Disorders of bilirubin and lipid metabolism
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Chapter 5

Short-term protein restriction at advanced age stimulates FGF21 signalling, energy expenditure and browning of white adipose tissue


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Keywords: Dietary protein restriction, FGF21 signalling, energy metabolism, thermogenesis, browning of white adipose tissue


Abstract

Dietary protein restriction has been demonstrated to improve metabolic health under various conditions. However, the relevance of ageing and age-related decline in metabolic flexibility on the effects of dietary protein restriction have not been addressed. Therefore, we investigated the effect of short-term dietary protein restriction on metabolic health in young and aged mice. Young adult (3 months old) and aged (18 months old) C57Bl/6J mice were subjected to a 3-month dietary protein restriction. Outcome parameters included FGF21 levels, muscle strength, glucose tolerance, energy expenditure, and transcriptomics of brown- and white adipose tissue. Here we report that a low protein diet had beneficial effects in aged mice by reducing some aspects of age-related metabolic decline. These effects were characterized by increased plasma levels of FGF21, browning of subcutaneous white adipose tissue, increased body temperature and energy expenditure, while no changes were observed in glucose homeostasis and insulin sensitivity. Moreover, the low protein diet used in this study was well tolerated in aged mice indicated by the absence of adverse effects on body weight, locomotor activity, and muscle performance. In conclusion, our study demonstrates that a short-term reduction in dietary protein intake can impact age-related metabolic health alongside increased FGF21 signalling, without negatively affecting muscle function. These findings highlight the potential of protein restriction as a strategy to induce energy expenditure and browning of white adipose tissue in aged individuals.
Introduction

Ageing and age-associated changes in lifestyle are known to affect a wide range of metabolic processes. Ageing itself is one of the main risk factors for the development of chronic metabolic diseases, including type 2 diabetes (T2D), cardiovascular disease and cancer. With the worldwide increase in life expectancy and median lifespan over the last few decades, understanding the mechanisms by which ageing affects metabolic processes has become an increasingly important research focus. Caloric restriction (CR) is one of the most effective strategies to delay the symptoms of ageing and to extend longevity in a wide variety of animals. CR, however, is difficult to maintain and its long-term success has been limited by the poor adherence to this diet. Recently, changing macronutrient balance has emerged as a more feasible alternative for CR. Long-term dietary protein restriction without reducing total caloric intake has been shown to have similar beneficial effects on metabolic health and extension of longevity in mice as CR. Within one week after the dietary switch, mice were protected against obesity and resistant to cold stress as a result of increased energy expenditure and increased utilization of free fatty acids and glucose in brown adipose tissue.

Mechanisms underlying the beneficial metabolic effects of protein restriction have been associated with multiple pathways including mTOR and FGF21 signalling. FGF21 has recently emerged as an endocrine signal associated with metabolic control, as it is increased in response to fasting, starvation, protein restriction and physical exercise as well as overfeeding, ageing, and metabolic diseases such as obesity, T2D, and non-alcoholic fatty liver disease. FGF21 signalling is associated with improved metabolic health and longevity as transgenic mice overexpressing FGF21 exhibit increased lifespan and share a number of beneficial phenotypes with long lived dwarf mice. Currently, clinical trials are approved for FGF21 analogues in T2D patients to lower body weight, insulin levels and plasma triglycerides. In response to a low protein (LP) diet, transcription of Fgf21 is rapidly induced in the liver by both PPARα signalling as well as the GCN2/eIF2 pathway, and FGF21 is subsequently secreted into the circulation. Work on (hepatic) FGF21-deficient mice showed that the LP diet-induced effects on basal- and cold-induced energy expenditure, insulin sensitivity, glucose metabolism, and fatty acid clearance were FGF21-dependent. Together, these studies suggest that protein restriction induced hepatic FGF21 production and activated brown adipose tissue (BAT) and white adipose tissue (WAT) to increase thermogenesis, resulting in increased energy expenditure.

Ageing is, however, associated with diminished lipid handling, defective thermogenesis and impaired de novo adipogenesis in subcutaneous WAT and BAT, which together contribute to the development of age-dependent insulin resistance and dyslipidaemia. During ageing, the expansion of subcutaneous WAT is accompanied by a decrease in thermogenic capacity of this depot, reflected by decreased expression of the key
thermogenic marker \( Ucp1 \)\(^{29}\) and a reduced ability to maintain constant body temperature in response to cold exposure \(^{30}\). Whether a short-term protein restriction, starting at an advanced age, has beneficial effects on metabolic health is not known. Here we asked whether a short-term dietary intervention with reduced protein content would be able to improve metabolic health at an advanced age. Using aged mice, we examined the effects of an LP diet on FGF21 levels, insulin sensitivity, energy expenditure, and thermogenesis. We found a strong increase in plasma FGF21 levels in response to low dietary protein intake in aged mice, accompanied by an improvement in whole body energy homeostasis and WAT thermogenesis. Together, these findings highlight the role of protein restriction in the amelioration of the age-related decline of adipose function.

**Methods**

**Animals**

Male C57BL/6J mice (Charles River, USA), bred in our facility, were used at 3 months (\( n=13 \)) and 18 months (\( n=22 \)) of age in this study. Animals were housed in a light- and temperature-controlled facility (12-hour light cycle, 21°C room temperature) with free access to water and standard chow (RM1; SDS Diets, Woerden, The Netherlands). Animal experiments were performed with the approval of the National Ethics Committee for Animal Experiments of The Netherlands, in accordance with relevant guidelines and regulations (including laboratory and biosafety regulations). Mice were excluded from the experiment if they reached humane endpoint (15% body weight loss or sustained inactivity) or had developed severe (liver) tumours at the end of the experiment. In total, less than 15% of the aged animals were excluded from the study, with no substantial differences between experimental groups.

**Animal experiments**

To study the short-term metabolic effects of protein intake, animals were given a semi-synthetic diet, varying in protein content, for twelve weeks. Prior to the experiment, mice were given a run-in diet (20% protein control diet) for two weeks, to normalize microbial status. All experimental diets were based on the AIN-93G breeding diet supplemented with cysteine (D10012G, Open Source Diets, New Brunswick, NJ, USA) containing a fixed fat content of 20% (% of total kcal). Experimental diets consisted of a low-protein diet (LP; 7% of total kcal), medium-protein diet (MP or control; 20% of total kcal), and high-protein diet (HP; 40% of total kcal), in which protein is replaced by an isocaloric amount of starch. For a detailed description of the diets, see Supplementary Table 1.

Aged animals (18 months of age) were divided into the experimental groups, normalized for body weight and 4h-fasting glucose, insulin, and cholesterol levels (Supplementary Figure 1). Young adult animals (3 months of age) were randomly assigned to one of the
three experimental diets. Animals were weighed weekly and body composition was
determined by nuclear magnetic resonance (NMR), both during the run-in period and after
eight weeks of the experimental diet, using the Bruker Minispec LF110 BCA-Analyzer
(Bruker BioSpin, Rheinstetten, Germany). Food intake was measured several times for a
72h period. Two days before termination, mice were injected subcutaneously with a
temperature sensor (IPITT300, BMDS, Seaford DE, USA), and body temperature was
measured the next day during the light phase. Animals were terminated after a 2-6 hour
fast, introduced one hour before the lights were turned on. A detailed schematic overview
of the experimental design is shown in Figure 1A.

Muscle strength, endurance and physical performance tests

The hanging wire test and grip strength test were performed during both the run-in
period and after eight weeks of the experimental diet to determine changes in physical
strength. Physical health and motor skill learning ability were tested using the rotarod test
after seven weeks of the experimental diet.

Grip strength test: Forelimb grip strength was measured using Amatek digital force
gauge (Chatillon DFE II, Elancourt, France). Mice were positioned to grasp the bar with
forelimbs only and then pulled horizontally until letting go. The test was performed six
times (two rounds of three trials each). Within rounds, mice were given minimal rest (30sec)
while between rounds, mice were returned to their home cage for >15min. The maximum
grip strength of the six trials was recorded.

