STUDY PROTOCOL

Study N°	**2**
Massey RMS Code N°	
Massey RM	**PR96444 RNIEL**

How does the Digestible Indispensable Amino Acid Score (DIAAS) influence Protein Turnover in Older Adults? An Evaluation of the Health Efficacy Potential of Combinatorial Proteins.

Short title: **Combining Dietary Protein Sources to Improve Amino-Acid Digestibility and Net Protein Balance in Older Adults - The DIPO Study**

Date: **October 2023**
Version: **Original**
N° **1**
Ethics number: **19566**

Sponsor: Riddet Institute, Massey University
Private Bag 11 222, Palmerston North 4442
New Zealand
Phone: +64 69517295

TABLE OF CONTENTS

1 INVESTIGATOR(S) SIGNATURE(S) PAGE

PRINCIPAL INVESTIGATOR

Prof. David S. Rowlands, PhD
School of Sport, Exercise, & Nutrition
College of Health
Massey University, Auckland
Tel: +64 (0) 272099383
Email: D.S.Rowlands@massey.ac.nz

_____
Date:

_____
Signature

PRINCIPAL INVESTIGATOR

Robin D. Nielsen, PhD. Candidate
School of Sport, Exercise, & Nutrition
College of Health
Massey University, Auckland
Tel: +64 212874100
Email: R.Nielsen@massey.ac.nz

_____
Date:

_____
Signature

SUB- INVESTIGATORS

Assoc. Prof. John Harrison
Chemistry
Massey University, Auckland

_____
Date:

_____
Signature

SUB- INVESTIGATORS

Prof. Rozanne Kruger
School of Sport, Exercise, & Nutrition
College of Health
Massey University, Auckland

_____
Date:

_____
Signature

SUB- INVESTIGATORS

Dr. Suzanne Hodgkinson
Riddet Institute
Massey University
Palmerston North

_____
Date:

_____
Signature

SUB- INVESTIGATORS

Dr. Carlos Montoya
Ag Research
Palmerston North

_____
Date:

_____
Signature

I have read this Protocol and agree that it contains all the necessary details for this study. I will conduct the study as outlined herein and will complete the study within the time designated. By my signature, I agree to conduct this study in compliance with the Protocol, Written Informed Consent, IRB / IEC procedures, the Declaration of Helsinki, ICH Good Clinical Practices guidelines, and the applicable local regulations governing the conduct of clinical studies.

I will provide copies of the Protocol and all pertinent information to all individuals responsible to me who assist in conducting this study. I will discuss this material with them to ensure they are fully informed regarding the study product and the conduct of the study.

I will use only the Written Informed Consent form approved by the Independent Ethics Committee (IEC) ethics committee. I will fulfil all responsibilities for submitting pertinent information to the IEC responsible for this study.

2 SPONSOR TEAM SIGNATURES PAGE – Not Applicable for Current Application

PROJECT MANAGER

Name and Title
Institution
Address
Tel:
Email:

_____
Date:

_____
Signature

MEDICAL DIRECTOR

Name and Title
Institution
Address
Tel:
Email:

_____
Date:

_____
Signature

BIOSTATISTICIAN

Name and Title
Institution
Address
Tel:
Email:

_____
Date:

_____
Signature

CLINICAL DATA MANAGER

Name and Title
Institution
Address
Tel:
Email:

_____
Date:

_____
Signature

CLINICAL PROJECT MANAGER

Name and Title
Institution
Address
Tel:
Email:

_____
Date:

_____
Signature

SPONSOR

Name and Title
Institution
Address
Tel:
Email:

_____
Date:

_____
Signature

COUNTRY LEGAL REPRESENTATIVE

Name and Title
Institution
Address
Tel:
Email:

_____
Date:

_____
Signature

CONTRACT RESEARCH ORGANIZATION

Name
Address
Tel:
Email:

_____
Date:

_____
Signature

3 PROTOCOL AMENDMENT

Applicable Section(s) Page(s)	Original text	New / revised text	Reason for change
Protocol version N° 1 –			
Protocol Amendment N° 1 –			

4 STUDY SITES

COUNTRY / CITY	INVESTIGATOR NAME	INSTITUTION, PHONE, EMAIL
New Zealand / Auckland	David S. Rowlands	Massey University, +64 (0) 27 2099 383, D.S.Rowlands@massey.ac.nz

5 STUDY CONTACT INFORMATION

ROLE	NAME	ADDRESS, PHONE, EMAIL
Central Laboratory	Not applicable	
Monitors	Not applicable	
Clinical Research Organization	Not applicable	
Central storage	Not applicable	
Medical Safety Officer	Not applicable	
Clinical Safety Manager	Not applicable	
Product Manager	Not applicable	

6 ABBREVIATIONS - DEFINITIONS

AE	Adverse Event
AUC	Area Under the Curve
CRA	Clinical Research Associate (synonym: monitor)
CRF	Case Report Form
CPM	Clinical Project Manager
DIAAS	Digestible Indispensable Amino Acids Score
GCP	Good Clinical Practice
IAA	Indispensable Amino Acids
IEC	Independent Ethics Committee
IRB	Institutional Review Board
ITT	Intent-To-Treat
MP	Method and Procedure: providing all the information necessary to carry out activities efficiently, safely, and in an authorized manner.
NB	Net Protein Balance (protein synthesis minus protein degradation)
PD	Protein Degradation (i.e., protein breakdown)
PP	Per-Protocol
PS	Protein Synthesis
SAE	Serious Adverse Event
SOP	Standard Operating Procedure: (Providing all the information necessary to use equipment efficiently, safely, and in an authorized manner)
SUSAR	Serious Unexpected Suspected Adverse Reaction (see definition in chapter 19.4.)
SMF	Study Master File

7 SYNOPSIS

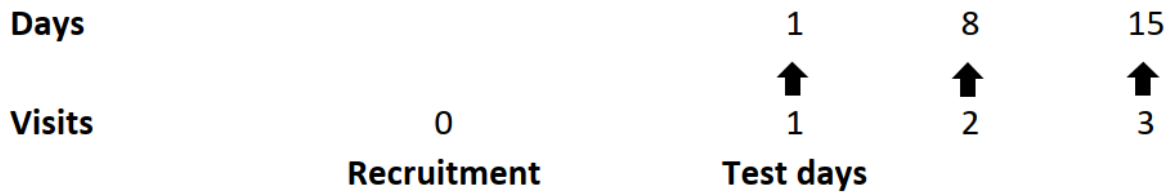
An innovative development in nutrition involves assessing dietary protein quality through the Digestible Indispensable Amino Acid Score (DIAAS). Compared to the traditional Protein Digestibility Corrected Amino Acid Score (PDCAAS), the DIAAS offers a more accurate approach to assessing dietary protein quality (FAO, 2013). The DIAAS is based on measuring the true ileal digestibility of each indispensable amino acid (IAA) using growing pig digesta model, which has been confirmed to predict human digestibility (Hodgkinson et al., 2022). It eliminates truncation of the protein quality score, allowing for a more accurate ranking system to identify dietary protein quality and complementarity within protein sources (Moughan, 2019; Bailey et al., 2019). The ratio between the first limiting IAA in a test protein with the corresponding IAA in a reference protein defines the DIAAS. The DIAAS score can be classified into one of three quality categories: <75 (no quality), 75-99 (high-quality), and ≥100 (excellent quality). A DIAAS score of ≥100 implies that the dietary protein source is effectively utilized and satisfies the estimated average requirement for protein (EAR: 0.66 g kg−1 d−1). However, the effect of DIAAS on whole-body protein turnover has yet to be verified in detail.

To answer the question of the influence of protein quality measured by the DIAAS on whole-body protein turnover and the efficacy potential of combinatorial proteins in a whole-foods matrix, we will conduct a nutritional crossover study to compare how incremental increases in protein quality, as defined by the FAO DIAAS criteria: 50% (no quality), 75% (high-quality), and ≥100% (excellent quality) affects protein kinetics in older men and women aged 67-77 years.

The study will compare 3 dietary protein interventions increasing in the DIAAS. The conditions will comprise meals with stepwise increases in DIAAS: 50%, 75%, and 100%. The protein-rich meals will be ingested orally on-site and be isocaloric (see Section 13.1). To measure splanchnic extraction, each meal will include oral ingestion of 84 mg of L-[^{15}N]-Phenylalanine (ingested with the juice). Administration and sampling will occur in an air-conditioned clinic room after an overnight fast between 7:00 and 9:00 am. The participants will follow a controlled dinner the evening before the interventional days. Infusion of stable isotopes L-[Ring-$^{2}H_5$]-Phenylalanine (Phe) and L-[Ring-$^{2}H_2$]-Tyrosine (Tyr) will be administrated over 5.5 hours to measure postprandial protein synthesis, breakdown, and protein net balance over 4 hours. Arterialized-venous blood will be collected from a hand vein at specified intervals for 5.5 hours (post-absorption and post-prandial) based on prior infusion studies.

