An investigation into macular pigment augmentation with all three macular carotenoids and their safety in humans

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DECLARATION

No part of the work described in this thesis, or the thesis itself, was submitted previously for a degree at this or any other institution. The work described in this thesis was performed entirely by the author, unless otherwise stated.

Signature:

Eithne Connolly

Date: 20th September 2013

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Finally, I would like to thank the other members of the MPRG for their help, support and friendship.

LIST OF ABBREVIATIONS

Adenosine triphosphate	ATP
Age related eye disease study	AREDS
Age-related macular degeneration	AMD
Apolipoprotein E	ApoE
Becton Dickinson	BD
Best corrected visual acuity	BCVA
Body mass index	BMI
Butylated hydroxytoluene	BHT
Case report form	CRF
Choroidal neovascularisation	CNV
Complement factor H	CFH
Contrast sensitivity function	CSF
Critical flicker fusion frequency	CFF
Customised heterochromatic flicker photometry	cHFP
Deoxyribonucleic acid	DNA
Eye disease case control study	EDCCS
Food frequency questionnaire	FFQ
Flavin adenine dinucleotide	$FADH_2$

Generally recognised as safe	GRAS
Geographic atrophy	GA
Heterochromatic flicker photometry	HFP
High density lipoproteins	HDL
High performance liquid chromatography	HPLC
Hydrogen peroxide	H_2O_2
Infra red	IR
International standard randomised controlled trial number register	ISRCTN
Liquid crystal display	LCD
Low density lipoproteins	LDL
Lutein	L
Macular mean sensitivity	MMS
Macular pigment	MP
Macular pigment optical density	MPOD
Meso-zeaxanthin ocular supplementation trial	MOST
Meso-zeaxanthin ocular supplementation trial in normals	MOST-N
Meso-zeaxanthin	*meso*-Z
National health and nutrition examination survey	NHANES
Nicotinamide adenine dinucleotide	NADH

No observable adverse effect level	NOAEL
Optical density unit	ODU
Oxygen	O_2
Pathologies Oculaires Liées á l'Age	POLA
Polyunsaturated fatty acids	PUFAs
Reactive oxygen intermediates	ROIs
Retinal pigment epithelium	RPE
Scottish collaborative group	SCG
Standard deviation	SD
Standard operating procedure	SOP
Statistical package for the social sciences	SPSS
Total lutein	TL
Total zeaxanthin	TZ
Water	H_2O
Zeaxanthin	Z

ABSTRACT

TITLE

An investigation into macular pigment augmentation with all three macular carotenoids and their safety in humans.

INTRODUCTION

The macula is located at the centre of the retina and is responsible for fine detailed and colour vision. The centre of the macula houses a protective pigment collectively referred to as macular pigment (MP). MP represents the highest concentration of the carotenoids lutein (L), zeaxanthin (Z) and *meso*-zeaxanthin (*meso*-Z) within the body. MP acts as a filter of short-wave length (blue) light and is a powerful antioxidant.

Age-related macular degeneration (AMD) is an eye disease that affects the central part of the retina called the macula and in its late form, results in loss of central vision. Although the pathogenesis of AMD remains poorly understood, it is believed that cumulative exposure to short-wave length (blue) light and reactive oxygen intermediates (ROIs) play an important role in the development of AMD. There is a hypothesis that MP can help protect from this disease by virtue of its protective properties.

OBJECTIVES

This research study was designed to:

1. Investigate macular and serum response to all three macular carotenoids L, Z and *meso*-Z in humans.
2. To investigate the response, safety and stability of supplementation with L, Z and *meso*-Z in combination in humans.

METHODS

Study One: Augmentation of macular pigment following supplementation with all three macular carotenoids: an exploratory study

Ten subjects were included in this study (five normal and five with early AMD). All subjects were instructed to consume a formulation containing 7.3 mg of *meso*-Z, 3.7 mg of L and 0.8 mg of Z per day over an eight week study period. The spatial profile of MP optical density (i.e. MPOD at 0.25°, 0.5°, 1° and 1.75°) was measured using customised heterochromatic flicker photometry (cHFP) and a blood sample was collected at each study visit in order to analyse serum concentrations of *meso*-Z, L and Z using high performance liquid chromatography (HPLC).

Study Two: Supplementation with all three macular carotenoids: response, stability and safety

Forty four healthy subjects were recruited into this randomised, placebo-controlled, clinical trial. Subjects consumed one tablet per day containing 10.6 mg of *meso*-Z, 5.9 mg

of L and 1.2 mg of Z (Intervention, I group) or Placebo (P group). The spatial profile of MPOD was measured using cHFP, and serum concentrations of L and Z were quantified using HPLC. Subjects were assessed at baseline, three and six months. Clinical pathology analysis was performed at baseline and six months.

RESULTS

Study One

There was a significant increase in serum concentrations of *meso*-Z and L after two weeks of supplementation ($p < 0.05$). Baseline serum carotenoid analysis (i.e. pre-supplementation) detected a small peak eluting at the same time as *meso*-Z in all subjects, with a mean ± standard deviation (SD) concentration of 0.02 ± 0.01μmol/L. We also report significant increases in MPOD at 0.25°, 0.5°, 1° and average MPOD across the spatial profile after just two weeks of supplementation with this formulation ($p < 0.05$, for all). Four subjects (one normal and three AMD) who had an atypical MPOD spatial profile at baseline (i.e. pre-supplementation), had the more typical MPOD spatial profile (i.e. highest MPOD at the centre) after eight weeks of supplementation with the study formulation.

Study Two

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°: $p = 0.001$; 0.5°: $p = 0.001$), 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

Study One

There was a significant increase in serum concentrations of *meso*-Z and L following supplementation with a formulation containing 7.3 mg *meso*-Z, 3.7 mg L and 0.8 mg Z and a significant increase in MPOD, including its spatial profile, after just two weeks of supplementation. Also, this study detected the possible presence of *meso*-Z in human serum pre-supplementation and the ability of this carotenoid formulation to rebuild central MPOD in subjects who have atypical profiles at baseline.

Study Two

Subjects supplemented with *meso*-Z, 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.

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 THE RETINA

The retina is made up of ten distinct layers which are bound externally by Bruch's membrane and on its internal aspect by the vitreous.

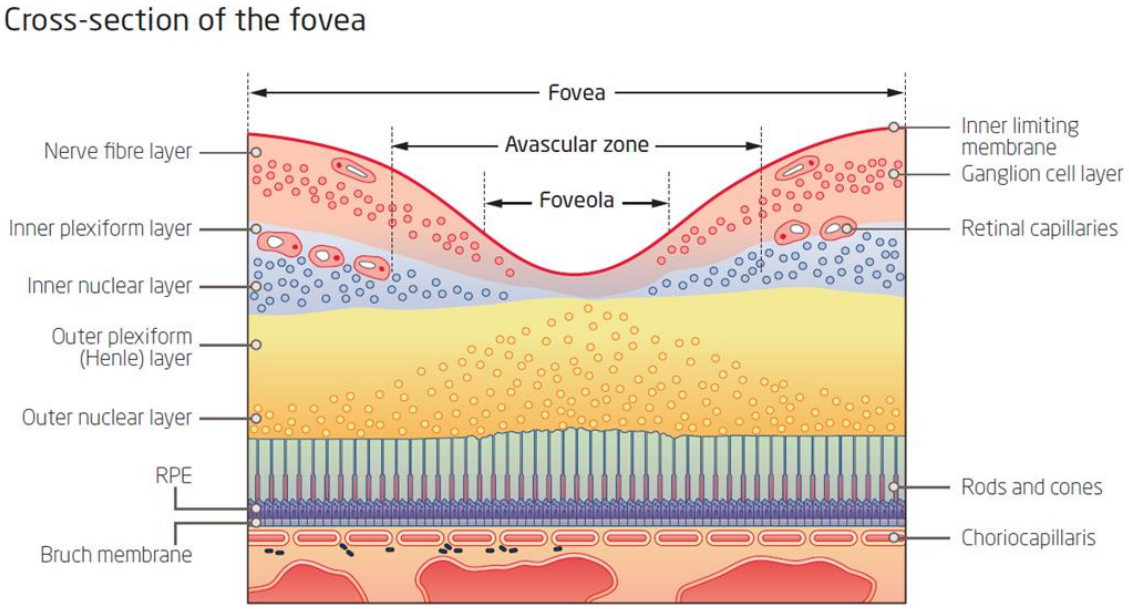


FIGURE 1.1 Schematic showing the different layers of the retina.[1]

1.1.1 Bruch's membrane

Bruch's membrane is located within the choroid and is located beneath the retinal pigment epithelium (Figure 1.1). Age related changes can occur within Bruch's membrane leading to the formation of drusen (Figure 1.6).

1.1.2 Retinal pigment epithelium

The retinal pigment epithelium (RPE) is the pigmented cell layer that nourishes the retinal visual cells, and is firmly attached to the underlying choroid and overlying retinal visual cells.

1.1.3 Photoreceptor layer

This layer is primarily made up of photoreceptors. There are two types of photoreceptors in the retina: rods and cones. Rods are responsible for sensing contrast, brightness and motion. Cones are responsible for fine resolution, spatial resolution and colour vision. The density of the rods and cones vary within different regions of the retina, the peripheral retina is dominated by rods and the macula is dominated by cones.

1.1.4 Outer limiting membrane

The outer limiting membrane is a layer that separates the inner segment portions of the photoreceptors from their cell nucleus.

1.1.5 Outer nuclear layer

The outer nuclear layer contains the nuclei and cell bodies of the rods and cones.

1.1.6 Outer plexiform layer

The outer plexiform layer is an area of the retina where important synaptic interactions occur. These synapses mainly occur between rod and cone cells upon various bipolar and horizontal cells. The most important synaptic interactions that take place are responsible for detecting light and dark backgrounds, and contrast between objects.

1.1.7 Inner nuclear layer

The inner nuclear layer is made up of a number of three types of closely packed cells; bipolar cells, horizontal cells, and amacrine cells.

1.1.8 Inner plexiform layer

The inner plexiform layer is the synapse between bipolar cells and the dendrites of the ganglion cells or amacrine cells.

1.1.9 Ganglion cell layer

These cells are situated between the innermost plexiform layer and the nerve fibre layer and are the last neural link in the visual pathway. The axons of these cells form the innermost surface of the nerve fibre layer.

1.1.10 Nerve fibre layer

The nerve fibre layer is made up of the axons of the ganglion cells that leave the eye and form the optic nerve.

1.1.11 Inner limiting membrane

The inner limiting membrane is the location where the foot processes of the müller cells come together.

1.2 THE FOVEA AND MACULA

The fovea is a region at the centre of the retina and is where the macula is located. It is a specialised region responsible for central, colour and detailed vision. It also houses the highest density of cone photoreceptors.[2] The macula is approximately 5.5 mm in diameter, which includes the fovea. The macula is characterised by a yellow colour (*macula lutea,* which is Latin for 'yellow spot'), which is attributable to the presence of macular pigment (MP).[3] The concentration of MP peaks at the centre of the macula (Figure 1.2).

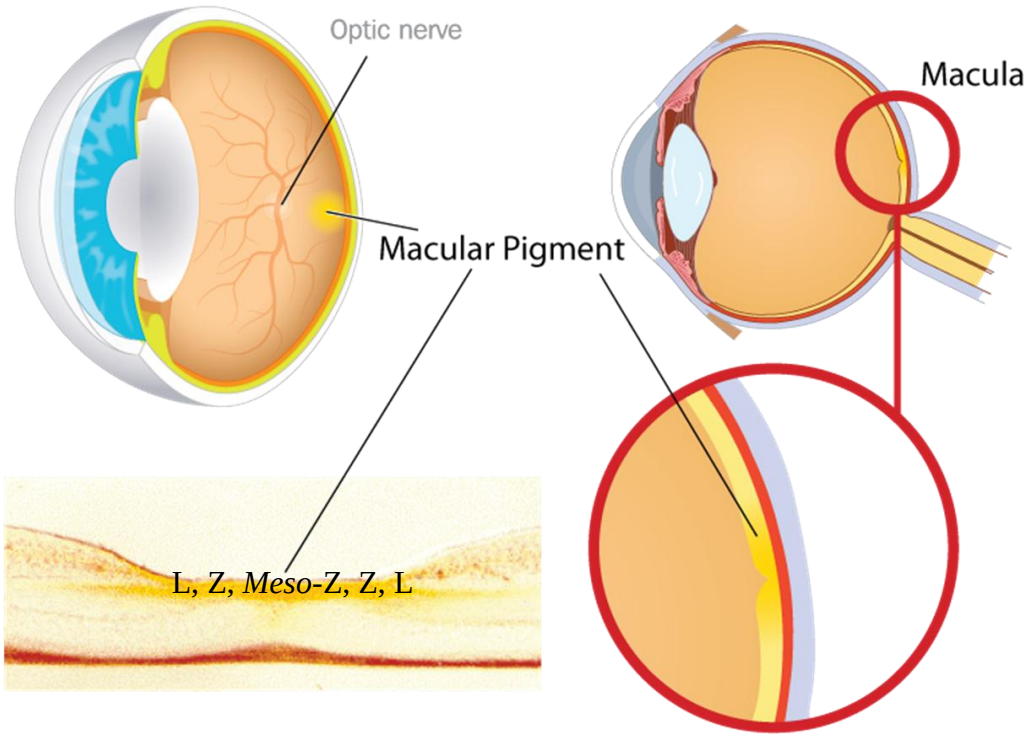


FIGURE 1.2 Location and distribution of the macular carotenoids at the fovea. Image courtesy of Prof. Max Snodderly and Prof. John Nolan.

1.3 THE OPTIC NERVE

The optic nerve is the convergence of ganglion cell axons at the optic disc where the nerve impulses are transmitted from the retinal cell layers to the brain.

1.4 AGE-RELATED MACULAR DEGENERATION

1.4.1 Definition

Age-related degeneration (AMD) is a degenerative disease of the macula.[4] It is the leading cause of blindness in people over the age of 50 years in the developed world.[5;6] AMD results in a loss of central and colour vision, however, individuals will retain their navigational ability with AMD as peripheral vision is not affected, regardless of stage, and

in the absence of any additional ocular pathology. Hence, an individual with AMD does not lose their vision completely. There is an eventual loss of central vision. This loss of central vision results in an inability to recognise faces, read, watch television and drive and therefore has a significant impact on an individual's independence and quality of life.

It is estimated that the number of people suffering from AMD in the Republic of Ireland is approximately 80,000 (7.9%), with over 417,000 (1.8%) people affected in the United Kingdom.[7] The prevalence of AMD is expected to rise steadily in the future primarily due to increasing longevity (Figure 1.3) and predicted world population growth (Figure 1.4).[8]

FIGURE 1.3 Male and female life expectancy 1950-2050. Figures from 1950 and 2011: Irish Department of Health and Children data; projected figures for 2028 and 2050: USA data

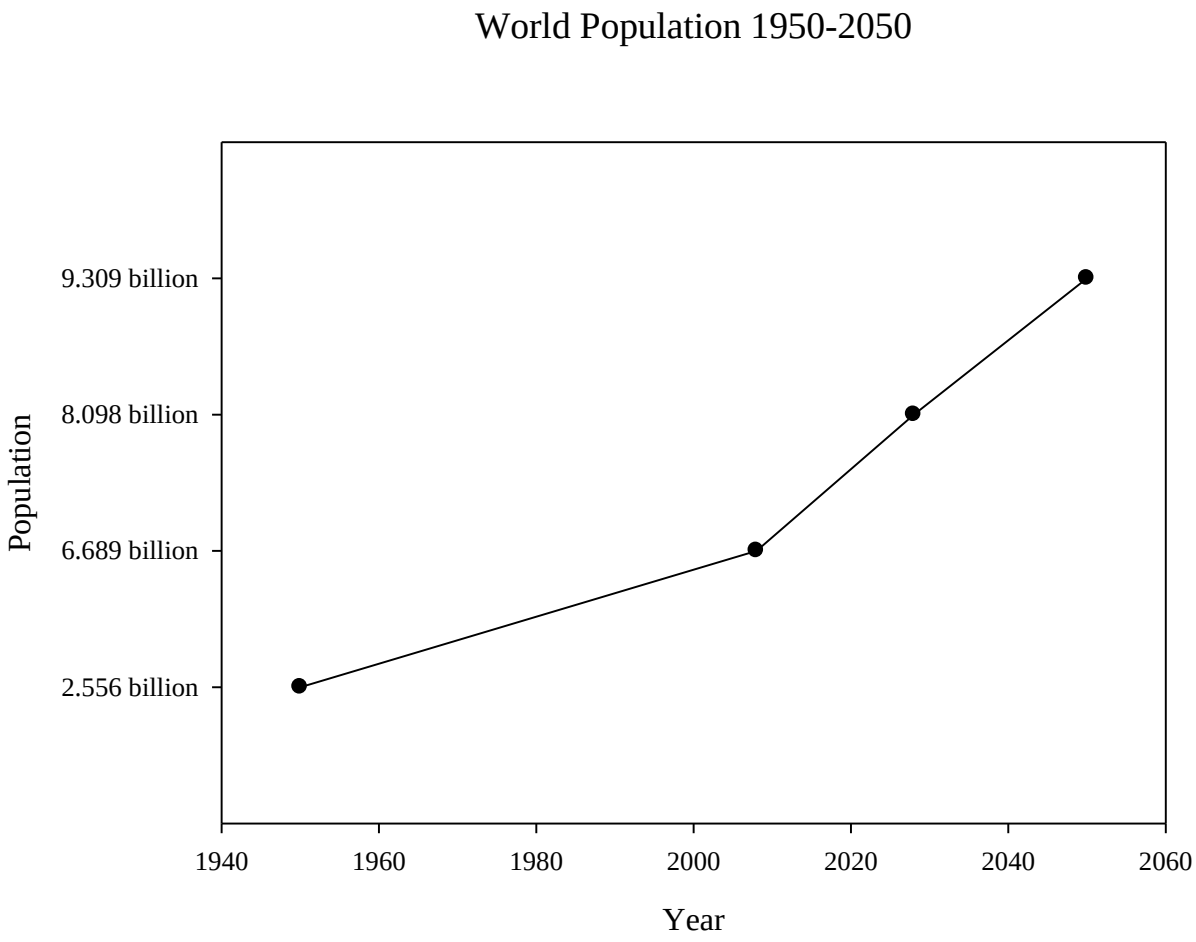


FIGURE 1.4 World population 1950-2050 (predicted)

1.4.2 Classification of age-related macular degeneration

In 1995, the International Age-Related Maculopathy Epidemiological Study Group developed a grading classification for AMD that defines the disease based on morphological changes, with visual acuity not a criterion for the presence or absence of AMD.[4] This was compiled in order to homogenise the system used to identify and classify disease in all future clinical and epidemiological studies (Figure 1.5).

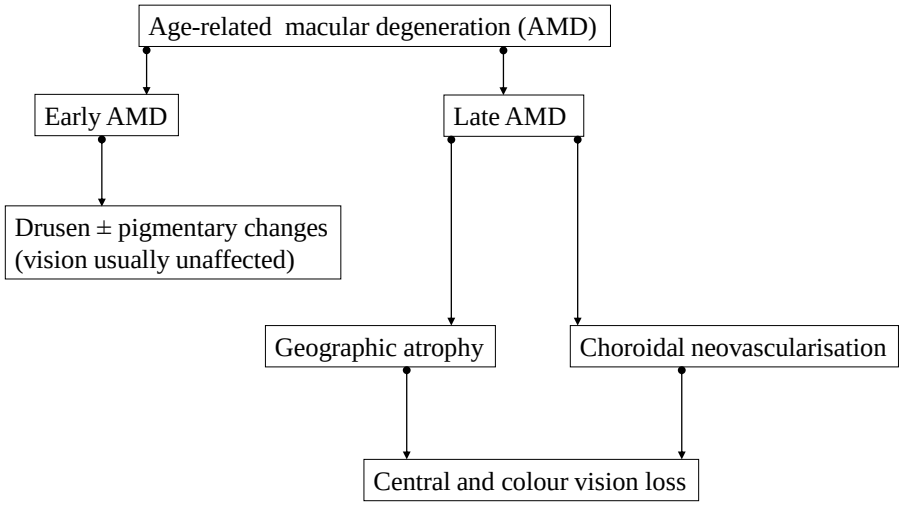


FIGURE 1.5 Schematic showing the different stages of AMD

AMD is defined as a disorder of the macular area, often clinically apparent after 50 years of age, and can be characterised by any of the following, which are not clinically related to other pathology:

1. Soft drusen ≥ 63 μm in diameter. Drusen are whitish-yellow spots that lie external to the neuroretina or RPE (Figure 1.6). Drusen may be soft and confluent, soft distinct or soft indistinct. Hard drusen do not alone characterise the disorder.
2. Areas of increased pigment (hyperpigmentation) in the outer retina or choroid associated with drusen.
3. Areas of depigmentation (hypopigmentation) of the RPE, most often more sharply demarcated than drusen, without visible choroidal vessels associated with drusen.

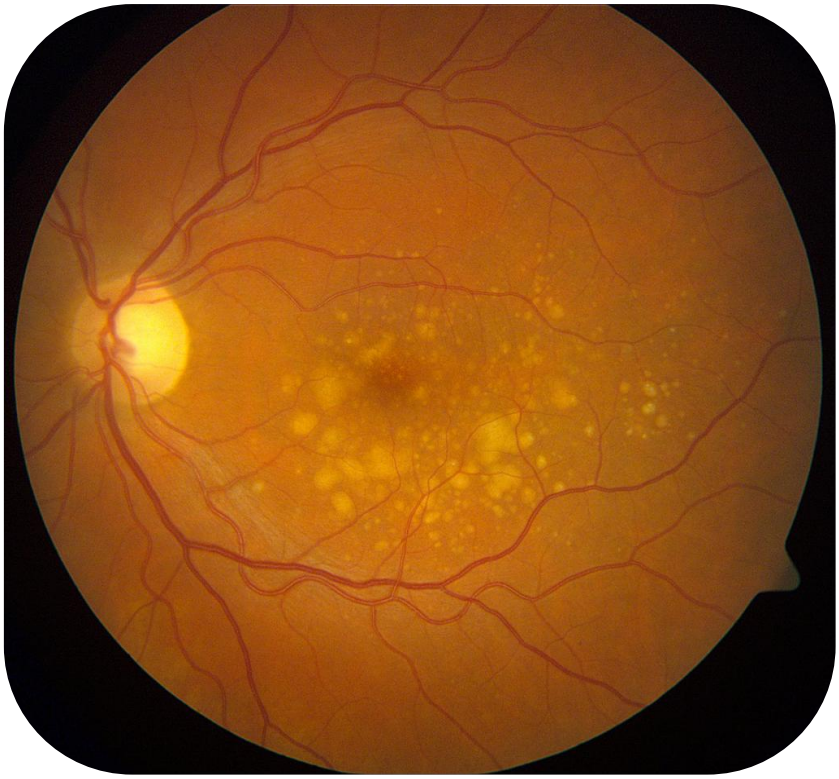


FIGURE 1.6 Macular drusen. Image courtesy of The Institute of Eye Surgery

These early changes at the central retina are associated with the progressive accumulation of drusen, and may predispose to the late stage of AMD.[9;10] Late AMD has been classified into two different types, either geographic atrophy or choroidal neovascularisation (CNV).

Geographic atrophy (GA) is characterised by the following signs, which are not clinically related to other pathology:

1. Any sharply delineated area of hyper or hypopigmentation or apparent absence of the RPE in which choroidal vessels are more visible than in surrounding areas that must be ≥ 175 µm in diameter (Figure 1.7).

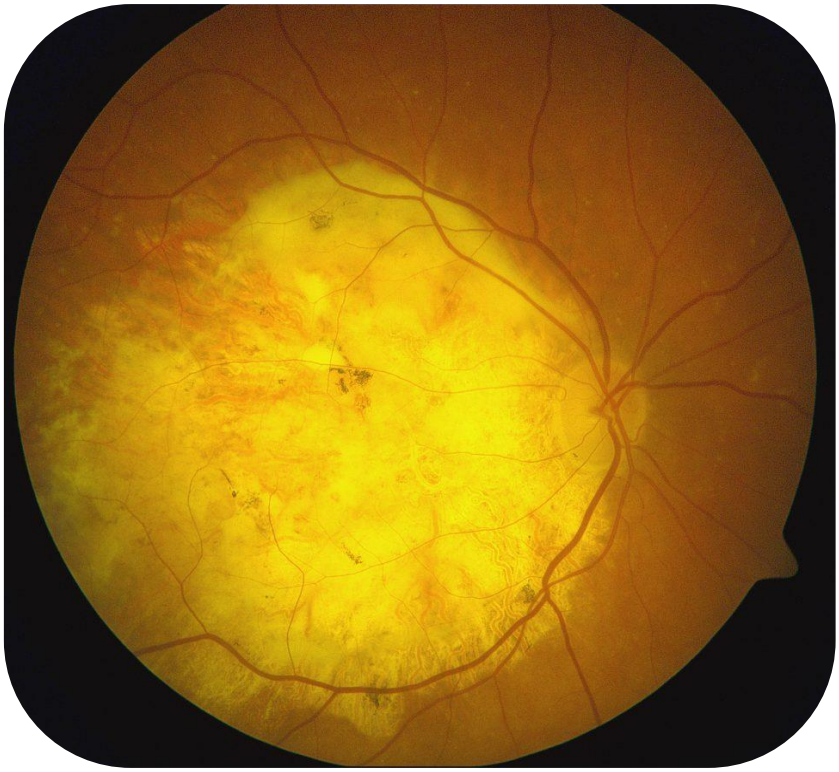


FIGURE 1.7 Geographic atrophy. Image courtesy of The Institute of Eye Surgery

Choroidal neovascularisation is characterised by any of the following signs, which are not clinically related to other pathology:

1. RPE detachment(s), which may be associated with neurosensory retinal detachment.
2. Subretinal or sub-RPE neovascular membrane(s).
3. Epiretinal, intraretinal, subretinal, or sub RPE scar tissue or fibrin-like deposits.
4. Subretinal haemorrhages (Figure 1.8).
5. Hard exudates within the macular area, related to any of the above, and not related to other retinal vascular disease.

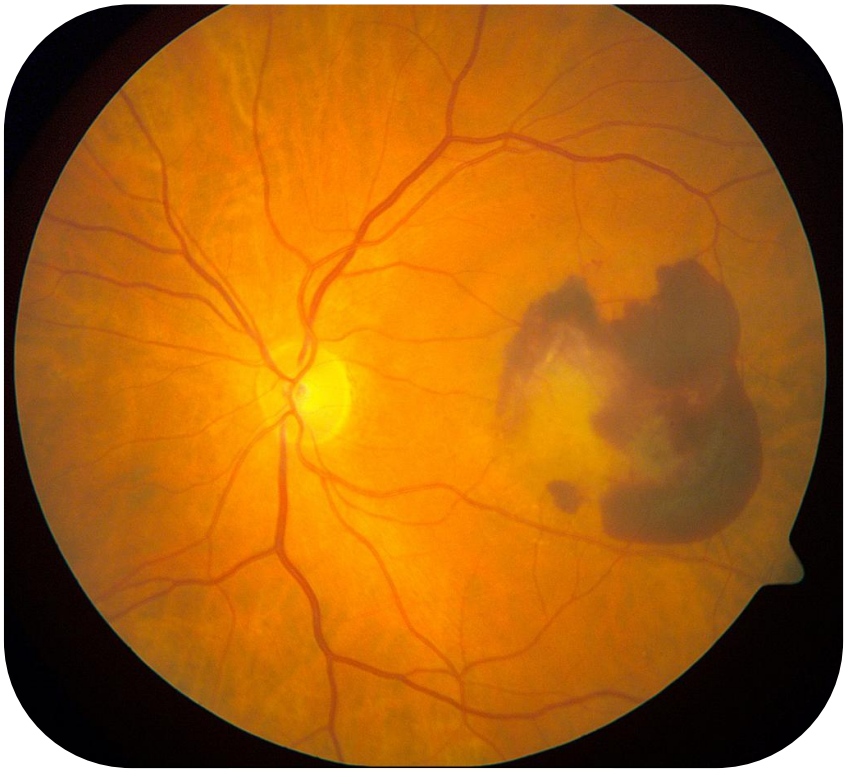


FIGURE 1.8 Choroidal neovascularisation, showing sub-retinal haemorrhage. Image courtesy of The Institute of Eye Surgery

1.4.3 Pathogenesis of age-related macular degeneration

It is now accepted that the pathogenesis of AMD is multifactorial. It is dependent on the interaction between an individual's genetic and environmental (lifestyle) background. Although the exact pathogenesis is unknown, there are well-established risk factors that contribute to the development of this disease. The established risk factors include increasing age, a positive family history of AMD, and cigarette smoking.[11-13] Therefore, cigarette smoking is the only proven environmental/lifestyle risk factor for this disease.[14;15] Putative risk factors include obesity,[16;17] sex, low macular pigment levels, and a diet deficient in fruit and vegetables,[18] particularly those containing the macular carotenoids, lutein (L) and zeaxanthin (Z).[19] Although the pathogenesis of AMD remains poorly understood, there is a growing body of evidence to suggest that one or more of the

following processes contribute to this condition: oxidative stress; inflammation; cumulative blue light damage; RPE cell dysfunction; reduced foveolar choroidal circulation.

1.4.3.1 *Oxidative stress*

Oxidative stress occurs when the level of oxidants (reactive oxygen intermediates; ROIs) in a system exceeds the detoxifying capacity of its antioxidants, thus leading to oxidative damage to macromolecules with consequential injury to cells/tissues.[20;21] Prolonged exposure to ROIs will result in cellular damage to DNA tissues and will eventually cause disease.[22] Antioxidants are a mechanism of defence within the body that scavenge ROIs and therefore protect against potential damage to tissues. With increasing age there is a positive increase in ROI levels, with a decrease in the level of antioxidants. It is now known that this imbalance leads to the increase in prevalence in age-related diseases, including AMD.[21]

1.4.3.1.1 *Oxidative processes*

The process of oxidation is essential if a cell is to provide energy for vital functions. Oxidation occurs when oxygen is metabolised in a process called cellular respiration. Cellular respiration is the process by which fuels such as carbohydrates, proteins and fats are oxidised to acetyl-Coenzyme A, which then acts as a substrate in the tri-carboxylic acid cycle where it is oxidised to carbon dioxide. The energy produced by this oxidative process is harnessed in the form of the electron carriers nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These electrons are transferred along

the electron transport chain and combine with oxygen (O_2) to form water (H_2O). In a process known as oxidative phosphorylation, energy released in the formation of H_2O is conserved in the form of adenosine triphosphate (ATP).[23] The major site for the production of ROIs is the electron transport chain within the mitochondria. It is thought that ROIs 'leak' from the active site of enzymes involved in the above process.[24]

At physiologic levels, ROIs function as signalling and regulatory molecules, whereas at pathologic levels they are highly deleterious and act as cytotoxic oxidants. Even at low concentrations, prolonged exposure to ROIs results in DNA mutation, tissue injury, and disease.[22] The body, however, has an inherent defence system, consisting of antioxidants and antioxidant enzymes, which act synergistically in scavenging ROIs and thus protecting the underlying tissues.

1.4.3.1.2 *Reactive oxygen intermediates*

Most ROIs are the inevitable by-products of normal and essential metabolic reactions, such as energy generation from smoking, excess consumption of alcohol and irradiation. However, pollution, asbestos, fungal or viral infections, cigarette smoking, short wavelength (blue) light, inflammation and ageing are all known to be associated with increased production of ROIs. ROIs can be classified according to their reactivity towards biological targets, their site of production, their chemical nature, or their free radical or non-radical sub groups. ROIs contain one or more unpaired electron in their outer orbits.[25] In order to achieve a stable state, these unstable molecules 'steal' electrons from other

molecules (e.g. lipids, proteins, DNA), which are themselves rendered unstable by this reaction and a cascade of cytotoxic reactions ensues (Figure 1.9).

The non-radical reactive oxygen derivatives contain their full complement of electrons, but in an unstable state. The most important among them is hydrogen peroxide (H_2O_2) and singlet oxygen (O_2). Hydrogen peroxide can generate free radicals through the Fenton reaction,[21] and singlet oxygen can damage molecules as it converts back to normal oxygen.

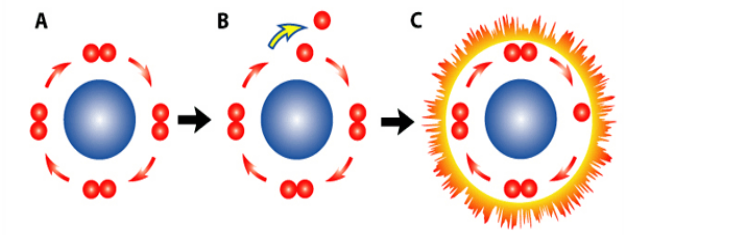


FIGURE 1.9 Production of reactive oxygen intermediates. A, stable molecule; B, electron is stolen; C, unstable molecule. Image courtesy of Prof. John Nolan

1.4.3.1.3 *ROIs and retinal cellular damage*

The retina is particularly vulnerable to damage by ROIs. The vulnerability of the retina is due to the extremely high metabolism of oxygen and therefore produces an equally high amount of ROI by-products. In addition, the photoreceptor outer segments contain a high concentration of polyunsaturated fatty acids (PUFAs), which are readily oxidised by ROIs because of their conjugate double bonds, thus generating a cytotoxic chain reaction of events, thereby producing yet more ROIs and further consequential oxidative injury.[21;26]

It is widely accepted that oxidative damage plays a significant role in the pathogenesis of AMD; however, the precise mechanism is not yet fully understood. AMD is characterised by a loss of photoreceptors and RPE cell dysfunction,[27] the latter being largely attributable

to an age-related accumulation of lipofuscin (yellow-brown pigment granules representing lipid-containing residues of lysosomal digestion).[28] Of note, the accumulation of lipofuscin within the RPE cells increases as a result of incomplete digestion of oxidatively damaged photoreceptor outer segment membranes.[29] In turn, liopfuscin acts as a chromophore (a compound which, when irradiated with light of an appropriate wavelength, emits an electron, thereby generating a ROI),[21;30] thus provoking further oxidative injury.[29;31]

Bruch's membrane is a permeable barrier situated between the choriocapillaris and the RPE. Its function is to regulate the movement of various substances between these two tissues. Bruch's membrane is known to accumulate lipid age deposits with increasing age.[32] These lipids originate from the PUFAs of photoreceptor outer segments,[32] and are thought to compromise the function of Bruch's membrane as well as provide an ideal substrate for the further generation of ROIs.[33]

1.4.3.2 Cumulative exposure to short wavelength light

Short wavelength (blue) light is situated in the lower part of the visible spectrum at a wavelength of approximately 458 nm. The visible spectrum extends from a wavelength between 400 – 700 nm. The lens and the cornea have the ability to filter and block the majority of light within the ultraviolet range (10 – 100 nm), in contrast the retina is very susceptible to blue light damage.[34] Blue light is very high in energy and has been shown to have a major impact on photoreceptor and RPE function, inducing photochemical damage and apoptotic cell death.[35]

Several studies have shown that blue light exerts the highest amount of damage to the retina when compared to other wavelengths of light.[36] It is also known that the photoreceptors house an abundance of photosensitisers, substances which produce ROIs when exposed to short wavelength light under aerobic conditions.[37] Lipofuscin, the lipid-protein aggregate that accumulates within the RPE cells, is derived from phagocytosed photoreceptor outer segments,[29] and is an ideal environment for the production of ROIs. Lipofuscin, because of its broadband light absorption spectrum and its constant exposure to light and oxygen within the RPE, leads to cellular dysfunction at the retina.[38] There have been many investigations into oxidative damage to RPE cells which has implicated A2E, a constituent of lipofuscin, as an initiator to light-induced cell apoptosis in the RPE.[39;40]

1.4.3.3 Inflammation

Inflammation and its exact biochemical mechanisms with respect to AMD has been the subject of widespread debate.[41] A study by Hollyfield *et al.* identified a mechanism of oxidative damage-induced inflammation which occurs at a retinal level and results in the formation of drusen like lesions, consistent with AMD.[42] In this study they immunised mice with a unique oxidation fragment of a photoreceptor PUFA which had been previously found adducted to proteins in the drusen of AMD eyes. These mice mounted an auto-immune response to this oxidation fragment and, following the inflammatory complement cascade, developed drusen like lesions. Most recently a study by Kauppinen *et al.*, 2012 concluded that oxidative stress can activate NLRP3 inflammasomes in RPE cells, which occupy centre stage in the pathogenesis of AMD.[43]

1.5 CAROTENOIDS

1.5.1 Definition

Carotenoids are a specialised group of naturally-occurring coloured pigments that possess protective properties. Carotenoids are synthesised *de novo* by all plants, where they play an important role in the photosynthetic process. Carotenoids are also found in non-photosynthetic micro-organisms (bacteria, yeast, and moulds), where they are known to protect against the detrimental effects of light and oxygen.[44] Carotenoids have many common properties: they are hydrophobic, they have little or no solubility in water; they are intensely coloured; they protect against blue light damage; and they protect against oxidative stress.[45;46]

1.5.2 Biochemical structure of carotenoids

Carotenoids have a basic 40-carbon ($C_{40}H_{56}$) structure from which all variations are derived. The central carbon chain of alternating single and double bonds carry cyclic or acyclic end groups. The extended system of conjugated double bonds contributes to their major biochemical functions, and is responsible for their colour. Carotenoids can be subdivided into two groups: carotenes composed of only carbon and hydrogen atoms, and the xanthophylls, which have at least one oxygen atom. β-carotene, α-carotene, and lycopene are members of the carotene group. L, Z, *meso*-zeaxanthin (*meso*-Z), α-cryptoxanthin, and astaxanthin are members of the xanthophylls group.

