THE ASSESSMENT OF PAEDIATRIC MACULAR PIGMENT OPTICAL DENSITY

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Abstract

Purpose: Dry age related macular degeneration (AMD) is the UK's leading cause of visual loss. Dry AMD accounts for approximately 90% of the 600,000 people affected by AMD in the UK. Macular pigment (MP) is believed to play a significant role in mitigating the onset and progression of AMD. Data available for paediatric MP is lacking, particularly in western ethnic populations.

Method: This thesis set out to investigate macular pigment optical density (MPOD) at the early stage of life, to assist in understanding the evolution of MPOD in the human life span. The Visucam 200 (an objective technique) and the MPS 9000 (a subjective technique) were used to collect data on subjects aged 4-16 years.

Results: The Visucam, a device which measures MPOD with an automated technique, produced consistently more repeatable measures than those recorded with the MPS. Paediatric MPOD was not significantly correlated with age, iris colour, refractive error or gender. Assessment of paediatric MPOD with the MPS did not show any correlation to lutein levels recorded in a three day diet history. Paediatric mean MPOD was found to be, Visucam 0.40 (SD 0.08) and MPS 0.43 (SD 0.13).

Conclusions: Paediatric MPOD as determined by objective and subjective measures yield similar values. The mean MPOD value was similar to adult MPOD levels as determined by previous adult based MPOD studies. This could infer MPOD levels develop to adult levels prior to the age of five years.

Key words: Coefficient of repeatability, Correlation coefficient, macular pigment optical density, MPS, Visucam.

Dedication

This doctorate is dedicated to my patients, colleagues, friends, late Granny (blinded by macular degeneration) and Professor Frank Eperjesi whose unswerving guidance was invaluable.

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Abbreviations:

AF	Autofluorescence
ARMD/AMD	Age-related macular degeneration
ARM	Age-related maculopathy
CC	Correlation coefficient
CoR	Coefficient of repeatability
dB	Decibels
FR	Fundus reflectometry
HFP	Heterochromatic flicker photometry
HRA	Heidelberg retinal angiograph
L	Lutein
Mcg	Micro grams
MP	Macular pigment
MPOD	Macular pigment optical density
MZ	Meso zeaxanthin
nm	Nanometers
OCT	Optical coherence tomography
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RRS	Resonance Raman spectroscopy
SD	Standard deviation
VA	Visual acuity
VEP	Visual evoked potential
Z	Zeaxanthin

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CHAPTER 1 - Definition, history and clinical relevance of macular pigment optical density

Overview

In this chapter macular pigment optical density (MPOD) will be defined with consideration of its anatomical and molecular basis. The history of MPOD and an introduction to MPOD assessment techniques will be given. An overview of previous MPOD studies will be discussed and summarised. The chapter will conclude with an outline of the current studies' objectives.

1.1
Introduction

Macular pigment optical density has become an increasingly popular area of research and investigation amongst optometrists, ophthalmologists and scientists over the past three decades (Snodderly, 1984; Bone and Landrum, 1985). Macular pigment optical density represents the unit term given as a numerical value of between 0-1.0. Higher/more dense levels of MPOD are represented by a higher numerical value.

The simplest psychophysical technique for measuring MPOD involves determining the detection threshold for foveal and parafoveal stimuli using one or more wavelengths absorbed by the MP and one or more not absorbed. The optical density (OD) or absorbance is calculated:

$A\lambda s = \text{Log}\ (Rf\lambda s/Rp\lambda\ 1) - \text{Log}\ (Rf1/Rp1)$

Where
A is absorbance,
R is radiance of the detection threshold
λs refers to a SW light in the absorption band of MP, e.g., the peak at 460 nm,
λ1 refers to a LW light outside the absorption band of MP, e.g., 540 nm
f refers to a retinal locus containing a significant concentration of MP, and
p refers to a parafoveal locus containing zero, or nearly so, MPOD
(Hammond et al. 2005)

Understanding the molecular and cellular basis of diseases is vital for dissecting the mechanisms of disease pathogenesis and for designing appropriate and effective treatments (Grover, 2013). Prior to discussing the history of MPOD the anatomy, physiology and chemistry underpinning MPOD will be outlined.

Definition of MPOD

Macular pigment (MP) is a yellow, oily substance located in the fibres of Henlé and the outer segment membranes of photoreceptors in the macular region of the human retina. The MP has some remarkable characteristics; it is uniquely located to protect the photoreceptors from damaging short wave light, it aids visual resolution by reducing chromatic aberrations and, like many carotenoids, acts as a powerful anti-oxidant (Murray, 2008). This combination of properties has resulted in research being undertaken to assess whether MP may play a role in protecting the eye from macular disease. There is increasing evidence to support this (Bone *et al.*1985; Beatty *et al.* 2001, and Koh *et al.*, 2004). This evidence will be considered in this and the following chapter.

The chemical and molecular basis of MPOD

There are two main classes of naturally occurring carotenoids: carotenes such as β-carotene and α-carotene - both hydrocarbons - are linear or cyclized at one or both ends of the molecule. Linear hydrocarbons are linear molecular structures in which each carbon atom is bonded to two other carbons atoms, in a linear arrangement, except for the terminal carbon which is only bonded to one other carbon atom. A cyclic hydrocarbon is a hydrocarbon in which the carbon chain joins to itself in a ring. The second main class of carotenoids are xanthophylls, the oxygenated derivatives of carotenes. All xanthophylls produced by higher plants, such as violaxanthin, antheraxanthin, zeaxanthin (Z), neoxanthin, and lutein (L) are also synthesized by green algae (Eon Seon *et al.* 2003; Sajilata *et al.* 2008). Macular pigment cannot be manufactured by the body. In humans and primates its origin is entirely diet derived. Sommerburg *et al.* (1998) aimed to determine which foodstuffs might be useful as dietary supplements. Dark green leafy vegetables, such as Brussels sprouts, spinach, broccoli and lettuce are a good source of L but contain very little, if any, Z (Viner, 2005). Naturally yellow/orange coloured foodstuffs (with the exception of pumpkins and carrots) contain more Z than their green counterparts (Viner, 2005). Landrum *et al.* (1997a) and Edwards *et al.* (1997) have demonstrated that alterations to diet can have an effect on the levels of MP, with potential enhancement of MP levels. Goji berries diet

supplementation (14g/day) have been shown to reduce soft drusen formation over a 90 day study period, compared to a control group (Bucheli *et al.* 2011).

The xanthophyll Z is an important carotenoid within the xanthophyll cycle. Synthesized in plants and some micro-organisms, it is the pigment that gives many plants their colour. Lutein is the dominant carotenoid in the peripheral macula, Z in the mid-peripheral macula, and meso zeaxanthin (MZ) at the epicentre of the macula (Bone *et al.* 1997). Meso zeaxanthin is a stereo isomer of L and the conversion of L into MZ requires a shift of one carbon-carbon double bond in the ε-ring of L (Bone *et al.* 2007b). The ε-ring of L is the 5th methyl (hydrocarbon) group. Xanthophylls such as L are found in highest quantity in the leaves of most green plants, where they act to modulate light energy. In humans, photo-oxidative damage affects cellular lipids, proteins and DNA and is involved in the patho-biochemistry of erythema formation, premature aging of the skin, development of photodermatoses and skin cancer. Evidence shows that β-carotene and L , can prevent UV-induced erythema formation and contribute to life-long protection against exposure to harmful effects of sunlight (Stahl and Sies, 2007).

Analysis of the chemical formula of Z reveals a long chain molecule:

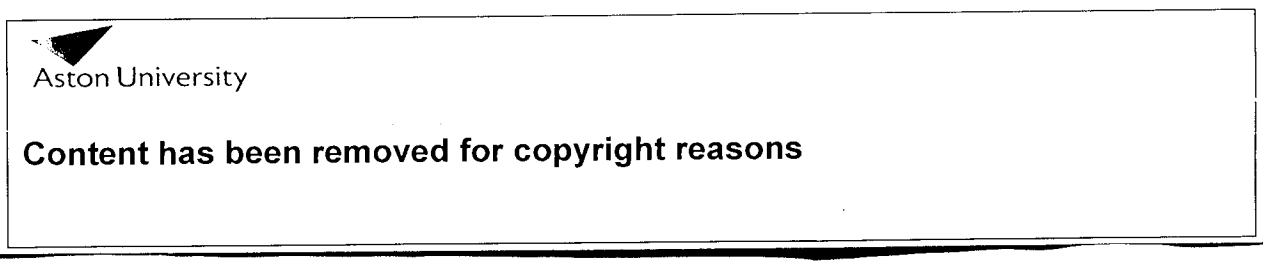


Figure 1.1 –Z long chain molecule, one double stranded bond in each ring (from http://areds-2.com/carotenoids.html)

Compounds that have the same molecular formula but different chemical structures are isomers. Stereoisomers have the same functional groups and connectivities, they differ only in the arrangement of atoms and bonds in space. Lutein is very similar to Z but the double bond location is different in one of the end rings. Lutein and Z have identical chemical formulas and are isomers, but not stereoisomers.

Figure 1.2 – Lutein – single stranded rings (source: http://areds-2.com/carotenoids.html)

Figure 1.2 shows this difference in double bond position gives L three chiral centers whereas Z has two. A chiral center is characterised by an atom that has four different groups bonded to it in such a manner that it has a non-superimposable mirror image (Hunt, 2015). Because of symmetry, the (3R,3'S) and (3S,3'R) stereoisomers of Z are identical. Therefore, Z has only three stereoisomeric forms. The (3R,3'S) stereoisomer is MZ.

The ratio of L to Z and MZ within 0.25 mm of the fovea is approximately 1: 2.4 (Bone *et al.,* 1988) but this reverses at the retinal periphery, where the ratio is 2: 1 (Bone et al., 1988). There is a 100-fold drop in the concentration of MP in the peripheral retina compared with the fovea, although levels vary considerably between individuals (Bone *et al.,* 1985).

1.12

Oxidative stresses of MPOD

During the development of AMD, high levels of oxidative stress - a harmful state defined by the presence of pathologic levels of reactive oxygen species (ROS) relative to the antioxidant defence, have been stated to play a role (Beatty *et al.* 2000b; Khandhadia *et al.* 2010). Reactive oxygen species are highly reactive ions and "free radicals" (chemicals containing atoms with an unpaired electron in its outer orbit) which involve oxygen molecules. Free radicals are present that do not contain oxygen, but ROS refers to free radicals containing oxygen molecules. They are characterised as short lived, unstable and react with other molecules to achieve stability. Environmental stresses lead to excessive production of ROS causing progressive oxidative damage and ultimately cell death (Sharma *et al.* 2012).

In healthy subjects, local retinal protection against free radicals is aided by the presence of MP, which is comprised of the carotenoid L and its isomers Z (Bone *et al.*1985) and MZ (Khachik *et al.* 2002). It has been suggested that these xanthophylls play a similar role in humans as in plants—i.e. as antioxidants and screeners of high-energy blue light (Krinsky *et*

al. 2002). In addition, the discovery that L and Z have been found in higher concentrations in the rod outer segments of the perifoveal retina, than the peripheral retina lends support to their proposed protective role against AMD (Rapp *et al.* 2000). These carotenoids are able toextinguish singlet oxygen - a potent oxidant - (Krinsky *et al.*1979) scavenge ROS,(di Mascio *et al.*1989) limit the peroxidation of membrane phospholipids (Lim *et al.*1992) and reduce lipofuscin formation (Sundelin *et al.* 2001; Qin *et al.* 2011). Lipofuscin is primarily composed of cross-linked protein residues, formed due to iron-catalysed oxidative processes. Since it is undegradable and cannot be removed via exocytosis, lipofuscin accumulation in postmitotic cells is inevitable, whereas proliferative cells efficiently dilute it during division. Lipofuscin accumulation may also diminish autophagocytotic capacity by acting as a sink for newly produced lysosomal enzymes and, therefore, interfere with recycling of cellular components. Lipofuscin, thus, may be directly related to cellular degeneration in old age (Brunk *et al*, 2002).

The pathogenesis of AMD relates to the disturbance of the relation between the photoreceptors and the nourishing tissues beneath the retina. Current thinking in the mechanisms underpinning AMD are represented in figure 1.3:

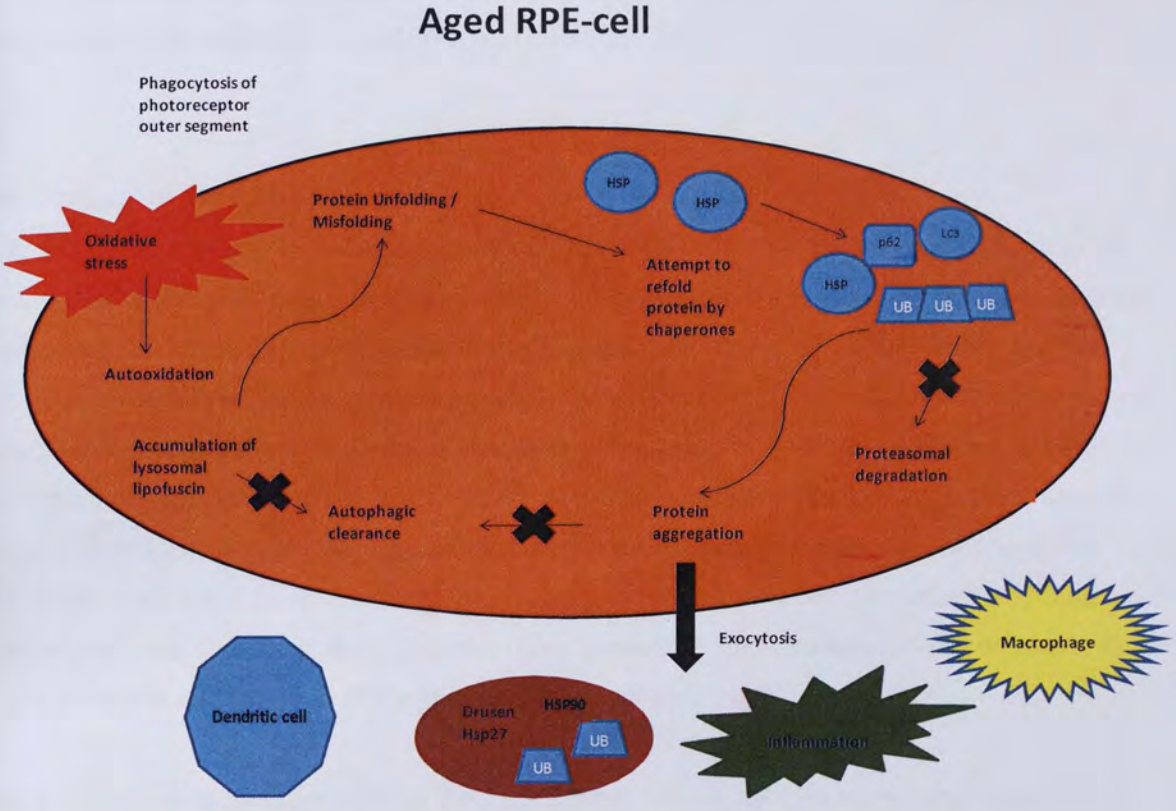


Figure 1.3, Schematic representation of protein aggregation in aged retinal pigment epithelial cells (RPE). HSP = Heat Sensitive Protein, P62= a multifunctional protein adapter -role in cell signalling, LC3 = Protein Light Chain 3, UB = UltraViolet B irradiation (Adapted from Kinunnen *et al.* 2012).

With reference to figure 1.3, RPE cells digest retinal outer-segment discs that are endocytosed and fused with lysosomes to be degraded. In aged RPE cells, lysosomal degradation is impaired resulting in accumulation of lipofuscin. The lipofuscin is an auto-oxidant material which inreases oxidative stress and protein damage in RPE cells. Heat sensitive proteins (HSP) attempt to repair protein damage, but this process is disturbed or weakened in aged cells. Simultaneously, proteasomal and autophagy protein clearance systems are not working as effectively as in young RPE cells. Proteins are most likely moved via exocytosis to the outside of the RPE cells. This material is considred to be involved in drusen formation together with chronic inflammation and inflammatory cells (Kinnunen *et al.*

2012). It is this drusen formation and collection of waste products within the macula which represents the antamocial and pathological basis of AMD.

1.2

The history of MPOD

Early work with monkey models heralded the critical thinking that MPOD and AMD may be significantly related. Macaque monkeys raised on xanthophyll-free diets were characterised by an absence of yellow pigmentation in the macula (Malinow *et al.*1980). These animals also developed more intense hyperfluorescence in fluorescein angiography than observed in controls fed a normal diet, and a more frequent occurrence of drusen-like bodies – the precursors of AMD (Malinow *et al.* 1980). The researchers were unclear as to the cause of the hyperflorescence, but postulated that the phenomenon arose from alterations at the RPE level. They went on to conclude that there was merit in the use of nonhuman primates for examining the significance of MP and, more generally, for investigating the effects of diet on the structure and function of the macula (Malinow *et al.* 1980).

Research gained momentum during the 1980s when measurement techniques explored varying methods of assessing MPOD. One such technique employed high performance liquid chromatography (HPLC) to determine the distribution of L and Z, the two major components of the MP (Bone *et al.* 1988). Snodderly (1995) concluded that carotenoids and antioxidant vitamins retarded some of the destructive processes in the retina and RPE that lead to AMD. Landrum and Bone (1997b) assesed the ability for MP to reduce the risk for individuals suffering from AMD. A study of autopsy eyes found 30% lower concentrations of L and Z in retinas with AMD compared to controls (Landrum *et al.* 2001). In two previous studies, these carotenoids had also been found to be less abundant in the serum and diet of those with neovascular AMD (Seddon *et al.*1994. However Mares-Perlman *et al.* (1995), found no relationship between serum L/Z and AMD. Further evidence of protection by the MP is found in studies of photopic injury during ophthalmic surgery (Jaffe *et al.* 1988; Michels *et al.*1992), in a study of central sparing in annular macular degeneration (Weiter *et al.* 1988), and in studies of age-related loss of short wavelength cone sensitivity (Hagerstrom-Portnoy, 1988).

These studies were followed up by Edwards *et al.* (1997) who supplemented diets with spinach and/or corn sources of L and Z. The average MPOD showed nearly a 20 % increase after four weeks of supplementation, followed by a decrease to baseline and another increase at 12 weeks. The researchers were unclear as to the fluctuation in subjects MPOD

levels but determined three types of responses to dietary supplementation: 1) Nonresponders in blood and retina 2) responders in blood but not retina and 3) responders in blood and retina. Landrum and Bone (1997b) investigated supplementation with L in two subjects over a 140 day period and found increases in MPOD of 39% and 21 %. The study used subjective flicker photometry, meaning all MPOD data provided was acquired via a subject psychometric testing procedure. The significant limitation of this study was that just two subjects were studied, so no statistical analysis could be applied.

1.21

MPOD and its relation to AMD

The terms age-related maculopathy (ARM) and age-related macular degeneration (AMD), were defined by the International ARM Epidemiological Study Group and a uniform grading system put forward (Bird *et al.* 1995). The term ARM is used to encompass all signs of age-related changes at the macula. It is characterised by any of the following: soft drusen and areas of hypo/hyperpigmentation associated with drusen. In advanced stages of ARM the term AMD is used. This encompasses wet and dry types. Choroidal neovascularisation, subretinal haemorrhage and RPE detachment are the defining criteria of wet AMD while dry AMD refers to geographic atrophy (Bird *et al.* 1995). In AMD the RPE cells are either incapable of mitosis (Fleming et al., 1996) or divide very slowly (Al-Hussaini et al., 2008) and therefore do not significantly regenerate so any damage is permanent. The relationship between MPOD and advancing AMD is shown in figure A1.1 (Appendix 1).

The study of MPOD in optometry and ophthalmology focusses on lower MPOD numerical values as a risk factor in AMD (Raman *et al.* 2012). Monitoring MPOD requires an understanding of how MPOD develops within the earliest measurable stage of life. Such data will assist in understanding the life cycle history of MPOD, and enable clinicians to better understand to what extent MPOD is genetically determined and which lifestyle factors may influence MPOD. This level of understanding may aid in the development of treatments that increase MPOD and negate risk factors for developing AMD. There is currently varying opinion as to whether MP degrades with age (Berendschot *et al.* 2005). In the absence of any other ocular or neurological pathology, MPOD at the youngest age of life may not correspond to MPOD at the advanced stages of life (Nolan *et al.* 2010). The majority of previous MPOD studies have used adult subjects (Nolan *et al.* 2007) who may or may not have been previously diagnosed with AMD (Kanis *et al.* 2007). To date, there has been very little research assessing MPOD at the early stage of life in infants and juveniles.

1.3

Introduction to MPOD assessment techniques

There are a number of methods which can be employed to assess MPOD. These fall into objective and subjective techniques. Objective methods include fundus reflectometry (FR) and autofluorescence (AF) whilst subjective methods include heterochromatic flicker photometry (HFP) and colour matching.

Heterochromatic flicker photometry

In conventional HFP, the participant eliminates flicker in a visual stimulus which alternates between two wavelengths, by altering the luminance of one of the wavelengths present. The use of HFP to measure MP levels was first described 35 years ago by Werner and Wooten (1979) but the technique was not fully implemented until 1987, in a paper by Werner et al. (1987). The principle underpinning HFP is through spectral absorption properties and retinal location of MP. In HFP the MPOD is calculated by initiating a light stimulus of two alternating wavelengths at the fovea and parafoveal location. One is a short wavelength (SW) blue light that is maximally absorbed by MP and the other is a long wavelength (LW) green /yellow light that is not absorbed by MP (Snodderly et al. 2004). If the colours are alternated at an appropriate frequency and the luminance of the two colours is not perceived to be equal by the subject, the stimulus will appear to flicker. The perceived colour of this light will be an amalgamation of the two source colours (Beatty et al. 2000a, Snodderly et al. 2004). The radiance (intensity) of the blue light is adjusted by the subject until the flicker is minimized (Tang et al. 2004; Stringham et al. 2008). This occurs when there is an equal luminance match between the blue and green lights (Loane et al. 2007; Kirby et al. 2009). The procedure is then repeated at a parafoveal locus where MP is minimal. More blue light will be absorbed by MP at the fovea than the parafovea, thus a higher radiance of blue light will be required at the fovea to perceive minimal flicker. It is the log ratio of the radiance of blue light stimulated at the fovea compared with that required at the parafovea which gives the assessment of maximal MPOD.

Colour matching

Colour matching of appropriate monochromatic stimuli is another psychophysical based technique for measuring MPOD which generates spectral absorption curves that match the extinction spectrum of xanthophylls. However, colour matching has not evolved as a

technique for measuring MPOD, and few instruments are available with this method (Beatty *et al.* 2008).

Fundus reflectometry

Fundus reflectometry is an objective technique that relates to the quantitative assessment of the amount of light reflected from the fundus, to give an MPOD value. This can be subdivided into one and two wavelength techniques. Of the two wavelength techniques, scanning laser ophthalmoscopy (SLO) has been used, which yields images at two wavelengths: one well absorbed (488 nm) by MPOD and one minimally absorbed (514 nm) (Berendschot, 2004). Dual-pass images are generated and digital subtraction of one of these two images provides an MPOD map from which the spatial profile can be obtained. However, measuring MPOD with SLO requires pupillary dilation, costly equipment, and technical support.

Autofluorescence (AF)

The AF approach exploits the fluorescent properties of lipofuscin within the RPE (Delori, 2004). Stimulation of the fluorescence above 550 nm, where MP has near zero absorption, provides a single-pass measurement of MPOD (Beatty *et al.* 2008). This technique also requires expensive specialist equipment, pupillary dilation, and technical support.

Dual-wavelength methods of AF can be used to assess MPOD. The AF method compares results from the region of maximum MPOD overlying the fovea to an area with no optically appreciable MP several degrees eccentric to the fovea, using two excitation wavelengths that are differentially absorbed by the MP (488-nm, well-absorbed, and 512 nm, minimally absorbed) (Lima *et al.* 2010). The fluorophore is the fluorescent chemical compound that can re-emit light upon light excitation. The sample is excited sequentially at these two wavelengths: one to optimally excite the fluorophore, along with the autofluorescent components of the cell — and the other principally to excite only the autofluorescence (Knight *et al.* 2001). The difference between the two fluorescence measurements obtained from illumination at the respective wavelengths is then equal to the fluorescence of MPOD.

A study using dual-wavelength analysis of AF images in 369 subjects demonstrated that ring like structures in the MP spatial profile are fairly common, show a high degree of bilaterality, and appeared inversely related with AMD (Dietzel *et al.* 2011b). A ring-like structure of MP was observed in 73 (19.8%) study subjects. The MP maximum of the ring was located on average at 0.85° and the minimum at 0.48° from the centre of the fovea.

Ring like structures were significantly more common in females and never smokers and were found significantly less often in eyes with AMD than in healthy eyes. This was determined even after adjustment for influential factors such as smoking and lifestyle (adjusted odds ratio, 0.347; 95% confidence interval, 0.196 –0.617). The authors requested future longitudinal studies investigate how the spatial distribution of MPOD changes individually over the course of time. These longitudinal studies could incorporate high-resolution spectral domain OCT to better understand whether changes in the spatial distribution of MP are the sequelae of developing AMD or whether the MP ring-like spatial distribution is protective against AMD (Dietzel *et al.* 2011b).

The details of MPOD acquisition techniques will be expanded upon in Chapter 2.

Much of the previous research undertaken into MPOD has used HFP mainly with adult subjects. A summary of previous research findings is given in Table 1.1

Study and year	Sample Size	Age Range(y)	Mean MPOD (SD)
Hammond & Caruso-Avery, 2000	217	17-92	0.22 (0.13)
Johnson et al, 2000	7	33-54	0.4 (0.05)
Iannoccone et al, 2007	183	69-86	0.34 (0.21)
Howells et al, 2013	169	18-30	0.43 (0.14)
Loane et al, 2007	121	20-70	0.39
Nolan et al, 2010	4373	50-93	0.20 (0.15)
Raman et al, 2011	300	20-39 30-39 40-49 50-59 60+	0.39 0.42 0.40 0.36 0.27
Yu et al, 2012	281	17-85	0.49 (0.18)
Zhu et al, 2012	94	6-12	0.56 (0.25)
McCorkle et al, 2013	66	7-10	0.62 (0.27)
Ciulla et al, 2001	280	18-50	0.21 (0.13)
Loughman et al, 2012 (MPS)	39	21-61	0.32 (0.16)
Loughman et al, 2012 (Densitometer)	39	21-61	0.40 (0.16)
Snodderly et al, 2004	54	50-79	0.42 (0.22)
Wooten et al, 1999	30	16-60	0.27 (0.15)

Table 1.1

A summary of previous research findings with HFP

Table 1.1 shows a vast range of previous MPOD data over the past 15 years with mean MPOD findings from 0.20 (SD 0.15) in Nolan et al's study (2010) up to 0.62 (SD 0.27) in McCorkle et al (2013) review of subjects aged 7-10 years. Many of the studies are small scale with sample sizes less than 50. Age ranges are typically between 17-89 years with numerous studies assessing MPOD between 50-89 years. This is a reasonable age range that relates to the typical age of onset of AMD, reported by the Framingham Eye study, which revealed that AMD affected 2% of Americans aged 52-64 years: 11% aged 65-74 years; and 28% aged 75 years and older (Leibowitz *et al.* 1980).

1.4

Conclusions

Research on MPOD has progressed from determining its existence in the 1980s, to techniques of measuring it in donor eyes, measuring it subjectively in human subjects in the late 1990s and new millennium and moving forwards to assessing it objectively via single/dual wavelength reflectometry and AF. An understanding of the structure and morphology of MP may assist in our understanding of drusen formation and this may lead to more successful AMD intervention strategies in the future.

Previous HFP MPOD studies in adults have given a range of values between 0.21 and 0.48, but sample sizes in these studies were usually small (less than 50) and age ranges tended to be wide for each study, or in mid-late adulthood. None of these studies have reported on MPOD in the age group below 17 years.

The aim of the current study is to provide objective paediatric MPOD in Studies' 1 and 2, and subjective paediatric MPOD in Studies' 3 and 4. Studies' 1 and 2 will use the Visucam. Studies' 3 and 4 will use the MPS.

In the next chapter, a review of the MPOD literature in children is reported. Further consideration will be given to the assessment of MPOD using FR techniques with details of alternative techniques, along with the study rationale.

CHAPTER 2- Literature review

This chapter comprises a review of the current literature relating to MPOD assessment techniques, in particular the use of FR techniques and specifically the Visucam. A detailed review of HFP and the MPS will also be provided and an outline of alternative MPOD assessment techniques. Studies which have previously documented MPOD levels in children will be reviewed. Concluding this chapter, will be the study rationale where the clinical and research indications for the current four studies of this thesis will be given.

2.1
MPOD assessment techniques

The assessment of MPOD can be broadly divided into two clinical methods- objective (less subject dependent) and subjective (subject dependent). The objective techniques for measuring MPOD are: fundus reflectometry (FR), autofluorescence (AF), resonance Raman spectroscopy (RRS) and electrophysiology using visual evoked potentials (VEP).
Of the subjective techniques, psychophysics in the form of HFP dominates. Psychophysics has been described as the scientific study of the relation between stimulus and sensation (Gescheider, 1997).

Objective techniques

MPOD assessment using Fundus Reflectometry
Quantitative measurement of light reflected from the fundus is known as FR, and Brindley and Wilmer (1952) were the first to adopt this technique. Their aim was to estimate MPOD *in vivo* by comparing light reflected at the macula with light reflected from a peripheral area of retina. Since then, FR has evolved to become the most widely used of the objective methods for MPOD measurement and refinements in techniques have facilitated more accurate MPOD assessment. Eisner *et al.* (1992) were the first to use a scanning laser ophthalmoscope (SLO) for the purpose of measuring MPOD. Subsequently it has become a popular FR method for measuring MP. One of the main advantages of using SLO over other FR techniques is its confocal optics, which help minimize stray light scatter.

The commonly noted assumptions for FR are:

Homogeneity of fundus tissues - The spectral characteristics, absorption, reflection and scattering properties of the various retinal tissues are assumed to be homogenous for the areas being assessed. Gellermann *et al.* (2004) have indicated this is a simplification. However, other researchers (Van der kraats *et al.* 2006) do not consider this to be a problem. Delori *et al.* (2001) concluded irregular RPE melanin distribution had no significant effect on MPOD as measured with their FR technique.

Bleaching of photoreceptor pigments - It has been established that 93–99% of cone photopigment and 59-85% of rod photopigment is bleached as a result of the level of illumination used prior to measurement, depending on the particular FR method (Chen *et al.* 2001; Delori *et al.* 2001; Bour *et al.* 2002; Van de Kraats *et al.* 2006). Bleaching is important in order to avoid light absorption by the pigments and their subsequent interference with MPOD. It is assumed that any remaining unbleached photopigment, particularly rhodopsin, has a minimal effect. This has been investigated by Chen *et al.* (2001), Delori *et al.* (2001) and Bour *et al.* (2002), and proven to be the case. In the case of the Visucam, Zeiss Germany (Technical Support) indicated that the instrument does not require the process of photopigment bleaching for MPOD acquisition.

Light scatter - Previous research has reported that if reflectance from pre- retinal and intra-retinal structures is not controlled for, the measured MPOD can be artificially low (Delori *et al.* 2001; Berendschot and Van Norren, 2004; Berendschot and Van Norren, 2005). This is because the reflectance method works on the principle that the entire incident light is reflected after passing through the MP. If some light is reflected before it reaches the MP, e.g. by the crystalline lens, then this will be collected as reflected light, but it will not actually have been affected by MP absorption, with a resultant misleading low MPOD (Wüstemeyer *et al.* 2002). Most FR devices aim to eliminate this problem although this could account for the low MPOD values in some studies (e.g. Bour *et al.* 2002; Wüstemeyer *et al.* 2003). Methods used to counteract this problem include separating the entrance and exit pupils, using confocal optics as found in SLOs, and the incorporation of stray light into optical models. In addition, for the comparison technique of FR, the use of a peripheral reference should account for crystalline lens scatter (Delori *et al.* 2001). This issue will not be a concern in the current studies of this thesis, as the presence of ophthalmic disease was one of the exclusion criteria.

The MPOD measured over the entire stimulus area - With psychophysical methods there is some variability regarding which part of the MP distribution is actually being

measured with the test stimulus. In most HFP studies MPOD is measured at interval points between 0-8 degrees from the fovea centralis. With FR there is a general consensus that the MPOD is the mean amount over the chosen detection field (Berendschot *et al.* 2000, Delori *et al.* 2001; Wüstemeyer *et al.* 2002; Berendschot and Van Norren, 2005). However, this assumption has not been verified.

FR - advantages and disadvantages

Advantages

1) Objective method
2) Quick measurement
3) Density maps of MP distribution can be plotted quickly
4) Reliability is good
5) It is suitable for children

Disadvantages

1) Pupil dilation may be required
2) Precise alignment is required
3) Control for light scatter, which can include considerable modelling
4) Costly and complicated instruments, although attempts have been made to produce less expensive reflectometers

With respect to reliability, Zagers *et al.* (2002) documented that FR variability in an intra-session MPOD study was the result of fixation errors, with less experienced subjects showing greater variability. Snodderly and colleagues (2004) indicated that intersession reliability is more valuable than intra-session reliability. Since results generally show higher variability between sessions, this is a more robust test for an instrument and was used in Study 1 of this thesis, where the repeatability of the Visucam was assessed. This in turn, should provide a more robust platform to present the results of Study 2 of this thesis.

In a review of MPOD assessment techniques, Howells *et al.* (2011) recommended using objective techniques based on FR or AF but conceded that commercial instruments capable of this were not available. Howells *et al.* reported that the development of such an instrument would aid research in this area and provide a better understanding of the relationship between MPOD and AMD, as well as supporting MPOD screening in optometric practice. Within just a few months of their recommendation the Zeiss Visucam became available for use in a clinical setting. The objective nature of the MPOD assessment with the Visucam

made it potentially suitable for use with children. This could allow the measurement of MPOD at an early stage of life and further the understanding of the natural history of MPOD through the human life cycle.

Objective techniques in the assessment of MPOD – The Visucam

Bartlett and Eperjesi (2010a) commented that the measurement of MPOD in the clinical environment may become more feasible with the clinical availability of objective techniques. The Visucam is such an objective technique and the repeatability of this technology was assessed in Study 1 of this thesis. Further, the objectivity of the measurement should allow better comparison of measurement of MPOD with other objective assessments. This is desirable as current MPOD assessment techniques are varied. Subjective techniques are dependent on the proficiency of whoever administers the test and the ability of the subject to respond accordingly.

Instrumentation: Visucam 200

In Studies' 1 and 2, a Visucam 200 was used to measure MPOD.

The Visucam® 200 (Carl Zeiss Ltd., Cambridge, UK) features a non-mydriatic colour fundus camera which allows imaging through a variable pupil aperture of minimum 3.3 mm. Stereo image handling and assessment of MPOD are combined with auto functions that allow reproducible fundus imaging. The Visucam 500 is the other Visucam in the range. This has an additional autoflourescence feature but the measurement of MPOD is identical.

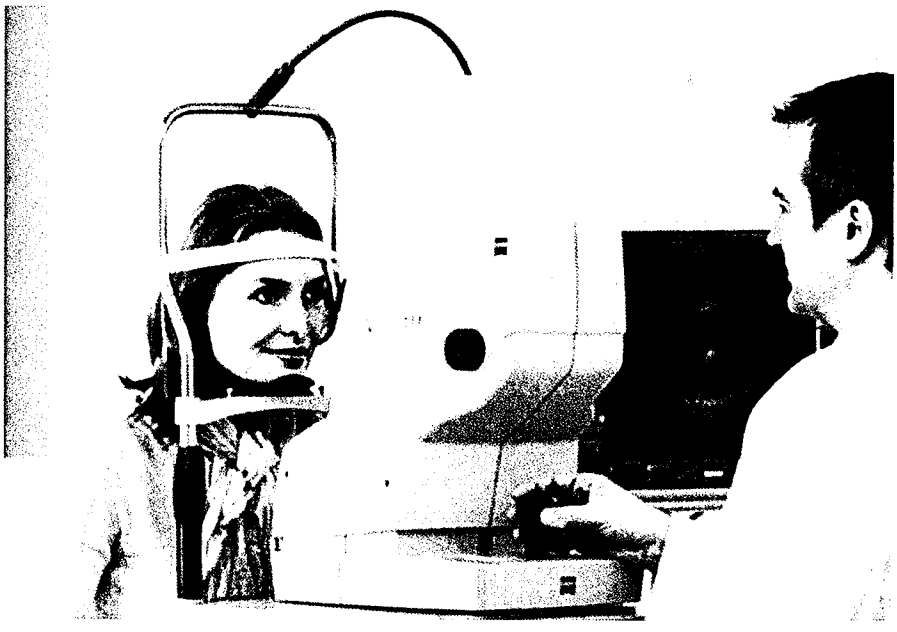


Figure 2.1: The Zeiss Visucam 200

Source: http://www.gemioptal.cz/overena-kvalita/

The MPOD is determined objectively by one-wavelength reflectometry. The basis for this determination is a 30° digital fundus image of the macula captured with blue light (460 nm). Measurement of MPOD occurs within a 7° radius of the fovea and software assesses the MPOD profile and provides a maximal MPOD measure from the profile, which is typically 1-2° from the fovea. This makes it difficult to compare to other MPOD techniques which measure MPOD within a set distance (e.g. 0.5 degrees) from the central macular.

Zeiss UK and Zeiss Germany declared no commercial interest in the study.

2.2

Principle of Visucam reflectometry

A standard retinal camera was used to capture the reflected light - Reflected paramacular (Rp) and Reflected macular (Rm). The MPOD and its profile are then calculated with a software algorithm. The principle of a one-wavelength method is that areas without structures (like vessels) are used as control points for calculating the MPOD profile. This is determined using a sophisticated algorithm function which automatically compensates for fundus reflectance at peripheral locations with the calculated measure as a reflection value per pixel. The optional MPOD module for the Visucam used the reflectance of a single blue 460-nm wavelength based on a single blue-reflection fundus image to determine MPOD and its spatial distribution. The subject was positioned in front of the fundus camera and instructed to look at a target inside. The fundus was illuminated by monochromatic blue light.

Figure 2.2: The basic optical principles underpinning the Visucam's calculation of MPOD (Source, Zeiss Germany)

The results consist of four parameters

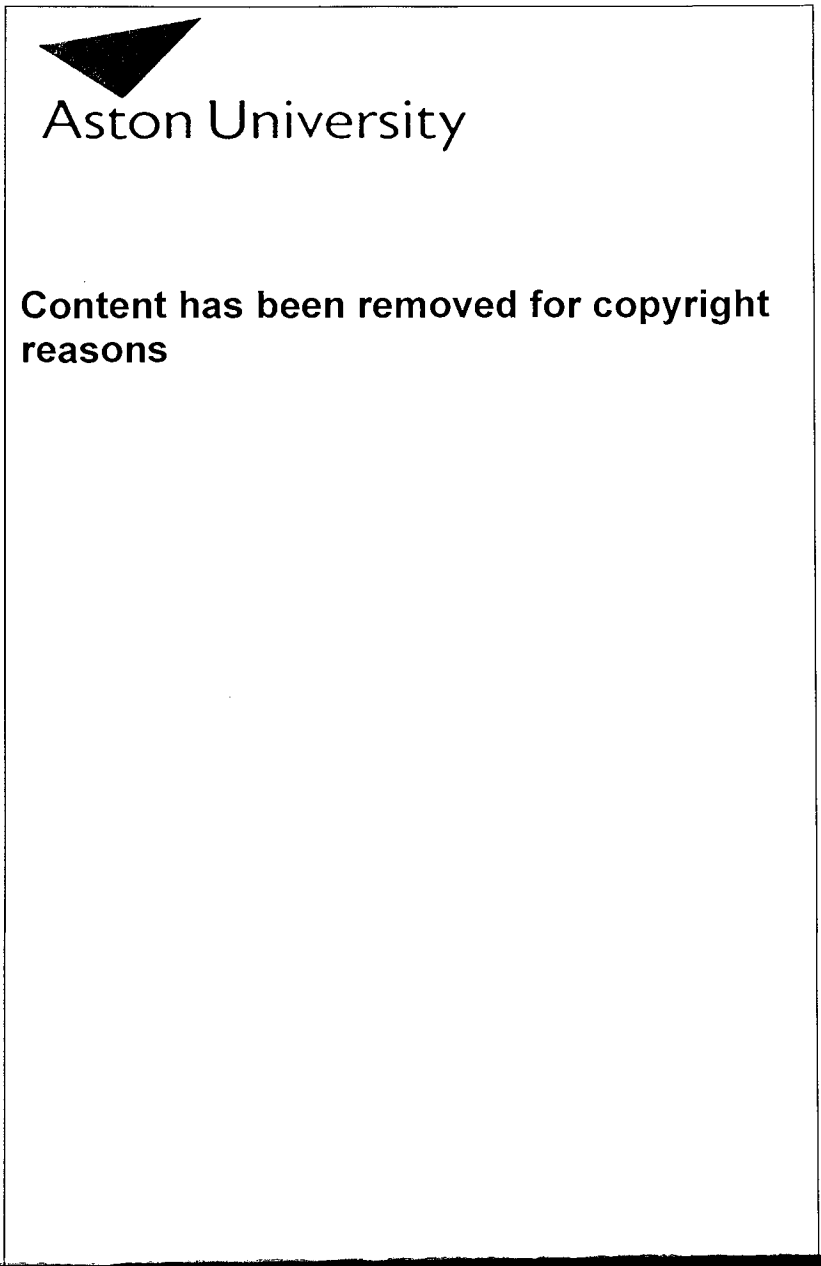
Max OD - maximum optical density, measured at 1-2° from the fovea (Zeiss, Germany – personal communication)

Mean OD - mean optical density (over entire 7 ° area of MP)

Area - where the MP could be detected- approximately 7 ° radius

Volume - the sum of all optical densities.

An example image of a typical Visucam image is shown in figure 2.3



In Study 1 and 2 of this thesis, the single right eye Max OD measure was the only measure taken (see upper point of green peak top right section of figure 2.3) this was due to the subsequent photophobia and resultant pupillary miosis which follows exposure to the Visucam bright flash on image acquisition. Previous investigators have determined a strong correlation between right eye and left MPOD values in infants using FR, $r=0.97$; $p<0.0001$, (Bernstein *et al.* 2013).

The Visucam internal hard drive provided safe storage for the acquired images. Images could be transferred via USB or DVD for further back-up. The following data were collected:

date, age, gender, max MPOD measure (RE only) and refractive error. Zeiss Engineers recommended that the camera was kept clean for optimal MPOD acquisition. Frequent causes of lens contamination were airborne dirt particles (e.g. dust). More serious contamination was caused by small drops of the subject's tear fluid sprayed on the front lens when blinking. It was important to avoid serious contamination caused by the participant sneezing or touching the lens surface with the finger or nose. Fatty residue of this kind resulted in distinct bright areas on the acquired image. Such fatty residue can only be removed by moist cleaning of the Visucam lens (see Appendix 2 figure A2.1).

