Amino Acids (2024) 56:55
https://doi.org/10.1007/s00726-024-03414-5

ORIGINAL ARTICLE

Evaluation of supplementary carnosine accumulation and distribution: an initial analysis of participants in the Nucleophilic Defense Against PM Toxicity (NEAT) clinical trial

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Received: 12 April 2024 / Accepted: 20 August 2024

Abstract

Carnosine is an endogenous dipeptide that buffers intracellular pH and quenches toxic products of lipid peroxidation. Used as a dietary supplement, it also supports exercise endurance. However, the accumulation and distribution of carnosine after supplementation has not been rigorously evaluated. To do this, we randomized a cohort to receive daily supplements of either placebo or carnosine (2 g/day). Blood and urine samples were collected twice over the subsequent 12 week supplementation period and we measured levels of red blood cell (RBC) carnosine, urinary carnosine, and urinary carnosine-propanol and carnosine-propanal conjugates by LC/MS–MS. We found that, when compared with placebo, supplementation with carnosine for 6 or 12 weeks led to an approximate twofold increase in RBC carnosine, while levels of urinary carnosine increased nearly sevenfold. Although there were no changes in the urinary levels of carnosine propanol, carnosine propanal increased nearly twofold. RBC carnosine levels were positively associated with urinary carnosine and carnosine propanal levels. No adverse reactions were reported by those in the carnosine or placebo arms, nor did carnosine supplementation have any effect on kidney, liver, and cardiac function or blood electrolytes. In conclusion, irrespective of age, sex, or BMI, oral carnosine supplementation in humans leads to its increase in RBC and urine, as well as an increase in urinary carnosine-propanal. RBC carnosine may be a readily accessible pool to estimate carnosine levels. **Clinical trial registration:** This study is registered with ClinicalTrials.gov (Nucleophilic Defense Against PM Toxicity (NEAT Trial)—Full Text View—ClinicalTrials.gov), under the registration: NCT03314987.

Keywords Carnosine · Carnosine propanol · Carnosine propanal · Measurement

Handling editor: J. Spaas.

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Published online: 31 August 2024

Introduction

Naturally occurring histidyl dipeptides such as carnosine (β-alanine-L-histidine) are present in high concentrations in glycolytically active tissues of humans, including skeletal muscle (8–10 mM), and the brain (1–2 mM) (Boldyrev et al. 2013; Hoetker et al. 2018; Zhao et al. 2020; Posa et al. 2023). These dipeptides have a unique chemical structure, allowing them to buffer intracellular pH ($[pH]_i$), neutralize lipid peroxidation products (Zhao et al. 2019; Baba et al. 2013), chelate transition metals (Zhao et al. 2019), and quench reactive oxygen species (Ihara et al. 2019). Given these properties, several studies have shown that increasing skeletal muscle carnosine content enhances the capacity for high-intensity exercise (Baguet et al. 2010; del Favero et al. 2012; Derave et al. 2007).

In addition, some preclinical studies have also demonstrated the therapeutic benefits of carnosine for the treatment of ischemic and neurodegenerative diseases. For instance, we have found that in mice, increasing myocardial carnosine protects against ischemia–reperfusion injury by buffering $[pH]_i$ and removing toxic aldehydes generated by the oxidation of membrane lipids (Zhao et al. 2020). In other human and animal studies, carnosine supplementation protected from cerebral ischemic injury, improved wound healing responses (Boakye et al. 2019; Kim et al. 2021), attenuated autoimmune neuroinflammation (Joshi et al. 2021; Spaas et al. 2021), and mitigated some of the adverse effects of exposure to fine airborne particulate matter ($PM_{2.5}$) (Abplanalp et al. 2019). The effects of carnosine supplementation on metabolic and cardiovascular disorders appear to be more nuanced. While some studies demonstrated improved glucose tolerance and enhanced removal of advanced glycation and lipo-oxidation products in obese or at-risk humans (de Courten et al. 2016; Hariharan et al. 2023; Regazzoni et al. 2016), the use of carnosine in another supplementation study did not appear to improve cardiac function or alleviate cardiovascular disease risk (Saadati et al. 2023). Nevertheless, despite studies suggesting the beneficial effects and therapeutic potential of carnosine supplementation, no large-scale human study has examined the efficacy of carnosine supplementation in increasing its levels in a diverse demographic cohort, and whether carnosine accumulation and distribution are affected by sex, age, and obesity.

In humans, after oral consumption and absorption into circulation, carnosine is rapidly hydrolyzed by serum carnosinase (CN1), resulting in a short half-life (1.20 ± 0.36 min) (de Jager et al. 2023). However, some of the ingested carnosine can localize in skeletal muscle, and studies have shown that skeletal muscle levels doubled in humans receiving carnosine supplements (Schon et al. 2023). In addition to skeletal muscle, ingested carnosine can enter red blood cells (RBCs), where it is prevented from degradation by CN1 (Oppermann et al. 2021). Additionally, carnosine can also be found in urine, either in its free form or as a stable covalent conjugate with products of lipid peroxidation. Previous work in humans has shown that approximately 14% of ingested carnosine is recovered in the urine for up to five hours (Gardner et al. 1991). How ingested carnosine is distributed among these pools, and whether this distribution is impacted by individual demographics (age, sex, BMI) is unknown. Furthermore, owing in part to these uncertainties, it is not clear how carnosine levels can be best measured in a facile and economical manner in large-scale clinical supplementation trials (Schon et al. 2023). Currently, carnosine levels are most often assessed by mass spectrometric analysis of muscle biopsy samples or by ^{1}H magnetic resonance spectroscopy (^{1}HMRS) of the same tissue (Hoetker et al. 2018; Baguet et al. 2011). However, neither of these approaches measure non-skeletal muscle pools of carnosine, and they are cumbersome and expensive. ^{1}HMRS requires specialized equipment and provides only relative measurements of carnosine, whereas muscle biopsies are not only expensive, but a major deterrent for many participants. Therefore, there is an urgent need for acceptable, facile, and economical methods to assess carnosine abundance and distribution in humans, especially in the context of large clinical studies evaluating the effects of oral carnosine supplementation.

The current analysis was therefore designed to examine how oral supplementation with carnosine affects its levels in RBCs and urine, as well as the levels of urinary carnosine conjugates. To address these issues, we collected and analyzed samples obtained from participants in a double-blind, randomized, placebo-controlled study (O'Toole et al. 2020) and evaluated the distribution and accumulation of carnosine, and how this might be affected by demographic characteristics.

