1 The effect of nutritional supplementation on the multi-focal electroretinogram in healthy eyes

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**Abstract**

Background

Previous studies have demonstrated an increase in macular pigment optical density (MPOD) with lutein (L)-based supplementation in healthy eyes. However not all studies have assessed whether this increase in MPOD is associated with changes to other measures of retinal function such as the multifocal ERG (mfERG). Some studies also fail to report dietary levels of L and zeaxanthin (Z). Because of the associations between increased levels of L and Z, and reduced risk for AMD, this study was designed to assess the effects of L-based supplementation on mfERG amplitudes and latencies in healthy eyes.

Methods

Multifocal ERG amplitudes, visual acuity (VA), contrast sensitivity (CS), MPOD and dietary levels of L and Z were assessed in this longitudinal, randomised clinical trial. Fifty two healthy eyes from 52 participants were randomly allocated to receive a L-based supplement (treated group), or no supplement (non-treated group).

Results

There were 25 subjects aged 18-77 (mean age ± SD; 48 ± 17) in the treated group and 27 subjects aged 21-69 (mean age ± SD; 43 ± 16) in the non-treated group. All participants attended for three visits. Visit one at baseline, visit two at 20 weeks and visit three at 40 weeks. A statistically significant increase in MPOD ($F$ 17.0, $p = <0.001$) and shortening of mfERG ring 2 P1 latency ($F$ 3.69, $p = 0.04$) was seen in the treated group

Conclusions

Although the results were not clinically significant, the reported trend for improvement in MPOD and mfERG outcomes warrants further investigation.

**Keywords**: lutein, macular pigment optical density, multifocal electroretinogram, randomised trial

## Introduction

Oxidative stress is implicated in the development of age-related macular degeneration (AMD), because of the high percentage of polyunsaturated fatty acids in the retina, which are readily oxidised [1], because of: retinal exposure to blue wavelengths of light [2], the retina is highly metabolically active [3] and phagocytosis within the retinal pigment epithelium is itself a free-radical producing process. As a result, interest has been raised in the impact of antioxidant supplementation, or dietary modification, on the onset and progression of AMD. The carotenoids lutein (L) and zeaxanthin (Z) are lipid soluble antioxidants found within the retina and together make up the macular pigment (MP). They are thought to reduce oxidative damage by filtering short wavelength blue light within the macula [4] and by quenching light-induced singlet oxygen and related free radicals [5]. Putative associations between increased levels of these carotenoids and reduced risk for development of AMD have been suggested [6-8]. Increased levels of MP optical density (MPOD) have been reported in healthy eyes and eyes with AMD when supplemented with L and Z [9-17]. Macular pigment reduces with eccentricity in the macular region [18], although is present at much lower levels in other parts of the retina and other ocular tissues [19]

Improvements in visual function in eyes with early AMD and non-exudative AMD have been reported in several studies involving carotenoids [20-23]. The Age Related Eye Disease Study 2 (AREDS2) investigators reported that substituting L and Z for beta-carotene (used in the AREDS 1 formulation) in a subset of participants resulted in the risk of progression of AMD being reduced by a further 18% over the 25 % reduction in risk already reported with the AREDS 1 formulation [24].

Visual acuity (VA) is commonly used as a measure of visual function in clinical trials, but it is not an ideal outcome measure as it is subjective, assesses a relatively small retinal area, and doesn’t offer an overview of macular function. A combination of objective and subjective testing is likely to provide a more robust assessment of visual and retinal function when diagnosing AMD and assessing treatment outcomes. The multifocal electroretinogram (mfERG) can be used to objectively evaluate specific retinal areas [25]. It simultaneously assesses electrical potentials from different retinal areas [26], is valuable in the identification of focal retinal pathology and in monitoring the impact of potential treatments [27] and can be used to evaluate bipolar cell function, with some contributions from photoreceptors and retinal ganglion cells [28].

The mfERG principally measures cone photoreceptor and bipolar cell function. Tubulin is found in the receptor axon layer of the cone-abundant fovea where it may selectively binds L and Z [29], leading to the MPOD increases seen on supplementation. The rationale was that an increase of L and Z binding to tubulin around the cone photoreceptor axons may have affected cone function which could be objectively assessed by the mfERG. The aim of this study was to assess the effect of a lutein (L)-based nutritional supplement on mfERG latency and amplitude in healthy eyes. Secondary outcome measures were VA, contrast sensitivity (CS) and MPOD.

## Materials and methods

The research was approved by Aston University Human Sciences Ethical Committee. The tenets of the declaration of Helsinki [30] and the consolidated standards of reporting trials (CONSORT) checklist [31] were followed. The study was registered with an International Standard Randomised Controlled Trial Number (ISRCTN 17842302) and all participants provided informed written consent.

