NORWEGIAN SCHOOL OF SPORT SCIENCES

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Are basal levels and regulation of amino acid transporters in muscle different in prostate cancer patients treated with androgen deprivation therapy compared to patients with normal testosterone levels?

A cross-sectional study in prostate cancer patients undergoing androgen deprivation therapy or treated with surgery.

Master thesis in Sports Science
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Abstract

Background: Prostate cancer patients receiving androgen deprivation therapy (ADT) often experience negative side effects influencing the quality of life, such as loss of muscle mass. Resistance exercise has been proposed to counteract this loss of muscle mass. However, literature shows a blunted anabolic response in patients on ADT when compared to the expected response to resistance exercise in elderly.

Purpose: The purpose of this study was to compare the basal levels of amino acid transporters in muscle and the acute regulation of amino acid transporters in response to protein supplementation and resistance exercise between prostate cancer patients receiving ADT treatment and prostate cancer patients with normal testosterone levels.

Method: The study was a single-blind, controlled acute study including 20 prostate cancer patients, 10 receiving ADT treatment (ADT group) and 10 treated with surgery (OPR group). The participants were subjected to an accustoming day before executing two resistance exercise sessions, and one acute day with protein supplementation, resistance exercise and muscle biopsies. Resistance exercise sessions consisted of 3 series of 10 repetitions in the exercises: one-legged leg press, one-legged knee extension, chest press, and seated row, where the left leg operated as control (not trained). On the acute day, participants received two standardized protein supplementations and completed one last resistance exercise session. Muscle biopsies were collected from *m. vastus lateralis*, first in a fasted state in the not trained leg, two hours post supplementation in both legs, and two hours post resistance exercise in combination with supplementation from both legs. Muscle tissue was fractionated and analysed by western blot for regulators of amino acid transporters eIF2α, and ATF4, and the amino acid transporters CD98-, and SLC38A9.

Results: There were no difference between groups in protein expression for eIF2α, ATF4, and SLC38A9 at baseline, but CD98 levels in the ADT group were higher in the nuclear fraction and lower in the cytosolic fraction compared to the OPR group. There were no differences between groups for protein expression in eIF2α, CD98, ATF4, and SLC38A9 post-supplementation. In the ADT group, there was an increased protein expression for eIF2α in the trained leg post resistance exercise in combination with

supplementation in the cytosolic fraction, and a decrease in the untrained leg in the membrane fraction. There were no differences between groups in protein expression for CD98, ATF4, and SLC38A9 post-resistance exercise in combination with supplementation.

Conclusion: ADT treated prostate cancer patients had lower basal levels of CD98 in the cytosolic fraction and higher levels in the nuclear fraction compared to patients treated with surgery, but there were no differences between groups in the response to protein supplementation and resistance training.

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Acknowledgements

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Mats Stadsnes

1. Introduction

Prostate cancer is according to the Norwegian cancer registry for 2022 the most common form of cancer in men, accounting for 26,5% of all cancer cases diagnosed in 2017-2021 (Norway, 2021; Silva, 2022). Depending on tumour localization and prevalence various treatments are available, the most common curative approaches involve surgery, radiation, and hormonal treatment (Kreftforeningen, 2023). Androgen deprivation therapy (ADT) results in reductions of testosterone levels equal to castrated levels to inhibit or reduce growth in androgen-sensitive tumours. However, the treatment is associated with numerous negative side effects, including a loss of muscle mass (Grossmann et al., 2013; Overkamp et al., 2023).

Resistance exercise has been suggested to counteract the loss of lean body mass in prostate cancer patients undergoing ADT (Keilani et al., 2017). However, several studies on the subject have reported a blunted anabolic response to resistance exercise in this population (Alberga et al., 2012; Houben et al., 2023; Newton et al., 2019; Nilsen et al., 2015). Given that prostate cancer predominantly affects older individuals, the reported response to resistance exercise in prostate cancer patients undergoing ADT could be influenced by age. In addition, the absence of androgens following ADT initiation could supress the role of testosterone in the anabolic response of humans (Basualto-Alarcón et al., 2013; Silva, 2022). Therefore, a better understanding of the anabolic response at a cellular level in prostate cancer patients undergoing ADT is needed to potentially influence muscle mass regulation in this population.

The balance between muscle protein synthesis (MPS) and protein degradation determines the net protein balance, and therefore regulates muscle mass (Paddon-Jones et al., 2004). The MPS stimulator protein mammalian target of rapamycin (mTORC1) can regulate protein translation and is influenced by various stimuli (Laplante & Sabatini, 2009; Wang et al., 2021). Mechanical stress, growth hormone, energy status, and amino acids have been shown to stimulate or inhibit mTORC1 activation through various signalling pathways (Dickinson et al., 2011; Laplante & Sabatini, 2009; Olsen et al., 2019; Wang & Proud, 2006).

The availability of amino acids can regulate MPS through mTORC1 stimulation (Laplante & Sabatini, 2009). The general amino acid control pathway (GAAC) is a nutrient-sensing pathway that can restore intracellular amino acid levels during amino acid deficiencies. This pathway involves the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) and subsequent upregulation of activating transcription factor 4 (ATF4), leading to a general reduction in translation but an increase in amino acid transporter transcription (Bröer & Bröer, 2017; Malmberg & Adams, 2008). In skeletal muscles, the main function of amino acid transporters is to transport amino acids from interstitial fluid to the cytoplasm and intracellular organelles of muscle cells (Roberson et al., 2020).

Testosterone may affect the regulation of muscle mass and amino acid availability in muscles in different ways. Houben et al. (2023) observed no additional benefits of protein supplementation following a resistance exercise intervention in prostate cancer patients undergoing ADT, but rather an increase in fat mass and fat percentage was reported. Furthermore, Haren et al. (2011) reported that access to testosterone increased mRNA levels of the amino acid transporter solute carrier family 38 member 2 (SNAT2). Taken together, these findings suggest a potential limitation in the handling of amino acids in the absence of testosterone, which may indicate reduced amino acid sensitivity and availability for prostate cancer patients receiving ADT.

To the best of authors knowledge, no studies have investigated the anabolic response to protein supplementation and resistance exercise in amino acid sensitivity and availability in prostate cancer patients receiving ADT. In addition, Haren et al. (2011) is the only study to investigate the effect of testosterone levels on amino acid transporters. Taken together, the role of amino acid sensitivity and availability in muscle mass regulation and potential influence on negative side effects in muscle mass following ADT initiation, in addition to lack of existing literature represent a need for future research on the subject.

1.1 Aims and hypothesis

The purpose of this study was to compare the basal levels of amino acid transporters and the response in transporters and their regulators to protein supplementation and resistance exercise in combination with protein supplementation in prostate cancer patients receiving ADT treatment with prostate cancer patients who underwent surgery. Consequently, we compared one group with castrate levels of testosterone with one group with normal levels of testosterone in protein expression of the regulators eIF2α, ATF4, and amino acid transporters CD98 and SLC38A9 in a fasted state (baseline), after protein supplementation and after resistance exercise in combination with protein supplementation.

The study had the following primary hypothesis:

The basal levels of amino acid transporters in muscle, and the acute regulation of amino acid transporters in response to protein supplementation and resistance exercise, will be inferior in prostate cancer patients receiving ADT treatment compared to prostate cancer patients with normal testosterone levels.

The primary hypothesis was operationalized to the following testable secondary hypothesises:

- Prostate cancer patients receiving ADT treatment will have lower protein expression of eIF2α in response to protein supplementation and resistance exercise in combination with supplementation, but similar at baseline.
- Prostate cancer patients receiving ADT treatment will have lower protein expression of ATF4 in response to protein supplementation and resistance exercise in combination with supplementation, but similar at baseline.
- Prostate cancer patients receiving ADT treatment will have lower protein expression of CD98 at baseline, and in response to protein supplementation, and resistance exercise in combination with protein supplementation.
- Prostate cancer patients receiving ADT treatment will have lower protein expression of SLC38A9 at baseline, and in response to protein supplementation, and resistance exercise in combination with protein supplementation.

2. Theory

2.1 Prostate cancer

Prostate cancer is the most ordinary form of cancer in men, with a median age of 70 years for diagnosis (Silva, 2022). Prostate cancer was diagnosed in 26,5% of cancer cases in 2017-2021 by all ages, 32,5% for the age group 50-69 years and 25,3% for the age group >70 years (Norway, 2021). The cause of prostate cancer is still unknown, but it occurs in glands located in the prostate (Kreftforeningen, 2023; Silva, 2022). Given the localization and growth of the prostate glands in prostate cancer cases, symptoms of the disease include weak and frequent urination, and difficulty emptying the urinary bladder. Less often but the occurrence of blood in urine and semen, and lower back pain may also be symptoms of prostate cancer (Kreftforeningen, 2023).

There is observed a trend in increasing incidences of prostate cancer spanning from 20th century to mid-21st century across various age groups, except for individuals between 0 and 54 years. Given that prostate cancer usually occurs in the elderly, increased life expectancy may be the main cause for this observed increase in new cases (Norway, 2021; Silva, 2022).

2.1.1 Examination and diagnosis

By suspicion of prostate cancer clinical examination of the prostate glands can be used to examine the size and form of the prostate glands. Additionally, a tissue sample from prostate and PSA concentration in the blood can be used for diagnosis. PSA-concentration measurements over time are used as an auxiliary mean to diagnosis cancer cases, and measure the effect of treatment (Kreftforeningen, 2023).

2.1.2 Treatment

Various treatments for prostate cancer are available but will often include negative side effects which may influence the quality of life for patients. Aggressivity and growth rate of the tumour, the potential spread of the tumour, the age, and condition of the patient, and additional diseases all play major roles in considering the form of treatment (Kreftforeningen, 2023).

Given a 95,5% five-year relative survival rate and the negative side effects following various treatments for prostate cancer, some patients in the low-risk specter of the disease are given the option of active surveillance (Kreftforeningen, 2023; Silva, 2022). By active surveillance, the development of the disease is monitored and includes frequent control examination of the patient (Kreftforeningen, 2023).

Based on localization and prevalence of the tumour, patients can be given the option of surgery or radiation as treatment. Surgery involves the surgical removal of the prostate glands, and potentially seminal vesicles and lymph nodes (Kreftforeningen, 2023). Radiation treatment differs between internal radiation (Brachytherapy), where radiation is placed within the prostate glands, and external radiation. External radiation is usually given in combination with hormonal treatment (Kreftforeningen, 2023). Hormonal treatment, or androgen deprivation therapy (ADT), results in testosterone level reduction to a castrated level to inhibit or reduce growth in androgen-sensitive tumours. However, ADT treatment is related to numerous negative side effects, including loss of muscle mass (Grossmann et al., 2013; Overkamp et al., 2023).

2.1.3 Androgen deprivation therapy

ADT uses hormonal therapy to reduce testosterone levels and mortality by suppressing androgen activity (Grossmann et al., 2013; Heidenreich et al., 2014; Pagliarulo et al., 2012). The effect is achieved by reducing the production of androgens by chemical castration, or the use of anti-androgens that block androgen receptors (AR) to inhibit signalling. Additionally, a combination of both approaches known as androgen blockade can be used as treatment (Grossmann et al., 2013; Heidenreich et al., 2014).

Activation of AR regulates the effect of androgens, mainly testosterone, and dihydro-testosterone. Thus, inhibition of AR may suppress the effect of androgens on androgen-sensitive tumours in prostate cancer patients (Gobinet et al., 2002; Grossmann et al., 2013). Huggins og Hodges (2002) displayed the positive effect of ADT possess as a treatment for prostate cancer. In contrast, ADT is also related to numerous negative side effects, such as the negative impact on sexual function, increased fat mass, decreased bone density, and loss in muscle mass (Grossmann et al., 2013).

As described, ADT can be viewed as an effective treatment to stop the progression of prostate cancer but may also lead to negative developments on various factors for prostate cancer patients. In a recent study by Overkamp et al. (2023), results show a significant decrease in muscle mass and an increase in fat mass during the five first months of ADT in prostate cancer patients. Additionally, a decrease in muscle strength, cardiometabolic health, physical activity levels, and quality of life was observed (Overkamp et al., 2023).

Lifestyle changes, such as diet and physical activity levels have the potential to affect body composition, muscle strength, aerobic capacity, and cardiometabolic health. Regarding Overkamp et al. (2023), energy intake did not seem to change but they observed a significant decline in habitual activity. Therefore, negative effects observed by ADT may be amplified by lifestyle changes following ADT initiation.

2.1.4 ADT and anabolic response

Regarding numerous negative side effects following ADT initiation, exercise has been suggested as a strategy to counteract the loss of lean body mass and increase in fat mass (Alberga et al., 2012). However, prostate cancer is seen to advance with old age as described earlier. Additionally, testosterone levels have been shown to play a key role in anabolic response in humans (Basualto-Alarcón et al., 2013). This may indicate that prostate cancer patients receiving ADT treatment may experience a blunted anabolic response given age-related influence in combination with the lack of androgens. Multiple articles have investigated the effects of exercise on body composition in prostate cancer patients receiving ADT treatment, as presented in Table 2.1.

Table 2.1: Schematic overview of articles investigating body composition in response to exercise in prostate cancer patients receiving ADT.

Author	Aim	Subjects	Intervention	Results
Alberga et al. (2012)	Examine the effects on body composition and fitness following a 24-week exercise program in prostate cancer patients.	Participants (n=121) were males scheduled to receive radiotherapy alone or with ADT, divided by age (<65 years or >65 years) and ADT (presence or absence).	Exercise training included either progressive aerobic or resistance exercise for three supervised sessions per week for 24 weeks.	Presence of ADT: • For the aerobic group percent body fat increased (p=0.004), and lean body mass decreased (p=0.003). • For the resistance group there were no significant changes in fat percent or lean body mass. Absence of ADT: No changes in body composition in any group.
Houben et al. (2023)	Examine the effect of resistance exercise with or without protein supplementation on body composition, muscle mass, muscle strength, physical performance, and aerobic capacity in prostate cancer patients receiving ADT.	Participants (n=60) undergoing ADT were assigned to a 20-week resistance exercise program receiving either a protein supplementation group (n=30) or placebo (n=30). Additionally, a separate control group (n=36) received the usual care.	Resistance exercise included a progressive whole-body training program for two supervised 60-minute sessions per week, for 20 weeks. Participants were either given a protein supplementation (31g whey protein) or a placebo immediately after exercise and each night before sleep.	No significant change in lean mass within groups from baseline. The exercise group receiving protein supplementation increased in fat mass and body fat percentage (both <0.005), and a larger increase in the control group (both <0.001)
Newton et al. (2019)	Examine effects of targeted resistance exercises on bone mineral density in prostate cancer	Participants (n=154) undergoing ADT were randomized into: • Impact loading +	• Impact loading + resistance exercise included two supervised	No significant differences in lean mass (kg) or fat mass (kg) for either group in absolute values or change

	patients undergoing ADT treatment.	resistance exercise supervised for 12 months (n=57). • Aerobic + resistance exercise supervised for 6 months, followed by a 6-month home-based program (n=50). Delayed aerobic exercise, 6 months receiving training information, following 6 months of supervised aerobic training (n=47).	sessions per week for 12 months. Additionally, two sessions of home exercise were completed weekly. • Aerobic + resistance exercise included two supervised sessions per week for 6 months, following home-based training for 6 months. Delayed aerobic exercise were given information about exercise for the initial 6 months, following two supervised aerobic sessions per week for the remaining 6 months.	over 6 and 12 months.
Nilsen et al. (2015)	Examine the effects of strength training on body composition, physical functioning, and quality of life in prostate cancer patients during ADT.	Participants (n=50) were randomized to either 16 weeks of high-load strength training (n=28) or usual care (n=30).	High-load strength training included three sessions per week for 16 weeks. After two weeks of familiarization, the training program followed a daily undulating periodization model, with a linear progression in volume.	No significant differences in total lean body mass between groups. However, significant effects were found in lean body mass for the upper and lower extremities (<0.01 and <0.05, respectively) when compared to usual care. No effect on fat mass, areal bone density, or health-related quality of life was observed.

Findings in Alberga et al. (2012) demonstrate the value of resistance exercise in elderly men with prostate cancer, the observed increase in percent body fat and reduction in lean body mass for the aerobic group contrasts with the resistance exercise group who preserved their lean body mass and had reduced increases in percent body fat. In conclusion, the study demonstrates the beneficial effects of resistance exercise regardless of the presence or absence of ADT in prostate cancer patients (Alberga et al., 2012).

The study by Houben et al. (2023) demonstrates that resistance exercise is well tolerated over long periods in prostate cancer patients receiving ADT. This is supported in the study by Newton et al. (2019) who followed exercise groups over the duration of 12 months. Findings in Houben et al. (2023), Newton et al. (2019) and Nilsen et al. (2015) show no significant differences in total lean mass following a resistance training regime in prostate cancer patients receiving ADT. In conclusion, mentioned articles display resistance exercise as a prominent way to reduce or counteract the negative side effects observed on body composition following ADT treatment.

However, a recent meta-analysis by Nilsen et al. (2022) compared the effect of long-term resistance exercise on body composition between prostate cancer patients receiving ADT and healthy elderly men. Findings support that resistance exercise mainly counteracts the negative effects following ADT treatment, as lean body mass was preserved in participants. In contrast, healthy elderly men have been observed to increase lean body mass following a resistance exercise intervention (Nilsen et al., 2022).

In summary, participating in resistance exercise displays positive outcomes for prostate cancer patients to counteract the negative side effects on body composition following ADT treatment (Alberga et al., 2012; Houben et al., 2023; Newton et al., 2019; Nilsen et al., 2015). However, findings may indicate a blunted anabolic response to resistance exercise in those patients receiving ADT, given the preservation in lean body mass versus an increase observed in healthy elderly males (Nilsen et al., 2022).

Regarding a blunted anabolic response, the term anabolic resistance is used to describe a reduced response to anabolic stimuli, such as resistance exercise or protein

supplementation (Wilkinson et al., 2018). According to Wilkinson et al. (2018), anabolic resistance may be caused by a variety of factors, including reduced availability of amino acids and alterations in anabolic signalling pathways.

Interestingly, Houben et al. (2023) observed no additional benefits of protein supplementation following a resistance exercise intervention, but an increase in fat mass and fat percentage in prostate cancer patients on ADT. Findings in Haren et al. (2011) indicate an influence of testosterone levels in amino acid sensitivity, due to an observed increase during testosterone availability in amino acid transporter SNAT2 mRNA. Taken together, this may suggest a limitation in the handling of amino acids in the absence of testosterone. Therefore, a reduced amino acid sensitivity and availability for prostate cancer patients receiving ADT could be a possible factor for the blunted anabolic response described earlier.

2.2 Muscle mass

2.2.1 Muscle protein synthesis

Muscle mass regulation involves the balance between muscle protein synthesis (MPS) and protein degradation. MPS is the process of building new proteins within muscle cells, while protein degradation is the process of breaking down existing proteins into their constituent amino acids, which determines the net protein balance and consequently regulates muscle mass (Paddon-Jones et al., 2004).

A prerequisite for MPS is the creation of mRNA through the process of transcription (Rennie et al., 2004). MPS produces chains of amino acids by peptide bindings which connect amino acids through carboxyl groups (Hershey et al., 2019). Decoding of the mRNA happens in ribosomes by translation (Wang & Proud, 2006). Ribosomes can bind to mRNA, where other enzymes can translate the message and deliver the correct amino acid for the amino acid chain (Hershey et al., 2019). MPS is regulated by intracellular mechanisms activated by various stimuli as shown in Figure 2.1 (Coffey & Hawley, 2007; Rennie et al., 2004; Wang & Proud, 2006).

The physiological effects of testosterone are mediated by its binding to intracellular androgen receptors and following translocation to the nucleus, which initiates transcription of specific genes, and inducing the expression of various biological responses (Gobinet et al., 2002; Horstman et al., 2012). Increased testosterone levels have shown to result in increased stimulation of MPS by various signalling pathways or activation of intracellular androgen receptors (Basualto-Alarcón et al., 2013; Dubois et al., 2012).

