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1 **The role of chronic muscle (in)activity on carnosine homeostasis: a study with**
2 **spinal-cord injured athletes**

3

4 **Running title:** Chronic muscle inactivity and muscle carnosine content

5

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35 **Abstract**

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37 To examine the role of chronic (in)activity on muscle carnosine (MCarn) and how
38 chronic (in)activity affects MCarn responses to β -alanine supplementation in spinal-
39 cord injured athletes, sixteen male athletes with paraplegia were randomized (2:1 ratio)
40 to receive β -alanine (n=11) or placebo (PL, n=5). They consumed $6.4 \text{ g}\cdot\text{d}^{-1}$ of β -alanine
41 or PL for 28 days. Muscle biopsies of the active *deltoid* and the inactive *vastus lateralis*
42 (VL) were taken before and after supplementation. MCarn in the VL was also compared
43 with the VL of a group of individuals without paraplegia (n=15). MCarn was quantified
44 in whole muscle and in pools of individual fibers by High-performance Liquid
45 Chromatography. MCarn was higher in chronically inactive VL vs. well-trained *deltoid*
46 (32.0 ± 12.0 vs. $20.5\pm 6.1 \text{ mmol}\cdot\text{kg}^{-1} \text{ DM}$; $p=0.018$). MCarn was higher in inactive vs.
47 active VL (32.0 ± 12.0 vs. $21.2\pm 7.5 \text{ mmol}\cdot\text{kg}^{-1} \text{ DM}$; $p=0.011$). In type-I fibers, MCarn
48 was significantly higher in the inactive VL than in the active *deltoid* (38.3 ± 4.7 vs.
49 $27.3\pm 11.8 \text{ mmol}\cdot\text{kg}^{-1} \text{ DM}$, $p=0.014$). MCarn increased similarly between inactive VL
50 and active *deltoid* in the β -alanine group (VL: $68.9\pm 55.1\%$, $p=0.0002$; *deltoid*:
51 $90.5\pm 51.4\%$, $p<0.0001$), with no changes in the PL group. MCarn content was higher in
52 the inactive VL than in the active *deltoid* and the active VL, but this is probably a
53 consequence of fiber type shift (type I to type II) that occurs with chronic inactivity.
54 Chronically inactive muscle showed an increase in MCarn after BA supplementation
55 equally to the active muscle, suggesting that carnosine accretion following β -alanine
56 supplementation is not influenced by muscle inactivity.

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59 **Keywords:** muscle inactivity, muscle activity, carnosine, β -alanine, homeostasis.

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69 **Introduction**

70 Carnosine is a multifunctional dipeptide abundantly expressed in human skeletal
71 muscle, where it is thought to play important physiological roles, including pH
72 regulation (1, 12) , Ca²⁺ handling (14) and reactive aldehyde detoxification (3, 9). The
73 availability of β-alanine, the rate-limiting precursor of carnosine synthesis, is the most
74 influential factor affecting muscle carnosine content (MCarn) (22). Studies have
75 consistently shown that β-alanine supplementation increases MCarn (33), which has
76 been associated with improved high-intensity exercise performance (34) and potentially
77 improved health (2). Conversely, decreased MCarn during the washout period following
78 β-alanine has been associated with the return of exercise tolerance to pre-
79 supplementation levels (37).

80 While β-alanine intake, either ingested from food or supplements, increases
81 MCarn in a dose-dependent, saturable fashion (33), it remains unclear whether other
82 stimuli can also affect MCarn. There is evidence indicating that exercise can modulate
83 carnosine homeostasis, with cross-sectional studies showing that sprint- and strength-
84 trained athletes have higher MCarn than sedentary and endurance-trained individuals
85 (31, 36). Although this is suggestive of an effect of high-intensity exercise on carnosine
86 synthesis, possibly due to an effect of long-term exposure to acidosis as an adaptive
87 trigger, several subsequent longitudinal studies did not confirm that chronic exercise
88 training can increase MCarn (4, 15, 20, 27-29). The lack of control over the dietary
89 intake of β-alanine, the lack of control for fiber type shifting alongside training, and
90 purportedly insufficient training stimuli might, however, limit the interpretation of these
91 studies. Type II fibers contains approximately 1.5 times more carnosine than type I
92 fibers (21, 23), which highlights the importance of accounting for changes in fiber type
93 distribution when fiber shift can occur.

94 De Salles Painelli et al. (11) showed that 12 weeks of high-intensity interval
95 training increased MCarn in vegetarians, a population that consumes virtually no β-
96 alanine in the diet. In contrast, Hoetker et al. (24) showed that low-intensity training
97 increased muscle carnosine, while high-intensity training decreased muscle carnosine.
98 The potential reasons for the disparity between these two studies are not immediately
99 apparent and it is clear that there is a requirement to further investigate whether or not
100 acute exercise can modulate the carnosine content in skeletal muscle.

101 It also remains uncertain whether exercise training or training status can affect
102 carnosine accretion in response to β-alanine supplementation. Bex et al. (7) showed that

103 muscle groups under higher training loads respond to β -alanine supplementation with
104 greater carnosine accretion in comparison with muscle groups under lower training
105 loads. Bex et al. (6) showed increased carnosine accretion in response to β -alanine
106 supplementation in individuals who undertook exercise training when compared with
107 non-trained controls. In both studies (6, 7), muscle carnosine was measured with
108 magnetic resonance spectroscopy, a method that has been shown to be less reliable than
109 more direct measures of carnosine content, such as chromatography-based techniques
110 from muscle biopsy samples (10). In contrast, using high-performance liquid
111 chromatography (HPLC), Kendrick et al. (28) showed similar carnosine accretion
112 between trained and untrained muscle groups in response to β -alanine supplementation.
113 Out of the inconsistent findings in the literature, to date, it remains unknown whether
114 physical activity modulates MCarn or whether it enhances MCarn responses to β -
115 alanine supplementation.

116 To understand the role of muscle activity on muscle carnosine homeostasis, we
117 examined the extremes of the muscle activity spectrum (*i.e.*, chronic long-term athletic
118 training *vs.* chronic long-term muscle inactivity) by investigating well-trained athletes
119 with spinal-cord injury. In these athletes we compared their chronically trained muscle
120 (*deltoid*) with their chronically inactive muscle (*vastus lateralis*), both at baseline and in
121 response to β -alanine supplementation. We hypothesized that paralyzed muscles would
122 display lower carnosine levels at baseline and reduced carnosine loading in response to
123 β -alanine supplementation, in comparison with active muscles.

