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Kinetics of Muscle Carnosine Decay after β-Alanine Supplementation: A 16-wk Washout Study

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[1]Applied Physiology and Nutrition Research Group, School of Physical Education and Sport, Rheumatology Division, Faculdade de Medicina FMUSP, Universidade de São Paulo, São Paulo, SP, BRAZIL; [2]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, BRAZIL; [3]Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, SP, BRAZIL; and [4]Musculoskeletal Physiology Research Group, Sport, Health and Performance Enhancement Research Centre, Nottingham Trent University, Nottingham, UNITED KINGDOM

ABSTRACT

YAMAGUCHI, G. C., K. NEMEZIO, M. L. SCHULZ, J. NATALI, J. E. CESAR, L. A. RIANI, L. S. GONÇALVES, G. B. MÖLLER, C. SALE, M. H. G. MEDEIROS, B. GUALANO, and G. G. ARTIOLI. Kinetics of Muscle Carnosine Decay after β-Alanine Supplementation: A 16-wk Washout Study. *Med. Sci. Sports Exerc.*, Vol. 53, No. 5, pp. 1079–1088, 2021. **Purpose:** This study aimed to describe the kinetics of carnosine washout in human skeletal muscle over 16 wk. **Methods:** Carnosine washout kinetics were studied in 15 young, physically active omnivorous men randomly assigned to take 6.4 g·d^{-1} of β-alanine (n = 11) or placebo (n = 4) for 8 wk. Muscle carnosine content (M-Carn) was determined before (PRE), immediately after (POST), and 4, 8, 12, and 16 wk after supplementation. High-intensity exercise tests were performed at these same time points. Linear and exponential models were fitted to the washout data, and the leave-one-out method was used to select the model with the best fit for M-Carn decay data. Repeated-measures correlation analysis was used to assess the association between changes in M-Carn and changes in performance. **Results:** M-Carn increased from PRE to POST in the β-alanine group only (+91.1% ± 29.1%; placebo, +0.04% ± 10.1%; $P < 0.0001$). M-Carn started to decrease after cessation of β-alanine supplementation and continued to decrease until week 16 (POST4, +59% ± 40%; POST8, +35% ± 39%; POST12, +18% ± 32%; POST16, −3% ± 24% of PRE M-Carn). From week 12 onward, M-Carn was no longer statistically different from PRE. Both linear and exponential models displayed very similar fit and could be used to describe carnosine washout, although the linear model presented a slightly better fit. The decay in M-Carn was mirrored by a similar decay in high-intensity exercise tolerance; M-Carn was moderately and significantly correlated with total mechanical work done (r = 0.505; P = 0.032) and time to exhaustion (r = 0.72; $P < 0.001$). **Conclusions:** Carnosine washout takes 12–16 wk to complete, and it can be described either by linear or exponential curves. Changes in M-Carn seem to be mirrored by changes in high-intensity exercise tolerance. This information can be used to optimize β-alanine supplementation strategies. **Key Words:** CARNOSINE, WASHOUT, β-ALANINE, HUMAN SKELETAL MUSCLE

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β-Alanine supplementation has been consistently shown to increase muscle carnosine content (M-Carn) by approximately 60%–80% on average following typical dosing regimens (~3–6 g·d^{-1} for ~4–10 wk) (1,2). Carnosine (β-alanyl-L-histidine) is primarily a cytoplasmatic dipeptide that is thought to play important physiological roles, including acid–base regulation (3–5), protection against oxidative damage (6,7), protein glycation and carbonylation (8), detoxification of reactive aldehydes (9), and regulation of intramuscular calcium transients (10,11). β-Alanine supplementation has become a popular nutritional strategy among athletes to improve performance (12) because of its well-demonstrated ergogenic effects, especially in high-intensity exercises where the increased buffering capacity brought about by increased M-Carn improves intramuscular pH regulation (13–16). In

contrast with the large number of studies consistently showing that almost all individuals respond to chronic β-alanine supplementation by increasing M-Carn (>20 studies with ~500 participants in total), much less is known about how M-Carn responds when β-alanine supplementation ceases. Precise information on how M-Carn responds to β-alanine cessation could provide valuable knowledge about the mechanisms controlling M-Carn in skeletal muscle as well as the basis for applying more effective supplementation strategies.

To date, only three studies have evaluated muscle carnosine washout (17–19), with conflicting data being reported. Harris et al. (19) were the first to study carnosine washout; using chromatographic carnosine determination in muscle biopsy samples, they reported an exponential decay for carnosine with a half-life ($t_{½}$) of 8.6 wk after β-alanine supplementation (6.4 g·d^{-1} for 4 wk). Subsequently, Baguet et al. (17) supplemented 4.8 g·d^{-1} of β-alanine for 5–6 wk, and using hydrogen magnetic resonance spectroscopy to quantify muscle carnosine, they reported linear (i.e., zero order) washout kinetics in M-Carn with a ~30% reduction in M-Carn occurring in the third week of washout; by the ninth week, mean carnosine had returned to presupplementation levels. However, the individuals who were considered high responders (i.e., those whose M-Carn had increased by more than 30%, n = 3) still exhibited elevated M-Carn in the 9th week and were predicted to reach presupplementation levels only in the 15th week. The low responders (i.e., those whose M-Carn had increased by <30%, n = 5), on the other hand, required only 6.5 wk to return to the presupplementation levels. Also using hydrogen magnetic resonance spectroscopy, Stellingwerff et al. (18) reported a longer washout time after an 8-wk β-alanine supplementation period (1.6 or 3.2 g·d^{-1}). The authors predicted that ~15–20 wk would be required for the complete washout of M-Carn, along with a calculated decay rate of ~2% per week. This was 40% slower than the decay rate reported by Baguet et al. (17). More recently, Spelnikov and Harris (20) used the available data from these studies (17–19) to propose a mathematical model for carnosine washout assuming an exponential decay described by first-order kinetics, leading to the assumption that the rate of carnosine decay is dependent on M-Carn levels.

The studies investigating M-Carn washout kinetics have some methodological limitations in addition to equivocal findings. The allotted time for washout may not have been sufficiently long to return M-Carn levels to the presupplementation values (6–9 wk, with a predicted washout time of ≥15 wk) (17). Two studies (17,18) used nuclear magnetic resonance spectroscopy to quantify M-Carn, a method that has been shown to have limited validity (21). Only two carnosine measurements were made across the washout period in these studies, which renders it impossible to describe the kinetic profile of washout.