Dust removal: Dust or other loose particles on the lens surface can be quickly removed with the LensPen (see figure 2.4) or the brush provided in the cleaning set.

Figure 2.4 Removal of dust from front lens
(Image courtesy of Zeiss, Germany)

If the desired result was still not achieved after repeated wiping, e.g. the bright spots remain visible, the lens surface needed to be moist cleaned. A typical reason for severe contamination was accidental contact between the front lens and the subject's nose when the instrument was not positioned far enough away from the subject. Finger touching of the lens was a risk with the young subjects in studies' 1 and 2. Further details of the care and cleaning of the Visucam lens are found in Appendix 2, figures A2.1-A2.2.

Besides the vasculature surrounding the macular, no significant bright spots should be visible in the resulting flash image. The figures 2.5 and 2.6 show examples of image degradation (brighter due to post-treatment). Experience within the two week familiarisation period with the Visucam confirmed that a compromised Visucam lens surface results in sub-optimal and lower MPOD results due to reduced light reflected at the paramacular and macular.

Figure 2.5 Contamination due to a fingerprint
(Image courtesy of Zeiss, Germany)

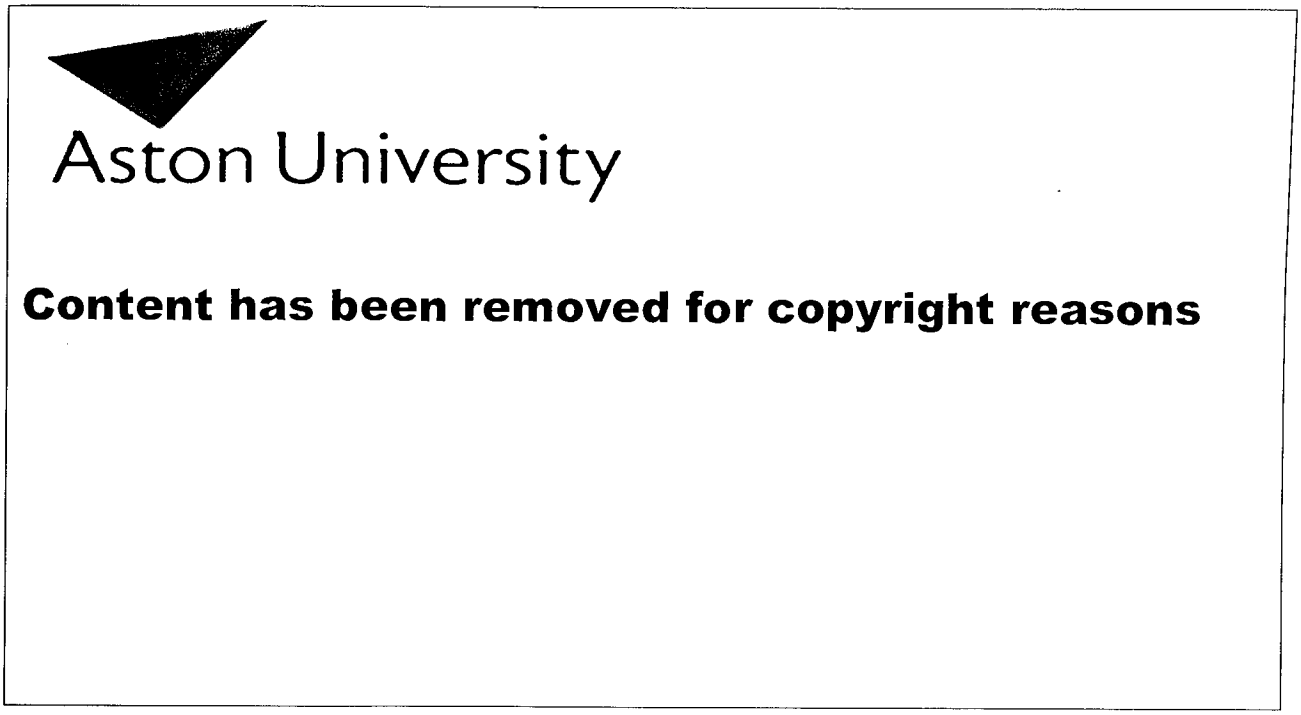


Figure 2.6 The bright spot was caused by contact with the finger or nose (Image courtesy of Zeiss, Germany)

2.3

How does the Visucam MPOD assessment compare to other methods of measuring MPOD?

With objective MPOD techniques becoming only recently available within a clinical setting, there are few comparative studies available. The Visucam for this current study stems from a device developed by Schweitzer *et al.* (2010), using single-wavelength reflectometry, which became commercially available in a fundus camera (Visucam 200® and Visucam 500®, Carl Zeiss Meditec AG). Previous studies have shown the results are comparable to those obtained by the two-wavelength AF with a correlation between the two methods of r^2=0.855 (Schweitzer *et al.* 2010). However, others have pointed out the values used in their analysis were defined as values found in 'an annulus with a radius of 0.5 deg' which is not available in the current commercial device (Creuzot-Garcher *et al.* 2014).

ɹzot-Garcher *et al.* (2014) compared MPOD in healthy young subjects with the modified ɹelberg Retina Angiograph (HRA) and the Visucam. Maximal and mean MPOD were ɜsured. The modified HRA used two-wavelength (488 and 514 nm) AF. The Visucam ɜsured the reflectance of a single 460 nm wavelength. A summary of the results is shown ɜble 2.1.

Table 2.1 A summary of the results from HRA versus Visucam study, n=67
Source: Creuzot-Garcher *et al.* (2014)

The correlation coefficient (cc) between the two methods was low for maximal and mean MPOD, $r^2 = 0.106$, $p = 0.007$ and $r^2 = 0.148$, $p = 0.001$, respectively. The investigators concluded that the agreement between modified HRA AF and FR Visucam was moderate for MPOD. Their results suggested that the two methods were not interchangeable. This is significant as it implies that monitoring MPOD with different technologies is not currently possible. Future studies could investigate whether a linear correction factor could be applied to allow the Visucam MPOD measures to be more accurately compared to other MPOD assessment techniques.

Birkeldh *et al.* (2014) reported no correlation between the Visucam and HFP in 33 subjects without ocular pathology (mean age 51.9 years ±17.8; 4 men and 29 women). There was a statistically significant difference ($p<0.001$) between the mean maximum MPOD in the healthy subjects measured with the Visucam (0.384 ±0.049) and with the Maculux® (0.559 ±0.133). No correlation ($r^2= 0.089$, $p=0.09$) was found between the two techniques. The

mean MPOD value with the Visucam in the Birkeldh study appears to be similar to other adult MPOD study values. The mean MPOD from the HFP Maculux appears to be some 20-30% higher than other adult MPOD studies results (see Table 1.1 and Table 2.2). It is therefore unsurprising that no correlation was determined between the Visucam and the Maculux.

Dennison *et al.* (2013) stated that the Visucam under-estimated MPOD values and obtained clustered MPOD values around 0.3-0.40 (see limitations below).

Other objective MPOD Studies

Previous studies reported an age-dependent decline of MPOD (Beatty *et al.* 2001, Yu *et al.* 2012) including more recent RRS studies (Obana *et al.* 2014) in which age dependence of MPOD was investigated. Analysis revealed that age and axial length were significantly correlated with low MPOD values (regression coefficient of -0.59 for age and -0.404 for axial length, respectively). Taken together with data indicating that L supplementation increased MPOD (Bone *et al.* 2010) the Obana study supported a rationale for supplementation of MP in people with AMD. Kaya *et al.* (2012) used a custom built FR to assess MPOD. A total of 85 healthy subjects and 96 subjects with AMD were included in this study. The healthy control subjects showed a wide range of ages (mean, 51.6 years; range, 21-79 years). Subjects with AMD were significantly older (mean, 71.2 years; range, 50-89 years). Spectral fundus reflectance of the fovea was measured in a 2.3° detection field with a custom built fundus reflectometer. Subjects with AMD showed a reduced MPOD (0.35 ± 0.12) as compared to the healthy control group (0.39 ± 0.12, $p = 0.01$ between groups). No age dependence of MPOD ($r = -0.14$, $p = 0.19$) was found in the healthy control group. In the AMD group, however, MPOD declined with age ($r = -0.24$, $p = 0.02$). This study indicated that MPOD was reduced in people with AMD. In addition, the data of the Obana study indicated that MPOD was age dependent in AMD patients, but not in healthy controls.

Yuying *et al.* (2015) assessed MPOD in a large healthy Chinese population sample of n=441, aged 3-81 years (242 male and 199 female subjects). Mean MPOD values were measured within a radius of 7°, using the Visucam. The MPOD values were reported as 'max'and 'mean' optical density. The average values were 0.303 ± 0.097 du (density units) for themax OD and 0.109 ± 0.031 du for the mean OD. A significant inverse relationship was found between age and MPOD (for max OD = 0.716, $p < 0.001$; for mean OD, = 0.669, $p < 0.001$).

Subjects with no lens opacities had higher MPOD values than those with moderate lens opacities ($p < 0.001$). This could be due to reduced FR occurring due to the presence of lenticular opacity. The MPOD values were not associated with gender, BMI or smoking status. The investigators concluded that MPOD within 7° of eccentricity, was found to decrease with increasing age.

2.4

Limitations of the Visucam

The disadvantage of single-wave FR is that it requires normal retinal architecture, therefore results may be erroneous in people with advanced AMD. The relevance of MP measurements in advanced AMD/geographic atrophy is questionable (where VA has reduced to less than 6/60). This limitation is therefore a minor one, particularly in a paediatric study where AMD is not a consideration. Dennison *et al.* (2013) believed the Visucam under-estimated MPOD values and obtained clustered MPOD values around 0.3-0.4. However, there was no record of calibration of their Visucam and no evidence of ensuring optical clarity of the Visucam lens during the data collection. Prior to Study 1 and 2, within the familiarisation period with the Visucam, it was noted that a compromised clarity of the Visucam optical media resulted in low and clustered MPOD values, most likely due to the reduced reflectance from the MP. Lens cleaning measures were implemented in Study 1 and 2, to ensure optimal clarity of the Visucam lens.

2.5

Alternative objective MPOD acquisition techniques

Autofluorescence

This relies on the intrinsic fluorescence, (AF) of lipofuscin in the RPE. Lipofuscin in the RPE is a waste product of photoreceptor outer segment phagocytosis and accumulates with age (Delori *et al.* 1994; Delori *et al.* 1995; Bernstein *et al.* 2010). When excited with light wavelengths of 400 to 590 nm, lipofuscin fluoresces, emitting light in the wavelength range 520–800 nm (von Rückmann *et al.* 1995). The most frequently used instrument for AF acquisition of MPOD is the confocal SLO, purpose-built (Berendschot and Van Norren, 2006) or a modified version of a clinically available SLO (Wüstemeyer *et al.* 2003; Trieschmann *et al.* 2006; Schmitz-Valkenberg *et al.* 2008). The SLO uses the imaging method of fundus AF. The subject fixes a target whilst multiple AF fundus images, usually taken over a 20° field, are obtained at wavelengths of 488 nm and 514 nm. A barrier filter

above or close to the threshold of MP absorption (e.g., 530 nm) is used to ensure that the emitted AF is only collected outside the absorption range of MP, thereby avoiding any further absorption and allowing a single-pass measurement rather than a double-pass as used in FR. All the AF images are aligned and averaged for each wavelength. A computer program digitally subtracts the averaged images at the two wavelengths and uses a greyscale index of intensity to generate a map of MPOD. There is currently limited clinical availability of AF based MPOD techniques, although Heidelberg Engineering introduced the Spectralis® AF device for measuring MPOD (AF-MPOD). The validity and repeatability of the AF Spectralis MPOD assessment technique has yet to be determined.

Canovas (2010) compared MPOD acquisition with AF to conventional HFP. The HRA uses confocal SLO to obtain MPOD measures. At all retinal eccentricities the HFP values were consistently lower than the HRA values ($p < 0.001$). Nevertheless, a significant correlation was found at almost all locations. The strongest correlation between the two methods was found at 1.75° from the centre of the fovea ($r = 0.73$). The researchers concluded that the modified-HRA AF methods for MPOD generated results which were highly correlated with the standard HFP method but consistently higher at all eccentricities. The findings suggested that HRA can be reliably used in patients unable to perform HFP (Canovas *et al.* 2010).

Resonance Raman Spectroscopy (RRS)

Resonance Raman Spectroscopy (RRS) was first described by Bernstein *et al.* in 1998.The technique uses L and Z's ability to exhibit a phenomenon called Raman scattering (Koyama and Hashimoto, 1988). The use of RRS to measure MPOD quickly gained popularity, with several papers published on its use (Gellerman *et al.* 2004; Neelam *et al.* 2005) although the momentum appears to be receding with the advent of the Spectralis AF MPOD device.

The principle of RRS is based on monochromatic light being directed at a molecule where a portion of the light is scattered. Most of the light is scattered elastically (Rayleigh scattering), but a small proportion is scattered inelastically (Raman scattering). The technique of RRS is different from almost all other MPOD assessment methods, in that it measures absolute levels of MP in a 1 mm (3.5°) area with no peripheral consideration at all. This is claimed to be acceptable because the signal is derived directly from the MP itself, rather than relying on light that must travel to deeper layers of the retina (Gellerman *et al.* 2002; Gellerman *et al.* 2004). Furthermore, the signal is only strong enough to register at carotenoid concentrations found in the maculae, rather than in structures such as the cornea and lens. However, RRS has drawn criticism from Hammond *et al.* (2005) in how it detects reduced MPOD with age. Hammond argued that this is an area where the validity of RRS is questionable, since with

other MPOD techniques, there appears to be little or no age-related MP decline (Berendschot and Van Norren, 2005; Hammond *et al.* 2005).

An independent study simulating incremental increases in lens yellowing and scatter found that the Raman signal intensity was significantly attenuated as the density of the yellow and scatter filters increased (Hogg *et al.* 2007b). The authors concluded that when using RRS to assess MPOD, the status of the lens needs to be factored into the MPOD assessment. The large decline in MPOD with age reported in many RRS studies may not hold validity. A review of MPOD in assessment in new-borns has employed a form of RRS, with some success and that lenticular opacity would be highly unlikely in this group (Bernstein *et al.* 2013).

Visual Evoked Potentials (VEP)

Using steady-state VEPs, Robson and Parry (2008) measured MPOD across a range of eccentricities in three subjects. Blue-green gratings on a colour monitor were used, and these same gratings were also used to measure MPOD with HFP. The VEP and HFP results were compared with each other in addition to MPOD measured by minimum motion photometry. This technique isolates the luminance channel by setting motion-based luminance nulls using annular stimuli. A correction factor was required for the VEP and HFP results, to allow for the overlapping phosphor emissions of the blue and green stimuli. The correlation between all three techniques was found to be excellent ($r \geq 0.94$, $p<0.0005$, in all cases). This indicates that steady-state VEPs have potential for objectively measuring MPOD but within a research only setting due to the large scale specialist equipment required.

Assessment of MP using the colour-specificity of VEP has been attempted (Moreland *et al.* 1998). The length of testing time ensures such techniques are of academic interest only and would be unlikely to find use in a clinical context. The authors concluded that large blue/green gratings produce VEPs particularly prone to contamination due to subject-specific variations in MP whilst red/green VEPs are relatively insensitive for measurement use.

In Study 3 and 4 of this thesis HFP was used in the assessment of paediatric MPOD.

2.6

Principles of conventional heterochromatic flicker photometry

The flicker is generated by alternating light of two different wavelengths, blue light, which is absorbed by MP, and green light, which is not absorbed by the MP. As a result of the pre-receptorial location of MP in the retina, incident light first passes through and is dissipated by the MP (peak absorption at λ ~460 nm) before stimulating the photoreceptors. When the short wavelength light is alternated at an appropriate frequency with a wavelength that is not absorbed by MP and the luminance of the two wavelengths is not perceived to be equal, the combined stimulus will appear to flicker (Snodderly et al. 2004; Loane et al. 2007).
The MP has its peak concentration foveally or just parafoveally, and decreases rapidly with eccentricity. Thus the HFP methods always use a central (peak MPOD) and peripheral (low or zero MPOD) measurement point, to subtract these values and obtain an individual MPOD value for each subject. The peripheral measurement is used as a reference point for the central measurement, to correct for the 'baseline' flicker sensitivity of each individual. Using HFP, subjects have to be instructed to fixate a stimulus and indicate when flicker is first observed (MPS) or when flicker is minimised (e.g . Macuscope).de Kinkelder *et al.,* (2010).

The MPOD value is derived by taking the logarithm of the ratio between blue and green luminance measured centrally and peripherally, as displayed in the equation:

$$\text{MPOD} = \log \frac{[Lbc]}{[Lgc]} - \log \frac{[Lbp]}{[Lgp]}$$

Lbc and Lgc are the luminances of the blue and green light, respectively, during the central measurement and Lbp and Lgp the luminances of blue and green light, respectively, during the peripheral measurements (de Kinkelder *et al.,* 2010).

Conventional HFP requires the observer to adjust the luminance ratio of the two wavelengths of light at both retinal locations until the flicker is perceived to disappear or be reduced to a minimum (Van de Kraats et al. 2006; Bone et al. 2007a). This can be practically difficult to achieve and so more recent variations of HFP involve the subject indicating when the flicker is first noticed - a more intuitive method for naïve subjects (Van der Veen et al. 2009b). Comparison of HFP with objective measures of MP using spectral fundus reflectance reports a high correlation between the two techniques ($r = 0.72$, $p = 0.001$, van der Veen et al. 2009b).

2.7
The MPS

Study 3 and 4 used the Macular Pigment Screener (MPS) - also known as the QuantifEYE or M:POD - to measure MPOD.

Figure 2.7: The MPS attached to a personal computer
Source: www.drnoragindi.com/quantifeye-testing.html (cited 8-6-15)

The MPS was supplied by Aston University, UK.

The MPS adopts a novel approach to the measure of MPOD. Instead of subjects responding to minimal or no flicker, as with the Macular Metrics device, they respond to the appearance of flicker as the alternation rate is decreased in 6 Hz steps from a starting level of 60 Hz (van der Veen et al.2009b). This is above the critical flicker fusion frequency for the test conditions and therefore, subjects do not initially perceive any flicker. Instead of the radiance of one wavelength being adjusted by the observer, a sequence of blue-green ratios is used and these are inverse-yoked to ensure that overall luminance remains constant. The device determines each subject's sensitivity to flicker prior to the measurement of MPOD.

Figure 2.8: The optical principles underpinning the MPS

Source: www.optometricmanagement.com (2016)

Figure 2.8 is a representation of the subject observing the alternation of the two test frequencies and how the MP affects the detection of these stimuli. The peripheral fixation test has no such modulation effect as MP is not present. The decibel difference between the central and peripheral measure gives the MPOD value. A peripheral measure was not used in Study 3 and 4 due to the limited attention span of subjects – instead an age matched value was used in the software algorithm of the MPS.

The numerical value of MPOD is calculated by the logarithm of the ratio between the luminance values made centrally and peripherally for the short and long wavelengths of light that make up the HFP target. For this reason, values for MPOD are scalar and presented without units. Table 2.2 summarises a selection of previous HFP studies that measured central MPOD.

Author	Year	Age range (yrs)	n	MPOD
Ciulla	2001	18-50	280	0.26 +-0.21
Beatty	2001	21-81	46	0.29+-0.15
Ciulla	2004	50-89	110	0.26 +-0.19
Mares	2006	70-74	413	0.38 +-0.21
Nolan	2010	50-86	79	0.23+-0.17
Yu	2012	17-85	281	0.49+-0.18
Zhu	2012	6-12	94	0.56+-0.25

Table 2.2 – Summary of previous HFP MPOD studies

The unweighted mean MPOD of the seven studies in table 2.2 is 0.35

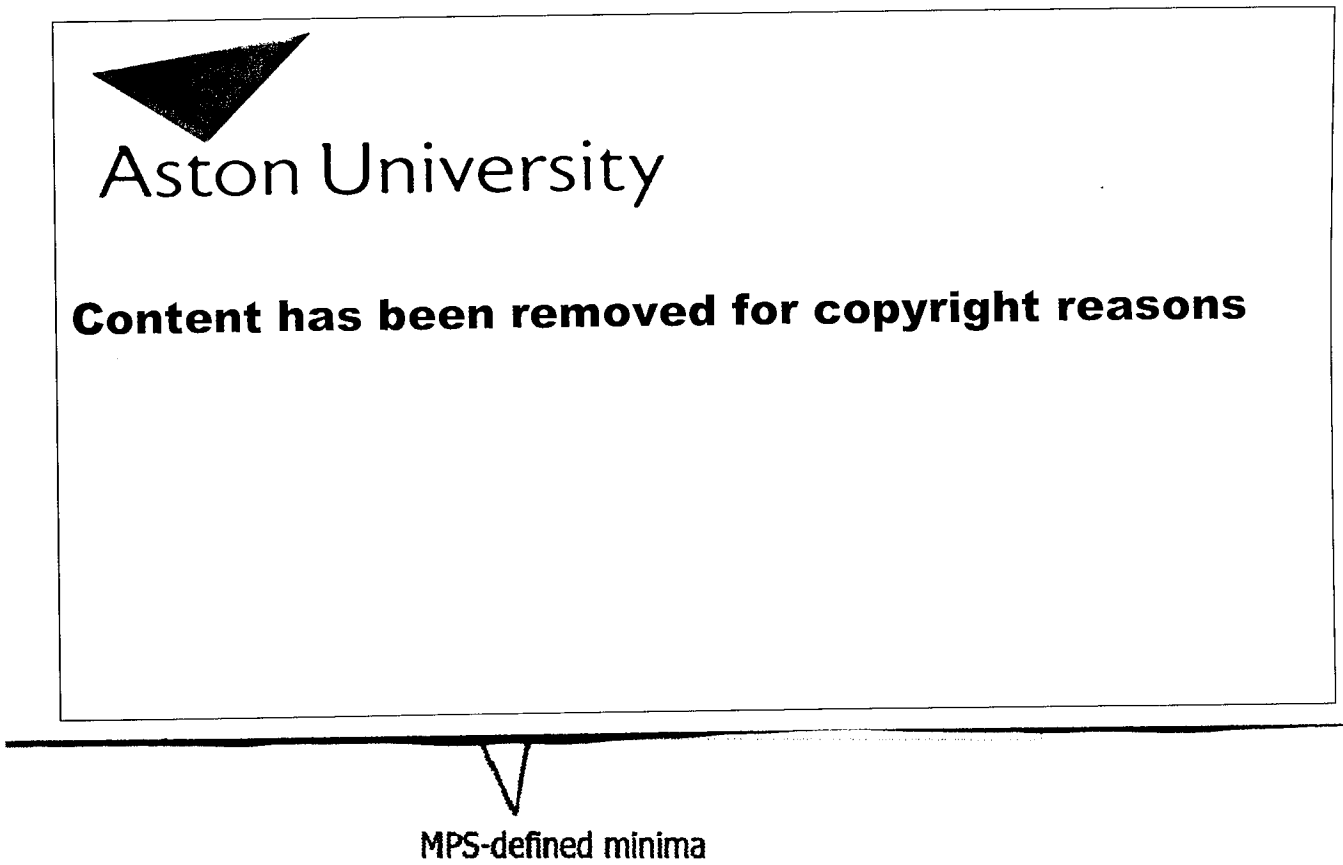


Figure 2.9: Optimal MPS V shaped curve (Source: Howells et al. 2013a)

The X axis gives the measure in decibels and the Y axis the flicker frequencies in Hz.

Figure 2.9 gives an example of an optimal central curve and an optimal peripheral curve – both V-shaped with well-defined minima. Only the blue central curve was tested in Study 3 and 4, due to the limited attention span of the subjects and the optically clear media. This type of curve was accepted for inclusion in Study 3 and 4. Subjects respond to the appearance of flicker as the blue-green alternation rate was automatically decreased in steps of 6 Hz from a starting level of 60 Hz. The C trend point denotes the MPS minima. The minimum point on the curve corresponds to equiluminance of the blue and green lights. The difference between the central and peripheral minima provides a decibel figure which can be manually assessed from a table to give the final MPOD value - the larger the difference, the higher the MPOD. An internal algorithm calculates the MPOD using the difference between the central and peripheral minima -age matched compensation software provided the peripheral minima in Study 3 and 4.

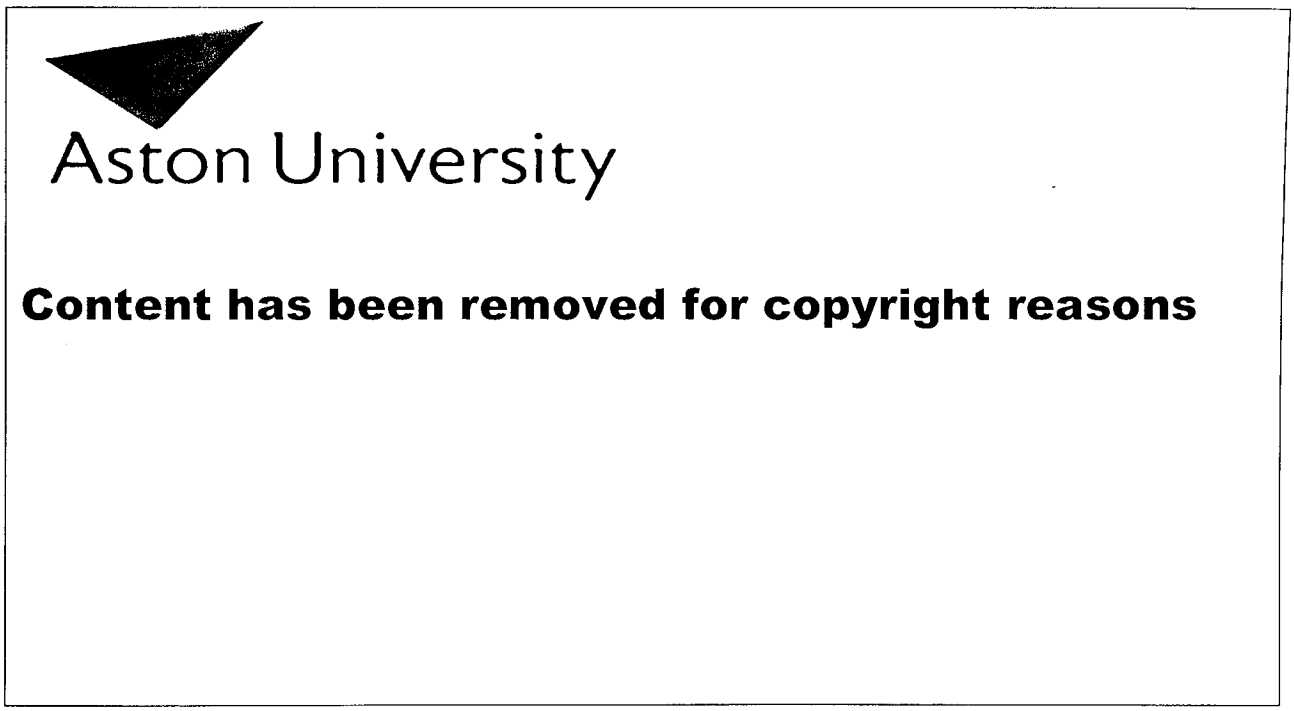


Figure 2.10: Sub optimal V shaped curve (Source: Howells et al. 2013a)

In figure 2.10 the central curve is less than optimal because it has several points all with similar flicker frequencies to the computer- chosen exact minimum at 6.80 dB, rather than an isolated, definite minimum. The peripheral curve has an exact minimum at 4.50 dB but, judging by eye, its right adjacent point has a very similar flicker frequency that could also be argued to be a minimum (at 4.70 dB).

'he MPS viewing piece was cleaned regularly and the calibration had been confirmed at \ston University prior to use. In addition there was a two week familiarisation period to ;nsure clear understanding in the operator use of the MPS.

2.8

Alignment of the MPS align with other methods of measuring MPOD

_oughman *et al.* (2012) found a high level of correspondence (r = 0.68) with the Macular Metrics Macular densitometer device which also uses HFP (See Appendix 2 figure A2.3). However, a paired-samples t-test showed a significant difference in mean values, with a bias of lower MPOD values from the MPS ($t = -4.103$, $p < 0.001$). Bland-Altman analysis indicated only moderate agreement between the two instruments, reflected in 95% limits of agreement of 0.1 ± 0.27. Inter-sessional repeatability, expressed as a CoR, ranged from 0.18 to 0.21 [mean (±SD): 0.19 (0.02)] for the MPS and from 0.11 to 0.12 [mean (±SD): 0.12 (0.01)] for the Macular Metrics. Loughman concluded that the MPS consistently obtained MPOD readings, which were lower than that found with the Macular Metrics, and that the MPS exhibited substantial test-retest variability.

Van der Veen *et al.* (2009b) compared MPS data with that from a retinal reflectometry-based instrument, the MP Reflectometer (MPR). This uses the MP spectrum to calculate MPOD. Hence, any correlation between the MPR and the MPS provides strong evidence that the MPS is selectively sensitive to the absorption spectrum of MP. The MPS data showed a relatively reduced MPOD level, but agreed almost exactly with the MPR when corrected by the 0.5° edge effect factor of 1.6. This edge effect correction factor represents the difference in MPOD measures by each device, as determined from the central fovea (van der Veen *et al.* 2009b). In addition, the paper contained the largest published MPOD data set. More than 5,000 eyes of patients from a wide age range under real world clinical conditions. The median and distribution of these data was similar to that from smaller samples.

de Kinkelder et al. (2011) compared three different techniques to assess MPOD. The right eyes of 23 healthy subjects (mean age 33.9±15.1 years) were measured. This study evaluated agreement with MPS, the Macuscope and a FR method (see Appendix 2, Figure A2.4 Macuscope). Repeatability of MPS was assessed in 20 other healthy subjects (mean age 32.1±7.3 years) and compared with measurements by a FR method in 10 subjects.

They found low agreement between test and retest measurements with the Macuscope. The average difference and the limits of agreement were −0.041±0.32. There was high agreement between test and retest measurements of MPS (−0.02±0.18) and the FR method (−0.04±0.18). The MPOD data obtained by the Macuscope and MPS showed poor agreement: −0.017±0.44. For the Macuscope and the FR method, the CC was r=0.05 (p=0.83). A significant correlation of r=0.87 (p<0.001) was found between MPS and the FR method. The authors concluded the repeatability of Macuscope measurements were low (i.e., wide limits of agreement) and MPOD values correlated poorly with FR, and agreed poorly with MPS. They felt the Macuscope protocol was unsuitable for studying MPOD.

2.9
Advantages and disadvantages of MPS

Advantages of MPS

Unlike all other HFP-based methods, the observer signals the appearance of flicker instead of its disappearance. This is a unique aspect of the technique. It is achieved by reducing the temporal frequency from above the critical flicker point. This ramping reduces Troxler (fading) effects, thus providing a well-defined endpoint for when flicker starts and ends. The designers of the MPS believe that detecting flicker is much easier than eliminating it, as is required in other HFP-based methods. Therefore the task is relatively easy, particularly for younger subjects (Murray et al, 2013b).

The MPS uses automatic customised HFP flicker rates. The subject carries out a simple pre-test flicker sensitivity test, pressing a button when they detect flicker, this selects their optimal temporal frequency for the test. This takes only a few seconds and is suitable for a naïve subject group.

The 'centre only' technique allows fast, convenient screening of subjects of all ages. The peripheral test is still available to expert/scientific users, and is recommended in cases where lens yellowing is abnormally rapid (e.g. established diabetes). The broad spectrum bright white background ensures the measurement is not affected by normal media yellowing. Such problems could potentially arise using a low level blue light background.

The MPS is easy to use. It does not require intensive operator training and can be operated by non-expert staff, however the acceptance and rejection of V shaped curve requires operator familiarity of the testing procedure and expected results.

Disadvantages of MPS

The main disadvantage of HFP is that it relies on the subject. The nature of HFP is potentially a conceptually difficult test to complete for the subject. Differences in operator training in the technique provided to subjects, or by differences in HFP instrumentation add further variables. Furthermore, donor eyes with high carotenoid levels in the peripheral retina and lens also have high macular levels (Bhosale et al. 2007). This has implications for the use of any technique that assumes little or no MP in the peripheral retinal areas. However, this would not affect long term monitoring of MPOD as the same subject would have the same up-take centrally and peripherally. Bartlett *et al.* (2010c) concluded that when MPOD was monitored over time any change less than 0.33 units should not be considered clinically significant due to measurement noise of the MPS. This makes the clinical application of commercially available HFP much more difficult to justify for long term monitoring of patients' MPOD levels.

2.10
Assessing MPOD in children

A number of previous studies have investigated MPOD in a range of different age groups and ethnicities. The majority of these studies have used HFP to calculate MPOD values, Snodderly *et al.* (2004); Loane *et al.* (2007); Tskia *et al.* (2011). The majority of these studies have been undertaken with adult subjects. As with any clinical test the nature of subjective tests give rise to criticism due to inter subjectivity and potential poor repeatability. Bartlett and Eperjesi (2010a) questioned the repeatability of HFP and reported a CoR for the MPS in adult subjects of 0.33.

There are few studies reported in the literature which have examined MPOD in children. This is primarily due to the sustained interest in assessing the relationship between MPOD and AMD.

Zhu *et al.* (2012) reported findings for a Chinese ethnic paediatric sample. A total of 94 healthy Chinese children, 6 to 12 years old were recruited. The MPOD was measured with HFP, minimum and central foveal thicknesses (MFT and CFT), were measured by OCT. The

mean MPOD was 0.56 ± 0.25, without any significant gender difference (p = 0.12). The MPOD showed no significant association with age, body mass index, spherical equivalent refraction or CFT. The figure of 0.56 represents a relatively higher MPOD value than has been found in previous western adult MPOD studies, which have ranged between 0.21 (Ciulla *et al.* 2001) to 0.45 (Johnson *et al.* 2000). The logical follow on question from this study is whether the higher Chinese MPOD value is due to the young age group (and MPOD declines with age) or ethnicity. Another Chinese, adult based HFP MPOD study indicates the latter, with the same mean value of 0.56, for the age range 17-85 years. They also found a gradual decline in MPOD with advancing age and a tendency for females to have a slightly lower MPOD than males (Yu *et al.* 2012).

McCorkle et al. (2015) assessed the repeatability of HFP with the macular densitometer in 66 Caucasian/mixed ethnic subjects aged 7-10 years. There was no significant difference between MPOD for the two sessions (p = 0.59, session 1: Mean MPOD 0.61 ± 0.28; session 2: Mean MPOD 0.63 ± 0.27) and no significant difference was found between boys and girls (p = 0.56). They reported an intersession reliability of 0.70 (Cronbach's α). The Cronbach value is a measure of the numerical coefficient of reliability. A Cronbach value of 0.7 is deemed borderline acceptable in terms of reliability (Nunnaly, 1978). McCorkle *et al.* (2015) recommended that the measure of MPOD in children should be expanded, and considered a surrogate measure of brain levels of xanthophylls, particularly L. They believed it would be beneficial for studies to investigate how L and Z relate to childhood cognitive function and development. In adult subjects with mild cognitive impairment, MPOD was related to cognition including the composite score on the language ability, attention and the assessment of neuropsychological status (Renzi *et al.* 2014). In a study assessing Infant autopsies and L levels in the brain, infants born preterm had significantly lower concentrations of L and Z compared with term infants in most of the brain regions analysed. If on average the total content of carotenoids in term infants was between 50–60 pmol/g, in preterm infant it was about 20 pmol/g, suggesting a potential deficiency in infants born before term (Vishwanathan *et al.* 2014). (Where pmol= Pico mole, a unit equal to 10^{-12} moles).

Bernstein *et al.* (2013) assessed MPOD in 51 infants using blue light reflectometry for the age group 0-7 years, including premature babies at the time of their retinopathy of prematurity screening. The investigators correlated MPOD with skin carotenoid levels measured by RRS, serum carotenoids measured by HPLC, and dietary carotenoid intake. The measured MPOD correlated significantly with age (r = 0.36; p = 0.0142), with serum L + Z (r = 0.44; p = 0.0049) and with skin carotenoid levels (r = 0.42; p = 0.0106). All premature

infants had undetectable MPOD, and most had unusually low serum and skin carotenoid concentrations (Bernstein *et al.* 2013). This indicates that infantile MPOD development aligns with the earliest phase of the visual critical period, where the works of Hubel and Wiesel indicated that the major component of visual development occurs (Hubel and Wiesel, 1970). Bernstein postulated that absent MPOD in preterm infants may be a consequence of low dietary intake and/or severe oxidative stress. However, there is uncertainty in aligning RRS methods of assessing MPOD to conventional HFP or FR techniques. Previous work has found a weak correlation between the two methods and RSS MPOD results to be significantly reduced when pupil size was smaller (Hogg *et al.* 2007a).

Vaegan and Taylor (1979) investigated the early visual critical period of the infant and found the effects of deprivation start at about four months of age and continue to a cumulative but decreasing degree throughout the first decade of life. Bernstein suggests a separate MP critical period may well initiate *in utero* and gain momentum within the first year or two of life (Bernstein *et al.* 2013). These data were published after the planning phase of the studies reported in this thesis. Isenberg *et al.* (1986) attempted to classify the ophthalmoscopic appearance of the developing macula in normal infants. The classification ranged from 34 weeks of gestational age when MP was first evident in the macula, through the development of the annular ring reflex of the macular and foveolar reflex, to a mature (adult-appearing) macula at 42 weeks.

The fact that MP is required for functional vision has been confirmed by Zhang et al. (2002) in a study of Stargardts macular dystrophy. A total of 16 subjects were recruited with a confirmed diagnosis of Stargardts using a SLO and divided into three MP groups. No MP, low MP and normal MP. All subjects with a VA of 60/60 or worse had no MP in the fovea. All patients with VA of 6/12 or better had a normal amount of MP in the fovea. Patients with low MP had intermediary VA values except for two eyes, one with 6/6 and another with 6/60 (Zhang et al. 2002).

Assessing infantile and paediatric MPOD may enhance knowledge of paediatric macular disease and have relevance to photophobia, scotopic sensitivity syndrome/visual dyslexia and strabismus/microtropia. Oka *et al.* (2013) documented structural asymmetry in the paediatric macula between the strabismic eye and dominant eye. They concluded that dominant eyes of the strabismic group exhibited thinner superior temporal ganglion cell complex thicknesses within a measured 3-mm region. Retinal ganglion cells in this region could be affected by efferent neural degeneration that originates in the visual pathway responsible for adaptations to the visual experience (Oka *et al.* 2013). With consideration of

these findings and in the case of strabismus, where the macula of the deviating eye may be seldom used, it is possible for an interocular difference in MPOD to develop where the deviating macula may develop subtle atrophic characteristics due to non-use. However, prior to these further factors being investigated, the establishment of normative objective paediatric MPOD values are required. This was the aim of the current study.

Central to the understanding of MPOD development, is the determination of MPOD at the earliest stage of life and Studies 1-4 investigate this. It is anticipated that this will add to previous research and may encourage community optometrists to consider the proposition of routinely assessing MPOD levels in all patients. With this advancement may follow an enhanced level of understanding of macular conditions and swifter detection of subtle disease. Early detection of most disease processes are associated with a more successful prognosis.

The logical follow-on to assessment of MPOD is the ability to maintain, manipulate or increase the value prior to the onset of disease process. Studies remain divided on the relation between increasing MPOD and improvement of visual function/reduction in prevalence of AMD. The Lutein Intervention Study Austria (LISA) found that supplementation of L had a statistically significant impact on MPOD within a six month time period. A significant correlation was found between the increase in MPOD after six months and the increase in VA after six months ($r = 0.27$, $p = 0.013$) (Weigert *et al.* 2011). Another study failed to confirm the inverse hypothetical relationship between MPOD and AMD (Dietzel *et al.*2011a).

2.11
Rationale

Most clinical research has focused on the potential roles of L and Z in preventing AMD, and much less is known about their function and physiology in the infant eye. This is due, in part, to the fact that the most commonly used methods to assess MPOD, such as HFP (Bone *et al.* 2004) and AF imaging (Canovas *et al.* 2010) are unsuitable for infants and young children because of their inability to participate in the required psychophysical testing and the lack of significant lipofuscin in their RPE (Bernstein *et al.*2013). Previous researchers have localised these dietary compounds and their metabolites within the eye from a very early age via high-performance liquid chromatography (HPLC) analysis of autopsy eyes (Bone *et al.* 1988).

Bernstein reported that MP could play a very important function in foveal development, enhancement of infant VA, or protection against light-induced oxidative damage (Bernstein *et al.* 2013). But without knowledge of normal MP levels and distributions in infants and children, progress on these potential functions cannot proceed (Bernstein *et al.* 2013).

It is within this context that the exploratory Study's 1, 2, 3 and 4 of this thesis were undertaken – to fill a gap left by previous researchers who have focussed on MPOD at a much later stage of life. Furthermore, within the context of advanced understanding of paediatric macular disease Bernstein and Hammond advocate paediatric MPOD assessments in neonates suspected of early onset retinal disease and particularly in oxidative stress-based disease such as retinopathy of prematurity (Bernstein *et al.* 2013).

The main questions that Study's1-4 aims to address are:

1) How does the repeatability of the Visucam and MPS compare in paediatric subjects?
2) What are the mean MPOD values in the sample paediatric population with these two techniques? How do these values compare to adult studies using similar techniques?
3) Does age, gender or iris pigmentation affect the mean MPOD value?
4) Does L intake have any relationship to MPOD in paediatric subjects?

Studies in this thesis

Exploratory Studies 1-4 aimed to assess MPOD in a consented paediatric sample between the ages of 4 and 16 years. Longitudinal follow-up tracking of MPOD through advancing years of this age group would be fraught with difficulty. Instead in the larger study (Study 2) it was decided to divide the age group into three subgroups of 4 to 8, >8 to 12, >12 to 16 years. Mean MPOD would be calculated and compared between these groups to assess for any change in MPOD that may occur with increasing age. Any relationship would then be confirmed with linear regression analysis. In respect of Bernstein *et al's* (2013) work (which became available after the completion of the current study) it has become evident that significant development of paediatric MPOD occurs within the preterm and direct post term period. Nevertheless this current study has value in defining objective paediatric MPOD. This may prove clinically useful for patients whose paediatric MPOD is lower or significantly lower than the expected age-matched mean level and that supplementation to achieve normative paediatric MPOD levels may be indicated. Routinely monitoring MPOD may become routine in optometric practice within the next decade. If optometrists are to gain a clearer

understanding as to how MPOD evolves through life, then it is necessary to understand what level of MPOD is typical within an early stage of life. The first two exploratory studies in this thesis will provide normative data of a predominantly White sample using an objective-based technique between the ages of 4 to 16 years. The subsequent two exploratory studies in this thesis will provide normative data of a predominantly White sample using a subjective based technique between the ages of 4 to 16 years. These two techniques are compared (discussion chapter 7). Study 4 investigated the relationship between L levels in the diet and paediatric mean MPOD.