124

125 **Methods**

126

127 Participants

128 Twenty-three men with spinal cord injury and paraplegia were screened for
129 eligibility. Inclusion criteria were: 1) participation in a structured exercise training
130 program for ≥ 6 h per week for ≥ 6 months prior to participation; 2) spinal-cord injury
131 with loss of motor function of the lower limbs corresponding to the American Spinal
132 Cord Injury Association Scale A or B for ≥ 1 year and 3) age 18-45 years. Exclusion
133 criteria were: 1) diagnosed with a chronic disease that would preclude participation in
134 the study and 2) use of supplements containing creatine or β -alanine ≤ 6 months prior to
135 participation. Before signing the written consent form, all participants were fully
136 informed about benefits and risks involved with participation. All procedures were

137 approved by the Institutional Ethics Committee before the commencement of the study
138 (#41495115.0.0000.5391) and complied with the Declaration of Helsinki.

139 One individual did not meet the inclusion criteria (sedentary) while six other
140 individuals were unavailable for participation. Sixteen volunteers were then randomly
141 allocated in a 2:1 ratio to receive β -alanine (n=11) or placebo (n=5). An unbalanced 2:1
142 allocation ratio was used due to the limited potential participant pool for this study, the
143 invasive nature of the study and to increase the number of observations in the β -alanine
144 group. Two participants (one from each group) dropped-out after the initial biopsy
145 session and therefore did not participate in the post-supplementation analyses. We were
146 unable to obtain usable muscle samples from the *vastus lateralis* of 3 participants (one
147 from the β -alanine group and 2 from the placebo group).

148 The participants had been training for 10.5 ± 3.7 years at the time of the study; all
149 of them reported that they performed upper-body strength, arm crank, mobility and
150 shoulder stability training 2-5 times per week in addition to their sport-specific training,
151 totalling 573 ± 102 min of training per week. All participants reported the use of passive
152 leg exercises, but none of them used electrical stimulation of the lower limbs.
153 Participant general characteristics are displayed in Table 1, while specific information
154 about individual spinal cord injury is displayed in Table 2.

155

156 **Table 1:** Participant general characteristics.

	β-alanine (n=11)	Placebo (n=5)	p
Age (y)	36.1 ± 3.9	34.6 ± 8.1	0.62
Body mass (kg)	71.9 ± 11.8	67.8 ± 11.9	0.52
Length (cm)	176 ± 8.6	172 ± 6.9	0.38
BMI (kg/m²)	23.2 ± 3.0	22.9 ± 3.8	0.89
Time since injury (y)	14.1 ± 3.7	13.4 ± 7.3	0.80
VO_{2peak} (L·min⁻¹)	1.3 ± 0.6	1.7 ± 0.8	0.30
Power_{max} (W)	71.2 ± 42.5	91.1 ± 53.7	0.44

157 BMI: body mass index; Power_{max}: maximum load attained in a progressive arm-crank
158 exercise test to volitional fatigue. p-values refer to independent samples *t* tests.

159

160

161

162

163 **Table 2:** Individual spinal cord injury characteristics and training status of each
 164 participant.

Participant	ASIA classification	Lesion level	Training status	Sport
β -alanine				
1	B	C6-C7	Amateur athlete	Artistic Gymnastics
2	A	T12	Competitive athlete	Cross-training
3	B	T12-L1	Competitive athlete	Wheelchair basketball
4	B	L1-L2	Competitive athlete	Wheelchair basketball
5	B	C6-C7	Amateur athlete	Brazilian Jiu Jitsu
6	B	T3	Competitive athlete	Endurance hand-bike
7	B	C6-C7	Competitive athlete	Wheelchair rugby
8	B	T2-T3	Competitive athlete	Wheelchair rugby
9	A	C7	Competitive athlete	Wheelchair rugby
10	A	C5	Competitive athlete	Wheelchair rugby
11	A	C6-C7	Competitive athlete	Wheelchair rugby
Placebo				
12	B	T12-L1	Competitive athlete	Paralympic archery
13	B	T5-T6	Competitive athlete	Endurance hand-bike
14	B	L2	Competitive athlete	Wheelchair basketball
15	B	T9	Amateur athlete	Endurance hand-bike
16	B	C7	Competitive athlete	Wheelchair rugby

165 ASIA: American Spinal Cord Injury Association.

166

167 Experimental design

168 This study was designed to test the influence of chronic exercise training and
 169 muscle inactivity on 1) MCarn and 2) MCarn accumulation in response to β -alanine
 170 supplementation. Thus, we first performed a cross-sectional analysis to compare MCarn
 171 in trained (*deltoid*) vs. paralyzed (*vastus lateralis*) muscles in highly trained athletes
 172 with paraplegia. Since this within-individual analysis only allows for the comparison
 173 between different muscle groups (*i.e.*, *deltoid* vs. *vastus lateralis*), we conducted a
 174 second cross-sectional analysis to compare MCarn between paralyzed vs. active muscles
 175 in the same muscle group (*i.e.*, *vastus lateralis*). Thus, we compared MCarn in athletes
 176 with paraplegia vs. MCarn in a group of physically active individuals without paraplegia
 177 (n=15 healthy, physically active men; age=28 \pm 5 y; body mass=76.2 \pm 17.3 kg;

178 BMI=24.8±3.9) who took part in another study that was being conducted at the same
179 time in our lab and was published elsewhere (37).

