BMJ Open

Does supplementation with carnosine improve cardiometabolic health and cognitive function in patients with pre-diabetes and type 2 diabetes? study protocol for a randomised, double-blind, placebo-controlled trial

Estifanos Baye,[1] Kirthi Menon,[1] Maximilian PJ de Courten,[2] Arul Earnest,[3] James Cameron,[4] Barbora de Courten[1,5]

To cite: Baye E, Menon K, de Courten MPJ, *et al*. Does supplementation with carnosine improve cardiometabolic health and cognitive function in patients with pre-diabetes and type 2 diabetes? study protocol for a randomised, double-blind, placebo-controlled trial. *BMJ Open* 2017;**7**:e017691. doi:10.1136/bmjopen-2017-017691

► Prepublication history and additional material are available. To view these files please visit the journal online (http://dx.doi.org/10.1136/bmjopen-2017-017691)

Received 10 May 2017
Revised 20 June 2017
Accepted 21 June 2017

For numbered affiliations see end of article.

Correspondence to
Professor Barbora de Courten; barbora.decourten@monash.edu

ABSTRACT

Introduction Carnosine, an over-the-counter food supplement, has a promising potential for the prevention and treatment of chronic diseases such as type 2 diabetes (T2DM), cardiovascular and neurodegenerative diseases through its anti-inflammatory, antiglycation, antioxidative and chelating effects. We have previously shown that supplementation with carnosine preserves insulin sensitivity and secretion in non-diabetic overweight and obese individuals. The effect of carnosine on cardiometabolic risk and related cognitive outcomes in patients with pre-diabetes and T2DM has thus far not been studied. We therefore aim to investigate whether supplementation with carnosine improves cardiometabolic health and cognitive function in patients with pre-diabetes and T2DM.

Methods and analysis We will employ a parallel design randomised controlled trial. Fifty participants with pre-diabetes (impaired fasting glycaemia and impaired glucose tolerance) and T2DM (with HbA1c level < 8%) aged between 18 to 70 years will be randomly assigned to the intervention or control group. At baseline, participants will undergo a medical review and series of tests including anthropometric measurements (body mass index, a dual X-ray absorptiometry and peripheral quantitative computed tomography scan), an oral glucose tolerance test, cardiovascular measurements (central blood pressure, endothelial function and arterial stiffness), cognitive function, physical activity measurement, heart rate variability and liver fibroscan as well as questionnaires to assess dietary habits, sleep quality, depression and quality of life. The intervention group will receive 2 g of carnosine daily in two divided doses while the control group will receive identical placebo capsules for 14 weeks. All baseline measurements will be repeated at the end of the intervention. The change in glycaemic, cardiovascular and cognitive parameters as well as other measures will be compared between the groups.

Ethics and dissemination This study is approved by the Human Research Ethics Committee of Monash Health and Monash University, Australia. The findings will be disseminated via peer-reviewed publications and conference presentations.

Strengths and limitations of this study

- Employs direct measures of clinical outcomes alongside blood analysis for comprehensive investigation of the potential mechanisms involved.
- Assesses possible confounders influencing cardiometabolic outcomes.
- Measures only surrogate markers for diabetes and cardiovascular diseases including fasting glucose and insulin, haemoglobin A1C, central blood pressure, endothelial function and arterial stiffness, but not hard clinical end points.

Trial registration NCT02917928; Pre-results.

INTRODUCTION

Diabetes has become a major global public health problem. The number of people with diabetes worldwide has more than doubled over the past three decades[1] and it is expected to rise to 592 million by 2035, with most of these cases being type 2 diabetes (T2DM).[2] The prevalence of pre-diabetes is also increasing worldwide and it is projected that more than 470 million people will have pre-diabetes in 2030.[3] It has been estimated that around 5%–10% of people with pre-diabetes become diabetic annually.[4] T2DM dramatically increases the risk of cardiovascular diseases (CVD) with ~80% of individuals who have both obesity and T2DM developing CVD.[5] T2DM and CVD are associated with increased risk of cognitive decline.[6–10] Furthermore, neurodegenerative diseases such as Alzheimer's and Parkinson's disease often occur in people with T2DM. Alzheimer's and Parkinson's diseases share

many common pathogenic features with T2DM and CVD including chronic low-grade inflammation (CLI), increased oxidative stress and accumulation of advanced glycation endproducts (AGEs).[11] Progression of these chronic diseases is worsened by a sedentary life-style. Lifestyle modifications not surprisingly are the mainstay of prevention and therapy of these chronic diseases.[12–17] However, these strategies are difficult and costly to achieve on a large scale and thus the T2DM epidemic continues unabated. Low-cost, accessible and easy to implement interventions, which are synergistic to lifestyle intervention, are therefore needed.

Carnosine, an over-the-counter food supplement, is currently used in exercise physiology to increase exercise performance.[18] Several animal and limited human studies suggest that carnosine has potential for the prevention and treatment of obesity, T2DM, CVD, and neurodegenerative diseases[19–22] through its anti-inflammatory, antioxidative, anti-glycating and chelating effects.[20 21 23–25] Our team has demonstrated for the first time that supplementation with carnosine prevented worsening of glucose metabolism in non-diabetic obese and overweight adults.[26] Importantly, as in previous trials,[27–30] there were no side effects associated with the carnosine supplementation. Another trial using carnosine combined with other supplements in overweight and obese non-diabetic individuals showed a decrease in fat mass and fasting glucose and an increase in fat free mass with a trend for improvement of insulin secretion.[31] However, in that trial, it is not clear if these effects were due to carnosine or the other supplements[31]. A beneficial effect of carnosine was also described in a recent intervention in patients on standard heart-failure therapy who were suffering from stable chronic heart failure and severe left-ventricular dysfunction.[32] Other trials reported the beneficial role of carnosine on cognitive performance in healthy individuals and patients with neurodegenerative diseases.[27 33–35] None of them, however, investigated the interplay between cardiometabolic risk and cognitive outcomes.

We therefore aim to conduct a randomised placebo-controlled clinical trial to determine the effect of carnosine on improving the cardiometabolic profile and cognitive function in patients with pre-diabetes and T2DM, and study the mechanisms involved.