The protocol used in this study has previously been reported by our research group in an investigation of the impact of L and Z supplementation on mfERG in eyes affected by age-related maculopathy [32]. We have included a description of the outcome measures here for completeness.

To assess the effects of an L-based supplement in healthy eyes, 52 healthy eyes from 52 participants were randomly allocated, using the Microsoft Excel random number generator, to receive a L-based supplement (treated group) or no supplement (non-treated group) at visit 1. All participants attended for three visits. Visit one at baseline, visit two at 20 weeks and visit three at 40 weeks. All the data was collected by one of the authors (EJB) who was masked as to which participants had been allocated to the treated or non-treated group. Participants reported to another of the authors (HEB) for their randomization code and allocation of supplement tablets where appropriate. Participants were asked to contact HEB if they had any queries about supplementation. It was explained to each participant that they should not disclose whether they were in the treated or non-treated group to EJB.

The supplement was Ocuvite Duo, provided by Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK. The supplement had two elements. A tablet and a capsule packed in blister packs. The tablets contained all of the supplement nutrients (see table 1) except for omega 3 which was contained in the capsules. The dosage was one tablet and one capsule taken orally twice per day. All the nutrients were within the safe upper levels as defined by the UK Food Standards Agency [33].

Insert table 1 about here.

*Sample size*

Two previous studies assessing the effects of nutritional supplementation on retinal function using focal electroretinograms (fERG) [34] and mfERG [22] demonstrated significant results between groups treated with a nutritional supplement compared with non-treated groups. The fERG study suggested a sample size of 8 healthy eyes for their healthy older group and 30 eyes for the age-related maculopathy group [34], which gave a power of 90% at $\alpha = 0.05$, for detecting a between group difference of 25 – 30% in amplitude or phase. The mfERG study by the CARMIS investigators, had 27 ARM eyes, 15 in the supplemented group and 12 in the non-supplemented group [22] and this gave a power of 90% at $\alpha = 0.05$, for detecting a between-group difference of ≥ 55% in mfERG amplitude. A 55% difference in mfERG amplitude was used as the effect size in sample size calculations for this study [22] (table 2).

Insert table 2 about here

Because there is paucity in the literature about mfERG latency changes with nutritional supplementation, effect sizes for mfERG latency were based on a study of vitamin A supplementation by Dolan et al., who noted a change in central and peripheral P1 latency of 6 ms with vitamin A supplementation in a single participant case study [35]. For mfERG latency the sample sizes used in this study gave a power of 80% at the 5% significance level.

*Inclusion criteria*

Suitability for inclusion was evaluated by questionnaire, fundus photographs and VA. For inclusion participants had to provide written informed consent and were required to have best corrected VA of at least 0.2 LogMAR (for good mfERG central fixation), clear optical media, no signs of retinal or optic nerve disease, good general health and not taking medication that affects the retina.

*Exclusion criteria*

Moderate to dense lens opacities, intraocular lens implants, corneal opacities, glaucoma or ocular hypertension, history of intraocular inflammation, retinal detachment or retinal disease, retinal laser, diabetes, alcoholism, systemic hypertension, epilepsy, ocular surgery (excluding LASIK/EK), ocular trauma or neurological disease in the studied eye. Participants with a history of taking medications that cause retinal toxicity (e.g. topiramate, metronidazole, quinolones, thioridazine, deferoxamine, cisplatin, oxazepam, vigabatrin, tamoxifen, digoxin) were also excluded.

Outcome measures

*Multifocal Electroretinography*

The VERIS science 6.1 (Electrodiagnostic imaging, San Mateo, California, USA) was used to record the mfERG. The multifocal stimulus, consisting of 61 scaled hexagons was displayed on a high-resolution, black-and-white cathode ray tube monitor 30cm wide and 30 cm high with a frame rate of 75 Hz. The hexagon stimulus radius subtended approximately 20° of visual field. Each hexagon was independently alternated between white (200 cd/m²) and black (1cd/m²) according to a pseudorandom binary m-sequence [36]. Total recording time was four minutes. Recording time was divided into eight, 30 second segments allowing for participant rests. Fixation target perception was confirmed before testing commenced. The fixation target size was 1.5% of the area of the central hexagon. Each subject's vision was optimally corrected with the VERIS system's refractor/camera system. To allow equal magnification of the stimulus array on the retina, the distance between the participant's eye and refractor/camera was adjusted by obtaining a sharp image on the observation monitor as per the

