

# ***Supplementation with all three macular carotenoids: response, stability and safety***

Eithne E. Connolly,<sup>1,2</sup> Stephen Beatty,<sup>1,2</sup> James Loughman,<sup>2,3,4</sup> Alan N. Howard,<sup>5,6</sup>

Michael S. Louw,<sup>7</sup> John M. Nolan<sup>1,2</sup>

<sup>1</sup>*Macular Pigment Research Group, Department of Chemical and Life Sciences, Waterford Institute of Technology, Waterford, Ireland;*

<sup>2</sup>*Institute of Vision Research, Whitfield Clinic, Cork Road, Waterford, Ireland;*

<sup>3</sup>*Department of Optometry, School of Physics, Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland;*

<sup>4</sup>*African Vision Research Institute, Faculty of Health Sciences, University of KwaZulu Natal, Durban, South Africa;*

<sup>5</sup>*Downing College, University of Cambridge, Cambridge, UK;*

<sup>6</sup>*Howard Foundation, Cambridge, UK;*

<sup>7</sup>*Biomnis Ireland, Dublin 18, Ireland.*

**Word Count: 3,995**

## **CORRESPONDING AUTHOR:**

John M. Nolan, Macular Pigment Research Group, Department of Chemical and Life Sciences, Waterford Institute of Technology, Waterford, Ireland; [jmnolan@wit.ie](mailto:jmnolan@wit.ie)

## **GRANT INFORMATION**

Supported by Macuvision Europe, Macuhealth, Macuhealth Canada and the Howard Foundation.

## **ABSTRACT**

### **Purpose**

This study was designed to investigate serum and macular response, and safety, to supplementation with *meso*-zeaxanthin (MZ), lutein (L) and zeaxanthin (Z), the carotenoids that constitute macular pigment (MP).

### **Materials and Methods**

44 healthy subjects were recruited into this randomized, placebo-controlled, clinical trial. Subjects consumed one tablet per day containing 10.6 mg MZ, 5.9 mg L and 1.2 mg Z (Intervention, I group) or placebo (P group). The spatial profile of MP optical density (MPOD) was measured using heterochromatic flicker photometry (HFP), and serum concentrations of L and Z were quantified using high performance liquid chromatography (HPLC). Subjects were assessed at baseline, three and six months. Clinical pathology analysis was performed at baseline and six months.

### **Results**

Serum concentrations of L and Z increased significantly in the I group ( $p = 0.001$  and  $0.003$ , respectively) and remained stable in the P group ( $p > 0.05$ ). There was a significant increase in central MPOD in the I group ( $0.25^\circ$ :  $p = 0.001$ ;  $0.5^\circ$ :  $p = 0.001$ ), with no significant change in the P group ( $p > 0.05$ ). Clinical pathology analysis confirmed that all variables remained within the normal reference range, with the exception of total cholesterol and low density lipoprotein (LDL), which exhibited baseline values outside the accepted normal reference range prior to supplementation.

### **Conclusion**

Subjects supplemented with MZ, L and Z exhibit significant increases in serum concentrations of these carotenoids, and a subsequent increase in central MPOD. Pathology analysis suggests no adverse clinical implications of consuming these carotenoids.

**KEYWORDS**

Macular pigment; lutein; *meso*-zeaxanthin; zeaxanthin; clinical pathology analysis

## INTRODUCTION

The macula houses a yellow pigment, attributable to the carotenoids *meso*-zeaxanthin (MZ), lutein (L), and zeaxanthin (Z). Indeed, this pigment lends its name to the *macula lutea* (Latin for yellow), and is more recently referred to as macular pigment (MP).<sup>1</sup> Interestingly, of the over 700 carotenoids identified in nature, these three dietary carotenoids selectively accumulate at the macula,<sup>1-3</sup> indicating an exquisite degree of biological selectivity in this retinal tissue.

An average western diet contains 1.3-3 mg/day of L and Z combined,<sup>4</sup> with substantially more L than Z (represented by an estimated ratio of circa 7:1). It has been reported that approximately 78% of dietary L and Z is sourced from vegetables, with L found in highest concentrations in dark green leafy vegetables.<sup>5</sup> It appears that humans ingest relatively low levels of MZ, although it should be noted that there has been no satisfactory published investigation of MZ concentrations in the foods of a typical diet. Interestingly, in spite of its absence or low concentrations in a normal diet, MZ accounts for about one third of total MP at the macula, consistent with the hypothesis that retinal MZ is produced primarily by isomerisation of retinal L at the macula.<sup>6</sup>

Age-related macular degeneration (AMD) is a degenerative condition of the macula, and its late form is the most common cause of blind registration in the developed world.<sup>7</sup> It is now accepted that AMD is the result of (photo) oxidative-induced retinal injury. However, the anatomic (central retinal),<sup>8</sup> biochemical (antioxidant)<sup>9</sup> and optical (short wavelength-filtering)<sup>10</sup> properties of MP, suggest that this pigment may confer protection against AMD (protective hypothesis).<sup>11</sup> Also, its optical (short wavelength-filtering) properties suggest that MP plays a role in visual performance and experience in the normal population (visual performance hypothesis).<sup>12</sup> The protective and visual performance hypotheses of MP have led to significant research in this area. However, questions asked by eye care professionals often relate to the response (in blood and at the macula), and safety, following supplementation with these carotenoids.

This study was designed to assess response, and also the safety of consumption of the macular carotenoids MZ, L and Z by analysing blood samples for changes in renal and liver function, as well as lipid profile, haematological profile, and markers of inflammation after six months of supplementation.

## **METHODS**

### **Study design**

The *meso*-zeaxanthin ocular supplementation trial in normals (MOST-N) is a double blind, randomized, placebo controlled, clinical trial registered with the International Standard Randomized Controlled Trial Number Register (ISRCTN60816411). All subjects signed an informed consent document and the experimental measures conformed to the tenets of the Declaration of Helsinki. The study was reviewed and approved by the Research Ethics Committee, South East Region, Waterford Regional Hospital, Waterford, Ireland and by the Ethics Committee at Waterford Institute of Technology, Waterford, Ireland.

44 healthy subjects were recruited into the study, which consisted of two groups: Intervention (I, n = 22) and Placebo (P, n = 22). The inclusion criteria were as follows: male or female between the age of 18 and 61 years; absence of ocular pathology by self report; no clinical signs of retinal pathology as assessed by expert assessment of fundus photographs; visual acuity of at least 20/60 in the study eye; not currently taking supplements containing MZ, L and Z; not pregnant.

### **Supplementation and examination procedures**

The formulation for this study was manufactured by Industrial Organica SA, Monterrey, Mexico by isomerising L obtained from marigold extracts. A proportion of L (60%) was converted into MZ, and the small quantity of Z in the extract remained unchanged. The resulting composition was microencapsulated after diluting with rice starch. Following consistency testing, it was

confirmed that the capsules contained 10.6 mg MZ, 5.9 mg L, and 1.2 mg Z (confirmed by high performance liquid chromatography [HPLC] analysis). The placebo consisted of rice starch and was microencapsulated to look identical to the carotenoid I capsule.

All subjects were instructed to take one capsule per day with a meal for six months. At baseline (V1), demographic, lifestyle and vision information was also collected from each subject including: name; contact information; age; sex; body mass index (BMI); smoking habits; lifestyle; medication and vision history. Baseline dietary intakes of L and Z were quantified using a self-administrated, semiquantitative food frequency questionnaire developed by the Scottish Collaborative Group at the University of Aberdeen (Scotland UK), recently described by O'Connell et al.<sup>13</sup>

Best corrected visual acuity (BCVA) was measured at baseline using a computer generated LogMAR test chart (Test Chart 2000 Pro; Thompson Software Solutions, Hatfield, UK) at a viewing distance of 4 metres, using the Sloan ETDRS letterset. BCVA was recorded using a letter-scoring visual acuity rating, with 20/20 visual acuity assigned a value of 100. BCVA was scored relative to this value, with each letter correctly identified assigned a nominal value of one, for example, a BCVA of 20/20<sup>+1</sup> equated to a score of 101, and 20/20<sup>-1</sup> to 99. The eye with better visual acuity was chosen as the study eye; however, where both eyes had the same corrected acuity, the right eye was chosen as the study eye.