1.5.2.1 Biochemical structure of the macular carotenoids

The stereochemistry of the xanthophyll carotenoids L, Z and *meso*-Z are similar and are the only carotenoids found at the macula.[46] These three macular carotenoids are structural isomers of one another, where L differs from Z and *meso*-Z by the positioning of a double bond in the six-carbon ring located on the right side of the carbon chain (Figure 1.10). The presence of *meso*-Z at the macula is believed to be due to an isomerisation of retinal L, the nature of which remains to be fully elucidated.[46]

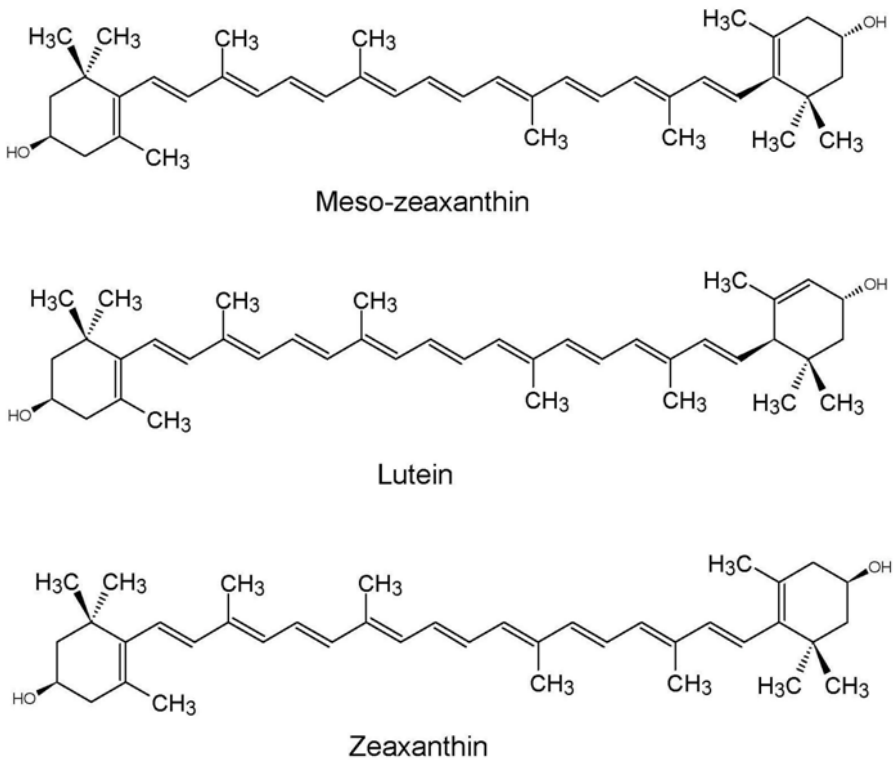


FIGURE 1.10 Chemical structures of *meso*-zeaxanthin, lutein and zeaxanthin.[1]

1.5.3 Sources of macular carotenoids

To date approximately 700 carotenoids have been identified and isolated in nature, with humans typically ingesting up to 40 of these carotenoids through the metabolism of carotenoid-rich foods. Humans are unable to synthesise L and Z *de novo*, which means they are entirely of dietary origin.[47]

Fruit and vegetables are the most important source of carotenoids in the human diet. An average western diet contains 1.3-3 mg/day of L and Z combined,[48] with significantly more L than Z (represented by an estimated ratio circa 7:1).[48;49] 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 (including spinach, broccoli, kale, and collard greens).[19] However, as most current dietary databases report intakes of L and Z combined, it has been difficult to assess the relative intakes and respective roles of the individual macular carotenoids at the macula. Recently, a study by Perry *et al.* did report concentrations of L and Z separately within the major food sources, as determined by the National Health and Nutrition Examination Survey (NHANES). This study, confirmed that green leafy vegetables are the richest source of L (e.g. cooked spinach and kale), whereas corn and corn products were confirmed as being a major source of Z.[50] Egg yolk is a source of highly bioavailable Z and L. The lipid matrix of egg yolk, containing cholesterol, triacylglycerols and phospholipids, provides a vehicle for the efficient absorption of the carotenoids.[50-53]

It appears that humans ingest relatively low levels of *meso*-Z (if any); however, research is ongoing in this area, given the recent interest in this centrally located macular carotenoid. To date, there has been no comprehensive analysis of the concentration of *meso*-Z in a typical diet. However, eggs from hens fed *meso*-Z are known to be a rich human dietary source.[54] Also, a study by Maoka *et al*. in 1986 reported that *meso*-Z and Z are present in 21 species of edible fish, shrimp, and sea turtles.[55] It is well known that in both rainbow trout and salmon, the colour of the flesh is due to the deposition of astaxanthin (a carotenoid from the same family as *meso*-Z). Analysis of skin from trout fed a diet rich in astaxanthin revealed significant quantities of *meso*-Z formed from the astaxanthin. Like the rainbow trout, the Atlantic salmon also deposits *meso*-Z within its skin.

The presence of *meso*-Z in the serum of unsupplemented individuals has never been demonstrated unambiguously. Efforts to extract and quantify *meso*-Z in human blood have demonstrated that if it is present, the concentrations are low.[46;56] Interestingly, in spite of its absence or low concentrations in a normal diet, *meso*-Z accounts for about one third of total MP at the macula, consistent with the hypothesis that retinal *meso*-Z is produced primarily by isomerisation of retinal L at the macula.[46]

1.6 MACULAR PIGMENT

1.6.1 Definition

At the macula, the carotenoids L, Z and *meso*-Z selectively accumulate in high concentrations, to the exclusion of all other carotenoids, and are collectively known as macular pigment (MP).[3;57] *Meso*-Z, L and Z are naturally occurring hydroxycarotenoid

plant pigments. L and Z are dietary in origin and are not synthesised *de novo* in humans, whereas *meso*-Z is not found in a conventional diet, but is primarily formed in the retina following conversion from L.[58]

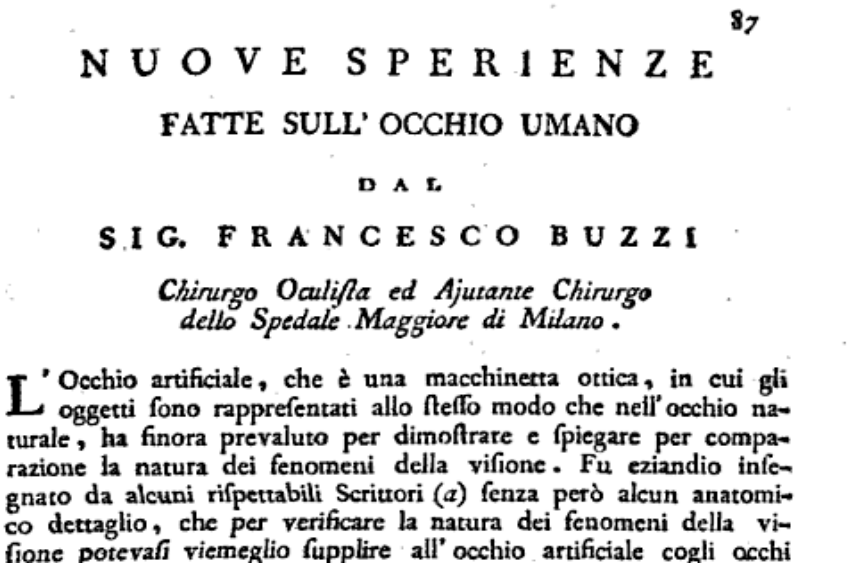
1.6.2 History of macular pigment

In 1792, an ophthalmologist called Francesco Buzzi was the first to describe the yellow colouration that was visible at the centre of the retina in the human eye. He reported this finding in his well-known work "*Nuovo sperienze fatte sull' occhio umano*" – new experiments on the human eye (Figure 1.11).[59] Almost concurrently, in 1795 Samuel Thomas von Soemmering independently discovered the *foramine centrali limbo luteo* (the central yellow-edged hole). His description of it was a "yellow round spot, and a small hole in the middle", and he published his finding in a communication in 1799.[60] Investigations intensified into the composition and function of this yellow pigment following a review written by Sir Everard Home, a British physician, in 1798. Sir Home carried out many experiments to investigate the presence of this pigment. He concluded that only human and monkey eyes contained the pigment.[61]

The term "macular pigment" was first coined by Walls *et al*. in 1933. In the late 19th and early 20th Century it was proposed that this pigment may have protective properties for the retina against short wavelength damage.[62] In 1855 James Clerk Maxwell published *"Experiments on colour as perceived by the human eye with remarks on colour-blindness"* This was the first indication that the "yellow spot" may play a role with respect to colour vision.[63]

This was first discussed by Schultze in 1866 who believed that macular yellow may reduce chromatic aberration and provide some protection against the hazards of short wavelength visible light.[64] In 1945, Wald examined the spectral properties of macular pigment, showing that it had a spectrum that related to carotenoid absorption which belonged to a family of xanthophylls found in green leaves. Extraction of this pigment yielded a hydroxyl carotenoid that Wald believed was L.[65]

It was not until 1985 that Bone and Landrum proposed that this pigment was composed of the carotenoids L and Z,[45] which was later confirmed by Handlemann *et al*.[66] An additional carotenoid, *meso*-Z, was identified later as the third carotenoid located at the central retina. It was also demonstrated that this was the most central carotenoid at the macula.[46] It was further proposed by Landrum *et al*. that *meso*-Z was primarily formed at the macula following isomerisation from retinal L.[67] This has since been confirmed in a study carried out by Neuringer *et al*. in 2004.[47]



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NUOVE SPERIENZE

FATTE SULL' OCCHIO UMANO

DAL

SIG. FRANCESCO BUZZI

Chirurgo Oculiſta ed Ajutante Chirurgo dello Spedale Maggiore di Milano.

L'Occhio artificiale, che è una macchinetta ottica, in cui gli oggetti ſono rappreſentati allo ſteſſo modo che nell'occhio naturale, ha finora prevaluto per dimoſtrare e ſpiegare per comparazione la natura dei fenomeni della viſione. Fu eziandio inſegnato da alcuni riſpettabili Scrittori (*a*) ſenza però alcun anatomico dettaglio, che per verificare la natura dei fenomeni della viſione potevaſi viemeglio ſupplire all'occhio artificiale cogli occhi

FIGURE 1.11 First description of the macula lutea.[59]

1.6.3 Retinal accumulation of the macular carotenoids

As mentioned above, the macular carotenoids are selectively accumulated at the macula (Figure 1.2). In humans this accumulation of macular carotenoids, MP, is found in its highest concentration at the fovea in the fibres of Henle, and at the parafovea in the inner and outer plexiform layers.[68] It has been shown *in vitro*, that a mixture of L, Z, and *meso*-Z, in a ratio of 1:1:1,[69] can quench more singlet oxygen than the individual carotenoids at the same total concentration, and may explain the exquisite biological selectivity and spatial distribution of these pigments within this specialised retinal tissue.[70]

1.6.4 Distribution of the macular carotenoids

The distribution of the macular carotenoids in the primate retina has been demonstrated to generally peak at the centre of the macula with a concentration of 1 mM at this location.[70-72] At the fovea Z is the most predominant carotenoid, with L predominating in the

parafoveal region. The concentration of *meso*-Z peaks centrally (*meso*-Z:Z ratio is 0.82 in the central retina [within 3 mm of the fovea] and 0.25 in the peripheral retina [11-21 mm from the fovea]) (Figure 1.12).[71] This distribution is most probably attributable to the fact that retinal *meso*-Z is produced primarily by isomerisation of retinal L, therefore accounting for lower relative levels of L, and higher relative levels of *meso*-Z, in the central macula, and vice versa in the peripheral macula.[46]

The spatial profile of MP in the majority of the population measured has been shown to peak centrally, and decline exponentially to optically undetectable levels at a retinal eccentricity between 7° to 10° from the fovea.[57;73] However, various research studies have reported, in some subjects, deviations from this typical exponential distribution within the central 1° of retinal eccentricity.[73-76]

For example, Delori *et al*., 2006, described bimodal spatial distributions of MP that were characterised by a ring-like pattern with high density values at approximately 0.7° retinal eccentricity from the fovea.[74] This was later confirmed by Berendschot *et al.*, who reported that both reflectance and auto fluorescence (AF) maps also displayed this ring-like distribution and suggested that such patterns follow the distribution of the inner plexiform layer.[75]

There is now a consensus that inter-individual variability in MP spatial profile does exist, however, the terminology used to classify such variation has differed due to the different methods used to measure MP.

With HFP, a cross section of MP is measured, prompting terms such as 'central dip'[77], 'minor flanking peaks'[73], or 'shoulder'[73] to describe profiles that do not exhibit the typical decline, approximately 40% of subjects measured have a 'central dip'. Using AF, the term 'ring-like' structure has been used to describe the profile measured. For this study, an atypical profile has been described as one where MPOD at 0.25° does not exceed MPOD at 0.5° by more than 0.04 optical density unit (ODU).

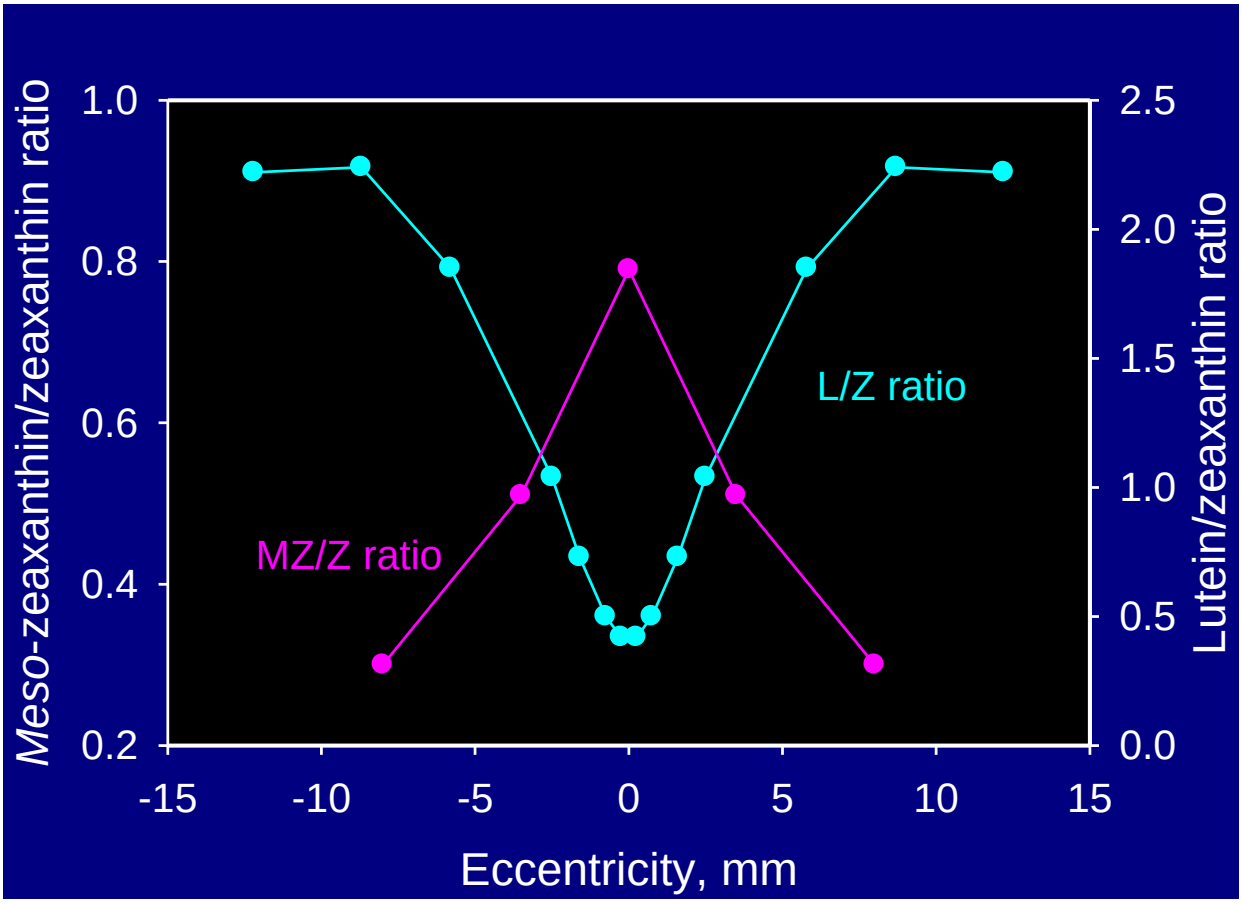


FIGURE 1.12 Distribution of the macular carotenoids where 0° eccentricity represents the centre of the fovea. Image courtesy of Prof. Richard Bone

1.6.5 Functions of macular pigment

It is now widely accepted that MP plays an important role in macular health; in particular its ability to act as an antioxidant[78] and a blue light filter.[3] It has also been suggested that it is important for visual performance.[79-81]

1.6.5.1 Antioxidant

The biochemical structure of the macular carotenoids L, Z and *meso*-Z (containing a high number of double bonds) allows them the capability to quench singlet oxygen, free radicals, and triple state photosensitisers, thus limiting phosopholipid peroxidation.[82-86] A study by Kirschfeld *et al.* was the first to propose the theory that MP may act as an antioxidant to protect the macula against oxidative stress.[87] Direct evidence that MP acts as an antioxidant was shown by Khachik *et al.*, who demonstrated the presence of oxidation products of L and Z in retinal tissues.[78]

An *in vitro* study conducted by Siems *et al.* demonstrated that L and Z are more resistant to degradation from oxidative stress than other carotenoids,[88] and a further study investigating the effects of oxidative stress on human RPE cells showed enhanced survival in the presence of Z and other antioxidants.[85] Of the three macular carotenoids, it appears that Z is a more potent antioxidant than L,[84;89] and *meso*-Z has been shown to be an even more potent antioxidant than Z, but only in the presence of a binding protein; however the situation is reversed if the binding protein is absent.[82] Animal studies have provided evidence of protection from light-induced photoreceptor damage in the presence of L and Z.[90;91] Most recently an investigation by Li *et al.* reported that the singlet oxygen

quenching ability of L, Z and meso-Z in a ratio of 1:1:1 can quench more singlet oxygen than the individual carotenoids.[69]

1.6.5.2 Optical filter

MP is a filter of blue light, as the macular carotenoids have an absorption spectrum of 458 nm; this filtration reduces the photo-oxidative damage to retinal cells.[92] It has been estimated that the quantity of visible light (~460 nm) incident upon the photo-receptors of the macula is substantially reduced as a result of the filtering properties of MP; this reduction is estimated at approximately 40%, but varies from 3-100% between individuals.[3;93] L allows the greatest protection from blue light incident on the retina because of its parallel and perpendicular orientation to the cell membrane, which allows it to filter light in all directions in comparison to Z and *meso*-Z, which are orientated parallel to the cell membrane.[83;92] However, it should be noted that the three macular carotenoids have different absorption spectra and, therefore, the combination of all three carotenoids at the macula results in the pre-receptoral absorption of more light than any individually (Figure 1.13).

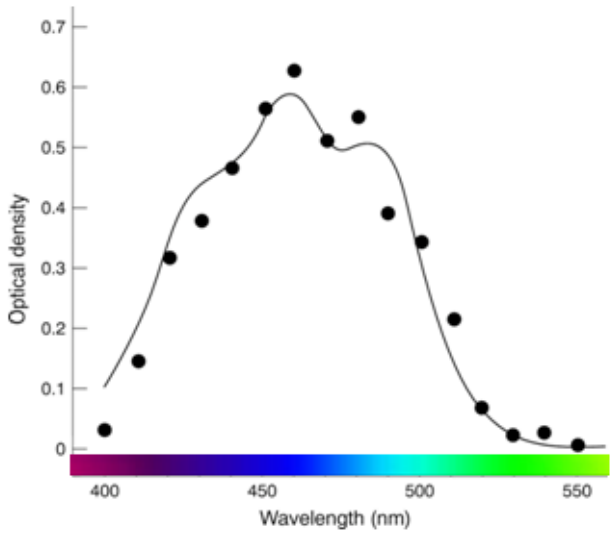


FIGURE 1.13 Absorbance spectrum of macular pigment. Image courtesy of Prof. John Nolan

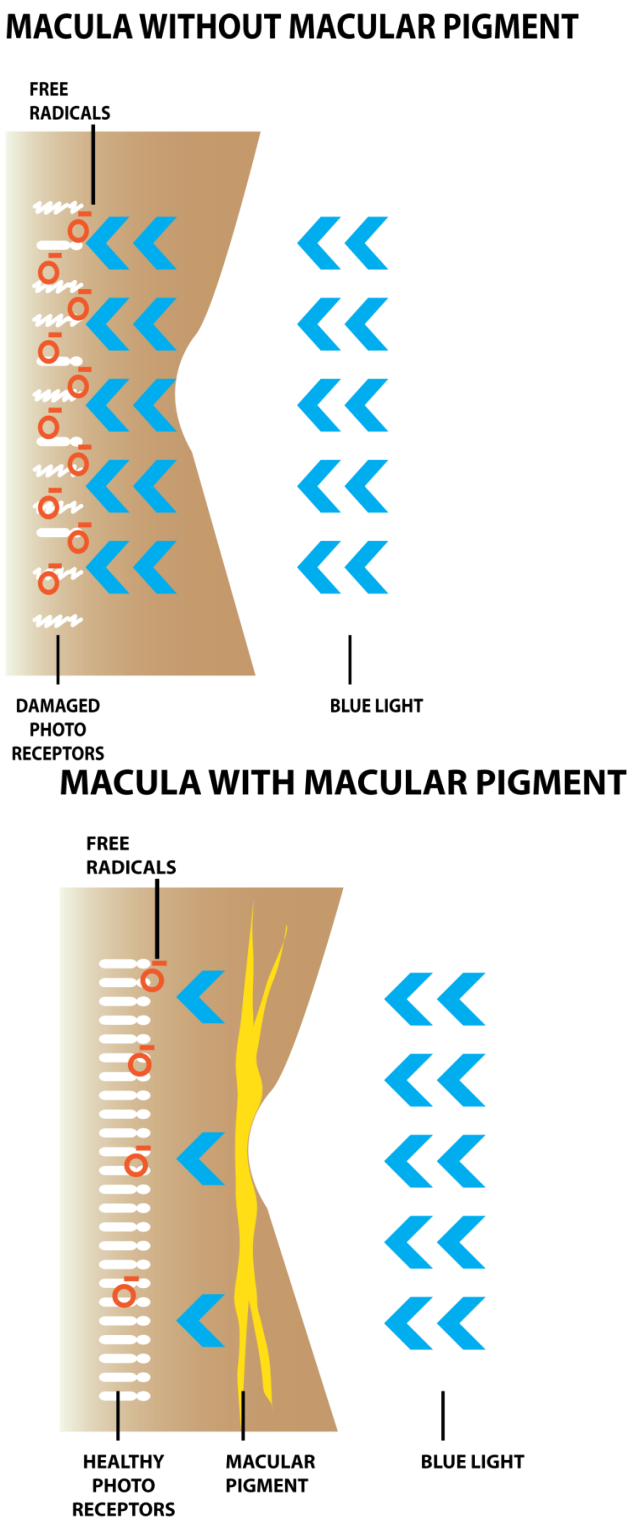


FIGURE 1.14 How macular pigment protects the retina. Image courtesy of Prof. John Nolan

1.6.6 Macular pigment: Protective hypothesis

The lens and the cornea have the capacity to absorb almost all UVA (320-400 nm) and UVB (320-290 nm) light. Light of longer wavelengths (400-520 nm) passes through the anterior media where they irradiate the macula. MP has a maximum absorbance of 460 nm (Figure 1.13), and therefore filters out damaging blue light at a pre-receptoral level, which allows MP to attenuate the amount of blue light incident on the macula.[93] It has been estimated that MP absorbs approximately 40% of blue light before it is incident on the photoreceptors.[94]

L is reported to be a superior filter of blue light when compared with Z, this is due to its orientation with respect to the plane of the phospholipid bilayer of the cell membrane,[83] which is both parallel and perpendicular. In contrast, Z and *meso*-Z only exhibit perpendicular orientation to this layer. However, it is the combination of the absorption spectra of all the three carotenoids that collectively offer optimal filtration of blue light at the macula, which would not be achieved by any of these carotenoids individually (Figure 1.14).[83;95] This ability to absorb blue light is an important function, as a high exposure to high energy wavelengths can result in photochemical retinal injury; this has most recently been demonstrated by Barker *et al.* in rhesus monkeys using low laser light (476 nm [blue]).[96] In this study, one group of monkeys had been deprived of the macular carotenoids from birth, giving them no detectable MP. The control group of monkeys had been fed a typical diet of L and Z from birth. The retinae of the monkeys deprived of the carotenoids was exposed to the low power laser light and then supplemented with either L or Z, six months later they were again exposed to the low power laser light. The relationship between lesion size and exposure energy was then analysed in both groups.

The control monkeys showed less light-induced damage in the foveal region compared to the parafovea (where there is no MP). In contrast, the monkeys who were deprived of the macular carotenoids showed no difference in light induced damage between the fovea and parafovea prior to supplementation. This finding helps to support the hypothesis that MP offers foveal photoprotection.

The peak concentration of MP at the centre of the fovea is also consistent with its role as an optical filter. It has been shown that short wavelength cones (s cones) suffer a loss in sensitivity with increasing age,[97] however, it has also been shown that this loss of sensitivity is reduced at the fovea, where MP peaks, suggesting a protective effect of this pigment.[98]

MP also displays antioxidant properties, including an ability to quench singlet oxygen, and inhibit the peroxidation of phospholipids.[82-86] The antioxidant properties of L and Z have been demonstrated in the retina.[78] Because of their readily available supply of electrons they are ideally located to quench ROIs, thus limiting membrane phospholipid peroxidation and attenuating oxidative damage.

1.6.7 Macular pigment: Visual performance hypothesis

In addition to the 'protective' hypothesis of MP, its optical (short wavelength-filtering) and anatomic properties suggest it plays a role in visual performance and experience in the normal population (visual performance hypothesis).[99-102] The antioxidant properties of MP may attenuate or prevent the deleterious effect of free radical damage on the physiological

functions of the photoreceptors and their axons. To date, there have been many studies carried out on the role MP plays in visual performance, whereby MP may enhance visual acuity, glare disability, photostress recovery, contrast sensitivity and colour vision.[79;81;100;102-104] However, not all of the studies carried out have been able to find significant findings in relation to visual performance. Most notably, Stringham *et al*., 2008, reported that MP is strongly related to improvements in glare disability and photostress recovery in a manner strongly consistent with its spectral absorption and spatial profile. Following four to six months of daily 12 mg L and Z supplementation there was a significant increase in MPOD, and visual performance in glare improved for most subjects.[81]

1.7 RISK FACTORS FOR AGE-RELATED MACULAR DEGENERATION

1.7.1 Introduction

AMD is the leading cause of visual impairment in the developed world.[5] It has been established that there are three main risk factors for the development of AMD which are: increasing age; cigarette smoking; positive family history of AMD. However, there are also additional putative risk factors which include: obesity; sex; low dietary intake of L and Z; low macular pigment levels; high levels of light exposure.[105]

1.7.2 Increasing age

Increasing age is the most important risk factor for AMD. Studies have shown that the progression and prevalence of AMD has been rising exponentially with increasing age.[106] This is consistent with the '*Free Radical Theory of Aging'* which states that with

increasing age, there is a rise in the oxidant load coupled with a decrease in antioxidant load within the body.[21;107] These actions can prove deleterious within the body, leading to increases in lipid peroxidation, the formation of age pigments (e.g. lipofuscin), DNA damage and reduced mitochondrial function. These processes have been hypothesised to play an important role in the development of AMD.[108-112]

1.7.3 Positive family history of age-related macular degeneration

It has been established that having a positive family history of AMD is a risk factor for this condition.[113] To date there have been several genes that have been associated with an increase in AMD, which include the Complement Factor H (CFH) gene, the Apolipoprotein E (Apo E) gene and the ARMS2.[114;115] CFH acts as a regulator of inflammation in the innate immune system. Variants of the gene which code for CFH have been associated with an increased risk of AMD.[114] Individuals with the Y402H variant of the CFH gene are unable to regulate the inflammatory response to damaged cells (i.e. oxidative damage), and as a result, further cell damage occurs. The ARMS2 gene, has also been associated with an increased risk of AMD.[115] However, the precise role of this gene has yet to be fully understood. The Apo E gene codes for particular apolipoproteins, which are transporters of lipids and cholesterol within the body. It has been suggested that the inheritance of a specific Apo E allele may contribute to drusen formation at the retina, thereby increasing the risk of AMD.[116]

1.7.4 Cigarette smoking

It is now accepted that cigarette smoking greatly increases an individual's risk of developing AMD as it increases the volume of ROIs and oxidative stress levels in the body. Almost all epidemiological studies carried out to date have shown an increased incidence and prevalence associated with cigarette smoking.[117] An animal model has shown that mice exposed to chronic levels of cigarette smoke develop evidence of oxidative damage with ultrastructural degeneration of the RPE and Bruch's membrane, and RPE cell apoptosis, in comparison to mice exposed to normal room air.[118] Interestingly, mice exposed to a more severe concentration of cigarette smoke, but for a shorter period, developed similar structural changes to Bruch's membrane and the choriocapillaris without any RPE changes. This may indicate that chronic exposure to cigarette smoke is required to cause changes to RPE cells.[117] It has also been suggested that cigarette smoking causes the same vascular damage in the eye to those seen in cardiovascular disease.[119] Therefore, cigarette smoking may simply represent an antecedent common to both atherosclerosis and AMD, as AMD has been putatively linked with cardiovascular disease.[21] Finally, many studies have consistently shown that cigarette smoking is associated with lower levels of MP.[105;120]

1.7.5 Obesity

Obesity is one of the putative risk factors for AMD. The rationale surrounding this is due to obesity being linked with poor diet; increased oxidative stress; undesirable cholesterol levels (cholesterol may hinder antioxidant transport); increased inflammation. However, studies investigating the relationship between obesity and AMD have reached conflicting conclusions. The majority of the studies looking at obesity have been cross sectional in

design. The Blue Mountains Eye Study reported that there was a higher prevalence of AMD in individuals with above average body mass index (BMI),[121] likewise the Age-Related Eye Disease Study (AREDS) Report no. 3 also found that neovascular AMD was more prevalent amongst those with a high BMI.[122] The Pathologies Oculaires Liées à l'Age (POLA) study also reported that obesity (assessed by BMI) resulted in an increased risk of developing early signs of AMD.[119]

1.7.6 Sex

It has been suggested that female sex is associated with a higher risk for AMD; however, the rationale for this association remains unclear. One suggestion is that it is linked to lower oestrogen levels that can lower haemodynamics, and consequentially, contribute to AMD, however studies supporting this relationship are conflicting.[123;124]

1.7.7 Low dietary intake of lutein and zeaxanthin

Briefly, L and Z are entirely dietary of origin and humans cannot synthesise them *de novo*. Individuals with a poor diet will therefore have lower MPOD levels. In the literature several case-control studies have reported on the relationship between dietary intake of carotenoids to both risk and prevalence of AMD. The Dietary and Ancillary Study of the Eye Disease Case Control Study (EDCCS) and AREDS Report no. 22, both found a strong association between high dietary intake of L and Z and reduced risk of developing AMD.[15;125]

1.7.8 Exposure to blue light

Cumulative (short wavelength) light damage represents an environmental factor, which is believed to be a risk factor for the development of AMD. [34;35] The visible spectrum of light extends from a wavelength of approximately 400-700 nm. Short wavelength blue light lies in the lower end of this spectrum, at a wavelength of approximately 458 nm. while the majority of light within the ultraviolet range (10-400 nm) is blocked out by the lens and the retina.[34] However, substantial quantities of high-energy visible light irradiates the retina, particularly in young people who have clearer lenses than elder people.

Investigations have frequently demonstrated that damage to the photoreceptors and the RPE of laboratory animals can be induced by ambient levels of visible light.[126] It has been shown that blue light exerts the most damage to the retina when compared to other wavelengths of light.[36]

Lipofuscin appears to be a key mediator of photo-oxidative stress, and has been shown to be a photo-inducible generator of ROIs, with the threshold for generation of these unstable molecules being lowest for light at the blue end of the visible spectrum.[127] The retina is an ideal location for the generation of singlet oxygen by lipofuscin due to its high exposure to light and oxygen. ROIs produced by photoreactive lipofuscin have been shown to cause RPE cellular dysfunction.[128] Specifically, the lipofuscin constituent A2E has been implicated as a mediator to light-induced oxidative damage to RPE cells.[39] In a cell culture model of human RPE cells, it was confirmed that A2E was an initiator of blue light-induced RPE cell apoptosis.[40]

1.8 SAFETY OF THE MACULAR CAROTENOIDS

1.8.1 Human studies

Prior to this research study being carried out there had been no human clinical trials reporting on the safety of supplemental macular carotenoids by conducting comprehensive clinical pathology analysis. A review by Shao *et al.* identified over 30 peer-reviewed human clinical trials involving L with a minimum dose of 2 mg/day.[129] A limited number of these studies investigating the beneficial effects of L have assessed any possible side effects and were mainly assessed by self report. There has been no published human study that has specifically focused on the safety of L, Z or *meso*-Z supplementation.

1.8.1.1 Lutein

The majority of studies investigating L have focused on short term use, and follow up was no longer than 12 months. The highest dose of L used in a human trial was 40 mg/day for nine weeks followed by 20 mg/day for 17 weeks.[130] To date only one randomised placebo-controlled clinical trial has investigated L response with safety (self reported) over a duration of 12 months at a dose of 10 mg/day.[131]

There is a high level of confidence in the safety of L following the absence of any pattern of adverse effects from published clinical trial data. There has only been one documented side effect of L supplementation called carotenoidermia. Carotenoidermia is a reversible condition characterised by a yellow discoloration of the skin. The condition most often occurs as a result of high β-carotene supplementation.[132-134] However, following clinical trials using L, two cases reported incidences of carotenoidermia following supplementation

with concentrations of 15 mg/day for 4-5 months.[129] The Institute of Medicines recognises carotenoidermia as a harmless biological effect of high carotenoid intake.[135]

1.8.1.2 Zeaxanthin

There have been a number of studies designed to investigate the pharmacokinetics of L and Z that did not necessarily include safety end-points. In a pharmacokinetic study that included five men and five women who were given capsules containing either 1 mg or 10 mg of Z per day for 42 days (corresponding to doses of approximately 0.014 and 0.14 mg/kg body weight (bw) per day for a 70 kg adult), clinical chemistry measures (haematology, blood chemistry and urine analysis) and adverse events were recorded.[133] In the group receiving the higher dose there were three adverse effects (one case of bilirubinaemia, one case of abnormal vision, and one case of abnormal accommodation) that were deemed to be remotely or possibly related to treatment. All the adverse events were rated as mild to moderate in severity. A variety of clinical chemistry measurements as well as any adverse events were recorded during the study. There has been a relatively large number of human studies that have examined correlations between AMD and exposure to L/Z via intake from traditional food or from dietary supplements, or via measurements of serum concentrations. Although these studies were designed to look for ocular effects, where clinical or biochemical parameters were also examined, no adverse effects of the xanthophylls were reported.

1.8.1.3 Meso-zeaxanthin

Human clinical trials reporting on the safety of *meso*-Z are limited to date, with the majority of them not reporting any safety information.