Materials and methods

Cohort and study design

The current study comprises an initial analysis of samples obtained from participants in the Nucleophilic Defense Against PM Toxicity (NEAT) clinical study (NCT03314987). A full description of NEAT study design, including sample size calculation, procedures, endpoints, and statistical evaluation has been previously published (O'Toole et al. 2020). For the initial screening of baseline carnosine levels (Visit 1), we consented 527 volunteers

from the greater Louisville, KY area. These volunteers were of both sexes, any ethnicity, and had an age range of 23–65 years. Those over the age of 65 were excluded due to a greater likelihood of confounders influencing the larger clinical endpoints of the NEAT study. After measurement of basal carnosine levels, individuals who had levels among the lowest in the distribution were invited to participate in the full study (Fig. 1). These included participants were randomized at Visit 2 to receive visually identical carnosine (Belle Chemical; 99% purity) or placebo (cornstarch) capsules (0.5 g/capsule) which were contained in blister packs of a larger drug card (supplied by the University of Colorado, School of Pharmacy). The participants were instructed to take 2 capsules each morning and evening (2 g/day) through their Visit 3, approximately 6 wk later, with no other dietary restrictions. At this visit, we performed clinical

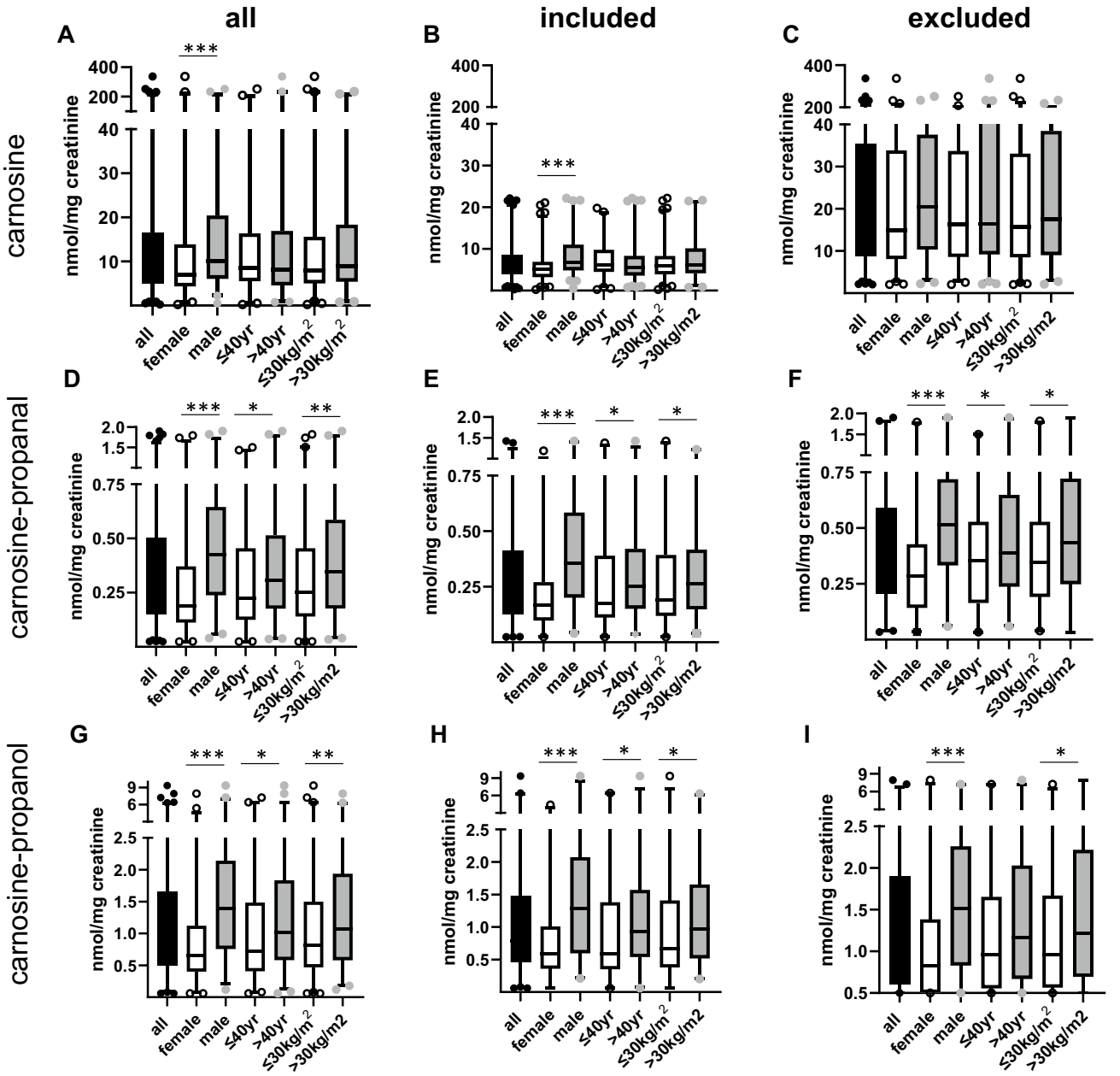

Fig. 1 Levels of carnosine and carnosine conjugates. Illustrated are levels of urinary carnosine (**A**–**C**), carnosine propanal (**D**–**F**), and carnosine propanol (**G**–I) in all screened participants (**A**, **D**, **G**), those included in the full study (**B**, **E**, **H**), and those excluded from the full study (**C**, **F**, **I**). Values were determined in samples collected at the screening visit, Visit 1. A Students's t-test was used after log-transformation to examine differences between groups. *$p < 0.05$; **$p \leq 0.001$; ***$p < 0.0001$

measurements, collected blood and urine, and assessed compliance by counting unused capsules in the drug card. The participants were also given a second drug card for the subsequent 6 weeks. After approximately 12 total weeks of supplementation, the participants returned at Visit 4 for final clinical measures and biospecimen collection. Of the 299 participants who initiated the study, 273 completed all 4 visits. Of the data obtained from those in the carnosine supplementation group, only that from those with greater than 80% compliance (n = 99 at Visit 3; n = 80 at Visit 4) was used for final analysis.

Measurement of RBC carnosine

For RBC isolation and sample preparation, blood samples (approximately 4 mL) were drawn into heparinized tubes (Becton Dickinson, Franklin Lakes, NJ), and incubated on ice for 30 min. These samples were then centrifuged at 1000×*g* for 15 min and the plasma and buffy coat discarded. An aliquot of the remaining RBCs (41 µL) was added to 375 µL of ice-cold water and used to determine protein concentration. An additional 41 µL aliquot was added to 375 µL of fixative solution (55% methanol, 45% water, 1 µM carnosine-d4 (CDN Isotopes). Both samples were mixed by vortexing and incubated on ice for 5 min. The fixed sample was then applied onto an Amicon Ultra-0.5 Centrifugal Filter Unit (Merck KGaA, Darmstadt, Germany), centrifuged at 14000×*g* for 42 min at 4 °C and the filtrate was collected and stored at −80 °C until analysis.

Filtrates from the lysed RBC samples were thawed and diluted tenfold with a solution of 75% acetonitrile:25% water, and analyzed by LC–MS. The analytes were eluted using a binary solvent system consisting of 10 mM ammonium formate, 0.125% formic acid in 5% acetonitrile: 95% water for mobile phase A and 10 mM ammonium formate, 0.125% formic acid in 95% acetonitrile: 5% water for mobile phase B at a flow rate of 0.55 mL/min. Initial conditions were 0.1: 99.9 A:B ramping to 99.9: 0.1 A:B over 5 min then quickly ramping to 0.1:99.9 A:B over 0.5 min. For quantification, carnosine, m/z 227 → 110, and carnosine-d4, m/z 231 → 110, transition ions as well as two confirmation ions were followed. Levels of carnosine in the participant samples were quantified using the peak ratio of carnosine and carnosine-d4 internal standard, interpolated using the standard curve, and expressed as nmoles carnosine/µg protein. For each batch of participant RBC samples analyzed, a carnosine standard curve was prepared in exactly the same way, serially diluting a high concentration of the analyte until a carnosine signal was not detected. The limit of detection (LOD) was estimated to be 4 fmoles for RBC samples. The limit of quantitation (LOQ) was defined to be the amount on column at which the integrated area of carnosine doubled as the amount injected onto the column doubled and was estimated to be 8fmoles for RBC samples. Out of 853 participant RBC samples analyzed, 108 were below the LOD and 241 were below the LOQ.

