High-intensity interval exercise increases humanin, a mitochondrial encoded peptide, in the plasma and muscle of men

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Running title: Exercise increases humanin expression

34 **ABSTRACT**

35 Humanin, a small regulatory peptide encoded within the 16S ribosomal RNA gene
36 (*MT-RNR2*) of the mitochondrial genome, has cellular cyto- and metabolo-protective
37 properties similar to that of aerobic exercise training. Here we investigated whether
38 acute high-intensity interval exercise or short-term high-intensity interval training
39 (HIIT) impacted skeletal muscle and plasma humanin levels. *Vastus lateralis* muscle
40 biopsies and plasma samples were collected from young healthy untrained men
41 (n=10, 24.5±3.7 y) before, immediately following, and 4 h following the completion
42 of 10 x 60 s cycle ergometer bouts at VO_{2peak} power output (untrained). Resting and
43 post-exercise sampling was also performed after six HIIT sessions (trained)
44 completed over 2 weeks. Humanin protein abundance in muscle and plasma were
45 increased following an acute high-intensity exercise bout. HIIT trended (p=0.063) to
46 lower absolute humanin plasma levels, without effecting the response in muscle or
47 plasma to acute exercise. A similar response in the plasma was observed for the small
48 humanin-like peptide 6 (SHLP6), but not SHLP2, indicating selective regulation of
49 peptides encoded by MT-RNR2 gene. There was a weak positive correlation between
50 muscle and plasma humanin levels, and contraction of isolated mouse EDL muscle
51 increased humanin levels approximately 4-fold. The increase in muscle humanin
52 levels with acute exercise was not associated with *MT-RNR2* mRNA or *humanin*
53 mRNA levels (which decreased following acute exercise). Overall, these results
54 suggest that humanin is an exercise sensitive mitochondrial peptide and acute
55 exercise-induced humanin responses in muscle are non-transcriptionally regulated and
56 may partially contribute to the observed increase in plasma concentrations.
57

58 **Key words:** mitochondrial derived peptides, mitokine, humanin, exercise, small

59 humanin-like peptides

60 **NEW AND NOTEWORTHY**

61 Small regulatory peptides encoded within the mitochondrial genome (mitochondrial
62 derived peptides) haven been shown to have cellular cyto- and metabolo-protective
63 roles that parallel those of exercise. Here we provide evidence that humanin and
64 SHLP6 are exercise-sensitive mitochondrial derived peptides. Studies to determine
65 whether mitochondrial derived peptides play a role in regulating exercise-induced
66 adaptations are warranted.

67 **INTRODUCTION**

68 Exercise activates cellular signaling processes that, if repeatedly activated, lead to
69 adaptations that enhance the functional capacity of various organs in the body (15,
70 16). Skeletal muscle is particularly plastic with its adaptive responses being
71 proportional to the nature of the exercise stress. Adaptative stress responses can
72 include, but are not limited to, the induction of muscle fiber hypertrophy, enhanced
73 mitochondrial function, and alterations in substrate utilization and storage (15, 20,
74 31). Mitochondria are best recognized for supplying ATP to contracting muscle and
75 responding to exercise training by increasing their volume/density (mitochondrial
76 biogenesis) (8). However, the role of mitochondria extends beyond that of energy
77 production to include control over some inflammatory responses, proteostasis,
78 adaptive stress responses and apoptosis (5, 35). Since mitochondria are sensitive to
79 cellular energy demands and have diverse roles in controlling homeostatic processes,
80 they are well placed to facilitate adaptive signaling in response to exercise stress.
81
82 Mitochondria have their own DNA (mtDNA), which contains only 13 protein-coding
83 genes, all of which encode essential subunits of the oxidative phosphorylation
84 (OXPHOS) complexes, CI, CIII, CIV and CV (1). As the remaining 72 subunits of
85 these complexes, and all other mitochondrial proteins are encoded by nuclear DNA
86 (13), and as such it is essential that the mitochondria communicate with the nucleus.
87 A number of mitocellular communication networks have been described, including
88 physical interactions, metabolites and signaling molecules (13). Recently, small
89 peptides encoded by short open reading frames (sORFs) within the mitochondrial
90 genome have been identified, and suggested to mediate a novel pathway by which the
91 mitochondrial genome can communicate directly with the cell nucleus and other cells

92 (7, 12, 14, 28). The first described mitochondrial derived peptide (MDP) was
93 humanin (14, 27), a 24-amino acid polypeptide encoded within the 16S ribosomal
94 RNA gene (*MT-RNR2*). Since humanin's initial identification through a cDNA library
95 generated from a human Alzheimer's disease brain (14), several more peptides have
96 been reported to be encoded within the 16S ribosomal RNA gene (small-humanin-like
97 peptides 1-6; SHLP1-6) (7) and one within the 12S ribosomal RNA (mitochondrial
98 open reading frame of the 12S rRNA-c; MOTS-c)(28).
99
100 Primarily, rodent and cell studies have shown the mitochondrial derived peptides are
101 largely cyto- and metabolo-protective (23) with humanin and its analogs initially
102 being shown to confer protection against amyloid-β-related cytotoxicity (14) as well
103 as having more generalized anti-apoptotic effects (12, 21). Humanin also has
104 beneficial impacts on cellular metabolism that parallel those of exercise, with
105 exogenous treatment of rodents prompting insulin sensitivity, glucose stimulated-
106 insulin release, fat mass loss and improved cognitive function (10, 25, 36, 43).
107 Furthermore, humanin and its analogs can promote mitochondrial respiration and
108 mitogenesis in various cell models (22, 24, 39).
109
110 Consistent with these observations there is some evidence that exercise training may
111 alter mitochondrial derived peptide levels, with 12 weeks of resistance training
112 elevating muscle humanin expression, while the more aerobic Nordic walking did not
113 induce the same response (9). However, whether acute exercise stress modulates
114 humanin expression, and thus potentially implicates humanin as an exercise-sensitive
115 peptide that may modulate metabolic adaptation to exercise training is not known.
116 Therefore, we investigated the hypothesis that acute high-intensity exercise and short-

117 term high-intensity interval training (HIIT) would increase humanin levels in plasma
118 and skeletal muscle. We report that acute high-intensity exercise induces an increase
119 in relative muscle and plasma humanin levels, and that short-term HIIT trended to
120 lower absolute levels of plasma humanin without affecting its response to acute
121 exercise. Furthermore, contraction of mouse EDL muscle in isolation increases
122 muscle levels of humanin.

