Kinetics of muscle carnosine decay after β-alanine supplementation: a sixteen-week washout study

Running title: Sixteen weeks of muscle carnosine washout

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ABSTRACT

Purpose: To describe the kinetics of carnosine washout in human skeletal muscle over 16 weeks. **Methods:** Carnosine washout kinetics were studied in fifteen young, physically-active omnivorous men randomly assigned to take 6.4 $g \cdot d^{-1}$ of β-alanine (n=11) or placebo (PL, n=4) for 8 weeks. Muscle carnosine content (M-Carn) was determined before (PRE), immediately after (POST) and 4, 8, 12 and 16 weeks 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%; PL:+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 onwards, 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 TWD ($r=0.505$; $p=0.032$) and TTE ($r=0.72$; $p<0.001$). **Conclusion:** Carnosine washout takes 12-16 weeks to complete, and it can be described either by linear or exponential curves. Changes in M-Carn appear to be mirrored by changes in high-intensity exercise tolerance. This information can be used to optimise β-alanine supplementation strategies.

Keywords: carnosine; washout; β-alanine; human skeletal muscle.

INTRODUCTION

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 $g \cdot d^{-1}$ for ~4-10 weeks) (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), due 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 weeks after β-alanine supplementation (6.4 $g \cdot d^{-1}$ for 4 weeks). Subsequently, Baguet et al. (17) supplemented 4.8 $g \cdot d^{-1}$ of β-alanine for 5-6 weeks and, using hydrogen magnetic resonance spectroscopy (1H-MRS) 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 pre-supplementation 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 ninth week, and were predicted to reach pre-supplementation levels only in the fifteenth week. The low-responders (i.e., those whose M-Carn had increased by less than 30%, n=5), on the other hand, required only 6.5 weeks to return to the pre-supplementation levels. Also using 1H-MRS, Stellingwerff et al. (2012) (18) reported a longer washout time after an 8-week β-alanine supplementation period (1.6 or 3.2 $g \cdot d^{-1}$). The authors predicted that ~15-20 weeks 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. (2009) (17). More recently, Dmitry & Harris (2018) (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 upon 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 pre-supplementation values (6-9 weeks, with a predicted washout time of ≥15 weeks) (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.

Since 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 weeks), and using a reference method for carnosine quantification. M-Carn decay was described using a Bayesian modelling approach. A secondary aim of this study was to examine whether changes in high-intensity exercise capacity mirrors 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·week^{-1}), healthy, omnivorous men aged 18-35 years were eligible to participate. Exclusion criteria were: 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; 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 y, 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 below 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, since our primary goal was descriptive and did not depend upon 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 (figure 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 were 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 prior 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 3 or 6 participants, with the groups matched for maximum cycling power output (W_{max}; β-alanine=264.6± 47.1W; PL=250.7± 61.7W) 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 weeks with either β-alanine (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 2nd and 3rd visits, the participants were familiarized with the cycling capacity test at 110% of their individual W_{max} ($CCT_{110\%}$). During the 4th visit (before supplementation – PRE) and in the 5th visit (after supplementation – POST), participants were assessed for $CCT_{110\%}$ and M-Carn content. The 6 remaining visits were carried out 1, 2, 4, 8, 12 and 16 weeks 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. Due to 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, although 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 4 times per day at 3-4 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 weeks 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 ($p = 0.6564$).

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, Brasil). Participants then performed a graded cycling capacity test to exhaustion to determine individual W_{max}. In the 2nd and 3rd 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 hours prior to the main trials, and to abstain from caffeine intake in the 16 hours prior to 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 hours prior to 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. Germany) to determine their individual maximum 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 rev·min^{-1}) 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 above 60 rev·min^{-1}. $W_{max.}$ 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. Germany). The test began with a 5-min warm up at 100 W, followed by a 3-minute 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 seconds, followed by 95% of W_{max} for 15 seconds 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 rev·min^{-1}) throughout the test, with exhaustion occurring when they could not maintain cadence above 60 rev·min^{-1}. 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

coefficient of variation between the two familiarization sessions was 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, UK), using the Bergstrom method (25) with suction. The samples were taken from the mid-portion of the *m. 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. Due to 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.

Quantification of M-Carn content

M-Carn content was determined in a liquid chromatographer connected to a UV diode array detector (Shimadzu®, Prominance UFLC 20AD, Tokyo, Japan) using the method described by Mora et al. (2007) (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 auto sampler 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 prior to each batch of analysis using known concentrations of 50, 100, 500, 1.000 and 2.500 $\mu mol \cdot l^{-1}$ 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, USA) attached to an Atlantis Silica column guard (4.6 x 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 CV 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-day 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 BIC 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-by-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 3 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 standard deviation. 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 ± standard deviation (with 95% confidence intervals – CI) and the significance level was set *a priori* at $p<0.05$.

Additionally, a linear and an exponential Bayesian fit 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 $f(x)=b_1e^{-b_2x}+b_3$), 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 pre-supplementation M-Carn mean (offset: 20.44 mmol·kg^{-1} DM) from the post-supplementation 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=0, sd=1) and, since the fit was performed after the removal of the offset, b_3 distribution was not relevant. Several other values were tested as priors, 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-by-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-week washout period. In the PL group, no significant differences were shown between any of the time points (all $p>0.05$). M-Carn after 12 and 16 weeks of washout were not statistically different from PRE. M-Carn loading and washout data are shown in figure 3, panels A 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.

