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AUTHOR

Furber, Matthew J.; Anton-Solanas, Ana; Koppe, Emma; et al.

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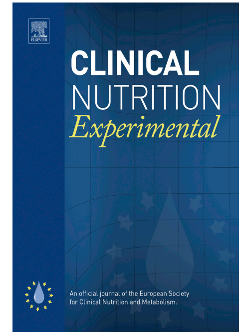

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1 # A 7-day high protein hypocaloric diet promotes
2 # cellular metabolic adaptations and attenuates
3 # lean mass loss in healthy males.

4 **Matthew Furber [1,2,*], Ana Anton-Solanas[1], Emma Koppe[3], Charlotte Ashby[3], Michael**
5 **Roberts[2] and Justin Roberts[4].**

6 [1] GSK Human Performance Lab, Unit 2 Brentside Executive Park, Great West Road, Brentford,
7 Middlesex, TW8 9DA, UK; matthew.x.furber@gsk.com, ana.x.anton-solanas@gsk.com
8 [2] University of Hertfordshire, Division of Sport, Health and Exercise Science, College Lane, Hatfield,
9 Hertfordshire, AL10 9AB, UK; m.g.roberts@herts.ac.uk
10 [3] GSK RD Platform Technology and Science, Gunnels Wood Rd, Stevenage SG1 2NY, UK;
11 emma.l.koppe@gsk.com, charlotte.x.ashby@gsk.com
12 [4] Anglia Ruskin University, Faculty of Science and Technology, Department of Life Sciences,
13 Cambridge Campus Cambridge CB1 1PT; justin.roberts@anglia.ac.uk
14
15
16 * Correspondence: matthew.x.furber@gsk.com; Tel.: +44-77967-07387

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17 **Abstract:** Mitochondrial quantity and density are associated with increased
18 oxidative metabolism. It has been demonstrated that a hypocaloric high fat/low
19 carbohydrate (HF/LC) diet can up-regulate transcriptional markers of mitochondrial
20 biogenesis; this was yet to be explored *in vivo* subsequent to a high protein/low
21 carbohydrate (HP/LC) diet. Thus the aims of the study were to explore such diets on
22 transcriptional markers or mitochondrial biogenesis, body composition and resting
23 metabolic rate (RMR). Forty-five healthy male participants were randomly assigned
24 one of four intervention diets: eucaloric high protein (PRO-EM), hypocaloric high
25 protein (PRO-ER), eucaloric high carbohydrate (CHO-EM) or hypocaloric high
26 carbohydrate (CHO-ER). The macronutrient ratio of the high protein diet and high
27 carbohydrate diets was 40:30:30% and 10:60:30 % (PRO:CHO:FAT) respectively.
28 Energy intake for the hypocaloric diets were calculated to match resting metabolic
29 rate. Participants visited the laboratory on 3 occasions each separated by 7 days. On
30 each visit body composition, resting metabolic rate and a muscle biopsy from the
31 vastus lateralis was collected. Prior to visit 1 and 2 habitual diet was consumed
32 which was used as a control, between visit 2 and 3 the intervention diet was
33 consumed continuously for 7-days. In the PRO-ER group a significant increase in
34 AMPK, PGC-1α, SIRT1 and SIRT3 mRNA expression was observed, the increase in
35 AMPK, PGC-1α was also than the other groups ($p < 0.05$). No change was observed
36 in any of the transcriptional markers in the other 3 groups. Despite ~30 % reduction
37 in calorie intake no difference in lean mass (LM) loss was observed between the
38 PRO-ER and CHO-EM groups. The results from this study demonstrated that a 7-
39 day a high protein hypocaloric diet resulted in increased AMPK, SIRT1 and PGC-1 α
40 mRNA expression at rest, also in a hypocaloric state increased dietary protein
41 attenuated LM mass loss.

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42

43 **Keywords**: High protein diet, PGC-1α, AMPK, hypocaloric, low carbohydrate diet

44

45 **Introduction**

46

47 Mitochondria are responsible for energy production via fatty-acid oxidation, Krebs
48 cycle and oxidative phosphorylation. Mitochondria quantity and density has been
49 linked with increased endurance performance [1], reduction in type 2 diabetes and
50 improved insulin sensitivity [2-6]. A seminal paper from Puigserver, Wu [7] first
51 described peroxisome proliferator-activated receptor gamma coactivator 1-alpha
52 (PGC1-α), in the subsequent years PGC-1α had been identified as key for the
53 regulation and co-activation of mitochondrial biogenesis [8].

54

55 Both AMP-activated protein kinase (AMPK) and silent information regulator T1
56 (SIRT1) mediated deacetylation [9] have been demonstrated to regulate expression
57 of PGC-1α in human muscle [10]. A substantial body of evidence suggests that
58 SIRT1 expression responds to decreases in nutrient availability [11] and increases in
59 energy expenditure [12]. Similarly AMPK is stimulated by cellular stressors that
60 causes a depletion of adenosine triphosphate (ATP) and elevation of AMP, such as
61 calorie restriction [13], hypoglycaemia [13] and exercise [14]. Subsequently,
62 increases in PGC-1α mRNA expression can be observed in direct response to such
63 stressors [13]. Acute hypocaloric HF/LC diets (50% fat, 30% carbohydrate and 20%,
64 protein) significantly increases AMPK phosphorylation and PGC-1α deacetylation,
65 this is not observed subsequent a hypocaloric low fat high carbohydrate (LF/HC) diet
66 (20:60:20 %) [15], suggesting increased dietary carbohydrate intake prevents the