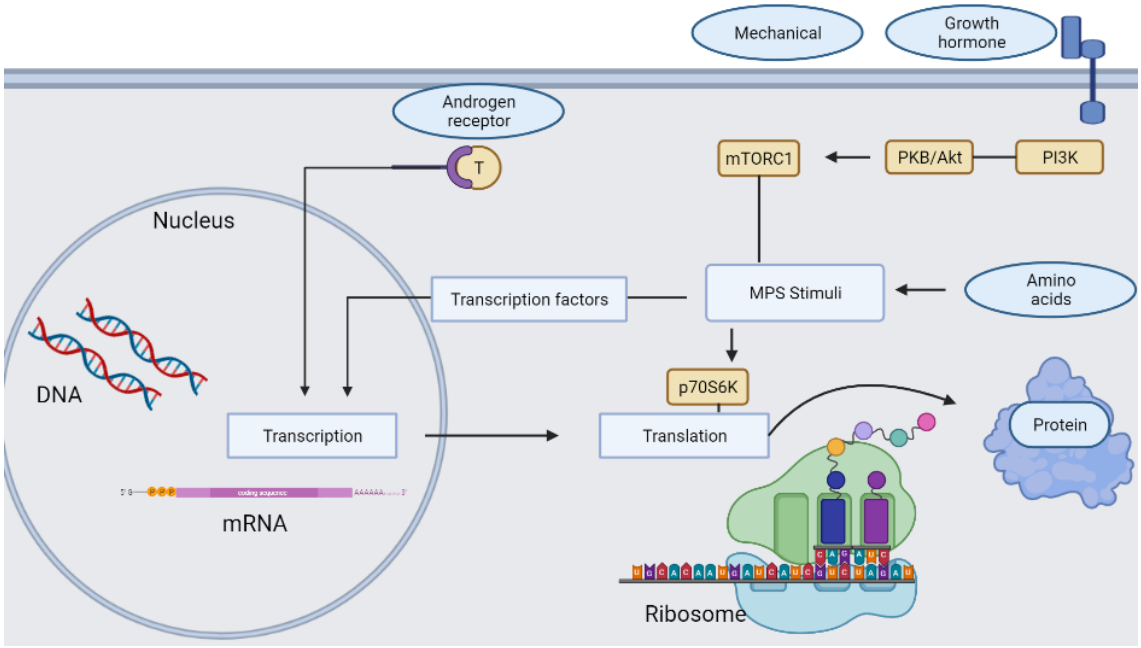


Figure 2.1: Simplified production of stimulating mechanisms for MPS. T = Testosterone. The figure is created with biorender.com and inspired by multiple articles (Basualto-Alarcón et al., 2013; Coffey & Hawley, 2007; Rennie et al., 2004; Wang & Proud, 2006).

2.2.2 Mammalian Target of Rapamycin Complex 1

The mammalian target of rapamycin complex 1 (mTORC1) can regulate protein translation through phosphorylation of ribosomal protein S6 kinase (p70S6K) and 4E-BP1 (Laplante & Sabatini, 2009; Wang et al., 2021).

The tuberous sclerosis complex (TSC), compromising hamartin (TSC1) and tuberin (TSC2), functions as a GTPase-activating protein (GAP) for Ras homolog enriched in brain (Rheb) and plays a key role in the regulation of mTORC1. Upon activated, the GTP-linked Rheb interacts with and activates mTORC1 (Laplante & Sabatini, 2009). Multiple signalling pathways have been identified to stimulate or inhibit mTORC1 activation in response to various stimuli, such as growth hormone, mechanical stress, energy status, and amino acids (Dickinson et al., 2011; Laplante & Sabatini, 2009; Olsen et al., 2019; Wang & Proud, 2006). During increased mechanical tension, focal adhesion kinase (FAK) activation can lead to subsequent phosphorylation events and

anabolic signalling (Olsen et al., 2019). The signalling pathway regarding growth hormone includes phosphoinositide 3-kinase (PI3K), and subsequent inactivation of TSC by protein kinase B (PKB/AKT), resulting in mTORC1 activation (Laplante & Sabatini, 2009). In conditions of low energy status, the adenosine monophosphate-activated protein kinase (AMPK) is activated as an response to the adenosine triphosphate (ATP) and adenosine diphosphate (ADP) ratio, which consequently phosphorylates TSC2, enhancing the GAP activity of TSC2 and Rheb, resulting in inhibition of mTORC1 activation (Laplante & Sabatini, 2009). Moreover, in the presence of amino acids, the association between Rag proteins, consisting of four GTPases, and Raptor promotes translocation and subsequently activation of mTORC1 (Laplante & Sabatini, 2009).

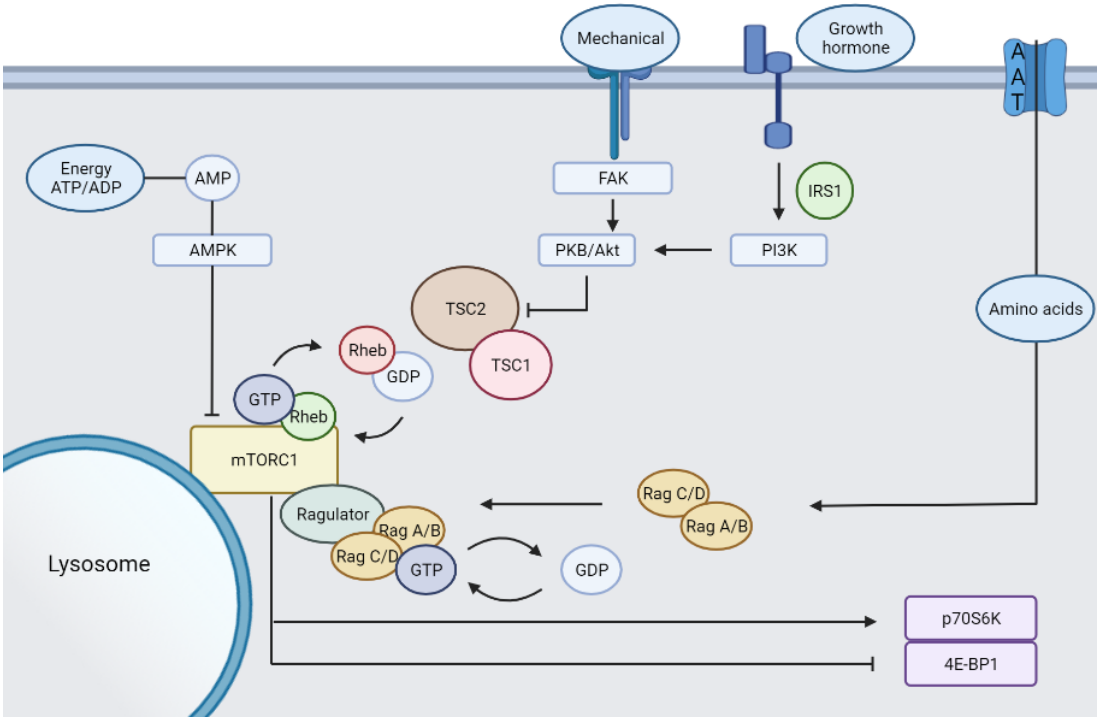


Figure 2.2: Simplified production of stimulating mechanisms for mTORC1. AAT = Amino acid transporter. IRS1: insulin receptor substrate 1. Figure is created with biorender.com and inspired by multiple articles (Dickinson et al., 2011; Laplante & Sabatini, 2009; Olsen et al., 2019; Wang & Proud, 2006).

2.2.3 General amino acid control pathway

The general amino acid control pathway (GAAC) is a nutrient-sensing pathway, that can restore intracellular amino acid levels during amino acid deficiencies. Through binding and activation of GCN2 by increased levels of uncharged tRNA, GCN2 phosphorylates eukaryotic translation initiation factor 2α (eIF2α) (Malmberg & Adams, 2008). Phosphorylation of eIF2α inhibits protein synthesis but increases levels of activating transcription factor 4 (ATF4).

ATF4 plays a crucial role in regulating gene expression in response to amino acid limitation (Kilberg et al., 2005). During amino acid insufficiency in cells, ATF4 is activated through phosphorylation and translation. Once activated, ATF4 binds to specific DNA sequences in the promotor regions of target genes and activates their transcription (Kilberg et al., 2005). Genes regulated by ATF4 include those involved in extracellular amino acid transport and metabolism, where activation leads to a general reduction in translation, but simultaneously an upregulation in amino acid transporter transcription (Bröer & Bröer, 2017). Thus, increases the de novo synthesis of nonessential amino acids and uptake capacity of both nonessential and essential amino acids, therefore counteracting amino acid insufficiency (Malmberg & Adams, 2008).

Overall, the activation of ATF4 is a key mechanism by which cells respond to amino acid insufficiency to maintain viability in challenging conditions (Chen et al., 2014; Kilberg et al., 2005). As shown by Chen et al. (2014), where glutamine starvation led to the activation of the GAAC pathway and consequently increased intracellular amino acids through increased levels of amino acid transporters. In summary, the GAAC pathway activates genes to counteract amino acid deficiencies and restore homeostasis as shown in Figure 2.3 (Malmberg & Adams, 2008).

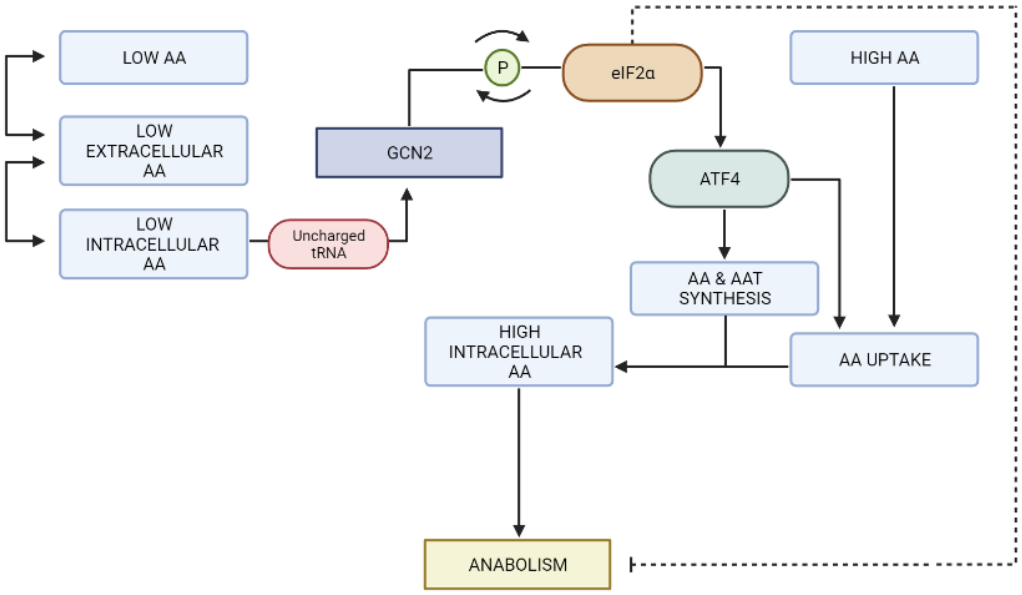


Figure 2.3: Simplified overview of the GAAC pathway following amino acid deficiency. AA = amino acids. AAT = amino acid transporter. P = phosphorylation. Figure is created with biorender.com and inspired by multiple articles (Kilberg et al., 2005; Malmberg & Adams, 2008).

2.2.4 Amino acid transporters

In skeletal muscles, amino acid transporter's main function is to transport amino acids from interstitial fluid to muscle cells cytoplasm and intracellular organelles (Roberson et al., 2020). The availability of amino acids has shown to regulate MPS through mTORC1 stimulation, where essential amino acids mainly are responsible for amino acid-induced mTORC1 activation (Laplante & Sabatini, 2009; Volpi et al., 2003). Additionally, Nicklin et al. (2009) showed that transport of the essential amino acid leucine is required for stimulation of mTORC1 and regulated by a glutamine-dependent mechanism. This finding indicates that mTORC1 activation may be dependent on intracellular glutamine levels. The amino acid transporters CD98 and SLC38A9 will be presented and analysed in this thesis.

2.2.5 CD98

Amino acid transporters solute carrier family 3 member 2 (SLC3A2) also known as CD98, forms a functional heterodimer with some of the members of solute carrier family 7, including solute carrier family 7 member 5 (SLC7A5), also known as LAT1

(Console et al., 2022). SLC7A5/SLC3A2 forms a bidirectional transporter that regulates the simultaneous transport of glutamine out of cells and the transport of leucine into cells (Console et al., 2022; Roberson et al., 2020). Amino acid transporter solute carrier family 38 member 2 (SLC38A2) also known as SNAT2, transports glutamine into the cell (Roberson et al., 2020). Therefore, CD98, LAT1, and SNAT2 contribute to the regulation of intracellular leucine and glutamine levels. Taken together with findings in Nicklin et al. (2009), the results demonstrate the importance of amino acid transporters in the regulation of MPS through amino acid availability.

2.2.6 SLC38A9

Amino acid transporters solute carrier family 36 member 1 (SLC36A1), also known as PAT1, and solute carrier family 38 member 9 (SLC38A9) are located within the lysosomal membrane, and plausibly function as sensors for lysosomal amino acids and mTORC1 translocation to the lysosomal membrane to increase mTORC1 activation (Roberson et al., 2020; Wang et al., 2021).

SLC38A9 is a lysosomal amino acid transporter for glutamine, arginine, and leucine. Additionally, SLC38A9 functions as an upstream regulator for the Ragulator-Rag GTPases complex, which is modulated by amino acids, whereas PAT1 plays a key role in the translocation of mTORC1 (Wang et al., 2021). Amino acid accumulation in lysosome may start signalling to Ragulator-Rag GTPase complex and regulate recruitment of mTORC1 to lysosome, and therefore impact mTORC1 activation as shown in Figure 2.4 (Wang et al., 2021).

The process where PAT1 recruits mTORC1 to the lysosome membrane includes the transportation of amino acids from the lysosome to the cytosol. Since one of the roles of PAT1 is to transport amino acids from lysosome to cytosol, an overexpression could lead to lower amino acid availability within the lysosome. This may indicate amino acid accumulation in lysosomes as a key factor for recruiting mTORC1 to lysosomes (Dickinson & Rasmussen, 2013; Zoncu et al., 2011). Vacuolar H+ ATPase is localized on the lysosome membrane, as shown in Figure 2.4. By amino acid accumulation in the lysosome, Vacuolar H+ ATPase may stimulate mTORC1 through interaction with the Ragulator-Rag GTPase complex (Dickinson & Rasmussen, 2013; Zoncu et al., 2011).

In summary, these findings demonstrate the ability for amino acids to stimulate protein synthesis through mTORC1 translocation and activation, and the importance of amino acid transporters in their regulatory role regarding intracellular amino acid levels and potential function as sensing mechanisms (Console et al., 2022; Dickinson & Rasmussen, 2013; Roberson et al., 2020; Zoncu et al., 2011).

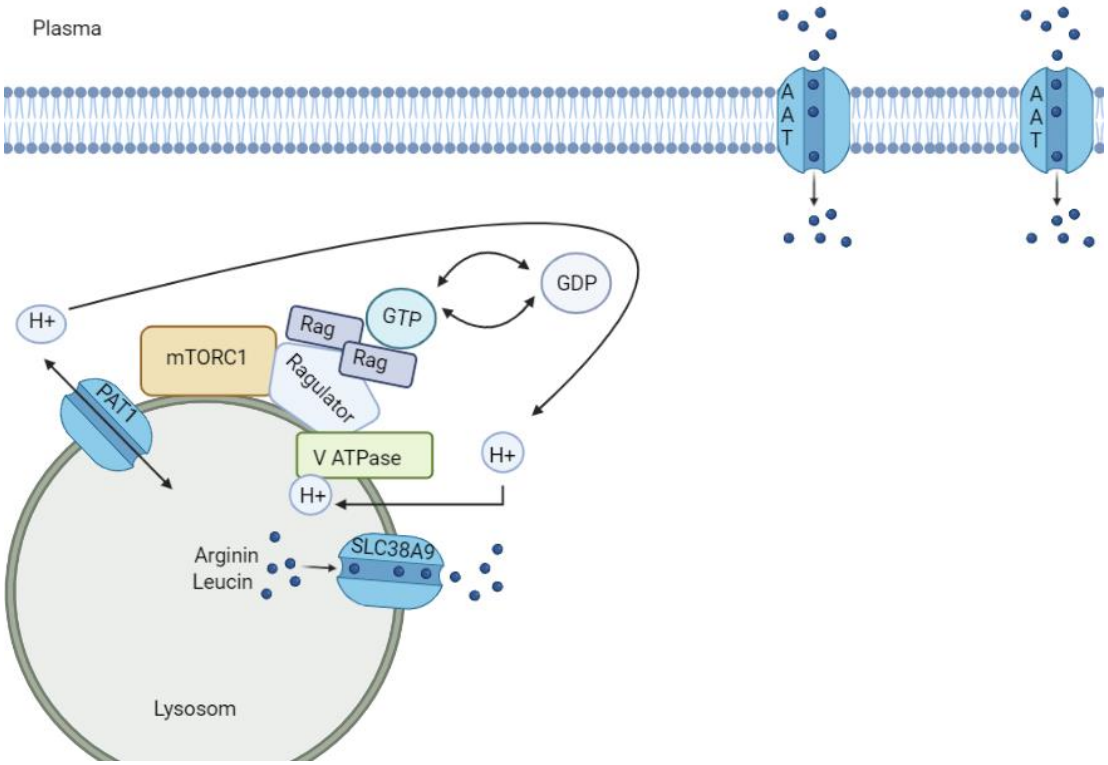


Figure 2.4: Simplified production of amino acid accumulation in lysosome, following signalling to Ragulator-Rag GTPase complex and recruitment of mTORC1 to the surface of lysosome. Figure is created with biorender.com and inspired by multiple articles (Roberson et al., 2020; Wang et al., 2021; Zoncu et al., 2011; Ögmundsdóttir et al., 2012).

2.3 Effect of resistance exercise and protein supplementation

Regarding the high adaptability of skeletal muscle in response to exercise and nutrition, and the possible variations in completion of resistance exercise, such as mode, intensity, and duration. Resistance exercise and protein supplementation present the potential to stimulate mTORC1 and regulate MPS through multiple of previously mentioned stimulus (Coffey & Hawley, 2007; Kumar et al., 2009).

2.3.1 Muscle protein synthesis and the effect of resistance exercise and protein supplementation

Baar og Esser (1999) showed a strong correlation between activation of p70S6K1 and increased muscle mass following resistance exercise. Thus, demonstrating activation of mTORC1 and following p70S6K1 phosphorylation as a key factor for MPS and resistance exercise-induced hypertrophy. However, amino acid deficiency post-resistance exercise may increase protein degradation and therefore result in a negative protein net balance (Burd et al., 2009). Biolo et al. (1997) investigated the response in muscle protein kinetics by amino acid supplementation at rest and 3h post heavy load resistance exercise. Results demonstrate an increased effect of amino acid supplementation on net muscle protein balance when following resistance exercise (Biolo et al., 1997).

In summary, resistance exercise and amino acid supplementation influence muscle protein synthesis. Response in net protein balance by resistance exercise is dependent on amino acid availability to stay positive. Additionally, the response may be enhanced by amino acid supplementation following resistance exercise (Biolo et al., 1997; Burd et al., 2009; Baar & Esser, 1999).

2.3.2 Amino acid availability and the effect of resistance exercise and protein supplementation

Amino acid transporters have as described earlier a key role in amino acid availability and following regulation of MPS, through amino acid accumulated stimulation of mTORC1 (Wang et al., 2021).

Drummond et al. (2011) investigated the effect of a single resistance exercise session on amino acid transporters, whether age influenced the response to resistance exercise. The findings where an increase in LAT1, SNAT2, and CD98 protein levels in response to

resistance exercise independent of age. Upregulation of amino acid transporters mRNA levels may be an acute regulation in response to resistance exercise to increase amino acid availability and MPS during recovery (Drummond et al., 2011).

Additionally, differences in ATF4 and signal transducer and activator of transcription 3 (STAT3) expression indicate different regulation of amino acid transporters in elderly and young adults. Jones et al. (2009) showed that interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) can increase mRNA and protein levels for SNAT2 through STAT3 in trophoblast cells. In addition, STAT3 showed a greater increase in the elderly when compared with younger adults (Drummond et al., 2011). It may be speculated that the reported increase in amino acid transporters expression to resistance exercise in elderly may be facilitated through a stress-indued response to resistance exercise stimulus (Dickinson & Rasmussen, 2013; Jones et al., 2009). The increase in SNAT2 mRNA levels in Drummond et al. (2011) was only significant in the elderly group, which supports indications for an alternative regulating mechanism in amino acid transporters in the elderly when seen together with the increase in STAT3.

