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1 **Title**

2 Exercise and training regulation of autophagy markers in human and rodent skeletal muscle.

3

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21

22 **Short running title:** Autophagy changes with exercise

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24 **Abbreviations**

25 LC3, Microtubule-associated proteins 1A/1B light chain 3; p62, ubiquitin-binding protein p62;
26 INH, inhibitors; MLSS, maximal lactate steady state; HIIE, high-intensity interval exercise;
27 HIIT, high-intensity interval training; MICE, moderate-intensity continuous exercise; SIE,
28 sprint-interval exercise; GXT, graded exercise test; $\dot{V}O_{2peak}$, peak oxygen uptake; $\dot{V}O_{2max}$,
29 maximal oxygen uptake W_{LT}, power at the lactate threshold; $\dot{W}_{max}$, maximal aerobic power.

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30 **Abstract**

31 Autophagy is a key intracellular mechanism by which cells degrade old or dysfunctional
32 proteins and organelles. In skeletal muscle, evidence suggests that exercise increases
33 autophagosome content and autophagy flux. However, the exercise-induced response seems to
34 differ between rodents and humans, and little is known about how different exercise
35 prescription parameters may affect these results. The present study utilised skeletal muscle
36 samples obtained from four different experimental studies using rats and humans. Here we
37 show that following exercise, in the soleus muscle of Wistar rats, there is an increase in LC3B-
38 I protein levels (+ 109%) immediately after exercise, and a subsequent increase in LC3B-II
39 protein levels (+ 97%) 3 hours into the recovery. Conversely, in human skeletal muscle, there
40 is an immediate exercise-induced decrease in LC3B-II protein levels (- 24%), independent of
41 whether exercise is performed below or above the maximal lactate steady state, which returns
42 to baseline 3.5 hours following recovery, while no change in LC3B-I protein levels is observed.
43 p62 protein levels did not change in neither rats nor humans following exercise. By employing
44 an *ex vivo* autophagy flux assay previously used in rodents we demonstrate that the exercise-
45 induced decrease in LC3B-II protein levels in humans does not reflect a decreased autophagy
46 flux. Instead, effect size analyses suggest a modest-to-large increase in autophagy flux
47 following exercise that lasts up to 24 hours. Our findings suggest that exercise-induced changes
48 in autophagosome content markers differ between rodents and humans, and that exercise-
49 induced decrease in LC3B-II protein levels do not reflect autophagy flux level.

50

51 **Keywords:** autophagy, exercise, LC3, skeletal muscle.

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52 **Introduction**

53 Autophagy is the cellular process by which an autophagosome (a double–membrane vesicle)
54 engulfs, and delivers to the lysosome, proteins or organelles that need to be degraded. It is the
55 recycling machinery of the cell and is important for the correct removal of intracellular
56 pathogens or misfolded proteins, among others, which may activate deleterious cellular
57 signalling pathways (e.g., inflammation) (1). In skeletal muscle, autophagy is important to
58 prevent mitochondrial damage (2), as well as promoting positive muscle regeneration (3),
59 optimal glucose metabolism (4), and for training-induced increases in mitochondrial proteins
60 and endurance performance (5). Thus, it is important to better understand the factors that
61 influence autophagy in skeletal muscle.

62 The autophagy machinery consists of a core set of autophagy-related (ATG) proteins (6).
63 Among these, the ATG8 family (which includes the subfamily members LC3A, LC3B, LC3C,
64 GABARAP, GABARAPL1, GABARAPL2) promote autophagosome formation and
65 autophagosome-lysosome fusion (7, 8). In skeletal muscle LC3s are abundantly expressed (9),
66 which makes LC3 a widely used marker of autophagosome content. In rodents, endurance
67 exercise acutely increases the levels of LC3-II (and the LC3-II/I ratio) as well as the appearance
68 of LC3 puncta in both skeletal and cardiac muscle (4). Similarly, endurance exercise to
69 exhaustion increases LC3-II protein levels in the tibialis anterior, along with a tendency for
70 increased exercise-induced autophagy flux, but unchanged protein levels of the autophagy
71 receptor p62 (10). Following a similar exercise session in mice LC3-I protein levels have also
72 been reported to increase (11). A study comparing two different exercise regimes in mice
73 showed that both low- and moderate-intensity exercise increased LC3A/B-II protein levels and
74 the LC3A/B-II/I ratio 3 hours following the end of exercise (12). Although findings are
75 inconclusive with regard to exercise-induced p62 protein changes, the LC3 findings
76 collectively suggest that autophagosome content, and possibly autophagy flux, are increased
77 after endurance exercise in rodents.

78 In contrast to rodent studies, human studies show a distinct pattern of exercise-induced changes
79 in autophagosome content markers. Protein levels of LC3B-II, and the LC3B-II/I ratio, have
80 been shown to decrease 0 to1 h following different types of endurance exercise and return to
81 baseline values after 3 to 4 h of recovery in human skeletal muscle (13-16). In contrast, 60 min
82 of exercise at 60% of $\dot{V}O_{2max}$ has been reported to increase the levels of LC3A/B-II protein
83 levels 2 h after the end of exercise (17). Following most types of endurance exercise, the protein

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84 levels of the autophagy receptor p62 remained unchanged (13, 15-17). In contrast, following 2
85 h at 70% of $\dot{V}O_{2peak}$, but not at 55% of $\dot{V}O_{2peak}$, the p62 protein levels decreased, which could
86 suggest an effect of exercise intensity on the exercise-induced p62 protein changes (14).
87 However, since both the exercise intensity and the total work completed were different, it is
88 difficult to isolate any of these factors. Other differences such as training status, timing of
89 biopsies, antibodies used, or sample size may also contribute to the reported discrepancies
90 between human studies. Whether exercise intensity distinctly affects the LC3B and p62 protein
91 levels changes following an exercise session remains to be fully elucidated.

