Furthermore, the same study showed that although these phosphorylation patterns were related to lean body mass gains, they were not related to the acute measures of MPS (Mayhew et al, 2009). While some find acute phosphorylation patterns to predict subsequent hypertrophy (Baar & Esser, 1999; Terziz et al, 2008; Mayhew et al, 2009), our findings suggest acute phosphorylation patterns of important translation signaling proteins downstream from mTOR is not predictive of reported outcome measures after 11 weeks of resistance training and

protein supplementation. This statement is limited by the fact that we only measured these phosphorylation patterns 2 hour post RE and protein intake.
In agreement with our findings, a large study including both young and old participants, showed that downstream mTOR-effectors such as p70, 4E-BP1 and eEF2 was not directly correlated to resistance exercise-induced changes in LBM (Phillips et al, 2013). Mitchell et al (2014) reported initial acute measures of MPS to be disconnected intracellular signaling and not predictive of hypertrophy after 16 weeks of resistance exercise. Similar to our study, Mitchell et al (2014) found no correlations on an intra-individual level between participants initial response to an exercise bout combined with protein intake (which they were supplemented with throughout the study), and DXA-measures of lean body mass and MRI-measures of muscle volume after 16 weeks of resistance training.

Several studies already described, clearly show a disconnect between phosphorylation-states of p70, 4E-BP1 and eEF2, concomitant MPS and hypertrophy (Atherton et al, 2010; Reitelseder et al, 2011; Kumar et al, 2009; Grenhaff et al, 2008; Mitchell et al, 2015; West et al, 2011; Mitchell et al, 2014; Farnfield et al, 2012). Thus, a model where MPS-increases follow from proportionate alterations in the phosphorylation-pattern of p70, 4E-BP1 and eEF2, leading to predictions of long-term strength-gains and hypertrophy seems to be an oversimplification. This agrees with our findings as phosphorylation-patterns of p70, 4E-BP1 and eEF2 were not able to explain our participants response to chronic resistance training and protein intake. As resistance exercise is shown to elevate MPS for at least 24 hours (Tang et al, 2008) and up to 48 hours (Phillips et al, 1997), pinpointing crucial time-points of measuring acute anabolic signaling responses, which furthermore predicts outcomes of long-term resistance training and protein supplementation seems difficult. Furthermore, one has to accept a clear limitation when analyzing muscle tissue collected from biopsies, that results might not be reflecting the processes on a whole muscle- and whole body-level.

There is a need for more studies implementing a longitudinal aspect in relation to acute measurements to better understand how and if, different points-in-time phosphorylation-patterns downstream of mTOR reflect and predict subsequent hypertrophy and strength gains. Most studies examining intracellular signaling

responses, link phosphorylation patterns to acute measures of MPS and lack the longitudinal outcome measures we present in this study. We acknowledge the limitations of our measured phosphorylation-patterns only reflecting the anabolic state in m. *vastus lateralis* 2 hours post RE and protein intake. Thus it is of interest to further elucidate various points-in-time measurements of the anabolic signaling pathways related to hypertrophy and strength gains. This should be executed in various populations with different training backgrounds and age-groups, and after different training regimes.

6.7. Conclusion

We conclude that supplementation with equal amounts of protein from native whey and milk, in combination with strength training resulted in similar signaling for muscle hypertrophy and similar gains in *m. vastus lateralis* thickness, muscle mass and muscle strength in elderly. We expected post-intervention signaling-responses to be of greater magnitude, but there was only a tendency of greater p70-phosphorylation on acute day 2 compared to day 1. Furthermore we show that strength training combined with protein supplementation significantly increases 1RM strength and hypertrophy in an elderly population of both genders. There were no group differences, which could be related to a large daily protein intake (>1.2 g per kg/body mass) in all participants, possibly confounding the hypothesized effect of a higher leucine content and faster absorption-rate in native whey. Moreover, the difference in leucine-content between NW and milk might have been too small to induce different responses between supplements in our study-design.

Several studies have been using p70-, 4E-BP1- and eEF2-phosphorylation as indexes of anabolic response to strength training and protein intake. Our long-term outcome-measures were not mirrored by individual signaling responses, emphasizing that acute measures of intracellular signaling should be interpreted with caution. To be considered reliable indexes of response to repeated exposures of strength training and protein intake, acute measures should be viewed in context with longitudinal outcome measures.

7. References

Aas, S. (2014). *Effekten av ulike melkeproteinprodukter på hypertrofisignalering etter en styrketreningsøkt.*(MSc). Norwegian school of sport sciences. http://hdl.handle.net/11250/219169

Adams, G.R, Bamman, M.M. (2012). *Characterization and regulation of mechanical loading-induced compensatory muscle hypertrophy*. Compr Physiol. Oct;2(4):2829-70. doi: 10.1002/cphy.c110066

Atherton, P.J., Etheridge, T., Watt, P.W., Wilkinson, D., Selby, A., Rankin, D., Smith, K., Rennie, M.J. (2010). *Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling*. Am J Clin Nutr. Nov;92(5):1080-8. doi: 10.3945/ajcn.2010.29819. Epub 2010 Sep 15.

Baar, K., Esser, K. (1999). *Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise*. Am J Physiol 276:C120–C127

Biolo, G., Tipton, K.D., Klein, S., Wolfe, R.R. (1997). *An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein*. Am J Physiol. Jul;273(1 Pt 1):E122-9.

Biolo, G., Maggi, S.P., Williams, B.D., Tipton, K.D., Wolfe, R.R. (1995*). Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans*. Am J Physiol;268:E514- 20.

Blomstrand, E., Eliasson, J., Karlsson, H.K., Kohnke, R. (2006). *Branched-chain amino acids activate key enzymes in protein synthesis after physical exercise*. J Nutr 136, 269S–273S.

Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J., Yancopoulos, G.D. (2001). *Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo*. Nat Cell Biol. Nov;3(11):1014-9.

Bohé, J., Low, J.F., Wolfe, R.R., Rennie, M.J. (2001). Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. J Physiol. Apr 15;532(Pt 2):575-9.

Boirie, Y., Gachon, P., Beaufrère, B. (1997). *Splanchnic and whole-body leucine kinetics in young and elderly men*. Am J Clin Nutr. Feb;65(2):489-95.
Bolster, D.R., Kubica, N., Crozier, S.J., Williamson, D.L., Farrell, P.A., Kimball, S.R., Jefferson, L.S. (2003). *Immediate response of mammalian target of rapamycin (mTOR)-mediated signalling following acute resistance exercise in rat skeletal muscle*.J Physiol. Nov 15;553(Pt 1):213-20. Epub 2003 Aug 22.

Borsheim, E., Cree, M.G., Tipton, K.D., Elliott, T.A., Aarsland, A., Wolfe, R.R. (2004). *Effect of carbohydrate intake on net muscle protein synthesis during recovery from resistance exercise*. J Appl Physiol 96: 674–678.

Burd, N. A., Yang, Y., Moore, D. R., Tang, J. E., Tarnopolsky, M. A., & Phillips, S. M. (2012). *Greater stimulation of myofibrillar protein synthesis with ingestion of whey protein isolate v. micellar casein at rest and after resistance exercise in elderly men*. Br J Nutr, 108(6), 958-962.

Burd, N. A., Holwerda, A. M., Selby, K. C., West, D. W., Staples, A. W., Cain, N. E., Cashaback, J.G.A., Potvin, J.R., Baker, S.K., Phillips, S.M. (2010). *Resistance exercise volume affects myofibrillar protein synthesis and anabolic signalling molecule phosphorylation in young men*. J Physiol, 588(Pt 16), 3119-3130.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., Sabatini, D.M. (1998). *RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1*. Proc Natl Acad Sci U S A. Feb 17;95(4):1432-7.

Casperson, S.L., Sheffield-Moore, M., Hewlings, S.J., Paddon-Jones, D. (2012). *Leucine supplementation chronically improves muscle protein synthesis in older adults consuming the RDA for protein*. Clin Nutr. Aug;31(4):512-9. doi: 10.1016/j.clnu.2012.01.005. Epub 2012 Feb 20.

