Hydrolyzed collagen supplementation prior to resistance exercise augments collagen synthesis in a dose-response manner in resistance-trained, middle-aged men

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Running head: Resistance exercise and collagen ingestion in middle-age

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New and noteworthy: This study is the first to document the dose-response effect of hydrolyzed collagen (HC) ingestion before resistance exercise (RE) on collagen turnover in middle-aged, resistance-trained men. Strikingly, RE alone did not increase collagen synthesis (suggesting connective tissue anabolic resistance) but ingesting 15g HC rescued the collagen synthesis response, and 30g augmented that response further. These results were associated with the increased bioavailability of collagen amino acids in the blood, which stimulate collagen synthesis.

ABSTRACT

Resistance exercise (RE) increases collagen synthesis in young and older men, while hydrolyzed collagen (HC) ingestion improves this response to RE in a dose-response manner in young men. However, the collagen synthesis response to RE with and without HC in *middle-aged* men is unknown. Eight resistance-trained men (age: 49±8 years; height: 1.78±0.02m; mass: 90±4kg) took part in this double-blind, crossover design study, and undertook 4×10 repetitions of lower-limb RE at maximum load, after consuming 0g, 15g, or 30g vitamin C-enriched HC. We analyzed venous blood samples for N-terminal propeptide of type 1 pro-collagen (PINP), β-isomerized C-terminal telopeptide of type 1 collagen (β-CTx) and 18 collagen amino acids throughout all three interventions. The serum PINP concentration×time area-under-the-curve (AUC) was higher following 30g (169±28 μg/mL×h) than 15g (134±23 μg/mL×h, $P<0.05$) HC ingestion, and both 15g and 30g were higher than 0g HC (96±23 μg/mL×h, $P<0.05$). RE with 0g HC showed no change in serum PINP concentration. The AUCs for glycine, proline, hydroxyproline, alanine, arginine, lysine, serine, leucine, valine and isoleucine were greater with 30g than 15g and 0g HC ingestion ($P<0.05$), and greater with 15g than 0g HC ingestion ($P<0.05$). Plasma β-CTx concentration decreased after RE independently of HC dose. Our study suggests connective tissue anabolic resistance to RE in middle-aged men but ingesting 15g HC rescues the collagen synthesis response, and 30g augments that response further. This dose-response is associated with the increased bioavailability of collagen amino acids in the blood, which stimulate collagen synthesis.

INTRODUCTION

Injury is a major concern for athletes of all ages, with injury rates to collagenous tissues such as muscle, ligament and tendon being similar between young, middle-aged and older male athletes (1). Chronic resistance exercise (RE), however, is a safe and effective method to reduce tendon injury risk (2). For example, chronic high intensity RE causes the tendon of healthy adults to adapt by increasing its mechanical (stiffness) and morphological (size) properties (3-6), which should result in reduced tissue stress during loading (7). Furthermore, increasing tendon stiffness can improve the rate of torque development (RTD) (8), which should enhance athletic performance (9, 10) by enabling force to be transmitted from the muscle to the bone more efficiently (11).

The RE-induced change in tendon stiffness can be explained by an increase in tendon cross-sectional area (12), an increase in collagen fibril density (13) and an increase in collagen fibril cross-linking (14, 15). For the first two of these factors to occur, an increase in collagen synthesis is likely required for collagen content to increase (16-19). As human tendon dry weight comprises 60-85% type I collagen (20, 21), measuring serum concentration of procollagen type I N-terminal propeptide [PINP, which is cleaved off during the maturation of procollagen to collagen] can indicate whether tendon collagen synthesis has increased following RE. Indeed, previous studies have found that serum PINP concentration increased following a single bout of RE (22, 23) and after 12 weeks' RE (24).

Given the importance of a positive net collagen turnover for RE-induced connective tissue adaptations to occur (16-19), exogenous collagen ingestion may enhance the collagen synthesis response to an acute bout of RE. Indeed, Lee et al. (23) recently showed that HC ingestion prior to high-intensity RE augmented the serum PINP concentration × time area under the curve (AUC) in a dose-response manner in resistance-trained, *young* men, i.e. 30 g

48 > 15 and 0 g. This response was likely related to the higher postprandial serum
49 concentrations of key amino acids associated with collagen composition and synthesis (e.g.
50 glycine, proline, and hydroxyproline) in the 30 g intervention compared to the 15 g and 0 g
51 interventions (23). However, this is the only study to date to have investigated the dose-
52 response relationship of collagen ingestion prior to RE on collagen synthesis, and this
53 relationship may be different in other populations. For example, it is not yet known how
54 much collagen is necessary to maximize collagen synthesis following RE in middle-aged
55 men.

56 Research typically contrasts extreme age groups to accentuate ageing effects on RE
57 and nutrition, leaving the middle-aged population (40 - 64 years) often overlooked. For
58 instance, compared to young adults (aged 18 – 39 years), tendons of older adults (aged > 65
59 years) show diminished extracellular matrix gene expression in response to acute RE (18).
60 While older tendon improves with chronic RE, the rate of adaptation is slower than younger
61 tendon (25). It is crucial, however, to examine responses in middle-aged individuals, to
62 provide meaningful exercise and nutritional recommendations for this population. Ageing is
63 known to blunt the anabolic response to RE and protein ingestion, doubling the protein intake
64 required to optimize muscle protein synthesis after RE in older adults compared to young (26,
65 27). Given that collagen-rich tissues such as skin exhibit reduced collagen synthesis with age
66 (28), it is possible that ageing also alters the collagen synthesis response to exogenous
67 collagen ingested before RE, although this remains to be explored.

68 The aim of this study was to investigate the effect of lower-limb RE with vitamin C-
69 enriched HC supplementation on serum PINP concentration in resistance-trained, middle-
70 aged men. The objective was to determine the optimal dose of HC required to stimulate
71 maximal whole body collagen synthesis following RE in this population. We hypothesized

that collagen synthesis would increase in response to RE, and that this effect would be augmented in a dose-dependent manner by supplementation with vitamin C-enriched HC.

