Cognitive impAiRmEnt Study (CARES)

Introduction

Macular pigment (MP), found at the central retina (macula), is comprised of the carotenoids lutein (L), zeaxanthin (Z) and *meso*-zeaxanthin (MZ). Research to date has demonstrated the importance of MP for protecting against age-related macular degeneration (AMD) progression(1-4), and has also established that MP plays an important role in enhancing visual function in both diseased(5-8) and non-diseased(9) eyes, via the optical (light-filtering) properties of this pigment(10;11). Of note, it has been shown that supplementation(12-15), and in particular supplementation with all three carotenoids (MZ, L and Z) in a mg ratio of 10:10:2 may offer the best means of enriching MP across its spatial profile(16-18), and impacts positively on visual function (e.g. contrast sensitivity and glare disability) in human subjects(9).

Recent research has identified that L and Z are also present in brain tissue, specifically the cerebellum, pons, and the frontal /occipital cortices(19;20). L and Z were also detected in the hippocampus and prefrontal and auditory cortices of human brain tissue(21). Interestingly, it has been found that MP levels correlate with concentrations of L and Z in the primate brain(20). This had led researchers to speculate that the macular carotenoids may also have an antioxidant role in the brain, similar to that in the human retina (the retina is part of the central nervous system). Possible functions of carotenoids in the brain include: antioxidant; anti-inflammatory; structural and functional enhancement of synaptic membranes and gap junction communication, and thereby may ultimately protect against insult to cognition(22;23). Recent work by our group has shown that patients with mild to moderate Alzheimer's disease (AD) exhibit significantly less MP, poorer vision and a higher occurrence of AMD when compared to control subjects(24). Moreover, in a subsequent clinical trial we found that supplementation with the macular carotenoids (MZ, Z, and L) benefited patients with AD, in terms of increases in MP and in terms of clinically meaningful improvements in visual function(25). In another example, Rinaldi *et al* have shown that in patients with AD, plasma concentrations of L and Z were lower in comparison to control subjects, with a significant and inverse relationship observed between L concentrations and dementia severity(26;27). In

another study, supplemental L resulted in improved performance in a range of cognitive tests in unimpaired older women(28). Indeed, it has been shown that a positive relationship exists between MP levels and cognitive performance in unimpaired and mildly cognitively impaired adults(29-31). Recent work has also shown that MP levels are significantly related to better global cognition, verbal learning and fluency, recall, and processing and perceptual speed, whereas serum L and Z levels were only significantly related to verbal fluency in older adults with normal cognitive function(32). Taken together, these studies suggest that MP's constituent carotenoids may play a role in cognitive function, and given the relationship between MP and brain carotenoid levels, it is reasonable to hypothesise that MP could be used as a biomarker for cognitive function and/or AD; however, additional study in this area is needed.

As cited above, as part of the Carotenoids and Age-Related Dementia Study (CARDS) Trials, our centre has already conducted and published studies for the Howard Foundation (HF) (research funders) reporting MP levels and associated health and functional parameters in patients with AD and patients of similar age without AD. One other aspect studied as part of the CARDS investigation was the study of lipids (poly unsaturated fatty acids) in AD patients and age-matched controls(33). This work has been achieved by collaboration with the MRC HNR, Elsie Widdowson Laboratory, CB1 9NL Cambridge, UK (Dr Albert Koulman). The rationale for conducting this work was influenced by a letter published by Mapstone *et al*(34), which suggested a link between lipids and AD. In our study, we found significant differences in lipids between AD and control subjects (similar to those reported by Mapstone *et al*) (PCaa [40:6], PCaa [38:6], PCae [38:4]), but we suggest that these differences are linked to diet. Indeed, our results suggest that lipids will provide poor biomarkers per se for AD, but instead reflect the effects of a potentially beneficial diet in the elderly. Epidemiological studies have suggested that diets high in omega-3, an essential fatty acid, have a protective role in maintaining cognitive function(35-37). This led to the role of omega-3 in dementia being examined, however, a number of randomised controlled trials have failed to show omega-3 as having any significant effect(38;39). Vitamin E is another powerful antioxidant which protects tissue cells from damage. It has been suggested that vitamin E can help reduce the progression of AMD among individuals at a high risk of developing the disease(1;40). Some studies suggest that vitamin E may also provide protection against a decline in brain

function(41) (42); however, others have found no effect(43). Overall, we feel that the evidence supports the addition of vitamin E to our intervention as the concentration level that we will be adding to the active supplement has been shown to be safe(44;45). In addition, it is known that vitamin E will help the active intervention to remain stable during the period of supplementation, given the unique antioxidant properties of this vitamin(45).