180

181 In addition to the cross-sectional analyses, we conducted a double-blind,
182 placebo-controlled trial with athletes with paraplegia to compare MCarn in response to
183 β -alanine supplementation in their trained *vs.* paralyzed muscle groups. To account for
184 the effects of chronic training and paralysis on fiber type distribution and for the
185 influence of the higher carnosine levels in type II fibers (11, 22), MCarn was
186 determined not only in whole muscle, but also in single muscle fibers. Groups were
187 equalized according to the $Power_{max}$ by ranking the participants within blocks of 4
188 individuals and using the block randomization method (www.random.org). All
189 participants with paraplegia were asked to attend to the laboratory on three different
190 occasions. In the first visit, body mass, body length and peak oxygen consumption were
191 measured. In a second visit, 2-7 days apart, muscle biopsies of the *deltoid* (trained
192 muscle) and the *vastus lateralis* (paralyzed muscle) were taken for pre-supplementation
193 (PRE) MCarn determination. A 28-day β -alanine supplementation period commenced
194 on the next day, after which (POST) another biopsy of the *deltoid* and the *vastus*
195 *lateralis* was taken. All athletes and coaches were instructed to maintain athletes'
196 training regimens and dietary habits across the supplementation period, which was
197 verbally confirmed upon their visit after supplementation. The experimental procedures
198 for the participants without paraplegia were described elsewhere (37), but in this study
199 we are only interested in their baseline MCarn as this will serve as a reference of typical
200 values in non-paralyzed *vastus lateralis*.

201

202 Preliminary Testing

203 Body mass was measured to the nearest 100 g on a digital scale (100 CH,
204 Welmy, São Paulo, Brazil). Wheelchair weight was first measured without the
205 volunteer. When stabilized, the scale display was reset and the volunteer was transferred
206 back to the wheelchair to determine body mass. Length was measured as the distance
207 between the top of the head of the bottom of the feet, with the individual laid down on a
208 gurney with their knees fully extended and their feet beside one another.

209 A maximal incremental test was conducted in a mechanically braked arm
210 ergometer (EB 4100, Cefise, Brazil) as previously described (32). The test was initiated
211 with a load corresponding to 25 W (0.3 kilopond at 85 rev·min⁻¹), followed by 25 W

212 increments every minute until volitional exhaustion or until the participant could no
213 longer maintain 85 rev·min⁻¹. Breath by breath gas exchange was measured in a gas
214 analyzer (Quark CPET, Cosmed, Rome, Italy) calibrated according to manufacturer
215 instructions. VO_{2peak} was considered the arithmetic mean of the least 30 seconds of the
216 test and Power_{max} was the maximum power attained.

217

218 Supplementation protocol

219 Participants consumed 6.4 g·d⁻¹ of either β-alanine (SR CarnoSyn®, Natural
220 Alternatives International, Inc, Carlsbad, CA) or placebo (maltodextrin, Natural
221 Alternatives International, Inc, Carlsbad, CA) for 28 days, totalling an accumulated
222 dose of 179.2 g. The total daily dose was split into 4 individual doses of 1.6 g provided
223 in 0.8 g sustained release tablets, identical in number and appearance. All participants
224 completed a supplementation log; compliance with supplementation was 98±2% of the
225 total dose in β-alanine group and 97±1.5% in placebo group. At the end of the
226 supplementation period, the participants reported which substance they believed they
227 were taking. Three participants in the β-alanine group and 2 in the placebo groups
228 correctly guessed their treatments (Fisher's exact: p=0.6). Two out of the 16 participants
229 reported slight symptoms of paresthesia, one from β-alanine group and the other from
230 the placebo group.

231

232 Muscle biopsies

233 Approximately 70-100 mg of wet muscle were obtained from the mid portion of
234 the *vastus lateralis* and from the most lateral and voluminous part of the *deltoid* under
235 local anesthesia (3 ml lidocaine 1%), using the percutaneous Bergstrom needle biopsy
236 technique (5) modified with suction. Samples were obtained PRE and POST
237 supplementation from the same locations, as close as possible to one another, and
238 immediately frozen in liquid nitrogen. Before analyses, the samples were freeze dried
239 overnight for 16 h, and then all visible blood, fat and connective tissue were removed.

240

241 Quantification of MCarn in whole muscle

242 Carnosine was quantified in extracts of whole muscle by High-performance
243 Liquid Chromatography (LC-10vp, Shimadzu, Japan) coupled with ultraviolet detection
244 at 214 nm (SPD-10vp, Shimadzu, Japan), using the method described by Mora et al.
245 (30). Approximately 3-5 mg of freeze-dried muscle were manually powdered and

246 deproteinized in an acid extraction solution [0.5 M HClO₄, 1 mM EDTA; 1:50 sample
247 weight (mg):volume (μl)] via intermittent vortex bursts for 10 min (30s on, 30 s resting
248 on ice). The extracts were then centrifuged (3 min, 5000 g at 4° C) and the supernatant
249 was then neutralized with 2.1 M KHCO₃ (1:4 v:v) and filtered through a 0.2 μm pore
250 size centrifugal filter tube. Chromatographic separation was performed using an Atlantis
251 HILIC silica column (4.6 × 150 mm, 3 μm; Waters, Massachusetts, USA) and an
252 Atlantis Silica column guard (4.6 × 20 mm, 3 μm). Briefly, the method uses mobile
253 phase A [0.65 mM ammonium acetate, in water/acetonitrile (25:75) (v/v)], and mobile
254 phase B [4.55 mM ammonium acetate, in water/acetonitrile (70:30)], both adjusted to
255 pH 5.5. The separation condition was: linear gradient from 0 to 100% of mobile phase B
256 for 13 min at a flow rate of 1.4 mL·min⁻¹. All samples were analyzed in duplicate with
257 the intra-assay coefficient variation being 2.03% and the coefficient of variation
258 between different biopsies from the same site being 3.95% (10).

