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Randomized Control Trials

Omega-3 fatty acid, carotenoid and vitamin E supplementation improves working memory in older adults: A randomised clinical trial

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SUMMARY

Background & aims: Accumulating evidence suggests that omega-3 fatty acids (ω-3FAs), carotenoids and vitamin E can improve cognitive performance. However, their collective impact on cognition has not yet been investigated in healthy individuals. This study investigated the combined effect of ω-3FA, carotenoid and vitamin E supplementation on the cognitive performance of older adults.

Methods: Cognitively healthy individuals aged ≥65 years consumed daily 1 g fish oil (of which 430 mg docosahexaenoic acid, 90 mg eicosapentaenoic acid), 22 mg carotenoids (10 mg lutein, 10 mg *meso*-zeaxanthin, 2 mg zeaxanthin) and 15 mg vitamin E or placebo for 24 months in a double-blind, placebo-controlled, randomised clinical trial.

Results: Following 24-month supplementation, individuals in the active group (n = 30; aged 69.03 ± 4.41years; 56.7% female) recorded significantly fewer errors in working memory tasks than individuals receiving placebo (n = 30; aged 69.77 ± 3.74 years; 70% female) (point estimate effect sizes ranged 0.090–0.105). Interestingly, as the cognitive load of the working memory tasks increased, the active group outperformed the placebo group. Statistically significant improvements in tissue carotenoid concentrations, serum xanthophyll carotenoid concentrations and plasma ω-3FA concentrations were also observed in the active group versus placebo (point estimate effect sizes ranged 0.078–0.589). Moreover, the magnitude of change of carotenoid concentrations in tissue and ω-3FA and carotenoid concentrations in blood were related to the magnitude of change in working memory performance.

Conclusion: These results support a biologically plausible rationale whereby these nutrients work synergistically, and in a dose-dependent manner, to improve working memory in cognitively healthy older adults. Increasing nutritional intake of carotenoids and ω-3FAs may prove beneficial in reducing cognitive decline and dementia risk in later life.

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1. Introduction

Due to the increasing prevalence of Alzheimer's disease (AD) and its associated economic, societal and caring burden, emphasis is now being placed on preventative strategies to delay its onset and reduce the risk of developing the disease. Accumulating evidence suggests that good nutrition (e.g. fruits, vegetables, fish) and healthy dietary patterns are important for improving cognitive performance [1,2], and are associated with a reduced risk of AD [3–5]. Importantly, advances in science and technology have increased our capacity to fully understand the unique neuroprotective mechanisms of specific nutrients that are likely driving these positive results. Some dietary components selectively accumulate in the brain where they play important physiological

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functions. These include omega-3 fatty acids (ω-3FAs) [6,7], xanthophyll carotenoids (oxygen-containing, plant-based pigments) and vitamin E [8–10]. Previous observational and interventional work that has separately examined the effects of these nutrients on cognitive function has yielded promising, yet mixed, results [9,11,12]. Overall, the evidence to date suggests that these nutrients can work independently to improve cognitive performance, primarily due to their antioxidant and anti-inflammatory properties. Interestingly, previous exploratory work has shown that a combination of the ω-3FA docosahexaenoic acid (DHA) and the xanthophyll carotenoid lutein can work synergistically to improve cognition in older women [13]. The present study, the Cognitive impAiRmEnt Study (CARES), was designed to examine the potential synergistic effects of a combination of ω-3FAs (namely DHA and eicosapentaenoic acid [EPA]), xanthophyll carotenoids (specifically lutein, zeaxanthin and *meso*-zeaxanthin) and vitamin E (D-α-tocopherol form) on the cognitive performance of cognitively healthy older adults.

2. Materials & methods

2.1. Classification of evidence

This study provides Class II evidence that 24-month supplementation with 430 mg DHA, 90 mg EPA, 10 mg lutein, 2 mg zeaxanthin, 10 mg *meso*-zeaxanthin and 15 mg vitamin E (D-α-tocopherol) is effective in improving cognitive performance, namely working memory, in cognitively healthy older adults.

2.2. Study design and procedures

CARES Trial 2 (Trial 1 published previously [14]) was a parallel group, double-blind, placebo-controlled, block-randomised clinical trial. Volunteers, primarily from the South-East catchment area of Ireland, were recruited through regional and national advertisement campaigns. Eligibility criteria included: age ≥65 years; no self or family collateral report of memory loss; no rapidly progressive or fluctuating symptoms of memory loss; no established diagnosis of early dementia; no consumption of cognitive enhancement therapies (e.g. cholinesterase inhibitors); no history of stroke disease; no depression (under active review); no psychiatric illness (under active review of psychotropic medications); no glaucoma (acute angle); not consuming carotenoid or fish/cod liver oil supplements; and no fish allergy.

Prior to enrolment, all individuals that expressed an interest in participating in the trial completed a screening assessment (performed by RP) to confirm eligibility. This included assessing cognition using the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) and the Montreal Cognitive Assessment (MoCA). Individuals that fulfilled the criteria for each assessment were invited to participate in the trial. Individuals with borderline scores were referred to a consensus panel consisting of a Consultant Geriatrician, Psychiatrist of Old Age and Clinical Neuropsychologist for assessment of eligibility [15,16]. Eligible individuals were invited to enrol into the study (Fig. 1).

Of the 60 participants enrolled at baseline, 9 were lost at follow-up and 1 was excluded. Among participants in the active group, 2 were no longer interested in participating. Among participants in the placebo group, 2 were no longer interested in participating, 2 developed early-stage age-related macular degeneration (AMD) and 2 developed other health issues. One adverse event was recorded during the trial. One participant (female, aged 77 years at baseline) reported severe diarrhoea 4 weeks after commencing the trial. Of note, this participant was a survivor of cancer of the rectum. Upon trial completion, details of the intervention code revealed that this participant was enrolled into the placebo group. Thus, an attrition rate of 15% was recorded. Final visit (i.e. 24-month) data of 1 participant (male, aged 77 years at baseline) in the placebo group were removed prior to rANOVA analysis as *meso*-zeaxanthin was detected in 24-month (but not 12-month) serum (0.186 μmol/L). The presence of *meso*-zeaxanthin suggested carotenoid supplementation and was retrospectively confirmed via telephone with the participant.

