



# Carnosine supplementation reduces plasma soluble transferrin receptor in healthy overweight or obese individuals: a pilot randomised trial

Estifanos Baye<sup>1</sup> · Jozef Ukropec<sup>2</sup> · Maximilian P. J. de Courten<sup>3</sup> · Timea Kurdiova<sup>2</sup> · Patrick Krumpolec<sup>2</sup> · José-Manuel Fernández-Real<sup>4</sup> · Giancarlo Aldini<sup>5</sup> · Barbara Ukropcova<sup>2,6,7</sup> · Barbora de Courten<sup>1</sup>

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## Abstract

Abnormalities of iron homeostasis have been linked to insulin resistance, type 2 diabetes and cardiovascular disease. Carnosine, an over-the-counter food supplement with chelating properties, has been shown to decrease serum iron and improve glucose metabolism in diabetic rodents. We have previously demonstrated that carnosine supplementation prevented worsening of glucose metabolism in healthy overweight and obese middle-aged adults. Yet, the impact of carnosine on markers of iron metabolism in humans has not been investigated. We aimed to determine whether carnosine supplementation has an effect on iron parameters in overweight and obese, otherwise healthy adults. We included 26 participants, who were randomly allocated to receive 1 g carnosine ( $n = 14$ ) or identical placebo ( $n = 12$ ) twice daily for 12 weeks. Iron parameters including iron, ferritin, transferrin, soluble transferrin receptor, total iron binding capacity and iron saturation were measured in serum or plasma by standard commercial assays. Carnosine supplementation decreased plasma soluble transferrin receptor compared to placebo (mean change difference  $\pm$  standard error:  $-0.07 \pm 0.03$  mg/l,  $p = 0.04$ ). None of the other iron parameters were different between carnosine and placebo groups. At follow-up, soluble transferrin receptor was associated inversely with urinary carnosine concentrations and positively with serum carnosinase-1 activity (both  $p < 0.02$ ). Our findings suggest that carnosine may modulate iron metabolism in high-risk groups which could ameliorate insulin resistance and prevent type 2 diabetes. Larger human clinical trials are required to confirm our results.

**Keywords** Iron metabolism · Soluble transferrin receptor · Carnosine · Insulin resistance · Type 2 diabetes

## Introduction

Iron is the most abundant transition metal in the body and a potent pro-oxidant that has been known to modulate glucose and lipid metabolism (Liu et al. 2009). Abnormalities of iron homeostasis have been shown to play a role in

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✉ Barbora de Courten  
barbora.decourten@monash.edu

- <sup>1</sup> Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University, 43-51 Kanooka Grove, Clayton, Melbourne, VIC 3168, Australia
- <sup>2</sup> Institute of Experimental Endocrinology, Biomedical Research Centre, Slovak Academy of Sciences, Bratislava, Slovakia
- <sup>3</sup> Centre for Chronic Disease, College of Health and Biomedicine, Victoria University, Melbourne, Australia

- <sup>4</sup> Department of Diabetes, Endocrinology and Nutrition, Biomedical Research Institute of Girona (IDIBGI), Girona, Spain
- <sup>5</sup> Department of Pharmaceutical Sciences, Università degli Studi di Milano, Milan, Italy
- <sup>6</sup> Institute of Pathological Physiology, Faculty of Medicine, Comenius University, Bratislava, Slovakia
- <sup>7</sup> Faculty of Physical Education and Sports, Comenius University, Bratislava, Slovakia