Hanging wire test: Strength and endurance were tested using the hanging wire test. A
wire (3 mm in diameter, 55 cm in length) was suspended between two supports, 35cm
above the tabletop. Mice were positioned at the centre of the wire, hanging by their
forelimbs. The test was performed six times (two rounds of three trials each), in which the
maximal hanging times were recorded. Within rounds, mice were given minimal rest (30sec)
while between rounds, mice were returned to their home cage to rest for >15min.
Whenever the mouse reached the end of the wire, it was immediately placed back at the
original position at the centre, and recording time continued. When the mouse turned and
fell by its hind limbs, the fall was considered voluntary and was not counted.

Rotarod test: Neuromuscular performance was determined with the rotarod apparatus
(Series 8, IITC Life Science, Woodland Hills, CA, USA), using a four-day protocol. On day
1, mice were trained to run on a rotating rod, with three runs accelerating in speed slowly
(1-5rpm in 180sec, 1-5rpm in 30sec, 4-10rpm in 60sec). A fourth run consisted of the
experimental protocol with acceleration from 4rpm to 20rpm in 180sec. The running time
was measured until the mice fell or until 300sec was reached. On days 2, 3 and 4, the
experimental procedure was repeated three times, with 15min rest between runs. For each
day, the maximal running time was recorded. Learning capacity was calculated as the
difference between the maximal running time on day 3/4 and the experimental run on
training day 1.
Indirect calorimetry

Real-time metabolic analyses were performed using a Comprehensive Laboratory Animal Monitoring System (TSE systems GmbH, Bad Homburg, Germany) at week 11 of the experimental diet. After a period of overnight acclimatization, carbon dioxide (vCO₂) production, oxygen (vO₂) consumption, respiratory exchange ratio (RER), energy expenditure (EE), food intake and activity were determined for 48h in individual mice. Infrared beams recorded locomotor activity according to the number of beam break events in the horizontal (x) and vertical (z) plane.

Glucose homeostasis

Glucose homeostasis and insulin sensitivity were determined at week 10 of the experimental diet, by performing an oral glucose tolerance test (OGTT) in combination with analysis of tracer based glucose kinetics. For this, 1.5 g/kg body weight D-glucose (25% w/v of which 5% w/w stable isotope labelled [6,6-2H₂]-glucose tracer (Cambridge Isotope Laboratories, Andover, MA)) was administered orally after an overnight (10h) fast. At 0, 5, 15, 30, 45, 60, 90, and 120 min after glucose administration, blood glucose levels were determined using an OneTouch Select Plus glucose meter (Lifescan, Zug, Switzerland) and bloodspots were collected on filter paper (Sartorius Stedim TFN 180g/m², The Netherlands) for tracer analysis. To determine insulin levels, bloodspots were collected at 0, 5, 15, 30, 60, 120 min after glucose administration. Insulin was extracted from the bloodspots and determined using the rat insulin ELISA kit from Crystal Chem and mouse insulin standard (Cat. 90010 and 90020, Zaandam, The Netherlands) according to the manufacturers’ protocol. The values for insulin, as measured in bloodspots, were adjusted by factor 1.28, based on matching samples from plasma. Total area under the curve was calculated for both glucose and insulin curves, over the periods 0 to 120min and 0 to 30min, respectively.

Fractional distribution of [6,6-2H₂]-glucose was determined by gas chromatography quadrupole mass spectrometry (Agilent 9575C inert MSD; Agilent Technologies, Amstelveen, The Netherlands) according to van Dijk et al. In short, glucose was extracted from the bloodspot and converted to its penta-acetate derivative. Positive chemical ionization with methane enabled monitoring of ions m/z 408-412 (corresponding to m0 - m4 mass isotopologues) which were corrected for the fractional distribution due to natural abundance of 13C by multiple linear regression to obtain the excess fractional distribution (M0 - M4) due to the dilution of administrated [6,6-2H₂]-glucose; M2 represents the fractional contribution of the administered tracer and was used in the calculations of blood glucose kinetics. To describe changes in blood glucose kinetics in mice, we used the Minimal Model that was adjusted for Mice (MiniM) as developed by van Dijk (supplementary methods). In short, to generate a sufficient number of samples that cannot be taken from mice, we used a mathematical approach using the measured values at
indicated time points. The model used allows estimations of peripheral glucose utilization rates, peripheral insulin sensitivity, as well as hepatic insulin sensitivities. Hepatic insulin sensitivity was calculated as a ratio of the endogenous glucose production and the insulin levels over the course of the experiment, and displayed as the delta area under the curve (t=0-120 min). Relevant equations are shown in the supplementary material.

Muscle mitochondrial content and capacity

To determine mitochondrial content and capacity, mitochondria were isolated from the quadriceps muscle by differential centrifugation as described previously. The O\textsubscript{2} fluxes in isolated mitochondria were measured using MiR05 buffer (respiration buffer) containing 110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 20 mM HEPES, 0.5 mM EGTA, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 3 mM MgCl\textsubscript{2}, 1 mg/mL bovine serum albumin (BSA), pH 7.1. Under three conditions, with different oxidizable substrates, 1) 2 mM pyruvate plus 2 mM malate, or 2) 2 mM pyruvate plus 2 mM malate plus 5mM glutamate, or 3) 25 µM palmitoyl-CoA plus 2 mM L-carnitine plus 2 mM malate, at 37°C using a two-channel high-resolution Oroboros Oxygraph-2k (Oroboros, Innsbruck, Austria). The O\textsubscript{2} fluxes were normalized for protein content, determined with the BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) and expressed as nmol/(min∙mg mitochondrial protein). Results were corrected for the ratio of citrate synthase activity (described below) in tissue homogenate to the isolated mitochondria, as a reflection of the enrichment of mitochondrial preparations.

Citrate synthase activity was determined, as a marker of mitochondrial content, in isolated mitochondria and quadriceps homogenates (15%, w/v in PBS) in PBS (pH 7.4), by measuring the formation of 5-thio-2-nitrobenzoic acid at 412 nm at 37 °C. In short, the assay mixture contained 0.1 M Tris (pH 8.1), 5 mM triethanolamine-HCl, 0.05 mM EDTA, 0.1% Triton-X100, 0.5 mM oxaloacetate, 0.1 mM dithionitrobenzoic acid, and the reaction was started with 0.5 mM acetyl-CoA. Enzyme activities were expressed as µmol/(min∙mg protein).

Plasma levels of FGF21, free fatty acids, cholesterol and amino acids

Plasma FGF21 levels were determined using the Mouse/Rat Quantikine ELISA (MF2100, R&D systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Plasma free fatty acids levels were determined using the Diasys NEFA FS kit (#15781, Holzheim, Germany) according to manufacturers’ protocol, measuring absorbance at 540 nm. Plasma cholesterol levels were determined according to manufacturers’ protocol (Roche 11489232, Mannheim, Germany), using Diasys Cholesterol standard (#113003010030, Holzheim, Germany). Amino acid levels were determined using cation-exchange high-performance liquid chromatography followed by post-column ninhydrin derivatization, on a Biochrom30 analyzer (Pharmacia Biotech,
Norleucine was used as an internal standard. A physiological amino acid calibration standard was used for calibration (Sigma-Aldrich, Darmstadt, Germany).

**Adipose tissue histology and immunohistochemistry**

For adipose tissue histology, brown adipose tissue (BAT) and subcutaneous white adipose tissue (scWAT) were fixed in 10% formalin overnight and paraffin-embedded sections (4μm) were stained with hematoxylin and eosin (H&E). Images of scWAT were taken with Aperio Image Scope (Leica Biosystems, Amsterdam, The Netherlands) for adipocyte size assessment, in which >100 adipocytes were randomly counted for each mouse, using ImageJ (NIH, USA).

To assess the thermogenic capacity of adipocytes, H&E staining of 4 consecutive slides, interspaced with 300-400μm, were scored for the presence of multilocular cells. To confirm a brown-like phenotype, UCP1 was detected in paraffin-embedded scWAT tissues by immunohistochemistry. The endogenous peroxidase activity was blocked with 1% v/v H2O2 in methanol. After a 15 minutes treatment with normal goat serum, sections were incubated overnight at 4°C with the primary antibody rabbit anti UCP1 (ab10983, Abcam, Cambridge, UK, 1:1000 diluted in PBS). For 30 minutes, the sections were incubated with the secondary antibody goat-anti-rabbit/biotin (BA-1000, Vector, 1:250), and subsequently with Vectastain ABC reagents (PK4000, Vector labs) for another 30 minutes. The reaction was visualized by incubating with 3,3′-Diaminobenzidine (DAB) solution for 10 minutes.

