UNIVERSITY OF COPENHAGEN
Faculty of Health and medical sciences

PhD Thesis

Jacob Bülow

The Ageing Skeletal Muscle:
Effects of Training and Protein supplementation

Institute of Sports Medicine Copenhagen, Bispebjerg Hospital

Primary supervisor: Professor Michael Kjær

Co-Supervisors: Professor Lars Holm and Professor Søren Balling Engelsen

The thesis has been submitted to the Graduate School of the Faculty of Health and Medical Sciences, University of Copenhagen March 31st, 2020

Table of content

1.0 Preface

This thesis is written on data derived from the interdisciplinary CALM-study which was conducted at the Institute of Sports Medicine at Bispebjerg Hospital from 2014-2018. It is written in order to obtain the PhD degree from the Faculty of Health and Medical Sciences at the University of Copenhagen.

I started my Ph.D. in 2017 where the inclusion of participants was completed. During my 3-years I have had the primary responsibility for the daily management in completing the study. Due to the large-scale and interdisciplinary nature of the CALM-study, I have been involved in many different tasks varying from conducting the human experiments and the laboratory work of preparing and analyzing blood and muscle tissue samples to communicate and collaborate with scientist from very different fields such as chemistry, agriculture, physiology, ethnologist and anthropologist.

The work presented in this thesis investigates the effect of 12 months of supplementation with proteins of different qualities and different types of training on the maintenance of skeletal muscle mass, glucose tolerance, skeletal muscle protein synthesis and the skeletal muscle metabolome within healthy elderly. Further, it uses the interdisciplinary nature of the CALM study design for the exploration of the relationship between the metabolism of the aging skeletal muscle and general measurements of metabolic health.

The work conducted is published or expected to be published in the 4 following papers:

-Paper I: **Jacob Bülow**, Stanley J. Ulijaszek, Lars Holm
Rejuvenation of the term Sarcopenia, DOI: 10.1152/japplphysiol.00400.2018

-Paper II: Kenneth H. Mertz, Søren Reitelseder, Rasmus Bechshoeft, **Jacob Bulow**, Grith Højfeldt, Mikkel Jensen, Simon R. Schacht, Mads Vendelbo Lind, Morten A. Rasmussen, Ulla R. Mikkelsen, Inge Tetens, Søren B. Engelsen, Dennis S. Nielsen, Astrid P. Jespersen, Lars Holm
The effect of daily protein supplementation with or without resistance training for 1 year on muscle size, strength and function in healthy older adults.
A Randomized Clinical Trial

-Paper III: **Jacob Bülow**, Mie Cecilie Faber Zillmer, Grith Højfeldt, Rasmus Bechshøft, Jakob Agergaard, Peter Schjerling, Lars Holm
Recommended long-term nutritional supplementation, irrespective of quality and additional training does not affect glucose tolerance differently than carbohydrate supplementation in healthy elderly: the CALM cohort

-Paper IV: **Jacob Bülow**, Bekzod Khakimov, Søren Reitelseder, Rasmus Bechshøft, Søren Balling Engelsen, Lars Holm
The effect of long-term nutritional supplementation with or without different types of training on the skeletal muscle protein synthesis rate and metabolome in healthy elderly: the CALM study.

In addition, I have been involved in the following spin-off papers which are not included in this thesis:

J.L. Castro-Mejía, B. Khakimov, Ł. Krych, **J. Bülow**, R.L. Bechshøft, G. Højfeldt, K.H. Mertz, E.S. Garne, S.R. Schacht, H.F. Ahmad, W. Kot, L.H. Hansen, F.J.A. Perez-Cueto, M.V. Lind, A.J. Lassen, I. Tetens, T. Jensen, S. Reitelseder, A.P. Jespersen, L. Holm, S.B. Engelsen & D.S. Nielsen. *Physical fitness in community dwelling older adults is linked to dietary intake, gut microbiota and metabolomic signatures*, *Aging Cell* (2020), e13105. (DOI: https://doi.org/10.1111/acel.13105)

Schacht SR, Lind MV, Mertz KH, **Bülow J**, Bechshøft R, Højfeldt G, Schucany A, Hjulmand M, Sidoli C, Andersen SB, Jensen M, Reitelseder S, Holm L, Tetens I. *Development of a Mobility Diet Score (MDS) and Associations With Bone Mineral Density and Muscle Function in Older Adults. Front Nutr. 2019 Sep 4; 6:114.* (DOI: 10.3389/fnut.2019.00114)

Funding for the project was provided by: *P.Carl Petersens foundation, Arla Foods Ingredients Group P/S, University of Copenhagen Excellence Program 2016 (UCPH-CALM), Region Hovedstaden*

2.0 Acknowledgements

I would like to thank my principal supervisor Michael Kjær for letting me be a part of the inspiring and exceptional research environment that you have created at the Institute of Sports Medicine at Bispebjerg and for introducing me to Lars holm 8 years ago.

I would also like to express my gratitude to all of my co-supervisors. Lars Holm, from the first day I went into your office 8 years ago as a young medical student, you have continued to inspire me with you enthusiastic and positive nature. You have always taken the time to critically discuss any of my ideas and supported me in pursuing the best of them. To you I owe most of what I have accomplished so far within research. Søren Balling Engelsen, I am grateful for the support you gave on my interest in conceptual ideas and the philosophy of science when it was most needed. Thank you for introducing me to the world of chemometrics and the multivariate nature of biology and for letting me be a part of your very inspiring department. With regards to the CALM study, I would like to thank all the participants and all of the scientific staff who have been involved. Especially, I want to express my gratitude towards those of you who were part of the CALM office as long as it lasted. A special thanks to Grith for all the (sometimes) good intellectual and personal discussion and for never abandoning me despite of my sometimes annoying nature. Søren Reitelseder, I cannot thank you enough for all that you have done for me. You have taught me how to run experiments properly, the joy of running and so many other things during the last 8 years. Kenneth, thank you for always listening to all of my stories and complaining's and for all the good scientific discussion through the last 7 years. For all the help that I received from all of the secretaries and especially Else and Maria I am very grateful. I would also like to thank both Camilla and Anja for all the help in the lab and for making the long days of sample preparation full of good stories. Bekzod Khakimov, thank you for all you help with setting up the measurements at Science and analyzing the data. You made my time at Science very fun and fruitful. Lastly, I would like to thank all of my family for always supporting me and cheering me up in the darkest hours. To Emilie, thank you for being a constant reminder of all the good that I have in my life, your patience with my fluctuating mood and for your ability to always make me smile and laugh at the end of long day. To Otto and Augusta, you have not made anything easier but thankfully you have made everything more meaningful.

Jacob Bülow, Copenhagen March 31st

3.0 List of abbreviations

AA	Amino acids
ASCA	ANOVA-simultaneous component analysis
ASM	Appendicular skeletal muscle mass
ASMI	Appendicular skeletal muscle mass index
AUC	Area under the curve
BCAA	Branch chain amino acids
BIA	Bio impedance analysis
CALM	Counter-acting Age-related Loss of Muscle mass
CSA	Cross-sectional area
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
DXA	Dual-energy x-ray absorptiometry
EAA	Essential amino acids
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
FBR	Fractional breakdown rate
FEV1	Forced Expired Volume in the first second
FSR	Fractional synthesis rate
FVC	Forced Expiratory Volume
GC-MS	Gas chromatography – mass spectrometry
GC-C-IRMS	Gas chromatography- combustion- isotope ratio mass spectrometry
HOMA	Homeostasis model assessment
ITT	Intention to treat
LBM	Lean body mass
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MPT	Muscle protein turnover
MRI	Magnetic resonance imaging
MSI-CE-MS	Multisegment injection-capillary electrophoresis-mass spectrometry
NB	Net balance
NNR	Nordic nutritional recommendations
OGTT	Oral glucose tolerance test
PCA	Principal component analysis
PP	Per protocol
qCSA	Quadriceps cross sectional area
RM	Repetition maximum
ROI	Region of interest
RT	Resistance training
SARC-F	Strength, Assistance walking, Rise from a chair, Climb stairs, and Falls screening tool
SD	Standard deviation
SEM	Standard error of the mean
SPPB	Short physical performance battery
UPLC-MS	Ultra performance liquid chromatography – mass spectrometry
WAT	White adipose tissue
WHO	World Health Organization

4.0 Abstracts

The phenomenon of Sarcopenia, originally defined as the age-related loss of muscle mass, has gained interest across several different scientific disciplines within the last three decades due to its association to increased morbidity and mortality. Several different explanations as well as interventions to counteract the sarcopenic process has been suggested and investigated in both epidemiological and short-term interventions studies. Especially different types of exercise and increased daily protein intakes has been investigated as strategies with promising results in the pursuit of maintaining skeletal muscle mass with age. However, most of these studies have been highly controlled and of shorter duration hereby questioning whether or not these results would be rediscovered in more applied studies of longer duration.

First, this thesis presents an analysis of the changes in the definition of Sarcopenia that has occurred since its introduction in 1989 and its consequences; Secondly, this thesis report different outcomes from the CALM study which is a large interdisciplinary study designed to investigate the effect of one-year of protein supplementation with or without different types of training in healthy older adults above 65 years of age. 208 healthy elderly above 65 years of age were randomized into one of following 5 groups: (CARB (20g of maltodextrin + 10g of sucrose) COLL (20g of collagen hydrolysate + 10g of sucrose) WHEY (20g of whey hydrolysate +10g of sucrose) LITW (Home-based light intensity training 3-5 times pr. week + the whey supplement) and HRTW (Center-based heavy resistance exercise 3 times pr. week+ the whey supplement). The participants were instructed to take the supplements twice daily at breakfast and lunch or in relation to their training sessions. The primary outcome in the CALM study were the cross-sectional area of the quadriceps muscle of the mid-thigh. Further, the effect of the different intervention on the glucose tolerance and body composition was evaluated. In a subgroup (n=66) of the 208 participants included, the effect of the intervention on the skeletal muscle protein synthesis in response to protein intake were also investigated. Lastly, we attempted to measure the skeletal muscle metabolome and exploratorily investigate the effect of the intervention.

The current definitions of Sarcopenia due no longer cover the phenomenon of age-related loss of muscle mass but are now including the two partially depending phenomena of muscle strength and physical function. No scientifically acceptable argument for the change in definition is forwarded and the primary argument of clinical relevance is tautological. Therefore, we suggest a return to the original definition.

We did not find any effect of one-year prolonged supplementation with proteins in comparison to an iso-caloric carbohydrate supplement with respect to the maintenance of skeletal muscle mass. The participants increased their body weight, fat% and HbA1c and no effect on the muscle protein synthesis nor the muscle metabolome were observed. One-year of prolonged heavy resistance exercise on top of protein supplementation had a minor effect on the maintenance of skeletal muscle mass in comparison to protein supplementation alone and home-based light load resistance exercise had no effect despite an observed higher adherence in comparison to the adherence in the heavy resistance exercise group.

In conclusion, the findings across the different measurements presented in this thesis were consistent and provide strong evidence against further increases in recommended daily protein intake within healthy and active elderly. It provides solid evidence for making realistically estimates of the long-term effects of resistance exercise in combination with protein supplementation. Further, light load resistance training is not enough if gains in muscle mass and function are desired within this population of healthy and active elderly. The results from the acute trial suggest that the measurement of muscle protein synthesis without measuring breakdown is not a usable method for investigating muscle development over time. Lastly, our analysis platform for measuring the skeletal muscle metabolome showed promising results for future studies.

5.0 Dansk resumé

I løbet af de foregående tre årtier er interessen for fænomenet Sarkopeni, oprindeligt defineret som alders-relateret tab af muskelmasse, inden for adskillige forskellige videnskabelige discipliner steget markant pga. fænomenets påviste sammenhæng med øget risiko for morbiditet og mortalitet. Flere forskellige forklaringer på fænomenet samt forslåede interventioner til at modvirke den sarkopene proces er blevet undersøgt i epidemiologiske og interventions studier af kortere varighed. Især forskellige typer af træning samt en øgning i det daglige protein indtag er blevet undersøgt med positive resultater i forsøget på at modvirke det alders-relaterede tab af muskelmasse. Størstedelen af de tidligere studier er dog yderst kontrollerede og af kortere varighed, og det er derfor stadig uvist, hvorvidt disse fund kan overføres til længevarende studier af mere repræsentativ karakter for dagligdagen hos ældre.

Denne afhandling præsenterer først en analyse af ændringerne i definitionen af fænomenet Sarkopeni der er forekommet siden begrebets introduktion i 1989 og de heraf følgende konsekvenser. Dernæst rapporteres forskellige resultater fra CALM-studiet, som er et stort interdisciplinært studie designet til at undersøge effekten af 1 års daglig proteintilskud med og uden træning i raske aktive ældre. 208 raske ældre blev randomiseret til én af de følgende fem grupper: CARB (20g maltodextrin + 10g sukrose) COLL (20g kollagen hydrolysat + 10g sukrose) WHEY (20g valle hydrolysat +10g sukrose) LITW (Hjemmebaseret let styrketræning 3-5 gange ugentligt + the tilskuddet) og HRTW (Centerbaseret tung styrketræning 3 gange ugentligt + valle tilskuddet). Deltagerne blev instrueret i at indtage tilskuddet to gange dagligt i forbindelse med morgenmad og frokost eller i forbindelse med træningssessionerne. Det primære endemål for CALM -studiet var ændringer i tværsnitsarealet af m. quadriceps målt midt på låret. Yderligere undersøgte vi effekten af interventionen på glukosetolerancen samt kropskompositionen. I en undergruppe bestående af 66 ud af de 208 deltagere undersøgte vi effekten af interventionen på muskelproteinsynteseresponset til protein indtag i akutte studier før og efter interventionen. Derudover forsøgte vi at måle muskelmetabolomet samt foretage en eksplorativ analyse af, hvorvidt interventionen havde en effekt på dette.

De nuværende definitioner af Sarkopeni dækker ikke længere udelukkende over fænomenet alders-relateret tab af muskelmasse men inkluderer nu yderligere de to delvist afhængige fænomener muskelstyrke of fysisk funktionalitet. Der er ikke fremført nogle videnskabelige acceptable argumenter for ændringen i definitionen, og argumentet angående de

nye definitioners kliniske relevans, hviler på tautologisk argumentation. På baggrund af dette, foreslår vi at gå tilbage til den oprindelige definition af fænomenet.

Vi fandt ingen effekt at 1-års proteintilskud i sammenligning med et isokalorisk kulhydratstilskud i forhold til bibeholdelse af muskelmasse. Deltagerne øgede deres kropsvægt, fedt% samt HbA1c som en effekt af tiden, og vi fandt ingen ændringer i hverken muskelproteinsyntesen eller muskelmetabolomet. Vi fandt en lille effekt at 1-års tung styrketræning i kombination med dagligt proteintilskud på bibeholdelsen af muskelmasse i sammenligning med dagligt proteintilskud alene, og hjemmebaseret let styrketræning havde ingen effekt på trods af en højere grad af adhærens til interventionen i forhold til den tunge styrketrænings gruppe.

Fundene i CALM-studiet var konsistente henover de forskellige mål, og de samlede resultater fremviser således solid evidens mod yderligere stigninger i det anbefalede daglige indtag af protein for raske aktive ældre. Yderligere giver resultaterne grobund for realistiske estimater for langtidseffekten af tung styrketræning. Derudover viser disse resultater at let hjemmebaseret styrketræning ikke er nok, hvis man vil øge muskelmassen i en population af raske aktive ældre. Resultaterne fra akutstudierne viser at muskelproteinsyntesemål uden samtidige mål af proteinnedbrydning ikke er en brugbar metode til at undersøge udviklingen i muskelmasse henover tid. Endvidere viste vores anvendte metode til at måle muskelmetabolomet lovende resultater med henblik på brugbarheden i fremtidige studier.

6.0 Introduction

As a kid I wakened each morning
with the world streaming toward me
like a sparkling boulevard.
Now I have to get up in winter darkness
and bring it in bit by bit
on my thin legs

Søren Ulrik Thomsen[1]

The poem by Søren Ulrik Thomsen beautifully expresses the essence of old age, with the comparison of the easiness and velocity of youth with the laborious work of just getting through the daily tasks of an older individual. In addition, the last line points towards one of the anatomical hall marks of aging which is perceived as one of the contributors to the essence of old age, namely the thin legs, i.e. the lowered mass of skeletal muscle.

The scientific interest in the age-related loss of skeletal muscle mass has gained a lot of attention during the last 3 decades. The societal legitimization of research within this field has found its main argument in the function of skeletal muscle mass with regards to bodily movement[2–7]. Further, the loss of physical function that follows the loss of muscle mass has been associated with increased risk of both morbidity, mortality and low quality of life[8–10].

Besides playing a crucial role in moving body limbs, the skeletal muscle is gradually being acknowledged its vital function in the maintenance of metabolic homeostasis. Due to its proportion of the total body weight (approximately 40 % in healthy lean individuals)[11] and the fact that it is the primary tissue responsible for variations in resting energy expenditure of normal healthy individuals[12], the alteration of the skeletal muscle metabolism has started to receive attention as a prognostic factor within several different diseases as well as within healthy aging[13–16].

This, in combination with the increased proportion of elderly citizens within the Westernized societies, has facilitated a still increasing interest in how to attenuate the loss of skeletal muscle mass in order to maintain physical function and metabolism.

The CALM study (Counteracting Age-related Loss of Muscle mass) was initiated in the pursuit of ways to prevent the loss of muscle mass[17].

The study was conducted with the purpose of clarifying whether or not recommending daily protein supplementations with or without different types of muscular resistance training would be a feasible strategy in counteracting the loss of skeletal muscle mass with age. Hence, the primary outcome was quadriceps muscle cross-sectional area (CSA) measured by golden standard methodology, MRI. Multiple other measurements were made ranging from imperceivably physiological measurements such as the gut microbiome, muscle protein synthesis and the metabolome, to personal factors such as independence and quality of life.

This thesis will introduce the concept of sarcopenia and address the results from the CALM study with a special emphasis on the metabolism of the aging skeletal muscle. Beside the primary outcome measures, data derived from the oral glucose tolerance test performed before and after the intervention by all participants, as well as the blood and muscle tissue samples obtained in a sub-group of participants completing an acute trial before and after the intervention will be used.

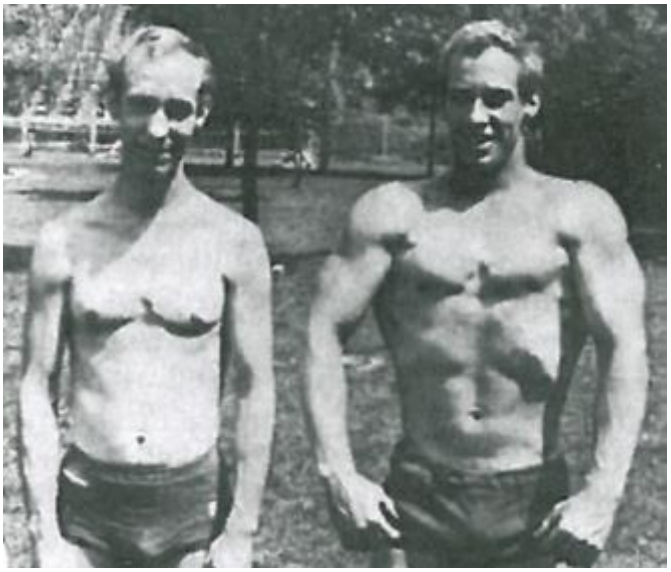
7.0 Background

7.1 The human skeletal muscle – structure and function

Skeletal muscle is a central organ within the human body. It is responsible for the movement of body limbs by contraction and is highly important in the maintenance of metabolic homeostasis. It consists of bundles of cells which are called fibers, and these fibers are kept together by sheets of connective tissue. A single fiber is consisting of a cell membrane with several nuclei distributed underneath the membrane along the length of the fiber. The cytoplasm of the muscle cell is constituted of thousands of myofibrils, which are the same length as the muscle fiber and is made up by small contractile units called sarcomeres. Sarcomeres are composed of actin and myosin filaments which during contractions slides across each other shortening the length of the fiber and hereby shortening the length of muscle leading to the movements of body limbs. In the human skeletal muscle, there are three different types of muscle fibers defined by 3 different isoforms of myosin which gives the fiber its functional and metabolic characteristics. Type I fibers, i.e. slow fibers, are characterized by a relative slow speed of contraction compared to Type IIx fibers, i.e. fast fibers, and Type IIa which are somewhere in between with regards to contraction velocity. Further, Type I fibers are relying on aerobic metabolism where especially Type IIx relies on anaerobic metabolism. As we age we experience a loss of muscle fibers.Since muscle fibers are unable to divide and form new fibers, the only way of gaining muscle mass is by increasing the diameter of the individual fiber. In addition, with advancing age the morphology of the muscle fibers are changing from being angular into having a more rounded shape as well as the clear differentiation between Type I and II fibers are lost[18]. The alteration in fiber type composition and distribution and the loss of fibers are thought to be partially responsible for the decline in physical performance seen with age.

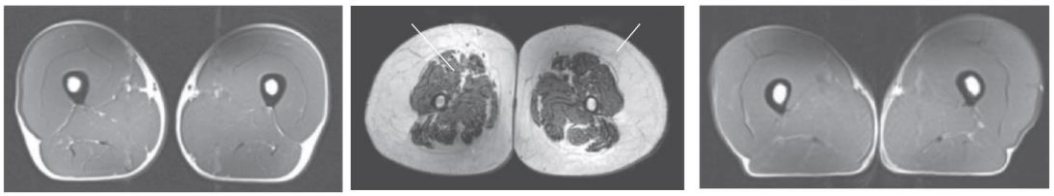
The human skeletal muscle has at least 3 different functions. First, it is responsible for bodily movements in collaboration with the nervous system, bones, joints and tendons. By contraction, the muscle is shortened, decreasing the length between the adhering ends of the muscle fibers, which are connected to bone and spanning across at least one joint hereby moving parts of the body. Secondly, it is vital for the maintenance of metabolic homeostasis. Under normal healthy conditions, the skeletal muscle mass is responsible for ~65% of the glucose disposal in response to insulin secretion[19], and it serves as the body's amino acid reservoir during fasting and states of disease[12]. Thirdly, it has recently (within the last 20 years) been acknowledge as a secretory endocrine organ, communicating with other organs through myokines[20].

Lastly, the skeletal muscle mass plasticity is another very important aspect. Even though there may be genetic factors limiting maximal attainable physical performance of a given individual, the picture underneath of two genetic twins (one is a marathon runner and the other one a heavy resistance exercise trained) highlight the skeletal muscles adaptability to different environmental stimulus.



Picture 1. Genetic identical twins. To the left a marathon runner, to the right a heavy resistance exercise trained. Adopted from Keul et al.[21]

This plasticity is not only present in young but is to some extent preserved throughout life. This is illustrated in the comparison of MRI cross-sectional scans of the thigh of a 40-year old triathlete, a 70-year-old sedentary and a 70-year-old triathlete illustrated underneath. In the 70-year-old triathlete the muscle structure is similar to the 40-year-old were the sedentary 70-year-old has a general loss of mass and a changed structure with massive fat infiltration and connective tissue.



Picture 2. MRI cross-sectional scans of the mid-thigh. To the left a 40-year-old triathlete, in the middle a 70-year-old sedentary, and to the right a 70-year-old triathlete. Adopted from Harridge et al.[22]

In conclusion, due to the skeletal muscle's various functions, its importance in both health and disease and its adaptability to different stimuli, makes it a highly relevant research

topic for both clinical and basic research. The significant decrease in skeletal muscle mass with age does not make it less interesting with respect to both society and the individual.

7.2 Sarcopenia

In 1989 the phenomenon of age-related loss of skeletal muscle mass, which had been known for centuries, were given the name Sarcopenia by Irwin Rosenberg[23]. He suggested the Greek name Sarcopenia (Sarco= Flesh, Penia= lack of) in order fuel research interest and general awareness of this phenomenon because as he wrote:

> *"There may be no single feature of age-related decline that could more dramatically affect ambulation, mobility, calorie intake, and overall nutrient intake and status, independence, breathing, etc. "*

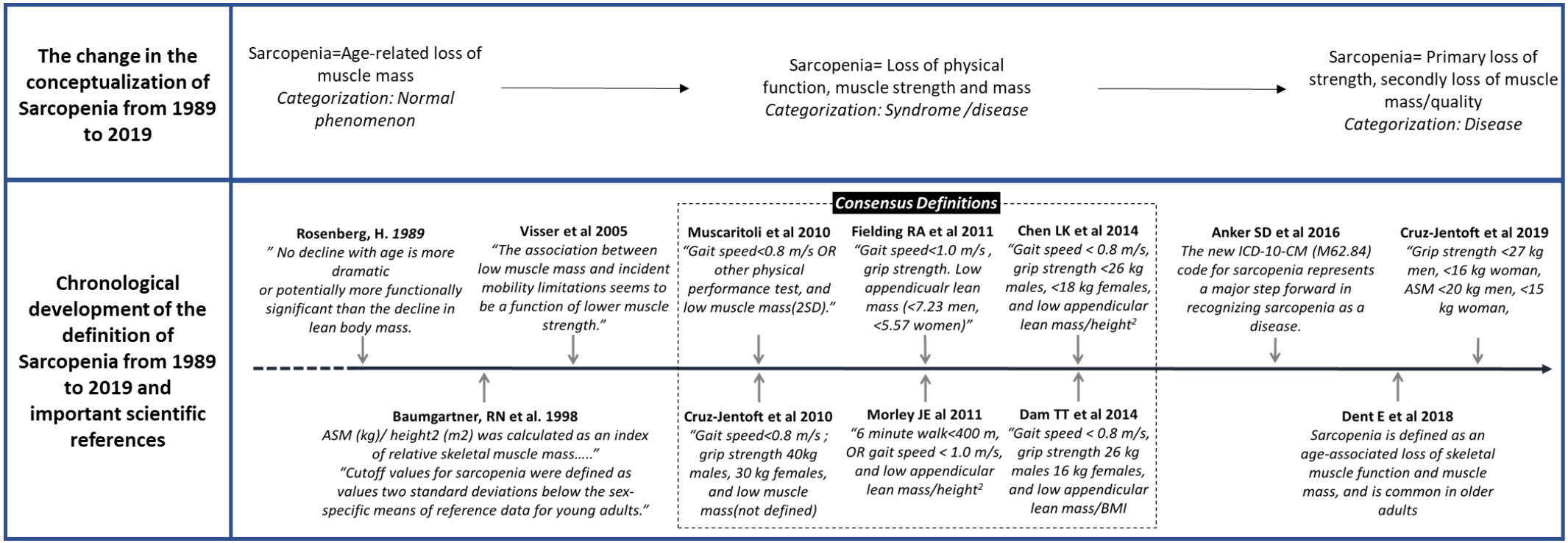
The baptism immediately sparked an increased interest in the phenomenon of age-related loss of muscle mass within the scientific communities and in 1998 Baumgartner reported an operational definition legitimized by its association to decreased physical function and mortality[24]. However, from around 2000 the focus changed from muscle mass to physical function and strength. The Health ABC study showed that muscle strength rather than muscle mass predicted loss of physical function and mortality[25,26], and based on these and other findings researchers suggested a separate name, Dynapenia (dynamis=power, penia=lack of), for the phenomenon of age-related loss of muscle strength[27,28]. They argued that the literature had put too much emphasis on muscle size in the attempt to explain the loss of physical function with age. Nevertheless, the 6 consensus definitions published between 2010 and 2014 kept the name sarcopenia but changed the definition instead[2–7]. These definitions broadly define Sarcopenia as a condition with low muscle mass and either low muscle strength or /and physical performance, and in 2016 the condition described using these definitions were assigned an ICD-10 code acknowledging it as a disease[29]. Recently an updated version of the most cited consensus definition, EWGSOP, were published categorizing Sarcopenia as a disease (the original from 2010 categorized it as a syndrome) with a new operational definition putting even more emphasis on the aspect of strength and making muscle mass and the undefined term muscle quality a secondary criteria[30].

Despite the vast amount of consensus articles and a newly published guideline for clinical practitioners[31], the concept of sarcopenia and especially how to define the phenomenon

is still controversial[32]. The consensus definitions are problematic in several ways. Firstly, the unambiguous name (i.e. loss /lack of flesh) is misleading according to the consensus definitions unspecific nature which includes three separate phenomena, i.e. loss of strength, loss of performance/functionality, loss of mass/quality, which provides a breeding ground for confusion. Secondly, the three separate phenomena are only partially related, which makes the separation of cause and effect practically impossible, e.g. practically any changes in health status can result in a decrease in physical activity which would secondly affect both strength and mass and vice versa. Thirdly, the very important role of skeletal muscle mass in the overall metabolic homeostasis of the human body and its crucial role in the state of disease is easily overlooked due to the current definitions primary criteria of strength and physical function. Lastly, the documentation of Sarcopenia being an independent state of disease is not convincing which is again due to the included phenomena's partially relation. Therefore, treating it as such could be problematic with regards to both scientific investigation and clinical practice. The development of the concept of Sarcopenia is illustrated in fig. 1.

Consequently, in this thesis the term Sarcopenia will refer to the very well documented natural phenomenon of age-related loss of muscle mass[26,33–35]. In this perspective, it is important to stress that Sarcopenia should be conceptualized as a dynamic process rather than a static condition. This process begins years before the individual will become aware of its possible negative consequences. Interventions strategies that is thought to attenuate the Sarcopenic process were the primary focus of the CALM-study. These strategies have the character of prevention rather than treatment, which again explain the included study population of otherwise healthy elderly. The included participants were not expected to fulfil the current non validated diagnostic criteria of Sarcopenia, but instead be representable of elderly people undergoing a sarcopenic process. Further, the included participants should be representing healthy elderly that are both capable and willingly to follow general health recommendations by the Danish authorities.

Figure 1. The change in the conceptualization of Sarcopenia and the chronological development of the definition and important scientific references from 1989 to 2019[2,4–7,23,24,29–31,36,37].



7.3 The CALM study - background

A linear decline in muscle mass in humans from the mid 30ties or mid 40ties until the mid 60ties have been reported by several studies[34,38–41]. Depending on the measurement, study- design and population, the annual rate of loss of muscle mass is estimated to be around 1-2%. After the age of 65 the deterioration starts to be progressive[34] with an increased rate above 2% in the lower limbs[42]. The loss of skeletal muscle mass is accompanied by a loss in physical performance[43] and a loss of muscle strength[26] which again has been associated with an increased mortality risk[44] and a decreased satisfaction with life[9]. Several explanations for the loss of muscle mass and function have been suggested ranging from insufficient intake of nutrients, changes in endocrine function, chronic low-grade inflammation, deterioration of the blood flow and simple disuse and lack of physical activity[45–48].

Regarding the otherwise healthy part of the elderly population, especially the possibility of adjusting daily protein intake has served as one of the main areas of interest in the quest of preventing or attenuating the sarcopenic process. Cross-sectional studies have shown an association between daily intake of protein and skeletal muscle mass in elderly males and females. Geirsdottir et al observed a difference in LBM of 2.3 kg between the highest (Q4 = 1.36 ± 0.19 g/kg/day) and lowest (Q1 0.63 ± 0.08 g/kg/day) quartiles of protein intake in 237 (age 65-92 years) community-dwelling healthy adults[49], Morris et al observed a positive linear relationship between daily protein intake and ASMI in physical active elderly ≥50 years in 636 obese participants[50], and Sahni et al observed a positive association (r=0.10, p=0.005) of leg lean mass and protein intake in 2675 healthy participants with a mean age of 59.2±9.5years (29-86 years)[51]. Further, in a prospective cohort with 2066 participants Houston et al found that the quantile (1.2g/kg/day) having the highest energy adjusted daily protein intake lost app. 40% less ALM compared to the lowest quantile (0.8g/kg/day) i.e. 0.5 kg and 0.88 kg respectively during a period of 3 years [52]. However, it is important to notice that these observed differences are most prominent when comparing the participants with the lowest to the highest intake, and that the lowest quartile in both Geirsdottir et al and Houston et al are having a protein intake below or exactly on the recommended level of 0.8g/kg/day.

These observational studies are supported by acute studies investigating the attenuated responsiveness of the aging skeletal muscle to the anabolic stimulus of protein intake[53]. Briefly since this will be elaborated in section 7.4, several studies have shown that

elderly requires a larger amount of amino acids compared to young in order maximize the skeletal muscle protein synthesis[54–56]. In a retrospective study, Moore et al compared the responsiveness to different doses of high quality dietary proteins intake in healthy older adults (~71 years) and young (~22 years)[57]. They observed, that healthy elderly needed ~0.40g/kg compared to ~0.24g/kg in young in order to maximize their MPS.

The observational data showing a higher LBM with higher intakes of protein in combination with the results from the acute studies have led committees and research groups to suggest changes in the nutritional recommendations adjusting the recommended daily intake of protein within elderly above 65 years of age[58–60]. The NNR recommends otherwise healthy elderly to have a daily intake of protein of 1.2g of protein/ kg BW/day[58] instead of the 0.83 g of protein/kg BW/day recommended by WHO[61]. However, studies investigating the effect of an increase in daily protein intake have shown diverse results[62–66], which is suggested to be at least partially explained by the duration of the studies being 6 months or less[60].

Therefore, the effect of increasing daily protein intake on both muscle mass and strength is still a debated topic though leaning towards an adjustment of recommendations towards an increase in daily intake[59,67,68]. Nonetheless, there is still a need for long-term human intervention studies investigating the effect of an adjusted protein intake within healthy elderly. Since it is difficult to change the diet composition without changing the diet habits, using protein supplements seems like a more feasible strategy in order to enhance the adherence to recommendations rather than selectively adjusting the individual's diet composition. Further, these studies should account for the possible difference between a recommendation and an actual effect of an adjusted protein intake in order to either refute or support the current trend for the increased recommended daily protein intake within elderly.

Another and very potent way of attenuating the sarcopenic process, is different kinds of physical activity. Conducting heavy resistance exercise is known to be the most effective way of stimulating muscle growth naturally within young as well as elderly[69–71]. Despite being the most potent way of stimulating muscle growth, it is needless to say that the growth is only achieved if the exercise is conducted at sufficient intensity and frequency. But, especially the adherence to different exercise interventions within elderly is an issue, which has not so far found any good solution[72]. In addition, it has been shown that some older adults prefer exercise modalities of lower intensity, lesser cost and more convenient when it comes to

locations on which the training is conducted such as a home-based setting[73,74]. Further, exercise modalities of lower intensity have actually shown capable of increasing muscle mass. Holm et al observed an increase of 3% in quadriceps cross-sectional area by MRI after completing 10 sets of 36 repetition (isolated knee extensions) at 15% 1RM 3 times a week for 12 weeks in healthy young sedentary[75], and Watanabe et al found an increase in the cross sectional area of the mid-thigh by MRI of 5% after completing 3 sets of 13 repetitions (isolated knee extensions) at 30% 1RM twice a week for 12 weeks[76]. Taking the adherence-issue into account and the fact that otherwise healthy and independently living elderly above 65 years are not seeking to maximize but instead maintaining their respective mass, lower intensity training modalities could therefore show to be an equally or better strategy than heavy resistance training in preventing or attenuating the progression of sarcopenia. Additionally, from the perspective of prevention and with respect to general recommendations by health authorities, home-based training modalities have the advances of easiness with regards to implementation and costs in comparison to center-based training regimes.

It should be noted however, that most of the studies investigating the effect of different types of training are short-term (≤6months) and often very strictly monitored. Long-term and more applied studies representing daily living are lacking.

7.4 Skeletal muscle protein synthesis

Skeletal muscle contains around 40% of the total amount of protein in an adult human body and with ~65% of the total amounts of muscle protein being the structural proteins actin and myosin[77]. The skeletal muscle is estimated to have a turnover rate around 1-2% pr. day[11], which is relatively low compared to plasma proteins such as albumin with a turnover rate of ~8.5% pr. day[78]. Despite this relatively slow turnover rate, the skeletal muscle still accounts for around 30-50% of the total protein turnover in the body and the synthesis and breakdown of proteins are responsible for 20% of the resting energy expenditure[11].

Muscle protein turnover (MPT) consist of the two different processes, muscle protein synthesis (MPS) and muscle protein breakdown (MPB). MPS and MPB fluctuates through the day due to different stimulus and it is the net balance (NB) between the two that determines whether we maintain our muscle mass (0 NB), gain muscle mass (+NB) or lose muscle mass (-NB)[79] (see illustration 1). MPS and MPB is under conditions of health in general affected by physical activity levels, feeding status (fed/fasted) and different hormones[80]. In

relation to the protein kinetics, the focus in this thesis will be on the anabolic effect of protein on MPS measured in the CALM acute studies.

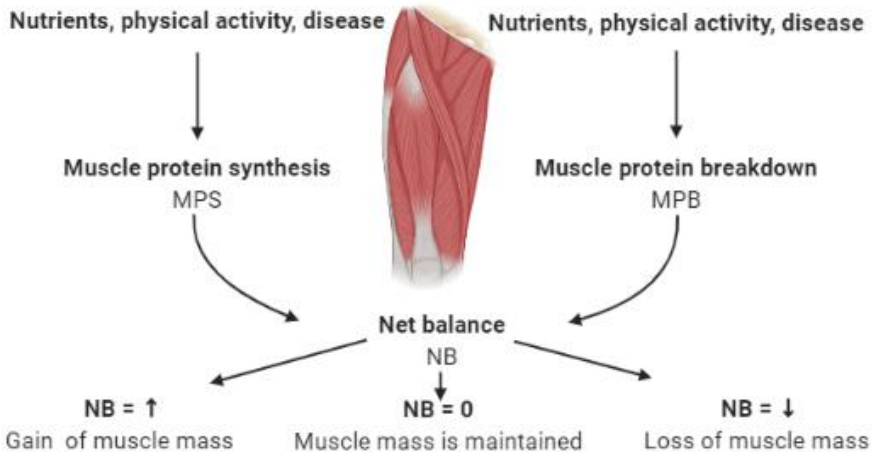


Illustration 1. Simple illustration of the theory of MPS, MPB and NB for the gain, loss and maintenance of skeletal muscle mass

The MPS of skeletal muscle can be measured by different methodologies with the use of tracers. A tracer is a compound that that can be distinguished from the normal occurring compound (tracee) but do not differ with regards to metabolic of chemical properties[81]. Measuring the MPS, stable isotope labelled amino acids is used, which only differs from the normal occurring amino acids with respect to a slightly higher mass due to the extra neutron(s) in its nucleus. With different kinds of mass spectrometry, the tracer appearance, dilution or incorporation in different pools or body compartments can hereby be measured and synthesis and breakdown rates can be estimated based on different assumptions. A common way of measuring the MPS is by the direct incorporation technique. The direct incorporation technique measures the incorporation of a given tracer into the structural proteins of the muscle during a specific time period. Based on the protein-bound tracer abundance at specific time points, knowledge on the abundance of tracer in the precursor pool for protein synthesis and several different underlying assumptions, the fractional synthesis rate (FSR) can be measured[79].
Despite there being several other ways of estimating muscle protein synthesis, the following will focus on studies using this method, since this is the method used in CALM.

The literature on FSR distinguishes between basal FSR representing the protein synthesis in the fasted state and response FSR which represents the protein synthesis after a given intervention such as protein intake or exercise. Therefore, any alteration in either basal or

response FSR with age could lead to a negative NB and hereby a loss of muscle mass the MPB is not altered in the opposite direction.

With regards to basal FSR the literature is inconsistent. While some have found a decreased basal FSR in older individuals compared to young[82,83], others have only found a tendency towards a decrease[84] and some has been unable to see any difference[85,86]. This discrepancy could be a result of methodological differences, e.g. choice of tracer, measuring period, etc.[87] or several other individual factors. Some studies has also found that middle aged females has a higher basal FSR in comparison with males[88,89], despite not differing on whole body protein turnover[90]. Nonetheless, most studies investigating FSR have been conducted on males, and there is a general lack of studies investigating differences between the genders in all age-groups[91], and this should be kept in mind when converting or extrapolating the interpretations of acute studies into recommendations.

It is well-known that protein intake leads to an increase in muscle FSR. FSR has been shown to be stimulated in dose-dependent but saturable way following amino-acid infusion or protein ingestion within both young and elderly[57,92], but the responsiveness with respect to FSR seems to be blunted in older individual compared to young[55–57,93,94]. Moore et al showed in a retrospective analysis of previous studies conducted by their research group that the FSR in both old and young exhibited this relationship only differing with respect to the amount of protein necessary to elicit maximal FSR[57]. Where young healthy males (n=65, 22y [18-37]) needed ~0.24g of protein pr. kg BW (0.25g/kg LBM) to maximize FSR and older males (n=43, 71y [65-80]) needed ~0.4g of protein pr. kg BW (0.61g/kg LBM) to maximize FSR[57]. These studies in combination, support the epidemiological studies described in section 7.3 on the relationship between protein intake and muscle mass. If the aging skeletal muscle is becoming less sensitive to protein intake with regards to FSR this would eventually lead to a more negative NB if the elderly is consuming the same amount of protein as young. Further, the increases in FSR after consumption of protein and the increased availability of amino acids in the circulation have been shown to last for at least 2-3 hours dependent on protein type provided[68,85], which makes the potential anabolic stimulus repeatable several times a day. However, ~50% of the daily protein intake in elderly is consumed at dinner, which results in the total amount of protein consumed at breakfast and lunch being below the levels that have shown to maximize muscle protein synthesis[68,95]. Taking this into considerations, it has been suggested that any

recommendations regarding protein intake within healthy elderly should be pr. meal instead of pr. day[96].

Despite these studies being highly valuable for our understanding of the development of sarcopenia, there are several limitations to their interpretations. An increase or decrease in either basal or response FSR is difficult to interpret with respect to its impact on NB since any changes in MPB needs to be accounted for. The specific measuring of MPB is unfortunately more difficult due to several different methodological circumstances[79]. However, with the respect to the reported negligible effect of AA and insulin on MPB, this issue with respect to the interpretation of FSR-data is thought of being a minor concern[97]. Even though, recently Kim IY et al found that not only alterations in PS relation to meal intake but also PB contributed significantly to the positive NB observed[98]. As stated by the authors, these findings illustrate that only measuring PS could lead to erroneous conclusion with respect to evaluating the anabolic response to a given stimulus or intervention. In addition, it is of interest that it has been impossible to link either basal or response FSR to muscle mass or hypertrophy. To my knowledge, only Mitchell et al has investigated a possible link with a negative result showing no association[99]. There could be several good explanations for the lack of congruence, but it raises the concern that the clinical or physiological relevance of the FSR measures have been overemphasized by the supporters of increased daily protein recommendations within elderly above 64 years of age[59,60,68,100–102]. Nonetheless, studies evaluating the FSR measurement with respect to its interpretation are highly needed. However, the regulation of FSR and the impaired FSR seen in response to different interventions with age is an indication of a loss of anabolic sensitivity, which may be one out of several causes of Sarcopenia.

7.5 The relation between the skeletal muscle and glucose metabolism

Under normal condition, the skeletal muscle mass is responsible for ~65% of the glucose disposal in response to insulin secretion[19]. Further, during physical activity the skeletal muscle mass is responsible of 95% of the increased energy expenditure which if rigorous can increase up to 25 fold compared to the resting metabolic rate.[103] This ability demands a high degree of flexibility and adaptability when it comes to substrate utilization. The mechanisms by which this change in substrate utilization is controlled, and the ability of the organism to change between substrates for fuel production has been investigated using the theoretical concept of metabolic flexibility[103]. Metabolic flexibility is basically referring to the organism's ability to

adapt to changes in metabolic or energy demands in the transition from fasted to fed or inactive to active, and is in general a specific application of Walter B. Cannons broader concept of physiological homeostasis from 1929[104]. With age and disease it has been shown that the metabolic flexibility of especially the skeletal muscle is impaired[103,105].

An important argument for the concept of metabolic flexibility are the glucose and fatty acid cycle first described by Randl et al. In 1963 Randl et al showed how the selection of substrate in fuel production and storage within skeletal muscle and white adipose tissue (WAT) were not only controlled by hormonal regulation but affected selectively by the specific substrates used for fuel production, i.e. glucose and fatty acids[106]. Through several different animal studies, Randl et al showed the inhibitory effects of glucose and insulin on the release and oxidation of fatty acids in both skeletal muscle and WAT and the suppression of glucose oxidation during periods of carbohydrate deprivation. Further, they showed that in a diabetic rat model this cycle was disturbed with an accelerated oxidation of fatty acids despite a delivery of both glucose and insulin leading to an impaired glucose uptake, glycolysis and impaired glucose oxidation. These results has since been proven, extended to other tissues within both animals and humans, and expanded in terms of both impact and the variety of mechanism by which fuel selection is controlled in conditions of health and disease[107].

Due to its known importance for metabolic homeostasis, the loss of skeletal muscle mass and quality is thought to play a crucial role in the decreasing metabolic flexibility seen with age. It is known that the glucose tolerance is lowered with advancing age[108] and that ~25% of all elderly between 75-80 years are fulfilling the criteria of being diagnosed with type 2 diabetes[109]. In type 2 diabetic patients, the accumulation of intramyocellular lipids are thought to be partially responsible for the loss of insulin sensitivity seen with this condition. The aging skeletal muscle show a somewhat similar characteristics with increased intramyocellular lipids[110]. Further, a lower skeletal muscle area of the abdominal muscles has been associated with an increasing plasma lipids and cholesterol levels[111]. However, a recent study by Chee et al. showed that inactivity rather than age is causing the lipid accumulation and insulin resistance observed in elderly[112]. Supporting these findings, resistance exercise training (RT) and muscle activity enhances insulin sensitivity[113–116], and light muscular intensity training such as aerobic training improves fasting plasma glucose levels, decreasing both glucose- and insulin AUC during an OGTT[117] in prediabetic patients. It is however, difficult to separate the effect of physical activity

from the effect of muscle mass and quality per se on both insulin sensitivity and increased intramyocellular lipids, since muscle mass and quality is decreasing with inactivity and vice versa[45] and that activity levels are known to decrease with advancing age. Interestingly, Solerte et al showed that 6 month of AA supplementation (8g/day) increased muscle mass and insulin sensitivity in 41 elderly non diabetic sarcopenic elderly (66-84years)[118], supporting that muscle mass per se might be important for the maintenance glucose homeostasis in this population. Further, Manders et al[119] showed a positive effect of protein consumption (0.3g/kg casein + 0.1g/kg leucine) on reducing post-meal hyperglycemia in 11 long-standing type 2 diabetic patients (58±1 years) underpinning that AA also work by other mechanism than its effect on skeletal muscle secondly improving insulin sensitivity as suggested by Solerte et al, such as the well-known insulinotropic effects of branched chain amino acids (BCAA)[120].

Therefore, the effect of different types of interventions such as exercise and protein supplementation thought to counteract sarcopenia might also be beneficial for improving glucose tolerance within elderly either by its direct effect on insulin secretion, the lowering of the concentration of intramyocellular lipids, or secondarily by the increase of muscle mass. However, it is important to notice that even though protein supplementation has shown to be beneficial with respect to glucose tolerance in short-term studies, high circulating plasma AA levels have recently also been associated with the risk of developing diabetes[121]. This in combination highlights that interventions studies investigating the long-term effect of different strategies expected to counteract sarcopenia should be aware of how these interventions affect the glucose tolerance.

7.6 The Omics-disciplines and the skeletal muscle metabolome

For the last three decades the omics disciplines have evolved dramatically, starting with genomics in the 1990ties and now including epigenomics, transcriptomics, proteomics, metabolomics and microbiomics[122]. Omics is derived from the Greek suffix-ome, which in the science of cellular and molecular biology refers to all constituents considered collectively within a given area of interest. The development of the different omics disciplines has primarily been driven by technological development making high-throughput analysis cost-efficient. However, the development has equally been depending on the ability to handle, analyze and interpret large and complex data-sets[123]. These analyses are based on pattern-recognition methods also known as multivariate data analysis or chemometrics[124]. An important aspect of the omics-disciplines is

the altered approach to biology, which is due to the analysis's strategy rather than the creation of big-data sets per se and could be categorized as a top-down in contrast to the traditional bottom-up approach, meaning that instead of starting with investigating specific parts of a given system and then use these results to inductively support the probability of a certain conclusion, the whole system is considered collectively, and the results are then used deductively for further interrogating specific parts of the system. If the underlying assumptions of the omics discipline is accepted, i.e. that the whole is actually measured, this new approach is offering a rejuvenation of the deterministic scientific approach and conceptual framework, which has otherwise been questioned and criticized[125,126]. Within this thesis going too much into detail with this theoretical discussion is beyond the scope, but it is important to keep these essentially different scientific approaches and their respective cons and pros in mind since both approaches are used (Traditional: Paper II+III (IV), Multivariate: Paper IV). The different omics disciplines and their respective relevance in interpreting biology is illustrated underneath.

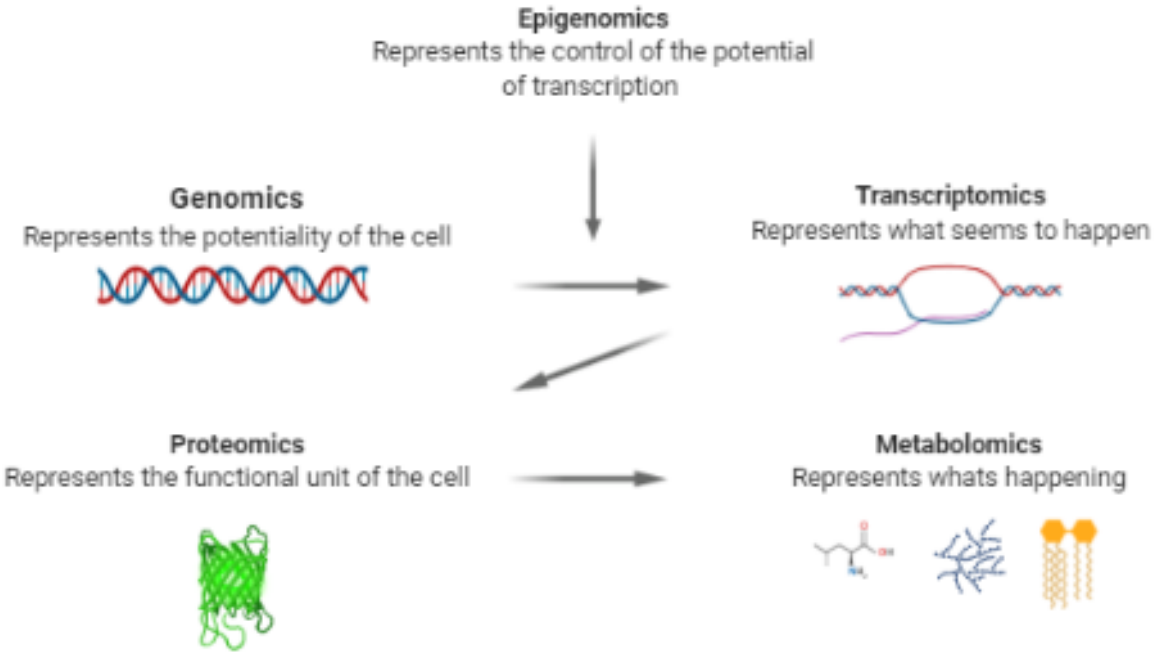


Illustration 2. Overview of the different omics-disciplines and their relation to biology.

A difficulty in measuring tissue specific metabolomes are the amount of tissue available. However, due to relatively recent advances within technology it is now possible to measure the

skeletal muscle metabolome despite the low amount of tissue normally available from human experiments[127]. Fazelzadeh et al show differences in the skeletal muscle metabolome between young, healthy elderly and frail elderly in a study using different targeted platforms. They showed differences in the metabolome at rest within metabolites related to mitochondrial function, fiber type and tissue turnover[128], and changes within both young and frail elderly with respect to the amino acid metabolism as an effect of 6 month of progressive resistance exercise. Using un-targeted platforms, Sato and colleagues observed daily variation and response to a 5 day high fat or high carbohydrate diets in the skeletal muscle metabolome in middle aged men (30-45 years of age)[129], and Saoi et al showed an effect of bicarbonate ingestion prior to strenuous interval exercise on different metabolic pathways in comparison to placebo in 7 active young men[130]. These three studies underline the possibilities of new insights in the metabolism of the skeletal muscle offered by the tissue specific application of metabolomics. It is important to note, that these studies were using both targeted[128] as well as un-targeted[130,131] platforms. In general, the aim of targeted platforms is to investigate known metabolites were as un-targeted platforms seeks to acquire as many metabolites as possible[132]. The targeted approach has the advantaged when it comes to absolute quantification, however it will only measure already identified metabolites. Contrary, the un-targeted approach is unbiased, meaning that it actually measures what is in the sample and not only the targeted metabolites, which may result in the discovery of new and unknown metabolites. The disadvantaged using un-targeted metabolomics are both identification and its relative and not absolute quantification. One could argue, that targeted metabolomics are not true to its name, i.e omics ≈ the whole, in comparison to un-targeted metabolomics, which at least is measuring as many as technically possible. Further, un-targeted metabolomics are more in line with the overall omics theory described above where the focus is on doing inductive hypothesis generating research rather than the classical hypothesis driven research.

As already demonstrated by the three previous studies measuring the skeletal muscle metabolome, this methodology is promising for understanding alterations in the skeletal muscle metabolism as well as for discovering new metabolites. Further, metabolites are as illustrated (illustration 2) the end point in the production chain starting with the genome. It is therefore thought, that the metabolome of a given tissue are closer related to the phenotype than the measured substances of the other omics disciplines, and it has therefore also great potential when it comes to the discovery of new biomarkers and for phenotyping patients within different

disease categories. Establishing and validating protocols for measuring the skeletal muscle metabolome can therefore contribute to both understanding of normal as well as diseased skeletal muscle and the diagnostics of different muscle diseases.

8.0 Aims and hypothesis

The aim of this PhD project was to evaluate the effect of 12 month of supplementation with or without different types of training on several different parameters related to the skeletal muscle within healthy males and females above 65 years of age representing the part of population anticipated of being both capable and willing to follow general health recommendations. The study was designed as an intention to treat study in order to make any possible findings transferable into general health recommendations. Further, the PhD project aimed at using the interdisciplinary design and the many test and measures performed to explain any differences possible observed as an effect of the intervention.

The primary study questions were as follows:

1) How is Sarcopenia defined? What is the argument for changing the definition? And what are the implications of the current definitions? (Paper I)
2) Is protein supplementation with or without different types of training an effective way of preventing or attenuating the sarcopenic process with respect to muscle mass and muscle strength? (Paper II)
3) How does supplementation with or without different types of training affect the glucose tolerance within healthy elderly? (Paper III)
4) How does prolonged supplementation with or without different types of training affect the MPS and the skeletal muscle metabolome and how should changes in muscle FSR be interpreted? (Paper IV)

Briefly, the hypothesis with respect to the primary outcome for the CALM-study were as follows. We hypothesized that for the PP-analysis, changes in qCSA in the training arm would be: HRTW > LITW > WHEY; and in the nutritional arm: WHEY>COLL>CARB
However, due to the hypothesis that the adherence would be higher in LITW compared to HRTW during the 12 months of intervention, the hypothesis of the ITT analysis for the training arm were: LITW≥HRTW>WHEY.

The hypothesis is illustrated in the figure 2 underneath.

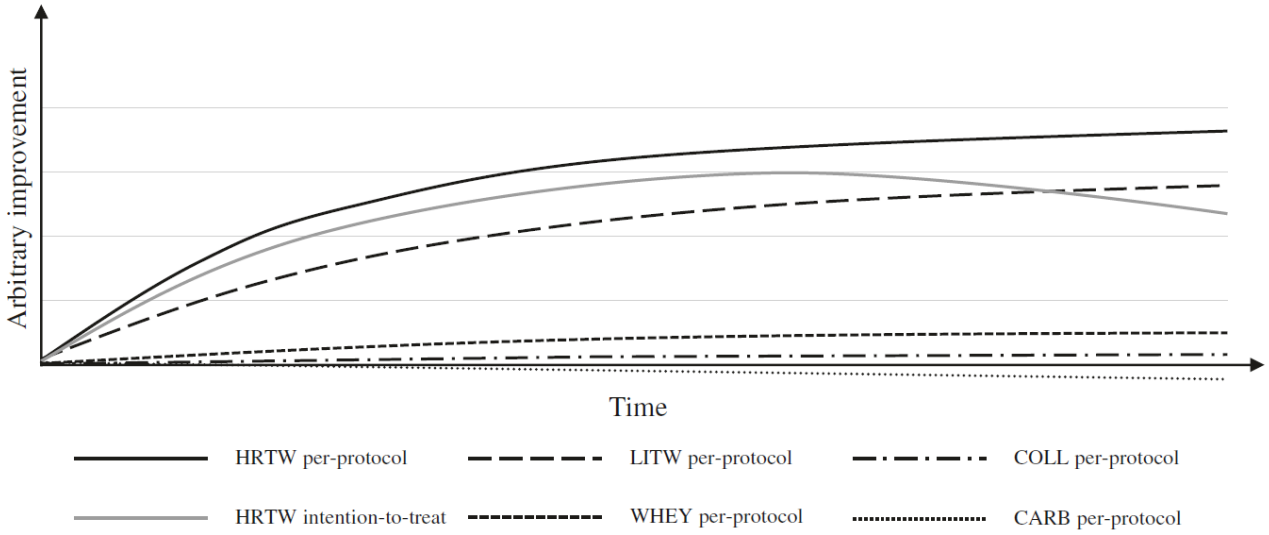


Figure 2. Hypothesized improvements over time with different interventions. Black lines mark the expected effect of per-protocol analysis: HRTW (solid line), LITW (long-dashed line), WHEY (short-dashed line), COLL (dashed-dotted line), and CARB (dotted line) interventions when analyzed per protocol. The gray line marks the expected effect intention-to-treat analysis of HRTW. Adopted from Bechshøft et al.[17]

9.0 Study design

9.1 The CALM study

The CALM study was designed as a randomized controlled trial investigating the effect of 5 different interventions thought to affect the skeletal muscle mass for 12 months. The 5 different interventions were split into two intervention arms. A nutritional arm containing 3 groups receiving a supplement 2 times daily for 12 months:

1) 20g of maltodextrin + 10g of sucrose (CARB-group)
2) 20g of collagen hydrolysate +10g of sucrose (COLL-group)
3) 20g of why hydrolysate+10g of sucrose (WHEY-group)

And a training arm containing 3 groups (WHEY-group is reused due to practical limitations) all receiving the whey supplement two times daily in addition to conducting either:

4) Home-based light intensity training 3-5 times pr. week. Supervised and adjusted monthly (LITW)
5) Center-based heavy resistance exercise 3 times pr. week. Continuously adjusted. (HRTW)

The nutritional supplementation arm (CARB, COLL, WHEY) investigated the effect of protein supplementing two times daily and the impact of protein quality in comparison to an isocaloric control. The participants were instructed to ingest the supplements before or during breakfast and lunch in order to increase satiety and thereby limiting a potential excessive calorie intake.

The training arm (HRTW, LITW, WHEY) investigated the effect of resistance training at two different intensities in combination with whey protein supplementation in comparison to supplementation with whey protein alone (WHEY group is reused as the control group in the training arm). The HRTW-group performed a supervised center-based heavy resistance exercise program 3 times weekly. The intensity was periodized into-3months cycles, increasing the load progressively from 3-sets of 12 repetition maximum (RM) with 2 minutes break in between, to 5 sets of 6 RM with 2 minutes break in between, in each cycle. The training

program consisted of 5 exercises, leg extension, leg press, leg curl, shoulder pull-down and arm push-up. The LITW-group performed light load home-based resistance training 3-5 times weekly (average of 4 times a week) using body weight and TheraBand® rubber bands (Hygenic Corp., Akron, OH, USA). The training sessions were supervised once a week for the first month and once a month for the remaining 11 months of the intervention in order to ensure correct execution. The training program consisted of 5 exercises, leg extension (1 minute exercise, 1 min break), chair stand/squat (1 minute exercise, 1 min break), leg curl (1 minute exercise, 1 min break), shoulder pull (1.5 minute exercise, 1.5 min break) and arm stretch (1.5 minute exercise, 1.5 min break).

Adherence to the supplementation and the training intervention in the LITW-group were registered by the participants in hard-copy diaries and the adherence to the training in the HRTW-group were registered by the training personnel. Participants with a registered supplementation adherence >75% corresponding to the consumption of 1.5 supplement pr. day were included in the PP-analysis. Participants with a registered training adherence >65% corresponding to participating in 2 training sessions per week in the HRTW-group and 3 training sessions per week in the LITW-group were included in the PP-analysis.

The study was designed as an modified intention-to-treat analysis in order to make the results applicable for future recommendations. This means that participants not following the intervention could continue their participation through-out the trial. However, adherence registrations were collected in order to conduct a per protocol analysis as well. It was conducted at the Institute of Sports Medicine at Bispbjerg Hospital from 2014-2018. The study design is illustrated in figure 3.

