BMJ Open

Assessing the effect of anthocyanins through diet and supplementation on cognitive function in older adults at risk for dementia: protocol for a randomised controlled trial

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To cite: do Rosario V, Lorzadeh E, Brodaty H, *et al*. Assessing the effect of anthocyanins through diet and supplementation on cognitive function in older adults at risk for dementia: protocol for a randomised controlled trial. *BMJ Open* 2024;**14**:e086435. doi:10.1136/bmjopen-2024-086435

► Prepublication history and additional supplemental material for this paper are available online. To view these files, please visit the journal online (https://doi.org/10.1136/bmjopen-2024-086435).

Received 14 March 2024
Accepted 27 August 2024

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ABSTRACT

Introduction Promising evidence is emerging for the procognitive, anti-inflammatory and neuroprotective properties of dietary flavonoids, particularly anthocyanins that provide red, purple and blue plant pigments.

Methods and analysis The 'Food for Thought' study is a multicentre, 6-month randomised, parallel 3-arm clinical trial. Its primary aim is to investigate whether anthocyanin consumption, either through diet or supplementation, can prevent memory loss progression and improve inflammatory and cardiovascular health in older adults at risk for dementia. Eligible participants will include those aged 60–85 years with a diagnosis of amnestic mild cognitive impairment or with a self-referral of memory concerns and scoring ≤13 on the Memory Index Score within the Telephone Montreal Cognitive Assessment screening test. Participants will be randomised to one of three arms: High anthocyanin ('purple foods') diet (aiming for a target of 250 mg anthocyanins/day); freeze-dried product derived from blackcurrants (250 mg anthocyanins/day); or control (coloured maltose powder). The primary outcome is auditory anterograde memory functioning assessed by the Buschke and Grober Free and Cued Selective Reminding Test-Immediate Recall. Secondary outcomes are additional cognitive functions including processing speed, working memory, aspects of executive functioning (attentional shifting and word generativity) and premorbid estimate as well as subjective memory problems and self-reported depression symptoms. Additional secondary outcomes are blood pressure, inflammatory biomarkers, brain-derived neurotrophic factor, fatty acid profile, apolipoprotein E and polyphenol metabolites, gut microbiota composition and function and vascular and microvascular endothelial function tests. Repeated measures analysis of variance and/or mixed linear modelling will evaluate changes over time, with the inclusion of covariates.

Ethics and dissemination Ethics approval has been obtained from the Greater Western Human Research Ethics Committee (2021/ETH12083). A Consumer Advisory Group was established to guide and review the protocol and dissemination strategy. The results of this trial are intended to be published in a peer-reviewed journal.

Trial sponsor National Health and Medical Research Centre Dementia Collaborative Research Centre.
Start date of clinical trial: 02 September 2022.
Expected end date: 11 October 2024.

Trial registration number ACTRN12622000065796.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ A novel clinical trial to investigate the effects of anthocyanins, either through diet or supplementation, on cognitive function of older adults at risk for dementia.
⇒ A battery of cognitive tests and questionnaires will be administered to investigate multiple domains of cognitive function from a subjective and objective standpoint.
⇒ A wide range of secondary outcomes will assess the mechanisms by which anthocyanins may exert their beneficial effects on brain function, including a questionnaire about mood, blood biomarkers, vascular reactivity tests and gut microbiome evaluation.
⇒ Background diet of participants will be collected before and during the study, allowing associations between cognitive function and usual anthocyanin intake to be determined, and to account for the potential confounding effect of the usual diet during the clinical trial.
⇒ As a limitation, participants are eligible to join the study by self-referral with memory problems (following an objective screening using a validated tool).

INTRODUCTION

Ageing can be accompanied by a gradual decline in cognitive functions, including memory.[1] Even in the absence of a diagnosable condition, such as mild cognitive impairment

(MCI) or dementia, the perception of increased forgetfulness and declining cognitive function can be a source of apprehension in older people and are associated with an increased risk of dementia.[2 3]

However, estimates suggest that only 2.6–18.6% of adults aged above 60 years with memory problems seek medical help,[4] with average delays of 1–3 years between the time of symptom onset and seeking medical support and diagnosis.[5] Once memory deficits are present, it is important to mitigate further cognitive decline as up to 50% of MCI patients who present to memory clinics progress to dementia within 4 years from diagnosis.[6–8]

Therefore, novel approaches that can contribute to the prevention of dementia are needed. Nutrition plays a major role in neuroprotection, especially in the case of polyphenols that are found in fruits, vegetables, whole grains and seeds.[9] Depending on the population, flavonoids account for around 60% of all polyphenols in the human diet, followed by phenolic acids (around 30%).[10] The subclass of flavonoids with the most promising benefits on brain health is anthocyanins, which provide the purple, blue and red pigments in many plant-based foods. Anthocyanins are phytonutrients that contribute to the antioxidant and anti-inflammatory activity of plant foods, reducing oxidative stress, cardiovascular disease and neuroinflammation.[11 12]