1.8.2 Animal studies

Most recently a study by Ravikrishnan *et al.* (2011) investigated the adverse effects of Lutemax 2020™ in acute and subchronic toxicity, and mutagenicity in Han Wistar rats;[136] this investigation demonstrated no lethality at 2000 mg Lutemax 2020™/kg bw. In the subchronic study, Han Wistar rats were administered L/Z at concentrations of 0, 4, 40, and 400 mg/kg bw/day for 90 days. Compared to the control group, administration of L/Z did not result in any significant changes in clinical observation, ophthalmic examinations, body weights, feed consumption, and organ weights. Following this test the no observed adverse effect level (NOAEL) for L/Z was determined as 400 mg/kg bw/day.

The World Health Organisation carried out a comprehensive report in 2006 which included all safety evaluations carried out to date on both L and Z. The conclusion for L was that in several studies of toxicity, including developmental toxicity, no adverse effects were documented in animals, including monkeys, or humans and the NOAEL was 200 mg/kg bw/day. The conclusion for Z was that it would be included in the acceptable daily intake at 0-2 mg/kg bw due to its spectral and physiological similarities with L.

Studies to date have investigated the safety of *meso*-Z using both animal models and humans. In 2006, Chang *et al.* carried out a study on the toxicitiy of *meso*-Z by administering oral doses of 2, 20 and 200 mg/kg/day to Han Wistar rats for 13 weeks.[137] The conclusion from this study was that no compound-related mortality, clinical signs of toxicity, changes in body weights, ophthalmology, clinical pathology, gross pathology, or histopathology were noted. Based on the results of this study, the NOAEL of *meso*-Z in rats is 200mg/kg/day when administered orally for 13 consecutive weeks.

Meso-Z has also been tested for mutagenic activity using Salmonella typhimurium tester strains TA98, TA100, TA1535 and TA1537 and Echerichia coli tester strain WP2uvrA in both the presence and absence of microsomal enzymes prepared from Aroclor™ induced rat liver. No dose produced mutagenetic activity was observed.[138]

1.9 STUDY OBJECTIVES

This body of research was designed to investigate human response to the macular carotenoids *meso*-Z, L and Z. *Meso*-Z was of particular interest as limited data was available at the time, including only animal models and two small research projects involving human subjects. One of these studies measured MPOD response and the second measured serum response. Therefore, a comprehensive study was designed to investigate how humans would respond to the combination of the macular carotenoids when given in the form of a supplement.

The first study, the *Meso*-zeaxanthin Ocular Supplementation Trial (MOST), was designed to evaluate MPOD response, including its spatial profile (i.e. 0.25°, 0.5°, 1°, and 1.75°), and serum carotenoid response, in 10 subjects (five normal and five AMD), following consumption of a dietary food supplement containing all three macular carotenoids: *meso*-Z, L and Z, in which *meso*-Z was the predominant carotenoid.

The second study (the *Meso*-zeaxanthin Ocular Supplementation Trial in Normals [MOST-N]) was designed as the first double blind, placebo-controlled randomised study, to investigate serum and macular responses following consumption of a dietary supplement containing all three macular carotenoids (*meso*-Z, L and Z). In addition, it was also designed to assess the safety of consumption of the macular carotenoids *meso*-Z, 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.

CHAPTER TWO

STUDY ONE

Augmentation of macular pigment following supplementation with all three macular carotenoids: an exploratory study

2.1 INTRODUCTION

This study was designed to investigate how individuals respond to a supplement containing *meso*-Z in combination with L and Z, as it has been reported that there is an association between AMD and MP profile and given that research has shown that *meso*-Z is generated at the retina following conversion from L. It is possible that individuals lacking centrally located MP require *meso*-Z to be provided in supplement form, as such individuals could (perhaps) lack the capacity to convert L to *meso*-Z within the retina, although this hypothesis remains speculative for now.

There are several published studies reporting on supplemental L and/or Z, and the impact of such supplementation on MP levels and/or serum concentrations of these carotenoids (Table 2.1). In 1997, Hammond *et al*. showed that a diet modified to resulting in increased consumption of L and Z, for as little as four weeks, could augment MP, with this effect being maintained for several months following resumption of a normal, unmodified diet.[139] Of note, two of the 11 subjects involved in that study did not show a significant rise in MP optical density (MPOD), despite a significant increase in serum L concentrations. These subjects were termed "retinal non-responders", and it has been hypothesized that this phenomenon may be due to a compromised ability to capture and/or stabilise the macular carotenoids in these individuals. Landrum *et al.* investigated the effect of L supplementation (30 mg per day) in two individuals over a 140 day period.[140] They found an increase in serum L concentrations in both individuals, coupled with a parallel increase in MPOD. Trieschmann *et al.,* 2007, reported on a commercially available L-based supplement with respect to macular and serum response in patients who displayed features of AMD and concluded that supplementation with 12 mg of L and 1 mg of Z, combined

with co-antioxidants, resulted in a significant increase in MPOD at 0.5° eccentricity in the majority of subjects (average increase ~ 0.1 optical density units [ODU]).[141]

Of note, prior to this study there had only been one study which has investigated the effects of supplemental *meso*-Z on MPOD levels in human subjects.[142] That study, which included 10 subjects, showed that a soya bean oil-based supplement containing 14.9 mg of *meso*-Z, 5.5 mg of L and 1.4 mg of Z resulted in an average increase of ~ 0.07 MPOD at 0.75° of eccentricity over a 120 day period. However, limitations of the study performed by Bone *et al.* include: MPOD was measured at only one point of retinal eccentricity (~ 0.75°) and would therefore not have been able to detect changes in MPOD, if any, occurring at other retinal eccentricities (e.g. 0.25°, 0.5°, 1°, 1.75°), including the more central eccentricities of 0.25° and 0.5°; no controls were included in the study; small sample size (n = 10); and serum concentrations of *meso*-Z were only measured for two subjects.

This study, the M*eso*-zeaxanthin Ocular Supplementation Trial (MOST), was designed to evaluate MPOD response, across its spatial profile (i.e. 0.25°, 0.5°, 1°, and 1.75°), and serum carotenoid response, in 10 subjects (five normal and five AMD), following daily consumption of a dietary food supplement containing all three macular carotenoids: *meso*-Z, L and Z, in which *meso*-Z was the predominant carotenoid.

TABLE 2.1 Studies reporting on macular pigment optical density response to supplementation with the macular carotenoids.

Principal Author	Year	n	Age	L mg/d	Z mg/d	MZ mg/d	Duration (weeks)	Tec	Retinal ecc	PF	MP rise	Sig.
NORMAL subjects - dietary modification												
Hammond et al.[139]	1997	10	30-65	11.2	0.6	0	15	HFP	0.5°	5.5°	~ 0.05	$p < 0.05$
		2	30-65	0.4	0.3	0	15	HFP	0.5°	5.5°	~ 0.05	-
		1	30-65	10.8	0.3	0	15	HFP	0.5°	5.5°	~ 0.05	$p < 0.05$
Johnson et al.[143]	2000	7	33-54	11.2	0.57	0	15	HFP	0.5°	5.5°	~ 0.07	$p < 0.05$
NORMAL subjects - supplement modification												
Landrum et al.[140]	1997	2	42-51	30	0	0	20	HFP	0.75°	8°	~ 0.20	-
Berendschot et al.[144]	2000	8	18-50	10	0	0	12	SLO	0.75°	14°	~ 0.05	$p = 0.022$
		8	18-50	10	0	0	12	SA	0.75°	-	~ 0.04	$p < 0.001$
Aleman et al.[145]	2001	8	11-59	20	0	0	24	HFP	0.17°	5-7°	0.07	$p = 0.04$
		8	11-59	20	0	0	24	HFP	0.5°	5-7°	0.07	-
		8	11-59	20	0	0	24	HFP	1°	5-7°	0.08	-
		8	11-59	20	0	0	24	HFP	2°	5-7°	0.04	-
Bone et al.[146]	2003	2	19-59	30	1.5	0	20	HFP	0.75°	8°	~ 0.20	-
		1	53	0	30	0	17	HFP	0.75°	8°	~ 0.07	-
		21	19-59	2.4	0	0	17	HFP	0.75°	8°	~ 0.04	-
		12	19-60	20	0	0	17	HFP	0.75°	8°	~ 0.06	$p < 0.05$
		2	26-27	5	0	0	17	HFP	0.75°	8°	~ 0.03	-
Koh et al.[145]	2004	6	64-81	20	0	0	20	HFP	0.5°	6°	0.07	$p > 0.05$
Bernstein et al.[147]	2004	8	<61	20	0	0	16	HFP	0.75°	8°	0.04	-
		8	<61	20	0	0	16	RRS	-	-	76RC	-
Bone et al.[142]	2007	10	21-58	5.5	1.4	15	17	HFP	0.75°	8°	~ 0.07	$p < 0.05$
Wenzel et al.[148]	2007	3	24-52	30	2.7	0	17	HFP	0.33°	7°	0.07	$p < 0.001$
		3	24-52	30	2.7	0	17	HFP	0.5°	7°	0.07	$p < 0.002$
		3	24-52	30	2.7	0	17	HFP	1°	7°	0.046	$p< 0.002$
		3	24-52	30	2.7	0	17	HFP	2°	7°	0	-
Schalch et al.[149]	2007	23	18-45	10.7	0.8	0	17	HFP	0.5°	5.5°	0.06	$p = 0.04$
		23	18-45	0	12.6	0	17	HFP	0.5°	5.5°	0.01	$p > 0.1$
		23	18-45	10.2	11.9	0	17	HFP	0.5°	5.5°	0.06	$p = 0.04$
Johnson et al.[150]	2008	11	60-80	12	0.5	0	16	HFP	1.5°	7°	-	$p < 0.05$

		11	60-80	12	0.5	0	16	HFP	3°	7°	-	p < 0.01
Stringham et al.[81]	2008	40	17-41	10	2	0	24	HFP	0.25°	10°	0.19	-
		40	17-41	10	2	0	24	HFP	0.5°	10°	0.16	-
		40	17-41	10	2	0	24	HFP	1°	10°	0.1	-
		40	17-41	10	2	0	24	HFP	3°	10°	0.07	-
		40	17-41	10	2	0	24	HFP	7°	10°	0.03	-
Connolly et al.[56]	2010	5	30-85	3.7	0.8	7.3	8	HFP	0.25°	7°	0.16	p < 0.05
		5	30-85	3.7	0.8	7.3	8	HFP	0.5°	7°	0.16	p < 0.05
Nolan et al.[151]	2011	61	18-41	12	1	0	52	HFP	0.25°	7°	0.12	p = 0.001
		62	18-42	12	1	0	52	HFP	0.5°	7°	0.11	p = 0.001
Nolan et al.[152]	2012	12	56±8	20	2	0	24	HFP	0.25°	7°	0.09	p = 0.092
	2012	12	51±13	10	2	10	24	HFP	0.25°	7°	0.13	p = 0.002
Tanito et al.[153]	2012	11	NA	10	0.8	0	12	RRS	-	-	1140RC	p = 0.0817
	2012	11	NA	0	10	0	12	RRS	-	-	329RC	p = 0.908
	2012	11	NA	10	0.8	0	12	AF	0.5°	7°	0.97	p = 0.2451
	2012	11	NA	0	10	0	12	AF	0.5°	7°	0.017	p = 0.7467

AMD subjects

Principal Author	**Year**	**n**	**Age**	**L** mg/d	**Z** mg/d	**MZ** mg/d	**Duration (weeks)**	**Tech**	**Retinal ecc.**	**PF**	**MP rise**	**Sig.**
Koh et al.[145]	2004	7	64-81	20	0	0	20	HFP	1°	6°	0.07	p > 0.05
Trieschmann et al.[154]	2007	108	51-87	12	1	0	24	AF	1°	6°	0.1	p < 0.001
Richer et al.[155]	2007	76	-	10	0	0	52	HFP	1°	7°	0.25	p < 0.05
Connolly et al.[56]	2010	5	30-85	3.7	0.8	7.3	8	HFP	0.25°	7°	1.6	p < 0.05
		5	30-85	3.7	0.8	7.3	8	HFP	0.5°	7°	1.6	p < 0.05
Weigert et al.[156]	2011	84	72±9	15	0	0	24	HFP	0.25°	7°	0.08	p <0.001
Richer et al.[157]	2011	25	76±9	0	8	0	52	HFP *	1°	7°	0.13†	p = 0.03
		25	74±11	9	8	0	52	HFP	1°	7°	0.20†	p = 0.06
		10	74±9	9	0	0	52	HFP	1°	7°	0.18†	p = 0.03
Sasamoto et al.[158]	2011	33	64±9	6	0	0	52	AF	0.5°	7°	0.008	p = 0.9558
Hammond et al.[159]	2012	246/63‡	55+	12	0.6	0	104	RRS	central 3°	-	61 (RC)	p < 0.001
Ma et al.[160]	2012	27	50-79	10	0	0	48	AF	central 2°	-	18	p < 0.05
	2012	27	50-79	0	10	0	48	AF	central 2°	-	22.4	p < 0.05

	2012	27	50-79	10	10	0	48	AF	central 2°	-	6.9	$p < 0.05$

Abbreviations: 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; 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; RC = Raman count = data unavailable. *modified HFP technique (QuantifEYE®) †measurements from right eyes in the study ‡246 at baseline, 63 at year 2

2.2 METHODS

2.2.1 Subjects

This was a non-randomized, open labelled study. Ten subjects were recruited for this study. Subjects were recruited following a locally advertised poster campaign (*Appendix 5)* and by word of mouth. Following a detailed explanation of all aspects of the study by the study investigator (Eithne Connolly, EC), all subjects signed an informed consent document (*Appendix 1*). All experimental measures conformed to the Declaration of Helsinki for research involving humans. The study was reviewed and approved by the Research Ethics Committee, Waterford Institute of Technology and the Research Ethics Committee, South East Region, Waterford Regional Hospital, Waterford, Ireland (*Appendix 2*).

This study consisted of two groups; Group 1 (n = 5), inclusion criteria: 18 and 60 years of age; best corrected visual acuity (BCVA) of at least 20/60 in the study eye. Exclusion criteria: pregnancy (self reported); presence of ocular pathology (assessed by fundus photography); currently taking supplements containing *meso*-Z, L or Z. Group 2 (n = 5), inclusion criteria: male or female; early AMD (defined using the International Classification and Grading System for Age-Related Maculopathy and Age-Related Macular Degeneration)[4] in at least one eye (assessed by vitreo retinal specialist) with BCVA of at least 20/60 in that eye *(see Section 2.2.3),* hereafter known as the study eye for this group. Exclusion criteria: currently taking supplements containing *meso*-Z, L, or Z; presence of ocular pathology other than AMD.

2.2.2 Lifestyle/Demographic questionnaire (EC)

The following details were recorded for each subject using a personal/lifestyle questionnaire in the study case report form (CRF) *(Appendix 3)*: demographic details; BCVA; family history of eye disease; smoking history (from which pack year consumption was calculated as: [daily cigarette consumption x number of years smoked ÷ 20]. 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);[161] personal ophthalmic and medical history; medication use; alcohol consumption (average units weekly); iris colour; body mass index (BMI); blood pressure; ethnicity; and education.

2.2.3 Visual acuity (EC)

BCVA was measured for each eye 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 $20/20^{+1}$ equated to a score of 101, and $20/20^{-1}$ to 99. Subjects with a score of less than 75 in the better eye were excluded from the study. The study eye was selected as the eye with the better BCVA, if both eyes were identical in score the right eye was selected for all subsequent tests.

2.2.4 SUPPLEMENTATION

The mixture of carotenoids was manufactured by Industrial Organica SA, Monterrey, Mexico by isomerising L obtained from marigold extracts. A proportion of the L was converted into *meso*-Z, and the small quantity of Z in the extract remained unchanged. The resulting composition was microencapsulated after diluting with rice starch. Each capsule contained 7.3 mg *meso*-Z, 3.7 mg L, and 0.8 mg Z. All subjects (in both groups) were instructed to take one capsule per day with a meal for 60 days.

2.2.5 Blood sample collection (EC)

Training and certification for intravenous venepuncture was performed at Waterford Regional Hospital, Waterford, Ireland (*Appendix 4).* Blood samples (8 mL) were collected from each subject at every visit, using a standard aseptic venepuncture technique, in an 8.5 mL Becton Dickinson (BD) Vacutainer™ SST™ II clot activator and gel tube (BD Vacutainer Systems, New Jersey, USA). The procedure for venepuncture is given in the standard operating procedure (SOP) (*Appendix 7*). Once the blood was taken it was placed in a refrigerator immediately, prior to centrifugation, using a DESAGA Starstedt – Gruppe, GC12 centrifuge (Desaga GmbH, Heidelberg, Germany) at 4000 RPM for 10 minutes. Following centrifugation, the serum was aliquoted into 1.5 mL amber light-sensitive microcentrifuge tubes (Brand GmbH, Wertheim, Germany) and stored at minus 70° C until time of analysis.

2.2.6 Measurement of macular pigment optical density using heterochromatic flicker photometry (EC)

Heterochromatic flicker photometry (HFP) was the first technique developed to measure MPOD and is one of the most widely used techniques for measuring MPOD *in vivo*. HFP is a psychophysical technique, based on the principle that MP absorbs short wave-length (blue) light, with maximum absorption occurring at a wavelength of 458 nm. During MPOD measurement the subject observes a series of flickering targets, which alternate in square-wave counterphase motion between a green light (564 nm; not absorbed by MP) and a blue light (460 nm; maximally absorbed by MP). The log ratio of the amount of blue light absorbed centrally (fovea), where MP peaks, to that absorbed at a peripheral retinal locus (parafovea; the 'reference point, 7°', where MPOD is assumed to be zero), gives a measure of the individual's MPOD (Figure 2.1). This method has been validated against the absorption spectrum of MP *in vitro*.[162]

MPOD was measured using the Macular Metrics Densitometer™, developed by Professor B. R. Wooten of Brown University, Providence, Rhode Island, USA, using the HFP method. The device was modified from the one described originally.[163;164] Two different techniques for measuring MPOD using this device were employed for normal subjects and AMD subjects, and are described below. We used the bracketing procedure for the AMD subjects as we find this procedure more suitable for older subjects (see below). All subjects were trained how to perform the HFP task at their first study visit. MPOD data was not recorded until subjects demonstrated a high level of understanding of the task. Reliability and reproducibility of MPOD measurements obtained using the Macular Metrics Densitometer™ have previously been reported.[165]

2.2.6.1 Background to method

In order to measure MPOD, the subject views a stimulus that alternates between a wavelength band absorbed by MP and one that is not. The radiance of the wavelength band absorbed by MP is adjusted in order to minimize the subjects' percept of flicker. The range of alternation rates where flicker is not perceived is called the null zone. Primarily because of inter-individual differences in temporal (e.g. flicker) sensitivity, it is optimal to customize the HFP task for each subject by selecting the alternation rate to achieve a null zone and a precise setting. This has been termed as customized HFP (cHFP).[166]

The first methodological consideration when using cHFP is selecting the appropriate flicker rate. Selecting the best flicker rate for each subject enables one to accommodate the variation in flicker sensitivity due to factors such as age and disease.[167] If differences among subjects in flicker sensitivity are not accounted for (i.e. a fixed flicker frequency is used), then a subject with low flicker sensitivity (i.e. low critical flicker fusion frequency – CFF) will most likely experience a large null flicker zone. Alternatively, a subject with a high CFF may not be able to eliminate flicker from the test target, which would make the task difficult to complete.

Predicted optimal HFP flicker frequencies were estimated in order to facilitate good subject performance and reduce measurement error. To achieve this, we used an age-guided algorithm to estimate optimal HFP flicker frequencies for all the measurements performed (i.e. the measurement locus at 0.25°, 0.5°, 1°, 1.75° and reference locus at 7°). This algorithm was informed by many years' experience with the Densitometer™ at several different laboratories (see Table 2.2).

The second methodological consideration involves a test stimulus configuration in which the radiances of the two alternating components are inverse-yoked. In other words, when the blue component is adjusted to be more intense, the luminance of the green component is commensurately decreased, and vice versa. This procedure keeps the brightness of the test stimulus relatively constant. This approach is regarded as an improvement, as some subjects find changes in brightness distracting when they perform the task.

This investigation measured the spatial profile of MP at four different retinal eccentricities: 0.25°, 0.5°, 1°, and 1.75° with a reference point at 7°. The targets and fixation points used for each retinal eccentricity measured were as follows: the 0.25° and 0.5° eccentricities were measured using a 0.5° and 1° diameter disc with a 5 minute black fixation point at the centre; the 1° and 1.75° eccentricities were measured using a 20 minute wide annulus with mean radius corresponding to those eccentricities, with a centrally fixated 5 minute black fixation point. The 7° reference measurement involved a 2° diameter disc located 7° to the left of fixation with reference to a 5 minute red fixation point (Figure 2.1). For the purpose of this investigation, we assume that flicker perception is dominated by the edges of the disc-shaped stimuli used in each target, although other research has suggested that this may not be the case.[73-75;164;168]

2.2.6.2 *Method of Adjustment (used for young subjects)*

Prior to MP measurement, subjects were shown a video that described the task in hand, with instructions. The first measurement of MP was carried out at 0.5° retinal eccentricity. The subject was instructed to place his/her study eye at the viewing eyepiece and the examiner ensured that the tilt of the main unit allowed comfortable viewing for the subject.

The appropriate flicker frequency was set for the subject's age. The examiner set the radiance button all the way to the left (i.e. lowest blue light intensity) or right (i.e. highest blue light intensity). The subject then pushed the first of the radiance control buttons (on the left of the control pad) that electronically, smoothly and continuously altered the blue/green ratio until the beginning of the null flicker zone was reached. The subject continued to hold down the button until the end of the null flicker zone was reached. Once the null flicker zone had been defined, the subject used the second radiance control button (on the right) to go back through the no flicker zone. The subject then used both radiance control buttons to go back and forth through the null flicker zone until the centre of the zone (i.e. their null flicker point) was identified, and the radiance value at this point was then recorded by the examiner. After each measurement, the examiner offset the radiance button to a random position and the subject repeated the test as above. This procedure was repeated on four more occasions and the radiance values were recorded in the MPOD log form. The same procedure was repeated for measurements (see above) at the following retinal eccentricities 0.25°, 1°, 1.75°, 7° (Figure 2.1). MPOD was then calculated using the log ratio of the measurement radiance values with respect to the reference radiance values obtained for each subject at 7°, using a method of adjustment MPOD calculator provided by Macular Metrics (Providence, Rhode Island, USA).

Of note, if the subject reported that there was no null flicker zone, the examiner increased the flicker frequency by two Hz. If the subject reported a very wide null zone then the flicker frequency was reduced by two Hz. These steps were repeated if necessary.

TABLE 2.2 Predicted optimal flicker frequency for Densitometer™ targets

Age*	OFF†	OFF 7°
18 - 20	19	13
21 - 30	19	12
31 - 40	18	11
41 - 50	16	10
51 - 60	14	9
61 - 70	13	8
71 - 80	12	7
81 +	11	6

* Age guided logarithm, † OFF = optimal flicker frequency for 0.25°, 0.5°, 1°, 1.75° retinal eccentricity; OFF 7° = reference location for measuring macular pigment optical density in the parafovea

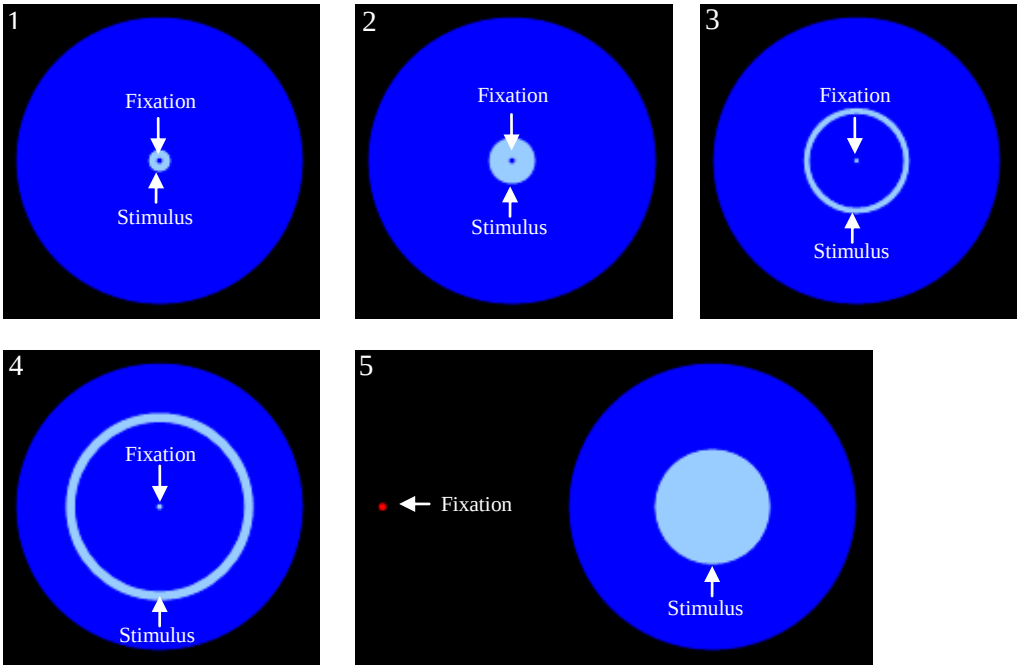


FIGURE 2.1 Targets used for measuring the spatial profile of macular pigment optical density; MPOD = macular pigment optical density; Fixation = fixation point for stimulus; Stimulus = stimulus viewed by subject; 1 = target used to measure MPOD at 0.25° retinal eccentricity (stimulus size = 0.5°); 2 = target used to measure MPOD at 0.5° retinal eccentricity (stimulus size = 1°); 3 = target used to measure MPOD at 1° retinal eccentricity (stimulus size = 2°); 4 = target used to measure MPOD at 1.75° retinal eccentricity (stimulus size = 3°); 5 = target used to measure MPOD at 7° retinal eccentricity (stimulus size = 2°).

2.2.6.3 *Bracketing Method (used for age-related macular degeneration subjects)*

The "bracketing method" developed by members of the Macular Pigment Research Group, Waterford, Ireland (Professor J. M. Nolan, Dr. E. Loane) and Professor B.R. Wooten of Brown University, USA (Densitometer™ inventor), allowed us to obtain quick, but accurate and customized, MPOD values for Group 2 and the procedure is described below.

A diagrammatic representation of the initial test stimulus was used to familiarise the subject with the nature of the task (Figure 2.1). The examiner selected the target required to measure MPOD at 0.5° eccentricity (1° stimulus [Figure 2.1]). The subject was instructed to place his/her study eye at the viewing eyepiece and the examiner ensured that the tilt of the main unit allowed comfortable viewing for the subject. The appropriate flicker frequency was set for the subject's age. The examiner set the radiance button all the way to the left (i.e. lowest blue light intensity). The examiner then pushed a button that electronically, smoothly and continuously altered the blue/green ratio until the subject reported that there was no flicker. The radiance value obtained was recorded and this same procedure was repeated on four more occasions and recorded in the MPOD log form. The examiner set the radiance button all the way to the right (i.e. highest blue light intensity) and repeated the test four times as above. This completed the first part of the measurement (10 radiance values obtained in total, five approaching from the lowest blue light intensity and five approaching from highest blue light intensity). The same procedure was repeated for measurements (see above) at the following retinal eccentricities 0.25°, 1°, 1.75°, 7°, and MPOD was calculated using the log ratio of the measurement radiance values with respect to the reference radiance values obtained for each subject at 7°, using a

bracketing procedure MPOD calculator provided by Macular Metrics (Providence, Rhode Island, USA).

Previous models of the densitometer (and most other similar devices) control the blue/green energy ratio with a rotary dial. Thus, the subject (if using method of adjustment) or the examiner (if using bracketing) turn the dial until the desired point of null flicker is reached. This works well for most subjects. However, some are prone to adjust the dial much too slowly. Others, on the other hand, make their adjustments too quickly. In the bracketing procedure, there are individual differences in the way different examiners control the dial. The current version of the densitometer avoids these potential sources of variability by substituting the dial with two push buttons: one button when depressed and held down causes the blue/green ratio to increase, whereas the other causes the blue/green ratio to decrease. Unlike the situation where a subject or examiner turns a dial, the rate of blue/green change is controlled entirely by the densitometer's electronics and was determined to be optimal (neither too fast or too slow) at 7 seconds for a sweep from one extreme to the other of the blue/green ratio. Preliminary studies have shown that this new procedure not only removes the aforementioned variability, but the task is qualitatively easier for the subjects. Although the bracketing method was introduced to aid ease of use, there was one AMD subject (subject 10) who was unable to complete the test, despite several attempts to explain the procedure. Results from this subject were unreliable (i.e. repeated measures or variation within measurement greater than 10%) and were therefore excluded from all MPOD analysis and presentation.

2.2.7 Serum total lutein and total zeaxanthin analysis – Assay 1 (EC)

A 400 μL aliquot of serum was pipetted into an amber light-sensitive microcentrifuge tube (1.5 mL total capacity). Ethanol (300 μL) containing 0.25 g/L butylated hydroxytoluene (BHT) and 200 μL internal standard (α-tocopherol acetate [0.25 g/L]) were added to each tube. Heptane (500 μL) was then added and samples were vortexed vigorously for 2 minutes followed by centrifugation at 2000 rpm for 5 minutes (MSC Micro Centaur, Davison & Hardy Ltd., Belfast, UK). The resulting heptane layer was retained and transferred to a second labelled amber light-sensitive microcentrifuge tube, and a second heptane extraction was performed. The combined heptane layers were immediately evaporated to dryness under nitrogen. These dried samples were reconstituted in 200 μL methanol (containing 0.25 g/L BHT), and 100 μL was injected for high-performance liquid chromatography (HPLC) analysis.

The instrumentation used to quantify the serum was an Agilent 1200 series (Agilent Technologies Ltd., Dublin, Ireland) system with photodiode array detection at a wavelength of 450 nm. A 5μm analytical/preparative 4.6 x 250 mm 201TP speciality reverse phase column (Vydac, Hesperia, CA) was used with an in-line guard column. The mobile phase consisted of 97% methanol and 3% tetrahydrofuran. The flow rate was 1 mL/min, and the total run time was 15 minutes.

DSM Nutritional Products (Basel, Switzerland) provided total L (TL) and total Z (TZ) standards to generate response factors which were used to calculate serum concentrations of TL and TZ. An internal standard: α-tocopherol acetate made up in ethanol (0.25 mg/L)

was used to correct for recovery of extractions for HPLC analysis and assist quantification. All chromatograms were integrated manually by drawing a baseline and dropping perpendicular lines to quantify the peaks of interest (Figure 2.2). All carotenoid peaks were integrated and quantified using Agilent ChemStation software.

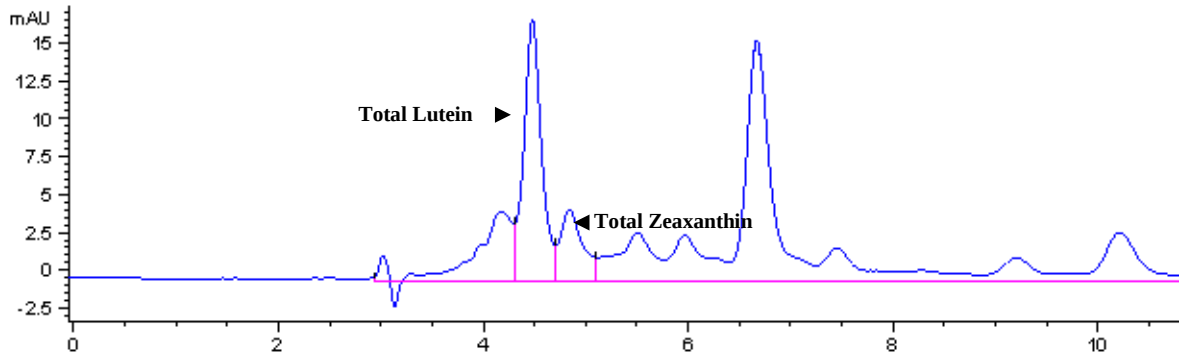


FIGURE 2.2 Chromatogram showing lutein and zeaxanthin peaks obtained from Assay 1 using high performance liquid chromatography as follows: column: 5 micron analytical/preparative 4.6 x 250 mm 201TP speciality reverse phase; detector: photo diode array and UV; wavelength: 450 nm; mobile phase: 97% methanol and 3% tetrahydrofuran; run time: 15 minutes; elution: isocratic. All peaks were integrated manually using Chemstation software

2.2.8 Serum *meso*-zeaxanthin analysis – Assay 2 (EC)

Assay 1 outlined above resulted in separation of TL and TZ. The eluent that corresponded to the peak of TZ from assay 1 was collected from the waste line (fraction 1) and evaporated to dryness under nitrogen. Fraction 1 also contained some TL, as TL and TZ eluted close together, which made it difficult to collect just TZ from the waste line. All dried down samples were then reconstituted in 50 µL of n-hexane-isopropanol (90:10) and 40 µL was injected onto the 10 µm Chiralpak™ AD column (250 x 4.6 mm; Chiral Technologies Europe, France) protected by a Chiralpak™ guard column and a 2 µm filter. In order to achieve separation of the Z isomers (Z and *meso*-Z), a flow rate of 0.8 mL/min with the following gradient elution: starting at 90% n-hexane and 10% isopropanol, and increasing to 95% n-hexane over 30 min was used.[8] Integration was manually carried out

on the resulting chromatogram from assay 2 by drawing a baseline between ~13 and ~30 minutes and then dropping a perpendicular line to quantify the proportions of Z and *meso*-Z from their peak areas. The proportions of the Z isomers were assumed to be the same as in the TZ fraction from the column of assay 1 which enabled calculation of the individual amounts of Z and *meso*-Z in the TZ fraction. A sample chromatogram showing the *meso*-Z and Z peaks is presented in Figure 2.3.

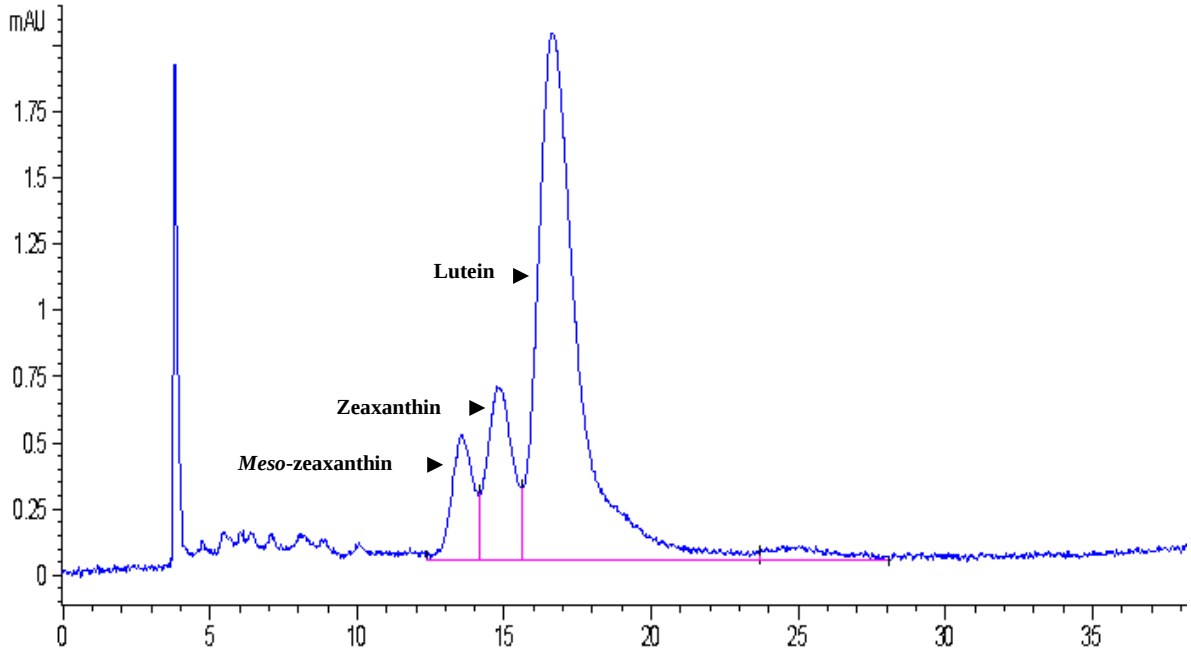


FIGURE 2.3 Chromatogram showing zeaxanthin isomer peaks (1 and 2) obtained from Assay 2. Peak 3 is a lutein peak collected into fraction 1. The eluate also contained some TL, as TL and TZ were not baseline separated in the first fractionation therefore it is not possible to collect just TZ from the waste line. Method used as follows: column: Chiralpak™ AD column (250 x 4.6 mm); detector: diode array; wavelength: 450 nm; mobile phase: isopropanol and n-hexane; run time: 30 minutes; elution: gradient, starting at 90% n-hexane and 10% isopropanol, increasing to 95% n-hexane. All peaks were integrated manually using Chemstation software.

