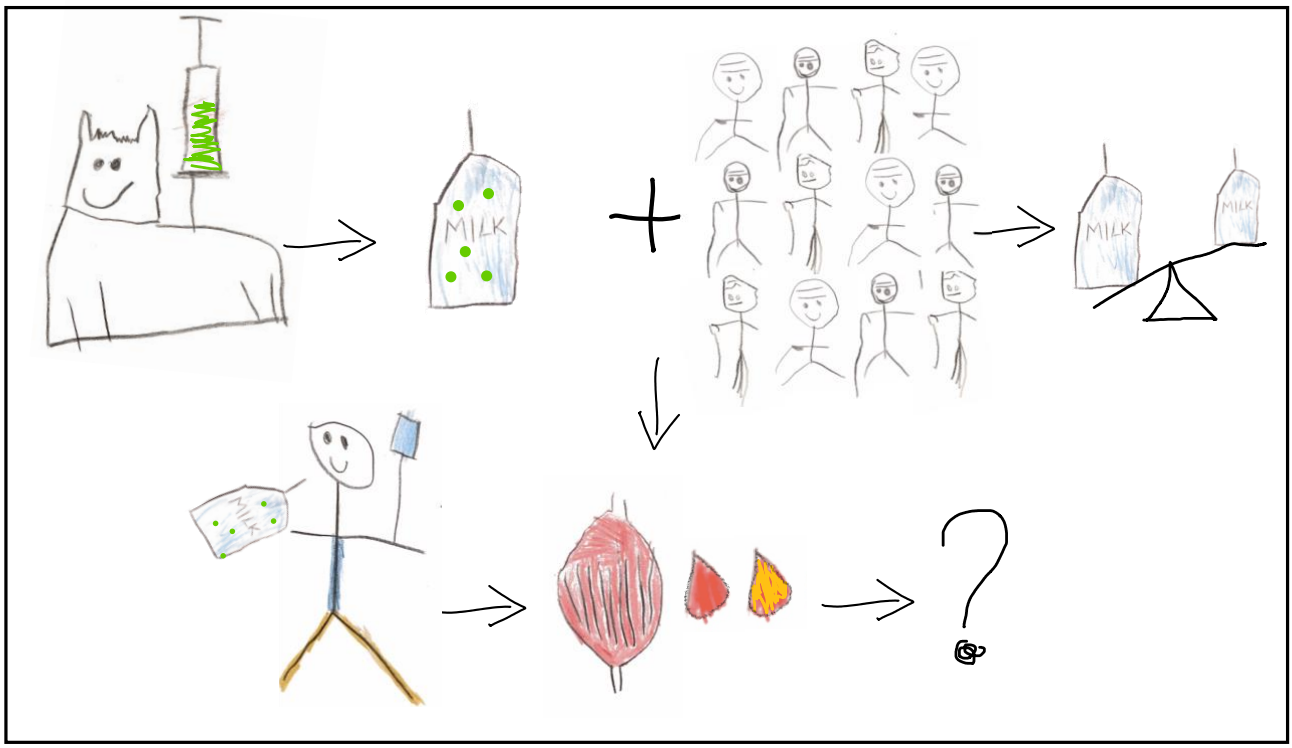
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

PhD Thesis

Grith Stougaard Højfeldt

Fate of nutrient-derived amino acids: influence of habituated levels of daily dietary intake on protein utilization



Institute of Sports Medicine Copenhagen, Bispebjerg Hospital

Primary Supervisor: Professor Gerrit van Hall
Co-Supervisors: Professor Lars Holm and Senior Researcher Peter Schjerling

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1.0 Preface

This thesis is written in order to obtain a PhD degree from the Faculty of Health and Medical Sciences, University of Copenhagen. The thesis is based on results from 3 years research at the Institute of Sports Medicine Copenhagen, Bispebjerg Hospital, and the human studies conducted at the Department of Clinical Physiology and Nuclear Medicine, Bispebjerg and Frederiksberg Hospital.

The work is expected to be published in the two following papers, which are both included at the end of the thesis with the co-authorship declarations:

- Paper 1 (manuscript): Grith Højfeldt, Jacob Bülow, Jakob Agergaard, Ali Asmar, Peter Schjerling, Lene Rørdam, Jens Bülow, Gerrit van Hall, Lars Holm. *Post-absorptive and post-prandial amino acid metabolism and whole body protein synthesis and degradation after 3 weeks habituation to a normal and high protein intake*
- Paper 2 (manuscript): Grith Højfeldt, Jacob Bülow, Jakob Agergaard, Lene Rørdam, Jens Bülow, Peter Schjerling, Gerrit van Hall, Lars Holm. *The postprandial plasma protein but not muscle protein synthesis is decreased after 20 days habituation to a high protein intake*

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Thanks to all of my other colleagues, you have all made it at pleasure going to work every day. Søren, Kenneth, Jacob and the entire CALM office, a special thanks to you for discussions and friendly competitions. To the Bechshøfts, thank you for continuous motivation, and help with last minute formalities. Mette, thank you for occasionally taking the pressure of, and making me realize that at times we are all clueless. Jakob Agergaard, I am not sure where you fit in, but somehow everywhere. For sure, I would have been lost on numerous occasions without you, thank you.

Lastly the greatest thanks to my family for always helping out in your own unique ways, as best you could. To Stefan for thinking that me working late is "badass" and never a pain, for solo parenting when I couldn't be there, and for reminding me that protein turnover is not all that life has to offer. Villum, Mie and Astrid, you definitely did not make this any easier, but you made my life outside work so much better and made the work mistakes and mishaps seem less significant. You will always be my greatest achievements!

Grith Stougaard Højfeldt, Copenhagen June 10th

3.0 List of abbreviations

Akt	Protein kinase B
Alat	Alanine-aminotransferase
ANOVA	Analysis of variance
APE	Atom percent excess
APTT	Activated partial thromboplastin time
Atrogin1	Atrophy-related gene 1
BW	Body weight
DXA	Dual x-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Endo	Endogenous
Exo	Exogenous
FOXO	Forkhead homeobox type O
FSR	Fractional synthesis rate
GC	Gas chromatograph
GCC-IR-MS	Gas Chromatograph Combustion Isotope-Ratio-Mass-Spectrometer
GI-tract	Gastro intestinal tract
HP	High protein
ICG	Indocyanine green
IGF1	Insulin like growth factor 1
IR	Tracer infusion rate
LAT-1	L-type amino acid transporter 1
LBM	Lean body mass
LC	Liquid chromatograph
LDL	Low density lipoproteins
LP	Low protein
MPE	Mole percent excess
MS/MS	Tandem mass spectrometer
MtBSTFA	N-methyl-N-(tert-butyldimethylsilyl) trifluroroacetamide
mTORC1	Mechanistic target of rapamycin complex1
MuRF1	Muscle ring finger 1
NAP	N-acetyl-n-propyl
PAT1	Proton-assisted amino acid transporter 1
PCR	polymerase chain reaction
PF	Plasma flow
PITC	phenylisothiocyanate
PTC	Phenylthiocarbamyl
pV	Partial volume of distribution
Ra	Rate of appearance
Rd	Rate of disappearance
RDA	Recommended daily allowance

SNAT2	Sodium neutral amino acid transporter 2
TSH	Thyroid-stimulating hormone
VLDL	Very low-density lipoproteins
WHO	World health organization

4.0 Abstract

Background: Old age appears to encompass an inevitable loss of lean body mass, and with this a loss of muscle strength, mobility, and function. This has led to a reevaluation of the official recommendation for protein intake, with suggestions of higher protein intakes for older adults. These suggestions are based on associations of positive effect of a higher compared to lower than recommended protein intakes for healthy adults. The aim in this PhD project was to elucidate the underlying mechanisms of those findings by investigating the fasted (post-absorptive) and acute postprandial-state after habituation to the officially recommended vs. higher protein intake.

Method: Twelve healthy non-medicated male participants (66.6±1.6 years of age) were included in this double-blinded cross-over intervention study, which consisted of a 20-day habituation period to the recommended and a 20-day habituation period to a high level of protein intake (1.1 g/kg lean body mass (LBM) and >2.1 g/kg LBM respectively). An experimental trial followed each habituation period, where the fasted (post-absorptive) and four-hour postprandial amino acid and protein kinetics were assessed on a whole body level as well as across the leg (skeletal muscle) and the splanchnic bed. This was done by a combination of tracer dilution, arterial-tissue venous differences, blood flow measures and direct incorporation methodologies. Further, the protein rich mixed meal contained intrinsically labeled D_5-Phenylalanine-whey and ^{15}N-Phenylalanine-caseinate proteins allowing the determination of meal-protein derived amino acid appearance rates.

Results: Habituation to a high compared to the recommended level of protein intake resulted in a higher fasting protein synthesis and breakdown rate but, most importantly, led to a lower net protein balance, which was not compensated for over a four-hour period after the intake of a protein dense meal. In addition, the acute four-hour post-prandial plasma fractional protein synthesis rates were lower after habituation to a high protein intake, and the oxidation rates were higher.

Discussion/Conclusion: Three weeks of habituation to a 2-fold higher than the recommended protein intake caused a more negative post-absorptive net protein balance, i.e. loss of lean body mass. The net protein balance in the four-hour post-prandial period was similar after high and recommended protein intake and plasma

protein fractional synthesis lower after habituation to high protein intake. Together, these results suggest being cautious advising protein intake to elderly far above the levels of the recommended protein needs for healthy adults. Whether this is equally true for the 24-hour protein balance remains to be investigated. It should be noted, that the participants in this study were healthy and a relatively young representation of older individuals. Hence, one limitation of the present study is that the results may not necessarily reflect conditions in older and frailer individuals. However, we hypothesize that regardless of age, supplementing older individuals with protein intakes which largely exceeds the current recommendations, will activate the body's ability to remove excess amino acids by breakdown/oxidation thereby lowering the nitrogen sparing capabilities. This will lead to higher protein loss in the fasted state, requiring a higher degree of compensation from meal intakes, this in a state where the postprandial ability to retain nitrogen is minimized.

5.0 Dansk resumé

Bagrund: Et øget fokus på det fald i muskelmasse, der ses med alderen, har, fra flere forskere, ført til en revurdering, af de officielle anbefalinger for proteinindtag. Dette har ført til, at flere foreslår et øget proteinindtag hos ældre over 65 år. Forslagene om et øget proteinindtag bygger på lang-tids og akutte studie, som viser en positiv effekt af højt proteinindtag i forhold til lavt. Formålet med dette studie var at bygge videre på de resultater der ligger til grund for de ny forslag. Dette ved at undersøge effekten af 20 dages tilvænning til højt proteinindtag versus 20 dages tilvænning til det officielt anbefalede og undersøge aminosyre kineetikker i faste og postprandial tilstand, samt evnen til at udnytte aminosyrerne fra et kost-protein.

Metode: 12 raske mandlige forsøgsdeltagere (66.6±1.6 år), som ikke fast indtog noget medicin, blev inkluderet i dette dobbeltblindede overkrydsnings studie, som bestod af 20 dages tilvænning til l.1 g protein/kg muskelmasse/dag og 20 dages tilvænning til >2.1 g protein/kg muskelmasse/dag. Hvert tilvænningsforløb blev efterfulgt af en forsøgsdag, hvor de fastende og postprandiale aminosyre kinetikker blev undersøgt på hel-krops niveau samt over benet og over splanchnicus gebettet. Dette blev gjort ved at analysere fortynding af sportsstofsmærkede aminosyre, arterie-vene forskelle og blod flows metodikker. Et proteinrigt måltid der indeholdt indre stabil-isotop-mærkede proteiner blevet givet og brugt til at bestemme proteinernes absorptions hastigheder.

Resultater: Tilvænning til højt protein indtag sammenlignet med det anbefalede niveau førte til en øget proteinsyntese og i faste tilstand også til forhøjet protein nedbrydning. Mest afgørende, resulterede tilvænning til højt protein indtag til en lavere protein-balance, i faste tilstand. Dermed et højere tab i protein, som et proteinrigt måltid ikke kunne kompensere for. Endvidere var plasma protein-syntesen i den fire timers postprandiale periode lavere efter tilvænning til høj, end tilvænning til lav protein.

Diskussion/Konklusion: Tre ugers tilvænning til et protein indtag der var to-fold højere end de officielle anbefalinger førte til en mere negative protein net-balance i faste, hvilket kan resultere i tab i muskelmasse. Dette viser, at man bør være påpasselige med at anbefale et protein indtag der markant overstiger de officielle anbefalinger. Om dette resultat også afspejler, en 24 timer protein net balance bør undersøges. Det bør påpeges at forsøgspersonerne i dette studie var raske, medicinerede og relativt unge repræsentanter for en ældre befolkning. Dermed kan

resultaterne fra dette studie ikke nødvendigvis overføres til meget ældre personer. Hypotesen fremadrettet er dog den at, uanset alder, vil et protein indtag der er markant højere end de officielle anbefalinger fører til en øget nedbrydning og oxidering af aminosyre. Dette vil fører til et højere tab af protein i fastende perioder og dermed stille højere krav til protein opbygning i de postprandiale periode. Dette i en tilstand hvor protein nedbrydning ellers er opreguleret.

6.0 Introduction

Several studies have evaluated the benefits of increased dietary protein intake in counteracting the age-associated degeneration of muscle mass [1–4], termed sarcopenia [5]. Sarcopenia may be inevitable but individual differences in the progression [2] indicate that outside factors can slow down the advancement. One commonly accepted factor is the significance of proper nutrition [6], specifically protein [1,7]. However contradictory conclusions were reached between individual studies investigating the effects of various protein level intakes, with claims of either positive [1,2,4] or neutral [8] effects.

The current recommended protein intake, by the Food and Nutrition board in the US [9], EU [10], as well as the World Health Organization [11] is 0.83 g/kg BW/day independent of age for an adult. The estimated average requirement (RDA) was found to be 0.66 g/kg/day. Thus, in order to ensure that the needs of the vast majority of the population should be met, the RDA was set at the 97.5th percentile i.e. 0.83 g/kg BW/day. The current recommendations are based on a meta-analysis made by Rand and colleagues in 2003 [12], which included numerous studies employing the nitrogen balance technique to estimate protein need. However, concurrently with a critique of the nitrogen balance technique [13–15], a tendency among researchers within this field was to generally recommend a higher protein intake up to between 1-1.2 g/kg BW/day [1,4,7,16]. A suggestion included in the newest recommendations for the Nordic countries from 2012. Recommendations which are based solely on epidemiological studies [17].

Therefore, this PhD project was designed to quantify the protein absorption and protein synthesis and breakdown rate and thus whole body and skeletal muscle net protein deposition with advanced technical and clinical methodology. This approach should provide detailed information on the underlaying mechanisms of habituation to a recommended and 2-fold higher protein intake and possibly provide rational for the apparent conflicting data in the literature.

7.0 Background

7.1 Muscle protein turnover

Muscle protein turnover rate includes the two different processes of muscle protein synthesis and breakdown rates. Muscle proteins have a turnover of 1-2% pr. day [18]. In spite of this low turnover rate as compared to for example blood proteins such as albumin (8-9 % pr. Day) [19] the total body skeletal muscles still account for approximately 30-50% of total protein turnover rate in the body due to its shear mass (~40% of body mass of a healthy lean individual) [18].

Protein synthesis and breakdown rates are very different processes but strictly inter-regulated by physiological stimuli such as exercise, feeding, fasting, and hormones [20]. The maintenance of muscle protein mass is obtained by a zero net balance meaning an equal rate of protein synthesis and breakdown. Any fluctuations favoring one or the other of the two kinetics will results in a change in protein, lean body, mass. In the postprandial state (hours following a meal) protein synthesis is dominant over protein breakdown leading to a gain in lean body mass and vice versa in the fasted states. Preferable leading to a zero net protein balance over an entire day [21].

Mechanistically, the regulation of protein synthesis is far better studied than the regulation of breakdown. In this study the expression of insulin like growth factor 1 gene (IGF1) is measured by PCR. The IGF-1 pathway is one of the most studied regulatory pathways, suggested to be involved in regulation of both synthesis and degradation [22]. However it should be noted that increased expression of these markers have primarily been seen with exercise [23–26], and augmented with exercise in combination with feeding [27–29]. Feeding alone has, to our knowledge, not been investigated. The mechanisms of IGF-1 works by binding to the IGF-1 receptor enabeling docking of the insulin receptor substrate which is required for most of the downstream mechanisms of IGF-1 [30]. Subsequently with IGF-1 receptor substrate binding a stimulation of the Akt (protein kinase B) pathway is seen [22,31,32] . Resulting in stimulation of synthesis and inhibition of breakdown [32,33]. The anabolic response of both IGF1 and Akt has been verified, by genetic activation of both IGF1 [34] and AKT [35] showing a muscle hypertrophic response. The protein synthesis stimuli occurs through downstream phosphorylation of the mechanistic target of rapamycin complex1 (mTORC1) [36], which, is a central regulator of cell metabolism. As a negative regulator of muscle synthesis myostatin expression causes inhibition of Akt

[37], which, can however be rescued by administering IGF-1[38]. Inhibition of Akt can both serve to inhibit synthesis and upregulate breakdown since Akt activity can suppress breakdown through suppression of the forkhead homeobox type O (FOXO) family of transcription factors [39] leading to a down-regulated expression of the atrophy-related genes, Atrogin1 and muscle RING finger 1 (MuRF1), both central for breakdown [40–42]. This is supported by findings that both Atrogin and MuRF1 knock out mice are resistant to muscle mass loss [43]. The downstream mechanisms of IGF-1 is illustrated in Figure 1.

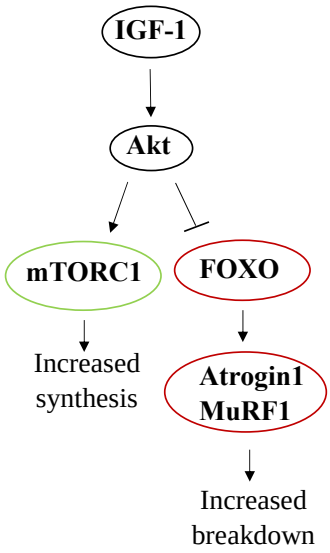

Figure 1: Downstream mechanisms of IGF-1.

7.2 Anabolic stimulus by nutrients

Two potent and feasible ways to stimulate the protein turnover are exercise [44] and nutrition [45,46]. The focus in this dissertation is the anabolic effect of nutrients, with the knowledge that the anabolic effect of proper nutrition can be augmented by exercise and vice versa [28,47].

Essential amino acids directly stimulate muscle protein synthesis [45,48–51], while non-essential amino acids does not appear to be required [52]. The stimulatory effect on skeletal muscle protein synthesis following infusion or ingestion of an amino acids or proteins occurs in a dose dependent but saturable manner [45,46,53]. An increase in amino acid availability, i.e. increased amino acids in the blood and likely intracellularly lead to a rapid, approximately two fold, increases in muscle protein synthesis [54]. This increase lasts about 2-3 hours when high excess abundance of amino acids (hyperaminoacidemia) is sustained [3,55] and longer (~5.5

h) if the hyperavailability is more moderate [56]. Mitchell et al. found that during the initial postprandial period the muscle protein synthesis rate in older male individuals (~70 years of age) did not benefit from a bolus of three grams of leucine 90 min following an intake of 15 grams essential amino acids. Thus, extra leucine does not further enhance the positive effect of an increased essential amino acid intake [57]. While the stimulatory effect of amino acid intake is transient and non-cumulative, it can be repeated more times a day. This is evident in the study by Areta investigating different protein intake patterns over a 12-hour period [58]. Areta et al. found that four 20 g-protein intakes every third hour was superior to eight small (10 g) every 1.5 hour with regards to the muscle fractional synthesis rate. Thus complimenting the finding of a non-cumulative effect. However, the intermediate protein intake was also superior to two larger protein (40 g) intakes, every 6th hour, over the 12-hour period. This indicates that a stimulatory response can be repeated within this period. If not, the effect of four times 20 grams, would be similar (if the maximum stimulation was reach with 20 g) or less than that of two times 40 grams.

The combination of a dose response relationship between amino acid ingestion and lack of cumulative abilities between several smaller meals has led to the notion that an optimal protein intake should be denoted pr. meal and not merely pr. day [54]. This in order to achieve optimal stimulation of protein synthesis and overall anabolism more times a day.

Protein is the primary stimulatory nutrient of body protein synthesis. However, ingesting protein as part of a mixed meal can be favorable. A key reason is the need for proper calorie intake and hormonal responses. In a hypocaloric state the muscle mass will decrease, as body proteins are being broken down and oxidized for energy use. In a study from 1977, Long and colleagues [59] showed a dose dependent inverse relationship between infused carbohydrates and nitrogen excretion in hospitalized patients. A dose dependency that reached steady state once the carbohydrate intake reached a calorie intake matching the requirements [59]. A lean body mass sparing effect can also be seen in a hypocaloric state if the circulating amino acids are increased [60]. Thus, when evaluating the stimulatory effect of a given nutrient stimulus, it is important that the energy balance is obtained, and that protein intake is well controlled.

Beyond securing proper energy balance, the carbohydrate in a mixed meal leads to an increase in insulin secretion. This might be favorable for synthesis, as an increase in insulin has been shown to augment the synthetic response seen with increased circulatory amino acids [61] although, only in concert with an increase in the circulatory amino acids [62]. Furthermore, insulin appears to inhibit protein breakdown independent of circulating amino acids [62,63], an effect that can be augmented by an increase in the circulating amino acids [64]. This anti-catabolic role of insulin on protein turnover, makes it an important anabolic player. The mechanism through which insulin alone exerts its effect on protein turnover, synthesis and breakdown, has to my knowledge not been investigated in vivo. However, Greenhaff et al. investigated graded, steady-state, insulin concentrations concomitant with a constant amino acid infusion [61]. This showed upregulation of mTOR1 at insulin concentrations of 30 mU/l compared to 15 mU/l, which decreased again with increasing insulin concentrations. Additionally, MuRF1 continued to stay downregulated at concentrations of 30,70 and 170 mU/l compared to 15 mU/l. The effects of isolated insulin in vitro where investigated by Alessi et al. who showed that in cultured myotubes insulin leads to a several fold increase in activation of Akt [65]. Collectively, the findings by Greenhaff and Alessi points to a mechanistic effect of insulin through the Akt/mTORC1 pathway. However, in a systemic review Trommels et al. looked at 40 different studies investigating the effect of exogenous insulin on muscle protein synthesis rates in young and older adults and found that the current scientific data do not support a stimulatory role of exogenous insulin administration on protein synthesis in vivo [66]. Unless the insulin level is supraphysiological [67].

7.3 Simulation of protein synthesis in young vs. older.

Nutrients that stimulate protein synthesis, are the same across ages. However, the potency of the anabolic response might be different. Moore and colleagues showed (2015) that older individuals needed a higher protein bolus in order to get the same anabolic response as younger [54]. This is an anabolic resistance, which is also seen in other studies [68–70]. There are some discrepancies however, as not all studies find this anabolic resistance. Symons and colleagues do not find any difference in synthesis rates following ingestion of 29.5 g of protein [71], possibly because the anabolic effect is saturated. However, they did find that circulating levels of amino

acids, following the protein ingestion was higher in older individuals, which have otherwise been shown to be correlated with increased synthesis [45]. Higher circulatory level of amino acids following protein ingestion may indicate either a faster absorption rate or a slower uptake from the circulation to the tissues – or both.

Studies to compare basal protein synthesis rates in absence of nutrient stimulation between young and old participants have also given rise to divergent conclusions. Some studies found an age-dependent reduction in baseline synthesis rate [68,72,73] while others did not find any difference [71,74,75]. The inconclusive knowledge of basal synthesis rates in young and older individuals might play a role in the divergent responses to anabolic nutrient stimuli. This may suggest individual differences that needs to be controlled before exploring nutrient intake response on protein turnover. The individual differences could be found in quality of the protein and/or caloric intake, or divergent habituated levels of protein intake. This will be further elaborated in the section 7.5.

The anabolic role of insulin, as a stimulator of protein synthesis, has more consistently been shown to differ between age groups. Insulin being more anabolic potent in younger compared to older individuals [76,77].

The causal mechanism for anabolic resistance to a nutrient intake is yet unknown. However, may be found in 1) difference in the ‘first pass extraction’ a process including protein digestion rate and splanchnic tissues amino acid utilization rates, 2) delivery of circulating amino acids to the tissue, and 3) the tissues ability to take up and incorporate the amino acids into proteins or the capacity to degrade them (Figure 2).

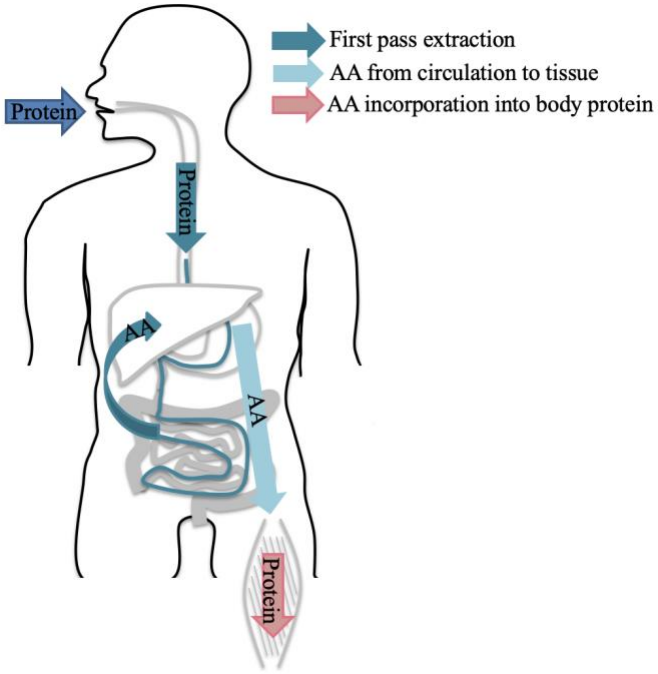

Figure 2: Overview of the route of protein through the body, from degradation to amino acids in the GI-tract and liver to uptake and incorporation by muscle.

