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RETINAL DISORDERS

Macular response to supplementation with differing xanthophyll formulations in subjects with and without age-related macular degeneration

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Abstract
Purpose Our aim was to investigate the macular response to three different supplements containing lutein (L), zeaxanthin (Z) and *meso*-zeaxanthin (MZ) in normal subjects and those with age-related macular degeneration (AMD).
Materials and Methods Macular pigment optical density (MPOD) and serum xanthophyll concentrations were measured in normal (n=31) and AMD subjects (n=32), randomly assigned to: group 1 (20 mg L, 2 mg Z, 0.3 mg MZ), group 2 (10 mg L, 2 mg Z, 10 mg MZ) or group 3 (3 mg L, 2 mg Z, 17 mg MZ). MPOD was measured at baseline, 2, 4, 6 and 8 weeks and at 0.25°, 0.5°, 1.0° and 1.75° of eccentricity using customised heterochromatic flicker photometry and serum xanthophylls by HPLC.
Results MPOD increased significantly at all eccentricities in each group (p<0.05), except at 1.75° in group 3 (p=0.242). There was no difference in MPOD measurements between AMD and normal subjects, except for group 2, where AMD subjects exhibited a greater response at 1.75° (p=0.012). Final serum concentrations of MZ were positively and significantly related to final MPOD values at each eccentricity in all subjects. Targeted analysis of those subjects receiving the MZ-containing supplements exhibited stronger relationships between serum MZ concentrations and MPOD at 0.25° in group 3 than group 2; in group 2 all associations were positive, but only significant at 1.75°.
Conclusions Serum concentrations of MZ were strongly correlated with MPOD after 8 weeks of supplementation with the group 3 formulation, but the inclusion of L in the group 2 formulation may result in greater MPOD augmentation across the spatial profile.

Keywords Age-related macular degeneration · Macular pigment · Lutein · Zeaxanthin · *Meso*-zeaxanthin

Abbreviations

AMD	Age-related macular degeneration
L	Lutein
Z	Zeaxanthin
MZ	Meso-zeaxanthin
MPOD	Macular pigment optical density

Introduction

Supplementation with the macular xanthophyll carotenoids and co-antioxidants has been associated with retardation of progressive macular diseases such as age-related macular degeneration (AMD) [1], the leading cause of blindness in the Western world [2]. Supplementation has also been shown to improve visual function and performance in diseased and non-diseased eyes [3–7]. These observed benefits have been attributed to the biochemical (anti-oxidant) and photochemical (blue-light filtration) [8] properties of 3R,3′R,6′R-lutein (L), 3R,3′R-zeaxanthin (Z), and 3R,3′S-*meso*-zeaxanthin (MZ), the macular pigment (MP)'s constituent xanthophylls

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[6, 9]. Furthermore, the importance of these compounds for neural efficiency, and consequently for visual processing, has also been recently reported [10].

L and Z are entirely of dietary origin, and can be sourced from a plethora of foodstuffs, including egg yolk and leafy green or yellow vegetables, such as spinach, sweet corn, peppers, etc. Although the presence of MZ has been demonstrated in certain food types [11], its content in a typical diet remains under investigation, and this carotenoid is generated (at least in part) as a result of endogenous bioconversion of retinal L [12, 13]. Importantly, the spatial distribution of the three constituent xanthophylls in MP is not uniform. Dissection studies of human maculae show that, relative to Z, the concentration of L in the adult neural retina increases with radial distance from the fovea, while that of MZ decreases. In the fovea, the ratio of MZ:Z:L is 1:1:1, while in the peripheral macula, L predominates over the zeaxanthins, reflecting the peak concentration of MZ at the central fovea [14, 15].

To date, research germane to MP has largely focused on the use of supplemental L and Z as a means of augmenting MP in an attempt to ameliorate the natural course of AMD [1, 16, 17] and as a means of optimising visual performance [18]. These studies have reported that MP can be augmented following such supplementation [15, 19]; however, the interpretation of such trials is limited by the absence of MZ in the macular carotenoid supplements used, and because MZ exhibits the greatest antioxidant capacity of the three macular xanthophylls [20]. Indeed, it is noteworthy that the combination of the three xanthophylls in a ratio of 1:1:1 has an in vitro antioxidant capacity to quench singlet oxygen that is superior to iso-molar equivalent amounts of the three xanthophylls individually [20]. This suggests there may be a need for all three xanthophylls to exert maximal antioxidant effect in defence of the macula. Furthermore, supplementation with all three of MP's constituent xanthophylls, in a MZ:L:Z ratio (mg) of 10:10:2, has been shown in normal, healthy subjects to result in a superior MP response in terms of augmentation, desirable modification of its spatial profile [7, 21], when compared with alternative formulae (UltraLutein®: 20 mg L, 2 mg Z, 0.3 mg MZ). More recently, we have shown that the same xanthophyll mixture was also superior in terms of serum bioavailability for capture by the retina, when compared with both UltraLutein® and a customised (commercially unavailable) high MZ formulation of 3 mg L, 2 mg Z, and 17 mg MZ [22].

This study was designed to investigate MP optical density (MPOD) response to differing formulations containing the macular xanthophylls in subjects with and without AMD. In addition, the relationships between augmented MPOD and serum concentrations of L, Z and MZ are reported.

Materials and methods

Subjects

This was a randomised and double-masked xanthophyll supplementation study in which MPOD and serum concentrations were determined. The serum measurements have already been described [22], so they will be only briefly outlined. All subjects signed an informed consent document, and the experimental design and execution conformed to the Declaration of Helsinki. This clinical trial (registration number: ISRCT N81595685) was registered on the website http://isrctn.org on 27 August 2009, and was initiated on 1 March 2011. The study was reviewed and approved by the Research Ethics Committee, South East Region, Waterford Regional Hospital, and the Ethics Committee of Waterford Institute of Technology, Waterford, Ireland.

We were interested in studying two different subject populations, those with and those without AMD. Normal subjects were in good general health with corrected distance visual acuity (CDVA) of 6/12 or better and absence of ocular pathology (fundus images were reviewed prior to enrolment by an ophthalmologist [S.B.]). Subjects suffering with early AMD were defined as those exhibiting drusen and pigmentary changes, and were identified at a pre-project enrolment and screening visit, conducted by an ophthalmologist with a special interest in retinal disease and experienced in the classification of AMD for research purposes (S.B.) [23]. Fundus images were graded, in a masked fashion, by an accredited reading centre at the University of Wisconsin. Exclusion criteria, for AMD and for normal subjects, were past or current use of supplemental macular xanthophylls and/or pregnancy. Changes in blood volume associated with pregnancy could potentially obscure any relationships between the serum xanthophyll and MPOD measurements especially in a trial of this size.