123 **MATERIALS AND METHODS**

124

125 ***Participants***

126 Ten healthy young males (age: 24.5 ± 3.7 y, BMI: 24.1 ± 2.1 kg/m^2, body mass, 79.7
127 ± 2.8 kg; VO_{2peak}: 41 ± 2 ml/kg/min) were recruited and gave written informed
128 consent to participate. All participants reported as non-smokers, free of chronic
129 illnesses including cardiovascular or metabolic disease and completed <4 hours of
130 physical activity/week. Participants were not taking any medication or dietary
131 supplements within the past 6 months. This study was approved by the Health and
132 Disability Ethics Committee, New Zealand (16STH116).

133

134 ***Exercise study protocol***

135 Acute exercise and exercise training experimental design are outlined in Fig. 1a. Prior
136 to the first acute exercise session participants completed a ramped (starting at 60 W
137 and increasing by 15 W per minute) exercise protocol to voluntary exhaustion using a
138 electromagnetically braked cycle ergometer (Velotron, RacerMate, WA, USA) with
139 open-circuit spirometry (Parvo Medics True One 2400, Sandy, Utah, USA) to
140 determine peak oxygen uptake (VO_{2peak}) and associated peak power output, and on a
141 separate day preformed a 16 km time trial at least 2 days prior to and following the
142 first and last acute exercise session, respectively. During the time trail participants
143 were instructed to complete a 16 km cycle ergometer ride as fast as possible, and they
144 received information on distance travelled but not time. In the acute exercise sessions
145 before (untrained) and following (trained) the short-term high-intensity interval
146 training (HIIT) period fasted (overnight) participants completed 10x 60 s cycling
147 intervals at VO_{2peak} power output interspersed with 75 s low-intensity (30 W)

148 intervals on a cycle ergometer. HIIT consisted of six training sessions, using the same
149 protocol as in the acute exercise bouts (starting with 8 intervals and increasing to 12
150 by the last session) on non-consecutive weekdays over two weeks (Fig. 1a).
151
152 ***Blood and muscle sampling***
153 Blood samples were collected during the acute exercise sessions from a cannula in an
154 antecubital vein into a K2 EDTA vacutainer tube before, after 5 exercise intervals
155 (Mid), immediately after and 4 h following exercise (Fig. 1a). Whole blood samples
156 were centrifuged immediately (2000 × g for 10 min at 4°C) and plasma was recovered
157 and placed on ice until storage at -80°C. Percutaneous muscle biopsies were obtained
158 from the vastus lateralis muscle (thigh) using a suction-modified Bergstrom biopsy
159 needle under local anaesthesia[1]. Muscle biopsy samples were either placed in ice-cold
160 histidine-tryptophan-ketoglutarate (HTK, in mM: 180 histidine, 30 mannitol, 18
161 histidine-HCl, 15 NaCl, 9 KCl, 4 MgCl, 2 tryptophan, 1 KH-2-oxoglutarate, 0.015
162 $CaCl_2$) transplant buffer (Custodiol®, Alsbech Hähnlein, Germany) for mitochondrial
163 respiration analysis or snap-frozen in liquid nitrogen and subsequently stored at -80°C
164 until further analysis. Mitochondrial respiration was measured in permeabilised
165 skeletal muscle fibres from basal (pre-exercise) samples before (untrained) and
166 following (trained) 2 weeks of HIIT using a Oroboros O2k oxygraph (Oroboros
167 Instruments, Innsbruck, Austria), as described previously in detail for this study with
168 full respiration data presented (17).
169
170 ***Plasma mitochondrial derived peptide (MDP) analysis***
171 Plasma humanin, SHLP 2 and 6 were extracted in 90% acetonitrile and 10% 1 N
172 HCl and measured using an in-house sandwich ELISA developed at the University

173 of California, Los Angeles (UCLA), USA (6, 7, 42). A standard curve was produced
174 using synthetic humanin, SHLP 2 and 6 (GenScript, HK), respectively, to allow
175 measurement of endogenous concentrations within a range of 0.1-50 ng/mL. Intra-
176 and inter-assay coefficient variations were reported as <10%. Following ELISA,
177 sample absorbances were measured on a plate spectrophotometer (Molecular Devices,
178 CA, USA) at 490 nm. For humanin the capture antibody used in the ELISA was a
179 rabbit anti-human analogue HNG anti-sera that was produced by Harlan Laboratories
180 (Indianapolis, IN, USA). IgG subclasses purified with a protein A column
181 chromatography (Pierce, Rockfold, IL). Total IgG was further purified with a
182 humanin peptide conjugated ligand affinity column and labeled with biotin. This
183 biotinylated ligand affinity purified IgG is used as detection antibody (6). There is no
184 similarity among SHLP fragments and the full length humanin, with details on these
185 peptides being reported previously (7). Antibodies we produced in the same way for
186 SHLP2 and SHLP6 (7). We have not detected SHLPs peptide with the humanin
187 ELISA, thus there is little cross-activity between these ELISA's.
188
189
190 ***Murine housing and isolated muscle experiments***
191 In-house bred male C57Bl/6j mice were maintained in a temperature-controlled
192 (20°C) animal facility with 12- hour light-dark cycle, and *ad libitum* access to water
193 and a standard rodent chow diet (Teklad TB 2018; Harlan, Madison, WI). All
194 experiments were approved by the University of Auckland Animal Ethics Committee,
195 New Zealand. At 12-20 weeks of age, littermates were anaesthetized using
196 pentobarbitone diluted in saline to a final concentration of 3mg/mL. Sufficient
197 surgical anesthesia was determined by lack of response to toe pinch, at which time the

198 extensor digitorum longus (EDL) muscle was excised and then mounted in an
199 incubation chamber (Radnoti, Monrovia, CA) containing Kreb-Henseleit buffer
200 (118.5 mM NaCl, 24.7 mM $NaHCO_3$, 4.74 mM KCl, 1.18 mM $MgSO_4$, 1.18mM
201 KH_2PO_4, 2.5 mM CaCl2, 8 mM mannitol, and 2 mM pyruvate, 0.1% BSA). As
202 described previously (32), muscles were brought to optimal tension, incubated for 10
203 min before being contracted [pulse durations: 350 ms at a frequency of 150 Hz, every
204 5 second for 10 min with an electrical stimulator (Radnoti, Monrovia, CA)] or left
205 under basal tension for 10 min. Immediately following rest/contraction period
206 muscles were snap frozen in liquid nitrogen and stored at -80°C until further analysis.
207
208 ***Immunoblotting***
209 Muscle protein samples were extracted with modified RIPA buffer (50 mM Tris, pH
210 8.0, 75 mM NaCl, 0.3% NP-40, 1% sodium deoxycholate, 0.1% SDS) with EDTA-
211 free protease and phosphatase inhibitors (Roche, USA). Protein concentration was
212 determined by BCA assay and sample concentrations were adjusted with Laemmli
213 buffer and then heated at 95°C for 5 minutes. Proteins were separated in 15% Tris-
214 glycine gels and transferred to 0.2μm PVDF membranes (Bio-Rad, USA) using a
215 semidry transfer system (Transblot Turbo; Bio-Rad, USA) at a constant 9V for 15
216 min. Membranes were blocked in 2% fish gelatin (Sigma #G7041) and then incubated
217 with primary antibody overnight at 4°C [Humanin (Sigma Aldrich #H2414, 1:500,
218 validated by peptide blocking, Suppl. Fig. 2), ERK1/2 phosphor-Thr202/Tyr204 (Cell
219 signaling technologies #9101, 1:1000), total ERK (Cell signaling technologies #9107,
220 1:5000), β-Actin (Sigma Aldrich #A-2228, 1:5000), and α-tubulin (Thermo Fisher
221 Inc. Scientific #A11126), 1:10,000]. The following day membranes incubated with
222 HRP-conjugated secondary antibodies (1:10,000) for 1 h at room temperature and

