THE ASSOCIATION BETWEEN MACULAR PIGMENT OPTICAL DENSITY AND GLARE RECOVERY TIME WITH SELECTED MACULAR DEGENERATION AND OCULAR VASCULAR PERFUSION RISK FACTORS

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Thesis Summary

Age-related macular degeneration (AMD) is the most common cause of severe visual impairment affecting older adults in the developed world. The pathogenesis of AMD is not fully understood.

This study sought to investigate the association between macular pigment optical density (MPOD) and glare recovery time (GRT) with a selection of other confirmed and putative AMD risk factors (RF): age, gender, body mass index (BMI), calculated percentage body fat (%BF), iris colour, family history (FH) of AMD, and ocular vascular perfusion (OVP) RF: migraine, Raynaud's phenomenon (Rph) and vascular dysregulation (VDys). Interocular comparison was assessed for MPOD and GRT. The effect of ocular dominance on MPOD and GRT, and GRT repeatability was also examined. The use of GRT as a surrogate measure for MPOD was assessed.

In this healthy, mixed-gender, White population no significant association was found between MPOD measured by heterochromatic flicker photometry (HFP) at 0.5° eccentricity and any AMD or OVP RF assessed by this study. No significant interocular difference in MPOD was found. No significant association was found between MPOD and ocular dominance.

GRT after 30-second duration bleach using the direct ophthalmoscope was significantly and positively associated with age. No significant association was found for any other AMD or OVP RF examined, after correction for age. No significant interocular difference was found. No significant association was found with ocular dominance. GRT intra-session repeatability was good and inter-session repeatability was moderate. This method of GRT was not found to be a good surrogate measure for MPOD.

This study generated three new theories: the possible association between the OVP RF migraine, Rph and VDys and AMD risk, the Müller cell (Mc) / neuroglial cell hypothesis for macular pigment, and the retinal theory for Meares-Irlen syndrome (MIS) also known as Visual Stress.

Keywords: cone-specific visual cycle, direct ophthalmoscope, intrinsically photosensitive retinal ganglion cells, MPS screener, and Müller cells.

To mum and dad

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List of abbreviations

AMD	Age-related macular degeneration
ApoE	Apolipoprotein
AREDS	First Age-Related Eye Disease Study
AREDS2	Second Age-Related Eye Disease Study
A2E	N-retinylidene-N-retinylethanolamine
BDES	Beaver Dam Eye Study
BMES	Blue Mountain Eye Study
BMI	Body mass index
CFB	Complement factor B
CFH	Complement factor H
CFI	Complement factor I
CI	Confidence interval
CRP	C-reactive protein
C1	Complement component 1
C2	Complement component 2
C3	Complement component 3
DA	Dark adaptation
DHA	Docosahexaenoic acid
EDCCS	Eye Disorders Case-Control Study
EMS	Eger Macular Stressometer
ET-1	Endothelin-1
FAF	Fundus autofluorescence
GA	Geographic atrophy
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
GST	Glutathione S-transferase
GRT	Glare recovery time
GWAS	Genome-wide association study
HC	Horizontal cell(s)
HDL	High density lipoprotein cholesterol
HFL	Henle fibre layer
ILM	Internal limiting membrane of retina
ipRGC	Intrinsically photosensitive retinal ganglion cell(s)
IS	Inner segments of retinal photoreceptors
L	Lutein
LDL	Low density lipoprotein cholesterol
LoA	Limit of agreement

Mc	Müller cell(s)
Mcc	Müller cell cone
MIS	Meares-Irlen syndrome
MZ	Meso-zeaxanthin
MZyg	Monozygotic
MP	Macular pigment
MPOD	Macular pigment optical density
MPS	Macular Pigment Screener
nAMD	Neovascular age-related macular degeneration
NHANES	The first National Health And Nutrition Examination Survey
Opn4	Melanopsin
OS	Outer segments of retinal photoreceptors
OVP	Ocular vascular perfusion
RAP	Retinal angiomatous proliferations
RF	Risk factor
ROS	Reactive oxygen species
Rph	Raynaud's phenomenon
RRS	Resonance Raman spectroscopy
R/RPE/C	Retina / retinal pigment epithelium / choroid
RS	Rotterdam Study
SLS	Sjögren-Larrson syndrome
SNPs	Single nucleotide polymorphisms
Td	Trolands
UV	Ultraviolet
VDys	Vascular dysregulation
VEGF	Vascular endothelial growth factor
WARMGS	Wisconsin Age-Related Maculopathy Grading System
Z	Zeaxanthin
%BF	Percentage body fat
1-WFAF	One-wavelength fundus autofluorescence
2-WFAF	Two-wavelength fundus autofluorescence

Chapter 1 Introduction

The following chapter will provide background information about age-related macular degeneration (AMD) including its epidemiology, pathogenic features, classification, risk factors (RF), including the macular pigments (MP) lutein (L) and zeaxanthin (Z), pathogenic mechanisms and visual consequences, including glare recovery time (GRT). Sections covering MP and glare recovery time (GRT) in greater detail are included. This information will be used to support the two experimental chapters and their conclusions.

1.1 Epidemiology of AMD

In epidemiological studies AMD is categorised as either "early", by the presence of soft indistinct or reticular drusen; any soft drusen type with retinal pigment epithelium (RPE) depigmentation or with increased retinal pigmentation, although the exact criteria is dependent on the classification system used by each study, or "late" if geographic atrophy (GA) or neovascular AMD (nAMD) is present. The subtypes of AMD will be discussed in greater detail in section 1.2.

1.1.1 Prevalence and incidence

A recent Bayesian meta-analysis of 31 population studies from Europe, North America and Australia with a combined population of 57,173, has estimated the prevalence and incidence of late AMD in the UK population aged 50 year and over.[1] The overall prevalence of late AMD was 2.4% (95% credible interval (CrI) 1.7% to 3.3%), equivalent to 513,000 cases (95% CrI 363,000 to 699,000). Approximately 52% of late AMD cases were GA. The estimated number of prevalent cases of late AMD were 314,000 for women and 192,000 for men. Incidence was estimated from age-specific prevalence data. 71,000 new cases of late AMD were estimated per year (table 1.1).[1]

Table 1.1 Estimated UK late AMD prevalence and incidence in those ≥ 50 years of age indicating the variation in AMD with age and gender

The prevalence of early and late AMD is variable within populations of similar and different ethnicity, however several general patterns are visible from the data displayed in tables 1.2 and 1.3. The prevalence of early AMD is greatest in Hispanic and White individuals and least in Asian and Black individuals.[2] Differences in AMD grading scales make inter-study

comparison of early AMD prevalence difficult.[3] The definition of late AMD is subject to less variability between grading scales than early AMD. Late AMD prevalence is greatest in Asian and White individuals and least in Black and Hispanic individuals. Higher levels of choroidal melanin associated with darker skin and iris colouration has been hypothesised to have a protective effect on the RPE, Bruch's membrane and the photoreceptors (PR).[4] The protective effect of choroidal melanin may be a combination of light / heat absorption; melanin has a broad absorption spectrum spanning ultraviolet (UV), visible and near infra-red (NIR),[5] and heavy metal ion and other chemical binding, free radical scavenging and antioxidant effects.[6, 7] Choroidal melanin exhibits an age-related decline throughout the retina after 40 years of age. In the macular region, RPE cells show a 35% reduction in melanin granules between the early and late decades of life.[8] The consequence of reduced melanin with age is a lower level of retinal and RPE protection.

The ratio of GA to nAMD is also variable within and between ethnicities; Asian (0-40% GA), Black (11-100% GA), Hispanic (0-69%), Indian (0-94% GA) and White (21-83% GA). The variability is in part due to the small numbers of late AMD detected, despite the large size of the study populations. The prevalence of early and late AMD from studies of Northern and Western European populations and the global prevalence of AMD from selected sources are summarised in tables 1.2 and 1.3.

Table 1.2 The prevalence of AMD from Northern and Western European studies

Study, Country (Date) Grading System, Digital Photography	Subjects Age (years)	Study Duration	Prevalence of Early AMD, % (95% CI)	Prevalence of Late AMD, % (95% CI) % of late AMD = GA
GHS, Germany[3] (2014) IC, DP	4,340 35-74	2007-2008	1a: 2.1 (1.7-2.6) 1b: 8.0 (7.2-8.8) 2a: 1.0 (0.7-1.3) 2b: 0.5 (0.3-0.7) 3: 0.3 (0.2-0.6)	GA: 0.1 (0.0-0.2) nAMD: 0.1 (0.0-0.2) %GA: 50%
TES, Norway[9] (2012) IC, DP	2,631 65-87	2007-2008	ID: 34.9 (33.1-36.8) SD: 24.1 (22.5-25.8)	GA: 1.0 (0.6-1.4) nAMD: 2.5 (1.9-3.1) %GA: 28%
SES, United Kingdom[10] (2011) IC	934 64-79 (men)	1979-1997	1: 42.8 (n/a) 2: 7.7 (n/a) 3: 1.5 (n/a) Total 9.2 (7.4-11.4)	0.5 (0.2-1.2) %GA: n/a
AGES, Iceland[11] (2011) mWARMGS, DP	5,272 66-91	2002-2006	SDD: 38.1 (36.8-39.5) HypoP: 11.2 (10.3-12.1) HyperP: 20.3 (19.2-21.4) Total 21.3 (20.1-22.5)	GA: 2.4 (2.0-2.8) nAMD: 3.3 (2.8-3.8) %GA: 42%
OMS, Norway[12] (2006) IC, DP	459	2002	ID: 26.6 (n/a) SDD: 2.8 (n/a) SID: 13.5 (n/a) HypoP: 12.6 (n/a) HyperP: 44.4 (n/a)	2.8 (n/a) %GA: 54%
RES, Iceland[13] (2003) IC	1,045	1996	ID: 9.9 (n/a) SDD: 4.9 (n/a) SID: 4.9 (n/a) HypoP: 5.1 (n/a) HyperP: 3.0 (n/a	GA: 3.2 (n/a) nAMD: 0.7 (n/a) %GA: 83%
RS, The Netherlands[14] (1995) WARMGS	6,251 55+	1990-1993	ID: 33.9 (1-9 drusen) ID: 5.5 (10+ drusen) LD: 6.8 (1-9 drusen) LD: 2.0 (10+ drusen)	1.7 (n/a) %GA: 35%
CCES, Denmark[15] (1995) WARMGS	946 60-79	1986-1988	-	12.2 (n/a) %GA: 21%
Laatikainen *et al*.[16] Finland (1995) IC	500 70+	1991	31 (n/a)	6.2 (n/a) %GA: 61%