92 Autophagy flux assays are considered the 'gold-standard' to assess autophagy levels (18).
93 Autophagy flux is the term used for the combined autophagy steps, which includes
94 autophagosome formation, maturation, fusion with lysosomes, and breakdown of the
95 autolysosome contents. One such assay aims to chemically block the fusion of autophagosomes
96 with the lysosome (the end-point of the degradation process) and to monitor the accumulation
97 of LC3-II (18). Performing an *in vivo* autophagy flux is not ethically possible in human tissues
98 and remains a limitation. This means that human studies have relied on markers of
99 autophagosome and autophagy receptor protein levels (13-17). Although not previously used
100 in humans, animal models have also utilised an *ex vivo* autophagy flux analysis (19).
101 Implementing this *ex vivo* LC3-II flux assay could provide a direct assessment of autophagy in
102 human studies and would avoid having to rely solely on indirect markers (i.e., LC3-II/I ratio).

103 Despite the increase in autophagy research in skeletal muscle, there is currently no consensus
104 on the exercise-induced regulation of autophagosome content in skeletal muscle. The aims of
105 the current study are multiple: 1) to assess potential differences in exercise-induced changes in
106 LC3B and p62 protein between rodents and humans; 2) to elucidate if the exercise-induced
107 LC3B and p62 protein changes are affected by exercise below or above the maximal lactate
108 steady state (MLSS) in humans; 3) to explore the effects of exercise training on the basal LC3B
109 and p62 protein levels in humans; and 4) to assess whether the exercise-induced changes in
110 LC3B-II protein levels are reflective of a decreased *ex vivo* autophagy flux in humans.

111 **Materials and Methods**

112 Four different studies were included in this manuscript: a single exercise session in rats (Study
113 1), exercise in humans at three different work-matched intensities above or below the maximal
114 lactate steady state (MLSS) (Study 2), a 3-week high-volume high-intensity interval training
115 in humans (Study 3), and a single exercise session in humans for the establishment of *ex vivo*

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116 autophagy flux (Study 4). All human participants were deemed healthy, and their
117 characteristics can be found in Table 1. Studies were performed at Victoria University
118 (Melbourne, Australia), and all analyses were performed under similar conditions in the same
119 laboratory. All studies were approved by the Victoria University Animal Ethics Committee and
120 the Victoria University Human Research Ethics Committee. Informed consent was obtained
121 from all human participants prior to study participation.

122 [Insert Figure 1 here]

123 **Study 1 – Exercise in rats**

124 **Overview**

125 Twenty-eight male Wistar rats (8 weeks old) were obtained from the Animal Resource Centre
126 (Perth, Australia). The Victoria University Animal Ethics Committee approved this study
127 (AEC 15/002). All procedures were performed according to the Australian Code of Practice
128 for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research
129 Council, Australia, 8th Edition). Rats were housed in groups of 2 to 4 in a temperature-
130 controlled room and maintained with a chow diet (Specialty Feeds, Perth, WA) and water ad
131 libitum on a 12:12 h light-dark cycle, 18-22 °C, with approximately 50% humidity. The animals
132 underwent acclimatisation over three days, using five separate 15 min running sessions
133 (ranging from being placed on a non-moving treadmill belt to running at a speed of 0.25 $m{\cdot}s^{-}$
134 ^{1}). At least 48 h before the experimental exercise session, the animals performed an incremental
135 exercise test. The incline of the treadmill was set at 10 degrees and the test was started at 0.16
136 $m{\cdot}s^{-1}$. The speed of the treadmill was increased 0.05 $m{\cdot}s^{-1}$ every three minutes. Animals were
137 removed from the treadmill when they could no longer keep up with the speed despite
138 encouragement (air puff).

139 **Experimental session**

140 On the experimental day, animals were exercised at 80% of their top speed achieved during the
141 incremental test (approximately 0.38 $m{\cdot}s^{-1}$ at a 10 degree incline) for seven 2-minute intervals
142 interspersed with 1 minute of rest. Rats were humanely killed using 90 $mg{\cdot}kg^{-1}$ i.p.
143 pentobarbitone prior to (REST), immediately after (+0 h), or 3 h after the completion of the
144 exercise protocol, and the soleus was removed and immediately frozen in liquid nitrogen and
145 stored at -80 °C.

146 **Study 2 – Exercise in humans: effects of exercise intensity**

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147 **Overview**

148 Ten healthy males volunteered for this study (Table 1). Participants were required to attend the
149 laboratory at Victoria University 8 to 11 times. For the first trial, participants underwent a
150 cycling graded exercise test (GXT) with 1-min increments as previously described (20). The
151 following visits were dedicated to determining the maximal lactate steady state (MLSS), which
152 was established by a series of 30-min constant power sessions. After the establishment of the
153 MLSS, participants completed two constant power exercise sessions to exhaustion at +6% of
154 the MLSS. Following this, they performed three experimental sessions that included skeletal
155 muscle biopsies.

156 **Experimental session**

157 The three experimental sessions were performed in a randomised order at -18%, -6% or +6%
158 of the MLSS. The MLSS was selected as the reference point because it is a critical intensity
159 that delineates heavy from severe exercise intensity (21), and three intensities (2 below and 1
160 above the MLSS) were chosen for the study. Participants were given 48 h of complete rest
161 before each trial, and at least 7 days between the successive experimental trials. They were
162 asked to maintain their normal diet and to replicate it on the day before and during the
163 experimental trials. Biopsies were taken from the *vastus lateralis* muscle at rest before the start
164 of exercise (REST), immediately upon completion of the exercise session (+ 0 h), and 3.5 h
165 after the end of the exercise (+ 3.5 h). Samples were immediately cleaned of excess blood, fat,
166 or connective tissue, and rapidly frozen in liquid nitrogen. Samples were stored at -80 °C until
167 subsequent analyses.

