

1 **Exercise training alters and β -alanine combined with exercise augments histidyl**
2 **dipeptide levels and scavenges lipid peroxidation products in human skeletal**
3 **muscle**

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40

41 **Abstract**

42 Carnosine and anserine are dipeptides synthesized from histidine and β -alanine by carnosine
43 synthase (ATPGD1). These dipeptides, present in high concentration in the skeletal muscle, form
44 conjugates with lipid peroxidation products such as 4-hydroxy *trans*-2-nonenal (HNE). Although
45 skeletal muscle levels of these dipeptides could be elevated by feeding β -alanine, it is unclear how
46 these dipeptides and their conjugates are affected by exercise training with or without β -alanine
47 supplementation. We recruited twenty physically active men, who were allocated to either β -alanine
48 or placebo-feeding group matched for VO_2 peak, lactate threshold, and maximal power (W_{max}).
49 Participants completed 2 weeks of conditioning phase followed by 1 week of exercise testing (CPET)
50 and a single session followed by 6 weeks of high intensity interval training (HIIT). Analysis of muscle
51 biopsies showed that the levels of carnosine and ATPGD1 expression were increased after CPET and
52 decreased following a single session and 6 weeks of HIIT. Expression of ATPGD1 and levels of
53 carnosine were increased upon β -alanine-feeding after CPET, while ATPGD1 expression decreased
54 following a single session of HIIT. The expression of fiber type markers myosin heavy chain (MHC) I
55 and IIa remained unchanged after CPET. Levels of carnosine, anserine, carnosine-HNE, carnosine-
56 propanal and carnosine-propanol were further increased after 9 weeks of β -alanine supplementation
57 and exercise training, but remained unchanged in the placebo-fed group. These results suggest that
58 carnosine levels and ATPGD1 expression fluctuates with different phases of training. Enhancing
59 carnosine levels by β -alanine feeding could facilitate the detoxification of lipid peroxidation products in
60 the human skeletal muscle.

61 **New and Noteworthy**

62 Carnosine synthase expression and carnosine levels are altered in the human skeletal muscle during
63 different phases of training.

64 During high intensity interval training, β -alanine feeding promotes detoxification of lipid peroxidation
65 products and increases anserine levels in skeletal muscle.

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67 **Introduction**

68 Carnosine (β -alanine-L-histidine) is a naturally occurring histidyl dipeptide, present at high
69 concentrations in the skeletal muscle, the olfactory bulb and the heart (14, 35, 36, 48). It is
70 synthesized by the enzyme carnosine synthase (ATPGD1), which catalyzes the ligation of β -alanine
71 to histidine *via* an ATP-mediated reaction (27). The homeostasis of carnosine in skeletal muscle is
72 maintained by a complex interplay of proton-coupled oligonucleotide transporters (PHT1 and PEPT2)
73 that transport amino acids across the biological membrane (29, 39, 40), as well as carnosinases
74 (CNDP1 and CNDP2), which hydrolyze carnosine to β -alanine and histidine (71). Several studies
75 have shown that β -alanine acts as a rate-limiting precursor of carnosine, and oral β -alanine
76 supplementation increases carnosine levels in the human skeletal muscle (9, 12, 23, 34, 66). It has
77 also been suggested that exercise training may augment carnosine levels in the human skeletal
78 muscle (44, 52) (38); but there is no consensus in the literature. In one study it was reported that 8
79 weeks of sprint cycle training increased carnosine levels in human skeletal muscle (68); however,
80 another study using a similar protocol, reported no effect of exercise training on carnosine
81 concentration (8). Similarly, no effect of exercise training on carnosine levels in the human skeletal
82 muscle was observed after another similar training intervention lasting for 10 to 16 weeks (8, 42, 43,
83 47). Therefore, the effects of exercise on carnosine levels and the underlying mechanisms that
84 regulate its homeostasis in the human skeletal muscle during exercise remains unclear and requires
85 further investigation.

86 It is widely believed that carnosine is the predominant histidyl dipeptide present in humans
87 (14), whereas its methylated analogue, anserine, is present in high concentrations in the skeletal
88 muscle of birds and rodents (14), but not in humans (20, 46). Anserine is synthesized by the

89 methylation of carnosine or by the condensation of N π -methyl-L-histidine with β -alanine - reactions that
90 are catalyzed by the enzymes carnosine N-methyl-transferase and ATPGD1, respectively (25). Both
91 carnosine and anserine contains a highly-reactive amine group, which confers to them the ability to
92 form stable conjugates with highly toxic lipid peroxidation products such as 4-hydroxy *trans*-2-nonenal
93 (HNE) and acrolein (1, 15, 51). Recent studies have shown that the formation of lipid peroxidation
94 products are increased in the skeletal and cardiac muscle during strenuous exercise (4, 5, 18, 24, 70,
95 75). Previous studies performed in rodents and humans have shown that the oral supplementation of
96 carnosine increases the extrusion of carnosine-aldehyde conjugates in urine, improves renal function
97 in obese Zucker rats (2) and improves glucose uptake in obese humans (21, 55). Furthermore, we
98 have recently reported that the perfusion of isolated mice hearts with carnosine protects against
99 ischemia-reperfusion injury (6), and that in apo-E^{-/-} mice the addition of carnosine to drinking water
100 decreases atherogenesis by removing reactive aldehydes (10). Collectively, these studies suggest
101 that carnosine detoxifies and removes lipid peroxidation products that play a pathogenic role in
102 several diseases (28, 30, 45, 50, 56, 62). Nevertheless, the role of carnosine in removing the
103 products of lipid peroxidation that accumulate in the skeletal muscle during exercise has not been
104 studied.

105 Given that β -alanine supplementation increases carnosine in humans and anserine levels in
106 birds (12, 23, 34, 57), we examined the effects of exercise training in physically-active individuals with
107 or without β -alanine supplementation on these dipeptides in human skeletal muscle. We tested the
108 hypothesis that β -alanine supplementation increases carnosine levels during exercise training and
109 that this increase in carnosine promotes the detoxification of toxic lipid peroxidation products
110 generated in skeletal muscle during strenuous exercise.

111 **Materials and Methods**

112 *Participant's recruitment and characteristics.*

113 Twenty healthy, physically-active male men were recruited for this study. Participants were informed
114 of any potential risks of participation in the study before the distribution of a health assessment
115 questionnaire and informed consent forms. Inclusion criteria for participation included at least 6
116 months of not using supplements (β -alanine, creatine, branched chain amino acids, chronic caffeine
117 supplementation). This study was performed at two Centers. Participants were recruited, tested and
118 trained at Victoria University and biochemical assays were performed at the University of Louisville.
119 This study was approved by the Victoria University Human Research Ethics Committee (HRE13-220).

120 *Study overview*

121 The study was divided into five phases: (a) familiarization phase; (b) baseline exercise testing; (c)
122 conditioning phase followed by 1-week of exercise testing (CPET); (d) exercise testing; and (e) high-
123 intensity interval training (HIIT) (Fig. 1).

124 *Familiarization phase.*

125 Before entering the study protocol, the participants were familiarized with the exercise testing by
126 visiting the exercise laboratory on three separate occasions. Participants were informed of any
127 potential risks of the study, and after consent they were asked to complete a health assessment
128 questionnaire and informed consent.

129 *Baseline Exercise testing*

130 A week after familiarization, baseline exercise testing was performed. Exercise testing and training
131 was conducted on a Velotron cycle ergometer (Racermate Inc., Washington, USA) and each session
132 was separated by at least 48 h. Testing consisted of an incremental cycling protocol to exhaustion to
133 determine lactate threshold (LT), maximal power (W_{max}) peak oxygen consumption (VO_2 peak), a
134 Cycling Capacity Test (CCT), and a muscle biopsy (as described below; Table 1). Participants were
135 then ranked for VO_2 peak, and matched pairs were randomly assigned to either β -alanine (CarnoSyn,
136 sustained-release β -alanine, Natural Alternatives International, San Marcos, USA) or placebo
137 (maltodextrin, Natural Alternatives International, San Marcos, USA) supplementation. All supplements

138 were tested by an independent drug surveillance laboratory (HFL Sport Science, Cambridgeshire, UK)
139 and tested negative for contamination from prohibited substances. The supplementation lasted 9
140 weeks. It involved ingesting 6.4 g per day (two 800 mg tablets, four times daily, at least 2 h apart) of
141 β -alanine or placebo with meals or snacks. All supplements for 7 days were contained in a sealed
142 opaque pill container and were distributed to the participants weekly by an independent individual not
143 involved with data collection.

144 *Conditioning phase and exercise testing (CPET)*

145 During the conditioning phase (first two weeks), participants completed six cycling training sessions
146 (separated by at least 48 h) that consisted of 1 h of cycling at 70% of their lactate threshold. The goal
147 of this phase was to improve the exercise tolerance of all participants, and to reduce the risk for injury
148 during the following HIIT phase. In the third week, the participants performed a second exercise
149 testing, which included an incremental cycling protocol to exhaustion, a CCT, and a 30-km cycling
150 time trial (30-km TT).