Because more precise information on the kinetics of carnosine washout can offer insightful physiological information of the mechanisms controlling the synthesis and degradation of carnosine in skeletal muscle, as well as help practitioners to better design β-alanine supplementation strategies, we studied the kinetics of carnosine washout in human skeletal muscle by measuring M-Carn monthly over a longer period (16 wk) and using a reference method for carnosine quantification. M-Carn decay was described using a Bayesian modeling approach. A secondary aim of this study was to examine whether changes in high-intensity exercise capacity mirror changes in M-Carn content, as this would serve as a confirmation of previous data suggesting an association between M-Carn and high-intensity exercise performance (22).

METHODS

Participants. Physically active (participation in exercise activities for ≥150 min·wk^{-1}), healthy, omnivorous men age 18–35 yr were eligible to participate. Exclusion criteria were as follows: current or previous use of β-alanine (for 6 months before the study) or creatine (for 3 months before the study), current or previous use of any other dietary supplement 3 months before the study, and smoking and admitted use of anabolic steroids or any other performance-enhancing drugs. One-hundred and twelve participants were initially screened for eligibility, of which 27 (age, 28 ± 4 yr; height, 1.74 ± 0.08 m; body mass, 76.0 ± 17.3 kg) were deemed eligible and randomly allocated to receive either β-alanine (n = 18) or placebo (dextrose; PL, n = 9) in a 2:1 ratio. Although unbalanced allocation ratios can reduce the statistical power of between-group interactions, this effect is small when ratios are less than 3:1, and it may be advantageous to gain more experience on a treatment whose effects are not well known (23). We thus opted for a 2:1 randomization ratio to maximize the number of participants receiving β-alanine, because our primary goal was descriptive and did not depend on comparisons with the PL group. Twelve participants (7 from the β-alanine group and 5 from the placebo group) dropped out the study after allocation for various reasons and were not included in the analyses (Fig. 1); therefore, 15 participants completed the study (β-alanine, n = 11; PL, n = 4; Table 1). All participants were requested to maintain their habitual dietary intake, as well as their habitual levels of physical activity throughout the study. Compliance with these requests was verbally confirmed with the participants several times throughout the study. They were also fully informed of the risks associated with participation and gave their signed informed consent before participation. The study was approved by the ethics committee of the School of Physical Education and Sport of the University of Sao Paulo (1.942.548) and complies with the standards established by the Declaration of Helsinki.

Experimental design. This was a double-blind, randomized, placebo-controlled, parallel-group study. Randomization was performed in a 2:1 ratio (β-alanine/placebo) in blocks of three or six participants, with the groups matched for maximal cycling power output (W_{max}: β-alanine, 264.6 ± 47.1 W; PL, 250.7 ± 61.7 W) using the block randomization method and a random sequence generator (www.random.org). The random allocation sequence was generated by a researcher who was not directly involved with, and therefore blinded to, the experimental sessions. After the completion of preliminary tests, the individuals were supplemented for 8 wk with either β-alanine

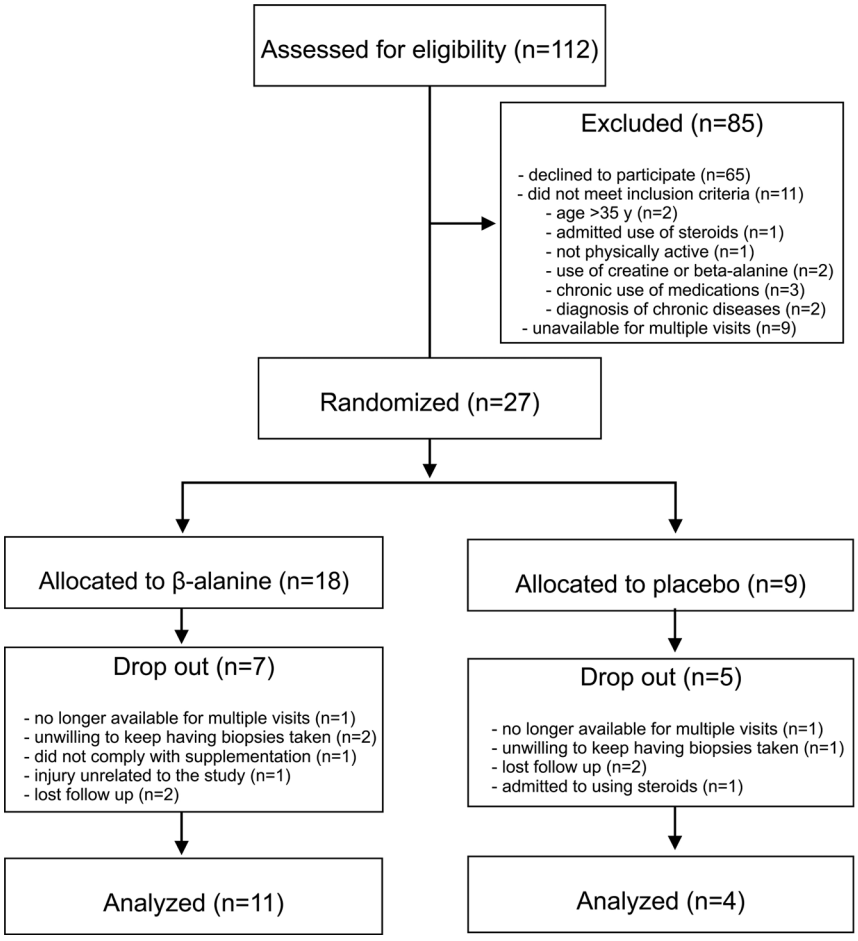

FIGURE 1—Flow diagram indicating participants' enrollment in the study.

(SR-CarnoSyn®; Natural Alternatives International, Inc., Carlsbad, CA) or placebo (maltodextrin; Natural Alternatives International, Inc.).

All participants were requested to attend the laboratory on 11 different occasions. During the first visit, cycling maximal power output (W_{max}) was determined. During the second and third visits, the participants were familiarized with the cycling capacity test at 110% of their individual W_{max} ($CCT_{110\%}$). During the fourth visit (before supplementation; PRE) and in the fifth visit (after supplementation; POST), participants were assessed for $CCT_{110\%}$ and M-Carn content. The six remaining visits were carried out 1, 2, 4, 8, 12, and 16 wk after the end of supplementation (POST1, POST2, POST4, POST8, POST12, and POST 16) where M-Carn was determined. $CCT_{110\%}$ was also determined at POST4, POST8, POST12, and POST16. Because of the large number of muscle biopsies required, several participants did not agree to partake in the POST1 and POST2 trials; hence, these two data sets were excluded from the mixed model analysis but were included in the mathematical modeling of washout kinetics. Figure 2 illustrates the experimental design and the final number of samples analyzed in each time point.