**Western analysis**

Homogenates of scWAT were prepared using RIPA lysis buffer (50mM Tris HCl, 150mM NaCl, 5mM EDTA, 30mM pyrophosphate, 50mM NaF, 1% (v/v) Triton x100, 1mM PMSF, 2 mM orthovanadate) with Halt protease- and phosphatase inhibitor cocktail (Thermo Scientific). Protein levels were determined, normalized using the BCA assay (Pierce), and subjected to a 4-15% gradient SDS–PAGE using the Mini Protean 3 system (Bio-Rad), followed by transfer to PVDF membranes. Standard western analysis was performed using 5% milk in PBS as blocking solution and antibodies to UCP1 (ab10983, 1.3 µg/mL (1:1000), Abcam, Cambridge, UK) and actin (a2066, 6 µg/mL (1:100), Sigma, The Netherlands). HRP-labelled secondary antibodies were used at 1:5000 dilution, and visualization was done by Chemidoc MP Imaging Systems (Bio-Rad).

**Gene expression analysis**

Total RNA was isolated from mouse liver and adipose tissues using phenol-chloroform extraction with TRIzol (Invitrogen). Transcript levels of synthesized cDNA (M-MLV reverse transcriptase, Thermo-Fisher Scientific, Waltham, USA) were measured with Fast Advance Taqman Mastermix (Applied Biosystems, Foster City, CA, USA) or Fast Start
SYBR green (Roche, Mannheim, Germany) on a QuantStudio 7 Flex Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Ct-values were normalized by a standard curve and presented as relative expression of housekeeping gene \(36b4\). qPCR primers are listed in Supplementary Tables 2 & 3.

**Transcriptome analysis**

Total RNA from scWAT and BAT was isolated for RNA sequencing using the RNeasy MINI Kit (Qiagen, Venlo, The Netherlands), according to manufacturer’s protocol. Quality control was performed using a fragment analyser (ProSize 3.0, Advanced Analytical Technologies, Inc.) confirming all scWAT and BAT samples had an RNA quality number (RQN) value greater than 6 and 8 respectively. A total of 1µg RNA was used for library preparation and sequencing, performed at Novogene Co. Ltd. Hong Kong using the Illumina HiSeq 2500 instrument on the PE150-platform. Paired-end clean reads were mapped to the reference genome (mmu_GRCm38.p6) using HISAT2 software. HTSeq was used to count the read numbers mapped of each gene, including known and novel genes. Fragments Per Kilobase of exon model per Million (FPKM) mapped reads of each gene was calculated based on the length of the gene and reads count mapped to this gene, as a means of normalizing for the effect of sequencing depth and gene length.

Differential Expression of Genes (DEG) analysis between two groups (n=4-5) was performed using DESeq2 R package, providing statistical routines using a model based on the negative binomial distribution. The resulting p-values were adjusted using the Benjamini and Hochberg’s approach for controlling the False Discovery Rate (FDR). Genes with a padj<0.1 found by DESeq2 were assigned as differentially expressed. For validation of the RNA sequencing data, expression of several genes was also determined by qPCR, and these were well in agreement with the RNA sequencing outcomes (Supplementary Figure 2).

PCA plots, with 95% confidence levels, were created using ClustVis (https://biit.cs.ut.ee/clustvis/), and Venn diagrams were made using http://www.venndiagrams.net/. For age-related comparisons, enrichment analysis was performed with DAVID version 6.8 (https://david.ncifcrf.gov/gene2gene.jsp), using all genes with padj<0.1. For dietary comparisons, enrichment analysis of metabolic pathways (of KEGG defined pathways) was performed manually using all genes with a p<0.05. Significance of pathway enrichment was determined by the Fisher exact test, using GraphPad Prism 8.3.0 software package (GraphPad Software, USA).
Statistical analysis

GraphPad Prism 8.3 software package (GraphPad Software, USA) was used to perform statistical analysis. Data were analysed by 2-way ANOVA, followed by Tukey multiple comparisons tests. Correlation graphs were created using a nonparametric Spearman correlation. Energy expenditure was correlated with body weight and analysis of energy expenditure with body weight as covariate (ANCOVA) was assessed according to the methods described on www.mmpc.org. Data are presented as Tukey boxplots or mean ± standard error of the mean (SEM), as indicated in the figure legends. Significance is indicated as *p<0.05, **p<0.01, ***p<0.001.

Results

Effect of dietary protein intake on physical health in young adult and aged mice.

To evaluate the effects of dietary protein content on metabolic health in the context of ageing, young adult (3 months old) and aged (18 months old) mice were given an isocaloric diet (in which protein was replaced by starch, a complex carbohydrate) with either a reduced protein content (LP, 7%) or an elevated protein content (HP, 40%) compared to a control diet with medium protein content (MP, 20%) (Figure 1A). Over the course of eight weeks, total intake of the LP diet was higher both in young adult and aged mice (10.0%, p=0.11 and 14.4%, p<0.001 compared to MP) (Figure 1B). As a result of increased intake, absolute protein intake was effectively reduced by 58.7% in young adult mice and by 57.7% in the aged mice on an LP diet (Figure 1C). An HP diet did not affect food intake significantly, thereby these mice consumed on average 188% (young adult mice) and 198% (aged mice) of protein, compared to littermates on an MP diet. Despite these substantial changes in protein intake, in LP-fed animals, plasma levels of all essential amino acids were maintained or even increased, except for the branched-chain amino acids, which were reduced (Figure 1D).

An important consequence of ageing is the increased risk of sarcopenia, a risk that is elevated due to reduced protein supply. To prevent the decline of muscle mass, protein supplementation is often given to elderly patients. To assess whether reduced protein intake, in our study, negatively affected physical strength in aged mice, we measured three markers of physical health and found no negative consequences. First, either LP or HP did not substantially affect body weight and body composition throughout the experiment. Aged mice did not lose weight on either LP or HP diet, whereas young adult mice displayed normal weight gain (Figure 1E). In addition, both lean and fat mass were not affected by either an LP or HP diet (Figure 1F-G, Table 1).

Second, muscle performance tests showed an age-related decline of muscle function, but these changes were not associated with dietary protein content. Physical performance, measured by the maximal running time on an accelerating rotarod, was significantly higher in young adult animals compared to aged animals (Figure 1H-I), but these were mainly...
associated with differences in body weight (R²=0.562, p<0.001), with no significant effect of the diet (Figure 1J). The motor skill learning capacity was measured over four consecutive days, and did not significantly change as a function of age or diet (Figure 1K).

Similar results were obtained with the hanging wire test and the grip strength test, in which age-related or body weight-dependent effects were observed, but no effect of dietary protein content (Table 1, Figure 1L-M).

Third, we found no significant effect of dietary protein content on muscle mitochondrial content as determined by citrate synthase activity or mitochondrial function measured by maximal oxidative capacity on different substrates (Table 2). Together, these findings indicate that overall physical health was not significantly affected by a reduced dietary protein intake in either young adult or aged mice and high protein intake in aged mice did not prevent an age-related decline in physical strength.

Figure 1: Body weight and other physiological characteristics in young adult and aged mice. (A) Design of the 12-week dietary intervention and diet composition of the experimental diets. Young adult mice (3 months of age) and aged mice (18 months of age) were normalized on a run-in diet for 2 weeks to standardize microbial status (young;
(B) Food intake, averaged of several measurements in 8 weeks. (C) Protein intake based on individual differences in food intake. (D) Heatmap of average amino acid levels in different experimental groups (young: n=8, old: n=12). (E) Changes in body weight, lean mass (F) and fat mass (G) after 8 weeks of the dietary treatment (young: n=13, old: n=19-22). Physical performance measured by rotarod test over 4 consecutive days (H), with maximal running time over day 2/3/4 (I) and learning capacity (K). Correlation plot between body weight and maximal running time (J) (young: n=13, old: n=19-22). Correlation plots between body weight and hanging time (L) or grip strength (M), measured after 8 weeks of the experimental diet (young: n=13, old: n=19-22). Results are shown as Tukey boxplots or as mean ± SEM (1B/1C/1H). In all graphs, LP=red, MP=grey, HP=blue. Statistical significance was determined by 2-way ANOVA, followed by Tukey multiple comparisons tests, and indicated as *p<0.05, **p<0.01, ***p<0.001. In the correlation plot, young adult mice are represented by open symbols, while aged mice are represented by solid symbols. Data was analysed using a nonparametric Spearman correlation.

