

Protein intake to support muscle health in a clinical setting

Citation for published version (APA):

Weijzen, M. (2022). *Protein intake to support muscle health in a clinical setting*. [Doctoral Thesis, Maastricht University]. Maastricht University. <https://doi.org/10.26481/dis.20221019mw>

Document status and date:

Published: 01/01/2022

DOI:

[10.26481/dis.20221019mw](https://doi.org/10.26481/dis.20221019mw)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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The studies presented in this thesis were performed within the NUTRIM, School of Nutrition and Translational Research in Metabolism at Maastricht University.

Design and layout: Tessa Schreurs

Printed by: Gildeprint

ISBN: 978-94-6469-012-5

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Protein intake to support muscle health in a clinical setting

DISSERTATION

To obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus, Prof. dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
on Wednesday 19 October 2022, at 16:00 hours

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Chapter 1

General introduction

Introduction

Adequate nutritional intake is essential to maintain nutritional status, support crucial body functions, maintain quality of life, and lower disease burden. Food intake, with protein ingestion in particular, and physical activity are important regulators of muscle mass (1-3). Skeletal muscle mass is in a constant state of turnover, generally maintaining a balance between muscle protein synthesis and muscle protein breakdown rates. Following the ingestion of dietary protein, plasma amino acid concentrations rise, providing precursors to support *de novo* muscle protein synthesis (3). Besides building blocks, amino acids are also signalling molecules that directly stimulate muscle protein synthesis. When muscle protein synthesis rates exceed muscle protein breakdown rates, a positive net protein balance will develop, resulting in net muscle mass gain (**Figure 1.1**).

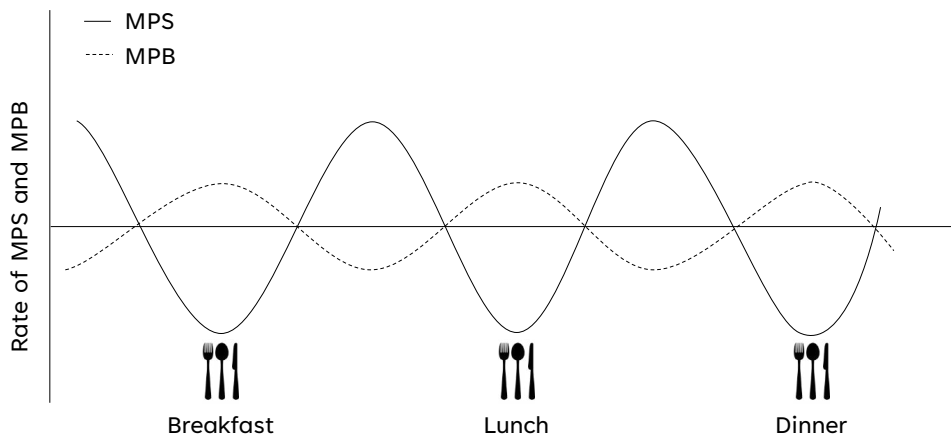


Figure 1.1 rate of muscle protein synthesis and muscle protein breakdown.

In situations where a decline in skeletal muscle mass is observed, such as during muscle disuse or illness, an imbalance between protein breakdown and synthesis rates results in a negative net muscle protein balance. To maintain muscle mass adequate amounts of protein should be consumed. Protein intake recommendation guidelines set by the world health organisation (WHO) are 0.8 g protein per kg bodyweight per day for adults of all ages (4). It has been suggested that older individuals or individuals with acute or chronic illness should consume 1.2-1.5 g protein/kg/d (5). Furthermore, it has been proposed that daily protein intake should be equally distributed throughout the day, with at least 20 g protein being provided with each main meal. The latter was based on the observation that the ingestion of 20 g protein is sufficient to maximize postprandial muscle protein synthesis rates in healthy young males (6, 7). However, older individuals may require more protein per meal to maximize muscle protein synthesis rates due to the anabolic resistance of aging (8-10). Anabolic resistance is defined as the reduced capacity of skeletal muscle tissue to increase muscle protein synthesis rates following an anabolic stimulus (i.e. protein ingestion). In general, habitual protein intake ranges between 0.9 and 1.1 g/kg/d in healthy, community-dwelling, older individuals (11, 12), with a considerable amount of individuals (14-45%) consuming below the recommended intake level of 0.8 g protein/kg/d (12). Though daily protein intake is generally sufficient in the active, healthy, older population, many older individuals experience periods where daily protein intake is compromised.

Food intake during hospitalization

Substantial changes in habitual food intake may occur when individuals are hospitalized. Food intake is typically reduced due to a variety of factors, such as restricted timing of food provision, adverse effects of medication, a reduced appetite, and prescribed periods of fasting (**Figure 1.2**) (13-15).

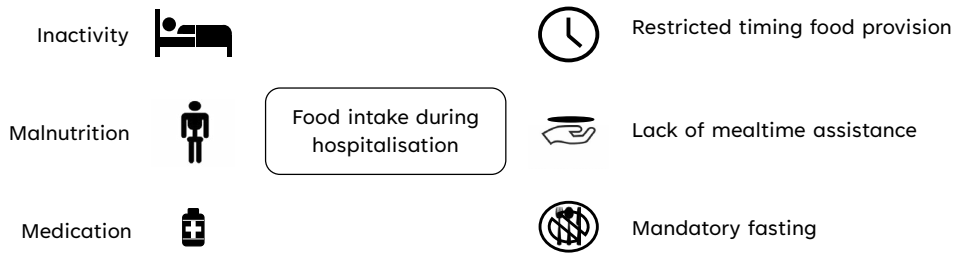


Figure 1.2 factors influencing food intake during hospitalization.

The reduced food intake can lead to malnutrition, which is defined as a deficiency in energy, protein, and/or micronutrients (16). When patients are admitted to the hospital they are screened for malnutrition using either the Short Nutritional Assessment Questionnaire (SNAQ) or the Malnutrition Universal Screening Tool (MUST) (17, 18). Patients are classified as low, medium, or high risk for malnutrition. During hospitalization, malnutrition is a critical and highly prevalent problem, as up to 40% of the patients have been reported to be malnourished during their hospital stay (19, 20). Malnutrition has been shown to prolong the length of stay (19, 21, 22), accelerate the loss of muscle mass (23), impair functional outcome (24), and increase the risk of morbidity and mortality (21, 22, 25). A dietician will be asked for a consult when patients are identified as high-risk for malnutrition and/or when the patient's nutritional status deteriorates during hospital stay. Usual care entails dieticians advising patients to consume more protein-rich foods, and/or provide oral nutritional supplements (ONS) in addition to their main meals. There are a variety of (logistical) concepts available to provide food during hospitalization. These logistical concepts differ between hospitals. A commonly used food concept is providing main meals, i.e. breakfast, lunch, and dinner, at strict timeslots, that patients have to order in advance. Unfortunately, there are few data available on energy and protein intake during hospital stay. Most studies have assessed food intake by estimating dietary intake or using food frequency questionnaires in hospitalized patients (26-28). Only few studies have measured the amount of food that was actually consumed (29, 30). It is important that the amount of food consumed is accurately measured, as earlier studies have shown that patients do not consume all food that is provided (31, 32). Clinical nutrition guidelines (ESPEN) recommend a protein intake level of 1.2-1.5 g/kg/d for malnourished patients or patients at risk for malnutrition due to acute or chronic illness as a means to support muscle mass and strength maintenance (5, 33). Previous work has shown that these guidelines are by no means met, as protein intake is generally low (~0.9 g/kg/d) in older patients (30), with less than 30% of the hospitalized patients meeting the recommended protein intake levels (30, 34).

Strategies to improve protein intake

Clearly, current strategies are not sufficient for patients to meet the recommended daily protein intake requirements of 1.2-1.5 g/kg/d. Strategies proposed to improve energy and/or protein intake during hospitalization are changing the meal ordering system, adding meal moment(s), fortifying products with high quality protein or adding protein rich supplements to the diet. A recent study shows that changing the hospital meal service from a traditional service (i.e. three strict meal moments where patients have to order in advance), to a service that comprises 6 small but protein-rich meals per day, provided directly at the bedside of the patient, improved dietary intake and patient satisfaction (35). Daily protein intake with the traditional meal service was 0.71 g/kg/d, while the novel food service resulted in a higher protein intake of 0.87 g/kg/d. The novel food service resulted in more patients meeting protein intake requirements when compared to the traditional meal service (23 vs 8%, respectively). In situations where it is not possible to change the hospital food system, main meals can be optimised by using more protein-rich or food products fortified with high quality protein. It has been shown that modifying the hospital diet by using fortified products improves dietary protein intake (36). Fortifying 'familiar' products, such as bread, soup, and yoghurt can increase daily protein intake during hospitalization (30, 37). Another frequently applied strategy to increase protein intake is to provide oral nutritional supplements (ONS). Previous work has shown that ONS provision can increase daily protein intake by as much as 8-30 g protein per day (38-40). Which type, source, and/or amount of protein is preferred may depend on the population and physical activity level of the population. It is generally assumed that higher quality protein sources, such as animal-based proteins, are preferred over plant-based proteins as they are more rapidly digested and absorbed and have a higher essential amino acid content (41, 42). Furthermore, protein hydrolysates may be preferred over intact proteins as they can augment amino acid absorption during the early postprandial phase (43, 44). In some conditions, such as critical illness, digestion and absorption might be impaired and an individualized approach may be required to optimize food intake and attenuate muscle mass and strength loss during hospitalization. Another strategy to increase daily protein intake levels could be to modulate the timing of consuming protein-rich products or supplements. Protein ingestion prior to sleep is followed by normal protein digestion and amino acid absorption kinetics and increases overnight muscle protein synthesis rates (45, 46). Protein ingestion prior to sleep may represent an opportunity to stimulate muscle protein synthesis rates in addition to the three main meals. At present, much work is being done to assess whether pre-sleep protein supplementation can increase daily protein intake and improve nutritional status in patients admitted to the hospital.

Physical activity

Aside from poor nutritional status, physical activity levels are also very low in patients during their hospitalization. Hospitalized patients spend 93-99% of their time sitting or lying down, with very little time standing or walking (47). There are observations that inpatients complete less than 1000 steps/d during their hospital stay (47). As periods of inactivity typically induce anabolic resistance (48), the combination of malnutrition and physical inactivity is very alarming, and can result in functional decline, loss of independence, and accelerated loss of muscle mass during hospital stay (49-52). The accelerated muscle loss during hospitalization can have a detrimental consequences on muscle strength and function in the older population, as many older adults do not recover fully from this catabolic event (**Figure 1.3**) (53).

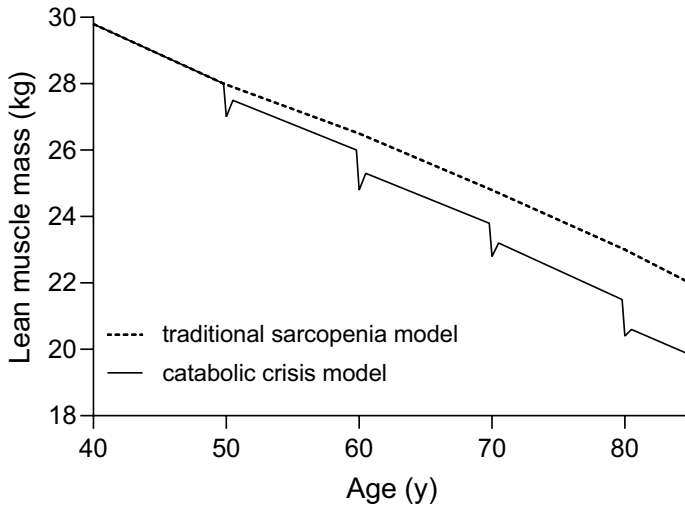


Figure 1.3 catabolic crisis model – muscle loss (adapted from English et al).

Muscle disuse has been studied using different lab-based models such as limb immobilization or bed rest. Previous studies show that even a few days of disuse already lead to significant loss of muscle mass and strength in both young and older individuals (54-61). Lab-based disuse models are used to mimic hospital stay. The average hospitalization stay ranges between 5 and 13 days (62, 63). Recent work shows that one week of hospital admission results in similar amounts (~5%) of muscle loss as lab-based disuse studies (51, 64). In more extreme conditions, such as during critical illness, more severe muscle wasting (~18% after 10 d) has been demonstrated (65). Moreover, survivors of critical illness can experience ICU-acquired muscle weakness up to 5 years following ICU admission (66). As every patient case is unique, an individualized approach is necessary to optimize food intake and maximize physical activity to help preserve muscle mass and strength during hospitalization.

Outline of dissertation

The present thesis investigates various aspects of nutrition and muscle health in clinically compromised patient groups as well as healthy volunteers. To determine how food intake can be optimized during hospitalization, we first needed to assess the nutritional status of patients admitted to our hospital. In Chapter 2 we describe food intake in older patients admitted to the hospital for elective total knee or hip replacement surgery. As we showed that patients admitted for elective orthopaedic surgery were malnourished during their hospital stay, we were interested whether more vulnerable patients showed similar (low) food intake levels during hospitalization. Therefore, we assessed food provision and consumption in patients at risk for malnutrition in Chapter 3. Thereafter, we assessed whether protein supplementation could attenuate muscle loss and/or augment muscle mass regain following 1 week of unilateral leg immobilization in healthy, young males (Chapter 4). As postprandial protein handling plays an important role in the anabolic response to feeding, we assessed the protein digestion and amino acid absorption kinetics and the subsequent muscle protein synthetic response of intact protein and free amino acids in a group healthy, young volunteers (Chapter 5). It has been suggested that protein digestion

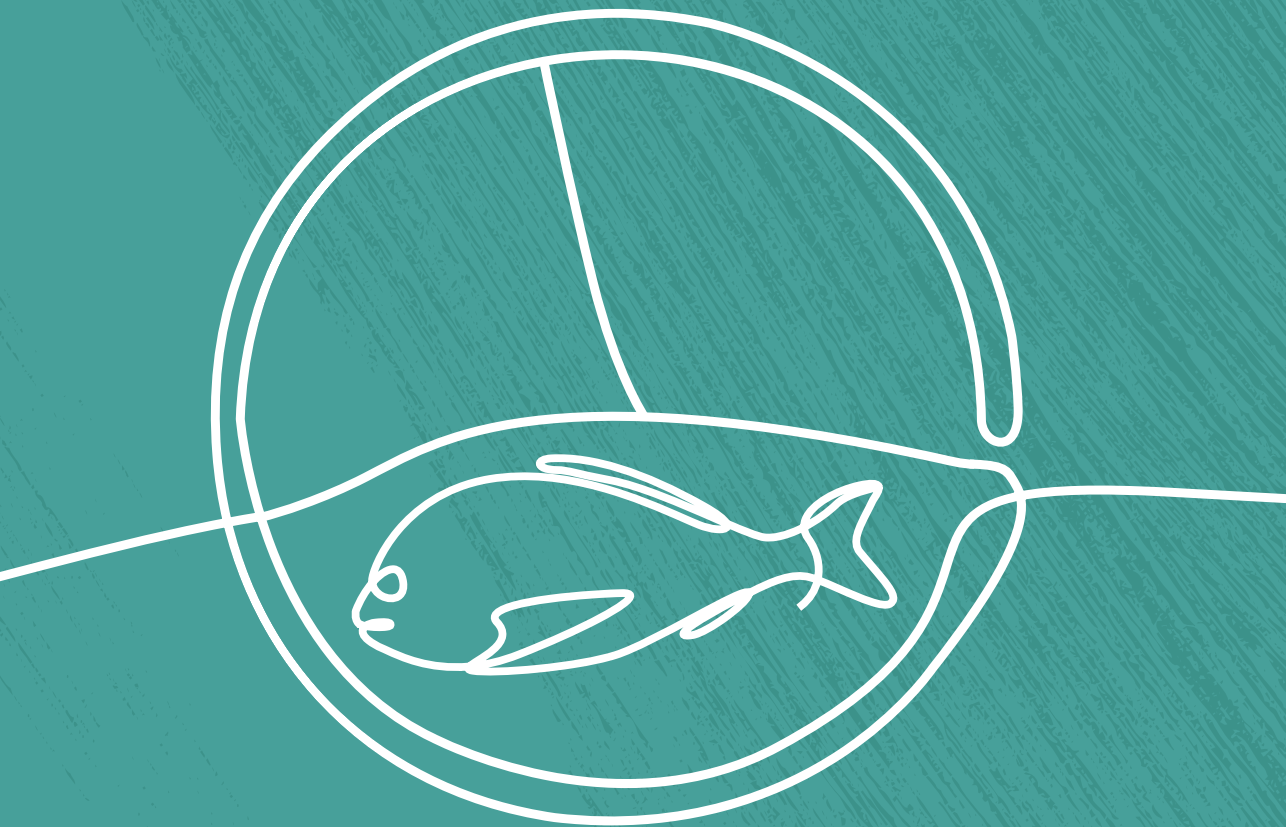
and amino acid absorption are compromised in intensive care unit patients, therefore we also assessed protein digestion and amino acid absorption kinetics in critically ill patients following the ingestion of intact protein and free amino acids (Chapter 6). Finally, in Chapter 7 we discuss the implications of the findings presented in this thesis and address key areas for future research.

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Chapter 2

Protein intake falls below $0.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in healthy, older patients admitted for elective hip or knee arthroplasty

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J Nutr Health Aging. 2019;23(3):299-305

Abstract

Background

Hospitalization is generally accompanied by changes in food intake. Patients typically receive hospital meals upon personal preference within the framework of the food administration services of the hospital.

Objective

In the present study, we assessed food provision and actual food and snack consumption in older patients admitted for elective hip or knee arthroplasty.

Methods

In this prospective, observational study, $n=101$ patients (age: 67 ± 10 y; hospital stay: 6.1 ± 1.8 d) were monitored during hospitalization at the Orthopaedic nursing ward of the Maastricht University Medical Centre+ following elective hip or knee arthroplasty. Energy and protein provided by self-selected hospital meals and snacks, and actual energy and protein (amount, distribution, and source) consumed by patients was weighed and recorded throughout 1-6 days.

Results

Self-selected meals provided 6.5 ± 1.5 MJ·d⁻¹, with 16, 48, and 34 En% provided as protein, carbohydrate, and fat, respectively. Self-selected hospital meals provided 0.75 ± 0.16 and 0.79 ± 0.21 g·kg⁻¹·d⁻¹ protein in males and females, respectively. Actual protein consumption averaged merely 0.59 ± 0.18 and 0.50 ± 0.21 g·kg⁻¹·d⁻¹, respectively. Protein consumption at breakfast, lunch, and dinner averaged 16 ± 8 , 18 ± 9 , and 20 ± 6 g per meal, respectively.

Conclusions

Though self-selected hospital meals provide patients with ~ 0.8 g·kg⁻¹·d⁻¹ protein during short-term hospitalization, actual protein consumption falls well below 0.6 g·kg⁻¹·d⁻¹ with a large proportion ($\sim 32\%$) of the provided food being discarded. Alternative strategies are required to ensure maintenance of habitual protein intake in older patients admitted for elective orthopaedic surgery.

Introduction

Hospitalization in older adults is accompanied by substantial changes in food intake. At present, the hospitalization duration in older patients is 5 days or longer [1, 2]. During such a short period of hospitalization, food intake is generally reduced due to periods of fasting, adverse effects of medication, strict timing of food provision, reduced appetite, and/or pain and discomfort [3-5]. Such a reduced food intake throughout the hospitalization period often results in a negative energy and/or protein balance. Low levels of energy and protein intake, also referred to as protein-energy malnutrition, result in accelerated loss of lean body mass, muscle strength, and impairments in functional capacity [6-8]. Moreover, malnutrition during hospitalization has been shown to increase the length of hospital stay, the risk for infections, the incidence of hospital readmissions, and mortality rates [9-12].

The negative health consequences of malnutrition are, at least partly, attributed to the concomitant loss of skeletal muscle mass and strength. Several studies from our group, as well as others, have shown significant declines in muscle mass and strength during short periods of immobilization [13-18] and hospitalization [19, 20]. It has been well-established that the loss of muscle tissue is accelerated when energy balance remains negative [21]. Apart from the negative effects of physical inactivity, the loss of muscle mass observed during hospitalization may be particularly attributed to an insufficient protein intake as a direct consequence of the lower energy intake. The current recommended dietary intake for protein has been set at $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for healthy adults of all ages. In the general population, older community-dwelling individuals consume well above ($\sim 1.0 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) these recommended protein intake levels [6, 22-24]. Recently updated guidelines suggest a protein intake of $1.2\text{-}1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for older individuals suffering from acute or chronic diseases in order to maintain muscle mass during hospitalization [25, 26]. Minimal requirements during hospitalization should be aimed at achieving energy balance and maintaining habitual protein intake. Currently, patients typically receive hospital meals upon personal request within the framework of the food administration services of the hospital. However, it is unknown whether these self-selected hospital meals provide sufficient energy and/or protein.

Whereas several studies have assessed energy and/or protein provision in patients during hospitalization [3, 9, 27-33], only few studies have measured the actual amount of energy and protein consumed by patients [4, 34-38]. In addition, snack consumption between meals is generally not reported. Recent studies show that less than 30% of the older hospitalized patients reach a protein intake of $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ [36, 39]. Moreover, protein intake has been shown to be well below these increased recommendation levels for at least one day in older patients during hospitalization [36, 38]. There is no data available on actual energy and protein intake during the entire hospitalization period in older patients. In addition, a clear quantification on the protein consumption pattern in hospitalized patients such as the protein amount per meal, protein distribution, and protein sources has not been reported. We hypothesized that older patients during several days of hospitalization consume well below the recommended protein intake level of $1.2\text{-}1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$.

In the present study, we assessed food provision as well as actual food and snack consumption in older patients ($n=101$) during short-term hospitalization following elective total hip or knee arthroplasty. We quantified the amount, distribution, and source of protein in self-selected hospital meals consumed at breakfast, lunch, dinner, and snacks during several days of hospitalization.

Materials and methods

Study design

This observational study assessed the nutritional content of self-selected hospital meals, and measured actual food and snack consumption in all patients undergoing elective hip or knee arthroplasty between April 2016 and August 2016. Patients were screened for malnutrition using the Malnutrition Universal Screening Tool (MUST) [40] upon arrival on the nursing ward as part of standard admission procedures (all included patients had a MUST=0 score upon hospital admission). Information concerning the project was given orally and patients gave consent to collect their food trays after meal consumption. Age, BMI, type of anesthesia used during surgery, and length of stay (LOS) were recorded. There was no extra burden on the patient during hospitalization. There were no exclusion criteria. The study was registered as NTR5942 (www.trialregister.nl). Observational food intake data and retrospective, blinded patient data were collected under the Agreement on Medical Treatment Act and the Personal Data Protection Act, according to Medical Ethical standards.

Provision of hospital meals

Hospital meals were provided at three strict timeslots every day; at breakfast, lunch, and dinner. In between the main meals, patients were provided with hot and/or cold drinks 3 times a day. There was mealtime assistance during the provision of all meals. Patients selected their meals upon request the day before and could indicate different portion sizes of 0.5, 1, or 2 portion(s). During the first day after surgery, when patients were not able to select their meals due to the time spent at the surgery room, they received standard hospital meals.

Consumption of hospital meals

On the serving tray, patients received a description of their ordered menu, which was collected for the study. When patients were finished eating, the serving tray was taken and all leftovers were weighed using a scale (Soehnle, Germany) and reported. To assess snack consumption, patients were asked to fill in a daily snack list during hospitalization that was collected daily. If patients were not able to complete the snack list, the researcher recalled their snack consumption in between meals and reported this. During the entire assessment period the researcher was present at the ward.

Nutritional content of hospital meals

Total energy (MJ), protein (g and En%), carbohydrate (g and En%), and fat (g and En%) were calculated for all provided and consumed food based upon product specifications provided by the food suppliers and the Dutch Food Consumption Database 2016 (NEVO; RIVM, Bilthoven, the Netherlands) [41]. The contribution of animal- and plant-based sources to dietary protein intake was determined and expressed as a percentage of total protein intake for all meals. Food intake was recorded from the day of hospital admission (day 0) until the day of hospital discharge (day 2-6). Data on food intake on the day of hospital admission and discharge was not included, since these days did not include all main meals. The reported intake was calculated in $n=101$ patients from the day of surgery (day 1) until day 2, in $n=98$ until day 3, in $n=52$ until day 4, and in $n=17$ until day 5.

To estimate patients' nutritional needs, energy requirements were calculated based upon resting energy expenditure using the Harris and Benedict equation [42]. A Physical Activity Level (PAL) of 1.3 for patients "not restricted to bed" and an Injury Factor (IF) of 1.2 for "minor operation" was used, resulting in a correction factor of 1.56 to estimate (minimal) energy requirements [43]. During hospital stay patients were encouraged to mobilize as soon as possible and received physiotherapy training for 30 min daily, from day 2 onwards.

Statistical analysis

All data was checked for normality and was normal distributed, except for energy and protein consumption on day of surgery (day 1). Data are expressed as mean±SD (for consistency; both normal and non-normal distributed data). Differences between provided and consumed food intake were analyzed using a paired Students t-test. Energy and protein intake during hospitalization and between main meals were analyzed using repeated measures ANOVA with time as within-group factor (either days or meals) and gender as between-group factor. Analyses for energy and protein intake were performed for patients hospitalized until day 3 (**Figure 2.1 and 2.2**) and analyses for protein intake distribution were performed excluding snack consumption (**Figure 2.3**). In case of a significant interaction between time and gender, separate analyses were performed to determine time-effects for males and females (one-factor repeated measures ANOVA) with a Bonferroni post-hoc test to locate these differences and between-group effects for each time-point (Students t-test). Statistical significance was set at $P<0.05$. All calculations were performed using the statistical software program SPSS (version 24.0, IBM Corp., Armonk, USA).

Results

Patients' characteristics

In total, 101 older patients were monitored (males/females: 37/64; age: 67 ± 10 y; hospital stay: 6.1 ± 1.8 d) during hospitalization following elective hip or knee arthroplasty. Patients' characteristics are presented in **Table 2.1**. Estimated resting metabolic rate averaged 7.15 ± 0.74 MJ·d⁻¹ in males and 5.81 ± 0.87 MJ·d⁻¹ in females. The estimated (minimal) energy requirements were calculated using a correction factor of 1.56 and averaged 11.15 ± 1.16 MJ·d⁻¹ in males and 9.06 ± 1.35 MJ·d⁻¹ in females.

Table 2.1: Patients' characteristics

	All patients (n=101)	Males (n=37)	Females (n=64)
Age (y)	67±10	66±8	68±10
Body weight (kg)	81.1±16.9	86.3±11.0	78.1±18.9*
Height (m)	1.69±0.09	1.75±0.07	1.65±0.08*
BMI (kg·m⁻²)	28.4±5.0	28.3±3.6	28.5±5.7
Length of stay (d)	6.1±1.8	6.1±1.8	6.1±1.9
Resting Metabolic Rate (MJ·d⁻¹)	6.30±1.04	7.15±0.74	5.81±0.87*
General Anesthesia (n)	68 (67%)	25 (68%)	43 (67%)
Spinal Anesthesia (n)	33 (33%)	12 (32%)	21 (33%)
THA (n and %)	57 (56%)	19 (51%)	25 (39%)
TKA (n and %)	44 (44%)	18 (49%)	39 (61%)

Values are mean±SD. BMI: body mass index, THA: total hip arthroplasty, TKA: total knee arthroplasty. Resting metabolic rate was calculated based upon gender, body weight, height and age, using the adjusted Harris and Benedict equation. Data were analyzed by a Students t-test. * Indicates a significant difference between males and females, $P < 0.05$.

Energy provision and consumption

Energy provision and consumption (MJ·d⁻¹) from self-selected hospital meals during hospitalization are presented in **Figure 2.1**. Hospital meals provided 6.90 ± 1.78 MJ·d⁻¹ in males (**Figure 2.1A**) and 6.25 ± 1.34 MJ·d⁻¹ in females (**Figure 2.1B**) during the entire hospitalization period, while energy consumption averaged 5.29 ± 1.77 and 3.96 ± 1.39 MJ·d⁻¹, respectively. On average, $32 \pm 17\%$ of the provided food (i.e. energy content) was not consumed ($P < 0.001$). For energy intake, a significant time x gender interaction was observed ($P < 0.001$). For both males and females, energy intake increased from day 1 (1.58 ± 1.87 and 1.02 ± 1.38 MJ·d⁻¹) to day 2 (6.45 ± 2.66 and 4.60 ± 2.00 MJ·d⁻¹; $P < 0.001$), with a further increase observed to day 3 (6.92 ± 2.77 and 5.24 ± 1.73 MJ·d⁻¹) although this only reached significance for females ($P = 0.001$).

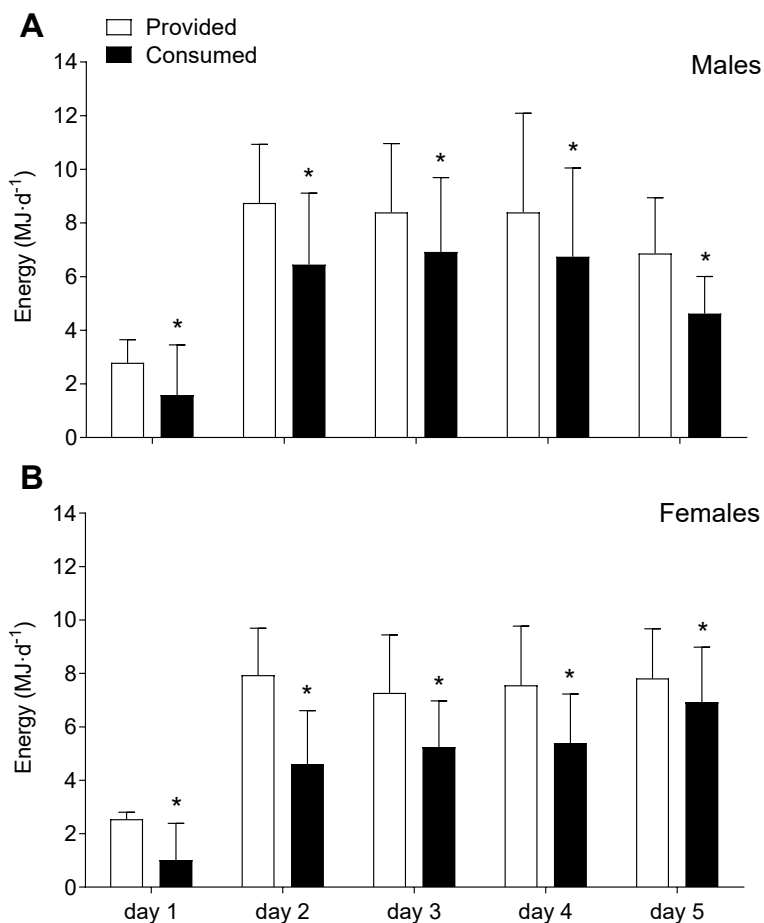


Figure 2.1 Mean (\pm SD) energy provision and consumption ($\text{MJ}\cdot\text{d}^{-1}$) during short-term hospitalization in older, hospitalized males (**A**) and females (**B**). Food intake was calculated in $n=101$ (MIF 37/64) patients until day 2, in $n=98$ (MIF 36/62) until day 3, in $n=52$ (MIF 17/35) until day 4, and in $n=17$ (MIF 6/11) until day 5. * Indicates a significant difference when compared with provided food, $P<0.001$.

Energy consumption was lower in females when compared with males on day 2 and 3 of hospitalization (both $P<0.001$) and tended to be lower in females on day 1 ($P=0.084$). Total macronutrient consumption (g and En%) in males and females are presented in **Table 2.2**.

Table 2.2 Macronutrient intake in older, hospitalized males (n=37) and females (n=64).

	Energy (MJ)		Carbohydrate (g and (En%))		Protein (g and (En%))		Fat (g and (En%))	
	Males	Females	Males	Females	Males	Females	Males	Females
Breakfast	1.90±0.64	1.46±0.61 *	49±17 (44%)	39±15 (46%)*	20±8 (18%)	14±7 (16%)*	18±8 (36%)	14±7 (36%)*
Lunch	2.17±0.80	1.59±0.62 *	56±22 (44%)	42±17 (45%)*	23±11 (18%)	16±7 (17%)*	21±9 (37%)	15±8 (36%)*
Diner	1.75±0.45	1.51±0.45*	53±16 (51%)	46±13 (51%)*	21±6 (20%)	19±6 (21%)	12±5 (26%)	10±5 (26%)
Snacks	0.80±1.06	0.54±0.30	30±30 (63%)	24±14 (73%)	3±5 (6%)	2±2 (6%)	6±14 (30%)	3±3 (20%)

Values are mean±SD. Data were analyzed by a Students t-test. * Indicates a significant difference between males and females, $P<0.05$.

Protein provision and consumption

Protein provision and consumption ($\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) from self-selected hospital meals during hospitalization are presented in **Figure 2.2**. Self-selected hospital meals provided $0.75\pm 0.16 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in males (**Figure 2.2A**) and $0.79\pm 0.21 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in females (**Figure 2.2B**) during hospitalization, while actual protein consumption averaged merely 0.59 ± 0.18 and $0.50\pm 0.21 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, respectively. The consumed amount of protein was $32\pm 19\%$ lower than the provided amount of protein at all days of hospitalization ($P<0.001$). Protein intake levels increased during hospitalization to a similar extent in males and females (time \times gender interaction effect, $P=0.306$, main time effect, $P<0.001$), with lower protein intakes on the day of surgery (day 1) when compared with day 2 and day 3 ($P<0.001$), and on day 2 when compared with day 3 ($P=0.007$). Protein intake during hospitalization was overall lower in females when compared with males (main gender effect, $P=0.028$).

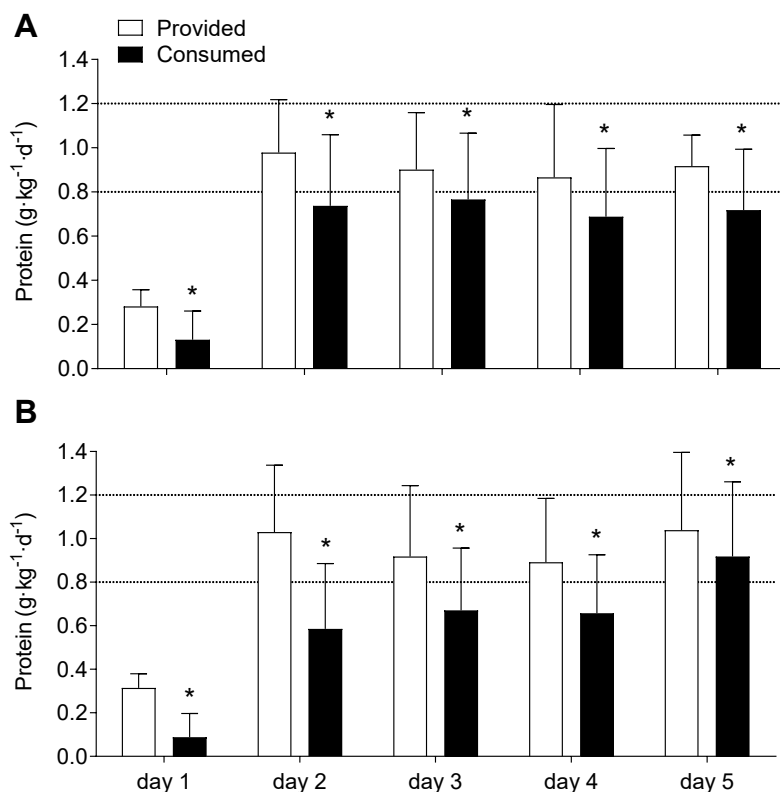


Figure 2.2 Mean (\pm SD) protein provision and consumption ($\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) during short-term hospitalization in older, hospitalized males (**A**) and females (**B**). Food intake was calculated in $n=101$ (MIF 37/64) patients until day 2, in $n=98$ (MIF 36/62) until day 3, in $n=52$ (MIF 17/35) until day 4, and in $n=17$ (MIF 6/11) until day 5. The dotted lines represent the recommended dietary intake of $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ and the recommended protein intake of $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ suggested for older, hospitalized individuals. * Indicates a significant difference when compared with provided food, $P<0.001$.

Protein intake per meal

Distribution of protein provision and consumption (g) across main meals is presented in **Figure 2.3**. Absolute protein intake across main meals is shown in **Table 2.2**. Self-selected hospital meals provided 26 ± 8 , 28 ± 10 , and 23 ± 4 g protein in males, and 23 ± 7 , 23 ± 6 , and 24 ± 4 g protein in females at breakfast, lunch, and dinner, respectively. Protein consumption was $32\pm 23\%$ lower than protein provision at breakfast ($P<0.001$), $27\pm 23\%$ at lunch ($P<0.001$), and $17\pm 18\%$ at dinner ($P<0.001$). A significant time \times gender interaction effect was observed for protein intake distribution during hospitalization ($P<0.001$). Protein intake in males averaged 20 ± 7 , 22 ± 11 , and 21 ± 6 g at breakfast, lunch, and dinner, respectively, with no differences between main meals ($P=0.157$). Protein intake in females differed between main meals and averaged 14 ± 7 , 16 ± 7 , and 19 ± 6 g, respectively ($P<0.001$). Post-hoc analyses in females showed that protein consumption was lower at breakfast and lunch when compared with dinner (both $P<0.001$), and lower at breakfast when compared with lunch ($P=0.042$). Protein consumption during hospital admission was lower in females when compared with males at breakfast and lunch (both $P<0.001$), but did not differ at dinner between genders ($P=0.120$). Snack consumption provided merely 3 ± 5 g protein per day in males and 2 ± 2 g in females, with no differences between genders (**Figure 2.3A and B**; $P=0.141$).

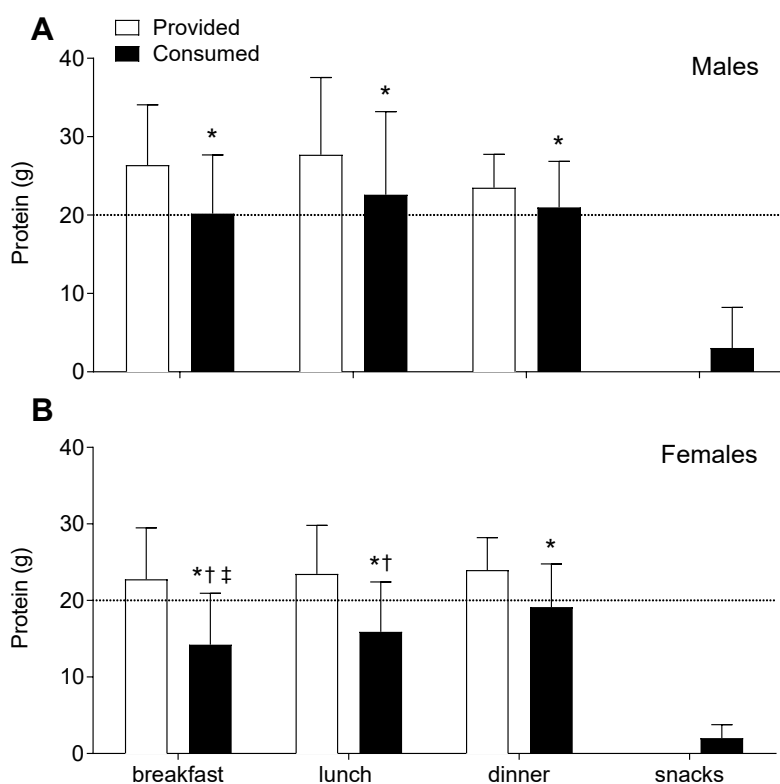


Figure 2.3 Mean (\pm SD) dietary protein provision and consumption (g) across main meals in older, hospitalized males (**A**; $n=37$) and females (**B**; $n=64$). * Indicates a significant difference when compared with provided, $P<0.001$; † Indicates a significant difference when compared with dinner, $P<0.001$; ‡ Indicates a significant difference when compared with lunch, $P<0.05$.