Drummond et al. (2012) investigated the effect of seven days bed rest on essential amino acid induced MPS, mTORC1 signalling, and amino acid transporters mRNA and protein expression. Results show an increase in LAT1 and SNAT2 protein levels as a response to amino acid supplementation before the intervention, but the increase was eliminated following seven days of bed rest (Drummond et al., 2012). Additionally, an amino acid-induced increase in ATF4 was also eliminated following the intervention. In contrast, amino acid-induced increase in amino acid transporters mRNA levels was not affected (Drummond et al., 2012). In summary, seven days of bed rest impairs MPS, mTORC1 signalling, and amino acid transporter protein levels in acute response to essential amino acid supplementation in elderly (Drummond et al., 2012).

Taken together, findings in Drummond et al. (2011) and Drummond et al. (2012) indicate that amino acid transporters are regulated based on various stimuli, such as physical activity and amino acid availability. Additionally, the maintained increase in amino acid transporters mRNA levels and eliminated increase in ATF4 supports indications for an alternative regulative mechanism for amino acid transporters in elderly individuals (Drummond et al., 2012).

Dickinson et al. (2014) investigated the effect of increased leucine supplementation during the first 24h of recovery following resistance exercise session in MPS, mTORC1 signalling, and amino acid transporters mRNA expression. Findings show that supplementation with increased leucine content may prolong the anabolic effect following resistance exercise in elderly individuals. It was observed a rapid and maintained increase in mRNA levels for LAT1, CD98, and PAT1 for the group with amplified leucine dosage (Dickinson et al., 2014). This indicates that increased leucine supplementation effect amino acid availability, which may lead to amino acid accumulation, and following mTORC1 stimulation. As previously mentioned, mTORC1 can regulate protein translation through ribosomal S6K1, and 4E-BP1 phosphorylation, which may explain the prolonged anabolic effect of resistance exercise observed in Dickinson et al. (2014) (Wang et al., 2021). The observed increase in amino acid transporters mRNA levels can be taken together with findings in Drummond et al. (2011) and Drummond et al. (2012), where a single session of resistance exercise and amino acid supplementation resulted in differences in amino acid transporters mRNA levels.

In summary, findings indicate amino acid transporters as adaptable mechanisms, which are regulated based on various stimuli such as resistance exercise and protein supplementation. Additionally, ample dosage of essential amino acid supplementation may prolong amino acid availability (Dickinson et al., 2014; Drummond et al., 2012; Drummond et al., 2011).

2.4 Testosterone

Testosterone is known as the male sex hormone and is mainly produced in the Leydig cells of the testicles. Testosterone is regulated through the production and release of Gonadotropin-realising hormone (GnRH) by the hypothalamus (Horstman et al., 2012). This stimulates the production and release of luteinizing hormone, which enters circulation and activates the synthesis and secretion of testosterone in the testicles (Horstman et al., 2012).

In summary, testosterone can be viewed as a central hormone for health in men, due to its biological effect on sexual function, bone density, muscle mass, and function (Barone et al., 2022; Matsumoto et al., 2008; Shin et al., 2018). A decline in testosterone levels is normally observed in elderly males, and is associated with lower production in the testicles, genetic factors, and disease (Barone et al., 2022; Shin et al., 2018).

2.4.1 Muscle protein synthesis and the effect of testosterone levels

Increased testosterone levels have been shown to result in increased stimulation of MPS and the inhibition of protein degradation by activation of PI3K (Dubois et al., 2012). Basualto-Alarcón et al. (2013) showed that testosterone may influence MPS through the PKB/mTORC1/p70S6K signalling pathway, or by binding to androgen receptors as shown in Figure 2.1. These findings indicate that testosterone levels affect anabolic signalling pathways, and potentially regulate the anabolic response to various stimuli.

2.4.2 Amino acid transporters and the effect of testosterone levels

The impact by ADT, and consequently testosterone availability equal to castrated levels, have shown to amino acid availability in cancer cells, by modulating the expression of LAT1 and LAT3 (Zhao et al., 2023). A reduction in AR activity has been observed to reduce the expression of LAT3, and consequently resulting in a decline in the availability of leucine. This reduction in leucine availability, may subsequently trigger an upregulation of LAT1 expression (Zhao et al., 2023).

Haren et al. (2011) investigated the effect of castration and testosterone replacement on differences in gene transcription in *m. gastrocnemius* in mice over 14 days. The purpose of the study was to investigate responses in transcription following castration and

testosterone replacement before measurable changes in muscle mass. The results show that testosterone levels affected mRNA expression for SNAT2, where access to testosterone increased mRNA levels (Haren et al., 2011). The findings indicate that testosterone levels may influence amino acid sensitivity in skeletal muscle, since SNAT2 transports amino acids in and out of the muscle and has been shown as an effective activator for mTORC1 (Wang et al., 2021).

In contrast, Ferrando et al. (2003) showed a reduction in amino acid-induced MPS by increased testosterone levels, despite an increase in lean mass and amount of phenylalanine when compared with placebo. Therefore, findings indicate that testosterone levels mainly contribute to hypertrophy by inhibition of protein degradation (Ferrando et al., 2003).

To the best of authors knowledge, Haren et al. (2011) is the only study to investigate the effect of testosterone levels in amino acid transporters, which represents a need for future research on the subject.

2.5 Summary

In summary, prostate cancer patients undergoing ADT seem to experience a blunted anabolic response to resistance exercise (Alberga et al., 2012; Houben et al., 2023; Newton et al., 2019; Nilsen et al., 2015). Additionally, findings in Houben et al. (2023) show an increase in fat mass and fat percentage for participants receiving protein supplementation. Taken together with results reported by Haren et al. (2011) considering testosterone levels influence in SNAT2, findings suggest a potential limitation in the handling of amino acids in the absence of testosterone. Thus, suggesting a reduced amino acid sensitivity and availability for prostate cancer patients receiving ADT. Amino acids have the ability to influence MPS through mTORC1 stimulation, and may therefore serve as a key factor in the regulation of muscle mass (Laplante & Sabatini, 2009). Reduced ability in the handling of amino acids may therefore influence the blunted anabolic response to resistance exercise observed in prostate cancer patients undergoing ADT.

3. Method

The data used in this master thesis was collected as a part of the project PROST100 which was conducted at the Institute of Physical Performance at the Norwegian School of Sports Sciences (NSSS).

3.1 Ethical considerations

This investigation followed ethical guidelines and was approved by the Regional ethical committee (REK) (REK #2016/640) and the Norwegian Medicines Agency (EUDRACT NO. 2016-005209-38).

3.2 Recruitment and inclusion

The participants were recruited through the urological department at AHUS, Oslo University Hospital (OUS), the Prostate cancer association (PROFO), and local media. To be a part of the study, potential participants had to fulfil the inclusion criteria, presented in Table 3.1. They were excluded according to the exclusion criteria.

Table 3.1: Schematic overview of inclusion- and exclusion criteria for participation in PROST100. ADT = Androgen deprivation therapy.

Inclusion criteria
• ADT group: Treated with ADT. • Control group: Not treated with ADT. • Age: <75 years. • Capable reading and writing capabilities in Norwegian. • Received and accepted informed consent. • Approved participation by an oncologist.
Exclusion criteria
• Regularly strength training (1 session per week for the last 6 months). • Uses medication for osteoporosis. • Use of Warfarin (Marevan). • Not recommended to quit the use of acetylsalicylic acid. Contradictions regarding exercise without adaption: • Unregulated hypertension (>160/95 mmHg). • Uncontrolled congestive heart failure (NYHA class >2). • Unstable angina pectoris. • Heart attack during last 6 months. • Cardio arrhythmia • Chronic obstructive lung disease. • Sincere asthma. • Epilepsy. • Insulin-addicted diabetes mellitus. • Skeleton metastases. Mental contradictions: • Sincere anxiety or depression. • Dementia. • Alcohol- or other abuse. • Mentally developmentally disabled. Relationships that influence the ability to complete workouts: • Uncontrolled pain. • Severe degree of osteoarthritis. • Planned for knee- or hip prosthetic surgery. • Amputation.

3.3 Participants

The participants in PROST100 were prostate cancer patients treated with either ADT or surgery. Participants who underwent surgery must not have been treated with hormonal therapy at any point to participate in the study. In total PROST100 included twenty-four participants, where twenty of these were included in this study. Due to low weight in collected muscle biopsies, four participants could not be included for fractionation and were therefore excluded from further analysis in this study. For the remainder of this study, only participants included will be presented further.

3.3.1 Participant characteristics

There were ten participants in both the ADT group and the surgery (OPR) group for this study. There was significant a difference in fat percentage (p=0.04) and duration from diagnosis (p=0.007) between the groups. There was no significant difference in age, body weight, height, lean body mass, fat-free mass, or total fat mass between groups as displayed in Table 3.2.

Table 3.2: Schematic overview of participant characteristics.

	ADT **(n=10)**	OPR **(n=10)**	Difference between groups
Age (years)	65.1 (±8.9)	65.6 (± 6.0)	(p=0.25)
Body weight (kg)	85.4 (±12.0)	82.1 (±12.5)	(p=0.56)
Height (cm)	176.2 (±3.8)	177.6 (±6.4)	(p=0.54)
Body mass index	27.4 (±3.4)	26.1 (±4.6)	(p=0.47)
Lean body mass (kg)	54.5 (±4.7)	57.0 (±6.6)	(p=0.34)
Fat-free mass (kg)	57.3 (±4.77)	60.2 (±6.8)	(p=0.30)
Fat-percentage (%)	*33.6 (±4.8)	27.5 (±7.0)	(p=0.04)
Total fat mass (kg)	28.2 (±7.8)	22.3 (±8.8)	(p=0.13)
Time from diagnosis (months)	34.6 (±44.9)	**99.9 (±44.5)	(p=0.007)
ADT duration (months)	19.1 (±18.8)		

3.4 Study design

The project PROST100 was designed as an acute, controlled cross-sectional study to investigate the response in muscle protein synthesis, plasma glucose, and insulin following supplementation and supplementation combined with resistance exercise in prostate cancer patients either treated with Zoladex (ADT) or surgery. The participants met at NIH on four occasions: one day for accustoming, two workouts, and an acute day for data collection as shown in Figure 3.1.

Participation in PROST100 included multiple tests and measurements, including the collection of different biological materials. The body composition of participants was measured by weight of lean mass, fat mass, and bone mass. Muscle strength in *m. Quadriceps femoris* was measured in different ways. Static muscle strength was measured in the exercise knee extension, and dynamic muscle strength was measured in the exercises leg press, chest press, and seated row. Blood sampling was conducted as part of the project, using a venous catheter to minimize the number of pricks. In PROST100 there was planned muscle biopsy collection on 3 occasions during the Acute day (Day 4), providing a total of 5 samples. In addition to physical tests and biological sampling, participants completed a questionnaire and 3 supervised resistance exercise sessions. Further on only methods and analyses relevant for this master thesis will be described.

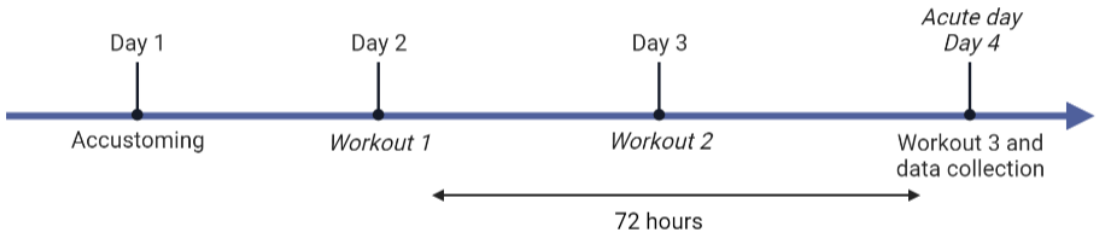


Figure 3.1: Overview of PROST100 study design. Time lapse from Accustoming (Day 1) to Workout 1 (Day 2) vary from 3 till 30 days. Time lapse from Workout 1 (Day 2) to Acute day (Day 4) is within 72 hours. Figure is created with biorender.com.

3.4.1 Protocol

The Accustoming (Day 1) was used as an introduction for the participants to test the procedures used in the PROST100 study. The participants first signed the declaration of consent, then completed Godin Exercise Lesure-Time Questionnaire containing background information including diet and physical habits. Following a dual x-ray absorptiometry (DXA) scan, maximal voluntary contraction (MVC) and 1 repetition maximum (1RM) in one-legged leg press, one-legged leg extension, chest press, and seated row were completed. To standardize the procedure participants only trained their right leg during the intervention, and the left leg was used for baseline measurements.

A general warm-up for the participants was completed prior to the MVC and 1RM tests, containing 10 minutes of cycling on an ergometer bike. In addition, a specific warm-up was completed by series of 10, 6, 3 and finally 1 repetition with increased load until failure was reached. Results from 1RM were used to calculate the load used by participants in Workout 1, 2, and 3. Workout 1 (Day 2) completion varied from 3 till 30 days following Accustoming (Day 1) at NIH.

A general warm-up prior to workouts was completed as previously described, and a specific warm-up contained 2 series of increased load in each exercise. Exercises, series, repetitions, load, and perceived exertion are demonstrated in Table 3.2. In addition to the completion of the workout, the participants were given an individualized dose of deuterium oxide during Workout 1 (Day 2). Workout 2 (Day 3) and Workout 3 (Day 4) were completed in the same manner and within 48 hours of Workout 1 (Day 2), except for the supplementation of a dose of deuterium oxide.

Table 3.3: Overview of content in workouts, including exercises, series, repetitions, load, and rate of perceived exertion. WU: warm-up, OL: one-legged, KG: kilograms, RPE: Rate of perceived exertion, rep: repetitions, 1RM: one repetition maximum.

Exercises	**WU 1: 10 rep**		**WU 2: 10 rep**		**1 set: 10 rep 70% 1RM**		**2 set: 10 rep 70% 1RM**		**3 set: 10 rep 70% 1RM**	
	KG	RPE	KG	RPE	KG	RPE	KG	RPE	KG	RPE
OL Leg Press										
OL Leg Extension										
Chest Press										
Seated Row										

The participants arrived in a fasted state at NIH at 08:00 on the Acute day (Day 4). The first muscle biopsy was conducted at 08:30 in the not trained leg before a standardized meal was given. The second muscle biopsies were conducted in both legs, 2 hours after completion of Meal 1. At 11:30 Workout 3 was completed in the same manner as previous workouts, with the exception that the load was increased to 10RM for each series in each exercise. In addition, MVCs were completed pre and post-Workout 3 to objectively measure fatigue and physical exertion by the workout. After Workout 3, the participants were given another standardized meal. The final muscle biopsies were conducted in both legs, 2 hours after completion of Meal 2. An overview of timeline and content of Acute day (Day 4) is shown in Figure 3.2.

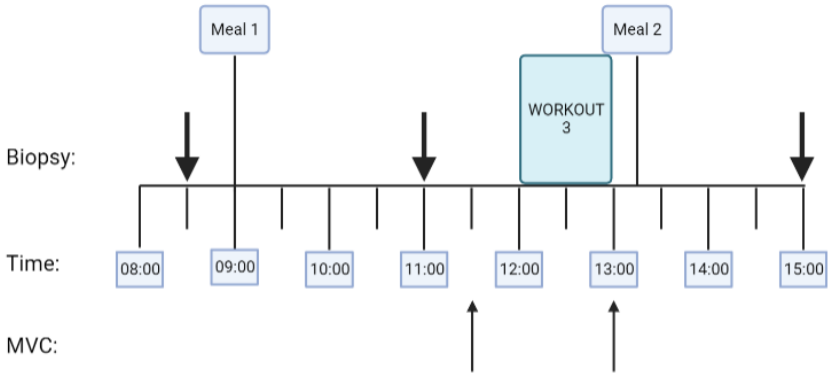


Figure 3.2: Overview of timeline and content in Acute day (Day 4). Figure is created with biorender.com.

3.5 Measurements

3.5.1 Supplement

The participants were given two standardized meals throughout the Acute day (Day 4). The first meal was given in a fasted state, and the second post Workout 3. The standardized meals consisted of breakfast cereal containing a high glycaemic index and a protein shake. Nutrients in both meals were calculated by the body weight of each participant to standardize supplementation given to each participant and contained 1.0g carbohydrate and 0.3g protein per kilogram bodyweight.

3.5.2 DXA

The DXA-scan was used to measure the body composition of the participants (Lunar IDXA, GE Healthcare, Madison, USA). Body composition was used to compare anthropometrical data between groups and in calculations of nutrients in standardized meals. Additionally, lean body mass (LBM) was used to calculate the of dose deuterium oxide given at Workout 1 (Day 2). Deuterium oxide was used to measure the speed of muscle protein synthesis.

3.5.3 MVC

The MVC was conducted by a maximal isometric contraction in *m. Quadriceps femoris* on the trained leg, using a knee extension apparatus (Gym2000) connected to a power cell (HBM U2AC2, Darmstadt, Germany). Power outputs were registered by the connected power cell and analysed using Labview 2017 (Labview 8.2, National Instr., Austin, Texas).

The specific warmup for the MVC contained isometric contractions of 50%, 70%, and 90% of maximal effort in the trained leg. Participants were instructed to create and hold maximal effort for 2-3 seconds at each attempt, and restitution between attempts varied from 30-60 seconds. The test was completed when a participant created two similar power outputs from different attempts, usually, this varied from 3-5 attempts. The purpose of the MVC was to independently control for fatigue and physical exertion by Workout 3 on the Acute day (Day 4), given a decline in power output from pre to post indicated fatigue.

3.5.4 Muscle biopsy

A total of five muscle biopsies from three timepoints were collected during the Acute day (Day 4), as shown in Figure 3.2. Muscle biopsies were collected from *m. Vastus lateralis*. To avoid tissue collecting from previous areas, both proximal and distal entrance was performed in the study.

Before muscle biopsy, local anaesthesia was set in the skin and connective tissue before disinfection of concerning area. Further on, a small incision was made in the skin and muscle fascia before muscle tissue was collected using a Bergstrom technique. Biopsies were performed using a biopsy needle connected to a vacuum pump. As the needle is in place, the vacuum will pull tissue inside the needle which is sliced off. Finally, collected muscle tissue is dissected, frozen in liquid nitrogen, and stored at negative 80 degrees Celsius.

3.6 Analyses

3.6.1 Fractionation

Fractionation of collected muscle biopsies was conducted using ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Cat. No.: 539790). The kit contained Extraction Buffer I, II, III, IV, Benzonase, and PINC, and was designed for subcellular extraction of proteins from cytosolic, membrane and organelles, core and cytoskeletal fractions in frozen pellets, cell culture, and other tissue. The four detergent based extraction buffers have increasing ionic strength for extraction of the four subcellular fractions cytosolic, membrane, nuclear, and cytoskeletal. In preparation for the procedure, tubes are marked in advance, and the weight of collected biopsies is controlled.

For the cytosolic fraction, there was added 1,0 ml Extraction Buffer I and 5 µl PINC before the sample was homogenized for 3 x 5 seconds. Homogenized samples were placed on ice, until every sample was homogenized and placed in Multi-Vortex V-32 (Grant-bio) for 10 minutes at 4 degrees Celsius and centrifuged at 1000G for 10 minutes at 4 degrees Celsius. The supernatant was then transferred to marked 1.5ml tubes, and the rest pellet was placed on ice. The supernatant was aliquoted by 25 ql in marked 0,2 ml tubes and placed in the freezer.

For the membrane fraction there was added 1,0 ml Extraction Buffer II and 5 µl PINC to the tube containing the rest pellet. The tube was then vortexed to resolve the pellet and placed in Multi-Vortex V-32 for 30 minutes at 4 degrees Celsius. The samples were then centrifuged at 6000G for 10 minutes at 4 degrees Celsius. The samples were then transferred, aliquoted, and frozen in the same manner as previously. For the nuclear fraction there was added 0,5 ml Extraction Buffer III, 5µl PINC, and 1,5µl Benzonase to the tube containing the rest pellet, which was vortexed to resolve the pellet. Following, the samples were placed in Multi-Vortex V-32 for 10 minutes at 4 degrees Celsius, before they were centrifuged by 7000G for 10 minutes at 4 degrees Celsius. The supernatant was then transferred, aliquoted, and frozen in the same manner as previously.

Finally, the tube containing the rest pellet was added 0,5 ml Extraction Buffer IV and 5µl PINC for the cytoskeletal fraction, and vortexed to resolve the pellet. To

homogenize the sample, samples were placed on a heating block for 5 minutes at 90 degrees Celsius and vortexed again. Samples were then centrifuged at 10 000G for 10 minutes at 20 degrees Celsius. The supernatant was then transferred to marked 1,5 ml tubes and stored at room temperature, before they were aliquoted, and frozen in the same manner as previously. Samples were distributed in bags, placed in marked boxes, and stored in a freezer at negative 80 degrees Celsius.