168 **Study 3 - Exercise training in humans: effect of high-volume training**

169 **Overview**

170 Participants completed 20 days of twice-a-day high-intensity interval training (HIIT), as
171 previously published (22, 23). Skeletal muscle biopsies were obtained at rest before (PRE) and
172 after (POST) the 20 days of high-volume of HIIT.

173 **Experimental sessions**

174 Participants were given 48 h of rest before the sample collection. All samples were obtained
175 from the *vastus lateralis* muscle and participants were provided standardised meals, as
176 previously described (23, 24). Biopsies were taken at rest and were immediately cleaned of

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177 excess blood, fat, or connective tissue, and rapidly frozen in liquid nitrogen and stored at -80
178 °C for subsequent analyses.

179 **Study 4 – Exercise-induced autophagy flux in human skeletal muscle**

180 **Overview**

181 Samples from five healthy participants from a larger study were analysed. The GXT protocol
182 utilised in this study was the same as in Study 2. Participants had been familiarised with the
183 exercise required as they had undertaken two GXTs and two exercise sessions in the two weeks
184 before the experimental session.

185 **Experimental session**

186 Two participants underwent the following exercise: six 30-s 'all-out' cycling bouts against a
187 resistance initially set at 0.075 kg·kg body mass^{-1} (~ 175% $\dot{W}_{max}$), interspersed with a 4-min
188 recovery period. The other three participants performed a session consisting of 90 minutes of
189 continuous cycling at ~ 42% of $\dot{W}_{max}$. Participants were given 72 h of rest before the
190 experimental session. All samples were obtained from the *vastus lateralis* muscle and
191 participants were provided standardised meals, as in previous studies (23, 24). Biopsies were
192 taken at rest before the start of exercise (REST), immediately upon completion of the exercise
193 bout (+ 0 h), 2.5 hours into the recovery (+ 2.5 h), and 24 hours after the initial skeletal muscle
194 sample (+ 24 h). Small muscle portions were immediately immersed into two separate vials
195 with 3 mL of oxygenated DMEM, and the autophagy flux assay was started (see below in *ex*
196 *vivo* autophagy flux assay). Once the protocol was finalised, samples were stored at -80 °C for
197 subsequent analyses

198 **Skeletal Muscle Analyses**

199 **Preparation of whole-muscle lysates**

200 Approximately 10 to 20 mg of frozen muscle was homogenised two times for two minutes at
201 a speed of 30 Hz with a TyssueLyser instrument (Qiagen, Canada) in an ice-cold lysis buffer
202 (1:20 w/v) containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM $Na_4P_20_7$, 1 mM
203 Na_3V0_4, 1 % NP-40, with added protease and phosphatase inhibitors at a 1:100 concentration
204 (Cell Signaling Technology). Protein concentration was determined using a commercial
205 colourimetric assay (Bio-Rad Protein Assay kit II, Bio-Rad Laboratories Pty Ltd, Gladesville,

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206 NSW, AUS) and lysates were then diluted with an equal volume in 2x Laemmli buffer
207 containing 10% Β-mercaptoethanol.

208

209 [Insert Table 1 here]

210

211 **Western blotting**

212 For each protein of interest, a signal linearity test was conducted to determine the ideal loading
213 amount. Muscle lysates were then loaded in equal amounts (10 to 20 μg) and separated by
214 electrophoresis for 1.5 to 2.5 h at 100 V using pre-cast stain-free SDS-PAGE gels (4-20%).
215 Once resolved, the gels were wet transferred onto LF PVDF membranes using a Turbo Transfer
216 system (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Membranes were blocked at
217 room temperature for 1 h using 5% skim milk or 5% bovine serum albumin (BSA) in tris buffer
218 saline (TBS) 0.1% tween-20 (TBS-T). After 3 x 5-min washes in TBS-T, membranes were
219 incubated overnight at 4 °C with gentle agitation in primary antibody solutions (1:1000
220 antibody in 5% BSA, plus 0.02% Na Azide). The antibody for LC3B was purchased from Cell
221 Signalling (#3868S) and the antibody for p62 from Abcam (#ab56416). The following
222 morning, membranes were washed 3 x 5-min in TBS-T and subsequently incubated under
223 gentle agitation at room temperature with the appropriate host species-specific secondary
224 antibody for 60-90 min in 1-5% skim milk in TBS-T. Membranes were washed again for 3 x
225 5-min in TBS-T before being immersed for 5 min under gentle agitation at room temperature
226 in Clarity ECL detection substrate (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS).
227 Protein bands were visualised using a Bio-Rad ChemiDoc imaging system and band densities
228 were determined using Bio-Rad ImageLab software (Bio-Rad Laboratories Pty Ltd,
229 Gladesville, NSW, AUS). All samples for each participant were loaded on the same gel, along
230 with different concentrations of a mixed-homogenate internal standard (IS), and a calibration
231 curve plotted of density against protein amount. From the subsequent linear regression
232 equation, protein abundance was calculated from the measured band intensity for each lane on
233 the gel. Total protein content of each lane was obtained from the stain-free image of the
234 membrane and was used for normalisation of the results.

235 ***Ex vivo* autophagy flux assay.**

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236 The following protocol was adapted from previous studies performing ex vivo autophagy flux
237 in rodents (19, 25). Upon collection of the skeletal muscle sample, two small pieces (~ 10 mg)
238 were placed in 3 mL of oxygenated DMEM CO_2 independent media (ThermoFisher
239 #18045088) at 37 °C. The tissues were then incubated with continuous oxygenation for 1 h
240 with ('treated' sample, with inhibitors), or without ('untreated' sample), 60 µL of NH_4Cl (20
241 $\mu L \cdot mL^{-1}$; 40 mM; Sigma Aldrich #S7653) and 30 µL Leupeptin (10 $\mu L \cdot mL^{-1}$; 100 uM; Sigma
242 Aldrich #L2884). Upon completion of a 1-h incubation, samples were snap-frozen and stored
243 at -80 °C until further analysis. Autophagy flux (Net LC3B-II flux) was obtained by subtraction
244 of the densitometric value of LC3B-II from treated compared to the untreated sample.

