Exercise alters and beta-alanine combined with exercise augments histidyl dipeptide levels and scavenges lipid peroxidation products in human skeletal muscle

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

*David Hoetker[1,2], *Weiliang Chung[3,4], Deqing Zhang[1,2], Jingjing Zhao[1,2], Virginia K Schmidtke[1,2], Daniel W. Riggs [1,2], Wim Derave[3], Aruni Bhatnagar[1,2], David Bishop[4,5], Shahid P. Baba[1,2],

[1]Diabetes and Obesity Center and [2]Envirome Institute, Department of Medicine, University of Louisville, Louisville, Kentucky. USA

[3]Department of Movement and Sports Sciences, University of Ghent Belgium.

[4]Institute of Health and Sport, Exercise and Active Living, College of Sports and Exercise Sciences, Victoria University, Australia and School of Medical and Health Sciences, Edith Cowan University, Joondahp, Australia.

Address Correspondence to: Shahid P. Baba, Ph.D.
Diabetes and Obesity Center
Envirome Institute
Department of Medicine
580 South Preston Street
Delia Baxter Building, Room 421A
University of Louisville
Louisville, KY 40202
Phone: 502-852-4274
Fax: 502-852-3663
Email: spbaba01@louisville.edu

Keywords: acrolein, carnosine, exercise, 4-hydroxy-*trans-2*-nonenal

* Authors contributed equally to the manuscript

Abstract

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

New and Noteworthy

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

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

Introduction

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

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

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

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

Materials and Methods

Participant's recruitment and characteristics.

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

Study overview

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

Familiarization phase.

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

Baseline Exercise testing

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

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

Conditioning phase and exercise testing (CPET)

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

High intensity interval training (HIIT)

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

Physical activity and nutritional control

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

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

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

Participants visited the exercise laboratory to perform an incremental cycling protocol to exhaustion for the purpose of determining their LT, W_{max}, and $VO_{2\ peak}$. The test consisted of 4-min stages, separated by 30-s of rest, until voluntary exhaustion or the inability to maintain a minimum pedal cadence of 60 rpm. The participants began cycling at 60 W with an increase of 30 W for each subsequent stage. Maximal power was determined using the following equation: W_{max} = Workload + (t/240) x 30 (where t-time completed during the final stage) (37). Venous blood samples were taken at the end of each stage *via* intravenous cannulation and analyzed using an automated cartridge-based gas analyzer (Radiometer, Copenhagen, Denmark). The LT was calculated using the modified D_{max} method (13) . Expired gases were analyzed every 15-s using a metabolic cart (Moxus Metabolic System, AEI Technologies, USA), which was calibrated using known gas concentrations prior to each test (20.93% O_2, 0.04% CO_2 and 16.10% O_2, 4.17% CO_2; BOC Gases, Australia). Oxygen consumption was recorded every 15-s and the two-highest consecutive 15-s values recorded during the test were averaged and recorded as the participant's $VO_{2\ peak}$.

Cycling Capacity Test

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

Muscle biopsies

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

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

Preparation of carnosine-aldehyde conjugates

Authentic standards for carnosine (*m/z 227)*, anserine *(m/z 241)* and tyrosine-histidine *(m/z 319)* were used to determine the multiple reaction monitoring transitions (MRMs). Carnosine-aldehyde conjugates used as standards were prepared as described previously (7). Briefly, acrolein was synthesized by acid hydrolysis of diethyl acetal acrolein (Sigma) in HCl (0.1 M) which was kept at room temperature (RT) for 30 min. The carnosine-propanal conjugate was synthesized by incubating 100 mM acrolein (10 µL) with 10 mM carnosine (990 µL) in water at RT for 2 h. Carnosine-propanol conjugate was synthesized by incubating 10 mM $NaBH_4$ (10 µL) in water with carnosine-propanal (500 µL) at RT. Carnosine-HNE conjugate was synthesized by incubating HNE (70 mM; Calbiochem) with 10 mM carnosine in water (10:1) at RT. The dipeptides and carnosine-aldehyde conjugates were individually infused into a stream of 0.55 mL/min 50:50 A: B UPLC solvent going into a Waters (Milford, MA) Xevo TQ-S micro triple quadrupole mass spectrometer. An automated optimization program IntelliStart (Waters, Milford, MA) was used to determine the optimal ionization voltages, collision energies and product ions. Maximum ionization conditions and optimal daughter ions were used to program sensitive MRMs.

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

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

were vortexed thoroughly to precipitate proteins, kept on ice for 15 min, and then centrifuged at 16,000 *x g* for 10 min at 4˚C. The supernatants were stored at -20˚C for further processing. Prior to injection into TQ-S micro mass spectrometer in positive mode, the samples were diluted in 75% acetonitrile: 25% water. Dipeptides and their aldehyde conjugates were separated and identified by using Waters ACQUITY UPLC H-Class System coupled with a Xevo TQ-S micro triple quadrupole MS. The analytes were separated by a Waters Acquity BEH HILIC column (1.7µm, 2.1x50 mm) equipped with an in-line frit filter unit. The analytes were eluted by using a binary solvent system consisting of 10 mM ammonium formate, 0.125% formic acid in 50% acetonitrile: 50% water for mobile phase A and 10 mM ammonium formate 0.125% formic acid in 95% acetonitrile: 5% water for 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: 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 held from 5.5-8 min to equilibrate the column for the next injection. Dipeptides were quantified using the LC/MS calibration curve of relative area of carnosine and anserine to tyrosine-histidine (IS), and their aldehyde conjugates were quantified using the peak ratio of histidyl-dipeptide and tyrosine-histidine (IS) and expressed as mole/mg wet weight. For carnosine *m/z 227→110*, anserine *m/z 241→109,* carnosine-HNE m*/z 383→110*, carnosine-propanal *m/z 283→110,* carnosine-propanol *m/z 285→110* and tyrosine-histidine *m/z* 319*→110,* MRM transitions were followed. We recorded the abundance of at least four confirmation transitions for each molecule.

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

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

Western blot analysis

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

Statistical Analyses

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

Results

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

muscle could also change due to a change in fiber type. However, our results showed that exercise training, at least, under the protocol used for this study, did not affect peptidyl transporters such as PHT1, hydrolases CNDP2, nor did it affect the levels of MHCI or MHCIIa, suggesting that neither an increase in transport and hydrolysis, nor a change in fiber type can account for the increase in carnosine levels after exercise. In contrast, our results showing that elevated carnosine levels in the skeletal muscle were accompanied by a greater abundance of its synthetic enzyme – ATPGD1 in both the placebo and β-alanine fed groups, provides circumstantial evidence that exercise increases carnosine levels in skeletal muscle by stimulating its synthesis, rather than its transport or uptake.

