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The Effect of nutritional supplementation on subjective and objective measures of visual and retinal function

A random controlled trial

Emma Berrow

THE EFFECT OF NUTRITIONAL SUPPLEMENTATION ON SUBJECTIVE AND OBJECTIVE MEASURES OF VISUAL AND RETINAL FUNCTION – A RANDOMISED CONTROLLED TRIAL

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In industrialised countries age-related macular disease (ARMD) is the leading cause of visual loss in older people. Because oxidative stress is purported to be associated with an increased risk of disease development the role of antioxidant supplementation is of interest. Lutein is a carotenoid antioxidant that accumulates within the retina and is thought to filter blue light. Increased levels of lutein have been associated with reduced risk of developing ARMD and improvements in visual and retinal function in eyes with ARMD.

The aim of this randomised controlled trial (RCT) was to investigate the effect of a lutein-based nutritional supplement on subjective and objective measures of visual function in healthy eyes and in eyes with age-related maculopathy (ARM) – an early form of ARMD. Supplement withdrawal effects were also investigated.

A sample size of 66 healthy older (HO), healthy younger (HY), and ARM eyes were randomly allocated to receive a lutein-based supplement or no treatment for 40 weeks. The supplemented group then stopped supplementation to look at the effects of withdrawal over a further 20 weeks. The primary outcome measure was multifocal electroretinogram (mfERG) N1P1 amplitude. Secondary outcome measures were mfERG N1, P1 and N2 latency, contrast sensitivity (CS), Visual acuity (VA) and macular pigment optical density (MPOD).

Sample sizes were sufficient for the RCT to have an 80% power to detect a significant clinical effect at the 5% significance level for all outcome measures when the healthy eye groups were combined, and CS, VA and mfERG in the ARM group.

This RCT demonstrates significant improvements in MPOD in HY and HO supplemented eyes. When HY and HO supplemented groups were combined, MPOD improvements were maintained, and mfERG ring 2 P1 latency became shorter. On withdrawal of the supplement mfERG ring 1 N1P1 amplitude reduced in HO eyes. When HO and HY groups were combined, mfERG ring 1 and ring 2 N1P1 amplitudes were reduced. In ARM eyes, ring 3 N2 latency and ring 4 P1 latency became longer. These statistically significant changes may not be clinically significant.

The finding that a lutein-based supplement increases MPOD in healthy eyes, but does not increase mfERG amplitudes contrasts with the CARMIS study and contributes to the debate on the use of nutritional supplementation in ARM.

Keywords: age-related macular disease, electrophysiology, lutein.

To Mom, Paul, Ellie and Tom

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Table of contents

List of tables **Page**

List of tables **Page**

List of tables **Page**

List of tables **Page**

List of tables **Page**

List of tables **Page**

List of tables **Page**

List of figures **Page**

List of Abbreviations

AMD	Age-related macular degeneration
ARM	Age-related maculopathy
ARMD	Age-related macular disease
BlamD	Basal laminar deposit
BlinD	Basal linear deposit
CNV	Choroidal neovascularisation
CR	Coefficient of repeatability
CRT	Cathode ray tube
CS	Contrast sensitivity
CV	Coefficient of variation
HFP	Heterochromatic flicker photometry
HO	Healthy older
HY	Healthy younger
ISCEV	International society for clinical electrophysiology of vision
L	Lutein
LED	Light emitting diode
mfERG	multifocal electroretinography
MP	Macular pigment
MPOD	Macular pigment optical density
MZ	Meso-zeaxanthin
PEDF	Pigment epithelium-derived factor
RPE	Retinal pigment epithelium

List of Abbreviations continued

VA	Visual acuity
VEGF	Vascular endothelial growth factor
VERIS	Visual evoked response imaging system
Z	Zeaxanthin

The effect of nutritional supplementation on subjective and objective measures of visual function – a randomised controlled trial

Chapter 1: Background

1.1 Prevalence and incidence of age-related macular disease

Age-related macular disease (ARMD) is the leading cause of visual loss in developed countries [1-4]. In Britain ARMD is a growing public health concern. Blind and partial sight registrations have increased in by approximately 30-40% over 40 years whereas registrations for cataract, glaucoma and optic atrophy have decreased [4]. The prevalence of ARMD will continue to increase as life expectancy increases [1, 5-7]. New ARMD treatments such as ranibizumab for slowing cell proliferation and reducing neovascularisation in 'wet' age-related macular degeneration (AMD), are costly to the National Health Service [8-10]. Government expenditure will also increase for managing ARMD as blind and partial sight registration, and years of lost working life rises. The psychological impact [11, 12] and reduced quality of life [13, 14] that occur during the later stages of the disease are devastating to individuals and their relatives, giving greater impetus for the need to examine systems for earlier diagnosis and monitoring of ARMD, and investigate potential preventative ARMD measures.

1.2 Retinal anatomy

Age-related macular disease affects the retinal pigment epithelium (RPE), Bruch's membrane, choriocapillaris and the photoreceptors which are located in the outer retina. The retina is a highly differentiated neuroectodermal tissue consisting of two distinct regions: the central retina (the macula) which is specialised for detailed vision, and the peripheral retina. The human retina is comprised of three layers of nerve cell bodies and two layers of synapses [15] (figure 1.1).

Figure 1.1: Section through the human eye with a schematic enlargement of the retina (Source - Webvision, http://webvision.med.utah.edu/imageswv/Sagschem.jpeg with permission).

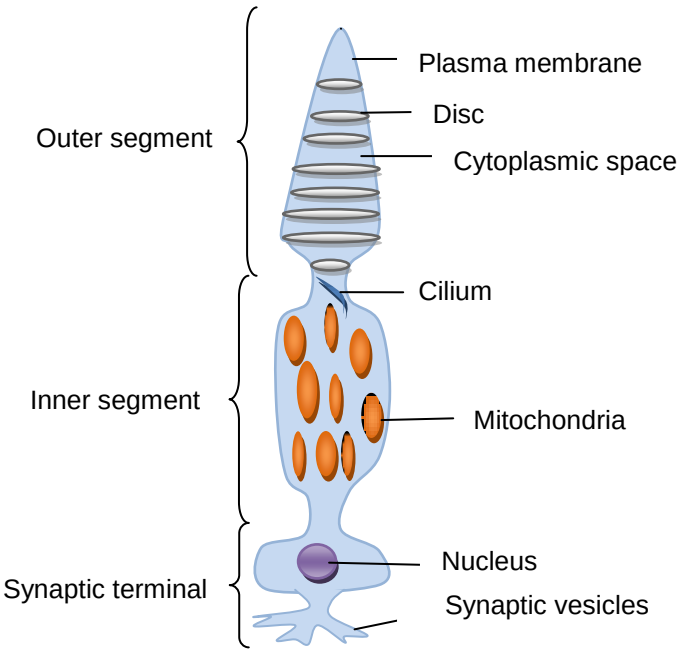
The outer nuclear layer contains photoreceptor cell bodies. The inner nuclear layer contains bipolar, horizontal and amacrine cell bodies. The ganglion cell layer contains ganglion cells and displaced amacrine cells [15].

1.3 Structure and function of the photoreceptors

Within the human retina there are two types of photoreceptors – rods and cones. There are approximately 92 million rods, predominantly found at the periphery of the retina, which contain the visual pigment rhodopsin and are required for night vision. There are approximately 4.6 million cones found predominantly in the macula which contain the red, green or blue opsin pigments necessary for colour vision and sharp visual acuity [16]. These visual pigments are located within the outer segment discs (figure 1.2) of the photoreceptors and are essential for light absorption. Photoreceptors are continually exposed to oxygen, and when they are exposed to light, free radicals are produced which over time cause injury to the photoreceptor discs. Photoreceptor outer segment discs consist of approximately 50% of polyunsaturated fatty acids (docosahexaenoic acid or DHA) with the remaining 50% comprising of proteins [17]. Photoreceptors selectively uptake DHA which is used to make new disc membranes [18]. Photoreceptor inner segments contain mitochondria and the necessary processes for transport of molecules to the outer segment and axonal parts of the cell. The inner segments form junctions

with the Müller cells and together comprise the outer limiting membrane. Photoreceptor basal axonal processes synapse with bipolar and horizontal cells (figure 1.1).

Figure 1.2: Schematic diagram of a cone photoreceptor (source - author's own).



1.4 The RPE, Bruch's membrane and choriocapillaris

The RPE plays a central role in regulating the environment surrounding the photoreceptors where phototransduction occurs [19]. Each RPE cell underlies approximately 30 photoreceptor cells. The RPE cell apical processes diurnally phagocytose the shed outer segments of photoreceptors allowing for constant turnover of photoreceptor membrane discs [20]. The RPE consists of a non-renewing monolayer of cuboidal-shaped cells, of which the apical surface is positioned next to the photoreceptors and the basal surface is situated adjacent to the collagen-rich Bruch's membrane. The correctly functioning RPE provides an outer blood-retinal barrier as RPE cell plasma membranes, linked by tight junctions, selectively allow nutrient and waste product exchange between the ocular vascular beds and retinal tissues, and prevents leakage of macromolecules and other potentially harmful products into the retina [21].

An abundance of melanosomes give the RPE its pigment which allows for the absorption of stray light – essential for image sharpness by minimising light scatter [22]. Within the visual cycle the RPE is involved in the retinoid processing cycle in the renewal of 11-cis retinal as all-

trans retinal is transported to the RPE and re-isomerized into 11-cis retinal which is transported back to the photoreceptors and combined with opsin. Retinal pigment epithelial cells reduce uniformly over time with increasing age within the retina, at a greater rate equatorially compared to centrally [23]. Melanosome number also consistently decreases with increasing age, possibly through the damaging effects of blue light irradiation [24].

Bruch's membrane lies between the RPE and choriocapillaris. It provides a semi-permeable filtration barrier through which major metabolic transfer occurs [25]. It consists of five layers – the basement membrane, inner collagen fibre layer, elastic fibre sheet, collagen fibre layer and the basement membrane of the capillary endothelial cells of the choriocapillaris [26].

The choriocapillaris is directly adjacent to Bruch's membrane and comprises of an intricate network of blood vessels which provide oxygen and nutrients to the RPE. It is among the most highly perfused tissues in the body [27]. Choriocapillaris density decreases and Bruch's membrane thickens with increasing age [28] and an ageing Bruch's membrane has been shown to decrease RPE phagocytosis [29].

1.5 The macula

The macula is defined as the portion of the posterior retina that contains xanthophyll and two or more layers of ganglion cells [30]. This region is approximately 6 mm in diameter and consists of two areas. The cone-dominated fovea is a depression in the inner retinal surface in the centre of the macula measuring 0.8mm in diameter. The parafovea encircles the fovea and is dominated by rod photoreceptors thus the macula overall has a larger cone concentration compared to the remainder of the eye but is not cone-dominant [16]. The central floor of the fovea is called the foveola and this is 0.35 mm thick. The foveola lies within a capillary free zone and thus has no retinal circulation [30]. The rod to cone ratio in the macula is 9:1 compared to the overall retina where it is 20:1[16].

The macula contains the xanthophyll carotenoids lutein (L), zeaxanthin (Z) and meso-zeaxanthin (MZ) which give the macula its characteristic yellow colour when observed on fundoscopy. Together, these carotenoids are termed macular pigment (MP) [31]. The ratio of L to Z is 1:2.4 in the central 0-0.25mm of the retina, to over 2:1 in the peripheral 8.7-12.2 mm of

the retina which is linearly correlated with the rod:cone ratio [32]. Meso-zeaxanthin is thought to be converted in the retina from L [33].

1.6 Age-related macular disease (ARMD)

Age-related macular disease is a degenerative disease of the macula, most common over the age of 50 years [34]. It is the leading cause of visual loss within western industrialised countries [1, 2, 4]. The number of blind registrations attributable to the disease increased by 30-40% between 1950-1990 in Britain [4] and cases each year are continuing to rise [1, 6, 7] as these populations have an increasing longevity. The RPE, Bruch's membrane, photoreceptors and choriocapillaris are affected in ARMD.

1.7 Definition of ARMD

The international age-related maculopathy group has defined an international classification system for quantifying and defining the different subgroups of ARMD in an attempt to permit easier comparison of research findings between groups [34].

Age-related maculopathy (ARM) as defined by the international age-related maculopathy group is a disorder of the macular area, most apparent after age 50 and is characterised by areas of:

- Drusen which are external to the neuroretina and RPE. They are soft and distinct or soft and indistinct. Hard drusen are not characteristic of ARM.
- Hyperpigmentation in the outer retina or choroid with drusen.
- Hypopigmentation of the RPE with drusen.

This early stage of the condition may not affect vision, but can predispose patients to visual loss (see figure 1.3 below).

Later stages of the condition are classified by the international age-related maculopathy group age-related macular degeneration (AMD). This form of the disease can occur with or without the involvement of new blood vessel growth. If new vessels are not involved, (non-exudative or dry AMD), clinical presentation is a sharply defined round or oval area of hypopigmentation

where choroidal vessels are more visible than the surrounding area, with a diameter greater than 175μm [34] (figure 1.4). This is also known as geographic atrophy (GA).

The term 'wet AMD', also termed disciform AMD, exudative AMD or neovascular AMD refers to the involvement of neovascularisation (figure 1.5) and has numerous manifestations:

- Choroidal neovascularisation (CNV)
- RPE detachment(s)
- Subretinal or sub-RPE neovascular membrane(s)
- Deposition of scarring, glial tissue or fibrin-like material within the epiretinal, intraretinal, subretinal or sub-RPE layers
- Subretinal haemorrhages (without other retinal vascular cause)
- Hard exudates (formed from lipid) associated with the above manifestations (without other retinal vascular cause).

Figure 1.3: ARM showing drusen.

Figure 1.4: AMD with GA.

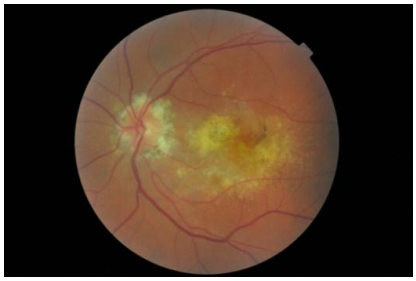
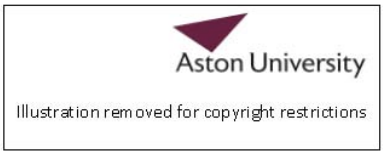


Figure 1.5: Exudative AMD.



(Source – figures 1.3, 1.4 author's own photos, figure 1.5 Webvision with permission).

In accordance with the international classification system the term ARMD will be used to encompass both ARM and AMD for the purposes of this thesis.

1.8 Physiology of ARMD

The RPE rests on Bruch's membrane and separates the neural retina from the choriocapillaris. The RPE phagocytoses the outer segment discs of the photoreceptors and is a point of metabolite and waste exchange, which is considered crucial to retinal function [21]. The initial signs of ARMD are variations within and below the RPE, seen as alterations in the pigmentation of the RPE, with or without the occurrence of drusen [35]. Drusen are discrete white-yellow spots containing abnormal extracellular lipoprotein deposits which accumulate between the RPE basal lamina and the inner collagenous layer of Bruch's membrane [36]. Within the ageing eye a build up of lipofuscin granules can be seen in the RPE [37], possibly caused by a reduced ability of the RPE's phagocytic-lysosomal system to efficiently digest photoreceptor outer segment membranes [38], leading to an accumulation of lipids from this material in Bruch's membrane reducing membrane permeability. This in turn may interrupt the supply of nutrients from the choroid to the retina ultimately leading to photoreceptor atrophy [39]. Oxidative stress causes injury and inflammation to the RPE and choriocapillaris affecting nutrient supply to the RPE and retina, possibly thus further damaging the RPE and retina, leading to the retinal atrophy seen in the later stages of ARMD [40].

1.9 Aetiology of ARMD

Although the precise aetiology of ARMD is currently unknown there are several hypotheses that have been postulated:

Oxidative stress

Ageing is associated with cumulative oxidative damage [41]. The retina is constantly under high oxygen tension and is thus susceptible to this damage. Reactive oxygen intermediates (ROI), a term used to describe hydrogen peroxide, singlet oxygen and free radicals, are synthesized as by-products of phototransduction and cell metabolism [42]. Phagocytosis of photoreceptor outer segments by the RPE produces ROIs, increasing oxidative stress. Outer segments of photoreceptors contain polyunsaturated fatty acids (PUFA) and vitamin A. Under high oxygen tension and light irradiation the outer segments undergo lipid peroxidation, especially within the macular area [43]. Light irradiation induces photoreceptor damage [44]. It has been suggested that lipid peroxidation may be involved in the cause of light induced retinal degeneration [45]. A healthy RPE is required for the correct functioning of the retina [46] but RPE changes occur with age as lipofuscin granules accumulate within RPE cells. Lipofuscin is composed of vitamin

A metabolites and lipid peroxides, and is constantly exposed to visible light (400-700nm) and high oxygen tension (~70mmHg) which cause reactive oxygen species synthesis and possible RPE membrane damage [47]. Lipofuscin accumulates in the human RPE from approximately 20 years of age and continues throughout life [48]. Lipofuscin is a photosensitizer that may increase the risk of retinal photodamage and contribute to the development of ARMD [49]. There are differing thoughts as to whether RPE melanosomes provide a protective effect to the RPE by scavenging reactive free radicals [50].Therefore their decline within the RPE with increasing age [24] may reduce free radical scavenging by these cells. However an increase in phototoxic melanin-lipofuscin complexes (melanolipofuscin) also occur with increasing age and may have a detrimental effect to the RPE as their accumulation more closely reflects the onset of AMD than lipofuscin accumulation alone [51].

Genetics

Several genes have been associated with an increased risk of developing ARMD and have been verified in further studies [52]. The LOC387715 variant [53] and complement factor H gene polymorphisms (Y402H) predispose individuals to an increased risk of developing ARMD [54]. Protective genes have also been identified such as the complement factor B and complement component 2 gene, although current knowledge is rudimentary and continued genetic research may yield further information [55]. Although the extent of heritability and the number of genes involved in ARMD is presently unknown [56] there has been evidence to suggest increased risk of disease development with a positive family history of the disease [57-60]. It appears likely that a combination of exposure to environmental stimuli and genetic predisposition to ARMD are implicated in the pathogenesis of the disease [4, 61].

Deterioration of Ruysch's complex

Ruysch's complex consists of the RPE, Bruch's membrane and choriocapillaris. The hydraulic conductivity of Bruch's membrane reduces with increasing age [35, 62, 63]. Bruch's membrane collagen solubility decreases with increasing age, particularly at the posterior pole, and is thought to interfere with the function of the RPE [64] whose cell attachment rates are decreased on an aged Bruch's membrane [65]. Cross-linking of collagen fibres within Bruch's membrane increases with increasing age and a rise in UV absorbance and fluorescence also occurs within the membrane [66].

In ARMD deposition of long-space collagen and basement membrane proteins can be observed between the RPE plasma membrane and RPE basement membrane [67]. These deposits are termed basal laminar deposits (BlamD). Basal linear deposits (BlinD) are found between the basement membrane of RPE cells and Bruch's membrane mostly comprising of membranous debris [68]. Histopathologically ARMD is characterized by occurrence of both deposits [68, 69]. The presence of BlamD is strongly associated with the presence of AMD [70] which compromises photoreceptor cell function [71] and BlinD are also specific for AMD [68]. Histopathological studies have correlated BlamD with CNV [72, 73] and a severely compromised RPE [67].

With increasing age Bruch's membrane progressively accumulates lipid content [25, 74] and fluid diffusion is slowed [62]. It is thought that the debris within Bruch's membrane is derived from RPE metabolic activity [25] and this rise in lipid and protein quantity within Bruch's membrane reduces permeability, thus impeding flow of macromolecules between the RPE and choroid [75]. This may lead to slowed regeneration of photopigment due to retinoid deficiency, ultimately causing photoreceptor loss [76]. Bruch's membrane thickens with increasing age, [77] which is associated with a decline in phagocytosis of photoreceptor outer segments by RPE cells [78] and increases the distance for oxygen transport between the choriocapillaris and outer retina, reducing the oxygen to the outer retina [79]. In the normally functioning RPE, vascular endothelial growth factor (VEGF) and pigment-epithelium derived factor (PEDF) proteins are optimally balanced within the RPE, with PEDF being an antagonist of VEGF. Oxidative stress and the accumulation of deposits within the RPE and Bruch's membrane may disrupt this balance [80, 81] contributing to the development of CNV [82-84]. The build up of debris within the RPE and Bruch's membrane is thought to trigger a chronic inflammatory response within the area and activation of the complement system which can result in chronic cellular damage [40].

There is evidence to suggest that choroidal circulation attenuation may play a role in the development of ARMD. Ninety percent of the oxygen requirement of the photoreceptors is provided by the choroidal circulation [85] and reduced choroidal blood flow has been associated with ARMD [86, 87]. Choriocapillaris density and lumen diameter reduce with age [28], which may decrease oxygen to the RPE and photoreceptors, and reduce clearance of waste products from Bruch's membrane, leading to its thickening with age [40]. Retinal hypoxia increases the release of VEGF within Ruysch's complex leading to CNV [88]. Vascular deficits are further

advanced in AMD [89] with a linear relationship between reduced choroidal blood flow and increased risk for development of CNV [90]. Retinal hypoxia drives the synthesis of VEGF which gives rise to the angiogenesis seen in CNV [91].

1.10 Risk Factors for ARMD

Although the pathogenesis of ARMD is still not fully understood genetic predilection together with environmental factors are implicated. Epidemiological studies have found conflicting findings between ARMD development and many potential risk factors. An in-depth account of all modifiable and non-modifiable risk factors associated with ARMD development has been accepted for publication and can be found in appendix 1.

Age, smoking and genetic factors appear to be consistently associated with an increased risk of developing ARMD. However, ageing and genetic disposition cannot be currently modified, leading to increased interest as to how other modifiable factors may reduce the risk of ARMD.

Nutrition as a risk factor for developing ARMD

Some research has found an association between certain nutrients and reduced risk for ARMD development (table 1.1). The first National Health and Nutrition Examination Study (NHANES) found dietary vitamin A provides a protective effect against AMD with no beneficial effect shown with vitamin C [92]. The Beaver Dam eye study found no association between vitamins A, C and E and reduced risk of developing ARM [93]. Another study of serum lycopene in the Beaver Dam eye study showed an increased risk of ARMD with reduced lycopene levels [94]. However, lower levels of L, Z and vitamin E were not related to an increased risk for ARMD development in this study. Conversely higher serum alpha tocopherol (a form of vitamin E) and an antioxidant index including ascorbic acid (a form of vitamin C), alpha tocopherol and beta carotene (a form of vitamin A), were found to be conducive to lower ARMD risk in the Baltimore longitudinal study [95]. The Physicians Health study and the Blue Mountains eye study did not find a protective effect for vitamin C, E and multivitamins [96], and vitamin E and beta carotene [97] against ARMD respectively. The Eye Disease Case Control Study (EDCCS) found a reduced risk of neovascular AMD with higher serum and dietary carotenoid levels [98]. An antioxidant index combining selenium, vitamin C, vitamin E and carotenoids also showed reductions in risk in this study. A further study from the EDCCS reported that spinach and collards, high in the carotenoids lutein (L) and zeaxanthin (Z), were most strongly associated

with a reduced risk for AMD ($p<0.001$) [99]. The carotenoids L and Z together make up macular pigment (MP). They are lipid soluble antioxidants, not produced within the body and only obtained through diet [31]. Collard greens are various loose-leafed vegetables of Brassica oleracea, the same species that produces cabbage and broccoli. They are genetically similar to kale and spring greens. High-dose vitamins C, E beta carotene and zinc were found to be effective in lowering the odds ratio of developing advanced AMD in eyes with intermediate drusen, large drusen and non-central GA in a large trial undertaken by the AREDS group [100]. Improvements in visual function in eyes with ARM or non-exudative AMD were reported in several studies involving carotenoids [101-104].

High levels of omega-3 fatty acid consumption (>75 percentile) have been shown to provide a protective effect against progression to AMD [105]. Lowering the dietary glycaemic index with higher omega-3 intake also showed a reduction in AMD progression in this study. The benefits of a low glycaemic diet in reducing ARM risk have been identified in other studies [105-107]. The Blue Mountains eye study found a lower risk of developing ARM when consuming omega-3 fatty acids in the form of one serving of fish per week [108]. Consumption of linoleic acid in the form of 1 to 2 servings of nuts per week was also associated with reduced ARM risk in this study. The AREDS study found a reduction in risk of progression from drusen to geographic atrophy in those with the highest dietary intake of omega-3 fatty acid [109] and reduced risk of developing neovascular AMD [110, 111]. It is thought that omega-3 provides a protective role within the retina by inhibiting oxidative stress and reducing inflammation within the retina [112].

Association between higher trans-unsaturated fat intake and increased prevalence of AMD was reported in a large study of 6734 participants [113]. Omega-3 fatty acids and olive oil were associated with a reduced prevalence of ARM and AMD in this study. However, the third NHANES results showed no association between dietary fat intake and ARM risk in 7883 participants [114] and this was echoed in 3654 participants from the Blue Mountains eye study [115]. Studies of mouse retinae have shown an increase in the accumulation of basal laminar deposits when consuming a high fat and cholesterol diet [116]. Some studies have shown that diets higher in fats have a propensity to be lower in essential nutrients and antioxidants [93, 117].

Table 1.1: Dietary supplementation and ARMD risk

Study	**Cohort sizes**	**Study design**	**Dietary component assessed**	**Outcome**
Beaver Dam eye study [118]	n = 1968	Retrospective longitudinal cohort design	Zinc, carotenoids, vitamin C, E	Higher zinc = lower ARM risk (odds ratio 0.6)
Mares-Perlman *et al.*,[94]	n = 127 ARM, 9 GA, 31 AMD, 167 controls	Nested case-control study within a population-based cohort	Carotenoids, tocopherols	Low lycopene = higher ARMD risk (odds ratio 2.2)
Baltimore longitudinal study [95]	n = 976	Retrospective and prospective longitudinal design	α-tocopherol, β-carotene, ascorbic acid	Protective effect of combined plasma levels of α-tocopherol, β-carotene, ascorbic acid against ARMD. Protective effect for ARMD with high plasma α-tocopherol levels (no odds ratio given)
Blue Mountains eye study [97]	n = 156	Case-controlled design	α-tocopherol, β-carotene	No protective effect of α-tocopherol or β-carotene against ARM
Eye disease case control study [98]	n = 421 AMD, 615 controls	Case-control design	Vitamin C, E, carotenoids, selenium	Protective effect of combined nutrients against AMD (no odds ratio given)
Seddon *et al.*, [99]	n = 356 AMD, 520 controls	Case-control design	Carotenoids, vitamin A,C,E	Higher carotenoids = lower risk of AMD (odds ratio 0.57)

Table 1.1 continued

Study	**Cohort sizes**	**Study design**	**Dietary component assessed**	**Outcome**
Age-related eye disease study [100]	n = 3640	Double-masked, prospective, clinical trial	Vitamin C, E, β-carotene, zinc	Vitamin C, E, β-carotene, zinc = reduced progression from ARM (and non-central GA) to AMD (odds ratio 0.72)
Chiu *et al.*, [105]	n = 2924	Observational study	Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA)	Higher DHA = lower progression of ARM to AMD (hazard ratio 0.73)
Blue Mountains eye study [108]	n = 2454	Observational study	Omega-3, linoleic acid	Higher omega-3 = reduced risk of incident ARM (relative risk 0.69), higher linoleic acid = reduced risk of incident ARM (relative risk 0.65)
Age-related eye disease study [109]	n = 2132	Prospective cohort study	DHA, EPA	Higher DHA and EPA = reduced risk of progression from ARM to GA (odds ratio 0.45)
Age-related eye disease study [110]	n = 4519	Cohort study	Omega-3	Omega-3 inversely associated with CNV (odds ratio 0.61)
Chong *et al.*, [113]	n = 6734	Cohort study	Omega-3, olive oil	Omega-3 (odds ratio 0.85) and olive oil (odds ratio 0.48) associated with reduced risk of progression to AMD

Table 1.1 continued

Study	Cohort sizes	Study design	Dietary component assessed	Outcome
Mares-Perlman *et al.*, [119]	n = 8222	Prospective cohort study	Lutein and zeaxanthin	Higher levels of lutein and zeaxanthin = lower odds for pigmentary changes (odds ratio 0.1)
Age-related eye disease study [120]	n = 4159	Prospective cohort study	Lutein and zeaxanthin, vitamin A,C,E	Lutein and zeaxanthin intake was inversely associated with CNV (odds ratio 0.65), GA (odds ratio 0.45) and large or extensive drusen (odds ratio 0.73)
Pathologies oculaires liees a l'age [121]	n = 2584	Prospective cohort study	Retinol, ascorbic acid, α-tocopherol	Higher α-tocopherol = decreased risk for ARM (odds ratio 0.72)
Van Leeuwen *et al.*, [122]	n = 4170	Prospective cohort study	Vitamin C, E, β-carotene and zinc	Vitamin C E, β-carotene and zinc levels associated with a 35% reduced risk of ARMD hazard ratio 0.65)

1.11 The role of nutritional supplementation in ARMD

Currently there are limited treatments available for delaying the course of ARMD and these are discussed in appendix 2. Because oxidative stress may be a factor in the pathogenesis of ARMD, the function of antioxidant supplementation in this disease is of interest. Current recommendation for the treatment of ARMD includes nutritional supplementation with antioxidants, vitamins and zinc in eyes with extensive intermediate drusen, one large druse or noncentral GA in one or both eyes, or if one eye has AMD [100].

The carotenoids L and Z together make up MP. They are lipid soluble antioxidants, not produced within the body and only obtained through diet [31]. Within the central fovea, MP is most concentrated within the photoreceptor axons and is also found in the inner plexiform layers external to the foveola [123]. It is also located in the fibres of Henle which largely consist of cone axons (Henle f.l. in figure 1.6) the fovea, the inner nuclear layer at the parafoveal area [124] in the RPE and choroid [125]. Tubulin is a protein located in cone photoreceptor cytoskeletons, which selectively binds to L and Z [126]. Macular pigment reduces with eccentricity to optically undetectable levels at six to eight degrees [127]. Zeaxanthin is more concentrated than L at the centre of the fovea with the situation reversed towards the peripheral retina [128]. Lutein and Z, and hence MP, are thought to reduce oxidative damage by filtering short wavelength blue light within the macula [123] and by quenching light-induced singlet oxygen and related free radicals [129].

Figure 1.6: A cross section through the monkey fovea demonstrating anatomy of the central retina – source Webvision, http://webvision.med.utah.edu/imageswv/monkfov.jpeg (with permission).GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, Henle f.l. = Henle fibre layer.

Relationships between AMD and low levels of L and Z concentrations within the body have been discovered [99, 130] and high intake of foods rich in L and Z are associated with a decreased likelihood of developing ARMD [119, 120]. Lutein supplementation has been correlated with improved visual function in healthy eyes and eyes with ARMD [101, 102, 104, 131-134]. Indeed, it has been suggested that increasing retinal antioxidants may improve the function of damaged photoreceptors [135].

Vitamin E, another antioxidant, is located in the photoreceptor outer segments [136] which may also provide some protective effect on the retina [137]. Alpha-tocopherol is the form of vitamin E that is preferentially absorbed and accumulated in humans [138]. Its deficiency is linked with damage of the retina [139], an increase in RPE accumulation of lipofuscin [140] and an increase in lipid peroxidation [136]. Vitamin E supplementation has improved the survival of photoreceptor cell nuclei in the retinae of a rat strain developed by the Royal College of Surgeons [141]. Rats deficient in vitamin E showed attenuation of the flash electroretinogram with reduced a- and b-wave amplitudes and delayed latencies [142]. A significant protective effect of alpha-tocopherol against the risk of developing ARMD was shown in a study of 226 cases of ARMD [143] and in the Pathologies Oculaires Liees a l'Age (POLA) study, although

this association was not found in other studies [94, 97]. Lipofuscin formation has been shown to decrease in rabbit and bovine RPE cells when supplemented with vitamin E [144]. Mice fed on a vitamin E deficient diet had accelerated degenerative changes in the retina compared to mice on vitamin E supplemented diets [145].

Vitamin C or L-ascorbic acid was found to reduce oxidative stress within rat retinae by inhibiting oxidation of rod outer segment membrane lipids during intense light which reduced photoreceptor cell loss [146]. Reduced phototoxic injury was also demonstrated in rat retinae in a more recent study when vitamin C was administered [147]. Ascorbic acid deficiency was also shown to increase oxidative stress in guinea pig retinae [148]. Vitamin C combined with vitamin E, carotenoids and selenium showed a significant association with a decreased likelihood of neovascular AMD in the EDCCS [130]. Another study assessing supplementation with vitamin C, vitamin E, zinc and beta-carotene found a 35% reduced risk of developing ARMD [122]. The POLA study found no association between vitamin C serum levels and reduced risk for ARMD development [121].Vitamin C was shown to protect against hypoxia induced cell apoptosis in bovine RPE cells [149].

Beta-carotene, a precursor to vitamin A has been linked with an increased risk of lung cancer in smokers [150] and is not included in the nutritional supplementation used for this study. However it has been found to provide a reduced risk in the progression of AMD when combined with zinc, vitamin C and vitamin E in a randomised controlled trial undertaken by the Age-related Eye Disease Study (AREDS) group [100]. This large scale study of 3640 participants showed that those with extensive intermediate drusen, large druse or non-central GA in 1 or both eyes, or advanced AMD in 1 eye should consider taking a high-dose supplement with vitamin C and E, beta carotene and zinc. A protective effect of beta-carotene, ascorbic acid and alpha-tocopherol against risk of developing ARMD was found in another study of 226 cases of ARMD [143]. However a protective effect of beta-carotene was not demonstrated in a further study [97]. The Blue Mountains eye study reported an increased risk of incident neovascular AMD associated with higher beta-carotene intake [151].

Docosahexaenoic acid (DHA) is a dietary omega-3 long chain polyunsaturated fatty acid (LCPUFA) from fish oil that cannot be made by the body but can only be obtained through the diet. It is located in the outer segment membranes of retinal photoreceptors [112] and is important for maintaining photoreceptor outer segment membrane fluidity [152]. Rod and cone

photoreceptors uptake DHA which is used as a building block for producing outer segment discs [18]. Increased intake of polyunsaturated fatty acids has been linked with a reduced incidence of ARM and reduced risk of progression from ARM to advanced AMD [108-111, 113, 115]. It is thought that omega–3 LCPUFAs may protect the retina against damage from ischaemia, light, oxygen, age and inflammation [111]. Neuroprotectin D1 is a bioactive product of DHA which protects RPE cells from oxidative stress [153]. Deficiency in DHA may lead to reduction in rod sensitivity and recovery with age [154]. Rhesus monkey retinas deficient in essential fatty acids have been shown to have abnormal full field electroretinograms (ffERG) with delayed rod implicit times and rod recovery [154]. The Inuit population have a diet high in fish oil, although paradoxically, the incidence of ARM and AMD was found to be higher in this population than in most other populations studied [155]. The authors did not provide possible mechanisms for the higher incidence of ARM and AMD in this study. The Inuit diet has been shown to have low fruit and vegetable consumption, giving rise to dietary deficiencies in antioxidants [156], which as already discussed, may be linked with an increased risk for developing ARMD.

Copper and zinc are trace minerals that are essential for retinal health. Oral supplementation of zinc oxide and copper was shown to reduce the risk of progression to advanced AMD in eyes with extensive intermediate drusen, large drusen, non-central GA and in fellow eyes of participants with AMD in one eye ($p = 0.009$) [100]. Zinc content in the macular RPE is reduced in ageing and ARMD [157]. It is found throughout the retina [158] and in the synaptic terminals of the photoreceptors which when released from the synapse, may modulate photoreceptor activity in vertebrates [159, 160]. Reduced zinc serum levels have been associated with AMD [161]. In the rat retina reduced zinc has been associated with increased oxidative stress [162] which, in the human retina, is associated with an increased risk for ARMD development. Excessive zinc consumption can lead to copper deficiency and thus copper is often found in supplements containing zinc. Copper is found in photoreceptor inner segments. Mouse retinae with copper, zinc superoxide dismutase deficiency show features of AMD [163] and a reduction of copper and zinc has been identified in human eyes with AMD [164].

The fact that nutrition is a modifiable risk factor in the pathogenesis of ARMD means that there are active steps that can be taken to potentially reduce the risk of developing the disease or to slow the progression of disease.

1.12 Measures of visual function in ARMD

In the majority of clinical studies of ARMD visual acuity (VA) is used as a measure of macular function. However this is far from ideal as it assesses one small area of the retina, does not provide an overall measure of macular function and relies on subjective patient responses. It may be preferable to undertake a combination of objective and subjective testing to assess how the disease affects other measures of visual and retinal function.

Subjective measures of visual function

Subjective cone-mediated tests that have shown attenuation in ARM include reduced distance and near VA, contrast sensitivity (CS), visual field loss, slowed cone adaptation dynamics, reduced microperimetry sensitivity, decreased foveal sensitivity and colour vision deficiency [165-181]. Administration of a lutein-based (L-based) supplement has shown some improvements in subjective measures of visual performance in eyes with AMD but not ARM [101, 102, 104] and effects of supplement withdrawal were not assessed in these studies. Slowed rod adaptation kinetics have been shown to be affected at least as much as (some say more than) cone adaptation kinetics [76, 182]. Contrast sensitivity and VA are cone-mediated measures and the functional status of cone photoreceptors can provide information about changes in the RPE and Bruch's membrane [183] as is seen in ARMD.

Objective measures of visual function

Objective measures of cone function have shown sensitivity to abnormality in ARMD, including the focal electroretinogram (fERG) which measures single areas of retinal function and the multifocal electroretinogram (mfERG) which measures many single areas of outer retinal function simultaneously. A review of the literature demonstrates that both tests provide a direct measure of retinal function and are a useful means to diagnose and monitor disease progression [184] (see full review published in Documenta Ophthalmologica – appendix 3). The RPE, Bruch's membrane, choriocapillaris and photoreceptor layer are affected in ARMD, and are all situated prior to the bipolar cell layer. Any damage to or before the bipolar cell layer as occurs in ARMD, will reduce the amplitude of the first order kernel of the conventional cone-driven mfERG [185]. The mfERG is a technique that can distinguish between inner and outer retinal disease such as ARMD, as inner retinal disturbance to amacrine and ganglion cells has shown no effect on mfERG amplitude with only a small effect on the mfERG waveform [185].

Improvements in central retinal function as determined by increased fERG and mfERG amplitudes have been witnessed in eyes with ARM when L-based supplementation was administered [103, 134], suggesting that antioxidant therapy may reduce outer retinal dysfunction in ARM. However, in these studies the amount of dietary L and Z, self-supplemented L and Z or retinal accumulation of L and Z (macular pigment optical density, or MPOD) were not quantified. Intervention with supplemented and dietary increases in L and Z have shown increases in MPOD in eyes with ARM [186, 187] in some studies but not in others [188].