245 **Statistical analysis**

246 All values are reported as mean ± standard deviation (SD). All statistical analyses were carried
247 out on the raw values normalised to the total protein loading and calibration curve. For Study
248 1, one-way repeated-measures of ANOVA with Holm-Sidak post-hoc were used. For Study 2,
249 two-way repeated measures of ANOVA were used, and main effects and interactions were
250 further analysed using Holm-Sidak post-hoc tests. For Study 3, a two-tailed paired student's t-
251 test was used. For Study 4, a one-way ANOVA with Holm-Sidak post-hoc tests were utilised.
252 Effect sizes (ES) were quantified and defined as: small (0.2), moderate (0.5), large (0.8), and
253 very large (1.3). Statistical significance was set at $p < 0.05$ for all analyses. GraphPad Prism
254 8.3 software was used for the statistical analysis.

255

256 **Results**

257 **Study 1 - Exercise-induced changes in LC3B and p62 protein changes in soleus muscle of**
258 **Wistar rat.**

259 There was a main effect of time for LC3B-I and LC3B-II protein levels (both $p = 0.01$), as well
260 as the LC3B-II/I ratio ($p = 0.0003$). Compared to REST, there was a significant increase in
261 LC3B-I protein level at + 0 h (+ 109 ± 103%; ES = 1.1; $p = 0.017$; Figure 2.A) and at + 3 h (+
262 82 ± 62%; ES = 1.1; $p = 0.04$; Figure 2.A). Compared to REST, LC3B-II protein level did not
263 significantly change at + 0 h (- 20 ± 46%; ES = - 0.32; $p = 0.63$; Figure 2.B), but significantly
264 increased at + 3 h (+ 97 ± 102 %; ES = 0.95; $p = 0.04$; Figure 2.B), and from + 0 h to + 3 h (+
265 159 ± 129%; ES = 1.2; $p = 0.02$; Figure 2.B). There was a significant decrease in the LC3B-
266 II/I ratio immediately (+ 0 h) after exercise (- 65 ± 12 %; ES = - 1.5; $p = 0.001$; Figure 2.C),

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267 followed by a significant increase from + 0 h to + 3 h (+ 164 ± 98%; ES = 1.5; p = 0.001;
268 Figure 2.C). Protein level of p62 did not significantly change at any time point (p > 0.05; Figure
269 2.D).

270

271 [Insert Figure 2 here]

272

273 **Study 2 - Effects of exercise intensity on exercise-induced LC3B and p62 protein changes**
274 **in human skeletal muscle.**

275 There was no main or interaction effect for LC3B-I protein levels (p > 0.05; Figure 3.A). There
276 was no time x intensity effect for LC3B-II protein levels (p = 0.85), but there was a main effect
277 of time (p < 0.0001). Compared to REST, there was a significant decrease at + 0 h (- 24 ± 16%;
278 90 % CI [- 28, -19%]; ES = - 0.82; p = 0.0001; Figure 3.B), but not at + 3 h (+ 6 ± 31%; 90 %
279 CI [- 4, 15%]; ES = 0.01; p > 0.99; Figure 3.B), and a significant increase between + 0 h and +
280 3 h (+ 40 ± 38%; 90 % CI [28, 51%]; ES = 0.85; p < 0.0001; Figure 3.B). There was no time x
281 intensity effect for LC3B-II/I ratio (p = 0.85), but there was a main effect of time (p < 0.0001).
282 Compared to REST, there was a significant decrease at + 0 h (- 21 ± 17%; 90 % CI [- 26, -
283 16%]; ES = - 0.65; p = 0.003; Figure 3.C), but no significant difference at + 3 h (+ 10 ± 45 %;
284 90 % CI [- 4, 24%]; ES = 0.18; p = 0.27; Figure 3.C), and a significant difference between + 0
285 h and + 3 h (+ 40 ± 45%; 90 % CI [27, 54%]; ES = 0.67; p = 0.0001; Figure 3.C). There was
286 no main or interaction effect for p62 protein levels (all p > 0.05; Figure 3.D).

287

288 [Insert Figure 3 here]

289

290 **Study 3 – Effects of high-volume HIIT on resting LC3B and p62 protein levels in human**
291 **skeletal muscle.**

292

293 Resting levels of LC3B-II protein levels significantly increased from PRE to POST (+ 132 ±
294 140%; 90 % CI [+ 55, 209%]; ES = 0.85; p = 0.04; Figure 4.A). There was no significant
295 training effect on LC3B-I, LC3B-II/I ratio, and p62 protein levels (all p > 0.05).

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296

297 [Insert Figure 4 here]

298

299 **Study 4 - Exercise-induced changes in autophagy flux in human skeletal muscle.**

300 There was a main effect for LC3B-II protein levels in untreated samples (p = 0.017). Compared
301 to REST there were no significant changes at + 0 h (- 26 ± 23%; 90 % CI [- 43, - 10%]; ES = -
302 0.77; p = 0.10), at + 2.5 h (+ 23 ± 27%; 90 % CI [3, 43%]; ES = 0.64; p = 0.24), or at + 24 h
303 (+ 8.7 ± 23%; 90% CI [-8, 26%]; ES = 0.26; p = 0.53). However, relative changes from REST
304 to + 0 h between the samples from Study 1 and those in Study 2 were comparable (- 26% vs -
305 24% respectively; and ES = - 0.77 vs - 0.82 respectively), suggesting a similar exercise-induced
306 response in LC3B-II across experiments in the untreated samples.

307

308 For net LC3B-II flux there was no main effect (p = 0.27). Compared to REST, effect size
309 analyses showed a large positive change at + 0 h (+ 117 ± 163%; 90 % CI [- 3, 237%]; ES =
310 0.82; p = 0.22), at + 2.5 h (+ 113 ± 178%; 90 % CI [- 18, 244%]; ES = 0.88; p = 0.22), and at
311 + 24 h (+ 93 ± 126%; 90% CI [1, 126%]; ES = 0.79; p = 0.22).