2.2.9 Body mass index (EC)

BMI was measured for each subject and was recorded in kg/m^2. Subjects were required to remove their footwear and their height was measured using a standard height measure

(Assist Creative Resources Ltd., Wrexham, UK) and recorded in centimetres. Weight was measured using a SECA Model 761 mechanical personal floor scales (SECA GmbH & Co., Germany) and recorded in kilograms.

2.2.10 Study visits (EC)

MPOD, including its spatial profile (i.e. 0.25°, 0.5°, 1°, 1.75°), was measured at baseline and at two week intervals (V1: Baseline; V2: 2 weeks; V3: 4 weeks; V4: 6 weeks; V5: 8 weeks) over the 60 day study period using cHFP.

2.2.11 Statistical analysis (EC, JN, JS)

The statistical software package SPSS 17.0 (SPSS Inc., Chicago, Illinois, USA) was used for analysis and SigmaPlot 8.0 (Systat Software Inc., Chicago, Illinois, USA) was used for graphical presentations. Between group differences (Normal [Group1] vs. AMD [Group 2] in age, BMI, baseline serum carotenoid concentration and baseline MPOD levels (Table 2.3) were investigated using independent samples t-tests. Between group differences in sex were investigated using the standard Chi square test. Pearson correlation coefficient analyses were conducted to investigate the relationship between bivariables. All quantitative variables investigated exhibited a typical normal distribution. Means ± SDs are presented in the text and tables. Repeated measures analysis of variance was conducted for MPOD including its spatial profile, and serum concentrations of *meso*-Z, TL, TZ and Z measured at each study visit using a general linear model approach. Differences between two time points, within subjects, were assessed using paired samples t-test. The 5% level of significance was used throughout the analysis.

2.3 RESULTS

2.3.1 Baseline

The baseline demographic, lifestyle, macular serum carotenoid concentrations and MPOD data for the entire study group (Normal subjects [Group 1] and AMD subjects [Group 2]) are presented in Table 2.3. As seen from this table, age was the only variable for which a statistically significant between group difference was observed (p = 0.001).

TABLE 2.3 Baseline characteristics for Group 1 and Group 2

Characteristic	Entire Group	Group 1	Group 2	*p* – value
Age	53 ± 21	35 ± 9	72 ± 11	p = 0.001
Sex[*]	5 M, 5 F	2 M, 3 F	3 M, 2 F	p = 0.527
BMI	27 ± 4	24.8 ± 1.8	29.4 ± 4.3	p = 0.075
Serum TL[†]	0.302 ± 0.103	0.314 ± 0.086	0.290 ± 0.126	p = 0.728
Serum TZ[‡]	0.131 ± 0.070	0.169 ± 0.78	0.093 ± 0.036	p = 0.082
Serum *meso*-Z[§]	0.023 ± 0.007	0.022 ± 0.005	0.023 ± 0.009	p = 0.735
Serum Z[ǁ]	0.108 ± 0.067	0.147 ± 0.74	0.070 ± 0.030	p = 0.063
0.25° MPOD	0.39 ± 0.19	0.45 ± 0.17	0.30 ± 0.20	p = 0.245
0.5° MPOD	0.38 ± 0.16	0.44 ± 0.13	0.30 ± 0.17	p = 0.205
1° MPOD	0.26 ± 0.15	0.30 ± 0.10	0.23 ± 0.21	p = 0.519
1.75° MPOD	0.14 ± 0.10	0.12 ± 0.08	0.17 ± 0.14	p = 0.556
Average MPOD	0.29 ± 0.13	0.33 ± 0.09	0.25 ± 0.18	p = 0.399

[*] M = male, F = female; [†] = Total lutein (μmol/L); [‡] = Total zeaxanthin (μmol/L); [§] = *Meso*-zeaxanthin (μmol/L); [ǁ] = Zeaxanthin (μmol/L); Mean ± SD; Group 1 = normal subjects; Group 2 = AMD subjects (Age-related Macular Degeneration); BMI = body mass index (kg/m^2); MPOD = macular pigment optical density (Optical density unit); 0.25° MPOD = MPOD measured at 0.25° retinal eccentricity; 0.5° MPOD = MPOD measured at 0.5° retinal eccentricity; 1.0° MPOD = MPOD measured at 1.0° retinal eccentricity; 1.75° MPOD = MPOD measured at 1.75° retinal eccentricity; Average MPOD = average MPOD of all degrees of retinal eccentricity (0.25°, 0.5°, 1.0° and 1.75 degrees of retinal eccentricity)

2.3.2 Alterations in serum macular carotenoid concentrations following supplementation

The serum concentrations for each study visit are summarised in Table 2.4; the p values displayed in the final column of this table were obtained using the Huynh-Feldt correction for sphericity. Use of the more conservative Greenhouse-Gesser correction would have led, in all cases, to the same conclusions regarding statistical significance. It is clear from Table 2.4 and the mean plots of Figures 2.4, 2.5 and 2.6 that the serum concentrations of *meso*-Z, TL and TZ increase significantly with time; whereas, there was no significant time effect for serum concentrations of Z ($p = 0.909$) (Table 2.4, Figure 2.7). Post hoc analysis (paired samples t-tests) revealed that the significant increase from baseline was present after two weeks of supplementation (TL: $p < 0.05$; TZ: $p < 0.05$, and *meso*-Z: $p = 0.01$). The data for each individual subject are presented in Table 2.5.

TABLE 2.4 Average serum results for all subjects at each study visit

	V1	**V2**	**V3**	**V4**	**V5**	**p - value**
Total lutein	0.30 ± 0.1	0.36 ± 0.12	0.40 ± 0.14	0.37 ± 0.12	0.36 ± 0.12	p = 0.002
Total zeaxanthin	0.13 ± 0.07	0.16 ± 0.06	0.18 ± 0.07	0.16 ± 0.05	0.17 ± 0.06	p = 0.003
***Meso*-zeaxanthin**	0.02 ± 0.01	0.06 ± 0.02	0.07 ± 0.03	0.06 ± 0.02	0.06 ± 0.03	p = 0.000
Zeaxanthin	0.11 ± 0.07	0.10 ± 0.05	0.11 ± 0.05	0.10 ±0.04	0.11 ± 0.04	p = 0.909

Values represent mean ± SD in μmol/L; N = 10; V1 = visit 1, V2 = visit 2, V3 = visit 3, V4 = visit 4, V5 = visit 5

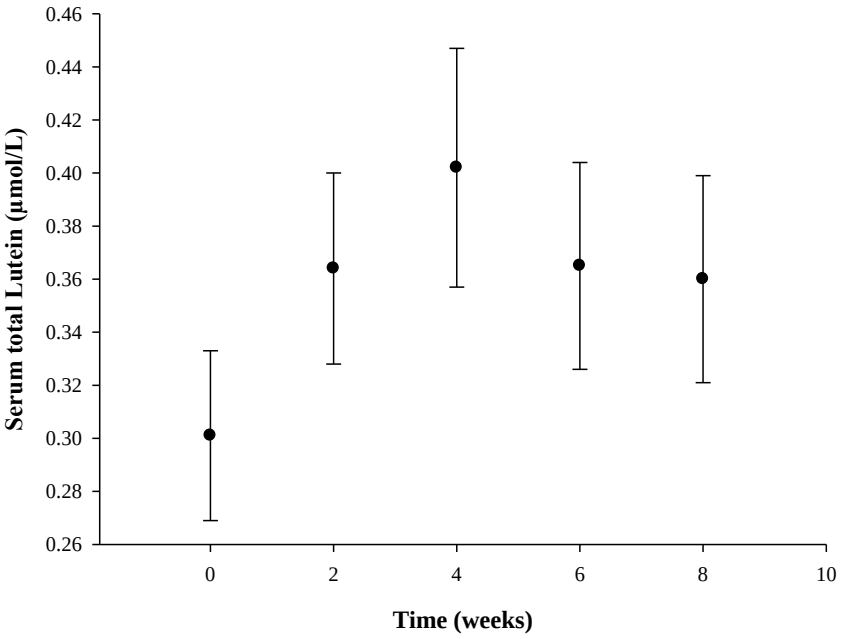


FIGURE 2.4 Longitudinal serum total lutein concentrations following supplementation with *meso*-zeaxanthin, lutein and zeaxanthin

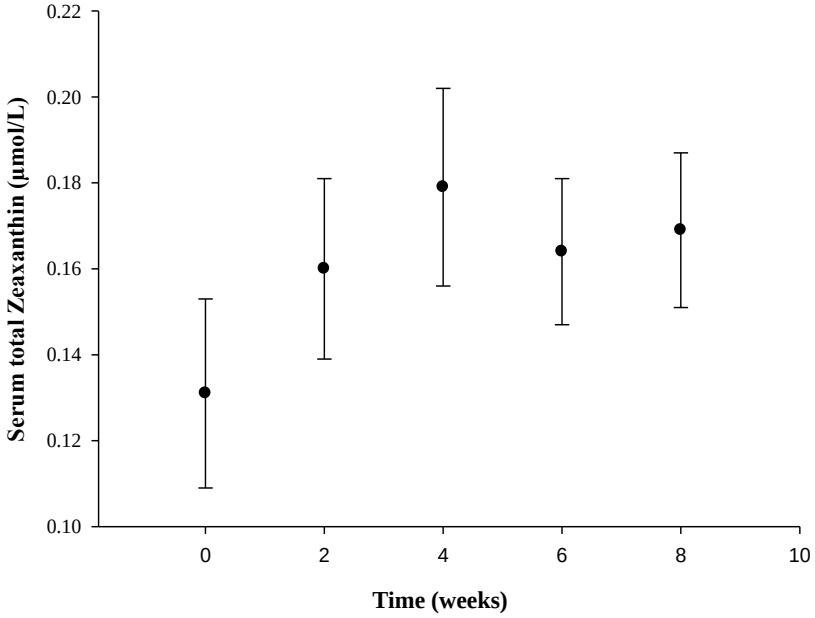


FIGURE 2.5 Longitudinal serum total zeaxanthin concentrations following supplementation with *meso*-zeaxanthin, lutein and zeaxanthin

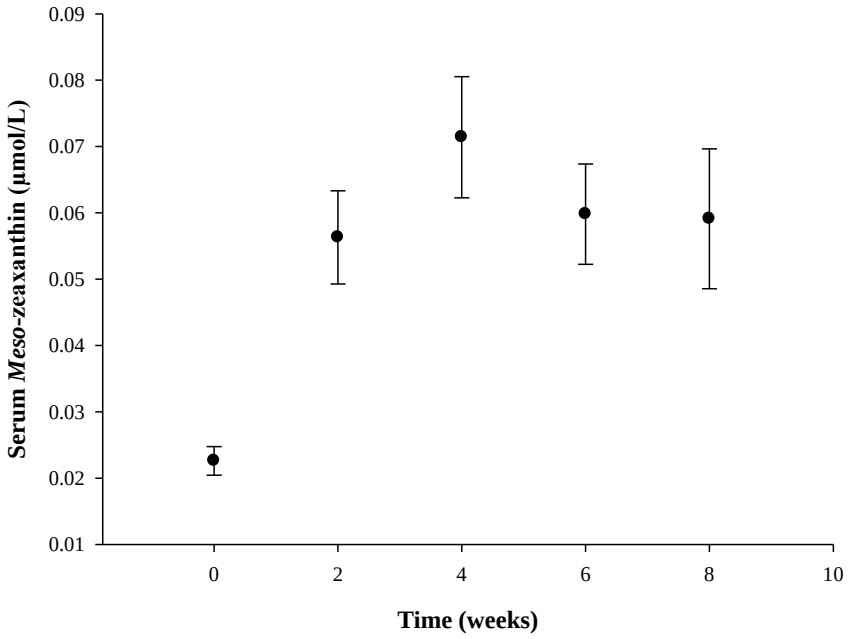


Figure 2.6 Longitudinal serum *meso*-zeaxanthin concentrations following supplementation with *meso*-zeaxanthin, lutein and zeaxanthin

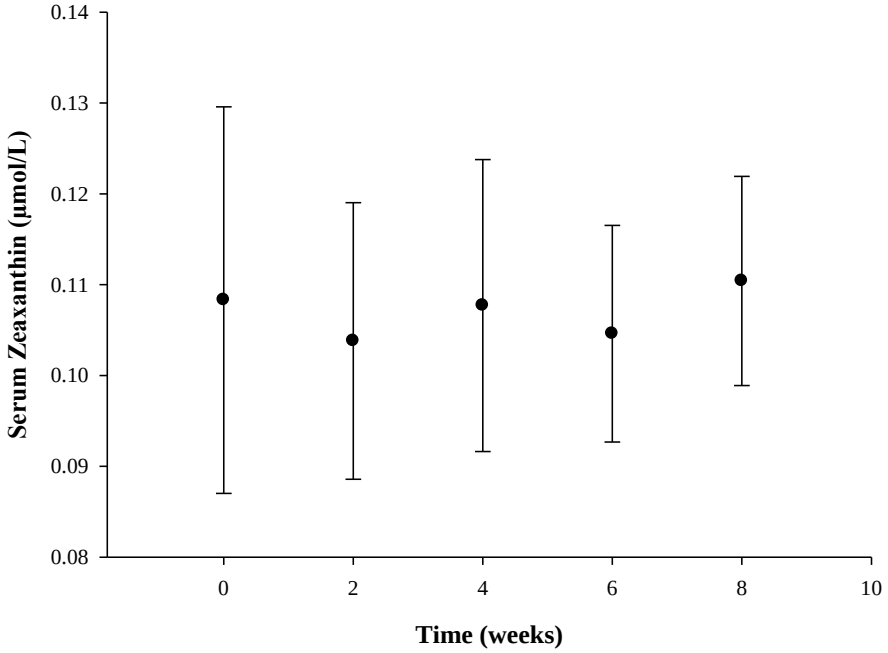


FIGURE 2.7 Longitudinal serum zeaxanthin concentrations following supplementation with *meso*-zeaxanthin, lutein and zeaxanthin

TABLE 2.5 Individual serum concentrations for total lutein, total zeaxanthin, *meso*-zeaxanthin and zeaxanthin at each study visit

S	Group	V1				V2				V3				V4				V5			
		TL	TZ	MZ	Z	TL	TZ	MZ	Z	TL	TZ	MZ	Z	TL	TZ	MZ	Z	TL	TZ	MZ	Z
1	Normal	0.30	0.18	0.03	0.15	0.29	0.17	0.03	0.13	0.34	0.22	0.06	0.17	0.34	0.21	0.06	0.14	0.33	0.18	0.07	0.11
2	Normal	0.25	0.13	0.02	0.11	0.36	0.18	0.07	0.11	0.37	0.20	0.08	0.12	0.25	0.14	0.03	0.11	0.25	0.12	0.03	0.09
3	Normal	0.29	0.13	0.02	0.11	0.35	0.15	0.05	0.11	0.32	0.14	0.06	0.08	0.34	0.16	0.07	0.09	0.31	0.14	0.04	0.10
4	Normal	0.27	0.11	0.02	0.09	0.46	0.23	0.09	0.13	0.59	0.23	0.11	0.12	0.45	0.20	0.08	0.12	0.55	0.24	0.12	0.12
5	Normal	0.46	0.30	0.03	0.27	0.55	0.28	0.08	0.20	0.62	0.32	0.11	0.21	0.51	0.27	0.09	0.18	0.49	0.27	0.07	0.20
6	AMD	0.49	0.14	0.03	0.11	0.55	0.18	0.07	0.11	0.58	0.20	0.10	0.10	0.58	0.20	0.10	0.10	0.53	0.22	0.12	0.10
7	AMD	0.22	0.10	0.02	0.08	0.31	0.15	0.06	0.09	0.34	0.16	0.07	0.09	0.29	0.14	0.05	0.09	0.32	0.16	0.04	0.11
8	AMD	0.31	0.10	0.04	0.07	0.32	0.13	0.06	0.07	0.39	0.17	0.06	0.11	0.39	0.14	0.06	0.09	0.34	0.14	0.04	0.10
9	AMD	0.16	0.05	0.02	0.03	0.24	0.07	0.03	0.04	0.19	0.06	0.03	0.03	0.18	0.07	0.03	0.04	0.22	0.09	0.03	0.06
10	AMD	0.27	0.07	0.02	0.05	0.23	0.07	0.03	0.04	0.30	0.09	0.03	0.06	0.33	0.14	0.04	0.10	0.27	0.14	0.03	0.11

Values represent mean ± SD in μmol/L; N = 10; S = Subject; V1 = visit 1, V2 = visit 2, V3 = visit 3, V4 = visit 4, V5 = visit 5; TL = total lutein, TZ = total zeaxanthin, MZ = *meso*-zeaxanthin, Z = zeaxanthin

2.3.3 Alterations in the spatial profile of MPOD following macular carotenoid supplementation

Mean MPOD values for each study visit are summarized in Table 2.6; the p values displayed in the final column of this table were obtained using the Huynh-Feldt correction for sphericity. Use of the more conservative Greenhouse-Gesser correction would have led, in all cases, to the same conclusions regarding statistical significance. It is clear from Table 2.6 and the mean plots of Figure 2.8 that MPOD at 0.25°, 1° and average MPOD across the retina all increase significantly with time; whereas, there was no significant time effect for MPOD at 0.5° and 1.75° throughout the study period ($p = 0.101$ and $p = 0.61$). Of note, the biggest increase seen in MPOD was nearest the centre (i.e. at eccentricity 0.25°) (see Table 2.7 and Figure 2.8).

Post hoc analysis (paired samples t-tests) revealed that a significant increase from baseline was present after two weeks of supplementation ($p < 0.005$, for all), with the exception of MPOD at 1.75° which was significantly different from baseline only at V3 ($p = 0.004$). The data for each individual subject is presented in Table 2.8.

TABLE 2.6 Average macular pigment optical density at each degree of eccentricity for all subjects

MPOD	V1	V2	V3	V4	V5	p - value
0.25	0.39 ± 0.19	0.49 ± 0.18	0.52 ± 0.22	0.57 ± 0.26	0.61 ± 0.26	$p < 0.017$
0.5	0.38 ± 0.16	0.43 ± 0.15	0.46 ± 0.16	0.44 ± 0.18	0.45 ± 0.18	$p < 0.101$
1.0	0.26 ± 0.15	0.29 ± 0.14	0.32 ± 0.10	0.32 ± 0.17	0.38 ± 0.14	$p > 0.030$
1.75	0.14 ± 0.10	0.17 ± 0.10	0.17 ± 0.06	0.16 ± 0.08	0.16 ± 0.09	$p > 0.610$
Total average	0.29 ± 0.13	0.34 ± 0.12	0.37 ± 0.12	0.37 ± 0.15	0.40 ± 0.15	$p < 0.019$

Values represent mean ± SD; N = 9 (as one subject (10) was unable to use the Densitometer and was therefore unable to have her MPOD measured); MPOD = macular pigment optical density; V1 = visit 1, V2 = visit 2, V3 = visit 3, V4 = visit 4, V5 = visit 5; 0.25 = 0.25° retinal eccentricity; 0.5 = 0.5° retinal eccentricity; 1.0 = 1° retinal eccentricity; 1.75 = 1.75° retinal eccentricity; < = less than; > = greater than

2.3.4 The relationship between alterations in MPOD spatial profile and alterations in serum carotenoid concentrations

In this study, the following showed significant increases with time: serum *meso*-Z, serum TL and serum TZ, MPOD at eccentricities at 0.25°, 1°, and also average MPOD across the retina (i.e. 0.25°, 0.5°, 1°, 1.75°) (see repeated measures results above, Figures 2.4, 2.5, 2.6, 2.8 and Tables 2.4 and 2.6, respectively).

However, investigating the relationship between *change* in serum concentrations (for V2-V1) in each of *meso*-Z, TL and TZ with respect to *change* in MPOD at 0.25°, 1° and average MPOD, we found that, in every case, there is an inverse correlation between these variables ($r = -0.538$ to -0.805 e.g. V2-V1 serum concentrations of *meso*-Z vs V2-V1 MPOD at 0.25°: $r = -0.538$, $p = 0.135$, Figure 2.9A). The fact that some of these correlations were not statistically significant can be ascribed to the small sample size of the

current study. Of note, the strongest inversed correlation was seen for TZ (*meso*-Z + Z combined), which was statistically significant (r = -0.805, p = 0.009).

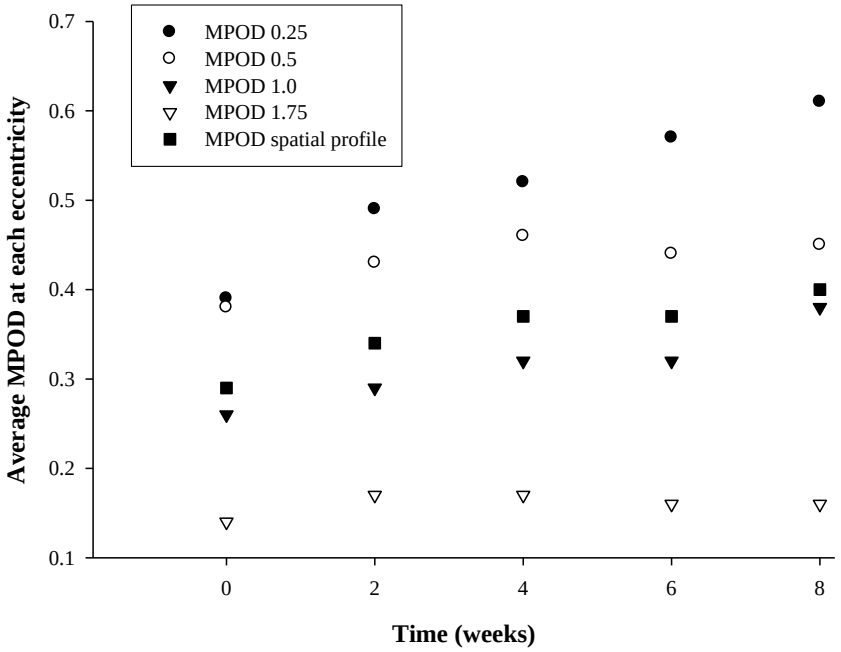


FIGURE 2.8 Longitudinal macular pigment optical density measurements following supplementation with *meso*-zeaxanthin, lutein and zeaxanthin.

Interestingly, however, and for *meso*-Z only, the correlation is much closer to zero when we compare V5-V1 change rather than V2-V1 change (i.e. V5-V1 serum concentrations of *meso*-Z vs V5-V1 MPOD at 0.25°: r = -0.028, p = 0.943, Figure 2.9B), whereas, for TL and TZ the change in serum concentrations of these carotenoids versus the change in MPOD at 0.25° remained inverse at visit 5 (r = -0.434 and -0.671, respectively).

TABLE 2.7 Individual macular pigment optical density values for each study visit

S	Group	V1					V2					V3					V4					V5			
		0.25	**0.5**	**1**	**1.75**	**Av**	**0.25**	**0.5**	**1**	**1.75**	**Av**	**0.25**	**0.5**	**1**	**1.75**	**Av**	**0.25**	**0.5**	**1**	**1.75**	**Av**	**0.25**	**0.5**	**1**	
1	Normal	0.39	0.37	0.26	0.16	0.30	0.70	0.48	0.34	0.19	0.43	0.93	0.65	0.40	0.17	0.54	1.10	0.65	0.38	0.20	0.58	1.14	0.74	0.49	
2	Normal	0.51	0.48	0.22	0.02	0.31	0.61	0.48	0.26	0.18	0.38	0.68	0.52	0.42	0.13	0.44	0.72	0.57	0.57	0.15	0.50	0.75	0.60	0.54	
3	Normal	0.30	0.27	0.21	0.09	0.22	0.44	0.30	0.20	0.05	0.25	0.42	0.36	0.32	0.10	0.30	0.44	0.32	0.28	0.08	0.28	0.42	0.32	0.29	
4	Normal	0.72	0.61	0.33	0.11	0.44	0.68	0.66	0.30	0.21	0.46	0.70	0.66	0.35	0.21	0.48	0.78	0.66	0.35	0.19	0.50	0.73	0.60	0.36	
5	Normal	0.35	0.49	0.46	0.22	0.38	0.53	0.59	0.42	0.14	0.42	0.42	0.57	0.35	0.18	0.38	0.55	0.56	0.39	0.18	0.42	0.66	0.48	0.47	
6	AMD	0.06	0.14	0.00	0.00	0.05	0.17	0.26	0.03	0.00	0.12	0.31	0.31	0.13	0.09	0.21	0.34	0.21	0.00	0.00	0.14	0.31	0.17	0.10	
7	AMD	0.36	0.36	0.19	0.19	0.27	0.35	0.33	0.25	0.16	0.27	0.36	0.31	0.26	0.16	0.27	0.35	0.29	0.19	0.17	0.25	0.44	0.38	0.34	
8	AMD	0.25	0.20	0.21	0.13	0.20	0.30	0.25	0.29	0.27	0.28	0.29	0.25	0.25	0.17	0.24	0.29	0.22	0.25	0.17	0.23	0.36	0.27	0.31	
9	AMD	0.53	0.52	0.50	0.34	0.47	0.60	0.51	0.54	0.32	0.49	0.60	0.52	0.43	0.30	0.46	0.57	0.52	0.47	0.30	0.46	0.65	0.51	0.51	

Values represent mean; N = 9 (as one subject (10) was unable to use the Densitometer and was therefore unable to have her MPOD measured); S = Subject; V1 = visit 1, V2 = visit 2, V3 = visit 3, V4 = visit 4, V5 = visit 5; 0.25 = 0.25° retinal eccentricity; 0.5 = 0.5° retinal eccentricity; 1 = 1° retinal eccentricity; 1.75 = 1.75° retinal eccentricity; Av = average MPOD across entire spatial profile (0.25°, 0.5°, 1°, 1.75°); MPOD measured in optical density units

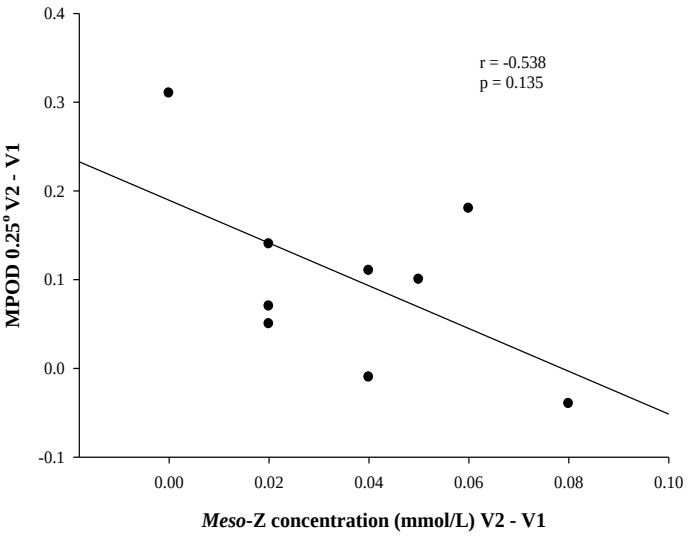


FIGURE 2.9 A Change in macular pigment optical density at 0.25° with respect to change in serum concentrations of *meso*-zeaxanthin between visits 1 and 2

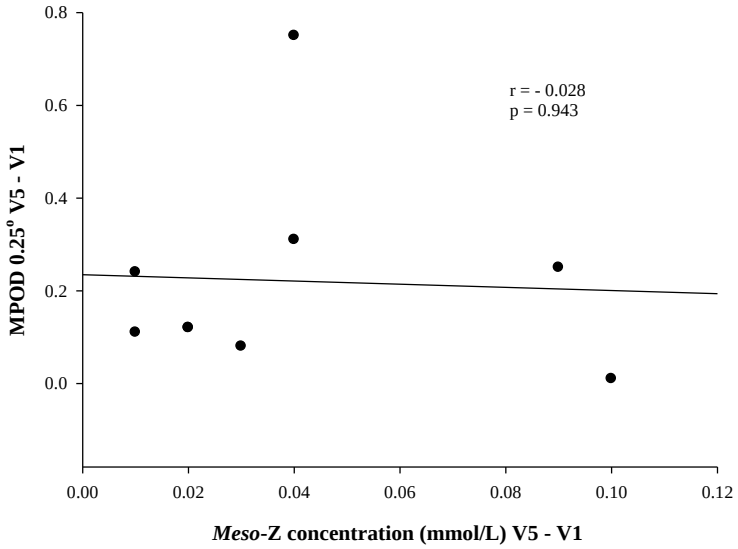


FIGURE 2.9 B Change in macular pigment optical density at 0.25° with respect to change in serum concentrations of *meso*-zeaxanthin between visits 1 and 5

2.3.5 Typical versus atypical MPOD spatial profile

Recent studies have been concerned with the spatial profile and distribution of MPOD.[104;139;140;143-150;155;169-174] Of note, in the present study, four subjects (one Normal subject [Group 1] - Subject 5; and three AMD subjects [Group 2] - Subjects 6, 7 and 9) who displayed an atypical MPOD spatial profile at baseline (i.e. pre-supplementation), had the more typical MPOD spatial profile (i.e. highest MPOD at the centre) after eight weeks of supplementation with *meso*-Z, L and Z (i.e. the formulation used in this study). The MPOD spatial profile, averaged for the above four subjects, at pre (baseline) and post-supplementation (after 8 weeks) is presented in Figure 2.10 and their individual spatial profiles, at these two time points, are presented in Figure 2.11.

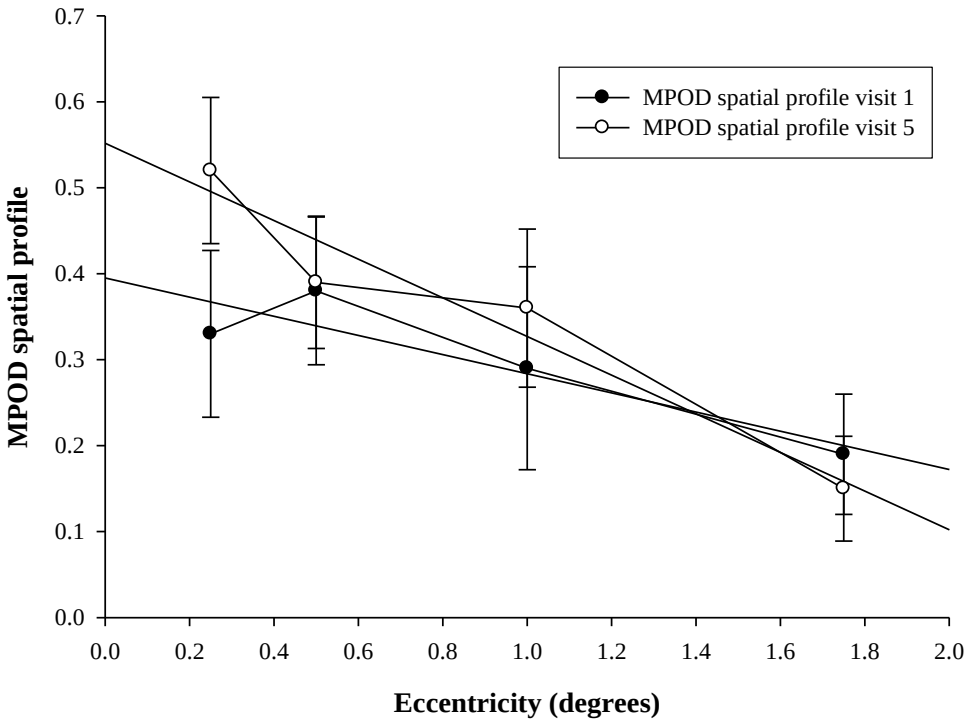


FIGURE 2.10 Change in MPOD spatial profile following macular carotenoid supplementation. This change was measured in 9 subjects over an eight week study period.

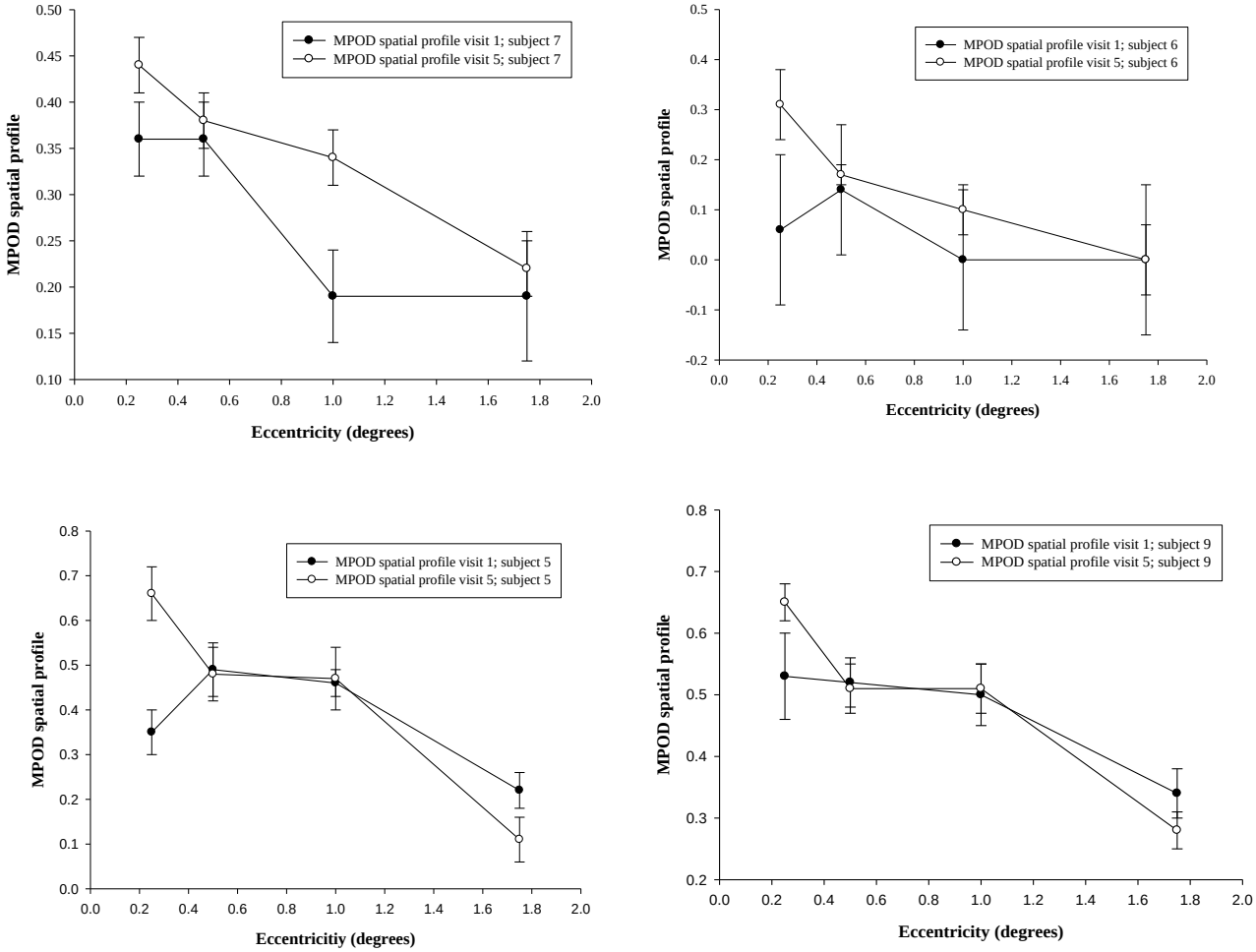


Figure 2.11 Individual macular pigment optical density spatial profiles for pre- and post-supplementation with *meso*-zeaxanthin, lutein and zeaxanthin

2.4 DISCUSSION

This study was designed to investigate macular and serum responses to supplementation with the three macular carotenoids (in which *meso*-Z predominates: 7.3 mg of *meso*-Z, 3.7 mg of L and 0.8 mg of Z), in normal healthy subjects and patients with early AMD. MPOD was measured using cHFP at 0.25°, 0.5°, 1°, and 1.75° retinal eccentricity with a reference point at 7°, every two weeks over a 60 day study period. A blood sample was also collected, at each study visit, in order to analyze serum concentrations of *meso*-Z, TL,

TZ and Z. The limitations of this investigation were: no controls were included into the study; small sample size (n = 10); however, the entire spatial profile of MPOD was assessed and serum concentrations of *meso*-Z were analysed for all 10 subjects.