Anthocyanins have been extensively researched due to their neuroprotective and procognitive characteristics, including improved working memory, processing speed, executive function and episodic memory in humans, in part through neurogenic actions in the hippocampus—a region of the brain implicated in learning and memory.[13 14] In observational and clinical trial studies, anthocyanins have shown procognitive, anti-inflammatory and neuroprotective properties and may improve microvascular function.[15] There is also preclinical evidence where anthocyanin-rich foods may reverse age-related decline in neuronal signal transduction, cognitive and motor behavioural deficits,[16] as well as improve cerebellar physiology and motor learning,[17] suggesting that polyphenolic compounds are able to cross the blood-brain barrier and localise in various brain regions important for learning and memory.[18] A systematic review including 20 randomised controlled trials (RCTs) found positive effects of anthocyanins on working memory, verbal memory and executive function;[19] however, such positive findings were not observed across all cognitive domains, and a few studies did not find any cognitive improvements. Another systematic review and meta-analysis of 13 RCTs evaluated the effects of anthocyanin-rich supplementation on the cognition of cognitively healthy middle-aged and older adults. A significant improvement with a small effect size in processing speed was found for the older adults (meta-analysis of four studies, while no significant differences were observed between intervention and control groups for memory, attention, executive function or psychomotor performance).[20] Therefore, considering discrepancies between study outcomes, with no single domain showing consistent improvement after anthocyanin supplementation and the usage of different food sources and dosages in clinical trials, it is not yet possible to provide evidence-based dietary guidance. Additionally, the question of whether cognition-enhancing benefits can be obtained using doses achievable through a mixed diet, rather than a synthetic supplement or single food source, has not been answered. Such differences on how best to deliver anthocyanins have led us to have two interventional arms, including both food sources of anthocyanins and anthocyanins provided as a supplemental fruit-derived product.

Several mechanisms may underlie the benefits of anthocyanins on cognitive function. Flavonoids and anthocyanins reduce the formation of neurofibrillary tangles and β-amyloid plaques (hallmarks of Alzheimer's disease) in rodent models, are neuroprotective and reduce neuroinflammation and increase brain blood flow in people with MCI.[21 22] The influence of anthocyanins on brain-derived neurotrophic factor (BDNF) may contribute to cognitive benefits. BDNF occurs in high concentrations in the hippocampus where it plays a critical role in memory formation (long-term potentiation), synaptic plasticity, neurogenesis and neuronal survival.[23 24] Anthocyanins and flavonoids increase hippocampal BDNF expression and stimulate neurogenesis by acting as a BDNF 'mimic', that is, exerting direct agonist effects on the BDNF receptor, tropomyosin-related kinase B.[25] In addition, anthocyanins can exert effects on gut bacteria through their prebiotic characteristics.[26] Prebiotics increase bacterial production of short-chain fatty acids, such as butyrate, which has been shown to improve memory function in an Alzheimer's disease (AD) mouse model,[27] and to stimulate neurogenesis in ischaemic brain rat models[28] by acting as a histone deacetylase inhibitor. Thus, these parameters are prime candidates to target in examining the mechanisms underlying the beneficial effects of anthocyanins on brain function.

Mechanisms that contribute to the procognitive effects of anthocyanins are likely to be broad, and may include improved microvascular function, suppression of neurodegeneration and increased neurogenesis, by reducing neuroinflammation and increasing BDNF. These actions may be partially mediated through interactions with gut microbiota. However, the focus in clinical trials to date has mainly been through single-food dietary interventions with high doses of anthocyanin or supplements over short periods of time (eg, 6–12 weeks). Populations in Western countries reportedly have an estimated anthocyanin consumption of 25–30 mg/day, but this is influenced by their cultural food choices, age and sex.[29] A study conducted in older Australian adults using weighed food records showed a lower anthocyanin intake of 7.0±9.2 mg/day.[25] If cognitive benefits of habitual anthocyanin-rich food intake are to be conferred, dietary support to achieve higher intake in these groups may be warranted. Hence, it is important to obtain evidence towards translation of experimental data into dietary guidance for longer-term consumption of various anthocyanin-rich foods in priority population groups.[30]

The aim of this multicentre, 6-month randomised, parallel 3-arm clinical trial is to investigate whether anthocyanin consumption, through dietary changes or supplementation, can prevent memory loss progression in older adults at risk for dementia. Secondary outcomes, such as vascular function, blood biomarkers and gut microbiome will be investigated to potentially elucidate the mechanisms by which anthocyanins may contribute to cognitive improvement and prevent further decline in people with memory problems.

METHODS AND ANALYSIS

Study objectives are:

1. To investigate the ability of dietary anthocyanins to prevent progressive loss of cognitive function and memory in older adults at risk for dementia.
2. To assess the mechanisms by which anthocyanins exert their beneficial effects.

Study design

This clinical trial comprises three arms, namely:

1. High anthocyanin diet ('purple diet'); or
2. Freeze-dried product derived from blackcurrants; or
3. Control freeze-dried product matched in macronutrients and vitamin C content to the active agent (placebo).