7.3.1 First pass extraction

Ingested proteins are degraded to smaller peptides and amino acids in the gastrointestinal tract that will either be used in the gastro intestinal cells or enter the hepatic portal system. The portal vein carries the amino acids and smaller peptides to the liver where they are taken up and further degraded, used in liver and blood protein synthesis or 'bypassed' and entering the circulation (The path is illustrated in Figure 2). The extraction of orally ingested amino acids before the remaining is released to the peripheral circulation, i.e. the "loss" from oral intake to release to the circulation via the hepatic veins, is the "first pass extraction". The degree of splanchnic extraction of amino acids differs between individuals and stimuli. Increased first pass splanchnic extraction has been associated with a high fat percentage body composition [78,79], increasing amount of protein intake [80], and age [81]. In fact, Boirie and colleagues showed in 1997 that older participants had a first-pass splanchnic extraction of leucine which was twice as high compared to that of young participants [78] resulting in lower peripheral leucine concentration.

'First bypass extraction is not easy to determine and cannot simply be determined from the amino acid intake and compared with the blood amino acids

levels. This is because the blood amino acid concentration is depending on: 1) The rates of amino acids from the ingested protein that enter the main circulation (including digestion rate and splanchnic tissues extraction). 2) The rates of amino acid uptake by peripheral tissues. 3) The rates of amino acid release from the peripheral tissues. In other words, it is mandatory to asses all these processes of amino acid appearance and disappearance rates rather than to rely on amino acid concentration. Hence the use of advance techniques in the thesis to quantify all these amino acid/protein fluxes.

7.3.2 Circulating amino acids and uptake by the tissue

Amino acids cannot readily diffuse across cell membranes and are thus dependent on amino acid transporters to carry them across the lipid bilayer [82]. In line with this the expression of some amino acid transporters have been shown to be transiently upregulated in response to increased circulating amino acids [83]. As described in a review by Bröer [84], the human genome encompass approximately 50 different amino acid transporters, which are required to carry out specialized roles in the different cell types and transport different types of amino acids. In this thesis the expression of genes encoding for three amino acid transporters found in muscle have been assessed by PCR. These amino acid transporters are: 1) LAT1 (L-type amino acid transporter 1), which transports branched chain amino acids [85]. For the transports of branched chain amino acids into the cells, LAT1 relies on exchange with glutamine being transported out of the cell. 2) The transporter SNAT2 (sodium neutral amino acid transporter 2), transports glutamine into the cell, thus facilitating this exchange with branched chained amino acids being transported into the cell by LAT1[85]. The expression level of SNAT2 depends on the amino acid concentration, with increased expression in amino acid depleted states [84]. 3) PAT1 (proton-assisted amino acid transporter 1) is thought to, not only transport amino acids, but also act as amino acid receptors through which they are proposed to be involved in the mTORC1 pathway [82].

At basal conditions, there is no difference between the common amino acid transporters in young vs. old. However, younger individuals show 40% and 63% increase in SNAT2, at 3 hours and 6 hours post exercise, respectively, where older participants fail to show any increase at these two time points. In contrast, older

participants have a constantly higher LAT1 expression [86]. Despite the difference in transporter expression, the absolute accumulation of intramuscularly leucine did not differ between groups in the study by Dickinson and colleagues [86].

7.3.3 Incorporation of amino acids into tissue protein

Amino acids transported from the circulation across the cell membranes contribute to the intracellular free amino acid pool. Incorporation of amino acids from this pool into proteins takes place during translation of mRNA, which are the transcribed products of the active genes in a given cell type. The mRNA product from transcription goes through 3 steps of translation: Initiation, elongation and termination. The initiation step is the most rate limiting and the best described in terms of mechanisms known to regulate translation, of which mTORC1 is an important player [87]. This is confirmed by the lack of mTORC1 expression when amino acids are depleted, and the reactivation, when amino acids are provided [88]. mTORC1 activation, sets of a cascade of phosphorylation's of downstream targets such as 4E-BP1 which are involved in increasing translation initiation and S6K1, which stimulates cell growth, both of which mediate an increase in protein synthesis [36,88]. This is illustrated in Figure 3, which builds upon the downstream mechanisms of IGF1 (Figure 1). As an indirect measure of synthesis, the expression of the various genes involved in the synthesis process can be determined by PCR and the expression of the proteins by western blot. However, the regulatory pathways involved in the changes in protein synthesis and degradation are difficult to determine, gives a poor time resolution and the interaction between various pathways are still not completely understood. Fortunately, the total end effect of all these regulatory pathways being the rate of muscle protein synthesis and breakdown, can be measured in vivo via tracer methodologies. Measuring the direct incorporation of ingested or infused labelled amino acids into proteins over a given period of time.

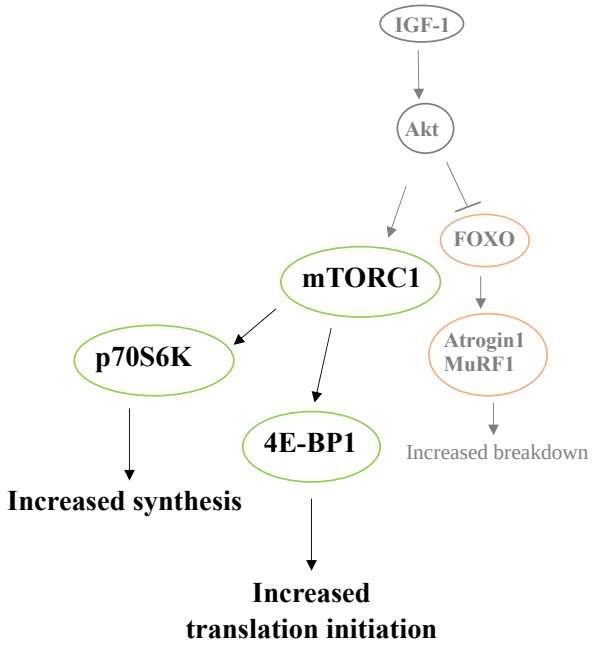

Figure 3: In continuation of figure 1, this figure shows the downstream effects of mTORC1 leading to increased protein synthesis.

7.3.4 Catabolic removal of amino acids from the circulation

Amino acids which are not utilized as building blocks for protein synthesis are metabolized or released from the cells into the main circulation. The major site for degradation of amino acids is the liver, were enzymes removing the amine group from the amino acids are most abundant. However these enzymes are also present in the kidneys and other tissues [89], e.g. in muscle which is also capable of degrading the branched chained amino acids. Degradation of amino acids starts with removal of the amino group, wherefrom the remaining carbon skeleton can be metabolized. The amino group is, for many amino acids, transferred to α-ketogluterate forming glutamate in a reversible transamination reaction. In the muscle, transamination also occurs forming glutamine, however the nitrogen is then transferred to pyruvate to form alanine, which can readily enter the circulation and is transported to the liver. In the liver alanine is back-transformed to pyruvate which can be used for gluconeogenesis, with removal of the amino group [90]. In the liver excess ammonia is combined with carbon dioxide to form urea, which is released to the blood and excreted by the kidneys into the urine. The capacity to breakdown excess amino acids to metabolites, such as urea, is limited, but exceeds the levels that can be uptaken through increased protein intake, and increased protein intakes are therefore not under

normal healthy conditions toxic [91]. However, a formation of higher amounts of urea in the liver in response to a high protein diet do enhance the necessity to excrete more urea via the kidneys and the glomerular filtration rate is increased with increasing protein intakes [92]. Whether a high protein intake can damage the kidneys is not known, but most patient presenting with chronic kidney disease ingest more protein than the official recommendations [92].

Urea is secreted mainly by the kidneys, and a large representative of secreted urea can thus be found by analyzing urea content of urine over 24 hours. The total urea production is difficult to measure, as in addition to the primary loss of nitrogen through urea/urine, nitrogen is also secreted through feces, sweat, and several smaller contributions such as through hair loss, tooth brushing, and exhaled ammonia [93,94]. The rate by which urea is produced, can be assessed by a constant infusing of stabile isotopically labeled urea tracer and analyzing the tracer abundance in the blood, which is then a measure of newly formed urea.

7.4 Recommended protein intake for older individuals

When estimating the body's protein need, the perspective is commonly divided into two different general views.

1) Estimation of the whole body protein needs, which accounts for all tissues.
2) Estimation of protein needs for optimal skeletal muscle growth.

The latter is a result of the desire to utilize protein intake as a mean to counteract the age-related loss of muscle mass. A comprehensible notion, as muscle consists predominantly of water and protein, and skeletal muscle accounts for 50-75% of total body protein [18].

7.4.1 Official recommendations and the nitrogen balance technique

The official recommendations by the Food and Nutrition board in the US [9], EU [10], as well as the World Health Organization [11] is 0.83 g/kg BW/day independent of age. However the recommendation by the Nordic countries from 2012 differ between adults (18-64 years of age) and older individuals (≥65 years of age) with an intake of 10-20 E% for adults and 15-20 E% for older adults, corresponding to 0.8-1.5 g protein/kg BW/day and 1.1-1.3 g protein/kg BW/day, respectively [17]. The official

recommendations are all based on whole body nitrogen balance studies, representing the first of the two above mentioned views.

The bulk of nitrogen in our food and body is found in amino acids and thus also protein. The difference between nitrogen input and output is the nitrogen balance. The nitrogen balance can therefore be used as a surrogate for protein turnover and thus protein need [95], where nitrogen input is nitrogen content in the nutrition and nitrogen output is the secreted nitrogen. As stated previously nitrogen secretion primarily occurs through urine, and less through feces, sweat, and miscellaneous [93,94]. A measure of a positive nitrogen balance will thus mean higher input than output, and thereby excess nitrogen intake. Vice versa a negative nitrogen balance will mean that the body secrets more nitrogen than it takes in. Based on this, the protein requirement as defined by Rand and colleagues [12] is the protein intake resulting in a nitrogen equilibrium, thus a nitrogen balance of zero, interpreted as protein homeostasis.

A drawback of using the nitrogen balance technique is that all participants need several weeks adaption to the protein level tested in order to avoid nitrogen pool size changes (retention or excretion). This is evident in the study by Campbell et al. who studied the nitrogen balance to an intake of 52.9 g protein/day for eight weeks and again following 14 weeks [13]. Campbell and colleagues showed a zero net balance, thus protein homeostasis, following 8 weeks ingestion of 52.9 g protein/day. However, keeping the participants on the diet for a total of 14 weeks resulted in a loss of lean mass. The nitrogen flux (intake and output) should be measured over several days following and adaption period while participants are tightly controlled with regards to intake of protein sources, macro nutrient consumption, energy, and their physical activity. This makes a quantitative protein homeostasis via the nitrogen balance method quite tedious and prone to error. In addition, the nitrogen balance technique has been criticized for underestimating the nitrogen loss as urea output is not necessarily equal to urea production due to urea recycling through the gut [95]. Underestimating urea production will lead to a positive nitrogen balances even at lower nitrogen intakes an in vivo negative protein balance.

7.4.2 Trending changes in dietary protein recommendations

Globally the aging population is growing due to an increase in population as such and in the average lifespan. This has led to an increased focus on the health and independency of the older generation. This meant an increased focus on the importance of muscle mass maintenance [1–4] and consequently focus on optimal protein intake to support healthy aging. Reevaluation of the current standards have identified the official dietary guidelines as an area which may need revision, as well as the methods used to generate these guidelines. With this there has been a shift in focus from whole body protein need to the protein need for optimal muscle maintenance, suggest to be 1.0-1.2 g protein/kg BW/ day [1,4,7,16]. These suggestions are primarily generated via acute studies, a term covering studies which assess the effect of a single or few boluses over a shorter period of time (several hours). Or via retrospective and long-term follow up studies which determine total loss or gain of muscle mass.

Acute responses to nutrition

The immediate/acute response to a nutrient/protein ingestion or infusion of amino acids has been studied on several occasions [45,46,50,53,54,96]. These studies are acute, short termed but allow for a very controlled setting. Common for these studies are the use of proteins (most often fast absorptive) or isolated amino acids, compromising possible findings that would have been seen with the higher energy intake, insulin secretions and such from a whole meal. In addition, the stimulus indifferent whether it is protein or amino acids are not matched to the participants habituated diet. Thus, the given stimulus may be higher or lower than what the participant is accustomed to. One can wonder whether a response to any stimulus that is out of the ordinary, might be exacerbated making it unsuitable for studying a "normal" physiological response.

The discoveries presented in section "7.2 Anabolic stimulus by nutrients" mostly used the ingestion of a single bolus of protein. Generally, amino acids increase protein synthesis in a dose dependent saturable manner without the accumulative ability. Thus the stimulus needed get the optimal synthesis response must be ingested or infused over a short period of time [58]. Moreover, these studies investigating acute responses to protein or amino acids are usually performed with the use of stable

isotope labeled amino acid tracers to measure muscle protein synthesis. The most common measure is the incorporation of tracer into muscle protein, allowing for calculations of the muscle fractional protein synthesis rates. Muscle protein breakdown is harder to measure and is thus often left out [54]. It has been shown, and reviewed by Rennie et al. that intake of isolated amino acid stimulates protein synthesis rate, while having negligible effect on breakdown rate [97]. However, as described in section "7.1 Muscle protein turnover", protein synthesis does not necessarily determine the whole body net anabolic or catabolic state, and if it stands alone, can thus not be used for estimating the whole body protein need. Even when accounting for breakdown and synthesis the deduction from an acute postprandial response to recommendations of long-term protein intakes still requires a lot of assumptions.

Retrospective and long term follow up

The above-mentioned acute, single meal intervention studies do not provide knowledge of the long-term effects of various protein intake doses. Moreover, short term studies do not allow measurements of changes in lean body mass, as is otherwise the primary concern when the focus is maintaining muscle mass. Therefor long-term cross sectional or follow up studies are introduced. In 2008 Houston and colleagues published data from a three year follow-up study including 3074 older (70-75 year) participants [2]. This study showed a significant lower loss in lean body mass, following ingestion of the two highest quintiles of protein (0.9 and 1.2 g/kg BW/day) as compared to the lowest (0.8 g protein/kg BW/day) [2]. In a cross-sectional study from 2016 Loenneke and colleagues found a significantly higher leg lean protein mass in participants habitually ingesting one to two meals with more than 30g of protein (daily intake of 1.0 and 1.4 g protein/kg BW/day) as compared to ingesting no meals with extra protein (daily intake of 0.6 g protein/day) [98]. These long term follow up habituation studies evaluating total LBM [99] or loss thereof [2,100] have the clear advantage of being long term, minimally invasive and the possibility for large cohorts. On the down side, these studies sometimes spanning several years, are difficult to control tightly. Additionally, getting valid dietary registrations over a day is difficult [101], let alone while assuming a constant diet over a period of several years

gives even more rise to error. On top of this, the cross-sectional studies are not paired, thus the difference in lean body mass, can have multiple explanations. For instance, divergent exercise level over time will likely impact the progression in LBM [102]

Conclusively across the span of long-term studies, whether it be retrospective, cross sectional or long term follow up, it seems that too little protein can be detrimental to muscle mass and that this can be counteracted by additional protein intake. However, it has not been examined whether simply adding more protein than current RDA will benefit muscle mass further.

7.5 Habituation to divergent protein intakes

Various research methods, which have been used for investigating the optimal protein intake for the older populations have led to contradictory conclusions. Importantly, very few – if any of the numerous studies – have actually shown that older adults need more protein than the current official recommendations. Longitudinal intervention and cross-sectional studies commonly find differences between the most distinct quintiles, but not between adjacent ranges of protein intake [2,98]. There can be many reasons for this: power issue, biological variation and individual responses due to e.g. different habituated levels of protein. In that context it must be emphasized that the RDA of 0.83 g/kg BW/d corresponds to the quantity of protein that resulted in a net balance of zero in 97.5% of all investigated participants, while 50% had a zero net balance already with 0.66 g/kg BW/d. In the light of this, it becomes very hard to show, statistically, that more protein is needed in the remaining 2.5% within a whole population group, even if it is a population of elderly individuals, who might require slightly higher protein intake. Turning to acute studies, investigating how a bolus of protein stimulates muscle protein synthesis, whole body turnover rates and affects net balance, these responses are not solely dependent on the protein dose. The handling of amino acids in the metabolism is highly adaptable and therefore (also) influenced by the level of protein intake that the individual is habituated to. To get a valid measure of the impact and response of any given protein intake, the research participants' habituated protein intake should be controlled. In neither the nitrogen balance studies nor the studies measuring the immediate responsiveness to protein intake presented above, such habituation levels have been controlled. Hence, the notion that response to a protein dose is dependent on the

habituated level is completely overlooked. Except from relying on different experimental methodologies, the contradictory results and conclusions from the numerous studies discussing whether older adults require more dietary protein could therefore, in part emerge from the fact that the participants are habituated to different levels of protein intakes. Indeed, there are findings that different populations have different eating patterns with regards to protein intake. Where studies from France [103] and the US [104] show that a higher number of older individuals do not ingest the recommended levels of protein, the older population in Denmark do ingest sufficient dietary protein [105].

Several studies have looked at the nitrogen balance response to divergent protein intakes with habituation periods spanning from seven days to three weeks [80,106–109]. All of these studies found that the nitrogen turnover is increased with increasing intakes of protein, however they also found increased nitrogen excretion. This was evident both in young (~27-30 years of age) [108] and older (68 ±9 years of age) [109] adults. The point that habituation to a high protein intake can hinder the ability to retain nitrogen from an amino acid intake is confirmed by Price and colleagues, in a study from 1993, using both nitrogen balance methods as well as analyzing amino acid turnover rates [110]. This showed that just as habituation to high protein causes increased nitrogen secretion, the amino acid oxidation was also increased. This finding that amino acid oxidations is increased following habituation to high protein intakes compared to low is confirmed in more studies [111,112]. Thus, the inability to retain nitrogen translates to the inability to retain amino acids.

In a recent study Gorrissen and colleagues habituated older individuals to a low protein intake (0.7 g/kg BW/day) or high protein (1.5 g/kg BW/day) for 14 days where after they measured their ability to take up and utilize 25 g of whey protein [113]. Of special interest they found that the postprandial circulating amino acid concentrations were higher following habituation to low protein vs. high. With reference to the protein dose-protein synthesis response studies, a high availability of circulating amino acids is favorable [45]. However, Gorissen et al found no impact on the muscle protein synthesis response [113]. In line with this, Walrand et al. showed that while the synthesis will not continue to increase when habituated to a high and a very high protein intake (1.5 vs. 3 g/kg FFM/day), the oxidation continues to increase [114].

Collectively, amino acid metabolism is affected by habituated levels of protein intake; so that high levels of dietary protein decreases nitrogen retention [108,115–117],

increases amino acid oxidation [114,118] and affects the basal rate of protein turnover [108,116,118]. Additionally, Price et al. show that habituation to high protein intake caused a lower fasting net balance. Importantly, the consequence of a lower net balance, as pointed out by Price, is that the demand for net balance repletion in the post prandial state is increased following habituation to high protein intakes. Fortunately, Quevedo et al. showed that the high nitrogen excretion seen following habituation to high protein intakes is reversible, and will decrease within a four day period, if the protein intake is markedly reduced (from 1.82 to 0.77 g protein/kg BW/day) [119].

To fill in the gap of the impact of habituated level on fed and fasted amino acid and protein kinetics, studies integrating all of the above measures while tracing amino acids from they are ingested as dietary proteins, to secretion in the circulation and uptake by the tissue are warranted.

8.0 Aims and hypotheses

The aim of this PhD project was to explore how whole body and muscle specific protein turnover rates as well as amino acid metabolism is affected by enhancing the dietary protein intake level beyond the current RDA in older males (66.6±1.6).

We designed a double-blind randomized controlled trial and applied extensive state-of-the-art tracer methodologies, extensive sampling sites and provided intrinsically isotope-labeled proteins to older men. This allowed the comparison of how the different levels of habituated dietary protein intake affected fasting amino acid and protein kinetics and the postprandial response as well as the investigation of metabolic routes involved in amino acid metabolism. We looked at this via urea concentrations and turnover rates, postprandial rate of appearance of dietary protein-derived amino acids, utilization of dietary amino acids for protein synthesis and degradation, amino acid oxidation, splanchnic bed and limb release and uptake of amino acids, and postprandial blood and muscle fractional protein synthesis rates.

The leading hypothesis of this PhD project is that habituation to an increased dietary protein intake improves meal protein utilization. Thus, resulting in higher

whole body, blood protein and muscle protein synthesis, as well as higher net protein balance.

9.0 Study design

The investigation was a double-blinded cross-over study including 12 healthy male participants between 65-70 years of age. They completed a 20 day habituation to a high level of protein intake (HP, >2.1 g protein /kg LBM/day) and to a recommended level of protein intake[11] (RP, 1.1 g protein/kg LBM/day, corresponding to 0.8 g/kg BW/day assuming ~30% fat). The second trial period commencing no sooner than 45 day after the first one. The participants underwent a trial day on day 21 following each habituation period where their ability to utilize amino acids from a protein rich mixed breakfast meal was determined. Figure 4 shows an overview of the design. The trial day was the same for both habituation periods.

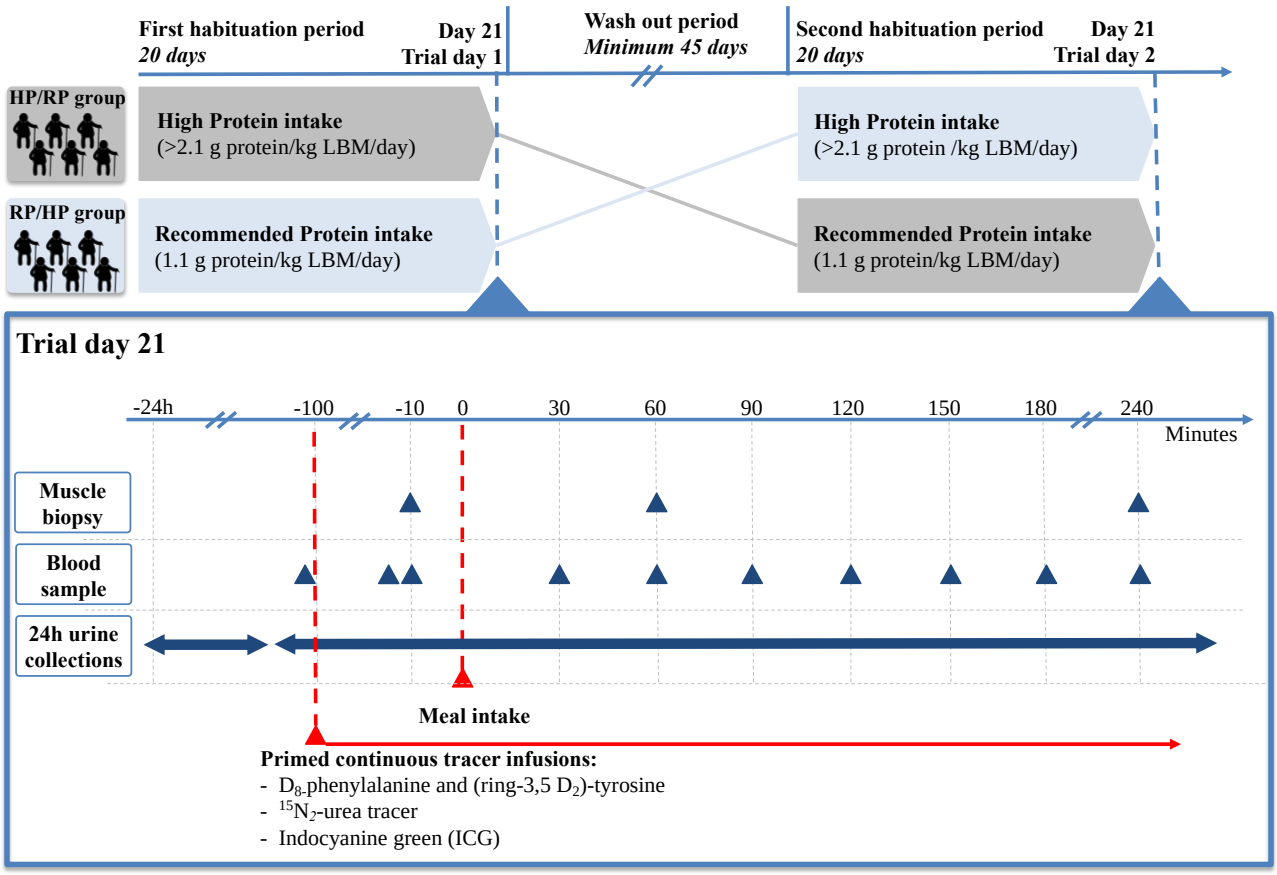

Figure 4: Study overview. The participants were randomly assigned to the HP/RP group or the RP/HP group, receiving either HP in the first 20-day habituation period and RP in the second 20-day habituation period or vice versa. The two habituation periods were interspaced by at least 45 days. 24-hour urine collections were performed on the last day of the intervention period (8 a.m. day 20 till 8 a.m. day 21), as well as during the trial day (8 a.m day 21-8 a.m. day 22). On the day 21 following each habituation period, there was an identical trial day, where the participants ability to utilize amino acids from a protein rich meal was assessed. This via a primed continuous infusion of stable isotope tracers and ICG and a mixed breakfast meal containing intrinsically labelled proteins were provided. Muscle biopsies were taken immediately pre, 60 min and 240 min post meal intake. Blood samples were taken prior to infusion start (-100), twice prior to meal intake (-12 and -10 min) and 30, 60, 90, 120, 150, 180 and 240 min after meal ingestion.