Participants will remain rested during sampling and may do computer work, watch TV, or relax. There will be a minimum 1-week washout between trials. The 4-hour post-prandial response will be calculated as the area under the curve (AUC) using the post-absorptive concentration as the baseline reference. The AUC will then be divided by time to determine the average increase in post-prandial plasma concentration. We will calculate the whole-body PS, PB, and NB (NB = PS - PB) using the standard steady-state isotope dilution equations (Jonker et al., 2019). Outcomes will be analysed using mixed model analysis of variance (SAS, Cary, NC). Fixed effects will be meal condition and time (where relevant). Random effects will be subject to an unstructured covariance matrix to account for correlated data within the crossover. Data will be log-transform to improve linearity and model fit and to express outcomes as percent differences. Primary outcomes (PS, PB, and NB) will be referenced to our estimate of the smallest important clinical changes. Data will be presented as the least-squares mean and uncertainty (confidence interval) and, where useful, the effect size as a standardized mean difference with modified Cohen d effect size descriptors for the mean and breadth of the confidence interval. Results will be used to determine the influence of protein quality in a whole-food matrix defined by DIAAS and the effect of combining proteins to modify DIAAS on the primary health outcome of whole-body protein net balance governing lean mass homeostasis in older adults.

8 STUDY PLAN OVERVIEW



Study plan showing the four visits to the lab. Days 1 through 15 assume weekly testing for men and women.

9 INTRODUCTION

In recent years, there has been a growing interest in shifting global protein consumption towards more sustainable food production based on the assumption that animal protein production impacts the environment significantly (Espinosa-Marrón et al., 2022). However, reducing dietary proteins from animal sources could potentially lower the protein quality of most diets, which is of crucial concern, given the vital role of dietary protein in human growth, maintenance, and physiological function (Wu, 2016). Unlike other macronutrients, dietary protein comprises all the nitrogen and amino acids required for synthesizing muscles and organs, the production of enzymes and hormones, and the functioning of the immune system (Wu, 2009).

Amino acids can be categorized as dispensable (DAAs) or indispensable amino acids (IAAs) based on their ability to be synthesized de novo in the body. Only DAAs can be synthesized effectively in humans, and IAAs must be obtained through dietary protein sources. In this context, assessing dietary protein quality should be based on the digestibility, absorbability, and bioavailability of IAAs. An innovative development in nutrition involves assessing dietary protein quality is through the Digestible Indispensable Amino Acid Score (DIAAS). Compared to the traditional Protein Digestibility Corrected Amino Acid Score (PDCAAS), the DIAAS offers a more accurate approach to assessing dietary protein quality (FAO, 2013). The DIAAS is based on measuring the true ileal digestibility of each amino acid using growing pig digesta, which has been confirmed to predict human digestibility (Hodgkinson et al., 2022). It eliminates truncation of the protein quality score, allowing for a more accurate ranking system to identify dietary protein quality and complementarity (Moughan, 2019; Bailey et al., 2019). The ratio between the first limiting IAA (LimAA) in a test protein with the corresponding IAA in a reference protein derives from the DIAAS. The DIAAS score can be classified into one of three quality categories: <75 (no quality), 75-99 (high-quality), and ≥100 (excellent quality). A DIAAS score of ≥100 implies that the dietary protein source is effectively utilized and satisfies the estimated average requirement for protein (EAR: 0.66 g kg−1 d−1).

It has been shown that higher-quality proteins (beef sirloin, pork loin, eggs) induce a greater increase in whole-body protein balance compared to lower-quality proteins (tofu, kidney beans, peanut butter, mixed nuts) due to greater IAA availability (Park et al., 2021). Even when consumed isocaloric (500 kcal) and isonitrogenous (26g protein from eggs and cereal) (Kim et al., 2018). However, these findings did not account for equal amounts of IAAs content. These differences in protein turnover can be attributed to limiting IAAs in lower-quality proteins, as indicated by their DIAAS values, which have been shown in cell cultures to decrease global rates of protein synthesis (Vaughan et al., 1971; van Venrooij et al., 1972; Pain & Henshaw, 1975). In addition, since the limiting amino acid also limits the use of all other dietary amino acids for protein synthesis, the body must oxidize excess amounts of these amino acids (Bos et al., 2003; Tujioka et al., 2011; Luiking et al., 2005). In contrast, if one increases the dietary amount of the first limiting amino acid, protein synthesis will increase, and so will the utilization of the other dietary amino acids, reducing their oxidation (Gorissen et al., 2016). These findings align with recent research indicating that elevated peripheral levels of IAAs are linked to enhanced muscle and whole-body protein synthesis, especially following meals (Church et al., 2020). Once the limiting amino acid requirement is reached, further increases in dietary intake will cause no further increase in protein synthesis nor a decrease in the oxidation of the other IAAs (Gaudichon & Calvez, 2021; FAO, 2007). Despite our growing understanding of protein quality and advancements in measuring dietary protein quality through the DIAAS, the relationship between the DIAAS and protein turnover has yet to be verified in humans. This knowledge gap is significant as dietary protein is crucial for

maintaining health, preventing disease, and mitigating protein malnutrition (Manary et al., 2016; Layman et al., 2008).

Integrating the DIAAS is paramount for the aging population, as progressive loss of muscle mass, strength, and function, strongly predicts disability and mortality in older adults aged 50-86 (Su et al., 2022; Xu et al., 2021). Slowing or preventing the progression of sarcopenia is of utmost importance for maintaining or improving the quality of life for older adults. It is well established that dietary protein intake stimulates an anabolic response (Volpi et al., 2003; Wolfe et al., 2008) mainly via stimulating protein synthesis by IAAs, which is predominantly blunted in older adults. Hence, the importance of the DIAAS could prove valuable to ensure optimal IAA intake for older adults to support skeletal muscle mass retention and whole-body protein net balance. However, the relationship between the calculated DIAAS of a mixed meal and whole-body protein net balance has yet to be verified in this population.

Based on current research investigating whole-body protein kinetics, we hypothesize that higher-quality proteins (DIAAS ≥100%) elicit larger changes in whole-body protein balance compared to stepwise lower-quality proteins (i.e., DIAAS: 75% and 50%) and this follows the first limiting amino acid as all IAAs are required for synthesising new proteins.

10 OBJECTIVES OF THE STUDY

10.1 Primary objective

To determine the relationship between stepwise increases in DIAAS (protein quality) derived from modifications to combinatorial proteins within a whole-foods matrix and whole-body protein kinetics (PS + PB = NB) as a metric for lean tissue mass homeostasis in older adults.

10.2 Primary outcome

Post-prandial PS, PB, and NB responses.

10.3 Secondary objectives

To validate DIAAS as a metric of bioavailability of key and limiting amino acids in the plasma following meal ingestion from analysis of total plasma IAA, leucine, and lysine concentrations and total 4-h post-prandial AUC. To study the glycemic response to meals as a measure of health index and the insulin response as part of the mechanisms driving PS.

10.4 Secondary outcomes

Post-prandial plasma:

1. AUC for total IAA concentration
2. AUC leucine (anabolic trigger for protein synthesis)
3. AUC lysine (first limiting amino acid)
4. Insulin
5. Glucose

Sensory analysis in response to the meals using linear Likert scales postprandial. This will be measured at 0 minutes preprandial and 30, 90, and 240 minutes postprandial.

11 STUDY DESIGN

11.1 Type of Study

A randomized controlled trial with a crossover design (cRCT) comprising three arms in a clinical laboratory setting.

11.2 Subjects, groups, and centres

This study will recruit 8 older male and female participants (67-77 years of age; BMI ≥23 to ≤30 kg/m^2. Participants will be recruited from the local Auckland community via general advertisement at the University, University participant databases, social media, community notice boards, web pages, and word of mouth.

The study will be a single-center, lab-based study.

Dropouts, that is, participants who start and do not complete, are predicted from several levels:

1. Illness
2. Unexpected life events

With the estimated sample size of 8 participants, the conservative target recruitment into the study (working backward) should be 10 (25% dropout) based on recent experience. Participants who withdraw will be replaced with two analyses available: full dataset (includes any partially completed participants) and per Protocol.

11.3 Expected study duration and milestones.

Participant recruitment and commencement of the study will be approximately 30th July 2023 and will proceed until complete. All data collection is anticipated to be completed by 15th April 2024. Sample analysis is anticipated to be completed by Nov 2024. Statistical analysis and write up early 2025. The analysis components will continue (stated Feb 2023 with the preparation of plasma amino acid assay) and continue during data collection through total and stable-isotopically labelled amino acid assay, with samples run in batches to cluster between-run variability.

- October to November 2023 – study protocol development and organization.
- November 2023 to January 2024 – study protocol submission to the ethical committee.
- November-Maj 2023-24 - recruitment period.
- Late February 2024 to October-2024 - testing/data collection period.
- 1st October to late 2024 - data analysis, manuscript/thesis chapter preparation, and reporting.

12 STUDY POPULATION

12.1 Description

8 healthy older male and female participants (67-77 years of age; BMI ≥23 to ≤30 kg/m^2. Further definition is provided within the inclusion/exclusion criteria sections *12.3 and 12.4*.