Protein distribution per main meal as % of the total consumed amount of protein is presented in **Supplemental Figure 2.1**. Breakfast, lunch, and dinner provided 30 ± 7 , 33 ± 8 , and $33\pm 7\%$ protein in males (**Supplemental Figure 2.1A**) and 27 ± 8 , 30 ± 7 , $39\pm 9\%$ protein in females (**Supplemental Figure 2.1B**). Snacks contributed for the remaining $4\pm 5\%$ protein in males and $4\pm 3\%$ in females.

Protein sources

The contribution of animal- and plant-based sources to total protein intake (%) is shown in **Figure 2.4**. In total, protein intake from self-selected hospital meals contained a higher amount animal-based protein sources when compared with plant-based protein sources ($66\pm 7\%$ and $34\pm 7\%$, respectively, $P<0.001$). Dairy products and eggs provided the largest amount of animal-based proteins ($35\pm 12\%$), followed by meat and fish ($30\pm 11\%$). For plant-based protein sources, bread, cereals, and potatoes provided the largest amount of protein ($26\pm 7\%$). Fruit, vegetables, and legumes provided only $4\pm 2\%$ of plant-based protein to the total diet, and $4\pm 3\%$ did not fit in any of these categories.

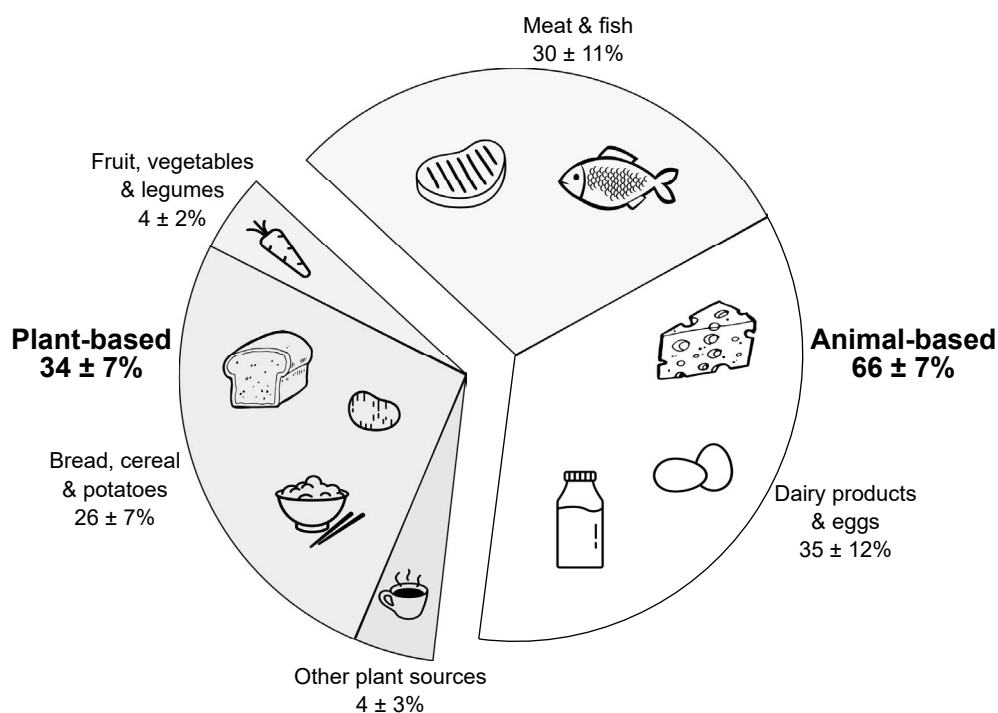


Figure 2.4 Contribution of animal- and plant-based sources to total dietary protein intake (expressed as a percentage of total protein intake) in older, hospitalized patients ($n=101$).

Discussion

Self-selected hospital meals provided patients with $\sim 0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ protein during short-term hospitalization, while actual protein intake was below $0.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ with more than 30% of the provided food being discarded. Absolute protein intake per main meal ranged from 14 to 22 g in all patients, with protein intake being particularly low at breakfast in females. A total of 34% of daily protein intake was derived from plant-based protein sources.

In the present study, we assessed actual energy and protein consumption patterns during short-term hospitalization in older patients admitted for elective hip and knee arthroplasty. Daily energy consumption averaged 5.29 ± 1.77 and $3.96\pm 1.39 \text{ MJ}\cdot\text{d}^{-1}$ in males and females, respectively, which is merely $\sim 50\%$ of the estimated energy requirements. Consequently, all patients remained in a negative energy balance throughout their hospitalization. At present, up to 30% of the older patients in Western-European hospitals have been identified as being malnourished during hospitalization [9-11, 44, 45]. Poor nutritional status in older hospitalized patients has been associated with accelerated weight loss, slower wound healing, an increase in length of hospital stay, higher mortality rates, and more frequent hospital readmission [9-11, 45, 46]. An energy deficit during hospitalization and bedrest accelerates the loss of skeletal muscle mass [21]. The muscle atrophy typically observed during hospitalization [19, 20, 47] may be largely attributed to the lack of sufficient protein consumed as a result of low(er) energy intake levels. We assessed dietary protein consumption in older patients during short-term hospitalization after total hip or knee arthroplasty. Self-selected hospital meals provided patients with merely $\sim 0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ protein, which is well below recommended daily intake levels of $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ [25, 26]. Since 30% of food provided was discarded, actual protein consumption was much lower and averaged $0.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. Protein intake was particularly low on the day of surgery when compared with subsequent hospitalization days (Figure 2.2). Though protein consumption increased on the following days of recovery on the orthopedic ward, protein consumption still remained far below recommended daily intake levels ($1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) throughout the entire hospitalization period. This seems to support previous work showing that protein consumption is insufficient in older patients during short-term hospitalization [36, 39]. Consequently, interventional strategies should be employed to effectively increase energy intake to match energy requirements and, as such, avoid energy malnutrition. Furthermore, protein intake should be maintained at habitual intake levels, which in the light of a reduced daily energy intake, requires the installment of a more protein-dense diet.

The ingestion of dietary protein, and more specifically the postprandial rise in plasma amino acid concentrations, stimulates muscle protein synthesis and inhibits muscle protein breakdown, thereby stimulating muscle protein accretion [48]. The postprandial increase in muscle protein synthesis rates forms an important factor in muscle mass maintenance. Ingestion of 20 g of a high quality protein has been shown to increase postprandial muscle protein synthesis rates in young adults [49, 50]. Due to anabolic resistance with aging, greater amounts of protein (>20 g per meal) are required to significantly stimulate muscle protein synthesis in older individuals [51-53]. In the present study, the amount of protein consumed at breakfast, lunch, and dinner varied between 14 and 22 g in both the male and female patients (Figure 2.3). While protein intake per meal was likely sufficient to induce an anabolic response in the male patients, protein intake remained well below 20 g for most meals in females. Particularly at breakfast, protein intake averaged only 14 ± 7 g in the female patients. This seems to agree with previous findings

showing that protein consumption is particularly low at breakfast in older individuals [22, 24, 54]. Given the suboptimal anabolic response with every main meal and the presence of anabolic resistance to protein ingestion in older individuals [53], the protein content of each main meal should be increased to allow ingestion of at least 20 g protein per main meal.

As animal-based proteins are generally regarded as being more anabolic than plant-based proteins [55, 56], we also assessed the contribution of animal- and plant-based protein sources to total dietary protein intake (Figure 2.4). Self-selected hospital meals contained a relative large amount of animal-based protein sources ($66\pm 1\%$) when compared with plant-based protein sources ($34\pm 1\%$). This is in line with the general Western diet in community-dwelling older adults [57]. As shown in Figure 2.4, protein intake in older patients was mainly derived from meat, fish, dairy products and eggs, which are the main protein sources in the aging population [6, 58, 59]. The relative contribution of animal versus plant-based protein sources in the hospital diet does not seem to require any modification.

Our data clearly show that even healthy patients admitted for elective hip or knee arthroplasty consume far less energy and protein than the estimated daily requirements. Since more than 30% of the provided food is not consumed, it is obvious that simply increasing food provision will not be effective to prevent energy and protein malnutrition during hospitalization. As the maintenance of habitual protein intake levels is key to attenuate muscle mass loss, a more protein-dense diet should be consumed as total energy intake is typically reduced during hospitalization. Various strategies can be applied to increase the relative protein intake in the diet, including the consumption of more protein-rich foods, supplementation with oral nutritional supplements (ONS), fortification of meals with protein isolates, and/or the provision of well-timed protein-rich snacks [36, 38, 60, 61]. To increase absolute protein intake in the diet, a first target for intervention should be breakfast. As protein consumption is typically low at breakfast (Figure 2.3), increasing protein intake at breakfast should be of greater benefit. In support, additional protein supplementation at breakfast has been shown to increase skeletal muscle mass and function in older, frail individuals [62, 63]. Another target would be increasing food consumption on the day of surgery. As energy and protein intake were hardly existing on the day of surgery, food provision during the pre- and post-surgery period could be installed within the restraints set by the surgical procedures. The provision of ONS may help to cover the energy and protein deficits during the first 1-2 days following surgery. During subsequent days of hospitalization, food fortification, provision of more protein-dense foods, and/or an adding an extra protein meal will likely be more appropriate. In line, provision of protein-enriched foods (such as bread, yoghurt, cake, fruit juice, and soup) or the use of more protein-dense foods throughout the day have been shown effective in improving protein intake during hospitalization [36, 38, 60]. In addition, the timing of protein-rich products serves as alternative strategy to increase protein intake levels during hospitalization. We have recently shown that protein ingestion prior to sleep increases overnight muscle protein synthesis rates in healthy, older men [64, 65], and supports muscle mass and strength gains during prolonged exercise training in young adults [66]. However, whether prolonged pre-sleep protein supplementation can attenuate muscle mass and/or strength loss in older patients during hospitalization remains to be assessed. Nutritional intervention strategies need to be assessed for their efficacy to increase energy and protein intake and, as such, to help preserve muscle mass and strength in older patients during hospitalization.

In conclusion, energy and protein intake levels remain well below requirements during hospitalization in older patients admitted for elective hip or knee arthroplasty. While patients are provided with $0.8 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, actual protein consumption does not even reach $0.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ with 30% of the provided food being discarded. Strategic interventions are required to increase energy intake and ensure maintenance of habitual protein intake levels in older patients admitted for elective orthopedic surgery.

Acknowledgements

We thank all medical staff and meal assistants of the Orthopedic nursing ward of the Maastricht University Medical Centre+ for their collaboration and assistance.

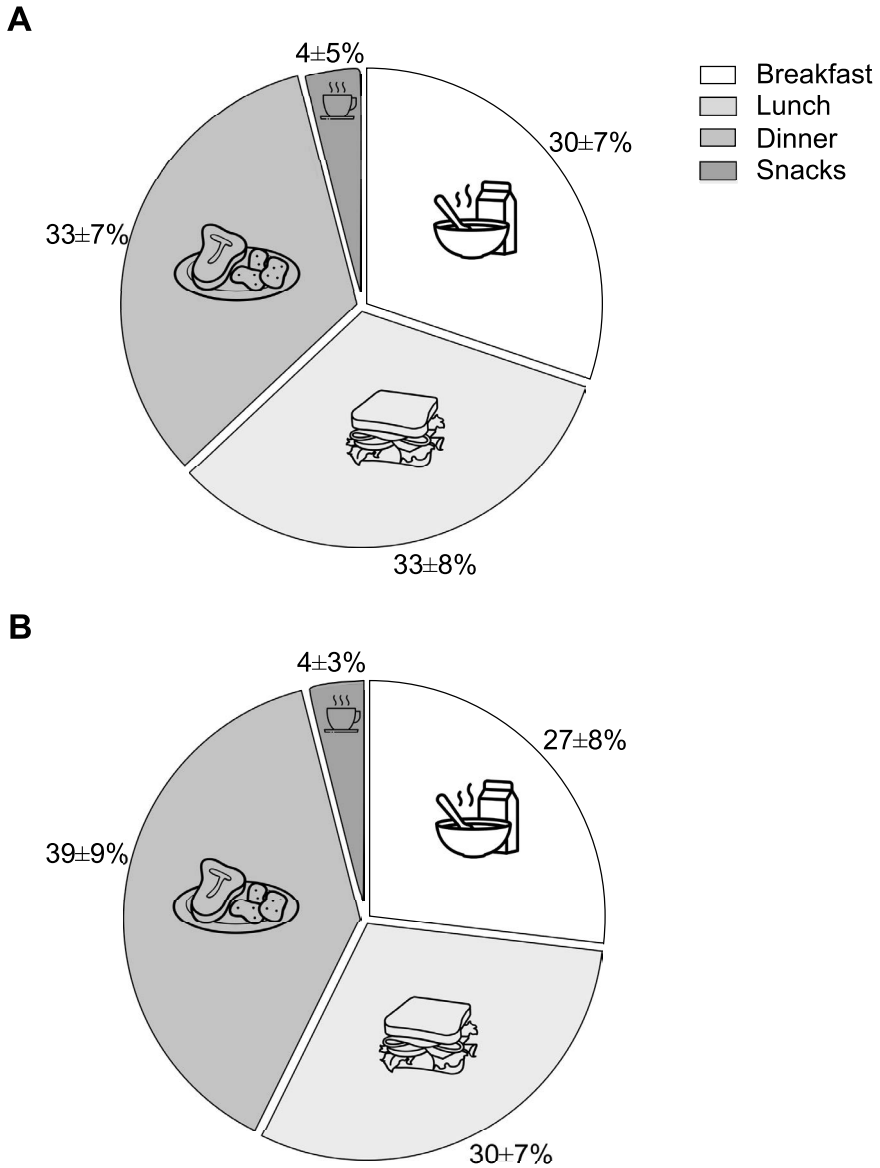
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Supplemental material



Supplemental figure 2.1 Distribution of protein intake across main meals and snacks (in % of the total amount of protein consumed) during short-term hospitalization in older, hospitalized males (**A**; n=37) and females (**B**; n=64).



Chapter 3

During hospitalization, older patients at risk for malnutrition consume <0.65 grams of protein per kilogram body weight per day

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Nutr Clin Pract. 2020 Aug;35(4):655-663

Abstract

Background

Malnutrition is prevalent in hospitalized patients. To support muscle maintenance in older and chronically ill patients, a protein intake of 1.2-1.5 g·kg⁻¹·d⁻¹ has been recommended during hospitalization. Common nutritional strategies to increase protein intake are the provision of more protein-dense foods and the use of oral nutritional supplements.

Objective

The aim of this study was to assess daily protein intake levels and distribution in older patients at risk for malnutrition during hospitalization.

Methods

In this prospective, observational study, we measured actual food and food supplement consumption in patients ($n=102$, age: 68 ± 14 y; hospital stay: 14[8-28] d) at risk of malnutrition during hospitalization. Food provided by hospital meals, oral nutritional supplements and snacks, and the actual amount of food (not) consumed were weighed and recorded for all patients.

Results

Hospital meals provided 1.03[0.77-1.26] g protein·kg⁻¹·d⁻¹, while actual protein consumption was only 0.65[0.37-0.93] g·kg⁻¹·d⁻¹. Protein intake at breakfast, lunch, and dinner was 10[6-15], 9[5-14], and 13[9-18] g, respectively. The use of oral nutritional supplements ($n=62$) resulted in a greater energy (1.26[0.40-1.79] MJ·d⁻¹) and protein intake levels (11[4-16] g·d⁻¹), without changing the macronutrient composition of the diet.

Conclusions

Despite a daily protein provision of ~1.0 g·kg⁻¹·d⁻¹, protein intake in hospitalized older patients remains well below these values (~0.65 g·kg⁻¹·d⁻¹) as 30-40% of the provided food and supplements are not consumed. Provision of oral nutritional supplements may increase energy and protein intake, but does not change the macronutrient composition of the diet. Current nutritional strategies to achieve the recommended daily protein intake in older patients during their hospitalization are not as effective as generally assumed.

Introduction

Hospitalization is accompanied by substantial changes in habitual food intake. Food intake during hospitalization is typically reduced due to restricted timing of food provision, adverse effects of medication, a reduced appetite, and prescribed periods of fasting [1-3]. Malnutrition during hospitalization is a critical and highly prevalent problem, as up to 40% of the patients have been reported to be malnourished during their hospital stay [4, 5]. Malnutrition is defined as a deficiency in energy, protein, and/or micronutrients [6, 7] and is generally accompanied by a more adverse clinical outcome during hospital stay. During hospitalization, malnutrition in patients has been shown to prolong the length of stay [4, 8, 9], accelerate the loss of muscle mass [10], impair functional outcome [11], and increase the risk of morbidity and mortality [8, 9, 12].

Accelerated loss of skeletal muscle mass and strength is, at least partly, attributed to the negative health consequences of malnutrition. Muscle mass loss during hospitalization results in functional decline and the loss of independence in older patients [13]. Muscle atrophy, already observed during a few days of hospitalization [14-16] is accelerated by the associated physical inactivity and/or an insufficient protein intake, as a direct consequence of the lower energy intake. The current recommended dietary intake for protein is set at $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for healthy adults of all ages according to the WHO [17]. Recent ESPEN nutritional guidelines recommend a higher protein intake level of $1.2\text{-}1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for malnourished patients or patients at risk for malnutrition due to acute or chronic illness as a means to support muscle mass and strength maintenance [18, 19]. Previously, we showed that during short-term hospitalization protein intake falls well below $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in patients undergoing elective orthopaedic surgery [20]. In line, several other studies have reported that hospitalized patients do not meet these recommended protein intake levels [21-23]. So far, most studies have assessed food intake by using estimated dietary intake records or food frequency questionnaires in hospitalized patients [23-25], whereas only a few studies have actually measured the amount of food that was consumed [20-22]. A difference of 30-40% between food provision and food consumption has been reported during hospital stay, resulting in low energy and protein intake levels in older patients [20, 26-28].

In current practice, patients at risk for malnutrition are referred to a dietitian during hospitalization. Consequently, various nutritional strategies are applied to increase protein intake. The use of more protein-rich foods in the diet [29], protein fortification of meals [21, 30], and/or the provision of oral nutritional supplements (ONS) [31-33] are being applied to increase protein intake during hospitalization. However, there is currently limited data on actual protein consumption in older patients at risk of malnutrition, their protein intake distribution pattern, and the actual consumption of the prescribed oral nutritional supplements during hospitalization. In the present study, we therefore assessed both food provision and food consumption from self-selected hospital meals and the actual intake of oral nutritional supplements, and snacks in 102 older patients at risk of malnutrition during several days of hospital admission. We hypothesized that despite the existing nutritional strategies to increase protein intake, older patients at risk for malnutrition do not achieve current protein intake guidelines during hospitalization.

Methods

Study design

In the current prospective observational study the nutritional content of hospital meals, snacks, and oral nutritional supplements was assessed, and actual food, snacks, and ONS consumption was measured in $n=102$ patients during their hospitalization. Three different nursing wards (respiratory $n=36$, geriatric $n=32$, and general surgery $n=34$) were selected to include patients between February 2017 and June 2017. The Malnutrition Universal Screening Tool (MUST) was used to screen patients for malnutrition 34 upon arrival on the nursing ward as a part of standard admission procedures. Patients gave consent to collect their food trays after meal consumption after information concerning the project was given orally. Patients were included if they were either screened as malnourished (MUST score 2), at risk for malnutrition (MUST score 1), or if they were indicated as at risk for malnutrition by a dietitian due to various reasons (i.e. recent weight loss, having nutritional support at home, or reporting low food intake during hospitalization). Age, gender, BMI, reason for admission, and length of stay (LOS) were recorded. Patients were excluded if they received exclusive or supplemental parenteral or enteral nutrition, or if their hospital stay was expected to be less than three days. There was no extra burden on the patients during hospitalization. Retrospective, blinded patient data, and observational food intake data were collected under the Agreement on Medical Treatment Act and the Personal Data Protection Act, according to Medical Ethical standards.

Provision of hospital meals

There were three strict timeslots every day where hospital meals were provided; breakfast (~8:00 AM), lunch (~12:00 noon), and dinner (~5:30 PM). Patients were provided with voluntary hot and/or cold drinks, snacks, and ONS three times a day in between the main meals (at set time points at 10:00 AM, 2:00 PM, and 7:00 PM). During the provision of all meals there was mealtime assistance. Patients chose their main meal and portion size (0.5, 1, or 2) the day before. Data was collected for a minimum of three days and data collection was stopped after a maximum 7 days (**Supplemental figure 3.1**).

Consumption of hospital meals

Researchers collected a description of the patients' ordered meal, which was available on the serving tray. This was done for breakfast, lunch, and dinner. The researchers collected the serving tray and all leftovers were weighed using a scale (Soehnle, Germany) when patients were finished eating. To assess snack and ONS consumption, snack- and supplement leftovers were weighed and the researcher collected wrappers and nursing notes, and communicated with food assistants, patients, and family. A researcher was present at the ward during the entire assessment period.

Oral nutritional supplements (ONS)

Of the total group of 102 patients, $n=62$ were prescribed with ONS. Energy- and/or protein ONS were provided as a cold beverage in between the main meals. To allow assessment of the data based on ONS use, data is presented for the non-ONS group and ONS group. ONS provision varied between one supplement per week to three supplements per day, which was a result of the prescription by the dietitian and provision by the food assistant. A variety of different flavours and energy- and/or protein ONS were available during the assessment period (for a full description of the prescribed ONS see **Supplemental table 3.1**).

Nutritional content of hospital meals

For all provided and consumed food total energy (MJ), protein (g and En% (percentage of energy provided by macronutrient)), carbohydrate (g and En%), and fat (g and En%) were calculated, based upon product specifications provided by the food suppliers and the Dutch Food Consumption Database 2016 (NEVO; RIVM, Bilthoven, the Netherlands [35]). The Harris and Benedict equation was used to estimate patients' basal energy requirements [36].

Statistical analysis

Data were checked for normality; all data were non-normally distributed, except for energy consumption and protein consumption at dinner. Data are expressed as mean \pm SD or median[IQR] when appropriate. Food provision and consumption data are expressed as median[IQR] (for consistency; both normal and non-normally distributed data). Differences between provided and consumed food intake were analysed using Wilcoxon signed-rank tests. Mann Whitney U tests were used to test for differences between the non-ONS group and ONS group. Spearman's rho test was used to calculate the relationship between daily energy and protein intake. Statistical significance was set at $P < 0.05$. All calculations were performed using the statistical software program SPSS (version 25.0, IBM Corp., Armonk, USA).

Results

Patients' characteristics

In total, 102 patients were monitored (males/females: 53/49; age: 68 ± 14 y; LOS: 14[8-28] d) during hospitalization. In **Table 3.1** Patients' characteristics are presented. Calculated resting metabolic rate averaged $5.61[5.11-6.44]$ MJ·d⁻¹ ($1340[1220-1540]$ kcal·d⁻¹). In total, 62 patients were prescribed with ONS (ONS) and 40 patients did not receive ONS (non-ONS). The ONS group had a longer hospital stay (18[11-35] d) than the non-ONS group (10[6-17] d) ($P < 0.001$).

Table 3.1 Patients' characteristics.

	All patients (n=102)	non-ONS group (n=40)	ONS group (n=62)
Age (y)	68±14	67±15	69±14
Gender (M/F)	53/49	20/20	33/29
Body weight (kg)	69.0±16.0	70.8±17.6	67.8±14.9
Height (m)	1.71±0.10	1.71±0.10	1.71±0.09
BMI (kg·m⁻²)	23.6±4.8	24.2±5.2	23.1±4.4
Length of stay (d)	14[8-28]	10[6-17]*	18[11-35]
Acute admission (%)	75	63	84
Elective admission (%)	25	37	16
Respiratory/Geriatric/ General Surgery ward (%)	35/31/34	28/35/38	40/29/31
Resting metabolic rate (MJ·d⁻¹)	5.61 [5.11-6.44]	5.66 [5.22-6.63]	5.55 [5.05-6.39]

Values are mean±SD or median [IQR] where appropriate. BMI: body mass index. Resting metabolic rate was calculated based upon the adjusted Harris and Benedict equation [36]* Indicates a significant difference between non-ONS and ONS group, $P < 0.05$.

Energy provision and consumption

Hospital meals provided $7.87[5.82-9.21]$ MJ·d⁻¹ ($1880[1390-2200]$ kcal·d⁻¹) during the entire hospital stay, while energy consumption averaged $4.98[3.05-6.41]$ MJ·d⁻¹ ($1190 [730-1530]$ kcal·d⁻¹). On average, 37% of the provided energy content in hospital meals and snacks was not consumed and was discarded ($P < 0.001$). Total macronutrient composition of the consumed hospital meals (g and En%) are presented in **Table 3.2**.

Table 3.2 Macronutrient intake in hospitalized patients (n=102).

	Energy (MJ)		Carbohydrate (g and (En%))		Protein (g and (En%))		Fat (g and (En%))	
	non-ONS	ONS	non-ONS	ONS	non-ONS	ONS	non-ONS	ONS
Breakfast	0.96 [0.65-1.25]	1.30 [0.75-1.69]	26 [19-37] (49)	36 [22-45] (47)	8 [6-13] (16)	12 [6-17] (15)	8 [5-13] (33)	13 [7-18] (36)
Lunch	1.04 [0.60-1.53]	1.16 [0.55-1.69]	31 [19-46] (50)	35 [16-46] (49)	8 [5-14] (14)	10 [5-16] (14)	9 [4-16] (34)	10 [5-17] (34)
Dinner	0.99 [0.62-1.52]	1.23 [0.91-1.65]	28 [19-49] (52)	40 [28-51] (53)	11 [7-17] (20)	13 [9-18] (17)	7 [4-12] (26)	9 [5-13] (28)
Snacks	0.56 [0.36-0.76]	0.56 [0.25-0.86]	25 [17-39] (75)	24 [9-35] (72)	1 [0-1] (8)	1 [0-4] (8)	1 [0-4] (17)	2 [0-5] (27)
ONS		1.26 [0.40-1.79]		37 [13-55] (55)		11 [4-16] (16)		9 [3-15] (31)

Values are expressed as median [IQR]; En% (percentage of energy provided by macronutrient), ONS (oral nutritional supplements) was prescribed in n=62.



Protein provision and consumption

Protein provision and consumption ($\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) from self-selected hospital meals, snacks and oral nutritional supplements during hospitalization are presented in **Figure 3.1**. Protein provision was $1.03[0.77-1.26]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ during hospitalization, while actual protein consumption was $0.65[0.37-0.93]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. The consumed amount of protein was 37% lower than the provided amount of protein ($P<0.001$). Only 4% of the patients had a protein intake equal to or above recommended protein guidelines (≥ 1.2 $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Thirty-five percent of the patients met the protein requirements set by the WHO (≥ 0.8 $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$).

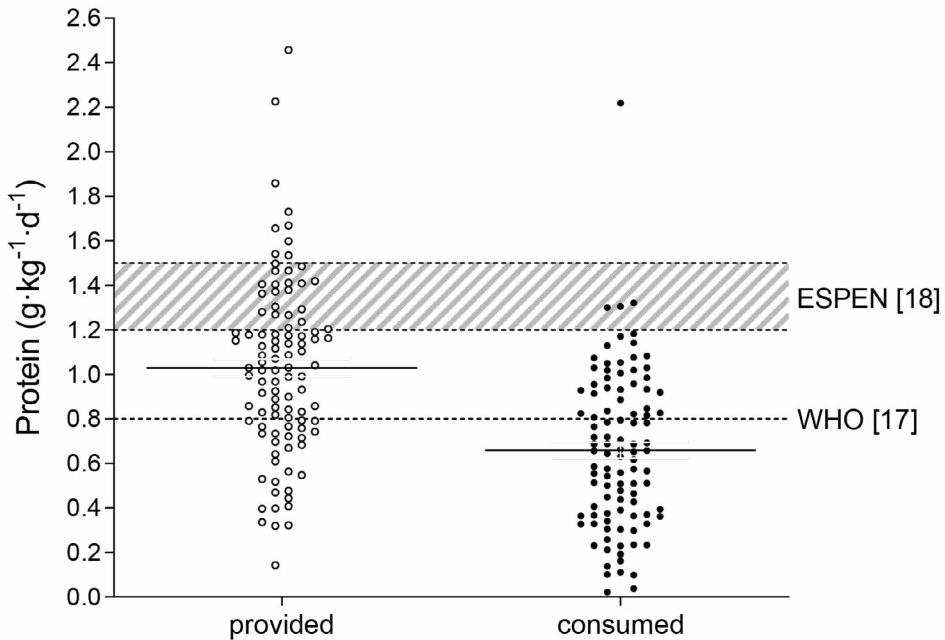


Figure 3.1 Protein provision and consumption ($\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) during hospitalization. Protein intake was measured in $n=102$ patients over on average 4.6 hospitalization days. The dotted line represents the recommended protein intake of 0.8 $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ suggested for healthy adults of all ages by the WHO [17]. The shaded area represents the recommended protein intake of $1.2-1.5$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ suggested for malnourished patients or patients at risk for malnutrition due to acute or chronic illness [18].

Energy provision and consumption between groups

Energy provision and consumption from self-selected hospital meals, snacks and oral nutritional supplements during hospitalization are presented in **Figure 3.2A-B**. Hospital meals provided $5.83[4.65-7.54]$ $\text{MJ}\cdot\text{d}^{-1}$ ($1390[1110-1800]$ $\text{kcal}\cdot\text{d}^{-1}$) and $8.82[7.51-9.95]$ $\text{MJ}\cdot\text{d}^{-1}$ ($2110[1790-2380]$ $\text{kcal}\cdot\text{d}^{-1}$) during the entire hospitalization period in the non-ONS (Figure 2A) and ONS (Figure 2B) group, respectively. Energy consumption was $3.22[2.73-5.27]$ $\text{MJ}\cdot\text{d}^{-1}$ ($770[650-1260]$ $\text{kcal}\cdot\text{d}^{-1}$) vs $5.67[3.67-7.15]$ $\text{MJ}\cdot\text{d}^{-1}$ ($1350[880-1710]$ $\text{kcal}\cdot\text{d}^{-1}$), respectively.

On average, 37% of the provided energy content was not consumed and was discarded ($P<0.001$). After correction for ONS consumption in the ONS group, there were no differences in total energy intake consumed from meals and snacks between the non-ONS and ONS group.

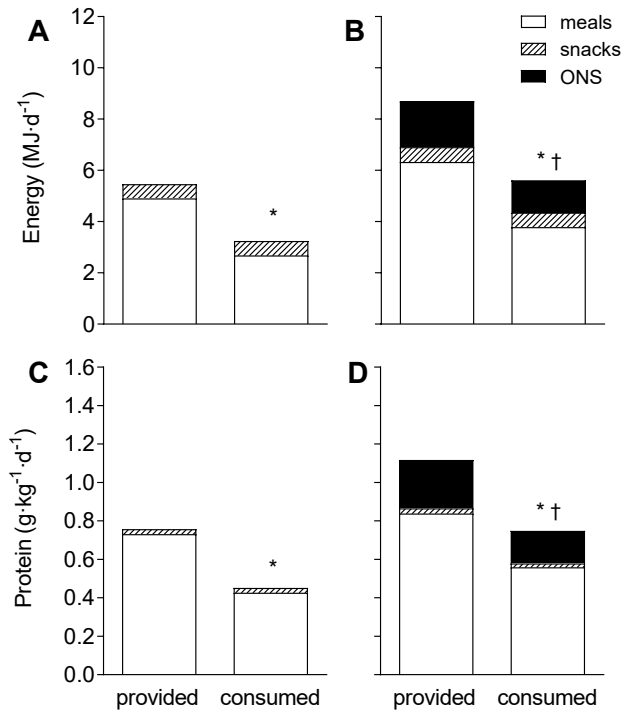


Figure 3.2 Total dietary energy provision and consumption ($\text{MJ}\cdot\text{d}^{-1}$) (**A** and **B**) and protein provision and consumption ($\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) (**C** and **D**) across main meals in patients during hospitalization. Total energy and protein intake in patients that were not prescribed with oral nutritional supplements (non-ONS; **A** and **C**; $n=40$) and patients that were prescribed with oral nutritional supplements (ONS; **B** and **D**; $n=62$). * Indicates a significant difference when compared with provided, $P<0.001$; † Indicates a significant difference when compared with non-ONS group, $P<0.001$.

Protein provision and consumption between groups

Protein provision and consumption ($\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) from self-selected hospital meals, snacks and ONS during hospitalization are presented in **Figure 3.2C-D**. Main meals provided $0.73[0.53-1.00]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ protein in the non-ONS group (Figure 2C) and $0.84[0.68-1.09]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ protein in the ONS group (Figure 2D). Snacks provided merely $0.03[0.01-0.04]$ and $0.03[0.01-0.07]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ protein in the non-ONS and ONS group, respectively. ONS added $0.25[0.14-0.34]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ protein to total protein provision. Protein consumption through the consumption of main meals and snacks in the non-ONS group was $0.43[0.31-0.65]$ and $0.02[0.01-0.04]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, respectively, while protein consumption in the ONS group was $0.56[0.38-0.72]$, $0.02[0.01-0.05]$, and $0.17[0.05-0.25]$ $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ from the consumption of main meals, snacks, and ONS, respectively. On average, 32% of the ONS provided was not consumed. Patients in the ONS group had a higher total protein intake when compared with patients in the non-ONS group ($P<0.001$). Protein intake from the consumed hospital meals and snacks did not differ between groups when ONS contribution was excluded in the ONS-group ($P=0.169$).

Protein intake distribution

Distribution of protein provision and consumption (g) across main meals is presented in Figure 3.3. Hospital meals provided 15[11-19], 15[12-20], and 20[15-24] g protein in the non-ONS group (**Figure 3A**) and 19[14-25], 18[13-23], and 21[19-24] g protein in the ONS group (Figure 3B) at breakfast, lunch, and dinner, respectively. Protein consumption was 35, 36, and 34% less than protein provided at breakfast, lunch, and dinner, respectively (all $P < 0.001$). As a result, actual protein intake was 8[6-13], 8[5-14], and 11[7-17] g in the non-ONS group and 12[6-17], 10[5-16], and 13[9-18] g in the ONS group at breakfast, lunch, and dinner, respectively. Protein intake at breakfast was higher in the ONS group when compared with the non-ONS group ($P = 0.044$). Snacks contributed only 0.3[0.1-0.9] g protein in the morning, afternoon, and evening in the non-ONS group, respectively. In the ONS group, snacks and oral nutritional supplements contributed 5[2-7], 4[2-6], and 3[1-6] g protein to total protein intake during the morning, afternoon, and evening, respectively.

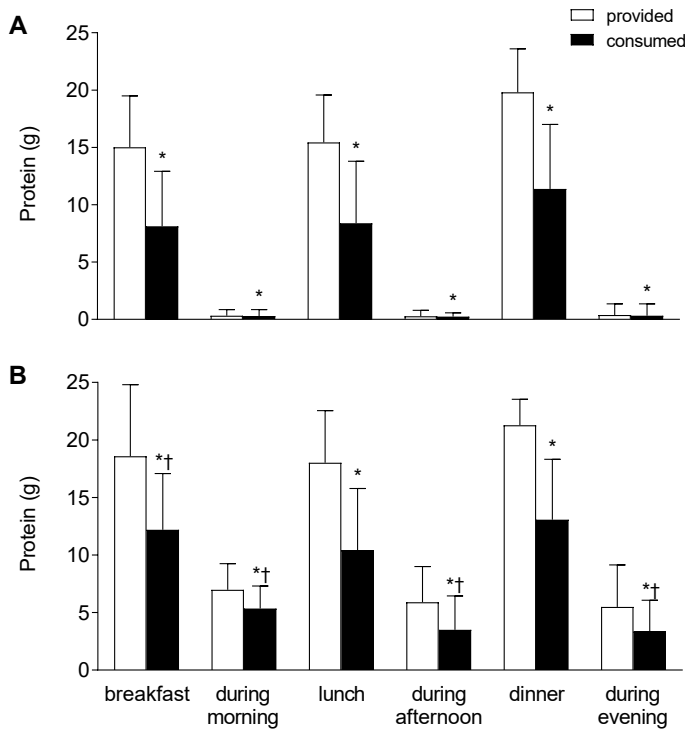


Figure 3.3 Mean \pm SD dietary protein provision and consumption (g) across main meals in patients in the non-ONS group (**A**; $n=40$) and ONS group (**B**; $n=62$) during hospitalization. * Indicates a significant difference when compared with provided, $P < 0.001$; † Indicates a significant difference when compared with non-ONS group, $P < 0.05$.

Protein intake distribution per main meal expressed as the percentage of the total amount of protein consumed is presented in **Figure 3.4**. Breakfast, lunch, and dinner provided 28 ± 13 , 26 ± 12 , and $39 \pm 17\%$ and snacks contributed $7 \pm 6\%$ of total daily protein intake in the non-ONS group (Figure 3.4A). In the ONS group, breakfast, lunch, and dinner provided 25 ± 10 , 19 ± 10 , and $27 \pm 13\%$ of the daily protein intake. Snacks contributed $6 \pm 6\%$ of daily protein intake and oral nutritional supplements contributed the remaining $23 \pm 18\%$ of daily protein intake in the ONS group (Figure 3.4B).

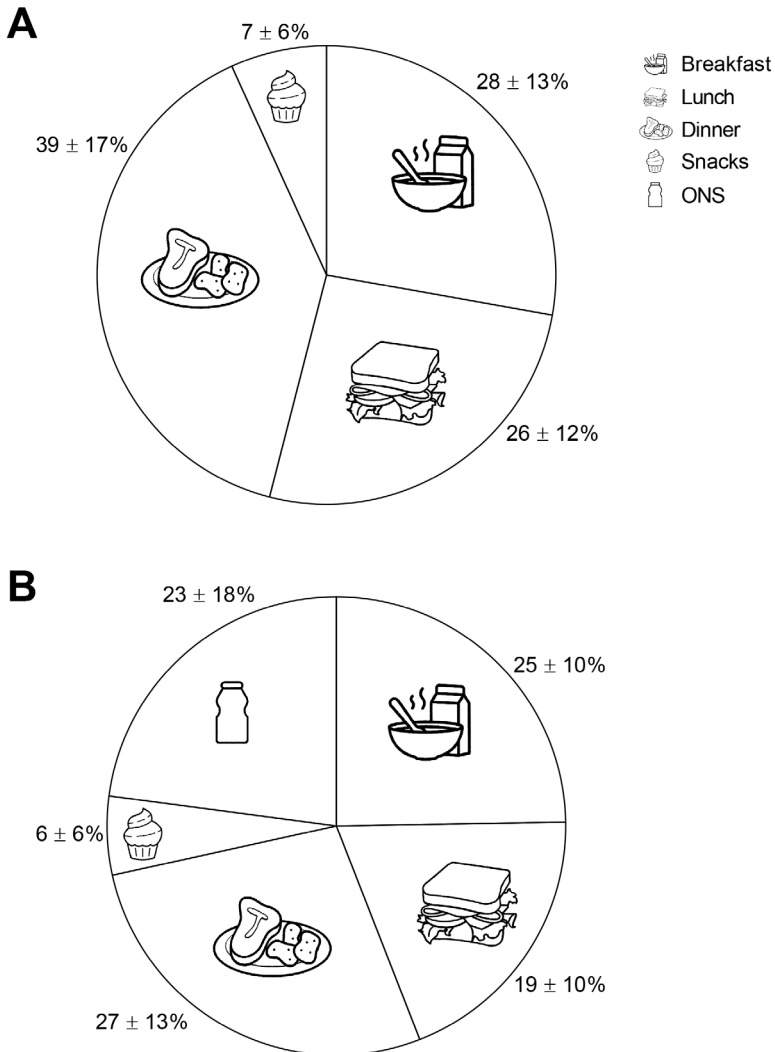


Figure 3.4 Distribution of protein intake across main meals, snacks, and oral nutritional supplements (ONS) (expressed as a percentage of the total amount of protein consumed) during hospitalization in non-ONS group (**A**; $n=40$) and ONS group (**B**; $n=62$).