151 *High intensity interval training (HIIT)*

152 After exercise testing, the participants underwent a single session of HIIT and then began an
153 individualized 6 week HIIT program (3 sessions per week). The exercise intensity of each session was
154 the intensity at the LT plus 40 to 90% of the difference between the LT and W_{\max} , in addition to each
155 participants LT. For example, a participant with a LT and W_{\max} of 200 and 280 W respectively would
156 have a training workload of 232 W, $(200 + 40/100 * (280 - 200) = 232)$. The number of intervals for
157 each session ranged from 4 to 14 intervals and there was a 2:1 min work to rest ratio. The training
158 plan was designed to mimic athletic training programs and to allow progression, while preventing
159 overtraining (Fig. 1).

160 *Physical activity and nutritional control*

161 Participants were instructed to maintain a normal dietary pattern and to keep high-intensity physical
162 activity to a minimum throughout the entire study. To minimize within-subject variability in muscle
163 metabolism, participants were instructed to consume identical dinners and not to have breakfast prior

164 to each muscle biopsy and exercise testing session. Participants were provided with a standardized
165 dinner (50 kJ.kg⁻¹ body mass, consisting of 60/20/20% of carbohydrate, fat and protein respectively)
166 and breakfast (40 kJ kg⁻¹ BM, consisting of 60/20/20% of carbohydrate, fat and protein respectively)
167 prior to the 30-km TT, which were consumed 15 h and 3 h prior to exercise.

168 *Maximal Power, Lactate Threshold (LT) and Peak Oxygen Consumption*

169 Participants visited the exercise laboratory to perform an incremental cycling protocol to exhaustion
170 for the purpose of determining their LT, W_{\max} , and $VO_{2\ peak}$. The test consisted of 4-min stages,
171 separated by 30-s of rest, until voluntary exhaustion or the inability to maintain a minimum pedal
172 cadence of 60 rpm. The participants began cycling at 60 W with an increase of 30 W for each
173 subsequent stage. Maximal power was determined using the following equation: $W_{\max} = \text{Workload} +$
174 $(t/240) \times 30$ (where t-time completed during the final stage) (37). Venous blood samples were taken
175 at the end of each stage *via* intravenous cannulation and analyzed using an automated cartridge-
176 based gas analyzer (Radiometer, Copenhagen, Denmark). The LT was calculated using the modified
177 D_{\max} method (13) . Expired gases were analyzed every 15-s using a metabolic cart (Moxus Metabolic
178 System, AEI Technologies, USA), which was calibrated using known gas concentrations prior to each
179 test (20.93% O₂, 0.04% CO₂ and 16.10% O₂, 4.17% CO₂; BOC Gases, Australia). Oxygen
180 consumption was recorded every 15-s and the two-highest consecutive 15-s values recorded during
181 the test were averaged and recorded as the participant's $VO_{2\ peak}$.

182 *Cycling Capacity Test*

183 High-intensity exercise performance was determined by time to exhaustion and total work done
184 (TWD) during the CCT at 110% W_{\max} (CCT_{110%}). After a warmup for cycling at 100 W for 10 min,
185 participants began cycling at a pedal cadence of 80 to 100 rpm at 80% of W_{\max} for 15 s before
186 increasing to 90% for 15 s and eventually 110% of W_{\max} , while maintaining a seated position.

187 *Muscle biopsies*

188 Resting muscle biopsies (approximately 150 to 300 mg wet weight) were taken from the *vastus*
189 *lateralis* muscle by an experienced medical practitioner using a Bergstrom needle. Muscle biopsies

190 were performed at least 48 h after the completion of baseline exercise testing, after the conditioning
191 plus exercise testing (at rest and immediately after the first HIIT session), and after 6 weeks of the
192 HIIT program. Participants rested in the supine position, and after injection of a local anesthetic into
193 the skin and fascia (1% xylocaine, Astra Zeneca) a small incision was made in the *vastus lateralis*.
194 Muscle samples were processed, cleaned of excess blood, fat, and connective tissue, and
195 immediately frozen in liquid nitrogen and stored at -80°C until analysis.

196 *Preparation of carnosine-aldehyde conjugates*

197 Authentic standards for carnosine (*m/z* 227), anserine (*m/z* 241) and tyrosine-histidine (*m/z* 319) were
198 used to determine the multiple reaction monitoring transitions (MRMs). Carnosine-aldehyde
199 conjugates used as standards were prepared as described previously (7). Briefly, acrolein was
200 synthesized by acid hydrolysis of diethyl acetal acrolein (Sigma) in HCl (0.1 M) which was kept at
201 room temperature (RT) for 30 min. The carnosine-propanal conjugate was synthesized by incubating
202 100 mM acrolein (10 µL) with 10 mM carnosine (990 µL) in water at RT for 2 h. Carnosine-propanol
203 conjugate was synthesized by incubating 10 mM NaBH₄ (10 µL) in water with carnosine-propanal (500
204 µL) at RT. Carnosine-HNE conjugate was synthesized by incubating HNE (70 mM; Calbiochem) with
205 10 mM carnosine in water (10:1) at RT. The dipeptides and carnosine-aldehyde conjugates were
206 individually infused into a stream of 0.55 mL/min 50:50 A: B UPLC solvent going into a Waters
207 (Milford, MA) Xevo TQ-S micro triple quadrupole mass spectrometer. An automated optimization
208 program IntelliStart (Waters, Milford, MA) was used to determine the optimal ionization voltages,
209 collision energies and product ions. Maximum ionization conditions and optimal daughter ions were
210 used to program sensitive MRMs.

211 *Identification of histidyl dipeptides and carnosine-aldehyde conjugates by LC/MS/MS*

212 Skeletal muscle biopsies from participants were homogenized in an extraction solution containing 10
213 mM HCl and 200 µM tyrosine-histidine as an internal standard (IS). The tyrosine-histidine was
214 purchased from Bachem. Homogenates were sonicated on ice for 10 s, centrifuged at 16,000xg for 10
215 min and supernatant was diluted with 3 volumes of ice-cold acetonitrile. Before analysis, the samples

216 were vortexed thoroughly to precipitate proteins, kept on ice for 15 min, and then centrifuged at
217 16,000 $\times g$ for 10 min at 4°C. The supernatants were stored at -20°C for further processing. Prior to
218 injection into TQ-S micro mass spectrometer in positive mode, the samples were diluted in 75%
219 acetonitrile: 25% water. Dipeptides and their aldehyde conjugates were separated and identified by
220 using Waters ACQUITY UPLC H-Class System coupled with a Xevo TQ-S micro triple quadrupole
221 MS. The analytes were separated by a Waters Acquity BEH HILIC column (1.7 μ m, 2.1 \times 50 mm)
222 equipped with an in-line frit filter unit. The analytes were eluted by using a binary solvent system
223 consisting of 10 mM ammonium formate, 0.125% formic acid in 50% acetonitrile: 50% water for
224 mobile phase A and 10 mM ammonium formate 0.125% formic acid in 95% acetonitrile: 5% water for
225 mobile phase B at a flow rate of 0.55 mL/min. Initial conditions were 0.1: 99.9 A: B ramping to 99.9:
226 0.1 A:B over 5 min then quickly ramping to 0.1:99.9 A:B over 0.5 min. This initial composition was
227 held from 5.5-8 min to equilibrate the column for the next injection. Dipeptides were quantified using
228 the LC/MS calibration curve of relative area of carnosine and anserine to tyrosine-histidine (IS), and
229 their aldehyde conjugates were quantified using the peak ratio of histidyl-dipeptide and tyrosine-
230 histidine (IS) and expressed as mole/mg wet weight. For carnosine m/z 227 \rightarrow 110, anserine m/z
231 241 \rightarrow 109, carnosine-HNE m/z 383 \rightarrow 110, carnosine-propanal m/z 283 \rightarrow 110, carnosine-propanol m/z
232 285 \rightarrow 110 and tyrosine-histidine m/z 319 \rightarrow 110, MRM transitions were followed. We recorded the
233 abundance of at least four confirmation transitions for each molecule.

234 The precision of the LC/MS/MS method was validated by replicate analysis of the samples
235 with highest and lowest concentrations of the analytes. Homogenates were pooled from lowest (1 mL)
236 (n=10) and highest (1 mL) carnosine concentration samples (n=10). Five aliquots from each sample
237 were processed and analyzed each day for three consecutive days. Relative variability was calculated
238 by calculating the coefficient of variation (CV) of replicates within one sample run and between
239 different sample runs as described by Chesher (19). The variability of inter- and intra-assay for
240 carnosine low was 3.4%, carnosine high 4.92%, anserine low 9.95%, anserine high 6.87%, carnosine-

241 propanal low 8.07%, carnosine-propanal high 6.25%, carnosine-propanol low 4.14%, carnosine-
242 propanol high 5.96%, carnosine-HNE low 12.14% and carnosine-HNE high 16.46%. The lower limit of
243 quantification (LOQ) for carnosine was 19 nM, anserine 24 nM, carnosine-propanal 275 nM,
244 carnosine-propanol 261 nM and carnosine-HNE 257 nM.