Cermak, N.M., Res, P.T., de Groot, L.C., Saris, W.H., van Loon, L.J. (2012*). Protein supplementation augments the adaptive response of skeletal muscle to resistance-type exercise training: a meta-analysis.* Am J Clin Nutr. Dec;96(6):1454-64. doi: 10.3945/ajcn.112.037556. Epub 2012 Nov 7.

Cuthbertson, D., Smith, K., Babraj, J., Leese, G., Waddell, T., Atherton, P., Wackerhage, H., Taylor, P.M., Rennie, M.J. (2005). *Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle*. FASEB J. Mar;19(3):422-4. Epub 2004 Dec 13.

Chevalier, S., Goulet, E.D., Burgos, S.A., Wykes, L.J., Morais, J.A. (2011). *Protein anabolic responses to a fed steady state in healthy aging*. J Gerontol A Biol Sci Med Sci.66:681–688.

Churchward-Venne, T.A., Burd, N.A., Mitchel,l C.J., West, D.W., Philp, A., Marcotte, G.R., Baker, S.K., Baar, K., Phillips, S.M. (2012). *Supplementation of a suboptimal protein dose with leucine or essential amino acids: effects on myofibrillar protein synthesis at rest and following resistance exercise in men*. J Physiol, 590(Pt 11), 2751-2765.

Coffey, V.G., Zhong, Z., Shield, A., Canny, B.J., Chibalin, A.V., Zierath, J.R., Hawley, J.A. (2005). *Early signaling responses to divergent exercise stimuli in skeletal muscle from well-trained humans*. FASEB J.20:190–192

Deer, R.R., Volpi, E. (2015). *Protein intake and muscle function in older adults*. Curr Opin Clin Nutr Metab Care. May;18(3):248-53. doi: 10. 1097/MCO. 0000000000000162.

Deldicque, L., De Bock, K., Maris, M., Ramaekers, M., Nielens, H., Francaux, M., Hespel, P. (2010). *Increased p70s6k phosphorylation during intake of a protein-carbohydrate drink following resistance exercise in the fasted state*. Eur J Appl Physiol. Mar;108(4):791-800.

Deldicque, L., Atherton, P., Patel, R., Theisen, D., Nielens, H., Rennie, M.J., Francaux, M. (2008). *Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise*. Eur J Appl.Physiol 104:57–65

Deutz, N.E., Bauer, J.M., Barazzoni, R., Biolo, G., Boirie, Y., Bosy-Westphal, A., Cederholm, T., Cruz-Jentoft, A., Krznariç, Z., Nair, K.S., Singer, P., Teta, D., Tipton, K., Calder, P.C. (2014). *Protein intake and exercise for optimal muscle function with aging: recommendations from the ESPEN Expert Group*. Clin Nutr. Dec;33(6):929-36. doi: 10.1016/j.clnu.2014.04.007. Epub 2014 Apr 24.

Dickinson, J.M., Gundermann, D.M., Walker, D.K., Reidy, P.T., Borack, M.S., Drummond, M.J., Arora, M., Volp,i E., Rasmussen, B.B. (2014). *Leucine-enriched amino acid ingestion after resistance exercise prolongs myofibrillar protein synthesis and amino acid transporter expression in older men*. J Nutr. Nov;144(11):1694-702. doi: 10.3945/jn.114.198671

Dickinson, J.M., Drummond, M.J., Fry, C.S., Gundermann, D.M., Walker, D.K., Timmerman, K.L., Volpi, E., Rasmussen, B.B. (2013). *Rapamycin does not affect post-absorptive protein metabolism in human skeletal muscle*. Metabolism. Jan;62(1):144-51. doi: 10.1016/j.metabol.2012.07.003. Epub 2012 Sep 6.

Dickinson, J.M., Fry, C.S., Drummond, M.J., Gundermann, D.M., Walker, D.K., Glynn, E.L., Timmerman, K.L., Dhanani, S., Volpi, E., Rasmussen, B.B. (2011). *Mammalian target of rapamycin complex 1 activation is required for the stimulation of human skeletal muscle protein synthesis by essential amino acids*. J Nutr. May;141(5):856-62. doi: 10.3945/jn.111.139485. Epub 2011 Mar 23.

Dreyer, H.C., Fujita, S., Glynn, E.L., Drummond, M.J., Volpi, E., Rasmussen, B.B. (2010). *Resistance exercise increases leg muscle protein synthesis and mTOR signalling independent of sex*. Acta Physiol.199:71–81. doi: 10.1111/j.1748-1716.2010.02074.x.

Dreyer, H.C., Drummond, M.J., Pennings, B., Fujita, S., Glynn, E.L., Chinkes, D.L, Dhanani, S., Volpi, E., Rasmussen BB. (2008). *Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle*. Am J Physiol Endocrinol Metab. Feb; 294(2): E392–E400.

Dreyer, H.C., Fujita, S., Cadenas, J.G., Chinkes, D.L, Volpi, E., Rasmussen, B.B. (2006). *Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle*. J Physiol. Oct 15;576(Pt 2):613-24. Epub 2006 Jul 27.

Drummond, M.J., Fry, C.S., Glynn, E.L., Timmerman, K.L., Dickinson, J.M., Walker, D.K., Gundermann, D.M., Volpi, E., Rasmussen, B.B. (2011). *Skeletal muscle amino acid transporter expression is increased in young and older adults following resistance exercise*. J Appl Physiol . Jul;111(1):135-42. doi: 10.1152/japplphysiol.01408.2010. Epub 2011 Apr 28.

Drummond, M.J., Glynn, E.L., Fry, C.S., Timmerman, K.L., Volpi, E., Rasmussen, B.B. (2010). *An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle*. Am J Physiol Endocrinol Metab. 2010 May;298(5):E1011-8. doi: 10.1152/ajpendo.00690.2009. Epub 2010 Feb 9.

Drummond, M.J., Fry, C.S., Glynn, E.L., Dreyer, H.C., Dhanani, S., Timmerman, K.L., Volpi, E., Rasmussen, B.B. (2009). *Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis*. J. Physiol. 587:1535–1546.

Drummond, M.J., Dreyer, H.C., Pennings, B., Fry, C.S., Dhanani, S., Dillon, E.L., Sheffield-Moore, M., Volpi, E., Rasmussen, B.B. (2008). *Skeletal muscle protein anabolic response to resistance exercise and essential amino acids is delayed with aging*. J Appl Physiol. May;104(5):1452-61. doi: 10.1152/japplphysiol.00021.2008. Epub 2008 Mar 6.

Etheridge, T., Philp, A., Watt, P.W. (2008). *A single protein meal increases recovery of muscle function following an acute eccentric exercise bout*. Appl Physiol Nutr Metab. Jun;33(3):483-8. doi: 10.1139/H08-028.

Evans, W.J., Phinney, S.D., Young, V.R. (1982). *Suction applied to a muscle biopsy maximizes sample size*. Med Sci Sports Exerc.;14(1):101-2.

Fry, C.S., Drummond, M.J., Glynn, E.L., Dickinson, J.M., Gundermann, D.M., Timmerman, K.L., Walker, D.K., Volpi, E., Rasmussen, B.B. (2013). *Skeletal muscle autophagy and protein breakdown following resistance exercise are similar in younger and older adults*. J Gerontol A Biol Sci Med Sci. May;68(5):599-607. doi: 10.1093/gerona/gls209. Epub 2012 Oct 22.

Fry, C.S., Drummond, M.J., Glynn, E.L., Dickinson, J.M., Gundermann, D.M., Timmerman, K.L., Walker, D.K., Dhanani, S., Volpi, E., Rasmussen, B.B. (2011). *Aging impairs contraction-induced human skeletal muscle mTORC1 signaling and protein synthesis*. Skelet Muscle. Mar 2;1(1):11. doi: 10.1186/2044-5040-1-11.

Fujita, S., Rasmussen, B.B., Bel,l J.A., Cadenas, J.G., Volp,i E. (2007a). *Basal muscle intracellular amino acid kinetics in women and men.* Am J Physiol Endocrinol Metab.Jan;292(1):E77-83. Epub 2006 Aug 8.

