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Title: Casein protein hydrolysate fed human serum from older adults regulates *in vitro* muscle protein synthesis, muscle protein breakdown and myotube growth

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Abstract

In this study we used a recently developed *ex vivo-in vitro* model to assess the effect of feeding older adults a casein protein hydrolysate (CPH) compared with non-bioactive non-essential amino acid (NEAA) supplement on Muscle Protein Synthesis (MPS) and Breakdown (MPB). Serum from six healthy older males following overnight fast and 60 min postprandial ingestion of CPH or NEAA (0.33 $g.kg^{-1}$ body mass) was used to condition C2C12 myotube media. CPH-fed serum significantly increased MPS compared to fasted serum. In addition, CPH-fed serum induced myotube growth and markedly suppressed atrogin-1, but not MuRF1, expression. Comparatively, no change in MPS, myotube growth and gene expression was observed following NEAA-fed serum treatment. CPH-fed serum from older adults stimulated *de novo* MPS, suppressed markers of protein breakdown and resulted in myotube growth, indicating a potential role for CPH as a dietary protein source to prevent age-related sarcopenia.

Keywords: Casein; Hydrolysate; Muscle protein synthesis; Serum; Skeletal muscle

Abbreviations: AA, amino acids; CPH, casein protein hydrolysate; EAA, essential amino acids; MPB, muscle protein breakdown; MPS, muscle protein synthesis; NEAA, non-essential amino acids; TAA, total amino acids

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1. Introduction

Aging is accompanied by a progressive reduction in muscle mass, strength and quality, contributing to frailty and mortality in older adults [1]. Therefore, preservation of muscle mass and function with advancing age is of high priority. Among many factors that contribute to age related decrease in skeletal muscle mass, physical inactivity and inadequate protein intake increase the risk of physiological and functional decline [2]. Consequently, numerous studies have shown beneficial effects of feeding amino acids (AA) and proteins from different sources on MPS in the elderly [3,4,5].

The stimulatory effect of essential amino acids (EAA), primarily leucine, on MPS is well documented [6,7,8]. Milk fractions, whey and casein, provide a complete EAA profile and the highest leucine content of dietary protein, 11% and 9.3%, respectively. Whey protein induces rapid and transient increase of aminoacidemia and stimulation of MPS, while casein protein suppresses muscle protein breakdown (MPB) and has more modest, but prolonged, effect on MPS due to slower AA absorption kinetics [7, 8, 9].

Several studies have found that the mode of delivery of dietary protein and digestion rate differentially modulates postprandial muscle protein accretion [9,10,11]. In this respect, prior modification by hydrolysis has been shown to improve protein digestion and absorption kinetics, increasing circulating AA concentration compared to intact protein [7,12,13,14], exemplified by greater incorporation of casein protein hydrolysate (CPH) over intact casein into muscle protein in the elderly [15].

This study employed an *ex vivo-in vitro* model [16,17] to evaluate the bioactivity of CPH for myotube growth, MPS and MPB in C2C12 skeletal muscle myotubes. Myotube growth, MPS and expression of genes linked to MPB were measured following incubation in media conditioned with (i) fasted serum, (ii) serum sampled following ingestion of CPH or (iii) ingestion of an isonitrogenous, non-bioactive mixture of non-essential amino acids (NEAA) in

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older adults. We hypothesized (i) anabolic potency measured *in vitro*, would be greater in myotubes incubated in media conditioned with CPH-fed serum than fasted-serum and (ii) the post-ingestion bioactivity evoked by feeding CPH would be greater than for an isonitrogenous mix of NEAA. These findings provide new insights into the effects of hydrolysed protein sources on the regulation of muscle protein balance during aging.

2. Materials and methods

2.1. Ethical Approval

The study was approved by the local ethics committee at the University of Limerick (EHSREC-_2013_01_13) and conformed to the standards set by the Declaration of Helsinki. Six old healthy male participants agreed to participate in the study, gave informed written consent and completed the intervention trial. Baseline participant characteristics were as follows: age 58±6.7y; weight 81.3±7kg, height 1.78±0.04m, body mass index 25.8±3kg·m^{-2}, body fat 22±3.6%.