METHODS AND ANALYSIS

Study design and setting

This study is a parallel design randomised double-blind placebo controlled trial (figure 1). We have followed the Standardised Protocol Interventions: Recommendations for Interventional Trials (SPIRIT) 2013 Statement which

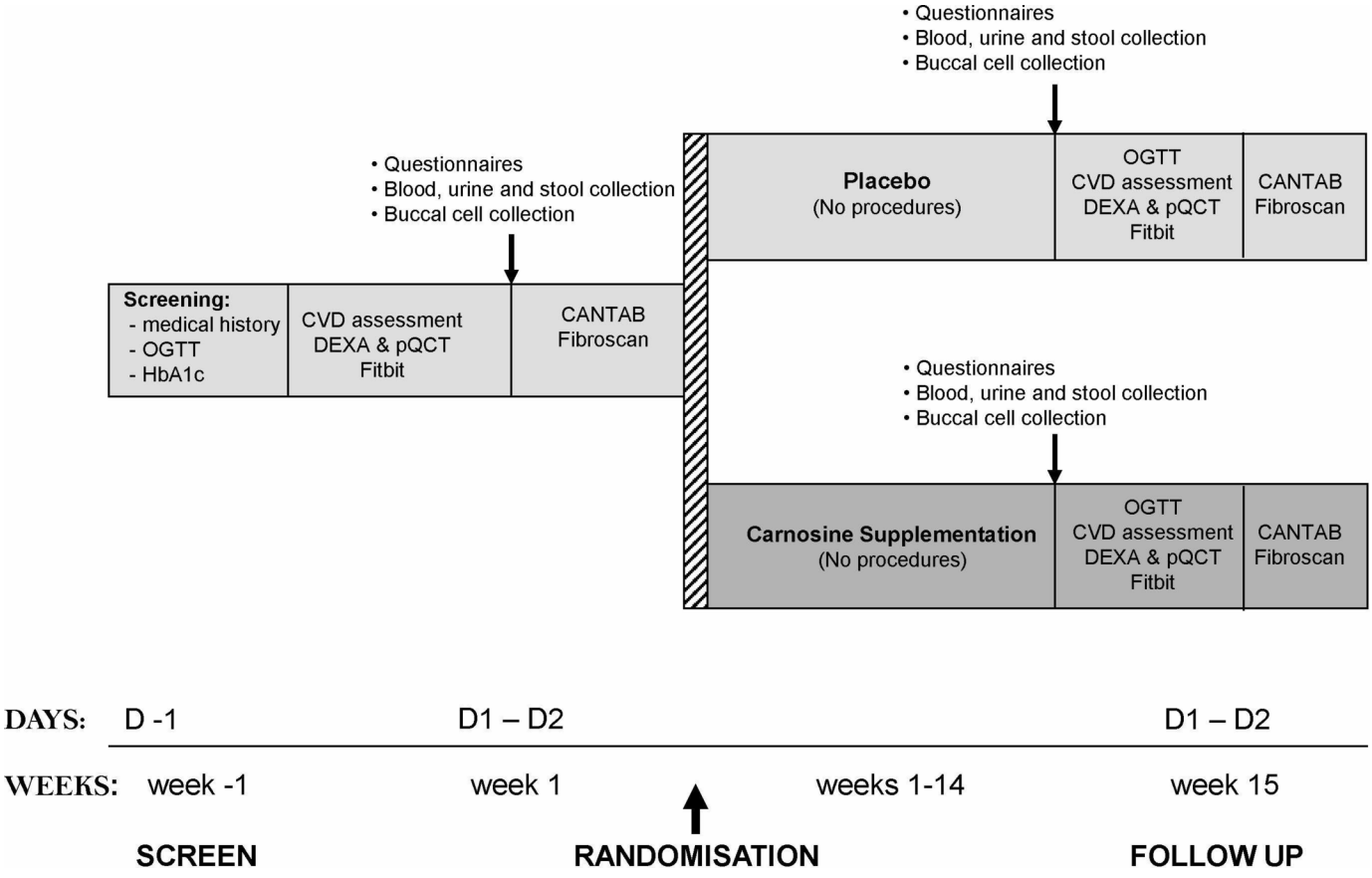

Figure 1 Study timeline. CANTAB, Cambridge neuropsychological test automated battery; CVD, cardiovascular disease; DEXA, dual energy X-ray absorptiometry; HbA1c, haemoglobin A1c; OGTT, oral glucose tolerance test; pQCT, peripheral quantitative CT scan.

defines standard protocol items for clinical trials (see online supplementary file 1).[36] Fifty patients with pre-diabetes and T2DM (diet controlled or on oral therapy) aged between 18 to 70 years will be included. Participants will be randomised to receive either 2 g daily carnosine in two divided doses or identical placebo for 14 weeks. Participants will be sought via several advertising strategies, including posters, flyers, newspapers, and email newsletters at Monash University and Monash Medical Centre in Melbourne, Australia, and via online advertising, including social media and popular community websites. Participants with T2DM will also be recruited from the National Diabetes Service Scheme – an initiative of the Australian Government administered with the assistance of Diabetes Australia.

Inclusion criteria

Inclusion criteria are (1) aged between 18 to 70 years, (2) have pre-diabetes (impaired fasting glycaemia and impaired glucose tolerance) or T2DM (previously diagnosed or on the basis of the screening oral glucose tolerance test or on medications for treatment of diabetes), (3) haemoglobin A1c (HbA1c) level in patients with T2DM below 8%, (4) stable body weight, having exhibited a weight change <5 kg in the last 6 months with no intention to lose weight or change physical activity in the study period, (5) patients with T2DM have to be either on diet or oral therapy without changes in treatment at least for the previous 3 months and will also be advised not to change their pre-existing therapy for the duration of the study if HbA1c does not exceed 8%.

Exclusion criteria

Exclusion criteria will include (1) morbid obesity (body mass index (BMI) >40 kg/m2), (2) current smoking and/or high alcohol use (more than four standard drinks per week for men and more than two standard drinks per week for women), (3) taking anti-inflammatory medications and any medications or supplements known to effect cardiometabolic or cognitive outcomes, (4) blood transfusion history in the past 3 months, (5) any renal (estimated glomerular filtration rate of <30 mL/min), cardiovascular, haematological, respiratory, gastrointestinal, endocrine or central nervous system diseases, psychiatric disorders, active cancer within the preceding 5 years, or the presence of acute inflammation or infection based on the medical history and the physical and laboratory examinations obtained at recruitment, and (6) pregnant or lactating women.

Sample size calculation

The sample size is calculated based on data from a similar cohort of subjects with pre-diabetes and T2DM studied in our laboratory, which had a mean (SD) fasting glucose value of 10 (2.0) and HbA1c of 7 (0.5). On this basis, a sample size of 22 completed subjects in each arm is required to detect a 20% difference in fasting glucose and an absolute change of 0.5% in HbA1c respectively. This effect size is clinically significant and similar to that observed after a 12 week carnosine supplementation in individuals with pre-diabetes.[26] Based on a type I error of 0.05 (two-tail) and a type II error of 0.10 (power = 90%), we would require 50 participants to complete the trial including an additional six participants to allow for non-completion.