MATERIALS AND METHODS

Participants

Participants were recruited from a population of resistance-trained, middle-aged men (i.e. posters advertising the study were placed in gymnasiums of the local area, and local sports clubs were emailed with the study information, which was disseminated to members). Recruitment began in November 2019 and data collection was completed in September 2021. To be eligible to participate, volunteers had to be male, have at least 12 months' resistance training experience (including lower limb resistance exercise [RE] performed at least once a week) and to be free from musculoskeletal injury. Volunteers were excluded if they had a history of patellar tendon pathology, were vegan (due to the bovine source of HC), consumed nutritional supplements or medication purported to have beneficial effects on muscle-tendon properties (e.g. antioxidants, protein, etc.), had sustained a lower limb injury in the previous six months, smoked or were <40 or >65 years-old. The required sample size was estimated before conducting the study with G*Power software (version 3.1.9.6, Heinrich-Heine-Universität Düsseldorf). The *a priori* estimation was performed using a large effect size ($\eta_p^2 = 0.22$), on the basis of the results from Shaw et al. (2017), which demonstrated a two-fold increase in the serum PINP concentration × time AUC after exercise with 15 g compared to 5 g gelatine ingestion. A minimum of eight participants was deemed necessary to detect an effect of HC dose [one-way repeated measures analysis of variance (ANOVA); α: 0.05; power: 0.80]. To account for participants withdrawing from the study, 13 resistance-trained, middle-aged men were recruited. However, three participants withdrew prior to commencement of the first intervention, one based on recommendation from his general

practitioner, and two due to injuries sustained independent of the study. Two more participants completed a portion of the study in March 2020, however, due to immediate laboratory closure during the COVID-19 national lockdown restrictions in Ireland, they could not complete all interventions and were subsequently excluded from the analysis. Therefore, eight men (mean ± SD: age, 49 ± 8 years; height, 178 ± 2 cm; body mass, 90 ± 4 kg; 10 repetition maximum (10-RM) leg press, 330 ± 88 kg) were included in the final analyses after providing written informed consent prior to study commencement (**Figure 1**). The study was registered at https://clinicaltrials.gov/ (identifier: NCT06236659), was approved by Liverpool John Moores University Research Ethics Committee (approval number: 19SPS049) and complied with the Declaration of Helsinki.

FIGURE 1 NEAR HERE

Experimental Design

The study was a double-blind, repeated measures crossover design. Participants reported to the laboratory on four separate occasions separated by 72 hours. The purpose of the initial visit was to establish each participant's 10-RM on a leg press machine (Samson, New Mexico, USA), and to familiarize them with the exercise protocol. Secondly, body mass and stature were measured using calibrated weighing scales (model 769, SECA, Birmingham, UK) and a wall mounted digital stadiometer (model 264, SECA, Birmingham, UK), respectively. The order of measurements during familiarization was the same as the order they appear below. During the subsequent three visits, participants reported to the laboratory at 07:00 after a 10 h overnight fast and ingested either 0, 15, or 30 g vitamin C-enriched hydrolyzed collagen (HC) supplement in a randomized order, prior to four sets of 10-RM on the leg press machine. This was followed by 6 h fasted, passive (seated) rest. Venous blood samples were collected at regular intervals for the duration of each intervention (**Figure 2**),

and serum/plasma samples were analyzed for the concentration of procollagen type I N-terminal propeptide (PINP, a marker of collagen synthesis), 18 collagen amino acids, and β-isomerized C-terminal telopeptide (β-CTx, a marker of collagen breakdown).

FIGURE 2 NEAR HERE

Leg Press 10 Repetition Maximum (10-RM) Protocol

Following a brief dynamic warm-up, comprising 5 min submaximal cycling and 5 min full body dynamic stretching, participants performed 2 – 4 sets' leg press RE (Samson, New Mexico, USA) with no additional load, in order to determine their individual foot placement and range of motion on the machine. The 10-RM was determined during four stages: participants first performed 1 – 2 sets of 5 – 10 repetitions at an estimated 50% - 70% of their perceived 10-RM. Following this, the load was increased by 5 – 20 kg on each subsequent set for a total of 4 – 6 attempts, separated by 3 min rest until the participant could only perform 10 repetitions or fewer. The final load, for which the participant successfully completed 10 repetitions, was deemed to be their 10-RM.

Dietary Control Measures

Habitual dietary behavior of all participants was measured to ensure participants maintained their normal diet throughout the three nutritional interventions, and to ensure the same meal was consumed on the night prior to each intervention. To provide an estimate of habitual macronutrient and energy intake, participants were required to complete a dietary log of all food and beverages consumed on two weekdays and one weekend day (e.g. Thursday, Friday and Saturday) during the week prior to the first intervention (**Table 1**). Instructions and reference guides to assist in estimating portion sizes were provided. Estimates of nutrient and

145 energy intake were assessed using nutritional software (Nutritics v5.80, Nutritics LTD,
146 Dublin, Ireland). Before each intervention, participants were requested to abstain from
147 caffeine and alcohol in the preceding 24 h and to finish consuming their final meal no later
148 than 21:00 in the evening. In between interventions, participants were asked to continue
149 monitoring their food intake, and to inform the researchers of any deviation from their food
150 diaries. Electronic reminders to maintain the same intakes were sent to each participant on the
151 days between interventions, where they were also asked to confirm their compliance. A final
152 electronic reminder was sent to each participant at 20:00 the night before each intervention,
153 stating that they should consume the same final meal and begin fasting from 21:00.
154 Participants were instructed to fast overnight (water could be consumed ad libitum) and
155 report to the laboratory at 07:00 the following morning.

156 **TABLE 1** NEAR HERE

157 ***Supplementation with vitamin C-enriched collagen hydrolysate***

158 The supplement beverages were made by a laboratory technician (independent to this study),
159 using opaque bottles to ensure that both the researchers and participants remained blinded.
160 The supplement in each of the three interventions contained either 0, 15, or 30 g unflavored
161 hydrolyzed collagen (HC) powder (Collagen Protein, MyProtein, Manchester, UK), together
162 with 50 mg vitamin C powder (Holland and Barrett, Dublin, Ireland) and 400 mL water. To
163 ensure the supplement in each of the three interventions remained isocaloric, 30.5 g and 15.3
164 g maltodextrin (MyProtein, Manchester, UK) were added to the 0 g and 15 g doses of HC,
165 respectively. To match each supplement for taste, 3 g non-caloric sweetener (Pure Via 100%
166 Xylitol, Mersiant UK LTD., Buckinghamshire, UK) was added to the 0 g dose, and 4 g was
167 added to both the 15 g and 30 g HC doses.

168 ***Resistance exercise intervention and blood sampling***

169 Upon arrival at the laboratory, participants rested for 15 min before an indwelling cannula
170 (BD Nexiva™, 22G, BD Medical, Berkshire, UK) was inserted into an antecubital vein to
171 allow for repeated blood sampling. A baseline blood sample was collected (-1 h), into 5 mL
172 serum and plasma separating tubes (BD Medical, Berkshire, UK), and the participant was
173 instructed to consume the entire contents of the supplement within 5 min of that blood sample
174 being taken. After ingestion, participants rested for 1 h while additional blood samples were
175 drawn at two more time points (-0.5 h and 0 h). Participants then completed a standardized
176 warm up, comprising dynamic stretching, and two ascending warm-up sets at 50-80% 10-RM
177 on the leg press machine (Samson, New Mexico, USA). The RE intervention comprised 4
178 sets of 10 repetitions at 90-100% 10-RM, with each set separated by 2 min rest. Having
179 completed the RE, participants were required to remain in a rested state in the laboratory for
180 the subsequent 6 h, while refraining from eating and drinking, apart from ingesting water *ad*
181 *libitum*. Blood samples were drawn at eight time points over the 7-h period (-1 h, -0.5 h, 0 h,
182 +0.5 h, +1 h, +2 h, +4 h, +6 h, **Figure 2**). The cannula was flushed with 5 mL saline solution
183 every 30 min to prevent clotting. Whole blood was collected in 5 mL serum separating tubes
184 (BD Medical, Berkshire, UK) at all time points, and allowed to clot for at least 30 min. At
185 time points -1 h, +0.5 h, + 2 h, and +6 h, additional whole blood samples were collected in 5
186 mL plasma separating tubes (BD Medical, Berkshire, UK). Whole blood samples were
187 centrifuged at 2000 revolutions per minute (RPM) for 10 min, before being aliquoted into 1.5
188 mL Eppendorf tubes and stored at −70°C until subsequent analysis.