the development of several chronic diseases such as type 2 diabetes (T2DM), cardiovascular disease (CVD), neurodegenerative diseases and non-alcoholic fatty liver disease (Liu et al. 2009; Zheng et al. 2008; Rajpathak et al. 2009a; Oshiro et al. 2011). We and others have shown that iron overload is associated with insulin resistance, cardiometabolic syndrome and T2DM via inducing oxidative stress (Liu et al. 2009; Fernandez-Real et al. 1998, 2002; Cooksey et al. 2010). High concentrations of ferritin (Gillum 2001; Lecube et al. 2008), transferrin (Podmore et al. 2016) and soluble transferrin receptor (sTfR) (Freixenet et al. 2009) have been observed in obesity and T2DM. This seems counterintuitive because sTfR levels are proportional to cellular expression of the membrane-associated transferrin receptors, reflecting cellular iron demands, and, therefore, the higher the body iron stores, the lower the sTfR concentrations. However, the findings about the direction of the relationship between cardiometabolic risk and sTfR are conflicting according to a recent meta-analysis (Orban et al. 2014). At least two studies reported positive associations of sTfR with waist circumference (Freixenet et al. 2009; Montonen et al. 2012), suggesting that the higher sTfR concentrations in abdominal obesity of hyperferritinemic subjects might be due to increased infiltration of visceral fat by macrophages. Unlike other cell types, macrophages increase their transferrin receptors as their cellular iron concentrations increase (Freixenet et al. 2009). In fact, circulating sTfR seems modulated by pleiotropic effects (Souto et al. 2003). This indicates that optimal concentration of iron parameters are required for glucose and lipid metabolism (Simcox and McClain 2013). Targeting iron metabolism has, therefore, become a compelling therapeutic strategy for the prevention and treatment of chronic diseases such as T2DM and CVD (Crielaard et al. 2017; Meng et al. 2017).

Carnosine has been shown to have chelating effects in addition to its anti-oxidative, anti-inflammatory and anti-glycating properties, thereby suggested for the prevention and treatment of T2DM and CVD (Baye et al. 2016; Boldyrev et al. 2013). In-vitro, supplementation with carnosine has prevented iron-mediated toxicity in endothelial and proximal tubular cells regardless of glucose levels, which the authors ascribed to chelation of iron (Zhang et al. 2016). Carnosine has also been shown to enhance the ferric reducing activity of human plasma by converting ferric to ferrous ( $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ ), indicating its anti-oxidant ability in the presence of transition metal ions (Mozdzan et al. 2005). In streptozotocin induced diabetic rats, administration of carnosine reduced serum iron, glucose and lipid concentrations which could be due to its chelating effects or ability to scavenge free radicals and deactivate reactive oxygen species (Soliman et al. 2007). A recent human study also showed that carnosine supplementation decreased malondialdehyde levels, a marker of oxidative

stress (Elbarbary et al. 2017). However, the studies showing the role of carnosine on iron and glycaemic parameters have been carried out either in vitro or in rodents. We and others have previously shown that carnosine supplementation improved glucose metabolism in humans (de Courten et al. 2016; Houjehani et al. 2017; Elbarbary et al. 2017). To our knowledge, the effect of carnosine on markers of iron metabolism in humans has thus far not been investigated. Hence, we aimed to examine whether the chelating properties of carnosine have effect on iron parameters in non-diabetic overweight and obese individuals.

## Materials and methods

### Study design and population

Details of the study protocol have been reported elsewhere (de Courten et al. 2016; Baye et al. 2017; Regazzoni et al. 2016). This study is a secondary analysis of data from a pilot parallel-design, randomised, double-blind, placebo-controlled trial which was conducted in 30 healthy overweight and obese individuals at the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia. All participants were non-diabetic based on a 75 g oral glucose tolerance test and had no sign of infection according to a physical examination and routine blood analyses. None of them were smokers, and had taken any medications or food supplements. All participants underwent a study protocol that involved anthropometric assessments, lipid profile and glycaemic measures, assessment of urinary carnosine concentrations and serum carnosinase-1 content and activity. Participants were randomly assigned either to receive 1 g oral carnosine (Flamma S.p.A, Italy) or identical placebo (1 g sucrose) twice daily for 12 consecutive weeks. All participants were asked to refrain from substantial life style changes throughout the study period as well as from vigorous physical activity and caffeine for 3 days prior their visits. Participants with significant weight change ( $\geq 5$  kg) in the study period were excluded from the analysis. In this analysis, we have excluded three participants (1 carnosine, 2 placebo) for non-compliance with the protocol, and one additional participant (1 carnosine) due to unusually high iron parameters. All metabolic testing, blood and urine collections were performed after a 12 h overnight fast and 12 h after carnosine ingestion.

All participants provided written informed consent before enrolment into the study. The study protocol was approved by the Ethics Committee of the University Hospital Bratislava, Comenius University, Slovakia, and it adheres to the Ethical Declaration of Helsinki.

## Assessment of dietary preference

Food preference questionnaire was used to determine the participants' dietary preference for high-fat/low-fat foods based on 72 different food items. Participants rate each food hedonically on a nine-point Likert scale, with one-dislike extremely, five-neutral, and nine-like extremely. Fat preference score was computed by dividing high-fat score by low-fat score.