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

We would like to thank Jay Rai for assistance with statistical analysis, Natural Alternatives International for providing β-alanine and Bioanalytical Core in Diabetes and Obesity Center for biochemical analysis.

Funding Sources

This work was supported by the National Institutes of Health, R01HL122581-01 (SPB), R01HL55477 and GM103492 (AB).

Disclosures

None.

References

1. **Aldini G, Carini M, Beretta G, Bradamante S, and Facino RM**. Carnosine is a quencher of 4-hydroxy-nonenal: through what mechanism of reaction? *Biochem Biophys Res Commun* 298: 699-706, 2002.
2. **Aldini G, Orioli M, Rossoni G, Savi F, Braidotti P, Vistoli G, Yeum KJ, Negrisoli G, and Carini M**. The carbonyl scavenger carnosine ameliorates dyslipidaemia and renal function in Zucker obese rats. *J Cell Mol Med* 15: 1339-1354, 2011.
3. **Anderson MM, Hazen SL, Hsu FF, and Heinecke JW**. Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanal, and acrolein. A mechanism for the generation of highly reactive alpha-hydroxy and alpha,beta-unsaturated aldehydes by phagocytes at sites of inflammation. *J Clin Invest* 99: 424-432, 1997.
4. **Aoi W, Naito Y, Sakuma K, Kuchide M, Tokuda H, Maoka T, Toyokuni S, Oka S, Yasuhara M, and Yoshikawa T**. Astaxanthin limits exercise-induced skeletal and cardiac muscle damage in mice. *Antioxid Redox Signal* 5: 139-144, 2003.
5. **Aoi W, Naito Y, Tokuda H, Tanimura Y, Oya-Ito T, and Yoshikawa T**. Exercise-induced muscle damage impairs insulin signaling pathway associated with IRS-1 oxidative modification. *Physiol Res* 61: 81-88, 2012.
6. **Baba SP, Hellmann J, Srivastava S, and Bhatnagar A**. Aldose reductase (AKR1B3) regulates the accumulation of advanced glycosylation end products (AGEs) and the expression of AGE receptor (RAGE). *Chem Biol Interact* 191: 357-363, 2011.

7. **Baba SP, Hoetker JD, Merchant M, Klein JB, Cai J, Barski OA, Conklin DJ, and Bhatnagar A**. Role of aldose reductase in the metabolism and detoxification of carnosine-acrolein conjugates. *J Biol Chem* 288: 28163-28179, 2013.
8. **Baguet A, Everaert I, De Naeyer H, Reyngoudt H, Stegen S, Beeckman S, Achten E, Vanhee L, Volkaert A, Petrovic M, Taes Y, and Derave W**. Effects of sprint training combined with vegetarian or mixed diet on muscle carnosine content and buffering capacity. *Eur J Appl Physiol* 111: 2571-2580, 2011.
9. **Baguet A, Reyngoudt H, Pottier A, Everaert I, Callens S, Achten E, and Derave W**. Carnosine loading and washout in human skeletal muscles. *J Appl Physiol (1985)* 106: 837-842, 2009.
10. **Barski OA, Xie Z, Baba SP, Sithu SD, Agarwal A, Cai J, Bhatnagar A, and Srivastava S**. Dietary carnosine prevents early atherosclerotic lesion formation in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* 33: 1162-1170, 2013.
11. **Bellia F, Amorini AM, La Mendola D, Vecchio G, Tavazzi B, Giardina B, Di Pietro V, Lazzarino G, and Rizzarelli E**. New glycosidic derivatives of histidine-containing dipeptides with antioxidant properties and resistant to carnosinase activity. *Eur J Med Chem* 43: 373-380, 2008.
12. **Bex T, Chung W, Baguet A, Stegen S, Stautemas J, Achten E, and Derave W**. Muscle carnosine loading by beta-alanine supplementation is more pronounced in trained vs. untrained muscles. *J Appl Physiol (1985)* 116: 204-209, 2014.
13. **Bishop D, Jenkins DG, McEniery M, and Carey MF**. Relationship between plasma lactate parameters and muscle characteristics in female cyclists. *Med Sci Sports Exerc* 32: 1088-1093, 2000.
14. **Boldyrev AA, Aldini G, and Derave W**. Physiology and pathophysiology of carnosine. *Physiol Rev* 93: 1803-1845, 2013.
15. **Carini M, Aldini G, Beretta G, Arlandini E, and Facino RM**. Acrolein-sequestering ability of endogenous dipeptides: characterization of carnosine and homocarnosine/acrolein adducts by electrospray ionization tandem mass spectrometry. *J Mass Spectrom* 38: 996-1006, 2003.
16. **Carvalho VH, Oliveira AHS, de Oliveira LF, da Silva RP, Di Mascio P, Gualano B, Artioli GG, and Medeiros MHG**. Exercise and beta-alanine supplementation on carnosine-acrolein adduct in skeletal muscle. *Redox Biol* 18: 222-228, 2018.
17. **Cecconi F, and Levine B**. The role of autophagy in mammalian development: cell makeover rather than cell death. *Dev Cell* 15: 344-357, 2008.
18. **Ceci R, Duranti G, Sgro P, Sansone M, Guidetti L, Baldari C, Sabatini S, and Di Luigi L**. Effects of tadalafil administration on plasma markers of exercise-induced muscle damage, IL6 and antioxidant status capacity. *Eur J Appl Physiol* 115: 531-539, 2015.
19. **Chesher D**. Evaluating assay precision. *Clin Biochem Rev* 29 Suppl 1: S23-26, 2008.
20. **Christman AA**. Factors Affecting Anserine and Carnosine Levels in Skeletal-Muscles of Various Animals. *International Journal of Biochemistry* 7: 519-527, 1976.
21. **de Courten B, Jakubova M, de Courten MP, Kukurova IJ, Vallova S, Krumpolec P, Valkovic L, Kurdiova T, Garzon D, Barbaresi S, Teede HJ, Derave W, Krssak M, Aldini G, Ukropec J, and Ukropcova B**. Effects of carnosine supplementation on glucose metabolism: Pilot clinical trial. *Obesity (Silver Spring)* 24: 1027-1034, 2016.
22. **Derave W, Everaert I, Beeckman S, and Baguet A**. Muscle carnosine metabolism and beta-alanine supplementation in relation to exercise and training. *Sports Med* 40: 247-263, 2010.
23. **Derave W, Ozdemir MS, Harris RC, Pottier A, Reyngoudt H, Koppo K, Wise JA, and Achten E**. beta-Alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. *J Appl Physiol (1985)* 103: 1736-1743, 2007.