It may be argued that the cognitive ability of AD/dementia patients may have deteriorated too rapidly to show any meaningful effect in the supplementation trial. Therefore, studying cognitive impairment at an earlier stage may prove more informative. Mild Cognitive Impairment (MCI) is an intermediate stage between the normal changes in cognition associated with ageing and very early dementia. Although MCI is a risk factor for AD, many individuals with this condition revert to normal or do not progress(46;47). Of note, it is a distinct entity from AD and is not a diagnosis of dementia. Individuals with the condition will exhibit a decline in their cognitive abilities (memory, thinking abilities); however, these changes are not significant enough to affect daily life or functioning. As a guideline for calculating scores on cognitive function tests, individuals with MCI typically score 1 to 1.5 standard deviations below the mean for their age and education matched counterparts(48). Therefore, individuals with MCI are a suitable population group to study cognition over time, and the potential role MP may have in terms of cognitive function.

In summary, what remains to be investigated is if supplementation with the macular carotenoids, fish oil and vitamin E will impact positively on macular pigment and cognitive function in patients with MCI. In many ways, the science conducted to date has led us to this important research question and the Cognitive impAiRmEnt Study (CARES) described below is uniquely designed to answer this important research question.

Main study objective

To investigate if supplementation with the macular carotenoids, fish oil and vitamin E in subjects with mild cognitive impairment (MCI) improves cognitive function.

Study recruitment

Subjects will be recruited under the guidance of Professor John Nolan, Project Manager, who will act as the Principal Investigator (PI). The study target numbers are 60 subjects with MCI and 60 subjects with no cognitive impairment aged over 65 years. To achieve this, it is expected that we will have to screen a significant number of subjects, and only those who meet strict eligibility criteria will be enrolled in the study:

a. Individuals with reported memory problems either self-reported or reported by a family member or carer
b. Individuals referred will also have to be functionally independent from a memory point of view, and able to execute their activities of daily living independently
c. Screening will be carried out by a trained researcher in the Research Centre located at Carriganore House
d. Reported symptoms of memory loss will be documented including time symptoms first noticed and chronology of symptoms
e. Medical co-morbidities will also be documented
f. Other lifestyle and clinical history will also be noted including
    - Family history of “significant memory problems”
    - Smoking history and alcohol intake
    - Educational and employment history
g. Medications will be listed and cross checked with the patients family or pharmacy

Information lectures to Geriatricians and Psychiatrists of Old Age in the region will be conducted to highlight CARES and to help identify subjects with MCI. Using standardised screening steps, pre-screening will be conducted by Geriatricians and Psychiatrists of Old Age to identify individuals that meet the inclusion criteria for the study. Individuals identified medically as potentially suitable for enrolment will be referred to the PhD researcher for an additional cognitive function screening assessment. Individuals with no cognitive impairment will be recruited through an organised advertising campaign using newspaper, radio and online adverts. Individuals that meet the eligibility criteria will attend our research centre in

Carriganore House where a cognitive assessment will be performed to confirm absence of cognitive impairment. Pre-screening, screening and follow-up study visits will be structured as follows:

Pre-screening:

1. Montreal Cognitive Assessment (MoCA). This has been validated in the setting of MCI. The MoCA test is a one-page 30-point test administered in approximately 10 minutes. It assesses several cognitive domains and has been validated in the MCI setting(49;50). The short-term memory recall task (5 points) involves two learning trials of five nouns and delayed recall after approximately 5 minutes. Visuospatial abilities are assessed using a clock-drawing task (3 points) and a three-dimensional cube copy (1 point). Multiple aspects of executive functions are assessed using an alternation task adapted from the trail-making B task (1 point), a phonemic fluency task (1 point), and a two-item verbal abstraction task (2 points). Attention, concentration and working memory are evaluated using a sustained attention task (target detection using tapping; 1 point), a serial subtraction task (3 points), and digits forward and backward (1 point each). Language is assessed using a three-item confrontation naming task with low-familiarity animals (lion, camel, rhinoceros; 3 points), repetition of two syntactically complex sentences (2 points), and the aforementioned fluency task. Finally, orientation to time and place is evaluated (6 points)(51;52). Individuals with MCI that obtain a final score between 19 and 25 will be considered for enrolment into the study. Individuals with no cognitive impairment must score ≥26 in order to be considered for enrolment into the study.