Supplementation protocol. Two 800-mg tablets of either β-alanine or PL were taken four times per day at 3- to 4-h intervals, totaling 6.4 $g \cdot d^{-1}$. Both β-alanine and PL tablets were indistinguishable and identical in size and overall appearance. The participants were instructed to consume their tablets along with main meals (i.e., breakfast, lunch, dinner) and before sleep. They were also requested to complete a log sheet to verify compliance with supplementation (β-alanine, 95% ± 4%; PL, 97% ± 2%). We defined *a priori* that any participant not meeting a minimum of 90% compliance with the supplementation protocol would be excluded from the study, which has been verified after 4 and 8 wk of supplementation. The efficacy of blinding procedures was verified by asking the participants whether they believed to have received β-alanine or PL at the end of the supplementation period. A Fisher's exact test showed no significant differences for the frequency of correct identification of groups from what was expected from random guesses ($P = 0.6564$).

TABLE 1. Participant characteristics at baseline.

	β-Alanine ($n = 11$)	Placebo ($n = 4$)	*P* Value (95% CI)[a]
Age, yr	28 ± 4	27 ± 6	0.74 (–7.9 to 10.3)
Weight, kg	77.21 ± 15.70	73.10 ± 23.54	0.76 (–30.9 to 39.4)
Height, m	1.75 ± 0.09	1.72 ± 0.08	0.44 (–0.6 to 0.1)
BMI, $kg \cdot m^{-2}$	24.99 ± 3.34	24.45 ± 5.95	0.87 (–8.4 to 9.5)
W_{max}	264.6 ± 47.1	250.7 ± 61.7	0.70 (–77.6 to 105.3)

Data are presented as mean ± SD.
[a]Independent-sample *t*-test with equal variances not assumed.
BMI, body mass index; W_{max}, maximal power output attained in a graded exercise test to exhaustion on a cycloergometer.

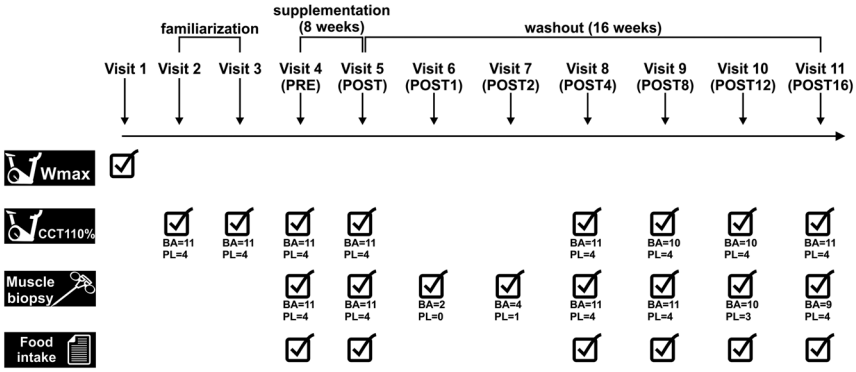

FIGURE 2—Overview of the study design. The numbers indicate how many participants showed up for muscle biopsies and for the $CCT_{110\%}$ at each time point in each group. BA, β-alanine group; $CCT_{110\%}$, TTE exercise tolerance test on a cycle ergometer at 110% of the W_{max}; PL, placebo group; W_{max}, maximal power output attained in a graded exercise test to exhaustion on a cycloergometer.

Preliminary tests and main trials. In the first visit, height was measured to the nearest 0.01 m using a stadiometer, and body mass was measured to the nearest 10 g in a digital scale (100 CH; Welmy, São Paulo, Brazil). Participants then performed a graded cycling capacity test to exhaustion to determine individual W_{max}. In the second and third visits, the participants were familiarized with the $CCT_{110\%}$, which was performed on the same cycle ergometer.

The participants were free to choose the most convenient period of day (morning, afternoon, or evening) for undertaking the tests; this was recorded and replicated individually in all remaining visits. All participants were instructed to abstain from alcohol intake and heavy exercise in the 24 h before the main trials and to abstain from caffeine intake in the 16 h before the main trials. Compliance with these requests was verbally confirmed in all visits. They were also requested to arrive in a well-fed and well-hydrated state, but avoiding large meals in the 2 h before the main trials. In all main trials, food intake was assessed at the participants' arrival, followed by the muscle biopsy, and then by the $CCT_{110\%}$. *Ad libitum* water intake was allowed throughout all trials.

Maximal incremental cycling test. All participants performed a maximal incremental exercise test on an electromagnetically braked cycle ergometer (Lode Excalibur®; Lode B.V., Groningen, Germany) to determine their individual maximal power output (W_{max}). The ergometer position and saddle height were recorded for each individual during the preliminary tests and replicated in all upcoming experimental sessions. Participants started the test by pedaling at a load of 100 W, which was increased by 6 W every 15 s (24). The participants pedaled at a constant, self-selected pedal cadence (60–100 rpm) throughout the test until volitional exhaustion. Strong standardized verbal encouragement was provided in all tests. Exhaustion was deemed to have occurred when the cadence could not be maintained greater than 60 rpm. $W_{max.}$ was determined by the last completed stage added to the proportion of the last stage not completed multiplied by 6.

High-intensity exercise tolerance test ($CCT_{110\%}$). The $CCT_{110\%}$ was performed on the same electromagnetically braked cycle ergometer (Lode Excalibur®; Lode B.V.). The test began with a 5-min warm-up at 100 W, followed by a 3-min resting interval, where the participants remained seated on the ergometer. The $CCT_{110\%}$ commenced at 80% of the previously determined W_{max} for the first 15 s, followed by 95% of W_{max} for 15 s and 110% of W_{max} for the rest of the test, until exhaustion. The participants pedaled at a constant, self-selected pedal cadence (60–100 rpm) throughout the test, with exhaustion occurring when they could not maintain cadence greater than 60 rpm. Strong verbal encouragement was given in all trials. Time to exhaustion (TTE) and total mechanical work done (TWD) were recorded and used as performance measurements. Test–retest coefficients of variation between the two familiarization sessions were 3.8% for TTE and 4.7% for TWD, which is in accordance with previous reports (24).

Muscle biopsies. Muscle biopsies were obtained with a 6-mm biopsy needle (Northern Hospital Supplies, Edinburgh, United Kingdom) using the Bergstrom method (25) with suction. The samples were taken from the midportion of the *musculus vastus lateralis* of the dominant leg, under local skin anesthesia (3 mL lidocaine 1%), as previously described (25). Samples of ~60–100 mg were immediately frozen in liquid nitrogen and stored in the vapor phase of liquid nitrogen until analysis. Because of the repeated biopsies over time, the location was slightly changed across visits (~1 cm inward and upward), as illustrated in the Supplemental Digital Content 1 (Figure, Supplemental Digital Content, location where the multiple biopsies were taken, http://links.lww.com/MSS/C197).