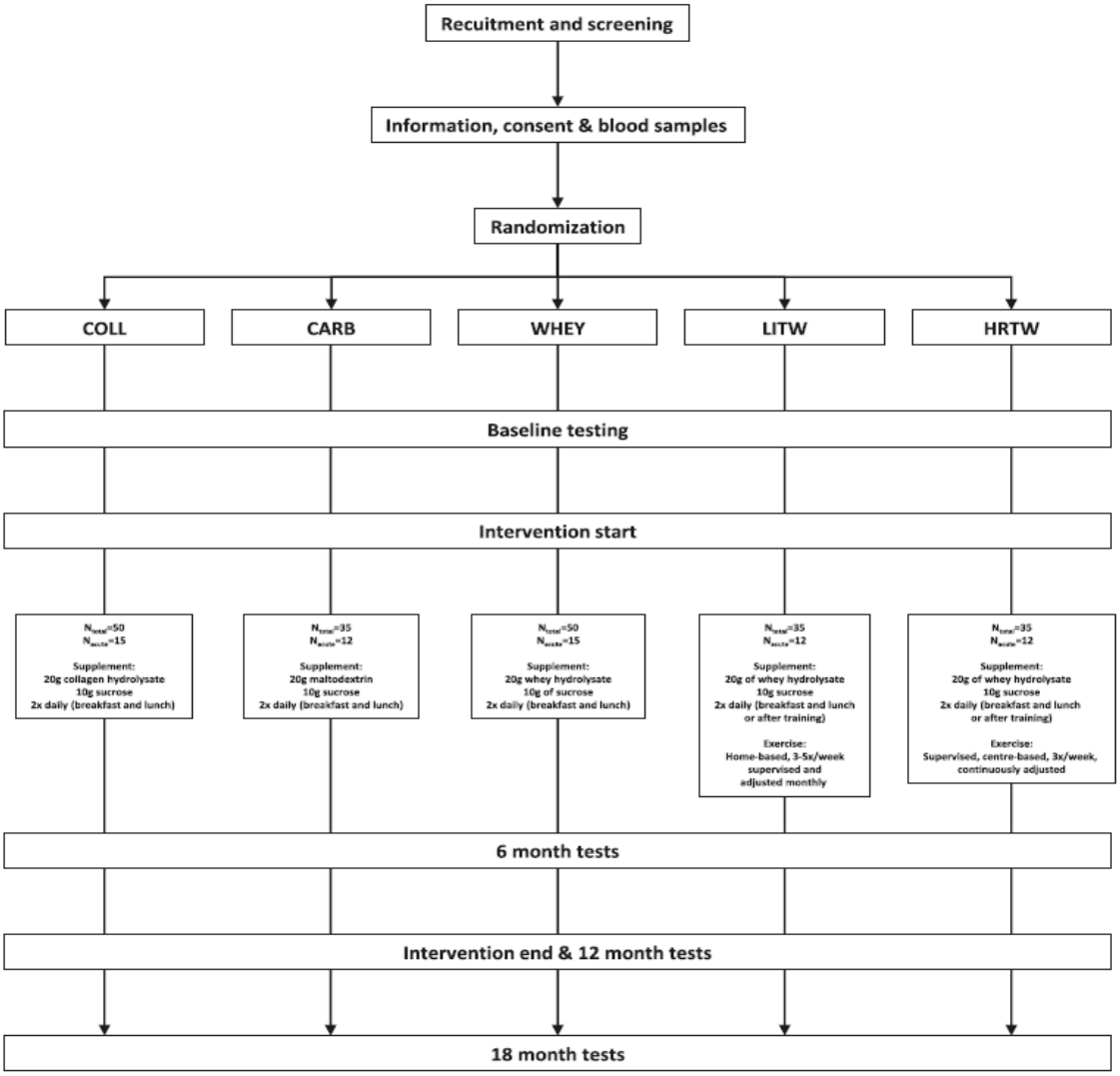
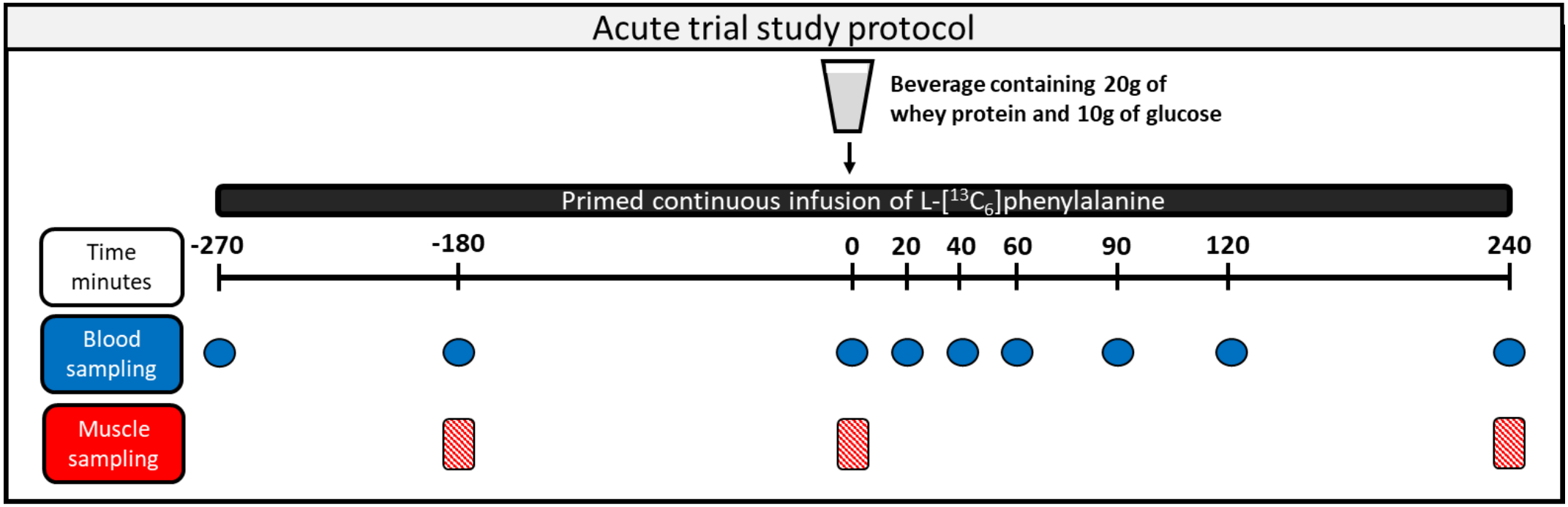


Figure 3. Participant flow. N_{total} represents the expected number of inclusions in each group. N_{acute} represents the expected number of participants who will complete the measurements of fractional synthesis rate at 0 and 12 months. COLL Collagen supplementation, CARB Carbohydrate supplementation, WHEY Whey supplementation, LITW Light intensity resistance training and whey supplementation, HRTW Heavy resistance training and whey supplementation. Adopted from Bechshøft et al.[17]

9.2 The acute trial

In addition to the general test battery, a subgroup of 66 participants (12 in COLL, LITW and HRTW, and 15 in CARB and WHEY) were also participating in an acute infusion trial at baseline and after the 12 months of intervention. Figure 4 shows an overview of the acute trial

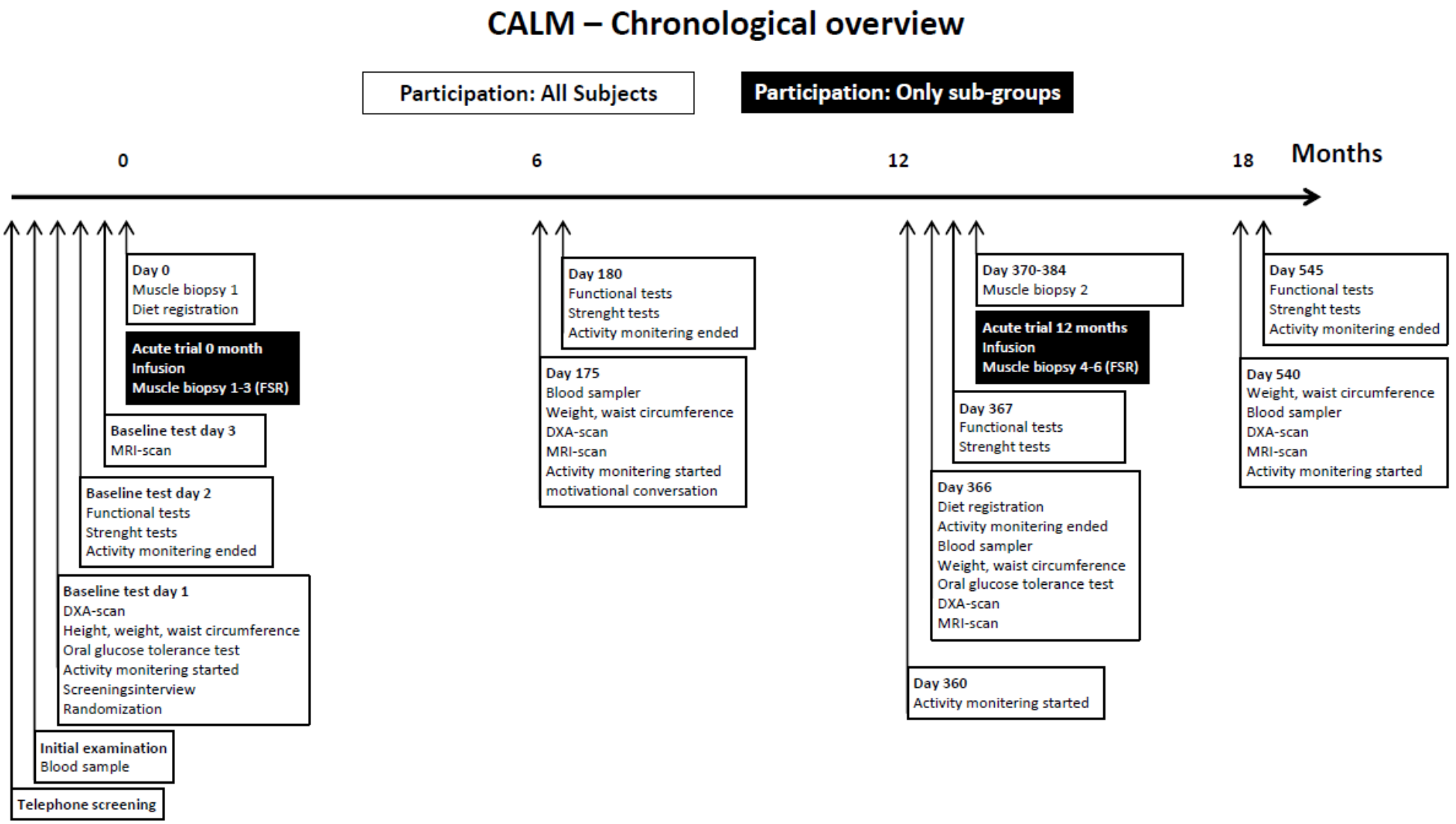
Figure 4. Acute study conducted at baseline and after 12 months of intervention in a randomly selected sub-set of participants from each study group. Adopted from paper IV.



9.3 Test and measures performed

As mentioned in section 1.0, the CALM study was conducted in collaboration between three different faculties at the University of Copenhagen, Faculty of Health and Medical Sciences, Faculty of Science and Faculty of Humanities. Due to the involvement of scientists from many different disciplines and interdisciplinary nature of the study great variety of different measurements and observation were performed at several different timepoints. For a chronological overview of the test performed in the CALM-study that are used in this thesis see figure 5 underneath. For the complete list see Bechshøft et al[17].

Figure 5. Chronological overview of the test conducted CALM



10.0 Methods and methodological considerations

10.1 Study population, exclusion criteria and randomization

We carried out the study in 208 otherwise healthy elderly (both males and females) living in the Greater Copenhagen area. We advertised for the project in local newspapers, magazines, radio programs, social media, senior centres and public events. Inclusion were started in 2014 and ended in 2016. Participants were excluded from participants by the following criteria:

- Care dependency
- Disability in lower extremities
- Arthritis or arthrosis in knee or hip joints, arthritis requiring medication, or other rheumatic diseases potentially affecting joints or muscles
- Diagnosed or suspected knee osteoarthritis (based on EULAR criteria: three symptoms and three signs); excluded if more than one of the following thee symptoms are found: morning stiffness <30 minutes, persistent knee pain, or functional limitations
- Bilateral knee alloplastic and hip alloplastic material
- Connective tissue disorders
- Severe COPD (FEV1/FVC ratio <70 % and FEV1 < 50 % of predicted value (GOLD stage 3 or 4)
- Unstable cardiac arrhythmias or decreased LVEF (<60 %)
- Gut diseases affecting food absorption
- Surgical diseases affecting ability to conduct heavy load strength exercise
- Embodied magnetic metal
- Endocrinological diseases potentially affecting muscles (diabetes mellitus, growth hormone-treated, sex hormone-treated, or untreated thyroid diseases)
- Alcohol consumption >21 U/week for men and 14 U/week for women (1 U = 15.2 ml of alcohol)
- Participation in studies using the same stable isotopically labeled tracers as this study (i.e., L-[ring-13C6] phenylalanine) within the last 6 months
- >1 h of weekly heavy strength training
- Dementia or other severe cognitive impairment

- Not holding Danish citizenship or not fluent in Danish

Further participants were excluded if they used the following medications:

- Systemic corticosteroids
- Sex hormone therapy, anti-sex hormone therapy,
- Anticoagulants (thrombin inhibitors, K-vitamin antagonists, heparins, pentasaccharides, factor Xa inhibitors, thrombocyte inhibitors except nonsteroidal anti-inflammatory drugs and acetylsalicylic acid).

Participants were initial screened by telephone. Participants not excluded underwent a physical examination including measuring of blood pressure and blood samples to determine if the participants could participate in the intervention safely. The participants also completed a 30-s chair stand test used for stratifying the randomization. The participants enrolled were then randomized into one of the 5 interventions groups described in section 9.1 using MinimPy 0.3[17,133]. Randomization was stratified by sex and number of completed repetitions on the 30-s chair stand test (<16 or ≥16). Participants in the supplement groups (WHEY, COLL, CARB), were blinded to which supplement they received. Training interventions were not blinded to the participants.

Table 1. Baseline characteristics of the included participants by group. Adopted from Paper II.

	CARB	COLL	WHEY	LITW	HRTW
Variable	(n = 36)	(n = 50)	(n = 50)	(n = 36)	(n = 36)
Demographics, Mean (SD)					
Age, y	69.6 (3.9)	70.4 (4.1	70.3 (4.3)	70.4 (4.0)	70.3 (3.1)
BMI, kg/m^2	26.0 (3.9)	25.4 (6.0)	25.2 (3.6)	25.7 (3.1)	25.9 (3.5)
Daily activity, Steps/day	10894 (5165)	10590 (3996)	10118 (3590)	10119 (3450)	9777 (3574)
Protein intake, g/kg/day	1.2 (0.3)	1.2 (0.4)	1.1 (0.3)	1.0 (0.3)	1.1 (0.4)
Energy intake, kJ/day	8442 (1804)	8150 (1952)	8529 (2092)	7445 (2220)	8268 (2146)
Body Composition					
Fat free mass, kg	48.5 (7.8)	49.2 (8.6)	50.0 (8.5)	48.1 (9.3)	48.8 (9.9)
Fat percentage, %	33.2 (9.3)	32.0 (9.1)	32.7 (7.5)	34.3 (7.5)	34.7 (7.1)

Quadriceps size, cm^2	56.6 (11.3)	56.0 (13.9)	54.5 (11.0)	56.7 (11.4)	55.4 (13.1)
Strength and function					
400 m gait time, s	248 (42)	243 (38)	242 (30)	242 (30)	251 (27)
30 s chair stand, reps	19.9 (5.7)	20.1 (5.3)	19.4 (4.6)	20.1 (4.6)	18.9 (4.9)
Leg extensor power, W	183.1 (56.2)	191.2 (67.2)	189.6 (59.6)	190.8 (61.4)	194.2 (65.8)
MVIC, Nm	158.9 (41.1)	169.0 (53.4)	177.6 (47.0)	171.5 (44.4)	165.0 (50.8)
SF-36					
MCS	59.3 (3.2)	57.3 (4.3)	57.6 (3.6)	57.1 (4.7)	57.5 (4.4)
PCS	55.3 (4.7)	56.0 (4.7)	56.8 (3.1)	56.4 (4.0)	56.5 (4.2)
Laboratory data					
Hba1c, mmol/mol	36.0 (2.2)	35.8 (3.4)	36.2 (3.5)	35.8 (2.9)	35.8 (2.7)
Total cholesterol, mmol/L	5.6 (0.9)	5.7 (1.0)	6.0 (1.2)	5.5 (1.0)	5.8 (0.9)
HDL Cholesterol, mmol/L	1.9 (0.5)	2.0 (0.6)	1.8 (0.5)	1.8 (0.5)	1.8 (0.5)
LDL Cholesterol, mmol/L	3.1 (0.8)	3.2 (1.0)	3.4 (0.9)	3.0 (1.0)	3.4 (1.0)
Triglycerides, mmol/L	1.3 (0.6)	1.4 (0.8)	1.7 (0.8)*	1.4 (0.6)	1.4 (0.6)
Creatinine, µmol/L	76.8 (14.7)	81.4 (15.9)	80.5 (11.6)	78.8 (14.7)	77.0 (12.7)

10.2 Quadriceps cross sectional area – Magnetic resonance imaging (MRI)

The primary outcome of the CALM-study was changes in cross-sectional area of the m. quadricep of the dominant thigh. Any changes would directly reflect the different interventions ability to affect the skeletal muscle mass.

MRI were chosen as the imaging modality due to its high sensitivity and validity[134] and its superiority to other modalities such as DXA scans[135]. MRI is an imaging modality which work by employing a strong magnetic field which align the protons in the body with the field. A radiofrequency current is then pulsed through the patient to make the protons spin out of equilibrium. The radiofrequency field is then turned off and the protons will then realign with the magnetic field under the release of energy. The time it takes for the protons to realign in combination with the energy released is measured by the MRI sensors and used for discriminating between different chemical molecules. Different tissues have different chemical molecules and by computational analysis of the measured signals by the MRI sensors specific and detailed imaging of different tissues can be produced[136].

The cross-sectional area of the m. quadriceps was chosen due to its importance for physical functionality and for the comparison between other measurements (e.g. biopsies) and studies. Scans were performed at baseline, 6, 12 and 18 months (see fig. 5). Participants were instructed to abstain from vigorous physical activity for 48 hours prior to each scan. The following description is adopted from paper II with minor modifications.

MRI scans of both thighs were performed in a Siemens Verio 3 Tesla scanner by blinded radiographers. Participants were scanned in supine position using a dedicated 32-channel body coil, and a phantom was placed parallel to the femur during the scans. The following protocol was used; 3 plane GRE scout (matrix res. 1.2.0x1.6x6.0 mm, FOV 330mm, TE 3.69ms, TR 7.8ms, scan time 27s); Axial T1 tse from the medial tibia plateau to the pubic symphysis (matrix res. 0.8x0.8x8.0mm, FOV 400mm, TE 8.4ms, TR 500, scan time 3:26).

Each scan consisted of six 8-mm thick axial slices separated by 60-mm gap, with the first slice being placed on the medial tibia plateau. Slice 4 was used for assessing the qCSA and all analysed scans were performed by the same blinded investigator using OsiriX v. 5.5.2 (OsiriX medical imaging software, Geneva, Switzerland). Each scan was analysed twice, showing a mean coefficient of variation between measurements of 0.7%. The mean of the two measurements were used for further analysis. Staff performing and analysing the MRI images as well as the strength and functional tests were blinded towards the interventions and time points of the scans.

The primary outcome was the assessment of the time interval between baseline and after 12 months of intervention. Scans performed at 6 months after the intervention start and scans performed at 18 months (6 months after ending the intervention) will not be reported in this thesis.

10.3 Body composition - Dual-energy x-ray absorptiometry (DXA)

DXA scans were performed to assess body composition at baseline 6, 12 and 18 months and bone mineral density at baseline and 12 months. Changes in body composition due to the intervention is of interest, but especially the interpretational information it provides with respect to other measures conducted in CALM is also highly valuable.

DXA scanners has the advantage of being less requiring with respect to costs, trained personnel and time in comparison to more advanced technology such as MRI or computed tomography (CT). Further, due to the low dose of radiation it is safe to use, and scans

can be conducted repeatedly, making it a valuable method in many different clinical as well as research settings. It was originally developed for assessing the bone mineral density in osteoporotic patients but are now also widely used for assessing body composition. Despite anthropometric data such as height, weight, waist circumference being strongly correlated with different tissue quantities, DXA-scans are preferred to assess body composition in trials of shorter duration due to its higher sensitivity[137].

The DXA-scan is an imaging modality which work by the attenuations of dual x-ray radiation energy range. By placing an individual between the x-ray emission source and an x-ray radiation detector it is possible to estimate the composition and mass of three different kind of tissues, i.e. bone-, fat-free- and fat mass. The mass and composition is determined by differences in attenuation of the x-ray beam in comparison to a known reference material[138]. The following description of the DXA scan procedure is adopted from paper III and slightly modified.

All DXA scans was performed in a Lunar DPX-IQ DXA scanner (GE Healthcare, Chalfont St. Giles, UK), and analyzed with the enCORE v.16 software package (Lunar iDXA; GE Medical Systems) by an investigator blinded to participants ID, time and intervention. At baseline and at 12 months, participants arrived in the morning having refrained from solid foods from 21:00 the day before, and the scanning was performed between 08:00 and 10:00. At the 12-month time point DXA scans were performed between 48 hours and 14 days after last training session. The DXA scans performed at 6 and 18 months were performed in the fed state at all time during the day with participants being euhydrated and instructed to void prior to the scan.

The DXA scan were auto segmented by the software and the regions of interest (ROI). This were then secondly adjusted by the blinded investigator according to predefined anatomically fixed points. For the upper body the most distal part of the chin, the armpit and caput humerus were used to separate the head, torso and arms. For the lower body a triangle was placed upside down with the two proximal corners being parallel with the most proximal part of the hip and the distal tip placed so the lines followed the lateral part of ramus ischiadikum in order to separate the legs. Unblinded personnel performed DXA scans and blood sampling, but analyses and interpretation of the data output from these were done by blinded researchers.

Measures of total fat free mass, leg and arm fat free mass, appendicular fat free mass (ASM), total fat mass, total fat %, visceral fat % and bone mass generated by the DXA-

scans a baseline and 12 months were used in this thesis. The appendicular skeletal muscle mass index (ASMI) were calculated as ASM/ height^2.

10.4 Oral glucose tolerance test (OGTT) – Insulin ELISA

An OGTT was performed at baseline and at 12 months in order to assess changes in glucose tolerance as an effect of the intervention. Any changes would be highly relevant with respect to the overall question of whether or not the interventions could have relevance for recommendations with respect to prevention of Sarcopenia.

The OGTT has previously and is still being used as an important test in diagnosing and monitoring diabetes, even though it was replaced by HbA1c as the primary diagnostic measurement in type 2 diabetes in 2011[139]. However, where HbA1c is a static measurement representing the mean glucose level in plasma across a timespan of ~3 months, i.e. the lifespan of an erythrocyte, the OGTT is a dynamic measurement of a 2-hours response period to 75 g of orally ingested glucose. The amount of blood samples drawn during an OGTT can vary, depending on the desired time resolution but normally include at least 2, e.g. a fasting value and the 2-hour post glucose intake value. HbA1c ≥ 48 mmol/mol / 6.5%, fasting plasma glucose ≥ 7.0 mmol/L or 2-hour OGTT plasma glucose ≥ 11.1 mmol/L are the current diagnostic cut-off values that defines type 2 diabetes.

Besides its diagnostic use, the OGTT can be used to estimate the insulin sensitivity. The euglycemic insulin clamp technique is considered being the golden standard regarding the measurement of insulin sensitivity. However, the euglycemic insulin clamp is a demanding and time-consuming technique in comparison to a simple OGTT due to its use of insulin and glucose infusions which requires experienced personnel and tightly monitoring. Matthews et al were the first to develop and validate an equation of estimating the insulin sensitivity based on fasting plasma glucose and insulin concentrations, known as the homeostasis model assessment (HOMA)[140]. Even though fairly good correlated with insulin-sensitivity ($r=0.69$, $p<0.0001$), Matsuda et al. developed and validated a calculation to derive estimates of insulin sensitivity based on insulin and glucose concentrations during an OGTT which correlated better ($r=0.73$, $p<0.0001$)[141]. In this thesis the following equations are used:

$$\text{HOMA-IR: } \frac{Insulin_0 * Glucose_0}{22.5}$$

Where $Insulin_0$/$Glucose_0$ is fasting plasma concentrations. Insulin concentrations are in μIU/mL and fasting glucose is in mmol/L.

Matsuda:

$$\frac{10{,}000}{\sqrt{(Insulin_0 * (Glucose_0 * 18) * ((mean\ OGTT\ insulin\ concentration)\ * (mean\ OGTT\ glucose\ concentration)))}}$$

Where $Insulin_0$ is the fasting insulin concentration in plasma, $Glucose_0$ is the fasting glucose concentration in plasma, mean OGTT insulin/glucose concentration is the mean insulin/glucose concentration calculated by dividing the AUC during the OGTT with 120min, using the timepoints 0,45 and 120min from the OGTT trial. Further, the Matsuda index were also calculated using the reduced timepoints equation from DeFronze et al[142] :

$$\frac{10{,}000}{\sqrt{(Insulin_0 * (Glucose_0 * 18) * (Glucose_{120} * 18)\ * Insulin_{120})}}$$

Where $Insulin_0$/$Glucose_0$ is the fasting insulin/glucose concentrations in plasma and $Insulin_{120}$/$Glucose_{120}$ is the 120min insulin/glucose concentrations in plasma. In both equations' glucose concentration is multiplied by 18 to get the concentration in mg/dL. Insulin concentrations is in μIU/mL.

The following description is adopted from paper III with minor modifications. The OGTT at baseline and 12 months were both conducted at Bispebjerg Hospital in the fasted state after the participants had completed the DXA-scan. An antecubital venous catheter was inserted, and a two basal venous blood sample was drawn. Then 75 g of anhydrous glucose dissolved in 250 ml of tap water was administered and 2 sets of blood samples were drawn in K3-EDTA vials at 45 and 120 min after the glucose consumption. One set of the plasma samples was sent to the biochemical department at the hospital and analyzed for glucose, HbA1c and proinsulin C-peptide. The other sample set were cooled on ice for 15 min and then centrifuged for 10 min at 3172 g at 4°C to isolate the plasma and aliquots was stored at -80°C for insulin analysis.

Glucose concentrations were measured at the biochemical department at Bispebjerg Hospital with absorption photometry using cobas® 8000 modular analyser, Roche Diagnostics.

The insulin concentrations were measured using an insulin enzyme-linked immunosorbent assay kit (ELISA) (ALPCO Diagnostics, Windham, NH, USA). The procedure was as follows: 200 µL plasma was converted to serum by adding 5µL 0.2unit/µL thrombin solution (T6884 – Sigma Aldrich). Samples were vortexed, left for 10 min at room temperature and spun at 1600 g for 10 min. The supernatant was collected for the insulin analysis. The ELISA was performed according to the manufacture's instruction. The kit was equilibrated to room temperature and 25 µL of each standard, control, and sample were loaded into each well followed by 100 µL Detection Antibody. All samples were loaded in duplicates and samples from the same subject were loaded on the same plate. Samples from subjects in the different groups were randomized on each plate. Standards and controls were loaded in triplicates. The plates were incubated for 1 h at room temperature, shaken at 800 rpm on a microplate shaker followed by 6 times washing. Thereafter, 100 µL of TMB substrate was added to each well to activate the fluorophore, the plate was incubated for further 15 min at room temperature shaking at 800 rpm on a microplate shaker. 100 µL of stop solution was added to each well and the plate was analyzed immediately after at 450 nm in the microplate reader (Multiskan FC, Fisher Scientific).

The area under the curve (AUC) for insulin and glucose concentrations were calculated using the trapezoidal method and used in the Matsuda and HOMA-IR equations to estimate changes in insulin sensitivity.

10.5 Muscle protein synthesis

The muscle protein synthesis was measured at baseline and at 12 months using the direct incorporation technique. We measured FSR during a period of 3 hour in a fasted state (basal FSR) and response period of 4 hour to a protein and carbohydrate drink containing 20g of whey hydrolysate and 10g of glucose (response FSR). Any differences between basal FSR and response FSR as well as any difference across the 12 months of intervention could provide valuable information with respect to explaining any observed differences in muscle mass as an effect of the intervention. Further, the number of individuals included as well as the inclusion of both males and females is very unique due to the high costs of experiments of this kind. Lastly, an evaluation regarding the interpretation of the FSR measure is also possible due to the diversity of measurements conducted in CALM.

10.5.1 Tracers

Tracer techniques has been used within metabolic research for around 100 years. Rudolf Schoenheimer were one of the pioneers within this field and his definition of a tracer is still holding today. In his well renowned thesis "The dynamic state of body constituents" he wright:

> *"In order to mark a compound for biological studies the label has to be of such nature that no change of physiological properties is affected by its introduction, but the experimenter must be able to estimate it in small amounts. Labels that satisfactorily fulfill these requirements are isotopes of elements that occur in organic matter: namely the less abundant isotopes of carbon, hydrogen, oxygen and nitrogen."*[81]

All atoms exist in various forms depending on the number of neutrons in the nucleus of the atom. These different variations of the same atoms are named isotopes. Isotopes can be divided into two forms, e.g. radioactive and stable isotopes. Stable isotopes have the advantage in comparison to the radioactive, that they do not disintegrate under the emission of radiation and are therefore safe to use in experiments. Further, the different stable isotopes natural abundance is very different. As an example, the stable isotope ^{12}C natural abundance is 98.9% in comparison to 1.1% for ^{13}C, which has one neutron more in its nucleus[81]. In stable isotope tracer techniques, the used properties are the low natural occurrence in combination with the slightly higher mass due to the extra neutron in the nucleus. In principle, a certain compound such as an amino acid can be marked with one or more ^{13}C, making the amino acid distinguishable from the natural occurring amino acid due to its slightly higher mass which can be measured by mass spectrometry. The tracer can either be administered orally or as an infusion, and by subsequently sampling from different tissues its path and destiny can be traced.

A wide range of stable isotopic labeled compounds exist, and selection of the tracer depends on the question (tissue, nutrient, metabolic pathway) of interest. Since skeletal muscle proteins consist of amino acids, MPS is measured by using different stable isotope labeled amino acids. For the direct incorporation technique (see section 10.5.5) by which it is possible to measure the FSR, leucine and phenylalanine with different stable isotope labeling are the most commonly used tracers. Despite that in theory, the measurement should be indifferent of the tracer used, it has been shown that the absolute FSR values obtained vary. Therefore,

results from studies using different infusion protocols cannot be compared directly. The variation is both due to the specific tracer used but also the choice of precursor and the period of time between biopsies[87]. However, changes in FSR are in general consistent with respect to both choice of tracer and infusion protocol.

In CALM we use L-[ring-$^{13}C_6$] phenylalanine was used as tracer. Herein, all six carbon atoms of the aromatic ring are labelled, ^{13}C.

10.5.2 Acute trial protocol and tissue sampling

The following description is adopted from paper IV with minor modifications. Participants arrived at the facility 8 a.m. in the morning by car or public transportation to avoid physical activity in an overnight fasted state from 9 p.m. the day before. They were instructed to abstain from strenuous physical activity 3 days prior to the trial. The participants were placed in a bed in a supine position and two venous catheters were inserted in an antecubital vein in each arm and a background blood sample were taken. Hereafter, at -270 minutes (see fig. 4), a continuous infusion with L-ring[$^{13}C_6$] phenylalanine tracer (Cambridge Isotope Laboratories, Tewksbury, MA, USA) at an infusion rate of 6.0 μmol·kg $FFM^{-1}·h^{-1}$ was started after injection of a priming dose 6.0 μmol·kg FFM^{-1} over 2 minutes. The tracers were dissolved in sterile saline water and filtered through 0.20-μm-pore disposal filters (Minisart, Sartorius Stedium Biotech, Gottingen, Germany) in the morning of the experiment. The tracer infusion rate was set to obtain a venous tracer-to-tracee ratio (TTR) of ~10%. After reaching steady state at -180 minutes another blood sample and the first biopsy were taken. The participants continued to rest in the supine position until another blood sample and biopsy were taken at 0 minutes. Immediately after, a drink containing 20 g of whey hydrolysate and 10 g of glucose was provided and finished immediately. Then blood samples were taken a 20 min, 40 min, 60 min, 90 min, 120 min and 240 min. At 240 min the last biopsy was taken, and the infusion stopped.

All blood samples were collected in 9 mL plasma Vacutainers containing EDTA, put at rest on ice for ≥10 min, and spun down at 3,200 g for 10 min at 4°C. Plasma were then transferred to eppendorf tubes and stored at -80°C until further analysis.

All three biopsies were obtained from vastus lateralis with individual incisions with ~3cm in-between with a 4-mm biopsy needle (Bergström, Stockholm, Sweden) using manual suction. At the beginning of the trial, the skin was shaved, and the thigh muscle were inspected and the incision sites for the three biopsies were marked. Before obtaining each biopsy, the area

was disinfected and local anesthetic (1% lidocaine) was administered. An ~1.5 cm incision was made before inserting the needle and obtaining the biopsy. An elastic band with a compression pad was used to compress the incision site for 30 min in order to avoid intramuscular hematoma. Before compression, the incisions were strapped with SteaStrips and covered with waterproof plaster. The muscle specimens were quickly cleansed from any visible blood, fat and connective tissue under a microscope, and then frozen in liquid N_2 and stored at -80°C until further analysis.

10.5.3 Sample preparation – FSR

The following description is adopted from paper IV with minor modifications. The muscle specimens were prepared for GC-C-IRMS analysis as follows. ~20 mg of the muscle sample was transferred to 2 mL lysing tube containing 10 lysing beads and two silicon carbide crystals. 1 mL of 4°C homogenizing buffer (Tris 0.02 M [pH 7.4], NaCl 0.15M, ED(G)TA 2 mM, TritonX-100 0.5%, sucrose 0.25 M) were added and the sample were homogenized 4 · 45 sec at speed 5.5 $m \cdot sec^{-1}$ with 2min pause in between (FastPrep 120A-230; Thermo Savant, Holbrook, NY, USA). The samples then rested for 3 hours at 5°C. They were then spun at 800 g for 20 min at 5°C and the supernatant discarded. 1.0mL of 4°C homogenizing buffer were added to the pellet and the sample were once again homogenized for 1 · 45sec at speed 5.5$m \cdot sec^{-1}$, left for 30 min at 5°C and then spun 800 g for 20 min at 5°C. The supernatant was again discarded and 1.5 mL KCl-buffer (KCl 0.7M, pyrophosphate ($Na_4P_2O_7$) 0.1M) added and the samples were vortexed and left overnight at 5°C. The sample were then vortex and spun at 1,600 g for 20 min at 5°C and the supernatant (the myofibrillar protein fraction) was then transferred to a Scot-glass and 2.3 mL ethanol 99% was added. The samples were then vortexed and left for 2 hours at 5°C. After a spin 1,600 g for 20 min at 5°C the supernatant was discarded and 1mL 70% ethanol was added to the pellet containing the myofibrillar protein fraction. The samples were vortexed and then spun at 1600 g for 20 min at 5°C and the supernatants were once again discarded. To hydrolyze the myofibrillar proteins 1 mL of 6 M HCL was added and the sample, vortexed and left overnight at 110°C. The constituent amino acids were then purified over Dowex resin (AG 50W-X8 resin; Bio-Rad Laboratories, Hercules, CA) columns using 2 M NH_4OH for elution and put under N_2 flow at 70°C until dried. Hereafter, the hydrolyzed the amino acids were derivatized as the N-acetyl-propyl (NAP) derivative. Briefly, the samples were dried under N_2 flow. Then 200 µL propyl acetate and 100 µL boron trifluoride propanol were added. Samples were then vortex and heated for 30 min at 120°C. The samples were then dried under N_2 flow.

Then 50 µL acetonitrile, 26 µL 1.4-dioxan, 38 µL triethylamine and 24 µL were added with vortex mixing in between. Samples were then heated in an oven at 55 °C for 15 minutes and then transferred to a 1.5 mL eppendorf tube. 50 µL chloroform and 2 x 75 µL 0.001 M $NaCHO_3$ was added with vortex mixing in between. Samples were then spun, and the top aqueous layer were removed, and the sample were stored as -20 °C until GC-C-IRMS analysis.

10.5.4. Mass spectrometry – GC-C-IRMS

As mentioned in section 10.5.1. the measurement of the abundance of tracer is conducted by the use of mass spectrometry (MS) coupled to different kind of separation modules. In CALM we use the gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) system for measuring the enrichment in myofibrillar protein fraction.

The GC-C-IRMS system is a combination of three separate modules, the GC module, the combustion oven, and the IRMS. The GC has the function of separating all of the metabolites injected to the column. The sample is injected with a carrier gas to the column and the gaseous compound interacts with the column wall and is then trapped until the column temperature reaches a certain level. During the heating of the oven in which the column is placed, the chemical compounds will be released from the column at different timepoints due to the physical and chemical interaction with the coating of the inner wall of the column. This is called the retention time. By knowing the retention time, the specific compounds of interest can then be followed through the next steps. After separation, the compound is then lead into the combustion oven with a temperature of 940 °C, yielding in the case of amino acids: CO_2, N_2 and H_2. The last step is the IRMS which is capable of measuring the $^{13}CO_2/^{12}CO_2$ ratio which is referenced to a calibration curve. When the CO_2, N_2 and H_2 is entering the IRMS it is bombarded with electrons to make the molecule charged. The molecule is then accelerated before it is lead into the magnetic field which will bend the curvature. Depending on the magnets current, either CO_2, N_2 or H_2 will be analyzed. The curvature of a given molecule is depending on its mass, and different masses are measured by detectors spaced from each other hereby giving the$^{13}CO_2/^{12}CO_2$ ratio. It is very important that the separation of molecules in the GC module is complete, since there is no further separation by mass in the following modules. In addition, using multiple labelling is clearly and advantage using IRMS, since the $^{13}CO_2/^{12}CO_2$ ratio becomes markedly different, despite the relatively small amounts of tracer infused during the acute trial.

For the analysis described above we used the GC-C-IRMS system provided by Hewlett Packard 5890-Finnigan GC combustion III-Finnigan Deltaplus; Finnigan MAT; Bremen; Germany. For a detailed description of settings etc. see the publication by Bornø et al.[143].

10.5.5. The direct incorporation technique – FSR calculation

The direct incorporation technique also known as the precursor-product used in CALM for calculating the FSR is the preferred way of measuring protein(s) specific changes. Basically, in order to calculate the FSR using this technique the following measures are needed:

1. The enrichment level in the precursor,
2. The enrichment level in the product.

The enrichment levels are normally given in mole per excess (MPE) which is calculated using the tracer-to-tracee ratio (TTR) measured by the IRMS as follows:

$$MPE = \frac{TTR}{1 + TTR}$$

The enrichment in the precursor represents the amount of tracer that has been available for the specific protein synthesis of interest. The enrichment level in the product represents the amount of tracer that has been incorporated into the specific proteins(s) of interest. For calculating a rate, a minimum of two samples is required. Depending on the choice of precursor, the number of samples can vary. A simple illustration of the precursor product model can be found underneath.

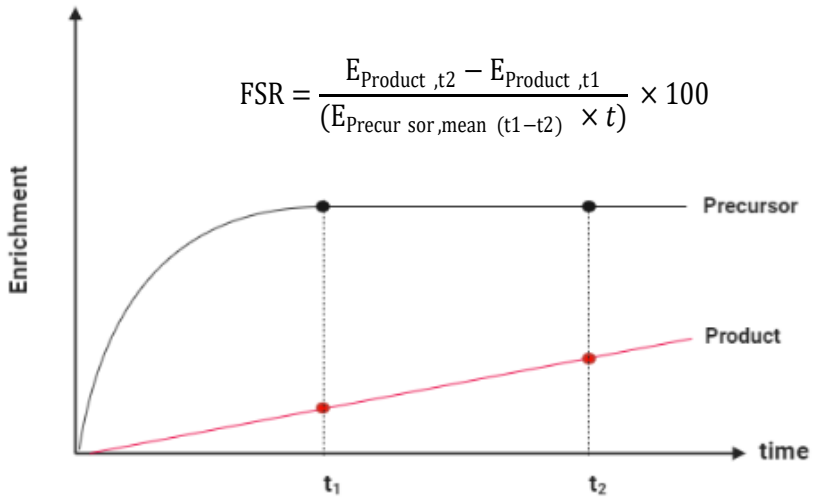


Illustration 3. A simple illustration of the precursor product technique. An infusion of a given tracer is started. Sampling is beginning after reaching steady state in the precursor level. Since time to reach steady state in the product is far longer than the precursor, the difference in enrichment between t_1 and t_2 will represent the synthesis rate. Black and red dots represent sampling from the precursor as well as the product pool. E is enrichment, t is time

Different assumption underlies the interpretation of FSR calculation as being representative of the specific protein synthesis[79]:

1. That the rate of incorporation of the tracee is constant during the incorporation period of tracer.
2. The tracer incorporated will not reappear due to breakdown during the time of the infusion protocol
3. The pool size of tracee bound protein is constant through the experiment
4. No significant time delay in the incorporation of amino acids into protein
5. The measured enrichment of the precursor is the actual enrichment of the true precursor.

These assumptions need to be considered when interpreting the certainty of any measurement of FSR actually representing the true synthesis of a given protein. However, in short term studies such as the acute trial in CALM measuring protein with a relatively slow turnover rate the assumptions are reasonable.

In CALM we use the L-[ring-$^{13}C_6$] phenylalanine as the tracer. We use a weighted mean of the plasma enrichment levels as precursor, since we did not have the possibility of measuring intracellular precursor enrichment level at more than one timepoint due to small tissue samples. Despite not qualifying for the 5th assumption listed above, there is some advantages of using the plasma enrichment instead of the intracellular enrichment in phenylalanine which is otherwise known as the best estimate of the true precursor[144]. Since, the time resolution is much higher due to the number blood samples obtained (7) in comparison to muscle samples (3), any unintended fluctuation in enrichment levels during the infusion trial will be captured using the plasma enrichment. The final equation for measuring the FSR in CALM were as follows:

$$\mathrm{FSR} = \frac{\mathrm{E}\Delta_{\text{Myofibrilar protein, Phe}}}{(\mathrm{E}_{\text{Plasma mean, Phe}} \times t)} \times 100$$

Where $E\Delta_{\text{Myofibrilar protein, phe}}$ is the difference in enrichment level between two biopsies either M_{240min} -M_{0min} or M_0 - $M_{-180min}$, $E_{\text{plasma mean, phe}}$ is the weighed mean of the plasma enrichment levels and t is the time between the biopsies in hours. A factor of 100 were used to express the FSR in %/h.

10.6 Amino acids (AA)

The plasma AA concentrations as well as the plasma enrichment with L-[ring-$^{13}C_6$] phenylalanine was measured at baseline and at 12 months. Both were measured in plasma samples obtained at -180, 0, 20, 40, 60, 90 and 240min during the infusion trial. The enrichment levels were used for calculating the FSR as described above. The AA concentrations would provide coarse information on uptake and metabolism kinetics for each individual after the ingestion of 20g of whey hydrolysate and 10g of glucose. Again, the number of individuals included as well as the inclusion of both males and females is very unique due to the high costs of experiments of this kind. The AA-profile is used as documentation of protein intake/uptake in study IV. Since, I have not been directly involved in the preparation of samples and measurements it will not be described in detail.

The plasma enrichments were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) using internal standards. LC-MS/MS is using liquid chromatography for separation of metabolites before the samples is lead into the MS/MS module. The metabolites

are sprayed into an interface (ESI probe, electron spray ionization) in order to make the metabolites charged. Then, they pass on through the first MS/MS modules which further separates and fragmentize the metabolites before they reach the detector. The plasma samples were prepared as follows. 100 µL of plasma was mixed with 100 µL combined internal standard and 120 µL of 50% acetic acid (Merck, Darmstadt, Germany) For more detailed description of the preparation and instrumentation see Bornø et al 2014[145].

For each time point listed above the following 20 AA were measured:, Aspartic acid (Asp), Glutamic acid (Glu), Serine (Ser), Glycine (Gly), Aspargine (Asn), Glutamine (Gln), Histidine (His), Threonine (Thr), Alanine (Ala), Proline (Pro), Arginine (Arg), Tau-Methylhistidine (Tau-MeHis), Tyrosine (Tyr), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Tryptophan (Trp) Phenylalanine (Phe) and Lysine (Lys).

10.7 Skeletal muscle metabolome

The skeletal muscle metabolome was measured at timepoint 0 min and 240 min at baseline and at 12 months using the biopsies obtained during the acute-infusion protocol. As described in section 7.6 few studies have investigated the skeletal muscle metabolome. The use of targeted[128] as well as untargeted[130,131] platforms has been used previously. All of them uses the same sample preparation protocol validated by Alves et al[127] with minor variations, but different MS-modalities (MSI-CE-MS, UPLC-MS, GC-MS). We use untargeted GC-MS TOF (time of flight) and a similar preparation protocol to the previous studies for measuring the skeletal muscle metabolome in CALM. Before analyzing the samples from the CALM study, we ran several tests runs with different preparation protocols using samples we had in excess from previous studies conducted by our research group. This were done in order to ensure that the preparation protocol as well as the GC-MS TOF protocol performed as expected.

Since the skeletal muscle metabolome analysis was not included as part of the original test battery planned in CALM, we were limited by the amount of tissue that was left after the planned analysis were conducted. Therefore, our main focus (paper IV) were:

1) to report on the methodological aspects of measuring the skeletal muscle metabolome using untargeted GC-MS TOF,

2) to explore whether or not we were capable of seeing an effect of the 20g of whey hydrolysate and 10g of glucose after 4 hours and

3) to explore if there were any alteration in the skeletal muscle metabolome as an effect of the intervention.

It should be noted, that the effect of the intervention was only explored in the supplemental arm (CARB n=6, COLL n=6, WHEY n=6), since we did not have enough samples in the training arm (LITW n=3, HRTW n=3).

10.7.1 Sample preparation

The following description are adopted from paper IV with minor modifications. Muscle samples were extracted using a similar method as described by Alves et al 2015[127], which is based methanol/chloroform/water at Vol:Vol ratio of X:Y:Z, respectively. The muscle specimens were prepared and analyzed as followed. The description is adopted from paper IV. ~25 mg of frozen muscle tissue was put into 2 mL lysing tubes containing 10 lysing beads and two silicon carbide crystals. 0.5 mL of 5°C solvent (50% methanol containing 20 ppm ribitol) was added. The biopsies were homogenized 4 x 1 min with 2 min pause in between at speed 5.5 $m \cdot sec^{-1}$ at 5°C (FastPrep 120A-230; Thermo Savant, Holbrook, NY, USA). Then, 300 µL of chloroform was added and the homogenized samples were vigorously vortexed for 10 min at room temperature. The samples rested on ice for 20 min and was then centrifugated for 15 min at 5°C at 16,000 g. 60 uL of the upper part of the aliquot (methanol part) and 40 uL of the lower part of the aliquot (chloroform part) was put into 200 uL glass inserts. The glass inserts were then dried under vacuum using a SpeedVac (Labogene, Lynge, Denmark) at 40°C for 3 hours. Samples were then derivatized in two steps, first by addition of 10 uL 20 $mg \cdot mL^{-1}$ methoxamine hydrochloride in dry pyridine (90 min at 45°C by agitating at 750 rpm) followed by trimethylsilylation (TMS) using trimethylsilyl cyanide (TMSCN), as described previously[146]. TMS derivatization was performed by addition 40 uL TMSCN and by agitating at 750 rpm for 40 min at 45°C. A total of 226 number of samples were analyzed in a randomized order in GC-MS, Y samples originate from this study design and Z samples were pooled control muscle samples run every 10^{th} sample in the sequence.

The control muscle sample were prepared as follows. 20 skeletal muscle samples of ~25mg frozen muscle tissue from previous studies were prepared as described above. However, the upper part of the aliquot (50% MeOH part) of all twenty 2 mL lysing tubes were transferred and mixed vigorously before 60 uL were transferred to each of the twenty 200 uL glass inserts. The lower part of the aliquot (Chloroform part) of all twenty 2 mL lysing tubes were mixed in

the same way before 40 uL were transferred to each of the twenty 200 uL glass inserts. This were done in order to secure that the samples were as similar as possible.

10.7.2 Gas chromatography - mass spectrometry – time of flight (GC/MS-TOF)

The GC/MS-TOF is basically functioning the same way as the variants of mass spectrometry modalities described above. Briefly, the samples are injected into the column using and inlet carrier gas. The column is then heated and depending on the chemical properties of the different metabolites they will be released at different timepoints and led forward to the MS-TOF. Here the metabolites will be ionized and accelerated to the same speed by an electric field. Then they pass to a chamber of vacuum with no electric field, and the ions with a high mass-to-charge (m/z) ratio will move slower than those with a low m/z ratio hereby separating the ions. The GC/MS-TOF has the advantaged that the flight times are very short for all of the ions making it possible to accumulate several thousand mass spectra in one second. This rapid accumulation of spectra leads to better reproducibility and signal-to-noise ratios and a better use of small quantities of samples.

Our samples were analyzed as follows. The description is adopted from paper IV with minor modifications. Sample derivatization and injection of 1 uL derivatized aliquot were automated using a Dual-Rail MultiPurpose Sampler (MPS) (Gerstel, Mülheim an der Ruhr, Germany) as described previously[147]. The GC–MS consisted of an Agilent 7890B gas chromatograph (GC) (Agilent Technologies, California, USA) coupled with a time-of-flight mass spectrometer, HT Pegasus TOF-MS, (LECO Corporation, Saint Joseph, USA). A GC column used was Restek ZB 5% Phenyl 95% Dimethylpolysiloxane column (30 m with I.D. 250 lm and film thickness 0.25 lm) with a 5 m inactive guard column (Phenomenex, Torrance, USA). A hydrogen generator, Precision Hydrogen Trace 500 (Peak Scientific Instruments Ltd, Inchinnan, UK) was used to supply a carrier gas at the constant column flow rate of 1.0 $mL \cdot min^{-1}$. The initial temperature of the GC oven was set to 40 C and held for 2 min followed by heating at 12 $°C \cdot min^{-1}$ to 320 °C and kept for an additional 8 min, making the total run time 33.3 min. A post run time at 40 °C was set to 5 min. Mass spectra was recorded in the range of 45–600 m/z with a scanning frequency of 10 scans sec^{-1}, and the MS detector and ion source was switched off during the first 6.4 min of solvent delay time. The transfer line and ion source temperature were set to 280 °C and 250 °C, respectively. The mass spectrometer was tuned according to manufacturer's recommendation using perfluorotributylamine (PFTBA). The MPS and GC–MS

was controlled using vendor software Maestro (Gerstel, Mülheim an der Ruhr, Germany) and ChromaTOF (LECO Corporation, Saint Joseph, USA).

10.7.3 Assignments and analysis strategy

The following description is adopted from paper IV with minor modifications. We processed and assigned the metabolites as follows. The raw GC-TOF-MS data was processed using Statistical Compare toolbox of the ChromaTOF software (Version 4.50.8.0) with following settings; the raw data was used without smoothing prior to peak deconvolution, baseline offset was set to 0.8, expected averaged peak width was set to 1.2 sec, signal-to-noise was set to ≥5, peak areas were calculate using deconvoluted mass spectra, common m/z ions of derivatization products were determined as 73, 75, and 147. Deconvoluted mass spectra were also used for peak identification using LECO-Fiehn and NIST11 libraries. The library search was set to return top 10 hits with EI-MS match of >75% using normal-forward search and with a mass threshold of 20. Deconvoluted peaks were aligned across all samples using following settings; retention time shift allowance of <3 sec, EI-MS match of >90%, mass threshold of >25, and present in >90% of all pooled samples.

In paper IV our analysis strategy was chosen in order to assess the following three questions: First and foremost, we wanted to validate and report the methodological aspect of measuring the skeletal muscle metabolome using untargeted GC/MS-TOF. Secondly, we investigated if we were able to detect any differences as an effect of 20g of whey hydrolysate and 10g of sucrose between the metabolome of the 0 min biopsy and the 240 min biopsy. Thirdly, we wanted to investigate whether or not we could detect any differences between the groups at 12 months. However, we categorized the last analysis as an exploratory analysis instead of a confirmatory due to the low number of samples available.

10.8 Statistical analysis

For all the planned analysis (Paper II, III and IV(only FSR)) we used the statistical analysis described in Bechshøft et al[17]. The sample size (paper II and III) was calculated based on our detection limit for the primary outcome of qCSA. We aimed to detect between-group differences in qCSA changes of 2% over the intervention period, corresponding to approximately 80 mm^2. Based on previous data from our lab[148], an SD of ~1.4 cm^2 for qCSA was expected. Thus, applying a level of significance of 0.05 and a power of 0.80, a group size of 30 participants

was required. Taking dropout rate into account we included 36 participants in HRTW, LITW and CARB groups and 50 participants in WHEY and COLL groups since we expected a higher dropout rates in these two groups due to taste issues and the lack of motivation to complete with no training[17]. The power calculation of sample size for the subgroups participating in the acute trial were based on previous data obtained in healthy elderly participants by our group since we are not aware of on any other data on MPS responsiveness over time. We used the following parameters for conducting a power calculation of the sample size in the sub groups: based on previous data we expected an average 4-h postprandial responsiveness to protein intake from basal condition of 0.016 $\%\cdot h^{-1}$ which would approximately correspond to an increase in muscle protein FSR of 50 % with an interparticipant SD on the increase of 0.017 $\%\cdot h^{-1}$. We aimed to be able to detect a change in responsiveness of 50% within a group after the 12-months intervention corresponding to a change of 0.008 $\%\cdot h^{-1}$ in the increase in the muscle protein FSR from 0.016 $\%\cdot h^{-1}$ to 0.024 $\%\cdot h^{-1}$. The SD for the change in responsiveness were set to 0.006 $\%\cdot h^{-1}$ based on previous findings. When including 10–12 participants, a power of at least 0.80 to detect a statistically significant difference within each intervention group over time would be obtained. When taking an the expected dropout into account, it were decided to include 15 in the WHEY and COLL groups 12 participants in the HRTW, LITW and CARB group.

We performed all analysis as intention-to-treat and per protocol in paper II, III and IV. Intention-to-treat analysis were performed on all participants completing the study irrespective of adherence to the intervention. Per protocol analysis were performed on the participants with a supplementation adherence >75% corresponding to 1.5 supplement pr. day and a training adherence >3 sessions pr. week for the LITW-group and >2 sessions pr. week in the HRTW-group. The following descriptions are adopted from their respective papers with minor modifications.

10.1 Paper II

Baseline data are summarized by group means ± standard deviations (SD) unless otherwise stated (table 1.). Effects of the interventions were investigated within each study arm, separately. The individual treatment effects are reported as the mean change and associated 95% confidence intervals (CI)) during the intervention. Between-treatment effects are reported as mean difference in treatment effect and associated 95% CI. The level of significance was set to

<0.05. The effects of the interventions were analysed as a modified intention-to-treat, including all participants that completed at least one test at the 12-month timepoint, irrespective of adherence to the interventions.

Changes from baseline to 12 months were investigated separately in the supplementation arm and in the training arm of the study, using a longitudinal mixed model with time (baseline and 12 month) and intervention group (three levels) as fixed predictors, including their interaction, and person as random term. Treatment inferences were based on significance test of the interaction term, and further investigated by contrasts of intervention group changes from baseline to 12 months between all pairs (CARB vs COLL vs WHEY, and WHEY vs LITW vs HRTW) of group combinations.

R (version 3.5.1) with the function lm() from the stats package (ver 3.5.1), lmer() from the lme4 package (ver. 1.1-20) and glth() from the multcomp package (ver. 1.4-8) were used for data analysis.

10.2 Paper III

Data was tested for normality by the Shapiro-Wilks normality test and for equal variance by the Brown-Forsythe test. All insulin, HOMA, proinsulin C-peptid and Matsuda data were log2-transformed to obtain normal distribution. Data was analyzed using two-way ANOVA with repeated measurements followed by the post Holm- Sidak test. P-values below 0.05 were considered significant and trends are reported for p-values between 0.05 and 0.1.

In the intention to treat group, we analyzed if changes in any of the measured parameters were associated with changes in HbA1c, insulin AUC, and glucose AUC. Furthermore, we looked for associations at baseline between our measured parameters and HbA1c, insulin AUC, and glucose AUC from a cross sectional perspective. Therefore, all subjects are pooled in this analysis. Due to multiple testing a p-value below 0.001 was considered significant. For the cross-sectional associations, we used the Bonferroni Correction for multiple testing $\alpha/45 = 0.05 / 45 = 0.001$. Therefore, a p-value below 0.001 was considered significant. All tests were performed in Prism (GraphPad) and all data are presented as means ± standard error (SE).

10.3 Paper IV

FSR data were analyzed with a one-way ANOVA on each intervention arm separately comparing the difference between delta fractional synthesis rates at 0month and at 12months (Δdelta FSR). Further, a paired t-test between the basal and response were performed at 0month for males and females separately. All FSR analysis were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA).

The MM data was subjected to univariate and multivariate statistical analysis prior to investigate possible effects according to the study design factors, including visit (0m and 12m), treatment (basal and response) and the intervention (CARB, COLL, WHEY, LITW and HRTW). Principal component analysis (PCA)[149] was performed prior to explore the MM data and evaluate an overall variation present in the dat. ANOVA-simultaneous component analysis (ASCA)[150]with permutation test, as described previously[151], was used to study significance of study design factors and their explained variations . Further, any single metabolite difference according to the design factors were analyzed using an ANOVA adjusted for multiple testing using false discovery rate (FDR) rate of 10%. Prior to PCA, ASCA and ANOVA, the MM data was normalized to the internal standard (ribitol) peak area. The MM data was mean centered (the mean of each column was subtracted from the corresponding variable) and divided by its standard deviation, also called “auto scaling” before PCA and ASCA. All statistical data analysis was conducted using MATLAB ver. 2016b (The Mathworks, Inc. USA) and custom MATLAB scripts written by the authors.

11.0 Results and discussion

11.1 Paper I (results and discussion)

This study investigates the current definitions made by several different consensus groups on sarcopenia and their given arguments. The primary study question in paper I were:

> How has the definition of Sarcopenia changed over time? What is the argument for changing the definition? And what are the implications of the current definitions?

As outlined in figure 1, the definition of Sarcopenia has changed significantly since its introduction in 1989. From pointing to the self-evident and natural phenomenon of age-related loss of muscle mass it is now considered as a clinical condition of disease with its own ICD-10 diagnostic code. According to the latest consensus report[30] and clinical guideline[31], published after Paper I, Sarcopenia is now defined by the following diagnostic algorithm:

1. Find case – Use the SARC-F screening questionnaire or clinical suspicion
2. Assess – Muscle strength (grip strength or chair stand test)
3. Confirm – Muscle quantity or quality (DXA, BIA, CT, MRI)
4. Severity – Physical performance (gait speed, SPPB)

If muscle strength (step 2) is below a certain reference value, the diagnosis should be confirmed by a measure of either muscle quantity or quality (step 3). The severity should then be categorized by the patient's physical performance (step 4). The latest algorithm is not markedly different from the previous consensus definitions except for changing the categorization from syndrome to disease and the clarity when it comes to clinically assessing possible patients.

Only three of the different consensus reports provide an argument for changing the definition of Sarcopenia from the original, i.e. low muscle mass, namely the lack of clinical relevance[3,4,6]. This argument is disconcerting for at least two reasons. First, if a well-defined phenomenon is not clinically relevant, changing the definition does not make it become clinically relevant. Instead, it changes the phenomenon under consideration. Secondly, every definition can become clinically relevant by adding a clinically relevant criteria to its definition.

Baumgartner et al proposed the first operational definition in 1998 which exclusively focused on muscle mass (fig. 1). They qualified the definition through its association to lowered physical function and morbidity. This changed the focus from 2000 and forth from concentration on the loss of muscle mass to be centered on the robustness of its association with

decreased physical function and mortality. At glance, this appears reasonable from a clinical perspective, e.g. to focus on the phenomenon with the strongest association to a negative health outcome. However, with the changed definition the outcome and the phenomenon are almost, if not exactly, the same. This completely undermines the argument of the new definition being clinically relevant due to the use of tautological reasoning which can be illustrated by the following three points:

1. **Definition:**
   Sarcopenia = Decreased physical function/strength + low mass
2. **Argument for its clinical relevance:**
   Sarcopenia is associated with decreased physical capability/function and mortality
3. **1(def.) + 2(argument):**
   Decrease in physical function/strength is associated with decreased physical capability/function

From a scientific perspective, the argument for including strength and physical function into the definition is therefore invalid.

The consequences of the changed definition are several. According to the algorithms the muscle mass/quality is secondary to strength/physical function and only of value if the first criteria is fulfilled. The crucial role of the skeletal muscle in maintaining metabolic homeostasis and its endocrine functions are likely overlooked clinically when the primary criteria inclusion criteria is strength/physical function. Likewise physical function is at risk of being reduced to the question of muscle mass/quality when both are directly coupled in the definition[27]. Furthermore, it reduces the relevance of the term in other clinical specialties such as nephrology, endocrinology and oncology, where muscle mass per se could be of clinical importance for both categorizing patients as well as in selecting treatment. Beside the reductionist understanding of the three different phenomena, i.e. loss of mass, loss of strength, loss of function, the new definitions also lead to general confusion of what is meant by the term sarcopenia, since it no longer covers one but three phenomena.

In conclusion, solid scientific research demands a high degree of control as well as clarity with respect to the definition of the phenomenon of interest. The current change in

definition has not contributed to neither clarity nor the possibility of conducting controlled experiments with homogeneity amongst included participants. This is underpinned by investigators reporting it to be a major challenge to recruit research participants who match the criteria for primary Sarcopenia[152]. Based on this, one could fairly speculate whether the loss of muscle mass is clinically relevant or at all a phenomenon of disease. This off course depends on the definition of disease, which again is a very difficult to define[153,154]. However, it seems troublesome to define phenomena in a way that makes them clinically relevant since all phenomena eventually could turn into being phenomena of disease. Instead, phenomena which are clinically relevant should be defined based on their phenotypical manifestations ensuring that the phenomenon of interest is clinically manifest, relevant and detectable.

11.2 Results - Paper II, III IV

11.2.1 Results Paper II

Paper II reports on the primary outcome of the CALM study. The study was designed in order to answer the following question:

> Is recommending protein supplementation with or without different types of training an effective way of preventing or attenuating the sarcopenic process with respect to muscle mass and muscle strength? (Paper II)

With the intention-to-treat study design the main purpose of the CALM study was to investigate the effect of recommendation rather than the intervention per se. Nevertheless, both an ITT as well as PP-analysis were conducted. Results descriptions are adopted from Paper II with minor modifications.

We had 1285 contacts from potential participants. 1148 were screened via telephone. Out of the 1148, 280 participants had a planned on-site screening visit of which 39 participants declined to participate and 33 were excluded prior to enrolment. Out of the 208 participants included and randomized into one of the five intervention groups. A total of 184 out of the 208 participants included completed the 12-month test-battery. In total, 24 participants dropped out during the study. 11 of the participants dropped out due to illness or injury unrelated to the intervention. 5 participants dropped out due to disliking the supplement. 3 participants dropped out due to the test-battery being to extensive, and participants dropped out 5 due to personal reasons. A consort diagram can be found in paper II.

Adherence and dietary registrations:

The adherence to the training and supplementation intervention can be seen in table 2. The adherence to the training intervention were significantly lower in the HRTW compared to the LITW group ($P < 0.01$). The adherence to the supplement did not differ significantly between groups ($P < 0.11$). However, it is important to notice that a relatively large fraction of the participants (34 subject) failed to report their supplement intake throughout the intervention. All of these participants came to our facilities as planned and received additional supplements, but they are not included in the adherence values due to their insufficient registration of supplement intake.

Table 2. Overview of the adherence to the intervention, supplements-non reporters and drop outs. Number are medians and [interquartile range] in percentage. Adopted from paper II

	CARB		COLL		WHEY		LITW		HRTW	
	ITT	PP	ITT	PP	ITT	PP	ITT	PP	ITT	PP
Training compliance	-	-	-	-	-	-	89% [77%, 96%]	94% [88%, 97%]	72% [62%, 78%]	78% [75%, 82%]
Supplement compliance	95% [77%, 97%]	96% [89%, 98%]	96% [86%, 99%]	96% [86%, 99%]	88% [82%, 93%]	90% [85%, 96%]	90% [77%, 94%]	93% [85%, 100%]	87% [79%, 97%]	94% [87%, 98%]
Supplement non-reporters (n=)	7		11		14		1		1	
Drop outs (n=)	2		6		6		6		4	
Included subjects (n=)	34	22	44	31	44	25	30	20	32	19

The changes between the collected 3-day (Wednesday to Friday) weighed dietary registrations at baseline and 11months after intervention start can be seen in table 3 (and the full table can be found in paper II). A detailed description of the dietary assessment is published by Rønnow et al[155]. Comparing the pre-intervention and 11-month dietary registration protein intake was increased at 11 month for COLL (29.0 ± 3.9 g·day^{-1}, $P<0.0001$), WHEY (25.7 ± 5.0 g/day, $P<0.0001$), LITW (23.8 ± 4.2 g·day^{-1}, $P<0.0001$), and HRTW (26.7 ± 3.8 g·day^{-1}, $P<0.0001$), while energy intake did not change significantly (COLL: 408 ± 266 kJ·day^{-1}, $P = 0.13$; LITW:

474 ± 437 kJ·day^{-1}, P = 0.29; HRTW: -41 ± 324 kJ·day^{-1}, P = 0.9. Energy intake increased for CARB, with no change in protein intake (Energy: 948 ± 428 kJ·day^{-1}, P=0.04; Protein: -4.9 ± 5.3 ·day^{-1}, P=0.37).