2.3. Randomisation and intervention

Eligible individuals were assigned to the active or placebo group using block randomisation with no stratification. Random allocation sequencing in block sizes of 10 and in a 1:1 randomisation ratio was performed using a trial management system (Trial Controller) designed by our research centre and overseen by a Statistician (JS). In addition to completing the random allocation sequencing for the trial, the Trial Controller was used to document patient information (name, study code and contact details), assist with the scheduling of study visits and support the organisation and management of active and placebo capsules used in the trial. Comprehensive security and access controls in relation to the storage of the electronic data and the prevention of unauthorised access were implemented for this software.

Capsule dispensing was performed by members (CK and LOB) of UPMC Whitfield Pharmacy, Waterford, Ireland. Using the Trial Controller system these individuals had access to patient study codes, assigned intervention group and capsule batch numbers. Importantly, pharmacy members had no contact with participants and no access to participant names or contact details. By comparison, researchers directly involved in CARES had access to participant details and study codes, but no access to information regarding intervention allocation or capsule batch numbers. The researcher (RP) received a box of tablets from the pharmacy members (CK or LOB) with a subject identification label (i.e. both the researcher and study participant were blinded to the intervention). The intervention code was only revealed at study completion.

Participants were randomised to either the active intervention (n = 30) containing 1 g fish oil (of which 430 mg DHA and 90 mg EPA), 22 mg xanthophyll carotenoids (of which 10 mg lutein, 10 mg *meso*-zeaxanthin and 2 mg zeaxanthin) and 15 mg vitamin E (D--tocopherol) (now commercially known as Memory Health) or placebo (sunflower oil) (n = 30) group for 24 months. Previous research has shown that the carotenoid formulation used in the present study is the most efficacious in terms of achieving a response in retinal tissue concentrations (i.e. in the macula lutea) [17,18]. Both the discs of retinal photoreceptors [19] and the grey matter of the brain [20] are enriched in phospholipids with DHA. In contrast, the presence of EPA in both visual and cognitive tissues is residual. Therefore, a fish oil formulation with the highest DHA content achievable to improve the DHA composition of these tissues was chosen. Fifteen milligrams of vitamin E was chosen as it is the maximum amount allowed by the European Food Safety Authority. Moreover, previous research has demonstrated a greater carotenoid response in blood when combined with fish oil [13,21]. Doses were provided via two oval-sized capsules. Active and placebo capsules were identical in colour and size. Each active capsule contained equal quantities of fish oil, carotenoids and vitamin E. Carotenoid and vitamin E concentrations were manufactured by Industrial Orgánica (Monterrey, Mexico), while fish oil concentrations were manufactured by Epax (Ålesund, Norway; product number: EPAX1050 TG/N non-tuna). Participants were instructed

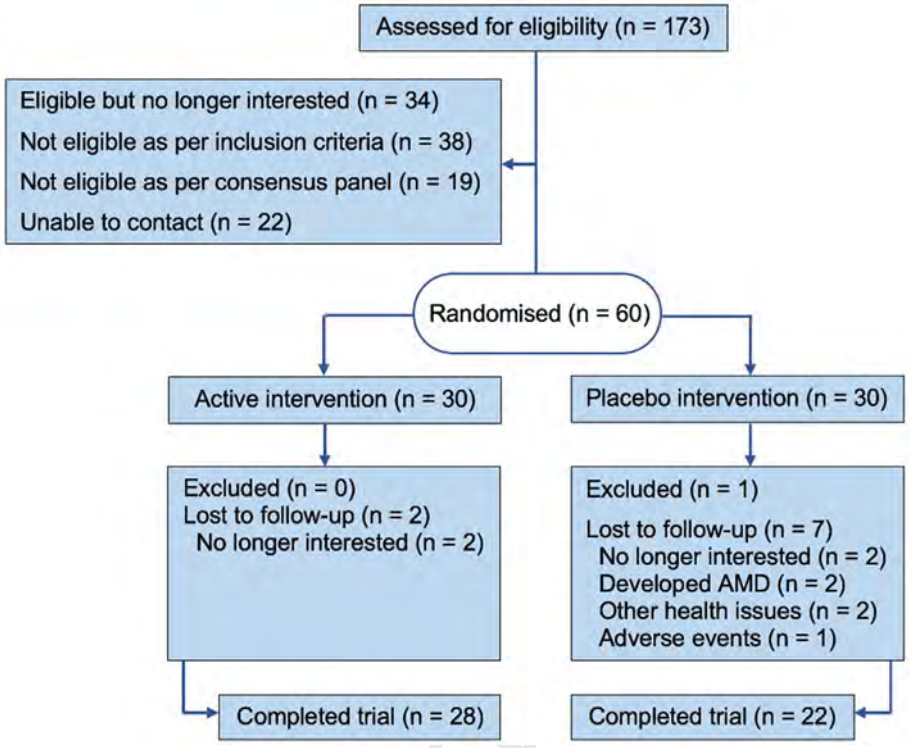

Fig. 1. Consolidated Standards of Reporting Trials flow diagram for CARES Trial 2.

to consume two capsules per day and in one sitting with a meal. Frequent phone calls were made to ensure compliance. Tablet counting was also performed at each follow-up visit to determine the overall level of compliance for both active and placebo groups. For each participant, the total number of capsules remaining at the end of the trial (i.e. the amount of capsules remaining after 12 months plus the amount remaining after 24 months) was divided by the total number of capsules issued for the trial. From this, a percentage was calculated. Study visits occurred at baseline, 12- and 24-months at a single site (Nutrition Research Centre Ireland). The trial commenced in March 2016 and concluded in June 2019 (i.e. last 24-month subject visit).