Subject BMI was calculated (kg/m^2), with subject height (m) being measured with the Leicester Height Measure, and weight (kg) being measured with the SECA weighing scales (SECA, Birmingham, UK). Smoking status was classed as: current smoker, ex-smoker or non-smoker.

A subject's weekly intake of carotenoid-rich foods (eggs, broccoli, corn, dark leafy vegetables) was inputted into the "L/Z screener" to yield a rough score of dietary xanthophyll intake and was primarily designed to detect subjects with low values (personal communication, Dr Elizabeth Johnson, Tufts University). Values were weighted for frequency of intake (i.e., a greater score was given for higher frequency), bioavailability (eggs three times greater than vegetables), and content (eggs < corn < broccoli < dark green leafy vegetables). The product is a ranking score in arbitrary units, reflecting the relative intakes of L and Z ranging from 0 to 75. After inputting those foods with known concentrations of L and Z

into the screener [24], the following categories were generated: low dietary carotenoid intake score, ranging from 0 to 15 (i.e., ≤ 2 mg/d); medium dietary carotenoid intake score, ranging from 16 to 30 (i.e., between > 2 and 13 mg/day); and high dietary carotenoid intake score, ranging from 31 to 75 (i.e., > 13 mg/day).

Of the 72 subjects originally enrolled in this study, sixty-three completed the full trial in accordance with the a priori study guidelines. Of these 63 subjects, 32 exhibited no ocular pathology (normal subjects) and 31 were diagnosed with AMD (AMD subjects). The 63 subjects were split into three different intervention groups, as follows: group 1: (*n* 23; 12 normal, 11 AMD; 20 mg of L and 2 mg of Z, 0.3 mg MZ ["Ultra Lutein™", provided by Nature's Plus, Natural Organics Inc., Melville, NY, USA]); group 2: (*n* 21; ten normal, 11 AMD; 10 mg L, 2 mg Z and 10 mg MZ [Macushield™, provided by MacuVision Europe Ltd, Solihull, UK]); group 3: (*n* 19; nine normal, ten AMD 3 mg L, 2 mg Z and 17 mg MZ [customised MZ formulation provided by Industrial Organica, Monterrey, Mexico; not available commercially]). Following the commencement of the current study, the group 1 supplement, which was intended to have zero MZ content, was found to contain a small amount of MZ (0.3 mg/capsule) [22]. All supplements used in this study were oil-suspended and non-esterified, and were provided in gelatin capsules. Each subject was required to consume one capsule daily, with a main meal, for the duration of the 8-week study period, with serum samples taken at baseline, four, six, and 8 weeks, and MPOD measured using customised heterochromatic flicker photometry (cHFP) every second week. At the fortnightly study visits, subjects were questioned on capsules consumed and supplement packs were checked at the final visit to confirm compliance by the tablet count.

Vision and MPOD measurements

MPOD was measured using the cHFP densitometer™ (Macular Metrics II, Rehoboth, MA, USA), a validated instrument, the reproducibility of which has been described [25]. The composite MPOD is the sum of the MPOD values at 0.25°, 0.5°, 1.0° and 1.75° eccentricities and is representative of the measured MP spatial profile.

Serum carotenoid analysis [22]

Briefly, serum (0.4 ml) was extracted into heptane using α-tocopheryl acetate as an internal standard. The heptane extract was dried and loaded onto an Ultracarb 250 x 4.6 mm ODS 3μmC18 column (Phenomenex, Cheshire, UK) for HPLC analysis. The peaks for L and total Z isomers were baseline separated and were quantified using appropriate standards. The Z peak was collected manually from the eluate, evaporated to dryness, and loaded onto a second HPLC column (5 μm Chiralpak™ AD 250 × 4.6 mm column; Apex Scientific Ltd, Maynooth, Kildare, Eire), which separated Z and MZ. The ratio of the MZ and Z peak areas obtained from Assay 2 was then applied to the concentration of total Z obtained from assay 1 in order to quantify MZ and Z individually (method of proportions technique). The accuracy of L and total Z concentrations was validated using the serum standard reference material (968d; National Institute for Standards and Technology, Gaithersburg, MD, USA).

Statistical analysis

SPSS ver. 19; SPSS Inc., Chicago, IL, was used for data analysis. The data was tested for normality, and although some of the serum data appeared skewed from the plotted histograms, Kolmogorov-Smirnov normality tests confirmed that the data satisfied the criteria for normality, and therefore standard normality statistical tests were used during analysis. Means ± SDs are presented in the text and tables. Between-(intervention)-group differences at baseline for numeric data (age, body mass index [BMI], diet score, serum carotenoid and MPOD levels) were investigated using ANOVA. Between-(intervention)-group differences at baseline for categorical variables (gender, smoking habits, sex and ocular status [normal or AMD]) were investigated using the standard χ^2 test. With respect to normal versus AMD subjects, statistical differences at baseline for numeric data (age, BMI, diet score and serum xanthophyll concentrations) were investigated using ANOVA, whereas differences between categorical variables (gender, smoking habits, sex) were investigated using the standard χ^2 test.

Repeated measures analysis (RMANOVA) was used to investigate MPOD response for each intervention group (at each eccentricity) over the five study visits. Time was a within-subjects factor, disease status and intervention group were between-subjects factors, with age and baseline serum Z as covariates, as there were some small differences in age and serum Z concentrations between the subject groups. Post-Hoc RMANOVA analysis was used to identify potential significant differences, in terms of MPOD response, between intervention groups. Changes in MPOD between baseline and 8 weeks for each intervention group were investigated using simple paired t-tests.

The relationship between serum concentrations of the macular carotenoids and MPOD was investigated using Pearson correlation coefficients (*r*), using all the data for investigation of L and Z, but excluding group 1 subjects for the investigation of MZ (as MZ was not present in meaningful amounts in group 1 intervention). Data for correlation analysis was also split by ocular disease status (AMD versus normal) for between-group investigation. A 5 % level of significance was adopted throughout the analysis, without adjusting for multiple testing.