Measurement of urinary carnosine and carnosine conjugates

Urine samples were aliquoted and frozen at −80 °C until analysis. The measurements of carnosine and its propanal- and propanol-conjugates were accomplished as previously described (Abplanalp et al. 2019). In brief, urine samples were diluted in a solution of 75% acetonitrile:25% water containing carnosine-d4 and tyrosine-histidine as internal standards. Samples were separated and carnosine and its conjugates were identified using LC–MS. The analytes were eluted using a binary solvent system consisting of 10 mM ammonium formate, 0.125% formic acid in 50% acetonitrile: 50% water for mobile phase A and 10 mM ammonium formate, 0.125% formic acid in 95% acetonitrile: 5% water for mobile phase B at a flow rate of 0.55 mL/min. Initial conditions were 0.1: 99.9 A: B ramping to 99.9: 0.1 A:B over 5 min then quickly ramping to 0.1:99.9 A:B over 0.5 min. Creatinine levels were measured on an Ace Axcel Clinical Chemistry Analyzer (Alfa Wassermann). Final carnosine and carnosine conjugate levels were quantified using the peak ratio of histidyl-dipeptide internal standard (carnosine d4 for carnosine; tyrosine-histidine for carnosine conjugates), interpolated using a standard curve and expressed as values normalized to creatinine (nmoles/mg creatinine). For each batch of urine samples analyzed, a carnosine standard curve was prepared in exactly the same way, serially diluting a high concentration of the analyte until a carnosine signal was not detected. The limit of detection (LOD) was estimated to be 1.5fmoles for urine samples, while the LOQ was estimated to be 3fmoles for urine samples. Of the 837 samples urine samples, no participant samples were below the LOQ.

Clinical and biochemical measures

Blood pressure measurements were taken at each visit, while the participants self-reported any noticeable side effects (tingling, nausea, skin rash) of their supplementation. The analysis of ALP, ALT, AST, bilirubin, albumin, BUN, creatinine, sodium, potassium, chloride, total glucose, cholesterol, and HbA1c was accomplished through commercial services (Quest Diagnostics).

Statistical analysis

Demographic characteristics are expressed as mean ± standard deviation (SD) for continuous variables, and frequency (%) for categorical variables. Student's t-test was used to detect statistical differences between different

groups. In all analyses, statistical significance was set at a p-value < 0.05. Mixed effects models were constructed to examine changes in carnosine levels as the dependent variables and the randomization group (carnosine vs. placebo) as the independent variable. To evaluate the impact of time (supplement duration: 6 and 12 weeks) and other potential interactions on the relationship between the randomization group and changes in carnosine and carnosine propanal levels, we adjusted the mixed effect models with 4 covariates (time, sex, age, BMI) and 6 interactions (time*sex, time*age, time*BMI, group*time, group*sex and group*BMI). During this model selection process, we found that time and other interactions had no or minimal effect on these relationships. Therefore, we removed all the interactions but kept the 4 covariates in the mixed effect models when performing the subgroup analysis**.**

The estimated fixed effect coefficients (β for the randomization group: carnosine vs. placebo) against the log transformed dependent variables were exponentiated to calculate the fold difference between two groups. Calculated fold differences between the carnosine and placebo group are presented as forest plots. All statistical analyses were performed using SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina). The forest plots were produced in Graph Pad Prism, version 9.1 (Graph Pad Software, La Jolla, California).

Results

Characteristics of study participants

To initiate the study, we screened 527 healthy participants (females: n = 298 and males: n = 229) at Visit 1. The characteristics of this cohort are listed in Table 1. Urinary carnosine levels in these participants showed a wide variation, with a mean of 18.7 ± 34.2 nmoles/mg creatinine, (Fig. 1A). Upon stratification, we found the levels of urinary carnosine were significantly higher in males (20.1 ± 30.8 nmoles/mg creatinine) than in females (17.6 ± 36.7 nmoles/mg creatinine) ($p < 0.0001$; Fig. 1A). There were no statistically significant differences among participants who were ≤ 40 or > 40 years of age or those with BMI ≤ 30 kg/m^2 or > 30 kg/m^2.

Like carnosine, the levels of carnosine propanal (0.50 ± 0.33 nmoles/mg creatinine) and carnosine propanol (1.70 ± 1.34 nmoles/mg creatinine) were significantly higher in males than in females (0.28 ± 0.27 nmoles/mg creatinine and 0.95 ± 0.90 nmoles/mg creatinine, respectively; Fig. 1D, G). The higher levels of carnosine conjugates in males could not be ascribed to higher levels of carnosine alone, because even though males had only 1.17-fold higher levels of carnosine, they had 1.78-fold higher levels of carnosine propanal and 3.40-fold higher levels of carnosine propanol. Unlike urinary carnosine however, urinary levels of these carnosine conjugates were sensitive to age and BMI. The urinary levels

Table 1 Characteristics of screened participants

Characteristics	Total (n = 527)	Randomized (n = 299)	Excluded (n = 228)	p value
Female n (%)	298 (56.6%)	173 (57.9%)	125 (54.8%)	0.486
Race n (%)				0.480
White	424 (80.5%)	243 (81.3%)	181 (79.4%)	
Black	52 (9.9%)	31 (10.4%)	21 (9.2%)	
Other	51 (9.7%)	25 (8.4%)	26 (11.4%)	
Age (avg yr ± SD)	44.4 ± 12.4	44.8 ± 12.3	43.8 ± 12.4	0.349
Income n (%)				0.227
< $45,000	126 (23.9%)	79 (26.4%)	47 (20.6%)	
$45,000-$89,999	200 (38.0%)	106 (35.5%)	94 (41.2%)	
> $90,000	201 (38.1%)	114 (38.1%)	87 (38.2%)	
Education n (%)				0.697
≤ High school	20 (3.8%)	13 (4.4%)	7 (3.1%)	
Some college to 4 year degree	303 (57.5%)	173 (57.9%)	130 (57.0%)	
≥ Masters	204 (38.7%)	113 (37.8%)	91 (39.9%)	
BMI (avg kg/m^2 ± SD)	29.7 ± 6.9	29.7 ± 6.9	29.8 ± 7.5	0.869

Listed are the characteristics of all screened individuals (Visit 1), those who were included and randomized for the full study and those who were excluded. Continuous variables (age, body mass index (BMI)) are presented as mean ± SD

Categorical variables (sex, race, income, education) are presented as percent values

of carnosine propanal and carnosine propanol were much higher in participants > 40 than ≤ 40 years of age and were also higher in those with BMI > 30 kg/m^2 than BMI ≤ 30 kg/m^2 (Fig. 1D, G).

Given that screened participants with high measured levels of urinary carnosine may be on a carnosine-rich diet, we excluded 228 participants from further study. As shown in Table 1, there were no statistically significant differences in sex, race, age, income, education, and BMI between those who were excluded from the study and those who were included and randomized. However, as expected, those excluded from the study had higher levels of urinary carnosine than those who were included (Fig. 1B, C). There were no major differences in levels of carnosine propanal (Fig. 1E, F) or carnosine propanol (Fig. 1H, I) between the included and excluded groups.