259

260 Quantification of MCarn in pools of individual fibers

261 Approximately 30-40 fibers were isolated and cut in two halves (0.5-1 mm). One
262 half was used for MHC determination while the remaining piece was weighed on a
263 quartz-fiber fish-pole balance to the nearest 0.01 μg, as previously described (11, 28).
264 Fibers of the same MHC type were pooled and metabolite was extracted by adding
265 ultrapure water and vortexing for 10 min (30-s bursts interspersed with 30-s cooling
266 periods on ice). Carnosine was then quantified by HPLC (Hitachi, Schaumburg, IL)
267 with pre-column derivatization coupled to a fluorescence detector as previously
268 described (13). Metabolite separation was undertaken in a Hypersil ODS analytical
269 column (3 μm, 150 x 4.6 mm I. D., Shandon, Runcom, UK) at 23 °C. A binary gradient
270 formed from solvent A [12.5 mM sodium acetate, pH 7.2 tetrahydrofuran (995:5, v/v)]
271 and solvent B [12.5 mM sodium acetate, pH 7.2 - methanol-acetonitrile (500:350:150,
272 v/v)] was used with the following gradient: 0 to 1.5 min, 0% solvent B; 1.5 to 10 min,
273 35% B; 10 to 26 min, 60% B; 26 to 30 min, 100% B; 30 to 35 min, 100% B; 35 to 45
274 min, 0% B. Flow-rate was 2.0 ml·min⁻¹ in the initial 10 minutes, and gradually
275 decreased to 1.0 ml·min⁻¹ at 26 min until finish. Excitation and emission wavelengths
276 were 340 nm and 450 nm. The derivatization reagent was kept in the dark at 2°C and
277 prepared by mixing 80 μL of OPA (40mg) plus absolute ethanol (800 μL) to 4 μL of β-
278 mercaptoethanol and 1 mL of a 0.4M borate buffer (pH 9.65). Extract and reagent (1:1
279 v:v) were reacted for 30 s prior to injection. Fresh derivatization reagent was used with

280 each new sample batch. All samples were analyzed in duplicate and the intra-assay
281 coefficient of variation was 8.8% in pools of single fibers. Quantification of both
282 chromatographic methods was performed by integrating peak areas.

283

284

285 Myosin heavy-chain isoform characterization

286 Myosin heavy-chain isoform was characterized in single fibers and in whole
287 muscle by dissolving (mixing with a vortex) a half fiber or ~3 mg of freeze-dried
288 muscle sample in extraction buffer (0.06 M tris-hydroxymethylaminomethane pH 6.8,
289 1% w/v sodium dodecyl sulphate, 0.6%w/v EDTA, 15% w/v glycerol, 5% v/v
290 mercaptoethanol, and bromophenol blue) followed by SDS-PAGE electrophoresis, as
291 described by Kendrick et al. (28). MHC isoform type was determined as described by
292 Galpin et al (16). Ten micro-litters of the extract were loaded in a polyacrylamide gel
293 and ran for ~28h at 4°C. Protein was revealed using a silver staining kit (PlusOne,
294 Cytiva). Band intensity was quantified in the ImageJ software and used for individual
295 fiber typing or for whole muscle MHC distribution.

296

297 Dietary β -alanine intake assessment

298 β -alanine intake was assessed with three food diaries undertaken on 3 non-
299 consecutive days (two weekdays and one weekend day) following the instructions of a
300 registered nutritionist. β -alanine intake through consumption of fish, poultry and meat
301 was estimated from the data of Jones et al. (25).

302

303 Statistical analysis

304 Participants general characteristics (age, body mass, length, body mass index,
305 VO_{2peak} , $Power_{max}$, and time since injury) were compared between groups with unpaired
306 t-tests. Linear mixed models (proc mixed, SAS University Edition) were used to
307 compare whole muscle MCarn between *deltoid* and *vastus lateralis* before and after β -
308 alanine or placebo supplementation. Group, time and muscle group were fixed factors,
309 whilst participants were random factors. Linear mixed models were also used for dietary
310 β -alanine intake data, with group and time being fixed factors. Four covariance matrices
311 (unstructured, compound symmetric, autoregressive and toeplitz) were tested, with the
312 best fit being chosen using the lowest BIC (Bayesian Information Criteria) value.

313 Hypothesis-driven, single degree-of-freedom contrast analysis was used to identify
314 significant differences whenever a significant main interaction effect was observed.
315 Delta post-pre supplementation was calculated and compared between muscle groups
316 with Welch unpaired t-test for unbalanced samples (RStudio v.4.0.0).

317 For MCarn measured in pools of single fibers, linear mixed models did not
318 converge (due to unbalanced sample sizes and missing sub-type fiber data, as most
319 samples had no type I or type II MHC), meaning that data sets for each fiber type were
320 analyzed separately. For the baseline data, β -alanine and placebo groups were pooled
321 and a Welch unpaired t-test (RStudio v.4.0.0) for unbalanced sample sizes was used to
322 compare MCarn between the *deltoid* and *vastus lateralis* muscles and to compare
323 MCarn in the *vastus lateralis* between athletes with paraplegia and individuals without
324 paraplegia; MCarn in type I vs. type II fibers were compared within the same muscle
325 group of the same individuals with t-test for dependent samples (RStudio v.4.0.0). For
326 the MCarn responses to β -alanine supplementation, fast MHC subtypes were pooled and
327 delta post-pre supplementation was deemed as the response variable (data from the
328 placebo group was excluded due to very low or zero number of observations in some
329 instances) and compared with a two-way general linear model (type III, for unbalance
330 samples) where muscle group (*deltoid* vs. *vastus lateralis*) and fiber type (type I vs. type
331 II) were fixed factors (SPSS v.14). Data are reported as mean \pm SD and the significance
332 level was set at $p<0.05$.

333

334 **Results**

335

336 Role of chronic muscle (in)activity on muscle carnosine

337 In the athletes with paraplegia, MCarn was significantly higher in the
338 chronically inactive *vastus lateralis* muscle in comparison with their well-trained
339 *deltoid* muscle (main effect of muscle group: $F=10.7$, $p=0.006$; figure 1, panel A).
340 Higher MCarn was also shown in the inactive *vastus lateralis* when compared to the
341 active *vastus lateralis* of individuals without paraplegia ($p=0.011$, 95%CI: 2.7–18.8;
342 figure 1, panel B).

343

344 **Figure 1:** Individuals (white circles) and mean \pm SD group values for carnosine content
345 in whole muscle. Panel A: cross-sectional analysis comparing the active *deltoid* muscle
346 and the paralyzed *vastus lateralis* muscle in athletes with paraplegia (p-value refer to

347 the main effect of muscle group in the mixed models). Panel B: cross-sectional analysis
348 comparing the paralyzed *vastus lateralis* muscle in athletes with paraplegia vs the active
349 *vastus lateralis* in a group of physically active participants without paraplegia that took
350 part in another study (data published in Yamaguchi et al. 2020 (37); p-value refer to
351 Welch unpaired t-tests for unbalanced samples).