3.6.2 Total protein

To standardize protein concentrations in sample preparation for the Western blot analysis, total protein analysis was conducted on each fraction in each sample. The DC protein method is a colorimetric analysis to measure protein concentration in each sample. Bovine Gamma Globulin (Bio-Rad, USA) 0.125, 0.25, 0.5, 1.0, and 1.5 were used as standard proteins. Samples consisted of cytosol, membrane, cytoskeletal, and nuclear fractions. Cytoskeletal fractions were diluted 1:6 with dH_2O to get a similar protein concentration as the standard proteins, the remaining fractions were pipetted as they were.

Type 1 water (dH_2O), protein standards, samples, and control were pipetted in triplets of 5 µl on a 96-well microplate (Greiner Bio-One International AG, Kremsmünster, Germany). Following, 25 µl reagent A* (4 ml DC Reagent A, 500-0113 + 80µl DC Reagent S, 500-0115), and 200µl reagent B (DC Reagent B, 500-0114) were added to each well. The microplate was then covered with a plastic film and incubated for 15 minutes in a dark location. After incubation, the plastic film was removed, and the back of the microplate was wiped using a precision cloth (Kimtech). Analysis was then conducted using a 690 nm of FC Multiscan™ (Thermo Scientific™). Protein concentrations were calculated by Thermo Scientific™ SkanIt™ software (version 1.01.16) and the accepted CV% deviation was set to <10% between sample triplets.

3.6.3 Western blot

Western Blot analysis was conducted on the nuclear, cytosolic, and membrane fractions for each fractionated sample. The amount of protein in both nuclear and cytosolic fraction for each well was set to 6 µg, giving a concentration of 0,4 µg/µl for each sample. For the membrane fraction, the protein amount for each well was set to 3,9 µg,

giving a concentration of 0,26 µg/µl for each sample. Fractions were usually run independently of one another given chosen proteins for the fraction.

In sample preparation, there was added sample buffer containing 4x Laemmli Sample Buffer (Cat. #161-0747, BioRad), dithiothreitol (DTT) (Cat. #161-0611, BioRad), and dH_2O water based on the individual protein concentration of each sample examined in total protein analysis. Samples were then vortexed and centrifuged before being placed on a heating block at 70 degrees Celsius for 10 minutes. Following, samples were pipetted in 15-well gels (Mini-Protean Stain-Free Gels, Cat. #4568096, Biorad). Gels were set up as duplicates for each run, usually containing five samples from two participants and two control samples. First, 5µl of weight marker (Precision Plus Protein Standards, All Blue, Cat. #161-0373, BioRad) was pipetted in the two outermost wells. Next, 15µl of sample or control sample were pipetted in the remaining wells. Control samples were either pipetted in wells 6 and 7, or for the duplicate gels in wells 6 and 13 to compare independent gels to one another. Electrophoreses were set at 200V for 34 minutes (PowerPac HC High-Current Power Supply, BioRad) before the gels were activated for 2,5 minutes using Chemidoc MP Imaging System (BioRad) stain-free gel program.

PVDF-membranes (Trans-Blot Turbo Transfer System RTA Transfer Kits 1704272, BioRad) was activated by methanol (1.06007.2500, Merck KGaA) for 30 seconds and washed twice for 30 seconds in dH_2O. Finally, membranes and transfer stacks were put in the transfer buffer for 5 minutes, respectively. Before blotting transfer stacks, membranes and gels were stacked and rolled over to remove potential air bubbles within. Blotting was conducted using 7 minutes Mixed program by Trans-Blot Turbo Transfer System (BioRad). Upon completion, a stain-free blot picture was taken of the membranes using the ChemiDoc MP Imaging System (BioRad), which is used for the normalization of total protein.

Due to different blocking solutions used in nuclear and cytosolic fractions, membranes were cut based on the molecular weight of chosen proteins before the blocking procedure. For the nuclear and cytosolic fractions, membranes were cut just under 75 kDa, in the middle of 50 kDa and 37 kDa, and at 25 kDa, respectively. Regarding membrane fractions, membranes were cut just under 50 kDa and at 25 kDa,

respectively. Membranes were so put in a blocking solution for 2 hours at room temperature. For CD98, eIF2α, and Rheb a 5% skimmed milk solution mixed with TBS-T (Tris-buffered saline, 10x TBS, Cat. #170-6435, Biorad & Tween 20, 437082Q, VWR) was used, and for ATF4 a 1:5 AFB (Animal-Free Blocking Solution, Cat. #15019L, Cell Signalling Technology) solution mixed with dH_2O water was used. For SLC38A9 a 3% BSA solution mixed with TBS-T was used.

After the blocking procedure membranes were washed and put in incubation tubes containing primary antibodies for chosen proteins. Incubation was conducted on a roller mixer overnight at 4 degrees Celsius. Primary antibody was diluted with a 1% skimmed milk solution for membranes blocked in milk, as AFB for membranes blocked in AFB, and 5% BSA for membranes blocked in BSA. However, A schematic overview of antibodies and dilutions for chosen proteins in this study is presented in Table 3.3.

The following day membranes were washed for 15 minutes in TBS-T and 3 x 5 minutes in TBS before 1h incubation with a secondary antibody regarding the host of the primary antibody, as shown in Table 3.4. Secondary antibodies were diluted with 1% skimmed milk solution for membranes blocked in milk, as 1:5 AFB solution for membranes blocked in AFB, and 1% BSA for membranes blocked in BSA. Upon completion of secondary antibody incubation, the washing procedure from primary antibody incubation was repeated.

Next, membranes were put in a chemiluminescent substrate (SuperSignal, 34076, Thermo Scientific) for 5 minutes, respectively, in a dark location. Finally, Chemi High Sensitivity pictures were taken using ChemiDoc MP Imaging System (Biorad). Further analysis and quantification of pictures were conducted using Image Lab 6.1.0 software. To account for variations in the procedure, the intensity of each protein band was normalized to the total amount of protein in the corresponding well.

Regarding CD98 and SLC38A9 for the membrane fraction, molecular weight between concerning proteins was considered too close for cutting. Therefore, membranes were first incubated and analysed for CD98. Following, membranes were stripped and re-blocked, and re-incubated for primary antibody overnight for SLC38A9. Membranes were put in stripping buffer (Restore™ Western Blot Stripping Buffer, 21059, Thermo

Scientific) for 20 minutes, before being quickly rinsed five times in TBS and washed 3 x 5 minutes in TBS. Following, membranes were re-blocked and re-incubated for primary antibody overnight for SLC38A9. The following day steps were completed in the same manner as described earlier.

Table 3.4: Schematic overview of primary- and secondary antibodies. Primary antibodies and related secondary antibodies used are displayed numerically (1,2,3,4).

Primary antibody	**Producer**	**Host**	**Dilution**	**Lot.nr**	**Cat. #**
eIF2α (1)	Cell Signalling Technology	Rabbit	1 : 1000	9	5324S
ATF4 (2)	Abcam	Goat	1 : 2000	GR3444345-2	1371
CD98 (3)	Sigma-Aldrich	Rabbit	1 : 1000	L6171	1400262
SLC38A9 (4)	Abcam	Rabbit	1 : 250	GR3232524-17	130398-1001
Secondary antibody					
Anti-rabbit IgC HRP-linked antibody (3)	Cell Signalling Technology	Goat	1 : 3000	31	7074S
Anti-rabbit IgC HRP-linked antibody (4)	Cell Signalling Technology	Goat	1 : 1000	31	7074S
Rb pAb to Goat IgC HRP-linked antibody (2)	Abcam	Rabbit	1 : 8000	GR3408788-2	6741
Anti-rabbit IgC HRP-linked antibody (1)	Invitrogen	Goat	1 : 30 000	TK274616	31460

3.7 Statistical analysis

The purpose of this study was to examine the acute anabolic response to protein supplementation and resistance exercise in combination with protein supplementation in amino acid sensitivity and availability in prostate cancer patients treated with surgery or ADT. This was done by investigating total protein expression in eIF2a, ATF4, CD98, and SLC38A9 between baseline, post-protein supplementation and post-resistance exercise in combination with protein supplementation in prostate cancer patients treated with surgery or ADT.

Statistical analysis and calculations were completed using a free trial period in GraphPad Prism 9.5.1 (Graphpad Software Inc., San Diego, CA, USA). Raw data from baseline were tested for normal distribution visually with a Q-Q plot and with the Shapiro-Wilk test prior to further analysis. Additionally, raw data from baseline were tested for lognormal distribution with the Shapiro-Wilk test. Values that failed the normality tests were transformed into lognormal values before further analysis.

To compare difference between groups in 1RM for included exercises and habitual training unpaired t-tests were used. Further on, to compare difference in MVC measured as maximal amplitude from pre- to post-resistance exercise paired t-tests were used.

To compare the difference between groups in protein expression at baseline unpaired t-tests were used. Further analysis to examine difference between groups, untrained leg, and trained leg from baseline to post-protein supplementation, and from post-protein supplementation to post-resistance exercise in combination with protein supplementation Two-way ANOVA analysis were used. Statistical differences were assessed as a p-value at or below 0.05.

4. Results

4.1 Muscle strength and habitual training

There were no significant differences between groups in 1RM strength tests. There was a tendency for difference in time spent in hard/moderate endurance activity (p=0.08) and the OPR group spent more time in light endurance activity than the ADT group (p=0.03).

Table 4.1: Strength measured as 1 repetition maximum (1RM) in two leg exercises and two upper-body exercises, and self-reported physical training given as min per week with endurance and strength training in the group treated with androgen deprivation therapy (ADT) and the group treated with surgery (OPR).

	ADT **(n=10)**	OPR **(n=10)**	Difference between groups
1RM Leg press (kg)	73.0 (±27.5)	76.0 (±21.7)	(p=0.79)
1RM Leg extension (kg)	34.8 (±4.9)	39.0 (±9.5)	(p=0.23)
1RM Chest press (kg)	57.3 (±9.7)	63.3 (±13.8)	(p=0.27)
1RM Seated row (kg)	39.6 (±7.9)	35.8 (±20.1)	(p=0.58)
Hard/Moderate endurance (min)	120 (±104)	249 (±193)	(p=0.08)
Light endurance (min)	81 (±170)	*235 (±124)	(p=0.03)
Resistance exercise (min)	31 (±65)	29 (±57)	(p=0.94)

4.2 MVC

There was a significant decrease in maximal force in the MVC test from pre- to post-resistance exercise in both the OPR group with a 10.7% decrease (p= 0.03; Figure 4.1A), and in the ADT group with a 11% decrease (p=0.004; Figure 4.1B).

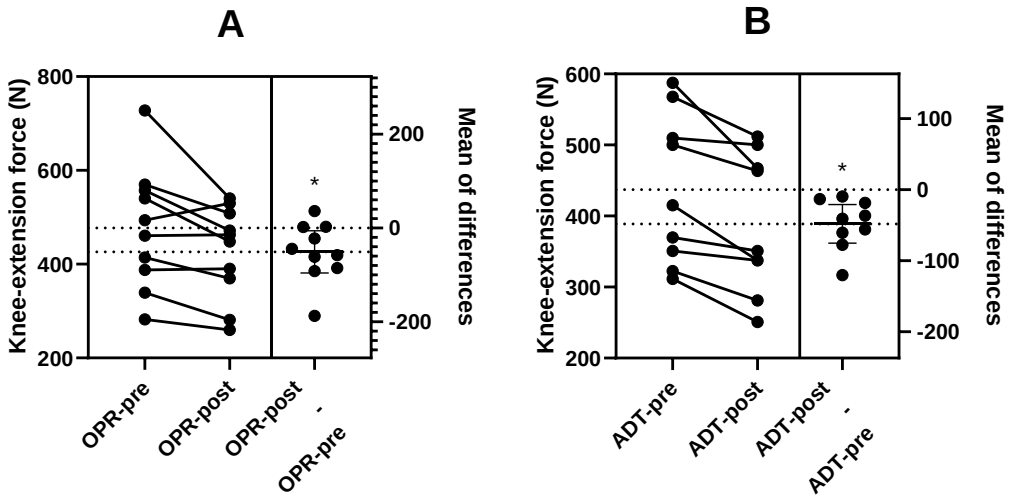


*Figure 4.1: Maximal isometric force in maximal voluntary contractions (MVC) pre- and post-resistance exercise in prostate cancer patients treated with surgery (OPR; A) and androgen deprivation therapy (ADT; B). *=statistically significant difference from pre to post. Results are presented as individual values with mean ± standard deviation for each group.*

4.3 Protein expression

4.3.1 Presentation of representative Western blot bands

Following tables (Table 4.2, Table 4.3, and Table 4.4) present representative Western blot bands analysed for regulators of amino acid transporters (ATF4 and eIF2α) and the two amino acid transporters CD98 and SLC38A9 in the different fractions analysed in the study.

Table 4.2: Representative Western Blot bands for proteins analysed in the nuclear fraction. 1L = First biopsy in the left leg (baseline). 2L = Second biopsy in the left leg (Untrained). 2R = Second biopsy in the right leg (Trained). 3L = Third biopsy in the left leg (Untrained). 3R = Third biopsy in the right leg (Trained).

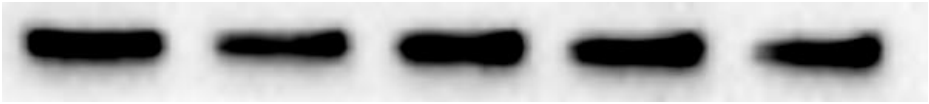
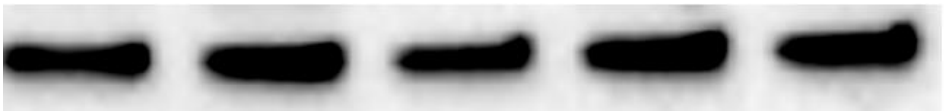
Nuclear			**ADT**		
Timepoint	**1L**	**2L**	**2R**	**3L**	**3R**
eIF2α					
CD98					
ATF4					
Nuclear			**OPR**		
Timepoint	**1L**	**2L**	**2R**	**3L**	**3R**
eIF2α					
CD98					
ATF4					

Table 4.3: Representative Western Blot bands for proteins analysed in the cytosolic fraction. 1L = First biopsy in the left leg (baseline). 2L = Second biopsy in the left leg (Untrained). 2R = Second biopsy in the right leg (Trained). 3L = Third biopsy in the left leg (Untrained). 3R = Third biopsy in the right leg (Trained).

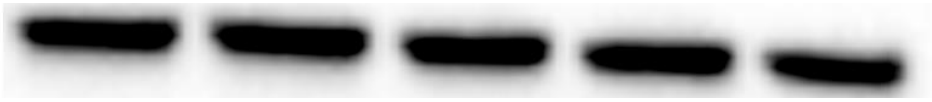
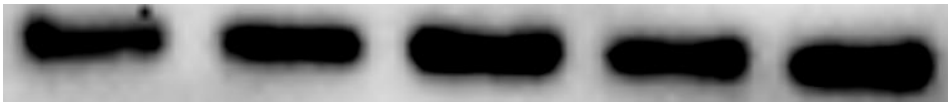
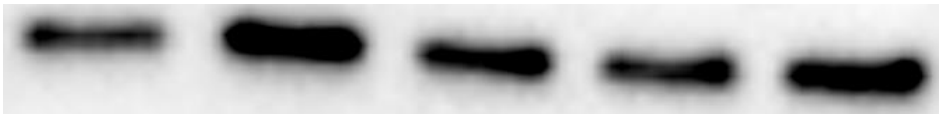
Cytosolic	**ADT**				
Timepoint	**1L**	**2L**	**2R**	**3L**	**3R**
eIF2α					
CD98					
ATF4					
Cytosolic	**OPR**				
Timepoint	**1L**	**2L**	**2R**	**3L**	**3R**
eIF2α					
CD98					
ATF4					

Table 4.4: Representative Western Blot bands for proteins analysed in the membrane fraction. 1L = First biopsy in the left leg (baseline). 2L = Second biopsy in the left leg (Untrained). 2R = Second biopsy in the right leg (Trained). 3L = Third biopsy in the left leg (Untrained). 3R = Third biopsy in the right leg (Trained).

Membrane	**ADT**				
Timepoint	**1L**	**2L**	**2R**	**3L**	**3R**
eIF2α					
SLC38A9					
Membrane	**OPR**				
Timepoint	**1L**	**2L**	**2R**	**3L**	**3R**
eIF2α					
SLC38A9					

4.3.2 eIF2α at baseline

There were no significant differences between groups at baseline in protein expression for eIF2α in the nuclear fraction (p=0.43; Figure 4.2A), cytosolic fraction (p=0.70; Figure 4.2B), or membrane fraction (p=0.61; Figure 4.2C).

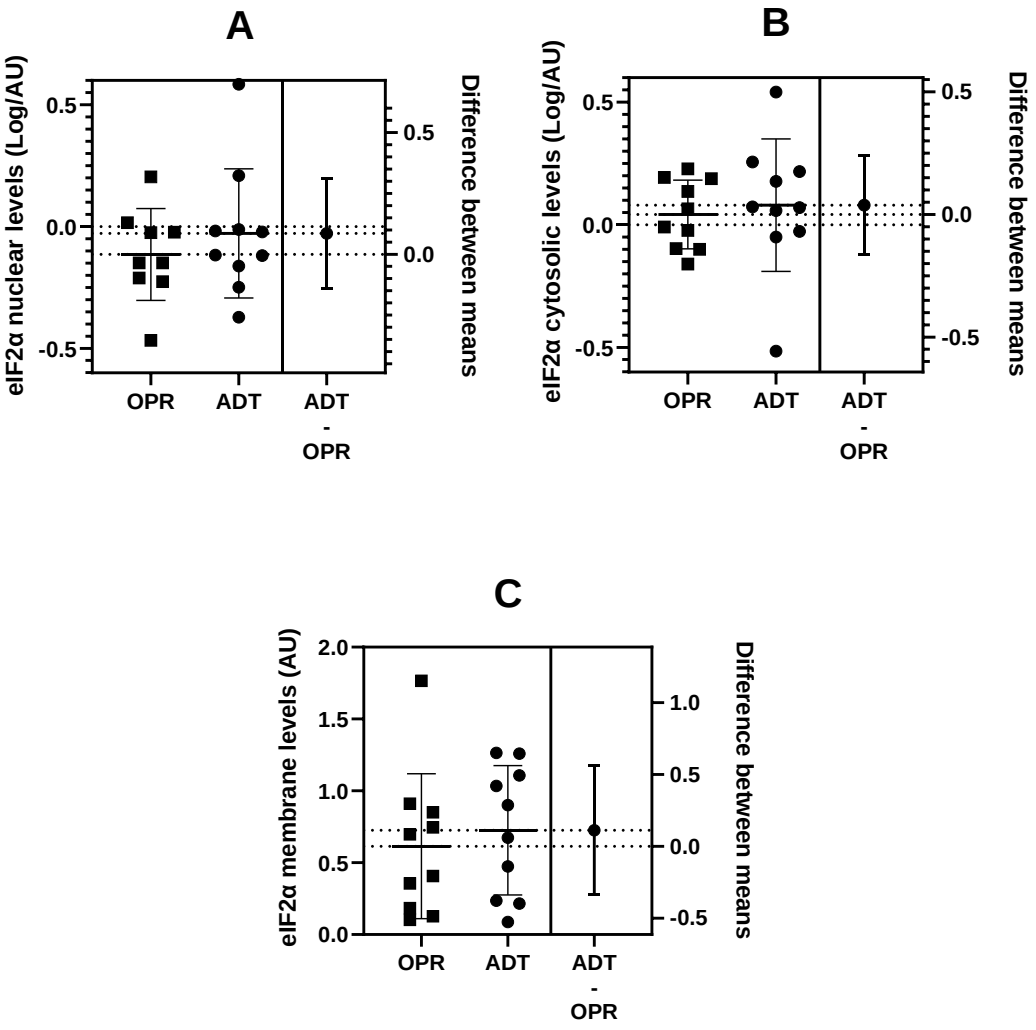


Figure 4.2: Total amount of eIF2α in the nuclear fraction (A), cytosolic fraction (B), and membrane fraction (C) at baseline for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. Results are presented as individual values with mean ± standard deviation for each group.

4.3.3 ATF4 at baseline

There were no significant differences between groups at baseline in protein expression for ATF4 in the nuclear fraction (p=0.29; Figure 4.3A) or in the cytosolic fraction (p=0.13; Figure 4.3B).

