

The Impact of Supplemental Macular Carotenoids in Alzheimer's Disease: A Randomized Clinical Trial

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Abstract.

Background: Patients with Alzheimer's disease (AD) exhibit significantly less macular pigment (MP) and poorer vision when compared to control subjects.

Objective: To investigate supplementation with the macular carotenoids on MP, vision, and cognitive function in patients with AD versus controls.

Methods: A randomized, double-blind clinical trial with placebo and active arms. 31 AD patients and 31 age-similar control subjects were supplemented for six months with either Macushield (10 mg meso-zeaxanthin [MZ]; 10 mg lutein [L]; 2 mg zeaxanthin [Z]) or placebo (sunflower oil). MP was measured using dual-wavelength autofluorescence (Heidelberg Spectralis®). Serum L, Z, and MZ were quantified by high performance liquid chromatography. Visual function was assessed by best corrected visual acuity and contrast sensitivity (CS). Cognitive function was assessed using a battery of cognition tests, including the Cambridge Neuropsychological Test Automated Battery (CANTAB)).

Results: Subjects on the active supplement (for both AD and non-AD controls) exhibited statistically significant improvement in serum concentrations of L, Z, MZ, and MP ($p < 0.001$, for all) and also CS at 1.2 cpd ($p < 0.039$). Also, for subjects on the active supplement, paired samples *t*-tests exhibited four significant results (from five spatial frequencies tested) in the AD group, and two for the non-AD group, and all indicating improvements in CS. We found no significant changes in any of the cognitive function outcome variables measured ($p > 0.05$, for all).

Conclusion: Supplementation with the macular carotenoids (MZ, Z, and L) benefits patients with AD, in terms of clinically meaningful improvements in visual function and in terms of MP augmentation.

Keywords: Age-related macular degeneration, Alzheimer's disease, cognitive function, contrast sensitivity, lutein, meso-zeaxanthin, randomized clinical trial, visual function, zeaxanthin

INTRODUCTION

We have recently reported in the Carotenoids and Age-Related Dementia Study (CARDS, report 1) that patients with mild to moderate AD exhibit significantly

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less macular pigment (MP), poorer vision, and a higher occurrence of age-related macular degeneration (AMD; another age-related disorder), when compared to control subjects [1].

MP, which is made up of the dietary carotenoids lutein (L), zeaxanthin (Z), and *meso*-zeaxanthin (MZ) [2], is found exclusively at the central macula and can be measured *in vivo* [3, 4]. Of note, the macula (the central part of the retina) is part of the central nervous system, and it is this specialized part of the retina that is responsible for central and detailed vision [5]. We know that the macular carotenoids, via their short-wavelength (blue) light filtering [6] and antioxidant properties [7, 8], play a protective role in AMD [9]. We also know that MP is positively related to visual function [10], and that enrichment of MP with nutritional supplements containing the macular carotenoids improves visual function in normal subjects (i.e., subjects without retinal disease) [11] and in subjects with early stages of AMD [12–15]. Indeed, the optical properties of MP, which include its preferential absorption of short-wavelength (blue) light, is likely to explain the visual benefits noted in previous clinical trials [16].

Of interest, we know from previous work that L and Z are present in the brain, including in the cerebellum, pons, and frontal and occipital cortices [17–19], and that their concentrations in the brain are positively correlated with retinal concentrations of these nutrients in primates [18] including humans [19]. Furthermore, there is a growing body of evidence suggesting a positive relationship between MP levels and cognitive function in humans [20–22] and Johnson et al. have reported that supplementation with the macular carotenoids impacts positively on cognitive function in older women [23].

Given the growing body of evidence showing that oxidative stress and inflammation contribute to cognitive impairment [24, 25] and AD pathogenesis [26, 27], it is plausible that carotenoids in the brain could protect against such stresses, given their proven antioxidant [7, 8] and anti-inflammatory properties [28, 29]. It has also been suggested that the carotenoids may play a beneficial role by enhancing gap junctional communication in the brain [30–32].

In summary, we have already reported that patients with AD have significantly less MP, lower serum concentrations of L and Z, poorer vision, and a higher occurrence of AMD when compared to control subjects. Also, there is a biologically plausible rationale, supported by a growing body of scientific evidence, which suggests that enrichment of retinal and brain nutrition with the carotenoids L, Z, and MZ will protect

and enhance cognitive function in humans. This study was conducted to investigate the impact of supplementation with the macular carotenoids on MP (primary outcome measure), and vision and cognitive function (secondary outcome measures) in patients with AD compared with controls of similar age, and is the first study to do so.

MATERIALS AND METHODS

Study design and subject recruitment

This clinical trial began in January 2013 (i.e., the first subject visit) and ended in September 2013 (i.e., last subject six month visit).

31 patients with mild to moderate AD (predominantly moderate) were recruited (through the Age-Related Care Unit at University Hospital Waterford [UHW]) into the study. Subjects recruited into this group (the AD group) were eligible if they had mild to moderate AD, which was defined as having an average Mini-Mental State Examination (MMSE) score of 14 to 24 with documented difficulties in other domains, such as carrying out activities of daily living, or behavioral changes. Subjects were excluded if they were currently taking supplements containing the macular carotenoids, or if they had done so over the previous 12 months. Other screening tests to check for eligibility included the clock drawing test and semantic fluency score. Co-morbid diagnoses were documented, including vascular risk factors and diabetes. Current medications were verified including cholinesterase inhibitors and glutamate receptor antagonists. Social histories were documented and collateral histories were collated from a family member or carer for all patients. Non-contrast computed tomography (CT) brain scan was performed to rule out radiological evidence of stroke disease.

Of note, 10 (32%) had also participated in the cross-sectional study previously reported by our group (CARDS1) [1] and 21 (68%) were newly recruited. Importantly, the subjects that had already participated in CARDS1 were re-examined at baseline of this study because of the time difference between the cross-sectional examination and the start of this clinical trial. Subjects with AD were randomly allocated, in a double-blind fashion to a supplement consisting of either Macushield™ (Macuvision Europe Ltd. Blythe Valley Innovation Centre, Central Boulevard, Blythe Valley Business Park, Solihull B90 8AJ, United Kingdom) ($n = 16$, active supplement containing 10 mg MZ; 10 mg L; 2 mg Z) or placebo ($n = 15$, sunflower oil).