2.4. Standard protocol approvals, registrations and patient consents

Written informed consent was obtained from all participants prior to enrolment. Ethical approval was granted by the Waterford Institute of Technology and University Hospital Waterford research ethics committees in Waterford, Ireland, in December 2015. CARES (trial registration number: ISRCTN10431469) adhered to the tenets of the Helsinki Declaration (as revised in 2013) and followed the full code of ethics with respect to recruitment, testing and general data protection regulations as set out by the European Parliament and Council of the European Union.

2.5. Sample size calculations and outcome measures

Sample sizes of 30 per group were determined from power analysis to be suitable in this study. Subjects were randomly allocated between the active and placebo intervention groups and a 5% level of significance was chosen (i.e. a 95% confidence level). Calculations were based on repeated measures analysis of variance (rANOVA) analysis between two time points (i.e. baseline and end of study). All tests were assumed to be two-sided. RBANS across all five cognitive domains (i.e. RBANS total scale score) was the primary outcome measure for CARES. As all RBANS domains were considered to be of equal significance, the average scores of the 5 domains were used for power analysis. Based on data provided from baseline, the mean RBANS score was 106 and mean standard deviation (SD) was 12. Assuming a correlation of 0.70 for within-subject RBANS scores between baseline and end of study, a statistical power of approximately 96% was estimated for an effect size of 10.60 (10% of baseline RBANS score) and 79% for an effect size of 7.95 (7.5% of RBANS score). Secondary outcome measures included change in the following variables: working memory, attention, episodic memory, macular pigment optical volume (MPOV), skin carotenoid score (SCS), plasma ω-3FA concentrations, and serum concentrations of xanthophyll carotenoids and vitamin E.

2.6. Measurements

2.6.1. Cognitive function

Global cognition was assessed using the MoCA version 7.1 [22] and the RBANS Record form A [23] at screening and at 12- and 24-month follow-up (performed by RP). The MoCA is a short (10-min) 30-item cognitive screening questionnaire used to detect cognitive impairment. It assesses multiple cognitive domains including visuospatial abilities, executive function, phonemic fluency, attention, immediate and delayed recall, language and orientation. From this a composite score is generated. A score $\geq$26 out of 30 was desirable for enrolment. The RBANS is a core diagnostic tool for detecting cognitive decline or improvement. It takes approximately 30 min to administer and assesses immediate memory, visuospatial ability, language, attention and delayed memory using 12 sub-tests. Scores from each domain are summed to determine a total index/scale score. The RBANS yields index standard scores that are based on the raw scores of each subtest. RBANS index scores are metrically scaled, with a mean of 100 and a SD of 15 for each age group. A score of 100 on any of these measures

equates to the average performance of individuals of similar age. Scores of 85 and 115 correspond to 1 SD below and above the mean, respectively, while scores of 70 and 130 are 2 SDs below and above the mean. Approximately 68% of all examinees score between 85 and 115 and circa 95% score in the 70 to 130 range [24]. In the present study, a score of $\geq$78 was desirable for enrolment as it is above the defined cut-off score (1.5 SD below the mean) for cognitive impairment based on RBANS population-based norms.

Additional assessments of specific cognitive domains were performed using the Cambridge neuropsychological test automated battery (CANTAB) Connect Research software (Cambridge Cognition, Cambridge, UK) [25]. This computerised software program was performed on an iPad and required a finger-operated response. The CANTAB battery included the motor control task (MOT) to determine comprehension, the spatial working memory task (SWM) to measure working memory, the reaction time task (RTI) to assess attention and the paired associated learning task (PAL) to assess episodic memory [26]. A description of each cognitive task and associated outcomes measures is provided in Supplementary eTable 1.

2.6.2. Tissue carotenoid concentrations

2.6.2.1. Macular pigment. The xanthophyll carotenoids lutein, zeaxanthin and *meso*-zeaxanthin selectively accumulate in the central retina where they are collectively referred to as macular pigment (MP). Given that retinal concentrations (i.e. MP) correlate with brain concentrations of lutein and zeaxanthin [27], and higher MP levels are associated with better cognitive performance [28,29], MP can be used as a non-invasive biomarker of brain nutrition and cognitive health. MP was measured by dual wavelength autofluorescence (AF) using the Spectralis HRA+OCT MultiColor (Heidelberg Engineering GmbH, Heidelberg, Germany). Pupillary dilation of one eye (using a drop of 0.5% proxymetacaine hydrochloride followed by a drop of 1% tropicamide) was performed prior to measurement and patient details were entered into the Heidelberg Eye Explorer (HEYEX version 1.7.1.0) software. Dual-wavelength AF in this device uses two excitation wavelengths; one that is well absorbed by MP (486 nm, blue) and one that is not (518 nm, green) [30]. The following acquisition parameters were used: high speed scan resolution, 2 s cyclic buffer size, internal fixation, 30-s movie and manual brightness control. Alignment, focus and illumination were first adjusted in infrared mode. Once the image was evenly illuminated, the laser mode was switched from infrared to blue plus green laser light AF. Using the HEYEX software, the movie images were aligned and averaged, and a MP density map was created. MPOV calculated as MP average times the area under the curve out to 7° eccentricity is reported here [31] and has been previously validated as an accurate and reliable assessment of MP [32]. A higher MPOV score was indicative of greater MP.

2.6.2.2. Skin carotenoid score. Carotenoid concentrations were also measured using the Pharmanex® BioPhotonic Scanner (Salt Lake City, UT, USA). This scanner measures carotenoid levels in human tissue at the skin surface using optical signals (resonant Raman spectroscopy) [33]. These signals identify the unique molecular structure of carotenoids, allowing their measurement without interference by other molecular substances. Participants placed a specific point (between the maximal and distal palmar creases, directly below the fifth finger) of their right hand (previously cleaned with hand sanitizer) in front of the scanner's low-energy blue light for 30 s. Following this, a SCS was generated, which provided an indication of the participants' overall carotenoid levels (ranging from zero to 90,000). A higher score was indicative of greater carotenoid intake. This technology has been previously validated for its safety and accuracy in measuring carotenoid status [34,35].