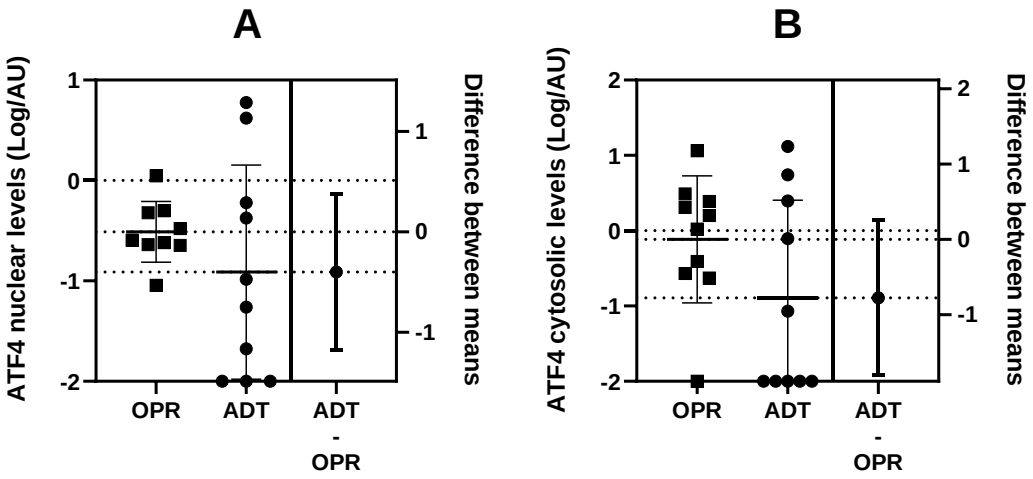


Figure 4.3: Total amount of ATF4 in the nuclear fraction (A) and cytosolic fraction (B) at baseline for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. Results are presented as individual values with mean ± standard deviation for each group.

4.3.4 CD98 at baseline

The expression of CD98 in the nuclear fraction was significantly higher at baseline in the ADT group than in the OPR group (p=0.047; Figure 4.4A). In contrast, in the cytosolic fraction the expression of CD98 was significantly higher at baseline in the OPR group than the ADT group (p=0.03; Figure 4.4B).

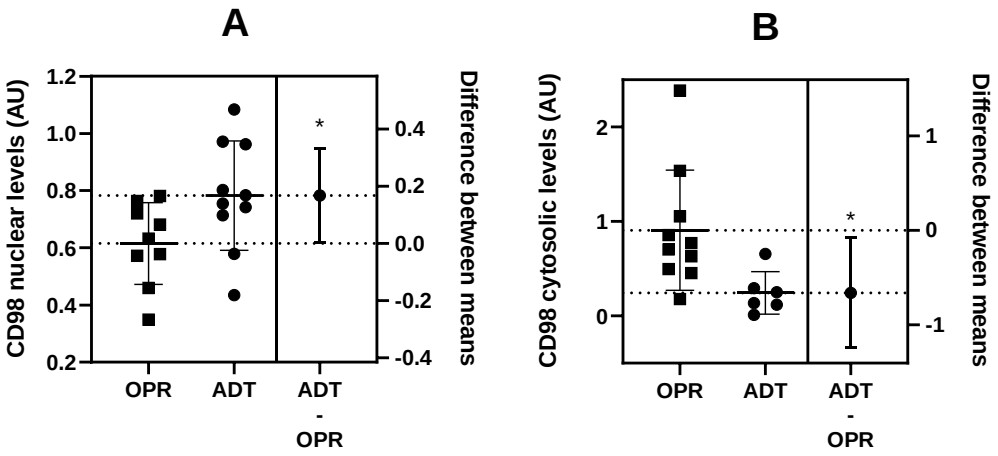


*Figure 4.4: Total amount of CD98 in the nuclear fraction (A) and cytosolic fraction (B) at baseline for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). AU=Arbitrary units. * = significant differences. Results are presented as individual values with mean ± standard deviation for each group.*

4.3.5 SLC38A9 at baseline

There were no significant differences between groups at baseline in protein expression for SLC38A9 in the membrane fraction (p=0.46; Figure 4.5A).

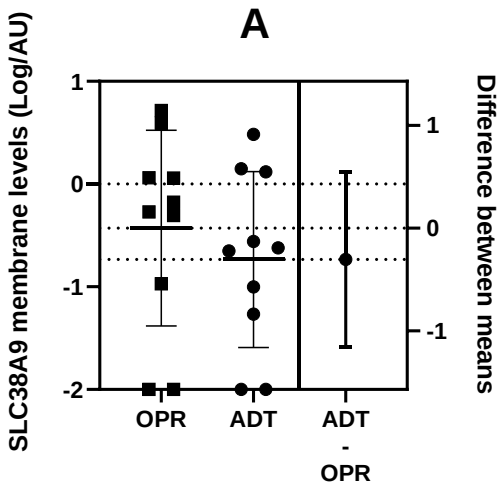


Figure 4.5: Total amount of SLC38A9 in the membrane fraction (A) at baseline for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. Results are presented as individual values with mean ± standard deviation for each group.

4.3.6 Effect of protein supplementation on eIF2α

There were no significant difference between groups when comparing protein expression for eIF2α at baseline with untrained and trained leg post-protein supplementation in the nuclear fraction (p=0.15; Figure 4.6A1, p=0.17; Figure 4.562), cytosolic fraction (p=0.51; Figure 4.6B1, p=0.19; Figure 4.6B2) and membrane fraction (p=0.15; Figure 4.6C1, p=0.87; Figure 4.6C2).

There was no significant difference in protein expression for eIF2α between baseline and untrained leg post-protein supplementation in the nuclear fraction (p=0.31; Figure 4.5A1), cytosolic fraction (p=0.62; Figure 4.6B1) and membrane fraction (p=0.20; Figure 4.6C1). Additionally, there was no significant difference in protein expression for eIF2α between baseline and the trained leg post-protein supplementation in the nuclear fraction (p=0.74; Figure 4.6A2), cytosolic fraction (p=0.30; Figure 4.6B2), and membrane fraction (p=0.85; Figure 4.6C2).

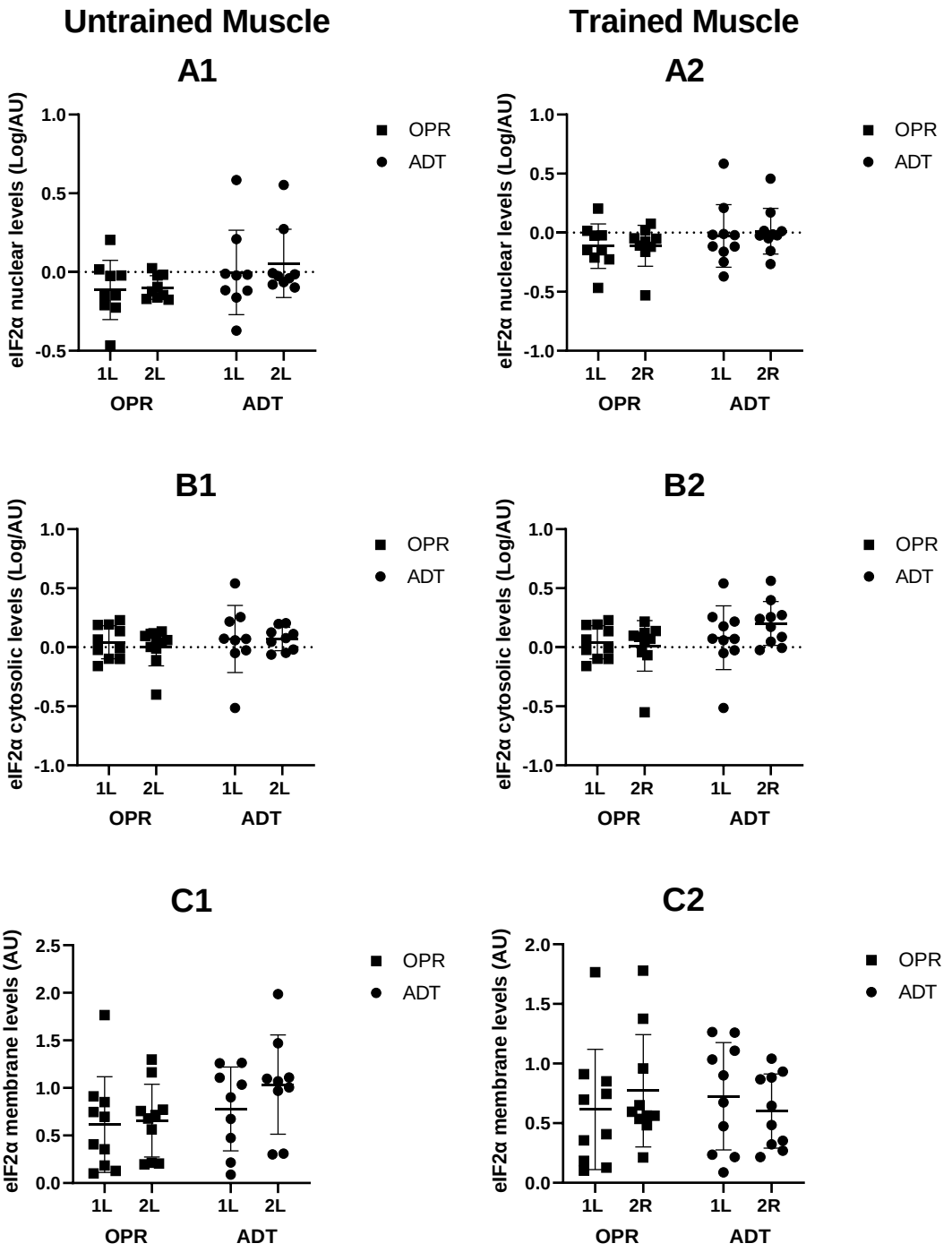


Figure 4.6: Protein expression for eIF2α at baseline and post-protein supplementation in the untrained leg (A1, B1, C1) and the trained leg (A2, B2, C2) in the nuclear fraction (A1, A2), cytosolic fraction (B1, B2), and membrane fraction (C1, C2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. Results are presented as individual values with mean ± standard deviation for each group.

4.3.7 Effect of protein supplementation on ATF4

There were no significant differences between groups when comparing protein expression for ATF4 at baseline with the untrained leg post-protein supplementation in the nuclear fraction (p= 0.19; Figure 4.7A1). There was a tendency for greater protein expression for ATF4 in the OPR group by total variation of 16,4% to the ADT group when comparing groups at baseline with the untrained leg post-protein supplementation in the cytosolic fraction (p= 0.055; Figure 4.7B1).

Additionally, there was a tendency towards a difference between groups when comparing baseline and the trained leg post-protein supplementation in the nuclear fraction (p= 0.08; Figure 4.7A2), where the difference between means (0.66 AU) indicates a greater protein expression for ATF4 in the OPR group post-protein supplementation. There were no significant differences between groups when comparing baseline and trained leg post protein supplementation in the cytosolic fraction (p= 0.11; Figure 4.7B2)

There were significant differences in protein expression for ATF4 between baseline and the untrained leg post-protein supplementation in the nuclear fraction (p= 0.047; Figure 4.7A1). When divided by groups results show no significant difference for ADT (p= 0.80; Figure 4.7A1), but a tendency towards an increase for ATF4 protein expression from baseline to the untrained leg post-protein supplementation for OPR (p= 0.053; Figure 4.7A1). There was no significant difference when comparing protein expression for ATF4 at baseline with the untrained leg post-protein supplementation in the cytosolic fraction (p= 0.17; Figure 4.7B1).

There were no significant differences in protein expression for ATF4 when comparing baseline with the trained leg post-protein supplementation in the nuclear fraction (p= 0.88; Figure 4.7A2), and cytosolic fraction (p= 0.68; Figure 4.7B2).

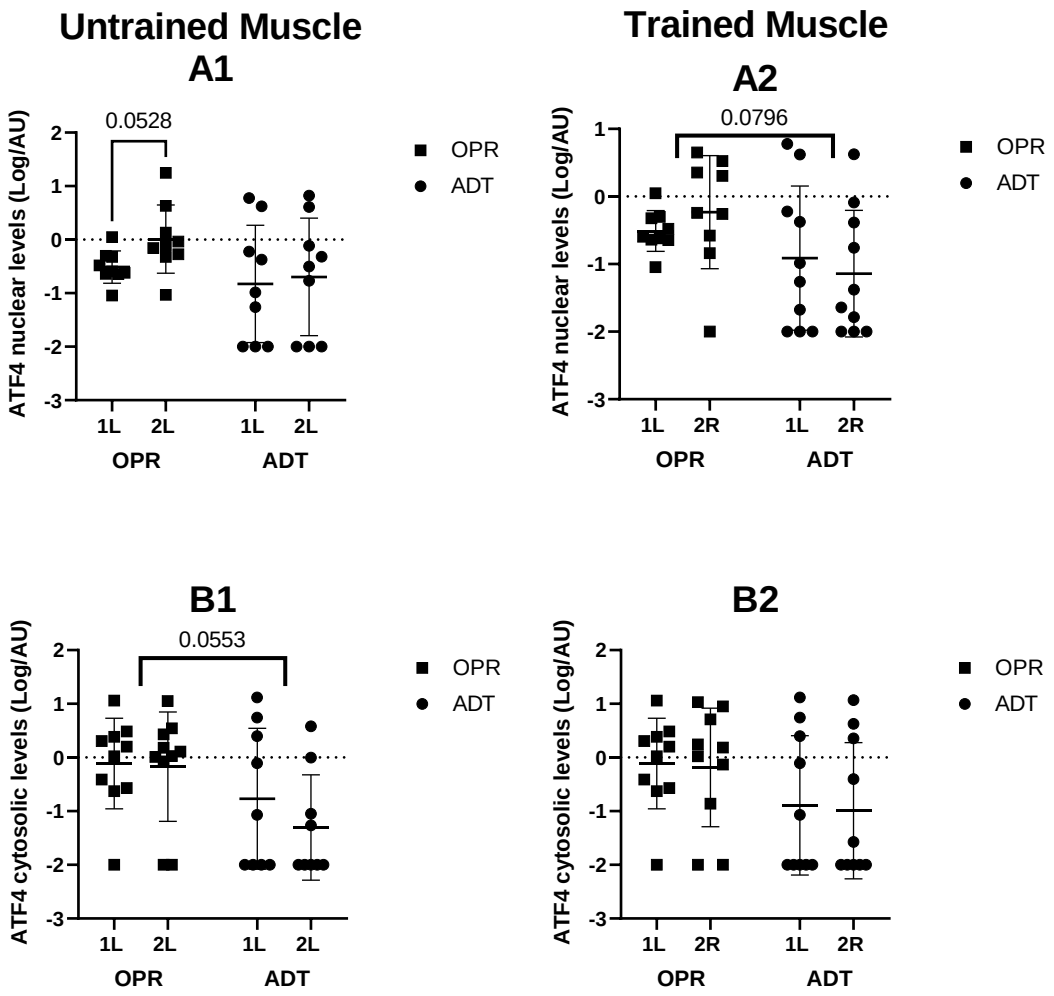


Figure 4.7: Protein expression for ATF4 at baseline and post-protein supplementation in the untrained leg (A1, B1) and the trained leg (A2, B2) in the nuclear fraction (A1, A2), and cytosolic fraction (B1, B2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. Tendencies are presented as p-value. Results are presented as individual values with mean ± standard deviation for each group.

4.3.8 Effect of protein supplementation on CD98

There were no significant differences between groups when comparing protein expression for CD98 at baseline with untrained and trained leg post-protein supplementation in the nuclear fraction (p=0.29; Figure 4.8A1, p=0.23; Figure 4.8A2), and cytosolic fraction (p=0.20; Figure 4.8B1, p=0.10; Figure 4.8B2).

There was no significant difference in protein expression for CD98 between baseline and untrained leg post-protein supplementation in the nuclear fraction (p=0.23; Figure 4.8A1), and cytosolic fraction (p=0.48; Figure 4.8B1).

There was a tendency towards an increase in protein expression for CD98 (0.10 AU) from baseline to the trained leg post-protein supplementation in the nuclear fraction (p= 0.08; Figure 4.8A2). However, when divided by groups the results show no tendency. There was no significant difference in protein expression for CD98 between baseline and the trained leg post-protein supplementation in the cytosolic fraction (p=0.42; Figure 4.8B2).

There was a significant difference in the interaction between baseline and the trained leg post-protein supplementation combined with differences between groups, as the changes went in opposite directions (OPR: down, and ADT: up) (1L vs 2R x OPR vs ADT; p=0.041; Figure 4.8B2).

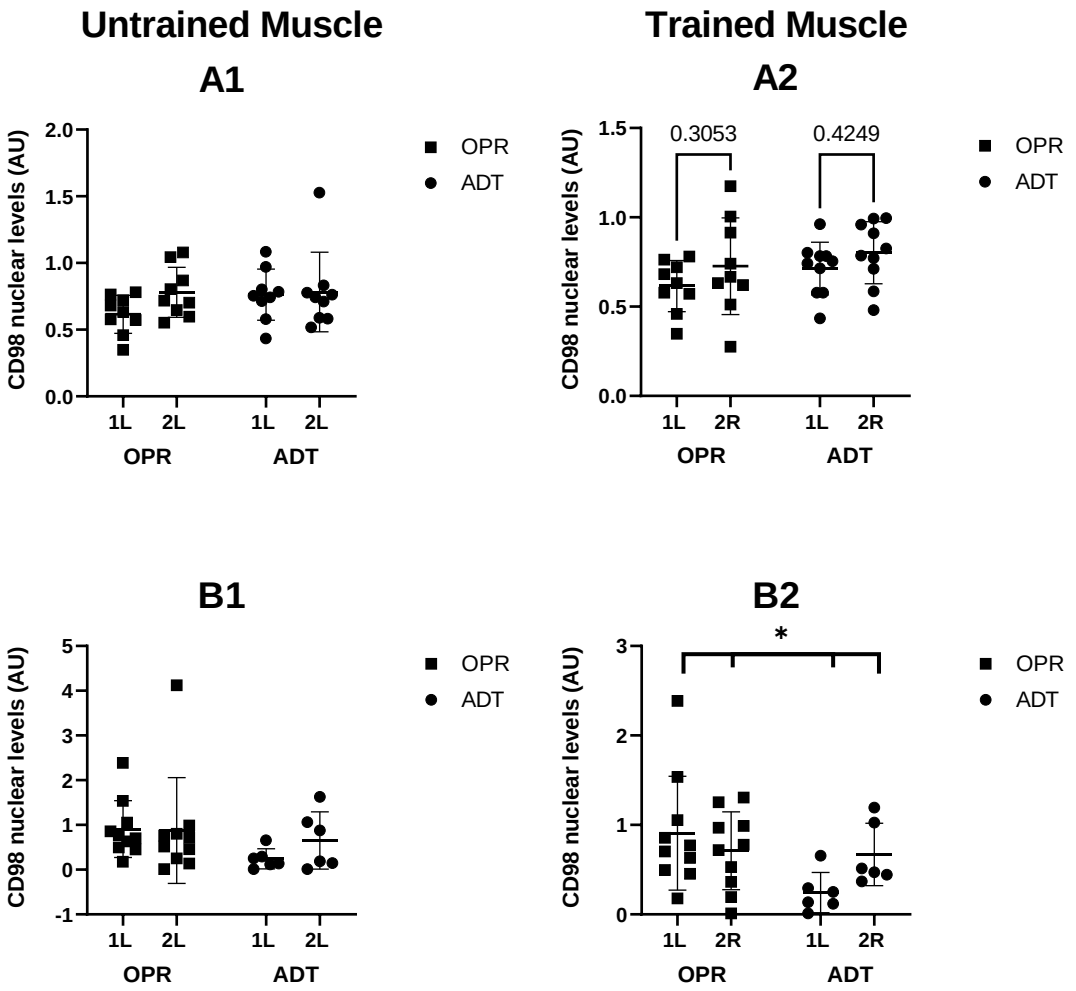


Figure 4.8: Protein expression for CD98 at baseline and post protein supplementation in untrained leg (A1, B1) and trained leg (A2, B2) in the nuclear fraction (A1, A2) and cytosolic fraction (B1, B2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). AU=Arbitrary units. Tendencies are presented as p-value. Results are presented as individual values with mean ± standard deviation for each group.

4.3.9 Effect of protein supplementation on SLC38A9

There were no significant differences between groups when comparing protein expression for SLC38A9 at baseline with the untrained and the trained leg post-protein supplementation in the membrane fraction (p= 0.86; Figure 4.9A1, p= 0,98; Figure 4.9A2).