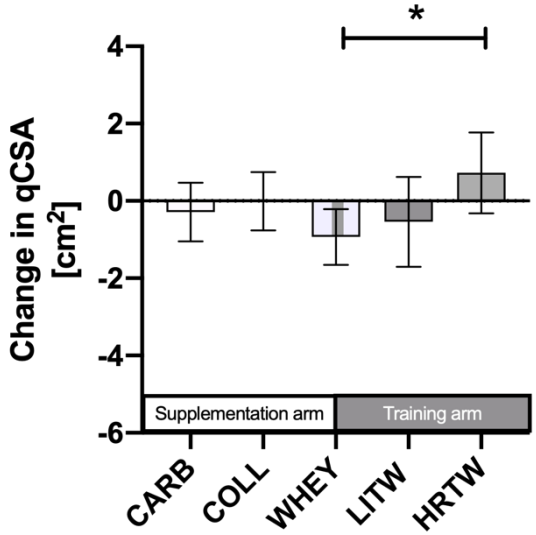
Table 3. Changes in protein and energy intake at baseline and 11 months after intervention start. * denotes a significant change from baseline $p<0.05$. Adopted and modified from paper II.

	CARB		COLL		WHEY		LITW		HRTW	
	ITT	PP	ITT	PP	ITT	PP	ITT	PP	ITT	PP
Changes from 0-12m	(n = 34)	(n=22)	(n = 44)	(n=31)	(n = 44)	(n=25)	(n = 36)	(n=20)	(n = 36)	(n=19)
Protein intake, g/day	-4.9 (5.3)	3.9 (5.9)	29.0 (3.9)*	27.2 (4.5)*	25.7 (5.0)*	31.4 (6.3)*	23.8 (4.2)*	26.9 (4.7)*	26.7 (3.8)*	34.6 (4.0)*
Protein intake excluding supplement, g/day	-4.9 (5.3)	3.9 (5.9)	-8.3 (3.6)	-9.8 (4.2)	-6.4 (4.3)	-5.0 (6.1)	-9.6 (3.9)	-9.8 (4.7)	-5.8 (3.2)	-2.3 (4.3)
Energy intake, kJ/day	948 (428)*	865.9 (474)*	408 (266)	343 (313)	517 (413)	900 (608)	474 (437)	874 (551)	-41 (324)	348 (418)
Energy intake excluding supplement, g/day	-81 (425)	-196 (466)	-649 (260)	-703 (304)	-389 (397)	-130 (603)	-472 (427)	-161 (550)	-961 (315)	-695 (431)

qCSA by MRI:

We did not observe any between-group differences in changes in qCSA, (P=0.17, figure 6) in the nutritional supplementation arm. In the training arm, HRTW was associated with a different change in qCSA compared to WHEY (Between-group difference [mean, 95% CI]: 1.68, 0.41 to 2.95 cm^2, P=0.03), but not compared to LITW (1.29 cm^2, -0.08 to 2.67 cm^2, P=0.16) and LITW was not significantly different compared to WHEY (0.39, -0.88 to 1.66 cm^2, P=0.82). When tested within each group separately with a t-test, neither HRTW ([mean, 95% SD]: 0-12-month change: +0.73, -0.32±1.77 cm^2) nor LITW ([mean, 95% SD]: -0.54, -1.70 ±0.62 cm^2) exhibited significant changes in qCSA whereas a significant decrease was observed for WHEY ([mean, 95% SD]: -0.93, -1.65±0.21 cm^2).

Figure 6. Changes from baseline to 12 months in m. quadriceps cross-sectional area (qCSA) measured by MRI. *: Significant between-group difference in changes over the intervention period. Adopted from Paper II.



11.2.2 Results Paper III

Paper III report the results from the OGTT, DXA scans and the fasting blood sample obtained prior to the OGTT. The primary research question was:

> How does supplementation with or without different types of training affect the glucose tolerance within healthy elderly?

The purposes of measuring OGTT were twofold. First, whether or not 1-year of protein supplementation with or without training would improve glucose tolerance. Secondly, to

discover potential negative effect of especially the one year of supplementation. Results descriptions are adopted from Paper III with minor modifications.

Out of the 184 completing the study (see section 11.2) 164 participants completed the OGTT before and after the intervention. The intention-to-treat (ITT) analysis included all of the participants (164) who completed the OGTT before and after the trial and the per protocol (PP) analysis included 100 participants with an registered self-reported supplementation adherence $\geq$ 75% and an average training adherence during the intervention $\geq$ 2.0 for the HRTW group and $\geq$3.0 for the LITW group. 34 participants out of the 164 participants were excluded from the PP analysis due to lack of registrations and 30 were excluded due to lack of adherence to the respective interventions.

Nutritional intervention arm (body composition, fasting blood samples, OGTT):

Body composition: The nutritional supplementation groups increased body weight (BW) (p=0.008) (WHEY 0.5±0.4 kg, COLL 0.5±0.3 kg, CARB 1.2±0.6 kg) and BMI (p=0.008) (WHEY 0.2±0.1 kg·m^{-2}, COLL 0.2±0.1 kg·m^{-2}, CARB 0.4±0.2 kg·m^{-2}) as a main effect of time with no group x time interaction after 12 months of nutritional supplements within the participants included in the ITT analysis (Table 4). The same was true when analyzing the participants qualified for the per protocol (PP) analysis with respect to BW (p=0.040) (WHEY 0.2±0.4 kg, COLL 0.3±0.4 kg, CARB 1.3±0.6 kg) and BMI(p=0.034) (WHEY 0.1±0.1 kg·m^{-2}, COLL 0.1±0.1 kg·m^{-2}, CARB 0.4±0.2 kg·m^{-2}) (Table 3). In addition, there was an increase in fat% in ITT(p=0.003) (WHEY 0.6±0.3%, COLL 0.5±0.3%, CARB 0.4±0.2%) and PP (p=0.017) (WHEY 0.5±0.4%, COLL 0.4±0.4%, CARB 0.8±0.4%) analysis as a main effect of time and no change in lean body mass were observed. Again, there was no significant difference between the groups. There were no changes in ASMI or daily step count neither between groups nor over time (Table 4, Table 5).

Fasting plasma samples: Fasting plasma glucose, proinsulin C-peptide and insulin concentrations were not different before and after the 12 months nutritional interventions and there was no difference between the groups within the participants included in the ITT analysis. HbA1c increased significantly with a main effect of time (p=0.001) (WHEY 0.1±0.4 mmol/mol, COLL 1.1±0.4 mmol·mol^{-1}, CARB 1.2±0.6 mmol/mol) with no difference between the groups within the participants included in the ITT analysis. (Table 4). The PP analysis showed no changes in fasting plasma glucose, insulin concentration, or proinsulin C-peptide (Table 5) but

the HbA1c showed a significant main effect of time (p=0.002) (WHEY 0.4±0.5 mmol/mol, COLL 1.2±0.5 mmol·mol^{-1}, CARB 1.7±0.5 mmol·mol^{-1}) (Table 5). Again, we saw no differences between the groups (Table 5).

OGTT: The plasma glucose AUC during the OGTT was not different between the nutritional supplement groups but the plasma glucose AUC increased as a main effect of time (p=0.049)(WHEY 21±15 mmol·mL-1 x 120min, COLL 33±20 mmol·mL^{-1}x 120min, CARB 18±28 mmol·mL^{-1}x 120min) after 12 months of nutritional supplements within the participants included in the ITT analysis (Table 4) and the plasma insulin AUC decreased as a main effect of time (p=0.032) (WHEY -691±414 uIU·mL^{-1}x 120min, COLL 228±390 uIU·mL^{-1}x 120min, CARB -121±370 uIU·mL^{-1}x 120min) with no differences between the groups (Table 4).No changes in HOMA-IR index and the Matsuda index after 12 months of nutritional supplementation within the participants included in the ITT analysis were observed(Table 4). No changes over time in the plasma glucose AUC, but a trend (p=0.051) for a main effect of time for a decrease in the insulin AUC (Table 5) were observed in the PP analysis. No effect of the interventions on the HOMA-IR or Matsuda indexes were observed (Table 5)

Table 4. Intention to treat analysis of the nutritional supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and no significant effects of the different interventions. COLL, collagen; CARB, carbohydrate; WHEY, whey. Adopted paper III

																ANOVA		
Nutrition arm ITT	**WHEY, N=39 (22 males, 17 females)**						**COLL, N=39 (23 males, 16 females)**						**CARB, N= 29 (15 males, 14 females)**				p-value	
	0 M		12 M		Delta change, number (%)		0 M		12 M		Delta change, number (%)		0 M		12 M		Change [absolute (%)]		Time	Group	Interaction
Subject characteristics																					
Age (year)	69.9	± 0.7					69.8	± 0.6					69.7	± 0.8						n.s.	
Body mass (kg)	74.0	± 2.1	74.5	± 2.2	0.5(0.7%)	± 0.4(0.5%)	75.1	± 2.1	75.5	± 2.1	0.5(0.7%)	± 0.3(0.5%)	73.6	± 2.3	74.8	± 2.5	1.2(1.5%)	± 0.6(0.8%)	0.008	n.s.	n.s.
BMI (kg/m2)	24.6	± 0.6	24.8	± 0.6	0.2(0.7%)	± 0.1(0.5%)	25.1	± 0.7	25.2	± 0.7	0.2(0.7%)	± 0.1(0.5%)	25.4	± 0.7	25.8	± 0.8	0.4(1.5%)	± 0.2(0.8%)	0.008	n.s.	n.s.
Fat mass (%)	32.0	± 1.2	32.6	± 1.2	0.6(2.3%)	± 0.3(1.1%)	30.8	± 1.4	31.2	± 1.5	0.5(1.6%)	± 0.3(1.1%)	31.6	± 1.7	32.4	± 1.8	0.8(2.7%)	± 0.4(1.2%)	0.003	n.s.	n.s.
Lean mass (kg)	48.6	± 1.4	48.5	± 1.4	-0.1(-0.1%)	± 0.2(0.3%)	50.0	± 1.4	50.0	± 1.4	-0.1(-0.1%)	± 0.2(0.3%)	48.4	± 1.4	48.5	± 1.5	0.1(0.2%)	± 0.2(0.4%)	n.s.	n.s.	n.s.
ASMI (kg/m2)	7.46	± 0.18	7.46	± 0.18	-0.002(0.02%)	± 0.003(0.4%)	7.66	± 0.20	7.68	± 0.20	0.01(0.2%)	± 0.03(0.4%)	7.63	± 0.20	7.65	± 0.21	0.02(0.3%)	± 0.03(0.4%)	n.s.	n.s.	n.s.
Daily steps	10515	± 590	10003	± 533	-343(-1.8%)	± 604(5.5%)	10607	± 675	10450	± 774	3(4.8%)	± 574(7.2%)	11404	± 1008	10107	± 863	2274(-10.7%)	± 1030(7.8%)	n.s.	n.s.	n.s.
Fasting blood analysis																					
Fasting glucose (mmol/L)	5.4	± 0.1	5.5	± 0.1	0.12(2.3%)	± 0.08(1.5%)	5.5	± 0.1	5.5	± 0.1	-0.02(-0.2%)	± 0.07(1.3%)	5.5	± 0.1	5.5	± 0.1	0.04(0.8%)	± 0.08(1.4%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	4.9	± 0.5	4.1	± 0.4	-0.8(-2.7%)	± 0.3(6.9%)	4.9	± 0.5	4.9	± 0.6	0.1(4.3%)	± 0.3(6.9%)	4.4	± 0.5	4.7	± 0.6	0.2(3.8%)	± 0.3(7.0%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	758	± 48	738	± 44	-27(0.1%)	± 28(3%)	680	± 35	703	± 44	22(3%)	± 24(3%)	686	± 47	715	± 68	29(4%)	± 38(5%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	36.2	± 0.6	36.3	± 0.6	0.1(0.4%)	± 0.4(1.0%)	35.3	± 0.5	36.5	± 0.5	1.1(3.6%)	± 0.4(1.5%)	35.7	± 0.4	36.9	± 0.3	1.2(3.6%)	± 0.4(1.1%)	0.001	n.s.	n.s.
OGTT																					
Glucose AUC (mmol/L x 120 min)	870	± 22	891	± 25	21(2.6%)	± 15(1.7%)	895	± 25	928	± 25	33(4.9%)	± 20(2.4%)	926	± 24	944	± 30	18(2.8%)	± 28(3.0%)	0.049	n.s.	n.s.
Glucose 45 min (mmol/L)	8.6	± 0.3	8.7	± 0.3	0.1(1.9%)	± 0.2(2.2%)	8.8	± 0.3	9.0	± 0.3	0.2(4.1%)	± 0.3(3.1%)	9.2	± 0.3	9.3	± 0.3	0.1(3.0%)	± 0.3(3.2%)	n.s.	n.s.	n.s.
Glucose 2h (mmol/L)	6.2	± 0.2	6.6	± 0.3	0.3(5.7%)	± 0.2(3.5%)	6.5	± 0.3	7.1	± 0.3	0.6(12.8%)	± 0.3(4.1%)	6.7	± 0.2	7.0	± 0.4	0.3(4.0%)	± 0.4(5.0%)	0.01	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	5066	± 718	4375	± 512	-691(-1.9%)	± 414(6.9%)	4645	± 532	4873	± 623	228(8.9%)	± 390(7.3%)	4047	± 394	3926	± 661	-121(-8.1%)	± 370(6.9%)	0.032	n.s.	n.s.
HOMA-IR	1.21	± 0.14	1.05	± 0.11	-0.15(-0.9%)	± 0.07(6.8%)	1.21	± 0.14	1.23	± 0.16	0.02(5.6%)	± 0.09(7.8%)	1.08	± 0.13	1.19	± 0.18	0.10(5.0%)	± 0.09(6.8%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	9.6	± 1.0	10.1	± 1.0	0.5(15.0%)	± 0.7(6.0%)	9.2	± 0.9	9.5	± 1.0	0.3(6.8%)	± 0.6(6.7%)	9.1	± 0.9	10.1	± 1.9	2.6(21.0%)	± 1.3(10.7%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	11.8	± 1.4	10.7	± 0.9	-1.1(3.4%)	± 0.9(6.0%)	11.3	± 1.3	10.7	± 1.5	-0.6(4.7%)	± 1.0(12.4%)	10.1	± 0.9	12.6	± 2.0	2.5(18.6%)	± 1.5(11.7%)	n.s.	n.s.	n.s.

Table 5. Per protocol analysis of the nutritional supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and no significant effects of the different interventions. COLL, collagen; CARB, carbohydrate; WHEY, whey. Adopted paper III

																ANOVA		
Nutrition arm PP	**WHEY, N= 21 (10 males, 11 females)**						**COLL, N=28 (16 males, 12 females)**						**CARB, N=15 (9 males, 6 females)**			p-value		
	0 M	12 M	Difference				0 M	12 M	Difference				0 M	12 M	Change [absolute (%)]	Time	Group	Interaction
Subject characteristics																		
Age (year)	69.7 ± 0.8						69.5 ± 0.6						69.1 ± 0.9				n.s.	
Body mass (kg)	72.6 ± 3.2	72.8 ± 3.1	0.2(0.5%)	±	0.4(0.6%)		76.0 ± 2.5	76.3 ± 2.6	0.3(0.4%)	±	0.4(0.6%)		72.2 ± 3.1	73.5 ± 3.1	1.3(1.9%) ± 0.6(0.8%)	0.040	n.s.	n.s.
BMI (kg/m2)	24.1 ± 0.8	24.2 ± 0.7	0.1(0.5%)	±	0.1(0.6%)		25.4 ± 0.9	25.5 ± 0.9	0.1(0.4%)	±	0.1(0.6%)		24.3 ± 0.9	24.7 ± 0.9	0.4(1.9%) ± 0.2(0.8%)	0.034	n.s.	n.s.
Fat mass (%)	31.0 ± 1.6	31.5 ± 1.6	0.5(1.7%)	±	0.4(1.4%)		31.6 ± 1.7	32.0 ± 1.7	0.4(1.7%)	±	0.4(1.4%)		29.1 ± 2.1	30.0 ± 2.0	0.9(3.9%) ± 0.4(1.6%)	0.017	n.s.	n.s.
Lean mass (kg)	48.2 ± 2.1	48.3 ± 2.0	0.1(0.3%)	±	0.2(0.4%)		50.0 ± 1.5	49.9 ± 1.5	-0.1(-0.3%)	±	0.2(0.4%)		49.3 ± 2.1	49.7 ± 2.1	0.4(0.8%) ± 0.2(0.4%)	n.s.	n.s.	n.s.
ASMI (kg/m2)	7.36 ± 0.23	7.39 ± 0.22	0.04(0.6%)	±	0.04(0.6%)		7.64 ± 0.24	7.64 ± 0.24	0.00(0.0%)	±	0.3(0.5%)		7.60 ± 0.35	7.61 ± 0.36	0.02(0.2%) ± 0.04(0.6%)	n.s.	n.s.	n.s.
Daily steps	11354 ± 771	10362 ± 797	-697(-1.1%)	±	916(8.1%)		11021 ± 753	10370 ± 975	-440(-2.7%)	±	676(7.7%)		10183 ± 1175	11055 ± 1256	323(8.8%) ± 822(9.8%)	n.s.	n.s.	n.s.
Fasting blood analysis																		
Fasting glucose (mmol/L)	5.3 ± 0.1	5.5 ± 0.2	0.1(2.7%)	±	0.1(2.3%)		5.5 ± 0.1	5.4 ± 0.1	-0.04(-0.7%)	±	0.1(1.3%)		5.6 ± 0.1	5.5 ± 0.1	-0.1(-1.2%) ± 0.1(1.9%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	3.9 ± 0.6	3.4 ± 0.4	-0.5(6.3%)	±	0.3(11.9%)		4.7 ± 0.5	4.9 ± 0.6	0.2(5.3%)	±	0.3(8.5%)		4.6 ± 0.8	4.5 ± 0.9	-0.1(1.5%) ± 0.4(9.5%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	694 ± 68	675 ± 50	-19(3.6%)	±	42(5.7%)		680 ± 41	705 ± 51	25(4.0%)	±	25(3.9%)		696 ± 70	739 ± 79	42(8.2%) ± 32(6.4%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	34.8 ± 0.7	35.2 ± 0.8	0.4(1.3%)	±	0.5(1.5%)		35.3 ± 0.6	36.5 ± 0.7	1.2(3.7%)	±	0.5(1.9%)		35.4 ± 0.5	37.1 ± 0.4	1.7(4.9%) ± 0.5(1.7%)	0.002	n.s.	n.s.
OGTT																		
Glucose AUC (mmol/L x 120 min)	834 ± 27	860 ± 35	27(3.1%)	±	20(2.4%)		910 ± 30	930 ± 29	20(3.4%)	±	24(2.9%)		951 ± 39	960 ± 39	9(2.3%) ± 41(4.4%)	n.s.	n.s.	n.s.
Glucose 45 min (mmol/L)	8.0 ± 0.3	8.2 ± 0.4	0.1(0.02%)	±	0.3(0.03%)		9.0 ± 0.4	9.0 ± 0.3	-0.04(1.8%)	±	0.3(3.6%)		9.6 ± 0.5	9.7 ± 0.3	0.1(3.2%) ± 0.5(4.9%)	n.s.	0.013	n.s.
Glucose 2h (mmol/L)	6.2 ± 0.3	6.6 ± 0.4	0.4(7.7%)	±	0.3(5.3%)		6.5 ± 0.3	7.1 ± 0.3	0.6(12.7%)	±	0.3(5.4%)		6.6 ± 0.4	6.8 ± 0.6	0.2(2.5%) ± 0.5(6.7%)	n.s.	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	4540 ± 1052	3442 ± 541	-1098(-6.7%)	±	616(10.4%)		5154 ± 719	5394 ± 844	240(11.0%)	±	550(10.2%)		4188 ± 655	4138 ± 1119	-51(-9.6%) ± 558(8.8%)	(0.0505)	n.s.	n.s.
HOMA-IR	0.93 ± 0.16	0.86 ± 0.14	-0.07(7.9%)	±	0.06(11.4%)		1.17 ± 0.13	1.22 ± 0.17	0.05(6.2%)	±	0.10(9.4%)		1.13 ± 0.21	1.13 ± 0.25	-0.01(0.1%) ± 0.10(9.3%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	11.7 ± 1.5	12.1 ± 1.4	0.5(16.6%)	±	1.2(9.4%)		8.7 ± 1.0	8.8 ± 1.1	0.1(5.9%)	±	0.7(7.3%)		8.6 ± 1.0	10.6 ± 1.7	2.0(21.5%) ± 1.3(14.5%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	14.3 ± 2.2	12.6 ± 1.3	-1.7(0.5%)	±	1.5(8.8%)		11.0 ± 1.5	10.2 ± 1.7	-0.8(4.9%)	±	1.3(16.9%)		9.6 ± 1.0	11.5 ± 1.9	1.9(13.0%) ± 1.3(16.9%)	n.s.	n.s.	n.s.

Training intervention arm (body composition, fasting blood samples, OGTT):

Body composition: No changes in body mass, BMI and fat% over time in the training intervention arm in either the ITT or PP analysis were observed (Table 6, Table 7). An increase in lean body mass over time were observed in the PP analysis (p=0.033) (WHEY 0.1±0.2 kg, LITW 0.3±0.3 kg, HRTW 0.6±0.3 kg) and no effect in the ITT analysis. This could indicate an effect of actual exercise. An effect of time on ASMI were observed in the HRTW group only (p=0.001) (HRTW 0.15±0.04 $kg \cdot m^{-2}$) in the ITT analysis (Table 6). A main effect of time on ASMI (p=0.001) (WHEY 0.04±0.04 $kg \cdot m^{-2}$, LITW 0.12±0.05 $kg \cdot m^{-2}$, HRTW 0.18±0.06 $kg \cdot m^{-2}$) and no differences between the groups (Table 7) were seen in the PP analysis. No changes in daily step count were observed during the intervention (Table 6, Table 7).

Fasting blood samples: Fasting plasma glucose, plasma insulin, and proinsulin C-peptide concentrations did not change after 12 months nutritional and exercise interventions in the participants included in the ITT analysis (Table 2). HbA1c concentration increased as a main effect of time (p=0.005)) (WHEY 0.1±0.4 $mmol \cdot mol^{-1}$, LITW 1.4±0.5 $mmol \cdot mol^{-1}$, HRTW 0.5±0.3 $mmol \cdot mol^{-1}$) in the participants included in the ITT analysis (Table 6). No changes in fasting plasma glucose, plasma insulin or proinsulin C-peptide concentrations were observed after the interventions in the PP analysis (Table 7). A main effect of time in the HbA1c concentrations (p=0.023)) (WHEY 0.4±0.5 $mmol \cdot mol^{-1}$, LITW 1.1±0.5 $mmol \cdot mol^{-1}$, HRTW 0.6±0.5 $mmol \cdot mol^{-1}$) were observed in the participants included in the PP analysis (Table 7).

OGTT: Plasma glucose and insulin AUC during the OGTT showed no effect of the 12 months interventions in the participants included in the ITT analysis (Table 6). No changes in the HOMA-IR and the Matsuda index were observed in the participants included in the ITT analysis (Table 6). A significant effect of time on the glucose AUC (p=0.037) (WHEY 27±20 $mmol \cdot mL^{-1}$ x 120min, LITW 29±16 $mmol \cdot mL^{-1}$ x 120min, HRTW 35±36 $mmol \cdot mL^{-1}$ x 120min), the glucose 2h concentration (p=0.019) (WHEY 0.4±0.3 $mmol \cdot L^{-1}$, LITW 0.5±0.3 $mmol \cdot L^{-1}$, HRTW 0.4±0.3 $mmol \cdot L^{-1}$) and a trend for a main effect of time for a decrease in the insulin AUC (p=0.06) were observed in the PP analysis (Table 7). No changes were observed in the HOMA-IR and the Matsuda indexes in the PP analysis (Table 7)

Table 6**:** Intention to treat analysis of the exercise and whey supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and a significant effect of the HRTW intervention on ASMI using the Holm-Sidak post hoc test p=0.001 (indicated by * in the table). HRTW, high resistance training + whey; LITW, low intensity training + whey; WHEY, whey. Adopted paper III

																ANOVA		
Training arm ITT	**WHEY, N=39 (22 males, 17 females)**						**LITW, N=28 (14 males, 14 females)**						**HRTW, N=29 (17 males, 12 females)**				p-value	
	0 M		12 M		Difference		0 M		12 M		Difference		0 M		12 M		Change [absolute (%)]		Time	Group	Interaction
Subject characteristics																					
Age (year)	69.9	± 0.7					70.6	± 0.8					70.8	± 0.6						n.s.	
Body mass (kg)	74.0	± 2.1	74.5	± 2.2	0.5(0.7%)	± 0.4(0.5%)	73.9	± 1.9	74.7	± 2.0	0.2(1.0%)	± 0.2(0.8%)	78.5	± 2.6	78.7	± 2.5	0.2(0.4%)	± 0.5(0.7%)	n.s.	n.s.	n.s.
BMI (kg/m2)	24.6	± 0.6	24.8	± 0.6	0.2(0.7%)	± 0.1(0.5%)	25.4	± 0.6	25.6	± 0.6	0.8(1.0%)	± 0.6(0.8%)	26.0	± 0.7	26.1	± 0.7	0.1(0.4%)	± 0.2(0.7%)	n.s.	n.s.	n.s.
Fat mass (%)	32.0	± 1.2	32.6	± 1.2	0.6(2.3%)	± 0.3(1.1%)	33.2	± 1.4	33.7	± 1.5	0.5(1.8%)	± 0.4(1.4%)	33.8	± 1.3	33.7	± 1.4	-0.1(-0.4%)	± 0.3(1.1%)	n.s.	n.s.	n.s.
Lean mass (kg)	48.6	± 1.4	48.5	± 1.4	-0.1(-0.1%)	± 0.2(0.3%)	48.0	± 1.7	48.1	± 1.7	0.1(0.3%)	± 0.2(0.4%)	50.3	± 1.9	50.7	± 1.9	0.4(0.8%)	± 0.2(0.4%)	n.s.	n.s.	n.s.
ASMI (kg/m2)	7.5	± 0.2	7.5	± 0.2	-0.002(0.02%)	± 0.003(0.4%)	7.55	± 0.21	7.63	± 0.22	0.08(1.1%)	± 0.05(0.6%)	7.72	± 0.25	7.87	± 0.26*	0.15(2.0%)	± 0.04(0.5%)	0.001	n.s.	0.02
Daily steps	10515	± 590	10003	± 533	-343(-1.8%)	± 604(5.5%)	10371	± 679	9482	± 476	-530(0.6%)	± 625(6.6%)	9599	± 653	8982	± 660	-595(-4.0%)	± 436(4.9%)	n.s.	n.s.	n.s.
Fasting blood analysis																					
Fasting glucose (mmol/L)	5.4	± 0.1	5.5	± 0.1	0.12(2.3%)	± 0.08(1.5%)	5.6	± 0.1	5.6	± 0.1	-0.01(0.5%)	± 0.1(2.0%)	5.6	± 0.1	5.7	± 0.1	0.1(1.6%)	± 0.1(1.1%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	4.9	± 0.5	4.1	± 0.4	-0.8(-2.7%)	± 0.3(6.9%)	4.2	± 0.4	4.6	± 0.4	0.4(19.2%)	± 0.4(11.0%)	4.5	± 0.5	4.4	± 0.5	-0.01(4.4%)	± 0.4(7.3%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	758	± 48	738	± 44	-27(0.1%)	± 28(3%)	661	± 41	711	± 41	50(14.3%)	± 45(7.0%)	682	± 43	714	± 48	35(6.0%)	± 29(4.2%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	36.2	± 0.6	36.3	± 0.6	0.1(0.4%)	± 0.4(1.0%)	35.5	± 0.6	36.9	± 0.7	1.4(4.1%)	± 0.5(1.4%)	35.7	± 0.5	36.2	± 0.5	0.5(1.6%)	± 0.3(1.0%)	0.005	n.s.	n.s.
OGTT																					
Glucose AUC (mmol/L x 120 min)	870	± 22	891	± 25	21(2.6%)	± 15(1.7%)	909	± 28	933	± 30	25(3.2%)	± 16(1.9%)	909	± 25	922	± 37	13(1.5%)	± 27(3.3%)	n.s.	n.s.	n.s.
Glucose 45 min (mmol/L)	8.6	± 0.3	8.7	± 0.3	0.1(1.9%)	± 0.2(2.2%)	8.6	± 0.4	8.9	± 0.4	0.2(4.7%)	± 0.3(3.5%)	8.9	± 0.3	8.8	± 0.4	-0.1(0.1%)	± 0.4(4.5%)	n.s.	n.s.	n.s.
Glucose 2h (mmol/L)	6.2	± 0.2	6.6	± 0.3	0.3(5.7%)	± 0.2(3.5%)	7.1	± 0.3	7.4	± 0.3	0.3(5.0%)	± 0.2(3.1%)	6.6	± 0.3	7.0	± 0.4	0.4(6.7%)	± 0.3(4.5%)	0.025	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	5066	± 718	4375	± 512	-691(-1.9%)	± 414(6.9%)	3988	± 449	3510	± 422	-479(-4.0%)	± 274(8.1%)	4154	± 809	3843	± 449	-311(9.6%)	± 663(11.4%)	n.s.	n.s.	n.s.
HOMA-IR	1.21	± 0.14	1.05	± 0.11	-0.15(-0.9%)	± 0.07(6.8%)	1.05	± 0.10	1.14	± 0.12	0.09(21.7%)	± 0.10(12.8%)	1.14	± 0.15	1.16	± 0.14	0.03(6.7%)	± 0.10(7.7%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	9.6	± 1.0	10.1	± 1.0	0.5(15.0%)	± 0.7(6.0%)	9.3	± 0.9	10.5	± 1.6	1.2(24.5%)	± 1.5(20.7%)	9.4	± 0.9	10.3	± 1.2	0.8(15.1%)	± 0.9(11.7%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	11.8	± 1.4	10.7	± 0.9	-1.1(3.4%)	± 0.9(6.0%)	9.5	± 0.8	9.2	± 1.3	-0.3(3.1%)	± 1.3(13.7%)	10.4	± 0.9	10.7	± 1.1	0.3(9.8%)	± 0.9(11.6%)	n.s.	n.s.	n.s.

Table 7: Per protocol analysis of the exercise and whey supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and no significant effects of the different interventions. HRTW, high resistance training + whey; LITW, low intensity training + whey Adopted paper III

																				ANOVA		
Training arm PP	**WHEY, N=21 (10 males, 11 females)**							**LITW, N=19 (9 males, 10 females)**							**HRTW, N=17 (10 males, 7 females)**					p-value		
	0 M			12 M			Difference			0 M			12 M			Difference			0 M			12 M			Change [absolute (%)]			Time	Group	Interaction
Subject characteristics																														
Age (year)	69.7	±	0.8					70.2	±	0.9								70.7	±	0.8								n.s.	
Body mass (kg)	72.6	±	3.2	72.8	±	3.1	0.2(0.5%) ± 0.4(0.6%)	72.8	±	2.2	73.6	±	2.0	0.8(1.2%)	±	0.6(0.9%)		77.9	±	3.5	78.1	±	3.4	0.2(0.5%)	±	0.8(1.0%)	n.s.	n.s.	n.s.
BMI (kg/m2)	24.1	±	0.8	24.2	±	0.7	0.1(0.5%) ± 0.1(0.6%)	25.2	±	0.8	25.4	±	0.7	0.2(1.2%)	±	0.2(0.9%)		26.1	±	0.9	26.2	±	0.9	0.1(0.5%)	±	0.3(1.0%)	n.s.	n.s.	n.s.
Fat mass (%)	31.0	±	1.6	31.5	±	1.6	0.5(1.7%) ± 0.4(1.4%)	34.0	±	1.8	34.5	±	1.8	0.5(2.0%)	±	0.5(1.6%)		33.3	±	1.7	33.0	±	1.9	-0.3(-1.2%)	±	0.5(1.4%)	n.s.	n.s.	n.s.
Lean mass (kg)	48.2	±	2.1	48.3	±	2.0	0.1(0.3%) ± 0.2(0.4%)	46.6	±	1.8	46.9	±	1.8	0.3(0.6%)	±	0.3(0.5%)		50.3	±	2.6	50.9	±	2.6	0.6(1.2%)	±	0.3(0.6%)	0.033	n.s.	n.s.
ASMI (kg/m2)	7.36	±	0.23	7.39	±	0.22	0.04(0.6%) ± 0.04(0.6%)	7.35	±	0.24	7.47	±	0.24	0.12(1.6%)	±	0.05(0.7%)		7.73	±	0.35	7.91	±	0.36	0.18(2.4%)	±	0.06(0.7%)	0.001	n.s.	n.s.
Daily steps	11354	±	771	10362	±	797	-697(-1.1%) ± 916(8.1%)	10376	±	833	9846	±	604	128(4.5%)	±	568(6.2%)		9573	±	972	8981	±	812	-592(-3.0)	±	420(4.5%)	n.s.	n.s.	n.s.
Fasting blood analysis																														
Fasting glucose (mmol/L)	5.3	±	0.1	5.5	±	0.2	0.1(2.7%) ± 0.1(2.3%)	5.7	±	0.1	5.6	±	0.1	-0.1(-0.6%)	±	0.1(2.4%)		5.7	±	0.1	5.8	±	0.1	0.1(1.6%)	±	0.1(1.5%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	3.9	±	0.6	3.4	±	0.4	-0.5(6.3%) ± 0.3(11.9%)	4.3	±	0.5	4.6	±	0.5	0.4(0.2%)	±	0.6(0.2%)		4.3	±	0.7	4.2	±	0.7	-0.1(1.7%)	±	0.5(10.7%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	694	±	68	675	±	50	-19(3.6%) ± 42(5.7%)	678	±	54	737	±	52	60(17.8%)	±	62(9.8%)		657	±	56	711	±	56	54(0.1%)	±	39(0.1%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	34.8	±	0.7	35.2	±	0.8	0.4(1.3%) ± 0.5(1.5%)	35.3	±	0.8	36.4	±	0.8	1.1(3.4%)	±	0.5(1.6%)		36.0	±	0.7	36.6	±	0.7	0.6(1.8%)	±	0.5(1.4%)	0.023	n.s.	n.s.
OGTT																														
Glucose AUC (mmol/L x 120 min)	834	±	27	860	±	35	27(3.1%) ± 20(2.4%)	924	±	40	953	±	38	29(3.8%)	±	16(1.9%)		916	±	34	951	±	46	35(4.4%)	±	36(4.4%)	0.037	n.s.	n.s.
Glucose 45 min (mmol/L)	8.0	±	0.3	8.2	±	0.4	0.1(0.02%) ± 0.3(0.03%)	8.8	±	0.5	9.0	±	0.5	0.2(4.1%)	±	0.3(3.2%)		8.8	±	0.4	9.1	±	0.5	0.3(4.0%)	±	0.4(5.4%)	n.s.	n.s.	n.s.
Glucose 2h (mmol/L)	6.2	±	0.3	6.6	±	0.4	0.4(7.7%) ± 0.3(5.3%)	7.1	±	0.3	7.6	±	0.4	0.5(7.5%)	±	0.3(3.7%)		6.9	±	0.4	7.3	±	0.4	0.4(7.9%)	±	0.3(5.8%)	0.019	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	4540	±	1052	3442	±	541	-1098(-6.7%) ± 616(10.4%)	4135	±	556	3701	±	574	-433(-4.0%)	±	322(10.4%)		4431	±	1310	3856	±	610	-575(5.6%)	±	1026(10.8%)	(0.0595)	n.s.	n.s.
HOMA-IR	0.93	±	0.16	0.86	±	0.14	-0.07(7.9%) ± 0.06(11.4%)	1.08	±	0.12	1.16	±	0.14	0.08(25.4%)	±	0.15(18.7%)		2.01	±	0.22	1.11	±	0.19	-0.01(3.5%)	±	0.14(10.9%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	11.7	±	1.5	12.1	±	1.4	0.5(16.6%) ± 1.2(9.4%)	8.8	±	0.9	10.6	±	2.2	1.8(32.1%)	±	2.2(29.2%)		9.9	±	1.3	11.1	±	1.8	1.2(18.0%)	±	1.4(16.6%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	14.3	±	2.2	12.6	±	1.3	-1.7(0.5%) ± 1.5(8.8%)	8.4	±	0.7	9.3	±	1.8	0.9(12.7%)	±	1.8(19.8%)		10.5	±	1.4	10.4	±	1.4	-0.1(10.0%)	±	1.3(17.1%)	n.s.	n.s.	n.s.

Correlations (BMI, ASMI, VAT, Fat% and daily steps):

We conducted an exploratory correlation analysis of different health parameters (BMI, ASMI, visceral adipose tissue%(VAT), fat%, Steps/day) with HbA1c, InsulinAUC and GlucoseAUC. AUC both as a delta between 0 and 12 month (suppl. 1) and at baseline (suppl. 2, 3 and 4). No correlation between changes in BMI, ASMI, VAT%, Fat%, Steps/day and changes within HbA1c, Insulin AUC and Glucose AUC after the 12 months of intervention (suppl. 1 in paper III). HbA1c was only significantly correlated with VAT in females ($p=0.0004$) at baseline. Further, InsulinAUC were correlated with both BMI and fat% for both males and females ($p<0.0001$, $p<0.0001$), and with VAT ($p<0.0001$) in males at baseline. No significant correlation between GlucoseAUC and the measured parameters were observed. For figures see supplemental figures in Paper III.

11.2.3 Results Paper IV

Paper IV report the FSR and skeletal muscle metabolome results from the acute trial. The primary research question was:

> How does prolonged protein supplementation with or without different types of training affect the MPS and the skeletal muscle metabolome and how should changes in muscle FSR be interpreted?

The purposes of measuring FSR were first and foremost to investigate the effect of prolonged interventions thought to affect the skeletal muscles anabolic sensitivity. Secondly, we wanted to evaluate the interpretation of the acute FSR, since the theoretical concept of anabolic resistance is partially relying on this interpretation. Further, we wanted to evaluate the measurement of the skeletal muscle metabolome using small amount of tissue samples on an un-targeted GC/MS-TOF platform. Lastly, we wanted to investigate the effect of an oral intake of 20g of whey hydrolysate and 10 g of glucose after 4 hours on the skeletal muscle metabolome, and the effect of the one-year intervention on the fasting skeletal muscle metabolome. The following results descriptions are adopted from Paper IV with minor modifications. Baseline characteristics of the subjects participating in the acute trial can be found in paper IV.

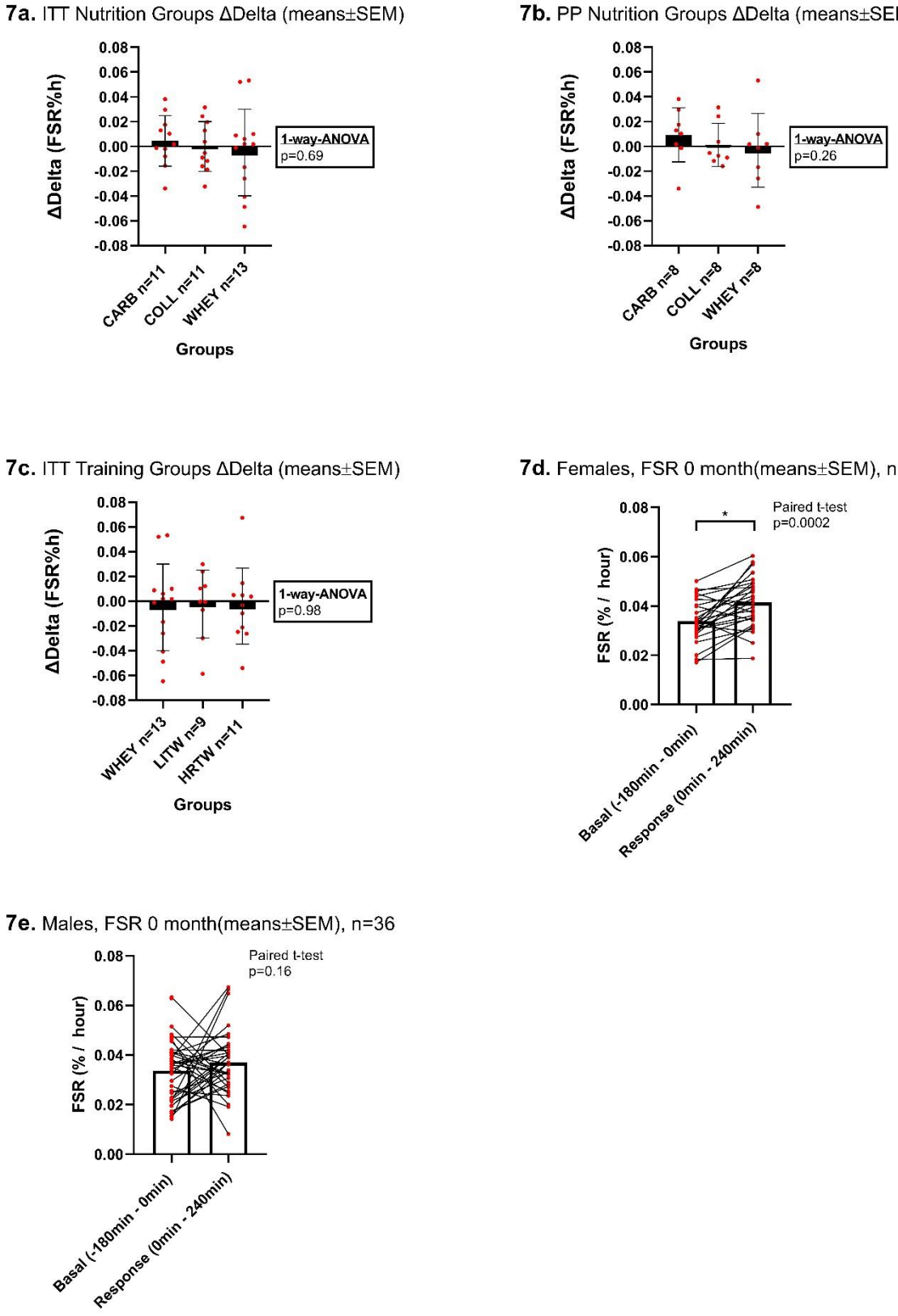
We included 66 participants (29 females and 37 males) with a mean age of 70 years [range; 65-80years]. Baseline subject characteristics for the 5 different intervention groups can be found in paper IV (table 1). All included subjects participated in the acute trial at 0 month and 64 out of the 66 particpants completed it. 2 participants (1 COLL, 1 LITW) had not the 240min

biopsy taken due to complications during the trial and they did therefore not participate in the acute trial at 12 months. 9 subjects did not participate in the acute trial at 12 months (1 (CARB), 3 (COLL), 2 (WHEY), 2 (LITW), 1 (HRTW)) due to complications or discomfort after the acute trial at baseline. This resulted in 55 subjects with complete sample sets at both baseline and 12 months. 6 participants in the HRTW-group had an adherence below 66%, 2 subjects had a supplementary adherence below 75%, and 2 subjects conducted their acute trial more than 14 days after the last training session. In the HRTW-group for both FSR and MM measures, only 3 subjects out of the 11 subjects had completed the acute trial at baseline and 12 months with sufficient adherence and time between intervention stop and the acute trial at 12 months to be included in the per protocol (PP) analysis. The training and supplementary mean adherence for these 3 subjects were 80% (SD±11%) respectively 86% (SD±10%). 4 participants in the LITW-group had an adherence below 66%, 2 participants had no training or supplementary registrations, and 2 participants conducted their acute trial more than 14 days after the last training session. In the LITW-group, for FSR only 4 and for the MM measures only 3 participants out of the 9 participants that had completed the acute trial at baseline and 12 months with sufficient adherence and time between intervention stop and the acute trial to be included in a PP analysis. The training and supplementary mean adherence for the 4 participants were 86% (SD±7%) 86% (SD±5%), respectively. In the WHEY-group, 8 participants completed the acute trial at baseline and 12month with sufficient adherence to be included in a PP analysis for the FSR measurements and 6 participants for the MM measurements. The supplementary mean adherence for the 8 participants were 94% (SD±5%). In the COLL-group, 9 subjects completed the 0 and 12month acute trial with sufficient adherence to be included in a PP analysis for the FSR measurements and 6 for the MM measurements. The supplementary mean adherence for these 9 participants were 91% (SD±7%). In the CARB-group, 8 participants completed the acute trial at baseline and 12 months with sufficient adherence to be included in a PP analysis for the FSR measurements and 6 participants for the MM measurements. The supplementary mean adherence for these 8 participants were 87% (SD±9%). All participants are included at baseline in the following analysis, and only those participants with an acceptable adherence are included in the analysis testing the effect of the intervention at 12 months.

FSR

The description is adopted from paper IV with minor modifications. No difference was seen irrespective of adherence to the intervention when we compared the Δdelta (Δdelta =($^{12month}FSR_{response}$ - $^{12month}FSR_{basal}$)- ($^{0month}FSR_{response}$ - $^{0month}FSR_{basal}$) FSR between groups in the nutrition supplementation arm (ITT: p=0.69; PP: p=0.26) (*ITT,mean[%±SEM]:* CARB 0.0045±0.006 , COLL -0.0001±0.006, WHEY -0.0049±0.01) (*PP, mean[%·h^{-1}±SEM]:* CARB 0.0094±0.008, COLL 0.0013±0.006, WHEY -0.0032±0.011) (figure 7a,7b). No difference was seen in the ITT-analysis when comparing the Δdelta FSR between groups in the training arm (p=0.98)) (*ITT,mean[%±SEM]:* WHEY -0.0049±0.01, LITW -0.0022±0.009, HRTW -0.0039±0.009; (figure 7c). Due to the low number of participants fulfilling the PP-criteria, we were not able to perform a PP analysis in the training arm (LITW: n=4; HRTW: n=3). A difference was observed between the basal and response period but only within females at baseline (Female: p=0.0002; males: p=0.16) (Females, mean*[%·h^{-1}±SEM]*: FSR_{basal} 0.035±0.002; $FSR_{response}$ 0.041±0.002) (Males, mean*[%·h^{-1}±SEM]*: FSR_{basal} 0.034±0.002; $FSR_{response}$ 0.037±0.002) (figure 7d,7e).

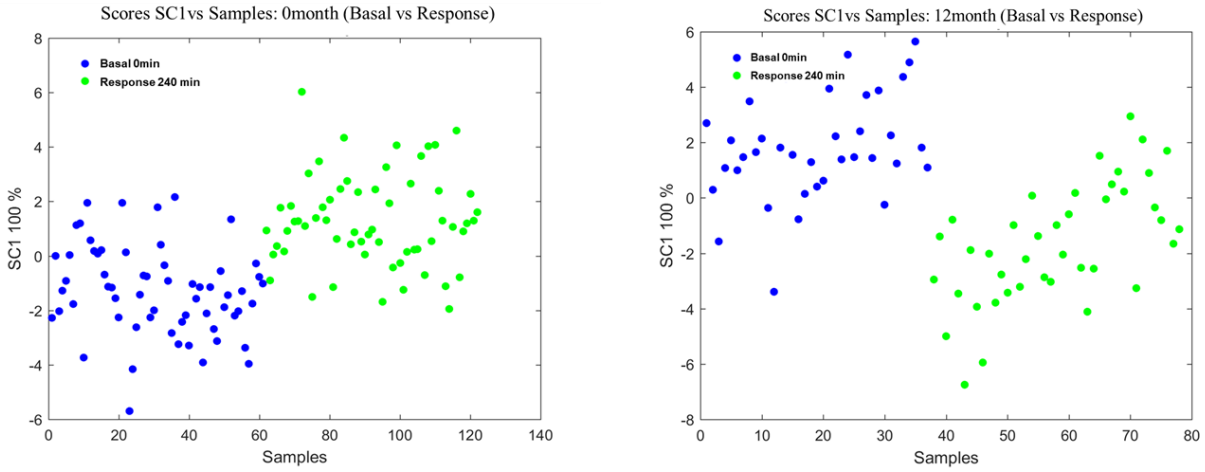
Figure 7. 7a) ITT analysis of Δdelta FSR in the nutritional arm, 7b) PP analysis of Δdelta FSR in the nutritional arm, 7c) ITT analysis of Δdelta FSR in the training arm, 7d) Females basal vs. response FSR at 0month, 7e) Males basal vs. response FSR at 0month. * denotes significant difference ($p<0.05$). Boxes are means±SEM for all plots. Figure is adopted from Paper IV



Skeletal muscle metabolome:

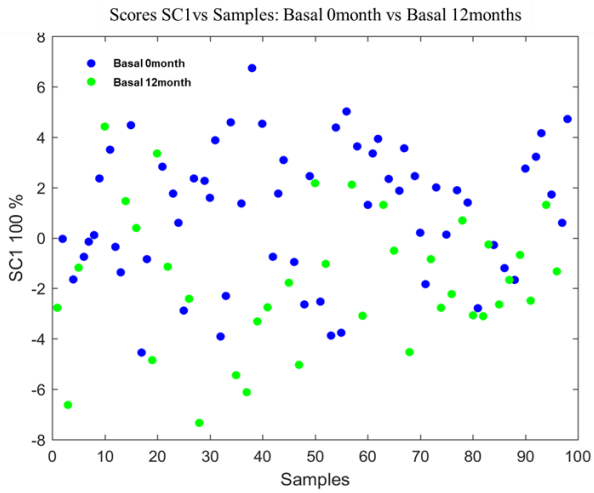
The description is adopted from paper IV with minor modifications. The final metabolite data (MM) contained 191 peaks resolved from the GC-MS data. Out of the 191 metabolites, we were able to identify 74 at level 2 based on Metabolomics Standards Initiative[156]. The identification criteria was set to EI-MS match of ≥ 750, RI match of ±20 and metabolites with labile protons being trimethylsilylated (TMS). The metabolites found corresponded to 17 amino acids, 12 fatty acids, 11 sugars, 9 organic acids, 7 sugar alcohols, 3 phenolics, and 10 other metabolites including 2 indole derivatives, ibuprofen, uric acid, and cholesterol (see S1 in paper IV for metabolite table). The principal component analysis (PCA) of the MM shows that up to 25% of variation is captured by the first three principal components of the PCA model. We saw no trend of separation of samples was observed according to visit, treatment, sex or the CALM intervention design in the PCA model as illustrated in figure 8. Supporting the lack of separation in the PCA model, an ASCA analysis revealed no effect of the treatment meaning that the basal (0 min) MM did not differ from the response MM (240 min) measured 240min after the ingestion of 20g of whey hydrolysate and 10g of sucrose at baseline (0 month) (p=0.42, n = 61). Neither were there any differences in the MM between 0 min and 240 min at the acute trial at 12 months (p=0.20 n= 37). This is illustrated in the SC1 plots in figure 9 showing no good separation between the metabolome at basal and at 240 min at either baseline (0month) or at 12 months.

Figure 9. SC1 scores at 0 and 12months coloured by basal(0min) and response(240min). The ASCA did not show any difference between the basal and response muscle metabolome (0month: p=0.42, n=61; 12month: p=0.20 n= 37). Figure is adopted from paper IV.



Although, two metabolites, 3-hydroxybutyric acid and 2-butenedioic acid, were significantly lower at 240min at both visits (ANOVA, 0m: p=0.0025, effect-size=13.8%; N=61; 12m: p=0.019 ; effect-size=18.1% ; N=39; respectively 0m: p=0.0025; effect-size=14.69; N=61; 12m: p=0.049; effect-size=14.75%; N=39) Similarly, ASCA analysis revealed no sign of significant effect in relation to the visit (basal 0 month versus basal 12 month) (p=0.62, n=61). This is illustrated in the SC1 plot in figure 10 showing no clear separation of the basal (0min) metabolome between baseline and 12 months.

Figure 10. SC1 scores for basal (0min) samples coloured by basal(0month) vs basal(12month). The ASCA did not show any difference between the basal 0 vs 12month (p=0.62, n=61) Figure is adopted from paper IV.



Further, an ASCA analysis investigating any differences in the basal MM after 12 months as an effect of the intervention showed no effect (p=0.68, CARB n=6, COLL n=6, WHEY n=6). Due to low number of participants in the training arm (LITW=3, HRTW=3) this analysis was only conducted in the nutritional intervention. The SC1 vs SC2 score plot is seen in figure 11.

Figure 11. SC1vs SC2 scores at 12months coloured by nutritional groups (CARB n=6, WHEY n=6, COLL n=6). The ASCA did not show any significant differences between the groups (p=0.68). Figure is adopted from paper IV.

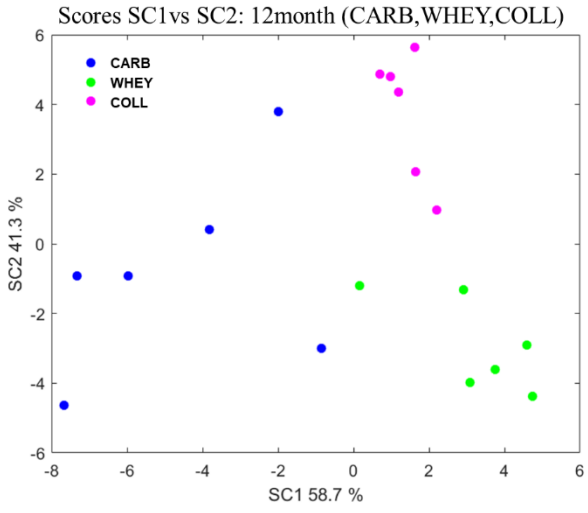
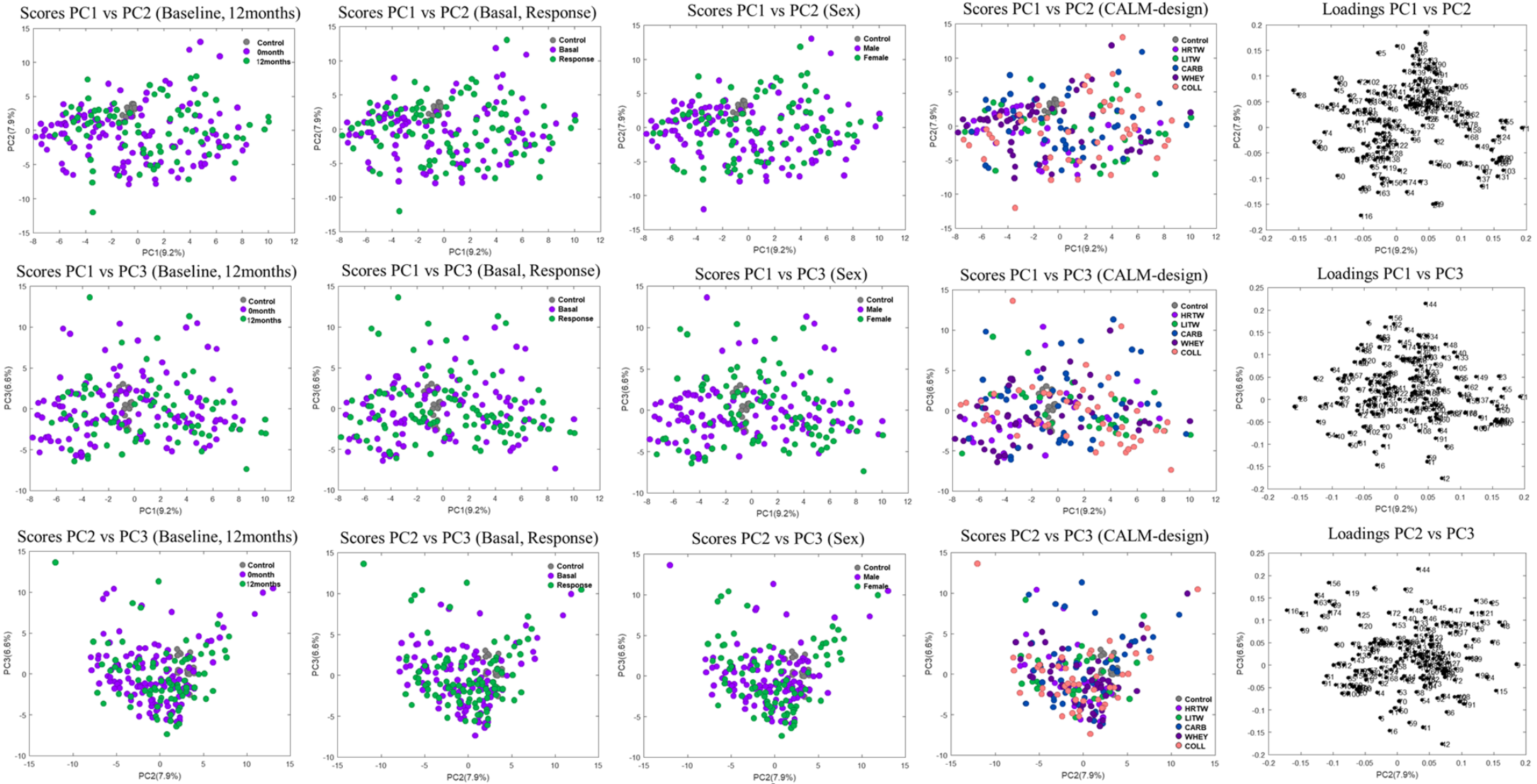


Figure 8. PCA model of the MM. Scores for PC1 vs PC2 (1st row), PC1 vs PC3(2nd row), PC2 vs PC3 (3rd row) colored according to baseline vs 12months (1st column), basal vs response (2nd column), sex (3rd column) and CALM design (4th column). Loadings are presented in the 5th column. 25% of variation is captured by the first three principal components of the PCA model, although no trend of separation of samples was observed according to visit, treatment, sex or the CALM intervention design. Control samples are grey and are clustered well in the PCA model. Adopted from paper IV.



11.4 Discussion

The purpose of the CALM study was first and foremost to evaluate the effect of recommending increased daily protein intake by supplementation with or without different types of exercise on the maintenance of skeletal muscle mass within otherwise healthy elderly. Secondly, the study were designed as an interdisciplinary study with various measurements ranging from ethnological field studies of social and everyday life to imperceivably physiological measurements such as the metabolome and protein turnover which would allow for not only an in depth physiological interpretation of any changes with respect to the primary outcome, i.e. muscle mass, but also providing explanations and knowledge on how elderly perceive and why they act or do not act on such interventions. In order to evaluate the effect off recommendation the study was designed as a randomized controlled trial and the collected data were planned to be analysed as a modified ITT as well PP. The following will discuss the results presented above.

11.4.1 The nutritional intervention

In this study, no beneficial effects of increasing daily protein intake (from ~1.1 $g \cdot kg^{-1}$ to ~1.5 $g \cdot kg^{-1}$) by protein supplementations were observed on the preservation of skeletal muscle mass measured by MRI in a population of healthy independently living elderly Danes. These results were irrespective of the analysis, i.e. ITT (figure 6) and PP (supplemental, paper II). This contrasts with the previous epidemiological studies showing an association between the daily protein intake and the maintenance of skeletal muscle mass in elderly[49–52]. However, it should be noticed that these studies only observers a clear effect of a higher protein intake when comparing the lowest quantile with the highest as described in section 7.3. As an example, Houston et al[52] did only detect significant differences between the highest (1.2 $g \cdot kg^{-1} \cdot day^{-1}$) and the lowest quantile (0.8 $g \cdot kg^{-1} \cdot day^{-1}$) with respect to changes in ASM over 3 years. The results from the CALM study provides strong evidence against the benefits of increased protein intakes on the maintenance of muscle mass, suggesting that the relationship observed in these studies may be caused by the lowest quantile protein intake being too low indicating a saturated rather than a linear relationship. It could be argued that the daily protein intake of the population included in this study were too high and not representative of the elderly population in general therefore resulting in the lack of effect of increased protein intake. However, Bhasin et al investigated the effect of 6 months of increased protein (0.8 $g \cdot kg^{-1}$ to 1.2 $g \cdot kg^{-1}$) intake on

skeletal muscle mass in 92 functionally limited men ≥65 years with a habitual protein intake of ≤0.83 $g \cdot kg^{-1}$ with or without testosterone therapy. This study is particularly interesting regarding the hypothesized positive effects of increasing protein intake on the maintenance of muscle mass within elderly, since it could be categorized as a proof of concept study due to its included population and intervention. If we assume that protein intake above the current RDA would benefit muscle mass, we would expect to see the largest effect in a population habitually having a (too) low intake. However, they found no effect of increased protein intake irrespective of additional testosterone therapy. These findings are in line with Gade et al[157] which did not find any effect of increased daily protein intake in combination with low-intensity resistance exercise on skeletal muscle mass in geriatric patients during and 12 weeks after hospitalization. Combined, the findings by Gade et al[157] and Bhasin et al[66] supports the results in CALM despite the different study populations investigated supporting the conclusion that increased protein intake is not a feasible strategy in counteracting the sarcopenic process.

The results from the DXA scan revealed an increase in fat percentage in all groups within the nutritional arm. This is in contrast with the findings by Bhasin et al[66] showing a loss of fat mass as an effect increased protein intake. However, in contrast to the CALM study the calorie intake we kept constant between the different intervention. This could indicate that the participants in the CALM study did not adjust their daily energy intake despite this only being significant different from baseline in the CARB group (table 3). This were in contrast to our hypothesis that the ingestion of a protein supplement would lead to a decreased energy intake in the following meal due to the previously shown satiety effect of protein intake[158]. Another explanation to the increased fat% could be changes in physical activity between baseline and after 12months. However, we did not observe any differences between daily step counts between baseline and 12 months on the 4 days accelerometer-based physical activity monitoring (table 4-7), suggesting that the increase in fat% observed were due to an increase in total energy intake rather than a decrease in physical activity. Nevertheless, since we did not include a group maintaining normal eating behaviour, we cannot exclude that this change could be due to the effect of one year of age rather than the effect of supplementation.

In general, one year of protein supplementation did not affect the glucose tolerance (HbA1c and insulin secretion and blood glucose during a glucose tolerance test) differently than supplementing with carbohydrates. We saw an increase in the HbA1c levels in the nutritional groups as an effect of time irrespective of analysing the data as ITT or PP (table 4 and 5) with a

gross average of ~1mmol·mol^{-1}. Across all groups the numerical changes in HbA1c were larger in the participants following the intervention (PP) which could indicate that it is the intervention rather than age causing this increase. Further, the increase of ~1mmol·mol^{-1} in the HbA1c levels after one year is relatively large compared to the descriptive literature reporting similar increase pr. decade[159]. In addition, none of the participants had a Hba1c above the diagnostic threshold of 48mmol·mol^{-1} at 12 months, indicating that this change is not clinically relevant. However, it is important to notice that even though clinicians work with diagnostic thresholds the condition of impaired glucose tolerance is a physiologically continuum. If assumed that the increases seen in this study are truly an effect of the intervention and not just age and random variation, ~30% of the included study population would fulfil the diagnostic criteria of type 2 diabetes within 10 years if they continued the intervention. Despite these indications, it is important to emphasize that we cannot exclude that this increase was due to age rather than the intervention since we did not include a group that did not receive any supplement. Nevertheless, this still stress the importance of measuring possible unintended side effects or harms when investigating and evaluating interventions of treatments, which is often forgotten and potentially leading to the phenomenon of overdiagnosis[160]. We did not see any changes in either HOMA-IR or any of the two Matsuda indexes which is could be explained by the inclusion of participants with normal glucose tolerance since these indexes were developed to assess insulin sensitivity in diabetic patients[140,141]. We only saw and increase in the 2h glucose concentration and glucose AUC and a decrease in Insulin AUC as an effect of time in the ITT and not the PP analysis. This differences between the analysis could be due to the reduced number of participants.