2.2. Study design

Participants reported to the lab following an overnight fast (>10 h) on two occasions separated by at least 7 d. A blood sample from the antecubital vein was collected at baseline (t=0 min) by a clinical nurse on each day as described previously [16]. Participants consumed 0.33g.kg-1 body mass of either an NEAA control beverage or a CPH beverage. Both beverages were prepared in 500 mL water (7.6% w/v) and their composition is reported in Table 1. As aminoacidemia and MPS have been previously shown to peak between 45-90 min following protein feeding [18,19], an additional blood sample was collected 60 min postprandial.

2.3. Amino acid analysis

AA profile of each participant using plasma samples collected at 0 and 60 min postprandial was determined using pre-established protocols [20] on the Agilent 1200 RP-UPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 1260 binary pump

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and a G1367C automated liquid handling system. AA separation, data acquisition and quantitative analysis was performed as discussed previously [16].

2.4. Metabolic marker analysis

Plasma insulin was determined using a commercial kit (Merck Millipore) on a MAGPIXTM Multiplex reader and processed using Bio-Plex ManagerTM MP.

2.5. Cell culture

For all experiments, C2C12 myoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (D6429; Sigma-Aldrich), supplemented with 10% fetal bovine serum (F7524, Sigma-Aldrich), 1% penicillin/streptomycin (P0781; Sigma-Aldrich) and 1 mM L-glutamine (G7513; Sigma-Aldrich), under standard conditions at 5% CO_2 in a humidified air atmosphere at 37 °C. Once cells reached 70-80% confluence, differentiation was induced by DMEM supplemented with 2% horse serum (H1270; Sigma-Aldrich) as previously described [16] for up to 7 days. Prior to treatment with human serum, fully differentiated myotubes were nutrient deprived in an amino acid (AA) and serum free DMEM medium (US biological, Salem, MA, USA), supplemented with 1 mM sodium pyruvate (GE Healthcare, Thermo-Fisher), 1% (v/v) penicillin/streptomycin solution, 1 mM L-glutamine, 6 mM D-glucose (Sigma-Aldrich), and 34 mM NaCl (Sigma-Aldrich) (pH 7.3).

2.6. Myotube diameter measurement

Images of myotubes were taken using a light microscope (Olympus CKX31). Images were saved as JPEG images and analysed using Image J software (National Institutes of Health, Baltimore, MD) to measure changes in myotube thickness. Three measurements of the diameter were taken along each myotube, in at least 6 different fields, for at least 100 myotubes per treatment condition.

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2.7. Electrical impedance measurement

Electrical impedance measurements were taken using the xCELLigence™ RTCA Instrument (ACEA Biosciences) and a microelectronic 16-well gold plated base sensor plate (ACEA Biosciences) [21]. C2C12 myoblasts were seeded on an electrode-containing plate at a density of 5000 cells per well in a volume of 0.2 mL. Cells were allowed to proliferate for 30 h and differentiation was induced as previously described [21]. Automated cell index readings were taken every 15 min during the myoblast proliferation and myotube formation and every 2 min throughout the experiment. Data were normalized to the start of the treatment phase of the experiment. Data are n=5 due to limited serum sample from one participant as a result of a haemolysed sample.

2.8. Muscle Protein Synthesis

Measurement of MPS in C2C12 following serum treatment was performed via the surface sensing of translation technique (SUnSET) [22]. Differentiated C2C12 myotubes were nutrient deprived in AA- and serum- free DMEM medium for 1 h and treated with 20% human serum in AA and serum free DMEM medium containing 1 µM puromycin (Merck Millipore Limited) for an additional 4 h. Cellular protein lysates were obtained as previously described [16]. MPS was determined by immunoblotting.

2.9. Immunoblotting

Protein lysates (30µg) were loaded on 4-15% linear gradient SDS-PAGE precast gels (Mini-Protean TGX Stain-free, Bio-Rad 456-8083) and subjected to electrophoresis at 200 V for up to 1 h. Following electrophoresis, total protein in each lane of the gel was determined using stain-free UV-induced fluorescence that activates tryptophan residues on the gel (UVITEC Cambridge Imaging system, UVITEC, Cambridge, UK). Proteins were transferred onto a 0.2 µm nitrocellulose membrane using a semi-dry transfer technique (Trans-blot® Turbo™ Bio-Rad), following which membranes were blocked for 1 h at room temperature with 5% bovine

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serum albumin (BSA) in 0.05% Tween-20 in Tris-buffered saline (TBS-T). Membranes were then incubated overnight at 4 °C with the primary antibody for puromycin (MABE343 anti-puromycin, clone 12D10 mouse monoclonal, Merck Millipore Limited). Following overnight incubation, membranes were probed with IRDye® 800CW goat anti-mouse IgG2a-specific secondary antibody (LI-COR Biosciences UK Ltd). Images were captured using UVITEC Cambridge Imaging system (UVITEC, Cambridge, UK) and whole-lane band densitometry was quantified using NineAlliance UVITEC Software (UVITEC, Cambridge, UK).