Contrast sensitivity was measured using a computer generated letter contrast test (Thompson Test Chart 2000 Pro; Thompson Software Solutions, Hatfield, UK), similar in design to a Pelli-Robson chart.<sup>14</sup>

Retinotopic ocular sensitivity was assessed by Microperimetry using a Nidek MP1 device. The central 6° of fixation were examined and are reported as Macular Mean Sensitivity (MMS) within 2°, 4° and 6° of the macula. Fundus photography was also performed at each study visit, and the photographs were assessed by a vitreo retinal specialist to confirm the absence of significant retinal pathology. MPOD, including its spatial profile (i.e. 0.25°, 0.5°, 1°, 2°, 4°, 6°, 8°, 10°, 12°, 14°, 16°, 18°, 20°), was also measured.

1.75°), was measured at V1, V2, and V3 using customised heterochromatic flicker photometry (cHFP) method previously described.<sup>15</sup>

A blood sample was collected at each study visit (i.e. baseline, three and six months [V1, V2, V3, respectively]) for serum carotenoid analysis of L and Z, using a method previously described by our group.<sup>15</sup> Additional blood samples were collected at V1 and V3 for clinical pathology analysis (see below).

### **Clinical pathology analysis**

Clinical pathology analysis was carried out on all subjects at V1 prior to supplementation and at V3 (i.e. after six months) in order to test for any change in renal and liver function, lipid profile, haematological profile, and markers of inflammation following supplementation with MZ, L and Z. To achieve this, non fasting blood samples were collected at both visits using standard venepuncture techniques. The blood was collected in three plastic collection tubes as follows: Tube 1 (serum): contained an added clot activator and gel layer; Tube 2 (glucose): contained sodium fluoride; Tube 3 (haematology): contained the anticoagulant dipotassium ethylene diamine tetra-acetic acid (K<sub>2</sub>EDTA). All collection tubes were labelled with the subject's number, visit and date, and were inverted a minimum of eight times to ensure appropriate mixing of the blood with each additive in the tubes.

The serum tube was centrifuged within two hours of collection and a 1 mL sample was aliquoted into a clean labelled plastic tube which was then transported with the other two tubes to Biomnis Ireland, Dublin, Ireland (Irish National Accreditation Board certified), for independent analysis. All pathology variables tested are outlined in Table 1. Analysis at Biomnis Laboratories was conducted using an Abbott Architect ci8200 (ABBOTT, Abbott Park, IL, USA) and Advia 120 (Siemens Healthcare Diagnostics, Deerfield, IL, USA), as appropriate. The reference ranges for this study were obtained from the insert kits for the instrumentation used by Biomnis laboratories. The only exceptions were the lipids (high density lipoproteins (HDL), LDL

total cholesterol and triglycerides) where the reference ranges come from the European Guidelines on Cardiovascular Disease Prevention<sup>16</sup> and glucose, whose reference range comes from the World Health Organisation.<sup>17</sup>

## **STATISTICAL ANALYSIS**

The statistical software package SPSS (version 17) was used for analysis and SigmaPlot (version 8.0) was used for graphical presentations. Means  $\pm$  SDs are presented in the text and tables. Between group differences in age, BMI, baseline serum carotenoid concentration and baseline MPOD levels were investigated using independent samples t-tests. Between group difference with respect to sex and smoking habits were investigated using the standard Chi square test. Pearson correlation coefficient analyses were conducted to investigate bivariate relationships. Repeated measures analysis of variance was conducted to investigate changes in serum concentrations of L and Z, and MPOD (including its spatial profile) across the three study visits, using a general linear model approach. Differences between two time points, within subjects, were assessed using paired samples t-test. We used the 5% level of significance throughout our analysis.

**Table 1.** Clinical pathology variables assessed at baseline (V1) and following six months' (V3) supplementation with meso-zeaxanthin, lutein and zeaxanthin in both the intervention and placebo groups.

Pathology variable	Function of test	Reference Range (Unit)	V1 I*	V3 I	p value I	V1 P†	V3 P	p value P
Sodium	Renal profile	135-145 (mmol/L)	139.42±1.68	139.26±2.08	0.51	139.26±2.05	139.26±1.69	1.00
<b>Potassium</b>	Renal profile	3.3-5.3 (mmol/L)	4.16±0.36	4.55±0.40	<b>0.01</b>	4.26±0.30	4.43±0.24	<b>0.04</b>
Chloride	Renal profile	98-107 (mmol/L)	104.05±2.55	98.89±21.35	0.32	104.05±1.72	103.11±1.97	0.15
Urea	Renal profile	2.5-7.7 (mmol/L)	4.72±1.16	5.03±1.11	0.23	5.31±1.40	5.37±1.53	0.76
Creatinine	Renal profile	40-90 (µmol/L)	75.11±14.13	76.84±11.70	0.42	77.00±14.36	74.68±14.97	0.15
Total protein	Liver profile	64-83 (g/L)	72.63±3.53	71.05±3.12	0.10	71.63±3.58	70.05±4.97	0.12
Albumin	Liver profile	37-52 (g/L)	44.47±1.84	44.58±2.67	0.82	43.53±1.98	44.21±3.78	0.30
<b>Globulins</b>	Liver profile	21-36 (g/L)	28.16±3.29	26.47±2.95	0.11	28.11±3.63	26.37±4.11	<b>0.07</b>
Total bilirubin	Liver profile	3.4-21.0 (µmol/L)	8.73±4.94	8.21±3.85	0.59	8.05±2.62	8.77±2.99	0.29
Alanine aminotransferase	Liver profile	0-55 IU/L	24.32±18.18	19.42±7.62	0.18	22.47±14.11	23.16±14.72	0.63
Aspartate aminotransferase	Liver profile	5-36 IU/L	20.37±4.68	19.05±4.59	0.16	22.16±8.25	21.89±10.13	0.81
Alkaline phosphate	Liver profile	40-150 IU/L	78.84±27.32	74.63±17.65	0.41	79.00±62.93	79.95±76.25	0.80
Gamma glytamyl transpeptidase	Liver profile	9-36 IU/L	33.84±40.39	25.05±17.25	0.29	25.16±12.33	23.89±11.55	0.42
<b>Cholesterol total</b>	Lipid profile	<5.0 (mmol/L)	5.21±0.92	5.24±0.91	0.79	5.26±0.93	4.92±0.86	<b>0.02</b>
Triglycerides	Lipid profile	0.60-1.70 (mmol/L)	1.38±0.75	1.66±0.93	0.13	1.10±0.44	1.09±0.68	0.93
HDL	Lipid profile	1.00-1.55 (mmol/L)	1.46±0.33	1.49±0.31	0.63	1.54±0.32	1.51±0.32	0.46
<b>Direct LDL</b>	Lipid profile	<3.0 (mmol/L)	3.03±0.75	3.25±0.80	<b>0.01</b>	3.13±0.84	2.98±0.80	0.23
Calcium	Bone profile	2.10-2.60 (mmol/L)	2.38±0.07	2.35±0.10	0.33	2.36±0.09	2.36±0.12	0.80
Phosphate	Bone profile	0.80-1.56 (mmol/L)	1.16±0.16	1.14±0.15	0.63	1.10±0.21	1.09±0.13	0.82
<b>Magnesium</b>	Bone profile	0.65-1.10 (mmol/L)	1.00±0.07	0.95±0.09	<b>0.01</b>	0.98±0.06	0.92±0.06	<b>0.01</b>
Uric Acid	Bone profile	155-394 (µmol/L)	263.47±94.34	273.47±85.91	0.19	274.68±88.78	271.74±85.68	0.76
Glucose	Bone profile	3.1-6.1 (mmol/L)	5.31±2.10	5.77±2.94	0.11	5.03±0.41	4.94±0.47	0.50
High sensitive reactive protein	Inflammation marker	<5.0 (mg/L)	4.00±7.36	3.31±4.88	0.57	1.49±1.25	4.18±13.40	0.40