There was no significant difference in protein expression for SLC38A9 when comparing baseline with the untrained leg (p= 0.24; Figure 4.9A1) and the trained leg (p= 0.66; Figure 4.9A2) post-protein supplementation in the membrane fraction.

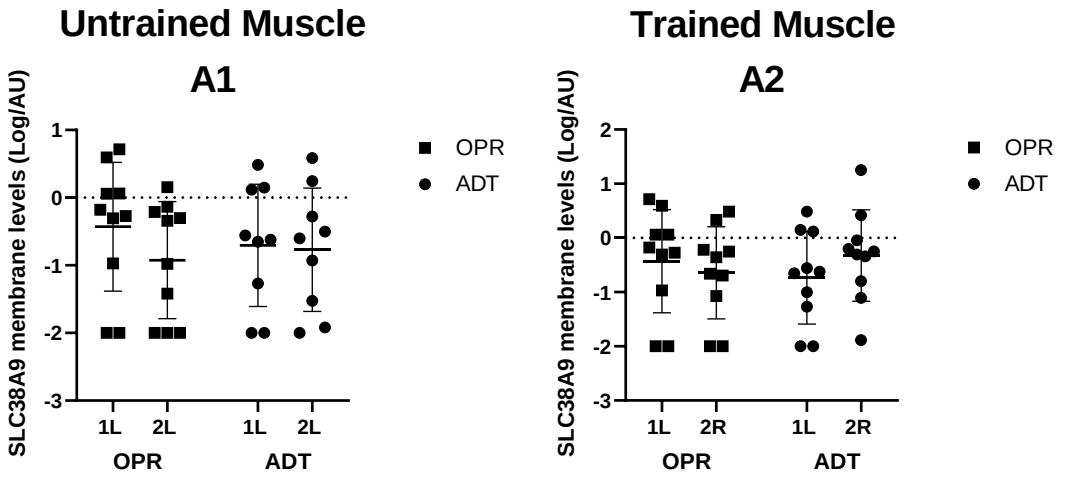


Figure 4.9: Protein expression for SLC38A9 at baseline and post-protein supplementation in the untrained leg (A1) and the trained leg (A2) in the membrane fraction (A1, A2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. Results are presented as individual values with mean ± standard deviation for each group.

4.3.10 Effect of resistance exercise in combination with protein supplementation in eIF2α

There was a tendency towards difference in protein expression for eIF2α between groups when comparing the untrained leg post-protein supplementation with the untrained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction (p= 0.08; Figure 4.10A1), where the difference between means (-0.14 AU) indicates a greater protein expression for eIF2α in the ADT group.

There were no significant differences in protein expression for eIF2α between groups when comparing the untrained leg post-protein supplementation and the untrained leg post-resistance exercise in combination with protein supplementation in the cytosolic fraction (p= 0.32; Figure 4.10B1), and membrane fraction (p= 0.65; Figure 4.10C1). There was no significant difference in protein expression for eIF2α between groups when comparing the trained leg post protein supplementation with the trained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction (p= 0.16; Figure 4.10A2), and membrane fraction (p= 0.74; Figure 4.10C2).

There was a significant difference in protein expression for eIF2α between groups when comparing the trained leg post-protein supplementation with the trained leg post-resistance exercise in combination with protein supplementation in the cytosolic fraction (p= 0.02; Figure 4.10B2), where the difference between means (-0.19 AU) showed a greater protein expression for eIF2α in the ADT group. There were no significant differences in protein expression for eIF2α when comparing the untrained leg post-protein supplementation and the untrained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction (p= 0.47; Figure 4.10A1) and cytosolic fraction (p= 0.30; Figure 4.10B1).

There was a significant difference in protein expression for eIF2α between the untrained leg post-protein supplementation and the untrained leg post-resistance exercise in combination with protein supplementation in the cytosolic fraction (p= 0.028; Figure 4.10C1). When divided by groups results show no significant difference in OPR (p= 0.95; Figure 4.10C1), but a significant decrease in protein expression for eIF2α in ADT (p= 0.006; Figure 4.10C1) from post-protein supplementation to post-resistance exercise in combination with protein supplementation in the cytosolic fraction.

There was no significant difference in protein expression for eIF2α when comparing the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction ($p= 0.18$; Figure 4.10A2), cytosolic fraction ($p= 0.83$; Figure 4.10B2), and membrane fraction ($p= 0.86$; Figure 4.10C2)

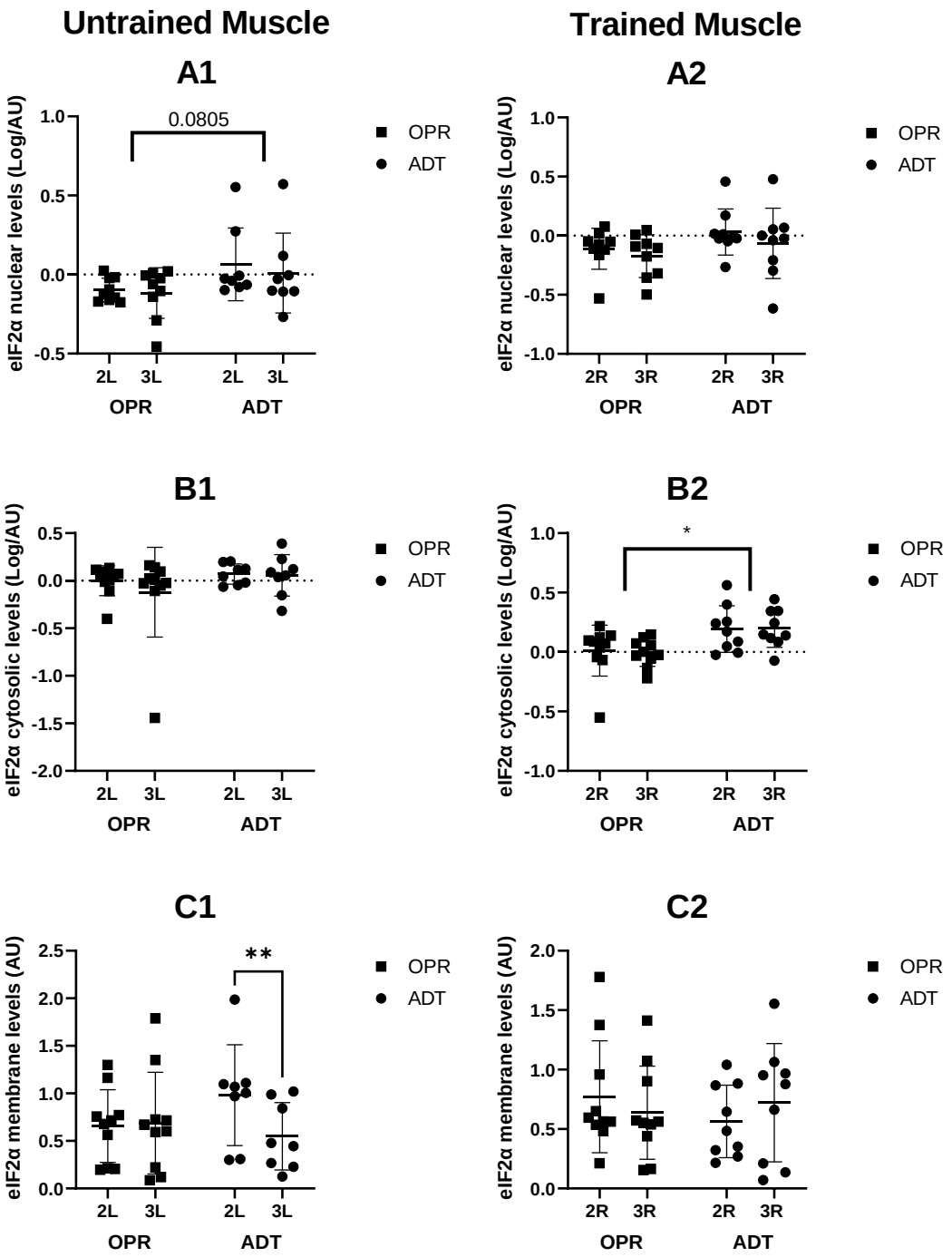


*Figure 4.10: Protein expression for eIF2α post protein supplementation and post resistance exercise in combination with protein supplementation in untrained legs (A1, B1, C1) and trained legs (A2, B2, C2) in the nuclear fraction (A1, A2), cytosolic fraction (B1, B2) and membrane fraction (C1, C2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. *=Significant differences. Tendencies are presented as p-value. Results are presented as individual values with mean ± standard deviation for each group.*

4.3.11 Effect of resistance exercise in combination with protein supplementation in ATF4

There were no significant differences between groups in protein expression for ATF4 when comparing the untrained leg post-protein expression and the untrained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction (p= 0.21; Figure 4.11A1), and cytosolic fraction (p= 0.16; Figure 4.11B1).

There was a tendency for difference in protein expression for ATF4 between groups when comparing the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction (p= 0.09; Figure 4.11A2), where the difference between the means (0.63 AU) indicates a greater protein expression for ATF4 in the OPR group. There were no significant differences between groups in protein expression for ATF4 when comparing the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the cytosolic fraction (p= 0.12; Figure 4.11B2).

There were no significant differences in protein expression for ATF4 between the untrained leg post-protein supplementation and the untrained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction (p= 0.91; Figure 4.11A1). There was a tendency for difference in protein expression for ATF4 between the untrained leg post-protein supplementation and the untrained leg post-resistance exercise in combination with protein supplementation in the cytosolic fraction (p= 0.08; Figure 4.11B1). When divided by groups results show no tendency for difference in the OPR group (p= 0.98; Figure 4.11B1), but a tendency for an increase in protein expression for ATF4 from post-protein supplementation to post-resistance exercise in combination with protein supplementation in the ADT group (p= 0,06; Figure 4.11B1).

There was a tendency for difference in protein expression for ATF4 between trained leg post protein supplementation and trained leg post resistance exercise in combination with protein supplementation in the nuclear fraction (p= 0.07; Figure 4.11A2). When divided by groups results show no tendency for difference in OPR (p= 0.79; Figure 4.11A2), but a tendency for an increase in protein expression for ATF4 from post-

protein supplementation to post-resistance exercise in combination with protein supplementation in the ADT group (p= 0.09; Figure 4.11A2). There were no significant differences in protein expression for ATF4 between the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the cytosolic fraction (p= 0.19; Figure 4.11B2).

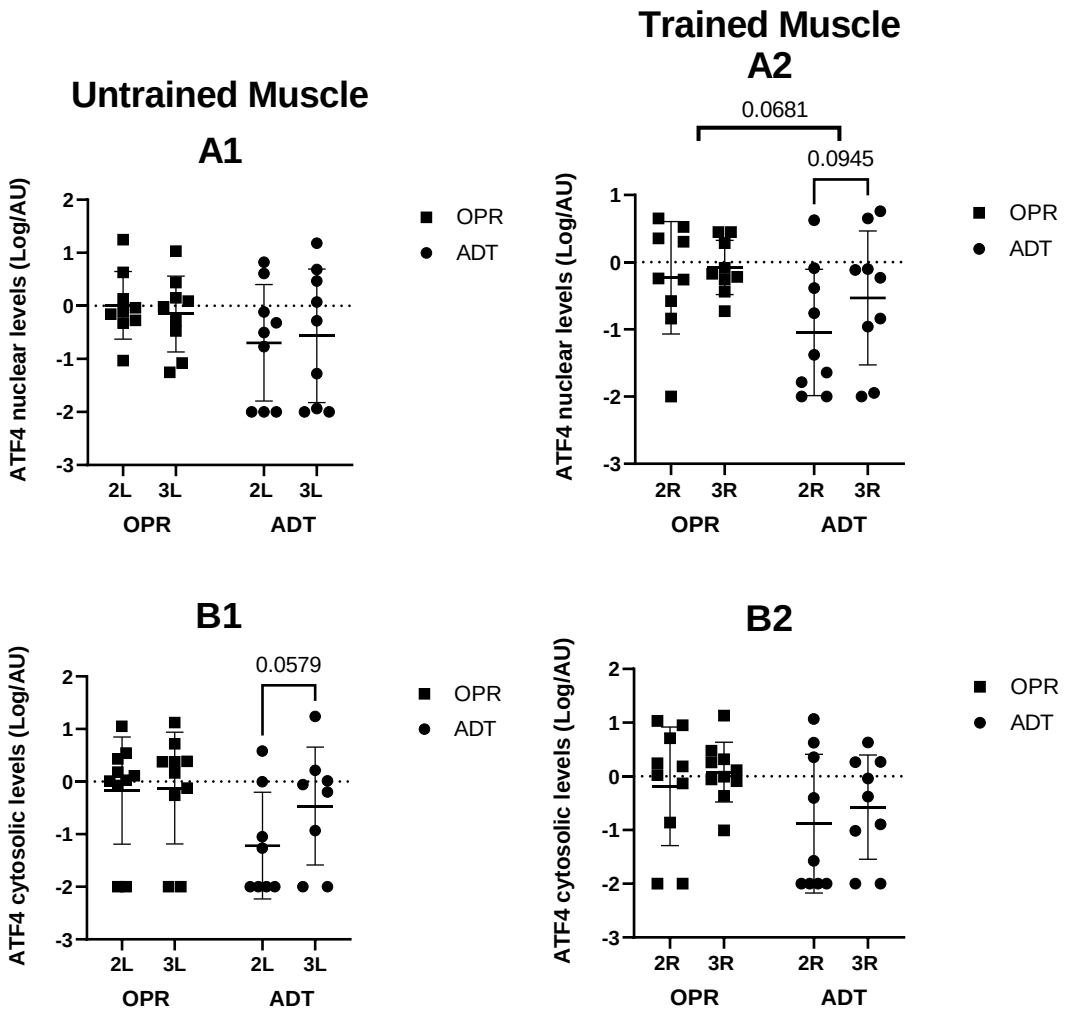


Figure 4.11: Protein expression for ATF4 post-protein supplementation and post-resistance exercise in combination with protein supplementation in the untrained legs (A1, B1) and the trained legs (A2, B2) in the nuclear fraction (A1, A2) and cytosolic fraction (B1, B2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. Tendencies are presented as p-value. Results are presented as individual values with mean ± standard deviation for each group.

4.3.12 Effect of resistance exercise in combination with protein supplementation in CD98

There was no significant difference between groups in protein expression for CD98 when comparing the untrained leg post-protein expression and the untrained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction ($p=0.87$; Figure 4.12A1), and cytosolic fraction ($p=0.29$; Figure 4.12B1). Additionally, there was no significant difference between groups in protein expression for CD98 when comparing the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction ($p=0.80$; Figure 4.12A2), and cytosolic fraction ($p=0.73$; Figure 4.12B2).

There were no significant differences in protein expression for CD98 between the untrained leg post-protein supplementation and the untrained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction ($p=0.25$; Figure 4.12A1), and cytosolic fraction ($p=0.44$; Figure 4.12B1).

There were no significant differences in protein expression for CD98 between the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction ($p=0.30$; Figure 4.12A2). There was a tendency for difference between the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the cytosolic fraction ($p=0.09$; Figure 4.12B2), where both groups indicate an increase in protein expression for CD98 post resistance exercise in combination with protein supplementation. However, when divided by groups the results show no tendency.

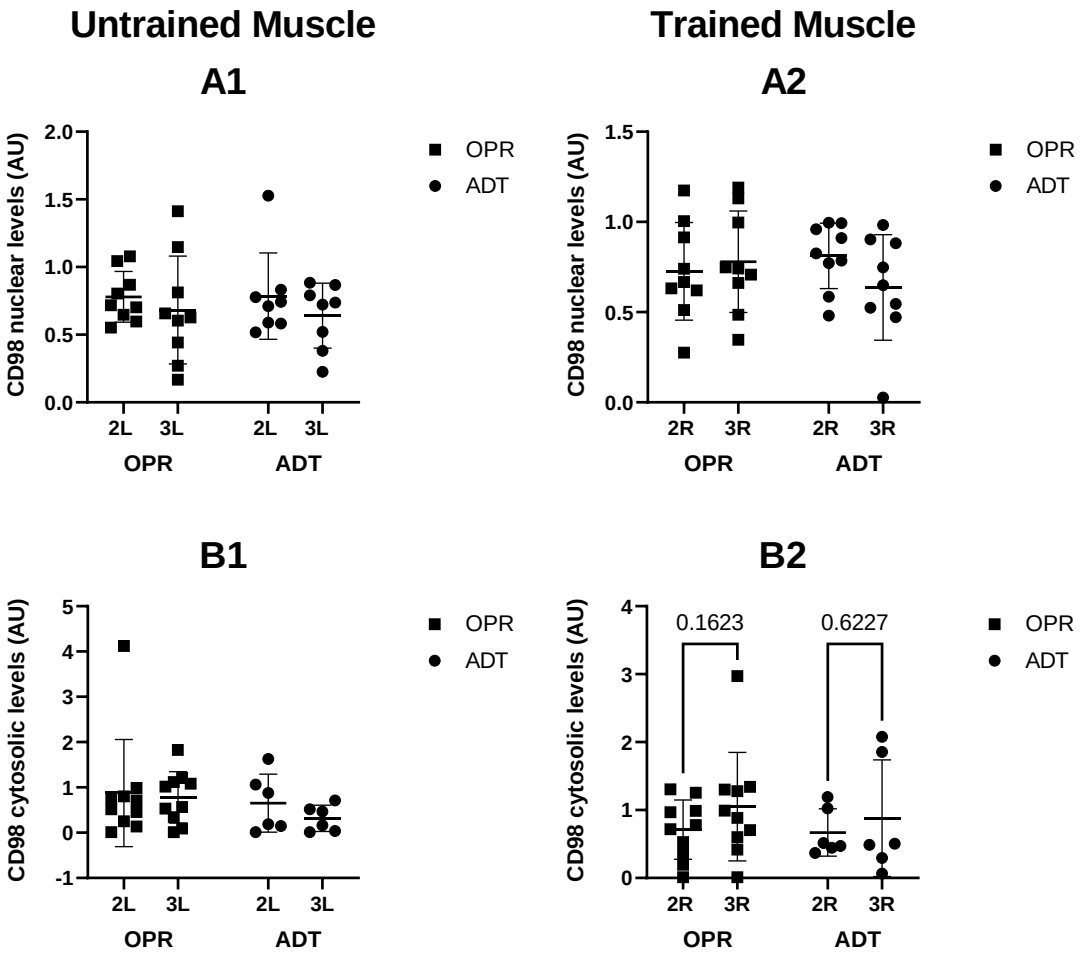


Figure 4.12: Protein expression for CD98 post protein-supplementation and post-resistance exercise in combination with protein supplementation in the untrained legs (A1, B1) and the trained legs (A2, B2) in the nuclear fraction (A1, A2), and cytosolic fraction (B1, B2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log/AU=Arbitrary units transformed to lognormal values. AU=Arbitrary units. Tendencies are presented as p-value. Results are presented as individual values with mean ± standard deviation for each group.

4.3.13 Effect of resistance exercise in combination with protein supplementation in SLC38A9

There were no significant differences between groups in protein expression for SLC38A9 when comparing the untrained leg post-protein expression and the untrained leg post-resistance exercise in combination with protein supplementation in the membrane fraction (p= 0.69; Figure 4.13A1). Additionally, there were no significant differences between groups in protein expression for SLC38A9 when comparing the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the membrane fraction (p= 0.72; Figure 4.13A2).

There were no significant differences in protein expression for SLC38A9 between the untrained leg post-protein supplementation and the untrained leg post-resistance exercise in combination with protein supplementation in the membrane fraction (p=0.47; Figure 4.13A1). Additionally, there were no significant differences in protein expression for SLC38A9 between the trained leg post-protein supplementation and the trained leg post-resistance exercise in combination with protein supplementation in the membrane fraction (p= 0.34; Figure 4.13A2).