352 Note: data from athletes with paraplegia refer to baseline (pre-supplementation) β -
353 alanine and placebo groups pooled.

354

355 Importantly, chronic muscle inactivity in spinal cord injury was associated with
356 a substantive increase in fast MHC isoform distribution (Figure 2), which could
357 influence whole muscle MCarn and become a confounding factor in our analysis, since
358 MCarn is ~ 1.5 times higher in type II than in type I fibers (21, 23). Therefore, we also
359 determined MCarn in single fibers. In type I fibers, MCarn was significantly higher in
360 the inactive *vastus lateralis* muscle in comparison with the active *deltoid* muscle
361 ($p=0.014$, 95%CI: -19.5—2.6; figure 3, panel A). There were no significant differences
362 in MCarn in type IIa between the active and inactive muscles ($p=0.851$, 95%CI: -12.7–
363 15.2; figure 2, panel B), as was the case for hybrid type IIx/IIa fibers ($p=0.53$, 95%CI: -
364 14.1–7.7; figure 3, panel C). We were unable to compare carnosine content in type IIx
365 fibers because they were present in only 2 out of the 16 samples.

366

367

368 **Figure 2.** Representative images of silver-stained electrophoresis gel for MHC typing in
369 whole muscle and in single fibers (panel A). MHC isoform distribution in the active
370 deltoid muscle and in the paralyzed *vastus lateralis* muscle in athletes with paraplegia
371 (β -alanine and placebo pooled) at baseline (panels B and C).

372

373

374 **Figure 3:** Individual (white circles) and mean \pm SD group values for muscle carnosine
375 content in pools of single fibers in the paralyzed *deltoid* muscle and in the active *vastus*
376 *lateralis* muscle in athletes with paraplegia. Panels A, B and C display comparisons
377 between muscle groups. Panels D and E display comparisons within muscle groups.

378 Note: Data refer to baseline (pre-supplementation) β -alanine and placebo groups pooled.

379 p-values for between-muscle group comparisons were calculated with Welch unpaired t-

380 tests for unbalanced samples while p-values for within-muscle group comparisons were

381 calculated with t-test for dependent samples. Different sample sizes are due to the
382 absence of fiber types in some of the samples.

383

384 Role of chronic muscle (in)activity on muscle carnosine responses to β -alanine
385 supplementation

386 Significant increases in MCarn were shown in both *deltoid* and *vastus lateralis*
387 muscles in the β -alanine, but not in the placebo group (group-by-time-by-muscle
388 interaction: $F=6.61$, $p=0.0045$; figure 4, panel A). Between-group contrast analyses
389 confirmed that post-supplementation MCarn was significantly higher in the β -alanine
390 group than in placebo group (*deltoid*: $p=0.0046$; *vastus lateralis*: $p=0.024$). MCarn
391 increased $68.9\pm 55.1\%$ in the *vastus lateralis* (within-group effect: $p<0.0001$) and
392 $90.5\pm 51.4\%$ in the *deltoid* (within-group effect: $p=0.0002$) in the β -alanine group. No
393 significant differences were shown for the delta changes between the *deltoid* and *vastus*
394 *lateralis* in either the β -alanine ($p=0.653$; 95%CI:-10.6–6.9; figure 4, panel B) or
395 placebo ($p=0.623$; 95%CI:-6.8–4.6; figure 4, panel B) group.

396

397

398 **Figure 4:** Individual (white circles) and mean \pm SD muscle carnosine content before and
399 after β -alanine supplementation in *deltoid* and *vastus lateralis* in the β -alanine and
400 placebo groups (panel A). Post-pre absolute delta changes in muscle carnosine in the β -
401 alanine and placebo groups (panel B).

402 Note: In panel A, group-by-time-by-muscle interaction effect: $F=6.61$, $p=0.0045$; p -
403 values were calculated with single-degree-of-freedom contrast analyses in the mixed
404 models. In panel B, p -values were calculated with Welch unpaired t-tests for unbalanced
405 samples.

406

407 To account for the effect of MHC isoform distribution on the MCarn responses
408 to β -alanine supplementation, MCarn accretion was compared in pools of single fibers
409 between the *deltoid* and *vastus lateralis*. No effects of fiber type ($F=0.40$, $p=0.53$),
410 muscle group ($F=0.19$, $p=0.68$) or fiber type by muscle group interaction ($F=2.37$,
411 $p=0.13$) were shown (figure 5).

412

413

414 **Figure 5:** Individual (white circles) and mean±SD single fiber muscle carnosine
415 accretion in response to β-alanine supplementation in *deltoid* and *vastus lateralis*. Type
416 II fibers represent IIa and hybrid IIa/IIx fibers pooled.

417 General linear model: fiber type (F=0.40, p=0.53); muscle group (F=0.19, p=0.68); fiber
418 type by muscle group interaction (F=2.37, p=0.13).

419

420 Dietary β-alanine intake

421 The estimated β-alanine intake was similar between groups and across the study
422 period (PRE: β-alanine=0.8±0.6g, placebo:0.4±0.3g; POST: β-alanine=1.1±1g;
423 placebo:0.4±0.2g) (main effect of group: F=2.71, p=0.128; group by time interaction:
424 F=0.2, p=0.664).

425

426 **Discussion**

427 By comparing a habitually highly active muscle (the *deltoid*) with a chronically
428 inactive muscle (the *vastus lateralis*) in athletes with spinal cord injury, we have been
429 able to show that not only chronic muscle inactivity does not result in a significant
430 reduction in MCarn, but that the MCarn content in the chronically inactive *vastus*
431 *lateralis* muscle was higher than in the highly active *deltoid* muscle. This result was
432 further confirmed in a retrospective analysis that showed higher MCarn in the paralyzed
433 *vastus lateralis* muscle of athletes with paraplegia than in the *vastus lateralis* muscle of
434 physically active individuals without paraplegia. Whilst this result might not necessarily
435 seem intuitive, previous studies have shown that prolonged muscle inactivity (>10
436 months) results in a dramatic muscle fiber type shift, from type I to type II (19, 35).
437 Indeed, we showed that 95% of the fibers in the chronically inactive *vastus lateralis*
438 muscle were fast MHC, whilst the distribution was closer to 50:50 (slow:fast MHC) in
439 the highly active *deltoid* muscle and it is typically 40:60 (slow:fast) in non-paralyzed
440 *vastus lateralis* (8). This suggests that higher MCarn content in the inactive *vastus*
441 *lateralis* could be, at least in part, explained by a type I to II shift, suggesting that it is
442 the predominance of type II muscle fibers in paralyzed muscles that could account for
443 the elevated MCarn to a much greater extent than muscle paralysis *per se*. Increased
444 type II fiber distribution can increase whole muscle MCarn because carnosine in type II
445 fibers is ~1.5 times higher than in type I fibers (11, 21, 23). To account for this effect,
446 we examined MCarn in pools of isolated fibers and showed no differences between

447 *vastus lateralis* and *deltoid* muscles in type II fibers.