We did not find any effect of the one-year protein supplementation on the skeletal muscle protein synthetic response to protein intake in comparison to the iso-caloric control. In contrast, Oikawa et al[161] have recently reported an acutely increased effect of whey protein in comparison to collagen protein on the FSR. However, no studies have to my knowledge investigated whether the effects of age and high- or low-quality proteins observed in acute settings are consistent before and after interventions. Further, it has so far not been possible to establish any correlations between these acute measurements of protein synthesis and muscle mass[162] or any other easy interpretable outcome. This, in combination with the recent findings by Kim et al[98] emphasizing the importance of measuring FBR when evaluating interventions thought to positively affect the NB, suggest that the measurement of FSR only may not be usable method for investigating the long-term effects on muscle mass. Its further questions the current

interpretation of the lack of responsiveness in FSR to protein ingestion observed in elderly as negative per se regarding the maintenance of skeletal muscle based on the results in CALM and the listed reasons above. It is important to notice, that these results further weaken the arguments for increasing protein recommendations since these are typically justified by the epidemiological studies in combination with the current interpretation of FSR with respect to muscle mass[59,68].

At baseline, we observed an increase in FSR in response to protein intake compared to basal FSR. However, this difference was only observed within females (figure 7D+E). This difference could be explained by the amount of protein pr.kg LBM they received during the acute trial. As described in section 7.4, Moore et al[57] showed that that a protein intake of ~0.61 g/kg LBM is needed in order to maximize the FSR in response in elderly. The amount of protein pr kg LBM significantly (un-paired t-test, $p<0.0001$) differed between males (n=36: 0.36±0.04 g/kg LBM; mean±SD) and females (n=29: 0.50±0.05 g/kg LBM; mean±SD) during the acute trial, as a result of all participants receiving 20g of whey hydrolysate and 10g of glucose. Females were hereby closer to receiving the amount of protein suggested to elicit the maximal response in FSR according to more. The lack of response within males, are in line with the previous studies showing an impaired/lack of response in FSR to hyoeraminoacidemia[55,94] and ingestion of EAA[56,93]. We did not observe any differences in the basal FSR between males and females (un-paired t-test, $p=0.75$; males: 0.034 ±0.013%/h; females: 0.035±0.009%/h, mean±SD). This is an interesting observation since previous studies has shown such sex dimorphism. Differences in basal FSR between males and females in both young and old has been reported by Henderson et al[88], and Smith[89] has found sex differences within elderly. The discrepancy between the previous and our results could be explained by the differences in the BMI (Henderson et al:~38, Smith et al: ~26, CALM: ~25) of the study populations, since increased adiposity has been associated with increases in protein metabolism[163]. However, the power in CALM is high, emphasizing that any sex dimorphism regarding basal FSR is highly unlikely.

The measuring of the human skeletal muscle metabolome has to my knowledge only been measured three times previously. Fazelzadeh et al[128] measured the skeletal muscle metabolome using targeted UPLC-MS/MS and GC-MS yielding 96 different metabolites. Saoi et al[130] found 84 metabolites using un-targeted MSI-CE-MS and Sato et al[129] found 625 metabolites using un-targeted GC-MS and UPLC-MS/MS. All of the three studies showed significant differences in the skeletal muscle metabolome as an effect of their respective interventions. As

described in the section 10.7 we used a similar sample preparation protocol but an untargeted GC-MS platform. We found 191 different metabolites which is more than two out of the three previous studies. This is fairly good taking into considerations we only used on analysis platform. The control samples are clearly centered in the PCA model and the first three components captures 25% of the variance in the dataset. In combination with the relatively high number of participants (n=65), this highlights the robustness of both the preparation protocol as well as the analysis platform. We did not observe any differences in the skeletal muscle metabolome as an effect of the protein and carbohydrate intake during the acute trial. This were consistent between baseline and 12months after the intervention and could be explained by one of the following two. First, that the amount of protein and glucose ingested were not server enough to elicit any alteration in the metabolome after 240 min; Secondly, that the measurement were not sensitive enough. However, the first explanation seems most plausible for the following reasons. Despite the increase in circulating plasma AA concentration following the nutrient intake, the concentrations peaks after ~60 minutes and are almost back to baseline levels after 240 min (see figure S2 in paper IV). In addition, we only see an increase in the FSR in females supporting that the effect of the nutrient intake on the protein synthesis was minor. Despite no global differences we observed a decrease in two metabolites, 3-hydroxybutyric acid and 2-butenedioic acid, 240 min after the intake. This finding was consistent between baseline and 12 months after the intervention. Keeping in mind that the subjects have been fasting for ~15 hours and that 3-hydroxybutyric acid is a ketone produced by the liver and used in extrahepatic tissue during fasting or glucose deprivations[164] it is therefore plausible that a substantial part of the nutrients provided has been used in supporting vital organs rather than stimulating muscle protein synthesis and hereby altering the skeletal muscle metabolism. Based on this, it is my conviction that the lack of effect of the nutrient intake regarding the metabolome is more likely to be caused by the intervention applied rather than the sensitivity of the analysis. What happened earlier in the postprandial period were not possible to assess due to the restricted number of biopsies obtained in the acute trial, but it is important to take these findings into considerations when designing future studies. We did not have enough samples to fully investigate the effect of the CALM design. Therefore, we were only capable of conducting an exploratory investigation within the nutritional arm using the 0 min biopsy at 12 months comparing the metabolome between groups. The ASCA analysis investigating the differences between the basal metabolome at 12 months between the CARB, COLL and WHEY group did

not show any significant differences, but this could be due to the low number of samples (n=6 for each group).

The findings in the nutritional arm of the CALM study were overall consistent across the different measurements showing no effect of one-year prolonged protein supplementation in comparison to an iso-caloric control group receiving carbohydrates. It is my conviction that the included study population reflects the part of the elderly population (at least in Denmark) which would both capable and willingly to follow recommendations by the authorities. Therefore, the results presented and discussed are providing strong evidence against further increases in the recommended daily protein intake for otherwise healthy older adults >65 years of age. Further, the results indicate that changing the recommendations might actually have unintended negative effects such as the increase in BW, BMI and fat%. This underline the importance of being cautious when changing recommendation and that such changes needs to considerate any possible unintended harms that this might cause.

11.4.2 The training intervention

This study also investigated the one-year effect home-based light load and center based heavy resistance exercise in combination with whey supplementation on skeletal muscle mass. Surprisingly, we did not see any significant changes in the qCSA measured by MRI. We observed a difference between HRTW and WHEY but not between HRTW and LITW nor LITW and WHEY. We only observed changes in qCSA from baseline in the WHEY group indicating that the between group differences were driven by the decrease in qCSA in this group rather than an increase in the HRTW group. This is in contrast to previous studies, which have shown increases of 5-10% in qCSA in studies of shorter duration (3-4 months)[165,166], despite that some studies have also had difficulties to induce muscle hypertrophy in elderly populations[167,168]. However, the results from this study seems as more realistic estimates of the effects of recommending resistance exercise for elderly due to the following: Despite, the median training adherence corresponded to an average of ~2 training sessions in the HRTW group, the training were interrupted by prolonged brakes since most of the participants were on vacation for 3-4 weeks during the intervention, which could potentially attenuate the increases in skeletal muscle mass. Further, the studies of shorter duration are more likely to maintain a high intensity throughout the study period than studies of longer duration. The increases of 5-10% in qCSA are therefore unlikely to be observed under real-life conditions with several different factors

interfering with both adherence and training intensity. Contrary to the hypothesis in CALM (see figure 2), regardless of the higher adherence in the LITW group we did not observe any changes in qCSA and the effects on the strength measures (not reported in this thesis, see paper II) were scarce and inferior to the those seen in the HRTW group. Therefore, these results suggest that LITW is not a feasible strategy for improving muscle mass, strength and function within this population, and instead future studies should focus on increasing the adherence to heavy resistance training programs. Again, it is important to emphasize that these results should not be extrapolated to other population groups such as frail or less active elderly.

In contrast to the results from the MRI scans, the DXA scans actually revealed a significant increase in the ASMI in the ITT analysis as an effect of time and a significant effect of the HRTW intervention (table 6). In the PP analysis, an increase in both ASM and LBM were observed as an effect of time. This discrepancy between the results from MRI and DXA can be explained by their different ROI's. The DXA were measuring whole body and limb specific changes in LBM where the MRI did only measure changes in qCSA. Even though the training intervention were focused on the lower limbs, two upper body exercises were included which could explain why we observed an effect of time in training arm on DXA but not MRI. Further, the results from the DXA scan indicates that the training intervention also prevented the increased BW, BMI and fat% since we did not observe a similar effect of time as in the nutritional arm. Again, this could be due to general changes in physical activity but as in the nutritional intervention arm, the activity levels were not different between baseline and 12 months suggesting an actual effect of the intervention.

Interestingly, the effect of time on ASMI and LBM observed did not affect the HbA1c levels which also increased as an effect of time in the training arm with a gross average of ~0.7 $mmol \cdot mol^{-1}$. This result was supported by the cross-sectional analysis only showing an association between BMI, fat% and VAT and not ASMI with respect to changes in the glucose metabolism (for figures see S1-4 in paper III). This contrasted with our hypothesis of a secondary positive effect increasing muscle mass on glucose tolerance and previous suggestions that improvements in glucose homeostasis might be due to an increase in lean body mass[118]. However, this discrepancy might be explained by a possible negative effect of the supplement outweighing the positive effects of an increased muscle mass, since we did not include a training group without supplementation. In addition, it should be noticed that the included study population were not diabetic and in general very active (>10.000 $steps \cdot day^{-1}$) and the results

should not be extrapolated to other population groups. Again, we cannot exclude that the changes observed with respect to the HbA1c levels were due to one-year of age rather than the intervention, since we did not include a group without training or supplementation.

Unfortunately, we were only able to investigate the effect of the training intervention on the FSR with an ITT analysis due to the low numbers of participants completing the training intervention with an acceptable adherence, time from last training session to the acute trial, and missing samples due to complications during the acute trial. The ITT analysis did not show any significant changes in FSR as an effect of the training intervention. As discussed above (section 11.4.1), these results suggest that the measurement of FSR only may not be a usable method for investigating the long-term effects on muscle mass.

In general, the results from the training arm in the CALM study were consistent across the different measurements showing small effects of one-year of heavy resistance exercise in combination with whey hydrolysate supplementation in comparison to supplementing alone. In line with our hypothesis, the adherence to home-based light load resistance exercise were higher compared to center-based heavy resistance exercise. Contrary to our hypothesis, the higher adherence did not result in improvements in comparison to high resistance exercise or supplementation alone, suggesting that heavy resistance exercise is needed for improving functional capacity in this healthy and highly active study population. However, it should be stress that these findings are not transferable to individuals in less active or healthy populations groups.

11.4.3 Limitations

There are several different limitations in the CALM study. Firstly, the inclusion of healthy and active elderly with an average daily protein intake above the current RDA in their habitual diet[155]. Hence, these results cannot be transferred other parts of the elderly population with either lower protein and energy intake and lower levels of activity. However, the study was designed to test the effect of changes in recommendation and not the effect of similar interventions in populations which are unlikely to follow recommendations from authorities. Further, we did not include a control group with no supplementation and a group conducting exercise alone. Therefore, we were not able to distinguish the effect of supplementation from the effect of one-year of age nor were we able to distinguish the effect of training with supplementation from training alone. The changes observed as an effect of the training

intervention may not only be attributed to the training but the interaction between supplementation and training, but due to the study design we were not capable of distinguishing between such possible interaction. Despite the increase in calorie intake and increases in BW, BMI and fat% we cannot exclude that this is an effect of age rather than the intervention, which is a clear restriction regarding the interpretation of the results.

Regarding the acute trial, the number of participants completing the acute trial at 12 months with and without an acceptable adherence were lower than expected due to both complications during the acute trial as well as practical and logistic circumstances conducting such a large trial. Therefore, we were no able to conduct a PP analysis on the FSR in the training arm, and the risk of the study being underpowered in the analysis investigating the changes as an effect of the intervention were at risk of being underpowered. Further, it has recently been shown that the underlying assumptions of the calculations of the FSR, i.e. no recycling of tracer, may not be fulfilled with the tracer used in the CALM study[169]. This were supported by the observation of a higher degree of variation during the basal period at 12 months compared to 0 months, which could be explained by a recycling of the tracer that has been incorporated during the acute trial at baseline.

12.0 Conclusion and perspectives

The investigation of the current definitions of Sarcopenia shows that these are not scientifically justified due tautological reasoning as well as lack of evidence for Sarcopenia being a distinct phenotype with disease like characteristics and not just a natural phenomenon of aging. Further, the current definitions contribute to confusion due to the inclusion of three separate phenomena making the separation of cause and effect impossible when scientifically investigating different interventions. Therefore, it is suggested to discard the current definition and use the original definition of the concept instead, i.e. age-related loss of skeletal muscle mass, in order to restore the scientific functionality of the concept of Sarcopenia. This study due not exclude that Sarcopenia in the future could be scientifically justified as a separate phenomenon of clinical relevance, but the current approach of making it so by incorporating clinically relevant measures into the definition are not scientifically valid. In addition, the lack of clinical relevance is not an argument against research within Sarcopenia but instead it should change the focus of interest from treatment to prevention in future studies.

In the CALM study, we did not observe any relevant differences 12 months of protein supplementation in comparison to iso-caloric carbohydrate supplement irrespective of the different methods applied in this thesis. However, we observed an unintended effect of time on both HbA1c, body weight and increases in fat% indicating that the participants did not adjust their calorie intake as expected. However, it was not possible to exclude that these changes were due to age rather than the intervention since we did not include a control group without supplementation. We only saw a minor effect of 12 months of protein supplementation in addition with heavy resistance exercise on the maintenance of skeletal muscle mass in comparison to protein supplementation alone. These results are probably more realistic estimates of the possible effects of heavy resistance exercise with respect to muscle mass and strength that can be expected if such types of training were recommended by the authorities than the effects observed in previous studies of shorter durations.

We did not find any changes in muscle protein synthesis in response to protein intake as an effect of the nutritional intervention. This provide some evidence against the frequent extrapolated interpretation of increases in FSR as positive per se with respect to muscle protein net balance and muscle mass. Further, our results suggest that the measurement of FSR without measuring FBR is not a usable method for investigating long-term developments in skeletal muscle mass. Interestingly, we were not capable of confirming the previous findings of sex dimorphism with respect to the basal FSR period in our study population. However, more studies specifically designed to investigate such differences are still needed. Unfortunately, we were not able to fully test the effect of the training intervention on the FSR due to low number of participants completing the 12 months acute trial with sufficient adherence.

We were able to measure the skeletal muscle metabolome using the relatively small amount of tissue normally available and hereby confirming the possibility of measuring the skeletal muscle metabolome from previous studies. Different to the previous studies, we used an un-targeted GC-MS as the analysis platform which yielded a relatively high amount metabolites (191) when accounting for the use of only one analysis platform. This underpins our analysis platform as promising for future studies. Interestingly, we consistently did not see any alterations in the skeletal muscle metabolome 240 min after the ingestion of 20g of whey and 10g of glucose. This finding should be taking into considerations when planning future studies investigating the effect on the skeletal muscle metabolome of different interventions.

In conclusion, the findings across the different measurements were consistent in both the nutritional and training intervention arm. This study provides strong evidence against the current trend of increasing recommendations of daily protein intake in the quest of preventing Sarcopenia defined as the age-related loss of muscle mass. Further, it provides solid evidence for making realistically estimates of the long-term effects of resistance exercise in combination with protein supplementation. Lastly, light load resistance training is not enough if gains in muscle mass and function are desired within this population of healthy and active elderly. Importantly, these findings due not exclude the possibilities of light load resistance training being an effective tool in other study populations.

13.0 References

1 Thomsen SU. Rystet spejl:digte. Gyldendal, 2011.

2 Muscaritoli M, Anker SD, Argilés J, *et al.* Consensus definition of sarcopenia, cachexia and pre-cachexia: joint document elaborated by Special Interest Groups (SIG) 'cachexia-anorexia in chronic wasting diseases' and 'nutrition in geriatrics'. *Clin Nutr* 2010; **29**: 154–9.

3 Studenski SA, Peters KW, Alley DE, *et al.* The FNIH Sarcopenia Project: Rationale, Study Description, Conference Recommendations, and Final Estimates. *Journals Gerontol Ser A Biol Sci Med Sci* 2014; **69**: 547–58.

4 Cruz-Jentoft AJ, Baeyens JP, Bauer JM, *et al.* Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing* 2010; **39**: 412–23.

5 Fielding RA, Vellas B, Evans WJ, *et al.* Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia. *J Am Med Dir Assoc* 2011; **12**: 249–56.

6 Morley JE, Abbatecola AM, Argiles JM, *et al.* Sarcopenia with limited mobility: an international consensus. *J Am Med Dir Assoc* 2011; **12**: 403–9.

7 Chen L-K, Liu L-K, Woo J, *et al.* Sarcopenia in Asia: consensus report of the Asian Working Group for Sarcopenia. *J Am Med Dir Assoc* 2014; **15**: 95–101.

8 Landi F, Liperoti R, Russo A, *et al.* Sarcopenia as a risk factor for falls in elderly individuals: Results from the ilSIRENTE study. *Clin Nutr* 2012; **31**: 652–8.

9 Friedman EM, Ryff CD. Living well with medical comorbidities: A biopsychosocial perspective. *Journals Gerontol - Ser B Psychol Sci Soc Sci* 2012; **67 B**: 535–44.

10 Alexandre T da S, Duarte YADO, Santos JLF, Wong R, Lebrao ML. Sarcopenia according to the European Working Group on Sarcopenia in Older People (EWGSOP) versus dynapenia as a risk factor for mortality in the elderly. *J Nutr Heal Aging* 2014; **18**: 751–6.

11 Rasmussen BB, Phillips SM. Contractile and nutritional regulation of human muscle growth. *Exerc Sport Sci Rev* 2003; **31**: 127–31.

12 Wolfe RR. The underappreciated role of muscle in health and disease. *Am J Clin Nutr* 2006; **84**: 475–82.

13 Chang KV, Hsu TH, Wu WT, Huang KC, Han DS. Association Between Sarcopenia and

Cognitive Impairment: A Systematic Review and Meta-Analysis. *J Am Med Dir Assoc* 2016; **17**: 1164.e7-1164.e15.

14 Bone AE, Hepgul N, Kon S, Maddocks M. Sarcopenia and frailty in chronic respiratory disease: Lessons from gerontology. Chron. Respir. Dis. 2017; **14**: 85–99.

15 Bahat G, Ilhan B. Sarcopenia and the cardiometabolic syndrome: A narrative review. *Eur Geriatr Med* 2016; **7**: 220–3.

16 Landi F, Onder G, Bernabei R. Sarcopenia and diabetes: Two sides of the same coin. *J Am Med Dir Assoc* 2013; **14**: 540–1.

17 Bechshøft RL, Reitelseder S, Højfeldt G, *et al.* Counteracting Age-related Loss of Skeletal Muscle Mass: a clinical and ethnological trial on the role of protein supplementation and training load (CALM Intervention Study): study protocol for a randomized controlled trial. *Trials* 2016; **17**: 397.

18 Andersen JL, Schjerling P, Saltin B. Muscle, genes and athletic performance. Sci. Am. 2000; **283**: 48–55.

19 Ng JM, Azuma K, Kelley C, *et al.* PET imaging reveals distinctive roles for different regional adipose tissue depots in systemic glucose metabolism in nonobese humans. *Am J Physiol - Endocrinol Metab* 2012; **303**. DOI:10.1152/ajpendo.00282.2012.

20 Pedersen BK, Febbraio MA. Muscles, exercise and obesity: Skeletal muscle as a secretory organ. Nat. Rev. Endocrinol. 2012; **8**: 457–65.

21 Keul J, Dickhuth HH, Simon G, Lehmann M. Effect of static and dynamic exercise on heart volume, contractility, and left ventricular dimensions. *Circ Res* 1981; **48**: I162-70.

22 Harridge SDR, Lazarus NR. Physical Activity, Aging, and Physiological Function. *Physiology* 2017; **32**: 152–61.

23 Rosenberg IH. Summary Comments. *Am J Clin Nutr* 1989; **50**: 1231–3.

24 Baumgartner RN, Koehler KM, Gallagher D, *et al.* Epidemiology of sarcopenia among the elderly in New Mexico. *Am J Epidemiol* 1998; **147**: 755–63.

25 Newman AB, Kupelian V, Visser M, *et al.* Strength, but not muscle mass, is associated with mortality in the health, aging and body composition study cohort. *Journals Gerontol - Ser A Biol Sci Med Sci* 2006; **61**: 72–7.

26 Goodpaster BH, Park SW, Harris TB, *et al.* The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. *J Gerontol A Biol Sci Med Sci* 2006; **61**: 1059–64.

27 Clark BC, Manini TM. Sarcopenia =/= dynapenia. *J Gerontol A Biol Sci Med Sci* 2008; **63**: 829–34.

28 Clark BC, Manini TM. What is dynapenia? *Nutrition* 2012; **28**: 495–503.

29 Anker SD, Morley JE, von Haehling S. Welcome to the ICD-10 code for sarcopenia. *J Cachexia Sarcopenia Muscle* 2016; **7**: 512–4.

30 Cruz-Jentoft AJ, Bahat G, Bauer J, *et al.* Sarcopenia: revised European consensus on definition and diagnosis. *Age Ageing* 2019; **48**: 16–31.

31 Dent E, Morley JE, Cruz-Jentoft AJ, *et al.* International Clinical Practice Guidelines for Sarcopenia (ICFSR): Screening, Diagnosis and Management. *J Nutr Heal Aging* 2018; **22**: 1148–61.

32 Suetta C, Maier AB. Is muscle failure a better term than sarcopenia? J. Cachexia. Sarcopenia Muscle. 2019; **10**: 1146–7.

33 Suetta C, Haddock B, Alcazar J, *et al.* The Copenhagen Sarcopenia Study: lean mass, strength, power, and physical function in a Danish cohort aged 20–93 years. *J Cachexia Sarcopenia Muscle* 2019; published online Aug 16. DOI:10.1002/jcsm.12477.

34 Janssen I, Heymsfield SB, Wang ZM, Ross R. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *J Appl Physiol* 2000; **89**: 81–8.

35 Larsson L, Degens H, Li M, *et al.* Sarcopenia: Aging-Related Loss of Muscle Mass and Function. *Physiol Rev* 2018; **99**: 427–511.

36 Visser M, Goodpaster BH, Kritchevsky SB, *et al.* Muscle Mass, Muscle Strength, and Muscle Fat Infiltration as Predictors of Incident Mobility Limitations in Well-Functioning Older Persons. *Journals Gerontol Ser A Biol Sci Med Sci* 2005; **60**: 324–33.

37 Dam T-T, Peters KW, Fragala M, *et al.* An Evidence-Based Comparison of Operational Criteria for the Presence of Sarcopenia. *Journals Gerontol Ser A Biol Sci Med Sci* 2014; **69**: 584–90.

38 Gallagher D, Heymsfield SB. Muscle distribution: Variations with body weight, gender, and age. In: Applied Radiation and Isotopes. 1998: 733–4.

39 Gallagher D, Visser M, De Meersman RE, *et al.* Appendicular skeletal muscle mass: Effects of age, gender, and ethnicity. *J Appl Physiol* 1997; **83**: 229–39.

40 Borkan GA, Hults DE, Gerzof SG, Robbins AH, Silbert CK. Age changes in body composition revealed by computed tomography. *Journals Gerontol* 1983; **38**: 673–7.

41 Sehl ME, Yates FE. Kinetics of human aging: I. Rates of senescence between ages 30 and

70 years in healthy people. *J Gerontol A Biol Sci Med Sci* 2001; **56**: B198-208.

42 Frontera WR, Hughes VA, Fielding RA, Fiatarone MA, Evans WJ, Roubenoff R. Aging of skeletal muscle: A 12-yr longitudinal study. *J Appl Physiol* 2000; **88**: 1321–6.

43 Bortz WM. How fast do we age? Exercise performance over time as a biomarker. *Journals Gerontol - Ser A Biol Sci Med Sci* 1996; **51**. DOI:10.1093/gerona/51A.5.M223.

44 Alexandre T da S, Duarte YADO, Santos JLF, Wong R, Lebrao ML. Sarcopenia according to the European Working Group on Sarcopenia in Older People (EWGSOP) versus dynapenia as a risk factor for mortality in the elderly. *J Nutr Heal Aging* 2014; **18**: 751–6.

45 Bortz WM. The disuse syndrome. *West J Med* 1984; **141**: 691–4.

46 Bortz W. Disuse and Aging, 2009. *Journals Gerontol Ser A Biol Sci Med Sci* 2010; **65A**: 382–5.

47 McKee A, Morley JE. Hormones and sarcopenia. *Curr Opin Endocr Metab Res* 2019; **9**: 34–9.

48 Rolland Y, Czerwinski S, Abellan Van Kan G, *et al.* Sarcopenia: its assessment, etiology, pathogenesis, consequences and future perspectives. *J Nutr Health Aging* 2008; **12**: 433–50.

49 Geirsdottir OG, Arnarson A, Ramel A, Jonsson P V., Thorsdottir I. Dietary protein intake is associated with lean body mass in community-dwelling older adults. *Nutr Res* 2013; **33**: 608–12.

50 Morris MS, Jacques PF. Total protein, animal protein and physical activity in relation to muscle mass in middle-aged and older Americans. *Br J Nutr* 2013; **109**: 1294–303.

51 Sahni S, Mangano KM, Hannan MT, Kiel DP, McLean RR. Higher Protein Intake Is Associated with Higher Lean Mass and Quadriceps Muscle Strength in Adult Men and Women. *J Nutr* 2015; **145**: 1569–75.

52 Houston DK, Nicklas BJ, Ding J, *et al.* Dietary protein intake is associated with lean mass change in older, community-dwelling adults: The Health, Aging, and Body Composition (Health ABC) study. *Am J Clin Nutr* 2008; **87**: 150–5.

53 Breen L, Phillips SM. Skeletal muscle protein metabolism in the elderly: Interventions to counteract the ‘anabolic resistance’ of ageing. Nutr. Metab. 2011; **8**: 68.

54 Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. Aging is associated with diminished accretion of muscle proteins after the ingestion of a small

bolus of essential amino acids. *Am J Clin Nutr* 2005; **82**: 1065–73.

55 Volpi E, Mittendorfer B, Rasmussen BB, Wolfe RR. The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. *J Clin Endocrinol Metab* 2000; **85**: 4481–90.

56 Cuthbertson D, Smith K, Babraj J, *et al.* Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *FASEB J* 2005; **19**: 422–4.

57 Moore DR, Churchward-Venne TA, Witard O, *et al.* Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. *Journals Gerontol - Ser A Biol Sci Med Sci* 2015; **70**: 57–62.

58 Nordic Council of Ministers NC of M. Nordic Nutrition Recommendations 2012. *Nord Nutr Recomm 2012*; published online Aug. DOI:10.6027/Nord2014-002.

59 Bauer J, Biolo G, Cederholm T, *et al.* Evidence-based recommendations for optimal dietary protein intake in older people: A position paper from the prot-age study group. *J Am Med Dir Assoc* 2013; **14**: 542–59.

60 Traylor DA, Gorissen SHM, Phillips SM. Perspective: Protein requirements and optimal intakes in aging: Arewe ready to recommend more than the recommended daily allowance? Adv. Nutr. 2018; **9**: 171–82.

61 World Health Organization. Protein and amino acid requirements in human nutrition. World Health Organ. Tech. Rep. Ser. 2007. http://www.ncbi.nlm.nih.gov/pubmed/18330140 (accessed Oct 29, 2019).

62 Markofski MM, Jennings K, Timmerman KL, *et al.* Effect of Aerobic Exercise Training and Essential Amino Acid Supplementation for 24 Weeks on Physical Function, Body Composition, and Muscle Metabolism in Healthy, Independent Older Adults: A Randomized Clinical Trial. *J Gerontol A Biol Sci Med Sci* 2019; **74**: 1598–604.

63 Børsheim E, Bui QUT, Tissier S, Kobayashi H, Ferrando AA, Wolfe RR. Effect of amino acid supplementation on muscle mass, strength and physical function in elderly. *Clin Nutr* 2008; **27**: 189–95.

64 Mitchell CJ, Milan AM, Mitchell SM, *et al.* The effects of dietary protein intake on appendicular lean mass and muscle function in elderly men: A 10-wk randomized controlled trial. *Am J Clin Nutr* 2017; **106**: 1375–83.

65 Norton C, Toomey C, McCormack WG, *et al.* Protein Supplementation at Breakfast and Lunch for 24 Weeks beyond Habitual Intakes Increases Whole-Body Lean Tissue Mass in

Healthy Older Adults. *J Nutr* 2016; **146**: 65–9.

66 Bhasin S, Apovian CM, Travison TG, *et al.* Effect of protein intake on lean body mass in functionally limited older men a randomized clinical trial. *JAMA Intern Med* 2018; **178**: 530–41.

67 Oliveira CLP, Dionne IJ, Prado CM. Are Canadian protein and physical activity guidelines optimal for sarcopenia prevention in older adults? Appl. Physiol. Nutr. Metab. 2018; **43**: 1215–23.

68 Volpi E, Campbell WW, Dwyer JT, *et al.* Is the optimal level of protein intake for older adults greater than the recommended dietary allowance? *Journals Gerontol - Ser A Biol Sci Med Sci* 2013; **68**: 677–81.

69 Stewart VH, Saunders DH, Greig CA. Responsiveness of muscle size and strength to physical training in very elderly people: A systematic review. Scand. J. Med. Sci. Sport. 2014; **24**: e1-10.

70 Morton RW, Murphy KT, McKellar SR, *et al.* A systematic review, meta-analysis and meta-regression of the effect of protein supplementation on resistance training-induced gains in muscle mass and strength in healthy adults. *Br J Sports Med* 2018; **52**: 376–84.

71 Bechshøft RL, Malmgaard-Clausen NM, Gliese B, *et al.* Improved skeletal muscle mass and strength after heavy strength training in very old individuals. *Exp Gerontol* 2017; **92**: 96–105.

72 Rivera-Torres S, Fahey TD, Rivera MA. Adherence to Exercise Programs in Older Adults: Informative Report. *Gerontol Geriatr Med* 2019; **5**: 233372141882360.

73 King AC. Interventions to Promote Physical Activity by Older Adults. *Journals Gerontol Ser A Biol Sci Med Sci* 2001; **56**: 36–46.

74 Gray PM, Murphy MH, Gallagher AM, Simpson EEA. Motives and barriers to physical activity among older adults of different socioeconomic status. *J Aging Phys Act* 2016; **24**: 419–29.

75 Holm L, Reitelseder S, Pedersen TG, *et al.* Changes in muscle size and MHC composition in response to resistance exercise with heavy and light loading intensity. *J Appl Physiol* 2008; **105**: 1454–61.

76 Watanabe Y, Madarame H, Ogasawara R, Nakazato K, Ishii N. Effect of very low-intensity resistance training with slow movement on muscle size and strength in healthy older adults. *Clin Physiol Funct Imaging* 2014; **34**: 463–70.

77 Rennie MJ, Tipton KD. PROTEIN AND AMINO ACID METABOLISM DURING AND AFTER EXERCISE AND THE EFFECTS OF NUTRITION. *Annu Rev Nutr* 2000; **20**: 457–83.

78 Levitt DG, Levitt MD. Human serum albumin homeostasis: A new look at the roles of synthesis, catabolism, renal and gastrointestinal excretion, and the clinical value of serum albumin measurements. Int. J. Gen. Med. 2016; **9**: 229–55.

79 Wolfe RR, Chinkes DL. Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis. Hoboken, NJ: John Wiley & Sons, 2005.

80 Rose AJ, Richter EA. Regulatory mechanisms of skeletal muscle protein turnover during exercise. J. Appl. Physiol. 2009; **106**: 1702–11.

81 Schoenheimer R. The dynamic state of body constituents. Cambridge: MA: Harvard University Press., 1942.

82 Welle S, Thornton C, Jozefowicz R, Statt M. Myofibrillar protein synthesis in young and old men. *Am J Physiol - Endocrinol Metab* 1993; **264**. DOI:10.1152/ajpendo.1993.264.5.e693.

83 Yarasheski KE, Zachwieja JJ, Bier DM. Acute effects of resistance exercise on muscle protein synthesis rate in young and elderly men and women. *Am J Physiol - Endocrinol Metab* 1993; **265**. DOI:10.1152/ajpendo.1993.265.2.e210.

84 Toth MJ, Matthews DE, Tracy RP, Previs MJ. Age-related differences in skeletal muscle protein synthesis: Relation to markers of immune activation. *Am J Physiol - Endocrinol Metab* 2005; **288**. DOI:10.1152/ajpendo.00353.2004.

85 Symons TB, Schutzler SE, Cocke TL, Chinkes DL, Wolfe RR, Paddon-Jones D. Aging does not impair the anabolic response to a protein-rich meal. *Am J Clin Nutr* 2007; **86**: 451–6.

86 Fry CS, Drummond MJ, Glynn EL, *et al.* Aging impairs contraction-induced human skeletal muscle mTORC1 signaling and protein synthesis. *Skelet Muscle* 2011; **1**. DOI:10.1186/2044-5040-1-11.

87 Smith GI, Patterson BW, Mittendorfer B. Human muscle protein turnover--why is it so variable? *J Appl Physiol* 2011; **110**: 480–91.

88 Henderson GC, Dhatariya K, Ford GC, *et al.* Higher muscle protein synthesis in women than men across the lifespan, and failure of androgen administration to amend age-related decrements. *FASEB J* 2009; **23**: 631–41.

89 Smith GI, Atherton P, Villareal DT, *et al.* Differences in muscle protein synthesis and anabolic signaling in the postabsorptive state and in response to food in 65-80 year old men and women. *PLoS One* 2008; **3**: e1875.

90 Tipton KD. Gender differences in protein metabolism. Curr. Opin. Clin. Nutr. Metab. Care. 2001; **4**: 493–8.

91 Markofski MM, Volpi E. Protein metabolism in women and men: Similarities and disparities. *Curr Opin Clin Nutr Metab Care* 2011; **14**: 93–7.

92 Bohé J, Low A, Wolfe RR, Rennie MJ. Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: A dose-response study. *J Physiol* 2003; **552**: 315–24.

93 Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *Am J Physiol Endocrinol Metab* 2006; **291**: E381-7.

94 Guillet C, Prod'homme M, Balage M, *et al.* Impaired anabolic response of muscle protein synthesis is associated with S6K1 dysregulation in elderly humans. *FASEB J* 2004; **18**: 1586–7.

95 Baum JI, Kim IY, Wolfe RR. Protein consumption and the elderly: What is the optimal level of intake? Nutrients. 2016; **8**. DOI:10.3390/nu8060359.

96 Moore DR, Churchward-Venne T a, Witard O, *et al.* Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. *J Gerontol A Biol Sci Med Sci* 2015; **70**: 57–62.

97 Rennie MJ, Wackerhage H, Spangenburg EE, Booth FW. Control of the size of the human muscle mass. *Annu Rev Physiol* 2004; **66**: 799–828.

98 Kim IY, Schutzler S, Schrader A, *et al.* Quantity of dietary protein intake, But not pattern of intake, Affects net protein balance primarily through differences in protein synthesis in older adults. *Am J Physiol - Endocrinol Metab* 2015; **308**: E21–8.

99 Mitchell CJ, Churchward-Venne TA, West DWD, *et al.* Resistance exercise load does not determine training-mediated hypertrophic gains in young men. *J Appl Physiol* 2012. DOI:10.1152/japplphysiol.00307.2012.

100 Wolfe RR. The role of dietary protein in optimizing muscle mass, function and health outcomes in older individuals. *Br J Nutr* 2012; **108**: S88-93.

101 Elango R, Humayun MA, Ball RO, Pencharz PB. Evidence that protein requirements have been significantly underestimated. Curr. Opin. Clin. Nutr. Metab. Care. 2010; **13**: 52–7.

102 Phillips SM, Chevalier S, Leidy HJ. Protein 'requirements' beyond the RDA: implications for optimizing health. Appl. Physiol. Nutr. Metab. 2016; **41**: 565–72.

103 Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. Cell Metab. 2017; **25**: 1027–36.

104 Cannon WB. ORGANIZATION FOR PHYSIOLOGICAL HOMEOSTASIS. *Physiol Rev* 1929; **9**: 399–431.

105 Galgani JE, Moro C, Ravussin E. Metabolic flexibility and insulin resistance. Am. J. Physiol. - Endocrinol. Metab. 2008; **295**: E1009.

106 Randle PJ, Garland PB, Hales CN, Newsholme EA. THE GLUCOSE FATTY-ACID CYCLE ITS ROLE IN INSULIN SENSITIVITY AND THE METABOLIC DISTURBANCES OF DIABETES MELLITUS. *Lancet* 1963; **281**: 785–9.

107 Hue L, Taegtmeyer H. The Randle cycle revisited: A new head for an old hat. Am. J. Physiol. - Endocrinol. Metab. 2009; **297**. DOI:10.1152/ajpendo.00093.2009.

108 DeFronzo RA. Glucose intolerance and aging. *Diabetes Care* 1981; **4**: 493–501.

109 Kirkman MS, Briscoe VJ, Clark N, *et al.* Diabetes in Older Adults. *Diabetes Care* 2012; **35**: 2650–64.

110 Crane JD, Devries MC, Safdar A, Hamadeh MJ, Tarnopolsky MA. The effect of aging on human skeletal muscle mitochondrial and intramyocellular lipid ultrastructure. *Journals Gerontol - Ser A Biol Sci Med Sci* 2010; **65**: 119–28.

111 Vella CA, Nelson MC, Unkart JT, Miljkovic I, Allison MA. Skeletal muscle area and density are associated with lipid and lipoprotein cholesterol levels: The Multi-Ethnic Study of Atherosclerosis. *J Clin Lipidol* 2020. DOI:10.1016/j.jacl.2020.01.002.

112 Chee C, Shannon CE, Burns A, *et al.* Relative contribution of intramyocellular lipid to whole-body fat oxidation is reduced with age but subsarcolemmal lipid accumulation and insulin resistance are only associated with overweight individuals. *Diabetes* 2016; **65**: 840–50.

113 Miller JP, Pratley RE, Goldberg AP, *et al.* Strength training increases insulin action in healthy 50- to 65-yr-old men. *J Appl Physiol* 1994; **77**: 1122–7.

114 Biensø RS, Olesen J, Gliemann L, *et al.* Effects of Exercise Training on Regulation of Skeletal Muscle Glucose Metabolism in Elderly Men. *Journals Gerontol Ser A* 2015; **70**:

866–72.

115 Joseph LJ, Farrell PA, Davey SL, Evans WJ, Campbell WW. Effect of resistance training with or without chromium picolinate supplementation on glucose metabolism in older men and women. *Metabolism* 1999; **48**: 546–53.

116 Iglay HB, Thyfault JP, Apolzan JW, Campbell WW. Resistance training and dietary protein: effects on glucose tolerance and contents of skeletal muscle insulin signaling proteins in older persons. *Am J Clin Nutr* 2007; **85**: 1005–13.

117 Slentz CA, Bateman LA, Willis LH, *et al.* Effects of exercise training alone vs a combined exercise and nutritional lifestyle intervention on glucose homeostasis in prediabetic individuals: a randomised controlled trial. *Diabetologia* 2016; **59**: 2088–98.

118 Solerte SB, Gazzaruso C, Bonacasa R, *et al.* Nutritional Supplements with Oral Amino Acid Mixtures Increases Whole-Body Lean Mass and Insulin Sensitivity in Elderly Subjects with Sarcopenia. *Am J Cardiol* 2008; **101**: 69E-77E.

119 Manders RJF, Praet SFE, Meex RCR, *et al.* Protein hydrolysate/leucine co-ingestion reduces the prevalence of hyperglycemia in type 2 diabetic patients. *Diabetes Care* 2006; **29**: 2721–2.

120 Manders RJ, Little JP, Forbes SC, Candow DG. Insulinotropic and muscle protein synthetic effects of branched-chain amino acids: Potential therapy for type 2 diabetes and sarcopenia. Nutrients. 2012; **4**: 1664–78.

121 Wang TJ, Larson MG, Vasan RS, *et al.* Metabolite profiles and the risk of developing diabetes. *Nat Med* 2011; **17**: 448–53.

122 Hasin Y, Seldin M, Lusis A. Multi-omics approaches to disease. Genome Biol. 2017; **18**. DOI:10.1186/s13059-017-1215-1.

123 Nicholson JK, Lindon JC. Systems biology: Metabonomics. *Nature* 2008; **455**: 1054–6.

124 Trygg J, Holmes E, Lundstedt T. Chemometrics in metabonomics. J. Proteome Res. 2007; **6**: 469–79.

125 Anderson PW. More is different. *Science* 1972; **177**: 393–6.

126 Laughlin RB, Pines D. The theory of everything. *Proc Natl Acad Sci U S A* 2000; **97**: 28–31.

127 Alves RDAM, Dane AD, Harms A, *et al.* Global profiling of the muscle metabolome: method optimization, validation and application to determine exercise-induced metabolic effects. *Metabolomics* 2015; **11**: 271–85.

128 Fazelzadeh P, Hangelbroek RWJ, Tieland M, *et al.* The Muscle Metabolome Differs between Healthy and Frail Older Adults. *J Proteome Res* 2016; **15**: 499–509.

129 Sato S, Parr EB, Devlin BL, Hawley JA, Sassone-Corsi P. Human metabolomics reveal daily variations under nutritional challenges specific to serum and skeletal muscle. *Mol Metab* 2018; **16**: 1–11.

130 Saoi M, Percival M, Nemr C, Li A, Gibala M, Britz-Mckibbin P. Characterization of the Human Skeletal Muscle Metabolome for Elucidating the Mechanisms of Bicarbonate Ingestion on Strenuous Interval Exercise. *Anal Chem* 2019; **91**: 4709–18.

131 Sato S, Parr EB, Devlin BL, Hawley JA, Sassone-Corsi P. Human metabolomics reveal daily variations under nutritional challenges specific to serum and skeletal muscle. *Mol Metab* 2018; **16**: 1–11.

132 Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted Metabolomics Strategies—Challenges and Emerging Directions. *J Am Soc Mass Spectrom* 2016; **27**: 1897–905.

133 Saghaei M, Saghaei S. Implementation of an open-source customizable minimization program for allocation of patients to parallel groups in clinical trials. *J Biomed Sci Eng* 2011; **04**: 734–9.

134 Mitsiopoulos N, Baumgartner RN, Heymsfield SB, Lyons W, Gallagher D, Ross R. Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography. *J Appl Physiol* 1998; **85**: 115–22.

135 Maden-Wilkinson TM, Degens H, Jones DA, McPhee JS. Comparison of MRI and DXA to measure muscle size and age-related atrophy in thigh muscles. *J Musculoskelet Neuronal Interact* 2013; **13**: 320–8.

136 Magnetic Resonance Imaging (MRI). https://www.nibib.nih.gov/science-education/science-topics/magnetic-resonance-imaging-mri#pid-946 (accessed March 5, 2020).

137 Marinangeli CP, Kassis AN. Use of dual X-ray absorptiometry to measure body mass during short- to medium-term trials of nutrition and exercise interventions. *Nutr Rev* 2013; **71**: 332–42.

138 Shepherd JA, Ng BK, Sommer MJ, Heymsfield SB. Body composition by DXA. *Bone* 2017; **104**: 101–5.

139 World Health Organisation (WHO). Use of Glycated Haemoglobin (HbA1c) in the

Diagnosis of Diabetes Mellitus: Abbreviated Report of a WHO Consultation. 2011 http://www.ncbi.nlm.nih.gov/pubmed/26158184 (accessed March 5, 2020).

140 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–9.

141 Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 1999; **22**: 1462–70.

142 DeFronzo RA, Matsuda M. Reduced time points to calculate the composite index. Diabetes Care. 2010; **33**. DOI:10.2337/dc10-0646.

143 Bornø A, Hulston CJ, Van Hall G. Determination of human muscle protein fractional synthesis rate: An evaluation of different mass spectrometry techniques and considerations for tracer choice. *J Mass Spectrom* 2014; **49**: 674–80.

144 Martini WZ, Chinkes DL, Wolfe RR. The Intracellular Free Amino Acid Pool Represents Tracer Precursor Enrichment for Calculation of Protein Synthesis in Cultured Fibroblasts and Myocytes. *J Nutr* 2004; **134**: 1546–50.

145 Bornø A, Van Hall G. Quantitative amino acid profiling and stable isotopically labeled amino acid tracer enrichment used for in vivo human systemic and tissue kinetics measurements. *J Chromatogr B Anal Technol Biomed Life Sci* 2014; **951**–**952**: 69–77.

146 Khakimov B, Motawia MS, Bak S, Engelsen SB. The use of trimethylsilyl cyanide derivatization for robust and broad-spectrum high-throughput gas chromatography-mass spectrometry based metabolomics. *Anal Bioanal Chem* 2013; **405**: 9193–205.

147 Khakimov B, Christiansen LD, Heins AL, *et al.* Untargeted GC-MS Metabolomics Reveals Changes in the Metabolite Dynamics of Industrial Scale Batch Fermentations of Streptoccoccus thermophilus Broth. *Biotechnol J* 2017; **12**: 1700400.

148 Holm L, Olesen JL, Matsumoto K, *et al.* Protein-containing nutrient supplementation following strength training enhances the effect on muscle mass, strength, and bone formation in postmenopausal women. *J Appl Physiol* 2008; **105**: 274–81.

149 Hotelling H. Analysis of a complex of statistical variables into principal components. *J Educ Psychol* 1933; **24**: 417–41.

150 Smilde AK, Jansen JJ, Hoefsloot HCJ, Lamers R-JJAN, van der Greef J, Timmerman ME. ANOVA-simultaneous component analysis (ASCA): A new tool for analyzing designed

metabolomics data. *Bioinformatics* 2005; **21**: 3043–8.

151 Khakimov B, Poulsen SK, Savorani F, *et al.* New nordic diet versus average Danish diet: A randomized controlled trial revealed healthy long-term effects of the new nordic diet by GC-MS blood plasma metabolomics. *J Proteome Res* 2016; **15**: 1939–54.

152 Vellas B, Fielding RA, Bens C, *et al.* Implications of ICD-10 for Sarcopenia Clinical Practice and Clinical Trials: Report by the International Conference on Frailty and Sarcopenia Research Task Force. *J frailty aging* 2018; **7**: 2–9.

153 Rattan SIS. Aging is not a disease: Implications for intervention. Aging Dis. 2014; **5**: 196–202.

154 Zhavoronkov A, Bhullar B. Classifying aging as a disease in the context of ICD-11. *Front Genet* 2015; **6**: 326.

155 Schacht SR, Lind MV, Bechshøft RL, *et al.* Investigating risk of suboptimal macro and micronutrient intake and their determinants in older Danish adults with specific focus on protein intake-a cross-sectional study. *Nutrients* 2019; **11**. DOI:10.3390/nu11040795.

156 Sumner LW, Amberg A, Barrett D, *et al.* Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* 2007; **3**: 211–21.

157 Gade J, Beck AM, Andersen HE, *et al.* Protein supplementation combined with low-intensity resistance training in geriatric medical patients during and after hospitalisation: A randomised, double-blind, multicentre trial. *Br J Nutr* 2019; **122**: 1006–20.

158 Soenen S, Westerterp-Plantenga MS. Proteins and satiety: Implications for weight management. Curr. Opin. Clin. Nutr. Metab. Care. 2008; **11**: 747–51.

159 Dubowitz N, Xue W, Long Q, *et al.* Aging is associated with increased HbA1c levels, independently of glucose levels and insulin resistance, and also with decreased HbA1c diagnostic specificity. *Diabet Med* 2014; **31**: 927–35.

160 Brodersen J, Schwartz LM, Heneghan C, O'Sullivan JW, Aronson JK, Woloshin S. Overdiagnosis: what it is and what it isn't. BMJ evidence-based Med. 2018; **23**: 1–3.

161 Oikawa SY, Kamal MJ, Webb EK, McGlory C, Baker SK, Phillips SM. Whey protein but not collagen peptides stimulate acute and longer-term muscle protein synthesis with and without resistance exercise in healthy older women: a randomized controlled trial. *Am J Clin Nutr* 2020; **111**: 708–18.

162 Mitchell CJ, Churchward-Venne TA, Parise G, *et al.* Acute post-exercise myofibrillar

protein synthesis is not correlated with resistance training-induced muscle hypertrophy in young men. *PLoS One* 2014; **9**. DOI:10.1371/journal.pone.0089431.

163 Henderson GC, Nadeau D, Horton ES, Nair KS. Effects of Adiposity and 30 Days of Caloric Restriction Upon Protein Metabolism in Moderately vs. Severely Obese Women. *Obesity* 2010; **18**: 1135–42.

164 Human Metabolome Database: https://hmdb.ca/metabolites/HMDB0000357#referencese accessed 24.03.2020. .

165 Esmarck B, Andersen JL, Olsen S, Richter EA, Mizuno M, Kjær M. Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. *J Physiol* 2001; **535**: 301–11.

166 Heisterberg MF, Andersen JL, Schjerling P, *et al.* Losartan has no additive effect on the response to heavy-resistance exercise in human elderly skeletal muscle. *J Appl Physiol* 2018; **125**: 1536–54.

167 Turpela M, Häkkinen K, Haff GG, Walker S. Effects of different strength training frequencies on maximum strength, body composition and functional capacity in healthy older individuals. *Exp Gerontol* 2017; **98**: 13–21.

168 Phillips BE, Williams JP, Greenhaff PL, Smith K, Atherton PJ. Physiological adaptations to resistance exercise as a function of age. *JCI insight* 2017; **2**. DOI:10.1172/jci.insight.95581.

169 Holm L, Dideriksen K, Nielsen RH, *et al.* An exploration of the methods to determine the protein-specific synthesis and breakdown rates in vivo in humans. *Physiol Rep* 2019; **7**. DOI:10.14814/phy2.14143.

14.0 Papers

-Paper I: **Jacob Bülow**, Stanley J. Ulijaszek, Lars Holm
Rejuvenation of the term Sarcopenia, DOI: 10.1152/japplphysiol.00400.2018

-Paper II: Kenneth H. Mertz, Søren Reitelseder, Rasmus Bechshoeft, **Jacob Bulow**, Grith Højfeldt, Mikkel Jensen, Simon R. Schacht, Mads Vendelbo Lind, Morten A. Rasmussen, Ulla R. Mikkelsen, Inge Tetens, Søren B. Engelsen, Dennis S. Nielsen, Astrid P. Jespersen, Lars Holm
The effect of daily protein supplementation with or without resistance training for 1 year on muscle size, strength and function in healthy older adults.
A Randomized Clinical Trial

-Paper III: **Jacob Bülow**, Mie Cecilie Faber Zillmer, Grith Højfeldt, Rasmus Bechshøft, Jakob Agergaard, Peter Schjerling, Lars Holm
Recommended long-term nutritional supplementation, irrespective of quality and additional training does not affect glucose tolerance differently than carbohydrate supplementation in healthy elderly: the CALM cohort

-Paper IV: **Jacob Bülow**, Bekzod Khakimov, Søren Reitelseder, Rasmus Bechshøft, Søren Balling Engelsen, Lars Holm
The effect of long-term nutritional supplementation with or without different types of training on the skeletal muscle protein synthesis rate and metabolome in healthy elderly: the CALM study

Paper I: **Jacob Bülow**, Stanley J. Ulijaszek, Lars Holm
Rejuvenation of the term Sarcopenia, DOI: 10.1152/japplphysiol.00400.2018

J Appl Physiol 126: 255–256, 2019.
First published July 12, 2018; doi:10.1152/japplphysiol.00400.2018.

VIEWPOINT

Rejuvenation of the term sarcopenia

Jacob Bülow,[1] Stanley J. Ulijaszek,[2] and Lars Holm[3,4]

[1]*Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery M, Bispebjerg Hospital, Copenhagen, Denmark;* [2]*Institute of Social and Cultural Anthropology, University of Oxford, United Kingdom;* [3]*Department of Biomedical Sciences, University of Copenhagen, Denmark; and* [4]*School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, United Kingdom*

Submitted 8 May 2018; accepted in final form 9 July 2018

It is our viewpoint that the recent consensus definitions of sarcopenia are dysfunctional for clinical and experimental practice as well as in theory. In 1989, the term sarcopenia was introduced to describe the phenomenon of age-related loss of lean body mass (10). Since 2010, six consensus definitions have been presented, and in 2016, it was assigned its own ICD-10 code (1, 3, 5, 6, 8, 9, 11). A comparison of the original definition with the new consensus definitions clarifies how the term sarcopenia no longer describes the phenomenon it originally addressed. Rather, the term is now caught in tautological association, which causes confusion and hinders rather than helps understanding of this condition.

The Original Definition

In 1989, Rosenberg (10) observed that the phenomenon of decreasing lean body mass with older age had not been given the scientific attention it deserved and drew attention to it by suggesting a name combining the two words *sarco* (meaning flesh) and *penia* (meaning loss) in accordance with the characteristic that it described. The focus of this original definition was the loss of muscle mass as a discrete phenomenon, with a leading interest in legitimizing clinical and scientific attention to it (10). This definition of sarcopenia was used descriptively with the purpose of defining and articulating the loss of skeletal muscle mass, as a concrete object.

The New Consensus Definitions

Between 2011 and 2014, six consensus definitions of sarcopenia were agreed upon (3, 5, 6, 8, 9, 11). These shifted the focus from the original phenomenon of loss of skeletal muscle mass to that of physical function. All of these definitions employ an algorithm with the same logic. Physical function capability is initially assessed (gait speed or grip strength) and, only if function is impaired below a cut-point, is muscle mass (as the appendicular lean mass) secondarily evaluated. Hence, low muscle mass is not a single stand-alone determinant by which sarcopenia is defined, and having only a low muscle mass is not an adequate criterion by which to be defined as being sarcopenic. Physical function is not synonymous with muscle function, although the concepts are sometimes used interchangeably in the six consensus articles. Physical function is an interplay between multiple organ systems that can be estimated through tests like gait speed, whereas skeletal muscle, other than having the capability of contracting and allowing movement, has many functions in metabolism and as an endocrine organ.

The consensus definitions were made by working groups, with representatives from different societies within the geriatric field in Europe, the United States, and Asia, two of them receiving partial funding from the pharmaceutical industry. Discussion surrounding these definitions focuses most strongly on determination of the exact cut-off values for both physical function tests and muscle mass measurements. Surprisingly, the theoretical framework underpinning the definitions is not discussed thoroughly in any of the articles and arguments for the inclusion of physical function is found in only three (5, 8, 11) of the six papers. They share one main argument only, that the original definition is not clinically relevant.

Questioning the Reasoning for Changing the Definition

The main argument for including physical function in the definition is at least twofold. First, if a well-defined phenomenon is not clinically relevant, changing the definition does not make it become clinically relevant. Instead, it changes the phenomenon under consideration. Second, every definition can become clinically relevant by adding a criterion that is clinically relevant, as in this case with physical function. The linking of loss of skeletal muscle mass to physical function reflects the logic behind the change of focus in the research field of sarcopenia, which is notably absent from the consensus articles. During the 1990s there was a research drive to develop operational criteria for cutoff values for categorizing adults as suffering from sarcopenia. The initial suggestion for an operational criterion and cutoff value was established by Baumgartner in 1998 (2), who legitimized the criterion by showing its association with a decrease in physical function and mortality. This initiated the shift in focus from muscle mass to physical function. From around 2000, the research focus shifted to considerations of how muscle strength and physical function such as gait speed have stronger association than low muscle mass to a decrease in physical function and mortality. Instead of concentrating on the loss of muscle mass, research interest centered on the robustness of the phenomenon's association with decreased physical function and mortality, thereby making physical function the primary object of interest.

From a clinical perspective it appears reasonable to focus on the phenomenon with the strongest association to a negative

Address for reprint requests and other correspondence: J. Bülow, Institute of Sports Medicine, Copenhagen M81, Bispebjerg Hospital (Building 8, level 1), Nielsine Nielsens Vej 11, 2400 Copenhagen NV, Denmark (e-mail: jacob.bulow@live.dk).

 8750-7587/19 Copyright © 2019 the American Physiological Society

Downloaded from www.physiology.org/journal/jappl at Copenhagen Univ Lib (130.226.229.016) on January 30, 2019.

health outcome. However, in this case, the outcome and the phenomenon is almost, if not exactly, identical, and the argument for the change of focus from muscle mass to physical function is a tautology—arguing that there should be a change in focus from decreased muscle mass to decreased physical function, since a decrease in physical function has a stronger association with a decrease in physical function.

There are several consequences of the change in definition. According to the algorithms used in the consensus definitions, skeletal muscle is only of value to the definitions if it is associated with bodily movement. If gait speed is not reduced, presence of a low muscle mass is irrelevant according to the consensus definitions. This is despite the fact that skeletal muscle is the largest metabolic organ of the body and is crucial in the endocrine regulation of metabolism as well as being the body's largest reservoir of amino acids (7). Such functions are likely to be overlooked clinically when the primary inclusion criterion for sarcopenia is physical function and not muscle mass. Likewise, physical function is at risk of being reduced to the question of muscle mass when both are directly coupled in the definition (4). Furthermore, it reduces the relevance of the term in other clinical specialties such as nephrology and endocrinology, where muscle mass per se could be of clinical importance for both categorizing patients as well as in selecting treatment. Beside the reductionist understanding of the two different phenomena, the new definitions also lead to general confusion of what is meant by the term sarcopenia, since it no longer covers one but two phenomena.

Conclusion

Since the reasoning behind the change in definition of sarcopenia rests upon a tautological association and that the meaning of the term has become misleading as it no longer corresponds with the phenomenon that it addresses, we suggest a return to the use of the original definition for future research. 'Sarcopenia' should exclusively be used as a descriptive term addressing age-related loss of muscle mass. This would return focus onto uncovering the causes and consequences of the phenomenon, and clinicians will hereby have an unambiguous and useful term. Perhaps returning to the original definition could cause confusion in relation to acceptance of age-related loss of muscle mass as a clinical relevant phenomenon. However, the theoretical foundations of the consensus definitions are tautological, and we anticipate that the consequences of these definitions would continue to create confusion. There may be other and better definitions than the original but since nobody will benefit from the current consensus definitions, breaking out of the tautology is necessary to allow science and clinical practice to move on.

GRANTS

Grant provided by P. Carl Petersen Fond, Stipend from RegionH.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.B. conceived and designed research; J.B., S.J.U., and L.H. drafted manuscript; J.B., S.J.U., and L.H. edited and revised manuscript; J.B., S.J.U., and L.H. approved final version of manuscript.

REFERENCES

1. **Anker SD, Morley JE, von Haehling S.** Welcome to the ICD-10 code for sarcopenia. *J Cachexia Sarcopenia Muscle* 7: 512–514, 2016. doi:10.1002/jcsm.12147.
2. **Baumgartner RN, Koehler KM, Gallagher D, Romero L, Heymsfield SB, Ross RR, Garry PJ, Lindeman RD.** Epidemiology of sarcopenia among the elderly in New Mexico. *Am J Epidemiol* 147: 755–763, 1998. doi:10.1093/oxfordjournals.aje.a009520.
3. **Chen L-K, Liu L-K, Woo J, Assantachai P, Auyeung T-W, Bahyah KS, Chou M-Y, Chen L-Y, Hsu P-S, Krairit O, Lee JSW, Lee W-J, Lee Y, Liang C-K, Limpawattana P, Lin C-S, Peng L-N, Satake S, Suzuki T, Won CW, Wu C-H, Wu S-N, Zhang T, Zeng P, Akishita M, Arai H.** Sarcopenia in Asia: consensus report of the Asian Working Group for Sarcopenia. *J Am Med Dir Assoc* 15: 95–101, 2014. doi:10.1016/j.jamda.2013.11.025.
4. **Clark BC, Manini TM.** Sarcopenia =/= dynapenia. *J Gerontol A Biol Sci Med Sci* 63: 829–834, 2008. doi:10.1093/gerona/63.8.829.
5. **Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel J-P, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M; European Working Group on Sarcopenia in Older People.** Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing* 39: 412–423, 2010. doi:10.1093/ageing/afq034.
6. **Fielding RA, Vellas B, Evans WJ, Bhasin S, Morley JE, Newman AB, Abellan van Kan G, Andrieu S, Bauer J, Breuille D, Cederholm T, Chandler J, De Meynard C, Donini L, Harris T, Kannt A, Keime Guibert F, Onder G, Papanicolaou D, Rolland Y, Rooks D, Sieber C, Souhami E, Verlaan S, Zamboni M; International Working Group on Sarcopenia.** Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia. *J Am Med Dir Assoc* 12: 249–256, 2011. doi:10.1016/j.jamda.2011.01.003.
7. **Janssen I, Ross R.** Linking age-related changes in skeletal muscle mass and composition with metabolism and disease. *J Nutr Health Aging* 9: 408–419, 2005.
8. **Morley JE, Abbatecola AM, Argiles JM, Baracos V, Bauer J, Bhasin S, Cederholm T, Coats AJS, Cummings SR, Evans WJ, Fearon K, Ferrucci L, Fielding RA, Guralnik JM, Harris TB, Inui A, Kalantar-Zadeh K, Kirwan B-A, Mantovani G, Muscaritoli M, Newman AB, Rossi-Fanelli F, Rosano GMC, Roubenoff R, Schambelan M, Sokol GH, Storer TW, Vellas B, von Haehling S, Yeh S-S, Anker SD; Society on Sarcopenia, Cachexia and Wasting Disorders Trialist Workshop.** Sarcopenia with limited mobility: an international consensus. *J Am Med Dir Assoc* 12: 403–409, 2011. doi:10.1016/j.jamda.2011.04.014.
9. **Muscaritoli M, Anker SD, Argilés J, Aversa Z, Bauer JM, Biolo G, Boirie Y, Bosaeus I, Cederholm T, Costelli P, Fearon KC, Laviano A, Maggio M, Rossi Fanelli F, Schneider SM, Schols A, Sieber CC.** Consensus definition of sarcopenia, cachexia and pre-cachexia: joint document elaborated by Special Interest Groups (SIG) "cachexia-anorexia in chronic wasting diseases" and "nutrition in geriatrics". *Clin Nutr* 29: 154–159, 2010. doi:10.1016/j.clnu.2009.12.004.
10. **Rosenberg IH.** Summary Comments. *Am J Clin Nutr* 50: 1231–1233, 1989. doi:10.1093/ajcn/50.5.1231.
11. **Studenski SA, Peters KW, Alley DE, Cawthon PM, McLean RR, Harris TB, Ferrucci L, Guralnik JM, Fragala MS, Kenny AM, Kiel DP, Kritchevsky SB, Shardell MD, Dam T-TL, Vassileva MT.** The FNIH sarcopenia project: rationale, study description, conference recommendations, and final estimates. *J Gerontol A Biol Sci Med Sci* 69: 547–558, 2014. doi:10.1093/gerona/glu010.