### Table 1: Physiological characteristics in young adult and aged mice.

Body weight and composition were determined after 8 weeks of the experimental diets. Physical capacity of young adult and aged mice were measured before and after 8 weeks of the experimental diet using the grip strength test and hanging wire test. Young adult animals: n=13, Aged animals: n=19-22.

<table>
<thead>
<tr>
<th>Body composition</th>
<th>Young animals</th>
<th>Aged animals</th>
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<tr>
<td></td>
<td>LP MP HP</td>
<td>LP MP HP</td>
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<tr>
<td>Body weight (g)</td>
<td>31.2 ± 1.18</td>
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<td>Fat mass (g)</td>
<td>5.5 ± 0.9</td>
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<td>(% of BW)</td>
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<td>Lean mass (g)</td>
<td>21.5 ± 0.27</td>
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<td>(% of BW)</td>
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### Grip Strength Test

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<td>MP</td>
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<td>HP</td>
<td>142 ± 4.6</td>
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### Hanging Wire Test

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<td>HP</td>
<td>39 ± 3.6</td>
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### Table 2: Mitochondrial capacity of liver and quadriceps in young adult and aged mice.

Citrate synthase activity was measured in tissue homogenate (liver or quadriceps) as a marker of mitochondrial content, expressed as µmol/(min⋅mg protein). Maximal oxygen consumption stimulated by different oxidizable substrates was expressed in nmol/(min⋅mg mitochondrial protein). PM: pyruvate/malate, PMG: pyruvate/malate/glutamate, P-CoA palmitoyl-CoA/malate/carnitine. Young adult animals: n=5-6, Aged animals: n=8-10, † significant compared to MP, ‡ significant LP vs HP.

<table>
<thead>
<tr>
<th>Quadriceps</th>
<th>Young animals</th>
<th>Aged animals</th>
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<tr>
<td></td>
<td>LP MP HP</td>
<td>LP MP HP</td>
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<tr>
<td>Citrate synthase</td>
<td>0.42 ± 0.03</td>
<td>0.43 ± 0.02</td>
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<tr>
<td>PM</td>
<td>132 ± 9.1</td>
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<td>PMG</td>
<td>162 ± 9.9</td>
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<td>P-CoA</td>
<td>50 ± 5.9</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Citrate synthase</td>
<td>0.12 ± 0.04</td>
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<td>PM</td>
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<tr>
<td>P-CoA</td>
<td>35 ± 1.8</td>
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Effects of reduced dietary protein intake on glucose homeostasis.

As previous studies have reported improved glucose homeostasis as a result of (periodic) short-term low-protein feeding \(^{10,11,34,35}\), we examined the effect of dietary protein intake on glucose homeostasis. Surprisingly, we did not observe major changes in fasting glucose or insulin levels, glucose tolerance, or glucose-stimulated insulin-secretion after low protein feeding in either young adult or aged mice (Figure 2A-B, Table 3). Only a small improvement in hepatic insulin sensitivity in young adult mice on an LP diet was observed by tracer-based glucose kinetics (Figure 2C-D). In line with this, mitochondrial oxidative capacity of the liver to oxidize pyruvate in the presence of malate was improved in young adult mice on an LP diet (Table 2). Overall, these results show that dietary protein intake does not have a major effect on glucose homeostasis in mice with a stable lean mass and body weight.

Figure 2: Glucose tolerance and hepatic insulin sensitivity in young adult and aged mice. Glucose tolerance (A) and insulin response (B) after oral administration of 1.5g/kg BW D-glucose (young: n=13, old: n=19). (C) Hepatic insulin sensitivity, before (dashed line) and after (solid line) 10 weeks of the experimental diet. (D) Hepatic insulin sensitivity, delta AUC (young: n=13, old: n=19). Results are shown as Tukey boxplots (Fig 2D), as mean (Fig 2C), or as mean ± SEM (Fig 2A/2B). In all graphs, LP=red, MP=grey, HP=blue. Young adult mice are represented by open symbols, while aged mice are represented by solid symbols. Statistical significance was determined by 2-way ANOVA, followed by Tukey multiple comparisons tests, and indicated as *p<0.05, **p<0.01, ***p<0.001.
Table 3: Effects of diet and age on the response to an oral glucose tolerance test. Mice were given oral administration of 1.5g/kg body weight D-glucose after an overnight (10h) fast. The area under the curve (AUC) was calculated for both glucose and insulin. Young adult animals: n=13, Aged animals: n=19-22.

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Effects of dietary protein intake on energy expenditure and body temperature.

Next, we examined the effect of dietary protein content on whole-body energy metabolism by indirect calorimetry. The respiratory exchange ratio (RER) during the dark phase was significantly increased due to LP feeding in both young adult and aged mice, indicating a shift in metabolism towards increased glucose utilization (Figure 3A-B). In contrast to previous studies, EE was not significantly increased in young adult mice after LP feeding (Figure 3C). However, an LP diet substantially affected EE in aged mice. Analysis of the EE data with body weight as covariant (ANCOVA) demonstrated a significant LP-induced increase in EE in aged mice during the dark phase compared to both MP and HP (Figure 3D). An HP diet did not affect EE in both young adult and aged mice. Furthermore, an LP diet did not significantly affect voluntary activity, suggesting that the increased EE was due to an enhanced basal metabolic rate (Figure 3E). In line with this, we found an age-related reduction in body temperature (p<0.001) and a moderate increase by reduced protein intake (p=0.067, p=0.062 for LP compared to HP diet in young adult and aged mice, respectively) (Figure 3F). Together, these results suggest that EE, and to a lesser extent body temperature, can be ameliorated by reduced dietary protein intake in aged mice.
Figure 3: Diet-induced changes in energy homeostasis and body temperature in aged mice. Respiratory exchange ratio (RER) of 48 hours in young adult and aged animals (A), average RER of both light and dark phase (B) (young; n=10, old; n=17). (C) Energy expenditure over 48h in both young adult and aged mice (young; n=10, old; n=17). (D) ANCOVA corrected energy expenditure of both light and dark phase in aged mice (adjusted body weight = 37.88g). (E) Average locomotor activity (cm/min) during both light and dark phase in young adult and aged mice (young; n=10, old; n=17). (F) Body temperature, measured subcutaneously during the light phase (young; n=10, old; n=17). (G) Plasma FGF21 levels in plasma (young; n=8, old; n=12). Fg21 expression levels in liver (H), BAT (I) and scWAT (J) (young; n=9, old; n=12). Correlation plot between plasma FGF21 levels and Fg21 mRNA levels in liver (K) and BAT (L) (young; n=7, old; n=10). Results are shown as Tukey boxplots or as mean ± SEM (3A/3C). In all graphs, LP=red, MP=grey, HP=blue. Statistical significance was determined by 2-way ANOVA, followed by Tukey multiple comparisons tests, and indicated as *p<0.05, **p<0.01, ***p<0.001. In the correlation plots, young adult mice are represented by open symbols, while aged mice are represented by solid symbols. Data was analysed using a nonparametric Spearman correlation.
Effects of low dietary protein intake on expression and circulating levels of FGF21.

Previously, it has been shown that the beneficial effects of protein restriction are mediated by increased hepatic expression and circulating levels of FGF21 \(^{13,22-24}\). In our study, we also observed an increase in plasma FGF21 levels by an LP diet in the aged mice (average 1638 pg/mL (LP) vs. 904 pg/mL (MP)), whereas young adult mice already displayed relatively high levels on an MP diet (average 1453 pg/mL (LP) vs. 1610 pg/mL (MP)) (Figure 3G). Conversely, an HP diet reduced FGF21 levels in both young adult and aged mice as compared to MP fed littermates.