Fujita, S., Dreyer, H.C., Drummond, M. J., Glynn, E. L., Cadena, J. G., Yoshizawa, F., Volpi E., Rasmussen, B. B. (2007b). *Nutrient signalling in the regulation of human muscle protein synthesis*. J Physiol, 582(Pt 2), 813-823.

Gingras, A.C., Gygi, S.P, Raught, B., Polakiewicz, R.D., Abraham, R.T., Hoekstra, M.F., Aebersold, R., Sonenberg, N. (1999). *Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism*. Genes Dev. Jun 1;13(11):1422-37.

Glynn, E.L., Fry, C.S., Drummond, M.J., Dreyer, H.C., Dhanani, S., Volpi, E., Rasmussen, B.B. (2010). *Muscle protein breakdown has a minor role in the protein anabolic response to essential amino acid and carbohydrate intake following resistance exercise*. Am J Physiol Regul Integr Comp Physiol. Aug;299(2):R533-40. doi: 10.1152/ajpregu.00077.2010. Epub 2010 Jun 2.

Goodman, C.A., Miu, M.H., Frey, J.W., Mabrey, D.M., Lincoln, H.C., Ge, Y., Chen, J., Hornberger, T.A. (2010). *A phosphatidylinositol 3-kinase/protein kinase B-independent activation of mammalian target of rapamycin signaling is sufficient to induce skeletal muscle hypertrophy*. Mol Biol Cell. Sep 15;21(18):3258-68. doi: 10.1091/mbc.E10-05-0454. Epub 2010 Jul 28.

Greenhaff, P.L., Karagounis, L.G., Peirce, N., Simpson, E.J., Hazell, M., Layfield, R., Wackerhage, H., Smith, K., Atherton, P., Selby, A., Rennie, M.J. (2008). *Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle*. Am J Physiol Endocrinol Metab ;295:E595–604.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., Yonezawa, K. (2002). *Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action*. Cell 110:177–189

Hornberger, T.A., Sukhija, K.B., Wang, X.R., Chien, S. (2007). *mTOR is the rapamycin-sensitive kinase that confers mechanically-induced phosphorylation of the hydrophobic motif site Thr(389) in p70(S6k).* FEBS Lett. Oct 2;581(24):4562-6. Epub 2007 Aug 31.

Hulmi, J.J., Tannerstedt, J., Selänne, H., Kainulainen, H., Kovanen, V., Mero, A.A. (2009). *Resistance exercise with whey protein ingestion affects mTOR signaling pathway and myostatin in men*. J Appl Physiol. May;106(5):1720-9. doi: 10.1152/japplphysiol.00087.2009.

Jacobs, B.L., You, J.S., Frey, J.W., Goodman, C.A., Gundermann, D.M., Hornberger, T.A. (2013). *Eccentric contractions increase the phosphorylation of tuberous sclerosis complex-2 (TSC2) and alter the targeting of TSC2 and the mechanistic target of rapamycin to the lysosome*. J Physiol 591:4611–4620

Janssen, I., Ross, R. (2005). *Linking age-related changes in skeletal muscle mass and composition with metabolism and disease*. J.Nutr. Health Aging, 9(6): 408–419. PMID:16395513.

Karlsson, H.K., Nilsson, P.A., Nilsson, J., Chibalin, A.V., Zierath, J.R., Blomstrand, E. (2004). *Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise*. Am J Physiol Endocrinol Metab 287:E1–E7

Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., and Wolfe, R. R. (2006). *A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly*. Am. J. Physiol. Endocrinol. Metab. 291, E381–E387.

Katsanos, C. S., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., & Wolfe, R. R. (2005). *Aging is associated with diminished accretion of muscle proteins after the ingestion of a small bolus of essential amino acids*. Am J Clin Nutr, 82(5), 1065-1073.

Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., Guan, K.L. (2008). *Regulation of TORC1 by Rag GTPases in nutrient response*. Nat Cell Biol;10:395–345. [PubMed: 18344988]

Kim, I.Y., Schutzler, S., Schrader, A., Spencer, H., Kortebein, P., Deutz, N.E., Wolfe, R.R., Ferrando, A.A. (2015). *Quantity of dietary protein intake, but not pattern of intake, affects net protein balance primarily through differences in protein synthesis in older adults*. Am J Physiol Endocrinol Metab. Jan 1;308(1):E21-8. doi: 10.1152/ajpendo.00382.2014. Epub 2014 Oct 28.

Koopman, R., Walrand, S., Beelen, M., Gijsen, A.P., Kies, A.K., Boirie, Y., Saris, W.H., van Loon, L.J.C. J Nutr. (2009). *Dietary protein digestion and absorption rates and the subsequent postprandial muscle protein synthetic response do not differ between young and elderly men*. Sep;139(9):1707-13. doi: 10.3945/jn.109.109173. Epub 2009 Jul 22.

Koopman, R., Beelen, M., Stellingwerff, T., Pennings, B., Saris, W.H.M., Kies, A.K., Kuipers, H., van Loon, L.J.C. (2007). *Coingestion of carbohydrate with protein does not further augment postexercise muscle protein synthesis*. Am J Physiol Endocrinol Metab 293: E833–E842, 2007

Kumar, V., Selby, A., Rankin, D., Patel, R., Atherton, P., Hildebrandt, W., Williams, J., Smith, K., Seynnes, O., Hiscock, N., Rennie, M.J. (2009). *Age-related differences in the dose-response relationship of muscle protein synthesis to resistance exercise in young and old men*. J Physiol, 587(Pt 1), 211-217.

Laahne, J. A. (2013). *Nativt myseprotein gir større og raskere økning av aminosyrer i blod enn behandlede mysefraksjoner og lettmelk, men ikke raskere restitusjon av muskelfunksjon.* (MSc.), Norwegian School of Sport Sciences, http://brage.bibsys.no/xmlui/handle/11250/171884.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., Hall, M.N. (2002). *Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control*. Mol Cell. Sep;10(3):457-68.

Mayhew, D.L., Kim, J.S., Cross, J.M., Ferrando, A.A., Bamman, M.M. (2009). *Translational signaling responses preceding resistance training-mediated myofiber hypertrophy in young and old humans*. J Appl Physiol. Nov;107(5):1655-62. doi: 10.1152/japplphysiol.91234.2008. Epub 2009 Jul 9.

Mitchell, W.K., Phillips, B.E., Williams, J.P., Rankin, D., Lund, J.N., Smith, K., Atherton, P.J. (2015). *A dose- rather than delivery profile-dependent mechanism regulates the "muscle-full" effect in response to oral essential amino acid intake in young men*. J Nutr. Feb;145(2):207-14. doi: 10.3945/ jn.114.199604. Epub 2014 Dec 10.

Mitchell, C.J., Churchward-Venne, T.A., Parise, G., Bellamy, L., Baker, S.K., Smith, K., Atherton, P.J., Phillips, S.M. (2014). PLoS One. *Acute post-exercise myofibrillar protein synthesis is not correlated with resistance training-induced muscle hypertrophy in young men*. Feb 24;9(2):e89431. doi: 10.1371/journal.pone.0089431. eCollection 2014. Erratum in: PLoS One. 210;9(5):e98731.

Moore, D.R., Churchward-Venne, T.A., Witard, O., Breen, L., Burd, N.A., Tipton, K.D., Phillips S.M. (2015). *Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men*. J Gerontol A Biol Sci Med Sci. Jan;70(1):57-62. doi: 10.1093/gerona/glu103. Epub 2014 Jul 23.

Moore, D.R., Atherton, P.J., Rennie, M.J., Tarnopolsky M.A., Phillips, S.M. (2011). *Resistance exercise enhances mTOR and MAPK signalling in human muscle over that seen at*

rest after bolus protein ingestion. Acta Physiol (Oxf). Mar;201(3):365-72. doi: 10.1111/j.1748-1716.2010.02187.x

Moore, D.R., J. E. Tang, N. A. Burd, T., Rerecich, M. A., Tarnopolsky M.A.,. Phillips S.M. (2009). *Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise*. J. Physiol. 597: 897-904.