245 *Western blot analysis*

246 Skeletal muscle biopsies collected from participants at different stages of training (n=4-8 in each
247 group) were homogenized in RIPA buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM
248 EGTA, 1% NP-40). Homogenates were centrifuged for 25 min at 13,000×g and the supernatants were
249 separated by SDS-PAGE. Immunoblots were analyzed using anti-ATPGD1, anti-CNDP2, anti-PHT1
250 anti-PEPT2, anti-glyceraldehyde 3-phosphate (GAPDH), anti-myosin heavy chain (MHC1) and MHCIIa
251 antibodies. Antibodies were purchased from Abcam and Thermo-fisher. Western blots were
252 developed using HRP substrate (ECL plus from Pierce) and scanned with Typhoon Bioimager (GE
253 healthcare). Band intensity was quantified by using Image Quant TL software (Amersham
254 Biosciences) that were normalized to GAPDH and Amido-black staining.

255 **Statistical Analyses**

256 Differences in histidyl dipeptides and histidyl-dipeptide conjugates between groups (β -alanine vs
257 placebo) and times (week) were estimated using linear mixed-effects models with a group and time
258 interaction variable. Statistical analyses were performed using SAS, version 9.4, software (SAS
259 Institute, Inc., Cary, North Carolina). For Western blot analysis, a one-way ANOVA was used followed
260 by Bonferroni corrections. Statistical analysis was done using GraphPad analysis software. Statistical
261 significance was accepted at $p < 0.05$. Group data are presented as mean \pm standard deviation (SD).

262 **Results**

263 *Exercise training in combination with β -alanine feeding increases carnosine levels*

264 To examine whether exercise training in combination with or without β -alanine supplementation
265 could affect carnosine levels in the human skeletal muscle, we collected muscle biopsies from the

266 participants at different phases of training, and analyzed for carnosine by LC/MS/MS. We identified
267 carnosine in the skeletal muscle on the basis of retention time, MRMs, and fragmentation pattern,
268 which accurately matched with the *ex-vivo* carnosine standard (Fig. 2 A, B). Our results showed that
269 at baseline, the levels of carnosine in the skeletal muscle were not statistically different between the
270 placebo (6.68 ± 1.81 nmol/mg tissue) and the β -alanine-fed groups (6.32 ± 1.41 nmol/mg tissue; Fig.
271 2 C, E). In the placebo-fed group, the levels of carnosine after 2 weeks of conditioning followed by 1
272 week of exercise testing (CPET) were increased ~24% (8.31 ± 1.92 nmol/mg tissue) compared with
273 the baseline ($p < 0.05$). However, carnosine levels were decreased after 6 weeks of HIIT compared
274 with the CPET (6.01 ± 1.34 nmol/mg tissue; $p < 0.05$, Fig. 2 C, E). The carnosine levels in all the
275 participants, except one was decreased, which was included in the analysis. In the β -alanine-fed
276 group, the levels of carnosine after CPET were increased by ~51% (9.53 ± 1.33 nmol/mg tissue;
277 $p < 0.0001$) compared with the baseline (Fig. 2 C, E) and reached 14.40 ± 4.38 nmol/mg tissue after an
278 additional 6 weeks of HIIT leading to a total ~127% increase in muscle carnosine over the 9 week
279 supplementation period and ~140% compared with the placebo fed group. Carnosine concentration in
280 the placebo-fed group was decreased following a single session of HIIT (6.74 ± 1.94 nmol/mg tissue)
281 compared with the CPET ($p < 0.05$) whereas its concentration in the β -alanine fed group increased
282 ~66% compared with the baseline (10.51 ± 2.82 nmol/mg tissue; $p < 0.001$) and ~10% compared with
283 pre-HIIT (Fig. 2 D, F). Collectively, these results demonstrate that carnosine levels fluctuate during
284 different phases of training (in the placebo fed condition) and further confirms the potent effect of
285 chronic β -alanine supplementation on muscle carnosine loading when combined with high-intensity
286 exercise training.

287 *ATPGD1 expression in the skeletal muscle is altered during different phases of exercise training*

288 Given that the carnosine levels were elevated in the skeletal muscle after 2 weeks of
289 conditioning followed by 1 week of exercise testing (CPET) and decreased after a single session and
290 6 weeks of HIIT, we next investigated whether there were any changes in the expression of enzymes

291 (ATPGD1 and CNDP2) or transporters (PHT1 and PEPT2) that determine carnosine levels in the
292 skeletal muscle (27, 29, 39, 40, 71). Our results showed that in the placebo-fed group, ATPGD1
293 expression increased ~1.5-fold after CPET compared with the baseline, which was decreased
294 following a single session and 6 weeks of HIIT (Fig. 3 A, B, C). We next determined whether ATPGD1
295 could respond to exercise stimulus, in the β -alanine-fed group and found that its expression was
296 increased ~1.2-fold after CPET compared with the baseline and decreased following a single session
297 of HIIT (Fig. 3 D, E). Expression of PHT1 and CNDP2 remained unchanged in all the groups at
298 different phases of training (Fig. 3 A B, D, E). PEPT2 was not detected in these tissues. To examine
299 whether changes in ATPGD1 expression and carnosine levels could be due to a shift in fiber type
300 during exercise, we next compared the expression of fiber type markers MHC I and IIa in tissues
301 collected at baseline and after CPET. In comparison with the baseline, the expression of both MHC I
302 and IIa remained unchanged after CPET (Fig. 3 F) suggesting that the changes in ATPGD1
303 expression and carnosine levels during exercise could not be attributed to changes in fiber types.
304 Collectively these results suggest that ATPGD1 responds to exercise stimulus and thus could be an
305 important regulator to maintain carnosine levels in the skeletal muscle during exercise.

306 *β -alanine feeding in combination with exercise training increases anserine level in the human skeletal*
307 *muscle*

308 To examine whether β -alanine could also increase anserine levels, we first examined whether
309 anserine is present in human skeletal muscle. Our LC/MS/MS analysis of human skeletal muscle
310 detected anserine, which had a similar retention time, MRMs and fragmentation pattern to the *ex-vivo*
311 anserine standard (Fig. 4 A, B). Our results showed that anserine levels at baseline were similar in the
312 placebo-(0.111 ± 0.025 nmol/mg tissue) and β -alanine-fed groups (0.101 ± 0.019 nmol/mg tissue),
313 which were increased ~29% after 9 weeks of β -alanine feeding compared with the placebo-treated
314 group (0.122 ± 0.024 vs 0.095 ± 0.015 nmol/mg tissue; $p < 0.05$ Fig. 4 C, E). However, after 6 weeks
315 of HIIT, the levels of anserine in the β -alanine-fed group were ~20 % higher than its baseline values

316 ($p<0.05$) and after CPET (0.111 ± 0.023 nmol/mg tissue $p<0.05$). Anserine levels were decreased in
317 the β -alanine fed group following a single session of HIIT compared to CPET, except one participant
318 that was included in the analysis, however the differences between the two groups did not reach to
319 statistical significance (0.097 ± 0.022 nmol/mg tissue, $p<0.13$). No changes in anserine levels were
320 observed in the placebo fed group following a single session of HIIT (Fig. 4 D, F).

321 *Carnosine promotes detoxification of lipid peroxidation products in human skeletal muscle*

322 Because, strenuous exercise has been reported to increase the production of lipid
323 peroxidation products in both cardiac and skeletal muscle (4, 5, 18, 24, 70, 75), we next examined
324 whether the increase in carnosine levels in the skeletal muscle following β -alanine supplementation
325 could scavenge these products. Our analysis of the skeletal muscle biopsies showed that at baseline,
326 the skeletal muscle had detectable levels of carnosine-aldehyde conjugates, carnosine-HNE (m/z
327 385; Fig. 5 A, B), carnosine-propanal (m/z 283; Fig. 5 C, D), and carnosine-propanol (m/z 285; Fig. 5
328 E, F), identified on the basis of their retention time, MRM, and fragmentation pattern.

329 When we analyzed the skeletal muscle, biopsies collected after 6 weeks of HIIT, we found
330 that carnosine-HNE levels were significantly higher (~58%) in the β -alanine-fed group than in the
331 placebo-fed group (β -alanine: 480 ± 214 vs placebo: 303 ± 178 fmols/mg tissue, $p<0.05$; Fig. 6 A, B).
332 The levels of the aldehyde conjugates in the β -alanine-fed group after 9 weeks of β -alanine feeding
333 and exercise training were increased ~104% compared with their baseline (480 ± 214 vs 236 ± 94
334 fmols/mg tissue; $p<0.0009$) and ~48% compared with the values recorded at CPET (325 ± 146
335 fmols/mg tissue; $p<0.04$; Fig. 6 A, B). Similarly, the generation of carnosine-propanal after 6 weeks of
336 HIIT was significantly increased in the β -alanine-fed group (~119%) compared with the placebo-fed
337 group (β -alanine; 980 ± 445 fmols/mg tissue vs placebo: 446 ± 114 fmols/mg tissue; $p<0.005$; Fig. 6
338 E, F). The levels of carnosine-propanal in the β -alanine-fed group after 9 weeks of feeding increased
339 ~88% compared with the baseline (521 ± 224 fmols/mg tissue; $p<0.001$) and ~68% compared with the

340 CPET (580 ± 130 fmols/mg tissue; $p < 0.007$; Fig. 6 E, F). No changes in these conjugates were
341 observed following the first session of HIIT (Fig. 6 C, D, G, and H).