While FGF21 is primarily produced by the liver, in response to metabolic challenges like fasting and starvation \(^{13}\), BAT and WAT have also been shown to produce FGF21 in response to cold exposure \(^{36,37}\). In the current study, we observed an effect of reduced protein intake on 
\(Fgf21\) in both liver and BAT. Correlation analysis indicates that 83.2\% of the variation in plasma FGF21 can be explained by stimulation of hepatic \(Fgf21\) mRNA expression (Figure 3H-I). In contrast, this induction of \(Fgf21\) in BAT did not correlate with plasma FGF21 levels (Figure 3J-K). No changes in \(Fgf21\) expression were observed in subcutaneous WAT in response to an LP diet in either young adult or aged mice (Figure 3L). In conclusion, reduced dietary protein intake increases plasma levels of FGF21 in aged mice and this is primarily caused by an induction of hepatic \(Fgf21\) expression.

Effects of dietary protein restriction and age on brown adipose tissue.

The increased energy expenditure, body temperature, and BAT \(Fgf21\) mRNA levels in response to a LP diet pointed towards changes in BAT activity. To investigate the effect of a LP diet on gene expression in BAT, we performed transcriptome analysis. PCA analysis of all differentially expressed genes (DEGs, p<0.05) indicated that the age-related effect was more profound than the diet-induced effect on gene expression (Figure 4A). Interestingly, a LP diet increased the variation in gene expression of both young adult and aged mice (Figure 3A), resulting in 297 and 64 (35 common) age-related DEGs (p<0.05, FDR=0.1) for respectively MP and LP diet, the majority of which were upregulated (Figure 4B-C). Genes affected by age were associated with low-grade inflammation, such as increased levels of the complement system, rather than downregulation of specific genes involved in BAT function (Figure 4D).

Reduced dietary protein intake resulted in 59 and 32 (10 common) DEGs in respectively young adult and aged mice (Figure 4E-F). No effect, however, was observed by a LP diet on the expression of thermogenic genes, including \(Ucp1\) (uncoupling protein 1), \(Ppargc1a\) (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), and \(Prdm16\) (PR domain containing 16) (Figure 4G-I), indicating that thermogenesis was not affected.

An alternative explanation for the observed increase in energy expenditure and body temperature could be the increased size of BAT and increased clearance of glucose and non-esterified fatty acids (NEFA) \(^{23}\). While reduced dietary protein intake slightly increased BAT weight both in young adult and aged mice (Figure 4J), we did not detect any
stimulation of proliferation, as indicated by levels of Ki67 mRNA (Figure 4L). In contrast, we observed minor differences in BAT morphology (Figure 4K) associated with increased levels of the lipid droplet marker Plin (Figure 4M), suggesting differences in BAT were most likely due to lipid accumulation. Interestingly, we found several other batokines next to Fig21 increased in response to a LP diet, including Bmp8b (increased 5.2-fold and 3.1-fold in young adult and aged mice, respectively), Lcn2 and Gdf15 (>3-fold increased expression levels after LP feeding) (Figure 4N-O). Other genes encoding batokines, however, such as Angplt8, Nrg4, Bmp4, and Bmp7, were not affected (Figure 4P). In conclusion, while we found no clear evidence for the increased thermogenic capacity of BAT other than increased BAT weight, a LP diet did increase the expression of selected batokines.
Figure 4: Activation of BAT secretory peptide profile by reduced protein intake. (A) PCA plot, plotting all genes with a significant difference (p<0.05) in either a diet- or ageing-induced comparison (3791 genes) (n=4-5). (B) Number of significant DEGs (p<0.1) either upregulated or downregulated in aged mice compared to young adult mice (n=4-5), with a Venn diagram showing overlap between different diets (C). (D) KEGG pathway analysis using DEGs (p<0.1) of age-related changes on an MP diet. (E) Number of significant DEGs (p<0.1) either upregulated or downregulated by a LP diet compared to MP diet (n=4-5), with a Venn diagram showing overlap between ages (F). (G) PCA plot, plotting 225 genes involved in KEGG pathway “Thermogenesis” (mmu04714) (n=4-5). mRNA expression levels of Ucp1 (H) and Pparα1/Pdra/Prdm16 (I) and Ki67 (L) in BAT (young; n=9, old; n=12). J) Brown adipose tissue weight, as percentage of total body weight (young; n=13, old; n=19-22). K) Representative pictures of BAT morphology, scale bar represents 100um (n=4). RNA seq expression levels of different Pli genes (M), Bmp8β (N) Len2 and Gdf15 (O) (n=4-5). P) RNAseq batokine expression profile, log² fold change (log²FC) differences between LP and MP fed animals. Results are shown as Tukey boxplots or mean ± SEM (4N,4O), with LP=red, MP=grey, HP=blue. Statistical significance was determined by 2-way ANOVA, followed by Tukey multiple comparisons tests, and indicated as *p<0.05, **p<0.01, ***p<0.001. In PCA plots, aged LP=light blue, aged MP=dark blue, young adult LP=light green, young adult MP=dark green.

Effects of dietary protein intake and age on subcutaneous white adipose tissue.

In the absence of major effects on BAT, we investigated whether the observed metabolic improvements could be mediated by changes in scWAT. We hypothesized that increased energy expenditure could be the result of browning of scWAT, mediated by FGF21. When analysing the transcriptome of scWAT, similar to BAT, the age-related effects on gene expression were more pronounced than the diet-induced effects (Figure 5A). Most age-related changes in gene expression were observed on a MP diet, while switching diets to either a LP or HP diet resulted in increased individual variation, reducing the number of significant DEGs (Figure 5B). On a MP diet, most genes found were downregulated by ageing, resulting in highly enriched KEGG pathways related to ribosomal function or innate immune cell signalling. In contrast, upregulated genes were involved in ECM-receptor interactions, focal adhesion, and metabolic pathways (Figure 5C-D).

Diet changed in total 1124 DEGs significantly in young adult mice, most of which were the result of HP feeding (Figure 5E-F). In contrast, most of the 50 DEGs significantly changed in aged mice were LP-mediated (Figure 5E-G). Pathway enrichment analysis highlighted changes in the thermogenic pathway (KEGG mmu04714), oxidative phosphorylation, Pparg-signalling, and TCA cycle, among others, as a result of LP feeding (Figure 5H). Further, increased levels of Ucp1 mRNA levels, an essential gene in adaptive thermogenesis, indicated upregulation of the thermogenic capacity of scWAT. Ucp1 levels were significantly increased in aged mice by a LP diet compared to both MP and HP diets (Figure 5I). Noticeably, young adult mice on either a LP or MP diet already exhibited high levels of Ucp1 compared to mice on an HP diet.

In line with these results, transcriptome data shows both age-related and diet-induced changes in the total thermogenic gene profile (Figure 5J). A clear age-induced difference in the thermogenic gene profile of MP-fed young adult and aged mice was found. However, when fed a LP-diet, we found a greater resemblance between young adult and aged mice, in line with a reversal of the age-related decline in thermogenic capacity. In contrast, an HP diet increased the individual variation between young adult mice, and combining these
results with reduced Ucp1 mRNA expression (Figure 5I–J), pointed to a low thermogenic potential. Altogether, these results suggest regulation of the thermogenic potential by dietary protein intake.

Figure 5: Increased thermogenesis in subcutaneous WAT by reduced protein intake. (A) PCA plot, plotting all genes with a significant difference (padj<0.1) in either a diet- or ageing-induced comparison (7447 genes), LP=blue, MP=green, HP=red, aged mice are shown in light while young adult animals are represented in dark colours (n=5). (B) Number of significant DEGs (padj<0.1) either upregulated or downregulated in aged mice compared to young adult mice, with a Venn diagram showing overlap between different diets (C). (D) KEGG pathway analysis using downregulated DEGs (padj<0.01; green) or upregulated DEGs (padj<0.01; red) between young adult and aged animals on an MP diet. (E) Significant DEGs (padj<0.1) either upregulated or downregulated by any dietary interaction, with a
Venn diagram showing overlap between the specific dietary comparisons in young adult mice (F) and aged mice (G).