Results

Baseline findings

Table 1 shows the demographic, lifestyle, ocular disease status, dietary xanthophyll scores, baseline serum xanthophyll concentrations and MPOD data (at four eccentricities: 0.25°, 0.5°, 1°, 1.75°, and composite sum of these eccentricities) for all three intervention groups. There were no significant differences in terms of these variables between the intervention and subject groups, except for age and serum Z concentrations. AMD subjects were older than the normal subjects (p=0.044), and there were significant differences in serum Z concentrations between intervention groups, with group 3 exhibiting higher concentrations than the other two groups (p=0.043). However, there was no difference in group 3 in serum Z concentrations between the AMD and normal subjects. Although ANOVA suggested that the age of those with AMD was greater than that of normal subjects, this only applied to intervention group 1 subjects, where the difference approached significance (p=0.064, independent 't' test). With respect to the dietary intake of xanthophylls by the treatment and subject groups, there were no differences in the xanthophyll scores and there was no interaction between disease

Table 1 Demographic, lifestyle, ocular status (normal or AMD), baseline MPOD data and baseline serum xanthophyll concentrations for the three intervention groups

Characteristic	All	SD	Group 1	SD	Group 2	SD	Group 3	SD	p#
Subjects	*n* 63	-	*n* 23	-	*n* 21	-	*n* 19	-	-
Normal	32	-	12	-	10	-	9	-	0.968*
AMD	31	-	11	-	11	-	10	-	-
Mean Age[†]									
Normal subjects	62	9	59	11	60	10	65	6	0.271[‡]
AMD subjects	66	9	67	7	66	10	65	10	0.908[‡]
BMI	27	3.3	28	4.3	26	2.4	28	2.9	0.139[‡]
Diet score	22	9	21	9.1	21	9.2	25	8.5	0.292[‡]
Sex									
Male	24	-	6	-	7	-	11	-	0.362*
Female	40	-	16	-	12	-	12	-	
Smoking habits									
Non-smoker	36	-	12	-	13	-	11	-	0.419*
Ex-smoker	22	-	9	-	5	-	8	-	
Current smoker	6	-	1	-	1	-	4	-	
Baseline mean MPOD									
0.25° eccentricity	0.45	0.20	0.43	0.20	0.44	0.18	0.48	0.22	0.734[‡]
0.5° eccentricity	0.35	0.17	0.34	0.19	0.35	0.16	0.37	0.19	0.856[‡]
1° eccentricity	0.24	0.13	0.23	0.13	0.23	0.12	0.27	0.14	0.568[‡]
1.75° eccentricity	0.13	0.10	0.13	0.10	0.11	0.09	0.15	0.10	0.434[‡]
Composite MPOD	1.15	0.52	1.12	0.57	1.10	0.45	1.28	0.54	0.650[‡]
Baseline mean serum carotenoids[§]									
Z Normal subjects	0.045	0.027	0.047	0.031	0.036	0.023	0.056	0.025	0.225
Z AMD subjects	0.053	0.032	0.046[a]	0.024	0.043[a]	0.027	0.078[b]	0.039	0.043[‡‖]
L Normal subjects	0.229	0.126	0.219	0.110	0.221	0.147	0.250	0.124	0.828[‡]
L AMD subjects	0.282	0.157	0.308	0.190	0.241	0.127	0.300	0.151	0.599

AMD age-related macular degeneration, *BMI* body mass index, *MPOD* macular pigment optical density, *L* lutein, *Z* zeaxanthin, *MZ meso*-zeaxanthin

group 1: high L group (20 mg L/day, 2 mg Z/day, 0.3 mg MZ/day); group 2: combination group (10 mg L/day, 2 mg Z/day, 10 mg MZ/day); group 3: high MZ group (3 mg L/day, 2 mg Z/day, 17 mg MZ/day)

#p values were results from ANOVA ([‡]) or χ^2 ([*]) tests between groups

[†] AMD subjects were significantly older than normal subjects, p=0.044; Data relating to BMI, sex, smoking habits and diet score did not differ between AMD and normal subjects, and were therefore reported as combined values

[§] Some serum samples were missing/not collected: group 1, all present; group 2, one missing; group 3, two missing

[‖] There were no differences in baseline serum concentrations, except for higher concentrations of Z observed in the AMD subjects in group 3. There was no serum MZ in the baseline samples

status and treatment group (p=0.893 and 0.174 for AMD and normal subjects, respectively).

MPOD response after 8 weeks: AMD and normal subjects, combined

Mean (SD) MPOD values at each study visit and change in MPOD over the 8-week study period for the three intervention groups are presented in Table 2. There was a significant increase in MPOD at each eccentricity for each intervention group, with the exception of that at 1.75° in group 3 (MPOD increase=12 %, p=0.242). Further analysis of the subject groups separately showed that this lack of response was predominantly attributable to the normal subjects in whom the mean change in MPOD was −7 % (p=0.604), while the mean increase amongst the AMD subjects almost reached significance (p=0.054; +28 %) (Table 3). With that exception, the increases in MPOD at all eccentricities were statistically comparable between the three treatment groups (Post Hoc RMANOVA, p>0.05 for all comparisons) (Table 2).

MPOD response after 8 weeks: AMD and normal subjects, analysed separately

Mean change in MPOD over the 8-week study period for the three intervention groups, for normal and AMD subjects separately, is presented in Table 3. There was a significant increase in MPOD values in both normal and AMD subjects at most eccentricities for each intervention group. The lowest (but still substantial) responses occurred in AMD subjects in treatment group 3, at 0.25° (increase =23 %, p=0.054) and

Table 2 Mean MPOD values at each visit for the three xanthophyll intervention groups