Screening

The study timelines are presented in figure 1. At visit 1, a signed informed consent form will be obtained from each participant followed by screening by a registered medical practitioner, who will take a medical history and perform a physical examination, including vital signs and anthropometric measures using the methods outlined below. A urine pregnancy test will be performed in females to exclude pregnancy. Participants will then undergo an oral glucose tolerance test (OGTT) with measurement of glucose levels in every 30 min for five time points. Both fasting and 2 hour glucose levels will be used to determine presence of either pre-diabetes or diabetes according to WHO guidelines.[37] Screening blood samples will be sent to Monash Pathology to be analysed for full blood count, HbA1c, kidney and liver function tests, lipid profile, concentration of calcium, magnesium and phosphate, and C-reactive protein (CRP) which will be used as a marker of inflammation.

Baseline assessments

Baseline assessments for eligible participants will commence at the second visit, which will involve measurement of endothelial function by EndoPAT, central blood pressure and arterial stiffness (Complior), and assessment of whole body composition (dual X-ray absorptiometry), and muscle and fat tissue density of the skeletal muscle in the non-dominant leg (peripheral quantitative CT). Physical function (dynamometer), physical activity (Fitbit) and heart rate variability using Biomodule BH3 will also be measured. At the third visit, participants' cognitive function using the Cambridge Neuropsychological Test Automated Battery for Prodromal Alzheimer's Disease (CANTAB test) and liver fibroscan will be assessed. Urine samples for measurement of AGEs, urinary carnosine and albumin-creatinine ratio (ACR), stool samples for microbiome and buccal samples for DNA analyses will be taken. Blood samples will also be collected for measurement of AGEs, carnosine levels, DNA damage tests and markers of inflammation. Participants will complete validated questionnaires to assess physical activity, dietary habits, knee and back pain, sleep quality, presence of depression and quality of life.

Randomisation

Following baseline assessments, participants will be randomised to commence supplementation with either carnosine or placebo. Randomisation will be performed in blocks of four by gender to ensure gender balance and stratified by treatment (on treatment versus not on

treatment). The randomisation codes will be generated by the study statistician and will be sent to the Monash Health Clinical Trial Pharmacy, Melbourne for allocation and dispensing. Non-transparent capsules of carnosine and placebo will be provided in clear containers and they will be identical and tasteless, to ensure that both participants and investigators are blinded to the treatment. To maximise compliance, participants will be contacted every 4 weeks by phone, and will be advised to return the empty containers at the end of the study.

Intervention

All participants will be instructed to consume orally four capsules of carnosine (Flamma S.p.A, Italy) or matching placebo daily (two capsules of 500 mg twice daily) for 14 weeks. The purity of carnosine (CarnoPure™) used in this study is greater than 99.5% and it is odourless, crystalline and fully synthetic. The daily dose of 2 g was selected on the basis of previous human trials and our pilot intervention, demonstrating changes in insulin sensitivity and secretion.[26] Participants will be asked to maintain their usual physical activity and make no dietary changes.

Follow-up visits

Participants will be scheduled for their follow-up visits at the end of the last week of the study while still being on the supplements. All the procedures that were performed during the baseline visits including blood pressure measurement, anthropometry, OGTT, body composition, central blood pressure, endothelial function and arterial stiffness, muscle and fat density in skeletal muscle, liver fibroscan, and heart rate variability will be repeated. Participants will again be asked to fill out the same questionnaires.

Safety considerations

During the screening, baseline and follow-up procedures, any new medical conditions or abnormal laboratory tests requiring urgent attention will be promptly discussed with the participant by a qualified medical practitioner involved in the study. If they do not require urgent medical attention, they will be discussed with participant at the end of the trial. Where appropriate, participants will be advised to visit their general practitioner for follow-up. All participants will be informed of the results of their medical review and blood tests after completion of the study. As a part of the trial, all participants will be advised on strategies to improve their diabetes and cardiovascular risk profile after completion of the trial.

Outcome assessment

The primary outcome of this trial is the difference (change) in glycaemic control (2 hour glucose) between carnosine and placebo groups. Secondary outcomes include changes in other metabolic parameters (fasting glucose, glucose and insulin area under the curve (AUC) after OGTT, HbA1c) body weight, body mass index, body fat composition, waist-to-hip ratio, resting systolic and diastolic blood pressure, endothelial function, arterial stiffness, lipid profile, liver fibroscan and cognitive function. Exploratory outcomes include markers of inflammation including interleukins (IL-1β, IL-6, IL-8 and IL-10), CRP, tumour necrosis factor α (TNFα), macrophage migration inhibitory factor (MMIF), monocyte chemoattractant protein-1 (MCP-1), and nuclear factor kappa beta (NF-κβ) activity. In addition, measurement of AGEs and ALEs, heart rate variability, self-reported questionnaire data and physical activity will also be included as exploratory outcome measures or co-variates.

Data collection and analysis

Anthropometry

BMI

Body weight (kg) and height (cm) will be measured using a digital scale (Tanita BWB-600,

Australia) and stable stadiometer (Seca 206, Australia), respectively, both at baseline and following the intervention period, during which participants will be lightly clothed and without shoes. BMI will be calculated as weight (kg)/height (m) square.

Waist-to-Hip Ratio (WHR)

Waist and hip circumference will be measured in duplicate using a constant-tension tape for measuring. Waist circumference will be measured at the midpoint between the upper iliac crest and the lowermost rib at the end of a normal expiration, while hip circumference will be determined at around the widest part of the buttocks. WHR will be determined as waist (cm)/hip (cm).

Body fat composition

Dual energy X-ray absorptiometry (DEXA) will be used to measure whole body fat composition. The fat tissue mass measured in this way consists of the sum of the fatty elements of all the soft tissue, not just adipose tissue, while the lean tissue mass reflects the sum of all chemical fat free tissue components. DEXA has been validated as an accurate, safe, and non-invasive method of measuring soft tissue body composition by region.[38]

Muscle and fat tissue density in skeletal muscle

Peripheral quantitative CT scan (pQCT) (Stratec XCT3000, Stratec, Germany) will be used to measure the muscle and fat tissue and bone density of the participants' non-dominant leg. It is a three-dimensional technique which determines volumetric bone mineral density, is capable of differentiating cortical and trabecular bone, and assessing three-dimensional geometric properties, at peripheral sites.[39] pQCT is also used to identify cross-sectional area and density of muscle and fat tissues.

Metabolic studies

OGTT

After a 10–12 hour overnight fast, participants will ingest 75 g of glucose over 2 min. Blood samples will be drawn at 0, 30, 60, 90, and 120 min to check plasma glucose and insulin concentrations. Glucose and insulin AUC will also be computed.