Dietary protein intake strongly correlated with daily energy intake in both the non-ONS group ($r=0.894$; $P<0.001$) and ONS group ($r=0.860$; $P<0.001$) (**Figure 3.5**). Relative contribution of protein to total energy intake (En% protein) did not differ between groups ($P=0.422$).

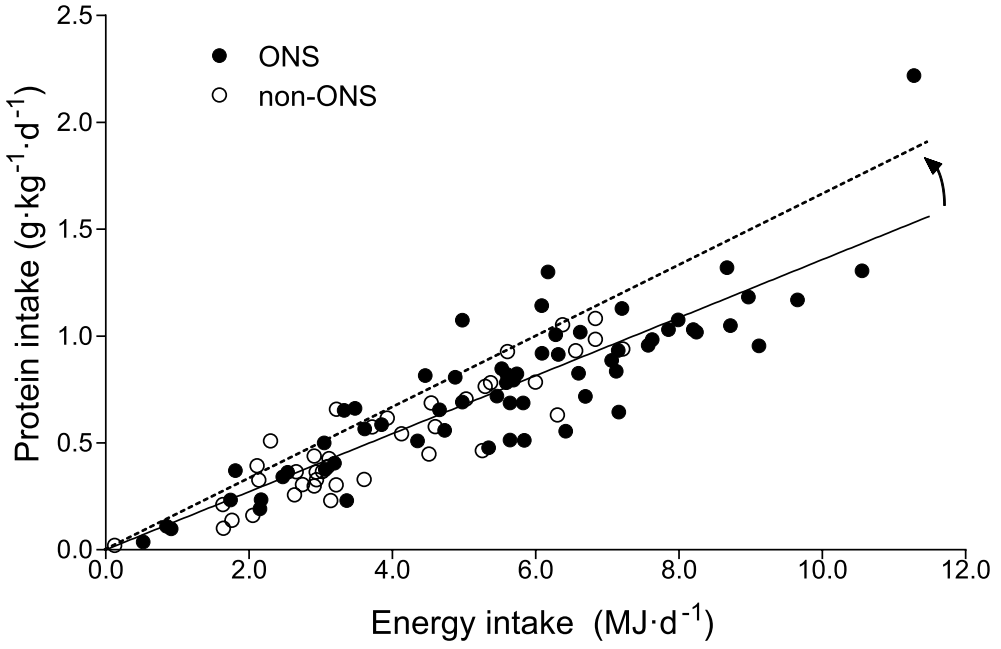


Figure 3.5 Daily energy and total protein intake in older patients at risk for malnutrition during hospitalization. The dotted line represents the association line between energy and protein intake if a more protein-dense diet would be provided.

Discussion

Despite a protein provision of $1.0 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, protein intake was merely $0.65 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in older patients who were deemed at risk for malnutrition during their hospitalization. In total, 37% of the provided food was discarded and 32% of the provided oral nutritional supplements were not consumed. Total energy and protein intake per day were greater in those patients receiving oral nutritional supplements, which did not affect the macronutrient composition of the diet. Median protein intake per main meal ranged from 8 to 13 g protein in all patients.

In the present study, we assessed the consumption of self-selected hospital meals, snacks, and oral nutritional supplements in patients who were classified at risk for malnutrition during their hospitalization. Daily energy intake was merely $5.0 \text{ MJ}\cdot\text{d}^{-1}$, which is well below their calculated resting metabolic rate ($5.6 \text{ MJ}\cdot\text{d}^{-1}$). Consequently, patients seemed to remain in a negative energy balance during their entire hospital stay. An energy deficit during hospitalization accelerates the loss of skeletal muscle mass and strength [37]. Muscle atrophy typically observed during hospitalization [14, 15] has been attributed to the lack of sufficient protein consumed as a direct consequence of a decline in food intake. Current guidelines suggest a protein intake of $1.2\text{-}1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ to support muscle mass maintenance in malnourished patients or patients at risk for malnutrition due to acute or chronic illness [18, 19]. In the present study, we show that protein consumption during long-term hospitalization does not even nearly reach the suggested protein intake guidelines. In fact, less than 4% of the patients consumed $\geq 1.2 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. Merely 35% of the patients consumed an amount of protein that was equal to or more than the required $0.8 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ that is prescribed by the WHO for healthy adults (Figure 3.1).

The amount of protein that was provided via the hospital diet was $1.0 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. Even if patients would have fully consumed all meals and oral nutritional supplements that were provided, the ESPEN guidelines on daily protein intake would not have been reached in 72% of the patients. With 30-40% of the provided food and supplements being discarded, daily protein intake did not even reach WHO guidelines on recommended protein intake. In our hospital, current practices to increase protein intake in these patients include the provision of energy and/or protein-rich snacks in between main meals, counselling to motivate patients to choose protein-rich products, and/or the prescription of oral nutritional supplements. Clearly though, these existing strategies are not effective enough to reach a protein intake at the level of WHO, let alone the ESPEN guidelines on protein intake for patients. Moreover, during hospitalization patients are less physically active and food intake is typically reduced. To maintain protein intake at habitual intake levels under conditions of a reduced energy intake, likely requires the instalment of a more protein-dense diet.

One of the often applied strategies to increase protein intake is to provide oral nutritional supplements. Previous work showed higher energy and protein intake in patients being prescribed with ONS, providing up to 8-30 g protein extra per day [31, 38, 39]. Our data extend on these findings showing that even though ~30% of the oral nutritional supplements was discarded, they still seemed effective in increasing energy and protein intake when compared to those patients not receiving ONS. However, providing patients with oral nutritional supplements did not change the protein density of the hospital diet (Figure 3.5). Though ONS provision did increase absolute daily energy and protein intake, the increase in protein intake would be greater if products with greater protein content were used. Using more protein dense ONS may represent an effective strategy to allow the diet to become more protein dense, with relatively more protein being

consumed at the same, or even a lower energy intake (Figure 5). This is especially required to allow patients to maintain their habitual protein intake level, which is necessary to attenuate muscle mass loss during hospitalization. As there are numerous types of ONS with varying protein contents for different patient populations, dietitians should make an informed decision on the optimal prescription of the right product(s) and matching diet for each individual patient.

Previous research has shown that ingestion of at least 20 g of a high quality protein is needed to increase postprandial muscle protein synthesis rates [40-44]. Our data clearly show that the amount of protein consumed at breakfast (10[6-15] g), lunch (9[5-14] g), and dinner (13[9-18] g) remains well below the proposed 20 g (Figure 3.3). As this is in line with previous work showing inadequate protein intake with breakfast and lunch, it is essential to increase the protein content of each meal [20, 45-47]. Apart from using protein-dense ONS as described above, the provision of more protein-dense products or fortifying main meals would allow patients to consume more protein per meal. Previous studies have shown that the provision of protein-fortified foods (such as bread, yoghurt, cake, fruit juice, and soup) or the use of more protein-dense foods (such as dairy, eggs, fish, meat) can be effective in increasing both absolute as well as relative protein intake during hospitalization [21, 29, 30]. Another potential strategy to increase total daily protein intake could be by creating an additional meal moment to consume a protein-rich snack or supplement. The ingestion of a protein-rich snack before sleep could serve as such an additional meal moment to increase protein intake. Our lab has recently shown that protein ingestion prior to sleep increases overnight muscle protein synthesis rates in older individuals [48, 49]. Whether these nutritional intervention strategies are effective to increase daily protein and/or energy intake during hospitalization remains to be assessed.

In conclusion, energy and protein intake levels are well below suggested guidelines in hospitalized patients at risk for malnutrition. As 30-40% of the provided food and supplements are not consumed, actual protein consumption remains well below the minimal requirements of $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ and far below recommended intake levels of $1.2\text{-}1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. While the provision of oral nutritional supplements increases habitual energy and protein intake, it does not affect the macronutrient composition of the diet. Current nutritional strategies to achieve the recommended daily protein intake in older patients during their hospitalization are not as effective as generally assumed and should be redesigned.

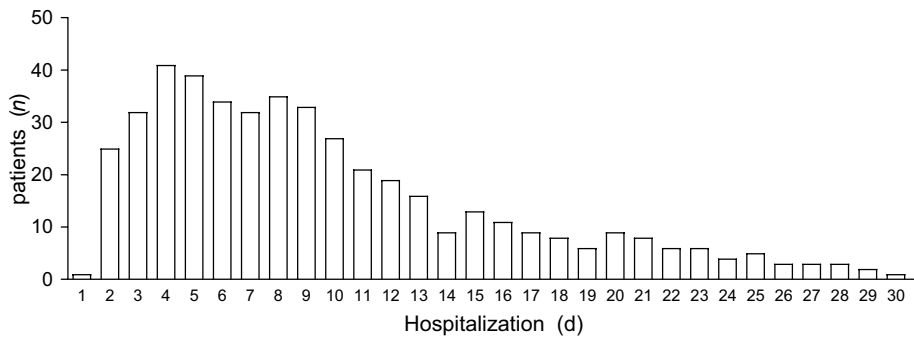
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Supplemental material



Supplemental figure 3.1 Patients (n) included and followed during their days of hospitalization. Patients were followed for a minimum of three hospitalization days and data collection was stopped after seven days.

Supplemental table 3.1 Macronutrient composition of oral nutritional supplements per serving.

	Volume (ml)	En (MJ)	Carbohydrate (g and En%)	Protein (g and En%)	Fat (g and En%)
Nutridrink compact	125	1260	37 (50)	12 (16)	12 (35)
Nutridrink compact Multifibre	125	1256	32 (42)	12 (16)	13 (39)
Nutridrink	200	1250	37 (49)	12 (16)	12 (35)
Nutridrink Multifibre	200	1260	37 (49)	12 (16)	12 (35)
Nutridrink Juicy style	200	1270	67 (89)	8 (10)	0 (0)
Nutridrink protein	200	1260	33 (45)	18 (24)	10 (31)
Renilon 4.0	125	1044	29 (47)	5 (8)	13 (45)
Renilon 7.5	125	1044	25 (40)	9 (15)	13 (45)

Supplemental table 3.2 Product options of generic hospital menu.

Breakfast / Lunch			
Bread	Toppings	Dairy	Other
White bread	Butter	Semi-skimmed milk Buttermilk	Fruit juice
Brown bread	Smoked beef	Semi -skimmed	Fruit
Whole grain bread	Chicken breast	Chocolate milk Low-fat yoghurt	Tomato salad
Rye bread	Ham	Fruit yoghurt	Fruit salad
Rusk	Cervelat	Curd cheese	Instant soup
Ginger bread	Pate	Vanilla custard	Boiled egg
Crispbread	Cheese	Chocolate custard	Coffee
Cornflakes	Cumin cheese		Tea
Muesli	Herb cheese Marmalade Chocolate sprinkles Chocolate spread Peanut butter Syrup		
Dinner			
Meat/ gravy	Starch products	Vegetables	Dessert
Baked fish	Pommes duchesse	Peas, carrots and corn	Vanilla and chocolate custard
Chicken breast	Boiled potatoes	Peas and carrots	Vanilla yoghurt
Turkey steak	Mashed potatoes	Spinach	Forest fruit custard cheese
Beef steak	Rice	Green beans	Low-fat yoghurt
Veal meatball	Macaroni	Beetroot	Apricot compote
Ham steak		Cauliflower	
Poached fish		Coleslaw	
Omelet		Apple sauce	
Vegetable spring roll			
Gravy			



Chapter 4

No differences between *Vicia Faba* peptide network and milk protein supplementation on changes in muscle mass during short-term immobilization and subsequent remobilization, but increases muscle protein synthesis rates during recovery

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Ready for submission

Abstract

Background

Muscle mass and strength rapidly decrease during short periods of immobilization (<7 d), after which they slowly recover during remobilization. Recent artificial intelligence applications have identified peptides that appear to possess anabolic properties in in vitro assays and murine models.

Objective

This study assessed the impact of supplementing with a plant-based peptide network on muscle mass, muscle strength, and myofibrillar protein synthesis rates during 7 days of disuse and 14 days of remobilization.

Methods

Thirty healthy, young (24±5 y) men were subjected to 7 days of one-legged knee immobilization followed by 14 days of ambulant recovery. Participants were randomly allocated to ingest either 10 g of *Vicia faba* peptide network (NPN_1; *n*=15) or an isonitrogenous control (milk protein concentrate; MPC; *n*=15) twice daily throughout the study protocol. One day before casting, directly after cast removal, and 14 d after cast removal, single-slice computed tomography scans and knee extension one-repetition maximum (1-RM) tests were performed to assess quadriceps cross sectional area (CSA) and maximal leg strength, respectively. Participants ingested deuterium oxide daily with saliva and muscle biopsy samples being collected to measure myofibrillar protein synthesis rates during the immobilization and recovery periods.

Results

Leg immobilization decreased quadriceps CSA from 81.9±10.6 to 76.5±9.2 cm² and from 74.8±10.6 to 71.5±9.8 cm² in the NPN_1 and MPC group, respectively (*P*<0.001). Remobilization partially recovered quadriceps CSA (77.3±9.3 and 72.6±10.0 cm², respectively; *P*=0.009), with no differences between groups (*P*>0.05). Knee extension 1-RM decreased during immobilization (*P*<0.001) and was partially recovered during remobilization (*P*=0.008), with no differences between groups (*P*>0.05). During immobilization, myofibrillar protein synthesis rates were lower in the immobilized leg (1.07±0.24 and 1.10±0.24 %/d, respectively) compared with the non-immobilized leg (1.55±0.27 and 1.52±0.20 %/d, respectively; *P*<0.001), with no differences between the NPN_1 and MPC group (*P*>0.05). During remobilization, myofibrillar protein synthesis rates in the immobilized leg increased more in the NPN_1 when compared with the MPC group (1.53±0.38 vs 1.23±0.36 %/d, respectively; *P*=0.027).

Conclusions

Vicia Faba peptide supplementation does not preserve muscle mass or strength during short-term immobilization, or augment muscle mass and strength regain during remobilization when compared to milk protein supplementation. NPN_1 supplementation did not differ from milk protein supplementation in modulating myofibrillar protein synthesis rates during immobilization, but further increased myofibrillar protein synthesis rates during remobilization.

Introduction

Recovery from injury or illness often requires a period of inactivity. Periods of inactivity strongly reduce muscle mass and strength [1, 2]. Importantly, even short periods of immobilization (<7 d) induce a substantial decline in muscle mass and strength [3-6], which is accompanied by reduced insulin sensitivity and impacts the time course of recovery [7-10]. The loss of muscle mass during disuse has been attributed to a decline in both basal [2, 11-13] and postprandial muscle protein synthesis rates [14], resulting in a negative net muscle protein balance.

So far, few nutritional strategies have been identified to preserve muscle mass during a period of immobilization. Amino acid supplementation and omega-3 fatty acid supplementation have been shown to attenuate muscle disuse atrophy during 7 and 14 days of limb immobilization [15, 16]. In contrast, protein, leucine, and creatine supplementation did not attenuate muscle disuse atrophy during 5-7 days of limb immobilization [4-6, 17]. Recently, a predictive machine learning approach has been applied to characterize peptides with potentially bioactive properties [18]. With this approach, a peptide network derived from *Vicia faba* (NPN_1) was identified and isolated for human consumption following enzymatic hydrolysis. Preclinical evidence indicated that peptides contained within the peptide network activated markers of protein synthesis, while reducing markers of protein degradation as well as TNF α secretion [19]. Follow-up work in mice has suggested that NPN_1 supplementation upregulated mTOR activity and attenuated soleus muscle atrophy in a disuse (hindlimb unloaded) model [19]. This implies that NPN_1 represents an alternative, plant-derived protein that may promote muscle health *in vivo* in humans. Consequently, we hypothesized that NPN_1 supplementation may attenuate muscle mass and strength loss by stimulating muscle protein synthesis rates during a short period of single-leg immobilization *in vivo* in humans.

Though a handful of studies have assessed the impact of a short period of disuse on muscle mass and strength, few studies have addressed changes in muscle protein synthesis and muscle mass and strength regain during subsequent remobilization. Previous work from our group has shown that muscle mass and strength can return to baseline values following 6 weeks of natural rehabilitation [20]. Shorter periods of ambulatory recovery (7-14 d) reported a partial recovery of muscle cross sectional area [4, 15, 21]. To date, the only nutritional supplement that was shown to accelerate recovery during active rehabilitation is creatine [22]. Other nutritional strategies failed to further augment muscle mass regain during ambulatory recovery [4, 15, 16, 21]. We hypothesized that NPN_1 supplementation during recovery from a period of immobilization may also support a more rapid regain of muscle mass and strength by stimulating muscle protein synthesis rates in healthy adults.

To test our hypotheses, we recruited 30 healthy young volunteers to be subjected to one week of single-leg immobilization, followed by a two-week recovery period. During this period participants were supplemented with the NPN_1 or an isonitrogenous control (milk protein) in a randomized, double-blind manner. Prior to and after immobilization and following two weeks of recovery, leg muscle mass, leg muscle strength, and daily muscle protein synthesis rates were assessed.

Material and methods

Participants

Thirty healthy, young men (24 ± 5 y) were included in the present study. Exclusion criteria included: (family) history of thrombosis, (family) history of Factor V Leiden, or other known thrombophilia (such as; protein C, protein S, antithrombin deficiency), lower limb, back or shoulder injuries that could interfere with the use of crutches, allergies to milk protein, participation in structured resistance exercise program, co-morbidities interacting with mobility and muscle metabolism of the lower limbs (e.g., arthritis, spasticity/rigidity, all neurological disorders and paralysis), use of any medications known to (or that may) affect protein metabolism, diagnosed diabetes, metabolic, cardiovascular or intestinal disorders, a history of neuromuscular problems, use of anti-coagulants, use of protein and/or fish-oil supplements, participation in a $^2\text{H}_2\text{O}$ study in the previous six months, and smoking. On the screening visit, all participants were fully informed about the nature and risks of the experimental procedures before providing informed consent. This study was approved by the local Medical Ethical Committee of Maastricht University Medical Centre+ and conforms to the principles outlined in the latest version of the Declaration of Helsinki for use of human subjects and tissue. This trial was registered at www.trialregister.nl as NTR7645. The study was independently monitored by the Clinical Trial Center Maastricht.

Experimental design

A schematic overview of the experimental design is depicted in **Figure 4.1**. Participants were randomly allocated to either the *Vicia faba* derived peptide network (NPN_1; $n=15$) or milk protein concentrate (MPC; $n=15$) supplemental groups.

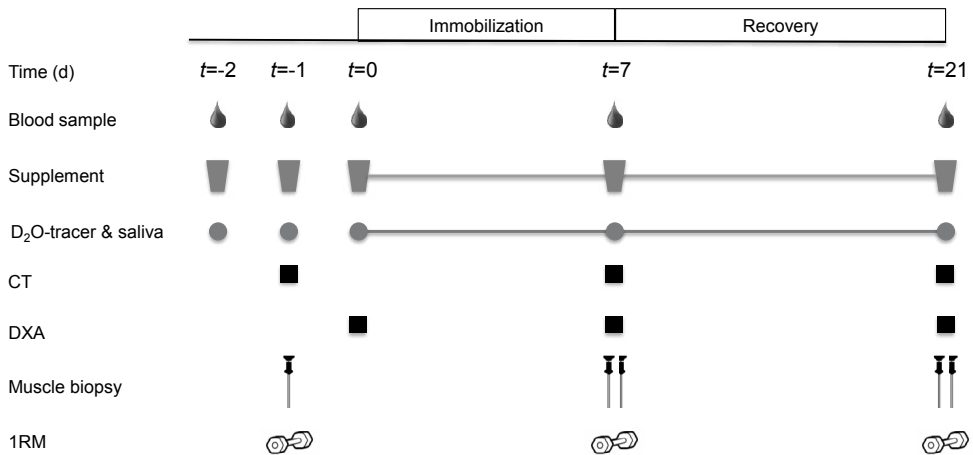


Figure 4.1 Outline of experimental protocol.

All participants were subjected to 7 days of knee immobilization on a randomized leg followed by 14 days of free-living (habitual) recovery. A series of measurements were performed one day prior to casting, directly after cast removal, and 14 days after cast removal. Single-slice computed tomography (CT) scans were performed at the mid-thigh of both legs, whole-body DXA scans were taken, muscle biopsies from both the immobilized and non-immobilized leg were taken, venous blood samples were collected, and 1-legged knee extension and leg press strength (one repetition maximum, 1-RM) was assessed for both legs separately. Participants underwent a deuterated water ($^2\text{H}_2\text{O}$)-dosing protocol, which started two days prior to casting and continued until the end of the experimental trial.

Leg immobilization and habitual recovery

Each participant was fitted for a full leg plaster cast in a randomized and counterbalanced (for leg) fashion to induce knee immobilization. The cast extended from 10 cm above the ankle to 25 cm above the patella and was set at a 30° flexion angle. Throughout the immobilization period, participants were provided with crutches to allow movement, but were instructed to not put any weight on the immobilized leg. They were instructed to perform a series of ankle exercises (i.e., plantar and dorsal flexion, and circular foot movements) to avoid developing deep vein thrombosis. Following cast removal, participants were transported by wheelchair until the muscle biopsy sample was collected. After the post-immobilization visit ($t = 7$ d), the 14-d recovery period started.

Protein supplementation

Supplementation began two days prior to casting. The NPN_1 group consumed a *Vicia faba* derived peptide network (NPN_1, Nuritas, Dublin, Ireland). The MPC group received an isonitrogenous milk protein concentrate (MPC; Friesland Campina, Wageningen, the Netherlands). NPN_1 was prepared as described by Cal et al. with some modifications [19]. Briefly, commercially available fava bean (*Vicia faba*) protein concentrate was resuspended in a food-grade buffer in a temperature-controlled bioreactor under constant agitation. Protein hydrolysis was initiated with the addition of a food-grade endoprotease. Hydrolysis progressed for a defined period, after which, enzyme activity was inactivated by heating to 80°C for 10 min. The resulting hydrolysate suspension was then spray-dried to a fine, free flowing powder. The amino acid composition for both supplements is displayed in **Table 4.1**. Supplements were consumed twice daily (after breakfast and before sleep) for the entirety of the experimental trial. Each 10 g serving contained 6 g protein resuspended in 200 mL of water.

Table 4.1 Protein content and amino acid composition.

	MPC	NPN_1
Protein content (%)	59	55
EAA (g/100g powder)		
Histidine	1.4	1.2
Isoleucine	2.8	2.1
Leucine	5.5	4.0
Lysine	4.6	3.2
Methionine	1.5	0.3
Phenylalanine	2.8	2.3
Threonine	2.4	1.8
Valine	3.5	2.3
ΣEAA	24.6	17.1
NEAA (g/100g powder)		
Alanine	1.8	2.2
Arginine	1.9	5.7
Aspartic acid ¹	3.5	4.7
Cysteine	0.1	0.2
Glutamic acid ²	12.6	8.9
Glycine	1.0	2.0
Proline	5.9	2.3
Serine	3.2	2.6
Tyrosine	3.1	1.9
ΣNEAA	33.2	38.6

EAA, essential amino acids; NEAA, nonessential amino acids; TAA, total amino acids.

Dietary intake and physical activity

Participants were instructed to refrain from strenuous physical activity and avoid alcohol intake and keep their diet as constant as possible for 2 days prior to the first experimental test day until the final test visit ($t = 21$ d). All participants received a standardized meal prior to test days on $t = -1, 7,$ and 21 d (2.9 MJ, 53 En% carbohydrate, 31 En% fat, 16 En% protein). Dietary intake and physical activity records were completed by the participants for three days prior to the immobilization period and during the final three days of the immobilization and recovery periods. Dietary intake records were analyzed using the Dutch Food Consumption Database 2019 (NEVO; RIVM, Bilthoven, the Netherlands) [23]. Daily steps were recorded over the same 3-day periods using a triaxial accelerometer (Actigraph GT3X; Actigraph LLC, Pensacola, FL, USA), worn on the waist. Data were included in the analysis if participants wore the Actigraph for a minimum of 2 days and at least 10 h per day.

Body composition

Body weight was measured with a digital balance with an accuracy of 0.1 kg (SECA GmbH, Hamburg, Germany). A single-slice CT scan (Siemens Definition Flash; Siemens, München, Germany) was performed to assess upper leg muscle cross-sectional area (CSA) as described previously [24]. Briefly, a 2-mm thick axial image was taken 15 cm proximal to the top of the patella with participants lying supine with their legs extended and feet secured. Image analyses was performed using ImageJ software (1.53k) and muscle CSA was determined for the whole-thigh, and for the quadriceps. Body composition (fat, fat free mass and bone mineral content) was determined by DXA scan (Hologic Discovery A, Marlborough, MA, USA). The system's software package APEX version 4.0.2 was used to determine whole body and regional (e.g. legs) lean mass, fat mass, and bone mineral content.

Leg strength

At the end of the test day, participants single-leg 1-RM was assessed. During screening, all participants were instructed and familiarized with safe lifting technique for the leg extension and leg press exercise. On the test day, after warming up, the load was set at 90% of the estimated 1-RM from the screening visit and increased after each successful lift until failure. Three min rest periods were allowed between lifts. A repetition was considered valid when the participant was able to complete the entire lift in a controlled manner, without assistance.

Deuterated water-dosing protocol

The deuterated water-dosing protocol consisted of 1 dosing day and 22 maintenance days. The dosing protocol was modified from previously published studies that have administered deuterated water in human participants [25-27]. Prior to dosing, baseline blood and saliva samples were collected. Participants then ingested two doses of 100 mL of 70% deuterium oxide (Cambridge Isotopes Laboratories, Andover, MA) separated by 30-60 min. Participants ingested 20 mL of 70% deuterium oxide every morning for the remainder of the trial. To assess body water enrichment ($^2\text{H}_2\text{O}$), participants collected saliva samples using a dental swab (Celluron, Hartmann, Germany) every evening for the entire experimental protocol. Participants were instructed to not eat or drink anything 30 min prior to saliva collection. Blood samples were collected at $t = -2, -1, 0, 7,$ and 21 d in EDTA-containing tubes and centrifuged at $1,000$ g for 10 min at 4°C . Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C . Prior to the immobilization period a muscle biopsy was collected from the vastus lateralis muscle of the leg identified as the leg that would not be immobilized (non-immobilized leg). On the visits following immobilization and recovery, muscle biopsies from both the immobilized and the non-immobilized leg were collected. Muscle biopsy samples were obtained from the middle region of the vastus lateralis, approximately 1–3 cm below the level that the CT scan was performed, by using the percutaneous needle biopsy technique [28]. Muscle samples were dissected carefully, freed from any visible non-muscle material, frozen in liquid nitrogen, and stored at -80°C until further analyses. A separate piece of muscle (~ 20 mg) was embedded in Tissue-Tek and immediately frozen in liquid nitrogen-cooled isopentane for later immunohistochemical analyses.

Plasma and saliva analyses

Creatinine, gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), triglycerides (TG), high-sensitivity C-reactive protein (hs-CRP), free fatty acids (FFA), insulin and glucose

concentrations were assessed in serum samples collected in a rested state on $t=-2, 0, 7,$ and 21 d. Serum creatinine, GGT, AST, ALT, LDH, CK, TG, and glucose concentrations were measured by using spectrophotometry (Cobas 8000 instrument; Roche Diagnostics). hs-CRP was measured by using particle-enhanced immuno-nephelometry (BN ProSpec; Siemens Healthineers). FFA concentrations were measured by using spectrophotometry (Alinity ci; Abbott), and insulin concentrations were determined by using chemiluminescent immunometric assay (XPI instrument; Siemens Medical Solutions Diagnostics). Plasma free [^2H]alanine enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 5975C MSD & 7890A GC, Wilmington, DE) on $t = -2, -1, 0, 7,$ and 21 d as described previously [25]. Body water enrichments were analyzed using the saliva samples collected throughout the experimental protocol. Samples were diluted 35-fold with ddH_2O and catalytic rods (Thermo Fisher Scientific, Bremen, Germany) were placed inside 12-ml glass vials (Labco Exetainer; Labco, Lampeter, UK). After dilution samples were prepared and analyzed as described by Holwerda et al. [25].

Muscle analysis

Myofibrillar protein-enriched fractions were extracted from ~ 70 mg wet muscle tissue by hand-homogenizing on ice using a pestle in a standard extraction buffer ($10 \mu\text{L}/\text{mg}$). The samples were centrifuged at 700 g and 4°C for 15 min. The pellet was washed with $400 \mu\text{L}$ extraction buffer and centrifuged at 700 g and 4°C for 10 min. Supernatant was removed and the pellet was washed with $500 \mu\text{L}$ milliQ water before vortexing and centrifugation at 700 g and 4°C for 10 min. Supernatant was removed and 1 mL of homogenization buffer was added and the material was suspended by vortexing before transferring into microtubes containing 1.4 mm ceramic beads and Lysing Matrix D (MP Biomedicals, Irvine, CA). The samples were shaken four times for 45 s at 5.5 m/s (FastPrep-24 5G, MP Biomedicals) to mechanically lyse the protein network. Samples were left to rest at 4°C for 3 h before centrifuging at 800 g and 4°C for 20 min. Supernatant was discarded and 1 mL of homogenization buffer was added. The microtubes were shaken once for 45 s and 5.5 m/s and left to rest at 4°C for 30 min before centrifuging at 800 g and 4°C for 20 min. Supernatant was discarded and 1 mL KCl buffer was added to the pellet and samples were left to rest overnight at 4°C . The next morning, samples were vortexed, transferred to new microtubes and centrifuged at $1,600$ g and 4°C for 20 min. The supernatant containing the myofibrillar proteins was collected. The myofibrillar protein was washed once with 100% ethanol, once with 70% ethanol and hydrolysed overnight in 2 mL of 6 M HCl at 110°C . The free amino acids from the hydrolysed myofibrillar protein pellet were dried under a continuous nitrogen stream while being heated at 120°C . The free amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG 50W-X8 resin columns (mesh size: $100\text{--}200$, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA), and eluted with 2 M NH_4OH . Thereafter, the eluate was dried, and the purified amino acids were derivatized to their $\text{N}(\text{O},\text{S})$ -ethoxycarbonyl ethyl esters (33). The derivatized samples were measured using a gas chromatography-isotope ratio mass spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60 m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE) and 5 m precolumn. Ion masses 2 and 3 were monitored to determine the $^2\text{H}/^1\text{H}$ ratios of muscle protein-bound alanine. A series of known standards was applied to assess linearity of the mass spectrometer and to control for the loss of tracer.

Immunohistochemistry

From all biopsies, 7- μ m-thick cryo-sections were cut at -20°C using a cryostat (CM 3050, Leica Biosystems, Nussloch, Germany). Samples were thaw-mounted onto uncoated pre-cleaned glass slides and samples of each individual subject were mounted on the same slide. Care was taken to properly align the samples for cross-sectional orientation of the muscle fibres. Samples were stained for muscle fibre typing, CSA as well as myonuclei as described previously [29]. In short, samples were air dried for 30 min after taking them out of the freezer. After 5 min fixation in acetone, the cryo-sections were incubated for 30 min with anti-myosin heavy chain type 1 (A4.840, DSHB, Iowa City, IA, 1:25) and anti-Laminin (L9393, Sigma-Aldrich, Darmstadt, Germany, 1:50) in a 0.05% Tween phosphate-buffered saline (PBS). Slides were then washed three times in the Tween/PBS solution. Appropriate secondary antibodies were then applied, GAMlgM Alexa 488 (A21426, Invitrogen, Grand Island, NY, 1:500) and GARlgG Alexa 647 (A21238, Invitrogen, 1:400) in combination with 4',6-diamidino-2-phenylindole (DAPI; D1306, Invitrogen, 1:100) for 30 minutes. After a final triple washing with PBS, slides were mounted with Mowiol (Calbiochem). Slides were viewed and automatically captured using 10x objective on a modified Olympus BX51 fluorescence microscope with a customized disk-spinning unit (DSU, Olympus, San Jose, CA, USA), computer-controlled excitation and emission filter wheels (Olympus), 3-axis high-accuracy computer-controlled stepping motor specimen stage (Grid Encoded Stage, Ludl Electronic Products, Hawthorne, NY, USA), ultra-high sensitivity monochrome electron multiplier CCD camera (C9100-02, Hamamatsu Photonics, Hamamatsu City, Japan) and controlling software (Stereoinvestigator; MBF BioScience, Williston, VT, USA). Before analyses, slides were blinded for both intervention and time point. All areas selected for analysis were free of 'freeze fracture' artefact, and care was taken such that longitudinal fibres were not used in the analysis. Quantitative analyses were performed using ImageJ software package (version 1.52p, National Institute of Health, MD, USA [30]). On average, 213 ± 133 muscle fibres were analyzed per muscle biopsy sample collected to determine muscle fibre type distribution, CSA, myonuclear content and domain size.

rtPCR

Total RNA was isolated and quantified from 10 to 20 mg of frozen muscle tissue using TRIzol® Reagent (Life Technologies, Invitrogen, Bleiswijk, the Netherlands), according to the manufacturer's protocol, as described previously [3]. Taqman primer/probe sets were obtained from Applied Biosystems: MAFBx (Hs01041408_m1), MuRF1 (Hs00261590_m1), FOXO1 (Hs01054576_m1), and 18S (Hs03003631_g1). Ct values of the target genes were normalized to Ct values of the internal control, and results were calculated as relative expression against the standard curve. The Ct values of all genes of interest were always within the lower and upper boundaries of the standard curve.

Calculations

Myofibrillar protein fractional synthetic rate (FSR) was determined using the incorporation of [²H]alanine into muscle proteins and the mean precursor [²H]alanine enrichment. The precursor [²H]alanine enrichment was estimated by correcting body water deuterium enrichments by a factor of 3.7 based on the deuterium labelling during de novo alanine synthesis [25]. As we assessed FSR for >14 d, the nonlinear equation was used to calculate FSR as described earlier [31,32]:

$$\text{FSR (\%/d)} = \frac{-\ln(1-f)}{t} \times 100\%$$

where f is calculated as the change in muscle protein-bound [²H]alanine enrichment divided by the mean precursor [²H]alanine enrichment, and t represents the time between biopsies on *days* -1 and 7, or between *days* 7 and 21.

Statistics

Data are expressed as mean±SD, unless stated otherwise. Data were checked and normality and sphericity were confirmed. An independent t test was used to assess differences in baseline characteristics between participants in the MPC and NPN_1 groups. The primary outcome variable was quadriceps CSA. A two-way repeated measures analysis of variance (ANOVA) with time as the within-subjects factor and treatment (MPC vs NPN_1) as between-subjects factor was applied to compare changes in quadriceps and whole thigh muscle CSA, muscle strength, muscle protein synthesis rates, and leg lean mass during immobilization and recovery. In case of significant interactions, separate analyses were performed in the NPN_1 and MPC groups; in case of significant main time effects, a Bonferroni post hoc test was applied to locate differences. Data were analyzed using SPSS version 27 (SPSS, IBM Corp., Armonk, NY, USA). Statistical significance was set at $P<0.05$.

Results

Participants

Baseline participants' characteristics did not differ between the MPC and the NPN_1 group (**Table 4.2**). Of the 30 participants, 29 completed the study ($n=14$ MPC, $n=15$ NPN_1). Data of one participant was excluded due to drop out during the recovery period due to the COVID-19 lockdown. A flowchart of participants is displayed in **Supplemental Figure 4.1**.

Table 4.2 Participants' characteristics.

	MPC (n=14)	NPN_1 (n=15)
Age (y)	26±5	23±4
Body weight (kg)	75.9±14.9	72.0±7.9
BMI (kg/m²)	24.4±3.1	22.6±1.3
Total lean mass (kg)	53.6±8.7	54.3±6.7
Leg (non-immobilized) lean mass (kg)	9.1±1.6	9.3±1.3
Leg (immobilized) lean mass (kg)	9.2±1.4	9.1±1.1
Fat (%)	26.0±6.4	21.6±3.7
Fasted glucose (mmol/L)	4.9±0.4	4.8±0.3
Systolic blood pressure (mmHg)	122±13	124±9
Diastolic blood pressure (mmHg)	70±10	67±9
Resting heart rate (beats/min)	64±7	65±9

Muscle cross sectional area

At baseline, quadriceps CSA did not differ between the groups ($P>0.05$). Leg immobilization significantly decreased quadriceps CSA (**Figure 2**, $P<0.001$), with no differences between the MPC (74.8±10.6 to 71.5±9.8 cm², -4.2±1.7%) and NPN_1 (81.9±10.6 to 76.5±9.2 cm², -5.4±2.4%; time x treatment, $P>0.05$). Following recovery, quadriceps CSA was greater vs post-immobilization ($P=0.009$) but remained lower vs pre-immobilization ($P<0.001$). The recovery in quadriceps CSA did not differ between the MPC (from 71.5±9.8 to 72.6±10.0 cm²; 1.9±2.4%) and NPN_1 group (from 76.5±9.2 to 77.3±9.3 cm²; 1.2±2.3%; $P>0.05$).

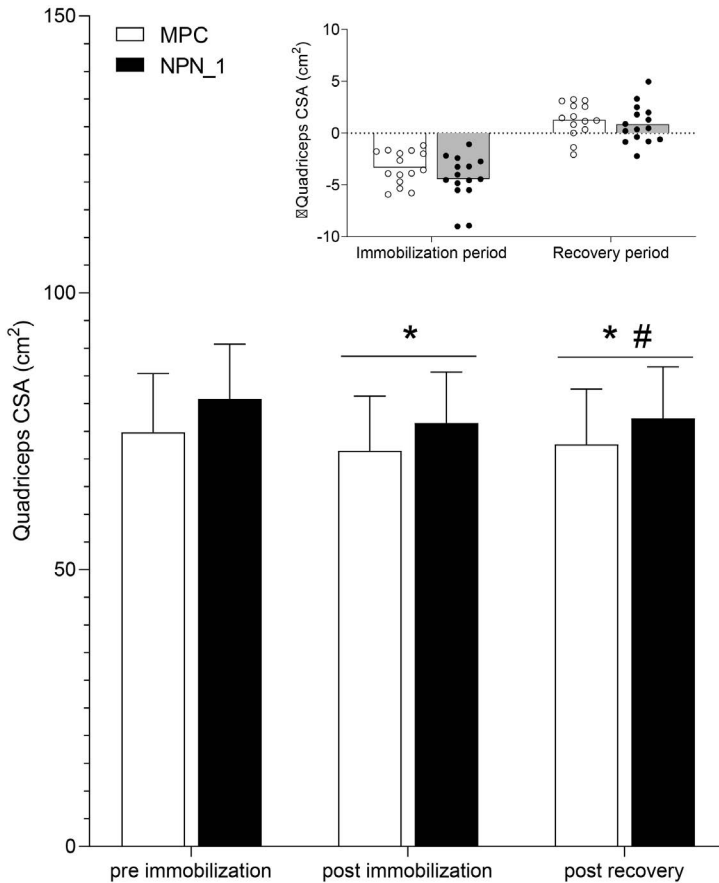


Figure 4.2 Cross sectional area (CSA) of *M. Quadriceps* in the MPC and NPN_1 group, before and after 7 days of single-legged immobilization, and after 14 days of free-living recovery, as measured by single-slice CT scan. Data were analyzed with a repeated measures ANOVA. Data are expressed as mean±SD. *Significantly different from pre-immobilization; #Significantly different from post-immobilization.