Modelling the kinetics of muscle carnosine washout

The leave-one-out information criterion (LOOIC) was used to estimate the prediction accuracy from two fitted Bayesian models of carnosine decay during 16 weeks, where one model was a linear decay and the other model was an exponential decay. LOOIC (lower values indicate better fit) was 395.27 (standard error=9.19) for the linear model and 398.22 (standard error=9.00) for the exponential model, with the difference between models being -2.95 (standard error=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 better than the exponential model (figure 3, panel C). The $t_{1/2}$ for M-Carn washout in the exponential decay model was calculated to be 4.6 weeks (95%CI:3.2-7.0).

Muscle carnosine content and exercise performance

A visual inspection of the absolute changes in performance during the washout period suggests a close association between performance changes with the changes in M-Carn content during the same period (figure 4, panels A and C). Repeated measures correlation analysis revealed a moderate, significant correlation between TWD and M-Carn ($r=0.505$, $p=0.032$; figure 4, panel B) and between TTE and M-Carn ($r=0.72$, $p<0.001$; figure 4, panel D). 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 are mirrored by similar changes in performance.

Dietary intake

No significant group-by-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) (Supplemental Digital Content 2).

DISCUSSION

In this study, we investigated the washout kinetics of muscle carnosine for 16 weeks 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 (HPLC), 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 muscle carnosine content is relatively stable over time (16-18). In our study, complete washout of carnosine occurred within a mean time of ~12 weeks, 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) weeks in the exponential decay model in our study, which is not too dissimilar to the 5.8 weeks reported by Baguet et al. (17), but somewhat shorter than the 8.6 weeks reported by Harris et al. (19). We also provided evidence for the association between M-Carn and high-intensity exercise performance following 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 weeks 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 upon 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 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-week washout period used in this study and up until M-Carn returns to the pre-supplementation levels. In the longer term, an exponential decay would probably better describe M-Carn washout kinetics as M-Carn tends to return to pre-supplementation 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 weeks 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 catalysed 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 towards 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 appears 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 favouring 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 non-specific and has 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 hydrolyse carnosine in murine tissues, such as 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 pre-supplementation 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. While 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 higher activity of CN2, therefore explaining the overall imbalance between carnosine synthesis and degradation in favour of degradation. Since 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 towards 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.

Since 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 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 is 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. Since 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 fibre type composition; thus, caution should be exercised when extrapolating our findings to other populations, such as athletes, women, and older individuals. Likewise, our data is limited to mixed muscle (i.e., vastus lateralis) and one should acknowledge that different muscle groups may respond differently. Moreover, we used an 8-week, 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 post-supplementation period.

To conclude, we showed that carnosine washout can be explained either by a linear or by an exponential decay over a 16-week 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 weeks and the $t_{1/2}$ is 4.6 weeks, 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 impact exercise performance, and that

494 interrupting supplementation for as long as 12 weeks may bring carnosine levels back to
495 pre-supplementation values, possibly abrogating its ergogenic effects.

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Figure 1. Flow diagram indicating participants' enrollment in the study.

635

636 **Figure 2.** Overview of the study design. The numbers indicate how many participants
637 showed up for muscle biopsies and for the $CCT_{110\%}$ at each time point in each group.
638 W_{max} = maximum power output attained in a graded exercise test to exhaustion on a
639 cyclo ergometer. CCT110% = time-to-exhaustion exercise tolerance test on a cycle
640 ergometer at 110% of the W_{max}. BA = β-alanine group. PL = placebo group.

641

642 **Figure 3. Panel A:** individual muscle carnosine responses to 8 weeks of β-alanine or
643 placebo supplementation followed by 16 weeks of washout. **Panel B:** mean ± standard
644 deviation responses to 8 weeks of β-alanine or placebo supplementation followed by 16
645 weeks of washout. **Panel C:** Linear and exponential fitted models for muscle carnosine
646 decay in the washout period. Gray areas represent the upper and lower limits of the
647 expected values of the posterior predictive distribution.
648 All results are expressed relative to dry muscle weight.
649 * significantly different from the previous time point (within-group effect)
650 # significantly different from PRE (within-group effect)
651 $ significantly different from β-alanine group in the same time point (between-group
652 effect)
653 $$ p=0.06 *vs.* β-alanine in the same time point (between-group effect)
654 ES=between-group Cohen's effect sizes

655

656 **Figure 4.** Absolute changes in muscle carnosine content are mirrored by changes in
657 performance, as assessed by total work (TW, panel A) and time to exhaustion (TTE,
658 panel C). Muscle carnosine content was moderately and significantly correlated with

TW and TTE, as depicted in the repeated measures correlation analysis chart (panels C and D).

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.

Supplemental Digital Content 1: Illustration of the location where the multiple biopsies were taken. In some participants, 6 or 7 biopsies were taken, depending on whether they showed up for biopsies 1 and 2 weeks in the washout period. Created with BioRender.com.

Supplemental Digital Content 2: Daily energy, macronutrient and β-alanine intake in the β-alanine and placebo groups across the study period. Data from the 3-day diaries were averaged and considered as daily intake. Data are presented as mean ± standard deviation.

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Conflict of interest: All authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. Although not directly related to this study, CS has received funding to support a PhD studentship relating to the effects of carnosine on cardiac function from Natural Alternatives International; a company formulating and manufacturing customised nutritional supplements, including (Carnosyn SR™) beta-alanine. The same company has also provided CS and BG with supplements for other studies free of charge and has contributed to the payment of open access publication charges for some manuscripts on beta-alanine supplementation. The results of the present study do not constitute endorsement by ACSM.

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