2.10. RNA isolation and Real-time PCR

Myotubes grown on 6-well plates were disrupted using QIAshredder spin column (Qiagen) and total RNA was extracted using the RNeasy® Mini kit (Qiagen) according to the manufacturer's instructions. Then, 1 μg of cDNA was synthesized using the Superscript VILO cDNA synthesis kit (11754050; Biosciences) in accordance with the manufacturer's instructions. Real-time PCR was conducted using Taqman Universal Master Mix II, no UNG (4440047; Thermo Fisher Scientific) and TaqMan® Gene Expression Assays (4331182; Thermo Fisher Scientific) in the Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Polymerase chain reaction was performed as follows: denaturation for 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s (annealing) and at 60 °C for 1 min (elongation). Negative control samples were always included in the amplification reactions to check for contamination. The messenger RNA (mRNA) expression of the target genes: muscle atrophy F-Box (atrogin-1) and muscle ring finger 1 (MuRF1) was normalized to two housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and peptidylprolyl isomerase A (PPIA).

2.11. Statistical analysis

GraphPad Prism v7.03 was used for statistical analysis. Unpaired and paired Sample *T*-tests were used to analyse myotube diameter, MPS, and mRNA expression responses to fed or fasted

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ex vivo human serum, and two-way ANOVA with Tukey's post-hoc test was used to analyse cell index. Level of significance was set at 95% ($P < 0.05$). Data presented are Mean ± SEM.

Table 1
Composition of CPH and isonitrogenous NEAA control protein drinks. Dose scaled per kg body mass, and amount reported in table would be typical dose for an 80 kg participant.

	CPH	**NEAA**

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Energy (kJ)	465	442
Degree of hydrolysis (%)	19.7	0
Macronutrient Content		
Protein or AAs (g)	26.4	26.4
Carbohydrate (g)	0.6	0
Fat (g)	-	0
EAA		
Leucine (g)	2.4	0
Isoleucine (g)	1.3	0
Valine (g)	1.6	0
Histidine (g)	0.7	0
Lysine (g)	2.4	0
Methionine (g)	0.8	0
Phenylalanine (g)	1.3	0
Threonine (g)	1.1	0
Tryptophan (g)	0.3	0
NEAA		
Alanine (g)	0.7	2.7
Arginine (g)	0.9	0
Aspartic Acid (g)	1.8	3.2
Cysteine (g)	0.1	0
Glutamic Acid (g)	5.5	9.6
Glycine (g)	0.5	1.1
Proline (g)	2.7	4.3
Serine (g)	1.4	3.6
Tyrosine (g)	1.3	2.0
Total BCAA (g)	5.3	0
Total EAA (g)	11.8	0
Total NEAA (g)	14.9	26.4

BCAA, branched chain amino acids; EAA, essential amino acids; NEAA, non-essential amino acids; AA, amino acids.

3. Results

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3.1. Plasma amino acid and insulin concentrations

Plasma samples from the volunteers were obtained after an overnight fast and 60 min post-prandial following CPH or NEAA ingestion, and levels of metabolic biomarkers and AA were determined (Table 2). Consumption of 0.33 $g.kg^{-1}$ body mass of the CPH beverage significantly increased the concentration of circulating EAA and NEAA compared to its corresponding fasted plasma (t=0) ($P< 0.05$). Comparatively, there was no increase in circulating EAA following NEAA ingestion (Table 2). Insulin concentrations increased in both feeding conditions but failed to reach statistical significance (Table 2).

3.2. Muscle protein synthesis

For *in vitro* experiments, differentiated myotubes were nutrient deprived for 1 h before carrying out treatment with AA- and serum- free medium conditioned with 20% *ex vivo* fasted or CPH/NEAA post-prandial serum for 4 h. The SunSET technique was adapted to measure MPS in C2C12 myotubes following treatment with *ex vivo* human serum as previously described [16]. Treatment with CPH-fed serum resulted in a significant increase in MPS ($P< 0.05$) compared to its corresponding fasted serum (Fig. 1A and B). Additionally, no significant difference in MPS in the NEAA group was observed (Fig. 1A and B).