Intervention arm 1

Intervention arm 1 will receive intensive dietary counselling supported by nutrition professionals to consume a 'purple diet' that includes foods that are rich in anthocyanin content that is convenient, tasty and affordable—to provide a target daily intake of 250 mg of anthocyanins per day, but at least a minimum of 100 mg per day. Dietary advice will consist of face-to-face counselling at the baseline and 3-month visits, as well as follow-up phone calls and emails to participants during the 6 months of intervention. At baseline, participants will receive the 'Purple Food Guide' a supermarket-friendly guide, as well as the 'Purple Food for Thought' cookbook to support their consumption of readily available anthocyanin-rich foods. These materials were developed and tested with a group of older adults and amended based on their feedback.[30] Participants will be asked to consume a minimum of two servings of 'low anthocyanin' foods or one serving of a 'high anthocyanin' food daily, either from the recipes provided or from lists of 'low' (<100 mg/100 g serve) and 'high' (≥100 mg/100 g serve) anthocyanin foods. Serves are derived from the portion size guide in the Australian Guide to Healthy Eating. Suggestions for seasonal anthocyanin-rich fresh produce will be discussed during phone calls and in hand-outs provided throughout the duration of the trial. Individual phone calls scripts will be developed by dietitians and administered by the researchers. If requested or necessary, participants may be referred to one of the dietitians in the research team for the individual phone consultations. During individual phone calls, participants will be encouraged to ask any questions about the material and information provided. Additional material will be sent to participants in follow-up emails, such as seasonal recipes and food purchasing tips, as has been shown to be successful in a recent Cochrane review.[31] Along with the 'Purple Food Guide' and the 'Purple Food for Thought' cookbook, these additional resources were developed with feedback from a Consumer Advisory Group[30] which found the following main concerns: Wanting traditional foods options to match food preferences and ensure familiarity of foods; recipes need to match cooking motivations; need information on seasonality and where to buy these foods. Additionally, as suggested by the advisory group, a $A100 gift voucher will be provided to participants to assist with food-related costs, given in two instalments of $A50 at the second and third study visit.

Intervention arm 2

Intervention arm 2 will receive 6 g of freeze-dried product derived from New Zealand grown blackcurrants (Neuroberries; Arepa Pty, Auckland, providing~250 mg anthocyanins/day, comprising approximately equal quantities of delphinidin and cyanidin subclasses). We have conducted preliminary investigations to evaluate the acceptance of this product among older adult consumers. This product can be consumed in various ways, including as a beverage added to water or in other foods, such as smoothies and yoghurt. Participants in this intervention arm will be guided to maintain their usual diet throughout the study.

Control

A freeze-dried product with similar colour, taste and texture and with identical packaging to the high-anthocyanin powder will be provided, matched in macronutrients and vitamin C content to the active product. Participants in the control group will be guided to maintain their usual diet throughout the study.

The study population comprises adults aged 60–85 years with self-referred memory problems or diagnosed with MCI who reside in the Greater Sydney or Illawarra regions of New South Wales, Australia.

Study population and recruitment

Inclusion criteria

1. (a) Diagnosis of amnestic MCI (single or multidomain) by a geriatrician, psychogeriatrician, neuropsychiatrist or neuropsychologist; or, (b) deemed as eligible following completion of the Telephone Montreal Cognitive Assessment (T-MoCA) screening test administered via a validated telephone interview tool.[32] A person scoring 13 or less on the Memory Index Score (MIS) within the T-MoCA will be considered eligible for the study.
2. Self-reported subjective memory problems.
3. English-speaking.
4. Aged 60–85 years.
5. Able to complete all or all but one of the following basic activities of daily living (unless for physical reason, g,

arthritis, poor vision): 'Do you need help to manage everyday activities such as: Personal hygiene?; Dressing?; Taking medication?; Shopping and preparing food?; Transport?; Telephoning?; Finances?; Housekeeping?'

Exclusion criteria

1. Non-English speaking.
2. History of psychiatric (other than mood) disorder in the past 5 years.
3. Underlying additional neurodegenerative condition, such as Parkinson's disease.
4. Other significant neurological history including head injury, epilepsy or tumour.
5. Scoring 11 or less at the overall T-MoCA or have been diagnosed with AD or any other dementia.
6. Taking any of the following medications: Donepezil, galantamine, rivastigmine or memantine.

Participants diagnosed with amnestic MCI by a geriatrician, psychogeriatrician, neuropsychiatrist or neuropsychologist will be recruited to the study without criteria based on T-MoCA scores. Other participants will be recruited to the study after being assessed by a trained researcher through a telephone interview—The Montreal Cognitive Assessment (T-MoCA).[32] The Montreal Cognitive Assessment (MoCA-30) is a widely used, extensively studied screening tool for distinguishing individuals who are cognitively unimpaired from those with MCI (average sensitivity 85%, average specificity 76%) and AD (average sensitivity 94%, average specificity 76%).[33] A validation study conducting equivalence testing found a sensitivity and specificity of 72% and 59% for the T-MoCA, respectively, compared with 70% and 75% for the MoCA-30, when examining the diagnostic ability to discriminate between MCI and normal cognition.[32]

Recruitment strategies

Recruitment strategies were informed by suggestions from the Consumer Advisory Group and include advertisements that will be placed in convenient locations where older adults may be present, including Men's Sheds, University of the Third Age, Community Centres and lifestyle villages for older adults. Wider advertisement of the study will occur through multiple online platforms including: Dementia Australia (https://www.dementia.org.au/); Australian Dementia Network (https://www.australiandementianetwork.org.au/); 'Step up for Dementia Research' (https://www.stepupfordementiaresearch.org.au/); through networks at the Universities of Wollongong and New South Wales; and through targeted social media (Facebook) advertisements.