**Manual pathway analysis, using all genes significant (p<0.05) in KEGG-defined pathways, differences between LP-MP in red and LP-HP in white.** (I) mRNA expression levels of Ucp1, LP=red, MP=grey, HP=blue (young; n=8, old; n=12). (J) PCA plots, plotting 225 genes involved in KEGG pathway "Thermogenesis", comparing ages at an LP, MP and HP diet, young adult mice in dark blue and aged mice in light blue (n=5). Statistical significance was determined by 2-way ANOVA, followed by Tukey multiple comparisons tests, and indicated as *p<0.05, **p<0.01, ***p<0.001.

**Effects of reduced dietary protein intake on age-related browning of scWAT.**

A rapid age-related decline of the thermogenic capacity of scWAT, as early as 4 months of age, has been previously shown in mice, including a loss of Ucp1 expression and reduced expression of other thermogenic markers such as Cidea, Cox7a1, and Cox8b. Similarly, we found an age-related downregulation of these genes under MP conditions (Figure 6A). We further investigated the browning capacity of the scWAT and found a strongly increased expression of the beiging-associated gene Elov13 due to LP feeding, to the same extent as Ucp1 (Figure 6B). However, other beiging-related genes, such as Cd137 (Tnfrsf9) and Tmem26, only showed a trend towards an increase in young adult LP-fed mice compared to HP-fed mice (Figure 6C). In contrast, classical brown adipocyte associated genes like Prdm16 and Pparc1a, were not altered in scWAT (Figure 6D).

To assess whether changes in Ucp1 mRNA levels were also accompanied by higher UCP1 protein levels, we determined protein levels by western analysis and found a positive correlation with Ucp1 mRNA levels (R2=58.2%, p<0.0001) (Figure 6E-F). Closer examination of the adipose tissue by UCP1 staining revealed heterogeneity of scWAT, with sporadic patches of brown-like adipocytes across the white adipose tissue. In animals on a LP diet, different locations in the tissue contained a high number of cells with multiple lipid droplets and high expression of UCP1 protein (representative picture in Figure 6G). The UCP1-rich patches were more frequently present in young adult animals compared to the aged animals (Figure 6H). However, a LP diet was able to increase the number of these patches in aged mice to levels similar to that of young adult mice.

In contrast to these patches of UCP1-rich cells, the surrounding non-thermogenic adipocytes did not change in morphology. Increased thermogenesis could potentially affect overall lipid oxidation, adipocyte size, and production of healthy adipokines. However, we found no differences in scWAT mass, adipocyte size or distribution upon analysis of representative pictures of non-thermogenic zones (Figure 6I-K). In addition, pathway analysis revealed that other functions such as adipokine signalling and lipolysis were not significantly altered in the total scWAT depot (Figure 5H). In line with this, plasma NEFA’s and triglycerides were also not significantly affected by protein restriction (Figure 6L,6M).

Taken together, while the thermogenic capacity of scWAT shows an age-related decline, this effect could be largely reversed by a LP diet. Spearman correlations connect Ucp1 mRNA levels to plasma FGF21 levels, energy expenditure as well as body temperature, suggesting that the reversal of the age-related decline in metabolic health starts with activating the thermogenic capacity in scWAT (plasma FGF21 levels; R2=22.6%,...
p=0.0006, energy expenditure; R²=9.7%, p=0.042, body temperature; R²=16.2%, p=0.002) (Figure 6N). Overall, these data suggest a localized browning in the scWAT, affecting age-related changes in energy expenditure and body temperature.

Figure 6: Selective induction of browning of subcutaneous WAT. (A) RNA seq expression levels of Cidea, Cox7a1 and Cox8b (n=5). mRNA expression levels of Elov3 (B) Cd37/Tmem26 (C) and Pparγ1a/Prdm16 (D), (young n=9, old n=12). (E) Representative western blots of UCP1 protein expression in scWAT (n=6). (F) Correlation plot between mRNA levels and protein levels of UCP1 (n=5-6). (G) Representative pictures of UCP1-staining showing patches of UCP1-rich cells in young adult and aged mice on a LP diet, scale bar represents 200µm, with quantification of the number of animals expressing certain patches (young n=13, old n=19-22). (H) Subcutaneous white adipose tissue weight, as percentage of total body weight (young n=13, old n=19-22). Average adipocyte size (J) and distribution (K) of both young adult and aged mice quantifying size of >100 adipocytes/mouse (young n=10, old n=13). (L) Plasma NEFA of terminal blood sample, fasted for 2-6 hours (young n=8, old n=13). (M) Plasma triglycerides (TG) of terminal blood sample, fasted for 2-6 hours (young n=13, old n=20). (N) Correlation plots between Ucp1 mRNA levels and plasma FGF21, energy expenditure and body temperature (young n=6-8, old n=9-11). Results are shown as mean ± SEM (6A/6K) or Tukey boxplots. In all graphs, LP=red, MP=grey, HP=blue. Statistical significance was determined by 2-way ANOVA, followed by Tukey multiple comparisons tests.
Discussion

In Western societies, the percentage of people over the age of 65 is rapidly increasing. With age, also the risk of obesity increases, and approximately one-third of the population develops obesity by the age of 60-70. Aging and obesity are prominent risk factors for many metabolic diseases; therefore, understanding how aging negatively affects energy metabolism and identifying strategies to reduce age-related decline in metabolic health are imperative. Dietary protein restriction has been shown to improve metabolic health in multiple species, including mice and humans. Long term dietary protein restriction in mice, starting directly after weaning, also indicated that longevity can be increased by reduced protein intake. However, whether a short-term intervention with a low protein diet starting at an advanced age also has beneficial effects on metabolic health is not known. Here we show that short-term dietary protein restriction in aged mice, starting at the age of 18 months, ameliorated the age-related decline of some aspects of metabolic health, including energy expenditure (EE), circulating levels of FGF21 and browning of subcutaneous white adipose tissue (scWAT) without noticeable adverse effects on physical health. Our findings indicate that interventions based on protein restriction have the potential to improve metabolic health when started at an older age. Previous studies into the impact of a late-life switch to caloric restriction reported either no effect or an increased survival. A recent study using dietary restriction at late-life in female mice showed no metabolic changes in WAT or BAT, indicating that the body has a memory of earlier nutrition. While these differences in outcome could have been influenced by differences in experimental design, such as starting age, duration of the diet, or gender, it also suggests that diet composition is an important factor for the effectiveness of late-life dietary intervention strategies.

Previously, FGF21 has been implicated in increasing energy expenditure and the browning of white adipose tissue via brain-mediated βKlotho signalling. Similarly, we found that plasma FGF21 levels correlated with markers of browning of subcutaneous WAT in our study. Both Ucp1 and Elovl3 mRNA levels in the scWAT were strongly increased by a low protein diet. ELOVL3 has been implicated as a key thermogenic enzyme as Elovl3-deficient mice are unable to activate their BAT under conditions of cold exposure due to a decreased capacity to elongate saturated fatty acyl-CoAs for heat generation. Other beige-related genes, such as Cd137 and Tmem26, have been implicated as markers of increased thermogenesis in WAT, noted by high expression levels in beige adipocytes compared to brown and white adipocytes and on specific white preadipocytes with great potential to activate adipogenesis with high thermogenic capacity. However, in this study, we only observed minor changes in these genes. In addition, classical brown adipocyte-associated genes, like Prdm16 and Pparg1a are thought to be involved in the recruitment of brown
preadipocytes, thereby activating thermogenesis from Myf5+ precursor cells. In our study, these genes were not altered in the total scWAT depot. As we determined mRNA levels in the total adipose depot, it could still be these genes are upregulated specifically in the Ucp1-rich patches. Furthermore, the finding of the specific UCP1-rich patches raises the question whether browning of these locations is due to differentiation of white adipocytes or infiltration of brown adipocytes. Closer examination of the areas in WAT with high Ucp1 expression is needed to provide further information on the mechanisms of browning.