Abbreviations: GHS: Gutenberg Health Study, IC: International Classification System for age-related maculopathy (ARM), DP: digital photography used to record retinal image, TES: Tromsø Eye Study, SES: Speedwell Eye Study, AGES: Age, Gene / Environment Susceptibility Study, mWARMGS: modified Wisconsin Age-Related maculopathy Grading System, OMS: Oslo Macular Study, RES: Reykjavik Eye Study, RS: Rotterdam Study, WARMGS: Wisconsin Age-Related maculopathy Grading System, CCES: Copenhagen City Eye Study, ID: intermediate drusen (63-125 µm), SD: soft drusen (> 125 µm), SDD: soft distinct drusen (> 125 µm), SID: soft indistinct drusen (> 125 µm), HypoP: hypopigmentation, HyperP: hyperpigmentation, LD: large drusen (≥ 125 µm), AMCW: macular changes without visual impairment. Early AMD definition. GHS: 1a: only soft distinct drusen (≤ 63 µm), 1b: only pigmentary abnormalities, 2a: only soft indistinct drusen (≥ 125 µm) or reticular drusen, 2b: soft distinct drusen (≥ 63 µm) with pigmentary abnormalities, 3: soft indistinct drusen (≥ 125 µm) or reticular drusen with pigmentary abnormalities, SES: IC, grade 1: soft distinct drusen or pigmentary irregularities, grade 2: soft indistinct or reticular drusen without pigmentary irregularities, or soft distinct drusen with pigmentary irregularities, grade 3: soft indistinct or reticular drusen with pigmentary irregularities. Where available age-standardised prevalence values are quoted.

Table 1.3 Global prevalence of AMD (ancestry presented alphabetically)

Study, Country (Date) Grading System, Digital Photography	Subjects Age (years)	Ancestry	Prevalence of Early AMD % (95% CrI / CI)	Prevalence of Late AMD % (95% CrI / CI) % of late AMD = GA
KNHANES, Korea[17] (2014) IC, DP	14.352 40+	Korean	6.02 (5.56-6.48)*	0.60 (0.45-0.75)*, %GA: 20%
SEEDS, Singapore[18] (2014) mWARMGS, DP	10,033 40-85	Indian Singaporean-Chinese Malaysian	4.5 (3.8-5.4)* 5.7 (5.8-7.8)* 3.7 (3.0-4.6)*	0.3 (0.2-0.7)*, %GA: 0% 0.6 (0.4-2.7)*, %GA: 20% 0.3 (0.2-0.9)*, %GA: 38%
NS, Japan[19] (2013) sAREDS, DP	5,595 50+	Japanese	22.8 (21.7-24.0)*	0.58 (0.36-0.80)*, %GA: n/a
Kulkarni *et al*., India[20] (2013) IC	19,140 50+	Indian	1.14 (0.99-1.29)*	0.24 (2.1-2.4)*, %GA: 48%
Mathenge *et al*., Kenya[21] (2013) IC, DP	4,414 50+	Kenyan	11.4 (n/a)	1.3 (n/a), %GA: 36%
Moon *et al*., Korea[22] (2012) IC, DP	10,449 50+	Korean	3.08% (n/a)	-
HES, China[23] (2011) mWARMGS, DP	6,581 30+	Chinese	3.0 (2.6-3.5)*	0.1 (0.0-0.12)*, %GA: 0%
Jenchitr *et al*., Thailand[24] (2011) IC, DP	10,788 50-98	Thai	2.7 (n/a)	0.3 (n/a), %GA: 26%
NHANES05-08, USA[25] (2011) mWARMGS, DP Prevalence values for participants ≥ 60 years of age	7081 40+	Mexican-American Non-Hispanic-Black Non-Hispanic-White	12.9 (2.0)** 5.0 (1.3)** 11.6 (1.1)**	0.4 (0.4)**, %GA: 0% 0.3 (0.3)**, %GA: 100% 2.6 (0.5)**, %GA: 62%
CIEMS, India[26] (2011) WARMGS, DP	4,542 30+	Indian	4.7 (4.1-5.4)*	0.18 (0.07-0.29)*, %GA: 44%
SEE, Spain[27] (2011) IC, DP	2,132 65-74	Spanish	10.3 (8.7-11.8)*	3.4 (2.5-4.3)*, %GA: 44%
BOSS, USA[28] (2010) mWARMGS, DP	2,810 48-92	European	3.4 (2.7-4.0)*	No late AMD detected
SMES, Singapore[29] (2008) mWARMGS, DP	3,265 40-80	Malaysian	3.5 (2.9-4.1)*	0.34 (0.29-0.34)*, %GA: 40%
EUREYE, Estonia, France, Greece, Italy, Norway, Spain, UK[30] (2006) IC, DP	4,753 65+	European	ARM grade 1: 36.5 (32.7-40.3)* 2: 10.1 (8.9-11.4)* 3: 2.5 (1.8-3.1)*	3.32 (2.52-4.13)*, %GA: 36%
BeiES, China[31] (2006) WARMGS, DP	4,377 40+	Chinese	2.9 (2.5-3.3)*	0.3 (0.1-0.4)*, %GA: 42% Corrected 2008 values
MESA, USA[32] (2006) mWARMGS, DP	6,176 45-85	Black Chinese Hispanic White	2.1 (n/a) 3.6 (n/a) 4.0 (n/a) 4.8 (n/a)	0.3 (n/a), %GA: n/a 1.0 (n/a), %GA: 14% 0.2 (n/a), %GA: n/a 0.6 (n/a), %GA: 78%
APEDS, India[33] (2005) IC	3,723 40-102	Indian	-	1.82 (1.39-2.25)*, %GA: 94%
Proyecto VER, USA[34] (2005) mWARMGS	3,178 50+	Hispanic	27.9 (26.2-29.6)*	0.50 (0.3-0.8)*, %GA: 69%
LALES, USA[35] (2004) mWARMGS	5,875 40+	Latino	9.4 (8.6-10.1)*	0.43 (0.26-0.60)*, %GA: 35%
CHS, USA[36] (2003) mWARMGS	2,361 69-97	Black White	8.8 (5.9-11.7)* 16.7 (15.1-18.4)*	0.3 (0.1-1.5)*, %GA: n/a 1.5 (0.9-2.0)*, %GA: n/a
VIP, Australia[37] (2000) IC	4,345 40+	European	15.1 (13.7-16.4)*	0.68 (0.3-1.00)* %GA: 41%

NHANESIII, USA[38] (1999) WARMGS	8,270 40+	Mexican-American Non-Hispanic Black Non-Hispanic White	7.54 (n/a) 8.27 (n/a) 9.1 (n/a)	0.06 (0.0-0.1)*, %GA: 24% 0.13 (0.0-0.4)*, %GA: 49% 0.50 (0.3-0.7)*, %GA: 63%
ARIC, USA[39] (1999) mWARMGS	11,532 48-72	Black White	3.7 (n/a) 5.4 (n/a)	0 (n/a), %GA: n/a 0.2 (n/a), %GA: n/a
BaltES, USA[40] (1999) IC	5,308 40+	Black White	19.91 (n/a) 22.53 (n/a)	0.19 (n/a), %GA: 50% 1.91 (n/a), %GA: 57%
BMES, Australia[41] (1995) mWARMGS	3,654 49+	European	7.2 (7.0-7.4)*	1.9 (1.5-2.4)*, %GA: 33%
BES, West Indies[42] (1995) IC	3,444 40-84	Black	23.5 (22.8-24.2)*	0.57 (0.55-0.59)*, %GA: 11%
BDES, USA[43] (1992) WARMGS	4,771 43-86	European	15.6 (n/a)	1.6 (n/a), %GA: 28%

Abbreviations: KNHANES: Korean National Health and Nutrition Survey, IC: International Classification System for ARM, DP: digital photography used to record retinal image, SEEDS: Singapore Epidemiology of Eye Disease Study, NS: Nagahama Study, mWARMGS: modified Wisconsin Age-Related maculopathy Grading System, WARMGS: Wisconsin Age-Related maculopathy Grading System, sAREDS: simplified Age-Related Eye Disease Study grading system, NKS: Nakuru Kenya Study, HES: Handan Eye Study, NHANES05-08: National Health and Nutrition Examination Survey 05-08, CIEMS: Central India Eye and Medical Study, SEE: Spanish Eyes Epidemiological Study, BOSS: Beaver Dam Offspring Study, SMES: Singapore Malay Eye Study, EUREYE: European Eye Study, BeiES: Beijing Eye Study, MESA: Multi-Ethnic Study of Atherosclerosis, APEDS: Andhra Pradesh Eye Disease Study, Proyecto VER: Proyecto Vision and Eye Research, LALES: Los Angeles Latino Eye Study, CHS: Cardiovascular Health Study, VIP: Visual Impairment Project, NHANESIII: third National Health and Nutrition Examination Survey, ARIC: Atherosclerosis Risk in Communities Study, BaltES: Baltimore Eye Survey, BDES: Beaver Dam Eye Study, BES: Barbados Eye Study, CBWS: Chesapeake Bay Waterman Study, CrI: credible interval, CI: confidence interval, GA: geographic atrophy. Search criteria: January 1992-March 2014, study population ≥ 2,000 included participants and international AMD grading system. Where available age-standardised prevalence values are quoted.
* 95% CI, ** standard error.

1.1.2 Projected increase in AMD over time

Wong *et al*. reported a worldwide prevalence of AMD of 8.69% (95% CrI 4.26 to 17.40) and projected that by 2020 approximately 196 million people will be affected by this disease. Asia accounts for 60% of the world population and will consequently have the largest projected number of AMD cases, despite the low AMD prevalence in this population. Europe represents 11% of the world population, but has the highest AMD and the second highest projected number of AMD cases.[2] Owen *et al*. estimated that the number of cases of late AMD in the UK would increase by 30.6% to 679,000 (approximately 52% GA) by 2020.[1] Similar estimates were reported by Minassian *et al*., 24.3% increase in AMD cases to 755,867 (31.8% GA) in the UK by 2020.[44] The results from recent studies estimating future AMD prevalence are given in table 1.4.