2.12
Conclusions

Studies have reported different levels of MPOD with various types of instrumentation and ethnicity. Much of the research focus has sought to examine the relationship between MPOD and AMD and has focussed on older age groups. The link between MPOD and AMD will be further discussed in Chapter 7 (Discussion). The technologies available to measure MPOD are increasing and there exists the potential for variability in assessment from one technique to another. Many of the techniques discussed represent large, costly technologies unlikely to be found within optometry or ophthalmology practices in their current form. Since there is no formal MPOD gold standard accepted by the ophthalmic community, then it is difficult to establish which measure represents the most accurate anatomical level. There are a significant number of assumptions being made for FR based techniques in acquiring the MPOD measure. The repeatability of this technology was compared to conventional HFP with the MPS (Study 3) in Chapter 5. This will allow determination of which device has the best repeatability and validity.

In the next chapter, the experimental design and results for Study 1 are described. This includes the repeatability of the Visucam in children, inclusion criteria, procedural elements of the study, and discussion of the results.

CHAPTER 3 - Study 1: Assessing the repeatability of the Visucam in a paediatric sample

In this chapter an account and discussion of the results from the repeatability study of the Visucam is presented. The purpose of Study 1 was to determine the repeatability of the Visucam in a paediatric sample.

3.1
Introduction

The purpose of Study 1 was to determine the coefficient of repeatability (CoR) of the Visucam in its assessment of MPOD in a paediatric sample attending an independent optometric practice. This relates to the emergence of assessing MPOD in clinical practice.

Repeatability refers to the variability in repeated measurements by one observer when all other factors are assumed constant. Reproducibility refers to the variability in repeated measurements when one or more factors, such as observer, instrument, calibration, environment or time is varied (McAlinden *et al.,* 2011).

In order to determine the sample size required for an agreement study it is necessary to determine the required accuracy of the limits of agreement in terms of confidence intervals. The following formula may be used:

$$1.96 \sqrt{\frac{3s^2}{n}} = \text{desired confidence interval of limits of agreement}$$

Where s= standard deviation of the differences, n= sample size

McAlinden *et al.,* (2011).

McAlinden *et al.,* (2011) recommend ideal sample sizes of 100 for agreement studies. Due to limited resources and time constraints in accessing the Visucam, a sample size of 16 was agreed for the pilot based Study 1. This sample size aligned with previous MPOD repeatability studies (Koh *et al.,* 2004, van der Veen *et al.,* 2009a+b).

Using G Power 3.1 and relating the correlation of the two MPOD measures: coefficient of determination 0.30, *post hoc* analysis of a sample size 16 would give an effect size (p) 0.55, critical t =1.76, to allow an α error probability of 0.05 and power= 0.8.

3.2
Participants

This research involved a paediatric sample of 16 naïve subjects aged 4-16 years (no younger than four, and no more than 16). Assessment of MPOD was determined with one measurement of the right eye (see procedure). Consent for inclusion was obtained from the parent/guardian and child (see Appendix 3, figures A3.1-3.3 consent and data collection paper sheets). The study was approved by the Ethics Committee of Aston University (see Appendix 3, figure A3.4). Full confidentiality of the records was maintained and participant numbers, not names, were used. Higher degrees of refractive error were excluded (see following exclusion/inclusion criteria). Subjects with any form of previous or current ophthalmic disease were excluded from the study to ensure a normative sample was being studied.

Refractive error was classified for this study as:

Emmetropia -0.25 to + 0.50 inclusive
Hyperopia > + 0.50 < +5.00
Myopia > -0.25 < -3.00

Inclusion criteria

Any ethnicity
Age of ≥ 4 to ≤16 years on MPOD acquisition
Clear fundus and media assessment
VA better than 6/9 in the measured eye

Exclusion criteria:

Under 4 years of age/poor co-op or over 16 years
Refractive error: More myopic than -3.00 D.S. and more hyperopic than +5.00 D.S. or more astigmatism than -2.50 D.C. (re: potential amblyopia/subnormal VA development, Ziylan, et al. 2007)

Diagnosed with any type or form of ophthalmic or retinal pathology (including strabismus)
Declined parent/guardian consent at any stage of the study
Any currently, suspected or previously diagnosed epilepsy.

Participant demographics

There were 16 participants, recruited in an optometric practice following an NHS eye examination. Mean age was: 10 years 1 month, with a range of 4 years, 2 months to 15 years, 11 months. SD 3 years 3 months. Table 3.1 summarises subject demographics for Study 1.

Participant demographic	***n***
Gender	
Male	9
Female	7
Refractive error	
Myopia	2
Emmetropia	9
Hyperopia	5
Iris pigmentation	
Light	9
Dark	7

Table 3.1: Subject demographics for Study 1

Materials

Zeiss Visucam 200 – supplied by Carl Zeiss Ltd, Cambridge, UK

3.3

Method

Prior to undertaking Study 1, there was a two-week familiarisation period with the Visucam in order to become used to the technique of MPOD image acquisition

The testing room had no windows and housed the Visucam. Total darkness was achievable within the room and the subjects were given a period of dark adaptation time of approximately two to three minutes prior to the MPOD measure being taken. This was to ensure good pupillary dilation to achieve a good quality image and MPOD calculation. The MPOD measure was only for the right eye, with the aim of obtaining a clear MPOD image with one attempt. This was due to the sub-optimal images that were achieved if repeated image capture was attempted (due to dazzling and pupillary spasm/miosis that required approximately 5-10 minutes to resolve). When the image quality was compromised or the subject moved their head very slightly during the MPOD image acquisition (resulting in an incomplete image acquisition) then the results were recorded but rejected due to the potential for substandard data. Before undertaking Study 1 and 2, there was a two-week familiarisation period for the examiner, in order to become familiar with the technique of MPOD image acquisition.

Clinical evaluation

The following measures were collected in Study 1:

Date, age, gender, MPOD measure with one measure attempt using Zeiss Visucam. The MPOD measure was repeated within a 2-4 weeks' time period.

Statistical analyses were performed with SPSS version 22 (SPSS IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) and Microsoft Excel 2010 (Microsoft, Redmond, Washington USA) and G Power 3.1 (Faul *et al.* 2009).

3.4

Results: Study 1

Subject	MPOD 1	MPOD2	Mean of MPOD1 and MPOD2	Diff-MPD1, MPD2
1	0.416	0.452	0.434	- 0.036
2	0.402	0.358	0.380	0.044
3	0.366	0.369	0.368	- 0.003
4	0.423	0.375	0.399	0.048
5	0.389	0.417	0.403	- 0.028
6	0.489	0.496	0.493	- 0.007
7	0.371	0.487	0.429	- 0.116
8	0.377	0.405	0.391	- 0.028
9	0.472	0.467	0.470	0.005
10	0.312	0.336	0.324	- 0.024
11	0.348	0.355	0.352	- 0.007
12	0.426	0.311	0.369	0.115
13	0.377	0.379	0.378	- 0.002
14	0.354	0.371	0.363	- 0.017
15	0.367	0.341	0.354	0.026
16	0.380	0.384	0.382	- 0.004
Sum Total	6.269	6.303	6.286	- 0.034
Mean	0.392	0.394	0.393	- 0.002
Max	0.489	0.496	0.493	0.115
Min	0.312	0.311	0.324	- 0.116
St Dev	0.045	0.055	0.044	0.049
CoR			1.96 x 0.049	0.095

CoR = 0.095 (approximating to 0.1)

Table 3.2: Study 1 Visucam repeatability results

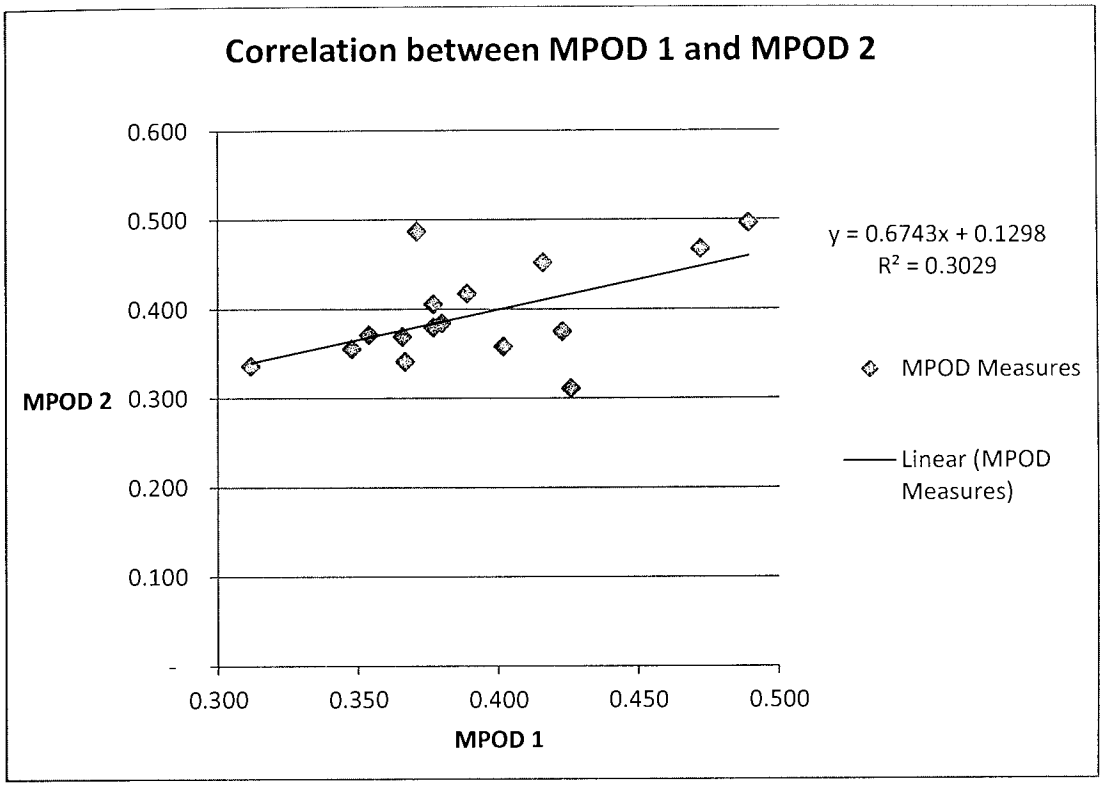


Figure 3.1 – Correlation coefficient between MPOD 1 and MPOD 2 for Study 1

R^2= Coefficient of determination

R= 0.55 (CC)

Correlation of MPOD 1 and MPOD 2. A paired t test was used to assess the agreement between the two measures, p=0.86, and therefore fail to reject the null hypothesis and there is a significant difference between the measures of MPOD 1 and MPOD 2 (see Appendix 3 table A3.4).

Through the use of G Power 3.1 and relating MPOD 1 to MPOD2, *post hoc* analysis, sample size of 16 with R^2 = 0.30, would give an effect size (p) 0.55, critical t =1.76, to allow an α error probability of 0.05 and 0.80 power.

Normality of data

Kolmogorov-Smirnov and Shapiro Wilk analysis gave >0.05 (see Appendix 3 table A3.3), and therefore the 'difference MPOD' distribution of data is normal, the histogram shows a relatively normal distribution of 'difference MPOD' and the Q-Q plot (see Appendix 3 figure A3.6) shows data that approximates to the diagonal line of normality. Assessment of normality in data is recommended prior to Bland Altman analysis.

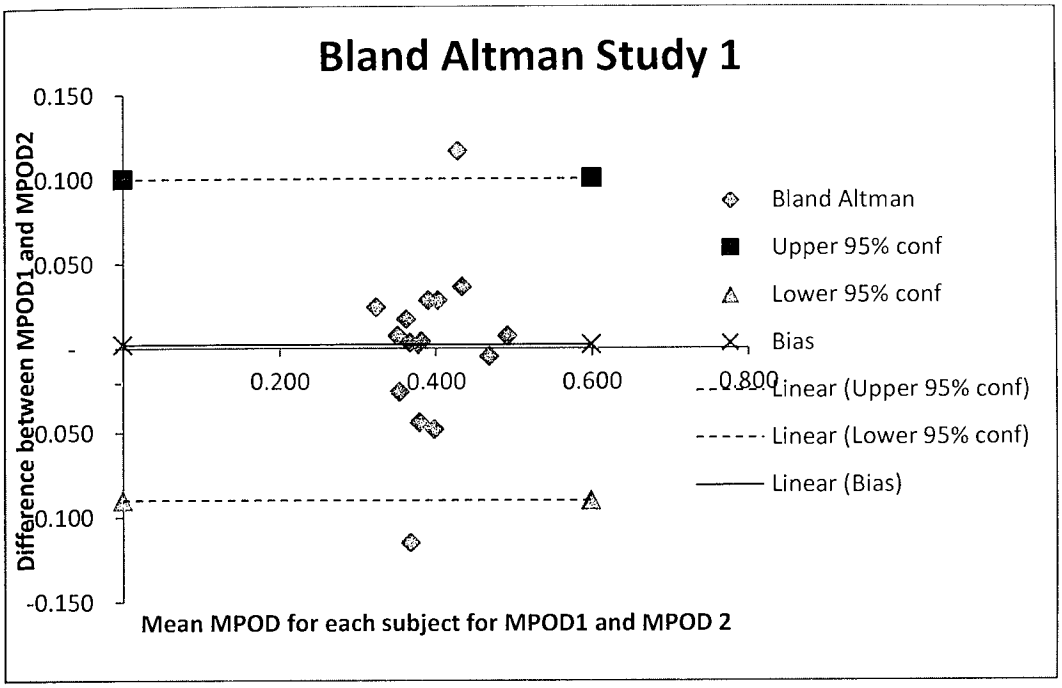


Figure 3.2: Bland and Altman Plot with data obtained from 16 paired MPOD readings from the Visucam. Correlation r = 0.047 (p>0.05), CoR 0.095

Data analysis

The mean for the first measure of Study 1 was 0.392 with a maximum of 0.489 and minimum of 0.312, SD 0.045. The mean for the second measure of Study 1 was 0.394 with a maximum of 0.496 and a minimum of 0.311, SD 0.055, indicating a slightly higher degree of variability in the second measure. This resulted in the mean for measure one and the mean for measure two being similar at 0.392 and 0.394 respectively. The mean test-retest difference was 0.043 or 10.9%.

Accurate analysis of test–retest data can be achieved using the CoR which gives the 95% confidence limits for the amount of difference between two sets of results (Bland *et al.* 1986; Elliot *et al.* 1988). It is calculated as 1.96 multiplied by the SD of the mean differences between the two sets of data.

The mean diff between measures was -0.02 (SD = 0.049). Therefore, 0.049 x 1.96= 0.1
(upper range CoR = 0.1 + 0.049 = 0.149, lower range CoR= 0.1 - 0.095 = 0.051)
The mean of the means of the two MPOD measures for the Visucam was 0.39
Therefore 0.1/0.39 is 25.6% - a moderate sized effect.

The CoR approximated to 0.1. In order for a change from one measurement to the next to be classed as clinically significant it needs to be greater than the CoR. The CoR allows consideration in the variation of measurements taken by a single person or instrument on the same subject and under the same conditions. A less-than-perfect test–retest reliability causes test–retest variability. Such variability can be caused by intra-observer variability.

3.5
Discussion

The assessment of the repeatability of a device is important if the device is to be used in a cross sectional study. The purpose of Study 1 was to determine the CoR of the Visucam in its assessment of MPOD in a paediatric sample.

The reason for the slightly higher degree of variability in the spread of the data for the second measure is unknown. A learning effect by the subject or the practitioner, would usually give more consistent data and a lower SD on the second measure. With reference to table 3.2, of the 16 repeat measures that were taken, five of them were lower on the second reading, the remainder 11 being higher. Differences in readings between subjects could be attributed to physiological between subject variability. Individual between subject differences are the measure of instrument repeatability. It is the mean SD of the algebraic differences between MPOD1 and MPOD2 of 0.049, which when multiplied by 1.96 gives the CoR value.

The CoR value is significant as it means any difference between two or more readings/groups of greater than 0.1 is not due to measurement noise. The mean of the two MPOD means for Study 1 was calculated to be 0.393 and 0.1/0.393 is approximately 25%. Therefore a 25% change would be deemed a moderate size effect. In a typical statistical analysis of repeatability results, the lower the CoR value the better. Relating this finding to Study 2 of this thesis - a difference of 0.1 or less (between individual subjects readings on a longitudinal basis) cannot be considered as anything other than instrument noise. A larger sample size would possibly result in outliers having a reduced effect on the CoR. Unpublished data has determined a CoR of 0.02 in a larger study with adults using the Visucam 500 (Personal communication – Dr Frank Eperjesi, Aston University, UK). Therefore it is apparent that there is greater variability in the repeated measures of child subjects when compared to adults. This variability in paediatric MPOD repeatability measures has also been reported by McCorkle *et al.* (2014). Determining what could be the

cause of the outliers in Study 1 of this thesis, remains contentious and an area for further larger scale study. By determining the cause of the outliers, measures can be taken to minimise the effects of these, improve the CoR and reducing instrument noise.

Reasonable concordance is demonstrated in the repeated measures, however Subject 7 and Subject 12 showed significant difference in their initial and follow up MPOD values. A further review of their images by the researcher and Zeiss clinical support (Germany) did not reveal a reason for this. There is a possibility that a difference in dietary intake between one measure and the second measure could go some way to explain the difference in MPOD values, however in such a short time period this would remain doubtful. Another possibility is that the amount of reflectance that has occurred when the image was taken has varied in some way due to set up - this would appear to be less likely as MPOD images for the two anomalous results appeared similar to the other images. This was confirmed through independent review by Zeiss Germany (personal communication - Elke Machalett, Zeiss, Germany) following email discussion. Zeiss proposed that the phenomenon of "juvenile macular reflex" could contribute to the variability in MPOD values obtained in Subject 7 and Subject 12. Examination of the literature reveals very little on juvenile macular reflex; there was a 1989 review by Noble *et al.* examining the golden tapetal sheen reflex in juvenile macular dystrophy. Bone *et al.* (2007b) documented that younger subjects were more prone to stronger reflections from the inner limiting membrane, leading to a pronounced foveal reflex.

Figure A3.7 (Appendix 3) is a summary of previous FR adult MPOD repeatability studies with an average test / re- test variability of approximately 11.5%. This correlates fairly well with Study 1 where the mean test-retest difference was 0.043 or 10.9% with a CoR of 0.10 (25%). In this respect it would appear that CoR in paediatric subjects is more variable to that from adults. Study 1 has a similar number of subjects compared with the studies in Figure A3.7 providing some useful comparison. Berendschot *et al.,* (2000) the first study listed in Figure A3.7 showed a within-subject variation of 10% of MPOD and 17% with spectral reflectance analysis, however this was within the context of a supplementation trial. Delori *et al.,* 2001 (second study, A3.7) should be interpreted with some caution. The larger group of 22 subjects in Delori *et al* study had MPOD readings taken 10-24 months apart. It is difficult to assess if the fluctuations in readings are due to instrument noise or other factors affecting the subjects' MPOD. The remaining studies were intra-session based repeatability studies and therefore cannot be directly compared to Study 1 which was an inter-session repeatability study. Few studies have assessed the inter-session reliability of their devices.

The probable reason - unlike HFP, the actual measurement time in FR is short and does not demand much effort from the subject – it is more convenient to take repeat measures within the same session. Zagers *et al.* (2002) hypothesized the variability in their intra-session MPOD results was the result of fixation errors, with less experienced subjects showing higher variability. Snodderly *et al.* (2004) documented, inter-session reliability was really more valuable than intra-session reliability. Since results generally show higher variability between sessions, this is a more robust test for an instrument. Study 1 aimed to fill a gap in knowledge relating to inter-session repeatability of FR in children. A study conducted by Bone *et al.* (2007b) can be compared to Study 1, although the subjects were aged 18-24 years. However the results are reported only in a Bland and Altman plot and a mean for MPOD was not recorded.

Westheimer and Liang (1995) considered light scatter in FR. The investigators noted that intra-ocular light scatter, known to increase with age, was variable at any given age. The majority of scattered light that reached the Visucam camera was due to back-scattering of the incident flash by the lens of the eye. This would result in a decrease in the contrast of the image and an under-estimate of MPOD. Additional stray light can arise from reflection at the inner limiting membrane. Delori and Pflibsen (1989) noted a significant increase in the estimate of MPOD when a small, inner limiting membrane reflectance was introduced to their model. It has been argued that the scattered light problem underlies an observed decrease in MPOD with age when determined by the Raman method (Hammond *et al.* 2005). In order to minimize this potential source of error, Bone *et al.* (2007b) restricted their study population to those less than 25 years old with clear optical media. However, they also conceded that younger subjects were more prone to stronger reflections from the inner limiting membrane, leading to a pronounced foveal reflex, referred to by engineers at Zeiss, Germany as the juvenile reflex (personal communication).

3.6
Conclusion

The mean MPOD for Study 1 first measures was 0.392, the mean for Study 1 second measures was 0.394. The mean of the two MPOD means for Study 1 was 0.393.

The ability to assess the repeatability of an instrument prior to undertaking a larger scale study (and using in clinical practice) provides a clear understanding as to what degree of instrument noise can be anticipated. In Study 1 a CoR of 0.1 was found for the Visucam.

The results of Study 1 will be compared to the repeatability of the MPS in determining paediatric MPOD. This is presented in Chapter 5 and is termed Study 3.

In the following chapter, the results of the main Visucam paediatric MPOD study are described. This has been termed Study 2 which investigated a normative MPOD value in a paediatric sample. Study 2 had a larger number of subjects than Study 1, and the data has been divided into different groups, according to age, refractive error, iris pigmentation and gender.

CHAPTER 4 - Study 2 - The assessment of mean MPOD in a paediatric sample with the Visucam

There have been a number of studies which have investigated MPOD in a range of different age groups and ethnicities, however the majority of these studies have been undertaken using the subjective technique of HFP (Snodderly et al. 2004; Loane et al. 2007; Tskia et al. 2011) and the vast majority of these studies have been undertaken with adult subjects (Iannoccone et al. 2007).

The difficulty in assessing MPOD using HFP in children is due to the concentration and co-operation of the subject creating a level of fatigue which may be difficult for naïve subjects (Van der Veen et al. 2009a). This chapter describes the assessment of paediatric MPOD with the Visucam which objectively measures MPOD with single wavelength FR. To the authors knowledge this is the first known study in the UK for the assessment of objective MPOD in this age group.

4.1
Introduction

Understanding the molecular and cellular basis of diseases is vital for determining the mechanisms of disease pathogenesis and for designing appropriate and effective treatments (Cardinale *et al.* 2014). Macular pigment optical density has become an increasingly popular area of research amongst optometrists, doctors and scientists over the past three decades (Bone *et al.* 1985; Snodderly *et al.* 1995).

Macular Pigment

Macular pigment is a yellow, oily substance located in the fibres of Henlé and the outer segment membranes of photoreceptors in the macular region of the human retina (see section 1.1 for further detail).

Assessing paediatric MPOD may increase understanding of juvenile macular disease and further enhance understanding of the ocular ageing process by providing MPOD values at an early stage of life. This could enable researchers and clinicians to gain a better understanding about the relationship between MPOD and age. Low levels of MPOD have been linked to the development of AMD (Nolan *et al.* 2007; Raman *et al.* 2012). Early detection of almost any disease processes tends to be associated with more successful treatment outcomes and long term prognosis.

4.2

Method

The study was approved by the Ethics Committee of Aston University (see Appendix 4 figure A4.6). Full confidentiality of the records was maintained and participant numbers, not names, were used.

Subjects

This study, termed Study 2, was carried out between January 2013 and May 2013. The method in Study 2 was similar to that used in Study 1, and is briefly described below.

Seventy-three naïve subjects were recruited from an optometric practice, following an eye examination. This number of subjects were similar to previous reflectometry studies: Chen *et al.* (2001) n= 54, Zagers *et al.* (2002) n=38 and subsequent Visucam studies Creuzot-Garcher *et al.* (2014) n= 67. Parent/Guardians were given information sheets regarding the nature and purpose of the study, such that informed consent could be granted. The child subject was also given an information sheet and verbal agreement that they could decline or withdraw at any time (see Appendix 4 figures A3.1-A3.3 for information sheets).

Using G Power 3.1 and relating MPOD to age, *post hoc* analysis of a sample size 73 gave an effect size (p) 0.16, critical t =1.29, to allow an α error probability of 0.1 and 0.54 power.

Inclusion criteria

Any ethnicity
Age of ≥ 4 to ≤ 16 years on MPOD acquisition
Clear fundus and media assessment
VA better than 6/9 in the measured eye

Exclusion criteria:
Under 4 years of age/poor co-op or over 16 years
Refractive error: More myopic than -3.00 D.S. and more hyperopic than +5.00 D.S. or more astigmatism than -2.50 D.C. (re: potential amblyopia/subnormal VA development, Ziylan, *et al.* 2007)

Diagnosed with any type or form of ophthalmic or retinal pathology (including strabismus)
Declined parent/guardian consent at any stage of the study
Any currently suspected or previously diagnosed epilepsy

Refractive error definitions
Emmetropia: -0.25 to +0.25 inclusive
Myopia: -3.00 to > -0.25 DS
Hyperopia >+0.25 to +5.00 DS inclusive

*Lower degrees of astigmatism were present within hyperopic or myopic refractive errors. Higher degrees of astigmatism (>-2.50 cyl) were excluded as per the study inclusion/exclusion criteria due to the risk of meriodonal amblyopia (Wallace *et al.* 2007).

Materials

The Visucam 200 (Carl Zeiss Ltd., Cambridge, UK)

In this study the max MPOD measure only was taken, for simplified data analysis.
The Visucam was supplied by Carl-Zeiss-Strasse, Germany and then quality assured by Carl Zeiss Ltd. Cambridge, UK. Zeiss UK and Zeiss Germany declared no direct commercial interest in this study.

The testing procedure was as described in Study 1.

The following data was collected: date, age, gender, MPOD measure (RE only) and refractive error.

Before undertaking Study 2, there was a two-week familiarisation period with the Visucam in order to become familiar with the technique of MPOD image acquisition. Study 2 followed the repeatability study (Study 1) which ran from December 2012 – January 2013.

4.3

Results

Seventy-three subjects were included in Study 2, 40 female and 33 male with a mean age of 11.1 years, (SD) 2.8 years. Statistical analyses were performed with SPSS version 22 (SPSS IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) and Microsoft Excel 2010 (Microsoft, Redmond, Washington USA) and G Power 3.1 (Faul *et al.* 2009).

The Shapiro-Wilk test was used to determine whether variables were normally distributed. For p-values < 0.05 the null hypothesis of normality was rejected (Graphical figures for the results are located within Appendix 4, figures A4.01-A4.18).

Variable	Age 4-8y	Age >8-12y	Age >12-16y
n	14	25	34
Max Age (y)	7.9	12	15.9
Min Age (y)	5.1	8.4	12.1
Mean Age (y)	6.5	10.45	13.5
SD (y)	0.8	1.1	1.25
Max MPOD	0.5	0.74	0.49
Min MPOD	0.32	0.3	0.3
Mean MPOD	0.4	0.4	0.39
SD	0.05	0.08	0.04

Table 4.1 Descriptive stats summary in age groupings for Study1

Number excluded: Six (Two from each age group, mainly due to juvenile reflex – excessive "sheen" at the macula). Exclusion rate: 6/79 = 7.6%

Overall mean of the three MPOD means: 0.396, SD 0.06

A single factor ANOVA was conducted to assess for any statistically significant difference in mean MPOD between the three age groups. The null hypothesis was accepted and there was no significant difference between the three age groups, p= 0.98 (F= 0.019, F crit 3.12, see Appendix 4 figure A4.17). This was confirmed with linear regression analysis with MPOD and age r=0.015, p=0.9. (see Appendix 4 figure A4.17-A4.18)

Using G Power 3.1 and relating MPOD to age, *post hoc* analysis of a sample size 73 would give an effect size (p) 0.16, critical t =1.29, to allow an α error probability of 0.1 and 0.54 power.

Emmetropia	**Hyperopia**	**Myopia**
n= 29	n= 36	n= 8
Max 0.50 Min 0.31	Max 0.74 Min 0.30	Max 0.44 Min 0.34
Mean 0.40 SD 0.05	**Mean 0.40** SD 0.07	**Mean 0.39** SD 0.03

Table 4.2 Refractive error summary in Study 2

Light	**Dark**
n= 49	n= 24
Max 0.49 Min 0.30	Max 0.74 Min 0.32
Mean 0.39 SD 0.04	**Mean 0.41** SD 0.08

Table 4.3 Iris pigmentation summary in Study 2

Light iris pigmentation was defined as blue, green, and grey.
Dark iris pigmentation was defined as light brown and dark brown irides.
Light iris pigmentation, n=49, had a slightly lower mean MPOD of 0.39 (SD 0.04).

Dark iris pigmentation had smaller subject numbers at n=24 and a slightly higher mean MPOD of 0.41 (SD 0.08). In addition dark iris pigmentation had a larger range of MPOD values of 0.74 to 0.32 and the highest recorded MPOD value of 0.74.

Female	Male
n=40	n=33
Max 0.50 Min 0.30	Max 0.742 Min 0.31
Mean 0.39 SD 0.04	**Mean 0.40** SD 0.07

Table 4.4 Gender summary in Study 2

Female subjects, n=40, had a very slightly lower mean MPOD value of 0.39 (SD 0.04) and a narrower range of MPOD values. Male subjects n=33 had a slightly higher mean MPOD of 0.40 (SD 0.07) indicating slightly higher spread of values in the male MPOD data collected.

Female Light	Male Light
n=29	n=20
Max 0.48 Min 0.30	Max 0.49 Min 0.31
Mean 0.39 SD 0.04	**Mean 0.39** SD 0.05
Female Dark	**Male Dark**
n=11	n=13
Max 0.50 Min 0.35	Max 0.74 Min 0.32
Mean 0.40 SD 0.04	**Mean 0.43** SD 0.1

Table 4.5 Gender, subdivision of iris pigmentation in Study 2

Gender subdivision of iris pigmentation revealed male dark irides (n=13) with the highest mean MPOD of 0.43 (SD 0.1). Male and female light irides groups had mean MPOD of 0.39.

The data were analysed using non-parametric methods: Mann Whitney U test indicated that for gender variable, p value 0.44 and the null hypothesis was accepted and there was no statistically significant difference between male and female MPOD (See Appendix 4, figure A 4.07).

The independent samples Mann Whitney U test was applied, this indicates that for iris pigmentation variable, p =0.12 and the null hypothesis was accepted and there was no

statistically significant difference between light and dark iris pigmentation in relation to mean MPOD (see Appendiix 4, figure A4.14).

Male dark vs female light

The greatest difference in mean group MPOD values was between the male dark pigmentation irides (0.43) and female light irides (0.39). Analysis was therefore undertaken to assess if this difference was statistically significant. The independent samples Mann Whitney U test was applied, this indicated that for iris pigmentation male dark vs female light, p value 0.11 and the null hypothesis was accepted and there was no statistically significant difference between light and dark iris pigmentation MPOD (see Appendix 4 figure A4.15).

An incidental finding was that the highest level of MPOD was detected within a 10 year old male, mildly hyperopic child with dark irides and of Eastern European descent with MPOD of 0.742. Ethnicity was not a recorded factor within the study although it was noted in this case due to the high level of MPOD.

4.4
Discussion

Study 2 was a pilot study aiming to collect objective MPOD values from a cross sectional sample of a UK paediatric population. This study provided information on the mean level of MPOD in three different age groups of a paediatric sample. The mean MPOD value of 0.40 was consistent across age grouping, refractive error and gender. In view of the limited previous studies, comparisons are made with older age samples with the Visucam or paediatric populations with alternative MPOD measurement techniques. This discussion has been structured into a discussion of the individual variables which were investigated in Study 2.

Visucam Studies

A study assessing MPOD with the Visucam in 129 Caucasian subjects (mean age 56.6 years) determined a mean MPOD of 0.353 (SD 0.009) in 228 eyes (Pipis *et al.* 2013). A medium positive correlation between age and maximum MPOD was also found ($r = 0.35$, $p < .0001$). Pipis *et al* did not identify any difference in MPOD values between men and women. The mean MPOD of 0.40 found in Study 2 of the current work aligns well with the mean MPOD of 0.35 reported by Pipis *et al.* The slight difference in mean values could be

explained by the difference in participant age between Study 2 (mean age of 11.1y; SD 2.8y) and Pipis 2013 *et al.*, (56.6y; SD 14.7y). The higher SD value for the mean age in the Pipis *et al.* study is due to the considerably larger spread of age in the participants. The lower mean MPOD in the Pipis *et al.* study, compared to Study 2 could be due to a decline in MPOD with age. This has been reported by Yuying *et al* (2015) who investigated MPOD with the Visucam in a healthy Chinese population of 441 participants aged 3-81 years. The mean MPOD was 0.303 ± 0.097. A significant inverse relationship was found between age and MPOD ($\beta = -0.716$, $p < 0.001$). Participants with no lens opacities had higher MPOD values than those with moderate lens opacities ($p < 0.001$). This level of MPOD is approximately 25% lower than the mean value of 0.40 in Study 2. There could be a number of reasons for this: different ethnicity between Yuying's study population and the western population of Study 2. In addition, another factor could relate to the older age group being studied in the Yuying study and MPOD decreases with age. A gradual decline in MPOD with advancing age has previously been reported (Yu *et al.* 2012).

Creuzot-Garcher *et al* (2014) examined mean MPOD with the Visucam in 67 participants aged 23-30 years and reported a mean MPOD 0.35 (SD not provided). This mean MPOD value is more similar to Study 2's mean MPOD value of 0.40. Participants in the Creuzot-Garcher study had a mean age of 25 years (SD not provided), nearly 15 years older than Study 2's participant mean age of 11.1 years (SD 2.8y). The difference in mean MPOD between the Study 2 and Creuzot-Garcher study is within the CoR value of 0.1, it is therefore difficult to draw conclusions. However, the possibility of age related decline in MPOD cannot be discounted.

In a small scale study undertaken by Dawczynski *et al.* (2011) with the Visucam 500, three subjects were compared, one healthy subject, one subject with dry AMD and one subject with exudative AMD. Age and subject characteristics were not documented. The results are shown in table A4.1 (end of Appendix 4). It is difficult to draw comparison to Study 2, as there were only three subjects in the Dawczynski study. Examining the max OD figures of 0.69 in the healthy patient compares to the very highest value in Study 2 of 0.74. The second subject in the Dawczynski study (with dry AMD) had a max OD MPOD value of 0.43.The third subject with exudative AMD had a max OD value of 0.35, approximately half the value of the first normal subject. The MPOD value of 0.43 in the dry AMD patient is higher than the mean MPOD value in Study 2 of 0.40. This could indicate that the dry AMD patient in the Dawczynski study when younger had significantly higher MPOD and development of AMD has started as the individuals MPOD has fallen. Dawczynski concluded that MPOD measures should be performed as soon as possible in patients with

AMD, using these measured changes over time as a progression parameter to forecast the future course of macular disease. Dawczynski believed that MPOD measurement could become a form of examination for AMD comparable to visual field tests or measurement of nerve fibre layer thickness for glaucoma. The study concluded that MPOD measurement could enable long-term monitoring and risk assessment of the progression of macular changes in a practical way. The most significant draw-back of this study was the obvious limitation of the number of subjects.

Other adult based FR studies are summarised in Table A4.3 (End of Appendix 4). The MPOD ranges were between 0.13 (Bour *et al.* 2002) to 0.8 (Brindley and Wilmer, 1952). The unweighted mean MPOD from the 14 listed studies in table A4.3 was 0.35. This is slightly lower than the mean MPOD from Study 2 of 0.40.

Paediatric MPOD Studies

As previously referred to, the Chinese HFP study (Zhu *et al.* 2012) reported paediatric MPOD to be relatively high - mean 0.56 (SD 0.25) with no significant gender differences. There are at least two possible explanations for the differences found in the Chinese paediatric study. Firstly, subjective techniques in Chinese six year olds could be potentially inaccurate due to test comprehension issues. Secondly, there exists the potential for ethnic differences in MPOD, and ethnic differences in the prevalence of macular degeneration.

Bernstein *et al.* (2013) examined 51 infants and children ranging from preterm to age seven years. The team used blue light reflectance to image the MP in premature babies with of retinopathy of prematurity (ROP) screening and in children aged less than seven years who were undergoing examinations under anaesthesia for other reasons. The investigators correlated the MPOD with skin carotenoid levels measured by resonance Raman spectroscopy, serum carotenoids measured by high-performance liquid chromatography, and dietary carotenoid intake. This was determined based on a three day diet history. Bottled breast milk and formula-fed infant L intakes were calculated based on volume consumed and HPLC analysis of mother's milk or manufacturers' reports of L content. The MPOD correlated significantly with age ($r = 0.36$; $p = 0.014$), with serum L + Z ($r = 0.44$; $p = 0.005$) and with skin carotenoid levels ($r = 0.42$; $p = 0.011$), but not with dietary L + Z intake ($r = 0.13$; $p = 0.50$). Bernstein found that all premature infants had undetectable MP, and most had unusually low serum and skin carotenoid concentrations. They found a steady rise in MPOD over the first seven years of life, eventually approaching average levels found in adult populations, and nearly all of the subjects with measurable MPOD had narrow, radially

symmetric MP patterns, in contrast to the more variable patterns reported in adults (Sharifzadeh *et al.* 2006).

Refractive error

In Study 2 there was a negligible difference in MPOD between myopia, hyperopia or emmetropia. For myopia n= 8, mean MPOD 0.39 (SD 0.03), hyperopia n= 40, mean MPOD 0.40 (SD 0.07) and emmetropia n=29 mean MPOD 0.40 (SD 0.05) These results align with the findings of Neelam *et al.* (2006) who investigated MP in relation to ocular biometry. The mean MPOD was 0.31 and 0.30 in the right and left eyes, respectively. No demonstrable relationship was observed between MPOD and axial length ($r = 0.09$, $p = 0.23$), anterior chamber depth ($r = 0.09$, $p = 0.23$) or vitreous chamber depth ($r = 0.15$, $p = 0.05$).

The previously discussed Chinese paediatric MPOD study carried out by Zhu *et al.* (2012) investigated MPOD with a Macular Metrics II Densitometer (Macular Metric, Providence, RI, USA) using a published protocol (Wooten *et al.*1999) with the measurement reference location set at 7°. Amongst other variables the research team assessed MPOD in relation to refractive error. Ninety-four healthy Chinese children, 6 to 12 years old, were recruited to the study. The spherical equivalent range of refractive error was –5.50 to +4.37 D as measured by auto refraction following cycloplegia. There was no significant correlation found between MPOD and spherical equivalent refraction ($r = –0.03$, $p = 0.84$) however there was an inverse relationship between MPOD and MFT ($R = –0.66$, $p = 0.028$) and a positive relationship between MPOD and CFT ($R = 0.67$, $p = 0.025$) for the myopia group. (See table A4.2 end of Appendix 4). Another study has correlated hyperopic refractive error and shorter axial length with AMD in an Asian population aged 40-80 years (Lavanya *et al.* 2010).

Other larger scale studies have found no association between refractive error and AMD development. Wang *et al.* (2004) as a subsection of the Blue Mountain study, examined refractive status and the 5-year incidence of ARM. They found no association between baseline spherical equivalent refraction and 5-year incidence of late or early AMD. Hyperopic right eyes had slightly higher incident rates for late (0.8%) and early (6.3%) AMD compared with myopic (0.4% and 4.1%, respectively) or emmetropic (0.5% and 5.0%, respectively) right eyes. After multivariable adjustment, the study found no significant association between hyperopia and the 5-year incidence of early or late AMD.

Gender

In Study 2 of this thesis there was no statistically significant difference between paediatric male and female MPOD ($p = 0.44$). This has been reported in previous adult based MPOD studies.

Yuying *et al.* (2015) using the Visucam have reported that both max OD and mean OD were higher in male subjects than in female subjects, but without a statistical difference (for max OD, $p = 0.018$; for mean OD, $p = 0.013$).

Other non Visucam studies have shown variable gender results with no difference in MPOD (Dietzel *et al.* 2011a, Delori *et al.* 2001) or higher MPOD for men (Hammond and Caruso-Avery, 2000) or women (Nolan *et al.* 2010).

Iannoccone *et al.* (2007) investigated MPOD in a large biracial American sample of normal 79.1 +/- 3.2-year-old adults living in the Mid-south ($n = 222$; 52% female; 23% black) and found the mean MPOD to be 0.34 (SD 0.21). No relationship between MPOD and gender, iris pigmentation or smoking status was found (Iannoccone *et al.* 2007).

Tang *et al.* (2004) assessed MPOD with HFP in a study which also included an initial repeatability study. The group-averaged MPOD was 0.48 (S.D. 0.23). They found no gender difference in MPOD. The coefficient of variability for the Tang study was 7.2-8.0% and the CoR was 0.12. The group concluded that the MPOD of Chinese subjects did not differ greatly from the reported MPOD in White subjects.

Howells *et al.* (2013b) in a HFP based study, reported that male subjects had a higher mean MPOD than females (0.47 ± 0.13 vs. 0.41 ± 0.14, $p < 0.01$) (Howells, 2013b) (See Appendix 4, figure A4.19). This finding is in common with others findings (Hammond *et al.* 2000; Nolan *et al.* 2007; Mellerio *et al.* 2002; Bartlett *et al.* 2010c; Hammond *et al.* 1996a; Wooten *et al.* 1999; Berendschot *et al.* 2002; Broekmans *et al.* 2002; Lam *et al.* 2005; Yu *et al.* 2012) However, the converse has been reported (Nolan *et al.* 2010).

Iris pigmentation

In the same Howells *et al.* (2013b) study, the authors concluded a possible association between MPOD and iris colour. Figure A4.20 (Appendix 4) shows that darker iris pigmentation was associated with higher levels of MPOD.

Howells *et al's* findings align with Study 2, which found light iris pigmentation of mean MPOD 0.39 (n=49, SD0.04), and dark iris pigmentation of mean MPOD 0.41 (n=24, SD 0.08), indicating a trend for slightly higher MPOD in dark iris pigmentation, but this did not reach statistically significant levels (p =0.122). A larger sample size in a wider scale study may clarify if darker iris pigmentation is truly related to higher levels of MPOD.