	Baseline Mean	SD	Week 2 Mean	SD	Week 4 Mean	SD	Week 6 Mean	SD	Week 8 Mean	SD	Mean change*	SD	% increase	$p^{\dagger}$
0.25° eccentricity														
Group 1	0.43	0.2	0.45	0.2	0.49	0.19	0.51	0.17	0.52	0.18	0.095	0.07	22	0.000
Group 2	0.43	0.16	0.5	0.16	0.52	0.17	0.55	0.17	0.58	0.16	0.147	0.08	34	0.000
Group 3	0.49	0.21	0.51	0.22	0.53	0.22	0.56	0.2	0.56	0.2	0.091	0.1	19	0.003
0.5° eccentricity														
Group 1	0.34	0.19	0.38	0.17	0.41	0.18	0.42	0.17	0.44	0.17	0.103	0.08	30	0.000
Group 2	0.35	0.14	0.39	0.15	0.43	0.15	0.44	0.15	0.48	0.15	0.129	0.08	37	0.000
Group 3	0.39	0.2	0.41	0.21	0.41	0.2	0.43	0.18	0.45	0.18	0.088	0.1	23	0.002
1° eccentricity														
Group 1	0.23	0.13	0.26	0.11	0.28	0.12	0.29	0.12	0.31	0.12	0.079	0.07	35	0.000
Group 2	0.23	0.11	0.26	0.11	0.29	0.12	0.29	0.12	0.3	0.13	0.072	0.05	31	0.000
Group 3	0.26	0.11	0.26	0.11	0.27	0.11	0.29	0.12	0.3	0.11	0.052	0.07	19	0.007
1.75° eccentricity														
Group 1	0.13	0.1	0.14	0.11	0.16	0.1	0.18	0.1	0.18	0.1	0.056	0.06	44	0.000
Group 2	0.1	0.08	0.12	0.07	0.15	0.08	0.15	0.08	0.16	0.07	0.057	0.04	55	0.000
Group 3	0.15	0.09	0.16	0.09	0.15	0.09	0.16	0.1	0.15	0.11	0.019	0.06	12	0.242
Composite MPOD[‡]														
Group 1	1.12	0.57	1.22	0.55	1.34	0.55	1.40	0.52	1.45	0.52	0.08	0.05	30	0.000
Group 2	1.10	0.45	1.26	0.44	1.38	0.48	1.42	0.48	1.51	0.49	0.10	0.05	37	0.000
Group 3	1.28	0.54	1.34	0.57	1.37	0.57	1.44	0.55	1.45	0.53	0.06	0.07	13	0.003

L, lutein; Z, zeaxanthin; MZ, *meso*-zeaxanthin

Group 1 (n 23): high L group (20 mg L/day, 2 mg Z/day, 0.3 mg MZ/day); Group 2 (n 21): combination group (10 mg L/day, 2 mg Z/day, 10 mg MZ/day); Group 3 (n 19): high MZ group (3 mg L/day, 2 mg Z/day, 17 mg MZ/day)

Data shown are mean (SD) MPOD values for each of the five study visits; *Mean change in MPOD refers to the change in MPOD between baseline and 8 weeks

[‡] Composite MPOD refers to the sum of measured MPOD values at each eccentricity. Repeated measures analysis was used to investigate changes in MPOD over the five study visits

MPOD readings for normal and AMD subjects were combined as there were no differences. Furthermore, increases in MPOD at all eccentricities were statistically comparable between intervention and subject groups (Post Hoc RMANOVA, p>0.05 for all comparisons)

[†] p values represent significance of the change between baseline and 8 weeks for normal and AMD subjects combined and each individual intervention group at each eccentricity measured (paired sample t-tests)

Table 3 Mean (SD) change and percentage increase in MPOD and serum lutein, zeaxanthin and *meso*-zeaxanthin concentrations for normal and AMD subjects separately and for each intervention group over the 8-week study period

	Normal Subjects				AMD Subjects			
MPOD	Mean change*	SD	% increase	$p^{\dagger}$	Mean change*	SD	% increase	$p^{\dagger}$
0.25° eccentricity								
Group 1	0.096	0.05	21	0.000	0.093	0.08	23	0.003
Group 2	0.145	0.08	36	0.000	0.148	0.08	32	0.000
Group 3	0.061	0.04	14	0.001	0.129	0.14	23	0.054
0.5° eccentricity								
Group 1	0.096	0.07	26	0.001	0.110	0.08	36	0.001
Group 2	0.097	0.05	29	0.000	0.161	0.09	45	0.000
Group 3	0.051	0.05	15	0.011	0.136	0.12	31	0.026
1° eccentricity								
Group 1	0.070	0.08	28	0.020	0.087	0.04	42	0.000
Group 2	0.061	0.04	27	0.002	0.083	0.06	35	0.001
Group 3	0.028	0.03	13	0.039	0.083	0.09	25	0.046
1.75° eccentricity								
Group 1	0.072	0.06	49	0.004	0.040	0.04	37	0.010
Group 2	0.031	0.03	28	0.011	0.081	0.04	83	0.000
Group 3	−0.009	0.05	−7	0.604	0.054	0.06	28	0.054
Composite MPOD[‡]								
Group 1	0.084	0.05	27	0.000	0.083	0.05	33	0.000
Group 2	0.086	0.03	33	0.000	0.118	0.05	41	0.000
Group 3	0.033	0.03	12	0.012	0.100	0.09	15	0.023
Serum concentration (μmol/L)			% Change	p (ANOVA)			% Change	p (ANOVA)
Lutein Group 1	0.493 ^{xy}	0.517	289		0.685 ^{x}	0.596	271	
Lutein Group 2	0.861^{x}	0.622	555	0.013	0.847 ^{x}	0.449	394	0.003
Lutein Group 3	0.025^{x}	0.060	20		0.014 ^{y}	0.127	17	
Zeaxanthin Group 1	0.018 ^{x}	0.041	106		0.040 ^{x}	0.034	107	
Zeaxanthin Group 2	0.036 ^{x}	0.036	105	0.003	0.030 ^{x}	0.026	116	0.012
Zeaxanthin Group 3	−0.022 ^{y}	0.028	−45		−0.004 ^{y}	0.019	−1	
meso-Zeaxanthin Group 1	0.012 ^{x}	0.022	-[‡‡]		0.007 ^{x}	0.007	-	
meso-Zeaxanthin Group 2	0.054 ^{x}	0.059	-	NS (0.096)	0.062 ^{y}	0.042	-	<0.001
meso-Zeaxanthin Group 3	0.027 $^{a, xy}$	0.047	-		0.098 $^{b, z}$	0.048	-	

L, lutein; Z, zeaxanthin; MZ, *meso*-zeaxanthin; AMD, age-related macular degeneration

Group 1 (*n* 22): high L group (20 mg L/day, 2 mg Z/day, 0.3 mg MZ/day); Group 2 (*n* 23): combination group (10 mg L/day, 2 mg Z/day, 10 mg MZ/day); Group 3 (*n* 19): high MZ group (3 mg L/day, 2 mg Z/day, 17 mg MZ/day)