10.0 Methods and methodological considerations

10.1 Human study

Participants: The study was carried out in healthy elderly males.

Inclusion criteria:

- Males age 65-70
- Independently dwelling

- No major health issues (blood pressure, cholesterol and lipid profile are measured and assessed within the normal range by a physician)
- Non-diabetic (based on Hemoglobin A1C levels within the normal range)
- No known current diseases
- No back pain
- Not regularly take any drugs or dietary supplements, which are known to influence muscle protein synthesis
- Alcohol intake below 21 units pr. week

The participants were recruited through adds in local papers and screened for the above-mentioned criteria during a phone screening, and a pre-examination day.

The study design, research questions as well as possible risk and discomforts were explained written and orally individually to all the participants, in accordance with the Helsinki declaration, before they signed a written consent form. The study was approved by the local ethical committee of the capital region of Denmark, protocol number H-15005598, and registered at clinicaltrials.gov under journal number NCT02587156.

At the pre-examination all participants had a blood sample taken and tested for hemoglobin, leukocyte with differential count, thrombocytes, alat (alanine-aminotransferase), APTT (activated partial thromboplastin time), basic phosphatase, cholesterol, LDL (low density lipoproteins), VLDL (very low density lipoproteins), triglycerides, hemoglobin A1c and TSH (thyroid-stimulating hormone). All participants also went through a dual X-ray absorptiometry (DXA)-scan (Lunar iDXA; GE Medical Systems, Pewaukee, WI, USA, with enCORE v.16 software) to determine their lean body mass (LBM). Participant characteristics are presented in Table 1.

Table 1: Participant characteristics assessed before the first intervention period (dietary intake is assessed before each of the intervention).

Participant characteristics N=12			
Age [years]	66.6	±	1.6
Height [m]	1.79	±	0.04
Body weight [kg]	84.3	±	10.9
BMI [kg/m2]	26.3	±	3.3
LBM [kg]	56.4	±	4.4
Fat %	29.9	±	5.7
Systolic blood pressure [mmHg]	137	±	25
Diastolic blood pressure [mmHg]	83	±	11
Protein intake [g/kg LBM/day]	1.5	±	0.3
Energy intake [kcal/LBM]	36.5	±	7.7
E% protein	18.4	±	2.6
Hemoglobin A1c [mmol/mol]	35.2	±	2.6
Thyrotropin (TSH) [x10-3IU/l]	1.5	±	0.8
Total Cholesterol [mmol/l]	5.5	±	0.7
HDL cholesterol [mmol/l]	1.7	±	0.4
LDL Cholesterol [mmol/l]	3.2	±	0.6
Triglyceride [mmol/L]	1.3	±	0.8

Data is mean ± SD

Habituation period.

The participants were randomly assigned to the HP/RP group or the RP/HP group, receiving either high protein in the first period and recommended protein intake in the second period or vice versa. The participants and all investigators were blinded for which supplement was given.

The participants received 4 daily supplements each consisting of 20 g whey protein and 10 g carbohydrate (sucrose) during the "high protein period" (HP). During the "recommended protein period" (RP) they received 4 daily supplements consisting of 30 grams of carbohydrates (20 g maltodextrin + 10 g sucrose). The two different supplements were isocaloric and ingested in a manner aiming at an equal protein content in all three main meals (e.g. two supplements with breakfast, one supplement with lunch and one supplement with dinner) in the high protein period.

Each habituation period consisted of a seven-day run in period and a 13 day tightly controlled period. In the seven-day run in period the participants ate their regular habitual diet as well as the 4 daily supplements. Dietary guidelines were given based on a three-days dietary registration done during the seven-day run in period, thus assuring a protein intake of ≈1.1 g protein/kg LBM/day for the remaining 13 day

tightly controlled period of the 20-days habituation. The participants did a second 3-day dietary registration during the first 3 days of the 13 days period in order to ensure that the dietary guidelines were well understood and followed. The exact intakes of protein in the two habituation periods are shown in Table 2.

Table 2: Daily protein intake for the two habituation periods in the 7 days run in period and in the 13 days tightly controlled period.

	20-day habituation period N=12				
RP period	Basic diet + carb. supplements (Only basic diet)				
Calorie intake day 8-20 [kcal/day]	2117	(1624)	±	84	(84)
Protein intake day 1-7 [g/kg LBM/day]	1.42	(1.42)	±	0.09	(0.09)
Protein intake day 1-7 [g/kg BW/day]	0.96	(0.96)	±	0.07	(0.07)
Protein intake day 8-20 [g/kg LBM/day]	1.11	(1.11)	±	0.03	(0.03)
Protein intake day 8-20 [g/kg BW/day]	0.75	(0.75)	±	0.03	(0.03)
HP period	Basic diet + protein supplements (Only basic diet)				
Calorie intake day 8-20 [kcal/day]	2207	(1666)	±	58	(58)
Protein intake day 1-7 [g/kg LBM/day] *	2.78	(1.35)	±	0.09	(0.07)
Protein intake day 1-7 [g/kg BW/day] *	1.87	(0.91)	±	0.08	(0.06)
Protein intake day 8-20 [g/kg LBM/day] *	2.53	(1.10)	±	0.05	(0.03)
Protein intake day 8-20 [g/kg BW/day] *	1.71	(0.74)	±	0.05	(0.02)

Values are mean ± SEM. Comparisons were made of intakes between periods. * denotes $p<0.001$ between habituation periods.

During the last 24 hours of the habituation period, i.e. from 8 a.m. on day 20 until 8 a.m. on day 21, the participants collected their urine in three-liter bottles kept at room temperature. The dinner on day 20 was provided for the participants ensuring that the only discrepancy in their meal before the experimental trial day was the different supplements. After the dinner until the trial the next day the participants were instructed to stay fasting.

Day 21 clinical trial

The participants arrived by taxi overnight fasted. They finished their first 24-hour urine collection upon arrival at the laboratory and the next 24-hour urine collection was started (day 21). The participants changed into clinical attire and were assigned to a bed until the end of the clinical trial. A catheter was placed anterograde in an antecubital vein where after the first blood samples were taken. Immediately

after these background sample were obtained, a primed continuous tracer infusion was started of D_8-phenylalanine (4 μmol/kg LBM prime, 4 μmol/kg LBM/h continuous), (ring-3,5 D_2)-tyrosine (2.2 μmol/kg BW prime, 0.42 mg/kg/BW/h continuous) and ^{15}N-Urea (84 μmol/kg BW prime, 9 μmol/kg BW/h continuous). Thirty minutes after the start of the tracer infusion a primed continuous infusion of indocyanine green (ICG) (1 mg prime, 14 mg/h continuous) was started. Sixty minutes after the start of the tracer infusion catheters were placed in retrograde position in a radial artery for blood sampling (coupled to a pressurized saline bag for flushing the catheter) and in a femoral artery for ICG infusion. In addition, a femoral vein catheter in antegrade position was placed for blood sampling. Lastly, a hepatic vein catheter was placed under local anesthesia and fluoroscopy guidance through the *vena femoralis* into one of the hepatic veins for blood sampling. An overview of the different catheters is shown in Figure 5. Once all catheters were placed, ICG infusion was moved from the antecubital vein to the femoral artery. Blood sampling was taken prior to the tracer infusion (-100 min, background), and twice immediately before feeding (-12 and -10, repeated sampling, hence analysis results of these are pooled. These are denoted -10), and post feeding at 30, 60, 90, 120, 150, 180 and 240 min. The blood samples were taken simultaneously from all three sites. Five ml was taken for blood gas measures and 10 ml arterial blood and 8 ml blood from the hepatic and femoral veins for tracer, insulin and ICG measures. These blood samples were collected in vials coated with either lithium heparin (samples for insulin and ICG analysis) or K3 EDTA (samples for tracer analysis). After transfer to the sample vials the blood samples were spun down at 3220xg for 10 min. The plasma was transferred to 500 μl eppendorf tubes, frozen and stored at -80°C until further analysis.

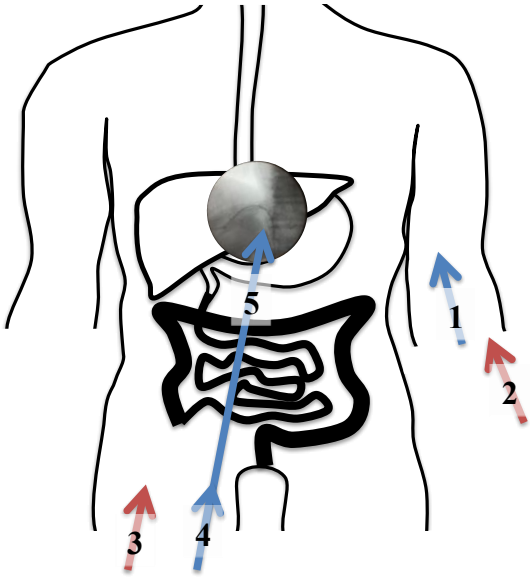

Figure 5: Overview of the catheter placement and usage. 1) Antecubital venous catheter for initial blood sampling and tracer infusion. 2) Radial artery catheter for blood sampling. 3) Femoral artery catheter for ICG infusion. 4)Femoral venous catheter for blood sampling, 5) Hepatic venous catheter for blood sampling.

The first muscle biopsy was taken immediately after the fasting blood samples (-10) was obtained. The second and third muscle biopsies were taken 60 min and 240 min postprandial. Biopsies were taken from the left *m. vastus lateralis*. The bulk midline of the muscle was identified and for each trial period biopsies were taken from the medial or lateral side. Furthermore, the biopsies were taken in random order between the most proximal, the middle and the distal part. There was always at least 4 cm between biopsy sites. The position from which the biopsies were taken can be seen in Figure 6.

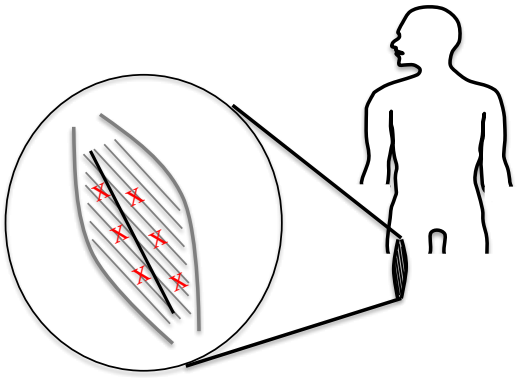

Figure 6: Biopsy locations. The bulk midline of the muscle was identified, and biopsies were taken from either the medial or lateral side pr. trial day. The biopsies were taken in random order between the most proximal, the middle and the distal part. There was always at least 4 cm between biopsy incision sites.

The biopsies were taken under local anesthesia by the percutaneous needle biopsy technique of Bergström [120], attained by a subcutaneous injection of 2-4 ml lidocain. A small incision was made in the skin and fascia and the biopsy was taken using a 5 mm Bergström needle with manual suction. The biopsy was rinsed in saline, put in a 2 ml cryo tube and snap frozen in liquid nitrogen, and stored at -80°C until further analysis. After the biopsies were taken, compression was placed on the incision to stop any bleeding, where after the incision was closed with strips and sterile bandages. Following the first biopsy the participants received a breakfast meal containing 0.61 g protein/kg LBM, of which the majority was intrinsically phenylalanine labeled protein. The intrinsically label allowed tracing the route of the ingested protein from amino acid appearance into the main circulation and the incorporation into proteins (liver synthesized blood protein and muscle). Of the 0.61 g protein/kg LBM, 0.31 g/kg LBM came from D_5-phenylalanine intrinsically labeled whey protein that was mixed with an equal amount of unlabeled whey protein product (Lacprodan® 80, Arla Foods Ingredients Group P/S, Viby J., Denmark) as well as 0.23 g/kg LBM ^{15}N-phenylalanine labeled caseinate protein also mixed with a similar amount of unlabeled caseinate protein (Miprodan® 40, Arla Foods Ingredients Group P/S, Viby J., Denmark). The whey protein was dissolved in water and served as a drink. Simultaneously with the caseinate which was mixed with raspberry jam and spread on a buttered bun. The macronutrient composition for the breakfast is seen in Table 3.

Table 3: Composition of the trial day breakfast.

	Trial day breakfast N=12			% of total kcal		
Calories [kcal]	493.19	±	25.54			
Protein [g/kg LBM]	0.61	±	0.00	28	±	0.75
- D_5-Phe labeled whey [g/kg LBM]	0.31	±	0.00			
- ^{15}N-phe labeled caseinate [g/kg LBM]	0.23	±	0.00			
Carbohydrates [g/kg LBM]	1.06	±	0.04	49	±	0.00
Fat [g/kg LBM]	0.21	±	0.01	21	±	0.54

Values are mean ± SEM.

The participants were instructed to ingest the breakfast within 10 minutes, and as the last bite or drink was swallowed the timer was started (defined as time 0 min).

Following the last biopsy at 240 min the tracer infusion was stopped and catheters were removed. The entrance places of the arterial catheters and the hepatic venous catheter were compressed for 20-30 min before the participant was allowed to sit up. Thereafter, the participants received a standardized lunch meal, containing 0.8 g protein/kg LBM. Where after they were encouraged to void for the last time and the total volume was determined and 3x2 ml was sampled and frozen. The remainder was mixed with the rest of the second 24-hour urine collection, covering 8 am on day 21 till 8 am on day 22.

The participants left the hospital by taxi, and at home they received a standardized dinner containing 0.61 g protein/kg LBM.
At 8 a.m. on day 22 the participants ended their 24-hour urine collection.

45+ day wash out period

Following the first trial, all participants had a wash out-period of at least 45 days. This was to allow time to return to their normal eating behavior and protein intakes as well as healing of the incisions form the muscle biopsies and catheters. During the washout period the participants continued their everyday life and dietary routines as they were prior to the first habituation period.

Second habituation period and trial

The second habituation period occurred exactly as the first one except for a change in supplements.

10.2 Tracers and infusion protocol

Stable isotopes are naturally occurring isotopes that do not disintegrate and exist in low quantities in all molecule and thus in the human body. The most commonly used stable isotopically labelled molecules in clinical studies are labelled with carbon (^{13}C), hydrogen (^{2}H, deuterium, D) or nitrogen (^{15}N). One or more atom(s) in for example an amino acid can be exchanged with one of these stable isotopes making it possible to distinguish that amino acid from the naturally amino acids. Thereby making it possible to trace that specific amino acid. Metabolites containing one or more stable isotope(s) are chemically and metabolically

indistinguishable from the naturally occurring metabolite. However, using mass spectrometry they can be quantified relative to the naturally occurring ones (the tracee). Thus, knowing the amount of the stable isotopically labeled compound (the tracer), they can be used to trace non-labeled amino acids direct incorporation into body proteins or appearance by measuring the dilutions of the tracers.

The tracers used for the current study are:

- L-[ring-D_5]-phenylalanine, denoted D_5-phenylalanine, used for intrinsically labeling whey protein.
- L-[^{15}N]-phenylalanine, denoted ^{15}N-phenylalanine, used for intrinsically labeling caseinate protein
- L-[D_8]-phenylalanine, denoted D_8-phenylalanine, infused
- L-[ring-3,5 D_2]-tyrosine, denoted D_2-tyrosine, infused
- $^{15}N_2$-urea, infused

The choice to use phenylalanine as the labeled amino acid was made for several reasons. Firstly, because it is an essential amino acid that cannot be synthesized in the body, any appearance of phenylalanine in the body must come from either food/protein intake or protein breakdown. Secondly, phenylalanine is not oxidized in skeletal muscle thus the only route of disappearance of phenylalanine from muscle is protein synthesis [121]. Phenylalanine can be degraded/oxidized, primarily by the liver thus whole body disappearance of phenylalanine is not protein synthesis but protein synthesis + breakdown. The first step in phenylalanine degradation is the irreversible hydroxylation of the 4th carbon in the phenol ring by the enzyme *phenylalanine hydroxylase*, forming tyrosine. The reaction is shown in Figure 7.

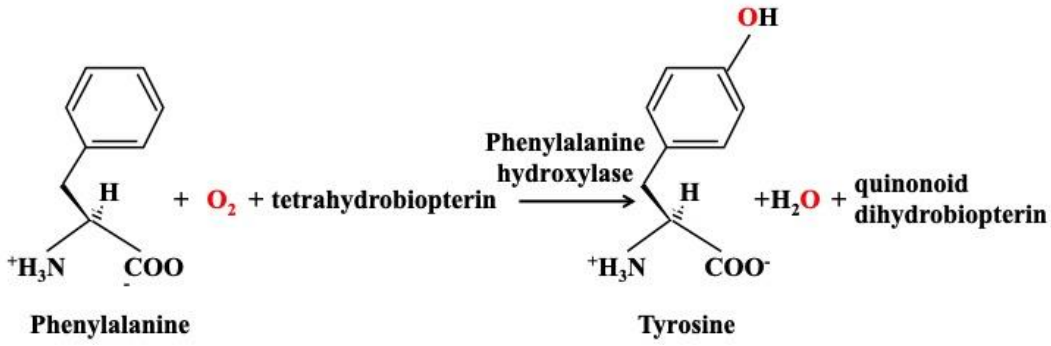

Figure 7: Conversion of phenylalanine to tyrosine[121].

In order to quantify how much of the phenylalanine disappearing from the blood is owed to phenylalanine breakdown to tyrosine, we need to know the conversion rate of phenylalanine to tyrosine. For this, we need to know the systemic tyrosine turnover rate, hence the primed-continuous infusion of D_2-tyrosine tracer. Moreover, the conversion of phenylalanine into tyrosine can be determined by the amount of the various labels, D_8, D_5 and ^{15}N, of phenylalanine into tyrosine (results in D_7, D_4 and ^{15}N-tyrosine, respectively).

Besides conversion to tyrosine, phenylalanine can also transaminate, i.e. reversibly aminate and deaminate, where phenylalanine's keto acid (phenylpyruvate) acts as an amino group acceptor or donor (see figure Figure 8)[122].

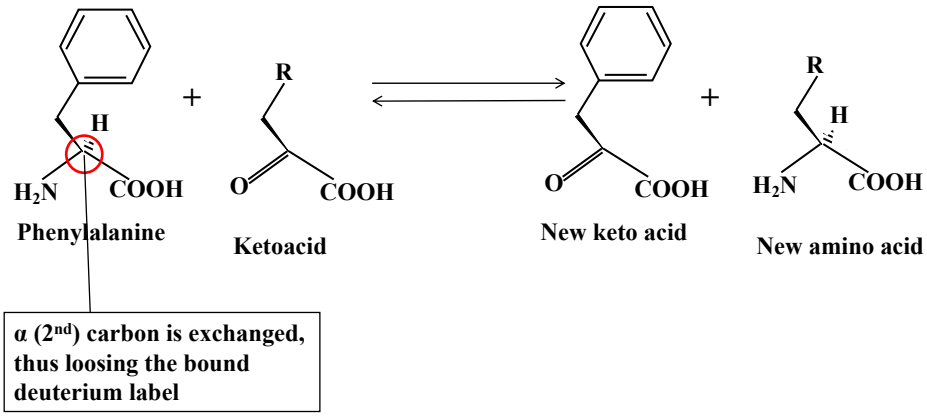

Figure 8: Transamination of phenylalanine to its α-keto acid. The exchange will transfer the deuterium label on α-carbon to the keto acid. Upon re-amination, hydrogen will attach, thus losing one deuterium labeling**.**

As a result, the infused D_8-phenylalanine is also present as D_7-phenylalanine and in turn D_6-tyrosine will be formed in the degradative pathway. For this reason, we have also measured the enrichment of D_7-phenylalanine and D_6-tyrosine.

The $^{15}N_2$-urea tracer allows us to assess the kinetics of urea from the meal, following habituation to the divergent protein intakes. Excess of amino acids are degraded yielding ammonia which to a large extent is converted to urea in the liver. The urea is released into the blood and excreted through the kidneys. As the urea is secreted with the urine, the amount excreted can be measured in urine samples. However, there can be differences between timing of liver urea production and urine excretion [123]. The appearance rate of urea over shorter periods of time can be assessed by a primed continuous infusion of a urea tracer.

All the tracer infusions are primed before infusion start in order to reach a steady state faster. The relationship seen between optimal priming dose and infusion rate is given by:

$$\frac{P}{IR} = \frac{C_u \cdot V}{R_a}$$

Where P=optimal prime, IR=infusion rate [µmol/kg/min], C_u=concentration of unlabeled tracee [µmol/ml], V=volume of distribution [ml/kg] and R_a=rate of appearance [µmol/min].

The urea turnover rate is approximately 9 %/h [124], which is why the infusion rate is relatively low (9 µmol/kg BW/hr) but the priming dose relatively large (84 µmol/kg BW) due to the large urea pool size. Practically, this means that the priming dose for urea is a determining factor for the level of enrichment achieved in the first few hours.

10.2.1 Production of intrinsically labeled proteins

The intrinsically labeled proteins that were used in this study were labelled with L-[ring-D_5]-phenylalanine for the whey protein and L-[^{15}N]-phenylalanine for the caseinate protein. The milk production took place at Aarhus University Foulum (Department of Animal Science) and the milk processing into whey and caseinate took place at Arla (Arla Foods, Nørre Virum, Denmark). The production was finalized prior to the start of this PhD. The production of the intrinsically labeled proteins have previously been described by Reitelseder et al. using the same procedure to produce L-[1-^{13}C]-leucine labelled whey and caseinate [125]. In brief steps, the production complied with the guidelines of the Danish Ministry of justice (Act No. 726, 1993). Five Danish Holstein Friesian cows with an average body weight of 676± 92 kg were used for the milk production. Two catheters were inserted in the right and left jugular vein under local anesthesia. One for tracer infusion and one for blood collection. Catheters were secured by skin sutures. Four cows were infused with L-[ring-D_5]-phenylalanine (180 g, 98 atom %; Cambridge Isotope Laboratories, Tewksbury, MA) and one cow with L-[^{15}N]-phenylalanine (180 g, 98 atom %; Cambridge Isotope Laboratories). The infusion solution per cow was made in 3x5 l of 0.9% NaCl. The infusions lasted 72 hours with an infusion rate of 208 ml/h (14.7 mmol/h for D_5-phenylalanine and 15.1 mmol/h for ^{15}N-phenylalanine), while the

cows had ad libitum feed and water. Milk was collected from 9 milkings, 3 pr. day, during infusion and 2 milkings after termination of the tracer infusions. The milk was stored at 2-3 ºC immediately post milking and once all milking's were done the milk was pasteurized (71-72ºC, 15 sec) and skimmed. The caseinate and whey protein fractions were purified and dried by Arla Foods (Arla Foods, Nørre Virum, Denmark). The dry powders were analyzed for quantity of label incorporation and amino acid content. Moreover, microbial quality was determined to allow for human ingestion.

10.3 Mass spectrometry

The analysis of amino acid concentrations and tracer enrichments were carried out by 3 different mass spectrometry systems. An overview of the machines is seen in Figure 9.

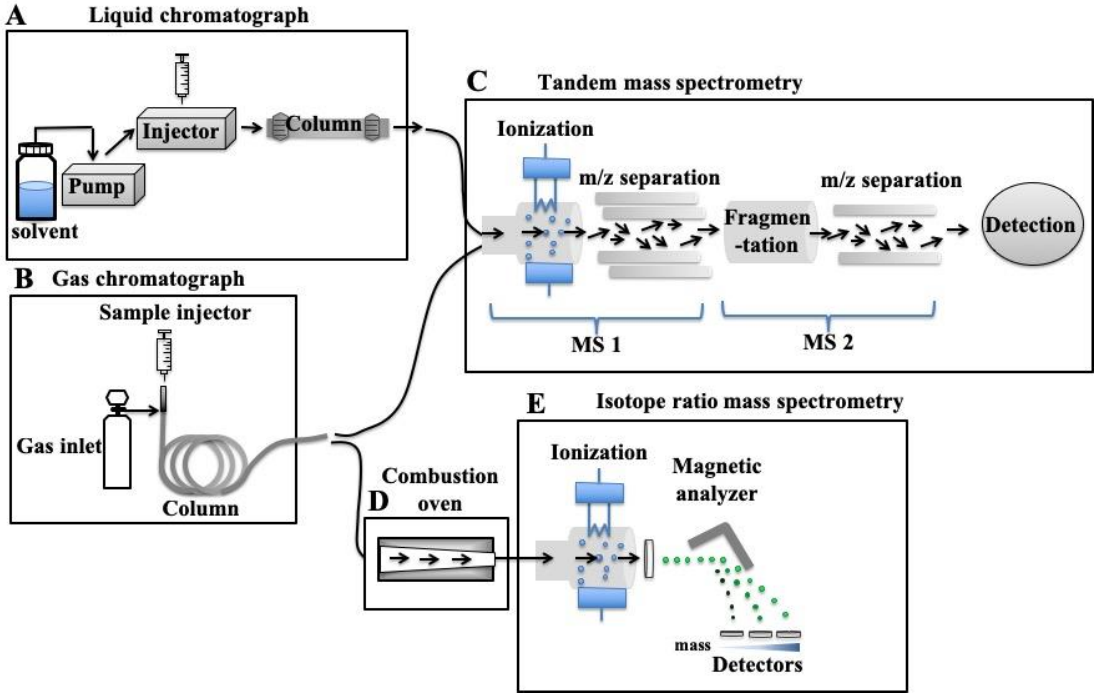

Figure 9: Overview of machines used for mass spectrometry analyses. A) liquid chromatograph, and B) Gas chromatograph both of which are coupled to a C) tandem mass spectrometer, D) Combustion oven (the combustion of molecules to CO_2 or N_2, and E) shows the isotope ratio mass spectrometer

The tandem mass spectrometer (Figure 9C) separates the metabolites by their mass to charge ratio. In case of a GC-MS/MS, this is done by bombarding the metabolites with electrons (electron impact) and separating the formed loaded fragments. Selected

fragments (mass/charge ratio) pass through to the next quadropole mass analyzer and are further fragmentated by a collision gas (argon). These fragments are then separated on the third quadrupole and detected. In case of a LC-MS/MS the liquid phase containing the metabolites is heated and sprayed into an interface (ESI probe, electron spray ionization) which give the metabolite a charge. Mass/charge separation and further fragmentation, separation and detection is similar as for the GC-MS/MS system.
The isotope ratio mass spectrometer is a sector magnet mass-spectrometer that works in a rather different way; described below.