3.3. Biomarkers of Muscle Protein Breakdown (mRNA expression)

The mRNA expression of MPB/atrophy related ubiquitin ligases, muscle atrophy F-Box (atrogin-1) and muscle ring finger 1 (MuRF1) was assessed to address the effects of CPH or NEAA post-prandial serum on MPB. CPH-fed serum treatment markedly reduced the expression of atrogin-1 relative to fasted ($P< 0.01$) and NEAA-fed ($P< 0.05$) serum (Fig. 2A), while no change in MuRF1 expression was observed (Fig. 2B). In addition, NEAA-fed serum had no effect on either, atrogin-1 or MuRF1 mRNA levels. These results suggest that CPH reduces MPB mediated through suppression of atrogin-1.

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3.4. Myotube size

Having previously optimized the performance of C2C12 skeletal muscle cells on the xCELLigence™ platform [21], it was used to evaluate changes in myotube diameter by measuring cellular electrical impedance. Cell index values are reported as the fed relative to the fasting conditions for both beverages. As seen in Fig. 3A, cell index measured during the treatment with CPH-fed serum showed marked increase at 1 ($P < 0.05$), 2 and 4 h treatment ($P < 0.01$) relative to its corresponding fasted serum. In addition, CPH-fed cell index values were also significant ($P < 0.05$) in comparison to NEAA-fed serum at 1 and 2 h treatment. To confirm if these results reflected changes in myotube diameter, images of myotubes were taken following 4 h treatment and measured using Image J. CPH-fed serum significantly increased myotube diameter compared to the effect of its corresponding fasted serum ($P < 0.01$) and to NEAA-fed serum ($P < 0.01$) (Fig. 3B).

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Table 2

Plasma insulin and amino acid profile of plasma samples at baseline and 60 min postprandial.

	CPH			NEAA		
Time (min)	**0**	**60**	**Δ (0-60)**	**0**	**60**	**Δ (0-60)**
Metabolic Biomarkers						
Insulin (pM)	168 ± 60	330 ± 115	**162 ± 116**	143 ± 33	195 ± 44	**52 ± 33**
EAA						
Leucine (µmol/L)	129 ± 8	459 ± 18*	**330 ± 16**$^{\#}$	133 ± 9	121 ± 7	**-12.4 ± 7**
Isoleucine (µmol/L)	58 ± 4	239 ± 4*	**182 ± 7**$^{\#}$	63 ± 4	56 ± 4	**-7 ± 3**
Valine (µmol/L)	209 ± 16	514 ± 8*	**305 ± 11**$^{\#}$	219 ± 10	211 ± 10	**-9 ± 11**
Histidine (µmol/L)	86 ± 7	121 ± 6*	**35 ± 5**$^{\#}$	86 ± 6	86 ± 5	**0 ± 4**
Lysine (µmol/L)	183 ± 15	418 ± 23*	**236 ± 20**$^{\#}$	191 ± 15	195 ± 13	**3 ± 12**
Methionine (µmol/L)	20 ± 2	84 ± 7*	**64 ± 6**$^{\#}$	21 ± 2	20 ± 2	**-1 ± 2**
Phenylalanine (µmol/L)	52 ± 3	119 ± 7*	**67 ± 6**$^{\#}$	54 ± 4	48 ± 2	**-6 ± 3**
Threonine (µmol/L)	101 ± 5	194 ± 6*	**93 ± 8**$^{\#}$	119 ± 8	160 ± 16*	**42 ± 12**
Tryptophan (µmol/L)	64 ± 4	114 ± 7*	**50 ± 4**$^{\#}$	61 ± 4	57 ± 2	**-5 ± 4**
NEAA						
Alanine (µmol/L)	240 ± 17	388 ± 26*	**148 ± 20**$^{\#}$	287 ± 38	575 ± 35*	**289 ± 33**
Arginine (µmol/L)	67 ± 4	148 ± 20*	**80 ± 10**$^{\#}$	71 ± 5	66 ± 5	**-5 ± 9**
Asparagine (µmol/L)	97 ± 6	182 ± 9*	**86 ± 9**$^{\#}$	98 ± 4	263 ± 23	**165 ± 22**
Aspartic acid (µmol/L)	0 ± 0	6 ± 2*	**6 ± 2**	0 ± 0	31 ± 12*	**31 ± 12**
Glutamine (µmol/L)	616 ± 35	814 ± 70*	**198 ± 46**	601 ± 19	731 ± 55*	**131 ± 44**
Glutamic acid (µmol/L)	69 ± 13	126 ± 21*	**57 ± 10**	67 ± 11	281 ± 76*	**214 ± 72**
Glycine (µmol/L)	179 ± 18	204 ± 16*	**25 ± 9**$^{\#}$	187 ± 10	351 ± 26*	**164 ± 25**
Tyrosine (µmol/L)	57 ± 4	191 ± 12*	**133 ± 10**$^{\#}$	61 ± 5	110 ± 10*	**48 ± 7**
EAA (µmol/L)	902 ± 59	2262 ± 64*	**1360 ± 64**$^{\#}$	947 ± 54	952 ± 47*	**5 ± 58**
NEAA (µmol/L)	1326 ± 60	2060 ± 92*	**734** ± 72	1367 ± 44	2368 ± 107*	**1001 ± 104**
TAA[a] (µmol/L)	2227 ± 108	4322 ± 112*	**2094 ± 113**$^{\#}$	2314 ± 96	3320 ± 164*	**1006 ± 150**