Members of the included population were randomized (at Visit 2) and instructed to take daily supplements of either carnosine (56% female) or placebo (56% female). A flow chart of study progress is shown in Fig. 2. Following randomization, we obtained evaluable carnosine measures from 230 participants at Visit 3 and from 214 participants at Visit 4. The characteristics of the participants at study Visits 2–4 are presented in Supplementary Table 1.

Carnosine supplementation enhances its accumulation in RBCs

In samples collected at Visit 2, levels of RBC carnosine in males (0.52 ± 0.40 nmole/μg protein) were 1.7-fold higher ($p < 0.05$) than those levels in females (0.39 ± 0.32 nmole/μg protein; Fig. 3). Levels of RBC carnosine were higher in males ≤ 40 than > 40 years of age (0.59 ± 0.39 nmole/μg protein versus 0.48 ± 0.40 nmole/μg protein; $p = 0.049$). However in females, carnosine levels were higher in those participants > 40 years (0.46 ± 0.38 nmole/μg protein) than in those ≤ 40 years of age (0.32 ± 0.23 nmole/μg protein; $p = 0.014$; Fig. 3). There was no effect of BMI in either sex.

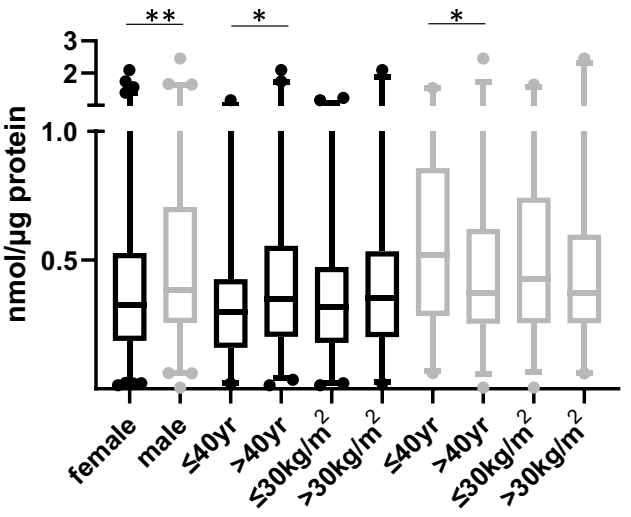

Fig. 3 RBC carnosine levels. Illustrated are levels of RBC carnosine in females (black bars) and males (gray bars) who were invited to participate in the full study (included population). Also illustrated are the levels in both sexes stratified according to age and BMI. Values were determined in samples collected at the randomization visit, Visit 2. A Students's t-test was used after log-transformation to examine differences between groups. *$p < 0.05$

At Visit 3, after approximately 6 weeks of supplementation, we found that, in comparison with placebo, carnosine supplementation led to a significant increase in RBC levels (placebo: 0.45 ± 0.32 nmole/μg protein; carnosine: 0.78 ± 0.40 nmole/μg protein; $p < 0.0001$, Figs. 4A; S1). Levels of RBC carnosine at Visit 4 were similar to those at

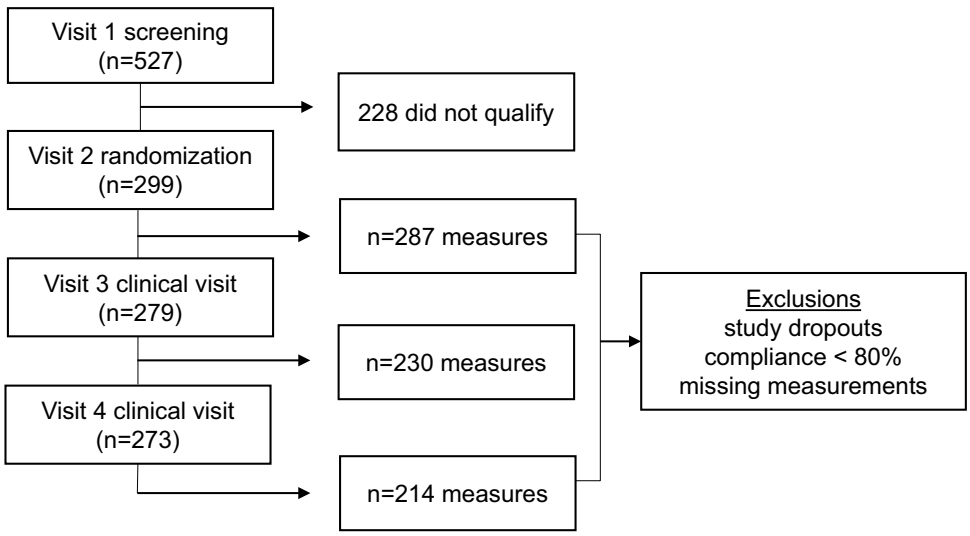

Fig. 2 Participant enrollment and carnosine analysis in the NEAT study. Illustrated is a schematic of the study progress at all clinical visits. The number of participants at each visit and the number of useable carnosine measures obtained at each visit are indicated