## Measurement of urinary carnosine and creatinine concentrations and serum carnosinase activity and content

Urine and blood samples were collected in the fasting state using pre-cooled containers and tubes, respectively, centrifuged and stored at  $-80^{\circ}\text{C}$  until analysis. Spot urine outcomes were corrected for creatinine concentrations. Urinary carnosine was measured using an internal standard and a triple quadrupole mass spectrometer (TSQ QUANTUM ULTRA, Thermo Scientific, Italy). Blank matrices for calibration curves set up were obtained by pooling urine from six volunteers aged 24–28 years who followed a lacto-ovo-vegetarian diet for 1 day before urine collection. Before pooling, biological fluids were analysed to ensure they did not contain the selected adduct above the 20% limit of quantitation. The internal standard, tyrosine hydroxylase (TH), was added at a final concentration of  $35\ \mu\text{M}$  for urine. Samples were then treated using analytical procedures as follows. Three independent samples were prepared for each level of the calibration curve and each was analysed in triplicate. Calibration curves were built by least square linear regression analysis by plotting the ratios between the peak areas of the analyte and the internal standard against the analyte's nominal concentration. For liquid chromatography–electrospray ionisation–mass spectrometry (LC–ESI–MS) analysis, urine samples were treated as follows: aliquots of  $150\ \mu\text{l}$  were mixed with  $150\ \mu\text{l}$  of an aqueous solution of 4% trichloroacetic acid (TCA) (v/v) and spiked with TH to reach a final concentration of  $35\ \mu\text{M}$ . The sample was then centrifuged ( $14,000g$ , 10 min,  $4^{\circ}\text{C}$ ), to remove any particulate matter. The urinary supernatant was collected and an aliquot was injected in the chromatography system and analysed by LC–ESI–MS in multiple reaction monitoring protocol as previously described (Aldini et al. 2011).

Urine creatinine was measured by a colorimetric assay based on Jaffe reaction (Cayman, USA) (Heinegard and Tidestrom 1973). The urine samples are diluted 1:10 with  $\text{H}_2\text{O}$  and then treated with an alkaline picrate solution. When the creatinine reacts with the picrate, a yellow/orange coloration is formed. The colour intensity is proportional to creatinine concentration and it is measured at 500 nm. The sample creatinine concentration is determined using a creatinine

standard curve. The colour derived by creatinine is then destroyed at acidic pH and the intensity is measured again as a factor of correction.

Serum carnosinase concentrations were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) developed by Adelman et al. (Adelman et al. 2012), as per instructions. Serum carnosinase activity was quantified by fluorometric determination of liberated histidine after carnosine addition. Briefly,  $10\ \text{mM}$  carnosine (Flamma, Italy) was added to serum and the reaction was stopped after 10-min incubation at  $37^{\circ}\text{C}$  by  $600\ \text{mM}$  TCA. For controls, TCA was added before carnosine. After centrifugation ( $4500\ \text{rpm}$ , 15 min, RT), supernatant was added to a mixture of OPA (incomplete phthaldehyde with 0.2% 2-mercaptoethanol) and  $4\ \text{M}$  sodium hydroxide and fluorescence were determined after 40 min (excitation: 360 nm and emission: 465 nm).