Paddon-Jones, D., Sheffield-Moore, M., Zhang, X.J., Volp,i E., Wolf, S.E, Aarsland, A., Ferrando, A.A., Wolfe, R.R. (2004). *Amino acid ingestion improves muscle protein synthesis in the young and elderly*. Am J Physiol Endocrinol Metab. Mar;286(3):E321-8. Epub 2003 Oct 28.

Paulsen, G., Mikkelsen, U.R., Raastad, T., Peake, J.M. (2012). *Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise?* Exerc Immunol Rev;18:42-97. Review

Peterson, M.D., Sen, A., Gordon, P.M. (2011). *Influence of resistance exercise on lean body mass in aging adults: a meta-analysis*. Med Sci Sports Exerc. 43(2):249–58. [PubMed: 20543750]

Peterson, M.D., Rhea, M.R., Sen, A., Gordon, P.M. (2010). *Resistance Exercise for Muscular Strength in Older Adults: A Meta-Analysis*. Ageing Res Rev. July; 9(3): 226–237. Published online 2010 April 10. doi: 10.1016/j.arr.2010.03.004

Pennings, B., Koopman, R., Beelen, M., Senden, J.M., Saris, W.H, van Loon L.J. (2011). *Exercising before protein intake allows for greater use of dietary protein-derived amino acids for de novo muscle protein synthesis in both young and elderly men*. Am J Clin Nutr. Feb;93(2):322-31. doi: 10.3945/ajcn.2010.29649. Epub 2010 Nov 17.

Phillips, B.E., Atherton, P.J., Varadhan, K., Limb, M.C., Wilkinson, D.J, Sjøberg, K.A., Smith, K., Williams, J.P. (2015). *The effects of resistance exercise training upon macro and micro-circulatory responses to feeding and skeletal muscle protein anabolism, in older men*. J Physiol. Apr 13. doi: 10.1113/JP270343. [Epub ahead of print]

Phillips, B.E., Williams, J.P., Gustafsson, T., Bouchard, C., Rankinen, T., Knudsen, S., Smith, K., Timmons, J.A.,. Atherton PJ. (2013). *Molecular Networks of Human Muscle*

Adaptation to Exercise and Age. PLoS Genet. March; 9(3): e1003389. Published online 2013 March 21. doi: 10.1371/journal.pgen.1003389

Phillips, S. M., Tipton, K. D., Ferrando, A. A., & Wolfe, R. R. (1999). *Resistance training reduces the acute exercise-induced increase in muscle protein turnover*. Am J Physiol, 276(1 Pt 1), E118-124.

Phillips, S. M., Tipton, K. D., Aarsland, A., Wolf, S. E., & Wolfe, R. R. (1997). *Mixed muscle protein synthesis and breakdown after resistance exercise in humans*. Am J Physiol, 273(1 Pt 1), E99-107.

Reidy, P.T., Walker, D.K., Dickinson, J.M., Gundermann, D.M., Drummond, M.J., Timmerman, K.L., Cope, M.B., Mukherjea, R., Jennings, K., Volpi, E., Rasmussen, B.B. (2014). *Soy-dairy protein blend and whey protein ingestion after resistance exercise increases amino acid transport and transporter expression in human skeletal muscle*. J Appl Physiol (1985). Jun 1;116(11):1353-64. doi: 10.1152/japplphysiol.01093.2013. Epub 2014 Apr 3.

Reitelseder, S., Agergaard, J., Doessing, S., Helmark, I.C., Lund, P., Kristensen, N.B., Frystyk, J., Flyvbjerg, A., Schjerling, P., van Hall, G., Kjaer, M., Holm, L. (2011). *Whey and casein labeled with L-[1-13C]leucine and muscle protein synthesis: effect of resistance exercise and protein ingestion*. Am J Physiol Endocrinol Metab. Jan;300(1):E231-42. doi: 10.1152/ajpendo.00513.

Rieu, I., Balage, M., Sornet, C., Giraudet, C., Pujos, E., Grizard, J., Mosoni, L., Dardevet, D. (2006). *Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia*. J Physiol. Aug 15;575(Pt 1):305-15. Epub 2006 Jun 15.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard , A.L., Nada, S., Sabatini, D.M. (2010). *Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids*. Cell. ;141:290–303. [PubMed: 20381137]

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., Sabatini, D.M. (2008). *The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1*. Science. ; 320:1496–1501. [PubMed: 18497260]

Smith, G.I., Reeds, D.N., Hall, A.M., Chambers, K.T., Finck, B.N., Mittendorfer, B. (2012). *Sexually dimorphic effect of aging on skeletal muscle protein synthesis*. Biol Sex Differ. May 23;3(1):11. doi: 10.1186/2042-6410-3-11.

Smith, G.I., Atherton, P., Reeds, D.N., Mohammed, B.S., Jaffery, H., Rankin, D., Rennie, M.J., Mittendorfer, B. (2009). *No major sex differences in muscle protein synthesis rates in the post absorptive state and during hyperinsulinemia-hyperaminoacidemia in middle-aged adults.* J Appl Physiol.107:1308–1315. doi: 10.1152/japplphysiol.00348.

Smith, G.I., Atherton, P., Villareal, D.T., Frimel, T.N., Rankin, D., Rennie, M.J, Mittendorfer, B. (2008). *Differences in Muscle Protein Synthesis and Anabolic Signaling in the Post absorptive State and in Response to Food in 65–80 Year Old Men and Women*. PLoS One. Mar 26;3(3):e1875. doi: 10.1371/ journal.pone.0001875.

Smith, K., Reynolds, N., Downie, S., Patel, A., Rennie, M.J. (1998). *Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein*. Am J Physiol. Jul;275(1 Pt 1):E73-8.

Symons, T. B., Sheffield-Moore, M., Wolfe, R. R., & Paddon-Jones, D. (2009). *A moderate serving of high-quality protein maximally stimulates skeletal muscle protein synthesis in young and elderly subjects.* J Am Diet Assoc, 109(9), 1582-1586.

Tang, J.E., Moore, D.R., Kujbida, G.W., Tarnopolsky, M.A., Phillips, S.M. (2009). *Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men*. J Appl Physiol 107: 987–992. First published July 9, 2009; doi:10.1152/japplphysiol.00076.2009.

Tang, J. E., Perco, J.G., Moore, D.R., Wilkinson, S.B., Phillips, S.M. (2008). *Resistance training alters the response of fed state mixed muscle protein synthesis in young men*. Am J Physiol Regul Integr Comp Physiol, 294(1), R172-178.

Terzis, G., Spengos, K., Mascher, H., Georgiadis, G., Manta, P., Blomstrand, E. (2010). *The degree of p70 S6k and S6 phosphorylation in human skeletal muscle in response to resistance exercise depends on the training volume*. Eur J Appl Physiol, 110(4), 835-843.

Terzis, G., Georgiadis, G., Stratakos, G., Vogiatzis, I., Kavouras, S., Manta, P., Mascher, H., Blomstrand, E. (2008). *Resistance exercise-induced increase in muscle mass correlates with*

p70S6 kinase phosphorylation in human subjects. Eur J Appl Physiol. Jan;102(2):145-52. Epub 2007 Sep 14.

Tipton, K.D., Ferrando, A.A., Phillips, S.M., Doyle, D., Wolfe, R.R. (1999). *Postexercise net protein synthesis in human muscle from orally administered amino acids*. American Journal of Physiology - Endocrinology and Metabolism Published 1 April Vol. 276 no. 4, E628-E634 DOI:

Verhoeven, S., Vanschoonbeek, K., Verdijk, L.B., Koopman, R., Wodzig, W.K., Dendale, P., van Loon, L.J. (2009). *Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men*. Am J Clin Nutr. May;89(5):1468-75. doi: 10.3945/ajcn.2008.26668. Epub 2009 Mar 25

Vigelsø, A., Dybboe, R., Hansen, C.N., Dela, F., Helge, J.W., Grau, AG. (2015). *GAPDH and ß-actin protein decreases with aging, making Stain-Free technology a superior loading control in Western blotting of human skeletal muscle.* J Appl Physiol 118: 386–394.

Volpi, E., Kobayashi, H., Sheffield-Moore, M., Mittendorfer, B., Wolfe, R.R. (2003). *Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults*. Am. J. Clin. Nutr., 78, 250–258.