24. **Diaz-Castro J, Guisado R, Kajarabille N, Garcia C, Guisado IM, De Teresa C, and Ochoa JJ**. Phlebodium decumanum is a natural supplement that ameliorates the oxidative stress and inflammatory signalling induced by strenuous exercise in adult humans. *Eur J Appl Physiol* 112: 3119-3128, 2012.
25. **Drozak J, Chrobok L, Poleszak O, Jagielski AK, and Derlacz R**. Molecular identification of carnosine N-methyltransferase as chicken histamine N-methyltransferase-like protein (hnmt-like). *PLoS One* 8: e64805, 2013.
26. **Drozak J, Piecuch M, Poleszak O, Kozlowski P, Chrobok L, Baelde HJ, and de Heer E**. UPF0586 Protein C9orf41 Homolog Is Anserine-producing Methyltransferase. *J Biol Chem* 290: 17190-17205, 2015.
27. **Drozak J, Veiga-da-Cunha M, Vertommen D, Stroobant V, and Van Schaftingen E**. Molecular identification of carnosine synthase as ATP-grasp domain-containing protein 1 (ATPGD1). *J Biol Chem* 285: 9346-9356, 2010.
28. **Eaton P, Li JM, Hearse DJ, and Shattock MJ**. Formation of 4-hydroxy-2-nonenal-modified proteins in ischemic rat heart. *Am J Physiol* 276: H935-943, 1999.
29. **Everaert I, De Naeyer H, Taes Y, and Derave W**. Gene expression of carnosine-related enzymes and transporters in skeletal muscle. *Eur J Appl Physiol* 113: 1169-1179, 2013.
30. **Glass CK, and Witztum JL**. Atherosclerosis. the road ahead. *Cell* 104: 503-516, 2001.
31. **Harris RC, Tallon MJ, Dunnett M, Boobis L, Coakley J, Kim HJ, Fallowfield JL, Hill CA, Sale C, and Wise JA**. The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. *Amino acids* 30: 279-289, 2006.
32. **Hartley DP, Ruth JA, and Petersen DR**. The hepatocellular metabolism of 4-hydroxynonenal by alcohol dehydrogenase, aldehyde dehydrogenase, and glutathione S-transferase. *Arch Biochem Biophys* 316: 197-205, 1995.
33. **He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, An Z, Loh J, Fisher J, Sun Q, Korsmeyer S, Packer M, May HI, Hill JA, Virgin HW, Gilpin C, Xiao G, Bassel-Duby R, Scherer PE, and Levine B**. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature* 481: 511-515, 2012.
34. **Hill CA, Harris RC, Kim HJ, Harris BD, Sale C, Boobis LH, Kim CK, and Wise JA**. Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. *Amino acids* 32: 225-233, 2007.
35. **Hipkiss AR**. Carnosine and its possible roles in nutrition and health. *Adv Food Nutr Res* 57: 87-154, 2009.
36. **Ivanisevic J, Epstein AA, Kurczy ME, Benton PH, Uritboonthai W, Fox HS, Boska MD, Gendelman HE, and Siuzdak G**. Brain region mapping using global metabolomics. *Chem Biol* 21: 1575-1584, 2014.
37. **Jamnick NA, Botella J, Pyne DB, and Bishop DJ**. Manipulating graded exercise test variables affects the validity of the lactate threshold and [Formula: see text]. *PLoS One* 13: e0199794, 2018.
38. **Juel C**. Muscle pH regulation: role of training. *Acta Physiol Scand* 162: 359-366, 1998.
39. **Kamal MA, Jiang H, Hu Y, Keep RF, and Smith DE**. Influence of genetic knockout of Pept2 on the in vivo disposition of endogenous and exogenous carnosine in wild-type and Pept2 null mice. *Am J Physiol Regul Integr Comp Physiol* 296: R986-991, 2009.
40. **Kamal MA, Keep RF, and Smith DE**. Role and relevance of PEPT2 in drug disposition, dynamics, and toxicity. *Drug Metab Pharmacokinet* 23: 236-242, 2008.
41. **Katakura Y, Totsuka M, Imabayashi E, Matsuda H, and Hisatsune T**. Anserine/Carnosine Supplementation Suppresses the Expression of the Inflammatory Chemokine CCL24 in Peripheral Blood Mononuclear Cells from Elderly People. *Nutrients* 9: 2017.
42. **Kendrick IP, Harris RC, Kim HJ, Kim CK, Dang VH, Lam TQ, Bui TT, Smith M, and Wise JA**. The effects of 10 weeks of resistance training combined with beta-alanine