1.13 Research rationale

Age-related macular degeneration is the leading cause of irreversible blindness in the western world [4], with the number of cases expected to increase as the population ages. Age-related maculopathy is an earlier stage of the condition that predisposes patients to visual loss, characterised by drusen (≥63μm) with or without RPE changes [34]. Age related macular disease is a term used to encompass both early and late stages of the disease (ARM and AMD). Although the pathogenesis of ARM is still not fully understood, genetic predilection together with environmental factors are implicated.

There are many modifiable and non-modifiable factors that have been inconsistently linked with increased risk for developing ARMD. Age, smoking and genetic susceptibility are largely consistent factors associated with an increased risk for developing the disease. Because it is purported that oxidative stress is a feature in the aetiology of the disease, research into the role of antioxidants such as L and Z may provide important answers for reducing risk of disease development and progression.

The potential benefits of L and Z supplementation on visual function require further investigation. Randomised-controlled trials (RCTs) are the most stringent methods to evaluate whether a cause-effect relationship exists between intervention with L and Z supplementation and non-intervention [189].

As subjective measures of visual function are prone to participant inconsistencies, objective measures of visual function may provide additional information when assessing ARMD.

Review of the literature provides evidence that the mfERG is an objective, sensitive tool for assessing retinal changes in ARMD and for monitoring effectiveness of clinical intervention in this disease. Indeed, mfERG measures were improved with lutein-based supplementation in eyes with ARM in a previous study, although retinal or dietary levels of L and Z were not quantified [103].

The limited treatments available for ARMD give greater impetus for the need to identify the disease earlier and assess treatments to slow disease progression. Improvements in visual function in ARM eyes taking a L-based supplement may imply a purpose for nutritional supplementation for delaying the onset and progression of the disease, and even remission of symptoms. Improvements in healthy eyes may be suggestive of a preventative or disease-delaying therapy, especially pertinent to those with a family history of ARMD or vulnerable to additional risk factors.

The aim of this study was to assess the effects of a L-based nutritional supplement on objective and subjective measures of visual function, and compare these to untreated eyes. The effects of supplement withdrawal were also assessed. This was undertaken in HY, HO and ARM-affected eyes.

The primary outcome measure for the trial was the N1P1 amplitude of first order kernel of the conventional mfERG. The rationale for this trial was to expand on the work done by the Carotenoids and antioxidants in age-related maculopathy study (CARMIS) investigators [103], who found that an L-based supplement increased central ring 1 and 2 mfERG N1P1 amplitudes after 6 and 12 months in 15 eyes with ARMD compared with 12 eyes of non-treated participants. Because the CARMIS investigators found improvements at 26 weeks of supplementation with 10 mg daily dose of lutein, a 12 mg daily dose of lutein was assessed over 20 weeks and 40 weeks for this study.

L-based supplement on mfERG N1P1 amplitude for healthy eyes or for different ages were not assessed by the CARMIS investigators. Modifiable and possible preventative strategies for reducing the risk of ARMD development and progression are of importance, hence the rationale for inclusion of healthy eyes in this trial.

The CARMIS investigators did not report whether mfERG N1, P1 and N2 latencies or other measures of visual function were affected by nutritional supplementation. Participant dietary levels of L and Z were also not quantified and may have contributed to the improvement in mfERG amplitudes rather than the supplement alone. Furthermore, retinal levels of L and Z (MPOD) were not measured throughout their study to determine whether increases in retinal L and Z were associated with the increases in mfERG amplitude.

Therefore, for this trial, secondary measures were first order mfERG N1, P1 and N2 latencies, retinal levels of L and Z (MPOD), VA and CS. Also, dietary analysis of the components found within the nutritional supplement were assessed over the study period to determine whether changes in visual function were due to dietary changes or could be attributed to the L-based supplement.

The hypothesis is that a lutein-based supplement may provide improvements in objective and subjective measures of visual and retinal function in healthy eyes and in eyes with early ARMD (ARM). Also, withdrawal of the supplement may show decreases in these measures.

1.14 Chapter one summary

The aim of this thesis is to extend on previous work by undertaking a RCT (study design methods discussed in chapter 3) investigating the effects of a L-based nutritional supplement on mfERG, CS, VA and MPOD in ARM eyes and healthy eyes (results in chapter 6), and to investigate the effects of withdrawal of the supplement on these measures in ARM and healthy eyes (results in chapter 7). Dietary levels of L and Z were also investigated so any findings could be clearly delineated as being due to the L-based supplement rather than dietary changes of L and Z. A literature review established the pertinence of the mfERG as a sensitive tool for objectively assessing ARMD and for demonstrating improvement with clinical intervention. A research rational for the study was put forward. Chapter 2 describes the literature review of the use of differing electroretinogram modalities as tools for diagnosing, monitoring and assessing treatment effects in ARMD.

Chapter 2: The role of electroretinography in ARMD

2.1 Introduction

In the majority of clinical studies of ARMD, visual acuity is used as a measure of macular function. However this is far from ideal as it does not provide an overall measure of macular function and relies on subjective patient responses. It is important to identify individuals who are at most risk of developing the ARMD, so that interventions and lifestyle modifications can be targeted appropriately. In asymptomatic eyes, sensitive, reproducible measures of macular function are important to highlight the earliest signs of ARM. The objectivity and the topographical mapping that multifocal electroretinography (mfERG) provides allows for assessment of localised retinal cell function deficits in ARM over time. Evidence also suggests that mfERG can be used to monitor effectiveness of surgical and clinical intervention. This review enumerates the efficacy of various types of electroretinogram (ERG) for assessing retinal function in ARMD.

2.2 Full field electroretinogram (ERG)

The full-field or flash ERG (ffERG) generally consists of a negative deflection, called the a-wave, which is mainly associated with the photoreceptors and a positive b-wave, thought to be produced by ON bipolar cell depolarisation. Scotopic ffERGs elicit rod–dominated photoreceptor function responses and photopic conditions elicit cone function responses. A 30 hertz light stimulus ERG also provides information about cone function, whilst oscillatory potentials reflect amacrine cell function [190].

The ffERG gives a massed retinal electrical response to a light stimulus but does not provide spatial retinal information. Thus small retinal lesions such as those seen in ARM may be missed by ffERG and so there have been contradictory findings reported for the appropriateness of using ffERG in the assessment of the condition [191-194] Holopigian *et al*., examined the effect of normal aging, ARM and AMD on the ffERG. They demonstrated reduction of amplitudes and increase in implicit times of the ffERG over time with increasing age for normal subjects, as well as those with ARM and AMD under photopic and scotopic conditions [194]. Similarities between older normal subjects and those with ARM highlight the importance of using age-matched normals for comparison when observing older subjects with ARMD. These results correlated with the findings of other studies showing a decrease in rod and cone ERG amplitudes with age [195] and a slowed inactivation of phototransduction in rod photoreceptors

with age when measuring the recovery of the a-wave using a paired flash ERG technique [191]. Marcus et al., [193] studied the b-wave of the ERG in 24 eyes of 12 subjects with ARMD and found low to normal b-wave amplitudes in diseased eyes and no correlation with clinical morphology. However, there was no control group comparison.

Along with reduced and delayed photopic cone-dominated a-wave responses, scotopic rod-dominated a- and b-wave responses have been examined in ARMD using the ffERG. These were also reduced and delayed, suggesting that ARMD affects rod as well as cone photoreceptors [192]. A review by Scullica and Falsini [196] identified that studies of retinal function in ARM and AMD have found substantial impairment of rod photoreceptor function. Inconsistency exists in the literature about the value of ffERG in ARMD. As the ffERG is a massed response the sensitivity of the test is limited when trying to assess small lesions as observed in ARM [197].

2.3 Pattern ERG

The pattern ERG (PERG) occurs with pattern reversal stimulation, typically a checkerboard pattern of mean overall luminance which isolates nonlinear retinal responses while cancelling linear responses [198]. This gives a direct measure of ganglion cell function and allows discrimination between optic nerve and macular disease [199]. Pattern ERG abnormalities have been seen in AMD and reduced PERG P50 amplitudes have been observed in maculopathies [199]. The PERG has been shown to be abnormal in macular dysfunction when there is no detectable change in the ffERG [200]. Since the PERG measures ganglion cell function [199] and ganglion cell function remains relatively preserved in AMD [201] it may be argued that PERG does not provide sufficient information about retinal function in ARMD, especially in ARM when only small areas of drusen are seen. The PERG elicits an inner retinal response whereas ARMD is a retinal degeneration primarily affecting the RPE and the choriocapilliaris [194].

The PERG has been effective at assessing retinal function in AMD when clinical interventions are undertaken. Neveu *et al.,* [202] demonstrated that a detectable PERG prior to photodynamic therapy (PDT) in eyes with choroidal neovascularisation (CNV) was the single best indicator for improvement in visual acuity following treatment. However in ARM the area of retinal lesion is usually smaller than the area of PERG recording making the PERG less effective at monitoring

ARM compared to angiography. The PERG fails to provide any spatial retinal information. Another study examining PERG at the early stages of PDT for CNV showed a reduction in amplitudes and delay in latencies of the P50 and N95 soon after PDT which resolved at one month [203]. Mackay *et al.,* [201] assessed longer term changes to the PERG over twelve months and found that the P50 and N95 amplitude reduced over the twelve months, without recovery. The P50 latency reduced over the year but then increased at twelve months. However this finding was not statistically significant.

2.4 Focal ERG

Unlike the ffERG, the focal ERG (fERG) has the ability to specifically stimulate the fovea - useful when evaluating macular disease [204, 205]. A flickering light stimulus is utilised to stimulate the macular region and measures macular cone photoreceptor and bipolar cell function [206]. Although there is no international procedural standard set for undertaking this ERG technique, a number of varying techniques for recording fERG have been described in the literature. Differing field sizes (3° to 18°) and light stimulus frequencies have been used. Seiple *et al*., [207] examined retinal function in ARMD using a range of stimulus frequencies (10-60 Hertz). This work showed fERG amplitude losses at high and low frequencies in patients with macular disease, but relative sparing of the mid-temporal frequencies although the type and severity of ARMD was not noted within the results or separated out from other macular diseases.

Research on fellow eyes of patients with unilateral CNV has suggested that these eyes have a normal foveal cone number but abnormal cone function, indicated by delayed implicit times. Results were adjusted for age, gender, iris pigmentation and spherical equivalent. [208] Another study confirmed these findings and is postulated to be due outer retinal ischaemia [209]. Normal fERG phase but decreasing amplitudes have been documented with increasing severity of non-exudative AMD [210].

Nutritional supplementation and its effects on macular function have been investigated in subjects with ARM and age-matched normals. Falsini and colleagues [134] found that at 180 days, normal and ARM eyes showed an increase in fERG amplitudes after lutein, vitamin E and nicotinamide supplementation. At 360 days amplitudes were maintained.

Binns and Margrain [211] proposed a fERG photostress modality to examine retinal function in ARM, using intense light adaptation to bleach the retina, followed by periodical fERG to examine the recoverability of retinal function and determine the rate of photopigment regeneration. Retinal function recovery rate was slower in those with ARM compared to controls suggesting impairment in the ability of the outer retina to regenerate cone photopigment.

The effect of age on the fERG has been detailed, showing decreasing amplitude with increasing age [205, 208] and increasing fERG implicit times [208], stressing the need for age-matched controls when investigating ARM and AMD with fERG.

Focal ERG is useful for assessing retinal function in ARM and AMD. However, good fixation is required and it is limited to a single area, thus giving no information about multiple areas of retinal function. Also, no international procedural standard has yet been determined.

2.5 Multifocal ERG

The multifocal ERG (mfERG), developed by Sutter and Tran [212] is based on a pseudorandom M-sequence stimulation technique that allows simultaneous recording of ERGs from many retinal areas at once [213]. Like PERG, fERG and ffERG it is an objective measurement of retinal function but in contrast to these tests the mfERG allows simultaneous measurements of multiple responses at different retinal locations [214, 215]. The first order kernel of the mfERG waveform comprises of a negative N1 component, a positive P1 component and a negative N2 component. Hood's very comprehensive literature on mfERG suggests that mfERG responses are similar to the ffERG in that the N1 of the first order kernel of the mfERG is cone-driven (as in a photopic ffERG) and that the P1 contains responses from the bipolar cells [216]. The mfERG is dominated by bipolar cell activity and so a disease that substantially decreases the mfERG amplitude must therefore be acting at or before the bipolar response [190]. The first order kernel is the most commonly measured parameter of the mfERG. The mfERG also contains a second order kernel which originates from induced components of the inner retina, more details of which can be understood in the work of Sutter. [217-219]. The mfERG maps retinal function within the central 30-50º of the retina and good fixation is required for accurate results, suggesting it may be better suited to assessment of retinal function in ARM where central vision is preserved.

Various studies have reported the efficacy of the mfERG in the assessment of ARMD [103, 134, 169, 201, 202, 214, 220-238]. Eyes with ARM have been found to have reduced foveal mfERG P1 amplitudes and increased N1 latencies when compared with age-matched normal controls [227]. Interestingly, asymptomatic fellow eyes of the ARM eyes in this study also exhibited the same findings, suggesting that the mfERG may be a sensitive means of detecting early ARM changes. Feigl *et al.,* [239] in contrast, found no such correlation, possibly attributable to non-uniform use of grading and classification systems and varying age range of the participants used in the different studies.

Research comparing the mfERG between exudative AMD, non-exudative ARM and normal controls has been undertaken [225] demonstrating reduction in the P1 and N1 amplitudes of both CNV and ARM eyes when compared with controls. However, although the average age of the CNV and ARM groups were similar in this study (64.4 years and 66.5 years respectively), the control group was younger (57.7 years). This may account for some amplitude reduction due to aging influence on the mfERG [240, 241]. Seiple *et al.,* demonstrated a significant linear relationship of 10.5% reduction in the N1 to P1 amplitude per decade [242], emphasizing the importance of using age-matched controls when interpreting mfERG results.

The mfERG guidelines described by the International Society for Clinical Electrophysiology of Vision (ISCEV) [243], measures cone-function. However rod-mediated mfERG can be recorded after dark adaptation. It is time consuming with poorer signal-to-noise ratios than cone mfERGs [244]. A study to compare rod- and cone-mediated mfERG in ARM showed reduced N1 and P1 amplitudes in ARM when compared to controls in both rod- and cone-mediated mfERGs [228]. Delayed rod-mediated mfERG P1 implicit times in ARM eyes were reported when compared with age-matched normal controls [230, 233, 234], implying that both rod and cone-function is affected in ARM. However further work using larger sample sizes would provide more definitive rod mfERG information, as to date conflicting results have been demonstrated.

Interesting work has been undertaken on the use mfERG to investigate the role of ischaemia in ARM. Hypoxia has been experimentally induced in younger and older healthy eyes resulting in a reduction in central and peripheral neuroretinal function indicated by reduced mfERG response densities [222, 245, 246]. This supports the hypothesis that post-receptoral vulnerability occurs during reduced oxygenation and ischaemia [233, 247].

Differing mfERG paradigms have been used to evaluate rod and cone systems in ARM. Global-flash mfERGs have been used in an attempt to overcome some of the conflicting findings that have been observed with the conventional mfERG and to better reflect adaptation deficits in ARM [231]. The findings indicate that the global-flash mfERG detects reduced adaptation responses before the conventional mfERG. Thus, it could be argued that global-flash mfERG may be more beneficial in identifying ARM sooner than the standard mfERG. More research is necessary to consolidate this hypothesis.

The slow flash mfERG is another paradigm that has been subtracted from the conventional fast flicker mfERG to assess nonlinear adaptive components within the retina in ARM [229]. Interestingly, neither conventional mfERG nor slow flash mfERGs discriminated between the ARM group and age-similar controls. However, the difference between the two paradigms showed a reduced late component waveform in the ARM group compared to the control group suggesting postreceptoral adaptation abnormalities in the ARM group.

Comparison between the conventional cone-mediated mfERG response and morphological changes in ARM have been examined [169, 221, 248], with outcomes suggesting slight increase in delayed implicit time with drusen progression, drusen regression with increasing RPE changes and in stable drusen. After more than two years the responses became more delayed with reduced response density. The mfERG changes were not limited to the drusen areas in these studies, suggesting retinal function does not correlate directly with morphology in ARM

The objectivity of the mfERG in assessing retinal function correlates well with subjective macular function tests in ARM and AMD [220], such as colour vision and microperimetry testing [169] and suggests they may be significantly related to retinal function in the cone-mediated mfERG in ARM.

2.6 Multifocal ERG in clinical intervention studies

mfERG has been investigated as a tool for assessing retinal function before and after PDT, a widespread treatment for predominant classic CNV prior to the introduction of intravitreal ranibizumab and bevacizumab.

Palmowski *et al*., [237] compared retinal function pre- and post-PDT in 16 eyes and found that after PDT the mfERG showed focal improvements in 13 eyes. In 10 eyes focal retinal function deteriorated in some locations. At fifteen weeks some areas of the mfERG did not demonstrate any improvement when compared with mfERGs obtained at three weeks post-PDT. They concluded that improvement in parafoveal function can be seen with mfERG and deemed it a suitable tool in assessing retinal function in AMD.

Short-term mfERG changes were assessed in 17 eyes with CNV before and after PDT with verteporfin by Jiang *et al.,* [236] The mfERG latencies and response amplitude densities remained largely unchanged within three days, and at one week post-PDT with verteporfin when compared with pre-PDT mfERG. Their data suggests that verteporfin therapy may not result in adverse effects within the outer retina, in contrast to other histopathological studies assessing verteporfin and PDT in the monkey retina [249].

Multifocal ERG recordings were performed before PDT, and at four days, two weeks and one month after PDT with verteporfin in a study by Lai *et al*., [223]. Their research showed a transient impairment in retinal function that resolved after one month. In contrast to the work by Jiang *et al*., there was a change at four days post-PDT with reduced N1 and P1 response amplitude densities and increased P1 latencies. However Lai's study contained only three AMD eyes with CNV, the other fourteen eyes being a mixture of myopic CNV, idiopathic CNV, polypoidal choroidal vasculopathy and central serous chorioretinopathy.

Studies at baseline and six weeks post-PDT were assessed with mfERG by Ruether *et al*., showing a trend towards reduced P1 amplitudes and delayed latency, although these effects were not statistically significant and comparison to a non-treated group did not occur [250].

Catala-Mora *et al*., [251] observed mfERG changes over a longer period post-PDT in twenty-three eyes. At two and six months after treatment the N1 and P1 amplitudes did not change and even improved in the more peripheral areas tested. They concluded that mfERG offers interesting non-subjective information about retinal sensitivity in macular diseases treated with PDT.

Mackay *et al.,* [238] examined the use of mfERG as a predictor of vision maintenance post-PDT in neovascular AMD using logistic regression models. Patients with an average of 6/30 vision were less likely to respond to PDT than those with poor vision. Relatively good contrast sensitivity and large central mfERGs increased the probability of a response to PDT. In a further study, mfERG was assessed in pre- and post-PDT in CNV. They found P1 response amplitude density increased at six months and then returned to baseline at twelve months [201]. Work undertaken by Moschos *et al.,* demonstrated that although at six months post-PDT seventy percent of vision remained stable, there was a demonstrable reduction of mfERG response amplitude density, highlighting the need for objective measures of retinal integrity when monitoring the efficacy of clinical intervention [252].

Feigl *et al.,* used the rod-mfERG and cone-mfERG to determine the effects of multiple PDT treatments in a case report of five eyes showing transient reduction in cone-mfERG waveforms and then recovery over time in all eyes and similar findings for rod-mfERG in four out of five eyes. However baseline rod-mfERG responses were poor, very small and the test demanding for patients [253].

With the recent implementation of costly intravitreal ranibizumab treatment for AMD with CNV, greater impetus has been given to looking at ways of diagnosing, monitoring and treating AMD earlier to preserve vision. In a study of nine patients who received intravitreal bevacizumab for the treatment of exudative AMD, mfERGs were performed pre- and post-treatment [235]. A linear relationship was found between visual acuity and P1 response amplitudes, suggesting that bevacizumab improved retinal function. mfERG parafoveal retinal response density improvement three months post-bevacizumab has been demonstrated in research by Moschos *et al.,* [254] when studying eighteen eyes with CNV. Correlation between mfERG retinal response density in the central 15 degrees and retinal thickness has been demonstrated in four eyes post-bevacizumab treatment, resulting in improvement of mfERG macular function responses with reduction in retinal thickness as measured by optical coherence tomography, although in this study ISCEV mfERG guidelines were replaced with a customised experimental m-sequence technique [255]. Ranibizumab efficacy has been investigated in a small study of three eyes using mfERG with a reduction in central and peripheral amplitudes being evident after three treatments when compared to age-matched normal eyes [256]. Further studies are required in the area of anti-vascular endothelial growth factor (anti-VEGF) agents,

both singularly and in combination treatments, to test the efficacy of different dosing regimens and the mfERG could have an important role for assessing retinal function within this area.

The role of nutrition in retinal health may prove to become an integral part in the management of ARM. Parisi *et al*., [103] looked at the influence of short-term carotenoid and antioxidant supplementation on retinal function in ARM. They demonstrated that in the central five degrees, ARM subjects treated with nutritional supplementation showed an increase in mfERG amplitudes after six months when compared with controls.

2.7 Chapter two summary

Sensitive but reproducible tests of macular function will become more important to detect the earliest signs of ARM. The objectivity and the topographical mapping of the mfERG allows localised retinal functional deficits in ARM to be observed over time. Evidence also suggests that mfERG can be used to monitor effectiveness of surgical and clinical intervention. Chapter 3 describes the randomised controlled trial clinical intervention design, using mfERG as the primary outcome measure.

Chapter 3: Study design

3.1 Randomised controlled trial design

Randomised controlled trials are the gold standard for detecting associations between interventions and outcomes [189]. The aim of this thesis is to investigate the effect of a L-based nutritional supplement on subjective and objective measures of visual function in healthy eyes and those with ARM, and to assess the effects of supplement withdrawal on these measures. A RCT was designed to investigate this research question and to attempt to make an original contribution to the literature in this area. The trial followed the Consolidated Standards of Reporting Trials (CONSORT) statement [257]. The CONSORT statement is designed to improve the reporting of RCTs and provides a 25 point checklist of required pieces of information when reporting randomised controlled trials (see table 3.1).

The study was a longitudinal, prospective, single-masked RCT. The principle investigator (EB) was masked from intervention and non-intervention assignment of participants. Aston university reception staff allocated participants to intervention or non-intervention groups depending on a randomly allocated number given by EB to the participants. The number was randomly generated using Microsoft (Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399 USA) excel random number generation.

This study compared the effect of a nutritional supplement on retinal function in 3 subgroups:

1) Under 50 years with healthy eyes (healthy younger, HY)

2) 50 years and over with healthy eyes (healthy older, HO)

3) 50 years and over with ARM (ARM).

For each group, participants were randomly allocated to an intervention or non-intervention group. The intervention group received a L-based nutritional supplement for 40 weeks, the remainder acting as controls. After 40 weeks, supplementation ceased. At 60 weeks only participants who were in the intervention group returned for assessment of the effects of

withdrawal of the supplement on visual function. Outcome measures were assessed at baseline (visit one), 20 weeks (visit two), 40 weeks (visit three) and 60 weeks (visit four – withdrawal).

Participants were asked to not discuss intervention with EB and were provided with another contact (HB) for contact to discuss concerns about supplementation. Tablets were provided free of charge by Bausch and Lomb (Bausch & Lomb House106-114, London Road, Kingston-Upon-Thames, Surrey KT2 6QJ, England). The study was also funded by Bausch and Lomb.

3.2 Participant recruitment

The study required the recruitment of people with and without ARM. Systems of recruitment included:

1. Leaflets for patients attending Aston Vision Sciences Department (appendix 4)
2. Leaflets for patients attending Aston University Day Hospital (appendix 4)
3. Information posters in Aston University and Solihull libraries (appendix 5)
4. Information poster at the Midland Eye Institute (appendix 5)
5. A research database composed comprising patients wishing to part in research from Aston University Optometry Clinic
6. Leaflets given to Professor Jonathan Gibson to hand to patients during a weekly clinical session at Aston University (appendix 4)

3.3 Research centre

The trial took place at a single research centre in the Vision Sciences department at Aston University. Enrolment, randomisation and data collection were carried out by EB. EB was masked to group assignment. HB provided the key code for randomisation. EB is a research ophthalmic physiologist and HB an optometrist and senior lecturer.

Table 3.1: CONSORT Statement 2010 25 item checklist.

Section/Topic	Item No	Checklist item	Reported on page/s
Title and abstract			
	1a	Identification as a randomised trial in the title	2
	1b	Structured summary of trial design, methods, results, and conclusions	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	39
	2b	Specific objectives or hypotheses	40, 41
Methods			
Trial design	3a	Description of trial design	41
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Not applicable
Participants	4a	Eligibility criteria for participants	58-60
	4b	Settings and locations where the data were collected	52
Interventions	5	Interventions for each group with sufficient details to allow replication	51,58

Table 3.1 continued.

Section/Topic	Item No	Checklist item	Reported on page/s
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	40,41,79-84
	6b	Any changes to trial outcomes after the trial commenced, with reasons	Not applicable
Sample size	7a	How sample size was determined	85-87
	7b	When applicable, explanation of any interim analyses and stopping guidelines	Not applicable
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	51
	8b	Type of randomisation	51
Allocation Concealment mechanism	9	Mechanism used to implement the random allocation sequence	51-52
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	52

Table 3.1 continued.

Section/Topic	Item No	Checklist item	Reported on page/s
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	51-52
	11b	If relevant, description of the similarity of interventions	Not applicable
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	113
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Not applicable
Results			
Participant flow	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	114,118,121,124
	13b	For each group, losses and exclusions after randomisation, together with reasons	66
Recruitment	14a	Dates defining the periods of recruitment and follow-up	62-64
	14b	Why the trial ended or was stopped	Not applicable
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	114,118,121,124

Table 3.1 continued.

Section/Topic	Item No	Checklist item	Reported on page/s
Numbers analysed	16	For each group, number of participants included in each analysis and whether the analysis was by original assigned groups	114,118,121,124
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	113-131,86,87
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Not applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	124-125
Harms	19	All important harms or unintended effects in each group	Not applicable
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	142-143
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	145-146
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	145-146

Table 3.1 continued.

Section/Topic	Item No	Checklist item	Reported on page/s
Other information			
Registration	23	Registration number and name of trial registry	67
Protocol	24	Where the full trial protocol can be accessed, if available	This thesis
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	52

3.4 Supplement composition

The supplement name was Ocuvite Duo, provided by Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK. The author has no proprietary interest in the supplement or company. The supplement was in tablet and capsule form. Participants took one capsule and one tablet together, once in the morning with water and this was repeated in the evening. The tablets and capsules were boxed and in blister packs. The nutrients contained within the supplement have been discussed in chapter 1 and are shown in table 3.2. All of the nutrients were within the safe upper levels as defined by the food standards agency [258].

Table 3.2: Supplement composition. All constituents were contained within the tablets except for omega 3 which was provided by the capsules.

Ingredient	**Safe upper levels per day [258]**	**Dosage per day**
Vitamin C	1000 mg (guidance only)	150 mg (per two tablets)
Copper	10 mg	400 µg (per two tablets)
Vitamin E	540 mg	15 mg (per two tablets)
Zinc	25 mg	20mg (per two tablets)
Lutein	Non established	12 mg (per two tablets)
Zeaxanthin	non established	0.6 mg (per two tablets)
Omega-3	non established	1080 mg (per two capsules)

3.5 Inclusion criteria

Suitability for inclusion was evaluated by questionnaire, fundus photographs and visual acuity. For inclusion participants had to provide written informed consent (see appendix 6) and were required to have:

For the early ARM, HO and HY groups in either eye or both eyes:

Best corrected visual acuity (VA) of 6/9.5 (Logarithm of the minimum angle of resolution, or LogMAR 0.2) or better (for good mfERG central fixation)

Clear optical media (as determined by fundal photography)

No signs of retinal or optic nerve disease other than ARM in the ARM group (as determined by fundal photography and questionnaire)

Good general health (as determined by health questionnaire)

No medication that affects the retina (as determined by health questionnaire)

For the ARM group in either eye or both eyes as defined by the international classification system described in chapter 1:

Drusen which are external to the neuroretina and RPE. They are soft and distinct or soft and indistinct. Hard drusen are not characteristic of ARM;

Or drusen with hyperpigmentation;

Or drusen with hypopigmentation

Rationale for ARM criteria

Without good central fixation mfERG results are prone to inaccuracies (as described in chapter 4). Thus eyes with AMD may be more susceptible to variable mfERG responses due to poor fixation. Therefore eyes with ARM were chosen for this trial. None of the participants progressed from ARM to AMD throughout the trial.

3.6 Exclusion criteria

Moderate to dense lens opacities

Intraocular lens

Corneal opacities

Glaucoma or ocular hypertension

Previous history of intraocular inflammation (e.g. uveitis)

Previous history of retinal detachment

Retinal disease (other than ARM in the ARM group)

Previous retinal laser

Diabetes

Systemic hypertension

History of ocular trauma

Neurological disease

Advanced AMD (CNV or GA) in the studied eye

Drugs causing retinal toxicity (chloroquine, cisplatin, oxazepam, vigabatrin)

Previous ocular surgery (excluding LASIK/EK)

Epilepsy

Intraocular lenses (IOLs) post-cataract surgery would not have necessarily affected the mfERG as it provides clarity where there was previous opacity. However as cataract occurrence increases with age, pseudophakic individuals were likely to be older participants which could not then be compared with younger phakic participants for baseline data (chapter 5).

Although mfERG has been undertaken to assess the use of vigabatrin on retinal function in participants with epilepsy [259], epilepsy was an exclusion criteria for this trial due to the flickering of the mfERG stimulus which may elicit an epileptic seizure, and due to some epileptic medication affecting the mfERG [259]. All other exclusion criteria were chosen as they may affect visual function or the mfERG.

Although unclear optical media can affect the interpretation of mfERG results [260] some participants for this study were over 50, thus it was not possible to completely exclude these effects. Therefore moderate to dense lens opacities were classed as an exclusion criterion. Participants with minimal nuclear sclerosis (N2 or less according to the lens opacities classification system II system [261]) were included in the trial, of which none progressed during the trial.

Food diaries (as described in chapter 4) were administered during the baseline visit and repeated at visit three to assess the dietary intake of L and Z of both the intervention and non-intervention groups. Health questionnaires were administered during the baseline visit (appendix 7) to assess smoking habits, past medical and past ophthalmic history, and again at visits 3 and 4 to assess whether smoking habits or health status had changed. Any participant changes in ocular or general health as per the exclusion criteria over the course of the study were excluded.

3.7 Data collection schedule

The participant schedule was composed, tested and modified to reduce participant visit time to increase efficiency and the likelihood that participants would return for further visits throughout the study:

1. Greet, call in and explain tests to participants, medical history, ophthalmic history, medication, cross check against inclusion / exclusion criteria. Obtain informed consent.

2. LogMAR VA – right and left (at first visit, then for the chosen study eye for subsequent visits)

3. Choose the eye with best corrected VA (at first visit and continue with the chosen study eye for subsequent visits)

Then on the inclusion eye only:

4. Macular pigment optical density measurement (MPS 9000, Tinsley Precision Instruments Ltd, Croydon, Essex, UK)

5. Check intra-ocular pressure (CT-80, Topcon, Newbury, Berkshire, UK)

6. Auto-refraction (NVision-K 5001, Shin-Nippon, Fukuyama, Hiroshima, Japan)

7. Contrast sensitivity at 1 metre (Pelli-Robson, Clement Clarke, Harlow, Essex, UK)

8. Dilate with Minims® 1% tropicamide (Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK)

9. Wait for a minimum of 20 minutes

10. Minims® Proxymetacaine 0.5% (Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK)

11. Multifocal ERG (Visual evoked response imaging system (VERIS) science 6.1, Electro-diagnostic Imaging inc, Redwood City, California, USA)

12. Check intraocular pressure post dilation

13. Slit lamp check for corneal and scleral integrity, and lens check

14. 45º Fundal photograph (TRC-NW8, Topcon, Newbury, Berkshire, UK)

15. Axial length measurement at visit three only (IOL master, Carl Zeiss, Welwyn Garden City Hertfordshire, UK)

16. Debrief and safety information regarding dilation

3.8 Recruitment and visit schedule

WEEKS	EVENTS
1-16	Preparation and organisation of resources
17-57	Recruitment and baseline measures
37-77	Participant second visits
57-108	Participant third visits. Unmasking occurs. Supplementation ceases
77-130	Participant fourth visits. Intervention groups only
130-156	Data analysis and thesis writing

Figure 3.1: Research timeline.

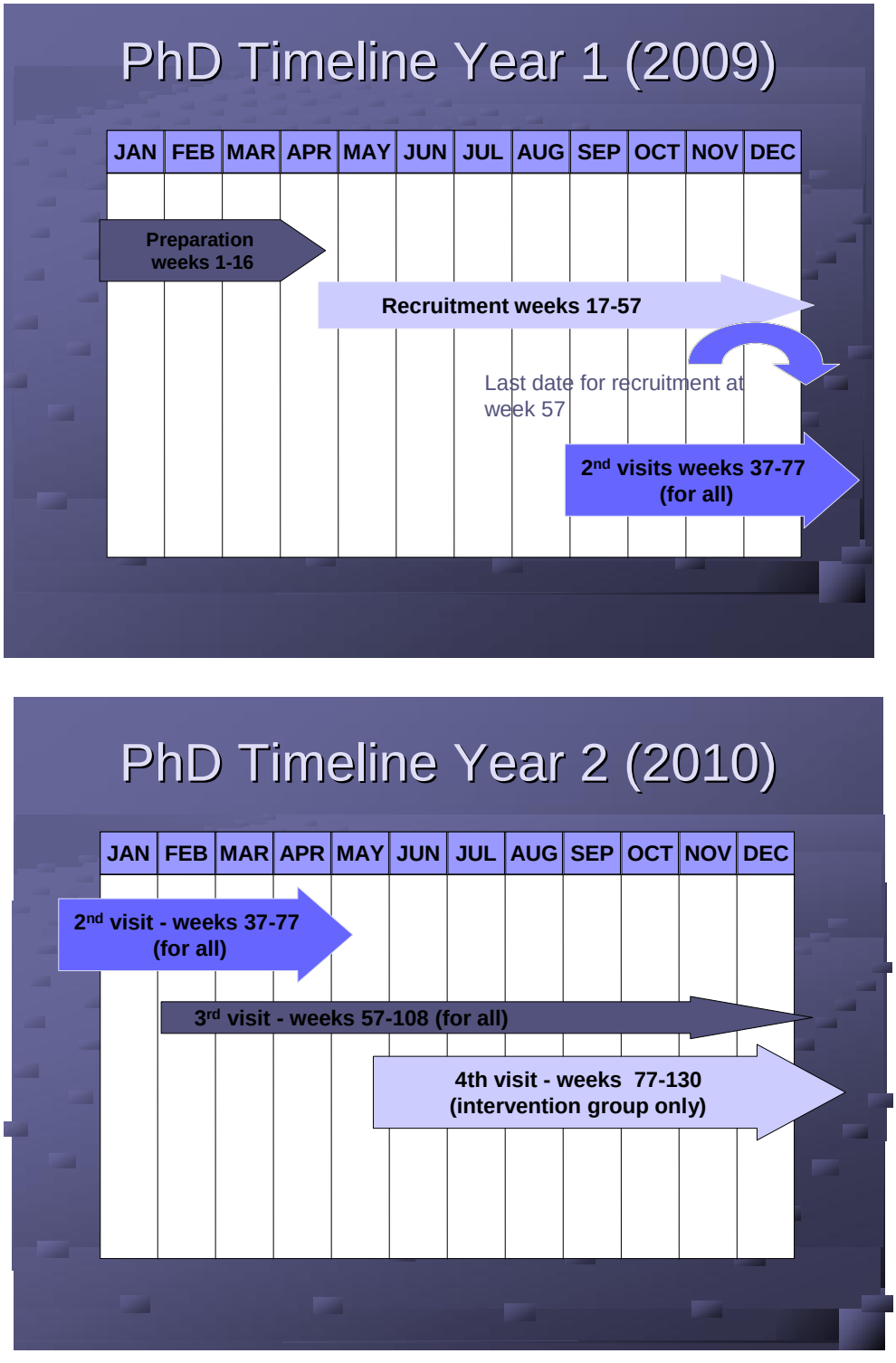
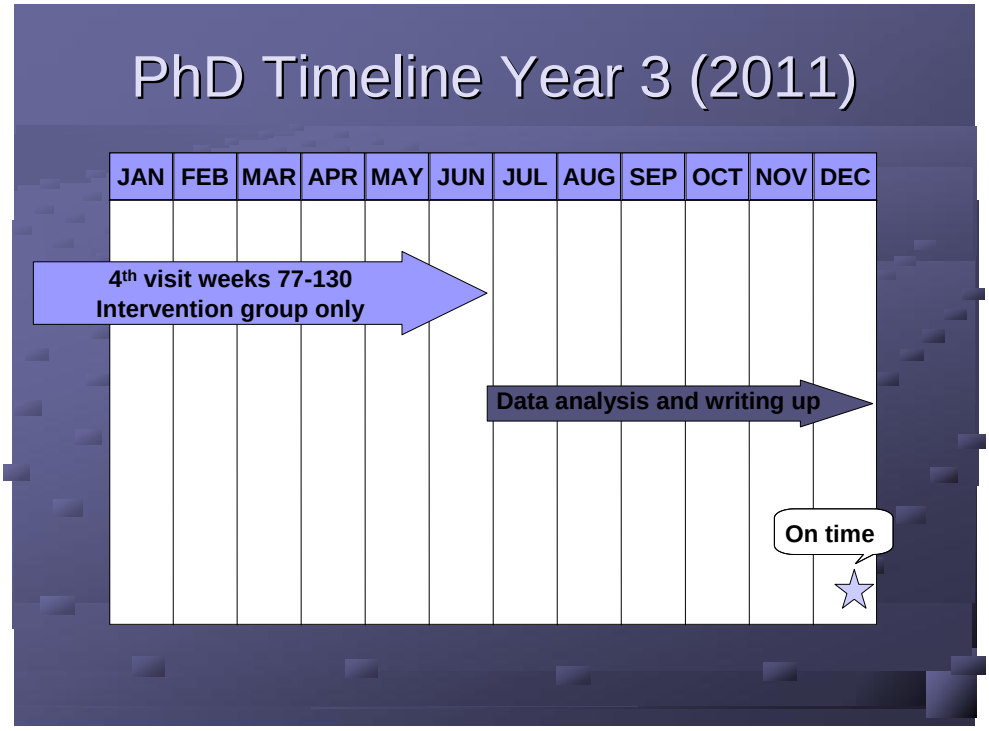


Figure 3.1: Research timeline continued.