342 We had previously reported that carnosine-propanal is enzymatically reduced by aldose reductase
343 (AR) to carnosine-propanol, and, in humans, carnosine-propanol is the most abundant carnosine-
344 aldehyde conjugate extruded in urine (7). In agreement with these results, we found that carnosine-
345 propanol was the most abundant carnosine-aldehyde conjugate (10.90 ± 4.02 pmols/mg tissue)
346 present in the human skeletal muscle. The levels of carnosine-propanol after 6 weeks of HIIT in the
347 β -alanine fed group increased ~86% compared with the placebo-fed group (β -alanine: 17.62 ± 8.74
348 pmols/mg tissue vs placebo: 9.48 ± 3.39 pmols/mg tissue; $p < 0.001$ Fig. 6 I, J). After 9 weeks, in the
349 β -alanine-fed group, the levels of carnosine-propanol conjugates were ~43% higher than their
350 baseline concentration (12.30 ± 6.90 pmols/mg tissue; $p < 0.06$) and ~40% compared with the CPET
351 (12.49 ± 6.09 pmols/mg tissue; $p < 0.06$ Fig. 6 I, J) respectively. No changes in these aldehyde
352 conjugates was observed after a single session of HIIT (Fig. 6 K, L).

353

354 Discussion

355 The major findings of this study are that low intensity endurance exercise training increases
356 carnosine levels, which were decreased after high intensity interval training, and that the magnitude of
357 this increase is augmented by β -alanine supplementation. The study also reports, for the first time that
358 significant levels of anserine are present in human skeletal muscle, and that like carnosine, the levels
359 of anserine also increase upon β -alanine supplementation. This increase in histidyl dipeptides levels
360 was accompanied by a parallel increase in their synthetic enzyme—ATPGD1, without significant
361 changes in the abundance of PHT1 and CNDP2 or in MHC1 and IIa, suggesting that changes in the
362 levels of histidyl dipeptides upon exercise and β -alanine supplementation could not be attributed
363 either to changes in skeletal muscle transport of the peptides and their precursors or to changes in
364 fiber type composition of the muscle. Importantly, we found that the increase in carnosine upon β -

365 alanine feeding and exercise led to a corresponding increase in several conjugates of carnosine with
366 the toxic products of lipid peroxidation such as HNE and acrolein. Taken together, these findings
367 support the notion that carnosine levels and ATPGD1 expression are altered during different phases
368 of exercise training and β -alanine feeding increases carnosine levels by stimulating its synthesis, and
369 that this increase in carnosine may have a protective role in preventing skeletal muscle damage due
370 to lipid peroxidation products generated as a result of increased oxidative stress during vigorous
371 exercise.

372 Previous studies have reported that the concentration of carnosine in the human skeletal
373 muscles ranges from 5-8 mmol.kg⁻¹ of wet muscle (14, 35, 52) or 20-30 mmol.kg⁻¹ in dry muscle (22,
374 31, 34). In this study, we found that carnosine levels in human skeletal muscle were within the range
375 (6.55±0.44 mmol.kg⁻¹ wet weight) reported by other investigators. The levels of carnosine in skeletal
376 muscle, while high, could be increased further by prolonged exercise. Previous studies suggest that
377 training may augment carnosine levels in the human skeletal muscle (38, 44, 52, 69). Our analysis of
378 the skeletal muscle showed that the carnosine levels were increased ~24% after CPET, which were
379 decreased following a single session and after 6 weeks of HIIT. In the β -alanine-fed group, carnosine
380 levels in the skeletal muscle were increased by ~40% after 3 weeks and ~140% after 9 weeks of β -
381 alanine supplementation, which are comparable with previous reports showing that carnosine
382 concentration in the skeletal muscle increased by ~40% after 4 weeks and by ~80% after 10 weeks of
383 β -alanine feeding either in combination with training or alone (23, 34, 42). Collectively these results
384 indicate that β -alanine supplementation is required to increase carnosine levels during the long-term
385 and stimulus from low endurance training and HIIT could alter carnosine levels, in the skeletal muscle.

386 Several mechanisms could account for the increase in carnosine levels in the skeletal
387 muscle after exercise. Carnosine levels in the skeletal muscle could increase due to greater uptake of
388 its precursors – β -alanine and histidine, or by the uptake of carnosine in the plasma into the skeletal
389 muscle. Because different fiber types contain different levels of carnosine(59), carnosine levels in the

390 muscle could also change due to a change in fiber type. However, our results showed that exercise
391 training, at least, under the protocol used for this study, did not affect peptidyl transporters such as
392 PHT1, hydrolases CNP2, nor did it affect the levels of MHCI or MHCIIa, suggesting that neither an
393 increase in transport and hydrolysis, nor a change in fiber type can account for the increase in
394 carnosine levels after exercise. In contrast, our results showing that elevated carnosine levels in the
395 skeletal muscle were accompanied by a greater abundance of its synthetic enzyme – ATPGD1 in both
396 the placebo and β -alanine fed groups, provides circumstantial evidence that exercise increases
397 carnosine levels in skeletal muscle by stimulating its synthesis, rather than its transport or uptake.

398 Although we did not see an increase in the abundance of peptide transporter proteins in the
399 skeletal muscle, it is possible that the carnosine levels in the skeletal muscle could be elevated due to
400 greater supply of carnosine in the plasma, potentially due to increased consumption of carnosine
401 containing foods such as red meat. This seems unlikely because the participants of our study were
402 requested not to change their diet. Nonetheless, even if the participants did not comply with our
403 instructions, the magnitude of the increase observed in our study could not be accounted by
404 increased consumption of red meat alone. The average meat consumed by Australians is ~ 280 g per
405 day and the carnosine concentration in red meat is ~ 450mg /100 g (23, 54), suggesting that the
406 average intake of carnosine per day is ~1.3 g, which is 4-5 fold less than that needed to increase
407 carnosine levels in the skeletal muscle within 21 days as seen in our study. Thus, an increase in
408 carnosine levels in the skeletal muscle most likely appears to be due to an increase in ATPGD1
409 expression. That both carnosine and ATPGD1 levels were decreased in tandem after 6 weeks of HIIT,
410 further reinforces the apparently close relationship between carnosine levels and ATPGD1 abundance
411 in the skeletal muscle. We did not pursue to measure ATPGD1 expression after 6 weeks of HIIT in
412 the β -alanine-fed group, because carnosine levels remained significantly elevated in this group due to
413 β -alanine feeding. However, despite this finding, the mechanisms by which exercise increases the
414 expression of ATPGD1 were not elucidated in the present study. Because the ATPGD1 expression

415 was increased in both the placebo and β -alanine fed humans, it is tempting to speculate that the
416 enzyme could be regulated by transcription factors that respond to exercise. However, this increase
417 in ATPGD1 was not sustained, and the abundance of the protein decreased after a single session and
418 6 weeks of HIIT in the placebo fed group. One possible explanation for the decrease in ATPGD1
419 protein levels could be that the protein degradation pathways such as autophagy or ubiquitin-
420 proteasome are activated following HIIT. Previous studies using animal models have provided
421 evidence that a single intense bout of forced treadmill or endurance training activates autophagy in
422 both the skeletal and cardiac muscle (33) (17). Similarly, it has been shown that in healthy, trained
423 humans, a single session of maximal eccentric resistance exercise induces autophagy and decreases
424 the expression of actin crosslinking protein filamin C gamma in the skeletal muscle (74). Collectively
425 our studies indicate that the changes in carnosine levels during different phases of exercise training
426 mirrors the ATPGD1 expression suggesting that this enzyme is one of the important regulators to
427 maintain carnosine levels.

428 The lack of increase in carnosine levels after 6 weeks of HIIT in our study is in contrast to a
429 recent report by Painelli *et.al.*(58) showing that the levels of carnosine are increased in the *vastus*
430 *lateralis* muscle of vegetarians after 12 weeks of HIIT, accompanied by a decrease in MHCI and an
431 increase in MHC IIa, with no change in ATPGD1 gene expression. In contrast to these findings, we
432 found that the expression of MHCI and IIa fibers remained unaltered after CPET, suggesting that the
433 increase in carnosine levels and ATPGD1 expression after CPET is independent of shifts in fiber type.
434 The reasons for these divergent results are not clear. However, this apparent disparity may be related
435 to differences in HIIT duration between the two studies. Our study was limited to 6 weeks, while in the
436 Painelli; study, a 12 week HIIT was used. Thus, a longer duration of exercise training, which led to
437 changes in fiber type could lead to a secondary, fiber-type dependent, increase in carnosine,
438 unrelated to changes in ATPGD1 levels. Despite these differences, both the studies indicate that the
439 carnosine responds to the stimulus induced by exercise training, but further work is required to

440 delineate the mechanisms that lead to an increase and decrease in the carnosine levels during early
441 and late phases of adaptation to exercise respectively.