2.6.3. Biochemical analysis

Non-fasting blood samples were collected at each study visit by standard venepuncture techniques. We used the same methodology as the one employed in a previous study [14] to extract lutein, zeaxanthin, *meso*-zeaxanthin and α-tocopherol from serum, and DHA and EPA from plasma; to quantify serum carotenoid and α-tocopherol concentrations and analyse amounts using high performance liquid chromatography (HPLC); and to quantify plasma concentrations of DHA and EPA and analyse amounts by gas chromatography coupled to flame ionisation detector (GC-FID).

2.6.4. Demographic, health and lifestyle data

Demographic, health and lifestyle data, medical history and current medication use were recorded via questionnaire. Height and weight measurements were recorded to calculate body mass index (kg/m^2). Colour fundus photographs were taken to assess the presence of ocular pathology (Zeiss Visucam 200, Carl Zeiss Meditec AG, Jena, Germany).

2.7. Statistical analysis

The statistical packages IBM SPSS version 25 and Minitab 19.2 were used, and the 5% significance level applied for all analyses. An adjustment for multiple comparisons was not carried out for this study as initial sample sizes were determined according to a 5% level of significance and 80% power for the primary outcome measure and pre-planned comparisons. For all analyses, point estimates and 95% confidence intervals were provided. Results were expressed as means ± SD for numeric data. Categorical data were expressed as percentages. Between-group differences were analysed using Independent Samples t-tests or Chi-square tests, as appropriate. rANOVA was used to assess Time and Time−Group interaction effects across 3 time points (i.e. baseline, 12- and 24-month follow-up) between both intervention groups for cognition and nutrition variables. Time effects examine whether or not a response variable is different at the time points of interest. Time−Group effects examine whether or not the time effect differs between the active and placebo groups. In cases where rANOVA showed interesting results, further statistical analyses were conducted using paired samples t-tests to examine statistical difference within groups at baseline and 24 months for both groups. A general linear model was used to assess (for dependent variables tissue carotenoid concentrations, serum carotenoid and plasma ω-3FA concentrations) the potential impact of sex, smoking habits, and alcohol consumption on Time and Time−Group effects. Effect size interpretations were based on parameters set by Cohen in 1988 [36,37] (i.e. 0.01, 0.06 and 0.14 for small, medium and large effect sizes, respectively for rANOVA analysis, and 0.20, 0.50 and 0.80 for small, medium and large effect sizes, respectively for paired samples t-test analysis. Finally, Spearman's rank correlation coefficient was used to investigate potential relationships between the observed changes in cognitive function variables and the observed changes in tissue carotenoid concentrations, serum carotenoid concentrations, and plasma ω-3FA concentrations. Effect size interpretations for Spearman's rank correlation coefficient were based on parameters set by Cohen in 1988 [37] (i.e. 0.20, 0.50, and 0.80 for small, medium and large effect sizes, respectively).

2.8. Data availability

Research protocols and anonymised data from CARES may be shared by written request from any qualified researchers for the purpose of replicating procedures and results.

Table 1A
Baseline demographic, health and lifestyle data of active and placebo intervention groups.

Variable	Active (n = 30)	Placebo (n = 30)
Demographic data		
Age (years)	69.03 ± 4.41	69.77 ± 3.74
Sex ([n] [% female])	17 (56.7%)	21 (70.0%)
Education (years)	16.47 ± 1.61	17.41 ± 2.69
Health and lifestyle data		
Medications	3.07 ± 2.55	3.27 ± 2.97
Exercise (min/week)	288.17 ± 308.23	313.67 ± 282.12
Smoking ([n]; [%])		
Never	12 (40.0%)	20 (66.7%)
Past	15 (50.0%)	9 (30.0%)
Current	3 (10.0%)	1 (3.3%)
BMI (kg/m^2)	28.92 ± 5.10	27.11 ± 4.18
Nutrition data		
MP (MPOV)	5325 ± 2206	5575 ± 2002
SCS	30,593 ± 8317	32,167 ± 11,354
Serum L	0.158 ± 0.060	0.203 ± 0.156
Serum Z	0.052 ± 0.014	0.059 ± 0.027
Serum MZ	0	0
Serum vitamin E	29.272 ± 5.576	28.280 ± 5.693
Plasma DHA	191.692 ± 82.881	214.406 ± 51.511
Plasma EPA	128.572 ± 68.841	127.327 ± 39.650

Data are presented as are mean ± standard deviation for numeric data and actual number and percentages for categorical data; Education: age (years) completed formal education; Medications: the number of prescribed medications consumed; Smoking status: never (smoked <100 cigarettes in lifetime), past smoker (smoked ≥100 cigarettes in lifetime and none in the past year) or current smoker (smoked ≥100 cigarettes in lifetime and at least 1 cigarette in the last year). BMI: body mass index; MP: macular pigment; MPOV: macular pigment optical volume, calculated as MP average times the area under the curve out to 7° eccentricity; SCS: skin carotenoid score; Serum lutein, zeaxanthin, *meso*-zeaxanthin, and plasma docosahexaenoic acid and eicosapentaenoic acid concentrations are expressed in μmol/L. Data missing in the active intervention group for the following variables: education (n = 1); MPOV (n = 1); SCS (n = 3); serum xanthophyll carotenoid and vitamin E concentrations (n = 4); plasma DHA and EPA (n = 3). Data missing in the placebo group for the following variables: serum xanthophyll carotenoid and vitamin E concentrations (n = 1).

Table 1B
Baseline cognition data of active and placebo intervention groups.