Cardiovascular studies

Blood pressure

Resting systolic and diastolic blood pressure and pulse rate will be measured using an automated oscillometric system (Omron, Australia)) after a 20 min seated rest. The average blood pressure derived from three measurements will be recorded.

Lipid profile

Lipid profile-related parameters to be measured include plasma total cholesterol, triglycerides, low-density and high-density lipoprotein cholesterol using a standard commercial enzymatic assay, a Beckman Coulter LX20PRO analyser and SYNCHRON Systems lipid and multi calibrators (Beckman Coulter Diagnostics, Australia).

Arterial stiffness and central pressure

Aortic (carotid–femoral) pulse wave velocity (aPWV) will be measured using the Complior device (Alam Medical, France). Pulse transit time - the time between successive arrivals of the foot (beginning of systole) of simultaneously recorded pressure waves – is averaged over 10 cycles; and velocity is derived from PWV = D/Δt (m/s), where D (distance) is measured in accordance with current guidelines of the European Working Group on Large Arteries.[40 41] Central blood pressure and aortic augmentation index will also be measured by this device.[42]

Endothelial function

Non-invasive peripheral arterial tomography (EndoPAT, Itamar Medical, Israel) will be used to record continuous plethysmographic signals of the finger arterial pulse wave.[43] Finger plethysmographic probes are placed on each index finger; and after a 5 min equilibration period, a blood pressure cuff on the non-dominant arm is inflated to 200 mmHg for 5 min and then deflated to induce reactive hyperaemia. Measurements of post-occlusion changes (reactive hyperaemia PAT: RH-PAT) are continued for 10 min. Results are normalised to the non-occluded arm, compensating for potential systemic changes (RH-PAT ratio).

Heart Rate and Heart Rate variability

The Zephyr Biomodule BH3 (Black Sensor, produced by Zephyr Technology) will be used to measure heart rate and heart rate variability for three consecutive days. It is a compact lightweight physiological monitoring telemetry device intended for monitoring of adults in the home, workplace and alternate care settings. The device consists of a BioPatch receptacle and an electronics module (monitoring and recording device). It is attached to the chest using standard adhesive electrocardiography (ECG) electrodes. The device stores and transmits vital sign data including ECG, heart rate (HRV), respiration rate, body orientation and activity. The BioModule BH3 provides the ability to detect and transmit single lead ECG signals to be received by IEEE 802.15.4/USB qualified ECG instruments. The BioModule BH3 collects and transmits measurements captured during both sedentary as well as rigorous activity for heart rate, posture and activity.

Cognitive tests

Participants' cognitive function will be assessed using the Cambridge Neuropsychological Test Automated Battery for Prodromal Alzheimer's Disease (CANTAB software, UK).[44] Motor screening task, reaction time, rapid visual information processing, paired associates learning, delayed matching to sample, pattern recognition and spatial working memory tests are included in the battery. The Victoria Stroop, Trail Making and Digit Symbol Substitution tests will also be used to supplement the CANTAB test.

Liver fibroscan

Non-invasive transient elastography (Fibroscan, Echo-Sens, France) will be used to assess liver fibrosis based on the measurement of liver fat and stiffness.[45] The technique uses an ultrasound to determine the speed of a shear wave emitted from a transducer probe directed towards the liver. The velocity of this wave is proportional to the density, stiffness or degree of fibrosis in the liver. Also, properties of the ultrasonic signals called controlled attenuation parameters measure the degree of liver fat.

Physical function and strength

Physical activity

A Fitbit Charge Heart Rate (Fitbit, USA) activity tracking monitor will be used to objectively measure participants' physical activity for five consecutive days. The feedback screen on this device will be obscured so as to avoid influencing participant activity levels. Physical activity data including steps/day, minutes of moderate/vigorous activity/day and average heart rate will be recorded. Participants will also be provided with a diary to complete during each monitoring period.

Hand grip strength

will be assessed for both hands using a Jamar hydraulic hand grip dynamometer (Lafayette Instrument Company, USA). The participant will hold the hand grip dynamometer with their elbow at a 90° angle and their opposite arm resting on their lap. The participant will apply as much force as possible for three seconds while in a seated position. This test will be completed three times with a 30 s rest between trials. The mean of the final two trials for each hand will be recorded as the criterion measure for hand grip strength.

Knee extension strength

will be measured in the dominant leg using a Baseline Cable Tensiometer (Fabrication Enterprises, USA). The participant sits upright on a tall stool with a seat back, and with their hands resting on their lap. The tensiometer is secured to the rear leg of the stool corresponding with the leg to be assessed. A Velcro strap is secured to the participant's ankle and they are instructed to apply a maximal force by attempting to move their leg in a

forward direction for three seconds. The tensiometer dial indicates the peak force achieved in kilograms. Three trials are performed in each leg with the mean score for each leg taken as the criterion value for quadriceps strength.

Self-Reported questionnaires

Record of habitual diet

Before and after intervention, dietary intake (energy, macronutrient, micronutrient, food groups) will be assessed through weighed 2×3 day food records, comprising three consecutive days (2 weekdays and one weekend day). Additional days or non-consecutive days will be analysed if considerable dietary variation is found. Food records will be analysed using Foodworks 7 Professional Dietary Software (Xyris Software, Australia) and Australian food composition data (NUTTAB 2010). Participants' preference for high-fat/low-fat foods will be also be determined using Food Preference Questionnaire. Participants will rate each food (n=72) hedonically on a 9-point Likert scale, with 1-dislike extremely, 5-neutral, and 9-like extremely. Fat preference score will be a ratio of high-fat score to low-fat score.

International Physical Activity Questionnaire (IPAQ)

The short version of IPAQ will be used to subjectively measure participants' physical activity in addition to the Fitbit. The short IPAQ asks participants to reflect on the past 7 days and report time spent on vigorous activity (eg, aerobics), moderate activity (eg, carrying light loads), walking, and sitting.[46]

Sleep quality

Pittsburgh Sleep Quality Index will be used as a general subjective measure of sleep, which will be completed by the participant before and after the intervention.[47] Participants will also be asked to complete the Pittsburgh Sleep Diary and Stanford Sleepiness Scale in the morning for 2 periods of 14 days (before and after).[48 49] The participants' usual sleep habits for the past 1 month will be assessed.

Depression scale

Personal Heath Questionnaire Depression Scale will be used to assess participants' depression level.[50] It is an 8-item self-report questionnaire that asks participants' to rate their feeling for the past 2 weeks from not at all (0) to nearly every day (3).