Downloaded from www.physiology.org/journal/jappl at Copenhagen Univ Lib (130.226.229.016) on January 30, 2019.

Paper II: Kenneth H. Mertz, Søren Reitelseder, Rasmus Bechshoeft, **Jacob Bulow**, Grith Højfeldt, Mikkel Jensen, Simon R. Schacht, Mads Vendelbo Lind, Morten A. Rasmussen, Ulla R. Mikkelsen, Inge Tetens, Søren B. Engelsen, Dennis S. Nielsen, Astrid P. Jespersen, Lars Holm

The effect of daily protein supplementation with or without resistance training for 1 year on muscle size, strength and function in healthy older adults.
A Randomized Clinical Trial

The effect of daily protein supplementation with or without resistance training for 1 year on muscle size, strength and function in healthy older adults.

A randomized controlled trial

Kenneth H. Mertz[1], MSc; Søren Reitelseder[1,2], PhD; Rasmus Bechshoeft[1], MD, PhD; Jacob Bulow[1], MD, PhD; Grith Højfeldt[1], PhD; Mikkel Jensen[1], MSc; Simon R. Schacht[3], MSc; Mads Vendelbo Lind[3], PhD; MSc; Morten A. Rasmussen[4], PhD; Ulla R. Mikkelsen, PhD[5]; Inge Tetens[3], PhD; Søren B. Engelsen[4], PhD; Dennis S. Nielsen[4], PhD; Astrid P. Jespersen[6], PhD; Lars Holm[1,2,7], PhD.

[1]Institute of Sports Medicine, Department of Orthopaedic Surgery M, Bispebjerg Hospital, Copenhagen, DK. [2]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, DK. [3]Vitality - Centre for Good Older Lives, Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, DK. [4]Department of Food Science, University of Copenhagen, Copenhagen, DK. [5]Arla Foods Ingredients Group P/S, Viby, DK. [6]Copenhagen Center for Health Research in the Humanities, Saxo-Institute, University of Copenhagen, Copenhagen, DK. [7]School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham, UK.

Corresponding author: Kenneth H. Mertz, Institute of Sports Medicine, Department of Orthopaedic Surgery M, Nielsine Nielsens Vej 11, Bispebjerg Hospital, 2400 Copenhagen, DK.
Email: Kenneth.hudlebusch.mertz@regionh.dk

Clinicaltrials.gov identifier: NCT02034760

Registration URL: https://clinicaltrials.gov/ct2/show/NCT020347600

Data described in the article will be made available upon request pending application to the CALM trial.

ABSTRACT

Background: Protein supplementation alone or combined with resistance training have been proposed to be effective strategies to counteract age-related losses of muscle mass and strength.

Objective: To investigate the effect of protein supplementation alone or combined with light intensity or heavy load resistance exercise on muscle size, strength and function in older adults.

Methods: In a 1-year randomized controlled trial (The CALM study), 208 healthy older adults (>65 years) were randomly assigned to one of five interventions: 1) Carbohydrate supplementation (CARB), 2) Collagen protein supplementation (COLL), 3) Whey protein supplementation (WHEY), 4) Home-based light-intensity resistance training with whey protein supplementation (LITW), 5) Center-based heavy-load resistance training with whey protein supplementation (HRTW). All intervention groups received the supplement twice daily. The primary outcome measure was change in m. quadriceps cross-sectional area (qCSA), assessed by magnetic resonance imaging. Secondary outcomes included isometric knee extensor strength (MVIC), 400 m gait speed, 30-s chair stand test, leg extensor power, and body composition.

Results: Protein supplementation did not affect qCSA, strength, body composition, or functional capabilities compared to CARB. Compared to WHEY, HRTW improved qCSA ([Between-group difference, 95% CI]; 1.68, 0.41 to 2.95 cm^2, P = 0.03) and MVIC (23.9, 14.2 to 33.6 Nm, $P< 10^{-5}$). LITW did not improve any measured parameter compared to WHEY.

Conclusions: Protein supplementation alone did not affect muscle size, strength or function. Based on this study, recommending protein supplementation as a stand-alone intervention for older individuals already exceeding daily protein intakes of >1.0 $g \cdot kg^{-1} \cdot day^{-1}$ appears to be ineffective in improving any of these parameters. Only HRTW was effective in preserving muscle mass and

increasing strength. Thus, we recommend that future studies aim to investigate strategies to increase long-term compliance to heavy resistance exercise in healthy older adults. This trial was registered at Clinicaltrials.gov as NCT02034760

Keywords: Protein supplementation, ageing, skeletal muscle, resistance training, randomized controlled trials, exercise

BACKGROUND

Progressive decline of muscle mass is a hallmark of ageing and is accompanied by decrements in muscle strength[1–3]. The loss of strength leads to a risk of developing functional limitations[4], with potential detrimental effects on health and autonomy of the individual. Thus development of feasible strategies to maintain muscle mass and strength is of great importance[5–7].

The progressive decline in muscle mass and function[8,9] has extensively been suggested to be counteracted by a higher protein intake and usage of muscle through exercise[10,11]. Cross-sectional and prospective cohort studies have shown that protein intake above the current recommended daily allowance (RDA) of 0.83 $g \cdot kg^{-1} \cdot day^{-1}$ [12] is associated with higher muscle mass[13–19], as well as a better preservation of muscle mass in older adults (>65 years)[20–22]. The latter leading to increased recommendations of 1.1-1.3 g protein$\cdot kg^{-1} \cdot day^{-1}$ for older adults in the recent edition of the Nordic Nutrition Recommendations[23]. However, intervention studies investigating the effect of increasing protein intake on muscle mass show mixed results[24–32]. The duration of intervention studies are generally short (≤6 months), and the discrepant findings might therefore be related to inadequate intervention lengths[33]. Furthermore, the importance of protein quality (evaluated by the digestible indispensable amino acid score, DIAAS[34,35]), when supplied as part of a mixed diet, is not known. Oikawa and colleagues[36] recently found that supplementation with a high quality protein supplement (whey) induced greater increases in both acute and 6-days integrated muscle protein synthesis compared to a lower-quality protein supplement (collagen). However, to the present authors´ knowledge, it has not been investigated whether whey protein supplementation results in better preservation of muscle mass compared to collagen during long-term supplementation. Thus, the impact of increasing dietary protein intake on muscle mass and strength in older adults

remains a debated topic, with an urgent need for long-term, well-conducted, human intervention studies[33,37–40].

While heavy resistance training is the most potent exercise modality to increase muscle mass and strength[41–44], some older adults prefer exercise interventions of lower intensity, expensiveness, and situated in more convenient locations like a home-based setting[45,46]. Lower intensity training modalities can be effective in enhancing muscle mass[47–49] and when accounting for adherence, a home-based low intensity exercise program might therefore be an equally (or more) effective long-term exercise intervention as heavy resistance exercise for older adults.

The aim of the present study was to investigate the effect of protein supplementation and resistance training by conducting a 1-year randomized controlled trial, partly single-blinded/partly double-blinded. The hypotheses were:

1) Supplementation with higher quality whey protein will benefit muscle size and strength more than supplementation with lower quality collagen protein in healthy older adults.

2) Adherence to home-based, light intensity resistance exercise is higher than adherence to center-based heavy resistance training, and thus exerts an equally beneficial long-term strategy for gaining/preserving muscle mass and strength.

METHODS

The Counteracting Age-Related Loss of Muscle Mass (CALM) trial was conducted at Bispebjerg Hospital, Copenhagen, Denmark between 2014 and 2018. The design of the trial and detailed descriptions of methods and exclusion criteria has been published previously[50]. The regional ethics committee approved the trial protocol (H-4-2013-070), and the subjects gave their written

informed consent to participate. The trial was registered at Clinicaltrials.gov (Identifier: NCT02034760).

Study participants:

208 community-dwelling adults aged 65 years and older were recruited. To be included the participants were not allowed to partake in >1 hour of heavy resistance training per week. Participants were not included if they had any medical condition potentially preventing them from safely completing the 1-year intervention[50].

Participant recruitment:

Recruitment was done through advertisements in newspapers, magazines, and social media, as well as presentations at senior centres and public events. After a brief telephone screening for exclusion criteria, the participants underwent a physical examination including blood samples and measurements of blood pressure to determine if the participants could perform the interventions safely. Subjects also performed a 30-s chair stand test that was used for stratifying randomization.

Randomization:

Following screening and health examination, participants were enrolled in the study and randomized into one of the following five groups using MinimPy 0.3[50,51]: 1) Carbohydrate supplementation (CARB; 20 g maltodextrin + 10 g sucrose), 2) Whey protein supplementation (WHEY; 20 g whey protein hydrolysate + 10 g sucrose), 3) Collagen protein supplementation (COLL; 20 g bovine collagen protein hydrolysate + 10 g sucrose), 4) Heavy resistance training with whey protein supplementation (HRTW), 5) Light-intensity training with whey protein supplementation (LITW). Randomization was done by an investigator not involved in interventions or not sensitive to blinding. We employed a stratified, biased coin minimization with 0.95 base probability, and

used allocation ratios corresponding to the group sizes (see sample size). Randomization was stratified by sex and number of completed repetitions on the 30-s chair stand test (<16 or ≥16).

Interventions:

The five intervention groups comprised the two arms of the study; A supplementation arm and a training arm. The supplementation arm investigated the effect of twice daily protein supplementation, and the impact of protein quality (WHEY, COLL, and CARB intervention groups). Subjects were instructed to ingest the supplements twice daily, at morning and midday, preferably just before or during meals to increase satiety, thereby limiting potential excessive caloric intake. All supplements were developed and packaged by Arla Foods Ingredients Group P/S, Viby J, Denmark. The other arm of the study, the training arm, investigated the effect of resistance training at two different intensities combined with whey protein supplementation against whey protein without training (HRTW, LITW, and WHEY). HRTW performed heavy resistance training 3 times weekly under supervision of trained personnel. Training intensity was periodized into 3-month cycles, increasing the load progressively from 12 repetition maximum (RM) to 6 RM in each cycle. LITW performed light load home-based resistance 3-5 times weekly, using TheraBand® rubber bands (Hygenic Corp., Akron, OH, USA) and bodyweight. To ensure proper execution, study personnel supervised LITW sessions once per week during the first month, and once per month during the remainder of the intervention. Training sessions were mainly focused on the lower extremities, but also included exercises for the shoulders and arms (see Bechshøft et al 2016)[50]. Adherence to HRTW was registered by staff, whereas LITW and supplementation interventions were registered by the participants in hard-copy diaries.

Primary outcome:

The primary outcome was change in midthigh m. quadriceps cross-sectional area (qCSA) of the dominant leg, measured by magnetic resonance imaging (MRI) scans. MRI is considered the gold standard for measuring muscle size, and detecting age-related atrophy[52,53]. MRI scans were performed in a Siemens Verio 3 Tesla scanner by blinded radiographers. Participants were scanned in supine position using a dedicated 32-channel body coil, and a phantom was placed parallel to the femur during the scans. The following protocol was used; 3 plane GRE scout (matrix res. 1.2.0x1.6x6.0 mm, FOV 330mm, TE 3.69ms, TR 7.8ms, scan time 27s); Axial T1 tse from the medial tibia plateau to the pubic symphysis (matrix res. 0.8x0.8x8.0mm, FOV 400mm, TE 8.4ms, TR 500, scan time 3:26). Subjects were instructed to avoid vigorous physical activity for 48 hours prior to the scans. Each scan consisted of six axial slices, with the first slice being placed in the medial tibia plateau. Each slice was 8 mm thick, separated by a 60 mm gap. Slice 4 on the dominant leg was used for assessing quadriceps cross-sectional area (qCSA). All scans were analysed by the same blinded investigator using OsiriX v. 5.5.2 (OsiriX medical imaging software, Geneva, Switzerland). Each scan was analysed twice, showing a mean coefficient of variation between measurements of 0.7%. The mean of the two measurements were used for further analysis.

Secondary outcomes:

To assess lower extremity strength, maximal voluntary isometric contraction (MVIC) of the knee extensors were measured at 70° knee flexion (0° = full extension) in an isokinetic dynamometer (Kinetic Communicator, model 500-11, Chattanooga, TN, USA). Furthermore, leg extensor power was measured in the Nottingham Power Rig (Queens Medical Center, Nottingham University, UK)[54]. The functional capabilities of the participants were assessed using the 400 m walk test[55] and 30-s chair stand test[56]. Assessments of functional capabilities as well as measures of lower

160 extremity strength and power have been described in detail elsewhere[57] Self-perceived quality of
161 life was measured using the Danish version of the 36-item Short Form Health Survey[58]. We report
162 the physical (PCS) and mental component scores (MCS) for baseline characteristics.

163 Body composition was assessed using dual-energy X-ray absorptiometry (Lunar iDXA, GE Medical
164 Systems, Pewaukee, WI, USA). Study participants arrived fasting from 21:00 the night before and
165 refrained from strenuous activities for 48 hours prior to the test. All scans were performed
166 between 08:00 and 10:00. From these scans we obtained lean tissue mass (LTM) as well as body
167 fat percentage. Regions of interest (ROIs) for the extremities and visceral body parts were set
168 based on the default definitions provided by the scanner software. The same examiner controlled
169 the default positioning of all regions, which were adjusted slightly when appropriate to take into
170 account inter-individual differences in body placement and body size.

171 Daily activity levels were measured by mounting an accelerometer-based activity monitor (activPal
172 3^{TM}, activPal 3c^{TM}, or activPal micro; PAL technologies, Glasgow, UK) mounted on the anterior
173 surface of the thigh[59]. The monitor was worn for 96 continuous hours covering a full weekend.
174 Data are represented as the average number of steps per day.

175 A detailed description of the dietary assessment can be found elsewhere[60]. Briefly, participants
176 weighed their dietary intake for three consecutive days (Wednesday to Friday), and wrote down
177 the information in food logs. Trained staff then quantified nutrient intake using a dietary
178 assessment tool (VITAKOST^{TM}, MADLOG ApS, Kolding, Denmark). Dietary assessments were
179 performed prior to the intervention, and after 11 months of the intervention. Nutrient intake was
180 assessed for foods only. Protein and Energy intake from the supplement was manually calculated
181 by multiplying the compliance to the supplement with the dietary content of the supplement. For

the participants who failed to report their compliance to the supplement, but who were still receiving the supplement, we used the median compliance rate from the respective groups.

Lastly, HbA1c, blood cholesterol and triglycerides, as well creatinine concentrations were monitored.

Blinding:

Participants in the supplement-only groups (WHEY, COLL, CARB), were blinded to which supplement they received. Training interventions were not blinded to the participants. Staff performing and analysing the MRI images as well as the strength and functional tests were blinded towards the interventions. Unblinded personnel performed DXA scans and blood sampling, but analyses and interpretation of the data output from these were done by blinded researchers.

Sample sizes:

We aimed to detect between-group differences in qCSA changes of 2% over the intervention period, corresponding to approximately 0.8 cm^2. Based on previous data from our lab[61], an SD of ~1.4 cm^2 for qCSA was expected. Thus, applying a level of significance of 0.05 and a power of 0.80, a group size of 30 participants was required. Taking dropout rate into account we included 36 participants in HRTW, LITW and CARB groups and 50 participants in WHEY and COLL groups[50].

Statistical analyses:

Baseline data are summarized by group means ± standard deviations (SD) unless otherwise stated. Effects of the interventions were investigated within each study arm, separately. The individual treatment effects are reported as the mean change and associated 95% confidence intervals (CI)) during the intervention. Between-treatment effects are reported as mean difference in treatment

effect and associated 95% CI. The level of significance was set to <0.05. The effects of the interventions were analysed as a modified intention-to-treat, including all participants that completed at least one test at the 12-month timepoint, irrespective of adherence to the interventions.

Changes from baseline to 12 months were investigated separately in the supplementation arm and in the training arm of the study, using a longitudinal mixed model with time (baseline and 12 month) and intervention group (three levels) as fixed predictors, including their interaction, and person as random term. Treatment inferences were based on significance test of the interaction term, and further investigated by contrasts of intervention group changes from baseline to 12 months between all pairs (CARB vs COLL vs WHEY, and WHEY vs LITW vs HRTW) of group combinations.

R (version 3.5.1) with the function lm() from the stats package (ver 3.5.1), lmer() from the lme4 package (ver. 1.1-20) and glth() from the multcomp package (ver. 1.4-8) were used for data analysis.

RESULTS

In total, we had 1285 contacts from potential participants of which 1148 were screened via telephone. 280 participants were scheduled for an on-site screening visit of which 39 participants declined to participate. 33 were excluded prior to enrollment in the study. Consort diagram is shown in **Figure 1**. 208 participants were randomized and 184 completed the 12-month tests Characteristics of the included subjects are presented in Table 1. 24 participants dropped out during the study; 11 due to illness or injury unrelated to the intervention, 5 due to disliking the supplement, 3 due to the testing being too extensive, and 5 due to personal reasons.

Compliance

Compliance to training was significantly higher in LITW compared to HRTW ([Median [Interquartile range]], LITW: 89% [77%, 96%]; HRTW: 72% [62%, 78%]; $P < 0.01$) (see **supplemental table 1**). Supplement compliance did not differ significantly between groups (CARB: 95% [77%, 97%]; COLL: 96% [86%, 99%]; WHEY: 88% [82%, 93%], P=0.11), however, a total of 34 participants failed to report their intake of the supplements throughout the intervention (supplemental table 1). These participants all came to the research facilities to receive additional supplements as planned, but they are not included in the compliance values due to their insufficient reporting of supplement intake.

Protein intake increased for COLL ([mean, 95% CI] +29.0, +21.1 to +36.8 g/day), WHEY (+25.7, +15.6 to +35.8 g/day), LITW (+23.9, +15.2 to +32.5 g/day), and HRTW (+26.7, +18.9 to +34.5 g/day) over the intervention period, while energy intake did not change significantly (COLL: +408, -130 to +947 kJ/day; WHEY: +518, -322 to +1358 kJ/day; LITW: +474, -427 to +1375 kJ/day; HRTW: -41, -707 to +625 kJ/day, (see **supplemental table 2**). Energy intake increased for CARB, with no change in protein intake (Energy: +948, +62 to +1835 kJ/day; Protein: -4.9, -15.8 to +6.1 g/day).

Quadriceps size

In the supplementation arm, we observed no between-group differences in changes in qCSA, (P=0.17, **Figure 2A**). In the training arm, HRTW was associated with a more positive change in qCSA compared to WHEY (Between-group difference [mean, 95% CI]: 1.68, 0.41 to 2.95 cm^2, P=0.03), but not compared to LITW (1.29 cm^2, -0.08 to 2.67 cm^2, P=0.16). Changes in qCSA were not significantly different for LITW compared to WHEY (0.39, -0.88 to 1.66 cm^2, P=0.82). Neither HRTW (0-12 month change: +0.73, -0.32 to +1.77 cm^2) nor LITW (-0.54, -1.70 to +0.62 cm^2)

247 exhibited marked changes in qCSA, whereas a decrease was observed for WHEY (-0.93, -1.65 to -
248 0.21 cm^2).

249 *Lower body strength and power*

250 No between-group differences were observed in the supplementation arm for neither MVIC (P =
251 0.13, **Figure 2B**) or leg extensor power (P = 0.94, **Figure 2C**). In the training arm, changes in MVIC
252 differed between groups, with HRTW inducing greater gains in MVIC compared to LITW (Between-
253 group difference: 16.8, 6.1 to 27.4 Nm, P = 0.01) and WHEY (23.9, 14.2 to 33.6 Nm, $P< 10^{-5}$).
254 However, changes in MVIC for LITW were not significantly different from WHEY (7.1 Nm, -2.8 to
255 17.1 Nm, P = 0.34). No between-group differences in changes in leg extensor power were
256 observed within the training arm (P = 0.73).

257 *Functional capabilities*

258 In the supplementation arm, between-group differences were observed in changes in 400 m gait
259 time (P = 0.99, **Figure 2D**), or number of repetitions on the 30 s chair stand test (P = 0.30, **Figure**
260 **2E**). In the training arm, changes in 400 m gait times were not significantly different between
261 groups (P = 0.14). However, gait times decreased for HRTW (0-12 months change: -7.8, -15.1 to -
262 0.45 s) and decreased nominally for LITW (-4.7, -9.9 to +0.6 s), with no apparent change in WHEY
263 (+0.1, -5.0 to +5.2 s). Changes in number of repetitions on the 30 s chair stand test did not differ
264 between groups in training arm (P = 0.82).

265 *Body composition*

266 In the supplementation arm, changes in fat percentage (P = 0.95, **Figure 2F**) and LTM (P = 0.29,
267 **Figure 2G**) did not differ between groups. However, in all supplementation groups increases fat
268 percentage were observed (CARB: +0.7, +0.1 to +1.5 percentage points (pp); COLL: +0.6, +0.0 to

+1.2pp; WHEY: +0.7, +0.1 to +1.2pp), with no marked changes in LTM (CARB: +0.18, -0.18 to +0.54 kg; COLL: -0.04, -0.32 to +0.25 kg; WHEY: -0.17, -0.48 to +0.14 kg). In the training study, between-group differences in changes in LTM did not reach significance (P = 0.09). Nominal increases in LTM were observed in HRTW (+0.39, -0.01 to +0.79 kg), whereas no apparent change was observed for LITW (+0.10, -0.33 to +0.54 kg). Between-group differences in changes in fat percentage did also not reach significance in the training arm (P = 0.10).

DISCUSSION

This study investigated the effect of two modifiable strategies to counteract age-related loss of muscle mass in older adults; protein supplementation alone and or combined with resistance exercise. Increasing daily protein intake from ~1.1 $g \cdot kg^{-1}$ to ~1.5 $g \cdot kg^{-1}$ by providing daily protein supplements to healthy home-dwelling older individuals had no beneficial effects in any of the performed measures. These results provide strong evidence that an increase in protein intake does not add a benefit in preserving muscle mass or strength in healthy older adults living independently and eating in accordance with current guidelines. Increasing protein content in an iso-caloric diet has been shown to result in loss of fat mass[24], but in the present study supplementation of any kind was associated with an increase in fat percentage. Although this finding was not controlled against normal eating behavior, gaining fat mass indicate that the older adults in the present study did not adjust energy intake and/or expenditure accordingly when supplemented with extra calories, irrespective of the source of supplemented calories (protein/carbohydrate).

Contrary to our hypothesis, WHEY was not associated with more positive changes in qCSA compared to the COLL or CARB. This finding is surprising and contradicts our hypothesis that

supplements with high-quality protein should be superior to lower-quality protein supplements in maintaining muscle mass. In a recent study from Oikawa and colleagues[36], it was found that whey protein supplementation induced greater acute and 6-day integrated muscle protein synthesis compared to collagen supplementation in healthy older women. While these findings are contradictory, it should be noted that acute changes in muscle protein synthesis are not well correlated with long-term changes in muscle mass[62]. Thus, while whey protein supplementation might increase muscle protein turnover to a greater extent than collagen protein supplementation, the present results indicate that this has no functional long-term effect in healthy older adults.

The impact of resistance exercise on top of whey supplementation was also investigated. The effects of LITW were sparse and inferior to those of HRTW, despite the higher compliance to LITW. While HRTW was effective in increasing muscle strength and the increments in MVIC were comparable to what has been previously observed[43,63–65], the lack of change in muscle mass was unexpected. Surprisingly, 1 year of supervised resistance training did not elicit significant increases in qCSA, which have been shown in several studies reporting 5-10% increments in qCSA after 3-4 months of training[66–68]. However, a number of other studies have also struggled to induce muscle hypertrophy in older adults[69–73]. In the present study, median training compliance corresponded to an average of ~2 training sessions per week in HRTW, which has been shown previously to induce hypertrophy in older adults[74]. However, during the present study, most participants went on vacation for 3-4 weeks during the intervention, causing prolonged breaks from training. These breaks from training are likely to attenuate the increases in muscle size, and thus could potentially explain the insignificant hypertrophy observed in the present results. Compared to the very intense 3-4 month training studies previously reported[66–68], we suggest that the present results

314 are more realistic estimates of the effects when recommending older adults to complete
315 resistance training for prolonged periods of time.

316 While our statistical analysis revealed no between-group differences in changes in functional
317 capabilities, it should be noted that we observed that HRTW improved 400 m gait times. The 400
318 m gait test has previously been shown to be a strong predictor of both functional capabilities and
319 risk of future mobility limitations in healthy older adults[55]. Furthermore, we have previously
320 shown that strength is a good predictor of functional capabilities in our cohort of older adults[57].
321 Albeit speculative in relation to the present results, our findings suggest that heavy resistance
322 exercise is capable of improving functional capacity even in active older adults.

323 LIMITATIONS

324 We recruited well-functioning home-dwelling healthy older adults with a rather active lifestyle. As
325 a group, they were well-nourished and ingested on average above current RDA of protein in their
326 habitual diet[60]. Hence, the present data cannot be extrapolated to other, more frail elderly people
327 and/or some eating less energy/protein in their normal diet.

328 Our study did not include training groups not receiving protein supplementation. Therefore, the
329 obtained results in the training groups therefore may not be solely attributed to the training per
330 se, and any interaction between protein supplementation and resistance training cannot be
331 derived from the present study. However, while protein supplementation has been shown to be
332 effective in improving adaptations to resistance training in young individuals[44], the additive effects
333 seem to be minor in older adults[44,75].

CONCLUSION

This 1-year intervention study does not support the hypothesis that protein supplementation benefits preservation of muscle mass and strength in healthy older adults already reaching daily protein intakes of >1.0 g protein·kg^{-1}·day^{-1}. Despite seemingly higher compliance, the addition of light resistance home-based training is not as effective as heavy load resistance training in increasing strength and function. Future research and innovation efforts should focus on improving long-term compliance to heavy resistance exercise in healthy older adults to obtain greater muscular benefits.

AKNOWLEGEMENTS

Author contributions: Mertz and Rasmussen had full access to all the data in the present study, and take responsibility for the integrity of the data analysis. *Study design:* Reitelseder, Bechshoeft, Højfeldt, Nielsen, Engelsen, Jespersen, Lind, Mikkelsen, and Holm. *Acquisition of data:* Mertz, Reitelseder, Bechshoeft, Bulow, Højfeldt, and Jensen. *Analysis and interpretation of data:* Mertz, Schacht, Tetens, Rasmussen, and Holm. *Drafting of manuscript*: Mertz and Holm. All authors critically revised the manuscript and had the opportunity to comment on the interpretation of results. All have approved the final version. *Statistical analysis:* Rasmussen. Obtained funding: *Holm, Jespersen, Nielsen, Engelsen.*

Funding: This work received funding from the University of Copenhagen Excellence Program for Interdisciplinary Research, the Danish Dairy Research Foundation, P. Carl Petersen´ Fund, Region H research fund, Arla Foods Ingredients P/S, and Innovation Foundation Denmark.

Role of the Sponsor: Arla Foods Ingredients P/S funded part of the study. Mikkelsen, employee at Arla Foods Ingredients was involved in study design, but was not involved in data acquisition or analysis.

357

358 REFERENCES

359 1. Goodpaster BH, Park SW, Harris TB, et al. The loss of skeletal muscle strength, mass, and
360 quality in older adults: the health, aging and body composition study. *J Gerontol A Biol Sci*
361 *Med Sci*. 2006;61(10):1059-1064. doi:10.1093/gerona/61.10.1059

362 2. Janssen I, Heymsfield SB, Wang ZM, Ross R. Skeletal muscle mass and distribution in 468
363 men and women aged 18-88 yr. *J Appl Physiol*. 2000;89(1):81-88.
364 doi:10.1152/jappl.2000.89.1.81

365 3. Frontera WR, Hughes VA, Fielding RA, Fiatarone MA, Evans WJ, Roubenoff R. Aging of
366 skeletal muscle: a 12-yr longitudinal study. *J Appl Physiol*. 2000;88(4):1321-1326.
367 doi:10.1152/jappl.2000.88.4.1321

368 4. Menant JC, Weber F, Lo J, et al. Strength measures are better than muscle mass measures in
369 predicting health-related outcomes in older people: time to abandon the term sarcopenia?
370 *Osteoporos Int*. 2017;28(1):59-70. doi:10.1007/s00198-016-3691-7

371 5. Holm L, Jespersen AP, Nielsen DS, et al. Hurrah for the increasing longevity: feasible
372 strategies to counteract age-related loss of skeletal muscle mass. *Scand J Med Sci Sports*.
373 2015;25(1):1-2. doi:10.1111/sms.12415

374 6. Witard OC, McGlory C, Hamilton DL, Phillips SM. Growing older with health and vitality: a
375 nexus of physical activity, exercise and nutrition. *Biogerontology*. 2016;17(3):529-546.
376 doi:10.1007/s10522-016-9637-9

377 7. Bauman A, Merom D, Bull FC, Buchner DM, Fiatarone Singh MA. Updating the Evidence for

Physical Activity: Summative Reviews of the Epidemiological Evidence, Prevalence, and Interventions to Promote “Active Aging”. *Gerontologist*. 2016;56 Suppl 2:S268-80. doi:10.1093/geront/gnw031

8. Tieland M, Trouwborst I, Clark BC. Skeletal muscle performance and ageing. *J Cachexia Sarcopenia Muscle*. 2018;9(1):3-19. doi:10.1002/jcsm.12238

9. Ni Lochlainn M, Bowyer RCE, Steves CJ. Dietary Protein and Muscle in Aging People: The Potential Role of the Gut Microbiome. *Nutrients*. 2018;10(7):1-19. doi:10.3390/nu10070929

10. Breen L, Stokes KA, Churchward-Venne TA, et al. Two weeks of reduced activity decreases leg lean mass and induces “anabolic resistance” of myofibrillar protein synthesis in healthy elderly. *J Clin Endocrinol Metab*. 2013;98(6):2604-2612. doi:10.1210/jc.2013-1502

11. Mikkelsen UR, Couppé C, Karlsen A, et al. Life-long endurance exercise in humans: circulating levels of inflammatory markers and leg muscle size. *Mech Ageing Dev*. 2013;134(11-12):531-540. doi:10.1016/j.mad.2013.11.004

12. World Health Organization (WHO). *Protein and Amino Acid Requirements in Human Nutrition - Report of a Joint WHO/FAO/UNU Expert Consultation*.; 2007. https://www.who.int/nutrition/publications/nutrientrequirements/WHO_TRS_935/en/.

13. Geirsdottir OG, Arnarson A, Ramel A, Jonsson P V., Thorsdottir I. Dietary protein intake is associated with lean body mass in community-dwelling older adults. *Nutr Res*. 2013;33(8):608-612. doi:10.1016/j.nutres.2013.05.014

14. Nilsson A, Montiel Rojas D, Kadi F. Impact of Meeting Different Guidelines for Protein Intake on Muscle Mass and Physical Function in Physically Active Older Women. *Nutrients*.

2018;10(9). doi:10.3390/nu10091156

15. Gregorio L, Brindisi J, Kleppinger A, et al. Adequate dietary protein is associated with better physical performance among post-menopausal women 60-90 years. *J Nutr Health Aging*. 2014;18(2):155-160. doi:10.1007/s12603-013-0391-2

16. Farsijani S, Morais JA, Payette H, et al. Relation between mealtime distribution of protein intake and lean mass loss in free-living older adults of the NuAge study. *Am J Clin Nutr*. 2016;104(3):694-703. doi:10.3945/ajcn.116.130716

17. Morris MS, Jacques PF. Total protein, animal protein and physical activity in relation to muscle mass in middle-aged and older Americans. *Br J Nutr*. 2013;109(7):1294-1303. doi:10.1017/S0007114512003133

18. Mangano KM, Sahni S, Kiel DP, Tucker KL, Dufour AB, Hannan MT. Dietary protein is associated with musculoskeletal health independently of dietary pattern: the Framingham Third Generation Study. *Am J Clin Nutr*. 2017;105(3):714-722. doi:10.3945/ajcn.116.136762

19. Sahni S, Mangano KM, Hannan MT, Kiel DP, McLean RR. Higher Protein Intake Is Associated with Higher Lean Mass and Quadriceps Muscle Strength in Adult Men and Women. *J Nutr*. 2015;145(7):1569-1575. doi:10.3945/jn.114.204925

20. Houston DK, Nicklas BJ, Ding J, et al. Dietary protein intake is associated with lean mass change in older, community-dwelling adults: the Health, Aging, and Body Composition (Health ABC) Study. *Am J Clin Nutr*. 2008;87(1):150-155. doi:10.1093/ajcn/87.1.150

21. Isanejad M, Mursu J, Sirola J, et al. Association of protein intake with the change of lean mass among elderly women: The Osteoporosis Risk Factor and Prevention - Fracture

Prevention Study (OSTPRE-FPS). *J Nutr Sci*. 2015;4:e41. doi:10.1017/jns.2015.31

22. McDonald CK, Ankarfeldt MZ, Capra S, Bauer J, Raymond K, Heitmann BL. Lean body mass change over 6 years is associated with dietary leucine intake in an older Danish population. *Br J Nutr*. 2016;115(9):1556-1562. doi:10.1017/S0007114516000611

23. Nordic Council of Ministers. *Nordic Nutrition Recommendations 2012*.; 2014.

24. Bhasin S, Apovian CM, Travison TG, et al. Effect of Protein Intake on Lean Body Mass in Functionally Limited Older Men: A Randomized Clinical Trial. *JAMA Intern Med*. 2018;178(4):530-541. doi:10.1001/jamainternmed.2018.0008

25. Norton C, Toomey C, McCormack WG, et al. Protein Supplementation at Breakfast and Lunch for 24 Weeks beyond Habitual Intakes Increases Whole-Body Lean Tissue Mass in Healthy Older Adults. *J Nutr*. 2016;146(1):65-69. doi:10.3945/jn.115.219022

26. Dillon EL, Sheffield-Moore M, Paddon-Jones D, et al. Amino acid supplementation increases lean body mass, basal muscle protein synthesis, and insulin-like growth factor-I expression in older women. *J Clin Endocrinol Metab*. 2009;94(5):1630-1637. doi:10.1210/jc.2008-1564

27. Ispoglou T, White H, Preston T, McElhone S, McKenna J, Hind K. Double-blind, placebo-controlled pilot trial of L-Leucine-enriched amino-acid mixtures on body composition and physical performance in men and women aged 65-75 years. *Eur J Clin Nutr*. 2016;70(2):182-188. doi:10.1038/ejcn.2015.91

28. Mitchell CJ, Milan AM, Mitchell SM, et al. The effects of dietary protein intake on appendicular lean mass and muscle function in elderly men: a 10-wk randomized controlled trial. *Am J Clin Nutr*. 2017;106(6):1375-1383. doi:10.3945/ajcn.117.160325

441 29. Børsheim E, Bui Q-UT, Tissier S, Kobayashi H, Ferrando AA, Wolfe RR. Effect of amino acid
442 supplementation on muscle mass, strength and physical function in elderly. *Clin Nutr*.
443 2008;27(2):189-195. doi:10.1016/j.clnu.2008.01.001

444 30. Tieland M, van de Rest O, Dirks ML, et al. Protein supplementation improves physical
445 performance in frail elderly people: a randomized, double-blind, placebo-controlled trial. *J*
446 *Am Med Dir Assoc*. 2012;13(8):720-726. doi:10.1016/j.jamda.2012.07.005

447 31. Markofski MM, Jennings K, Timmerman KL, et al. Effect of Aerobic Exercise Training and
448 Essential Amino Acid Supplementation for 24 Weeks on Physical Function, Body
449 Composition and Muscle Metabolism in Healthy, Independent Older Adults: A Randomized
450 Clinical Trial. *J Gerontol A Biol Sci Med Sci*. 2018;XX(Xx):1-7. doi:10.1093/gerona/gly109

451 32. Zhu K, Kerr DA, Meng X, et al. Two-Year Whey Protein Supplementation Did Not Enhance
452 Muscle Mass and Physical Function in Well-Nourished Healthy Older Postmenopausal
453 Women. *J Nutr*. 2015;145(11):2520-2526. doi:10.3945/jn.115.218297

454 33. Traylor DA, Gorissen SHM, Phillips SM. Perspective: Protein Requirements and Optimal
455 Intakes in Aging: Are We Ready to Recommend More Than the Recommended Daily
456 Allowance? *Adv Nutr*. 2018;9(3):171-182. doi:10.1093/advances/nmy003

457 34. Food and Agriculture Organization of the United Nations. *Dietary Protein Quality Evaluation*
458 *in Human Nutrition. Report of an FAQ Expert Consultation*. Vol 92. Rome; 2013.
459 http://www.fao.org/ag/humannutrition/35978-02317b979a686a57aa4593304ffc17f06.pdf.

460 35. Burd NA, McKenna CF, Salvador AF, Paulussen KJM, Moore DR. Dietary Protein Quantity,
461 Quality, and Exercise Are Key to Healthy Living: A Muscle-Centric Perspective Across the

462 Lifespan. *Front Nutr*. 2019;6(June):83. doi:10.3389/fnut.2019.00083

463 36. Oikawa SY, Kamal MJ, Webb EK, McGlory C, Baker SK, Phillips SM. Whey protein but not
464 collagen peptides stimulate acute and longer-term muscle protein synthesis with and
465 without resistance exercise in healthy older women: a randomized controlled trial. *Am J Clin*
466 *Nutr*. January 2020:1-11. doi:10.1093/ajcn/nqz332

467 37. Volpi E, Campbell WW, Dwyer JT, et al. Is the optimal level of protein intake for older adults
468 greater than the recommended dietary allowance? *J Gerontol A Biol Sci Med Sci*.
469 2013;68(6):677-681. doi:10.1093/gerona/gls229

470 38. Campbell WW, Crim MC, Dallal GE, Young VR, Evans WJ. Increased protein requirements in
471 elderly people: new data and retrospective reassessments. *Am J Clin Nutr*. 1994;60(4):501-
472 509. doi:10.1093/ajcn/60.4.501

473 39. Oliveira CLP, Dionne IJ, Prado CM. Are Canadian protein and physical activity guidelines
474 optimal for sarcopenia prevention in older adults? *Appl Physiol Nutr Metab*.
475 2018;43(12):1215-1223. doi:10.1139/apnm-2018-0141

476 40. Bauer J, Biolo G, Cederholm T, et al. Evidence-based recommendations for optimal dietary
477 protein intake in older people: a position paper from the PROT-AGE Study Group. *J Am Med*
478 *Dir Assoc*. 2013;14(8):542-559. doi:10.1016/j.jamda.2013.05.021

479 41. Stewart VH, Saunders DH, Greig CA. Responsiveness of muscle size and strength to physical
480 training in very elderly people: a systematic review. *Scand J Med Sci Sports*. 2014;24(1):e1-
481 10. doi:10.1111/sms.12123

482 42. Fiatarone MA, O'Neill EF, Ryan ND, et al. Exercise training and nutritional supplementation

for physical frailty in very elderly people. *N Engl J Med*. 1994;330(25):1769-1775. doi:10.1056/NEJM199406233302501

43. Bechshøft RL, Malmgaard-Clausen NM, Gliese B, et al. Improved skeletal muscle mass and strength after heavy strength training in very old individuals. *Exp Gerontol*. 2017;92:96-105. doi:10.1016/j.exger.2017.03.014

44. Morton RW, Murphy KT, McKellar SR, et al. A systematic review, meta-analysis and meta-regression of the effect of protein supplementation on resistance training-induced gains in muscle mass and strength in healthy adults. *Br J Sports Med*. 2018;52(6):376-384. doi:10.1136/bjsports-2017-097608

45. King AC. Interventions to promote physical activity by older adults. *J Gerontol A Biol Sci Med Sci*. 2001;56 Spec No(Ii):36-46. https://www.nutrition.org.uk/nutritionscience/life/older-adults.html?limitstart=0.

46. Gray PM, Murphy MH, Gallagher AM, Simpson EE. Motives and Barriers to Physical Activity Among Older Adults of Different Socioeconomic Status. *J Aging Phys Act*. 2016;24(3):419-429. doi:10.1123/japa.2015-0045

47. Csapo R, Alegre LM. Effects of resistance training with moderate vs heavy loads on muscle mass and strength in the elderly: A meta-analysis. *Scand J Med Sci Sports*. 2016;26(9):995-1006. doi:10.1111/sms.12536

48. Holm L, Reitelseder S, Pedersen TG, et al. Changes in muscle size and MHC composition in response to resistance exercise with heavy and light loading intensity. *J Appl Physiol*. 2008;105(5):1454-1461. doi:10.1152/japplphysiol.90538.2008

49. Watanabe Y, Madarame H, Ogasawara R, Nakazato K, Ishii N. Effect of very low-intensity resistance training with slow movement on muscle size and strength in healthy older adults. *Clin Physiol Funct Imaging*. 2014;34(6):463-470. doi:10.1111/cpf.12117

50. Bechshøft RL, Reitelseder S, Højfeldt G, et al. Counteracting Age-related Loss of Skeletal Muscle Mass: a clinical and ethnological trial on the role of protein supplementation and training load (CALM Intervention Study): study protocol for a randomized controlled trial. *Trials*. 2016;17(1):397. doi:10.1186/s13063-016-1512-0

51. Saghaei M, Saghaei S. Implementation of an open-source customizable minimization program for allocation of patients to parallel groups in clinical trials. *J Biomed Sci Eng*. 2011;04(11):734-739. doi:10.4236/jbise.2011.411090

52. Mitsiopoulos N, Baumgartner RN, Heymsfield SB, Lyons W, Gallagher D, Ross R. Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography. *J Appl Physiol*. 1998;85(1):115-122. doi:10.1152/jappl.1998.85.1.115

53. Maden-Wilkinson TM, Degens H, Jones D a., McPhee JS. Comparison of MRI and DXA to measure muscle size and age-related atrophy in thigh muscles. *J Musculoskelet Neuronal Interact*. 2013;13(3):320-328. http://www.ncbi.nlm.nih.gov/pubmed/23989253.

54. Bassey EJ, Short AH. A new method for measuring power output in a single leg extension: feasibility, reliability and validity. *Eur J Appl Physiol Occup Physiol*. 1990;60(5):385-390. doi:10.1007/BF00713504

55. Newman AB, Simonsick EM, Naydeck BL, et al. Association of long-distance corridor walk

525 performance with mortality, cardiovascular disease, mobility limitation, and disability.
526 *JAMA*. 2006;295(17):2018-2026. doi:10.1001/jama.295.17.2018

527 56. Jones CJ, Rikli RE, Beam WC. A 30-s chair-stand test as a measure of lower body strength in
528 community-residing older adults. *Res Q Exerc Sport*. 1999;70(2):113-119.
529 doi:10.1080/02701367.1999.10608028

530 57. Mertz KH, Reitelseder S, Jensen M, et al. Influence of between-limb asymmetry in muscle
531 mass, strength, and power on functional capacity in healthy older adults. *Scand J Med Sci*
532 *Sports*. 2019;(July):1-8. doi:10.1111/sms.13524

533 58. Bjorner JB, Thunedborg K, Kristensen TS, Modvig J, Bech P. The Danish SF-36 Health Survey:
534 translation and preliminary validity studies. *J Clin Epidemiol*. 1998;51(11):991-999.
535 doi:10.1016/S0895-4356(98)00091-2

536 59. Ryan CG, Grant PM, Tigbe WW, Granat MH. The validity and reliability of a novel activity
537 monitor as a measure of walking. *Br J Sports Med*. 2006;40(9):779-784.
538 doi:10.1136/bjsm.2006.027276

539 60. Rønnow Schacht S, Vendelbo Lind M, Bechshøft RL, et al. Investigating Risk of Suboptimal
540 Macro and Micronutrient Intake and Their Determinants in Older Danish Adults with
541 Specific Focus on Protein Intake-A Cross-Sectional Study. *Nutrients*. 2019;11(4).
542 doi:10.3390/nu11040795

543 61. Holm L, Olesen JL, Matsumoto K, et al. Protein-containing nutrient supplementation
544 following strength training enhances the effect on muscle mass, strength, and bone
545 formation in postmenopausal women. *J Appl Physiol*. 2008;105:274-281.

546 doi:10.1152/japplphysiol.00935.2007

547 62. Mitchell CJ, Churchward-Venne T a, Parise G, et al. Acute post-exercise myofibrillar protein
548 synthesis is not correlated with resistance training-induced muscle hypertrophy in young
549 men. *PLoS One*. 2014;9(2):e89431. doi:10.1371/journal.pone.0089431

550 63. Moro T, Brightwell CR, Deer RR, et al. Muscle Protein Anabolic Resistance to Essential
551 Amino Acids Does Not Occur in Healthy Older Adults Before or After Resistance Exercise
552 Training. *J Nutr*. 2018;148(6):900-909. doi:10.1093/jn/nxy064

553 64. Geirsdottir OG, Chang M, Jonsson P V, Thorsdottir I, Ramel A. Obesity, Physical Function,
554 and Training Success in Community-Dwelling Nonsarcopenic Old Adults. *J Aging Res*.
555 2019;2019:5340328. doi:10.1155/2019/5340328

556 65. Heisterberg MF, Andersen JL, Schjerling P, et al. Losartan has no additive effect on the
557 response to heavy-resistance exercise in human elderly skeletal muscle. *J Appl Physiol*.
558 2018;125(5):1536-1554. doi:10.1152/japplphysiol.00106.2018

559 66. Esmarck B, Andersen JL, Olsen S, Richter E a., Mizuno M, Kjær M. Timing of postexercise
560 protein intake is important for muscle hypertrophy with resistance training in elderly
561 humans. *J Physiol*. 2001;535:301-311. doi:10.1111/j.1469-7793.2001.00301.x

562 67. Lange KHW, Andersen JL, Beyer N, et al. GH administration changes myosin heavy chain
563 isoforms in skeletal muscle but does not augment muscle strength or hypertrophy, either
564 alone or combined with resistance exercise training in healthy elderly men. *J Clin Endocrinol*
565 *Metab*. 2002;87(2):513-523. doi:10.1210/jcem.87.2.8206

566 68. Welle S, Totterman S, Thornton C. Effect of age on muscle hypertrophy induced by

567 resistance training. *J Gerontol A Biol Sci Med Sci*. 1996;51(6):M270-5.
568 http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L263940
569 75.

570 69. Greig CA, Gray C, Rankin D, et al. Blunting of adaptive responses to resistance exercise
571 training in women over 75y. *Exp Gerontol*. 2011;46(11):884-890.
572 doi:10.1016/j.exger.2011.07.010

573 70. Phillips BE, Williams JP, Greenhaff PL, Smith K, Atherton PJ. Physiological adaptations to
574 resistance exercise as a function of age. *JCI insight*. 2017;2(17):1-16.
575 doi:10.1172/jci.insight.95581

576 71. Vincent KR, Braith RW, Feldman RA, et al. Resistance exercise and physical performance in
577 adults aged 60 to 83. *J Am Geriatr Soc*. 2002;50(6):1100-1107. doi:10.1046/j.1532-
578 5415.2002.50267.x

579 72. Brook MS, Wilkinson DJ, Mitchell WK, et al. Synchronous deficits in cumulative muscle
580 protein synthesis and ribosomal biogenesis underlie age-related anabolic resistance to
581 exercise in humans. *J Physiol*. 2016;594(24):7399-7417. doi:10.1113/JP272857

582 73. Turpela M, Häkkinen K, Haff GG, Walker S. Effects of different strength training frequencies
583 on maximum strength, body composition and functional capacity in healthy older
584 individuals. *Exp Gerontol*. 2017;98(August):13-21. doi:10.1016/j.exger.2017.08.013

585 74. McCartney N, Hicks AL, Martin J, Webber CE. A longitudinal trial of weight training in the
586 elderly: continued improvements in year 2. *J Gerontol A Biol Sci Med Sci*. 1996;51(6):B425-
587 33. doi:10.1093/gerona/51a.6.b425

588 75. Thomas DK, Quinn MA, Saunders DH, Greig CA. Protein Supplementation Does Not
589 Significantly Augment the Effects of Resistance Exercise Training in Older Adults: A
590 Systematic Review. *J Am Med Dir Assoc*. 2016;17(10):959.e1-9.
591 doi:10.1016/j.jamda.2016.07.002

592

593

594

595

596

597

598 FIGURE LEGENDS

599 Figure 1: **CONSORT diagram showing the flow of participants in the CALM trial.**

600 CARB: Carbohydrate supplementation; COLL: Collagen protein supplementation; WHEY: Whey
601 protein supplementation; LITW: Light-intensity training with whey protein supplementation;
602 HRTW: Heavy resistance training with whey protein supplementation.

603

604 Figure 2: **Changes in muscle size, strength and function over the intervention period.**

605 Changes from baseline to 12 months in A) m. quadriceps cross-sectional area (qCSA). B) Knee
606 extensor maximal voluntary isometric contraction (MVIC) C) Lean tissue mass (LTM). D) Fat
607 percentage. E) 400 m gait time. F) Leg extensor power. G) Reps on the 30-s chair stand test.
608 Results are shown as mean changes [± 95% CI] from baseline to 12 months of intervention. *:
609 Significant between-group difference in changes over the intervention period. CARB: Carbohydrate
610 supplementation; COLL: Collagen protein supplementation; WHEY: Whey protein
611 supplementation; LITW: Light-intensity training with whey protein supplementation; HRTW: Heavy
612 resistance training with whey protein supplementation.

613

614

615

616

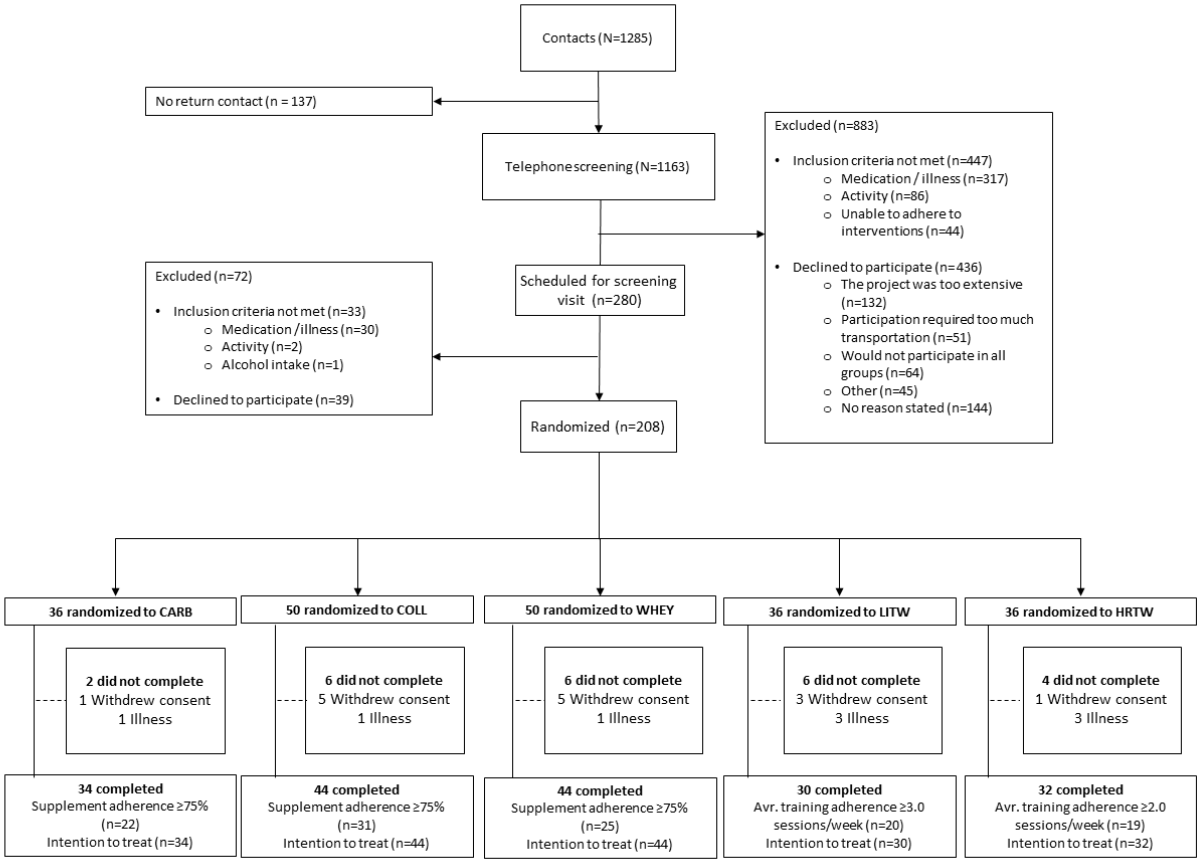
617 **Table 1. Baseline characteristics of the included participants by group.**

	CARB	COLL	WHEY	LITW	HRTW
Variable	(n = 36)	(n = 50)	(n = 50)	(n = 36)	(n = 36)
Demographics, Mean (SD)					
Age, y	69.6 (3.9)	70.4 (4.1	70.3 (4.3)	70.4 (4.0)	70.3 (3.1)
BMI, kg/m^2	26.0 (3.9)	25.4 (6.0)	25.2 (3.6)	25.7 (3.1)	25.9 (3.5)
Daily activity, Steps/day	10894 (5165)	10590 (3996)	10118 (3590)	10119 (3450)	9777 (3574)
Protein intake, g/kg/day	1.2 (0.3)	1.2 (0.4)	1.1 (0.3)	1.0 (0.3)	1.1 (0.4)
Energy intake, kJ/day	8442 (1804)	8150 (1952)	8529 (2092)	7445 (2220)	8268 (2146)
Body Composition					
Lean tissue mass, kg	48.5 (7.8)	49.2 (8.6)	50.0 (8.5)	48.1 (9.3)	48.8 (9.9)
Fat percentage, %	33.2 (9.3)	32.0 (9.1)	32.7 (7.5)	34.3 (7.5)	34.7 (7.1)
Quadriceps size, cm^2	56.6 (11.3)	56.0 (13.9)	54.5 (11.0)	56.7 (11.4)	55.4 (13.1)
Strength and function					
400 m gait time, s	248 (42)	243 (38)	242 (30)	242 (30)	251 (27)
30 s chair stand, reps	19.9 (5.7)	20.1 (5.3)	19.4 (4.6)	20.1 (4.6)	18.9 (4.9)

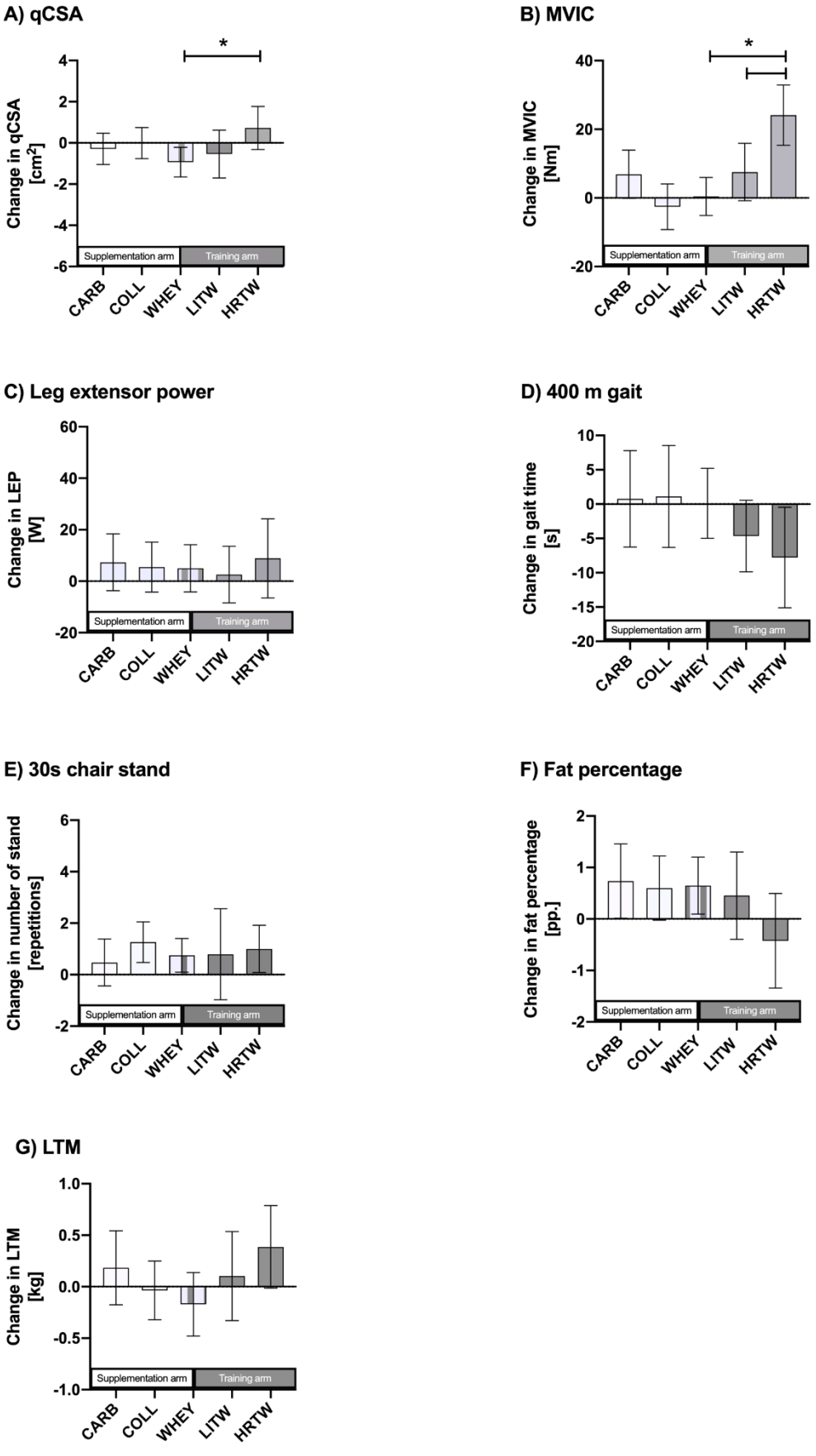
Leg extensor power, W	183.1 (56.2)	191.2 (67.2)	189.6 (59.6)	190.8 (61.4)	194.2 (65.8)
MVIC, Nm	158.9 (41.1)	169.0 (53.4)	177.6 (47.0)	171.5 (44.4)	165.0 (50.8)
SF-36					
MCS	59.3 (3.2)	57.3 (4.3)	57.6 (3.6)	57.1 (4.7)	57.5 (4.4)
PCS	55.3 (4.7)	56.0 (4.7)	56.8 (3.1)	56.4 (4.0)	56.5 (4.2)
Laboratory data					
Hba1c, mmol/mol	36.0 (2.2)	35.8 (3.4)	36.2 (3.5)	35.8 (2.9)	35.8 (2.7)
Total cholesterol, mmol/l	5.6 (0.9)	5.7 (1.0)	6.0 (1.2)	5.5 (1.0)	5.8 (0.9)
HDL Cholesterol, mmol/l	1.9 (0.5)	2.0 (0.6)	1.8 (0.5)	1.8 (0.5)	1.8 (0.5)
LDL Cholesterol, mmol/l	3.1 (0.8)	3.2 (1.0)	3.4 (0.9)	3.0 (1.0)	3.4 (1.0)
Triglycerides, mmol/l	1.3 (0.6)	1.4 (0.8)	1.7 (0.8)*	1.4 (0.6)	1.4 (0.6)
Creatinine, µmol/l	76.8 (14.7)	81.4 (15.9)	80.5 (11.6)	78.8 (14.7)	77.0 (12.7)

618

619 Figure 1: **CONSORT diagram showing the flow of participants in the CALM trial.**



620 Figure 2: **Changes in muscle size, strength and function over the intervention period.**



Supplemental table 1. **Overview of compliance to interventions.**

	CARB		COLL		WHEY		LITW		HRTW	
	ITT	**PP**	**ITT**	**PP**	**ITT**	**PP**	**ITT**	**PP**	**ITT**	**PP**
Training compliance (Median [IQR])	-	-	-	-	-	-	89% [77%, 96%]	94% [88%, 97%]	72% [62%, 78%]	78% [75%, 82%]
Supplement compliance (Median [IQR])	95% [77%, 97%]	96% [89%, 98%]	96% [86%, 99%]	96% [86%, 99%]	88% [82%, 93%]	90% [85%, 96%]	90% [77%, 94%]	93% [85%, 100%]	87% [79%, 97%]	94% [87%, 98%]
Supplement non-reporters (n=)	7		11		14		1		1	
Drop outs (n=)	2		6		6		6		4	
Included subjects (n=)	34	22	44	31	44	25	30	20	32	19

Participants were included in per protocol analysis if supplement compliance exceeded 75%, and training compliance exceeded 75% for LITW and 66% for HRTW. ITT: Intention -to-treat analysis. PP: Per protocol analysis. CARB: Carbohydrate supplementation. COLL: Collagen protein supplementation. WHEY: Whey protein supplementation. LITW: Light intensity training with whey protein supplementation. HRTW: Heavy resistance training with whey protein supplementation.