10.3.1 Gas chromatography combustion -isotope ratio- mass spectrometry

The Gas chromatography combustion isotope ratio mass spectrometry, GC-C-IRMS is illustrated in Figure 9B, D, E. First and important step is the perfect separation of all metabolites by the use of gas chromatography. The heating program used is often a long program in order to ensure perfect separation before the metabolite enter the oxidation ovens. The combustion oven (940 °C) yielding for amino acids N_2, CO_2 and H_2. Therefore, it is clear that each metabolite has to be pure before it enters the combustion oven as there is no molecule separation on mass (mass/charge) on such a system but only measurement of CO_2, N_2 or H_2. Following combustion, the CO_2, N_2 or H_2 charged by bombardment with electrons and subsequently accelerated before it come at the magnet were the curvature of the heavier molecule $^{13}CO_2$, ^{15}N and $^{2}H_2$ is less than the lighter CO_2, N_2 and H_2. Two detectors, that are spaced from each other, count the number of molecules of the heavier and lighter molecule. Depending on the magnet current either CO_2, N_2 or H_2 can be analyzed.

In this study the GC-C-IRMS is used to measure ^{15}N-phenylalanine in blood protein and myofibrillar proteins. Thus, the atom of interest following combustion is nitrogen gas, N_2. As amino acids only have one nitrogen atom, this is optimal for the GC-C-IRMS analysis. The GC-C-IRMS cannot differentiated between molecules, thus when looking for the labeled nitrogen, this will not be diluted by other nitrogen atoms from the same amino acids, as would have been the case if we were looking e.g. at a carbon or worse hydrogen labels. However, as the detector detects N_2, it takes

two labeled phenylalanine molecules for one “hit”, thus this setup requires relatively large quantities of sample for analysis.

10.4 Sample preparation flow overview for mass spectrometry analysis

As seen in Figure 10, the different samples are treated similar independent of being muscle or plasma samples. However, the muscles need to go through a homogenization step, and either freeze drying and cleaning or fractioning.

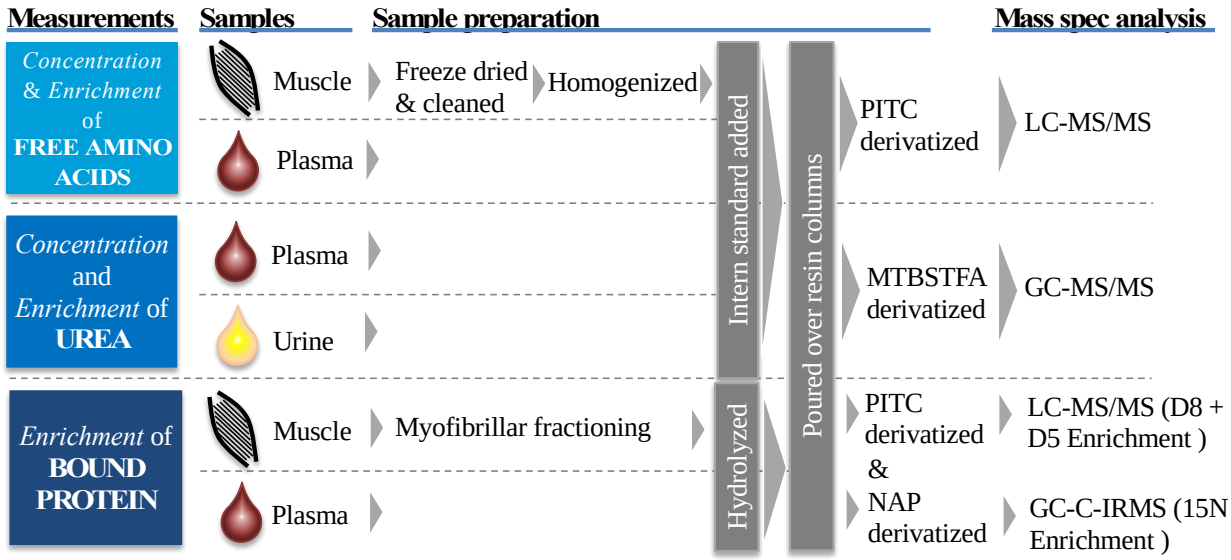

Figure 10: Overview of sample preparation for Mass spec analysis. Muscle samples undergo a few extra steps in preparation for mass spectrometry compared to plasma and urine samples. Concentration and enrichments of free amino acids is analyzed using LC-MS/MS. Concentration and enrichments of urea is analyzed using GC-MS/MS. Enrichments of bound protein is analyzed by both LC-MS/MS and GC-C-IRMS.

10.4.1 Preparation to analysis of tracer-protein bound abundance

The muscle specimens were first homogenized by placing the frozen muscle piece in a lysing matrix tube containing 10 bullets (Lysing matrix D Bulk, MP Biomedicals) and 2 silicon carbide beads (BioSpec), as well as 1 ml of homogenization buffer (0.02 M, pH7.4 Tris, to maintains steady pH; 0.15M NaCl to keep solution saline, 2mM EDTA + 2mM EGTA which chelates metal and salt ions; 0.5% TritonX-100 which permeabilizes membranes; 0.25 M sucrose, which decreases protein solubility of especially myosin heavy chain). Thereafter shaking them 5 times 45 seconds and speed 5.5 on a Fastprep (FastPrep®-24 machine, MO Biomedials, Illkirck, France)

and spun at 800xg, at 4°C for 20 min. The pellet containing the structural proteins was kept and the supernatant discarded.

The second step was to solubilize and separate the myofibrillar proteins and keep the collagenous proteins precipitated, by adding 1.5 ml Pyrophosphate/Potassium-buffer to the protein pellet [126], homogenizing it once more, and spinning at 800 g, 4°C for 20 min. This step was repeated, with the sample being left in the PyP-buffer over-night (0.1 M to solubilize myofibrillar proteins and 0.7M Potassium chloride, 0.7 as the high salt solution precipitates collagen protein). Following the centrifugation on day 2 the supernatant (the myofibrillar protein fraction) was transferred to new vials and 2.3 x volume of 99% ethanol was added and vortexed. This causes the proteins to precipitate. The sample was left for 2 hours, where after it was spun down at 1600xg, 4°C for 20 min. The supernatant was discarded, and the pellet was washed in 1 ml 70% ethanol, vortexed and spun at 1600xg, 4°C for 20 min. The supernatant was discarded and the myofibrillar proteins in the pellet saved. An overview of the myofibrillar fractioning procedure can be seen in Figure 11.

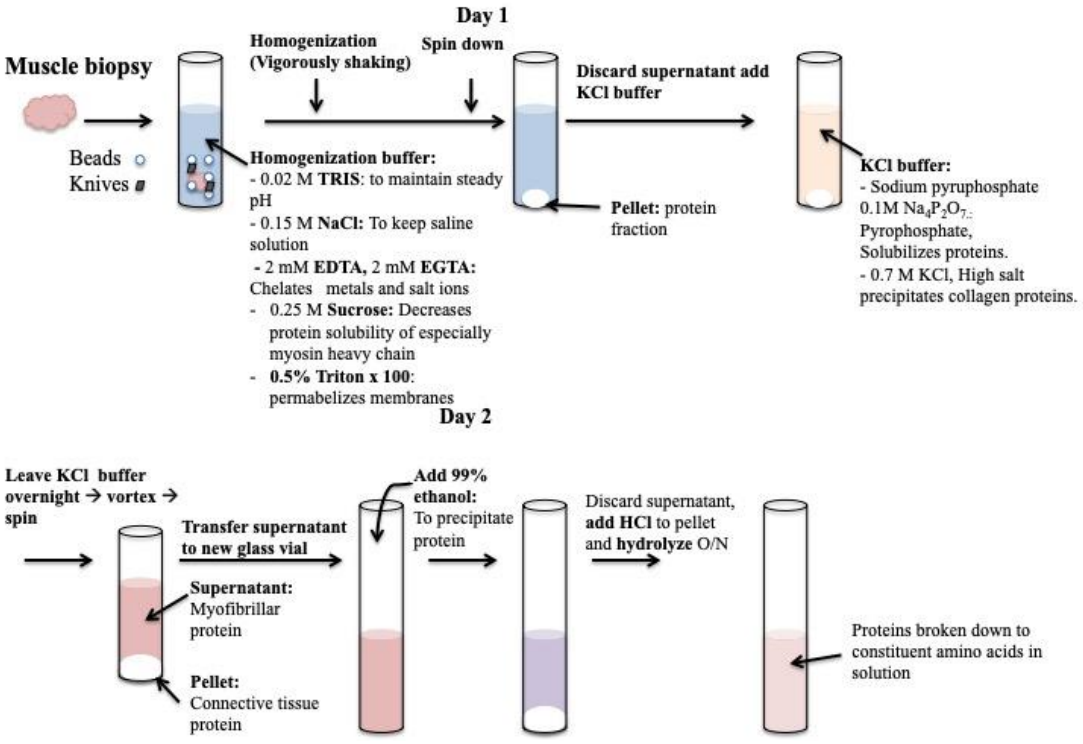

Figure 11: Muscle fractioning overview.

Having isolated the proteins of interest, the proteins were hydrolyzed into their constituent free amino acids. This was achieved by the addition of 1 ml 1 M HCl + 1

ml resin slurry and left overnight at 110°C. The final hydrolyzes step is the same for plasma protein and myofibrillar protein, and from this step the liberated amino acids were purified over cation exchange resin.

The hydrolyzed myofibrillar or plasma proteins were poured over pre-prepped resin columns. Firstly, the resin was pre-prepped by adding 1 ml of acidic acid to the resin columns. The sample was added, and the column thoroughly washed with 5x1.5 ml dH_2O. Following the washes, the amino acids were eluted from the resin by addition of 2x1 ml 2M NH_4OH, which was collected in vials. The samples were subsequently dried under a nitrogen flow at 70 °C. The procedure is depicted in Figure 12.

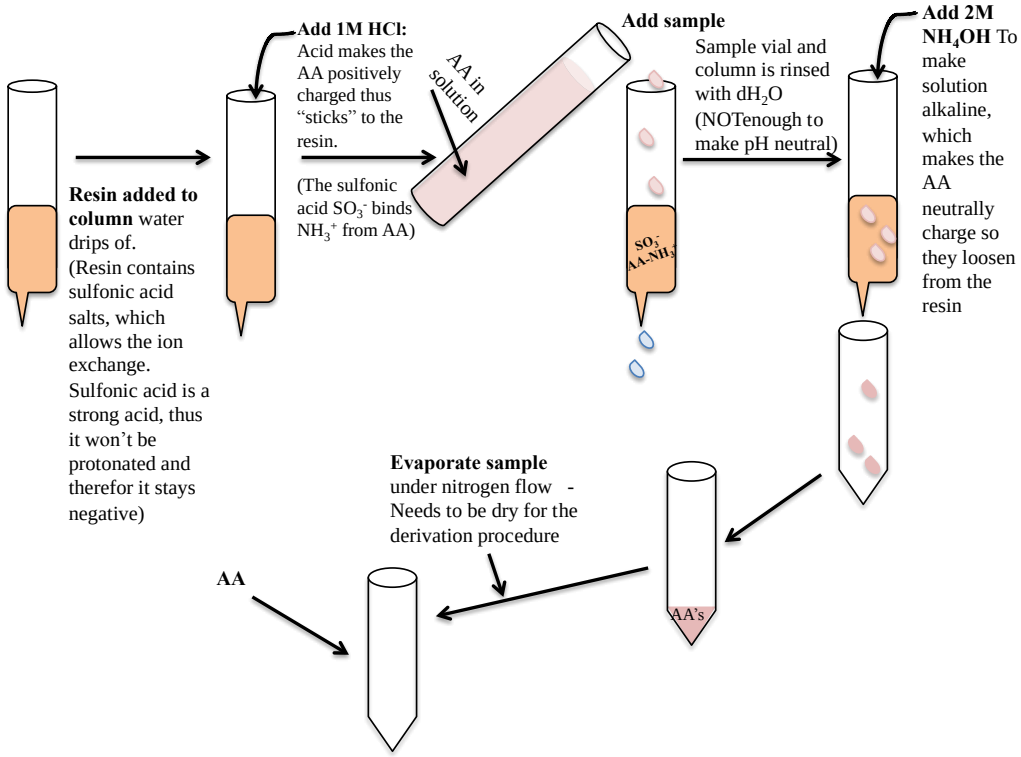

Figure 12: Overview of resin purification

The purified amino acids were then derivatized. For LC-MS/MS analyses the PITC derivatization procedure was applied[127]. Firstly, 20µl coupling buffer (Methanol: Milli-Q Water: Triethylamin (2:2:1, %, v/v)) was added to each sample, which was then vortexed. To remove excess reagent, samples were completely evaporated under a N_2 stream, for approximately 10-15 min. 20 µl fresh derivatization solution

(triethylamin: Milli-Q water: PITC:methahol (1:1:1:7, %, v/v)) was added and the samples vortexed and incubated at room temperature for 30 min to convert the amino acids into their phenylthiocarbamyl (PTC) derivatives. The PTC derivatives were dried under N_2 stream for approximately 30 min at room temperature, and re-dissolved in 100 µl mixture of acetonitrile, methanol, Mili- Q® purified water and 0.1 M ammonium acetate.

For GC-C-IRMS analysis (^{15}N-phenylalanine) NAP derivatization was applied. The hydrolyzed fractionated muscle and blood samples were dried completely under N_2 flow. 200 µl Propyl Acetate and 100 µl Boron Triflouride Propanol was added and samples were vortexed and heated at 120 °C for 30 min. After this the solvent was evaporated under N_2-flow in a heading block at 70°C for 8-10 min. While the samples were in the heating block 100 µl Boron Triflouride Propanol was added and dried. Following this 50 µl Acetonitril, 26 µl 1.4-Dioxan, 38 µl Triethylamine and 24 µl Acetic Anhydride was added with vortex mixing in between. The samples were put in a 55 °C oven for 15 min. All sample material was transferred to a 1.5 ml Eppendorf tube, and 50 µl Chloroform and 2x 75 µl 0.001 M $NaHCO_3$ was added with vigorous vortex mixing in between. The samples were spun down and the top aqueous layer was removed yielding the derivative which was saved at -20 °C until further analysis.

For blood and urine urea analysis to 50 µL of sample was added 50 µl internal standard and 500 µl acetic acid. The samples were then transferred onto a cation exchange column and after separation and overnight drying under a N_2 stream the samples were derivatized by adding 40 µl acetonitrile and 40 µl N-methyl-N-(tert-butyldimethylsilyl) trifluroroacetamide (MtBSTFA) + 1% tert-butyl-dimethylchlorosilane (tBDMCS). The samples were then vortexed and kept at 70ºC for 30 minutes. The derivatized samples were transferred to a GC-vial and injected into the GC-MS/MS system.

10.4.2 Preparation to analysis of free amino acids in muscle and plasma

The muscle biopsies were freeze dried and dissected free of visible blood, connective tissue and fat under a microscope in a 18°C room with <30% humidity. To the cleaned fibres 1 ml perchloric acid and internal amino acid standard mixture was

added before homogenization on ice using a glass rod for double extraction of free amino acids. The samples were centrifuged, and the supernatant was collected following each of the two centrifugations steps.

For the plasma samples, 100 µl combined internal standard and 120 µl 50% acetic acid (Merch, Darmstadt Germany) was mixed with 100 µl plasma.

Acidified plasma and muscle were then loaded on a resin column, following same procedure as explained for the preparation of muscle and plasma protein bound samples, illustrated in Figure 12.

The dried purified amino acids were also converted in to their ((PTC) derivatives, as explained for the preparation of muscle and plasma protein bound samples.

10.5 Metabolite kinetic calculations

Human *in vivo* metabolite kinetics quantitation by means of metabolite dilution techniques for the whole body or for a particular tissue or tissues is complex and awareness of limitations of the various modes/models is mandatory. With regard to the whole human body metabolite kinetics the body is usually viewed as a single pool (bag of water) when using the primed continuous tracer infusion methodology. Under steady state condition this model is fine. However in non-steady state conditions precautions needs to be taken as mixabilities and metabolism of the metabolites in the various pools can effect changes measured in the blood. With the same technique but with measurement across a tissue bed(s) a two pool model (arterial-tissue venous differences) or a three pool model can be used (arterial-tissue venous + tissue biopsy) to determined metabolite kinetics for a tissue(s), as depicted in Figure 13:

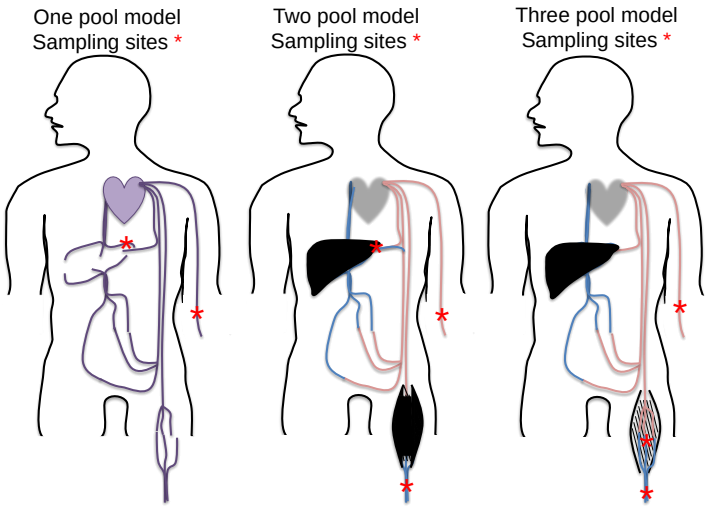

Figure 13: Model of 1, 2 and 3 pool models. The purple color in the one pool model represents that the whole body is viewed as one pool. In the two pool model the black boxes on the muscle and liver illustrates that we know what is occurring across the tissue, but not within. The reveling of the muscle in the three-pool model, represents knowledge of fluxes within the muscle. The red stars show sampling sites for each of the models.

Amino acid concentration with mass-spectrometry

Mass-spectrometry as such is not entirely quantitative but with the use of labelled metabolite internal standards it has become the gold standard in bioanalysis. The concentrations of the amino acids are calculated as the ratios between the peak area of the internal standards, with at known concentration, and the peak area of the individual amino acids.

The concentrations of the labeled amino acids are in turn calculated as the concentration of the unlabeled amino acid multiplied by the enrichment of the labeled amino acids, given in tracer-to-trace ratio (TTR), thus:

$$Conc_{labelled\ aa} = Conc_{unlabelled\ aa} \cdot \frac{Labelled\ aa}{unlabelled\ aa}$$

For all further calculations, the enrichment is given as mole percent excess (MPE=TTR/(1+TTR)).

10.5.1 One pool model

Measurements of whole body amino acid kinetics assume a constant well mixed single pool from where the samples are taken, in this case the blood/plasma. This steady state perturbated by any intervention, which feeds the system, i.e. feeding

like in the present study. In this case the intake of a breakfast meal high in protein causes the amount of circulating amino acids to increase which will have many effects that may change the uptake of amino acids by- and release from-tissues and possible the intracellular tissues storage. In other words, the exchange to and from the blood is not necessarily in equilibrium with the tissues. This non-steady state situation has been recognized for glucose kinetics by Steele [128], who proposed calculations accounting for the non-steady. The shift in pool size, seen in none steady state is assumed to be similar in the all tissues. However, it was noticed that not all tissues seemed to participate and this was accounted for by using the partial volume of distribution with the ratio between 'change in concentration of tracee (Δc [μmol/l])' and the 'change in time (Δt [min])', thus:

$$Pool\ size = pV \cdot \frac{\Delta c}{\Delta t}$$

pV, the partial volume of distribution is set at 0.125. Defined as the total volume that the target of interests is distributed in. If there is an even distribution between plasma and tissue, the volume of distribution can be defined as the total amount of tracer in the body (i.e. the infused and ingested tracer) divided by the tracer concentration in the plasma, thus $V=tracer_{body}/tracer_{plasma}$.

We have applied the Steele equation with modification introduced by Proietto et al. [129] . An approach which have been applied several times previously for amino acid and protein kinetics [113,130–132]. The modified Steele equations were used for all one-pool kinetics calculations of samples taken in the four-hour postprandial period. This requires calculations between two time points, thus for these measurements, values are calculated to the fasting time point -10, and postprandial at time 15, 45, 75, 105, 135, 165 and 210 min.

Amino acid uptake kinetics

Amino acids, absorbed from the oral intake of the protein rich breakfast, enter the circulation via the liver. Thus, when assessing the rate at which the amino acids from the breakfast meal enter the circulation, these calculations are based on enrichments and concentrations in plasma from the hepatic vein.

To calculate the rate of appearance (R_a) of phenylalanine from the intrinsically labeled whey and caseinate (the exogenous R_a (Exo R_a)) the total R_a is needed. Both are given by:

In the fasted, steady-state

$$Total\ R_a = \frac{IR}{E_{(t)}}$$

$$Exo\ R_a = \frac{Total\ R_a \cdot\ dE_{OT(t)}}{E_{protein}}$$

In the postprandial, non-steady state

$$Total\ R_a = \frac{IR}{\hat{E}_{(t)}} - \frac{pV \cdot \left[\frac{\hat{C}_{(t)}}{1+\hat{E}_{(t)}}\right] \cdot \left[\frac{dE_{(t)}}{dt}\right]}{\hat{E}_{(t)}}$$

$$Exo\ R_a = \frac{Total\ R_a \cdot\ dE_{OT(t)} + pV\ \cdot dC_{(t)} \cdot \left[\frac{dE_{(t)}}{dt}\right]}{E_{protein}}$$

Where IR= tracer infusion rate [µmol/kg LBM/min], $E_{(t)}$= enrichment [MPE] at time, t; $dE_{OT(t)}$= delta/change in plasma enrichment of the oral tracer (D_5-phe or ^{15}N-phe) at time, t; $\hat{C}_{(t)}$= average concentration (labeled and unlabeled) between values surrounding time, t [µmol/l]; $\hat{E}_{(t)}$=average enrichment between values surrounding time, t; $dE_{(t)}$=delta enrichment in plasma between values surrounding time, t; dt=delta time; and $E_{Protein}$=the enrichment of the intrinsic label in the ingested protein.

Whole body protein turnover

Protein turnover is a term covering the kinetics of synthesis of new body proteins and breakdown of existing body proteins. The difference between the two kinetic rates is the net balance. As noted in section "10.2 Tracers and infusion protocol", phenylalanine cannot be synthesized in the body. Thus, any appearance of phenylalanine that is not coming from the ingested or infused must originate from breakdown. Thus, as a measure of whole body breakdown rate, we must calculate the endogenous (Endo) R_a, given by:

$$Endo\ R_a = Total\ R_a - Exo\ R_a - IR$$

The formula is the same in the fasted and postprandial state however, the appearance rates used are the ones calculated in the fasted, steady state and in the postprandial, non-steady state, respectively.

Removal of phenylalanine occurs by incorporation into body proteins, or by oxidation and thus conversion to tyrosine (phe→ tyr). Calculated by:

In the fasted, steady-state

In the postprandial, non-steady state

$$Phe \rightarrow tyr = Total\ R_a \cdot \frac{E_{tyr}}{E_{phe}} \qquad Phe \rightarrow tyr = Total\ R_a \cdot \frac{\hat{E}_{tyr}}{\hat{E}_{phe}}$$

$$Total\ R_d = Total\ R_a \qquad Total\ R_d = pV \cdot \frac{dC}{dt} + Total\ R_a$$

$$Endo\ R_d = Total\ R_d - (Phe \rightarrow tyr)$$

10.5.2 Tissue metabolite kinetics

Samples taken from both a hepatic and femoral vein allow analysis of exchanges of phenylalanine across the splanchnic bed and across the leg simultaneously. The two-pool model will not give us an insight as to what happens inside the tissue with respect to potential changes in concentration and hence enrichment. However, as we only need blood samples a high time resolution is achieved with relative noninvasive methods.