In addition to increased thermogenesis of scWAT, specific activation of BAT by FGF21 has been shown previously to increase glucose/NEFA uptake, improve insulin sensitivity, and increase Ucp1 expression. However, our LP intervention did not affect overall insulin homeostasis or Ucp1 expression levels in BAT. Even though we cannot exclude the possibility of increased glucose/NEFA clearance by BAT, our results do not indicate that heat production in BAT is activated. Besides Fg21 mRNA expression, we did find increased expression of other adipokines such as Bmp8b, Lcn2, and Gdf15 in our transcriptomics data, which can promote the thermogenic potential of BAT and WAT. Whether BAT-derived FGF21 reaches the circulation remains controversial, however, it could have indirect effects in this study by stimulating batokine production. BMP8b is part of a large family of BMPs that are involved in the differentiation of white and brown adipocytes, with BMP8b contributing to maximal thermogenic capacity by remodelling of the neuro-vascular network. Similarly, LCN2 and GDF15 are involved in inducing thermogenesis, as suggested by knockout-studies and cold-exposure experiments. However, as the thermogenic potential measured by Ucp1 expression was not induced in BAT after LP intervention, the role of these batokines in the current study needs to be further evaluated.

Insulin resistance plays a central role in the development of metabolic diseases. Aging is associated with insulin resistance due to age-related changes in adipose tissue function, mass and distribution. These changes are accompanied by diminished lipid handling, low-grade inflammation, defective thermogenesis, and impaired de novo adipogenesis. In our study, aged mice displayed insulin resistance, increased adiposity, and decreased thermogenesis. Short-term LP feeding did not impact glucose tolerance, insulin sensitivity or adiposity at an advanced age, and only mildly improved hepatic insulin sensitivity in young adult mice, without differences in body weight. In contrast to our observations, some studies have reported improved glucose homeostasis as a result of LP feeding both in (young) mice and humans, but these latter findings were accompanied by decreased body weight, potentially explaining the improved insulin sensitivity. Our results, however, show that energy expenditure and thermogenesis can be improved by LP feeding in aged mice, indicating that these LP-induced effects are independent of insulin sensitivity and body weight changes. Interestingly, while aged mice respond well to a 7% low protein diet regarding EE, suggesting that energy expenditure and insulin sensitivity could be distinct features of protein restriction in aged mice, the effects of LP on EE could not be
reproduced in young adult animals. In young mice, food intake is increased as a result of LP feeding, and animals do not gain weight, but changes in EE are not detectable. Subtle changes in energy expenditure, nutrient absorption, activity or a combination of these might explain this discrepancy in energy balance, however a larger cohort is needed to provide answers. In addition, a previous study shows a divergent effect of 5% or 10% \(^{54}\), suggesting a threshold of protein intake to improve metabolic parameters. This hypothesis could also explain why young adult mice on 7% protein restriction did not display increased energy expenditure. The fact that aged mice did respond to a 7% protein restriction suggests that this threshold could be age-specific.

Epidemiological studies suggest that diets high in protein and low in carbohydrates are associated with increased mortality \(^{55}\). Data from the Third National Health and Nutrition Examination Survey (NHANES III), stratified for age indicated that high protein diets (>20%) were associated with increased mortality and cancer in respondents under the age of 65 years, and with a 5-fold increase in diabetes mortality across all ages \(^{56}\). In respondents over 65, however, high-protein intake was associated with reduced overall mortality. A possible explanation is that at an advanced age, low protein intake might result in a metabolic decline due to decreased availability of amino acids, protein anabolic resistance, and immobility. In agreement with the effect of aging turning the beneficial effects of protein restriction on mortality into negative effects, feeding 2-year old mice a 4% protein diet led to a rapid weight loss of 10% in 15 days \(^{56}\). Although not measured, this was likely due to loss of muscle mass, an age-related risk that is elevated with reduced protein supply \(^{57}\). To avoid muscle mass deterioration at an advanced age, our study was conducted with a less restricted protein content of 7%. Dietary protein restriction reduced plasma levels of branched-chain amino acids, which are often associated with metabolic diseases \(^{58,59}\), but increased levels of most other amino acids, suggesting no signs of amino acid deficiencies. In our study, aged mice displayed no reduction in body weight, lean mass, or fat mass during the 3-month intervention with different protein diets. Physical tests determining muscle mass and function revealed only an age-related decline and no negative effects of the LP diet, indicating that moderate protein restriction of 7% is well tolerated at an advanced age in mice. Interestingly, feeding an HP diet for 3 months at advanced age did not ameliorate the age-related decline in muscle strength, suggesting a discrepancy with the common consensus in literature \(^{31–33}\). However, the short duration of the diet and the age at which the diet is started could have an effect on the outcome. Starting the diet at 18 months of age, when the negative effects on grip strength and hanging wire are still relatively small, the impact of a high protein diet may not have been noticeable, suggesting the consequences of protein intake might be age-specific.

Important to mention is the possible role of carbohydrate intake on the diet-induced effects on energy metabolism in this study. Firstly, a change in the protein-carbohydrate ratio changes glucose oxidation, resulting in increased RER. However, whether these
changes in RER directly affect energy expenditure remains unknown. Secondly, carbohydrate intake could also have influenced hepatic \textit{Fgf21} expression by regulation of the transcription factor ChREBP in conditions of high sucrose intake \textsuperscript{60,61}. In our study, the higher sucrose content of the AIN-93G semisynthetic diet, compared to chow, might indeed have stimulated hepatic \textit{Fgf21} transcription at the start of the experiment. Still, it does not explain the differences between our experimental groups. In our diet, the protein content has been substituted with starch, not sucrose. Therefore, mechanisms underlying \textit{Fgf21} stimulation are unlikely the result of changes in ChREBP regulation by carbohydrate intake. More likely, the amino acid composition affects FGF21 levels and improves metabolic health \textsuperscript{10,34}. Several diets with reduced levels of essential amino acids such as methionine or branched-chain amino acids (leucine, isoleucine, or valine) mimic the effects of a LP diet \textsuperscript{10,62,63}. Whereas the branched-chain amino acids were significantly reduced in plasma, most other amino acid levels were increased by dietary protein restriction in both ages, which is in line with previous studies \textsuperscript{8}. It needs to be further determined whether mechanisms of hepatic \textit{Fgf21} transcription are the result of selective amino acid deprivation.

**Conclusion**

Taken together, it is evident that a LP diet provides several benefits on metabolic health in aged mice. Our study indicates that an short-term intervention with an optimal balance of dietary protein and carbohydrates can positively impact metabolic health in aged individuals as we find increased energy expenditure and thermogenesis, via browning of white adipose tissue, without any negative reduction in muscle function. We speculate that FGF21-mediated changes in scWAT are crucial to achieve metabolic health in aged individuals, suggesting that age-related decline of adipose function can be effectively improved by targeting FGF21 signalling. Altogether, this study highlights the possibility of browning of scWAT to improve metabolic health by LP feeding in the context of advanced age.

**Acknowledgments**

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• Chapter 5

Author contribution
MBD, MB, JKK and JWJ conceived the study and designed the experiments. MBD, MB and MAVL conducted experiments and performed data analysis. HK, MHK and AG analysed samples. RPvO provided animals. THvD performed the modeling of glucose kinetics. VWB performed data analysis. MBD, MB, MAVL, BMB, JKK and JWJ interpreted the data. MBD, JKK and JWJ wrote the manuscript. JKK and JWJ supervised the study. All authors critically reviewed and edited the manuscript.

Conflict of Interest
The authors declare no conflict of interest.
**Supplementary information**

**Supplementary table 1. Dietary composition.** Diets varying in protein/carbohydrate ratio were used to investigate the metabolic effects in the context of ageing. Diets were based on the AIN-93G breeding diet, obtained from Open Source Diets, containing 7% (low protein), 20% (medium protein) of 40% (high protein).