67 activation of the AMPK/SIRT – PGC-1α axis in skeletal muscle that would otherwise
68 be activated by a low calorie diet.
69
70 The preservation of lean mass is important for the maintenance of quality of life [16],
71 it is generally accepted that weight loss strategies which preserve LM are
72 preferential to those that results in skeletal muscle atrophy [17]. During hypocaloric
73 diet-induced weight loss approximately 20 – 30% of mass lost is lean mass (LM)
74 [18], increasing dietary protein is one method which has been demonstrated to
75 attenuate skeletal muscle atrophy in a hypocaloric state [19]. The manipulation of
76 carbohydrate intake as a regulator of weight maintenance / loss is well documented
77 demonstrating improved lipid profile and fat oxidation [20, 21]. However, the majority
78 of literature manipulates dietary fat – protein remains constant. A small number of
79 metabolic perturbation murine studies have manipulated protein intake and it has
80 been shown that high protein intake (35 % protein) increases PGC-1α expression
81 relative to a control diet (15% protein) [22].
82
83 In this field most research focuses on increasing dietary fat to restrict carbohydrate
84 intake. However it is well documented the increased protein intake can attenuate LM
85 loss and may be a preferable choice during weight loss. The impact high protein
86 diets have on transcriptional markers of mitochondrial biogenesis is not fully
87 explored. This study was designed to investigate the impact of increasing protein
88 independently or alongside calorie restriction on metabolic adaptation, body
89 composition and RMR.
90
91 **Materials and Methods**

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92

93 **Participants**

94

95 Forty-eight healthy males volunteered to participate in the study with forty-five
96 completing (mean ± SD: age 26.0 ± 5.1 years; body mass 74.9 ± 10.2 kg; height
97 179.5 ± 5.9 cm). One participant was removed due to non-dietary adherence and
98 two did not complete the study. Participants were initially screened against pre-
99 determined criteria to ensure they met the inclusion criteria and were free from any
100 medical condition that would preclude participation in the study. Participants could
101 not be following a restrictive dietary regime (vegetarian, vegan) or suffer from any
102 food allergies/intolerances. Participants could not be participating in physical activity
103 > 2 times per week and could not be consuming or have consumed any dietary or
104 protein supplements in the previous 2 weeks. The experimental procedures and
105 potential risks associated with the study were explained and the participants gave
106 written informed consent prior to participation. None of the participants had a history
107 of any neurological disease or musculoskeletal abnormality. The study was approved
108 by the University of Hertfordshire School of Life and Medical Sciences ethics
109 committee LMS/PG/UH/00196.

110

111 **Study Protocol**

112

113 In a randomised repeated-measures study design, the participants were assigned to
114 one of four groups: energy matched high protein (PRO-EM, n = 11), energy
115 restricted high protein (PRO-ER, n = 12), energy matched high carbohydrate (CHO-
116 EM, n = 12) or energy restricted high carbohydrate (CHO-ER, n = 10) (Table 1). The

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117 participants attended the laboratory on three occasions, each separated by 7 days.
118 Before visit 1 (baseline) and visit 2 (pre) all participants remained on their habitual
119 diet, between visits 2 and visit 3 (post) the participants consumed the randomly
120 assigned intervention diet. All experimental procedures were completed in the same
121 order at the same time of the day. The baseline and pre assessments were used as
122 a control to demonstrate stability of the measures assessed, where no differenced
123 were observed between these measures the pre measures were used for analysis.

124

125 Table 1 Prescribed macronutrient breakdown for each group.

	Prescribed calorific intake	Carbohydrate (%)	Protein (%)	Fat (%)
PRO-EM	Matched to estimated daily energy requirements	30	40	30
PRO-ER	Restricted to resting metabolic rate	30	40	30
CHO-EM	Matched to estimated daily energy requirements	60	10	30
CHO-ER	Restricted to resting metabolic rate	60	10	30

126 (% = percentage of total daily calorific intake)

127

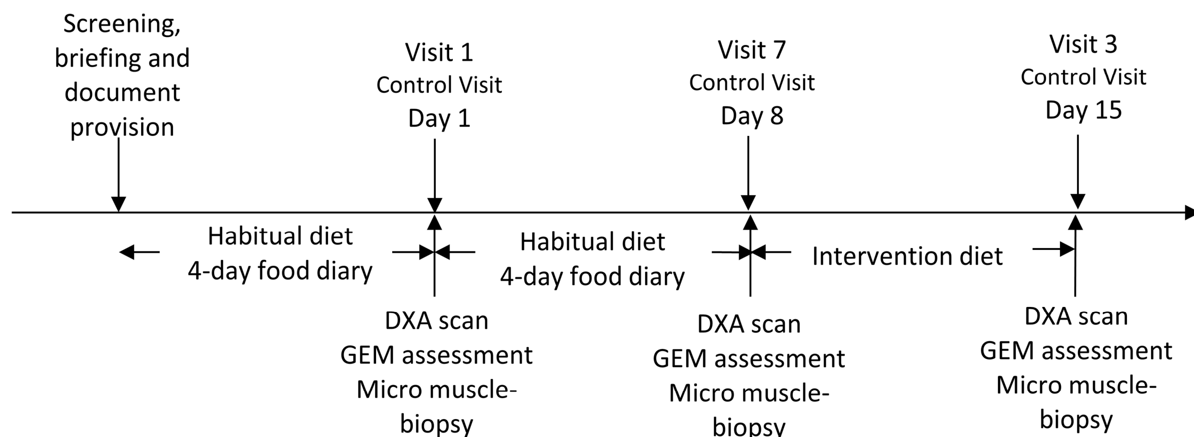
128 **Experimental protocol.**

129

130 Following an overnight fast the participants arrived at their allocated timeslot (start
131 time range: 07:30 – 09:30 am) each testing visit. The participants were requested to
132 arrive at the laboratory using the same mode of transport. Morning physical activity
133 was not permitted. Prior to the pre and baseline visits the participants were

134 completed a standardised 4 day food dairy. The food diary was visually assessed for
135 accuracy by the investigator and further detail was requested if required. An
136 assessment of body composition (DXA scan) was then undertaken, followed by the
137 measurement of resting metabolic rate. Finally a micro-muscle biopsy was collected
138 (Figure 1).