12.2 Subject screening

Participants recruited will be individually screened via interview to establish availability and electability.

12.3 Subject inclusion criteria

All participants must comply with the following inclusion criteria:

- Men and women aged 67 to 77.
- BMI: ≥23 and ≤30
- HbA1c within the non-diabetic or pre-diabetic range of <40 mmol/mol.
- Sedentary to moderate physical activity.
- Obtained his/her informed consent.

12.4 Subject exclusion criteria

Participants representing one or more of the following criteria are excluded from participation in the study:

- Planning on leaving the city or proximity to participate in all 3 study arms for the entire study duration, or any other foreseen factor that may prevent completion of the study.
- Other foreseen factors that may prevent the completion of the study.
- Criteria-defined sedentary due to a precluding disability.
- Missing hands (for arterialized-venous blood sampling)
- Active malignancy (cancer) within the past six months.
- Unwilling to ingest meat alternatives.
- Allergy to experimental foods (i.e., gluten, lectin, and allergens).
- Any gastrointestinal disease or disorder that may affect the study outcomes.
- Gastrointestinal bypass surgery or congenital gastrointestinal issues.
- Chronic inflammatory disease (rheumatoid arthritis, psoriasis, psoriatic arthritis, Crohn's disease, ulcerative colitis, and ankylosing spondylitis).
- Taking supplements or medications that are thought to interfere with the study outcomes.
- Physical activity level (PAL) >2.25 (below vigorious physical activity).
- Currently participating or having participated in another clinical study during the last four weeks prior to the beginning of this study that may affect results.

12.5 Subject withdrawal criteria

The study participants are entitled to withdraw from the study at any time voluntarily and for any other reason without affecting their access to the treatment or their future treatment by the Investigator. In addition, a participant may be withdrawn from the study at any time for reasons including, but not limited to, the following:

- Investigator's medical decision when continuing the study would compromise the safety of the participant.
- The participant is unwilling or unable to adhere to protocol requirements.
- In the event of injury.
- Non-compliance with the dietary intervention protocols (pre-intervention control diet and interventional diet).
- Observation of adverse effects.
- Absence from more than two testing sessions.

Participants will be notified by the Investigator prior to withdrawal from the study/investigational product in writing with a full explanation for their withdrawal. Depending on the time of withdrawal from the study protocol, participants will be followed up as follows:

- Prior to or following participation in any trials, participants will be provided standard notification of reasons for withdrawal.

- During the trials, participants will be withdrawn following non-compliance to the interventional diets, inability to maintain a normal diet and allowed physical activity patterns, or any observed adverse effect. Participants' condition will be monitored for 24 hours following withdrawal if due to adverse effects.

Participants withdrawn during the study will be replaced in randomized sequence order until n=8 is complete or a maximum dropout replacement of 2 is met or exceeded.

13 STUDY INTERVENTION

13.1 Study intervention description

The intervention combinatorial proteins (CP) meals will comprise three isocaloric (810 kcal) and isonitrogenous (22g protein) with stepwise increases in DIAAS (DIAAS: 50%, 75%, and 100%). Intervention foods will be commercially bought and stored at the lab location. An in-depth overview of the macronutrient and caloric composition of the three meals is shown in Table 1.

- CP1: Chickpeas, Quinoa, Quorn, and Bread (DIAAS 105%)
- CP2: Chickpeas, Quinoa, Tofu, and Bread (DIAAS 75%)
- CP3: Chickpeas, Quinoa, and Bread (DIAAS 50%)

	CP1	**CP2**	**CP3**
Bread, g	62	147	230
Chickpeas, g	30	44	54
Quinoa, g	55	22	23
Quorn, g	104		
Tofu, g		26	
DIAAS, %	105	75	50
First limiting amino acid	Lysine	Lysine	Lysine
Protein, g	22	21	22
Protein, kcal	88	84	88
Ingested IAA, g	9	9	9
Digested IAA, g	8	8	8
Ingested Leu, g	1.6	1.6	1.6
Digested Leu, g	1.5	1.5	1.5
Ingested Lys, g	1.2	0.9	0.7
Digested Lys, g	1.1	0.8	0.5
Carbohydrates, g	131	132	131
Carbohydrates, kcal	524	528	524
Fats, g	22	22	22
Fats, kcal	198	198	198
Fiber, g	13	7	9
Total kcal	810	810	810

Table 1: An overview of the macronutrient and caloric composition for the intervention meals with an overview of ingested and bioavailable IAAs, Leu (leucine), and Lys (lysine).

13.1.1 Composition

The macronutrient composition of each meal will consist of ~810 kcal using juice (carbohydrates) and olive oil (fats) to balance the macronutrient profiles (amounts need is shown in Table 2). The macronutrient profile will be ~11% protein, ~65% carbohydrates, and ~24% fats, as recommended by the Institute of Medicine (Manore, 2005). All interventional meals will contain 6-13g of fiber which is within normal per-meal fiber range (Table 1).

CP1: Chickpeas, Quinoa, Quorn, and Bread (DIAAS 105)		
Baker's Mango and Passionfruit juice	245	ml
Olive oil	15	ml
CP2: Chickpeas, Quinoa, Tofu, and Bread		
Baker's Mango and Passionfruit juice	140	ml
Olive oil	13	ml
CP3: Chickpeas, Quinoa, and Bread		
Baker's Mango and Passionfruit juice	7	ml
Olive oil	12	ml

Table 2: An overview of the number of juice (carbohydrates) and olive oil (fats) to balance the macronutrient profiles for every experimental meal to reach ~810 kcal.

Form and dosage

The meals will be ingested orally on-site and calibrated to meet the per-meal protein requirement (0.27 g /kg) for light to moderate physically active individuals (FAO, 2001). As 80% of total energy is assumed to be derived from larger meals (snacks subtracted), the calculated per-meal protein requirement is: 1.0 g/kg/d x 0.80 (80%) = 0.80 g/kg/d. Assuming that three larger meals are consumed daily, 0.8 g/kg/d is divided by three = 0.27 g/kg/meal, resulting in 22g protein/meal for the average weight of the NZ population (80.5 kg) (MoH, 2017-2020: body size). The 1.0 g/kg/d to calculate the per-meal requirements was derived from reviewed literature for light to moderate physically active individuals (Park et al., 2021; Luiking et al., 2005; Tang et al., 2009; Volek et al., 2013; Pinckaers et al., 2022). The calculation of the per-meal IAA requirements was adjusted with a correction factor of 1.52 (1.0 g/kg/d divided by 0.66 g/kg/d = 1.52) (Table 3) as the FAO 2013 IAA requirements (mg/kg/d) is based on the 0.66 g/kg/d model, and a 1.0 g/kg/d model is used in this study.

IAAs	FAO (2013) IAA requirements, mg/kg/day	FAO (2013) IAA requirements, mg/day	FAO (2013) IAA requirements, mg/meal	IAA requirements (CF), mg/meal
Threonine	15	1208	322	488
Valine	26	2093	558	846
Isoleucine	20	1610	429	651
Leucine	39	3140	837	1268
Phenylalanine (AAA)	25	2013	537	813
Tyrosine (AAA)				
Histidine	10	805	215	325
Lysine	30	2415	644	976
Methionine (SAA)	15	1208	322	488
Cysteine (SAA)				
Tryptophan	4	322	86	130

Table 3: The numbers are based on an 80.5 kg individual. The corrected IAA requirements (mg/meal) are the FAO IAA requirements per meal multiplied by the correction factor (CF) 1.52. AAA: aromatic amino acids, SAA: sulfur amino acids, IAAs: indispensable amino acids.

13.1.2 Quality control

The investigators will perform quality control, ensuring that the food items are within the expiry dates and in good condition (no breaches). All foods will be commercially bought from the same brand and supplier throughout the study.

13.1.3 Packaging and labelling

The food items will be packed with the identity of treatment and frozen on-site or dry-stored depending on the food item need. Unique codes are placed on each food item according to the sequence code (*13.1.5*).

13.1.4 Randomization technique and coding

The participants and investigators will be aware of the treatment assignments. Three abbreviation codes will be applied. The codes will be (CP1) Quinoa, Quorn, chickpeas, and bread, (CP2) Chickpeas, quinoa, tofu, and bread, and (CP3) Chickpeas, quinoa, and bread. The abbreviations are CP1, CP2, and CP3. Participants will be allocated to sequences based on the Youden square design. The randomization list with participant number and the code sequence will be shared with the study investigators to select the abbreviation label for each participant on each intervention day.

13.2 Study product administration

13.2.1 Amount, composition, and preparation

As mentioned above, one of three meal conditions will be ingested once within 15 min, along with 300 ml of water, followed by blood sample collection. The investigators will prepare the meals on-site in a research kitchen under hygienic conditions. The amount of food being ingested will be 22g of protein, 132g of carbohydrates, and 22g of fats to meet the macronutrient composition stated in *13.1.1*.