supplementation on whole body strength, force production, muscular endurance and body composition. *Amino acids* 34: 547-554, 2008.
43. **Kendrick IP, Kim HJ, Harris RC, Kim CK, Dang VH, Lam TQ, Bui TT, and Wise JA**. The effect of 4 weeks beta-alanine supplementation and isokinetic training on carnosine concentrations in type I and II human skeletal muscle fibres. *Eur J Appl Physiol* 106: 131-138, 2009.
44. **Kim JH K, Changkeun K, Harris RC, Harris DB, Sale C, Wise JA**. Effect on muscle fibre morphology and carnosine content after 12 days training of korean speed skaters. . *Med Sci Sports Exerc* 37: S-192, 2006.
45. **Lusis AJ**. Atherosclerosis. *Nature* 407: 233-241, 2000.
46. **Mannion AF, Jakeman PM, Dunnett M, Harris RC, and Willan PL**. Carnosine and anserine concentrations in the quadriceps femoris muscle of healthy humans. *Eur J Appl Physiol Occup Physiol* 64: 47-50, 1992.
47. **Mannion AF, Jakeman PM, and Willan PL**. Effects of isokinetic training of the knee extensors on high-intensity exercise performance and skeletal muscle buffering. *Eur J Appl Physiol Occup Physiol* 68: 356-361, 1994.
48. **Margolis FL**. Carnosine in the primary olfactory pathway. *Science* 184: 909-911, 1974.
49. **Marnett LJ**. Lipid peroxidation-DNA damage by malondialdehyde. *Mutat Res* 424: 83-95, 1999.
50. **Musatov A, Carroll CA, Liu YC, Henderson GI, Weintraub ST, and Robinson NC**. Identification of bovine heart cytochrome c oxidase subunits modified by the lipid peroxidation product 4-hydroxy-2-nonenal. *Biochemistry* 41: 8212-8220, 2002.
51. **Orioli M, Aldini G, Benfatto MC, Facino RM, and Carini M**. HNE Michael adducts to histidine and histidine-containing peptides as biomarkers of lipid-derived carbonyl stress in urines: LC-MS/MS profiling in Zucker obese rats. *Anal Chem* 79: 9174-9184, 2007.
52. **Parkhouse WS, McKenzie DC, Hochachka PW, and Ovalle WK**. Buffering capacity of deproteinized human vastus lateralis muscle. *J Appl Physiol (1985)* 58: 14-17, 1985.
53. **Pegova A, Abe H, and Boldyrev A**. Hydrolysis of carnosine and related compounds by mammalian carnosinases. *Comp Biochem Physiol B Biochem Mol Biol* 127: 443-446, 2000.
54. **Purchas RW, Rutherfurd SM, Pearce PD, Vather R, and Wilkinson BH**. Concentrations in beef and lamb of taurine, carnosine, coenzyme Q(10), and creatine. *Meat Sci* 66: 629-637, 2004.
55. **Regazzoni L, de Courten B, Garzon D, Altomare A, Marinello C, Jakubova M, Vallova S, Krumpolec P, Carini M, Ukropec J, Ukropcova B, and Aldini G**. A carnosine intervention study in overweight human volunteers: bioavailability and reactive carbonyl species sequestering effect. *Sci Rep* 6: 27224, 2016.
56. **Rittner HL, Hafner V, Klimiuk PA, Szweda LI, Goronzy JJ, and Weyand CM**. Aldose reductase functions as a detoxification system for lipid peroxidation products in vasculitis. *J Clin Invest* 103: 1007-1013, 1999.
57. **Sale C, Saunders B, and Harris RC**. Effect of beta-alanine supplementation on muscle carnosine concentrations and exercise performance. *Amino acids* 39: 321-333, 2010.
58. **Salles Painelli V, Nemezio KM, Jessica A, Franchi M, Andrade I, Riani LA, Saunders B, Sale C, Harris RC, Gualano B, and Artioli GG**. HIIT Augments Muscle Carnosine in the Absence of Dietary Beta-Alanine Intake. *Med Sci Sports Exerc* 2018.
59. **Sewell DA, Harris RC, Marlin DJ, and Dunnett M**. Estimation of the carnosine content of different fibre types in the middle gluteal muscle of the thoroughbred horse. *J Physiol* 455: 447-453, 1992.
60. **Siems WG, Grune T, and Esterbauer H**. 4-Hydroxynonenal formation during ischemia and reperfusion of rat small intestine. *Life Sci* 57: 785-789, 1995.

61. **Srivastava S, Chandra A, Wang LF, Seifert WE, Jr., DaGue BB, Ansari NH, Srivastava SK, and Bhatnagar A**. Metabolism of the lipid peroxidation product, 4-hydroxy-trans-2-nonenal, in isolated perfused rat heart. *J Biol Chem* 273: 10893-10900, 1998.
62. **Srivastava S, Dixit BL, Cai J, Sharma S, Hurst HE, Bhatnagar A, and Srivastava SK**. Metabolism of lipid peroxidation product, 4-hydroxynonenal (HNE) in rat erythrocytes: role of aldose reductase. *Free Radic Biol Med* 29: 642-651, 2000.
63. **Srivastava S, Vladykovskaya E, Barski OA, Spite M, Kaiserova K, Petrash JM, Chung SS, Hunt G, Dawn B, and Bhatnagar A**. Aldose reductase protects against early atherosclerotic lesion formation in apolipoprotein E-null mice. *Circ Res* 105: 793-802, 2009.
64. **Srivastava S, Watowich SJ, Petrash JM, Srivastava SK, and Bhatnagar A**. Structural and kinetic determinants of aldehyde reduction by aldose reductase. *Biochemistry* 38: 42-54, 1999.
65. **Stadtman ER**. Role of oxidant species in aging. *Curr Med Chem* 11: 1105-1112, 2004.
66. **Stellingwerff T, Decombaz J, Harris RC, and Boesch C**. Optimizing human in vivo dosing and delivery of beta-alanine supplements for muscle carnosine synthesis. *Amino acids* 43: 57-65, 2012.
67. **Stevens JF, and Maier CS**. Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease. *Mol Nutr Food Res* 52: 7-25, 2008.
68. **Suzuki Y IO, Takahashi H, Takamatsu K**. The Effect of Sprint Training on Skeletal Muscle Carnosine on Humans. *Sprint Training and Muscle Carnosine* 2: 105-110, 2004.
69. **Tallon MJ, Harris RC, Boobis LH, Fallowfield JL, and Wise JA**. The carnosine content of vastus lateralis is elevated in resistance-trained bodybuilders. *J Strength Cond Res* 19: 725-729, 2005.
70. **Teixeira V, Valente H, Casal S, Marques F, and Moreira P**. Antioxidant status, oxidative stress, and damage in elite trained kayakers and canoeists and sedentary controls. *Int J Sport Nutr Exerc Metab* 19: 443-456, 2009.
71. **Teufel M, Saudek V, Ledig JP, Bernhardt A, Boularand S, Carreau A, Cairns NJ, Carter C, Cowley DJ, Duverger D, Ganzhorn AJ, Guenet C, Heintzelmann B, Laucher V, Sauvage C, and Smirnova T**. Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem* 278: 6521-6531, 2003.
72. **Tjalkens RB, Cook LW, and Petersen DR**. Formation and export of the glutathione conjugate of 4-hydroxy-2, 3-E-nonenal (4-HNE) in hepatoma cells. *Arch Biochem Biophys* 361: 113-119, 1999.
73. **Toninello A, Pietrangeli P, De Marchi U, Salvi M, and Mondovi B**. Amine oxidases in apoptosis and cancer. *Biochim Biophys Acta* 1765: 1-13, 2006.
74. **Ulbricht A, Gehlert S, Leciejewski B, Schiffer T, Bloch W, and Hohfeld J**. Induction and adaptation of chaperone-assisted selective autophagy CASA in response to resistance exercise in human skeletal muscle. *Autophagy* 11: 538-546, 2015.
75. **Zhang Q, Zheng J, Qiu J, Wu X, Xu Y, Shen W, and Sun M**. ALDH2 restores exhaustive exercise-induced mitochondrial dysfunction in skeletal muscle. *Biochem Biophys Res Commun* 485: 753-760, 2017.