Essential amino acids (EAA); Total amino acids (TAA). Data represents Means ± SEM (N=6);* within groups (P< 0.05), $^{\#}$ between groups (P< 0.05)

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INSERT FIG 1 HERE

Fig. 1. Effect of *ex vivo* human serum on MPS in skeletal muscle cells. C2C12 myotubes were nutrient deprived for 1 h followed by treatment with *ex vivo* fasted (Fast) or 60 min postprandial (Fed) human serum for 4 h. Postprandial serum was obtained 1 h after ingesting CPH or isonitrogenous NEAA. (A) Densitometric analysis of MPS before and after treatment with media conditioned by CPH or NEAA-fed relative to corresponding fasted serum. (B) Representative immunoblot of MPS (measured by puromycin incorporation) (left) relative to total protein (loading control) (right). Data reported as Mean±SEM.*relative to corresponding fasted serum $P< 0.05$.

INSERT FIG 2 HERE

Fig. 2. Effect of *ex vivo* human serum on MPB in skeletal muscle cells. C2C12 myotubes were nutrient deprived for 1 h followed by treatment with 20% *ex vivo* fasted (Fast) or 60 min postprandial (Fed) human serum for 4 h (n=6). Quantification of (A) atrogin-1 and (B) MuRF1 mRNA expression after treatment with CPH or NEAA *ex vivo* fed relative to fasted serum. Data reported as Mean±SEM. ** $P< 0.01$ relative to corresponding fasted serum; # $P< 0.05$ relative to CPH-fed serum.

INSERT FIG 3 HERE

Fig. 3. Effect of *ex vivo* human serum on cell index and myotube diameter in skeletal muscle cells. C2C12 myotubes were nutrient deprived for 1 h followed by treatment with 20% *ex vivo* fasted (Fast) or 60 min postprandial (Fed) human serum for 4 h. (A) Plot of changes in normalized cell index response to CPH or NEAA-fed serum relative to fasted serum measured by xCELLigence™ system (N=5). (B) Quantification of myotube diameter taken post treatment as measured by microscopy (N=6). Data reported as Mean±SEM.*, **relative to corresponding fasted serum $P< 0.05$, $P< 0.01$ respectively; and $^{\#}$, $^{\#\#}$between groups $P< 0.05$, $P< 0.01$ respectively i.e. between CPH-fed and NEAA-fed serum.

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4. Discussion

The beneficial effect of dietary protein on muscle mass and function in the elderly has been well established [3,4,5,23]. Optimizing food intake to maximise stimulation of postprandial MPS is, therefore, a valid nutrient strategy to offset protein loss during aging. The kinetics of protein digestion, aminoacidemia and AA absorption are known to impact postprandial rates of MPS [9,10,15]. In addition, specific hydrolysis of protein prior to ingestion has been shown to augment post-prandial MPS response compared with intact protein [7,15]. Since CPH has been shown to have digestion and absorption similar to those of WPH [24], we investigated its anabolic potency in C2C12 skeletal muscle myotubes by using *ex vivo* human serum from CPH-fed older adults.