Contrary to the increase in AMD prevalence with time forecast by predictive studies, real data from the 2005 to 2008 National Health and Nutrition Examination Survey (NHANES 2005-2008) indicated a reduction in the prevalence of all AMD compared to the third NHANES (NHANESIII) study reporting data from 1988 to 1994. The reduction in AMD prevalence in the USA between these studies may indicate improvements in public health (reduced smoking and blood pressure, improved diet and increased exercise), although differences in study method should also be considered.[25] Rudnicka *et al*. however, reporting from a large systematic review and meta-analysis (n = 57,173) of populations with European ancestry found no evidence of an alteration of late AMD prevalence with time.[45] Despite the increase in population numbers, new treatments for nAMD and possibly GA are likely to reduce future AMD prevalence, suggesting a more positive outlook for the ocular

health of individuals and the resources of health care agencies than that suggested by predictive studies.

Table 1.4 Estimated future increase in AMD

Study	Early AMD			Late AMD		
	Date	Actual Number or Millions	% increase	Date	Actual Number or Millions	% increase
Worseley *et al*. (2015)[46]	2014	167,500	-	2014	7,600	-
New Zealand	2026	189,200	13.0%	2026	8,600	13%
Wong *et al*. (2014)[2]		Millions			Millions	
Africa	2014	15.36	-	2014	0.77	-
	2020	18.47	20.0%	2020	0.93	20.8%
	2040	35.53	131.3%	2040	1.80	133.8%
Asia	2014	55.51	-	2014	4.59	-
	2020	66.29	19.4%	2020	5.52	20.3%
	2040	105.76	90.5%	2040	9.92	116.1%
Europe	2014	47.81	-	2014	2.57	-
	2020	50.87	6.4%	2020	2.79	8.6%
	2040	58.65	22.7%	2040	3.69	43.6%
Latin America & Caribbean	2014	19.87	-	2014	0.86	-
	2020	23.59	18.7%	2020	1.02	18.6%
	2040	36.95	86.0%	2040	1.61	87.2%
Northern America						
	2014	14.77	-	2014	0.76	-
	2020	16.70	13.1%	2020	0.90	18.4%
	2040	21.30	44.2%	2040	1.36	78.9%
Oceana						
	2014	1.21	-	2014	0.09	-
	2020	1.43	18.2%	2020	0.11	22.2%
	2040	2.07	71.1%	2040	0.19	111.1%
Lindekleiv *et al*. (2013)[47]	2012			2012	187,000	-
Scandinavia	2020	-	-	2020	212,000	13.3%
	2040			2040	328,000	75.4%
Owen *et al*. (2012)[1]						
UK	2020	-	-	2020	670,000	30.6%
Minassian *et al*. (2010)[44]	2010	-	-	2010	608,213	-
UK	2020			2020	755,867	24.3%

1.1.3 Limitations of studies examining AMD prevalence and incidence

Bayesian meta-regression analyses indicated that 50% of the variance between studies in late AMD prevalence was attributable to study design, whereas 20% was attributable to differing age profiles between studies.[45] The prevalence and incidence of AMD are significantly associated with age, therefore values should be age-corrected. Gender is not generally thought to be associated with AMD, however greater female longevity will bias the results towards higher female AMD rates in the older age range.[1] Classification systems for AMD grading have evolved over time, making it difficult to compare AMD stages between studies using different grading systems. The inclusion of studies with AMD grading systems other than the International Classification (IC) and the Wisconsin Age-Related Maculopathy (WARM) grading systems, and variable inclusion of fundus imaging in a systematic review / meta-analysis will lead to variability in late AMD prevalence values and contribute to increased levels of heterogeneity.[2, 45] The different AMD grading systems are summarised in section 1.2.

Optical coherence tomography (OCT) may be used to differentiate nAMD from retinal neovascularisation associated with PCV, however, this has not been used in the majority of

studies to date. Reliance on photographic grading may lead to an overestimation of early AMD and nAMD prevalence and higher ratios of nAMD to GA for Asians.[48] New treatments for AMD released during the course of the study period will reduce AMD prevalence, as it would be unethical to withhold more effective treatments from longitudinal study participants.[48] Blindness certification rates in the UK due to nAMD have fallen since the introduction of vascular endothelial growth factor (VEGF) inhibitors from 2006 onwards.[49]

1.2 Lesions and pathogenic features of AMD

1.2.1 Features of early AMD

Early AMD is characterised by the presence of drusen and / or RPE pigment abnormalities. These features, depending on their severity are considered to increase the risk of an individual developing late AMD. Subretinal drusenoid deposits are less visible using colour photographic methods, but also represent an increased risk of progression to late AMD.[50]

1.2.1.1 Drusen

Drusen are sub-RPE and subretinal deposits containing esterified cholesterol and phosphatidylcholine (40% of drusen contents).[51] The constituents of drusen vary with age, drusen size and retinal location. Drusen were reported to contain; opsins derived from photoreceptor outer segment phagocytosis, dendritic cells, proteins, lipids, sugar-containing molecules, non-fibrillar amyloid-β, the main constituent of amyloid plaques in Alzheimer disease (AD), N-retinylidene-N-retinylethanolamine (A2E), a lipofuscin chromophore that produces singlet oxygen in response to photo-excitation with visible light wavelengths, advanced glycation end products suggesting oxidative stress and inflammatory mediators, including vitronectin, amyloid A, C5 and C5b-9 terminal complexes, HLA-DR, fibrinogen, factor X, prothrombin, glial fibrillary acidic protein (GFAP) a sensitive marker for retinal stress and Müller cell (Mc) activation, and in some cases immunoglobulin.[52-55]

Drusen are a hallmark feature of AMD, assumed to develop in association with risk polymorphisms in the complement factor H (CFH) gene, but they are not exclusive to AMD.[54, 56] Drusen also develop as a consequence of normal ageing. Comparative analysis of drusen taken from normal and AMD donors revealed higher levels of crystallin and oxidative protein modifications including; cross-linked species of tissue metalloproteinase inhibitor 3 and vitronectin, carboxymethyl lysine and carboxyethyl pyrrole protein adducts (uniquely generated from the oxidation of lipids containing the docosahexaenoic acid (DHA) derivative, docosahexaenoate). These differences suggest that oxidative injury leading to oxidative protein modifications may be involved in drusen formation associated with AMD.[57]

Drusen are observed ophthalmoscopically in 15-30% of all individuals, increasing to 80% of

those aged over 60 years, but are rare in individuals below 40 years of age.[56] There is a great individual variety in the appearance of drusen, which may be described in terms of; number, size (small < 63 µm, medium 63 µm to 125 µm and large > 125 µm), margins (distinct or indistinct), and texture (soft or hard).[54] Table 1.5 contains a summary of drusen types.

Table 1.5 Classification of drusen

Hard (nodular) drusen	Small, well-defined yellow deposits, usually < 50 µm. Visible ophthalmoscopically. Minimal risk of soft drusen occurrence and / or progression to late AMD unless large numbers (> 8) are present.[58] Located sub-RPE.[56]
Soft (exudative) drusen	Larger and ill defined yellow deposits. Visible ophthalmoscopically. Higher risk of progression to late AMD. Located sub-RPE.[56]
Basal linear deposits (diffuse) drusen Soft laminar drusen may also be described as diffuse drusen	Extensive deposits between the basement membrane of the RPE and the inner collagenous zone of Bruch's membrane. Visible in histological sections. The presence of choroidal filling delays with fluorescein angiography (FA) indicates the presence of diffuse drusen. When associated with early AMD with soft drusen, eyes with diffuse drusen tend to develop GA rather than nAMD.[59]
Cuticular drusen (formerly basal laminar drusen)	Small, round, multiple, densely packed, yellow-white deposits. Contain the same constituents as soft drusen. Visible ophthalmoscopically, but better visualised by their hyperfluorescence with FA ("starry-sky" fundus).[60] Common in individuals with mis-sense mutations in fibulin-5 and those with high-risk Y402H alleles in CFH.[61] Located sub-RPE.
Subretinal drusenoid deposits (formerly reticular pseudodrusen, reticular drusen)	Yellow interlacing networks. Not always visible ophthalmoscopically. Better visualised using blue or IR wavelengths or spectral domain OCT. Reticular pseudodrusen are a stronger predictor for progression to late AMD than classical drusen. Located subretinally (like vitelliform lesions) not sub-RPE.[50]

The examination of drusen over time has revealed a variety of spontaneous changes that may occur. Hard drusen may enlarge and progress to soft drusen ("drusen softening"). Soft drusen may enlarge and become confluent, leading to pigment epithelium detachment (PED). Drusen may exhibit spontaneous regression, as drusen material is phagocytosed by macrophages and cleared from the sub-RPE space. Drusen material that is not removed becomes calcified, appearing ophthalmoscopically as refractile, crystalline structures containing cholesterol crystals.[56, 62]

Several case reports from non peer-reviewed publications have suggested that MP supplements may lead to drusen resolution.[63-65] While there may be biologically plausible arguments for supplement-induced drusen resolution, these results need to be controlled for spontaneous drusen resolution, reported to occur in 20% of cases over seven years.[66] Another study found that soft drusen resolved spontaneously in 3.5% of cases over 5.9 years.[67] Analysis by drusen size revealed a 10-year regression for small (< 63 µm) drusen of 48.9%, for medium drusen (≥ 63 µm to < 125 µm) of 17.8%, and for larger drusen (≥ 125 µm to < 250 µm) and (≥ 250 µm) of 30.4% and 30.8% respectively.[58] The later stages of drusenoid pigment epithelium detachment (DPED) life cycle may involve varying degrees of drusen collapse and regression associated with RPE degeneration, clinically observed as hypopigmentary change.[68]