2. Alzheimer's Questionnaire (AQ). The AQ is a clinician-administered and informant-based screening tool used to detect cognitive impairment. It is regarded as a time efficient and sensitive measure for detecting MCI using structured interview-based questions. The AQ consists of 21 yes/no questions in a weighted format relevant to five different domains: memory, orientation, functional ability, visuospatial and language. The total score is calculated by summing the number of items with a "yes" response. Clinical symptoms known to be highly predictive of AD are given a greater weight in the total score. Individuals with MCI that score between 5 and 14 points will be identified as potentially suitable for enrolment. Individuals with no cognitive impairment must score between 0 and 4 points in order to be deemed

suitable for enrolment. The AQ has been previously validated and has shown high sensitivity and specificity for detecting MCI(53;54). In the event where an informant is not present during the pre-screening assessment, the family member or carer will be contacted via telephone to complete the AQ. In circumstances where no informant is available, the clinician will administer the AQ to the patient themselves.

3. 4 Mountains test (4MT)

The 4MT assesses spatial perception and memory using subtests referred to as place perception (PP) and place memory (PM). Participants are presented with a computer-generated landscape containing 4 mountains with a semi-circular mountain range in the background. Participants are shown a sample landscape along with a panel of four landscapes, consisting of the original landscape seen from another viewpoint and three similar but incorrect images. Participants must identify which of the four images shows the same landscape as in the previous image.
For the perceptual task, this panel is presented at the same time as the target image, whereas for the memory task the panel is presented after a 2 second delay. In the PP task, a maximum of 30 seconds is given for a forced choice match-to-sample. In the PM task, a 4MT landscape is shown for 10 seconds followed by a 2 second delay(55;56). The test takes approximately 20 minutes to complete and has been used previously among individuals with MCI and AD(57). A study by Moodley *et al* suggested that a total 4MT score of ≤8 was associated with 100% sensitivity and 90% specificity for detecting early AD when tested in a UK population, and associated with 100% sensitivity and 50% specificity for detection of MCI and AD when tested in an Italian population group.

Screening:

1. Cognition assessment: Repeatable Battery for the Assessment of Neuropsychological Status (RBANS). This is a neuropsychological assessment initially introduced in 1998(58). It consists of ten subtests which give five scores, one for each of the five domains tested (immediate memory, visuospatial/constructional, language, attention, delayed memory). There is no assessment of executive function, category fluency and motor responses. It takes approximately 30 minutes to administer(59). It was originally introduced in the screening for dementia. Individuals with MCI that obtain an index score below 78 i.e. 1.5 standard deviations below the

mean in at least one of the five cognitive domains assessed will be considered as eligible to participate in the study. For individuals with no cognitive impairment (controls), an index score between 85 and 115 in each cognitive domain is desirable i.e. ±1 standard deviation from the mean.

2. Function assessment: Level of function will be assessed using Bristol Activities of Daily Living Scale (BADLS). This is a 20-item questionnaire designed to measure the ability of someone with dementia to carry out daily activities such as dressing, preparing food and using transport(58-60). It takes a carer (professional or family) 15 minutes to complete. It is sensitive to change in dementia and short enough to use in clinical practice (carers may fill it in while clinicians are performing direct assessment of patients). It is regularly used as an outcome measure in clinical trials, where it is world leading as a dementia-specific measure. This outcome is among those recommended by a consensus recommendation of outcome scales for nondrug interventional studies in dementia(61). Similar to the AQ, in the event where an informant is not present, they will be contacted via telephone to complete the BADLS. In circumstances where no informant is available, the trained researcher will administer the questionnaire to the patient themselves**.**

Pre-screening and screening for non-MCI subjects will be performed by the PhD researcher at Carriganore House.

Candidates who meet eligibility criteria for CARES will be held and all symptoms, co morbidities, and collateral history will be reviewed. Scores of the RBANS and BADLS will be collated and eligible participants will then be invited into the study. Candidates with borderline scores from the screening assessments (RBANS and/or BADLS) will be referred to a consensus panel for assessment of eligibility(62;63).

Subsequent study visits (baseline, 12- and 24-months)
The RBANS is the only cognitive function assessment tool from the screening battery that will be repeated at 12- and 24-month follow-up visits.