**Full blood count**

White cell count	Haematology	3.88-10.49 (10e9/L)	7.07±2.00	6.79±1.49	0.24	5.97±1.24	6.92±2.34	0.10
Red cell count	Haematology	3.73-5.02 (10e12/L)	4.53±0.43	4.58±0.40	0.35	4.64±0.36	4.58±0.36	0.30
<b>Haemoglobin</b>	Haematology	11.3-15.2 (g/dL)	14.23±1.35	13.91±1.37	<b>0.03</b>	14.46±1.46	13.85±1.28	<b>0.01</b>
<b>Haematocrit</b>	Haematology	0.323-0.462 (L/L)	0.40±0.04	0.41±0.04	<b>0.01</b>	0.40±0.04	0.41±0.03	0.38
<b>MCV‡</b>	Haematology	83.1-99.1 (fL)	87.93±4.33	90.41±4.54	<b>0.01</b>	87.06±3.02	89.42±3.22	<b>0.01</b>
<b>MCH§</b>	Haematology	28.3-33.9 (pg)	31.42±1.51	30.38±1.54	<b>0.01</b>	31.15±1.58	30.28±1.34	<b>0.01</b>
<b>MCHC  </b>	Haematology	32.1-36.6 (g/dL)	35.75±0.98	33.62±0.93	<b>0.01</b>	35.78±1.36	33.88±1.09	<b>0.01</b>
<b>Platelets</b>	Haematology	164-382 (10e9/L)	295.47	287.00	0.24	313.28	299.00	<b>0.08</b>
<b>Differential White Cell Count</b>								
Neutrophils	Haematology	1.91-7.16 (10e9/L)	4.39±1.57	4.05±1.01	0.15	3.44±0.72	4.18±2.02	0.16
<b>Lymphocytes</b>	Haematology	1.01-3.13 (10e9/L)	1.85±0.67	1.86±0.57	0.92	1.72±0.65	1.87±0.73	<b>0.04</b>
Monocytes	Haematology	0.19-0.68 (10e9/L)	0.42±0.10	0.39±0.80	0.23	0.36±0.08	0.40±0.14	0.21
Eosinophils	Haematology	0.05-0.51 (10e9/L)	0.25±0.20	0.27±0.15	0.62	0.24±0.17	0.23±0.12	0.79
Basophils	Haematology	0.02-0.15 (10e9/L)	0.07±0.03	0.07±0.02	0.71	0.10±0.07	0.07±0.04	0.10
<b>Large unstained cells</b>	Haematology	0.00-0.30 (10e9/L)	0.14±0.03	0.13±0.03	0.81	0.12±0.04	0.16±0.06	<b>0.01</b>

Paired samples t-tests were carried out on all variables between baseline and six months. This table shows mean ± SD for all variables tested.

\* Intervention group

† Placebo group

‡ Mean corpuscular volume

§ Mean corpuscular hemoglobin

|| mean corpuscular hemoglobin concentration

## RESULTS

### Baseline

The demographic, lifestyle, dietary intake of L and Z (mg/day), serum concentrations of L and Z ( $\mu\text{mol/L}$ ), and MPOD data at baseline for the I and P groups (n=44) are presented in Table 2.

There was no statistically significant difference between groups in terms of any of these variables at baseline ( $p > 0.05$ , for all). Statistically significant relationships between variables, at baseline, are presented in Table 3 and Figure 1.

**Table 2.** Baseline characteristics of the intervention and placebo group.

Characteristic	Intervention (n = 22)	Placebo (n = 22)
<b>Age (n)</b>	43 $\pm$ 13	45 $\pm$ 12
18-30	5	4
31-40	3	3
41-50	6	6
51-60	6	9
61	2	0
<b>BMI*</b>	27.2 $\pm$ 6.1	26.8 $\pm$ 5
<b>BCVA<sup>†</sup></b>	116 $\pm$ 7.8	116 $\pm$ 7.9
<b>Log letter contrast sensitivity</b>	1.61 $\pm$ 0.17	1.60 $\pm$ 0.25
<b>Microperimetry MMS2<sup>o‡</sup></b>	13.43 $\pm$ 2.0	13.09 $\pm$ 2.3
<b>Microperimetry MMS4<sup>o</sup></b>	13.05 $\pm$ 1.8	12.63 $\pm$ 1.7
<b>Microperimetry MMS6<sup>o</sup></b>	11.05 $\pm$ 1.9	10.69 $\pm$ 1.8
<b>Dietary Lutein (mg/day)</b>	1.33 $\pm$ 0.76	1.19 $\pm$ 0.74
<b>Dietary Zeaxanthin (mg/day)</b>	0.19 $\pm$ 0.07	0.21 $\pm$ 0.16
<b>Serum Lutein</b>	0.40 $\pm$ 0.12	0.40 $\pm$ 0.17
<b>Serum Zeaxanthin</b>	0.18 $\pm$ 0.07	0.20 $\pm$ 0.08
<b>Macular pigment optical density</b>		
0.25 <sup>o</sup>	0.45 $\pm$ 0.21	0.45 $\pm$ 0.19
0.5 <sup>o</sup>	0.37 $\pm$ 0.18	0.38 $\pm$ 0.19
1 <sup>o</sup>	0.26 $\pm$ 0.13	0.23 $\pm$ 0.12
1.75 <sup>o</sup>	0.13 $\pm$ 0.08	0.09 $\pm$ 0.09
<b>Sex (n)</b>		
Male	8	9
Female	14	13
<b>Smoking habits<sup>§</sup> (n)</b>		
Current	5	4
Past	8	4
Never	9	14

Data are presented as mean  $\pm$  SD unless otherwise noted.\*BMI Body mass index defined as body weight

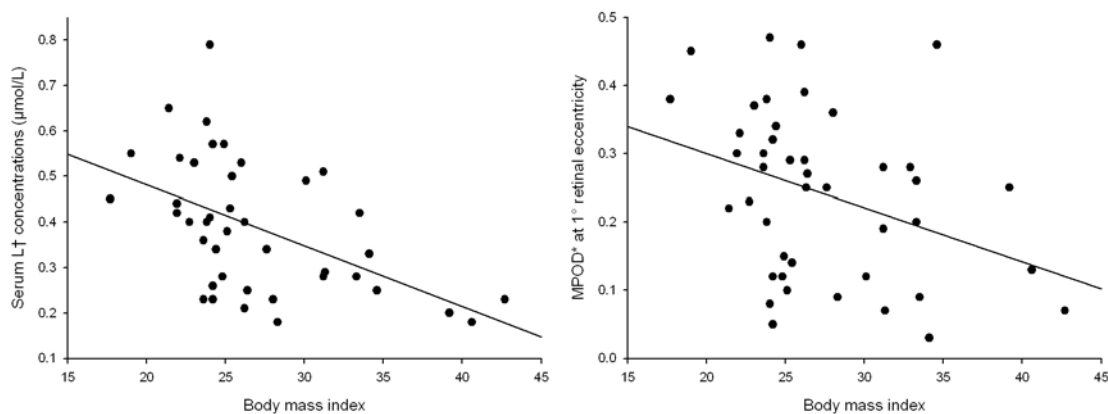
in kilograms divided by height in squared meters ( $\text{kg}/\text{m}^2$ ); <sup>†</sup>BCVA Best corrected visual acuity (recorded using a letter-scoring visual acuity rating, with 20/20 visual acuity assigned a value of 100. BCVA was scored relative to this value, with each letter correctly identified assigned a nominal value of one, for example, a BCVA of 20/20<sup>+1</sup> equated to a score of 101, and 20/20<sup>-1</sup> to 99); <sup>‡</sup>MMS Macular mean sensitivity (as defined by the mean retinotopic ocular sensitivity within 2°, 4°, 6° of the macula); <sup>§</sup>Smoking habits (never smokers had smoked less than 100 cigarettes in their lifetime. Past smokers had smoked at least 100 cigarettes in their lifetime, but had not smoked for at least one year prior to investigation. Current smokers had smoked at least 100 cigarettes in their lifetime and had at least one cigarette in the year prior to investigation). Independent samples t-test resulted in no statistical difference between groups and differences between smoking and gender was analysed using chi square analysis.

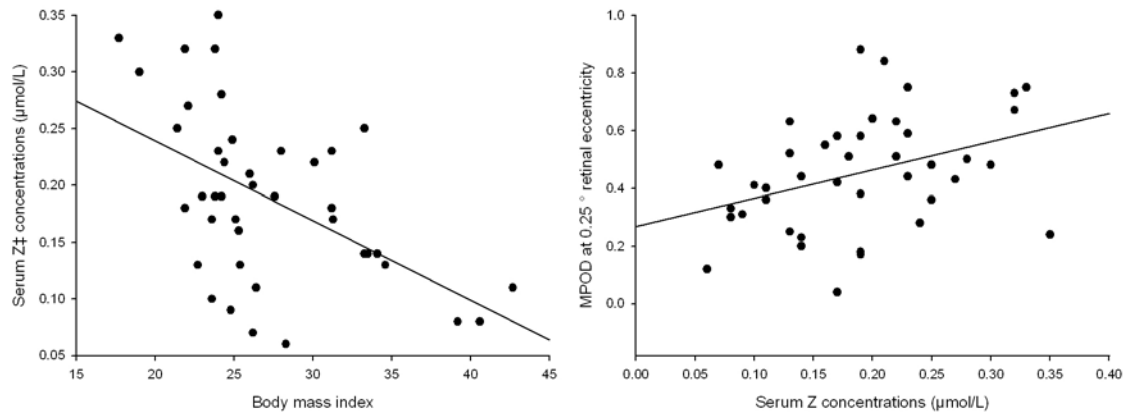
**Table 3.** Significant relationships between baseline variables for the entire study group before intervention (n = 44)