312

313 [Insert Figure 5 here]

314

315 **Discussion**

316 Our study shows that: 1) exercise-induced changes in LC3B protein levels differs between
317 rodents and humans; 2) exercise-induced changes in LC3B and p62 protein levels appear to be
318 independent of exercising below or above the MLSS in human skeletal muscle; and 3) the
319 exercise-induced decrease in LC3B-II protein levels observed in humans were not reflective of
320 a decrease in autophagy flux.

321 The results of the present study showed that the exercise-induced changes in LC3B protein
322 levels differ between rodents and humans. In our rat study, there were increased LC3B-I protein
323 levels 0 to 3 hours following a single endurance exercise session (Figure 2). This was not
324 observed in our human study, in accordance with previous literature (16). An increase of

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325 LC3B-I protein levels may stem from increased LC3B mRNA translation. In fact, LC3B
326 mRNA expression has been shown to increase following exercise to exhaustion in mice (10),
327 and an exercise-induced increase in LC3-I protein levels has also been shown in rodents (11).
328 However, it is difficult to compare across studies as not many studies report the changes of
329 LC3-I protein levels.

330 In the present study, LC3B-II protein levels were unaltered immediately after exercise in rats
331 but were significantly increased 3 hours into the recovery. This is in line with previous research
332 showing that LC3-II is significantly increased in rodents 80 to 180 min from the start of
333 exercise (4, 16, 26). Due to the incomplete information regarding the antibodies used, it was
334 impossible to recapitulate the findings for the LC3 subfamily members used in some of the
335 rodent studies. Future research should address whether the different LC3 subfamily members
336 are similarly modified following exercise in rodents. Our results show that the protein levels
337 of the autophagy receptor p62 remained unchanged at all time points in rats. While this is in
338 contrast to some rodent studies (4, 27), it is in agreement with other findings (10-12, 28). A
339 possible explanation may relate to the duration of the exercise in the different studies, as the
340 only two studies reporting an exercise-induced decrease in p62 protein levels did exercise for
341 at least 110 min (4, 27), and a decrease in p62 was not seen at earlier time points or in the
342 recovery period (27). On the other hand, p62 protein level has been previously shown to be
343 decreased 6 hours into the recovery from both low- and moderate-intensity exercise (12),
344 suggesting a delayed lysosomal degradation of autophagosomes, which may have been missed
345 by most studies including the present study. It is important to mention that other proteins can
346 also act as autophagy receptors (e.g., NBR1, OPTN (29)), and how these are altered by exercise
347 requires further investigation.

348 In contrast to rodents, the findings from our human study show that, independently of
349 exercising below or above the MLSS, LC3B-II protein levels and the LC3B-II/I ratio were
350 decreased following exercise and returned to baseline 3.5 hours into the recovery (Figure 3).
351 This was in accordance with most human studies (13-16), with one exception (17). A major
352 difference with the study of Brandt, Gunnarsson (17) was the protein analysed. In contrast to
353 the present study and others where an antibody targeting the LC3B subfamily was utilised (13-
354 16), Brandt, Gunnarsson (17) used an antibody targeting a combination of LC3A and LC3B.
355 Whether the protein levels of the different the LC3 subfamily members are differentially
356 regulated following exercise remains to be elucidated. Interestingly, a proteomic analysis of
357 human skeletal muscle studies only detected LC3A (30), which may indicate a greater protein

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358 abundance of LC3A when compared to the other subfamily members in skeletal muscle. The
359 present findings also show that LC3B-I protein levels were not altered following exercise,
360 consistent with previous studies in humans (13, 15). The unchanged LC3B-I protein levels
361 could be due to unchanged mRNA expression of LC3B or rapid conjugation of LC3-I into
362 LC3-II and increased autophagosome degradation. The finding of unchanged p62 protein levels
363 following exercise, independent of exercise intensity, were in accordance with most studies
364 (13, 15-17). Furthermore, the previously reported role of exercise intensity on p62 protein
365 changes (14) may not be due to exercise intensity differences between protocols, but possibly
366 related to other factors such as total work performed.

367 Our data demonstrated that following three weeks of high-volume HIIT there was an increase
368 in basal LC3B-II protein level, suggesting an increase in autophagosome content (Figure 4).
369 To the best of our knowledge, this is the first study suggesting that HIIT can lead to increased
370 autophagosome content but our findings are in line with the results of a previous study where
371 three weeks of one-legged knee extensor training led to an increase in LC3B-II protein levels
372 (16). However, others have not shown any effect of endurance training on LC3A/B-II protein
373 levels, despite an increase in LC3A/B-I protein levels (17). Whether training volume or
374 intensity are more important for the training-induced changes in LC3B-II requires further
375 research.

376 A limitation of human studies to date is the use of LC3B-II and p62 protein levels to infer
377 changes in autophagy flux. This has led to the idea that a decrease in LC3B-II protein levels
378 following exercise could be reflective of a temporary decrease in autophagy flux (13). In the
379 present study, a protocol adapted from a rodent study (19) was used to examine, for the first
380 time, the effects of exercise on *ex vivo* autophagy flux in human skeletal muscle. The results
381 showed that autophagy flux (measured as net LC3B-II flux) did not decrease immediately after
382 0, 2.5, or even 24 hours after exercise (Figure 5.A). Although limited by the low number of
383 participants, the effect size analyses suggested a moderate-to-large increase in *ex vivo*
384 autophagy flux following exercise (+ 93-117%; ES = 0.79-0.88). Our findings in humans were
385 in agreement with those from a rodent study showing a similar exercise-induced fold-change
386 in autophagy flux (10). These findings would suggest that exercise-induced autophagy flux is
387 similar between rodents and humans (Figure 5.B), despite a different exercise-induced LC3B
388 protein regulation. The use of an *ex vivo* autophagy flux assay in future human studies will
389 allow researchers to overcome the limitation of solely relying on static protein markers. Future

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390 research should interrogate the autophagy flux response to different stimuli (e.g., inactivity) in
391 skeletal muscle and with larger sample sizes.