Volpi, E., Mittendorfer, B., Wolf, S. E., and Wolfe, R. R. (1999). *Oral amino acids stimulate muscle protein anabolism in the elderly despite higher firstpass splanchnic extraction.* Am. J. Physiol. 277, E513–E520.

Wall, B.T., Cermak, N.M., van Loon, L.J. (2014). *Dietary protein considerations to support active aging*. Sports Med.Nov;44 Suppl 2:S185-94. doi: 10.1007/s40279-014-0258-7.

Wall, B.T., Hamer, H.M., de Lange, A., Kiskini, A., Groen, B.B., Senden, J.M., Gijsen, A.P., Verdijk, L.B., van Loon, L.J. (2013). *Leucine co-ingestion improves post-prandial muscle protein accretion in elderly men*. Clin Nutr. Jun;32(3):412-9. doi: 10.1016/j.clnu.2012.09.002. Epub 2012 Sep 20.

Wang, X., Proud, C.G. (2008). *A novel mechanism for the control of translation initiation by amino acids, mediated by phosphorylation of eukaryotic initiation factor 2B.* Mol Cell Biol. 2008 Mar;28(5):1429-42. Epub 2007 Dec 26.

Wang, X., Beugnet, A., Murakami, M., Yamanaka, S., Proud, C.G. (2005). *Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins.* Mol Cell Biol. Apr;25(7):2558-72

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., Proud, C.G. (2001). *Regulation of elongation factor 2 kinase by p90 (RSK1) and p70 S6 kinase*. EMBO J. Aug 15;20(16):4370.

West, D.W., Burd, N.A., Churchward-Venne, T.A., Camera, D.M., Mitchel,l C.J., Baker, S.K., Hawley, J.A., Coffey, V.G., Phillips, S.M. (2012). *Sex-based comparisons of myofibrillar protein synthesis after resistance exercise in the fed state*. J Appl Physiol. Jun;112(11):1805-13. doi: 10.1152/ japplphysiol. 00170.

West, D.W., Burd, N.A., Coffey, V.G., Baker, S.K., Bruke, L.M., Hawley, J.A., Moore, D.R., Stellingwerff, T., Phillips, S.M. (2011). *Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise*. Am J Clin Nutr. Sep;94(3):795-803. doi: 10.3945/ajcn.111.013722. Epub 2011 Jul 27

West, D.W., Kujbida, G.W., Moore, D.R., Atherton, P., Burd, N.A., Padzik, J.P., De Lisio, M., Tang, J.E., Parise, G., Rennie, M.J., Baker, S.K., Phillips, S.M. (2009). *Resistance exercise-induced increases in putative anabolic hormones do not enhance muscle protein synthesis or intracellular signalling in young men*.J Physiol. 2009 Nov 1;587(Pt 21):5239-47. doi: 10.1113/jphysiol.2009.177220. Epub 2009 Sep 7.

Yang, Y., Churchward-Venne, T.A., Burd, N.A., Breen, L., Tarnopolsky, M.A., Phillips, S.M. (2012a). *Myofibrillar protein synthesis following ingestion of soy protein isolate at rest and after resistance exercise in elderly men*. Nutr Metab (Lond). Jun 14;9(1):57. doi: 10.1186/1743-7075-9-57.

Yang, Y., Breen, L., Burd, N.A., Hector, A.J., Churchward-Venne, T.A., Josse, A.R., Tarnopolsky, M.A., Phillips, S.M. (2012b). *Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men*. Br. J. Nutr. 2012, 108, 1–9.

You, J.S., Lincoln, H.C., Kim, C.R., Frey, J.W., Goodman, C.A., Zhong, X.P., Hornberger, T.A. (2014). *The role of diacylglycerol kinase zeta and phosphatidic acid in the mechanical activation of mammalian target of rapamycin (mTOR) signaling and skeletal muscle hypertrophy*. J Biol Chem 289:1551–1563

8. Tables

9. Figures

10. Abbreviations

Akt/PKB	protein kinase B
1RM	one repetition maximum
4E-BP1	eukaryotic translation initiation factor 4E binding protein 1
BCAA	branched chain amino acid
BMD	bone mineral density
BMI	body mass index
CSA	cross sectional area
DGK	diacylglyserol kinase
DXA	dual X-ray absorptiometry
EEA	essential amino acid
eEF2	eukaryot elongation factor 2
eIF4E	eukaryotic initiation factor of translation-4E
GTP	guanosine triphosphate
FFM	fat free mass
LAT1	large neutral amino acid transporter 1
LBM	lean body mass
MPS	muscle protein synthesis
MPB	muscle protein breakdown
mTORC1/2	mammalian target of rapamycin complex 1/2
MVC	maximal voluntary contraction
p70S6K	70-kD S6 protein kinase
PA	phosphatic acid
PDK-1	3-phosphoinositide-dependant protein kinase 1
PI3-K	phosphatidylinositol-3-kinase
Rag GTPase	ras-related guanosin-triphosphate
RE	resistance exercise
RFD	rate of force development
RCT	randomized controlled trial
Rheb	ras homolog enriched in brain
RM	repetition maximum
SNAT2	sodium coupled neutral amino acid transporter 2
Thr	threonine

tRNA	transfer ribo nucleic acid
TSC 1/2	tuberous sclerosis 1/2
UPS	ubiquitin-proteasome system
MuRF1	muscle specific RING Finger 1
MAFbx	muscle atrophy F-box
FOXO	Forkhead box (transcription factors FOXO1, FOXO3a, FOXO4)

11. Appendix 1

Forsøkspersoner søkes!
«Styrketrening og melkeprotein»

Prosjektet "**Hvordan påvirker forskjellige melkeproteiner muskelproteinbalanse hos eldre?**" har til hensikt å undersøke effekten av forskjellige melkeproteiner på muskelvekst gjennom en 12 ukers treningsperiode hos yngre og eldre.

Vi søker etter kvinner og menn over 70 år som ikke trener regelmessig, men ønsker å komme i bedre fysisk form

Studien vil foregå i perioden september-desember 2014 og innebærer en rekke tester før og etter treningsperioden. Det vil bli ca. 4 oppmøter på 2-3 timer i forkant og etterkant av treningsperioden for testing.

Treningen vil foregå 3 ganger per uke i grupper på 3 med personlig trener på alle økter ved Norges idrettshøgskole.

Før og etter treningsperioden vil det bli tatt blodprøver og muskelbiopsier

Er du interessert, ta kontakt med :
Håvard Hamarsland: 93445916; haavardh@nih.no

12. Appendix 2

Forespørsel om deltakelse som forsøksperson

Hvordan påvirker inntak av forskjellige melkeproteiner muskelproteinbalanse hos eldre?

Dette skrivet er til alle potensielle forsøkspersoner. Vi ber om din deltakelse i prosjektet, så fremt du oppfyller kriteriene: Du må være 70 år eller eldre, være normalt aktiv, og ellers kunne gjennomføre styrketrening. Du kan ikke bruke spesifiserte medikamenter eller kosttilskudd (proteinpulver, kreatin eller lignende). Hvis du bruker slike kosttilskudd kan du likevel delta som forsøksperson ved at du slutter med tilskuddet senest én uke før prosjektstart. Du kan ikke delta om du er laktoseintolerant, har melkeallergi eller er allergisk mot lokalbedøvelse (tilsvarende det man får hos tannlegen).

Bakgrunn og hensikt med forsøket

Sarkopeni (aldersrelatert muskelsvinn) har de siste årene fått mye oppmerksomhet da det i tillegg til å redusere funksjon og livskvalitet i hverdagen også disponerer for flere livsstilssykdommer (bla. type II diabetes og osteoporose). Styrketrening og et økt inntak av proteiner har vist seg å kunne motvirke muskelsvinnet. Inntak av proteiner har i seg selv en umiddelbar muskeloppbyggende effekt ved at proteinsyntesen øker; og kombinerer vi proteininntak med styrketrening får vi en vesentlig kraftigere effekt. Økningen i proteinsyntesen bestemmes i stor grad av mengden og kvaliteten på proteinet, samt hvor raskt proteinet tas opp i blodet. I tillegg til proteinsyntesen vil også proteinnedbrytningen til enhver tid spille inn på proteinomsetningen i muskulaturen. Sammenliknet med proteinsyntesen vet vi lite om hvordan proteinnedbrytningen påvirkes av proteininntak etter styrketrening. Ny kunnskap om dette kan gi oss bedre forutsetninger for å maksimere utbyttet av

styrketrening, som vil være av stor interesse for eldre med tanke på livskvalitet og funksjon i hverdagen.