442 In addition to carnosine, skeletal muscle of different animals shows a wide variety of histidyl
443 dipeptides such as carbinine, homocarnosine, anserine, ophidine, and acetylcarnosine (14).
444 Reasons for such diversity of peptides remains unclear. Nonetheless, previous work has shown that
445 anserine, which is the methylated analogue of carnosine is the predominant histidyl dipeptide
446 present in the avian skeletal muscle (14), whereas in human skeletal muscle, carnosine is believed to
447 be the sole histidyl dipeptide (20, 46). However, our LC/MS/MS analysis showed that in addition to
448 high levels of carnosine, anserine could also be detected in human skeletal muscle at a concentration
449 of $0.106 \pm 0.003 \text{ mmol.kg}^{-1}$ wet muscle, which accounts for approximately 2% of the total histidyl
450 dipeptide content of the muscle. This is consistent with other mammalian species, nearly all of which
451 synthesize anserine, in addition to carnosine. However, anserine had not been detected in previous
452 study with human muscle, which may be due to the lack of a sensitive and selective methods of
453 detection(46). The LC/MS/MS method used in the present study was sensitive enough for the
454 quantification of 10 fmol of anserine in the skeletal muscle and it also provided unambiguous
455 structural identification of the dipeptide, which is not possible with the previous measurement
456 techniques such as HPLC attached with UV detector. In this regard, it is important to point out that a
457 catalytically active analog of the avian carnosine N-methyltransferase - UPF0586 is expressed in
458 humans (26), which further supports the possibility that humans can synthesize anserine from
459 carnosine.

460 We found not only that anserine was present in human skeletal muscle, but that its levels
461 were increased (to $0.122 \pm 0.024 \text{ mmol.kg}^{-1}$) after β -alanine supplementation combined with the
462 exercise training. Because anserine is synthesized by the methylation of carnosine (catalyzed by
463 carnosine-N-methyltransferase) (25), it is not surprising that the levels of both these peptides change
464 in parallel. Moreover, even though the magnitude of relative increase in anserine after 9 weeks of β -

465 alanine feeding was lower (~20%) than the increase in carnosine (~130%), the anserine levels were
466 significantly higher compared with the placebo-fed group. Because anserine is not rapidly hydrolyzed
467 by serum carnosinase (11, 53), its increase might provide benefits not provided by an increase in
468 carnosine. That supplementation of anserine/carnosine (3:1) to elderly people preserves verbal
469 episodic memory (41) , suggests that it may have potent salutary effects in humans. However,
470 additional studies are warranted to test whether anserine could be more beneficial than carnosine,
471 whose effectiveness may be limited by serum carnosinase (71).

472 A major finding of our study is that the increase in carnosine levels in the skeletal muscle
473 was accompanied by elevated levels of several conjugates of carnosine with lipid peroxidation
474 products such as acrolein and HNE. Previous studies have shown that strenuous exercise increases
475 the production of reactive oxygen species (ROS), which lead to increased peroxidation of unsaturated
476 fatty acids in the membrane (4) (5, 24, 70). Peroxidation of membrane lipids results in the formation
477 of stable end-products such as HNE and acrolein, which are believed to be toxic second messengers
478 that amplify and prolong tissue damage under oxidative stress (49, 60, 65). That the production and
479 accumulation of lipid peroxidation products is increased in skeletal muscle after exercise is supported
480 by the observation that protein-HNE adducts accumulate in the skeletal muscle during strenuous
481 exercise (4, 5, 75). By themselves, lipid peroxidation products such as HNE are highly toxic, and
482 therefore to minimize their toxicity and protein-modification reactions, most tissue metabolize HNE
483 and related aldehyde via several enzymatic pathways catalyzed by aldehyde dehydrogenases
484 (ALDHs) and aldo-keto reductases (AKRs) (62-64). When overexpressed in skeletal muscle ALDH2
485 (32, 61, 72) has been reported to stimulate the removal of HNE during an exercise challenge (75),
486 suggesting that this pathway is indeed an important mode of eliminating HNE and related aldehydes.
487 Nevertheless, in addition to enzymatic detoxification, reactive aldehydes such as HNE are also
488 removed by carnosine and anserine (1, 2, 15, 35). Therefore, our studies showing that the oral intake
489 of β -alanine and HIIT increased the abundance of carnosine-HNE, carnosine-propanal, and

490 carnosine-propanol conjugates supports the hypothesis that lipid peroxidation products could
491 accumulate in the human skeletal muscle during intense exercise and that carnosine could facilitate
492 their detoxification by forming covalent adducts with the aldehydes. This is further supported by data
493 published by Carvalho *et.al* showing that HIIT and β -alanine supplementation increases the levels of
494 carnosine-acrolein conjugates (m/z 303) in skeletal muscle (16). Reactive aldehydes such as HNE
495 and acrolein are generated by the oxidation of polyunsaturated fatty acids, whereas acrolein could
496 also be generated from the oxidation of amino acids by myeloperoxidase (3), metabolism of biogenic
497 amines, (73), tobacco smoke, diesel exhaust, and several foods (67). Hence, our results showing that
498 exercise training increases carnosine-propanal and carnosine-propanol suggests that in addition to
499 lipid peroxidation, inflammation and biogenic amine metabolism could also contribute to the
500 generation of these conjugates during exercise training. We had previously reported that the
501 carnosine-propanal conjugate is metabolized to carnosine-propanol by a reductive reaction catalyzed
502 by aldose reductase. In humans, carnosine-propanol is the predominant form of the carnosine-
503 aldehyde conjugate that is excreted out in urine. Therefore, our present data showing that carnosine-
504 propanol is the predominant conjugate in the skeletal muscle further extends the concept that
505 reduction of carnosine-aldehyde conjugates by aldose reductase is an important step in the metabolic
506 transformation of these conjugates (7). Taken together, our observations and reports from other
507 laboratories suggest that β -alanine feeding enhances the generation of carnosine-aldehyde
508 conjugates and thus enhancing carnosine levels in the skeletal muscle could potentially ameliorate
509 the toxic effects of reactive aldehydes during strenuous exercise.

510 In conclusion, the results of this study suggest that carnosine levels and ATPGD1
511 expression are altered during different phases of exercise training and ATPGD1 could regulate
512 carnosine homeostasis in the human skeletal during exercise. These findings demonstrate and
513 confirm the efficacy of β -alanine in enhancing carnosine and reveals its potential to act as a precursor
514 for anserine during long-term exercise trainings. Our finding that β -alanine feeding and exercise

515 training increase the levels of carnosine-aldehyde conjugates in the skeletal muscle suggests that β -
516 alanine supplementation, in addition to its potential effects on exercise capacity, might be an effective
517 strategy to diminish the aldehyde-induced toxicity in the skeletal muscle during strenuous exercise.

518

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526 **Disclosures**

527 None.

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742 **Figure Legends**

743 **Fig.1 Experimental design.** Twenty physically-active individuals (n=20) were familiarized with all
744 exercise testing followed by the assessment of baseline characteristics, these included the lactate
745 threshold (LT), peak oxygen consumption (VO₂ peak), maximal power (W_{max}) and a cycling capacity

746 test at 110% W_{max} (CCT 110%). Participants then underwent a 2 week conditioning phase (1 h at 70%
747 LT, 3 times per week), followed by a repeat of all baseline exercise testing plus a 30-km cycling time
748 trial (30-Km TT), in week 3. Participants then began high-intensity interval training (HIIT, 2:1 min work
749 rest ratio at 40% difference between LT and W_{max}) at the start of week 4. From weeks 4 to 9
750 participants completed HIIT 3 times per week for a total of 17 sessions. Muscle biopsies were
751 collected at baseline, at rest after the 2 week conditioning phase followed by 1 week exercise testing,
752 immediately following the first session of HIIT (at the start of week 4), and after 6 weeks of HIIT.

753 **Fig. 2 Carnosine levels are altered in the skeletal muscle during different phases of training in**
754 **the placebo fed group and enhanced in the β -alanine group.** Representative single ion
755 chromatogram of (A) carnosine in the skeletal muscle of one participant involved in the study (i) inset
756 shows the fragmentation pattern of carnosine in the skeletal muscle (B) representative fragmentation
757 pattern of *ex-vivo* carnosine standard. Individual levels of carnosine (C) at baseline, after CPET (3)
758 and after 6 weeks of HIIT in the β -alanine and placebo-fed groups (9). (D) Individual carnosine levels
759 after CPET (3), followed by a single session of high intensity interval training (3') in the β -alanine and
760 placebo-fed groups. (E, F) Absolute values of carnosine in the placebo and β -alanine-fed groups; data
761 is presented as mean \pm SD, n=10 subjects in each group. * p <0.01 vs baseline (placebo), $^{\delta}$ p <0.01 vs
762 baseline (β -alanine), $^{\#}$ p <0.05 vs 9 weeks of placebo, $^{\$}$ p <0.01 vs 3 weeks of β -alanine, $^+$ p <0.001 vs 3'
763 weeks of placebo-fed group.