<table>
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<tr>
<th>Ingredient</th>
<th>D17041406</th>
<th>D17041407</th>
<th>D17041408</th>
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<td>220</td>
<td>441</td>
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<tr>
<td>Cysteine</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>Corn Starch</td>
<td>471</td>
<td>368</td>
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<td>altodextrin 10</td>
<td>156.4</td>
<td>122.2</td>
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<tr>
<td>Sucrose</td>
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<td>107.078</td>
<td>107.078</td>
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<tr>
<td>Cellulose</td>
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<td>50</td>
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<tr>
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<td>t-butylhydroquinone</td>
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<td>Mineral Mix S10022C</td>
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<td>3.5</td>
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<tr>
<td>Calcium Carbonate</td>
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<td>12.495</td>
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<td>Potassium Citrate, H2O</td>
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<tr>
<td>Potassium Phosphate, monobasic</td>
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<td>7.763</td>
<td>0</td>
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<tr>
<td>Calcium Phosphate, dibasic</td>
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<tr>
<td>Sodium Chloride</td>
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<tr>
<td>Choline Bitartrate</td>
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<td>FD&amp;C Yellow Dye #5</td>
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<td>FD&amp;C Red Dye #40</td>
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<td>Total</td>
<td>999.446</td>
<td>1000.963</td>
<td>998.955</td>
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<td>kcal/gm</td>
<td>4.1</td>
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Supplementary figure 1: Normalization of aged animals into experimental groups. Physiological parameters were measured after a 4h fast prior to the experiment, including body weight (A), blood glucose (B), plasma insulin (C) and total plasma cholesterol (D) (n=22). E) Mean, SEM and N of the experimental diets excluding animals who reached humane endpoint. F) p-values, using Ordinary one-way ANOVA between different experimental diets. In all graphs, LP=red, MP=grey, HP=blue.
Supplementary figure 2: Validation of RNA sequencing data. Comparing RNA sequencing data (n=4-5) of different genes to qPCR results (young; n=9, old; n=12), including Fgf21 (A) and Bmp8b (B) in brown adipose tissue, and Ucp1 (C) and Elovl3 (D) for scWAT. All graphs show mean ± SEM as well as individual samples, LP=red, MP=grey, HP=blue.
Supplementary methods

Calculation of Glucose Kinetics

Tracer concentrations were calculated as the product of the blood glucose concentration and the fractional contribution of the tracer at each time point (Suppl. figure 3B, equation 1). To describe changes in blood glucose kinetics in mice, we used the minimal model for glucose metabolism in humans as introduced by Bergman et al. that was further developed for oral glucose tests by Dalla Man and Cobelli et al. (Suppl. figure 3A)\textsuperscript{1-6}. We have further modified this model for use in mice by adjusting the restrictions in the total sample volume that can be taken in experiments in mice. To generate sufficient input data, measured data of blood glucose, insulin and tracer concentrations were fitted into equation 2 (Suppl. figure 3B) (SAAM II v2.1, The Epsilon Group, Charlottesville, VA, USA). By interpolations, concentrations of all three metabolites were estimated at multiple time points (t). The bioavailability (F) (Suppl. figure 3B, equation 3) and delay (d) of the bolus were estimated from tracer curves. The model (Minimal Model for Mice, i.e., MiniM) is presented in Figure A. Insulin-mediated, glucose-mediated, and -independent glucose utilization fluxes, peripheral and hepatic insulin sensitivity as well as glucose effectiveness upon administration of a glucose bolus were estimated using the SAAM II software (SAAM II v2.3.1.1, The Epsilon Group, Charlottesville, VA, USA).

For execution of this model, additional parameters were introduced. For flexibility of the model, delay time (d) and bioavailability (F) were introduced as Bayesian values (1.0 ± 0.1) called adjustment parameters $A_d$ and $A_F$, respectively. Next to this, the volume of the accessible glucose pool, $V_g$, was adapted from literature on humans\textsuperscript{7,8} and the insulin independent glucose utilization under basal conditions ($U_{iig}$) was set to three times the insulin dependent glucose utilization ($U_{idg}$)\textsuperscript{9,10}. Furthermore, the independent glucose utilization under basal conditions was set to flux at 45% ($F_{iig}$) of the endogenous glucose production (EGP), rather than an absolute value as was used in humans. In our hands, an absolute value that was valid for all mice strings under very different conditions could not be generated. Above parameters were set as Bayesian values in the model including 10% standard deviation. As result, the fractional turnover rates $k_0$ to $k_4$ were estimated as well as the fractional turnover rates determining the utilization fluxes, i.e., independent flux ($k_{ic} + k_{ic}$), glucose mediated flux ($k_{ig}$) and insulin mediated flux ($k_{ib}$).
Supplementary Figure 3: Compartmental model for calculating kinetic parameters of glucose metabolism upon an OGTT in mice. A) An oral gavage was given, which passes the liver to contribute to the accessible plasma glucose pool \( Q_p \) and to the inaccessible “tissue” glucose pool \( Q_t \). Additionally, glucose is produced/released by the tissues such as the intestine and liver also contribute to \( Q_p \). As such, two rates of appearances can be distinguished, namely that of exogenous injected glucose \( (R_{a\text{exo}}) \) and of endogenous glucose (EGP). The \( Q_p \) is in equilibrium with \( Q_t \) via two rate constants \( (k_1 \) and \( k_2 \)). Disposal of glucose \( (U) \) from the model can be divide into insulin-independent glucose-dependent disposal \( (U_{iig}) \), insulin-independent constant disposal \( (U_{iic}) \), insulin-dependent constant disposal \( (U_{idc}) \) and insulin-dependent disposal \( (U_{idi}) \). As with glucose, insulin is also distributed over two compartments in this model, namely the accessible plasma insulin pool \( I_p \) and the inaccessible tissue insulin pool \( I_t \). B) Insulin action, as well as peripheral and hepatic insulin sensitivity were estimated using varying fluxes and insulin concentrations, in which \( b \) indicates basal values measured before the experiment and \( 0 \) indicates the estimated value at the time of injection. Abbreviations; \( k_0-k_4 \): fractional turnover rates, \( V_G \): volume of the accessible pool, \( k_{iac}-k_{ia}-k_{idc} \): fractional turnover rates determining the utilization fluxes.

Equations for assessing glucose metabolism, using kinetic parameters:

- **Equation 1:** Calculation of tracer concentrations from blood glucose concentration and the fractional contribution of the tracer at each time point.

- **Equation 2:** Generation of sufficient input, based on measured data of glucose, insulin and tracer concentration.

- **Equation 3:** Bioavailability of the bolus.

- **Equation 4:** Glucose clearance rate of basal level.

- **Equation 5:** Endogenous glucose production.

- **Equation 6:** Insulin action.

- **Equation 7:** Peripheral insulin sensitivity.

- **Equation 8:** Hepatic insulin sensitivity.

- **Equation 9:** Hepatic glucose responsiveness.
Supplemental References


### Supplementary table 2: Sybr Green primer sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
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<tr>
<td>36B4</td>
<td>acidic ribosomal phosphoprotein</td>
<td>GCT CCA AGC AGA TGC AGC A</td>
<td>CCG GAT GTG AGG CAG CAG</td>
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<tr>
<td>Bmp8b</td>
<td>Bone Morphogenetic Protein 8b</td>
<td>TCA ACA CAA CCC TCC ACA TCA</td>
<td>AGA TCG GAG CGT CTG AAG ATC</td>
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<tr>
<td>Cld137</td>
<td>Tumor Necrosis Factor Receptor Superfamily Member 9</td>
<td>CGT GCA GAA CTC CTG TGA TAA C</td>
<td>GTC CAC CTA TGC TGG AGA AGG</td>
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<tr>
<td>Cidea</td>
<td>Cell Death-Inducing DFFA-Like Effector A</td>
<td>GCC GTG GTT AAG GAA TCT GC</td>
<td>GTA TGT GCC CTC ATA GAC CA</td>
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<tr>
<td>Elovl3</td>
<td>Elongation Of Very Long Chain Fatty Acids Protein 3</td>
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## Supplementary table 3: Taqman primer sequence

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<tr>
<td>Fgf21 fibroblast growth factor 21</td>
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<td>TGA CAC CCA GGA TTT GAA TGA C</td>
<td>CCT GGC TTC AAG GCT TTG AGC TCC A</td>
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<tr>
<td>Ppargc1a peroxisome proliferative activated</td>
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<td>GGC CTG CAG TTC CAG AGA GT</td>
<td>CCC CAT TTG AGA ACA AGA CTA TTG AGC GAA CC</td>
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<td>receptor, gamma, coactivator 1</td>
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</table>
References