I denne studien ønsker vi å undersøke om et nyutviklet myseprotein produsert av Tine® kan bedre effekten av styrketrening (føre til større økning i muskelmasse og styrke). Dette nye myseproteinet vil sammenliknes med effekten av vanlig lettmelk.

Dette er et dobbelt blindet, randomisert, kontrollert studie, som betyr at verken du eller forskerne du kommer i kontakt med vet hvilken drikk du inntar.

Gjennomføringen av forsøket

Forsøket går kort fortalt ut på å gjennomføre en treningsperiode på 12 uker med styrketrening tre ganger i uken. Gjennom denne perioden inntas det to enheter på 0,6 l daglig med enten melk eller nativ myse. Du vil bli tilfeldig trukket (randomiseres) til én av gruppene. Før og etter treningsperioden vil det gjennomføres en rekke tester (se under) for å se på effekten av de forskjellige drikkene.

Før treningsperioden

Du skal møte på Norges idrettshøgskole 4 ganger for tilvenning til tester og treningsøvelser, styrketester, måling av kroppssammensetning (DXA), en legesjekk og muskelbiopsier i ukene før forsøket. I tillegg må du møte for en MR-analyse hos Curato røntgen. Tidspunkter for de ulike oppmøtene avtales individuelt. Under følger et eksempel på tidsplan for tester:

Dag 1: Underskrevet samtykke og helseerklæring. Fastende blodprøve, DXA-scan, medbrakt frokost, tilvennig til styrketester og funksjonelle tester (ca. 2 timer).
Minimum 2 dager hvile.
Dag 2: Gjennomføring av styrketester og funksjonelle tester (ca 1 time).
Minimum 2 dager hvile.
Dag 3: MR hos Curato røntgen (ca. 30 minutter).
Dag 4: Akuttforsøk (ca. 8timer) eller prebiopsi (ca 45 minutter).

Før treningsstudien må du også gjennomføre to kostintervju, en tilsvarende

kostregistrering vil gjentas mot slutten av treningsperioden. **I de to siste dagene før tester og biopsi(er) må du avstå fra all krevende fysisk aktivitet (trening)**.

Akuttforsøk

Ti deltakere fra hver gruppe trekkes tilfeldig ut til å gjennomføre et akuttforsøk før og etter treningsperioden, dette innebærer 2 biopsier før treningsperioden og 2 biopsier etter treningsperioden (totalt 4 biopsier). De resterende deltakerne i hver gruppe deltar ikke i akuttstudien og tar bare én biopsi før og én etter treningsperioden. Hensikten med akuttforsøket er å måle hvordan muskulaturens respons til trening forandres over treningsperioden og hvordan inntak av de to drikkene påvirker dette. Oppstart denne dagen vil være mellom kl. 0800 og 0900, og forsøket er ferdig mellom kl. 1530 og 1630. Før vi gjennomfører treningsøkten vil vi ta en biopsi og gjennomføre en styrketest i et kneekstensjonsapparat. Treningsøkten vil være identisk med noen av øktene som gjennomføres senere i treningsperioden. Etter treningsøkten vil du innta en av de to drikkene, og det vil bli tatt en biopsi tre timer etter økten. Det vil også bli tatt blodprøver (gjennom venekateter) flere ganger i løpet av dagen, og gjennomført styrketester rett etter økten, 3 timer etter økten og 24 timer etter økten, for å måle restitusjon. Dermed vil du måtte sette av en hel dag til testdagen (fra 0700-0800 frem til ca. 1530-1630) og 30 min til styrketesting og blodprøve dagen etter.

Treningsperioden

Treningsperioden starter når man har gjennomført alle testene, og den varer i 12 uker. I disse 12 ukene skal det trenes styrke tre ganger i uken (mandag, onsdag og fredag) i grupper på tre deltakere med oppfølging av en personlig trener på alle økter. Drikkene inntas to ganger om dagen; etter trening og på kvelden på treningsdager, og morgen og kveld på treningsfrie dager.

Etter treningsperioden gjennomføres alle testene på nytt for å måle endringer.

Tester

DXA: ved et av oppmøtene før testingen gjøres en DXA-analyse for å måle kroppssammensetningen som vil danne grunnlaget for de standardiserte måltidene ved testgjennomføringen. Denne testen innebærer at deltakerne ligger stille i ca. 10 minutter.

MR: for å måle muskelvekst i lår- og overarmsmuskulaturen benyttes en MR-analyse. Denne testen innebærer at du må ligge i ro ca. 15 minutter.

1RM tester: for å måle styrke vil det testes hvor mye du kan løfte maksimalt en gang i to øvelser som heter beinpress og brystpress.
Muskelfunksjonstest: testingen av muskelfunksjonen gjøres i et kneekstensjonsapparat som er låst ved 90° i kneleddet.
Funksjonelle tester: en test av hvor raskt du kan reise seg fra en stol fem ganger på rad, samt en test av hvor raskt du kan gå opp en trapp vil bli brukt til å si noe om funksjon i hverdagen og mobilitet.
Blodprøver: blodprøvene vil tas i sammenheng med biopsiene og vil gjøres gjennom venekatetrene slik at det ikke blir noen ekstra stikk for blodprøver.
Biopsier: For de som tilfeldig velges til å være med på akuttforsøket blir det to biopsier før og to biopsier etter treningsperioden. For de som ikke skal være med på akuttforsøket blir det én biopsi før og én etter treningsperioden.
Biopsiene tas ut på følgende måte:

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

Eventuelle ulemper ved å delta

Deltakelse i prosjektet vil kreve en del tid og oppmerksomhet.
Trening skal gjennomføres med stor belastning, og vil medføre en viss risiko for skade og følelse av sårhet/stølhet i muskulaturen.
Venekateter medfører en liten infeksjonsfare og det kan oppleves ubehagelig.
Vevsprøvetakninger (biopsier) medfører en liten infeksjonsfare, og ubehag/smerter kan oppleves under inngrepet. Du kan også oppleve lette til moderate smerter i 1-2 døgn etter inngrepet.
Du vil få et lite arr etter snittet i huden; arret vil sakte bli mindre tydelig. Enkelte personer vil kunne få en fortykning av huden i arrområdet.

Personvern

Vi vil kun lagre informasjon om deg under ditt forsøkspersonnummer. Undervis i forsøket vil vi oppbevare en kodeliste med navn og forsøkspersonnummer. Denne

kodelisten vil fysisk være låst inne, slik at det er kun forskerne tilknyttet studien som har adgang til den. Alle som får innsyn i informasjon om deg har taushetsplikt. Innsamlet data vil bli anonymisert etter 15 år (kodelisten destrueres).

Alle prøver vil analyseres "blindet", det vil si at forskerne som utfører den enkelte analysen ikke vet hvilken forsøksperson prøven kommer fra (verken forsøkspersonnummer eller gruppe). Prøver vil bli analysert ved NIH (biopsier), Universitet i Oslo (ernæringsinstituttet; biopsier og blod) og Universitetet i Arkansas, USA (biopsier og blod). Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

Biobank

Biopsiene og blodprøvene vil bli oppbevart i en forskningsbiobank uten kommersielle interesser (vurdert av Regional Etisk Komite). Hvis du sier ja til å delta i studien, gir du også samtykke til at det biologiske materialet og analyseresultater inngår i biobanken. Prøvene vil bli lagret til år 2020. Ansvarlig for biobanken er Professor Truls Raastad ved Seksjon for fysisk prestasjonsevne ved NIH. Det biologiske materialet kan bare brukes etter godkjenning fra Regional komité for medisinsk og helsefaglig forskningsetikk (REK). Hvis du sier ja til å delta i studien, gir du også ditt samtykke til at prøver og avidentifiserte opplysninger utleveres til ernæringsinstituttet ved universitetet i Oslo og universitetet i Arkansas.