3.9 Intervention compliance assessment

At visit 3 unmasking occurred. Participants were asked to return any boxes of the supplement that were not taken. Those who forgot to bring back the tablets were asked to contact EB after counting tablets at home. Patient compliance was elicited using supportive language to minimise the number of participants concealing supplement non-adherence [262], and reporting lower levels of remaining tablets than was actually the case. The sole reason for non-adherence was forgetfulness.

3.10 Adverse effects of intervention

Participants were contacted by telephone within the first month of receiving the intervention by HB to encourage compliance and ask if there were any problems taking the supplements. Participants were provided with a contact number (HB) to communicate any problems with the supplement. No adverse effects were reported with regards to the lutein-based supplement throughout the course of the trial or after the trial had ceased.

3.11 Enrolment and follow-up

Of the 112 that were assessed for enrolment, 81 met the inclusion criteria and underwent testing at visit 1. At visit 2 there were 69 participants remaining. At visit 3 there were 66 participants remaining (table 3.3). The study at visit 4 was designed to assess the effects of supplement withdrawal. Thus only those who were taking the supplement for visits 1,2 and 3 were required to attend for visit 4, of which there were 30 participants. Reasons for participant exclusion and withdrawal are laid out on table 3.4.

Table 3.3 Enrolment and follow-up numbers.

	HY	HO	ARM
Visit 1 (baseline)	37	27	17
Visit 2	32	23	14
Visit 3	30	22	14
Visit 4 (previously supplemented eyes only)	12	10	8

Table 3.4: Numbers and reasons for participant exclusion and withdrawal.

	HY	**HO**	**ARM**
Excluded			
Visit 1 - visit 2	1 Hypertension 1 Ocular hypertension	1 LASEK	0
Visit 2 - visit 3	0	0	0
Visit 3 – visit 4	1 Ocular hypertension	0	0
Withdrew			
Visit 1 – visit 2	1 Illness (from the treated group) 1 Migraine post mfERG (from the non-treated group) 1 Participant to contact (from the treated group)	3 Participant wanted to call back when appropriate for them and did not (2 from the non-treated group, 1 from the treated group)	1 No reason given (from the treated group) 2 Participant to contact (from the non-treated group)
Visit 2 – visit 3	2 Moved away (1 from the non-treated and 1 from the treated group)	1 Illness (from the non-treated group)	0
Visit 3 – visit 4	1 Illness	1 Illness	0

3.12 Resources

Ongoing technical difficulties with the VERIS mfERG system delayed the study by 3 months. Technical support was not available in the UK. The computer was remotely accessed by Electrodiagnostic imaging (EDI), San Mateo, California, USA, who managed to solve issues as they arose. These included 'slow error messages' and the VERIS software quitting when data analysis was undertaken. Due to differing time zones between UK and California, USA, prompt assistance was not always available. A fault not able to be rectified via remote access in March 2010 led to the computer being sent to the USA as per EDI request. The computer was not usable from March 2010 to May 2010. On return the fault remained. Thus due to technical difficulties mfERG parameters were not measured for all participants for each visit. After various new components were tried including a new switchbox, cabling and motherboard, the VERIS resumed operation.

3.13 Ethics

The research was approved by Aston University Human Sciences Ethical Committee (reference 300608/PF1 - see appendix 8**).** The tenets of the declaration of Helsinki and [263] the CONSORT checklist [257] were followed. The study was registered with International Standard Randomised Controlled Trial (ISRCTN) register (number 17842302).

3.14 Research Training

Refresher skills in electrophysiology were undertaken at the Birmingham and Midland Eye Centre with Dr Peter Good. Definitive mfERG techniques were observed at Nottingham Queens Medical Centre with guidance from Dr Yaqin Wen. Electrode insertion techniques were observed at Aston University and through online study.

3.15 Chapter three summary

This chapter described the RCT design used for assessing the effects of a L-based nutritional supplement on objective and subjective measures of visual function for this trial. The CONSORT reporting standards were met and described in conjunction with this trial. In the next chapter the protocol for obtaining primary and secondary outcome measures will be outlined, and factors affecting the primary outcome measure will be described.

Chapter 4: Outcome measures

4.1 Primary outcome measure - the mfERG

The mfERG was introduced almost two decades ago by Sutter and Tran [212]. As previously discussed the mfERG allows for simultaneous recording of electrical responses from many retinal areas at once. This provides a better understanding of the focal effects of disease processes on the outer retina and allows retinal areas to be grouped together for analysis, depending upon the disease of interest.

4.2 The mfERG stimulus

When undertaking the conventional cone-mediated mfERG, the retina is stimulated using a number of hexagons in photopic conditions. The hexagons are inversely scaled with cone density [16] in order to obtain an approximately uniform retinal response across the visual field being stimulated. The hexagons are commonly presented on a cathode ray tube (CRT) monitor. The drawbacks of CRT monitors include signal artefacts due to the time required for completion of raster scanning for each frame and time lags in phosphor decay [264]. Other stimulus presentation methods such as liquid crystal displays (LCD) and light emitting diode (LED) displays have been researched in an attempt to reduce some of these shortcomings but have their own limitations that need to be assessed before these methods are introduced [265, 266].

Each hexagon within the group of hexagons is independently controlled by a mathematical sequence called a binary m-sequence [213], where each hexagon independently has a 50% chance of being black or white in each frame. These sequences are computer-generated. A different, pre-determined sequence drives each hexagon within the group of hexagons. The sequences consist of a group of 0's and 1's which switch the hexagons to black or white for each frame. Each retinal response is derived from cross-correlating the raw signal data from the m-sequence controlling that hexagon sequence. Because the waveforms are very small, many signals are averaged together. Responses are obtained by adding all signals for when each hexagon is white (on) for that retinal area and subtracting all signals for when the hexagon is black (off) for the same area. Thus the resulting retinal response is a mathematical extraction rather than an immediate retinal response to the stimulus. Each frame is typically changed every 13.33 ms (75Hz) and appears as a random flickering of hexagons. A higher spatial resolution is obtained by increasing the number of hexagons stimulating the same area of the retina. This can

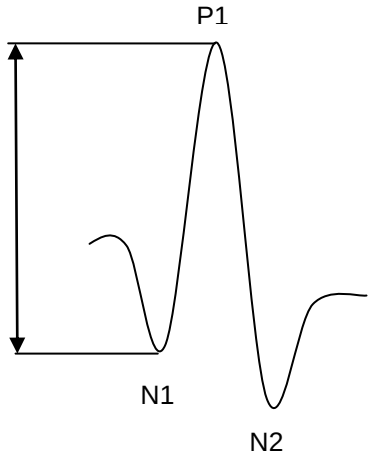
produce finer detail but at a cost of a poorer signal to noise ratio and longer testing times for patients [226].

4.3 The mfERG response

Because the magnitude of mfERG responses are very small (in the order of nanovolts), they have to be amplified. The waveform of the first-order kernel of the cone-mediated mfERG is comprised of an initial negative (N1), a positive (P1) and a second negative (N2) deflection

(figure 4.1). Work undertaken by Hood *et al.* using pharmacological dissection of the retinae of rhesus monkeys and comparison with human retinae demonstrated that N1 is generated by hyperpolarisation of OFF-bipolar cells with a lesser contribution attributable to photoreceptor hyperpolarisation [267]. ON-bipolar cell depolarisation with OFF-bipolar and photoreceptor cell recovery forms the leading edge of P1 with the trailing edge demonstrating ON-bipolar cell recovery [267]. Although there is paucity in the literature as to the cellular derivation of the N2, one study suggested it to be a composite response dominated by the interaction between two successive stimuli and the P1 component of a second stimulus delayed one frame from the first stimulus [268]. Another mfERG study, on porcine retinae found that the N2 is dominated by ON-bipolar cell activity and also shaped by OFF-bipolar cell activity although this needs to be interpreted with caution as there are retinal differences between primate and porcine retinas [269]. Hood *et al.* stated that the N1, P1 and N2 of human mfERG components are largely influenced by the different states of the bipolar cells [185].

Figure 4.1: A normal mfERG response. The double ended arrow demonstrates N1P1 amplitude (source - authors own drawing).



The second-order kernel is thought to be associated with inner retinal cell contributions [270, 271] with lesser outer retinal contributions [269, 272]. However, Hood *et al.* highlighted that these responses are non-linear, i.e. second-order and higher-order responses are influenced by retinal adaptation to successive hexagon flashes and thus cannot be linked directly to cellular responses but only to adaptive circuitry within the retina [185]. Sutter gives a more in-depth analysis of mfERG second-order kernels in his literature [218]. For the purposes of the L-based supplement study, and for the supplement withdrawal study, first-order kernel waveforms were assessed due to their ability to detect retinal dysfunction in ARMD.

The International society for clinical electrophysiology of vision (ISCEV) has published guidelines for recording cone-mediated mfERG in an attempt to reduce inconsistencies between laboratories [243]. These have been adhered to for the trial as described within the text of this chapter.

4.4 The mfERG analysis

Because mfERG signals are so small they are very susceptible to noise. Some studies have analysed retinal areas stimulated by a single hexagonal element [221, 273] although each waveform (trace array) from each retinal area stimulated by each hexagon is usually too noisy to reveal data of significance. Thus it is usual to group together traces from areas where the responses are considered to be similar, presenting accurate waveform estimates for different retinal regions. Traces can be grouped together in a variety of ways depending on the pathology of interest, for example a concentric ring analysis to examine ARMD due to the circinate nature of the disease. Once mfERG signals have been recorded the groups of averages can then be analysed by the computer software in several ways:

Normalised – Waveform amplitudes for each stimulus element (hexagon) in a chosen group are combined and then divided by the root mean square, providing the same SD and amplitude (approximately) for each group. This simplifies waveform comparisons for each group and is useful for latency comparisons between groups.

Sum of groups – Waveform amplitudes are added together providing a cumulative response for that group, allowing comparison of equal areas or for comparing the total retinal response of one eye to another (unit = nV).

Root Mean Squared (RMS) – The RMS is composed from the sum of squares, making this measure consistently positive. It compares each trace with its own template which reduces biasing but is sensitive to noise and can lead to overestimation of amplitude due to inability to discriminate between signal and noise. Any noise makes a positive contribution, falsely making amplitude larger.

Scalar product – This is a correlation between a template and an individual waveform and not a direct measure of amplitude or latency and this can be seen as a 3D topography. Waveforms are averaged over groups chosen to create templates. Then a calculation of the average of individual traces and the group template traces are correlated. This is less susceptible to noise than the root mean squared (RMS) method [274].

Response density scaled - is the scalar product divided by the area of the element or hexagon (units = nV/deg^2). Each trace is scaled to compensate for stimulus size and is an accurate representation of response amplitudes of hexagon group averages. Each hexagon is inversely scaled with the concentration of cone photoreceptors throughout the retina (response density scaled) to give similar amplitudes for each area stimulated [212]. The amplitudes of each waveform in a chosen group are added together and then divided by the hexagons within that group. This gives a scaled average group response to compensate for stimulus (hexagon) size, allowing a precise analysis of the response amplitude for each group. Values are unit area of stimulus per degree squared (nV/deg^2).This gives an accurate view of actual response amplitudes. Due to its accuracy this method of analysis was chosen for the purposes of our studies. This was also the method of analysis chosen in the CARMIS study [103].

5.5 Factors affecting the mfERG response

There are numerous technical and biological factors that have been shown to affect mfERG amplitude and latency [274, 275]. It is essential that when reporting mfERG findings in research and clinical settings these factors are controlled where possible or adjusted for when

uncontrollable. The use of stringent inclusion and exclusion criteria can minimise biological discrepancy but may reduce sample sizes and thus the power of research findings.

Electrode type and position

The type of electrode used has an impact on the size of the mfERG response with the largest amplitudes being recorded from contact lens electrodes [276], although these electrodes can give rise to corneal and conjunctival abrasion, especially when used for long periods.

For this trial Dawson Trick Litzkow (DTL) electrodes were used as they do not impede the optics of the eye unlike contact lens electrodes, and provide greater participant comfort while providing reproducible results [277, 278]. They are silver-impregnated microfiber corneal electrodes with small adhesive sponge pads at each end that are secured to the nasal and temporal canthi while the microfiber thread is positioned either along the lower lid, or draped into the lower fornix. The DTL electrodes used in this study were placed along the upper margin of this lower lid as per the ISCEV guidelines, which state that positioning the fibre into the fornix may reduce variability but results in smaller amplitudes [279].

Amplifiers and filters

Filtering is required to eliminate unwanted electrical noise from the mfERG signal but can attenuate the mfERG signal. Han *et al.* found that mfERG signal filtering restricted between 10 and 100 Hz contained less noise, a higher signal to noise ratio and less intersubject variability than signals filtered at 10-300 Hz. However Keating *et al.* recommend a bandwidth as wide as possible [274]. The mfERG first order kernel waveform is concentrated at around 19 and 47 Hz [280]. However, ISCEV guidelines recommend a high pass filter between 3 and10 Hz and a low pass filter of between 100 and 300 Hz. Our filters were set in line with the ISCEV guidelines and VERIS manufacturer (Electrodiagnostic imaging, Redwood City, California, USA) recommendation at 10-300Hz [243]. The CARMIS study used a 1-100 Hz bandpass filter in their study assessing mfERG amplitudes with a lutein-based supplement [103]. Amplifiers amplify mfERG signals prior to their conversion from analogue to digital. Although ISCEV do not provide a figure for amplifier gain they state that the amplifier should produce recognisable signals without saturation. Our amplifier setting was set at 100,000 as per the CARMIS study [103]. Our amplifier and filter settings remained constant for all participant visits.

Stimulus and background luminance

Stimulus type has already been described but the luminance level of the stimulus also affects mfERG waveforms. Therefore it is vital to periodically check the calibration of the stimulus luminance as per the ISCEV calibration guidelines when assessing differences in mfERG waveforms between groups and over time [281]. A study assessing different mfERG stimulus luminance at 150, 300 and 500 candelas per meter squared (cd/m^2) found that there was a 20% increase in mfERG amplitude when luminance increased from 150 to 500 cd/m^2 and latency decreased by 1.5ms [282]. This was reproduced in another study demonstrating a linear increase in P1 latency and reduction in N1P1 amplitude with decreases in mean luminance of the mfERG stimulus [283]. Guidelines for mfERG luminance as per ISCEV should be 100-200cd/m^2 for the white hexagon stimulus and the black hexagon should have low enough luminance to provide ≥90% contrast. Our equipment was calibrated as per the VERIS manufacturer's guidelines at monthly intervals using the VERIS autocalibration software/hardware system. A photometric sensor, mounted in casing was placed over the refractor camera and screen luminance was automatically calibrated by the VERIS system. Because monitor brightness can change considerably during warm-up this was done at least 20 minutes after the equipment was switched on. Equally, all participants were not tested until at least 20 minutes after the mfERG had been switched on for this trial. The luminance (L) and contrast settings were:

White hexagons (Lmax) = 200 cd/m^2

Black hexagons (Lmin) = 1 cd/m^2

ISCEV calibration guidelines

Mean luminance = $\frac{(Lmax + Lmin)}{2}$ = 100.5 cd/m^2

Contrast = $\frac{(Lmax-Lmin)}{(Lmax+Lmin)} \times 100 = 99.0\ \%$

Brightness of background room lighting has also been shown to affect mfERG amplitude and latency with reduced amplitudes and shorter P1 latencies occurring as room lighting was

increased from darkness [284]. Therefore it is necessary for the eye to be light adapted for at least 15 minutes prior to testing [243]. The mfERG recordings were undertaken in the same room for all participant visits, with each participant being light adapted in this room for at least 20 minutes during pupil dilation prior to mfERG testing.

Stimulus size

There are various stimulus hexagon sizes that can be used to provide varying degrees of spatial resolution when recording the mfERG, usually ranging from 19 to 241 hexagons. However there are trade-offs when deciding on stimulus hexagon size. Decreasing the size of the stimulus hexagons over the same field will yield greater resolution, good for highlighting small defects that may otherwise be missed. However the signal to noise ratio will be decreased, requiring longer testing times and greater discomfort for the patient. Lower spatial resolution (larger stimulus hexagon size) increases the signal to noise ratio, therefore less recording time is necessary for the patient although small areas of attenuation of retinal function may be missed. In a study by Heinemann-Vernaleken *et al.* [226] different resolutions of mfERG were assessed in 20 eyes of 14 patients with ARM. They found that 16 of the 20 eyes showed focal retinal dysfunction at both 103 and 241 stimulus element spatial resolution. However in the higher resolution recordings, a reduced signal to noise ratio meant that 3 recordings could not be analysed. They concluded that although higher resolution provides greater sensitivity for detecting focal abnormality, the lower resolution recordings provided better signal to noise ratios and were more appropriate in the clinical setting. We used a 61 hexagon stimulus as recommended by ISCEV mfERG guidelines to balance the necessity for participant comfort while providing adequate assessment of macular function [243]. This also replicates the CARMIS study stimulus type [103].

Areas of analysis

Studies have shown that mfERG waveforms change with retinal area. In healthy eyes conventional cone-mediated mfERG amplitudes are greatest at the centre and decrease with eccentricity [285] due to the high density of cone photoreceptors in the fovea which decline with eccentricity [16]. Asymmetry has been found between the inferior versus superior, and nasal versus temporal retinal areas in some studies but not others. Larger mfERG amplitudes and shorter N1 and P1 latencies in the superior retina compared to the inferior retina were recorded in one study [285] but no significant differences were seen between nasal and temporal areas in this study. Conversely, another study found larger amplitudes in the temporal retina

compared to the nasal retina but no difference between superior and inferior areas [286]. Other work has shown no difference between inferior and superior, or temporal and nasal areas for mfERG measures in healthy eyes, eyes with ARM, or when comparing healthy eyes to ARM eyes [227]. However, when concentric ring analysis was used, attenuation of central mfERG waveforms were found in ARM eyes when compared to healthy eyes [221, 287].

Concentric ring analysis was used for the L-based supplementation study and the supplement withdrawal study, based on its appropriateness in detecting ARM and its ability to detect changes in retinal function in ARM with nutritional supplementation in the CARMIS study [103].

Indeed, baseline differences of the participants were found in the mfERG measures between ARM eyes and healthy age-matched eyes using concentric ring analysis as described in chapter five. Single retinal areas stimulated by individual localised hexagons were not assessed because of poor signal to noise ratios as previously described, lack of correlation with drusen morphology [169, 221, 287] and the subtle nature of ARM.

Participant cooperation

Any deviation in fixation during recording of the mfERG has an effect on the spatial accuracy of the mfERG waveforms because as the eye moves, different areas of the retinal field will be stimulated by different hexagons rather than 1 fixed hexagon per retinal area when the eye is stationary. One study by Chisholm *et al.* demonstrated that around 51% of participants fixation fell within 1.2 degrees from the point of fixation suggesting fixation quality was adequate when the central element subtended 2.4 degrees or greater (typically a 61 hexagon stimulus pattern or less). Greater resolution (smaller, greater numbers of hexagons) means that any deviation in fixation will have a greater effect on mfERG waveforms as any eye movement will cause the retina to be stimulated by a larger number of hexagonal stimulus elements [274, 288]. Another study demonstrated that central mfERG amplitudes were most affected by fixation deviation, showing reduced amplitudes. These amplitudes became increasingly affected the further from the centre the fixation deviation occurred [289]. The investigators also suggested that mfERG amplitude was not greatly attenuated if fixation remained within the central hexagon. However they used a 103 hexagon stimulus pattern in contrast to Chisholm *et al's* study [288], although

stimulus distance was adjusted so the central hexagon subtended 2.4 degrees as per Chisholm *et al's* work.

Poor visual acuity can limit the ability to focus on the central fixation marker throughout a mfERG recording. This can lead to reduction in central mfERG amplitudes [289, 290]. Because fixation has such an impact on mfERG waveforms one of the inclusion criteria for this study was for all participants to have a logarithmic minimum angle of resolution (logMAR) distance visual acuity of 0.2 or better in order to see the fixation cross throughout mfERG recordings. This was required for HO, HY and ARM eyes. The mfERG system had a refractor camera, used to monitor fixation throughout the recording process. Any recordings affected by loss of fixation or blink artefact were eliminated and repeated. All participants positively responded when asked if they could see the fixation cross before mfERG recordings commenced.

Age

Age is a significant factor that affects the mfERG. This is described further in chapter five where a baseline study was undertaken to obtain normative values for the laboratory and to compare the effects of age and disease on retinal function using the mfERG. This was done prior to participants being allocated to treated and non-treated groups for the L and Z supplementation trial.

Pupil size

Pupil size controls the amount of light reaching the retina and therefore has a significant effect on the size of the mfERG response. Studies have shown that when pupil size is reduced mfERG N1P1 amplitude is significantly reduced and P1 latency delayed [274, 291].Therefore ISCEV mfERG guidelines recommend that pupils are dilated prior to mfERG recording [243]. For this study each participant at each visit was dilated with tropicamide 1% (Bausch and Lomb, Kingston-Upon-Thames, UK) for consistent pupil dilation for each visit.

Media opacity and Intraocular lenses (IOLs)

Any disturbance to the passage of light through to the retina will have an impact on mfERG waveforms. Therefore when assessing retinal responses with the mfERG it is important to

exclude participants with significant cataract or intraocular lenses as these factors affect the transmission of light through to the retina. Increasing severity of cataract has been associated with reduced mfERG amplitudes in the central retina [292]. Studies have compared mfERG responses before and after cataract surgery, and have found that mfERG N1P1 amplitudes significantly increased centrally after cataract surgery when compared with amplitudes prior to surgery [293, 294]. The effects of differing strengths of light scattering acrylic filters on mfERG waveforms have been studied, again largely showing central reduction in amplitude [295] although this was not statistically significant. Other work using a liquid crystal light diffuser also showed a statistically significant reduction in macular mfERG amplitudes [260]. Another study found that mfERG amplitudes were significantly reduced peripherally as well as centrally in eyes with cataract when compared to post-operative amplitudes [296].

Eyes with IOLs were excluded from this study because we wanted to obtain normative values for our laboratory and assess baseline differences between healthy younger, healthy older and ARM eyes. Because young eyes do not routinely require IOLs, exclusion of any eyes with IOLs meant that any mfERG differences between young and old eyes, or young and ARM eyes could be attributed to age or macular disease rather than a change of optics caused by the IOLs.

Axial length and refractive error

Increasing severity of myopia has been associated with reduced mfERG amplitudes and delayed latencies. One study found a reduction in central mfERG N1 amplitude and both central and paracentral mfERG P1 amplitudes with increasing degrees of myopia and axial length. Refractive error was correlated with axial length in this study [297]. Other work assessing the effects of myopia in adults and children found that central and peripheral mfERG N1, P1 and N2 amplitudes reduced and N1, P1 and N2 latencies increased in adults with myopia. However, this relationship was not demonstrated in children with myopia except for P1 latency. This increased with myopia in children for all areas analysed together but not when peripheral and central areas were analysed separately [298]. The authors attributed mfERG differences in the adult group to changes in retinal function over time with long-term myopia. A study by Chen *et al* found prolonged P1 latencies for all areas in myopic eyes when compared to emmetropic eyes [299]. They found that axial length accounted for 15% of the latency variance while refractive error was responsible for 27% of the variation in latency between groups. They suggested the remainder of the variance may be caused by other effects such as inter-subject variability. The authors suggested that this may have accounted for the lack of amplitude

difference between myopes compared to emmetropes in their study. Greater loss of peripheral mfERG P1 amplitudes were seen in another study of myopes when compared to an emmetropic/low myopic group [300] which the authors suggested may be due to a coarser distribution of cones in the periphery of the retina in eyes with an increased axial length. Some studies have attributed attenuated mfERG responses in myopia to reduced cone and outer retinal function [299, 300]. However, other non-conventional mfERG protocols [301], and conventional mfERG protocols combined with structural retinal tests [302] have also shown inner retinal function attenuation in myopia.

As described in the results chapter there was no statistically significant difference between treated and non-treated groups for axial length or spherical equivalent. Therefore we did not need to adjust for this in our analysis. All participants' refractive error was corrected throughout mfERG recordings using the refractor camera that accompanies the VERIS system.

Drugs and diseases affecting retinal function

The mfERG has been successfully used as a tool to assess the effects of drug toxicity on retinal function. Mercury toxicity in the retinas of 10 patients gave reduced P1, N1 and N2 amplitudes for all areas, with faster N1 latencies and delayed P1 latencies centrally in mfERG recordings [303]. Ethambutol is used as an effective treatment for treating tuberculosis. Its retinal toxicity has been demonstrated with central mfERG N1 amplitudes being reduced in these eyes when compared to a control group in one study [304] and N1 and P1 central amplitudes reductions seen in a case study [305]. The case study demonstrated that after 3 months of ceasing ethambutol treatment mfERG amplitudes recovered, providing supporting evidence of the usefulness of mfERG as a tool for assessing the effects of systemic drugs on retinal function. Hydroxychloroquine and chloroquine are anti-malarial medications but are also used to treat rheumatoid arthritis, systemic lupus erythematosus and Sjögren's Syndrome. Significant correlations between hydroxychloroquine dosage and central and/or paracentral mfERG amplitudes have been demonstrated with reductions in amplitude with increasing dosage of the drug [306-310]. The mfERG was also shown to be useful in demonstrating the reversal of toxic retinal effects when the medication was ceased [311]. Vigabatrin is an anti-epileptic drug that has been associated with attenuated retinal function as shown by mfERG testing [312]. Peripheral (8-23 degrees) mfERG amplitude reductions were demonstrated in one study in 6 of 12 patients taking vigabatrin [313]. Other studies have also shown mfERG abnormalities which persist after cessation of vigabatrin [314].

There are systemic diseases that can affect retinal function as assessed by the mfERG. Diabetic and hypertensive participants were excluded from our studies due to the effects of diabetic [315-319] and hypertensive retinopathy [320] on mfERG waveforms. As mfERG measures retinal function, retinal disease or damage will have an impact on mfERG amplitudes and/or latencies (see table 4.1) [216, 321-324].

Hence any participants taking medications that affect retinal function, or who had retinal disease (other than ARM in the ARM group) were excluded from this study.

Table 4.1: Site and mechanism of retinal damage and effects on mfERG waveforms (adapted from Hood *et al.*, 2000 [216]).

Damage	Mechanism	mfERG P1 amplitude	mfERG P1 latency
Cone photoreceptor	Outer segment damage / cell loss	Smaller Smaller	Moderate delay Normal
Outer plexiform layer	Altered synaptic transmission	Normal or larger	Large delay
On-bipolar cells	Cell loss	Smaller	Moderate delay
Off-bipolar cell	Cell loss	Larger	Slightly faster?
Inner plexiform layer	Altered synaptic transmission	Approximately normal (waveform changes)	Small delay (<3ms)
Ganglion cell	Cell loss	Approximately normal	Approximately normal

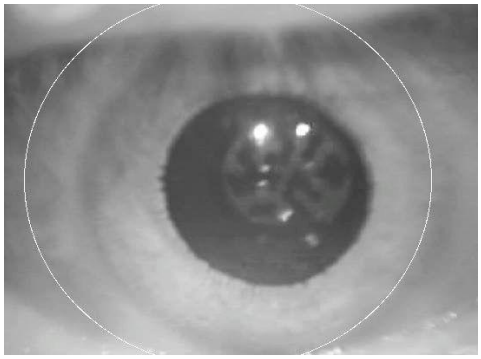
4.6 Experimental protocols

Multifocal Electroretinography

The VERIS science 6.1 (Electrodiagnostic imaging, San Mateo, California, USA) was used to record the mfERG. The multifocal stimulus, consisting of 61 scaled hexagons was displayed on a high-resolution, black-and-white cathode ray tube monitor 30cm wide and 30 cm high with a

frame rate of 75 Hz. The hexagon stimulus radius subtended approximately 20° of visual field. Each hexagon was independently alternated between white (200 cd/m²) and black (1cd/m²) according to a pseudorandom binary m-sequence [213]. Total recording time was four minutes. Recording time was divided into eight, 30 second segments allowing for participant rests. Fixation target perception was confirmed before testing commenced. The fixation target size was 1.5% of the area of the central hexagon. Each subject's vision was optimally corrected with the VERIS system's refractor/camera system. Participants were positioned 44cm from the stimulus, giving a stimulus viewing field of approximately 39 degrees. To allow equal magnification of the stimulus array on the retina, the distance between the participant's eye and refractor/camera was adjusted by obtaining a sharp image on the observation monitor as per the manufacturer's recommendation. The participant's eye was monitored throughout testing using this system (figure 4.2). Pupils were maximally dilated with tropicamide 1% (Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK). Gold cup electrodes filled with signa gel (Parker laboratories, Fairfield, New Jersey, USA) were applied to the forehead (ground electrode) and approximately one centimetre posterior to the temporal canthus (reference electrode) of the tested eye after these areas were abraded and cleaned using nuprep (Weaver and company, Aurora, USA). A DTL fibre electrode (Diagnosys Ltd, Impington, Cambridge, UK) was used as the active electrode and was placed along the sclera adjacent to the lower eyelid. The participant was asked to blink to ensure that the electrode found the same natural position for each visit. These electrodes were used for the trial for participant comfort [325] and test-retest reliability [277, 278] while not obscuring vision. Proxymetacaine hydrochloride 0.5% (Bausch and Lomb, Kingston-Upon-Thames, Surrey, UK) was instilled to minimise blinking throughout the recording. Any recordings contaminated with artefact were discarded and repeated. The untested eye was obscured throughout the procedure. In order to remove signal artefacts and improve the signal to noise ratio but without attenuating mfERG waveforms, one iteration of artefact removal was performed for each mfERG recording. No spatial averaging was performed because this would reduce spatial resolution as each retinal area stimulated by a hexagon is averaged with $1/6^{th}$ of its neighbouring hexagons. Thus the waveform of each single retinal area stimulated by a single hexagon would lose some of its own identity as it was averaged with surrounding waveforms.

Figure 4.2: A picture of the refractor camera monitoring participant fixation.



Contrast sensitivity

The Pelli-Robson CS test (Clement Clarke International Ltd, Harlow, Essex, UK.) was measured at a 1 meter distance as per the manufacturer guidelines, with distance correction when required, as a quick and reliable method for testing contrast sensitivity [326]. Contrast sensitivity provides another tool for testing visual performance. Studies have shown it to be repeatable to within 3 letters or +/- 0.15 log units [327] and suggest that a significant change in CS score would be +/- 0.30 log units (1 line, or 6 letters) in healthy eyes [327, 328] and eyes with ARM [329]. Therefore this value was used as the effect size to calculate sample size in the power calculations for this trial. For consistency CS was undertaken in the same room for each visit with a background luminance of (142 lux) throughout the trial. The Pelli-Robson chart is a good measure of medium to low spatial frequencies [330].

Macular pigment optical density

In order to obtain values for retinal accumulation of L and Z throughout the trial MPOD was obtained using the MPS 9000. The MPS 9000 uses the principle of heterochromatic flicker photometry (HFP). Participants were required to make flicker matches between two wavelengths of light, a blue light (~465nm), and green light (~530nm). Flicker matches were initially obtained centrally (1°). Flicker rate was gradually reduced from above the critical fusion frequency (60 Hz) by 6 Hz per second until the participant observed the flicker and pressed a response buzzer accordingly. This procedure continued for a series of pre-set blue-green ratios. Once flicker was detected the luminance of the blue and green light was changed by 0.2dB increasing the blue light and decreasing the green light while the overall mean

luminance was kept constant. Then the temporal frequency was reset to 60 Hz and the frequency reduced by 6 Hz per second again. The sequence continued for a series of blue-green ratios until a V-shaped curve was obtained. The minimum value of this curve was where the blue and green lights were of equal luminance. This whole process was repeated peripherally (8°) and again a V-shaped curve was obtained, providing a minimum value where blue a green lights were equiluminant. Because MP selectively absorbs blue light and is found centrally but not peripherally, the central minimum value differed from the peripheral minimum value. MPOD was determined by dividing central minimum blue light intensity by peripheral minimum blue light intensity and log10 of this value using MPS 9000 computer software. Greater detail of this technique is described by Van Der Veen *et al.* [331]. Both the background and target luminance was set to 250 cd/m². Participants wore distance glasses for the test if required, and were instructed to fixate on the central target for obtaining central values. For peripheral testing participants were asked to blink frequently and adopt a more relaxed fixation at 8° around a 1.75° red fixation target to reduce Troxler's effect. Troxler's effect is named after the person who discovered this phenomenon, Ignaz Paul Vital Troxler in 1804 as cited by Martinez-Conde *et al.* [332]. When fixating at a single point for longer than 20 seconds, any stimulus away from that point will fade and disappear. This is due to neural adaptation. Small eye movements around the peripheral fixation target improve peripheral stimulus visibility, reducing Troxler's effect when obtaining peripheral MPOD values [332]. Instructions were given to the participant prior to the test and a practice run was undertaken for each visit before undertaking the main test. A study assessing the repeatability of the MPS 9000 within the laboratory at Aston University demonstrated a coefficient of repeatability of 0.33, suggesting that a clinically significant change in MPOD would be 0.33 units or greater [333]. Therefore this value was chosen as the effect size to calculate sample size in the power calculations for this trial.

Visual acuity

LogMAR distance VA testing using a 3 meter ETDRS 750 lux retro-illuminated chart was undertaken for each participant (Sussex Vision, Rustington, West Sussex, UK). The eye with the best corrected VA was determined at the participant's first visit and this eye was assessed for the subsequent 3 visits. In the case of the ARM participants, the eye with ARM was studied. If both eyes had ARM, the eye with the best corrected visual acuity was included in the study to ensure good mfERG fixation. Only one eye was chosen per participant because of intraclass correlation - environmental and genetic risk factors for ARMD such as smoking, age and genetic disposition, act on the individual and thus have an impact on the probability of the

disease occurring in both eyes, even if not clinically visible in both eyes [334]. Significance testing where total sample size (eyes) exceeds the number of participants is invalid and prone to false positive findings [335]. Confidence intervals and SDs would also be misleading due to a mixture of between-subject and within-subject variation [335].The advantage of using logMAR charts are that they have five letters per line with 0.1 logMAR progression per line, whereas Snellen charts do not provide this linear scale and have an increasing number of letters per line as the letter size decreases. LogMAR charts are reported to be an accurate tool for assessing VA [336]. Repeatability of logMAR VA tests has already been reported in the literature, with test-retest variability ranging between 0.07-0.19 [336-340].

Intraocular pressure

Non-contact intraocular pressure readings (Topcon CT-80 non-contact tonometer, Topcon, Newbury, Berkshire, UK) were taken prior to instillation of tropicamide 1%. If readings of 21mmHg or above were recorded, the participant was advised to see an optometrist for further measurements and advice, and was excluded from the study. This happened for one participant. If under 21mmHg, tropicamide 1% was instilled for the mfERG. Intraocular pressure was rechecked at the end of each participant visit.

Fundus photography

A central 45 degree fundal photograph was taken with the Topcon TRC-NW8, (Topcon, Newbury, Berkshire, UK) for each visit to determine any change in fundus or media opacity. Participants were instructed to fixate on a central fixation target for each visit to ensure identical fundus positioning. None of the healthy eyes progressed to ARM throughout the trial. None of the ARM participants progressed to AMD throughout the trial.

Food Diaries

In order to assess whether any changes in outcome measures were due to the lutein-based nutritional supplement rather than changes in dietary intake of nutrients, each participant was provided with a food diary (see appendix 9). The diary was filled in over two week days and one weekend day. Participants were given food diaries at visits one, three and four to complete. The data from the food diaries was inputted into the Weighted Intake Software Package (WISP, Tinuviel, Llanfechell, Anglesey, UK). Lutein values for foods were taken from the United

States department of agriculture (USDA) nutrient databank, release 23 (http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/SR23/nutrlist/sr23a338.pdf).

4.7 Chapter four summary

This chapter described the primary and secondary outcome measures that were used to assess the effects of a L-based nutritional supplement on objective and subjective measures of visual and retinal function. The many factors that can affect mfERG waveforms were explained. The methods employed to reduce or eliminate these factors, while providing a comfortable mfERG recording experience for each participant were discussed. Experimental protocols were described for transparency and in order for the experiments to be replicated by other research groups.

Because it is necessary for all laboratories to obtain normative mfERG data, the next chapter reports the normative data for HY and HO eyes. This is compared with mfERG values for ARM eyes to ascertain differences in mfERG values due to age and age-related eye disease. Coefficient of variability and repeatability for mfERG values are also determined for N1P1 amplitude, and N1, P1 and N2 latency. Sample size calculations for the primary and secondary outcome measures are also reported in chapter five. Because there are conflicting findings as to whether dietary and retinal levels of L are reduced in individuals with ARMD, baseline dietary and MPOD analysis between HY, HO and ARM groups are also reported.

Chapter 5: Baseline data analysis

5.1 Sample size

Sample sizes were calculated as per Lehr [341]. Two previous studies assessing the effects of nutritional supplementation on retinal function using focal electroretinograms (fERG) [134] and mfERG [103] demonstrated significant results between groups treated with a nutritional supplement compared with non-treated groups. The fERG study suggested a sample size of 8 healthy eyes for their healthy older (HO) group and 30 eyes for the age-related maculopathy (ARM) group [134], providing a 90% power, at $\alpha = 0.05$, for detecting a between group difference of 25 – 30% in amplitude or phase. The mfERG study by the CARMIS investigators, suggested 27 ARM eyes, 15 in the supplemented group and 12 in the non-supplemented group [103], providing a power of 90%, at $\alpha = 0.05$, for detecting a between-group difference of $\geq$ 55% in mfERG amplitude. As such the 55% difference was used as the effect size in sample size calculations for this trial [103] (table 5.1 and 5.2).

Because there is paucity in the literature about mfERG latency changes with nutritional supplementation, effect sizes for mfERG latency were based on a study for vitamin A supplementation by Dolan et al., who noted a change in central and peripheral P1 latency of 6 ms with vitamin A supplementation in a single participant case study [342]. Thus, the sample sizes were adequate for the primary outcome measure in this thesis in providing an 80% power at the 5% significance level.

Table 5.1: Group sizes required to have 80% power at the 5% significance level for VA, CS, mfERG amplitude and MPOD for healthy eyes. The mean and standard deviation (SD) data were calculated from 52 healthy eyes at visit 1.

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

^^ Based on VA repeatability studies [336-340].

^ Based on Elliott *et al's* paper [327].

* Repeatability value from Bartlett et al's of HFP repeatability paper [333]

** Based on Parisi *et al's* paper of a 55% change in mfERG amplitude [103]

*** Based on Dolan *et al's* paper [342].

Table 5.2: Group sizes required to have 80% power at the 5% significance level for VA, CS, mfERG amplitude and MPOD for ARM eyes. The mean and SD data were calculated from 16 ARM eyes at visit 1.