448

449 Although the increase in whole muscle MCarn seemed to occur mainly due to
450 fiber type shifts, in type I fibers a significant higher MCarn was shown, thereby
451 suggesting that chronic muscle inactivity could increase MCarn, at least in type I fibers.
452 Carnosine is typically higher in type II than in type I fibers (11, 21, 23), which we
453 confirmed in the active deltoid muscle in the present study. Interestingly, in the
454 paralyzed *vastus lateralis*, MCarn in type I fibers was not only comparable to type II
455 fibers in the same paralyzed muscle, but also to the MCarn typically shown in type II
456 fibers in other studies (11, 23). These data further reinforce the notion that chronic
457 inactivity may increase MCarn in type I fibers only. Hence, increased MCarn in type I
458 fibers can also account, although to a lesser extent, for the increased whole muscle
459 MCarn. It is important to highlight, however, that type I fibers were not present in many
460 of the samples obtained; in fact, only 4 samples had type-I fibers to be analysed and
461 caution should be exercised in interpreting these data.

462 In spite of the limited strength of this evidence, we can speculate that the
463 apparent higher carnosine levels in type I fibers in the inactive muscle might arise from
464 an adaptive mechanism to help protect against excessive ROS formation that is typically
465 found in atrophic conditions (18). This mechanism might involve improved efficiency
466 of β -alanine transport into cells. Jung et al. (26) found that TauT expression, a major β -
467 alanine transporter, is elevated in spinal cord motor neurons of Amyotrophic Lateral
468 Sclerosis transgenic (G93A) mice, suggesting that TauT expression partially protects
469 motor neurons by compensating for oxidative stress. Since oxidative stress is one of the
470 main processes involved in muscle atrophy after spinal cord injury (18), these findings
471 provide some support to the hypothesis that similar mechanisms can occur in
472 chronically paralyzed skeletal muscle. We, unfortunately, were unable to assess protein
473 and gene expression levels in our samples due to the very limited availability of usable
474 material in the muscle specimens, which we acknowledge as a limitation of our study.
475 Thus, this hypothesis remains to experimentally confirmed.

476 Importantly, β -alanine supplementation increased MCarn to a similar extent in
477 both the active *deltoid* and the inactive *vastus lateralis*. This indicates that muscle
478 (in)activity has no influence on the muscles ability to respond to β -alanine
479 supplementation. This was confirmed in both type I and type II fibers, although the type
480 I fiber dataset is limited due to the reduced number of type I fibers in the paralyzed

481 *vastus lateralis*. Our data are in agreement with the study of the Kendrick et al. (28) that
482 showed similar carnosine accretion between trained and untrained muscle groups in
483 response to β -alanine supplementation. On the other hand, our data disagrees with those
484 by Bex et al. (7) who showed superior carnosine accretion in muscle groups under
485 higher training loads in comparison with muscle groups under lower training loads. Our
486 data also seem to contradict those by Bex et al. (6) who showed a tendency for
487 increased carnosine accretion in response to β -alanine supplementation in individuals
488 undergoing exercise training vs. non-trained controls. In their study, however, this
489 difference was not confirmed when the two muscle groups (gastrocnemius and soleus)
490 were analysed separately, suggesting a lack of consistency in the effects. In both studies
491 (6, 7), MCarn was determined with magnetic resonance spectroscopy, a method that is
492 less reliable for muscle carnosine quantification than the chromatographic methods used
493 in our (10) and in Kendrick's studies (27, 28). Importantly, our study was the first to
494 address this question using a single fiber approach, where differences in fiber type
495 distribution are controlled. The similar increase between muscle groups and fiber types
496 further indicates that chronic (in)activity has no effect on MCarn accretion in response
497 to β -alanine supplementation, regardless of fiber type distribution. This corroborates
498 early findings by Harris et al. (22) who showed that type I and type II fibers can equally
499 increase MCarn during β -alanine supplementation in active muscle, and also expands
500 this notion to a condition in which muscle activity is virtually absent.

501 The lack of difference in carnosine accretion between active and inactive
502 muscles in response to β -alanine supplementation may seem to be in contrast with our
503 hypothesis of increased β -alanine transport efficiency in type I fibers following chronic
504 muscle inactivity, since the low availability of β -alanine to muscle cells is the most
505 limiting factor for carnosine synthesis (22). However, this seems not to hold true when
506 β -alanine is abundantly available due to supplementation (17). In cases where β -alanine
507 availability is high, β -alanine transport efficiency seems no to influence carnosine
508 synthesis rates and the activity of carnosine synthase appears to become a rate-limiting
509 factor for intramuscular carnosine synthesis (17). This might explain why carnosine
510 content is higher in type I fiber of paralyzed muscles under no β -alanine
511 supplementation (hypothetically due to increased TauT expression), but no differences
512 in carnosine loading was shown following supplementation, even in type I fibers.

513

514 To conclude, we demonstrated that MCarn is higher in an inactive than in an
515 active, well-trained muscle. However, this is most likely a reflection of higher type II-
516 fiber distribution resulting from muscle fiber shifts in the inactive muscle. Although we
517 showed some evidence that chronic inactivity increases MCarn in type-I fibers, the
518 strength of the evidence is limited due to the low number of observations. Finally, we
519 provided robust evidence that carnosine accretion in chronically inactive muscle in
520 response to β -alanine supplementation was similar to that of an active, well-trained
521 muscle.