764 **Fig. 3 Carnosine synthase (ATPGD1) expression is enhanced after 2 weeks conditioning phase**
765 **followed by 1 week of exercise testing (CPET) and decreased after high intensity interval**
766 **training (HIIT).** Representative Western blots of ATPGD1, proton/histidine transporter (PHT1), and
767 carnosinase (CNDP2), in the skeletal muscle biopsies obtained from the (A) placebo and (D) β -
768 alanine-fed groups at baseline, after CPET and followed by a single session of HIIT (3' HIIT). Bands
769 normalized to amido-black (AB) and (B, E) data are presented as mean \pm SD, n=4 samples in each
770 group. (C) ATPGD1 expression in biopsies collected from placebo-fed group after CPET and after 6

771 weeks of HIIT. Bands were normalized to GAPDH and lower panel shows the quantitative analysis of
772 ATPGD1. Data are presented as mean \pm SD, n = 4-8 samples in each group. (F) Western blots of
773 MHC I and II at baseline and after CPET from placebo-fed group. Lower panel shows the quantitative
774 analysis of MHCI and II normalized to GAPDH. Data are mean \pm SD, n=4-8 in each group, * $p < 0.05$ vs
775 baseline and, # $p < 0.05$ vs CPET.

776 **Fig. 4 β -alanine feeding combined with exercise enhances anserine levels in the human**
777 **skeletal muscle.** Representative single ion chromatogram of (A) anserine in the skeletal muscle of
778 one participant involved in the study, (i) inset shows the fragmentation pattern of anserine present in
779 skeletal muscle. (B) Representative fragmentation pattern of the *ex-vivo* anserine standard. Individual
780 anserine levels (C) at baseline, after the 2 week conditioning phase followed by 1 week of exercise
781 testing (CPET) (3) and after 6 weeks of high intensity interval training (HIIT) in the β -alanine- and
782 placebo-fed groups (9). (D) Individual anserine levels after CPET (3) followed by a single session of
783 HIIT (3'). (E, F) Mean \pm SD of anserine in the placebo- and β -alanine-fed groups. N=10 participants in
784 each group, * $p < 0.01$ vs pre-supplementation (placebo), # $p < 0.05$ vs 9-weeks of placebo
785 supplementation, \$ $p < 0.05$ vs 3-weeks of β -alanine supplementation.

786 **Fig.5 Tandem mass spectra (MS/MS) of carnosine-aldehyde conjugates detected in the human**
787 **skeletal muscle and ex-vivo.** Representative single ion chromatogram from one participant involved
788 in the study and fragmentation pattern of conjugates synthesized *in-vitro* (A, B) carnosine-HNE (C, D)
789 carnosine-propanal and (E, F) carnosine-propanol.

790 **Fig. 6 Carnosine-aldehyde conjugates are enhanced by β -alanine feeding.** Individual levels and
791 absolute values of (A, B) carnosine-HNE (m/z 383), (E, F) carnosine-propanal (m/z 283), and (I, J)
792 carnosine-propanol (m/z 285) detected in the human skeletal muscle by LC/MS/MS at baseline, after
793 2 weeks of conditioning phase followed by 1 week of exercise testing (CPET) (3), and after 6 weeks
794 of HIIT in the placebo and β -alanine fed groups (9). Individual and absolute values of (C, D)
795 carnosine-HNE, (G, H) carnosine-propanal and (K, L) carnosine-propanol after CPET (3), followed by

796 a single session of HIIT (3'). Data are presented as mean±S.D, n=10 subjects in each group, * $p<0.05$
797 vs baseline (β -alanine-fed group), # $p<0.05$ vs 9-weeks of placebo feeding, # $p<0.05$ vs 3-weeks of β -
798 alanine feeding.

799 **Table. 1 Participant characteristic**

800 Characteristic of the participants (n=20), age (y), mass (kg), peak oxygen consumption (VO_2 peak),
801 lactate threshold (LT) and maximal power (W_{max}).