	VA (logMAR)	CS (log units)	MPOD	Central mfERG N1P1 amplitude (nV/deg^2)	Central mfERG P1 latency (ms)
Mean	0.03	1.77	0.36	120.14	30.83
Standard deviation (SD)	0.06	0.15	0.24	38.82	1.72
Effect size (E)	0.10^^	0.30^	0.33*	66.08**	6***
E/SD	1.67	2.00	1.38	1.70	3.48
$(E/SD)^2$	2.79	4.00	1.89	2.90	12.16
Sample size = $16/(E/SD)^2$ (two sided)	6	4	9	6	2

^^ Based on VA repeatability studies [336-340].

^ Based on Elliott *et al's* paper [327].

* Repeatability value from Bartlett et al's of HFP repeatability paper [333].

** Based on Parisi et al's paper of a 55% change in mfERG amplitude [103].

*** Based on Dolan et al's paper [342].

5.2 Investigating the effect of age and ARM, on mfERG measures - baseline values

5.21 Purpose

The mfERG response is affected by many variables as previously described in chapter 4. As an outcome measure for assessing the effects of a nutritional supplement versus non-supplementation in ARM eyes and healthy eyes, it was important to establish normative mfERG data for comparison with diseased eyes.

5.22 Methods

Eighty one eyes from 81 participants aged between 18-83 (mean ± sd; 50.3 ± 18.1) were recruited over a nine month period from Aston University (Birmingham, UK) optometry department patients, and from staff and students from within the University. They were divided into three groups: a healthy younger (HY) group of 37 participants aged between 18-48, (mean age ± sd; 32.9 ± 9.0 years), a healthy older group (HO) of 28 participants aged between 50-77, (mean age ± sd; 63.4 ± 8.1 years) and an ARM group of 16 participants aged between 52-83 (mean age 67.2 ± 8.5 years). Age-related maculopathy was defined as per the international classification system described in chapter 1 [34]. Multifocal ERG outcome measures were; N1P1 amplitude, measured from the N1 trough to the P1 peak; N1 latency, measured from the start of the trace to the N1 trough; P1 latency, measured from the start of the trace to the P1 peak; and N2 latency, measured from the start of the trace to the N2 trough (see figure 4.1 in chapter 4). Multifocal ERG testing was carried out as per the experimental protocols set out in chapter 4.

Kruskal-Wallis and one-way between groups analysis of variance (ANOVA) using statistical packages for social sciences - SPSS 16.0 (SPSS UK Ltd, West Street, Woking, Surrey) were conducted to explore the impact of age and ARM on retinal function of five retinal areas (see figures 5.1 and 5.2) using the mfERG. Each data set was checked for normality using the Shapiro-Wilk statistic which assesses the normality of distribution of the data. A non-significant result indicates normality and therefore ANOVA was used for analysis with Tukey's post-hoc range test. When parametric assumptions were not met according to Shapiro-Wilk tests for normality (i.e. there was a statistical significance for the Shapiro-Wilk test), Kruskal-Wallis one-way analysis of variance for independent groups was performed with Mann-Whitney U tests demonstrating post-hoc differences between groups (tables 5.3, 5.4, 5.5 and 5.6).

Figure 5.1: Grouping of the mfERG areas analysed. Ring 1 - central hexagon (approximately 0.0-2.5º), ring 2 (approximately 2.5-5.0º) surrounds the central hexagon, ring 3 (approximately 5.0-10.0º) surrounds ring 2, ring 4 (approximately 10.0-15.0º) surrounds ring 3 and ring 5 (approximately 15.0-20.0º) surrounds ring 4.

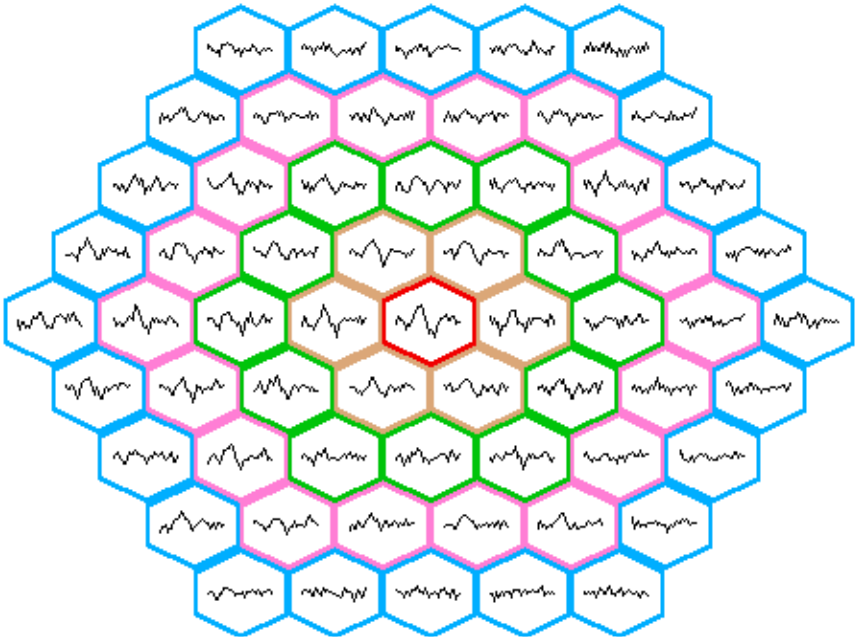
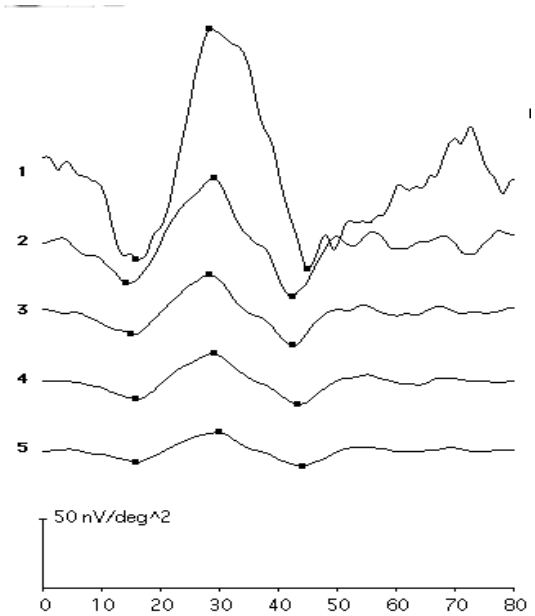


Figure 5.2: mfERG waveforms. Each trace corresponds to the average of each ring of hexagons analysed. N1 is denoted by the first cursor, P1 by the second, and N2 by third cursor. The difference between the first two cursors denotes N1P1 amplitude. The x-axis is in milliseconds.



5.23 Results

An independent-samples t-test demonstrated no significant difference in age between the ARM (mean ± sd: 67.2 ± 8.5 years) and HO (63.4 ± 8.1 years) groups; $t = 1.45$, $p = 0.16$. There was a significant difference using ANOVA in spherical equivalent refraction ($F = 3.43$, $p = 0.04$), between the HY (mean ± sd: -0.23 ± 1.90), HO (0.78 ± 2.39) and the ARM (1.29 ± 2.17) groups with post-hoc analysis demonstrating a difference between ARM and HY groups; $p=0.02$ but no difference between HY and HO eyes, or between HO and ARM eyes. A Chi-squared test for independence using SPSS 16.0 software indicated a significant difference between ethnicity and groups, with HO and ARM groups exclusively containing 28 and 16 caucasians respectively and the HY group containing 8 asians and 29 caucasians ($\chi^2 = 10.56$, $p = 0.01$, $p = 0.01$). There was no significant difference between gender and groups ($\chi^2 = 0.14$, $p= 0.93$) with 13 males and 24 females in the HY group, 9 males and 19 females in the HO group and 6 males and 10 females in the ARM groups.

Table 5.3: Baseline comparison data for multifocal electroretinogram rings 1-5 N1P1 amplitudes, between HY, HO and ARM cohorts using one way between-groups ANOVA and Kruskal-Wallis one-way analysis of variance for independent groups when parametric assumptions were not met.

	Ring 1	**Ring 2**	**Ring 3**	**Ring 4**	**Ring 5**
N1P1 Amplitudes	$\chi^2 = 18.626$ (p = <0.001*)	$\chi^2 = 15.360$ (p = <0.001*)	$\chi^2 = 9.258$ (p = <0.010*)	$\chi^2 = 8.166$ (p = 0.017*)	$\chi^2 = 7.223$ (p = 0.027*)
Post-hoc analysis					
HY / HO	p = 0.107 Mann-Whitney U	p = 0.059 Mann-Whitney U	p = 0.251 Mann-Whitney U	p = 0.234 Mann-Whitney U	p = 0.366 Mann-Whitney U
HO / ARM	p = 0.004* Mann-Whitney U	p = 0.021* Mann-Whitney U	p = 0.062 Mann-Whitney U	p = 0.078 Mann-Whitney U	p = 0.080 Mann-Whitney U
HY / ARM	p = <0.001* Mann-Whitney U	p = <0.001* Mann-Whitney U	p = 0.002* Mann-Whitney U	p = 0.005* Mann-Whitney U	p = 0.007* Mann-Whitney U

* Statistically significant where p=<0.05

Table 5.4: Baseline comparison data for multifocal electroretinogram rings 1-5 N1 Latencies between HY, HO and ARM eyes using one way between-groups ANOVA and Kruskal-Wallis one-way analysis of variance for independent groups when parametric assumptions were not met.

	Ring 1	**Ring 2**	**Ring 3**	**Ring 4**	**Ring 5**
N1 latency	$\chi^2 = 12.630$ (p = 0.002*)	$\chi^2 = 11.917$ (p = 0.003*)	$\chi^2 = 15.348$ (p = <0.001*)	$\chi^2 = 8.059$ (p = 0.018*)	$\chi^2 = 13.444$ (p = 0.001*)
Post-hoc analysis					
HY / HO	p = 0.024* Mann-Whitney U	p = 0.033* Mann-Whitney U	p = 0.070 Mann-Whitney U	p = 0.152 Mann-Whitney U	p = 0.115 Mann-Whitney U
HO / ARM	p = 0.071 Mann-Whitney U	p = 0.104 Mann-Whitney U	p = 0.006* Mann-Whitney U	p = 0.093 Mann-Whitney U	p = 0.018* Mann-Whitney U
HY / ARM	p = 0.001* Mann-Whitney U	p = 0.001* Mann-Whitney U	p = <0.001* Mann-Whitney U	p = 0.007* Mann-Whitney U	p = <0.001* Mann-Whitney U

* Statistically significant where p=<0.05

Table 5.5: Baseline comparison data for multifocal electroretinogram rings 1-5 P1 Latencies between HY, HO and ARM eyes using one way between-groups ANOVA and Kruskal-Wallis one-way analysis of variance for independent groups when parametric assumptions were not met.

	Ring 1	**Ring 2**	**Ring 3**	**Ring 4**	**Ring 5**
P1 latency	$\chi^2 = 16.184$ (p = 0.001*)	$\chi^2 = 21.806$ (p = <0.001*)	$\chi^2 = 19.090$ (p = <0.001*)	$\chi^2 = 15.759$ (p = <0.001*)	$\chi^2 = 28.491$ (p = <0.001*)
Post-hoc analysis					
HY / HO	p = <0.001* Mann-Whitney U	p = <0.001* Mann-Whitney U	p = <0.001* Mann-Whitney U	p = <0.001* Mann-Whitney U	p = <0.001* Mann-Whitney U
HO / ARM	p = 0.807 Mann-Whitney U	p = 0.864 Mann-Whitney U	p = 0.740 Mann-Whitney U	p = 0.892 Mann-Whitney U	p = 0.186 Mann-Whitney U
HY / ARM	p = 0.003* Mann-Whitney U	p = 0.002* Mann-Whitney U	p = 0.003* Mann-Whitney U	p = 0.005* Mann-Whitney U	p = <0.001* Mann-Whitney U

*Statistically significant where p=<0.05

Table 5.6: Baseline comparison data for multifocal electroretinogram rings 1-5 N2 Latencies between HY, HO and ARM eyes using one way between-groups ANOVA and Kruskal-Wallis one-way analysis of variance for independent groups when parametric assumptions were not met.

	Ring 1	**Ring 2**	**Ring 3**	**Ring 4**	**Ring 5**
N2 latency	$\chi^2 = 7.634$ (p = 0.022*)	F = 10.247 (p = <0.001*)	$\chi^2 = 20.985$ (p = <0.001*)	$\chi^2 = 18.457$ (p = <0.001*)	$\chi^2 = 14.794$ (p = 0.001*)
Post-hoc analysis					
HY / HO	p = 0.014* Mann-Whitney U	p = 0.002*	p = <0.001* Mann-Whitney U	p = <0.001* Mann-Whitney U	p = <0.002* Mann-Whitney U
HO / ARM	p = 0.706 Mann-Whitney U	p = 0.756	p = 0.652 Mann-Whitney U	p = 0.777 Mann-Whitney U	p = 0.263 Mann-Whitney U
HY / ARM	p = 0.041* Mann-Whitney U	p = 0.001*	p = <0.001* Mann-Whitney U	p = 0.004* Mann-Whitney U	p = 0.002* Mann-Whitney U

*Statistically significant where p=<0.05

Table 5.7: Mean values for mfERG N1P1 amplitude, N1 latency, P1 latency and N2 latency between HY, HO and ARM eyes for 5 rings of eccentricity.

	Ring 1	**Ring 2**	**Ring 3**	**Ring 4**	**Ring 5**
Mean amplitudes ± SD (nV/deg^2)					
HY	178.65 ± 54.02	72.11 ± 19.84	41.88 ± 12.46	28.09 ± 8.51	17.12 ± 5.48
HO	155.77 ± 38.82	61.83 ± 15.12	37.43 ± 10.15	24.70 ± 7.19	15.82 ± 5.69
ARM	120.14 ± 38.82	52.00 ± 15.30	32.10 ± 9.31	21.96 ± 6.39	13.21 ± 4.43
Mean N1 latencies ± SD (ms)					
HY	15.16 ± 2.45	14.08 ± 1.53	14.80 ± 1.13	14.95 ± 0.96	14.62 ± 1.42
HO	15.58 ± 1.91	15.18 ± 1.97	15.39 ± 0.95	15.17 ± 1.13	14.95 ± 1.52
ARM	15.60 ± 3.06	15.21 ± 1.54	15.78 ± 1.38	15.36 ± 1.22	15.00 ± 1.69
Mean P1 latencies ± SD (ms)					
HY	28.45 ± 1.47	27.12 ± 1.50	27.64 ± 1.52	28.24 ± 1.37	28.83 ± 1.07
HO	30.71 ± 2.88	29.17 ± 1.59	29.28 ± 1.18	29.38 ± 1.13	30.21 ± 1.00
ARM	30.14 ± 3.09	28.85 ± 2.58	29.06 ± 2.27	29.69 ± 1.52	30.83 ± 1.72
Mean N2 latencies ± SD (ms)					
HY	42.95 ± 1.45	42.31 ± 1.68	41.50 ± 1.06	41.66 ± 1.69	42.11 ± 1.45
HO	44.28 ± 2.11	43.26 ± 2.09	42.65 ± 0.95	42.88 ± 1.28	43.16 ± 1.39
ARM	43.93 ± 1.80	43.57 ± 1.86	42.86 ± 0.91	43.04 ± 1.55	43.69 ± 1.62

N1P1 amplitudes were significantly reduced in the ARM group compared with the HO group for rings 1 and 2. Amplitudes were reduced for all 5 rings in the ARM group compared to the HY group (table 5.3 and 5.7).

N1 latencies were significantly longer in HO group compared to the HY group in rings 1 and 2. The ARM group showed longer N1 latencies in rings 3 and 5 than in the HO group and for all 5 rings when compared to the HY group (table 5.4 and 5.7).

P1 latencies were significantly longer in HO and in ARM eyes when compared to HY eyes for all 5 rings (table 5.5 and 5.7).

N2 latencies were also significantly longer for all 5 rings in HO and ARM eyes compared to HY eyes for all 5 rings (table 5.6 and 5.7).

5.24 Discussion

Because mfERG was going to be used to assess the longitudinal effects of a nutritional supplement on retinal function in healthy and diseased eyes, the aim of this study was to compare the effects of age and ARM on mfERG N1P1 amplitudes and N1, P1 and N2 latencies at baseline, and to establish a normative database for our laboratory as recommended by ISCEV guidelines [243].

The effect of age on retinal function as assessed by the mfERG

Mean amplitudes were reduced for all 5 rings in HO eyes when compared to HY eyes but this did not reach statistical significance in this study. There were statistically significantly prolonged N1 latencies in rings 1 and 2 in HO eyes compared to HY eyes. There were prolonged P1 latencies in rings 1-5 of HO eyes when compared to HY eyes. N2 latencies for all 5 rings were also delayed in HO eyes compared to HY eyes. Nabeshima *et al.,* found reduced mfERG N1P1 amplitudes in all 5 rings in subjects over 50 years compared to younger subjects, but not prolonged P1 latencies. Their study included 52 eyes and assessed rings of retinal eccentricity with 61 hexagonal stimuli as used in this study [240]. Our study compared a larger

number of 65 healthy eyes. However, in a further study by Nabeshima *et al.,* reduced N1P1 amplitudes and delayed P1 latencies in 93 normal eyes of 56 participants were seen, although it was not stated whether ring analysis was used or which areas were affected [343]. Jackson *et al.,* also found lower mean amplitudes and prolonged N1 and P1 latencies in older adults compared to younger adults, with mfERG ageing changes being greatest at the fovea and decreasing with eccentricity in a seven ring 103 hexagon analysis [344] and this was echoed in other studies [345, 346]. Anzai *et al.,* found a significant inverse correlation between amplitude and age in the central retina up to 8 degrees but not at other locations [347], although there was no statistically significant change in latency with age in this study. Seiple *et al.,* found that the rate of amplitude reduction was greatest in the central 3º [348] but found that age had less influence on latency. Conversely a small number of studies have found no difference in mfERG amplitude with age [241, 349].

It is thought that ageing-related mfERG changes are due to preretinal optical and neural contributions [350-353]. We used the conventional mfERG for this study, which is cone-driven. It has been shown that cone and rod photoreceptor density decline with age, with rods declining at a greater rate than cones [354]. There are significant interactions between rods and cones, with evidence to suggest that rods are required for cone survival [355] and secrete factors that enhance cone survival [356]. Thus, rod and cone photoreceptor decline with age may explain age-related changes within the mfERG.

The effect of ARM on retinal function as assessed by the mfERG

This study demonstrated significantly reduced amplitudes in rings 1 and 2, and prolonged N1 latencies in rings 3 and 5 in eyes with ARM when compared to age, gender and ethnicity-matched HO eyes. Foveal (ring 1) amplitude reductions and delayed N1 latency was found in 15 eyes with ARM in a study by Li *et al.,* using a 6 ring analysis of 103 hexagonal stimuli [227]. A similar result was seen in another study with ring 1 and 2 amplitudes being reduced in 15 ARM eyes, although N1, P1 and N2 latency results were not reported here [357]. Gerth *et al.,* were able to demonstrate reduced amplitudes and prolonged N1, P1 and N2 latencies in 31 ARM eyes extending out to 25 degrees in radius in some eyes [221] when using 103 hexagonal stimuli. However they did not use ring analysis for their study but rather individual retinal area per hexagon analysis. No N1P1 amplitude reduction or prolonged N1 or P1 latency was found in a 61 hexagonal stimuli study of 24 ARM eyes when using 5 ring analysis, although the summed responses of all 5 rings showed reduced N1 amplitude which is a different measure

than the N1P1 amplitude used in our analysis [228]. N2 latency was not reported in their study. Another study using both 103 and 61 hexagonal stimuli and using a curve-fitting data analysis method also found no statistical significant mfERG changes in ARM eyes, although ring analysis was not carried out here but a central versus peripheral analysis [233].

As described in chapter 1, the RPE phagocytoses the outer segment discs of the photoreceptors and is a point of metabolite and waste exchange [21]. In ARM there are variations within and below the RPE, seen as alterations in the pigmentation of the RPE, with or without the occurrence of drusen [35]. Drusen may cause displacement of photoreceptor outer segments and disturb nutrient exchange between the choriocapillaris and photoreceptors [358]. Accumulation of lipofuscin within the RPE and lipids in Bruch's membrane occurs with age [37], possibly caused by the reduced ability of the RPE's phagocytic-lysosomal system to efficiently digest photoreceptor outer segment membranes [38]. This accumulated material may interrupt the supply of nutrients from the choroid to the retina ultimately leading to photoreceptor atrophy [39]. Oxidative stress causes injury and inflammation to the RPE and choriocapillaris which may lead to an altered extracellular matrix, also affecting nutrient supply to the RPE and retina, further damaging the RPE and retina, leading to the retinal atrophy seen in the later stages of ARMD [40].

There is some evidence to suggest that rods are the first photoreceptors to degenerate in ARMD [358, 359]. As mentioned previously, cone photoreceptor function is dependent on the support of rod photoreceptors. Rods are more prolific within the peripheral retina than the central retina and support cone photoreceptor function. Cone photoreceptors drive the conventional cone-mediated mfERG. It may be that the prolonged N1 latencies found for rings 3 and 5 in ARM eyes in this study may be due to attenuation of rod function, on which cone function relies.

Results showed prolonged N1 (rings 1 and 2), P1 (rings 1-5) and N2 latencies (rings 1-5) with ageing. However, with ARM, prolonged ring 3 and 5 N1 latencies were seen and were accompanied by a reduction in central mfERG amplitudes when compared to age-matched healthy eyes. According to Gerth *et al.,* [221] and Hood *et al.,* [216] (see table 4.1) altered synaptic transmission results in delayed mfERG latency whereas cell loss reduces mfERG N1P1 amplitude. This may suggest that photoreceptor synaptic transmission slows with age, whereas photoreceptor damage and dysfunction as seen in ARMD causes the central amplitude reductions seen here in ARM eyes [221].

It may appear obvious that due to the effects of age and disease there were prolonged N1, P1 and N2 latencies, and reduced N1P1 amplitudes for all 5 rings in the ARM group compared with the HY group in post-hoc tests for our study. However for transparency of research reporting the data was included. A mean difference in refractive error was found between the HY and ARM group of 1.52 dioptres. A large variation in refractive error (greater than +3.00 dioptres) does have a significant impact on mfERG N1P1 amplitude. However, small variations such as ours have minimal responses on mfERG measures [360]. Furthermore, any refractive error was corrected for with the refractor camera when measuring the mfERG in our study. There is no evidence in the literature to suggest that the mfERG varies between differing ethnic groups. Therefore the difference between group ethnicity in our study should not have affected the results.

5.25 Conclusion

The aim of this study was to establish a normative database for our laboratory as recommended by ISCEV guidelines and to compare the effects of age and ARM on mfERG N1P1 amplitudes and N1, P1 and N2 latencies at baseline prior to undertaking a study to assess the effects of a nutritional supplement on retinal function in healthy eyes and eyes with ARM.

This baseline study demonstrated age-related changes in the mfERG which emphasised the importance of having age-matched groups for comparing the effects of intervention when using the mfERG to assess retinal function. It also revealed changes in retinal function in ARM, highlighting the sensitivity of the mfERG as a tool for detecting central outer retinal dysfunction in ARM.

5.3 Investigating the coefficient of variation and coefficient of repeatability of mfERG measures for different rings of retinal eccentricity between younger, older and diseased eyes

5.31 Purpose

The previous section demonstrated that mfERG responses were age-dependent. Further to ageing, ARM caused N1P1 amplitude reductions in rings 1 and 2, and N1 latency delays in rings 3 and 5 when compared to age-matched HO eyes.

As described in chapter 4, mfERG amplitudes can show a high degree of variability depending on technical and participant factors. Thus the purpose of this study was to assess the reliability of recordings within the laboratory by comparing the coefficient of variation (CV) for the HY, HO and ARM groups for N1P1 amplitudes and N1, P1 and N2 latencies for five rings of eccentricity. Coefficient of repeatability (CR) was also assessed in healthy eyes.

Short and medium term CR were assessed to compare repeatability of mfERG over time for healthy eyes. Medium term CR was also assessed in ARM eyes to compare repeatability of mfERG between healthy and ARM eyes.

The CV is a ratio of the SD to the mean (μ) (SD / μ) and is useful for measuring mfERG amplitude and latency variability between the differing rings of eccentricity and for examining variation between groups of data for a single visit. The CR can be used to analyse the repeatability of a single measurement method over two visits [361]. It is calculated as 1.96 multiplied by the SD of the mean differences between two sets of data from the mfERG (one set per visit in this case) and this provides 95% confidence limits for the difference between two sets of data. In this study the CR values demonstrated the amount of change in mfERG measures between visits which could be put down to measurement noise.

5.32 Methods

Coefficient of variation (CV): 81 eyes from 81 participants as per the characteristics described in section 4.22 were recruited over a nine month period from Aston University (Birmingham, UK) optometry department patients, and from staff and students from within the university. They were divided into three groups: a HY group of 37 participants, a HO group of 28 participants and an ARM group of 16 participants.

Coefficient of repeatability (CR): Repeatability of the mfERG was assessed over the short (CRS and medium term (CRM) in healthy eyes. Medium term mfERG repeatability (CRM) was also assessed for ARM eyes.

Short term CR (CRS) for healthy eyes: Eight healthy eyes, aged between 19 and 57 (mean ± sd; 35.8 ± 11.7 years) were available to evaluate the CRS. Multifocal ERG was performed at two visits, the second visit being within seven days of the first (mean ± sd; 2.63 ± 2.72 days).

Medium term CR (CRM) for healthy eyes: Twenty six healthy eyes, aged between 21 and 69 (mean ± sd; 43.7 ± 16.4 years) were available to evaluate the CRM. Multifocal ERG was performed at two visits, the second visit being approximately 5 months after the first (mean ± sd; 4.92 ± 0.63 months).

Medium term CR (CRM) for ARM eyes: Four eyes with ARM, aged between 61 and 70 (mean ± sd; 65.0 ±3.9 years) were available to evaluate CRM for this group. Multifocal ERG was performed at two visits, the second visit being approximately 5 months after the first (mean ± sd; 5.00 ± 0.82 months). Multifocal ERG was carried out as per the mfERG methods in chapter 4.

5.33 Results

All repeatability measures were calculated using Microsoft Excel software (Microsoft Corporation, Microsoft Way, Redmond, WA, USA).

The CV for all parameters for all five rings of eccentricity can be seen in tables 5.8, 5.9, 5.10 and 5.11. The mean overall variability for mfERG N1P1 amplitude was 30% for the HY group, 29% for the HO group and 31% for the ARM group. The mean overall variability for N1 latency was 10% for the HY group, 10% for the HO group and 12% for the ARM group. The mean overall variability for P1 latency was 5% for the HY group, 5% for the HO group, and 8% for the ARM group. The mean overall variability for N2 latency was 3% for the HY and HO groups and 4 % for the ARM group.

The CRS for all mfERG measures for five rings of eccentricity can be seen in table 5.12. The overall mean area CRS was 1.30 ms for N1 latency, 1.90 ms for P1 latency, 1.72 ms for N2 latency and 40.45 nV/deg^2 for N1P1 amplitude. The CRM for all mfERG measures for five rings of retinal eccentricity can be seen in table 5.13. The total mean area CRM was 2.12 ms for N1 latency, 2.54 ms for P1 latency, 2.74 ms for N2 latency and 43.36 nV/deg^2 for N1P1 amplitude.

The CRM for ARM eyes for all mfERG measures for five rings of retinal eccentricity is shown in table 5.14. The total mean area CRM for ARM eyes was 2.83 ms for N1 latency, 2.82 ms for P1 latency, 2.89 ms for N2 latency and 15.58nV/deg^2 for N1P1 amplitude.

Table 5.8: Coefficient of variability (CV) for N1P1 amplitude for each of the five areas of retinal eccentricity and mean CV for all rings

CV for N1P1 amplitude (%)	Ring 1	Ring 2	Ring 3	Ring 4	Ring 5	total area mean CV
HY (n=37)	29.58	27.24	29.52	30.27	30.68	29.46
HO (n=28)	25.10	24.63	27.25	27.67	37.36	28.40
ARM (n=16)	29.46	28.64	28.16	28.59	32.34	29.40

Table 5.9: Coefficient of variability (CV) for N1 latency for each of the five areas of retinal eccentricity and mean CV for all rings

CV for N1 latency (%)	Ring 1	Ring 2	Ring 3	Ring 4	Ring 5	total area mean CV
HY (n=37)	7.04	7.80	5.39	5.31	5.62	6.23
HO (n=28)	6.16	5.83	3.94	4.23	4.98	5.03
ARM (n=16)	9.45	6.84	6.18	5.89	5.82	6.84

Table 5.10: Coefficient of variability (CV) for P1 latency for each of the five areas of retinal eccentricity and mean CV for all rings.

CV for P1 latency (%)	Ring 1	Ring 2	Ring 3	Ring 4	Ring 5	total area mean CV
HY (n=37)	4.62	5.48	5.75	4.28	3.53	4.73
HO (n=28)	4.88	4.32	4.11	3.83	2.67	3.96
ARM (n=16)	7.71	7.93	6.62	4.80	5.26	6.46

Table 5.11: Coefficient of variability (CV) for N2 latency for five areas of retinal eccentricity and mean CV for all rings.

CV for N2 latency (%)	Ring 1	Ring 2	Ring 3	Ring 4	Ring 5	total area mean CV
HY (n=37)	3.64	3.47	3.18	2.38	3.45	3.22
HO (n=28)	4.06	3.66	2.77	2.37	2.47	3.07
ARM (n=16)	3.95	3.94	2.28	3.66	3.79	3.52

Table 5.12: Short term coefficient of repeatability (CRS) for N1, P1 and N2 latency and N1P1 amplitude for all rings.

CRS healthy eyes (n=8)	N1 latency (ms)	P1 latency (ms)	N2 Latency (ms)	N1P1 amplitude (nV/deg^2)
Ring 1	2.31	2.22	1.74	96.83
Ring 2	1.24	2.54	1.50	44.49
Ring 3	0.00	1.74	2.04	28.73
Ring 4	1.74	1.37	1.36	17.40
Ring 5	1.23	1.62	1.95	14.78
Total area mean CRS	1.30	1.90	1.72	40.45

Table 5.13: Medium term coefficient of repeatability (CRM) for N1, P1, and N2 latency and N1P1 amplitude for all rings

CRM healthy eyes (n=26)	N1 latency (ms)	P1 latency (ms)	N2 Latency (ms)	N1P1 amplitude (nV/deg^2)
Ring 1	1.90	3.20	4.48	125.56
Ring 2	3.18	2.26	2.40	40.97
Ring 3	1.86	2.08	1.87	20.52
Ring 4	1.89	2.68	2.66	16.48
Ring 5	1.76	2.48	2.27	13.27
Total mean area CRM	2.12	2.54	2.74	43.36

Table 5.14: Medium term coefficient of repeatability (CRM) for N1, P1, N2 and N1P1 amplitude for all rings for ARM eye

CRM ARM eyes (n=4)	N1 latency (ms)	P1 latency (ms)	N2 Latency (ms)	N1P1 amplitude (nV/deg^2)
Ring 1	4.50	2.05	4.08	13.97
Ring 2	1.56	4.29	3.62	18.31
Ring 3	2.06	3.63	0.94	19.31
Ring 4	4.00	2.05	4.23	14.37
Ring 5	2.05	2.06	1.56	11.96
total mean area	2.83	2.82	2.89	15.58

5.34 Discussion

The aim of this study was to determine the variability and repeatability of all first-order mfERG measures in HY, HO and ARM eyes.

The mean overall CV for N1P1 amplitude was 29% in HY eyes and 28% in HO eyes which correlates well with a another study showing an overall N1P1 amplitude CV of 28% in a group of 70 eyes aged 9-80, and a P1 latency CV of 7% [346], although CV for N1 or N2 latency was not reported in that study. Comparatively, P1 latency for this study was less variable at 5% for HY and 4% for HO eyes. Other mfERG variability studies have shown differing figures for N1P1 amplitude CV in healthy eyes, ranging from 10% to 47% [283, 286, 362-364], suggesting quite a wide-ranging degree of variability. Some of these studies did not report CV for mfERG latency. However Harrison *et al.* found CV for mfERG latencies varied from 2.2% to 4.3% with average whole area latencies of 3.0%, although it was not stated whether these were N1, P1 or N2 latencies [364]. Another study found a CV for N1 and P1 latency of 12% and 8% respectively [363], higher than this study's findings. Different electrode types and methods of mfERG analysis may account for variability between studies, along with attenuation in stimulus retinal position, signal to noise ratio and electrode placement.

Interestingly, Gundogan *et al.* demonstrated a reduction in CV with increasing eccentricity [363]. No such reduction in CV for any mfERG measures was found in this study. This may suggest consistency throughout the retinal field being stimulated and good central fixation as central CV was largely consistent with peripheral CV.

There is scant reporting about the CV of mfERG responses and macular disease. A study by Tosha *et al*. [365] looked at the variability of mfERG responses in Stargardt's disease (a form of juvenile macular degeneration). They found a larger CV in mfERG P1 amplitude in this group when compared to a normal group, with variation being larger in the central region in eyes with Stargardt's disease whereas the variation in normal eyes was greatest in the periphery. In eyes with Stargardt's disease the central amplitude and latency variation was twice that of normal eyes (22.8% vs 10.9% for amplitude and 2.11% vs 0.85% for latency), which the authors attributed to unsteady fixation. The average VA of the Stargardt's diseased eyes was 6/18 in that study, whereas the VA of the ARM eyes in this study was 6/9.5 or better and therefore able to see the fixation cross for recording the mfERG.

As already described in chapter 4, although contact lens electrodes give the highest recorded amplitudes when undertaking mfERG, for this study DTL plus electrodes were used as the active corneal electrode for participant comfort [325] and test-retest reliability [277], while not obscuring vision. A study by Mohidin *et al.* [276] showed that the CV of mfERG amplitudes was 23% with DTL electrodes and not significantly different to the CV of contact lens or gold foil electrodes, although N1, P1 and N2 latency CV was not reported here. Another study has demonstrated a greater inter-session reliability using DTL electrodes when compared to Burian-Allen bipolar contact lens electrode [366].

The CRS and CRM for N1P1 amplitude in our study did reduce with retinal eccentricity which is in accordance with another study [367]. There is paucity in the literature about mfERG CR data, with a greater reporting of CV data. This may be because of the high variation in mfERG recordings over time which are affected by changes in signal to noise ratios, electrode position and retinal stimulus position, whereas the CV is a normalised index (SD / mean) of a single visit.

5.35 Conclusion

This study demonstrated comparable mfERG CVs between HY, HO and ARM-affected eyes, and with increasing eccentricity. It would appear that latencies are more stable than amplitudes over recording sessions with regards to CV and CR in healthy and ARM-affected eyes. Maintaining constant calibration of the equipment as per the ISCEV guidelines [281], using the same person to undertake mfERG measurements on each participant for each visit, the same mfERG technique, electrode type and electrode placement for each participant and carefully monitoring participant fixation for each visit minimises mfERG variability between visits and groups. This is essential when trying to determine differences between groups over time and when trying to assess the effectiveness of an intervention on mfERG measures such as nutritional supplementation.

5.4 Baseline measures of dietary, supplemented and retinal lutein and zeaxanthin

Because MP is a modifiable factor potentially linked with reduced risk for ARMD, and ageing and smoking are predominant risk factors for developing ARMD, a study was undertaken to determine whether there were any baseline differences in dietary, supplemented L and Z, and retinal L and Z (MPOD) between HY, HO and ARM eyes. Baseline smoking pack years were also assessed between groups. This work has been published (see appendix 10) [368]. No statistical significance was found in dietary L and Z between HO, HY or ARM eyes in this baseline study. There was a significant difference in supplementary L and Z in ARM eyes compared to HY and HO eyes but no statistical significance for MPOD between all three groups even when the 3 L and Z supplemented eyes in the ARM groups were removed from the analysis. Based on participant's current intake of dietary and supplemental L and Z, the results did not support the theory that ARM develops as a result of L and Z deficiency because the ARM group consumed similar levels of L and Z as the other groups in this study. It could be that historically ARM participants consumed low levels of L and Z and this may have predisposed them to ARM, although it seems likely that higher pack years smoked by this group could be a factor in the development of the disease.

5.5 Analysis of the remainder of the supplement components, and non-supplement nutrients

The nutritional supplement also contained vitamin C and E, copper, zinc and omega 3, which have been associated with reduced risk of developing ARMD (as described in chapter 1). Dietary levels of these nutrients were analysed at baseline to ascertain any differences between HY, HO and ARM groups. Although not contained within the nutritional supplement, dietary levels of carotene (plant forms of vitamin A) and retinol (animal forms of vitamin A) were also analysed because of the potential protective effect of vitamin A against ARMD described in chapter 1. Methods of data collection and analysis were as for published work in section 5.4 (full details in appendix 10) [368]. No significant difference was demonstrated between groups for any of the nutrients at baseline (table 5.15).

Table 5.15: Dietary nutrient levels between HO, HY and ARM groups

		Mean	**nutrient**	**Values ± SD**
		HY	HO	ARM
Copper (mg)	F=2.124 p=0.333	0.93 ± 0.27	1.36 ± 0.83	1.13 ± 0.37
Zinc (mg)	F=2.097 p=0.147	6.37 ± 1.94	7.69 ± 1.85	8.40 ± 3.39
Vitamin C (mg)	F=3.043 p=0.059	68.00 ± 45.53	116.94 ± 65.81	103.55 ± 50.55
Vitamin E (mg)	F=1.873 p=0.167	4.38 ± 1.91	5.92 ± 2.63	5.84 ± 2.55
Lutein and zeaxanthin (μg)	X^2=4.983 p=0.083	1316.81 ± 1623.62	1916.03 ± 1505.83	1924.36 ± 1054.13
omega 3 (g)	F=0.314 p=0.732	0.18 ± 0.12	0.14 ± 0.15	0.15 ± 0.13
Carotene (μg)	F=1.582 p=0.218	1608.07 ± 1715.61	2707.18 ± 1917.65	2184.27 ± 1314.31
Retinol (mg)	F=0.960 p=0.392	178.14 ± 114.16	641.47 ± 1435.84	377.82 ± 283.70

5.6 Chapter five summary

Sample sizes were calculated for healthy and ARM eyes to provide 80% power at the 5% significance level for primary and secondary outcome measures.

The results of baseline data analysis of mfERG measures between HY, HO and ARM eyes emphasised the importance of having age-matched groups when comparing healthy eyes to diseased eyes, or when comparing intervention to non-intervention groups using the mfERG to assess retinal function. The data also revealed changes to retinal function in ARM, highlighting the sensitivity of the mfERG as a tool for detecting central retinal dysfunction in ARM.

As the primary outcome measure, reliability and repeatability of mfERG measures were assessed and were found to be comparable to other studies. Equipment calibration, electrode position, fixation monitoring and mfERG recordings were carried out by the same personnel which gave the results seen in this study, highlighting the need for consistency when undertaking mfERG.