1.2.1.2 Pigmentary abnormalities

Hypo- and hyperpigmentation of the RPE is another ophthalmoscopically visible indicator of early AMD. Pigmentary changes are due to deposits between the inner collagenous layer of

Bruch's membrane and the basement membrane of the RPE. Hypopigmented areas may also result from RPE cell death and hyperpigmented areas reflect proliferating (hypertrophic) RPE cells or RPE cells containing phagocytosed pigmented material from neighbouring cells that have been lost.[69]

1.2.1.3 Subretinal drusenoid deposits

Subretinal drusenoid deposits (SDD) are located subretinally, rather than sub-RPE. They form a yellow interlacing network visible in some patients ophthalmoscopically or using colour fundus photography, but are better visualised using blue or IR wavelengths, fundus autofluorescence (FAF) or spectral domain OCT.[54] The constituents of SDD are similar to soft drusen, but do not contain lectin-binding disaccharide bridges and opsins associated with photoreceptors, GFAP and cellular retinal-binding proteins (CRALBP) associated with Mc, and CRALBP associated with the RPE.[70, 71] Subretinal drusenoid deposits are more likely to be observed in the perifovea (90.1% of a sample of 20 eyes with AMD) compared to the fovea (9.9%). Photoreceptor changes (outer segment (OS) shortening or loss and inner segment (IS) deflection or absence, and choroidal alterations (choriocapillary ghosts, choroidal thinning, loss of large vessels and hyalinisation of the macular stroma were also associated with SDD.[54]

1.2.1.4 Risk of progression from early to late AMD

The presence of large (> 125 μm) drusen is considered an important marker for progression to nAMD and GA. Focal RPE hyperpigmentation may indicate impending nAMD.[72] The first Age-Related Eye Disease Study (AREDS) report number 18 described a simple way to assess the 5-year risk of developing late AMD by grading the presence of the early AMD signs; large drusen and any pigmentary changes in one or both eyes (one point for either sign per eye). The scores and risk of progression to late AMD were simplified to; 0, 0.5%; 1, 3%; 2, 12%; 3, 25%; 4, 50%.

Although SDD are not included within the International Classification of AMD, they are strong predictors for progression to nAMD and GA.[54] Hogg *et al*. reported that SDD are associated with the development of nAMD in the fellow eye of individuals with unilateral nAMD, after correction for age and gender; odds ratio (OR) 5.5 (95% CI 1.1-28.8) and all eyes that developed GA during follow-up had visible SDD at baseline. Subretinal drusenoid deposits appear to be a stronger predictor for progression to late AMD than classical drusen.[50] The prevalence of SDD and consequently their importance to AMD risk have been under-estimated in older studies due to the reliance on colour photography for grading AMD features.

1.2.2 Pathogenic features of GA

Geographic atrophy is characterised by the loss of RPE, PR and choriocapillaris.[73] It has

been suggested that GA may represent the end stage of the drusen life cycle in eyes with AMD,[74] however, to reach this stage eyes would have to avoid the competing risk of nAMD, which normally obscures the process of drusen regression and GA formation.[75] Klein *et al*. reported that the site of the initial appearance of GA was previously occupied by large drusen in 96% of cases, very large drusen (≥ 250 μm) in 83% of eyes and confluent drusen. Geographic atrophy was nearly always preceded by hyperpigmentation overlying drusen, followed by regression of the drusen and pigment and the appearance of hypopigmentation, occasionally accompanied by refractile deposits (23% of cases) representing residual material not removed by macrophages. The average time for GA to develop in individuals with large or confluent drusen was approximately 5-6 years, whereas GA developed an average of 2.5 years in the presence of hypopigmentation.[75] Less frequently, GA may develop after drusenoid PED (50% risk after seven years),[76] regression of nAMD following treatment with VEGF inhibitors (72% of eyes),[77] or following RPE rupture.[78]

Histopathological studies suggest that RPE cells are the primary target in GA and their death results in choriocapillaris atrophy. Bruch's membrane, a five-layered structure which includes the RPE basement membrane, calcifies and doubles in thickness due to deposits of collagen, lipids and debris, leading to reduced fluid permeability and nutrient transport, while the choriocapillaris and choroidal thickness is halved with age.[79] Lipofuscin accumulation within RPE cells combined with lipid peroxidation products resulting from oxidative stress is thought to cause dysfunction and ultimately GA,[73] although a cause and effect relationship between RPE lipofuscin accumulation and AMD has not been established.[80]

Lipofuscin levels within RPE cells increase with age, increasing from 12% to 19% in the macular retina between the ages of 50 and 90 years.[81] At the GA perimeter (junctional zone) the lysosomal compartment of RPE cells may contain much higher levels of lipofuscin, leading to the characteristic hyperfluorescence observed with FAF (figure 1.1).[82, 83] Higher levels of autofluorescence in the rim area bordering the area of GA (normally devoid of autofluorescence) was associated with faster lesion progression.[84] Using OCT, fast progression was associated with a marked separation between the RPE / Bruch's membrane complex, possibly correlated with basal laminar deposits which may to promote RPE cell death.[85]

Geographic atrophy is also associated with dysfunction of PR, with rods affected before cones.[86] The loss of PR appears to be secondary to changes of, and beneath the RPE, however PR loss may be evident outside the area of GA over normal appearing RPE cells.[87] Activation of Mc, microglia and macrophage activity are also features of GA.[73] Individuals with GA have significantly lower choroidal blood flow. Choroidal malperfusion

has been implicated as a potential cause of SDD, considered to be a greater predictor for GA than classical drusen.[50, 73]

Figure 1.1 Fundus photograph and fundus FAF images of GA

1.2.3 Pathogenic features of nAMD

Wound repair in most tissues is associated with new blood vessel growth (neovascularisation). New vessels sprout from existing vessels to repair or replace damaged vasculature. In the retina neovascularisation is counterproductive, leading to more damage rather than less.[88] Subretinal neovascularisation associated with nAMD originates from choroidal neovascularisation (CNV), which sprout from choroidal vessels and extend through Bruch's membrane and the RPE to reach the subretinal space. A second source of subretinal neovascularisation may also be observed in cases with nAMD; retinal angiomatous proliferation (RAP) originating from the deep capillary bed located in the inner nuclear layer of the retina, grow through the photoreceptor layer to reach the subretinal space.[88] Choroidal neovascularisation may remain beneath the RPE, leading in some cases to the development of vascularised PED. Polypoidal choroidal vasculopathy (PCV), a condition characterised by aneurysmal or polypoidal dilations of the inner choroidal vasculature is also associated with choroidal neovascularisation. The clinical appearance of PCV is often difficult to distinguish from nAMD and may represent a subtype of nAMD.[89]

New vessels, whether retinal or choroidal in origin are deficient in tight junctions compared to normal retinal vessels and therefore leak plasma (fluid) in to the surrounding tissue. New vessels are also more fragile leading to the formation of haemorrhages.[88]

Neovascular AMD represents one of two possible end points in the progression of AMD. The factors that determine whether an eye develops nAMD or GA have not been fully

elucidated, however, certain conditions are known to predispose an eye to neovascularisation. Examination of Bruch's membrane from eyes with nAMD revealed higher levels of calcification and fragmentation compared to eyes with non-exudative AMD.[90] Breaks in Bruch's membrane, whether secondary to pathological processes (e.g. angioid streaks) are associated with CNV.[91]

The major clinical features of active nAMD include; subretinal haemorrhage and / or fluid, sub-RPE haemorrhage and / or fluid (from vascularised or serous PED), RPE pigment alterations and hard exudates. Chronic nAMD is characterised by subretinal fibrosis, with or without the features of active nAMD listed (figure 1.2).[92] A study using SD-OCT has described five signs preceding the development of new-onset nAMD by at least one month; new RPE defects, new PR defects, drusen touching the PR layer and the external limiting membrane (ELM), and hyper-reflective spots possibly representing new growing vessels.[93]

Figure 1.2 Photographic and spectral-domain OCT images of active CNV attributed to nAMD

1.2.4 Classification of AMD

The terminology used to describe AMD has evolved over the years.[94, 95] The lack of a standard agreement on the definition of specific AMD lesions and an accepted method of AMD classification has led to the development of several AMD classification schemes used predominantly for the assessment of AMD prevalence and progression risk in epidemiological studies, but later simplified for use in the clinical setting.

The Wisconsin Age-Related Maculopathy Grading System (WARMGS) published in 1991, was the first AMD grading scheme designed specifically for use in epidemiological studies and clinical trials.[96] Early and late features of AMD, termed early and late age-related maculopathy (ARM) were based on the presence and severity of 13 features including; centrally located drusen, pigmentary abnormalities, GA and nAMD assessed from retinal

photographs.

The complexity and number of scales used in WARMGS led to a consensus group meeting in the mid-nineties and the development of the International Classification System for Age-related Maculopathy (IC),[94] also referred to as a modified version of WARMGS (mWARMGS).[23] This photographic-based system attempted to distinguish early features of AMD; drusen and pigmentary abnormalities (termed ARM) from late features of AMD; GA and nAMD (termed AMD).[97] The WARMGS and IC systems have been modified to simpler forms in many studies (tables 1.2 and 1.3).

The first Age-Related Eye Disease Study, a four-stage, photographic grading system was designed to examine the benefit of nutritional supplementation in individuals with no or early AMD in either eye and individuals with late AMD in one eye.[98] The AREDS grading system introduced the term "advanced AMD" to describe GA and nAMD. This was simplified to a clinically achievable, four-stage classification system from which an AMD progression risk algorithm was developed (sAREDS).[99] Another clinically-based AMD grading scheme; the Clinical Age-Related Maculopathy Staging System (CARMS) has been used in several studies. This scheme used a five-point scale to differentiate "early AMD" and "intermediate AMD" (grades 1-3) from "late AMD" (grade 4: GA and grade 5: nAMD or PED).[100]

The latest clinical AMD classification system resulted from a Delphi review of the current AMD classification criteria. The term "AMD" was preferred to other terms such as "ARM" and "ARMD" (a longer abbreviation of age-related macular degeneration). The confusing term "dry AMD", previously used to describe early AMD and GA was limited to the description of GA only. The term "drupelets" (the small units of aggregate fruit found in raspberries or blackberries) was introduced to describe small drusen (< 63 μm), not considered to increase risk of AMD progression, in order to differentiate these from intermediate and larger drusen that are associated with significant risk of progression. This five-stage clinical AMD classification system was the first to differentiate normal ageing changes, not classified as AMD, from early AMD.[101] A summary of the main AMD classification systems and their modified / simplified forms is given in table 1.6.