Eyes with light-coloured irides transmit 100 times as much light as do those with dark brown irises (Van den Berg *et al.* 1991). In addition, light-coloured irides are associated with low choroidal melanin (Weiter *et al.* 1985; Menon *et al.* 1992). The melanin of the iris and choroid has been shown in animal experiments to confer substantial protection against light damage by reducing retinal irradiance (Rapp *et al.* 1980).

In a study by Hammond and Snodderly (1996b) 95 subjects were assessed; iris colour, MPOD, plasma concentrations of L and Z and beta-carotene, dietary intake of L and Z and beta-carotene, and total fat intake. Iris colour was determined by self-assessment and classified as blue or grey (group I), green or hazel (group II) or brown or black (group III). The MPOD was determined by measuring foveal and parafoveal sensitivities to lights of 460 and 550 nm, using HFP. Plasma carotenoid concentrations were measured using reverse-phase high-performance liquid chromatography. Dietary intake was determined by a detailed food-frequency questionnaire. Despite similarities in diet and in blood concentrations of carotenoids, significant differences in MPOD ($p < 0.02$) were found for different coloured irides (group I, n = 38, MPOD = 0.25; group II, n = 26, MPOD = 0.32; group III, n = 31, MPOD = 0.38). The difference between Hammonds and Snodderly light group I MPOD 0.25 and dark group III MPOD 0.38 represents a difference of approximately 35%, compared to Study 2's difference of 5%. This difference could be in part explained by the different method of stratifying the iris pigmentation with the addition of a group with green and hazel iris pigmentation. In Study 2, green iris pigmentation was classified into the lighter iris pigmentation group, whilst hazel iris pigmentation was classified into the dark iris pigmentation group. The use of HFP will give different values for MPOD determination in comparison to FR.

Hammond and Snodderly (1996b) postulated that the relationship between MPOD and iris colour may be the result of one or two factors: the evolution of a shared tendency to accumulate melanin and carotenoids due to similar environmental pressures (e.g. light and oxygen); and/or MPOD might be depleted due to the tendency for eyes with light irides to transmit more light than eyes with dark irides, causing increased oxidative stress. This second factor aligns with more recent hypothesis's relating the development of high UV

exposure to AMD. The MP was believed to limit retinal oxidative damage by absorbing incoming blue light and/or quenching ROS (Beatty *et al.* 2000b).

In a systematic review by Chalam *et al.* (2011) the authors examined previous epidemiologic evidence, which indicated a trend associating severity of light exposure and AMD. The Beaver Dam Eye study provided epidemiologic evidence that exposure to sunlight may be associated with the development of early AMD. The amount of time spent outdoors was significantly associated with the development of AMD (odds ratio [OR] 2.26; 95% confidence interval [CI] 1.06-4.81). Subjects exposed to the summer sun for more than five hours a day during their teens, in their 30s, and at the baseline examination were at a higher risk of developing increased retinal pigment (risk ratio [RR], 2.99; 95% confidence interval [CI], 1.18-7.60; p =.02) and early ARM (RR, 2.20; 95% CI, 1.02-4.73; p =.04) [corrected] ten years sooner, than those exposed to less than two hours per day during the same periods (Tomany *et al.* 2004).

Limitations of study

Study 2 was limited in number of subjects and constrained due to the time limited availability of resources. More subjects would allow a greater statistical power. Through the use of G Power 3.1 and relating MPOD to age, *post hoc* analysis a sample size of 73 would give an effect size (p) 0.16, critical t =1.29, to allow an α error probability of 0.1 and 0.54 power. Tripling the sample size and assuming the same effect size (p=0.16) gives 0.87 power. A small sample size, results in a difficulty in finding significant relationships from the data. Statistical tests normally require large sample sizes to ensure a representative distribution of the population and to be considered representative of groups of people to whom results can be generalised or transferred. Increased sample size and longer term follow up in subsequent larger scale paediatric MPOD studies is recommended. The findings from the pilot based Study 2 should help future researchers to determine necessary sample sizes (see chapter 7 discussion section 7.5).

Measures used to collect the data

There has been emerging criticism of the validity in using the Visucam to assess MPOD. This criticism has been led by Dennison *et al.* (2013) in their study which assessed concordance of MP measures using HFP, dual-wavelength AF, and single-wavelength FR. Dennison *et al.* (2013) reported that the Visucam under-estimated MPOD values and obtained clustered MPOD values around 0.30-0.40. However, there was no documentation of calibration of their Visucam and no evidence of ensuring optical clarity of the Visucam lens during the data collection. During Study 2, within the familiarisation period with the Visucam,

it was noted that a compromised clarity of the objective lens in the Visucam resulted in low and clustered MPOD values, most likely due to the reduced reflectance from the MP that the Visucam sensor was able to detect (see Visucam Chapter 3).

Examiner measurement bias

Whether conscious or not, this is the instrumentation bias that follows an examiners individual technique and can result in consistent inaccuracy of measurement. If the examiners technique is sub-optimal, then it follows that the data collected may be inconsistent. The two week familiarisation period with the Visucam was specifically implemented to reduce the effects of examiner measurement bias.

Future research

Further paediatric MPOD studies are required. Lack of prior paediatric MPOD research studies results in scarce data available on paediatric MPOD, as previously documented by both Bernstein (2013) and Zhu (2012). The lack of paediatric MPOD studies compounds the need for further studies in this area. Larger scale assessment as to how well HFP, FR, AF provide repeatable measures would be beneficial to the research community. Larger scale studies, with greater financial resources, would allow greater statistical power.
Studies using FR and the Visucam in older "normal" subjects and older subjects with macular pathology are also required. This would enhance knowledge of MPOD measured from FR and any predictive value it may have in macular disease processes.

Newer methods of objectively assessing MPOD may be considered with the Heidelberg Spectralis Modified Scanning Laser Ophthalmoscope. Dennison reported that agreement was strong between the Densitometer and Spectralis at all central eccentricities (e.g. at 0.25° eccentricity: accuracy = 0.97, precision = 0.90, corrected cc = 0.87) (Dennison *et al.* 2013).

Additional Visucam studies with diet and or L based supplementation will provide valuable information in the ability for FR to chart efficacy of diet/treatment interventions. Studies which provide MP density profiles in relation to mapped AMD profiles may provide further support in MP having a protective role in preventing AMD development.

4.5

Conclusions

In view of the limited power of Study 2 it is difficult to draw definitive conclusions. Lighter irides tended to be associated with a slightly lower MPOD although this did not reach statistically significant levels. Darker irides tend to be associated with a slightly higher MPOD although this did not reach statistically significant levels. Refractive error and gender were independent of MPOD. A single factor ANOVA showed mean MPOD did not vary between the three different age groups of 4-8, 8-12, 12-16 years ($p=0.98$). This was confirmed with linear regression analysis. This could indicate that MPOD develops to near adult levels by 0 - 4 years of age and remains constant during the infantile and juvenile period, further larger scale studies wielding greater power could further investigate this.

CHAPTER 5 – Study 3 - Assessing the repeatability of the MPS in a paediatric sample

In this chapter an account and discussion of the results of the repeatability study of the MPS are described. This study was termed Study 3. The purpose of Study 3 was to determine the consistency of the MPS in providing MPOD measures. This would enable an understanding of the degree of instrument noise with the MPS. The optical principles underpinning the MPS are discussed in Chapter 2. The method and use of the MPS are reported in the procedural section of this chapter. The results have been documented in tabular format, in Bland and Altman plots and a discussion of how they relate to previous repeatability studies. Limitations are also discussed. A conclusion, with further consideration of the implications of the results culminates this chapter.

5.1
Introduction

The purpose of Study 3 was to determine the CoR of the MPS in its measurement of MPOD in a paediatric sample. The justification for pursuing this research relates to the emergence of assessing MPOD in clinical practice. Measuring paediatric MPOD may increase understanding of juvenile macular disease. In addition it should further enhance understanding of the ocular ageing process by providing MPOD figures at the earliest (measurable) stage of life. This second consideration should enable researchers and clinicians to gain an enhanced understanding as to how MP levels alter as we age.

Prior to the wide spread use of devices for measuring paediatric MPOD, the accuracy and repeatability of these instruments are required. Chapter 5 consists of a study investigating the repeatability of the MPS, a commercial device designed to measure MPOD in the clinical environment.

5.2
Method

Eleven naïve subjects underwent two MPOD measures, separated by two to four weeks. Due to limited resources and time constraints in accessing the MPS, a sample size of 11 was agreed by the research team for the pilot based Study 3. This sample size aligned with previous MPOD repeatability studies (Koh *et al.,* 2004, van der Veen *et al.,* 2009a, van der Veen *et al.,* 2009b). The measurement of MPOD with the MPS is more time consuming than objective MPOD techniques.

Using G Power 3.1 and relating the correlation of the two MPOD measures, coefficient of determination 0.55, *post hoc* analysis of a sample size 11 would give an effect size (p) 0.74, critical t =1.83, to allow an α error probability of 0.05 and power= 0.95.

The measurements were conducted by the same researcher (the author) in the same environment. The study was conducted between August and November 2014. Prior to undertaking Study 3, there was a two-week familiarisation period with the MPS in order to become conversant with the technique.

Research Subjects

Recruitment of a consented paediatric sample between the age of 4-16 years (no younger than four, no older than 16) was undertaken. MPOD was measured using the MPS, with two measurements for the right eye, the mean of these two results were recorded (see clinical procedure).

Consent for inclusion was gained from the parent/guardian and the subject. The study was approved by the Ethics Committee of Aston University (see Appendix 5 figure A5.1). Full confidentiality of the records was maintained and subject numbers, not names, were used. Higher degrees of refractive error were excluded (see following exclusion/inclusion criteria). Subjects with any form of previous or current ophthalmic disease were excluded from the study to ensure a normal sample was being studied.

Parents/guardians were given an information sheet detailing the nature of the research, allowing informed consent to be given. (See Appendix 5, A5.3 - Information sheet/consent form). In addition a child information sheet was also provided to ensure subjects understood how the MPOD procedure would occur. A signature for consent was also obtained from the

subject. (See Appendix 5, A5.4 child Info sheet and child consent form). The parent and/or child were free to withdraw from the research at any given time, without prejudice or sanction.

Subjects Inclusion/exclusion criteria

Subjects for this study were recruited at an optometric practice, following an NHS eye examination.

Inclusion criteria:

- Any ethnicity
- Age of ≥ 4 - ≤16 years on MPOD acquisition
- Clear and consistent data acquisition to allow computerised assistance in the determination of the MPOD measurements
- Clear fundus and media assessment
- VA better than 6/9 in the measured eye

Exclusion criteria:

- Under 4 years of age/poor co-operation or over 16 years
- Refractive error > -3.00 and >+5.00 or >-2.50 cylinder (re potential amblyopia/subnormal VA development
- Blurred/poor quality/inconsistent data acquisition
- Diagnosed with any type or form of ophthalmic or retinal pathology (including strabismus)
- Declined parent/guardian consent at any stage of the study
- Any currently, suspected or previously diagnosed epilepsy

Materials

MPS

Figure 5.1: The MPS attached to a personal computer, simulated within the practice environment

The MPS was supplied by Topcon, Newbury, U.K to the Aston University Ophthalmic Research Group.

5.2.2

Procedure

The following measures were obtained in Study 3.

Date, age, gender, MPOD measured twice using the MPS and the mean measure represented MPOD 1. The MPOD measure was repeated within a 2-4 week time period and again measured twice using the MPS and the mean measure represented MPOD 2.

The testing room was an Optometry consulting room 4 m x 3 m squared with blackout blinded windows and housed the MPS attached to a standard desktop PC running Windows 7. Total darkness was achievable within the room.

Subjects were instructed in the operation of the MPS device. The room was then darkened. This was to minimise potential distractions for the subject. The subject remained seated and

positioned their right eye into the viewing piece of the MPS. The MPOD measure attempt was only for the right eye, with the aim of obtaining a clear MPOD within two measurement attempts only, to provide a mean figure. Should the result have been compromised with sustained inconsistent subject responses, then the results were recorded but rejected due to the potential for substandard data acquisition.

The eye not being tested was occluded, subjects wore their habitual refractive correction and were asked to place their forehead in position ensuring the test eye was centred on the appropriate target within the instrument. The central target was a 1° circular stimulus composed of blue (465 nm) and green (530 nm) LEDs. The subject fixated directly at the stimulus while the alternation rate between the blue and green was reduced down from 60 Hz. Subjects were required to make flicker matches using two wavelengths of light, one of which was absorbed by the MP (465 nm) and another (530 nm) which was not. Subjects were instructed to press a button as soon as flicker was detected, in contrast to the more conventional HFP approach where they are required to adjust a green-blue luminance ratio until flicker is minimised. At the point when they first detected flicker, the subject immediately pressed a response button and this plotted a point on a graph of the MPS software, which was visible to the examiner on a computer screen. Once the flicker had been perceived, the process started again. The first five responses were used to ascertain the flicker sensitivity of the subject. Based on this, the main part of the test began and the subject responded to a series of green–blue ratios until a V-shaped curve was plotted on the computer screen. The minimum point on the curve corresponded to equiluminance of the blue and green lights.

In view of the limited attention of the subjects and the optically clear media of each subject, instead of plotting a peripheral MPOD curve, age matched compensation was taken to give the final MPOD figure. This facility was available within the MPS software, and was agreed upon prior to undertaking Study 3 (see Appendix 5, figure A5.2, no significant lenticular yellowing in the studied age group). The calibration equation is based on published data from Pokorny *et al.*, (1997) and Murray *et al.*, (2013c) observations of different age groups. It has been demonstrated theoretically and empirically (Makridaki *et al.*, 2009) that the minimum of the peripheral flicker settings is age dependent, and will shift rightwards along the x-axis (green blue ratio in dB) of a peripheral flicker settings graph, according to the following expression:

Min_p (dB) - 4 + age * 0.02

Where Min_p is the minimum flicker point in dBs for peripheral viewing.

Murray *et al.,* (2013c) have reported a close relationship between the age estimated and true MPOD from central and peripheral measurement. The mean for their age-estimate method was 0.38±0.14, and the mean for the true MPOD based on central and peripheral minima was 0.37±0.14. There was no statistical difference between the two measures (p=0.641) and the Pearson r was 0.89 (p<0.001). The co-efficient of determination (r^2) was 0.8 indicating that 80%of the variability in the estimated MPOD can be accounted for in terms of the variability in the central vs peripheral measurement. Murray *et al.,* (2013c) concluded that the age-estimate technique represents the actual MPOD extremely well. This, considered in conjunction with the potential limited attention span of the paediatric subjects in Study 3, resulted in the age matched compensation software being used in Study 3.

The follow-up MPOD measure (MPOD 2) was taken between two and four weeks following the first MPOD assessment. In both visits it was the author who was the MPS operator. This was to eliminate the possibility of inter practitioner variability on obtaining the MPOD measures. It also allowed exactly the same procedure to be followed for both visits.

5.3

Results

The results of Study 3 (n=11) are given in raw data format in table 5.1. The mean age was 8 years 5 months (SD 2 years 9 months) and the gender distribution as four males and seven females. The mean MPOD 1 (first assessment) was 0.43 (SD 0.08) and mean MPOD 2 (repeat assessment) was 0.39 (SD 0.1).

Subject	Mean MPOD 1	Mean MPOD2	MPOD 1 minus MPOD 2	Mean MPOD	Age (months)	Gender
1	0.48	0.43	0.05	0.46	182	F
2	0.58	0.38	0.20	0.48	122	M
3	0.38	0.43	-0.05	0.41	182	F
4	0.29	0.24	0.05	0.27	163	F
5	0.43	0.43	0.00	0.43	176	M
6	0.48	0.53	-0.05	0.51	149	M
7	0.34	0.14	0.20	0.24	71	F
8	0.43	0.48	-0.05	0.46	135	F
9	0.53	0.43	0.10	0.48	178	F
10	0.43	0.43	0.00	0.43	131	F
11	0.38	0.43	-0.05	0.41	150	M
Sum Total	4.75	4.35	0.40	4.55	1639	7F
Mean	0.43	0.40	0.04	0.41	149	4M
Max	0.58	0.53	0.20	0.51	182	
Min	0.29	0.14	-0.05	0.24	71	
St Dev	0.08	0.11	0.10	0.09	34	
Lower limits			-0.15			
Upper limits			0.22			

Table 5.1 Results of Study 3

Since Kolmogorov-Smirnov and Shapiro Wilk have values both >0.05 (Appendix 5 table A5.2) the difference in MPOD distribution of data is normal, the histogram shows a relatively normal distribution of MPOD and the Q-Q plot (see Appendix 5, figure 5.5 and figure A5.6) shows data that approximates to the diagonal line of normality. Assessment of normality in data is recommended prior to Bland Altman analysis.

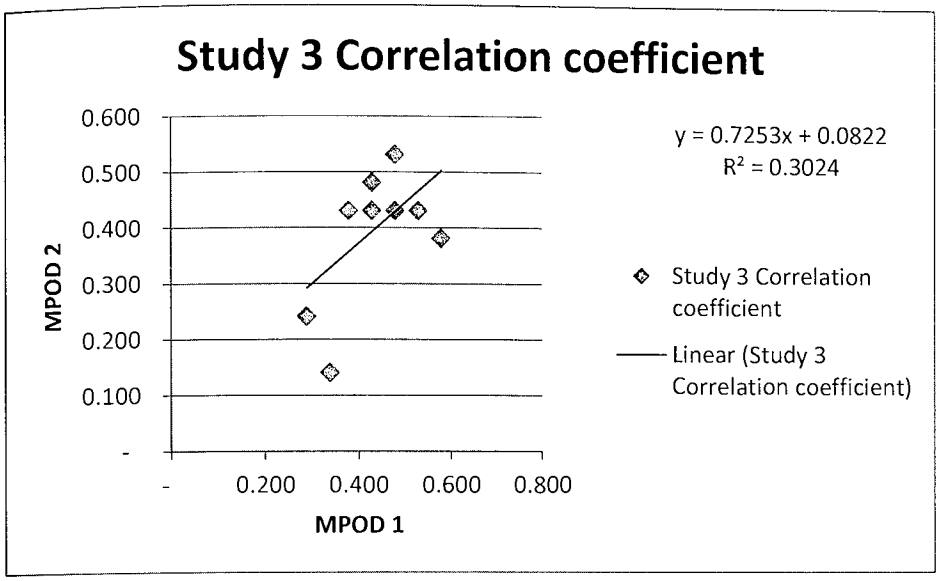


Figure 5.2 Correlation of MPOD 1 and MPOD 2 in Study 3

Correlation of MPOD 1 and MPOD 2, where R^2 represents the coefficient of determination, which gives a correlation coefficient of 0.55. A paired t test was used to assess the agreement between the two measures, p=0.23, and therefore fail to reject the null hypothesis and there is a significant difference between the measures (see Appendix 5, table A5.3).

Study 3 had a correlation coefficient, for MPOD 1 and MPOD 2 - of +0.55 indicating some limited correlation between the two measures. The Bland Altman plot in figure 5.3 shows all subjects fall within the upper and lower 95% confidence limits but the observed spread of data was greater than the Visucam Bland Altman in Study 1.

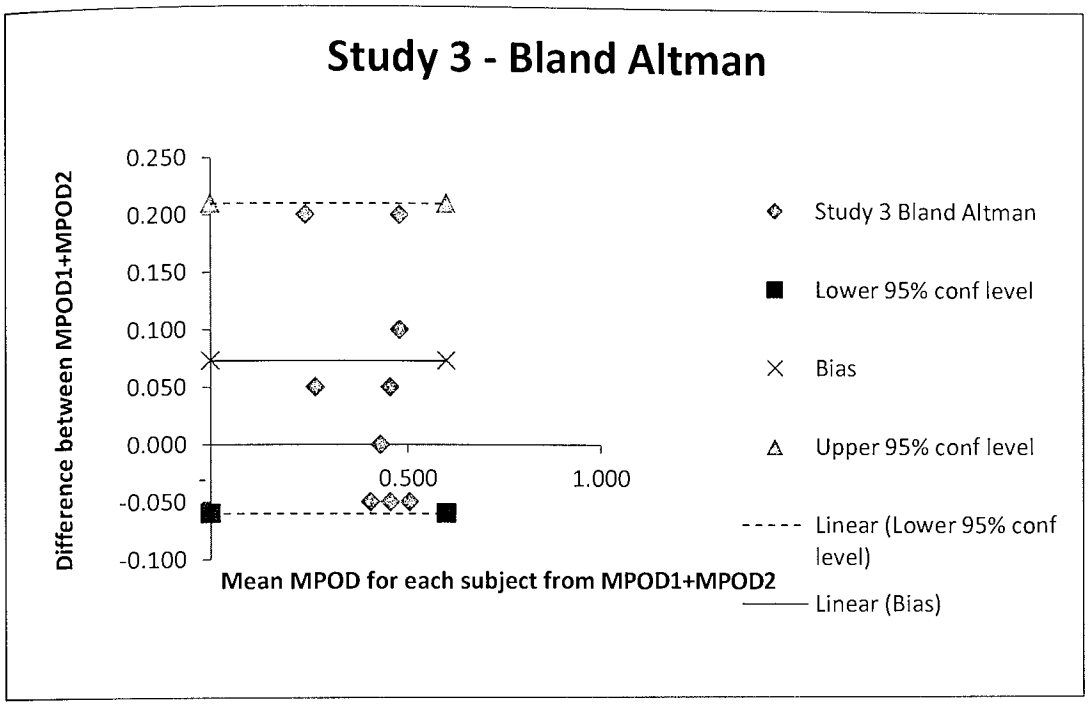


Figure 5.3 - Bland Altman for repeated measures with MPS with upper and lower 95% confidence limits

Accurate analysis of test–retest data can be achieved using the CoR which gives the 95% confidence limits for the amount of difference between two sets of results (Bland *et al.* 1986; Elliot *et al.* 1988). It is calculated as 1.96 multiplied by the SD of the mean differences between the two sets of data.

The mean difference between measures was 0.036 (SD = 0.095). Therefore, 0.095 x 1.96= 0.18 (upper range CoR = 0.18 + 0.095 = 0.275, lower range CoR= 0.18 - 0.095 = 0.085)
The mean of the means of the two MPOD measures for the MPS was 0.41
Therefore 0.18/0.41 is 43.9% - a moderate to large sized effect.

5.4
Discussion

The assessment of the repeatability of an instrument is critical if the instrument is to be used longitudinally to monitor a measured value. Determining instrument noise allows clinicians to understand when a clinically significant difference in measurement has occurred. Assessing the repeatability of the MPS in paediatric MPOD provides useful clinical data at an early stage of life and how suitable it may be to use the MPS for this purpose.

Previous research conducted by Bartlett *et al.* (2010c) assessed the MPS with 40 subjects (age range 18 to 50 years, mean 25.4±8.2 years) and suggested that only increases or decreases in MPOD of more than 0.33 can be classed as clinically significant. Study 3 found a CoR of 0.18, and 0.18/0.41 is 43.9% - a moderate to large sized effect.

This means that any difference in consecutive MPOD measures needs to be greater than approximately 0.18 to be considered as anything other than instrument noise. Study 3 (and especially the Bartlett study) indicates that the MPS is a poor instrument for assessing MPOD on a longitudinal basis; large differences in MPOD need to occur in order for them to be considered anything other than instrument noise. Caution is required when interpreting the results of studies which use the MPS to assess the efficacy of dietary treatment supplements on MPOD. In addition, the Bartlett study also documented that if two different operators are assessing MPOD with the MPS intra-session, only increases or decreases in MPOD of more than 0.26 can be classed as clinically significant. This indicated that different examiners techniques can result in an additional 0.26 variation in MPOD. This factor, coupled with the CoR of 0.18 indicate that different clinicians measuring MPOD longitudinally will have significant difficulty in determining whether differences in MPOD are clinically significant or attributable to instrument noise.

Study	n	Age range	Ocular health status	Experience of with HFP	Reliability statistics
Hammond and Fuld 1992	10	19-42	Healthy	7 naïve subjects	CoR 0.06
Hammond, Fuld and Curran-Celentano 1995/96a	20	19-22	Healthy	All naive	CoR 0.20
Hammond *et al.* 1997	13	30-65	Healthy	Not reported	CoR 0.14
Hammond and Caruso-Avery 2000	8 (217 in full study)	17-92	Healthy	All naive	CoR 0.08
Snodderly *et al.* 2004	48	50-79	15 self-reported eye disease	All naive	CoR 0.19
Koh *et al.* 2004	13	Mean age ARM patients 70 +- 7 years Mean age matched controls 67 +- 5 years	ARM and healthy controls	Not reported	CoR 0.04
Tang *et al.* 2004	6 (68 subjects in full study)	22-23	Healthy	Not reported	CoR 0.12
van der Veen *et al.* 2009a+b	11 (26 subjects in full study)	22-64	Healthy	Not reported	CoR 0.09
Bartlett, Acton and Eperjesi 2010c	40	18-50	Healthy	All naive	CoR 0.33 MPS
Bartlett *et al.* 2010b	38		Healthy	All naive	CoR 0.58 Macuscope

Table 5.2 – Summary of previous HFP CoR studies

Adapted from Bartlett *et al.* 2010a

Table 5.2 shows a range of CoR between 0.06 (Hammond and Fuld, 1992) to 0.33 Bartlett *et al.* 2010c. These statistics relate to adult based repeatability studies with HFP. The variation

in the results could be attributable to different operator techniques (and the acceptance of V shaped curves) and different technologies being employed to assess HFP MPOD.

McCorkle *et al.* (2015) assessed the agreement in measures in 66 subjects, with HFP using the Macular Densitometer. Among the full sample, there was no significant difference between MPOD at the two sessions ($p = 0.59$—session 1: 0.61 ± 0.28; session 2: 0.63 ± 0.27). They reported a Cronbach alpha value of 0.70 between the two sessions. The Cronbach alpha value is a measure of the numerical CoR. A Cronbach alpha value of 0.7 is deemed borderline acceptable in terms of reliability (Nunnaly, 1978). This was lower than other studies with the same device, reporting intersession Cronbach values of 0.89 (Wooten *et al*,. 1999) and 0.97 (Hammond & Caruso-Avery, 2000). McCorkle *et al.* (2015) also reported that limiting the examiners for test-retest improves the reliability of the measurement in children from low to moderate (Cronbach α 0.57 and 0.72 respectively).

Study 3 CoR results are higher than the results found by Beatty *et al.* (2001), who reported a coefficient of reproducibility of 0.08 and a CoR of 0.09 for an instrument employing HFP to measure MPOD. The absorption spectrum for MP was generated by taking optical density measurements, with central and peripheral fixation, between 450 and 560 nm. Reproducibility of a method/test can be defined as the closeness of the agreement between independent results obtained with the same method on an identical subject. In mathematical terms, it is the variability of the average values obtained by several observers while measuring the same item (inter-observer variability) Slezak *et al.* (2010). As acknowledged by Bartlett *et al.* these differences may be due to the use of an HFP instrument manufactured for a clinical setting rather than an HFP instrument designed for research purposes.

In another repeatability study of the MPS, the authors reported a cc of 0.97 ($p<0.001$) for re-test data on 11 subjects aged 20-90 years (van der Veen *et al.* 2009a). Mean test-retest variability was 0.0195 ± 0.047. The research team also calculated the repeatability of measurements provided by the mean of differences divided by the mean values from the two estimates. In this case this was 11.7%. Bartlett *et al* criticised the van der Veen study, in that the measurement was repeated five times at the first data collection session. Bartlett *et al* suggested that their own study was more realistic from a clinical perspective, in which subjects were allowed just one practice run. This provided a more realistic impression of repeatability of the MPS in a clinical, rather than research, setting.

In a study undertaken by Loughman *et al.* (2012) the test–retest variability for the MPS was investigated alongside the Macular Metrics Densitometer. Twenty five subjects aged 21-61 years, had MPOD measured on three occasions over a one week period. All data was collected by a single operator. A CoR for the MPS was found, ranging from 0.18 to 0.21 (mean 0.19 ± 0.02). This correlates very well with the CoR of approximately 0.18 with the paediatric age group found in Study 3. The slightly higher variability in older subjects could be due to different reaction times and response criterions set by different age groups.

In the Loughman *et al.* (2012) study, agreement between the MPS and the Macular Densitometer was also studied. The mean difference between instruments was statistically significant, with a bias of lower MPOD with the MPS, reflected in 95% limits of agreement of 0.1 ± 0.27, indicating only moderate agreement between the two sets of readings (Bland & Altman 1986). The underestimation in MPOD values yielded by the MPS was in the range 0.05–0.15. The authors also considered subjects whose results were statistical outliers. Contrary to the observations of van der Veen *et al.* (2009a, 2009b), the observed discrepancy between the MPS and the Macular Metrics Densitometer was not systematic and not amenable to adjustment by means of a correction factor. The finding of outlier results, was also found in Study 3, where subject 2 and subject 7 had a between visit difference of 0.2. Determining what the reason for these differences may allow a correction factor to be calculated in the future. This may then allow more direct comparisons between the MPS and other MPOD assessment technologies, with the application of a correction factor if required.

In Study 3, the centre only measure was taken; the peripheral measure was estimated from the age of the subject and their expected level of lens yellowing (Makridaki et al. 2009). Peripheral measures were not taken, due to the ocular media of the paediatric subjects being uniformly clear and a documented minimal to non-existent lenticular yellowing in this age group (see Appendix 5, figure A5.2, slides 1-9).

Peripheral viewing with the MPS uses a similar method to central viewing, with a reference location at 8 degrees retinal eccentricity (van der Veen *et al.* 2009b). An internal algorithm calculates the MPOD using the difference between the central and peripheral minima (the larger the difference, the higher the MPOD), although this can also be calculated manually.

The MPS has been criticised for its assessment of the peripheral measure by Loughman *et al.* (2012). The MPS employs one stimulus for the central and peripheral measures, whereas the Macular Densitometer employs two stimuli for the peripheral measurement only and one

target centrally. Invariably, subjects reported difficulty completing the peripheral measurement, when using the MPS, whereas no such difficulty was reported for the Macular Densitometer. Loughman *et al.* (2012) hypothesized that the difference in peripheral stimulus size was contributing to the greater relative difficulty experienced by subjects using the MPS and could explain the exclusion of three subjects unable to complete the peripheral measures using the MPS. Loughman hypothesized that the difference in eccentricity of the peripheral reference target (7° for the Macular Densitometer and 8° for the MPS) could be a potential source of differences in the calculated MP values (as the calculation of MP is based on the log ratio of central versus reference values). As the MPS employs a more eccentric reference stimulus, it might be expected that this technique would derive higher MP values, if the effect was significant. The MPS, however, appears to underestimate MPOD in comparison with the Macular Densitometer, so it is unlikely that the difference in reference location can explain the mean difference between devices.

A potential source of variability in the repeatability of the MPS, relates to the technique it employs to collect data. A suprathreshold flicker rate is gradually reduced at a set rate of 6 Hz per second, and the subject responds by pressing a button to indicate the point at which flicker is detected. The rate of flicker decrease is a compromise between test time and differences in subject reaction times (van der Veen *et al.* 2009a). Although it has previously been shown that reaction times vary little across age (Porciatti *et al.* 1999), response times are more difficult to account for. It is possible that subject threshold criteria could change during the course of a measurement session, particularly as task complexity increases from the central to peripheral target testing (Madden & Allen 1995; Hommel *et al.* 2004). This change in response criterion could potentially be difficult for an examiner to detect and could contribute to poor results on either the first measure or the follow up repeat measure.

Furthermore, the MPS is unable to provide a useful measure of subject performance reliability. It is at the examiners discretion whether to accept the MPOD result or not by assessing whether a 'typical' V-shaped flicker response curve has been generated. The accompanying documentation with the MPS has stated that 'irregularities in data' were typical with the result that the shape of the V curve can vary between individual subjects. This makes interpretation of the curve, and reliability of the result, dependent on examiner skill and training and liable to significant variation, depending on the criterion individual examiners set to accept or reject individual subjects result. Loughman *et al.* (2012) have postulated that this subjective decision making by examiners could represent a partial explanation for the poor CoR reported by Bartlett *et al.* (2010c). It has been suggested that the number of subjects in the Bartlett study with significant variation in test–retest MPOD

values represents operator error (inappropriate acceptance of low-quality V-shaped flicker response functions), rather than measurement noise (Murray *et al.* 2011). Loughman *et al.* (2012) supported Bartlett *et al.* (2010c) that MPOD values obtained using the MPS may be significantly affected by examiner skill level and training, and this considered in conjunction with the limited means to determine patient performance acceptability, provides an MPOD result with the MPS which can be considered unreliable and potentially misleading.

In response to this, Howells *et al.* (2013a) conducted a study with a protocol for improved MPS reliability in terms of inter-session repeatability, in 27 subjects aged 19-52 years. After short central and peripheral practices, each subject completed two or three central and peripheral tests at each visit (visits were 7-14 days apart). The total procedure time per visit was between 10 and 20 minutes. This protocol resulted in an inter-session CoR of 0.08.

Howells *et al.* (2013a) also conducted an intra-session repeatability study. Each subject had their right eye (RE) and left eye (LE) assessed alternately, with three repeats per eye. The total procedure time, including regular, short breaks to help avoid fatigue, was approximately 45 minutes. Although the Howells protocol may have provided approximately 40% improvement in the CoR, the lengthy testing times implemented to achieve this would make it difficult to recommend the protocol in community optometric practice.

Considering the MPS results in relation to the Visucam in Study 1, which found a CoR of 0.1 and mean MPOD 0.39, = 0.1/0.39= 25.6%, the MPS at CoR 0.18 and 43.9% resulted in nearly twice the level of variation in MPOD readings/instrument noise. As previously documented, this is likely due to the subjective method in gathering data. This, considered in conjunction with the examiners' judgement as to what constitutes an acceptable V shaped, results in a higher degree of variability, particularly for younger subjects.

The findings from Study 3 partially support the concerns raised by Bartlett *et al.* (2010) that if MPOD is being monitored longitudinally to assess the effect of an intervention, any change less than 0.33 units should not be considered clinically significant as it is very likely to be due to measurement noise. Study 3 indicates that this value may be closer to 0.18. If research optometrists have been criticised by one of the MPS designers (Murray *et al.* 2011) then it is does not seem plausible that community optometrists or their support staff would be able to achieve reliable and repeatable results with the MPS.

Limitations of study

As with other HFP studies, Study 3 was limited by the subjective method of the testing procedure for the paediatric participants. The subjective nature of the examiner in accepting or rejecting V shaped curves added further variability.

5.5

Conclusion

The CoR for the MPS was 0.18, giving 43.9% - a moderate to large sized effect. This finding is slightly lower than previous studies (Bartlett *et al.* 2010c, Loughman *et al.* 2012). The MPS has too poor repeatability as an instrument for the assessment of paediatric MPOD in a longitudinal study.

CHAPTER 6 – Study 4: Assessing mean MPOD in a paediatric sample with the MPS

In this chapter an account and discussion of the results of the paediatric MPOD MPS Study with diet history will be given. The purpose of Study 4 was to determine the mean value of MPOD in a consented paediatric sample using the MPS. In addition the relationship between MPOD and L consumption was investigated. The L intake was calculated with a three day diet history. Study 4 recruited 23 subjects following an NHS eye examination.

6.1
Introduction

The purpose of Study 4 was to determine the mean MPOD value in a consented paediatric sample, using the MPS. A three day diet history was used to retrospectively calculate the estimated mean daily L intake (see Appendix 6, figure A6.01 and A6.02.) This study was a follow on from Study 2 described in Chapter 4 in which the paediatric mean MPOD values using the Visucam were determined. The justification for pursuing this research relates to the emergence of assessing MPOD in clinical practice and to what extent this technology provides an accurate measure in a paediatric population. This information was requested by Bernstein *et al.* in their 2013 study where they concluded that a more complete knowledge of infant ocular carotenoid status would have considerable value in enhancing the diagnosis and treatment of a variety of ocular disorders throughout the human lifespan. (Bernstein *et al.* 2013) Diseases of aging such as AMD may depend on light damage and oxidative stress that accumulate over a lifetime, including the childhood years (Nussbaum *et al.* 1981; Loane *et al.* 2008; Ma *et al.* 2012; Sangiovanni *et al.* 2012). Studies providing normative data for paediatric MPOD will assist in fulfilling this gap. Furthermore, information relating to L intake and MPOD has been requested by Trieschmann *et al.* (2007). Study 4 was designed to add to the data addressing these requests.

6.2
Method

Subjects and inclusion/exclusion criteria

There were 23 subjects (11 of which had partaken in Study 3 and their visit one results were included), the MPOD was measured with the MPS, and each subject completed a three day diet history. This was carried out between August and November 2014. This number of subjects was agreed upon due to limited resources and time constraints in accessing the

MPS. This sample size aligned with previous MPOD HFP studies (Koh *et al.*, 2004, van der Veen *et al.*, 2009a, van der Veen *et al.*, 2009b). The measurement of MPOD with the MPS is more time consuming than objective MPOD techniques.

Post hoc analysis for MPOD related to age with G Power 3.1 for sample size n=23 and effect size p=0.31, alpha 0.1, critical t=1.32 and power=0.6. This indicates some under power of Study 4, however since this was a pilot based study this element of under power was unforeseen as the calculation of power required initial availability of results – at the time of study design there was no availability of paediatric MPOD data within a predominantly White population.

The subjects age ranged between 4-16 years (no younger than four, no older than 16). The MPOD was determined with two measurements of the right eye, the mean of these two results was the final recorded MPOD (see clinical procedure). Subjects for this study were recruited within an optometric practice, following an NHS eye examination.

Inclusion criteria:
Any ethnicity
Age of ≥ 4 - ≤16 years on MPOD acquisition
Clear fundus and media assessment
VA better than 6/9 in the measured eye

Exclusion criteria:
Under 4 years of age/poor co-op or over 16 years
Refractive error > -3.00 and >+5.00 or >-2.50 cylinder (re: potential amblyopia/subnormal VA development)
Blurred/poor quality/inconsistent data acquisition
Diagnosed with any type or form of ophthalmic or retinal pathology (including strabismus)
Declined parent/guardian consent at any stage of the study
Any suspected or previously diagnosed epilepsy

Consent for inclusion was gained from the parent/guardian and child themselves. The study was approved by the Ethics Committee of Aston University (see Appendix 5, figure A5.1). Full confidentiality of the records was maintained and subject numbers, not names, were used.

Parents/guardians were given an information paper sheet detailing the nature of the research, allowing informed consent to be given (see Appendix 6 figure A6.01). In addition a child information sheet was also provided to ensure the subjects understood how the MPOD procedure would occur, and a signature for consent was also obtained from the child. The parent and/or child were free to withdraw from the research at any given time, without prejudice or sanction – this was made clear in the information sheets supplied to parent/guardians and child subjects.

Materials – MPS, Diet History information sheets – (Appendix 6 figure A6.01)

Determination of MPOD

The MPS determines MPOD using HFP, with software incorporated into a desktop computer. The computer's internal hard drive provided safe, consistent data storage for the acquired results. The optical principles underpinning the MPS determining MPOD are discussed elsewhere (chapter 2).

Procedure

The following data was collected in Study 4: Date, age, gender, two MPOD measures using the MPS, three day diet history completed by parent/guardian.

The procedure and method was identical to Study 3. The mean MPOD value which was used in data analysis was the mean of two MPOD measures obtained in one sitting.

In view of the potential for limited attention of the subjects and the optically clear media of each subject, instead of plotting a peripheral MPOD curve, age matched compensation was taken to give the final MPOD figure. This facility was available within the MPS software (see Appendix 5 figure A5.2, no significant lenticular yellowing in the studied age group). During the testing procedure, the parent/guardian completed the three day diet history information. If the parent/guardian declined to provide this information (or supplied incomplete diet histories) the child was allowed to complete the test but the results were not included in the subsequent data analysis.

Lutein intake was determined through a completed three day diet history using Alacalc software (Alacalc, York's, UK). An estimated portion size of food was possible using Alacalc software in conjunction with L + Z content of foods (See Appendix 6, figure A6.02 – diet history data sheet).

Results analysis was undertaken with SPSS 21 and Microsoft Excel 2010 edition.

6.3
Results

Subject	MPOD 1	MPOD2	Mean	MPOD 1 minus MPOD 2	Age (months)	Gender	Mean daily lutein intake (mcgs)
1	0.77	0.77	0.77	0.00	171	F	792
2	0.53	0.50	0.52	0.03	96	F	405
3	0.77	0.62	0.70	0.15	173	F	588
4	0.59	0.58	0.59	0.01	190	M	807
5	0.24	0.19	0.22	0.05	191	F	1,585
6	0.34	0.29	0.32	0.05	114	M	874
7	0.38	0.53	0.46	-0.15	149	F	745
8	0.46	0.56	0.51	-0.10	130	F	384
9	0.38	0.29	0.34	0.09	160	F	460
10	0.34	0.34	0.34	0.00	83	M	685
11	0.38	0.38	0.38	0.00	99	M	593
12	0.34	0.34	0.34	0.00	118	M	247
13	0.48	0.43	0.46	0.05	182	F	833
14	0.58	0.38	0.48	0.20	122	M	500
15	0.38	0.43	0.41	-0.05	182	F	325
16	0.29	0.24	0.27	0.05	163	F	141
17	0.43	0.43	0.43	0.00	176	M	189
18	0.48	0.53	0.51	-0.05	149	M	161
19	0.34	0.14	0.24	0.20	71	F	269
20	0.43	0.48	0.46	-0.05	135	F	485
21	0.53	0.43	0.48	0.10	178	F	271
22	0.43	0.43	0.43	0.00	131	F	26
23	0.38	0.43	0.41	-0.05	150	M	407
Sum Total	10.27	9.74	10.00	0.53	3,504	14F 9M	11,771
Mean	0.45	0.42	0.43	0.02	144		512
Max	0.77	0.77	0.77	0.20	191		1,585
Min	0.24	0.14	0.22	-0.15	71		26
Std Devn	0.13	0.14	0.13	0.09	35		338

Table 6.1 Study 4 Results

Table 6.1 shows the raw data for Study 4, n=23.The mean MPOD was 0.43 (SD 0.13), the mean age was 12 years (SD 2 years 11 months) the mean L intake was 512 Mcg (SD 338Mcg) and the gender split was 14 females to 9 males.