CP1, CP2, and CP3 contain chickpeas, tofu (only CP2), and Quorn (only CP1) will also be pan-fried. Quinoa will be simmered in water until cooked (approx. 10 min). All pan-fried foods will be seasoned with salt and paper in small quantities. The bread included in all interventional meals will be heated in an oven at 200°C for 5-10 minutes.

Other interventional foods that makeup the isocaloric cut-off of 810 kcal will be served cold and as fluid (uncooked).

The controlled pre-intervention evening diet, consisting of dinner (pre-packed), snack (muesli bar), and fluid (300 mL water) will be commercially bought from the same supplier throughout the study. The macronutrient composition will be 25g of protein, 70g of carbohydrates, and 30g of fats translating to 560 kcal. Participants are allowed 133 ml/hour if male, and 122 ml/hour if female during the 5.5-hour infusion period.

13.2.2 Familiarization trials

N/A.

13.2.3 Route of administration

Meals will be ingested orally on-site under investigator supervision within 15 minutes.

13.2.4 Subject compliance

Arrival on time or within reasonable limits. Ingesting meals within 15 min. Maintaining a normal diet and physical activity pattern within the intervention period with no strenuous or vigorous physical activity two days before interventions and comply with the controlled diet the day before intervention.

13.3 Study product handling

13.3.1 Storage and distribution

The interventional food items will be stored in a freezer and dry-stored on-site with limited access.

13.3.2 Study product accountability and reconciliation

The Investigator agrees not to supply the test food items to anyone except the subjects participating in this study.

14 ASSESSMENT OF EFFICACY

Efficacy will be assessed upon completion of the data collection and full statistical report and will be addressed according to the primary and secondary objectives listed above.

15 ASSESSMENT OF SAFETY

Safety will be continuously assessed based on the AE/SAE reporting and medical supervision ensured by the Principal Investigator or a designated person.

16 CONDUCT OF THE STUDY

16.1 Subject recruitment

A total of 8 men and women (67-77 years of age) will be required to complete the study, with contingency for another 2 if required due to dropout. The target is 4 in each gender, with contingency to complete 5 and 3 of either gender combination, depending on recruitment. Participants will be recruited from the local Auckland community via general advertisement at the University, University participant databases, social media, community notice boards, web pages, and word of mouth. Each participant will have the study outline and requirements explained in detail. At this point, they will be allowed to ask questions regarding the study and answer a health questionnaire. An inclusion checklist will be completed after the subject has agreed to participate by signing the consent. Subjects will be enrolled after fulfilling all inclusion criteria and presenting none of the exclusion criteria.

16.2 Visit 0 (day 0)

In-person screening will be conducted during the visit (V0), and consent will be obtained. Afterward, baseline parameters (personal data, history, anthropometric measures, and HbA1c) will be recorded with instructions.

HbA1c will be analysed via a Cobas B 101 system with finger prick blood collection. This process enables the measurement of HbA1c levels, ensuring they fall within the designated inclusion range. All evaluations will take place after the signing of informed consent. In addition, each participant will be required to answer three questionnaires to collect data on habitual protein intake, attitudes towards consumption of meat and meat alternatives, and sustainable diet patterns.

16.3 Visit 1 (day 1)

It will comprise the completion of the first arm of the test protocol.

- Participants will report to the lab/clinic after an overnight fast between 7-9:00h.
- The lab environment will be air-conditioned at 21-22°C.
- Each participant will report at a consistent time across the study to account for circadian variability.
- The stable isotope infusion protocol will last for 5.5 hours, during which participants will remain in a semi-supine or seated position. A calibrated syringe infusion pump will administer a primed-constant continuous infusion of stable isotopes via a catheter inserted into the antecubital vein in one arm and a second catheter will be inserted in a collateral dorsal hand vein to obtain arterialized-venous blood samples following the positioning of the hand in a customized heated-hand box (overview 16.5). The primary outcome measure for this study will be the determination of net whole-body protein balance, which will be assessed using the stable isotopes L-[Ring-$^{2}H_5$]-Phenylalanine and L-[Ring-$^{2}H_2$]-Tyrosine. Infusion rates for L-[Ring-$^{2}H_5$]-Phenylalanine and L-[Ring-$^{2}H_2$]-Tyrosine will be 270 µmol/h^{-1} and 85.5 µmol/h^{-1}, respectively, with priming doses of 270 µmol and 85.5 µmol. To prime the phenylalanine-derived plasma tyrosine pool, a bolus dose of L-[Ring-$^{2}H_4$]-tyrosine will be administered (prime = 23.25 µmol) (Jonker et al., 2014; Jonker et al., 2019). To measure splanchnic extraction, each meal will include oral ingestion of 84 mg of L-[^{15}N]-Phenylalanine (half about halfway through meal and the rest at the end of the meal co-ingested with the juice). During the study at time point 0 minutes (preprandial), 30, 90, and 240 minutes (postprandial) the participants will be asked about sensory information in response to the meal on a 10 cm Likert scale. Participants are allowed 133 ml/hour if male, and 115 ml/hour if female during the 5.5-hour infusion period (Yamada et al., 2022).

16.4 Visits 2-3 (days approx. 8 to 15)

- A repeat of Visit 1 but with another randomized order of treatment, allowing for a minimum period of 7 days of washout between visits to minimize any carry-over effects.

16.5 Biological samples

Arterialized venous blood will be collected from a dorsal hand vein heated hand method in a Perspex chamber (~55°C air temperature) (Gallen and Macdonald 1990). Blood samples will be collected from a superficial dorsal vein of the hand and placed in a thermostatically controlled box to mimic arterial samples (time points for blood sample collection can be viewed in Figure 1). Triplicate arterialized-venous blood samples will be collected at -20, -10 and 0 minutes after the infusion begins to measure pre-prandial enrichment of amino acids and glucose and insulin plasma concentrations. Post-prandial measurements will be collected between time 0-240 minutes. Each intervention will involve a protein meal given 90 minutes after the start of the infusion, and approximately 140 mL of blood will be collected per intervention. An overview of the infusion protocol is provided below (Figure 1).

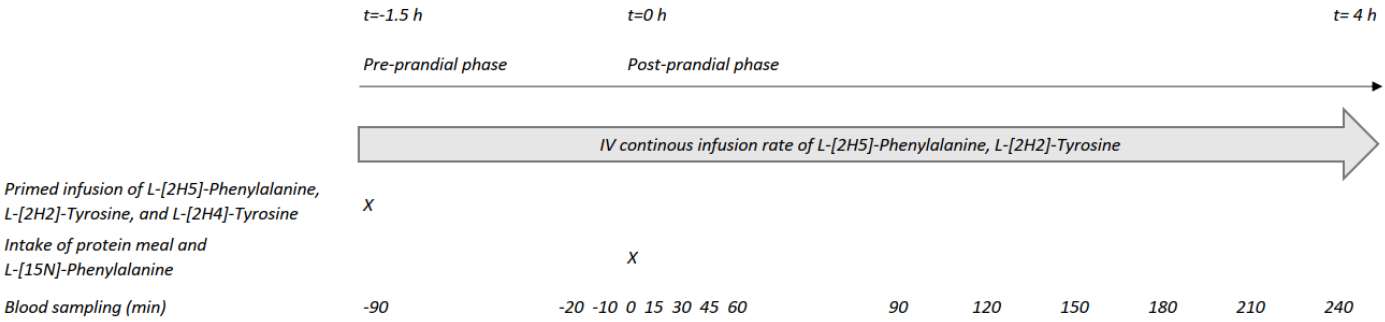


Figure 1. Overview of the study protocol. In randomized order, participants receive one of the five meals at time=0 (x). A primed continuous infusion of Phe and Tyr stable isotopic tracers is used to calculate whole-body protein synthesis, breakdown, and net balance of the 4-h post-prandial period.

Blood samples will be collected and placed in heparinized tubes to prevent clotting. To minimize potential reactions, the samples will be immediately placed on ice, followed by deproteinization of the blood plasma with Trichloroacetic (TCA) (Appendix 2), the remaining untreated blood plasma will also be collected. The deproteinized and untreated plasma will be frozen and stored at -80°C until further analysis. To analyse the samples, we will use a liquid chromatography-electrospray ionization-tandem mass spectrometry quadrupole-time of flight (LC/MS-QTOF) system to determine isotope tracer enrichments, concentrations, and insulin. The amino acid enrichment will be expressed as a tracer:tracee ratio corrected for natural background abundance. An automatic analyser will determine plasma glucose concentrations. We analyse the samples in batches to minimize potential variability, and all procedures follow established protocols and guidelines. Quality control measures will be taken throughout the analysis to ensure the accuracy and precision of the results. Additionally, some blood samples might be analysed overseas.

16.6 Follow-up

Following the completion of the study, each participant will be given a contact number at Massey University, where they can contact a member of the experimental team should any adverse effects develop.