392 The main limitation of the present study is the low sample size and the two different exercises
393 utilised in our *ex vivo* autophagy flux experiments. Nonetheless, our findings highlight the
394 value of using this assay in human skeletal muscle studies. Furthermore, our study was limited
395 to LC3B, whereas the role of other ATG8 family members in exercise and skeletal muscle
396 autophagy remains unexplored.

397 In conclusion, the results of the current study showed that exercise-induced LC3B protein
398 changes differ between rodents and humans. This indicates caution must be taken when
399 extrapolating autophagy protein results from rodents to humans. Furthermore, findings from
400 the current study show that a reduction in LC3B-II protein levels following exercise in humans
401 was consistent across exercise intensities but was not indicative of a decrease in autophagy
402 flux. This suggests that studies should avoid looking at 'static' levels of LC3 protein levels and
403 include autophagy flux assays to provide a more valid assessment of dynamic changes in
404 autophagy with exercise.

405 **Acknowledgements**

406 The authors would like to thank all the participants for volunteering to take part in these studies.

407 **Conflict of Interest statement**

408 All authors involved in this research declare no conflict of interest.

409 **Funding**

410 This work was supported by an ARC grant to D.J.B (DP140104165).

411 **Author contributions**

412 J.B, C.G, N.A.J, A.J.G, M.L, and D.J.B designed the research. J.B, C.G, N.A.J, A.J.G, E.P,
413 T.J, and A.G performed the research. J.B, M.L, and D.J.B wrote the manuscript. J.B, C.G,
414 N.A.J, A.J.G, E.P, T.J, A.G, M.L, and D.J.B revised and approved the manuscript.

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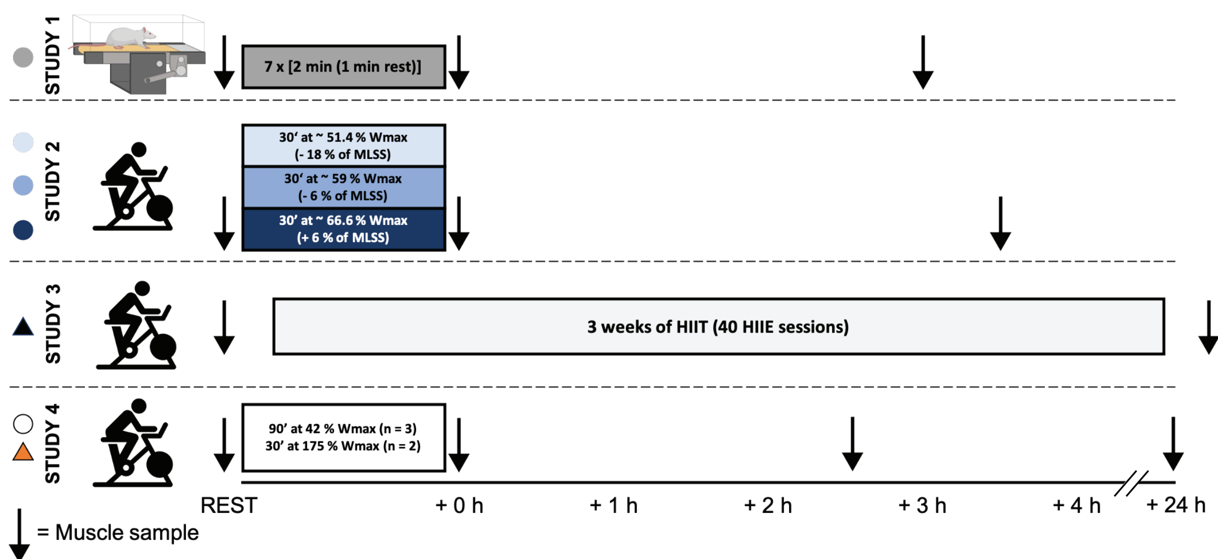

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514 Figure 1. Schematic representation of the four different experimental studies with the time-
515 course of the muscle samples collected. MLSS = Maximal lactate steady state; HIIT = High-
516 intensity interval training; HIIE = High-intensity interval exercise; $\dot{W}_{max}$ = maximal aerobic
517 power determined from a graded exercise test. Created with BioRender.com

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519 Table 1. Descriptive data of the human participants recruited for studies 2, 3, and 4. Data are
520 mean ± SD.

	Age (y)	$\dot{V}O_{2peak}$ (mL·min^{-1}·kg^{-1})	Trial	Relative Exercise Intensity (% $\dot{W}_{max}$)	Absolute Exercise Intensity (W)
Study 2 (n = 10)	27.5 ± 7.7	55.8 ± 10.0	- 18 % MLSS	51 ± 4	181 ± 39
			- 6 % MLSS	59 ± 4	207 ± 45
			+ 6 % MLSS	67 ± 5	234 ± 51
Study 3 (n = 9)	22.4 ± 5.2	47.0 ± 7.5	-		
Study 4 (n = 5)	30.0 ± 7.3	48.1 ± 4.4	SIE	175 ± 21	444 ±179
			MICE	42 ± 2	142 ± 35