Innsynsrett og oppbevaring av materiale

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

Informasjon om utfallet av studien

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

Forsikring

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

Finansiering

Prosjektet er fullfinansiert av Tine® og Norges forskningsråd.

Publisering

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

Samtykke

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

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

Dersom du ønsker flere opplysninger kan du ta kontakt med Håvard Hamarsland på tlf: 93 445 916 eller e-post: haavardh@nih.no, Gøran Paulsen på tlf: 93429420, eller Truls Raastad på tlf: 23 26 23 28 el. 913 68 896

Vennlig hilsen

Håvard Hamarsland (Stipendiat)

Gøran Paulsen (forsker)

Truls Raastad (Professor)

Samtykke til deltakelse i studien Hvordan påvirker inntak av forskjellige melkeproteiner muskelproteinbalanse hos eldre?

Jeg er villig til å delta i studien

(Signert av prosjektdeltaker, dato)

Jeg bekrefter å ha gitt informasjon om studien

(Signert, rolle i studien, dato)

13. Appendix 3

Week	Exercise	Monday			Wednesday			Friday		
		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
1-3	**Hammersquat**	2	12	RM	2	10	90% of 12RM	1	8	RM
	Leg press	1	12	RM	2	10	90% of 12RM	2	8	RM
	Kne extensions	2	12	RM	2	10	90% of 12RM	2	8	RM
	Calf raise	2	12	RM	2	10	90% of 12RM	2	8	RM
	Chest press	1	12	RM	1	10	90% of 12RM	1	8	RM
	Seated row	1	12	RM	1	10	90% of 12RM	1	8	RM
	Close grip pull-down				1	10	RM			
	Shoulder press	1	12	RM	1	10	90% of 12RM	1	8	RM
	Back extensions	1	max 20		1	max 20		1	max 20	
	Ab crunch	1	max 20		1	max 20		1	max 20	
4-6		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
	Hammersquat	2	10	RM	2	10	90% of 10RM	1	6	RM
	Leg press	1	10	RM	2	10	90% of 10RM	2	6	RM
	Kne extensions	2	10	RM	2	10	90% of 10RM	2	6	RM
	Calf raise	2	10	RM	2	10	90% of 10RM	2	6	RM
	Chest press	1	10	RM	1	10	90% of 10RM	1	6	RM
	Seated row	1	10	RM	1	10	90% of 10RM	1	6	RM
	Close grip pull-down				1	10	RM			
	Shoulder press	1	10	RM	1	10	90% of 10RM	1	6	RM
	Back extensions	1	max 15		1	max 15		1	max 15	
	Ab crunch	1	max 15		1	max 15		1	max 15	
7-9		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
	Hammersquat	2	10	RM	2	10	90% of 10RM	2	6	RM
	Leg press	2	10	RM	3	10	90% of 10RM	3	6	RM
	Kne extensions	3	10	RM	2	10	90% of 10RM	3	6	RM
	Calf raise	2	10	RM	2	10	90% of 10RM	2	6	RM
	Chest press	2	10	RM	2	10	90% of 10RM	2	6	RM
	Seated row	2	10	RM	2	10	90% of 10RM	2	6	RM
	Close grip pull-down				2	10	RM			
	Shoulder press	2	10	RM	2	10	90% of 10RM	2	6	RM
	Back extensions	1	max 15		1	max 15		1	max 15	
	Ab crunch	1	max 15		1	max 15		1	max 15	
10-12		Sets	Reps	Load	Sets	Reps	Load	Sets	Reps	Load
	Hammersquat	3	8	RM	3	8	90% of 8RM	2	6	RM
	Leg press	2	8	RM	3	8	90% of 8RM	3	6	RM
	Kne extensions	3	8	RM	2	8	90% of 8RM	3	6	RM
	Calf raise	2	8	RM	2	8	90% of 8RM	2	6	RM
	Chest press	3	8	RM	2	8	90% of 8RM	2	6	RM
	Close grip pull-down				2	10	RM			
	Seated row	2	8	RM	2	8	90% of 8RM	2	6	RM
	Shoulder press	2	8	RM	2	8	90% of 8RM	2	6	RM
	Back extensions	1	max 10		1	max 10		1	max 10	
	Ab crunch	1	max 10		1	max 10		1	max 10	

14. Appendix 4

WESTERN BLOT - PROTOKOLL

Forberedelser

SDS-running buffer: 200ml SDS + 1800ml dH2O
Tris/glycine Transfer buffer: 200 ml metanol + 200ml Tris/glycine + 1600 ml dH2O
TBS: 100mL TBS + 900 mL ultrarent vann
TBS-T: 200 mL TBS + 1800 mL ultrarent vann + 2 mL tween 20.
Ultrarent vann: 3 L

- Lag 5 % melkeløsning: 20 g melkepulver + 400 mL TBS-T (røres med magnet)
- Mengde beregnet for 1 boks (2 membraner). Settes i kjøleskap over natt.

DAG 1

KLARGJØRING. BLANDE PRØVER

- Ta TBS, TBS-T og melkeblanding ut av kjøleskapet.
- Sett varmeblokk på 60° C (faktisk temperatur: 70° C). Dobbeltsjekk at den faktisk er påslått, og at riktig blokk står i. Hent ut prøver fra fryseren.
- Lag sample buffer: Bland 336 uL Laemmli sample buffer og 14 uL 5MDT i et eget 1,5 ml rør.
- Bland prøve, H_20, sample buffer i henhold til «sample preparation mal».
- «Sample preparation mal» finner man under *SFP group -> analyselab -> SOP-prosedyrer*.
- Print også ut «NuPAGE ELFO WB»-skjema.
- Sett merkede eppendorfrør på varmeblokk i 10 min ved 70°. Sett på stoppeklokke!

MENS PRØVEN STÅR PÅ VARMEBLOKK

- Finn fram: 4 geler til hver boks som skal kjøres, running buffer, elektroforeseboks (den største av boksene) og GeneOn markør
- Husk prøvene på varmeblokka!
- Klipp opp plasten på gelene, og tørk av. Ta av grønn tape under gelen og fjern kammen.
- Sett gelene i boksen med ”brønnene innover”. Dytt gelene godt ned og lukk igjen klemmen.

ELEKTROFORESE

- Fyll indre kammer med SDS running buffer (sjekk at det ikke lekker i ytre kammer).
- Sett 5 uL markør i brønn 1 og 10 på begge sider (bruk lilla tynn pipette)
- Sett 30 uL prøve i de øvrige brønnene. (Bruk lilla tynn pipette)
- Ytre kammer fylles 2/3 fullt med running buffer.
- Sett på lokk, montér ledninger, slå på og still inn volt (200 V) og tid ca 40 min
- Sjekk at strømmen lager bobler i indre bufferkammer.

MENS ELEKTROFORESEN GÅR – AKTIVERE PVDF-MEMBRANENE
(husk å ta bilder av gelene etter elektroforesen)

- Klipp opp 4 PVDF-membraner per elektroforese-kammer og legg disse i lyseblå boks. Skriv nummer øverst i venstre hjørne av membranen og klipp et hakk øverst i høyre hjørne.
- Finn frem 24 filterpapir og 4 pads.
- Pads legges i transfer buffer i lilla boks i >15 min (klem ut luftbobler).
- PVDF-membraner aktiveres på følgende måte (i en tilstrekkelig romslig boks):
    - 30 sek i metanol
    - 30 sek i dH2O
    - 1-2 min i nytt dH2O
    - 10-15 min i transferbuffer
- Filterpapir legges i transferbuffer i lilla boks like før bruk.