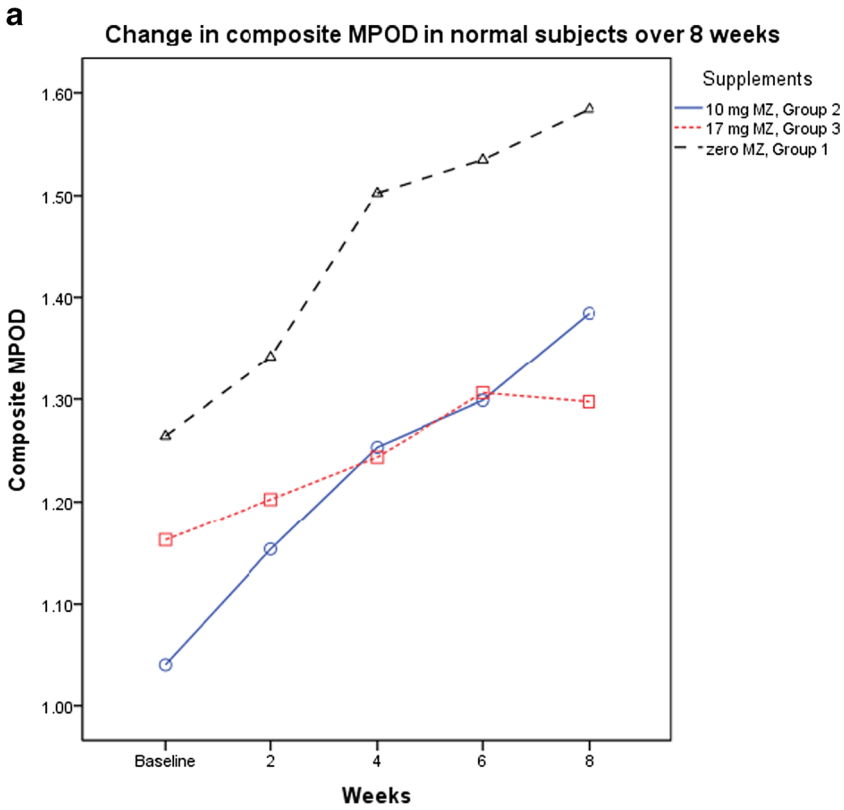
*Mean change in MPOD refers to the change in MPOD between baseline and 8 weeks

† Significance (*p)* values were calculated using paired sample t-tests, and represent significance for the change between baseline and 8 weeks for each individual intervention group at each eccentricity measured. Increases in MPOD at all eccentricities were statistically comparable between interventions (Post Hoc RMANOVA, $p>0.05$ for all comparisons

‡ Composite MPOD refers to the sum of measured MPOD values at each eccentricity. Serum xanthophyll concentrations were the mean differences between baseline and week 8. There were no differences in serum responses between normal and AMD subjects, except for MZ concentrations in group 3, which were greater in AMD subjects than in normal subjects ($^{a, b}$ $P<0.008$, independent sample *t*-test). Differences between the individual xanthophyll changes and in response to the three treatments were tested using ANOVA, followed by an LSD test. Unlike superscripts (x, y, z) indicate significant differences ($P<0.05$)

‡‡ Not possible to calculate % increase in MZ as MZ not present in baseline samples

1.75° (increase =28 %, $p=0.054$) eccentricities. The observed changes in MPOD, for normal and AMD subjects at each eccentricity, were statistically comparable between interventions (Post Hoc analyses, $p>0.05$ for all comparisons). Figure 1a and b show graphical representations of the changes in composite MPOD values for normal and AMD subjects separately. The figures illustrate the comparable increases in the MPOD spatial profile with time, both between treatment



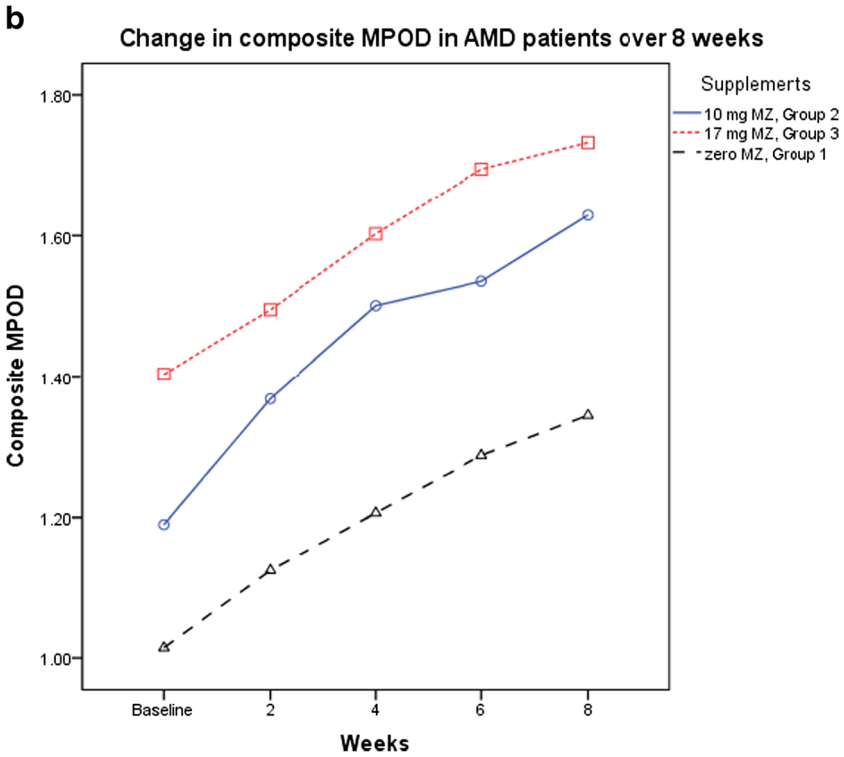


Fig. 1 **a**: Graphical representation of composite MPOD responses of normal subjects over 8 weeks. Composite MPOD values represent the sum of measured MPOD values at 0.25°, 0.5°, 1.0° and 1.75° eccentricities, over the 8-week supplementation period. Group 1 (*n 12*): high L group (20 mg L/day, 2 mg Z/day, 0.3 mg MZ/day); group 2 (*n 10*): combination group (10 mg L/day, 2 mg Z/day, 10 mg MZ/day); group 3 (*n* 9): high MZ group (3 mg L/day, 2 mg Z/day, 17 mg MZ/day). There was no difference in the changes in the composite MPOD values, at each eccentricity and for all groups (Post Hoc RMANOVA, $p>0.05$ for all comparisons). **b**: Graphical representation of composite MPOD responses of subjects with AMD over 8 weeks. These data are similar to that shown in Fig 1a for normal subjects and the statistical results were the same. Subject numbers were 11, 11 and ten for groups 1, 2 and 3, respectively. There were no significant differences in composite MPOD responses between normal (Fig. 1a) and AMD subjects (Fig. 1b) at any time point

groups, and between normal and AMD subjects (Post Hoc RMANOVA, $p>0.05$ for all comparisons).