Because the nutrients in the nutritional supplement are thought to reduce the risk of developing ARMD, dietary levels of these were determined for each group. Baseline levels of dietary and retinal levels of L and Z between HY, HO and ARM eyes found no statistically significant difference for any group. Also no significant difference was found between groups for copper, zinc, omega 3 and vitamins C or E. Although not contained within the nutritional supplement, dietary vitamin A levels were also analysed between groups because of its potentially protective effect against risk of developing ARMD.

Chapter six reports the results from a prospective longitudinal randomised controlled trial, assessing the effects of a nutritional supplement on retinal and visual function in HY, HO and ARM eyes. Dietary levels of these nutrients are also assessed over the trial to clearly delineate whether any changes in retinal and/ or visual function are due to dietary levels of nutrients, or the nutritional supplement.

Chapter 6: Randomised controlled trial supplementation results

A mixed between-within subjects analysis of variance (ANOVA) using SPSS 16.0 software was conducted to explore the effects of nutritional supplementation compared with no treatment on retinal function over three time periods using mfERG amplitudes and latencies for different areas of retinal eccentricity as the main outcome measures. This provided analysis of the between-subjects variable (treated and non-treated group), and within-subject variable (time) on the outcome measures (dependent variable). There is no non-parametric alternative to the mixed between-within ANOVA.

6.1 Healthy older group result post-unmasking

There were 22 participants in the healthy older (HO) group that completed all three visits before unmasking occurred; 11 in the treated (T) group and 11 in the non-treated (NT) group. The treated group age range was 52-77 years and the non-treated group age range was 51-69 years. There were six females (55%) and five males (45%) in the treated group. There were nine females (82%) and two males (18%) in the non-treated group. A chi-squared test for independence demonstrated no significant difference between treated and non-treated groups for gender (X^2 = 1.886, p = 0.170). Due to a three month fault with the VERIS mfERG equipment only 14 participants (seven in the treated group and seven in the non-treated group) undertook this test for all three visits. However the visual acuity (VA), contrast sensitivity (CS) and macular pigment optical density (MPOD) were undertaken on all 22 participants. All participants in the HO cohort were Caucasian. A summary of differences in baseline characteristics between treated and non-treated HO participants are detailed in table 6.1 below and were analysed using independent-samples t-tests using SPSS 16.0. Of the treated group seven participants (64% of the 11 treated participants) returned their baseline dietary questionnaire. Of the non-treated group 10 returned their baseline dietary questionnaire (91% of the 11 non-treated participants).

Table 6.1: A summary of baseline characteristics for the HO group using independent-samples t-tests.

Variable	Treated group (T) (n=11)		Non-treated group (NT) (n=11)			
	mean	±SD	mean	±SD	t	p
Age (years)	65.00	9.76	60.45	6.74	1.27	0.22
Smoking (pack-years)*	2.69	4.14	4.82	8.08	-0.78	0.45
Spherical equivalent (D)	0.40	2.90	0.65	2.23	-0.23	0.82
Axial length (mm)	23.49	1.48	23.64	1.17	-0.28	0.79
Baseline dietary questionnaires	Treated group (n=7)		Non-treated group (n=10)			
	mean	±SD	mean	±SD	t	p
Dietary copper (mg)	1.76	1.11	1.08	0.43	1.78	0.96
Dietary zinc (mg)	8.23	1.56	7.31	2.01	1.01	0.33
Dietary retinol (µg)	1181.86	2212.99	263.20	118.95	1.10	0.31
Dietary carotene (µg)	2455.00	1303.92	2883.70	2305.52	-0.44	0.67
Dietary Vitamin E (mg)	5.84	1.85	5.98	3.17	-0.10	0.92
Dietary Vitamin C (mg)	120.57	73.27	114.40	64.06	0.18	0.86
Dietary lutein and zeaxanthin (µg)	1393.05	542.31	2297.25	1926.20	-1.41	0.19
Dietary Omega 3 (g)	0.17	0.15	0.13	0.15	0.56	0.59

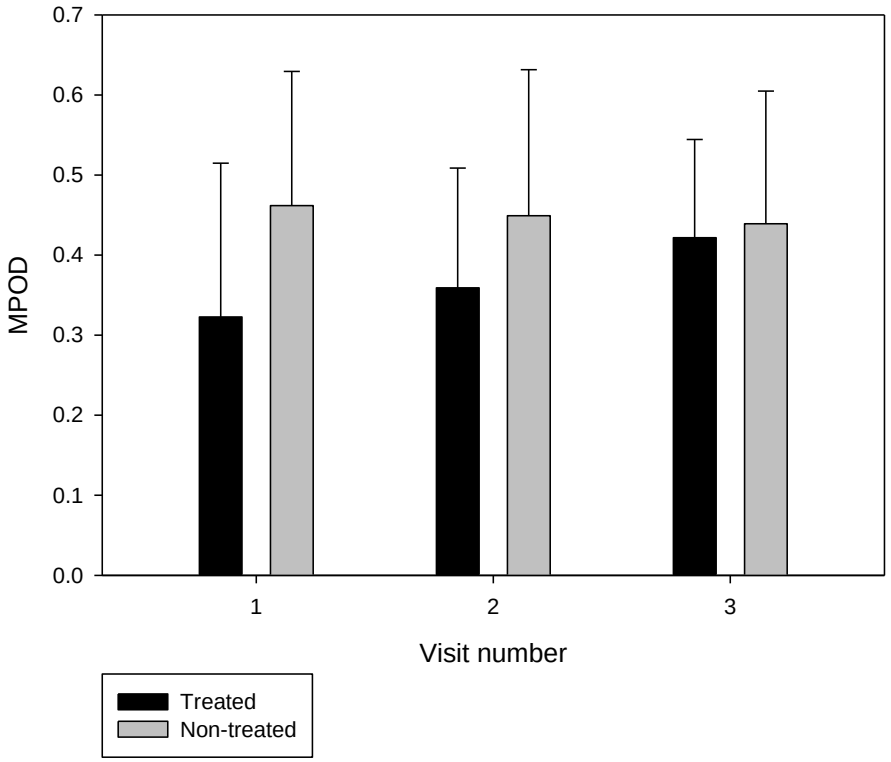
*pack-years= (cigarettes smoked per day x years smoked) / 20

The HO cohort ANOVA results for mfERG are displayed in table 6.2 (in appendix 11). For all mfERG outcome measures for the HO group there was no significant interaction between treated and non-treated groups over three time periods or for group (treated versus non-treated) for any mfERG parameters. There was no significant effect for time for any mfERG parameter except for ring 1 P1 latency with longer latencies for both groups at visit two (tables 6.2 and 6.3 in appendix 11).

All participants (11 in the treated group and 11 in the non-treated groups) undertook VA, CS and MPOD measurements at all three visits (table 6.4 in appendix 11).

For VA and CS for the HO cohort there was no significant interaction between treated and non-treated groups over three time periods. There was no significant effect for time or group. For MPOD there was no significant effect for time or group but a significant interaction between time and group. There was an increase in MPOD of 0.1 over the three visits in the treated group compared to a reduction in MPOD 0.02 in the non-treated group (figure 6.1 below and table 6.5 in appendix 11).

Figure 6.1: Differences between mean MPOD values over three visits between treated and non-treated groups for HO eyes.



Of the 7 people in the HO treated group who completed the baseline dietary questionnaire, four completed a further questionnaire at visit three. Of the 10 people in the HO non-treated group who completed the baseline dietary questionnaire, seven completed a further questionnaire at visit three. A paired-samples t-test using SPSS 16.0 demonstrated no significant difference for any of the dietary components between visits one and three in the HO treated group or for the HO non-treated group (tables 6.6 and 6.7 in appendix 11).

6.2 Healthy younger group result post-unmasking

There were 30 participants in the healthy younger (HY) cohort that completed all three visits before unmasking occurred; 14 in the treated group aged 18-46 years and 16 in the non-treated group aged 21-48 years. All participants in the treated group were Caucasian, consisting of nine females (64.3%) and five males (35.7%). In the non-treated group there were 11 females (68.8%) and 5 males 32.1%) of five South Asians (31.2%) and 11 Caucasians (68.8%). A chi-squared test for independence demonstrated a significant difference between treated and non-treated groups for ethnicity (X^2 = 5.250, p = 0.022) but not for gender (X^2 = 0.067, p = 0.796). Due to a three month fault with the VERIS mfERG equipment 11 participants (eight in the treated group and three in the non-treated group) underwent this test for all three visits. However the VA, CS and MPOD were undertaken on all 30 participants. A summary of differences in baseline characteristics are detailed in table 6.8 below and were analysed using independent-samples t-tests using SPSS 16.0. Of the treated group seven participants returned their baseline dietary questionnaire (50% of the 14 treated participants). Of the non-treated group seven returned their baseline dietary questionnaire (44% of the 16 non-treated participants).

Table 6.8: A summary of baseline characteristics for the HY group using independent-samples t-tests.

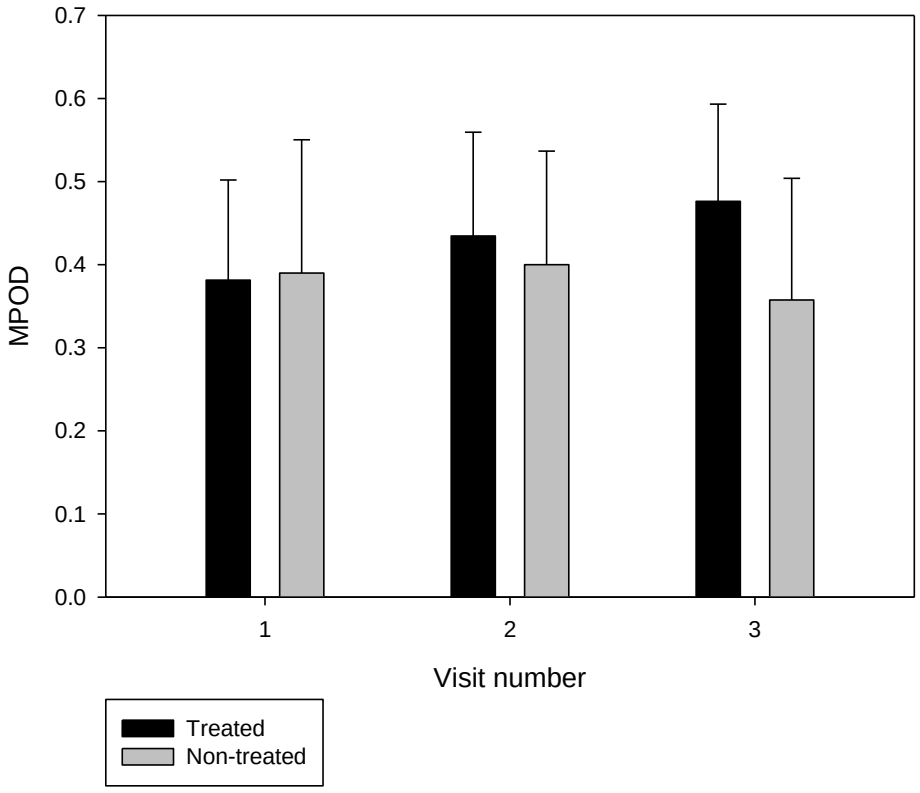
Variable	Treated group (n=14)		Non-treated group (n=16)			
	mean	±SD	mean	±SD	t	p
Age (years)	35.86	8.93	32.56	9.14	1.00	0.33
Smoking (pack-years)*	1.49	2.53	1.29	3.88	0.17	0.87
Spherical equivalent (D)	0.83	1.56	-0.07	2.26	1.25	0.22
Axial length (mm)	23.17	0.78	23.71	1.03	-1.59	0.12
Baseline dietary questionnaires	Treated group (n=7)		Non-treated group (n=7)			
	mean	±SD	mean	±SD	t	p
Dietary copper (mg)	0.98	0.27	0.88	0.28	0.74	0.48
Dietary zinc (mg)	6.57	2.30	6.17	1.66	0.37	0.72
Dietary retinol (µg)	229.29	126.15	127.00	78.87	1.82	0.09
Dietary carotene (µg)	1124.43	686.43	2091.71	2315.22	-1.06	0.31
Dietary Vitamin E (mg)	3.67	1.25	5.09	2.26	-1.45	0.17
Dietary Vitamin C (mg)	50.29	28.77	85.71	54.15	-1.53	0.16
Dietary lutein and zeaxanthin (µg)	1197.62	1238.45	1614.90	2293.37	-0.42	0.68
Dietary Omega 3 (g)	0.17	0.09	0.19	0.15	-0.35	0.74

The HY cohort ANOVA results for mfERG are displayed in table 6.9 in appendix 11. All participants (14 in the treated group and 16 in the non-treated groups) undertook VA and CS measurements at all three visits (table 6.11 in appendix 11). One participant in the treated group was unable to perform the MPOD at one of their visits (therefore, n=13 for the treated group and n=16 in the non-treated group for this outcome measure).

For the HY group there was no statistically significant difference between treated and non-treated eyes for any mfERG measures or for CS (tables 6.9 – 6.12 in appendix 11). There was a

significant difference over time in VA for both treated and non-treated groups, VA reducing by approximately two letters from baseline to visit two and then improving by two letters from visit two to visit three (table 6.12 in appendix 11). As the test was undertaken in the same room for each visit it could be that the background illumination of the logMAR chart fluctuated. This change of two letters is well within the repeatability of ETDRS visual acuity testing for adults [369]. There was a significant interaction effect between time and group for MPOD, with MPOD increasing over the three visits by 0.1 in the treated group compared with a 0.03 reduction in MPOD in the non-treated group between visits one and three (table 6.11 and 6.12 in appendix 11 and figure 6.2 below).

Figure 6.2: Differences between mean MPOD values over three visits between treated and non-treated groups for HY eyes.



Of the seven people in the HY treated group who completed the baseline dietary questionnaire, four completed a further questionnaire at visit three. Of the seven people in the HY non-treated group who completed the baseline dietary questionnaire, three completed a further questionnaire at visit three. An independent samples t-test using SPSS 16.0 demonstrated no significant

difference for any of the dietary components between visits one and three in the HY treated group except for carotene, or for the HY non-treated group except for retinol (tables 6.13 and 6.14 respectively in appendix 11). Dietary carotene levels for the HY treated group were lower at visit one (mean ± SD: 1138.25 ± 561.10) compared to visit three (mean ± SD: 2097.75 ± 466.31). Dietary retinol levels for the HY non-treated group were lower at visit 1 (mean ± SD: 69.33 ± 34.36) compared to visit three (mean ± SD: 316.67 ± 117.69).

6.3 Healthy younger and healthy older group combined result post-unmasking

To fully exploit the data and provide greater statistical power for VA and mfERG measures the HY and HO groups were also combined. There were 52 participants in the combined HO and HY groups that completed all three visits before unmasking occurred; 25 in the treated group aged 18-77 years and 27 in the non-treated group aged 21-69 years. All participants in the treated group were Caucasian, consisting of 15 females (60.0%) and 10 males (40.0%). In the non-treated group there were 20 females (74.1%) and seven males (25.9%) of five Asians (18.5%) and 22 Caucasians (81.5%). A chi-squared test for independence demonstrated a significant difference between treated and non-treated groups for ethnicity (X^2 = 5.122, p = 0.024) but not for gender (X^2 = 1.169, p = 0.280). Due to a three month fault with the VERIS mfERG equipment only 25 participants (15 in the treated group and 10 in the non-treated group) underwent this test for all three visits. However the VA, CS and MPOD were undertaken on all 52 participants. A summary of differences in baseline characteristics are detailed in table 6.15 below and were analysed using independent-samples t-tests. Of the treated group 14 participants returned their baseline dietary questionnaire (56.0%). Of the non-treated group 17 returned their baseline dietary questionnaire (63.0%).

Table 6.15: A summary of baseline characteristics for combined HY and HO groups using independent-samples t-tests.

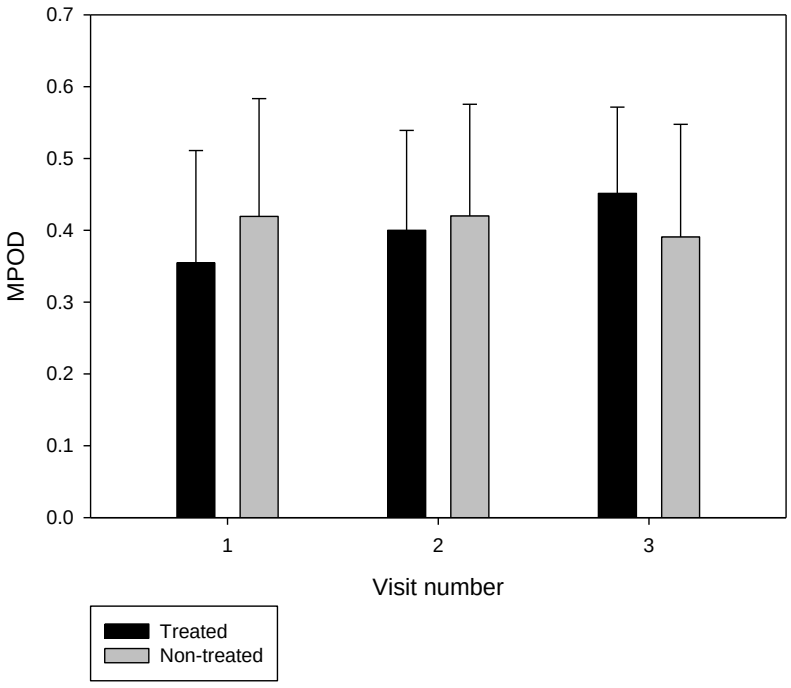
Variable	Treated group (n=25)		Non-treated group (n=27)			
	mean	±SD	mean	±SD	t	p
Age (years)	48.68	17.35	43.93	16.15	1.02	0.31
Smoking (pack-years)*	2.02	3.32	2.73	6.08	-0.52	0.61
Spherical equivalent (D)	0.64	2.21	0.22	2.24	0.68	0.50
Axial length (mm)	23.31	1.12	23.68	1.07	-1.23	0.23
Baseline dietary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

The combined HO and HY cohort ANOVA results for mfERG are displayed in table 6.16 in appendix 11. For all mfERG outcome measures for the combined HY and HO group there was no significant interaction between treated and non-treated groups over three time periods or for group (treated versus non-treated) for any mfERG parameters. There was no significant effect for time for any mfERG parameter except for ring 2 P1 and ring 2 N2 latency (table 6.17 in appendix 11). Ring 2 N2 latency became longer over the three visits for both groups. Ring 2 P1 latency became shorter over three visits in the treated group.

All participants (25 in the treated group and 27 in the non-treated groups) undertook VA and CS measurements at all three visits (table 6.18 in appendix 11). One participant in the treated group was unable to perform the MPOD at one of their visits (therefore n=24 for the treated group and n=27 in the non-treated group for this outcome measure).

For the combined HO and HY group there was no statistically significant difference between treated and non-treated eyes for VA or CS (table 6.18 and 6.19 in appendix 11). There was a significant interaction effect between time and group for MPOD, with MPOD increasing over the three visits by 0.1 in the treated group compared with a 0.03 reduction in MPOD in the non-treated group between visits one and three (figure 6.3 below and tables 6.18 and 6.19 in appendix 11).

Figure 6.3: Differences between mean MPOD values over three visits between treated and non-treated groups for HY and HO eyes.



Of the 14 people in the combined HY and HO treated group who completed the baseline dietary questionnaire, eight completed a further questionnaire at visit three. Of the 17 people in the combined HY and HO non-treated group who completed the baseline dietary questionnaire, 10 completed a further questionnaire at visit three. A paired-samples t-test using SPSS 16.0

demonstrated no significant difference for any of the dietary components between visits one and three in the combined HY and HO treated group or for the combined HY and HO non-treated group (tables 6.20 and 6.21 respectively in appendix 11).

6.4 Age-Related Maculopathy group result post-unmasking

There were 14 participants in the ARM cohort that completed all three visits before unmasking occurred; eight in the treated group and six in the non-treated group. The treated group age range was 56-81 years and the non-treated group age range was 61-83 years. There were five females (62.5%) and three males (27.5%) in the treated group. There were five females (83%) and one male (17%) in the non-treated group. A chi-squared test for independence demonstrated no significant difference between treated and non-treated groups for gender (X^2 =0.729, p = 0.393). Due to a three month fault with the VERIS mfERG equipment, seven participants (four in the treated group and three in the non-treated group) undertook this test for all three visits. However the VA, CS and MPOD were undertaken on all 14 participants. All participants in the ARM group were Caucasian. A summary of differences in baseline characteristics are detailed in table 6.22 below and were analysed using independent-samples t-tests. Of the treated group seven participants (88% of the eight treated participants) returned their baseline dietary questionnaire. Of the non-treated group four returned their baseline dietary questionnaire (67% of the six non-treated participants). None of the ARM eyes tested developed into AMD throughout the study or they would have been excluded. None of the ARM eyes clinically changed over the study period as recorded by 45º fundus photographs.

Table 6.22: A summary of baseline characteristics for the ARM group using independent-samples t-tests.

Variable	Treated group (n=8)		Non-treated group (n=6)			
	mean	±SD	mean	±SD	t	p
Age (years)	65.50	9.27	69.67	7.52	-0.87	0.40
Smoking (pack-years)*	7.04	9.42	13.5	15.86	-0.96	0.36
Spherical equivalent (D)	0.72	2.27	0.96	2.30	-0.20	0.85
Axial length (mm)	23.11	0.86	23.48	1.25	-0.65	0.53
Baseline dietary questionnaires	Treated group (n=7)		Non-treated group (n=4)			
	mean	±SD	mean	±SD	t	p
Dietary copper (mg)	1.08	0.30	1.22	0.51	-0.60	0.57
Dietary zinc (mg)	8.07	2.43	8.98	5.06	-0.41	0.69
Dietary retinol (µg)	492.00	347.89	288.25	97.02	0.78	0.46
Dietary carotene (µg)	2399.71	1530.05	1807.25	882.10	0.70	0.50
Dietary Vitamin E (mg)	5.06	1.60	7.21	3.55	-1.41	0.19
Dietary Vitamin C (mg)	98.71	49.42	112.00	59.02	-0.40	0.70
Dietary lutein and zeaxanthin (µg)	1606.50	686.20	2550.02	1557.50	-1.42	0.19
Dietary Omega 3 (g)	0.14	0.10	0.17	0.18	-0.35	0.73

*pack-years=(cigarettes smoked per day x years smoked) / 20

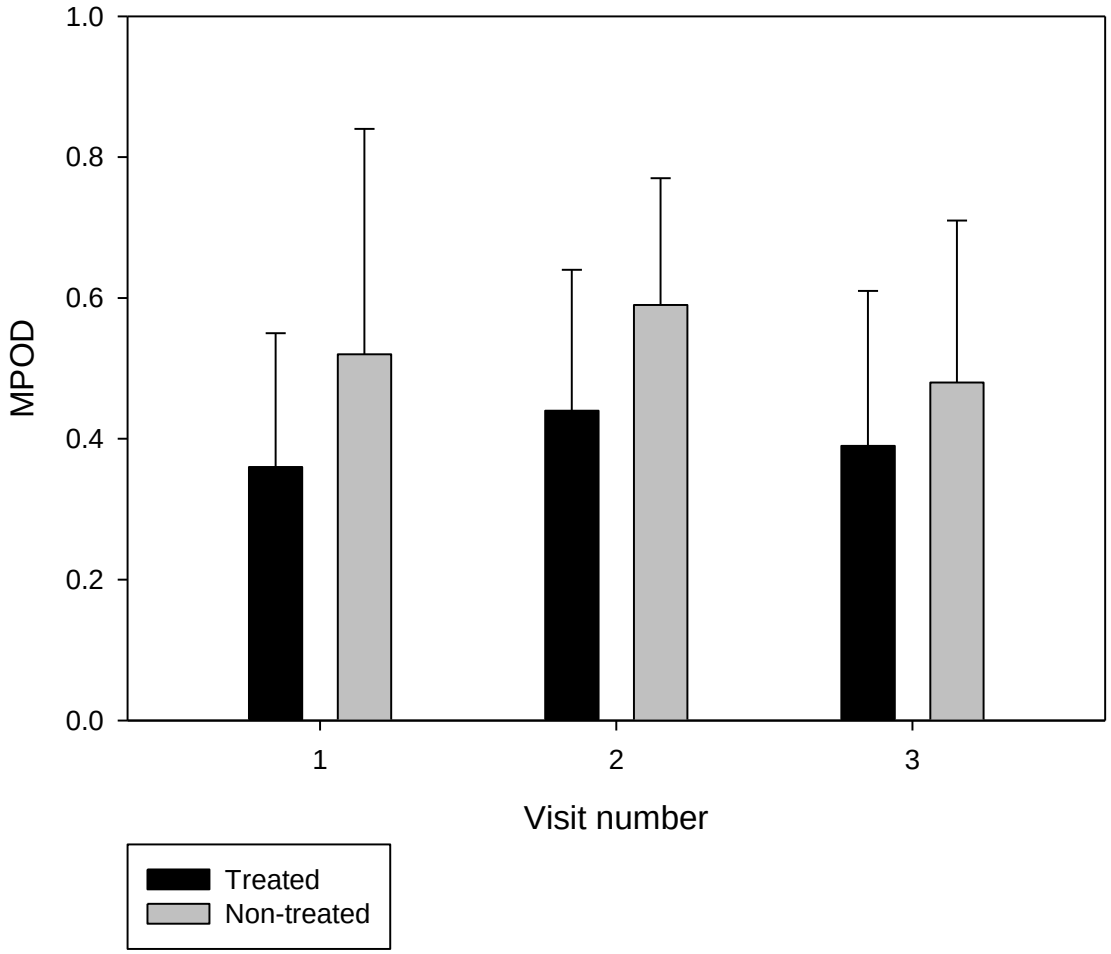
The ARM cohort ANOVA results for mfERG are displayed in table 6.23 in appendix 11. For all mfERG outcome measures for the ARM group there was no significant interaction between treated and non-treated groups between visits one and three. There were no significant effects for time or group (treated versus non-treated), except for ring 3 and ring 4 N1P1 amplitude for time (tables 6.23 and 6.24 in appendix 11) with the amplitudes of both rings for both groups reducing between visits one and two, and increasing between visits two and three.

All eight treated participants underwent mfERG at visit one and visit three. Therefore to exploit this greater sample size, paired-samples t-tests were carried out using SPSS 16.0. This analysis also showed statistical significance for ring 3 N1P1 amplitude, with amplitude increasing by 6.67 nV/deg^2 (table 6.25 in appendix 11).

Five eyes in the non-treated group had mfERG at visit one and three so an independent-samples t-test between treated and non-treated groups was undertaken to assess the differences between visit one and three for the eight treated and five non-treated ARM eyes for mfERG measures (table 6.26 in appendix 11). No statistically significant differences were seen in differences between treated and non-treated groups between visits one and three for any mfERG measures except for ring 3 N1 latency with the non-tested group ring 3 N1 latency having a greater difference between visit one and three (2.17 ms) than the treated group (0.31 ms). This is a spurious result as N1 latency improved in the non-treated group between visits 1 and 3 when compared to the treated group.

All participants (eight in the treated group and six in the non-treated group) undertook VA, CS and MPOD measurements at all three visits (table 6.27 in appendix 11). There was no significant interaction between treated and non-treated groups for VA, CS and MPOD in the ARM group (figure 6.4) between visits one and three. Furthermore, there was no significant effect for time or group (tables 6.27 and 6.28 in appendix 11).

Figure 6.4: Differences between mean MPOD values over three visits between treated and non-treated groups for ARM eyes.



Of the seven people in the ARM treated group who completed the baseline dietary questionnaire, five completed a further questionnaire at visit three. Of the four people in the ARM non-treated group who completed the baseline dietary questionnaire, four completed a further questionnaire at visit three. An independent samples t-test using SPSS 16.0 demonstrated no significant difference for any of the dietary components between visits one and three in the ARM treated group or for the ARM non-treated group (tables 6.29 and 6.30 respectively in appendix 11).

Although our sample size was small, a study by Koh *et al.,* [186] found a statistically significant increase in MPOD of 0.07 in seven patients with ARM supplemented with 10mg of lutein daily over 18-20 weeks. Our L supplement contained 12mg of lutein and 0.6mg of zeaxanthin. All participants from HO, HY and ARM groups were asked to provide details of any additional nutritional supplementation at baseline. Not all participants were able to specify the amounts of nutrients within their supplements. Therefore chi-squared tests for independence were carried out to investigate differences between treated and non treated groups. There were no statistically significant differences at baseline between treated and non-treated groups for participants own supplements (table 6.31). There were no changes to participants own supplement use throughout the study when questioned at visits two and three.

6.5 Study nutritional supplement compliance

Compliance was assessed by asking participants to return any boxes of the supplement that were not taken and remaining tablets were counted. Those who forgot to bring back the tablets were asked to contact the principle investigator after counting tablets at home (table 6.32). Patient compliance was elicited using supportive language to minimise the number of participants concealing supplement non-adherence [262], and reporting lower levels of remaining tablets than was actually the case. The sole reason for non-adherence was forgetfulness. A one way ANOVA using SPSS 16.0 found no statistically significant difference between groups for supplement compliance during the study (see table 6.32) ($F = 0.396$, $p = 0.676$).

Table 6.31: Summary of the baseline differences between treated (T) and non-treated (NT) participants own supplement use using chi-squared tests for independence.

Supplement	HO T	HO NT	X^2	p	HY T	HY NT	X^2	P	ARM T	ARM NT	X^2	p
Multivitamin	0	3	1.544	0.214	1	1	<0.001	1.000	2	0	0.304	0.581
Vitamin C	1	1	<0.001	1.000	0	0			1	0	<0.001	1.000
Vitamin E	0	0			0	0			0	0		
Vitamin D	0	0			0	0			0	0		
Vitamin B12	1	0	<0.001	1.000	0	1	<0.001	1.000	0	0		
Zinc	1	0	<0.001	1.000	0	0			0	0		
Selenium	0	0			0	0			0	0		
Calcium	0	0			0	0			1	1	<0.001	1.000
Cod liver oil	1	2	<0.001	1.000	0	2	0.404	0.525	1	1	<0.001	1.000
GLA	0	0			0	0			0	0		
Ginkgo biloba	1	1	<0.001	1.000	0	1	<0.001	1.000	0	1	0.022	0.881

Table 6.31 continued.

Supplement	HO T	HO NT	X^2	p	HY T	HY NT	X^2	P	ARM T	ARM NT	X^2	p
Omega 3	1	0	<0.001	1.000	0	0			2	1	<0.001	1.000
Glucosamine	2	2	0.550	0.458	0	1	<0.001	1.000	4	3	<0.001	1.000
Folic acid	0	0			0	0			1	0	<0.001	1.000
Starflower oil	0	0			0	0			0	0		
Garlic	1	1	<0.001	1.000	0	0			0	0		
Evening primrose	0	0			0	1	<0.001	1.000	0	0		
Royal jelly	0	0			0	0			0	0		
Ginseng	0	0			0	0			0	0		
Flaxseed	0	0			0	0			0	0		
I-caps	0	0			0	0			1	0	<0.001	1.000
Visionace	0	0			0	0			0	0		
Saw palmetto	0	1	<0.001	1.000	0	0			0	0		
Rosehip	0	1	<0.001	1.000	0	0			0	0		

Table 6.31 continued.

Supplement	HO T	HO NT	X^2	p	HY T	HY NT	X^2	P	ARM T	ARM NT	X^2	p
Iron	0	1	<0.001	1.000	0	0			1	0	<0.001	1.000
Lutein	0	0			0	0			1	1	<0.001	1.000
Aloe	0	1	<0.001	1.000	0	0			0	0		
Chondroitin	0	1	<0.001	1.000	0	1	<0.001	1.000	0	1	0.022	0.881
Magnesium	0	0			0	0			1	0	<0.001	1.000
MSM	0	0			0	0			2	0	0.304	0.581

Table 6.32: Summary of trial duration with independent t tests (mean ± SD) and participant compliance (% ± SD).

Mean trial duration (months)	**HY T**	**HY NT**	**t test (p value)**	**HO T**	**HO NT**	**t test (p value)**	**ARM T**	**ARM NT**	**t test (p value)**
VISIT 1-2	5.1 ± 0.9	5.1 ± 0.6	0.304 (0.764)	4.9 ± 0.8	4.7 ± 0.7	0.573 (0.573)	5.6 ± 0.9	5.3 ± 0.8	0.617 (0.549)
VISIT 1-3	10.7 ± 1.5	10.7 ± 1.2	0.055 (0.957)	10.5 ± 0.9	10.5 ± 1.7	0.000 (1.000)	10.4 ± 1.1	10.5 ± 1.4	-0.192 (0.851)
Mean compliance (% tablets taken)	76.9 ± 14.8	-		82.1 ± 17.0	-		81.1 ± 13.0	-	

6.6 Chapter six summary

For all mfERG outcome measures for the HO group there was no significant interaction between treated and non-treated groups over three time periods or for group (treated versus non-treated). There was no significant effect for time for any mfERG parameter except for ring 1 P1 latency with longer latencies for both groups at visit two. This was not clinically significant based on the CR studies undertaken in chapter five. For VA and CS for the HO cohort there was no significant interaction between treated and non-treated groups over three time periods. There was also no significant effect for time or group. For MPOD there was no significant effect for time or group but a significant interaction between time and group. There was an increase in MPOD of 0.1 over the three visits in the treated group compared to a reduction in MPOD 0.02 in the non-treated group.

For the HY group there was no statistically significant difference between treated and non-treated eyes for any mfERG measures or for CS. There was a significant difference over time in VA for both treated and non-treated groups, with VA reduced by approximately two letters from baseline to visit two and then improved by two letters from visit two to visit three. As the test was undertaken in the same room for each visit it could be that the background illumination of the logMAR chart fluctuated. This is not clinically significant as a change of 2 letters is well within the repeatability of ETDRS visual acuity testing [369]. There was a significant interaction effect between time and group for MPOD, with MPOD increasing over the 3 visits by 0.1 in the treated group compared with a 0.03 reduction in MPOD in the non-treated group between visits 1 and 3.

In order to exploit the larger sample size, data from HY and HO eyes were also combined for analysis. There was no significant interaction between treated and non-treated groups over three time periods or for group (treated versus non-treated) for any mfERG parameters. There was no significant effect for time for any mfERG parameter except for ring 2 P1 and ring 2 N2 latency. Ring 2 N2 latency became longer over the three visits for both groups. Ring 2 P1 latency became shorter over three visits for treated eyes and became longer and then shorter for non-treated groups. There was no statistically significant difference between treated and non-treated eyes for VA or CS. There was a significant interaction effect between time and group for MPOD, with MPOD increasing over the three visits by 0.1 in the treated group compared with a 0.03 reduction in MPOD in the non-treated group between visits 1 and 3.

For all mfERG outcome measures for the ARM cohort there was no significant interaction between treated and non-treated groups over three time periods for mfERG measures. There were no significant effects for time or group, except for ring 3 and ring 4 N1P1 amplitude for time with the amplitudes of both rings for both groups reducing between visits one and two, and increasing between visits two and three. For VA, CS and MPOD there was no significant interaction between treated and non-treated groups over three time periods. Furthermore there was no significant effect for time or group.

No effect of antioxidant supplementation was demonstrated for any outcome measures over 40 weeks in HO, HY, combined HY and HO or ARM groups except for an increase in MPOD over the study period in HY and HO eyes, and in combined HO/HY eyes.

The next chapter describes the results of 20 weeks of withdrawal of the lutein-based supplement on the primary and secondary outcome measures.

Chapter 7: Supplementation withdrawal results

Once unmasking occurred at visit 3 a further study was undertaken to assess the effects of supplement withdrawal on visual function. This was done for all participants in the HO, HY and ARM groups who took the supplement. These participants attended for a fourth visit at 60 weeks where mfERG, MPOD, VA and CS were repeated.

The data were analysed with SPSS 16.0. using paired-samples t-tests when parametric assumptions were met according to normality as shown by non-significance for the Shapiro-Wilk test. The Wilcoxon Signed Rank test was carried out when parametric assumptions were not met.

7.1 Healthy older group

Although there were 11 treated participants at visit three, there were 10 participants in the HO group that completed both visit three and four, with one participant dropping out due to illness. The age range was 54-77 years (mean ± SD; 64.0 ± 9.02 years). There were six females (60%) and four males (40%). Due to a three month fault with the mfERG equipment, eight undertook this test for both visits. However the VA, CS and MPOD were undertaken on all 10 participants. All participants were Caucasian. Four of the 10 participants completed a dietary questionnaire at visit three, of which three completed a further questionnaire at visit four. Spherical equivalent did change from a mean ± SD of 0.14 ± 3.15 at visit three to 0.43 ± 3.16 dioptres of sphere at visit four ($t = -3.183$, $p = 0.011$). This 0.29 change is not clinically significant and well within the repeatability values of the NVision K5001 autorefractor based on Davies *et al's* work [370].

A summary of the changes in objective and subjective outcome measures of visual function and changes in dietary differences of participant characteristics between visits three and four are detailed in table 7.1, 7.2 and 7.3 respectively in appendix 12.

After withdrawal of the nutritional supplement for 20 weeks, there was no statistically significant change for any outcome measure except for mfERG ring 1 N1P1 amplitude which reduced on supplementation withdrawal. There was also no change in dietary intake of nutrients during the withdrawal period.

7.2 Healthy younger group

Although there were 14 treated participants at visit three, there were 12 participants in the HY group that completed both visit three and four, with one participant unable to return and another developing ocular hypertension. The age range was 18-46 years (mean ± SD = 36.0 ± 8.96 years). There were seven females (58%) and five males (42%). Due to a three month fault with the mfERG equipment, nine undertook this test for both visits. However the VA, CS and MPOD were undertaken on all 12 participants. All participants were Caucasian. Three of the 12 participants completed a dietary questionnaire at visit three, of which two completed a further questionnaire at visit four. The spherical equivalent did not change between visit three (mean ± SD; 0.42 ± 0.99 dioptres of sphere) and visit four (mean ± SD; 0.49 ±0.92 dioptres of sphere).

A summary of the changes in objective and subjective outcome measures of visual function and changes in dietary differences of participant characteristics between visits three and four are detailed in table 7.4, 7.5 and 7.6 respectively in appendix 12. The data were analysed with SPSS 16.0 using paired-samples t-tests when parametric assumptions were met according to normality as shown by non-significance for the Shapiro-Wilk test. The Wilcoxon Signed Rank test was carried out when parametric assumptions were not met.

After withdrawal of the nutritional supplement for 20 weeks, there was no statistically significant change for any outcome measure except for mfERG ring 3 P1 latency which reduced with supplementation withdrawal. There was also no change in dietary levels of nutrients during the withdrawal period.