Quantification of M-Carn content. M-Carn content was determined in a liquid chromatographer connected to a UV diode array detector (Shimadzu®; Prominance UFLC 20 AD, Tokyo, Japan) using the method described by Mora et al. (26). Skeletal muscle samples were freeze-dried, dissected free of visible blood and connective tissue, and powdered. Approximately 3 mg of the powdered dry muscle was deproteinized with perchloric acid and subsequently neutralized with potassium bicarbonate as previously described (27). Muscle extracts were filtered with syringe filters (Hexis®; PVDF, 13 mm, 02 μm) and injected into the chromatographer via an autosampler using the cut injection method (total aspirated volume of 5 μL). All samples and

standards were analyzed in duplicates. Standard curves for carnosine were performed before each batch of analysis using known concentrations of 50, 100, 500, 1.000, and 2.500 μmol·L^{-1} of carnosine (coefficient of linearity $r^2 > 0.99$). Separation was performed at room temperature using an Atlantis HILIC silica column (4.6 × 150 mm, 3 μm; Waters, Milford, MA) attached to an Atlantis Silica column guard (4.6 × 20 mm, 3 μm) under the following conditions: linear gradient from 0% to 100% of mobile phase A (ammonium acetate 0.65 mmol·L^{-1} in water/acetonitrile 25:75 v/v, pH 5.5) to mobile phase B (ammonium acetate 4.55 mmol·L^{-1} in water/acetonitrile 70:30 v/v, pH 5.5) at a flow rate of 1.4 mL·min^{-1}. Separation was monitored using a UV detector at 214 nm. The column was equilibrated for 5 min under the initial conditions before each injection. Quantification was performed using peak areas and the obtained concentration adjusted to each sample weight. The intra-assay coefficient of variation of carnosine measurement between the duplicate injections was 3.6%. All samples were analyzed with the experimenters being blind to the group, time point, and the participant.

Dietary intake. Dietary intake was assessed by a trained nutritionist using 3-d food diaries at the following time points: PRE, POST, POST4, POST8, POST12, and POST16. All participants were instructed by a nutritionist on how to complete a diary. All diaries were verified with the participant upon their return, with any inconsistencies being resolved individually whenever necessary. Data were calculated using nutrition software containing nutrient information of Brazilian food (Avanutri® Online, Rio de Janeiro). Total caloric intake as well as carbohydrate, protein, and fat intake were calculated. The dietary intake of β-alanine was estimated based on data available in the literature (3,28).

Statistical analysis. Linear mixed models (proc mixed; SAS University Edition) were used to analyze M-Carn and dietary intake data, with group (β-alanine vs PL) and time (PRE, POST1, POST4, POST8, POST12, and POST16) being fixed factors and participants being random factors. Four different covariance matrix structures were tested (unstructured, autoregressive lag-1, toeplitz, and compound symmetric), and the Bayesian information criterion (lowest Bayesian information criterion value) was used to choose the structure that best fit to each data set. Where there were significant group or time main effects, or group–time interaction, a hypothesis-driven single-degree of freedom contrast analysis was used to locate within- and between-group differences. The association between carnosine content and performance across time was assessed in β-alanine group using the repeated-measures correlation (rmcorr, R 3.5.1), with data at three different time points (POST, POST8, and POST16) being used to represent low, medium, and high M-Carn before and after supplementation. Cohen's *d* effect sizes were calculated between groups for our main outcome (i.e., M-Carn) as the mean difference between β-alanine and PL divided by the pooled SD. Baseline participants' characteristics were compared between groups using independent-sample *t*-tests with equal variances not assumed (SPSS version 17). The proportion of participants correctly/incorrectly guessing the substance they were taking was tested with the Fischer's exact test. Data are presented as mean ± SD (with 95% confidence intervals (CI)), and the significance level was set *a priori* at $P < 0.05$.

In addition, linear and exponential Bayesian fits of the M-Carn content over the washout weeks were performed. First, the data were prepared by removing, for each participant, the M-Carn level before supplementation (i.e., PRE) from all other measurements (i.e., POST to POST16). All following Bayesian analyses were performed with the brms package (29) in the R software (R Core Team, 2018). For the exponential fit (defined as $f(x) = b_1 e_2^{-bx} + b_3$), the b_1 prior distribution was based on our group's previous measurements of M-Carn in omnivores. More specifically, the parameters were calculated by subtracting the presupplementation M-Carn mean (offset, 20.44 mmol·kg^{-1} DM) from the postsupplementation M-Carn (34.66 ± 12.85 mmol·kg^{-1} DM), resulting in a normal distribution of 14.22 ± 12.61. For b_2, a generic normal distribution was used (mean (SD) = 0 (1)), and because the fit was performed after the removal of the offset, b_3 distribution was not relevant. Several other values were tested as previously described, but they did not change the overall results. These fits were analyzed and compared using the leave-one-out information criteria (LOOIC), where the smaller values are associated with better fits.

RESULTS

Muscle carnosine content. M-Carn significantly increased 91.1% ± 29.1% from PRE to POST supplementation in the β-alanine group (group–time interaction, $P < 0.0001$; within-group effect, β-alanine, $P < 0.0001$), but not in the PL group (+0.04% ± 10.1%; within-group effect, $P = 0.999$; between-group effect, $P < 0.0001$). In the β-alanine group, M-Carn started to decrease after the end of the supplementation period, being significantly lower at all time points in comparison with the previous time point (all, $P < 0.05$), indicating a continuous decrease in M-Carn throughout the 16-wk washout period. In the PL group, no significant differences were shown between any of the time points (all, $P > 0.05$). M-Carn values after 12 and 16 wk of washout were not statistically different from PRE. M-Carn loading and washout data are shown in Figures 3A and B. The β-alanine-to-carnosine conversion ratio was 4.4% ± 2.0%, which was calculated assuming that 40% of body mass was muscle mass and that 70% of muscle mass was water.

Modeling the kinetics of muscle carnosine washout. The LOOIC was used to estimate the prediction accuracy from two fitted Bayesian models of carnosine decay for 16 wk, where one model was a linear decay and the other model was an exponential decay. LOOIC (lower values indicate better fit) values were 395.27 (SE = 9.19) for the linear model and 398.22 (SE = 9.00) for the exponential model, with the difference between models being −2.95 (SE = 4.5). This indicates that both models provide a similar degree of fit with the data set and that the linear model predicts carnosine decay slightly