223 then developed and imaged using Clarity Western ECL (Bio-Rad, USA)
224 chemiluminescence and Chemidoc XRS+ system (Bio-Rad). Band intensities were
225 quantified using ImageJ software and each band was normalized to a gel control
226 sample in each respective gel and then normalized to β-actin as a loading control
227 protein.

228

229 ***Real-time polymerase chain reaction***

230 Total RNA from muscle was extracted using an AllPrep® DNA/RNA/miRNA
231 Universal Kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's
232 instructions. Total RNA was fractioned into small (<200 nucleotides) and large RNAs
233 (>200 nucleotides) using a Zymo RNA Clean & Concentrator Kit (Cat # R1016,
234 Zymo Research, Irvine, CA) as per the manufacturer's instructions. Total RNA used
235 for SYBR green quantitative real-time PCR (qRT-PCR) was reverse transcribed using
236 a High-Capacity RNA-to-cDNA™ kit (Life Technologies, Carlsbad, CA). Fractioned
237 RNA (50ng) used for TaqMan® small RNA assays were reverse transcribed using a
238 TaqMan® microRNA RT kit #4366596 (Life Technologies, Carlsbad, CA) and
239 multiplexed with a combined pool of the small RNA assay targets (Humanin, RNU44
240 and RNU48)(26, 33). Quantification of genes of interest from both total and
241 fractioned samples were measured by a QuantStudio 6 PCR System using SYBR
242 green select master mix (Applied Biosystems, Foster City, CA) and TaqMan
243 Universal PCR Master Mix with UNG (PE Applied Biosystems), respectively. Each
244 sample was loaded in triplicate and normalized to the geometric mean of two
245 reference genes for both total and small RNA gene expression assays (B2M and 36B4
246 for SYBR green and RNU44 and RNU48 genes for small RNA assays respectively).
247 Genes of interest were expressed using the delta-delta Ct (ΔΔCt) method. Primer

248 sequences for genes SYBR green assays and assay ID's for Taqman assays in
249 supplementary table's 1 and 2.
250
251 ***Cell culture***
252 143B human osteosarcoma and 143B Rho 0 cells obtained from Dr Mike Murphy
253 (University of Otago, Dunedin, New Zealand) and cultured in MEM media
254 supplemented with 10% fetal calf serum (FCS), D-Glucose 3.5mg/ml, HEPES 20mM,
255 Sodium Pyruvate 1mM, and Uridine 50µg/ml. Cells were incubated at 37°C with 5%
256 CO2. 143B Rho 0 cells, which are devoid of mtDNA, were generated by culturing
257 143B cells in low doses of ethidium bromide, and characterized and described
258 previously (2, 19).
259
260 ***Statistical Analyses***
261 Statistical analyses were performed using Prism 8 (GraphPad Software Incorporated,
262 California, USA), with statistical significance determined as $p \leq 0.05$. Data are
263 presented as individual data points and mean ± standard error of the mean (SEM).
264 Statistical significance was determined with linear regression, one-way (time) or two-
265 way (time x training status) ANOVA with Holm-Sidak's post-hoc analysis, or
266 student's t-test as indicated.

267 **RESULTS**

268 **Humanin levels increase in muscle and plasma with acute exercise**

269 To determine the effect of high-intensity interval exercise on acute responses to
270 exercise, skeletal muscle samples were collected before and following acute exercise
271 prior (untrained) to and after (trained) short-term high-intensity interval training
272 (HIIT) (Fig. 1a). Acute high-intensity exercise substantially increased mRNA
273 expression of the exercise responsive gene *PPARGC1A* (PGC1α) and short-term HIIT
274 improved exercise performance and muscle mitochondrial function (Fig. 1b-d and
275 (17)), indicating that the exercise protocol induced a potent training stimulus.

276

277 Neither acute exercise nor exercise training altered the absolute expression of
278 humanin in skeletal muscle (Fig. 2a; time effect= 0.193, training effect 0.870),
279 however relative (individual fold change from pre exercise) muscle humanin
280 increased with acute exercise (time effect $p=0.019$), most prominently 4 h following
281 exercise for trained (post-hoc $p<0.013$ vs pre exercise; Fig. 2b). Humanin absolute
282 and relative plasma concentration increased with acute exercise (time effect $p<0.05$),
283 and absolute plasma levels tended to be lower overall following short-term HIIT
284 (trained; $p=0.063$) despite showing a similar acute exercise response (Fig. 2c-d).

285

286 To determine if the effect of exercise on mitochondrial derived peptides was unique to
287 humanin, we measured the plasma concentrations of small humanin-like peptides
288 (SHLP) 2 and 6, which are also encoded within the 16S ribosomal RNA (MT-RNR2)
289 (7). In contrast to humanin, SHLP2 plasma concentrations were not affected by acute
290 exercise or exercise training (Fig. 3a-b). However, SHLP6 showed a significant time
291 effect with acute exercise ($p<0.001$) appearing to increase with acute exercise then

292 return to baseline during recovery (Fig. 3c-d). Similar to humanin, short-term HIIT
293 lead to an overall lower plasma concentration of SHLP6 (Fig. 3c; training effect
294 p=0.008) but did not change the response to exercise (Fig. 3d). Plasma SHLP6 levels
295 were below the detection limit of the assay at various time points for three
296 participants when they were untrained (before HIIT), and for six participants when
297 they were trained (following HIIT). These data points were not included in analysis
298 (Fig. 3c), and data for all time-points have been removed for fold change data (Fig.
299 3d) if pre-exercise sample was below detection limit.
300
301 **Skeletal muscle humanin correlates with plasma humanin during acute exercise**
302 Having observed that humanin responds to exercise in muscle and in plasma we next
303 correlated humanin muscle and plasma, finding there was no relationship between
304 plasma and muscle humanin at rest (Fig. 4a), and a weak association between muscle
305 and plasma humanin (R^2=0.07, p=0.04) levels when exercise samples were also
306 considered (Fig. 4b). We then tested for the accumulation of humanin within
307 exercising muscle by electrically stimulating contraction of isolated mouse extensor
308 digitorum longus (EDL) muscle *ex vivo*. EDL humanin levels were 4-fold higher
309 immediately following 10 minutes of contraction compared to basal EDL levels (Fig.
310 4c).
311
312 **Skeletal muscle transcriptional regulation of humanin during acute exercise**
313 Since humanin appeared to be produced locally in muscle tissue during acute
314 exercise/contraction, we next investigated its transcriptional regulation by using
315 primers designed against *MT-RNR2* mRNA (16S rRNA) and specifically against the
316 humanin sequence within the 16S rRNA (Fig. 5a). *MT-RNR2* mRNA expression was