Quality of life

EuroQol five dimensions questionnaire (EQ-5D) is a standardised instrument for measuring general health status in terms of five dimensions; mobility, self-care, usual activities, pain/discomfort, and anxiety/depression.[51] Participants will rate their level of severity in each dimension using three level scale.

Laboratory measurements

Measurement of carnosine and carnosinase

Serum and urine carnosine levels will be quantitatively analysed with high-performance liquid chromatography/electrospray ionisation tandem mass spectrometry (HPLC-ESI-MS systems) (triple quadruple and orbitrap MS analyser); metabolites of carnosine from covalent detoxification of the reactive carbonyl species (precursors of AGEs and ALEs) will be profiled similarly. Plasma carnosinase protein content will be measured by ELISA for human carnosinase one with a monoclonal antibody (clone ATLAS, Abcam) and peroxidase substrate.[52]

Measurement of AGEs & ALEs

Plasma and urinary AGEs and ALEs will be measured by LC–MS and ELISA tests.[53] Circulating receptor for AGEs will be measured by ELISA.[54] Protein modifications and the effect of carnosine supplementation will be determined by proteomic approaches. Systemic oxidative status will be determined by measuring the advanced oxidation protein products and by measuring the cysteinylated form of albumin by mass spectrometry. Mercapturic acid adducts with the main reactive carbonyls species will also be quantitatively determined by LC-ESI-MS/MS.[53]

Inflammatory markers

Plasma inflammatory markers (IL-1β, IL-6, IL-8 and IL-10, TNFα, MMIF, and MCP-1) will be measured using a bead-based multiplex assay (Multi-analyte flow assay kit, LEGENDPlex, BioLegend, CA, USA), while plasma CRP will be analysed via highly sensitive near infrared particle immunoassay rate methodology and a Beckman Coulter Synchron LX system chemistry analyser (Beckman Coulter Inc., Australia).

NF-κβ activity

Nuclear extracts of white blood cells will be obtained and analysed for the binding capacity of the p50/p65 subunit of NF-κβ to an NF-κβ oligonucleotide consensus sequence as per the manufacturer's instructions (Active Motif, CA, USA).

DNA damage tests

Telomere length and micronuclei will be measured in DNA isolated samples according to the protocol previously described.[55 56] Frozen isolated lymphocytes and buccal cells will be used for DNA damage tests to measure telomere length and mitochondrial DNA deletions. Buccal cells will be collected in a minimally invasive manner from the inside of the cheek/mouth using a soft toothbrush.

Statistical analysis

Changes in glycaemic control, and other parameters across interventions, will be compared using the Analysis of Covariance (ANCOVA) model to account for covariates such as age, sex, obesity, physical activity, and carnosinase activity if necessary. The assumptions for the tests will be assessed and data transformation and non-parametric tests applied when appropriate. Categorical variables will be compared between the two arms using the Chi-squared test or Fisher's Exact test wherever appropriate. *P* values of <0.05 will be taken as statistically

significant. Analysis will be performed by intention to treat principle. A formal interim analysis is planned when 25 patients have been recruited and have the endpoints successfully assessed. The trial will be stopped early for efficacy when the t-statistic crosses the 2.9626 level for both endpoints (Fasting glucose and HbA1c), according to the sequential O'Brien-Fleming alpha spending function proposed by Lan and DeMets.[57] Data analysis will be performed in Stata V14.0 (Stata Corp, College Station, Tx, USA).

ETHICS AND DISSEMINATION

Ethics approval

This trial has obtained ethical approval from the Human Research Ethics Committee of Monash Health (ID number: 16061A) and Monash University (ID number: 7787), Melbourne, Australia. The trial has commenced in October 2016 and currently recruiting participants.

Informed consent

All participants will receive adequate information about the nature, purpose, possible risks and benefits of the trial before enrolment. A written informed consent form is required for enrolment.

Dissemination

The study findings will be disseminated in national and international conferences, and peer-reviewed publications.

DISCUSSION

This clinical trial will investigate the effect of carnosine supplementation on cardiometabolic health and cognitive outcomes in patients with pre-diabetes and T2DM, and comprehensively investigating the mechanisms including CLI and AGEs.

Recent literature suggest that carnosine has a potential to prevent or delay the development of T2DM and its complications. Animal studies show that carnosine supplementation reduces, in a dose-dependent manner, blood glucose, HbA1c and increases insulin sensitivity and secretion along with β-cell mass.[19–22 58] Data from db/db mice demonstrated that carnosine supplementation can delay the development of T2DM.[19] In rodents, carnosine has also been shown to reduce proteinuria and renal vascular permeability[59] and plasma corticosterone level,[60] and prevented microvascular complications of diabetes including renal,[61] retinal[62] and neural damages.[63]

Two human clinical trials showed promising therapeutic benefits of carnosine. We have previously shown in non-diabetic overweight and obese individuals that carnosine supplementation increased urinary carnosine levels, and prevented worsening of glucose metabolism.[26] In a subgroup of individuals with impaired glucose tolerance, both 2 hour glucose and insulin levels were reduced after carnosine supplementation compared with placebo.[26] Another study has shown that carnosine supplement together with chromium and cinnamon decreased fasting glucose[31] and increase fat free mass[31] in patients with pre-diabetes. Chromium and cinnamon have both been shown to have positive effects on T2DM and therefore could have exaggerated the effect of the supplement on glucose metabolism. The effect of carnosine alone in a representative sample of patients with pre-diabetes needs to be confirmed. In spite of these findings, high quality human clinical trials investigated the role of carnosine on glucose metabolism remain scarce. This trial will thus inform and advance this field.

Evidence from animal studies showed that administration of carnosine reduced weight, blood pressure, lipid levels and atherosclerotic plaque instability[19–22 64] and inhibited the development of atherosclerosis[58 65] and hypertension.[22] An intervention study conducted in patients on a standard heart failure therapy who were suffering from stable chronic heart failure and severe left ventricular dysfunction demonstrated that chronic supplementation of carnosine showed a trend to increased end-diastolic volume.[32] More recently, supplementation with chicken extract, which is rich in carnosine and anserine, was shown to reduce heart rate variability in young females.[66] Nevertheless, human clinical trials with larger sample sizes investigating the role of carnosine on the prevention of cardiovascular diseases are lacking. This trial can therefore provide valuable insights on the effect of carnosine on surrogate measures of cardiovascular diseases in humans.