189 ***Blood analyses***

190 The methods for measuring markers of collagen synthesis and breakdown, and concentrations
191 of circulating collagen amino acids, have been described in detail previously (23), and are
192 therefore described here in brief. PINP analyses were performed at South East Technological

University, while β-CTX and amino acid profile analyses were performed at the Bioanalytical Facility, University of East Anglia.

PINP and β-CTx

Serum samples at rest prior to HC ingestion, 0.5 h-post RE, 1-h post RE, 2 h-post RE, 4 h-post RE and 6 h-post RE were used to measure serum PINP concentrations using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Cloud-Clone Corp, Wuhan, China). The intra-assay coefficient of variation (CV) was <10% and the inter-assay CV was <12%, with a detection range of 2.47-200 $\mu g \cdot L^{-1}$, and sensitivity of <0.91 $\mu g \cdot L^{-1}$. The ELISA absorbance readings were performed at 450 nm, using a Versamax microplate reader (Molecular Devices Corporation, Sunnyvale, California USA). The concentration × time total area under the curve (AUC) for PINP and amino acids (see below) were calculated using Prism software (version 9.4.1, GraphPad Inc., San Diego, San Diego, California USA). EDTA plasma concentrations of β-CTx were measured using electrochemiluminescence immunoassay on a Cobas e601 analyzer (Roche Diagnostics, Germany). The inter-assay coefficient of variation (CV) for β-CTx was ≤ 3% between 0.2 and 1.5 μg/L with the sensitivity of 0.01 μg/L.

Amino acid profile by LC-MS/MS analysis

Eighteen amino acids associated with collagen composition (glycine, proline, hydroxyproline, glutamic acid, alanine, arginine, phenylalanine, leucine, serine, lysine, valine, histidine, threonine, tyrosine, isoleucine, methionine, glutamine and ornithine) were measured simultaneously using anionic ion-pair reverse phase liquid chromatography tandem mass spectrometry system following derivatization of the amino acid with *n*-butanol hydrogen chloride. The assay range was 0 – 2000 nmol/L for alanine, glutamine, glutamic acid, glycine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine,

whereas for arginine, histidine, hydroxyproline, isoleucine, methionine and ornithine the assay range was 0 – 500 nmol/L. Inter-assay precision coefficient of variation (CV) for all amino acids studied were between 3.3% to 10.3%.

Statistical Analysis

All statistical analyses were conducted while researchers were still blinded to participant information and supplement dose. Data were analyzed using GraphPad Prism, version 9 (San Diego, California, USA) and are presented as mean ± SD unless otherwise stated. Serum amino acid concentrations were analyzed using two-factor (time × supplement dose) repeated measures ANOVAs. The same analyses were performed on total PINP concentrations over time, and Tukey's post hoc test was computed for post hoc comparisons. The concentration × time total area under the curve (AUC) was computed for serum PINP and for each amino acid, and compared between interventions using a one-way repeated measures ANOVA with Tukey's post-hoc analyses performed for multiple pairwise comparisons.

RESULTS

Effects of collagen supplementation combined with resistance exercise on collagen synthesis in men

Serum PINP concentration at baseline did not differ between interventions (0 g: 11.7 ± 9.3 µg/L; 15 g: 13.3 ± 7.3 µg/L; 30 g: 11.3 ± 8.7 µg/L; $F_{2,9} = 0.126$, $P = 0.882$). There was a main effect of time ($F_{7,49} = 6.51$, $P < 0.001$) and dose ($F_{2,14} = 18.3$, $P < 0.001$). There was also a dose × time interaction effect ($F_{14,98} = 3.54$, $P < 0.001$), as there were no changes across time in the 0 g HC intervention, but there were changes in serum PINP concentration during the 15 g and 30 g HC interventions. Serum PINP concentration was higher at all time points after RE during the 30 g HC intervention compared with the 0 g intervention (**Figure 3A**, $P <$

241 0.05), whereas 15 g was higher than 0 g at + 0.5 h and 1 h only ($P < 0.05$). In both the 15 g
242 and 30 g HC interventions, PINP concentrations peaked at 2 h after RE. Peak PINP values
243 were 70 % and 146 % higher than 0 g at 2 h post-exercise in the 15 g and 30 g HC
244 interventions, respectively. Compared with 0 g HC, the PINP concentration × time total AUC
245 (**Figure 3B**) was 1.4 times greater in the 15 g HC intervention ($P = 0.024$), and 1.8 times
246 greater in the 30 g HC intervention ($P < 0.001$). Finally, the AUC was 1.2-fold greater in the
247 30 g compared with the 15 g HC intervention ($P = 0.033$).

248 **FIGURE 3** NEAR HERE

249 ***Effects of collagen supplementation combined with resistance exercise on collagen***
250 ***breakdown***

251 Plasma β-CTx concentration did not differ at baseline between the three interventions (P
252 <0.0001, **Figure 4**). There was a main effect of time ($F_{1,8} = 16.4$, $P = 0.004$), i.e. during all
253 three interventions, plasma β-CTx decreased immediately after RE and remained lower for
254 the duration of the interventions. Additionally, at 6 h post-exercise (the final sample time
255 point in each intervention), β-CTx concentration was greater than at +0.5 h ($P < 0.001$) and
256 +2 h ($P < 0.001$), but lower than baseline ($P < 0.001$). There was no main effect of dose ($F_{2,13}$
257 $= 0.911$, $P = 0.421$), and there was no dose × time interaction ($F_{3, 24} = 0.675$, $P = 0.595$) on
258 plasma β-CTx concentration. Finally, there was no difference in the β-CTx concentration ×
259 time total AUC between interventions ($F_{2,14} = 1.24$, $P = 0.320$; **Figure 4B**).

260 **FIGURE 4** NEAR HERE

261 ***Serum Amino Acid Profile***

262 **Figure 5** displays the serum concentrations of 18 amino acids that constitute type I collagen
263 over the 7 h period after ingestion of 0, 15, or 30 g HC. There were main effects of time for

glycine, proline, hydroxyproline, glutamic acid, alanine, arginine, lysine, serine, leucine, valine, phenylalanine, threonine, isoleucine, tyrosine and methionine ($P < 0.05$). There were main effects of dose for glycine, proline, hydroxyproline, alanine, arginine, lysine, serine, leucine, valine and isoleucine, with 15 and 30 g HC displaying greater concentrations of these amino acids than 0 g HC ($P < 0.05$), and 30 g HC displaying greater concentrations than 15 g HC ($P < 0.05$). There were dose × time interactions for all but 3 amino acids (aspartic acid, histidine, and glutamine).