317 not affected by acute exercise (untrained) and there was no correlation between *MT-*
318 *RNR2* expression and muscle humanin levels (Fig. 5b-c). To determine if humanin
319 transcripts exist independent of *MT-RNR2*, we enriched a fraction for small (<200
320 nucleotides) and which was separated from large (>200 nucleotides) mRNA fractions
321 and used a custom TaqMan small RNA assay with a probe designed specifically
322 against humanin sequence to measure *Humanin* mRNA (Fig. 5d). Control
323 experiments show that this probe specifically detects a mitochondrial sequence
324 because no amplification was evident in cells devoid of the mitochondrial genome
325 (143B Rho 0 cells; Suppl. Fig. 1a). The expression of *Humanin* mRNA was greater in
326 the large fraction, which only contains sequences >200 nt such as the full-length *MT-*
327 *RNR2* mRNA, than the small fraction (Suppl. Fig. 1b). With acute exercise the
328 *Humanin* mRNA was decreased in the small fraction, but similar to *MT-RNR2*
329 *Humanin* mRNA did not change in the large fraction (Fig 5e-f).
330

331 **Muscle *BAX* mRNA expression correlates with plasma humanin concentration**
332 Some of the reported primary downstream targets of humanin are BAX and ERK
333 (27). Acute HIIT did not significantly affect muscle ERK phosphorylation, however
334 *BAX* mRNA decreased immediately following exercise and returned to baseline levels
335 during recovery (Fig. 6a-b). Since B*AX* mRNA similar response pattern in response
336 to acute exercise as humanin, we correlated *BAX* with plasma and muscle humanin
337 levels. There was a significant but weak ($p=0.025$, $R^2=0.251$) correlation between
338 plasma humanin and *BAX* mRNA, but not *BAX* mRNA and muscle humanin levels
339 (Fig. 6 c-d). Neither plasma nor muscle humanin levels were associated with
340 phosphorylated-ERK ($p > 0.60$, $R^2 < 0.02$; data not shown).
341

DISCUSSION

The mitochondrial derived peptide, humanin, has been shown to respond to cellular stress (23) and can facilitate mitochondrial and metabolic adaptions when given exogenously to mice (22, 24, 39). Here, we provide evidence that muscle and plasma abundance of humanin increase in response to acute high-intensity exercise in young men, and this appears to be independent of transcription. Furthermore, short-term high-intensity interval training (HIIT) tends to lower absolute plasma humanin and SHLP6 levels but does not affect the acute increase in response to exercise. Finally, we provide evidence that humanin plasma concentrations during exercise correlate with muscle humanin levels, and an increase in humanin levels also occurred in isolated contracting mouse EDL muscle.

Our finding that humanin is an exercise sensitive mitochondrial peptide is partially supported by the observation that 12 weeks of resistance exercise training increases skeletal muscle humanin expression which also correlated with parameters of metabolic health in older men (54±7 years) with pre-diabetes (9). While we did not observe an effect of short-term HIIT on intramuscular humanin levels, a trend for decrease in plasma concentration was observed with short-term HIIT. Since humanin plasma levels have been reported to decrease with advancement of metabolic disease (9, 29, 38), it is likely that participants in the Gidlund et al. study (9) had lower baseline humanin levels than the younger cohort in this study, and the increase observed related to resistance exercise-associated improvements in metabolic health. This is unlikely to have occurred in our already healthy cohort that only undertook short-term (2 weeks) training. Alternatively, this may reflect the differences in exercise modalities, with short-term HIIT having a greater aerobic component than

367 resistance training or the duration of training period (2 vs. 12 weeks). Interestingly
368 within the same study 12 weeks of Nordic walking, which is primarily aerobic
369 endurance exercise, also did not alter muscle humanin levels (9). Further studies are
370 therefore required to more comprehensively identify the regulation of humanin in
371 response to differing durations, intensities and modes of exercise, in both healthy and
372 metabolically unhealthy individuals.
373
374 Compared to high-intensity interval training, acute exercise had a more pronounced
375 effect on humanin muscle and plasma levels. This suggests that the increase in
376 humanin levels during acute exercise is likely to be in response to acute exercise-
377 related mitochondrial stress and might explain why the increase is transient, with
378 humanin levels generally trending towards baseline 4 h post-exercise. This is in
379 agreement with the short (<30 minutes) half-life of humanin (6). Similarly, the
380 mitochondrial peptide MOTS-c has been shown to rapidly (within 30-minutes) and
381 transiently translocate from the mitochondria to the nucleus in response to metabolic
382 stress (glucose restriction and oxidative stress) to directly interact with DNA and
383 facilitate an antioxidant response (28). While the current study was not designed to
384 elucidate the molecular targets of humanin, we did observe a weak association
385 between plasma humanin and muscle *BAX* mRNA levels. Humanin can form a
386 complex with BAX and inhibit its translocation to the mitochondrial outer membrane,
387 preventing its pro-apoptotic function (12, 34). While we do not have cellular
388 localization data, it is possible that by decreasing BAX's pro-apoptotic role, this feeds
389 back to the nucleus to reduce transcription. Therefore, it is tempting to speculate that
390 exercise-induced increases in humanin may play a role in regulating exercise-related
391 protection against mitochondrial-mediated apoptosis (18).

392

393 The weak correlation between muscle and plasma humanin levels during acute
394 exercise may indicate that either humanin from the circulation (potentially be released
395 other tissues) is entering the muscle or that humanin is being released from the muscle
396 into the circulation. While both processes are possible and implicate humanin as a
397 'mitokine', the increase in humanin levels following contraction of isolated EDL
398 muscle strongly suggests that the muscle is capable of intrinsically increasing
399 humanin expression within minutes of contraction. Surprisingly, 16S rRNA (MT-
400 RNR2) gene transcription was not affected by exercise and small *Humanin mRNA*
401 levels decreased. This suggests that acute regulation of humanin expression during
402 exercise/contraction is non-transcriptional, but the decrease in transcription may begin
403 to explain why a reduction in plasma humanin was observed following HIIT. Since
404 expression of muscle humanin increased quickly during exercise/contraction (within
405 10 minutes), this suggests exercise/contraction may be acting to suppress the
406 degradation of humanin leading to its increase in muscle and in circulation. While
407 humanin has been reported to colocalize with lysosomes (11), its endogenous
408 expression appears to be primarily regulated via ubiquitin-mediated degradation with
409 binding of TRIM11 (Tripartite Motif Containing 11) promoting proteolysis (37).
410 TRIM11 is highly expressed in skeletal muscle (3, 41), and ubiquitin–proteasome-
411 dependent proteolysis appear to be down regulated by endurance exercise, while in
412 contrast, muscle disuse upregulates the ubiquitin–proteasome pathway (40). However,
413 whether TRIM11 activity is modulated by exercise/contraction is not known.