BLOTTING

- Finn frem: blottedelen til boksene, lilla bokser, pinsett, grønn spade, penn, tørkepapir, rulle og gul avfallsboks.
- Slå av, trekk ut ledninger. Ta ut en gel om gangen, og tørk av. Ta ut den fremste gelen først.
- Knekk opp med en spade. Løft av den minste platen fra motsatt side av buen.
- Fjern brønnene. Skjær av nedre del av gelen, og lag sandwich (blank del liggende i plastbrett dekket av transferbuffer):
    - Pad
    - Filterpapir + membran (bruk rulle for god kontakt)
    - Gel
    - Filterpapir
    - Pad
- Skyll elektroforesekammer i dH2O, og monter sandwichen i kammeret. Svart mot svart, blank mot rødt.
- Fyll indre kammer med transferbuffer.
- Legg i roterende magnet i bunnen i ytre kammer og legg i frossent kjøleelement.
- Fyll kammer fullt med transfer buffer (så det dekker øvre del av padsene i sandwichen)
- Sett på lokk, montér ledninger. Sjekk at elektrodene sitter i sporene slik de skal.
- Blotting: 100V, 30 min.

NÅR BLOTTING ER IGANGSATT

- Rydd det som kan ryddes.

NÅR BLOTTINGEN ER FERDIG: Ta bilder av membran og geler!
Etter bilder: KUTTING

- Bruk pasteurpipette til å fukte glassplaten

- Legg membranen på glassplaten med proteinsiden opp, og bruk linjal og blyant for å tegne opp hvor det skal kuttes. Kutt på 120, på 82, rett under 62 og rett under 26.
- Skriv nytt navn til høyre på hver membrandel, og kutt i vei.
- Proteinvekt: eEF2: ca 95 kDA
  P70: ca 70 kDA
  4E-BP1: 15-20 kDA

BLOKKERING

- Tøm 5 % melkeløsning i en lyseblå boks. Fyll dH2O i en lilla boks.
- Slå av, trekk ut ledninger, ta ut og åpne blottmodulen over lilla plastboks.
- Legg pads i dH2O (må ikke komme i kontakt med deconex). Filtrene kastes. Membraner legges i en boks med TBS.
- Dekk til med parafilm, og sett boksen på gyrorockeren i 2 timer (hastighet: 20).
- Finn glassplate, kniv, linjal, blyant, pasteurpipette, markørvektskjema, og boks med TBS.
- Finn fram rør for primært antistoff-løsning, og merk disse med antistoff, initialer og dato.
- Vask elektroforese-kamrene.
- **1 time pause.**

VASKING

- Tøm ut melkeløsningen fra boksen med membranene.
- Skyll 2 ganger i romtemperert TBS-T.
- Skyll 2 x 2 min i TBS-T på gyrorocker (hastighet: 65).

INKUBERING I PRIMÆRT ANTISTOFF (FOSFO-PROTEINER)

- Finn fram antistoff for de fosfoproteinene som skal studeres.
- Lag 1 % melkeløsning: Bland 10 mL 5 % melkeløsning med 40 mL TBS-T.
- Antistoff tines og spinnes ned.
- Pipetter 5 mL 1 % melkeløsning i hvert rør, og pipetter primært antistoff i riktig rør.
- Ta membranene opp av TBS-T-løsningen og rull dem med proteinsiden inn (tallet inn).
- Legg rørene på roller mikser i kjøleskap over natt (hastighet: 7).
- Sett TBS, TBS-T, dH2O og 5 % melkeløsning i kjøleskap.

DAG 2

VASKING

- Ta TBS, TBS-T og melkeløsning ut av kjøleskapet (TBS og TBS-T røres og varmes).
- Bruk pinsett for å ta membranene ut av rørene. Ta vare på rørene med antistoffer (?).
- Legg membranene i lyseblå plastboks og skyll to ganger i TBS-T.
- 1 x 15 min i TBS-T på gyrorocker (hastighet: 65).
- 3x 5 min i TBS på gyrorocker (hastighet: 65).

INKUBERING I SEKUNDÆRT ANTISTOFF

- Lag 1 % melkeløsning: Bland 20 mL 5 % melkeløsning med 80 mL TBS-T med magnet.
- Tilsett 30 mL 1 % melkeløsning i forholdsvis liten plastboks, og tilsett 10 uL sekundært antistoff (goat-anti-rabbit, 1:3000 fortynning).
- Følgende membraner skal ha goat-anti-rabbit som sekundært antistoff: fosfo p70, fosfo eEF2, og fosfo 4E-BP1.
- Legg membranene i løsningen med proteinsiden opp, og inkubér i 1 time på gyrorocker (hastighet: 20). Bruk lokk/parafilm.
- **60 min pause.**

VASKING

- Tøm ut melkeløsningen fra boksen med membranene.
- Skyll 2 ganger i romtemperert TBS-T.
- 1 x 15 min i TBS-T på gyrorocker (hastighet: 65).
- 3 x 5 min i TBS på gyrorocker (hastighet: 65).

BILDER

- Mens membranene vaskes: Finn fram et lite rør og pakk det inn i aluminiumsfolie.
- Bland substratvæske, vend på røret og sett i skap (omtrent 2 mL total mengde per bilde).
- Lag oppsett for billedtagningen. Velg: new single channel → blots → chemi hi sensitivity
- Ta bilder med proteinsiden opp. Velg et bilde og lagre.

STRIPPING, VASKING OG BLOKKERING

- Strip membranene med «Restore PLUS Western blot stripping buffer» i 10 min på gyrorocker
- Skyll membranene 5 x 1 min i TBS. Benytt ny plastboks.
- 3 x 5 min i TBS på gyrorocker (hastighet: 65).
- Blokkér i 5 % melkeløsning i 120 min.
- 1 % melkeløsning: Bland 10 mL 5 % melkeløsning med 40 mL TBS-T (forberedelse til antistoff-inkubering)
- Finn fram rør for primært antistoff-løsning, og merk disse med antistoff, initialer og dato.

VASKING

- Tøm ut melkeløsningen fra boksen med membranene.
- Skyll 2 ganger i romtemperert TBS-T.
- Skyll 2 x 2 min i TBS-T på gyrorocker (hastighet: 65).

INKUBERING I PRIMÆRT ANTISTOFF (TOTAL-PROTEINER)

- Finn fram antistoff for de total-proteiner som skal studeres.
- Antistoff tines og spinnes ned.
- Pipetter 5 mL eller 10 uL 1 % melkeløsning i hvert rør/boks, samt riktig primært antistoff.

- Ta membranene opp av TBS-T-løsningen og rull dem med proteinsiden inn (tallet inn).
- Legg rørene på roller mikser i kjøleskap over natt (hastighet: 7).
- Sett TBS, TBS-T og 5 % melkeløsning i kjøleskap.

DAG 3

VASKING

- Ta TBS, TBS-T og melkeløsning ut av kjøleskapet (TBS og TBS-T røres og varmes).
- Bruk pinsett for å ta membranene ut av rørene. Ta vare på rørene med antistoffer (?)
- Legg membranene i lyseblå plastboks og skyll to ganger i TBS-T.
- 1 x 15 min i TBS-T på gyrorocker (hastighet: 65).
- 3 x 5 min i TBS på gyrorocker (hastighet: 65).

INKUBERING I SEKUNDÆRT ANTISTOFF

- Lag 1 % melkeløsning: Bland 20 mL 5 % melkeløsning med 80 mL TBS-T med magnet.
- Tilsett 30 mL 1 % melkeløsning i forholdsvis liten plastboks, og tilsett 10 uL sekundært antistoff (goat-anti-rabbit, 1:3000 fortynning).
- Følgende membraner skal ha goat-anti-rabbit som sekundært antistoff: Total p70, Total eEF2 og Total 4E-BP1.
- Legg membranene i løsningen med proteinsiden opp, og inkubér i 1 time på gyrorocker (hastighet: 20). Bruk lokk/parafilm.

VASKING

- Tøm ut melkeløsningen fra boksen med membranene.
- Skyll 2 ganger i romtemperert TBS-T.
- 1 x 15 min i TBS-T på gyrorocker (hastighet: 65).
- 3 x 5 min i TBS på gyrorocker (hastighet: 65).

BILDER

- Mens membranene vaskes: Finn fram et lite rør og pakk det inn i aluminiumsfolie.
- Bland substratvæske, vend på røret og sett i skap (omtrent 2 mL total mengde per bilde).
- Lag oppsett for billedtagningen. Velg: new single channel → blots → chemi hi sensitivity
- Ta bilder med proteinsiden opp. Velg et bilde og lagre.