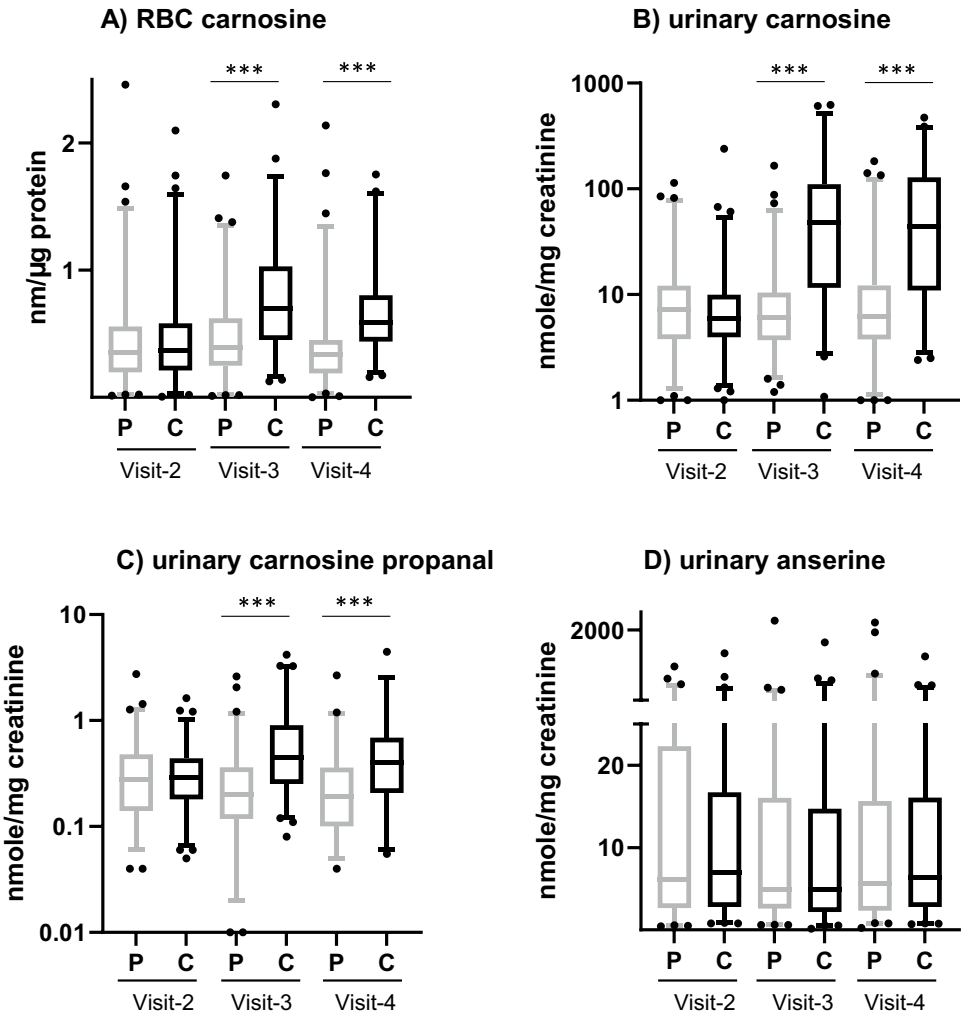

Fig. 4 Time-dependent evaluation of the levels of histidyl dipeptides and carnosine-propanal excretion. Illustrated are box plots of RBC carnosine levels (**A**), urinary carnosine levels (**B**), urinary carnosine propanal levels (**C**), and urinary anserine levels (**D**) in subjects of the placebo (gray: P) and carnosine (black: C) supplementation groups at Visits 2–4. ***$p < 0.0001$

Visit 3 (placebo: 0.39 ± 0.31 nmole/µg protein; carnosine: 0.66 ± 0.34 nmole/µg protein; $p < 0.0001$; Fig. 4A).

Carnosine supplementation enhances urinary excretion of the propanal conjugate

At Visit 1, urinary carnosine levels were an average of 18.7 ± 34.2 nmoles/mg creatinine in the whole population (Fig. 1A) and 7.1 ± 4.6 nmoles/mg creatinine in the randomized participants (Fig. 1B). When we analyzed samples obtained at Visit 3, carnosine levels increased significantly for those in the carnosine arm versus the placebo arm (placebo: 11.0 ± 18.2 nmoles/mg creatinine; carnosine: 89.1 ± 118 nmoles/mg creatinine; $p < 0.0001$; Figs. 4B; S1). A similar difference between the placebo and carnosine groups was observed at Visit 4 (placebo: 15.4 ± 27.8 nmoles/mg creatinine; carnosine: 89.1 ± 104 nmoles/mg creatinine, $p < 0.0001$; Fig. 4B). There were no differences in levels of urinary anserine levels between the supplementation groups at any time point (Figs. 4D; S1).

To examine the effects of carnosine supplementation on the excretion of carnosine conjugates, we measured the levels of carnosine propanal and carnosine propanol. As shown in Figs. 4C and S1 the levels of carnosine propanal increased in the carnosine arm versus placebo arm at Visit 3 (placebo: 0.31 ± 0.34 nmoles/mg creatinine; carnosine: 0.74 ± 0.78 nmoles/mg creatinine; $p < 0.0001$), and at Visit 4 (0.30 ± 0.33 nmoles/mg creatinine in the placebo arm and 0.63 ± 0.70 nmoles/mg creatinine in the carnosine arm; $p < 0.0001$). While carnosine propanol was the predominant urinary conjugate at Visit 1 (Fig. 1G, H, I), there was no significant increase in its levels at Visits 3 and 4 in either the carnosine or the placebo arm.

Finally, we found that urinary carnosine was weakly, but positively associated with RBC carnosine at both Visit 3 ($R^2 = 0.078$, $p = 0.002$) and Visit 4 ($R^2 = 0.055$, $p = 0.015$) (Fig. 5A, B). Likewise, we found that urinary carnosine propanal was positively associated with RBC carnosine at Visit 3 ($R^2 = 0.067$, $p = 0.005$), (Fig. 5C). There was no significant association between these variables at Visit 4 (Fig. 5D).

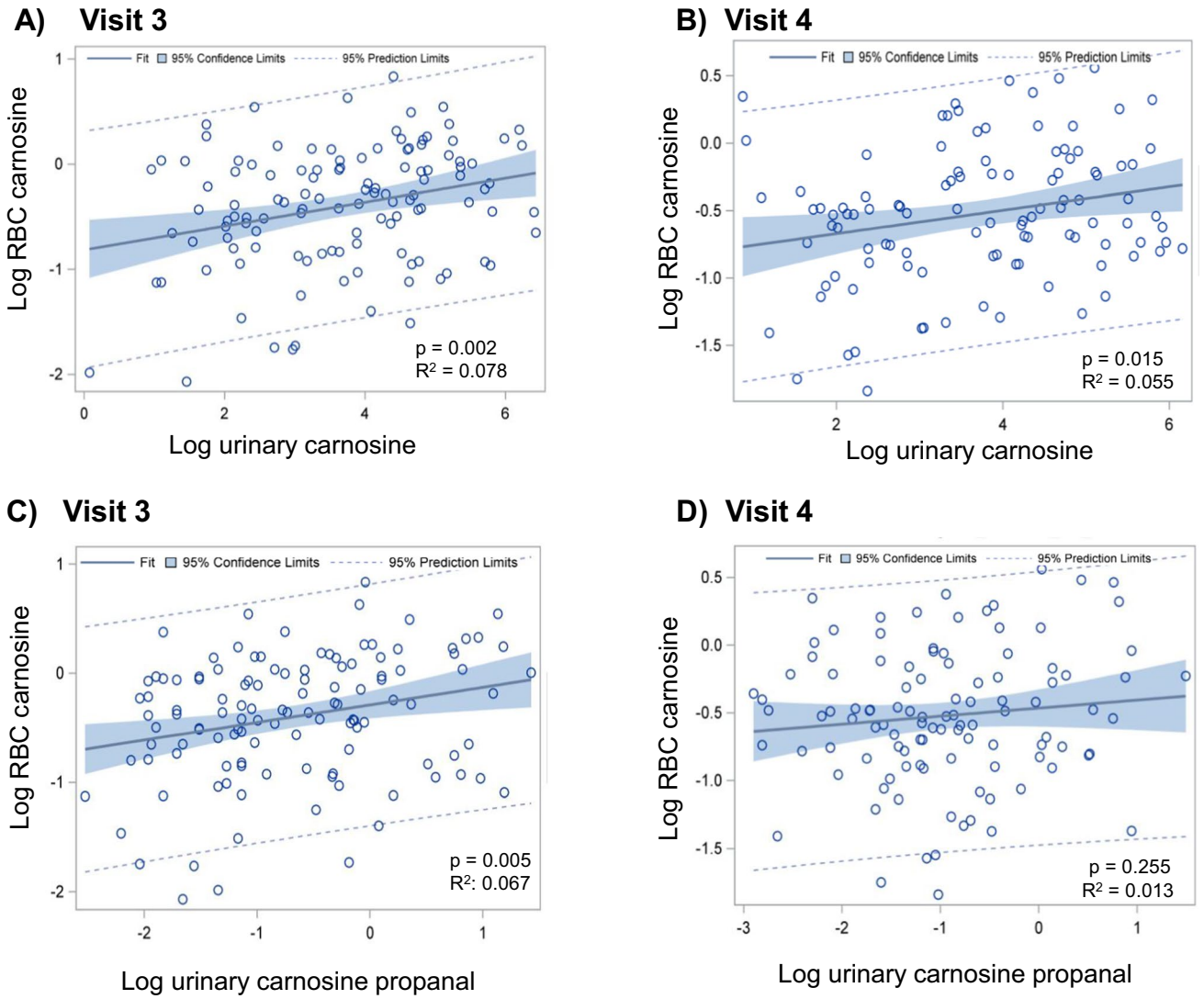

Fig. 5 Linear regression analysis of RBC carnosine, urinary carnosine and urinary carnosine propanal. Illustrated are the relationships between and RBC carnosine and urinary carnosine at Visit 3 (**A**) and Visit 4 (**B**). Also illustrated are the relationships between RBC carnosine and urinary carnosine-propanal at Visit 3 (**C**) and Visit 4 (**D**)