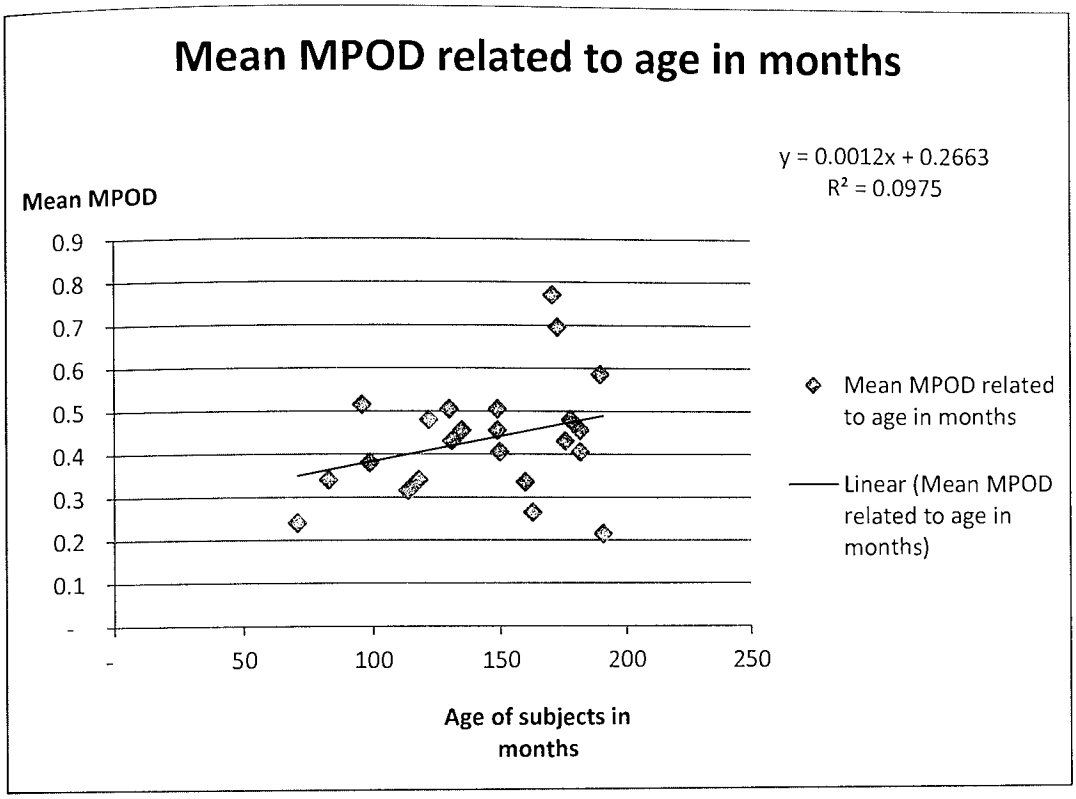


Figure 6.1 - Mean MPOD in relation to age

Correlation coefficient: 0.31

A weak positive correlation with age was found (0.31) and a paired sample t-test analysis did not reach statistical significance p=0.157 (see Appendix 6, table A6.4).

Post hoc analysis for MPOD related to age with G Power3.1 for sample size n=23 and effect size p=0.31, alpha 0.1, critical t=1.32 and power=0.6.

Gender split

Male MPOD, n=9 MPOD=0.42 (Max: 0.585 Min: 0.315, SD: 0.09)

Female MPOD, n= 14 MPOD=0.44 (Max: 0.77 Min 0.215, SD: 0.16)

A two-sample Student's t-test assuming unequal variances using a pooled estimate of the variance was performed to test the hypothesis that mean MPODs for male and female were equal. The mean MPOD of male subjects (mean = 0.42, SD = 0.09, n = 9) was not significantly different from females (mean = 0.44 SD = 0.16, n = 14), t (21) = 0.44, p = 0.65 (see Appendix 6, table A6.2)

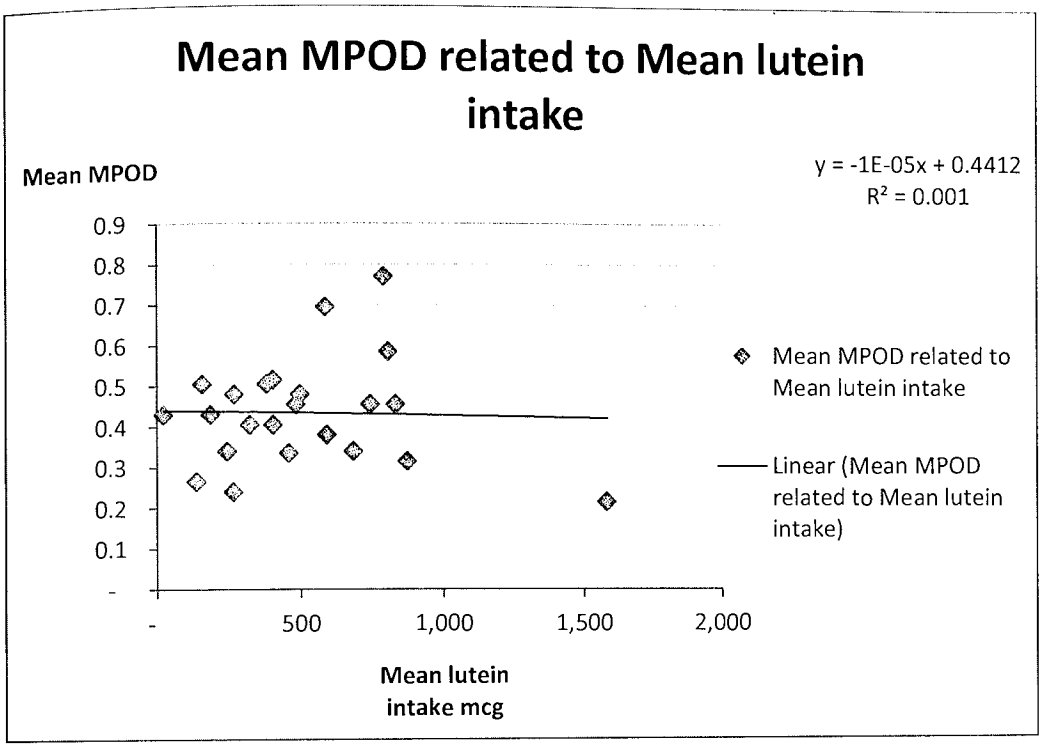


Figure 6.2: Mean MPOD in relation to mean L intake

cc: -0.032

A t-test assessing mean MPOD to L intake (correlation -0.03) found no significant relationship between the two variables, p =0.865 (see Appendix 6, table A6.6).

Gender split

Male mean L intake: 496 Mcg (Max: 874 Min: 161 SD: 265)

Female mean L intake: 521 Mcg (Max: 1585 Min: 25.5 SD: 387)

Analysis of male and female mean daily L intake showed a normal distribution for both (see Appendix 6, Figures A6.14-A6.19). A two-sample Student's t-test assuming unequal variances using a pooled estimate of the variance was performed to test the hypothesis that mean L intake for male and female were equal. There was no statistically significant difference between male and female mean L intake (t= 0.31, p= 0.76). (See Appendix 6, table A6.3)

6.4
Discussion

Assessing paediatric MPOD using HFP provides data for MPOD at an early stage of life. The assessment of L, using a prior three day diet history provided a method to assess the relationship between L intake and MPOD. The results of Study 4 show a mean MPOD of 0.43 (SD 0.13), with no statistically significant gender difference (p=0.65).

The findings of the current study are similar to those of Bartlett *et al.* (2010c), with minimal correlation between MPOD and age (See figure 6.2, cc: 0.31), although Bartlett was studying an older and wider age group, aged 18-50 years. The age/MPOD relationship has also been investigated using HFP in other studies; some have reported a positive correlation between the two variables, (Hammond *et al.*2000; Beatty *et al.*2001) while others reported no relationship (Werner *et al.*1987; Bone *et al.*1988; Ciulla *et al.*2004).

Zhu *et al.* using HFP examined 94 subjects aged 6-12 years of Chinese ethnicity. The mean MPOD was 0.56 ± 0.25, without any significant difference between genders (p = 0.12). The mean MPOD showed no significant association with age, body mass index, intra-ocular pressure, spherical equivalent refraction or central foveal thickness (Zhu *et al.* 2012). The mean MPOD value of 0.56 is a higher MPOD value than has been found in previous western adult MPOD studies, which have ranged between 0.21 (Ciulla *et al.* 2001) and 0.45 (Johnson *et al.* 2000). Previous studies investigating adult population mean MPOD with the MPS have given values ranging from 0.32±0.15 (Loughman *et al.*2012) to 0.35±0.14 (Bartlett *et al.*2010c). These values are approximately 15% lower than the mean MPOD in Study 4 of 0.43 (SD 0.13). This 15% difference could be due to population differences, age MPOD differences, dietary L intake differences or measurement/threshold setting differences.

In a study conducted by Tskia *et al.* (2011) using the MPS, the mean MPOD was 0.52 (SD 0.15). Participants with unilateral wet AMD had a higher level of mean MPOD in their fellow eye compared to those with bilateral dry AMD (0.58, SD 0.14 versus 0.48, SD 0.14; p = 0.026). Mean MPOD of patients with bilateral dry AMD was found not to differ significantly from that of healthy older subjects (0.48, SD 0.14 versus 0.50, SD 0.14; p = 0.865). No correlation with age was observed, females had slightly but significantly higher levels of MPOD (0.55 versus 0.49, p = 0.029). (SD not reported for gender MPOD).

In Study 4, the mean MPOD for males was 0.42 (SD 0.09), female was 0.44 (SD 0.16), with no statistically significant difference in these values (t=0.44, p=0.65). Bartlett *et al.* (2010c) reported the mean MPOD for their study was 0.35±0.14, with the mean MPOD for females (n=32) as 0.33±0.12 and for males (n=8) 0.45±0.19. The mean male/female MPOD values in Bartlett *et al.* 2010c study were significantly different (t=-2.393, p=0.022) but of note was the significant asymmetry in gender split in their study, with approximately four times as many female subjects as male subjects. This could potentially result in a small number of outliers in the male group skewing the male MPOD results.

Table 6.1 shows that the mean L intake was 512 Mcg (max 1585, min 25.5, SD: 338). The relatively high SD indicates a wide spread of values and this effect is magnified due to the small sample size. The mean L intake was approximately half that reported by Curran-Celentano in their 2001 study with 18-50 year old subjects. This difference could be due to regional and/or age differences in dietary L intake.

Bernstein *et al.* (2013) have related L intake to MPOD in their infant blue light study.

Total serum carotenoids and serum L and Z were significantly associated with MPOD; individually, serum L and Z also correlated with MPOD. But this correlation with MPOD did

not extend to dietary intakes of these nutrients, as shown in the second (upper right) graph of Figure 6.3.

In Study 4 there was no correlation found between mean L intake and MPOD, with a cc -0.03 (see figure 6.2). This finding may be due to the small sample size and variable L intake of the subjects. There was no statistically significant difference between male and female mean L intake ($t= 1.0$, $p > 0.05$). The levels of L detected in Study 4 subjects' diet, may have been insufficient to significantly affect MPOD values.

Johnson *et al.* (2010) found an increasing level of L intake throughout adult life, peaking at 1.0mg in the 70+ age group. The L intake in the Johnson *et al* study younger age group of 4-8 years was 0.31mg/day and 9-13 years of 0.33mg/day which was slightly less than found in Study 4 of 0.51mg/day.

Curran-Celentano *et al.* (2001), assessed MPOD in relation to dietary intake of L, examining 280 subjects aged 18-50 years. The MPOD was determined psychophysically with a 460-nm, 1 degree test stimulus. Mean MPOD was 0.21 +/- 0.13. Average intakes of L and Z were 1.1 +/- 0.84 mg/day. Although dietary intake variables of L and Z differed by sex, no significant sex differences were found in serum concentrations of L and Z or MPOD. Serum L and Z and dietary intake of L and Z were significantly correlated and significantly related to variations in MPOD ($r = 0.21$, $p < 0.001$, and $r = 0.25$, $p < 0.001$, respectively). The study concluded that, MPOD was associated with L and Z in the diet and the serum. Retinal concentrations, however, were influenced by other unknown factors. They recommended that to understand the effect of dietary L and Z intake on the retina and risk of age-related eye disease, future studies should include measures of macular concentrations of these pigments.

The lack of relation between MPOD and L in the diet in Study 4 is supported by Wenzel *et al.* (2007a) who investigated the relationship of diet, serum and retinal concentrations of L and Z in married couples. Fifty participants, 25 male-female married couples, between 29 and 70 years of age participated in the study. The MPOD was measured with HFP at 4 retinal loci. Dietary intake of L and Z in the sample was related to serum L and Z concentrations ($p = .01$). Dietary intake ($p = 0.04$) and serum concentrations ($p = 0.003$) of L and Z were significantly related to MPOD at 30' eccentricity. They concluded lipoprotein concentrations and genes, which are not shared by spouses, appear to influence MPOD more than factors such as diet and body mass index.

In conflict to with the finding reported here of no relationship between L intake and MPOD, Burke *et al.* (2005) previously have reported a correlation between MPOD and L intake. The MPOD was measured at 4 loci with HFP in 98 adults, 45-73 years old. Dietary L + Z were positively correlated with MPOD: 0.2° ($r = 0.24$, $P = 0.02$), 0.5° ($r = 0.237$, $p = 0.02$), 1° ($r = 0.27$, $p = 0.009$), and 2° ($r = 0.25$, $p = 0.02$) eccentricity. The lowest fruit and vegetable consumers had lower MPOD at 0.5° ($p = 0.01$), 1° ($p = 0.03$), and 2° ($p = 0.006$) eccentricity compared with the highest consumers. They concluded that carotenoid-rich diets and serum carotenoids positively contribute to MP status. The difference in findings between Study 4 and Burke *et al.* findings could be in part explained by difference in HFP technology to assess MPOD and difference in recording techniques of diet history and L and Z content calculations.

Examining the relationship between L and MPOD in the literature revealed a significant number of L supplementation studies. These have consistently reported a link between supplemented L in the diet and MPOD (Bone and Landrum, 2010; Murray *et al.* 2013a; Yao *et al.* 2013). Tanito *et al.* (2012) found L supplementation increased MPOD levels within the fovea more effectively than did Z.

Sasamoto *et al.* (2011) showed that daily supplementation with 6 mg of L for one year did not affect the MPOD level, indicating that 6 mg of L may be insufficient to increase the MPOD level. Subjects with low baseline MPOD were more likely to exhibit a dramatic rise in MPOD, or to exhibit no rise in MPOD, in response to supplements than subjects with medium to high baseline MPOD values.

Trieschmann *et al.* (2007) have previously reported that supplementation with 12 mg L and 1 mg Z, combined with co-antioxidants, resulted in an increase of MPOD at 0.5° eccentricity in a majority of subjects, including those with AMD. However, in a substantial proportion of subjects, in spite of rises in serum concentrations of L and Z, no MPOD increase could be detected over the study period. The authors hypothesised that this indicated intestinal malabsorption of these carotenoids was not responsible for the lack of a macular response to such supplements. These results indicated that saturable mechanisms may play a role in the retinal capture and/or stabilisation of the macular carotenoids. This provides further explanation for there not being a statistically significant relation between mean daily L intake and measured levels of MPOD in Study 4. Other metabolic and absorption factors may relate to L intake and MPOD levels. The research and investigation of these factors may facilitate development of L supplementation that maximises MPOD.

In a study by Johnson *et al.* (2008) only the peripheral measure of MPOD responded to L supplementation. The research team hypothesized a biochemical interaction between adipose tissue and the retina in L metabolism. They concluded that gender differences in L metabolism may be an important factor in tissue interactions and in determining MPOD

A significant correlation was found between the increase in MPOD and the increase in VA after 6 months ($r = 0.27$, $p = 0.013$) in the LISA study. The study concluded that patients who show a pronounced increase in MPOD also benefit in terms of visual function. This was significant as it was one of the major studies that linked MPOD to visual function (Weigert *et al.* 2011).

Wenzel *et al.* (2007b) assessed the effect of L supplementation on MPOD measured using HFP in a small scale study. For 120 days, three subjects consumed 30 mg of L and 2.7 mg of Z supplement per day. The MPOD was measured with HFP at 20', 30', 60' and 120' eccentricity. High-performance liquid chromatography was used to measure serum carotenoid concentrations in blood samples collected at baseline and at 30-day intervals. At the two most central loci, The MPOD significantly increased in all three subjects with a mean change of approximately 0.09 log units at 20' eccentricity and 0.08 log units at 30' eccentricity. MPOD significantly increased in two subjects at 60' eccentricity, and in one subject at 120' eccentricity. The study concluded that changes in MPOD indicated that carotenoid deposition occurred linearly and may be biased towards the central retina. Furthermore, carotenoid deposition may occur outside the central fovea in interventions with pharmacological doses of carotenoid, resulting in underestimations of psychophysical measures of MPOD.

Limitations of Study 4

A larger scale study would have greater statistical power. Through the use of G Power 3.1 and *post hoc* analysis, n=23, effect size p=0.31, alpha error 0.1, critical t=1.32 and power=0.6. Doubling the sample size with other factors held constant would increase power to 0.81. Small sample size pilot studies are required prior to larger scale studies being undertaken.

Due to the subjective method of determining MPOD with HFP, there is inherent variability in subjects setting thresholds for flicker detection, reaction times and the examiners thresholds for accepting or rejecting the V shaped curve results. Diet history recorded by parents and

estimation of portion sizes brings potential variability in the calculation of mean daily L intake.

6.5
Conclusion

The mean MPOD with the MPS in this paediatric sample was 0.43 (SD 0.13). The mean L intake was 512 Mcg (SD 338) and no correlation was found between MPOD and mean L intake ($p = 0.865$). Although previous studies have found a link between L and MPOD (Trieschmann *et al.* 2007; Bone and Landrum, 2010; Weigert *et al.* 2011) other metabolic and absorption factors may relate to L intake and MPOD levels. The research and investigation of these factors may facilitate development of L supplementation that maximises MPOD. This will require further biochemical and pharmacological collaboration.

The next chapter is the main discussion chapter of this thesis, which gives a detailed overview of the main findings and research implications of the four studies with recommendations for future research.

CHAPTER 7 - Discussion

7.1

Introduction

To the author's knowledge the results of these four studies are the first UK reporting of paediatric MPOD within the ophthalmic community. These pilot based studies provide an indication that paediatric MPOD levels in the 4-16 year age group are likely to be similar to those of adults. Logical extrapolation of the results indicates that should a critical period for the development of MPOD exist, then it is postulated to occur in the 0-4 year age period. These findings align well with Bernstein *et al.* (2013) and provide useful paediatric MPOD measures for comparative purposes. The assessment of diet history was also a unique aspect of Study 4 and to the author's knowledge has not been reported in any other UK paediatric MPOD research.

This chapter is a summary of each of the four studies undertaken, a discussion of key findings, clinical implications, suggestions for future research and final conclusions. Initially this research was aimed at increasing understanding of juvenile MPOD and the repeatability of an evolving objective MPOD technique, the Visucam. This is an objective single wavelength reflectometer that uses blue light 460nm to determine MPOD from a fundus image over a 30 degree retinal area centred on the fovea. An area 7 degrees in radius from the centre of the fovea is analysed and the maximal MPOD measure was taken within 1-2 degrees from the central fovea.

The scope of the project was increased to include a subjective technique of MPOD assessment - the MPS, with the same age group. The MPS uses a psychophysical technique known as HFP to measure MPOD. Subjects were instructed to respond to a series of green-blue ratios until a V-shaped curve was plotted on the computer screen. The minimum point on the curve corresponded to equiluminance of the blue and green lights. The difference between the central and pre-calculated peripheral minima, determined the MPOD – the larger the difference, the higher the MPOD. The MPS based research included a three day diet history which allowed the investigation of the relationship between MPS MPOD and dietary L. In the Visucam studies (Study 1+2) relationships between MPOD and age, iris pigmentation, refractive error and gender were determined. It was anticipated that the information collected in this study would supplement the paediatric MPOD studies of Zhu

et al. (2012) and Bernstein *et al.* (2013) by providing further paediatric MPOD data to enhance understanding of MPOD development in early life.

The results from this study allowed the Visucam and the MPS to be compared in terms of their CoR, which is an indicator of measurement noise. Determining which technology has the lower CoR assists in understanding which technique is most suitable for use in a clinical setting. This was reported in the test-retest studies: Study 1 (Visucam) and Study 3 (MPS). Study 2 (Visucam) and Study 4 (MPS) provided a paediatric mean MPOD. The data were compared to other paediatric and adult MPOD studies. Such information may have value in enhancing understanding of the pathological basis and natural history of AMD. The diet history component of the research was unique in accompanying paediatric MPOD data, with a calculated daily mean L intake of 0.51 mg (SD 0.34) and mean MPOD of 0.43 (SD 0.13) for n=23.

The data distribution of Study 1 (Visucam), 2 (Visucam) and 4 (MPS) were found to be non-normal and non-parametric assessment was used to assess data. Study 3 (MPS) had normal and non-normal data distributions and parametric and non-parametric analysis was used respectively. Where two variables were compared with one being a normal distribution and the other being non-normal distribution, to err on the side of caution the data was assessed with non-parametric assessment (Campbell, 2006).

7.2

Key findings

In Chapters 1 and 2 the concept of assessing paediatric MPOD was introduced with an over view of previous paediatric MPOD studies (Zhu *et al.* 2012; Bernstein *et al.* 2013) and adult based MPOD studies (Seddon *et al.* 1994; Landrum *et al.* 1997a; Nolan *et al.* 2010; Weigert *et al.* 2011). The pharmacological structure of L and Z was explored and the pathological process of AMD and its relation to MPOD was evaluated.

Different techniques used to measure MPOD were described along with the variability associated with MP measurement. Heterochromatic flicker photometry was the most widely used method of measurement of MP. The literature review which comprised Chapter 2 focussed on the limited previous studies which have investigated paediatric MPOD. It discussed in detail the principles of FR techniques in assessing MPOD and other objective techniques. The Visucam was described, including the optical principles underpinning its measure of MPOD. The technique of measurement and previous Visucam based MPOD

studies were reported. The MPS was also described, including the optical principles underpinning its measure of MPOD. The technique of measurement and previous MPS based MPOD studies were reported. The rationale for the four studies in this project was developed.

Thus, the main questions posed by this thesis were:

How does the repeatability of the Visucam and MPS compare in paediatric subjects?

What are the mean MPOD values in the sample paediatric population with these two techniques? How do these values compare to adult studies using similar techniques?

Does age, gender or iris pigmentation affect the mean MPOD value?

Does L intake have any relationship to MPOD in paediatric subjects?

The research chapters aimed to answer these questions.

Chapter 3 (Study 1) consisted of a report on the test-retest repeatability of the Visucam. The Visucam was simple and swift for the operator and the subjects. The Visucam mean MPOD was 0.40 (SD 0.06) and the CoR 0.1 and 0.1/0.40 was approximately 25%. A change of MPOD has to be greater than 25% for it to be genuine change and not one due to measurement noise. This is a medium size effect.

A cross-sectional study was conducted in Chapter 4 (Study 2) with 73 subjects, 40 female and 33 male with a mean age of 11.1 years (SD 2.8 years). The aim of this study was to collect objective MPOD values from a paediatric sample and to assess whether MPOD had a significant relationship with age, gender, iris pigmentation or refractive error. A single factor ANOVA was conducted to assess for any statistically significant difference in mean MPOD between the three age groups. The null hypothesis was accepted and there was no significant difference between the groups, p= 0.98. This was confirmed with linear regression analysis with MPOD and age r=0.015, p=0.9. This indicated that MPOD appears to develop to near adult levels during the 0 - 4 years age of life time period and remains relatively constant during the infantile and juvenile period thereafter (although power was limited to 0.54 and further study was recommended). The greatest difference in mean group MPOD values was between the male dark pigmentation irides (0.43) and female light irides (0.39). Analysis showed this did not reach statistically significant levels, (p 0.109). MPOD was

found to be independent of refractive error (0.39-0.40 across the three refractive categories) and gender (p = 0.444).

In Chapter 5 (Study 3) the repeatability of the MPS was reported. The CoR was calculated as 0.18. Therefore 0.18/0.41 results in 44% - a moderate to large size effect. Measures obtained using the Visucam in Chapter 3 (Study 1) were found to be repeatable and the mean MPOD was similar to those taken using the MPS (Visucam=0.40 vs MPS=0.43) however the SD varied considerably, (Visucam=0.06 vs MPS=0.13). Therefore the two methods should not be used interchangeably as the MPS exhibited a greater spread of data. The CoR value of 0.18 from Chapter 5 (Study 3) indicated the MPS had significant measurement noise for assessment of paediatric MPOD and would not be suitable to use in a longitudinal paediatric MPOD study, unless investigating gross (>0.18) changes in MPOD. Of the two instruments, the Visucam would be preferred for assessing MPOD in children due to its lower CoR value.

In Chapter 6 (Study 4) mean MPOD measurement in a group of children using the MPS, was reported. *Post hoc* analysis for MPOD related to age with G Power3.1 for sample size n=23 and effect size p=0.31, alpha 0.1, critical t=1.32 and power=0.6. This level of power may have been insufficient to make definite conclusions. There were 23 subjects aged 4-16 years and a three day diet history. From this a daily L intake was calculated using Alacalc software (Alacalc, York's, UK). The mean L intake was 0.51mg (SD 0.34). A t-test assessing mean MPOD to L intake (correlation -0.03) found no significant relationship between the two variables, p =0.00. The results of Study 4 gave a mean MPOD of 0.43 (SD 0.13), with no statistically significant gender difference (p=0.65). A weak positive correlation with age was found (0.31) and a paired sample t-test analysis did not reach statistical significance p=0.00.

Study 4 indicated that MPOD and L intake do not relate to age or gender and may relate to other metabolic or life style factors. The levels of L detected in Study 4 subjects' diet, may have been insufficient to significantly affect MPOD values, as assessed by the MPS. McCorkle *et al.* (2015) hypothesized that the measure of MPOD in children, a surrogate measure of brain levels of xanthophyll's, particularly L, would be beneficial for studies looking to investigate how L and Z relate to childhood cognitive function and development.

7.3
Strengths of studies 1-4

There was a significant gap in the literature relating to paediatric MPOD and Bernstein *et al.* (2013) and Zhu *et al.* (2012) encouraged further studies in this area. The current studies provide objective and subjective data for paediatric MPOD. Study 1 (Chapter 4) provided data on the test-retest ability of the Visucam in a paediatric sample. To the authors knowledge this has not been reported before. Dennison *et al.* (2013) stated that the Visucam under-estimated MPOD values and obtained clustered MPOD values around 0.3-0.4. However, there was no documentation of calibration of the Visucam used in that study. There was also no evidence of ensuring optimal optical clarity of the Visucam lens during the data collection. If the Visucam lens becomes compromised, MPOD readings become lower and clustered (see Visucam instrumentation Chapter 3). As reported in Chapter 3, Study 1 had measures in place to ensure optimum optical clarity of the Visucam lens.

Study 2 (Chapter 4) described a cross sectional paediatric sample based objective measure of MPOD with the Visucam. This was a novel study aiming to fill a research gap of objective based measurement of paediatric MPOD. The accuracy of the results in Study 2 was supported by the test-retest study conducted in Study 1. Segregating results in to age groups provided some information and enhanced understanding of the natural history of MPOD in early life. Obana *et al.* (2014) found MPOD levels declined by more than 10 % through each decade of life. Study 2 indicated that MPOD levels remained stable through childhood at mean 0.4 (SD 0.07).

Study 3 (Chapter 5) described a test-retest study of the subjective assessment of paediatric MPOD with the MPS. This enabled a direct comparison to the objective test-retest of the Visucam in Study 1. The results of Study 3 (Chapter 5) provided a CoR of 0.18, giving 44% - a moderate to large sized effect. This finding was in agreement with previous adult based studies (Bartlett *et al.* 2010c, Loughman *et al.* 2012).

Study 4 (Chapter 6) investigated a cross sectional White paediatric sample based subjective measure of MPOD with the MPS. It provided data on MPOD where very limited data was available. The mean MPOD with the MPS was 0.43 (SD 0.13). The findings differed to Zhu *et al.* (2012) who examined Chinese school-aged children with HFP and determined a mean MPOD of 0.56 ± 0.25, without any significant difference between boys and girls ($p = 0.12$). This MPOD difference may in part be explained by genetic factors, lifestyle or dietary L intake. It can be noted, however, that the mean MPOD in Study 4 (Chapter 6) when

considered in conjunction with the CoR of 0.18 indicate that the MPOD values still fall within the range of CoR and as such may be a consequence of the variability of the device within a paediatric population.

The calculation of the paediatric L intake was a unique element of Chapter 6. The mean L intake was 0.51mg (SD 0.34). This was higher than that found by Johnson *et al.* (2010) in their study of L intake in differing age groups. They found L intake was approximately 0.33-0.40mg for a similar age group. In addition they found a gradual increase in L intake with increasing age, to 0.9mg during the 30-50 years of life. In Study 4 no relationship was found between MPOD and mean L intake ($r= -0.032$, $p= 0.865$). This finding was in agreement with Bernstein *et al.* 2013 (see Chapter 6, figure 6.4).

7.4

Limitations of Studies 1-4

In Study 1 (Chapter 3) the small sample size in the test-retest study resulted in statistical outliers having a greater impact on the CoR. An increased sample size would ensure statistical outliers have a reduced effect on the calculation of the CoR. Through the use of G Power 3.1 and *post hoc* analysis, a sample size of 16 would give an effect size (p) of 0.24, critical t 1.34, to allow an α error probability of 0.1.The margin of error is $1.34/ (2 \sqrt{16}) = 0.168$, or about 16.8%. A larger sample size of 50 would give a margin of error $1.34/ (2 \sqrt{50}) = 0.09$ or about 9%. This demonstrates that even in a repeatability study, a larger sample size study would give a lower margin of error and increase the confidence in, and significance of the results.

The cross sectional Study 2 (Chapter 4) had a lower number of subjects in the 4-8 (n=14) group compared to the other >8-12 (n= 25) and >12-16 (n= 34) groups. The author was aware of the apprehension of the younger subjects and the relatively protective position of the parent/guardians. This was observed during recruitment where parents of younger subjects were wary of allowing their child to partake in the study. The main limitation of this study was reduced numbers for analysis of this individual age group; however this was difficult to determine during the study design phase, as very few previous studies have reported paediatric MPOD. Study 4 was underpowered and therefore definite conclusions cannot be drawn. Using G Power 3.1 and relating MPOD to age, *post hoc* analysis of a sample size of 73 would give an effect size (p) 0.16, critical t =1.29, to allow an α error probability of 0.1 and 0.54 power.

In addition there has been some criticism of the validity of the Visucam. This criticism has been led by Dennison and Nolan in their study which assessed concordance of MP measures using HFP, dual-wavelength AF, and single-wavelength FR. Dennison *et al.* (2013) determined in their study (n=63) that the Visucam under-estimated MPOD values and obtained clustered MPOD values around 0.3-0.4. There was no information in their study detailing the calibration of their Visucam and no evidence of ensuring optical clarity of the Visucam lens during the data collection. In the absence of these measures not being reported, there is a significant possibility of them not being implemented. This, considered in conjunction with the lower subject numbers in the Dennison study, make the Dennison *et al.* study potentially less robust. In addition, Dennison et al (2013) did not report on the repeatability of the Visucam. Instead, they carried out a regression analysis of the Visucam to compare to the macular densitometer and HRA spectralis and found little correlation between the Visucam and the other two methods: Visucam max MPOD on Densitometer central MP: R^2=0.008, p=0.843. Regression analysis also demonstrated a weak relationship between MP measured by the Spectralis and Visucam (Visucam max MPOD on Spectralis central MP: R^2=0.047, p=0.348). However, the Visucam assesses maximal MPOD within a 3 degrees radius region of the fovea – i.e. the measured peak may be at a slightly different point between subjects. Whereas the Spectralis and Densitometer measure MPOD at set intervals (e.g. 0.25 degrees or 0.5 degrees). This difference in location in measured MPOD between the Visucam and the Densitometer/Spectralis brings another variable into the comparisons. Such comparisons are therefore flawed and potentially misleading.

In Study 3 (Chapter 5) and Study 4 (Chapter 6) the MPS was used to measure MPOD. There was potential variability in subjects setting thresholds for flicker detection, reaction times and the examiners' threshold for accepting or rejecting the V shaped curve results. Poor technique could result in random error or bias. This could benefit from further examination in future studies where different examiners assess MPOD on the same subjects on different days using the same MPS. If the examiners technique is sub-optimal, then it follows that the data collected will also be sub optimal. The two week familiarisation period with the MPS was used to reduce the effects of examiner measurement bias. However the accepting or rejecting of V shaped curves remains a significant variable factor. As discussed in Chapter 7, Howells *et al.* (2013a) recommended a protocol that could be applied within a research setting to reduce variability in the data. However, the additional time and subject fatigue associated with this protocol would likely be incompatible with paediatric subjects in a clinical setting.

In Study 4 (Chapter 6) the data presented in this body of work showed that L in the diet was not related to MPOD measured with the MPS. Diet history recorded by parents and estimation of portion sizes brings potential variability in the calculation of mean daily L intake. Serum blood measures of L would be preferable due to its objectivity. However, this procedure is likely to be too invasive for children.

7.5
Future Research

Study sample sizes and future recommendations

In Study 1, using G Power 3.1 and relating the correlation of MPOD 1 to MPOD 2, with R^2 = 0.30, *post hoc* analysis of sample size of 16 would give an effect size (p) 0.55, critical t =1.76, to allow an α error probability of 0.05 and 0.81 power. The margin of error is 1.76/ (2 $\sqrt{}$ 16) = 0.22, or about 22%. A larger sample size of 50 would give a margin of error 1.76/ (2 $\sqrt{}$ 50) = 0.12 or about 12%. This demonstrates that even in a repeatability study, a larger sample size study would give a lower margin of error and increase the confidence in, and significance of the results.

Study 2 was limited in number of subjects and constrained due to availability of resources. More subjects would allow a greater statistical power. Through the use of G Power 3.1 and relating MPOD to age, *post hoc* analysis of sample size of 73 would give an effect size (p) 0.16, critical t =1.29, to allow an α error probability of 0.1 and 0.54 power. Tripling the sample size to 219 and assuming the same effect size (p=0.16) gives 0.87 power.

Study 3, Through the use of G Power 3.1 and relating the correlation of MPOD1 and MPOD2 measures, coefficient of determination 0.55, *post hoc* analysis of a sample size 11 would give an effect size (p) 0.74, critical t =1.83, to allow an α error probability of 0.05 and power= 0.95. The margin of error is 1.83/ (2 $\sqrt{}$ 11) = 0.27, or about 27%. A larger sample size of 50 would give a margin of error 1.83/ (2 $\sqrt{}$ 50) = 0.13 or about 13%.

Study 4, A larger scale study would yield greater statistical power. Through the use of G Power 3.1 and *post hoc* analysis, n=23, effect size p=0.31, alpha error 0.1, critical t=1.32 and power=0.6. Doubling the sample size to n=46 with other factors held constant would increase power to 0.82.

In view of these calculations, the repeatability studies are adequately powered, however for ultimate caution, the margin of error could be reduced in both repeatability studies by increasing the sample size to approximately 50.

With respect to the main studies (Study 2 and Study 4) both were underpowered at 0.54 and 0.60 respectively. By increasing the sample size to 250 for Study 1 (to allow approximately 10% rejection of poor image capture) the power would be increased to 0.87. For Study 4, increasing the sample size to 46 would increase the power to 0.82. The reason that Study 2 requires a significantly larger sample size (compared to Study 4) is due to the lower R^2 value when relating MPOD to age.

Since Studies 1-4 were pilot studies, with no previous paediatric MPOD data available at study design, there was no *A priori* made. However the data made available from Studies 1-4 should allow future researchers to develop *A priori* based on these findings.

Alternative techniques and study designs

The Macular Densitometer is an HFP device which measures MP at 0.5 degrees from the fovea. It is currently being used at over 40 research centres around the world, including the National Institute of Health in the US, and the data generated by this instrument has been used for MP measurements in over 100 peer-reviewed scientific publications (Dennison *et al.* 2013). It is the only MP-measuring device that has been validated i.e. by comparing the data it generates with the *in vitro* spectral absorption curve of the macular carotenoid (Wooten *et al.* (1999); Stringham *et al.* (2008). Technologies which correlate strongly with the Macular Densitometer may be preferable for future MPOD studies.

Studies assessing the relationship between L intake and MPOD are required, with strict calculation of the L intake within a controlled environment. Such facilities have been developed within the University of Ulster, Coleraine, Ireland. Resources available include a specially-designed residential suite with accommodation, in which up to 12 human volunteers may be accommodated for days or weeks at a time (University of Ulster, Research Studies Institute, 2016).

Another research possibility exists with evaluating MPOD and L intake within the strict confines of The European Prospective Investigation into Cancer and Nutrition (EPIC) study,

which could yield significant and valuable data. The EPIC project is one of the largest cohort studies in the world, with more than half a million (521, 000) subjects recruited across 10 European countries and followed for almost 15 years. This large scale study based in multiple centres in the UK and Europe was designed to investigate the relationships between diet, nutritional status, lifestyle and environmental factors, and the incidence of cancer and other chronic diseases.

Vishwanathan *et al.* (2014) reported significantly lower concentrations of L and Z in preterm infants compared with term infants, in most of the brain regions analysed. There may therefore be value in assessing objective MPOD in preterm infants and considering supplementary L measures to ensure optimal retinal and neurological development. Lutein has also been shown to enhance gap junctional communication (Stahl and Sies, 2001), which may be important for the development of the brain and visual processing (Johnson, 2014). It has been reported that L was able to stimulate the differentiation of human stem cells into neural cells in vitro simulating the process of brain development and maturation (Kuchan *et al.* 2013).

There was a tendency for lower MPOD in lighter irides in Study 2, with light iris pigmentation, n=49, had a slightly lower mean MPOD of 0.39 (SD 0.04). Dark iris pigmentation had lower subject numbers at n=24 and a slightly higher mean MPOD of 0.41 (SD 0.08). Bernstein *et al.* (2013) reported one patient in their study with oculocutaneous albinism and nystagmus (subject 44) which had no detectable MP in either eye. Assessing MPOD in patients with albinism may be worthwhile and supplementary L measures may become routinely instituted at the very earliest stage of life, in people with this condition in the future. McCorkle *et al.* (2015) recommended studies assessing paediatric MPOD in conjunction with neurological development, relating MPOD to L levels in the brain. Assessing objective MPOD in attention deficit hyperactivity disorder (ADHD) or autistic children may be considered in the future.

Studies 1-4, highlight that further paediatric MPOD studies are required. Assessment as to how well HFP, FR and AF provide accurate, precise and repeatable measures would be beneficial to the research community. Larger scale studies, with greater resources, may yield greater statistical significance. Studies using the Visucam in older normal subjects and older subjects with macular pathology are also required. This would enhance knowledge of MPOD measured from FR and any predictive value it may have in macular disease processes. Additional adult Visucam and MPS studies with diet and or L based supplementation will provide valuable information on the ability for FR and HFP to chart efficacy of diet/treatment programs.

Studies which provide MP density profiles in relation to the same subjects' mapped AMD profiles may provide further support for MP having a protective role in restricting AMD development.

7.6
Final Conclusions

The results confirm significant variability in the repeatability of MPS in the measurement of paediatric MPOD. The Visucam had approximately half the variability of the MPS (CoR 0.10 vs 0.18).

With the Visucam the mean MPOD 0.40 (SD 0.08) did not vary between the three different age groups of 4-8, 8-12, 12-16 years. A single factor ANOVA confirmed no statistically significant difference in mean MPOD between the three reported age groups ($p=0.98$). This was confirmed with linear regression analysis with MPOD and age $r=0.015$, $p=0.9$. However, power was limited in this pilot based Study to 0.54. Further larger scale studies of several hundred participants would yield greater power and could further investigate the mean MPOD in these age groups. Measured MPOD was statistically independent of age, gender, iris colour and refractive error.

The mean MPOD with the MPS was 0.43 (SD 0.13). The mean L intake was 0.51 mg (SD 0.34) and no relationship was found between MPOD and mean L intake ($p =0.865$).
The Visucam had good repeatability in assessing paediatric MPOD. It is the authors preferred instrument for assessing paediatric MPOD from this study. There is still a need for the development of an instrument providing an objective measure of paediatric MPOD with repeatability at adult levels. This, coupled with the ability to view spatial distribution profiles of MP with good portability, would increase the usefulness of this technology in a clinical setting. Routine longitudinal monitoring of MPOD in patients with an instrument that has a low CoR should remain the future aim of researchers and clinicians. This would promote an increased understanding of the relationships between MPOD, AMD and visual acuity.

REFERENCES

Al-Hussaini, H., Kam, J.H., Vugler, A., Semo, M., Jeffery, G., (2008) Mature retinal pigment epithelium cells are retained in the cell cycle and proliferate in vivo. *Mol. Vis.* **14**, 1784–1791.

Bartlett H., Howells O., Eperjesi F. (2010a) The role of macular pigment assessment in clinical practice: a review. *Clin & Exp Optom.* **93**, 300–308.

Bartlett H., Acton J., Eperjesi F. (2010b) Clinical evaluation of the MacuScope macular pigment densitometer. *Br. J. Ophthalmol.* **94**, 328–331.

Bartlett H., Stainer L., Singh S., Eperjesi F., Howells O. (2010c) Clinical evaluation of the MPS Macular Pigment Screener. *Br. J. Ophthalmol.* **94**, 753–756.

Beatty S., Koh, H.H., Carden D., Murray I.J. (2000a) Macular pigment optical density measurement: A novel compact instrument. *Ophthal. Phys. Optics.* **20**, 105–111.

Beatty S., Koh H.H., Phil M., Henson, D., Boulton M. (2000b) The Role of Oxidative Stress in the Pathogenesis of Age-Related Macular Degeneration. *Surv. Ophthalmol.* **45**, 115–134.

Beatty S., Murray I.J., Henson D.B., Carden D., Koh H., Boulton M.E. (2001) Macular pigment and risk for age-related macular degeneration in subjects from a Northern European population. *Invest. Ophthalmol. Vis. Sci.* **42**, 439–446.

Beatty S., Van Kuijk F.J.G.M., Chakravarthy U. (2008) Macular pigment and age-related macular degeneration: longitudinal data and better techniques of measurement are needed. *Invest. Ophthalmol. Vis. Sci.* **49**, 843-5.

Berendschot T.T.J.M., Goldbohm R.A., Klöpping W.A., van de Kraats J, van Norel J, van Norren D. (2000) Influence of lutein supplementation on macular pigment, assessed with two objective techniques. *Invest. Ophthalmol. Vis. Sci.* **41**, 3322–3326.

Berendschot T.T.J.M., Willemse-Assink J.J.M., Bastiaanse M, de Jong PT, van Norren D. (2002) Macular pigment and melanin in age-related maculopathy in a general population. *Invest. Ophthalmol. Vis. Sci.* **43**,1928–1932.

Berendschot T.T.J.M., van Norren D. (2004) Objective determination of the macular pigment optical density using fundus reflectance spectroscopy. *Arch Biochem. Biophys.* **430**, 149–155.

Berendschot T.T.J.M., van Norren D. (2005) On the age dependency of the macular pigment optical density. *Exp. Eye Res.* **81**, 602–609.