Variable	Active (n = 30)	Placebo (n = 30)
Global cognition		
MoCA	27.53 ± 1.76	27.03 ± 1.16
RBANS immediate memory	108.37 ± 13.30	107.0 ± 13.38
RBANS visuospatial	112.73 ± 13.20	115.40 ± 14.16
RBANS language	102.30 ± 10.28	101.40 ± 9.18
RBANS attention	100.83 ± 9.75	99.30 ± 15.24
RBANS delayed memory	107.30 ± 9.91	106.13 ± 10.94
RBANS total scale	107.70 ± 11.41	108.03 ± 11.34
Comprehension (MOT)		
Latency (millisecond)	941.81 ± 219.17	1013.22 ± 234.54
Working memory (SWM)		
Between errors stage 4	0.70 ± 1.47	1.13 ± 1.81
Between errors stage 6	3.87 ± 3.56	5.27 ± 3.77
Between errors stage 8	12.0 ± 4.81	11.07 ± 3.18
Between errors all stages	16.07 ± 7.67	16.63 ± 6.67
Total errors stage 4	0.87 ± 2.27	1.20 ± 2.09
Total errors stage 6	4.0 ± 3.63	5.57 ± 4.20
Total errors stage 8	12.50 ± 5.23	11.28 ± 3.13
Total errors all stages	16.86 ± 8.02	17.07 ± 6.68
Strategy	8.45 ± 2.28	9.27 ± 2.29
Reaction time (RTI)		
Simple reaction time (millisecond)	371.62 ± 52.74	371.52 ± 46.22
Simple error score (millisecond)	0.97 ± 1.43	0.93 ± 1.02
5-choice reaction time (millisecond)	416.89 ± 45.37	425.91 ± 48.61
5-choice error score (millisecond)	0.23 ± 0.57	0.60 ± 1.0
Episodic memory (PAL)		
First attempt memory score	10.40 ± 3.88	10.07 ± 3.26
Total errors adjusted stage 2	0.27 ± 0.69	0.13 ± 0.51
Total errors adjusted stage 4	1.33 ± 2.26	1.87 ± 2.53
Total errors adjusted stage 6	6.77 ± 5.48	6.80 ± 4.38
Total errors adjusted stage 8	17.0 ± 9.56	14.90 ± 9.38
Total errors adjusted all stages	25.37 ± 15.42	23.70 ± 13.05

Data are presented as are mean ± standard deviation. MoCA: Montreal Cognitive Assessment; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status; MOT: motor control task; SWM: spatial working memory; RTI: Reaction time; PAL: paired associated learning. Data missing in the active intervention group for the following variables: SWM between errors stage 8 score (n = 2); SWM between errors total score (n = 1); SWM total errors stage 8 score (n = 2); SWM total errors across all stages (n = 1); SWM strategy score (n = 1). Data missing in the placebo group for the following variables: SWM between errors stage 8 score (n = 1); SWM total errors stage 8 score (n = 1).

3. Results

3.1. Baseline data

Demographic, health and lifestyle, nutrition and cognitive function data were statistically comparable between active and placebo groups at baseline (Tables 1A and 1B).

3.2. Level of compliance

On average, the level of compliance to the intervention was 87% among individuals in the active group (n = 27) and 91% in the placebo group (n = 21). Level of compliance was statistically comparable between both groups.

3.3. Observed change in cognitive function

Table 2 summarises the observed change over time in cognitive function and nutrition variables for active and placebo intervention groups, based on rANOVA analysis.

3.3.1. Global cognition

The RBANS total scale score (i.e. the primary outcome measure) improved in both groups after 24 months (+3% versus +6% for active and placebo groups, respectively). The Time effect was statistically significant ($\eta^2 = 0.135$, 90% confidence interval [CI] [0.037, 0.232]) however, no Time−Group effect was noted ($\eta^2 = 0.054$, 90% CI [0, 0.131]). Individuals in the active group performed better over time in comparison to individuals in the placebo group in the RBANS language domain. A statistically significant Time−Group interaction effect was also observed for the RBANS immediate memory domain, but this improvement was seen in the placebo group (Table 2).

3.3.2. Working memory

Medium to large Time and Time−Group effect sizes were recorded by individuals receiving the active intervention for working memory tasks, with small effect sizes noted among individuals in the placebo group. Following the 24-month intervention period, individuals in the active group significantly reduced the number of total errors made at stage 8 of the SWM task by 38%, while individuals receiving placebo declined by 1% after 24 months ($\eta^2 = 0.090$, 90% CI [0.005,0.189]). Additionally, individuals consuming the active intervention recorded 26% fewer total errors post intervention in comparison to individuals receiving placebo where the number of errors increased by 14% after 24 months ($\eta^2 = 0.105$, 90% CI [0.012, 0.207]) (see Fig. 2A and B, and Table 2).

Errors bars: +/− 1 standard error. Lower score is indicative of better performance. Spatial working memory total errors: the number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the individual. Stage 8 involves 8 boxes, and all stages is the sum of stages 4, 6 and 8. Statistical significance was observed between active and placebo

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Table 2
Observed changes over time in cognitive function and nutrition variables for active and placebo intervention groups using repeated measures analysis of variance.