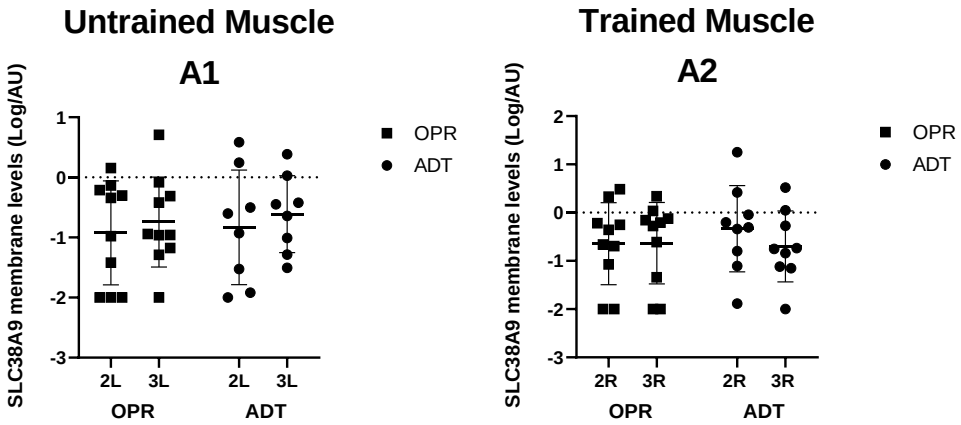


Figure 4.13: Protein expression for SLC38A9 post protein supplementation and post resistance exercise in combination with protein supplementation in untrained legs (A1) and trained legs (A2) in the membrane fraction (A1, A2) for prostate cancer patients treated with surgery (OPR) and androgen deprivation therapy (ADT). Log(Y)/AU=Arbitrary units transformed to lognormal values. Results are presented as individual values with mean ± standard deviation for each group.

5. Discussion

5.1 Methodical considerations

Methodical considerations will be discussed prior to the discussion of obtained results.

5.1.1 Internal validity

Internal validity refers to if the results in a given study can be accounted for by the intervention and not outer factors which cannot be controlled for (Drageset & Ellingsen, 2009). To reduce the probability of interference by outer factors the following considerations were applied in this study.

Before inclusion in the project, the author completed a pilot of all tests and workout protocols to prevent variation in the completion of project procedures across different test leaders. Additionally, author was blinded to which treatment participants had received until Western blot analysis was completed to prevent potential influence in tests and analysis, therefore reducing the potential of influencing the results. This was done by digitizing participants as they were included in the project, biopsy tubes and future analysis were all labelled by the individual number given to each participant. Thus, minimizing the risk for conscious, or unconscious influence by the author in data collection and analysis of samples in this study. Participants were not blinded, given they knew which treatment they had received prior to the intervention.

There was a significant decrease in both groups in the MVC test from pre- to post-resistance exercise in both groups in this study. This indicates an increased state of fatigue following resistance exercise, thus strengthening the validity of the study by accomplishing the wanted state in response to resistance exercise in participants. Findings confirm a resistance exercise-induced state of fatigue and present an objective measurement of desired response to resistance exercise in both groups.

5.1.2 External validity

External validity refers to the generalizability of the participants for a given study. To which degree are the participants included in a study representative for the population investigated, thus representative to which degree the results can be transferred to the population in question (Drageset & Ellingsen, 2009).

In a national mapping survey of habitual physical activity among adults and the elderly, results of daily activity levels show 246 (±74) min of light physical activity, 37 (±27) min of moderate physical activity, and 1.3 (±3.6) min of hard physical activity in men aged >65 years (Hansen et al., 2023). In addition, results from a national quality-of-life examination show that 27% of the elderly (67-79 years) report completion of resistance exercise at least once a week (Støren, 2021).

In this study participants treated with ADT (n=10) reported 120 (±104) min of hard/moderate endurance training weekly, and 5/10 reported time spent weekly doing resistance exercise. Participants treated with surgery (n=10) reported 249 (±193) min of hard/moderate endurance training weekly, and 4/10 reported time spent weekly doing resistance exercise. Taken together with results from the national mapping survey and quality-of-life examination findings, physical habitual exercise data indicate that participants included in this study are among the more physically active part of the population (Hansen et al., 2023; Støren, 2021).

There was a significant difference in time spent in light endurance activity, with 81.0 (±170.1) min for the ADT group, and 235.0 (±124.2) for the surgery group (p=0.03). A decline in light endurance activity by undergoing ADT treatment can be supported by findings in Overkamp et al. (2023), which reported a significant decrease of -1,207 (±1,942) steps a day following five months of ADT treatment.

The implementation of specific inclusion and exclusion criteria in this study was intended to reduce potential confounding factors and minimize the likelihood of undesirable incidents during data collection. Nevertheless, considering the habitual physical behaviours and comorbidities frequently observed within the studied population, the applied methodological and design approach used in this study may be more applicable to the more physically active segment of the population (Overkamp et al., 2023). As a result, the generalizability of our findings to other parts of the prostate cancer population may be restricted.

5.1.3 Reliability

Reliability refers to how accurate and reliable the data material of a given study are, thus representative for the reproducibility and repeatability for a given intervention or

analysis is (Drageset & Ellingsen, 2009). Regarding this study the following factors may influence the reproducibility and repeatability.

Due to inclusion of an ongoing project, strength tests and workout protocol were completed by different test leaders. Even tough considerations were applied to reduce the probability of interference, potential differences in workout intensity cannot be excluded.

Western blotting as a quantitative method is debatable, due to the various steps, techniques, reagents, and detection methods to obtain associated data used in the analysis. Thus, highlighting the importance for standardized procedures in combination with updated reagents and detection methods to obtain reliable quantitative data (Taylor et al., 2013). However, the various steps and techniques used in the method may present challenges with reproducibility and repeatability for the obtained results.

A critical step during the procedure is sample preparation and pipetting of sample onto gels. During sample preparation individual amounts of sample is pipetted into tubes to dilute and standardize protein concentrations for every sample. Next, a given amount of prepared sample is pipetted into wells on the gel. This presents potential for human error and differences in amount of sample pipetted into wells. To control for variations in amount of sample, intensity of each protein band was normalized to the total amount of protein in corresponding well. Control samples are pipetted into two wells which is later used to determine control variation (CV). Raw data is later divided by control samples, thus presenting the opportunity to compare samples across different gels and runs. In addition, gels were set up as duplicates to increase the accuracy and reproducibility of obtained results. After dividing raw data by control samples, the mean of each individual sample was calculated using duplicate values.

Accepted CV were set to 20% between control samples. In several cases CV were too high (>20%), which had an observed tendency to occur when control samples were pipetted into well 6 and 13. Regarding the pressure of time, thus an inability to run concerning analysis again, concerning values were evaluated by differences of one timepoint to another for the same participant and only included if duplicates matched. Values from seven participants across all fractions did not have duplicates, but these

were only included since CV were below accepted variation (<20%). However, this represents a methodological weakness in the study which may restrict the reproducibility and repeatability of obtained results. Obtained results for CD98 in the membrane fraction were excluded from the study, due to methodological errors and challenges during analyses.

In addition, the occurrence of analysed proteins below detection limit was observed in several cases during analysis. Cases of proteins below detection limit were only included in statistical analysis in cases where CV were below accepted variation (<20%) and duplicates matched. To maintain the statistical power of the study and not exclude these participants, values of analysed proteins below detection limit were set to 0.01 AU for further statistical analysis. This may have been avoided by using gels with a higher ability to load proteins, but due to limit of time this was not possible. Thus, presenting a restrictive factor which should be considered when interpreting obtained results for this study.

5.1.4 Summary

In summary, participants included in this study may be among the more physically active segment of the population. Thus, restricting the generalizability of obtained results to the rest of prostate cancer population. Taken together with various issues mentioned concerning western blot analysis, reader is recommended to interpret obtained results with care.

5.2 Protein expression

To the best of authors knowledge, no study has examined muscle content of amino acid transporters and regulator proteins in prostate cancer patients treated with ADT. Additionally, only the study by Haren et al. (2011) seem to have investigated the effect of testosterone levels on amino acid transporters. This was noteworthy done in mice and only a single amino acid transporter in muscle was part of the analysis. This represents a distinct weakness since animal studies not necessarily is generalizable to humans regarding anabolic cellular mechanisms in response to supplementation and exercise, and a lack of research on the subject.

Therefore, discussion of obtained results will be by studies that have investigated amino acid sensitivity and availability in a similar manner or in elderly individuals. However, the lack of existing literature on the influence of testosterone on amino acid sensitivity and availability limits this part of the discussion.

5.2.1 Baseline

The results showed no difference between groups in protein expression for eIF2α in the nuclear fraction, cytosolic fraction, or membrane fraction at baseline, as expected regarding this study hypothesis. Additionally, there was no difference in protein expression for ATF4 in the nuclear fraction and cytosolic fraction, also supporting this study hypothesis. However, the expression of CD98 in the nuclear fraction was significantly higher at baseline in the ADT group than the OPR group, which in contrast was significantly higher at baseline in the cytosolic fraction in the OPR group than the ADT group. There were no differences between groups in protein expression for SLC38A9 in the membrane fraction, which contradict this study hypothesis.

In accordance with this study hypothesis and findings in other studies, the protein expression of eIF2α, ATF4 were similar at baseline (Dickinson et al., 2014; Drummond et al., 2012; Drummond et al., 2011). Drummond et al. (2012) reported no difference in protein expression for regulator ATF4, and amino acid transporters LAT1 and SNAT2 at baseline in elderly adults. In a fasted state there may be amino acid insufficiency in cells, which could lead to the phosphorylation of eIF2α and activation of ATF4. Once activated, this could result in an upregulation in amino acid transporter transcription (Bröer & Bröer, 2017). As shown in Chen et al. (2014), where glutamine starvation led

to the activation of the GAAC pathway and increased intracellular amino acids through increased levels of amino acid transporters.

In this study, results show significant differences at baseline in protein expression for CD98 between groups in the nuclear and cytosolic fraction. In contrast to this study hypothesis, the ADT group had a higher protein expression for CD98 in the nuclear fraction at baseline than the OPR group. Whereas the opposite was observed in the cytosolic fraction. Interestingly, these findings may indicate that protein expression and location for CD98 is influenced by the absence of testosterone. Testosterone have shown to stimulate LAT3 transcription in cancer cells, and ADT treatment will therefore supress this initiation, and among other things result in reduced leucin availability in cancer cells (Zhao et al., 2023). It is plausible that the reduced LAT3 synthesis also occurs in muscle cells, thus reducing leucin availability. To compensate for this, it could be speculated that muscle cells increase synthesis of other amino acid transporters, such as LAT1 and CD98 (Zhao et al., 2023). This would still not explain the opposite findings in the nuclear fraction and cytosolic fraction but indicates that protein expression for CD98 could be influenced by ADT through inhibition of LAT3 synthesis. It could be speculated that increased amount in the nuclear fraction indicate an increased requirement for transportation at the nuclear membrane, as increased CD98 accumulation is observed at the nuclear membrane in some types of virus infections (Hirohata et al., 2015).

It should be considered that for the cytosolic fraction only six participants in the ADT group were included in this study. Therefore, as a potential error, concluding there is a difference between groups should be interpreted with care. In addition, a potential influence by testosterone levels could be explanatory regarding these findings. Drummond et al. (2011) reported no difference at baseline in protein expression for CD98 between young adults and elderly adults. It could be expected that elderly adults have lower testosterone levels then young adults, since a decline in testosterone levels are normally observed in elderly males, and are associated with low production in the testicles, genetical factors and disease (Barone et al., 2022; Shin et al., 2018). In this study, the ADT group have testosterone levels at a castrated level and therefore not directly comparable to findings in Drummond et al. (2011).

5.2.2 Effect of protein supplementation

For the response to protein supplementation and resistance exercise in combination with protein supplementation the term protein localization will be used, as it may be limited as to which degree the synthesis of new protein is responsible in obtained results, given that the duration between supplementation and biopsies may be insufficient. It is therefore reasonable to assume that observed differences in analyses is mainly due to translocation across different fractions rather than increased synthesis. Synthesis may however contribute and should therefore not be excluded but the main mechanisms for rapid differences across fractions is evaluated to be translocation in this study.

In contrast to this study hypothesis, there were no significant difference in protein location for eIF2α and ATF4 between prostate cancer treated with surgery or ADT in response to protein supplementation. However, there was a tendency towards an increased protein localization for ATF4 in the OPR group post protein supplementation for untrained leg in the cytosolic fraction, and for trained leg in the nuclear fraction. Given that the GAAC pathway activation is in response to low availability of amino acids it seem paradoxically that regarding proteins should increase in response to protein supplementation and high availability of amino acids, as the phosphorylation of eIF2α inhibits protein synthesis but increases levels of ATF4, or potentially stimulate a translocation of ATF4 to the nuclear fraction (Luo et al., 2013; Malmberg & Adams, 2008).

However, Drummond et al. (2012) reported increased levels of ATF4 1h and 3h post essential amino acid ingestion, indicating that some regulatory mechanisms to high amino acid availability would increase uptake to cells. This is however speculatively, and these mechanisms in response to high amino acid availability is to the best of authors knowledge not yet understood. It is also noteworthy that Drummond et al. (2012) analysed homogenate fractions of samples and therefore mainly the total amount of proteins, as this study investigated across different fractions, and consequently a possible translocation may influence our results in a higher degree. The amino acid induced increase in ATF4 reported by Drummond et al. (2012) was removed after 7-days bed rest, this may be speculated as a regulatory mechanism regarding physical

activity levels and consequently the need for amino acids in cell. While this would not be directly comparable to this study, considering different testosterone levels in participants, and it seem improbable that sedentary activity influences a difference in protein localization in this study, due to the completion of two resistance exercise sessions by our participants prior to the acute day. However speculative, a potential influence cannot be excluded, given the low levels of habitual activity reported in this study.

In this study, it is plausible that scheduled time between biopsies were insufficient to show response in synthesis of ATF4, but differences across fractions could provide information regarding function of eIF2α and ATF4. However speculatively, but differences between fractions could be due to translocation, as changes in localization could provide indications of effect by protein supplementation. Since a nuclear translocation of ATF4 could indicate phosphorylation of eIF2α, as no change in localization of ATF4 indicate no change in phosphorylation status of eIF2α. It could potentially have happened a response to resistance exercise in phosphorylated levels of eIF2α, but this was not measured in this study. Originally, measurements of phosphorylated levels of eIF2α were intended in this study but was excluded as analysis-protocol not yet is optimized.

In contrast to this study hypothesis there were no difference in protein localization for CD98 and SLC38A9 between prostate cancer patients treated with surgery or ADT in response to protein supplementation. However, there was a tendency towards increased protein localization for CD98 in both groups when comparing baseline to trained leg post protein supplementation in the nuclear fraction. These findings could indicate that previous resistance exercise may have influenced protein localization, since there only was a tendency for increased protein levels in the trained leg. Drummond et al. (2011) demonstrated that a single session of resistance exercise has the potential to influence protein expression in amino acid transporters. Specifically regarding CD98, no difference was reported in protein expression in response to resistance exercise at 3h, 6h or 24h in elderly adults. This finding indicates that duration after resistance exercise is insufficient to observe increased levels of CD98, and plausibly SLC38A9, due to increased synthesis and consequently no observed difference in this study. Considering that participants completed two resistance exercise sessions prior to the acute day, there

could be expected differences between the untrained and the trained leg already at baseline. However, in younger adults there was a significant increase in protein expression for CD98 at 24h, suggesting a potentially delay in the response of CD98 protein expression to resistance exercise and therefore a possible influence of age (Drummond et al., 2011). An influence by prior resistance exercise in this study would therefore seem improbable, given the population of participants included but cannot be excluded.

As mentioned earlier, it is paradoxically that increased availability to amino acids would increase levels of amino acid transporters, given that stimulation of ATF4 is a response to low amino acid availability. However, regulatory mechanisms of amino acid transporters in response to high amino acid availability cannot be excluded. As CD98 forms a functional heterodimer with LAT1, which together forms bidirectional transport of leucine into cells (Console et al., 2022; Roberson et al., 2020). It could be expected that the observed increase in LAT1 by Drummond et al. (2012) in response to amino acid ingestion would be observed in amino acid transporters investigated in this study as well. However, Drummond et al. (2012) also demonstrated the influence of physical activity in amino acid induced muscle protein anabolism, as 7-days bed rest blunted the acute anabolic response to amino acid ingestion in amino acid sensitivity and availability. As described earlier, this is not directly comparable to findings in this study and improbable given resistance exercise completed prior to the Acute day, but a potential influence in the response to protein supplementation cannot be excluded.

5.2.3 Effect of resistance exercise in combination with protein supplementation

In this study, there was a tendency towards a greater in protein location for eIF2α in untrained leg in the nuclear fraction in the ADT group post resistance exercise in combination with protein supplementation. In contrast to this study hypothesis, there was a significantly greater protein location for eIF2α in the trained leg in the cytosolic fraction in the ADT group. However, the untrained leg in the cytosolic fraction showed a significant decrease in protein location for eIF2α in the ADT group, and no difference for the OPR group post-resistance exercise in combination with protein supplementation response. Drummond et al. (2011) reported increased levels of phosphorylated eIF2α in young adults in response to resistance exercise. Elderly adults however showed

increased levels only at 6h and 24h, and a tendency at 3h (Drummond et al., 2011). Increased levels of phosphorylated eIF2α indicate a sufficient stress response by resistance exercise, even though this response may be weaker or delayed in elderly. However speculatively, but this could result in increased ATF4 activation and eventually increased transcription of amino acid transporters genes, and consequently increase protein levels of amino acid transporters. However, these findings indicate that sufficient duration for increased protein levels by synthesis may be multiple hours.

In addition, participants in Drummond et al. (2011) did not receive protein supplementation, and this may have influenced the GAAC pathway in response to resistance exercise. Since phosphorylation of eIF2α and following activation of ATF4 can restore amnio acid levels during amino acid deficiencies, it may be plausible that this study have had seen a more clear response to resistance exercise without protein supplementation (Malmberg & Adams, 2008). This does not however explain a tendency in protein location for ATF4 for a greater response between biopsies in the ADT group than the OPR group in the trained leg in the nuclear fraction, and the untrained leg in the cytosolic fraction post-resistance exercise in combination with protein supplementation. It may however be speculated, that the ADT group by various reasons have an inferior amino acid availability than the OPR group, as the resistance exercise induced stress response could lead to a greater translocation of ATF4 to the nuclear fraction. These speculations may be supported by reported differences in CD98 at baseline in this study.

There was a tendency in the OPR group for higher protein expression for ATF4 than the ADT group in the trained leg post-resistance exercise in combination with protein supplementation in the nuclear fraction. This finding can be viewed with findings in Drummond et al. (2012) which reported an amino acid induced increase in protein expression for ATF4 1h and 3h post supplementation in elderly adults. Drummond et al. (2011) reported no difference in protein expression for ATF4 in elderly post-resistance exercise, but an increase in protein expression for STAT3 3h and 6h post-resistance exercise. Taken together with the reported increase in amino acid transporters, it may be speculated that amino acid availability in elderly is facilitated through a stress-induced response to resistance exercise. This is further supported by Paddon-Jones et al. (2004), which reported an increase in SNAT2 through STAT3 in trophoblast cells. These

findings indicate alternative regulatory response to resistance exercise in amino acid transporters in elderly. In this study, this could be reflected in comparisons between the trained- and the untrained leg, as a clear influence by prior resistance exercise sessions would result in higher levels of amino acid transporters in trained leg compared with untrained leg.

The difference response in phosphorylated levels of eIF2α and protein expression for ATF4 between young and elderly adults observed in Drummond et al. (2011) may indicate a potential influence by testosterone levels, since elderly adults are expected to have lower testosterone availability than young adults (Barone et al., 2022). Unfortunately, this was not controlled for, and testosterone levels are not directly comparable to participants in this study. However, observed difference between groups in Drummond et al. (2011) cannot be explained by testosterone levels with certainty, but a potential influence can neither be excluded.

Results show a tendency for an increase in protein expression for CD98 in trained leg post resistance exercise in combination with protein supplementation in both groups in the cytosolic fraction. However speculatively, but this finding can be viewed together with the suggested regulatory mechanism mentioned earlier, as a potential influence in amino acid transporter levels by a stress-induced response to resistance exercise. Given that only six participants were analysed in the cytosolic fraction, observed results from this fraction should be interpreted with caution. Despite this observed tendency, results show no significant difference post resistance exercise in combination with protein supplementation in protein expression for CD98 and SLC38A9. Considering a potential type-II error regarding number of participants analysed, interpretation of these findings remain unclear, but contrasts with similar studies investigating amino acid transporters in response to resistance exercise and protein supplementation (Dickinson et al., 2014; Drummond et al., 2012; Drummond et al., 2011).

6. Conclusion

The purpose of this study was to compare the basal levels of amino acid transporters in muscle and the acute regulation of amino acid transporters in response to protein supplementation and resistance exercise between prostate cancer patients receiving ADT treatment and prostate cancer patients with normal testosterone levels. Consequently, one group with levels of testosterone equal to castration was compared with one group with normal levels of testosterone in protein expression for the regulators eIF2α, ATF4, and amino acid transporters CD98 and SLC38A9 at baseline, after protein supplementation and after resistance exercise in combination with protein supplementation.