manufacturer's recommendation. The participant's eye was monitored throughout testing using this system. Pupils were dilated with tropicamide 1% (Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK). Gold cup electrodes filled with signa gel (Parker Laboratories, Fairfield, New Jersey, USA) were applied to the forehead (ground electrode) and approximately 1 cm posterior to the temporal canthus (reference electrode) of the tested eye after these areas were abraded and cleaned using Nuprep (Weaver and Company, Aurora, USA). A Dawson Trick Litzkow (DTL) fibre electrode was used as the active electrode and was placed along the sclera adjacent to the lower eyelid. The participant was asked to blink to ensure that the electrode found the same natural position for each visit. These electrodes (Diagnosys UK ltd) were used for the trial for participant comfort [37] and test-retest reliability [38] while not obscuring vision. Proxymetacaine hydrochloride 0.5% (Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK) was instilled to minimise blinking throughout the recording. Any recordings contaminated with artefact were discarded and repeated. The untested eye was obscured throughout the procedure. In order to remove signal artefacts and improve the signal to noise ratio but without attenuating mfERG waveforms, one iteration of artefact removal was performed for each mfERG recording. This removed small eye movement artefacts. No spatial averaging was performed because this would reduce spatial resolution as each retinal area stimulated by a hexagon is averaged with 1/6$^{th}$ of its neighbouring hexagons. Thus the waveform of each single retinal area stimulated by a single hexagon would lose some of its own identity as it was averaged with surrounding waveforms. The mfERG measures were N1, P1 and N2 latency and N1 P1 amplitude (figure 1) and these were assessed for five rings of retinal eccentricity (figure 2).

Insert figures 1 & 2 about here.

We used a 61 hexagon stimulus as recommended by ISCEV mfERG guidelines to balance the necessity for participant comfort while providing adequate assessment of macular function [39]. This also replicates the CARMIS study stimulus type [22].

*Contrast sensitivity*

The Pelli-Robson CS test (Clement Clarke International Ltd, Harlow, Essex, UK) was used at 1 m as per the manufacturer guidelines, with distance refractive correction when required. For consistency CS was undertaken in the same room for each visit with a background illumination of 142 lux throughout the trial.

*Macular pigment optical density*

In order to obtain values for retinal accumulation of L and Z., MPOD was obtained using the Macular Pigment Screener 9000 (MPS) which uses the principle of heterochromatic flicker photometry (HFP).

Participants were required to make flicker matches between two wavelengths of light, a blue light (~465nm), and green light (~530nm). Flicker matches were initially obtained centrally (1°). Flicker rate was gradually reduced from above the critical fusion frequency (60 Hz) by 6 Hz until the participant observed the flicker and pressed a response button accordingly. This procedure continued for a series of pre-set blue-green ratios. Once flicker was detected the luminance of the blue and green light was changed by 0.2dB increasing the blue light and decreasing the green light while the overall mean luminance was kept constant. Then the temporal frequency was reset to 60 Hz and the frequency reduced by 6 Hz again. The sequence continued for a series of blue-green ratios until a V-shaped curve was obtained. The minimum value of this curve was where the blue and green lights were of equal luminance. This whole process was repeated peripherally (8°) and again a V-shaped curve was obtained, providing a minimum value where blue a green lights were equiluminant. Because MP selectively absorbs blue light and is found centrally but not peripherally, the central minimum value differed from the peripheral minimum value. Macular Pigment Optical Density was determined by dividing central minimum blue light intensity by peripheral minimum blue light intensity and log10 of this value using MPS computer software. Greater detail of this technique is described by Van Der Veen *et al.* [40]. The background and target luminance was set to 250 cd/m². Participants wore distance glasses for the test if required, and were instructed to fixate on the central target for obtaining central values. For peripheral testing participants were asked to blink frequently and adopt a more relaxed fixation at 8° around a 1.75° red fixation target to reduce Troxler's effect.

*Visual acuity*

LogMAR distance VA testing using a 3 m ETDRS 750 lux retro-illuminated chart was undertaken for each participant (Sussex vision, Rustington, West Sussex, UK). The eye with the best corrected VA was determined at the participant's first visit and this eye was assessed for the subsequent visits.

*Intraocular pressure*

Non-contact intraocular pressure readings (Topcon CT-80 non-contact tonometer, Topcon, Newbury, Berkshire, UK) were taken prior to instillation of tropicamide 1%.

*Fundus photography*

A central 45° fundal photograph was taken with the Topcon TRC-NW8, (Topcon, Newbury, Berkshire, UK) at each visit to determine any change in fundus or media opacity. Participants were instructed to fixate on a central fixation target for each visit to ensure identical fundus positioning. Any changes in fundus or media opacity would have resulted in exclusion from the study.