414

415 For the first time, we also assessed plasma levels of the small humanin-like peptides
416 (SHLP) 2 and 6 following exercise. Interestingly, while SHLP6 showed a similar

417 trend as humanin (increasing in the plasma with acute exercise but showing lower
418 absolute levels following training) SHLP2 concentration was not altered by acute
419 exercise or HIIT. This suggests independent and stimulus-specific regulation of
420 peptides encoded within the same region (16S rRNA) of the mitochondrial genome.
421 Consistent with this, SHLP2 and 6 are differentially expressed across mouse tissues,
422 and like humanin, SHLP2 appears to have a greater cytoprotective role than SHLP6
423 (7), and both humanin and SHLP2 can lower several markers of age associated
424 metabolic conditions (30). One reason potentially why humanin and SHLP6 showed a
425 similar response to exercise is that they are both encoded by the heavy strand of
426 mitochondrial DNA, while SHLP2 is encoded by the light strand (7). We note that a
427 number of humanin homologues open reading frames (ORF) reside within the nuclear
428 genome, and could also transcribe humanin-like peptides (4). However, the antibodies
429 used in the ELISA for humanin and SHLPs, and the humanin mRNA probe do not to
430 detect these peptides in cells missing mitochondrial DNA (143B Rho 0 cells) ((7) and
431 Suppl. Fig. 1a), indicating that our results can be attributed to primarily
432 mitochondrially transcribed peptides.
433
434 The results of our study demonstrate that the mitochondrial encoded peptide,
435 humanin, is regulated by acute high-intensity exercise and short-term high-intensity
436 interval training (HIIT) in young healthy males. Furthermore, we suggest that the
437 increase in plasma humanin levels is partially the result of increased levels of
438 humanin within skeletal muscle, which may be regulated independent of transcription.
439
440

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AUTHOR CONTRIBUTIONS

TLM, CJM, AJH and DCS conceived the experiments. JSTW, CPH, RFD, JW, CJM and TLM performed the experiments and analyzed the data. PC, MVB, DCS and AJH provided unique reagents and facilities to undertake experiments. TLM and JSTW wrote the manuscript, and all authors approved the final manuscript version.

AUTHOR CONFLICTS OF INTEREST

All authors declare no competing interests.

SUPPLEMENTARY DATA

Supplementary data can be accessed via the following link:

https://github.com/TMerryNZ/Humain_JAP_Supps.git

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608
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610

611 **FIGURE LEGENDS**

612 **Figure 1. Human exercise experimental design**. Overview of the acute exercise 613 bout and high-intensity interval training (HIIT) study design **(a)**. The effect of acute 614 exercise protocol on muscle *PPARGC1A* (PGC1α) mRNA expression **(b)**, and HIIT 615 period (trained vs untrained) on 16 km time trial performance **(c)** and mitochondrial 616 respiration **(d)**. **p<0.01 vs Pre-TR; ***p<0.001 vs untrained or Pre acute exercise; 617 Pre, Pre-acute exercise; Post, post-acute exercise; 4 h, 4 h-post acute exercise; 618 untrained, before HITT period; Trained, after HIIT training period; CI oxphos, 619 complex 1 oxidative phosphorylation; CII oxphos, complex 2 oxidative 620 phosphorylation.

621

622 **Figure 2. The effect of acute high-intensity exercise and short-term HIIT on** 623 **skeletal muscle and plasma humanin levels**. *Vastus lateralis* humanin absolute **(a)** 624 and relative **(b)** muscle levels**,** and absolute **(c)** and relative **(d)** plasma levels at rest 625 (Pre), after five exercise intervals (Mid), immediately (Post) and 4 h (4 h) following 626 acute high-intensity exercise before (untrained) and after (trained) short-term high- 627 intensity interval training (HIIT). Horizontal dotted line represents Pre-exercsie. 628 Representative western blots (a and b) are from two different participants. Significant 629 main 2-way ANOVA effects are given in figures and *p<0.05 for post-hoc vs Pre of 630 same training status.

631

632 **Figure 3. The effect of acute high-intensity exercise and short-term HIIT on** 633 **skeletal muscle and plasma SHLP levels**. Absolute and relative plasma SHLP2 **(a-** 634 **b)** and SHLP6 **(c-d)** concentrations at rest (Pre), after five exercise intervals (Mid), 635 immediately (Post) and 4 h (4 h) following acute high-intensity exercise before

636 (untrained) and after (trained) short-term high-intensity interval training (HIIT).
637 Horizontal dotted line represents Pre-level. Significant main 2-way ANOVA effects
638 are given in figures and $^{\#}p<0.05$ for post-hoc vs untrained at same time-point. SHLP,
639 small humanin-like peptides.

640

641

642 **Figure 4. Correlation of plasma and muscle Humanin levels and increased**
643 **humanin expression within isolated mouse EDL muscle during contraction**.
644 Correlation between plasma and muscle humanin levels at rest **(a)** and in response to
645 exercise **(b)** from trained and untrained acute high intensity exercise. Muscle humanin
646 levels in mouse extensor digitorum longus (EDL) **(c)** muscles at rest (basal) and
647 following *ex vivo* contractions (contract). Curved dotted lines represent 95%
648 confidence intervals. *p<0.05 vs basal for student t-test.

649

650 **Figure 5. mRNA levels of MT-RNR2 and humanin following acute high-intensity**
651 **exercise untrained**. Primers were used against sequences within the *MT-RNR2* gene
652 that were independent of (MT-RNR2 primer) or matched to the humanin mRNA
653 sequence (humanin primer) **(a)**. *MT-RNR2* mRNA muscle levels **(b)** and association
654 with humanin muscle levels **(c)** in response to untrained acute high interval exercise.
655 Muscle mRNA from acute high-intensity exercise pre-training was fractioned into
656 small (<200 nucleotides) and large (>200 nucleotides) mRNA fragments **(d)** and these
657 fragments were assayed with the humanin primer probe **(e-f)**. Curved dotted lines
658 represent 95% confidence intervals (c). Horizontal dotted line represents Pre-level (e-
659 f). n=7-8 for small mRNA faction assay due to limited amount of sample available.
660 *p<0.05 **p<0.01 vs pre for one-way ANOVA.