FIGURE 5 NEAR HERE

DISCUSSION

This is the first study to demonstrate that whole body collagen synthesis increases in response to resistance exercise (RE) in combination with the ingestion of vitamin C-enriched hydrolyzed collagen (HC) in resistance-trained, *middle-aged* men. Our data support our hypothesis, as the nutritional supplementation (both 15 and 30 g hydrolyzed collagen) was sufficient to increase serum PINP concentration, and the PINP concentration × time AUC more than RE alone in a 6 h period post RE. Interestingly, the 30 g HC intervention led to a ~27 % greater PINP total AUC compared to the 15 g HC intervention, which led to a ~39 % greater total AUC than the 0 g HC intervention. This HC dose-response effect on whole body collagen synthesis was reflected by a similar HC dose-response effect on serum collagen amino acid availability. These data therefore show that high-intensity lower-limb RE alone is insufficient to stimulate collagen synthesis in resistance-trained, middle-aged men. However, supplementing RE in this population with either 15 or 30 g HC (enriched with vitamin C) rescues this apparent anabolic resistance to RE, probably by collagen amino acids stimulating collagen synthesis independently of RE.

287 In our study design, we chose to use *high intensity* leg press RE to stimulate the
288 quadriceps muscle-tendon unit (MTU), as this mode of RE is considered a primary stimulus
289 to enhance the material and mechanical properties of human patellar tendon (3). There are no
290 other studies, to our knowledge, that have described the collagen synthesis response to RE in
291 *middle aged* men. Surprisingly, we observed no increase in collagen synthesis following RE
292 alone, which was unexpected given various types of exercise, including running, skipping,
293 and RE have all been shown to increase collagen synthesis in young men and women
294 independently of nutritional supplementation (23, 29-31). In the time since our study was
295 initiated, 12 high intensity sets of RE have been shown to increase the fractional synthetic
296 rate (FSR) of muscle connective tissue protein in older men (32). Furthermore, the collagen
297 FSR of older tendon has very recently been shown to increase after 4 weeks' moderate
298 intensity leg press RE using 12 weekly sets of leg-press RE (25). Taken together these
299 findings imply that a greater volume (i.e. greater number of sets) may be required to
300 maximize the serum PINP response in *middle-aged* men. However, Lee et al. (23) observed
301 an increase in whole body collagen synthesis (also measured via serum PINP concentration)
302 following RE alone in *young* men, using a very similar RE protocol to that used in our study.
303 Thus, our data suggest a blunted collagen synthesis response to RE alone in *middle-aged* men
304 for a given volume and relative intensity of RE. Nevertheless, the addition of a HC
305 supplement (containing at least 15 g HC) appears to 'rescue' this blunted collagen synthesis
306 to RE in this population.

307 There is a parallel body of research describing the interaction between RE, dietary
308 protein supplementation and muscle protein synthesis (MPS) responses (33). The term
309 'anabolic resistance' describes how older skeletal muscle is not as responsive as young
310 muscle to RE (34). The provision of additional whey protein can assist in mitigating this issue
311 and recovering MPS rates after RE in older tissues, but the dose required can be double that

312 which maximizes MPS in young (26). At least 15 g collagen supplementation rescued the
313 collagen synthesis response in our study, and the advantage observed during the 30 g
314 intervention may be indicative of a need for higher doses as age increases. This is likely
315 given that in young men, collagen synthesis increased after RE alone and increased further
316 with ingestion of 30 g HC but with no benefit of a 15 g dose (23). It is interesting that just 15
317 g HC was sufficient to augment collagen synthesis in *middle-aged* men, which suggests that
318 the collagen synthesis response may already be close to maximized by RE alone in younger
319 populations. Furthermore, ageing is associated with reduced collagen synthesis (at rest) in
320 men (35), which may help explain the importance of exogenous collagen supplementation,
321 even in relatively small amounts, to augment collagen synthesis in *middle-aged* men
322 following RE. It is noteworthy, however, that peak serum PINP concentration (which
323 occurred after RE) and total PINP AUC were lower in *middle-aged* men, and more
324 comparable with the *pre-exercise* levels observed in young men (23), further suggesting a
325 blunted response of older connective tissue to collagen ingestion and RE. It remains to be
326 seen whether ingestion of >30 g HC would further recover the post RE collagen synthesis
327 response in middle-aged men.

328 Compared to fasted levels in the current study, there was a 2 – 2.9-fold increase in
329 peak PINP concentration following RE with HC ingestion. Additionally, we have shown that
330 collagen feeding alone increases collagen synthesis, albeit for a short duration and to a much
331 lesser extent than during the post RE period. The increases in serum PINP concentration in
332 response to both exogenous collagen supplementation and RE correspond to the dose-
333 response elevation (30 > 15 > 0 g) in serum amino acid concentration (**Figure 5**). The key
334 amino acids found in collagen (e.g. glycine, proline, and hydroxyproline) peaked in our
335 serum samples between 1 and 2 h after ingestion of the supplement (**Figure 5)**, which is in
336 agreement with previous studies in young healthy men and women (23, 29, 36-38). There are

several mechanisms by which exogenous collagen ingestion may have promoted collagen synthesis. Firstly, while glycine is a non-essential amino acid, the amount synthesized per day in humans may be insufficient for optimal collagen synthesis (39), and the abundance of dietary glycine supplied by collagen supplements (and confirmed by our amino acid profiles) may make up for this shortcoming. Secondly, in a bovine model, 10 g/day of dietary glycine increased collagen synthesis in articular chondrocytes, which are specialized cells in the ECM of cartilage (40), suggesting a collagen synthesis-stimulating role of glycine. Additionally, proline and hydroxyproline have been shown *in vitro* to regulate TGF-β (transforming growth factor-β) in fibroblasts, leading to downstream phosphorylation of protein kinase B (Akt) and mammalian target of rapamycin complex I (mTORCI), the key regulatory pathway leading to collagen protein synthesis (41). Due to the greater post-exercise responses in our data (compared to the pre-exercise postprandial period), it is conceivable that the increased levels of collagen synthesis in the 15 g and 30 g HC interventions were the result of cumulative signaling from both the RE induced mechanical loading (19) and amino acid induced stimulation of growth factors (41) within the ECM of target connective tissue (e.g. in the tendon and muscle).