Dependent variable	Independent variable	Pearson coefficient (r)	Significance (p)
MPOD <sup>†</sup> 0.5°	BMI	-0.322	0.035
MPOD 1°	BMI	-0.355	0.019
MPOD 1.75°	BMI	-0.322	0.035
Serum Lutein	BMI	-0.516	0.001
Serum Zeaxanthin	BMI	-0.524	0.001
MMS 2° <sup>‡</sup>	Age	-0.409	0.007
MPOD 0.25°	Serum Zeaxanthin	0.373	0.016
MMS 2°	MPOD 0.25°	0.304	0.050
MPOD 1°	Serum Zeaxanthin	0.343	0.028
Serum Lutein	Age	0.318	0.040
Total Cholesterol	Age	0.439	0.004
BCVA <sup>§</sup>	Serum Lutein	0.318	0.040
Serum Lutein	Diet Lutein	0.374	0.017

\*BMI Body mass index; <sup>†</sup>MPOD Macular pigment optical density; <sup>‡</sup>MMS Macular mean sensitivity (as defined by the mean within 2° of the macula); <sup>§</sup>BCVA Best corrected visual acuity

**Figure 1.** Statistically significant relationships between baseline variables (n=44)





\*MPOD = Macular pigment optical density; †L = Lutein; ‡Z= Zeaxanthin

### Compliance to study visits

Of the 44 subjects recruited into this study, 18 subjects from the I group, and 17 subjects from the P group, attended and completed all study visits (i.e. V1, V2 and V3). Four subjects were lost to follow-up (personal reasons [e.g. death in family]), and the remainder did not attend V2.

### Retinal findings

There was no significant change observed in retinal sensitivity at six months for any of the microperimetry tests performed (i.e. MMS 2°, MMS 4°, MMS 6°,  $p > 0.05$ , for all tests). There was no noticeable change in retinal findings at six months (confirmed by a vitreo retinal specialist).

### Lutein and zeaxanthin response in serum

There was a statistically significant increase in serum concentrations of L and Z ( $\mu\text{mol/L}$ ) from baseline at three months (L:  $p = 0.001$ ; Z:  $p = 0.001$ ) and six months (L:  $p = 0.001$ ; Z:  $p = 0.001$ ) in the I group. There was no significant change from baseline in serum concentrations of L or Z ( $\mu\text{mol/L}$ ) in the P group over this period ( $p > 0.05$ , for both). These findings are consistent with

repeated measures analysis of variance which showed a statistically significant time/arm interaction effect ( $p = 0.001$  for L and  $p = 0.003$  for Z) [see Figure 2a].

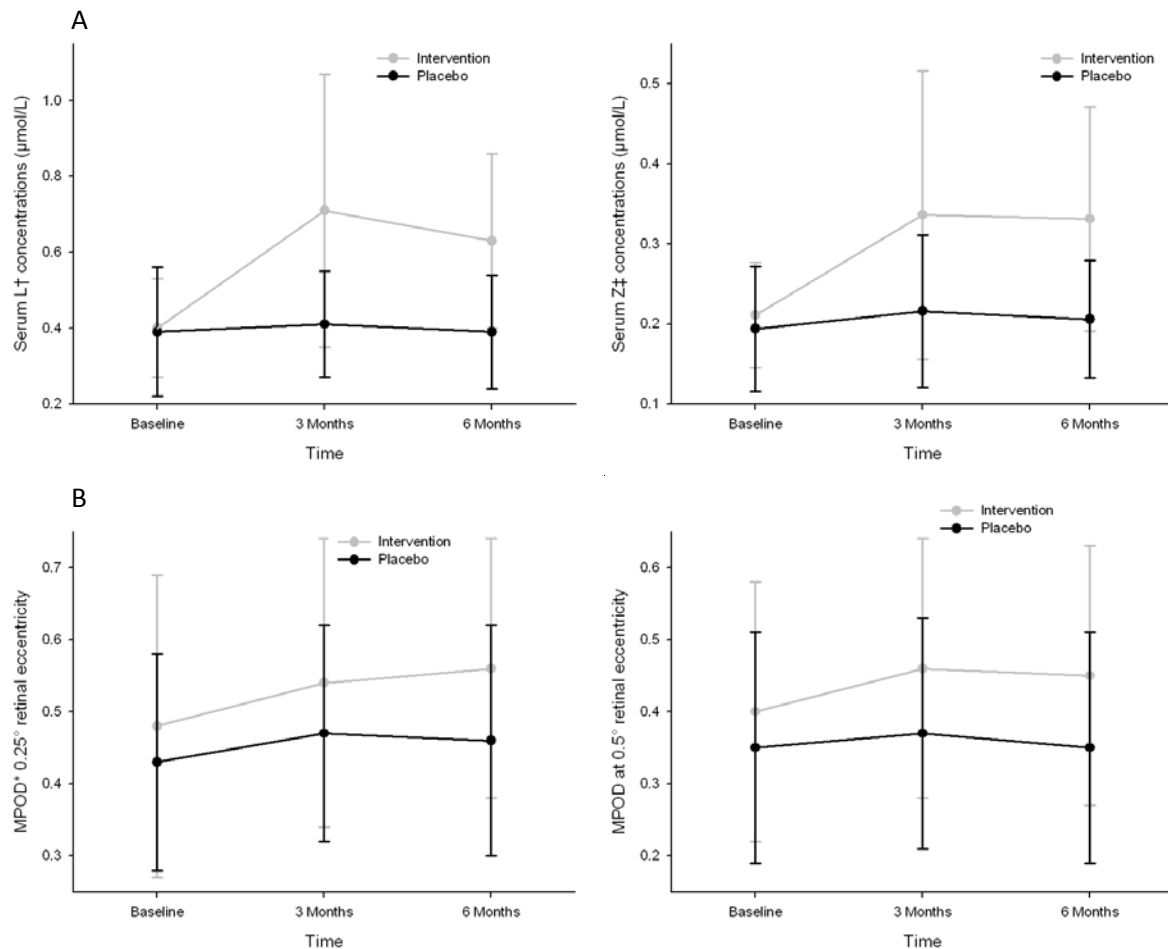
### **Macular pigment optical density response**

There was a statistically significant increase in MPOD at  $0.25^\circ$  retinal eccentricity from baseline at three months and six months in the I group ( $p = 0.001$ , for both). There was no significant change from baseline in MPOD at  $0.25^\circ$  retinal eccentricity in the P group at either three months or six months ( $p > 0.05$ , for both). Repeated measures analysis did not show a statistically significant time/arm interaction effect ( $p > 0.05$ ) [see Figure 2b].

There was a statistically significant increase in MPOD at  $0.5^\circ$  retinal eccentricity from baseline at three months and six months in the I group ( $p = 0.001$  and  $0.01$ , respectively). There was no significant change observed at this eccentricity in the P group at three months or six months ( $p > 0.05$ , for both). Repeated measures analysis showed a significant time/arm interaction effect ( $p = 0.016$ ) [see Figure 2b].

There was no statistically significant increase at  $1^\circ$  and  $1.75^\circ$  retinal eccentricity from baseline at three months or six months in either the I or P group ( $p > 0.05$ , for all).

**Figure 2** Change in central macular pigment optical density and serum lutein and zeaxanthin concentrations for the intervention and placebo group.



\*MPOD = Macular pigment optical density; <sup>†</sup>L = Lutein; <sup>‡</sup>Z = Zeaxanthin; Note: data presented here is mean  $\pm$  SD for subjects that attended each study visit (n = 18, I group; n = 17, P group).

### Clinical pathology analysis

We report statistically significant variation from baseline to six months (in both positive and negative directions) in 8 of the 25 variables assessed in the I group and 9 of the 25 variables assessed in the P group following supplementation with the macular carotenoids (Table 1).

However, all variables remained within the normal reference range, with the exception of total cholesterol and LDL, which had a baseline value outside the accepted normal reference range in both the I and P groups prior (i.e. at baseline) to supplementation with the macular

carotenoids (high density lipoproteins [HDL] and LDL, the total, cholesterol reference ranges were taken from the European Guidelines on Cardiovascular Disease Prevention.

## **DISCUSSION**

The MOST-N study was designed to measure serum and macular response to a dietary supplement containing all three macular carotenoids (MZ, L, and Z) in the normal population (Irish Republic), as part of a randomized, double blind, placebo controlled, clinical trial, and to assess safety of consumption of these carotenoids by performing clinical pathology analysis.