Berendschot T.T.J.M., van Norren D. (2006) Macular pigment shows ringlike structures. Invest. Ophthalmol. Vis. Sci. 47, 709–714.

van den Berg T.J., IJspeert J.K. & de Waard, P.W. (1991) Dependence of intraocular straylight on pigmentation and light transmission through the ocular wall. *Vis Res.* **31**, 1361–1367.

Bernstein P.S., Yoshida M.D., Katz N.B., McClane R.W., Gellermann W. (1998) Raman detection of macular carotenoid pigments in intact human retina. *Invest Ophthalmol & Vis Sci.* **39**, 2003–2011.

Bernstein P.S., Delori F.C., Richer S., van Kuijk F.J., Wenzel A.J. (2010) The value of measurement of macular carotenoid pigment optical densities and distributions in age-related macular degeneration and other retinal disorders. *Vis. Res.* **50**, 716–728.

Bernstein P.S., Sharifzadeh M., Liu A., Ermakov I., Nelson K., Sheng X., Panish C., Carlstrom B., Hoffman R.O., Gellermann W. (2013) Blue-light reflectance imaging of macular pigment in infants and children. *Invest. Ophthalmol. Vis.Sci.* **54**, 4034–40.

Bird A.C., Bressler S.B., Chisholm I.H., Coscas G., Davis M.D., de Jong P.T.V.M., Klaver C.C.W., Klein R., Mitchell P., Sarks J.P., Sarks S.H., Soubrane G., Taylor H.R., Vingerling J.R. (1995) An international classification and grading system for age-related maculopathy and age-related macular degeneration. The international ARM epidemiological study group. *Surv. Ophthalmol.* **39**,367-374.

Bhosale P., Zhao D.Y., Bernstein P.S. (2007) HPLC measurement of ocular carotenoid levels in human donor eyes in the lutein supplementation era. *Invest. Ophthalmol. Vis. Sci.* **48**, 543–549.

Birkeldh U., B, Wahlberg-Ramsay B.M., Nilsson M., Brautaset R . Evaluation of two methods for measurements of macular pigment optical density (MPOD) *Acta. Ophthalmol.* **92.**1755-3768.

Bland J.M., Altman D.G. (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*, **1**, 307–10.

Bone R.A., Landrum J.T. & Tarsis, S.L. (1985) Preliminary identification of the human macular pigment. *Vis. Res.* **25**, 1531–1535.

Bone R.A., Landrum J.T., Fernandez L., Tarsis S.L. (1988) Analysis of the macular pigment by HPLC: retinal distribution and age study. *Invest. Ophthalmol. Vis. Sci.* **29**, 843–849.

Bone R. A., Landrum J.T., Friedes L.M., Gomez C.M., Kilburn M.D., Menendez E., Vidal I., Wang W. (1997) Distribution of lutein and zeaxanthin stereoisomers in the human retina. *Exp. Eye Res.* **64**, 211–218.

Bone R.A., Landrum, J.T. (2004) Heterochromatic flicker photometry. *Arch Biochem Biophys.* **430**, 137–142.

Bone R., Landrum J.T., Cao Y., Howard A.N., Alvarez-Calderon F. (2007a) Macular pigment response to a supplement containing meso-zeaxanthin, lutein and zeaxanthin. *Nutr. Metab.* **4**, 12.

Bone R.A., Brener, B. & Gibert, J.C. (2007b) Macular pigment, photopigments, and melanin: Distributions in young subjects determined by four-wavelength reflectometry. *Vis. Res.* **47**, 3259–3268.

Bone R.A., Landrum J.T. (2010) Dose-dependent response of serum lutein and macular pigment optical density to supplementation with lutein esters. *Arch Biochem. Biophys.* **504**, 50–55.

Bour L.J., Koo L., Delori F.C., Apkarian P., Fulton A.B. (2002) Fundus photography for measurement of macular pigment density distribution in children. *Invest. Ophthalmol. Vis. Sci.* **43**,1450–1455.

Brindley G. S., Wilmer E.N. (1952) The reflexion of light from the macular and peripheral fundus oculi in man. *J. Physiol* .**116**, 350-356

Broekmans W.M.R., Berendschot T.T.J.M., Klöpping-Ketelaars I.A., de Vries A.J., Goldbohm R.A., Tijburg L.B., Kardinaal A.F., van Poppel G. (2002) Macular pigment density in relation to serum and adipose tissue concentrations of lutein and serum concentrations of zeaxanthin. *Am. J. Clin. Nutr*, **76**, 595–603.

Brunk U.T. & Terman, A. (2002) Lipofuscin: mechanisms of age-related accumulation and influence on cell function. *Free Rad. Biol. Med.* **33**, 611–619.

Bucheli P., Vidal K., Shen L., Gu Z., Zhang C., Miller L.E., Wang J. (2011) Goji berry effects on macular characteristics and plasma antioxidant levels. *Optom Vis Sci.* 2011 **88**, 257-262.

Burke J.D., Curran-Celentano, J. & Wenzel, A.J. (2005) Diet and serum carotenoid concentrations affect macular pigment optical density in adults 45 years and older. *J. Nutr.* **135**, 1208–1214.

Campbell M.J. (2006) http://www.healthknowledge.org.uk/public-health-textbook/research-methods/1b-statistical-methods/parametric-nonparametric-tests (cited 8-10-15)

Canovas R., Lima V.C., Garcia P., Morini C., Prata T.S., Rosen R.B. (2010) Comparison between macular pigment optical density measurements using two-wavelength autofluorescence and heterochromatic flicker photometry techniques. *Invest. Ophthalmol. Vis. Sci.* **51**, 3152–3156.

Cardinale A., Chiesa R., Sierks M. (2014) Protein Misfolding and Neurodegenerative Diseases. *Inter. J. Cell Biol.* Article ID 217371, 2p

Chalam K. V., Khetpal V., Rusovici R., Balaiya S. (2011) A review: role of ultraviolet radiation in age-related macular degeneration. *Eye & contact lens*, **37**, 225–232.

Chen S.F., Chang, Y. & Wu, J.C. (2001) The spatial distribution of macular pigment in humans. *Curr. Eye Res.* **23**, 422–434.

Ciulla T.A., Curran-Celantano J., Cooper D.A., Hammond B.R. (Jr)., Danis R.P., Pratt L.M., Riccardi K.A., Filloon T.G. (2001) Macular pigment optical density in a midwestern sample. *Ophthalmology.* **108**, 730–737.

Ciulla T.A., Hammond B.R. (Jr) (2004) Macular pigment density and aging, assessed in the normal elderly and those with cataracts and age-related macular degeneration. *Am. J. Ophthalmol.* **138**, 582–587.

Creuzot-Garcher C., Koehrer P., Picot C., Aho S., Bron A.M. (2014) Comparison of two methods to measure macular pigment optical density in healthy subjects. *Invest Ophthalmol Vis Sci.* 55(5):2941-6.

Curran-Celentano J., Hammond B.R. (Jr)., Ciulla T.A., Cooper D.A., Pratt L.M., Danis R.B. (2001) Relation between dietary intake, serum concentrations, and retinal concentrations of lutein and zeaxanthin in adults in a Midwest population. *Am. J. Clin. Nutr.* **74**, 796–802.

Dawczynski J, Jentsch S., Schweitzer D., Hammer M., Strobel J. (2011) Macular Pigment Density Measurement in Patients with Age-related Macular Degeneration. *Euro. Ophth. Rev.* **5**,141-142,

Delori F.C., Pflibsen, K.P. (1989) Spectral reflectance of the human ocular fundus. *App. Optics.* **28**, 1061–1077.

Delori F.C. (1994) Spectrophotometer for noninvasive measurement of intrinsic fluorescence and reflectance of the ocular fundus. *App. Optics.* **33**, 7439–7452.

Delori F.C., Dorey C.K., Staurenghi G., Arend O., Goger D.G., Weiter J.J. (1995) *In vivo* fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. *Invest. Ophthalmol. Vis. Sci.* **36**, 718–729.

Delori F.C., Goger D.G., Hammond B.R., Snodderly D.M., Burns S.A. (2001) Macular pigment density measured by autofluorescence spectrometry: comparison with reflectometry and heterochromatic flicker photometry. *J. Optic. Soc. Amer. A. Optics, Image Sci. Vis.* **18**, 1212–1230.

Delori F.C. (2004) Autofluorescence method to measure macular pigment optical densities fluorometry and autofluorescence imaging. **430**, *Arch. Biochem. Biophys.* 156–162.

Dennison J.L., Stack J., Beatty S., Nolan J.M. (2013) Concordance of macular pigment measurements obtained using customized heterochromatic flicker photometry, dual-wavelength autofluorescence, and single-wavelength reflectance. *Exp. Eye. Res.* **116**,190–198.

Dietzel M., Zeimer M., Heimes B., Claes B., Pauleikhoff D., Hense H.W. (2011a) Determinants of macular pigment optical density and its relation to age-related maculopathy: results from the Muenster Aging and Retina Study (MARS). *Invest. Ophthalmol. Vis. Sci.* **52**, 3452–3457.

Dietzel M., Zeimer M., Heimes B., Pauleikhoff D., Hense H.W. (2011b) The ringlike structure of macular pigment in age- related maculopathy: Results from the Muenster Aging and Retina Study (MARS). *Invest. Ophthalmol. Vis. Sci.* **52**, 8016–8024.

Edwards, R.B., Hammond B.R. Jr, Johnson E.J., Russell R.M., Krinsky N.I., Yeum K.J., Snodderly D.M. (1997) Dietary modification of macular pigment density. *Invest. Ophthalmol. Vis. Sci.* **38**,1795-1801.

Eisner E., Burns S.A., Hughes G.W., Webb R.H. (1992) Reflectometry with a scanning laser ophthalmoscope. *Appd. Optics*, **31**, 3697–710.

Eon Seon Jin. (2003) Microalgal biotechnology: Carotenoid production by the green algae Dunaliella salina. *Biotechnol. Bioprocess Eng* . **8**, 6, 331-337.

Faul F., Erdfelder E., Buchner A., Lang A.G. (2009) Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. *Behav Res Meth.* **41**, 1149-1160.

Fleming, P.A., Braekevelt, C.R., Harman, A.M., Beazley, L.D., (1996) Retinal pigment epithelium and photoreceptor maturation in a wallaby, the Quokka. *J. Comp. Neurol.* **370**, 47–60.

Gellermann W., Ermakov I.V., Ermakova M.R., McClane R.W., Zhao D.Y., Bernstein P.S. (2002) *In vivo* resonant Raman measurement of macular carotenoid pigments in the young and the aging human retina. J. Optic. Soc. Amer. A. Optics, Image Sci. Vis. **19**, 1172–1186.

Gellermann W., Bernstein, P.S. (2004) Noninvasive detection of macular pigments in the human eye. *J. Biomed. Optics.* **9**, 75–85.

Gescheider G. (1997) *Psychophysics: the fundamentals* (3rd ed.). Lawrence Erlbaum Associates. p. ix. ISBN 0-8058-2281-X

Group, A.-R.E.D.S. 2 R. (2013) Lutein + zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. *J. Amer. Med. Assocn.* **309**, 2005–2015

Grover M. (2013) Identification of novel therapeutics for complex diseases from genome wide association data. BIGDATA 2013 presentation. School of Life and Environmental Sciences, Deakin University.

Haegerstrom-Portnoy G. (1988) Short-wavelength-sensitive-cone sensitivity loss with aging: a protective role for macular pigment? J. Optic. Soc. Amer. A. Optics, Image Sci. Vis. 5, 2140–2144.

Hammond B.R. (Jr). & Fuld, K. (1992) Interocular differences in macular pigment density. *Invest. Ophthalmol. Vis. Sci.* **33**, 350–355.

Hammond B.R. (Jr). Fuld K., Curran-Celentano J. (1995) Macular pigment density in monozygotic twins. *Invest. Ophthalmol. Vis. Sci.* **36**, 2531–2541.

Hammond B.R. (Jr)., Curran-Celentano J., Judd S., Fuld K., Krinsky N.I., Wooten B.R., Snodderly D.M. (1996a) Sex differences in macular pigment optical density: Relation to plasma carotenoid concentrations and dietary patterns. *Vis. Res.* **36**, 2001–2012.

Hammond B.R. (Jr)., Fuld, K. & Snodderly, D.M. (1996b) Iris color and macula pigment optical density. *Exp. Eye. Res.* **62**, 293-297.

Hammond B.R., (Jr)., Wooten, B.R. & Snodderly, D.M. (1996c) Cigarette smoking and retinal carotenoids: Implications for age-related macular degeneration. *Vis. Res.* **36**, 3003–3009.

Hammond B.R., (Jr)., Wooten, B.R., Snodderly, D.M. (1997) Individual variations in the spatial profile of human macular pigment. J. Optic. Soc. Amer. A. Optics, Image Sci. Vis. **14**, 1187–1196.

Hammond B.R. (Jr)., Caruso-Avery, M. (2000) Macular pigment optical density in a southwestern sample. *Invest. Ophthalmol. Vis. Sci.* **41**,1492–1497.

Hammond B.R. (Jr)., Wooten, B.R., Smollon, B. (2005) Assessment of the validity of *in vivo* methods of measuring human macular pigment optical density. *Optom. Vis. Sci.* **82**, 387–404.

Hogg R.E., Anderson R.S., Stevenson M.R., Zlatkova M.B., Chakravarthy U. (2007a) *In vivo* macular pigment measurements: a comparison of resonance Raman spectroscopy and heterochromatic flicker photometry. *Br. J. Ophthalmol.* **91**, 485–490.

Hogg R.E., Zlatkova M.B., Chakravarthy U., Anderson, R.S.(2007b) Investigation of the effect of simulated lens yellowing, transparency loss and refractive error on *in vivo* resonance Raman spectroscopy. *Ophthal. Phys. Optics.* **27**, 225–231.

Hommel B., Li K.Z.H., Li, S.-C. (2004) Visual search across the life span. *Dev Psychol.* **40**, 545–558.

Howells O., Eperjesi F., Bartlett H. (2011) Measuring macular pigment optical density *in vivo*: a review of techniques. *Graef. Arch. Clin. Exp. Ophthalmol.* **249**, 315–347.

Howells O., Eperjesi F., Bartlett H. (2013a) Improving the repeatability of heterochromatic flicker photometry for measurement of macular pigment optical density. *Graef. Arch. Clin. Exp. Ophthalmol.* **251**, 871–880.

Howells O., Eperjesi F., Bartlett H. (2013b) Macular pigment optical density in young adults of South Asian origin. *Invest. Ophthalmol. Vis. Sci.* **54**, 2711–19

Hubel D.H., Wiesel, T.N. (1970) The period of susceptibility to the physiological effects of unilateral eye closure in kittens. *J. Physiol.* **206**, 419–436.

Hunt I. (2015) Lecture notes in Stereochemistry, University of Calgary. Alberta, Canada. (source:http://www.chem.ucalgary.ca/courses/350/Carey5th/Ch07/ch7-3.html) cited 5-10-15.

Iannaccone A., Mura M., Gallaher K.T., Johnson E.J., Todd W.A., Kenyon E., Harris T.L., Harris T., Satterfield S., Johnson K.C., Kritchevsky S.B. (2007) Macular pigment optical

density in the elderly: findings in a large biracial Midsouth population sample. *Invest. Ophthalmol. Vis. Sci.* **48**,1458–1465.

Isenberg S.J. (1986) Macular development in the premature infant. *Am. J. Ophthalmol.*, **101**, 74–80.

Jaffe G.J., Irvine A.R., Wood I.S., Severinghaus J.W., Pino G.R., Haugen C. (1988) Retinal phototoxicity from the operating microscope. The role of inspired oxygen. *Ophthalmol.* **95**, 1130–1141.

Johnson E.J., Hammond B.R., Yeum K.J., Qin J., Wang X.D., Castaneda C., Snodderly D.M., Russell R.M. (2000) Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density. *Am. J. Clin. Nutr.* **71**, 1555–1562.

Johnson E.J. Chung H.Y., Caldarella S.M., Snodderly D.M. (2008) The influence of supplemental lutein and docosahexaenoic acid on serum, lipoproteins, and macular pigmentation. *Am. J. Clin. Nutr.* **87**, 1521–1529.

Johnson E.J., Maras J.E., Rasmussen H.M., Tucker K.L. (2010) Intake of lutein and zeaxanthin differ with age, sex, and ethnicity. *J. Amer. Diet. Assoc.* **110**,1357–1362.

Johnson E.J. (2014) Role of lutein and zeaxanthin in visual and cognitive function throughout the lifespan. *Nutr. Rev.* **72**, 605–612.

Kanis M.J., Berendschot, T.T.J.M., van Norren, D. (2007) Influence of macular pigment and melanin on incident early AMD in a white population. *Graef. Arch. Clin. Exp. Ophthalmol.* **245**, 767–773.

Kaya S., Weigert G., Pemp B., Sacu S., Werkmeister R.M., Dragostinoff N., Garhöfer G., Schmidt-Erfurth U., Schmetterer L. (2012) Comparison of macular pigment in patients with age-related macular degeneration and healthy control subjects - A study using spectral fundus reflectance. *Act. Ophthalmol.* **90**, 399-403.

Khachik F., de Moura F.F., Zhao D.Y., Aebischer C.P., Bernstein P.S. (2002) Transformations of selected carotenoids in plasma, liver, and ocular tissues of humans and in nonprimate animal models. *Invest. Ophthalmol. Vis. Sci.* **43**, 3383–3392.

Khandhadia S., Lotery, A. (2010) Oxidation and age-related macular degeneration: insights from molecular biology. *Expert Rev. Molec. Med.* **12**, p.e34.

Kilbride P.E., Alexander K.R., Fishman M., Fishman G.A. (1989) Human macular pigment assessed by imaging fundus reflectometry. *Vision Res.* **29**:663-74.

King A., Gottlieb E., Brooks D.G., Murphy M.P., Dunaief J.L. (2004) Mitochondria-derived reactive oxygen species mediate blue light-induced death of retinal pigment epithelial cells. *Photochem. Photobiol.* **79**, 470–475.

de Kinkelder, R., van der Veen R.L., Verbaak F.D., Faber D.J., van Leeuwen T.G., Berendschot T.T.J.M. (2011) Macular pigment optical density measurements: evaluation of a device using heterochromatic flicker photometry. *Eye.* **25**,105–112.

Kinnunen K., Petrovski G., Moe M.C., Berta A., Kaarniranta K.(2012) Molecular mechanisms of retinal pigment epithelium damage and development of age-related macular degeneration. *Act. Ophthalmol.*, **90**, 299–309.

Kirby M.L., Galea M., Loane E., Stack J., Beatty S., Nolan J.M. (2009) Foveal anatomic associations with the secondary peak and the slope of the macular pigment spatial profile. *Invest. Ophthalmol. Vis. Sci.* **50**, 1383–1391.

Knight A.W., Billinton, N. (2001) Distinguishing GFP from cellular autofluorescence. *Biophotonics Inter.* **8**(7) 42–50.

Koh H., Murray I.J., Nolan D., Carden D., Feather J., Beatty S. (2004) Plasma and macular responses to lutein supplement in subjects with and without age-related maculopathy: a pilot study. *Exp. Eye Res.* **79**: 21– 27.

Koyama Y., Hashimoto H. (1988) Time-resolved resonance Raman spectroscopy of triplet .beta.-carotene produced from all-trans, 7-cis, 9-cis, 13-cis, and 15-cis isomers and high-pressure liquid chromatography analyses of photoisomerization via the triplet state. *J. Phys. Chem.* **92**, 2101–2108

van de Kraats J., Berendschot T.T.J.M., Valen S., van N. (2006) Fast assessment of the central macular pigment density with natural pupil using the macular pigment reflectometer. *J. Biomed. Opt.* **11**, 060431.

Krinsky N.I. (1979) Carotenoid protection against oxidation. *Pure App. Chem.* **51**, 649–660.

Krinsky N.I. (2002) Possible biologic mechanisms for a protective role of xanthophylls. *J. Nutr.* **132,** 540S–542S.

Kuchan M., Wang F., Geng Y., Feng B., Lai C. (2013) Lutein Stimulates the Differentiation of Human Stem Cells to Neural Progenitor Cells In Vitro. Presented at Advances and Controversies in Clinical Nutrition, Washington, DC. Abstract No. 23.

Lam R.F., Rao S.K., Fan D.S., Lau F.T., Lam D.S. (2005) Macular pigment optical density in a Chinese sample. *Curr. Eye Res.* **30**, 799–805.

Landrum J.T., Bone R.A., Kilburn M.D. (1997a) The macular pigment: a possible role in protection from age-related macular degeneration. Adv. Pharm. 38, 537-56.

Landrum J.T., Bone R.A., Joa H., Kilburn M.D., Moore L.L., Sprague K.E. (1997b) A one year study of the macular pigment: the effect of 140 days of a lutein supplement. *Exp. Eye. Res.* **65**, 57–62.

Landrum J.T., Bone R.A., Mayne S.T., Gomez C.M., Tibor S.E., Twaroska E.E. (2001) Macular pigment in donor eyes with and without AMD: a case-control study. Invest. Ophthalmol. Vis. Sci. 42, 235–240.

Lavanya R., Kawasaki R., Tay W.T., Cheung G.C., Mitchell P., Saw S.M., Aung T., Wong T.Y. (2010) Hyperopic refractive error and shorter axial length are associated with age-related macular degeneration: The Singapore Malay eye study. *Invest. Ophthalmol. Vis. Sci.* **51**, 6247–6252.

Leibowitz H.M., Krueger D.E., Maunder L.R., Milton R.C., Kini M.M., Kahn H.A., Nickerson R.J., Pool J., Colton T.L., Ganley J.P., Loewenstein J.I., Dawber T.R. (1980) The Framingham Eye Study monograph: An ophthalmological and epidemiological study of cataract, glaucoma, diabetic retinopathy, macular degeneration, and visual acuity in a general population of 2631 adults, 1973-1975. *Surv. Ophthalmol.* **24**(Suppl) 335–610.

Lim B.P., Nagao A., Terao J., Tanaka K., Suzuki T., Takama K. (1992) Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. *Biochimica et Biophysica Acta*, **1126**, 178–184.

Lima V.C., Rosen R.B., Maia M., Prata T.S., Dorairaj S., Farah M.E., Sallum J. (2010) Macular pigment optical density measured by dual-wavelength autofluorescence imaging in diabetic and nondiabetic patients: a comparative study. *Invest. Ophthalmol. Vis. Sci.* **51**, 5840–5845.

Loane E., Stack J., Beatty S., Nolan J.M. (2007) Measurement of macular pigment optical density using two different heterochromatic flicker photometers. *Curr. Eye Res.* **32**, 555–564.

Loane E., Nolan J.M., O'Donovan O., Bhosale P., Bernstein P.S., Beatty S. (2008) Transport and Retinal Capture of Lutein and Zeaxanthin with Reference to Age-related Macular Degeneration. *Surv. Ophthalmol.* **53**, 68–81.

Loughman J., Scanlon G., Nolan J.M., O'Dwyer V., Beatty S. (2012) An evaluation of a novel instrument for measuring macular pigment optical density: The MPS. *Act. Ophthalmol.* **90**, e90–7.

McAlinden C., Khadka J., Pesudovs K. (2011) Statistical methods for conducting agreement (comparison of clinical tests) and precision (repeatability or reproducibility) studies in optometry and ophthalmology. *Ophth. Physiol. Opt.* **31(4)**:330-8.

McCorkle S.M., Raine L.B., Hammond B.R. (Jr)., Lisa Renzi-Hammond L.R., Hillman C.H., Khan N.A. (2015) Reliability of Heterochromatic Flicker Photometry in Measuring Macular Pigment Optical Density among Preadolescent Children. *Foods.* **4**, 594-604.

Ma L., , Dou H.L., Wu Y.Q., Huang Y.M., Huang Y.B., Xu X.R., Zou Z.Y., Lin X.M. (2012) Lutein and zeaxanthin intake and the risk of age-related macular degeneration: a systematic review and meta-analysis. *Br. J. Nutr.* **107**, 350–9.

Madden D.J., Allen P.A. (1995) Aging and the speed/accuracy relation in visual search: evidence for an accumulator model. *Optom. Vis. Sci.* **72**, 210–216.

Makridaki M., Carden D. & Murray I.J. (2009) Macular pigment measurement in clinics: Controlling the effect of the ageing media. *Ophth. Phys. Optics.* **29**, 338–344.

Malinow M.R., Feeney-Burns L., Peterson L.H., Klein M.L., Neuringer M. (1980) Diet-related macular anomalies in monkeys. *Invest. Ophthalmol. Vis. Sci.* **19**, 857–863.

Mares-Perlman J.A., Brady W.E., Klein R., Klein B.E., Bowen P., Stacewicz-Sapuntzakis M., Palta M. (1995) Serum antioxidants and age-related macular degeneration in a population-based case-control study. *Arch.Ophthalmol*, **113**, 1518–1523.

Mares J.A., LaRowe T.L., Snodderly D.M., Moeller S.M., Gruber M.J., Klein M.L., Wooten B.R., Johnson E.J., Chappell R.J; CAREDS Macular Pigment Study Group and Investigators. (2006) Predictors of optical density of lutein and zeaxanthin in retinas of older women in the Carotenoids in Age-Related Eye Disease Study, an ancillary study of the Women's Health Initiative. *Am J Clin Nutr*, **84**, 1107–1122.

di Mascio P., Kaiser, S. & Sies, H. (1989) Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochem. Biophys.* **274**, 532–538.

Mellerio J., Ahmadi-Lari S., van Kuijk F., Pauleikhoff D., Bird A., Marshall J. (2002) A portable instrument for measuring macular pigment with central fixation. *Curr. Eye Res.* **25**, 37–47.

Menon I.A., Wakeham D.C., Persad S.D., Avaria M., Trope G.E., Basu P.K. (1992) Quantitative determination of the melanin contents in ocular tissues from human blue and brown eyes. *J.Oc. Pharm.* **8**, 35–42.

Michels M., , Lewis H., Abrams G.W., Han D.P., Mieler W.F., Neitz J. (1992) Macular phototoxicity caused by fiberoptic endoillumination during pars plana vitrectomy. *Am. J. Ophthalmol.* **114**, 287-96

Moreland J.D., Robson A.G., Soto-Leon N., Kulikowski J.J. (1998) Macular pigment and the colour-specificity of visual evoked potentials. *Vis. Res.* **38**, 3241–3245.

Murray I. J. (2008) The Mpod – a new instrument for measuring macular pigment. *Optician*, 25-01-08, 32-34.

Murray I.J. (2011) Letter response to editor: MPS and Bartlett Study. *Br. J. Ophthalmol.* 2011;**95**:431-432

Murray I.J., Makridaki M., van der Veen R.L., Carden D., Parry N.R., Berendschot T.T.J.M. (2013a) Lutein supplementation over a one-year period in early AMD might have a mild beneficial effect on visual acuity: The CLEAR study. *Invest. Ophthalmol. Vis. Sci.* **54**,1781–1788.

Murray I.J., Carden D. (2013b) Measuring macular pigment. *Optician* 8-3-13, 23-25.

Murray I.J., Hassanali B., Carden D. (2013c) Macular pigment in ophthalmic practice; a survey. *Graefe's Arch. for Clin. Exper. Ophthalmol.* **251**, 2355-2362

Neelam K., O'Gorman N., Nolan J, O'Donovan O, Wong HB, Au Eong KG, Beatty S. (2005) Measurement of macular pigment: Raman spectroscopy versus heterochromatic flicker photometry. *Invest. Ophthalmol. Vis. Sci.* **46**,1023–1032.

Neelam K. Nolan J., Loane E., Stack J., O'Donovan O., Au Eong K.G., Beatty S. (2006) Macular pigment and ocular biometry. *Vis. Res.* **46**, 2149–2156.

Noble K.G., Margolis S., Carr R.E. (1989) The golden tapetal sheen reflex in retinal disease. *Am. J. Ophthalmol.* **107**, 211-17.

Nolan J., O'Donovan O., Kavanagh H., Stack J., Harrison M., Muldoon A., Mellerio J., Beatty S. (2004) Macular pigment and percentage of body fat. *Invest. Ophthalmol. Vis. Sci.* **45**, 3940–3950.

Nolan J.M., Stack J., O' Donovan O., Loane E., Beatty S. (2007) Risk factors for age-related maculopathy are associated with a relative lack of macular pigment. *Exp. Eye. Res.* **84**, 61–74.

Nolan J.M., Kenny R., O'Regan C., Cronin H., Loughman J., Connolly E.E., Kearney P., Loane E., Beatty S. (2010) Macular pigment optical density in an ageing Irish population: The Irish Longitudinal Study on Ageing. *Ophthalmic Res.* **44**, 131–139.

Nunnaly J. (1978) Psychometric theory. New York: *McGraw-Hill.*

Nussbaum J.J., Pruett, R.C. & Delori, F.C. (1981) Historic perspectives. Macular yellow pigment. The first 200 years. *Retina.* **1**, 296–310.

Obana A., Gohto Y., Tanito M., Okazaki S., Gellermann W., Bernstein P.S., Ohira A. (2014) Effect of age and other factors on macular pigment optical density measured with resonance Raman spectroscopy. *Graef. Arch. Clin. Exp. Ophthalmol.* **252**, 1–8.

Oka M., Yamashita T., Ono S., Kubo I., Tabuchi A. (2013) Quadrantal macular retinal thickness changes in strabismus subjects with abnormal binocular vision development. *Jap. J. Ophthalmol.* **57**, 225–32.

Pipis A., Touliou, E., Augustin, A.J. (2013) Macular pigment optical density in a Central European population. *Ophthalmic Surg. Las Imag Retina.* **44**, 260–7.

Pokorny, J., Xu, J., Smith, V.C. (1997) Optical density of the human lens. *J. Opt. Soc. Am.* **14**, 953-960.

Porciatti V., Fiorentini A., Morrone M.C., Burr D.C. (1999) The effects of ageing on reaction times to motion onset. *Vis. Res.* **39**, 2157–2164.

Qin L., Bartlett H., Griffiths H.R., Eperjesi F., Armstrong R.A., Gherghei D. (2011) Macular Pigment Optical Density is Related to Blood Glutathione Levels in Healthy Individuals. *Invest. Ophthalmol. Vis. Sci* .**52**, 5029-5033.

Raman R., Biswas S., Gupta A., Kulothungan V., Sharma T. (2012) Association of macular pigment optical density with risk factors for wet age-related macular degeneration in the Indian population. *Eye*, **26**, 950–957.

Rapp L.M., Williams T.P. (1980) A parametric study of retinal light damage in albino and pigmented rats. In: Williams TP, Baker BN. *The effects of constant light on visual processes.* New York: Plenum Press,135-59.

Rapp L.M., Maple, S.S., Choi, J.H. (2000) Lutein and zeaxanthin concentrations in rod outer segment membranes from perifoveal and peripheral human retina. *Invest. Ophthalmol. Vis. Sci.* **41**,1200–1209.

Renzi L.M., Dengler M.J., Puente A., Miller L.S., Hammond B.R.,(Jr). (2014 Relationships between macular pigment optical density and cognitive function in unimpaired and mildly cognitively impaired older adults. *Neurobiol. Aging,* **35**: 1695–1699.

Robson A.G., Parry N.R.A. (2008) Measurement of macular pigment optical density and distribution using the steady-state visual evoked potential. *Vis. Neurosci,* **25**, 575–83.

von Rückmann A., Fitzke, F.W. & Bird, A.C. (1995) Distribution of fundus autofluorescence with a scanning laser ophthalmoscope. *Br. J. Ophthalmol.* **79**, 407–412.

Sajilata M.G., Singhal, R.S., Kamat, M.Y. (2008) The carotenoid pigment zeaxanthin - A review. *In Comprehensive Reviews in Food Science and Food Safety.* **7**, 29–49.

SanGiovanni J.P., Neuringer, M. (2012)The putative role of lutein and zeaxanthin as protective agents against age-related macular degeneration: Promise of molecular genetics for guiding mechanistic and translational research in the field. *Am. J. Clin. Nutr.* **96**, 1223(S) – 1233(S)

Sasamoto Y., Gomi F., Sawa M., Tsujikawa M., Nishida K. (2011) Effect of 1-year lutein supplementation on macular pigment optical density and visual function. *Graef. Arch. Clin. Exp. Ophthalmol.* **249**, 1847–1854.

Schmitz-Valckenberg S., Holz F.G., Bird A.C., Spaide R.F. (2008) Fundus autofluorescence imaging: review and perspectives. *Retina.* **28**, 385–409.

Schweitzer D., Jentsch S., Dawczynski J., Hammer M., Wolf-Schnurrbusch U.E., Wolf S. (2010) Simple and objective method for routine detection of the macular pigment xanthophyll. *J. Biomed. Opt.* **15**, 061714.

Seddon J.M., Ajani U.A., Sperduto R.D., Hiller R., Blair N., Burton T.C., Farber M.D., Gragoudas E.S., Haller J., Miller D.T. (1994) Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. *J. Amer. Med. Assoc.* **272**, 1413–1420.

Sharifzadeh M., Bernstein, P.S. & Gellermann, W. (2006) Nonmydriatic fluorescence-based quantitative imaging of human macular pigment distributions. *J. Optic. Soc. Amer. A. Optics, Image Sci. Vis.* **23**, 2373–2387.

Sharma P., Jha A.B., Dubey R.S., Pessarakli M. (2012) Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *J. Botany*. **2012**, 1–26.

Slezak P, I., Waczulikova I. (2010) Reproducibility and Repeatability. *Physiol. Res.* **60**, 203-205, 2011

Snodderly D.M., Brown P.K., Delori F.C., Auran J.D. (1984) The macular pigment. I. Absorbance spectra, localization, and discrimination from other yellow pigments in primate retinas. *Invest. Ophthalmol. Vis. Sci.* **25**, 660–673.

Snodderly D.M. (1995) Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am. J. Clin. Nutr.* **62**, 1448S–1461S.

Snodderly D.M., Mares J.A., Wooten B.R., Oxton L., Gruber M., Ficek T.; CAREDS Macular Pigment Study Group (2004) Macular pigment measurement by heterochromatic flicker photometry in older subjects: the carotenoids and age-related eye disease study. *Invest. Ophthalmol. Vis. Sci.* **45**, 531–538.

Sommerburg O., Keunen J.E.E., Bird A.C., Van Kuijk F.J.G.M. (1998) Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. *Br. J. Ophthalmol.* **82**: 907-910.

Stahl W., Sies H. (2001) Effects of carotenoids and retinoids on gap junctional communication. *Biofactors* **15**: 95–98.

Stahl W., Sies, H. (2007) Carotenoids and flavonoids contribute to nutritional protection against skin damage from sunlight. *Molec. Biotechnol.* **37**, 26–30.

Stringham J.M. Hammond B.R. (Jr)., Nolan J.M., Wooten B.R., Mammen A., Smollon W., Snodderly D.M. (2008) The utility of using customized heterochromatic flicker photometry (cHFP) to measure macular pigment in patients with age-related macular degeneration. *Exp. Eye. Res.* **87**, 445–453.

Sundelin S.P., Nilsson S.E.G. (2001) Lipofuscin-formation in retinal pigment epithelial cells is reduced by antioxidants. *Free Radical Biol. Med.* **31**, 217–225.

Tang C.-Y.Y., Yip H.S., Poon M.Y., Yau W.L., Yap M.K. (2004) Macular pigment optical density in young Chinese adults. *Ophth. Physiol. Optics.* **24**, 586–593.

Tanito M., Obana A., Gohto Y., Okazaki S., Gellermann W., Ohira A. (2012) Macular pigment density changes in Japanese individuals supplemented with lutein or zeaxanthin: Quantification via resonance Raman spectrophotometry and autofluorescence imaging. *Jap. J. Ophthalmol.* **56**, 488–496.

Tomany S.C., Cruickshanks K.J., Klein R., Klein B.E., Knudtson M.D. (2004) Sunlight and the 10-year incidence of age-related maculopathy: the Beaver Dam Eye Study. *Arch. Ophthalmol.* **122**, 750–757.

Trieschmann M., Heimes B., Hense H.W., Pauleikhoff D. (2006) Macular pigment optical density measurement in autofluorescence imaging: comparison of one- and two-wavelength methods. *Graef. Arch. Clin. Exp. Ophthalmol.* **244**, 1565–1574.

Trieschmann M., Beatty S., Nolan J.M., Hense H.W., Heimes B., Austermann U., Fobker M., Pauleikhoff D. (2007) Changes in macular pigment optical density and serum concentrations of its constituent carotenoids following supplemental lutein and zeaxanthin: The LUNA study. *Exp. Eye. Res.* **84**, 718–728.

University of Ulster, Research Institute (2016) http://biomed.science.ulster.ac.uk/research-institute/niche/facilities/ cited 2-3-16

Vaegan., Taylor, D. (1979) Critical period for deprivation amblyopia in children. *Trans. Ophthalmol. Soc. U. K.* **99**, 432–439.

van der Veen R.L.P., Berendschot T.T.J.M., Hendrikse F., Carden D., Makridaki M., Murray I.J. (2009a) A new desktop instrument for measuring macular pigment optical density based on a novel technique for setting flicker thresholds. *Ophth. Physiol. Optics.* **29**, 127–137.

van der Veen R.L.P. , Berendschot T.T.J.M., Hendrikse F., Carden D., Makridaki M., Murray I.J. (2009b) Correspondence between retinal reflectometry and a flicker-based technique in the measurement of MP spatial profiles. *J. Biomed. Opt.* 14: 064046

Viner C. (2005) Measuring macular pigment levels: An in-practice procedure? Optician-online, http://www.opticianonline.net/measuring-macular-pigment-levels-an-in-practice-procedure/ cited 19-4-16

Vishwanathan R., Kuchan M.J., Sen S., Johnson E.J. (2014) Lutein and preterm infants with decreased concentrations of brain carotenoids. *J. Pediatr. Gastroenterol. Nutr.* **59**: 659–665.

Wallace D., Chandler D.L. , Beck R.W., Arnold R.W, Bacal D.A., Birch E.E, Felius J., Frazier M., Holmes J.M, Hoover D., Klimek D.A., Lorenzana I., Quinn G.E., Repka M.X., Suh D.W., Tamkins S. (2007) Treatment of Bilateral Refractive Amblyopia in Children 3 to <10 Years Old. *Am J Ophthalmol.* **144**, 487–496.

Wang J.J., Jakobsen K.B., Smith W., Mitchell P. (2004) Refractive status and the 5-year incidence of age-related maculopathy: The Blue Mountains Eye Study. *Clin. Exp. Ophthalmol.* **32**, 255–258.

Weigert G., Kaya S., Pemp B., Sacu S., Lasta M., Werkmeister R.M., Dragostinoff N., Simader C., Garhöfer G., Schmidt-Erfurth U., Schmetterer L. (2011) Effects of Lutein Supplementation on Macular Pigment Optical Density and Visual Acuity in Patients with Age-Related Macular Degeneration. *Invest. Ophthalmol. Vis. Sci.*, **52**, 8174–8178.

Weiter J.J., Delori F.C., Wing G.L., Fitch K.A., (1985) Relationship of senile macular degeneration to ocular pigmentation. *Am. J. Ophthalmol.* **99**, 185–187.

Weiter J.J., Delori F., Dorey C.K. (1988) Central sparing in annular macular degeneration. *Am. J. Ophthalmol.* **106**, 286–292.

Wenzel A.J., Sheehan J.P., Burke J.D., Lefsrud M.G., Curran-Celentano J. (2007a) Dietary intake and serum concentrations of lutein and zeaxanthin, but not macular pigment optical density, are related in spouses. *Nutr. Res.* **27**, 462–469.

Wenzel A.J., Sheehan J.P., Gerweck C., Stringham J.M., Fuld K., Curran-Celentano J. (2007b) Macular pigment optical density at four retinal loci during 120 days of lutein supplementation. *Ophth. Physiol. Optics.* **27**, 329–335.

Werner J.S., Wooten B.R. (1979) Opponent chromatic mechanisms. Relation to photopigments and hue naming. *J. Optic. Soc. Amer. A. Optics, Image Sci. Vis.* **69**, 422–434.

Werner J.S., Donnelly S.K., Kliegl, R. (1987) Aging and human macular pigment density. Appended with translations from the work of Max Schultze and Ewald Hering. *Vis. Res.* **27**, 257–268.

Westheimer G., Liang J. (1995) Influence of ocular light scatter on the eye's optical performance. *J. Optic. Soc. Amer. A. Optics, Image Sci. Vis.* **12**, 1417–1424.

Wooten B.R., Hammond B.R. (Jr),, Land R.I., Snodderly D.M. (1999) A practical method for measuring macular pigment optical density. *Invest. Ophthalmol. Vis. Sci.* **40**, 2481–2489.

Wüstemeyer H., Jahn C., Nestler A., Barth T., Wolf S. (2002) A new instrument for the quantification of macular pigment density: first results in patients with AMD and healthy subjects. *Graef. Arch. Clin. Exp. Ophthalmol.* **240**, 666–671.

Wüstemeyer H., Moessner A., Jahn C., Wolf S. (2003) Macular pigment density in healthy subjects quantified with a modified confocal scanning laser ophthalmoscope. *Graef. Arch. Clin. Exp. Ophthalmol.* **241**, 647–651.

Yao Y., Qiu Q.H., Wu X.W., Cai Z.Y., Xu S., Liang X.Q. (2013) Lutein supplementation improves visual performance in Chinese drivers: 1-year randomized, double-blind, placebo-controlled study. *Nutr.* **29**, 958–964.

Yu J., Johnson E.J., Shang F., Lim A., Zhou H., Cui L., Xu J, Snellingen T., Liu X., Wang N., Liu N. (2012) Measurement of macular pigment optical density in a healthy Chinese population sample. *Invest. Ophthalmol. Vis. Sci.* **53**, 2106–2111.

Yuying Ji, Zhang X., Wu K., Su Y., Zuo C., Chen H., Li M., Wen F. (2015) Macular pigment optical density in a healthy Chinese population. *Act. Ophthalmol.* **93**, 550–555.

Zagers N.P.A., van de Kraats J., Berendschot T.T.J.M., van Norren D. (2002) Simultaneous measurement of foveal spectral reflectance and cone-photoreceptor directionality. *App. Optics.* **41**, 4686–4696.