Randomisation and blinding

Block randomisation of participants will be conducted using a computer application by a member of the research team who is not involved in data collection. Sets of nine participant numbers will be randomised into the three groups, that is, three of each. Treating clinicians and scientific investigators will be blinded to participants' randomised allocation (control or intervention arms 1 and 2). After randomisation, the names and details of participants will be provided to the commercial partners, Arepa, who will send the freeze-dried products directly to home addresses (intervention arm 2 and control). To monitor compliance (intervention arms 1 and 2), the following procedures will be conducted: participants will be asked how much powder they have left in follow-up emails and phone calls; phenolic and anthocyanin metabolites will be analysed in blood samples; follow-up emails and calls will be conducted to monitor how participants are dealing with consuming the supplement powder. Participants allocated to the purple foods dietary intervention arm cannot be blinded (intervention arm 1).

Procedures

Once participants are included in the study, a researcher will contact them to arrange an introductory video-call to answer their questions, collect consent to participate in the study and provide assistance to complete the online dietary assessment tool.[34] Following the introductory video-call and collection of dietary intake information, participants will be allocated to the most appropriate clinical site according to their home location, either University of Wollongong (UOW) Clinical Trials Unit or the Centre for Healthy Brain Ageing at the University of New South Wales. Participants will attend one of the clinical sites for baseline measurements prior to commencing the trial (approximately 2 hours), involving neuropsychological testing, blood collection, anthropometry and vascular measures (UOW site only). All assessment procedures will be repeated at 12 weeks and 24 weeks (see figure 1). The

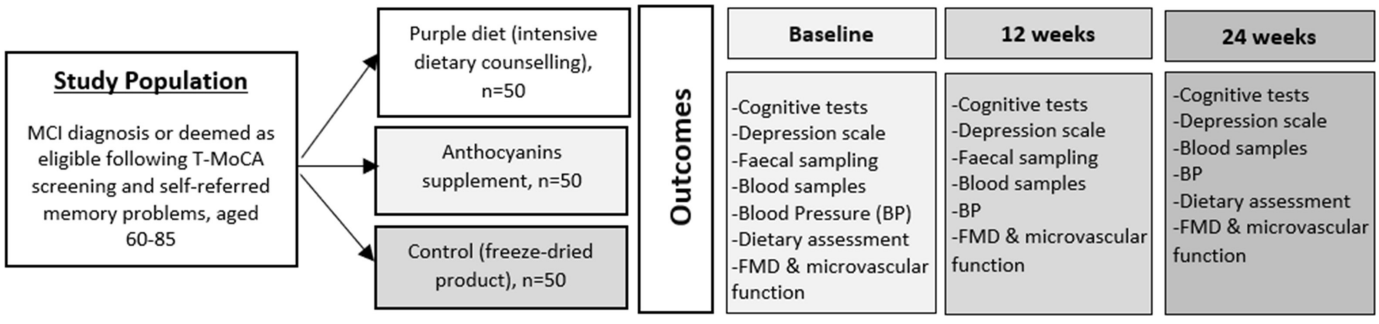

Figure 1 Flowchart of procedures conducted during the study. FMD, flow-mediated dilatation; MCI, mild cognitive impairment; T-MoCA, Telephone Montreal Cognitive Assessment.

study commenced in September 2022 and is expected to be completed in late 2024. On the advice of the Consumer Advisory Group, ineligible participants will be offered additional information or support. For those screened as having probable dementia (rather than MCI), a referral will be offered to further support diagnosis.

Outcomes

Primary outcome (cognitive function)

- Buschke and Grober Free and Cued Selective Reminding Test-Immediate Recall[35] (auditory anterograde memory functioning): Participants are presented with 16 pictures, 4 at a time and asked to identify the item that belongs to a said category (eg, 'point to the fruit' for grape). After each set of four pictures, participants recall the items when provided with each category cue. Three-free recall trials with interference (ie, counting backwards) are then conducted, with selective cueing for items missed. One final-free recall trial is performed 20–30 min later. The measures evaluated are: total-free recall (cumulative sum of free recall from the three trials; range 0–48), total recall (cumulative sum of free recall+cued recall from the three trails, range 0–48), delayed-free recall (free delayed recall, range 0–16) and delayed total recall (free delayed recall+cued delayed recall, range 0–16).

Secondary outcomes

Cognitive function

Oral Symbol Digit Test (processing speed)[36]

A coding key with nine abstract symbols is presented, each paired with a number between 1 and 9. Participants are presented with a sheet of symbols without the numbers, and are asked to use the key provided to indicate the number associated with each symbol as quickly as possible for 2 min. The task measures the speed of processing, which is a fundamental determinant of performance on many cognitive tasks. Scores are calculated from the number of correct responses in 2 min.