We measured plasma β-CTx concentration as a marker of whole-body collagen breakdown, and observed a sustained decrease after supplement ingestion (regardless of HC dose) and RE, suggesting a positive net collagen turnover following RE with or without HC ingestion. In a recent study, Aussieker and colleagues (37) found that ingestion of both 30 g whey protein and 30 g HC with RE appeared to inhibit whole body collagen breakdown (i.e. serum CTX-I concentration decreased) 2 h after RE in young adults. However, this was not the case when water alone was ingested with RE, where serum CTX-I concentration remained unaltered throughout the intervention. This may suggest a positive effect of protein ingestion (regardless of protein type) on whole body collagen breakdown were it not for the

362 fact that plasma β-CTx concentration decreased in our 0 g HC intervention. In our study, we
363 calorie matched both the 0 g and 15 g HC beverages to the energy of the 30 g HC
364 supplement, thus all of our RE interventions took place with the same energy availability. It
365 is likely, therefore, that the inhibited collagen breakdown observed in young adults (37), and
366 in our study of middle-aged men, was the result of calorie intake, regardless of macronutrient
367 composition, which is in line with previous work by Henriksen et al. (42). Furthermore, given
368 that plasma β-CTx concentration decreased 90 min after supplement ingestion (time: 08:30)
369 in our study, and remained lower than fasted concentrations (time: 07:00) for the remainder
370 of all three interventions (**Figure 4**), it is unlikely that the decrease we observed in plasma β-
371 CTx concentration was associated with diurnal variation, as previously suggested (43).

372 It has previously been shown that procollagen type I C-terminal propeptide
373 concentration (another marker of collagen synthesis) and Achilles tendon collagen content
374 (estimated from echo intensity) simultaneously increased after two months' resistance
375 training in healthy young men, which corresponded to an increase in Achilles tendon stiffness
376 (44). This suggests that enhanced tendon collagen synthesis and content are required to
377 improve the mechanical properties of the MTU in response to progressive overload. Thus,
378 given our findings, we can speculate that repeated stimulation (through RE and 30 g HC
379 supplementation) would result in greater increases in tendon size and stiffness in *middle-aged*
380 men over a prolonged period. Emerging data in young men (45, 46) and young women (47)
381 suggest positive outcomes in certain morphological and mechanical tendon properties when
382 HC is ingested in combination with resistance training in young populations. However, it is
383 not yet known if similar outcomes would be found in *middle-aged* men and women. One
384 previous study, however, did show that daily supplementation with 10 g collagen for 12
385 weeks did not increase serum PINP concentration in middle-aged men (48). However, it is

386 unclear if increasing the dose of collagen and/or supplementing in combination with RE
387 would alter this response.

388 *Limitations*

389 Human tendon (30) and serum (49) PINP concentration are known to increase after an acute
390 bout of exercise, and serum PINP remains elevated for up to four days after RE in humans
391 (50), which is in accordance with the elevated collagen fractional synthetic rate in skeletal
392 muscle and tendon following RE (51). Thus, although we did not measure collagen synthesis
393 directly within the MTU, measuring a biomarker of collagen synthesis in the form of PINP,
394 which is cleaved off during the maturation of procollagen to collagen (52), is a reliable
395 alternative. Finally, this study included solely male participants and, considering the potential
396 effects of estrogen on collagen synthesis (53), it is not known if we would have seen different
397 results in resistance-trained, middle-aged, pre- or postmenopausal women.

398 *Conclusion*

399 Our data show that the combination of high-intensity, lower-limb resistance exercise and
400 hydrolyzed collagen supplementation increases collagen synthesis in middle-aged, resistance-
401 trained men, in a dose-response manner, i.e. 30 g > 15 g > 0 g. Crucially, this important
402 finding was reflected in a collagen dose-response effect on the bioavailability of collagen
403 amino acids known to independently stimulate collagen synthesis. Given the lack of response
404 to resistance exercise alone, our findings suggest the sensitivity of collagen rich tissues, such
405 as tendon and ligament, to resistance exercise may be reduced in middle-age, which can be
406 recovered with the ingestion of hydrolyzed collagen.

407

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DISCLAIMERS

The content is solely the authors' responsibility and does not necessarily represent the official views of the authors' affiliations.

AUTHOR CONTRIBUTIONS

CDN and RME designed the research; CDN conducted the research; CDN, JCYT, JD, RD, WDF and RME analyzed the data; and CDN and RME wrote the paper. RME had primary responsibility for final content. All authors read, edited, and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data described in the article, code book, and analytic code will be made available upon request from the corresponding author pending application and approval.

IRB STATEMENT

The study was registered at https://clinicaltrials.gov/ (identifier: NCT06236659), was approved by Liverpool John Moores University Research Ethics Committee (approval number: 19SPS049) and complied with the Declaration of Helsinki. All participants provided written informed consent prior to study commencement.

SUPPLEMENTAL MATERIAL

Supplemental Table S1: DOI. 10.6084/m9.figshare.26947501

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610 FIGURE LEGENDS

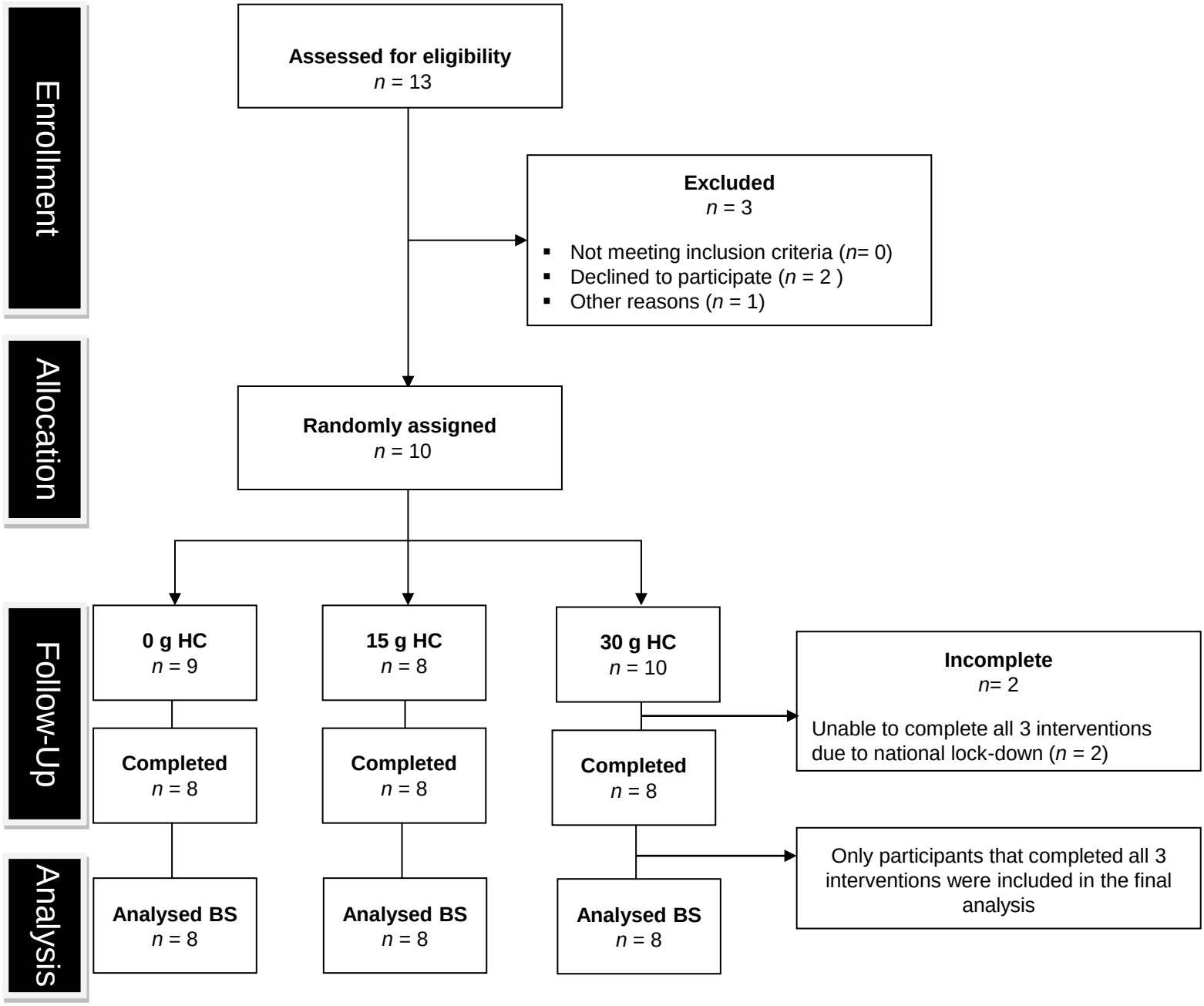
611 **Figure 1**. CONSORT diagram. *HC,* hydrolyzed collagen intervention; *BS*, blood sample.