802

Fig.1

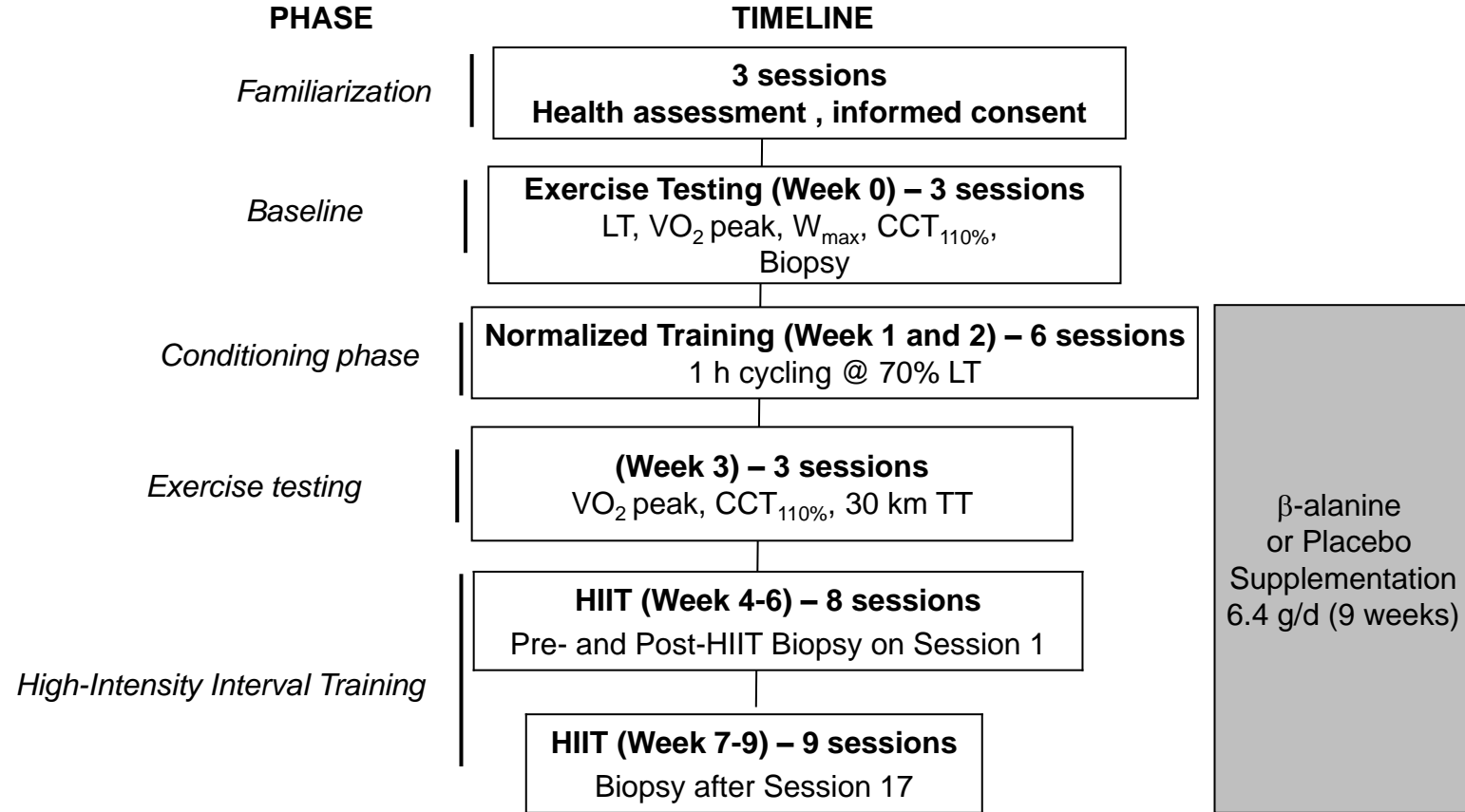


Fig.2

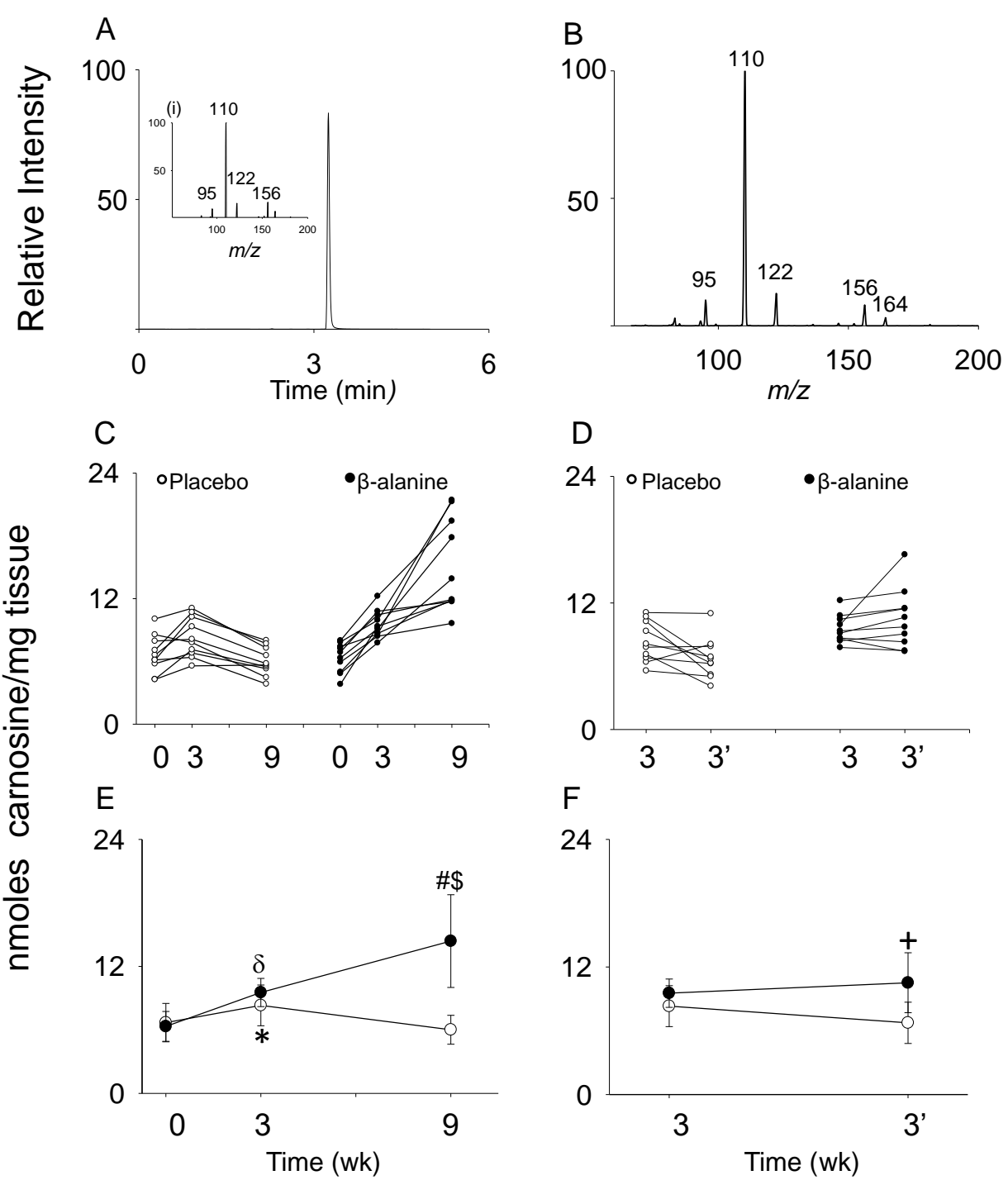


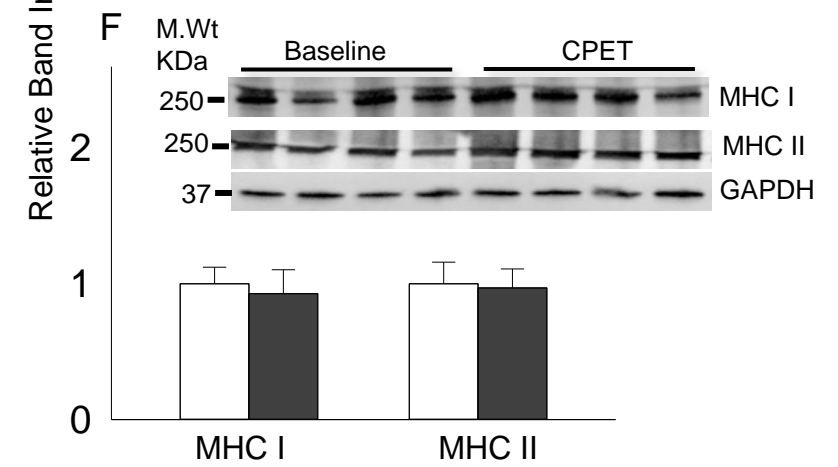
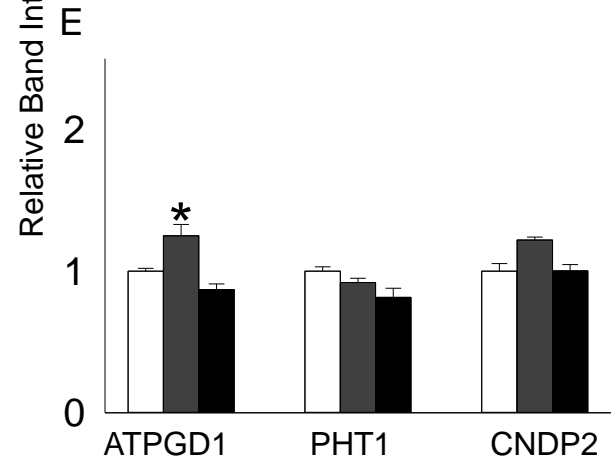
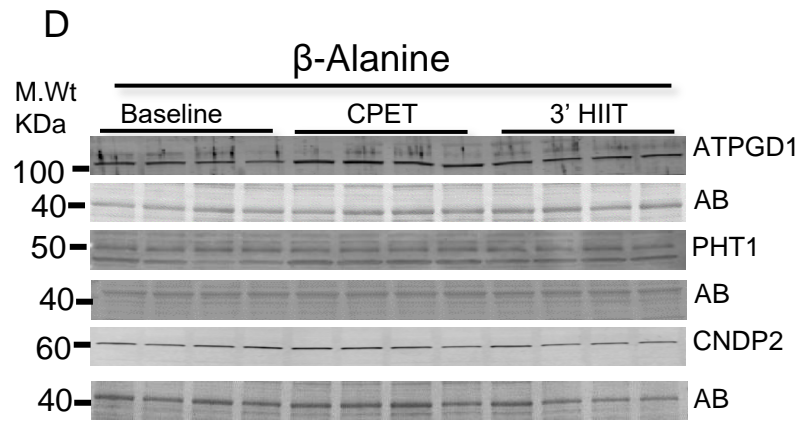
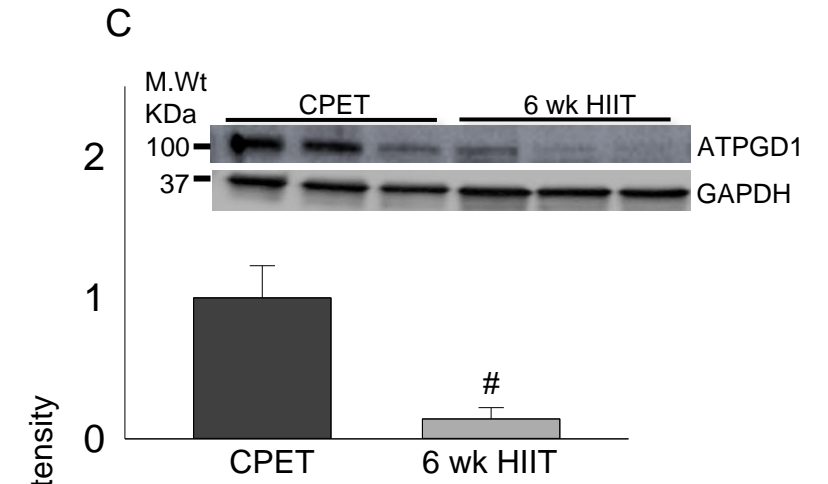
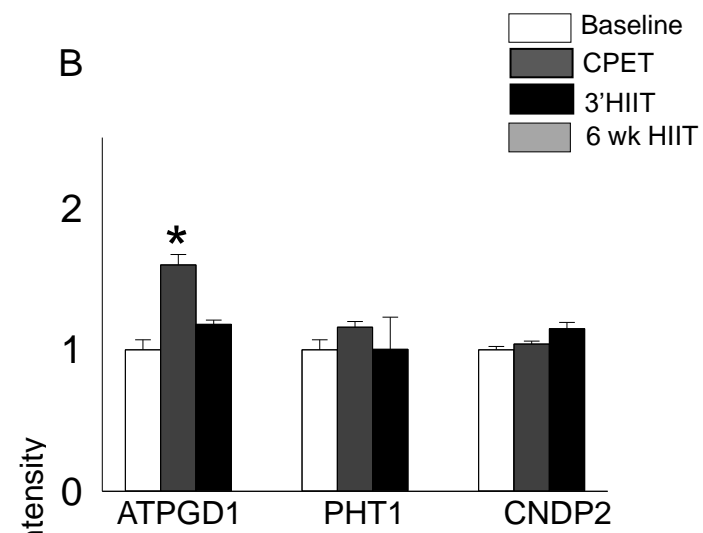
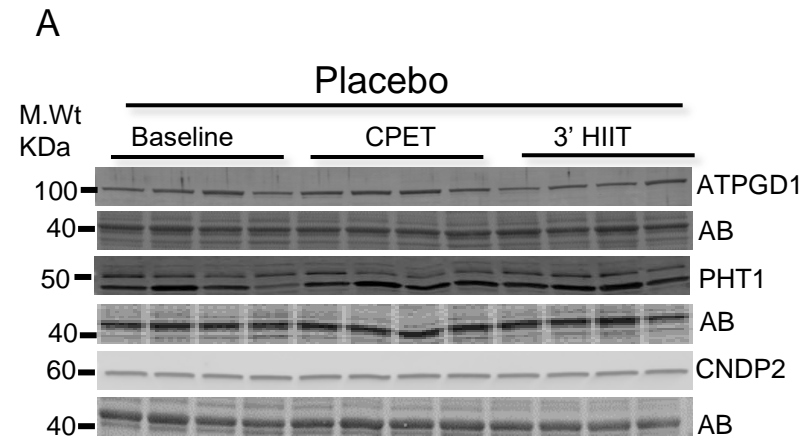
Fig.3