139



140

141 Figure 1. Schematic of experimental protocol.

142

143 **Measurement of habitual dietary intake**

144

145 A 4 day food diary using estimated household measures was used to collect habitual
146 dietary intake prior to the pre and baseline visits. In the study briefing the participants
147 were provided with a comprehensive example of the food diary and were instructed
148 on how to complete it. The importance of accuracy and detail were emphasised, as
149 was the importance of maintaining current dietary habits and documenting all food
150 and drink consumed. Participants were requested to record intake on 3 week days
151 and 1 weekend day each week, a total of 8 days dietary intake was collected over
152 the 2 week prior visit 1 and visit 2.

153

154 **Body composition assessment: Dual energy x-ray absorptiometry (DXA) scan**

155

156 All metal and piercings were removed and fasted body mass (BM) was measured
157 using a digital column scale (seca 704, seca Ltd., Hamburg, Germany). A full body
158 DXA scan (GE Lunar iDXA, GE Healthcare, UK) was performed in accordance with
159 the manufacturer's guidelines for patient positioning and was analysed using
160 enCORE Software, version 14.10 (GE Healthcare, Bucks, UK). The scan measured
161 BM, total LM and total fat mass (FM). All scans were performed by a fully trained
162 operator. In addition to regular machine calibration, a standard quality assurance
163 procedure was performed each testing day.

164

165 **Resting metabolic rate (RMR)**

166

167 RMR was measured using indirect calorimetry (coefficient of variation = 1.48 %). All
168 assessments were completed in a temperature controlled environmental chamber
169 (temperature set to 24°C). The participants arrived at the laboratory in the morning
170 following an overnight fast. The participants were requested to expel as little energy
171 as possible prior to arrival. The participant lay in a supine position on a couch and a
172 large towel was placed over their body to maintain comfort. Once comfortable a
173 metabolic hood (Gas Exchange Measurement (GEM), GEMNutrition Ltd, UK) was
174 placed over the participants head. The participant was instructed to relax, breathe
175 normally and not to move or fall asleep. The GEM was set to collect respiratory bins
176 every 60 seconds for 20 minutes, the data analysed was from the final 5 minutes of

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177 colleciton. Throughout the test the participant was observed through a window to
178 check for adherence. RMR was calculated using the modified Weir equation [23]:
179
180 $$\text{“RMR} = 1.44 \times (3.9 \times \dot{V}O_2 + 1.1 \times \dot{V}CO_2)\text{”}$$
181
182 Respiratory exchange ratio was calculated to assess for changes in resting substrate
183 utilisation by dividing the $\dot{V}CO_2$ value (L•min^{-1}) by the $\dot{V}O_2$ value (L•min^{-1}) from each
184 minute bin collected on the GEM.
185
186 **Micro muscle biopsy**
187
188 Muscle biopsies were obtained from the midpoint on the lateral aspect of the right
189 vastus lateralis muscle. The biopsy site was cleaned using Betadine (Pardue
190 Products, USA) and samples were obtained under local anaesthesia, 2 ml of 1 % v/v
191 without adrenaline Lidocaine Hydrochloride (Hameln Pharmaceuticals: cat. no
192 PL01502) was injected into the subcutaneous fat of the selected biopsy site. Once
193 the anaesthetic had taken effect (~5 minutes), a 14 gauge co-axial was inserted ~2
194 cm into the muscle, a disposable core biopsy instrument (TSK Stericut Biopsy
195 Needle 14 Gauge, TSK Laboratories, Japan) was then inserted through the co-axial
196 and activated. A single biopsy pass was used collecting approximately 10 - 20 mg of
197 muscle tissue. The biopsy instrument was immediately removed from the leg and
198 within 10 seconds the muscle tissue was removed from the biopsy instrument using
199 a sterile scalpel and flash frozen in liquid nitrogen. The muscle sample was placed in
200 an RNAse free plastic vial and stored at -80 OC under the HTA license number
201 12202 until analysis.

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202

203 **Dietary prescription**

204

205 For the two energy restricted (ER) groups the participants were provided an
206 intervention diet with an energy intake that met RMR. RMR was calculated from the
207 GEM result attained from Visit 1. For the energy matched (EM) groups, energy
208 intake was calculated according the World Health Organisation [24] lifestyle
209 dependent physical activity multiplier:

210

211 "EI = PAF x RMR"

212 EI = Energy intake, PAF = physical activity factor, RMR = resting metabolic rate

213

214 RMR was attained from the GEM result on visit 1, and PAF was selected following a
215 series of lifestyle questions concerning daily activity levels, type of work and exercise
216 activity (Table 2).

217

218 Table 2. Lifestyle dependent physical activity multiplier used to calculate energy
219 intake [24].

Activity level	Calculation
Low	1.53 x RMR
Moderate	1.77 x RMR
Active	2.25 x RMR

220

221 A total of 34 (17 high carbohydrate, 17 high protein) different diet menus were
222 created starting at 1,075 kcal, increasing by 150 kcal for each different diet up to
223 3,325 kcal. Participants were assigned the diet closest to their calculated

224 interventional energy intake. The greatest difference between estimated intervention
225 diet and prescribed diet was 75 kcal per day.
226
227 All intervention meals were created by Soulmatefood® (Waterfoot, Lancashire, UK)
228 and directly delivered to the participant's door (Table 3). Each participant received
229 two deliveries; the first delivery contained three day's food, the second four day's
230 food. Each day consisted of 5 pre packaged/cooked meals (breakfast, morning
231 snack, lunch, afternoon snack and dinner). Included with the delivery was a daily
232 menu with consumption instructions. The participants were required to refrain from
233 consuming any additional food and just drink water or fluids free from caffeine or
234 energy.
235
236 Table 3. Prescribed daily calorie intake and macronutrient breakdown for each group
237 throughout the 7-day intervention.