## Measurement of markers of iron metabolism

Serum iron concentration was measured by a standard commercial rate assay (Iron Kit Cat. No. 467910, Beckman Coulter Diagnostics, New South Wales, Australia). The coefficient of variation (CV) was 2.48% at  $11.14\ \mu\text{mol/l}$  and 3.8% at  $43.56\ \mu\text{mol/l}$ . The reference interval was  $13\text{--}35\ \mu\text{mol/l}$ . Both ferritin and transferrin concentrations were measured in plasma by standard commercial rate assays (Access/DXI Ferritin Reagent Pack Cat. No. 33020 and Transferrin Kit Cat. No. 467942, Beckman Coulter Diagnostics, New South Wales, Australia). The CV of ferritin was 4.99% at  $41.21\ \mu\text{g/l}$  and 8.27% at  $117.51\ \mu\text{g/l}$ . The reference interval for males aged above 10 years was  $20\text{--}300\ \mu\text{g/l}$  and for females aged 10–50 years was  $15\text{--}80\ \mu\text{g/l}$ . The CV of transferrin was 4.96% at  $1.96\ \text{g/l}$  and 4.64% at  $2.56\ \text{g/l}$ , and its reference range was  $2.0\text{--}3.6\ \text{g/l}$ . Measurement of iron, ferritin and transferrin were performed using a Beckman Coulter DXC800 Analyser. Plasma sTfR concentrations were quantitatively determined by a sandwich immunoenzymatic assay (Access sTfR assay Cat. No. A32493, Beckman Coulter Diagnostics, New South Wales, Australia) using the Access Immunoassay Systems performed on a Beckman Coulter Unicel DXI 800 analyzer. The CV was 4.90% at  $0.71\ \text{mg/l}$ , 4.81% at  $1.87\ \text{mg/l}$  and 4.26% at  $6.81\ \text{mg/l}$ . The reference interval was  $0\text{--}1.55\ \text{mg/l}$  as recommended by Beckman Coulter. Total iron binding capacity was computed by multiplying the transferrin concentrations by 25.1; while iron saturation was calculated using the formula:  $(3.982 * \text{iron})/\text{transferrin}$ . The reference intervals of iron binding capacity and iron saturation were  $45\text{--}76\ \mu\text{mol/l}$  and  $15\text{--}46\%$ , respectively.

## Measurement of markers of inflammation

High-sensitivity C-reactive protein (hsCRP) was measured in serum using an immunoturbidimetric assay

(Randox, UK). The reference range was 0.477–10 mg/dl. Serum IL-6 was quantified by a bead-based multiplex assay (LEGENDplex™ Human Metabolic Panel, Biolegend, San Diego, CA). The reference interval of IL-6 was 0–114.2 pg/ml. The CVs for hsCRP and IL-6 were < 10%.

## Statistical analyses

The sample size calculation, where insulin sensitivity was the primary outcome of the trial, has been reported elsewhere (de Courten et al. 2016). Kolmogorov–Smirnov tests and visual inspection of probability and dot plots were used to assess normality of data. Appropriate data transformation was performed when needed. Results were presented as means  $\pm$  standard deviations. Independent *t* tests were employed to determine baseline as well as change from baseline differences between carnosine and placebo groups. Pearson correlations were used to assess the relationships between markers of iron metabolism and carnosine measurements. Linear regressions were performed to determine the presence of associations between iron parameters and carnosine measurements after adjusting for age, sex and body mass index. Data analyses were performed using SAS Studio 3.4 (SAS Institute Inc., Cary, North Carolina).

## Results

Baseline characteristics of the participants are presented in Table 1. Twenty-six participants were included in this analysis. Five of them were females (2 carnosine, 3 placebo). Overall mean age and body mass index of the participants were  $42.7 \pm 8.1$  years and  $31.9 \pm 4.4$  kg/m<sup>2</sup>, respectively, with no significant differences between carnosine and placebo groups (both  $p > 0.6$ ). As previously described (de Courten et al. 2016), there were no baseline differences between groups in glycaemic parameters, blood pressure, lipid profiles, dietary preference, resting energy expenditure, and hsCRP (all  $p > 0.1$ ). Both groups also did not differ in baseline urinary carnosine-creatinine ratio ( $0.12 \pm 0.08$  carnosine vs  $0.11 \pm 0.08$  placebo,  $p = 0.8$ ) or serum carnosinase-1 activity ( $1.8 \pm 0.8$   $\mu$ mol/ml/hr carnosine vs  $2.1 \pm 0.6$   $\mu$ mol/ml/hr placebo,  $p = 0.4$ ) or content ( $57.7 \pm 23.5$  ng/ml carnosine vs  $65.2 \pm 37.5$  ng/ml placebo,  $p = 0.5$ ). Markers of iron metabolism such as iron, ferritin, transferrin, sTfR, total iron binding capacity and iron saturation were not different between carnosine and placebo groups at baseline (all  $p > 0.2$ ; Table 2). None of the iron parameters were different by gender (all  $p > 0.1$ ). Change in dietary preference score did not differ between carnosine and placebo groups (mean difference  $\pm$  standard deviation:  $-0.72 \pm 10.58$  carnosine vs  $-0.88 \pm 27.3$  placebo,  $p = 0.9$ ).