Supplementation studies to date have previously reported on serum response to supplementation with the macular carotenoids, with the majority of these studies reporting significant increases in serum concentrations of L and/or Z following supplementation with these carotenoids. Consistent with these previous studies, this study reports statistically significant increases in *meso*-Z, and TL, after just two weeks of supplementation. Of note, average serum TL concentrations exhibited the highest average increase following supplementation with the study formulation, when compared to the other carotenoids (*meso*-Z and TZ). The finding of a 1.3 fold increase in serum concentrations of L is consistent with previous studies, as this study formulation contained only 3.7 mg. For example, Bone *et al.*, supplemented two subjects with 5 mg of L per day for 120 days and reported a 3 fold increase in serum concentrations of this carotenoid.[175] Similarly, Berendschot *et al.* supplemented 8 subjects with 10 mg of L per day for 12 weeks and reported a 5 fold increase in serum concentrations of this carotenoid.[144]

In this study, serum concentrations of Z showed no significant increase over the study period, and this may be attributable to the low amount of Z in the formulation (only 0.8 mg per capsule). Previous studies have reported significant increases in serum concentrations of Z following supplementation, albeit with a higher concentration of this carotenoid (e.g. Schalch *et al*. 2007: 12.6 mg Z for 17 weeks showed an increase of ~ 1.09 μmol/L;[149] Bone

et al. 2003: 30 mg of Z for one year showed an increase of 0.52 μmol/L) in the preparation.[146]

There was a statistically significant increase in serum concentrations of *meso*-Z, with a 3 fold increase observed over the eight week study period (i.e. mean *meso*-Z: V1 = 0.02 ± 0.01 μmol/L; mean *meso*-Z V2 = 0.06 ± 0.02 μmol/L). However, it is important to point out that while *meso*-Z demonstrated a 3 fold increase in serum (from its baseline value), that following supplementation, when absolute average *meso*-Z serum concentrations is compared with average absolute serum L concentrations, we see that there is significantly more circulating L than *meso*-Z in serum (mean ± SD: 0.36 ± 0.12 μmol/L versus 0.06 ± 0.03 for L and *meso*-Z, respectively). Also, when the concentration increase is compared to the studies carried out by Thurnham *et al.*[171] and Bone *et al*.,[142] it can be seen that our serum *meso*-Z response was much lower when compared to those studies (i.e. mean ± SD serum *meso*-Z concentration μmol/L = 0.209 ± 0.128 and 0.094 ± 0.071, respectively. However, it should be noted that the supplement used in the study by Thurnham *et al.* was suspended in oil; whereas, our study used a micro-encapsulated form of the supplement suspended in starch, which may account for, at least in part, the low serum response reported here, given that oil has been shown to promote carotenoid absorption.[171]

The investigation by Thurnham *et al.* 2008 reported an average increase of 0.209 ± 0.128 μmol/L in serum concentrations of *meso*-Z (following supplementation with 8 mg per day of this carotenoid over a 22 day study period). Similarly, the study by Bone *et al.* observed augmented average serum concentrations of *meso*-Z (0.094 ± 0.071 μmol/L) following supplementation with 14 mg per day of this carotenoid over a 120 day period.[142] The study

conducted by Thurnham *et al.* 2008 reported on the absorption of *meso*-Z following supplementation with this carotenoid.[171] Also, they compared the plasma responses to supplementation with a formulation containing *meso*-Z (Lutein Plus®) with formulations containing L and Z (but not *meso*-Z), and reported that the increases seen in plasma L and Z concentrations were similar for each formulation, suggesting that *meso*-Z has little effect on absorption of L and/or Z. However, although Thurnham *et al*. reported that *meso*-Z did not decrease the absorption of L and Z, it is important to note that the study formulation used in that study contained more L than *meso*-Z (8mg *meso*-Z, 10.8 mg L and 1.2 mg Z) whereas, in this study, the formulation contained more *meso*-Z than L (i.e. 7.3 mg *meso*-Z, 3.8 mg L and 0.8 mg Z). Thus, it may not be possible to extrapolate directly the effects of *meso*-Z on the absorption of L and Z to this study without further work. Of note, the studies conducted by Thurnham *et al*. and Bone *et al.* are the only two studies to date that have investigated serum carotenoid response following supplementation with a preparation containing *meso*-Z, making any discussion with respect to this finding difficult. Also, no study to date has investigated and/or reported on histology or retinal function in response to *meso*-Z supplementation.

To date, no study has reported the presence of *meso*-Z in human serum pre-supplementation with this carotenoid. This notion is unsurprising, given that *meso*-Z is not found in a typical western diet (with the exception of some unusual foods and shellfish).[55] However, in the current study, the possible presence of *meso*-Z was detected, albeit in minute concentrations, in all 10 subjects (mean ± SD *meso*-Z in μmol/L: 0.023 ± 0.007). The possibility that *meso*-Z was in serum at baseline is a novel and interesting finding and may be explained as follows: *meso*-Z may be present in carotenoid containing foods but as chiral chromatography is needed to separate *meso*-Z from Z, *meso*-Z may not have been

detected since it is rarely used. Alternatively, *meso*-Z may be generated in serum following L transformation. However, the paucity of studies investigating any aspect of *meso*-Z in the diet and/or serum renders any discussion with respect to this finding that *meso*-Z is present in the serum of unsupplemented subjects difficult, and further study is warranted to fully investigate this assumption.

This study is the first investigation into the spatial profile of MPOD (i.e. at 0.25°, 0.5°, 1°, 1.75°) following supplementation with all three macular carotenoids (*meso*-Z, L and Z), which enabled us to measure change, if any, at the above degrees of retinal eccentricity, including the more central locations where *meso*-Z is located.[18] We report increases in MPOD at 0.25°, 0.5°, 1°, and average MPOD across the retina (i.e. average of 0.25°, 0.5°, 1°, and 1.75°) during the study period, which became significant after just two weeks of supplementation. The rapid increase seen in MPOD in the current study is a somewhat novel finding as, previous studies have not measured and/or reported on MPOD after two weeks of supplementation. In other words, previous studies to date have only reported on change in MP levels, if any, after four weeks of supplementation and beyond.

The findings of this study are consistent with a study conducted by Hammond *et al.* in 1997, who reported significant MPOD augmentation following dietary modification (i.e. corn 0.4 mg L and 0.3 mg Z and spinach 10.8 mg L and 0.3 mg Z) after just four weeks of dietary intervention.[139] This study is also consistent with previous reports that have investigated MP response to macular carotenoid supplementation (Table 2.1). In contrast, however, there was no significant augmentation of MPOD at 1.75° eccentricity. Also, and of interest, it was observed that the greatest increase in MPOD at 0.25°, with a mean ± SD

increase of 0.16 ± 0.05 ODU at this eccentricity. Of note, no study to date has measured MPOD at this eccentricity following supplementation with *meso*-Z, L and Z, and, therefore, it is difficult to make direct comparisons with other reports. It is likely that the significant increase seen in central MPOD in this study may be due to either *meso*-Z and/or L, especially given that *meso*-Z and L demonstrated significant responses in serum concentrations. However, with respect to *meso*-Z, this novel finding is interesting given that *meso*-Z is the dominant carotenoid in the study formulation (i.e. 7.3mg [62%]) and given that the ratio of *meso*-Z to L, and the ratio of *meso*-Z to Z, is greater at the centre of the fovea. For example, in 1997, Bone *et al*. reported that the proportions of *meso*-Z: Z in the central 3mm of the macula was 0.83 which decreased with increasing distance from the fovea.[70] Also, it is important to note that although the mean concentration of *meso*-Z was only 0.06 µmol/L at visit 2, this represents ~160 $x10^3$ ng of *meso*-Z per 5 litres of blood. This observation is important, given that the amount of *meso*-Z in human donor eyes has been reported as ~7.7 ng and also given that an active binding protein for Z and *meso*-Z have been identified in retinal tissue.[142] There has only been one other study to date that has measured MPOD following daily supplementation with *meso*-Z. That study, recently performed by Bone *et al.*, in 2007, included 10 normal subjects, who were supplemented with 14.9 mg of *meso*-Z, 5.5 mg of L and 1.4 mg of Z, for 120 days. Bone and co-workers reported a significant increase in MPOD at 0.75° of retinal eccentricity (mean increase = 0.07 ODU at this eccentricity) over the study period. However, in their study, MP was measured at only one retinal location (0.75°).[142]

As mentioned above, previous studies reporting on MPOD response to supplemental L and Z have reported parallel increases between these variables. In 1997, Hammond *et al*. showed MPOD augmentation following dietary modification after four weeks.

Interestingly, two of the 11 subjects in that study did not respond at the macula, despite a significant increase found in serum concentrations of L and Z. Hammond *et al.* referred to these subjects as 'retinal non-responders'.[139] The findings from this study are consistent with this, it was observed that one of the 10 subjects recruited (Subject 4) into this trial did not respond at the macula, despite significant increases found in serum concentrations of *meso*-Z and L. In fact, and of particular interest, this subject displayed one of the highest increases in serum macular carotenoid concentrations. Also, this subject displayed a "typical" MPOD spatial profile and had the highest MPOD level (of subjects in this study), at baseline (i.e. 0.72 ODU at 0.25° retinal eccentricity). It is possible that this subject's macula was saturated with MP, thus precluding the possibility of MP augmentation in response to supplementation. However, a longer supplementation period and follow up may have resulted in MPOD augmentation, for this subject.

Unexpectedly, there was an inverse trend between rises in serum concentrations of *meso*-Z, TL and TZ (V2-V1) and increases in MPOD at 0.25°, 0.5°, 1° eccentricity and in average MPOD across the retina (V2-V1). Interestingly, however, this trend disappeared when the relationship between change in MPOD (at 0.25°) was investigated from V5 and V1 and change in serum *meso*-Z from V5 and V1, whereas, it remained inverse for the relationship between change in MPOD (at 0.25°) from V5 and V1 and change in serum TL and TZ from V5 and V1. This somewhat unexpected and apparently contradictory finding may simply be explained by the fact that circulating *meso*-Z was captured by tissues more rapidly in subjects with depleted levels of this carotenoid at the macula and/or other target tissues (e.g. fat cells). This hypothesis is supported by this finding that the observed inverse trend between change in MPOD and change in serum *meso*-Z did not persist

beyond V3. The above findings must, however, be interpreted with appreciation of the small sample size of this study and additional study into this relationship is merited.

Another interesting finding from this study was the observation that four subjects (one normal and three AMD) exhibited an atypical MPOD spatial profile at baseline (i.e. secondary peak). Interestingly, however, following supplementation with *meso*-Z, all subjects exhibited the more typical MPOD spatial profile (exponential like decline), after just 8 weeks of supplementation. In other words, it is tempting to hypothesise that the subjects who displayed the atypical MPOD spatial profile at baseline were exhibiting a relative lack of MP centrally (and therefore *meso*-Z), perhaps due to an inability to convert L to *meso*-Z at this location, but were able to rebuild their central MP peak with a supplement containing *meso*-Z.

While the findings of this study are interesting, it is important to note the limitations inherent in the study design, and these include: the sample size of this trial was small (n = 10), it was a non-blind open labelled study, and the period of follow-up was only 8 weeks (60 days). Future research is warranted in a double-blind, randomised, placebo-controlled trial of longer duration to investigate serum and macular response, in a larger cohort to a supplement containing the three macular carotenoids *meso*-Z, L and Z.

CHAPTER THREE

STUDY TWO

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

3.1 INTRODUCTION

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

To date, there has been no clinical trial that has performed safety analysis of the macular carotenoids, in particular *meso*-Z, in human subjects. However, a number of human intervention studies have monitored adverse events following supplementation with high doses of L for extended periods of time, with no adverse effects reported. Now that *meso*-Z has become more commercially available it has enabled studies to evaluate human responses in clinical trials. This research was conducted as it was seen as one of the next steps to be taken in this area of research. To date, no study has measured these parameters and it we felt it was important to investigate and report on such outcomes. Of particular importance was the information regarding the safety as currently there is widespread controversy surrounding *meso*-Z. It has been shown that *meso*-Z is present at the centre of the macula in high concentrations and also that it is due to a biochemical conversion from L, but there is debate surrounding the ability to increase *meso*-Z levels through supplementation. From the limited research to date there appears to be no apparent safety issues associated with *meso*-Z. A small toxicity animal model reported a NOAEL for *meso*-Z as 200 mg/kg/day, and the absence of mutagenicity has also been shown. This study has been designed to investigate the safety of consumption of *meso*-Z by measuring renal and liver function, lipid profile, haematological factors or markers of inflammation.

This research will help inform the scientific community on the safety of *meso*-Z in supplement form.

3.2 METHODS

3.2.1 Study design

The *meso*-zeaxanthin ocular supplementation trial in normals (MOST-N) was a double blind, randomized, placebo controlled, clinical trial registered with the International Standard Randomized Controlled Trial Number Register (ISRCTN60816411).

3.2.2 Subjects

Forty four healthy subjects were recruited into the study, and were randomised into one of two groups: Intervention (I, n = 22) and Placebo (P, n = 22). Following a detailed explanation of all the study procedures by the study investigator (EC), each subject signed an informed consent document (*Appendix 1*). All experimental measures conformed to the tenets of the Declaration of Helsinki. The study protocol 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 (*Appendix 2*).

Subjects were recruited following a locally advertised poster campaign (*Appendix 5*), by word of mouth, and by advertisement on local newspaper and radio. The inclusion criteria for participation in this study were as follows: between the age of 18 and 60 years; absence

of ocular pathology by self report; no clinical evidence of retinal pathology as assessed by expert assessment (vitreo retinal specialist) of fundus photographs; visual acuity of at least 20/60 in the study eye; not currently taking supplements containing *meso*-Z, L and Z; not pregnant.

3.2.3 Supplement formulation

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 *meso*-Z, 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 *meso*-Z, 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 Intervention capsule. All subjects were instructed to take one capsule per day with a meal for six months.

3.2.4 Lifestyle/Demographic Questionnaire (EC)

A detailed description of the questionnaire can be found in Chapter 2 (*Section 2.2.2 – Lifestyle/Demographic Questionnaire*).

3.2.5 Best corrected visual acuity (EC)

A detailed description of how best corrected visual acuity was measured can be found in Chapter 2 (*Section 2.2.3 – Best Corrected Visual Acuity*).

3.2.6 Food frequency questionnaire (EC)

Dietary intake of L and Z was assessed by a self-administered, semi-quantitative food frequency questionnaire (FFQ) developed by the Scottish Collaborative Group (SCG) at the University of Aberdeen, Scotland, UK. The questionnaire is designed to assess a subject's dietary intake over the preceding two-three months. It consists of 170 foods and drinks, grouped into 21 sections (*Appendix 6).* Portion sizes are specified at the beginning of the FFQ (Figure 3.1) and subjects are required to indicate how many portions they consume per day and how often they consume that type of food, ranging from 'rarely or never' to '7 days per week'. The questionnaire was completed by the subject in front of the investigator, following detailed instructions.

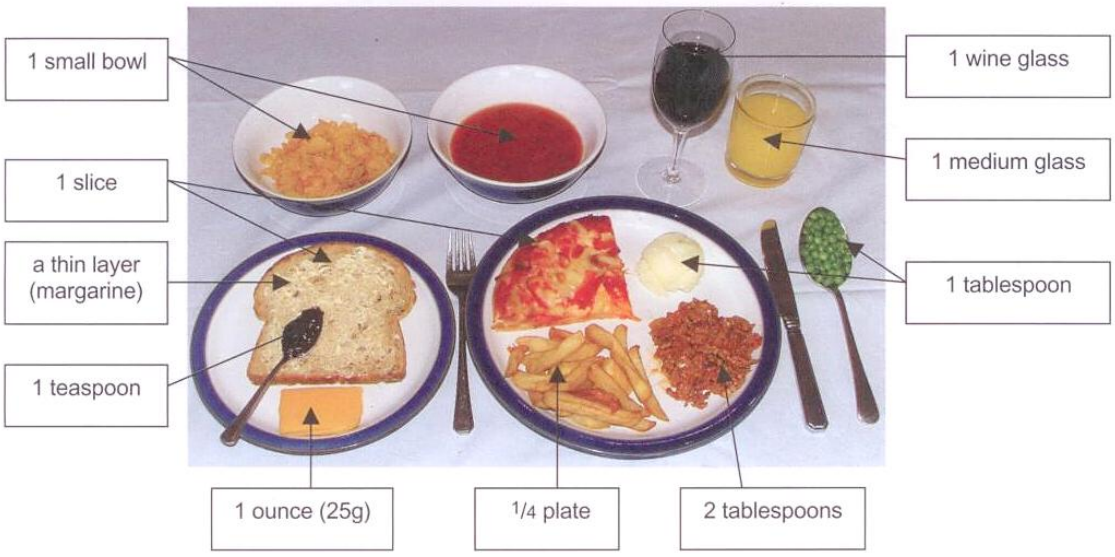


FIGURE 3.1 Photograph showing the portion sizes referred to in the Diet and Lifestyle questionnaire.[176]

Once completed, all FFQs were manually inputted into a Microsoft™ Access® spreadsheet and was then emailed to the SCG for analysis. Dietary intake of L and Z was calculated using food composition data from UK, European and US data sources using standard principles or criteria for the matching of food items, and standardised recipes or manufacturer's ingredient information where necessary.

3.2.7 Contrast sensitivity (EC)

3.2.7.1 Background to method

Contrast sensitivity is a physical dimension referring to the light-dark transition at a border or an edge of an image that delineates the existence of a pattern or object. Contrast is defined as the ratio of the difference in the luminance of these two values.[177;178] Contrast sensitivity is a useful tool for measuring visual function. This study used non-periodic patterns (i.e. letters on a chart where dark targets are presented on a spatially extended white background, contrast is typically defined as luminance of the background minus the luminance of the letter, divided by the luminance of the background).[179] The amount of contrast a person needs to see a target is called the contrast threshold. In clinical research contrast threshold is usually expressed as contrast sensitivity, where sensitivity is the reciprocal of threshold. Contrast sensitivity is typically expressed on a logarithmic scale.[178]

Visual acuity, is a measure of the spatial-resolving ability of the visual system presented at near 100% contrast, in other words, all targets are shown at the same contrast, but their sizes vary during the test.[180] Contrast sensitivity on the other hand involves altering the

level of contrast of the target for a range of spatial frequencies (e.g. 1.5, 3, 6, 12, 18 cycle per degree [cpd]). The contrast sensitivity at each spatial frequency observed by an individual can be plotted as contrast sensitivity function (CSF).

3.2.7.2 Testing procedure

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

Letters were presented in triplets of constant size, at a viewing distance of one metre, and with a dominant spatial frequency of 1.5 cpd. As with the Pelli-Robson chart, contrast reduced in 0.15 log unit steps between each letter triplet, with letters within the same triplet maintaining the same average contrast. The letters were presented on a liquid crystal display (LCD) monitor which was γ corrected using the function provided with the Test Chart 2000 software.

At a constant room illuminance of 870 lux, and with distance correction being worn, each subject was asked to identify letter triplets in decreasing contrast (Figure 3.2). Testing was conducted monocularly. Subjects were allowed sufficient time to facilitate letter recognition, and testing ceased once two or more letters within a triplet at a particular contrast level were either not seen or incorrectly identified. A letter by letter scoring system was used, with each letter assigned a score of 0.05 log units, and minimum contrast levels were converted and recorded as a log contrast sensitivity value.

FIGURE 3.2 Example of triplets shown for letter contrast test. Image courtesy of Thomson Software Solutions

3.2.8 Microperimetry (EC)

3.2.8.1 Background to method

Retinotopic ocular sensitivity was assessed by Microperimetry using a Nidek MP1 (Nidek Instruments Inc., Padova, Italy). Microperimetry is an automatic technique used to quantify the sensitivity of the visual field using different light sensitivities. The MP1 uses an infrared camera with a 45° field of view. The examination is performed using a LCD that projects the target and stimuli for the subject to view. Background luminance is set at 1.27 cd/m^2; stimuli intensities ranged from 0 to 20 dB, where 0 dB represents the brightest and 20 dB represents the faintest stimuli. There is also a tracking system in place to compensate for eye movements while the test is being carried out. Prior to testing, an infra red (IR) image is captured to identify areas of high contrast (i.e. large vessels or disc margin) that are then used to track the movement of the test eye in real time. During the test, this landmark is automatically tracked every 40 ms to allow correction of the stimulus position on the internal LCD, thus to maintaining the same test location on the fundus.[181]

Once the exam has been completed a colour fundus photograph is captured and the IR image is digitally overlaid with the colour fundus image to produce a colour sensitivity map for that test eye (Figure 3.3).

FIGURE 3.3 Colour sensitivity map of a test carried out on the Nidek MP1. Image taken from screenshot of Nidek MP1

3.2.8.2 Testing procedure

For the purpose of this study, the central 6° of fixation were examined and reported as Macular Mean Sensitivity (MMS) within the central 2°, 4° and 6° of the macula. Microperimetry was performed on the study eye (i.e. eye with best visual acuity) in a dark

room and the fellow eye was occluded using a patch. No dilation drops were used, however, subjects were dark adapted for at least 30 minutes prior to the examination. All examinations were carried out by one experienced examiner (EC).

All patients received brief training before the examination to practice recognising the stimulus targets and using the response button. The test stimuli were white Goldmann size I, the interval between stimuli was 200 ms, and the threshold strategy used was 4-2-1. A 1° red cross was used as a fixation target. The study pattern consisted of 49 projection points radiating in a concentric pattern projecting 7° from the macula (Figure 3.4).

Follow up examinations were carried out at all study visits using the follow up software on the Microperimeter – Nidek MP1. This is done using results of the previous exam and allows for record and examination continuity.

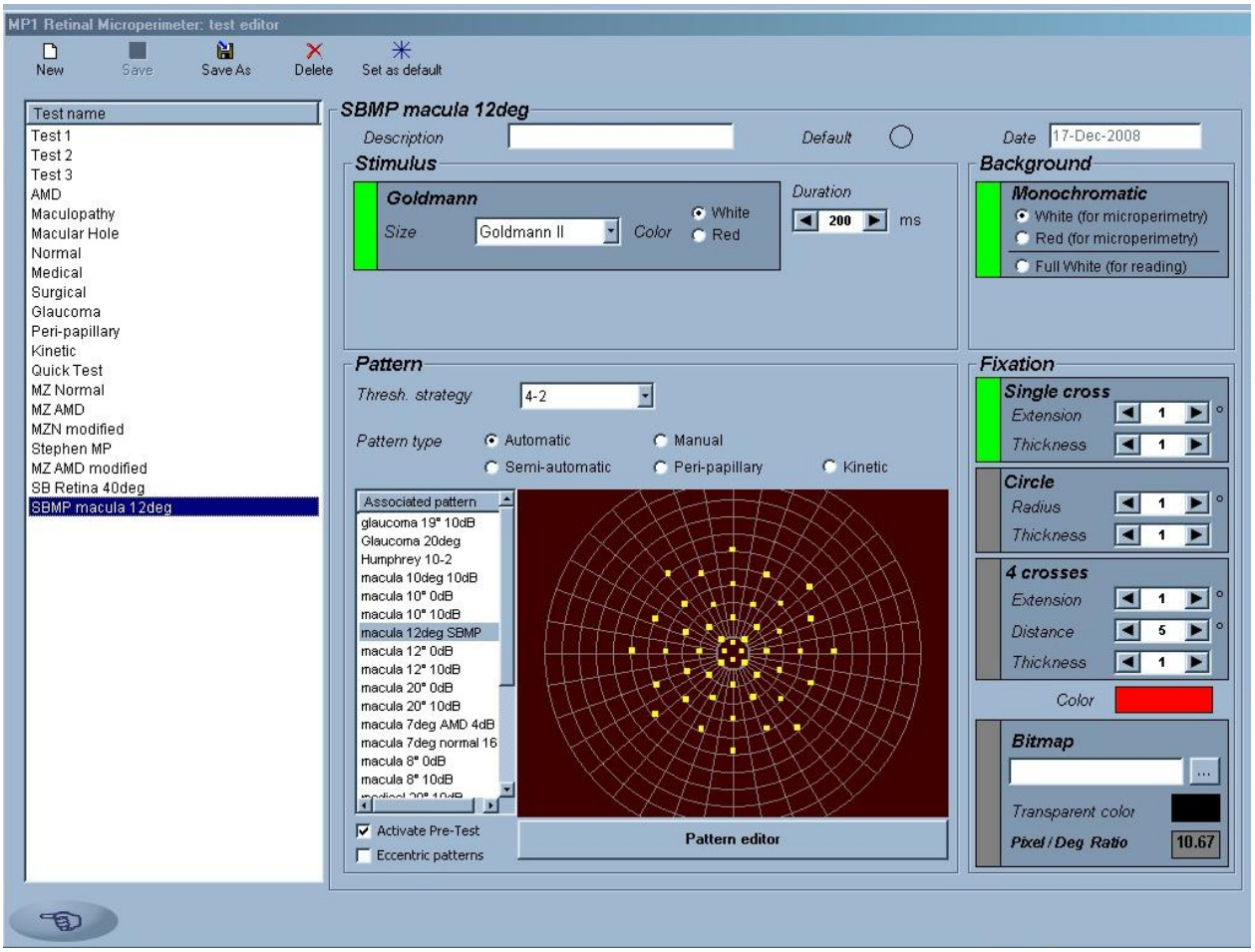


FIGURE 3.4 Example of the pattern used to measure macular sensitivity Image taken from screenshot of Nidek MP1

3.2.9 Macular pigment optical density measurement (EC)

MPOD, including its spatial profile (i.e. 0.25°, 0.5°, 1°, 1.75°), was measured at visit 1 (V1), visit 2 (V2), and visit 3 (V3) using cHFP method previously described in *Chapter 2 – Section 2.2.6 Macular Pigment Optical Density Measurement*.

3.2.10 Blood collection (EC)

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 the method previously described in *Chapter 2 – 2.2.5 Blood Sample Collection*. Additional blood samples were collected at V1 and V3 for clinical pathology analysis (see below).

3.2.11 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 *meso*-Z, L and Z. To achieve this, non-fasting blood samples were collected at both visits using standard venepuncture techniques (EC). 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_2EDTA). 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 3.1.

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 reference ranges for the lipids (high density lipoproteins [HDL], low density lipoproteins [LDL], total cholesterol and triglycerides), which were obtained from the European Guidelines on Cardiovascular Disease Prevention and glucose, whose reference range comes from the World Health Organisation.[182;183]

3.2.12 Statistical analysis (EC, JN, JS)

The statistical software package SPSS (version 17) was used for analysis and SigmaPlot (version 8.0) was used for graphical presentations. Means ± 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. The 5% level of significance was used throughout the analyses.

TABLE 3.1 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
Potassium	Renal profile	3.3-5.3 (mmol/L)	4.16±0.36	4.55±0.40	**0.01**	4.26±0.30	4.43±0.24	**0.04**
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
Globulins	Liver profile	21-36 (g/L)	28.16±3.29	26.47±2.95	0.11	28.11±3.63	26.37±4.11	**0.07**
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
Cholesterol total	Lipid profile	<5.0 (mmol/L)	5.21±0.92	5.24±0.91	0.79	5.26±0.93	4.92±0.86	**0.02**
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
Direct LDL	Lipid profile	<3.0 (mmol/L)	3.03±0.75	3.25±0.80	**0.01**	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
Magnesium	Bone profile	0.65-1.10 (mmol/L)	1.00±0.07	0.95±0.09	**0.01**	0.98±0.06	0.92±0.06	**0.01**
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
Haemoglobin	Haematology	11.3-15.2 (g/dL)	14.23±1.35	13.91±1.37	**0.03**	14.46±1.46	13.85±1.28	**0.01**
Haematocrit	Haematology	0.323-0.462 (L/L)	0.40±0.04	0.41±0.04	**0.01**	0.40±0.40	0.41±0.03	0.38
MCV‡	Haematology	83.1-99.1 (fL)	87.93±4.33	90.41±4.54	**0.01**	87.06±3.02	89.42±3.22	**0.01**
MCH§	Haematology	28.3-33.9 (pg)	31.42±1.51	30.38±1.54	**0.01**	31.15±1.58	30.28±1.34	**0.01**
MCHC‖	Haematology	32.1-36.6 (g/dL)	35.75±0.98	33.62±0.93	**0.01**	35.78±1.36	33.88±1.09	**0.01**
Platlets	Haematology	164-382 (10e9/L)	295.47	287.00	0.24	313.28	299.00	**0.08**
Differential White Cell Count								
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
Lymphocytes	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	**0.04**
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
Large unstained cells	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	**0.01**

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 haemoglobin; ‖ mean corpuscular; hemoglobin concentration

3.3 RESULTS

3.3.1 Baseline

Baseline demographic, lifestyle, dietary intake of L and Z (mg/day), serum concentrations of L and Z (μmol/L), and MPOD data for the I and P groups (n = 44) are presented in Table 3.2. There was no statistically significant difference between groups with respect to baseline variables ($p > 0.05$, for all). Statistically significant relationships between variables, at baseline, are presented in Table 3.3 and Figure 3.5.

3.3.2 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.

3.3.3 Retinal findings

There were no significant change observed in retinoptic ocular sensitivity at six months for any of the areas examined (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).

TABLE 3.2. Baseline characteristics of the intervention and placebo group.

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

Data are presented as mean ± SD unless otherwise noted.*BMI Body mass index defined as body weight in kilograms divided by height in squared meters (kg/m^2); †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 $20/20^{+1}$ equated to a score of 101, and $20/20^{-1}$ to 99); [‡]MMS Macular mean sensitivity (as defined by the mean retinotopic ocular sensitivity within 2°, 4°, 6° of the macula); [§]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 3.3 Significant relationships between baseline variables for the entire study group before intervention (n = 44)

Dependent variable	**Independent variable**	**Pearson coefficient (r)**	**Significance (p)**
MPOD[†] 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°[‡]	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[§]	Serum Lutein	0.318	0.040
Serum Lutein	Diet Lutein	0.374	0.017

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

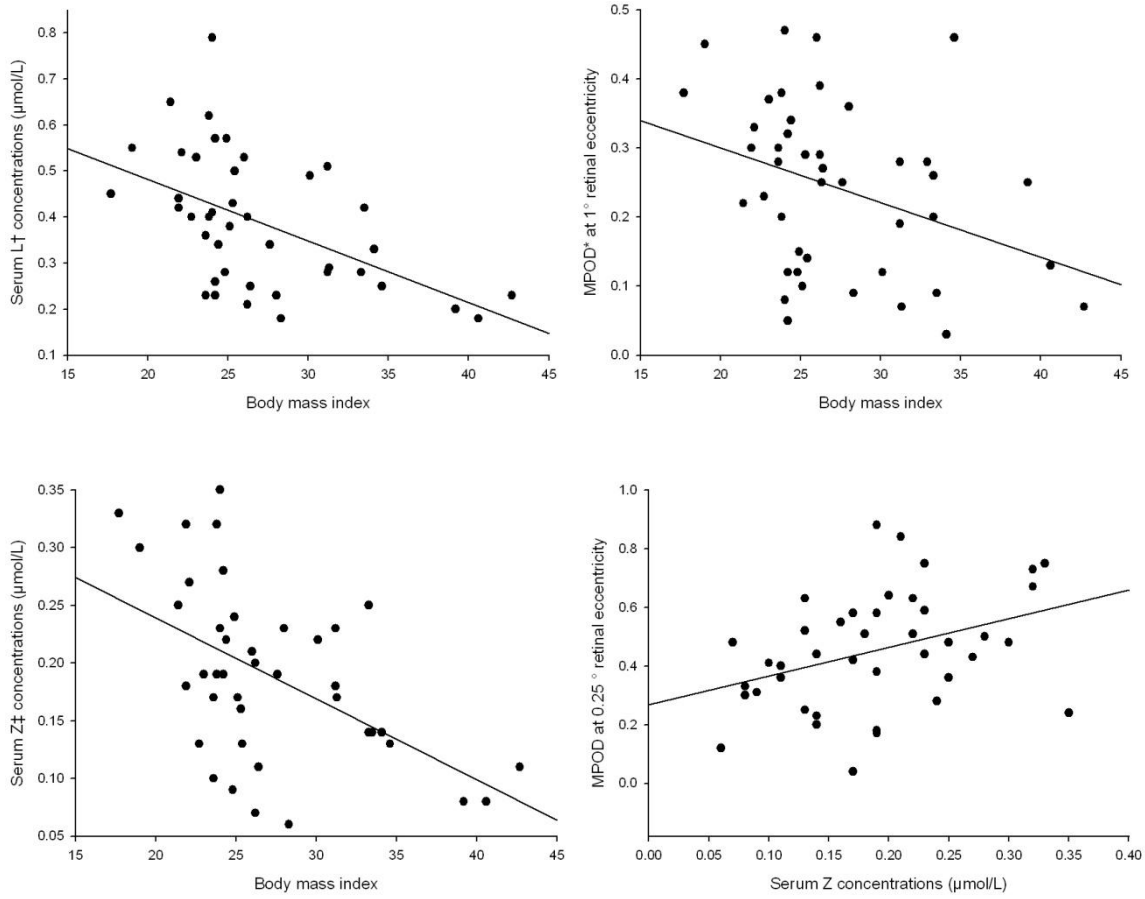


FIGURE 3.5 Statistically significant relationships between baseline variables (n=44)
*MPOD = Macular pigment optical density; †L = Lutein; ‡Z= Zeaxanthin

3.3.4 Lutein and zeaxanthin response in serum

There was a statistically significant increase in serum concentrations of L and Z (µmol/L) from baseline at three months ($p = 0.001$, for both) and six months ($p = 0.001$, for both) in the I group. There was no significant change from baseline in the P group over the six months ($p > 0.05$, 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 3.6A].

3.3.5 Macular pigment optical density response

There was a statistically significant increase in MPOD at 0.25° retinal eccentricity 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° 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 3.6B].

There was a statistically significant increase in MPOD at 0.5° retinal eccentricity 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 either 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 3.6B].

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

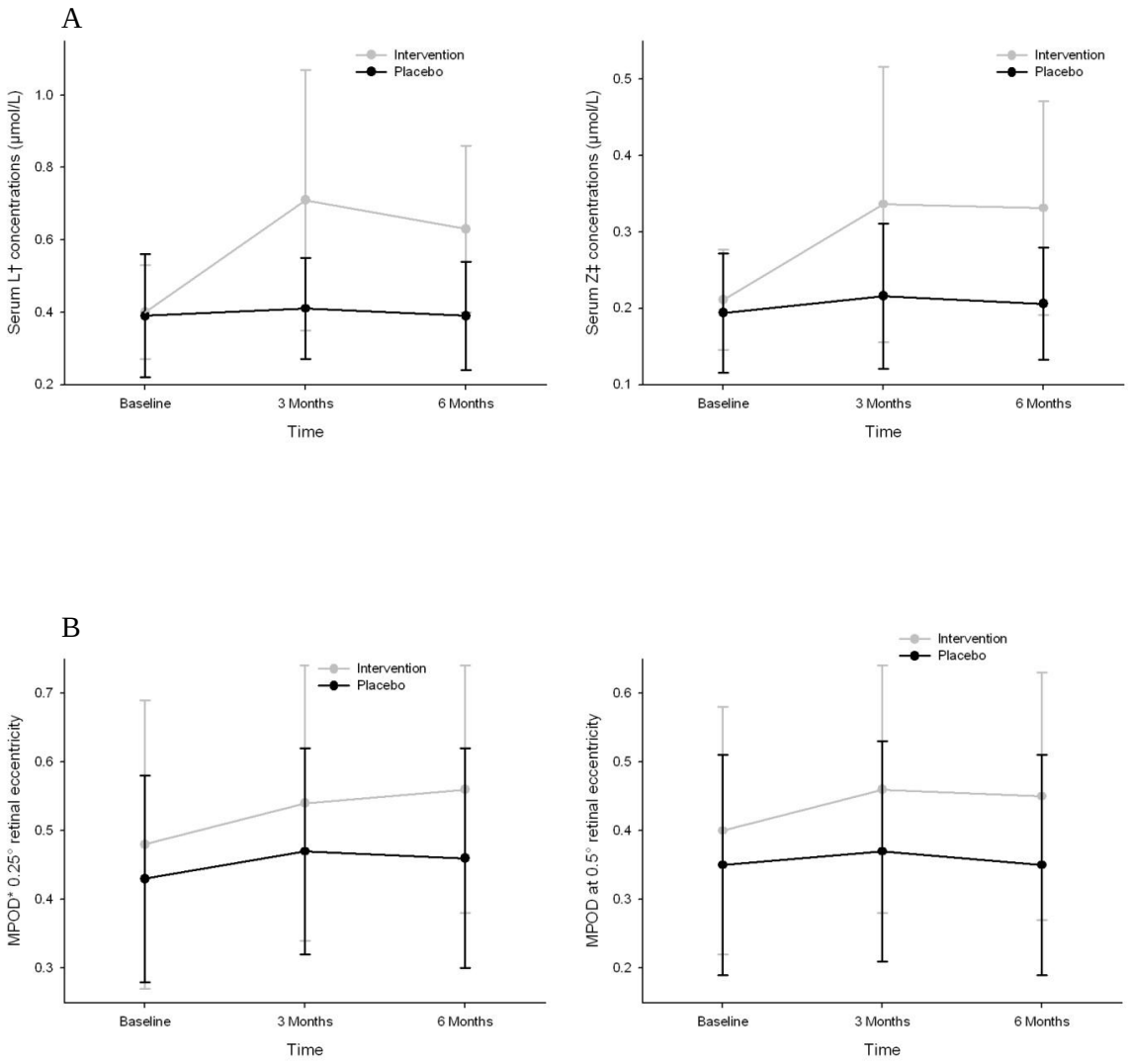


FIGURE 3.6A/B Change in central macular pigment optical density and serum lutein and zeaxanthin concentrations for the intervention and placebo group

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

3.3.6 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 in 9 of the 25 variables assessed in the P group following supplementation with the macular carotenoids (Table 3.1). However, all variables remained within their respective normal reference ranges, 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 to supplementation with the macular carotenoids.