Subgroup analysis

Using a fixed effects model, we found that, for males and females in the carnosine supplementation group, there was a ~ 2.10- and ~ 1.84-fold increase in RBC carnosine compared to those levels in the placebo group (Fig. 6A). RBC carnosine accumulation over time in the stratified age groups (≤ 40 years, > 40 years) and in the stratified BMI groups (non-obese: ≤ 30 kg/m^2; obese: > 30 kg/m^2) had similar fold increases in the carnosine versus placebo arms (6 weeks: 1.87 fold; 12 weeks: 1.90 fold; ≤ 40 year old: 2.12-fold; > 40 year old: 1.92-fold; ≤ 30 kg/m^2: 1.90-fold; > 30 kg/m^2: 1.94-fold; Fig. 6A).

Likewise, using a mixed effects model, we found that males and females in the carnosine supplementation group had levels of urinary carnosine that were ~ 5.69- and ~ 9.06-fold greater, respectively, than those levels in the corresponding placebo group (Fig. 6B). Females had a somewhat higher increase because of lower-baseline levels, but the two groups had overlapping confidence intervals. Levels of urinary carnosine in participants ≤ 40 and > 40 years of age in the carnosine group increased by ~ 6.42- and ~ 7.87-fold respectively, compared with their counterpart placebo groups (Fig. 6B). Similarly, there was little effect of BMI or time of supplementation (Fig. 6B).

The excretion of carnosine propanal in individuals of the carnosine arm was not affected by sex (Fig. 6C). However, the increase in carnosine propanal in participants ≤ 40 yr (~ 1.61-fold) was slightly lower than participants > 40 years of age (~ 2.61-fold; Fig. 6C), suggesting that excretion

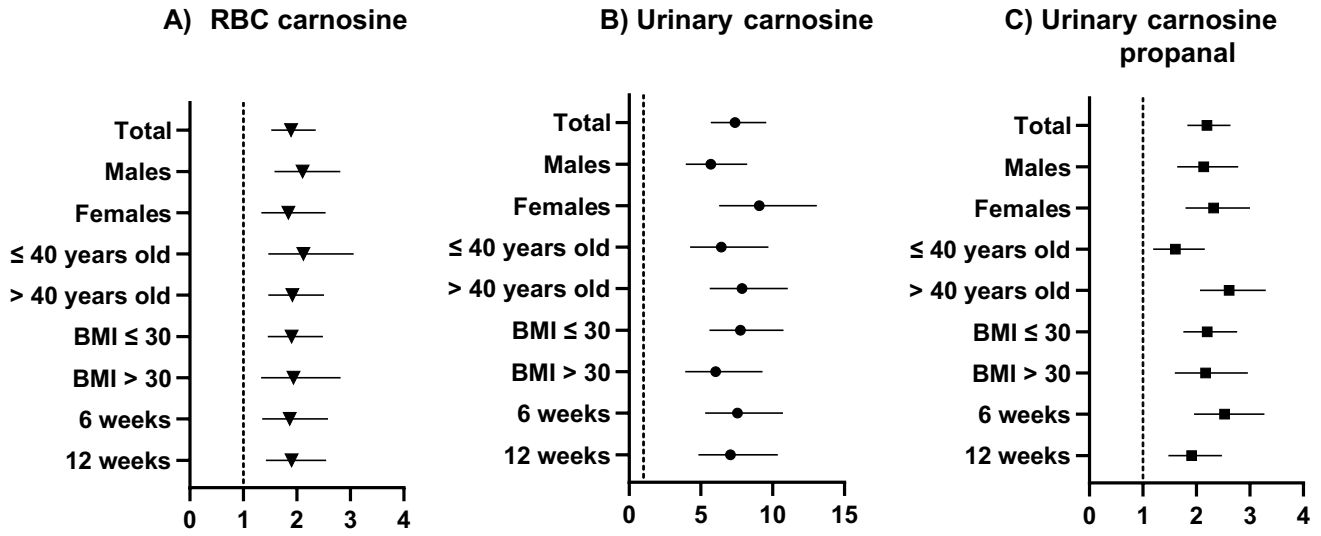

Fig. 6 Stratified analysis of carnosine and carnosine propanal levels. Illustrated are Forest plots depicting fold differences (versus placebo group) based on mixed effects models. Models were adjusted for sex, age, BMI, and supplement duration with changes in carnosine levels as the dependent variables and stratified groups as independent variables. Changes in the levels of RBC carnosine (**A**), urinary carnosine (**B**), and urinary carnosine propanal (**C**) are illustrated

of this conjugate may be increased with age. In the non-obese and obese participants, carnosine propanal increased by ~2 and 2.20-fold versus the placebo group counterparts (Fig. 6C), while there was a slight decrease in the levels of this conjugate with time. We did not perform a mixed effects analysis of carnosine propanol as there were no changes in this conjugate between Visits 3 and 4. Among all participants, while there were no differences in RBC carnosine accumulation at Visit 3 between all stratified groups, we did observe significant differences in accumulated levels of urinary carnosine in females versus males ($p = 0.021$), and in carnosine propanal levels in females ≤ 40 versus > 40 years of age (0.5-fold, $p = 0.024$) (Supplementary Table 2). Mixed effects models showed that time did not affect the relationship between the randomization group (carnosine vs. placebo) and changes in carnosine or carnosine propanal (Supplementary Table 3).

Toxic side effects of carnosine supplementation

Carnosine supplementation did not affect indices of systemic metabolism (HbA1c, cholesterol), renal function (BUN, creatinine, albumin), or hepatic function (ALT, AST, ALP) (Table 2). We did observe statistically significant increases in both systolic and diastolic blood pressure at Visit 4, as well as minor changes in electrolyte balance (Cl, K) (Table 2). The magnitude of the blood pressure changes (average of 4.4 mM Hg for systolic; average of 2.6 mM HG for diastolic) is small and in the range of daily fluctuation, and such small changes give rise to little medical concern. We also observed decreased bilirubin levels at both visits (Table 2). The participants self-reported only a few side effects of supplementation, the major being a tingling sensation (n = 3–11 among the supplementation groups and visits) and nausea (n = 3–13).