Group	Dietary intake ($kcal•day^{-1}$)	CHO ($•day^{-1}$)		PRO ($•day^{-1}$)		FAT ($•day^{-1}$)	
		g	$g•kg^{-1}$	g	$g•kg^{-1}$	g	$g•kg^{-1}$
PRO-EM	2828 ± 331	217 ± 26	3.0 ± 0.5	289 ± 34	4.0 ± 0.7	96 ± 11	1.3 ± 0.2
PRO-ER	1876 ± 116	143 ± 9	1.9 ± 0.3	191 ± 12	2.5 ± 0.4	64 ± 4	0.8 ± 0.1
CHO-EM	2881 ± 213	444 ± 33	6.1 ± 0.6	75 ± 6	1.0 ± 0.1	97 ± 7	1.3 ± 0.1
CHO-ER	1735 ± 246	267 ± 37	3.7 ± 0.7	45 ± 6	0.6 ± 0.1	59 ± 8	0.8 ± 0.2

238
239
240 **Quantitative PCR (qPCR)**
241

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242 *RNA Isolation*: Human muscle biopsies were homogenised in 700 µL MagNA Pure
243 LC RNA Isolation Tissue Lysis buffer (Roche, Mannheim, Germany) in Roche
244 MagNA Lyser Green bead tubes at 6500 rpm for 30 seconds. After homogenisation,
245 the tubes were centrifuged for 10 min at 13,000g before 350 µL of each homogenate
246 was used for RNA extraction. Total RNA was extracted using the MagNA Pure 96
247 Cellular RNA Large Volume Kit on a MagNA Pure 96 (Roche, Mannheim, Germany),
248 in an elution volume of 50 µL according to the manufacturer's protocol. RNA was
249 stored at -80 ^{O}C. RNA concentrations were determined (A260) using a NanoQuant
250 plate on a Tecan Infinite 200PRO. RNA quality (RNA integrity number equivalent,
251 RINe) was assessed using RNA ScreenTape on a 2200 TapeStation (Agilent, Santa
252 Clara, USA).
253
254 *Reverse Transcription*: As a result of the large sample number and the automated
255 process, RNA concentration was not taken into account for each individual reaction.
256 14 µL of each RNA sample was reverse transcribed in a total volume of 20 µL using
257 the High capacity cDNA reverse transcription kit (without RNase inhibitor, Applied
258 Biosystems, Thermo Fisher, Loughborough, UK). The average RNA concentration
259 across the 142 samples was 32.4 ng•µL^{-1} equating to an average of 453ng
260 RNA/reverse transcription reaction. Reactions were performed in 96 well PCR plates
261 on a PTC-225 Peltier thermal cycler (MJ Research, Quebec, Canada) using the
262 following profile; 25°C for 10 minutes, 37°C for 60 minutes, 85°C for 5 minutes, 4°C
263 hold. Minus RT control reactions were set up for 14 samples, in which the RNA
264 component was replaced with nuclease free water (Ambion, Fisher Scientific,
265 Loughborough, UK).
266

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267 *qPCR*: For quantitative real-time PCR (qPCR), Human TaqMan® gene expression
268 assays were purchased as a 20X assay ready stock from Life Technologies
269 (Carlsbad, USA) (primers 18 mM and probes 5 mM) (Table 4). 1 μL cDNA was
270 added to each qPCR reaction mixture which also contained gene expression assay
271 mix (primers 900 nM final, probe 250 nM final), LightCycler 480 Probes Master and
272 nuclease free water to give a 5 μL total reaction volume. Reactions were prepared in
273 white multiwell 384 plates (Roche, Mannheim, German) using a Mosquito HV (TTP
274 Labtech, Melbourn, UK). The plates were sealed using optical seals (Roche,
275 Mannheim, Germany) and centrifuged at 290g for 2 min before being run on a
276 LightCycler® 480 instrument (Roche, Mannheim, Germany) with the following
277 thermal cycling parameters: initial de-naturation step 95℃ for 10 min, followed by 45
278 cycles of denaturation at 95℃ for 10 s and primer annealing/extension at 60℃ for
279 30 s. A cooling step at 40℃ for 30 s was the final stage of the run.

280

281 Table 4. Taqman Assays on Demand information (Applied Biosystems)

Gene name	Gene abbreviation	Assay on Demand number
Silent information regulator-T1 (SIRT1)	SIRT1	Hs01009005_m1
Silent information regulator-T3 (SIRT3)	SIRT3	Hs00953477_m1
AMP-activated protein kinase 1 (AMPK)	PRKAA1	Hs01562315_m1
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)	PPARGC1	Hs01016719_m1
Peroxisome proliferator-activated receptor delta (PPARδ)	PPARδ	Hs00987011_m1

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Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GAPDH	Hs99999905_m1

282

283 The crossing point (Cp) value for each sample was calculated using the second
284 derivative maximum method applied directly by the Roche software to the real-time
285 amplification curves. This value represents the cycle at which the increase of
286 fluorescence is highest and where the logarithmic phase of a PCR begins.

287

288 Amplification Efficiency of qPCR assays: The efficiency of each of the Taqman®
289 assays on demand was confirmed by performing standard curves in a 384 well
290 qPCR assay on the LightCycler® 480 under the same conditions as already
291 described. Six point standard curves with 1 in 10 serial dilutions were prepared in
292 nuclease free water for human plasmid DNA for each of the genes being tested
293 alongside Human genomic DNA with a top concentration of 3e5 copies/μL and 1e5
294 copies/μL respectively. qPCR reactions were performed in triplicate and the
295 amplification efficiency calculated for each Taqman® assay on the basis of the
296 equation $E = (10–1/slope –1) \times 100$ with the logarithm of the template concentration
297 on the x axis and the average Cp plotted on the y axis.

298

299 **Statistical analysis**

300

301 The RMR and body composition data were analysed using a 2-way repeated
302 measures ANOVA with dietary intake (between) and time point (within) as the main
303 variables. Significant interaction effects were analysed with the use of LSD post hoc
304 test to determine the location of the pairwise differences within (time) and/or between

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305 (dietary intake). The muscle biopsy qPCR analysis raw Cp values were normalised
306 to the house keeper gene (GAPDH) using an analysis of covariance method, before
307 relative fold change over baseline (visit 1) were calculated using $2^{-\Delta\Delta CT}$. Visit one and
308 visit two were used as control. Statistical analysis of the qPCR fold change data was
309 assessed using a paired sample t-test to determine the difference between the
310 control trials, where no difference was observed a paired sample test was run to
311 assess pre-post intervention relative fold change (time) difference. Pre – post
312 intervention group difference was assessed using a one way ANOVA. Statistical
313 significance was set at $p < 0.05$. Statistical analysis was conducted using the
314 statistical package for the Social Science software program (SPSS; version 22, IBM,
315 Armonk, NY). All data are presented mean ± SEM unless specified.