The artery will deliver amino acids to a tissue, where they can be incorporate as proteins. Thus, a positive arterial – tissue venous (A-V) concentration difference across the leg will mean that amino acids have disappeared, i.e. increased net protein synthesis. In contrast, a negative A-V concentration difference across the leg, will represent higher net protein breakdown, due to an increased release of amino acids from the leg. We must know the blood flow to the leg in order to assess the net protein balance. More information on blood flow can be found in section "10.6 Plasma flow". The net balance across the leg is given by:

$$Femoral\ NB = \left(C_a - C_{fv}\right) \cdot PF_{femoral}$$

Where C_a and C_{fv} are the amino acid concentration (labeled and unlabeled) in the artery and femoral vein respectively, and $PF_{femoral}$= leg plasma flow [l/min].

Historically differnet approaches have been employed when assessing the rate of appearance and rate of disappearance using the two-pool model. Smith et al. compared various calculations with the use of enrichments given in TTR and MPE and reached the conclusion that two sets of equations could be used, with the enrichments givens as MPE [133]. The sets of equations were 1) a set of equations

proposed by Thompson in 1989 [134], calculating the R_a first and then calculating the R_d as the sum of R_a and the net balance. However this method was only valid with the correction factor proposed by Nair et al. in 1992 [135] 2) The set of equations proposed by Smith et al. calculating the R_d and R_a is calculated as the R_d-NB.

Both sets of calculations were done with the exact same results. The equations presented below are the equations proposed by Smith et al [133].

The rate of disappearance of phenylalanine across the leg was given by:

$$Femoral\ R_d = (C_a \cdot E_a\ - C_v \cdot E_v\ ) \cdot\ PF/E_a$$

Where E_a and E_v is the D_8-phenylalanine enrichment in the artery and femoral vein, given in MPE.

The rate of appearance of phenylalanine across the leg was calculated as the difference between the rate of disappearance and the net balance:

$$Femoral\ R_a = Femoral\ R_d - NB$$

A-V differences across the splanchnic bed.

The intend in adding a hepatic venous catheter was to gain information about the amino acid secretion from the first pass splanchnic extraction. The amino acids enter the circulation from the hepatic vein, thus a negative net balance, where the venous concentrations is higher than the arterial concentration, represents secretion of amino acids into the circulation and a positive net balance will represent increased removal of phenylalanine by the liver, e.g. by oxidation to tyrosine. However, the results can be hard to interpret when looking at the net balance across the splanchnic bed, as the entire GI tract has a venous outflow and the hepatic artery has arterial outflow into the liver. This means that not only does amino acids from digestive tract have an outflow in the liver, but also the recirculation of amino acids in the artery, and this before reaching the hepatic vein, which is the sampling site.

The net balance across the splanchnic bed is given by:

$$Splanchnic\ NB = (C_{hv} - C_a) \cdot PF_{Splanchnic}$$

Where C_{hv}=the amino acid concentration (labeled and unlabeled) in hepatic vein, and $PF_{splanchnic}$= splanchnic plasma flow [l/min].

Three pool model

The three-pool model looks at the fluxes of amino acids between arterial and venous blood as well as the intramuscular amino acids concentration and enrichments as depicted in Figure 14.

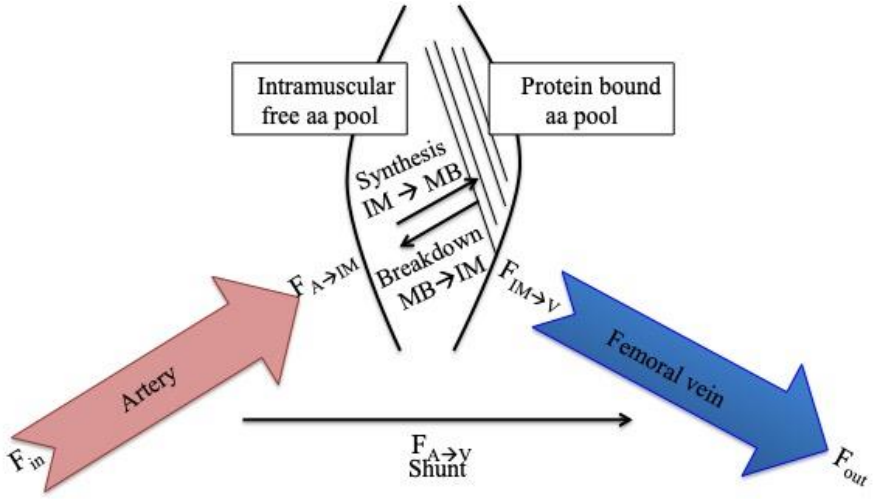

Figure 14: Overview of the three pools in the three-pool model, constituting the arterial pool, the intramuscular pool and the venous pool. F denotes flow, and the subscript is the flow direction e.g. $F_{A\rightarrow IM}$ is the flow from artery to intramuscular compartment.

The assumptions of the three-pool model are similar to the assumption for whole body calculation with regard to the choice of amino acid tracers. The labeled amino acid must not by synthesized in the body and may not be oxidized in the tissue of interest, in this case the muscle.

The metabolite uptake by and release from the different compartments are given by:

$$F_{In} = C_A \cdot PF_{femoral}$$

$$F_{A\rightarrow IM} = \left(\left(\frac{E_{IM} - E_V}{E_A - E_{IM}}\right) \cdot C_V + C_A\right) \cdot PF_{Femoral}$$

$$Net\ balance = (C_A - C_V) \cdot PF_{Femoral}$$

$$Synthesis_{IM\rightarrow MB} = Breakdown_{MB\rightarrow IM} + NB$$

$$Breakdown_{MB\rightarrow IM} = F_{A\rightarrow IM} \cdot \left(\left(\frac{E_A}{E_{IM}}\right) - 1\right)$$

$$F_{out} = C_V \cdot PF_{femoral}$$

$$Shunt\ F_{A\rightarrow V} = F_{IN} - F_{A\rightarrow IM}$$

$$Efficacy = \frac{S_{IM \rightarrow MB}}{F_{IN}}$$

Where F_{in}=the flow of phenylalanine in the artery feeding the muscle tissue, C_A=phenylalanine concentration in the artery, $PF_{femoral}$=Plasma flow in leg, $F_{A \rightarrow IM}$=flow of phenylalanine from the artery to the intramuscular compartment, $E_{IM/V/A}$= $D_{7/8}$-phenylalnine Enrichment in the intracellular compartment, vein or artery respectively (IM/V/A), $Synthesis_{IM \rightarrow MB}$=flow of phenylalanine from the intramuscular interstitial space to the muscle-bound protein pool, thus a measure of synthesis. The $Breakdown_{MB \rightarrow IM}$=is the flow of phenylalanine from the muscle-bound protein pool to the interstitial space, thus a measure of breakdown. F_{out}=flow of phenylalanine in the vein draining the muscle, Shunt $F_{A \rightarrow V}$=the flow of phenylalanine being shunted directly from the artery to the vein and lastly the Efficacy= a measure of how much of the phenylalanine presented to the muscle via the artery is actually used for muscle protein synthesis.

Direct incorporation

Direct incorporation entails measuring the protein bound enrichment in the tissue, in this case we analyze incorporation into myofibrillar and plasma protein. We do this by measuring the enrichment (given in MPE for the D_5-phenylalanine enrichment from whey, and in APE for the ^{15}N-phenylalanine enrichment from caseinate) and thus the presence of phenylalanine from the whey and the caseinate in the breakfast meal in the myofibrillar and plasma proteins in the four-hour postprandial period. We also calculated the myofibrillar and plasma fractional synthesis rate (FSR) based on the infused D_8-phenylalanine (thus $D_{8/7}$-phenylalalnine enrichments). The myofibrillar FSR is based on the biopsies taken before and 240 min postprandial, and the plasma FSR is based on blood samples taken before as well as 60 and 240 min postprandial. This allows us to determine the FSR in the whole postprandial period (0-4h) and the immediate postprandial period (0-1h). FSR is given by:

$$FSR = (\frac{\Delta E\ Myofibrillar/plasma\ [MPE]}{E\ Precursor\ pool\ [MPE]} * \Delta time\ [h]) * 100\%$$

Where the ΔE Myofibrillar/plasma= the difference in myofibrillar/plasma $D_{8/7}$-phenylalanine enrichments between the two time points of interest, Δtime=time between biopsies and E Precursor pool MPE = the precursor enrichment in the two time points of interest. For the plasma FSR the precursor pool is the enrichment in blood taken form the hepatic vein. For myofibrillar FSR, the best estimate of the real precursor pool is the enrichment of the free amino acid tracer in the intramuscular compartment [136]. However, the enrichment is only measured in the intramuscular compartment at the time points of the biopsy, and thus there is an assumption of steady state in the period of interest. However, due to dilution by the meal intake, the infused $D_{8/7}$-phenylalanine tracer is not in steady state throughout the entire trial period. In order to correct for this, a higher time resolution for the enrichment is needed. The relationship between the intramuscular enrichment and the arterial enrichment to the biopsy sampling times, where multiplied with the weighed mean of the arterial enrichment in the entire FSR period. Thus the precursor pool for the myofibrillar FSR used in this study was calculated as:

$$Precursor\ pool = \left(\overline{mean}\left(\frac{Intramuscular\ E[MPE]}{Artery\ E\ [MPE]}\right)\right) * \overline{mean}\ artery\ E\ [MPE]$$

Where $\overline{mean}$= the weighed mean over the period of interest.

10.6 Plasma flow

Plasma flow across the leg and the splanchnic bed was measured using a primed continuous infusion of indocyanine green (ICG) into a femoral artery. ICG is a dye, which binds tightly to plasma proteins and is confined to the vascular system. Removal of ICG occurs exclusively through the liver, with a half-life of 3-4 min. Measurements of flow by ICG relies on the Fick principle as previously described by Henriksen et al. [137]. Stating that the release or uptake of a substance, in this case ICG, is the product of blood flow multiplied with the A-V difference. Thus, solving for flow, flow can be measured by:

$$Plasma\ flow = \frac{ICG\ influx}{C_{ICGa} - C_{ICGv}}$$

The plasma ICG concentration was determined by measuring the absorbance by spectrophotometry at ICG peak absorbance at wavelengths 805 and the subtracting the sample turbidity at measured at 900 nm for each sample. The concentrations in each sample was assessed by comparing the sample absorbance with the absorbance of a standard curve with known concentrations. The calculation of flow was based on the difference in ICG concentration found in the artery and hepatic and femoral venous blood, thus the dilution across the splanchnic and femoral bed was calculated.

10.7 Insulin

Plasma samples, converted to serum by use of 1unit thrombin pr. 200 µl EDTA plasma, were analyzed for insulin, using ALPCO Insulin ELISA assay (Catalog number: 80-INSHU-E01.1, E10.1, ALPCO). The exact reagents used by ALPCO are not known, however briefly, the procedure was that 25 µl serum sample was loaded onto a 96 well plate. 100 µl insulin detection antibody was added, and the plate was incubated for one hour at room temperature at mild shaking. Afterwards the wells were washed and 100 µl TMB substrate (fluorescent substrate) was added and the plate was incubated for 15 minutes at room temperature, mild shaking. Following this 100µl stop solution was added and the plate was read at 450 nm exactly 5 minutes after.

An identical standard curve with known concentrations was added to all plates, and the absorbance with backgrounds subtracted were converted to insulin concentration and plotted on a double logarithmic scale. Based on the standard curve the concentrations of the samples were calculated. A control samples with known concentration was added to each plate, as well as one sample, made from a mix of serum from all participants, was loaded on all plates to check for plate to plate deviations.

10.8 PCR

The mRNA expression in the muscle tissues of genes coding for growth factors (IGF1, MGF and Myostatin), breakdown (Atrogin1 and MURF1), amino acid transporters (SNAT2, LAT1 and PAT1), and myokine related proteins (FGF21) was

tested using real time RT-PCR. The selected targets and their corresponding primers are listed in table Table 4.

Table 4: Primers for real time PCR analyses

mRNA	Sense	Antisense	Ref sequence
RPLP0	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG	NM_053275.3
GAPDH	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT	NM_002046.4
IGF1	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC	NM_000618.3
MGF	GCCCCCATCTACCAACAAGAACAC	CGGTGGCATGTCACTCTTCACTC	NM_001111283.2
Myostatin	TGCTGTAACCTTCCCAGGACCA	GCTCATCACAGTCAAGACCAAAATCC	NM_005259.2
Atrogin1	TGTTACCCAAGGAAAGAGCAGTATGGA	ACGGAGCAGCTCTCTGGGTTATTG	NM_058229.3
MURF1	TGGGGGAGCCACCTTCCTCT	ATGTTCTCAAAGCCCTGCTCTGTCT	NM_032588.3
PAT1	ACCCCAGCCACCTCCCCTTG	GAACTTCCGAGGATCCTTCATTTTG	NM_078483.4
SNAT2	AAACACCACCTTAACACAGCCAACA	TGAAAAGATCAGAATTGGCACAGCA	NM_018976.5
LAT1	GTGGCTCCTCCAGGGCATCT	CTCGGCCTCCTGGCTATGTCTC	NM_003486.7

10.8.1 Muscle homogenization and RNA isolation

Approximately 10 mg of frozen muscle was transferred to 2ml tube (Biospec), which contained 1000 µl TriReagent (Molecular research center, Cincinnati, OH, USA), five stain-less steelbeads (2.3 mm, BioSpec Products, Bartlesville, Oklahoma, USA), and one silicon-carbide sharp particle of 1mm (BioSpec Products). For every two samples added to the tube, the tubes were placed in a FastPrep®-24 machine (MO Biomedials, Illkirck, France) and run for 15 seconds at speed 4. Once all samples had gone thorough one round in the fast prep, all samples went through a second and third round. Following homogenization, 100 µl bromo-chloropropane was added. Following vortex and a spin at 1200xg for 15 minutes, samples were separate into an upper aqueous RNA containing phase, a DNA containing interphase and lower organic red phase containing the RNases. The upper phase was transferred to fresh 1.5 ml Eppendorf tubes, and an equal volume of isopropanol was added to precipitate the RNA. The samples were spun at 12000xg for 8 minutes, where after supernatant was removed. The pellet was dissolved in first 96 % and washed in 75 % ethanol, where after it was dried and dissolved in 20 µl RNase free water. A small portion was used to measure the RNA concentration. This was done by measuring the absorbance at

240, 260, 280 and 320 nm on a spectrophotometer in each sample, and a gel electrophoresis was performed to ensure good RNA integrity.

10.8.2 cDNA synthesis and Real time PCR

In order to quantify the mRNA by real-time RT-PCR complementary DNA (cDNA) was synthesized from 500 ng RNA via reverse transcription using OmniScript reverse transcriptase (Qiagen, California, USA), in the presence of a mixture of dNTP's (nucleotides, OmniScript kit). A poly dT primer (Invitrogen, Naerum, Denmark) was used as to give the polymerase reaction a point of initiation. The samples were vortexed and put on a 37°C heating block for an hour allowing annealing of primers and synthesis of cDNA, followed by 30 min incubation at 70°C to deactivate the enzyme.

Real time PCR was used to quantify the amplification of cDNA as it occurred. cDNA transcribed from the 500 ng RNA was 20 x diluted. Each 5 µl of diluted cDNA sample was mixed with 20µl quantitect SYBR Green master mix (Qiagent, Hilden, Germany) containing the specific primers. The real time PCR method relies on thermal cycling, first denaturing the DNA, secondly the primers anneal to the target sequence, and thirdly DNA is enzymatically replicated. This was done on a real-time PCR machine (MX3005P, Stratagene, La Jolla, Ca, USA) using the thermal profile: 10 min, 95°C followed by 50 x [15 sec, 95°C → 30 sec, 58°C → 90 sec, 63°C (signal collection)], and then a melting curve.
During each thermal cycle the amount of DNA is doubled increasing the product in an exponential fashion. The increase is quantified by the SYBR green dye, a fluorescent marker, which binds to the newly formed double-stranded DNA causing a large increase in the fluorescence intensity. The signal was collected at the 63°C step and the threshold cycle (Ct) values were defined within the exponential phase and related to a standard curve made from the corresponding synthetic oligonucleotides for each target.

The specificity of the PCR product was confirmed by a melting curve analysis after the amplification.

As a loading control a housekeeping gene was run for all samples. A house keeping gene is a gene of which, the expression is not expected change independent

on intervention or timing of the biopsy material used for the analysis. The house keeping genes of choice in this study was RPLP0, which was validated by the use of the house keeping gene GAPDH.

All data calculated from the standard curve was firstly divided by RPLP0 to normalize samples based on a loading control, after which all samples were divided by the baseline sample (defined as the sample taken at baseline in the recommended habituation period) in order to look at the change from background. The binary logarithm on all data has been taken before analysis are done.

10.9 Experimental deviations

Three participants did not have constant infusion of D_2-tyrosin, thus they are not included in measurements of phenylalanine conversion to tyrosine, whole body protein synthesis or whole body protein net protein balance.

One participant was excluded from all postprandial calculations due to mixing error with the labeled proteins.

D_7-phenylalalnine and D_6-tyrosine (deriving from transamination of D_8-phenylalalnine and conversion from D_7-phenylalanine to D_6-tryosine) was only measured for 5 participants. Based in the known D8-phe/D7-phe and D7-tyr/D6-tyr ratios for these 5 participants, the D7-phe and D6-tyr have been calculated for all participants.

For the flow measurements one participant had hyperlipidemia, causing unreliable flow measurements. Thus, this participant is excluded for all two and three-pool analysis. Further for the leg flow, for one participant the ICG was faultily infused in a vein instead of an artery, and for two other participants there were missing values, causing these participants to be excluded for two and three pool measures across the leg. Leaving 7 participants for these analyses.

10.10 Statistical analysis

The response to feeding over time, following the two habituation periods were compared using Two-way ANOVA with repeated measures. When there was an

intervention or interaction effect a SIDAK post hoc test was performed. When there was a time effect Dunnet's post hoc test was performed comparing post prandial time points to fasting. The fasting samples and postprandial myofibrillar FSR measurements were compared using a paired t-test. The insulin data is presented as the numerical values however, the values were not normally distributed, tested using Shapiro Wilk test, and visualized by plotting the residuals, thus statistical analysis are performed on log transformed data. For all PCR data the binary logarithm has been taken prior to analysis.

GraphPad Prism 8.0 was used for all other statistical test than the PCR analysis for which Sigma Plot version 12.0 (Systat Software Inc., San Jose, CA) was used. For subject characteristics, data is means ±SD. mRNA data is geometric mean ± backtransformed SEM. All other data is presented as means ±SEM. Significant level was set to $p<0.05$ and whenever $0.1>p>0.05$ tendencies was discussed.

11.0 Results and discussion

In the attempt to counteract age-related loss of muscle mass, the current recommendations for daily dietary protein intake for adults (>65 years of age) have been re-evaluated for its suitability for older individuals. This has resulted in suggestions for higher protein intake pr. day based on the optimal intake for muscle mass maintenance [1]. Whereas the current official recommendations for adults are based on securing whole body protein balance, not merely muscle [12]. In this study, we compared how amino acid metabolism and whole body and muscle protein synthesis and degradation, i.e. protein turnover, were affected following three weeks habituation to the current official recommendations for protein intake (RP, 1.1 g protein/kg LBM/day) compared with a three week habituation to high protein intake (HP >2.1 g protein/kg LBM/day) in 12 healthy males between 65-70 years of age. Following each of the habituation periods the overnight fasted and postprandial whole body, first splanchnic bypass, and skeletal muscle amino acid kinetics were assessed. This was done using infusion of stable isotopes, ingestion of intrinsically labelled proteins combined with sampling from arterial blood, hepatic venous blood, femoral venous blood and muscle biopsies.

The very elaborate set up allows for tracing the route of protein-bound dietary

and body protein amino acids. These are traced from their availability in the main circulation from protein digestion and splanchnic tissue utilization rates (first splanchnic bypass), and subsequent disappearance from the main circulation. This enables assessments of both whole body and skeletal muscle protein synthesis and degradation and fractional synthesis rates of blood and myofibrillar proteins.

11.1 First splanchnic bypass

During first splanchnic bypass, ingested proteins are digested and degraded into their constituent amino acids. Amino acids which are not used by the gastro intestinal tract, are absorbed across the epithelial cells of the gut and transported via the portal vein, into the liver. The amino acids which are not oxidized or degraded in the liver, i.e. those that 'escape' first splanchnic bypass, are secreted to the circulation via the hepatic vein. We have measured the concentration of amino acids directly in the hepatic vein and in a peripheral radial artery (see Figure 5 in methods). The blood in the peripheral arteries contain the metabolites and nutrients recycled from peripheral tissues mixed with those nutrients delivered via the hepatic vein including food nutrients. Table 5 shows the amino acid concentrations in the artery and the arteriovenous difference of concentrations measured in the radial artery and a hepatic vein ($A\text{-}V_h$). A negative $A\text{-}V_h$ difference across the splanchnic bed means that the amino acid secretion is higher than the amino acid uptake by the splanchnic tissues. Thus, a negative $A\text{-}V_h$ difference is expected in the post-prandial state because a high amount of amino acids originating from the digested meal proteins escape first splanchnic bypass.

At baseline, no differences in arterial concentrations of any amino acids except from glycine was apparent after RP and HP habituation. The arterial concentration of all of the amino acids increased with time after meal intake (Table 5, Figure 15). To visualize this, the sum of all amino acid concentrations is pictured in Figure 15. Participants habituated to RP diet had higher levels of glycine (Gly), asparagine (Asn), alanine (Ala), tyrosine (Tyr) and isoleucine (Ile) at several timepoints compared with participants following HP diet. In addition, tendencies for higher concentrations were also observed for lysine (Lys), tryptophan (Trp), methionine (Met) and threonine (Thr) ($P<0.07$) in RP versus HP habituated

participants. Thus, RP habituated participants either have a higher appearance or slower disappearance from the main circulation in the early post-prandial phase.

The measured A-V_h differences revealed no intervention (RP versus HP) effect. For all but two amino acids there was a significant time effect, with the A-V_h differences becoming more negative in the postprandial period. This with exception of glutamine (Gln) and glutamic acid (Glu).