*Food Diaries*

In order to assess whether any changes in outcome measures were due to the L-based nutritional supplement rather than changes in dietary intake of nutrients, each participant was provided with a

food diary. The diary was filled in over two week days and one weekend day. Participants were given food diaries at visits one and three. The data from the food diaries was analysed using the Weighted Intake Software Package (WISP, Tinuviel, Llanfechell, Anglesey, UK). Lutein values for foods were taken from the United States Department of Agriculture (USDA) nutrient databank, release 23 (http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/SR23/nutrlist/sr23a338.pdf).

## Results

There were 25 participants (mean age ± SD; 48.68 ± 17.35, range 18-77 years) in the treated group and 27 (mean age ± SD; 43.93 ± 16.15, range 21-69 years) in the non-treated group. All participants in the treated group were Caucasian, consisting of 15 females and 10 males. In the non-treated group there were 20 females and 7 males, 5 South Asians and 22 White. A chi-squared test for independence demonstrated a significant difference between treated and non-treated groups for ethnicity ($X^2$ = 5.122, p = 0.024) but not for gender ($X^2$ = 1.169, p = 0.280). There was no significant difference between treated and non-treated groups for VA, CS or MPOD.

Due to technical difficulties mfERG parameters were not measured for all participants for each visit. Only 25 participants (15 in the treated group and 10 in the non-treated group) underwent mfERG for all three visits. However the VA, CS and MPOD were undertaken on all 52 participants. A summary of differences in baseline characteristics are detailed in table 3 and were analysed using independent-samples t-tests. Of the 25 participants in the treated group 14 participants returned their baseline dietary questionnaire (56.0%). Of the 27 participants in the non-treated group 17 returned their baseline dietary questionnaire (63.0%)

Insert table 3 about here.

Each data set was checked for normality using the Shapiro-Wilk statistic which assesses the normality of distribution of the data. A non-significant result indicates normality and therefore ANOVA was used for analysis with Tukey's post-hoc range test. When parametric assumptions were not met according to Shapiro-Wilk tests for normality Kruskal-Wallis one-way analysis of variance for independent groups was performed with Mann-Whitney U tests demonstrating post-hoc differences between groups.

In addition, a mixed between-within subjects ANOVA using SPSS 16.0 software (IBM, North Harbour, Portsmouth, UK) was conducted to explore the effects of nutritional supplementation compared with no treatment on retinal function over three time periods using mfERG amplitudes and latencies for different areas of retinal eccentricity. This provided analysis of the between-subjects variable (treated and non-treated group), and within-subject variable (time) on the outcome measures (dependent variable). This test is considered to be tolerant of data distributions that vary from normal [41]. Post-

hoc testing was used in lieu of a Bonferroni correction. The significant ANOVA results for mfERG are displayed in table 4.

Insert table 4 about here.

For all mfERG outcome measures there was no significant interaction between treated and non-treated groups over three time periods or for group (treated versus non-treated) for any mfERG parameters. There was no significant effect for time for any mfERG parameter except for ring 2 P1 and ring 2 N2 latency (see table 5a). Ring 2 N2 latency became longer over the three visits for both groups. Ring 2 P1 latency became statistically significantly shorter over three visits in the treated group (see table 5b), although this change was not clinically significant based coefficient of repeatability (CR) values obtained within our laboratory.

Insert table 5a about here

Insert table 5b about here.

All participants (25 in the treated group and 27 in the non-treated groups) undertook VA and CS measurements at all three visits. One participant in the treated group was unable to perform the MPOD at one of their visits (therefore n=24 for the treated group and n=27 in the non-treated group for this test). There was no statistically significant difference between treated and non-treated eyes for VA or CS. There was a significant interaction effect between time and group for MPOD, with MPOD increasing over the three visits by 0.1 in the treated group compared with a 0.03 reduction in MPOD in the non-treated group between visits one and three ($F = 17.00$, $p < 0.001$).

Insert table 6 about here.

Insert figure 3 about here.

Of the 14 people in the treated group who completed the baseline dietary questionnaire, eight completed a further questionnaire at visit three. Of the 17 people in the non-treated group who completed the baseline dietary questionnaire, 10 completed a further questionnaire at visit three. A paired-samples t-test using SPSS 16.0 demonstrated no significant difference for any of the dietary components between visits one and three in the treated group and the non-treated group ($p > 0.05$).

1 Compliance was assessed by asking participants to return any boxes of the supplement that were not
2 taken and remaining tablets were counted. Those who forgot to bring back the tablets were asked to
3 contact the principle investigator after counting tablets at home. Patient compliance was elicited using
4 supportive language to minimise the number of participants concealing supplement non-adherence
5 [42], and reporting lower levels of remaining tablets than was actually the case. The sole reason for
6 non-adherence was forgetfulness. A one way ANOVA using SPSS 16.0 found no statistically significant
7 difference between groups for supplement compliance during the study (see table 7) ($F = 0.40$, $p =$
8 $0.68$).