Discussion

Although previous studies have shown that levels of skeletal muscle carnosine could be increased by oral ingestion of carnosine or its precursor β-alanine (Boldyrev et al. 2013), a systematic evaluation of carnosine accumulation and distribution following supplementation, particularly in non-muscle tissues, is lacking. In this study we found that a ~6-week supplementation with 2 g/day carnosine was sufficient to increase its level in RBCs and extending the supplementation to ~12 weeks did not lead to further increases. Significantly, supplementation also increased the levels of urinary carnosine and carnosine-propanal conjugates, suggesting that oral supplementation with carnosine may be an effective strategy for increasing systemic levels and for removing toxic products of lipid peroxidation and inflammation such as acrolein (Anderson et al. 1997; Stevens and Maier 2008). The observed increase in measured carnosine is likely due to supplementation and not a consequence of dietary factors. In addition to carnosine, another histidyl dipeptide, anserine, is abundant in beef and chicken and can be used as an indicator of meat consumption (Yeum et al. 2010). However, we observed no differences in anserine levels between the treatment groups at any time point. Carnosine supplementation was equally effective in increasing its levels independent of

Table 2 Blood pressure and plasma metabolic profile

Measure	Normal range	Visit-2		Visit-3		Visit-4	
		Placebo	Carnosine	Placebo	Carnosine	Placebo	Carnosine
HbA1c (%)	5.7–6.4	5.24 ± 0.65	5.38 ± 0.70	5.31 ± 0.70	5.34 ± 0.60	5.30 ± 0.61	5.42 ± 0.77
Cholesterol (mg/dL)	< 200	200.8 ± 43.5	197.9 ± 44.5	196.0 ± 47.5	199.5 ± 41.6	203.4 ± 42.3	204.7 ± 42.5
Systolic BP (mm Hg)	< 120	124.5 ± 13.1	128.9 ± 13.4	124.1 ± 12.8	127.6 ± 13.9	125.4 ± 13.4	129.8 ± 14.4**
Diastolic BP (mm Hg)	< 80	79.9 ± 9.1	82.5 ± 9.8	79.7 ± 9.7	81.9 ± 8.9	80.5 ± 9.0	83.1 ± 9.0**
ALT (U/L)	4–36	22.6 ± 14.6	22.1 ± 13.9	23.1 ± 15.6	22.9 ± 17.7	22.3 ± 13.4	21.2 ± 11.2
AST (U/L)	8–33	20.8 ± 8.1	21.9 ± 14.4	21.7 ± 10.8	21.8 ± 11.1	20.6 ± 7.7	20.9 ± 10.1
ALP (U/L)	20–133	66.0 ± 19.1	70.7 ± 23.4	69.2 ± 24.0	72.2 ± 26.3	67.6 ± 23.4	70.5 ± 23.2
Bilirubin (mg/dL)	0.1–1.2	0.559 ± 0.271	0.467 ± 0.214	0.579 ± 0.399	0.461 ± 0.223*	0.539 ± 0.312	0.450 ± 0.225**
BUN (mg/dL)	6–20	14.0 ± 4.1	14.3 ± 3.8	14.0 ± 4.2	14.4 ± 3.9	13.9 ± 3.9	14.4 ± 4.0
Creatinine (mg/dL)	0.6–1.3	0.848 ± 0.180	0.852 ± 0.157	0.867 ± 0.183	0.852 ± 0.160	0.872 ± 0.193	0.872 ± 0.163
Albumin (g/dL)	3.4–5.4	4.51 ± 0.29	4.43 ± 0.31	4.51 ± 0.30	4.47 ± 0.33	4.50 ± 0.35	4.41 ± 0.27
Cl (mEq/L)	96–106	102.0 ± 2.4	102.4 ± 2.8	102.0 ± 2.4	102.8 ± 2.4*	101.9 ± 3.1	102.5 ± 2.5
Na (mEq/L)	135–145	139.2 ± 2.3	138.8 ± 2.2	139.3 ± 2.1	139.5 ± 2.3	139.4 ± 2.6	139.3 ± 2.4
K (mEq/L)	3.70–5.20	4.16 ± 0.42	4.20 ± 0.34	4.19 ± 0.36	4.28 ± 0.41	4.16 ± 0.38	4.27 ± 0.43**

Listed are measured values at randomization (Visit 2) and at Visits 3 and 4. Values are avg ± SD. *$p < 0.05$ in placebo vs. carnosine groups at Visit 3; **$p < 0.05$ in placebo vs. carnosine groups at Visit 4. *HbA1c* hemoglobin A1c; *BP* blood pressure; *ALT* alanine transaminase; *AST* aspartate transaminase; *ALP* alkaline phosphatase; *BUN* blood urea nitrogen; *Cl* chloride; *Na* sodium; *K* potassium

sex, age, and BMI, and seems to have no adverse effects on hepatic, renal, or vascular function. These findings suggest that carnosine supplementation is a robust, well-tolerated intervention to increase its levels and reduce the burden of toxic aldehydes. Our results further suggest that evaluating carnosine accumulation could be readily followed by measuring its levels in RBCs. Measurement of RBC carnosine could be a more economically feasible, and minimally invasive approach to assess the efficacy of carnosine supplementation especially in large-scale clinical trials.

Basal carnosine levels

In our cohort of 527 individuals, we detected carnosine in a range from 0.27 to 337.5 nmol/mg creatinine (mean concentration = 18.7 ± 34.2 nmol/mg creatinine), which is a somewhat larger range than previously reported (Tsuruta et al. 2010). This may be due to greater variability and larger sample size of our study. The reasons for the appearance of carnosine in the urine and for its variability remain unclear. In humans, ingested carnosine, is rapidly hydrolyzed by carnosinase and only that which escapes this hydrolysis (usually 1% of the ingested dose (Abe et al. 1993)) is excreted in the urine (Everaert et al. 2012). Carnosine is also released from tissues and its concentration in urine is increased upon prolonged strenuous activity (Corsetti et al. 2016). As borne out in Supplemental Table 2, demographic factors (sex, age) may have an impact on levels of urinary carnosine and carnosine propanal but, interestingly, not on levels of RBC carnosine. Additional physiological factors (diet, level of exercise), environmental factors (exogenous aldehyde/acrolein exposure) factors, hydrolysis by carnosinase (CN1), and uptake in different tissues may also impact the levels of urinary carnosine and carnosine conjugates differently than they impact RBC carnosine levels. Hence, it is not altogether unexpected that the relationship between RBC and urinary measures may show a somewhat weak correlation (Fig. 5).

Sex, age and BMI dependence of carnosine levels and carnosine uptake

Men generally have higher intramuscular carnosine content than women (Mannion et al. 1995), which may be attributed to the greater percentage of fast twitch muscle fibers, greater cross-sectional area of type II fibers, and higher testosterone concentrations in men, all of which are positive determinants of intramuscular carnosine (Everaert et al. 2013; Posa et al. 2023). The present study also shows that men have higher RBC carnosine levels than women. We found that upon supplementation, females excrete higher levels of carnosine in the urine than males (Supplementary Table 2). This may be because females have higher carnosinase activity, or lower muscle mass which acts as the sink for ingested carnosine. Thus, our current work clearly shows that not only do females have lower carnosine in their urine and RBC at baseline, but even when given equal doses of supplements, excrete more carnosine than males. Despite this difference, after supplementation with an equal dose, RBC carnosine levels in males and females were comparable

(Supplementary Table 2), suggesting that females are likely to benefit as much as males from carnosine supplementation.