Table 1.6 Summary of the main AMD grading schemes

AMD Classification system Tables 1.2 and 1.3 list studies that used these systems	Early and late AMD grading
WARMGS[96] Used by longitudinal epidemiological studies to predict progression to late AMD. Not suitable for clinical purposes. Subclinical lesions easily overlooked by ophthalmoscopy are graded. AMD features graded using a set of standard circles of varying diameter (C0 = 1/24 DD, 63μm, C1 = 1/12 DD, 125μm, C2 = 1/6 DD, 250μm, I1 = 1.6% of inner subfield, I2 = 6.3% of inner subfield, O1 = 1.6% of outer subfield, O2 = 6.3% of outer subfield) on a transparent sheet, from stereoscopic transparencies (slides) viewed using a	Early AMD: drusen are graded by size (0-8), type (0-8), area (0-8), confluence (0 - ≥50% per subfield), RPE degeneration (0 - ≥50% per subfield), subretinal gray / black pigment (0 - ≥ circle C2). Late AMD: GA area ≥ circle I1 (0 - ≥50% of subfield), subretinal or sub-RPE haemorrhages (absent or present, graded as one lesion per subfield), subretinal and sub-RPE detachment (mostly subretinal, mostly sub-RPE or both), subretinal fibrous scar (absent to ≥50% per subfield). Drusen confluence was graded.

fluorescent viewing box at x15 magnification. A grid consisting of three concentric circles with diameters; 2/3 DD (1000μm), 2 DD (3000μm) and 4 DD (6000μm) similar to that used by ETDRS and divided into nine subfields is superimposed onto one of the stereo images, centred on the fovea.	No differentiation between hyper and hypopigmentation.
IC[94] (a modified version of WARMGS) Used by longitudinal epidemiological studies to predict progression to late AMD. Not suitable for clinical purposes. Subclinical lesions easily overlooked by ophthalmoscopy are graded. AMD features graded using a set of standard circles of varying diameter (C0 = 1/24 DD, 63μm, C1 = 1/12 DD, 125μm, C2 = 1/8.6 DD, 175μm, C3 = 1/6 DD, 250μm, C4 = 1/3 DD, 500μm) on a transparent sheet, from stereoscopic transparencies (slides) viewed using a fluorescent viewing box at x15 magnification. The same grid used in WARMGS is superimposed onto one of the stereo images, centred on the fovea.	Early AMD: drusen are graded by morphology (0-8), prominent drusen type within outer circle (0-8), number of drusen (0-8), size of drusen (1-8), main location of drusen (1-8), area covered by drusen (1-8 per subfield), hyperpigmentation (0-8), hypopigmentation (0-8), main location of hyper / hypopigmentation (1-8). Late AMD: GA: presence (0-8), location (1-8), area covered (1-8), nAMD: presence (0-8), typifying features (1-8), location (1-8), area covered (1-8). Drusen confluence was graded as soft indistinct drusen. Differentiation between hyper and hypopigmentation. Reticular drusen not graded.
AREDS[98] (a modified version of WARMGS) Used by longitudinal epidemiological studies to predict progression to late AMD. Not suitable for clinical purposes. Subclinical lesions easily overlooked by ophthalmoscopy are graded. AMD features graded using a set of standard circles of varying diameter (C-0: 0.042 (1/24) DD, 63μm, C-1: 0.083 (1/12) DD, 125μm, C-2: 0.167 (1/6) DD, 250μm, I-1: 0.120 DD, I-2: 0.241 DD, O-1: 0.219 DD, O-2: 0.439 DD) on a transparent sheet, from stereoscopic transparencies (slides) viewed using a fluorescent viewing box at x15 magnification. The same grid used in WARMGS is superimposed onto one of the stereo images, centred on the fovea. Central zone = central and four inner subfields.	Early AMD: drusen graded separately within grid, centre and inner subfields and centre subfield, presence and maximum size (0-8), type (0-8), area (0-8), presence outside grid (0-8), reticular drusen (0-8), calcified drusen (0-8), hyperpigmentation (0-8), hypopigmentation (0-8). Early AMD grades: Grade 1: drusen size <63μm, total area <125μm. Grade 2: drusen size ≥63μm, <125μm, total area ≥125μm and RPE abnormalities in the central or inner subfields. Grade 3: one or more of the following; drusen size >125μm, soft indistinct drusen size ≥63μm, total area > circle I2. soft distinct drusen size ≥63μm, total area > circle O2. GA within the grid, but not at the central macula. Late AMD: GA: graded separately within grid, centre and inner subfields and centre subfield (0-8), retinal elevation: presence or absence graded by subfield, drusenoid PED (0-8), fibrovascular / serous PED (0-8), serous / haemorrhagic sensory retinal detachment (0-8), hard exudates (0-8), subretinal / sub-RPE haemorrhage (0-8), subretinal fibrous tissue / fibrin (0-8), photocoagulation for AMD (0-8). Grade 4: late AMD (GA and nAMD).
sAREDS[99] Suitable for clinical use. Graded within 2DD of the fovea. Five-year risk of developing advanced AMD in one or both eyes without advanced AMD in either eye (unbracketed percentages) and with advanced AMD in one eye (bracketed percentages). The diameter of the normal retinal vein at the optic disc margin is approximately 125μm.	Graded 0-2 for each eye: large drusen (= ≥125μm): no = 0, yes = 1, pigmentation, no = 0, yes = 1. Five year risk of advanced AMD: Grade 0: 0.4% Grade 1: 3.1% Grade 2: 11.8% (14.8%) Grade 3: 25.9% (35.4%) Grade 4: 47.3% (53.1%)
CARMS[102] (a modified version of AREDS) Suitable for clinical use. Graded within a 3000μm radius centred on the foveal centre.	Early AMD: Grade 1: no signs associated with grades 2 to 5. Grade 2: extensive (≥15) small drusen (<63μm), non-extensive (<20) intermediate drusen (≥63μm, <125μm) or pigment abnormalities associated with AMD. Grade 3: extensive intermediate or large (≥125μm) drusen. Late AMD: Grade 4: GA. Grade 5: PED or CNV.
Ferris III et al. (2013)[101] Suitable for clinical use. Graded within 2DD of the fovea for patients aged over 55 years.	Early AMD: Grade 1: No age changes: no drusen and no AMD pigmentary abnormalities. Grade 2: Normal age changes: only drupelets (small drusen ≤63μm) and no AMD pigmentary abnormalities. Grade 3: Early AMD: medium drusen (>63μm, ≤125μm) and no AMD pigmentary abnormalities. Grade 4: Intermediate AMD: large drusen (>125μm) and / or any AMD pigmentary abnormalities. Late AMD: Grade 5: GA or nAMD.

WARMGS: Wisconsin Age-Related Maculopathy Grading System, DD: disc diameters, ETDRS: Early Treatment Diabetic Retinopathy Study, IC: International Classification and Grading System for Age-related Maculopathy (ARM) and AMD, AREDS: Age-Related Eye Disease Study Research Group Severity Scale for AMD, sAREDS: simplified Age-Related Eye Disease Study Research Group Severity Scale for AMD, CARMS: Clinical Age-Related Maculopathy Staging System. C1-3: in this context refers to central 1-3, not to be confused with complement components 1-3. All classification system information reproduced with permission.

Inter-observer agreement (κ-statistic) for four of the major AMD grading systems; WARMGS, IC, AREDS and CARMS suggested moderate to substantial agreement for most

features of AMD.[100] Comparison between studies using different grading schemes is more problematic, however, due to differences in definitions of AMD stages, terminology, image quality and treatment of the data (e.g. correction for age and gender).[103]

An attempt has been made to harmonise AMD classification between several of the major epidemiological studies; Beaver Dam Eye Study (BDES), Blue Mountains Eye Study (BMES), Los Angeles Latino Eye Study (LALES), and Rotterdam Study (RS), all of which used WARMGS or a modified version of WARMGS (mWARMGS). Grading of the same 60 images by the four respective centres revealed a variation in the exact agreement of AMD severity of between 61% and 84%, with weighted kappa scores ranging from 0.66 to 0.86 indicating moderate to substantial levels of agreement. Applying a correction for age and gender increased the prevalence of early AMD in all four studies, but did not affect the prevalence of late AMD. The authors concluded that despite harmonisation, it was difficult to correct for systematic differences in grading.[103] Comparisons between WARMGS and IC revealed no evidence of a difference in the definition of late AMD.[45]

Digital photography (DP) has been used with the current AMD grading systems developed for use with non-digital images, in papers published since 2006 (tables 1.2 and 1.3). Bartlett *et al*. reported that DP can be used with the commonly used AMD grading systems (WARMGS, IC and AREDS).[100] van Leeuwen *et al*. reported that digital imaging was as good as 35-mm film for grading AMD in epidemiological studies. The weighted κ value for between-technique agreement (a quantitative measure of the magnitude of agreement between observers) ranged from 0.41 (fair agreement) for number of drusen < 63 μm to 0.79 (substantial agreement) for drusen type and area occupied by drusen. The weighted κ values for GA (0.87) and nAMD (0.94) showed almost perfect agreement.[104]

The use of DP has allowed the development of automated methods of AMD classification. Kankanahalli *et al*. reported a high level of accuracy (92.3% to 98.0%) between manual grading by expert graders and automated retinal image analysis (ARIA) of AMD using the AREDS grading scheme, graded from 1 to 4. Accuracy was calculated in this study by dividing the sum of true positives and true negatives by the sum of true positives, false positives, true negatives and false negatives.[105] Future development of machine learning models in the area of ARIA may combine clinical signs with patient characteristics using "white box" methods (logistic regression and decision trees) and "black box" methods (support vector machines, random forests and AdaBoost).[106] This may allow real-time assessment of AMD grading for epidemiological studies without the need to send images to off-site grading centres, as well as affording practitioners a more detailed assessment of the stage of AMD, and allowing the development of more complex algorithms to predict AMD progression and outcome in the clinical setting.