Also shown in Table 3 are the changes in serum xanthophyll concentrations over the 8-week intervention study, obtained by subtracting the baseline concentration from the final values except for MZ. There was no MZ in the sera at baseline, so the MZ concentrations shown were final values. There were no differences between the serum responses in the

normal and AMD subjects, except for serum MZ concentrations in group 3 where the mean serum MZ at 8 weeks was significantly higher in the AMD subjects than in the normal subjects ($p<0.008$). Surprisingly, there was no difference in the mean serum MZ concentration following supplementation with formulation 2 (10 mg MZ/day) and 3 (17 mg MZ/day) in the normal subjects. It was not surprising, however, that the group 3 supplement also produced the lowest responses in both serum L and Z concentrations. Treatments 1 (20 mg L, 2 mg Z) and 2 (10 mg L, 2 mg Z) produced comparable increases in serum concentrations of Z and L.

Correlations between MPOD and serum xanthophyll concentrations

Table 4 shows that baseline MPOD values at each eccentricity was significantly correlated with baseline serum L concentrations in normal subjects, but not in those with AMD (except at 0.5° eccentricity). In contrast, baseline MPOD in subjects with AMD was correlated with baseline serum Z concentrations, but not in those with normal maculae (again, except at 0.5° eccentricity). Any relationship with MZ could not be investigated, as there was no MZ in baseline serum.

We examined relationships between MPOD values at 8 weeks and serum L, Z and MZ concentrations also obtained at 8 weeks. There were no correlations between MPOD and serum concentrations of L or Z at this time point in either AMD subjects or normal subjects. However, serum MZ concentrations were positively correlated with MPOD (at each eccentricity) in subjects with AMD but not the controls. Since the MZ concentration at 8 weeks actually represents the change in MZ concentration over 8 weeks, we also investigated the relationship between changes in MPOD and changes in serum concentrations of L, Z and MZ. Only two significant correlations were found. These were negative correlations between changes in MPOD at 0.5° and 1.75° eccentricities and serum concentrations of MZ in normal subjects.

We realised that the distribution of serum MZ results at week 8 was negatively skewed because there was no appreciable amount of MZ in the high L, group 1 supplement. We therefore restricted our examination of relationships between MPOD and serum MZ to groups 2 and 3 only. Table 5 shows the relationships between serum MZ concentrations and the MPOD measurements at week 8 for groups 2 and 3, individually and combined, and for subjects with and without AMD separately. All values indicated a positive relationship between the variables, but MPOD tended to show the strongest relationships in subjects with AMD who received the high MZ supplement (Group 3, 17 mg MZ/day); yet, as numbers were small in this subgroup ($n=7$), the results were not significant. There were no significant correlations when normal subjects were examined separately. Figure 2 illustrates some of these observations: the positive relationship between MPOD at 0.25° eccentricity and serum MZ concentrations at 8 weeks and the individual regression lines for the data for subjects supplemented with formulations 2 and 3. As the strength of the relationship between MPOD and serum concentrations of MZ was weakest at 1.75° eccentricity, we also examined the relationship between MPOD at all eccentricities and serum L concentrations at week 8 (and the change in serum L concentrations over the 8 weeks), but no significant correlations were identified (data not shown). Finally, we looked at the change in MPOD over the 8 weeks against final MZ concentrations in groups 2 and 3 combined (as these were the only groups supplemented with MZ). No significant correlations were identified at any eccentricity (data not shown).

Discussion

This study was designed to investigate MPOD responses to three differing macular carotenoid formulations in subjects with and without AMD over a short-term (8-week) period. In addition, the current study also investigated the relationship between MPOD at four eccentricities and serum concentrations of its constituent carotenoids (L, Z and MZ). In brief, we report that MPOD increased in response to supplementation in eyes of subjects with and without AMD, and serum MZ concentrations correlated positively with augmented MPOD values in subjects with AMD (Table 6).

For central MPOD (0.25° eccentricity), we report that groups 1, 2 and 3 achieved significant augmentation, with group 2 tending to show the greatest (but not significantly greater) response (34 %, Table 2). The failure to achieve a significantly greater augmentation of MPOD in group 2 than in groups 1 and 3 in this exploratory study may be attributable to small sample sizes. For example, taking 0.45 as the central MPOD baseline mean and 0.20 as the standard deviation, group 2 improved by approximately 15 % (of the 0.45 mean value) more than group 3. This corresponds to a MPOD of 0.0675, which represents 0.375 standard deviations. Indeed, to achieve power of 80 % at the 5 % level of significance using a two-tailed test, the sample size required to detect an effect size of 0.375 standard deviations would be 112 in each of the two groups [25], For the example cited (groups with 21 and 19 subjects, 5 % level of significance, two-tailed independent samples *t*-test), the actual power for detecting a difference of 0.375 standard deviation is only 21 %.

Group 3 exhibited the lowest degree of augmentation at 0.25° eccentricity (19 %), and although this was not significantly less than the other intervention groups, it was a somewhat unexpected finding, given that this formulation contained high amounts of MZ, and given that MZ reaches its peak concentration centrally. This seemingly counterintuitive finding may be attributable to the low content of L (3 mg)

Table 4 Correlation between serum xanthophyll concentrations and MPOD at baseline, at 8 weeks and with changes in MPOD in subjects with and without AMD