Variable	Baseline				12 months				24 months				
	Active		Placebo		Active		Placebo		Active		Placebo		
	n	M ± SD	n	M ± SD	M ± SD	%Δ; Outcome	M ± SD	%Δ; Outcome	M ± SD	%Δ; Outcome	M ± SD	%Δ; Outcome	η^2 (CI)
Cognition													
SWM TE8	22	11.32 ± 4.67	17	11.06 ± 3.25	11.45 ± 5.31	+1; Declined	11.76 ± 4.37	+6; Declined	7.05 ± 4.13	−38; Improved	11.18 ± 4.83	+1; Declined	0.090 (0.005, 0.189)
SWM TE all stages	22	16.77 ± 7.83	18	14.33 ± 6.37	16.59 ± 7.91	−1; Improved	16.06 ± 6.85	+12; Declined	12.45 ± 6.06	−26; Improved	16.39 ± 5.38	+14; Declined	0.105 (0.012, 0.207)
RBANS immediate	28	109.64 ± 12.37	21	106.57 ± 14.89	110.46 ± 13.11	+1; Improved	110.67 ± 11.66	+4; Improved	113.07 ± 12.23	+3; Improved	119.10 ± 10.66	+12; Improved	0.083 (0.008, 0.169)
RBANS language	28	102.86 ± 10.27	21	101.33 ± 9.42	103.11 ± 9.80	+0.2; Improved	105.14 ± 9.22	+4; Improved	110.82 ± 10.82	+8; Improved	105.52 ± 10.56	+4; Improved	0.094 (0.014, 0.184)
Nutrition													
MPOV	26	5154 ± 2221	21	5399 ± 1668	7338 ± 2704	+42; Improved	5403 ± 1847	+0.01, Improved	8505 ± 2972	+65; Improved	5063 ± 1808	−6; Declined	0.589 (0.472, 0.660)
SCS	24	30,458 ± 8552	20	33,750 ± 12,615	41,125 ± 11,468	+35; Improved	32,250 ± 11,170	−4; Declined	38,542 ± 12,420	+27; Improved	33,650 ± 12,861	−0.3; Declined	0.253 (0.118, 0.361)
Lutein	23	0.157 ± 0.064	19	0.207 ± 0.190	0.689 ± 0.346	+339; Improved	0.204 ± 0.153	−1; Declined	0.550 ± 0.361	+250; Improved	0.218 ± 0.146	+5; Improved	0.392 (0.245, 0.494)
Zeaxanthin	23	0.051 ± 0.014	19	0.064 ± 0.031	0.085 ± 0.035	+67; Improved	0.068 ± 0.042	+6; Declined	0.075 ± 0.033	+47; Improved	0.069 ± 0.032	+8; Improved	0.167 (0.050, 0.276)
MZ	23	0	19	0	0.052 ± 0.032	-; Improved	0	0; Unchanged	0.035 ± 0.031	-; Improved	0	0; Unchanged	0.420 (0.274, 0.519)
Vitamin E	23	29.060 ± 5.715	19	28.646 ± 5.912	30.251 ± 5.557	+4; Improved	29.922 ± 6.810	+4; Improved	28.803 ± 5.399	−1; Disimproved	30.231 ± 7.217	+6; Improved	0.024 (0, 0.085)
DHA	24	190.991 ± 85.894	19	207.415 ± 50.085	304.303 ± 95.382	+59; Improved	204.695 ± 61.975	−1; Declined	319.740 ± 111.854	+67; Improved	227.305 ± 58.274	+10; Improved	0.256 (0.120, 0.366)
EPA	24	125.704 ± 67.679	19	116.687 ± 32.506	142.532 ± 54.126	+13, Improved	105.883 ± 41.300	−9; Dieclined	166.272 ± 77.310	+32; Improved	118.095 ± 36.092	+1; Improved	0.078 (0.003, 0.169)

Data are presented as are mean ± standard deviation; %Δ at 12 months: 12-month visit minus baseline visit expressed as a percentage; %Δ at 24 months: 24-month visit minus baseline visit expressed as a percentage; Outcome: interpretation of direction of result (i.e. improved, declined or remained unchanged over time); η^2: effect size; CI: 90% confidence interval (lower limit, upper limit); SWM TE8: spatial working memory total errors at stage 8, the number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the individual at stage 8 of the assessment; SWM TE all stages: spatial working memory total errors across all stages, the number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the individual, calculated across all stages of the assessment; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status; RBANS immediate: immediate memory domain of the RBANS; MPOV: macular pigment optical volume; SCS: skin carotenoid score; MZ: meso-zeaxanthin; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid.

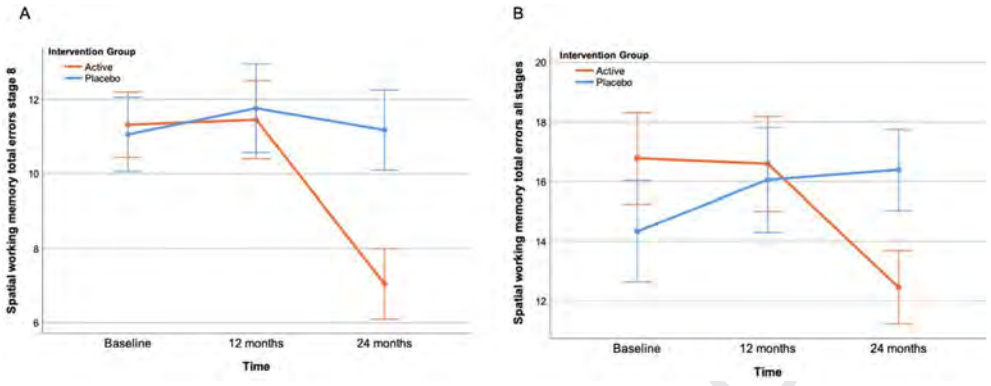

Fig. 2. Line graphs illustrating change in spatial working memory errors over 24 months.

groups for both SWM total errors at stage 8 and SWM total errors across all stages.

Of note, the number of total errors made at stage 4 of the SWM task were statistically comparable ($\eta^2 = 0.005$, 90% CI [0, 0.032]) for active (mean ± SD baseline 0.93 ± 2.34; final visit 0.86 ± 1.46) and placebo (baseline 0.80 ± 2.29; final visit 0.30 ± 0.98) groups. Interestingly, as the cognitive load of the task increased (i.e. from 4 to 6 tokens, and from 6 to 8 tokens), individuals in the active intervention (stage 6: baseline 4.50 ± 3.67, final visit 2.27 ± 2.27; stage 8: baseline 11.32 ± 4.67, final visit 7.05 ± 4.13) outperformed individuals receiving placebo (stage 6: baseline 4.26 ± 4.27, final visit 4.74 ± 3.05; stage 8: baseline 11.06 ± 3.25, final visit 11.18 ± 4.83), with $\eta^2 = 0.059$, 90% CI (0, −0.141) for stage 6 and $\eta^2 = 0.090$, 90% CI (0.005, 0.189) for stage 8.

3.3.3. Post hoc analysis

As outlined previously, paired samples t-tests were conducted in cases where rANOVA showed interesting results (see Supplementary eTable 2). Medium to large effect sizes were observed over time among individuals in the active intervention group for working memory (between errors at stage 8 and total errors at stage 6) and reaction time, with small effect sizes recorded among individuals consuming placebo. The nutritional intervention had a small effect on the RBANS composite score (d = −0.282, 95% CI [−0.573, −0.002]). In contrast, a large effect size was noted in the placebo group for this global cognition assessment (d = −0.578, 95% CI [−0.914, −0.274]).