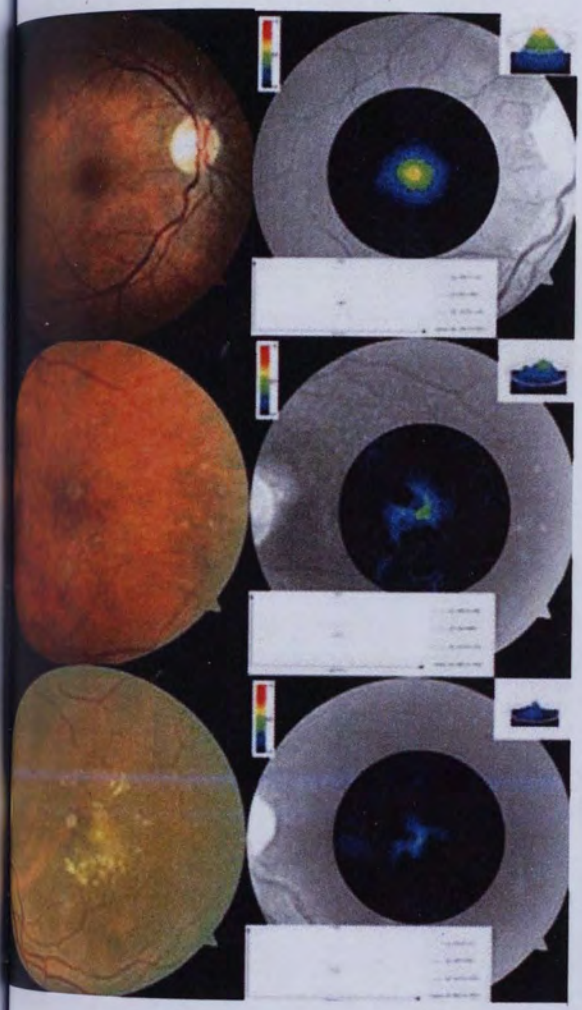
Zhang X., Hargitai J., Tammur J., Hutchinson A., Allikmets R., Chang S., Gouras P. (2002) Macular pigment and visual acuity in Stargardt macular dystrophy. *Graef. Arch. Clin. Exp. Ophthalmol.* **240**, 802–809.

Zhu J., Zheng W., Zhang Z., He G., Ke B. (2012) MPOD and its Relationship with Refractive Status and Foveal Thickness in Chinese School-aged Children. *Curr. Eye Res.* **38**, 1–6

Ziylan S., Yabas O., Zorlutuna N., Serin D. (2007) Isoametropic amblyopia in highly hyperopic children. *Acta. Ophthalmol. Scand.* **85**:111-3.

Appendix 1 Definition, history and clinical relevance of MPOD

Fundus Images and MPOD Analyses of:



Healthy Patient

Patient with dry AMD

Patient with Exudative AMD

Figure A1.1 - Introduction to MPOD: maps of three eyes included in a 2011 study, note the erosion and damage of the MP in advancing AMD

Source: Dawczynski J. *et al.* (2011) Macular Pigment Density Measurement in Patients with Age-related Macular Degeneration. *Euro. Ophth. Rev.* **5**(2):141-2,

Appendix 2 Literature review

The Visucam and FR

Care of the Visucam used in Study 1+2

Figure A2.1 Preparing for cleaning with the folded cleaning cloth

(Image courtesy of Zeiss, Germany)

Figure A2.2 Cleaning by circular movements

(Image courtesy of Zeiss Germany)

Figure A2.3 – The Macular Densitometer (Macular Metrics) sometimes referred to as the gold standard HFP

The original densitometer was intended for research and measure MPOD at 4 different foveal loci, providing the ability to map the foveal distribution of macular pigment
The current model is intended for clinical use but is still suitable for use in research settings, measures MPOD at two foveal loci, the standard locus of 0.5 degrees of visual angle from the centre of the fovea, and a second locus at 0.25 degrees of visual angle. This locus has been dubbed the meso locus due to the larger distribution of meso-zeaxanthin in this retinal area. The densitometer can be set to include either or both foveal measurements in a test.

Source: http://www.macularmetrics.us/what-is-macular-densitometer.php

Figure A2.4: Macuscope. The MacuScope uses the psychophysical technique of heterochromatic flicker photometry to measure MPOD.

Source: http://www.lensneye.com/macuscope/

Appendix 3 Assessing the repeatability of the Visucam in a paediatric sample

Parent Consent Form

Aston University
Birmingham

Consent Form

Michael Hope 955299

Research workers, School and subject area responsible:

Dr Eperjesi, Life & Health Sciences, Vision Sciences, Aston University
Mr Michael Hope, Life & Health Sciences, Vision Sciences, Aston University and Staff Optometrist at Coleman Opticians.

Project Title

A prospective analysis of the level of macula pigment density in a paediatric population.

Invitation

Your child is being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

What is the purpose of the study?

The purpose of this study is to assess the pigment structure within the retina of children's eyes. This will enable doctors and optometrists in the future to gain a better understanding as to what constitutes a normal level of retinal pigment. The analysis will look at children's age and gender to assess if these have any effect. The information will be useful in comparing to older peoples change in the retina as they age and may assist in further developing future treatment strategies for vision loss in the elderly.

Why have I been chosen?

You have been chosen as your child is a patient at the practice and has an age matched requirement for the study.

What will happen to me if I take part?

By volunteering for your child to participate you will be giving the research team consent to carry out an additional retinal photo of your child's eyes. The photo is completely safe and will take just 2-3 minutes of the appointment time. They will not be required to sit for any further tests. The research is being undertaken between Coleman Opticians, Norwich and Aston University, in Birmingham. It is anticipated that the study will last 18 months and run between September 2012 and January 2014. The study will collate results of the structure of children retinal images within the practice.

Are there any potential risks in talking part in the study?

The only real risk, as with all clinical research, is the risk of breaching privacy and confidentiality in relation to your child's ophthalmic health. This risk will be minimised by keeping your data anonymous at all times.

Do I have to take part?

No, you do not have to participate if you do not wish to do so. You are free to withdraw your child at any time from this research. No sanctions will be taken against any patient or parent/guardian who refuses to participate in or withdraws from the research.

Expenses and payments

There are no expenses or payments for participation in this research.

Will my taking part in this study be kept confidential?

Yes, your child's participation in the study will be strictly confidential through the use of reference numbers rather than names. Other members of the research team will only be given access to the research database after your identity has been removed. There will be no way to link any research

data to any individual participant. Data will be collected in paper form and recorded on study record sheets. Retinal images will be analysed with software and the results of this will be recorded on study data paper sheets. Data will be held on no more than 3 computer based storage devices. On completion of the study in 2016, data relating to the study will be erased from all computer sources within a 6 month time period and all written data will be shredded; this will be independently verified by two further optometrists/doctors in both cases.

What will happen to the results of the research study?

The results of this study will undergo statistical analysis in order to assess for patterns and trends in what factors may affect the change in the retina structure as we age. This may help future optometrists to further refine the diagnosis of sight threatening disease. Full patient confidentiality will be maintained at all times. We aim to publish the results of this research in professional journals. A copy of the published research will be made available during 2016-2017 within Coleman Optometrists practice, Norwich and Aston University, Birmingham. However, there will be no reference to any individual's identifiable details in any publication.

Who is organising and funding the research?

Funding of this research is through the following sources:

Coleman Optometrists
James Paget University Hospital, Norfolk
College of Optometrists
Michael Hope - Research Optometrist

The research is being organized by Michael Hope/Coleman Optometrists and Aston University.

Who has reviewed the study?

The research has been submitted for approval by Aston University's Ethics Committee.

Who do I Contact if Something Goes Wrong or I need Further Information?

Please contact: Mr M J Hope BSc (Hons) BMedSc, MCOptom

Aston University

Content has been removed for copyright reasons

Who do I contact if I wish to make a complaint about the way in which the research is conducted?

If you have any concerns about the way in which the study has been conducted, then you should contact Secretary of the University Research Ethics Committee on j.g.walter@aston.ac.uk or telephone 0121 204 4665.

Children Consent Form

VOLUNTEER CONSENT FORM

Title of Project: A prospective analysis of the level of macula pigment density in a paediatric population

Name of Chief Researcher: Mr M J Hope BSc (Hons) BMedSc, MCOptom

		Tick Box
1	I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2	I understand that my child's participation is voluntary and that I am free to withdraw them at any time without giving any reason, without my medical care or legal rights being affected.	
3	I agree to take part in the above study.	

_____ _____ _____

Name of parent/guardian Date Signature

Figure A3.1 – Study 1+2 parental consent form

Children's Agreement Form Michael Hope 955299

The people doing this research are:

Dr Eperjesi and Mr Michael Hope with Aston University, Birmingham.

Project Title

A study looking at children's eyes and how they compare to adult and old people's eyes.

Invitation

We are asking you to take part in a new study of children's eye health.

What is the reason for the study?

The information we collect may help to better understand how children's and adult's eyes are affected by getting old.

Why have I been chosen?

You have been chosen as you are the right age for the study.

What will happen to me if I take part?

You will have a special machine take a picture of your eyes. Your optometrist will stay with you all the time and your parents can watch if they like. The test is completely safe and will take just 2 or 3 minutes of time. It is similar to what your parents may sometimes have when they have an eye test.

Are there any reasons not to take part in the study?

We will keep all your results as secret as possible, nobody will be able to tell your eye pictures from other children's eye pictures. If you don't like the camera machine during the test you can ask for it to stop and it will stop straight away.

Do I have to take part?

No, you do not have to if you don't want to ☺

Will my results be kept secret?

Yes, all your results will be kept secret. Other people may see your results but only once your name has been removed. We will keep your results on a password protected computer.

What will happen to the results of the research study?

The results of this study will be studied to look for patterns and reasons in how children's eyes change towards becoming adults eyes. It will help doctors and optometrists to understand how normal eyes are different in children and adults and compare with problem eyes. This is so that we can understand how to help people with problem eyes in the future.

Who is arranging and paying for this study?

College of Optometrists
Coleman Optometrists
James Paget University Hospital, Norfolk
Michael Hope - Research Optometrist
Zeiss UK

The research is being organized by Michael Hope/Coleman Optometrists and Aston University.

Who has reviewed the study?

The study has been agreed to by Aston University's Ethics Committee.

Who do I speak to if I'm not happy?

Please speak with your optometrist you are with if you are not happy, or send a letter to Michael Hope at Coleman Opticians.

If you are still not happy you can contact: Aston University: j.g.walter@aston.ac.uk or telephone 0121 204 4665.

CHILDRENS AGREEMENT FORM

Aston University
Birmingham

Title of Project: A study looking at children's eyes and how they compare to adult and old people's eyes

Name of Study Researcher: Mr M J Hope BSc (Hons) BMedSc, MCOptom

		Tick Box
1	I agree that I have read this and understand it.	
2	I know that I don't have to take part in the study if I don't want to.	
3	I agree to take part in this study.	

Your name _____ Date _____ Signature _____

Figure A3.2 – Study 1+2 Children's consent info form

Data Collection Sheet

PAEDIATRIC MACULAR PIGMENT STUDY: Prelim DATA COLLECTION SHEET

Optometrist :

Today's date:

Pt Ref number: Male/Female Age: Years Months

Iris pigmentation: Light/Dark

Refraction:

R L

Macular Pigment Data via Visucam 200, Visit 1:

Right eye

Macular Pigment Data via Visucam 200: Visit 2,

date:

Right eye

---------- Iris: Light/Dark

Figure A3.3 – Study 1+2 data collection sheet

Aston University
Birmingham

Life and Health Sciences Research Ethics Committee's Decision Letter

Thank you for your resubmission. The additional information for the above proposal has been considered by the Chair of the LHS Ethics Committee.

Please see below for details of the decision and the approved documents.

Reviewer's recommendation: Approved

Please see the tabled list below of approved documents:

Documentation	Version/s	Date	Approved
Parent's information sheet and consent form	Parent consent form1c	31/04/2014	√
Children's information sheet	Children's agreement form1c	31/04/2014	√
Response to Referees	Referees response to 604	31/04/2014	√
Food Questionnaire	food_diary_hope	19/02/2014	√

After starting your research please notify the LHS Research Ethics Committee of any of the following:

Substantial amendments. Any amendment should be sent as a Word document, with the amendment highlighted. The amendment request must be accompanied by all amended documents, e.g. protocols, participant information sheets, consent forms etc. Please include version number and amended date to the file name of any amended documentation (e.g. "Ethics Application #100 Protocol v2 amended 17/02/12.doc").

Please email all notifications and reports to lhs_ethics@aston.ac.uk and quote the original project reference number with all correspondence.

Ethics documents can be downloaded from: http://www.ethics.aston.ac.uk/documents-all. Please note that these documents can ONLY be opened using Mozilla Firefox or the latest Internet Explorer version (IE9).

Statement of Compliance

The Committee is constituted in accordance with the Government Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK. In accord with University Regulation REG/11/203(2), this application was considered to have low potential risk and was reviewed by three appropriately qualified members, including the Chair of the Life and Health Sciences Ethics Committee.

Yours sincerely,

CMSpickett

Dr Corinne M Spickett
Chair of the LHS Ethics Committee

Figure A3.4– Ethics Committee approval for Study 1 and Study 2

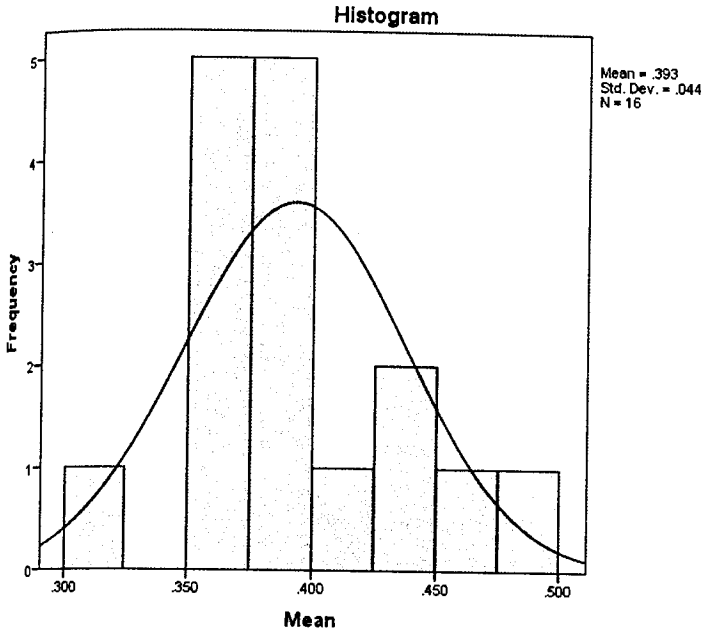


Figure A3.5 - Study 1 Normality distribution of Subjects difference in MPOD (measures 1+2)

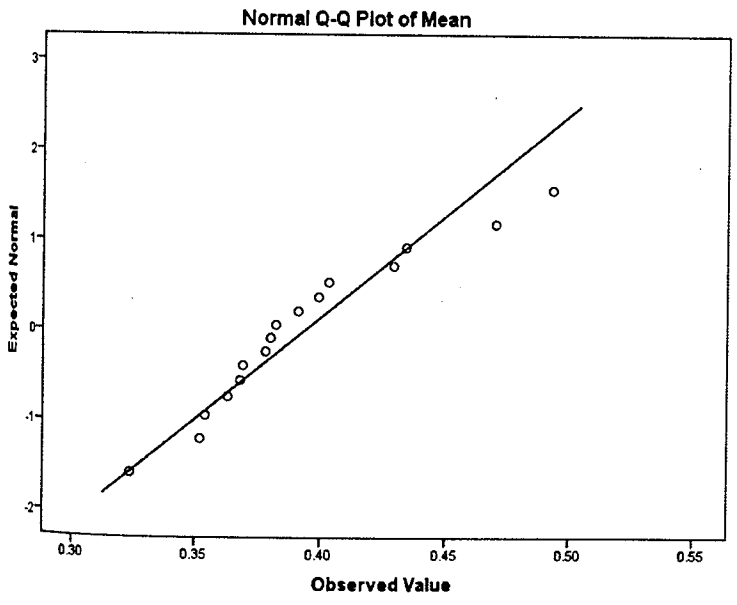


Figure A3.6 – Study 1- Q-Q plot of Subjects difference in MPOD (MPOD1+MPOD2)

A list of all fundus reflectometry (FR) studies to date that have given statistical data on reliability

Study/year	Number of subjects	Age range; eye health status; inter or intra session reliability	Reliability statistics
Berendschot *et al.*/ 2000	8	18–50; normal; intra-session.	Two measurements. Within subjects variation and coefficients of repeatability were 10% and 0.17 with a SLO, and 17% and 0.27 with a purpose-built reflectometer.
Delori *et al.*/2001	9	21–72; normal; both.	Two sessions, four subjects on different days & the other five on the same day. Mean absolute difference between 1st and 2nd session 0.039±0.029. Mean absolute test–retest difference (as a percentage of mean density)= 22%.
Delori *et al.*/2001	22	22–78; normal; inter-session.	Two sessions, 10-24 months apart. Mean absolute difference between sessions 0.042±0.042. Mean absolute test–retest difference=19%.
Berendschot *et al.*/ 2002	17	Average age 67.5; six ARM, 11 normal; intra-session.	Two sets of measurements. Coefficient of repeatability 0.11. Mean relative difference between measurements=10%.
Bour *et al.*/2002	'6 eyes'	23 subjects in full study, 6–20; normal; intra-session.	Two sets of measurements with a fundus camera. Correlation coefficient between measures was r=0.77 (p<0.05).
Wüstemeyer *et al.*/ 2002	10	16–43; normal; intra-session.	Two sessions with a SLO, no more than 30 mins apart. Mean within subjects coefficient of variation=6.2%. As a percentage of mean density, as used by Delori *et al.* [56], mean test–retest difference=3.1%.
Zagers *et al.*/2002	21	18–27 (n=15) & 40–74 (n=6); normal; intra-session.	Twenty-five measurements all in the same sitting with the FRA. Coefficient of repeatability 0.084.
Berendschot and van Norren/2005	53	19–76; normal; intra-session.	Five measurements (same sitting). Mean within subjects variation and coefficients of repeatability were 5.5% and 0.078 with the FRA 1, and 7.0% and 0.09 with the FRA 2.
van de Kraats *et al.*/ 2006	10	20 subjects in full study, 18–79; normal; both.	Two sessions on different days with the MPR. Test–retest correlation was r=0.94 (p<0.001). Five spectra measured in each test condition (intra-session), gave a mean within subjects variation of 'typically' 7%

Figure A3.7 – A Summary of previous MPOD repeatability studies in comparison to Study 1 (Source: Howells *et al.* 2011)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.237799
R Square	0.056548
Adjusted R Square	-0.01084
Standard Error	0.048846
Observations	16

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.002002	0.002002	0.839125	0.375157
Residual	14	0.033404	0.002386		
Total	15	0.035406			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-0.10011	0.112267	-0.89167	0.387636	-0.34089	0.140683	-0.34089	0.140683
X Variable 1	0.260211	0.284062	0.916038	0.375157	-0.34904	0.869463	-0.34904	0.869463

Table A3.1 - ANOVA

Descriptives

			Statistic	Std. Error
Mean	Mean		.39306	.011110
	95% Confidence Interval for Mean	Lower Bound	.36938	
		Upper Bound	.41674	
	5% Trimmed Mean		.39135	
	Median		.38100	
	Variance		.002	
	Std. Deviation		.044441	
	Minimum		.324	
	Maximum		.493	
	Range		.169	
	Interquartile Range		.058	
	Skewness		.909	.564
	Kurtosis		.604	1.091

Table A3.2 Study 3 Descriptive statistics

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mean	.162	16	.200*	.932	16	.259

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table A3.3 – Test of normality

t-Test: Paired Two Sample for Means

	MPOD 2	*MPOD 1*
Mean	0.3939375	0.3918125
Variance	0.003074329	0.002048429
Observations	16	16
Pearson Correlation	0.550385497	
Hypothesized Mean Dif	0	
df	15	
t Stat	0.174955523	
P(T<=t) one-tail	0.43172714	
t Critical one-tail	1.753050356	
P(T<=t) two-tail	0.863454279	
t Critical two-tail	2.131449546	

Table A3.4

A paired T test assessing statistical significance in relation between MPOD 2 and MPOD 1, p = 0.86, reject null hypothesis.

Appendix 4 Assessing mean MPOD in a paediatric sample with the Visucam 200

Gender: MPOD **Female** Assessment of normality in data

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
MPOD	.085	40	.200*	.974	40	.467

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure A4.01 Study 2 Visucam MPOD in paediatric sample n=73

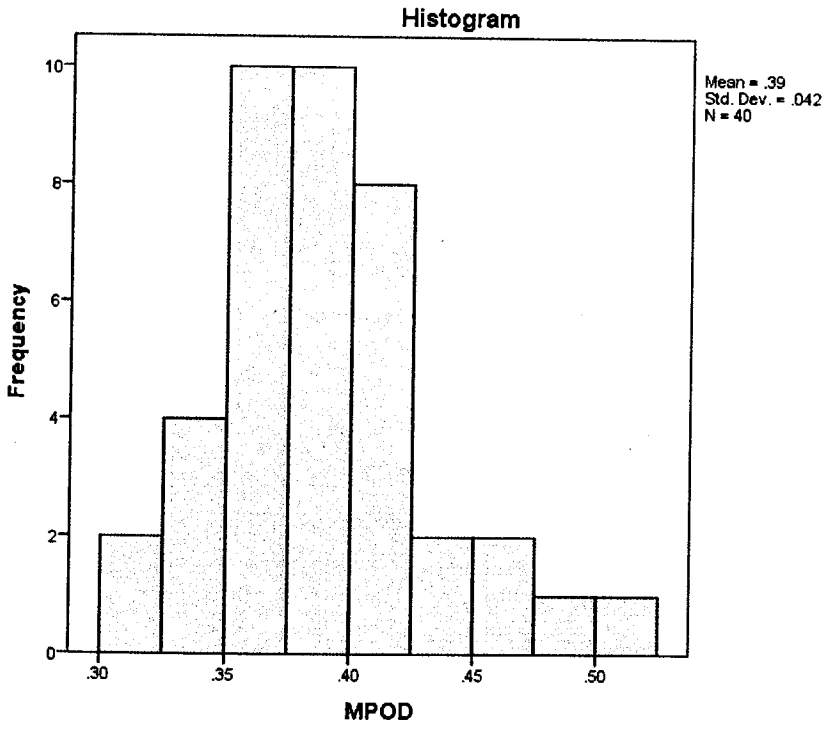


Figure A4.02 Study 2 -Histogram - MPOD Female Assessment of normality in data

Gender: MPOD **female** assessment of normality in data

Kolmogorov-Smirnov and Shapiro Wilk were both >0.05, the female distribution of data is normal, the histogram shows a relatively normal distribution of MPOD and the Q-Q plot (see Appendix 4 figure A4.0 – A4.02) shows data that approximates to the diagonal line of normality.

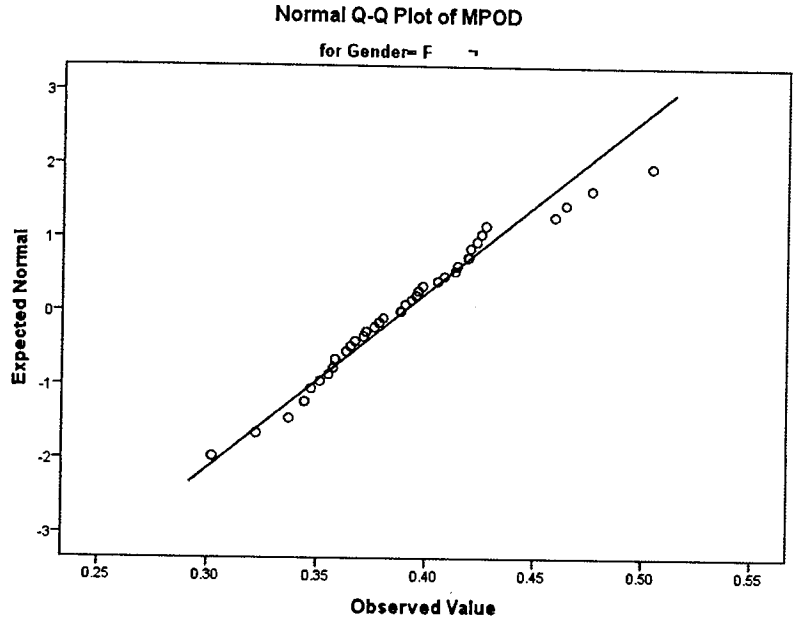


Figure A4.03 Study 2 – Normal Q-Q plot - MPOD Female Assessment of normality in data

Since Kolmogorov-Smirnov and Shapiro Wilk have figures both >0.05 then the female distribution of data is normal, the histogram shows a relatively normal distribution of MPOD and the Q-Q plot (see figure x Appendix) shows data that is fairly true to the diagonal line of normality.

Gender: MPOD **Male** Assessment of normality in data

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
MPOD	.171	33	.015	.743	33	.000

a. Lilliefors Significance Correction

Figure A4.04 Study 2_MPOD Male Assessment of normality in data

Since Kolmogorov-Smirnov and Shapiro Wilk have figures both <0.05 the male distribution of data is not normal.

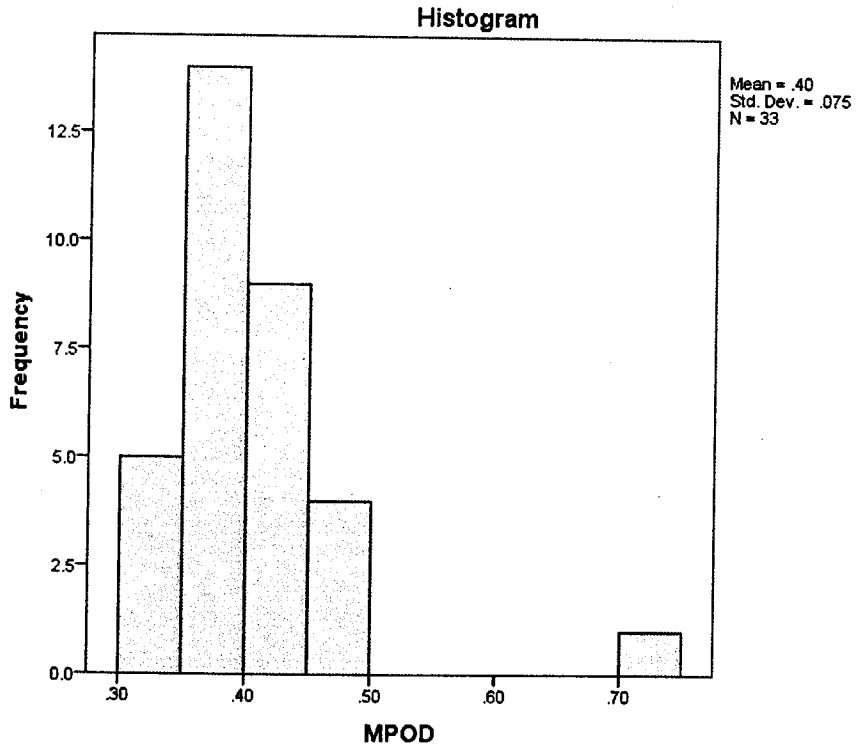


Figure A4.05 Study 2 – Histogram - MPOD Male Assessment of normality in data

It is clear that the one outlier in the male MPOD data may be contributing to the lack of normality in the male MPOD distribution.

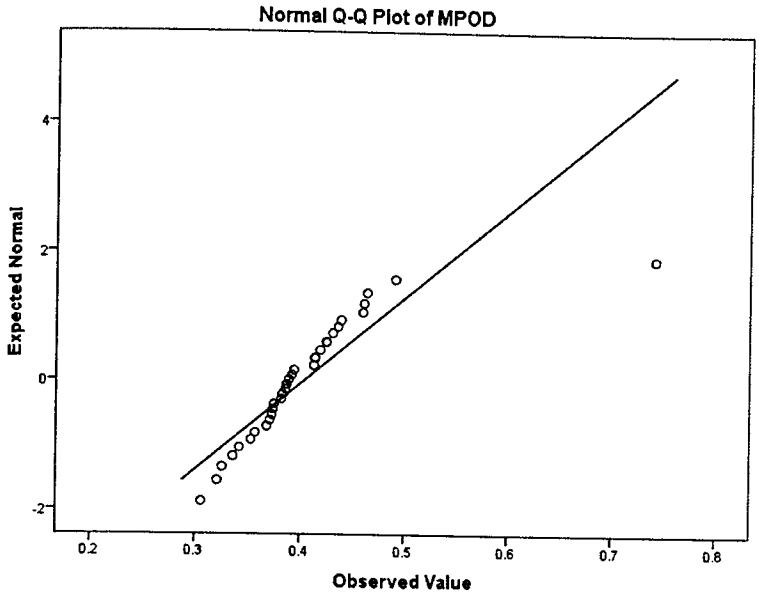


Figure A4.06 Study 2 – Normal Q-Q plot - MPOD Male Assessment of normality in data

Gender: MPOD **male** assessment of normality in data

Kolmogorov-Smirnov and Shapiro Wilk were both <0.05, the male distribution of data is not normal

Despite these conflicting results, an assessment of statistical significance for MPOD data between males and females has been assessed with non-parametric methods: Mann Whitney U test. (As the overall assessment of the distribution of data is non normal)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The medians of MPOD are the same across categories of Gender.	Independent-Samples Median Test	.915	Retain the null hypothesis.
2	The distribution of MPOD is the same across categories of Gender.	Independent-Samples Mann-Whitney U Test	.444	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure A4.07 Study 2 – Hypothesis test summary – statistically significant difference in MPOD between males and females

The Mann-Whitney U test results when two distributions have a different shape - comparing mean ranks rather than medians. This is what happens when data has violated Assumption #4 of the Mann-Whitney U test. Therefore the independent samples Mann Whitney U test is applied, this indicates that for gender variable p value (0.444) >0.05 and the null hypothesis is accepted and there is no statistically significant difference between male and female MPOD.

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
MPOD	.252	24	.000	.677	24	.000

a. Lilliefors Significance Correction

Figure A4.08 Dark pigmentation

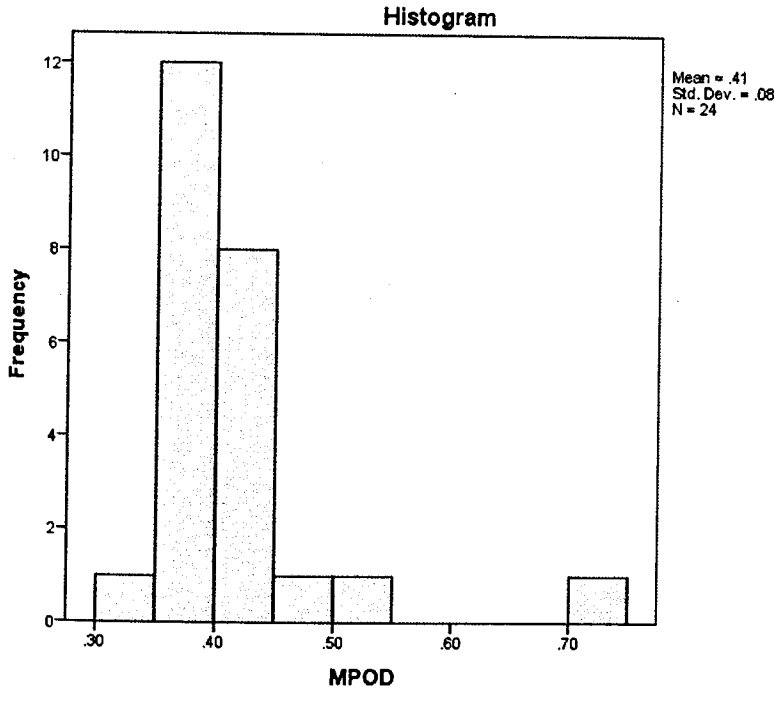


Figure A4.09 Histogram - MPOD Dark pigmentation- Assessment of normality in data

Since Kolmogorov-Smirnov and Shapiro Wilk have figures both <0.05 then the distribution of dark pigmentation irides data is not normal.

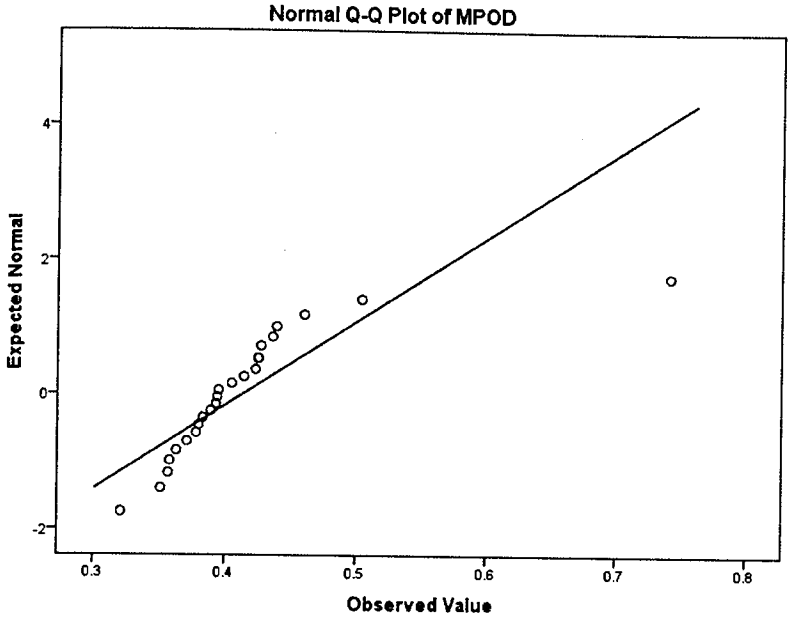


Figure A4.10 Study 2- Normal Q-Q plot - MPOD Dark pigmentation- Assessment of normality in data

Dark pigmentation

Since Kolmogorov-Smirnov and Shapiro Wilk were both <0.05, the distribution of dark pigmentation irides data is not normal (see Appendix 5, figure A5.07- A5.09).

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
MPOD	.072	49	.200*	.978	49	.490

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure A4.11 Light pigmentation

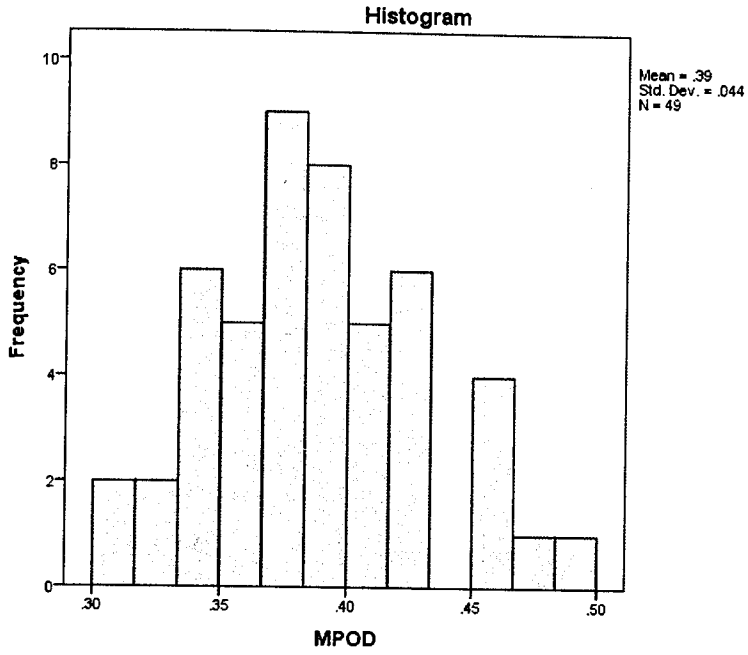


Figure A4.12 Study 2 - Histogram - MPOD Light pigmentation - Assessment of normality in data

Since Kolmogorov-Smirnov and Shapiro Wilk have figures both >0.05 the light iris pigmentation distribution of data is normal, the histogram shows a relatively normal distribution of MPOD and the Q-Q plot (below) shows data that is fairly true to the diagonal line of normality.

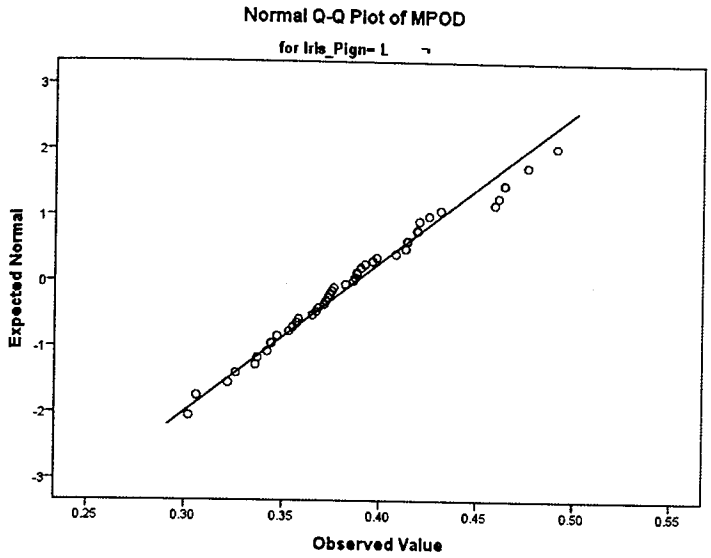


Figure A4.13 – Study 2- Normal Q-Q plot - MPOD Light pigmentation - Assessment of normality in data

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of MPOD is the same across categories of Iris_Pign.	Independent-Samples Mann-Whitney U Test	.122	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure A4.14 – Study 2, hypothesis testing for statistical significance in iris pigmentation and MPOD levels

The independent samples Mann Whitney U test is applied, this indicates that for iris pigmentation variable, p =0.122 and the null hypothesis is accepted and there is no statistically significant difference between light and dark iris pigmentation in relation to mean MPOD.

Male Dark vs Female Light

The greatest difference in mean group MPOD values was between the male dark pigmentation irides (0.43) and female light irides (0.39). Analysis was therefore undertaken to assess if this difference was statistically significant.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of MPOD is the same across categories of Iris_Pign.	Independent-Samples Mann-Whitney U Test	.109[1]	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

[1]Exact significance is displayed for this test.

Figure A4.15 – Study 2, assessing for statistical significance between male dark and female light iris pigmentation, in relation to mean MPOD.

The independent samples Mann Whitney U test was applied, this indicated that for iris pigmentation male dark vs female light, p =0.109 and the null hypothesis is accepted and there is no statistically significant difference between light and dark iris pigmentation MPOD.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
4-8yrs	14	5.59	0.399286	0.002392
>8-12yrs	25	9.91	0.3964	0.007032
>12-16yrs	34	13.45	0.395588	0.001486

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000137	2	6.84E-05	0.019228	0.980961	3.127676
Within Groups	0.248907	70	0.003556			
Total	0.249044	72				

Figure A4.16 ANOVA MPOD between 3 different age groups

Correlations

		MPOD	Age_Months
Pearson Correlation	MPOD	1.000	-.015
	Age_Months	-.015	1.000
Sig. (1-tailed)	MPOD	.	.450
	Age_Months	.450	.
N	MPOD	73	73
	Age_Months	73	73

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.015[a]	.000	-.014	.05958

a. Predictors: (Constant), Age_Months

b. Dependent Variable: MPOD

Figure A 4.17 – Linear regression analysis relating age of subjects to MPOD

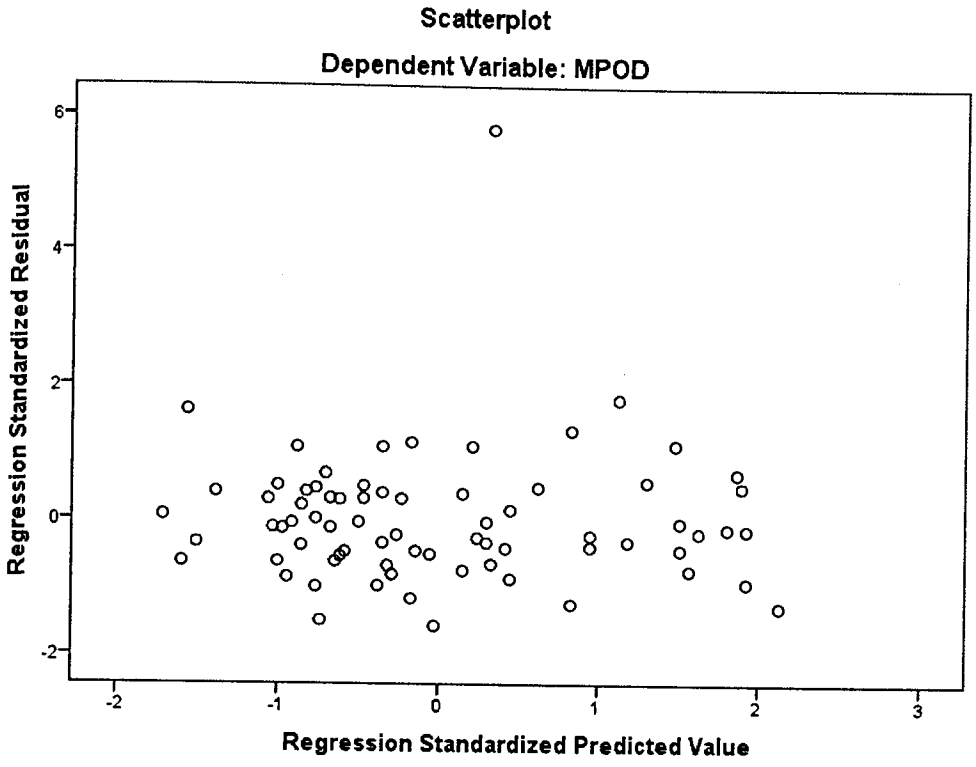


Figure A 4.18 - Linear regression analysis relating age of subjects to MPOD, showing bird nest distribution

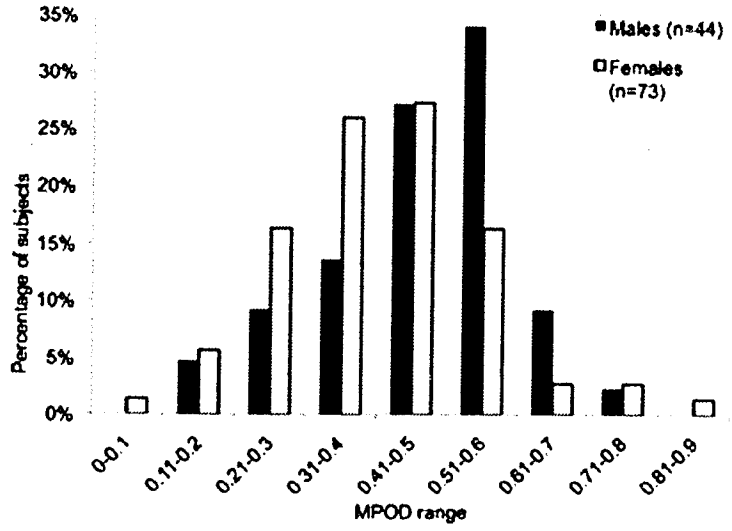
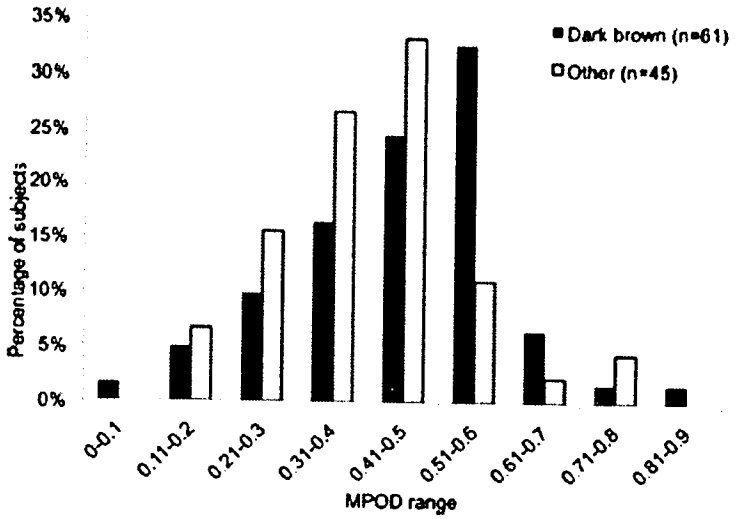


Figure A4.19: Frequency distribution of MPOD related to gender in Howells 2013 Study (Source: Howells *et al*, 2013)



The frequency distribution of MPOD for individuals with dark brown eyes and other colored eyes (brown, light brown, hazel, green) in group

Figure A4.20 Frequency distribution of MPOD related to iris pigmentation in Howells 2013 study(Source: Howells *et al*, 2013)

Appendix 4 (cont)

Figure	Mean OD (du)	Max OD (du)	Volume (du x pixel)	Area (pixel)
1B (healthy patient)	0.245	0.689	15.611	63.643
1D (patient with dry AMD)	0.173	0.430	7.506	43.463
1F (patient with) exudative AMD	0.123	0.349	3.001	24.440

Table A 4.1 reporting MPOD levels in Dawczynski study in relation to Study 2's findings

Source: Dawczynski *et al*, 2011

	Foveal thickness	R	p
Myopia	MFT	−0.66[a]	0.028[a]
	CFT	0.67[b]	0.025[b]
Emmetropia	MFT	−0.19[a]	0.34[a]
	CFT	0.20[b]	0.32[b]
Hyperopia	MFT	0.48[a]	0.14[a]
	CFT	−0.45[b]	0.17[b]

[a]Adjusted for age, BMI, SE, IOP, and CFT.
[b]Adjusted for age, BMI, SE, IOP, and MFT.
p Values and correlation coefficients of partial correlation analysis.