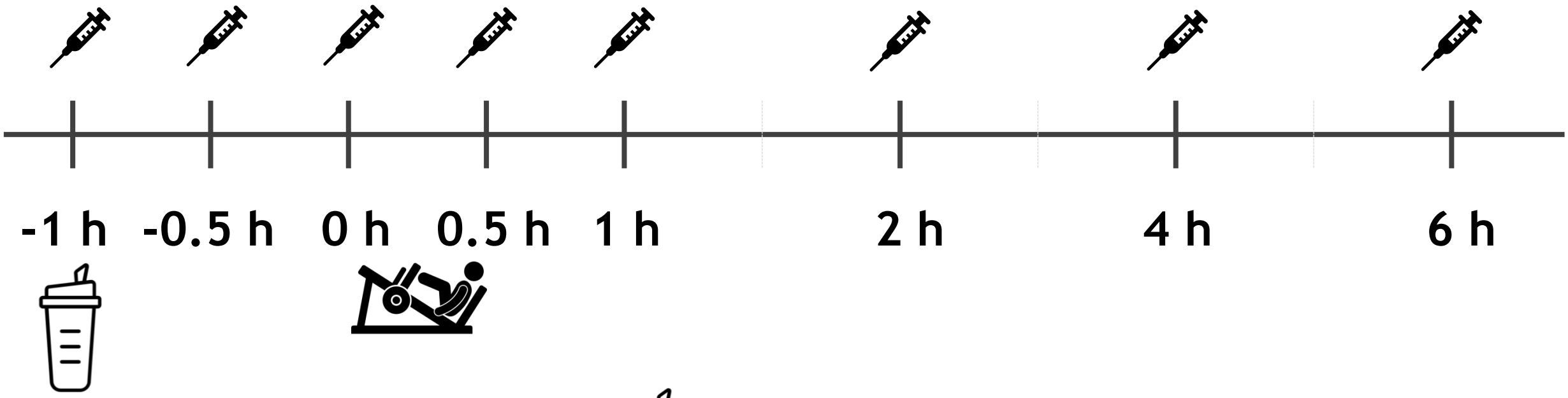
Figure 2. Schematic timeline of data collection.

Figure 3. A. Mean ± SEM serum PINP concentration over time (n = 8). Supplementation was provided at exercise -1 h (0 g black circles, 15 g green squares, 30 g pink triangles). Resistance exercise was completed between 0 h and +0.5 h; * 15 g and 30 g greater than 0 g; † 30 g greater than 15 g; ** 30g, not 15 g, greater than 0g. **B**. The serum PINP concentration × time total area under the curve (AUC) in response to resistance exercise combined with 0 g, 15 g, and 30 g hydrolyzed collagen supplementation (n = 8). Data are mean ± SEM. * Greater than 0 g, † 30 g greater than 15 g ($P < 0.05$).

Figure 4. A. Mean ± SEM plasma β-CTx concentration over time (n = 8). Supplementation was provided at exercise -1 h (0 g black circles, 15 g green squares, 30 g pink triangles). Resistance exercise was completed between 0 h and +0.5 h; * lower than -1 h; † lower than -1h and +6h ($P < 0.05$). **B.** Plasma β-CTx concentration × time total area under the curve (AUC) in response to resistance exercise combined with 0 g, 15 g, or 30 g hydrolyzed collagen supplementation (n = 8).

Figure 5. Mean ± SEM serum amino acid concentration across time after ingestion of 0 g (black circles), 15 g (green squares) and 30 g (pink triangles) of hydrolyzed collagen. Hydrolyzed collagen ingestion took place at -1 h, and resistance exercise began at 0 h.