Fig.4

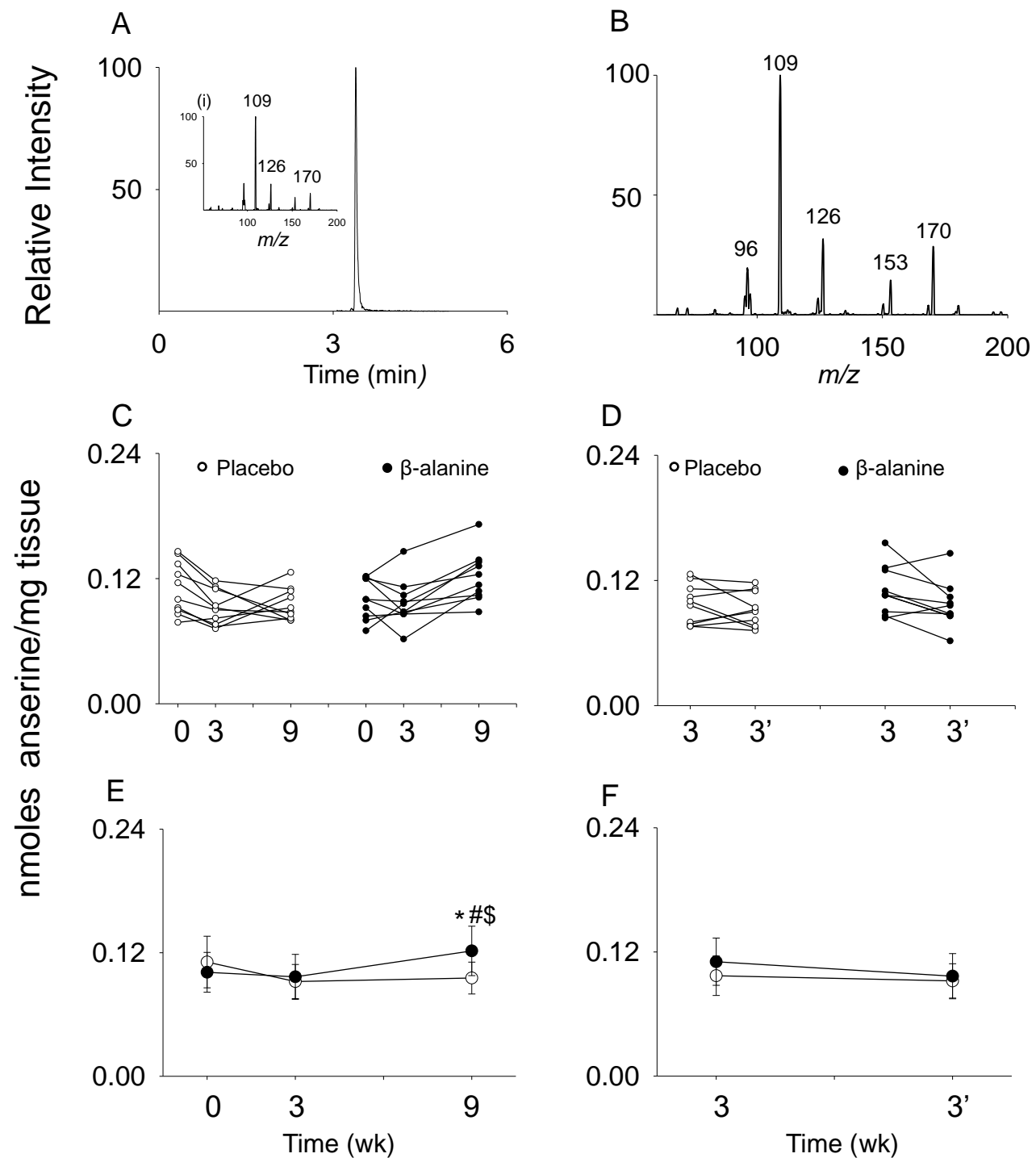


Fig.5

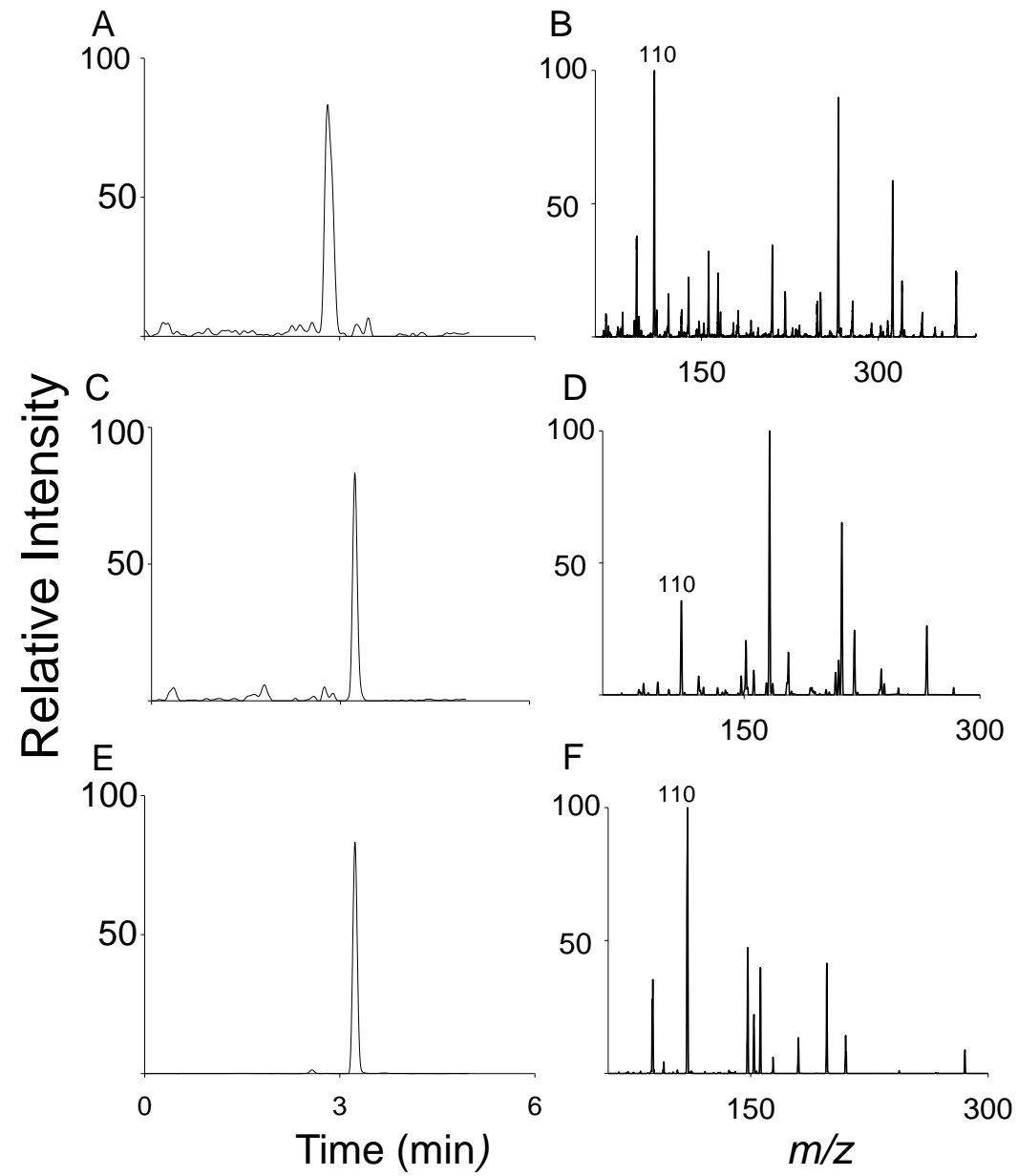


Table 1

Characteristic	Group	
	Placebo (n = 10)	β -alanine (n=10)
Age (y)	25.3 \pm 7.7	29.4 \pm 7.0
Mass (kg)	78.7 \pm 10.8	71.4 \pm 10.5
VO ₂ peak (mL.min ⁻¹ .kg ⁻¹)	53.1 \pm 7.9	53.1 \pm 9.0
Lactate threshold (LT)	201 \pm 50	204 \pm 59
W _{max} (W)	285 \pm 49	282 \pm 65