Carnosine supplementation diminishes carbonyl stress

One of the chemical properties of carnosine is its ability to form conjugates with reactive aldehydes, such as acrolein and 4-hydroxynonenal (4HNE), (Aldini et al. 2002; Anderson et al. 2018; Menini et al. 2012; Hoetker et al. 2018). These ubiquitous compounds are products of normal metabolism and produced during the process of uncontrolled lipid peroxidation (Ayala et al. 2014). In addition, acrolein is a cyclophosphamide metabolite, and individuals can also be exposed to environmental sources such as that generated from cigarette usage, overheated cooking oil, and fossil fuel combustion (Henning et al. 2017; Haberzettl et al. 2021; Liu et al. 2020). Because of the highly electrophilic nature of these aldehydes, they react readily with the nucleophilic functional groups present on proteins and DNA (Uchida et al. 1998; Ishii et al. 2007; Minko et al. 2009). In cells, there is an elaborate detoxification system to remove reactive aldehydes either via enzymatic or non-enzymatic mechanisms. The major enzymatic pathways of aldehyde detoxification, includes oxidation by aldehyde dehydrogenase (Chen et al. 2014; Gomes et al. 2014), reduction by aldo–keto reductases (Zhang et al. 2019; Baba et al. 2018), and the non-enzymatic conjugation with glutathione (Conklin et al. 2015) or carnosine (Zhao et al. 2020). Removal of reactive aldehydes by carnosine is unique because only the levels of carnosine within tissues can be enhanced by supplementation (Zhao et al. 2020; Hoetker et al. 2018). We found that at baseline, the levels of carnosine-conjugates (both propanal and propanal) were lower in females than in males, which may reflect the lower basal levels of tissue carnosine in females. Nevertheless, when given a comparable dose of carnosine supplements, the levels of urinary carnosine propanal in both males and females were comparable (Supplementary Table 2), reinforcing the view that carnosine supplementation in females is as effective in removing acrolein as in males. At baseline, the levels of carnosine conjugates were higher in an older (> 40 years) group than in a younger (≤ 40 years) group, and in those with a higher (> 30 kg/m^2) rather than a lower (≤ 30 kg/m^2) BMI. These differences may reflect higher levels of oxidative stress or systemic inflammation in older or obese individuals. Upon carnosine supplementation, while there was no impact of BMI on carnosine conjugate levels in males or females, there was a greater increase of carnosine propanal in older than younger females. These observations suggest that individuals of any BMI may benefit from carnosine supplementation and reduction of carbonyl stress and that older females are likely to benefit more than younger females.

A new method of measuring carnosine accumulation in humans

Although carnosine is rapidly hydrolyzed by CN1 in plasma (Teufel et al. 2003), several studies have nevertheless demonstrated beneficial effects of oral supplementation (de Courten et al. 2016; Regazzoni et al. 2016). For example, when given to type 2 diabetics or pediatric patients with diabetic nephropathy, carnosine ameliorated high glucose levels and increased the excretion of advanced glycation end products (Elbarbary et al. 2018; de Courten et al. 2016). Our results here showing that urinary carnosine levels were enhanced up to eightfold, after ~ 6 and ~ 12 weeks of supplementation suggests that carnosine is absorbed across the gastrointestinal tract in intact form and protected from CN1 degradation. The most important evidence demonstrating that carnosine degradation could be prevented in humans is our observation that carnosine levels were approximately doubled in RBCs after oral ingestion. To our knowledge, this is the first report of a placebo-controlled, randomized study using a large human cohort showing carnosine accumulation in RBCs after ingestion. These results are consistent with a recent report showing human RBCs protected carnosine from CN1 degradation and carnosine treatment increased its levels in isolated RBCs (Oppermann et al. 2021). One shortcoming of the study is that we did not measure urinary or RBC levels of other physiologically important histidyl di-peptides, such as N-acetylcarnosine (Van der Stede et al. 2023). These levels may also be potentially useful as indices of the efficacy of supplementation.

An additional strategy for increasing carnosine levels in humans is supplementation with β-alanine (Stellingwerff et al. 2012; Blancquaert et al. 2017; Hoetker et al. 2018), which was reported to increase skeletal muscle carnosine levels by ~ 40–80% (Stellingwerff et al. 2012; Blancquaert et al. 2017; Hoetker et al. 2018; Baguet et al. 2009; Derave et al. 2007). We also reported that β-alanine supplementation in humans for 6–8 weeks doubled the carnosine content in skeletal muscle (Hoetker et al. 2018), and a recent study with carnosine itself showed similar results (Schon et al. 2023). Although we did not measure carnosine accumulation in skeletal muscle in this study, our results showing that carnosine content is doubled in RBCs suggests a corresponding increase in skeletal muscle. No differences in RBC carnosine accumulation were noted between Visits 3 and 4 of supplementation. Thus, it appears carnosine uptake reaches a steady-state by at least 6 weeks and that continued supplementation does not lead to a further increase. However, continued supplementation may

be required to maintain these levels, as they may return to baseline within weeks of cessation (Baguet et al. 2009). Regardless, our current results demonstrate the utility of RBC carnosine measures and suggest that this approach can be used to assess tissue carnosine accumulation in a pool that is protected from CN1 degradation.

Summary

This initial analysis of our large randomized, double-blind clinical trial shows that even though the levels of carnosine and carnosine conjugates vary with sex, age, and BMI, oral supplementation with carnosine was equally efficacious in increasing its levels and in promoting the urinary excretion of conjugates. Likewise, despite baseline differences in RBC carnosine due to age and sex, levels were uniformly and equally increased by oral supplementation, regardless of age, sex, or BMI. Moreover, carnosine supplementation was well tolerated with few reported adverse effects or large changes in plasma metabolic profile. One limitation of the study is the lack of dietary control. Thus, recent consumption of food with abundant carnosine content (e.g., meat) may contribute to inflated carnosine levels. Although this may be true for a few individuals, we do not feel this is common in our randomized groups. Only a small number of participants (4–6) in the placebo group demonstrated abnormally high carnosine levels at Visits-3 and 4, potentially due to meat consumption, and we assume that individual levels of meat consumption to be similar in both randomization groups. Finally, this study demonstrates the utility of measuring carnosine in RBCs, a minimally invasive approach to assess changes in tissue carnosine in large clinical trials. Importantly, the ability of oral carnosine supplementation to increase the removal of acrolein, a highly toxic α,β-unsaturated aldehyde, widely present in the environment and implicated in atherogenesis (Srivastava et al. 2011) and muscle wasting (Chen et al. 2019), suggests that supplementation with this compound could bolster innate defense and delay the onset of cardiovascular diseases.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00726-024-03414-5.

Author contributions SPB: Conceptualization, Writing—original draft, Writing—review & editing. AAA: Conceptualization, Data curation, Investigation Project administration, Supervision. DH: Data curation, Investigation, Methodology, Validation. HG: Data curation, Formal analysis, Validation. DG: Formal analysis, Investigation. JZ: Formal analysis, Investigation. MFW: Methodology, Resources. PJR: Methodology, Resources. APD: Conceptualization, Methodology. SNR: Conceptualization, Formal analysis, Validation. CAP: Conceptualization, Funding acquisition. AB: Conceptualization, Funding acquisition, Project administration, Writing—original draft, Writing—review & editing. TEO: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing—original draft, Writing—review & editing.

Funding This work was supported by a grant from the National Institutes of Health (ES019217). This organization had no role in the study design, data analysis and interpretation, or preparation of this manuscript.

Data availability Data described in the manuscript, code book, and analytic code will be made available upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This study was approved by the University of Louisville IRB (#20.0258) and all participants gave written consent.

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