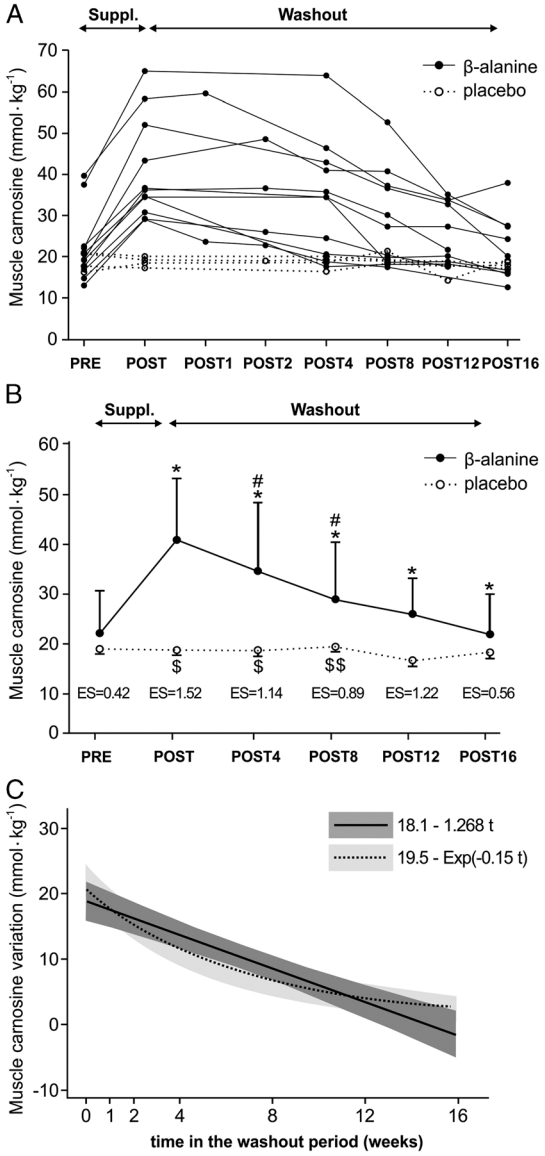

FIGURE 3—A, Individual muscle carnosine responses to 8 wk of β-alanine or placebo supplementation followed by 16 wk of washout. B, Mean ± SD responses to 8 wk of β-alanine or placebo supplementation followed by 16 wk of washout. C, Linear and exponential fitted models for muscle carnosine decay in the washout period. Gray areas represent the upper and lower limits of the expected values of the posterior predictive distribution. All results are expressed relative to dry muscle weight. *Significantly different from the previous time point (within-group effect). #Significantly different from PRE (within-group effect). $Significantly different from β-alanine group in the same time point (between-group effect). $$$P = 0.06$ vs β-alanine in the same time point (between-group effect). ES, between-group Cohen's effect sizes.

better than the exponential model (Fig. 3C). The $t_{1/2}$ for M-Carn washout in the exponential decay model was calculated to be 4.6 wk (95% CI, 3.2–7.0).

M-Carn and exercise performance. A visual inspection of the absolute changes in performance during the washout period suggests a close association between performance changes and the changes in M-Carn content during the same period (Figs. 4A, C). Repeated-measures correlation analysis revealed a moderate, significant correlation between TWD and M-Carn ($r = 0.505$, $P = 0.032$; Fig. 4B) and between TTE and M-Carn ($r = 0.72$, $P < 0.001$; Fig. 4D). These data indicate that the increase in M-Carn with β-alanine supplementation followed by the return to the baseline levels after the washout period is mirrored by similar changes in performance.

Dietary intake. No significant group–time interactions were shown for the daily intakes of carbohydrate ($P = 0.434$), protein ($P = 0.254$), lipids ($P = 0.861$), total energy ($P = 0.915$), or β-alanine ($P = 0.499$; Table, Supplemental Digital Content 2, Daily energy, macronutrient, and β-alanine intake in the β-alanine and placebo groups across the study period, http://links.lww.com/MSS/C198).

DISCUSSION

In this study, we investigated the washout kinetics of muscle carnosine for 16 wk after the cessation of β-alanine supplementation using multiple assessments of M-Carn in the washout period; we also used the high-performance liquid chromatography, a reference method for muscle carnosine quantification, and parallel assessments of high-intensity exercise performance. In alignment with the existing literature, we confirmed that carnosine washout in skeletal muscle is a slow process, thereby confirming that skeletal M-Carn is relatively stable over time (16–18). In our study, complete washout of carnosine occurred within a mean time of ~12 wk, although significant individual variation existed. Previous studies predicted both shorter (17) and longer (18) washout periods. We also showed that carnosine washout can be described by a linear decay, although an exponential model can also describe the washout kinetics just as well as the linear model. The calculated $t_{1/2}$ for M-Carn was 4.6 (95% CI, 3.2–7.0) wk in the exponential decay model in our study, which is not too dissimilar to the 5.8 wk reported by Baguet et al. (17) but somewhat shorter than the 8.6 wk reported by Harris et al. (19). We also provided evidence for the association between M-Carn and high-intensity exercise performance after both supplementation and washout.

The study of the kinetic properties can reveal important features of biological systems. In the case of carnosine washout, the literature has been controversial as to whether carnosine decay displays a linear or exponential function (17,20). To address this question, we fitted two Bayesian predictive models and used the LOOIC to select which one better describes the carnosine washout kinetics during the 16 wk after the cessation of β-alanine supplementation. This approach aimed to make use of the advantages of Bayesian statistics, such as the incorporation of prior information and the capacity of making predictions based on posterior probabilities, to shed a new light to the carnosine washout dynamic. Our data showed that both models displayed remarkably similar fits. Because the linear model is simpler and uses less terms, it would be mathematically preferred over the exponential model. On the other hand, linear decays are unusual in biological systems as they would predict, in the long-term, that concentrations would fall