The intervention and placebo supplements were identical in external appearance and therefore the two treatments were indistinguishable from each other.

An equal number ($n=31$) of age-similar controls free of AD (the none-AD Group) were similarly allocated to Macushield ($n=15$) or Placebo ($n=16$). Subjects were eligible for this group if they were aged (years) ≥ 65 . Subjects were excluded if they were currently taking supplements containing the macular carotenoids, or if they had done so over the previous 12 months.

Main study visits were at baseline and after six months of supplementation. All measurements were performed at the Vision Research Centre, Carriganore House, Waterford, Ireland. This clinical trial facility offers a very efficient and calm environment to conduct clinical trials. For consistency, all measurements were performed by the same researcher (EL) who was suitably trained on all aspects and technologies for this clinical trial.

Significant efforts were made to ensure subject compliance to the study supplements. Compliance was assessed on an ongoing basis (house visits and phone calls to care givers) by the study nurse (MB) for the AD subjects, and by the researchers (NO and EL) for the control subjects (mainly phone calls directly to the subjects). In addition, compliance was assessed by examining pill sleeves at the six month visit and by assessing serum carotenoid response using high performance liquid chromatography (HPLC, see below). The code was broken at 6 months, and the statistical analysis was performed by the Study Statistician (JS) and Principal Investigator (JMN). The results of this analysis are presented below. The methodology used to measure MP, visual function and cognitive function has already been described in detail (see CARDS report 1), so only a brief account of each method is presented below.

Ethics

The project was conducted in accordance with full sensitivity to the ethical requirements of the subjects recruited. The study objectives and methodology complied fully with the widely recognized international text and codes of practice such as the Declaration of Helsinki. A protocol was developed specifically for this study by the Principal Investigator (JMN) and Consultant Geriatrician (RM) at UHW to ensure that informed consent was obtained appropriately, and in keeping with the ethical code germane to obtaining consent from vulnerable subjects (which includes patients with AD). Ethical approval was granted from the local

Waterford South East (of Ireland) Region Ethics Committee prior to the study commencing.

Demographic, medical, ophthalmic, and lifestyle assessment

A demographic, medical, ophthalmic, and lifestyle case history was obtained for each subject at baseline. Body mass index (BMI) was calculated (kg/m^2) with subject height (m) measured with the Leicester Height Measure, and weight (kg) measured with the SECA weighing scales (SECA, Birmingham, UK). Smoking status was classed as either current smoker (i.e., smoked ≥ 100 cigarettes in lifetime and at least one cigarette within the last 12 months) or non-smoker (everybody else). Exercise was assessed by calculating the total exercise for any sporting activity measured as minutes per week. Diabetes was assessed by self-report and also by measuring HbA1c in blood (analysis conducted offsite at Biomnis Ireland, Three Rock Road, Sandyford Business Estate, Dublin 18, Ireland).

Cognitive function assessment

Cognition was assessed using a selection of validated measures. The MMSE was used to measure the severity of cognitive impairment. A semantic fluency score was obtained using 'Animal' as the category (as many exemplars as possible in one minute) and phonemic fluency was measured using the 'FAS Test' (as many words as possible starting with each letter, one minute per letter) [33]. Also, three tasks were chosen from the Cambridge Neuropsychological Test Automated Battery (CANTAB) [34]. All were administered using a finger-operated touch-screen tablet PC using a set of scripted instructions. The Paired Associates Learning task was selected to assess visual learning and memory [35]. A modified version of the Verbal Recognition Memory task was selected to assess verbal learning and memory [33]. In the modified version a free recall format was used instead of a recognition format. The CANTAB Motor Screening Task was used to assess motor speed and accuracy by instructing the subject to touch the center of a series of crosses that are presented on the screen [36].

Best corrected visual acuity and contrast sensitivity

The eye with best BCVA was selected as the study eye for vision testing. If both eyes had the same BCVA, the right one was selected. BCVA was measured with a

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235 computerized LogMAR ETDRS test chart (Test Chart
236 2000 Xpert; Thomson Software Solutions) viewed at
237 4 meters (m). The Sloan Early Treatment Diabetic
238 Retinopathy Study (ETDRS) letterset was used for this
239 test. Letter contrast sensitivity (CS) was assessed using
240 the computerized LogMAR ETDRS test chart (Test
241 Chart 2000 Pro; Thomson Software Solutions) at five
242 different spatial frequencies (1.2, 2.4, 6.0, 9.6, 15.15
243 cpd) [37]. Both these methods have been described in
244 more detail elsewhere [10, 38, 39].

245 *Retinal photograph assessment*

246 45 degree monoscopic color photographs, centered
247 on the macula, were taken in both eyes using a Zeiss
248 Visucam 200 (Carl Zeiss Meditec AG, Jena, Germany).
249 Retinal photographs were assessed for the presence
250 or absence of early AMD, in accordance with the
251 International Classification and Grading System for
252 Age-Related Macular Degeneration by a consultant
253 ophthalmologist (SB) with a special interest in retinal
254 disease and with a published track record in grading
255 this condition [40, 41]. In brief, the presence of soft
256 drusen and/or hypo-/hyper-pigmentary changes at the
257 macula were classed as early AMD.

258 *Macular pigment measurement*

259 MP was measured using the Heidelberg Spectralis®
260 HRA+OCT Multicolor (Heidelberg Engineering
261 GmbH, Heidelberg, Germany). This new technology
262 utilizes confocal scanning laser ophthalmoscopy
263 (cSLO) imaging with diode lasers and uses dual-
264 wavelength autofluorescence (AF) for measuring MP
265 [4, 42]. Dual-wavelength AF in this device uses two
266 excitation wavelengths, one that is well-absorbed by
267 MP (488 nm, blue), and one that is not well absorbed
268 by the pigment (518 nm, green). Of note, the AF
269 method utilized in this study has previously been
270 compared with the customized heterochromatic flicker
271 photometry (cHFP) technique for measuring MP, and
272 the measurements recorded from these two devices
273 exhibited excellent concordance [4]. However, the
274 physical (objective) AF device was deemed more
275 appropriate for this study, because patients with
276 AD might not have been able to use the subjective
277 (non-physical) cHFP device.

278 The Heidelberg Spectralis® AF method provides an
279 image of MP across its spatial profile, but here we
280 report just central MP (at 0.23 degrees eccentricity)
281 and MP volume (calculated as MP average times the
area under the curve out to 8 degrees eccentricity).