Primary hypothesis:

The basal levels of amino acid transporters in muscle, and the acute regulation of amino acid transporters in response to protein supplementation and resistance exercise, will be inferior in prostate cancer patients receiving ADT treatment compared to prostate cancer patients with normal testosterone levels.

Secondary hypothesis:

- Prostate cancer patients receiving ADT treatment will have lower protein expression of eIF2α in response to protein supplementation and resistance exercise in combination with supplementation, but similar at baseline.
    - There were no differences between groups in protein expression for eIF2α post-supplementation, hypothesis was therefore not confirmed.

- Prostate cancer patients receiving ADT treatment will have lower protein expression of ATF4 in response to protein supplementation and resistance exercise in combination with supplementation, but similar at baseline.
    - There were no differences between groups in protein expression for ATF4 post-supplementation, and post-resistance exercise in combination with supplementation, hypothesis was therefore not confirmed.

- Prostate cancer patients receiving ADT treatment will have lower protein expression of CD98 at baseline, and in response to protein supplementation, and resistance exercise in combination with protein supplementation.
    - There were no differences between groups post-supplementation, and post-resistance exercise in combination with supplementation, but we observed lower levels in the cytosolic fraction and higher levels in the nuclear fraction at baseline. The hypothesis was therefore partly confirmed.

- Prostate cancer patients receiving ADT treatment will have lower protein expression of SLC38A9 at baseline, and in response to protein supplementation, and resistance exercise in combination with protein supplementation.
    - There were no differences between groups in protein expression for SLC38A9 at baseline, post-supplementation, and post-resistance exercise in combination with protein supplementation, hypothesis was therefore not confirmed.

None of secondary hypothesis of differences between prostate cancer patients treated with surgery and ADT was confirmed. There was observed differences in basal levels for CD98, but with opposite findings for the nuclear and cytosolic fraction, and it remains uncertain what these findings represent. The primary hypothesis of inferior basal levels of amino acid transporters, and acute regulation of amino acid transporters in response to protein supplementation and resistance exercise in combination with protein supplementation was not confirmed. Regarding methodical considerations, more studies are needed to clarify observed differences and tendencies in obtained results.

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Table overview

Figure overview

Abbreviations

ADT	Androgen deprivation therapy
MPS	Muscle protein synthesis
mTORC1	Mammalian target of rapamycin
GAAC	General amino acid control pathway
eIF2α	Eukaryotic translation initiation factor 2α
ATF4	Activating transcription factor 4
SNAT2	Solute carrier family 38 member 2
AR	Androgen receptor
p70S6K	Protein S6 kinase
TSC	Tuberous sclerosis complex
TSC1	Hamartin
TSC2	Tuberin
GAP	GTPase-activating protein
Rheb	Ras homolog enriched in brain
FAK	Focal adhesion kinase
PI3K	Phosphoinositide 3-kinase
PKB/AKT	Protein kinase B

AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
CD98	Solute carrier family 3 member 2
LAT1	Solute carrier family 7 member 5
PAT1	Solute carrier family 36 member 1
SLC38A9	Solute carrier family 38 member 9
GnRH	Gonadotropin-realising hormone
NSSS	Norwegian School of Sports Sciences
REK	Regional ethical committee
OUS	Oslo University Hospital
PROFO	Prostate cancer association
MVC	Maximal voluntary contraction
DXA	Dual x-ray absorptiometry scan
1RM	one repetition maximum

Appendix

Western blot Protocol w/ Trans-blot Turbo Transfer System – BioRad

Reagent preperations:

Running buffer: 1,5 L (4 gels)

- 150 ml 10xTGS buffer + 1350 ml dH_20

Transfer buffer: 1L

- 200 ml 5x Transfer buffer + 600 ml dH_20 + 200 ml methanol

TBS: 2L

- 200 ml TBS + 1800 ml dH_20

TBS-T: 2L

- 200 ml TBS + 1800 ml dH_20 + 2 ml Tween 20

Sample preparation:

- Heating block is set to 70 degrees Celsius.
- Calculations based on protein concentration is used to individually dilute each sample with dH_20 and Sample buffer.
- Samples are mixed thoroughly using vortex and centrifuge, before heated on heating block for 10 minutes.

Electrophoreses preparation:

- Find gels, Running buffer, electrophoreses chamber and weight marker.
- Take gels out of packaging, remove comb at the upper side and the tape at the underside.

- Visually inspect that wells are intact.
- Use a Pasteur pipette to rinse the wells with Running buffer.
- Place gels in container with wells facing inwards, fill container with Running buffer to control for leaks.

Electrophoreses:

- Pipette 5 µl of weight marker in outermost wells, for duplicates centre and outermost well.
- Pipette samples in wells using desired loading volume.
- Fill outer chamber with Running buffer.
- Put on lid, install wiring, and turn on Power Pacer.
- Adjust Volt to 200 V and start electrophoreses.
- Control for bubbles in inner chamber, note amps (A) value and visually inspect that proteins wander parallel to each other down the gel.
- The blue line should be at the bottom of the chamber when electrophoreses is completed (25-40 minutes).

Blotting preparations:

- Find PVDF-membranes and transfer stacks.
- Cut upper left corner of membranes and write a number on each membrane.
- Activate PVDF-membranes:
    - 30 seconds in methanol.
    - 30 seconds x 2 in dH_20.
    - 2-3 minutes in Transfer buffer, respectively.
- Transfer stacks are moistened in Transfer buffer for 2-3 minutes, respectively.

Activation of gels:

- Note A value and duration of electrophoreses. Press stop and turn off Power Pacer.
- Take gels out and use a metal tool to open plastic surrounding gels.
- Remove one of the plastic plates and wells on the upper side of the gels.

- Cut the upper left corner of the gel.
- Put gel in dH_20 and remove the other plastic plate.
- Follow point. 6 in procedure ***ML I-18 – ChemiDoc MP Imaging System***.
- Save picture and put gel back in dH_20.

Blotting procedure:

- Take out a drawer of Trans-Blot Turbo Transfer System.
- Place a moist transfer stack in the bottom and roll over to remove air bubbles.
- Place a moist membrane on top of the transfer stack and roll over to remove air bubbles.
- Place gel on top of the membrane and roll carefully over to remover air bubbles.
- Place a new moist transfer stack on top of the gel and roll carefully over to remover air bubbles.
- Place top of the drawer on top of the drawer and lock.
- Put back in Trans-Blot Turbo Transfer System and start wanted program.

Blocking solution:

- Control according to concerning proteins covering letter the use of best suited blocking solution.
    - Bovine Serum Albumin (BSA).
    - Milk powder.
- Mix according to dilution with TBS-T.

Picture of membrane and gel after blotting:

- Put gels in dH_20 and membranes in Transfer buffer upon completion of the blotting procedure.
- Take picture of membranes and gels by using ChemiDoc Imaging System.
    - Follow point. 7 in procedure ***ML I-18 – ChemiDoc MP Imaging System***.

Blocking procedure:

- Put membranes in blocking solution.
- Incubate membranes using calm movements on a shaker for 2 hours in room temperature.
- Wash membranes to remove potential leftovers of blocking solution.
    - Rinse x2 in TBS-T.
    - Wash for 2 minutes x 2 in TBS-T
    - Put membranes temporarily in TBS before incubation with primary antibody.

Primary antibody:

- Control dilution according to antibody covering letter.
- Use container suitable for total volume made of primary incubation solution.
- Place membranes in container and incubate using a roller mixer or shaker over night in 4 degrees Celsius. Membranes are incubated using calm movements.

Washing procedure post primary antibody incubation:

- Wash membranes to remover potential leftovers of primary incubation solution.
    - Rinse x2 in TBS-T.
    - Wash for 15 minutes in TBS-T.
    - Wash for 5 minutes x 3 in TBS.

Secondary antibody:

- Choose secondary antibody considering host of the primary antibody.
- Control dilution according to antibody covering letter.
- Use container suitable for total volume made of secondary incubation solution.
- Place membranes in container and incubate using a roller mixer or shaker for 1 hour in room temperature.
- Upon completion of secondary incubation, repeat washing procedure from primary incubation.

Stripping:

- Put membranes in Stripping buffer for 20 minutes in room temperature using a shaker.
- Rinse x5 in TBS, then wash for 5 minutes x3 in TBS.
- Repeat blocking procedure, primary incubation procedure and secondary incubation procedure.

Detection:

- Mix substrate solution (5 ml Reagent A + 5 ml Reagent B), the solution is sensitive to light, use therefore a lightproof container or put in a dark location.
- Put membranes in substrate solution for 5 minutes, respectively.
- Turn on PC and ChemiDoc Imaging System.
- Follow point. 8 in procedure ***ML I-18 – ChemiDoc MP Imaging System***.
- Close program and turn off ChemiDoc Imaging System and PC.

Analyses

- Protein bands can be quantified by using Image Lab 6.1 Software.
- Follow point. 9 in procedure ***ML I-18 – ChemiDoc MP Imaging System***.
- Save results in project folder considering your project.

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Anne Schiøtz Kavli	22845512	21.09.2016	2016/640/REK sør-øst A
			Deres dato:	Deres referanse:
			08.06.2016	

Vår referanse må oppgis ved alle henvendelser

Truls Raastad
Norges idrettshøgskole

2016/640 Hvordan påvirker hormonbehandling for prostatakreft muskelrespons på trening og mat?

Vi viser til søknad om forhåndsgodkjenning av ovennevnte forskningsprosjekt. Søknaden ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK sør-øst) i møtene 28.04.2016 og 25.08.2016. Vurderingen er gjort med hjemmel i helseforskningsloven § 10, jf. forskningsetikkloven § 4.

Forskningsansvarlig: Norges idrettshøgskole

Prosjektleder: Truls Raastad

Opprinnelig prosjektbeskrivelse (redigert av REK)

Formålet med prosjektet er å undersøke om hormonbehandling ved prostatakreft påvirker den normale stimuleringen av muskelproteinsyntese etter måltid og trening samt hvordan hormonbehandling påvirker muskulatur og videre hvordan styrketrening kan tilrettelegges for å motvirke potensielt negative effektene av hormonbehandling.

En vanlig bivirkning av hormonbehandling er tap av muskelmasse og økning i fettmasse. Særlig kan endringen i kroppssammensetning påvirke muskulaturens sensitivitet for insulin, som igjen kan påvirke blodsukkerregulering. Normalt stimuleres muskulaturen etter inntak av mat og etter styrketrening, men tidligere studier tyder på at denne responsen er hemmet under hormonbehandling. I denne studien vil prostatakreftpasienter på hormonbehandling sammenlignes med pasienter som ikke får hormonbehandling.

Det planlegges å inkludere 40 pasienter med prostatakreft fra Oslo universitetssykehus, 20 som gjennomgår hormonbehandling og 20 pasienter som ikke mottar hormonbehandling.

Pasientene vil informeres om prosjektet ved poliklinikken, og interesserte vil få utfyllende informasjon om prosjektet av prosjektmedarbeidere.

Deltakelse i prosjektet medfører at man må møte to ganger på Norges idrettshøgskole. Den første dagen vil deltakerne få informasjon om hva som skjer dag 2, og muskelstyrke i ben og kroppssammensetning måles. Det antas at dette tar omtrent to timer.

Dag 2 må pasientene møte fastende klokken 07.00. Det skal gjennomføres styrketreningsøkter og målinger av muskelstyrke på ulike tidspunkt i løpet av dagen. Det skal settes inn to venekateter. I løpet av dagen blir det tatt blodprøver på en rekke ulike tidspunkt samt infusjon av stabile isotoper. Det skal videre tas muskel biopsi på fire ulike tidspunkt. Deltakerne vil bli servert to standardiserte måltider og proteintilskudd i løpet av dagen.

Saksbehandling

Søknad om forhåndsgodkjenning ble behandlet av komiteen i møte den 28.04.2016, der søknaden ble avslått med hjemmel i helseforskningsloven § 10, jf. § 5.

Følgende inngikk i komiteens vurdering, jf. brev av 19.05.2016:

«*I prosjektet skal problemstillingen knyttet til hvorvidt trening kan påvirke tap av muskelmasse undersøkes. I så måte anser komiteen at forskningsspørsmålene som reises er høyst relevante.*

Imidlertid er det en rekke forhold ved prosjektet som medfører at komiteen ikke kan godkjenne prosjektet slik det er planlagt gjennomført.

Komiteen vil innledningsvis bemerke at dette er forskning på en sårbar gruppe pasienter, kreftpasienter i aktiv behandling. Komiteen anser, på prinsipielt grunnlag, at det må stilles strenge krav til forskning på sårbare grupper av pasienter. Slik komiteen forstår prosjektet er belastningen kreftpasientene utsettes for i prosjektet relativt stor.

Prøvetakingen i seg selv ansees som omfattende og forbundet med stort ubehag for pasientene. Det vises her til innsetting av venekateter, infusjon av isotoper og spesielt gjennomføring av fire muskelbiopsier. Etter komiteens syn er det ikke redegjort for det vitenskapelige grunnlaget for den omfattende prøvetakingen i prosjektet eller hvordan nytteverdien ved innhenting av et så stort antall prøver kan veie opp for den ulempe det må være for deltakerne å gjennomføre disse.

Videre er det ikke redegjort for hvorvidt undersøkelsene som skal gjennomføres kan forenkles. Etter komiteens syn er det til stor belastning for deltakerne å måtte møte fastende ved et såpass tidlig tidspunkt for deretter å gjennomføre en lang dag med prøvetaking og treningsintervensjon. Det planlegges ikke for å dekke reiseutgifter eller gi deltakerne annen kompensasjon for å delta i prosjektet. Selv for friske, unge mennesker vil deltakelse være utfordrende, og man kan stille spørsmålstegn ved om ikke mange av deltakerne i denne pasientgruppen vil oppleve undersøkelsesdagen som utmattende.

Det fremgår hverken av søknad eller protokoll hvorfor man ønsker å undersøke problemstillingen rundt hvordan hormonbehandling påvirker proteinsyntese i muskel hos menn med prostatakreft. Det antas at deltakere ikke vil ha noen direkte nytte av å være med i prosjektet. Det er videre uklart for komiteen i hvilken grad resultatene av prosjektet vil ha nytte for pasientgruppen, og heller ikke om det vil være mulig å gjennomføre prosjektet på mindre sårbare personer. Andre grupper, som må ansees som friske og dermed mindre sårbare, mottar hormonbehandling for ulike tilstander, og det bør redegjøres for hvorvidt prosjektet kan gjennomføres i andre grupper av personer. Dette omfatter fore eksempel kvinner i overgangsalder.

Etter en samlet vurdering har komiteen kommet til at prosjektet av disse grunner ikke kan godkjennes slik det er beskrevet i søknad og protokoll.»

Prosjektleder har klaget på komiteens vedtak, i klage mottatt 08.06.2016. Supplerende informasjon om revisjon av informasjonsskrivet er mottatt i e-post av 24.08.2016, der det informeres om at setningen «samtykke til bruk av data i andre prosjekter» er slettet, at man ønsker å oppbevare biologisk materiale til 2025 samt at forventet prosjektslutt er i 2020.

Om klagen

Prosjektleder redegjør i klagen utfyllende om hvorfor man ønsker å undersøke problemstillingen. Det argumenteres for at prostatakreftpasienter på Androgen Deprivasjons terapi (ADT) opplever store negative endringer i kroppssammensetning som medfører redusert muskelmasse og økt fettmasse. Disse endringene er direkte relatert til bortfall av testosteron og gir redusert funksjonsnivå og en betydelig økt risiko for en rekke

følgesykdommer. Forskning som kan fremskaffe kunnskap om hvordan man best kan motvirke bivirkningene av ADT vil potensielt ha stor betydning for pasientenes funksjonsnivå, risiko for følgesykdommer og livskvalitet.

Det begrunnes hvorfor denne problemstillingen ikke kan undersøkes hos mindre sårbare grupper, og hvorfor antall tester og biopsier ikke kan reduseres uten å redusere nytten av prosjektet. Testdagen legges opp slik at den skal oppleves som minst mulig slitsom for deltakerne. De vil få rikelig tid til å slappe av og gjøre andre ting mellom testene og biopsiene. Blodprøver tas der de befinner seg slik at det er mulig å lese, se på TV eller prate med andre deltakere mens blodprøvene tas. De kan fritt bevege seg rundt i lokalet under infusjon av isotoper.

Man forventer at de prostatakreftpasienter som melder seg som deltakere til prosjektet vil være i relativt god form, og man understreker at man vil gi god informasjon om hva testdagen innebærer.

Vurdering av klagen

Klagen ble behandlet av komiteen i møtet 25.08.2016.

Komiteen har vurdert klagen og finner at søker har gitt nye opplysninger knyttet til relevans av forskningen for pasientgruppen. Belastningen pasientene utsettes for i prosjektet vurderes etter prosjektleders tilbakemelding som akseptabel.

Komiteen har imidlertid enkelte kommentarer til informasjonsskrivet som må etterkommes.

Det er kun vedlagt ett informasjonsskriv. Dersom man planlegger å bruke dette til pasienter både med og uten hormonbehandling må det settes inn en setning om at det skal inkluderes pasienter som mottar hormonbehandling og pasienter som ikke har fått hormonbehandling i prosjektet slik at det er klart for begge grupper hvorfor de forespørres om å delta i prosjektet.

Det søkes om opprettelse av en ny spesifikk biobank, «AXASP-Study» med ansvarshavende Truls Raastad ved Norges idrettshøgskole. Det er oppgitt ulik dato for prosjektslutt og biobank. Det gjøres oppmerksom på at en prosjektspesifikk biobank ikke kan ha varighet utover prosjektets sluttdato. Komiteen antar at dette betyr at man ønsker en prosjektperiode med varighet til 2025.

I informasjonsskrivet er dato for sletting av kodeliste/koblingsnøkkel oppgitt til 2036. Av dokumentasjonshensyn skal opplysningene oppbevares i 5 år etter prosjektslutt. Opplysningene skal oppbevares avidentifisert, dvs. atskilt i en nøkkel- og en datafil. Opplysningene skal deretter slettes eller anonymiseres. Årstallet for sletting av kodeliste må derfor endres til 2030.

I søknadsskjemaet oppgis det at blodprøver og muskelvev er planlagt analysert i Danmark, USA, Storbritannia og New Zealand. I informasjonsskrivet står det imidlertid kun at prøvene vil bli analysert i USA og Norge. Dersom det planlegges å analysere prøver i andre land enn Norge og USA må dette tydelig fremkomme av informasjonsskrivet.

Prosjektet godkjennes på vilkår av at informasjonsskrivet revideres i henhold til komiteens merknader.

Vedtak:

Prosjektet godkjennes med hjemmel i helseforskningsloven §§ 9 og 33 under forutsetning av at ovennevnte vilkår om revisjon av informasjonsskriv oppfylles.

Det bes om at revidert informasjonsskriv innsendes til vårt arkiv.

Komiteen godkjenner opprettelse av en spesifikk forskningsbiobank, «AXASP-Study» med ansvarshavende Truls Raastad ved Norges idrettshøgskole. Biobankregisteret ved Nasjonalt Folkehelseinstitutt vil få kopi av dette brev.

Det innsamlede biologiske materialet skal oppbevares avidentifisert og destrueres ved prosjektperiodens utløp.

Med hjemmel i helseforskningsloven § 29 tillater komiteen at humant biologisk materiale utføres til utlandet.

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

Godkjenningen gjelder til 31.12.2025.

Komiteens avgjørelse var enstemmig.

Forskningsprosjektets data skal oppbevares forsvarlig, se personopplysningsforskriften kapittel 2, og Helsedirektoratets veileder for «Personvern og informasjonssikkerhet i forskningsprosjekter innenfor helse- og omsorgssektoren».

Av dokumentasjonshensyn skal opplysningene oppbevares i 5 år etter prosjektslutt. Det skal i denne perioden ikke forskes på opplysningene. De skal oppbevares avidentifisert, dvs. atskilt i en nøkkel- og en datafil. Opplysningene skal deretter slettes eller anonymiseres.

Dersom det skal gjøres endringer i prosjektet i forhold til de opplysninger som er gitt i søknaden, må prosjektleder sende endringsmelding til REK, jf. helseforskningsloven § 11.

Prosjektet skal sende sluttmelding på eget skjema, jf. helseforskningsloven § 12, senest et halvt år etter prosjektslutt.

Klageadgang

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

Med vennlig hilsen

Knut Engedal
Professor dr. med.
Leder

Anne Schiøtz Kavli
Seniorkonsulent

Kopi til: turid.sjostedt@nih.no; Norges idrettshøgskole ved øverste administrative ledelse: postmottak@nih.no; Biobankregisteret: biobankregisteret@fhi.no