At baseline, whole thigh CSA did not differ between the groups ($P>0.05$). Leg immobilization significantly decreased whole thigh CSA (both $P<0.001$), with no differences detected between the MPC ($-2.8\pm 1.8\%$) and NPN_1 ($-4.4\pm 2.6\%$) group ($P>0.05$). Following recovery, whole thigh CSA was greater vs post-immobilization ($P=0.001$), but smaller than pre-immobilization ($P=0.010$). The recovery in whole thigh CSA did not differ between groups ($P>0.05$).

Muscle strength

At baseline, single-legged 1-RM leg extension strength did not differ between groups ($P>0.05$). Leg immobilization significantly decreased 1-RM leg extension strength (**Figure 4.3**, $P<0.001$), with no differences between the MPC (58 ± 10 to 51 ± 11 kg, $-13\pm 8\%$) and NPN_1 (56 ± 10 to 49 ± 9 kg, $-13\pm 7\%$) groups ($P>0.05$). Following recovery, single-leg 1-RM leg extension was greater vs post-immobilization ($P=0.008$) but remained lower vs pre-immobilization ($P=0.002$). No differences were detected on the increase in single-leg 1-RM leg extension between the MPC ($2.2\pm 6.7\%$) and NPN_1 group ($1.5\pm 7.3\%$, $P>0.05$) during recovery. Similar findings were

observed for 1-RM leg press strength, with no differences in the decline following immobilization or in the increase following recovery.

Muscle fiber characteristics

Muscle fiber characteristics are displayed in **Table 4.3**. Data of 8 participants were excluded or missing due to low fiber count or missing samples. Prior to immobilization, no differences were observed in type I, type II, and mixed muscle fiber CSA between groups ($P>0.05$). Leg immobilization tended to reduce mixed muscle fiber CSA ($P=0.091$), with no differences detected between the MPC (5479 ± 704 to 5108 ± 916 μm^2) and NPN_1 (5826 ± 1110 to 5094 ± 1006 μm^2 , $P>0.05$) groups. Following recovery, mixed muscle fiber CSA was lower when compared to baseline ($P=0.010$) but not different when compared to the post-immobilization timepoint ($P>0.05$). No differences in the decrease in mixed muscle fiber CSA were detected between groups ($P>0.05$).

Table 4.3 Muscle fiber characteristics.

	Pre-immobilization		Post-immobilization		Post-recovery	
	MPC (<i>n</i> =10)	NPN_1 (<i>n</i> =12)	MPC (<i>n</i> =10)	NPN_1 (<i>n</i> =12)	MPC (<i>n</i> =10)	NPN_1 (<i>n</i> =12)
Muscle fiber (<i>n</i>)						
Fiber type mixed	211±78	186±83	279±100	247±115	307±77	230±188
Fiber type I	60±24	75±41	93±37	95±61	106±44	81±68
Fiber type II	151±65*	111±52*	186±80*	152±59*	201±67*	149±127*
Muscle fiber CSA (μm^2)						
Fiber type mixed	5479±704	5826±1110	5108±916	5094±1006	4620±854#	5230±1262#
Fiber type I	5060±1102	5686±1246	5083±1384	4936±1140	4393±1071#	5036±1455#
Fiber type II	5636±696	5924±1134	5124±837	5225±1033	4608±943#	5395±1374#

* significantly different from fiber type I value ($P < 0.05$).

significantly different from pre-immobilization value ($P < 0.05$).

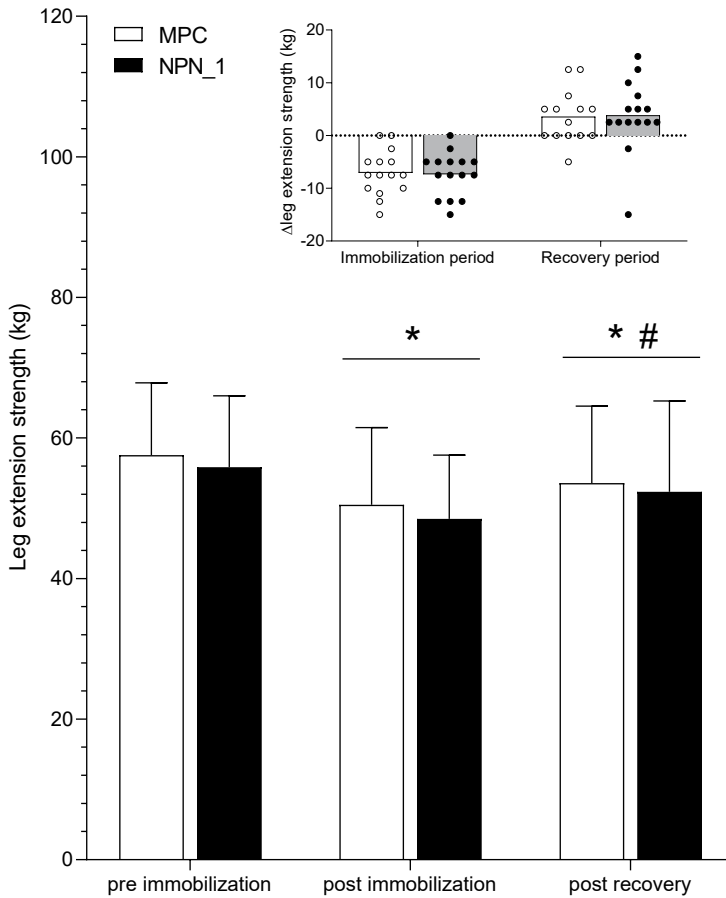


Figure 4.3 Leg extension strength (kg) in the MPC and NPN_1 group, before and after 7 days of single-legged immobilization, and after 14 days of free-living recovery, as measured by leg extension. Data were analyzed with a repeated measures ANOVA. Data are expressed as mean \pm SD. *significantly different from pre-immobilization; #significantly different from post-immobilization.

Muscle fractional synthesis rates

During immobilization, myofibrillar protein synthesis rates were lower in the immobilized leg (1.10 ± 0.24 and 1.07 ± 0.24 %/d) when compared to the non-immobilized leg (1.52 ± 0.20 and 1.55 ± 0.27 %/d) in the MPC and NPN_1 group, respectively ($P<0.001$), with no differences between groups ($P>0.05$, **Figure 4.4**). During remobilization, myofibrillar protein synthesis rates were lower in the non-immobilized leg (1.31 ± 0.34 and 1.42 ± 0.28 %/d in the MPC and NPN_1 group, respectively) when compared to the immobilization period ($P=0.030$), with no differences between groups ($P>0.05$).

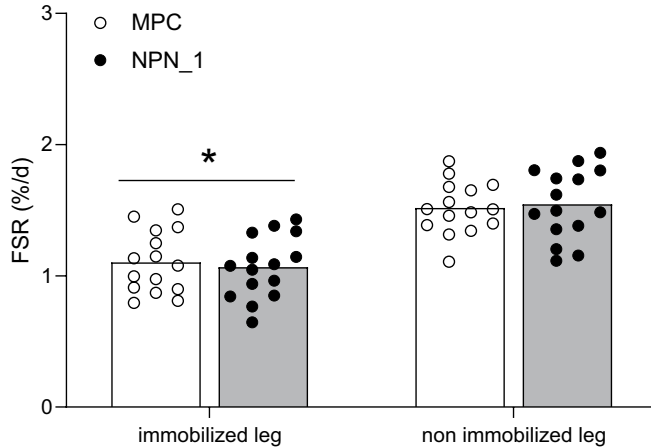


Figure 4.4 Myofibrillar protein fractional synthetic rates (%/day) calculated from saliva precursor pools. Myofibrillar FSR assessed during 7d of single-leg immobilization in the non-immobilized and immobilized leg in the MPC and the NPN_1 group. Data were analyzed with a repeated measures ANOVA. Bars are means (MPC n=15; NPN_1 n=15) and dots represent individual values. *significantly different from non-immobilized leg.

During remobilization, myofibrillar protein synthesis rates in the previously immobilized leg were higher (1.23 ± 0.36 vs 1.53 ± 0.38 %/d in the MPC and NPN_1 group) when compared to the period during immobilization ($P=0.001$). The increase in myofibrillar protein synthesis rates following remobilization was greater following NPN_1 (50 ± 46 %; from 1.07 ± 0.24 to 1.53 ± 0.38 %/d) when compared with MPC supplementation (13 ± 38 %; from 1.12 ± 0.24 to 1.23 ± 0.36 %/d, time x treatment $P=0.027$, **Figure 4.5**).

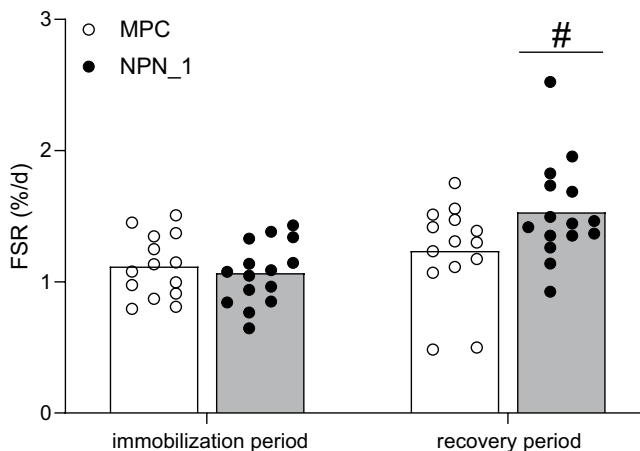


Figure 4.5 Myofibrillar protein fractional synthetic rates (%/day) calculated from saliva precursor pools. Myofibrillar FSR assessed during 7 d of single-leg immobilization and 14d of free-living recovery in the immobilized leg in the MPC and NPN_1 group. Data were analyzed with a repeated measures ANOVA. Bars are means (MPC n=14; NPN_1 n=15) and dots represent individual values. #significant time x treatment interaction.

mRNA expression

Following immobilization, FOXO (**Figure 4.6A**), MuRF1 (**Figure 4.6B**), and MAFBx (**Figure 4.6C**) mRNA expression were increased vs baseline ($P<0.001$), with no differences between groups (all $P>0.05$). Following recovery, FOXO, MuRF1, and MAFBx mRNA expression were lower vs post-immobilization ($P<0.001$), with no differences detected between groups ($P>0.05$). Whereas FOXO and MAFBx mRNA expression returned to baseline levels following recovery ($P>0.05$), MuRF1 mRNA expression remained elevated when compared to baseline ($P=0.016$).

Blood parameters

Blood parameters were assessed at baseline, prior to and following immobilization, and following remobilization. Data were analyzed for 20 participants ($n=9$ MPC, $n=11$ NPN_1). Data for 10 participants were excluded due to missing samples or participants not being fasted. Creatinine, GGT, AST, ALT, LDH and CK concentrations are displayed in **Supplemental Figure 4.2**. TG, hs-CRP, FFA, insulin and glucose concentrations are displayed in **Supplemental Figure 4.3**. There were no changes over time and between groups for GGT, AST, ALT, LDH, CK, TG, hs-CRP, FFA, and insulin (all $P>0.05$). Creatinine concentrations were lower following immobilization when compared to baseline and prior to immobilization (both $P<0.03$), but not different when compared to post-recovery. Creatinine was higher in the NPN_1 group when compared to the MPC group ($P<0.001$). Glucose concentrations were lower following immobilization when compared to baseline ($P=0.040$), with no differences between groups ($P>0.05$).

Dietary intake and physical activity

Dietary intake and step count data are displayed in **Table 4.4**. Data of 9 participants were excluded due to insufficient wear time. Prior to immobilization, no differences were observed in energy and protein intake between groups ($P>0.05$). During immobilization, total energy intake tended to be lower ($P=0.089$) and total protein intake was lower ($P=0.022$) when compared to pre-immobilization. During recovery, total energy and protein intake were lower when compared to pre-immobilization ($P<0.05$), but not different from during immobilization ($P>0.05$). Prior to immobilization, no differences were observed in step count between groups ($P>0.05$). During immobilization, step count reduced from 6056 ± 1923 to 1981 ± 1306 steps in the MPC group and from 6596 ± 2066 to 2400 ± 1338 steps in the NPN_1 group ($P<0.001$). During recovery step count improved back to pre-immobilization level ($P>0.05$). No differences in step count were detected between groups ($P>0.05$).

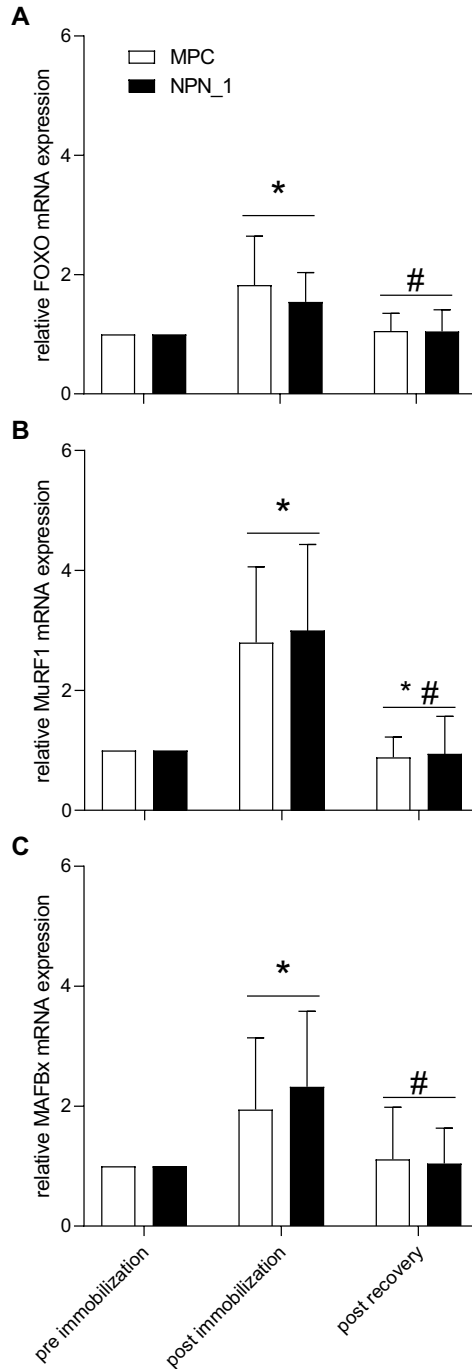


Figure 4.6 Skeletal muscle mRNA expression of selected genes of interest in the MPC and NPN_1 group, before and after 7 days of single-legged immobilization, and after 14 days of free-living recovery. Data were analyzed with a repeated measures ANOVA. Data are expressed as mean \pm SD. *significantly different from pre-immobilization; #significantly different from post-immobilization.

Table 4.4 Nutrition and physical activity.

	Pre-immobilization		Immobilization period		Recovery period	
	MPC	NPN_1	MPC	NPN_1	MPC	NPN_1
Nutrition						
Energy intake (MJ/d)	9.7±2.9	10.6±2.7	8.1±1.7	9.7±2.2	8.6±1.8*	8.9±2.3*
Carbohydrate (En%)	45±5	43±10	48±7	45±5	46±6	50±8
Fat (En%)	36±7	34±5	34±9	37±5	36±7	32±7
Protein (En%)	16±3	17±4	16±4	16±4	16±4	16±3
Protein intake (g/d)	93±32	106±23	73±20*	89±33*	79±22*	81±21*
Protein intake (g/kg/d)	1.2±0.4	1.5±0.4	1.0±0.3*	1.3±0.5*	1.1±0.3*	1.2±0.2*
Physical activity						
Step count	6056±1923	6596±2066	1981±1306*	2400±1338*	4947±2961#	6081±1914#

Nutrition data is displayed for n=14 MPC, n=15 NPN_1; Physical activity data is displayed for n=8 MPC, n=9 NPN_1
 * significantly different from pre-immobilization value (P<0.05); # significantly different from post-immobilization (P<0.05).

Discussion

The present study shows that 7 days of single-leg immobilization resulted in a substantial decline in daily muscle protein synthesis rates and a loss of muscle mass and strength, which were only partially recovered following 14 days of remobilization. When compared to milk protein supplementation, twice daily *Vicia faba* peptide network supplementation did not preserve muscle mass or strength during short-term immobilization, or augment muscle mass and strength regain during remobilization. However, NPN_1 supplementation resulted in significantly higher muscle protein synthesis rates during the remobilization period when compared to milk protein supplementation.

In the present study, 7 days of single-leg immobilization resulted in a substantial loss of muscle mass ($-5\pm 2\%$) and strength ($-13\pm 7\%$). These observed rates of muscle mass ($-0.7\pm 0.3\%$ per day) and strength loss ($-1.8\pm 1.0\%$ per day) confirm previous observations over similar durations of limb immobilization in our laboratory as well as others [3, 4, 13, 15, 17, 24, 33]. Our data clearly illustrate the impact of short periods of muscle disuse on muscle mass and strength. Recently, a predictive machine learning approach has been applied to identify peptides from *Vicia faba* with anabolic and anti-catabolic properties observed in in vitro and rodent models [18]. In the current trial, participants were provided with either 6 g protein dose of NPN_1 or an isonitrogenous control consisting of milk protein concentrate, provided twice daily. Daily NPN_1 consumption did not differ from MPC in the retention of muscle mass or strength throughout the 7 days of disuse (Figure 2). These data are in line with earlier work showing that supplementing high-quality protein sources does not preserve muscle mass or strength throughout 5-7 days of limb immobilization in otherwise healthy adults [5, 6, 17]. Conversely, previous work reported that supplementation with selected essential and non-essential amino acids could mitigate muscle mass loss following 7 days of immobilization when compared to an energy-matched carbohydrate control [16]. The discrepancy between findings might be explained by differences in the amounts of amino acids provided in the studies, as we provided 12 g protein of the NPN_1, whereas Holloway et al. provided ~ 70 g of the amino acid supplement and pre-loaded for 7 days. However, Kilroe et al. showed no differences in muscle loss when consuming either a high, low or no protein diet during 3 days of single-leg immobilization [34]. Clearly, more work will be needed to determine the various factors that determine the efficacy of amino acid and/or protein supplementation to attenuate muscle mass loss during muscle disuse.

Despite the clinical importance of recovery following such a short period of immobilization, only few studies have assessed muscle mass and strength regain during remobilization. Here, we observed only a partial regain in muscle mass ($1.5\pm 2.3\%$) and leg extension strength ($7.8\pm 11.9\%$) following 14 days of ambulant recovery. This seems to be in line with previous work showing only partial recovery of muscle cross sectional area following short periods (7-14 d) of ambulatory recovery [4, 15, 21]. When extrapolating the data on muscle mass loss, muscle mass would have returned to pre-immobilization levels after ~ 7 weeks of remobilization. This seems to agree with previous work showing full recovery of muscle mass and strength following 6 weeks of natural rehabilitation [20]. This illustrates that there are opportunities to shorten recovery periods using exercise and/or nutritional interventions. To date, there are no nutritional strategies that have proven effective in accelerating muscle mass regain during ambulatory recovery. Here, we assessed the impact of supplementing a food-derived peptide network that was shown preclinically to possess anabolic properties [19]. However, we did not show any differences in muscle mass

or strength regain when comparing the NPN_1 supplementation to supplementation with an isonitrogenous amount of milk protein. Within the applied study design, we cannot directly determine whether protein per se accelerated muscle mass or strength regain. However, the extent of muscle mass regain in our study is similar to earlier work showing that protein or amino acid supplementation does not augment muscle mass regain during ambulant recovery [16, 21]. The latter may not be surprising under conditions where ample amounts of protein are consumed throughout the day in otherwise healthy, active young adults not involved in any exercise rehabilitation program (Table 4).

In addition to our assessments of skeletal muscle mass and strength, we also provided participants with deuterium oxide to assess muscle protein synthesis rates during both immobilization as well as subsequent remobilization. We observed ~30 % lower myofibrillar protein synthesis rates in the immobilized leg during short-term disuse when compared to the non-immobilized leg (Figure 4). These data are in line with Kilroe et al. showing a similar reduction in myofibrillar protein synthesis rates during 7 days of disuse [13, 34]. Though muscle loss during disuse has been attributed to declines in both postabsorptive [2, 11, 35] as well as postprandial [12, 14, 35] protein synthesis rates, there are also indications of greater muscle protein breakdown during short periods of immobilization [21]. In agreement, we demonstrated a robust upregulation of protein breakdown markers in muscle (FOXO, MuRF1 and MAFBx; Figure 6). Again, no differences were observed between treatments, so we were unable to show any anabolic or anti-catabolic properties of the NPN_1 supplementation beyond the capacity of milk protein supplementation during the immobilization period. However, it should be noted that changes in mRNA expression are transient and merely represent snapshots in time. With protein intake levels having shown not to impact the decline in daily muscle protein synthesis rates during a short period of single-leg immobilization [34], it seems evident that other more intrinsic anabolic stimuli are required to preserve muscle mass during a period of disuse [24, 36-38]. Local muscle contraction during immobilization, by active stimulation or the application of exercise mimetics, is likely an essential component in effective strategies to attenuate muscle mass and strength loss during immobilization.

During subsequent 14 days of ambulant remobilization, daily myofibrillar protein synthesis rates increased when compared to the levels observed during immobilization. Despite the absence of differences in muscle mass and strength regains between treatments, we observed significantly (50 vs 13%) higher myofibrillar protein synthesis rates following NPN_1 supplementation when compared to MPC (Figure 5). We can only speculate on the relevance of these findings, but it is possible that the remobilization period was too short for us to allow detection of small, but clinically relevant, increases in muscle mass and/or strength regain following NPN_1 supplementation. Higher myofibrillar protein synthesis rates during recovery from a period of immobilization would be indicative of greater or more rapid reconditioning of muscle. However, whether these differences are specific for myofibrillar and/or other muscle protein fractions and whether they can translate to clinical benefits remains to be addressed in further studies. Though most pharmacological and nutritional strategies to preserve muscle mass and strength during a period of disuse and accelerate muscle mass and strength regain during remobilization have been unsuccessful [4-6, 17, 21, 39], it is evident that such strategies may be more effective in more clinically compromised populations where habitual physical activity and daily food intake are less than optimal. In the present study, habitual physical activity and sufficient daily protein consumption were evident in our healthy, young adults (Table 4). This may compromise the

ability to detect relevant improvements in the rate of muscle mass and strength regain following nutritional and/or pharmacological interventions in a research model of limb immobilization.

In conclusion, NPN_1 supplementation does not attenuate muscle mass or strength loss during short-term immobilization, or augment muscle mass and strength regain during remobilization when compared to the ingestion of an isonitrogenous amount of milk protein. NPN_1 supplementation did not differ from milk protein supplementation in modulating myofibrillar protein synthesis rates during immobilization, but further increased myofibrillar protein synthesis rates during subsequent remobilization. The relevance of the latter findings will require further investigation.

Acknowledgements

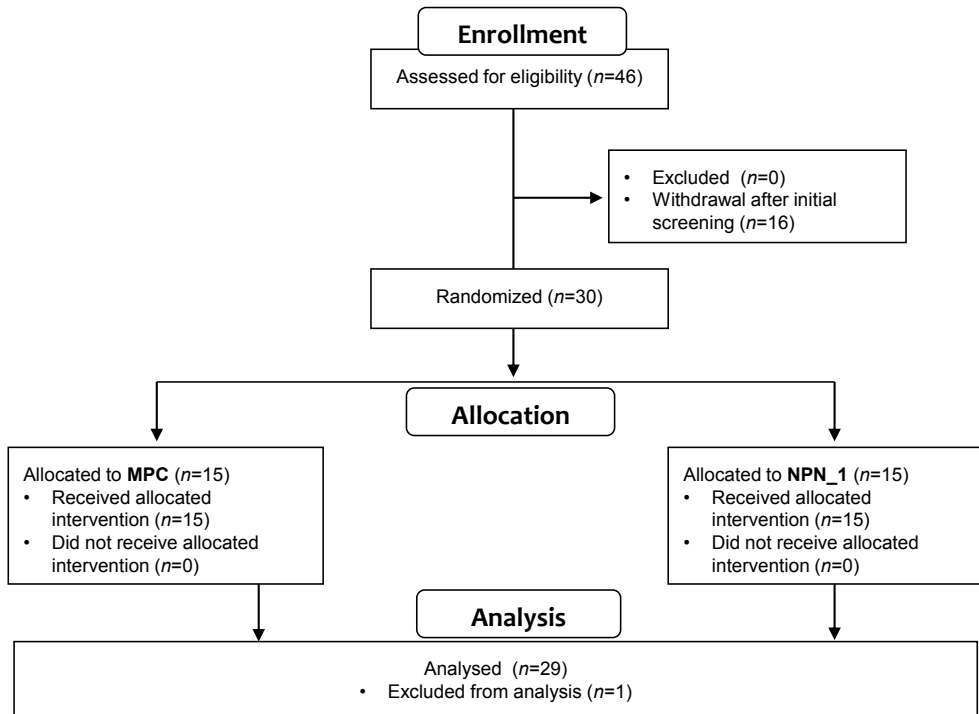
We greatly appreciate the technical expertise from Antoine Zorenc, Joy Goessens and Annemie Gijzen during the sample analyses. The practical assistance of Thorben Aussieker, Quinn Kruger, Tom Janssen, Laura Schenaarts, and Kira Schording was greatly appreciated.

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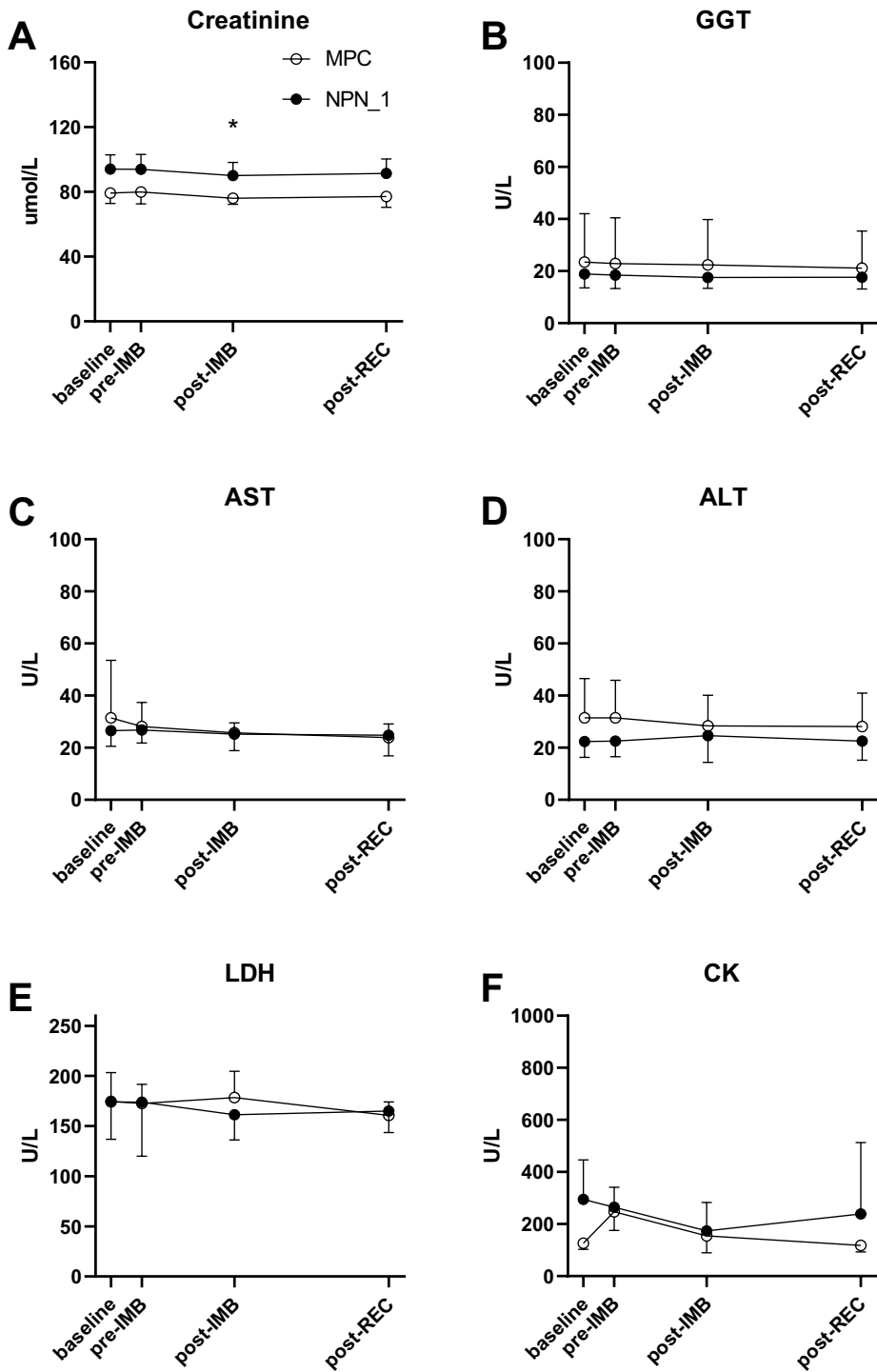
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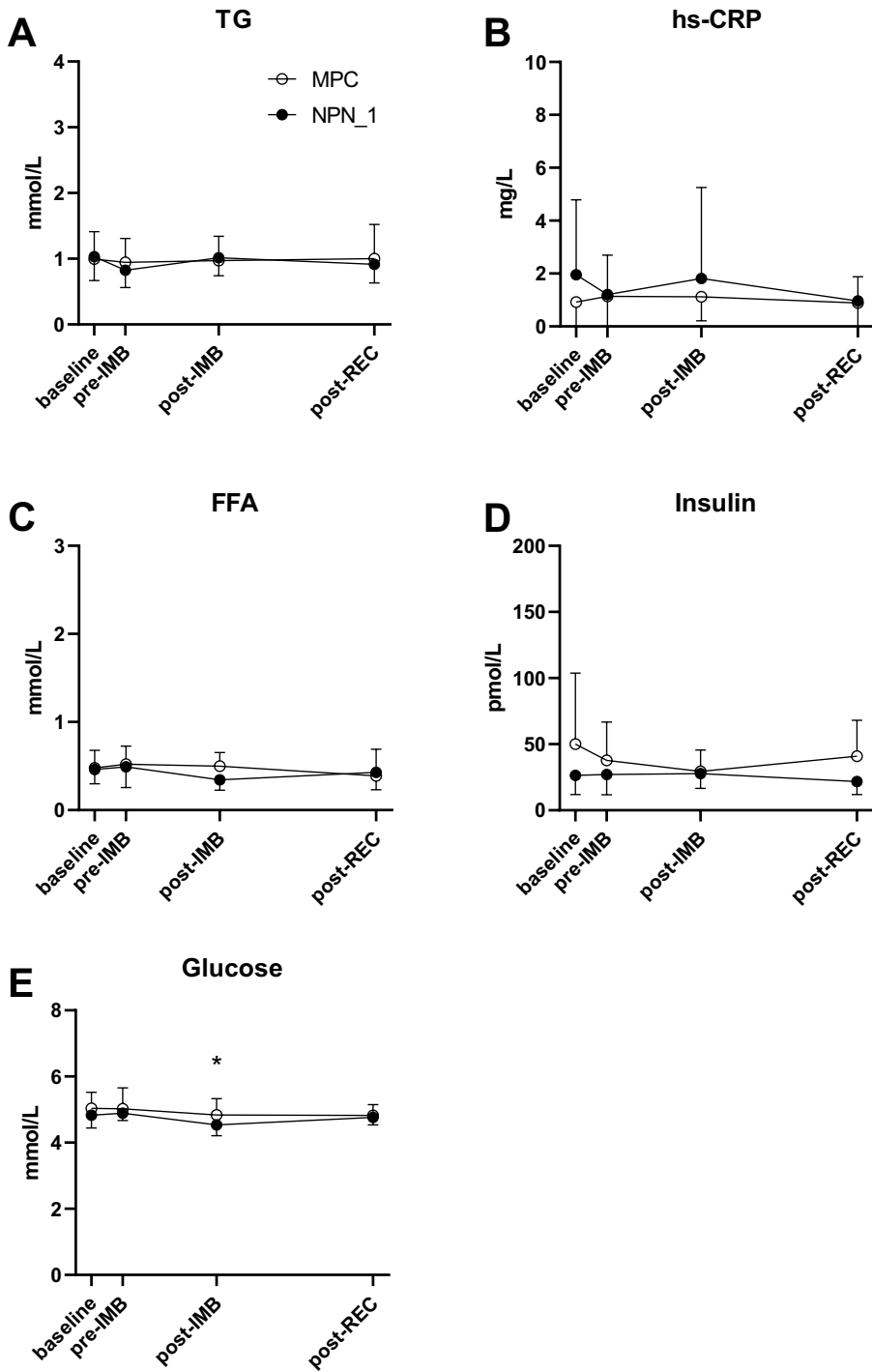
Supplemental material



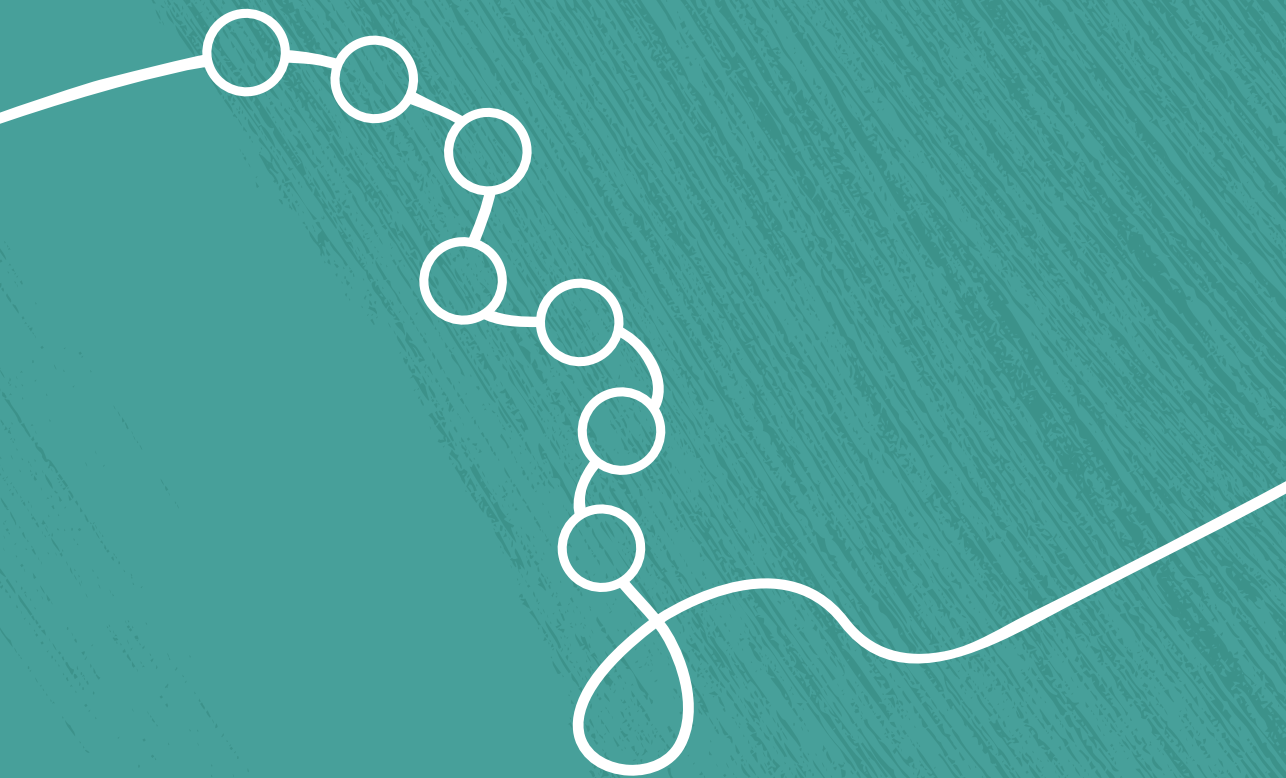
Supplemental figure 4.1 Participant flow chart.



Supplemental figure 4.2 blood parameters.



Supplemental figure 4.3 blood parameters.



Chapter 5

Ingestion of free amino acids compared with an equivalent amount of intact protein results in more rapid amino acid absorption and greater postprandial plasma amino acid availability without affecting muscle protein synthesis rates in young adults in a double-blind randomized trial

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J Nutr. 2022 Jan 11;152(1):59-67

Abstract

Background

The rate of protein digestion and amino acid absorption determines the postprandial rise in circulating amino acids and modulates postprandial muscle protein synthesis rates.

Objective

To compare protein digestion and amino acid absorption kinetics and the postprandial muscle protein synthetic response following ingestion of intact milk protein versus an equivalent amount of free amino acids.

Methods

Twenty-four healthy, young participants (age: 22 ± 3 y; BMI: 23 ± 2 kg/m²; sex: 12M/12F) received a primed continuous infusion of L-[ring-²H₅]-phenylalanine and L-[ring-3,5-²H₂]-tyrosine, after which they ingested either 30 g intrinsically L-[1-¹³C]-phenylalanine labeled milk protein or an equivalent amount of free amino acids labeled with L-[1-¹³C]-phenylalanine. Blood samples and muscle biopsies were obtained to assess protein digestion and amino acid absorption kinetics (secondary outcome), whole-body protein net balance (secondary outcome), and mixed muscle protein synthesis rates (primary outcome) throughout the 6 h postprandial period.

Results

Postprandial plasma amino acid concentrations increased after ingestion of intact milk protein and free amino acids (both $P<0.001$), with a greater increase following ingestion of the free amino acids when compared with intact milk protein (P -time \times treatment <0.001). Exogenous phenylalanine release into plasma, assessed over the 6 h postprandial period, was greater in the free amino acids ($76\pm 9\%$) compared with milk protein treatment ($59\pm 10\%$; $P<0.001$). Ingestion of free amino acids and intact milk protein increased mixed muscle protein synthesis rates (P -time <0.001), with no differences between treatments (from 0.037 ± 0.015 to $0.053\pm 0.014\%/h$ and 0.039 ± 0.016 to $0.051\pm 0.010\%/h$, respectively; P -time \times treatment = 0.629).

Conclusions

Ingestion of a bolus of free amino acids leads to more rapid amino acid absorption and greater postprandial plasma amino acid availability when compared to the ingestion of an equivalent amount of intact milk protein. Ingestion of free amino acids may be preferred over intact protein in conditions where protein digestion and amino acid absorption are compromised.