Supplemental table 2. **Changes from 0 to 12 months in Intention-to-treat analysis and per protocol analysis**

	CARB		COLL		WHEY		LITW		HRTW	
	ITT	PP	ITT	PP	ITT	PP	ITT	PP	ITT	PP
Changes from 0-12m	(n = 34)	(n=22)	(n = 44)	(n=31)	(n = 44)	(n=25)	(n = 36)	(n=20)	(n = 36)	(n=19)
Demographics, Mean (SE)										
Daily activity, Steps/day	-1662 (896)	434 (670)	330 (589)	-132 (716)	-91 (554)	-267 (823)	-322 (582)	113 (536)	-368 (411)	-381 (403)
Protein intake, g/day	-4.9 (5.3)	3.9 (5.9)	29.0 (3.9)*	27.2 (4.5)*	25.7 (5.0)*	31.4 (6.3)*	23.8 (4.2)	26.9 (4.7)	26.7 (3.8)	34.6 (4.0)
Protein intake excluding supplement, g/day	-4.9 (5.3)	3.9 (5.9)	-8.3 (3.6)	-9.8 (4.2)	-6.4 (4.3)	-5.0 (6.1)	-9.6 (3.9)	-9.8 (4.7)	-5.8 (3.2)	-2.3 (4.3)
Energy intake, kJ/day	948 (428)	865.9 (474)	408 (266)	343 (313)	517 (413)	900 (608)	474 (437)	874 (551)	-41 (324)	348 (418)
Energy intake excluding supplement, g/day	-81 (425)	-196 (466)	-649 (260)	-703 (304)	-389 (397)	-130 (603)	-472 (427)	-161 (550)	-961 (315)	-695 (431)
Body Composition										
Fat free mass, kg	0.2 (0.2)	0.4 (0.2)	0.0 (0.1)	-0.1 (0.2)	-0.2 (0.2)	-0.1 (0.2)	0.1 (0.2)	0.2 (0.3)	0.4 (0.2)	0.6 (0.3)
Fat percentage, %	0.7 (0.4)	0.7 (0.3)	0.6 (0.3)	0.6 (0.4)	0.7 (0.3)	0.6 (0.3)	0.5 (0.4)	0.5 (0.5)	-0.4 (0.5)	-0.8 (0.7)
Quadriceps size, cm^2	-0.3 (0.4)	-0.1 (0.5)	0.0 (0.4)	-0.1 (0.4)	-0.9 (0.4)	-1.1 (0.4)	-0.5 (0.6)	-0.2 (0.5)	0.7 (0.5) &	0.8 (0.7)
Strength and function										
400 m gait time, s	0.8 (3.5)	0.5 (2.9)	1.1 (3.7)	5.5 (4.6)	0.11 (2.52)	-4.48 (3.18)	-4.66 (2.55)	-6.79 (3.00)	-7.78 (3.59)	-13.32 (2.94)
30 s chair stand, reps	0.5 (0.5)	0.5 (0.6)	1.3 (0.4)	1.3 (0.5)	0.8 (0.3)	1.0 (0.4)	0.8 (0.9)	1.1 (1.3)	1.0 (0.4)	0.8 (0.3)
Leg extensor power, W	7.3 (5.4)	8.0 (6.9)	5.5 (4.8)	5.7 (6.2)	5.0 (4.6)	12.5 (6.2)	2.6 (5.4)	2.8 (6.3)	8.9 (7.5)	10.7 (10.9)
MVIC, Nm	6.9 (3.5)	10.5 (3.5)	-2.6 (3.3)	1.1 (3.8)	0.4 (2.8)	0.5 (3.3)	7.5 (4.1)	8.7 (4.8)	24.1 (4.3) &, §	29.4 (6.1) &,§
Laboratory data										

Hba1c, mmol/mol	1.06 (0.33)	1.52 (0.42)	0.98 (0.40)	1.03 (0.50)	0.16 (0.33)	0.48 (0.44)	1.27 (0.47)	1.10 (0.51)	0.50 (0.35)	0.42 (0.51)
Total cholesterol, mmol/l	-0.38 (0.12)	-0.41 (0.17)	-0.57 (0.11)	-0.74 (0.12)	-0.54 (0.10)	-0.67 (0.13)	-0.62 (0.09)	-0.69 (0.12)	-0.59 (0.1)	-0.58 (0.14)
HDL Cholesterol, mmol/l	-0.12 (0.05)	-0.17 (0.06)	-0.21 (0.03)	-0.23 (0.04)	-0.14 (0.05)	-0.22 (0.07)	-0.20 (0.05)	-0.20 (0.06)	-0.10 (0.05)	-0.10 (0.06)
LDL Cholesterol, mmol/l	-0.14 (0.10)	-0.12 (0.15)	-0.23 (0.12)	-0.36 (0.13)	-0.16 (0.08)	-0.25 (0.1)	-0.28 (0.08)	-0.34 (0.12)	-0.33 (0.09)	-0.28 (0.15)
Triglycerides, mmol/l	-0.23 (0.07)	-0.24 (0.10)	-0.40 (0.08)	-0.41 (0.10)	-0.52 (0.08)*	-0.43 (0.09)	-0.29 (0.09)	-0.28 (0.12)	-0.39 (0.09)	-0.47 (0.11)
Creatinine, µmol/l	3.71 (1.41)	4.14 (1.88)	3.37 (1.17)	2.19 (1.24)	-0.41 (1.07) $	-0.96 (1.25)	0.87 (1.20)	-0.35 (1.63)	2.50 (1.13)	0.47 (1.09)

* P < 0.05 vs CARB

$ P < 0.05 vs COLL

& P < 0.05 vs WHEY

§ P < 0.05 vs LITW

Participants were included in per protocol analysis if supplement compliance exceeded 75%, and training compliance exceeded 75% for LITW and 66% for HRTW. ITT: Intention-to-treat analysis. PP: Per protocol analysis. CARB: Carbohydrate supplementation. COLL: Collagen protein supplementation. WHEY: Whey protein supplementation. LITW: Light intensity training with whey protein supplementation. HRTW: Heavy resistance training with whey protein supplementation.

PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Jacob Bülow
E-mail	jacob.bulow@live.dk
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	The Ageing Skeletal Muscle: Effects of Training and Protein supplementation

2. The declaration applies to the following article	
Title of article	The effect of daily protein supplementation with or without resistance training for 1 year on muscle size, strength and function in healthy older adults. A randomized controlled trial
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☒ Date: 6.3.2020	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	F
2. Development of the key methods	F
3. Planning of the experiments and methodology design and development	F
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	C
5. Conducting the analysis of data	E
6. Interpretation of the results	D
7. Writing of the first draft of the manuscript	E
8. Finalisation of the manuscript and submission	D

Provide a short description of the PhD student´s specific contribution to the article.[i]
The PhD student has been the primary responsible of the daily mangement of the CALM study and been involved in both the clinical studies and the collection of data. The PhD student has discussed the results with Kenneth Mertz during the analysis and assisted with the interpretation. Further, the PhD student has contributed with critical revisions from the first drafts to the submitted manuscript

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	
If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another.	

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	25/3-20	Kenneth Hudlebusch Mertz	Msc	Kenneth Mertz
2.		Lars Holm	Professor	Skriv tekst her
3.				
4.				
5.				
6.				
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 30-3-2020
Principal supervisor: Michael Kjær

Name: Michael Kjær
Principal supervisor

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 27-03-2020
PhD student: Jacob Bülow

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

[i] This can be supplemented with an additional letter if needed.

[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."

[iii] If more signatures are needed please add an extra sheet.

Paper III: **Jacob Bülow**, Mie Cecilie Faber Zillmer, Grith Højfeldt, Rasmus Bechshøft, Jakob Agergaard, Peter Schjerling, Lars Holm
Recommended long-term nutritional supplementation, irrespective of quality and additional training does not affect glucose tolerance differently than carbohydrate supplementation in healthy elderly: the CALM cohort

Title Page

Title

Recommended long-term nutritional supplementation, irrespective of quality and additional training does not affect glucose tolerance differently than carbohydrate supplementation in healthy elderly: the CALM cohort

Authors

Jacob Bülow[1], Mie Cecilie Faber Zillmer[1], Grith Højfeldt[1], Rasmus Bechshøft[1], Jakob Agergaard[1], Peter Schjerling[1], Lars Holm[2,3]

Author affiliations

[1]Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery M, Bispebjerg Hospital, Copenhagen, Denmark
[2]Dept. of Biomedical Sciences, University of Copenhagen, Denmark
[3]School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham, United Kingdom

Corresponding Author

Jacob Bülow

Mailing address:
Institute of Sports Medicine Copenhagen M81,
Bispebjerg Hospital
Nielsine Nielsens Vej 11, 1st floor
DK-2400 Copenhagen NV
Denmark

Tel: +45 38 63 53 98

e-mail: jacob.buelow.02@regionh.dk

Sources of support

University of Copenhagen Excellence Programme for Interdisciplinary Research 2016; Arla Foods Ingredients P/S; Danish Dairy Research Foundation; Grant provided by P. Carl Petersen Fond, Stipend from The Capital Region of Denmark.

Word count

4701 incl. references

Conflict of interest declaration

None of the authors declared any conflict of interest.

ABSTRACT:

Aim:

The aim was to determine whether long-term nutritional protein supplements and exercise administered to counteract age-related loss of muscle mass in healthy elderly subjects benefits glucose regulation.

Methods:

164 healthy elderly subjects were recruited to complete 52 weeks of either nutritional supplementation (30 g carbohydrate (CARB),20 g whey (WHEY) or collagen (COLL) hydrolysate added 10 g sucrose) or training interventions (low (LITW) or high (HRTW) load resistance exercise training, both supplemented with 20 g whey hydrolysate and 10 g sucrose). Before and after the intervention period, the subjects completed a DXA scan and an oral glucose tolerance test (OGTT). Blood samples were collected before, at 45 min and 120 min after intake of 75 g glucose (OGTT). The results were analyzed both as intention to treat (ITT) and per protocol (PP).

Results:

In the nutritional supplementation arm (CARB,COLL,WHEY) general increases in body weight, BMI, fat%, and HbA1c in both ITT and PP analyses were observed as an effect of time. Furthermore, glucose area under the curve (AUC) and 120 min glucose level increased while insulin AUC decreased in the ITT analysis after one year in the nutritional supplementation arm. In the training arm (WHEY,HRTW,LITW) we observed an increase in appendicular skeletal muscle index (ASMI), HbA1c, and 120min glucose concentrations in ITT analysis, whereas LBM, ASMI, HbA1c, glucose AUC and 120min glucose increased in the PP analysis.

Conclusion:

The present results showed no improvement on the glucose regulation after 52 weeks of whey, collagen, or carbohydrate supplementation or whey combined with different training interventions in healthy and physically active older adults. The nutritional supplements resulted in increases in body weight, BMI and fat %. Therefore, recommending protein supplements to healthy older adults could have negative consequences.

Keywords:

Glucose metabolism - aging – nutritional supplementation – training – body composition

Introduction

Glucose tolerance is known to decrease with advancing age[1], and at age 75-80 years ~25% of all older adults meet the criteria of type 2 diabetes[2]. Inactivity, increasing adipose tissue, and low grade inflammation are factors contributing to increased insulin resistance with aging[3]. Skeletal muscle is the primary tissue of insulin-dependent glucose uptake and a reduced muscle mass could therefore further impair the insulin sensitivity[4]. Thus, sarcopenia defined as age-related loss of muscle mass[5] might be closely related to insulin resistance and type 2 diabetes[6].

Two strategies currently acknowledged to counteract the sarcopenic processes are higher protein intake[7] and heavy-load resistance exercise.

The positive effects of a high protein intake, is mainly seen when compared to intakes in the lowest quintile of protein intake[8], and may be dependent on the protein source[9]. In this perspective, the quality of the protein has been in focus. One way of evaluating protein quality is by using the digestible indispensable amino acid score (DIAAS) where the digestibility and the amino acid composition are the primary factors determining the score[10]. Based on DIAAS, milk proteins are high quality proteins particularly the whey fraction which is both easily digested and absorbed and has a favorable amino-acid composition with regards to the composition of essential amino acids (EAA) in comparison to other proteins[11]. With benefits to older people, EAA alone have been shown to stimulate protein synthesis[12] and supplementation with EAA reduces insulin resistance in both elderly subjects with sarcopenia[13] and with type 2 diabetes[14]. The effect is suggested to be mediated by an increased muscle protein synthesis and utilization of amino acids for restoring muscle mass and an increasing energy expenditure sensitizing the muscle to insulin and increasing the glucose uptake. Further, some essential amino acids have insulinotrophic effects enhancing the insulin secretion[15] reducing the prevalence of hyperglycemia in people with long-standing type 2 diabetes[16]. Although there is evidence of positive effects of supplementing elderly with high quality

protein or EAA on glucose tolerance the long-term effect of supplementation on glucose tolerance remains un-investigated.

Resistance exercise training (RT) is another approach to increase lean body mass in elderly[17,18] and muscle activity enhances insulin sensitivity[19–22] emphasizing the high potential for short-term RT +to lower insulin resistance in the aged population. Even though heavy resistance training is without doubt the most efficient way to stimulate muscle growth, the tolerability and adherence is considered to challenge long-term feasibility[23]. Adherence to physical activities that are convenient and exercises of moderate intensity are associated with increased participation[24] and some evidence seems to exist for the beneficial effect of moderate resistance training regimes in stimulating muscle growth as intention-to-treat analysis[25] in elderly men. While light intensity training such as aerobic training improves fasting plasma glucose levels, decreasing both glucose- and insulin AUC during an OGTT[26], it is not known whether long-term resistance training with light-load intensity impacts glucose tolerance.

This study investigates the long-term (52 weeks) effect of protein supplements with different quality and different exercise regimens on glucose metabolism during an OGTT, fasting glucose metabolism biomarkers and body composition measured with DXA scans. We hypothesized that the glucose metabolism would improve when comparing supplementation of proteins of different quality with an isocaloric carbohydrate drink. Furthermore, we hypothesized that low intensity or high resistance training plus the high-quality whey protein would lead to further improvements compared to whey protein alone on the glucose metabolism in the elderly. We performed intention to treat (ITT) and per protocol (PP) analysis to test the effect of both the different recommendations and interventions.

Methods

Study design

The study protocol was approved by The Danish Regional Committees of the Capital Region on the 4th of July 2013 (number H-4-2013-070) and all participants gave their written informed consent in accordance with the Declaration of Helsinki II. The results presented in this paper are part of the CALM study[27] registered at clinicaltrials.gov journal number: NCT02115698

Participants were randomized into one of five intervention groups consisting of nutritional supplements or nutritional supplements and exercise comprising the two analytical arms presented in this paper. The nutritional arm consists of carbohydrate supplementation (CARB; 20 g maltodextrin + 10 g sucrose), collagen supplementation (COLL; 20 g bovine collagen protein hydrolysate (Atpro-200) + 10 g sucrose), and whey supplementation (WHEY; 20 g whey protein hydrolysate (LACPRODAN DI-9224, + 10 g sucrose). All supplements were packaged by Arla Foods Ingredients Group P/S, Viby J, Denmark. The training arm includes all groups supplemented with the whey supplements, either without training (WHEY; the same group used in the supplementation arm), with light load home-based resistance training four times weekly (LITW), or with center-based heavy resistance training three times weekly (HRTW). The training protocols are described in detail elsewere[27]. The supplements were consumed twice daily, in the morning and at midday, just before or during meals. On training days, participants in the exercise groups were asked to ingest one of the supplements immediately after exercise. Adherence to nutritional supplements and exercise was registered in diaries by the participants. Per-protocol analysis was made on subjects with >75% supplements adherence corresponding to 1.5 or more drinks per day

and 2 or more training sessions per week for HRTW and 3 or more training sessions per week for LITW training adherence.

Assessments

Body composition was determined by whole-body DXA performed in a Lunar DPX-IQ DXA scanner (GE Healthcare, Chalfont St. Giles, UK), and analyzed with the enCORE v.16 software package (Lunar iDXA; GE Medical Systems). The investigator was blinded to participant, time-point and intervention. When the DXA-scans were performed at baseline and 12-month, the participants arrived in the morning having refrained from solid foods from 21:00 the day before, and the scanning was performed between 08:00 and 10:00. At the 12-month time point DXA scans were performed between 48 hours and 14 days after the last training session. Immidiately after the subjects completed the DXA-scan they underwent the OGTT.

The OGTT was performed as described in Bechshøft et al. 2016[27]. An antecubital venous catheter was inserted, and a set of basal venous blood sample was drawn. Then 75 g of anhydrous glucose dissolved in 250 ml of tap water was administered and 2 sets of blood samples were drawn in K3-EDTA vials at 45 and 120 min after the glucose consumption, respectively. One set of the plasma samples was analyzed for glucose, HbA1c and proinsulin C-peptide at the Department of Clinical Biochemistry, Bispebjerg Hospital. HbA1c and proinsulin C-peptide were only measured in the basal sample. The other set of samples was cooled on ice for 15 min and then centrifuged for 10 min at 3172 g at 4°C to isolate the plasma and aliquots was stored at -80°C for insulin analysis.

Analyses

The DXA scans were auto segmented by the software and the regions of interest (ROI) were secondly adjusted by the blinded investigator according to predefined anatomically fixed points. For

the upper body the most distal part of the chin, the armpit and caput humeri were used to separate the head, torso and arms. For the lower body a triangle was placed upside down with the two proximal corners being parallel with the most proximal part of the hip and the distal tip placed so that the lines followed the lateral part of ramus ischiadicum in order to separate the legs. The appendicular skeletal muscle mass index (ASMI) were calculated as appendicular lean mass/height^2.

Insulin was measured using the Insulin ELISA kit (ALPCO Diagnostics, Windham, NH, USA). 200 µl plasma was converted to serum by adding 5 µl 0.2 unit/µl thrombin solution (T6884, Sigma Aldrich, MO, USA). Samples were vortexed, left for 10 min at room temperature and spun at 1600 g for 10 min. The supernatant was collected for the insulin analysis. The ELISA was performed according to the manufacture's instruction. The kit was equilibrated to room temperature and 25 µl of each standard, control, and sample were loaded into each well followed by 100 µl Detection Antibody. All samples were loaded in duplicates and samples from the same subject were loaded on the same plate. Samples from subjects in the different groups were randomized on each plate. Standards and controls were loaded in triplicates. The plates were incubated for 1 h at room temperature, shaken at 800 rpm on a microplate shaker followed by 6 times washing. Thereafter, 100 µl of TMB substrate was added to each well to activate the fluorophore, the plate was incubated for further 15 min at room temperature shaking at 800 rpm on a microplate shaker. 100 µl of stop solution was added to each well and the plate was analyzed immediately after at 450 nm in a microplate reader (Multiskan FC, Fisher Scientific, MA, USA). AUCs of the glucose and insulin curves were calculated by the trapezoid method. HOMA-IR and Matsuda indices were calculated based on the obtained glucose and insulin values. HOMA-IR was calculated using the equation by Matthews et al[28]: $HOMA\ IR = \frac{Fasting\ insulin\ x\ fasting\ glucose}{22.5}$

where insulin concentration is in µIU/ml and fasting glucose is in mmol/L.

Matsuda index for timepoints 0, 45 and 120 min was calculated using the equation from Matsuda and DeFronzo 1999[29]:

$$\frac{10{,}000}{\sqrt{(Insulin_0 * (Glucose_0 * 18) * (mean\ OGTT\ insulin\ concentration\ * (mean\ OGTT\ glucose\ concentration)))}}$$

Matusda index for timepoints 0 and 120 min was calculated using the equation from DeFronzo and Matsuda 2010[30]:

$$\frac{10{,}000}{\sqrt{(Insulin_0 * (Glucose_0 * 18) * (Glucose_{120} * 18)\ * Insulin_{120}))}}$$

Where insulin is in μIU/mL and glucose is mmol/L. The glucose concentration is multiplied with 18 to get the glucose concentration in mg/dL, and the mean insulin- and mean glucose concentration is calculated by dividing the AUC during the OGTT by 120. Our Matsuda Index is calculated based on an oral glucose tolerance test with only three timepoints (0, 45min and 120min) and it should be noted that the obtained Matsuda values should only be compared to values calculated based on the same time points[31].

Associations

In the intention to treat group, changes in any of the measured parameters were associated with changes in HbA1c, insulin AUC, and glucose AUC. Furthermore as a cross sectional at baseline, associations between the measured parameters and HbA1c, insulin AUC, and glucose AUC was performed. Therefore, all subjects are pooled in this analysis. Due to multiple testing a p-value below 0.001 was considered significant.

Statistical analyses

All insulin, HOMA-IR, proinsulin C-peptid and Matsuda data were log2-transformed to obtain normal distribution. Data was tested for normality by the Shapiro-Wilks normality test and for equal variance by the Brown-Forsythe test. Data was analyzed using two-way ANOVA with repeated measurements for time. Significant effects of group, time or group x time interaction were followed by the post-hoc Holm-Sidak test. P-values below 0.05 were considered significant and trends are reported for p-values between 0.05 and 0.1. For the cross-sectional associations, Bonferroni Correction for multiple testing was applied; $\alpha/45 = 0.05 / 45 = 0.001$. Therefore, a p-value below 0.001 was considered significant. All tests were performed in Prism (GraphPad, CA, USA) and all data are presented as means ± standard error (SE).

Results

To the study, 208 participants were randomized of which 184 participants completed the 12-month tests (further information see Mertz et al(UNPUBLISHED). The intention-to-treat (ITT) analysis included the participants (n=164) who completed the OGTT before and after the trial. The per protocol (PP) analysis included the participants (n=100) with self-reported adherence to the supplementation $\geq 75\%$ and an average training adherence ≥ 2.0 for the HRTW group and ≥ 3.0 for the LITW group. 34 out of the 164 participants were excluded from the PP analysis due to lack of registrations and 30 participants were excluded due to lack of adherence to the respective interventions.

Nutrition intervention arm (body composition):

For the ITT analysis, the nutritional supplementation groups increased body weight (BW) (p=0.008) (WHEY 0.5±0.4 kg, COLL 0.5±0.3 kg, CARB 1.2±0.6 kg) and BMI (p=0.008) (WHEY 0.2±0.1

246 kg/m^2, COLL 0.2±0.1 kg/m^2, CARB 0.4±0.2 kg/m^2) as a main effect of time with no group x time
247 interaction after 12 months of nutritional supplements (Table 1). The same was true for the per
248 protocol (PP) analysis BW (p=0.040) (WHEY 0.2±0.4 kg, COLL 0.3±0.4 kg, CARB 1.3±0.6 kg)
249 and BMI (p=0.034) (WHEY 0.1±0.1 kg/m^2, COLL 0.1±0.1 kg/m^2, CARB 0.4±0.2 kg/m^2) (Table 3).
250 Furthermore, there was an increase in fat% in ITT (p=0.003) (WHEY 0.6±0.3%, COLL 0.5±0.3%,
251 CARB 0.4±0.2%) and PP (p=0.017) (WHEY 0.5±0.4%, COLL 0.4±0.4%, CARB 0.8±0.4%)
252 analysis as a main effect of time and no change in lean body mass. Again, there was no significant
253 difference between the groups. There were no changes in ASMI or daily step count neither between
254 groups nor over time (Table 1, Table 3).
255
256 **Training intervention arm (body composition):**
257 Body mass, BMI and fat% did not change significantly over time in the training intervention arm in
258 either the ITT or PP analysis (Table 2, Table 4). We found an increase in lean body mass over time
259 in the PP analysis (p=0.033) (WHEY 0.1±0.2 kg, LITW 0.3±0.3 kg, HRTW 0.6±0.3 kg) and no
260 effect in the ITT analysis. The ITT analysis of ASMI showed an effect of time in the HRTW group
261 only (p=0.001) (HRTW 0.15±0.04 kg/m^2) (Table 2). PP analysis showed a main effect of time on
262 ASMI (p=0.001) (WHEY 0.04±0.04 kg/m^2, LITW 0.12±0.05 kg/m^2, HRTW 0.18±0.06 kg/m^2) and
263 no differences between the groups (Table 4). The daily step count did not change over time (Table
264 2, Table 4).
265
266 **Nutrition intervention arm (fasting blood samples):**
267 In the ITT analysis, fasting plasma glucose and insulin concentrations were not different before and
268 after the 12 months nutritional interventions and there was no difference between the groups.
269 Fasting proinsulin C-peptide did not change either. In contrast, HbA1c increased significantly with

a main effect of time (p=0.001) (WHEY 0.1±0.4 mmol/mol, COLL 1.1±0.4 mmol/mol, CARB 1.2±0.6 mmol/mol) with no difference between the groups (Table 1). The PP analysis showed no changes in fasting plasma glucose, insulin concentration, or proinsulin C-peptide (Table 3). Like in the ITT analysis, HbA1c showed a significant main effect of time (p=0.002) (WHEY 0.4±0.5 mmol/mol, COLL 1.2±0.5 mmol/mol, CARB 1.7±0.5 mmol/mol) (Table 3). Again, we saw no differences between the groups (Table 3).

Training intervention arm (fasting blood samples):

In the ITT analysis, the fasting plasma glucose, plasma insulin, and proinsulin C-peptide concentrations did not change after 12 months nutritional and exercise interventions (Table 2). HbA1c concentration increased as a main effect of time (p=0.005) (WHEY 0.1±0.4 mmol/mol, LITW 1.4±0.5 mmol/mol, HRTW 0.5±0.3 mmol/mol) (Table 2). The PP analysis showed no changes in fasting plasma glucose, plasma insulin or proinsulin C-peptide concentrations after the interventions (Table 4). There was a main effect of time in the HbA1c concentrations (p=0.023) (WHEY 0.4±0.5 mmol/mol, LITW 1.1±0.5 mmol/mol, HRTW 0.6±0.5 mmol/mol) (Table 4).

Nutrition intervention arm (oral glucose tolerance test):

In the ITT analysis, the plasma glucose AUC during the OGTT was not different between the nutritional supplement groups but the plasma glucose AUC increased as a main effect of time (p=0.049) (WHEY 21±15 mmol/Lx120min, COLL 33±20 mmol/Lx120min, CARB 18±28 mmol/Lx120min) after 12 months of nutritional supplements (Table 1). Furthermore, the plasma insulin AUC decreased as a main effect of time (p=0.032) (WHEY -691±414 uIU/mLx120min, COLL 228±390 uIU/mLx120min, CARB -121±370 uIU/mLx120min) with no differences between the groups (Table 1). The HOMA-IR index and the Matsuda index did not change after 12 months

of nutritional supplementation (Table 1). The PP analysis showed no changes over time in the plasma glucose AUC, but a trend (p=0.051) for a main effect of time for a decrease in the insulin AUC (Table 3). The PP analysis showed no effect of the interventions on the HOMA-IR or Matsuda indexes (Table 3).

Training intervention arm (oral glucose tolerance test):

The ITT analysis of the plasma glucose and insulin AUC during the OGTT showed no effect of the 12 months interventions (Table 2). The HOMA-IR index and the Matsuda index did not change either (Table 2). The PP analysis showed a significant main effect of time on the glucose AUC (p=0.037) (WHEY 27±20 mmol/Lx120min, LITW 29±16 mmol/Lx120min, HRTW 35±36 mmol/Lx120min). For the glucose 120 min concentration (p=0.019) (WHEY 0.4±0.3 mmol/L, LITW 0.5±0.3 mmol/L, HRTW 0.4±0.3 mmol/L) a trend for a main effect of time was seen as a decrease in the insulin AUC (p=0.06) (Table 4). Interestingly, the HOMA-IR and the Matsuda indexes did not change after the interventions in the PP analysis (Table 4).

Correlations (BMI, ASMI, VAT, Fat% and Daily Steps):

We also conducted an exploratory correlation analysis of different health parameters (BMI, ASMI, visceral adipose tissue%(VAT), fat%, Steps/day) with HbA1c, Insulin AUC and Glucose AUC, both as a delta between 0 and 12 month (suppl. 1) and at baseline (suppl. 2, 3 and 4). We found no correlation between changes in BMI, ASMI, VAT%, Fat%, Steps/day and changes within HbA1c, Insulin AUC and Glucose AUC after the 12 months of intervention (suppl. 1). At baseline, HbA1c was only significantly correlated with VAT in females (p=0.0004), Insulin AUC were correlated with both BMI and fat% for both males and females (p<0.0001, p<0.0001), and with VAT

317 ($p<0.0001$) in males. We found no significant correlation between Glucose AUC and the measured
318 parameters.
319
320 ## Discussion
321 The current study investigated the effects of one year of different nutritional and exercise
322 interventions on HbA1c and glucose metabolism. Both an intention to treat (ITT) and per protocol
323 (PP) analysis was applied allowing us to test the effect of a recommendation and an actual
324 intervention of nutrient/protein supplementation with or without training on the glucose in healthy
325 elderly. The main finding is that long-term protein supplementation does not affect glucose
326 tolerance (HbA1c and insulin secretion and blood glucose during a glucose tolerance test)
327 differently than long-term isocaloric carbohydrate in healthy elderly individuals. Moreover, there
328 was no difference in HbA1c and glucose regulation when adding long-term training on top of whey
329 supplements in healthy elderly adults.
330 In general, the HbA1c levels increased with ~1mmol·mol^{-1} (gross avr of all groups) after 12 months
331 of intervention. Looking at the numerical changes in the ITT to PP analysis, the numerical increase
332 in HbA1c were higher in the PP analysis in the WHEY (0.1 and 0.4 mmol/mol, respectively),
333 COLL (1.1 and 1.2 mmol/mol, respectively) CARB (1.2 and 1.7 mmol/mol, respectively) and
334 HRTW (0.5 and 0.6 mmol/mol, respectively) suggesting an effect of the intervention rather than
335 time on the HbA1c levels.
336 We would have expected that the increased protein intake would regulate the overall daily caloric
337 intake due to a satiating effect[32]. However, we observed a significant increase in fat% in the
338 nutritional intervention arm where BW and BMI also increased significantly. In the training arm, no
339 significant changes were observed, which seemed to be driven by the HRTW group since both
340 WHEY and LITW nummerically increased the fat%, BW and BMI. Further, HRTW is the only

group with numerical reduction with respect total fat% compared to WHEY and LITW. Furthermore, the significant effect of time regarding the increase in LBM and ASMI in the PP analysis of the training intervention arm, suggests that the HRTW intervention has a positive effect on the body composition compared to the other interventions. However, this change in body composition in HRTW is not reflected in the HbA1c values of neither the ITT nor PP analysis. Fasting glucose was not affected by any of the interventions, but the 120 min glucose AUC in the OGTT tended to increase in all groups. Contrary to our hypothesis, the glucose tolerance was in general not positively affected by the nutritional or training interventions. There are several potential reasons for the lack of positive changes in the glucose metabolism; inclusion of healthy, independent older adults with adequate protein intake of ~1.1 $g \cdot kg^{-1} \cdot day^{-1}$[33] and general high activity levels represented by a daily step count above 10.000 steps. Shorter exercise interventions in middle-aged and older men did not affect the glucose tolerance, but only increased insulin sensitivity[20–22]. In contrast to our study, these subjects did not receive any nutritional supplementation. The addition of protein supplementation with the addition of 10 g of glucose to the training groups in our study might explain why we see a decrease in glucose tolerance as reflected by an increase in glucose AUC response to the OGTT. This decrease in glucose tolerance was further supported by an increase in 120 min glucose levels for both ITT and PP analysis of the training intervention arm and ITT analysis of the nutritional intervention arm. Thus, while the increase in LBM in the PP analysis of the training arm was hypothesized to improve glucose tolerance, this appeared inadequate to counteract the impairing effect of concomitant protein supplementation.

The insulin response to an OGTT seemed to change after the 12-month intervention, irrespective of intervention arms. All groups receiving whey protein had a numerical decrease in mean insulin AUC after the interventions, which could be explained by whey protein supplementation being

suggested to have insulin lowering effect[34]. No changes in glucose AUC or insulin AUC in the ITT analysis of the training intervention arm compared to the PP analysis was observed, indicating an effect of the intervention rather than an effect of age on these parameters.

Changes in the insulin response to an OGTT can be affected by insulin clearance[35] or beta cell function[36]. Interestingly, the changes in insulin sensitivity was not translated into positive effects on the glucose tolerance in general, and a decrease in insulin AUC and an increase in glucose AUC may be an early sign of decreased beta cell function and insulin secretion/production[37]. C-peptide concentrations could have revealed an indication of beta cell function as the insulin levels also can be affected by the rate of clearance[38]. Unfortunately, we did not measure the proinsulin C peptide levels during the OGTT. The Matsuda Index and HOMA-IR values did not change after the interventions indicating no changes in the general glucose metabolism. Again, it should be mentioned that the research participants were healthy and relatively active. Whether similar effects will be present in T2D patients, elderly with inadequate protein intake or low physical activity or sarcopenic elderly remain to be determined. At least, results from this type of individuals should not be extrapolated to other population groups.

Strengths of the present study are the high number of participants and a long intervention period. Furthermore, in the present study both ITT and PP analyses are being reported allowing for analysis of both the recommendation and the actual effects of the interventions. A limitation of the present study is the lack of abilityto distinguish between the effect of time and the supplement itself as we did not include a group receiving nothing . Further, the power calculation was made based on another outcome of this study[27].

We also exploratorily analyzed the data for any associations between changes in glucose metabolism, body composition, physical activity, and fat metabolism from a cross sectional perspective (see supplemental figures 1-4). No association between changes in BMI, ASMI, fat%,

VAT and daily steps after correcting for multiple testing was seen (supplemental fig. 1A). A general difference between males and females can be seen on the baseline cross sectional supplemental figures (supplemental fig. 2-4). Associations between BMI and HbA1c (r^2=0.056, p=0.0006, supplemental fig. 2A), VAT and Glucose AUC (r^2=0.129, p<0.0001, supplemental fig. 4C), Fat% and insulin AUC (r^2=0.185, p<0.0001, supplemental fig. 3D) were observed. This suggests that the changes in BMI, fat% and VAT rather than lean mass affect changes in glucose metabolism. This contrasts with what have previously been proposed[13,14], namely that improvements in glucose homeostasis might be due to an increase in lean body mass. Therefore, one should aim for a reduction in fat%, when trying to improve the glucose metabolism. This support weight loss and increased physical activity as the predominant approach to treat type 2 diabetes in older adults already being rather physically active[39].

In summary, we found deteriorations in HbA1c and glucose tolerance after one year in healthy older adults, irrespective of supplementing with high or low protein quality or isocaloric carbohydrate or conducting resistance exercise training with heavy or low intensity. In conclusion, protein supplementation, irrespective of quality and additional training, does not affect glucose metabolism differently than carbohydrate supplementation.

Acknowledgement

Author contributions: Bülow and Zillmer had full access to all the data in the present study and take responsibility for the integrity of the data analysis. Study design: Bechshøft and Holm. Acquisition of data: Bülow, Bechshøft, Højfeldt and Zillmer. Analysis and interpretation of data: Bülow, Zillmer, Schjerling and Holm. Drafting of manuscript: Bülow and Zillmer. All authors critically revised the manuscript and had the opportunity to comment on the interpretation of results.

Statistical analysis: Bülow, Zillmer Schjerling and Holm. Obtained funding: Bülow, Bechshøft and Holm.

Reference list

1. DeFronzo RA. Glucose intolerance and aging. *Diabetes Care*. 1981;4(4):493-501. http://www.ncbi.nlm.nih.gov/pubmed/7049632. Accessed February 20, 2019.
2. Kirkman MS, Briscoe VJ, Clark N, et al. Diabetes in Older Adults. *Diabetes Care*. 2012;35(12):2650-2664. doi:10.2337/dc12-1801
3. Lee PG, Halter JB. The Pathophysiology of Hyperglycemia in Older Adults: Clinical Considerations. *Diabetes Care*. 2017;40(4):444-452. doi:10.2337/dc16-1732
4. Defronzo RA, Tripathy D. Skeletal Muscle Insulin Resistance Is the Primary Defect in Type 2 Diabetes. *Diabetes Care*. 2009;32:S157-163. doi:10.2337/dc09-S302
5. Bülow J, Ulijaszek SJ, Holm L. Rejuvenation of the term sarcopenia. *J Appl Physiol*. 2019;126(1):255-256. doi:10.1152/japplphysiol.00400.2018
6. Wolfe RR. The underappreciated role of muscle in health and disease. *Am J Clin Nutr*. 2006;84(3):475-482. http://www.ncbi.nlm.nih.gov/pubmed/16960159.
7. Norton C, Toomey C, McCormack WG, et al. Protein Supplementation at Breakfast and Lunch for 24 Weeks beyond Habitual Intakes Increases Whole-Body Lean Tissue Mass in Healthy Older Adults. *J Nutr*. 2016;146(1):65-69. doi:10.3945/jn.115.219022
8. Houston DK, Nicklas BJ, Ding J, et al. Dietary protein intake is associated with lean mass change in older, community-dwelling adults: The Health, Aging, and Body Composition (Health ABC) study. *Am J Clin Nutr*. 2008;87(1):150-155. doi:10.1093/ajcn/87.1.150
9. Berrazaga I, Micard V, Gueugneau M, Walrand S. The role of the anabolic properties of plant-versus animal-based protein sources in supporting muscle mass maintenance: a critical review. *Nutrients*. 2019;11(8). doi:10.3390/nu11081825
10. *Food and Agriculture Organization of the United Nations.Dietary Protein Quality*

Evaluation in Human Nutrition. Report of an FAO Expert Consultation.; 2011. http://www.nutrinfo.com/biblioteca/libros_digitales/fao_protein_quality.pdf. Accessed March 17, 2020.

11. *Joint FAO/WHO Expert Consultation on Protein Quality Evaluation (1989: Bethesda, Maryland), World Health Organization & Food and Agriculture Organization of the United Nations. (1991). Protein Quality Evaluation : Report of the Joint FAO/WHO Expert Co*. https://apps.who.int/iris/handle/10665/38133.

12. Cuthbertson D, Smith K, Babraj J, et al. Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *FASEB J*. 2005;19(3):422-424. doi:10.1096/fj.04-2640fje

13. Solerte SB, Gazzaruso C, Bonacasa R, et al. Nutritional Supplements with Oral Amino Acid Mixtures Increases Whole-Body Lean Mass and Insulin Sensitivity in Elderly Subjects with Sarcopenia. *Am J Cardiol*. 2008;101(11 SUPPL.):69E-77E. doi:10.1016/j.amjcard.2008.03.004

14. Solerte SB, Gazzaruso C, Schifino N, et al. Metabolic effects of orally administered amino acid mixture in elderly subjects with poorly controlled type 2 diabetes mellitus. *Am J Cardiol*. 2004;93(8A):23A-29A. doi:10.1016/j.amjcard.2003.11.006

15. Manders RJ, Little JP, Forbes SC, Candow DG. Insulinotropic and muscle protein synthetic effects of branched-chain amino acids: Potential therapy for type 2 diabetes and sarcopenia. *Nutrients*. 2012;4(11):1664-1678. doi:10.3390/nu4111664

16. Manders RJF, Praet SFE, Meex RCR, et al. Protein hydrolysate/leucine co-ingestion reduces the prevalence of hyperglycemia in type 2 diabetic patients. *Diabetes Care*. 2006;29(12):2721-2722. doi:10.2337/dc06-1424

17. Peterson MD, Sen A, Gordon PM. Influence of resistance exercise on lean body mass in

aging adults: a meta-analysis. *Med Sci Sports Exerc*. 2011;43(2):249-258. doi:10.1249/MSS.0b013e3181eb6265

18. Tracy BL, Ivey FM, Hurlbut D, et al. Muscle quality. II. Effects of strength training in 65- to 75-yr-old men and women. *J Appl Physiol*. 1999;86(1):195-201. doi:10.1152/jappl.1999.86.1.195

19. Iglay HB, Thyfault JP, Apolzan JW, Campbell WW. Resistance training and dietary protein: effects on glucose tolerance and contents of skeletal muscle insulin signaling proteins in older persons. *Am J Clin Nutr*. 2007;85(4):1005-1013. doi:10.1093/ajcn/85.4.1005

20. Miller JP, Pratley RE, Goldberg AP, et al. Strength training increases insulin action in healthy 50- to 65-yr-old men. *J Appl Physiol*. 1994;77(3):1122-1127. doi:10.1152/jappl.1994.77.3.1122

21. Biensø RS, Olesen J, Gliemann L, et al. Effects of Exercise Training on Regulation of Skeletal Muscle Glucose Metabolism in Elderly Men. *Journals Gerontol Ser A*. 2015;70(7):866-872. doi:10.1093/gerona/glv012

22. Joseph LJ, Farrell PA, Davey SL, Evans WJ, Campbell WW. Effect of resistance training with or without chromium picolinate supplementation on glucose metabolism in older men and women. *Metabolism*. 1999;48(5):546-553. doi:10.1016/s0026-0495(99)90048-3

23. Daley AJ, Maynard IW. Preferred exercise mode and affective responses in physically active adults. *Psychol Sport Exerc*. 2003;4(4):347-356. doi:10.1016/S1469-0292(02)00018-3

24. King AC. Interventions to Promote Physical Activity by Older Adults. *Journals Gerontol Ser A Biol Sci Med Sci*. 2001;56(Supplement 2):36-46. doi:10.1093/gerona/56.suppl_2.36

25. Holm L, Reitelseder S, Pedersen TG, et al. Changes in muscle size and MHC composition in response to resistance exercise with heavy and light loading intensity. *J Appl Physiol*. 2008;105(5):1454-1461. doi:10.1152/japplphysiol.90538.2008

26. Slentz CA, Bateman LA, Willis LH, et al. Effects of exercise training alone vs a combined exercise and nutritional lifestyle intervention on glucose homeostasis in prediabetic individuals: a randomised controlled trial. *Diabetologia*. 2016;59(10):2088-2098. doi:10.1007/s00125-016-4051-z

27. Bechshøft RL, Reitelseder S, Højfeldt G, et al. Counteracting Age-related Loss of Skeletal Muscle Mass: a clinical and ethnological trial on the role of protein supplementation and training load (CALM Intervention Study): study protocol for a randomized controlled trial. *Trials*. 2016;17(1):397. doi:10.1186/s13063-016-1512-0

28. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28(7):412-419. http://www.ncbi.nlm.nih.gov/pubmed/3899825. Accessed February 21, 2019.

29. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care*. 1999;22(9):1462-1470. http://www.ncbi.nlm.nih.gov/pubmed/10480510. Accessed February 20, 2019.

30. DeFronzo RA, Matsuda M. Reduced time points to calculate the composite index. *Diabetes Care*. 2010;33(7). doi:10.2337/dc10-0646

31. DeFronzo RA, Matsuda M. Reduced time points to calculate the composite index. *Diabetes Care*. 2010;33(7):e93. doi:10.2337/dc10-0646

32. Weigle DS, Breen PA, Matthys CC, et al. A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations. *Am J Clin Nutr*. 2005;82(1):41-48. doi:10.1093/ajcn.82.1.41

33. Rønnow Schacht S, Vendelbo Lind M, Bechshøft RL, et al. Investigating Risk of Suboptimal

513 Macro and Micronutrient Intake and Their Determinants in Older Danish Adults with
514 Specific Focus on Protein Intake-A Cross-Sectional Study. *Nutrients*. 2019;11(4).
515 doi:10.3390/nu11040795
516 34. Graf S, Egert S, Heer M. Effects of whey protein supplements on metabolism. *Curr Opin*
517 *Clin Nutr Metab Care*. 2011;14(6):569-580. doi:10.1097/MCO.0b013e32834b89da
518 35. Bojsen-Møller KN, Lundsgaard A-M, Madsbad S, Kiens B, Holst JJ. Hepatic Insulin
519 Clearance in Regulation of Systemic Insulin Concentrations-Role of Carbohydrate and
520 Energy Availability. *Diabetes*. 2018;67(11):2129-2136. doi:10.2337/db18-0539
521 36. Kahn SE, Larson VG, Schwartz RS, et al. Exercise training delineates the importance of B-
522 cell dysfunction to the glucose intolerance of human aging. *J Clin Endocrinol Metab*.
523 1992;74(6):1336-1342. doi:10.1210/jcem.74.6.1592879
524 37. Abdul-Ghani MA, Tripathy D, DeFronzo RA. Contributions of beta-cell dysfunction and
525 insulin resistance to the pathogenesis of impaired glucose tolerance and impaired fasting
526 glucose. *Diabetes Care*. 2006;29(5):1130-1139. doi:10.2337/diacare.2951130
527 38. Leighton E, Sainsbury CA, Jones GC. A Practical Review of C-Peptide Testing in Diabetes.
528 *Diabetes Ther*. 2017;8(3):475. doi:10.1007/S13300-017-0265-4
529 39. Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2
530 diabetes with lifestyle intervention or metformin. *N Engl J Med*. 2002;346(6):393-403.
531 doi:10.1056/NEJMoa012512
532
533

Table 1:

Nutrition arm ITT	WHEY, N=39 (22 males, 17 females)			COLL, N=39 (23 males, 16 females)			CARB, N= 29 (15 males, 14 females)			ANOVA p-value		
	0 M	12 M	Delta change, number (%)	0 M	12 M	Delta change, number (%)	0 M	12 M	Change [absolute (%)]	Time	Group	Interaction
Subject characteristics												
Age (year)	69.9 ± 0.7			69.8 ± 0.6			69.7 ± 0.8				n.s.	
Body mass (kg)	74.0 ± 2.1	74.5 ± 2.2	0.5(0.7%) ± 0.4(0.5%)	75.1 ± 2.1	75.5 ± 2.1	0.5(0.7%) ± 0.3(0.5%)	73.6 ± 2.3	74.8 ± 2.5	1.2(1.5%) ± 0.6(0.8%)	0.008	n.s.	n.s.
BMI (kg/m2)	24.6 ± 0.6	24.8 ± 0.6	0.2(0.7%) ± 0.1(0.5%)	25.1 ± 0.7	25.2 ± 0.7	0.2(0.7%) ± 0.1(0.5%)	25.4 ± 0.7	25.8 ± 0.8	0.4(1.5%) ± 0.2(0.8%)	0.008	n.s.	n.s.
Fat mass (%)	32.0 ± 1.2	32.6 ± 1.2	0.6(2.3%) ± 0.3(1.1%)	30.8 ± 1.4	31.2 ± 1.5	0.5(1.6%) ± 0.3(1.1%)	31.6 ± 1.7	32.4 ± 1.8	0.8(2.7%) ± 0.4(1.2%)	0.003	n.s.	n.s.
Lean mass (kg)	48.6 ± 1.4	48.5 ± 1.4	-0.1(-0.1%) ± 0.2(0.3%)	50.0 ± 1.4	50.0 ± 1.4	-0.1(-0.1%) ± 0.2(0.3%)	48.4 ± 1.4	48.5 ± 1.5	0.1(0.2%) ± 0.2(0.4%)	n.s.	n.s.	n.s.
ASMI (kg/m2)	7.46 ± 0.18	7.46 ± 0.18	-0.002(0.02%) ± 0.003(0.4%)	7.66 ± 0.20	7.68 ± 0.20	0.01(0.2%) ± 0.03(0.4%)	7.63 ± 0.20	7.65 ± 0.21	0.02(0.3%) ± 0.03(0.4%)	n.s.	n.s.	n.s.
Daily steps	10515 ± 590	10003 ± 533	-343(-1.8%) ± 604(5.5%)	10607 ± 675	10450 ± 774	3(4.8%) ± 574(7.2%)	11404 ± 1008	10107 ± 863	2274(-10.7%) ± 1030(7.8%)	n.s.	n.s.	n.s.
Fasting blood analysis												
Fasting glucose (mmol/L)	5.4 ± 0.1	5.5 ± 0.1	0.12(2.3%) ± 0.08(1.5%)	5.5 ± 0.1	5.5 ± 0.1	-0.02(-0.2%) ± 0.07(1.3%)	5.5 ± 0.1	5.5 ± 0.1	0.04(0.8%) ± 0.08(1.4%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	4.9 ± 0.5	4.1 ± 0.4	-0.8(-2.7%) ± 0.3(6.9%)	4.9 ± 0.5	4.9 ± 0.6	0.1(4.3%) ± 0.3(6.9%)	4.4 ± 0.5	4.7 ± 0.6	0.2(3.8%) ± 0.3(7.0%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	758 ± 48	738 ± 44	-27(0.1%) ± 28(3%)	680 ± 35	703 ± 44	22(3%) ± 24(3%)	686 ± 47	715 ± 68	29(4%) ± 38(5%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	36.2 ± 0.6	36.3 ± 0.6	0.1(0.4%) ± 0.4(1.0%)	35.3 ± 0.5	36.5 ± 0.5	1.1(3.6%) ± 0.4(1.5%)	35.7 ± 0.4	36.9 ± 0.3	1.2(3.6%) ± 0.4(1.1%)	0.001	n.s.	n.s.
OGTT												
Glucose AUC (mmol/L x 120 min)	870 ± 22	891 ± 25	21(2.6%) ± 15(1.7%)	895 ± 25	928 ± 25	33(4.9%) ± 20(2.4%)	926 ± 24	944 ± 30	18(2.8%) ± 28(3.0%)	0.049	n.s.	n.s.
Glucose 45 min (mmol/L)	8.6 ± 0.3	8.7 ± 0.3	0.1(1.9%) ± 0.2(2.2%)	8.8 ± 0.3	9.0 ± 0.3	0.2(4.1%) ± 0.3(3.1%)	9.2 ± 0.3	9.3 ± 0.3	0.1(3.0%) ± 0.3(3.2%)	n.s.	n.s.	n.s.
Glucose 2h (mmol/L)	6.2 ± 0.2	6.6 ± 0.3	0.3(5.7%) ± 0.2(3.5%)	6.5 ± 0.3	7.1 ± 0.3	0.6(12.8%) ± 0.3(4.1%)	6.7 ± 0.2	7.0 ± 0.4	0.3(4.0%) ± 0.4(5.0%)	0.01	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	5066 ± 718	4375 ± 512	-691(-1.9%) ± 414(6.9%)	4645 ± 532	4873 ± 623	228(8.9%) ± 390(7.3%)	4047 ± 394	3926 ± 661	-121(-8.1%) ± 370(6.9%)	0.032	n.s.	n.s.
HOMA-IR	1.21 ± 0.14	1.05 ± 0.11	-0.15(-0.9%) ± 0.07(6.8%)	1.21 ± 0.14	1.23 ± 0.16	0.02(5.6%) ± 0.09(7.8%)	1.08 ± 0.13	1.19 ± 0.18	0.10(5.0%) ± 0.09(6.8%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	9.6 ± 1.0	10.1 ± 1.0	0.5(15.0%) ± 0.7(6.0%)	9.2 ± 0.9	9.5 ± 1.0	0.3(6.8%) ± 0.6(6.7%)	9.1 ± 0.9	10.1 ± 1.9	2.6(21.0%) ± 1.3(10.7%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	11.8 ± 1.4	10.7 ± 0.9	-1.1(3.4%) ± 0.9(6.0%)	11.3 ± 1.3	10.7 ± 1.5	-0.6(4.7%) ± 1.0(12.4%)	10.1 ± 0.9	12.6 ± 2.0	2.5(18.6%) ± 1.5(11.7%)	n.s.	n.s.	n.s.

Intention to treat analysis of the nutritional supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and no significant effects of the different interventions. COLL, collagen; CARB, carbohydrate;

Table 2:

Training arm ITT	WHEY, N=39 (22 males, 17 females)			LITW, N=28 (14 males, 14 females)			HRTW, N=29 (17 males, 12 females)			ANOVA p-value		
	0 M	12 M	Difference	0 M	12 M	Difference	0 M	12 M	Change [absolute (%)]	Time	Group	Interaction
Subject characteristics												
Age (year)	69.9 ± 0.7			70.6 ± 0.8			70.8 ± 0.6				n.s.	
Body mass (kg)	74.0 ± 2.1	74.5 ± 2.2	0.5(0.7%) ± 0.4(0.5%)	73.9 ± 1.9	74.7 ± 2.0	0.2(1.0%) ± 0.2(0.8%)	78.5 ± 2.6	78.7 ± 2.5	0.2(0.4%) ± 0.5(0.7%)	n.s.	n.s.	n.s.
BMI (kg/m2)	24.6 ± 0.6	24.8 ± 0.6	0.2(0.7%) ± 0.1(0.5%)	25.4 ± 0.6	25.6 ± 0.6	0.8(1.0%) ± 0.6(0.8%)	26.0 ± 0.7	26.1 ± 0.7	0.1(0.4%) ± 0.2(0.7%)	n.s.	n.s.	n.s.
Fat mass (%)	32.0 ± 1.2	32.6 ± 1.2	0.6(2.3%) ± 0.3(1.1%)	33.2 ± 1.4	33.7 ± 1.5	0.5(1.8%) ± 0.4(1.4%)	33.8 ± 1.3	33.7 ± 1.4	-0.1(-0.4%) ± 0.3(1.1%)	n.s.	n.s.	n.s.
Lean mass (kg)	48.6 ± 1.4	48.5 ± 1.4	-0.1(-0.1%) ± 0.2(0.3%)	48.0 ± 1.7	48.1 ± 1.7	0.1(0.3%) ± 0.2(0.4%)	50.3 ± 1.9	50.7 ± 1.9	0.4(0.8%) ± 0.2(0.4%)	n.s.	n.s.	n.s.
ASMI (kg/m2)	7.5 ± 0.2	7.5 ± 0.2	-0.002(0.02%) ± 0.003(0.4%)	7.55 ± 0.21	7.63 ± 0.22	0.08(1.1%) ± 0.05(0.6%)	7.72 ± 0.25	7.87 ± 0.26*	0.15(2.0%) ± 0.04(0.5%)	0.001	n.s.	0.02
Daily steps	10515 ± 590	10003 ± 533	-343(-1.8%) ± 604(5.5%)	10371 ± 679	9482 ± 476	-530(0.6%) ± 625(6.6%)	9599 ± 653	8982 ± 660	-595(-4.0%) ± 436(4.9%)	n.s.	n.s.	n.s.
Fasting blood analysis												
Fasting glucose (mmol/L)	5.4 ± 0.1	5.5 ± 0.1	0.12(2.3%) ± 0.08(1.5%)	5.6 ± 0.1	5.6 ± 0.1	-0.01(0.5%) ± 0.1(2.0%)	5.6 ± 0.1	5.7 ± 0.1	0.1(1.6%) ± 0.1(1.1%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	4.9 ± 0.5	4.1 ± 0.4	-0.8(-2.7%) ± 0.3(6.9%)	4.2 ± 0.4	4.6 ± 0.4	0.4(19.2%) ± 0.4(11.0%)	4.5 ± 0.5	4.4 ± 0.5	-0.01(4.4%) ± 0.4(7.3%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	758 ± 48	738 ± 44	-27(0.1%) ± 28(3%)	661 ± 41	711 ± 41	50(14.3%) ± 45(7.0%)	682 ± 43	714 ± 48	35(6.0%) ± 29(4.2%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	36.2 ± 0.6	36.3 ± 0.6	0.1(0.4%) ± 0.4(1.0%)	35.5 ± 0.6	36.9 ± 0.7	1.4(4.1%) ± 0.5(1.4%)	35.7 ± 0.5	36.2 ± 0.5	0.5(1.6%) ± 0.3(1.0%)	0.005	n.s.	n.s.
OGTT												
Glucose AUC (mmol/L x 120 min)	870 ± 22	891 ± 25	21(2.6%) ± 15(1.7%)	909 ± 28	933 ± 30	25(3.2%) ± 16(1.9%)	909 ± 25	922 ± 37	13(1.5%) ± 27(3.3%)	n.s.	n.s.	n.s.
Glucose 45 min (mmol/L)	8.6 ± 0.3	8.7 ± 0.3	0.1(1.9%) ± 0.2(2.2%)	8.6 ± 0.4	8.9 ± 0.4	0.2(4.7%) ± 0.3(3.5%)	8.9 ± 0.3	8.8 ± 0.4	-0.1(0.1%) ± 0.4(4.5%)	n.s.	n.s.	n.s.
Glucose 2h (mmol/L)	6.2 ± 0.2	6.6 ± 0.3	0.3(5.7%) ± 0.2(3.5%)	7.1 ± 0.3	7.4 ± 0.3	0.3(5.0%) ± 0.2(3.1%)	6.6 ± 0.3	7.0 ± 0.4	0.4(6.7%) ± 0.3(4.5%)	0.025	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	5066 ± 718	4375 ± 512	-691(-1.9%) ± 414(6.9%)	3988 ± 449	3510 ± 422	-479(-4.0%) ± 274(8.1%)	4154 ± 809	3843 ± 449	-311(9.6%) ± 663(11.4%)	n.s.	n.s.	n.s.
HOMA-IR	1.21 ± 0.14	1.05 ± 0.11	-0.15(-0.9%) ± 0.07(6.8%)	1.05 ± 0.10	1.14 ± 0.12	0.09(21.7%) ± 0.10(12.8%)	1.14 ± 0.15	1.16 ± 0.14	0.03(6.7%) ± 0.10(7.7%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	9.6 ± 1.0	10.1 ± 1.0	0.5(15.0%) ± 0.7(6.0%)	9.3 ± 0.9	10.5 ± 1.6	1.2(24.5%) ± 1.5(20.7%)	9.4 ± 0.9	10.3 ± 1.2	0.8(15.1%) ± 0.9(11.7%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	11.8 ± 1.4	10.7 ± 0.9	-1.1(3.4%) ± 0.9(6.0%)	9.5 ± 0.8	9.2 ± 1.3	-0.3(3.1%) ± 1.3(13.7%)	10.4 ± 0.9	10.7 ± 1.1	0.3(9.8%) ± 0.9(11.6%)	n.s.	n.s.	n.s.

Intention to treat analysis of the exercise and whey supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and a significant effect of the HRTW intervention on ASMI using the Holm-Sidak post hoc test p=0.001 (indicated by * in the table). HRTW, high resistance training + whey; LITW, low intensity training + whey;

Table 3:

Nutrition arm PP	WHEY, N= 21 (10 males, 11 females)			ANOVA p-value		
	0 M	12 M	Difference	Time	Group	Interaction
Subject characteristics						
Age (year)	69.7 ± 0.8				n.s.	
Body mass (kg)	72.6 ± 3.2	72.8 ± 3.1	0.2(0.5%) ± 0.4(0.6%)			
BMI (kg/m2)	24.1 ± 0.8	24.2 ± 0.7	0.1(0.5%) ± 0.1(0.6%)			
Fat mass (%)	31.0 ± 1.6	31.5 ± 1.6	0.5(1.7%) ± 0.4(1.4%)			
Lean mass (kg)	48.2 ± 2.1	48.3 ± 2.0	0.1(0.3%) ± 0.2(0.4%)			
ASMI (kg/m2)	7.36 ± 0.23	7.39 ± 0.22	0.04(0.6%) ± 0.04(0.6%)			
Daily steps	11354 ± 771	10362 ± 797	-697(-1.1%) ± 916(8.1%)			
Fasting blood analysis						
Fasting glucose (mmol/L)	5.3 ± 0.1	5.5 ± 0.2	0.1(2.7%) ± 0.1(2.3%)			
Fasting insulin (uIU/ml)	3.9 ± 0.6	3.4 ± 0.4	-0.5(6.3%) ± 0.3(11.9%)			
ProInsulin C-peptid (pmol/l)	694 ± 68	675 ± 50	-19(3.6%) ± 42(5.7%)			
HbA1c (mmol/mol)	34.8 ± 0.7	35.2 ± 0.8	0.4(1.3%) ± 0.5(1.5%)			
OGTT						
Glucose AUC (mmol/L x 120 min)	834 ± 27	860 ± 35	27(3.1%) ± 20(2.4%)			
Glucose 45 min (mmol/L)	8.0 ± 0.3	8.2 ± 0.4	0.1(0.02%) ± 0.3(0.03%)			
Glucose 2h (mmol/L)	6.2 ± 0.3	6.6 ± 0.4	0.4(7.7%) ± 0.3(5.3%)			
Insulin AUC (uIU/ml x 120 min)	4540 ± 1052	3442 ± 541	-1098(-6.7%) ± 616(10.4%)			
HOMA-IR	0.93 ± 0.16	0.86 ± 0.14	-0.07(7.9%) ± 0.06(11.4%)			
Matsuda Index (0,45,120 min)	11.7 ± 1.5	12.1 ± 1.4	0.5(16.6%) ± 1.2(9.4%)			
Matsuda Index (0,120 min)	14.3 ± 2.2	12.6 ± 1.3	-1.7(0.5%) ± 1.5(8.8%)			

Nutrition arm PP	COLL, N=28 (16 males, 12 females)			ANOVA p-value		
	0 M	12 M	Difference	Time	Group	Interaction
Subject characteristics						
Age (year)	69.5 ± 0.6					
Body mass (kg)	76.0 ± 2.5	76.3 ± 2.6	0.3(0.4%) ± 0.4(0.6%)			
BMI (kg/m2)	25.4 ± 0.9	25.5 ± 0.9	0.1(0.4%) ± 0.1(0.6%)			
Fat mass (%)	31.6 ± 1.7	32.0 ± 1.7	0.4(1.7%) ± 0.4(1.4%)			
Lean mass (kg)	50.0 ± 1.5	49.9 ± 1.5	-0.1(-0.3%) ± 0.2(0.4%)			
ASMI (kg/m2)	7.64 ± 0.24	7.64 ± 0.24	0.00(0.0%) ± 0.3(0.5%)			
Daily steps	11021 ± 753	10370 ± 975	-440(-2.7%) ± 676(7.7%)			
Fasting blood analysis						
Fasting glucose (mmol/L)	5.5 ± 0.1	5.4 ± 0.1	-0.04(-0.7%) ± 0.1(1.3%)			
Fasting insulin (uIU/ml)	4.7 ± 0.5	4.9 ± 0.6	0.2(5.3%) ± 0.3(8.5%)			
ProInsulin C-peptid (pmol/l)	680 ± 41	705 ± 51	25(4.0%) ± 25(3.9%)			
HbA1c (mmol/mol)	35.3 ± 0.6	36.5 ± 0.7	1.2(3.7%) ± 0.5(1.9%)			
OGTT						
Glucose AUC (mmol/L x 120 min)	910 ± 30	930 ± 29	20(3.4%) ± 24(2.9%)			
Glucose 45 min (mmol/L)	9.0 ± 0.4	9.0 ± 0.3	-0.04(1.8%) ± 0.3(3.6%)			
Glucose 2h (mmol/L)	6.5 ± 0.3	7.1 ± 0.3	0.6(12.7%) ± 0.3(5.4%)			
Insulin AUC (uIU/ml x 120 min)	5154 ± 719	5394 ± 844	240(11.0%) ± 550(10.2%)			
HOMA-IR	1.17 ± 0.13	1.22 ± 0.17	0.05(6.2%) ± 0.10(9.4%)			
Matsuda Index (0,45,120 min)	8.7 ± 1.0	8.8 ± 1.1	0.1(5.9%) ± 0.7(7.3%)			
Matsuda Index (0,120 min)	11.0 ± 1.5	10.2 ± 1.7	-0.8(4.9%) ± 1.3(16.9%)			

Nutrition arm PP	CARB, N=15 (9 males, 6 females)			ANOVA p-value		
	0 M	12 M	Change [absolute (%)]	Time	Group	Interaction
Subject characteristics						
Age (year)	69.1 ± 0.9					
Body mass (kg)	72.2 ± 3.1	73.5 ± 3.1	1.3(1.9%) ± 0.6(0.8%)	0.040	n.s.	n.s.
BMI (kg/m2)	24.3 ± 0.9	24.7 ± 0.9	0.4(1.9%) ± 0.2(0.8%)	0.034	n.s.	n.s.
Fat mass (%)	29.1 ± 2.1	30.0 ± 2.0	0.9(3.9%) ± 0.4(1.6%)	0.017	n.s.	n.s.
Lean mass (kg)	49.3 ± 2.1	49.7 ± 2.1	0.4(0.8%) ± 0.2(0.4%)	n.s.	n.s.	n.s.
ASMI (kg/m2)	7.60 ± 0.35	7.61 ± 0.36	0.02(0.2%) ± 0.04(0.6%)	n.s.	n.s.	n.s.
Daily steps	10183 ± 1175	11055 ± 1256	323(8.8%) ± 822(9.8%)	n.s.	n.s.	n.s.
Fasting blood analysis						
Fasting glucose (mmol/L)	5.6 ± 0.1	5.5 ± 0.1	-0.1(-1.2%) ± 0.1(1.9%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	4.6 ± 0.8	4.5 ± 0.9	-0.1(1.5%) ± 0.4(9.5%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	696 ± 70	739 ± 79	42(8.2%) ± 32(6.4%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	35.4 ± 0.5	37.1 ± 0.4	1.7(4.9%) ± 0.5(1.7%)	0.002	n.s.	n.s.
OGTT						
Glucose AUC (mmol/L x 120 min)	951 ± 39	960 ± 39	9(2.3%) ± 41(4.4%)	n.s.	n.s.	n.s.
Glucose 45 min (mmol/L)	9.6 ± 0.5	9.7 ± 0.3	0.1(3.2%) ± 0.5(4.9%)	n.s.	0.013	n.s.
Glucose 2h (mmol/L)	6.6 ± 0.4	6.8 ± 0.6	0.2(2.5%) ± 0.5(6.7%)	n.s.	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	4188 ± 655	4138 ± 1119	-51(-9.6%) ± 558(8.8%)	(0.0505)	n.s.	n.s.
HOMA-IR	1.13 ± 0.21	1.13 ± 0.25	-0.01(0.1%) ± 0.10(9.3%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	8.6 ± 1.0	10.6 ± 1.7	2.0(21.5%) ± 1.3(14.5%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	9.6 ± 1.0	11.5 ± 1.9	1.9(13.0%) ± 1.3(16.9%)	n.s.	n.s.	n.s.