522

523

524 **Perspectives and significance**

525 By investigating athletes with spinal cord injury, resulting in paraplegia and
526 lower limb immobilization, we demonstrated that sustained muscle (in)activity
527 increases muscle carnosine content, which we suggest is most likely due to type I to
528 type II fiber shift. We also provided some evidence that muscle inactivity might
529 increase carnosine content in type I fibers, possibly as a compensatory effect for the
530 increased oxidative stress that accompanies immobilization. Importantly, carnosine
531 accretion was similar in active and inactive muscles in response to β -alanine
532 supplementation, indicating that chronic (in)activity has no impact on the molecular
533 machinery involved in carnosine synthesis and homeostasis in skeletal muscle. This also
534 suggests that β -alanine supplementation, irrespective of exercise, is the main strategy to
535 increase muscle carnosine.

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548 **References**

- 549 1. **Abe H.** Role of histidine-related compounds as intracellular proton buffering
550 constituents in vertebrate muscle. *Biochemistry Biokhimiia* 65: 757-765, 2000.
- 551 2. **Artioli GG, Sale C, and Jones RL.** Carnosine in health and disease. *European*
552 *journal of sport science* 19: 30-39, 2019.
- 553 3. **Baba SP, Hoetker JD, Merchant M, Klein JB, Cai J, Barski OA, Conklin**
554 **DJ, and Bhatnagar A.** Role of aldose reductase in the metabolism and detoxification of
555 carnosine-acrolein conjugates. *The Journal of biological chemistry* 288: 28163-28179,
556 2013.
- 557 4. **Baguet A, Everaert I, De Naeyer H, Reingoudt H, Stegen S, Beeckman S,**
558 **Achten E, Vanhee L, Volkaert A, Petrovic M, Taes Y, and Derave W.** Effects of
559 sprint training combined with vegetarian or mixed diet on muscle carnosine content and
560 buffering capacity. *European journal of applied physiology* 111: 2571-2580, 2011.
- 561 5. **Bergstrom J, Furst P, Noree LO, and Vinnars E.** Intracellular free amino acid
562 concentration in human muscle tissue. *J Appl Physiol* 36: 693-697, 1974.
- 563 6. **Bex T, Chung W, Baguet A, Achten E, and Derave W.** Exercise training and
564 Beta-alanine-induced muscle carnosine loading. *Front Nutr* 2: 13, 2015.
- 565 7. **Bex T, Chung W, Baguet A, Stegen S, Stautemas J, Achten E, and Derave**
566 **W.** Muscle carnosine loading by beta-alanine supplementation is more pronounced in
567 trained vs. untrained muscles. *Journal of applied physiology* 116: 204-209, 2014.
- 568 8. **Burnham R, Martin T, Stein R, Bell G, MacLean I, and Steadward R.**
569 Skeletal muscle fibre type transformation following spinal cord injury. *Spinal cord* 35:
570 86-91, 1997.
- 571 9. **Carvalho VH, Oliveira AHS, de Oliveira LF, da Silva RP, Di Mascio P,**
572 **Gualano B, Artioli GG, and Medeiros MHG.** Exercise and beta-alanine
573 supplementation on carnosine-acrolein adduct in skeletal muscle. *Redox biology* 18:
574 222-228, 2018.
- 575 10. **da Eira Silva V, Painelli VS, Shinjo SK, Ribeiro Pereira W, Cilli EM, Sale**
576 **C, Gualano B, Otaduy MC, and Artioli GG.** Magnetic Resonance Spectroscopy as a
577 Non-invasive Method to Quantify Muscle Carnosine in Humans: a Comprehensive
578 Validity Assessment. *Scientific reports* 10: 4908, 2020.
- 579 11. **De Salles Painelli V, Nemezio KM, Pinto AJ, Franchi M, Andrade I, Riani**
580 **LA, Saunders B, Sale C, Harris RC, Gualano B, and Artioli GG.** High-Intensity
581 Interval Training Augments Muscle Carnosine in the Absence of Dietary Beta-alanine
582 Intake. *Medicine and science in sports and exercise* 50: 2242-2252, 2018.
- 583 12. **Dolan E, Saunders B, Dantas WS, Murai IH, Roschel H, Artioli GG, Harris**
584 **R, Bicudo J, Sale C, and Gualano B.** A Comparative Study of Hummingbirds and
585 Chickens Provides Mechanistic Insight on the Histidine Containing Dipeptide Role in
586 Skeletal Muscle Metabolism. *Scientific reports* 8: 14788, 2018.
- 587 13. **Dunnnett M, and Harris RC.** High-performance liquid chromatographic
588 determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-
589 methylhistidine in equine and camel muscle and individual muscle fibres. *Journal of*
590 *chromatography B, Biomedical sciences and applications* 688: 47-55, 1997.
- 591 14. **Dutka TL, and Lamb GD.** Effect of carnosine on excitation-contraction
592 coupling in mechanically-skinned rat skeletal muscle. *Journal of muscle research and*
593 *cell motility* 25: 203-213, 2004.
- 594 15. **Edge J, Eynon N, McKenna MJ, Goodman CA, Harris RC, and Bishop DJ.**
595 Altering the rest interval during high-intensity interval training does not affect muscle
596 or performance adaptations. *Experimental physiology* 98: 481-490, 2013.