3.4 DISCUSSION

The MOST-N study was designed to measure serum and macular response to a dietary supplement containing all three macular carotenoids (*meso*-Z, L, and Z) in the normal healthy population (Irish Republic), as part of a randomised, double blind, placebo-controlled, clinical trial, and to concurrently assess the safety of consuming these carotenoids through 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 3.4). Recent studies have reported and confirmed significant serum *meso*-Z response following supplementation with this carotenoid. Consistent with these previous studies, this study has reported statistically significant increases in serum concentrations of L and Z in the I group; with no such increases observed in the P group over the study period. *Meso*-Z was not quantified separately as part of the current study; however, *meso*-Z 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 ± 0.15 µmol/L; Final: 0.50 ± 0.22 µmol/L), and a 1.6-fold increase in serum concentrations of Z (Baseline: 0.21 ± 0.03 µmol/L; Final: 0.72 ± 0.11 µmol/L), which are somewhat poorer responses than other

studies supplementing with similar amounts of these carotenoids.[56;171] Possible reasons for this lower than normal responses are discussed below, following our discussion on MPOD.

TABLE 3.4 Serum carotenoid response following supplementation with the macular carotenoids

Principal Author	Journal	Year	N	L (mg/day)	Z (mg/day)	*meso*-Z (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
Normal Subjects																
Berendschot et al.[144]	IOVS	2000	8	10	0	0	12	0.18 ± 0.08	0.9 ± 0.18	400	-	-	-	-	-	-
Johnson et al.[143]	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.[184]	JID	2000	21	15	0	0	4	0.37	1.753	374	-	-	-	-	-	-
Bone et al.[146]	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.[145]	EER	2004	6	10	0	0	19	0.27 ± 0.1	1.95 ± 1.06	622	-	-	-	-	-	-
Zhao et al.[185]	AJCN	2006	8	12	0	0	8	0.17	0.874	514	-	-	-	-	-	-
Schalch et al.[149]	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.[142]	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.[148]	OPO	2007	3	30	2.7	0	17	-	-	~1500	-	-	~278	-	-	-
Thurnham et al.[171]	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.[150]	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.[186]	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.[56]	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.[151]	VR	2011	61	12	1	0	48	0.57	1.40	146	0.36	0.39	8	-	-	
														-	-	
AMD Subjects													-	-	-	
Koh et al.[145]	EER	2004	7	10	0	0	19	0.32 ± 0.22	1.89 ± 0.29	491	-	-	-	-	-	-
Khachik et al.[173]	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	-		
Trieschmann	EER	2007	97	12	1	0	36	0.158	0.44	178	-	-	-	-	-	-

et al.[187]																
Huang et al.[188]	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	-		
Connolly et al.[56]	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); *meso*-Z = *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

There were significant increases in MPOD at 0.25° and 0.5° retinal eccentricity, at three and six months in the I group; whereas, there were no significant changes in the P group. This is consistent with previous studies that also measured central MPOD and supplemented with similar concentrations of the macular carotenoids over a three month period. However, at six months, we report a slightly lower than normal MPOD response at 0.25° (Baseline = 0.45 ± 0.21, six months = 0.50 ± 0.18).

Given that the supplement used in the current study had higher amounts of *meso*-Z (10.6 mg) than L (5.9 mg) or Z (1.2 mg), we feel it is important to make a direct comparison to previous studies that also supplemented with *meso*-Z. 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 *meso*-Z, 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. The pilot study investigated 10 subjects (4 with AMD, 5 without AMD) who were assessed over an eight week study period following supplementation with 7.3 mg *meso*-Z, 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.

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 a *meso*-Z dominant supplement was used. Given the known anatomical (central retina),[57] biochemical (antioxidant),[3] and optical (short-wavelength filtering)[93] properties of MP, it is hypothesised that this pigment may confer protection against AMD, rendering 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 2.1 and 3.4) 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 encapsulated (e.g. oil versus microencapsulated); whether consumed alone or in the presence of other antioxidants; non-compliance.

Further analysis of serum and MPOD response in the study revealed the following: that there was one serum non-responder for L and Z (subject 28), which was not due to a lack of compliance (confirmed by retinal measurement 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 at the macula, as a result of a relative need for 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 *meso*-Z at the central macula from retinal L (although the exact mechanism remains unclear). One explanation rests on the possibility that this subject cannot generate *meso*-Z from retinal L (hence the lack of central baseline MP in this subject; MPOD at 0.25° = 0.18 and at 0.5° = 0.11), but could respond to a supplement containing *meso*-Z. Indeed, this notion is consistent with the findings of our previous pilot study reporting on *meso*-Z.[56] It is also possible that this subject initially consumed the macular carotenoid supplement, containing *meso*-Z, which caused an increase in his MP levels; 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', it was 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). It is possible this observed non-response in these two subjects may rest on their high baseline MPOD values of 0.73 and 0.51, respectively (i.e. possibly 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, three subjects with 'central dips' in their baseline MP spatial profiles were identified in this study (see publications by Kirby *et al*., 2008,[164] and Connolly *et al*., 2010,[56] for discussion on central dips in MP spatial profiles), these atypical profiles were normalized following supplementation with *meso*-Z, L and Z. This, again, is consistent with the hypothesis that these subjects are unable to generate *meso*-Z from L at the macula, but do respond to a supplement containing *meso*-Z. Moreover, and consistent with the above suggestion that family history of AMD and smoking cigarettes may inhibit *meso*-Z 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 *meso*-Z supplement resulting in a "normal" MP profile following supplementation.

The most novel aspect of the current study was the investigation with respect to safety of consumption of the macular carotenoids, determined through clinical pathology analysis, 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 their normative reference ranges, 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(i.e. 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 the safety of supplemental macular carotenoids through 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).[170;189] Indeed, doses of 20 mg/day for up to six months were not associated with any side effects.[140] Even doses of 30 mg/day for five months[130] or 40 mg/day over two months were not associated with any adverse effects.[132-134] 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.[135] Carotenedermia, is itself not known to be associated with any specific adverse effects on

human health and only results from excessive intake of L.[135] The majority of studies assessing safety of supplemental Z involving humans have also been observational in design, and have not included appropriate clinical pathology safety testing. Of note, none of these studies reported any adverse effects or ocular toxicity following supplementation with this carotenoid.[143;146;190-196] However, there has been one (unpublished) pharmacokinetic study in humans involving five men and five women designed to assess safety of Z consumption.[197] In this study conducted by Hoffmann-La Roche (now DSM Nutritional Products Ltd.), the subjects 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.[197]

In the animal model, there have been two investigations into the possibility of toxicological and/or mutagenic effects of *meso*-Z. A toxicity study carried out by Chang *et al*. in 2006, investigated the effect of administering 2, 20, and 200 mg/kg/day of *meso*-Z for thirteen weeks consecutively.[137] This study reported that *meso*-Z was well tolerated, and concluded that the no-observed-adverse-effect-level (NOAEL) of *meso*-Z 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 *meso*-Z.[138]

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.[198] 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.[199] 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).[200;201] Also, ocular toxicity studies have been performed on monkeys which also reported no evidence of treatment related changes.

CHAPTER FOUR

CONCLUSION

4.1 CONCLUSION

The first conclusion to be drawn from my research is that there was a significant increase in serum concentrations of *meso*-Z, L and Z following supplementation with these carotenoids. This is important because it demonstrates that humans respond to taking a food supplement containing the macular carotenoids, including the central macular carotenoid *meso*-Z. We also observed an increase in MPOD across its spatial profile, following supplementation with all three macular carotenoids. These observations are important, as they have shown, in a placebo-controlled clinical fashion, that individuals have the ability to respond to a dietary supplement designed to enrich this nutritional pigment at the eye. These findings, were observed in patients with and without AMD, which has important implications for both populations. For patients with AMD it is important as it has been previously shown that such individuals are lacking in MP, and there is a scientific rational suggesting that MP protects against AMD via its antioxidant and light filtering properties. This is an important finding as it confirms that the known lack of MP seen in individuals afflicted with, or at high risk of, developing AMD is not due to an inability of such subjects to respond to carotenoid consumption, and is therefore due to either a defective capture of circulating carotenoids by stabilization within the central retina. For normal subjects it is important because research has shown that macular pigment can enhance visual performance and experience in this population.

Secondly, and the most novel aspect of my research, was the investigation into the safety of consumption of all three carotenoids, including the central *meso*-Z. Mutagenicity and toxicology studies have previously shown that consumption of these carotenoids have no ill adverse effects in animal models. Of note, therefore, this was the first human trial to assess pathology parameters following consumption of these carotenoids. My data is

consistent with the findings of the animal studies, which suggest no adverse effects following consumption of these carotenoids in human subjects.

Thirdly, this study detected the possible presence of *meso*-Z in human serum pre-supplementation and the ability of this carotenoid formulation to rebuild central MPOD in subjects who display atypical profiles at baseline. *Meso*-Z in serum at baseline is a novel finding; however, it must be viewed with caution and will require further study to fully investigate this claim. This was the first study to investigate the rebuilding of an atypical spatial profile following supplementation with the three macular carotenoids. It is believed that patients who have an atypical profile of MP may be at increased risk of developing AMD. Importantly, by measuring the spatial profile in this study it helped to determine patients who displayed this atypical profile and, using a formulation containing the most central carotenoid (*meso*-Z), rebuild the central peak to a more normal profile. Importantly, my research has shown that even though an individual may be lacking in central MP (suggestive of a lack of *meso*-Z) their profiles can be normalised following supplementation with all three macular carotenoids.

4.2 FUTURE CONSIDERATIONS

This research has helped further our understanding of human response to supplementation with a combination of the three macular carotenoids in healthy individuals and individuals who suffer with AMD. Future investigation of enrichment of central MP in normal and AMD subjects is essential and ideally should probably be conducted in a much larger study cohort, in an effort to replicate and further elucidate the findings of this research. We

would encourage future research in this area and suggest that it include assessment of visual performance, grading of fundus photography to measure disease progression in AMD patients, serum concentrations of all three macular carotenoids and continuing analysis of safety parameters following consumption of *meso*-Z, L and Z.

Of note, and following the recommendations of this research study there is currently an investigation entitled 'The Central Retinal Enrichment Supplementation Trials (CREST)' designed to assess the benefit of supplementation with respect to the protective and visual performance hypotheses over a 24 and 12 month supplementation period, respectively. In addition, clinical pathology analysis is also ongoing to continue to assess the safety of consumption of these carotenoids. The design of CREST has been informed by this research, which as evidenced by the two scientific peer-reviewed publications from my work, has contributed significantly to this important field. Further research into the central macular carotenoids and its importance for vision is merited.

CHAPTER FIVE

PUBLICATIONS AND PRESENTATIONS

5.1 PEER-REVIEWED PUBLICATIONS

1. Sabour-Pickett S, Beatty S, **Connolly E**, Loughman J, Stack J, Howard A, Klein R, Klein BE, Meuer SM, Myers CE, Nolan JM.

Supplementation with three different macular carotenoid formulations in patients with early age-related macular degeneration

Currently under review

2. Meagher KA, Thurnham DI, Beatty S, Howard AN, **Connolly E**, Cummins W, Nolan JM.
Serum response to supplemental macular carotenoids in subjects with and without age-related macular degeneration
British Journal of Nutrition. 2013 Jul 28;110(2):289-300.

3. Loughman J, Nolan JM, Howard AN, **Connolly E**, Meagher K, Beatty S.
The impact of macular pigment augmentation on visual performance using different carotenoid formulations.
Investigative Ophthalmology & Visual Science. 2012 Nov 29;53(12):7871-80

4. Nolan JM, Feeney J, Kenny RA, Cronin H, O'Regan C, Savva GM, Loughman J, Finucane C, **Connolly E**, Meagher K, Beatty S.

Education is positively associated with macular pigment: the Irish Longitudinal Study on Ageing (TILDA).

Investigative Ophthalmology & Visual Science. 2012 Nov 27;53(12):7855-61

5. **Connolly EE**, Beatty S, Loughman J, Howard AN, Louw MS, Nolan JM.

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

Investigative Ophthalmology and Visual Science. 2011 Nov 29;52(12):9207-17.

6. Kirby ML, Beatty S, Loane E, Akkali MC, **Connolly EE**, Stack J, Nolan JM.

A central dip in the macular pigment spatial profile is associated with age and smoking.

Investigative Ophthalmology and Visual Science. 2010 Dec;51(12):6722-8.

7. Nolan JM, Kenny R, O'Regan C, Cronin H, Loughman J, **Connolly EE**, Kearney P, Loane E, Beatty S.

Macular pigment optical density in an ageing Irish population: The Irish Longitudinal Study on Ageing.

Ophthalmic Research. 2010;44(2):131-9.

8. **Connolly EE**, Beatty S, Thurnham DI, Loughman J, Howard AN, Stack J, Nolan JM.

Augmentation of macular pigment following supplementation with all three macular carotenoids: an exploratory study. Current Eye Research. 2010 Apr;35(4):335-51.

9. Nolan JM, O'Reilly P, Loughman J, Stack J, Loane E, **Connolly E**, Beatty S.

Augmentation of macular pigment following implantation of blue light-filtering intraocular lenses at the time of cataract surgery.

Investigative Ophthalmology and Visual Science. 2009 Oct;50(10):4777-85. 23.

5.2 NON PEER-REVIWED PUBLICATIONS

1. Nolan JM, **Connolly E**, Loane E, Kirby M, Beatty S.

Protecting against AMD: The role of the macular carotenoids.

Ophthalmology Times Europe. 2008 Jul/Aug;4(6):28-31

5.3 ORAL PRESENTATIONS

1. Meso-zeaxanthin Ocular Supplementation Trial in Normals (MOST N)

Macular Carotenoids and AMD Conference, Downing College, Cambridge, UK, July 2011

2. Online AMD research study for optometrists: current practice in the Republic of Ireland and the United Kingdom

Macular Carotenoids and AMD Conference, Downing College Cambridge, UK, July 2011

3. The Risk for Age-Related Macular Degeneration amongst Construction Workers in Ireland: Construction Workers Health Trust (CWHT)

Castlemartyr Resort, Cork, AMD Week, April 2009

4. Carotenoids and co-antioxidants in Age-Related Macular Degeneration: Age-Related Macular Degeneration Pathogenesis and Treatment

IV International Symposium of the German Ophthalmological Society, Baden Baden, Germany, September 2007

5.4 POSTER PRESENTATIONS

1. Effect Of Carotenoid Supplementation On Macular Pigment Optical Density And Visual Performance In Normal Observers: The Most Vision Trial

Loughman J, Beatty S, Howard A, **Connolly E**, John M. Nolan

Association for Research in Ophthalmology (ARVO) Conference, Fort Lauderdale, Florida, USA, May 2012

2. Macular Pigment Response To Three Different Macular Carotenoid Interventions In Patients With Early Age-Related Macular Degeneration

Nolan JM, Sabour-Pickett S, **Connolly E**, Loughman J, Howard A, Beatty S

Association for Research in Ophthalmology (ARVO) Conference, Fort Lauderdale, Florida, USA, May 2012

3. Comparison Of Macular Pigment Optical Density Measurements Using Customised Heterochromatic Flicker Photometry Versus Reflectance

Loughman J, Beatty S, Stack J, **Connolly E**, Nolan JM

Association for Research in Ophthalmology (ARVO) Conference, Fort Lauderdale, Florida, USA, May 2011

4. *Meso*-Zeaxanthin Ocular Supplementation Trial: MOST

Connolly EE, Beatty S, Loughman J, Nolan JM

Association for Research in Ophthalmology (ARVO) Conference, Fort Lauderdale, Florida, USA, May 2010

5. Augmentation of macular pigment following supplementation with all three macular carotenoids: a pilot study.

Connolly EE, Beatty S, Thurnham DI, Loughman J, Stack J, Howard AN, Nolan JM

Waterford Institute of Technology, Research Day, Waterford, Ireland, May 2010

6. *Meso*-zeaxanthin ocular supplementation trial: MOST

Connolly EE, Beatty S, Loughman J, Howard A, Nolan JM

Waterford Institute of Technology, Research Day, Waterford, Ireland, May 2010

7. Augmentation of macular pigment following supplementation with all three macular carotenoids: an exploratory study.

Connolly EE, Beatty S, Thurnham DI, Loughman J, Stack J, Howard AN, Nolan JM

Retina Conference, Dublin, Ireland, October 2009

8. Macular pigment levels increase following blue light-filtering intraocular lens implantation.

Nolan JM, O'Reilly P, **Connolly E**, Stack J, Loane E, Loughman J, Beatty S

Association for Vision and Ophthalmology, Fort Lauderdale, Florida, USA, May 2009

9. Longitudinal Relationships Between Macular Pigment and Serum Lutein in Patients Enrolled in the CARMA Clinical Trial (Carotenoids and Co-antioxidants in Age-Related Maculopathy)

Beatty S, Stevenson M, Nolan JM, Woodside J, The CARMA Study Group

Association for Vision and Ophthalmology, Fort Lauderdale, Florida, USA, May 2009

10. Macular pigment levels increase with AcrySof Natural SN60AT intraocular lens

O'Reilly P, Beatty S, **Connolly E**, Stack J, Loane E, Loughman J, Nolan JM

European Association for Vision and Eye Research Conference, Portoroz, Slovenia, October 2008

CHAPTER SIX

APPENDICES

6.1 APPENDIX 1: Sample consent form

NutraSight Consultancy Ltd

Macular and Serum Responses to Supplemental Meso-zeaxanthin, Lutein, and Zeaxanthin (Macushield™/MacuHealth with LMZ)

Date: _____ Subject Number: _____

- I confirm I have read and understand the Information Leaflet regarding this study. I further attest that the relevant information has been discussed fully in non-technical terms, and all my questions have been replied to with full satisfaction.
- I understand that my participation is voluntary and that I am free to withdraw at any time, without my medical care or legal rights being affected.
- I understand that my data concerning this study will be entered on a computer in order to be analysed together with the data obtained from other patients. My identity will always be protected. I give permission for this analysis.
- I understand that responsible authorities within the Macular Pigment Research Group may look at my data collected for this study where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.
- I agree to take part in the above study and hereby give my consent to have a blood sample collected for serum analysis.

_____	_____	_____
Name of Volunteer	Date	Signature of Volunteer
_____	_____	_____
Name of Witness	Date	Signature of Witness

I have no objection to being contacted by the Macular Pigment Research Group about future research studies.

I give permission for any data collected today to be used for other research studies carried out by the Macular Pigment Research Group.

6.2 APPENDIX 2: Ethics documents

Waterford Institute *of* Technology WIT

Institiúid Teicneolaíochta Phort Láirge
Waterford, Ireland.
TEL: +353-51-302000
WEB: www.wit.ie
EMAIL: info@wit.ie

Ref: 10/CLS/01

23rd September, 2011.

Ms. Eithne Connolly,
Macular Pigment Research Group,
Department of Chemical & Life Sciences,
WIT.

Dear Eithne,

Thank you for submitting your outstanding documentation in relation to your project *'Macular and Serum Responses to Supplemental Meso-zeaxanthin, Lutein and Zeaxanthin (Macushield™/MacuHealth with LMZ)'* to the WIT Research Ethics Committee.

We wish you well in the work ahead.

Yours sincerely,

pp Suzanne Kiely
Dr. John Wells,
Chairperson,
Research Ethics Committee.

cc: Dr. John Nolan
Mr. Stephen Beatty
Dr. James Loughman

Waterford Institute *of* Technology WIT

Institiúid Teicneolaíochta Phort Láirge
Waterford, Ireland
TEL +353-51-302000
WEB www.wit.ie
EMAIL info@wit.ie

Ref: 10/CLS/01

1st June, 2010.

Ms. Eithne Connolly,
Macular Pigment Research Group,
Department of Chemical & Life Sciences,
WIT.

Dear Eithne,

Thank you for bringing your project *'Macular and Serum Responses to Supplemental Meso-zeaxanthin, Lutein and Zeaxanthin (Macushield™/MacuHealth with LMZ)'* to the attention of the WIT Research Ethics Committee.
I am pleased to inform you that we approve WIT's participation in this project which we will convey to Academic Council.

However, we would ask you to make the following amendments:

Amend your application form to indicate that participants will complete an informed consent form.
In the publication section of the application form, please state the publication agreement in place between all researchers involved.
Please also outline a contingency plan in your application form in relation to the storage of blood samples.

Please note that for future consent forms, tick boxes should be used for participants to tick as appropriate and that any personal data collected should be adequate, relevant and non excessive.

Please re-submit your amended form to Suzanne Kiely for our records.

Yours sincerely,

pp Suzanne Kiely
Dr. John Wells,
Chairperson,
Research Ethics Committee.

cc: Dr. John Nolan
Mr. Stephen Beatty
Dr. James Loughman

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Waterford Regional Hospital,
Dunmore Road,
Waterford,
Ireland.

Telephone 051 848000
Fax 051 848572

RESEARCH ETHICS COMMITTEE.
HEALTH SERVICE EXECUTIVE, SOUTH EASTERN AREA.

Name & Address:
Ms. Eithne Connolly
Office Manager & Senior Serum Analyst
Macular Pigment Research Group
Department of Chemical and Life Science
Waterford Institute of Technology
Cork Road
Waterford

Study Title: ***"Macular and serum responses to supplemental meso-zeaxanthin, Lutein and zeaxanthin (Macushield™)"***

Date: 11th August 2008

Dear Ms. Connolly

The Research Ethics Committee Coordinator and/or the Chairperson, HSE, South East reviewed the above study on the 28th July 2008.

Expedited approval has been granted in advance of the REC meeting and constitutes full ethical approval.

In addition this study will be outlined at the next planned Research Ethics Committee Meeting for the HSE, South Eastern Area by the Research Ethics Committee Coordinator on Monday 15th September 2008 and any comments made at this meeting in relation to your study shall be communicated to you in writing.

The following documents were reviewed and approved:

1. (✓) Ethics Application Form
2. (✓) Protocol/ Research Proposal
3. ( ) Amendment
4. (✓) Participant Information Leaflet
5. (✓) Participant Consent Form
6. (✓) Recruitment Literature
7. ( ) Indemnity Form
8. ( ) Investigator brochure
9. (✓) Investigator(s) CV (s)
10. ( ) Investigators MDU/Insurance
11. ( ) Sponsor insurance
12. ( ) Funding for the study

It is a requirement of the REC, HSE, South East that you send copy of your study to the Research Ethics Office on completion.

Yours sincerely,

Caroline Lamb

Ms Caroline Lamb
Research Ethics Committee Coordinator
Health Service Executive, South Eastern Area

Cc:

The Research Ethics Committee, HSE, South East is a recognized Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human use) Regulations 2004 and as such is authorized to undertake ethical review of clinical trials of all descriptions and classes for the Republic of Ireland.

The Research Ethics Committee, HSE, South East issues ethical approval on the basis of information provided. It is the responsibility of the researcher to notify the Research Ethics Office of any changes to a study to ensure that the approval is still relevant.

6.3 APPENDIX 3: Sample case report form

Meso-Zeaxanthin Trial (MZT)

NutraSight Consultancy Ltd

CRF

Case Report Form

Investigator Parties:

1. Macular Pigment Research Group
   Waterford Institute of Technology

Study procedures

DESCRIPTION	EST. TIME MINS
A. Information leaflet discussion and informed consent	5
B. Collection of blood for serum carotenoid analysis	5
C. Demographic, medical history, lifestyle and vision case history questionnaires	20
D. Visual acuity	5
E. Macular pigment optical density spatial profile measurement	35
F. Dietary questionnaire	30
G. Microperimetry	15
H. Fundus photographs	5
Total	120

A. Informed consent

Was the patient given a copy of his/her consent? yes ☐ no ☐

If yes,

Date of informed consent: _____ Obtained by: _____

(DD/MM/YYYY)

Signature of person obtaining consent: _____

B. Blood extraction record sheet

Was a blood sample taken from the subject:

yes ☐ no ☐

If yes,

Time of blood extraction: _____

Time of subject's last meal: _____

Was this sample centrifuged, the serum extracted and stored in duplicate at -70^{0}C? yes ☐ no ☐

If yes,

Time of centrifugation: _____

Name of person obtaining blood: _____

Signature of person obtaining blood: _____

C. Demographic, medical history, lifestyle and vision case history questionnaires

Forename: _____ Surname: _____

Address: _____

_____

_____

Contact No(s): _____

Email: _____

Date of birth: _____ Age: _____(years)

Please circle number corresponding to correct answer. All questions must be answered unless otherwise specified.

1. Sex

Male 1

Female 2

If female, stage of Menopause (please circle):

Pre 1

During 2

Post 3

2. Race

White 1

Black 2

Asian 3

Spanish or Hispanic 4

Mixed race..... 5

3. Marital status

Are you now:

Married (or cohabiting)……	1
Widowed ……..	2
Single ……..	3
Divorced or separated.....	4

4. Education

Briefly describe your educational background:

_____

5. Occupation

Briefly describe your occupation:

_____

6. Smoking

a) Which best describes your smoking habits (whether cigarette, cigar, pipe etc.)?

Never smoker (smoked < 100 cigs in lifetime)	1
Ex-smoker (smoked ≥ 100 cigs in lifetime and none in past year)	2
Current smoker (smoked ≥ 100 cigs in lifetime and at least 1 cig in last year)	3

b) Have you smoked at least 100 cigarettes in your life? yes ☐ no ☐ If no skip to question 6.1

c) How long has it been since you last smoked?

Less than 1 day	☐
Less than 7 days	☐
Less than 1 month	☐
Less than 3 months	☐
Less than 6 months	☐
6 months to a year	☐
Greater than 1 year	☐

d) What is the average number of cigarettes you smoke (or smoked) on a daily basis? _____

e) For how many years have you smoked (or did you smoke)? _____

6.1 Are you commonly exposed to second-hand smoke at home or in the work place? yes ☐ no ☐

7. Alcohol

a) Regarding alcohol, which of the following statements best describes the way you drink?

I never drink	1
I drink only on special occasions	2
I drink once or twice a month	3
I drink once or twice a week	4
I drink every day	5
I drink twice a day or more	6

b) What is your average alcohol consumption on a weekly basis?

1 unit a week	1
2-5 units a week	2
6-10 units a week	3
> 10 units a week	4

8. Medical History

Have you any of the following medical conditions?	Yes	No
Diabetes	1	2
High blood pressure	1	2
High cholesterol	1	2
Angina	1	2
Stroke	1	2

If yes for any of the above please give details in the space provided below (e.g. year it occurred, treatment, medication etc.)

_____

_____

9. History of Eye Disease

	Yes	No
Have you ever been told by a doctor that you have Cataract?	1	2
Have you had an operation for Cataract?	1	2
Have you ever been told by a doctor that you have Macular Degeneration?	1	2
Have you ever been told by a doctor that you have Glaucoma?	1	2
Other?	1	2

If yes for any of the above please give details in the space provided below (e.g. year it was diagnosed, doctor etc.)

_____

_____

	Yes	No
Have you a family history of any of the above eye diseases?	1	2

(e.g. age-related macular degeneration, glaucoma etc.)

If a family member, what is their relation to you, and what eye disease do/did they have?

_____

10. Exercise

Do you perform any of the following physical activities?	Yes	No
Walking……..	1	2
Running……	1	2
Cycling……..	1	2
Swimming……	1	2
Gym-based work-outs……..	1	2
Team sport……	1	2
Other……..	1	2

If "Team sport" or "Other", please describe in the space provided

_____

How many times a week do you carry out the above exercise? [ ] **time(s)/week**

If you exercise, how long would each session usually last? [ ] **minutes**

11. Body Mass Index (BMI)

Please record the subject's weight and height in the spaces provided

Weight..... ☐ **Kg**

Height..... ☐ **M**

BMI..... ☐ **Kg/M²**

12. Blood pressure

Please record the subject's blood pressure level in the space provided. _____ **mmHg**

13. Vision case history

Approximately how long since your last eye examination? _____

Do you currently wear spectacles and/or contact lenses? Yes No

1 **2**

If yes, for what? _____

since when? _____

any problems with? _____

Have you ever undergone any ocular treatment or surgery (including Laser eye surgery)?

Yes No

1 **2**

If yes, for what? _____

when? _____

any complications? _____

Were you required to wear an eye patch as a child? Yes No

1 2

If yes, at what age? _____

for how long? _____

which eye? _____

Do you have any current problems with your vision? Yes No

1 2

If yes, please describe in the space provided

_____

D. High contrast visual acuity and refractive error

1: High Contrast (HC) Visual Acuity (VAR)

Please record the subject's unaided VA and aided VA (own spectacles/contact lenses if appropriate) in the spaces provided:

Unaided VA R [ ] L [ ]

Habitual VA (own spx or pinhole) R [ ] L [ ]

2: Study Eye

Please indicate which eye will be used for the current study (eye with best corrected HC VA)

Note: The study eye is the eye with best corrected visual acuity. If corrected visual acuity is the same in both eyes then use the right eye as the study eye.

R [ ] L [ ]

E. Macular Pigment Optical Density Spatial Profile

Eye:_____ **Radiance value**

	0.25 deg			
Predicted CFF				
Actual CFF				**MPOD =**
	0.5 deg			
Predicted CFF				
Actual CFF				**MPOD =**
	1 deg			
Predicted CFF				
Actual CFF				**MPOD =**
	1.75 deg			
Predicted CFF				
Actual CFF				**MPOD =**
	7 deg			
Predicted CFF				
Actual CFF				**MPOD =**

Note: Please attach graph.

F. Diet Questionnaire

Note: Please attach complete dietary questionnaire.

G. Fundus photographs

Fundus images taken from:

Right eye	**Yes**	**No**
	1	**2**
Left eye	**Yes**	**No**
	1	**2**

Comments

Signature of person obtaining images: _____

6.4 APPENDIX 4: Certificate of venepuncture training

6.5 APPENDIX 5: Example of advertising poster

The Macular Pigment Research Group (MPRG) needs your help!

The MPRG is looking for **volunteers** to help prevent blindness!

The MPRG is carrying out a research study to investigate blood and eye responses to supplemental *meso*-zeaxanthin, lutein, and zeaxanthin (Macushield™).

Background:

- Age-related macular degeneration (AMD) is the commonest cause of blindness in people over 50 years of age in the western world
- There is a dietary pigment found at the back of the eye, called macular pigment, which is believed to be protective against the development of AMD
- Macular pigment is also important for one's quality of vision (e.g. visual performance and visual comfort)
- This research study will investigate if taking a commercially available dietary supplement (Macushield™) increases your macular pigment level and improves your quality of vision

Who can volunteer?

- Anybody between the age of 18 to 60 years

What's involved?

- You will be required to make four visits to the MPRG lab over a nine month period

- During a typical study visit, the MPRG will obtain information on the following:
  - General health and lifestyle: by questionnaire
  - Diet: by questionnaire
  - Macular pigment level: using a flickering light test
  - The health of the back of your eye: by taking a photograph
  - Blood concentrations of the macular pigments: by taking a blood sample

If you are between the age of 18-60 years, please call

Ms. Eithne Connolly at 051-845505 or email econnolly@wit.ie

6.6 APPENDIX 6: Food frequency questionnaire

FOOD FREQUENCY QUESTIONNAIRE

Macular Pigment Research Group (MPRG) Department of Chemical & Life Sciences Waterford Institute of Technology	
MPRG-SOP-008 Issue 1	Issued by: Dr. Edward Loane Reviewed by: Dr. John Nolan Issue Date: 26/07/2007

Familiarize yourself with the first two pages of the Scottish Collaborative Group Food Frequency Questionnaire (version 6.5) so that you can summarize the important points for your volunteer.

ADDITIONAL POINTS TO EMPHASIZE:

1. The questionnaire refers to the volunteer's diet over the previous 2-3 months **only**
2. Foods that were eaten at least once a week are recorded by circling the average number of days per week that the food was consumed, followed by the average number of measures consumed **per day**
3. Foods that were eaten *less than once a week, but at least once a month* are recorded by circling **M**, followed by the average number of measures consumed **for the entire month, rather than per day**
4. Foods that were eaten either *only once, twice, or never in the last 2-3 months* are recorded by circling **R.** In this case there is no need to circle a measure
5. If the volunteer makes a mistake, they should put an X through the incorrect option and circle the correct option
6. *An option must be circled for each and every question*
7. It is wise to guide the volunteer through the first 1-2 pages of the questionnaire to ensure that they have grasped the above points. The investigator should be available to answer any queries that the volunteer may have while filling out the questionnaire

8. Once the volunteer has completed the questionnaire, carefully look through every page to ensure that they have filled it out correctly and have not missed any questions

Scottish Collaborative Group Food Frequency Questionnaire version 6.5

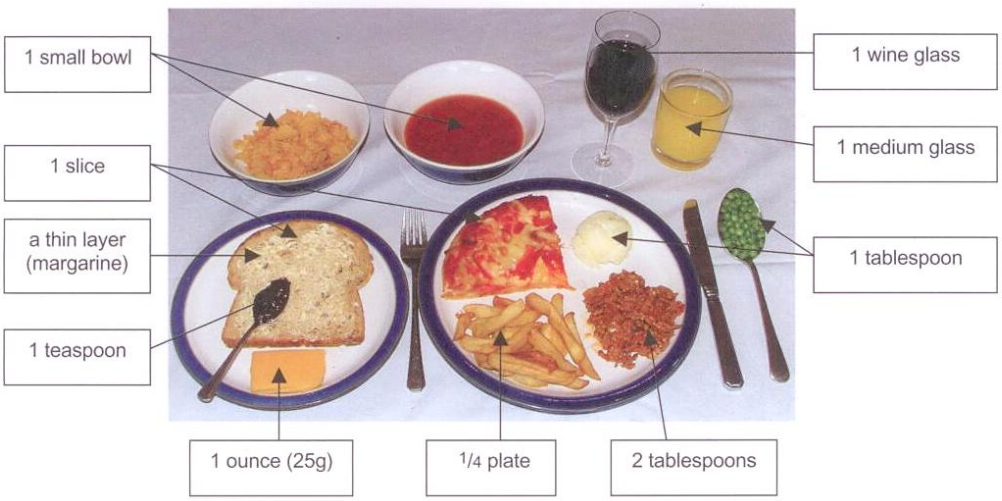
Diet Questionnaire

Thank-you for agreeing to complete this questionnaire.
It should take 20-30 minutes to complete.

Please take a few minutes to read the instructions carefully.

We would like you to describe your usual diet over the last 2-3 months. This should include all your main meals, snacks and drinks which you had at home or away from home e.g. at work, at restaurants or cafes and with friends and family.

The questionnaire lists 170 foods and drinks, and for each one a measure is given to help you estimate how much you usually have. The photograph below shows examples of some of these measures:



Please use **black or blue pen** to complete the questionnaire: do not use pencil.

How to complete the questionnaire

For **every line** in the questionnaire, we would like you to answer two things.

- **how much** of the food you had in a day you ate the food, and
- **how many** days a week you had the food.

To estimate **how much** of the food you had, you should circle a number under 'Measures per day'. Each food is described in common measures such as slices, glasses or tablespoons as illustrated in the photograph. *Please note that the measures are designed to be quite small, so your usual portion may easily be 2 or more measures.*

To estimate **how many** days a week you had the food, you should circle a letter or number under 'Number of days per week'.