Figure Legends

Fig.1 Experimental design. Twenty physically-active individuals (n=20) were familiarized with all exercise testing followed by the assessment of baseline characteristics, these included the lactate threshold (LT), peak oxygen consumption (VO_2 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 ± 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± 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 ± 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 ± 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 ± 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

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

Table. 1 Participant characteristic

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

Fig.1

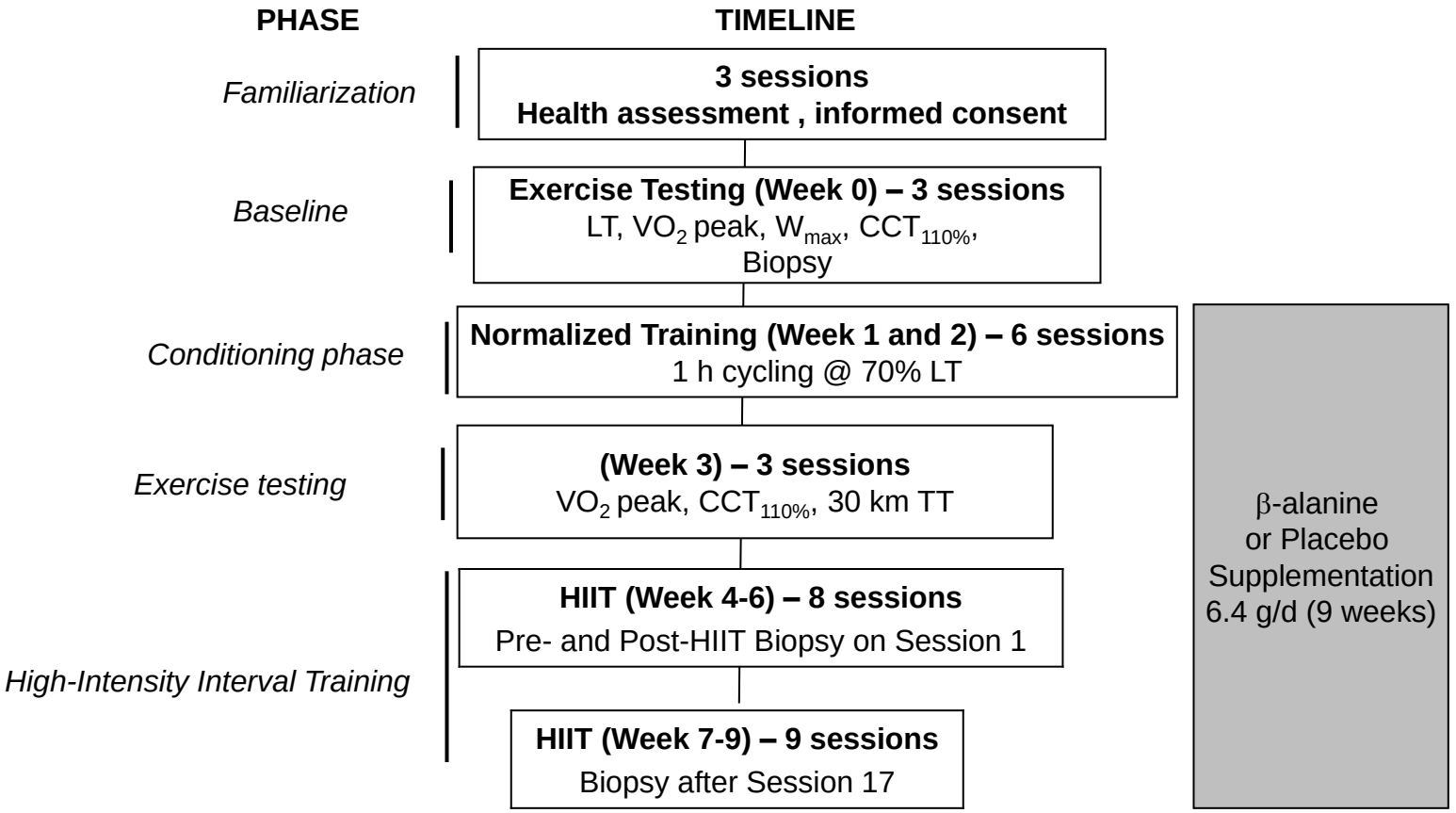
PHASE
TIMELINE
Familiarization
3 sessions
Health assessment , informed consent
Baseline
Exercise Testing (Week 0) – 3 sessions
LT, VO2 peak, Wmax, CCT110%,
Biopsy
Conditioning phase
Normalized Training (Week 1 and 2) – 6 sessions
1 h cycling @ 70% LT
Exercise testing
(Week 3) – 3 sessions
VO2 peak, CCT110%, 30 km TT
High-Intensity Interval Training
HIIT (Week 4-6) – 8 sessions
Pre- and Post-HIIT Biopsy on Session 1
HIIT (Week 7-9) – 9 sessions
Biopsy after Session 17
β-alanine
or Placebo
Supplementation
6.4 g/d (9 weeks)