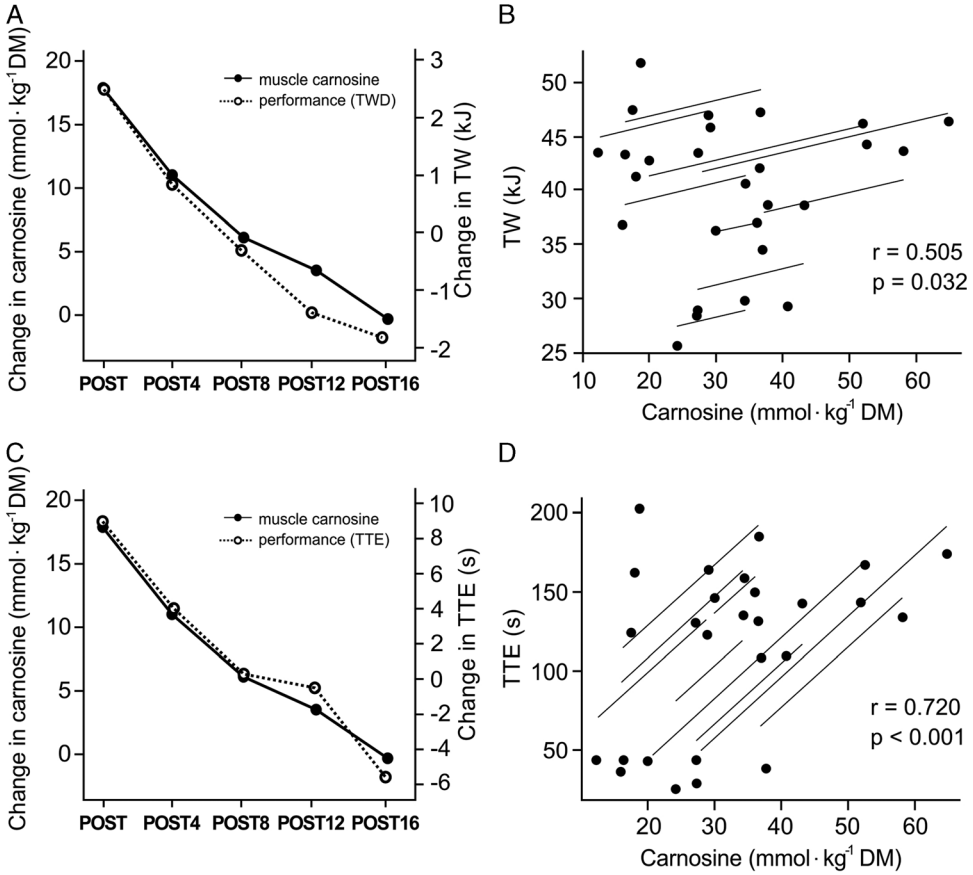

FIGURE 4—Absolute changes in M-Carn are mirrored by changes in performance, as assessed by total work (TW; A) and TTE (C). M-Carn was moderately and significantly correlated with TW and TTE, as depicted in the repeated-measures correlation analysis chart (B and D).

below zero. In the case of M-Carn, the linear decay can only be assumed to be accurate within a well-defined time period. Thus, we can only affirm that the decay in M-Carn is linear within the 16-wk washout period used in this study and up until M-Carn returns to the presupplementation levels. In the longer term, an exponential decay would probably better describe M-Carn washout kinetics, as M-Carn tends to return to presupplementation levels instead of keeping falling indefinitely. Nevertheless, both models indicate that carnosine levels have little influence on the rate of carnosine decay.

In our study, 8 wk of β-alanine supplementation led to a ~90% increase in M-Carn, which is in accordance with other studies using similar total doses of β-alanine (30). The effects of β-alanine supplementation on M-Carn are highly consistent in the literature (1). Carnosine synthesis in skeletal muscle is catalyzed by the enzyme carnosine synthase, a ligase that presents lower affinity for β-alanine than for histidine (31,32). Because the intramuscular concentrations of β-alanine are fairly low (~2 $\mu mol \cdot L^{-1}$) (33) and far smaller than those of histidine (~400 $\mu mol \cdot L^{-1}$) ((34), the carnosine synthesis rate is thought to be limited by β-alanine availability. Upon the ingestion of typical supplemental doses, β-alanine rapidly reaches the bloodstream and then enters the skeletal muscle, where its concentrations increase ~3-fold (33). The higher substrate availability probably leads to a transient increase in the activity of carnosine synthase, thereby increasing carnosine accretion; this increase, however, seems to occur in a saturable fashion (35) and the exceeding β-alanine is likely to be diverted toward oxidation (36). The relatively low catalytic efficiency of carnosine synthase seems to explain the rather slow increases in M-Carn in response to β-alanine supplementation and the mere ~5% β-alanine-to-carnosine conversion rates that have been consistently reported in the literature (2,36).

Although carnosine synthesis rates are not the sole factor that regulates intramuscular carnosine, it seems that higher activity of carnosine synthase induced by increased β-alanine availability predominates over other factors during supplementation periods, thereby leading M-Carn to increase in virtually all individuals. When β-alanine supplementation ceases, this mechanism driving carnosine accretion stops and then an imbalance favoring carnosine degradation/removal from skeletal muscle starts to predominate over carnosine synthesis, ultimately leading to a slow process of returning carnosine to baseline levels. When baseline levels are reached, a balance between carnosine synthesis/degradation and movement in to or out of the muscle cells seems to occur. At least three different mechanisms may account for carnosine washout, namely, intramuscular carnosine degradation by tissue dipeptidases, transport of the intact dipeptide out of muscle cells, and carnosine quenching via reaction with reactive species.

However, it is still uncertain whether these mechanisms can occur *in vivo* in human skeletal muscle, except for carnosine quenching by reactive species, which have been demonstrated to occur in humans (9,37), although to an extent that is too low to significantly contribute to carnosine washout.

Tissue carnosine dipeptidase 2 (CN2) is the only known enzyme capable of degrading carnosine in skeletal muscle. However, CN2 is nonspecific and has a low affinity for carnosine (38). Moreover, the literature is controversial as to whether CN2 has catalytic activity toward carnosine under physiological conditions. Teufel et al. (39) demonstrated that CN2 can degrade carnosine into its constituent amino acids in alkaline (pH 9.5) conditions but not at a physiologically relevant pH (7.5), leading the authors to suggest that carnosine is not a substrate of CN2 *in vivo*. Different results were reported by Margolis et al. (40), however, who showed that CN2 can hydrolyze carnosine in murine tissues, such as the kidney, skeletal muscle, and brain at pH 7.5. Interestingly, the catalytic activity in muscle, despite being low, slightly increased under high carnosine concentrations (40). If we were to assume that skeletal muscle CN2 operates in a similar fashion in humans, then the slow carnosine decay might be explained by an increase in CN2 activity driven by increased substrate (i.e., higher carnosine levels), which tends to return to its baseline activity by the time that M-Carn reaches presupplementation levels. Alternatively, carnosine decay could also be attributed to the activity of dipeptide transporters, mostly by PHT1, which has been shown to be expressed in human skeletal muscle (41) and could result in carnosine being exported from the muscle cells to the bloodstream. Although it remains to be experimentally determined whether carnosine can be transported out of muscle cells by PHT1, circumstantial evidence suggests this may occur in conditions such as intensive exercise (37), although other studies did not confirm this mechanism (9).

As for the washout mechanism, we therefore propose that, with the cessation of β-alanine supplementation, the reduced β-alanine availability would reduce carnosine synthase activity, thereby leading carnosine synthesis rates to quickly return to baseline levels. Carnosine degradation rates, on the other hand, would be still be above basal. Thus, increased carnosine in muscle would result in a higher activity of CN2, therefore explaining the overall imbalance between carnosine synthesis and degradation in favor of degradation. Because the catalytic efficiency of CN2 is poor and the rate of carnosine degradation is subsequently low, the increase in CN2 activity due to the increased substrate availability would be just sufficient to unbalance carnosine homeostasis toward degradation, but not sufficiently fast to result in an observable exponential curve that is clearly distinguishable from a linear curve. The notion that only minor differences between carnosine synthesis and degradation underpin the slow washout pattern, leading to a remarkable similarity between linear and exponential decays, can explain the inconsistencies between previous studies in describing the kinetics of carnosine decay (17–19). The proposed mechanisms underlying carnosine loading and washout are illustrated in Figure 5.