9

10 Insert table 7 about here

11

12 **Discussion**

13 This randomised controlled trial was designed to assess the effect of a nutritional supplement containing
14 12 mg L, 0.6 mg Z, 150 mg vitamin C, 15 mg of vitamin E, 400 µg copper, 20 mg zinc and 1080 mg
15 omega-3 fatty acids on objective and subjective clinical measures of visual and retinal function in
16 healthy eyes.

17 There was a statistically significant increase in MPOD and shortening of ring 2 P1 latency, with
18 supplementation. It is important to also consider the clinical significance of these findings. Clinical
19 significance is related to the reliability of the instrumentation being used. The reliability is quantified
20 using the coefficient of repeatability and was calculated for each outcome measure within our
21 laboratory. If the change in outcome recorded is less that the coefficient of repeatability, then it is not
22 possible to conclude that the change is not simply due to measurement noise. Although neither of these
23 statistically significant changes can be considered clinically significant based on coefficient of
24 repeatability data from our laboratory, the trends demonstrated may warrant further investigation.

25 Many studies have demonstrated an increase in MPOD with L-based supplementation in healthy eyes
26 [9-11,43,12-17]. However not all studies have assessed whether this increased retinal accumulation of
27 MP is associated with changes to other measures of visual and retinal function such as CS, VA or
28 mfERG [11,10,43,13-15,17]. Some studies also failed to report dietary levels of L and Z during
29 supplementation [10,11,13-16].

30 There have been conflicting results within the literature with regards to the effects of nutritional
31 supplementation on visual function in healthy eyes. A study by Sasamoto *et al.* assessing the effects of
32 a nutritional supplement on MPOD and visual function showed that MPOD did not significantly increase
33 over 12 months, although improvements in CS were seen [44]. It is difficult to compare this with the
34 current study as different methods of assessing MPOD (autofluorescence spectrometry) and CS (area
35 under the log contrast sensitivity function) were used, and supplement formulation (6mg L), ethnicity

1 (Japanese) and study design were not the same. Also some of the participants in Sasamoto *et al's*
2 study consisted of healthy fellow eyes of those with AMD or central serous chorioretinopathy which may
3 have been subject to subtle retinal changes not clinically visible.

4 The Collaborative Optical Macular Pigment Assessment Study (COMPASS) investigators concluded
5 that supplementing with 12 mg of L, 1 mg of Z and antioxidants in healthy eyes significantly increased
6 MPOD over 12 months, but this did not correspond with an improvement in VA or photopic CS [45].
7 This is in agreement with our study but a different method of CS assessment was used (contrast
8 sensitivity function), the sample size was larger at 121 subjects and the supplement formulation differed.

9 A 12 week study of 37 healthy eyes found no statistically significant improvement in VA or central CS
10 when supplementing with 6 or 12 mg of L [46]. Improvements were noted at wider fields of CS analysis.
11 This study differed from the current one in supplement formulation, ethnicity (Chinese) and methods of
12 CS (automated contrast glare tester) assessment.

13 A study by Bartlett and Eperjesi concluded that supplementing with 6 mg of L combined with vitamins
14 and minerals did not improve CS or VA over 9 or 18 months [47]. The same methods of VA and CS
15 assessment were used, and a similar study design as the current study although supplement
16 formulation differed.

17 To the authors' knowledge, the literature provides no information with regards to the effects of nutritional
18 supplementation on mfERG measures in healthy eyes. Ring 2 P1 latency of the mfERG became
19 statistically significantly shorter over time in those taking the supplement. Although there was a
20 difference between treated and non-treated groups for ethnicity for the current study there is no reason
21 to believe that the results would have been different if the treated group had contained similar numbers
22 of Asians as the non-treated group.

23 The CARMIS investigators found that a 10 mg L-based supplement over 12 months increased N1P1
24 amplitudes in rings 1 and 2 of the mfERG in 15 eyes with ARM or non-central geographic atrophy [22].
25 We did not find any mfERG amplitude increase in our healthy supplemented eyes, although the
26 supplement composition was not the same as the CARMIS supplement formula. Unlike the current
27 study, the CARMIS investigators did not report dietary levels of L and Z throughout the study period,
28 thus it is difficult to determine if the mfERG changes seen were due supplementary or dietary changes
29 in L and Z. Retinal accumulation of L and Z (MPOD) was not measured in the CARMIS study, thus it is
30 impossible to ascertain if increased retinal levels of L and Z were related to the increased central mfERG
31 amplitudes reported in their study.