To date there have been many published studies in the scientific literature that have reported on the effect of macular carotenoid supplementation on serum concentrations of these carotenoids, with the majority of these studies reporting significant increases in serum concentrations of L and Z following supplementation with these carotenoids (see Table 4), and recent studies have reported and confirmed significant serum MZ response following supplementation with this carotenoid. Consistent with these previous studies, we report statistically significant increases in serum concentrations of L and Z in the I group; whereas, as expected, the P group remained stable over the study period. MZ was not quantified separately as part of the current study; however, MZ response is detected as part of the Z peak in the HPLC assay used herein. Indeed, we report a 1.5-fold increase in serum concentrations of L (Baseline:  $0.39 \pm 0.15 \mu\text{mol/L}$ ; Final:  $0.50 \pm 0.22 \mu\text{mol/L}$ ), and a 1.6-fold increase in serum concentrations of Z (Baseline:  $0.21 \pm 0.03 \mu\text{mol/L}$ ; Final:  $0.72 \pm 0.11 \mu\text{mol/L}$ ), which are somewhat poorer responses than other studies supplementing with similar amounts of these carotenoids.<sup>18;19</sup> Possible reasons for this lower than normal responses are discussed below, following our discussion on MPOD.

**Table 4.** Serum carotenoid response following supplementation with the macular carotenoids

Principal Author	Journal	Year	N	L (mg/day)	Z (mg/day)	MZ (mg/day)	Duration (weeks)	Baseline L (µmol/L)	Final L (µmol/L)	Rise (%) L	Baseline Z (µmol/L)	Final Z (µmol/L)	Rise (%) Z	Baseline MZ (µmol/L)	Final MZ (µmol/L)	Rise (%) MZ
<i>Normal Subjects</i>																
Berendschot et al.	IOVS	2000	8	10	0	0	12	0.18 ± 0.08	0.9 ± 0.18	400	-	-	-	-	-	-
Johnson et al.	AJCN	2000	7	19.7	1	0	15	0.37 ± 0.05	0.67 ± 0.11	81	0.06 ± 0.01	0.07 ± 0.01	17	-	-	-
Hughes et al.	JID	2000	21	15	0	0	4	0.37	1.753	374	-	-	-	-	-	-
Bone et al.	JN	2003	21	2.4	0	0	24	0.245 ± 0.12	0.484 ± 0.176	98	-	-	-	-	-	-
			2	30	1.5	0	20	0.158	2.06	1204	-	-	-	-	-	-
			2	0	30	0	12	-	-	-	0.09	0.52	478	-	-	-
Koh et al.	EER	2004	6	10	0	0	19	0.27 ± 0.1	1.95 ± 1.06	622	-	-	-	-	-	-
Zhao et al.	AJCN	2006	8	12	0	0	8	0.17	0.874	514	-	-	-	-	-	-
Schalch et al.	ABB	2007	18	10.7	0.8	0	24	0.16 ± 0.07	1.104	590	0.05 ± 0.02	0.145	190	-	-	-
			16	0	12.6	0	24	0.13 ± 0.04	0.303	133	0.04 ± 0.03	1.09	2625	-	-	-
			19	10.2	11.9	0	24	0.17 ± 0.07	0.63	270	0.06 ± 0.03	0.81	1250	-	-	-
Bone et al.	NM	2007	10	5.5	1.4	14.9	17	0.31 ± 0.13	0.38 ± 0.12	23	0.097 ± 0.05	0.26 ± 0.07	168	0	94.5	-
Wenzel et al.	OPO	2007	3	30	2.7	0	17	-	-	~1500	-	-	~278	-	-	-
Thurnham et al.	BJN	2008	19	10.8	1.2	8	3	0.28 ± 0.13	0.88 ± 0.33	221	0.05 ± 0.02	0.37 ± 0.15	640	0	0.21 ± 0.13	-
Johnson et al.	AJCN	2008	11	12	0.5	0	16	0.28 ± 0.04	0.60	114	-	-	-	-	-	-
			16	0.32 ± 0.04	0.81	153	-	-	-	-	-	-	-	-		
Bone et al.	ABB	2010	24	20	0	0	20	0.199	1.62	714	-	-	-	-	-	-
			14	20	0	0	20	0.289	1.35	367	-	-	-	-	-	-
			22	10	0	0	20	0.301	1.01	235	-	-	-	-	-	-
			17	5	0	0	20	0.289	0.743	157	-	-	-	-	-	-
Connolly et al.	CER	2010	5	3.7	0.8	7.3	8	0.31 ± 0.086	0.386	25	0.17 ± 0.78	0.19	12	0.02 ± 0.01	0.066	230
Nolan et al.	VR	2011	61	12	1	0	48	0.57	1.40	146	0.36	0.39	8	-	-	-
<i>AMD Subjects</i>																
Koh et al.	EER	2004	7	10	0	0	19	0.32 ± 0.22	1.89 ± 0.29	491	-	-	-	-	-	-
Khachik et al.	IOVS	2006	15	2.5	0.13	0	24	0.28 ± 0.03	0.5 ± 0.11	79	0.057 ± 0.01	0.095 ± 0.01	67	-	-	-
			15	5	0.25	0	24	0.21 ± 0.03	0.72 ± 0.11	243	0.057 ± 0.01	0.095 ± 0.01	67	-	-	-

			15	10	0.5	0	24	0.21 ± 0.03	1 ± 0.11	376	0.057 ± 0.01	0.095 ± 0.01	67	-		
<b>Trieschmann et al.</b>	EER	2007	97	12	1	0	36	0.158	0.44	178	-	-	-	-	-	-
<b>Huang et al.</b>	IOVS	2008	20	10	2	0	24	0.316	0.877	177	0.08	0.19	138	-	-	-
			20	10	2	0	24	0.369	0.650	76	0.08	0.15	88	-		
<b>Connolly et al.</b>	CER	2010	5	3.7	0.8	7.3	8	0.29 ± 0.13	0.336	17	0.093 ± 0.036	0.15	61	0.02 ± 0.01	0.052	160

L = Lutein (mg/day); Z = Zeaxanthin (mg/day); MZ = *Meso*-zeaxanthin (mg/day); n = Number of subjects participating in study; Age = Age range (years) of subjects in study; Duration = duration of supplementation; ABB = Archives of Biochemistry and Biophysics; BJN = British Journal of Nutrition; IOVS = Investigative Ophthalmology and Visual Science; AJCN = American Journal of Clinical Nutrition; JN = Journal of Nutrition; JID = Journal of Infectious Diseases; VR = Vision Research; EER = Experimental Eye Research; CER = Current Eye Research; NM = Nutrition and Metabolism; OPO = Ophthalmic and Physiological Optics; - = data unavailable.\* includes MZ supplementation

**Table 5.** Studies reporting on macular pigment optical density response to supplementation with the macular carotenoids.