**Table 1** Baseline characteristics

Parameters	Carnosine ( <i>n</i> = 14)	Placebo ( <i>n</i> = 12)	<i>p</i> value
Age (years)	42.4 $\pm$ 7.2	43.2 $\pm$ 9.2	0.78
Body mass index (kg/m <sup>2</sup> )	31.51 $\pm$ 4.52	32.35 $\pm$ 4.44	0.63
Systolic blood pressure (mmHg)	118 $\pm$ 11.19	125.16 $\pm$ 12.71	0.13
Diastolic blood pressure (mmHg)	77.35 $\pm$ 6.73	82.66 $\pm$ 8.98	0.09
Fasting glucose (mmol/l)	5.21 $\pm$ 0.38	5.19 $\pm$ 0.37	0.86
Fasting insulin (mU/l)	12.18 $\pm$ 7.33	14.29 $\pm$ 5.21	0.41
2 h glucose (mmol/l)	6.43 $\pm$ 2.11	7.77 $\pm$ 2.44	0.14
2 h insulin (mU/l)	99.72 $\pm$ 79.25	133.7 $\pm$ 83.46	0.29
HOMA-IR (mU/l)/(mmol/l)	2.91 $\pm$ 2.05	3.29 $\pm$ 1.18	0.57
Total cholesterol (mmol/l)	5.45 $\pm$ 0.94	5.35 $\pm$ 0.85	0.78
HDL cholesterol (mmol/l)	1.23 $\pm$ 0.41	1.20 $\pm$ 0.24	0.84
Triglycerides (mmol/l)	1.76 $\pm$ 1.04	1.53 $\pm$ 0.52	0.50
HsCRP (mg/dl)	3.31 $\pm$ 2.88	4.43 $\pm$ 4.47	0.44
Urinary carnosine-creatinine ratio	0.12 $\pm$ 0.08	0.11 $\pm$ 0.08	0.79
Serum carnosinase-1 activity ( $\mu$ mol/ml/h)	1.88 $\pm$ 0.81	2.08 $\pm$ 0.63	0.48
Serum carnosinase-1 content (ng/ml)	57.71 $\pm$ 23.54	65.23 $\pm$ 37.57	0.55
Dietary preference (high fat/low fat score)	95.11 $\pm$ 14.09	86.15 $\pm$ 11.82	0.09
Resting energy expenditure (kcal/kg/day)	30.25 $\pm$ 3.57	32.30 $\pm$ 6.27	0.31

Means and standard deviations were reported. Independent *t* tests were computed to determine the *p* values HDL high-density lipoprotein, HOMA-IR homeostatic model assessment of insulin resistance, HsCRP hyper-sensitivity C-reactive protein

**Table 2** Effect of carnosine supplementation on circulating iron parameters

Parameters	Carnosine ( <i>n</i> = 14)		Placebo ( <i>n</i> = 12)		Change difference (Mean ± SE)	<i>p</i> *	<i>p</i> †
	Baseline	Change from baseline	Baseline	Change from baseline			
Iron, (μmol/l)	19.14 ± 3.77	-3.71 ± 4.21	20.25 ± 7.02	-2.66 ± 12.94	-1.05 ± 3.66	0.61	0.77
Transferrin, (g/l)	2.44 ± 0.27	-0.19 ± 0.17	2.48 ± 0.31	-0.16 ± 0.17	-0.03 ± 0.07	0.72	0.72
Ferritin, (μg/l)	85.21 ± 49.67	-18.57 ± 20.55	82.08 ± 75.90	-17.33 ± 20.74	-1.23 ± 8.12	0.90	0.88
Soluble transferrin receptor, (nmol/l)	1.17 ± 0.25	-0.13 ± 0.09	1.15 ± 0.13	-0.06 ± 0.06	-0.07 ± 0.03	0.84	0.04
Iron binding capacity, (μmol/l)	61.33 ± 6.83	-4.80 ± 4.43	62.35 ± 7.88	-4.18 ± 4.48	-0.62 ± 1.75	0.72	0.72
Iron saturation, (%)‡	0.03 ± 0.01	-0.24 ± 0.72	0.04 ± 0.01	-0.06 ± 0.15	-0.18 ± 0.21	0.65	0.40

Means and standard deviations were reported unless otherwise indicated

SE standard error

\*Baseline mean differences between carnosine and placebo groups

†Mean change differences between carnosine and placebo groups

‡Data transformation was performed. Independent *t* tests were computed to determine the *p* values