282 *Dietary intake of carotenoids*

283 A subject's weekly intake of carotenoid-rich foods
284 (eggs, broccoli, corn, dark leafy vegetables) was
285 inputted into the "L/Z screener" to give a carotenoid-
286 based diet score. The L and Z values used in the
287 screener were those reported by Perry et al. [43]. This
288 method of assessing and controlling for dietary intake
289 of carotenoids has been used with success elsewhere
290 [12]. Values are weighted for frequency of intake of
291 the food and for bioavailability of L and Z within these
292 foods. A ranking score reflecting the relative intakes
293 (representing arbitrary units) was generated and used
294 in analysis. For the AD subject, dietary habits were
295 confirmed by a family member or carer.

296 *Serum carotenoid assessment*

297 Non-fasting blood samples were collected in 9 ml
298 vacuette tubes containing a 'Z Serum Sep Clot Acti-
299 vator'. The blood samples were allowed to clot at
300 room temperature for approximately 30 min and then
301 centrifuged at 2700 rpm for 10 min in a Gruppe GC
302 12 centrifuge (Desaga Sarstedt) to separate the serum
303 from the whole blood. The resulting serum samples
304 were stored at circa -80°C until the time of batch
305 analysis using HPLC.

306 First, the serum samples were analysed for L and
307 total Z (co-eluted Z and MZ) using a reversed-phase
308 HPLC method (Assay 1, for details of method see pub-
309 lication by Nolan et al. [1]). The mixed Z fraction was
310 automatically collected from Assay 1 using an Agilent
311 1260 fraction collector. The eluent was dried under a
312 solvent concentrator (MiVac, GeneVac, Mason Tech-
313 nologies, Dublin, Ireland) and analyzed on Assay 2
314 for quantification of Z and MZ (Assay 2, for details of
315 method see publication by Thurnham et al. [44]).

316 *Statistics*

317 The statistical packages IBM SPSS version 21 was
318 used for statistical analysis. Random numbers (for the
319 allocation of subjects to active supplement or placebo)
320 were generated in Minitab version 16; block random-
321 ization was used. This study was very close in design
322 to a 2^2 factorial design (two factors each at two levels:
323 Macushield/Placebo and AD/Control) with 15 subjects
324 per cell. Such a study has statistical power of 81% to
325 detect a main effect of 0.75 standard deviations, and
326 power of 70% to detect an interaction effect of the
327 same magnitude, at the 5% level of significance [45].

328 Outcome variables analyzed included serum
329 carotenoids, MP, visual function measures, and

cognitive function measures. Between-group differences in these outcome variables at baseline (e.g., AD versus controls) were analyzed using Independent Samples *t*-tests or chi-squared tests as appropriate. Differences at baseline in demographic and lifestyle variables were also investigated, and controlled for in subsequent analyses, as appropriate.

The main focus of the present study was the investigation of change in the outcome variables over time (i.e., from baseline to six months). In other words, did supplementing with Macushield lead to improvements in these outcome variables, relative to the Placebo, and did the supplement work differently for AD and control subjects? Both of these research questions were addressed using Repeated Measures Analysis of Variance, with supplement [Macushield versus Placebo] and Group [AD yes/no] as between-subjects factors, and age and diet score as covariates. These covariates were included in the analyses because age and diet score were significantly different between AD and controls at baseline. For some cognitive scores, the assumptions required for Repeated Measures Analysis of Variance were violated, and in these cases we resorted to informal comparisons of change in score between AD and control subjects and between supplements.

In reporting findings in tables and figures, however, we considered that it would be more informative to report the results of paired *t*-tests, separately within each Supplement/Group patient category.

The 5% level of significance was used throughout all analyses, without adjustment for multiple comparisons. On standard assumptions (5% level of significance, two-tailed tests), the paired *t*-test subgroup analyses reported here, with about 15 subjects in each subgroup, had adequate power (82%) to detect "large" effect sizes (0.8 standard deviations, on Cohen's definition [46]). In general, however, it should be borne in mind that this small exploratory study was under-powered for the detection of smaller effect sizes and for the other analyses reported.

RESULTS

Baseline

Table 1 below presents baseline statistics for the AD and control groups. Of note, the sample and data presented here is slightly different to our already published cross-sectional paper (CARDS1), given that the sample was not precisely the same for CARDS1 and CARDS2. However, the conclusions are the same. We confirm that, at baseline in CARDS2, AD subjects have

significantly lower MP, poorer vision, poorer cognitive function, and a significantly higher prevalence of AMD, when compared to the control group.

Although we had attempted, when recruiting subjects for this study, to match the AD and control groups in terms of age, it can be seen in Table 1 that the AD group is significantly older (on average), and we therefore controlled for age in any analysis comparing outcome variables in AD and control groups. We also adjusted for diet score, the other variable which differed significantly at baseline between AD and control groups.

Dropouts

Control group

All 16 subjects on placebo completed their six month study visit, whereas there were 2 dropouts (n61 and n68) in the active (Macushield) group, resulting in 13 subjects in this arm of the study. Reasons given for dropout include: logistical difficulties (e.g., transport) and did not want to continue (willingness to participate).

AD group

12 subjects on placebo completed their six month study visit and there were 3 dropouts. Reasons for dropout include: logistical difficulties and did not want to continue (ADCD7 and ADN33); moved to nursing home and could not continue (ADN30). Also, 12 subjects in the active (Macushield) group completed their six month visit and there were 4 dropouts. Reasons for dropout include: logistical difficulties and did not want to continue (ADCD13, ADN22, ADN35, and ADN36).

Compliance

All subjects returned their capsule box and sleeves at their six month assessment visit. Assessment of capsule sleeves indicated that all subjects were consuming the supplements over the six-month study period. Also, serum carotenoid response confirmed that subjects in the active group were consuming the carotenoid intervention and that subjects in the placebo group exhibited no change in their serum carotenoid concentrations.