17 DATA MANAGEMENT

17.1 Electronic data storage

Data collected are listed in sections 16.2 to 16.5.
The researchers will capture all data required in the Protocol into a secure web-based password-protected repository only accessible by the researchers. Hard copies of laboratory data sheets and mass spec output feed will be scanned and saved, and the hard copies will be saved in swipe-card access or locked office areas.

17.1.2 Access rights

Designated researchers will be provided with a username and password to access the study database. This username/password pair may be used by a single individual only; passwords must not be shared with anyone else.

17.2 Audit trail

The clinical data management systems developed for this study comply with Good Clinical Practice (GCP) predicate rule requirements, laws, and regulations (Personal data protection) and allows an audit of actions performed by users.

18 AUDIT OF ACTIONS PERFORMED BY USERS STATISTICS

18.1 Effects to be estimated.

Protein net balance parameters, amino acid concentration AUC, sensory Likert scales.

18.2 Sample size calculations

The sample size was determined from the only relevant available NB data of Kim et al., 2018 where the egg vs. cereal difference (expected effect size between no and excellent quality) was 7 g net protein accretion in 165 min of infusion time, and the standard error derived from the p-value of 1.53 g (Kim et al., 2018). With the smallest important change of 2.2 g protein, using traditional null hypothesis significance testing with 5% type-1 and 20% type-2 error rates, a sample size of 8 was required (Hopkins, 2006), which we felt provided sufficient precision for the 30% of the anticipated difference between high and excellent protein quality, relative to the no quality contrast.

18.3 Randomization

Treatment sequences will be randomly assigned to participants in the Youden square sequence for n=3 treatments.

18.4 Interim Analysis

Not applicable

18.5 Datasets to be analysed

18.5.1 Full analysis dataset

All participants provided at least 2 complete treatments.

18.5.2 PP analysis dataset

All participants providing a full set of 3 treatments finished.

18.5.3 Missing values and outliers

No imputations are foreseen. The mixed model will consider incomplete sequences as missing at random.

18.6 Statistical analysis

18.6.1 Primary analysis

Outcomes will be analysed using a mixed model analysis of variance. Fixed effects will be meal condition and time (where relevant). The random effect will be subject to an unstructured covariance matrix to account for correlated data within the crossover. Data will be log-transformed to improve linearity and model fit and to express outcomes as percent differences. Primary outcomes (PS, PD, and NB) will be referenced to our estimate of the smallest important clinical changes. Data will be presented as the least-squares mean and uncertainty (95% confidence interval) and, where useful, the effect size as a standardized mean difference.

18.6.2 Secondary analyses

The statistical analysis on secondary outcomes will be as for the primary, except sensory scale data will not be log-transformed.

19 HANDLING OF ADVERSE EVENTS

19.1 Definition: Adverse event

An adverse event is defined as any untoward occurrence in a patient or clinical investigation subject administered an investigational product and which does not necessarily have to have a causal relationship with this treatment.

Adverse events are illnesses, signs, or symptoms (including an abnormal laboratory finding) occurring or worsening during the study. Adverse events can be serious or non-serious. They may or may not lead to the withdrawal of the subject/patient from the study. All reported adverse events must be documented and assessed for a relationship to the study.

Investigators must know and record the following information about adverse events:

- Subject and date
- Description of event
- Reporting source
- Suspect product
- Duration
- Frequency
- Intensity
- Seriousness
- Action taken
- Outcome and sequel
- Relationship to test product

19.2 Intensity

Mild: Symptoms hardly perceived, only slight impairment of general well-being.

Moderate: Clearly noticeable symptom, but tolerable without immediate relief.

Severe: Overwhelming discomfort.

19.3 Seriousness

A serious adverse event is any untoward medical occurrence at any dose:

- results in death,
- is life-threatening,
- requires inpatient hospitalization or prolongation of existing hospitalization,
- results in persistent or significant disability/incapacity,
- is a congenital anomaly/congenital disability, or
- is otherwise medically significant.

A non-serious event is all other adverse events not corresponding to the definition of a serious adverse event.

19.4 Unexpected or expected SAE

The evaluation of expectedness is based on current knowledge and applicable product information and will be assessed by the researchers. An unexpected AE is an AE in which the nature, severity, or frequency is inconsistent with information about the condition under study and/or inconsistent with information on the investigational product.

All adverse events suspected to be related to an investigational product, both unexpected and serious, are considered SUSARs (Suspected Unexpected Serious Adverse Reactions). A SUSAR is to be reported to the regulatory authority on short notice.

19.5 Relation to study

The reporting healthcare professional will assess the possibility of a link between the study and an adverse event based on the following criteria:

Unrelated:

- An adverse event that is not related to interventional administration.

Unlikely:

- The temporal relationship to product administration makes a causal relationship improbable.
- Other drugs, chemicals, or underlying diseases explain the event more plausibly.

Probable:

- There is a reasonable time relationship to product administration.
- The event is unlikely to be attributed to concurrent disease, drugs, or chemicals.
- The event should follow a clinically plausible response on withdrawal (dechallenge).
- Rechallenge information is not required to fulfil this definition.

Related:

- There is a plausible time relationship to product administration.
- It cannot be explained by concurrent disease or other drugs or chemicals.
- The response to the withdrawal of the product (dechallenge) should be clinically plausible.
- Confirmation must include a satisfactory rechallenge procedure.

19.6 Reporting and Documentation

Serious adverse event

In the scope of this study, the PI or designee should enter any Serious Adverse Events (SAE) in the EDC system within 24 hours of knowledge. Once an AE is considered serious and the corresponding SAE form has been captured, an email will be sent to the ethics committee and any appropriate University Committee.

Notification does not depend on whether there is a causal relationship with the study.

Non-serious adverse event

Adverse events must be documented for inclusion in study reporting and records.

19.7 Follow up

All SAEs must be followed up until the SAE outcome is known.

A follow-up visit may be required if an SAE(s) persist beyond study termination. Further analyses are required to evaluate a potential cause-effect relationship between the study and the adverse event. In that case, all examinations, laboratory analyses, and their results will be documented in the case report forms or an attached file. Any SAE occurring within 30 days after the last study product intake will be similarly reported within 24 hours.

19.8 Notification

The University, under the Principal Investigator, is responsible for the ongoing safety evaluation of the investigational product(s).

The Principal Investigator should promptly notify all concerned and the regulatory authority(ies) of findings that could adversely affect subjects' safety, impact the study's conduct, or alter the IRB/IEC's approval/favourable opinion to continue the study.

20 CONCOMITANT DIET AND TREATMENT

20.1 Permitted concomitant diets/treatments/medications

Participants will be allowed to consume habitual foods, if they avoid taking food other than their standard diet. However, they must comply with a control diet the evening before the test days.

20.2 Unauthorized concomitant diets/treatments/medications

Any special diet affecting nutrient metabolism (nutritional supplements, multivitamins, etc.) will not be permitted in the 15 days preceding and during the study.

No concomitant treatments or prescribed medications that may affect gastrointestinal function, kidney, skeletal muscle, or liver function that are thought to affect study outcomes are allowed other than painkillers.

20.3 Concomitant diets/treatments/medications record

All concomitant special diets, treatments, or medication will be recorded in the source documents and transposed into the database.

21 REGULATORY AND ETHICAL PREREQUISITES

21.1 Competent Authority requirements

The study will be conducted according to the relevant legal requirements.

21.2 IRB / IEC requirements

21.2.1 Ethics Committee and Registered Clinical Trial Approval

The Investigator will submit the study protocol for examination to the Institutional Review Board (IRB) / Independent Ethics Committee (IEC). The trial will be registered with the Australian New Zealand Clinical Trials Registry (ANZCTR). Commencement of the clinical study is not permitted without the written approval of the ethics committee and ANZCTR.

The IRB / IEC must be notified of all subsequent additions or changes in the study protocol.

21.2.2 Protection of the subject's confidentiality

Confidentiality of all study participants will be maintained; codes for subject identification will be utilized.

21.2.3 Written Informed Consent

Written Informed Consent will be obtained from the potential subject prior to any study-related activities and in accordance with all applicable regulatory requirements.

The Investigator and/or his/her designee will inform the subject, in addition to the Written Informed Consent, about all aspects of the subject's study participation. The written Informed Consent must be approved by the competent Ethic Committee and competent regulatory authority if applicable. Any amendments to these documents must be approved by the competent Ethic Committee and competent regulatory authority if applicable.

The Investigator and/or his/her designee and the subject must sign and date the Written Informed Consent before any study-related activities are performed. The subject must complete the printed name and enter the date of signature themselves

The ICF will be signed in double, and the subject will obtain one original of the signed Written Informed Consent. The second original is filed with the study documents at the investigational site.

The decision to participate in the study is entirely voluntary by the subject. The Investigator and/or his/her designee must emphasize to the subject that the consent to participate can be withdrawn at any time without penalty or loss of benefits to which the subject is otherwise entitled.

21.2.4 Declaration of Helsinki

This study will be conducted according to the principles and rules laid down in the Declaration of Helsinki (Appendix 1) and its subsequent amendments.