316

317 **Results**

318

319 **Habitual dietary intake**

320 No time or group interactions were observed for habitual total calorie intake or
321 macronutrient breakdown (Table 5). The mean daily calorific intake across all groups
322 was 2,340 ± 473 kcal•day^{-1} (range 2,192 ± 574 to 2423 ± 330 kcal•day^{-1}).

323

324 Table 5. Average daily habitual calorific intake and macronutrient breakdown for
325 each group prior to intervention diet. (mean ± SD and percentage (%) of total energy
326 intake).

	Mean daily calorie intake	CHO	PRO	FAT

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	(kcal•day-[1])	g•day-[1]	%	g•day-[1]	%	g•day-[1]	%
PRO-EM	2192 ± 173	232 ± 22	42.4	95 ± 6	17.2	86 ± 6	35.2
PRO-ER	2391 ± 133	267 ± 16	44.7	100 ± 8	16.7	93 ± 5	35.0
CHO-EM	2355 ± 152	274 ± 23	46.6	103 ± 9	17.5	89 ± 8	33.8
CHO-ER	2407 ± 107	307 ± 16	51.7	97 ± 7	16.0	84 ± 5	31.4

327 A total of 8 days dietary intake was collected over the 2 week prior visit 1 and visit 2;
328 participants were requested to record intake on 3 week days and 1 weekend day
329 each week using the estimated household measures method. (CHO = carbohydrate,
330 PRO = protein, FAT = fat). % = percentage of dietary intake
331
332
333 **Gene expression**
334
335 No significant difference was observed between TP1 and TP2 in any of the genes,
336 thus it can be assumed that any post intervention changes observed were due to the
337 intervention rather than the internal variability of the measure.
338
339 No significant group interactions were observed in any of the genes, however it is
340 important to note trends towards group interactions were observed within both PGC-
341 1α (f = 2.654, p = 0.063) and AMPK (f = 2.771, p = 0.056) expression. Within the
342 PRO-ER group significant pre-post intervention time point difference was observed
343 in PGC-1α (fold increase = 1.27, p = 0.0402), AMPK (fold increase = 2.09, p =
344 0.027), SIRT1 (fold increase = 1.50, p = 0.026) and SIRT3 (fold increase = 1.19, p =
345 0.010) mRNA expression). No time point gene expression changes were observed in
346 any other dietary group (Figure 2 A-E), or in the expression of PPAR mRNA.
347
348 A significant change in LM was observed post intervention as were significant group
349 difference. Post intervention 3 or the groups lost LM (p < 0.05) (Figure 3A), the CHO-

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350 ER losing the greatest amount (-1.26 ± 0.14 kg), the PRO-ER losing -0.82 ± 0.3 kg
351 and CHO-EM losing -0.53 ± 0.19 kg respectively. LM was maintained in the PRO-EM
352 group (-0.21 ± 0.17 kg). The CHO-ER group lost significantly more LM than both the
353 PRO-EM and CHO-EM groups ($p < 0.05$), no difference in LM was observed
354 between the PRO-ER and CHO-EM groups.
355
356 The PRO-ER and CHO-ER groups lost greater ($p < 0.05$) BM than both CHO-EM
357 and PRO-EM (Figure 3B). No difference was observed between groups matched for
358 energy intake. All groups exhibited a significant loss in FM ($p < 0.01$) post 7-days
359 dietary intervention (Figure 1B). The PRO-ER lost the greatest amount of FM (-0.99
360 ± 0.11 kg) and the CHO-EM group lost the least (-0.50 ± 0.14 kg). The difference
361 between these two groups was the only significant group difference observed ($p <$
362 0.05),
363
364

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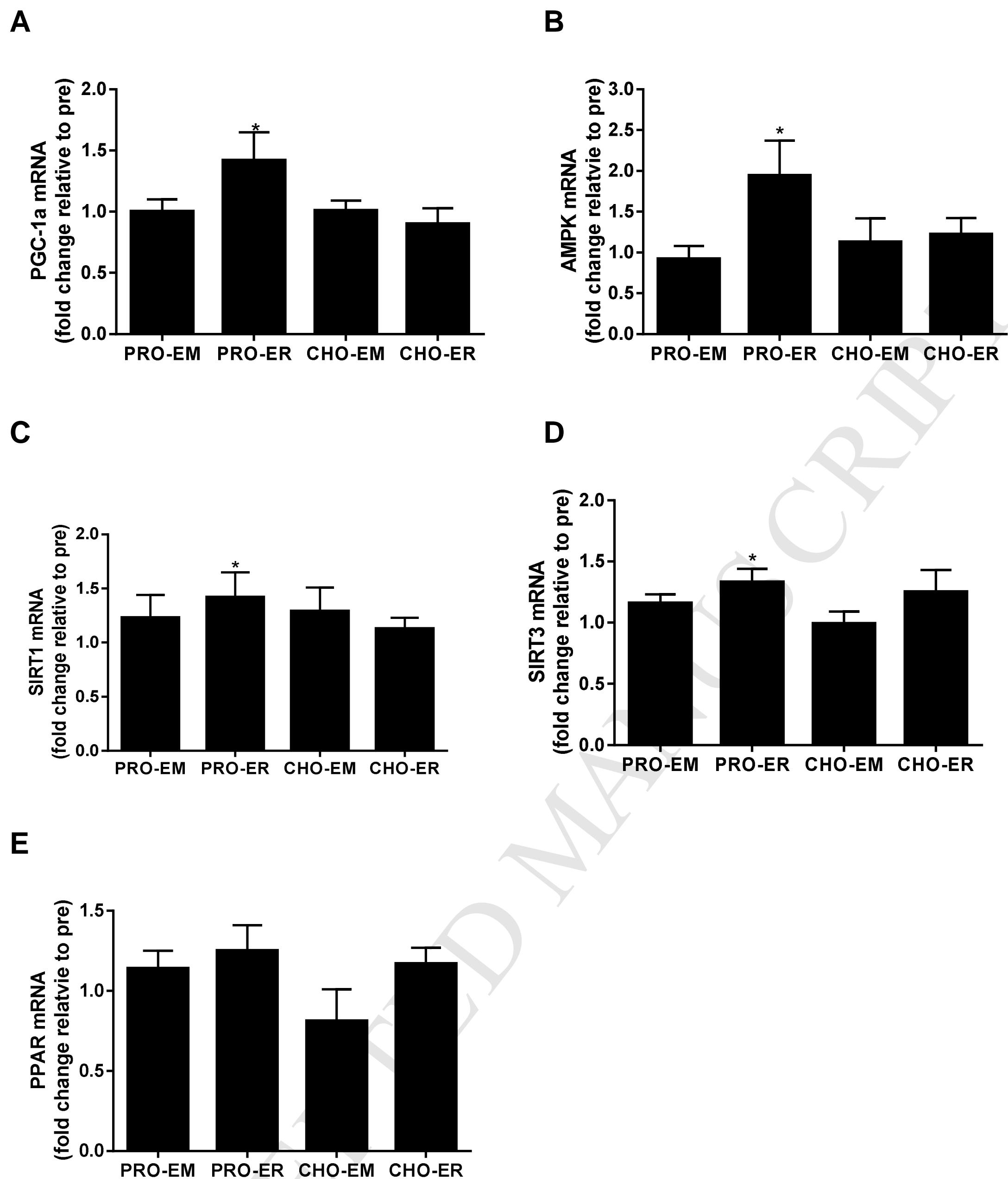