Changes from baseline to six months

Serum concentrations of lutein, zeaxanthin, and meso-zeaxanthin after six months of supplementation

In the Repeated Measures Analysis of change in serum L, the within-subjects Time*Supplement

Table 1
Demographic, lifestyle, vision, and cognition data of the AD and control subjects at baseline

Variables	AD (n = 31)	Control (n = 31)	Sig.
<i>Demographic and Health</i>			
Age (years)	80 ± 7.8	76 ± 6.6	0.031
Body mass index (Kg/m ²)	24.6 ± 5.8	26.4 ± 3.4	0.174
Exercise (total minutes of exercise per week)	174 ± 218	226 ± 16	0.304
Diet (estimated lutein and zeaxanthin intake)	16 ± 8	24 ± 14	0.008
Serum lutein (μmol/L)	0.232 ± 0.113	0.297 ± 0.179	0.104
Serum zeaxanthin (μmol/L)	0.051 ± 0.035	0.074 ± 0.042	0.03
Education (total years in education)	11 ± 4	14 ± 4	0.003
Smoking (% current)	8.60%	9.70%	0.88
Gender (% female)	58%	42%	0.203
<i>Vision</i>			
MP 0.23	0.41 ± 0.21	0.57 ± 0.17	0.002
MP vol	4074 ± 2585	6326 ± 2258	0.001
BCVA	88.9 ± 11.4	95.8 ± 8.4	0.009
CS1.2 (cpd)	1.49 ± 0.23	1.75 ± .22	<0.001
CS2.4 (cpd)	1.47 ± 0.25	1.79 ± 0.21	<0.001
CS6.0 (cpd)	1.19 ± 0.31	1.42 ± 0.24	0.004
CS9.6 (cpd)	0.94 ± 0.30	1.18 ± 0.26	0.005
AMD (% with AMD)	48.00%	16.00%	0.007
<i>Cognition</i>			
MMSE	19 ± 3.7	29 ± 1.7	<0.001
Semantic fluency score	6.0 ± 3.2	15.4 ± 5.2	<0.001
Phonemic fluency score	15.7 ± 10.3	32.5 ± 13.8	<0.001
VRM (phase 1)	1.4 ± 1.2	5.1 ± 2.6	<0.001
VRM (phase 2)	2.5 ± 1.6	7.3 ± 2.7	<0.001
VRM (phase 3)	3.4 ± 2.1	8.1 ± 2.9	<0.001
VRM Delayed Recall	0.4 ± 1.2	6.6 ± 3.3	<0.001
VRM Savings Score	0.1 ± 0.2	0.8 ± 0.5	<0.001
PAL (total errors adjusted)	136 ± 14.5	69 ± 39	<0.001
PAL (total errors adjusted 6 shapes)	28.5 ± 5.8	17.7 ± 10.7	<0.001
PAL Stages Complete	0.5 ± 0.9	4 ± 1.7	<0.001
PAL Patterns Reached	2.5 ± 0.9	7.2 ± 4.9	<0.001
PAL First Trial Memory Score	0.8 ± 2.3	9.7 ± 5.5	<0.001

Data displayed are mean ± standard deviation for interval data and percentages for categorical data. Variables, variables analyzed in the study; AD, subjects recruited into the study confirmed as having mild to moderate Alzheimer's disease; Control, subjects free of mild to moderate AD and of similar age to the AD subjects; Sig., the statistical difference (*p* value) between AD and control subjects assessed using independent samples *t*-tests or chi-squared depending on the variable of interest; Exercise, total exercise for any sporting activity measured as minutes per week; Diet, estimate of dietary intake of L and Z; Serum lutein, serum concentrations of lutein in μmol; Serum zeaxanthin, serum concentrations of zeaxanthin in μmol/L; Smoking, current (smoked ≥100 cigarettes in lifetime and at least one cigarette within the last 12 months) or non-smoking (smoked ≤100 cigarettes in lifetime and none within the last 12 months); MP 0.23, central macular pigment measured at 0.23 degrees eccentricity measured using the Heidelberg Spectralis®. MP vol, a volume of MP calculated as MP average times the area under the curve out to 8 degrees eccentricity (measured using the Heidelberg Spectralis®); BCVA, best corrected visual acuity; CS 1.2, CS 2.4, CS 6.0, and CS 9.6 = letter contrast sensitivity measured using the Thomson Software Solutions at 1.2, 2.4, 6.0, and 9.6 cycles per degree; AMD; age-related macular degeneration; MMSE, Mini-Mental State Examination; Semantic fluency score, a semantic fluency (categorical verbal fluency) score obtained from the number of animals named by the subject in 1 minute; Phonemic fluency score, a phonemic fluency (word fluency) score generated by the total number of words produced for the each of the letters F, A, and S, in 1 minute. MOT (mean latency), motor screening task measures the subject's speed of response; MOT (mean error), motor screening task measures the accuracy of the subject's pointing at cross targets; VRM (phase 1), VRM (phase 2), VRM (phase 3), Verbal Free Recall Memory immediate, three consecutive trials; VRM Delayed Recall, Verbal Free Recall Memory of the previous words after a delay period; VRM Savings Score, Delayed verbal recall divided by phase 3 immediate recall; PAL, Paired Associates Learning test which measure visual memory and new learning of the subjects; PAL (total errors adjusted), the adjusted score and includes an adjustment made for any stages not reached, allowing it to be comparable to all subjects even if the task was ended prematurely due to cognitive limitation; PAL (total errors adjusted 6 shapes), total errors made at the 6-pattern stage, adjusted for subjects who did not reach this stage; PAL Stages Complete, The number of stages successfully completed; PAL Patterns Reached, The number of patterns on the last problem in the task that the subject completed successfully; PAL First Trial Memory Score, The number of patterns correctly located after the first trial, summed across the stages completed.

426 interaction effect was significant ($p < 0.001$). Nei-
427 ther the Time*Group interaction ($p = 0.65$) nor the
428 Time*Supplement*Group interaction ($p = 0.97$) was

significant. Thus, there was a significant increase in
serum L concentrations after 6 months for subjects
on the active (Macushield) supplement compared with

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432 subjects on the placebo supplement, no significant dif-
 433 ference over time between AD and controls, and no
 434 evidence that the supplement worked differently over
 435 time for AD versus controls.

436 Similar results were obtained for serum concentra-
 437 tions of Z, in that the Time*Supplement effect was
 438 significant ($p = 0.007$), but not the others; and for MZ
 439 ($p < 0.001$ for Time*Supplement interaction). Thus, in
 440 short, we report that the active supplement significantly
 441 increases serum concentrations of L, Z and MZ, and it
 442 does so for both AD and control subjects.