Introduction

Protein ingestion is an important anabolic stimulus that regulates muscle mass maintenance [1-3]. The increase in muscle protein synthesis rates following protein ingestion has been attributed to the postprandial rise in circulating (essential) amino acids, with the rise in plasma leucine concentration being of particular relevance [4]. As the postprandial rise in circulating amino acids represents the driver of the muscle protein synthetic response to feeding, it is evident that protein digestion and amino acid absorption kinetics form a key determinant of the muscle protein synthetic response to protein intake [5-8]. In agreement, the greater postprandial increase in circulating amino acids after the ingestion of whey compared with micellar casein has been proposed to be, at least partly, responsible for the greater anabolic properties of whey versus casein [5, 6, 9, 10]. As such, more rapidly digestible proteins (like whey) are considered more potent in stimulating muscle protein synthesis than more slowly digestible proteins (like micellar casein).

Early work in pigs has shown that following protein digestion and absorption about 50% of dietary protein-derived amino acids are extracted by the splanchnic tissues in order to maintain its functional mass [11]. In agreement, studies in humans have reported that 50-60% of dietary protein-derived leucine and phenylalanine is released in the circulation throughout a 5-6 h postprandial period, with the remainder likely retained in the splanchnic area [3, 12, 13]. Interestingly, the percentage of the dietary protein-derived amino acids extracted by the splanchnic tissues seems to depend on various factors, including protein digestion and amino acid absorption kinetics [14]. In support, we have previously shown that ingestion of a protein hydrolysate leads to greater release of protein-derived phenylalanine into the circulation when compared to the ingestion of its intact protein, which tended to further increase postprandial muscle protein synthesis rates [8]. As free amino acids exhibit more rapid intestinal absorption when compared with dietary protein-derived amino acids [15], it could be speculated that postprandial splanchnic amino acid retention is less following ingestion of free amino acids when compared to the ingestion of an equivalent amount of intact protein. Consequently, ingestion of free amino acids may result in a greater postprandial release of amino acids in the circulation, thereby further increasing postprandial muscle protein synthesis rates when compared to the ingestion of an equivalent amount of intact protein.

In this study, we compare protein digestion and amino acid absorption kinetics and the subsequent muscle protein synthetic response following the ingestion of intact milk protein versus an equivalent amount of free, crystalline amino acids. We hypothesized that the ingestion of free amino acids would result in more rapid amino acid absorption, greater postprandial exogenous amino acid release into the circulation, and a greater rise in mixed muscle protein synthesis rates when compared with the ingestion of an equivalent amount of intact milk protein *in vivo* in humans. To test this hypothesis, healthy, young individuals received continuous infusion with L-[ring- $^2\text{H}_5$]-phenylalanine while consuming either a single bolus of specifically produced intrinsically L-[1- ^{13}C]-phenylalanine labeled milk protein [16-18] or an equivalent amount of free amino acids enriched with free L-[1- ^{13}C]-phenylalanine. Combining the ingestion of free or (milk) protein bound L-[1- ^{13}C]-phenylalanine with continuous intravenous infusion of L-[ring- $^2\text{H}_5$]-phenylalanine allows us to assess protein digestion and amino acid absorption kinetics, quantify the amount of exogenous amino acids being released into the circulation, assess the postprandial rise in muscle protein synthesis rates, and directly assess the metabolic

fate of the ingested free or protein bound L-[1-¹³C]-phenylalanine [16]. This is the first study to provide a comprehensive overview of the differences in postprandial protein digestion and amino acid absorption kinetics and postprandial muscle protein synthesis rates following the ingestion of a single bolus of dietary protein versus an equivalent amount of free, crystalline amino acids *in vivo* in humans.

Material and methods

Study design

Twenty-four healthy, young participants (22 ± 3 y; 23 ± 2 kg/m²; 12M/12F) were recruited via advertisements on poster boards and social media to participate in a stable isotope infusion trial. This double-blind, randomized, parallel-group trial was conducted between February and August 2018 at Maastricht University Medical Centre+, the Netherlands. A flowchart of subject enrolment is shown in Supplemental Figure 5.1. Participants' characteristics are presented in Table 5.1. Participants were informed about possible risks of the experimental procedures prior to providing written informed consent. This study was approved by the local Medical Ethical Committee of Maastricht University Medical Centre+ and conforms to the principles outlined in the latest version of the Declaration of Helsinki for use of human subjects and tissue. This trial was registered at <http://www.trialregister.nl> as NTR6941. The study was independently monitored by the Clinical Trial Center Maastricht.

Table 5.1 Participants' characteristics.

	PRO (n=12)	AA (n=12)
Age (y)	22±2	23±3
Sex; M/F (n)	6/6	6/6
Weight (kg)	70.0±8.6	68.1±10.6
BMI (kg/m²)	22.8±1.1	22.5±2.9
Body fat (%)	25.3±6.6	24.2±7.1
Appendicular lean mass (kg)	22.9±5.5	22.5±5.7
Lean body mass (kg)	50.9±10.0	49.9±10.1

Values are means±SD. Data were analyzed with Student's T-test. No differences were detected between groups. AA, free amino acids; BMI, body mass index; F, female; M, male; PRO, milk protein.

Pretesting

All participants were screened to assess body weight, height, body composition, and blood pressure. Body composition was determined by a dual-energy x-ray absorptiometry (DXA) scan (Hologic Inc., Discovery A, QDR series, software package: APEX version 4.0.2 (Marlborough, MA, USA). Whole-body and regional lean mass and fat mass were determined using reference values from the NHANES population-based dataset [19].

Intact milk protein and free amino acid mixture

Intrinsically L-[1-¹³C]-phenylalanine-labeled milk protein was obtained by infusing lactating Holstein cows with L-[1-¹³C]-phenylalanine for 48 h while collecting milk, as described previously [16, 17, 20]. A mixture of free amino acids (Ajinomoto, Raleigh, USA) was produced with an identical amino acid composition as the milk protein, and enriched with free L-[1-¹³C]-phenylalanine (Cambridge Isotope Laboratories, Andover, MA, USA) to achieve the same enrichment level. The L-[1-¹³C]-phenylalanine enrichment in both drinks was 38 mole percent excess (MPE). The milk protein and free amino acid mixture met all chemical and

bacteriological specifications for human consumption. Participants consumed 30 g intrinsically L-[1-¹³C]-phenylalanine labeled milk protein (PRO) or an equivalent amount of free amino acids (AA), dissolved in 300 mL water (**Table 5.2**). Randomization was performed by using a computerized random-number generator, and stratified by sex. An independent person was responsible for random assignment and drink preparation.

Diet and activity before testing

All participants were instructed to refrain from exhaustive physical activity and/or exercise and maintain their usual dietary habits 3 days prior to the test day. All participants consumed a standardized meal (2.9 MJ; composed of 18 percent energy (En%) protein, 54 En% carbohydrate, and 28 En% fat) the evening prior to the experiment. Female subjects were tested in the first 10 days (follicular phase) of their menstrual cycle (standardized). There was an equal balance between males and females in each condition (6 M/F per condition).

Experimental protocol

At 07.30 AM, following an overnight fast, participants arrived at the laboratory by car or public transport. A Teflon cannula was inserted into an antecubital vein for isotope infusion and a second cannula was inserted into the dorsal hand vein of the contralateral arm for arterialized blood sampling [21]. After a basal blood sample was collected ($t=-210$), the plasma phenylalanine and tyrosine pools were primed with a single intravenous dose of L-[ring-²H₅]-phenylalanine (2 $\mu\text{mol/kg}$) and L-[ring-3,5-²H₂]-tyrosine (0.613 $\mu\text{mol}\cdot\text{kg}^{-1}$). Subsequently, the continuous infusion was started (infusion rate: 0.05 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ L-[ring-²H₅]-phenylalanine and 0.015 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ L-[ring-3,5-²H₂]-tyrosine) and maintained for 9.5 h. Arterialized blood samples were collected during infusion at $t=-120, -60, -30, 0, 15, 30, 45, 60, 90, 120, 180, 240, 300,$ and 360 min relative to drink ingestion for the analysis of plasma amino acid concentrations and enrichments, and insulin concentrations. Blood samples (10 mL) were collected in EDTA-containing tubes and centrifuged at 1000g for 10 min at 4°C. Skeletal muscle biopsies were collected at $t=-120$ and 0 min for the determination of basal muscle protein synthesis rates. Immediately following the second biopsy ($t=0$ min, from the contralateral leg), participants ingested a single bolus of 30 g intact milk protein or an equivalent amount of free amino acids. Additional muscle biopsies were collected at $t=120$ and 360 min to determine postprandial muscle protein synthesis rates. Muscle biopsy collection was randomized between legs, and biopsies were collected from the middle region of the *M. vastus lateralis* (15 cm above the patella) using the Bergström needle technique [22]. All biopsy samples were freed from any visible adipose tissue and blood. Aliquots of plasma and muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until subsequent analysis.

Table 5.2 Amino acid contents of PRO and AA treatments.

	PRO	AA
EAA (% of TAA)		
Histidine	2.7	2.6
Isoleucine	4.9	4.8
Leucine	9.1	9.1
Lysine	7.7	9.5
Methionine	2.7	2.6
Phenylalanine	4.4	4.4
Threonine	4.2	4.2
Tryptophan	1.2	1.2
Valine	6.4	6.4
ΣEAA	43.2	44.7
NEAA (% of TAA)		
Alanine	3.0	2.9
Arginine	4.0	3.9
Aspartic acid ¹	7.3	7.2
Cysteine	0.8	0.8
Glutamic acid ²	19.8	19.6
Glycine	1.8	1.8
Proline	9.9	9.6
Serine	5.3	5.3
Tyrosine	4.9	4.3
ΣNEAA	56.7	55.3

AA, free amino acids; EAA, essential amino acids; NEAA, nonessential amino acids; PRO, milk protein; TAA, total amino acids. ¹aspartic acid includes asparagine ²glutamic acid includes glutamine.

Plasma and muscle tissue analysis

The Supplemental Methods section present details of analyses related to the determination of plasma (glucose, insulin, amino acids, L-[1-¹³C]- and L-[ring-²H₅]-phenylalanine enrichments, L-[1-¹³C]-, L-[ring-3,5-²H₂]-, and L-[ring-²H₄]-tyrosine enrichments) as well as muscle derived (mixed muscle protein L-[1-¹³C]- and L-[ring-²H₅]-phenylalanine enrichments) data.

Western blotting

The Supplemental Methods section present details of analysis related to the western blotting performed on muscle tissue samples (at $t=0$, 2, and 6 h).

Calculations

Intravenous infusion of L-[ring-²H₅]-phenylalanine and L-[ring-3,5-²H₂]-tyrosine combined with the ingestion of free or (milk) protein bound L-[1-¹³C]-phenylalanine, arterialized blood sampling, and skeletal muscle biopsy collection, allowed us to assess postprandial protein handling [23]. Specifically, metabolic calculations based on tracer kinetics were applied to assess whole-body amino acid kinetics (phenylalanine rate of appearance, exogenous phenylalanine rate of appearance, endogenous phenylalanine rate of appearance, phenylalanine rate of disappearance, and the fraction of dietary protein derived phenylalanine release into the circulation), whole-body protein metabolism (whole-body protein synthesis, breakdown, and oxidation rate as well as whole-body net protein balance), and mixed muscle protein synthesis (muscle protein fractional synthesis rate and incorporation of exogenous protein derived phenylalanine in muscle protein) in the basal and/or postprandial state have been described in detail previously [23-25]. Mixed muscle protein fractional synthetic rates were calculated using the weighted mean plasma L-[ring-²H₅]-phenylalanine enrichment during the incorporation period.

Statistics

All data are expressed as mean \pm SD. Normality of the data was verified using visual inspection of QQ plots and Shapiro-Wilk tests. No major violations for specific 2-way ANOVA assumptions were observed; in case of non-sphericity, the Greenhouse-Geisser correction was used. Differences in baseline characteristics were determined using unpaired, two-tailed Student's t-tests. Peak values and time to peak were calculated for all plasma time curves and unpaired, two-tailed Student's t-test were applied to identify differences in peak values or time to peak between groups. Two-way ANOVA with time as within-group factor and treatment as between-group factor was used to compare differences between groups over time in plasma insulin, amino acid concentrations and enrichments, whole-body phenylalanine appearance rates, and FSR. In case of a significant interaction between time and treatment, separate analyses were performed to determine time-effects for each group (one-factor repeated measures ANOVA) with a Bonferroni post-hoc test to locate these differences and between-group effects for each time-point (Students t-test). Based upon previous studies [5, 8, 26], the expected difference in MPS between interventions would be 0.009 %/h with a standard deviation of 0.0065 (or ~20% when expressed as relative difference between interventions). A sample size of 12 participants per group including a 10% dropout rate was calculated, using a 2-sided statistical test ($P<0.05$, 80% power), to detect differences in FSRs between groups (primary outcome). The effect size that can be detected was 1.38. Statistical significance was set at $P<0.05$. All calculations were performed using the statistical software program SPSS (version 26.0, IBM Corp., Armonk, USA).

Results

Plasma insulin and amino acid concentrations

Plasma insulin concentrations increased after the ingestion of the 30 g bolus of protein (PRO) or the equivalent amount of free amino acids (AA; P -time<0.001), with a greater rise in plasma insulin concentrations following the ingestion of AA compared with PRO (P -treatment=0.014; P -time x treatment=0.001; data not shown). Peak plasma insulin concentrations averaged 35 ± 15 and 24 ± 9 $\text{mU}\cdot\text{L}^{-1}$ in the AA and PRO group, respectively ($P=0.044$). Plasma phenylalanine and leucine concentrations increased rapidly after the ingestion of milk protein or the equivalent amount of free amino acids (P -time<0.001; **Figure 5.1**). Plasma phenylalanine (P -treatment=0.009; P -time x treatment<0.001) and leucine (P -treatment<0.001; P -time x treatment<0.001) concentrations increased to a greater extent following AA compared with PRO ingestion. Peak plasma phenylalanine and leucine concentrations were higher in the AA compared with the PRO treatment (129 ± 7 vs 87 ± 4 and 501 ± 42 vs 326 ± 59 $\mu\text{mol}\cdot\text{L}^{-1}$, respectively; $P<0.001$). In line, the incremental area under the curve (iAUC) of plasma phenylalanine and leucine concentrations were greater in the AA compared with PRO treatment (9.8 ± 4.8 vs 5.9 ± 1.4 $\mu\text{mol}\cdot 360\text{min}\cdot\text{L}^{-1}$ and 57.5 ± 15.2 vs 40.1 ± 8.1 $\mu\text{mol}\cdot 360\text{min}\cdot\text{L}^{-1}$, respectively; $P<0.05$). Postprandial plasma essential (**Figure 5.1C**) and non-essential (**Figure 5.1D**) amino acid concentrations increased following free amino acids and protein ingestion (P -time<0.001; P -treatment ≤ 0.010) and increased to a greater extent following free amino acids compared with intact milk protein ingestion (P -time x treatment<0.001).

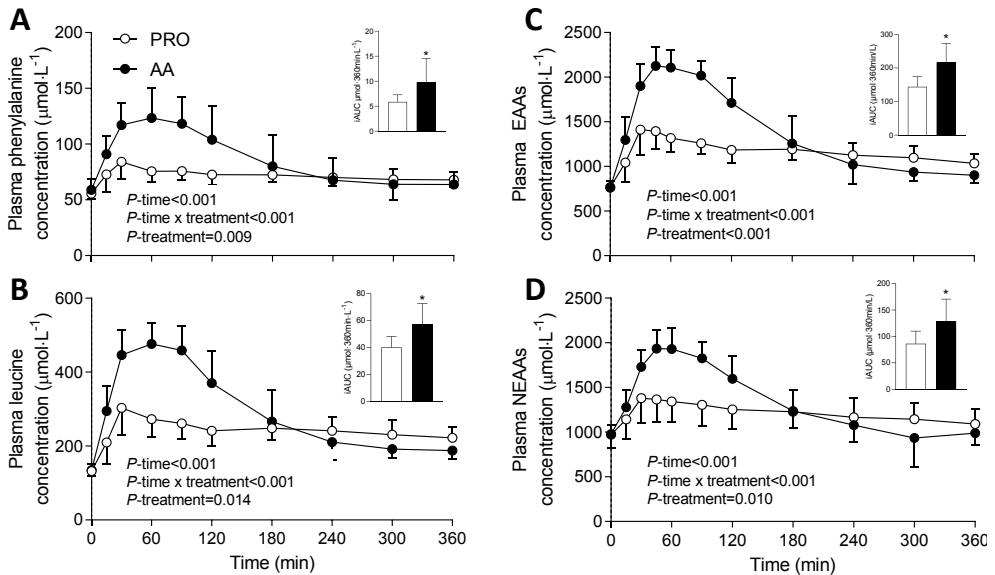


Figure 5.1 Plasma phenylalanine (A), leucine (B), EAAs (C), and NEAAs (D) concentrations and iAUC after ingesting 30 g milk protein (PRO) or an equivalent amount of free amino acids (AA). Values are mean \pm SD, $n=12$. Plasma concentrations were analyzed with the use of repeated measures ANOVA, iAUC were analyzed with independent Student's t -tests. *different from PRO (A: $P=0.043$; B: $P=0.003$; C: $P=0.006$; D: $P<0.001$). AA, free amino acids; EAA, essential amino acid; NEAA, nonessential amino acid; PRO, milk protein.

Plasma amino acid enrichments

Plasma L-[1-¹³C]-phenylalanine (tracer ingested as free L-[1-¹³C]-phenylalanine or as intrinsically L-[1-¹³C]-phenylalanine labeled milk protein) enrichments increased following free amino acids or protein ingestion (P -time<0.001; **Figure 5.2A**). Peak plasma L-[1-¹³C]-phenylalanine enrichments were reached at $t=90$ min in both the AA and PRO treatments. The increase in plasma L-[1-¹³C]-phenylalanine enrichments differs between treatments (P -time x treatment<0.001), with higher values following free amino acids compared with intact milk protein ingestion until $t=180$ min ($P<0.05$). In contrast, plasma L-[1-¹³C]-phenylalanine enrichments were higher in the PRO compared with the AA treatment following $t=240$ min ($P<0.05$). Plasma L-[ring-²H₅]-phenylalanine (infused tracer) enrichments did not differ between treatments prior to free amino acids and protein ingestion ($t=0$ min; $P>0.05$; **Figure 5.2B**). Plasma L-[ring-²H₅]-phenylalanine enrichments were higher following intact milk protein compared with free amino acids ingestion between $t=30$ until $t=90$ min ($P<0.05$). Plasma L-[ring-²H₅]-phenylalanine enrichments were higher in AA compared with PRO at $t=300$ min ($P=0.040$).

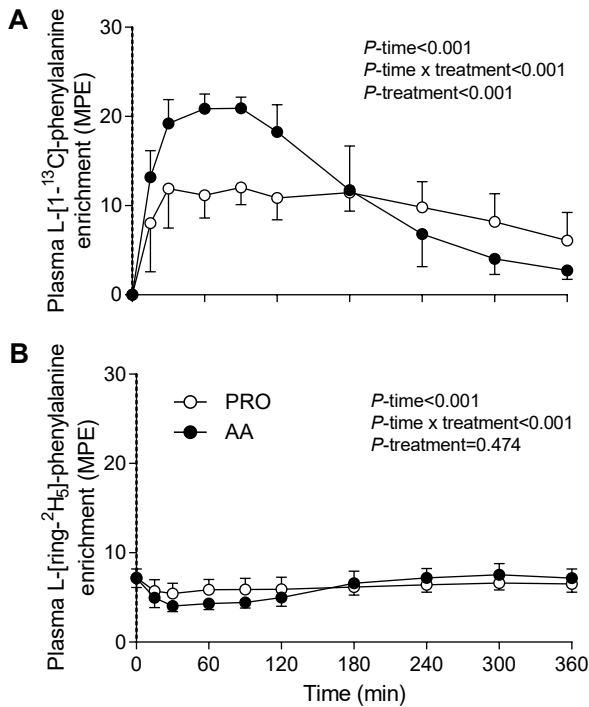


Figure 5.2 Plasma L-[1-¹³C]-phenylalanine (**A**) and L-[ring-²H₅]-phenylalanine (**B**) after ingesting 30 g milk protein (PRO) or an equivalent amount of free amino acids (AA). Values are mean \pm SD, $n=12$. Data were analyzed with the use of repeated measures ANOVA. AA, free amino acids; MPE, mole percent excess; PRO, milk protein.

Whole-body amino acid kinetics

Cumulative dietary-derived amino acids released in the circulation (**Figure 5.3A**) increased following both free amino acid and protein ingestion (P -time <0.001), with a greater increase following AA compared with the PRO treatment (P -time \times treatment <0.001). Exogenous plasma phenylalanine availability (**Figure 5.3B**), calculated as the percentage of the total amount of phenylalanine consumed as free amino acids or milk protein that was released in the circulation during the 6 h postprandial period, was significantly higher following AA versus PRO ingestion ($76\pm 9\%$ vs $59\pm 10\%$; $P<0.001$).

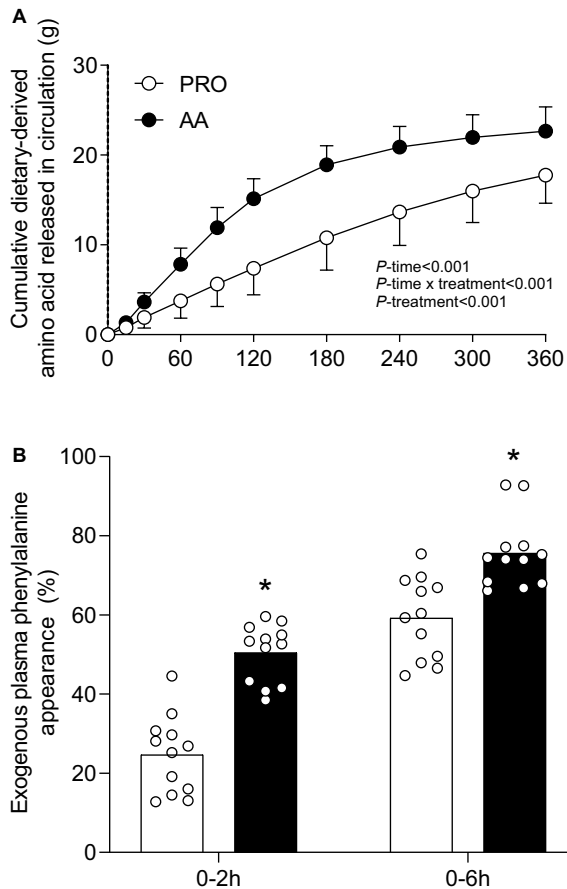


Figure 5.3 Cumulative dietary-derived amino acid released into the circulation (**A**), and postprandial exogenous phenylalanine availability (**B**), after ingesting 30 g milk protein (PRO) or an equivalent amount of free amino acids (AA). Bars are means ($n=12$) and dots represent individual values. Data were analyzed with (A) repeated measures ANOVA and (B) Independent Student's t -tests. *Significantly different from PRO ($P<0.001$). AA, free amino acids; PRO, milk protein.

In line with these findings, the exogenous phenylalanine R_a (**Supplemental Figure 5.2A**) increased following AA and PRO ingestion (P -time <0.001), with peak levels being reached at $t=30$ min in both treatments (0.75 ± 0.11 vs 0.38 ± 0.13 $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, respectively). Exogenous phenylalanine R_a increased to a greater extent in the AA compared with the PRO treatment (P -time \times treatment <0.001). Endogenous phenylalanine R_a (**Supplemental Figure 5.2B**) decreased following free amino acids and protein ingestion (P -time <0.001), with a greater decline at $t=60$ -120 min following free amino acids as opposed to protein ingestion (P -time \times treatment $=0.004$). Free amino acids and intact milk protein ingestion both increased total phenylalanine appearance and disappearance rates (P -time <0.001), with higher rates observed following free amino acids when compared with intact milk protein ingestion (P -time \times treatment <0.001 ; **Supplemental Figure 5.2C and D**). Whole-body protein turnover rates are presented in **Figure 5.4**. Basal whole-body protein turnover rates did not differ between treatments (all $P>0.05$). During the 6 h postprandial period, whole-body protein synthesis rates increased, protein breakdown rates decreased, and oxidation rates increased to a similar extent in both treatments (P -time <0.001 ; P -treatment >0.05 ; P -time \times treatment >0.05). Whole-body net protein balance increased from the basal to the postprandial state in both treatments (P -time <0.001), with a greater increase following the ingestion of AA compared with PRO (15.3 ± 4.1 vs 10.6 ± 2.2 $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, respectively; P -treatment $=0.002$; P -time \times treatment $=0.002$).

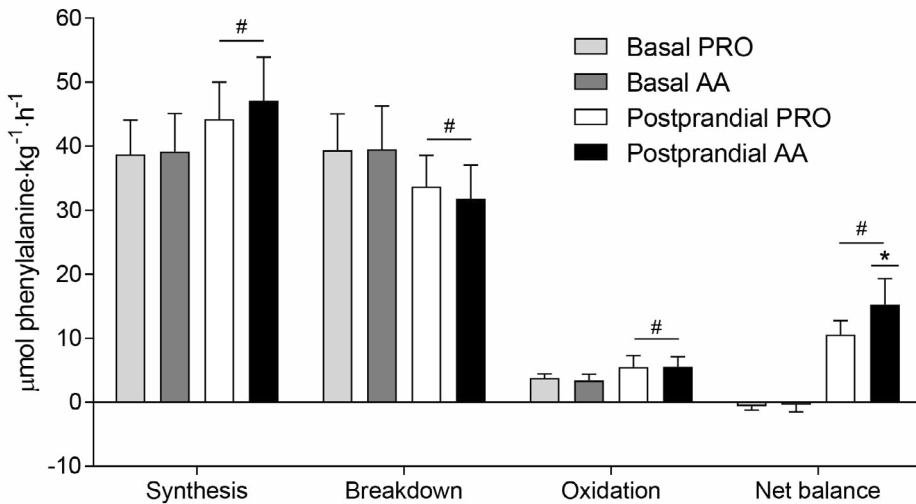


Figure 5.4 Whole-body protein synthesis, breakdown, oxidation, and net protein balance under basal conditions and after ingesting 30 g milk protein (PRO) or an equivalent amount of free amino acids (AA). Values are mean \pm SD, $n=12$. Data were analyzed with repeated measures ANOVA. *Significantly different from PRO ($P=0.002$); #Significantly different from basal ($P<0.001$). AA, free amino acids; PRO, milk protein.

Muscle protein synthesis and muscle protein-bound enrichments

Basal mixed muscle protein fractional synthesis rates (based on L-[ring- $^2\text{H}_5$]-phenylalanine) averaged 0.037 ± 0.015 and 0.039 ± 0.016 $\% \cdot \text{h}^{-1}$ in the AA and PRO treatment, respectively, with no differences between treatments ($P=0.868$; **Figure 5.5**). Following ingestion of free amino acids or intact milk protein, mixed muscle protein synthesis rates increased to 0.063 ± 0.021 vs 0.060 ± 0.026 $\% \cdot \text{h}^{-1}$ (between $t=0-2$ h) and 0.050 ± 0.016 vs 0.047 ± 0.017 $\% \cdot \text{h}^{-1}$ (between $t=2-6$ h) in the AA and PRO treatment, respectively ($P\text{-time}=0.002$), with no differences between treatments ($P\text{-time} \times \text{treatment}=0.827$). Mixed muscle protein synthesis rates assessed over the entire 6 h postprandial period (0.053 ± 0.014 vs 0.051 ± 0.010 $\% \cdot \text{h}^{-1}$ following AA vs PRO ingestion, respectively) were significantly higher than basal mixed muscle protein synthesis rates ($P<0.001$), with no differences between treatments ($P\text{-time} \times \text{treatment}=0.629$). In the present study we included male and female subjects without the intend of making a specific gender comparison. However, for general interest, muscle protein synthesis rates in the male versus the female volunteers are presented separately in **Supplemental Table 5.1**.

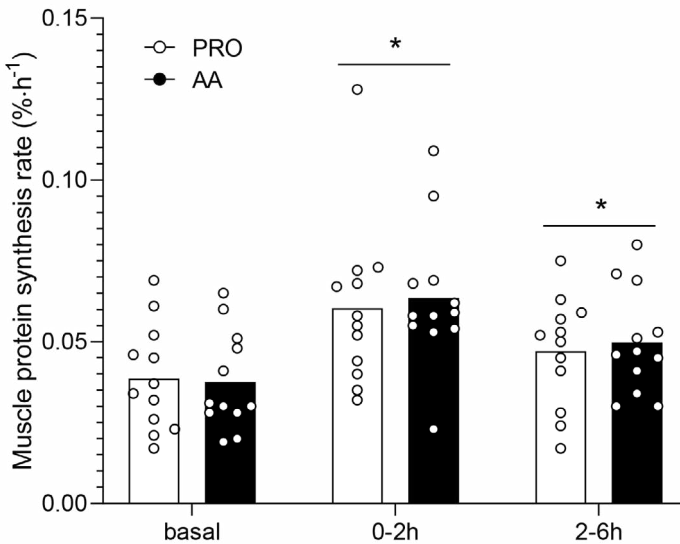


Figure 5.5 Mixed muscle protein fractional synthetic rates calculated based on L-[ring- $^2\text{H}_5$]-phenylalanine tracer during the basal state and over the 6 h postprandial period after ingesting 30 g milk protein (PRO) or an equivalent amount of free amino acids (AA). Bars are means ($n=12$) and dots represent individual values. *Significantly different from basal ($P<0.05$). AA, free amino acids; PRO, milk protein.

Muscle protein-bound L-[1-¹³C]-phenylalanine enrichments significantly increased following free amino acids and protein ingestion to 0.012 ± 0.006 vs 0.008 ± 0.006 MPE (at $t=2$ h) and 0.033 ± 0.011 vs 0.024 ± 0.008 MPE (at $t=6$ h), respectively (P -time <0.001 ; P -time x treatment $=0.060$; **Figure 5.6**). No significant differences in muscle protein-bound L-[1-¹³C]-phenylalanine enrichments were observed between treatments at 2 h ($P=0.105$). However, significantly higher muscle L-[1-¹³C]-phenylalanine enrichments were observed following free amino acids compared with intact milk protein ingestion at 6 h ($P=0.043$).

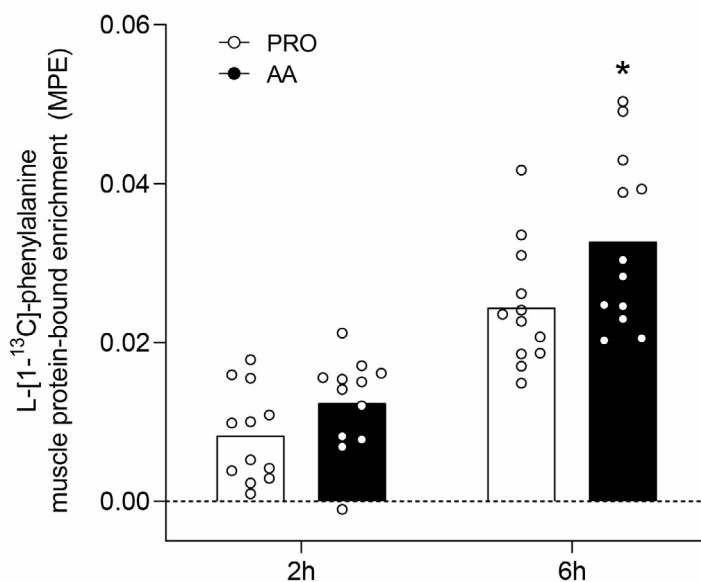


Figure 5.6 L-[1-¹³C]-phenylalanine incorporation into muscle protein after ingesting 30 g milk protein (PRO) or an equivalent amount of free amino acids (AA). Bars are means ($n=12$) and dots represent individual values. *Significantly different from PRO ($P=0.043$). AA, free amino acids; MPE, mole percent excess; PRO, milk protein.

Muscle molecular signaling responses

The phosphorylation status (ratio of phosphorylated to total protein) of key signaling proteins involved in the initiation of muscle protein synthesis are shown in **Supplemental Figure 5.3**. No significant changes were observed over time for muscle mTOR (Ser²⁴⁴⁸) phosphorylation status and no differences were observed between treatments (**Supplemental Figure 5.3A**). A significant time x treatment interaction was observed for muscle p70S6K (Thr³⁸⁹) phosphorylation status ($P=0.007$) (**Supplemental Figure 5.3B**). Muscle p70S6K (Thr³⁸⁹) phosphorylation status significantly increased from 0 to 2 h ($P=0.003$) following AA when compared with PRO ingestion. Muscle rpS6 (Ser^{235/236}) phosphorylation status (**Supplemental Figure 5.3C**) was significantly higher at the 2 h time point compared to 0 and 6 h for both the AA and PRO treatment (P -time $=0.009$), with no differences between treatments (P -treatment $=0.591$; P -time x treatment $=0.495$). No changes and differences between treatments were observed for muscle 4E-BP1 (Thr^{37/46}) phosphorylation status over time (**Supplemental Figure 5.3D**).

Discussion

The present study showed more rapid amino acid absorption following ingestion of free amino acids when compared to the ingestion of an equivalent amount of intact milk protein in young, healthy adults. The higher rate of amino acid absorption resulted in greater postprandial phenylalanine availability with 76% versus 59% of the ingested amino acids being released into the circulation following ingestion of free amino acids compared with intact milk protein. Ingestion of both intact milk protein and the equivalent amount of free amino acids robustly increased mixed muscle protein synthesis rates, with no differences between treatments.

Previous work has shown that more rapid protein digestion and amino acid absorption leads to greater postprandial amino acid release into the circulation, and a greater stimulation of muscle protein synthesis rates [5, 6, 8]. Consequently, it is generally assumed that ingestion of free amino acids as opposed to intact protein will further augment postprandial plasma amino acid availability, resulting in a more pronounced muscle protein synthetic response [5, 8, 27]. However, only few studies compared the postprandial release of amino acids into the circulation following the ingestion of free amino acids versus intact protein and/or its subsequent impact on postprandial muscle protein synthesis rates [27, 28]. No study has ever quantified postprandial release of exogenous amino acids and the subsequent muscle protein synthetic response following the ingestion of intact milk protein compared with an equivalent amount of free amino acids *in vivo* in humans.

In the present study, we observed a greater rise in circulating amino acids following the ingestion of 30 g free amino acids compared with the equivalent amount of intact milk protein (Figures 1-2). The plasma amino acid responses (incremental AUC) assessed over the entire 6 h postprandial period were substantially greater following ingestion of free amino acids when compared with intact milk protein. As amino acid levels did not reach baseline values with the 6 h postprandial period, it is likely that the iAUC could have increased more if the assessment period would have been extended further [14]. Peak plasma leucine concentrations were nearly twofold higher following ingestion of free amino acids when compared with intact milk protein (Figure 1B), despite the fact that both treatments provided exactly the same amount of leucine. The present study extends on previous work by combining continuous intravenous L-[ring- $^2\text{H}_5$]-phenylalanine infusion with the ingestion of intrinsically L-[1- ^{13}C]-phenylalanine labeled milk protein or an equivalent amount of free amino acids enriched with free L-[1- ^{13}C]-phenylalanine, which allows us to quantify protein digestion and amino acid absorption kinetics [14]. Ingestion of free amino acids showed a more rapid rise in plasma L-[1- ^{13}C]-phenylalanine enrichments when compared with intact milk protein (Figure 5.3A). In line, exogenous phenylalanine appearance rates were substantially higher following ingestion of free amino acids when compared with the ingestion of intact milk protein during the first 3 hours of the postprandial period (Supplemental figure 5.2A). Ingestion of amino acids consumed as free crystalline amino acids as opposed to intact milk protein allowed ~17% more of the provided phenylalanine to become available in the circulation, substantially increasing the availability of exogenous amino acids as substrates for peripheral tissues. Of course, the observed differences in amino acid absorption kinetics following protein vs free amino acid ingestion may be modulated when protein or free amino acids are ingested within the matrix of a product or meal containing other (macro)nutrients and fibres [25, 29, 30].

On a whole-body level, the ingestion of free amino acids or intact milk protein increased protein synthesis rates and lowered protein breakdown rates, resulting in a net positive protein balance (Figure 5.4). The greater exogenous amino acid release into the circulation following free amino acid versus intact milk protein ingestion resulted in a greater positive net protein balance (Figure 5.4), indicating greater postprandial tissue protein accretion. However, whole-body protein kinetics do not necessarily reflect skeletal muscle per se [24, 31, 32]. As we also collected several skeletal muscle biopsies we were able to assess the impact of the greater postprandial amino acid release following free amino acid versus intact milk protein ingestion on muscle protein synthesis rates and the direct incorporation rate of dietary (protein-)derived phenylalanine into muscle protein [16, 33]. In short, despite the greater release of amino acids and higher plasma leucine concentrations, no differences were observed in postprandial muscle protein synthesis between treatments.

It has been well established that the ingestion of 20 g protein is sufficient to maximize postprandial muscle protein synthesis rates in young, active individuals [34, 35]. Therefore, it could be speculated that the 30 g bolus of protein or free amino acids provided in the present study prevented us from detecting differences in postprandial muscle protein synthesis rates between treatments. The difference in anabolic response between treatments was lacking despite a much greater postprandial plasma amino acid release (Figure 5.3) and concomitant greater p70S6 phosphorylation (Supplemental figure 5.3) observed following free amino acid versus intact milk protein ingestion. The proposed benefits of greater postprandial plasma amino acid release on stimulating muscle protein synthesis may become more evident in conditions where less than 20 g protein is ingested or in those populations that suffer from anabolic resistance. In the presence of anabolic resistance, ingestion of more protein is required to maximize postprandial muscle protein synthesis rates [36, 37]. Ingestion of free amino acids as opposed to intact milk protein could provide some benefits in this setting as more of the ingested amino acids will become available to the muscle, thereby compensating, at least partly, for some of the anabolic resistance. This is supported by the observation that more of the ingested L-[1-¹³C]-phenylalanine was released into the circulation and used as precursor for de novo muscle protein accretion when ingested as free, crystalline phenylalanine as opposed to protein-bound phenylalanine (Figure 5.6). This will be even more relevant in conditions where anabolic resistance is attributed to impairments in protein digestion and/or amino acid absorption, such as in intensive care unit patients and patients suffering from gastrointestinal diseases [38, 39]. Follow-up studies should focus on the proposed benefits of free amino acid provision in situations where protein intake is restricted and/or in older and more clinically compromised patient populations. In conclusion, ingestion of a single bolus of free amino acids is followed by more rapid amino acid absorption, greater postprandial plasma amino acid release into the circulation, and greater dietary phenylalanine incorporation into mixed muscle protein when compared with the ingestion of an equivalent amount of intact milk protein. The postprandial increase in muscle protein synthesis rate does not differ following the ingestion of 30 g intact milk protein or the equivalent amount of free amino acids *in vivo* in healthy, young adults.

Acknowledgements

We greatly appreciate the medical assistance of Lisanne Houben in the execution of the experiments. Technical expertise from Antoine Zorenc, Joan Senden and Annemie Gijzen during the sample analyses was greatly appreciated.