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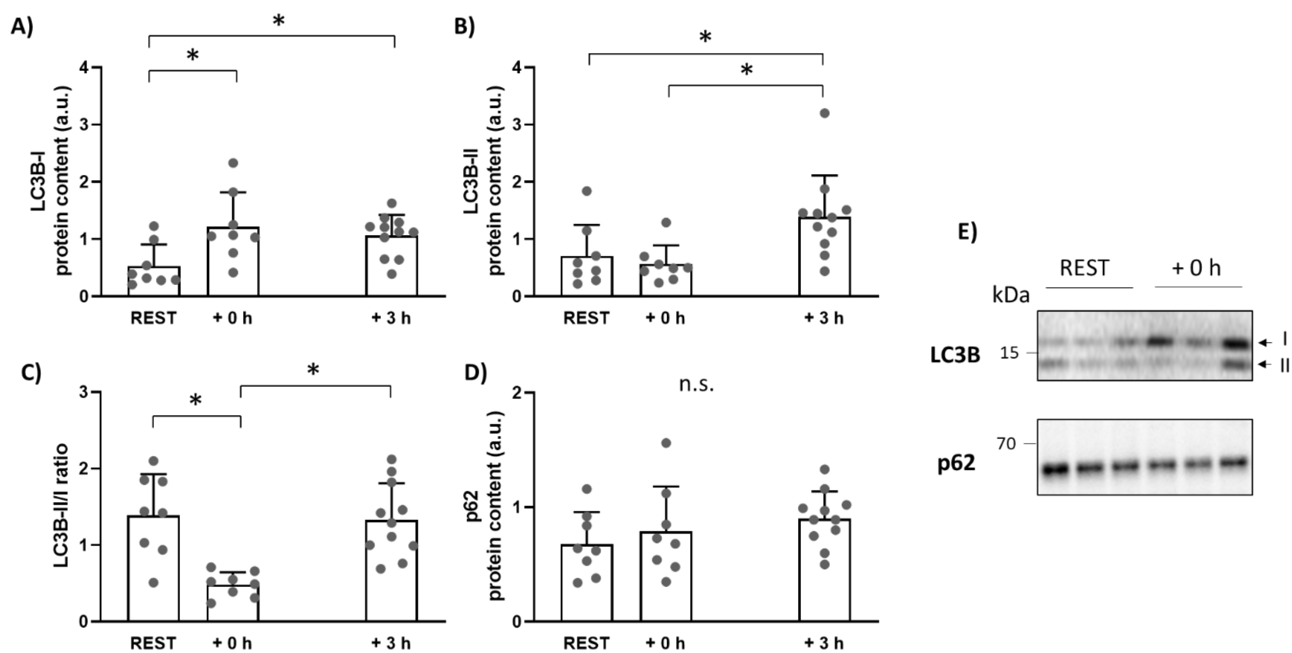

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525 Figure 2. Effects of exercise on A) LC3B-I and; B) LC3B-II protein levels; C) the LC3BII/I
526 ratio and; D) p62 protein levels in the soleus muscle of Wistar rats; E) representative blots of
527 LC3B and p62 protein. Data were analysed using one-way ANOVA; * = $p < 0.05$. Bars are
528 shown as mean + SD; n.s = not significant.

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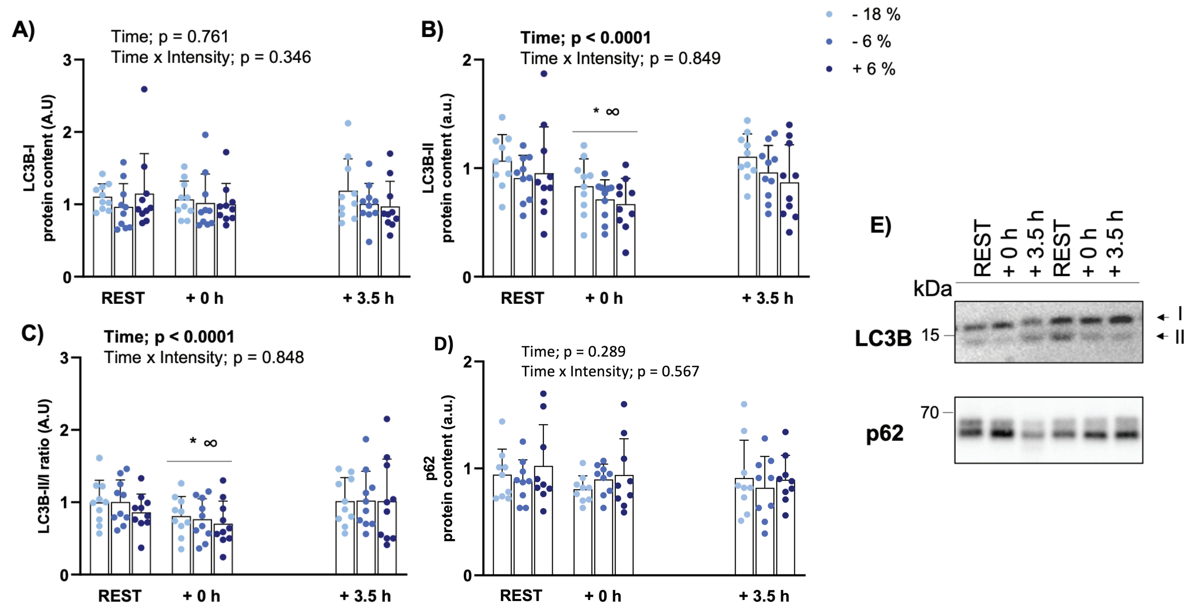

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531 Figure 3. Protein levels of A) LC3B-I and; B) LC3B-II at rest, as well as 0 h and 3.5 h after the
532 end of exercise; C) the LC3BII/I ratio and; D) p62 protein levels at rest, 0 h and 3.5 h after the
533 end of exercise; E) representative blots of LC3B and p62 protein. Participants performed the
534 exercise at three different intensities (-18% = light blue, -6% = normal blue, and +6% = dark
535 blue of the individually determined maximal lactate steady state). n = 9 for p62, n = 10 for
536 LC3B. * = different than REST; ∞ = different from + 3.5 h. Bars shown are mean + SD.