3.4. Observed change in nutritional status

Large Time−Group effect sizes were observed for individuals receiving the active intervention for carotenoid concentrations in tissue, with mean percentage increases of +65 and + 27 after 24 months recorded for MPOV and SCS variables, respectively. Medium to large Time−Group effect sizes were recorded for serum concentrations of lutein, zeaxanthin and *meso*-zeaxanthin (mean percentage increases of +250 for lutein and +47 for zeaxanthin) and plasma concentrations of DHA and EPA (mean percentage increases of +67 and + 32, respectively) after the 24-month intervention period. There was no evidence to suggest statistical significance for a Time or Time−Group effect for serum α-tocopherol concentrations in either group (Table 2). Of note, the observed increases in blood concentrations of xanthophyll carotenoids and ω-3FAs were all independently related to the observed increases in tissue carotenoid concentrations (MPOV and SCS), with the exception of EPA and SCS (Supplementary eTable 3).

3.5. Effects of demographic and other lifestyle variables

The possibility of an interaction effect for sex, education, BMI, smoking status and alcohol consumption on the statistically significant Time−Group effects observed for nutrition variables was examined using a general linear model. The dependent variables in these analyses included change in: MPOV; SCS; serum concentrations of lutein, zeaxanthin, *meso*-zeaxanthin and α-tocopherol; and plasma DHA and EPA concentrations. No statistically significant interactions were found. Thus, for example, changes in MPOV did not differ by sex, BMI, smoking status nor alcohol consumption.

3.6. Relationships between change in nutrition status and change in cognitive function

Spearman's rank correlation coefficient was used to investigate whether or not the observed changes in cognitive scores were

Table 3
Relationships between observed changes in nutritional status and observed changes in cognitive function using Spearman's rank correlation coefficient.

Observed change in nutritional status	Observed change in SWM total errors at stage 8			Observed change in SWM total errors across all stages			Observed change in simple reaction time		
	r	CI	n	r	CI	n	r	CI	n
MPOV	−0.452	−0.699, −0.164	45	−0.458	−0.671, −0.175	45	−0.353	−0.585, −0.068	48
Serum Lutein	−0.375	−0.627, −0.051	38	−0.332	−0.592, −0.010	39	−0.209	−0.482, 0.100	43
Serum *meso*-zeaxanthin	−0.388	−0.637, −0.066	38	−0.352	−0.607, −0.031	39	−0.220	−0.491, 0.090	43
Plasma DHA	−0.446	−0.679, −0.131	38	−0.408	−0.649, −0.093	39	−0.156	−0.438, 0.153	43
Plasma EPA	−0.310	−0.578, 0.018	38	−0.317	−0.580, 0.007	39	−0.365	−0.606, −0.063	43

Observed change: exit visit data minus baseline visit data; CI: 95% confidence interval (lower limit, upper limit); MPOV: macular pigment optical volume; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; Total errors: the number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the individual; Simple reaction time: the duration between the onset of the stimulus and the time at which the individual released the button. Calculated for correct trials, where the stimulus could appear in one location only.

related to the observed changes in tissue and serum concentrations of xanthophyll carotenoids and plasma concentrations of ω-3FAs (Table 3). Overall, medium to large-strength relationships were recorded, with the exception of relationships between reaction time and some nutritional variables. Individuals with higher concentrations of MPOV, lutein, *meso*-zeaxanthin, DHA or EPA after 24 months recorded fewer errors in the working memory task in comparison to individuals with lower changes in serum and tissue concentrations of xanthophyll carotenoids and lower changes in plasma ω-3FA concentrations.

4. Discussion

4.1. Summary of findings

Following 24-month supplementation, individuals in the active intervention exhibited improvements in working memory. Improvements in attention and global cognition were also recorded. The observed improvements in cognition are consistent with previous observational [11,29,38] and interventional [39,40] studies.

4.2. Working memory

With specific reference to working memory, individuals in the active group made significantly fewer errors in the final and combined stages of the SWM task in comparison to individuals receiving placebo. Of note, the observed changes in tissue and serum carotenoid concentrations, and in plasma ω-3FAs concentrations were directly related to the observed improvements in this working memory task. Previous studies have also reported a relationship between higher carotenoid and ω-3FA intake and better executive function [9,41,42]. Working memory is a key component of executive function that is responsible for the temporary holding of information for later access and application (e.g. holding a person's telephone number or address in mind, listening and responding to information spoken during a conversation). Brain regions involved in working memory include the prefrontal cortex, parietal regions, and the hippocampus. More specifically, working memory involves the encoding of stimuli (e.g. words, pictures) and can involve attending to just one feature of a stimulus (i.e. selective attention) (e.g. tuning out the various sounds in a busy restaurant to listen to your friend tell a story), or attending to multiple features of a stimulus (i.e. divided attention or multi-tasking) (e.g. singing along to a song on the radio while driving). While the span of our working memory is quite short (10–15 s) [43], it is vital for learning, retaining and responding to information. In the present study, the encoding and retrieval of information was comparable between active and placebo groups during a working memory task with few stimuli (i.e. stage 4 of the SWM task where the individual had to locate 4 tokens). Importantly, as the cognitive load increased (i.e. from 4 to 6 tokens, and from 6 to 8 tokens) individuals in the active intervention out-performed individuals in the placebo group, with better performance in stage 8 and summed stages where the cognitive load was at its highest. This suggests that the working memory capacity of individuals in the active group was favourably altered over time and that these positive changes may be attributed to the enrichment of ω-3FAs and carotenoids, given that the magnitude of change in cognition was related to the magnitude of change in nutrition levels and given that these nutrients have been previously shown to be neuroprotective [44]. In terms of clinical significance, the observed improvements in working memory can translate into practical benefits for day-to-day function. An improved working memory can enhance the capacity to retain information and prioritise the steps needed to make decisions and solve problems. Enhancing working memory can also aid individuals in focusing on the task at hand such as planning and prioritising tasks for the day ahead or remembering key information (e.g. keeping appointment).