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Supplemental material

Supplemental methods

Plasma analysis

Plasma insulin concentrations were determined with radioimmunoassay (RIA) kits (Human Insulin specific RIA, Millipore Corporation, MA, USA). Plasma amino acid concentrations were measured using ultra-performance liquid chromatograph-mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). Fifty μL plasma was deproteinized using 100 μL of 10% SSA with 50 μM of MSK-A2 internal standard (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 μL of ultra-pure demineralized water was added and samples were centrifuged. After centrifugation, 10 μL of supernatant was added to 70 μL of Borate reaction buffer (Waters, Saint-Quentin, France). In addition, 20 μL of AccQ-Tag derivatizing reagent solution (Waters, Saint-Quentin, France) was added after which the solution was heated for 10 min at 55°C. Of this 100 μL derivative 1 μL was injected and measured using UPLC-MS.

Plasma L-[ring- $^2\text{H}_5$]-phenylalanine and L-[ring-3,5- $^2\text{H}_2$]-tyrosine enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Little Falls, USA). Specifically, internal standards of [U- $^{13}\text{C}_9$ ^{15}N]-phenylalanine and [U- $^{13}\text{C}_9$ ^{15}N]-tyrosine were added to the plasma samples. Plasma samples were deproteinized on ice with 45% 5-sulfosalicylic acid solution. Free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin, 100–200 μm dry mesh size; Bio-Rad Laboratories, Hercules, USA). The purified amino acids were converted into tert-butyldimethylsilyl (TBDMS) derivatives with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) before analysis by GC-MS. The amino acid concentrations were determined using electron impact ionization by monitoring ions at mass/charge (m/z) 336 and 346 for unlabeled and [U- $^{13}\text{C}_9$ ^{15}N] labeled-phenylalanine respectively, and 466 and 476 for unlabeled and [U- $^{13}\text{C}_9$ ^{15}N]-tyrosine, respectively. Plasma phenylalanine and tyrosine ^{13}C or ^2H enrichments were determined by using electron ionization and selective ion monitoring at mass/charge (m/z) 336, 337, and 341 for unlabeled and labeled ($1\text{-}^{13}\text{C}$ and ring- $^2\text{H}_5$) phenylalanine, respectively, and m/z 466, 467, 468, and 470 for unlabeled and labeled ($1\text{-}^{13}\text{C}$, ring- $^2\text{H}_2$, and ring- $^2\text{H}_4$) tyrosine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Muscle analysis

For the measurement of mixed muscle protein-bound and muscle free L-[ring- $^2\text{H}_5$]-phenylalanine and L-[$1\text{-}^{13}\text{C}$]-phenylalanine enrichments, ~50 mg of wet muscle tissue was freeze-dried. Collagen, blood, and other non-muscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (8 mg) was weighed, and 35 volumes (7 times dry weight of isolated muscle fibers wet:dry ratio) of ice-cold 2% perchloric acid were added. The tissue was then sonicated and centrifuged. After centrifugation, the supernatant was collected for determination of L-[ring- $^2\text{H}_5$]-phenylalanine and L-[$1\text{-}^{13}\text{C}$]-phenylalanine enrichments in the muscle-free amino acid pool and processed in the same manner as the plasma samples for isotopic enrichment using GC-MS analysis. The protein pellet was washed with 3 additional 1.5 mL washes of 2% PCA, hydrolysed with 6M HCl at 120°C for 15-18 h,

and then dried under a nitrogen steam while heated to 120°C. Next, 50% acetic acid solution was added, and the hydrolyzed protein was passed over a cation exchange resin (Dowex AG 50W-X8, 100–200 mesh hydrogen form; Bio-Rad, Hercules, CA) using 2M NH₄OH. The eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters. For measurement of L-[1-¹³C]-phenylalanine, derivatized samples were measured using a GC-C-IRMS (MAT 253; Thermo Fisher Scientific, Bremen, Germany) equipped with DB5MS 30m column (No. 122–4762; Agilent) and GC-Isolink monitoring of ion masses 44, 45. To determine the L-[ring²H₅]-phenylalanine enrichment of mixed muscle protein, the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters [23]. The derivatized samples were measured using a gas chromatography-pyrolysis-isotope ratio mass spectrometer (GC-P-IRMS) (MAT 253; Thermo Fisher Scientific, Bremen, Germany) equipped with a DB17MS 60m column with 5 m pre-column (No. 122–4762; Agilent) and GC-Isolink, monitoring of ion masses ¹H and ²H. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Western blotting

Western blot analysis was performed on muscle tissue (at *t*=0, 2, and 6 h). A portion of each muscle sample frozen for biochemical analyses was homogenized in seven volumes Tris buffer (20 mm Tris-HCL, 5 mm EDTA, 10 mm Na pyrophosphate, 100 mm NaF, 2 mm Na₃VO₄, 1% Nonident P-40; pH 7.4) supplemented with protease and phosphatase inhibitors: aprotinin 10 µg/mL, leupeptin 10 µg/mL, benzamidin 3 mm and phenylmethylsulphonyl fluoride 1 mm. After homogenization, each muscle extract was centrifuged for 10 min at 10 000 g (4 °C) and sample buffer was added to the supernatant to final concentrations of 60 mm Tris, 10% glycerol, 20 mg/mL SDS, 0.1 mm dithiothreitol, and 20 µg/mL bromophenol blue. The supernatant was then heated for 5 min at 100°C and immediately placed on ice. Immediately before analyses, the muscle extraction sample was warmed to 50°C and centrifuged for 1 min at 1000g at room temperature. The total amount of sample loaded on the gel was based on protein content. After a Bradford assay, 30 µg protein were loaded in each lane. With the exception of mTOR, protein samples were run on a Criterion Precast TGX 4–20% gel (Order No. 567–1094; Bio-Rad) ± 90 min at 150 V (constant voltage) and transferred onto a Trans-blot Turbo 0.2 µm nitrocellulose membrane (Order No. 170–4159; Bio-Rad) in 7 min at 2.5 A and 25 V. The mTOR proteins were run and blotted for 10 min at 2.5 A and 25 V but on a Criterion Precast XT 3–8% Tris-acetate gel (Order No. 345-0130; Bio-Rad). Specific proteins were detected by overnight incubation at 4°C on a shaker with specific antibodies in 50% PBS Odyssey blocking buffer (Part No. 927-40 000; Li-Cor Biosciences, Lincoln, NE, USA) after blocking for 60 min at RT in 50% in PBS Odyssey blocking buffer. Polyclonal primary phospho-specific antibodies, anti-phospho-mTOR (Ser²⁴⁴⁸), anti-phospho-S6K1 (Thr³⁸⁹), anti-phospho-rpS6 (Ser²³⁵/Ser²³⁶) and anti-phospho-4E-BP1 (Thr^{37/46}) were purchased from Cell Signaling Technology (Danvers, MA, USA). In addition, anti-mTOR, anti-S6K1, anti-RS6 and anti-4E-BP1 were also purchased from Cell Signaling Technology. Following incubation, membranes were washed three times 10 min in 0.1% PBS-Tween 20 and once for 10 min in PBS. Next, samples were incubated on a shaker (1 h at RT) with infrared secondary antibodies, donkey anti-rabbit IRDYE 800 (dilution 1:10 000; Cat. No. 611-732-127; Rockland Immunochemicals, Pottstown, PA, USA) and donkey anti-mouse IRDYE 800CW (dilution 1:10 000; Cat. No. 626–32 212; Li-Cor Biosciences) dissolved in 50% PBS Odyssey blocking buffer. After a final wash step (3 × 10 min) in 0.1% Tween

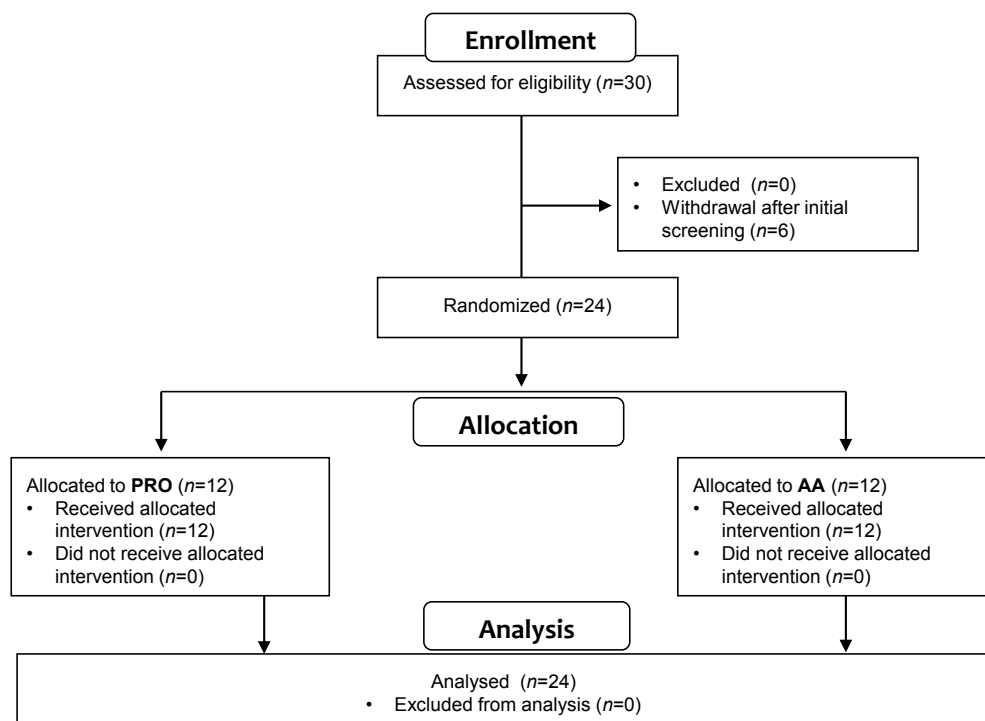
20-PBS and once 10 min in PBS, protein quantification was performed by scanning on an Odyssey Infrared Imaging System (Li-Cor Biosciences). Ponceau S staining was used to standardize for the amount of protein loaded. Phosphorylation status as a proxy of activation of the signaling proteins was expressed relative to the total amount of each protein.

Supplemental results

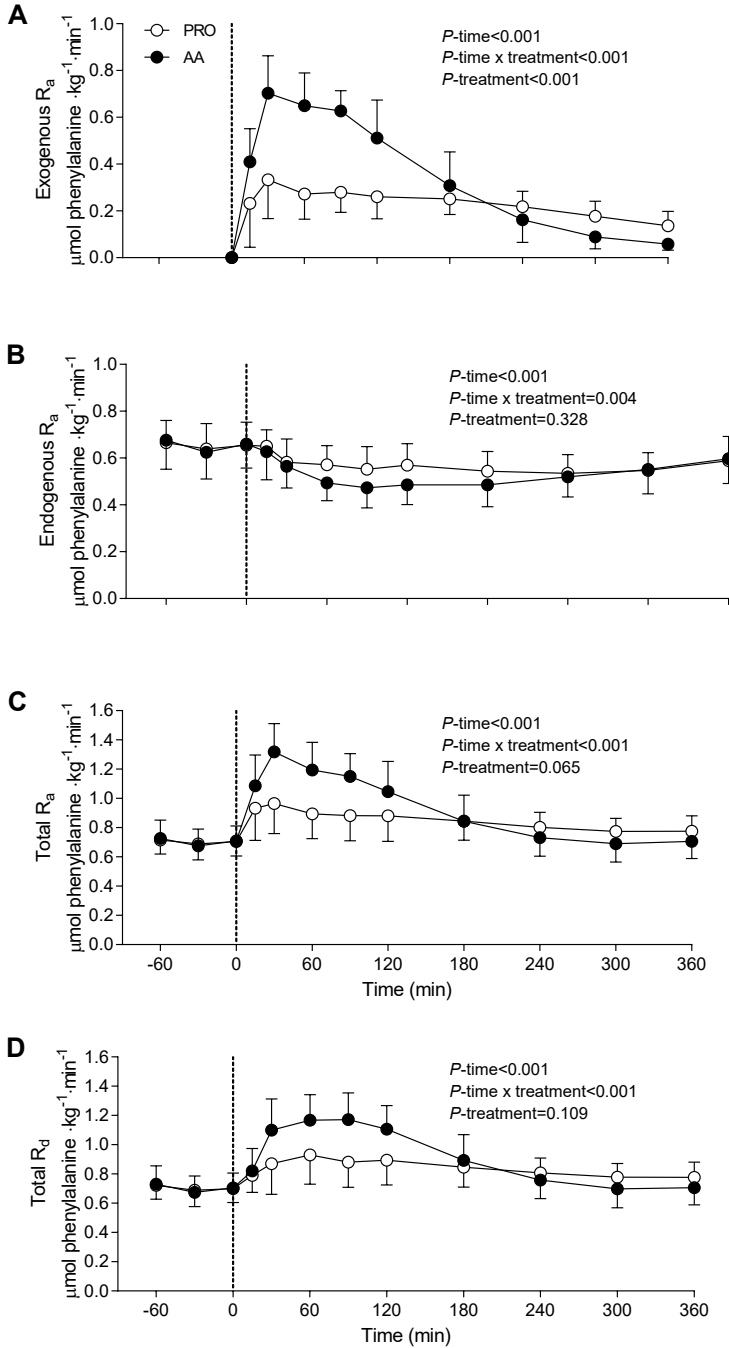
Supplemental table 5.1 MPS data for males and females.

	Males		Females	
	PRO (n=6)	AA (n=6)	PRO (n=6)	AA (n=6)
Basal (%·h⁻¹)	0.030±0.002		0.046±0.005	
0-2h (%·h⁻¹)	0.049±0.012	0.060±0.005	0.072±0.031	0.067±0.031
2-6h (%·h⁻¹)	0.048±0.020	0.043±0.009	0.046±0.015	0.056±0.020

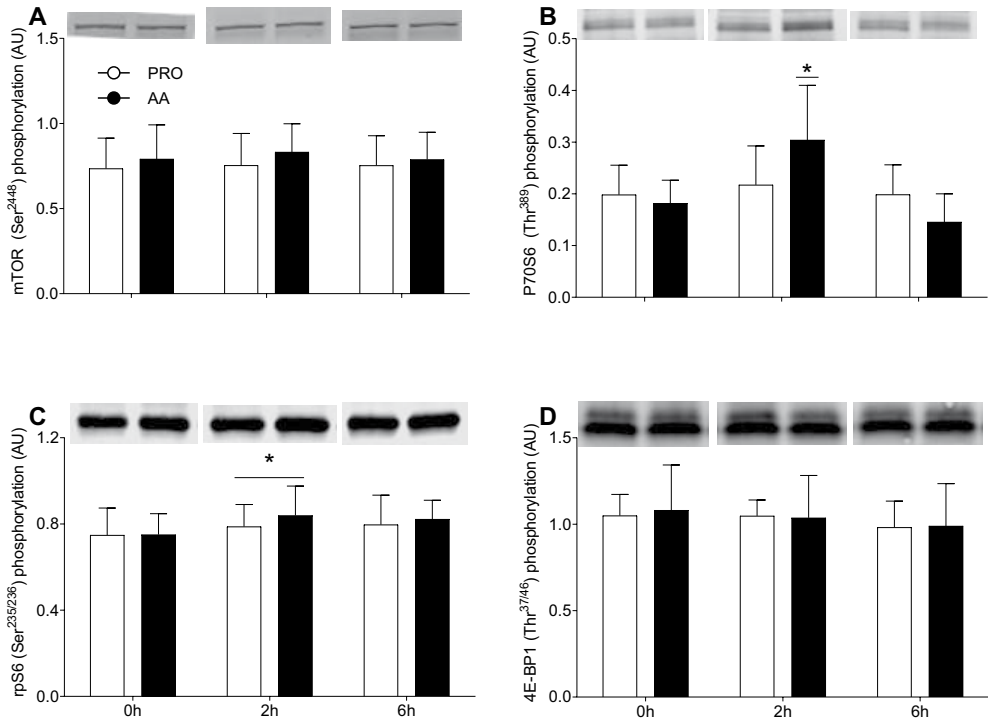
AA, free amino acids; PRO, milk protein.



Supplemental figure 5.1 Participant flow chart.



Supplemental figure 5.2 Exogenous rate of appearance (R_a ; **A**), endogenous R_a (**B**), Total R_a (**C**), and Total rate of disappearance (R_d ; **D**) after ingesting 30 g milk protein (PRO; $n=12$) or an equivalent amount of free amino acids (AA; $n=12$). The dashed line refers to drink ingestion. Data were analyzed with repeated measures ANOVA. AA, free amino acids; PRO, milk protein.



Supplemental figure 5.3 Skeletal muscle phosphorylation status (ratio of phosphorylated to total protein) of mTOR (Ser²⁴⁴⁸) (A), p70S6K (Thr³⁸⁹) (B), rpS6 (Ser^{235/236}) (C) and 4E-BP1 (Thr^{37/46}) (D) in the basal state, (t=0 h) and at 2 and 6 h after ingesting 30 g milk protein (PRO; n=12) or an equivalent amount of free amino acids (AA; n=12). *Significantly different from t=0 h (P<0.05). AA, free amino acids; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein¹; mTOR, mammalian target of rapamycin; p70S6K, p70S6 protein kinase 1; PRO, milk protein; rpS6, ribosomal protein S6.



Chapter 6

Administration of free amino acids improves exogenous amino acid availability when compared with intact protein in critically ill patients

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Ready for submission

Abstract

Background

Protein digestion and amino acid absorption appear compromised in critical illness. Provision of enteral feeds with free amino acids rather than intact protein may provide a strategy to improve postprandial amino acid availability.

Objective

To quantify systemic amino acid availability of diet-derived amino acids following enteral administration of intact protein versus an equivalent amount of free amino acids *in vivo* in critically ill patients.

Methods

Sixteen mechanically ventilated ICU patients at risk for malabsorption received a primed continuous infusion of L-[ring- $^2\text{H}_5$]-phenylalanine and L-[ring-3,5- $^2\text{H}_2$]-tyrosine after an overnight fast. Patients were randomly allocated to receive 20 g intrinsically L-[1- ^{13}C]-phenylalanine labelled milk protein or an equivalent amount of amino acids labelled with free L-[1- ^{13}C]-phenylalanine via a nasogastric tube over a 2-hour period. Protein digestion and amino acid absorption kinetics and whole-body protein net balance were assessed throughout a 6 h period.

Results

Following enteral nutrient infusion, both plasma phenylalanine and leucine concentrations increased (P -time <0.001), with a more rapid and greater rise following free amino acid compared with intact protein administration (P -time \times treatment $=0.003$). Diet-derived phenylalanine released into the circulation was 25% greater after free amino acids vs intact protein administration (68.7% [CI 62.3-75.1%] vs 43.8% [CI 32.4-55.2%], respectively; $P<0.001$). Whole-body protein net balance became positive following nutrient administration (P -time <0.001) and tended to be more positive following free amino acid in provision (P -time \times treatment $=0.07$).

Conclusions

Administration of free amino acids as opposed to intact protein increases post-prandial plasma amino acid availability in critically ill patients, allowing more diet-derived amino acids to become available to peripheral tissues.

Introduction

Nutritional support is part of routine care for mechanically ventilated patients admitted to the intensive care unit [1]. It has been postulated that adequate provision of nutrients, in particular protein, can help attenuate skeletal muscle wasting and improve long-term clinical outcomes [2-4]. Multiple studies have demonstrated that the uptake of enterally provided nutrients is impaired during critical illness [5-8]. It has been suggested that protein digestion and amino acid absorption are particularly compromised, based on an attenuated postprandial rise in circulating essential amino acid concentrations following protein administration in ICU patients [9, 10].

During critical illness both protein digestion and amino acid absorption may be compromised [11]. Exocrine pancreatic insufficiency and impaired intestinal uptake of nutrients are prevalent among ICU patients and may contribute to incomplete digestion and subsequent intestinal malabsorption of dietary protein [12, 13]. Especially in patients with diarrhea, daily fecal nutrient losses can amount to substantial daily protein-energy losses [14]. As intestinal function is not routinely measured, protein-energy malabsorption often occurs occult and is generally not accounted for in dosing or in the composition of enteral nutrition [15]. Although evidence suggests that uptake of dietary protein derived amino acids is impaired in critically ill patients, actual protein (mal) digestion and amino acid (mal)absorption has never been quantified in these patients. Using specifically produced intrinsically labelled protein, we can directly quantify protein digestion and amino acid absorption rates *in vivo* in human subjects [16, 17]. We hypothesize that protein digestion and amino acid absorption kinetics are strongly reduced in critically ill patients. If the ability of critically ill patients to digest dietary protein is indeed severely hampered, provision of free amino acids may be required to overcome maldigestion. We hypothesize that provision of free amino acids as opposed to intact protein improves diet-derived amino acid digestion and absorption and increases postprandial systemic amino acid availability in mechanically ventilated patients at risk of malabsorption.

To test our hypotheses, mechanically ventilated patients at risk for malabsorption were selected to receive continuous infusions with L-[ring- $^2\text{H}_5$]-phenylalanine while receiving either 20 g intrinsically L-[1- ^{13}C]-phenylalanine labelled milk protein concentrate or an equivalent amount of amino acids labelled with free L-[1- ^{13}C]-phenylalanine added. Combining the ingestion of free or (milk) protein bound L-[1- ^{13}C]-phenylalanine with the continuous intravenous infusion of L-[ring- $^2\text{H}_5$]-phenylalanine and L-[ring-3,5- $^2\text{H}_2$]-tyrosine allows us to assess protein digestion and amino acid absorption kinetics, quantitate the amount of diet-derived amino acids being released in the circulation, and assess its impact on whole-body protein turnover [18].

Material and methods

Subjects

A total of 16 adult (>18 year of age), critically ill patients admitted to our intensive care unit were randomly allocated to receive either 20 g intrinsically labeled milk protein or the equivalent amount of free amino acids. The study was conducted between March 2018 and November 2019 at a tertiary intensive care unit (Maastricht University Medical Centre+, the Netherlands). Patients were eligible to participate if they were mechanically ventilated, able to tolerate full enteral nutrition and had diarrhea defined as more than 350 g fecal weight per day. Exclusion criteria were any proven (pre-existing) bowel or pancreatic disease, contra-indications to enteral nutrition, any form of extracorporeal treatment on the study day (i.e. dialysis or extra-corporeal membrane oxygenation), renal or hepatic insufficiency and bodyweight below 50 kg or above 100 kg.

Written informed consent was obtained from the legal representative (i.e. next of kin) before participation in the study. If subjects regained capacity for consent, subjects were informed about participation and asked for written informed consent to use the data and materials collected. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre+ and conformed to standards for the use of human subjects in research as outlined in the most recent version of the Helsinki Declaration. The study was registered at clinicaltrials.gov (identifier NCT04791774).

Stable-isotope tracers

Stable-isotope labeled phenylalanine, leucine, and tyrosine tracers (L-[ring-²H₅]-phenylalanine, L-[1-¹³C]-phenylalanine, L-[1-¹³C]-leucine and L-[ring-²H₂]-tyrosine) were obtained from Cambridge Isotope Laboratories Inc. and tested for chemical and isotopic purity. For intravenous infusion, sterile solutions of L-[ring-²H₅]-phenylalanine, L-[1-¹³C]-leucine and L-[ring-²H₂]-tyrosine were prepared by A15 pharmacy and tested for sterility and non-pyrogenicity prior to use.

Intact milk protein and free amino acid mixture

For the intact protein group, intrinsically L-[1-¹³C]-phenylalanine labeled milk protein concentrate (80% protein content) was obtained by infusing a lactating cow with L-[1-¹³C]-phenylalanine, as described elsewhere [19-22]. Isotopic enrichment of L-[1-¹³C]-phenylalanine in the milk protein concentrate used was 38.3 molar percent excess (MPE). A custom amino acid mixture (Ajinomoto, Raleigh, USA) was produced with an amino acid composition identical to the milk protein (**Supplemental Table S1**) and enriched with free L-[1-¹³C]-phenylalanine (Cambridge Isotope Laboratories, Andover, MA, USA) to match the L-[1-¹³C]-phenylalanine enrichment level of the intact protein (38.3 MPE). The intact protein and free amino acid mixture met all chemical and bacteriological specifications for human consumption. The 20 g intrinsically L-[1-¹³C]-phenylalanine labeled milk protein as well as the equivalent amount of free amino acids were both dissolved in 250 mL sterile water and infused via a naso-gastric feeding tube over a 2-hour period. Randomization was performed using a computerized random-number generator assigned by an independent person.

Experimental protocol

On the day of study participation, ongoing enteral nutrition was ceased 6 hours prior to the start of the study protocol. Both intravenous tracer infusion and arterial blood sampling were performed using venous and arterial catheters already in place for routine care respectively. After collection of baseline blood samples ($t=-90$ min relative to intervention), plasma phenylalanine and tyrosine pools were primed with a single intravenous bolus ($2 \mu\text{mol/kg}$ L-[ring- $^2\text{H}_5$]-phenylalanine and $0.61 \mu\text{mol/kg}$ L-[ring-3,5- $^2\text{H}_2$]-tyrosine), followed by a continuous intravenous isotope infusion for the duration of the experimental protocol ($0.05 \mu\text{mol/kg/min}$ L-[ring- $^2\text{H}_5$]-phenylalanine and $0.015 \mu\text{mol/kg/min}$ L-[ring-3,5- $^2\text{H}_2$]-tyrosine).

After 90 min of isotope infusion, 20 g intrinsically labeled protein or an equivalent dose of labeled free amino acids was infused via a naso-gastric feeding tube over a 2 h period ($t=0$ until $t=120$ min). Additional arterial blood samples were collected during infusion at $t=-30, 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300,$ and 360 min. Blood samples (3.5 mL) were collected in EDTA-containing tubes and centrifuged (1200g, 10 min at 4°C). Aliquots of plasma were immediately stored at -80°C for future analysis. Fecal samples were collected on the study day and the day after via a fecal collection system (Flexi SealTM) already in place for routine care. Fecal samples were weighed, homogenized, and aliquots were stored at -80°C for later analysis.

Sample analysis

Plasma L-[ring- $^2\text{H}_5$]-phenylalanine and L-[ring-3,5- $^2\text{H}_2$]-tyrosine enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Little Falls, USA). Specifically, internal standards of [U- $^{13}\text{C}_9$ ^{15}N]-phenylalanine and [U- $^{13}\text{C}_9$ ^{15}N]-tyrosine were added to the plasma samples. Plasma samples were deproteinized on ice with 45% 5-sulfosalicylic acid solution. Free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin, 100–200 μm dry mesh size; Bio-Rad Laboratories, Hercules, USA). The free amino acids were converted to their N-tert-butyl dimethylsilyl (MTBSTFA) derivative before analysis by GC-MS. The amino acid concentrations were determined using electron impact ionization by monitoring ions at mass/charge (m/z) 336 and 346 for unlabeled and [U- $^{13}\text{C}_9$ ^{15}N] labeled-phenylalanine respectively, and 466 and 476 for unlabeled and [U- $^{13}\text{C}_9$ ^{15}N]-tyrosine, respectively. Plasma phenylalanine and tyrosine ^{13}C or ^2H enrichments were determined by using electron ionization and selective ion monitoring at mass/charge (m/z) 336, 337, and 341 for unlabeled and labeled ($1\text{-}^{13}\text{C}$ and ring- $^2\text{H}_5$) phenylalanine, respectively, and m/z 466, 467, 468, and 470 for unlabeled and labeled ($1\text{-}^{13}\text{C}$, ring- $^2\text{H}_2$, and ring- $^2\text{H}_4$) tyrosine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis.

Fecal analysis

Fecal elastase concentrations in the fecal homogenates was determined through an enzyme-linked immunosorbent assay (ELISA) and analyzed on a DYNEX analyzer (DYNEX TECHNOLOGIES, Inc. Chantilly, USA). Fecal elastase concentrations < 0.100 mg/g were considered to represent severe exocrine pancreatic insufficiency [12]. Nitrogen content was determined in the fecal homogenates according to the Dumas method using a Variomax CN-analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany). For amino acid analysis, 200 mg of fecal samples were weighed, aliquoted and dissolved in hydrochloric acid (6 M) and stored overnight

at 110 °C. Hydrolyzed, dried samples were dissolved in 500 µL of 0.1 M hydrochloric acid, vortexed and centrifuged for 5 min at 10,000 g. The supernatant was collected and used for amino acid analysis. Total amino acid concentrations and [1-¹³C]-phenylalanine enrichments in the fecal supernatant were determined in the same manner as the plasma samples as described above.

Calculations

Enteral provision of L-[1-¹³C]-phenylalanine combined with intravenous infusion of L-[ring-²H₅]-phenylalanine and L-[ring-²H₂]-tyrosine and arterial blood sampling allowed us to assess amino acid kinetics in non-steady state conditions. Total, endogenous and exogenous phenylalanine rate of appearance as well as systemic plasma availability of diet-derived amino acids (i.e. the fraction of diet-derived phenylalanine that appeared in the circulation) were calculated using modified Steele's equations [28-30]:

$$(1) \text{ Total } R_a = \frac{F_{iv} - \left[pV \times C(t) \times \frac{dE_{iv}}{dt} \right]}{E_{iv}(t)}$$

Total phenylalanine rate of appearance (Total R_a) was calculated using the first equation, where F_{iv} is the intravenous L-[ring-²H₅]-phenylalanine tracer infusion rate (µmol/kg/min), pV (0.125 L/kg) is the distribution volume for phenylalanine [28], $C(t)$ is the mean plasma phenylalanine concentration between 2 consecutive time points, dE_{iv}/dt is the time-dependent change in plasma L-[ring-²H₅]-phenylalanine enrichment and $E_{iv}(t)$ is the mean plasma L-[ring-²H₅]-phenylalanine enrichment between 2 consecutive time points.

$$(2) \text{ Exogenous } R_a = \frac{\text{Total } R_a \times E_{po}(t) + \left[pV \times C(t) \times \frac{dE_{po}}{dt} \right]}{E_{protein}}$$

$$(3) \text{ Endogenous } R_a = \text{Protein breakdown} = \text{Total } R_a - \text{Exo } R_a - F_{iv}$$

Rate of appearance (Exo R_a) represents the rate at which diet-derived phenylalanine enters the circulation, where $E_{po}(t)$ is the mean plasma of L-[1-¹³C]-phenylalanine enrichment between 2 consecutive time points, dE_{po}/dt is the time-dependent change in plasma L-[1-¹³C]-phenylalanine enrichment, and $E_{protein}$ is the L-[1-¹³C]-phenylalanine enrichment of the dietary protein (38.3 MPE). Endogenous phenylalanine rate of appearance (Endo R_a) represents the plasma appearance of phenylalanine derived from whole-body protein breakdown.

$$(4) \text{ Plasma availability} = \frac{\text{AUC}_{\text{Exogenous } R_a}}{\text{Phe}_{\text{protein}}} \times \text{BM} \times 100$$

The fraction of diet-derived phenylalanine appearing in the systemic circulation (plasma availability) can be calculated using equation 4, where $AUC_{\text{Exogenous Ra}}$ represents the AUC of exogenous R_a , $\text{Phe}_{\text{protein}}$ is the amount of dietary phenylalanine ingested (μmol), and BM is the participant's body mass. Total phenylalanine rate of disappearance (R_d in $\mu\text{mol Phe/kg/min}$) equals the rate of phenylalanine hydroxylation (first step in the conversion of phenylalanine to tyrosine) to the rate of phenylalanine utilization for protein synthesis. These parameters are calculated as follows:

$$(5) \text{ Total } R_d = \text{Total } R_a - pV \times \frac{dC}{dt}$$

$$(6) \text{ Phe hydroxylation} = \text{Tyr } R_a \times \frac{E_{\text{tyr}}(t)}{E_{\text{phe}}(t)} \times \frac{\text{Phe}R_d}{F_{\text{phe}} + \text{Phe}R_d}$$

$$(7) \text{ Protein synthesis} = \text{Total } R_d - \text{Phe hydroxylation}$$

$$(8) \text{ Phe net balance} = \text{Protein synthesis} - \text{Endo } R_d$$

Where $\text{Tyr}R_a$ is the total rate of tyrosine appearance based on the L-[ring-3,5- $^2\text{H}_2$]-tyrosine infusion and plasma enrichment. $E_{\text{tyr}}(t)$ and $E_{\text{phe}}(t)$ represent the mean plasma L-[ring- $^2\text{H}_4$]-tyrosine and L-[ring- $^2\text{H}_5$]-phenylalanine enrichment between 2 consecutive time points, respectively. F_{phe} is the intravenous infusion rate of L-[ring- $^2\text{H}_5$]-phenylalanine ($\mu\text{mol/kg/min}$).

Statistical analysis

Statistical analysis and visualization were performed using R v3.4.4. Data are expressed as median [1st, 3rd quartile] (tables and text) or means \pm SE (graphs) for visual purposes unless indicated otherwise. Baseline characteristics and non-time dependent variables between groups were compared using Mann-Whitney U test or Fisher's exact test as appropriate. To analyze serial measurements over time (plasma amino acids concentrations, enrichments and fluxes) we used linear mixed-effects models with a random intercept, using time, group and their interaction as fixed effects, and subjects as random effects, to compute differences over time and between groups [26]. The analysis was carried out for the period between $t=0$ (start of nutrition) and $t=360$ min. ANOVA tables were generated from the LME models to provide summary estimates for the fixed effects using the lmerTest package in R [27]. Based upon previous studies in both healthy and ICU patients [9, 16], a sample size of 8 subjects per group, including accounting for a 10% dropout rate, was calculated to be able to detect relevant differences in systemic availability between groups with statistical significance (2-sided test, $P < 0.05$, 80% power).

Results

Subjects

A total of 16 mechanically ventilated patients admitted to the intensive care unit were randomized between either the intact protein or free amino acid administration (**Figure 6.1**). Two patients had to be excluded due to loss of the arterial line during enteral infusion ($n=1$ protein group) or due to an error in priming the phenylalanine pool ($n=1$ free amino acid group).

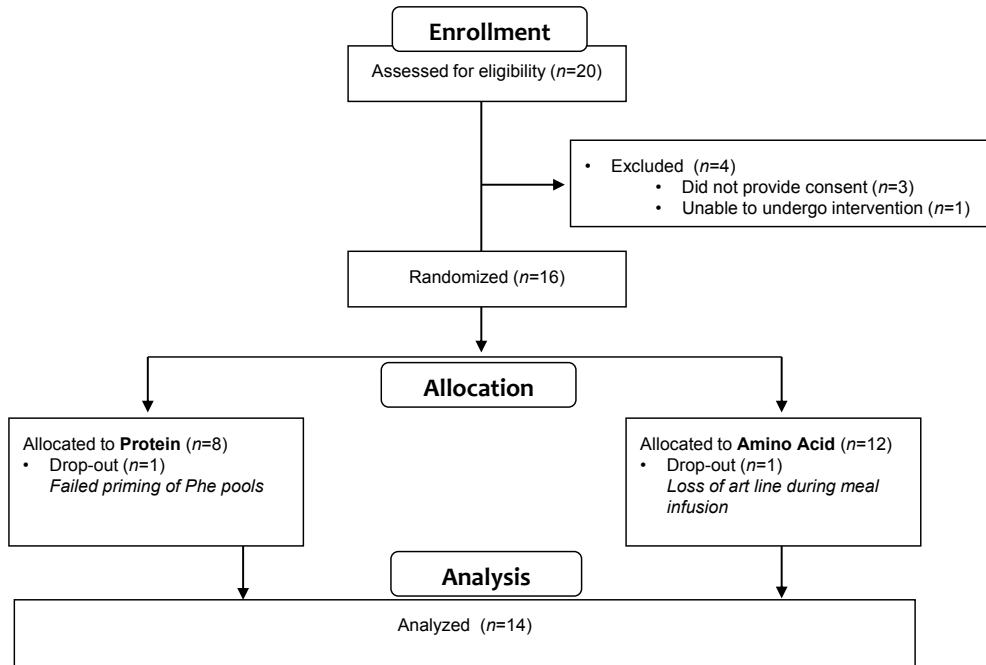


Figure 6.1 patient flowchart.

Baseline characteristics of the 14 patients included in the final analysis are presented in **Table 6.1**. There were 9 males included and the median [1st - 3rd quartile] age of the participants was 60 [47 - 71] y. There were no significant differences in admission characteristics or disease severity scores between the intervention groups, with a median APACHE II score of 16 [11 - 19] on admission. The study test day occurred on a median of day 15 [10 - 22] of ICU admission. Total ventilator days and hospital length of stay were 32 [18 - 47] and 66 [60 - 87] days respectively. At the test day, median fecal volume was 475 [365 - 600] mL/day, with no significant differences in fecal volume or weight between intervention groups.

Table 6.1 Baseline patients' characteristics.

	Overall (n=14)	Nutrition group		P
		Protein (n=7)	Amino acid (n=7)	
Age (y)	60 [47, 71]	55 [45, 60]	71 [54, 73]	0.10
Sex (male)	9 (64)	4 (57)	5 (71)	1.00
Weight (kg)	86 [75, 90]	85 [78, 90]	87 [75, 92]	1.00
BMI (kg/m ²)	26.6 [24.8, 28.9]	26.3 [25.7, 27.7]	26.9 [24.4, 29.3]	0.95
Origin of admission				0.43
ER	8 (57)	5 (71)	3 (42)	
Other hospital	5 (35)	2 (28)	3 (42)	
Ward	1 (7)	0 (0)	1 (14)	
APACHE II score	16 [11, 19]	18 [12, 20]	14 [12, 18]	0.65
SAPS II score	27 [21, 33]	28 [19, 30]	26 [23, 37]	0.41
Admission labs				
C-reactive protein (mg/L)	15.0 [7.0, 232.0]	20.5 [10.5, 180.5]	12.0 [6.0, 209.5]	0.62
Leucocyte count (10E ⁹ /L)	18.2 [15.0, 20.2]	19.7 [15.7, 23.2]	18.1 [15.9, 19.0]	0.52
Hemoglobin (mmol/L)	7.6 [6.7, 8.1]	7.9 [7.5, 9.1]	6.6 [6.2, 7.8]	0.14
Urea (mmol/L)	6.8 [4.5, 8.8]	5.3 [4.2, 7.4]	7.0 [6.8, 10.6]	0.09
Creatinine (umol/L)	85.0 [69.8, 113.5]	75.0 [62.5, 103.0]	94.0 [85.0, 113.0]	0.25
Albumin (g/L)	21.9 [16.8, 30.6]	27.6 [21.4, 31.8]	19.6 [16.8, 25.8]	0.52
Bilirubine (umol/L)	8.4 [6.3, 9.5]	7.1 [4.4, 8.5]	9.5 [7.3, 17.6]	0.09
Study day labs				
Day of ICU admission on test day	15.0 [10.0, 21.8]	15.0 [11.0, 26.0]	15.0 [8.0, 21.5]	0.56
C-reactive protein (mg/L)	80.5 [45.2, 150.2]	109.0 [53.5, 151.0]	59.0 [49.5, 118.5]	0.75
Leucocyte count (10E ⁹ /L)	12.0 [10.3, 14.0]	13.2 [12.6, 15.2]	10.4 [9.8, 11.6]	0.05
Hemoglobin (mmol/L)	5.4 [5.0, 5.8]	5.4 [5.0, 6.2]	5.4 [5.0, 5.6]	0.61
Trombocytes (10E ⁹ /L)	420.0 [236.5, 423.5]	422.0 [348.5, 440.0]	377.0 [220.0, 420.5]	0.25
Urea (mmol/L)	9.7 [8.5, 12.9]	9.0 [8.3, 9.7]	12.4 [8.6, 14.2]	0.30
Creatinine (umol/L)	64.0 [40.8, 81.0]	49.0 [34.5, 55.0]	81.0 [75.0, 94.0]	0.01
Albumin (g/L)	18.5 [13.5, 19.5]	17.6 [12.7, 18.6]	19.4 [15.8, 20.1]	0.51
Bilirubine (umol/L)	5.8 [4.6, 8.2]	4.6 [3.9, 7.4]	7.0 [5.6, 10.0]	0.22
Discharge demographics				
Total ventilator days	32.0 [18.0, 47.0]	20.0 [13.5, 42.0]	40.5 [34.8, 52.2]	0.19
ICU length of stay (days)	39.0 [26.2, 45.8]	41.0 [23.5, 52.5]	38.0 [27.5, 42.5]	0.75
Hospital length of stay (days)	66.0 [60.0, 86.8]	61.0 [54.5, 66.0]	80.5 [67.2, 98.0]	0.14
Hospital mortality (death)	2 (14.3)	1 (14.3)	1 (14.3)	0.58

Data are presented as median [1st quartile, 3rd quartile]. Between group differences tested using Mann-Whitney U test or Fisher's exact test as appropriate. ER = Emergency Room, APACHE = Acute Physiology And Chronic Health Evaluation, SAPS = Simplified Acute Physiology Score, ICU = Intensive Care Unit.