While elevated levels of EAA strongly increase anabolic signalling and MPS [8,25], NEAA do not have a stimulatory effect on MPS *in vitro* [17] or *in vivo* [6,26,27]. It has been demonstrated that significant stimulation of MPS requires at least a two-fold increase of plasma aminoacidemia above basal levels [28]. As expected, following CPH ingestion, plasma EAA concentrations were significantly elevated by 2.5-fold. Among EAA, leucine plays a unique role in the regulation of MPS [8,26]. Following CPH ingestion, plasma leucine was increased by 3.6-fold while no increase was observed following feeding with NEAA. These data demonstrate that ingestion of a CPH results in significant aminoacidemia, suggesting absorption of EAA is not compromised in these older adults. There has been much debate on whether insulin has a stimulatory effect on MPS in humans. Increased post-prandial insulin has no significant effect on MPS, and only in the presence of sufficient AA concentrations, insulin acts to increase AA delivery to skeletal muscle [29,30]. In this study, CPH and NEAA ingestion resulted in modest increases in insulin concentrations, but importantly, there were no significant differences between fed and fasted serum of both groups. Overall, our data

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demonstrates that CPH-fed serum stimulated MPS in C2C12 myotubes was most likely due to an increase in post-prandial circulating EAA levels.

Current knowledge of the nutrient effect on MPB is limited, partly due to the methodological difficulties in measuring protein breakdown. MPB is highly regulated by complex proteolytic systems, but primarily by the ubiquitin-proteasome system. Muscle specific ubiquitin ligases, atrogin-1 and MuRF1, play critical roles in atrophy by catalysing the ubiquitination and degradation of key muscle proteins [31]. Changes in molecular signalling of MPB pathways were assessed by analysing the mRNA expression of atrogin-1 and MuRF1 in C2C12 cells following treatment with *ex vivo* fasted and CPH or NEAA-fed human serum. A marked decrease in mRNA expression of atrogin-1, but not MuRF1, following treatment with CPH-fed serum relative to fasted and NEAA-fed serum was observed. Post-prandial NEAA serum did not change the expression of either atrogin-1 or MuRF1 mRNA. Several studies suggest that branched-chain amino acids (BCAA) and leucine attenuate protein breakdown via regulation of atrogin-1 and MuRF1 expression [32,33,34]. Of note, atrogin-1, and not MuRF1, has been previously shown to strongly correlate with the rate of MPB, indicating its tight association with breakdown of myofibrillar proteins [35]. In addition, insulin has been shown to exert its anabolic potency via attenuating skeletal MPB [36]. However, we did not see any significant differences in plasma insulin levels, making it unlikely that insulin contributed to the suppression of atrogin-1 expression following treatment with CPH-fed serum. Therefore, our data suggests that suppression of protein breakdown in C2C12 myotubes in response to feeding with CPH, is mediated by increased post-prandial levels of EAA.

In conclusion, CPH-fed serum from older adults stimulated MPS as measured by puromycin incorporation and suppressed markers of MPB that potentially led to myotube growth in C2C12 mytoubes *in vitro*. Conversely, the isonitrogenous non-bioactive NEAA-fed serum had no anabolic effect in this *in vitro* model. However, there are some limitations in this study.

Myotubes were treated with only 20% *ex vivo* human serum, while this gives an insight into the bioactivity of fed human serum this concentration may not elicit maximal stimulation of protein synthesis. Although we have provided new insights into the nutritional regulation of MPB, this was assessed by measuring changes in mRNA of only two markers of skeletal muscle breakdown. Finally, while media was conditioned by CPH fed serum from older adults, we did not measure the effects of CPH conditioned media on aged muscle cells which may provide further insights to the impact of CPH in ageing. Nevertheless, our findings provide new insights into the postprandial anabolic response of hydrolysed protein sources that could be used to improve more effective nutrient strategies for prevention of muscle loss during aging.

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Author contributions

Martina Pauk: Investigation, Formal analysis, Writing - Original Draft; **Miryam Amigo-Benavent**: Investigation, Writing - Review & Editing; **Bijal Patel**: Investigation, Writing - Review & Editing; **Philip M. Jakeman**: Conceptualization, Methodology, Writing - Review & Editing, Supervision; **Brian P. Carson**: Conceptualization, Methodology, Formal analysis, Writing - Review & Editing, Supervision.

Conflict of interest

The authors declare that there is no conflict of interest.

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