Fig.2

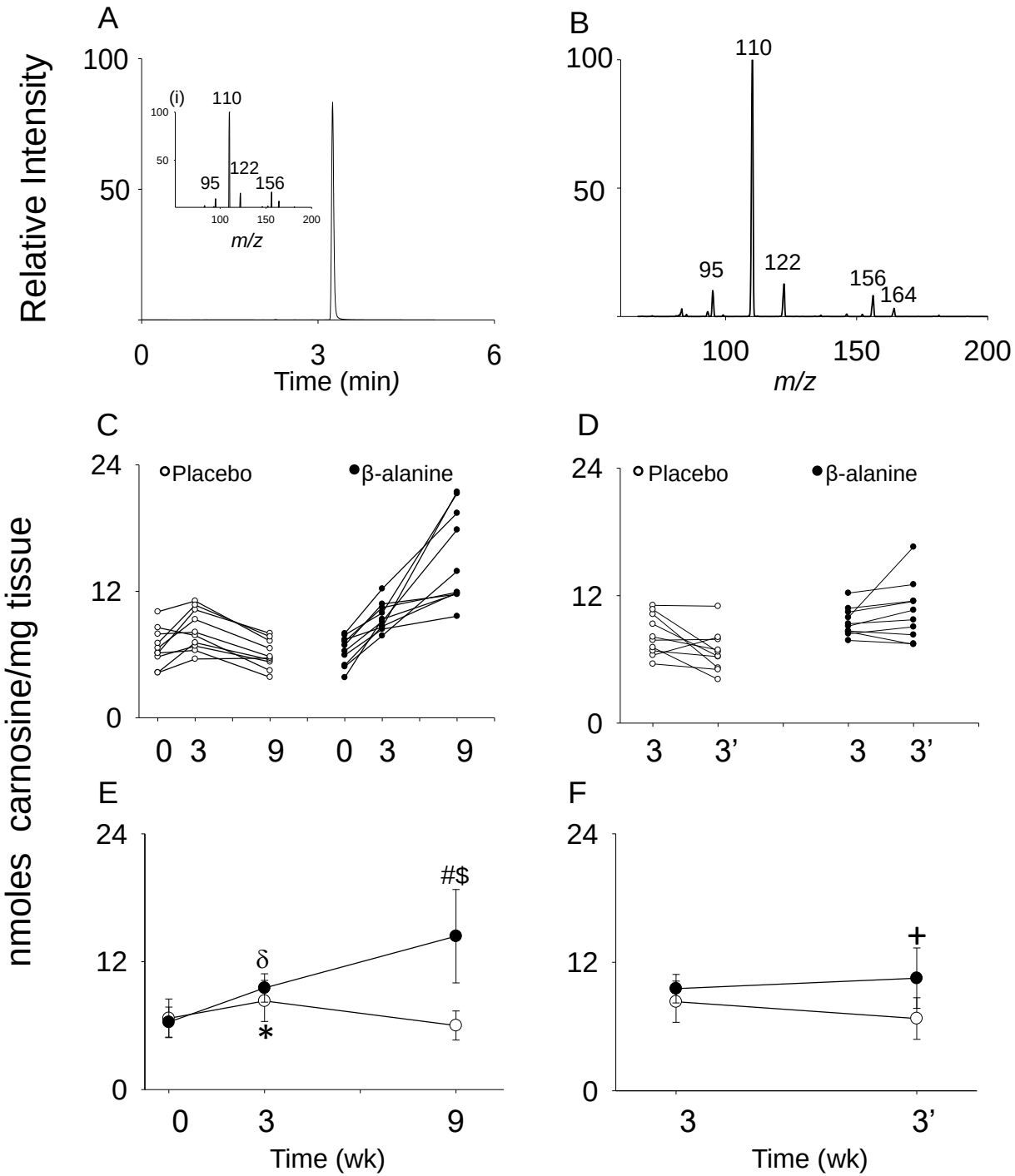
A
B
C
D
E
F
Relative Intensity
100
50
0
3
6
Time (min)
(i)
110
95
122
156
100
150
200
m/z
164
Placebo
β-alanine
24
12
0
3'
nmoles carnosine/mg tissue
#$
δ
*
+
Time (wk)

Fig.3

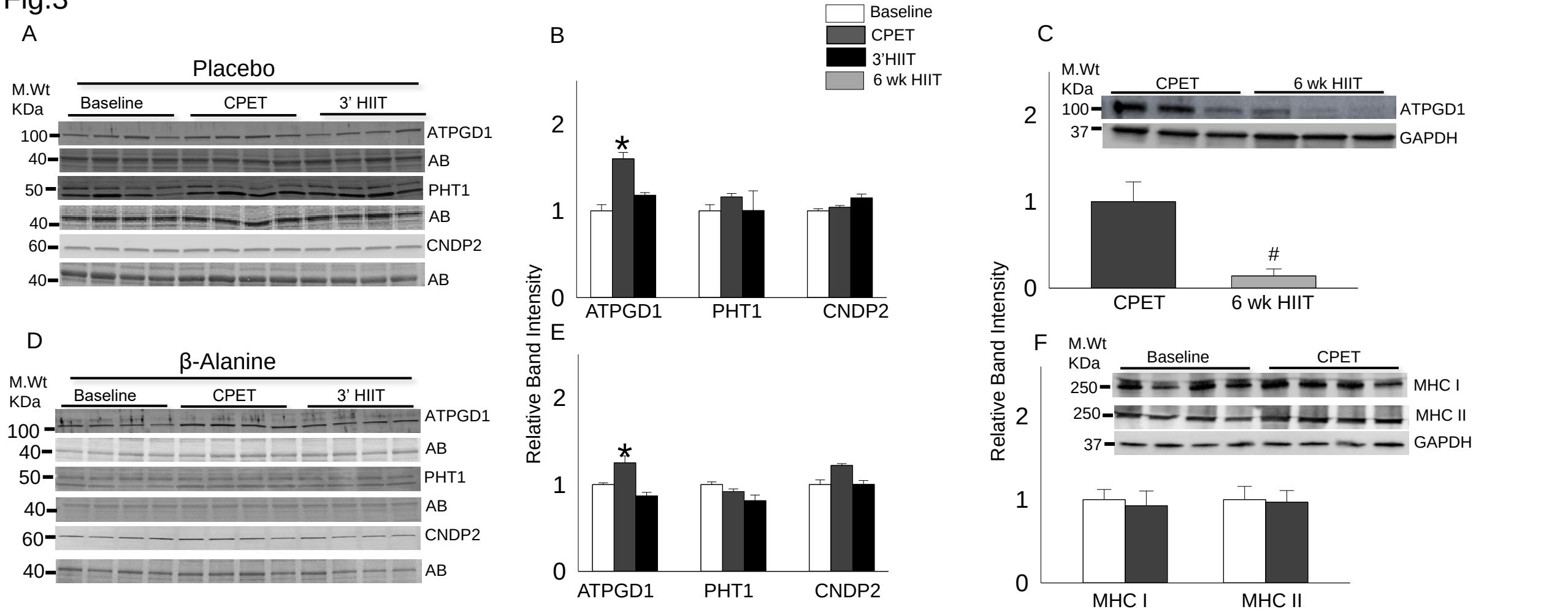
A
Placebo
M.Wt
KDa
Baseline
CPET
3' HIIT
100
ATPGD1
40
AB
50
PHT1
40
AB
60
CNDP2
40
AB
D
β-Alanine
M.Wt
KDa
Baseline
CPET
3' HIIT
100
ATPGD1
40
AB
50
PHT1
40
AB
60
CNDP2
40
AB
B
Baseline
CPET
3'HIIT
6 wk HIIT
Relative Band Intensity
2
1
0
*
ATPGD1
PHT1
CNDP2
E
Relative Band Intensity
2
1
0
*
ATPGD1
PHT1
CNDP2
C
M.Wt
KDa
CPET
6 wk HIIT
100
ATPGD1
37
GAPDH
Relative Band Intensity
2
1
0
#
CPET
6 wk HIIT
F
M.Wt
KDa
Baseline
CPET
250
MHC I
250
MHC II
37
GAPDH
Relative Band Intensity
2
1
0
MHC I
MHC II