Principal Author	Year	Journal	N	Age	L mg/d	Z mg/d	MZ mg/d	Duration (weeks)	Tec	Retinal ecc	PF	MP rise	Sig.
<b>NORMAL subjects - dietary modification</b>													
Hammond et al.	1997	IOVS	10	30-65	11.2	0.6	0	15	HFP	0.5°	5.5°	~ 0.05	p < 0.05
Hammond et al.	1997	IOVS	2	30-65	0.4	0.3	0	15	HFP	0.5°	5.5°	~ 0.05	-
Hammond et al.	1997	IOVS	1	30-65	10.8	0.3	0	15	HFP	0.5°	5.5°	~ 0.05	p < 0.05
Johnson et al.	2000	AJCN	7	33-54	11.2	0.57	0	15	HFP	0.5°	5.5°	~ 0.07	p < 0.05
<b>NORMAL subjects - supplement modification</b>													
Landrum et al.	1997	EER	2	42-51	30	0	0	20	HFP	0.75°	8°	~ 0.20	-
Berendschot et al.	2000	IOVS	8	18-50	10	0	0	12	SLO	0.75°	14°	~ 0.05	p = 0.022
Berendschot et al.	2000	IOVS	8	18-50	10	0	0	12	SA	0.75°	-	~ 0.04	p < 0.001
Aleman et al.	2001	IOVS	8	11-59	20	0	0	24	HFP	0.17°	5-7°	0.07	p = 0.04
Aleman et al.	2001	IOVS	8	11-59	20	0	0	24	HFP	0.5°	5-7°	0.07	-
Aleman et al.	2001	IOVS	8	11-59	20	0	0	24	HFP	1°	5-7°	0.08	-
Aleman et al.	2001	IOVS	8	11-59	20	0	0	24	HFP	2°	5-7°	0.04	-
Bone et al.	2003	JN	2	19-59	30	1.5	0	20	HFP	0.75°	8°	~ 0.20	-
Bone et al.	2003	JN	1	53	0	30	0	17	HFP	0.75°	8°	~ 0.07	-
Bone et al.	2003	JN	21	19-59	2.4	0	0	17	HFP	0.75°	8°	~ 0.04	-
Bone et al.	2003	JN	12	19-60	20	0	0	17	HFP	0.75°	8°	~ 0.06	p < 0.05
Bone et al.	2003	JN	2	26-27	5	0	0	17	HFP	0.75°	8°	~ 0.03	-
Koh et al.	2004	EER	6	64-81	20	0	0	20	HFP	0.5°	6°	0.07	p > 0.05
Bernstein et al.	2004	ABB	8	<61	20	0	0	16	HFP	0.75°	8°	0.04	-
Bernstein et al.	2004	ABB	8	<61	20	0	0	16	RRS	-	-	76RC	-
Bone et al.	2007	NM	10	21-58	5.5	1.4	15	17	HFP	0.75°	8°	~ 0.07	p < 0.05
Wenzel et al.	2007	OPO	3	24-52	30	2.7	0	17	HFP	0.33°	7°	0.07	p < 0.001
Wenzel et al.	2007	OPO	3	24-52	30	2.7	0	17	HFP	0.5°	7°	0.07	p < 0.002
Wenzel et al.	2007	OPO	3	24-52	30	2.7	0	17	HFP	1°	7°	0.046	p < 0.002
Wenzel et al.	2007	OPO	3	24-52	30	2.7	0	17	HFP	2°	7°	0	-
Schalch et al.	2007	ABB	23	18-45	10.7	0.8	0	17	HFP	0.5°	5.5°	0.06	p = 0.04
Schalch et al.	2007	ABB	23	18-45	0	12.6	0	17	HFP	0.5°	5.5°	0.01	p > 0.1
Schalch et al.	2007	ABB	23	18-45	10.2	11.9	0	17	HFP	0.5°	5.5°	0.06	p = 0.04
Johnson et al.	2008	AJCN	11	60-80	12	0.5	0	16	HFP	1.5°	7°	-	p < 0.05
Johnson et al.	2008	AJCN	11	60-80	12	0.5	0	16	HFP	3°	7°	-	p < 0.01
Stringham et al.	2008	OVS	40	17-41	10	2	0	24	HFP	0.25°	10°	0.19	-
Stringham et al.	2008	OVS	40	17-41	10	2	0	24	HFP	0.5°	10°	0.16	-
Stringham et al.	2008	OVS	40	17-41	10	2	0	24	HFP	1°	10°	0.1	-
Stringham et al.	2008	OVS	40	17-41	10	2	0	24	HFP	3°	10°	0.07	-
Stringham et al.	2008	OVS	40	17-41	10	2	0	24	HFP	7°	10°	0.03	-
Connolly et al.	2010	CER	5	30-85	3.7	0.8	7.3	8	HFP	0.25°	7°	0.16	p < 0.05
Connolly et al.	2010	CER	5	30-85	3.7	0.8	7.3	8	HFP	0.5°	7°	0.16	p < 0.05
Nolan et al.	2011	VR	61	18-41	12	1	0	52	HFP	0.25	7°	0.12	p = 0.001
Nolan et al.	2011	VR	62	18-42	12	1	0	52	HFP	0.5	7°	0.11	p = 0.001
<b>AMD subjects</b>													
Principal Author	Year	Journal	No.	Age	L mg/d	Z mg/d	MZ mg/d	Duration (weeks)	Tech	Retinal ecc.	PF	MP rise	Sig.
Koh et al.	2004	EER	7	64-81	20	0	0	20	HFP	1°	6°	0.07	p > 0.05
Trieschmann et al	2007	EER	108	51-87	12	1	0	24	AF	1°	6°	0.10	p < 0.001
Richer et al.	2007	OPT	76	-	10	0	0	52	HFP	1°	7°	0.25	p < 0.05

Connolly et al.	2010	CER	5	30-85	3.7	0.8	7.3	8	HFP	0.25°	7°	0.16	p < 0.05
Connolly et al.	2010	CER	5	30-85	3.7	0.8	7.3	8	HFP	0.5°	7°	0.16	p < 0.05

L = Lutein (mg/day); Z = Zeaxanthin (mg/day); MZ = *Meso*-zeaxanthin (mg/day); Tec = technique used to measure MPOD (macular pigment optical density); n = Number of subjects participating in study; Age = Age range (years) of subjects in study; Retinal ecc.= retinal eccentricity; PF = Parafovea stimulus; AJCN = American Journal of Clinical Nutrition; IOVS = Investigative Ophthalmology and Visual Science; ABB = Archives of Biochemistry and Biophysics; OPO = Ophthalmic and Physiological Optics; EER = Experimental Eye Research; NM = Nutrition and Metabolism; OPT = Optometry; JN = Journal of Nutrition; OVS = Optometry and Vision Science; RC = Raman counts; ODU = Optical density units; HFP = Heterochromatic flicker photometry; AF = Autofluorescence; SLO = Scanning Laser ophthalmoscope; SA = Spectral Analysis; AMD = Age related Macular Degeneration; RRS = Resonance Raman Spectroscopy; - = data unavailable.

We report significant increases in MPOD at 0.25° and 0.5° retinal eccentricity, at three and six months in the I group; whereas, as expected, MPOD remained stable in the P group. This is consistent with previous studies that also measured central MPOD and supplemented with similar amounts of the macular carotenoids at three months; however, at six months we report slightly lower than normal MPOD response at 0.25° (see Table 5).

Given that the supplement used in the current study had higher amounts of MZ (10.6 mg) than L (5.9 mg) or Z (1.2 mg), we feel it important to make direct comparison to previous studies that also supplemented with MZ. To date, there have been only two published studies that have reported on MPOD response following supplementation with this carotenoid in humans. Bone et al. carried out a study on 10 subjects supplemented with a soya bean oil-based supplement containing 14.9 mg MZ, 5.5 mg L and 1.4 mg Z, and reported an average increase of ~ 0.07 (~17%) ODU at 0.75° retinal eccentricity over a 120 day period. A pilot study by our group, where 10 subjects (5 with AMD, 5 without AMD) were assessed over an eight week study period following supplementation with 7.3 mg MZ, 3.7 mg L and 0.8 mg Z, and reported an average increase of ~ 0.16 (56%) ODU in MPOD at 0.25° retinal eccentricity.<sup>15</sup>

Also, it is interesting to note that only central MPOD, as discussed above, increased significantly in the I group, which is most likely due to the fact that we used a MZ dominant supplement. Given the known anatomic (central retina)<sup>3</sup>, biochemical (antioxidant)<sup>20</sup>, and optical (short-wavelength filtering)<sup>3</sup> properties of MP, there is a consensus that this pigment may confer

protection against AMD, making the above findings with respect to central MP augmentation important for patients with, or at risk of developing, AMD.

The differing serum carotenoid and MP responses reported between studies (again, see Tables 4 and 5) may be due to several factors, such as: dose of carotenoids consumed per day; type of carotenoids in the supplement (e.g. free versus ester); matrix in which carotenoids are consumed (e.g. oil versus microencapsulated); whether consumed alone or in the presence of other antioxidants; non-compliance to the study supplement

In-depth analysis of our study data with respect to non-response in serum and MPOD confirms the following. We found that there was one non-responder in serum for L and Z (subject 28), which was not due to a lack of compliance to the supplement (confirmed by retinal and tablet counting). Surprisingly, however, this subject did show a significant response in central MPOD. This finding is difficult to explain, but may indicate that this subject exhibited a rapid uptake of the carotenoids to the retina (suggesting a need of the macula to uptake these carotenoids). This finding is also provocative given that this subject had a confirmed family history of AMD, and was a current cigarette smoker. These two risk factors have been suggested to prevent the formation of MZ at the central macula from retinal L (although the exact mechanism remains unclear). One explanation rests on the possibility that this subject cannot generate MZ from retinal L (hence the lack of central baseline MP in this subject; MPOD at  $0.25^\circ = 0.18$  and at  $0.5^\circ = 0.11$ ), but could respond to a supplement containing MZ. Indeed, this notion is consistent with our previous pilot study reporting on MZ.<sup>15</sup> It is also possible that this subject initially consumed the macular carotenoid supplement, containing MZ, which had a positive effect on his MP; however, given that serum levels provide information on recent carotenoid intake, it is possible that this subject did not comply to taking the supplement by three or six months, explaining the appeared 'non-response' in this subject's serum.