22 QUALITY CONTROL AND QUALITY ASSURANCE

22.1 Monitoring – No Monitoring is Applicable for the current study

An appropriate monitoring visit by sponsor representatives will be made during the study. All detailed monitoring information is described in the related Monitoring Plan.

Monitoring will begin with an initiation visit prior to study commencement to clarify all aspects of the Protocol and documentation. The purpose of later visits during the implementation period will be to evaluate the study's progress and adherence to Protocol. The monitor will check CRFs for completeness, clarity, and consistency with the information in the subject's file (source data checking). At the end of the study, the monitor will make a study closing visit to all sites to ensure that all documentation is complete. In all cases, it is the responsibility of the CPM / monitors to maintain subject confidentiality.

Protocol deviations will be reported in the monitoring report, and the corresponding corrective action plan will be implemented. The main protocol deviations that will be looked for are:

- Written Informed Consent process not adequately performed;
- Violation of Inclusion / Exclusion criteria;
- Non-compliance with IP storage, dispensation, allocation, use, or return requirements;
- Sampling procedures incorrectly performed;
- Intake of unauthorized concomitant diets/treatments/medications;
- SAE and AE reporting requirements not followed;
- Study visit schedule not followed;
- Any other GCP non-compliance

The monitor will communicate any detected protocol deviation to the Investigator reported in a protocol deviation form. The signed form will be sent to the Clinical Project Manager.

22.2 Source documents

N/A

22.3 Quality Control

22.3.1 Quality control of essential documents

The research study team will ensure quality control of essential documents.

22.4 Audits and inspections

In addition to the routine monitoring procedures, IRB/IEC and other University audits and inspections may occur.

22.5 Responsibilities of Investigator

The investigators are responsible for the following:

- Obtaining the written and dated approval of the local ethics committee (and other local regulatory agencies, if any) prior to the beginning of the study.
- Selection of participants in accordance with the inclusion and exclusion criteria; obtaining the Written Informed Consent of the subject or legal guardian.
- Maintain confidentiality of subjects and potential subjects in accordance with the Declaration of Helsinki.
- Adherence to the study protocol and the spirit of Good Clinical Practice. If a modification becomes necessary, the rationale will be provided in a protocol amendment signed by the Investigator and sponsor for submission to the ethics committee.

- Accurate, complete, and timely data reported to the Riddet Institute.
- During the study, provide subjects with any information that may be relevant to them.
- Identification of adverse events with notification to the University, ethics committee, and health authorities, as applicable.
- Co-operation with monitoring visits, audits, and regulatory inspections. Providing direct access to source data and documents.
- Investigators may select a second contact at their study center to assist in implementing the study. However, in all cases, the main responsibility for all aspects of this implementation rests with the principal and co-investigators.
- Archiving of the Investigator's file (including all subjects' original signed Written Informed Consent forms) for at least ten years after the study's end or termination.

23 STUDY END PROCEDURES

23.1 Premature termination of the study

Should it be necessary to discontinue the study permanently before completion, the investigators will notify the IRB / IEC / ANZCTR of the rationale.

23.2 Termination of study

After the study's completion or termination, the Investigator will inform the Ethical Committee and Clinical Trials Register of the end of the study. A certificate of study closure will be established.

The study products will be destroyed after the last subject's last visit and in agreement with the Product Manager. Certificates of destruction will be issued and filed in the Study Master File.

The remaining biological samples will be destroyed after the final statistical report or publication in agreement with the Project Manager.

REFERENCES

- BAILEY, H. M. & STEIN, H. H. 2019. Can the digestible indispensable amino acid score methodology decrease protein malnutrition. Anim Front, 9, 18-23.
- BOS C, METGES CC, GAUDICHON C, PETZKE KJ, PUEYO ME, MORENS C, EVERWAND J, BENAMOUZIG R, TOMÉ D. Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. J Nutr. 2003 May;133(5):1308-15. doi: 10.1093/jn/133.5.1308. PMID: 12730415.
- CHURCH DD, HIRSCH KR, PARK S, KIM I-Y, GWIN JA, PASIAKOS SM, WOLFE RR, FERRANDO AA. Essential Amino Acids and Protein Synthesis: Insights into Maximizing the Muscle and Whole-Body Response to Feeding. Nutrients. 2020; 12(12):3717. https://doi.org/10.3390/nu12123717
- ESPINOSA-MARRÓN, A., ADAMS, K., SINNO, L., CANTU-ALDANA, A., TAMEZ, M., MARRERO, A., BHUPATHIRAJU, S. N., & MATTEI, J. (2022). Environmental Impact of Animal-Based Food Production and the Feasibility of a Shift Toward Sustainable Plant-Based Diets in the United States. Frontiers in Sustainability, 3. https://doi.org/10.3389/frsus.2022.841106
- FAO, 2001. Human Energy Requirements: Report of a Joint FAO/ WHO/UNU Expert Consultation. Food and Nutrition Bulletin, vol. 26, no. 1, 2005, p. 166.
- FAO, 2007. Joint WHO/FAO/UNU Expert Consultation. Protein and amino acid requirements in human nutrition. World Health Organ Tech Rep Ser. 2007;(935):1-265, back cover. PMID: 18330140.
- FAO, 2013. Dietary protein quality evaluation in human nutrition. Report of an FAQ Expert Consultation. FAO Food Nutr Pap. 2013;92:1-66. PMID: 26369006.
- GAUDICHON C, CALVEZ J. Determinants of amino acid bioavailability from ingested protein in relation to gut health. Curr Opin Clin Nutr Metab Care. 2021 Jan;24(1):55-61. doi: 10.1097/MCO.0000000000000708. PMID: 33093304; PMCID: PMC7752214.
- GORISSEN, S. H. M., HORSTMAN, A. M. H., FRANSSEN, R., CROMBAG, J. J. R., LANGER, H., BIERAU, J., RESPONDEK, F., & VAN LOON, L. J. C. (2016). Ingestion of wheat protein increases in vivo muscle protein synthesis rates in healthy older men in a randomized trial. Journal of Nutrition, 146(9), 1651–1659. https://doi.org/10.3945/jn.116.231340
- HODGKINSON, S. M., STROEBINGER, N., VAN DER WIELEN, N., MENSINK, M., MONTOYA, C., HENDRIKS, W. H., DE VRIES, S., STEIN, H. H. & MOUGHAN, P. J. 2022. Comparison of True Ileal Amino Acid Digestibility between Adult Humans and Growing Pigs. J Nutr, 152, 1635-1646.
- Hopkins WG. (2006). Sample sizes for magnitude-based inferences about clinical, practical or mechanistic significance (Abstract 2746). Medicine & Science in Sports & Exercise 38, S528-S529
- JONKER, R., DEUTZ, N. E. P., ERBLAND, M. L., ANDERSON, P. J., & ENGELEN, M. P. K. J. (2014). Hydrolyzed casein and whey protein meals comparably stimulate net whole-body protein synthesis in COPD patients with nutritional depletion without an additional effect of leucine co-ingestion. Clinical Nutrition,33 (2), 211–220. https://doi.org/10.1016/j.clnu.2013.06.014
- JONKER R, DEUTZ NEP, SCHOLS AMWJ, VELEY EA, HARRYKISSOON R, ZACHRIA AJ, ENGELEN MPKJ. Whole body protein anabolism in COPD patients and healthy older adults is not enhanced by adding either carbohydrates or leucine to a serving of protein. Clin Nutr. 2019 Aug;38(4):1684-1691. doi: 10.1016/j.clnu.2018.08.006. Epub 2018 Aug 16. PMID: 30150004; PMCID: PMC6377853.
- KIM, I. Y., SHIN, Y. A., SCHUTZLER, S. E., AZHAR, G., WOLFE, R. R., & FERRANDO, A. A. (2018). Quality of meal protein determines anabolic response in older adults. Clinical Nutrition, 37(6), 2076–2083. https://doi.org/10.1016/j.clnu.2017.09.025
- LAYMAN, D. K., CLIFTON, P., GANNON, M. C., KRAUSS, R. M. & NUTTALL, F. Q. 2008. Protein in optimal health: heart disease and type 2 diabetes. Am J Clin Nutr, 87, 1571S-1575S.
- LUIKING YC, DEUTZ NE, JÄKEL M, SOETERS PB. Casein and soy protein meals differentially affect whole-body and splanchnic protein metabolism in healthy humans. J Nutr. 2005 May;135(5):1080-7. doi: 10.1093/jn/135.5.1080. PMID: 15867285.the RESMENA project. Nutrition, 30, 424-9.
- MANARY, M., CALLAGHAN, M., SINGH, L. & BRIEND, A. 2016. Protein Quality and Growth in Malnourished Children. Food Nutr Bull, 37 Suppl 1, S29-36.