Table A 4.2 correlating refractive error to paediatric MPOD in Zhu 2012 study

Source: Zhu *et al*, 2012

Macular pigment optical density (MPOD) determined in different studies using fundus reflectance spectroscopy

Study	*N*	Methods	Foveal width	MPOD	SD	Age rang	Age effe
Brindley and Willmer	2	Comparison foveal-periphery	1.1	0.8			
Kilbride *et al.* [1989]	7	Spectral analysis	0.7	0.34	0.07		
Delori and Pflibsen [1989]	10	Comparison foveal-periphery	1.4	0.19	0.06		
Delori and Pflibsen [1989]	10	Spectral analysis	1.4	0.21	0.05		
Elsner *et al.* [1992]	1	SLO	Peak	0.35			
Van de Kraats *et al.* [27]	10	Spectral analysis	1.6	0.54	0.12		
Bour *et al.* [2002]	10	Photographs	Peak	0.13	0.04		
Delori *et al.* [2001]	159	Comparison foveal-periphery	2	0.23	0.07	16–	+
Chen *et al.* [2001]	54	CCD	Peak	0.23	0.07	20–	No
Berendschot *et al.* [2002]	435	Spectral analysis	2.3	0.33	0.15	60–	+
Broekmans *et al.* [2002]	376	Spectral analysis	1.5	0.33	0.15	18–	No
Wustemeyer *et al.* [2003]	109	SLO	Peak	0.16	0.06	18–	—
Berenschot [2004]	138	Spectral analysis	1.9	0.48	0.13	18–	No
Zagers [2002]	38	Directional analysis	1.9	0.51	0.14	18–	No

Table A4.3 Summary of previous FR MPOD studies in relation to Study 2 (Visucam)

Source, Berendschot 2004, Archives of Biochemistry and Biophysics 430 (2004) 149–155

Appendix 5 Assessing the repeatability of the MPS in a paediatric sample

Aston University
Birmingham

Life and Health Sciences Research Ethics Committee's Decision Letter

Aston University

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Thank you for your resubmission. The additional information for the above proposal has been considered by the Chair of the LHS Ethics Committee.

Please see below for details of the decision and the approved documents.

Reviewer's recommendation: Approved

Please see the tabled list below of approved documents:

Documentation	Version/s	Date	Approved
Parent's information sheet and consent	Parent consent form1c	31/04/2014	√
Children's information sheet	Children's agreement form1c	31/04/2014	√
Response to Referees	Referees response to 604	31/04/2014	√

Food Questionnaire	food_diary_hope	19/02/2014	√

After starting your research please notify the LHS Research Ethics Committee of any of the following:

Substantial amendments. Any amendment should be sent as a Word document, with the amendment highlighted. The amendment request must be accompanied by all amended documents, e.g. protocols, participant information sheets, consent forms etc. Please include a version number and amended date to the file name of any amended documentation (e.g. "Ethics Application #100 Protocol v2 amended 17/02/12.doc").

New Investigators The end of the study
Please email all notifications and reports to lhs_ethics@aston.ac.uk and quote the original project reference number with all correspondence.

Ethics documents can be downloaded from:
http://www.ethics.aston.ac.uk/documents-all. Please note that these documents can ONLY be opened using Mozilla Firefox or the latest Internet Explorer version (IE9).

Statement of Compliance

The Committee is constituted in accordance with the Government Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK. In accord with University Regulation REG/11/203(2), this application was considered to have low potential risk and was reviewed by three appropriately qualified members, including the Chair of the Life and Health Sciences Ethics Committee.

Yours sincerely,

Dr Corinne M Spickett

Chair of the LHS Ethics Committee

Figure A5.1 – Ethics Committee Clearance for Study 3 and 4

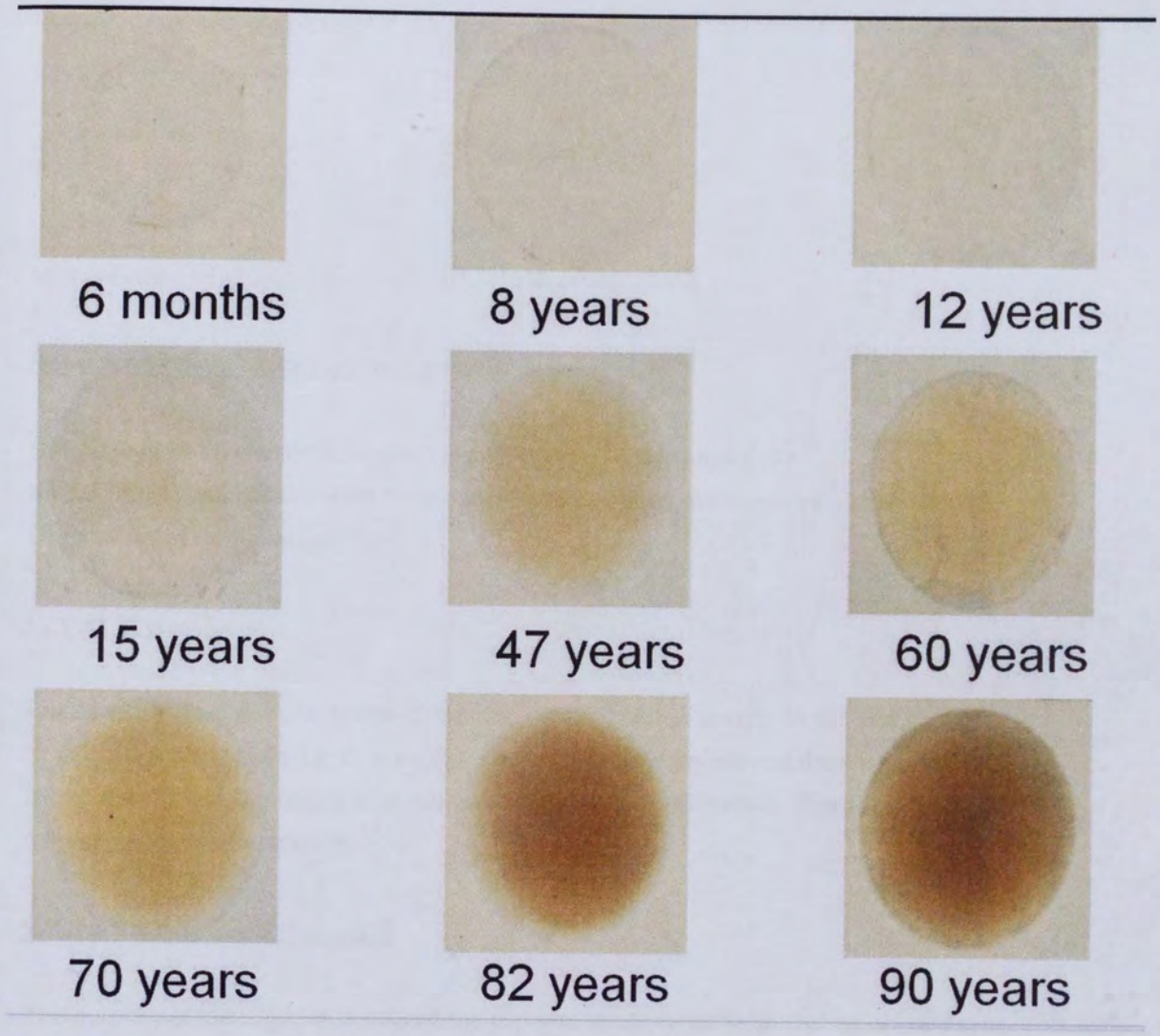

Figure A5.2 – Study 3 and 4 - demonstrating clear optical media of young subjects, therefore negating the need for peripheral measure

Lens transmission, (courtesy of Prof Marshall, UCL Institute of Ophthalmology, London)

Aston University
Birmingham

Consent Form

Aston University

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Project Title

A prospective analysis of the level of macular pigment optical density in children Invitation
Your child is being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully.

What is the purpose of the study?

The purpose of this study is to assess the pigment structure within the retina of children's eyes. This will enable doctors and optometrists in the future to gain a better understanding as to what constitutes a normal level of retinal pigment. The analysis will look at children's age and gender to assess if these have any effect. The information will be useful in comparing to older peoples change in the retina as they age and may assist in further developing future treatment strategies for vision loss in the elderly.

Why have I been chosen?

You have been chosen as your child is a patient at the practice and have an age matched requirement for the study.

What will happen to me if I take part?

By volunteering for your child to participate you will be giving the research team consent to carry out an additional test of your child's eyes. The test is similar to visual field test you may have undertaken in the past, it will take around 5 minutes. They will not be required to sit for any further tests. The

research is being undertaken between Coleman Opticians, Norwich and Aston University, in Birmingham. It is anticipated that the study will last 18 months and run between March/April 2014 and May 2014. The study will collate results of the children's test results within the practice.

You will also be asked to complete a short food diary relating to what your child has eaten over the previous 3 days. This is to look for how different foods may affect the retina. We will keep this information confidential.

Are there any potential risks in talking part in the study?

There are some flashing lights but the risk of epilepsy is very low and no greater than that of playing computer games.

The only other consideration, as with all clinical research, is the risk of breaching privacy and confidentiality in relation to your child's ophthalmic health. This risk will be minimised by keeping your data anonymous at all times.

Do I have to take part?

No, you do not have to participate if you do not wish to do so. You are free to withdraw your child at any time from this research. The future care of your child at the practice will not be affected by a decision to take part or not take part.

Expenses and payments:

There are no expenses or payments for participation in this research.

Will my taking part in this study be kept confidential?

Yes, your child's participation in the study will be strictly confidential through the use of reference numbers rather than names. Other members of the research team will only be given access to the research database after your identity has been removed. There will be no way to link any research data to any individual participant. Data will be collected in paper form and recorded on study record sheets. Test results will be analysed with software and the results of this will be recorded on study data sheets. Data will be held on no more than 3 computer based storage devices. On completion of the study in 2015, data relating to the study will be erased from all computer sources within a 6 month time period and all written data will be shredded; this will be independently verified by two further optometrists/doctors in both cases.

What will happen to the results of the research study?

The results of this study will undergo statistical analysis in order to assess for patterns and trends in what factors may affect the change in the retina structure as we age. This may help future optometrists to further refine the diagnosis of sight threatening disease. Full patient confidentiality will be maintained at all times. We aim to publish the results of this research in professional journals. A copy of the published research will be made available during 2015 within Coleman Optometrists practice, Norwich and Aston University, Birmingham. However, there will be no

reference to any individual's identifiable details in any publication.

Who is organizing and funding the research?

Funding of this research is through the following sources:

College of Optometrists Coleman
Optometrists
Michael Hope - Research Optometrist

The research is being organized by Michael Hope/Coleman Optometrists and Aston University.

Who has reviewed the study?

Aston University

Content has been removed for copyright reasons

Who do I contact if I wish to make a complaint about the way in which the research is conducted?

If you have any concerns about the way in which the study has been conducted, then you should contact Secretary of the University Research Ethics Committee on j.g.walter@aston.ac.uk or telephone 0121 204 4665.

VOLUNTEER CONSENT FORM

Title of Project: A prospective analysis of the level of macular pigment optical density in children

Name of Chief Researcher: Mr. M J Hope BSc (Hons) BMedSc, MCOptom

		Tick Box
1	I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions	
2	I understand that my child's participation is voluntary and that I am free to withdraw them at any time without giving any reason, without my medical care or	
3	I agree to take part in the above study.	

Name of parent/guardian Date Signature

Signature of Researcher:

Copy for: Parent/Researcher (delete as applicable) Version 1b

Figure A5.3

Parental consent and info form for Study 3 and 4

Aston University
Birmingham

Children's Agreement Form Michael Hope 955299

The people doing this research are:

Dr Eperjesi and Mr. Michael Hope with Aston University.

Project Title

A study looking at children's eyes and how they compare to adult eyes.

Invitation

We are asking you to take part in a new study of children's eye health.

What is the reason for the study?

The information we collect may help to better understand how children's and adult's eyes are affected by getting old.

Why have I been chosen?

You have been chosen as you are the right age for the study. We need children aged 4-16 years of age to take part. The reason for this is that we are studying details of the eye at a young age.

What will happen to me if I take part?

You will do a test where you have to press a button on a machine when you notice a light flicker on or off. Your optometrist will stay with you all the time and your parents can watch if they like. The test is completely safe and will take just 5 minutes of time. It is similar to what your parents may sometimes have when they have an eye test.

You will be asked to complete a food diary in which you tell us what you have had to eat and drink in the three days before your eye test.

Do I have to take part?

No, you do not have to if you don't want to

If you decide to take part and you don't like the machine during the test you can ask for it to stop and it will stop straight away.

Will my results be keptsecret?

Yes, all your results will be kept secret. Other people may see your results but onlyonce your name has been removed.

CHILDRENS AGREEMENT FORM

Title of Project: A study looking at children's eyes and how they compare to adult and old people's eyes

Name of Study Researcher: Mr. M J Hope BSc (Hons) BMedSc, MCOptom

		Tick Box
1	I agree that I have read this and understand it.	
2	I know that I don't have to take part in the study if I don't want to.	
3	I agree to take part in this study.	

Your name _____ Date _____ Signature _____

Signature of Researcher:

Copy for: Parent/Researcher (delete as applicable)

Version 1.2

Figure A5.4

Children Consent form – Study 3 and 4

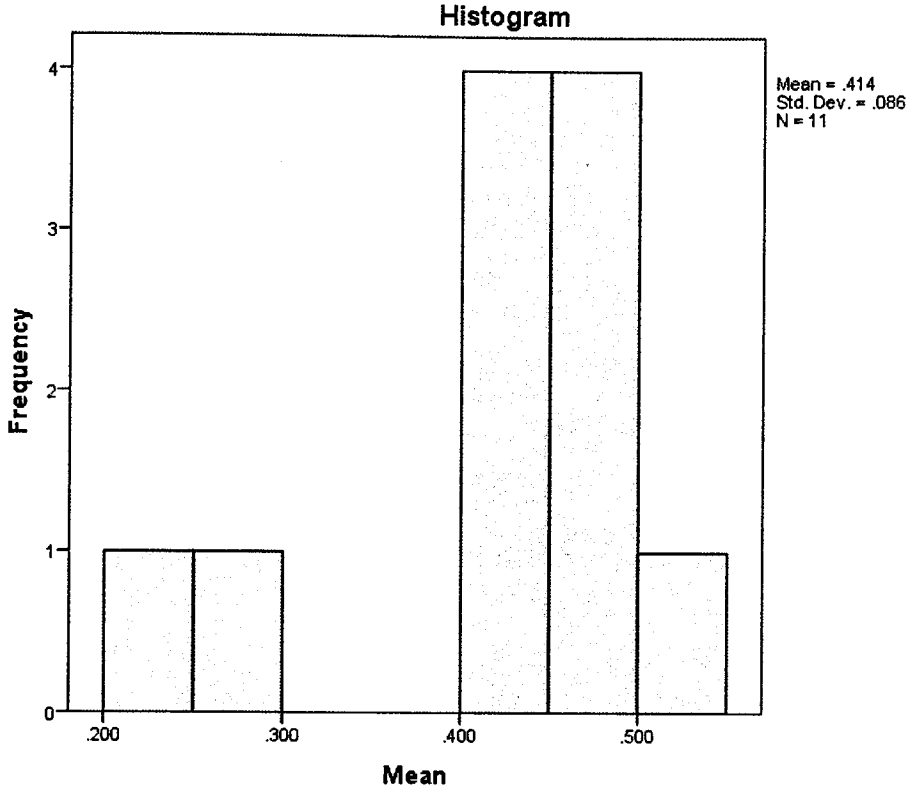

Figure A5.5 – Study 3 Histogram mean MPOD distribution

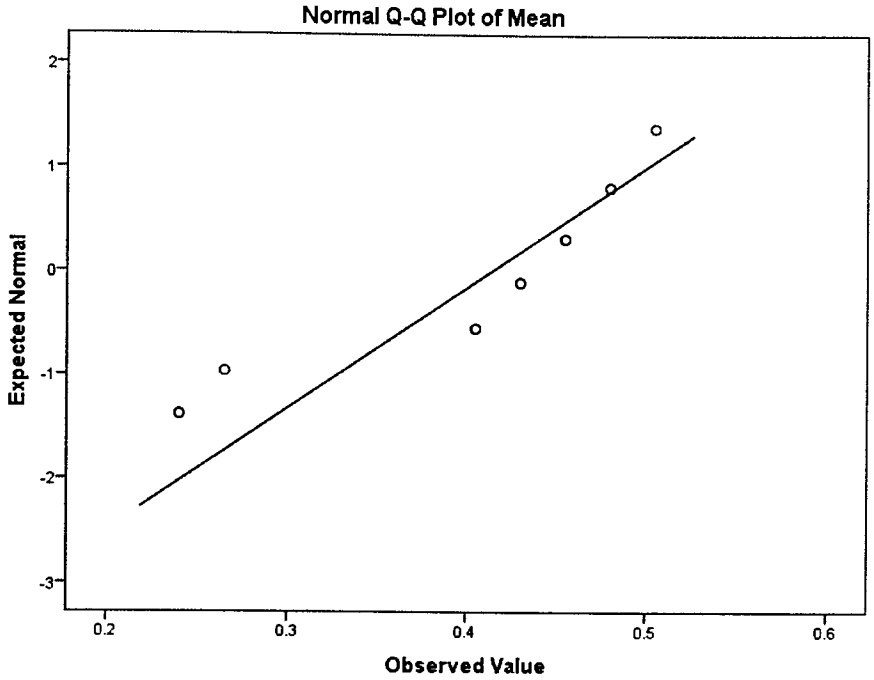

Figure A5.6 – Study 3 Q-Q plot of mean MPOD distribution

Tables…

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Mean	11	52.4%	10	47.6%	21	100.0%

Descriptives

			Statistic	Std. Error
Mean	Mean		.41364	.025850
	95% Confidence Interval for Mean	Lower Bound	.35604	
		Upper Bound	.47123	
	5% Trimmed Mean		.41821	
	Median		.43000	

	Variance	.007	
	Std. Deviation	.085735	
	Minimum	.240	
	Maximum	.505	
	Range	.265	
	Interquartile Range	.075	
	Skewness	-1.362	.661
	Kurtosis	.962	1.279

Table A5.1 – Study 3 – Descriptive stats

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mean	.278	11	.017	.820	11	.017

a. Lilliefors Significance Correction

Table A5.2 – Tests of normality in Study 3

t-Test: Paired Two Sample for Means

	Mean MPOD 1	*Mean MPOD2*
Mean	0.431818182	0.395454545
Variance	0.007016364	0.012207273
Observations	11	11
Pearson Correlation	0.549888758	
Hypothesized Mean D	0	
df	10	
t Stat	1.26808525	
P(T<=t) one-tail	0.116747507	
t Critical one-tail	1.812461123	
P(T<=t) two-tail	0.233495014	
t Critical two-tail	2.228138852	

Table A5.3 T test comparing measure MPOD1 and MPOD2

Appendix 6 Assessing mean MPOD in a paediatric sample with the MPS

Diet History: Info for Parents

Aston University
Birmingham

We are collecting information relating to your child's diet in order to assess for any link between nutrients in food and nutrients/chemicals located within the retina of the eye.

Why are we interested in this?

This information helps us to better understand the pigment within the retina at the early stage of life and how foods may affect this. The data can then be compared to adult and elderly peoples' retinal pigment. This in turn may help us to better understand how macular degeneration occurs in the later stage of life (wear and tear/aging of the retina).

How much detail is required?

Please provide a good level of detail, e.g. ham and tomato sandwich rather than just sandwich, cornflakes- semi skimmed milk and sugar rather than just cereal.

Do I have to provide this information?

No you do not. If you do not wish to provide this information then we will not pursue it.

Will this information be held confidential?

We take your confidentially very seriously. Your child's data is made anonymous via reference numbers and only the lead researcher will have access to the full data, though the academic findings will be made available to Aston University, Birmingham, UK.

Any questions please do not hesitate to contact the lead research Optometrist: Michael Hope

Thank you for working with Coleman Optometrists and Aston University to further our understanding of retinal development.

Figure A 6.01- Study 4 – Info for parents sheet

DIET HISTORY – We are collecting information on your child's diet, in order to assess for a link between pigments consumed within foods and pigments located within the retina of the eye. We take the issue of confidentiality seriously. All information provided is held strictly confidential between the lead researchers and Aston University. As a parent/guardian it is your right to decline to provide this diet information, if you so wish.

LUTEIN ZEXANTHIN

Day before yesterday

Breakfast

Lunch

Dinner/Tea

Snack through the day

Drinks

Yesterday

Breakfast

Lunch

Dinner/Tea

Snack through the day

Drinks

Today (so far)

Breakfast

Lunch

Dinner/Tea

Snack through the day

Drinks

Figure A 6.02 Study 4– Diet History data sheet

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mean	.187	14	.200*	.932	14	.325

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure A6.04 – Study 4 - Female MPOD – normality distribution

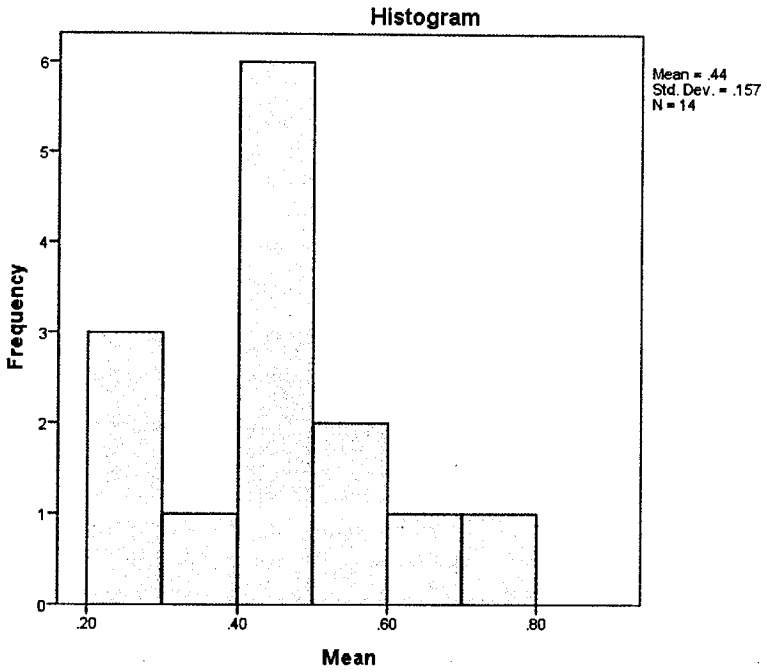

Figure A6.05–Study 4- female normality distribution histogram

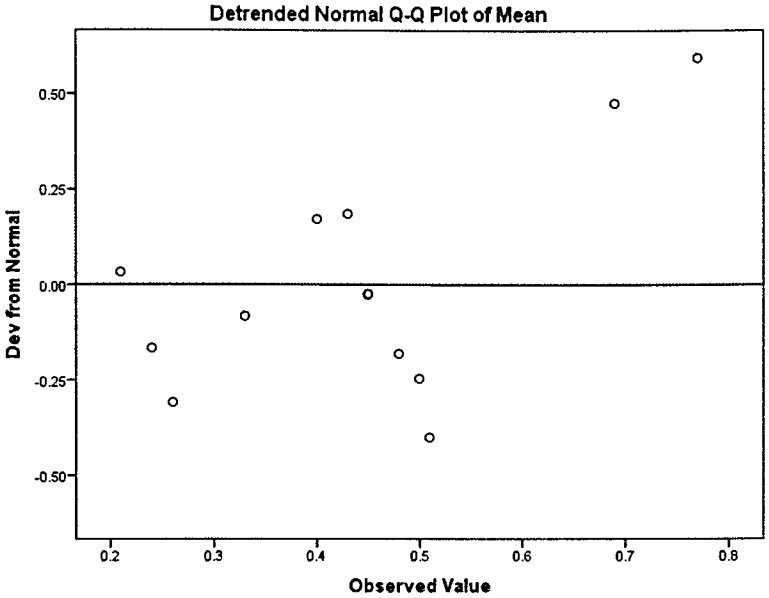

Figure A6.06 – Study 4 - female MPOD normality distribution Q-Q plot

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mean	.144	9	.200*	.946	9	.651

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure A6.07- Study 4 - Male MPOD – normality distribution

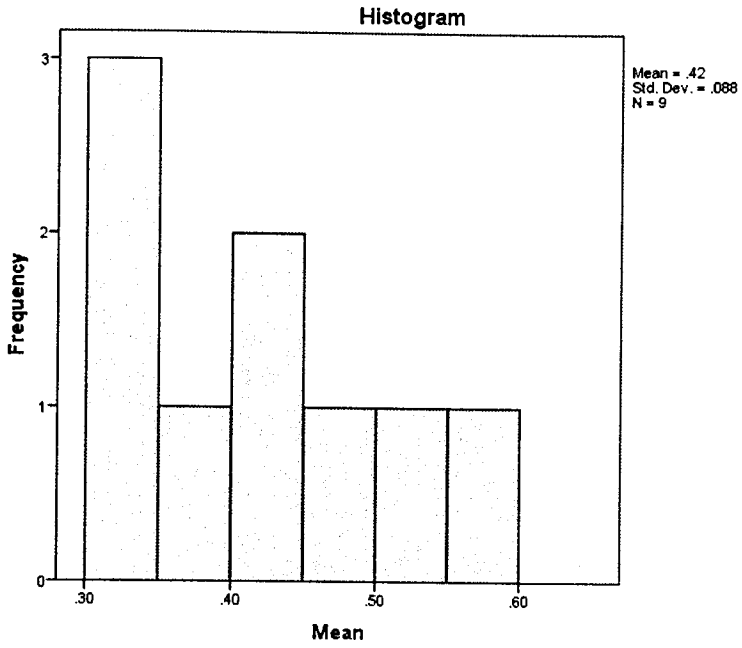

Figure A6.08 Study 4 – Male MPOD normality distribution histogram

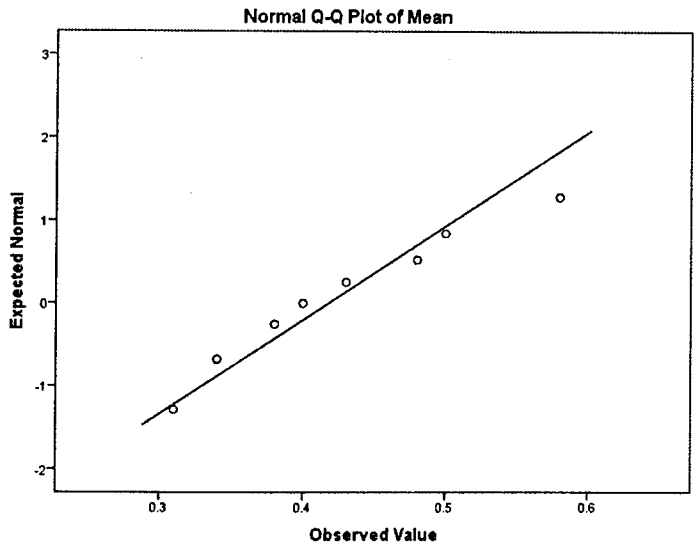

Figure A6.09 Study 4 – Male MPOD normality distribution Q-Q plot

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Mean	.147	23	.200*	.952	23	.326

*. This is a lower bound of the true significance.

a.Lilliefors Significance Correction

Figure A6.10 Study 4 - Overall MPOD distribution – all subjects

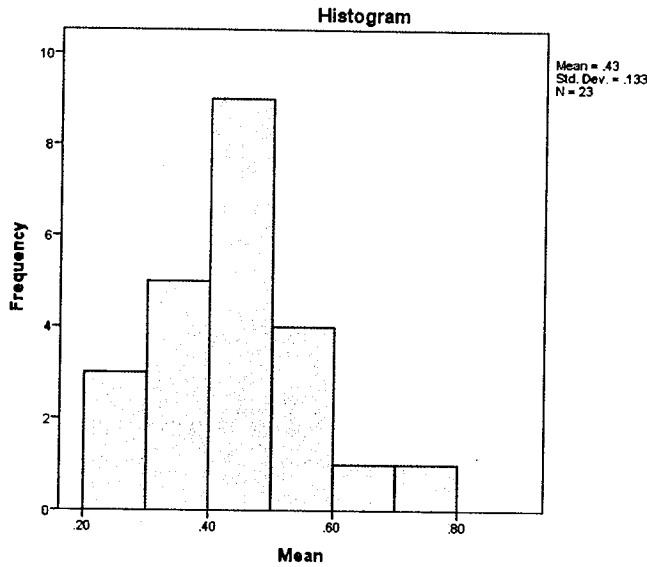

Figure A6.11 Study 4 – All subjects normality distribution histogram

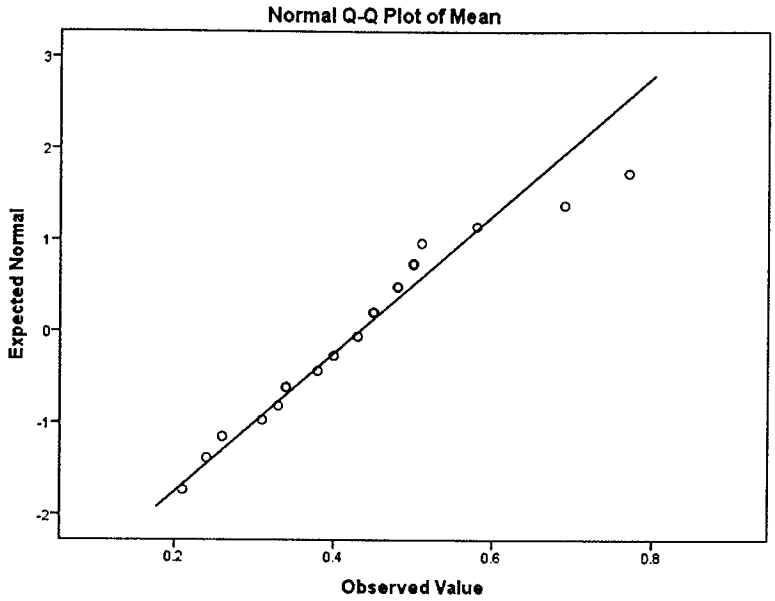

Figure A6.12 Study 4– All subjects normality distribution Q-Q plot

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The categories of Mean occur with equal probabilities.	One-Sample Chi-Square Test	.998	Retain the null hypothesis.
2	The distribution of FMean is normal with mean 0.441 and standard deviation 0.16.	One-Sample Kolmogorov-Smirnov Test	.713	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure A6.13- Study 4 Gender: Non parametric analysis of Male and Female MPOD

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Meandailyluteinintake	.160	9	.200*	.936	9	.538

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure A6.14 Study 4- Male Lutein intake – assessment of normality in data

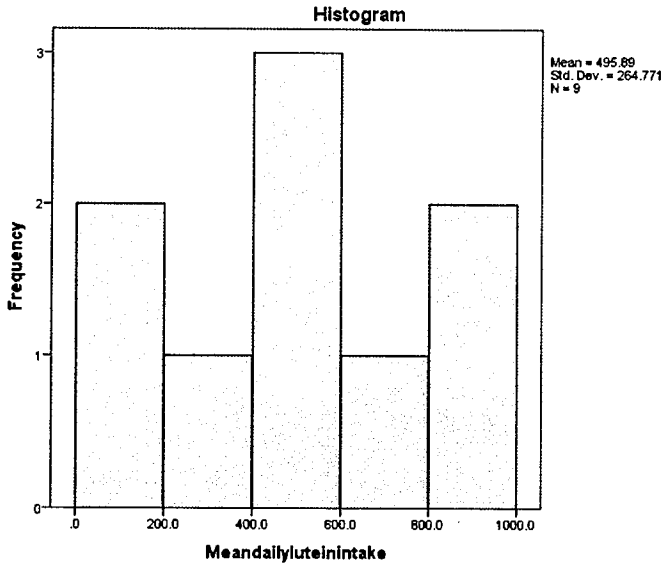

Figure A6.15 Male Lutein intake assessment of normality in data - histogram

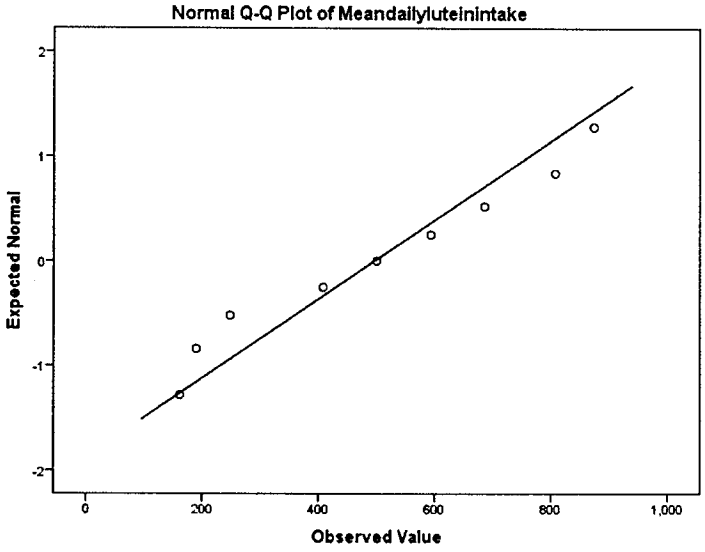

Figure A6.16 Study 4- Male Lutein intake assessment of normality in data – Q-Q plot

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Meandailyluteinintake	.181	14	.200*	.872	14	.045

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure A6.17 Study 4 -Female Lutein intake – assessment of normality in data

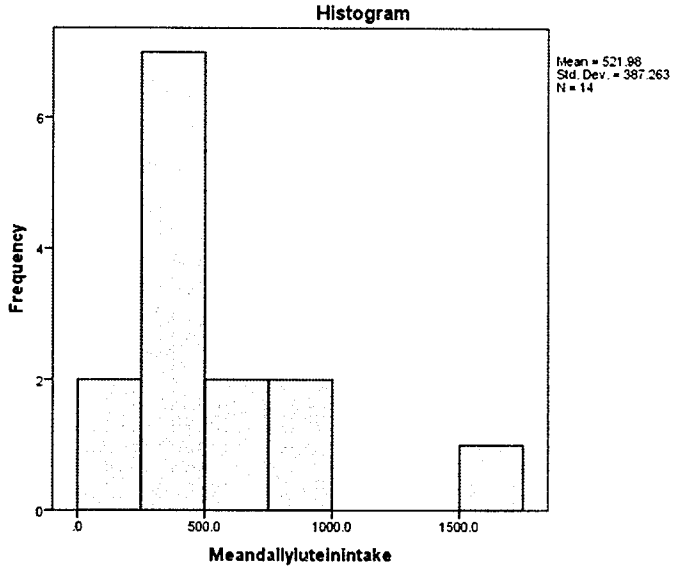

Figure A6.18 Study 4 -Female Lutein intake – assessment of normality in data - histogram

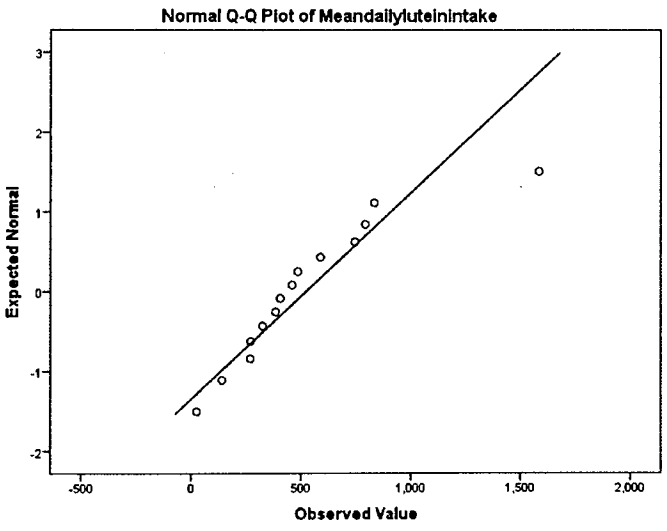

Figure A6.19 Study 4- Female Lutein intake – assessment of normality in data – Q-Q plot

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The categories of Meandailylutеinintake occur with equal probabilities.	One-Sample Chi-Square Test	1.000	Retain the null hypothesis.
2	The distribution of Femalelutein is normal with mean 521.979 and standard deviation 387.26.	One-Sample Kolmogorov-Smirnov Test	.749	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure A6.20 Study 4- Assessing for statistical significance between male and female mean lutein intake

Tables

Subject	MPOD 1	MPOD2	Mean	Diff-MPD1,MPD2	Age (months)	Gender	Mean daily lutein intake (mcg)
1	0.770	0.770	0.770	-	171.000	F	792.000
2	0.530	0.500	0.515	0.030	96.000	F	405.000
3	0.770	0.620	0.695	0.150	173.000	F	588.000
4	0.590	0.580	0.585	0.010	190.000	M	807.000
5	0.240	0.190	0.215	0.050	191.000	F	1,585.000

6	0.340	0.290	0.315	0.050	114.000	M	874.000
7	0.380	0.530	0.455	0.150	149.000	F	745.000
8	0.455	0.555	0.505	0.100	130.000	F	384.000
9	0.380	0.290	0.335	0.090	160.000	F	460.000
10	0.340	0.340	0.340	-	83.000	M	685.000
11	0.380	0.380	0.380	-	99.000	M	593.000
12	0.340	0.340	0.340	-	118.000	M	247.000
13	0.480	0.430	0.455	0.050	182	F	833
14	0.580	0.380	0.480	0.200	122.000	M	499.500
15	0.380	0.430	0.405	0.050	182.000	F	325.000
16	0.290	0.240	0.265	0.050	163.000	F	140.500
17	0.430	0.430	0.430	-	176.000	M	189.400
18	0.480	0.530	0.505	0.050	149.000	M	161.100
19	0.340	0.140	0.240	0.200	71.000	F	268.700
20	0.430	0.480	0.455	0.050	135.000	F	485.000
21	0.530	0.430	0.480	0.100	178.000	F	271.000
22	0.430	0.430	0.430	-	131.000	F	25.500
23	0.380	0.430	0.405	0.050	150.000	M	407.000
Sum Total	10.265	9.735	10.000	1.430	3,504.000	14F 9M	11,770.700

Mean	0.446	0.423	0.435	0.062	144.043		511.770
Max	0.770	0.770	0.770	0.200	191.000		1,585.000
Min	0.240	0.140	0.215	-	71.000		25.500
Std Devn	0.135	0.144	0.132532	0.062228	35.38808		338.056342

Table A6.1 – Study 4- MPS MPOD data for n=23, raw data table for MPOD, age, gender and mean daily lutein intake

t-Test: Two-Sample Assuming Unequal Variances

	792	*807*
Mean	501.2077	457
Variance	155927.2	64563.12
Observations	13	8
Hypothesized Mean Difference	0	
df	19	
t Stat	0.312091	
P(T<=t) one-tail	0.379185	
t Critical one-tail	1.729133	
P(T<=t) two-tail	0.758371	
t Critical two-tail	2.093024	

Table A6.2 Study 4 - A two-sample Student's t-test assuming unequal variances using a pooled estimate of the variance to test the hypothesis that mean lutein intake for male and female were equal

t-Test: Two-Sample Assuming Unequal Variances

	0.77	*0.585*
Mean	0.419231	0.399375
Variance	0.017095	0.004739
Observations	13	8
Hypothesized Mean Difference	0	
df	19	
t Stat	0.454642	
P(T<=t) one-tail	0.327259	
t Critical one-tail	1.729133	
P(T<=t) two-tail	0.654519	
t Critical two-tail	2.093024	

Table A6.3 – Study 4- A two-sample Student's t-test assuming unequal variances using a pooled estimate of the variance to test the hypothesis that mean MPODs for male and female were equal

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Mean	.4317	23	.13252	.02763
	Agemonths	144.04	23	35.388	7.379

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Mean & Agemonths	23	.305	.157

Table A6.4 – Study 4 – Relating MPOD to age – correlation

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Mean - Agemonths	-143.61174	35.34786	7.37054	-158.89730	-128.32618	-19.485	22	.000

Table A6.5 – Study 4 – Relating MPOD to age – T test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Mean	.4317	23	.13252	.02763
	Meandailyluteinintake	511.770	23	338.0563	70.4896

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Mean & Meandailyluteinintake	23	-.037	.865

Table A6.6 – Study 4 – Relating MPOD to L intake – correlation

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Mean - Meandailyluteinintake	-511.33783	338.06133	70.49066	-657.52651	-365.14914	-7.254	22	.000

Table A6.7 – Study 4 – Relating MPOD to mean daily L intake – T test