Plasma phenylalanine and leucine concentrations

Total plasma phenylalanine and leucine concentrations increased after enteral infusion of 20 g intact protein or the equivalent amount of free amino acids (P -time<0.001; **Figure 6.2**). Plasma phenylalanine (P -time x treatment=0.003) and leucine (P -time x treatment=0.003) responses were greater following free amino acid administration (AA) compared with intact protein (PRO). Peak plasma phenylalanine and leucine concentrations were higher in the free amino acid (AA) compared with the intact protein (PRO) group (141 [95%CI 137-161] $\mu\text{mol/L}$ vs 111 [95%CI 103-119] $\mu\text{mol/L}$ and 300 [95%CI 273-327] $\mu\text{mol/L}$ vs 223 [95%CI 202-244] $\mu\text{mol/L}$, respectively).

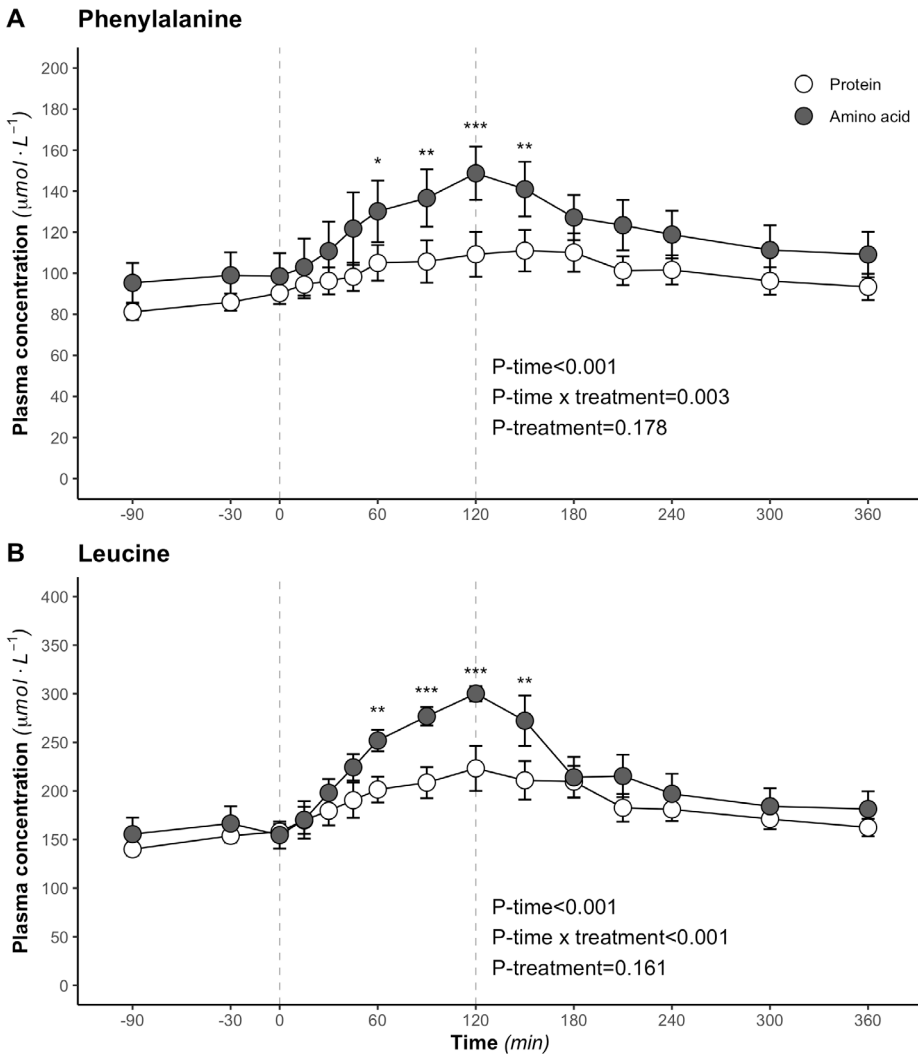


Figure 6.2 Total plasma phenylalanine (**A**) and leucine (**B**) concentrations over time after administration of 20 g milk protein or an equivalent amount of free amino acids. Data presented as mean \pm SE. Vertical dashed lines represent start ($t=0$ min) and end ($t=120$ min) of enteral nutrient administration. Significant differences in plasma amino acid concentrations between groups derived from the linear mixed effects model are marked with * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

Plasma amino acid enrichments

Plasma L-[1-¹³C]-phenylalanine enrichments, the enteral tracer included in the test meal, increased following the start of nutrient infusion (P -time <0.001) with a more rapid and greater post-prandial rise following free amino acid compared with intact protein administration (P -time \times treatment = 0.003). Peak plasma L-[1-¹³C]-phenylalanine enrichments were reached at the end of nutrient infusion ($t=120$ min) and were higher for the amino acid group when compared to the protein group (10.6 95% CI [8.6-12.6] vs 5.6 95% CI [3.8-7.4] MPE, respectively; $P=0.001$; **Figure 6.3A**). Plasma L-[ring-²H₅]-phenylalanine and L-[ring-²H₂]-tyrosine enrichments derived from the intravenous tracer infusion did not differ between treatments throughout the study period ($t=0$ min; $P>0.05$; **Figure 6.3BC**).

Plasma amino acid kinetics

Exogenous phenylalanine R_a (**Figure 6.4A**) increased following infusion of both intact protein and the equivalent amount of free amino acids (P -time <0.001), with greater increases observed in the amino acid group compared with protein group (P -time \times treatment=0.015). Endogenous phenylalanine R_a (**Figure 6.4B**), derived from whole-body protein breakdown, decreased following enteral nutrient infusion (P -time <0.001). There was no significant overall between-group difference in endogenous phenylalanine R_a for the entire study period (P -time \times treatment=0.723). Total phenylalanine rate of appearance temporarily increased during nutrient infusion, without any overall differences between groups (P -time <0.001 and P -time \times treatment=0.65, **Figure 6.3C**). Phenylalanine rate of disappearance slightly increased during nutrient infusion without any between group differences over time (P -time <0.001 , P -time \times treatment=0.84, **Figure 6.3D**).

Diet-derived phenylalanine release

Systemic availability of diet-derived phenylalanine, calculated as the percentage of dietary phenylalanine that was released into the systemic circulation, increased over time (P -time <0.001 , **Figure 6.5A**) and was greater following free amino acid administration when compared to intact protein provision (P -time \times treatment <0.001). In agreement, overall release of diet-derived amino acids, assessed over the entire 6 h postprandial period, was 25% greater following administration of free amino acids when compared to intact protein (69% [95% CI 62-75%] vs 44% [95% CI 32-55%], respectively; $P<0.001$, **Figure 6.5B**).

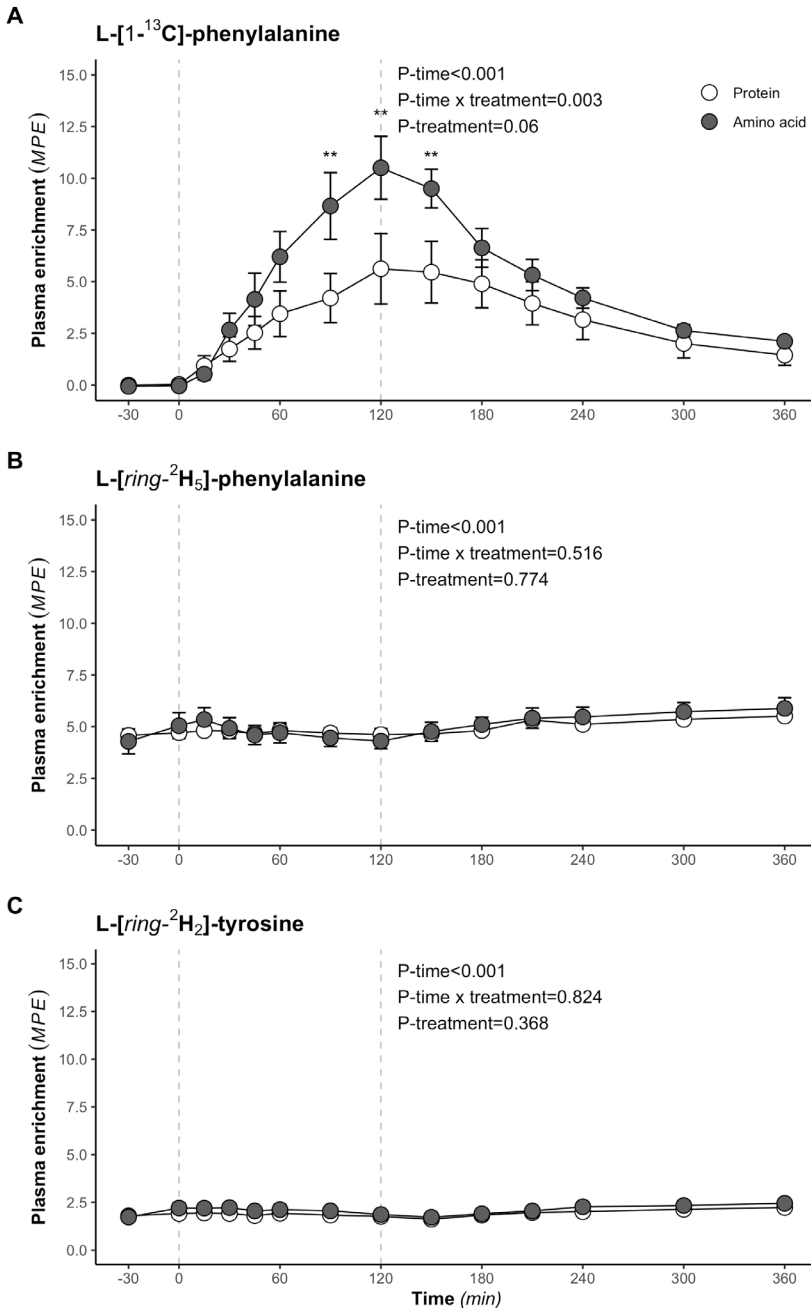


Figure 6.3 Plasma L-[ring-²H₅]-phenylalanine (A), L-[ring-²H₂]-tyrosine (B) and L-[1-¹³C]-phenylalanine (C) enrichments following administration of 20 g intrinsically L-[1-¹³C]-phenylalanine labelled milk protein or an equivalent amount of free amino acids labelled with [1-¹³C]-phenylalanine. Data are presented as mean ± SE. Vertical dashed lines represent start (t=0 min) and end (t=120 min) of enteral nutrient infusion. Significant differences in plasma concentrations between groups derived from the linear mixed effects model are marked with * (P < 0.05), ** (P < 0.01) or *** (P < 0.001).

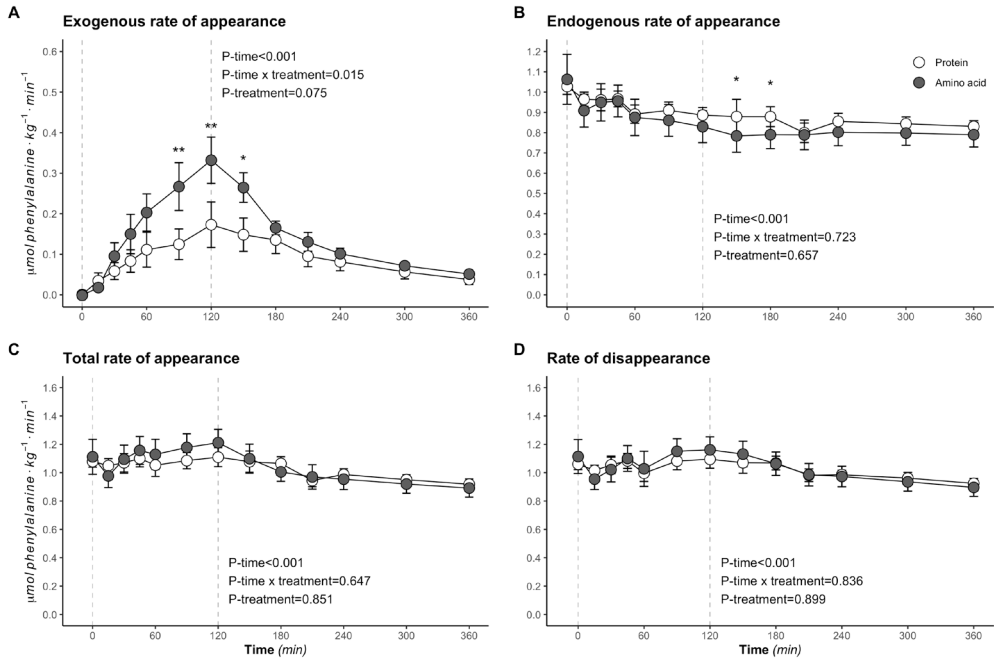


Figure 6.4 Whole-body phenylalanine kinetics, assessed as exogenous phenylalanine rate of appearance (Ra) (A), endogenous phenylalanine Ra (B), total phenylalanine Ra (C) and phenylalanine rate of disappearance (D) after administration of 20 g milk protein or an equivalent amount of free amino acids. Data are presented as mean \pm SE. Vertical dashed lines represent start (t=0 min) and end (t=120 min) of enteral nutrient infusion. Significant differences observed between groups derived from the linear mixed effects model are marked with * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

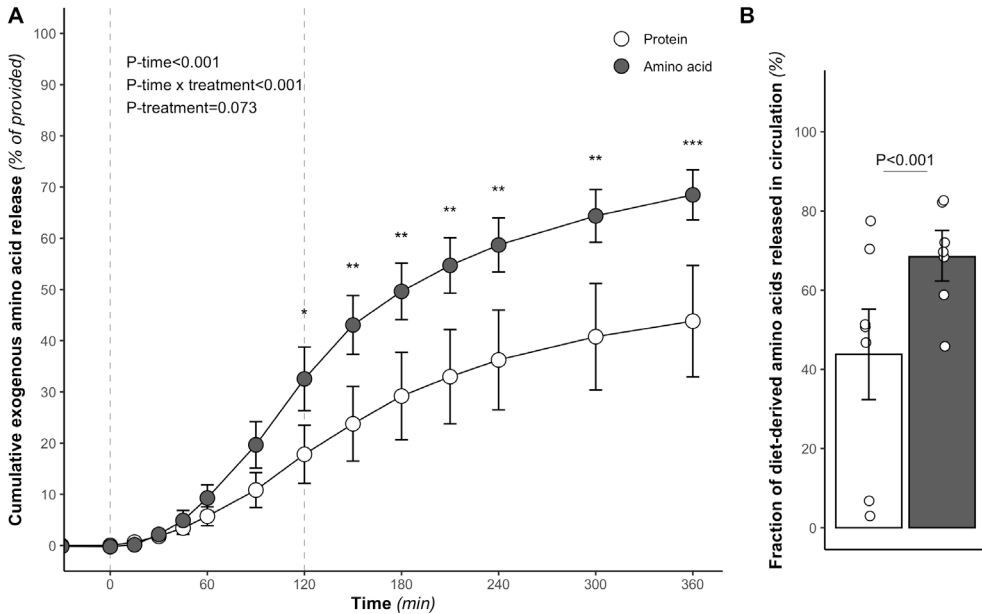


Figure 6.5 Cumulative fraction of diet-derived amino acids released in the circulation over time (A) and total release throughout the 6 h post-prandial period (B). Data are presented as mean \pm SE (A) or mean \pm 95% CI (B). Vertical dashed lines represent start ($t=0$ min) and end ($t=120$ min) of enteral nutrient infusion. Significant differences in cumulative exogenous amino acid release between groups derived from the linear mixed effects model are marked with * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

Whole-body protein turnover

Whole-body postabsorptive and postprandial protein turnover rates are summarized in **Figure 6.6**. Following administration of either free amino acids or protein, there was a slight decrease in whole-body protein synthesis and a robust decrease in whole-body protein breakdown without significant differences between treatments (P -time \times treatment 0.88 and 0.72 respectively, **Figure S6.1**). Whole-body protein oxidation slightly increased following nutrient administration, without any between group differences (P -time \times treatment = 0.21). Consequently, whole-body protein net balance, representing the net effect of protein synthesis and breakdown, was greater following amino acid provision in both groups (P -time < 0.001, **Figure S6.1**). Whole-body protein net balance tended to be higher following free amino acid versus intact protein (P -time \times treatment = 0.07).

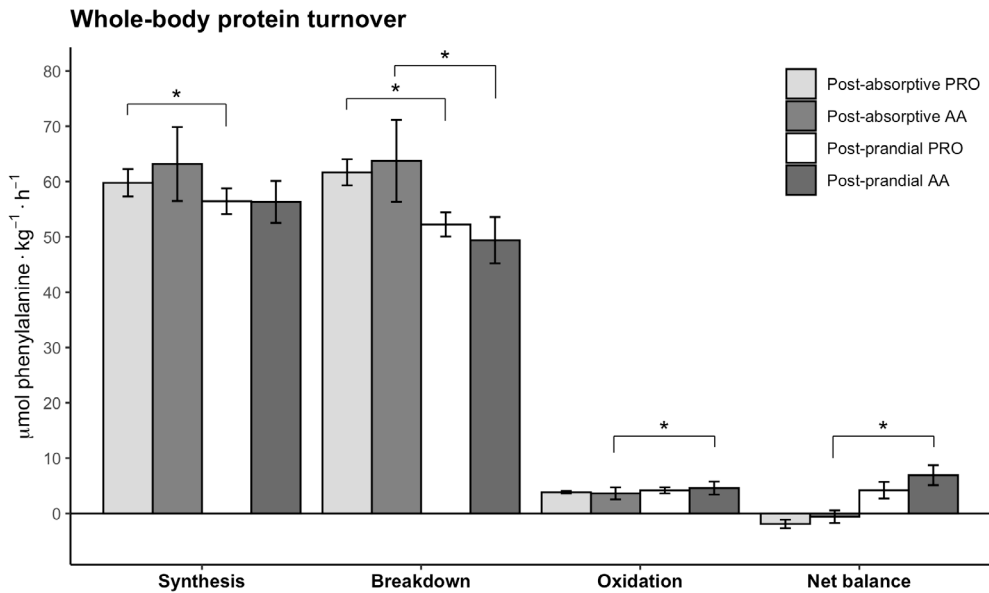


Figure 6.6 Whole-body protein synthesis, breakdown, oxidation and net protein balance under post-absorptive conditions and after administration of 20 g milk protein (PRO) or an equivalent amount of free amino acids (AA). Data are presented as median [1st – 3rd quartile] and between group differences test with Mann-Whitney U test, over time differences tested with Wilcoxon-signed rank test. Significant differences are marked with * ($P < 0.05$).

Faeces analysis

Median feces production during the study period was 240 [210, 281] g per day, with no between-group differences. Results from the feces analysis are presented in **Table 6.2**. Based on fecal elastase measurements, two patients met the criteria for severe exocrine pancreatic insufficiency (fecal elastase < 0.100 mg/g), both were in the free amino acids group. Based on nitrogen content, approximately 5% of fecal weight represents protein. Consequently, median fecal protein loss amounted to approximately 12.8 g of protein per day, an equivalent of 19% of the standard daily enteral protein dose administered to patients (1.2 g/kg/d). We did not observe any differences in fecal L-[1-¹³C]-phenylalanine enrichments between patients provided either the intrinsically L-[1-¹³C]-phenylalanine labeled protein or the equivalent amount of free amino acids enriched with free L-[1-¹³C]-phenylalanine.

	Overall (n=14)	Protein (n=7)	Amino acid (n=7)	P
Fecal weight (g/day)	240 [210, 281]	256 [218, 293]	240 [212, 261]	0.86
Fecal elastase (mg/g)	0.26 [0.23, 0.31]	0.26 [0.24, 0.28]	0.29 [0.16, 0.31]	0.85
Exocrine Pancreatic Insufficiency?				0.45
No EPI	12 (87.7)	7 (100)	5 (71.4)	
Severe EPI	2 (14.3)	0 (0.0)	2 (28.6)	
Fecal protein content (%)	4.9 [4.4, 5.8]	4.9 [4.4, 5.6]	4.9 [4.0, 6.0]	0.75
Daily fecal protein loss (g/day)	12.8 [8.9, 15.4]	11.2 [7.6, 15.1]	14.4 [9.8, 15.3]	0.66
Fecal protein loss (% of daily enteral protein dose)	19.0 [15.0, 23.3]	16.5 [13.3, 24.7]	19.5 [18.1, 21.6]	0.75
[1-¹³C]-phenylalanine enrichment (MPE)	0.06 [0.01, 0.11]	0.05 [0.01, 0.15]	0.09 [0.03, 0.11]	0.75

Data presented as median [1st quartile, 3rd quartile]. Between group differences tested using Mann-Whitney U test or Fisher's exact test as appropriate.

Discussion

In the present study we observed a rapid release of diet-derived amino acids into the systemic circulation following the onset of enteral feeding in mechanically ventilated ICU patients. A more rapid and greater release of diet-derived amino acids was observed following the administration of free amino acids when compared to the equivalent amount of intact protein. A total of 69% of the administered free amino acids were released into the circulation whereas 44% of the administered amino acids provided as intact protein were released throughout the 6-hour postprandial period. Both protein and free amino acid administration increased whole-body protein net balance with a trend towards a more positive net balance following the administration of free amino acids when compared with intact protein.

Protein-energy malabsorption is believed to be common among critically ill patient and limits the amount of diet-derived amino acids reaching the systemic circulation and becoming available for peripheral tissues such as the muscle and increase net protein losses associated with poor outcome in patients [11]. In our study, the percentage of dietary protein-derived amino acids that was released into the circulation as of fraction of the total amount provided was 44% following provision of 20 g intact milk protein, which appears to be similar to the 45-65% reported previously following milk protein ingestion by healthy adults under various conditions [16]. These data confirm recent observations that protein digestion and amino acid absorption are not severely compromised in critically ill patients [28].

We selected intensive care patients believed to be at an increased risk for severe protein-energy malabsorption based on the presence of diarrhea [14]. However, our data clearly show that protein digestion and amino acid absorption were not severely compromised in these patients [9]. To confirm the absence of maldigestion we also sampled fecal mass for several days throughout the experimental period and observed that 5% of the collected fecal mass consisted of protein, which is similar to fecal protein contents reported previously following the analyses of stool samples collected in both ICU patients and healthy adults [29, 30]. Patients in our study produced a median of 240 g feces per day, which would provide an estimated amount of 13 g protein being excreted daily via the fecal route. This would correspond with ~19% of habitual daily protein provision, with patients typically being administered 1.2 g protein per 24 h via continuous enteral infusion. However, most of the protein excreted in the feces is derived from microbiota and cell shedding [30] and therefore does not reflect intestinal protein loss. We also assessed the presence of L-[1-¹³C]-phenylalanine in fecal samples obtained throughout and up to 14 h after the 6 h assessment period, to see whether (some of) the administered protein was detectable in the feces. Though we did detect the presence of L-[1-¹³C]-phenylalanine in the stool-derived protein fraction, the enrichment levels were negligible. This further supports the observation that protein maldigestion and amino acid malabsorption are not prominent in critically ill patients.

Although protein digestion and amino acid absorption rates were not severely compromised following provision of intact protein in ICU patients, there appears to be room to accelerate and further improve the systemic availability of diet-derived amino acids. Following administration of 20 g free amino acids, we observed a greater increase in plasma leucine and phenylalanine concentrations when compared to the administration of an equivalent amount of milk protein. This was attributed to a more rapid and greater release of exogenous amino acids into the circulation, with the total amount of exogenous amino acids released into the circulation nearly

25% greater following free amino acid compared with intact protein provision within the 6-h post-prandial period (69% vs 44%; $P < 0.001$; Figure 6.5). Therefore, even in the absence of protein maldigestion a greater postprandial plasma amino acid availability can be achieved when free amino acids are being provided when compared to the administration of an equivalent amount of intact protein. Consequently, the amount of exogenous amino acids that became available as substrates for peripheral tissues was strongly increased.

Using continuous intravenous L-[ring- $^2\text{H}_5$]-phenylalanine and L-[ring- $^2\text{H}_2$]-tyrosine infusion, we were able to assess the impact of both protein and free amino acid administration on whole-body protein turnover rates. In a fasted state, whole-body protein synthesis and breakdown rates tend to be higher in ICU patients when compared with healthy controls [31-34]. The whole-body turnover rates observed in the present study are in line with those previously reported in ICU patients (Figure 6.6), indicating that critical illness is characterized by a state of increased protein flux [9, 35-37]. We did not observe a significant postprandial increase in whole-body protein synthesis rates, but protein breakdown rates clearly decreased following both free amino acid and intact protein administration. Consequently, without a substantial increase in phenylalanine hydroxylation rates, overall protein net-balance improved following protein and amino acid administration. The postprandial improvements in whole-body protein balance tended to be greater following free amino acid compared with intact protein provision ($P = 0.07$; Figure S6.1). Although our study was not powered to detect differences in whole-body turnover rates, this trend indicates that increased postprandial availability of diet-derived amino acids could potentially further increase the anabolic response to feeding in these patients. However, whole-body protein kinetics do not necessarily reflect skeletal muscle protein turnover [21]. Previous work has shown that a more rapid and greater postprandial release of protein-derived amino acids generally augments postprandial muscle protein synthesis rates in health [38, 39]. During critical illness, postprandial increase in muscle protein synthesis rates is blunted [28]. Whether increased release of amino acids such as those achieved following the provision of free amino acids can overcome this anabolic resistance remains to be determined, as we did not collect muscle biopsies in this study.

The current study is the first to assess protein digestion and amino acid absorption rates of either intact milk protein or an equivalent amount of free amino acids in critically ill patients. Our results suggest a potential role for the use of elemental feeds containing free amino acids or oligopeptides, as the greater release of diet-derived amino acids found in the free amino acid group provides a novel avenue to increase the anabolic potential of protein feeds in critically ill patients [40]. While elemental feeds are commercially available for use in critical care, studies have only investigated their effects on patient tolerability or incidence of diarrhea [41, 42]. Although these feeds may not improve gastro-intestinal tolerance of patients, our results demonstrate that feeds containing free amino acids result in a 25% greater postprandial plasma availability of diet-derived amino acids. This would mean that administering free amino acids at a rate of 1.3 g/kg/day according to the current ESPEN guidelines, would be equivalent to providing 1.6 g/kg/day of standard feeds containing milk protein [1] at least with regards to the postprandial delivery of amino acids to the periphery. This is of particular interest considering emerging evidence suggesting that increased protein targets and subsequent higher plasma amino acid concentrations could be beneficial for critically ill patients [43-45]. Providing feeds with free amino acids rather than protein could represent a more efficient way to achieve higher protein targets in patients, or to reduce volume required for nutrition in patients at risk for fluid-overload [46]. However, robust

prospective evidence for increased protein targets and indeed the role of elemental feeds therein are lacking and require further investigation.

In conclusion, enteral provision of free amino acids allows a more rapid and greater absorption of diet-derived amino acids when compared with the provision of intact protein in critically ill patients. More of the diet-derived amino acids are released in the circulation when amino acids are provided as free amino acids when compared to the administration of intact protein in critically ill patients. Provision of free amino acids as opposed to intact protein improves amino acid absorption and, as such, may further improve protein balance and attenuate muscle mass loss in critically ill patients.

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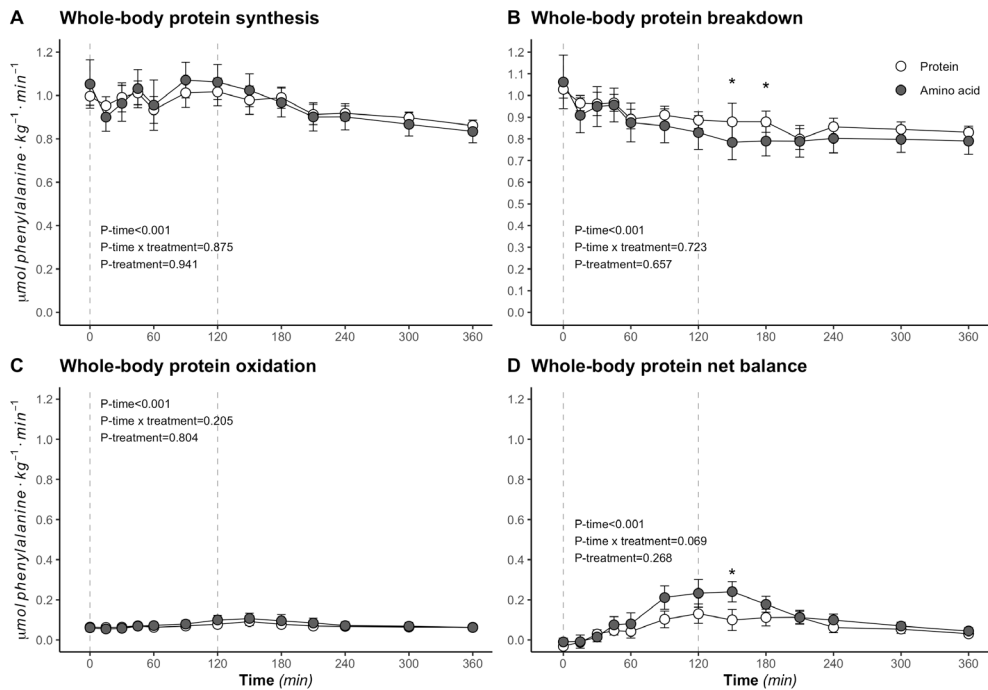
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Supplemental material

Supplemental table 6.1 Amino acid content of the intact milk protein concentrate and free amino acid mixture provided.

	Intact protein	Amino acids
EAA (%)		
Histidine	2.7	2.6
Isoleucine	4.9	4.8
Leucine	9.1	9.1
Lysine	7.7	9.5
Methionine	2.7	2.6
Phenylalanine	4.4	4.4
Threonine	4.2	4.2
Tryptophan	1.2	1.2
Valine	6.4	6.4
ΣEAA	43.2	44.7
NEAA (%)		
Alanine	3.0	2.9
Arginine	4.0	3.9
Aspartic acid	7.3	7.2
Cysteine	0.8	0.8
Glutamic acid	19.8	19.6
Glycine	1.8	1.8
Proline	9.9	9.6
Serine	5.3	5.3
Tyrosine	4.9	4.3
ΣNEAA	56.7	55.3
TAA (%)		
ΣTAA	100.0	100.0

Values are presented as percentage (%) of total amino acids. EAA, essential amino acids; NEAA, non-essential amino acids; TAA, total amino acids; PRO, milk protein; AA, free amino acids.



Supplemental figure 6.1 Whole-body protein synthesis (A), Breakdown (B), Oxidation (C) and net protein balance (D) after administration of 20 g milk protein or an equivalent amount of free amino acids. Data are presented as mean \pm SE. Vertical dashed lines represent start ($t=0$ min) and end ($t=120$ min) of enteral nutrient infusion. Significant differences observed between groups derived from the linear mixed effects model are marked with * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).



Chapter 7

General discussion

General discussion

When individuals are admitted to the hospital, their nutritional status generally deteriorates. At present, up to 30% of the older patients in Western-European hospitals have been identified as being malnourished during hospitalization [1-5]. This entails a suboptimal energy intake as well as specific protein malnutrition. Protein requirements set by the ESPEN organisation for older adults with chronic or acute diseases are $1.2 - 1.5 \text{ g}\cdot\text{kg}^{-1} \text{ bodyweight}\cdot\text{d}^{-1}$ to accommodate skeletal muscle mass maintenance and preserve muscle function [6, 7]. Unfortunately, only a very small subset of patients actually meets these protein requirements [8-11]. It is essential that we come up with more effective strategies to improve food intake during hospitalization. There are already plenty of strategies proposed to optimize food intake, and increase protein intake in particular, such as including more protein-rich products in hospital menus [10, 12, 13], changing the logistical food provision concept [14], and providing nutritional supplements or protein-rich snacks [15-17]. In the final chapter of this thesis, we elaborate on the implications of suboptimal nutritional intake in hospitalized patients and focus on future opportunities to improve nutritional status in relation to protein (muscle) metabolism in the clinical setting.

Daily protein ingestion is an important requirement for muscle mass maintenance. Throughout the day, there is a constant turnover (i.e., build-up and breakdown) of skeletal muscle tissue. When muscle protein synthesis rates exceed muscle protein breakdown rates, muscle mass will increase. In contrast, during catabolism, muscle protein breakdown rates exceed muscle protein synthesis rates, resulting in net muscle loss. Protein ingestion is a potent anabolic stimulus that increases muscle protein synthesis rates and lowers muscle protein breakdown rates in both young and older adults [18-20]. As such, protein ingestion represents one of the main factors affecting the balance between muscle protein synthesis and breakdown rates. Ingestion of 20 g of a high quality protein source has been shown to increase muscle protein synthesis rates in young adults [21, 22]. Due to anabolic resistance with aging, greater amounts of protein (>20 g per meal) are required to significantly stimulate muscle protein synthesis in older individuals [23-26]. This is even more relevant in a clinical setting, with patients being inactive and suffering from (acute) illness, likely contributing to the anabolic resistance.

As clearly shown in chapters 2 and 3, protein intake is insufficient in older patients during hospitalization, with average intake merely reaching $\sim 0.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ [8, 9]. This is in line with previously published data reported by various research groups, indicating that such low intakes represent a generic, global problem [10, 27, 28]. Daily protein intake during hospitalization is already below the general WHO recommendation of $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for adults. More importantly though, it only represents half or even less of the recommendations provided by the ESPEN group at $1.2-1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. In attempting to improve this obvious protein malnutrition during hospitalization, it is important to understand the hospital logistics. Currently, patients are offered food at three main meal moments; breakfast, lunch, and dinner. In addition, small snacks are offered in between the main meal moments. To increase protein intake, simply increasing meal size will not be sufficient, as already 30% of the provided food in a single meal is typically not consumed and discarded (Chapter 2, 3). Therefore, we need to think of alternative means on how to provide more protein to our patients. The time between dinner and breakfast (i.e., in the evening and during the night) forms a long period during which patients do not consume any food, and as a such, will remain in a catabolic state for a prolonged 12-15 hour period. Previous studies have shown that protein ingested prior to and administered during sleep is properly

digested and absorbed, allowing protein-derived amino acids to stimulate muscle protein synthesis during overnight sleep [29, 30]. The benefits of pre-sleep protein supplementation have been confirmed in long term training studies in healthy adults, but have not been assessed in a hospital setting in patients. Even though consuming pre-sleep protein may serve as a great opportunity to overcome the long catabolic period throughout the night, it remains to be assessed whether the implementation of protein provision in the evening prior to sleep modulates food intake throughout the (subsequent) day. Adding a protein-rich snack prior to sleep will only improve daily protein intake if food consumption throughout the subsequent day is not reduced. Therefore, we are currently investigating whether providing a pre-sleep snack is effective at increasing total daily protein intake throughout hospitalization.

Selecting appropriate foods or food products to offer patients as a (pre-sleep) protein snack can be challenging. The current hospital snacks generally provide only a small amount of protein (<5 g). Either the protein density of the products is too low, or the total amount of product is insufficient. For this reason, underfed patients are often prescribed with oral nutritional supplements (ONS). These supplements are commonly used in patients that are malnourished, or at risk for malnutrition to improve energy and protein intake. Unfortunately, acceptability and intake of the provided supplements is often suboptimal, with many patients who do not like the flavour, texture and/or smell. Furthermore, provision after a main meal, as opposed to pre-sleep, generally lowers actual consumption, as meals are generally not fully consumed. Attractive presentation of the supplements is essential to improve compliance and intake. Providing a pre-sleep ONS as a cold drink, in a glass, preferably with ice cubes or mixed with other products such as chocolate milk or ice cream might improve the taste and ultimately improve consumption, especially when optimal timing is taken into account. Yet, whether oral nutritional supplements are consumed and will effectively increase daily protein intake when provided prior to sleep also remains to be assessed.

In a clinical setting, protein supplements can act as an in-between solution, to temporarily increase protein and energy intake during hospital stay. However, our aim should be to use food products that are readily available for everyone. In this way, we encourage patients to incorporate a pre-sleep protein-rich snack in their habitual diet, beyond the scope of the hospital setting (e.g. at home). There are some food products available that can be used as a pre-sleep snack, such as cheese, quark, or unsalted nuts. However, there are limited suitable products available, creating opportunities for the food industry. Developing products suitable as a pre-sleep snack is challenging, as there are certain characteristics that these products should have (**Figure 7.1**).

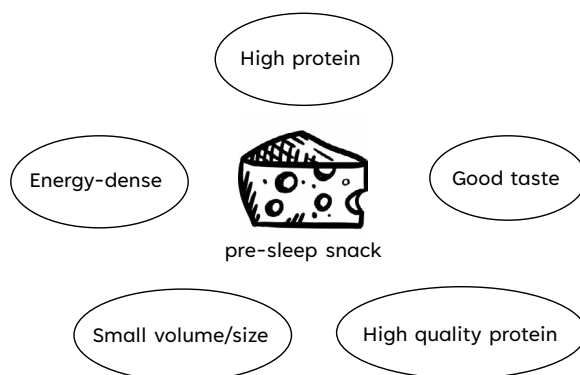


Figure 7.1 suggested characteristics of a pre-sleep snack.

First of all, the product should be protein-rich, as the goal of adding a pre-sleep snack is to increase daily protein intake. Furthermore, protein quality is an important factor. Proteins with high(er) essential amino acid contents are generally considered high(er) quality proteins and are also more likely to strongly stimulate postprandial muscle protein synthesis [31]. As shown in chapters 5 and 6 of this thesis, adding free amino acids results in an accelerated amino acid absorption and increased postprandial amino acid availability. Simultaneously, in chapter 5 we showed no difference in the anabolic response between a free amino acid mixture and intact protein. This could imply that adding free amino acids does not have an added value when the amino acid composition is good (e.g. ample essential amino acids), or in a situation where sufficient protein is provided. Products low in protein, or in essential amino acids could be improved by fortification with essential amino acids, with leucine in particular.