Table 5:Amino acid concentrations

			Conc. [µmol/L]							
			0 min	30 min	60 min	90	120	150	180	240 min
Asp	Arterial $	RP	4.5 ± 0.7	9.9 ± 1.0	12.0 ± 2.7	6.1 ± 0.7	9.4 ± 4.5	5.8 ± 1.1	4.9 ± 0.8	7.9 ± 3.3
		HP	4.7 ± 0.5	9.6 ± 0.9	7.3 ± 0.7	5.0 ± 0.4	5.0 ± 0.6	4.7 ± 0.4	4.6 ± 0.4	4.7 ± 0.5
	A-V $	RP	-3.2 ± 0.5	-15.5 ± 3.3	-5.2 ± 2.8	-4.2 ± 0.7	0.7 ± 3.7	-5.3 ± 2.4	-3.2 ± 0.7	-4.9 ± 2.0
		HP	-2.6 ± 0.3	-10.7 ± 2.1	-4.9 ± 1.8	-2.8 ± 0.3	-2.0 ± 0.6	-1.7 ± 0.4	-1.9 ± 0.3	-1.8 ± 0.4
Glu	Arterial $	RP	111 ± 27	127 ± 14	126 ± 19	125 ± 25	110 ± 20	125 ± 31	106 ± 23	107 ± 23
		HP	93 ± 10	114 ± 7	104 ± 8	96 ± 6	99 ± 15	91 ± 9	89 ± 8	92 ± 10
	A-V	RP	-95 ± 14	-122 ± 20	-114 ± 14	-108 ± 16	-106 ± 21	-113 ± 31	-104 ± 14	-117 ± 19
		HP	-80 ± 8	-108 ± 15	-86 ± 22	-94 ± 12	-71 ± 18	-73 ± 10	-80 ± 8	-84 ± 12
Ser	Arterial $	RP	113 ± 15	167 ± 15	183 ± 23	147 ± 14	144 ± 27	138 ± 19	120 ± 15	130 ± 20
		HP	102 ± 5	147 ± 7	131 ± 8	114 ± 7	104 ± 4	106 ± 6	104 ± 7	105 ± 6
	A-V $	RP	16 ± 30	-40 ± 60	2 ± 90	10 ± 120	26 ± 150	-7 ± 180	-5 ± 240	-13 ± 14
		HP	18 ± 30	-22 ± 60	-3 ± 90	10 ± 120	11 ± 150	13 ± 180	8 ± 240	9 ± 8
Gly	Arterial $	RP	*223 ± 27	* 245 ± 27	* 263 ± 32	* 234 ± 22	* 219 ± 25	* 234 ± 27	209 ± 21	*229 ± 25
		HP	184 ± 11	187 ± 12	176 ± 13	169 ± 12	166 ± 9	179 ± 13	180 ± 16	191 ± 15
	A-V $	RP	25 ± 6	-7 ± 11	20 ± 8	17 ± 12	19 ± 7	-3 ± 24	-8 ± 8	-17 ± 16
		HP	17 ± 5	-10 ± 17	8 ± 18	6 ± 12	-2 ± 10	4 ± 16	0 ± 9	0 ± 17
Asn	Arterial $	RP	45 ± 7	86 ± 11	*91 ± 11	* 79 ± 7	64 ± 7	* 73 ± 12	64 ± 9	60 ± 8
		HP	44 ± 3	77 ± 5	71 ± 5	62 ± 6	56 ± 4	57 ± 4	56 ± 5	55 ± 5
	A-V $	RP	9 ± 2	-28 ± 7	-14 ± 4	1 ± 5	-3 ± 4	-6 ± 5	-9 ± 3	-7 ± 3
		HP	8 ± 1	-19 ± 10	-7 ± 9	-3 ± 5	-4 ± 3	-1 ± 5	-2 ± 3	-1 ± 4
Gln	Arterial $	RP	707 ± 78	857 ± 100	893 ± 105	840 ± 76	721 ± 68	798 ± 83	722 ± 74	741 ± 76
		HP	648 ± 29	747 ± 41	756 ± 51	718 ± 50	646 ± 45	692 ± 47	687 ± 49	735 ± 52
	A-V	RP	108 ± 24	34 ± 35	68 ± 24	140 ± 41	85 ± 31	84 ± 56	46 ± 26	16 ± 43
		HP	98 ± 14	9 ± 60	52 ± 72	57 ± 41	43 ± 33	88 ± 48	73 ± 22	56 ± 51
His	Arterial $	RP	77 ± 9	101 ± 11	110 ± 11	100 ± 9	88 ± 8	97 ± 11	89 ± 11	91 ± 10
		HP	73 ± 3	93 ± 5	94 ± 5	85 ± 5	79 ± 3	83 ± 6	84 ± 5	88 ± 6
	A-V $	RP	8 ± 2	-12 ± 5	-9 ± 3	4 ± 5	0 ± 2	-6 ± 8	-9 ± 4	-12 ± 6
		HP	4 ± 2	-15 ± 9	-7 ± 9	-3 ± 5	-4 ± 5	-2 ± 6	-4 ± 3	-4 ± 6
Thr	Arterial $	RP	118 ± 17	217 ± 23	234 ± 25	216 ± 18	182 ± 17	199 ± 25	175 ± 21	166 ± 18
		HP	111 ± 6	197 ± 10	183 ± 9	165 ± 11	156 ± 13	145 ± 8	137 ± 9	134 ± 8
	A-V $	RP	18 ± 3	-74 ± 17	-40 ± 7	-9 ± 12	-12 ± 9	-18 ± 17	-17 ± 6	-13 ± 10
		HP	19 ± 4	-53 ± 27	-23 ± 24	-8 ± 14	7 ± 14	7 ± 12	4 ± 5	6 ± 9
Ala	Arterial $	RP	266 ± 29	* 407 ± 50	* 493 ± 54	* 487 ± 43	402 ± 40	* 431 ± 39	396 ± 38	401 ± 42
		HP	225 ± 17	323 ± 29	361 ± 28	362 ± 34	336 ± 24	349 ± 29	356 ± 31	363 ± 30
	A-V $	RP	144 ± 18	22 ± 25	6 ± 19	88 ± 33	90 ± 20	84 ± 26	76 ± 18	91 ± 25
		HP	123 ± 14	27 ± 36	34 ± 39	64 ± 28	80 ± 15	102 ± 35	101 ± 18	109 ± 26
Pro	Arterial $	RP	167 ± 18	290 ± 25	322 ± 31	332 ± 27	293 ± 25	335 ± 28	306 ± 25	300 ± 24
		HP	155 ± 11	277 ± 18	281 ± 20	278 ± 22	263 ± 15	271 ± 20	272 ± 26	269 ± 19
	A-V $	RP	9 ± 4	-96 ± 21	-77 ± 10	-55 ± 21	-67 ± 29	-84 ± 41	-81 ± 17	-73 ± 22
		HP	4 ± 5	-95 ± 35	-68 ± 38	-68 ± 25	-57 ± 19	-38 ± 23	-40 ± 10	-34 ± 17
Arg	Arterial $	RP	84 ± 11	138 ± 14	139 ± 16	128 ± 12	105 ± 10	119 ± 15	102 ± 11	95 ± 10
		HP	70 ± 5	116 ± 7	105 ± 7	96 ± 6	86 ± 4	88 ± 7	84 ± 7	81 ± 5
	A-V $	RP	12 ± 2	-29 ± 9	-12 ± 3	-1 ± 7	-10 ± 9	-12 ± 13	-13 ± 4	-11 ± 7
		HP	9 ± 2	-21 ± 16	-9 ± 13	-7 ± 10	-2 ± 5	1 ± 9	1 ± 3	-1 ± 7
Tyr	Arterial $	RP	62 ± 7	125 ± 12	* 132 ± 13	* 125 ± 10	105 ± 10	* 116 ± 12	* 102 ± 10	95 ± 10
		HP	64 ± 3	114 ± 7	110 ± 7	98 ± 5	88 ± 4	87 ± 4	84 ± 6	81 ± 4
	A-V $	RP	9 ± 3	-30 ± 12	-8 ± 4	3 ± 7	-3 ± 6	-3 ± 11	-1 ± 4	-4 ± 7
		HP	10 ± 2	-23 ± 18	-11 ± 15	-3 ± 10	0 ± 4	2 ± 8	5 ± 3	2 ± 6
Val	Arterial $	RP	235 ± 28	409 ± 38	429 ± 40	417 ± 30	349 ± 29	388 ± 42	341 ± 37	323 ± 33
		HP	260 ± 11	427 ± 22	419 ± 21	386 ± 20	344 ± 18	345 ± 22	326 ± 21	315 ± 18
	A-V $	RP	8 ± 5	-102 ± 26	-74 ± 8	-26 ± 20	-49 ± 22	-51 ± 32	-56 ± 11	-55 ± 18
		HP	0 ± 6	-109 ± 48	-70 ± 49	-66 ± 25	-51 ± 20	-26 ± 25	-27 ± 9	-29 ± 21
Met	Arterial $	RP	21 ± 2	51 ± 5	51 ± 5	45 ± 2	35 ± 3	39 ± 5	34 ± 3	28 ± 3
		HP	20 ± 1	47 ± 3	40 ± 3	34 ± 3	30 ± 1	29 ± 2	27 ± 2	25 ± 2
	A-V $	RP	5 ± 1	-15 ± 4	-5 ± 2	2 ± 3	-1 ± 2	-2 ± 3	-2 ± 1	-1 ± 2
		HP	4 ± 1	-11 ± 8	-3 ± 5	-1 ± 4	0 ± 2	2 ± 3	2 ± 1	2 ± 2
Ile	Arterial $	RP	52 ± 8	177 ± 22	175 ± 21	* 152 ± 13	112 ± 12	*129 ± 21	111 ± 17	91 ± 11
		HP	55 ± 4	176 ± 14	154 ± 14	121 ± 8	108 ± 10	91 ± 5	82 ± 5	74 ± 4
	A-V $	RP	0 ± 1	-82 ± 19	-48 ± 6	-20 ± 11	-34 ± 12	-26 ± 14	-27 ± 10	-23 ± 6
		HP	-2 ± 2	-77 ± 33	-51 ± 26	-34 ± 11	-13 ± 12	-16 ± 8	-16 ± 4	-14 ± 6
Leu	Arterial $	RP	124 ± 14	376 ± 36	394 ± 41	336 ± 28	258 ± 23	283 ± 38	242 ± 33	201 ± 24
		HP	135 ± 9	400 ± 33	358 ± 30	287 ± 17	242 ± 16	220 ± 17	203 ± 20	180 ± 15
	A-V $	RP	5 ± 3	-166 ± 40	-90 ± 12	-31 ± 22	-51 ± 19	-54 ± 21	-51 ± 6	-34 ± 8
		HP	-5 ± 3	-172 ± 64	-98 ± 49	-61 ± 23	-32 ± 19	-26 ± 16	-23 ± 7	-12 ± 8
Tryp	Arterial $	RP	54 ± 7	97 ± 10	106 ± 12	98 ± 9	79 ± 7	86 ± 9	72 ± 8	65 ± 7
		HP	51 ± 2	87 ± 5	85 ± 4	76 ± 3	67 ± 3	66 ± 4	61 ± 4	58 ± 3
	A-V $	RP	3 ± 1	-20 ± 7	-9 ± 2	2 ± 5	-4 ± 5	-4 ± 9	-4 ± 3	-6 ± 4
		HP	1 ± 1	-21 ± 10	-7 ± 9	-5 ± 5	-4 ± 3	0 ± 5	0 ± 2	-1 ± 4
Phe	Arterial $	RP	52 ± 6	91 ± 7	92 ± 8	85 ± 5	73 ± 5	83 ± 8	73 ± 7	71 ± 6
		HP	58 ± 2	90 ± 4	83 ± 4	79 ± 4	74 ± 4	75 ± 4	73 ± 5	72 ± 3
	A-V $	RP	7 ± 2	-16 ± 5	-4 ± 3	2 ± 4	-3 ± 4	-5 ± 7	-6 ± 2	-5 ± 4
		HP	6 ± 1	-15 ± 10	-4 ± 8	-4 ± 6	-3 ± 4	0 ± 7	0 ± 2	-1 ± 5
Lys	Arterial $	RP	177 ± 19	368 ± 33	374 ± 34	325 ± 21	260 ± 20	295 ± 34	257 ± 26	231 ± 22
		HP	187 ± 9	363 ± 19	318 ± 17	273 ± 15	241 ± 9	242 ± 13	230 ± 15	219 ± 12
	A-V $	RP	18 ± 4	-123 ± 33	-44 ± 12	-8 ± 17	-22 ± 19	-22 ± 22	-29 ± 9	-24 ± 12
		HP	17 ± 6	-106 ± 54	-31 ± 39	-16 ± 23	-11 ± 13	-2 ± 22	-4 ± 8	-6 ± 17
BCAA	Arterial $	RP	411 ± 49	962 ± 93	997 ± 96	904 ± 65	719 ± 61	800 ± 98	694 ± 85	614 ± 66
		HP	450 ± 21	1003 ± 61	931 ± 59	794 ± 40	693 ± 33	655 ± 40	611 ± 42	569 ± 33
	A-V $	RP	13 ± 9	-351 ± 84	-213 ± 23	-78 ± 51	-134 ± 51	-130 ± 65	-133 ± 24	-112 ± 31
		HP	-8 ± 11	-358 ± 143	-219 ± 120	-161 ± 58	-97 ± 48	-67 ± 49	-67 ± 19	-54 ± 29
ESS - BCAA	Arterial $	RP	591 ± 67	1078 ± 99	1118 ± 107	1009 ± 69	835 ± 66	934 ± 105	820 ± 83	764 ± 72
		HP	577 ± 24	1010 ± 46	922 ± 45	818 ± 43	744 ± 23	739 ± 38	709 ± 43	689 ± 33
	A-V $	RP	78 ± 14	-307 ± 81	-128 ± 27	-9 ± 54	-51 ± 50	-71 ± 78	-79 ± 29	-70 ± 45
		HP	68 ± 16	-254 ± 138	-89 ± 112	-45 ± 69	-13 ± 42	8 ± 65	1 ± 25	-2 ± 51
Total	Arterial $	RP	2703 ± 313	4337 ± 423	4618 ± 470	4273 ± 326	3605 ± 308	3971 ± 432	3523 ± 365	3430 ± 336
		HP	2541 ± 93	3992 ± 193	3836 ± 203	3501 ± 197	3186 ± 127	3220 ± 181	3137 ± 206	3139 ± 177
	A-V $	RP	308 ± 64	-929 ± 277	-464 ± 87	3 ± 222	-145 ± 177	-256 ± 343	-306 ± 127	-314 ± 209
		HP	245 ± 62	-862 ± 478	-390 ± 440	-244 ± 258	-116 ± 172	35 ± 257	-10 ± 105	-5 ± 212

A-V is the arterio-hepatic venous difference (A-V_h). $ denotes significant change from fasting, * denotes significant difference between RP and HP ($P<0.05$). N=11, data is mean ± SEM.

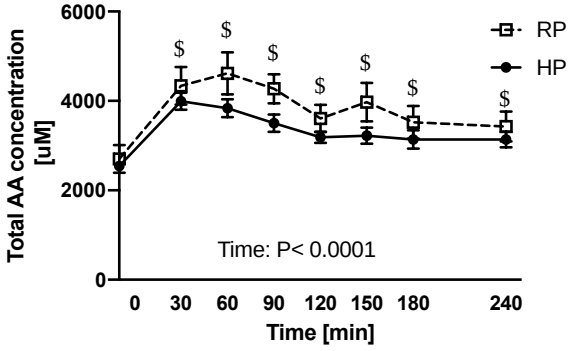

Figure 15: The sum of all amino acid concentrations in the arteries. -10 denotes immediately pre meal ingestions and 30, 60, 90, 120, 150, 180 and 240 min postprandial. Values are also given in table 5. $ denotes significant change from fasting ($p<0.05$). N=11, values are mean ± SEM

The net balances of the various amino acids across the splanchnic bed were calculated by multiplying the A-V_h differences with the splanchnic plasma flow shown in Figure 16. The mean splanchnic plasma flow was 1.1 l/min with neither intervention nor time effects.

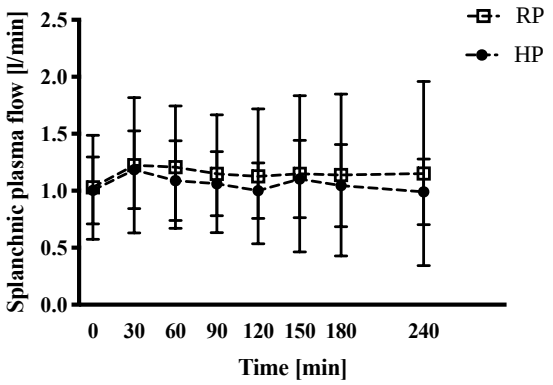

Figure 16: The splanchnic plasma flow in the immediate pre and post prandial period. N=10, values are mean ± SEM.

The product of the A-V_h differences and the splanchnic flow, i.e. the net balance of glutamine (Gln), alanine (Ala), phenylalanine (Phe) and the essential amino acids (EAA) are shown in Figure 17. These reveal no significant intervention effect for any of the amino acids and all showed a significant change from fasting to the post-prandial state except glutamine (Figure 17A). For alanine the net uptake was significantly lower post-prandially at time points 30, 60 and 90 min post prandial (Figure 17B). For phenylalanine the net uptake in fasting reversed to a net release at

30 min post prandial and seemed to do so for the total four hours, most notable following habituation to RP intake whereas following habituation to HP intake the balance was nearly zero, i.e. no net exchange of phenylalanine (Figure 17C). A very similar pattern was seen for the total essential amino acids (Figure 17D).

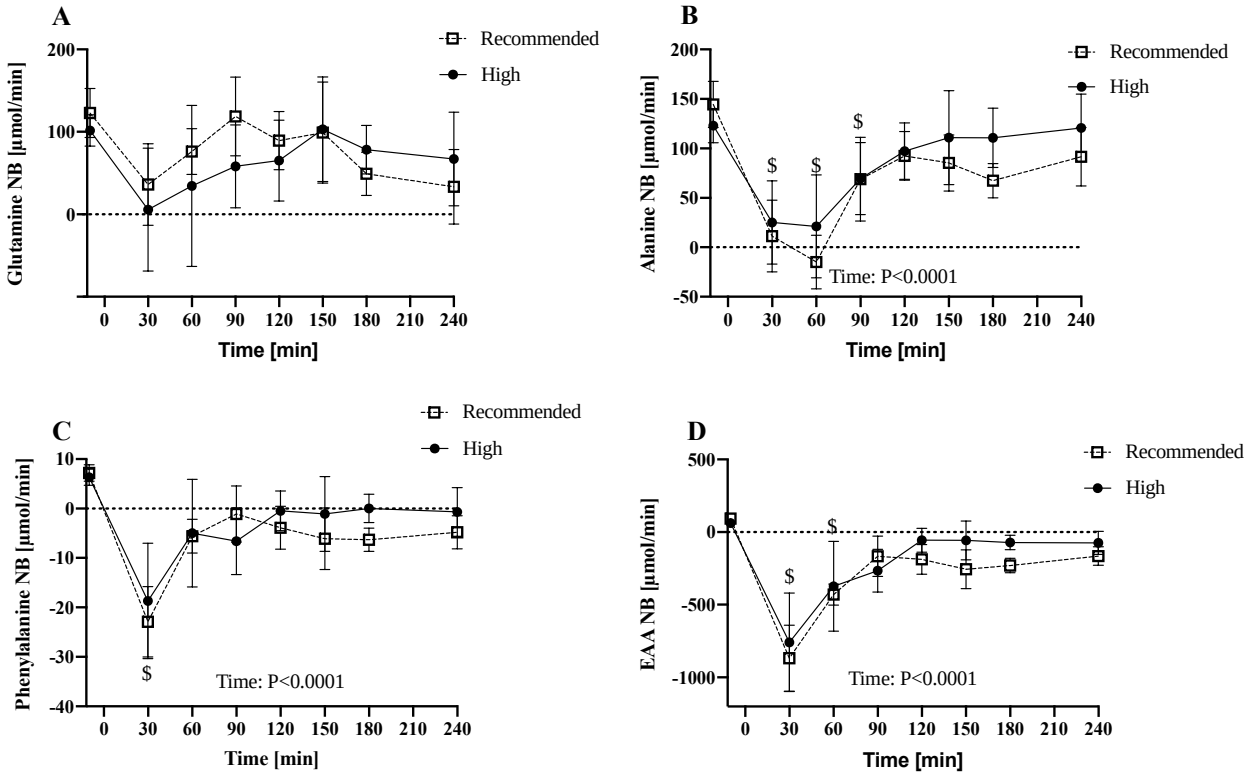

Figure 17: Splanchnic net uptake of glutamine A) alanine (B), phenylalanine (C), and the essential amino acids, EAA (D). The net balance change with time for the last three, and for all the other amino acids not pictured. $ denotes significant change from fasting ($P<0.05$). N=7, data is mean ± SEM.

The rate at which phenylalanine from the ingested whey and caseinate appears in the main circulation (measured as the appearance in the hepatic vein) can be calculated due to the intrinsic labeling of the ingested proteins. The whey protein was labeled with D_5-phenylalanine and the caseinate protein was labeled with ^{15}N-phenylalanine. The appearance rates are shown in Figure 18.

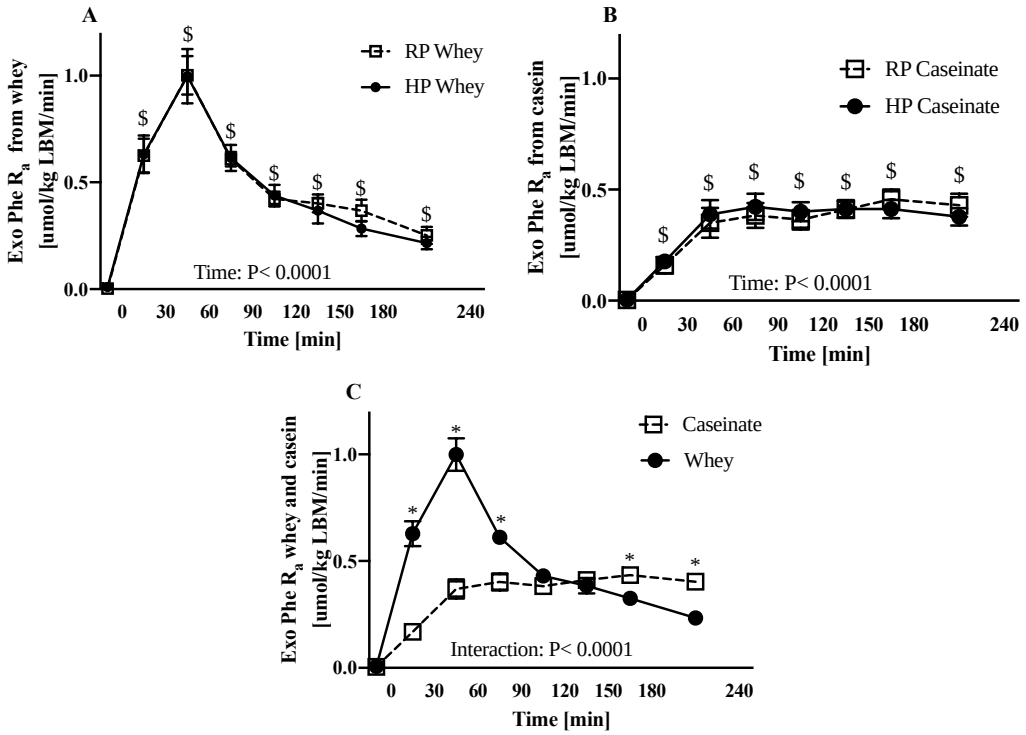

Figure 18: The exogenous rate of appearance of the ingested protein in the hepatic vein, representing the rate at which the intrinsic labels of phenylalanine from the milk proteins enter the circulation. Figure A) represents the appearance rate of phenylalanine from the whey protein, B) represents the appearance rate of phenylalanine from the caseinate protein. $ denotes significant difference from fasting (P<0.0005). Figure C) represents the appearance rate of phenylalanine from the whey and caseinate protein pooled from the two habituation periods. * denotes significant difference between the two habituation periods (P<0.05). (N=11, values are mean ± SEM)

There was a significant time effect in the appearance rate of the phenylalanine from the protein rich breakfast during the four-hour postprandial period (P<0.0001), albeit no difference between the interventions of habituation to RP and HP diets. The data from the RP and HP was pooled to compare the appearance rate of phenylalanine deriving from the whey and caseinate protein as there was no differences between habituation periods. This showed a significant interaction (P<0.0001) between the phenylalanine appearance from whey and caseinate protein. The increase in circulating phenylalanine from whey protein increased more rapid, and to a higher degree than the rate of appearance of phenylalanine from caseinate. The increased rate of appearance of phenylalanine from caseinate was sustained at a higher level for a longer period of time, reaching significance in the late postprandial phase (130 and 210 minutes postprandial). The ingested protein derived phenylalanine appearance from neither whey nor caseinate returned to baseline levels within the four-hour post-

prandial period. This means that we cannot compare the total first bypass extraction of phenylalanine, i.e. meal protein amino acids, between the two proteins, as we do not monitor the entire absorption period.

It should be noted, with regards to the measures of rate of appearance of the intrinsically labeled proteins, that this method has very recently been under critique. The claim for the critique was made by Wolfe and colleagues, who states that R_a is systematically underestimated. This underestimation is due to faulty assumptions of zero tracer dilution in the GI-tract and across the splanchnic bed as well as there being no recycling of the tracer [138]. While these are valid points, there has been made no suggestions for an alternative approach. In addition, the method used in this study has been used in several peer reviewed published articles [113,130,139–141]. Hence, interpretation of these data was done with attentiveness, but confidently.

The initial response to substantial increase of amino acids in the circulation is a secretion of insulin [142]. The insulin secretory response to amino acids are amplified by the presence of carbohydrates as the proteins in this study are administered as part of a mixed meal [143]. A significant increase in the insulin concentration is seen through the first three hours of the postprandial period (Figure 19). With no differences between the two habituation periods.

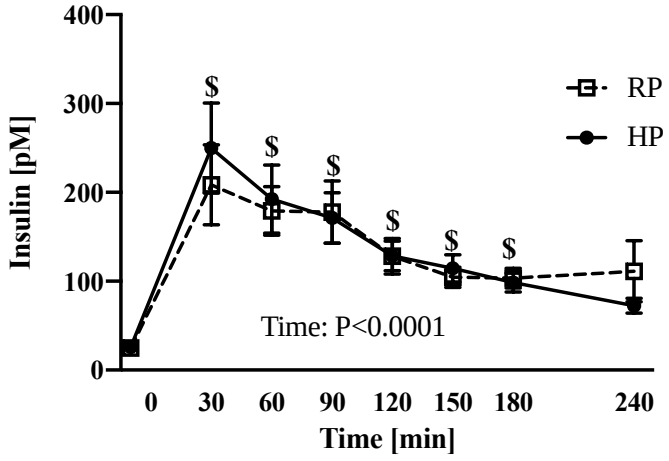

Figure 19: The insulin concentrations in the fasting (-10 min) and 240 min postprandial period. Significant time effects are determined by Dunnett's post hoc test. $ denotes significant difference from fasting ($P<0.05$) (N=11 values are mean ± SEM)

The splanchnic bed comprises many tissues with very different functions and metabolisms towards amino acids. Ingested proteins are hydrolyzed in the intestine to

amino acids and small polypeptides. These can be used by bacteria or taken up by the intestinal cells and can either be utilized for protein synthesis or metabolized. The amino acids which are not utilized in the gastro intestinal tract till be released into the portal vein. Next, the liver will take up apportion of these amino acids for liver cell protein synthesis and production of numerous proteins the liver produces and subsequently excrete together with the not utilized amino acids, into the main circulation via a hepatic vein. Therefor the total amount of ingested protein derived amino acids will not end up in the circulation. The postprandial A-V_h differences showed an increased release of ingested protein derived amino acids into the main circulation for all amino acids but glutamine (Figure 17A). This indicates that the glutamine metabolism in the splanchnic tissues matches the glutamine intake in the meal. This is in agreement with previous findings from Stoll et al., estimating that in young piglets the gut tissue uses more than 95% of ingested dietary glutamine [144]. That glutamine is important for the gastro intestinal tract, is further supported by the findings from Neis et al., who found that glutamine is taken up by the small intestines in the fasted state [145]. Hence, glutamine is considered the preferred energy source instead of glucose for rapidly dividing cells among others gastro intestinal cells. Moreover, glutamine is a key nitrogen carrier produced in large quantities and taken up by liver for urea production. Hence glutamine uptake by the liver is high and the expected small excess glutamine from meal protein digestion into the portal vein can easily be utilized by the liver and not escape into the main circulation. Alanine net splanchnic tissue uptake/release was also shown for its specialized role as a nitrogen carrier as well as the role of alanine in gluconeogenesis through the Cori cycle[146]. One could imagine that the metabolism of alanine might have been enhanced in the fasted state in response to weeks of high protein intake. However, no difference was observed suggesting that gluconeogenesis from alanine was not affected by high protein habituation.