Demographic questionnaire: this will include a means of contact and address for both the study subject and a family member for secretarial purposes.

Lifestyle information: lifestyle factors (e.g. tobacco use) will be recorded by questionnaire. Medical and ocular history will also be recorded. This will allow us to control for any confounding variables.

Health information: blood pressure levels and body mass index will also be recorded for each subject.

Dietary questionnaire: a basic food questionnaire will be used to estimate the intake of L and Z in the diet. Fish and nut consumption will also be assessed by questionnaire.

Cognitive function:

1. **CANTAB battery:** A battery of tests from the Cambridge Neuropsychological Test Automated Battery (64;65) (CANTAB, Cambridge Cognition, Cambridge, UK) will be used to assess cognitive response in subjects. This computerised software program will assess attention, memory and executive function and decision making.
2. **EEG system:** Brain function will also be measured using an electroencephalogram or EEG system. This non-invasive device uses sensors which are placed along the scalp to record the tiny electrical impulses being emitted constantly from the brain. EEG has proven itself to be very useful in helping to understand brain function and is commonly used to diagnose epilepsy, sleep disorders and other disorders/diseases of the brain. EEG has also been used among MCI patients(66-68).

Blood samples: Five and three separate blood samples will be collected from each MCI and control patient, respectively, at each visit, in order to measure the level of carotenoids in serum (including MZ), the level of vitamin E in serum, the lipid profile and red cell omega3 fatty acids (DHA and EPA) before and after supplementation, and for additional blood tests (see below). The methodology that will be used to conduct both these analytical assessments has been previously published(69-72). Serum carotenoid concentrations will also be measured using the Pharmanex® BioPhotonic Scanner. This scanner measures carotenoid levels in human tissue at the skin surface using optical signals. These signals identify the unique molecular

structure of carotenoids, allowing their measurement without interference by other molecular substances. The individual is provided with their own skin carotenoid score which provides an indication of their overall antioxidant levels. This technology is safe and has been previously validated(73).

Additional blood tests (MCI group only): These will include a full blood count, sodium, potassium, chloride, urea, creatinine, thyroid function assessment (Thyroid Stimulation Hormone (TSH) and Free T4), vitamin B12, Folate, homocysteine, cholesterol (lipid profile), and high sensitivity C-reactive protein. Analysis of these samples will be performed by BIOMNIS Ireland, an accredited medical testing service provider.

Serum lipid assessment: Serum lipid profile assessment will be conducted at the MRC HNR, Elsie Widdowson Laboratory, CB1 9NL Cambridge, UK (under the supervision of Dr Albert Koulman) using previously published methods(70;71).

Red cell DHA and EPA assessment: Red cell DHA and EPA assessment will also be conducted at the MRC HNR, Elsie Widdowson Laboratory (under the supervision of Dr Albert Koulman) using a method published by Ferguson et al in 2014.(70)

Complement factor assessment: Complement factor assessment (Membrane Attack Complex (MAC) and Complement component 3 (C3)) will be conducted at the MRC HNR, Elsie Widdowson Laboratory (following a recommendation by Dr David Thurnham).

Serum carotenoid and vitamin E assessment: Serum carotenoid and vitamin E assessment will take place at Carriganore House, Waterford, Ireland. Non-fasting blood samples will be collected in 9ml vacuette tubes containing a ‘Z Serum Sep Clot Activator’. The blood samples will be allowed to clot at room temperature for approximately 30 minutes and then centrifuged at 2700 rpm for 10 minutes in a Gruppe GC 12 centrifuge (Desaga Sarstedt) to separate the serum from the whole blood. The resulting serum samples will be stored at circa −80°C until the time of batch analysis using HPLC. First, the serum samples will be analysed for L, total Z (co-eluted Z and MZ), and vitamin E using a reversed-phase HPLC method (Assay

1, for details of method see publication by Nolan et al.(24)). The mixed Z fraction is then automatically collected from Assay 1 using an Agilent 1260 fraction collector. The eluent will be dried under a solvent concentrator (MiVac, GeneVac, Mason Technologies, Dublin, Ireland) and analysed on Assay 2 for quantification of Z and MZ (Assay 2, for details of method see publication by Thurnham et al.(72)).

Neuroimaging: Where indicated, Computerised Tomography (CT) scans will be performed to out rule vascular or other causes of memory loss or word finding difficulty.