### Effect of carnosine supplementation on markers of iron metabolism

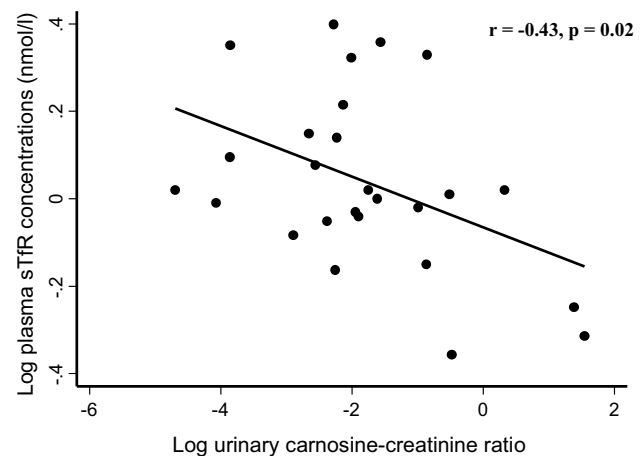
Carnosine supplementation significantly decreased the concentration of plasma sTfR compared to placebo (mean change difference ± standard error:  $-0.07 \pm 0.03$  mg/l,  $p = 0.04$ ; Table 2). Circulating serum iron, plasma ferritin, plasma transferrin, plasma iron binding capacity and serum iron saturation concentrations did not change after supplementation with carnosine compared to placebo (all  $p > 0.5$ ; Table 2).

### Associations between sTfR and carnosine measures and cardiometabolic parameters at follow-up

Plasma sTfR was correlated negatively with urinary carnosine-creatinine ratio ( $r = -0.43$ ,  $p = 0.02$ ; Fig. 1) and positively with serum carnosinase-1 activity ( $r = 0.61$ ,  $p = 0.0009$ ; Fig. 2). These associations were remained significant after adjustment for age, sex, body mass index and intervention group (urinary carnosine-creatinine ratio;  $\beta$ -coefficient:  $-0.06$  nmol/ml, 95% confidence interval:  $-0.12$  to  $-0.001$ ,  $p = 0.04$ ; serum carnosinase-1 activity;  $\beta$ -coefficient:  $0.21$  μmol/ml/hr; 95% confidence interval:  $0.08$ – $0.34$ ;  $p = 0.003$ ). Serum carnosinase-1 content was not related to plasma sTfR concentrations ( $r = 0.36$ ,  $p = 0.07$ ). Inflammatory markers such as hsCRP and IL-6, glycaemic or cardiovascular parameters were also not associated with plasma sTfR (all  $p > 0.1$ , data not shown).

## Discussion

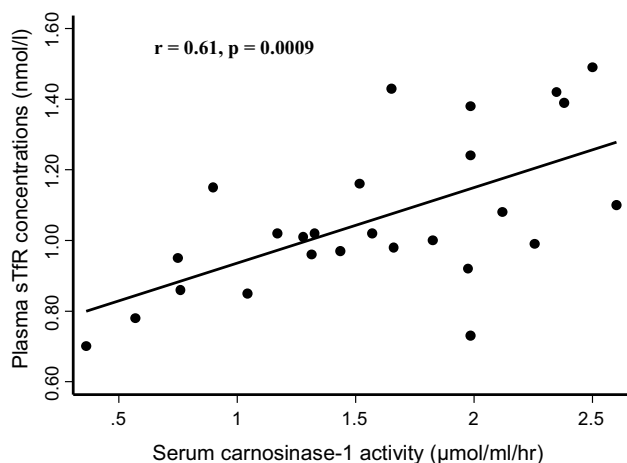
We investigated whether supplementation with carnosine changes markers of iron metabolism in overweight and obese, otherwise healthy adults. We have shown for the first



**Fig. 1** Correlation graph shows the relationship between urinary carnosine-creatinine ratio and plasma soluble transferrin receptor concentrations at follow-up ( $n = 26$ )

time that carnosine supplementation decreased plasma sTfR concentrations compared to placebo, and plasma sTfR was inversely associated with urinary carnosine-creatinine ratio and positively associated with serum carnosinase-1 activity at follow-up.