Eccentricity	Lutein						Zeaxanthin						*Meso*-zeaxanthin			
	Baseline		Week 8		Change		Baseline		Week 8		Change		Week 8		Change	
MP0.25°	AMD	Normal	AMD	Normal	AMD	Normal	AMD	Normal	AMD	Normal	AMD	Normal	AMD	Normal	AMD	Normal
r	0.290	0.381	0.169	0.233	0.026	−0.06	0.496	0.288	0.286	0.270	−0.318	0.114	0.520	0.241	0.037	−0.239
p	0.135	0.029	0.390	0.241	0.894	0.765	0.007	0.104	0.140	0.174	0.099	0.557	0.005	0.226	0.851	0.213
MP0.5°																
r	0.430	0.488	0.280	0.191	0.140	−0.211	0.430	0.409	0.372	0.263	−0.129	0.072	0.494	0.175	0.085	−0.447
p	0.023	0.004	0.149	0.340	0.477	0.291	0.022	0.018	0.051	0.185	0.512	0.712	0.007	0.383	0.665	0.015
MP1.0°																
r	0.240	0.382	0.120	0.197	0.196	0.108	0.532	0.307	0.355	0.144	−0.104	0.159	0.452	0.072	−0.135	−0.055
p	0.218	0.028	0.543	0.324	0.318	0.591	0.004	0.082	0.064	0.473	0.598	0.410	0.016	0.720	0.492	0.778
MP1.75°																
	0.240	0.425	0.001	0.203	0.068	0.112	0.489	0.310	0.208	0.101	−0.264	0.198	0.486	−0.258	0.171	−0.623
	0.218	0.015	0.997	0.309	0.732	0.587	0.008	0.085	0.289	0.615	0.175	0.313	0.009	0.194	0.384	<0.001

For abbreviations, group descriptions and subject numbers, see Table 2. Pearson correlation (r) and significance (p) values were calculated using the bivariate technique. Baseline MPOD at different eccentricities was correlated against the baseline serum xanthophyll concentrations. Week 8 MPOD values were correlated with the serum concentrations of L, Z and MZ obtained at 8 weeks, and the changes in MPOD over 8 weeks were correlated with the change in serum xanthophyll concentrations over the 8 weeks. Highlighted values were significantly correlated

Table 5 Correlations between serum *meso*-zeaxanthin concentrations and MPOD measurements at week 8 in subjects receiving only the supplements containing MZ (groups 2 and 3)

		meso-Zeaxanthin versus MPOD				
Eccentricities		Composite	0.25°	0.5°	1.0°	1.75°
Groups 2 and 3, all subjects	n					
r	35	0.461	0.438	0.448	0.440	0.360
p		0.005	0.009	0.007	0.008	0.034
Groups 2 and 3, AMD subjects only						
r	17	0.491	0.402	0.408	0.505	0.558
p		0.045	0.110	0.104	0.039	0.020
Groups 2 and 3, Normal subjects only						
r	18	0.343	0.379	0.386	0.298	0.029
p		0.163	0.121	0.114	0.229	0.910
Group 2 only, all subjects						
r	20	0.331	0.244	0.272	0.347	0.443
p		0.154	0.301	0.247	0.131	0.050
Group 3 only, all subjects						
r	15	0.573	0.602	0.607	0.530	0.333
p		0.026	0.018	0.016	0.042	0.225

For abbreviations and group descriptions, see Table 2. 'Composite' represents the sum of MPOD measurements at the four eccentricities. There were no differences between subjects with and without AMD, so the data were combined

Pearson correlation and significance values were calculated using the bivariate method. Highlighted values were significantly correlated, p=<0.05

in the group 3 formulation, reflected in the failure of that formulation to augment MP at 1.75° eccentricity (where L is the dominant carotenoid).

A similar result with respect to L was obtained in an earlier exploratory study, in which we used a Macushield supplement containing 3.7 mg L, 0.8 mg Z and 7.3 mg MZ as an encapsulated powder formulation [26]. Over the 8-week trial, there were significant increases in MPOD in the central eccentricities and there was no difference between normal and AMD subjects (five of each). However, there was no change in MPOD at 1.75° eccentricity and there was only a weak serum L response. The low content of L and lack of oil in the supplement may well have impaired the L response, as there was no increased pigmentation of the peripheral macula (1.75°) where L is known to be located.

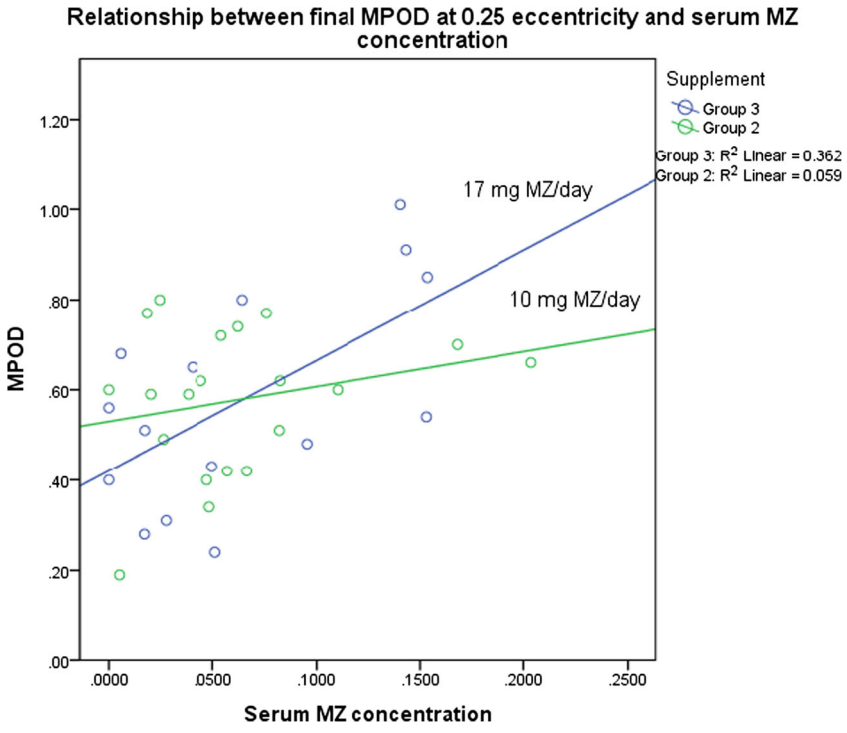


Fig. 2 Scatter-plot representation of the correlation (at 8 weeks) between serum MZ and MPOD at 0.25° eccentricity for AMD and normal subjects combined. Subjects in groups 2 (n=20; 10 mg MZ/day; r^2=0.059) and 3 (n=15; 17 mg MZ/day; r^2=0.362) only are presented and are displayed in different colours

Table 6 Main points of interest reported for the serum xanthophyll and MPOD measurements following 8 weeks of supplemention with 3 xanthophyll in subjects with and without early AMD