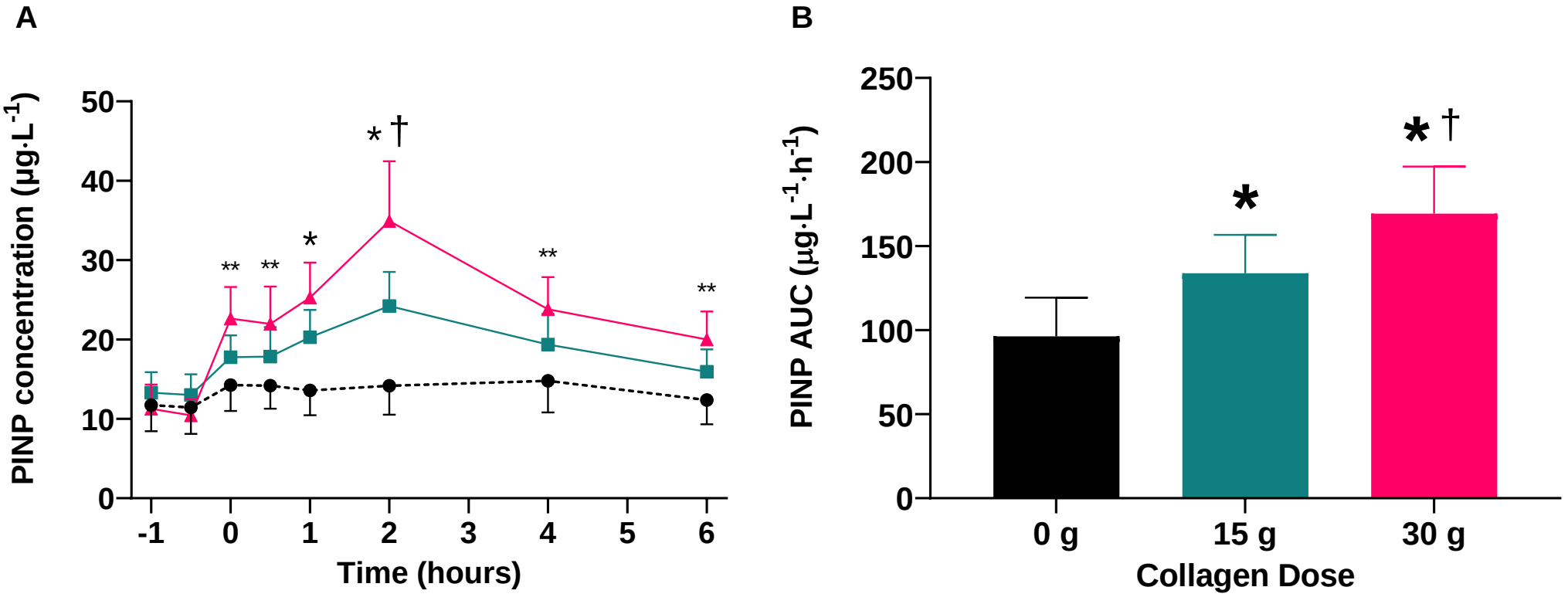

Enrollment
Assessed for eligibility
n = 13
Excluded
n = 3
Not meeting inclusion criteria (n= 0)
Declined to participate (n = 2)
Other reasons (n = 1)
Allocation
Randomly assigned
n = 10
Follow-Up
0 g HC
n = 9
15 g HC
n = 8
30 g HC
n = 10
Incomplete
n= 2
Unable to complete all 3 interventions due to national lock-down (n = 2)
Completed
n = 8
Completed
n = 8
Completed
n = 8
Analysis
Only participants that completed all 3 interventions were included in the final analysis
Analysed BS
n = 8
Analysed BS
n = 8
Analysed BS
n = 8