- If you had the food less than once a month, you should circle **R** (for **R**arely or never). *For these foods you do not need to fill in the number of measures per day.*
- If you had the food more than once a month but less than once a week, you should circle **M** (for **M**onth).
- If you had the food on average 1-6 days a week, you should circle 1-6 as appropriate.
- If you had the food every day, you should circle 7.

The example below shows the answers for someone who had 4 slices of bread every day, 1 apple 5 days a week, 1/2 a plate of chips (i.e. two 1/4 plates) once or twice a month but rarely or never had tomato juice:

	Measure	Measures per day	Number of days per week
a) Bread (including toast & sandwiches)	1 medium slice	1 2 3 **(4)** 5+	R M 1 2 3 4 5 6 **(7)**
b) Apples	1 medium apple	**(1)** 2 3 4 5+	R M 1 2 3 4 **(5)** 6 7
c) Chips from a chip shop or restaurant	1/4 plate	~~1~~ **(2)** 3 4 5+	R **(M)** 1 2 3 4 5 6 7
d) Tomato juice	1/2 medium glass	1 2 3 4 5+	**(R)** M 1 2 3 4 5 6 7

If you want to change an answer, please put a **cross** through the wrong answer and circle the new answer (see example above).

If there are any foods or drinks that you eat regularly which do not appear on the questionnaire, please list them in section 20 ('other foods and drinks').

It is very important that you give an answer for every line.

If you rarely or never have a food, please make sure that you circle R.

1. Breads

		Measure	Measures per day	Number of days per week
a)	Bread (including toast & sandwiches)	1 medium slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Bread roll or bun	1 roll or bun	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Croissants, butteries or garlic bread	1 roll or 2 pieces	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Other breads (pitta, naan, soft tortillas)	1 pitta or 1/2 naan	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Which type(s) of bread do you usually eat? **Please tick one or more boxes.**	White ☐	Brown / granary ☐	Wholemeal ☐

2. Breakfast Cereals

		Measure	Measures per day	Number of days per week
a)	Cornflakes, Special K, Rice Krispies etc.	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Bran Flakes, Sultana Bran, All Bran etc.	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Shredded Wheat, Weetabix etc.	1 biscuit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Coco Pops, Frosties, Sugar Puffs, Crunchy Nut Cornflakes etc.	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Muesli (all types)	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f)	Porridge or Ready Brek	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7

3. Milk *(including milk on cereals and in drinks, but not in cooked foods)*

		Measure	Measures per day	Number of days per week
a)	Full fat milk	1/4 pint	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Semi-skimmed milk	1/4 pint	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Skimmed milk	1/4 pint	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Soya milk	1/4 pint	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Dried milk or creamer	1 teaspoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7

4. Cream and Yogurt

		Measure	Measures per day	Number of days per week
a)	Low fat yogurt (plain or fruit)	1 pot (125 ml)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Full fat yogurt (e.g. Greek)	1 pot (125 ml)	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

	Measure	Measures per day	Number of days per week
c) Low calorie yogurt (plain or fruit)	1 pot (125 ml)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d) Fromage frais (plain or fruit)	1 pot (125 ml)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e) Cream (all types)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7

5. *Cheese*

	Measure	Measures per day	Number of days per week
a) Full fat hard cheese (e.g. Cheddar, Gruyere, Wensleydale, Gouda)	1 oz. (25g) or 1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b) Medium fat cheese (e.g. (Edam, Brie, Camembert, Feta, cheese spreads)	1 oz. (25g) or 1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c) Full fat cream cheese (e.g. Philadelphia, Boursin, Danish Blue)	1 oz. (25g) or 1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d) Low fat cheese (e.g. low fat cream cheese, low fat hard cheese)	1 oz. (25g) or 1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e) Cottage cheese (all types)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7

6. *Eggs*

	Measure	Measures per day	Number of days per week
a) Boiled or poached eggs	1 egg	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b) Fried eggs	1 egg	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c) Scrambled eggs or omelette	1 egg	1 2 3 4 5+	R M 1 2 3 4 5 6 7

7. ***Meats*** *(Meat substitutes e.g. Quorn or soya are listed in section 10)*

	Measure	Measures per day	Number of days per week
a) Mince or meat sauce (e.g. bolognese)	2 tablespoons	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b) Sausages (pork, beef or frankfurters)	1 sausage	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c) Burgers (beef, lamb, chicken or turkey)	1 burger	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d) Beef (roast, grilled, casseroled or fried)	2 tablespoons, 2 slices or 1 steak	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e) Pork or lamb (roast, grilled, casseroled or fried)	2 tablespoons, 2 slices or 1 chop	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

		Measure	Measures per day	Number of days per week
f)	Chicken or turkey (roast, grilled, casseroled or fried)	1 wing or thigh, 1/2 breast or 2 slices	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g)	Bacon or gammon	1 medium slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h)	Liver, liver sausage or liver pate	1 serving	1 2 3 4 5+	R M 1 2 3 4 5 6 7
i)	Haggis or black pudding	2 tablespoons or 1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
j)	Meat or chicken pies, pasties or sausage roll	1 individual pie or 1 roll	1 2 3 4 5+	R M 1 2 3 4 5 6 7
k)	Cold meats (e.g. ham, corned beef, chicken roll)	1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
l)	Salami or continental sausage	1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7

8. *Fish*

		Measure	Measures per day	Number of days per week
a)	Fish fingers	1 finger	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	White fish (e.g. haddock, cod, plaice or scampi) fried or cooked in batter	1 small fillet or 1 serving	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Grilled, poached or baked white fish	1 small fillet	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Smoked white fish	1 small fillet	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Fish cakes, fish pie	1 cake or 2 tablespoons	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f)	Fried oily fish (e.g. salmon, herring, fresh tuna or mackerel)	1 small fillet	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g)	Grilled, poached or baked oily fish	1 small fillet	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h)	Smoked oily fish (kipper, mackerel or salmon)	1 small fillet or 1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
i)	Tinned salmon	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
j)	Tinned tuna	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
k)	Sardines, pilchards or rollmop herrings	2 small fish or 1 large fish	1 2 3 4 5+	R M 1 2 3 4 5 6 7
l)	Prawns, crab etc.	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
m)	Mussels, oysters, cockles, scallops	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

9. Potatoes, Rice and Pasta

		Measure	Measures per day	Number of days per week
a)	Boiled or baked potatoes	1 medium or 1/2 large	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Mashed potatoes	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Oven chips or potato waffles	1/4 plate or 1 waffle	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Home-cooked chips	1/4 plate	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Chips from a chip shop or restaurant	1/4 plate	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f)	Roast or fried potatoes	1/4 plate	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g)	White rice	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h)	Brown rice	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
i)	Pasta (all types) or couscous	1/4 plate	1 2 3 4 5+	R M 1 2 3 4 5 6 7
j)	Noodles (all types)	1/4 plate or 1 pot	1 2 3 4 5+	R M 1 2 3 4 5 6 7

10. Savoury foods, Soups and Sauces

		Measure	Measures per day	Number of days per week
a)	Pizza	1 slice or 1/2 a small pizza	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Quiche or savoury flan	1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Savoury pancakes	1 pancake	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Baked beans	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Nut roast, nut burgers or vegetable burgers	1 slice or burger	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f)	Quorn products (all types)	1 tablespoon, slice or sausage	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g)	Soya beans, TVP, Tofu or soya meat substitute	1 tablespoon or 1 sausage	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h)	Other beans (kidney, butter, chick peas)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
i)	Lentils (excluding soup)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
j)	Soups (home-made)	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
k)	Soups (tinned)	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
l)	Soups (dried or instant)	1 small bowl or mug	1 2 3 4 5+	R M 1 2 3 4 5 6 7
m)	Gravy	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

		Measure	Measures per day	Number of days per week
n)	Tomato -based sauces (e.g. for pasta)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
o)	Other savoury sauces (white, cheese etc.)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
p)	Bottled sauces (e.g. ketchup)	1/2 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
q)	Mayonnaise or salad cream	1 teaspoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
r)	Oil & vinegar dressing	1 teaspoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
s)	Pickled vegetables or chutneys	1 teaspoon or 1 pickle	1 2 3 4 5+	R M 1 2 3 4 5 6 7

11. ***Vegetables*** *(including fresh, frozen and tinned vegetables)*

		Measure	Measures per day	Number of days per week
a)	Mixed vegetable dishes (e.g. stir-fry, curry or bake)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Tinned vegetables (all kinds)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Peas or green beans	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Carrots	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Cabbage (all kinds)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f)	Brussels sprouts	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g)	Broccoli	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h)	Spinach or spring greens	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
i)	Leeks or courgettes	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
j)	Cauliflower, swede (neeps) or turnip	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
k)	Sweetcorn	1 tablespoon or 1 piece	1 2 3 4 5+	R M 1 2 3 4 5 6 7
l)	Onions	1 tablespoon or 1/2 onion	1 2 3 4 5+	R M 1 2 3 4 5 6 7
m)	Tomatoes	1/2 medium or 2 small	1 2 3 4 5+	R M 1 2 3 4 5 6 7
n)	Sweet peppers	1/4 pepper	1 2 3 4 5+	R M 1 2 3 4 5 6 7
o)	Other salad vegetables (lettuce, cucumber etc)	2 leaves or 4 slices	1 2 3 4 5+	R M 1 2 3 4 5 6 7
p)	Potato salad	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
q)	Coleslaw or other veg. salads in dressing	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

12. *Fruit* (including fresh, cooked, frozen and tinned fruits)

	Measure	Measures per day	Number of days per week
a) Fresh fruit salad	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b) Tinned fruit (all kinds)	1 tablespoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c) Apples	1 fruit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d) Bananas	1 fruit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e) Oranges, satsumas or grapefruit	1 small or 1/2 large fruit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f) Pears	1 fruit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g) Peaches or nectarines	1 fruit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h) Kiwi fruit	1 fruit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
i) Dried fruit (e.g. raisins, dates or figs)	1 tablespoon or 1 oz (25g)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
j) All other fruits (grapes, strawberries, melon etc)	1 tablespoon or 1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7

13. *Puddings*

	Measure	Measures per day	Number of days per week
a) Milk-based puddings (e.g. rice, semolina)	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b) Sponge puddings (e.g. (steamed, syrup, jam)	1 small bowl	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c) Gateau or cheesecake	1 slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d) Fruit-based puddings (e.g. pie, tart, crumble)	1 pie, 1 slice or 2 tablespoons	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e) Mousse, blancmange, trifle, meringue	2 tablespoons or 1 meringue	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f) Custard or other sweet sauces	2 tablespoons	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g) Wrapped ice creams (Cornetto, Solero, Magnum etc.)	1 ice cream	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h) Other ice cream (all flavours)	1 scoop or small tub	1 2 3 4 5+	R M 1 2 3 4 5 6 7

14. *Chocolates, Sweets, Nuts and Crisps*

	Measure	Measures per day	Number of days per week
a) Chocolate bars (e.g. Mars, Dairy Milk)	1 bar or 2 oz. (50g)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b) Chocolate sweets, toffees or fudge	2 sweets	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

		Measure	Measures per day	Number of days per week
c)	Boiled sweets, mints	2 sweets	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Fruit gums, pastilles, jellies or chewy sweets	2 sweets	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Salted nuts (peanuts, cashews etc.)	1 small packet or 1 oz. (25g)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f)	Unsalted nuts	1 small packet or 1 oz. (25g)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g)	Crisps	1 small bag (25g)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
h)	Reduced fat crisps	1 small bag (25g)	1 2 3 4 5+	R M 1 2 3 4 5 6 7
i)	Other savoury snacks (Quavers, tortilla chips, popcorn etc.)	1 small bag	1 2 3 4 5+	R M 1 2 3 4 5 6 7

15. Biscuits

		Measure	Measures per day	Number of days per week
a)	Plain (e.g. Rich Tea, digestive)	1 biscuit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Sweet (e.g. ginger, custard creams)	1 biscuit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Shortbread	1 biscuit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Chocolate coated biscuits	1 biscuit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Savoury biscuits, (crackers, crispbreads)	1 biscuit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
f)	Oatcakes	1 biscuit	1 2 3 4 5+	R M 1 2 3 4 5 6 7
g)	Cereal bars, flapjacks	1 bar or slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7

16. Cakes

		Measure	Measures per day	Number of days per week
a)	Plain cakes (sponge, madeira, ginger etc.)	1 medium slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Sponge cakes with jam, cream or icing	1 medium slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Fruit cakes (all kinds)	1 medium slice	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Pastries, doughnuts or muffins	1 piece	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Pancakes or scones	1 pancake or scone	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

17. Spreads and Sugar

		Measure	Measures per day	Number of days per week
a)	Jam, honey, or marmalade	1 teaspoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Yeast or meat extract (Marmite, Bovril etc.)	1/2 teaspoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Peanut butter or chocolate spread	1 teaspoon	1 2 3 4 5+	R M 1 2 3 4 5 6 7

d) How many teaspoons of table sugar did you use each day in drinks and on cereals or deserts? ☐☐
(If you did not use any table sugar, please enter 0).

e) Did you use any butter, margarine or other fat spread or oil on bread? Yes ☐ No ☐

If yes, please give full details of the one or two types you used most (e.g. Asda Sunflower buttery spread).
If you did not spread any fat or oil on bread, please go straight on to question g.

_____ Office Code ☐☐

_____ Office Code ☐☐

f) How much did you normally spread on one slice of bread? (**Please tick one answer).**
(an example of a thin layer is shown in the photograph on the front cover).

a scrape ☐ a thin layer ☐ a thick layer ☐

g) Did you use any fat or oil for home frying or cooking? Yes ☐ No ☐

If yes, please give full details of the one or two types you used most (e.g. Tesco Pure Vegetable Oil).
If you did not use any fat or oil for home frying or cooking, please go straight on to section 18.

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18. Beverages and Soft Drinks

		Measure	Measures per day	Number of days per week
a)	Tea (regular)	1 cup or mug	1 2 3 4 5+	R M 1 2 3 4 5 6 7
b)	Herbal, fruit or decaffeinated tea	1 cup or mug	1 2 3 4 5+	R M 1 2 3 4 5 6 7
c)	Instant coffee (regular)	1 cup or mug	1 2 3 4 5+	R M 1 2 3 4 5 6 7
d)	Decaffeinated coffee	1 cup or mug	1 2 3 4 5+	R M 1 2 3 4 5 6 7
e)	Filter, espresso or cappuccino coffee	1 cup or mug	1 2 3 4 5+	R M 1 2 3 4 5 6 7

Please make sure you have given an answer for every line before leaving this page

		Measure	Measures per day					Number of days per week							
f)	Pure fruit juice (orange, apple, etc.)	1/2 medium glass	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
g)	Tomato juice	1/2 medium glass	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
h)	Blackcurrant squash (e.g. Ribena)	1 medium glass	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
i)	Other fruit squash	1 medium glass	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
j)	Diet fizzy drinks (Cola, lemonade etc.)	1 can	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
k)	Regular fizzy drinks	1 can	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
l)	Mineral water	1 medium glass	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
m)	Tap water (not in other drinks)	1 medium glass	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
n)	Hot chocolate	1 cup or mug	1	2	3	4	5+	R	M	1	2	3	4	5	6	7
o)	Horlicks or Ovaltine	1 cup or mug	1	2	3	4	5+	R	M	1	2	3	4	5	6	7

19. Alcoholic Drinks

Please estimate your average intake of alcohol over the last 2-3 months. If your intake varied from week to week, please try to give an overall estimate which allows for weeks with high or low intake. If you had less than one measure a week on average, please circle 0.

	Drink	Measure	Number of measures per week								
a)	Low alcohol lager or beer	1/2 pint	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
b)	Dark beer (Export, bitter or stout)	1/2 pint	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
c)	Light beer (lager or continental beers)	1/2 pint	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
d)	White wine	1 wine glass	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
e)	Red wine	1 wine glass	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
f)	Sherry, port etc.	1 sherry glass	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
g)	Spirits or liqueurs	1 pub measure	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
h)	Alcopops (e.g. Bacardi Breezer)	1 bottle	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+
i)	Cider	1 bottle or 1/2 pint	0	1-2	3-4	5-9	10-14	15-19	20-29	30-39	40+

Please make sure you have given an answer for every line before leaving this page

20. Other Foods and Drinks

Please enter details of any foods or drinks which you had **more than once a week** in the last 2-3 months which you have not included in the questionnaire above. If you do not want to add any foods, please leave this section blank and go to section 21.

	Food description	Measure	Measures per day	Number of days per week
a)	_____	_____	1 2 3 4 5+	1 2 3 4 5 6 7
b)	_____	_____	1 2 3 4 5+	1 2 3 4 5 6 7
c)	_____	_____	1 2 3 4 5+	1 2 3 4 5 6 7
d)	_____	_____	1 2 3 4 5+	1 2 3 4 5 6 7

21. Vitamin, Mineral and Food Supplements

Please give details and brand name of any supplements (e.g. multivitamins, iron tablets, cod liver oil, evening primrose oil, Complan, wheatgerm, bran) which you took in the last 2-3 months.

	Supplement type	Measure	Measures per day	Number of days per week
a)	_____	_____	1 2 3 4 5+	R M 1 2 3 4 5 6 7
	Brand name and details_____			
b)	_____	_____	1 2 3 4 5+	R M 1 2 3 4 5 6 7
	Brand name and details_____			
c)	_____	_____	1 2 3 4 5+	R M 1 2 3 4 5 6 7
	Brand name and details_____			
d)	_____	_____	1 2 3 4 5+	R M 1 2 3 4 5 6 7
	Brand name and details_____			

22. Other Information

Any other information or comments on your diet in the last 2-3 months

Date of completing the questionnaire _____

Thank-you very much for completing this questionnaire.

Please return it to the investigators as requested.

6.7 APPENDIX 7: HUMAN PHLEBOTOMY standard operating procedure

Macular Pigment Research Group (MPRG) Department of Chemical & Life Sciences Waterford Institute of Technology	
MPRG-SOP-009 Issue 2	Issued by: Dr. Edward Loane Updated by: Eithne Connolly Reviewed by: Dr. John Nolan Issue Date: 08/01/2011

1. REQUIREMENTS

A. Alcohol swabs
B. Sharps disposal container
C. Tourniquet
D. Vacutainer® needle system (Becton Dickinson Vacutainer™ Systems)
    i. Vacutainer® (BD, Plymouth, U.K.)
    ii. Sterile needles (preferably BD Vacutainer® Flashback Blood Collection Needles, 21G; Ref. 301746; BD, Plymouth, U.K.)
E. Blood tubes
    i. BD Vacutainer® SST™ II Advance Ref. 367958 8.5 ml
F. Cotton wool
G. Tape

2. PREPARATION

A. Select appropriate blood tubes and quantities of each
B. Invite your volunteer to sit comfortably in one of the armchairs and enquire if they have a 'good arm' for taking blood from. This is often the case
C. Appear confident! Being well organized and mentally visualizing the steps involved will promote your own confidence! Idle chit-chat may also help to relax your volunteer
D. Prepare a small ball of cotton wool and a length of tape, approximately 10cm long

3. PROCEDURE

A. Apply the tourniquet above the elbow of the selected arm and adjust so that it is tight, but not uncomfortable
B. **Clean your hands thoroughly using the handwashing technique set out by the Health Executive Authority**
C. Feel for an appropriate vein in the volunteer's ante-cubital fossa. An appropriate vein will feel firm but compressible compared to surrounding tissues. Remember that the vein you will select to take blood from will generally NOT be one of the visible superficial veins; i.e. 'go for the vein you can feel, not the vein you can see'. This rule will occasionally be broken, and only experience will guide this decision
D. **Clean the skin thoroughly overlying the selected vein with an alcohol swab**
E. Feel again for the selected vein and make note of the direction in which it is passing
F. Close the port on the end of the vacutainer by pressing the white tab
G. Take a sterile needle and twist off the end to reveal a narrow grey rubber tube that covers one end of the needle. Attach this end of the sterile needle to the vacutainer by screwing it on. **Be very careful not to touch the grey tubing**
H. Ask your volunteer to look away and tell them that you will let them know when there will be a sharp pinch. Remind them that they should hold their arm very steady and not jump
I. Remove the other end of the sterile needle to reveal a bare needle. **Make sure not to touch the needle!**
J. Ensure that the bevel (or 'opening') of the needle is facing away from the volunteer's arm before inserting the needle
K. Stabilise the distal end (the side closer to your volunteer's hand) of the vein with the thumb of your left hand (for right-handed phlebotomists), by gently drawing the skin back towards your volunteer's hand
L. Holding the plastic vacutainer with your right hand, smoothly and firmly insert the needle through the skin overlying the selected vein at an angle of approximately 30 degrees to the skin surface. Don't forget to remind your volunteer that they will feel a sharp pinch!
M. Watch the clear plastic part at the base of the needle for the 'flash-back' (blood entering this part of the needle). Once this happens, stop advancing the needle and hold it precisely in this position with your left hand (having switched hands at this point). One may also appreciate a slight 'give' once the vein has been correctly entered

N. Apply the tourniquet above the elbow of the selected arm and adjust so that it is tight, but not uncomfortable
O. **Clean your hands thoroughly using the handwashing technique set out by the Health Executive Authority**
P. Feel for an appropriate vein in the volunteer's ante-cubital fossa. An appropriate vein will feel firm but compressible compared to surrounding tissues. Remember that the vein you will select to take blood from will generally NOT be one of the visible superficial veins; i.e. 'go for the vein you can feel, not the vein you can see'. This rule will occasionally be broken, and only experience will guide this decision
Q. **Clean the skin thoroughly overlying the selected vein with an alcohol swab**
R. Feel again for the selected vein and make note of the direction in which it is passing
S. Close the port on the end of the vacutainer by pressing the white tab
T. Take a sterile needle and twist off the end to reveal a narrow grey rubber tube that covers one end of the needle. Attach this end of the sterile needle to the vacutainer by screwing it on. **Be very careful not to touch the grey tubing**
U. Ask your volunteer to look away and tell them that you will let them know when there will be a sharp pinch. Remind them that they should hold their arm very steady and not jump
V. Remove the other end of the sterile needle to reveal a bare needle. **Make sure not to touch the needle!**
W. Ensure that the bevel (or 'opening') of the needle is facing away from the volunteer's arm before inserting the needle
X. Stabilise the distal end (the side closer to your volunteer's hand) of the vein with the thumb of your left hand (for right-handed phlebotomists), by gently drawing the skin back towards your volunteer's hand
Y. Holding the plastic vacutainer with your right hand, smoothly and firmly insert the needle through the skin overlying the selected vein at an angle of approximately 30 degrees to the skin surface. Don't forget to remind your volunteer that they will feel a sharp pinch!
Z. Watch the clear plastic part at the base of the needle for the 'flash-back' (blood entering this part of the needle). Once this happens, stop advancing the needle and hold it precisely in this position with your left hand (having switched hands at this

point). One may also appreciate a slight 'give' once the vein has been correctly entered

AA. Click-on each blood tube into the plastic vacutainer, by pushing the coloured end of each tube onto the needle that is covered by the grey tubing within the vacutainer

BB. Each blood tube should fill automatically. Allow this to happen to the required amount

CC. Once the required number of tubes have been filled, **RELEASE THE TOURNIQUET**

DD. Gently rest a ball of cotton wool over the end of the needle where it enters your volunteer's skin, and then quickly and smoothly remove the needle and immediately apply firm pressure with your thumb over the ball of cotton wool

EE. Ask your volunteer to take over from you in applying pressure over the cotton wool

FF. Immediately dispose of the needle into the sharps disposal container by pressing the green tab on the vacutainer, releasing the needle

GG. Tape down the ball of cotton wool onto your volunteer's arm and encourage them to continue applying pressure for at least one minute. This will minimize any potential bruising

HH. Ensure that your volunteer feels ok, and then give yourself a mental 'pat on the back'!

6.8 APPENDIX 8: Standard operating procedure for measuring macular pigment optical density

MZ protocol for measuring macular pigment optical density using the Macular Densitometer™ II

INSTRUMENT PREPARATION

1. Perform a complete visual inspection of the Macular Densitometer™ II to ensure that all connections are in place and the instrument is switched on (switch at rear of main control unit)
2. Turn the dial on the right-hand side of the control unit to position 1

SUBJECT PREPARATION

1. Have the subject sit in the chair in front of the instrument (subject should wear their distance corrective lenses, if required). Note that reading glasses should not be worn during testing. If bifocals or varifocals are worn, the subject should view the targets through the upper part of their glasses (i.e. the part through which they would normally look during distance vision)
2. Have the subject place their test eye (the eye with best visual acuity, or else their right eye if visual acuity is equal in both eyes) at the viewing eyepiece and ensure that the tilt of the main unit allows comfortable viewing for the subject (i.e. the Densitometer™ II is at the correct height for the subject)
3.

SETTING THE FLICKER FREQUENCY (FF) FOR TESTING

1. Turn the dial on the left-hand side of the control unit to the appropriate flicker frequency setting for the subject's age (see table below). For example, to begin macular pigment measurement on a 65 year old subject measuring at target 1, the flicker frequency should be set to 13. Please see frequency table for appropriate frequency setting for each target/age

Age	FF at Target 1
18-30	19
31-40	18
41-50	16
50-60	14
61-70	13
71-80	12
81+	11

MACULAR PIGMENT OPTICAL DENSITY TESTING

Examiner to subject: We are now ready to measure your macular pigment. There are five parts to this test. The first part requires you to look at the small black dot on the center of the target (see Figure 1 on laminated card). You may continue to blink normally throughout the test, but it is essential that you keep focused on the small black dot. I will adjust the settings on the instrument and I want you to tell me when the flickering of the target surrounding the small black dot appears to stop. I will take six separate readings at the first target. You may cover your eye, which is not being tested, with your hand if you wish

1. Dim the room lights
2. Set the radiance to its starting position by pushing and holding the left button on the radiance control box until the radiance reaches its lowest value (~ 9) and ensure that the dial on the right-hand side of the control unit is set to position 1. Set the flicker frequency to the appropriate setting 5(see table above)
3. Ensure that the series controller is reset prior to testing (i.e. when you look into the optical unit you will see the smallest target (see figure 1)
4. The examiner presses and holds the right-hand button until the subject reports that there is no flicker and the examiner releases the button
5. Record the radiance value in the log sheet low (top right corner of the control unit)

6. Set the radiance to its new starting position by pushing and holding the right button on the radiance control box until the radiance reaches its highest value (~ 3000) and ensure that the dial on the right-hand side of the control unit is set to position 1.
7. The examiner presses and holds the left-hand button until the subject reports that there is no flicker and the examiner releases the button
8. Record the radiance value in the log sheet high (top right corner of the control unit)
9. Repeat the steps 2-7 two more times for target 1
10. Repeat all of the above for targets 2, 3 and 4 as required. Please note, that the required targets are selected using the series control by pressing the red button (see figures as appropriate)

Age	FF at Target 5
20-18	13
30-20	12
40-30	11
40-50	10
50-60	9
61-70	8
71-80	7
81+	6

***Examiner to subject:** Well done, we have completed the first part(s) of this test! Now we will take readings with the last target.*

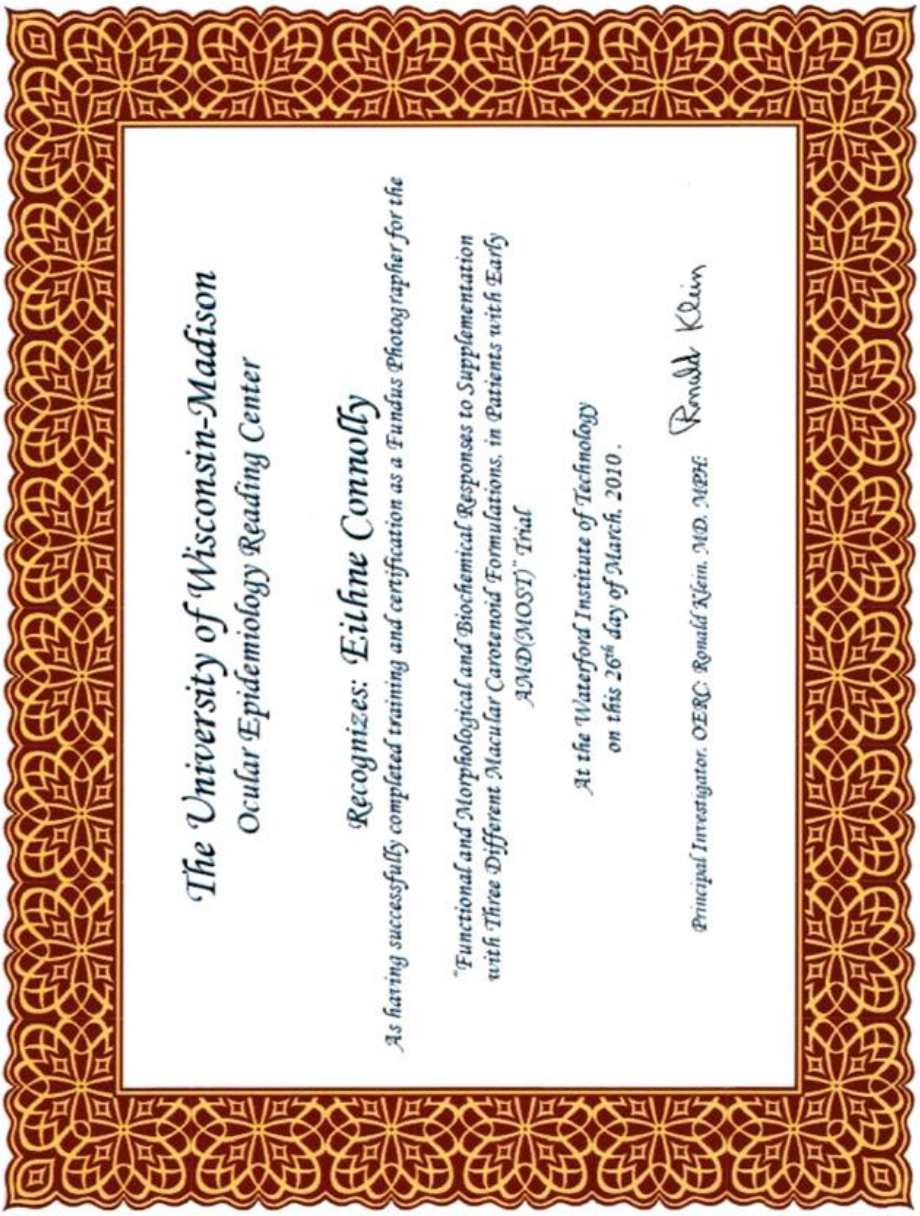
For this target, you are required to look at a small red fixation dot to the left of the flickering disc-shaped target (see Figure 5 on laminated card). You may continue to blink normally throughout the test, but it is essential that you keep focused on the small red fixation dot. I will adjust the settings on the instrument and I want you to tell me when the test disc appears to stop flickering. It is essential that you do not look at the flickering disc at any point for target 5, but keep fixated on the red fixation dot throughout. I will take six separate readings at this target also. You may cover your eye, which is not being tested, with your hand if you wish.

11. Dim the room lights
12. Set the radiance to its starting position by pushing and holding the left button on the radiance control box until the radiance reaches its lowest value (~ 9) and ensure that the dial on the right-hand side of the control unit is set to position 2. Set the flicker frequency to the appropriate setting (see table above)
13. Select target 5 by pressing the red button on the series controller (see figure 5)
14. The examiner presses and holds the right-hand button until the subject reports that there is no flicker and the examiner releases the button
15. Record the radiance value in the log sheet low (top right corner of the control unit)
16. Set the radiance to its new starting position by pushing and holding the right button on the radiance control box until the radiance reaches its highest value (~ 3000) and ensure that the dial on the right-hand side of the control unit is set to position 1.
17. The examiner presses and holds the left-hand button until the subject reports that there is no flicker and the examiner releases the button
18. Record the radiance value in the log sheet high (top right corner of the control unit)
19. Repeat the steps 2-7 two more times for target 5

POINTS TO NOTE

1. Record which eye is tested
2. Throughout testing, reassure the subject that they are doing the test correctly, and repeat instructions to the subject, as required
3. Do not rush the subject
4. Remind the subject to blink normally (about once every 3-4 seconds) throughout the test
5. If the subject reports that there is no null flicker zone, increase the flicker frequency by two Hz (using the dial on the left-hand side of the control unit) and proceed with macular pigment measurement. Repeat this step if necessary
6. If the experimenter identifies a very wide null zone (e.g. > 20% of mean) decrease the flicker frequency by two Hz (using the dial on the left-hand side of the control unit) and proceed with macular pigment measurement. Repeat this step if necessary
7. The rubber end of the eyepiece may be cleaned with an alcohol wipe between each subject. Avoid wiping the lens of the eyepiece, as this may leave streaks on the lens obscuring the subject's view

6.9 APPENDIX 9: Certificate for stereo fundus photography



The University of Wisconsin-Madison
Ocular Epidemiology Reading Center

Recognizes: Eilhme Connolly

As having successfully completed training and certification as a Fundus Photographer for the

"Functional and Morphological and Biochemical Responses to Supplementation with Three Different Macular Carotenoid Formulations, in Patients with Early AMD(MOST)" Trial

At the Waterford Institute of Technology
on this 26th day of March, 2010.

Principal Investigator, OERC: Ronald Klein, MD, MPH: Ronald Klein

6.10 APPENDIX 10: Peer-reviewed publication

Clinical Trials

Supplementation with All Three Macular Carotenoids: Response, Stability, and Safety

Eibne E. Connolly,[1,2] Stephen Beatty,[1,2] James Loughman,[2,3,4] Alan N. Howard,[5,6] Michael S. Louw,[7] and John M. Nolan[1,2]

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

Methods. Forty-four 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 with customized heterochromatic flicker photometry (cHFP), and serum concentrations of L and Z were quantified by using high performance liquid chromatography (HPLC). Subjects were assessed at baseline and at 3 and 6 months. Clinical pathology analysis was performed at baseline and 6 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°: $P = 0.001$; 0.5°: $P = 0.001$), 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 before supplementation.

Conclusions. Subjects supplemented with MZ, L, and Z exhibited significant increases in serum concentrations of these carotenoids and a subsequent increase in central MPOD. Pathology analysis suggested no adverse clinical implications of consuming these carotenoids. (http://isrctn.org number, ISRCTN60816411.) (*Invest Ophthalmol Vis Sci.* 2011;52:9207–9217) DOI:10.1167/iovs.11-8025

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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 has been more recently referred to as macular pigment (MP).[1] Interestingly, of the more than 700 carotenoids identified in nature, these three dietary carotenoids selectively accumulate at the macula,[1–3] indicating an exquisite degree of biological selectivity in this retinal tissue.

An average Western diet contains 1.3 to 3 mg/d of L and Z combined,[4] with substantially more L than Z (represented by an estimated ratio of ~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.[5] 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, despite 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 isomerization of retinal L at the macula.[6]

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.[7] It is now accepted that AMD is the result of (photo) oxidation-induced retinal injury. However, the anatomic (central retinal),[8] biochemical (antioxidant),[9] and optical (short wavelength–filtering)[10] properties of MP suggest that this pigment may confer protection against AMD (protective hypothesis).[11] 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).[12] 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 to (in blood and at the macula) and safety of 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 analyzing blood samples for changes in renal and liver function, as well as lipid profile, hematologic profile, and markers of inflammation after 6 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 Register. All subjects signed an informed consent document, and the experimental

6.11 APPENDIX 11: Peer-reviewed publication

Retina

A Central Dip in the Macular Pigment Spatial Profile Is Associated with Age and Smoking

Mark L. Kirby, Stephen Beatty, Edward Loane, Mukunda C. Akkali, Eithne E. Connolly, Jim Stack, and John M. Nolan

Purpose. To investigate the relationship between specific macular pigment (MP) spatial profiles and risk factors for age-related macular degeneration (AMD).

Methods. The MP spatial profile of 484 healthy subjects was measured with customized heterochromatic flicker photometry (cHFP) and categorized into one of two profile types: typical exponential or atypical "central dip." Data on risk factors for AMD were obtained with a general health and lifestyle questionnaire. Dietary and serum concentrations of lutein (L) and zeaxanthin (Z) were also assessed.

Results. The presence of the central dip MP spatial profile was significantly more common in older subjects (the mean ± SD age of subjects with a central dip MP spatial profile was 46.9 ± 12 years, whereas the mean age of subjects with a typical MP spatial profile was 41.8 ± 12 years; $P = 0.004$) and in current cigarette smokers ($P = 0.031$). Also, there was a significant age-related decline in central MP optical density (MPOD; 0.25° retinal eccentricity), but in the men only ($r = -0.146$, $P = 0.049$).