7.3 Healthy younger and healthy older groups combined

In order to provide an adequate sample size for VA, HY and HO groups were combined for all outcome measures. Although there were 25 treated participants at visit three, there were 22 participants in the combined HY and HO groups that completed both visit three and four. The age range was 18-77 years (mean ± SD = 51.0 ± 17.31 years). There were 13 females (59%) and nine males (41%). Due to a three month fault with the mfERG equipment, 17 undertook this test for both visits. However the VA, CS and MPOD were undertaken on all 22 participants. All participants were Caucasian. Seven of the 22 participants completed a dietary questionnaire at visit three, of which five completed a further questionnaire at visit four. The spherical

equivalent did not change between visit 3 (mean ± SD; 0.50 ± 2.23 dioptres of sphere) and visit four (mean ± SD; 0.46 ± 2.17 dioptres of sphere), t = -0.094 p = 0.926.

A summary of the changes in objective and subjective outcome measures of visual function and changes in dietary differences of participant characteristics between visits three and four are detailed in table 7.7, 7.8 and 7.9 respectively in appendix 12.

After withdrawal of the nutritional supplement for 20 weeks, there was no statistically significant change for any outcome measure except for mfERG ring 1 and ring 2 N1P1 amplitudes which reduced on supplement withdrawal. There was also no change in dietary levels of nutrients during the withdrawal period except for increased omega-3 levels.

7.4 Age-related maculopathy group

There were eight treated ARM participants at visit three, and they were all able to complete visit four. The age range was 56-81 years (mean ± SD = 65.50 ± 9.27 years). There were five females (62.5%) and three males (27.5%). All eight undertook all tests for both visits. All participants were Caucasian. Four of the eight participants completed a dietary questionnaire at visit three, of which three completed a further questionnaire at visit four. The spherical equivalent did not change between visit three (mean ± SD; 1.02 ± 2.15 dioptres) and visit four (mean ± SD; 0.85 ± 2.15 dioptres), Z = -0.980 p = 0.327.

A summary of the changes in objective and subjective outcome measures of visual function and changes in dietary differences of participant characteristics between visits three and four are detailed in table 7.10, 7.11 and 7.12 respectively in appendix 12.

After withdrawal of the nutritional supplement for 20 weeks, there was no statistically significant change for any outcome measure except for mfERG ring 3 N2 latency and ring 4 P1 latency which increased on supplementation withdrawal. There was no change in dietary levels of nutrients during the withdrawal period.

There appears to be a trend in HY, HO and combined HY/HO eyes for all N1P1 amplitudes to be reduced for all five rings after supplementation withdrawal. This is also true for ARM eyes for rings 1, 3 and 4.

7.5 Chapter seven summary

After 20 weeks of supplement withdrawal there were no changes in any outcome measures for any groups except for; a reduction in ring 1 N1P1 amplitude in HO eyes; a reduction in ring 3 P1 latency in HY eyes; a reduction in ring 1 and ring 2 N1P1 amplitude when HY and HO eyes were combined; and an increase in mfERG ring 3 N2 latency and ring 4 P1 latency in ARM eyes. There was a trend for reduced N1P1 amplitudes for all rings in HY and HO eyes and in rings 1, 3 and 4 in ARM eyes.

Chapter 8 Discussion

8.1 Main outcomes

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

Participants were divided into HY, HO and ARM groups. The primary outcome measure was mfERG N1P1 amplitude. The secondary outcome measures were mfERG N1, P1, and N2 latency, VA, CS and MPOD.

Supplementation effects in healthy eyes

Lutein and Z within the supplement was found to accumulate in the retinae of both HO and HY eyes over a 40 week period, in the form of a statistically significant increase in MPOD.

When HO and HY groups were combined, the increase in MPOD over three visits remained in the treated eyes, and mfERG ring 2 P1 latency became statistically significantly shorter over the three visits.

Many studies have demonstrated an increase in MPOD with L-based supplementation in healthy eyes [132, 133, 186, 371-377]. However not all studies have assessed whether this increased retinal accumulation of MP is associated with changes to other measures of visual and retinal function such as CS, VA or mfERG [186, 371-376]. Some studies also fail to report dietary levels of L and Z during supplementation [371, 372, 374-377].

There have been conflicting results within the literature with regards to the effects of L-based nutritional supplementation on visual function in healthy eyes. A recent prospective interventional study by Sasamoto *et al.*, assessing the effects of a L-based nutritional supplement on MPOD and visual function in 43 eyes of 43 subjects, showed that MPOD did not significantly increase over 12 months, although improvements in CS were seen [378]. It is difficult to compare Sasamoto's work with this (the Aston) study as different methods of assessing MPOD (autofluorescence spectrometry) and CS (area under the log contrast sensitivity function) were used, and supplement formulation (6mg L), ethnicity (Japanese) and

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

The Collaborative Optical Macular Pigment Assessment Study (COMPASS) investigators concluded that supplementing with 12 mg of L, 1 mg of Z and antioxidants in healthy eyes significantly increased MPOD over 12 months, but this did not correspond with an improvement in VA or photopic CS [379] in this prospective interventional study. This is in agreement with the Aston study but a different method of CS assessment was used (contrast sensitivity function), the sample size was larger at 121 subjects and the supplement formulation differed.

A 12 week randomised, double blind, placebo-controlled intervention study of 37 healthy eyes found no statistically significant improvement in VA or central CS when supplementing with 6 or 12 mg of L [380]. Improvements were noted at wider fields of CS analysis. This study differs from the Aston study in supplement formulation, ethnicity (Chinese) and methods of CS (automated contrast glare tester) assessment.

A double-masked, randomised controlled trial of 46 eyes by Bartlett and Eperjesi concluded that supplementing with 6 mg of L combined with vitamins and minerals did not improve CS or VA over 9 or 18 months [381]. This is in concordance with the Aston study. The same methods of VA and CS assessment were used, and a similar study design, but supplement formulation differed.

As previously discussed, the mfERG principally measures cone photoreceptor and bipolar cell function. Tubulin is found in the cone photoreceptor axon layer of the fovea where it may selectively bind to L and Z [126], leading to the MPOD increases seen on supplementation. The rationale was that an increase of L and Z binding to tubulin around the cone photoreceptor axons may have affected cone function which could be objectively assessed by the mfERG.

To the author's knowledge, the literature provides no information with regards to the effects of L-based supplementation on mfERG measures in healthy eyes. Ring 2 P1 latency of the mfERG became statistically significantly shorter over time in those taking the supplement. However this was not clinically significant.

Supplementation effects in ARM eyes

The nutritional supplement had no effect on any outcome measure for ARM eyes for this study.

Three studies have demonstrated an increase in MPOD with L-based supplementation in eyes with ARMD [102, 186, 375]. However not all studies have assessed whether this increased retinal accumulation of MP causes changes to other measures of visual and retinal function such as CS, VA or mfERG [186, 375]. Some studies also failed to report dietary levels of L and Z during supplementation [375].

The Lutein Nutrition Effects Measured by Autofluorescence (LUNA) prospective interventional study found that although a 12 mg L-based supplement increased MPOD in 100 eyes with ARM and atrophic AMD over 6 months overall, some participants did not show augmentation of MPOD even though serum levels of L and Z increased [375]. The larger sample size, mixed ARMD categories, differing supplement formula and dissimilar assessment of MPOD make it difficult for the LUNA study to be compared to the Aston study.

A study prospective interventional by Richer [104] concluded that L supplementation in 14 males with atrophic AMD improved CS and VA, although CS (CS function) and VA (Snellen) assessment, ARMD type and gender composition differed from the Aston study. Supplement formulation was incompatible, with spinach used for 11 subjects, and the remainder receiving a L-based antioxidant.

The Lutein Antioxidant Supplementation Trial (LAST) investigators concluded that a 12 month prospective randomised, double masked, placebo-controlled, supplementation trial with 10 mg L, or 10 mg L with other carotenoids, antioxidants and minerals, improved MPOD and CS (CS function) [102]. A larger sample size of 90 participants was enrolled in the LAST, and was predominantly male with atrophic AMD, thus incompatible with the Aston study.

A randomised controlled trial by Bartlett and Eperjesi found that supplementation with 6 mg of L combined with vitamins and minerals was not beneficial for improving CS in 15 eyes with ARM or atrophic AMD over 9 months [382]. Although the method of assessing CS was comparable to the Aston study, supplementation formulation differed.

The Taurine, Omega-3 fatty acids, Zinc, Antioxidant, Lutein (TOZAL) prospective, double-blind study concluded that 76.7% of 37 eyes with atrophic AMD had stable or improved VA at 6 months when taking an 8 mg L-based supplement. Although VA assessment was the same as the Aston study the type and ARMD and supplement composition differed [101].

The CARMIS randomised controlled trial found that a 10 mg L-based supplement over 12 months increased N1P1 amplitudes in rings 1 and 2 of the mfERG in 15 eyes with ARM or non-central geographic atrophy [103]. As the supplement composition and ARMD categories were not the same as for the Aston study, it is difficult to compare the CARMIS study with the Aston study. Unlike the Aston study, the CARMIS study did not report dietary levels of L and Z

throughout the study period, thus it is difficult to determine if the mfERG changes seen were due supplementary or dietary changes in L and Z. Retinal accumulation of L and Z (MPOD) were not measured in the CARMIS study, thus it is impossible to ascertain if increased retinal levels of L and Z were related to the increased central mfERG amplitudes reported in the study.

Withdrawal effects

Of those who were assigned to taking the supplement for the supplementation trial, a further study was undertaken to assess the effects of withdrawal of the supplement 20 weeks after supplementation had ceased.

On withdrawal of the supplement ring 1 N1P1 amplitudes were reduced in HO eyes and ring 3 P1 latency became shorter in HY eyes. When HY and HO eyes were combined, increasing sample size, ring 1 and ring 2 N1P1 amplitudes were reduced. In ARM eyes ring 3 N2 latency and ring 4 P1 latency became longer. There were no changes to any other outcome measures or for any other groups.

There is paucity in the literature with regard to the effects of L supplement withdrawal on measures of visual function. A further LUNA study concluded that 9 months after discontinuation of a L-based supplement, MPOD levels were still elevated in 108 participants, of which 100 had ARMD and 8 were healthy [383]. This is in concordance with the Aston study, with no statistically significant reduction in MPOD seen for HO, HY, combined HO and HY, and ARM eyes after supplement withdrawal. However, it is difficult to analogise the Aston and LUNA studies due to the larger sample size, combining of healthy and ARMD eyes, and differing supplement withdrawal time period assessed in the LUNA study.

Cystic fibrosis (CF) is associated with decreased carotenoid absorption and hence reduced retinal levels of L and Z [384]. Although this is not the same as supplement withdrawal, it may provide some information about the effects of reduced levels of retinal carotenoids on retinal and visual function. A study of 10 participants with CF demonstrated that although MPOD was significantly reduced in these eyes when compared to healthy controls, CS and mfERG measures were no different in CF eyes compared to healthy eyes [384]. This was echoed in another study of rhesus monkeys whose mfERG was normal even when L and Z were excluded from their diets from birth to 10-18 years [385].

A trend became apparent for reduced mfERG N1P1 amplitudes for all 5 rings for the HO group, HY group and combined HO and HY groups on withdrawal of the supplement. This was also true for rings 1, 3 and 4 in ARM eyes. A possible reason for this is that changes to retinal

function may occur more rapidly than depletion of retinal levels of L and Z. It may also be that the variability of the MPOD equipment masked any subtle reductions in L and Z levels. The other components of the supplement rather than L and Z may deplete faster in the retina on supplement withdrawal which may cause the reduction in mfERG amplitudes. However, if this was the case amplitudes may have increased during supplementation.

A possible mechanism for the trend for reduced N1P1 amplitudes is that although L and Z may deplete from around the tubulin binding sites in cone axons on supplement withdrawal, variability in MPOD equipment may mask any subtle reductions in L and Z levels. Therefore no reduction in retinal levels of L and Z (MPOD) are seen but subtle changes in retinal function are found in the form of reduced mfERG amplitudes. The trend for selective reduction in N1P1 amplitudes in ARM (rings 1, 3 and 4) may be due to inconsistent accumulation and depletion of L and Z in the diseased retina due to the disruption of nutrient and waste exchange as discussed in chapter 1.

8.2 Limitations

Technical difficulties affected sample sizes for mfERG measures. However this was compensated for by combining HY and HO eyes which increased the sample size. This also provided an adequate sample size for VA analysis. Paired t-tests for treated ARM eyes between visits one and three also provided an adequate sample size for mfERG analysis. All other outcome measures had sufficient sample sizes for data analysis with the exception of MPOD in the ARM group. However, a study by Koh *et al*. found a statistically significant mean rise of 0.07 in MPOD with supplementation of 10mg of free lutein in 7 ARM eyes over 18-20 weeks [186], a smaller sample size than for the Aston study.

The initial aim was to recruit 120 participants to obtain 40 HO, 40 HY and 40 ARM eyes. The recruitment process was extensive as described in section 2.2 and more difficult than predicted. On reflection, older participants and those with ARMD had more mobility problems than younger participants. Financial recompense for time and travel expenses may have encouraged older participants and those with ARMD to participate, especially as many visits were required.

Food diaries were prospective and were completed over several days in order to provide detailed dietary information. Return rates were lower than anticipated. Increased return rates may have occurred if a small fee was attached to each returned diary. A recall food diary would have provided a greater number of returned questionnaires as participants could have completed these during their visit to Aston. However, this may not have been as accurate due to participants having to remember their food intake over a 3 day period.

It may be argued that using a combination of supplement components does not allow for the assessment of individual effects of each active ingredient on measures of visual function. However many eye supplements available are combination supplements due to the synergistic nature of ingredients. Appropriate examples of these synergistic relationships include copper and zinc combinations required for copper-zinc superoxide dismutase, a part of the antioxidant system within RPE and retina [164], and increased bioavailability of L with the addition of certain fats, including olive and peanut oils [386, 387]. Because the causes of ARMD are multi-faceted it may be that multi-ingredient supplement is of more benefit than a single nutrient. Indeed, the AREDS investigators found that zinc and antioxidants reduced the relative risk of developing advanced AMD by 21% and 17% respectively. When zinc and antioxidants were combined, the risk reduced further to 25% [100].

A further limitation may be that the study population characteristics did not precisely replicate those of the general population. It may be that study participants who choose to partake in research do so because they have a greater interest in their own health than those within the general population. They may be more proactive in taking care of their health.

Serum L and Z levels could have been monitored throughout the trial. However, this would not necessarily reflect the levels accumulated within the retina [371] and the invasive nature of the procedure may have hindered recruitment further.

The lack of placebo in the non-treated group may be considered a limitation. The time constraints of the trial, and the costly and time consuming nature of placebo manufacture meant that placebo allocation was not feasible. It may be argued that participants not receiving a treatment may bias the results by not engaging in the study as enthusiastically as those given a treatment or placebo. However the results of the trial did not reflect this.

Supplement compliance was the same for treated ARM eyes, older eyes and younger eyes, averaging around 80%. However, better compliance for all groups may have provided different results. Forgetfulness was the sole reason for this level of compliance. Participants were required to take a tablet and a capsule in the morning and repeat this of an evening. Compliance may have improved if a supplement of single dosage was provided.

8.3 Confounding variables

Randomised controlled trials are the gold standard for assessing whether a cause-effect relationship exists between interventions and outcomes [189]. This is because random allocation of participants to intervention groups limits the influence of confounding variables. The trial

was single-masked, eliminating the possibility that observed effects were due to investigator bias.

For the supplementation trial, there were no differences between treated and non-treated groups for gender, age, smoking, spherical equivalent, axial length, dietary copper, zinc, L and Z, retinol, carotene, vitamin C, vitamin E or omega 3. This was true for HO, HY, combined HY and HO and ARM groups. There was a difference between treated and non-treated groups for ethnicity in the HY and combined HY and HO groups, but not for the HO or ARM group. There is no reason to believe that the results would have been different if the treated group had contained similar numbers of Asians as the non-treated group.

Although not contained within the nutritional supplement, dietary levels of carotene (plant forms of vitamin A) and retinol (animal forms of vitamin A) were also analysed throughout supplementation and withdrawal trials because of the potential protective effect of vitamin A against ARMD described in chapter 1. It could be argued that the increased dietary retinol levels (table 6.14 in appendix 11) in the non-treated group, and increased dietary carotene levels (table 6.13 in appendix 11) in the treated group of HY eyes during the supplementation trial (chapter six) confounded the results of the study. This is not likely to be the case as no clinically significant changes were seen for any measure between treated and non-treated HY eyes. Statistically significant changes were seen for MPOD measures. However MPOD measures accumulation of L and Z within the retina and not carotene or retinol.

It may also be argued that the increase in dietary omega-3 in the combined HY and HO eyes during the supplement withdrawal trial confounds the results. Omega 3 has a potentially protective effect on the retina as discussed in chapter one. Therefore any dietary increase may have masked any changes caused by supplement withdrawal. However there was a statistically significant reduction in ring 1 and ring 2 mfERG N1P1 amplitudes for this group. Nevertheless this reduction was not clinically significant.

8.4 Improvements and future work

A recent study found that nine months after the withdrawal of a L-based supplement MPOD was still elevated [383]. However the study participants were a mix of ARM and healthy eyes, and other measures of visual function were not assessed. Therefore extended supplement withdrawal trial duration may have identified further changes in outcome measures.

The method of MPOD collection was HFP. This is a subjective measure. Objective measures of retinal accumulation of MP such as fundus autofluorescence or fundus reflectometry may be more appropriate to compare with objective measures of retinal function such as the mfERG.

8.5 Conclusions

The study found statistically significant improvements in MPOD in HY and HO groups treated with a L-based supplement. When HY and HO groups were combined MPOD improvements were maintained, and mfERG ring 2 P1 latency became shorter.

On withdrawal of the supplement mfERG ring 1 N1P1 amplitude reduced in HO eyes. When HO and HY eyes were combined, mfERG ring 1 and ring 2 N1P1 amplitudes were reduced. In ARM eyes, ring 3 N2 latency and ring 4 P1 latency became longer.

While there were some statistically significant changes in outcome measures during supplementation and withdrawal, none of these changes were clinically significant. The trial was sufficiently powered to assess the effect of the supplement on all outcome measures for all groups with the exception of MPOD in ARM-affected eyes.

The results suggest that there is no clinically significantly beneficial effect of supplementation with 12 mg L, 0.6 mg Z, 150 mg vitamin C, 15 mg of vitamin E, 400 µg copper, 20 mg zinc and 1080 mg omega-3 fatty acids on visual or retinal function in healthy eyes or eyes with ARM. There is also no clinically significantly detrimental effect of supplement withdrawal on visual or retinal function in healthy eyes or eyes with ARM in this study. However, the statistically significant trend for increase in MPOD is encouraging and suggests that macular pigment does accumulate within the retina on L-based supplementation. It may be that any beneficial effect of accumulated macular pigment on retinal and visual function is not seen for many years. Longer-term studies may yield greater information.

The Aston study adds to literature in several ways. The CARMIS investigators concluded that central mfERG amplitudes were augmented after 12 months with a 10 mg L-based supplement in ARMD-affected eyes [103]. The Aston study extended on the CARMIS study by assessing the effects of a L-based supplement on mfERG latency, subjective measures of visual function in ARM and healthy eyes, by assessing accumulation of L and Z within the retina, by assessing dietary levels of supplement nutrients and by assessing the effects of supplement withdrawal on visual and retinal function.

Unlike the CARMIS study, the Aston study contributes to the debate about the use of nutritional supplementation in healthy younger and healthy older eyes, before the commencement of ARMD. Any positive effects of the nutritional supplement in the HY or HO cohort may have implied that there is a function for nutritional supplementation in delaying or possibly preventing the onset of ARMD. Any negative effects from supplement withdrawal may have also supported this hypothesis. However, while the trend for an increase in MPOD with supplementation is encouraging, it did not reach clinical significance to support this hypothesis. Any negative effects from withdrawal of the supplement in ARM eyes may have supported the use of nutritional supplementation in delaying progression of the disease. However, there were no clinically significant findings to support this theory.

The pathogenesis of ARMD although currently unclear, is purported to be a combination of genetic predisposition and environmental factors, leading to eventual photoreceptor death with accompanying visual loss. Sensitive, objective methods for early diagnosis of ARMD, and strategies for disease prevention need to be developed.

Randomised controlled trials remain the gold standard for investigating cause-effect relationships. Large-scale, long-term RCTs such as the AREDS 2 (www.areds2.org) will provide clinicians with more information about the effects of L and omega 3 on visual function and on the risk of developing ARMD.

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Appendix 1 - Risk factors for ARMD development

Risk Factors for Age-related Macular Degeneration

Emma J. Berrow, Hannah E. Bartlett , Frank Eperjesi, Jonathan M. Gibson

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Risk factors for ARMD development

The odds ratio is the ratio of the odds of ARMD occurring in those exposed to a risk factor to the odds of it occurring in those not exposed to a risk factor. The relative risk is a ratio of the probability of the ARMD occurring in a risk factor exposed group versus a non-exposed group.

Modifiable risk factors

Smoking

Smoking is the one modifiable risk factor that has been largely consistently associated with an increased risk of developing ARMD [1-9]. The Rotterdam study found that the higher the pack-years smoked, the higher the risk of developing neovascular AMD, with a 6.6 fold increase in risk for developing neovascular AMD when compared to non-smokers [10]. The Pathologies Oculaires Liees a l'Age (POLA) study found an increased risk of neovascular AMD and geographic atrophy in those who smoked for more than 20 years (odds ratio 3.0 for 20-39 pack years and 5.2 for 40 pack years). The risk remained elevated until 20 years after smoking cessation [11]. Smoking was not associated with risk for ARM development in this study. Increase of ARMD development risk with increase in smoking was also demonstrated in a study by Seddon *et al.,* [12] in a 12 year prospective study of 31843 females and in a 12 year prospective study of 21157 males by Christen *et al.,* [13]. The Rotterdam study results are echoed in a study on Japanese males [14]. All of these studies show that even previous smokers who had ceased smoking still had an elevated risk of ARMD development when compared with non-smokers, but not as elevated as current smokers. A study undertaking pooled and separate analysis of 14,752 participants from the Beaver Dam eye study, Rotterdam study and Blue Mountains eye study showed that apart from age, smoking was the only consistent risk factor associated with any form of ARMD [15]. This pooled study was taken from three different continents (North America, Europe and Australia). Another study based on 3271 Australians also highlighted this consistent association for ARM and AMD [16]. A review of the literature in conjunction with New Zealand morbidity and smoking prevalence data found an associated risk of AMD with smokers in New Zealand [17]. In the United Kingdom a two-fold risk of

ARMD has been associated with smoking when compared with non-smoker in 28000 individuals [18]. Cigar smoking in India has also been linked with a higher risk for developing AMD [19]. A study of Latino subjects also demonstrated an association between smoking and advanced AMD in the Hispanic population [20]. The effects of passive smoking on AMD risk have been examined in a United Kingdom study, comparing 435 end-stage AMD subjects with 280 healthy controls. The results showed an odds ratio of 1.87 in passive smoking exposure in non-smokers [21]. This prolific evidence across continents and differing ethnicities suggests that smoking is highly toxic to the retina, although the pathogenic mechanisms between smoking and retinal toxicity still remains unclear.

There are approximately 4000 toxic components in cigarette smoke one of which is nicotine. A study assessing the effects of nicotine on the vascular smooth muscle cells confirmed that nicotine increases CNV size and severity in laser induced CNV in the mouse eye model [22]. Nicotine attaches to nicotinic acetyl choline receptors on photoreceptors, bipolar, horizontal and ganglion cells [23]. Tar within cigarette smoke, contains hydroquinone – an oxidant, which in the mouse eye has been shown to encourage sub-RPE deposits and thickening of Bruch's membrane [24]. Cadmium is another toxic oxidant found in cigarette smoke, of which higher urinary levels in smokers is linked with an increased risk of AMD [25]. It accumulates 2.5 times higher in the choroid-RPE complex of smokers compared with non-smokers [26] and increases reactive oxygen species, alters RPE cell morphology and decreases cell survival [27]. Studies on mice eyes have demonstrated that chronic exposure to smoke causes changes to the RPE similar to those observed in AMD, with RPE apoptosis, increased oxidative damage [28], DNA damage to the RPE and increased inflammatory activity [29].

The effects of the combination of smoking and genetics of ARMD have been studied with many studies showing an additive effect of smoking for increased risk of developing ARMD when there is a genetic disposition for the disease [30-34] .

Alcohol intake

Studies assessing the association between ARMD risk and alcohol intake have shown inconsistent findings [8, 35, 36]. A relationship between beer consumption and risk for CNV has been identified in the Beaver Dam eye study, although no such relationship was seen with wine or spirit consumption [37]. This was echoed in subjects taken from the Latino community

with beer consumption and high alcohol intake being linked with a greater risk of developing the disease [20]. Conversely, no association between any type of alcohol and ARMD risk has also been shown in other studies [20, 38-40]. Interestingly, moderate wine consumption has been associated with a decreased risk of developing AMD [41] and in the Reykjavik eye study alcohol consumption decreased the risk for drusen formation [42] suggesting a protective effect of alcohol against ARMD. Chronic, heavy alcohol consumption is linked with an increased accumulation of ethyl esters and an increase in laser induced CNV of 28% within the rat choroid models [43]. Ethanol is the key component of alcohol, and when exposed to ethanol photoreceptor outer segment growth in the Zebrafish retina is inhibited, leading to poor photoreceptor function as demonstrated by reduced a- and b- wave amplitudes of the ERG [44]. Red wine has a high level of phenolic compounds that increase antioxidant activity which may reduce oxidative stress and abnormal proliferation of the RPE [45].

Socioeconomic factors

Socioeconomic factors have been inconsistently associated with an increased risk of developing ARMD. A Canadian study looking at socioeconomic status and CNV found that the severity of CNV was associated with lower socioeconomic status [46]. However in another Brazilian study assessing AMD in two differing socioeconomic populations no association between AMD and socioeconomic background was seen [47]. No association was demonstrated between ARMD and socioeconomic factor in a case-control study by Hyman et al., [48] or in the Framingham eye study [49], the Beaver Dam eye study [50] and the third NHANES Study [51].

Education

The Age-Related Eye Disease Study (AREDS) report number three found that education was inversely linked with drusen, GA and CNV [52]. The Eye Disease Case-Control Study Group (EDCCS) found a similar trend for education and neovascular AMD risk, although no statistical significance was demonstrated in their final multiple regression model [1]. The first NHANES study also demonstrated this association but statistical significance was lost on logistic regression modelling [53].

Nutrition

Nutrition as an associated risk factor for developing ARMD has also been subject to conflicting findings within the literature. The first NHANES study found dietary vitamin A provides a protective effect against AMD with no beneficial effect shown with vitamin C [53]. The Beaver Dam eye study found no association between vitamins A, C and E and reduced risk of developing ARM [54]. Another study of serum lycopene in the Beaver Dam eye study showed an increased risk of ARMD with reduced lycopene levels [55]. However, lower levels of lutein, zeaxanthin and vitamin E were not related to an increased risk for ARMD development in this study. Conversely higher serum alpha tocopherol and an antioxidant index including ascorbic acid, alpha tocopherol and beta carotene, were found to be conducive to lower ARMD risk in the Baltimore longitudinal study [56]. The Physicians Health study and the Blue Mountains eye study did not find a protective effect for vitamin C, E and multivitamins [57], and vitamin E and beta carotene [58] against ARMD respectively. The EDCCS found a reduced risk of neovascular AMD with higher serum and dietary carotenoid levels [59]. An antioxidant index combining selenium, vitamin C, vitamin E and carotenoids also showed reductions in risk in this study. A further study from the EDCCS reported that spinach and collards, high in the carotenoids lutein and zeaxanthin, were most strongly associated with a reduced risk for AMD ($p<0.001$) [60]. Collard greens are various loose-leafed vegetables of Brassica oleracea, the same species that produces cabbage and broccoli. They are genetically similar to kale and spring greens. High-dose vitamins C, E beta carotene and zinc were found to be effective in lowering the odds ratio of developing advanced AMD in eyes with intermediate drusen, large drusen and non-central GA in a large trial undertaken by the AREDS group [61]. Improvements in visual function in eyes with ARM or non-exudative AMD were reported in several studies involving carotenoids [62-65].

High levels of omega-3 fatty acid consumption (>75 percentile) have been shown to provide a protective effect against progression to AMD [66]. Lowering the dietary glycaemic index with higher omega-3 intake also showed a reduction in AMD progression in this study. The benefits of a low glycaemic diet in reducing ARM risk have been identified in other studies [66-68]. The Blue Mountains eye study found a lower risk of developing ARM when consuming omega-3 fatty acids in the form of one serving of fish per week [69]. Consumption of linoleic acid in the form of 1 to 2 servings of nuts per week was also associated with reduced ARM risk in this study. The AREDS study found a reduction in risk of progression from drusen to geographic atrophy in those with the highest dietary intake of omega-3 fatty acid [70] and reduced risk of developing neovascular AMD [71, 72]. It is thought that omega-3 provides a protective role within the retina by inhibiting oxidative stress and reducing inflammation within the retina [73].

Association between higher trans-unsaturated fat intake and increased prevalence of AMD was reported in a large study of 6734 participants [74]. Omega-3 fatty acids and olive oil were associated with a reduced prevalence of ARM and AMD in this study. However, the third NHANES results showed no association between dietary fat intake and ARM risk in 7883 participants [75] and this was echoed in 3654 participants from the Blue Mountains eye study [76]. Studies of mouse retinae have shown an increase in the accumulation of basal laminar deposits when consuming a high fat and cholesterol diet [77]. Some studies have shown that diets higher in fats have a propensity to be lower in essential nutrients and antioxidants [54, 78]

Body mass index (BMI)

A high BMI has been inconsistently linked with risk for developing ARMD. The Blue Mountains eye study found an odds ratio of 1.78 for risk of early ARM in obese subjects compared to those with a normal BMI [79]. The Age-related Eye AREDS group reported that a higher BMI was associated with a risk for developing neovascular AMD [80] and GA (odds ratio of 1.93) [81]. A 2.29-fold risk of AMD and a 1.54-fold risk of pigmentary abnormalities were demonstrated in the Pathologies Oculaires Liees a l'Age (POLA) study in obese subjects [82]. The relative risk was 2.35 for a BMI of 30 or more and 2.32 in a BMI of 25 to 29 for developing AMD in this study. Larger waist circumference and a larger waist-hip ratio have also been associated with an increasing relative risk for AMD development [83]. An inverse relationship between BMI and retinal levels of L and Z (macular pigment optical density, or MPOD) was reported [84]. The authors also assessed dietary L and Z intake and found that individuals with the highest BMI consumed lower amounts of L and Z. They concluded that lower dietary intake of L and Z, and/or competition between adipose tissue and retina for L and Z uptake were likely to affect retinal levels of L and Z [84]. Conversely, associations between lean males and dry AMD have been found [85]. A pooled study of 14,752 participants from the Beaver Dam eye study, Rotterdam study, and Blue Mountains eye study did not report any consistent association between BMI and risk for any forms of ARMD [15] and this was echoed in other studies [9, 86].

Cardiovascular disease

Cardiovascular disease (CVD) has been associated with risk for developing ARMD in several studies and discounted in others. The Beaver Dam eye study showed no association between CVD and neovascular AMD or geographic atrophy [87]. Arterial stiffness - an indicator for CVD, has been shown to be associated with the presence of AMD [88]. The Blue Mountains

eye study did show associations between CVD (relative risk 1.57) for early incident ARM. Higher HDL cholesterol levels were protective for late AMD, and high total/HDL cholesterol ratio was linked to increased risk of late AMD and GA [89]. C-reactive protein (CRP) is an inflammatory marker for CVD. Some studies have shown increased levels of CRP in ARMD [90-92] suggesting an inflammatory role in the development of ARMD. Conversely better cardiovascular health was associated with reduced risk of ARMD in the Cardiovascular Health and Age-Related Maculopathy (CHARM) study [93]. The POLA study showed a reduced risk (odds ratio of 0.72) for developing drusen and no association between AMD and a history of cardiovascular disease [82]. No association between CVD and ARMD risk was reported in the AREDS studies [52, 81], EDCCS study [1] or Smith *et al's.,* pooled analysis from the Rotterdam, Blue Mountains and Beaver Dam eye studies [15]. Hyman *et al.,* found a link between ARMD and CVD [48] in an earlier study but not in a later study [94].

Hyman *et al.*, also found an association between moderate to severe hypertension (diastolic >95 mmHg) and risk for developing neovascular AMD, especially in those receiving antihypertensive medication. The same association was not found for GA and hypertension, leading the authors to suggest that comparable disease processes may occur in neovascular AMD and hypertension [94]. Reduced choroidal blood flow in hypertensive individuals with neovascular AMD may account for this relationship [95]. The Framingham eye study [49] and the first NHANES study [53], reported links between ARMD development risk and hypertension. The AREDS group found increased risk for developing neovascular AMD and large drusen in those with hypertension and those taking hypertensive treatment [52], although no association with incident neovascular AMD was seen in their further study [81]. Hypertensive disease severity has been linked with neovascular AMD, with doubled odds in the severest of hypertension [96]. The Beaver Dam eye study [97, 98], Blue Mountains eye study [89], EDCCS [1] and others [99, 100] found no evidence to suggest that ARMD development risk and hypertension was linked.

Cholesterol levels and treatment

Links between cholesterol levels, cholesterol-lowering treatments and risk of ARMD development have been conflicting. A possible protective effect of statins and lipid-lowering treatments against ARMD has been found in a number of studies [101-105]. Some studies have suggested statins protect the vascular endothelium from oxidative damage [106] and reduce basal linear deposit accumulation in Bruch's membrane by reducing cholesterol [107]. Conversely, a review, assessing pooled data of the use of statins and lipid-lowering treatments did not show a reduced risk of developing ARMD when using statins [108]. Pooled data

analysis of the Beaver Dam, Rotterdam and Blue Mountains eye studies did not report effects of statins on ARMD risk [15]. Other studies have found no association between statin use and reduced risk for developing ARMD [82, 109-111]. Furthermore, some have reported an increased risk of ARMD development in those taking statins [112]. In the EDCCS higher levels of cholesterol were associated with an increased risk of neovascular AMD (≥6.7 mm/L = odds ratio of 4.1) [1], but no information about statins was presented in this study. The AREDS study also did not provide data about statin use or cholesterol levels in their study [52]. Because the benefits of statins for reducing heart disease and lowering cholesterol were not largely reported and routinely used until 1994 [113] this is the likely reason for lack of data before this period. The first NHANES study found no association between cholesterol levels and risk for ARMD development, but again statins were not assessed here as the study results were published in 1988 [53]. High-density lipoprotein (HDL) cholesterol levels were inversely related to AMD and a raised total/HDL ratio predicted AMD in the Blue Mountains Eye Study [89]. Statins and aspirin were found to be associated with reduced rates of CNV in a retrospective study of 326 patients with ARMD [102]. Aspirin was not found to be related to an increased risk of ARM or AMD development in the AREDS study [52] but was positively correlated in a later AREDS study [81]. It was not linked in other studies [114-116] or its effects were not reported [1, 48, 53].

Medication

Conflicting associations between the use of other medication and risk for developing ARMD have been reported. Those with GA were more likely to take antacids, and those with large drusen or extensive intermediate drusen were more likely to take hydrochlorothiazide diuretics in one AREDS report [52]. Antacid use and increased incidence of GA was also seen in another AREDS group study [81]. This report also highlighted an association between anti-inflammatory medication and increased incidence of GA. In a further study assessing the use of antacids and thiazide diuretics in ARMD no relationship was found for increased risk of the disease and either medication [117]. Van Leeuwen *et al.*, found an increase in risk for ARM development in those taking antihypertensive treatment and a decreased risk in women taking tricyclic antidepressants [118].

Hormones

The use of differing hormones has been associated with risk for developing ARMD. Thyroid hormones were associated with an increased risk of GA in the AREDS study, although the use

of oestrogen and progesterone in women was not associated with any form of ARMD in this study [81]. Thyroid and antithyroid hormones were not associated with ARMD in another study [117]. There was also no association between the use of hormone replacement therapy (HRT), hysterectomy or oopherectomy in women and ARMD risk in the POLA study [119]. However, a protective effect of HRT was found in another study with a 48% lower risk of developing CNV compared to those who had never used HRT, although no protective effect was found for ARM [120]. Reduced risk of developing ARM was seen in another study in women taking HRT [118]. Lack of oestrogen was shown to be associated with an increase basal laminar deposits and thickened bruch's membranes in mice retinae [121].The authors postulated that oestrogen down-regulated matrix metalloproteinase-2 – which is responsible for breaking down bruch's membrane and RPE basement membranes. Another study demonstrated that lack of oestrogen up-regulates a glycoprotein called YKL-40, leading to CNV. The function of YKL-40 in the retina is unknown [122].

Type II diabetes

Inconsistent links between type II diabetes and ARMD risk have been described. The Blue Mountains eye study also found a relationship between type II diabetes and development of GA after ten years with a relative risk of 3.89, but no relationship for neovascular AMD [89]. Type II diabetes was associated with an increased risk for developing ARMD compared to type I diabetics and controls [123]. The European Eye study (EUREYE) and AREDS group demonstrated a relationship between type II diabetes and risk of neovascular AMD development but not for GA and type II diabetes [81, 124]. Conversely, a study assessing the ten year follow-up of 133 newly diagnosed type II diabetic participants and 144 controls found no significant difference between groups in risk for ARMD development over the ten years [125]. No relationship between type II diabetes and ARMD was seen in the POLA study [82] a further Blue Mountains eye study [79], or reported by others [1, 15]. The mechanisms for any association between diabetes and ARMD are unknown. Hyperglycaemia in diabetes has been associated with reduced choroidal circulation within the foveal area [126, 127]. This may reduce the exchange of oxygen, nutrients and waste products within the outer retina which may increase susceptibility to ARMD.

Sunlight exposure

There are contradictory findings in the literature about the relationship between exposure to sunlight and risk for ARMD development. No statistically significant associations were reported

in a Brazilian study [47], Italian study [128] or studies from other global locations [1, 52, 129, 130]. Intriguingly, two studies have demonstrated a protective effect of light against ARMD [131, 132]. However, other studies have shown a detrimental effect of sunlight with increased risk of ARMD development. Blue light exposure was associated with a risk of developing GA in a study of 838 watermen [133]. The Beaver Dam eye study found a relationship between high sunlight exposure and a higher 10-year incidence and progression of ARM [134], with sunglasses and headwear providing protection against drusen development and RPE depigmentation. The Blue Mountains eye study found that abnormal skin sensitivity to sunlight was associated with AMD but not ARM [135]. Retinal photochemical injury occurs cumulatively over a long period to tolerable light levels. Sunlight damages the RPE-photoreceptor complex causing the formation of free radicals which peroxidise the fatty acids within the photoreceptor outer segments, leading to RPE and photoreceptor dysfunction and death [136]. Free radicals also increase the production of lipofuscin in RPE cells. A2E, a major fluorophore of lipofuscin, generates free radicals in response to light which leads to RPE apoptosis [137].