1.3 Risk factors for AMD

Studies have revealed many clinical RF associated with increased or decreased risk of AMD development and progression. Seventy three RF were identified of which 16 factors considered to be readily measured in a non-specialist setting, were investigated in a systematic review of 18 cross-sectional and prospective, and six case control studies by Chakravarthy *et al*.[107] Risk factor associations with AMD were ranked as; strong and consistent, moderate and consistent and weaker and inconsistent (table 1.7). The list of RF discussed in this section is not exhaustive. Other putative AMD RF include; socio-economic status, refractive error, cup / disc ratio, chlamydia pneumoniae infection, reproductive and related factors, and alcohol consumption.

Table 1.7 Summary of RF (excluding age) associated with late AMD

AMD Risk Factors	Cross-Sectional Studies Studies (n): OR (95% CI)	Case Control Studies Studies (n): OR (95% CI)	Prospective Cohort Studies Studies (n): RR (95% CI)
Strong and consistent: Current cigarette smoking	7: 3.58 (2.68-4.79)	5: 1.78 (1.52-2.09)	6: 1.86 (1.27-2.73)
Previous cataract surgery	5: 1.59 (1.08-2.34)	2: 1.54 (1.24-1.91)	3: 3.05 (2.05-4.55)
Family history of AMD	1: 3.95 (1.35-11.54)	2: 6.18 (0.98-38.9)*	0: -
Moderate and consistent: Higher BMI	6: 1.21 (0.97-1.53)*	2: 1.52 (1.15-2.00)	7: 1.28 (0.98-1.67)*
Cardiovascular disease	7: 1.12 (0.86-1.47)*	4: 2.20 (1.49-3.26)	5: 1.22 (0.92-1.63)*
Hypertension	7: 1.15 (0.88-1.51)*	3: 1.48 (1.22-1.78)	5: 1.02 (0.77-1.35)*
High plasma fibrinogen	2: 1.45 (1.22-1.73)	0: -	1: 1.03 (0.81-1.32)*
Weaker and inconsistent: Gender (female)	2: 1.06 (0.78-1.44)*	2: 1.00 (0.83-1.21)*	2: 1.01 (0.89-1.16)*
Ethnicity (White vs. other)	2: 1.09 (0.09-13.56)*	1: 4.20 (2.23-8.00)	2: 0.91 (0.49-1.69)*
Diabetes	3: 1.09 (0.61-1.92)*	1: 0.55 (0.06-4.87)*	3: 1.66 (1.05-2.63)
Iris colour (brown vs. blue)	1: 0.88 (0.65-1.17)*	2: 0.60 (0.12-2.98)*	3: 0.98 (0.72-1.32)*
Cerebrovascular disease	6: 1.10 (0.69-1.75)*	0: -	3: 1.54 (0.82-2.90)*
Serum total cholesterol level	6: 0.94 (0.84-1.04)*	1: 4.66 (1.35-16.11)	5: 0.99 (0.95-1.03)*
Serum HDL cholesterol level	6: 1.06 (0.80-1.39)*	1: 3.35 (0.92-12.23)*	5: 1.00 (0.97-1.02)*
Serum triglyceride level	3: 1.08 (0.89-1.30)*	1: 0.90 (0.25-3.24)*	2: 1.03 (0.81-1.32)*

Abbreviations: OR: odds ratio, RR: relative risk, CS: cross-sectional, CC: case control, PC: prospective cohort, BMI: body mass index, HDL: high-density lipoprotein.
* Indicates a non-significant association. Adapted from Chakravarthy *et al*. (2010) (Open Access).

1.3.1 Strong and consistent late AMD RF

Age, current cigarette smoking, previous cataract surgery and potentially (as the OR derived from the case control studies was not significant) family history (FH) of AMD were ranked as strong and consistent RF for AMD. Sections summarising AMD risk genes and fellow eye involvement are included after AMD FH.

Table 1.8 Strong and consistent late AMD RF

Age
All studies reviewed by Chakravarthy *et al*. concluded that increasing age was a strong and consistent RF for AMD. The prevalence of AMD increased with age; 50-59 years (approximately 0.3%), 60-69 years (approximately 0.5%), 70-79 years (approximately 2.5%) and ≥ 80 years (approximately 9%).[107] A recent systematic review of populations with a European ancestry confirmed this association (table 1.1).[1] Age is the most important risk factor for AMD. For all forms of AMD, prevalence, incidence and progression escalate rapidly with increasing age.[107] Physiological changes associated with ageing are mirrored in AMD pathophysiology (section 1.4).[108] AMD is closely associated with age, however this condition is not inevitable with increasing age. Approximately 88% of those aged 80 years and over are unaffected by advanced AMD.[1]
Smoking
Smoking is the most consistently documented personal RF after age, and is therefore the strongest modifiable RF for all types of AMD,[109-112] reported to account for 32% of the risk for the development of AMD.[113] Smoking is classed as a personal RF, however passive smoking may be described as an environmental RF. Smoking may facilitate AMD onset and progression through retinal oxidative stress, reduced choroidal blood flow, increased ischaemia, hypoxia and micro-infarctions, provocation of CNV and reduction of serum antioxidants.[114]
Previous cataract surgery
Chakravarthy *et al*. reported a significant association between AMD and previous cataract surgery from the combined results of cross-sectional, case control and prospective cohort studies,[107] however, the association between cataract extraction and subsequent development of AMD is controversial.[97] BDES concluded that late, but not early AMD was strongly associated with previous cataract surgery (OR 1.93, 95% CI 1.28 to 2.9). This association was retained after controlling for high-risk genetic status (CFH Y402H and age-related maculopathy susceptibility 2; ARMS2) and other RF.[115] The 10-year results from the BMES also concluded that cataract surgery eyes had a higher risk of developing late AMD (OR 3.3, 95% CI 1.1 to 9.9).[116]
AMD FH
The BMES study reported an increase in AMD risk associated with a family history of AMD. After adjustment for age, gender and current smoking status the following OR were reported; no AMD (OR 1.0 (index)), early AMD (OR 2.17, 95% CI 1.04 to 4.55), late AMD (OR 3.92, 95% CI 1.34 to 11.46). When analysed separately, nAMD was significantly and positively associated with AMD FH (OR 4.30, 95% CI 1.37 to 13.45).[117] Combined data from two case control studies was not significantly associated with AMD risk,[107] however each study reported a significant association. Hyman *et al*. reported a significant association between AMD and a self-reported history of AMD in brothers and sisters (OR 2.9, 95% CI 1.4 to 5.9) and sisters only (OR 2.4, 95% CI 1.2 to 8.9), but not brothers only (OR 2.4, 95% CI 0.9 to 6.9).[118] Klaver *et al*. found a significant association, independent of other RF between early (OR 4.8, 95% CI 1.8 to 12.2) and late AMD (OR 19.8, 95% CI 3.1 to 126.0), confirmed photographically and a FH of AMD in first-degree relatives.[119] Concordance of AMD was 90% for monozygous twin pairs compared to 70% for spouses of the twins in another study (chi-square test, p = 0.028).[120]
AMD risk genes
Complement factor H (function: inflammation and immune system) and ARMS2 (function: inflammation, immune system and extracellular matrix) gene polymorphisms account for over 50% of the risk of developing AMD.[121] Many other genes are reported to associate with AMD including those related to inflammation and immunity (e.g. C2, C3, CFB, CFI), extracellular matrix and cell adhesion (e.g. ACE, COL8A1, TIMP3), lipid / protein metabolism and transport (e.g. ABCA1, ABCA4, APOE, ELOVL4, LIPC), angiogenesis (e.g. HTRA1, IL8, VEGFA) and cellular stress and toxicity (e.g. ABCA4, ACE, APOE).[122] There is evidence that the stage of AMD development is influenced by particular gene polymorphisms; ABCA1 was associated with lower risk of intermediate and large drusen, and nAMD and GA. ARMS2 / HTRA1 was linked to late AMD development and increased nAMD lesion size. LIPC single nucleotide polymorphisms (SNPs) were also associated with nAMD.[123, 124]
Fellow eye risk
Although not investigated by Chakravarthy *et al*. in their systematic review, individuals with unilateral nAMD have a 4-12% per year cumulative risk of developing nAMD in their fellow eye.[125] Slower recovery from glare was reported to be an independent RF for CNV in fellow eyes of patients with unilateral nAMD.[126]

Abbreviations. C3: complement factor 3, CFI: complement factor I, ACE: angiotensin 1 converting enzyme, COL8A1: collagen type VIII α 1, TIMP3: tissue inhibitor of metalloproteinase 3, ABCA1: ATP-binding cassette subfamily A (ABC1) member 1, ABCA4: ATP-binding cassette subfamily A member 4, APOE: apolipoprotein E, ELOVL4: ELOVL fatty acid elongase 4, LIPC: hepatic lipase, HTRA1: HtrA serine peptidase 1 high temperature requirement factor 1, IL8: interleukin 8, VEGFA: vascular endothelial growth factor A.

1.3.2 Moderate and consistent late AMD RF

Moderate and consistent RF for advanced AMD include; higher BMI, cardiovascular disease, hypertension and raised plasma fibrinogen levels.