Measurement	Time point	Observation
Serum xanthophyll concentrations	Baseline	No differences between subjects with AMD and controls
	Following treatment	As above, except in group 3 where serum MZ was greater in AMD than controls
MPOD measurements	Response to supplementation with 3 treatments	All significantly increased at all eccentricities, except at 1.75° in response to the group 3 supplement only
Correlations between serum xanthophyll concentrations and MPOD	Baseline	Serum L correlated positively with MPOD in controls, but not AMD subjects Serum Z correlated positively with MPOD in AMD subjects, but not the controls
	8 weeks	Serum MZ correlated positively with MPOD in AMD subjects, but not the controls
	Changes between baseline and 8 weeks.	There were no significant correlations, except at 1.75° in controls with serum MZ concentrations

The three supplements were Ultralutein® (group 1), Macushield® (group 2) and a high MZ preparation (group 3). MPOD, macular pigment optical density; AMD, age-related macular degeneration; L, Z and MZ: lutein, zeaxanthin and *meso*-zeaxanthin, respectively

It is possible that augmentation of MP across the full spatial profile depends on supplementation with sufficient quantities of all three of its constituent carotenoids, and that augmentation of MP centrally (where MZ reaches its peak concentration) requires adequate reserves of MP peripherally (at 1.75° eccentricity, where L is the dominant carotenoid), as MZ is derived (at least in part) from retinal L. This is consistent with our finding in the current study that the greatest augmentation of composite MPOD values across the spatial profile was seen for group 2 (mean [%] change: group 2: 0.369 [36]; group 1: 0.290 [28]; group 3: 0.170 [14], Table 2), but it should be appreciated that these results were not statistically different.

In brief, therefore, we report that supplementation with a formulation containing high concentrations of MZ, but very low concentrations of L (group 3 and the earlier Macushield formulation [26]), fails to augment MP in the peripheral macula (where L is the dominant carotenoid). Consistent with this, supplementation with a formulation containing high concentrations of L, but little or no MZ (group 1), results in augmentation of MP in the peripheral and central macula, the central augmentation probably attributable to the presence of Z in that formulation.

At baseline and at the final visit, the relationship between serum carotenoid concentrations and MPOD was investigated separately for AMD and normal subjects (Table 4). This analysis was performed across intervention groups, in order to investigate the impact of disease status on the relationships (if any) between MPOD (at each eccentricity) and serum xanthophyll status following supplementation and regardless of intervention. Not surprisingly, at baseline, a significant and positive relationship was found between serum L and MPOD at each eccentricity for the normal (but not for AMD) subjects, supporting the view that MPOD in healthy subjects is influenced by the major xanthophyll in the blood [27, 28]. Unexpectedly, serum Z concentrations were mostly correlated with MPOD in the subjects with AMD. The concentration of Z in serum is low, but the significant correlations with MPOD in the AMD subjects may indicate attempts by patients with this condition to replace MZ in the macula with Z due to inefficiency in the bio-conversion of endogenous retinal L to retinal MZ [12]. However, relationships between the serum xanthophyll concentrations and MPOD at baseline were all positive, and it is therefore possible that small sample sizes may have contributed to our somewhat unexpected observations.

There were no correlations between the serum concentrations of L or Z or changes in their concentration at 8 weeks with MPOD measurements in subjects with or without AMD. The factors responsible for the lack of correlation may have been the insufficient time for a equilibrium to be achieved between the macular pigment and the elevated serum xanthophyll concentrations following supplementation, or the presence of MZ in two of the supplements caused alterations in pigment distribution within the macula, or both. The fact that a correlation between serum MZ and MPOD at 8 weeks was observed in the subjects with AMD, but not in those with good vision, may be support for the 'alteration in pigment distribution' hypothesis.

The relationship between the final serum MZ correlations and MPOD at 8 weeks, at each eccentricity in subjects with AMD but not those without this condition, was somewhat unexpected. The initial analyses, however, included MPOD measurements for subjects in all treatment groups. The high L supplement (group 1) contained only trace amounts of MZ, and therefore the impact of the high L in this supplement may have attenuated any direct effects of supplemental MZ on MPOD augmentation. Accordingly, when we excluded group 1 subjects from the analyses, we observed stronger

relationships between serum MZ concentrations and final MPOD measurements at all eccentricities (Table 5). More detailed analysis of these relationships confirmed that the greatest augmentation of MPOD was in the patients with AMD and that the group 2 supplement (containing equal amounts of MZ and L) resulted in the strongest relationship between serum MZ concentrations and MPOD at 1.75 eccentricity. Surprisingly, final serum concentrations of L (or even the changes in serum concentrations of L) were not correlated with MPOD in groups 2 and 3 (combined) at 1.75° eccentricity (data not shown), suggesting that supplementation with MZ in combination with L may represent a formulation better suited to augment MPOD across its spatial profile. In this short trial, subjects supplemented with the high MZ formulation exhibited the strongest correlations with MPOD at the central eccentricities, and it was also clear that subjects supplemented with both group 2 and group 3 formulations exhibited a positive relationship between MPOD and serum concentrations of MZ. However, it should be appreciated that the small sample sizes of each intervention group may have prevented some statistically significant associations amongst normal subjects or those receiving the group 2 supplement.

Following supplementation, AMD subjects no longer exhibited the positive and significant relationship between MPOD and serum Z that was observed at baseline. Furthermore, the changes in MPOD were not correlated with the changes in either serum L or Z concentrations. However, the change in MZ concentrations exhibited a significant relationship with MPOD, mainly amongst subjects with AMD, at all eccentricities. This finding is important, as it indicates that MP augmentation in AMD subjects may respond to increases in serum concentrations of MZ when this xanthophyll is provided in supplement form, and suggests that its provision may be an important means of MP augmentation in eyes where the bio-conversion of retinal L to MZ may be impaired.

Conclusion

Serum MZ response is positively related to MPOD following supplementation in AMD subjects, and a formulation containing equal amounts of L and MZ (10 mg of each) appears to result in a non-significantly greater augmentation of MP across the measured spatial profile, when compared with formulations lacking MZ or with only low doses of L, suggesting that provision of supplemental L and MZ in equal doses (possibly 10 mg of each as used here), with or without Z, may be an important determinant of MP augmentation in AMD-afflicted eyes.

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Declarations of Interest J. M. N. and S. B. do consultancy work for nutraceutical companies, in a personal capacity, and as directors of Nutrasight Consultancy Ltd. D. I. T. is a consultant to the Howard Foundation and receives consulting fees for same. A. N. H., D. I. T., S. B. and J. M. N. are named inventors on a patent application for "Improvements in or relating to visual performance and/or macular pigmentation", application number: PCT/GB2012/051567 held by the Howard Foundation, which is a UK charity established to support biomedical research.

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