Fig.4

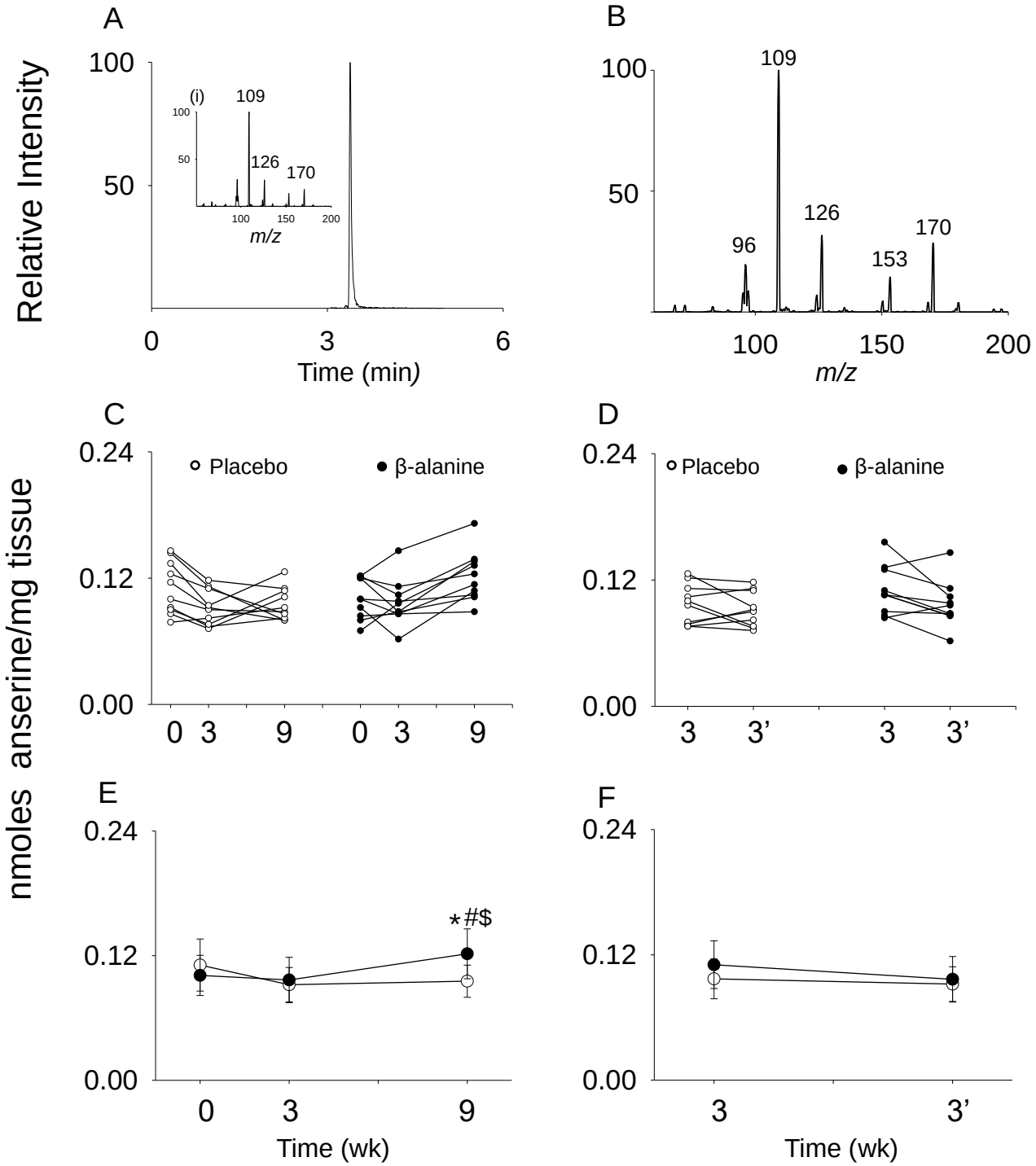
A
B
C
D
E
F
Relative Intensity
100
50
0
3
6
Time (min)
(i)
109
126
170
m/z
96
153
nmoles anserine/mg tissue
0.24
0.12
0.00
Placebo
β-alanine
3'
*#$
Time (wk)