Apart from protein density and protein quality, total energy content is a relevant characteristic. As many patients are malnourished during hospital stay [32], energy intake should (also) be improved and in such conditions it would be appropriate that the (pre-sleep) snack is also energy-dense. Another important characteristic is taste, the product should be tasteful and there needs to be some variation (e.g. both sweet and savoury products), as patients should continue to consume such a pre-sleep snack daily. Finally, the product should preferably be provided in a small volume, as patients generally consume less fluids throughout the evening to reduce the need to visit the restroom during the night. Ideally, there should be a wide variety of appropriate products available in the near future. This will enable us to offer our patients different product options to improve their daily protein and/or energy intake.

It is important that patients consume sufficient amounts of energy and protein, as malnutrition in patients has been shown to prolong the length of stay [2, 33, 34], accelerate the loss of muscle mass [35], impair functional outcome [36], and increase the risk of morbidity and mortality [5, 33, 34]. Patients should strive to meet protein intake levels of 1.2-1.5 g·kg⁻¹·d⁻¹ as a means to support muscle mass and strength maintenance [6, 7]. As mentioned before, providing a snack prior to sleep serves as a convenient opportunity to improve daily protein intake in patients during their hospital stay. Apart from establishing whether pre-sleep protein can actually increase total protein intake, future studies should evaluate how such an improved nutritional state may improve clinical health outcomes both on the short and on the long term. Moreover, in view of

the importance of nutritional status, we should not limit our focus on only adding a pre-sleep snack, but rather improve the entire (hospital) food concept (**Figure 7.2**).

The preferred hospital diet should provide at least 3 main meals with 20-30 g high-quality protein per meal. Protein-rich snacks should be offered in-between meal moments. Importantly, patients need to be stimulated to be more physically active (between the various meal moments) to sensitize the muscle to the anabolic properties of protein administration [37]. Furthermore, the food supply logistics need to enable patients to order food with no evident time restrictions. Food assistants should receive proper training to enable them to educate patients on more healthy and/or higher protein-content food choices. Lastly, providing a pre-sleep snack should form an essential part of such a novel food concept, allowing patients to increase daily protein intake and reduce overnight muscle proteolysis.

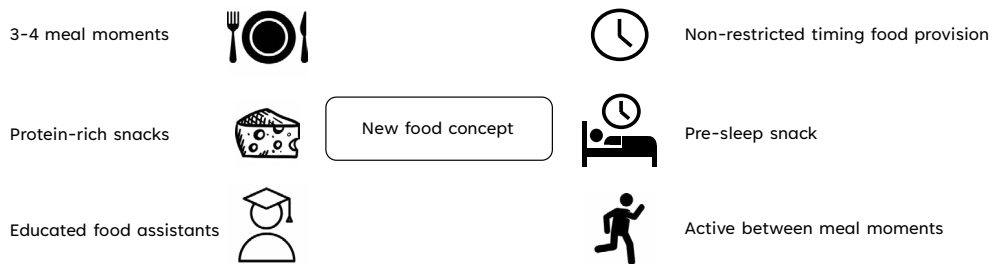


Figure 7.2 suggested parts of improved hospital food concept.

To develop such a novel hospital food concept and establish its efficacy to increase protein intake and preserve skeletal muscle mass and function during (and after) hospitalisation several questions still need to be resolved:

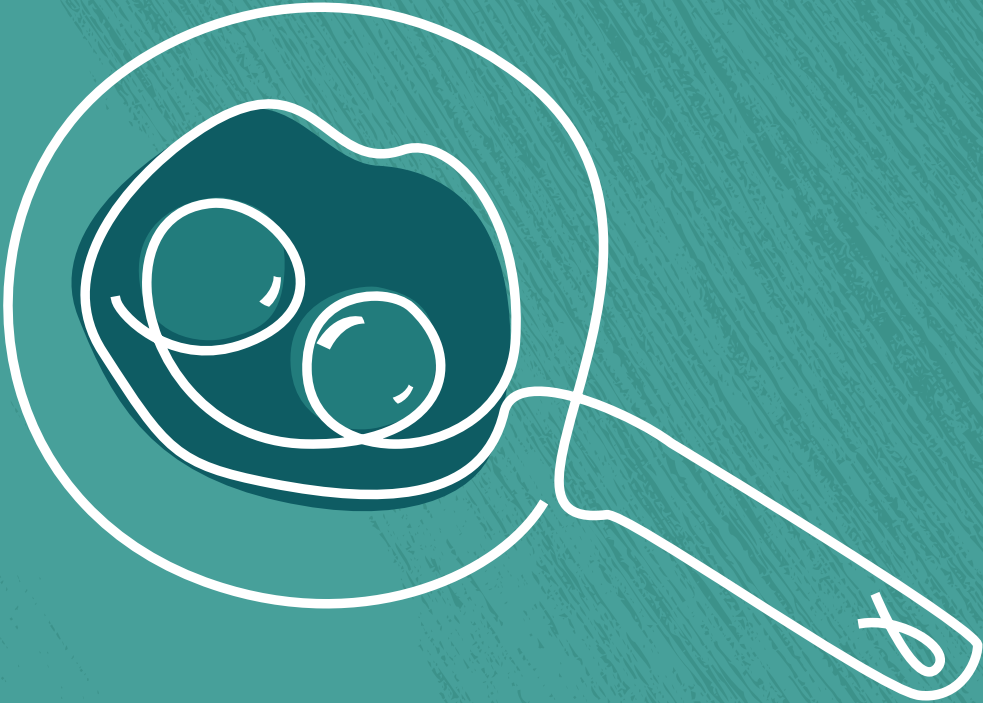
- Does the provision of a pre-sleep snack improve daily protein intake and prevent muscle loss in hospitalized (older) patients
- How much protein should a (pre-sleep) snack contain to stimulate muscle protein synthesis rates during overnight sleep in (older) hospitalized patients
- Which food products should be offered to patients to further augment daily protein intake during hospital stay
- How can a change in food provision logistics augment daily protein intake and attenuate skeletal muscle and strength loss in hospitalized patients
- What is the minimal amount of protein patients should consume to preserve muscle mass
- What is the minimal amount of physical activity that is required to preserve muscle mass and strength.

In conclusion, there is a growing aging population that is admitted to the hospital for elective and acute care. During hospitalisation, nutritional status deteriorates and patients are inactive, resulting in loss of muscle mass and function. In order to facilitate healthy aging and maintain quality of life, it is essential to incorporate lifestyle (nutrition and exercise) in the treatment prior to, during and post hospitalisation. With the right approach and attitude, we can improve our current health care system immensely.

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Chapter 8

Addendum

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Financial support

Summary

Food intake, physical activity and muscle mass maintenance

Adequate nutritional intake is essential to maintain nutritional status, support essential body functions, maintain quality of life, and lower disease burden. Food intake, with protein ingestion in particular, and physical activity are important regulators of muscle mass. Maintenance of skeletal muscle mass is of important clinical relevance in both health and disease. Having a low muscle mass at an older age increases the risk for hospitalization, impairs physical function, and results in a longer length of hospital stay, a worsened disease prognosis, and higher mortality rates during hospitalization.

Protein consumption

In **Chapter 2**, we assessed food provision and actual food consumption in 101 hospitalized, older patients admitted for elective orthopaedic surgery. We showed that 35-40% of the provided hospital food was not consumed, resulting in extremely low energy and protein intake levels. Protein intake averaged $0.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ during short-term (6 day) hospitalization. As we showed that patients admitted for elective orthopaedic surgery were malnourished during their hospital stay, we were interested whether even more vulnerable patients showed similar (low) food intake levels during hospitalization. In **Chapter 3** we showed that patients at risk for malnutrition consumed $\sim 0.7 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. Malnourished patients are often provided with oral nutritional supplements to improve total energy and protein intake. Providing patients with oral nutritional supplements did not increase the protein density of the hospital diet. Our findings demonstrate that protein intake is insufficient in patients during hospitalization. We need to think of alternative strategies to increase the protein density of the hospital meals and, as such, increase daily energy and protein consumption.

Muscle loss and regain

Besides protein intake, physical activity is an important regulator of muscle mass maintenance. Short periods of physical inactivity, lead to significant loss of muscle mass and strength. Strategies should aim to preserve muscle mass during periods of disuse. Therefore, in **Chapter 4**, we assessed whether protein supplementation can attenuate muscle loss and/or augment muscle mass regain following one week of unilateral leg immobilization. Healthy, young males were provided with either a peptide with proposed anabolic properties or milk protein concentrate. Participants ingested deuterium oxide daily with saliva and muscle biopsy samples being collected to measure myofibrillar protein synthesis rates during the immobilization and recovery periods. We demonstrated that peptide supplementation does not preserve muscle mass or strength during short-term immobilization, or further augment muscle mass and strength regain during remobilization when compared to milk protein supplementation. In line, peptide supplementation did not differ from milk protein supplementation in modulating myofibrillar protein synthesis rates during immobilization. Interestingly, we did observe higher rates of muscle protein synthesis following peptide when compared with milk protein supplementation during recovery. Higher myofibrillar protein synthesis rates during the remobilization period may be indicative of more rapid skeletal muscle reconditioning.

Anabolic properties of protein intake

As postprandial protein handling plays an important role in the anabolic response to feeding, we assessed the protein digestion and amino acid absorption kinetics and the subsequent muscle protein synthetic response following the ingestion of intact protein and free amino acids in a group healthy, young volunteers (**Chapter 5**). Ingestion of a bolus of 30 g free amino acids led to more rapid amino acid absorption and greater postprandial plasma amino acid availability when compared to the ingestion of an equivalent amount of intact milk protein. The greater amino acid availability did not lead to measurable differences in the muscle protein synthetic response following ingestion of free amino acids versus intact milk protein. It could be speculated that the ingestion of a 30 g bolus of protein or free amino acids provided, prevented us from detecting differences in postprandial muscle protein synthesis rates between treatments. The effect of greater postprandial plasma amino acid release on stimulating muscle protein synthesis may become more evident in conditions where less than 20 g protein or free amino acids are ingested and/or assessed in more clinically compromised populations suffering from anabolic resistance. In **Chapter 6** we assessed protein digestion and amino acid absorption kinetics in critically ill patients following the ingestion of intact protein and an equivalent amount of free amino acids. Administration of free amino acids resulted in a more rapid and greater postprandial plasma amino acid availability when compared to the administration of intact protein. Future studies should assess whether the greater postprandial amino acid availability results in greater postprandial muscle protein synthesis rates in critically ill patients.

Future directions

In the final chapter of this dissertation, we elaborate on the implications of less than optimal nutritional intake in hospitalized patients and focus on future opportunities to improve muscle protein balance in the clinical setting. We present the proposed benefits of incorporating a pre-sleep snack to the hospital diet. The main benefit of providing a protein-rich snack prior to sleep is that it may attenuate overnight proteolysis. Furthermore, we discuss which characteristics (e.g. high-protein, small volume. etc.) such a product should have to be the ideal pre-sleep protein snack. Lastly, we give suggestions on how to improve the entire (hospital) food concept, rather than only limiting our focus on adding a pre-sleep protein snack.

Samenvatting

Voedingsinname, fysieke activiteit en behoud van spiermassa

Het is belangrijk om voldoende voeding, met name eiwit, te consumeren om ziektelast te verlagen, en essentiële lichaamsfuncties en kwaliteit van leven te behouden. Het is met name belangrijk om spiermassa te behouden tijdens veroudering en ziekte. Een lage spiermassa verhoogd namelijk het risico op ziekenhuisopname, verminderd fysiek functioneren, resulteert in langere ziekenhuisopnames, verslechterd ziekteprognose en verhoogd het risico op overlijden tijdens ziekenhuisopname. Om spiermassa te behouden zijn er twee belangrijke anabole stimuli, eiwitinname en fysieke activiteit.

Eiwitname

Het is van belang dat tijdens opname in het ziekenhuis, patiënten voldoende blijven eten en bewegen. In **hoofdstuk 2** hebben we het voedingsaanbod en de voedingsinname in 101 oudere patiënten gemeten na een electieve, orthopedische operatie. In dit onderzoek kwam naar voren dat 35-40% van het verstrekte eten niet werd opgegeten, wat resulteerde in een te lage energie en eiwitname. Tijdens het kortdurende verblijf in het ziekenhuis was de gemiddelde eiwitname in deze relatief gezonde populatie slechts $0.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, ver onder de geadviseerde eiwitbehoefte van $1.2\text{-}1.5 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$. Vervolgens waren we geïnteresseerd of een meer kwetsbaardere patiëntenpopulatie ook een soortgelijke (lage) eiwitname heeft tijdens ziekenhuisopname. In **hoofdstuk 3** laten we zien dat patiënten met een risico op ondervoeding gemiddeld $\sim 0.7 \text{ g} \text{ eiwit} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ consumeren. Drinkvoeding wordt vaak voorgeschreven aan ondervoede patiënten om de energie en eiwitname te verbeteren. Helaas zagen wij dat het verstrekken van drinkvoeding de eiwitdichtheid van het ziekenhuisdieet niet veranderd. Deze resultaten tonen aan dat de eiwitname onvoldoende is tijdens ziekenhuisverblijf en dat er strategieën moeten worden bedacht om de eiwitdichtheid van ziekenhuismaaltijden te verhogen, om daarmee de totale energie en eiwitname te verbeteren.

Verlies van spiermassa en herstel

Naast eiwitname is fysieke activiteit een belangrijke anabole stimulus. Korte perioden van inactiviteit leiden al tot significant verlies van spiermassa en spierkracht. We moeten streven naar behoud van spiermassa en spierkracht tijdens zulke (korte) perioden van verminderde fysieke activiteit. In **hoofdstuk 4** hebben we onderzocht of door middel van eiwittoeslag het spiermassaverlies kon worden tegengegaan tijdens een korte periode van immobilisatie, en/of het herstel van spiermassa kon worden versneld tijdens de daaropvolgende herstelperiode. Tijdens de immobilisatieperiode van 1 week werd 1 been geïmmobiliseerd middels een gipskoker in een groep gezonde proefpersonen. Proefpersonen namen twee keer per dag een eiwittoeslag in de vorm van een peptide met anabole eigenschappen of een gelijke hoeveelheid melkeiwit. Dit peptide was niet in staat spiermassa of spierkracht te behouden tijdens immobilisatie, toename in spiermassa tijdens een periode van herstel werd ook niet verder versneld door inname van dit peptide in vergelijking met inname van het melkeiwit. We hebben ook de spiereiwitsynthese kunnen bepalen tijdens de immobilisatie en herstelperiodes, doordat de proefpersonen dagelijks deuterium oxide innamen en op meerdere dagen speeksel en spierbiopten werden verzameld. We zagen geen verschil in de spiereiwitsynthese snelheid tussen de 2 supplementen tijdens de immobilisatieperiode. Echter, tijdens de herstelfase zagen we dat er een hogere eiwitsynthese snelheid was na inname van het peptide in vergelijking met het melkeiwit. Deze hogere eiwitsynthese snelheid

kan een indicatie geven dat er een sterkere reconditionering van de spier plaatsvond tijdens de herstelperiode.

Anabole eigenschappen van eiwitinname

Vertering van eiwit en de opname van de hierbij vrijgekomen aminozuren spelen een belangrijke rol in de anabole respons op de inname van voeding. In **hoofdstuk 5** hebben wij gekeken naar de verhoging van de spiereiwsynthese na inname van vrije aminozuren in vergelijking met intact eiwit. Inname van 30 g vrije aminozuren leidt tot een snellere aminozuuropname en een grotere aminozuurbeschikbaarheid in het bloed in vergelijking met de inname van een gelijke hoeveelheid intact eiwit. Deze grotere aminozuurbeschikbaarheid resulteerde niet in meetbare verschillen in de spiereiwsynthese snelheid tussen de groepen. Het effect van de grotere aminozuurbeschikbaarheid op de spiereiwsynthese wordt wellicht duidelijker in situaties waar minder dan 20 g eiwit wordt ingenomen, of bij mensen die anabool resistent zijn. Om deze reden hebben wij in **hoofdstuk 6** gekeken naar de eiwitvertering en aminozuuropname van vrije aminozuren en intact eiwit in kritisch zieke patiënten. Het toedienen van vrije aminozuren resulteerde in een snellere en grotere postprandiale aminozuurbeschikbaarheid ten opzichte van de toediening van intact eiwit. Dit laat zien dat meer aminozuren beschikbaar komen in de bloedbaan en beschikbaar worden voor perifere weefsels, zoals de skeletspier. Toekomstig onderzoek zal moeten kijken of een grotere aminozuurbeschikbaarheid leidt tot een hogere spiereiwsynthese in deze kritisch zieke patiënten.

Toekomstig onderzoek

In het laatste hoofdstuk van dit proefschrift bespreken we de gevolgen van een suboptimale voedingsinname in ziekenhuispatiënten en richten we ons op mogelijkheden om de voedingsstatus te verbeteren in relatie tot (spier)eiwitmetabolisme in de klinische setting. Verder worden de voordelen besproken om een snack voor het slapengaan aan het ziekenhuismenu toe te voegen. Gedurende de nacht is er een lange periode dat een patiënt in een katabole staat is. Het belangrijkste voordeel van een snack voor het slapengaan is dat het deze lange katabole periode tegengaat. Ook bespreken we welke eigenschappen, zoals hoog-eiwit en een klein volume, een dergelijk product moet bezitten om een ideale snack voor het slapengaan te zijn. Tot slot geven we suggesties hoe het totale voedingsconcept in het ziekenhuis kan worden verbeterd, waarbij we ons niet beperken tot alleen een snack voor het slapengaan.

Impact

What is the main objective of the thesis, and what are the most important results and conclusions?

The number of individuals older than 65 y has grown rapidly over the last decades. The increased life-expectancy is a great achievement in biomedicine and healthcare. However, the rise of the aging population is associated with greater health care costs, with higher expenses for disease and injury prevention, treatment and rehabilitation. With increasing age, hospital admissions increase as well. The average hospital stay is 5 days, however, the length of stay increases with advancing age. During hospitalization, patients lose a substantial amount of muscle mass and strength. The amount of muscle tissue lost during hospitalization predicts mortality and clinical outcomes. Furthermore, the loss of muscle mass and strength results in physical and functional declines. As there is a trend starting that older individuals need to live at home for a longer period of time, it is important that they stay independent and self-reliant. Therefore, it is crucial that we develop strategies to prevent muscle mass, strength and function loss and augment recovery in older (clinical) populations.

Nutrition and physical activity are the two main anabolic stimuli for muscle mass maintenance. In order to develop strategies to prevent muscle mass, strength and function loss, it is essential to know the current nutritional and physical status of clinical populations. In **chapters 2 and 3** we assessed energy and protein intake in relatively healthy patients, as well as older patients at risk of malnutrition. We showed insufficient levels of protein and energy intake during hospitalization. This clearly demonstrates the urgent need to improve the patients' food intake throughout hospitalization. In addition, more studies are needed to further investigate the relation between protein intake and muscle loss during hospitalization.

To understand what is happening at a skeletal muscle level during disuse, we, in **chapter 4**, assessed muscle mass and strength loss during short-term immobilization. Disuse models are used to mimic a hospitalized setting without the disease-burden. A short period of muscle disuse already resulted in a significant amount of muscle being lost. We provided individuals with a designed peptide with proposed anabolic properties or a milk protein concentrate. Ingestion of the novel peptide did not attenuate muscle mass or strength loss during short-term limb immobilization when compared with milk protein concentrate supplementation. Furthermore, supplementation did not augment the regain of muscle mass and strength during recovery. Interestingly, we did observe higher rates of muscle protein synthesis following the peptide supplementation. Though this did not result in more rapid muscle mass and/or strength regain with a short recovery period, it may be of interest in a setting of more prolonged recovery.

To better understand the effect of protein type on muscle growth, we compared protein digestion and amino acid absorption kinetics of an intact protein supplement versus a free amino acid mixture in healthy, young adults (**chapters 5**). We demonstrated that a free amino acid mixture is more rapidly digested and absorbed, but this did not result in differences in muscle protein synthesis rates between supplements. This implies that a more rapid digestion and absorption does not necessarily result in higher rates of muscle protein synthesis. However, the difference in protein digestion and amino acid absorption kinetics may be relevant in conditions where anabolic resistance is attributed to impairments in protein digestion

and/or amino acid absorption. Therefore, in **chapter 6** we assessed protein digestion and amino acid absorption kinetics in intensive care unit patients. We showed that administration of free amino acids results in more rapid and greater post-prandial plasma amino acid availability when compared to intact protein administration. Future work will assess the health benefits of greater post-prandial amino acid availability in these patients.

What is the contribution of the research results to science and society?

The results presented in this thesis contribute to the scientific field of clinical nutrition and skeletal muscle metabolism. They help to better understand the relation between protein intake and muscle metabolism. With this knowledge, researchers can develop and investigate more effective strategies to increase protein intake and improve muscle health in clinical populations. Those strategies will be implemented in clinical practice to improve food intake and prevent muscle loss in clinical populations. If we can attenuate or prevent muscle mass, strength and function loss, we may reduce hospital stay and shorten the time needed to recover from hospital admission(s). Furthermore, when older individuals leave the hospital in a fitter state (e.g. well-fed and with better muscle health), they will more rapidly regain the capacity to live independently for a longer period. This can reduce the burden on our healthcare system, as less individuals require (additional) care (e.g. nurses at home, first-line dietitians and physical therapists) and will ultimately improve quality of life.

To whom are the research results relevant?

Besides the relevance for the scientific society, the results of this thesis are of considerable interest to dietitians. Dietitians need to have a good understanding of the current nutritional state of patients to prescribe a fitting diet. Ideally, patients should be informed about the importance of (sufficient) protein intake and muscle mass maintenance prior to hospital stay. Therefore, not only dietitians working in hospitals will benefit from the research results, our data are also relevant for dietitians who advise patients prior to and during recovery from hospital admission.

Furthermore, the presented results are relevant to (health care) policy makers. We clearly demonstrated that most patients are malnourished during their hospital stay. It is essential to implement strategies to make sure patients are able to meet their nutritional needs. To change the current logistical food concept, investments need to be made to change the infrastructure and evaluate the food concept. The presented work is also of interest to the general public, as every individual should recognize the importance of consuming sufficient protein and maintaining muscle mass prior to, during and after hospitalization. In addition, the research results are also relevant for physicians, nurses and food assistants, as they need to be educated on the importance of (the lack of appropriate) nutrition in a clinical setting.

Lastly, new insights into the relationship between protein intake and muscle health are of interest to the food industry. New strategies will be developed to prevent the loss of muscle mass, strength and function during hospital stay, which requires input from food companies to produce healthy, protein-rich snacks that can be effectively applied in novel food concept for patients.

Several communication strategies are applied to inform the various relevant stakeholders. The results are or will be published in international, peer-reviewed journals. The studies have been presented at several conferences and symposia, which increases the visibility of the results and contributes to new insights and ideas for future research. In addition, the results have been communicated during seminars for dieticians and during general lectures to increase public awareness of the importance of sufficient protein intake and the attenuating of muscle mass.

Dankwoord

En dan het slotstuk van dit proefschrift, de meest gelezen pagina's. Promoveren doe je niet alleen, en ik had dit mooie traject nooit af kunnen ronden zonder de hulp van velen.

Allereerst, mijn promotor, Luc, als masterstudent kwam ik bij jou binnenlopen in het najaar van 2015, zonder éinig benul wat onderzoek doen eigenlijk écht inhoudt. In dat eerste gesprek heb je mij al het een en ander duidelijk gemaakt, maar tijdens de stage heb jij (samen met Imre) mij echt enthousiast gekregen voor het onderzoek. De mogelijkheid om de voedingsdata verzameld tijdens mijn stage te presenteren op een internationaal congres was de kers op de taart voor mijn stage. Direct was dit ook het startschot voor mijn PhD. In het gesprek daar in Kopenhagen gaf jij aan dat "iemand intrinsiek gemotiveerd moet zijn voor het onderzoek wat je doet. Het wordt ergens zwaar, en dan moet je daar aan terug kunnen denken." Ja, daar heb je zeker gelijk in gehad. Maar wat ben ik jouw dankbaar dat ik binnen jouw onderzoeksgroep een plekje heb gekregen om te mogen promoveren. Dankjewel voor jouw steun, kritische noot, en oneindige steun.

Dan mijn copromotor, Lex, waar ik met Luc vele gesprekken heb gehad, hebben wij elkaar iets minder gesproken. Toch stond jouw deur altijd open, en wist ik dat ik altijd bij jou terecht kon met vragen, twijfels of gewoon om even te sparren. Je bent altijd geïnteresseerd geweest in hoe de studies verliepen, en bij ieder manuscript heb ik met jou alle statistiek (meerdere malen vaak) besproken. Dankjewel voor je interesse, hulp en deur die altijd open stond.

To the members of the Assessment Committee, Prof. dr. Caren Faber, Prof. dr. Stef Kremers, Prof. dr. Daisy Jonkers, Prof. dr. Arthur van Zanten, en Prof. dr. Olav Rooyackers, thank you for taking the time to carefully evaluate this dissertation and for participating in the Supervisory Committee.

Floris en Lisanne, wat ben ik blij dat jullie allebei enthousiast ja zeiden op de vraag of jullie mijn paranimfen wilde zijn. Met jullie deel ik mijn liefde voor klinisch onderzoek. Lisanne, dankjewel voor de gesprekken, theepauzes, lunch-wandelingen, maar vooral de steun die je mij tijdens mijn PhD hebt gegeven. Floris, het heeft even geduurd, maar we hebben een weg naar elkaar gevonden. Wat heb ik het ontzettend fijn gevonden om met jou samen de FEBO studie uit te voeren. Midden in de nacht samen op OK staan schept toch een band. Dankjewel voor de fijne samenwerking, goede gesprekken, en oprechte interesse. In jullie beide zie ik een vriend voor het leven.

Imre, jij hebt mij met Luc samen enthousiast gekregen voor het (klinische) onderzoek. Jij hebt mij aan het begin van mijn promotietraject bij de hand genomen, en ik kon echt álles aan je vragen. Ik ben dankbaar voor alles wat je mij hebt geleerd wat betreft de uitvoering van humane (patiënt gerelateerde) onderzoeken, maar ook zeker wat er op een congres (na alle officiële sessies) gebeurd! Andy, I want to thank you for our collaboration during our immobilization trial. It was tough at some times, but you always reminded me about the very cool data we were collecting, and that it would all be worth it in the end (which is definitely true!). I appreciate all the scientific discussions we had, but you also thought me it's "hockey" and not "ice-hockey" and every time I hear "Still haven't found what I've been looking for" I will think of you... Same goes for "It's beginning to look a lot like Christmas", Cas, Andy, this is definitely our office song... En over die muziek..., Cas, serieus, ik ben zo verrast dat jouw oren nog altijd werken!

Damn, die techno muziek staat HARD! Ik zal het toch missen. Ook jouw lange lijst met bijnamen voor iedereen, vele anekdotes, en imitaties van accenten hebben voor de nodige lachbuien gezorgd. Guys, thanks for all the good conversations, laughs and a listening ear when necessary. I could not have asked for better roomies, I'll miss you.

Antoine, Cleo en Claudia, bedankt voor jullie gezelligheid, interesse en onuitputtelijke steun. Of het nu een borrel, carnaval, of de kerstmarkt was... het werd altijd later dan gepland. Uit het oog is niet uit het hart.

Dan mijn andere dierbare M3 collega's, wat vind ik het jammer dat mijn tijd met jullie erop zit. Bedankt voor alle gezellige M3-uitjes, etentjes, BBQ bij Luc in de achtertuin, gesprekken op kantoor of bij een borrel. Philippe, jij stond altijd klaar om mij weer van Excel advies te voorzien, of om mij helemaal terug te begeleiden op de fiets toen het toch nét wat te warm was. Joey, ik heb jouw nuchtere kijk op het (klinische) werk altijd kunnen waarderen. Stefan, bedankt voor jouw hulp bij de MILK-AA data. Joy, het was altijd gezellig om bij jou te buurten, of het nu op kantoor was, het SIRC (met de goede muziek!) of op de 4e in het ziekenhuis... bedankt voor alle antwoorden op mijn vragen en de (soms ook serieuze) gesprekken. Maarten en Kevin, wat hebben wij een gezellige tijd gehad tijdens onze stage in het 'hok' tegenover het kantoor van Imre, Cas en Andy. Wes, ook al kom jij soms niet stop, bedankt voor je hulp, interesse en oneindige gespreksonderwerpen. Jorn, de CaRe studie was een leuke ervaring en waar ik jou kennis heb laten maken met Within Temptation en Ed Sheeran, weet ik wie Peter Selie is... Joan, Janneau, Joy, Antoine en Annemie, bedankt voor al jullie hulp en input bij de vele metingen in plasma, spier, of ander weefsel. Ale, thanks for all your help with the presleep study. Milan, Thorben, I hope I will still receive an invite for a Mullins pubquiz. Also Glenn, Tim, Cindy, Kristin and Jean, thanks for the interest and chats. En Rob, ook al zat jij niet bij M3, jij ook bedankt voor de fijne samenwerking de afgelopen jaren.

Dan wil ik ook nog alle studenten bedanken die mij geholpen hebben bij mijn onderzoeken. Maud, David, Daniel, Guus, Mihkel, Debbie, Kevin, Dion, Ilse, Laura, Sterre, Damian, Casper, Lieke en Maud, zonder jullie hulp had ik nooit alle data (binnen dit tijdsbestek) kunnen verzamelen. Guus, jij bedankt voor al jouw inzet bij de immobilisatie studie.

Lieve HB collega's, dank voor de leuke dagjes weg, kerstdiners, paasbrunches, verjaardag-vlaai en 'gewone' praatjes op de gang. Hasibe, Wendy, en Loek, jullie bedankt voor de analytische ondersteuning. Kelly, jou wil ik nog extra bedanken, gelijk starten met je PhD en beide een grote verbouwing uitvoeren... het creëert toch een band. Dankjewel voor het fijne contact.

Dan wil ik ook de afdeling Diëtetik bedanken voor de interesse, vragen en hulp bij sommige onderzoeken in mijn proefschrift. In het bijzonder wil ik Phil en Maxime bedanken.

Phil, bij jou kon ik altijd binnenlopen om te sparren, klagen, lachen of huilen. Zonder jou was ik in 2015 niet bij Luc uitgekomen. "Als je iets met eiwit wilt doen voor je stage moet je wel met Luc gaan praten". Dankjewel voor deze link. Toch mooi dat je mijn promotie nog mee mag maken als hoofd Diëtetik. Maxime, jouw interesse, input en hulp tijdens de verschillende studies waardeer ik enorm. Jij hebt mij veel inzicht gegeven in de zorg wat een diëtist levert. We hebben het er al vaak over gehad, maar er is nog zo veel onderzoek nodig waarmee we de patiëntenzorg (m.b.t. voeding) kunnen verbeteren. Wat ben ik blij dat we de presleep studie samen hebben kunnen doen. Hopelijk is dit slechts het begin van nog vele studies in de toekomst.

En natuurlijk wil ik graag alle van alle patiënten en proefpersonen bedanken die ingestemd hebben om deel te nemen aan wetenschappelijk onderzoek. Zonder jullie had dit proefschrift er nooit kunnen komen!

Dan naar wat dichters bij huis...

Maud, wat heb jij mij veel aan moeten horen.. Enthousiaste verhalen, maar soms ook een heel klaagzang, als het toch even niet lekker liep. Dankjewel voor je luisterend oor en bemoedigende woorden. We zijn ver gekomen van 2e klas Trevi naar nu. Door dik en dun, in voor en tegenspoed... "some friendships age like fine wine".

Anique en Mandy, lieve schatten, ik hoef niet veel woorden op papier te zetten. Jullie weten hoe dierbaar jullie voor me zijn. Altijd daar als ik jullie nodig heb. BEDANKT.

Sanne, Luc, Tessa, Danny, Toby en Loes, bedankt voor de nodige afleiding met spelletjesavonden, weekendjes weg of speciaal bier-avonden. In het bijzonder wil ik Tessa nog bedanken. Dikke merci voor je hulp met het ontwerp en lay-out van mijn proefschrift! Ik heb het al een paar keer gezegd, maar ik ben er echt ontzettend blij mee!

Mieke, Hub., jullie zijn er ook altijd voor mij geweest. Jullie hebben in het jaar dat Tim en ik bij jullie zijn komen wonen veel mee gekregen van mijn promotietraject. Ik weet dat jullie niet altijd begrepen waarom ik weer zo lang bezig was, of weer op zaterdag en zondag naar Maastricht ging, maar bedankt voor al jullie begrip. Hub. ik weet dat jij ook heel trots zou zijn. In gedachten ben je erbij <3

Daan, wat heb jij mij veel aan de telefoon gehad... na het werk, in de auto... vaak heel enthousiast, maar soms, net zoals Maud, ook met een klaagzang, of met een heftig verhaal. Lieve Daniëlle, Roberto, dankjewel voor jullie interesse, begrip en tijd.

Pap, Mam, jullie hebben mij altijd gesteund om te doen wat ik leuk vindt. Ik hoop dat jullie weten hoe dankbaar ik jullie hiervoor ben! Ook al snapten jullie in het begin niet aan wat voor traject ik ging beginnen, jullie toonden altijd interesse (soms tot het vervelende aan toe... ja mam, ik ben nu EINDELIJK klaar) en stonden jullie altijd voor mij klaar. Wat ben ik blij dat ik deze mijlpaal met jullie mag vieren.

Tot slot, lieve Tim, waar te beginnen. Mijn rots in de branding, met jouw rust, kalmte en nuchtere kijk op het leven kon jij mijn stress (om vaak onbenullige dingen) altijd weerleggen. Zonder jou had ik dit traject niet kunnen afronden. De timing was niet ideaal, onze verbouwing combineren met het afronden van mijn promotietraject, maar we hebben het gered. Bedankt voor jouw onvoorwaardelijke steun, ik houd van jou. Op naar het volgende avontuur...

Curriculum Vitae

Michelle Weijzen was born on October 21, 1992 in Sittard, the Netherlands. She completed her secondary school at Trevianum Scholengroep in Sittard in 2011. She obtained her bachelor's degree in biomedical sciences at Maastricht University in 2014. In 2014, she continued at Maastricht University to obtain her MSc in Health Food Innovation Management in 2016. During her MSc, she conducted an internship in the Muscle Metabolism Maastricht (M3) group of Prof. Luc van Loon at the Department of Human Biology at Maastricht University. In October 2016, she started her PhD under the supervision of Prof. dr. Luc van Loon and Dr. Lex Verdijk, focusing on food intake in clinical populations. In the years that followed, she performed several human intervention studies that focused on protein and muscle metabolism in healthy and clinical populations. Michelle was awarded with international Emerging leader in Nutrition Science Award from the American Society for Nutrition (ASN), a Gatorade Sports Science Nutrition Award at the European Congress of Sports Science (ECSS), Best Abstract (top 5) Award and Travel Grant at European Society for Clinical Nutrition and Metabolism (ESPEN), and a poster prize at the Nationaal Voedingscongres. From November 2022 onwards, Michelle will be working as a Postdoctoral fellow with Prof. Frank Vandenabeele at the University of Hasselt.

Publications

Weijzen MEG, Kouw IWK, Verschuren AAJ, Muyters R, Geurts JA, Emans PJ, et al. Protein Intake Falls below $0.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ in Healthy, Older Patients Admitted for Elective Hip or Knee Arthroplasty. *The journal of nutrition, health & aging*. 2019;23(3):299-305. doi: 10.1007/s12603-019-1157-2.

Weijzen MEG, Kouw IWK, Geerlings P, Verdijk LB, van Loon LJC. During Hospitalization, Older Patients at Risk for Malnutrition Consume <0.65 Grams of Protein per Kilogram Body Weight per Day. *Nutr Clin Pract*. 2020 Aug;35(4):655-663. doi: 10.1002/ncp.10542.

Trommelen J, **Weijzen MEG**, van Kranenburg J, Ganzevles RA, Beelen M, Verdijk LB, van Loon LJC. Casein Protein Processing Strongly Modulates Post-Prandial Plasma Amino Acid Responses *In Vivo* in Humans. *Nutrients*. 2020 Jul 31;12(8):2299. doi: 10.3390/nu12082299.

Weijzen MEG, van Gassel RJJ, Kouw IWK, Trommelen J, Gorissen SHM, van Kranenburg J, Goessens JPB, van de Poll MCG, Verdijk LB, van Loon LJC. Ingestion of Free Amino Acids Compared with an Equivalent Amount of Intact Protein Results in More Rapid Amino Acid Absorption and Greater Postprandial Plasma Amino Acid Availability Without Affecting Muscle Protein Synthesis Rates in Young Adults in a Double-Blind Randomized Trial. *J Nutr*. 2021 Oct 12:nxab305. doi: 10.1093/jn/nxab305.

Submitted for peer review:

Weijzen MEG, Holwerda AM, Jetten GHJ, Houben LHP, Kerr A, Davis H, Keogh B, Khaldi N, Verdijk LB, van Loon LJC. No differences between Vicia Faba peptide network and milk protein supplementation on changes in muscle mass during short-term immobilization and subsequent remobilization, but increases muscle protein synthesis rates during recovery.

Oral presentations

2022: Symposium oncologie diëtisten: "Access to a pre-sleep protein snack increases daily energy and protein intake in hospitalized patients"

ESTROT congress, Maastricht, the Netherlands: "Trabecular, but not cortical, bone tissue protein synthesis rates in the femoral head are reduced following an intracapsular hip fracture"

ECSS congress, Seville, Spain: "Vicia Faba peptide supplementation does not differ from milk protein in modulating muscle mass during immobilization and recovery, but increases muscle protein synthesis rates during recovery"

Diëtisten Overleg Regio Maastricht (DORM) Sterk Samen: "Presleep studie in het MUMC+"

2020: Nutrition 2020, online: "Ingestion of free amino acids as opposed to intact protein increases amino acid absorption but does not further augment postprandial muscle protein synthesis rates" Emerging leader in nutrition science ESPEN congress, online: "Ingestion of a bolus of free amino acids is followed by more rapid amino acid absorption"

and greater postprandial plasma amino acid availability when compared to the ingestion of an equivalent amount of intact protein". Best abstract session (top 5) and travel grant winner

ECSS congress, online: "Ingestion of free amino acids as opposed to intact protein increases amino acid absorption but does not further augment postprandial muscle protein synthesis rates. Gatorade Sport Sciences Institute (GSSI), Sports Nutrition Award: 2nd price

2017: Dutch Nutritional Science days: "Distribution of protein consumption in hospitalized, malnourished, older patients"

Workshop Strategieconferentie: "Patiënt, voeding en bewegen"

Poster presentations

2022 ESPEN congress, Vienna, Austria: "Vicia Faba peptide supplementation does not differ from milk protein in modulating muscle mass loss during immobilization and recovery, but increases muscle protein synthesis rates during recovery"

2021: Science day MUMC+: "Food consumption is ~40% lower than food provision in older patients during long-term hospitalization"

2019: Annual NUTRIM day: "Ingestion of free amino acids as opposed to intact protein results in a great post-prandial rise in plasma amino acid availability"

2017 ESPEN congress, The Hague, The Netherlands: "Distribution of protein consumption in hospitalized, malnourished, older patients"

Nationaal voedingscongres: "Distribution of protein consumption in hospitalized, malnourished, older patients"

Annual NUTRIM day: "Food consumption is ~40% lower than food provision in older patients during long-term hospitalization"

2016: ESPEN congress, Copenhagen, Denmark: "Protein provision versus protein consumption of self-selected hospital meals: an observational study measuring food intake in older patients"

Financial support by the following companies for the publication of this dissertation is gratefully acknowledged