Macular pigment: MP levels will be measured by dual-wavelength autoflourescence using the Spectralis HRA + OCT Multicolour (Heidelberg Engineering GmbH, Heidelberg, Germany). This method has been described in detail elsewhere (74-76).

Visual Acuity: BCVA will be measured using the Early Treatment Diabetic Retinopathy Study (ETDRS) logarithm of the minimum angle of resolution (LogMAR) chart (Test Chart 2000 PRO™; Thomson Software Solutions, UK) viewed at 4 metres.

Letter Contrast Sensitivity: Letter contrast sensitivity (CS) will be assessed using the LogMAR EDTRS (Test Chart 2000 PRO™; Thomson Software Solutions, UK) chart at five different spatial frequencies (1.2 [cycles per degree] cpd, 2.4cpd, 6.0cpd, 9.6cpd, 15.15cpd) viewed at 4 metres.

Retinal Photographs: Colour fundus photographs will be taken using a Zeiss Visucam 200 (Carl Zeiss Meditec AG, Jena, Germany) to assess the presence of ocular pathology.

Study design

Subjects with MCI (n=60) will be randomised in a 50:50 masked fashion to either the active supplement (i.e. Macushield containing 10 mg L; 10 mg MZ; 2 mg Z), fish oil (1gram) and vitamin E (15mg) or placebo containing sunflower oil for 24-months. Subjects with no cognitive impairment (n=60) will also be randomised in a 50:50

masked fashion to either active supplement or placebo for 24-months. Study visits will take place at baseline, 12-months, and 24-months.

Study Outcomes

The study outcome measures will be as follows:

Primary outcome measure: cognitive function

Secondary outcome measures: macular pigment; serum carotenoid assessment; serum vitamin E assessment; visual function; serum lipid assessment; red cell DHA and EPA assessment; development of AD.

Eligibility (see above screening)

Inclusion

Subjects that will be eligible for the trial include:

- Subjects aged 65 years and over
- Self or family member reported memory loss
- Functionally independent in activities of daily living BADLS
- Fulfils criteria for minimal cognitive impairment as per the RBANS
- Consensus panel agreement on the diagnosis of MCI (where applicable)

Exclusion

- Active depression (under active review)
- Established diagnosis of early dementia (on cognitive enhancement therapy)
- Current psychiatric illness (under active review of psychotropic medications)
- Stroke disease (clinical stroke or stroke on CTB)
- Rapidly progressive or fluctuating symptoms of memory loss
- Acute angle glaucoma
- Already consuming carotenoid supplements (e.g. Macushield) or fish oil supplements (e.g. Souvenaid)
- Already on cholinesterase inhibitors or NMDA receptor antagonists
- Fish allergy
- Daily consumption of cod liver oil

Ethics

The project will respect all ethical requirements in its objectives and methodologies. We will strictly comply with widely recognised international text and codes of practice such as the Declaration of Helsinki. Ethical approval will be requested from the local Waterford south east region (of Ireland) ethics committee and the Waterford Institute of Technology ethics committee prior to the study commencing. Written informed consent will be obtained from each subject prior to the study commencing.

Ensuring tablet compliance

Frequent phone calls and reminder text messages will be sent to subjects to ensure compliance. Tablet counting will be performed at follow-up visits.

Supplement management

All supplements for the trial will be provided by the study sponsor (HF). It is agreed that supplements will be shipped from the USA (organised by Industrial Organica) to Pharmacy at Whitfield Clinic, Waterford, Ireland. A customised clinical trial software management program (Trial Controller) has been developed for this trial. This robust administration system will be used to document patient information (name and contact details), support the organisation and management of tablets required for the clinical trial and assist with the scheduling of study visits. Randomisation to the active or placebo intervention group will also be performed by the system. Comprehensive security and access controls in relation to the storage of the electronic data and the prevention of unauthorised access have been implemented. Members of Whitfield Pharmacy and the MPRG will have individual usernames and passwords and their log in details and activity will be recorded. Both Whitfield Pharmacy and the MPRG will have censored log in's i.e. Whitfield Pharmacy will only have access to patient codes, intervention group and tablet batch numbers, and will not have access to patient names or contact information. Similarly, as this clinical trial is double-blind, the MPRG will not have access to the assigned intervention group, but will be able to view patient codes, names, contact details and study visit appointments.

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