443 These findings are presented in Table 2 and Fig. 1.
 444 In Table 2, all subjects on the active supplement
 445 (Macushield) exhibit significantly increased serum
 446 concentrations of L, Z, and MZ, in both control and
 447 AD subjects at six months.

448 The placebo categories exhibit no significant change
 449 over this time period, with the exception of a statisti-
 450 cally significant increase in serum concentrations of L
 451 in the AD group. Although this increase observed in
 452 the placebo group, for subjects with AD, was statisti-

453 cally significant, it was small (only 17%) compared
 454 to the large increase (291%) observed in the active
 455 (Macushield) group for subjects with AD.

456 *Macular pigment at baseline and after six months*
 457 *of supplementation*

458 In the Repeated Measures Analysis of
 459 change in MP (at 0.23⁰ eccentricity), the
 460 within-subjects Time*Supplement inter-
 461 action effect was significant ($p < 0.001$).
 462 Neither the Time*Group interaction ($p = 0.92$) nor
 463 the Time*Supplement*Group interaction ($p = 0.39$)
 464 was significant. Thus, there was a significant increase
 465 in central MP after 6 months, for subjects on the
 466 active supplement compared with subjects on the
 467 placebo supplement, no significant difference over
 468 time between AD and controls, and no evidence that
 469 the supplement worked differently over time for AD
 470 and controls.

Table 2

Serum concentrations of lutein and zeaxanthin at baseline and following six months of supplementation with either active or placebo intervention

Group	Intervention	Measurement	Mean ± SD at baseline	Mean ± SD at six months	% Change	Sig.
Control	placebo	serum L (μmol/L)	0.319 ± 0.188	0.280 ± 0.118	-12	0.381
Control	active	serum L (μmol/L)	0.288 ± 0.177	1.05 ± 0.361	+265	$p < 0.001$
AD	placebo	serum L (μmol/L)	0.174 ± 0.057	0.203 ± 0.074	+17	0.035
AD	active	serum L (μmol/L)	0.261 ± 0.142	1.02 ± 0.655	+291	$p < 0.001$
Control	placebo	serum Z (μmol/L)	0.082 ± 0.047	0.07 ± 0.030	-15	0.321
Control	active	serum Z (μmol/L)	0.068 ± 0.036	0.126 ± 0.04	+85	0.003
AD	placebo	serum Z (μmol/L)	0.042 ± 0.024	0.062 ± 0.035	+48	0.145
AD	active	serum Z (μmol/L)	0.048 ± 0.035	0.109 ± 0.076	+127	0.02
Control	placebo	serum MZ (μmol/L)	0	0	-	-
Control	active	serum MZ (μmol/L)	0	0.082 ± 0.059	-	0.001
AD	placebo	serum MZ (μmol/L)	0	0	-	-
AD	active	serum MZ (μmol/L)	0	0.081 ± 0.089	-	0.009

Data displayed are mean ± standard deviation. % change, the calculated percentage change from baseline to six months, calculated as baseline value minus the six month value divided by baseline value, multiplied by 100 (- = negative change and + = positive change); Sig., the p value for paired-sample t testing between baseline and six months for each group split by intervention; AD, Alzheimer's disease; Active, Macushield™: 10 mg lutein, 10 mg meso-zeaxanthin, and 2 mg zeaxanthin; Placebo, sunflower oil.

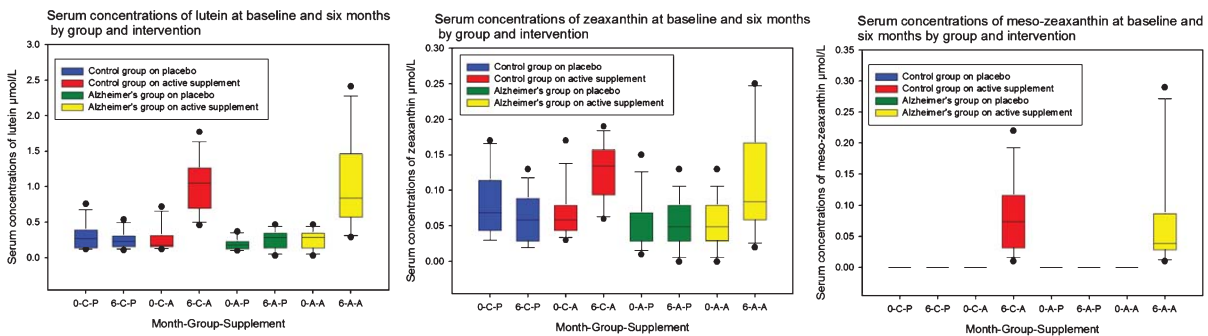


Fig. 1. Serum concentrations of lutein, zeaxanthin, and meso-zeaxanthin at baseline and six months by group and intervention. 0, baseline; 6, six months; C, control group; A, Alzheimer's group; P, placebo supplement; A, active supplement.

471 Similar results were obtained for MP volume, in
 472 that only the Time*Supplement effect is significant
 473 ($p < 0.001$). Thus, in short, we report that the supplement
 474 works to increase MP, both centrally and across

the spatial profile, and it does so for both AD and
 non-AD (control) subjects.

These findings are presented in Table 3 and Fig. 2.
 In Table 3, all subjects on the active supplement

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Table 3
 Macular pigment at baseline and following six months of supplementation with either active or placebo intervention

Group	Intervention	Measurement	Mean \pm SD at baseline	Mean \pm SD at six months	% Change	Sig.
Control	placebo	MP at 0.23°	0.58 \pm 0.18	0.54 \pm 0.18	-7	0.300
Control	active	MP at 0.23°	0.58 \pm 0.18	0.68 \pm 0.19	+17	0.002
AD	placebo	MP at 0.23°	0.40 \pm 0.17	0.38 \pm 0.18	-5	0.86
AD	active	MP at 0.23°	0.41 \pm 0.26	0.48 \pm 0.19	+17	0.009
Control	placebo	MP volume	6543 \pm 2150	6473 \pm 2131	-1	0.394
Control	active	MP volume	6593 \pm 2116	8291 \pm 2692	+26	$p < 0.001$
AD	placebo	MP volume	4008 \pm 2084	4327 \pm 1948	+7	0.304
AD	active	MP volume	3804 \pm 2255	5408 \pm 3130	+42	0.001

Data displayed are mean \pm standard deviation. % change, the calculated percentage change from baseline to six months, calculated as baseline value minus the six month value divided by baseline value, multiplied by 100 (- = negative change and + = positive change); Sig., the p value for paired-sample t testing between baseline and six months for each group split by intervention; MP at 0.23°, macular pigment at 0.23 degrees eccentricity; MP volume, MP average times the area under the curve out to 8 degrees eccentricity; AD, Alzheimer's disease; active, Macushield™: 10 mg lutein, 10 mg meso-zeaxanthin, and 2 mg zeaxanthin; placebo, sunflower oil.