Figure 2. Effect of feeding 7-days diet intervention on mRNA expression at rest.

Effect of 7-days high protein energy matched (PRO-EM), high protein energy restricted (PRO-ER), high carbohydrate energy matched (CHO-EM) or high carbohydrate energy restricted (CHO-ER) diet in healthy lean individuals on PCG-1α (**A**), AMPK (**B**), SIRT1 (**C**), SIRT3 (**D**) and PPAR (**E**) mRNA expression. Muscle samples were obtained following an overnight fast prior to the intervention and repeated again following 7-days dietary intervention (Post). Values are expressed as fold change (pre to post) after normalization to the reference gene (GAPDH) and are reported as the mean ± SE. For abbreviations of genes see Table 4. *p < 0.05 versus Pre intervention.

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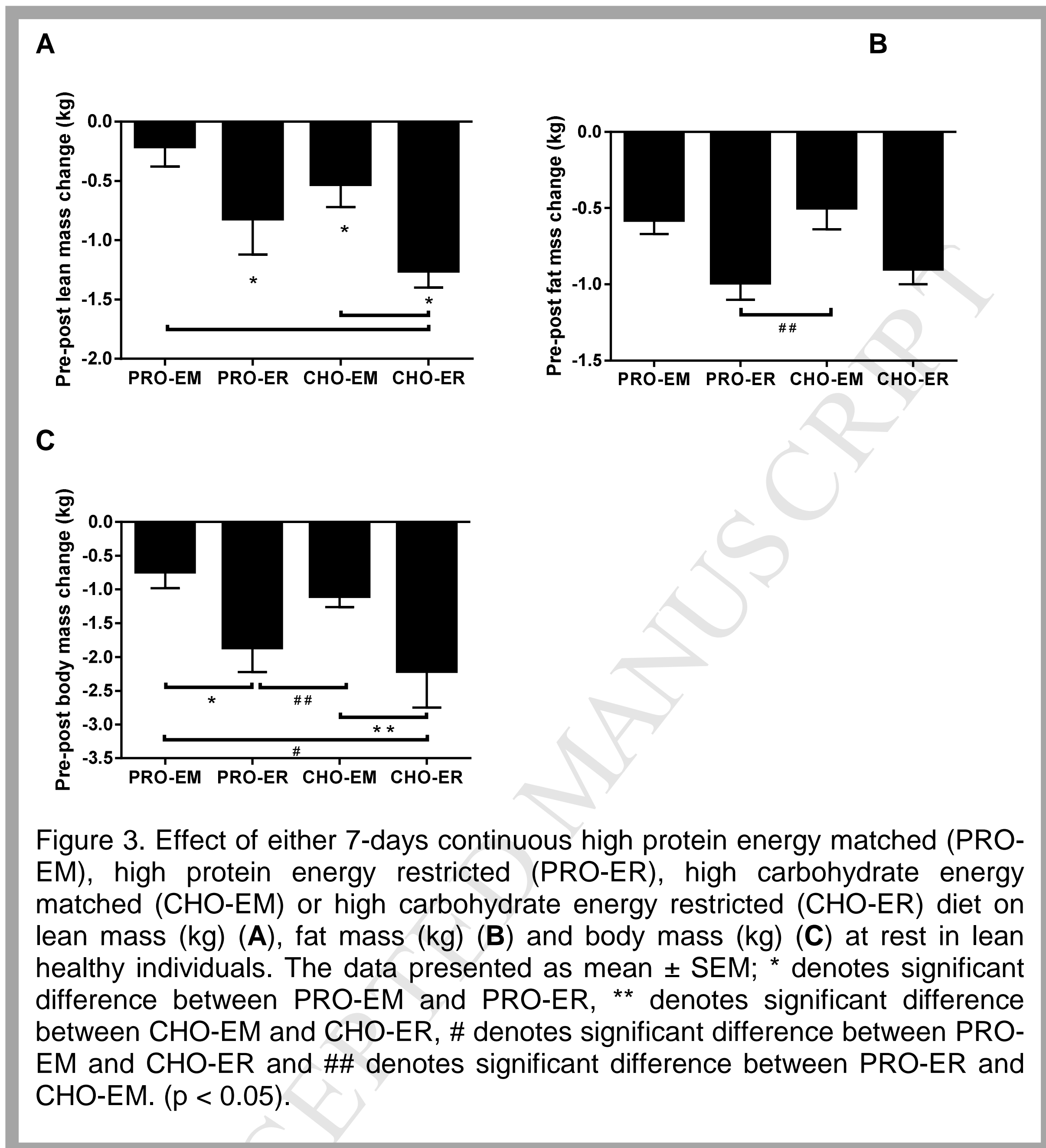