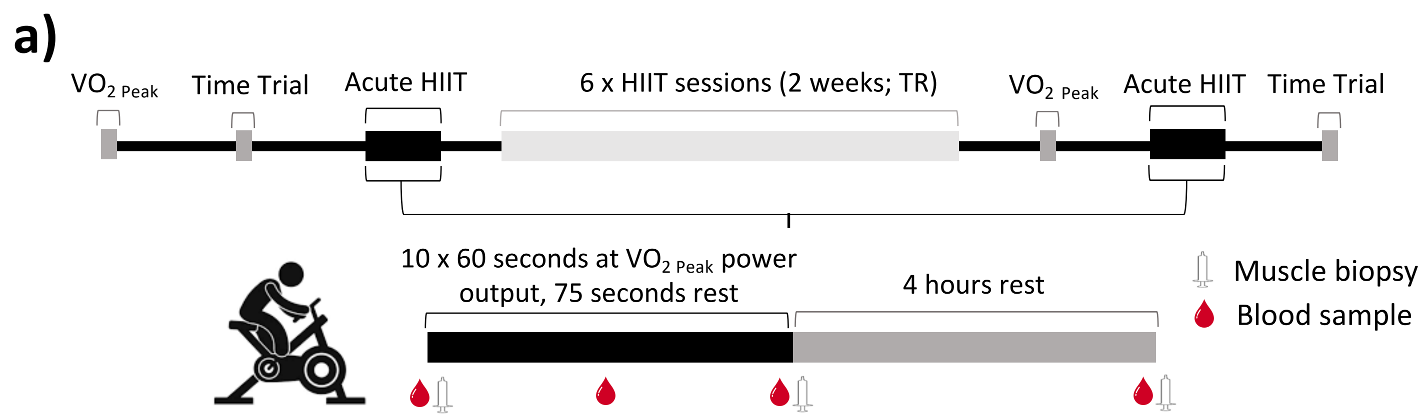
661

662 **Figure 6. Association between muscle and plasma humanin levels and ERK**
663 **phosphorylation and *BAX* mRNA expression in untrained**. ERK (extracellular
664 signal-regulated kinases) phosphorylation **(a)** and *BAX* mRNA **(b)** expression at rest
665 (Pre), immediately (Post) and 4 h (4 h) following untrained acute high-intensity
666 exercise before HIIT. Correlations between humanin plasma and muscle levels and
667 BAX mRNA expression **(c-d)** in response to acute high-intensity exercise before
668 HIIT. Curved dotted lines represent 95% confidence intervals. **p<0.01 vs pre for
669 one-way ANOVA.

670

Figure 1

a)



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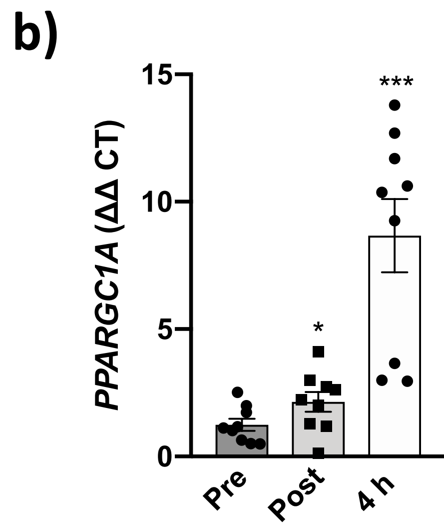

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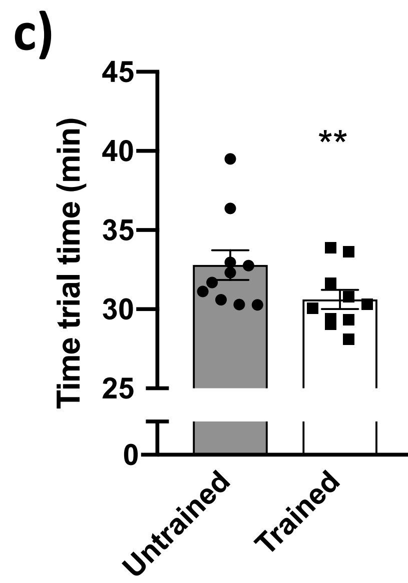

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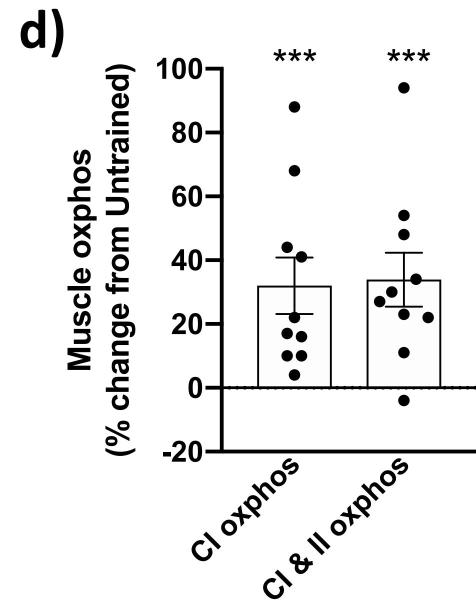