Miscellaneous

Other, less reported modifiable risk factors inconsistently associated with ARMD include parity greater than zero. Increased risk of neovascular AMD has been seen with parity greater than zero in the EDCCS study [1, 138] but this relationship is not apparent in another study [139]. Conversely parous women were found to have a 26% lower risk of developing ARM [120] in a more recent study. Although not clear, hormonal mechanisms such as the effects of oestrogen mentioned earlier may play a role.

Non-modifiable risk factors

Increasing age is strongly linked with a higher risk of developing ARMD [15, 19, 52, 128, 140-146], but there are other non-modifiable risk factors for developing the disease which are inconsistent within the literature.

Cataracts and intraocular lenses

Cataracts are known to protect the retina by reducing the amount of ultraviolet and blue light entering the eye. Thus after cataract extraction the retina is subjected to increased light levels

and increased photochemical damage. The Blue Mountains eye study and the Beaver Dam eye study found an increased risk for developing ARMD in eyes that had undergone cataract surgery [147-149]. This was evident in other work, showing an increased risk of AMD in eyes post-cataract extraction [150, 151]. Intraoperative photic damage and surgical inflammation have also been discussed as possible mechanisms for increasing AMD risk post-cataract extraction [152]. Other studies such as the AREDS group report 25 found no risk of ARMD progression after cataract surgery [153, 154]. It has been postulated that the cataract itself increases the risk of developing ARMD. In pooled findings from three studies severe cataract was associated with higher prevalence of ARMD [155]. Studies assessing the risk of ARMD development associated with the use of newer intraocular lenses with short-wavelength blue light filtering properties may provide more information in the coming years.

Cognitive impairment

Evidence from the AREDS group showed a link between reduced cognitive impairment and increased risk of AMD development [156]. This was resonated in ARM subjects in the Cardiovascular Health Study [157], a weak association in another study [158], and in AMD subjects in an Australian population [159]. The Rotterdam study demonstrated that tobacco and atherosclerosis may play a role in the pathogenesis of both ARMD and Alzheimer's disease [160]. Amyloid beta peptide is found in the neuritic plaques in Alzheimer's disease and also in drusen. It contributes to inflammatory processes in both of these diseases [161] and in many neurodegenerative diseases of ageing such as Parkinson's disease, arthritis, atherosclerosis and myocardial infarction [162]. Many people with ARMD reduce their physical and mental activity levels which is associated with cognitive decline [163]. Conversely, no significant relationship was established between Alzheimer's disease and ARMD [164] in 33 Alzheimer's cases when compared to 24 controls. The authors believe that small sample size and age-differences between the groups may have accounted for the lack of any relationship. They did not specify between ARM or AMD for their study.

Genetics

Although knowledge about the role of genetic variants in ARMD is currently rudimentary, many genes have been identified as providing either deleterious or protective effects against the disease [165]. A review of the many genes identified [166] found that genes within the complement cascade (CFH, C2, BF and C3), ARMS2 and HTRA1 are most strongly associated with ARMD development. Many studies have assessed familial predisposition to ARMD by

looking at monozygotic and dizygotic twins [167-172] with monozygotic twins showing a stronger concordance than dizygotic twins. The higher prevalence of ARMD in first-degree relatives of those with the disease than those without the disease further strengthens the case that genetic factors may play a part in ARMD pathogenesis [173-175]. More may be learnt over time as genetic marker testing becomes increasingly sophisticated, identifying greater numbers of genes associated with ARMD.

Gender

Female gender has been associated with increased risk for development of ARMD, although no consensus seems to prevail. A Croatian study of 6617 patients found that ARMD incidence was slightly increased in females compared to males [176]. This was echoed by the AREDS group with ARM being more apparent in females [52] and other work [141]. However this was not replicated in pooled analysis from the Beaver Dam, Rotterdam and Blue Mountains Eye studies [15, 177]. Males were more likely to undergo photodynamic therapy than females for neovascular AMD in an Israeli study [178], and were more likely to have AMD than women in two Japanese studies [179, 180] although the authors suggest that this may be due to the significantly higher proportion of Japanese men who smoke. A recent study of the Beaver Dam offspring study also showed that being male was associated with ARM [9].

Arthritis

An association between arthritis and increased likelihood of ARM was reported in one AREDS study [52], whereas another AREDS study suggested a weak association between anti-inflammatory medications and progression to AMD [81]. One study found subjects with rheumatoid arthritis (RA) had less prevalence of AMD and suggested anti-inflammatory agents, commonly used to manage the symptoms of RA, provide a protective effect against development of ARMD [181] since there is some evidence that inflammation may play a role in the development of ARMD [182]. However environmental and genetic factors may also be relevant as RA is commonly a disease of the young and ARMD more apparent over 50 years of age [183].

Ethnicity

Higher prevalence of ARMD has been shown in white people when compared with blacks although genetics, culture and diet may play a role in these differences. Darker iris pigmentation may also confer some protective effect in the black population [184]. The AREDS group found a higher risk of developing large drusen and CNV in whites [52]. A further AREDS study echoed these results for incident CNV [81]. However no such association was found in a Brazilian study of 107 participants with ARMD [47]. In the Salisbury Eye Evaluation (SEE) project the risk of developing large drusen and RPE pigmentation was higher in whites than blacks but the risk of developing GA or CNV was no different than for blacks [185]. A south Indian study found a prevalence of AMD in its population similar to other developed countries [19]. A Japanese population study reported similar prevalence of ARM to the white population of the Blue Mountains eye study. This similarity also held true for AMD in Japanese males, but AMD prevalence was lower in Japanese women when compared to the Blue Mountains Eye Study population. This disparity was assumed to occur due to a high proportion of Japanese male smokers according to the authors [179]. Another Japanese study suggested that the 9 year incidence of AMD was lower among the Japanese than among white people, but higher than among black people [180]. The prevalence of ARM in South Koreans was also found to be similar to other studies but the prevalence of AMD was lower [146]. Exudative AMD was found to be higher in Chinese compared to whites in a study assessing four different ethnic groups, even when smoking age, gender, pupil size, BMI, alcohol intake, diabetes and hypertension were adjusted for [186]. A putative mechanism for reduced risk of ARMD in blacks compared to whites is the protective effect of the darker pigmentation of the iris [184] and higher concentrations of melanin within the choroid of blacks compared to whites [187]. Melanin acts as an antioxidant, scavenging free radicals and reducing oxidative stress [188].

Iris Pigmentation

Iris pigmentation has been inconsistently associated with an increased risk for ARMD with the EDCCS demonstrating no association between iris colour and neovascular AMD [1, 151], incident ARMD [81] or GA [151]. Conversely light irises were associated with increased risk for ARMD in other studies [143, 189, 190]. Blue iris colour was linked with increased risk of both ARM and AMD in the Blue Mountains eye study [191]. However, five years later longitudinal data did not support this association. A study of 1000 Danes also showed no difference between light iris and dark iris colour for AMD [192].

Hypermetropia

Hypermetropia and its associated shorter axial length have been linked with increased ARMD development risk [48, 193-197]. An association between ARM risk and hypermetropia was found in the Blue Mountains Eye Study [198] and the Rotterdam study [199]. In a further Blue Mountains eye study no association was found between hypermetropia and the 5-year incidence of ARM [200]. Large drusen, extensive intermediate drusen and CNV were associated with hypermetropia in the AREDS study [52]. Other studies have reported no effect of hypermetropia on risk for developing ARMD [47, 201]. A biological mechanism for increased risk of ARMD with hypermetropia has not yet been elucidated. One study suggests shorter, thicker eyes with increased scleral rigidity decreases choroidal blood flow and thus retinal nutrient and waste exchange leading to increased oxidative stress [199].

Miscellaneous

Other, less reported non-modifiable risk factors inconsistently associated with ARMD include hand-grip strength, optic disc appearance and birth weight. A couple of studies have linked decreased hand grip strength to increased risk for AMD [48, 49]. Unusual optic disc appearance has been associated with ARMD risk [202] but repealed in other studies [203, 204]. Babies with increased birth weight were found to have a higher possibility of developing AMD than those with lower birth weight in one study [205] and this was echoed in another study but only in white participants for ARM although AMD risk was not assessed in this study [206].

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Appendix 2 – Current treatment of ARMD

Currents treatment of ARMD

Treatment for ARMD has changed significantly in the last decade. Current recommendation for the first line treatment of CNV according to the Royal College of Ophthalmologists (RCO) is intravitreal anti-vascular endothelial growth factor (anti-VEGF) such as ranibizumab (Lucentis, Novartis) and pegaptanib (Macugen, Pfizer) which binds to and inhibits the action of VEGF-A, thus inhibiting neovascularisation [1].

Evidence from the ANCHOR and MARINA randomised controlled trials (RCTs) showed a mean improvement in visual acuity after treatment with ranibizumab. The ANCHOR study compared intravitreal injection of ranibizumab with sham photodynamic therapy (PDT) to PDT treatment with sham injection in classic CNV. At a dose of 0.5 mg of ranibizumab with sham PDT treatment, mean visual acuity improved by 11.3 letters compared to a drop of 9.5 letters in the PDT group with sham injection at 12 months [2]. In the MARINA study intravitreal ranibizumab was compared with a sham injection. At a dose of 0.5mg of ranibizumab mean visual acuity improved by 7.2 letters but in the sham injection group visual acuity dropped by 10.4 letters. [3]. The primary outcome measure for both studies was the loss of fewer than 15 letters of visual acuity. Both the MARINA and ANCHOR studies found that significantly more patients lost less than 15 letters of visual acuity when receiving 0.5mg of ranibizumab when compared with sham injection [3] or PDT [2]. This was echoed in the PIER [4] and FOCUS RCTs [5]. The RCO recommend the use of intravitreal anti-VEGF to treat all CNV lesion types (classic, predominantly classic, minimally classic, occult and retinal angiomatous lesions). Extrafoveal CNV (lesions 200μm or greater from the centre of the foveal avascular zone) may be treated with focal argon laser photocoagulation, or anti-VEGF therapy if it is decided that laser induced scotoma may disrupt normal visual function [1].

The national institute of health and clinical excellence (NICE) current recommendations for the treatment of CNV is a 0.5mg dose of ranibizumab via intravitreal injection at 1 per month for 3 consecutive months followed by further monthly injections if a further visual acuity loss of greater than 5 letters occurs [6]. Treatment can only be administered if:

- best corrected visual acuity is between 6/12 and 6/96

- there is no permanent structural damage to the fovea

- the lesion is ≤ 12 disc areas in size

- there is evidence of disease progression

Although no long-term comparative studies have been executed it appears that ranibizumab has greater efficacy than pegaptanib according to RCO and NICE comparisons of studies between these 2 treatments [1]. On balance NICE noted statistically significant mean gains of letters for ranibizumab treatment while pegaptanib largely only reduced mean loss of letters [6]. Some studies have looked at combination treatments for CNV but currently there is insufficient evidence to suggest they are superior to monotherapy with ranibizumab and may increase safety concerns [7].

Other anti-angiogenic therapy includes anecorvate acetate (Alcon), an angiostatic steroid and bevacizumab (Avastin, Genentech) derived from the same antibody as ranibizumab. Both of these treatments have not been licensed for use in the UK. Although ranibizumab is the gold standard for treating CNV, injections cost the NHS approximately £10,700 per patient for a course of 14 with the drug manufacturer paying additional costs if further injections are required [6].

Treatments for non-neovascular AMD are limited but include antioxidant supplementation [8], counselling and rehabilitation. Surgical options include the IOL-Vip system where a -64.0 dioptre intraocular lens (IOL) is implanted in the capsular bag and a +53.0 IOL is implanted into the anterior chamber, reproducing a Galilean telescope and providing a distance magnification of 1.3. The lenses divert the image away from the diseased macular to a non-diseased area. Studies have shown improved visual acuity in patients with low vision due to end-stage macular degeneration although those with hyperopia were left with a high residual refraction [9, 10]. Another implantation system called the implantable miniature telescope (IMT) showed improved visual acuity of 2 lines or more after one year after in 77% of patients [11]. Improvements were also seen in another study [12], although it has been demonstrated that strict criteria need to be met for eye selection when undertaking telescopic implantation for patient satisfaction [13]. Surgical telescope treatments have very select inclusion criteria and require a specific post-operative visual rehabilitation programme with possible periodic endothelial cell

count due to the position of the anterior chamber IOL [14]. Miniature intraocular telescope surgery is not available on the NHS currently and is costly at around £6000 per eye.

Although the last decade has shown promising changes in the treatment of CNV, these limited and costly therapies are not appropriate for ARM or non-neovascular AMD. This has prompted interest in developing preventative measures and exploring supplementary methods to recognise ARM earlier and monitor treatment efficacy on disease outcomes.

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Appendix 3 – pages 195-206 published:

Berrow E, Bartlett H, Eperjesi F & Gibson J (2010) The electroretinogram: a useful tool for the evaluation of age-related macular disease? Documenta Ophthalmologica 121(1):51-62.

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Appendix 4 – Patient leaflet

Aston University
Life & Health Sciences

Assessment of nutritional intervention on the electrophysiology of the retina

Within the Ophthalmic Research Group we are investigating the effect of taking an ocular nutritional supplement on eye health. It is thought that this supplement might be useful for people with age-related macular disease, as well as for those with a family history of this condition. We are looking for volunteers of all ages to take part. The project will involve visiting the Optometry department every five months for a period of twenty months. Each visit will last for about one hour.

If you are interested in hearing more about the project, please contact Mrs Emma Berrow on 0121 204 4208, or berrowej@aston.ac.uk

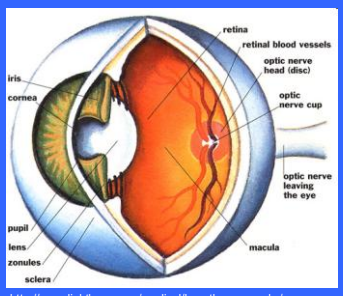
Appendix 5 – Patient poster

What is age-related macular degeneration?

Emma Berrow
Telephone 0121 204 4208

What is the macula?

The macula is found near the centre of the retina at the back of your eye. It is very important because it is responsible for what we see right in front of us. It gives us vision for fine detail that helps with reading, sewing and recognising faces.



http://www.lighthouse.org/medical/how-the-eye-works/

What is age-related macular degeneration?

Age-related macular degeneration (AMD) is a disease associated with aging that affects the central vision.

Broadly speaking, there are 2 forms of AMD, "wet" and "dry".

It is not yet fully understood as to what causes AMD

Risk factors for AMD

- Aging
- Family history
- Genetics
- Drusen (yellow deposits in the retina)
- High fat intake
- High blood pressure
- High cholesterol
- Obesity
- Smoking
- Exposure to sunlight
- Nutrition

Symptoms Include

Blurred vision

Distorted vision –wavy lines

Areas of missing vision

If you notice any of these symptoms you should make an appointment to see an optometrist or GP who will refer you to an eye specialist.

Treatment

Dry AMD does not tend to affect vision as much as wet AMD.

There is currently no treatment for the dry form of AMD but it is thought that nutritional supplements may help slow progression of the condition.

There is treatment available within the NHS that is suitable for some people with wet AMD. The treatment involves a course of injections. There is also a form of laser light therapy available.

Studies at Aston University

At Aston University the Ophthalmic Research Group are very interested at looking into the effects of nutritional supplementation on the early stages of AMD.

We require volunteers of all ages who have no signs of the disease and those who have early signs of AMD to see whether lutein, omega-3 and antioxidants may be beneficial to your eyes.

The tests take around an hour and a half and can be arranged at a time convenient to you.

If you are interested in taking part in this study please do not hesitate to contact Emma Berrow on 0121 204 4208 or berrowej@aston.ac.uk

Appendix 6 - Participant consent form

ASTON UNIVERSITY **REG/06/288(1)**

HUMAN SCIENCE ETHICAL COMMITTEE

CONSENT FORM FOR VOLUNTEERS

PROJECT TITLE

Clinical trial of Ocuvite Duo ocular nutritional supplements for retinal health

RESEARCH WORKERS, SCHOOL AND SUBJECT AREA RESPONSIBLE

Mrs Emma Berrow, Life & Health Sciences, Vision Sciences

Dr Hannah Bartlett, Life & Health Sciences, Vision Sciences

EXPLANATION OF ANY POSSIBLE HAZARDS AND THE PROCEDURES TO BE USED

We are assessing the effect of taking nutritional supplements on the health of the eye. We are doing this to try and determine whether taking these supplements is useful for people with and without age-

related macular degeneration, as well as people with a family history of this condition.

You will be randomly allocated to either take the supplement or not. The name of the supplement is Ocuvite Duo, and it is commonly available worldwide. You will also be asked to provide information about your diet, lifestyle, health, medications, and whether or not you currently take nutritional supplements. All of this information will be treated confidentially.

You will be asked to attend the Aston University Optometry Clinic once every 20 weeks for a period of 80 weeks. This visit will take around 1 hour in total and during the visit various measurements will be taken. Most of the measurements are similar to those that you would undergo in a routine eye test with your optician. We will undertake measurements of your visual acuity, contrast sensitivity, eye pressure and take a photograph of the back of your eye. You will be able to take regular breaks between tests.

Two of the tests are slightly different to what you might have experienced during an eye examination. One simply involves looking at a screen with your chin in a chin rest and pressing a button when you see blue lights flickering. This measurement will take around three minutes.

The other test that differs from a standard eye examination, is one called the multi-focal electroretinogram. This test tells us about how well the tissues at the back of your eyes are functioning. This is test takes around 15 minutes. The whole visit will last about 1hour in total.

It will be necessary to put drops into your eyes to dilate the pupils (i.e. make your pupils larger; the drops are called tropicamide). It will also be necessary to put in drops to numb the front of your eye to help you stop blinking (i.e. a mild surface anaesthetic; the drops are called proxymetacaine).

A small electrode is taped on the skin of the temple and another electrode is taped onto the forehead after both areas have been cleansed. A very fine fibre is then placed just inside your lower lid in order to take the measurements. You will then be asked to look into a bowl and look steadily at a central target while different patterns hexagon shapes alternate quickly from black to white. We take our measurements while you are looking at these patterns for intervals of around 15 seconds – we will ask you not to blink during this time. We will repeat the sequence of measurements 12 times with short breaks at appropriate intervals. The whole procedure normally does not last longer than 15 minutes. If you suffer from dry eyes during the procedure we can use artificial tears to make you more comfortable.

The tests are not diagnostic and do not constitute a full eye examination. However, we will inform you if we find any obvious abnormality, and will advise you about what type of healthcare professional you should consult to have any abnormality checked. You are perfectly free to ask any questions about any aspect of the study before deciding whether or not to take part. You are also free to withdraw from the study at any time, without giving a reason.

Explanation of potential hazards

Some individuals are sensitive to the type of flickering patterns that are presented in the study. It is important that you tell us whether you are sensitive in this way and **very important** that you tell us before the tests if you are epileptic or have had tests for epilepsy.

The drops that we are using are the same drops they use in hospitals and by optometrists to make your pupil larger. After receiving the drops there is a very slight risk of them causing pressure to build up in your eye. We will check that this does not happen by measuring the pressure before and after the measurements. However if the pressure does increase significantly we will ensure that you receive treatment at an eye department immediately.

Due to the dilating drops instilled in your eyes you will find that your vision may be blurred for a period of up to 6 hours (especially for reading) and that you are more sensitive to bright light such as sunlight. Because of this it is necessary that you do not drive or operate heavy or moving machinery during this period. General care with your environment and bright sunlight especially (i.e. it may be necessary to wear sunglasses) is also advised. If after the examination you find that you are experiencing any pain or discomfort with your eyes please contact your local eye department or any of the individuals listed below.

The eye drops used to numb your eyes can also produce in some individuals a reaction in the cornea that causes blurry vision. The reaction is very rare but if it is going to occur it will occur within 15 to 30 minutes so we will be able to detect it. In this case we will not continue with the measurements and the condition of the cornea will be monitored until it has recovered – which usually takes around two hours. It is important not to rub your eyes for an hour after having the numbing drops as you may scratch you eye without feeling it.

There is also a very slight risk of scratching the front of your eye when placing the cotton fibre in the lower lid. This risk however is minimal as the procedure is carried out carefully. Nevertheless the eyes of all individuals will be checked with an instrument following an eye drop that will show up any damage (called fluorescein). This is a standard procedure in everyday optometric practice.

CONFIDENTIALITY OF INFORMATION

The confidentiality of personal information and the anonymity of all volunteers involved in this investigation will be preserved by storage of the data in a locked filing cabinet, and will be accessible only to the investigators.

VOLUNTEER STATEMENT

I have read and understand the above explanation. I have had the opportunity to discuss it with the investigators and to ask any questions. I agree to take part in the above project and I have been informed that I am free to withdraw at any time. I understand that I will not benefit financially from taking part in the research or from its outcomes. I agree that any invention or other intellectual property that arises from the research will belong to Aston University. I agree to the information being used for research purposes, some of which may be included in scientific materials for publication.

Signed: .....

Name (block capitals): .....

Date: .....

Appendix 7

Health questionnaire

Please answer the following questions in block capitals

1. Name_____

2. Male/Female _____

3. Date of Birth _____

4. Please list any general health problems you have (e.g. blood pressure or diabetes)
   _____
   _____
   _____
   _____
   _____

5. Do you take any tablets or medicines? If so, please list the names below

   _____
   _____
   _____
   _____
   _____

6. Do you take any nutritional supplements (e.g. vitamins)? If so, please list the name or brand below

   _____
   _____
   _____

7. Do you smoke? _____

8. If so, how many cigarettes per day_____

9. If you don't smoke at the moment but you have done previously, please give details below_____
   _____
   _____

10. Please give information about any previous visits to an eye clinic or eye specialist

_____
_____
_____
_____

11. If you know the name of your eye condition, please give details below

_____
_____

12. How does your eye condition affect your vision?

_____
_____

13. Have you always lived in the UK? _____

14. If you have lived abroad for more than a year please give details of where, when and for how long

_____
_____
_____
_____

Contact address:

_____
_____
_____
_____
_____

Telephone number:

_____

Thank you for your time.

Appendix 8 – Ethics approval letter

Response from the LHS School Ethics Committee

11th July 2008

Project title: ***Assessment of nutritional intervention on the electrophysiology of the retina***

Reference Number: 300608/PF1

Researchers: Dr Bartlett, Dr Eperjesi and Dr Wolffsohn

I am pleased to inform you that the School LHS Ethics Committee has approved the above named project.

The details of the investigation will be placed on file. You should notify The Committee of any difficulties experienced by the volunteer subjects, and any significant changes which may be planned for this project in the future.

Yours sincerely

School LHS Ethics Committee

Appendix 9 – Food diary

OCULAR HEALTH AND LIFESTYLE

As part of our research portfolio here at Aston, we are interested in investigating the effect of diet and lifestyle on ocular factors. Please take the time to complete the following food diary and ocular comfort questionnaire to help us with our studies. We are particularly interested in macular pigment, which is made up of carotenoids that are only available in the food we eat. We believe that the macular pigment may protect against sight-threatening age-related eye disease, and are currently testing a new method of measurement. We would really appreciate your participation in this project, and will arrange a convenient time for you to attend.

The food diary will help us to assess the relationships between macular pigment and dietary intake. Please fill out this sheet and bring it with you to your appointment.

Thanks again for your time

DATE_____

TIME_____

Instructions on how to fill in your food diary

Please fill out this food diary for three days. Every time you eat or drink something write it down in the diary provided under the correct day.

Try and describe the food as accurately as possible:

For example:

One small or large bowl of cornflakes with skimmed milk

Two slices of toast thinly or thickly spread with butter

Wholemeal, white or brown bread

Skimmed or semi-skimmed milk

Large, medium or small banana

Try to give rough estimates of the food and drink consumed:

For example:

One small cup of tea or one large cup of coffee

Two or three chocolate biscuits

Two or three tablespoons of baked beans

Try to be as accurate as possible (it would be great if you could include weights!).

Remember to include all foods and drinks consumed at home and at other places such as restaurants and friend's houses etc.

Try to fill in the diary as you eat, instead of leaving it till the end of the day. This ensures that you won't forget what you have eaten.

THANK YOU FOR YOUR TIME

NAME_____

Day 1 (weekday)	
Breakfast:	Supper:
Lunch:	Snacks:

Day 2 (weekday)	
Breakfast:	Supper:
Lunch:	Snacks:

Day 3 (weekend day)	
Breakfast:	Supper:
Lunch:	Snacks:

Appendix 10 – pages 223-227, publication:

Berrow EJ, **Bartlett H** & Eperjesi F (2011) Do lutein, zeaxanthin, and macular pigment optical density differ with age or age-related maculopathy? European e-Journal of Clinical Nutrition and Metabolism 6:e197-e201.

Appendix 10 – pages 223-227, publication:

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Appendix 11 - Supplementation results tables

Table 6.2: Mixed between-within ANOVA for mfERG N1P1 amplitude, N1 latency, P1 latency and N2 latency over 3 visits for 5 areas of retinal eccentricity between treated and non-treated groups for HO eyes. The shaded area indicates statistical significance.

Outcome measure	Main effect: time		Main effect: group (treated/non-treated)		Interaction effect	
	F	p	F	p	F	p
Ring 1 N1-P1 amplitude	1.287	0.315	0.454	0.513	0.352	0.711
Ring 2 N1-P1 amplitude	2.081	0.171	0.098	0.759	0.367	0.701
Ring 3 N1-P1 amplitude	1.277	0.317	0.001	0.976	1.260	0.322
Ring 4 N1-P1 amplitude	0.410	0.673	0.081	0.781	1.225	0.331
Ring 5 N1-P1 amplitude	0.297	0.749	1.315	0.274	0.664	0.534
Ring 1 N1 latency	0.864	0.448	0.904	0.361	0.345	0.716
Ring 2 N1 latency	1.249	0.324	1.603	0.230	0.935	0.422
Ring 3 N1 latency	1.384	0.291	2.565	0.135	0.571	0.581
Ring 4 N1 latency	0.406	0.676	2.919	0.113	0.168	0.848
Ring 5 N1 latency	0.873	0.445	2.251	0.159	0.546	0.594
Ring 1 P1 latency	5.928	0.018	0.517	0.486	0.016	0.984
Ring 2 P1 latency	3.077	0.087	0.709	0.416	3.060	0.088
Ring 3 P1 latency	0.178	0.840	3.978	0.069	0.106	0.901
Ring 4 P1 latency	0.079	0.924	1.389	0.261	0.242	0.789
Ring 5 P1 latency	0.323	0.731	0.371	0.554	3.300	0.075
Ring 1 N2 latency	1.030	0.389	0.509	0.489	0.416	0.670
Ring 2 N2 latency	1.567	0.252	1.331	0.271	0.035	0.966
Ring 3 N2 latency	0.831	0.461	2.790	0.121	1.437	0.279
Ring 4 N2 latency	0.947	0.417	0.194	0.667	1.556	0.254
Ring 5 N2latency	0.278	0.762	1.617	0.228	1.497	0.266

Table 6.3: Mean values ± SD for mfERG measures for 5 rings (R) of eccentricity for HO treated and non-treated groups over 3 visits. The shaded areas indicate statistical significance.

		Visit 1 HO	**Visit 2 HO**	**Visit 3 HO**
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R1 N1 latency	Treated	15.36 ± 1.43	15.95 ± 1.12	15.83 ± 1.27
(ms)	Non-treated	15.28 ± 2.56	15.60 ± 1.15	14.88 ± 0.89
R1 P1 latency	Treated	29.29 ± 1.48	30.24 ± 1.15	30.12 ± 1.22
(ms)	Non-treated	29.64 ± 1.43	30.71 ± 0.89	30.48 ± 1.16
R1 N2 latency	Treated	44.88 ± 1.89	44.29 ± 1.89	45.36 ± 1.35
(ms)	Non-treated	44.40 ± 2.13	44.05 ± 2.43	44.28 ± 1.22
R1 N1P1 amplitude	Treated	154.25 ± 27.39	149.86 ± 46.47	144.86 ± 32.40
(nV/deg^2)	Non-treated	153.64 ± 46.53	130.29 ± 39.32	132.71 ± 29.63
R2 N1 latency	Treated	15.95 ± 0.89	15.48 ± 1.06	15.24 ± 1.05
(ms)	Non-treated	15.00 ± 0.68	15.24 ± 1.24	14.88 ± 0.89
R2 P1 latency	Treated	29.29 ± 1.62	29.29 ± 1.63	29.29 ± 1.63
(ms)	Non-treated	28.45 ± 1.22	29.40 ± 0.79	28.33 ± 1.18
R2 N2 latency	Treated	43.33 ± 1.86	44.17 ± 1.36	44.29 ± 1.55
(ms)	Non-treated	42.74 ± 1.78	43.45 ± 1.12	43.45 ± 1.31
R2 N1P1 amplitude	Treated	63.13 ± 16.10	59.43 ± 14.39	58.71 ± 12.66
(nV/deg^2)	Non-treated	64.79 ± 16.42	55.29 ± 17.49	55.14 ± 13.12
R3 N1 latency	Treated	15.24 ± 0.79	15.71 ± 0.75	15.83 ± 0.68
(ms)	Non-treated	15.00 ± 0.48	15.12 ± 1.12	15.12 ± 0.57
R3 P1 latency	Treated	29.76 ± 0.93	29.65 ± 0.94	29.52 ± 1.58
(ms)	Non-treated	28.57 ± 1.15	28.69 ± 1.72	28.33 ± 1.28
R3 N2 latency	Treated	43.10 ± 1.04	43.21 ± 1.22	43.69 ± 1.26
(ms)	Non-treated	42.14 ± 0.66	42.86 ± 1.79	42.38 ± 0.89
R3 N1P1 amplitude	Treated	34.60 ± 9.17	34.86 ± 9.15	33.57 ± 7.55
(nV/deg^2)	Non-treated	37.96 ± 10.13	32.00 ± 9.76	33.43 ± 8.54

Table 6.3 continued:

		Visit 1 HO	Visit 2 HO	Visit 3 HO
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R4 N1 latency (ms)	Treated	15.48 ± 0.81	15.71 ± 0.89	15.83 ± 0.68
	Non-treated	15.00 ± 0.48	15.24 ± 1.15	15.12 ± 0.89
R4 P1 latency (ms)	Treated	29.76 ± 0.79	29.64 ± 1.51	29.76 ± 0.93
	Non-treated	28.93 ± 1.50	29.40 ± 1.33	29.05 ± 1.01
R4 N2 latency (ms)	Treated	43.09 ± 1.34	41.31 ± 5.46	43.81 ± 1.06
	Non-treated	42.86 ± 1.06	43.57 ± 1.42	42.86 ± 0.66
R4 N1P1 amplitude (nV/deg^2)	Treated	22.35 ± 6.83	23.29 ± 5.43	23.71 ± 6.13
	Non-treated	23.63 ± 6.01	20.71 ± 5.53	22.57 ± 7.76
R5 N1 latency (ms)	Treated	15.71 ± 0.75	15.83 ± 0.96	15.71 ± 0.75
	Non-treated	14.88 ± 0.57	15.60 ± 1.15	15.24 ± 0.93
R5 P1 latency (ms)	Treated	29.76 ± 0.79	29.64 ± 1.58	30.11 ± 0.89
	Non-treated	30.36 ± 0.81	30.48 ± 1.66	29.64 ± 1.06
R5 N2 latency (ms)	Treated	43.57 ± 1.15	43.33 ± 1.08	43.57 ± 0.93
	Non-treated	42.74 ± 1.05	43.45 ± 0.89	42.86 ± 0.44
R5 N1P1 amplitude (nV/deg^2)	Treated	15.28 ± 6.36	17.00 ± 3.42	14.29 ± 4.96
	Non-treated	14.26 ± 3.90	13.00 ± 4.32	13.14 ± 5.01

Table 6.4: Mixed between-within ANOVA for VA, CS and MPOD over 3 visits between treated and non treated groups in HO eyes. The shaded area indicates statistical significance.

Outcome measure	Main effect: time		Main effect: group (treated/non-treated)		Interaction effect	
	F	p	F	p	F	p
VA	1.352	0.282	0.236	0.632	1.429	0.264
CS	1.877	0.180	0.367	0.551	0.192	0.827
MPOD	2.254	0.132	1.509	0.234	5.176	0.016

Table 6.5: Mean values ± SD between treated and non-treated HO eyes for VA, CS and MPOD over 3 visits. The shaded areas indicate statistical significance.

	Visit 1 HO	Visit 2 HO	Visit 3 HO
	Mean ± SD	Mean ± SD	Mean ± SD
VA (logMAR units)			
Treated	-0.05 ± 0.07	-0.05 ± 0.06	-0.05 ± 0.08
Non-treated	-0.11 ± 0.17	-0.03 ± 0.06	-0.06 ± 0.09
CS (log units)			
Treated	1.79 ± 0.16	1.81 ± 0.16	1.81 ± 0.16
Non-treated	1.81 ± 0.16	1.84 ± 0.15	1.87 ± 0.14
MPOD (optical density units)			
Treated	0.32 ± 0.19	0.36 ± 0.15	0.42 ± 0.12
Non-treated	0.46 ± 0.17	0.45 ± 0.18	0.44 ± 0.17

Table 6.6: HO treated group dietary questionnaire analysis showing no difference between visits 1 and 3 using paired-samples t-tests.

Dietary component	t	p
Copper	1.070	0.363
Zinc	-0.867	0.450
Vitamin E	-0.494	0.655
Vitamin C	1.154	0.332
Retinol	1.017	0.384
Carotene	1.771	0.175
Lutein and zeaxanthin	-0.316	0.773
Omega 3	-1.189	0.320

Table 6.7: HO non-treated group dietary questionnaire analysis showing no difference between visits 1 and 3 using paired samples t-tests.

Dietary component	t	p
Copper	0.297	0.776
Zinc	-0.320	0.760
Vitamin E	-0.159	0.879
Vitamin C	-0.217	0.835
Retinol	-0.683	0.520
Carotene	0.163	0.876
Lutein and zeaxanthin	0.854	0.426
Omega 3	0.263	0.801

Table 6.9: Mixed between-within ANOVA for mfERG N1P1 amplitude, N1 latency, P1 latency and N2 latency over 3 visits for 5 areas of retinal eccentricity between treated and non-treated groups for HY eyes.

Outcome measure	Main effect: time		Main effect: group (treated/non-treated)		Interaction effect	
	F	p	F	p	F	p
Ring 1 N1-P1 amplitude	0.522	0.612	0.048	0.831	1.250	0.337
Ring 2 N1-P1 amplitude	1.279	0.330	0.369	0.558	0.774	0.493
Ring 3 N1-P1 amplitude	1.415	0.298	0.000	0.993	0.093	0.912
Ring 4 N1-P1 amplitude	0.723	0.514	0.022	0.884	0.137	0.874
Ring 5 N1-P1 amplitude	0.835	0.468	0.072	0.794	0.652	0.547
Ring 1 N1 latency	1.533	0.273	3.268	0.104	2.401	0.153
Ring 2 N1 latency	0.325	0.732	1.427	0.263	0.609	0.567
Ring 3 N1 latency	0.190	0.830	0.591	0.462	0.316	0.737
Ring 4 N1 latency	0.251	0.784	2.435	0.153	0.005	0.995
Ring 5 N1latency	1.462	0.288	1.767	0.216	0.522	0.612
Ring 1 P1 latency	0.229	0.800	0.347	0.570	1.498	0.280
Ring 2 P1 latency	0.949	0.427	0.840	0.383	3.053	0.103
Ring 3 P1 latency	1.643	0.253	0.019	0.894	0.145	0.868
Ring 4 P1 latency	1.461	0.288	0.529	0.486	0.632	0.556
Ring 5 P1 latency	1.265	0.333	0.056	0.819	1.635	0.254
Ring 1 N2 latency	2.616	0.134	1.753	0.218	0.293	0.754
Ring 2 N2 latency	1.673	0.247	1.300	0.284	0.115	0.893
Ring 3 N2 latency	2.079	0.187	0.835	0.385	0.141	0.871
Ring 4 N2 latency	2.508	0.143	1.404	0.266	1.998	0.198
Ring 5 N2 latency	3.295	0.090	0.314	0.589	0.836	0.468

Table 6.10: Mean values ± SD for mfERG measures for 5 rings (R) of eccentricity for HY treated and non-treated groups over 3 visits.

		Visit 1 HY	**Visit 2 HY**	**Visit 3 HY**
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R1 N1 latency (ms)	Treated	15.00 ± 0.77	15.10 ± 0.53	15.10 ± 0.69
	Non-treated	15.28 ± 0.48	14.44 ± 0.96	13.87 ± 0.96
R1 P1 latency (ms)	Treated	28.85 ± 1.47	28.75 ± 1.72	28.33 ± 1.00
	Non-treated	28.61 ± 1.73	29.17 ± 3.00	29.72 ± 0.48
R1 N2 latency (ms)	Treated	42.50 ± 1.72	42.92 ± 1.72	44.17 ± 1.09
	Non-treated	43.06 ± 1.27	44.72 ± 3.76	45.00 ± 1.67
R1N1P1 amplitude (nV/deg^2)	Treated	178.81 ± 40.62	168.75 ± 50.51	171.38 ± 31.14
	Non-treated	154.58 ± 55.70	187.00 ± 43.86	194.00 ± 60.80
R2 N1 latency (ms)	Treated	15.10 ± 1.13	14.79 ± 0.59	15.31 ± 0.99
	Non-treated	14.72 ± 0.48	14.17 ± 2.21	14.44 ± 1.27
R2 P1 latency (ms)	Treated	27.92 ± 1.00	27.81 ± 0.99	26.67 ± 0.89
	Non-treated	27.78 ± 1.92	28.33 ± 2.20	28.33 ± 1.67
R2 N2 latency (ms)	Treated	41.46 ± 1.39	42.08 ± 1.72	42.40 ± 1.22
	Non-treated	42.22 ± 1.92	43.33 ± 1.67	43.33 ± 1.67
R2 N1P1 amplitude (nV/deg^2)	Treated	74.05 ± 20.37	64.13 ± 17.87	70.13 ± 10.09
	Non-treated	71.35 ± 21.55	75.67 ± 23.76	81.67 ± 30.27
R3 N1 latency (ms)	Treated	14.90 ± 1.13	14.90 ± 0.53	15.00 ± 0.77
	Non-treated	15.56 ± 0.96	15.00 ± 0.00	15.00 ± 0.00
R3 P1 latency (ms)	Treated	27.40 ± 1.57	28.23 ± 1.13	27.92 ± 1.41
	Non-treated	27.50 ± 2.50	28.61 ± 0.96	27.78 ± 2.10
R3 N2 latency (ms)	Treated	41.04 ± 0.86	41.67 ± 1.48	41.98 ± 1.17
	Non-treated	41.94 ± 1.73	42.22 ± 1.74	42.50 ± 1.67
R3 N1P1 amplitude (nV/deg^2)	Treated	42.10 ± 14.33	35.75 ± 7.27	40.13 ± 5.79
	Non-treated	39.48 ± 12.36	37.33 ± 14.74	41.00 ± 16.37

Table 6.10 continued.