32 The study formulation also contained omega-3 essential fatty acids, which may also be implicated in
33 the statistically significant change in Ring 2 P1 latency. Observational studies support the hypothesis
34 that omega 3 poly-unsaturated fatty acids (PUFA) are protective in the eye. A 2008 meta-analysis of
35 epidemiological studies reported that a high intake of omega-3 fatty acids and fish intake at least twice
36 a week may reduce the primary risk of early and late AMD by up to 38%. [48]. In a 5-year follow-up
37 study of the Blue Mountains Eye Study cohort, fish consumption at least once a week was associated

with a 40% reduction in incident early age-related macular degeneration, and more frequent consumption of fish (3 times a week) was found to be protective against late ARM. [49]. Other studies have found similar findings. [24], [25] [26], [16]. Some prospective studies also reveal a decreased likeliness of AMD progression in those with early AMD. [7, 17, 18].

*Study limitations*

The age range of participants (18-77 years) may have implications for the interpretation of results, as it has been demonstrated that mfERG parameters are correlated with age [50,51]. In addition, the use of ring averages to estimate effects may have resulted in underestimation of the true magnitude of effect due to age effect variability between individual rings [52].

Food diaries were prospective and were completed over several days in order to provide detailed dietary information. Return rates were low. A recall food diary may have provided a greater number of returned questionnaires as participants could have completed these during their visits. However, this may not have been as accurate due to participants having to remember their food intake over a three day period. Serum analysis would have provided a more accurate overview of compliance and dietary habits. It would have been useful to record body mass index (BMI) for all participants, as a BMI greater than 27 has been associated with reduced retinal uptake of Z and so may have confounded the results [53]. Use of a placebo for the non-treated group would have strengthened the study, but the cost of this was prohibitive.

Using a combined-nutrient formulation does not allow for any evaluation of the impact of individual nutrients. It is common, however, to find nutritional supplements that contain a combination of several nutrients; this is because of the synergistic relationships between them. Examples include copper and zinc combinations required for copper-zinc superoxide dismutase, a part of the antioxidant system within the retinal pigment epithelium and retina [54], and increased bioavailability of L with the addition of certain fats, including olive and peanut oils [55,56]. Use of multi-nutrient formulations in AMD is supported by the results of the AREDS, in which zinc and antioxidants reduced the relative risk of developing advanced AMD by 21% and 17% respectively. When zinc and antioxidants were combined, the risk reduced further to 25% [57].

## Conclusion

The current study adds to literature in several ways. It expands on the CARMIS study by assessing the effects of an L-based supplement in healthy eyes. The pertinence of this lies in any potential protective role the supplement may have in delaying onset of AMD in healthy eyes. The assessment of dietary intake over the study period is important in such studies so as to be able to attribute any changes in study outcome measures to supplementation rather than diet. Subjective measures of visual function were undertaken alongside objective measures of retinal function in order to assess whether

improvements in retinal function may have been associated with improved subjective measures of visual function.

The statistically significant accumulation of L and Z within the retina, and improved retinal function in healthy eyes with L-based supplementation are encouraging. It may be that further beneficial effects of accumulated MP on retinal and visual function may be witnessed over a longer term and/or with higher supplement dosage.

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**Conflict of Interest**: All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

**Ethical approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent:** Informed consent was obtained from all individual participants included in the study.

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1

2

## Tables

| Ingredient | Safe upper levels per day [33] | Dosage |
|---|---|---|
| Vitamin C | 1000 mg (guidance only) | 150 mg |
| Copper | 10 mg | 400 µg |
| Vitamin E | 540 mg | 15 mg |
| Zinc | 25 mg | 20mg |
| Lutein | none established | 12 mg |
| Zeaxanthin | none established | 0.6 mg |
| Omega-3 | none established | 1080 mg |

Table 1: supplement composition. These amounts were provided by taking two tablets and two capsules per day.

1

| | VA (logMAR) | CS (log units) | MPOD | Central mfERG N1P1 amplitude (nV/deg$^2$) | Central mfERG P1 latency (ms) |
|---|---|---|---|---|---|
| Mean | -0.11 | 1.89 | 0.39 | 173.17 | 29.09 |
| Standard deviation (SD) | 0.11 | 0.12 | 0.16 | 50.12 | 1.43 |
| Effect size (E) | 0.10^^ | 0.30^ | 0.33* | 95.24** | 6.00*** |
| E/S | 0.91 | 2.50 | 2.06 | 1.90 | 4.20 |
| $(E/SD)^2$ | 0.83 | 6.25 | 4.25 | 3.61 | 17.60 |
| Sample size = $16/(E/SD)^2$ (two sided) | 19 | 3 | 4 | 4 | 1 |

2 Table 2: Group sizes required to have 80% power at the 5% significance level for VA, CS, mfERG
3 amplitude and MPOD for healthy eyes. The mean and standard deviation (SD) data were calculated
4 from 52 healthy eyes at visit 1.