With regard to cognitive outcomes, carnosine supplementation was shown to prevent the development of ischaemic vascular dementia[67] and Alzheimer's disease[68] in rodents. Beneficial psychological effects of carnosine have also been demonstrated in humans. Supplementation with carnosine improved cognitive impairment in patients with Parkinson's disease[27] and Gulf War Illness,[69] and improved executive function and strategic efficiency and reduced perseverative errors in patients with schizophrenia.[28] In autistic children, carnosine has also been shown to improve their receptive speech, socialisation and behaviour.[29] None of the previous studies investigated the role of carnosine on cognitive outcomes associated with patients with impairment of glucose metabolism despite the presence of a strong link between the two. High quality clinical trials, such as this, should therefore be warranted to determine whether carnosine improves cognitive decline in patients with pre-diabetes and T2DM.

Chronic inflammation, oxidative stress and AGEs have been shown to play a role in the development of chronic diseases such as T2DM, CVD, and neurodegenerative diseases.[54 70 71] Mechanisms of action of carnosine include chronic inflammation, oxidative stress and AGEs, which have been thus far only been investigated in animal studies. Carnosine has been shown to reduce the levels of inflammatory cytokine production in mice.[20 72 73] In addition to its direct effect on cytokines, carnosine also plays a significant role as an antioxidant[73–76] by acting as

a free radical scavenger.[76 77] The anti-glycation properties of carnosine have also been demonstrated in several studies.[78–80] Carnosine suppresses the formation of AGEs by acting as a sacrificial molecule and protecting protein amino groups from glycation by highly reactive carbonyl compounds.[78 79] The inhibitory effects on AGEs and ALEs, which act as ligands and activators of receptor for AGE receptors,[81] further contribute to the anti-inflammatory effects of carnosine. Furthermore, carnosine has also been shown to inhibit insulin signalling pathways and sympathetic nervous system.[82] Human studies investigating the role of carnosine on these mechanisms of action are limited. High-quality human clinical trials such as this one are therefore essential to understand the role of carnosine in humans.

CONCLUSION

As a result of the steady increase in obesity, poor diet, and sedentary lifestyle across all demographic levels in modern society, chronic diseases such as T2DM continue to spread alarmingly. There is an urgent need for safe, effective, accessible, innovative, low-cost interventions aimed at the risk factors for chronic diseases to enable prevention of cardiometabolic disease. Several animal and few human studies suggest the beneficial role of carnosine supplementation for the prevention and treatment of chronic diseases. A well-designed human trials which investigate the effect of carnosine on cardiometabolic health and cognitive outcomes however are lacking. If we can demonstrate that carnosine optimises cardiometabolic health and cognitive function in patients with pre-diabetes and T2DM, our study would have substantial implications for public health and could lead to simple, low-cost, and effective strategies for prevention and treatment of T2DM, CVD and neurodegenerative diseases.

Author affiliations
[1]Monash Centre for Health Research and Implementation, School of Public Health and Preventive Medicine, Monash University, Melbourne, Victoria, Australia
[2]Centre for Chronic Disease, College of Health and Biomedicine, Victoria University, Melbourne, Victoria, Australia
[3]Department of Epidemiology and Preventive Medicine, School of Public Health and Preventive Medicine, Monash University, Melbourne, Victoria, Australia
[4]Monash Cardiovascular Research Centre, Monash Heart, Monash Health, Melbourne, Victoria, Australia
[5]Diabetes and Vascular Medicine Unit, Monash Health, Melbourne, Victoria, Australia

Acknowledgements EB is a recipient of Monash Graduate Scholarship and Monash International Postgraduate Scholarship. BdC is supported by National Heart Foundation Future Leader Fellowship (100864).

Contributors EB involved in the conception and development of the study protocol, wrote the first draft of the manuscript and revised the subsequent drafts. KM involved in the development of the study and critical revision of the manuscript. BdC conceived and developed the study protocol, and co-wrote the manuscript with EB. JC and MPJdC contributed to the conception and development of the study protocol, and critical revision of the manuscript. AE provided input on the sample size and analysis plan, and critical revision of the manuscript. All authors read and approved the manuscript.

Funding This study is supported by the Royal Australasian College of Physicians, Diabetes Australia Research Trust, Foundation for High Blood Pressure Research, CASS foundation, Australian National Heart Foundation and Australian Diabetes Society. Carnosine supplement (CarnopureTM) is received from Flamma S.p.A, Italy. These funding bodies have no role in the design of the study and collection, analysis, and interpretation of data and in writing of the manuscript.

Competing interests None declared.

Ethics approval Monash Health and Monash University, Melbourne, Australia.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. Danaei G, Finucane MM, Lu Y, *et al*. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *Lancet* 2011;378:31–40.
2. IDF. *Diabetes Atlas Sixth Edition Update: international Diabetes Federation*, 2014.
3. Tabák AG, Herder C, Rathmann W, *et al*. Prediabetes: a high-risk state for diabetes development. *Lancet* 2012;379:2279–90.
4. Forouhi NG, Luan J, Hennings S, *et al*. Incidence of type 2 diabetes in England and its association with baseline impaired fasting glucose: the Ely study 1990-2000. *Diabet Med* 2007;24:200–7.
5. Fox CS, Pencina MJ, Wilson PW, *et al*. Lifetime risk of cardiovascular disease among individuals with and without diabetes stratified by obesity status in the Framingham heart study. *Diabetes Care* 2008;31:1582–4.
6. Luchsinger JA. Type 2 diabetes and cognitive impairment: linking mechanisms. *J Alzheimers Dis* 2012;30(Suppl 2):S185–98.
7. Sandyk R. The relationship between diabetes mellitus and Parkinson's disease. *Int J Neurosci* 1993;69:125–30.
8. Barbeau A, Pourcher E. New data on the genetics of Parkinson's disease. *Can J Neurol Sci* 1982;9:53–60.
9. Boyd AE, Lebovitz HE, Feldman JM. Endocrine function and glucose metabolism in patients with Parkinson's disease and their alternation by L-Dopa. *J Clin Endocrinol Metab* 1971;33:829–37.
10. Lipman IJ, Boykin ME, Flora RE. Glucose intolerance in Parkinson's disease. *J Chronic Dis* 1974;27:573–9.
11. de la Monte SM, Wands JR. Alzheimer's disease is type 3 diabetes-evidence reviewed. *J Diabetes Sci Technol* 2008;2:1101–13.
12. de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. *Lancet Neurol* 2006;5:525–35.
13. Hirsch MA, Farley BG. Exercise and neuroplasticity in persons living with Parkinson's disease. *Eur J Phys Rehabil Med* 2009;45:215–29.
14. Jankovic J, Poewe W. Therapies in Parkinson's disease. *Curr Opin Neurol* 2012;25:433–47.
15. Marinus J, Visser M, Stiggelbout AM, *et al*. Activity-based diary for Parkinson's disease. *Clin Neuropharmacol* 2002;25:43–50.
16. Marques-Aleixo I, Oliveira PJ, Moreira PI, *et al*. Physical exercise as a possible strategy for brain protection: evidence from mitochondrial-mediated mechanisms. *Prog Neurobiol* 2012;99:149–62.
17. Singer C. Managing the patient with newly diagnosed Parkinson disease. *Cleve Clin J Med* 2012;79(Suppl 2):S3–S7.
18. Hobson RM, Saunders B, Ball G, *et al*. Effects of β-alanine supplementation on exercise performance: a meta-analysis. *Amino Acids* 2012;43:25–37.
19. Sauerhöfer S, Yuan G, Braun GS, *et al*. L-carnosine, a substrate of carnosinase-1, influences glucose metabolism. *Diabetes* 2007;56:2425–32.
20. Lee YT, Hsu CC, Lin MH, *et al*. Histidine and carnosine delay diabetic deterioration in mice and protect human low density lipoprotein against oxidation and glycation. *Eur J Pharmacol* 2005;513:145–50.
21. Nagai K, Tanida M, Niijima A, *et al*. Role of L-carnosine in the control of blood glucose, blood pressure, thermogenesis, and lipolysis by autonomic nerves in rats: involvement of the circadian clock and histamine. *Amino Acids* 2012;43:97–109.
22. Aldini G, Orioli M, Rossoni G, *et al*. The carbonyl scavenger carnosine ameliorates dyslipidaemia and renal function in Zucker obese rats. *J Cell Mol Med* 2011;15:1339–54.