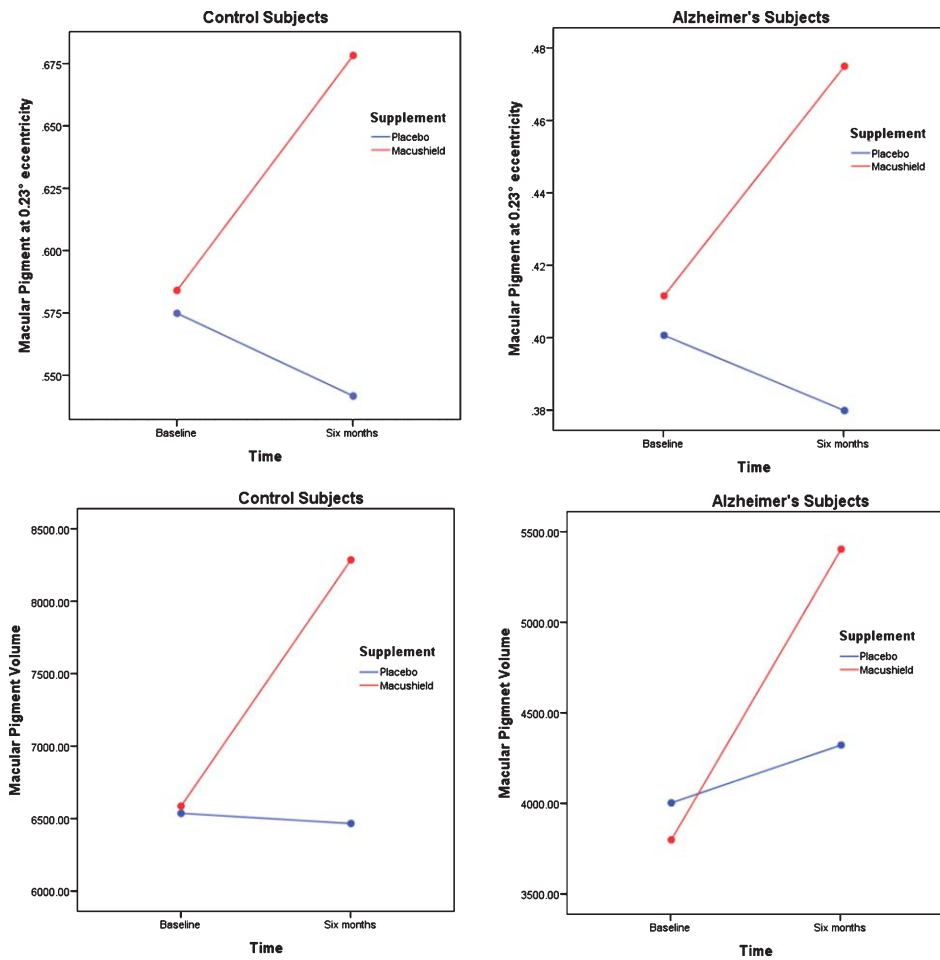


Fig. 2. Mean macular pigment at baseline and after six months of supplementation with either active supplement (Macushield) or placebo in subjects with Alzheimer's disease and control subjects.

479 (Macushield) exhibit significantly increased MP, in
480 both non-AD (control) and AD subjects, at six months.
481 The placebo subjects exhibit no significant change over
482 this time period.

483 *Visual function at baseline and after six months of* 484 *supplementation*

485 *Best corrected visual acuity*

486 Repeated Measures Analysis of change in BCVA
487 produced just one statistically significant effect, the
488 Time*Supplement interaction effect ($p = 0.005$). Fur-
489 ther examination shows that this effect arises because,
490 unexpectedly, in both AD subjects and controls, aver-
491 age BCVA increased slightly with time in the placebo
492 subjects, but declined in the Macushield subjects. Of
493 note, however, the change observed here, although sta-
494 tistically significant, does not represent a meaningful
495 change (clinically) in BCVA. Moreover, the paired
496 samples t -test produced no significant results for either
497 group, regardless of supplement ($p > 0.05$, for all).

498 *Contrast sensitivity*

499 In the Repeated Measures Analysis of change in
500 CS at 1.2 cpd, the within-subjects Time*Supplement
501 interaction effect was significant ($p < 0.039$). Nei-
502 ther the Time*Group interaction ($p = 0.23$) nor the
503 Time*Supplement*Group interaction ($p = 0.90$) was
504 significant. Thus, there was a significant increase in

505 CS at 1.2 cpd after 6 months for subjects on the
506 active supplement compared with subjects on the
507 placebo supplement, no significant difference over
508 time between AD and non-AD (control) subjects, and
509 no evidence that the supplement worked differently
510 over time for AD and non-AD (control) subjects.

511 No statistically significant findings were observed,
512 from the Repeated Measures analysis, for CS at other fre-
513 quencies. Examining the paired t -test results in Table 4
514 and Fig. 3, however, subjects on the active supple-
515 ment exhibited four significant results (from five spatial
516 frequencies tested) in the AD group, and two for the non-
517 AD group, and all indicating improvements in CS.

518 *Cognitive function at baseline and after six months* 519 *of supplementation*

520 We found no statistically significant main or interac-
521 tion effects ($p > 0.05$, for all variables analyzed) from
522 the Repeated Measures Analysis of any of the cognitive
523 function outcome variables measured (see Table 1 for
524 list of variables analyzed). Thus, supplementation with
525 Macushield, over the six months of the study, did not
526 significantly improve any of these cognitive function
527 scores, in either AD or non-AD (control) subjects.