5 ^^ Based on VA repeatability studies [58-62].

6 ^ Based on Elliot *et al's* paper [63].

7 * Repeatability value from Bartlett et al's of HFP repeatability paper [64]

8 ** Based on Parisi *et al's* paper of a 55% change in mfERG amplitude [22]

9 *** Based on Dolan *et al's* paper [35]

10

1

| Variable | Treated group (n=25) | | Non-treated group (n=27) | | | |
|---|---|---|---|---|---|---|
| | mean | ±SD | mean | ±SD | t | p |
| Age (years) | 48.68 | 17.35 | 43.93 | 16.15 | 1.02 | 0.31 |
| Smoking (pack-years)* | 2.02 | 3.32 | 2.73 | 6.08 | -0.52 | 0.61 |
| Spherical equivalent (D) | 0.64 | 2.21 | 0.22 | 2.24 | 0.68 | 0.50 |
| Axial length (mm) | 23.31 | 1.12 | 23.68 | 1.07 | -1.23 | 0.23 |
| Baseline dietary questionnaires | Treated group (n=14) | | Non-treated group (n=17) | | | |
| | mean | ±SD | mean | ±SD | t | p |
| Dietary copper (mg) | 1.37 | 0.87 | 1.00 | 0.38 | 1.60 | 0.12 |
| Dietary zinc (mg) | 7.40 | 2.08 | 6.84 | 1.91 | 0.78 | 0.44 |
| Dietary retinol (µg) | 705.57 | 1584.91 | 207.12 | 122.74 | 1.17 | 0.26 |
| Dietary carotene (µg) | 1789.71 | 1216.07 | 2557.59 | 2272.45 | -1.20 | 0.24 |
| Dietary Vitamin E (mg) | 3.67 | 1.25 | 5.09 | 2.26 | -1.45 | 0.17 |
| Dietary Vitamin C (mg) | 85.43 | 64.73 | 102.59 | 60.16 | -0.76 | 0.45 |
| Dietary lutein and zeaxanthin (µg) | 1295.33 | 924.07 | 2016.29 | 2044.30 | -1.30 | 0.21 |
| Dietary Omega 3 (g) | 0.17 | 0.12 | 0.15 | 0.15 | 0.30 | 0.76 |

2 Table 3: A summary of baseline characteristics for treated and non-treated healthy eyes using
3 independent-samples t-tests.

4

| Outcome measure | Main effect: time | | Main effect: group (treated/non-treated) | | Interaction effect | |
|---|---|---|---|---|---|---|
| | F | p | F | p | F | p |
| Ring 2 P1 latency | 5.067 | 0.015 | 0.188 | 0.668 | 3.694 | 0.041 |
| Ring 2 N2 latency | 3.622 | 0.044 | 0.191 | 0.666 | 0.095 | 0.910 |

1 Table 4: Significant results (shaded) from a mixed between-within ANOVA for mfERG N1P1
2 amplitude, N1 latency, P1 latency and N2 latency over three visits for five areas of retinal eccentricity
3 between treated and non-treated healthy eyes. The shaded areas indicate statistical significance.

4

1

| Outcome measure | Main effect: time | | Main effect: group (treated/non-treated) | | Interaction effect | |
|---|---|---|---|---|---|---|
| | F | p | F | p | F | p |
| Ring 1 N1-P1 amplitude | 0.311 | 0.736 | 0.617 | 0.440 | 0.067 | 0.935 |
| Ring 2 N1-P1 amplitude | 1.642 | 0.216 | 0.058 | 0.812 | 0.034 | 0.966 |
| Ring 3 N1-P1 amplitude | 1.659 | 0.213 | 0.112 | 0.741 | 0.063 | 0.939 |
| Ring 4 N1-P1 amplitude | 1.408 | 0.266 | 0.212 | 0.650 | 0.068 | 0.934 |
| Ring 5 N1-P1 amplitude | 0.007 | 0.993 | 1.894 | 0.182 | 0.193 | 0.826 |
| Ring 1 N1 latency | 1.203 | 0.319 | 1.072 | 0.311 | 0.957 | 0.400 |
| Ring 2 N1 latency | 0.579 | 0.569 | 1.868 | 0.185 | 0.253 | 0.778 |
| Ring 3 N1latency | 0.196 | 0.823 | 0.280 | 0.602 | 0.513 | 0.606 |
| Ring 4 N1 latency | 0.695 | 0.510 | <0.001 | 0.998 | 0.101 | 0.905 |
| Ring 5 N1 latency | 0.481 | 0.624 | 0.057 | 0.813 | 0.543 | 0.588 |
| Ring 1 P1 latency | 3.172 | 0.062 | 1.973 | 0.174 | 1.294 | 0.294 |
| Ring 2 P1 latency | 5.067 | 0.015 | 0.188 | 0.668 | 3.694 | 0.041 |
| Ring 3 P1 latency | 1.401 | 0.268 | 0.365 | 0.552 | 0.118 | 0.889 |
| Ring 4 P1 latency | 1.046 | 0.368 | 0.011 | 0.917 | 0.081 | 0.923 |
| Ring 5 P1 latency | 1.495 | 0.246 | 1.430 | 0.244 | 1.319 | 0.288 |
| Ring 1 N2 latency | 2.761 | 0.085 | 0.216 | 0.646 | 0.869 | 0.433 |
| Ring 2 N2 latency | 3.622 | 0.044 | 0.191 | 0.666 | 0.095 | 0.910 |
| Ring 3 N2 latency | 3.053 | 0.068 | <0.001 | 0.999 | 1.042 | 0.369 |
| Ring 4 N2 latency | 1.700 | 0.206 | 1.517 | 0.230 | 0.607 | 0.554 |
| Ring 5 N2latency | 1.490 | 0.247 | 0.057 | 0.814 | 0.509 | 0.608 |