Because the ergogenic effects of β-alanine supplementation are already well documented (12), our study design did not prioritize the assessment of the performance-enhancing properties of β-alanine. However, it is particularly interesting to note that we showed a significant association between M-Carn and high-intensity exercise tolerance, suggesting

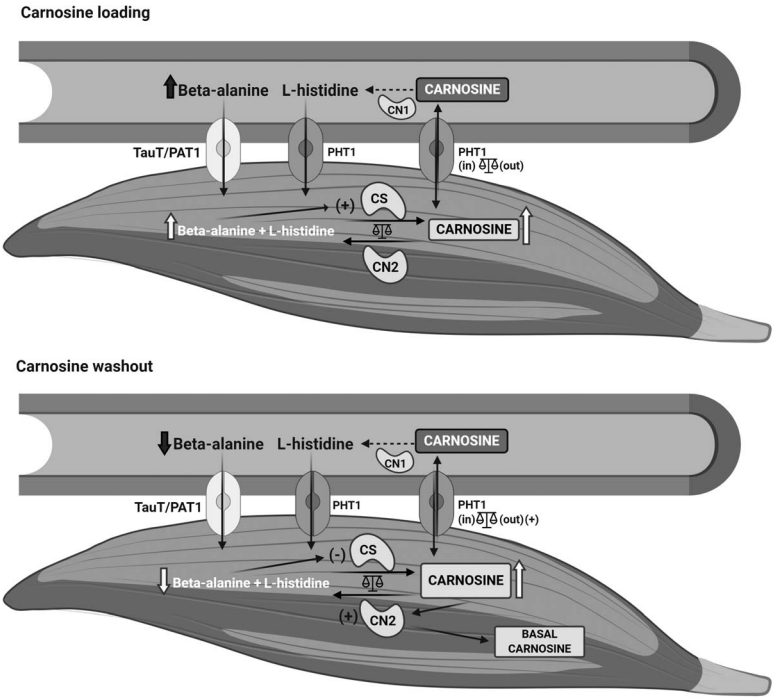

FIGURE 5—Illustration of the hypothetical mechanisms underlying carnosine loading during β-alanine supplementation (top illustration) and carnosine washout (bottom illustration). Created with BioRender.com.

that the performance-enhancing effects of M-Carn are dose dependent. This seems to strengthen the notion that pH regulation is a major ergogenic mechanism of carnosine (5,42,43) and is also aligned with previous literature indicating an association between M-Carn and performance (12,44), although further experimental evidence is warranted.

A limitation of our study is that we were unable to rigidly control the level of physical activity of our participants through the course of the study, although they verbally confirmed to have maintained their regular exercise routines. Because emerging evidence suggests that exercise might play a role in M-Carn homeostasis (37,45), we cannot rule out the possibility that physical activity had some influence on the rates of carnosine decay. M-Carn homeostasis is also influenced by sex, age, and fiber-type composition; thus, caution should be exercised when extrapolating our findings to other populations, such as athletes, women, and older individuals. Likewise, our data are limited to mixed muscle (i.e., vastus lateralis), and one should acknowledge that different muscle groups may respond differently. Moreover, we used an 8-wk, high-dose, supplementation protocol, providing a total accumulated β-alanine dose of ~360 g, which resulted in ~90% increase in M-Carn. Both the total accumulated dose and the carnosine accrual in our study were substantially greater than the doses (~90 to 180 g) and the increases in M-Carn (<40% to 60%) shown in previous studies (17–19). This might account, at least in part, for some of the differences between our results and those previously reported (17–19). Another limitation is that most participants refused to have biopsies taken at weeks 1 and 2 during the washout period, which has limited the resolution of our kinetic analysis in the early postsupplementation period.

To conclude, we showed that carnosine washout can be explained either by a linear or by an exponential decay over a 16-wk washout period. Although the linear decay presents a slightly better fit, the exponential model is more consistent with the physiological processes underlying carnosine homeostasis in skeletal muscle. The total washout time is ~12 wk and the $t_{1/2}$ is 4.6 wk, although interindividual variability exists. We also showed that changes in M-Carn correlate with changes in performance. From a practical perspective, athletes on β-alanine supplementation should consider that refraining from supplementation may negatively affect exercise performance and that interrupting supplementation for as long as 12 wk may bring carnosine levels back to presupplementation values, possibly abrogating its ergogenic effects.

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REFERENCES

1. Rezende NS, Swinton P, De Oliveira LF, et al. The muscle carnosine response to beta-alanine supplementation: a systematic review with Bayesian individual and aggregate data E-Max model and meta-analysis. *Front Physiol*. 2020;11:913.
2. Saunders B, De Salles Painelli V, De Oliveira LF, et al. Twenty-four weeks of β-alanine supplementation on carnosine content, related genes, and exercise. *Med Sci Sports Exerc*. 2017;49(5):896–906.
3. Abe H. Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. *Biochemistry (Mosc)*. 2000;65(7):757–65.
4. Artioli GG, Gualano B, Smith A, Stout J, Lancha AH Jr. Role of beta-alanine supplementation on muscle carnosine and exercise performance. *Med Sci Sports Exerc*. 2010;42(6):1162–73.
5. Dolan E, Saunders B, Dantas WS, et al. A comparative study of hummingbirds and chickens provides mechanistic insight on the histidine containing dipeptide role in skeletal muscle metabolism. *Sci Rep*. 2018;8(1):14788.
6. Smith-Ryan AE, Fukuda DH, Stout JR, Kendall KL. The influence of β-alanine supplementation on markers of exercise-induced oxidative stress. *Appl Physiol Nutr Metab*. 2014;39(1):38–46.
7. Smith AE, Stout JR, Kendall KL, Fukuda DH, Cramer JT. Exercise-induced oxidative stress: the effects of β-alanine supplementation in women. *Amino Acids*. 2012;43(1):77–90.
8. Ghodsi R, Kheirouri S. Carnosine and advanced glycation end products: a systematic review. *Amino Acids*. 2018;50(9):1177–86.
9. Carvalho VH, Oliveira AHS, de Oliveira LF, et al. Exercise and β-alanine supplementation on carnosine–acrolein adduct in skeletal muscle. *Redox Biol*. 2018;18:222–8.
10. Zapata-Sudo G, Sudo RT, Lin M, Nelson TE. Calcium-sensitizing function for the dipeptide carnosine in skeletal muscle contractility. *Cell Physiol Biochem*. 1997;91–2.
11. Dutka TL, Lamb GD. Effect of carnosine on excitation–contraction coupling in mechanically-skinned rat skeletal muscle. *J Muscle Res Cell Motil*. 2004;25(3):203–13.
12. Saunders B, Elliott-Sale K, Artioli GG, et al. β-Alanine supplementation to improve exercise capacity and performance: a systematic review and meta-analysis. *Br J Sports Med*. 2017;51(8):658–69.
13. de Andrade Kratz C, de Salles Painelli V, de Andrade Nemezio KM, et al. Beta-alanine supplementation enhances judo-related performance in highly-trained athletes. *J Sci Med Sport*. 2017;20(4):403–8.