Figure 3. Effect of either 7-days continuous high protein energy matched (PRO-EM), high protein energy restricted (PRO-ER), high carbohydrate energy matched (CHO-EM) or high carbohydrate energy restricted (CHO-ER) diet on lean mass (kg) (**A**), fat mass (kg) (**B**) and body mass (kg) (**C**) at rest in lean healthy individuals. The data presented as mean ± SEM; * denotes significant difference between PRO-EM and PRO-ER, ** denotes significant difference between CHO-EM and CHO-ER, # denotes significant difference between PRO-EM and CHO-ER and ## denotes significant difference between PRO-ER and CHO-EM. ($p < 0.05$).

366

367

368 A reduction in BM was observed post intervention in all groups ($p < 0.01$, Figure 3C).
369 The PRO-EM group lost the least BM (-0.74 ± 0.24 kg) whereas the CHO-ER lost the
370 greatest (-2.21 ± 0.17 kg). Significant post intervention group differences were
371 observed ($p < 0.01$), this was not seen in grouped matched for energy intake,
372 however both ER groups lost significantly more BM than EM groups.

373

374 No time or group interaction effects were observed in RMR (Table 6). Mean RMR
375 across all groups pre was 1910 ± 180 kcal vs. 1894 ± 184 post intervention.

376

377 Table 6. Resting metabolic rate (RMR) (kcal•day^{-1}) pre and post 7-day dietary
378 intervention (Mean ± SEM).

	RMR PRE (kcal•day^{-1})	RMR POST (kcal•day^{-1})
PRO-EM	1916 ± 73	1910 ± 70
PRO-ER	1904 ± 37	1950 ± 54
CHO-EM	1972 ± 38	1946 ± 44
CHO-ER	1848 ± 69	1768 ± 52

379

380

381

382 **Discussion**

383

384 This is the first acute dietary intervention study in healthy sedentary males to
385 demonstrate that increased dietary protein intake in a hypocaloric state positively
386 increased mRNA expression of PGC-1α and AMPK.

387

388 In a myoblast cell line it has previously been demonstrated that both calorie and
389 carbohydrate restriction independently increase transcriptional markers of
390 mitochondrial biogenesis. Following 36- hours incubation in a low glucose solution (5
391 mM) a > 2.5 fold increase in PGC-1α transcriptional activity was observed relative to

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392 a high glucose solution (25 mM), additionally the same authors also demonstrated
393 that following a 20-hour fast, relative PGC-1α mRNA levels were increased > 2 fold,
394 suggesting positive activity *in vitro* of energy and glucose restriction independently
395 [25]. However, *in vivo* results from the current study demonstrated no change in
396 mRNA expression in the PRO-EM (carbohydrate restriction) or CHO-ER (energy
397 restriction) groups, suggesting that acute carbohydrate restriction and energy
398 restriction independently do not exert sufficient metabolic stress to elicit metabolic
399 changes at an mRNA level. Furthermore the results from this study showed that
400 subsequent to a continuous, hypocaloric, high protein diet (40% protein (~ 2.5 $g{\bullet}kg^{-}$
401 ^{1}•day^{1}) and ~33 % energy deficit) a small, but significant, increase in resting mRNA
402 expression of AMPK, SIRT1, SIRT3 and PGC-1α in the vastus lateralis muscle was
403 observed. Furthermore, although non-significant, a tread towards a group interaction
404 of AMPK ($p = 0.056$) and PGC-1α ($p = 0.063$) gene expression was observed. Due
405 to two lost samples in the CHO-ER group at TP3 this resulted in unbalanced groups.
406 When statistics were re-run with phantom values for these two missing points the
407 groups interactions were apparent with the POR-ER group eliciting a significantly
408 greater response in post intervention gene expression relative to all other groups.
409 This would also suggest that the study may have been slightly under powered.
410 These results imply that, in contrast to independent restriction, the combination of
411 energy and carbohydrate restriction provides sufficient metabolic stress to cause
412 skeletal muscle adaptation to cope with the energy demands.
413
414 These results are corroborated by similar studies investigating the impact of high fat
415 feedings on mitochondrial biogenesis [15, 25]. It was shown that five days on a
416 hypocaloric HF/LC diet positively increased phosphorylation of AMPK and

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417 deacetylation of PGC-1α, where as the hypocaloric LF/HC diet negatively reduced
418 the phosphorylation of AMPK and deacetylation of PGC-1α [15]. These results, along
419 with the findings from the current study suggest that high carbohydrate intake
420 prevents the activation of the AMPK-SIRT1 axis that is otherwise observed in a
421 hypocaloric state. Furthermore, and importantly the method of carbohydrate
422 restriction does not seem important, increasing both dietary fat and/or protein result
423 in similar up-regulation of metabolic markers of mitochondrial biogenesis.
424
425 It is considered that weight loss strategies that result in maintenance of LM and
426 promote greater FM loss are preferential [4]. It has previously been demonstrated
427 that ~70 % of weight lost, induced by a hypocaloric diet, is attributed to FM and 30 %
428 from LM [26]. Furthermore it has been reported in obese women that increased
429 protein intake (1.6 g•kg^{-1}•day^{-1} vs. 0.8 g•kg^{-1}•day^{-1}) increased FM loss and improved
430 maintenance of lean mass [27]. The results from this study corroborate this in a
431 healthy male population. No between group difference was observed in energy-
432 matched conditions for BM, FM or LM. The CHO-ER group lost significantly more LM
433 than the CHO-EM group, interestingly however no difference in LM loss was
434 observed between the PRO-ER and CHO-EM groups. The PRO-EM group had a
435 higher protein intake relative to the CHO-EM group (2.5 g•kg^{-1}•day^{-1} vs. 1.0 g•kg^{-}
436 ^{1}•day^{-1}). Thus, although the calorie intake of the PRO-ER group was ~33 % less than
437 that of the CHO-EM group (total deficit of ~7,000 kcal over the 7-day intervention) no
438 change in LM was observed between groups, further highlighting the key role for
439 protein in LM maintenance. The mechanism of this is still not fully defined. The
440 dietary protein intake of the CHO-EM group was greater than the current reference
441 nutrient intake in the UK for adults of 0.75 g•kg^{-1}•day^{-1} [28], however it has been