23. Hipkiss AR, Michaelis J, Syrris P. Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. *FEBS Lett* 1995;371:81–5.
24. Aldini G, Granata P, Carini M. Detoxification of cytotoxic alpha,beta-unsaturated aldehydes by carnosine: characterization of conjugated adducts by electrospray ionization tandem mass spectrometry and detection by liquid chromatography/mass spectrometry in rat skeletal muscle. *J Mass Spectrom* 2002;37:1219–28.
25. Pavlov AR, Revina AA, Dupin AM, *et al*. The mechanism of interaction of carnosine with superoxide radicals in water solutions. *Biochim Biophys Acta* 1993;1157:304–12.
26. de Courten B, Jakubova M, de Courten MP, *et al*. Effects of carnosine supplementation on glucose metabolism: pilot clinical trial. *Obesity* 2016;24:1027–34.
27. Boldyrev A, Fedorova T, Stepanova M, *et al*. Carnosine [corrected] increases efficiency of DOPA therapy of Parkinson's disease: a pilot study. *Rejuvenation Res* 2008;11:821–7.
28. Chengappa KN, Turkin SR, DeSanti S, *et al*. A preliminary, randomized, double-blind, placebo-controlled trial of l-carnosine to improve cognition in schizophrenia. *Schizophr Res* 2012;142:145–52.
29. Chez MG, Buchanan CP, Aimonovitch MC, *et al*. Double-blind, placebo-controlled study of L-carnosine supplementation in children with autistic spectrum disorders. *J Child Neurol* 2002;17:833–7.
30. Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. *Physiol Rev* 2013;93:1803–45.
31. Liu Y, Cotillard A, Vatier C, *et al*. A Dietary Supplement Containing Cinnamon, Chromium and Carnosine decreases fasting plasma glucose and increases lean mass in overweight or obese Pre-Diabetic subjects: a Randomized, Placebo-Controlled Trial. *PLoS One* 2015;10:e0138646.
32. Lombardi C, Carubelli V, Lazzarini V, *et al*. Effects of oral administration of orodispersible levo-carnosine on quality of life and exercise performance in patients with chronic heart failure. *Nutrition* 2015;31:72–8.
33. Chengappa KN, Turkin SR, DeSanti S, *et al*. A preliminary, randomized, double-blind, placebo-controlled trial of L-carnosine to improve cognition in schizophrenia. *Schizophr Res* 2012;142:145–52.
34. Chez MG, Buchanan CP, Aimonovitch MC, *et al*. Double-blind, placebo-controlled study of L-carnosine supplementation in children with autistic spectrum disorders. *J Child Neurol* 2002;17:833–7.
35. Hisatsune T, Kaneko J, Kurashige H, *et al*. Effect of Anserine/Carnosine supplementation on Verbal Episodic memory in Elderly People. *J Alzheimers Dis* 2015;50:149–59.
36. Chan AW, Tetzlaff JM, Gøtzsche PC, *et al*. SPIRIT 2013 explanation and elaboration: guidance for protocols of clinical trials. *BMJ* 2013;346:e7586.
37. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998;15:539–53.
38. Svendsen OL, Haarbo J, Hassager C, *et al*. Accuracy of measurements of body composition by dual-energy x-ray absorptiometry in vivo. *Am J Clin Nutr* 1993;57:605–8.
39. Engelke K, Adams JE, Armbrecht G, *et al*. Clinical use of quantitative computed tomography and peripheral quantitative computed tomography in the management of osteoporosis in adults: the 2007 ISCD Official Positions. *J Clin Densitom* 2008;11:123–62.
40. Zoungas S, Cameron JD, Kerr PG, *et al*. Association of carotid intima-medial thickness and indices of arterial stiffness with cardiovascular disease outcomes in CKD. *Am J Kidney Dis* 2007;50:622–30.
41. Martin CA, Cameron JD, Chen SS, *et al*. Two hour glucose post loading: a biomarker of cardiovascular risk in isolated clinic hypertension. *J Hypertens* 2011;29:749–57.
42. Narayan O, Davies JE, Hughes AD, *et al*. Central Aortic Reservoir pressure analysis in the ANBP2 aortic mechanics Sub-study. *Hypertension* 2015. In Press.
43. Cheng K, Cameron JD, Tung M, *et al*. Association of left ventricular motion and central augmentation index in healthy young men. *J Hypertens* 2012;30:2395–402.
44. Egerházi A, Berecz R, Bartók E, *et al*. Automated neuropsychological test battery (CANTAB) in mild cognitive impairment and in Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry* 2007;31:746–51.
45. Afdhal NH. Fibroscan (transient elastography) for the measurement of liver fibrosis. *Gastroenterol Hepatol* 2012;8:605–7.
46. Craig CL, Marshall AL, Sjöström M, *et al*. International physical activity questionnaire: 12-country reliability and validity. *Med Sci Sports Exerc* 2003;35:1381–95.
47. Buysse DJ, Reynolds CF, Monk TH, *et al*. The Pittsburgh sleep quality index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989;28:193–213.
48. Hoddes E, Zarcone V, Smythe H, *et al*. Quantification of sleepiness: a new approach. *Psychophysiology* 1973;10:431–6.
49. Monk TH, Reynolds CF, Kupfer DJ, *et al*. The Pittsburgh Sleep Diary. *J Sleep Res* 1994;3:111–20.
50. Kroenke K, Strine TW, Spitzer RL, *et al*. The PHQ-8 as a measure of current depression in the general population. *J Affect Disord* 2009;114:163–73.
51. EuroQol Group. EuroQol--a new facility for the measurement of health-related quality of life. *Health Policy* 1990;16:199–208.
52. Everaert I, Taes Y, De Heer E, *et al*. Low plasma carnosinase activity promotes carnosinemia after carnosine ingestion in humans. *Am J Physiol Renal Physiol* 2012;302:F1537–F1544.
53. Aldini G, Facino RM, Beretta G, *et al*. Carnosine and related dipeptides as quenchers of reactive carbonyl species: from structural studies to therapeutic perspectives. *Biofactors* 2005;24:77–87.
54. Forbes JM, Sourris KC, de Courten MP, *et al*. Advanced glycation end products (AGEs) are cross-sectionally associated with insulin secretion in healthy subjects. *Amino Acids* 2014;46:321–6.
55. Thomas P, Holland N, Bolognesi C, *et al*. Buccal micronucleus cytome assay. *Nat Protoc* 2009;4:825–37.
56. Fenech M. Cytokinesis-block micronucleus cytome assay. *Nat Protoc* 2007;2:1084–104.
57. Lan KKG, DeMets DL. Discrete Sequential Boundaries for clinical trials. *Biometrika* 1983;70:659–63.
58. Brown BE, Kim CH, Torpy FR, *et al*. Supplementation with carnosine decreases plasma triglycerides and modulates atherosclerotic plaque composition in diabetic apo E(-/-) mice. *Atherosclerosis* 2014;232:403–9.
59. Peters V, Schmitt CP, Zschocke J, *et al*. Carnosine treatment largely prevents alterations of renal carnosine metabolism in diabetic mice. *Amino Acids* 2012;42:2411–6.
60. Li YF, He RR, Tsoi B, *et al*. Anti-stress effects of carnosine on restraint-evoked immunocompromise in mice through spleen lymphocyte number maintenance. *PLoS One* 2012;7:e33190.
61. Riedl E, Pfister F, Braunagel M, *et al*. Carnosine prevents apoptosis of glomerular cells and podocyte loss in STZ diabetic rats. *Cell Physiol Biochem* 2011;28:279–88.
62. Pfister F, Riedl E, Wang Q, *et al*. Oral carnosine supplementation prevents vascular damage in experimental diabetic retinopathy. *Cell Physiol Biochem* 2011;28:125–36.
63. Kamei J, Ohsawa M, Miyata S, *et al*. Preventive effect of L-carnosine on changes in the thermal nociceptive threshold in streptozotocin-induced diabetic mice. *Eur J Pharmacol* 2008;600:83–6.
64. Mong MC, Chao CY, Yin MC. Histidine and carnosine alleviated hepatic steatosis in mice consumed high saturated fat diet. *Eur J Pharmacol* 2011;653:82–8.
65. Barski OA, Xie Z, Baba SP, *et al*. Dietary carnosine prevents early atherosclerotic lesion formation in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* 2013;33:1162–70.
66. Young H, Benton D, Carter N. The effect of chicken extract on mood, cognition and heart rate variability. *Nutrients* 2015;7:887–904.
67. Ma J, Xiong JY, Hou WW, *et al*. Protective effect of carnosine on subcortical ischemic vascular dementia in mice. *CNS Neurosci Ther* 2012;18:745–53.
68. Corona C, Frazzini V, Silvestri E, *et al*. Effects of dietary supplementation of carnosine on mitochondrial dysfunction, amyloid pathology, and cognitive deficits in 3xTg-AD mice. *PLoS One* 2011;6:e17971.
69. Baraniuk JN, El-Amin S, Corey R, *et al*. Carnosine treatment for gulf war illness: a randomized controlled trial. *Glob J Health Sci* 2013;5:69–81.
70. Minihane AM, Vinoy S, Russell WR, *et al*. Low-grade inflammation, diet composition and health: current research evidence and its translation. *Br J Nutr* 2015;114:999–1012.
71. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. *Int J Biomed Sci* 2008;4:89–96.
72. Tsai SJ, Kuo WW, Liu WH, *et al*. Antioxidative and anti-inflammatory protection from carnosine in the striatum of MPTP-treated mice. *J Agric Food Chem* 2010;58:11510–6.
73. Liu WH, Liu TC, Yin MC. Beneficial effects of histidine and carnosine on ethanol-induced chronic liver injury. *Food Chem Toxicol* 2008;46:1503–9.
74. Alpsoy L, Akcayoglu G, Sahin H. Anti-oxidative and anti-genotoxic effects of carnosine on human lymphocyte culture. *Hum Exp Toxicol* 2011;30:1979–85.
75. Kim MY, Kim EJ, Kim YN, *et al*. Effects of α-lipoic acid and L-carnosine supplementation on antioxidant activities and lipid profiles in rats. *Nutr Res Pract* 2011;5:421–8.