List sorting (working memory)

Participants are presented with a series of stimuli (ie, animals or pieces of food) on an iPad, both visually and via audio. Items are displayed one at a time for 2 s. This is followed by a blank screen. For the first component, participants are only presented with items from one category (ie, food or animals) and are required to recite the names of all items in size order from smallest to largest. For the second component, participants are presented with items from both categories and asked to recite the food in size order and then the animals in size order. Participants are initially presented with only two stimuli. An incorrect response leads to a second trial of the same number of stimuli in the string. A correct response leads to a string with one additional item. The task is discontinued when the participant provides incorrect responses on two trials with the same number of stimuli in the string, or when the participant correctly sequences seven stimuli. List sorting scores are based on a sum of the total correct scores across two lists, which comprises the List Sorting 'Total Score'. The raw sum score is then transformed into an age-adjusted standard score (M=100 and SD=15).[37]

Trail Making Test A and B (processing speed/executive functioning)

Both parts of the Trail Making Test consist of 25 circles distributed over an A4 sheet of paper. In Part A, the circles are numbered 1–25, and participants are instructed to draw a line to connect the numbers in ascending order. In Part B, the circles include both numbers (1–13) and letters (A–L) and participant are instructed to draw a line to connect the circles in an ascending pattern, but with the added task of alternating between the numbers and letters (ie, 1-A-2-B-3-C). On both tasks the participant is instructed to connect the circles as quickly as possible without lifting the pen or pencil from the paper. Any errors made by the participant are immediately identified, and the participant is re-directed to the last correct circle to continue. Errors only affect the participant's score in that the correction of errors is included in the completion time for the task. The task is terminated if not completed within 5 min. Scores are derived from the time taken to complete each task, with higher scores indicating poorer performance. Part A is a measure of psychomotor speed. The difference in time taken between Parts A and B is taken as a measure of executive control, particularly attentional task-switching ability.[38]

Letter fluency (executive functioning)

This task provides a sensitive measure of executive dysfunction and control processes. The task requires the participant to produce as many words starting with a particular letter as possible in 1 min (F, A, then S). Numbers, proper nouns and word derivatives are not permitted. The number of correct words across the three trials are summed, with higher scores indicating greater executive functioning.[39]

Spot the word-2 (premorbid estimate)

This task assesses premorbid verbal abilities, using a robust lexical decision task. The test involves presenting an individual with pairs of items comprising one word and one non-word, for example, 'flonty – xylophone'. The individual is required to point to the real word in the pair. This format allows lexical decisions to be made through multiple methods including meaning, familiarity, appearance and sound of words. Scores are calculated from the number of correctly identified real words.[40]

Subjective memory problems

MAC-Q

This questionnaire was devised to assess subjective age-related memory decline. It comprises six questions related to memory functioning in everyday situations (eg, to remember a telephone number that they use at least once a week) in which the subject is asked to compare and rate their current ability to when they were 40 years

old. The total score ranges from 7 to 35, where greater scores indicate greater subjective memory loss. Scores ≥25 have been found to be suggestive of age-associated memory impairment.[41]

Mood

Geriatric Depression Scale—Short Form

The Short Form Geriatric Depression Scale consists of 15 questions in which participants respond yes or no to items related to their feeling over the past week. Of the 15 items, 10 indicated the presence of depression when answered positively, while the rest (question numbers 1, 5, 7, 11, 13) indicated depression when answered negatively. Scores of 0–4 are considered normal, depending on age, education and complaints; 5–8 indicate mild depression; 9–11 indicate moderate depression; and 12–15 indicate severe depression. It takes about 5–7 min to complete.[42]

Dietary intake

Participants will use Intake24, a validated computer-based programme[34] to collect their 24 hours recall dietary intake on three different days at baseline, and again for 3 days between the 12 and 24-week visits. Participants will be guided by a trained nutrition professional via an online Zoom call to record their dietary intake for 2 weekdays and 1 weekend day for the first data collection point. Thereafter, only those needing assistance to complete subsequent computerised dietary assessments will be offered Zoom calls guided by the nutrition professional. On the advice of the Consumer Advisory Group, participants who are unable to use a computer or who have difficulty even with guidance in online calls will be offered the option of completing the dietary assessment over the telephone, with the research member entering their responses directly into the programme.

To determine flavonoid and anthocyanin intake, each food item will be assigned a total flavonoid and subclass content value using the 'PhenolExplorer' polyphenol food composition database. The flavonoid subclasses to be included are flavones, flavonols, flavanones, flavan-3-ols, isoflavones and anthocyanins. The PhenolExplorer is a comprehensive and freely available database that contains more than 35 000 content values for 500 different polyphenols in over 400 foods.[43] In the case of flavonoid values for a food not being available in PhenolExplorer, the USDA database[44] for the flavonoid content of selected foods will be used. Each selected food item from the 24 hours dietary recall will be manually cross-referenced with these databases. Foods listed in the 24 hours dietary recall that are not in these databases will be assumed to contain no flavonoids. Flavonoid and subclass intakes from each food will be measured by multiplying the serving size of food consumed (grams) by its flavonoid content (mg per gram edible weight). Individual flavonoids from the six subclasses will be summed to provide a total value for each subclass, and data for total flavonoids will be calculated as the sum of these subclasses. An average daily dietary intake of each flavonoid subclass and total flavonoid intake will be calculated from the three dietary recalls at each collection time for each individual.