2 Table 5a: Mixed between-within ANOVA for mfERG N1P1 amplitude, N1 latency, P1 latency and N2
3 latency over 3 visits for 5 areas of retinal eccentricity between treated and non-treated groups for
4 combined HY and HO eyes. The shaded areas indicate statistical significance.

5

1

| | | Visit 1 | Visit 2 | Visit 3 |
|---|---|---|---|---|
| | | **Mean ± SD** | **Mean ± SD** | **Mean ± SD** |
| **R2 P1 latency (ms)** | Treated (n=15) | 28.56 ± 1.46 | 28.50 ± 1.48 | 27.89 ± 1.83 |
| | Non-treated (n=10) | 28.25 ± 1.38 | 29.08 ± 1.33 | 28.33 ±1.24 |
| **R2 N2 latency (ms)** | Treated (n=15) | 42.33 ± 1.84 | 43.06 ± 1.85 | 43.28 ± 1.65 |
| | Non-treated (n=10) | 42.58 ± 1.73 | 43.42 ± 1.21 | 43.42 ± 1.33 |

2 Table 5b: Mean values ± SD for significantly changed mfERG measures between treated and non-
3 treated healthy eyes over three visits.

4

| | Visit 1 | Visit 2 | Visit 3 |
|---|---|---|---|
| | Mean ± SD | Mean ± SD | Mean ± SD |
| **VA (logMAR units)** | | | |
| Treated | -0.09 ± 0.09 | -0.07 ± 0.08 | -0.10 ± 0.09 |
| Non-treated | -0.12 ± 0.12 | -0.06 ± 0.08 | -0.10 ± 0.09 |
| **CS (log units)** | | | |
| Treated | 1.88 ± 0.13 | 1.88 ± 0.13 | 1.89 ± 0.12 |
| Non-treated | 1.89 ± 0.12 | 1.89 ± 0.12 | 1.92 ± 0.10 |
| **MPOD (optical density units)** | | | |
| Treated | 0.35 ± 0.16 | 0.40 ± 0.14 | 0.45 ±0.12 |
| Non-treated | 0.42 ± 0.16 | 0.42 ± 0.16 | 0.39 ± 0.16 |

5 Table 6: Mean values ± SD between treated and non-treated eyes for VA, CS and MPOD over 3 visits.
6 The shaded areas indicate statistical significance.

7

1

| | | Treated | Non-treated |
|---|---|---|---|
| **Mean trial duration (months)** | **VISIT 1-2** | 5.0 ± 0.9 | 4.9 ± 0.7 |
| | **VISIT 1-3** | 10.6 ± 1.2 | 10.6 ± 1.4 |
| **Mean compliance (% tablets taken)** | | 79.5 ± 15.9 | N/A |

2 Table 7: Summary of trial duration (mean ± SD) and participant compliance (% ± SD).

3

4 **Figure legends**

5 Figure 1: A normal mfERG response. The double ended arrow demonstrates N1P1 amplitude (source
6 - authors own drawing).

7 Figure 2: Grouping of the mfERG areas analysed. Ring 1- red hexagon, ring 2 - beige hexagons, ring
8 3 - green hexagons, ring 4 - pink hexagons, ring 5 - blue hexagons.

9 Figure 3: Differences between mean MPOD values over three visits between treated and non-treated
10 groups for healthy eyes.