528 DISCUSSION

529 This report (CARDS 2) presents findings from a
530 six-month macular carotenoid interventional, double-

Table 4
Contrast sensitivity at baseline and following six months of supplementation with either active or placebo intervention

Group	Intervention	Measurement	mean \pm SD at baseline	mean \pm SD at 6 months	% Change	Sig.
Control	placebo	CS at 1.2cpd	1.83 \pm 0.154	1.82 \pm 0.150	-0.01	0.84
Control	active	CS at 1.2cpd	1.76 \pm 0.254	1.88 \pm 0.249	4.9	0.006
AD	placebo	CS at 1.2cpd	1.51 \pm 0.273	1.55 \pm 0.320	2.9	0.108
AD	active	CS at 1.2cpd	1.47 \pm 0.254	1.63 \pm 0.237	11	0.04
Control	placebo	CS at 2.4cpd	1.81 \pm 0.180	1.83 \pm 0.185	1.1	0.6
Control	active	CS at 2.4cpd	1.73 \pm 0.350	1.82 \pm 0.290	5	0.038
AD	placebo	CS at 2.4cpd	1.47 \pm 0.420	1.48 \pm 0.402	0.7	0.461
AD	active	CS at 2.4cpd	1.48 \pm 0.226	1.55 \pm 0.241	4.9	0.048
Control	placebo	CS at 6.0cpd	1.37 \pm 0.240	1.46 \pm 0.205	3.3	0.275
Control	active	CS at 6.0cpd	1.57 \pm 0.196	1.59 \pm 0.161	0.3	0.687
AD	placebo	CS at 6.0cpd	1.34 \pm 0.263	1.34 \pm 0.309	0	0.785
AD	active	CS at 6.0cpd	1.17 \pm 0.255	1.29 \pm 0.303	10	0.16
Control	placebo	CS at 9.6cpd	1.16 \pm 0.286	1.16 \pm 0.350	0	0.919
Control	active	CS at 9.6cpd	1.27 \pm 0.222	1.32 \pm 0.171	4	0.38
AD	placebo	CS at 9.6cpd	1.03 \pm 0.266	1.04 \pm 0.300	0.9	0.84
AD	active	CS at 9.6cpd	0.87 \pm 0.308	1.00 \pm 0.340	16	0.011
Control	placebo	CS at 15.15cpd	0.89 \pm 0.31	0.83 \pm 0.32	-7	0.39
Control	active	CS at 15.15cpd	0.75 \pm 0.36	0.88 \pm 0.25	16	1.76
AD	placebo	CS at 15.15cpd	0.85 \pm 0.16	0.79 \pm 0.16	-7	0.471
AD	active	CS at 15.15cpd	0.68 \pm 0.24	0.85 \pm 0.24	25	0.047

Data displayed are mean \pm standard deviation. Sig., the p value for paired-sample t testing between baseline and six months for each group split by intervention; CS, contrast sensitivity; cpd, cycles per degree; AD, Alzheimer's disease; active, MacushieldTM: 10 mg lutein, 10 mg meso-zeaxanthin, and 2 mg zeaxanthin; placebo, sunflower oil.

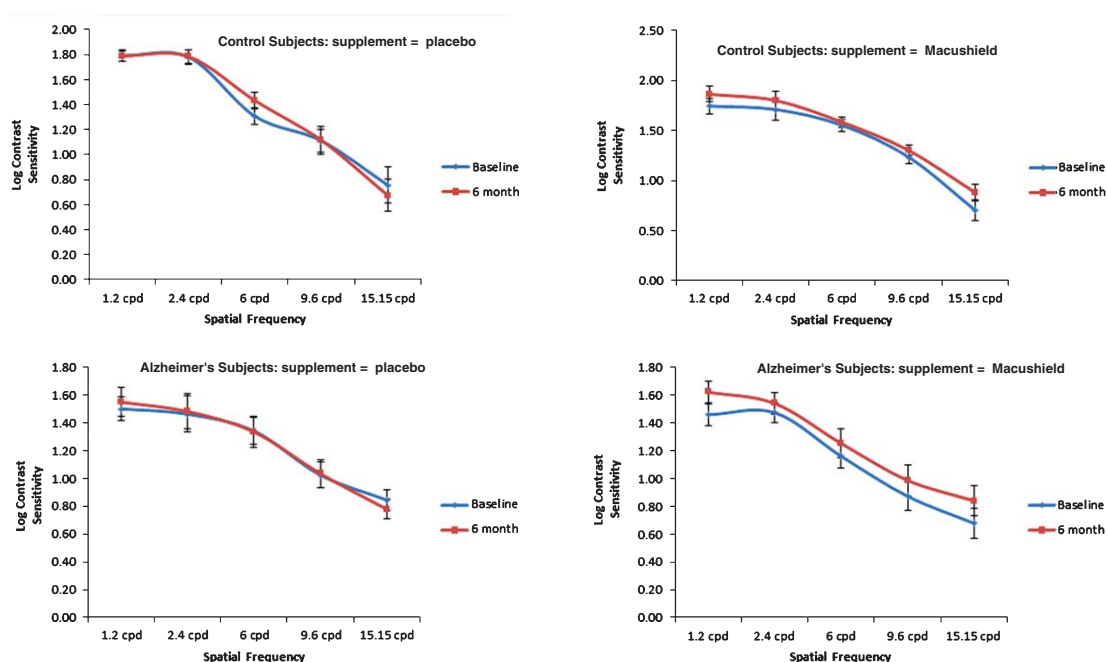


Fig. 3. Contrast sensitivity curve at baseline and after six months of supplementation with either active supplement (Macushield) or placebo in subjects with Alzheimer's disease and control subjects.

531 blind, placebo-controlled, randomized, clinical trial, in
 532 subjects with mild to moderate AD (AD subjects) com-
 533 pared with controls of similar age (control subjects).
 534 The rationale for conducting this experiment follows
 535 on from the previously reported finding that patients
 536 with moderate AD have significantly lower MP, and
 537 significantly poorer visual function, when compared to
 538 control subjects of similar age. Also, given that enrich-
 539 ment of MP has been shown to improve visual function,
 540 in both diseased [12] and non-diseased retinæ [11],
 541 it was logical to investigate whether a similar effect could
 542 be achieved in patients with AD, where baseline visual
 543 function was sub-optimal. Of note, this is the first study
 544 of its kind to attempt to answer this important research
 545 question.