With respect to MPOD 'non-response', we found that only two subjects (subjects 1 and 15) demonstrated little, or no, response in MP (although both these subjects demonstrated

significant response in serum concentrations of these carotenoids). We suggest that a simple explanation for this non-response in these two subjects rests on their high baseline MPOD values of 0.73 and 0.51, respectively (i.e. we suggest that they were already at their saturation points of MP). Other interesting findings with respect to MPOD response can be seen in the MPOD spatial profiles of subjects in this study. In brief, we identified three subjects with 'central dips' in their baseline MP spatial profiles (see publications by Kirby et al., 2008,<sup>21</sup> and Connolly et al., 2010,<sup>15</sup> for discussion on central dips in MP spatial profiles), which were normalized following supplementation of MZ, L and Z. This, again, is consistent with the hypothesis that these subjects are unable to generate MZ from L at the macula, but do respond to a supplement containing MZ. Moreover, and consistent with our above suggestion that family history of AMD and smoking cigarettes may inhibit MZ generation from L at the macula, the subjects in the current study who exhibited baseline central dips in their MP spatial profiles had either a positive family history of AMD or a history of smoking cigarettes, but, importantly, did respond to the MZ supplement resulting in a "normal" MP profile following this carotenoid intervention.

The most novel aspect of the current study concerns the efforts made to investigate safety of consumption of the macular carotenoids by performing clinical pathology analysis to assess renal and liver function, lipid profile, haematological profile, and markers of inflammation in subjects at baseline (V1) and after six months (V3). Although clinical pathology analysis demonstrated significant statistical variation from baseline to six months (in both positive and negative directions) in 8 of the 25 variables assessed in the I group and 9 of the 25 variables assessed in the P group, it is important to point out that all variables remained within the normal reference range given, with the exception of total cholesterol and LDL in the I group ( $p = 0.01$ ), which had a baseline value outside the accepted normal reference range before carotenoid supplementation commenced. Adverse events were also monitored during the study period; each subject was questioned at each visit regarding any adverse effects arising from consuming

the supplements. There were no adverse events recorded or reported by any subject taking part in the study following supplementation with all three macular carotenoids.

Of note, there are currently no published clinical trials performed in human subjects, which have assessed safety of supplemental macular carotenoids by conducting comprehensive clinical pathology analysis, such as that performed in the current study. However, a number of human intervention studies have been conducted involving supplementation with high doses of L for extended periods of time, with no adverse effects reported (assessment limited by self report).<sup>22-24</sup> Indeed, doses of 20 mg/day for up to six months were not associated with any side effects.<sup>25;26</sup> Even doses of 30 mg/day for five months<sup>27</sup> or 40 mg/day over two months were not associated with any adverse effects.<sup>28</sup> The only side effect reported as a result of L supplementation in humans has been carotenedermia, which is a harmless and reversible cutaneous hyperpigmentation of the skin.<sup>22;29;30</sup> Carotenedermia, is itself not known to be associated with any specific adverse effects on human health and only results from excessive intake of L.<sup>31</sup> The majority of studies assessing safety of supplemental Z involving humans have also been observational in design, and did not include appropriate clinical pathology safety testing. Of note, none of these studies reported any adverse effects or ocular toxicity following supplementation with this carotenoid.<sup>32-40</sup> However, there has been one (unpublished) pharmacokinetic study in humans involving five men and five women designed to assess safety of Z consumption.<sup>41</sup> In this study conducted by Hoffmann-La Roche (now DSM Nutritional Products Ltd.), the men and women were given capsules containing either 1 mg or 10 mg per day of Z for 42 days. Clinical chemistry measures and adverse events were recorded. Several clinical laboratory results fell outside the normal ranges, but there was only one adverse event where the possibility of an association with dosing was deemed even remotely plausible. The conclusion from this study was that all the adverse events were rated as mild to moderate in severity and unlikely to be related to the supplement.<sup>41</sup>

In the animal model, there have been two investigations into the possibility of toxicological and/or mutagenic effects of MZ. A toxicity study carried out by Chang in 2006, investigated the effect of administering 2, 20, and 200 mg/kg/day of MZ for thirteen weeks consecutively.<sup>42</sup> In their study, Chang et al. reported that MZ was tolerated well, and the no-observed-adverse-effect-level (NOAEL) of MZ in rats is >200 mg/kg/day when administered orally for thirteen consecutive days. The potential for mutagenic activity has also been tested using the Salmonella typhimurium tester strains TA98, TA100, TA1535, and TA1537 and Escherichia coli tester strain WP2uvrA in both the presence and absence of microsomal enzymes prepared from Acoclor™ induced rat liver. This report also found no mutagenic effect with various doses of MZ.<sup>43</sup>

Kruger et al. published a review on the safety of consumption of a crystalline L product (FloraGLO®) and concluded that crystalline L is safe and a Generally Recognized As Safe (GRAS) source of L, corroborated also by animal toxicology studies, and therefore suitable for human consumption.<sup>44</sup> This is also, consistent with a recent publication in Wistar rats that demonstrated no toxicological significant treatment-related changes in clinical observations, ophthalmic examinations, body weight gains, feed consumption, and organ weight following oral gavage administration of lutein/zeaxanthin concentrate to rats during 13 weeks at levels up to 400mg/kg/day.<sup>45</sup> A published report by the International Programme on Chemical Safety by the World Health Organization, Geneva, summarizes some clinical, toxicological and mutagenicity tests that have been carried out on animals with Z.<sup>46</sup> This report presented findings from a thirteen week study on mice and rats receiving oral doses of Z, who received 250, 500, 1000 mg/kg per day of Z for thirteen weeks. It was reported that there was no treatment-related effects observed throughout the study. In addition, haematology, blood chemistry and urine analysis measurements showed no evidence of toxicity. The NOAEL for this study was 1000 mg/kg per day of Z (i.e. the highest dose tested).<sup>47</sup> Also, ocular toxicity studies have been performed on monkeys which also reported no evidence of treatment related changes.<sup>48;49</sup>

In conclusion, we have shown that subjects supplemented with all three macular carotenoids, including MZ, demonstrate a statistically significant increase in serum concentrations of L and Z, and central MPOD, over a six month study period. Moreover, clinical pathology analysis following supplemental MZ, L and Z is not suggestive of associated toxicity.

## **ACKNOWLEDGEMENTS**

We thank Mr. Jonathon Oates, Head of Pharmacy, Waterford Regional Hospital, Dunmore Road, Waterford, Ireland for his help with randomization, labelling and packaging of the study supplements. We also thank Biomnis Ltd., Dublin, Ireland for their support and many discussions on the analysis and interpretation of the clinical pathology data. We also thank the Howard Foundation for supporting the clinical pathology analysis component of this research. Finally, we acknowledge the main study sponsors for supporting this clinical trial, which include: Macuvision Europe Limited; Macuhealth USA; Macuhealth Canada.

## Reference List

1. Bone RA, Landrum JT, Hime GW, et al. Stereochemistry of the Human Macular Carotenoids. *Investigative Ophthalmology & Visual Science* 1993;34:2033-40.
2. Bone RA, Landrum JT, Tarsis SL. Preliminary identification of the human macular pigment. *Vision Res* 1985;25:1531-5.
3. Snodderly DM, Brown PK, Delori FC, Auran JD. The Macular Pigment .1. Absorbance Spectra, Localization, and Discrimination from Other Yellow Pigments in Primate Retinas. *Investigative Ophthalmology & Visual Science* 1984;25:660-73.
4. Nebeling LC, Forman MR, Graubard BI, Snyder RA. The impact of lifestyle characteristics on carotenoid intake in the United States: The 1987 National Health Interview Survey. *Am J Public Health* 1997;87:268-71.
5. Sommerburg O, Keunen JEE, Bird AC, van Kuijk FJGM. Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. *Br J Ophthalmol* 1998;82:907-10.
6. Johnson EJ, Neuringer M, Russell RM, et al. Nutritional manipulation of primate retinas, III: effects of lutein or zeaxanthin supplementation on adipose tissue and retina of xanthophyll-free monkeys. *Investigative Ophthalmology Visual Science* 2005;46:692-702.
7. Congdon N, O'Colmain B, Klaver CC, et al. Causes and prevalence of visual impairment among adults in the United States. *Arch Ophthalmol* 2004;122:477-85.