442 demonstrated the proteolysis of skeletal muscle is suppressed when consuming ≥
443 1.5 g•kg^{-1}•day^{-1} of protein [29]. The PRO-ER group consumed 2.5 g•kg^{-1}•day^{-1} of
444 protein, significantly above this threshold.
445
446 With regards to weight loss ratios, the results demonstrate that in a hypocaloric diet
447 dietary protein intake can positively impact LM and FM loss. Following a continuous
448 hypocaloric diet (~33% energy deficit) and independent of macronutrient content
449 both groups lost a significant amount of LM. Approximately 44% of total BM loss in
450 the PRO-ER group was attributed to LM loss compared to 56% in the CHO-ER
451 group. This was coupled with a greater percentage of FM loss in the PRO-ER group
452 (56% vs. 44% respectively). As such, increased dietary protein intake (2.5 g•kg^{-1} vs.
453 0.6 g•kg^{-1}) attenuated LM loss by ~12 % relative to a hypocaloric diet low in protein.
454 A recent meta-analysis [30] investigating the role of dietary protein intake and long
455 term body composition changes (12 ± 9 weeks) concluded higher protein diets led to
456 greater BM loss (-0.79 kg), FM loss (-0.87 kg) and preserved more LM (+0.43 kg)
457 relative to lower protein diets. Although over a shorter period, the body composition
458 results from the hypocaloric groups observed in this study followed the same trends
459 with -0.35 kg BM, -0.09 kg FM and +0.44 kg LM body composition changes
460 observed. It has been demonstrated that a positive curvilinear (r^2 = 0.92) relationship
461 exists between LM and RMR [31], and RMR is known to decline during prolonged (>
462 9 months) periods of energy restriction [32]. The thermic effect of macronutrients
463 should also be considered, protein has a greater thermic effect compared with the
464 equivalent energy intake of fat or carbohydrate [33]. However the acute nature of the
465 intervention was not long enough the elicit change in RMR and no time point
466 difference or group interaction was observed in RMR, thus it may be assumed that

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467 the duration of the study was not long enough to elicit changes in RMR, as such
468 metabolic rate is not impacted by such a short dietary intervention.
469
470 A marked reduction in RER post intervention was observed in both ER groups;
471 however caution should be taken when drawing strong conclusions from this data.
472 Pre intervention both ER groups had a higher RER (PRO-ER = 0.89, CHO-ER =
473 0.85, PRO-EM = 0.83, CHO-EM = 0.83) and post intervention all groups had an RER
474 of 0.79. Thus a greater relative change was observed pre-post intervention in the ER
475 groups, but no group interaction was apparent. In mice and subsequent to 8 weeks
476 on a hypocaloric diet a significant reduction in RER was observed compared to
477 relative to energy matched diets [34]. Similar to the results from this study, RER was
478 reduced to the same level in the hypocaloric group's independent of dietary protein
479 intake (5% vs. 33% vs. 60%). Solon-Biet, Mitchell [34] also reported a significant
480 reduction in RER in the eucaloric high protein group relative to the eucaloric high
481 protein diet, however this was not to the amplitude of the any of the hypocaloric
482 diets. This suggests that in an eucaloric state restricted carbohydrate (increased
483 protein intake) results in a reduction in RER, however calorie restriction has a
484 greater metabolic impact, independent on macronutrient ratio.
485
486 Interestingly this study raises further considerations for dietary prescription studies
487 and methods used to measure energy intake. The mean calorie intake calculated
488 from 2 x 4 day food diaries for each participant in the PRO-EM and CHO-EM groups
489 were 2,192 ± 574 kcal•day^{-1} and 2,355 ± 527 kcal•day^{-1}, respectively. However,
490 mean calculated energy intake from RMR X Activity Factor (AF) were 2,828 ± 331
491 kcal•day^{-1} and 2,881 ± 213 kcal•day^{-1}, a difference of 636 and 526 kcal•day^{-1},

492 respectively. Despite meticulous effort to match the dietary intake of the PRO-EM
493 and CHO-EM to energy expenditure the results demonstrated a small reduction in
494 BM in both group (0.74 kg and 1.1 kg respectively), as such dietary prescription was
495 slightly underestimated. Despite this, habitual diet, as recorded using estimated
496 household measures, under predicted energy intake calculated using RMR x PA by
497 ~20%, a similar percentage to previous findings in athletes [35] (~28 %) and
498 sedentary participants [36] (~20%).

499

500 **1.1 Conclusions**

501

502 Mitochondrial quantity and density have been linked with increased endurance
503 performance [1] and a reduction in type 2 diabetes [2-6]. Also, it is suggested that
504 dietary strategies that maintain LM are preferential. This is the first study to
505 demonstrate the impact of increased protein intake in a hypocaloric diet on metabolic
506 adaptation. The results from this study demonstrated that a 7-day a high protein
507 hypocaloric diet resulted in increased AMPK, SIRT1, SIRT3 and PGC-1α mRNA
508 expression at rest, all of which are upstream markers of mitochondrial biogenesis.
509 Additionally increased protein intake helped to maintain LM mass in a hypocaloric
510 state. Thus, it may be concluded that a high protein, hypocaloric diet is preferential to
511 a high carbohydrate, hypocaloric diet with respect to cellular markers of fat
512 metabolism and also the maintenance of lean mass. However future research is
513 needed to understand the long-term impact of a high protein hypocaloric diet on
514 markers of mitochondrial biogenesis and body composition.

515

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