- 597 16. **Galpin AJ, Raue U, Jemiolo B, Trappe TA, Harber MP, Minchev K, and**
598 **Trappe S.** Human skeletal muscle fiber type specific protein content. *Analytical*
599 *biochemistry* 425: 175-182, 2012.
- 600 17. **Goncalves LS, Kratz C, Santos L, Carvalho VH, Sales LP, Nemezio K,**
601 **Longobardi I, Riani LA, Lima MMO, Saito T, Fernandes AL, Rodrigues J, James**
602 **RM, Sale C, Gualano B, Geloneze B, de Medeiros MHG, and Artioli GG.** Insulin
603 does not stimulate beta-alanine transport into human skeletal muscle. *American journal*
604 *of physiology Cell physiology* 318: C777-C786, 2020.
- 605 18. **Gorgey AS, Witt O, O'Brien L, Cardozo C, Chen Q, Lesnfsky EJ, and**
606 **Graham ZA.** Mitochondrial health and muscle plasticity after spinal cord injury.
607 *European journal of applied physiology* 119: 315-331, 2019.
- 608 19. **Grimby G, Broberg C, Krotkiewska I, and Krotkiewski M.** Muscle fiber
609 composition in patients with traumatic cord lesion. *Scandinavian journal of*
610 *rehabilitation medicine* 8: 37-42, 1976.
- 611 20. **Gross M, Boesch C, Bolliger CS, Norman B, Gustafsson T, Hoppeler H, and**
612 **Vogt M.** Effects of beta-alanine supplementation and interval training on physiological
613 determinants of severe exercise performance. *European journal of applied physiology*
614 114: 221-234, 2014.
- 615 21. **Harris RC, Dunnett M, and Greenhaff PL.** Carnosine and taurine contents in
616 individual fibres of human vastus lateralis muscle.
Journal of Sports Sciences 16: 1998.
- 617 22. **Harris RC, Tallon MJ, Dunnett M, Boobis L, Coakley J, Kim HJ,**
618 **Fallowfield JL, Hill CA, Sale C, and Wise JA.** The absorption of orally supplied beta-
619 alanine and its effect on muscle carnosine synthesis in human vastus lateralis. *Amino*
620 *acids* 30: 279-289, 2006.
- 621 23. **Hill CA, Harris RC, Kim HJ, Harris BD, Sale C, Boobis LH, Kim CK, and**
622 **Wise JA.** Influence of beta-alanine supplementation on skeletal muscle carnosine
623 concentrations and high intensity cycling capacity. *Amino acids* 32: 225-233, 2007.
- 624 24. **Hoetker D, Chung W, Zhang D, Zhao J, Schmidtke VK, Riggs DW, Derave**
625 **W, Bhatnagar A, Bishop DJ, and Baba SP.** Exercise alters and beta-alanine combined
626 with exercise augments histidyl dipeptide levels and scavenges lipid peroxidation
627 products in human skeletal muscle. *Journal of applied physiology* 2018.
- 628 25. **Jones G, Smith M, and Harris R.** Imidazole dipeptide content of dietary
629 sources commonly consumed within the British diet. *Proceedings of the Nutrition*
630 *Society* 70: E363, 2011.
- 631 26. **Jung MK, Kim KY, Lee NY, Kang YS, Hwang YJ, Kim Y, Sung JJ, McKee**
632 **A, Kowall N, Lee J, and Ryu H.** Expression of taurine transporter (TauT) is modulated
633 by heat shock factor 1 (HSF1) in motor neurons of ALS. *Molecular neurobiology* 47:
634 699-710, 2013.
- 635 27. **Kendrick IP, Harris RC, Kim HJ, Kim CK, Dang VH, Lam TQ, Bui TT,**
636 **Smith M, and Wise JA.** The effects of 10 weeks of resistance training combined with
637 beta-alanine supplementation on whole body strength, force production, muscular
638 endurance and body composition. *Amino acids* 34: 547-554, 2008.
- 639 28. **Kendrick IP, Kim HJ, Harris RC, Kim CK, Dang VH, Lam TQ, Bui TT,**
640 **and Wise JA.** The effect of 4 weeks beta-alanine supplementation and isokinetic
641 training on carnosine concentrations in type I and II human skeletal muscle fibres. *Eur J*
642 *Appl Physiol* 106: 131-138, 2009.
- 643 29. **Mannion AF, Jakeman PM, and Willan PL.** Effects of isokinetic training of
644 the knee extensors on high-intensity exercise performance and skeletal muscle
645

- 646 buffering. *European journal of applied physiology and occupational physiology* 68:
647 356-361, 1994.
- 648 30. **Mora L, Sentandreu MA, and Toldra F.** Hydrophilic chromatographic
649 determination of carnosine, anserine, balenine, creatine, and creatinine. *Journal of*
650 *agricultural and food chemistry* 55: 4664-4669, 2007.
- 651 31. **Parkhouse WS, McKenzie DC, Hochachka PW, and Ovalle WK.** Buffering
652 capacity of deproteinized human vastus lateralis muscle. *Journal of applied physiology*
653 58: 14-17, 1985.
- 654 32. **Pires FO, Lima-Silva AE, Hammond J, Franchini E, Dal' Molin Kiss MA,**
655 **and Bertuzzi R.** Aerobic profile of climbers during maximal arm test. *International*
656 *journal of sports medicine* 32: 122-125, 2011.
- 657 33. **Rezende NS, Swinton P, De Oliveira LF, Da Silva RP, DA Silva VE,**
658 **Nemezio K, Yamaguchi G, Artioli GG, Gualano B, Saunders B, and Dolan E.** The
659 Muscle Carnosine Response to Beta-Alanine Supplementation: A Systematic Review
660 with Bayesian Individual and Aggregate Data EMax Model and Meta-Analysis.
661 *Frontiers in physiology* 11: 2020.
- 662 34. **Saunders B, Elliott-Sale K, Artioli GG, Swinton PA, Dolan E, Roschel H,**
663 **Sale C, and Gualano B.** beta-alanine supplementation to improve exercise capacity and
664 performance: a systematic review and meta-analysis. *British journal of sports medicine*
665 51: 658-669, 2017.
- 666 35. **Scelsi R, Marchetti C, Poggi P, Lotta S, and Lommi G.** Muscle fiber type
667 morphology and distribution in paraplegic patients with traumatic cord lesion.
668 Histochemical and ultrastructural aspects of rectus femoris muscle. *Acta*
669 *neuropathologica* 57: 243-248, 1982.
- 670 36. **Tallon MJ, Harris RC, Boobis LH, Fallowfield JL, and Wise JA.** The
671 carnosine content of vastus lateralis is elevated in resistance-trained bodybuilders.
672 *Journal of strength and conditioning research* 19: 725-729, 2005.
- 673 37. **Yamaguchi GC, Nemezio K, Schulz ML, Natali J, Cesar JE, Riani LA, de**
674 **Souza Goncalves L, Moller GB, Sale C, de Medeiros MHG, Gualano B, and Artioli**
675 **GG.** Kinetics of Muscle Carnosine Decay after beta-alanine Supplementation: A 16-
676 Week Washout Study. *Medicine and science in sports and exercise* 2020.

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