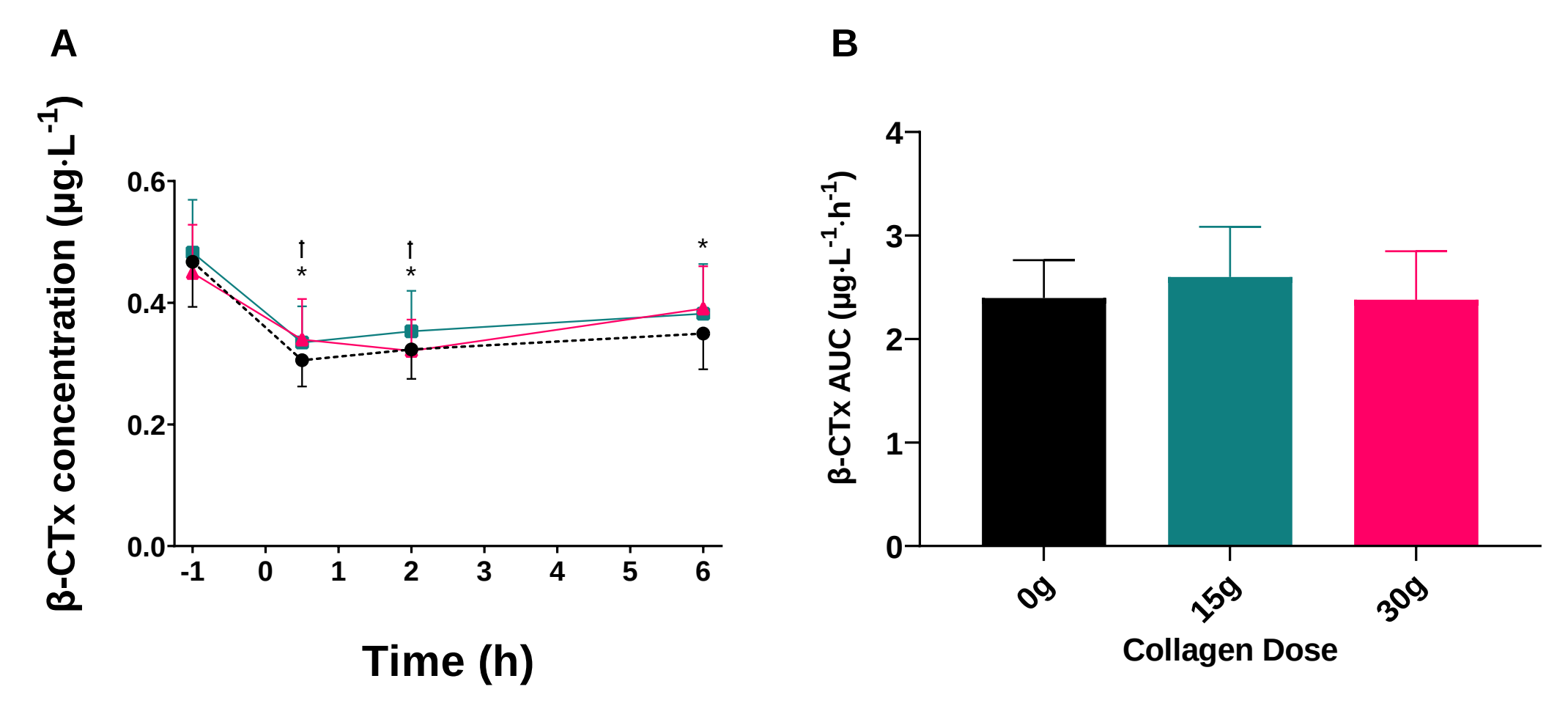


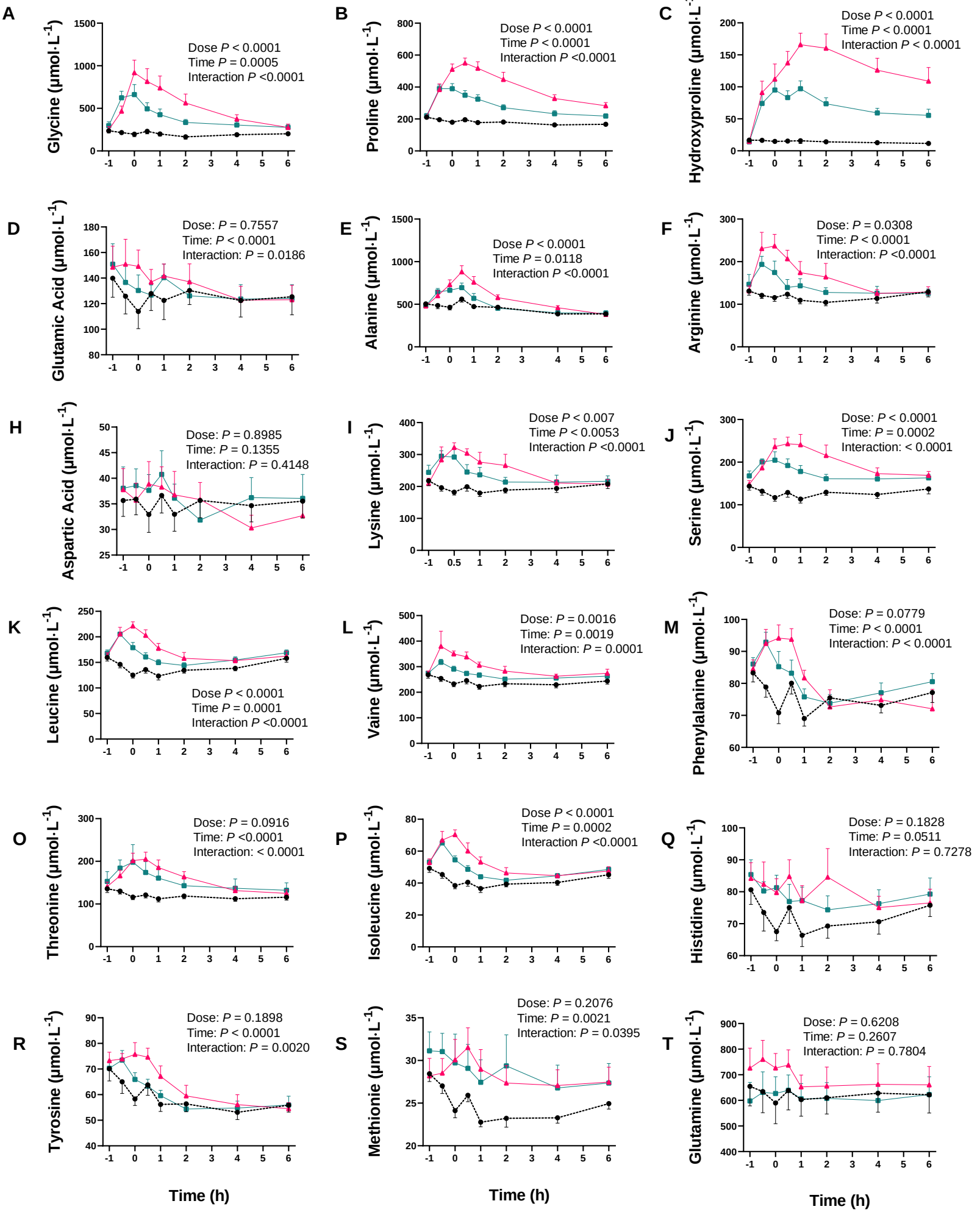
Supplementation protocol: 0g, 15g, or 30g of Hydrolysed Collagen

Blood sample

Leg Press Protocol: 4 sets x 10 RM


A
PINP concentration (µg·L-1)
50
40
30
20
10
0
-1
0
1
2
3
4
5
6
Time (hours)
**
**
*
* †
**
B
PINP AUC (µg·L-1·h-1)
250
200
150
100
50
0
*
* †
0 g
15 g
30 g
Collagen Dose


A
β-CTx concentration (µg·L-1)
0.6
0.4
0.2
0.0
-1
0
1
2
3
4
5
6
†
*
†
*
*
Time (h)
B
β-CTx AUC (µg·L-1·h-1)
4
3
2
1
0
0g
15g
30g
Collagen Dose

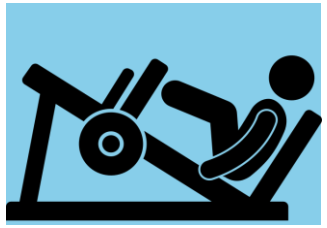

A
Glycine (μmol·L-1)
Dose P < 0.0001
Time P = 0.0005
Interaction P <0.0001
B
Proline (μmol·L-1)
Dose P < 0.0001
Time P < 0.0001
Interaction P <0.0001
C
Hydroxyproline (μmol·L-1)
Dose P < 0.0001
Time P < 0.0001
Interaction P < 0.0001
D
Glutamic Acid (μmol·L-1)
Dose: P = 0.7557
Time: P < 0.0001
Interaction: P = 0.0186
E
Alanine (μmol·L-1)
Dose P < 0.0001
Time P = 0.0118
Interaction P <0.0001
F
Arginine (μmol·L-1)
Dose: P = 0.0308
Time: P < 0.0001
Interaction: P <0.0001
H
Aspartic Acid (μmol·L-1)
Dose: P = 0.8985
Time: P = 0.1355
Interaction: P = 0.4148
I
Lysine (μmol·L-1)
Dose P < 0.007
Time P < 0.0053
Interaction P <0.0001
J
Serine (μmol·L-1)
Dose: P < 0.0001
Time: P = 0.0002
Interaction: < 0.0001
K
Leucine (μmol·L-1)
Dose P < 0.0001
Time P = 0.0001
Interaction P <0.0001
L
Vaine (μmol·L-1)
Dose: P = 0.0016
Time: P = 0.0019
Interaction: P = 0.0001
M
Phenylalanine (μmol·L-1)
Dose: P = 0.0779
Time: P < 0.0001
Interaction: P < 0.0001
O
Threonine (μmol·L-1)
Dose: P = 0.0916
Time: P <0.0001
Interaction: < 0.0001
P
Isoleucine (μmol·L-1)
Dose P < 0.0001
Time P = 0.0002
Interaction P <0.0001
Q
Histidine (μmol·L-1)
Dose: P = 0.1828
Time: P = 0.0511
Interaction: P = 0.7278
R
Tyrosine (μmol·L-1)
Dose: P = 0.1898
Time: P < 0.0001
Interaction: P = 0.0020
S
Methionie (μmol·L-1)
Dose: P = 0.2076
Time: P = 0.0021
Interaction: P = 0.0395
T
Glutamine (μmol·L-1)
Dose: P = 0.6208
Time: P = 0.2607
Interaction: P = 0.7804
Time (h)
Time (h)
Time (h)

METHODS

n = 8 males
49 ± 8 years

0 g **15 g** **30 g**

Hydrolyzed Collagen Supplement
Randomly assigned

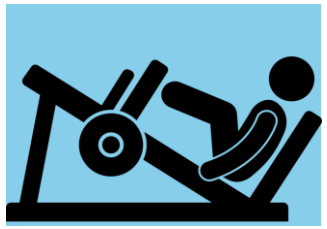


High intensity resistance exercise

PINP (collagen synthesis marker)

β-CTx (collagen breakdown marker)

OUTCOME



0 g PINP ↔ β-CTx ↓

15 g PINP ↑ β-CTx ↓

30 g PINP ↑ β-CTx ↓

CONCLUSION

Resistance exercise alone does not increase collagen synthesis in resistance trained, middle-aged men. Supplementation with hydrolyzed collagen increases post-exercise collagen synthesis in a dose-dependant manner, with no effect on collagen breakdown.