Per protocol analysis of the nutritional supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and no significant effects of the different interventions. COLL, collagen; CARB, carbohydrate;

Table 4:

Training arm PP	WHEY, N=21 (10 males, 11 females)			LITW, N=19 (9 males, 10 females)			HRTW, N=17 (10 males, 7 females)			ANOVA p-value		
	0 M	12 M	Difference	0 M	12 M	Difference	0 M	12 M	Change [absolute (%)]	Time	Group	Interaction
Subject characteristics												
Age (year)	69.7 ± 0.8			70.2 ± 0.9			70.7 ± 0.8				n.s.	
Body mass (kg)	72.6 ± 3.2	72.8 ± 3.1	0.2(0.5%) ± 0.4(0.6%)	72.8 ± 2.2	73.6 ± 2.0	0.8(1.2%) ± 0.6(0.9%)	77.9 ± 3.5	78.1 ± 3.4	0.2(0.5%) ± 0.8(1.0%)	n.s.	n.s.	n.s.
BMI (kg/m2)	24.1 ± 0.8	24.2 ± 0.7	0.1(0.5%) ± 0.1(0.6%)	25.2 ± 0.8	25.4 ± 0.7	0.2(1.2%) ± 0.2(0.9%)	26.1 ± 0.9	26.2 ± 0.9	0.1(0.5%) ± 0.3(1.0%)	n.s.	n.s.	n.s.
Fat mass (%)	31.0 ± 1.6	31.5 ± 1.6	0.5(1.7%) ± 0.4(1.4%)	34.0 ± 1.8	34.5 ± 1.8	0.5(2.0%) ± 0.5(1.6%)	33.3 ± 1.7	33.0 ± 1.9	-0.3(-1.2%) ± 0.5(1.4%)	n.s.	n.s.	n.s.
Lean mass (kg)	48.2 ± 2.1	48.3 ± 2.0	0.1(0.3%) ± 0.2(0.4%)	46.6 ± 1.8	46.9 ± 1.8	0.3(0.6%) ± 0.3(0.5%)	50.3 ± 2.6	50.9 ± 2.6	0.6(1.2%) ± 0.3(0.6%)	0.033	n.s.	n.s.
ASMI (kg/m2)	7.36 ± 0.23	7.39 ± 0.22	0.04(0.6%) ± 0.04(0.6%)	7.35 ± 0.24	7.47 ± 0.24	0.12(1.6%) ± 0.05(0.7%)	7.73 ± 0.35	7.91 ± 0.36	0.18(2.4%) ± 0.06(0.7%)	0.001	n.s.	n.s.
Daily steps	11354 ± 771	10362 ± 797	-697(-1.1%) ± 916(8.1%)	10376 ± 833	9846 ± 604	128(4.5%) ± 568(6.2%)	9573 ± 972	8981 ± 812	-592(-3.0) ± 420(4.5%)	n.s.	n.s.	n.s.
Fasting blood analysis												
Fasting glucose (mmol/L)	5.3 ± 0.1	5.5 ± 0.2	0.1(2.7%) ± 0.1(2.3%)	5.7 ± 0.1	5.6 ± 0.1	-0.1(-0.6%) ± 0.1(2.4%)	5.7 ± 0.1	5.8 ± 0.1	0.1(1.6%) ± 0.1(1.5%)	n.s.	n.s.	n.s.
Fasting insulin (uIU/ml)	3.9 ± 0.6	3.4 ± 0.4	-0.5(6.3%) ± 0.3(11.9%)	4.3 ± 0.5	4.6 ± 0.5	0.4(0.2%) ± 0.6(0.2%)	4.3 ± 0.7	4.2 ± 0.7	-0.1(1.7%) ± 0.5(10.7%)	n.s.	n.s.	n.s.
ProInsulin C-peptid (pmol/l)	694 ± 68	675 ± 50	-19(3.6%) ± 42(5.7%)	678 ± 54	737 ± 52	60(17.8%) ± 62(9.8%)	657 ± 56	711 ± 56	54(0.1%) ± 39(0.1%)	n.s.	n.s.	n.s.
HbA1c (mmol/mol)	34.8 ± 0.7	35.2 ± 0.8	0.4(1.3%) ± 0.5(1.5%)	35.3 ± 0.8	36.4 ± 0.8	1.1(3.4%) ± 0.5(1.6%)	36.0 ± 0.7	36.6 ± 0.7	0.6(1.8%) ± 0.5(1.4%)	0.023	n.s.	n.s.
OGTT												
Glucose AUC (mmol/L x 120 min)	834 ± 27	860 ± 35	27(3.1%) ± 20(2.4%)	924 ± 40	953 ± 38	29(3.8%) ± 16(1.9%)	916 ± 34	951 ± 46	35(4.4%) ± 36(4.4%)	0.037	n.s.	n.s.
Glucose 45 min (mmol/L)	8.0 ± 0.3	8.2 ± 0.4	0.1(0.02%) ± 0.3(0.03%)	8.8 ± 0.5	9.0 ± 0.5	0.2(4.1%) ± 0.3(3.2%)	8.8 ± 0.4	9.1 ± 0.5	0.3(4.0%) ± 0.4(5.4%)	n.s.	n.s.	n.s.
Glucose 2h (mmol/L)	6.2 ± 0.3	6.6 ± 0.4	0.4(7.7%) ± 0.3(5.3%)	7.1 ± 0.3	7.6 ± 0.4	0.5(7.5%) ± 0.3(3.7%)	6.9 ± 0.4	7.3 ± 0.4	0.4(7.9%) ± 0.3(5.8%)	0.019	n.s.	n.s.
Insulin AUC (uIU/ml x 120 min)	4540 ± 1052	3442 ± 541	-1098(-6.7%) ± 616(10.4%)	4135 ± 556	3701 ± 574	-433(-4.0%) ± 322(10.4%)	4431 ± 1310	3856 ± 610	-575(5.6%) ± 1026(10.8%)	(0.0595)	n.s.	n.s.
HOMA-IR	0.93 ± 0.16	0.86 ± 0.14	-0.07(7.9%) ± 0.06(11.4%)	1.08 ± 0.12	1.16 ± 0.14	0.08(25.4%) ± 0.15(18.7%)	2.01 ± 0.22	1.11 ± 0.19	-0.01(3.5%) ± 0.14(10.9%)	n.s.	n.s.	n.s.
Matsuda Index (0,45,120 min)	11.7 ± 1.5	12.1 ± 1.4	0.5(16.6%) ± 1.2(9.4%)	8.8 ± 0.9	10.6 ± 2.2	1.8(32.1%) ± 2.2(29.2%)	9.9 ± 1.3	11.1 ± 1.8	1.2(18.0%) ± 1.4(16.6%)	n.s.	n.s.	n.s.
Matsuda Index (0,120 min)	14.3 ± 2.2	12.6 ± 1.3	-1.7(0.5%) ± 1.5(8.8%)	8.4 ± 0.7	9.3 ± 1.8	0.9(12.7%) ± 1.8(19.8%)	10.5 ± 1.4	10.4 ± 1.4	-0.1(10.0%) ± 1.3(17.1%)	n.s.	n.s.	n.s.

Per protocol analysis of the exercise and whey supplementation interventions. Values are mean ± SEM. The ANOVA showed significant effects of time and no significant effects of the different interventions. HRTW, high resistance training + whey; LITW, low intensity training + whey

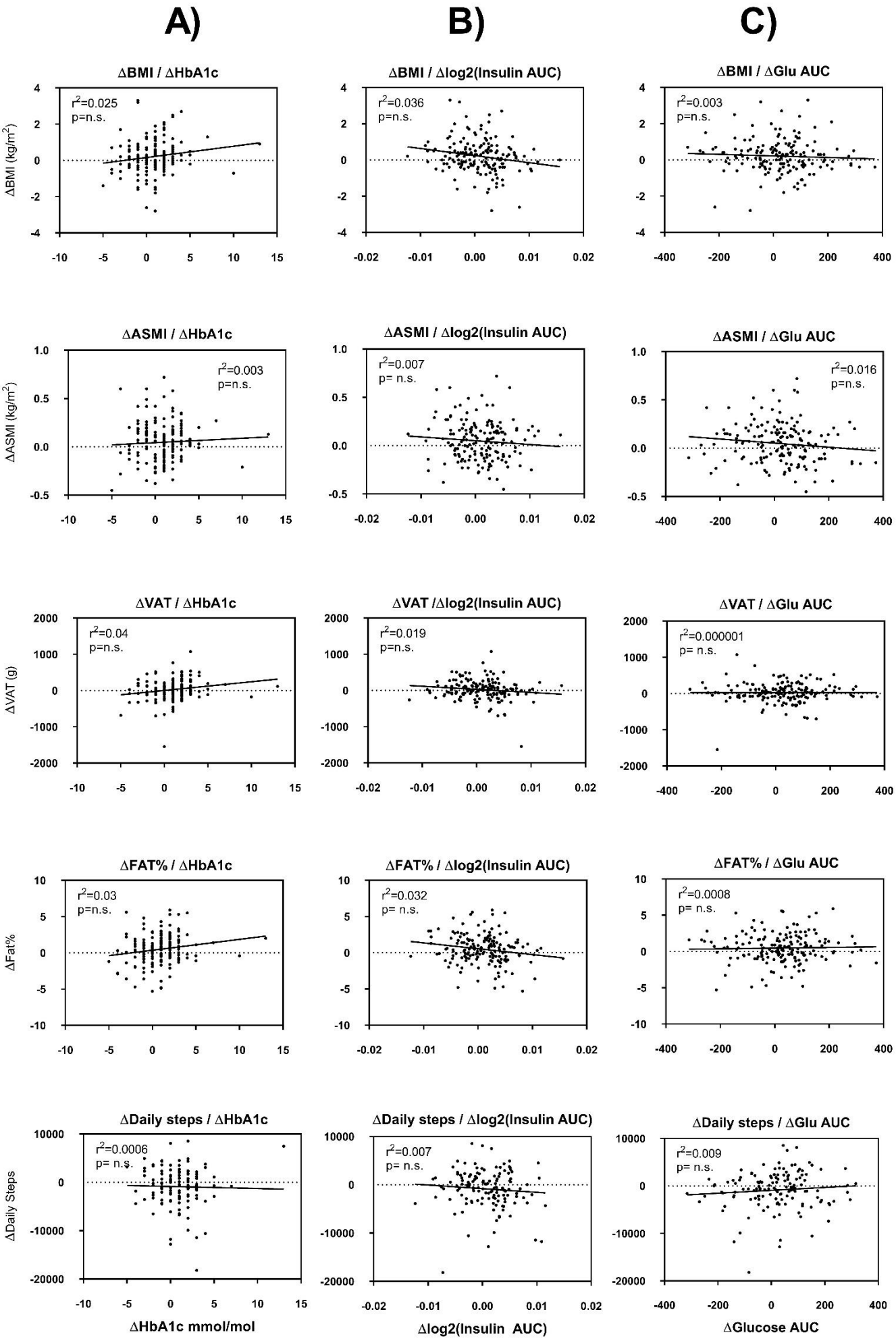
Supplemental figure 1: Correlations between changes from baseline to 12 months after intervention start in BMI, ASMI, VAT, Fat%, and daily steps and a) changes in HbA1c, b) changes in insulin AUC, and c) changes in glucose AUC from pre intervention to post intervention. P-values below 0.001 were considered significant.

Supplemental figure 2: Correlations between a) BMI, b) ASMI, c) VAT, d) Fat%, and e) daily steps and HbA1c at baseline in females and males. P-values below 0.001 were considered significant.

Supplemental figure 3: Correlations between a) BMI, b) ASMI, c) VAT, d) Fat%, and e) daily steps and insulin AUC response to an OGTT at baseline in females and males. P-values below 0.001 were considered significant.

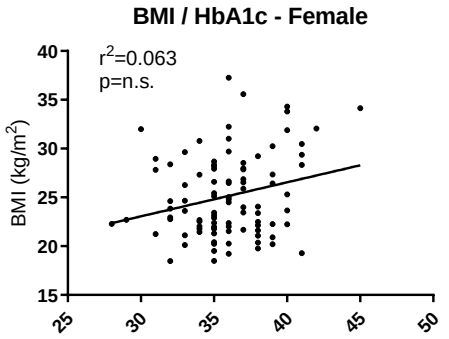
Supplemental figure 4: Correlations between a) BMI, b) ASMI, c) VAT, d) Fat%, and e) daily steps and glucose AUC response to an OGTT at baseline in females and males. P-values below 0.001 were considered significant

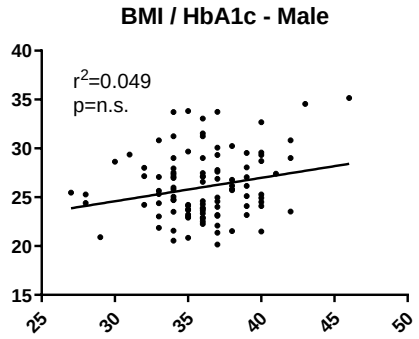
Supplemental figure 1



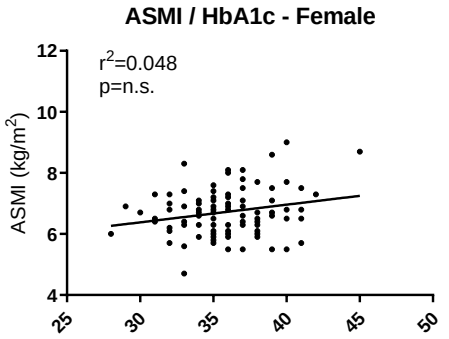
Supplemental figure 2

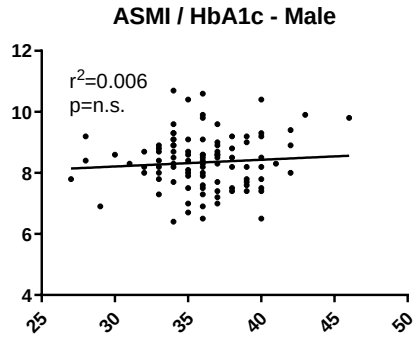
A)



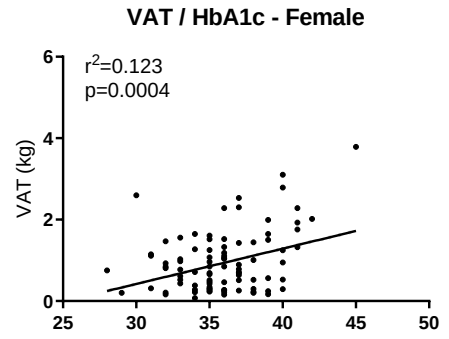


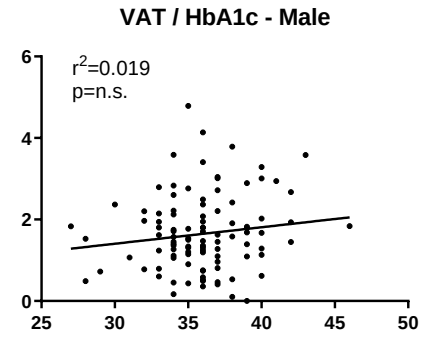
B)



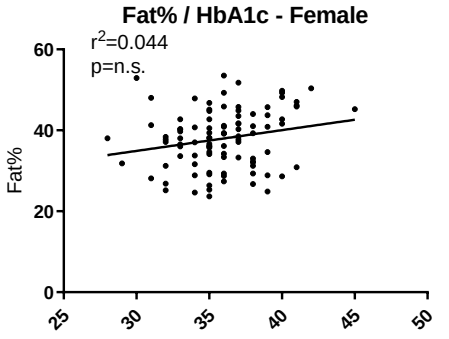


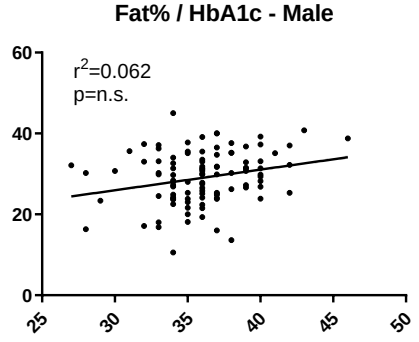
C)



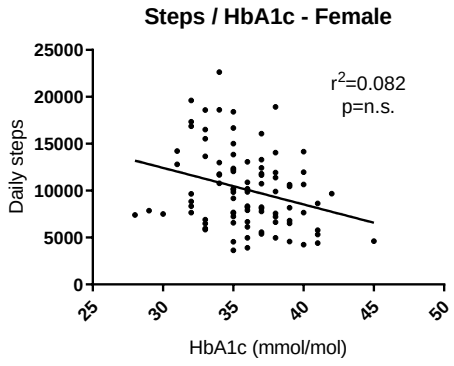


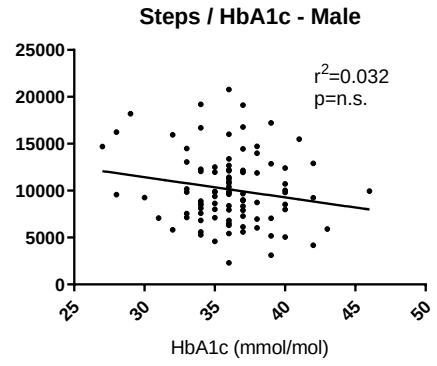
D)





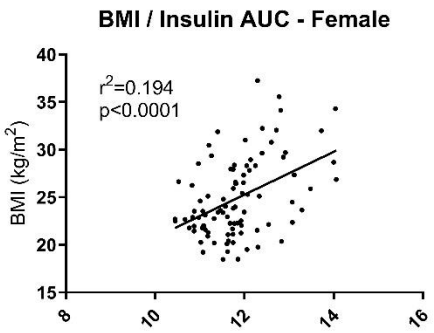
E)

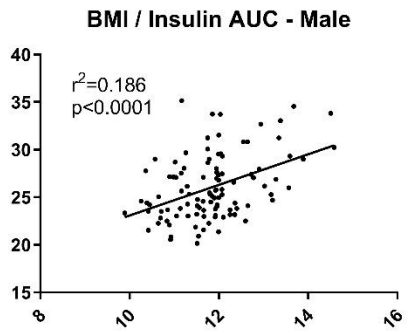




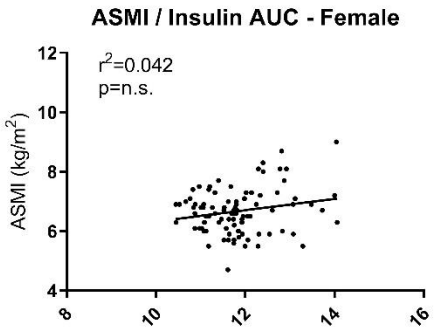
Supplemental figure 3

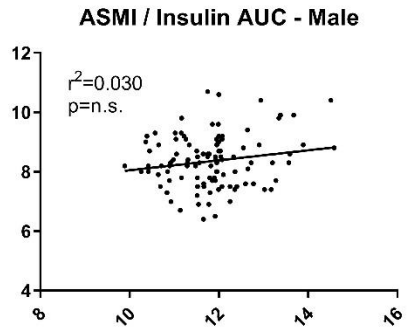
A)



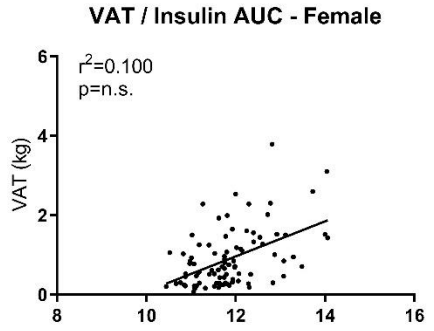


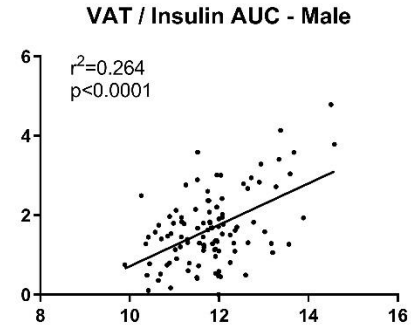
B)



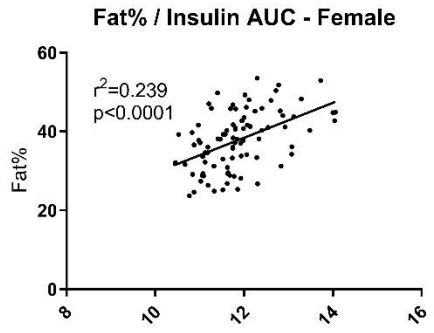


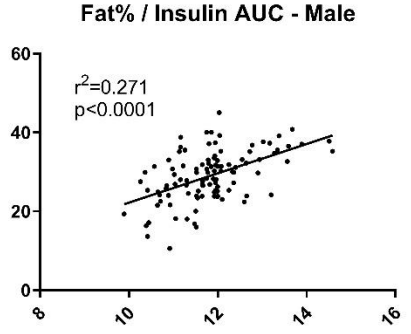
C)



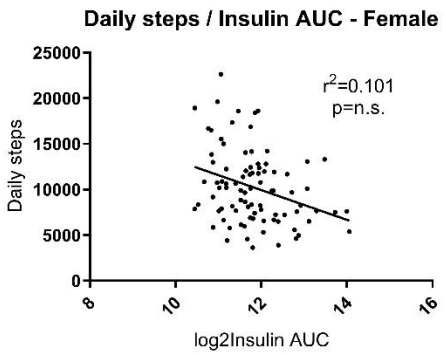


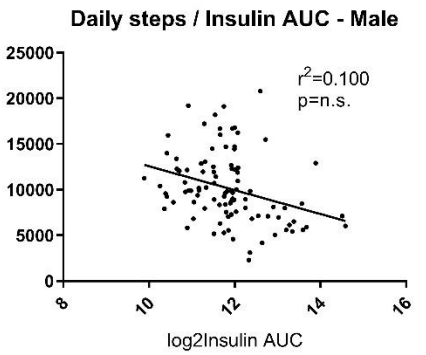
D)





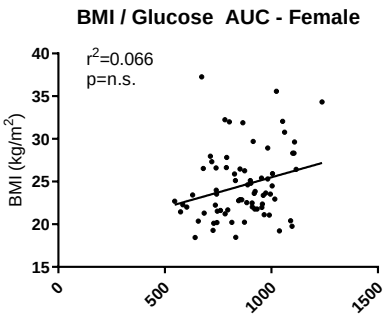
E)

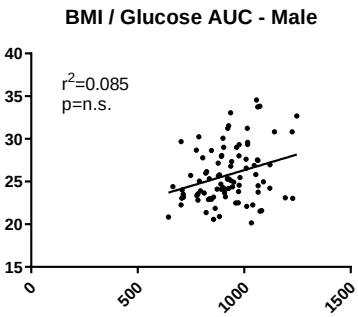




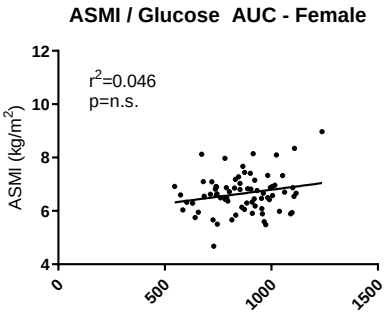
Supplemental figure 4

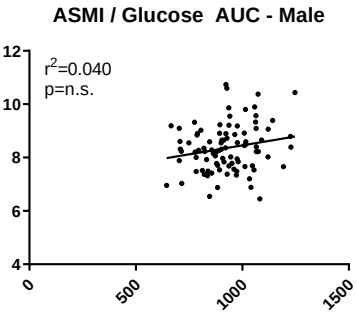
A)



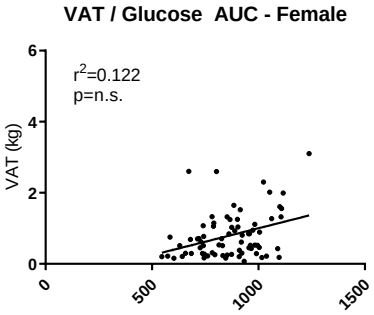


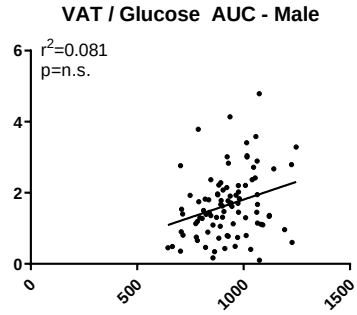
B)



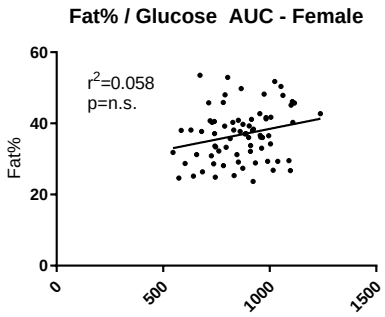


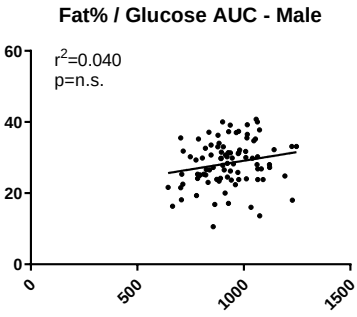
C)



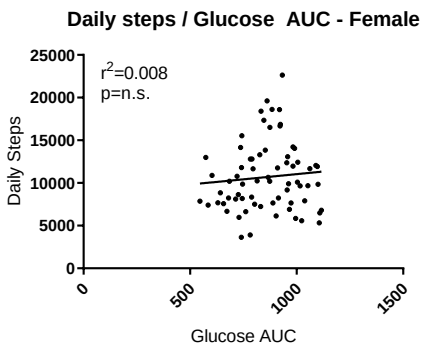


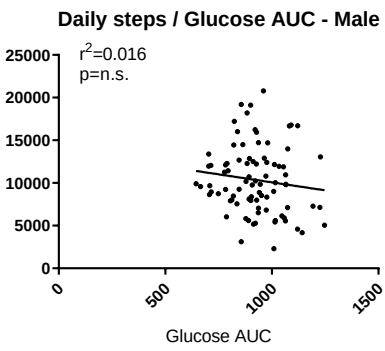
D)





E)





GRADUATE SCHOOL OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Jacob Bülow
E-mail	jacob.bulow@live.dk
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	The Ageing Skeletal Muscle: Effects of Training and Protein supplementation

2. The declaration applies to the following article	
Title of article	Recommended long-term nutritional supplementation, irrespective of quality and additional training does not affect glucose tolerance differently than carbohydrate supplementation in healthy elderly: the CALM cohort
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☒
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	C
2. Development of the key methods	C
3. Planning of the experiments and methodology design and development	E
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	C
5. Conducting the analysis of data	B
6. Interpretation of the results	B
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	B

Provide a short description of the PhD student´s specific contribution to the article.[i]
The PhD student has been the primary responsible of the daily mangement of the CALM study and been involved in both the clinical studies and the collection of data. The PhD student did all of the analysis in collaboration withcco-author Mie Zillmer, wrote the first draft and incorporated the feed-back from co-authors into the manuscript.

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	
If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another.	

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.		Mie Cecilie Faber Zillmer	msc	18-MAR-2020
2.		Grith Højfeldt	PhD	18.03.2020
3.	18-3-2020	Rasmus Bechschøft	MD, PhDD	
4.		Jakob Agergaard	PhD	20/3-20
5.	18-3-2020	Peter Schjerling	PhD	
6.	21-3-2020	Lars Holm	Professor	
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 30-3-2020
Principal supervisor: Michael Kjær

Name: Michael Kjær
Principal supervisor

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 22-3-2020
PhD student: Jacob Bülow

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

[i] This can be supplemented with an additional letter if needed.

[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."

[iii] If more signatures are needed please add an extra sheet.

Paper IV: **Jacob Bülow**, Bekzod Khakimov, Søren Reitelseder, Rasmus Bechshøft, Søren Balling Engelsen, Lars Holm
The effect of long-term nutritional supplementation with or without different types of training on the skeletal muscle protein synthesis rate and metabolome in healthy elderly: the CALM study

Title Page

Title

The effect of long-term protein supplementation with or without different types of training on the skeletal muscle protein synthesis rate and metabolome in healthy elderly Danes: the CALM study.

Authors

Jacob Bülow[1], Bekzod Khakimov[2], Søren Reitelseder[1,3], Rasmus Bechshøft[1], Søren Balling Engelsen[2], Lars Holm[3,5]

Author affiliations

[1]Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery M, Bispebjerg Hospital, Copenhagen, Denmark
[2]Dept. of Food Sciences, University of Copenhagen, Denmark
[3]Dept. of Biomedical Sciences, University of Copenhagen, Denmark
[4]Clinical Metabolomics Core Facility Dept. of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark.
[5]School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham, United Kingdom

Corresponding Author

Jacob Bülow

Mailing address:
Institute of Sports Medicine Copenhagen M81,
Bispebjerg Hospital (Building 8, level 1),
Nielsine Nielsens Vej 11
2400 Copenhagen NV
Denmark

Tel: +45 38 63 53 98

e-mail: jacob.buelow.02@regionh.dk

Sources of support

The study is funded by the Excellence Programme for Interdisciplinary Research 2016 University of Copenhagen. Grant provided by P. Carl Petersen Fond, Stipend from RegionH.

Word count

Conflict of interest declaration

None of the authors declared any conflict of interest.

ABSTRACT:

Background:

The suggestion of increasing the recommended daily protein intake in elderly <65 years to counteract sarcopenia has partly been justified by acute studies indicating a positive effect of increased protein intake with or without exercise on muscle protein synthesis. However, this effect has not previously been investigated in long-term intervention studies

Objective:

To investigate, the effect of long-term protein supplementation with or without exercise on muscle protein synthesis and the acute effect of protein and carbohydrate intake on the skeletal muscle metabolome.

Methods:

We randomized a subgroup of 66 healthy elderly from the CALM study to participate in an acute trial before and after 12 months of either 1) Carbohydrate supplementation (CARB), 2) Collagen protein supplementation (COLL), 3) Whey protein supplementation (WHEY), 4) Home-based light-intensity resistance training with whey protein supplementation (LITW), 5) Center-based heavy-load resistance training with whey protein supplementation (HRTW), all receiving the supplement twice daily. At the acute trial we measured the basal and postprandial FSR in response to 20g of whey hydrolysate and 10g of glucose. Further, we measured the skeletal muscle metabolome by GC-MS at basal and 4 hours after the protein and carbohydrate intake

Results:

1 year of protein supplementation in comparison to carbohydrate did not affect the muscle protein synthesis. No difference in basal FSR between males and females were observed, which is in contrast to previous findings (p=0.75; males: 0.034 ±0.013%/h; females: 0.035±0.009%/h, mean±SD). No alterations in the skeletal muscle metabolome were observed 4h after the intake of protein and carbohydrate.

Conclusion:

12 months of protein or carbohydrate supplementation did not alter the basal and protein-stimulated postprandial muscle protein synthesis rate in healthy elderly above 65 years of age. In contrast to previous findings, no differences in basal protein synthesis rates between males and females were observed. The applied methodology for measuring the skeletal muscle metabolome by GC-MS were robust and provided measurement of 191 metabolites of which 74 were identified at level 2. It seems as a promising method for future muscle metabolome investigations.

Keywords:

Muscle metabolome – protein synthesis - aging – protein supplementation – training – body composition – GC-MS.

Introduction

As we age, our physiological functions are gradually declining eventually compromising health, function and independence at old age[1,2]. Sarcopenia defined as the loss of skeletal muscle mass with age[3] is a crucial part in the explanation of the age-related functional decline due to its important role in both metabolism and physical functionality[4–6] Therefore, the maintenance of a well-functioning muscle mass is vital in the quest to remain independent, prevent falling[7] and to sustain quality of life at old age[8].

It is well known that both intake of proteins as well as exercise are required for maintaining and improving muscle mass and when absent detrimental muscle loss follows[2,5]. Muscle mass and changes herein are determined by the balance between muscle protein breakdown and synthesis, which are processes affected differently by various actions and interventions during the day[9]. Both intake of proteins as well as conduction of exercise are effective ways of stimulating of muscle protein synthesis processes in both young and old age[10,11] and consensus exist that such interventions primarily stimulate synthesis rates and that this is the main responsible for determining the net balance under such conditions[12]. The increase from basal condition rate in muscle protein synthesis after intake of a sub-optimal protein amount appears diminished in older people compared to young peers[13]. In accordance, observational studies reveal that elderly people with a higher than currently recommended daily protein intake have a larger lean body mass compared to those with a low protein intake[14–16]. Thus, some indications exist supporting a need for a high(er) protein intake for older adults.

Conduction of exercise also has a significant impact of muscle protein synthesis rates[10]. Fasting muscle protein synthesis rates are enhanced acutely, within hours[17,18], and prolonged, up to 72 hours[19], after completion of resistance exercise and postprandial increments in muscle protein

synthesis are enhanced for up to 48 hours in an exercised muscle[20]. Thus, when intervened acutely in research participants, exercise has an acute and a prolonged effect on muscle protein synthesis.

However, the responsiveness across a long-term intervention with high protein and exercise on the basal overnight fasted as well as the postprandial response to protein intake on muscle protein synthesis is unknown. We recently showed in elderly men, that the acute whole-body response to a standardized meal was impaired when habituated for three weeks to a diet high in protein intake compared to a diet with recommended protein intake (Højfeldt et al IN REVISION). Hence, muscle protein synthesis could be susceptible for adaptation and studies investigating the long-term effects of increased protein intakes as well as exercise on the protein synthesis are important in order to interpret the muscle protein synthesis process correctly.

Protein turnover in general is a demanding process in a cell and hence, changes in the skeletal muscle myofibrillar protein synthesis rate should be reflected in the general metabolome of the muscle. Yet, the literature on general alterations in the metabolome of the skeletal muscle in humans is scarce. Due to recent advances within the field of metabolomics and analytical technology it is however now possible to measure the muscle tissue specific metabolome despite the low amount of tissue available from human trials[21]. Fazelzadeh et al were able to show differences in the skeletal muscle metabolome between young, healthy elderly and frail elderly at rest within metabolites related to mitochondrial function, fiber type and tissue turnover[22]. Further, they did also show changes within the amino acid metabolism as an effect of 6 month of progressive resistance exercise in both healthy and frail elderly. Sato and colleagues also recently reported daily variation and response to a 5 day high fat or high carbohydrate diets in the skeletal muscle metabolome in middle aged men (30-45 years of age)[23]. Both of these studies underline the possibilities of new insights in the metabolism of the skeletal muscle offered by the tissue specific application of metabolomics.

The aim of the present study is to investigate the changes in the muscle protein synthesis rate in healthy elderly above 65 years of age after 12 months of intervention with daily protein supplementation in comparison to an isocaloric control. Furthermore, the effect of 12 month of resistance exercise with high or low intensity with protein supplementation in comparison to protein supplementation alone is investigated. We hypothesized i) that basal and protein-stimulated postprandial muscle protein synthesis rates are elevated in the exercise training groups after 12 months of intervention; ii) that prolonged intake of protein of different quality would not affect the acute muscle protein synthetic response to protein intake. In addition, the impact of the different interventions on the skeletal muscle metabolome is explored using untargeted GC-MS metabolomics adapted to muscle tissue[24]. Metabolic effects from dietary interventions are usually not trivial and multivariate data analysis is typically required to reveal effects from such investigations[25].

Methods

The CALM trial (Counteracting Age-Related Loss of Muscle Mass) was conducted at Bispebjerg Hospital between 2014 and 2018. It is designed as an intention-to-treat randomized controlled study. The study reported in this paper was conducted on a subgroup of subjects which in addition to the general measurements performed in the CALM trial also participated in an acute trial before and after the intervention. Further information and detailed description of purpose, methods and exclusion criteria in the CALM trial has been published previously[26]. The trial protocol (H-4-2013-070 and H-4-2013-070.3) was approved by the regional ethics committee and all participants provided written informed consent. The trial protocol for this study is registered at clinicaltrials.gov journal number: NCT02115698

Participants

We included 66 healthy elderly above 65 years of age. All participants were screened by a physician prior to enrollment. Participants were excluded if they performed >1 hour of heavy resistance training per week and if they had any medical condition potentially preventing them from completing the 1-year intervention. Participants were allowed to be medicated against hypertension, hypercholesterolemia and thyroid dysfunction. For an exact list of accepted medication see the trial protocol.

Study design

After enrollment, participants were randomized and stratified by sex and number of completed repetitions on the 30-s chair stand test (<16 or ≥16) into one of five intervention groups. 1) Carbohydrate supplementation (CARB; 20g maltodextrin + 10g of sucrose), 2) Collagen protein supplementation (COLL; 20g bovine collagen protein hydrolysate (ATpro 200) + 10g sucrose), 3) Whey protein supplementation (WHEY; 20g whey protein isolate (LACPRODAN, Aral Foods Ingredients P/S, Viby J, Denmark) +10g of sucrose), 4) Heavy resistance training with whey protein supplementation (HRTW), 5) Light-intensity training with whey protein supplementation (LITW). All groups were instructed to take their respective supplement 2 times daily at breakfast and at lunch. All supplements were developed and packaged by Arla Foods Ingredients Group P/S, Viby J, Denmark. The HRTW group were offered a supervised center-based progressive heavy resistance exercise program 3 times weekly and the LITW group were instructed to do a homebased non-supervised progressive light-load resistance training program 3-5 times weekly using TheraBand® rubber bands (Hygenic Corp., Akron, OH, USA) and bodyweight. For further details see prior publication (Mertz 2020). Before and after the intervention all participants went through a thorough

test battery (for further information see previous publication[26]) and an acute stable isotope labeled infusion trial. The present paper is primarily reporting results from the acute trials (see below).

Acute trial

Participants arrived at the facility 8 a.m. in the morning by car or public transportation to avoid physical activity in an overnight fasted state from 9 p.m. the day before. They were instructed to abstain from strenuous physical activity 3 days prior to the trial. The participants were placed in a bed in a supine position and two venous catheters were inserted in an antecubital vein in each arm and a background blood sample were taken. Hereafter at -270 minutes (see figure 1), a continuous infusion with L-[$^{13}C_6$] phenylalanine tracer (Cambridge Isotope Laboratories, Tewksbury, MA, USA) at an infusion rate of 6.0 µmol·kg $FFM^{-1}\cdot h^{-1}$ was started after injection of a priming dose 6.0 µmol·kg FFM^{-1} over 2 minutes. The tracers were dissolved in sterile saline water and filtered through 0.20-µm-pore disposal filters (Minisart, Sartorius Stedium Biotech, Gottingen, Germany) in the morning before the participants arrived. The tracer infusion rate was set to obtain a venous tracer-to-tracee ratio (TTR) of ~ 10%. After reaching steady state at -180 minutes another blood sample and the first biopsy were taken. The participants continued to rest in the supine position until another blood sample and biopsy were taken at 0 minutes. Immediately after, a drink containing 20 g of whey hydrolysate and 10 g of glucose was provided and finished immediately. Then blood samples were taken a 20 min, 40 min, 60 min, 90 min, 120 min and 240 min. At 240 min the last biopsy was taken, and the infusion stopped.

Blood samples

All blood samples were collected in 9-mL plasma Vacutainers containing EDTA, put at rest on ice for ≥10 min, and spun down at 3,200 *g* for 10 min at 4°C. Plasma were then transferred to eppendorf tubes and stored at -80°C until further analysis.

Muscle Biopsies

All three biopsies were obtained from vastus lateralis with individual incisions with ~3cm in-between with a 4-mm biopsy needle (Bergström, Stockholm, Sweden) using manual suction. At the beginning of the trial, the skin was shaved, and the thigh muscle were inspected and the incision sites for the three biopsies were marked. Before obtaining each biopsy, the area was disinfected and local anesthetic (1% lidocaine) was administered. An ~1.5cm incision was made before inserting the needle and obtaining the biopsy. An elastic band with a compression pad was used to compress the incision site for 30 min in order to avoid intramuscular hematoma. Before compression, the incisions were strapped with SteaStrips and covered with waterproof plaster. The muscle specimens were quickly cleansed from any visible blood, fat and connective tissue under a microscope, and then frozen in liquid N_2 and stored at -80°C until further analysis.

FSR

The muscle protein fractional synthesis rate (FSR) were calculated for two periods (see figure 1) using the precursor-product model as illustrated:

$$\mathrm{FSR} = \frac{\mathrm{E}\Delta_{\text{Myofibrilar protein, Phe}}}{(\mathrm{E}_{\text{Plasma mean, Phe}} \times t)} \times 100$$

Where $\mathrm{E}\Delta_{\text{Myofibrilar protein, Phe}}$ is the change in myofibrillar protein bound phenylalanine enrichment between two consecutive biopsies with *t* hours in-between; $\mathrm{E}_{\text{Plasma mean, Phe}}$ is the plasma weighted mean phenylalanine enrichment between the two biopsies. The 3-hour basal synthesis rate was calculated using the biopsies and blood samples at -180 min and 0 min, and the 4-hour synthesis rate in response to protein intake using the biopsies at 0 min and 240 min and a weighed mean of the plasma enrichment levels measured in the blood samples from 0, 20, 60, 90 and 240 min. A factor of 100 were used to express FSR in percent per hour ($\%\cdot h^{-1}$)[27]. The muscle specimens were prepared as follows. ~20 mg of the muscle sample was transferred to 2 mL lysing tube containing 10 lysing beads and two silicon carbide crystals. 1 mL of 4°C homogenizing buffer

(Tris 0.02M [pH 7.4], NaCl 0.15M, ED(G)TA 2mM, TritonX-100 0.5%, sucrose 0.25M) were added and the sample were homogenized 4 · 45 sec at speed 5.5 $m \cdot sec^{-1}$ with 2 min pause in between (FastPrep 120A-230; Thermo Savant, Holbrook, NY, USA). The samples then rested for 3 hours at 5°C. They were then spun at 800 *g* for 20 min at 5°C and the supernatant discarded. 1.0 mL of 4°C homogenizing buffer were added to the pellet and the sample were once again homogenized for 1 · 45sec at speed 5.5 $m \cdot sec^{-1}$, left for 30 min at 5°C and then spun 800 *g* for 20 min at 5°C. The supernatant was again discarded and 1.5 mL KCl-buffer (KCl 0.7M, pyrophosphate ($Na_4P_2O_7$) 0.1M) added and the samples were vortexed and left overnight at 5°C. The sample were then vortex and spun at 1,600 *g* for 20 min at 5°C and the supernatant (the myofibrillar protein fraction) was then transferred to a Scot-glass and 2.3 mL ethanol 99% was added. The samples were then vortexed and left for 2 hours at 5°C. After a spin 1,600 *g* for 20 min at 5°C the supernatant was discarded and 1mL 70% ethanol was added to the pellet containing the myofibrillar protein fraction. The samples were vortexed and then spun at 1600 *g* for 20 min at 5°C and the supernatants were once again discarded. To hydrolyze the myofibrillar proteins 1mL of 6M HCL was added and the sample, vortexed and left overnight at 110°C. The constituent amino acids were then purified over Dowex resin (AG 50W-X8 resin; Bio-Rad Laboratories, Hercules, CA) columns using 2M NH_4OH for elution and put under N_2 flow at 70°C until dried. Hereafter, the amino acids were derivatized as the N-acetyl-propyl (NAP) derivative as described in detail previously[28]. After derivatization, the samples were analyzed using a gas chromatography combustion isotope ration mass spectrometry (GC-C-IRMS) system (Hewlett Packard 5890-Finnigan GC combustion III-Finnigan Deltaplus; Finnigan MAT; Bremen; Germany). Briefly, 1 µL of sample were injected using a solvent split mode programmed-temperature vaporization (PVT) inlet. A detailed description of settings etc. has been published previously[27]. The plasma enrichments were analyzed using liquid chromatography-

tandem mass spectrometry (LC-MS/MS). Plasma samples were prepared and analyzed as described by Bornø et al 2014[29].

Muscle Metabolome

The muscle metabolome was measured using biopsies at time point 0 min and 240 min, both at 0 month and after 12 months of intervention. Muscle samples were extracted using a similar method as described by Alves et al 2015[21], which is based methanol/chloroform/water at Vol:Vol ratio of X:Y:Z, respectively. The muscle specimens were prepared and analyzed as followed. ~25 mg of frozen muscle tissue was put into 2 mL lysing tubes containing 10 lysing beads and two silicon carbide crystals. 0.5 mL of 5°C solvent (50% methanol containing 20 ppm ribitol) was added. The biopsies were homogenized 4 x 1 min at speed 5.5 $m \cdot sec^{-1}$ at 5°C (FastPrep 120A-230; Thermo Savant, Holbrook, NY, USA) with 2 min pause in between to avoid heating. Then, 300 µL of chloroform was added and the homogenized samples were vigorously vortexed for 10 min at room temperature. The samples rested on ice for 20 min and was then centrifugated for 15 min at 5°C at 16,000 g. 60 uL of the upper part of the aliquot (methanol part) and 40 uL of the lower part of the aliquot (chloroform part) was put into 200 uL glass inserts. The glass inserts were then dried under vacuum using a SpeedVac (Labogene, Lynge, Denmark) at 40°C for 3 hours. Samples were then derivatized in two steps, first by addition of 10 uL 20 $mg \cdot mL^{-1}$ methoxamine hydrochloride in dry pyridine (90 min at 45°C by agitating at 750 rpm) followed by trimethylsilylation (TMS) using trimethylsilyl cyanide (TMSCN), as described previously[30]. TMS derivatization was performed by addition 40 uL TMSCN and by agitating at 750 rpm for 40 min at 45°C. A total of 206 number of samples were analyzed in a randomized order in GC-MS, Y samples originate from this study design and Z samples were pooled control muscle samples run every 10^{th} sample in the sequence. Sample derivatization and injection of 1 uL derivatized aliquot were automated using a Dual-Rail MultiPurpose Sampler (MPS) (Gerstel, Mülheim an der Ruhr, Germany) as described previously[31].

The GC–MS consisted of an Agilent 7890B gas chromatograph (GC) (Agilent Technologies, California, USA) coupled with a time-of-flight mass spectrometer, HT Pegasus TOF-MS, (LECO Corporation, Saint Joseph, USA). A GC column used was Restek ZB 5% Phenyl 95% Dimethylpolysiloxane column (30 m with I.D. 250 lm and film thickness 0.25 lm) with a 5 m inactive guard column (Phenomenex, Torrance, USA). A hydrogen generator, Precision Hydrogen Trace 500 (Peak Scientific Instruments Ltd, Inchinnan, UK) was used to supply a carrier gas at the constant column flow rate of 1.0 $mL \cdot min^{-1}$. The initial temperature of the GC oven was set to 40°C and held for 2 min followed by heating at $12°C \cdot min^{-1}$ to 320°C and kept for an additional 8 min, making the total run time 33.3 min. A post run time at 40°C was set to 5 min. Mass spectra was recorded in the range of 45–600 m/z with a scanning frequency of 10 $scans \cdot sec^{-1}$, and the MS detector and ion source was switched off during the first 6.4 min of solvent delay time. The transfer line and ion source temperature were set to 280°C and 250°C, respectively. The mass spectrometer was tuned according to manufacturer's recommendation using perfluorotributylamine (PFTBA). The MPS and GC–MS was controlled using vendor software Maestro (Gerstel, Mülheim an der Ruhr, Germany) and ChromaTOF (LECO Corporation, Saint Joseph, USA). The raw GC-TOF-MS data was processed using Statistical Compare toolbox of the ChromaTOF software (Version 4.50.8.0) with following settings; the raw data was used without smoothing prior to peak deconvolution, baseline offset was set to 0.8, expected averaged peak width was set to 1.2 sec, signal-to-noise was set to ≥5, peak areas were calculate using deconvoluted mass spectra, common m/z ions of derivatization products were determined as 73, 75, and 147. Deconvoluted mass spectra were also used for peak identification using LECO-Fiehn and NIST11 libraries. The library search was set to return top 10 hits with EI-MS match of >75% using normal-forward search and with a mass threshold of 20. Deconvoluted peaks were aligned across all samples using following settings;

retention time shift allowance of <3 sec, EI-MS match of >90%, mass threshold of >25, and present in >90% of all pooled samples.

Statistical Analysis

FSR were analyzed according to the clinical trial registration with a one-way ANOVA on each intervention arm separately comparing the difference between delta fractional synthesis rates at 0month and at 12months (Δdelta FSR). Further, a paired t-test between the basal and response were performed at 0month for males and females separately. All FSR analysis were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA). The muscle metabolome data was subjected to univariate and multivariate statistical analysis prior to investigate possible effects according to the study design factors, including visit (0m and 12m), treatment (basal and response) and the intervention (CARB, COLL, WHEY, LITW and HRTW). Principal component analysis (PCA)[32] was performed prior to explore the MM data and evaluate an overall variation present in the dat. ANOVA-simultaneous component analysis (ASCA)[33] with permutation test, as described previously[24], was used to study significance of study design factors and their explained variations. Further, any single metabolite difference according to the design factors were analyzed using an ANOVA adjusted for multiple testing using false discovery rate (FDR) rate of 10%. Prior to PCA, ASCA and ANOVA, the MM data was normalized to the internal standard (ribitol) peak area. The muscle metabolome data was mean centered (the mean of each column was subtracted from the corresponding variable) and divided by its standard deviation, also called “auto scaling” before PCA and ASCA. All statistical data analysis was conducted using MATLAB ver. 2016b (The Mathworks, Inc. USA) and custom MATLAB scripts written by the authors.

Results

Participants

Out of the 66 participants included, 29 were females and 37 were males with a mean age of 70 years [range; 65-80years]. Baseline subject characteristics for the 5 different intervention groups are presented in table 1. 66 subjects participated in the acute trial at 0 month and 64 completed it. 2 participants (1 COLL, 1 LITW) had not the 240 min biopsy taken due to complications during the trial and they did not participate in the acute trial at 12 months. Further, 9 subjects did not participate at 12 months (1 CARB, 3 COLL, 2 WHEY, 2 LITW, 1 HRTW) due to complications or discomfort after the acute trial at 0 month, resulting in 55 subjects with complete sample sets. In the HRTW-group 6 subjects had an adherence below 66% corresponding to 2 training sessions pr. week, 2 subjects had a supplementary adherence below 50% corresponding to 1 supplement pr. day, and 2 subjects conducted their acute trial more than 14 days after the last training session. In the HRTW-group for both FSR and MM measures, only 3 subjects out of the 11 subjects had completed 0 and 12month acute trial with sufficient compliance and time between intervention stop and the acute trial to be included in a per protocol (PP) analysis. The training and supplementary mean adherence for these 3 subjects were 80% (SD±11%) respectively 86% (SD±10%). In the LITW-group 4 subjects had an adherence below 66% corresponding to 3 training sessions pr. week, 2 subjects had no training or supplementary registrations, and 2 subjects conducted their acute trial more than 14 days after the last training session. In the LITW-group, for FSR only 4 and for the MM measures only 3 subjects out of the 9 subjects that had completed 0- and 12-months acute trial with sufficient compliance and time between intervention stop and the acute trial to be included in a PP analysis. The training and supplementary mean adherence for these 4 subjects were 86% (SD±7%) 86% (SD±5%), respectively. In the WHEY-group, 8 subjects completed the 0 and 12month acute trial with sufficient compliance to be included in a PP analysis for the FSR

measurements and 6 for the MM measurements. The supplementary mean adherence for these 8 subjects were 94% (SD±5%). In the COLL-group, 9 subjects completed the 0 and 12month acute trial with sufficient compliance to be included in a PP analysis for the FSR measurements and 6 for the MM measurements. The supplementary mean adherence for these 9 subjects were 91% (SD±7%). In the CARB-group, 8 subjects completed the 0 and 12month acute trial with sufficient compliance to be included in a PP analysis for the FSR measurements and 6 for the MM measurements. The supplementary mean adherence for these 8 subjects were 87% (SD±9%). All subjects are included at 0month in the following analysis, and only those subjects with an acceptable compliance are included in the analysis testing the effect of the intervention at 12 months.

FSR

Comparing the Δdelta (Δdelta =($^{12month}FSR_{response}$ - $^{12month}FSR_{basal}$)- ($^{0month}FSR_{response}$ - $^{0month}FSR_{basal}$) FSR between groups in the nutrition supplementation arm, no difference was seen irrespective of adherence to the intervention (ITT: p=0.69; PP: p=0.26) (*ITT,mean[%±SEM]:* CARB 0.0045±0.006 , COLL -0.0001±0.006, WHEY -0.0049±0.01) (*PP, mean[%±SEM]:* CARB 0.0094±0.008, COLL 0.0013±0.006, WHEY -0.0032±0.011) (figure 2a,2b). Comparing the Δdelta FSR between groups in the training arm, no difference was seen for the ITT-analysis (p=0.98) (*ITT,mean[%±SEM]:* WHEY -0.0049±0.01, LITW -0.0022±0.009, HRTW -0.0039±0.009; (figure 2c). The PP analysis were not possible to perform in the training arm due to the low number of participants fulfilling the PP-criteria (LITW: n=4; HRTW: n=3). At 0 month, a difference was observed between the basal and response period but only within females (Female: p=0.0002; males: p=0.16) (Females, mean[%±SEM]: FSR_{basal} 0.035±0.002; $FSR_{response}$ 0.041±0.002) (Males, mean[%±SEM]: FSR_{basal} 0.034±0.002; $FSR_{response}$ 0.037±0.002) (figure 2d,2e).

Muscle Metabolome

The metabolite data of the muscle metabolome contained 191 peaks resolved from the GC-MS data. Out of these, 74 were identified at level 2 based on Metabolomics Standards Initiative[34], and identification criteria was set to EI-MS match of ≥ 750, RI match of ±50 and metabolites with labile protons being trimethylsilylated (TMS). These metabolites corresponded to 17 amino acids, 12 fatty acids,
11 sugars, 9 organic acids, 7 sugar alcohols, 3 phenolics, and 10 other metabolites including 2 indole derivatives, ibuprofen, uric acid, and cholesterol (Table S1). A PCA of the metabolite table shows that up to 25% of variation is captured by the first three principal components of the PCA model, although no trend of separation of samples was observed according to visit, treatment, sex or the CALM intervention design (figure 3). Supporting the above results, ASCA analysis also revealed no effect of the treatment, 0 min did not differ at 240min after the ingestion of 20g of whey hydrolysate and 10g of sucrose at the 0month visit ($p=0.42$, $n = 61$) (figure 4a). Neither were there any differences in the muscle metabolome between 0min and 240min at 12month visit ($p=0.20$ $n= 37$) (figure 4b). Although, two metabolites, 3-hydroxybutyric acid and 2-butenedioic acid, were significantly lower at 240min at both visits (ANOVA, 0m: $p=0.0025$, effect-size=13.8%; N=61; 12m: $p=0.0194$; effect-size=18.1% ; N=39; respectively 0m: $p=0.0025$; effect-size=14.69; N=61; 12m: $p=0.0494$; effect-size=14.75%; N=39) Similarly, ASCA analysis revealed no sign of a significant effect in relation to the visit (basal 0 month versus basal 12 month) ($p=0.62$, $n=61$) (figure 4c). Further, ASCA analysis showed no effect when comparing the different groups at 12 months ($p=0.68$, CARB n=6, COLL n=6, WHEY n=6) (figure 4d).

Discussion

This study investigates the one-year effect of two applicable strategies for counteracting sarcopenia, i.e. supplementation with proteins alone or in combination with resistance exercise, on the skeletal

muscle protein synthesis rates at basal overnight fasting level and the 4-hour postprandial response to 20 g of whey hydrolysate and 10 g of glucose. Further, we wanted to explore the impact of the different interventions, i.e. one-year effect and the effect of 20g of whey hydrolysate and 10g of glucose on the skeletal muscle metabolome after 4 hours. We did not find any impact of the 1-year supplementation with proteins of different quality or the addition of training on the muscle protein synthetic response to protein intake in comparison to the iso-caloric control irrespective of the choice of analysis (ITT for both protein and training interventions, PP for only protein interventions). Unfortunately, we were not able to perform an PP analysis in the training arm due to low number of participants fulfilling the PP criteria.

Regarding the characterization of the skeletal muscle metabolome, we were first of all able to confirm the possibility of measuring the skeletal muscle metabolome using a low amount of tissue samples (~25mg). This was done by application of an un-targeted GC-MS platform, which to our knowledge has only been done once before[35]. This led to the semi-quantitative characterization of the muscle metabolome, including 191 metabolite of which 74 were directly identified. However, we were not able to detect any effect on the skeletal muscle metabolome 4 hour post an intake of 20 g of whey hydrolysate and 10 g of glucose, neither before or after 12 month of intervention. Moreover, the analysis of the muscle metabolome did not reveal any effect of the nutritional supplementation group on the skeletal muscle metabolome after 12 months.

Evaluating the impact of intake of 20 g whey protein and 10 g glucose at inclusion to the study, we saw an increased FSR in response protein intake compared to the basal period, but only in women. The difference between males and females could be explained by the amount of protein pr. kg lean body mass they received during the acute trial. It has previously been shown, that an amount of 0.61 g/kg LBM (95% CI[0.32;0.89]) is needed in order to maximize the FSR in response to protein intake in healthy elderly males[36]. In this study, females received significantly (un-paired t-test,

p<0.0001) more protein pr. kg LBM than males (females, n=29: 0.50±0.05 g/kg LBM; males, n=36: 0.36±0.04 g/kg LBM; mean±SD) and were hereby closer to receive the amount of protein suggested to maximize the FSR response. The indication, that the males may not have received a sufficient amount of protein to stimulate protein synthesis is further supported by studies showing an impaired protein synthesis in response to hyperaminoacidemia[37,38] or orally intake of essential amino acids[39,40] in elderly. Another important observation in this study, is the lack of difference in basal FSR between males and females. In general, only few of the studies investigating differences between elderly and young analysis sex-differences separately. Henderson et al[41] found a difference in the basal FSR between males and females in both young (18-31 years) and old (≥60 years), and Smith et al[42] found a difference in basal FSR between elderly(65-80 years) males and females. However, we did not find any difference in basal FSR between males and females (un-paired t-test, p=0.75; males: 0.034 ±0.013%/h; females: 0.035±0.009%/h, mean±SD). This discrepancy could be explained by different study populations since the BMI of the participants in the two studies were higher (BMI: ~38[42], ~26[41]) than the participants in this study (~25), since it has been shown that adiposity is associated with increased protein metabolism[43]. The power in the present comparison is high, which emphasize that in the investigated cohort it is very unlikely that there is a sex difference. Nonetheless, studies specifically designed to investigate potential sex dimorphism are required if this should be explored in depth.

Contrary to our hypothesis, we did not find any effect of the different interventions with respect to the FSR. We did not see any sign of adaptations after one-year of intervention irrespective of the different supplements. Even though Oikawa et al[44] recently demonstrated an acute increased effect on FSR with respect to proteins of different quality, no studies have to our knowledge investigated if the measured differences between high and low quality proteins or the difference between young and old measured in acute settings is consistent over time or after a certain intervention period. In

addition, no studies have so far been able to demonstrate any correlation between the acutely measured FSR, muscle mass[45] or any other easy interpretable outcome. Despite that the maintenance of muscle mass depends on the balance of both synthesis and breakdown, fractional breakdown rates (FBR) are less frequently reported due to the notorious methodological difficulties of measuring FBR compared to the FSR. However, Kim et al recently demonstrated the importance of measuring muscle protein breakdown rates when evaluating the effect of an intervention with respect to muscle protein net balance[46]. This suggest that the measurement of FSR without measuring FBR may not be a usable method for investigating long-term effects on muscle mass development, and that the current interpretation of the lack of responsiveness in FSR to protein ingestion seen in elderly cannot be deemed either positive or negative in term of muscle mass development. In conclusion, these findings suggest that the transferability of FSR measurement and the responsiveness into general recommendations of living and eating is invalid and may have been overemphasized in the literature.

To our knowledge, only three studies measuring the skeletal muscle metabolome in humans have so far been conducted[22,35,47]. Fazelzadeh et al[22] found 96 different metabolites using targeted platforms (UPLC-MS/MS, GC-MS) in young (n=30m), healthy (n=47m/19f) and frail elderly (n=25m/18f), Sato et al[35] found 625 different metabolites using un-targeted GC-MS and UPLC-MS/MS in 7 overweight (BMI>27) middle aged men, and Saoi[47] found 84 metabolites using un-targeted MSI-CE-MS. We found 191 metabolites using un-targeted GC-MS, which is more than previous studies, despite the fact that we only used one analysis platform. Furthermore, we had a relatively high number of participants (n=65 (36m/29f) at baseline) which highlights the robustness of our analysis. Interestingly, we did not find any difference between the fasting biopsy and the biopsy taken 240 min post protein and glucose intake, with the result being consistent at both 0 and 12 months. This can be explained by two factors. Firstly, that our measurement is not sensitive enough; Secondly,

that the food supplement may not have been sufficient to alter the metabolome of the skeletal muscle after 240 min. We adhere to the second explanation due to the following: Even though we observed a significant increase in the plasma AA concentration during the acute trial, the plasma concentrations peaks after ~60 minutes and are almost down to fasting levels at 240 min (Figure S2). Further, we only saw an increase in FSR in females indicating that the effect of the protein and glucose intake on the muscle protein turnover was minor, although it could have affected other metabolic pathways in the muscle. The only alteration of the muscle metabolome was the decrease in the two metabolites 3-hydroxybutyric acid and 2-butenedioic acid concentrations in 240 min biopsy, which were consistent between 0 and 12 months. 3-hydroxybutyric acid is a ketone produced by the liver and used in extrahepatic tissue during fasting or glucose deprivations[48]. Keeping in mind that the subjects have been fasting for ~15 hours when receiving the protein and glucose drink, it is plausible that a substantial part of the nutrients has been used in supporting vital organs rather than stimulating muscle protein synthesis and hereby altering the skeletal muscle metabolism. The lack of findings is therefore not per se an argument against the sensitivity, but are more likely caused by the intervention applied, i.e. the acute trial. However, the finding emphasizes that the limited intake in terms of energy (CALC how much in kJ and relate it to their normal daily intake from dietary recordings) had very little impact on the muscle metabolome 240 min after intake. What has happened earlier in the postprandial period, we cannot of cause say anything about.

Unfortunately, we did not have enough samples at 12 months to fully investigate the effect of the intervention on the muscle metabolome. We conducted an exploratory analysis in the nutritional arm where we had six 0 min samples in each of the three groups which did not display any significant differences. This is in contrast to the previous studies all finding effects of their respective interventions on the skeletal muscle metabolome[22,35,47]. This can be explained by the

severity of the interventions applied in comparison to the intervention in this study, and the relatively low number of samples available in this study. However, when including both 0 min and 240 min biopsies in an ASCA analysis, there is an effect of group at 12 months ($p=0.04$) but not at baseline ($p=0.30$), which could indicate that there might be an effect that we cannot detect due to too low power. However, these indications should be cautiously interpreted since the analysis does not account for the representation of each subject twice in the data set. This again suggest, that the method is robust, and the lack of alterations in the skeletal muscle metabolome are rather caused by the intervention and study design applied. In conclusion, this study yielded a relatively high number of metabolites in comparison to the previous studies despite the use of only one analysis platform. This in combination with the simple sample preparation protocol and the low amount of tissue makes it a promising method for future investigation of the skeletal muscle metabolome.