All amino acids apart from glutamine showed a significant change in the net splanchnic tissue balance post feeding in the sense that more amino acids are released in the main circulation contributing to the raise in the arterial concentration. Interestingly, the arterial amino acid concentrations were higher post habituation to RP for five of the amino acids (Table 5). Besides the 5 amino acids which were higher following RP, there was a tendency ($P<0.07$) for increased concentration of four additional amino acids. The higher amino acid concentrations following RP diet can

be explained by lower first bypass extraction, reduced peripheral clearance and/or increased peripheral production.

The fact that the differences between habituation periods are not conveyed in the A-V_h differences indicates a higher first splanchnic extraction following the HP diet. However, this is not (at least not solely) an explanation for the lower arterial amino acid concentrations also in keeping with a similar meal protein derived phenylalanine appearance in RP and HP. The splanchnic net amino acid balances are quantitively small and thus more variable and based on three measurement (A-V_h and flow) as compared to one arterial amino acids concentration. Thus, significant difference might have been lost. So, we cannot rule out a higher splanchnic extraction post habituation to HP due to an increased usage in the splanchnic tissue. But the cause for the observed higher amino acid concentration under post-absorptive condition after HP habituation could also be the consequence of an increased peripheral tissue amino acid uptake or decreased release.

Differential uptake between whey and caseinate

The rate of appearance of phenylalanine from the whey protein and caseinate protein do not differ based on habituated protein intake. However, their individual uptake patterns differ substantially. Both whey and caseinate are milk proteins but quite different in their amino acid composition and physical properties. Branched chain amino acids (BCAA) are considerably more abundant in whey protein [147]. In addition, whey proteins are acid-soluble and rapidly emptied by the stomach whereas the caseinate proteins precipitation in the stomach by the gastric acid resulting in coagulation and slower gastric emptying [148]. Consequently, the amino acid appearance into the circulation is delayed from an intake of caseinate compared to whey as shown here and previously [125]. The whey and caseinate proteins were ingested together thus the concentrations of total phenylalanine in the circulation spanning the 240 min postprandial period is a mean from the circulating phenylalanine deriving from whey and caseinate and endogenous protein degradation.

Insulin response to long term whey supplementation

Besides the caseinate and whey co-ingestion, the proteins were ingested as part of a mixed meal. This will cause a higher rise in insulin secretion as opposed to

protein ingestions alone. There were no differences in the insulin concentration between habituations, suggesting that weeks of habituation to HP intake does not affect insulin secretion or the co-ingested carbohydrate intake causes such a high insulin concentration per see that it mask potential small effects from the amino acids (higher in RP) from the protein intake. Whey protein has been shown and recently reviewed to acutely lower the insulin secretory response to a meal [149]. There have not been any long-term studies in healthy normal weight adults. However, Baer and colleagues showed that 23 weeks supplementation of whey in overweight resulted in lowered fasting insulin secretion [150]. However, this was seen concomitant with an increased weight loss, and no decrease in glucose concentration indicating an increased insulin sensitivity. Therefore, whether their finding was due to the whey intake or weight loss cannot be concluded.

11.2 From circulation to tissue

Amino acids will be removed from the blood by either tissue uptake where they can be incorporated into proteins or metabolized via deamination/oxidation or to other metabolites including other amino acids. In this section, we will look at the indirect markers of amino acid kinetics and in relation protein synthesis and breakdown.

The phenylalanine fluxes (representing non-metabolized essential amino acid fluxes) across the leg were measured based on tracer dilution from the artery to the femoral vein (V_f) (the 2-pool model described in Methods). The A-V_f differences were increased 30 min postprandial as compared to fasting (P=0.02, Figure 20A). However when multiplied by the leg plasma flow, i.e. calculating the net balance (Figure 20D), the statistical difference disappeared, and only a tendency was seen (P=0.09). The rate of disappearance into the leg, a measure of muscle protein synthesis, is increased 30 and 60 min postprandial compared to fasting (Figure 20C), but with no significant difference between interventions. Visually, it appears as if the increased synthesis is maintained for a longer period following the RP period. However statistically there is no significance nor tendencies (interaction, and intervention: P=0.5).

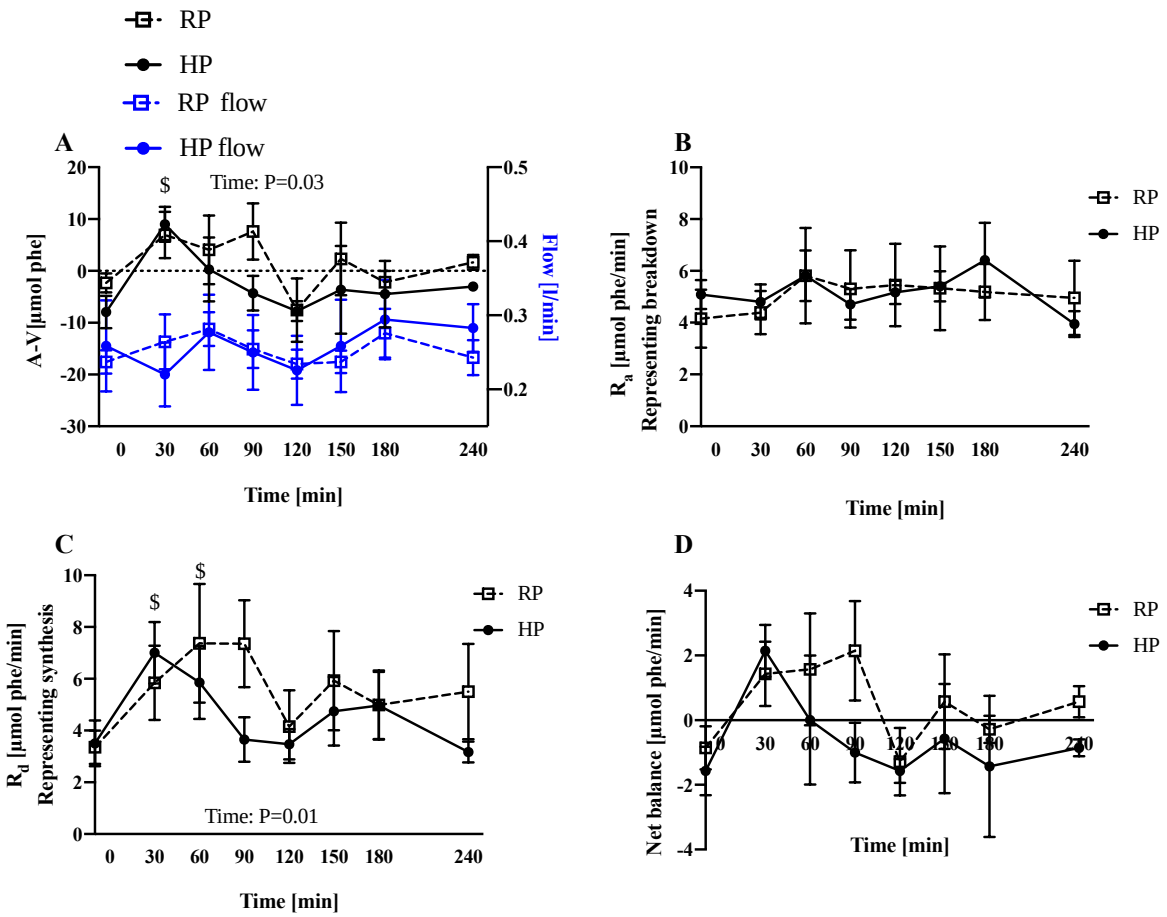

Figure 20: Phenylalanine fluxes across the leg measured using the 2 pool model. The A-V differences and leg plasma flow (figure A), the rate of appearance representing breakdown across the muscle (figure B), the rate of disappearance representing synthesis across the leg (figure C), and the difference between synthesis and breakdown, the net balance (Figure D). $ denotes difference from fasting (P≤0.03). Values are mean ±SEM, N=7.

The flux rates of free amino acids within the intramuscular compartment are calculated using the 3-pool model approach. This model requires knowledge of the intramuscular tracer enrichments and can thus only be applied at the time points where muscle biopsies are taken. Figure 21A represents the flux of amino acids from breakdown of tissue proteins into the intramuscular pool. Figure 21B represents the flux of amino acid from the intramuscular compartment to the tissue bound proteins and is thus a measure of synthesis. We show neither and effect of a protein rich meal nor habituated protein intake.

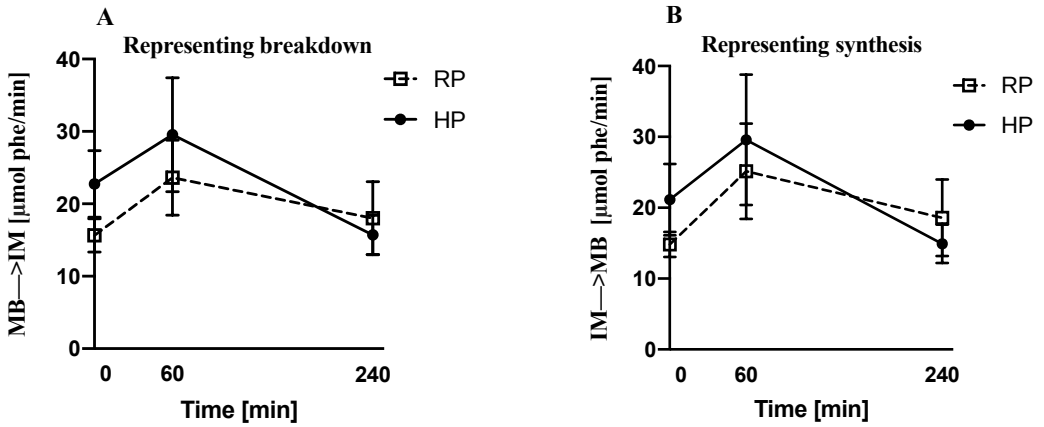

Figure 21: Fluxes of free amino acids into the intramuscular compartment as an estimate of protein synthesis (B), as well as the flux from the intramuscular pool into the venous site of circulation as an estimate of protein breakdown (A). There is no time nor intervention effects. The values are mean ± SEM, N=7.

The muscle biopsies were among others used to determine the expression levels of various genes. Expression of the atrogenes Atrogin1 and MuRF1 was measured as markers of protein breakdown (Figure 22 B and C). The expression of both genes were decreased four hours after a meal intake compared to fasting levels (time: P=0.02), but their expression pattern did not reveal any interaction effects. This suggests that meal intake may cause a decreased muscle protein breakdown, but the habituated protein intake has no effect.

Expression of three growth factors myostatin, IFG1Ea and MGF was determined in the same muscle biopsies taken in the fasted and four-hour post prandial state as markers of protein synthesis in muscle tissue (Figure 22D, E and F). The data show a tendency towards higher fasting expression of the myostatin gene (Figure 22D, P=0.07) following habituation to HP compared to RP, however with an increase postprandial only for RP. Likewise, for IGF-1Ea expression (Figure 22E, P=0.09), with a mild reduction in expression following habituation to HP. For MGF there is a significant time effect (Figure 22F, P=0.008), driven by a large relative decrease in expression four hours postprandial following habituation to HP.

Figure 22G, H and I shows the expression of mRNA in muscle tissue of three amino acid transports PAT1 (Figure 22G), SNAT2 (Figure 22H), and LAT1 Figure 22I). We show neither a time nor an intervention effect for any of the amino acid transporters.

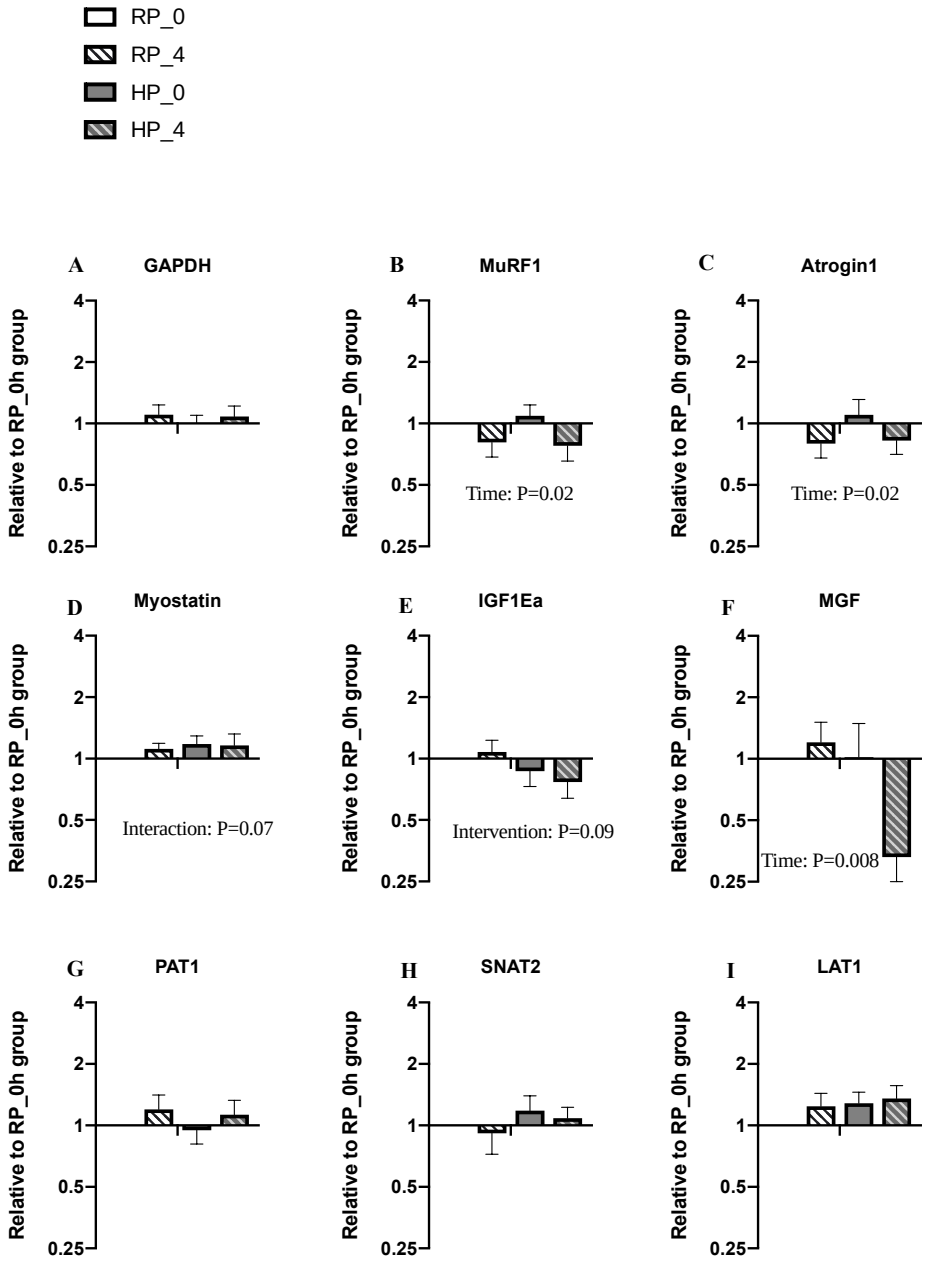

Figure 22: mRNA expression before and four hours postprandial, shown relative to fasting in the RP period. The expression of MuRF1 (B), Atrogin1 (C), Myostatin (D), IGF1Ea (E), MGF (F), PAT1 (G), SNAT2 (H) and LAT1 (I). Values are shown on binary logarithmic scales as the geometric mean ± backtransformed SEM, N=9.

Amino acids are dependent on carriers to transport them across cell membranes and into tissues [82]. We did not find that the amino acid specific transporters PAT1, SNAT2 and LAT1 were upregulated in the fasted state following three weeks of exposure to recommended or higher levels of proteins. Furthermore, none of the transporters were affected in response to increased levels in circulatory amino acids. A previous study has shown that in response to protein intake, the gene

expression of these transporters are only altered when combined with resistance exercise [85]. Further, the use of tissue biopsies always risks suffering from timing issues. Drummond et al.[83] found that the transcriptional upregulation is evident one hour after amino acid ingestion and returns to baseline level two hours following amino acid intake. Based on these existing data, any increased expression in mRNA coding for amino acid transporters appears to occur within the first hours following feeding. Therefore, the lack of change in expression at four hours postprandially in this study, might be due to timing of biopsy collection.

Muscle specific turnover, thus synthesis and breakdown were investigated both by employing the two pool and the three pool model, as well as looking for expression of muscle specific markers associated with synthesis and breakdown [23–26]. The muscle specific protein synthesis measured using the two pool model showed a significant increase in synthesis from the fasted to the immediate postprandial state. However, this was not seen for the same period when using the three-pool model. In both cases, there was no difference between habituate level of protein intake. The same was evident for the expression of myostatin, IGF1 and MGF, however there was a tendency towards an intervention effect for IGF1 with lower expression following habituation to high protein, and a tendency towards and interaction for myostatin where the tendency showed that only habituation to RP intake caused a postprandial increase in myostatin. Those results are contradicting as increased IGF1 has been associated with increased protein synthesis [32,33] and increased myostatin is known to inhibit synthesis [37]. However, it should be kept in mind that neither result did reach statistical significance. The expression of the IGF1 splice variant MGF decreased significantly postprandially, unlike what was expected based on the increased synthesis seen using the two pool model. Visually it seems that this is driven by a large decrease following habituation to HP, however there was no interaction. The fact that the expression of the markers for synthesis points in divergent directions and that there is no change in expression between fasting and fed, where we otherwise see an increase in synthesis in the two pool model, points to them as not being reflective markers for muscle specific protein synthesis in the fed state. This is confirmed, at least for myostatin by Mikkelsen et al. [151] and Agergaard et al. [152] who also do not find any change in myostatin with feeding alone.

The ingestion of the protein rich breakfast meal caused suppression of the atrogenes, Atrogin1 and MuRF1 (Figure 22B and C), indicating that feeding alone is

able to suppress muscle specific breakdown. Similar results were found by Mikkelsen et al. in a study where Rheumatroid arthritis patients received a whey supplement following one-legged exercise. In their study, the expression of both Atrogin1 and MuRF1 was decreased 1.5 hours after whey intake in the non-exercised control leg [151]. Likewise, Agergaard et al. showed in young males participants that the expression of Atrogin1 was decreased in muscle tissue from biopsies taken following ingestion of a mixed meal [152]. Despite our findings, that expression of Atrogin1 and MuRF1 is decreased in response to feeding, we did not observe a decrease in the rate of appearance across the leg, nor the muscle, both measures of muscle protein breakdown. However, it should be noted that an increased expression of genes affecting protein degradation at a certain time point does not or not necessarily translate into a decreased protein breakdown rate, especially not at that same time point but may be seen at a later point in time. Our findings seem different from what could have been expected. In a previous study by Nygren et al. (2003) it is evident that breakdown across the leg is significantly reduced following infusion of amino acids and insulin[153]. As we provide a mixed meal high in protein, the circulating amino acids coupled with the insulin secretion would, based on the findings by Nygren et al., be expected to result in a decreased breakdown, compared to fasting. A combination of the study by Nygren and the decrease in gene expression of breakdown markers, could indicate that the lack of a feeding effect on the breakdown measured across the leg and muscle, might be due to a lack of ability to detect the changes in breakdown.

The two-pool model A-V_f, leg model, is a commonly used measurement of metabolite and amino acids fluxes of skeletal muscle [133]. However, one of the underlying assumptions for both the two-pool and the three-pool model (an all other tracer measurements) is that there is a steady state in the infused tracer (in this case the D_8-phenylalanine) used for the calculations. The arterial enrichment of the infused D_8-phenylalanine, is the sum of D_8-phenylalanine and D_7-phenylalanine (the D_7-label is resultant from phenylalanine de- and re-amination). The enrichments are seen in Figure 23. There is a significant time effect, with the enrichment being lower at 30, 60 and 90 min postprandial compared to fasting. The decreased enrichment is the result of the phenylalanine from the breakfast meal diluting the tracer. This is a common issue which can be partially corrected by adding a dose of the infused tracer to the

meal, or by increasing the infusion rate of the tracer. We increased the infusion rate of D_8-phenylalanine following the meal intake. However, evidently not sufficient to overcome the dilution completely. From the equation used to calculate the rate of disappearance ($Femoral\ R_d = (C_a \cdot E_a\ - C_v \cdot E_v\ ) \cdot\ PF/E_a$) it is apparent that this rate will increase if the arterial enrichment decreases (assuming that the nominator remains unchanged because the enrichment in both artery and vein decreases). This will cause a somewhat overestimation of the rate of appearance ($Femoral\ R_a = Femoral\ R_d - NB$), thus potentially making it difficult to detect any decreases in rate of appearance in non-steady state conditions. However, in view of the relatively small transient changes in arterial enrichment it might not affect these measurements in a major way.

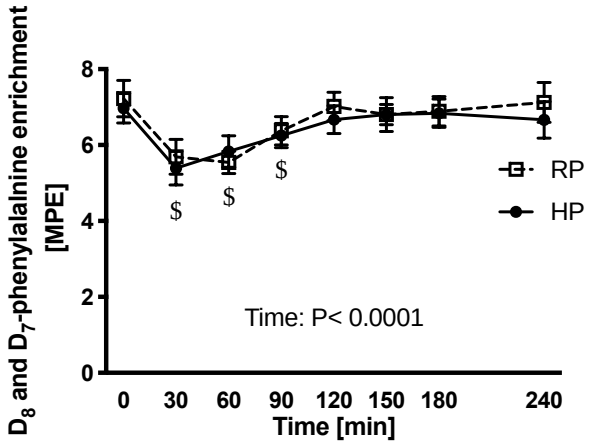

Figure 23: The sum of arterial enrichments from the infused D_8-phenylalanine and the D_7-phenylalanine resulting from transamination. $ denotes difference from fasting P<0.004. Values are mean ± SEM, N=11.

11.3 Incorporation into body proteins

The rate of incorporation of amino acids into proteins, the fractional protein synthesis rate (FSR), is determined for myofibrillar proteins (muscle biopsies) and plasma proteins (Figure 24). For the myofibrillar protein FSR there was no difference between the two interventions. However, plasma protein FSR revealed an intervention effect (P=0.04) in the form of a higher FSR across the four-hour postprandial period for participants habituated to the RP diet.

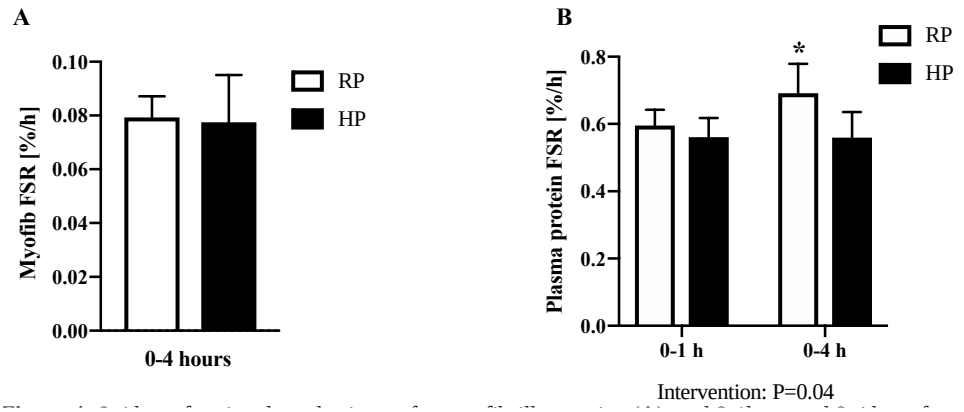

Figure 24: The fractional synthesis rate of myofibrillar protein (A) and plasma protein (B) in the postprandial period. * denotes difference between habituations P=0.0009. Values are mean ± SEM, N=9 (A) and N=11 (B).

The direct incorporation of phenylalanine from the intrinsically labelled whey (D_5-phenylalanine) and caseinate (^{15}N-phenylalanine) proteins ingested in the breakfast meals are shown in Figure 25. Values are given in MPE (mole percent excess) for D_5-phenylalanine and APE (atom percent excess) for ^{15}N-phenylalalnine. There was a significantly higher incorporation of both D_5- and ^{15}N-phenylalanine four hours postprandial compared to one hour postprandial ($p<0.0001$). Additionally, there was a significantly higher incorporation of D_5-phenylalanine into plasma proteins four hours post prandial following habituation to RP compared to HP (P=0.0007).