546 It is known that patients with dementia and AD have
 547 poor diets lacking in fruit and vegetables [47–49] and
 548 therefore we know that, on average, patients with AD
 549 consume less carotenoids than patients free of AD.
 550 Furthermore, it has been shown that high serum con-
 551 centrations of L+Z are associated with a lower risk of
 552 AD mortality in adults [50] and that plasma antioxi-
 553 dants are depleted in mild cognitive impairment and in
 554 AD when compared to subjects with normal cognitive
 555 function [51]. Indeed, our data is consistent with the
 556 above studies, as we confirm that (at baseline) patients
 557 with AD have significantly lower (33% lower) dietary

558 intake of foods known to contain the carotenoids (L
 559 and Z) when compared to control subjects of compa-
 560 rable age. Also, we found that serum concentrations
 561 of L and Z were significantly lower in subjects with
 562 AD when compared to control subjects (21% lower
 563 for L and 31% lower for Z). These findings in diet and
 564 serum were reflected in the MP data, with AD sub-
 565 jects exhibiting significantly lower MP (28% lower
 566 on average) when compared to control subjects. Finally,
 567 our data also confirms findings from our earlier publi-
 568 cation [1], in that subjects with AD have significantly
 569 poorer vision when compared to the control subjects
 570 (e.g., for CS at 2.4 cpd, subjects with AD have lower
 571 CS [17.9%] when compared to controls).

572 The main findings from our study are that AD
 573 sufferers who were supplemented with a carotenoid
 574 formulation containing 10 mg MZ, 10 mg L, and 2 mg
 575 of Z, exhibited significant increases in serum con-
 576 centrations of MZ, L, and Z, and in MP, with consequen-
 577 tial improvements in visual function (in terms of CS);
 578 whereas, the placebo groups exhibited no significant
 579 change in any of these outcome measures. Of note, the
 580 increases observed in MP (and serum concentrations
 581 of its constituent carotenoids) were comparable
 582 between AD and non-AD (control) subjects. Indeed, at
 583 six months, subjects receiving the active intervention
 584 (10 mg MZ, 10 mg L, and 2 mg Z) were comparable

585 in terms of average circulating serum concentrations
586 of L, Z, and MZ, irrespective of whether they were in
587 the AD or non-AD (control) groups, with no signif-
588 icant difference between these groups for any of the
589 carotenoids at this point ($p > 0.05$ for all comparisons).
590 The importance of this finding rests on the logical
591 conclusion that the observed and relative lack of circ-
592 ulating serum carotenoid concentrations and MP in AD
593 [1] is not attributable to an inability of these patients to
594 respond to carotenoid intake (e.g., they are not compro-
595 mised in terms of carotenoid absorption, transport, or
596 uptake). In other words, the findings are consistent with
597 the view that the reason why patients with AD have
598 lower MP compared to control subjects is likely due to
599 an associated poor dietary intake of foods containing
600 carotenoids (fruits and vegetables).

601 With respect to the serum and MP response exhib-
602 ited in both AD and non-AD (control) groups, our data
603 is consistent with previous studies where a supple-
604 ment containing all three of the macular carotenoids
605 (10 mgMZ, 10 mgL, and 2 mgZ) was used [11, 12,
606 52, 53]. Indeed, it is noteworthy from previous studies
607 that carotenoid supplements that do not contain MZ
608 in their formulation did not augment MP significantly
609 at six months [38, 54]. It appears, therefore, that best
610 results in terms of increasing serum carotenoid concen-
611 trations (for MZ, L, and Z), and MP augmentation, is
612 achieved when all three of the macular carotenoids are
613 included in the formulation, and this observation also
614 holds true for patients with AD. Further, supplementa-
615 tion with macular carotenoids, and consequential MP
616 augmentation, is associated with risk reduction for
617 AMD, a particularly important benefit as intervention
618 with current treatment modalities (i.e., monthly injec-
619 tions, under local anesthesia, into the eye) would be
620 problematic in this patient group.

621 We believe that it is important to draw attention to
622 our findings pertaining to visual function. Firstly, we
623 confirm that CS is significantly lower in AD subjects
624 when compared to non-AD controls. Addressing this
625 sensory defect in vulnerable AD patients should be
626 a priority for those involved in the care of patients
627 with this form of cognitive impairment, and routine
628 and frequent assessment of visual function should be
629 incorporated into the delivery of that care. For example,
630 improvements seen among AD subjects supplemented
631 with MZ, L, and Z were clinically meaningful at
632 spatial frequencies of 1.2 cpd and 15.15 cpd, equat-
633 ing to approximately one line of improvement on
634 standard Pelli-Robson chart, and likely to enhance
635 visual appreciation of small and large targets by these
636 subjects. We suggest that further studies may con-

637 sider other measures of visual function (e.g., glare
638 disability and photostress recovery), however, the fea-
639 sibility of including these measures will need to be
640 considered given the time required to perform the
641 tests and the ability of the subject to perform each
642 test.

643 Of note, no improvements in cognitive function were
644 demonstrated as a result of supplementation in either
645 AD or non-AD control subjects, a finding that is neither
646 surprising nor counter-intuitive. The rationale whereby
647 antioxidants are important for cognition rests on their
648 ability to prevent or attenuate oxidative damage, as
649 opposed to tissue repair. In other words, there is a
650 biologically plausible rationale, supported by emerg-
651 ing evidence, that antioxidant intake is protective for
652 cognition, but the notion that established cognitive
653 impairment could be reversed by supplementation with
654 antioxidants is less probable, especially in the context
655 of a short period of intervention (as reported herein).
656 Therefore, to investigate properly if supplementation
657 with the carotenoids L, Z, and MZ impact positively
658 on cognitive health/function, we suggest that subjects
659 with very early signs of cognitive decline, and subjects
660 of comparable age with no signs of cognitive decline
661 are selected, and are followed for at least 3 years. The
662 current study confirms that AD patients respond to
663 carotenoid supplements in the same way as normal
664 controls, and therefore it is possible that supplemen-
665 tation with these nutrients, if achieved early enough,
666 may support and protect cognitive health.

667 In conclusion, our data suggests that supplementa-
668 tion with the macular carotenoids (MZ, Z, and L) may
669 benefit patients with AD, in terms of clinically mean-
670 ingful improvements in visual function and in terms
671 of MP augmentation (and consequential risk reduction
672 for AMD). The impact of sustained supplementation
673 on cognition and visual function in AD subjects, and
674 on risk for AD, both warrant further study.

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