Soluble transferrin receptor is responsible for the cellular uptake of iron (Speeckaert et al. 2010), and is elevated in obesity (Freixenet et al. 2009) and insulin resistance (Fernandez-Real et al. 2007). Importantly, high sTfR was associated with increased risk of T2DM in overweight and obese individuals (Fernandez-Cao et al. 2017; Rajpathak et al. 2009b). Lifestyle intervention has been shown to reduce sTfR in high-risk groups. A study in overweight and obese children and adolescents showed a decrease in plasma sTfR after intervention with 8-month physical exercise compared to controls (Coimbra et al. 2017). Dietary weight loss with



**Fig. 2** Correlation graph shows the relationship between serum carnosinase-1 activity and plasma soluble transferrin receptor concentrations at follow-up ( $n = 26$ )

resistance training for 16 weeks also reduced serum sTfR in obese individuals compared to dietary weight loss alone and no weight loss (Fernandez-Real et al. 2009). Furthermore, a decrease in sTfR was observed after a 1-year dietary weight loss intervention in overweight and obese children compared to controls (Gong et al. 2014). In our study, we have demonstrated that concentration of plasma sTfR was reduced after carnosine supplementation compared to placebo. However, we did not find any association between sTfR and glycaemic parameters. This could be due to that all the participants were non-diabetic or the small sample size of the study. Nonetheless, we have previously shown that carnosine supplementation hampered insulin resistance and improved glucose tolerance in non-diabetic overweight and obese adults with and without impaired glucose tolerance (de Courten et al. 2016). Our data suggest that carnosine could be another strategy to prevent or delay the development of insulin resistance and T2DM in high risk groups via reducing sTfR concentrations.

Soluble transferrin receptor expression on erythroid precursors is determined by cellular iron demand (Speckaert et al. 2010). Circulating sTfR concentration in serum reflects total body sTfR concentration (Skikne 2008). It has been suggested as a better marker of iron overload in obese individuals (Mast et al. 1998). High concentration of sTfR was commonly reported in obese people (Fernandez-Cao et al. 2017; Freixenet et al. 2009; Coimbra et al. 2017; Sharma et al. 2009; Lecube et al. 2006), which could be due to functional iron deficiency, a state in which there is insufficient iron incorporation into erythroid precursors in the case of apparently adequate body iron stores (Macdougall et al. 1989). Carnosine has been suggested to downregulate transferrin receptor protein expression in vitro (Zhang et al. 2016). In humans, low plasma carnosinase activity promotes

circulating carnosine after supplementation with carnosine (Everaert et al. 2012). Consistent with this, we find that sTfR was associated inversely with urinary carnosine-creatinine ratio after adjustment for age, sex and body mass index. We have previously shown that carnosine supplementation increased urinary carnosine concentrations, but did not change serum carnosinase 1 activity or content (de Courten et al. 2016). Our findings may suggest that carnosine supplementation may improve iron uptake by erythroid precursors, thereby reducing the concentration of sTfR. Future mechanistic studies are needed to confirm this.

We report that sTfR was positively associated with serum carnosinase-1 activity after adjustment for age, sex and body mass index. Recent studies have shown that carnosinase-1 activity is modulated through an allosteric mechanism involving cysteine residue at position 102 (Cys102) (Peters et al. 2017). In particular, thiol-containing compounds, such as glutathione and cysteine, reduce carnosinase-1 activity through the formation of mixed disulfides. Since sTfR is involved in the redox homeostasis and hence in the protein cysteinylaton process, we suggest that carnosinase-1 activity may be modulated allosterically by the formation of Cys102 mixed disulfides, a reaction which is most likely related to plasma sTfR concentrations.

Carnosine has been shown to chelate heavy metal ions such as copper and zinc (Boldyrev et al. 2013). This has been shown to inhibit oxidative and inflammatory pathways (Nagai et al. 2012). In-vitro, carnosine prevented iron mediated toxicity in renal tubular cells likely due to chelating effects of carnosine (Zhang et al. 2016). Carnosine administration also resulted in decrease in serum iron concentration and improved glucose metabolism in streptozotocin induced diabetic rats (Soliman et al. 2007). In our study, however, carnosine supplementation did not change iron concentrations, which suggests that carnosine has no direct chelating effects on iron in healthy overweight or obese individuals.