Conclusions. A central dip in the MP spatial profile, seen in older subjects and in cigarette smokers, may represent an undesirable feature of macular pigmentation. Further research is needed in this area. (*Invest Ophthalmol Vis Sci.* 2010;51: 6722–6728) DOI:10.1167/iovs.10-5344

The macula contains the highest density of cone photoreceptors in the retina and is responsible for detailed central color vision.[1] Age-related macular degeneration (AMD) is the leading cause of age-related blindness in the developed world.[2,3] Increasing age, family history of AMD, and cigarette smoking[4–6] are the three major risk factors for AMD; other putative risk factors include being of the female sex, obesity, light iris color, low dietary intake and low serum concentrations of lutein (L) and zeaxanthin (Z), and low macular pigment optical density (MPOD).[7–9]

At the macula, the carotenoids L, Z, and *meso*-Z (generated from retinal L) accumulate at high concentrations (to the exclusion of all other carotenoids) and are collectively referred to as macular pigment (MP).[10–12] MP is a short-wavelength (blue) light filter[1] and a powerful antioxidant[11] and is therefore believed to protect against AMD.[13,14] Consistent with the suggested protection that MP may afford against AMD, a recent study has shown that risk factors for AMD (including the three established risk factors: increasing age, family history of AMD, and cigarette smoking) are associated with a relative lack of MP[7]; however, the relationship between the spatial profile of MP and risk factors for this disease, if any, is not yet known.

To date, studies investigating the spatial profile of MP have reported its distribution as a first-order exponential decline with increasing retinal eccentricity.[15–18] However, variations in its distribution have been reported.[15,16,19] Recently, it has been shown that atypical MP spatial profiles are reproducible, when measured with customized heterochromatic flicker photometry (cHFP).[19]

The importance of such variations, if any, in the spatial profile of MP (e.g., the presence of a central dip) is not yet known, but may be related to the putative protective role of this pigment. For example, reduced MPOD at the center of the macula (i.e., the presence of a central dip) may be associated with increased risk of AMD (given the lower antioxidant activity and short-wavelength light-filtering capacity of the affected individual, when compared with an individual without such a central dip). Indeed, and consistent with this hypothesis, Trieschmann et al.,[20] in a study of 400 subjects (253 with signs of early AMD, 147 without AMD), reported that eyes with AMD are more likely to display low central MPOD when compared with non-AMD eyes.

It appears that L, Z, and *meso*-Z play central roles in the macula; however, determinants of their concentration and factors that influence their spatial distribution remain unclear. The purpose of this study was to investigate the association, if any, between the MP spatial profile and established (and putative) risk factors for AMD.

Methods

Subjects

Four hundred eighty-four subjects were recruited for this single-visit study. The subjects were recruited by a local poster campaign, by word of mouth in the college community, and by invitation. The study was approved by the Research Ethics Committee of Waterford Institute of Technology. The subjects were required to sign an informed-consent document before participating, and all experimental procedures adhered to the tenets of the Declaration of Helsinki.

Inclusion criteria for participation were as follows: Caucasian race, age between 18 and 70 years, no evidence of ocular disease, visual acuity 6/12 or better in the study eye, and no current consumption of L and/or Z dietary supplements.

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Investigative Ophthalmology & Visual Science, December 2010, Vol. 51, No. 12

6.12 APPENDIX 12: Peer-reviewed publication

Augmentation of Macular Pigment following Implantation of Blue Light–Filtering Intraocular Lenses at the Time of Cataract Surgery

John M. Nolan,[1] Philip O'Reilly,[1] James Loughman,[2] Jim Stack,[1] Edward Loane,[1] Eithne Connolly,[1] and Stephen Beatty[1]

PURPOSE. (Photo)-oxidative stress is believed to play a role in the pathogenesis of age-related macular degeneration (AMD), with the threshold for retinal damage being lowest for short-wavelength (blue) light. Macular pigment (MP), consisting of the carotenoids lutein (L), zeaxanthin (Z) and *meso*-Z, has a maximum absorption at 460 nm and protects the retina from (photo)-oxidative injury. This study was designed to investigate whether the blue light–filtering properties of the Alcon AcrySof Natural intraocular lens (ANIOL) implanted during cataract surgery affects MP optical density (MPOD).

METHODS. Forty-two patients scheduled for cataract surgery were recruited for the study. These patients all had a preoperative best corrected visual acuity rating (BCVAR) of at least 0.5 (logMAR) in the study eye. The patients were randomized to have either the standard Alcon AcrySof three-piece acrylic intraocular lens (AIOL) (controls) or the ANIOL implanted at the time of cataract surgery. The spatial profile of MPOD (i.e., at 0.25°, 0.5°, 1.0°, and 1.75° eccentricity) was measured with customized heterochromatic flicker photometry (cHFP) 1 week before and 1 week after surgery, and at 3, 6, and 12 months after surgery. Serum concentrations of L and Z were also measured at each study visit.

RESULTS. There was a highly significant and positive correlation between all MPODs (e.g., at 0.25°) recorded 1 week before and after surgery in eyes with an AIOL implant ($r = 0.915$, $P < 0.01$; paired samples *t*-test, $P = 0.631$) and in those ANIOL implants ($r = 0.868$, $P < 0.01$; paired samples *t*-test, $P = 0.719$). Average MPOD across the retina increased significantly with time (after 3 months) in the ANIOL group (repeated-measures, general linear model, $P < 0.05$), but remained stable in the AIOL group (repeated-measures, general linear model, $P > 0.05$). There were no significant time or lens effects observed for serum L over the study period ($P > 0.05$). There was a significant time effect for serum Z over the study period ($P < 0.05$), but not a significant time/lens interaction ($P > 0.05$).

CONCLUSIONS. Customized HFP can reliably measure the MPOD spatial profile in the presence of lens opacity, and cataract surgery does not artifactually alter MPOD readings. This study also provides evidence that implanting an IOL that filters blue light is associated with augmentation of MPOD in the absence of raised serum concentrations of L and Z. However, further and longitudinal study is needed to assess whether the observed increase in MPOD after implantation of blue-filtering IOLs is associated with reduced risk of AMD development and/or progression. (*Invest Ophthalmol Vis Sci.* 2009;50:4777–4785) DOI:10.1167/iovs.08-3277

Age-related macular degeneration (AMD), which damages central vision, is the most common cause of age-related blindness in the western world.[1,2] Although the pathogenesis of AMD remains unclear, there is a growing body of evidence suggesting that oxidative stress is important in the pathogenesis of this condition and that cumulative short-wavelength (blue) light damage plays a role.[3–5]

Macular pigment (MP), which is entirely of dietary origin, and composed of the xanthophyll carotenoids: lutein (L), zeaxanthin (Z), and *meso*-Z, is thought to protect against AMD because it absorbs short-wavelength (blue) light at a preretinal level and because of its antioxidant properties.[6,7] The absorption spectrum of MP peaks at 460 nm and may therefore limit photo-oxidative damage to retinal cells.[8] MP levels are maximum within the photoreceptor axons of the foveola and the plexiform layers of the macula.[6,9] Of importance, both the absorptive characteristics of MP and its location in the anterior portion of individual photoreceptors enables the pigment to attenuate the amount of blue light incident on the photoreceptor.

It has been hypothesized that cataracts provide protection against AMD by absorbing blue light, and thus reducing photo-oxidative damage to the retina.[10] However, this protective effect, if any, would be restricted to certain types of lens opacity, such as nuclear sclerosis. In contrast, however, some studies have shown increased risk of cataract in association with AMD, which may reflect the fact that these conditions share antecedents (such as age).[11–13] The positive association of AMD and cataract is considered to be an effect of similar causation and risk factors of both disorders. Although some studies have failed to find a link between cumulative sunlight exposure and the risk of development of AMD,[14–16] many other studies have found a positive association between lifetime exposure to sunlight and AMD.[17–20] Recently, the age-related maculopathy and macular degeneration in elderly European populations (EUREYE) study has provided evidence of a link between cumulative (lifetime) sunlight exposure (in the presence of low antioxidant levels) and the risk of AMD.[21] Those individuals with high cumulative lifetime exposure to sunlight but who were in the lowest quartile for combined antioxidant levels (especially vitamin C, zeaxanthin, vitamin E

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6.13 APPENDIX 13: Peer-reviewed publication

Original Paper

Ophthalmic Research

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Macular Pigment Optical Density in an Ageing Irish Population: The Irish Longitudinal Study on Ageing

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James Loughman[d] Eithne E. Connolly[a, b] Patricia Kearney[c] Edward Loane[a]
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Key Words
Age-related macular degeneration · Irish Longitudinal Study on Ageing · Macular pigment optical density

Abstract
Purpose: The 3 carotenoids lutein, zeaxanthin, and *meso*-zeaxanthin, which account for the 'yellow spot' at the macula and which are referred to as macular pigment (MP), are believed to play a role in visual function and protect against age-related macular degeneration (AMD) via their optical and antioxidant properties. This study was undertaken to compare MP optical density (MPOD) in a population aged ≥50 years with MPOD values from a normative database of subjects aged 18–60 years. ***Methods:*** Seventy-nine subjects were recruited into this pilot study (The Irish Longitudinal Study on Ageing-TILDA). MPOD was measured using heterochromatic flicker photometry. Retinal fundus photographs, lifestyle data and general health data, were also obtained. ***Results:*** The mean ± SD age of the 79 subjects recruited into this study was 65 ± 11 years. There was a moderate, but statistically significant, age-related decline in MPOD at 0.5° in the TILDA data ($r = -0.251$, $p = 0.045$), which remained upon merging with a normative database of an additional 462 subjects aged between 18 and 67 years ($r = -0.179$, $p = 0.000$). ***Conclusions:*** We report an inverse association between MPOD and increasing age. Longitudinal data in a larger cohort of participants are required to satisfactorily investigate the relationship between the optical density of this pigment and age, and with risk for development and/or progression of AMD. This pilot study represents a first step in this endeavour.

Introduction

Age-related macular degeneration (AMD) is the advanced form of age-related maculopathy (ARM), and is the leading cause of blindness in people over 50 years of age in the developed world [1, 2]. The number of adults registered blind as a result of AMD in industrialized countries continues to rise, primarily due to increasing longevity [3, 4]. Beyond its inevitable impact on the individual sufferer, AMD poses a growing socioeconomic challenge to modern society [5–7].

Three dietary carotenoids, lutein, zeaxanthin and *meso*-zeaxanthin, accumulate at the macula, where they are collectively referred to as macular pigment (MP). Lutein and zeaxanthin are present in many foods, whereas *meso*-zeaxanthin is not found in a conventional diet, al-

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6.14 APPENDIX 14: Peer-reviewed publication

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ORIGINAL ARTICLE

Augmentation of Macular Pigment Following Supplementation with All Three Macular Carotenoids: An Exploratory Study

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ABSTRACT

Purpose: At the macula, the carotenoids *meso*-zeaxanthin (MZ), lutein (L), and zeaxanthin (Z) are collectively referred to as macular pigment (MP). This study was designed to measure serum and macular responses to a macular carotenoid formulation.

Materials and Methods: Ten subjects were recruited into this study (five normal and five with early age-related macular degeneration [AMD]). Subjects were instructed to consume a formulation containing 7.3 mg of MZ, 3.7 mg of L, and 0.8 mg of Z everyday over an eight-week period. The spatial profile of MP optical density (i.e., MPOD at 0.25°, 0.5°, 1°, and 1.75°) was measured using customized heterochromatic flicker photometry, and a blood sample was collected at each study visit in order to analyze serum concentrations of MZ, L, and Z.

Results: There was a significant increase in serum concentrations of MZ and L after two weeks of supplementation ($p < 0.05$). Baseline serum carotenoid analysis detected a small peak eluting at the same time as MZ in all subjects, with a mean ± SD of 0.02 ± 0.01 μmol/L. We report significant increases in MPOD at 0.25°, 0.5°, 1°, and average MPOD across its spatial profile after just two weeks of supplementation ($p < 0.05$, for all). Four subjects (one normal and three AMD) who had an atypical MPOD spatial profile (i.e., central dip) at baseline had the more typical MPOD spatial profile (i.e., highest MPOD at the center) after eight weeks of supplementation.

Conclusion: We report significant increases in serum concentrations of MZ and L following supplementation with MZ, L, and Z and a significant increase in MPOD, including its spatial profile, after two weeks of supplementation. Also, this study has detected the possible presence of MZ in human serum pre-supplementation and the ability of the study carotenoid formulation to rebuild central MPOD in subjects who have atypical profiles at baseline.

KEYWORDS: Age-related macular degeneration; Lutein; Macular pigment; Meso-zeaxanthin; Supplementation

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INTRODUCTION

Age-related macular degeneration (AMD) is an eye disease that affects the central part of the retina called the macula and in its late form, results in loss of central vision. Late AMD is the most common cause of

6.15 APPENDIX 15: Peer-reviewed publication

Augmentation of Macular Pigment following Implantation of Blue Light–Filtering Intraocular Lenses at the Time of Cataract Surgery

John M. Nolan,[1] Philip O'Reilly,[1] James Loughman,[2] Jim Stack,[1] Edward Loane,[1] Eithne Connolly,[1] and Stephen Beatty[1]

Purpose. (Photo)-oxidative stress is believed to play a role in the pathogenesis of age-related macular degeneration (AMD), with the threshold for retinal damage being lowest for short-wavelength (blue) light. Macular pigment (MP), consisting of the carotenoids lutein (L), zeaxanthin (Z) and *meso*-Z, has a maximum absorption at 460 nm and protects the retina from (photo)-oxidative injury. This study was designed to investigate whether the blue light–filtering properties of the Alcon AcrySof Natural intraocular lens (ANIOL) implanted during cataract surgery affects MP optical density (MPOD).

Methods. Forty-two patients scheduled for cataract surgery were recruited for the study. These patients all had a preoperative best corrected visual acuity rating (BCVAR) of at least 0.5 (logMAR) in the study eye. The patients were randomized to have either the standard Alcon AcrySof three-piece acrylic intraocular lens (AIOL) (controls) or the ANIOL implanted at the time of cataract surgery. The spatial profile of MPOD (i.e., at 0.25°, 0.5°, 1.0°, and 1.75° eccentricity) was measured with customized heterochromatic flicker photometry (cHFP) 1 week before and 1 week after surgery, and at 3, 6, and 12 months after surgery. Serum concentrations of L and Z were also measured at each study visit.

Results. There was a highly significant and positive correlation between all MPODs (e.g., at 0.25°) recorded 1 week before and after surgery in eyes with an AIOL implant ($r = 0.915$, $P < 0.01$; paired samples *t*-test, $P = 0.631$) and in those ANIOL implants ($r = 0.868$, $P < 0.01$; paired samples *t*-test, $P = 0.719$). Average MPOD across the retina increased significantly with time (after 3 months) in the ANIOL group (repeated-measures, general linear model, $P < 0.05$), but remained stable in the AIOL group (repeated-measures, general linear model, $P > 0.05$). There were no significant time or lens effects observed for serum L over the study period ($P > 0.05$). There was a significant time effect for serum Z over the study period ($P < 0.05$), but not a significant time/lens interaction ($P > 0.05$).

Conclusions. Customized HFP can reliably measure the MPOD spatial profile in the presence of lens opacity, and cataract surgery does not artifactually alter MPOD readings. This study also provides evidence that implanting an IOL that filters blue light is associated with augmentation of MPOD in the absence of raised serum concentrations of L and Z. However, further and longitudinal study is needed to assess whether the observed increase in MPOD after implantation of blue-filtering IOLs is associated with reduced risk of AMD development and/or progression. (*Invest Ophthalmol Vis Sci.* 2009;50:4777–4785) DOI:10.1167/iovs.08-3277

Age-related macular degeneration (AMD), which damages central vision, is the most common cause of age-related blindness in the western world.[1,2] Although the pathogenesis of AMD remains unclear, there is a growing body of evidence suggesting that oxidative stress is important in the pathogenesis of this condition and that cumulative short-wavelength (blue) light damage plays a role.[3–5]

Macular pigment (MP), which is entirely of dietary origin, and composed of the xanthophyll carotenoids: lutein (L), zeaxanthin (Z), and *meso*-Z, is thought to protect against AMD because it absorbs short-wavelength (blue) light at a prereceptoral level and because of its antioxidant properties.[6,7] The absorption spectrum of MP peaks at 460 nm and may therefore limit photo-oxidative damage to retinal cells.[8] MP levels are maximum within the photoreceptor axons of the foveola and the plexiform layers of the macula.[6,9] Of importance, both the absorptive characteristics of MP and its location in the anterior portion of individual photoreceptors enables the pigment to attenuate the amount of blue light incident on the photoreceptor.

It has been hypothesized that cataracts provide protection against AMD by absorbing blue light, and thus reducing photo-oxidative damage to the retina.[10] However, this protective effect, if any, would be restricted to certain types of lens opacity, such as nuclear sclerosis. In contrast, however, some studies have shown increased risk of cataract in association with AMD, which may reflect the fact that these conditions share antecedents (such as age).[11–13] The positive association of AMD and cataract is considered to be an effect of similar causation and risk factors of both disorders. Although some studies have failed to find a link between cumulative sunlight exposure and the risk of development of AMD,[14–16] many other studies have found a positive association between lifetime exposure to sunlight and AMD.[17–20] Recently, the age-related maculopathy and macular degeneration in elderly European populations (EUREYE) study has provided evidence of a link between cumulative (lifetime) sunlight exposure (in the presence of low antioxidant levels) and the risk of AMD.[21] Those individuals with high cumulative lifetime exposure to sunlight but who were in the lowest quartile for combined antioxidant levels (especially vitamin C, zeaxanthin, vitamin E

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6.16 APPENDIX 16: Non peer-reviewed publication

Clinical

Investigating macular pigment

Trials on meso-zeaxanthin are under way at the Macular Pigment Research Group. **Eithne Connolly** and **Dr John Nolan** describe how the work could shed more light on the importance of nutritional influence over macular degeneration progression

The macula is the central part of the retina responsible for detailed central vision. Age-related macular degeneration (AMD) is a disease of the macula that results in loss of central vision. AMD is the leading cause of age-related blindness in the western world. It is estimated that AMD affects approximately 417,000 people in the UK and 80,000 people in the Republic of Ireland.

The increasing prevalence of AMD worldwide is largely attributable to increasing longevity and lifestyle changes associated with western society. It is predicted that the current AMD prevalence figures will double by 2020. People with AMD lose their ability to read, recognise faces, watch television, and drive, and, therefore, lose their independence and quality of life. In addition, the cost of vision loss and visual impairment to society and to health care providers continues to rise, with significant economic implications.

Macular pigment (MP) is a yellow pigment found at the macula. MP is composed of the dietary carotenoids lutein, zeaxanthin, and meso-zeaxanthin. MP absorbs short-wavelength (blue) light pre-receptorally and scavenges and neutralises free radicals. It is unsurprising, therefore, that this pigment is believed to protect against AMD, because both blue light damage and free radicals are known to contribute to this condition. There is now an increasing body of scientific evidence which supports such a notion. For example, recent research by the Macular Pigment Research Group (MPRG) (www.wit.ie/mprg) has shown that individuals between the ages of 20 and 60 years (without ocular disease), who are at increased risk of developing AMD (eg cigarette smokers, people with a family history of AMD, people with poor diets lacking in antioxidants, people who are overweight and so on) have a relative lack of MP.

Meso-zeaxanthin trials

Meso-zeaxanthin is only found at the macula, whereas lutein and zeaxanthin are also found in a typical diet, serum, and several other tissues throughout the body. Meso-zeaxanthin is not found in a regular diet; however, it is generated at the macula following biochemical conversion from lutein, and it is also found in some foods such as sea food (eg shrimp and crab) and fish (eg trout and salmon).

Trevor McCormack (distributor of Macushield) and Dr John Nolan

Macushield is the only dietary supplement available that contains all three of the macular carotenoids, including meso-zeaxanthin. The importance of meso-zeaxanthin and its presence in MP can be summarised as follows: meso-zeaxanthin is the central portion of MP; meso-zeaxanthin is a more powerful antioxidant than either lutein or zeaxanthin; meso-zeaxanthin facilitates a wider range of short-wavelength light absorption; meso-zeaxanthin is more closely related to vulnerable photoreceptors at an anatomic level than either lutein or zeaxanthin.

The MPRG, at the Waterford Institute of Technology, Ireland is about to embark on two clinical trials with meso-zeaxanthin in the form of Macushield. The first clinical trial will be conducted in a randomised, double-blind, placebo-controlled, fashion and will be carried out on normal subjects (without ocular pathology). This trial is designed to investigate macular and serum responses to supplemental meso-zeaxanthin, lutein, and zeaxanthin (Macushield).

The second clinical trial will be conducted in a randomised, comparison (lutein, zeaxanthin versus meso-zeaxanthin, lutein and zeaxanthin [Macushield]) controlled fashion and will be carried out on patients with early AMD (presence of drusen and/or pigmentary changes). This trial is designed to investigate retinal sensitivity changes and AMD related pathology changes at the macula, if any, in response to MP augmentation.

These meso-zeaxanthin trials are essential to test, in full, the putative protective benefits that this carotenoid may offer our AMD patients, and individuals at risk of this condition. Also, recent case studies have shown that supplementation with Macushield results with significant MP augmentation in the central portion of the MP and resolution of centrally located drusen, and improvement in visual acuity, in two patients with AMD.

These individual case studies are positive, but properly conducted clinical trials, such as those outlined above, are essential to further our understanding of the importance of MP, and in particular the role of meso-zeaxanthin, for ocular health and AMD prevention.

● **Eithne Connolly** is a postgraduate and **Dr John Nolan** head of the Macular Pigment Research Group, Department of Chemical and Life Sciences, Waterford Institute of Technology, Waterford, Ireland

6.11 APPENDIX 11: Poster

Meso-zeaxanthin Ocular Supplementation Trial in Normals (MOST N)

Eithne E. Connolly[1, 2], Stephen Beatty[1, 2], James Loughman[3], John M. Nolan[1, 2]

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PURPOSE

The centre of the macula has a distinct yellow colour attributable to the presence of a yellow pigment known as macular pigment (MP). MP is made up of the three lipid-like carotenoids meso-zeaxanthin (MZ), lutein (L) and zeaxanthin (Z) (ratio at the macula: 1: 1: 1).[1; 2]

The MOST N (ISRCTN60816411) study was designed to investigate, in a double-blind, randomized placebo controlled fashion, changes in MP optical density (MPOD), and serum concentrations of the macular carotenoids in response to a supplement containing MZ, L and Z , in normal subjects.

METHODS

44 healthy subjects were recruited for this study. 22 subjects were randomized to consume a formulation containing 10.9 mg of MZ, 5.9 mg of L and 1.2 mg of Z (Intervention group [I]), and 22 subjects consumed a placebo containing corn starch and rice flour (Placebo group [P]) everyday with a meal over a six month study period.

At each study visit (Baseline [V1], 3 months [V2], 6 months [V3]) MPOD (at 0.25°, 0.5°, 1° and 1.75° degrees eccentricity) was measured using customized heterochromatic flicker photometry (cHFP) [Fig. 1].

Blood samples were also collected to analyze serum concentrations of L and TZ (total zeaxanthin which includes MZ and Z) by high performance liquid chromatography (HPLC) [Fig. 2]. Additional blood samples were collected at V1 and V3 and sent to Claymon Biomnis Laboratories, Ireland, for full clinical pathology analysis to assess the safety of MZ, L and Z consumption in humans [Table 1].

For MPOD and serum carotenoid data, we conducted repeated measures analysis using a general linear model approach. For the clinical pathology analysis, we used paired-samples t-tests to test for statistical differences between V1 and V3.

Figure 1. cHFP

Figure 2. HPLC

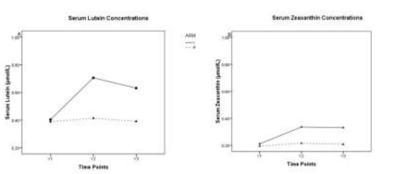
RESULTS

Serum Carotenoid Analysis

There was a statistically significant increase in serum concentrations of L and TZ* (μmol/L) from baseline at V2 and V3 in the I group ($p < 0.005$, for all). As expected, there was no statistically significant change from baseline in serum concentrations of L and Z in the P group over the study period ($p > 0.05$, for all) [Fig. 3A and B].

TZ* = total Z concentrations which includes MZ and Z

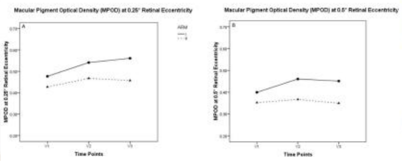
Figure 3. Serum Concentrations of Lutein and Zeaxanthin



Macular Pigment Optical Density

There was a statistically significant increase in MPOD at 0.25° and 0.5° retinal eccentricity from baseline at V2 and V3 in the I group of the study ($p < 0.005$, for all). As expected, there was no statistically significant change in MPOD over the study period, at any eccentricity measured, in the P group ($p > 0.05$, for all) [e.g. Fig. 4A and B].

Figure 4. Macular Pigment Optical Density



Clinical Pathology Analysis

Statistically significant differences from V1 to V3 were identified in 10 of the variables assessed and are highlighted in yellow in Table 1 (some increased and some decreased from baseline). However, and most importantly, all variables at each visit (with the exception of LDL, which had a baseline value outside the normal reference range) were compared with biological variation data tables at http://www.westgard.com/biodatabase1.htm, and were found to be within the normal reference range provided.

While LDL also appeared to show a significant difference between visits in the I group ($p = 0.01$), total cholesterol did not show any significant difference between visits ($p = 0.79$), and therefore it is unlikely to be of any medical significance. Further investigation is ongoing to confirm this.

Table 1. Clinical Pathology Variables

		INTERVENTION GROUP			PLACEBO GROUP		
Pathology variable	Ref. Range (Unit)	V1	V3	p value	V1	V3	p value
Sodium	135-145 (mmol/L)	139.42	139.26	0.51	139.26	139.26	1.00
Potassium	3.3-5.3 (mmol/L)	4.16	4.55	0.01	4.26	4.43	0.04
Chloride	98-107 (mmol/L)	104.05	98.89	0.32	104.05	103.11	0.15
Urea	2.5-7.7 (mmol/L)	4.72	5.03	0.23	5.31	5.37	0.76
Creatinine	40-90 (μmol/L)	75.11	76.84	0.42	77.00	74.68	0.15
Total protein	64-83 (g/L)	72.63	71.05	0.10	71.63	70.05	0.12
Albumin	37-52 (g/L)	44.47	44.58	0.82	43.53	44.21	0.30
Globulins	21-36 (g/L)	28.16	26.47	0.11	28.11	26.37	0.07
Total bilirubin	3.4-21.0 (μmol/L)	8.73	8.21	0.59	8.05	8.77	0.29
Alanine Aminotransferase	0-55 (IU/L)	24.32	19.42	0.18	22.47	23.16	0.63
Aspartate Aminotransferase	5-36 (IU/L)	20.37	19.05	0.16	22.16	21.89	0.81
Alkaline Phosphate	40-150 (IU/L)	78.84	74.63	0.41	79.00	79.95	0.80
Gamma GT	9-36 (IU/L)	33.84	25.05	0.29	25.16	23.89	0.42
Cholesterol total	**<5.0 (mmol/L)**	5.21	5.24	0.79	**5.26**	**4.92**	**0.02**
Triglycerides	0.60-1.70 (mmol/L)	1.38	1.66	0.13	1.10	1.09	0.93
HDL	1.00-1.55 (mmol/L)	1.46	1.49	0.63	1.54	1.51	0.46
Direct LDL	**<3.0 (mmol/L)**	**3.03**	**3.25**	**0.01**	3.13	2.98	0.23
Calcium	2.10-2.60 (mmol/L)	2.38	2.35	0.33	2.36	2.36	0.80
Phosphate	0.80-1.56 (mmol/L)	1.16	1.14	0.63	1.10	1.09	0.82
Magnesium	0.65-1.10 (mmol/L)	1.00	0.95	0.01	0.98	0.92	0.00
Uric Acid	155-394 (μmol/L)	263.47	273.47	0.19	274.68	271.74	0.76
Glucose	3.1-6.1 (mmol/L)	5.31	5.77	0.11	5.03	4.94	0.50
High Sens. Reactive Protein	<5.0 (mg/L)	4.00	3.31	0.57	1.49	4.18	0.40
Full Blood Count							
White cell count	3.88-10.49 (10e9/L)	7.07	6.79	0.24	5.97	6.92	0.10
Red cell count	3.73-5.02 (10e12/L)	4.53	4.58	0.35	4.64	4.58	0.30
Haemoglobin	**11.3-15.2 (g/dL)**	**14.23**	**13.91**	**0.03**	**14.46**	**13.85**	**0.01**
Haematocrit	**0.323-0.462 (L/L)**	**0.40**	**0.41**	**0.01**	0.40	0.41	0.38
MCV	**83.1-99.1 (fL)**	**87.93**	**90.41**	**0.00**	**87.06**	**89.42**	**0.00**
MCH	**28.3-33.9 (pg)**	**31.42**	**30.38**	**0.00**	**31.15**	**30.28**	**0.00**
MCHC	**32.1-36.6 (g/dL)**	**35.75**	**33.62**	**0.00**	**35.78**	**33.88**	**0.00**
Platlets	164-382 (10e9/L)	295.47	287.00	0.24	313.28	299.00	0.08
Diff. White Cell Count							
Neutrophils	1.91-7.16 (10e9/L)	4.39	4.05	0.15	3.44	4.18	0.16
Lymphocytes	1.01-3.13 (10e9/L)	1.85	1.86	0.92	1.72	1.87	0.04
Monocytes	0.19-0.68 (10e9/L)	0.42	0.39	0.23	0.36	0.40	0.21
Eosinophils	0.05-0.51 (10e9/L)	0.25	0.27	0.62	0.24	0.23	0.79
Basophils	0.02-0.15 (10e9/L)	0.07	0.07	0.71	0.10	0.07	0.10
L. unstained cells	**0.00-0.30 (10e9/L)**	0.13	0.13	0.81	**0.12**	**0.16**	**0.00**

CONCLUSION

This double-blind, randomized placebo controlled trial showed significant increases in serum concentrations of the macular carotenoids, and MPOD at 0.25° and 0.5° retinal eccentricity, following supplementation with a formulation containing 10.9 mg MZ, 5.9 mg L and 1.2 mg Z.

Of note, MOST N is the first study to investigate, and report on, the safety of human consumption of all three macular carotenoids, including MZ. We conclude that the consumption of MZ, L and Z does not produce any medically significant changes in the pathology variables tested.

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6.12 APPENDIX 12: Poster

Augmentation of macular pigment following supplementation with all three macular carotenoids: an exploratory study

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PURPOSE

Age-related macular degeneration (AMD) is an eye disease that affects the central part of the retina called the macula and in its late form, results in loss of central vision. It is now believed that both oxidative stress (i.e. free radical damage) and cumulative exposure to short-wavelength (blue) light are involved in the aetiopathogenesis of AMD.

The centre of the retina has a distinct yellow colour attributable to the presence of a pigment known as macular pigment (MP). MP comprises three dietary carotenoids *meso*-zeaxanthin (MZ), lutein (L) and zeaxanthin (Z). There is now a biologically plausible rationale, supported by a growing body of evidence, in support of the view that MP protects against AMD due to its ability to filter damaging blue light and quench free radicals. This study was designed to measure serum and macular responses to a macular carotenoid formulation.

METHODS

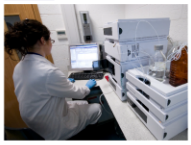
10 subjects were recruited into this open labeled study, which consisted of two groups: Group 1: 5 normal subjects with no ocular pathology; Group 2: 5 subjects with AMD in at least one eye (study eye). Subjects were instructed to take a formulation containing 7.3mg MZ, 0.8mg Z, and 3.7mg L every day for eight weeks.

Subjects were assessed at five separate study visits as follows: baseline (V1); 2 weeks (V2); 4 weeks (V3); 6 weeks (V4); 8 weeks (V5). MP optical density (MPOD) and serum concentrations of L and Z were assessed at each study visit using customised heterochromatic flicker photometry (cHFP, Fig. 1) and high performance liquid chromatography (HPLC, Fig. 2), respectively.

Figure 1

cHFP

Figure 2



HPLC

RESULTS

Serum

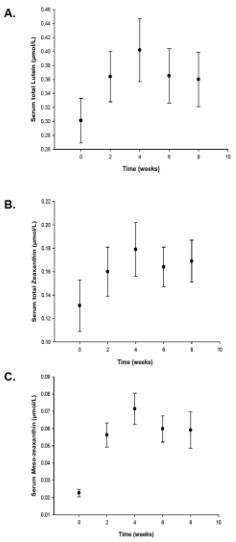
There was a significant increase in serum concentrations of MZ, TL and TZ over the study period, present after just two weeks of supplementation (Repeated measures analysis; MZ: $p = 0.01$; TL: $p < 0.05$; and TZ: $p < 0.05$) (Table 1 and Figs. 3A, B and C).

Table 1 Serum Macular Carotenoid Concentrations

	V1	V2	V3	V4	V5	p - value
Total lutein	0.30 ± 0.1	0.36 ± 0.12	0.40 ± 0.14	0.37 ± 0.12	0.36 ± 0.12	p = 0.002
Total zeaxanthin	0.13 ± 0.07	0.16 ± 0.06	0.18 ± 0.07	0.16 ± 0.05	0.17 ± 0.06	p = 0.003
***Meso*-zeaxanthin**	0.02 ± 0.01	0.06 ± 0.02	0.07 ± 0.03	0.06 ± 0.02	0.06 ± 0.03	p = 0.000
Zeaxanthin	0.11 ± 0.07	0.10 ± 0.05	0.11 ± 0.05	0.10 ±0.04	0.11 ± 0.04	p = 0.909

Values represent mean ± SD in μmol/L; N = 10

Figure 3 Serum Concentrations of L, Z and MZ



MPOD spatial profile

MPOD increased significantly at all eccentricities measured after just two weeks of supplementation, with the exception of MPOD at 1.75° (Table 2 and Fig. 4).

Table 2 Average MPOD

MPOD	V1	V2	V3	V4	V5	p - value
0.25	0.39 ± 0.19	0.49 ± 0.18	0.52 ± 0.22	0.57 ± 0.26	0.61 ± 0.26	p < 0.017
0.5	0.38 ± 0.16	0.43 ± 0.15	0.46 ± 0.16	0.44 ± 0.18	0.45 ± 0.18	p < 0.101
1.0	0.26 ± 0.15	0.29 ± 0.14	0.32 ± 0.10	0.32 ± 0.17	0.38 ± 0.14	p > 0.030
1.75	0.14 ± 0.10	0.17 ± 0.10	0.17 ± 0.06	0.16 ± 0.08	0.16 ± 0.09	p > 0.610
Total average	0.29 ± 0.13	0.34 ± 0.12	0.37 ± 0.12	0.37 ± 0.15	0.40 ± 0.15	p < 0.019

Values represent mean ± SD; V1 = 0.25 ° retinal eccentricity; 0.5 = 0.5° retinal eccentricity; 1.0 = 1 ° retinal eccentricity; 1.75 = 1.75 ° retinal eccentricity.
Total average = average MPOD for all eccentricities

Typical versus atypical MPOD spatial profile

Four subjects (one from Group 1 and three from Group 2) who displayed an atypical MPOD spatial profile at baseline (i.e. pre-supplementation), had the more typical MPOD spatial profile (i.e. highest MPOD at the centre) after eight weeks of supplementation with MZ, L and Z (Fig. 5).

Figure 4 MPOD Spatial Profile

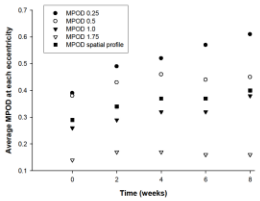
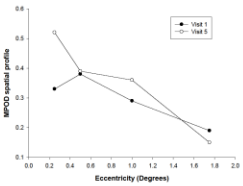


Figure 5 Change in MPOD spatial profile



CONCLUSION

In conclusion, we report significant increases in serum concentrations of the macular carotenoids following supplementation with a formulation containing 7.3 mg MZ, 3.7 mg L and 0.8 mg Z and also a significant increase in MPOD (and alteration of its spatial profile). Also, this exploratory study has identified the ability of this carotenoid formulation to rebuild central MPOD in subjects who display atypical profiles at baseline.

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Poster presented at Retina 2009, Dublin, Ireland; and the final manuscript has been accepted for publication by Current Eye Research.

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6.13 APPENDIX 13: Certificate in first aid

CHAPTER SEVEN

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