76. Ma XY, Jiang ZY, Lin YC, *et al*. Dietary supplementation with carnosine improves antioxidant capacity and meat quality of finishing pigs. *J Anim Physiol Anim Nutr* 2010;94:e286–95.
77. Dursun N, Taşkın E, Oztürk F. Protection against adriamycin-induced cardiomyopathy by carnosine in rats: role of endogenous antioxidants. *Biol Trace Elem Res* 2011;143:412–24.
78. Alhamdani MS, Al-Azzawie HF, Abbas FK. Decreased formation of advanced glycation end-products in peritoneal fluid by carnosine and related peptides. *Perit Dial Int* 2007;27:86–9.
79. Burcham PC, Kaminskas LM, Fontaine FR, *et al*. Aldehyde-sequestering drugs: tools for studying protein damage by lipid peroxidation products. *Toxicology* 2002;181-182:229–36.
80. Yan H, Harding JJ. Carnosine protects against the inactivation of esterase induced by glycation and a steroid. *Biochim Biophys Acta* 2005;1741:120–6.
81. Stinghen AE, Massy ZA, Vlassara H, *et al*. Uremic toxicity of Advanced Glycation End Products in CKD. *J Am Soc Nephrol* 2016;27:354–70.
82. Baye E, Ukropcova B, Ukropec J, *et al*. Physiological and therapeutic effects of carnosine on cardiometabolic risk and disease. *Amino Acids* 2016;48:1131–49.