Blood pressure

Blood pressure (BP) will be measured using a validated and calibrated automated device. Participants will be rested in supine position for 5 min, in a quiet room with a correctly fitting arm cuff. BP will be measured on both arms, and a repeat measure taken on the arm with the higher reading, with the average of the two readings recorded from the same arm.[45]

Blood biomarkers

Blood samples will be collected from participants after an overnight fast (8–12 hours). Two 4 mL EDTA tubes and one 5 mL serum-separating tube will be collected, processed and stored at—80°C for the biomarker analyses that include: Plasma levels of pro-inflammatory cytokines related to dementia pathology (tumour necrosis factor-alpha, interleukin-1 and interleukin-6 (IL-1, IL-6)), (50); C-reactive protein; circulating levels of butyrate (produced by microbiota) and BDNF; vascular markers of endothelial function including, vascular cell adhesion molecule 1 and E-selectin; as well as full fatty acid profile and apolipoprotein E (Cardinal Laboratories, Queensland). One of the 4 mL EDTA will be processed to produce two 1 mL plasma aliquots, with formic acid (conc 2%, so 20 µL in 1 mL plasma) added to analyse polyphenol metabolites using a microelution solid phase extraction of the plasma coupled with ultra-high-performance liquid chromatography quadrupole-time-of-flight mass spectrometry and standards for quantification, as described previously.[46]

Gut microbiota

Faecal samples will be collected at baseline and 12 weeks using commercial sampling kits (Microba, QLD) that use a proprietary method that preserves DNA within the microbial sample without cold storage (UOW site only). Participants will receive the kit and instructions to collect the faecal samples at their first visit at UOW, which will be mailed to the laboratory ('paid' postage envelope) when collected. The company will provide DNA extraction, library preparation and shotgun metagenomic sequencing of samples. Libraries will be prepared with an optimised high-throughput format of the manufacturer's protocol for sequencing on the NovaSeq 6000 (Illumina). Pools will be sequenced to a target depth of 3 Gb per sample. Downstream profiling and bioinformatic analysis will be processed by removing duplicate reads using 'clumpify.sh' from BBMap,[47] and then FastP[48] with default parameters to remove or trim the low-quality reads. Host contamination will be removed by mapping reads against the human reference genome with Minimap2.[49] Taxonomic profiles will be generated with MetaPhlAn 4[50] to generate strain level resolution annotation by mapping reads against clade-specific marker genes. Microbial functional pathways and metabolic potential will be analysed with HUMAnN 4.[51 52]

Vascular and microvascular function

Vascular measurements will be performed for participants at the UOW site only due to the availability of equipment. Vascular function will be using flow-mediated endothelium-dependent vasodilation (FMD) following standard guidelines,[53] by a trained researcher. FMD of the brachial artery will be measured using a uSmart 3300 ultrasound system (Terason, Massachusetts, USA) in combination with a semi-automated computerised analysis system (FMD Studio, QUIPU, Pisa, Italy). The brachial artery will be imaged longitudinally at 2–10 cm proximal to the antecubital fossa. Video recording will collect beat-to-beat measures of the diameter and velocity for 1 min, and the average will be used as the baseline. A BP cuff will be placed around the forearm and inflated to 60 mm Hg above resting systolic BP. Blood flow will be restricted for 5 min, then the cuff will be rapidly released, resulting in reactive hyperaemia. The FMD response will be calculated as the relative diastolic diameter change from baseline compared with the peak diastolic diameter following hyperaemia and expressed as a percentage. This FMD protocol is routinely performed in our laboratory using the methods outlined by Francois *et al*,[54] with intrasubject coefficients of variation of 4.96% for %FMD.

Microvascular cutaneous vascular reactivity will be measured using a laser speckle contrast imaging system with a laser wavelength of 785 nm (PeriCam PSI System, Perimed AB, Järfälla, Sweden). The image acquisition rate will be 21 images/s, and the distance between the laser head and the skin surface will be fixed at 25±0.5 cm. The skin of the volar side of the left arm will be gently cleaned with 70% isopropyl alcohol swabs. Three equidistant skin areas (region of interest) of approximately 80 mm^2 will be selected in the central volar part for the left arm, avoiding any skin mark or bulge areas. Participants will be instructed to avoid any movement, and not to speak or breathe deeply during the record. During the post-occlusive reactive hyperaemia (PORH) test, the baseline perfusion will be measured in the volar side of the left forearm for 2 min, followed by an arterial occlusion maintained for 3 min using a BP cuff around the upper arm inflated to a pressure of 50–60 mm Hg above systolic pressure reading.[55 56] After the BP cuff is released, the PORH response will be recorded for 3 min. The following parameters will be extracted: baseline flow (BF), biological zero (BZ) and peak value (PV). The maximum PORH perfusion (PORHmax) will be calculated as PV – (BF – BZ).[57 58] This PORH protocol is routinely performed in our laboratory; with intrasubject coefficients of variation of 7.7% for PV and 11.4% for PORHmax.

Analysis and power calculation

Cognitive outcomes, as well as data from the biomarkers and vascular tests will be evaluated using linear or generalised linear mixed models. Restricted maximum likelihood estimation will be used in the mixed model to incorporate partial data sets when subjects have been lost to follow-up or have missing data. Correlation and regression methods will be used to examine the relationships between measures, particularly between changes in cognitive measures and biomarkers and vascular outcomes. In all cases, significance will be considered as a p value<0.05. For gut microbiota analyses, Analysis of Compositions of Microbiomes with Bias Correction 2 will be used.[59 60]

Primary analyses will be conducted based on the intention-to-treat principle. Secondary analyses will be conducted including participants with a minimum compliance of interventions as following: averaging a minimum daily consumption of 75 mg of anthocyanins for participants allocated to arm 1 (based on the Intake24 reports); minimum intake of 800 g (out of 1008 g, >80%) across the 6 months of intervention for participants allocated to arms 2 and 3. Participants will be excluded from secondary analyses if they discontinue the intervention for a period longer than 14 days (all arms) or report adverse events deemed to be related to the intervention products.