Figure 2

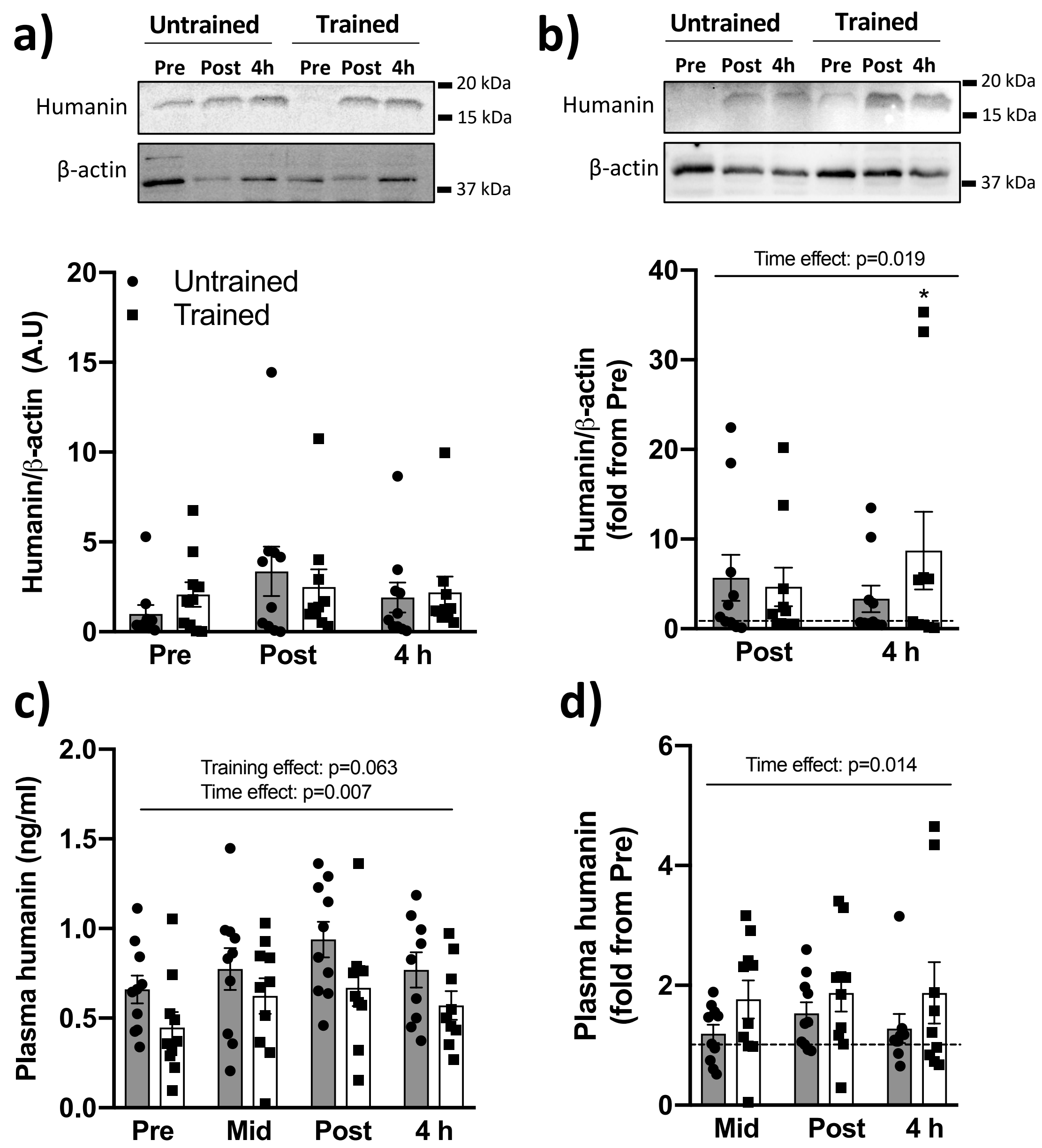

Figure 3

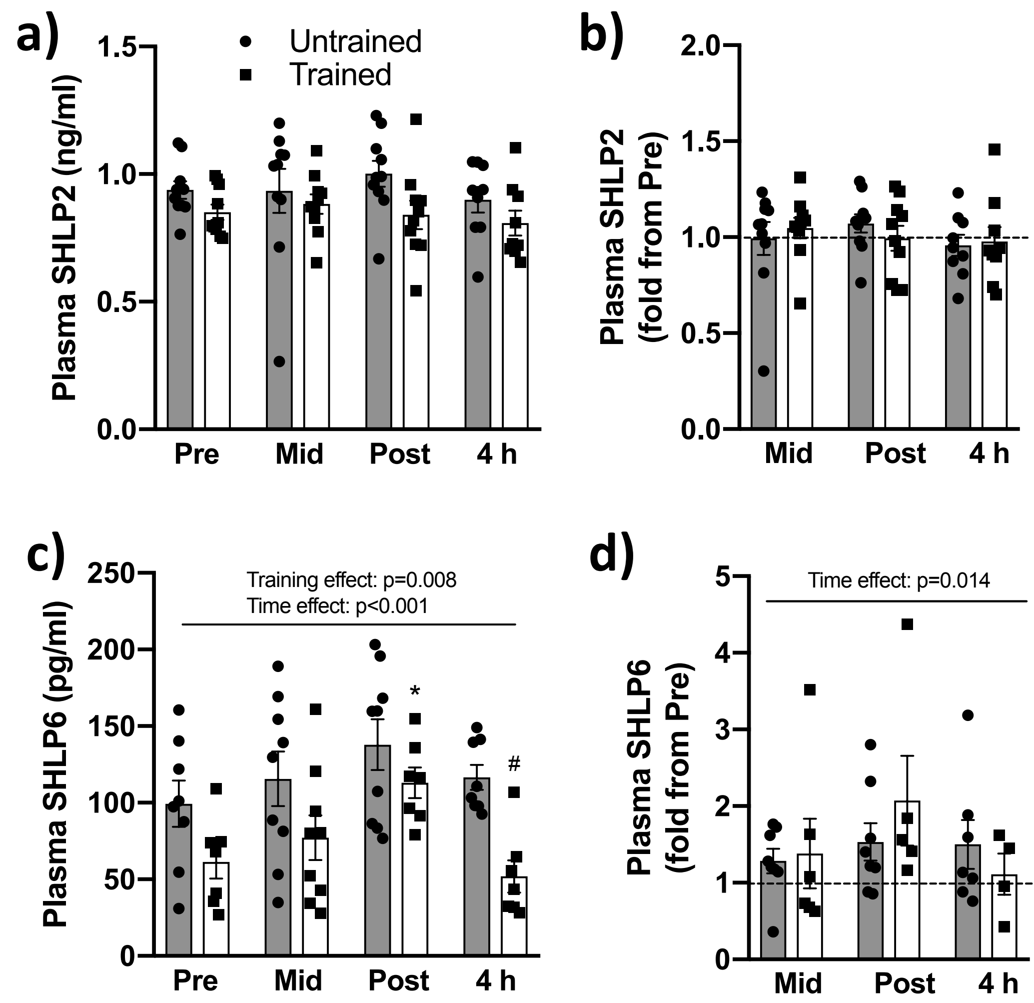