Limitations

There are some limitations to this study. The number of participants completing the 12-months acute trial with an acceptable adherence to the intervention were lower than expected. We were therefore not able to conduct a PP analysis on the FSR in the training arm as originally planned. Further, the power calculations conducted prior to the investigation were not based on similar studies since they do not exist. It is therefore likely, that this study is under-powered. Lastly, it has recently been pointed out that there could be issues regarding the assumptions of recycling for the FSR calculations[49]. We observed a larger variation in the basal period at 12 months, which could be caused by the tracer incorporated in the structural proteins at 0 month are being recycled at 12 months. This should be taking into considerations when planning future studies.

Conclusions

12 months of protein or carbohydrate supplementation did not alter the basal and protein-stimulated postprandial muscle protein synthesis rate in healthy elderly above 65 years of age. Unfortunately, we were not able to evaluate the effect of 12 months of exercise on the FSR due to lower completion and adherence than expected. In contrast to previous findings, no differences in basal protein synthesis rates between males and females were observed. The applied GC-MS methodology for measuring the skeletal muscle metabolome were robust, and it seems as a promising method for future investigation.

Acknowledgments

Author contributions: Bülow and Khakimov had full access to all the data in the present study and take responsibility for the integrity of the data analysis. Study design: Reitelseder, Bechshoeft, and Holm. Acquisition of data: Bülow, Reitelseder, and Bechshoeft. Analysis and interpretation of data: Bülow, Khakimov, Engelsen and Holm. Drafting of manuscript: Bülow. All authors critically revised the manuscript and had the opportunity to comment on the interpretation of results. All have approved the final version. Statistical analysis: Bülow, Khakimov, Engelsen and Holm. Obtained funding: Bülow, Khakimov, Holm, Engelsen.

Funding: This work received funding from the University of Copenhagen Excellence Program for Interdisciplinary Research, the Danish Dairy Research Foundation, P. Carl Petersen´ Fund, Region H research fund, Arla Foods Ingredients P/S, and Innovation Foundation Denmark.

Role of the Sponsor: Arla Foods Ingredients P/S funded part of the study. Mikkelsen, employee at Arla Foods Ingredients was involved in study design, but was not involved in data acquisition or analysis.

References

1 Sehl ME, Yates FE. Kinetics of human aging: I. Rates of senescence between ages 30 and 70 years in healthy people. *J Gerontol A Biol Sci Med Sci* 2001; **56**: B198-208.

2 Bortz W. Disuse and Aging, 2009. *Journals Gerontol Ser A Biol Sci Med Sci* 2010; **65A**: 382–5.

3 Bulow J, Ulijaszek SJ, Holm L. Rejuvenation of the term Sarcopenia. *J Appl Physiol* 2018; : japplphysiol.00400.2018.

4 Baumgartner RN, Koehler KM, Gallagher D, *et al.* Epidemiology of sarcopenia among the elderly in New Mexico. *Am J Epidemiol* 1998; **147**: 755–63.

5 Wolfe RR. The underappreciated role of muscle in health and disease. *Am J Clin Nutr* 2006; **84**: 475–82.

6 Goodpaster BH, Park SW, Harris TB, *et al.* The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. *J Gerontol A Biol Sci Med Sci* 2006; **61**: 1059–64.

7 Landi F, Liperoti R, Russo A, *et al.* Sarcopenia as a risk factor for falls in elderly individuals: Results from the ilSIRENTE study. *Clin Nutr* 2012; **31**: 652–8.

8 Friedman EM, Ryff CD. Living well with medical comorbidities: A biopsychosocial perspective. *Journals Gerontol - Ser B Psychol Sci Soc Sci* 2012; **67 B**: 535–44.

9 Patterson BW. Isotope tracers in metabolic research. Principles and practice of kinetic analysis, 2nd edition Robert R. Wolfe and David L. Chinkes. *J Am Soc Mass Spectrom* 2005; **16**: 977–8.

10 Biolo G, Maggi SP, Williams BD, Tipton KD, Wolfe RR. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am J Physiol - Endocrinol Metab* 1995; **268**. DOI:10.1152/ajpendo.1995.268.3.e514.

11 Donges CE, Burd N a, Duffield R, *et al.* Concurrent resistance and aerobic exercise stimulates both myofibrillar and mitochondrial protein synthesis in sedentary middle-aged men. *J Appl Physiol* 2012; **112**: 1992–2001.

12 Little JP, Phillips SM. Resistance exercise and nutrition to counteract muscle wasting. Appl. Physiol. Nutr. Metab. 2009; **34**: 817–29.

13 Moore DR, Churchward-Venne T a, Witard O, *et al.* Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. *J Gerontol A Biol Sci Med Sci* 2015; **70**: 57–62.

14 Houston DK, Nicklas BJ, Ding J, *et al.* Dietary protein intake is associated with lean mass change in older, community-dwelling adults: The Health, Aging, and Body Composition (Health ABC) study. *Am J Clin Nutr* 2008; **87**: 150–5.

15 Isanejad M, Mursu J, Sirola J, *et al.* Association of protein intake with the change of lean mass among elderly women: The Osteoporosis Risk Factor and Prevention - Fracture Prevention Study (OSTPRE-FPS). *J Nutr Sci* 2015; **4**: 1–8.

16 Mcdonald CK, Ankarfeldt MZ, Capra S, Bauer J, Raymond K, Heitmann BL. Lean body mass change over 6 years is associated with dietary leucine intake in an older Danish population. *Br J Nutr* 2016; **115**: 1556–62.

17 Burd N a, West DWD, Staples AW, *et al.* Low-load high volume resistance exercise stimulates muscle protein synthesis more than high-load low volume resistance exercise in young men. *PLoS One* 2010; **5**: e12033.

18 Churchward-Venne T a, Burd N a, Phillips SM. Nutritional regulation of muscle protein synthesis with resistance exercise: strategies to enhance anabolism. *Nutr Metab (Lond)* 2012; **9**: 40.

19 Miller BF, Olesen JL, Hansen M, *et al.* Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol* 2005; **567**: 1021–33.

20 Phillips SM, Tipton KD, Aarsland A, Wolf SE, Wolfe RR. Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am J Physiol Metab* 1997; **273**: E99–107.

21 Alves RDAM, Dane AD, Harms A, *et al.* Global profiling of the muscle metabolome: method optimization, validation and application to determine exercise-induced metabolic effects. *Metabolomics* 2015; **11**: 271–85.

22 Fazelzadeh P, Hangelbroek RWJ, Tieland M, *et al.* The Muscle Metabolome Differs between Healthy and Frail Older Adults. *J Proteome Res* 2016; **15**: 499–509.

23 Sato S, Parr EB, Devlin BL, Hawley JA, Sassone-Corsi P. Human metabolomics reveal daily variations under nutritional challenges specific to serum and skeletal muscle. *Mol Metab* 2018; **16**: 1–11.

24 Khakimov B, Poulsen SK, Savorani F, *et al.* New nordic diet versus average Danish diet: A randomized controlled trial revealed healthy long-term effects of the new nordic diet by GC-MS blood plasma metabolomics. *J Proteome Res* 2016; **15**: 1939–54.

25 Khakimov B, Gürdeniz G, Engelse SB. Trends in the application of chemometrics to foodomics studies. *Acta Aliment* 2015; **44**: 4–31.

26 Bechshøft RL, Reitelseder S, Højfeldt G, *et al.* Counteracting Age-related Loss of Skeletal Muscle Mass: a clinical and ethnological trial on the role of protein supplementation and training load (CALM Intervention Study): study protocol for a randomized controlled trial. *Trials* 2016; **17**: 397.

27 Bornø A, Hulston CJ, Van Hall G. Determination of human muscle protein fractional synthesis rate: An evaluation of different mass spectrometry techniques and considerations for tracer choice. *J Mass Spectrom* 2014; **49**: 674–80.

28 Bechshoeft R, Dideriksen KJ, Reitelseder S, Scheike T, Kjaer M, Holm L. The anabolic potential of dietary protein intake on skeletal muscle is prolonged by prior light-load exercise. *Clin Nutr* 2012; : 1–9.

29 Bornø A, Van Hall G. Quantitative amino acid profiling and stable isotopically labeled amino acid tracer enrichment used for in vivo human systemic and tissue kinetics measurements. *J Chromatogr B Anal Technol Biomed Life Sci* 2014; **951**–**952**: 69–77.

30 Khakimov B, Motawia MS, Bak S, Engelsen SB. The use of trimethylsilyl cyanide derivatization for robust and broad-spectrum high-throughput gas chromatography-mass spectrometry based metabolomics. *Anal Bioanal Chem* 2013; **405**: 9193–205.

31 Khakimov B, Christiansen LD, Heins AL, *et al.* Untargeted GC-MS Metabolomics Reveals Changes in the Metabolite Dynamics of Industrial Scale Batch Fermentations of Streptoccoccus thermophilus Broth. *Biotechnol J* 2017; **12**: 1700400.

32 Hotelling H. Analysis of a complex of statistical variables into principal components. *J Educ Psychol*

1933; **24**: 417–41.

33 Smilde AK, Jansen JJ, Hoefsloot HCJ, Lamers R-JAN, van der Greef J, Timmerman ME. ANOVA-simultaneous component analysis (ASCA): a new tool for analyzing designed metabolomics data. *Bioinformatics* 2005; **21**: 3043–8.

34 Sumner LW, Amberg A, Barrett D, *et al.* Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* 2007; **3**: 211–21.

35 Sato S, Parr EB, Devlin BL, Hawley JA, Sassone-Corsi P. Human metabolomics reveal daily variations under nutritional challenges specific to serum and skeletal muscle. *Mol Metab* 2018; **16**: 1–11.

36 Moore DR, Churchward-Venne TA, Witard O, *et al.* Protein ingestion to stimulate myofibrillar protein synthesis requires greater relative protein intakes in healthy older versus younger men. *Journals Gerontol - Ser A Biol Sci Med Sci* 2015; **70**: 57–62.

37 Volpi E, Mittendorfer B, Rasmussen BB, Wolfe RR. The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. *J Clin Endocrinol Metab* 2000; **85**: 4481–90.

38 Guillet C, Prod'homme M, Balage M, *et al.* Impaired anabolic response of muscle protein synthesis is associated with S6K1 dysregulation in elderly humans. *FASEB J* 2004; **18**: 1586–7.

39 Cuthbertson D, Smith K, Babraj J, *et al.* Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *FASEB J* 2005; **19**: 422–4.

40 Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *Am J Physiol Endocrinol Metab* 2006; **291**: E381-7.

41 Henderson GC, Dhatariya K, Ford GC, *et al.* Higher muscle protein synthesis in women than men across the lifespan, and failure of androgen administration to amend age-related decrements. *FASEB J* 2009; **23**: 631–41.

42 Smith GI, Atherton P, Villareal DT, *et al.* Differences in muscle protein synthesis and anabolic signaling in the postabsorptive state and in response to food in 65-80 year old men and women. *PLoS One* 2008; **3**: e1875.

43 Henderson GC, Nadeau D, Horton ES, Nair KS. Effects of Adiposity and 30 Days of Caloric Restriction Upon Protein Metabolism in Moderately vs. Severely Obese Women. *Obesity* 2010; **18**: 1135–42.

44 Oikawa SY, Kamal MJ, Webb EK, McGlory C, Baker SK, Phillips SM. Whey protein but not collagen peptides stimulate acute and longer-term muscle protein synthesis with and without resistance exercise in healthy older women: a randomized controlled trial. *Am J Clin Nutr* 2020; **111**: 708–18.

45 Mitchell CJ, Churchward-Venne TA, Parise G, *et al.* Acute post-exercise myofibrillar protein synthesis is not correlated with resistance training-induced muscle hypertrophy in young men. *PLoS One* 2014; **9**. DOI:10.1371/journal.pone.0089431.

46 Kim IY, Schutzler S, Schrader A, *et al.* Quantity of dietary protein intake, But not pattern of intake, Affects net protein balance primarily through differences in protein synthesis in older adults. *Am J Physiol - Endocrinol Metab* 2015; **308**: E21–8.

47 Saoi M, Percival M, Nemr C, Li A, Gibala M, Britz-Mckibbin P. Characterization of the Human Skeletal Muscle Metabolome for Elucidating the Mechanisms of Bicarbonate Ingestion on Strenuous Interval Exercise. *Anal Chem* 2019; **91**: 4709–18.

48 Human Metabolome Database: https://hmdb.ca/metabolites/HMDB0000357#referencese accessed 24.03.2020. .

49 Holm L, Dideriksen K, Nielsen RH, *et al.* An exploration of the methods to determine the protein-specific synthesis and breakdown rates in vivo in humans. *Physiol Rep* 2019; **7**. DOI:10.14814/phy2.14143.

Figure 1. Acute trial study protocol conducted at before and after the 12month of intervention.

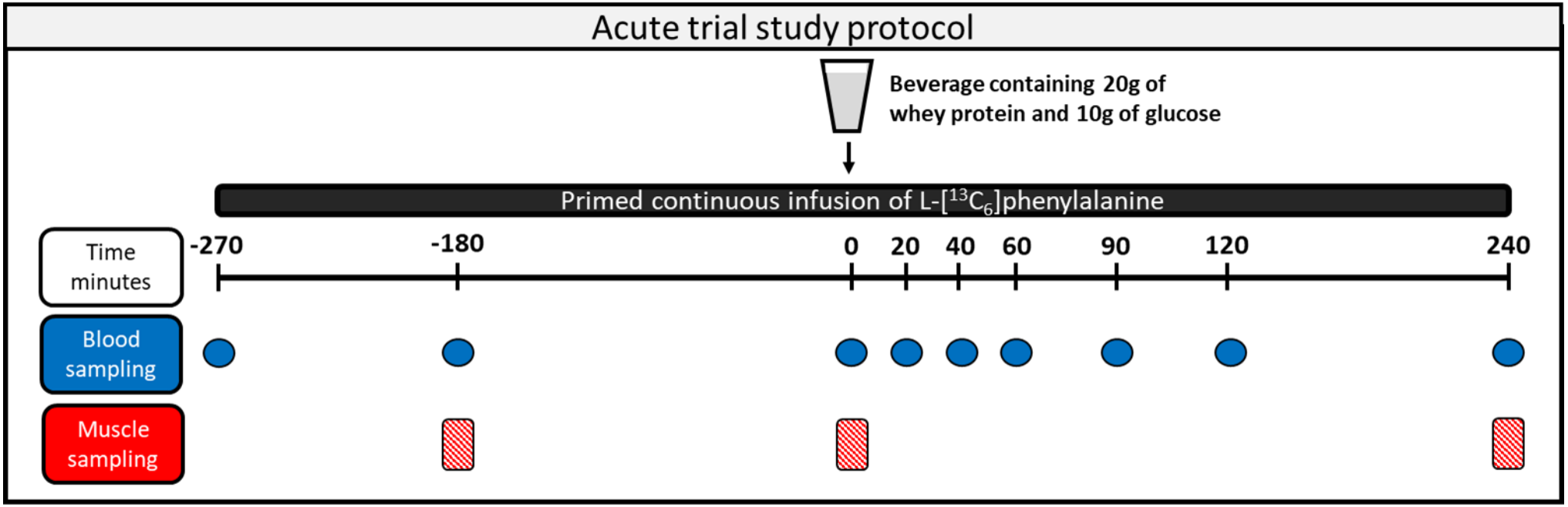
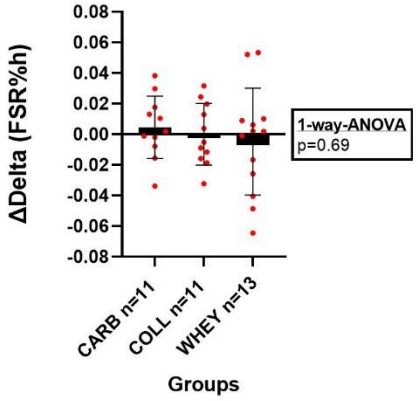


Table 1. Subject characteristics baseline. Means ± SD

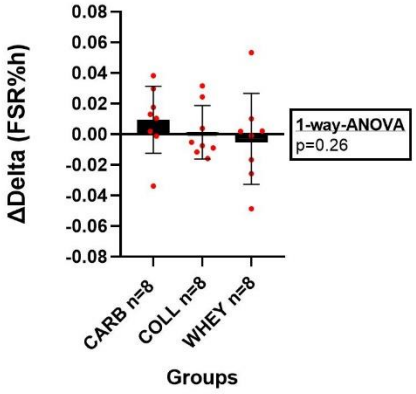
Subject characteristics at baseline (0 m)	CARB	COLL	WHEY	LITW	HRTW	One-way ANOVA
N (males /females)	12 (6/6)	15 (8/7)	15 (9/6)	12 (6/6)	12 (8/4)	
Age (y)	69 ± 4	70 ± 4	71 ± 5	69 ± 3	69 ± 3	P = 0.71
Height (m)	1.70 ± 0.06	1.72 ± 0.07	1.74 ± 0.07	1.72 ± 0.09	1.74 ± 0.09	P = 0.66
Weight (kg)	74.3 ± 11.3	77.0 ± 11.4	70.7 ± 8.5	73.2 ± 9.8	78.1 ± 14.7	P = 0.43
BMI (kg/m^2)	25.6 ± 3.6	26.2 ± 3.5	23.3 ± 1.9	24.8 ± 2.4	25.5 ± 3.3	P = 0.12
Fat free mass (kg)	48.3 ± 7.2	49.6 ± 9.3	48.2 ± 8.0	47.7 ± 10.4	52.8 ± 10.4	P = 0.64
30s chair rise test (repetitions)	21 ± 7	19 ± 4	19 ± 4	20 ± 4	21 ± 3	P = 0.74
Systolic blood pressure (mmHg)	154 ± 15	146 ± 24	145 ± 17	144 ± 19	149 ± 24	P = 0.74
Diastolic blood pressure (mmHg)	91 ± 10	82 ± 9	80 ± 9	85 ± 11	84 ± 9	P = 0.06
Glucose fasted (mmol/L)	5.5 ± 0.5	5.6 ± 0.4	5.5 ± 0.5	5.4 ± 0.3	5.6 ± 0.5	P = 0.78
Glucose 2 h OGTT (mmol/L)	6.8 ± 1.3	6.6 ± 1.5	6.1 ± 1.0	6.6 ± 1.1	6.0 ± 1.2	P = 0.40
Haemoglobin A1c (mmol/mol)	36 ± 2	36 ± 4	35 ± 3	34 ± 3	36 ± 2	P = 0.35
Total cholesterol (mmol/L)	5.9 ± 1.1	5.8 ± 1.3	5.7 ± 0.8	5.7 ± 0.9	5.9 ± 0.6	P = 0.97
HDL cholesterol (mmol/L)	1.9 ± 0.5	1.9 ± 0.5	2.0 ± 0.6	1.8 ± 0.4	1.9 ± 0.5	P = 0.90
LDL cholesterol (mmol/L)	3.4 ± 1.0	3.2 ± 1.2	3.1 ± 0.6	3.4 ± 1.0	3.5 ± 0.6	P = 0.78

Figure 2. 2a) ITT analysis of Δdelta FSR in the nutritional arm, 2b) PP analysis of Δdelta FSR in the nutritional arm, 2c) ITT analysis of Δdelta FSR in the training arm, 2d) Females basal vs. response FSR at 0month, 2e) Males basal vs. response FSR at 0 month. * denotes significant difference ($p<0.05$). Boxes are means±SEM for all plots.

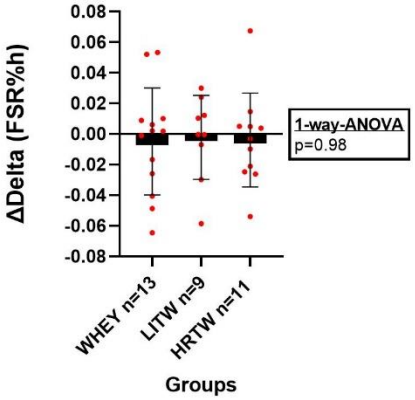
2a. ITT Nutrition Groups ΔDelta (means±SEM)



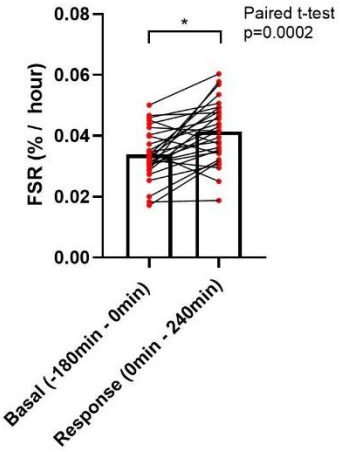
2b. PP Nutrition Groups ΔDelta (means±SEM)



2c. ITT Training Groups ΔDelta (means±SEM)



2d. Females, FSR 0 month(means±SEM), n=36



2e. Males, FSR 0 month(means±SEM), n=36

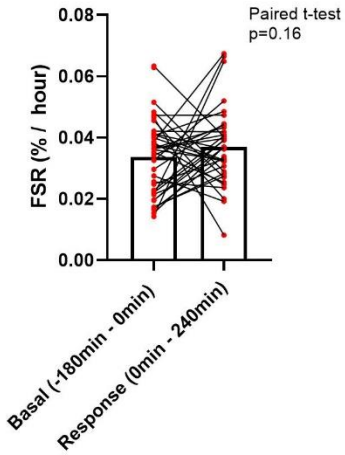


Figure 3. PCA model of the MM. Scores for PC1 vs PC2 (1st row), PC1 vs PC3(2nd row), PC2 vs PC3 (3rd row) colored according to baseline vs 12months (1st column), basal vs response (2nd column), sex (3rd column) and CALM design (4th column). Loadings are presented in the 5th column. 25% of variation is captured by the first three principal components of the PCA model, although no trend of separation of samples was observed according to visit, treatment, sex or the CALM intervention design. Control samples are grey and clustered well in all plots.

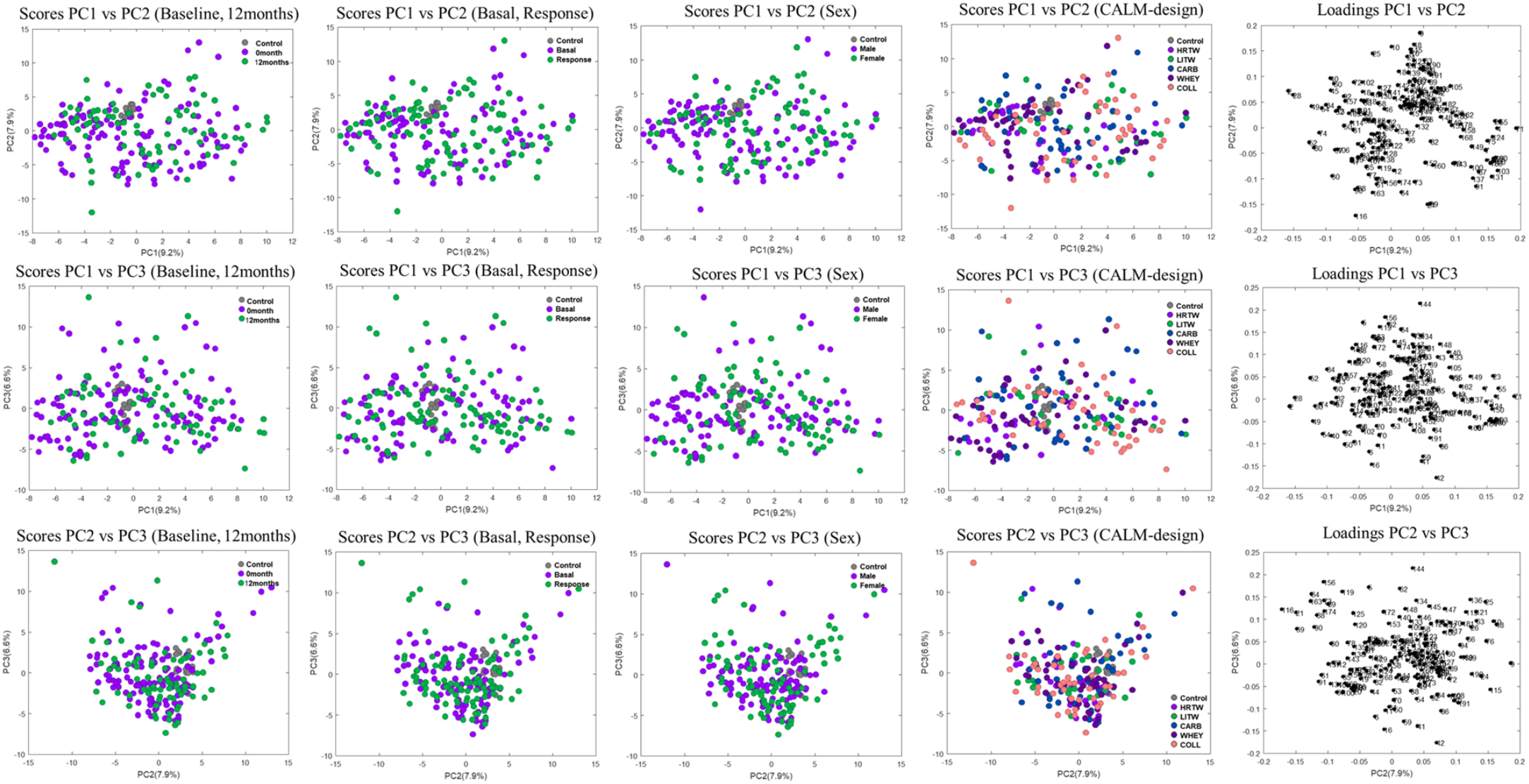
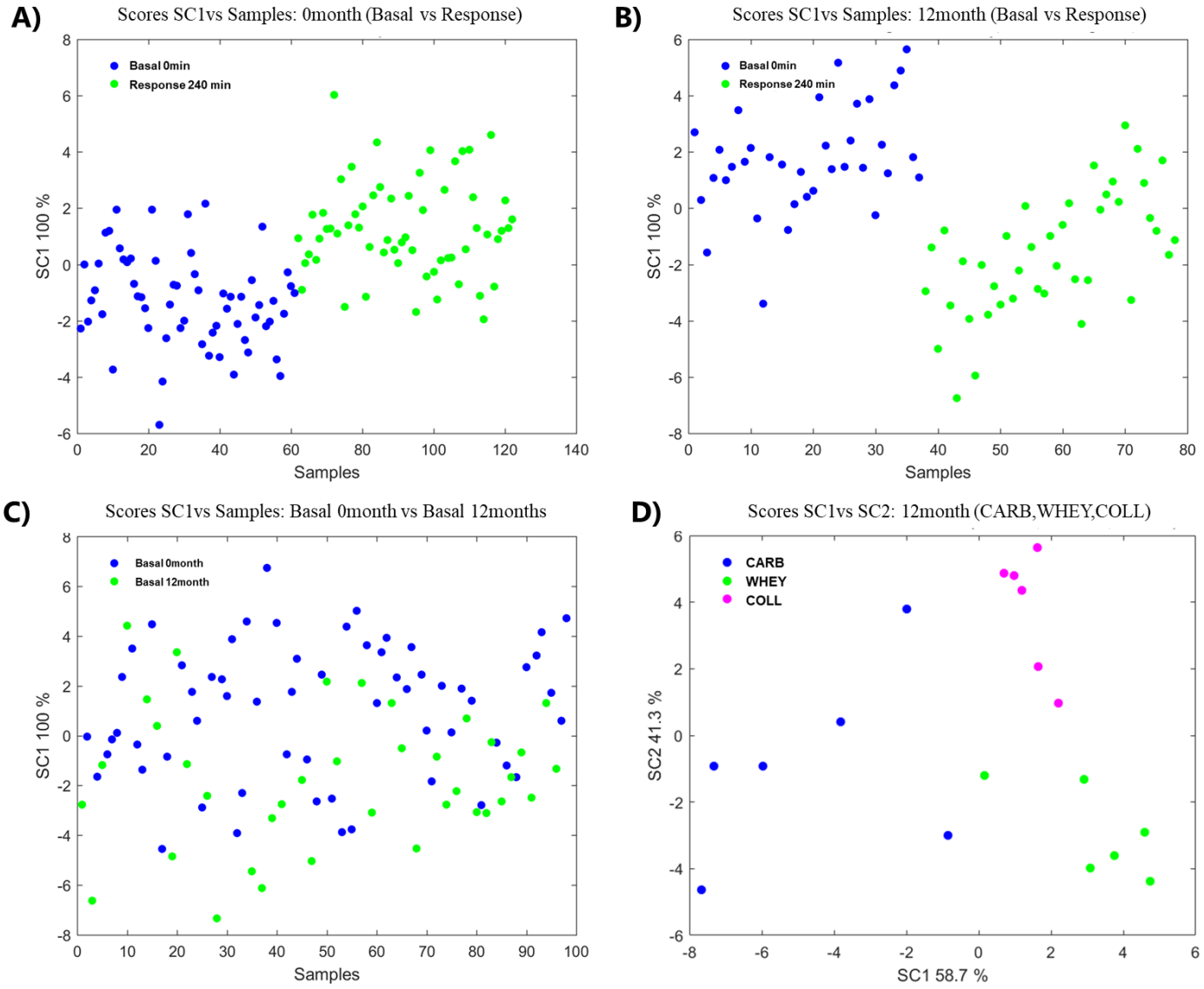


Figure 4. **A)** Scores SC1 0 month colored according to basal and 240 min response (ASCA: p=0.42). **B)** Scores SC1 12 month colored according to basal and 240 min response (ASCA: p=0.20). **C)** Scores SC1 colored according to basal at 0 and 12 months (p=0.62). **D)** Scores SC1 vs SC2 of basal at 12 months nutritional arm only colored according to CALM study design (ASCA: p=0.68)



Supplemental 1. Metabolite table. We measured 191 metabolite. 74 metabolites were identified at level 2 according to Sumner et al[34].

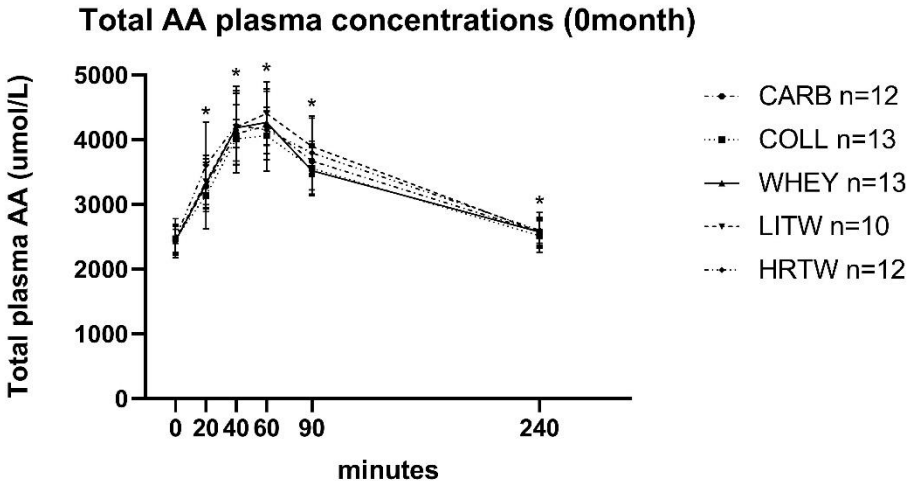
Variable #	Tentative Names	Final Names	Peak #	RI	RT	M/Z	good_mets_id	occurance	EI-MS from # sa	R of EI-MS from # samples	SN
1	Disiloxane, hexamethyl-	U1	2	1000.0013	412.4112	174	2	75.1111	87	0.95723	197.3424
2	N-(Trimethylsilyl)acetamide	Formamide, N,N-bis(trimethylsilyl	3	1000.0013	414.8244	116	3	53.7778	86	0.97851	563.1332
3	Analyte 2	U2	4	1000.0013	415.0115	237	4	72.4444	152	0.74302	965.8242
4	1-Hexene, 4,5-dimethyl-	2-Nonene, 3-methyl-, (E)-	5	1000.0013	415.761	71	5	55.5556	73	0.97476	154.5201
5	Disilathiane, hexamethyl-	Disilathiane, hexamethyl-	6	1000.0013	418.5392	163	6	97.7778	220	0.98845	1096.4126
6	Silanamine, N,N'-methanetetraylbis[1,1,1-tr	Disilathiane, hexamethyl-	8	1000.0013	420.0501	171	8	98.6667	210	0.99869	51706.7642
7	Analyte 17	U3	9	1000.0013	420.8275	77	9	53.7778	120	0.9579_a	228.6778
8	Disiloxanamine, 1,1,3,3,3-pentamethyl-N-(t	U4	10	1010.7212	430.5673	132	12	98.2222	143	0.9503	1348.5896
9	Cyclotetrasiloxane, octamethyl-	Cyclotetrasiloxane, octamethyl-	11	1012.9426	432.2824	281	13	99.5556	223	0.89556	518.0359
10	Tris(trimethylsilyl)borate	1,2-Bis(trimethylsiloxy)ethane	12	1014.9958	433.8675	221	14	96.4444	196	0.96871	461.9426
11	Analyte 35	U5	13	1016.5895	435.098	114	15	92.4444	178	0.88552	1828.2895
12	Silane, (hexyloxy)trimethyl-	Silane, (hexyloxy)trimethyl-	14	1019.4351	437.2949	159	17	69.3333	149	0.92185	163.5263
13	Disiloxane, hexamethyl-	2-Pentenoic acid, 4-oxo-, methyl e	15	1024.1669	440.9481	113	18	81.3333	177	0.8604	289.3013
14	1,2-Bis(trimethylsiloxy)ethane	U6	16	1024.9713	441.5691	235	19	33.7778	55	0.93516	39.9603
15	Analyte 51	U7	18	1028.746	444.4833	89	21	60	117	0.92386	190.8439
16	Propanetriol, 2-methyl-, tris-O-(trimethylsil	Propane, 2-methyl-1,2-bis(trimeth	19	1029.8347	445.3239	219	22	70.6667	152	0.97673	1324.2764
17	Analyte 52	U8	20	1032.3829	447.2913	281	23	90.2222	175	0.71655	150.0853
18	Analyte 53	U9	21	1033.2973	447.9972	185	24	59.5556	123	0.7436	140.7024
19	Analyte 55	U10	22	1035.9711	450.0615	133	25	56	98	0.88658	56.9422
20	Analyte 56	U11	24	1038.4447	451.9712	59	27	84.8889	137	0.94346	179.8458
21	Analyte 58	U12	26	1043.1102	455.5732	232	29	56.8889	125	0.91093	525.6997
22	Disiloxane, hexamethyl-	U13	29	1046.4524	458.1536	172	32	93.3333	206	0.88996	318.4354
23	Methyltris(trimethylsiloxy)silane	U14	30	1057.6349	466.787	207	33	55.5556	122	0.99427	3731.1869
24	Cyclohexene, 1-methyl-4-(1-methylethenyl)	Cyclohexene, 1-methyl-4-(1-meth	31	1059.8603	468.5051	68	35	97.7778	201	0.94964	135.5719
25	Analyte 81	U15	32	1065.0809	472.5357	140	36	59.1111	66	0.76839	88.4013
26	Methyltris(trimethylsiloxy)silane	U16	33	1066.7717	473.8411	207	38	100	224	0.98122	3451.1413
27	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trim	lactic acid-1TMS	34	1067.4143	474.3372	117	39	38.2222	36	0.9622	498.1359
28	Methyltris(trimethylsiloxy)silane	U17	35	1074.108	479.5051	207	40	43.5556	98	0.99453	2522.5536
29	Propanoic acid, 2-[(trimethylsilyl)oxy]-, trim	lactic acid-2TMS	36	1076.0516	481.0056	117	42	99.1111	221	0.9949	8003.9569
30	Bis-N,N-(trimethylsilyl)formamide	Oxalamic acid	39	1079.7819	483.8857	190	45	99.1111	133	0.99081	1380.7159
31	Silane, (2-ethoxyethoxy)trimethyl-	U18	40	1082.7713	486.1935	103	46	68.8889	112	0.98559	1090.8986
32	Mercaptoacetic acid, bis(trimethylsilyl)-	Borane, tris(trimethylsiloxy)-	41	1083.2468	486.5607	221	47	92.4444	206	0.96123	1517.4936
33	Hexanoic acid, trimethylsilyl ester	Hexanoic acid, trimethylsilyl ester	42	1088.8573	490.8922	173	48	77.3333	150	0.89433	137.3451
34	Analyte 108	U19	45	1093.163	494.2165	102	52	49.7778	90	0.90979	119.3774
35	Analyte 112	U20	46	1098.4823	498.3233	91	54	38.6667	74	0.91308	39.4518
36	Analyte 118	U21	49	1106.2117	504.2908	154	60	83.1111	179	0.92139	218.186
37	Acetic acid, [(trimethylsilyl)oxy]-, trimethyls	Oxalic acid, bis(trimethylsilyl) este	50	1114.4143	510.6236	204	61	39.1111	66	0.8767	79.1863
38	l-Alanine, N-(trimethylsilyl)-, trimethylsilyl e	Alanine, di-TMS	51	1114.6831	510.8311	116	62	91.1111	204	0.98851	3501.8802
39	Analyte 128	U22	52	1118.2932	513.6183	100	64	41.3333	68	0.88925	140.9357
40	Glycine, N-(trimethylsilyl)-, trimethylsilyl est	Glycine, N-(trimethylsilyl)-, trimeth	57	1131.4653	523.7878	102	69	76.4444	83	0.98507	1159.8716

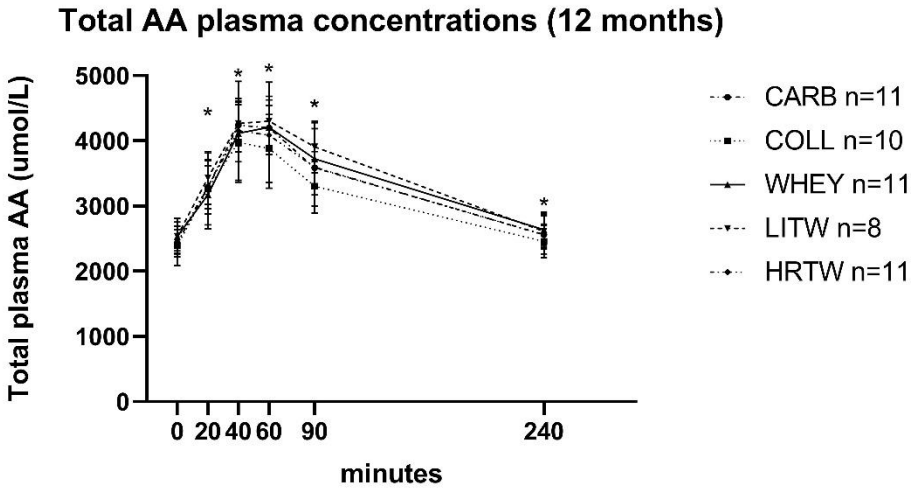
41	Tris(trimethylsilyl)amine	U23	58	1133.7484	525.5504	218	71	99.1111	122	0.9907	9462.1483
42	4,6-Dioxa-5-aza-2,3,7,8-tetrasilanonane, 2,2	U24	61	1141.6424	531.645	206	76	100	225	0.97151	776.1931
43	Cyclopentasiloxane, decamethyl-	U25	63	1146.7702	535.6039	267	80	34.2222	66	0.94417	52.9492
44	Analyte 155	U26	64	1148.2488	536.7455	124	81	97.7778	136	0.98729	295.6754
45	l-Leucine, trimethylsilyl ester	l-Isoleucine, trimethylsilyl ester	67	1164.3424	549.1706	86	87	42.6667	93	0.92856	189.0649
46	(R)-3-Hydroxybutyric acid, trimethylsilyl eth	Butanoic acid, 3-[(trimethylsilyl)ox	68	1166.1256	550.5473	117	88	82.2222	166	0.93757	112.0263
47	Toluene	n-Amylbenzene	70	1170.9506	554.2724	91	91	59.5556	89	0.9401	56.7897
48	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(	U27	73	1175.1538	557.5175	281	94	98.6667	218	0.97929	428.0411
49	Butanoic acid, 2-[(trimethylsilyl)amino]-, tri	Butanoic acid, 2-[(trimethylsilyl)an	74	1177.1609	559.0671	130	96	42.2222	93	0.91378	203.627
50	Phenyl-pentamethyl-disiloxane	Phenyl-pentamethyl-disiloxane	75	1180.8902	561.9463	209	97	56.8889	120	0.85335	185.4223
51	Phosphoric acid, bis(trimethylsilyl)monome	O-Methyl phosphate	76	1182.0225	562.8205	241	98	93.7778	208	0.84539	453.5068
52	Analyte 193	U28	79	1184.32	564.5942	246	102	57.7778	109	0.74039	110.18
53	Analyte 199	U29	82	1191.0181	569.7655	211	106	89.3333	189	0.83194	134.0948
54	Analyte 201	U30	83	1194.7806	572.6703	168	107	45.3333	93	0.91564	67.0471
55	Analyte 205	U31	85	1196.7206	574.1681	115	109	51.5556	104	0.94919	75.6103
56	Analyte 208	trans-3-Hexen-1-ol, tert-butyldime	86	1200.5028	577.047	157	111	76.8889	150	0.79034	58.3689
57	Analyte 209	U32	87	1204.2559	579.6366	246	112	91.1111	195	0.94687	188.2408
58	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(	U33	88	1211.2947	584.4933	281	116	100	224	0.9878	420.6674
59	Analyte 214	U34	89	1215.8733	587.6526	185	117	98.6667	196	0.96313	597.7134
60	L-Valine, N-(trimethylsilyl)-, trimethylsilyl es	L-Valine, N-(trimethylsilyl)-, trimet	90	1218.7471	589.6355	144	118	68.8889	102	0.82544	310.2387
61	1-Dimethyl(isopropyl)silyloxypropane	Carnitine	91	1220.0865	590.5597	117	119	99.5556	222	0.98631	1647.6422
62	Analyte 220	U35	92	1223.6883	593.045	227	120	50.6667	73	0.97705	56.4725
63	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(	U36	93	1229.3458	596.9486	281	121	97.3333	214	0.96653	217.4709
64	Analyte 228	U37	95	1238.9058	603.545	100	123	66.6667	143	0.96039	432.6445
65	Benzothiazole	Benzothiazole	96	1241.9154	605.6217	135	124	49.7778	85	0.90744	64.5704
66	Hydrazine, 1-ethyl-1-(2-methylpropyl)-	Diethylene glycol, bistrimethylsilyl	97	1246.0593	608.4809	73	125	78.6667	113	0.96931	72.0553
67	Benzoic acid trimethylsilyl ester	Benzoic acid trimethylsilyl ester	98	1252.1097	612.6557	179	127	99.5556	148	0.94651	232.8145
68	Urea, N,N'-bis(trimethylsilyl)-	Serine, bis(trimethylsilyl)-	99	1260.1907	618.2316	116	129	81.3333	97	0.926	116.9786
69	Octanoic acid, trimethylsilyl ester	Octanoic acid, trimethylsilyl ester	101	1267.5612	623.3172	117	132	94.2222	122	0.94226	80.0922
70	Silanol, trimethyl-, phosphate (3:1)	phosphate	103	1275.933	629.0938	133	134	97.7778	87	0.97986	5429.2322
71	Analyte 255	U38	104	1279.7839	631.7509	169	135	61.3333	138	0.99804	1197.6674
72	l-Threonine, O-(trimethylsilyl)-, trimethylsily	l-Threonine, O-(trimethylsilyl)-, tri	106	1297.1555	643.7373	130	138	78.6667	101	0.95368	112.9623
73	Glycine, N,N-bis(trimethylsilyl)-, trimethylsil	Glycine, N,N-bis(trimethylsilyl)-, tri	108	1309.1282	651.9985	174	140	36.4444	47	0.93843	216.4993
74	Analyte 267	U39	109	1310.0961	652.6663	84	141	53.7778	43	0.97633	135.5397
75	Analyte 271	U40	110	1311.0712	653.3391	197	142	41.7778	62	0.85815	63.2841
76	Butanedioic acid, bis(trimethylsilyl) ester	Butanedioic acid, bis(trimethylsilyl	112	1315.2085	656.1939	75	144	100	98	0.98316	186.1413
77	Analyte 285	Oxamimidic acid	114	1330.1281	666.4884	102	146	91.1111	158	0.9463	248.9708
78	á-Hydroxypyruvic acid, trimethylsilyl ether,	U41	116	1337.1286	671.3188	103	148	35.5556	73	0.96109	62.1547
79	Analyte 294	U42	117	1341.5974	674.4022	198	149	96	172	0.99342	4034.0694
80	Analyte 297	U43	118	1347.036	678.1549	320	152	59.5556	109	0.90136	51.4646
81	2-Butenedioic acid (E)-, bis(trimethylsilyl) es	2-Butenedioic acid (Z)-, bis(trimeth	119	1350.9194	680.8344	245	153	87.5556	190	0.84111	207.0125
82	Analyte 306	U44	120	1353.1108	682.3464	111	154	46.6667	45	0.96026	75.0194
83	N,N-Dimethylglycine, trimethylsilyl ester	U45	121	1356.8062	684.8963	58	157	99.1111	223	0.9953	2976.2553
84	Mercaptoacetic acid, bis(trimethylsilyl)-	U45	122	1359.7636	686.9369	221	159	88	183	0.96719	108.2735
85	Nonanoic acid, trimethylsilyl ester	Nonanoic acid, trimethylsilyl ester	123	1361.7391	688.3	117	160	100	183	0.99223	468.0082
86	Analyte 317	U46	124	1370.3921	694.2706	131	162	100	200	0.99306	6903.4971
87	Butanoic acid, 3-methyl-2-[(trimethylsilyl)ox	U47	125	1379.9302	700.8519	201	165	48	87	0.96107	263.9235
88	Analyte 328	U48	127	1390.2501	707.9726	126	169	97.3333	218	0.99664	2920.0345
89	Hexasiloxane, tetradecamethyl-	U49	128	1391.3129	708.7059	221	170	61.7778	132	0.93818	121.7981
90	Analyte 334	U50	131	1396.6395	712.3813	83	173	97.7778	214	0.97236	398.8362

91	Butanoic acid, 3-methyl-2-[(trimethylsilyl)ox	U51	132	1398.6609	713.776	145	174	55.5556	68	0.97373	217.9278
92	l-Aspartic acid, bis(trimethylsilyl) ester	l-Aspartic acid, bis(trimethylsilyl) e	136	1422.7863	728.9688	160	180	39.1111	63	0.92274	80.9993
93	Analyte 364	U52	139	1441.4076	740.6295	153	185	99.5556	222	0.99916	2717.9881
94	Decanoic acid, trimethylsilyl ester	Decanoic acid, trimethylsilyl ester	140	1457.9687	751	117	187	90.2222	180	0.93037	59.804
95	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-t	U53	143	1474.032	761.0588	281	193	90.6667	165	0.96881	68.884
96	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(t	U54	144	1487.2593	769.3418	101	196	92	75	0.97748	85.32
97	Analyte 392	U55	145	1491.8492	772.216	221	198	65.3333	123	0.9761	64.6886
98	Analyte 393	3,4-Dimethylbenzoic acid, trimeth	146	1493.789	773.4307	163	199	56.8889	102	0.76693	40.1995
99	Benzoic acid, 4-[(trimethylsilyl)oxy]-, methy	Benzoic acid, 4-[(trimethylsilyl)oxy	147	1496.0387	774.8394	209	200	60.8889	120	0.75619	107.3734
100	Analyte 409	U56	151	1509.191	783.0754	159	205	51.5556	84	0.96023	365.3134
101	L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethy	L-Proline, 5-oxo-1-(trimethylsilyl)-,	152	1520.0352	789.866	156	208	99.1111	221	0.99045	2528.0312
102	Analyte 422	U57	154	1530.6018	796.4829	84	210	77.7778	89	0.97639	434.8738
103	Analyte 430	U58	157	1537.7336	800.9488	173	214	36.8889	51	0.97061	192.7489
104	Analyte 435	1,3-Benzoxazol-2-amine-ditms	158	1539.559	802.0919	263	216	95.5556	209	0.93392	324.6661
105	Disiloxane, hexamethyl-	U59	161	1549.5305	808.336	221	219	89.7778	189	0.96364	94.6367
106	2,3,4-Trihydroxybutyric acid tetrakis(trimetl	U60	164	1557.5457	813.3551	73	222	39.1111	60	0.97822	109.8483
107	Analyte 453	U61	165	1564.4673	817.6894	225	223	100	168	0.99852	369.0536
108	Diethyl Phthalate	Diethyl Phthalate	167	1589.0069	833.0561	149	228	100	219	0.99549	1483.561
109	Analyte 472	U62	170	1605.0321	842.7494	204	233	93.7778	208	0.98044	1497.2565
110	Benzothiazole, 2-(methylthio)-	Benzothiazole, 2-(methylthio)-	171	1614.6977	848.1457	181	234	93.7778	135	0.96048	400.2211
111	Decamethyltetrasiloxane	U63	173	1632.2023	857.9185	221	238	39.5556	36	0.97525	61.7908
112	Cyclooctasiloxane, hexadecamethyl-	U64	174	1634.3875	859.1386	355	239	57.3333	93	0.97037	71.9338
113	Propanoic acid, 2-oxo-, trimethylsilyl ester	Dodecanoic acid, trimethylsilyl est	175	1650.635	868.2095	117	241	92	187	0.94811	72.2378
114	Analyte 502	U65	177	1662.2526	874.6956	276	243	99.1111	122	0.98754	662.8052
115	Analyte 505	U66	178	1673.4057	880.9224	153	244	97.3333	149	0.99833	405.8138
116	Analyte 510	U67	180	1693.16	891.9512	225	246	99.1111	210	0.99879	1930.7747
117	Analyte 515	U68	181	1703.1347	897.5201	221	250	85.7778	174	0.96272	61.6405
118	3-Pentanamine	U69	186	1733.9243	914.7099	58	259	58.2222	114	0.99799	513.3305
119	Phosphoric acid, bis(trimethylsilyl) 2,3-bis[(t	U70	189	1759.2783	928.8651	299	263	89.7778	73	0.98715	432.9142
120	Disiloxane, hexamethyl-	Ribitol, 1,2,3,4,5-pentakis-O-(trime	197	1826.5924	965.0757	73	272	65.3333	66	0.97812	120.5407
121	Tetradecanoic acid, trimethylsilyl ester	Tetradecanoic acid, trimethylsilyl e	201	1846.497	975.1623	117	276	98.2222	200	0.96503	198.4957
122	1,5-Anhydro-D-sorbitol, tetrakis(trimethylsi	U71	203	1849.0281	976.445	217	278	88.8889	174	0.98011	82.4511
123	Analyte 570	U72	204	1853.4108	978.6659	221	279	73.3333	136	0.97668	54.9598
124	D-Pinitol, pentakis(trimethylsilyl) ether	U73	206	1861.8967	982.9662	149	281	37.7778	32	0.93783	66.0444
125	2-Propanamine, N-methyl-	U74	208	1877.9959	991.1244	58	283	99.1111	223	0.99819	1393.2382
126	Analyte 581	D-Glucose, 2,3,4,5,6-pentakis-O-(t	209	1890.42	997.4203	204	284	40.4444	72	0.95514	210.6592
127	Nonadecane, 2-methyl-	U75	213	1914.7673	1009.7583	71	290	50.2222	74	0.93043	37.0309
128	Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	Ribitol, 5TMS	216	1926.605	1015.7571	205	293	90.6667	66	0.98352	40.1469
129	D-Mannitol, 1,2,3,4,5,6-hexakis-O-(trimethy	d-Galactose, 2,3,4,5,6-pentakis-O-	218	1935.518	1020.2738	205	295	99.5556	117	0.99792	3630.1913
130	Heptacosane	Nonadecane, 2-methyl-	220	1956.712	1031.0138	71	298	33.7778	49	0.9309	26.4561
131	Ethanol, 2-ethoxy-	Pentanamide	222	1971.2372	1038.3745	59	301	37.7778	72	0.98946	237.238
132	á-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(tr	D-Glucose, 2,3,4,5,6-pentakis-O-(t	223	1974.7217	1040.1402	204	302	42.2222	80	0.98094	249.9379
133	Analyte 615	U76	226	1999.4109	1052.6515	221	306	74.2222	124	0.97933	51.4754
134	10-Undecenoic acid, trimethylsilyl ester	Oleic acid, trimethylsilyl ester	228	2021.4848	1062.8985	117	309	58.2222	85	0.93952	70.9589
135	Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsily	Chizo-Inositol, per(trimethylsilyl) e	229	2025.062	1064.555	217	311	99.1111	171	0.99676	258.9738
136	Hexadecanoic acid, trimethylsilyl ester	Hexadecanoic acid, trimethylsilyl e	231	2044.3862	1073.503	117	313	100	220	0.99826	4517.9033
137	1-Undecene, 11-nitro-	9-Octadecenal	236	2081.9537	1090.8987	122	320	36.4444	62	0.93584	86.7878
138	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimeth	Myo-Inositol, 1,2,3,4,5,6-hexakis-C	237	2090.5201	1094.8653	217	321	99.5556	215	0.99764	2681.6539
139	Dodecane, 2-methyl-	U77	239	2125.8815	1111.2394	71	323	94.6667	124	0.97628	92.8165
140	Analyte 653	U78	242	2145.6229	1120.3807	221	326	70.6667	128	0.98435	46.13

141	2-Bromotetradecane	Stearyl iodide	245	2167.1	1130.3256	71	329	79.5556	79	0.95114	45.6706
142	Analyte 660	U79	246	2172.197	1132.6858	191	330	75.1111	56	0.84872	68.1501
143	Pentanamide	U80	247	2179.8223	1136.2167	59	331	70.6667	150	0.98791	277.7702
144	9,12-Octadecadienoic acid (Z,Z)-, trimethyls	U80	249	2207.7212	1148.8415	79	334	92.8889	47	0.97543	314.6022
145	Oleic acid, trimethylsilyl ester	trans-Oleic acid	250	2214.4616	1151.7062	117	335	99.5556	113	0.99515	1283.8822
146	Oleic acid, trimethylsilyl ester	cis-9-Octadecenoic acid, trimethyl	251	2220.7465	1154.3772	129	336	74.2222	45	0.93991	212.1862
147	Octadecanoic acid, trimethylsilyl ester	Octadecanoic acid, trimethylsilyl e	252	2241.7283	1163.2945	117	337	100	197	0.99772	2321.6506
148	Analyte 682	U81	254	2287.8517	1182.897	221	340	82.6667	142	0.93591	59.0387
149	Analyte 685	U82	255	2302.6018	1189.1658	57	341	37.7778	59	0.92143	32.6326
150	Analyte 688	U83	256	2315.8502	1194.7963	165	342	35.1111	74	0.96953	383.5612
151	2-methyltetracosane	Sulfurous acid, dodecyl pentyl est	257	2336.8628	1203.7267	71	343	86.2222	127	0.94775	75.2394
152	9-Octadecenamide, (Z)-	U84	264	2364.4945	1215.4702	59	351	79.5556	93	0.96202	944.7889
153	Sulfurous acid, 2-ethylhexyl hexyl ester	U85	267	2377.5574	1221.0219	57	354	39.5556	33	0.94293	34.6045
154	Hexanedioic acid, bis(2-ethylhexyl) ester	Hexanedioic acid, bis(2-ethylhexyl	268	2389.1892	1225.9654	129	355	99.1111	222	0.98995	2178.6528
155	Analyte 717	U86	272	2500.6786	1239.9936	221	360	76.8889	151	0.97499	48.9042
156	Glycerol, tris(trimethylsilyl) ether	Diglycerol	275	2596.0701	1248.9318	103	363	85.3333	175	0.9579_b	117.7555
157	Analyte 728	U87	277	2617.7604	1257.3899	207	366	63.1111	104	0.84174	29.2871
158	Diethylene glycol dibenzoate	U88	278	2620.577	1258.6728	105	367	55.1111	84	0.95431	101.3647
159	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,1	U89	279	2622.478	1259.5388	73	368	53.3333	105	0.99331	100.9203
160	Analyte 734	U90	281	2632.0813	1263.913	233	370	87.5556	98	0.98118	1034.7701
161	Tetradecane, 1-iodo-	U91	283	2664.9552	1278.8871	57	373	76.4444	53	0.94995	64.0469
162	Analyte 749	U92	285	2674.8164	1283.3789	221	376	85.3333	182	0.97951	54.0882
163	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)o	Hexadecanoic acid, 2,3-bis[(trimet	287	2687.5374	1289.1733	371	379	96.4444	98	0.98181	107.4469
164	1-Iodo-2-methylundecane	U93	288	2698.8474	1294.325	57	380	43.5556	48	0.94822	58.4199
165	Analyte 760	U94	289	2706.244	1297.6942	73	381	70.6667	76	0.995	148.2395
166	Analyte 763	U95	291	2721.4763	1304.6324	465	383	98.2222	143	0.99495	116.2192
167	Analyte 769	U96	292	2748.6061	1316.9901	221	384	89.7778	191	0.98182	59.2824
168	Nonadecane	U97	293	2753.0107	1318.9964	57	385	36.8889	41	0.98612	90.0926
169	Analyte 774	U98	294	2764.6882	1324.3155	141	386	85.7778	175	0.905	70.1041
170	Analyte 775	U99	295	2769.595	1326.5505	243	387	52	112	0.84993	65.1106
171	Analyte 777	U100	296	2773.2404	1328.211	221	388	67.1111	138	0.98797	78.2023
172	1-Monooleoylglycerol trimethylsilyl ether	Oleoylglycerol	297	2776.8977	1329.8769	101	389	41.7778	19	0.88527	34.75
173	Heptacosane	U101	298	2779.7786	1331.1891	69	391	77.3333	52	0.92242	28.6583
174	Octadecanoic acid, 2,3-bis[(trimethylsilyl)ox	Stearin, 1-mono-	299	2788.0811	1334.9709	73	392	84.4444	92	0.93167	43.689
175	9-Octadecenamide, (Z)-	U102	300	2793.22	1337.3117	59	393	96.8889	173	0.97899	421.4532
176	Heptacosane	U103	301	2800.9412	1340.5954	57	394	35.1111	62	0.98169	94.1214
177	Squalene	Squalene	302	2818.1035	1344.1592	69	395	99.5556	219	0.99452	453.3256
178	Analyte 790	U104	303	2820.529	1344.6628	221	396	78.2222	168	0.95762	63.1773
179	Heptasiloxane, hexadecamethyl-	U105	304	2865.8365	1354.071	73	398	74.6667	88	0.99328	90.5812
180	Analyte 799	U106	305	2899.341	1361.0282	71	399	40.8889	46	0.97611	54.9109
181	Analyte 801	U107	306	2915.515	1364.3867	243	400	47.1111	55	0.90848	43.7324
182	Heptasiloxane, hexadecamethyl-	U108	308	2945.0145	1370.5123	221	402	84.8889	173	0.97055	58.1095
183	Heptadecane, 2,6,10,15-tetramethyl-	U109	309	2964.855	1374.6321	71	403	64.4444	57	0.92805	39.7517
184	Heptasiloxane, hexadecamethyl-	U110	310	2988.1251	1379.4642	73	404	67.1111	74	0.99176	83.6331
185	Analyte 817	U111	312	3066.6099	1397.6399	221	406	72	133	0.9586	46.4073
186	Heptadecane, 2,6,10,15-tetramethyl-	U112	313	3096.4772	1404.6842	57	407	34.2222	40	0.97019	47.8818
187	Heptasiloxane, hexadecamethyl-	U113	314	3103.1264	1406.2524	73	408	52.4444	61	0.9869	54.4761
188	Analyte 822	alpha-Tocopherol	316	3143.6793	1415.8168	237	410	50.6667	88	0.94127	56.4285
189	Analyte 823	U114	317	3153.7416	1418.19	119	411	93.7778	184	0.98659	377.9998
190	Cholesterol trimethylsilyl ether	Cholesterol trimethylsilyl ether	318	3174.9029	1423.1808	129	412	99.5556	223	0.99756	1538.1991
191	Analyte 846	U115	326	3582.2998	1561.5376	57	424	96.4444	194	0.99368	286.2417

Supplemental 2. Total plasma amino acid concentrations at timepoint 0, 20, 40, 60, 90 and 240 min at 0 and 12 months. No effect of groups were observed at 0 month (p=0.58) or 12 months (p=0.44). * denotes significantly different from 0 min, p<0.0001. Analyzed using mixed effects analysis with Dunnets multiple comparison test in GraphPad Prism v. 8.0.0.





PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.

1. Declaration by	
Name of PhD student	Jacob Bülow
E-mail	jacob.bulow@live.dk
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	The Ageing Skeletal Muscle: Effects of Training and Protein supplementation

2. The declaration applies to the following article	
Title of article	The effect of long-term protein supplementation with or without different types of training on the skeletal muscle protein synthesis rate and metabolome in healthy elderly Danes: the CALM study.
Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☐ Date:	Manuscript not submitted ☒
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

3. The PhD student's contribution to the article ***(please use the scale A-F as benchmark)*** Benchmark scale of the PhD-student's contribution to the article **A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant	**A, B, C, D, E, F**
1. Formulation/identification of the scientific problem	F
2. Development of the key methods	B
3. Planning of the experiments and methodology design and development	F
4. Conducting the experimental work/clinical studies/data collection/obtaining access to data	C
5. Conducting the analysis of data	A
6. Interpretation of the results	A
7. Writing of the first draft of the manuscript	A
8. Finalisation of the manuscript and submission	F

Provide a short description of the PhD student´s specific contribution to the article.[i]
The PhD student has been the primary responsible of the daily mangement of the CALM study and been involved in both the clinical studies and the collection of data. The PhD student has been involved in the collecting and preparation of half of the samples for FSR analysis. The PhD student has been involved in the development used for measuring the skeletal muscle metabolome. The PhD student has conducted all of the analysis regarding the FSR. The PhD student has been assisted by Bekzod Khakimov on the practical aspects of the analysis of the skeletal muscle metabolome. The PhD student has conducted the interpretation and writing of the manuscript

4. Material from another thesis / dissertation[ii]	
Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)?	Yes: ☐ No: ☒
If yes, please state name of the author and title of thesis / dissertation.	
If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another.	

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	28-03-2020	Bekzod Khakimov	PhD	
2.	27-03-2020	Søren Reitelseder	PhD	
3.	28-3-2020	Rasmus Bechshøft	MD, PhD	
4.	26-03-2020	Søren Balling Engelsen	Professor	
5.	25.03.2020	Lars Holm	Professor	
6.				
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 30-3-2020
Principal supervisor: Michael Kjær

Name: Michael Kjær
Principal supervisor

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 28-3-2020
PhD student: Jacob Bülow

Please learn more about responsible conduct of research on the Faculty of Health and Medical Sciences' website.

[i] This can be supplemented with an additional letter if needed.

[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."

[iii] If more signatures are needed please add an extra sheet.