G*Power was used to calculate power for the critical interaction between group and time according to our previous studies in which the primary outcome measure was a similar learning and memory task—the Rey Auditory Verbal Learning Test. Our previous studies have demonstrated a strong effect of anthocyanin treatment on this measure in groups with memory impairment.[61] Power is set conservatively (0.95) to detect a small to medium effect (f=0.15) on this measure, requiring 96 participants (32 participants per experimental condition). To account for a large drop-out rate in this patient group, 50 participants will be recruited per arm. We estimate 5–10 eligible patients to be recruited at each of the clinical sites monthly.

ETHICS AND DISSEMINATION

Ethics

Ethics approval has been obtained from the Greater Western Human Research Ethics Committee (2021/ETH12083). Adverse events, unintended effects and other ethical considerations will be assessed and reported to ethics committee, as well as recorded/tabulated and presented yearly in ethics progress reports. The trial was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR). Protocol amendments will be submitted to the ethics committee through the 'Research Ethics and Governance Information System' and updated in the ANZCTR.

Confidentiality, privacy and data integrity

The data will be owned by UOW and be managed as sensitive data in a password-protected secure Microsoft platform. Researchers will collect a wide range of data from participants through telephonic and face-to-face appointments, including socio-demographic, anthropometric, cognitive tests scores, dietary intake and physical activity.

Additional data will be derived from laboratory analyses and biomedical equipment.

Following the inclusion and randomisation of participants in the study, all data collected will be de-identified by one of the associated investigators. Hard copies of questionnaires will be stored in a locked cabinet, to which only investigators will have access. Hard copies will be destroyed 5 years following the end of the study (placed in a closed recycling bin for confidential material). Data will be stored in an appropriate university approved secure electronic storage for sensitive data, and only the researchers will have access.

The clinical trial manager and research assistants will conduct validation checks and post-check actions on all entered data. All collected data will be reviewed by study investigators. Anthropometric and vascular function tools will be calibrated according to manufacturer's recommendations.

Patient and public involvement

The patients and public involvement (PPI) on the development of the study protocol consisted of three different methods: Stakeholder interviews, focus groups and input from a Consumer Advisory Group. These activities were conducted prior to commencement of the clinical trial.

Stakeholder interviews: compliance to intervention arm 1 (purple diet) and supports needed

In this study, two hypothetical personas were developed to allow the participants (older adults, n=5) to put themselves in the shoes of the target audience and consider how older adults with MCI may have managed to change dietary behaviours using the resources provided. Participants were asked to respond to and provide feedback two 'personas' (Bill and Nancy) and how they might experience the demands of the study protocol and what types of support they might need to successfully participate in the trial. A user persona is a hypothetical archetype of an actual or ideal user. Persona modelling is a consumer research method involving the use of a hypothetical 'typical' user of a product. User personas are used as a stimulus to support research participants to engage deeply with the topic.[62] They rely on the idea that 'someone will project their own feelings or beliefs onto an imaginary person or situation', enabling them to access thoughts or feelings that direct questioning may not.[62 63] This included requirements for data collection and their potential compliance with the 'Purple Diet' arm of the study. Stimulus materials included stock photos of 'Bill' and 'Nancy' with a short description of their situation as it related to their living and functional status (see online supplemental appendix). These stimulus materials were used to prompt participants to reflect on the two personas and discuss how they thought the protocol might need to be adapted to accommodate their needs. Recommendations from this study that were incorporated in the study protocol included: Provide recipes and advice in smaller amounts/drop feed information and process monitoring for compliance (follow-up emails and calls); provide transport and/or transport information on how to get to the research facilities independently; and provide additional recipes with lower prices foods that are in season.

Focus groups: development and pilot testing of intervention arm 1 (purple diet) resources

Focus groups with older adults explored the barriers and enablers to consuming an anthocyanin-rich diet for cognitive health.[30] Enabling factors for consuming anthocyanin-rich foods for cognitive health included personal motivation, taste preferences, social support and food availability. Common barriers involved budget constraints, food preferences and limited access to some foods. To promote anthocyanin-rich food consumption, it was recommended that strategies should be provided to participants to enhance their knowledge and skills, particularly around increasing access to these foods and where to buy them. 'The Purple Food for Thought' cookbook was developed by the research team and includes a collection of anthocyanin-rich recipes, a simplified synopsis of the science to date, shopping lists, food exchange lists, shopping and cooking tips and seasonal guides. This resource was distributed, and pilot-tested in a group[30] of older adults who were not cognitively impaired (n=20) who then participated in a series of focus groups to explore their experiences of interacting with the resources. The use of the two personas mentioned above ('Bill' and 'Nancy') were also used in focus groups to help tailor the recommendations to people with cognitive impairment. Feedback gathered during focus groups informed revisions on the structure of 'The Purple Food for Thought' cookbook, such as the inclusion of simplified recipes based on familiar and affordable anthocyanin-rich foods and clearer recommendations on recommended serving sizes for anthocyanin-rich foods. Based on this feedback, a second resource 'The Purple Food Guide' was created to help participants quickly and easily quantify anthocyanin-rich food intake and set daily goals, and this was tested by the same older adults. In follow-up focus groups, participants reported the guide was user-friendly and they appreciated the promotion of simple food exchange lists (ie, swap green grapes for purple grapes) and the use of grouping foods into simple 'High' and 'Low' sources of anthocyanin-rich foods with clear portion sizes based on the Australian Guide to Healthy Eating. Phenol-Explorer was used to create lists of 'High' anthocyanin foods (>100 mg per serve), and 'Low' anthocyanin foods (<100 mg per serve). The use of large print and colourful food-related images also contributed to its usefulness. Lastly, 'The Purple Food Diary' was developed by the research team to quantify consumption of anthocyanin-rich food and measure compliance to the dietary intervention arm 1. Feedback from focus groups was that participants thought recording anthocyanin-rich food intake in a diary each