Figure 4

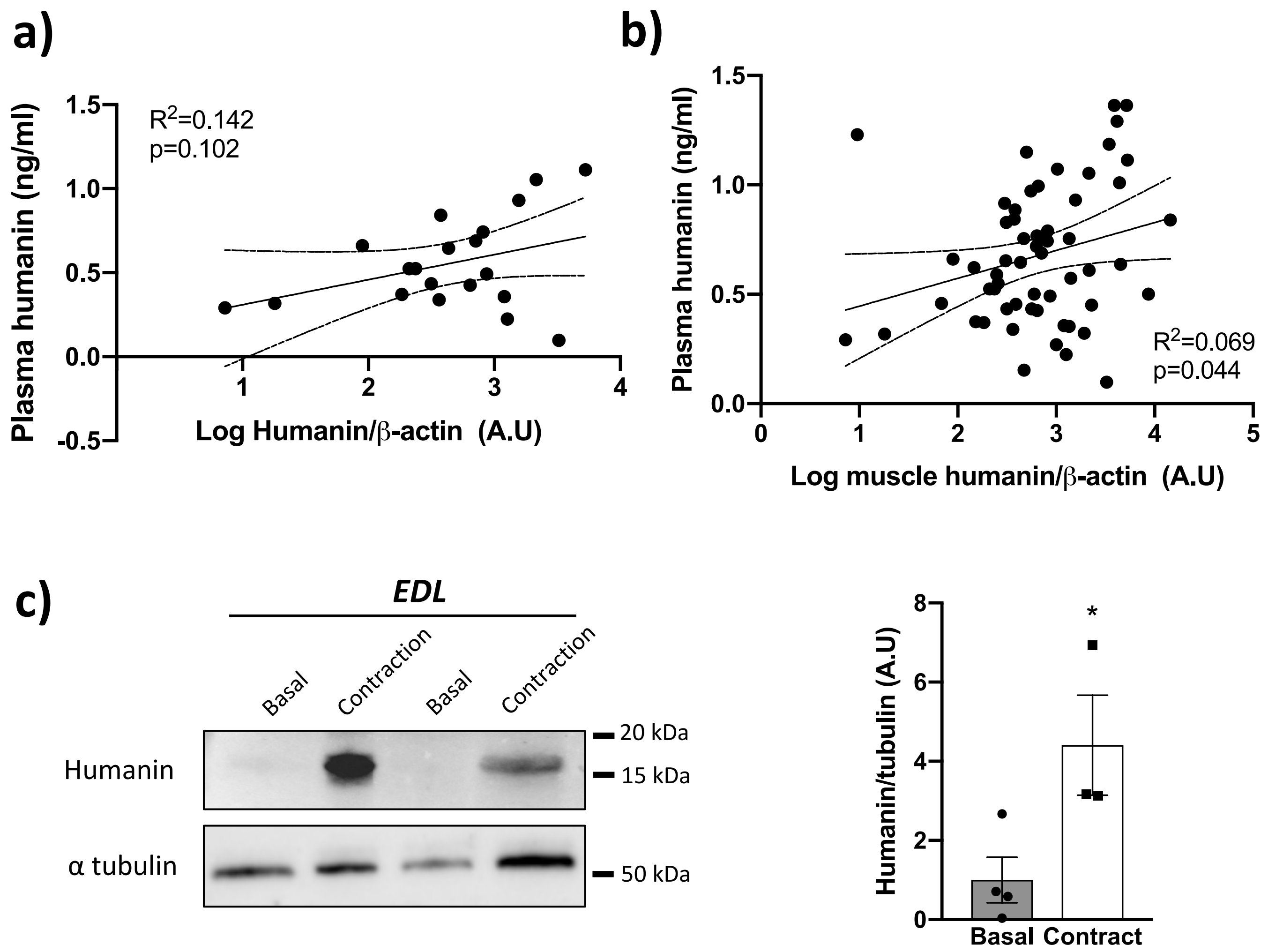

Figure 5

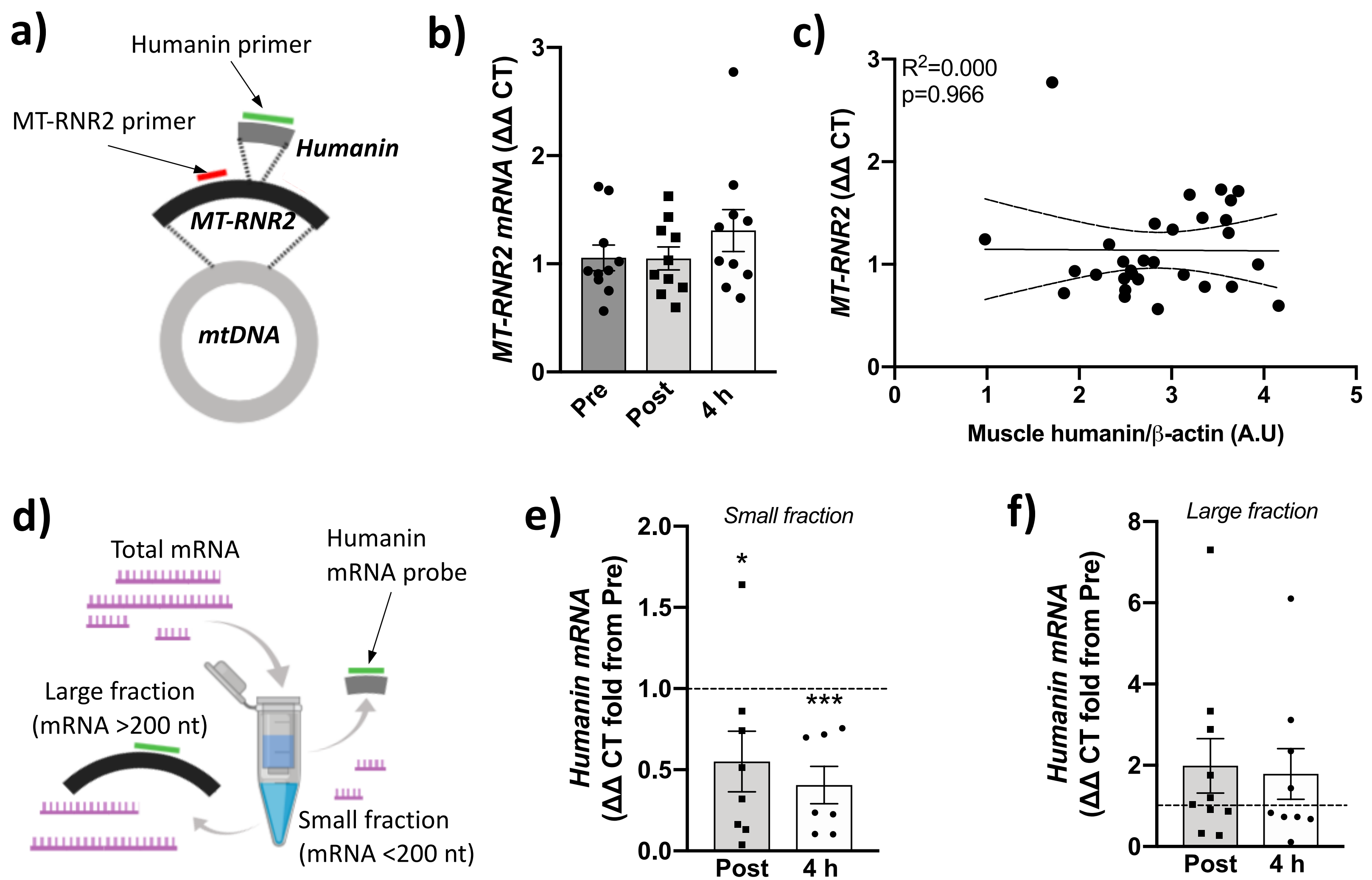

Figure 6