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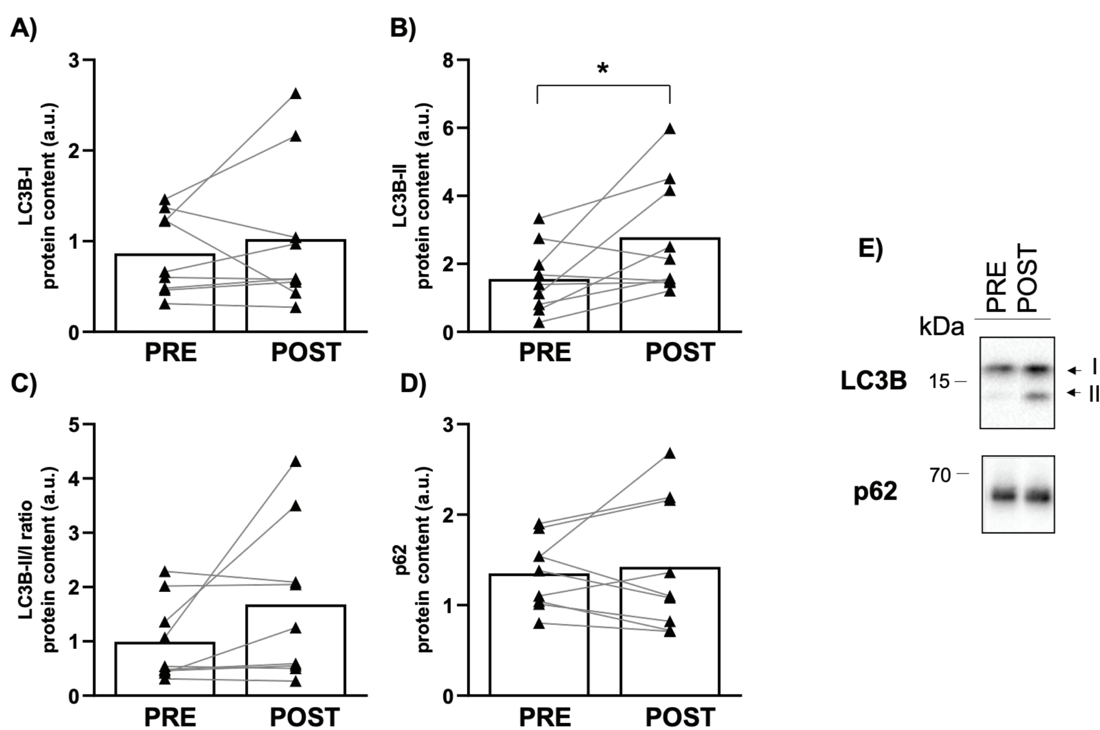

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539 Figure 4. Protein levels of A) LC3B-I and B) LC3B-II in PRE and POST training samples; C)
540 the LC3BII/I ratio and D) p62 protein levels in PRE and POST training samples; E)
541 representative blots of LC3B and p62 proteins. * = significantly different than PRE training
542 sample ($p < 0.05$). Individual and mean changes are shown.

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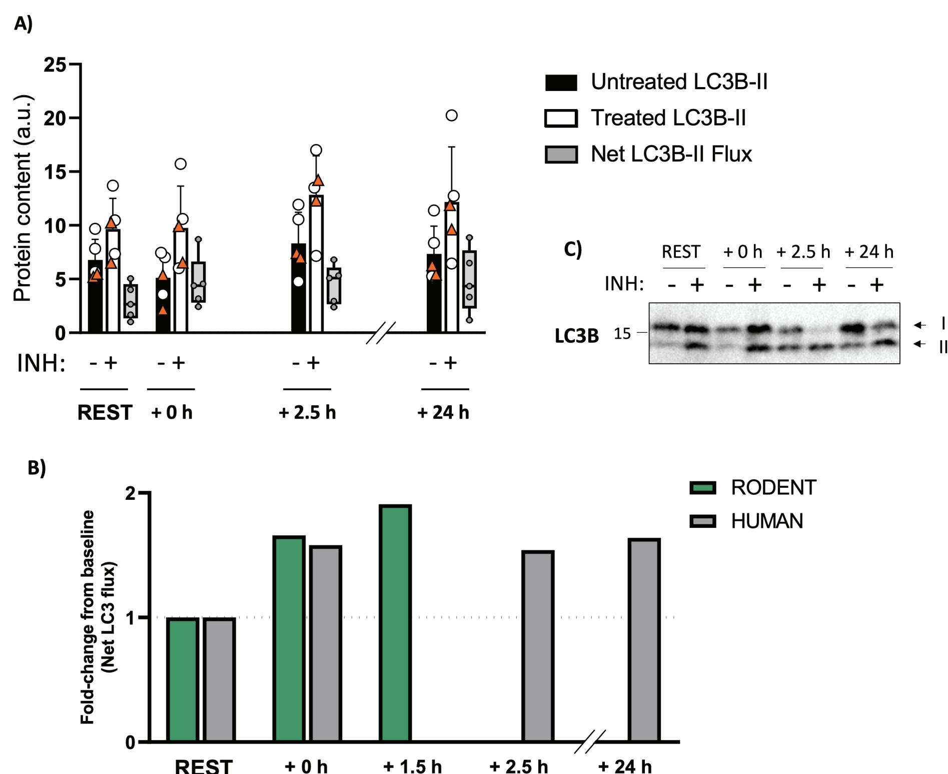

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545 Figure 5. A) LC3B-II protein levels from untreated (black bars) and treated samples (white
546 bars), and the net LC3B-II flux (in grey; calculated by subtracting untreated LC3B-II protein
547 levels from treated sample). C) Representative blot. Orange triangles represent participants
548 performing SIE (n = 2); white circles represent participants performing MICE (n = 3). B) Fold-
549 change following exercise in the net LC3-II flux from the present study (LC3B-II, human) and
550 a published rodent study (LC3-II, adapted from (10)). Bars for the treated and untreated
551 samples display the mean + SD. Individual data points along with box and whisker plots are
552 shown for net LC3B-II flux. INH = inhibitors NH_4Cl (40 mM) and Leupeptin (100 µM) added
553 to the treated sample.