Fig.5

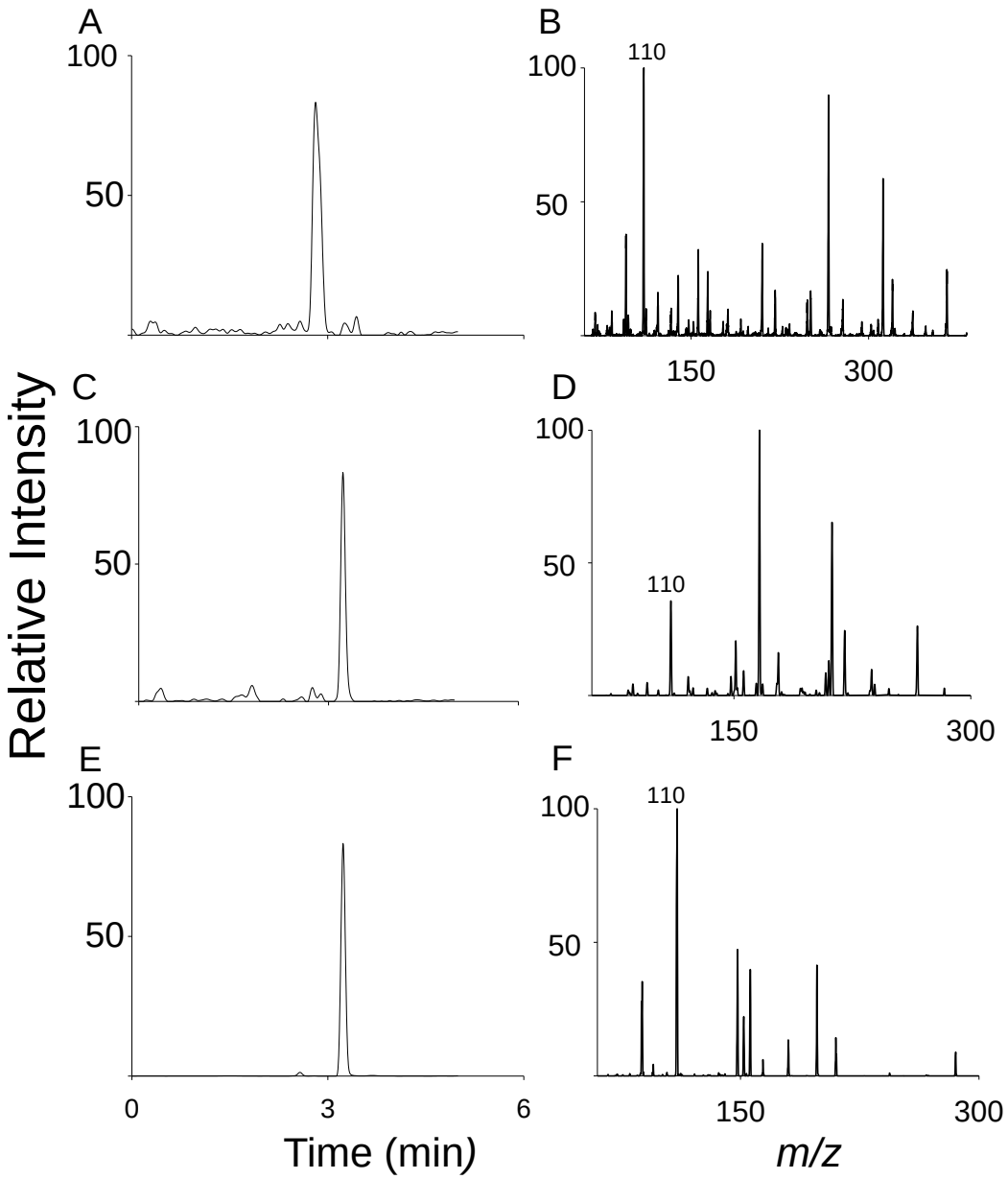

Fig.6

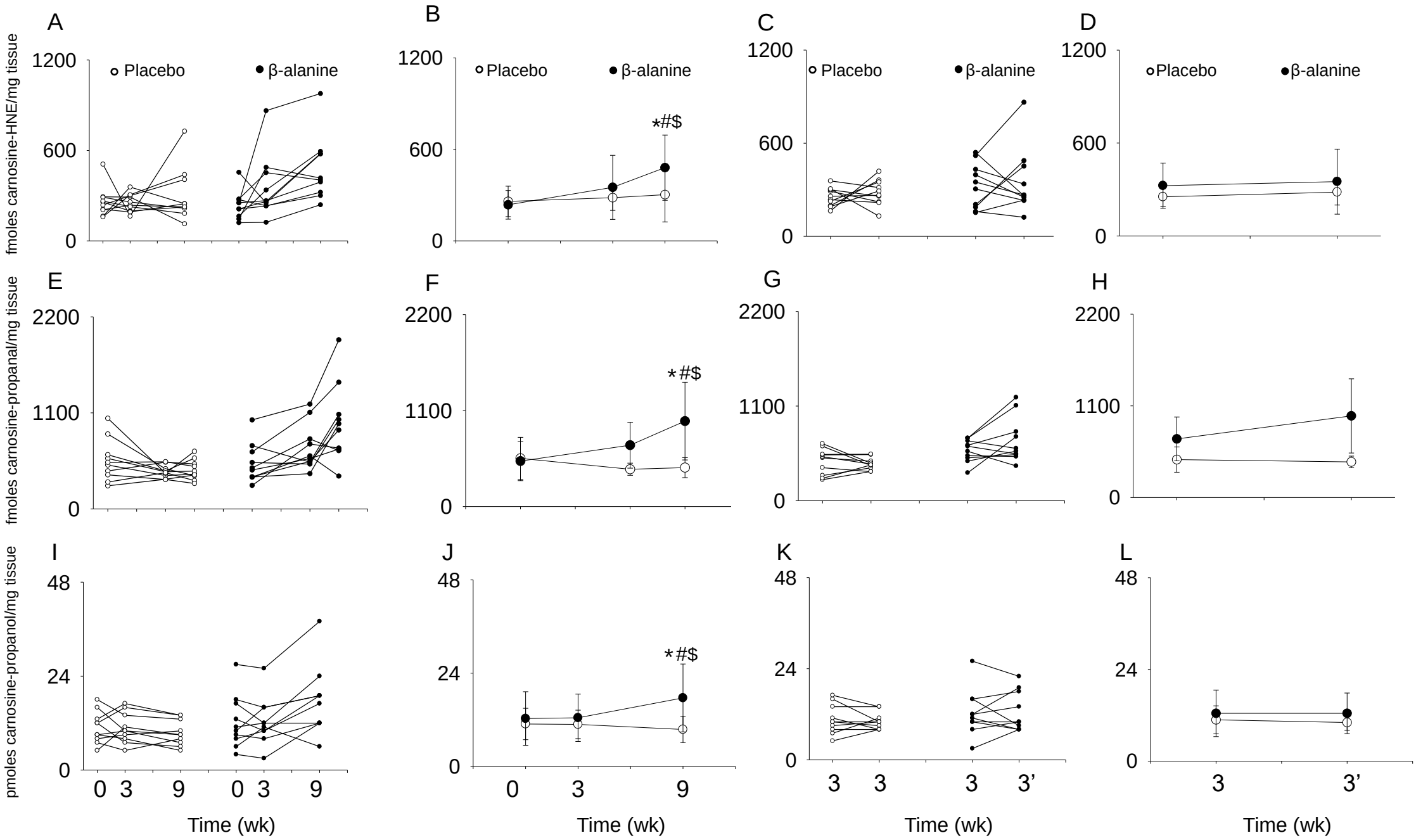
A
B
C
D
E
F
G
H
I
J
K
L
Placebo
β-alanine
*#$
fmoles carnosine-HNE/mg tissue
fmoles carnosine-propanal/mg tissue
pmoles carnosine-propanol/mg tissue
1200
600
0
2200
1100
48
24
0 3 9 0 3 9
3 3 3 3'
3 3'
Time (wk)

Table 1

Characteristic	Group	
	Placebo (n = 10)	β-alanine (n=10)
Age (y)	25.3 ± 7.7	29.4 ± 7.0
Mass (kg)	78.7 ± 10.8	71.4 ± 10.5
VO2peak ($mL.min^{-1}.kg^{-1}$)	53.1 ± 7.9	53.1 ± 9.0
Lactate threshold (LT)	201 ± 50	204 ± 59
W_{max} (W)	285 ± 49	282 ± 65