Table 1.9 Moderate and consistent late AMD RF

Raised BMI
The results from Chakravarthy *et al*. were not conclusive. The combined data from two case control studies (AREDS no. 3 and Hogg *et al*., 2008)[127, 128] revealed a significant association between raised BMI and AMD (OR 1.52, 95% CI 1.15 to 2.00), however, the association of the combined results from seven prospective cohort and from six cross-section studies did not reach significance. The authors offered the caveat that the association may be due to shared RF (e.g. hypertension) or unmeasured confounders (e.g. nutritional status). One of the most common consequences of obesity is dyslipidemia (increase in low-density lipoprotein, LDL and reduced HDL cholesterol).[129]
Cardiovascular disease
After correction for confounding variables such as age and gender; coronary heart disease, stroke and cardiovascular mortality were associated with AMD in some,[128, 130, 131] but not all studies.[110, 132] Combined the results from five prospective cohort (RR 1.22, 95% CI 0.92 to 1.63), seven cross-sectional (OR 1.12, 95% CI 0.86 to 1.47) and four case control studies (OR 2.20, 95% CI 1.48 to 3.26), returned a significant association for the case control studies only, with approximately twice the odds of late AMD in those with cardiovascular disease.[107]
Hypertension
Other population based, cross-sectional studies reported no association with AMD. Blue Mountains Eye Study, corrected for age, gender, current smoking and FH of AMD found no association with early or late AMD, and the Atherosclerosis Risk in Communities study (ARIC), adjusted for age and gender found no significant association (at p = 0.001 level) with early AMD.[133] Combined data from three case controlled studies identified a significant association between hypertension and late AMD (OR 1.48, 95% CI 1.22 to 1.78), although the combined results from seven cross-sectional and from five prospective cohort studies did not achieve significance.[107]
Raised plasma fibrinogen levels
Fibrinogen is an inactive protein involved in blood coagulation via its conversion to fibrin. Plasma fibrinogen levels were positively associated with late but not early AMD in BMES.[133] Combined results from two cross-sectional studies revealed a significant association between higher fibrinogen levels and late AMD (OR 1.45, 95% CI 1.22 to 1.73), however a single prospective cohort study (BMES) did not produce a significant result.[134]

1.3.3 Weaker and inconsistent late AMD RF

Weaker and inconsistent RF for AMD include; female gender, White ethnicity, diabetes, lighter iris colour, cerebrovascular disease, raised total and HDL cholesterol levels and raised plasma triglyceride levels.

Table 1.10 Weaker and inconsistent late AMD RF

Female gender
Chakravarthy *et al*. reported no significant association between female gender and late AMD from the combined results from two prospective studies (RR 1.01, 95% CI 0.89 to 1.16), two cross-sectional studies (OR 1.06, 95% CI 0.78 to 1.44) and two case control studies (OR 1.00, 95% CI 0.83 to 1.12).[107] A recent meta-analysis with combined data from 57,173 participants of European ancestry found that late AMD was slightly more common in women than men (OR 1.13, 95% Crl 1.01 to 1.28), gender differences were not significant for GA (OR 0.99, 95% Crl 0.78 to 1.26) or nAMD (OR 1.24, 95% Crl 0.99 to 1.54) when analysed separately.[45]
White ethnicity
Several studies have reported that AMD is more common among Whites than Blacks. Klein *et al*. reported late AMD prevalence for Blacks, Hispanics, Chinese and Whites as 0.3%, 0.2%, 1.0% and 0.6% respectively. These differences were maintained despite correction for age, gender, pupil size, BMI, smoking, alcohol consumption, diabetes and hypertension status.[32] Chakravarthy *et al*. reported a significant, positive association between White ethnicity and late AMD in one case control study (AREDS),[127] although the combined results from two cross-sectional studies (the third National Health and Nutrition Examination Survey, NHANES III and LALES) and two prospective cohort studies (the Multi-Ethnic Study of Atherosclerosis, MESA and the Cardiovascular Health Study, CHS) were not significant.[107] Europeans tend to have a higher prevalence of GA, compared to Africans, Asians and Hispanics, whereas, Asians generally have a higher prevalence of nAMD compared to other ethnicities.[2] The higher prevalence of nAMD in Asians is thought to be a consequence of polypoidal choroidal vasculopathy (PCV) being more common in these individuals. Approximately 25% of all late AMD detected in Asians from photographic grading may be PCV.[135]
Diabetes
The combined results from three prospective cohort studies (BDES, the Barbados Eye Study, BES and BMES) revealed a significant, positive association between the presence of diabetes and late AMD (RR 1.66, 95% CI 1.05-2.63), however the results from one case control study and the combined results from three cross-sectional studies were not significant.[107] The latest results from BMES indicated that fasting plasma glucose ≥ 5.6 mmol/L, or previous diagnosis or specific treatment for Type 2 diabetes was significantly associated with late AMD (p = 0.003), but not early AMD in White individuals aged 70 years and under.[136] The European Eye Study (EUREYE) reported a significant, positive association with diabetes and nAMD (OR 1.81, 95% CI 1.10 to 2.98), but not GA, suggesting a difference in pathogenesis for the two advanced forms of AMD.[137] Tromsø Eye Study, however, found no association between diabetes and GA (OR 1.92, 95% CI 0.70 to 5.28) or nAMD (OR 0.93, 95% CI 0.41 to 2.13).[138]
Lighter iris colour
A recent meta-analysis including the combined data from prospective cohort (RR 0.98, 95% CI 0.72 to 1.32), cross-sectional (OR 0.88, 95% CI 0.65 to 1.17) and case-control (OR 0.60, 95% CI 0.12 to 2.98) studies found no significant protective effect of brown versus blue irides.[107] A similar conclusion was reached by the Irish Nun Eye Study (INES). Comparing brown to blue irides for risk of any AMD revealed; unadjusted analysis (OR 0.73, 95% CI 0.44 to 1.22) and after correction for age, BMI, mean arterial blood pressure and refraction (OR 0.74, 95% CI 0.44 to 1.24). For late AMD the results were; unadjusted (OR 0.35, 95% CI 0.20 to 1.77) and adjusted for age, BMI, mean arterial blood pressure and refraction (OR 0.61, 95% CI 0.20 to 1.86). This study also reported no association between iris colour and retinal vessel caliber, and AMD status and retinal vessel caliber, after correction for age, BMI mean arterial blood pressure and refraction.[139]
Cerebrovascular disease
Cerebrovascular disease is a group of conditions that includes; stroke, transient ischaemic attack (TIA), carotid stenosis, subarachnoid haemorrhage and vascular dementia. Shared RF for cerebrovascular disease and AMD include age, hypertension, smoking and possibly AD.[140-143] Stroke, especially resulting from intracerebral haemorrhage, has been associated with nAMD.[144-147] Ocular ischaemic syndrome is associated with subfoveal choroidal thinning indicating impaired choroidal circulation.[148]
Serum total cholesterol, HDL cholesterol and triglycerides
There is some evidence to suggest that dietary fat consumption, especially saturated fat and cholesterol is associated with an increased risk of atherosclerosis and it is plausible that this may result in an increased risk of AMD, particularly nAMD.[109] Hogg *et al*. reported a significant association between nAMD and total, but not HDL cholesterol.[128] The Eye Disease Case-Control Study (EDCCS) found that compared to low (≤ 4.888 mmol / L) levels of total cholesterol, those with medium (4.889 to 6.748 mmol / L) and high (≥ 6.749 mmol / L) levels were associated with an increased risk of nAMD, (OR 2.2, 95% CI 1.3 to 3.4) and (OR 4.1, 95% CI 2.3 to 7.3) respectively, after controlling for other factors.[110] Combined data from BMES, BDES and RS also reported an inverse association between total cholesterol and nAMD (OR 0.92 per 10 mg / dL, 95% CI 0.88 to 0.99).[36] The latest results from BMES indicated that high serum triglyceride level (≥ 1.7 mmol/L) was associated with early (p = 0.009) and late AMD (p = 0.047), in a White population aged 70 years or younger. Low levels of serum HDL cholesterol (< 1.03 mmol/L in men and < 1.29 mmol/L in women) was, however, not associated with early or late AMD.[136]

1.3.4 Other relevant AMD RF

Other AMD RF relevant to this thesis include; sunlight exposure, ocular dominance, dietary MP and conditions associated with reduced or altered choroidal circulation.