8. Snodderly DM, Auran JD, Delori FC. The macular pigment. II. Spatial distribution in primate retinas. *Investigative Ophthalmology & Visual Science* 1984;25:674-85.
9. Khachik F, Bernstein PS, Garland DL. Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. *Investigative Ophthalmology & Visual Science* 1997;38:1802-11.
10. Bone RA, Landrum JT, Cains A. Optical-Density Spectra of the Macular Pigment In vivo and In vitro. *Vision Research* 1992;32:105-10.
11. Loane E, Kelliher C, Beatty S, Nolan JM. The rationale and evidence base for a protective role of macular pigment in age-related maculopathy. *Br J Ophthalmol* 2008;92:1163-8.
12. Loughman J, Davidson PA, Nolan JM, et al. Macular pigment and its contribution to visual performance and experience. *Journal of Optometry* 2010;3:74-90.
13. O'Connell ED, Nolan JM, Stack J, et al. Diet and risk factors for age-related maculopathy. *Am J Clin Nutr* 2008;87:712-22.
14. Elliott DB, Bullimore MA. Assessing the reliability, discriminative ability, and validity of disability glare tests. *Invest Ophthalmol Vis Sci* 1993;34:108-19.
15. Connolly EE, Beatty S, Thurnham DI, et al. Augmentation of macular pigment following supplementation with all three macular carotenoids: an exploratory study. *Curr Eye Res* 2010;35:335-51.
16. European Guidelines on Cardiovascular Disease Prevention. 2007.  
Ref Type: Report

17. World Health Organisation. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia. 2006.  
Ref Type: Report
18. Khachik F, de Moura FF, Chew EY, et al. The effect of lutein and zeaxanthin supplementation on metabolites of these carotenoids in the serum of persons aged 60 or older. *Invest Ophthalmol Vis Sci* 2006;47:5234-42.
19. Berendschot TTJM, Goldbohm RA, Klopping WAA, et al. Influence of lutein supplementation on macular pigment, assessed with two objective techniques. *Investigative Ophthalmology & Visual Science* 2000;41:3322-6.
20. Khachik F, Beecher GR, Goli MB. Separation and identification of carotenoids and their oxidation products in the extracts of human plasma. *Analytical Chemistry* 1992;64:2111-22.
21. Kirby ML, Galea M, Loane E, et al. Foveal Anatomic Associations with the Secondary Peak and the Slope of the Macular Pigment Spatial Profile. *Invest Ophthalmol Vis Sci* 2008;20..
22. Olmedilla B, Granado F, Southon S, et al. A European multicentre, placebo-controlled supplementation study with alpha-tocopherol, carotene-rich palm oil, lutein or lycopene: analysis of serum responses. *Clin Sci (Lond)* 2002;102:447-56.
23. Zhao X, Aldini G, Johnson EJ, et al. Modification of lymphocyte DNA damage by carotenoid supplementation in postmenopausal women. *Am J Clin Nutr* 2006;83:163-9.
24. Richer S, Stiles W, Statkute L, et al. Double-masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation in the intervention of atrophic age-related macular

- degeneration: the Veterans LAST study (Lutein Antioxidant Supplementation Trial). *Optometry* 2004;75:216-30.
25. Aleman TS, Duncan JL, Bieber ML, et al. Macular Pigment and Lutein Supplementation in Retinitis Pigmentosa and Usher Syndrome. *Investigative Ophthalmology Visual Science* 2001;42:1873-81.
  26. Duncan JL, Aleman TS, Gardner LM, et al. Macular pigment and lutein supplementation in choroideremia. *Experimental Eye Research* 2002;74:371-81.
  27. Landrum JT, Bone RA, JOA HILD, et al. A one year study of the macular pigment: the effect of 140 days of a lutein supplement. *Experimental Eye Research* 1997;65:57-62.
  28. Dagnelie G, Zorge IS, McDonald TM. Lutein improves visual function in some patients with retinal degeneration: a pilot study via the Internet. *Optometry* 2000;71:147-64.
  29. Granado F, Olmedilla B, Gil-Martinez E, Blanco I. Lutein ester in serum after lutein supplementation in human subjects. *Br J Nutr* 1998;80:445-9.
  30. Olmedilla B, Granado F, Gil-Martinez E, Blanco I. Supplementation with lutein (4 months) and alpha-tocopherol (2 months), in separate or combined oral doses, in control men. *Cancer Lett* 1997;114:179-81.
  31. ves-Rodrigues A, Shao A. The science behind lutein. *Toxicol Lett* 2004;150:57-83.
  32. Seddon JM, Ajani UA, Sperduto RD. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. *JAMA* 1994;272:1413-20.

33. Mares-Perlman JA, Brady WE, Klein R, et al. Serum antioxidants and age-related macular degeneration in a population based case control study. *Arch Ophthalmol* 1995;113:1518-23.
34. Khachik F, Spangler CJ, Smith JC, Jr., et al. Identification, quantification, and relative concentrations of carotenoids and their metabolites in human milk and serum. *Anal Chem* 1997;69:1873-81.
35. Beatty S, Boulton M, Henson D, et al. Macular pigment and age related macular degeneration. *Br J Ophthalmol* 1999;83:867-77.
36. Richer S. ARMD--pilot (case series) environmental intervention data. *J Am Optom Assoc* 1999;70:24-36.
37. Bone RA, Landrum JT, Dixon Z, et al. Lutein and zeaxanthin in the eyes, serum and diet of human subjects. *Experimental Eye Research* 2000;71:239-45.
38. Johnson EJ, Hammond BR, Yeum KJ, et al. Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density. *Am J Clin Nutr* 2000;71:1555-62.
39. Schalch W, Cohn W, Aebischer C-P. Pilot study on the dose response to lutein formulated as beadlets in capsules: plasma kinetics and accumulation in the macula after oral lutein administration under defined dietary conditions in humans. Unpublished report No. 1003951 from F. Hoffmann-La Roche Ltd, Basle, Switzerland. 2001.  
  
Ref Type: Report
40. Bone RA, Landrum JT, Guerra LH, Ruiz CA. Lutein and Zeaxanthin Dietary Supplements Raise Macular Pigment Density and Serum Concentrations of these Carotenoids in Humans. *J Nutr* 2003;133:992-8.

41. Cohn W, Hartmann D, Thurmann P, et al. Multiple oral dose pharmacokinetics in healthy subjects at two dose levels of zeaxanthin, formulated as beadlets and incorporated in capsules, module 1. Unpublished report No. 1007403 from Hoffmann-La Roche Ltd, Basle, Switzerland. 2002.  
  
Ref Type: Report
42. Chang CJG. Thirteen-week oral (gavage) toxicity of mesozeaxanthin in Han Wistar rats with a 4-week recovery. Gene Logic Study Number: 1567-04370, 1-344. 2006.  
  
Ref Type: Report
43. Mecchi MS. Salmonella-Escherichia coli/Mammalian-microsome reverse mutation assay with a confirmatory assay with mesozeaxanthin. 7609-100, 1-27. 2006.  
  
Ref Type: Report
44. Kruger CL, Murphy M, DeFreitas Z, et al. An innovative approach to the determination of safety for a dietary ingredient derived from a new source: case study using a crystalline lutein product. Food Chem Toxicol 2002;40:1535-49.
45. Ravikrishnan R, Rusia S, Ilamurugan G, et al. Safety assessment of lutein and zeaxanthin (Lutemax 2020): Subchronic toxicity and mutagenicity studies. Food Chem Toxicol 2011.
46. WHO (World Health Organisation). Safety and Evaluation of Certain Food Additives; Prepared by the Sixty-third Meeting of the joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series, No. 54; ISBN-13: 9789241660549. 2006.  
  
Ref Type: Report

47. Ettlin R, Steiger A, Hummler H. Tolerance study of zeaxanthin administered orally as a feed admixture to mice over 13 weeks. Unpublished report No. B-93'153 from F. Hoffmann-La Roche Ltd, Basle, Switzerland. 1980.

Ref Type: Report

48. Pfannkuch F, Wolz EACP, Schierle J, et al. Ro 01-9509 (zeaxanthin 10%) and Ro 15-3971 (lutein 10%): combined 52-week oral (gavage) pilot toxicity study with two carotenoids in the cynomolgus monkey (Roche project No. 904V98). Unpublished report No. B-171'423, Amendment to Final Report No. 1, dated December 18. Submitted to WHO by Roche, Basle, Switzerland. 2000.

Ref Type: Report

49. Pfannkuch F. Comprehensive overview on eye examinations on: combined 52-week oral (gavage) pilot study with two carotenoids in the cynomolgus monkey. Unpublished report No. 1004238 from F. Hoffmann-La Roche Ltd, Basle, Switzerland. 2001.

Ref Type: Report