14. Brisola GM, Artioli GG, Papoti M, Zagatto AM. Effects of four weeks of β-alanine supplementation on repeated sprint ability in water polo players. *PloS One*. 2016;11(12):e0167968.
15. de Salles Painelli V, Saunders B, Sale C, et al. Influence of training status on high-intensity intermittent performance in response to β-alanine supplementation. *Amino Acids*. 2014;46(5):1207–15.
16. Painelli Vde S, Roschel H, Jesus Fd, et al. The ergogenic effect of beta-alanine combined with sodium bicarbonate on high-intensity swimming performance. *Appl Physiol Nutr Metab*. 2013;38(5):525–32.
17. Baguet A, Reyngoudt H, Pottier A, et al. Carnosine loading and washout in human skeletal muscles. *J Appl Physiol*. 2009; 106(3):837–42.
18. Stellingwerff T, Anwander H, Egger A, et al. Effect of two β-alanine dosing protocols on muscle carnosine synthesis and washout. *Amino Acids*. 2012;42(6):2461–72.
19. Harris RC, Jones GA, Kim CK, Price KA, Wise JA. Changes in muscle carnosine of subjects with 4 weeks supplementation with a controlled release formulation of beta-alanine (Carnosyn™), and for 6 weeks post. *FASEB J*. 2009;23(1).
20. Spelnikov D, Harris RC. A kinetic model of carnosine synthesis in human skeletal muscle. *Amino Acids*. 2019;51(1):115–21.
21. da Eira Silva V, Painelli VS, Shinjo SK, et al. Magnetic resonance spectroscopy as a non-invasive method to quantify muscle carnosine in humans: a comprehensive validity assessment. *Sci Rep*. 2020; 10(1):4908.
22. Parkhouse WS, McKenzie DC, Hochachka PW, Ovalle WK. Buffering capacity of deproteinized human vastus lateralis muscle. *J Appl Physiol (1985)*. 1985;58(1):14–7.
23. Torgerson DJ, Torgerson KJ. Unequal randomisation. In: *Designing Randomised Trials in Health, Education and the Social Sciences*. London: Palgrave Macmillan; 2008.
24. Saunders B, Sale C, Harris RC, Morris JG, Sunderland C. Reliability of a high-intensity cycling capacity test. *J Sci Med Sport*. 2013;16(3):286–9.
25. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest*. 1975;35(7):609–16.
26. Mora L, Sentandreu MA, Toldra F. Hydrophilic chromatographic determination of carnosine, anserine, balenine, creatine, and creatinine. *J Agric Food Chem*. 2007;55(12):4664–9.
27. de Salles Painelli V, Nemezio KM, Pinto AJ, et al. High-intensity interval training augments muscle carnosine in the absence of dietary Beta-alanine intake. *Med Sci Sports Exerc*. 2018; 50(11):2242–52.
28. Jones GA, Smith M, Harris RC. Imidazole dipeptide content of dietary sources commonly consumed within the British diet. *Proc Nutr Soc*. 2011;70(OCE6).
29. Bürkner P. brms: An R Package for Bayesian Multilevel Models Using Stan. *Journal of statistical software*. 2017;80(1).
30. Hill CA, Harris RC, Kim HJ, et al. Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. *Amino Acids*. 2007;32(2):225–33.
31. Baumann L, Ingvaldsen T. Concerning histidine and carnosine. The synthesis of carnosine. *J Biol Chem*. 1918;35:263–76.
32. Drozak J, Veiga-da-Cunha M, Vertommen D, Stroobant V, Van Schaftingen E. Molecular identification of carnosine synthase as ATP-grasp domain-containing protein 1 (ATPGD1). *J Biol Chem*. 2010;285(13):9346–56.
33. Goncalves LS, Kratz C, Santos L, et al. Insulin does not stimulate beta-alanine transport into human skeletal muscle. *Am J Physiol Cell Physiol*. 2020;318(4):C777–86.
34. Bergstrom J, Furst P, Noree LO, Vinnars E. Intracellular free amino acid concentration in human muscle tissue. *J Appl Physiol*. 1974; 36(6):693–7.
35. Bakardjiev A, Bauer K. Transport of beta-alanine and biosynthesis of carnosine by skeletal muscle cells in primary culture. *Eur J Biochem*. 1994;225(2):617–23.
36. Blancquaert L, Baba SP, Kwiatkowski S, et al. Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by β-alanine transamination. *J Physiol*. 2016;594(17):4849–63.
37. Hoetker D, Chung W, Zhang D, et al. Exercise alters and beta-alanine combined with exercise augments histidyl dipeptide levels and scavenges lipid peroxidation products in human skeletal muscle. *J Appl Physiol*. 2018;125(6):1767–78.
38. Okumura N, Takao T. The zinc form of carnosine dipeptidase 2 (CN2) has dipeptidase activity but its substrate specificity is different from that of the manganese form. *Biochem Biophys Res Commun*. 2017;494(3–4):484–90.
39. Teufel M, Saudek V, Ledig JP, et al. Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem*. 2003;278(8):6521–31.
40. Margolis FL, Grillo M, Brown CE, Williams TH, Pitcher RG, Elgar GJ. Enzymatic and immunological evidence for two forms of carnosinase in the mouse. *Biochim Biophys Acta*. 1979;570(2):311–23.
41. Everaert I, Stegen S, Vanheel B, Taes Y, Derave W. Effect of beta-alanine and carnosine supplementation on muscle contractility in mice. *Med Sci Sports Exerc*. 2013;45(1):43–51.
42. Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. *Physiol Rev*. 2013;93(4):1803–45.
43. Harris RC, Tallon MJ, Dunnett M, et al. The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. *Amino Acids*. 2006;30(3):279–89.
44. Hobson RM, Saunders B, Ball G, Harris RC, Sale C. Effects of β-alanine supplementation on exercise performance: a meta-analysis. *Amino Acids*. 2012;43(1):25–37.
45. Salles Painelli V, Nemezio KM, Pinto AJ, et al. HIIT augments muscle carnosine in the absence of dietary beta-alanine intake. *Med Sci Sports Exerc*. 2018;50(11):2242–52.