		Visit 1 HY	**Visit 2 HY**	**Visit 3 HY**
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R4 N1 latency (ms)	Treated	14.69 ± 0.76	14.90 ± 0.53	14.90 ± 0.82
	Non-treated	15.28 ± 0.96	15.56 ± 0.96	15.56 ± 1.27
R4 P1 latency (ms)	Treated	28.13 ± 0.86	28.75 ± 0.89	28.13 ± 1.07
	Non-treated	29.16 ± 1.44	28.89 ± 1.27	28.33 ± 2.20
R4 N2 latency (ms)	Treated	41.67 ± 0.77	41.98 ± 1.25	41.88 ± 0.86
	Non-treated	41.67 ± 0.84	42.50 ± 0.83	43.06 ± 0.96
R4 N1P1 amplitude (nV/deg^2)	Treated	27.77 ± 9.42	23.63 ± 4.87	25.75 ± 3.49
	Non-treated	26.46 ± 7.40	24.67 ± 10.02	27.67 ± 11.59
R5 N1 latency (ms)	Treated	14.69 ± 0.99	14.48 ± 0.43	14.69 ± 0.88
	Non-treated	15.28 ± 0.96	14.72 ± 0.48	15.56 ± 1.27
R5 P1 latency (ms)	Treated	28.54 ± 0.97	29.27 ± 0.70	28.44 ± 1.04
	Non-treated	29.16 ± 1.44	28.89 ± 0.96	28.61 ± 2.09
R5 N2 latency (ms)	Treated	41.77 ± 1.13	42.19 ± 0.43	42.29 ± 1.07
	Non-treated	41.94 ± 1.27	42.22 ± 1.27	43.06 ± 0.96
R5 N1P1 amplitude (nV/deg^2)	Treated	17.01 ± 5.79	15.38 ± 2.62	16.50 ± 3.34
	Non-treated	13.54 ± 3.44	16.00 ± 4.36	17.67 ± 6.51

Table 6.11: Mixed between-within ANOVA for VA, CS and MPOD over 3 visits between treated and non treated groups in HY eyes. The shaded areas indicate statistical significance.

Outcome measure	Main effect: time		Main effect: group (treated/non-treated)		Interaction effect	
	F	p	F	p	F	p
VA	8.037	0.002	0.050	0.825	0.006	0.994
CS	2.010	0.167	0.009	0.925	0.009	0.925
MPOD	2.726	0.84	0.990	0.329	11.476	<0.001

Table 6.12: Mean values ± SD between treated and non-treated HY eyes for VA, CS and MPOD over 3 visits. The shaded areas indicate statistical significance.

	Visit 1 HY	Visit 2 HY	Visit 3 HY
	Mean ± SD	Mean ± SD	Mean ± SD
VA (logMAR units)			
Treated	-0.12 ± 0.08	-0.08 ± 0.09	-0.13 ± 0.08
Non-treated	-0.13 ± 0.08	-0.09 ± 0.08	-0.13 ± 0.08
CS (log units)			
Treated	1.95 ± 0.00	1.93 ± 0.08	1.95 ± 0.00
Non-treated	1.95 ± 0.00	1.93 ± 0.08	1.95 ± 0.00
MPOD (optical density units)			
Treated	0.38 ± 0.12	0.43 ± 0.12	0.48 ± 0.12
Non-treated	0.39 ± 0.16	0.40 ± 0.14	0.36 ± 0.15

Table 6.13: Dietary questionnaire analysis between visits 1 and 3 for the treated group of HY eyes using paired-samples t-tests. The shaded area indicates statistical significance.

Dietary component	t	p
Copper	0.935	0.419
Zinc	-0.488	0.659
Vitamin E	-0.745	0.510
Vitamin C	0.125	0.909
Retinol	0.245	0.823
Carotene	-5.706	0.011
Lutein and zeaxanthin	0.640	0.568
Omega 3	0.225	0.836

Table 6.14: Dietary questionnaire analysis between visits 1 and 3 for the non-treated group of HY eyes using paired-samples t-tests. The shaded area indicates statistical significance.

Dietary component	t	p
Copper	0.916	0.456
Zinc	-0.516	0.657
Vitamin E	-1.033	0.410
Vitamin C	-0.032	0.977
Retinol	-4.919	0.039
Carotene	0.626	0.595
Lutein and zeaxanthin	0.516	0.657
Omega 3	-0.173	0.878

Table 6.16: Mixed between-within ANOVA for mfERG N1P1 amplitude, N1 latency, P1 latency and N2 latency over 3 visits for 5 areas of retinal eccentricity between treated and non-treated groups for combined HY and HO eyes. The shaded areas indicate statistical significance.

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

Table 6.17: Mean values ± SD for mfERG measures for 5 rings (R) of eccentricity for combined HO and HY treated and non-treated groups over 3 visits. The shaded areas indicate statistical significance.

		Visit 1 HO & HY	**Visit 2 HO & HY**	**Visit 3 HO & HY**
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R1 N1 latency (ms)	Treated	15.17 ± 1.10	15.50 ± 0.93	15.44 ± 1.04
	Non-treated	15.28 ± 2.10	15.25 ± 1.18	14.58 ± 0.98
R1 P1 latency (ms)	Treated	29.05 ± 1.44	29.44 ± 1.63	29.17 ± 1.41
	Non-treated	29.33 ± 1.51	30.25 ± 1.76	30.25 ± 1.04
R1 N2 latency (ms)	Treated	43.61 ± 2.13	43.56 ± 1.88	44.72 ± 1.32
	Non-treated	44.00 ± 1.95	44.25 ± 2.68	44.50 ± 1.32
R1 N1P1 amplitude (nV/deg^2)	Treated	167.35 ± 36.15	159.93 ± 47.92	159.00 ± 33.50
	Non-treated	153.93 ± 46.18	147.30 ± 47.00	151.10 ± 47.78
R2 N1 latency (ms)	Treated	15.50 ± 1.08	15.11 ± 0.88	15.28 ± 0.98
	Non-treated	14.92 ± 0.61	14.92 ± 1.55	14.75 ± 0.97
R2 P1 latency (ms)	Treated	28.56 ± 1.46	28.50 ± 1.48	27.89 ± 1.83
	Non-treated	28.25 ± 1.38	29.08 ± 1.33	28.33 ±1.24
R2 N2 latency (ms)	Treated	42.33 ± 1.84	43.06 ± 1.85	43.28 ± 1.65
	Non-treated	42.58 ± 1.73	43.42 ± 1.21	43.42 ± 1.33
R2 N1P1 amplitude (nV/deg^2)	Treated	68.95 ± 18.72	61.93 ± 15.94	64.80 ± 12.42
	Non-treated	66.76 ± 17.12	61.40 ± 20.65	63.10 ± 21.97
R3 N1 latency (ms)	Treated	15.05 ± 0.97	15.28 ± 0.75	15.39 ± 0.82
	Non-treated	15.17 ± 0.66	15.09 ± 0.92	15.08 ± 0.47
R3 P1 latency (ms)	Treated	28.50 ± 1.76	28.89 ± 1.25	28.67 ± 1.66
	Non-treated	28.25 ± 1.59	28.67 ± 1.48	28.15 ± 1.46
R3 N2 latency (ms)	Treated	42.00 ± 1.40	42.39 ± 1.54	42.78 ± 1.47
	Non-treated	42.08 ± 0.98	42.67 ± 1.70	42.42 ± 1.07
R3 N1P1 amplitude (nV/deg^2)	Treated	38.60 ± 12.40	35.33 ± 7.91	37.07 ± 7.26
	Non-treated	38.41 ± 10.14	33.60 ± 10.89	35.70 ± 11.03

Table 6.17 continued.

		Visit 1 HO & HY	Visit 2 HO & HY	Visit 3 HO & HY
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R4 N1 latency (ms)	Treated	15.06 ± 0.86	15.28 ± 0.81	15.33 ± 0.88
	Non-treated	15.208± 0.61	15.34 ± 1.05	15.25 ± 0.97
R4 P1 latency (ms)	Treated	28.89 ± 1.16	29.17 ± 1.26	28.89 ± 1.29
	Non-treated	29.00 ± 1.41	29.25 ± 1.27	28.83 ± 1.37
R4 N2 latency (ms)	Treated	42.33 ± 1.27	41.67 ± 3.70	42.78 ± 1.36
	Non-treated	42.50 ± 1.11	43.25 ± 1.33	42.92 ± 0.71
R4 N1P1 amplitude (nV/deg^2)	Treated	25.24 ± 8.50	23.47 ± 4.96	24.80 ± 4.83
	Non-treated	24.48 ± 6.17	21.90 ± 6.81	24.10 ± 8.72
R5 N1 latency (ms)	Treated	15.17 ± 1.01	15.11 ± 0.99	15.17 ± 0.96
	Non-treated	15.00 ± 0.68	15.33 ± 1.05	15.33 ± 0.98
R5 P1 latency (ms)	Treated	29.11 ± 1.07	29.45 ± 1.16	29.22 ± 1.28
	Non-treated	30.00 ± 1.11	30.00 ± 1.62	29.33 ± 1.40
R5 N2 latency (ms)	Treated	42.61 ± 1.44	42.72 ± 0.97	42.89 ±1.17
	Non-treated	42.50 ±1.11	43.08 ± 1.12	42.92 ± 0.59
R5 N1P1 amplitude (nV/deg^2)	Treated	16.20 ± 5.91	16.13 ± 3.02	15.47 ± 4.17
	Non-treated	14.05 ± 3.59	13.90 ± 4.33	14.50 ± 5.56

Table 6.18: Mixed between-within ANOVA for VA, CS and MPOD over 3 visits between treated and non treated groups in HY and HO eyes. The shaded areas indicate statistical significance.

Outcome measure	Main effect: time		Main effect: group (treated/non-treated)		Interaction effect	
	F	p	F	p	F	p
VA	0.852	0.440	0.287	0.594	0.002	0.998
CS	1.615	0.209	0.425	0.517	0.144	0.866
MPOD	4.235	0.020	0.040	0.842	16.998	<0.001

Table 6.19: Mean values ± SD between treated and non-treated combined HO and HY eyes for VA, CS and MPOD over 3 visits. The shaded areas indicate statistical significance.

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

Table 6.20: Dietary questionnaire analysis between visits 1 and 3 for the treated group of HY and HO eyes using paired-samples t-tests.

Dietary component	t	p
Copper	1.702	0.133
Zinc	0.221	0.831
Vitamin E	-0.011	0.992
Vitamin C	1.137	0.293
Retinol	1.107	0.305
Carotene	1.058	0.325
Lutein and zeaxanthin	-0.155	0.881
Omega 3	-0.543	0.604

Table 6.21: Dietary questionnaire analysis between visits 1 and 3 for the non-treated group of HY and HO eyes using paired-samples t-tests.

Dietary component	t	p
Copper	0.519	0.617
Zinc	-0.841	0.422
Vitamin E	0.053	0.959
Vitamin C	-0.265	0.797
Retinol	-1.113	0.295
Carotene	-0.191	0.853
Lutein and zeaxanthin	0.102	0.921
Omega 3	0.867	0.408

Table 6.23: Mixed between-within ANOVA for mfERG N1P1 amplitude, N1 latency, P1 latency and N2 latency over three visits for five areas of retinal eccentricity between treated and non-treated groups for ARM eyes. The shaded areas indicate statistical significance.

Outcome measure	Main effect: time		Main effect: group (treated/non-treated)		Interaction effect	
	F	p	F	p	F	p
Ring 1 N1-P1 amplitude	3.470	0.134	0.666	0.452	0.432	0.677
Ring 2 N1-P1 amplitude	3.188	0.149	0.774	0.419	1.778	0.280
Ring 3 N1-P1 amplitude	11.963	0.021	0.599	0.474	1.768	0.282
Ring 4 N1-P1 amplitude	13.957	0.016	1.273	0.310	4.785	0.087
Ring 5 N1-P1 amplitude	1.363	0.354	0.378	0.566	0.871	0.303
Ring 1 N1 latency	0.616	0.585	2.133	0.204	0.241	0.796
Ring 2 N1 latency	0.441	0.671	0.019	0.896	0.116	0.894
Ring 3 N1 latency	2.341	0.212	< 0.001	0.999	2.613	0.188
Ring 4 N1 latency	0.420	0.683	0.732	0.431	0.080	0.924
Ring 5 N1latency	1.548	0.318	0.627	0.464	0.032	0.969
Ring 1 P1 latency	0.549	0.616	2.092	0.208	0.061	0.941
Ring 2 P1 latency	0.317	0.745	0.099	0.766	1.333	0.360
Ring 3 P1 latency	1.365	0.353	0.031	0.867	0.036	0.965
Ring 4 P1 latency	0.922	0.469	0.086	0.781	0.768	0.522
Ring 5 P1 latency	3.422	0.136	0.402	0.554	2.475	0.200
Ring 1 N2 latency	0.245	0.794	3.018	0.143	0.135	0.878
Ring 2 N2 latency	0.951	0.459	1.530	0.271	0.403	0.693
Ring 3 N2 latency	0.990	0.447	0.052	0.828	1.000	0.444
Ring 4 N2 latency	0.586	0.598	0.181	0.688	0.023	0.977
Ring 5 N2latency	0.075	0.929	0.021	0.891	0.078	0.926

Table 6.24: Mean values ± SD for mfERG measures for 5 rings (R) of eccentricity for ARM treated and non-treated groups over 3 visits. The shaded areas indicate statistical significance.

		Visit 1 ARM	**Visit 2 ARM**	**Visit 3 ARM**
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R1 N1 latency (ms)	Treated	16.46 ± 1.42	15.42 ± 1.08	15.63 ± 0.80
	Non-treated	15.28 ± 1.74	15.00 ± 0.00	15.00 ± 2.21
R1 P1 latency (ms)	Treated	30.56 ± 1.47	31.11 ± 0.96	31.25 ± 1.99
	Non-treated	28.61 ± 2.09	29.72 ± 2.68	30.00 ± 1.67
R1 N2 latency (ms)	Treated	44.38 ± 1.05	45.00 ± 2.63	45.21 ± 1.25
	Non-treated	43.61 ± 2.41	43.33 ± 0.00	44.72 ± 2.68
R1N1P1 amplitude (nV/deg^2)	Treated	115.52 ± 34.92	97.75 ± 16.13	125.75 ± 40.34
	Non-treated	111.19 ± 19.27	83.67 ± 24.79	100.00 ± 11.36
R2 N1 latency (ms)	Treated	15.21 ± 1.43	15.00 ± 0.68	15.42 ± 1.74
	Non-treated	15.00 ± 0.83	15.00 ± 0.00	15.83 ± 0.84
R2 P1 latency (ms)	Treated	28.96 ± 1.58	29.59 ± 0.83	28.75 ± 1.73
	Non-treated	29.44 ± 2.40	27.78 ± 0.48	29.17 ± 1.44
R2 N2 latency (ms)	Treated	43.96 ± 0.80	43.54 ± 1.05	44.59 ± 0.48
	Non-treated	43.06 ± 1.93	43.33 ± 0.00	43.61 ± 1.27
R2 N1P1 amplitude (nV/deg^2)	Treated	47.52 ± 9.47	46.50 ± 9.15	53.00 ± 13.17
	Non-treated	50.08 ± 3.52	35.67 ± 1.53	47.00 ± 5.20
R3 N1 latency (ms)	Treated	15.21 ± 0.79	15.21 ± 0.42	15.42 ± 1.44
	Non-treated	16.39 ± 0.48	15.28 ± 0.48	14.16 ± 1.44
R3 P1 latency (ms)	Treated	28.13 ± 1.05	28.96 ± 1.05	28.96 ± 1.25
	Non-treated	28.34 ± 1.44	29.17 ± 0.84	28.89 ± 1.27
R3 N2 latency (ms)	Treated	42.71 ± 0.42	42.71 ± 0.79	42.71 ± 1.05
	Non-treated	42.78 ± 0.48	43.05 ± 0.48	41.94 ± 1.27
R3 N1P1 amplitude (nV/deg^2)	Treated	29.58 ± 4.78	28.75 ± 5.91	36.25 ± 7.18
	Non-treated	30.00 ± 0.94	26.00 ± 1.73	31.00 ± 2.00

Table 6.24 continued.

		Visit 1 ARM	Visit 2 ARM	Visit 3 ARM
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
R4 N1 latency (ms)	Treated	15.00 ± 0.00	15.21 ± 0.42	15.83 ± 1.17
	Non-treated	15.56 ± 1.27	15.28 ± 1.27	15.84 ± 1.44
R4 P1 latency (ms)	Treated	29.38 ± 0.80	29.79 ± 0.79	29.17 ± 0.00
	Non-treated	29.44 ± 0.96	29.72 ± 1.27	29.72 ± 1.27
R4 N2 latency (ms)	Treated	42.29 ± 0.42	43.13 ± 0.80	43.13 ± 1.42
	Non-treated	42.78 ± 2.10	43.33 ± 0.84	43.33 ± 1.67
R4 N1P1 amplitude (nV/deg^2)	Treated	19.94 ± 4.17	19.25 ± 2.50	25.25 ± 4.50
	Non-treated	19.48 ± 0.48	17.33 ± 2.31	20.00 ± 2.00
R5 N1 latency (ms)	Treated	15.63 ± 0.80	15.21 ± 0.79	16.04 ± 1.05
	Non-treated	15.28 ± 0.48	15.00 ± 0.83	15.83 ± 0.84
R5 P1 latency (ms)	Treated	30.21 ± 1.43	30.63 ± 1.25	29.59 ± 0.48
	Non-treated	31.67 ± 1.44	30.56 ± 1.93	30.28 ± 2.41
R5 N2 latency (ms)	Treated	43.33 ± 1.80	43.34 ± 0.96	43.33 ± 0.68
	Non-treated	43.33 ± 0.84	43.05 ± 0.48	43.33 ± 1.67
R5 N1P1 amplitude (nV/deg^2)	Treated	12.08 ± 2.70	12.50 ± 3.42	15.50 ± 2.65
	Non-treated	12.75 ± 1.69	11.67 ± 3.21	12.67 ± 2.08

Table 6.25: Paired-samples t-tests analysing mfERG measures in 8 ARM treated eyes between visit 1 and visit 3. The shaded areas indicate statistical significance.

Visit 1 and 3 mfERG	t	p
Ring 1 N1 latency	1.000	.350
Ring 1 P1 latency	-.513	.624
Ring 1 N2 latency	-.241	.817
Ring 1 amplitude	-.897	.399
Ring 2 N1 latency	.799	.451
Ring 2 P1 latency	-.148	.887
Ring 2 N2 latency	-1.819	.112
Ring 2 amplitude	-.318	.760
Ring 3 N1 latency	.753	.476
Ring 3 P1 latency	-.595	.570
Ring 3 N2 latency	-.801	.449
Ring 3 amplitude	-2.787	.027
Ring 4 N1 latency	-1.488	.180
Ring 4 P1 latency	-.757	.474
Ring 4 N2 latency	-1.985	.088
Ring 4 amplitude	-2.271	.057
Ring 5 N1 latency	-1.420	.199
Ring 5 P1 latency	-.142	.891
Ring 5 N2 latency	-1.221	.262
Ring 5 amplitude	-1.081	.316

Table 6.26: Independent-samples t-tests for differences between mfERG measures between visits 1 and 3 for treated and non-treated eyes. The shaded areas indicate statistical significance.

Visit 1 and 3 mfERG	t	p
Ring 1 N1 latency	0.299	0.770
Ring 1 P1 latency	-0.543	0.598
Ring 1 N2 latency	1.226	0.246
Ring 1 amplitude	0.265	0.796
Ring 2 N1 latency	1.006	0.336
Ring 2 P1 latency	-0.086	0.933
Ring 2 N2 latency	0.526	0.610
Ring 2 amplitude	-0.017	0.987
Ring 3 N1 latency	-2.970	0.013
Ring 3 P1 latency	-0.959	0.358
Ring 3 N2 latency	0.146	0.886
Ring 3 amplitude	-0.498	0.628
Ring 4 N1 latency	-0.838	0.420
Ring 4 P1 latency	-0.347	0.735
Ring 4 N2 latency	-0.590	0.567
Ring 4 amplitude	-0.190	0.853
Ring 5 N1 latency	-1.069	0.336
Ring 5 P1 latency	-0.853	0.412
Ring 5 N2 latency	-0.593	0.565
Ring 5 amplitude	-0.034	0.973

Table 6.27: Mixed between-within ANOVA for VA, CS and MPOD over three visits between treated and non treated groups in ARM eyes.

Outcome measure	Main effect: time		Main effect: group (treated/non-treated)		Interaction effect	
	F	p	F	p	F	p
VA	0.564	0.584	0.843	0.377	0.174	0.843
CS	1.444	0.277	0.392	0.543	2.463	0.131
MPOD	1.383	0.291	1.395	0.260	0.519	0.609

Table 6.28 Mean values ± SD between treated and non-treated ARM eyes for VA, CS and MPOD over 3 visits.

	Visit 1 ARM	Visit 2 ARM	Visit 3 ARM
	Mean ± SD	Mean ± SD	Mean ± SD
VA (logMAR units)			
Treated	0.06 ± 0.08	0.07 ± 0.08	0.05 ± 0.10
Non-treated	0.03 ± 0.02	0.06 ± 0.03	0.03 ± 0.08
CS (log units)			
Treated	1.76 ± 0.16	1.69 ± 0.11	1.80 ± 0.16
Non-treated	1.75 ± 0.15	1.70 ± 0.12	1.68 ± 0.15
MPOD (optical density units)			
Treated	0.36 ± 0.19	0.44 ± 0.20	0.39 ± 0.22
Non-treated	0.52 ± 0.32	0.59 ± 0.18	0.48 ± 0.23

Table 6.29: ARM treated group dietary questionnaire analysis showing no difference between visits 1 and 3 using paired-samples t-tests.

Dietary component	t	p
Copper	-1.462	0.218
Zinc	-0.949	0.396
Vitamin E	0.264	0.805
Vitamin C	-0.914	0.413
Retinol	-1.642	0.176
Carotene	-0.330	0.758
Lutein and zeaxanthin	-1.047	0.354
Omega 3	-0.040	0.970

Table 6.30: ARM non-treated group dietary questionnaire analysis showing no difference between visits one and three using paired-samples t-tests.

Dietary component	t	p
Copper	0.794	0.485
Zinc	0.406	0.712
Vitamin E	0.783	0.491
Vitamin C	0.797	0.483
Retinol	0.260	0.812
Carotene	-0.182	0.867
Lutein and zeaxanthin	0.565	0.611
Omega 3	-0.501	0.651

Appendix 12 – Withdrawal results tables

Table 7.1: A summary of the change in each mfERG outcome measure for the HO group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal. Shaded areas show statistical significance.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
Ring 1 N1 latency (ms)	15.42 ± 1.09	15.73 ± 1.37	t = -0.436	0.676
Ring 1 P1 latency (ms)	29.90 ± 1.13	28.79 ± 1.07	Z = -0.544	0.586
Ring 1 N2 latency (ms)	44.69 ± 1.09	44.79 ± 1.88	t = -0.152	0.884
Ring 1 N1P1 amplitude (nV/deg^2)	151.38 ± 26.20	124.88 ± 29.33	t = 2.411	0.047
Ring 2 N1 latency (ms)	15.00 ± 0.89	14.90 ± 1.04	Z = -0.414	0.679
Ring 2 P1 latency (ms)	28.85 ± 1.17	28.33 ± 1.67	t = 1.174	0.279
Ring 2 N2 latency (ms)	43.65 ± 1.17	43.54 ± 1.39	Z = -0.213	0.832
Ring 2 N1P1 amplitude (nV/deg^2)	60.50 ± 9.71	53.00 ± 9.13	t = 2.243	0.060
Ring 3 N1 latency (ms)	15.52 ± 0.76	15.10 ± 0.53	Z = -1.000	0.317
Ring 3 P1 latency (ms)	29.06 ± 1.50	29.17 ± 1.18	t = -0.317	0.760
Ring 3 N2 latency (ms)	43.13 ± 1.24	42.92 ± 0.77	t = 0.805	0.447
Ring 3 N1P1 amplitude (nV/deg^2)	35.75 ± 6.41	31.00 ± 7.25	t = 1.952	0.092
Ring 4 N1 latency (ms)	15.52 ± 0.62	15.21 ± 0.86	Z = -0.530	0.596
Ring 4 P1 latency (ms)	29.48 ± 0.88	29.90 ± 1.04	t = -1.183	0.275
Ring 4 N2 latency (ms)	43.44 ± 1.22	42.81 ± 1.08	t = 1.657	0.141
Ring 4 N1P1 amplitude (nV/deg^2)	25.87 ± 5.69	22.50 ± 6.46	t = 1.863	0.105
Ring 5 N1 latency (ms)	15.52 ± 0.62	15.52 ± 0.62	Z = 0.000	1.000
Ring 5 P1 latency (ms)	29.70 ± 0.97	29.69 ± 0.76	Z = -0.137	0.891
Ring 5 N2 latency (ms)	43.44 ± 0.94	43.33 ± 1.26	t = 0.244	0.814
Ring 5 N1P1 amplitude (nV/deg^2)	15.63 ± 4.44	14.75 ± 3.96	t = 0.665	0.527

Table 7.2: A summary of the change in subjective outcome measures for the HO group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
VA (logMAR units)	-0.06 ± 0.09	-0.02 ± 0.06	Z = -1.247	0.212
CS (log units)	1.80 ± 0.16	1.80 ± 0.16	Z = 0.000	1.000
MPOD (optical density units)	0.42 ± 0.13	0.42 ± 0.16	t = 0.056	0.957

Table 7.3: A summary of the change in dietary outcomes for the HO group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
Dietary copper (mg)	1.31 ± 0.37	1.45 ± 0.66	t = -0.229	0.840
Dietary zinc (mg)	9.27 ± 1.21	10.87 ± 1.16	t = -1.391	0.299
Dietary retinol (µg)	350.00 ± 114.67	420 ± 103.59	t = -0.962	0.437
Dietary carotene (µg)	1641.00 ± 1179.75	1880.67 ± 1099.55	Z = 0.000	1.000
Dietary Vitamin E (mg)	8.86 ± 5.56	7.13 ± 3.60	t = 0.461	0.690
Dietary Vitamin C (mg)	120.33 ± 64.03	114.00 ± 29.51	t = 0.152	0.893
Dietary lutein and zeaxanthin (µg)	1843.98 ± 996.67	1386.11 ± 669.07	t = 1.785	0.216
Dietary Omega 3 (g)	0.21 ± 0.27	0.97 ± 0.44	t = -3.827	0.062

Table 7.4: A summary of the change in each mfERG outcome measure for the HY group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal. Shaded areas show statistical significance.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
Ring 1 N1 latency (ms)	14.63 ± 1.03	15.55 ± 1.02	t = -1.752	0.118
Ring 1 P1 latency (ms)	28.70 ± 1.03	28.33 ± 1.61	t = 0.594	0.569
Ring 1 N2 latency (ms)	44.07 ± 1.21	43.33 ± 2.24	t = 0.948	0.371
Ring 1 N1P1 amplitude (nV/deg^2)	179.00 ± 29.00	165.11 ± 35.71	t = 1.060	0.320
Ring 2 N1 latency (ms)	15.00 ± 1.02	14.44 ± 1.02	t = 1.206	0.262
Ring 2 P1 latency (ms)	27.04 ± 1.11	27.50 ± 1.56	t = -0.679	0.516
Ring 2 N2 latency (ms)	42.50 ± 1.25	41.85 ± 1.30	t = 1.075	0.314
Ring 2 N1P1 amplitude (nV/deg^2)	68.89 ± 10.14	62.33 ± 16.31	t = 1.485	0.176
Ring 3 N1 latency (ms)	15.00 ± 0.72	14.72 ± 0.72	Z = -0.966	0.334
Ring 3 P1 latency (ms)	28.06 ± 1.38	27.22 ± 1.32	Z = -1.975	0.048
Ring 3 N2 latency (ms)	41.67 ± 0.83	41.48 ± 0.70	Z = -1.300	0.194
Ring 3 N1P1 amplitude (nV/deg^2)	39.56 ± 6.11	36.89 ± 10.81	t = 1.055	0.322
Ring 4 N1 latency (ms)	14.91 ± 0.77	15.09 ± 0.50	Z = -0.707	0.480
Ring 4 P1 latency (ms)	28.06 ± 1.18	28.43 ± 0.78	Z = -1.511	0.131
Ring 4 N2 latency (ms)	41.85 ± 0.70	41.76 ± 1.28	Z = -0.333	0.739
Ring 4 N1P1 amplitude (nV/deg^2)	25.44 ± 3.61	24.11 ± 5.97	t = 0.669	0.522
Ring 5 N1 latency (ms)	14.72 ± 0.93	14.91 ± 0.65	t = -0.390	0.707
Ring 5 P1 latency (ms)	28.70 ± 1.11	27.97 ± 1.27	t = 2.198	0.059
Ring 5 N2 latency (ms)	42.41 ± 0.97	41.95 ± 0.59	Z = -1.035	0.301
Ring 5 N1P1 amplitude (nV/deg^2)	16.67 ± 2.83	15.56 ± 3.81	Z = -1.053	0.292

Table 7.5: A summary of the change in subjective outcome measures for the HY group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
VA (logMAR units)	-0.14 ± 0.05	-0.14 ± 0.05	t = -0.223	0.828
CS (log units)	1.95 ± 0.00	1.95 ± 0.00	Z = 0.000	1.000
MPOD (optical density units)	0.44 ± 0.05	0.44 ± 0.05	Z = 0.061	0.952

Table 7.6: A summary of the change in dietary outcomes for the HY group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
Dietary copper (mg)	0.90 ± 0.13	0.75 ± 0.00	Z = -1.342	0.180
Dietary zinc (mg)	9.80 ± 0.28	7.3 ± 0.00	Z = -1.342	0.180
Dietary retinol (µg)	357.00 ± 12.73	298.50 ± 3.54	Z = -1.342	0.180
Dietary carotene (µg)	1705.50 ± 191.63	2582.00 ± 9.90	Z = -1.342	0.180
Dietary Vitamin E (mg)	2.79 ± 0.45	3.40 ± 0.22	Z = -1.342	0.180
Dietary Vitamin C (mg)	39.50 ± 4.95	58.50 ± 3.54	Z = -1.342	0.180
Dietary lutein and zeaxanthin (µg)	1035.74 ± 566.43	1116.02 ± 34.87	Z = -0.447	0.655
Dietary Omega 3 (g)	0.24 ± 0.05	0.41 ± 0.00	Z = -1.342	0.180

Table 7.7: A summary of the change in each mfERG outcome measure for the HY and HO groups combined between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal. Shaded areas show statistical significance.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
Ring 1 N1 latency (ms)	15.00 ± 1.10	15.64 ± 1.16	t = -1.476	0.159
Ring 1 P1 latency (ms)	29.27 ± 1.21	29.02 ± 1.54	t = 0.621	0.544
Ring 1 N2 latency (ms)	44.36 ± 1.16	44.02 ± 2.15	t = 0.674	0.510
Ring 1 N1P1 amplitude (nV/deg^2)	166.00 ± 30.37	146.18 ± 37.98	t = 2.323	0.034
Ring 2 N1 latency (ms)	15.00 ± 0.93	14.66 ± 1.02	t = 1.166	0.261
Ring 2 P1 latency (ms)	27.89 ± 1.45	27.89 ± 1.62	t = 0.000	1.000
Ring 2 N2 latency (ms)	43.04 ± 1.32	42.65 ± 1.57	t = 0.926	0.368
Ring 2 N1P1 amplitude (nV/deg^2)	64.94 ± 10.55	57.94 ± 13.88	t = 2.561	0.021
Ring 3 N1 latency (ms)	15.24 ± 0.76	14.90 ± 0.65	Z = -1.393	0.163
Ring 3 P1 latency (ms)	28.53 ± 1.49	28.14 ± 1.57	Z = -1.244	0.214
Ring 3 N2 latency (ms)	42.35 ± 1.26	42.16 ± 1.02	Z = -1.786	0.074
Ring 3 N1P1 amplitude (nV/deg^2)	37.76 ± 6.40	34.12 ± 9.52	t = 2.114	0.051
Ring 4 N1 latency (ms)	15.20 ± 0.75	15.15 ± 0.67	Z = -0.100	0.921
Ring 4 P1 latency (ms)	28.73 ± 1.18	29.12 ± 1.16	Z = -1.756	0.079
Ring 4 N2 latency (ms)	42.60 ± 1.25	42.26 ± 1.28	Z = -1.372	0.170
Ring 4 N1P1 amplitude (nV/deg^2)	25.65 ± 4.55	23.35 ± 6.06	t = 1.714	0.106
Ring 5 N1 latency (ms)	15.10 ± 0.88	15.20 ± 0.69	t = -0.335	0.742
Ring 5 P1 latency (ms)	29.21 ± 1.16	28.78 ± 1.36	t = 1.730	0.103
Ring 5 N2 latency (ms)	42.89 ± 1.07	42.60 ± 1.17	Z = -0.884	0.376
Ring 5 N1P1 amplitude (nV/deg^2)	16.18 ± 3.59	15.18 ± 3.78	Z = -1.071	0.284

Table 7.8: A summary of the change in subjective outcome measures for the HY and HO groups combined between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
VA (logMAR units)	-0.10 ± 0.08	-0.08 ± 0.08	t = -1.466	0.157
CS (log units)	1.88 ± 0.00	1.88 ± 0.00	Z = 0.000	1.000
MPOD (optical density units)	0.43 ± 0.11	0.44 ± 0.14	Z = -0.201	0.840

Table 7.9: A summary of the change in dietary outcomes for the HY and HO groups combined between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	**Visit 4 (mean ± SD)**	**Test statistic**	**p**
Dietary copper (mg)	1.15 ± 0.35	1.17 ± 0.60	Z = -0.674	0.500
Dietary zinc (mg)	9.48 ± 0.91	9.44 ± 2.42	t = 0.034	0.975
Dietary retinol (µg)	352.80 ± 81.42	371.40 ± 98.98	t = -0.365	0.733
Dietary carotene (µg)	1666.80 ± 840.44	2161.20 ± 867.23	t = -.956	0.393
Dietary Vitamin E (mg)	6.43 ± 5.16	5.63 ± 3.27	t = 0.372	0.729
Dietary Vitamin C (mg)	88.00 ± 63.38	91.80 ± 36.91	t = -0.160	0.880
Dietary lutein and zeaxanthin (µg)	1520.69 ± 879.13	1278.07 ± 496.00	t = 1.033	0.360
Dietary Omega 3 (g)	0.22 ± 0.19	0.75 ± 0.44	t = -2.918	0.043

Table 7.10: A summary of the change in each mfERG outcome measure for the ARM group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal. Shaded areas show statistical significance.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
Ring 1 N1 latency (ms)	15.63 ± 1.32	15.49 ± 0.41	Z = -0.594	0.553
Ring 1 P1 latency (ms)	29.86 ± 2.34	30.31 ± 1.26	Z = -0.853	0.394
Ring 1 N2 latency (ms)	43.86 ± 2.04	44.13 ± 2.14	t = -2.084	0.076
Ring 1 N1P1 amplitude (nV/deg^2)	123.00 ± 29.94	107.88 ± 27.38	t = 1.191	0.272
Ring 2 N1 latency (ms)	15.10 ± 1.21	15.52 ± 0.62	Z = -0.750	0.453
Ring 2 P1 latency (ms)	28.23 ± 1.37	29.38 ± 1.88	t = -2.201	0.064
Ring 2 N2 latency (ms)	43.54 ± 1.53	44.04 ± 0.67	t = -0.810	0.444
Ring 2 N1P1 amplitude (nV/deg^2)	47.50 ± 11.26	50.00 ± 7.71	t = -0.514	0.623
Ring 3 N1 latency (ms)	15.42 ± 1.00	15.73 ± 0.94	t = -0.708	0.502
Ring 3 P1 latency (ms)	28.64 ± 1.41	28.75 ± 1.41	Z = -0.677	0.498
Ring 3 N2 latency (ms)	42.61 ± 0.94	43.33 ± 0.77	t = -2.496	0.041
Ring 3 N1P1 amplitude (nV/deg^2)	32.25 ± 8.66	29.63 ± 3.66	t = 1.050	0.329
Ring 4 N1 latency (ms)	15.73 ± 0.83	15.24 ± 0.86	Z = -1.033	0.302
Ring 4 P1 latency (ms)	29.06 ± 0.54	29.90 ± 0.69	Z = -2.414	0.016
Ring 4 N2 latency (ms)	43.13 ± 1.16	43.44 ± 1.21	t = -1.002	0.350
Ring 4 N1P1 amplitude (nV/deg^2)	22.00 ± 6.02	20.75 ± 3.20	t = 0.610	0.561
Ring 5 N1 latency (ms)	16.04 ± 0.86	16.00 ± 0.71	Z = -0.141	0.888
Ring 5 P1 latency (ms)	30.00 ± 1.18	29.90 ± 1.13	t = 0.160	0.878
Ring 5 N2 latency (ms)	43.75 ± 1.09	43.65 ± 0.99	t = 0.206	0.842
Ring 5 N1P1 amplitude (nV/deg^2)	12.63 ± 3.66	12.88 ± 2.36	t = -0.251	0.809

Table 7.11: A summary of the change in subjective outcome measures for the ARM group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
VA (logMAR units)	-0.10 ± 0.08	-0.08 ± 0.08	t = -0.060	0.954
CS (log units)	1.80 ± 0.16	1.73 ± 0.14	Z = -0.980	0.327
MPOD (optical density units)	0.40 ± 0.24	0.47 ± 0.20	t = -0.606	0.567

Table 7.12: A summary of the change in dietary outcomes for the ARM group between visit 3 (40 weeks) and visit 4 (60 weeks) during supplement withdrawal.

	Visit 3 (mean ± SD)	Visit 4 (mean ± SD)	Test statistic	p
Dietary copper (mg)	2.58 ± 1.89	0.91 ± 0.23	Z = -1.069	0.285
Dietary zinc (mg)	7.93 ± 3.41	9.40 ± 2.15	t = -1.257	0.336
Dietary retinol (µg)	3309.33 ± 3001.13	260.33 ± 59.00	t = 1.779	0.217
Dietary carotene (µg)	3200.33 ± 2149.15	2607.33 ± 851.27	t = 0.635	0.591
Dietary Vitamin E (mg)	5.20 ± 0.87	4.36 ± 1.65	t = 0.973	0.433
Dietary Vitamin C (mg)	176.00 ± 19.67	149.33 ± 43.02	t = 1.648	0.241
Dietary lutein and zeaxanthin (µg)	2840.22 ± 2332.53	2304.62 ± 1247.67	t = 0.435	0.706
Dietary Omega 3 (g)	0.07 ± 0.05	0.03 ± 0.06	Z = -0.816	0.414