- MANORE, MELINDA M. PHD, RD. Exercise and the Institute of Medicine Recommendations for Nutrition. Current Sports Medicine Reports 4(4):p 193-198, August 2005. | DOI: 10.1097/01.CSMR.0000306206.72186.00
- MOUGHAN, P. J. 2019. Measuring protein's punch: DIAAS is the new PDCAAS, The world of food ingredients, October/November 2019.
- MOUGHAN PJ. Population protein intakes and food sustainability indices: The metrics matter. Global Food Security. 2021/06/01/ 2021;29:100548. doi:https://doi.org/10.1016/j.gfs.2021.100548
- PAIN VM, HENSHAW EC. Initiation of protein synthesis in Ehrlich ascites tumour cells. Evidence for physiological variation in the association of methionyl-tRNAf with native 40-S ribosomal subunits in vivo. Eur J Biochem 1975;57(2):335–42.
- PARK, S., CHURCH, D. D., SCHUTZLER, S. E., AZHAR, G., KIM, I. Y., FERRANDO, A. A. & WOLFE, R. R. 2021. Metabolic Evaluation of the Dietary Guidelines' Ounce Equivalents of Protein Food Sources in Young Adults: A Randomized Controlled Trial. J Nutr, 151, 1190-1196.
- PINCKAERS, P. J. M., HENDRIKS, F. K., HERMANS, W. J. H., GOESSENS, J. P. B., SENDEN, J. M., JMX, V. A. N. K., WODZIG, W., SNIJDERS, T. & LJC, V. A. N. L. 2022. Potato Protein Ingestion Increases Muscle Protein Synthesis Rates at Rest and during Recovery from Exercise in Humans. Med Sci Sports Exerc, 54, 1572-1581.
- SU YC, CHANG SF, TSAI HC. The Relationship between Sarcopenia and Injury Events: A Systematic Review and Meta-Analysis of 98,754 Older Adults. J Clin Med. 2022 Oct 31;11(21):6474. doi: 10.3390/jcm11216474. PMID: 36362701; PMCID: PMC9654071.
- TANG, J. E., MOORE, D. R., KUJBIDA, G. W., TARNOPOLSKY, M. A. & PHILLIPS, S. M. 2009. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. J Appl Physiol (1985), 107, 987-92.
- TUJIOKA K, OHSUMI M, HAYASE K, YOKOGOSHI H. Effect of the quality of dietary amino acids composition on the urea synthesis in rats. J Nutr Sci Vitaminol (Tokyo). 2011;57(1):48-55. doi: 10.3177/jnsv.57.48. PMID: 21512291.
- VAN VENROOIJ WJ, HENSHAW EC, HIRSCH CA. Effects of deprival of glucose or individual amino acids on polyribosome distribution and rate of protein synthesis in cultured mammalian cells. Biochem Bio-phys Acta 1972;259(1):127–37.
- VAUGHAN MH, PAWLOWSKI PJ, FORCHHAMMER J. Regulation of protein synthesis initiation in HeLa cells deprived of single essential amino acids. Proc Natl Acad Sci USA 1971;68:2057–61
- VOLEK, J. S., VOLK, B. M., GOMEZ, A. L., KUNCES, L. J., KUPCHAK, B. R., FREIDENREICH, D. J., ARISTIZABAL, J. C., SAENZ, C., DUNN-LEWIS, C., BALLARD, K. D., QUANN, E. E., KAWIECKI, D. L., FLANAGAN, S. D., COMSTOCK, B. A., FRAGALA, M. S., EARP, J. E., FERNANDEZ, M. L., BRUNO, R. S., PTOLEMY, A. S., KELLOGG, M. D., MARESH, C. M. & KRAEMER, W. J. 2013. Whey protein supplementation during resistance training augments lean body mass. J Am Coll Nutr, 32, 122-35.
- VOLPI, E.; KOBAYASHI, H.; SHEffiELD-MOORE, M.; MITTENDORFER, B.; WOLFE, R.R. Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. Am. J. Clin. Nutr. 2003, 78, 250–258. DOI: 10.1093/ajcn/78.2.250
- Yamada Y, Zhang X, Henderson MET, Sagayama H, Pontzer H, Watanabe D, Yoshida T, Kimura M, Ainslie PN, Andersen LF, Anderson LJ, Arab L, Baddou I, Bedu-Addo K, Blaak EE, Blanc S, Bonomi AG, Bouten CVC, Bovet P, Buchowski MS, Butte NF, Camps SG, Close GL, Cooper JA, Cooper R, Das SK, Dugas LR, Eaton S, Ekelund U, Entringer S, Forrester T, Fudge BW, Goris AH, Gurven M, Halsey LG, Hambly C, El Hamdouchi A, Hoos MB, Hu S, Joonas N, Joosen AM, Katzmarzyk P, Kempen KP, Kraus WE, Kriengsinyos W, Kushner RF, Lambert EV, Leonard WR, Lessan N, Martin CK, Medin AC, Meijer EP, Morehen JC, Morton JP, Neuhouser ML, Nicklas TA, Ojiambo RM, Pietiläinen KH, Pitsiladis YP, Plange-Rhule J, Plasqui G, Prentice RL, Rabinovich RA, Racette SB, Raichlen DA, Ravussin E, Redman LM, Reilly JJ, Reynolds RM, Roberts SB, Schuit AJ, Sardinha LB, Silva AM, Sjödin AM, Stice E, Urlacher SS, Valenti G, Van Etten LM, Van Mil EA, Wells JCK, Wilson G, Wood BM, Yanovski JA, Murphy-Alford AJ, Loechl CU, Luke AH, Rood J, Westerterp KR, Wong WW, Miyachi M, Schoeller DA, Speakman JR; International Atomic Energy Agency (IAEA) Doubly Labeled Water (DLW) Database Consortium. Variation in human water turnover associated with environmental and lifestyle

factors. Science. 2022 Nov 25;378(6622):909-915. doi: 10.1126/science.abm8668. Epub 2022 Nov 24. PMID: 36423296; PMCID: PMC9764345.

- XU J, WAN CS, KTORIS K, REIJNIERSE EM, MAIER AB. Sarcopenia Is Associated with Mortality in Adults: A Systematic Review and Meta-Analysis. Gerontology. 2022;68(4):361-376. doi: 10.1159/000517099. Epub 2021 Jul 27. PMID: 34315158.
- WOLFE, R. R.; MILLER, S.L.; MILLER, K.B. Optimal protein intake in the elderly. Clin. Nutr. 2008, 27, 675–684. DOI: 10.1016/j.clnu.2008.06.008
- WU, G. 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids, 37, 1-17.
- WU, G. 2016. Dietary protein intake and human health. Food & Function, 7, 1251-1265.

APPENDIX 1

DECLARATION OF HELSINKI

WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI
Ethical Principles for Medical Research Involving Human Subjects

Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the:
29th WMA General Assembly, Tokyo, Japan, October 1975
35th WMA General Assembly, Venice, Italy, October 1983
41st WMA General Assembly, Hong Kong, September 1989
48th WMA General Assembly, Somerset West, Republic of South Africa, October 1996
52nd WMA General Assembly, Edinburgh, Scotland, October 2000
53th WMA General Assembly, Washington 2002 (Note of Clarification on paragraph 29 added)
55th WMA General Assembly, Tokyo 2004 (Note of Clarification on Paragraph 30 added)
59th WMA General Assembly, Seoul, October 2008

A. INTRODUCTION

1. The World Medical Association (WMA) has developed the Declaration of Helsinki as a statement of ethical principles for medical research involving human subjects, including research on identifiable human material and data.

   The Declaration is intended to be read as a whole and each of its constituent paragraphs should not be applied without consideration of all other relevant paragraphs.

2. Although the Declaration is addressed primarily to physicians, the WMA encourages other participants in medical research involving human subjects to adopt these principles.

3. It is the duty of the physician to promote and safeguard the health of patients, including those who are involved in medical research. The physician's knowledge and conscience are dedicated to the fulfilment of this duty.

4. The Declaration of Geneva of the WMA binds the physician with the words, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act in the patient's best interest when providing medical care."

5. Medical progress is based on research that ultimately must include studies involving human subjects. Populations that are underrepresented in medical research should be provided appropriate access to participation in research.

6. In medical research involving human subjects, the well-being of the individual research subject must take precedence over all other interests.

7. The primary purpose of medical research involving human subjects is to understand the causes, development and effects of diseases and improve preventive, diagnostic and therapeutic interventions (methods, procedures, and treatments). Even the best current interventions must be evaluated continually through research for their safety, effectiveness, efficiency, accessibility, and quality.

8. In medical practice and in medical research, most interventions involve risks and burdens.

9. Medical research is subject to ethical standards that promote respect for all human subjects and protect their health and rights. Some research populations are particularly vulnerable and need special protection. These include those who cannot give or refuse consent for themselves and those who may be vulnerable to coercion or undue influence.

10. Physicians should consider the ethical, legal, and regulatory norms and standards for research involving human subjects in their own countries as well as applicable international norms and standards. No national or international ethical, legal, or regulatory requirement should reduce or eliminate any of the protections for research subjects set forth in this Declaration.

B. BASIC PRINCIPLES FOR ALL MEDICAL RESEARCH

11. It is the duty of physicians who participate in medical research to protect the life, health, dignity, integrity, right to self-determination, privacy, and confidentiality of personal information of research subjects.

12. Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and adequate laboratory and, as appropriate, animal experimentation. The welfare of animals used for research must be respected.

13. Appropriate caution must be exercised in the conduct of medical research that may harm the environment.

14. The design and performance of each research study involving human subjects must be clearly described in a research protocol. The Protocol should contain a statement of the ethical considerations involved and should indicate how the principles in this Declaration have been addressed. The Protocol should include information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest, incentives for subjects and provisions for treating and/or compensating subjects who are harmed as a consequence of participation in the research study. The Protocol should describe arrangements for post-study access by study subjects to interventions identified as beneficial in the study or access to other appropriate care or benefits.

15. The research protocol must be submitted for consideration, comment, guidance, and approval to a research ethics committee before the study begins. This committee must be independent of the researcher, the sponsor, and any other undue influence. It must take into consideration the laws and regulations of the country or countries in which the research is to be performed as well as applicable international norms and standards but these must not be allowed to reduce or eliminate any of the protections for research subjects set forth in this Declaration. The committee must have the right to monitor ongoing studies. The researcher must provide monitoring information to the committee, especially information about any serious adverse events. No change to the Protocol may be made without consideration and approval by the committee.

16. Medical research involving human subjects must be conducted only by individuals with the appropriate scientific training and qualifications. Research on patients or healthy volunteers requires the supervision of a competent and appropriately qualified physician or other health care professional. The responsibility for the protection of research subjects must always rest with the physician or other health care professional and never the research subjects, even though they have given consent.

17. Medical research involving a disadvantaged or vulnerable population or community is only justified if the research is responsive to the health needs and priorities of this population or community and if there is a reasonable likelihood that this population or community stands to benefit from the results of the research.

18. Every medical research study involving human subjects must be preceded by careful assessment of predictable risks and burdens to the individuals and communities involved in the research in comparison with foreseeable benefits to them and to other individuals or communities affected by the condition under investigation.

19. Every clinical trial must be registered in a publicly accessible database before recruitment of the first subject.

20. Physicians may not participate in a research study involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians must immediately stop a study when the risks are found to outweigh the potential benefits or when there is conclusive proof of positive and beneficial results.

21. Medical research involving human subjects may only be conducted if the importance of the objective outweighs the inherent risks and burdens to the research subjects.

22. Participation by competent individuals as subjects in medical research must be voluntary. Although it may be appropriate to consult family members or community leaders, no competent individual may be enrolled in a research study unless he or she freely agrees.

23. Every precaution must be taken to protect the privacy of research subjects and the confidentiality of their personal information and to minimize the impact of the study on their physical, mental, and social integrity.

24. In medical research involving competent human subjects, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail, and any other relevant aspects of the study. The potential subject must be informed of the right to refuse to participate in the study or to withdraw consent to participate at any time without reprisal. Special attention should be given to the specific information needs of individual potential subjects as well as to the methods used to deliver the information. After ensuring that the potential subject has understood the information, the physician or another appropriately qualified individual must then seek the potential subject's freely-given informed consent,

preferably in writing. If the consent cannot be expressed in writing, the non-written consent must be formally documented and witnessed.

25. For medical research using identifiable human material or data, physicians must normally seek consent for the collection, analysis, storage and/or reuse. There may be situations where consent would be impossible or impractical to obtain for such research or would pose a threat to the validity of the research. In such situations the research may be done only after consideration and approval of a research ethics committee.

26. When seeking informed consent for participation in a research study the physician should be particularly cautious if the potential subject is in a dependent relationship with the physician or may consent under duress. In such situations the informed consent should be sought by an appropriately qualified individual who is completely independent of this relationship.

27. For a potential research subject who is incompetent, the physician must seek informed consent from the legally authorized representative. These individuals must not be included in a research study that has no likelihood of benefit for them unless it is intended to promote the health of the population represented by the potential subject, the research cannot instead be performed with competent persons, and the research entails only minimal risk and minimal burden.

28. When a potential research subject who is deemed incompetent is able to give assent to decisions about participation in research, the physician must seek that assent in addition to the consent of the legally authorized representative. The potential subject's dissent should be respected.

29. Research involving subjects who are physically or mentally incapable of giving consent, for example, unconscious patients, may be done only if the physical or mental condition that prevents giving informed consent is a necessary characteristic of the research population. In such circumstances the physician should seek informed consent from the legally authorized representative. If no such representative is available and if the research cannot be delayed, the study may proceed without informed consent provided that the specific reasons for involving subjects with a condition that renders them unable to give informed consent have been stated in the research protocol and the study has been approved by a research ethics committee. Consent to remain in the research should be obtained as soon as possible from the subject or a legally authorized representative.

30. Authors, editors, and publishers all have ethical obligations with regard to the publication of the results of research. Authors have a duty to make publicly available the results of their research on human subjects and are accountable for the completeness and accuracy of their reports. They should adhere to accepted guidelines for ethical reporting. Negative and inconclusive as well as positive results should be published or otherwise made publicly available. Sources of funding, institutional affiliations and conflicts of interest should be declared in the publication. Reports of research not in accordance with the principles of this Declaration should not be accepted for publication.

C. ADDITIONAL PRINCIPLES FOR MEDICAL RESEARCH COMBINED WITH MEDICAL CARE

31. The physician may combine medical research with medical care only to the extent that the research is justified by its potential preventive, diagnostic or therapeutic value and if the physician has good reason to believe that participation in the research study will not adversely affect the health of the patients who serve as research subjects.

32. The benefits, risks, burdens, and effectiveness of a new intervention must be tested against those of the best current proven intervention, except in the following circumstances:

    - The use of placebo, or no treatment, is acceptable in studies where no current proven intervention exists; or
    - Where for compelling and scientifically sound methodological reasons the use of placebo is necessary to determine the efficacy or safety of an intervention and the patients who receive placebo or no treatment will not be subject to any risk of serious or irreversible harm. Extreme care must be taken to avoid abuse of this option.

33. At the conclusion of the study, patients entered into the study are entitled to be informed about the outcome of the study and to share any benefits that result from it, for example, access to interventions identified as beneficial in the study or to other appropriate care or benefits.

34. The physician must fully inform the patient which aspects of the care are related to the research. The refusal of a patient to participate in a study or the patient's decision to withdraw from the study must never interfere with the patient-physician relationship.

35. In the treatment of a patient, where proven interventions do not exist or have been ineffective, the physician, after seeking expert advice, with informed consent from the patient or a legally authorized representative, may use an unproven intervention if in the physician's judgment it offers hope of saving life, re-establishing health, or alleviating suffering. Where possible, this intervention should be made the object of research, designed to evaluate its safety and efficacy. In all cases, new information should be recorded and, where appropriate, made publicly available.

22.10.2008

APPENDIX 2

Laboratory Quality Control handbook Center for Translational Research in Aging and Longevity		
Last update:	**Description**	**SOP 1010**
2018_0221 2021_0119	Preparation of plasma for LC-MS analysis of amino acids in TCA matrix	

Chemicals: Trichloroacetic acid, 99 %

Materials: Eppendorf Safe-Lock Tubes, 1.5 mL

A. Preparation of trichloroacetic acid (TCA) matrix cups for <u>100 µl plasma</u>:

1. Weigh 5.0 grams of TCA.
2. Add 10 mL of the Milli-Q water to TCA*
3. Transfer accurately **10 µl** of the TCA solution into an Eppendorf cup (Check your pipet upfront).
4. Close the cup
5. Mark the cup on the cap with a red dot
6. Store the TCA cups at -20°C until usage.
7. **For 50µl plasma: make TCA cups with 5µl TCA solution**
8. **For 250 µl plasma: make TCA cups with 25µl TCA solution.**
9. **For 500 µl plasma: make TCA cups with 50µl TCA solution.**

B. Blood preparation

(all steps need to be carried out at 4°C and within one hour after blood collection):

1. Take 200 (**or resp 100, 500,1000**) µl of Li-heparinized whole blood.
2. Centrifuge the blood for 5 min at 8000 g and 4°C.
3. Transfer 100 (**or resp 50, 250, 500**) µl supernatant (=plasma) into a TCA cup.
4. Vortex good until a 'milky' look.
5. Freeze the samples in liquid nitrogen or dry-ice
6. Store the samples at –80°C, until analyses.

*The resulting TCA solution can be reported in manuscripts as 33.3% (w/w). The final volume after dissolving TCA is quite a bit more than 10 mL. When measured on 1/19/2021, it was 12.5 +/- 0.1 mL. Based on 12.5 mL, it is (5 * 100/12.5 =) 40% (*w/v*), instead, but preferred is to report it as 33.3% (w/w). The formula weight of TCA is 163.39 g/mol. Thus, the molarity *before adding plasma* is (400 g/L / 163.39 g/mol = 2.4481) 2.45 M. After adding 10 volumes of plasma, it is (2.4481 mol/L / 11 =) 223 mM. Some TCA will serve as counterions for proteins, thus, <223 mM in supernatant.