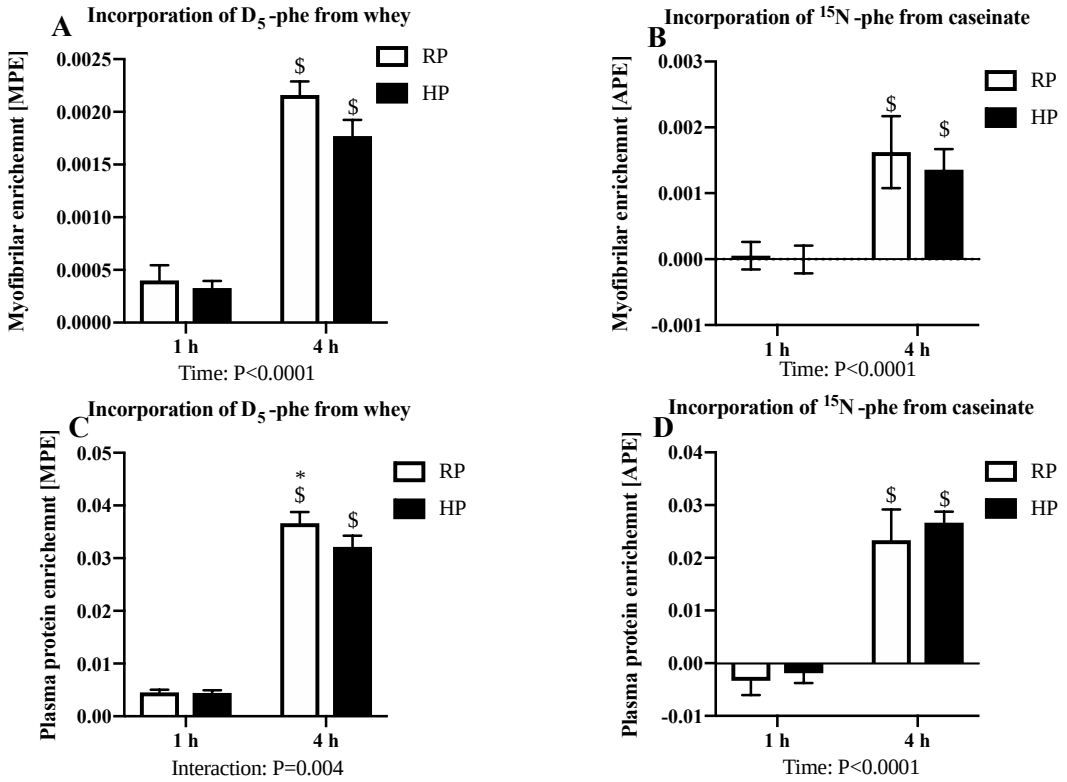

Figure 25: Incorporation of phenylalanine from whey (D_5-phenylalanine) and caseinate (^{15}N-phenylalanine) in the myofibrillar protein (A and B) and in the plasma proteins (C and D). $ denotes changes from one hour post prandial (P< 0.0001). * denotes changes between interventions (P=0.0007). Values mean ± SEM, N=9 (A and B) and N=11 (C and D)

Collectively, measurement of both the FSR (Figure 24) and the direct incorporation of isotope-labelled phenylalanine (Figure 25) showed that habituation to a HP compared to RP intake does not change the skeletal muscles ability to utilize dietary proteins in terms of changes in muscle protein synthesis rate and direct incorporation of dietary protein derived amino acids into muscle protein.

Gorissen and colleagues (2016) found the same in a similar setup, that habituation to 0.7 g protein/kg/day vs. 1.5 g protein/kg/day did not result in any changes to FSR in a five-hour post prandial period. In addition, they found no differences in the incorporation of phenylalanine from an intrinsically labelled whey protein despite finding higher circulating amino acid concentrations following habituation to a low protein intake as in the present study [113]. Bohe et al. [45] found a dose response relationship between the concentration of circulating essential amino acids and muscle protein synthesis rates, fitting the curve: MPS = 2.68 x [EAA]/(1.51 + [EAA]). However, neither this study, nor the study by Gorissen et al. [113] saw an

increased stimulatory effect on the muscle's synthesis rate with higher circulating amino acids.

Although we applied a cross-over design the inclusion of only nine participants for these particular analysis, albeit quite common for these kinds of studies, means that we need larger differences in order to reach significance as compared if we had a larger cohort.

For the total plasma proteins, we observed an intervention effect showing a significantly higher FSR following RP diet compared to HP, and in line with this a higher incorporation of phenylalanine from the whey protein four hours postprandially. Like the finding in this study, that the incorporation of phenylalanine from caseinate did not elicit any changes between habituation, Morens and colleagues looked at incorporation of amino acids from milk proteins (thus mix of caseinate and whey protein) into plasma proteins 8 hours postprandially [107]. The study by Morens et al. included young (~29 years of age) participants who were habituated to 1 g protein/kg/day or 2 g protein/kg/day and also did not observe differences between habituated level of protein intakes.

Taken together habituation to RP intakes compared to HP seems preferable with regard to utilizing amino acids from fast absorptive proteins for increased synthesis and incorporation into body proteins. This conclusion is in keeping with methodologic limitations and physiological interpretations on time effects inherent to these kinds of studies and may be responsible for some disagreement between the few studies conducted also because they are different in clinical and methodological design.

11.4 Excess amino acids

Amino acids that are not used for protein synthesis are metabolized via oxidation or conversion to other metabolites including other amino acids. Oxidation but also general amino acid metabolism is preceded by deamination and gives raise to urea production. The urea excretion was quantified in urine collected during the final day of the habituation (day 20) as well as during the trial day (day 21).

On day 20 (Figure 26A), there was a significantly higher urine urea quantity following habituation to the HP diet (P=0.01). On day 21, where all participants had the exact same protein intake (independent of prior habituation period), the urea excretion was identical during the 8-hour trial period independent of habituation. However, over the entire 24-hour period on day 21, a higher urea excretion was measured for participants habituated to a HP diet.

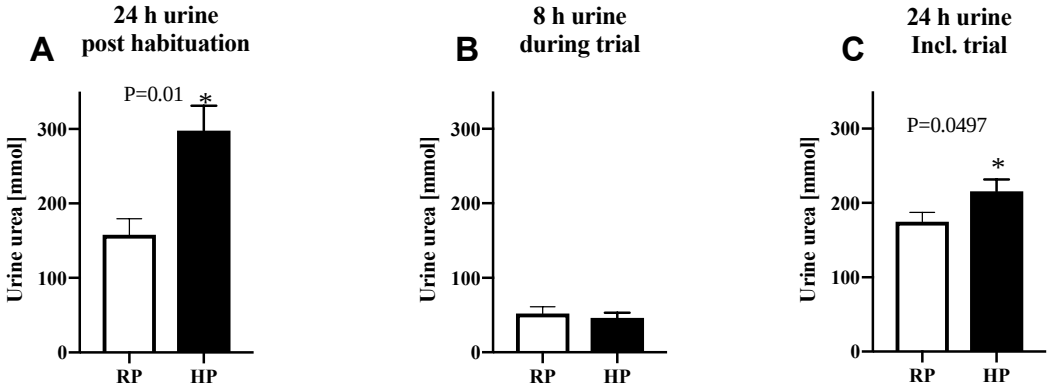

Figure 26: Urea excretion in the urine. A) 24-hour urine collection on day 20, B) 8-hour urine collection during the trial, including lunch, and C) 24-hour urine collection on day 21, including the 8-hour trial period. * denotes significant differences between interventions (A and B: N=10, C: N=12, values are mean ± SEM).

The rate at which urea appears into the circulation is shown in Figure 27. The rate of appearance of urea at fasting measured prior to the meal on the trial day was significantly higher for HP habituated participants (Figure 27A, P=0.0003) and continued to be so throughout the postprandial period with an increasing appearance rate for both HP and RP (Figure 27B, time and intervention: P<0.0001). No difference in change from fasting levels between the habituation periods, tested via two-way ANOVA of delta values from fasting (data not shown).

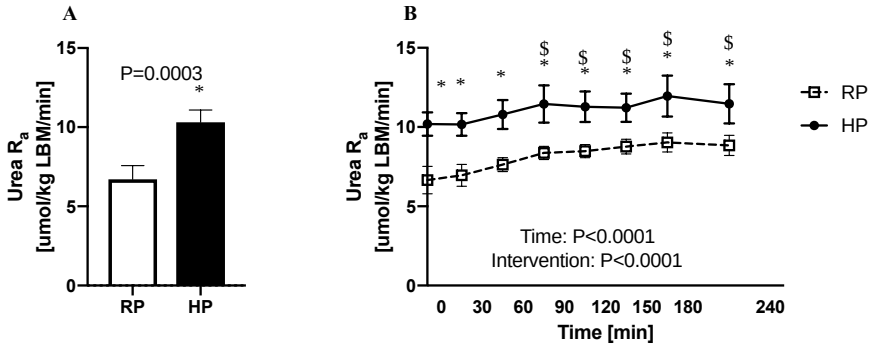

Figure 27: Urea rate of appearance in the post habituation, fasted state (A), and in the four-hour postprandial state (B). $ denotes significant changes from fasting (P≤0.01), *denotes significant difference between interventions (P<0.004). Values are mean ± SEM, N=10.

The conversion rate of phenylalanine to tyrosine, first and rate limiting step in phenylalanine oxidation, followed the same pattern as the urea rate of appearance. Habituation to HP compared to RP resulted in a significantly increased conversion rate in the fasted sate (Figure 28A, P=0.001), which was maintained in the postprandial state with a significant time and intervention effect (Figure 28B, time: P<0.0001, intervention: P=0.0002). The relative change from fasting is the same, tested via two-way ANOVA of delta values from fasting.

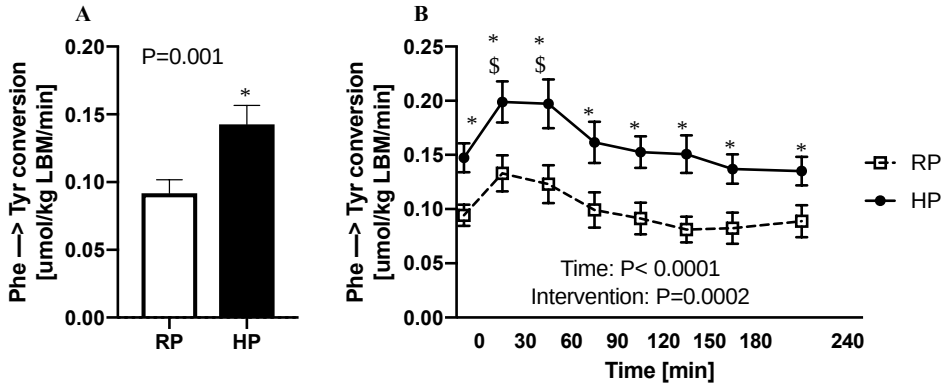

Figure 28: Phenylalanine conversion to tyrosine in the fasted state after 20 days of habituation (A) and in the four-hour postprandial state (B). $ denotes significant changes from fasting (P<0.0001), *denotes significant differences between interventions (P<0.0001). Values are mean ± SEM, N=8.

A high compliance to the dietary guidelines is supported by the urea quantity in the urine collected following the habituation period (day 20, Figure 26A). With a mean difference of 140 mmol urea, this is almost a two-fold increase from RP diet to HP diet, which is expected when adhering to a diet high in protein. This difference is not due to a different concentration of circulating amino acids in the fasted state because except for glycine, there are no fasting differences in amino acid concentration between RP and HP (Table 5).

The increased urea production and phenylalanine oxidation suggest faster removal rates of amino acids following habituation to a HP diet. This is in agreement with studies by Juliet and colleagues [80].

Despite an identical protein intake on day 21 the urine collected for 24 hours on day 21 (Figure 26C) had a substantially higher urea content after habituation to HP than RP diet. In addition, the rate of appearance of plasma urea remained elevated throughout trial period after HP (Figure 28B). These observations mean that more

nitrogen is produced in the HP habituated state despite the same intake suggesting an upregulated capacity to breakdown excess amino acids after habituation to high protein. After the HP period this resulted in a reduced ability to retain amino acids and nitrogen. The effects on urea production and excretion are supported by the phenylalanine hydroxylation rate. Despite similar protein intake at trial-day 21 a significantly increased phenylalanine hydroxylation rate was observed in HP habituated elderly. This increased oxidation rate of phenylalanine after HP habituation is in agreement with observations made while comparing two high levels of protein intake for leucine oxidations studied in both older (~70 years of age) and younger (~24 years of age) participants [114].

Conclusively, the increased urea production and phenylalanine conversion fit the hypothesis that HP diet leads to an increased splanchnic extraction and breakdown of amino acids as well as higher use of plasma protein production by the liver. Foe et al. proposed that an increase in plasma protein incorporation may be an effort to minimize the irreversible oxidation [154]. Thus, using the increased plasma protein synthesis as a storage of excess amino acids from the diet until needed.

11.5 Whole body amino acid and protein kinetics

The fasting whole body endogenous phenylalanine rate of disappearance and appearance, i.e. measures of whole body protein synthesis and breakdown, were increased in a fasted state following habituation to HP compared to RP (Figure 29, R_d: P=0.01, R_a: P=0.03). Thus, the total phenylalanine, protein, turnover rate was increased. However, the net phenylalanine balance was more negative in the fasted state following habituation to HP thus higher net loss of body proteins in spite of a higher turnover rate (Figure 29, P=0.03).

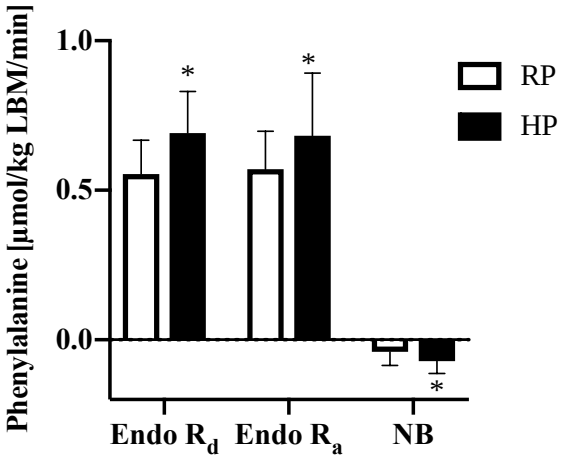

Figure 29: Fasting endogenous phenylalanine rate of disappearance (a measure of whole body synthesis), endogenous phenylalanine rate of appearance (a measure of breakdown) and the net phenylalanine balance (measure of a net loss or gain of body protein mass). * denotes significant difference between RP and HP conditions (P≤0.03) Values are mean ± SEM, N=9.

The different fasting protein turnover rates after HP as compared to RP causes a different starting point to study the response to the breakfast meal (Figure 30). Following both habituation periods there is a significant meal effect (time: P<0.0001) with the synthesis rate being increased from 45-210 min postprandial, with a intervention effect showing higher synthesis rates following habituation to HP compared to RP (Figure 30A, intervention: P=0.047). The whole body breakdown rates are decreased postprandial (Figure 30B, time: p<0.0001), with no differences between interventions despite the difference in the fasted state. The difference between synthesis and breakdown, i.e. the resultant net balance changed from being negative in the fasted state to positive over the course of the entire post prandial period (Figure 30C, time: P<0.0001), with no differences between interventions.

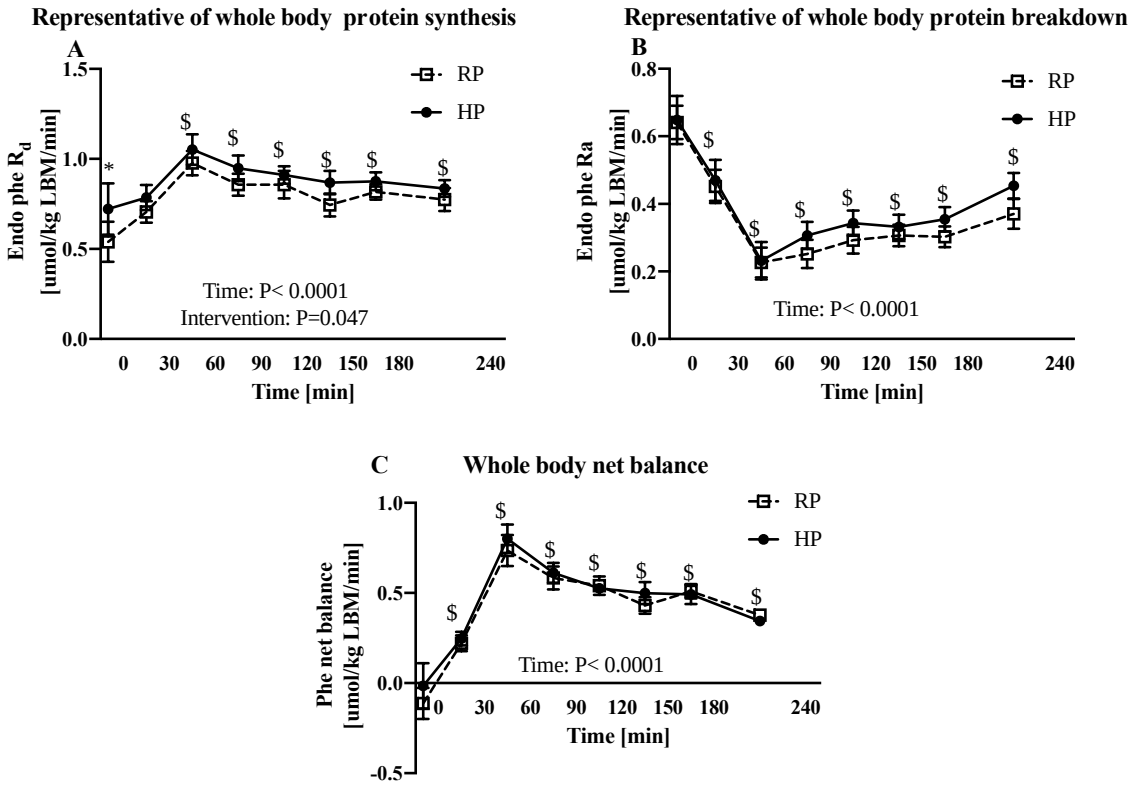

Figure 30: Whole body turnover in the immediate pre and four-hours postprandial phase. The endogenous rate of disappearance (A), the endogenous rate of appearance (B), and the whole body net balance (C). $ denotes significant changes from fasting (P<0.04) and * denotes significant differences between interventions with the Sidak post hoc test (P=0.005). Values are mean ± SEM, N=8

Habituation to HP compared to RP intake resulted in a higher breakdown and synthesis rate in the fasted state and higher synthesis in the postprandial state. Thus, the whole body turnover rate was higher following habituation to HP. The absolute mean difference in the net balances between HP and RP was 0.03 µmol/kg LBM/min. The average lean body mass of the 9 included participants was 55.7 kg and the molecular weight of phenylalanine is 165.19 g/mol. This results in a difference of 0.017 g phenylalanine/hour. Assuming that body proteins consists of 4.5% phenylalanine [155] and that humans, conservatively, undergo 8 hours fasting during a day, the net protein loss is 3 g of whole body proteins/day (0.2%/year) following HP diet compared to LP diet. This larger protein loss in the HP condition was not compensated for by the four-hour postprandial period (here exemplified by the protein rich breakfast), evident as the postprandial net balance was similar following both HP and RP intakes. However, our study conditions are experimental, controlled and short, whereas individuals with a habitual high protein intake would most likely ingest more proteins during the entire day, causing a higher postprandial diurnal positive net protein balance. Hence, compensating for the extra protein losses in the fasting

period. Alternatively, the results emphasize that once habituated to higher dietary protein intakes, one better not stop ingesting meals high in protein.
Pannemans and colleagues (1995), also observed an increased protein turnover following habituation, in a study where young [108] and older [109] participants were habituated to either 12 or 21 E% protein for three weeks. In a newer study with a similar set up, Gorissen and colleagues (2016) habituated older male participants to 0.70 g protein/kg BW/day or 1.53 g protein/kg BW/day for 14 days and determined fasting and postprandial response to a meal [113]. They could not find significant differences between habituation to protein intake [113]. However, they observed trends that dietary habituation to low protein compared to high protein amounts seemed to result in a postprandial lower protein breakdown, no difference in protein synthesis, but improved net protein balance. These results correspond well with our findings, except for the lack of protein synthesis response. The reason for the different results regarding protein synthesis might be due to the given stimuli. We provide ~34 g protein as part of a mixed meal (0.61 g protein/kg LBM), where Gorissen et al. provide 25 g isolated whey protein (~0.42 g protein/kg LBM). Moore and colleagues showed that for older individuals it took 0.61 g protein/kg LBM to reach plateau level protein synthesis [54]. Therefore, lack of a response in protein synthesis may be expected to be higher in our study than the study by Gorrisen. The peak mean synthesis in this study is found to be 1.05 μmol/kg LBM/min. With an average body weight of the included participants being 81.8 kg, this results in 63.0 μmol/kg BW/h. The mean peak synthesis in the study by Gorrissen et al. found approximately 34 μmol/kg BW/h and was thus substantially lower. This could indicate that a difference in the synthesis response in habituation to divergent protein intakes is either easier to detect due to less variability at higher protein synthesis rates, or that habituation to HP results in an increased ability to utilize very high dietary protein intakes compared to habituation to RP intake.

Conclusively, whole body protein turnover is increased following habituation to HP compared to RP protein intake. However, the fasting net protein balance is more negative, i.e. losing more body protein in the HP habituated state. This loss is not compensated for in the four hours following ingestion of a protein rich meal.

12.0 Conclusion and perspectives

A high protein diet, HP, with ~220% increased protein ingestion compared to a WHO-recommended diet, RP, did not lead to an effect on skeletal muscle specific protein kinetics, neither when measured by tracer dilution within the muscle (3-pool model), tracer dilution across the leg (2-pool model), myofibrillar fractional synthesis rate nor direct incorporation of amino acids derived from dietary protein. For plasma protein habituation to RP intake led to increased incorporation of amino acids deriving from fast absorptive whey protein as well as an increased fractional synthesis rate. The vast majority of plasma proteins are synthesized in the liver indicating an increased liver FSR following habituation to RP intake. However, on a whole body level, the HP compared to RP intake resulted in an increased protein turnover at fasting. This was due to an increase in both protein synthesis and protein breakdown rates. Most importantly in terms of lean body mass, the overall effect was a more negative net protein balance during fasting following a HP diet. The relatively lower net balance seen following HP diet compared to RP diet resulted in a delta net loss of 3 g of protein pr. day. A protein loss which was not counterbalanced by the protein rich meal over a four-hour period resulting in a net loss of lean body mass. In addition, both the fasting and postprandial response showed higher amino acid wasting through increased use of amino acids for oxidations as well as increased urea production, even following identical relative protein intakes. This shows that habituation to HP intake inhibits nitrogen retention whereas habitation to RP intake show indications of amino acid sparing effects.

With this, our hypothesis that habituation to an increased protein intake would improve meal protein utilization, result in higher whole body, blood protein and muscle protein synthesis, as well as higher net protein balance can be completely rejected. Quite contrarily, it seems that three weeks of habituation to a high protein intake activates the body's ability to remove excess amino acids by both oxidation and thus urea production. Thereby, lowering the nitrogen sparing capabilities as well as the ability to synthesize new proteins when 'normal' quantities of meal amino acids are available.

This study shows that we should be cautious to advise 'higher than needed' protein intakes, thus ensuring that we do not increase the bodies removal of amino acid by oxidation and urea production. However, this study cannot conclude on recommendations for protein intake, which require either long-term studies or at least 24h whole body net balance measurements. Firstly, as the postprandial response investigated was a response to a very protein dense meal, which cannot be expected to be comparable to a typical diet ingested for individuals habituated to the recommended level of protein intakes. Secondly, we do not assess the diurnal net balance, but merely the response to one single meal in the four-hour postprandial period. Thus, it is possible that in a real life setting the post-prandial net balance would be lower in participants habitually ingesting the recommended level of protein intake as their meals would contain less protein. Likewise, the lower net balance seen following HP intake might be compensated by the intake of several protein rich meals during an entire diurnal period. This is substantiated by the finding by Kim et al, that following four days habituation to 0.8 g protein/kg BW/day vs. 1.5 g protein/kg BW/day a higher post prandial net balance was seen, when the participants ingest the level of protein intake they are habituated to. Thus the net balance response is the result of divergent protein intakes [156].

For future studies, the differences we found in net protein balance in the fasted states after habituation to different levels of protein intake, emphasizes that these measurements are a prerequisite for reliable measures of acute protein handling with meal intake. Further, the fact that the we see a response on the whole body level, but not on the skeletal muscle level, indicates that studies investigating the body's protein needs based on whole body protein metabolism cannot be used to give recommendations on optimal protein intake for muscle mass maintenance and vice versa.

It should be noted that our elderly volunteers were healthy and a relatively young representative of the older population (66.6±1.6 years of age). Hence, the finding of the present study may not necessarily be valid for much older individual. However, we feel that the general effects of this study with respect to habituation to a high protein intake is likely applicable to all ages. Therefore, our recommendation for future studies is to investigate; 1) detailed amino acid and protein metabolism over 24

hours with multiple meal/protein intake; 2) confirm our findings in young healthy individuals and preferably ~80 years of age; 3) investigate the effects on elderly with common disease and adverse drug effect like hypertension, high cholesterol and type 2 diabetes as their protein needs or amino acids utilization efficiency may be different. This with the hypothesis that regardless of age, supplementing older individuals with protein intakes which largely exceeds the current recommendations, will activated the body's ability to remove excess amino acids thereby lowering the nitrogen sparing capabilities. This will lead to higher protein loss in the fasted state, that will not be compensated for by meal intakes, as the postprandial ability to retain nitrogen is minimized.

13.0 References

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14.0 Papers

Paper 1 (manuscript): Grith Højfeldt, Jacob Bülow, Jakob Agergaard, Ali Asmar, Peter Schjerling, Lene Rørdam, Jens Bülow, Gerrit van Hall, Lars Holm. *Post-absorptive and post-prandial amino acid metabolism and whole body protein synthesis and degradation after 3 weeks habituation to a normal and high protein intake*

Paper 2 (manuscript): Grith Højfeldt, Jacob Bülow, Jakob Agergaard, Lene Rørdam, Jens Bülow, Peter Schjerling, Gerrit van Hall, Lars Holm. *The postprandial plasma protein but not muscle protein synthesis is decreased after 20 days habituation to a high protein intake*