Iron as a transition metal can generate reactive oxygen species via Fenton reaction, leads to the formation of a variety of toxic by-products including hydroxynonenal, and induces oxidative stress (Liu et al. 2009). sTfR to ferritin ratio was associated with urinary 8-hydroxydeoxyguanosine excretion, a marker of oxidative damage in vivo (Tuomainen et al. 2007). Furthermore, markers of oxidative stress such as malondialdehyde and total reactive oxygen species were positively correlated with serum iron parameters in patients with  $\beta$ -thalassemia (Choudhuri et al. 2013). Carnosine has the ability to interrupt the production of hydroxyl radicals by trapping the Fenton reaction (Velez et al. 2008; Price et al. 2001), decrease malondialdehyde formations (Liu et al. 2008), and inhibit oxidative reactions (Nagai et al. 2012), which could subsequently improve iron and glucose metabolism. It has also been shown that carnosine is the most effective and selective hydroxynonenal sequestering agent

available (Aldini et al. 2014). The anti-oxidant properties of carnosine could, therefore, be a possible mechanism for the reduced sTfR.

Inflammatory cytokines including interleukins and tumour necrosis factor alpha have been shown to upregulate sTfR expression in vitro (Tsuji et al. 1991; Johnson et al. 2004). sTfR was positively associated with interleukin-6 (Coimbra et al. 2017) and C-reactive protein concentrations (Sharma et al. 2009). In rodents, carnosine suppressed the production and release of interleukin-6 and tumour necrosis factor alpha as well as C-reactive protein (Yan et al. 2009; Tsai et al. 2010). A recent human study in patients with T2DM showed that tumour necrosis factor alpha was reduced after carnosine supplementation (Houjehani et al. 2017). In our study, we did not find any association of sTfR with IL-6 and hsCRP. This may be due to that all participants had no sign of inflammation at enrolment or the small sample size of the study. Although future studies are needed to confirm the exact mechanism, it may be possible that the reduction of chronic low-grade inflammation after carnosine supplementation may downregulate the expression of sTfR.

Advanced glycation end products (AGEs) have been shown to associate with iron overload (Choudhuri et al. 2013). AGEs are also known to contribute to increased oxidative stress and inflammation (Vlassara 2001). A recent study in patients with  $\beta$ -thalassaemia showed positive associations between serum AGE concentrations and iron indices (Choudhuri et al. 2013). We have not measured AGEs in our study. Nonetheless, several experimental studies demonstrated that carnosine has the ability to inhibit AGEs formation produced through a non-enzymatic glycation and a direct reaction with reactive carbonyl species generated by lipid and sugar oxidation (Baye et al. 2016). In uremic patients undergoing peritoneal dialysis, supplementation with carnosine has also been shown to prevent AGEs formation by acting as a sacrificial molecule protecting protein amino groups from glycation by highly reactive carbonyl compounds (Alhamdani et al. 2007). This finding suggests that the antiglycating ability of carnosine may contribute to the reduction in sTfR concentration; however, this has to be confirmed by future mechanistic studies.

In this pilot clinical trial, comprehensive metabolic profiling of the study participants were performed; rigorous randomisation process was employed; allocation of treatment to the intervention groups was concealed; healthy participants who did not take any medications or supplements were studied; and no baseline differences between groups were observed, which all restricts the effect of confounding on reported outcome measures. We have measured several markers of iron metabolism including iron, ferritin, transferrin, sTfR, total iron binding capacity and iron saturation. We did not measure hepcidin concentrations and the participants' actual dietary intake, which could be a limitation

of this study. The small sample size of the study could be another limitation of this trial, where sample size calculation was not computed based on the outcome of iron parameters. The study was performed in overweight or obese individuals; hence, results cannot be extrapolated to another target groups, including individuals with normal weight. Larger sample size trials are therefore needed to confirm the effect of carnosine supplementation on iron metabolism.

In conclusion, we have demonstrated for the first time that carnosine supplementation reduced plasma sTfR in healthy overweight and obese humans. This suggests that carnosine may improve iron metabolism in high-risk groups and thereby prevent and delay the development of T2DM and CVD; however, future larger clinical trials are required to confirm our findings.

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**Author contributions** EB conceived the study idea, performed data analysis and interpretation, wrote first draft of the manuscript and revised subsequent drafts until publication. BdC designed the study, contributed to the study idea and data analysis, and critically reviewed the manuscript. MPJdC co-designed the study with BdC and contributed to the review of the manuscript. JU & BU assisted with design of the study, performed all clinical measurement and contributed to review of the manuscript. TK & PK contributed to the clinical study performance and data collection. JMFR contributed to review of the manuscript. GA performed laboratory measurement and reviewed the manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Written informed consent was obtained from all individual participants included in the study.

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