4.3. Carotenoid and omega-3 fatty acid synergy

An additional and interesting findings from this work relates to the positive and significant relationships observed between blood concentrations of ω-3FAs and carotenoids, and tissue carotenoid concentrations. Previous carotenoid intervention studies [45,46] have shown that increases in carotenoid concentrations in serum/plasma do not necessarily lead to a response in tissue (i.e. MP). Many researchers have hypothesised why the carotenoid response in tissue is less reliable than the response in blood, with some suggesting that genetics [47] or lifestyle factors [48] may explain the variation in MP augmentation. Previous work has illustrated improvements in serum DHA and lutein concentrations following 4-month supplementation with lutein-only, DHA-only, and lutein plus DHA in comparison to individuals consuming placebo [49]. However, only individuals consuming lutein exhibited statistically significant improvements in MP. In the present study, all individuals in the active intervention group exhibited an increase in MP in comparison to individuals consuming placebo (with the exception of 1 patient where acquisition of MP was poor and therefore questionable). We suggest that the consistency in tissue response is due to the presence of ω-3 FAs. While this conclusion cannot be tested directly due to the lack of carotenoid- and omega-only groups, this hypothesis is supported by the positive and significant relationships between ω-3FA and carotenoid concentrations in blood and tissue carotenoid concentrations (Supplementary eTable 1). Interestingly, and in support of this hypothesis, it has been shown that carotenoid density at the centre of the macula is directly associated with ω-3FA index [50] and plasma concentrations of docosapentaenoic acid [51]. Given this likely relationship, the suggestion that DHA facilitated a more consistent response in tissue carotenoid concentrations warrants further investigation in the future work.

4.4. Null and unexpected findings

While individuals receiving the active intervention responded positively to ω-3FA and carotenoid supplementation, improvements in α-tocopherol were not observed. Reasons underlying the poor vitamin E response following supplementation remain unclear. While in accordance with international recommended dietary allowances, it is possible that the daily dosage of vitamin E used in the present study was too low (in comparison to other interventional studies [52]) to have any meaningful effect.

In addition to working memory, small-scale improvements in attention, language and global cognition were also recorded. These findings are also consistent with the literature [11,29,41,53]. An unusual finding from our research included statistically significant improvements in the RBANS immediate memory domain in the placebo group. This was unexpected and may be a true result due to a learning/practice effect for this cognitive domain or driven by the poor performance of a small number of subjects in the active intervention group at their final study visit. Given that ω-3FAs [54] and carotenoids [40,55] have been shown to positively impact episodic memory, the null findings for episodic memory in the present study were surprising. This may be due to a lack of statistical power or due to the age of the sample (combined aged of 69.40 ± 4.07 years). Given that age-related changes in episodic memory accelerate after age 60 and that changes in the relevant neuro-circuitry may have already occurred, nutritional supplementation may have been too late to exhibit an effect.

4.5. Strengths and limitations

Strengths of CARES include its double-blind, placebo-controlled, randomised design, strict eligibility criteria which ensured a clean dataset to test hypotheses of interest (e.g. no previous consumption of carotenoid supplements), and the comprehensive assessment of cognition using sensitive and validated diagnostic measurement tools. However, it is important to acknowledge that the results of this trial are not necessarily generalisable to the overall population and may be subject to selection bias, given that data were collected at a single-site (Nutrition Research Centre Ireland) and involved a study sample that was primarily recruited from the same geographical area. Despite these limitations, this study found improvements in cognitive performance, xanthophyll carotenoid concentrations in tissue and serum, and plasma concentrations of ω-3FAs following a 24-month nutritional intervention trial, with the observed improvements in cognition related to the observed increases in the nutrients of interest.

5. Conclusion

In conclusion, this research has shown improvements in working memory following 24-month supplementation with ω-3FAs, xanthophyll carotenoids and vitamin E in cognitively healthy older adults. These results support a biologically plausible rationale whereby these nutrients work synergistically, and in a dose-dependent manner, to improve cognitive performance. These findings illustrate the importance of nutritional enrichment in improving cognition and enabling older adults to continue to function independently, and highlight how a combination of ω-3FAs and xanthophyll carotenoids may prove beneficial in reducing cognitive decline and/or delaying Alzheimer's disease onset in later life.

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Author contributions

Rebecca Power: Data curation, formal analysis, investigation, project administration, visualisation, roles/writing – original draft, writing – review & editing.

John M. Nolan: Formal analysis, methodology, resources, supervision, visualisation, roles/writing – original draft, Writing – review & editing.

Alfonso Prado-Cabrero: Methodology, resources, supervision, validation, roles/writing – original draft, Writing – review & editing.

Warren Roche: Data curation, formal analysis, software, writing – review & editing.

Robert Coen: Methodology, validation, visualisation, writing – review & editing. Tommy Power: Validation, writing – review & editing. Ríona Mulcahy: Methodology, resources, supervision, validation, visualisation, roles/writing – original draft, writing – review & editing.

Conflict of Interest

Rebecca Power: RP has performed consultancy work for MacuHealth LLC™ (Birmingham, MI, USA). RP is funded in part by the Howard Foundation (registered with the Charity Commission of England & Wales #285822), hereafter "Howard Foundation". These organisations have an interest in commercially available supplements containing the macular carotenoids. RP is also funded by a joint research centre grant from Science Foundation Ireland (SFI) and the Department of Agriculture, Food, and Marine on behalf of the government of Ireland under grant #16/RC/3835—VistaMilk to develop commercial dairy products enriched in carotenoids. John M. Nolan does consultancy work as a Director of NOW Science Consultancy Ltd. for companies with an interest in food supplements. Alfonso Prado-Cabrero: APC has performed consultancy work for MacuHealth LLC™ and the Howard Foundation. APC has also been involved in a Commercialisation Fund Programme from Enterprise Ireland to develop a biotechnological process to produce carotenoids and the fatty acids EPA and DHA. APC is currently supported by grant #16/RC/3835—VistaMilk. Robert Coen, Warren Roche and Tommy Power declare no conflicts of interest. Ríona Mulcahy does consultancy work on behalf of the Howard Foundation.

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Appendix A. Supplementary data

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