day would be too stressful to complete over a long period and could potentially lead to misreporting and misuse of the diary. Overall, these findings support the usability and acceptability of the Purple Food Guide and Cookbook, but the limited use and support for the Purple Food Diary suggested that was unlikely to be appropriate for application in a clinical trial setting and was hence omitted. Approval for the focus groups was granted by Western Sydney University Human Research Ethics Committee (H14660).

Consumer Advisory Group: study protocol feedback and recommendations to promote engagement

To minimise potential barriers to participation and to maximise participant retention in the study, we sought the feedback of a Consumer Advisory Group which comprised one older male carer of a person with dementia, a female hospital dietitian who mainly supported older patients and who had an interest in dementia risk reduction, and a female PhD student who cared for an elderly parent with MCI. The group reviewed the entire protocol as part of a 2-hour online discussion group, and provided valuable advice to enhance planned recruitment strategies, to promote perceived benefits to participants and to enhance support especially for those involved in the 'purple diet' arm.

Recommendations that were adopted included: Information to be presented in flyers, posters and social media campaigns to recruit participants; access to seasonal recipes and low-cost foods; allow for the presence of a support person during visits when needed; referral and other supports provided to people showing important cognitive impairment during the screening process; and confirmation that using a computer application to assess dietary intake was feasible.

Dissemination of results

In addition to PPI methods used to develop the protocol, the project team will consult with representatives from non-governmental organisations (ie, Dementia Association, U3A), local aged care providers and service organisations (ie, Meals on Wheels, People Care) at the completion of the study. Both will provide channels for research transfer into policy and practice. At the start of the project, feedback from the Consumer Advisory Group and focus groups with older adults were used to gain practical recommendations to promote compliance in the dietary counselling arm. At study completion, participants in the dietary intervention arm will be invited to participate in online focus groups facilitated by a social scientist with extensive experience in dementia research (LP). Participants will be asked about their current perceptions related to diet in dementia prevention and treatment, as well as identify how and through which channels to best communicate study findings to relevant stakeholders. The focus groups will inform the dissemination of key messages from the study findings via channels suitable to reach older target audiences and service providers.

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Acknowledgements The authors would like to acknowledge the role of Tracy McCaffery for support and co-ordination of dietary intake assessment using a computer-based application, and Emma Rita who supported the development and refinement of dietary resources used in this study. We acknowledge the Albert Einstein College of Medicine (Bronx, New York, USA) for sourcing the 'Buschke and Grober FCSRT-IR' test to be used in this clinical trial.

Contributors KChar, HB, KJA, SR, VB, KK, JP and KW-G designed the study. KK and KChar developed the dietary resources with support from dietetics research students. LP designed the social marketing component. VdR developed the study protocol, logistics and completed ethics committee applications and is the clinical trial manager. KChan is a project officer and oversees data collection at UNSW site. MEF will supervise vascular function tests and analysis (FMD). MJB developed the statistical plan and will supervise analysis. XJ will conduct microbiome analysis. JG is responsible for anthocyanin analyses in freeze-dried powders. EL is responsible for dietary assessments and dietary analyses. KChar and VdR are responsible for the overall content of the manuscript as guarantors. All authors revised drafts and contributed to the final manuscript.

Funding This research is funded by a grant awarded to lead CI Charlton by the NHMRC Dementia Centre for Research Collaboration under the World Class Research Project Grant funding scheme. KJA is funded by ARC FL190100011. Freeze-dried product of active and placebo freeze-dried powders is provided free-of-charge by Arepa, Auckland, New Zealand. There is no affiliation/association or financial interest between the researchers and the sponsor/funding body. There is no affiliation/association or financial interest between the researchers and the company donating the freeze-dried product. There is no affiliation/association or financial interest between the researchers and the developers of the Intake24 dietary assessment programme.

Competing interests HB is or has been an advisory board member or consultant to Biogen, Eisai, Eli Lilly, Medicines Australia, Roche and Skin2Neuron. He is a Medical/Clinical Advisory Board member for Montefiore Homes and Cranbrook Care. There are no other conflicts of interest among the other authors.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Ethics and dissemination section for further details.

Patient consent for publication Consent obtained directly from patient(s).

Provenance and peer review Not commissioned; externally peer reviewed.

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