Table 1.11 Other relevant AMD RF

Sunlight exposure
The Chesapeake Bay Waterman Study (CBWS) examined the association between exposure to visible and UV light over the preceding 20 years, with several ocular conditions including AMD. Pterygia and climatic droplet keratopathy, but not cataract were associated with increased ocular exposure to blue or visible radiation. Advanced AMD (GA or nAMD) was associated with higher exposure to blue or visible light (OR 1.36, 95% CI 1.00 to 1.85). No relationship was found between AMD and UV-A or UV-B.[149] The BMES reported an increased risk of GA in participants with very fair skin.[150] Fletcher reported that there is weak evidence for an association between sunlight exposure and AMD.[151] The EDCCS found no association between sunlight exposure and AMD.[110] Klein *et al*. reported that the incidence of large drusen (≥ 125 μm) and early AMD was higher for individuals with high sun exposure in their thirties (hazard ratio HR 1.38, p = 0.03) and (HR 1.25, p = 0.02), respectively, however neither was significant after adjustment for multiple comparisons. Sun exposure was not significantly associated with late AMD.[152]
Ocular dominance
Ocular dominance is not normally cited as a RF for AMD, however the dominant eye is likely to be exposed to a greater lifetime retinal light exposure as it has been reported that the non-dominant eye is closed to reduce glare when exposed to sunlight.[153] The right eye is more likely to be dominant with 65% to 71% of right eyes exhibiting ocular dominance compared to left eyes, despite the use of a variety of ocular dominance tests.[154-156] Pterygia are more likely to develop in the dominant eye.[153, 157] As with AMD, there is no consensus regarding the pathogenesis of pterygia,[158] but UV radiation from sunlight is thought to be a major factor in their development.[159] Conversely, results from the Salisbury Eye Examination (SEE) estimated that only 13% of attributable risk of cortical cataract was due to UV exposure.[160] Pterygia and pinguecula have been used as surrogate markers for prolonged sunlight exposure.[161] Pterygia were associated with a two to three-fold increase risk of early and late AMD.[162] Weak support was reported by BMES for an association between pinguecula and cortical cataract.[161, 163]
Dietary MP
Humans are unable to synthesise L, Z therefore serum levels are dependent on dietary intake.[164] Serum levels of L and Z may be increased by consuming greater amounts of dietary MP, [165, 166] or by taking MP supplements.[167] Seddon *et al*. reported from EDCCS that after controlling for known AMD confounders, the highest quintile of carotenoid intake was associated with a 43% reduction in risk for AMD compared to the lowest quintile (OR 0.57, 95% CI 0.35 to 0.92). The MP L and Z were most strongly associated with a reduced risk for AMD (p = 0.001).[168] Mares-Perlman *et al*. (NHANESIII) reported significantly less pigmentary abnormalities for non-Hispanic Whites, aged 40-59 years for those with the highest levels of dietary (but not serum levels) of L and Z, after correction for known confounders. No significant associations were found for other ethnicities (Mexican Americans or non-Hispanic Blacks) or any other age range (60-79 years and ≥ 80 years).[169] Gale *et al*. reported from a UK study that low plasma levels of Z was associated with AMD (early and late combined) (OR 2.0, 95% CI 1.0 to 4.1), whereas plasma levels of MP or L alone were not significantly associated with AMD.[170] Delcourt *et al*. (POLA) reported that early AMD was inversely associated with higher plasma levels of MP (OR 0.21, 95% CI 0.05 to 0.79), however, higher plasma levels of Z were associated with a lower risk of developing early AMD (OR 0.07, 95% CI 0.01 to 0.58).[171] Snellen *et al*. reported significantly higher prevalence of nAMD in individuals with the lowest dietary intake of L and Z (OR 5.3, 95% CI 1.5 to 18.4), after correction for known confounders.[172]
Macular pigment optical density (MPOD)
Lutein serum concentrations are correlated with MP levels in the retina.[173] Macular pigment levels can be increased by consuming greater amounts of dietary L and Z,[166, 173] or by taking MP in supplemental form.[167, 174] Bone *et al.* analysed L and Z levels from three concentric parafoveal regions (0° to 5°, 5° to 19° and 19° to 38°), from donor retinas taken from 56 eyes with AMD and 56 controls, using high performance liquid chromatography (HPLC). L and Z levels were lower in all regions for the AMD eyes. The central and paracentral MP deficit may have been partly attributable to the disease and therefore a comparison using the peripheral region was considered more reliable. In this region the highest quartile of L and Z had an 82% lower risk of AMD compared with the lowest quartile, corrected for age and gender (OR 0.18, 95% CI 0.05 to 0.64). These results suggested an inverse association between AMD risk and retinal L and Z, rather than a loss of retinal L and Z due to the destructive effects of AMD.[175]
Migraine
Migraine is a multifactorial (both biological and psychological) biobehavioural disorder,[176] and one of the most common primary headaches with a one-year prevalence of about 12%. Prevalence is three times higher in women than men and peaks in the 30 to 39 year age range.[177] Migraine with aura accounts for one third of migraine cases.[178] Interictal migraine is associated with foveal choroidal thinning thought to be associated with chronic ischaemic insult.[179] During the ictal period of the migraine the choroid has been variously described as significantly thinner,[180, 181] and significantly thicker,[182, 183] compared to interictal measurements. The reason for this inconsistency is unclear but may be an indication of ischaemia / reperfusion. Migraine is not known to be associated with AMD directly, but there is increasing evidence suggesting that migraine, especially migraine with aura, is a RF for stroke and possibly other cardiovascular disease events.[178, 184] Stroke, especially resulting from intracerebral haemorrhage, has been associated with nAMD.[144-147] Other shared RF for migraine and AMD include; increased body weight, hypercholesterolaemia, coronary heart disease, high homocysteine levels, increased levels of C-reactive protein (CRP) and oxidative stress.[185] It has been proposed that migraine attacks are characterised by ictal dopamine release in an individual with dopamine

receptor hypersensitivity due to a chronic dopaminergic deficit.[186, 187] In addition to operating as an antioxidant and neuroprotectact,[188-191] dopamine, along with melatonin may play an important role in PR OS disc shedding and phagocytosis. Shedding and phagocytosis of rod PR OS discs follows a circadian pattern with melatonin activating and dopamine inhibiting disc shedding. Melatonin-mediated rod PR OS shedding occurs primarily in the morning and is mostly completed within one and a half hours after light-onset (cone PR OS shedding occurs at light-offset in the evening). For the remainder of the day shedding and phagocytosis are suppressed by dopamine.[192] Low retinal dopamine may fail to adequately suppress rod PR OS shedding and phagocytosis, leading to an increased potential for the formation of drusen and RPD.

No previous studies examining HFP-derived MPOD in migraine sufferers was found, however migraine sufferers were reported to have significantly higher MPOD measurements using FAF (0.34 SD 0.15), compared to controls (0.20 SD 0.13, p = 0.006).[193]

Raynaud's phenomenon (Rph)

The pathogenesis of Rph is still not completely understood,[194] but has been divided into three broad pathophysiological mechanisms; vascular (nitric oxide, endothelin-1 (ET-1), angiotensin), neural (impaired vasodilation, increased vasoconstriction) and intravascular (platelet activation, fibrinolysis and reactive oxygen species, ROS).[195]

Despite the acknowledgement of Rph as a cause of reduced ocular perfusion pressure and ischaemia,[196] the author is unaware of any studies examining the association between Rph and AMD, MPOD or GRT. Shared RF for Rph and AMD include a lack of exercise, smoking, oxidative stress and raised serum homocysteine levels,[197-202] although the effects of controlling these RF on Rph have not been confirmed by randomised controlled trials.[203, 204]

Vascular dysregulation (VDys)

Vascular dysregulation may be classified as primary (pVDys) or secondary (sVDys). Patients with pVDys, formerly known as vasospastic syndrome, have an inborn difference in their response of their vascular system to cold temperature and, mechanical and physical stress.[205, 206] The prevalence of pVDys was reported to be 10% for women and 3% for men in a Swiss population, however population differences are likely.[207] Cold extremities were more commonly reported in a Swiss population; 31% of women and 7% of men.[208]

Patients with pVDys tend to be female, with symptoms manifesting at puberty and reducing with age.[208] Sufferers also tend to have low blood pressure, especially at night.[209, 210] They exhibit less desire to drink due to the anti-dipsogenic effects of prostaglandin E_2 on the hypothalamus, secondary to slightly raised levels of ET-1.[211, 212] Sleep onset is often delayed and sleep interrupted, especially if the feet are cold.[213] Systemic drug sensitivity is abnormal with pVDys cases requiring a reduced dose of some drugs (beta-blockers and calcium channel blockers) and possibly higher doses of others (e.g. painkillers).[212]

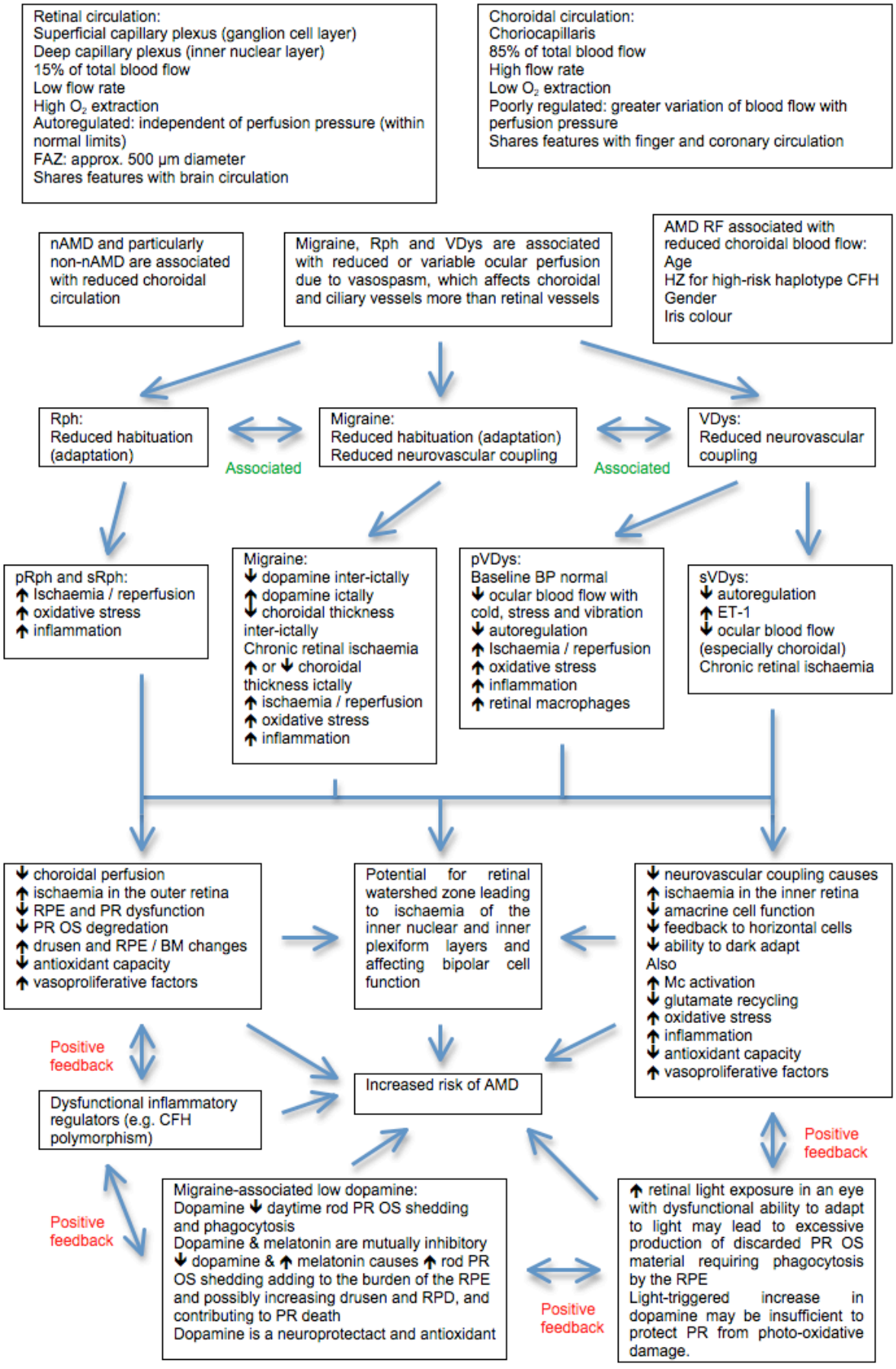
The pathogenesis of pVDys is not fully understood, but it is known that the autonomic nervous system and the endothelium of un-innervated retinal vessels are involved. Mitochondria are also involved, but it is unclear whether their influence is primary or secondary. An imbalance of ET-1 and nitric oxide (NO) is likely. Primary vascular dysregulation is associated with glial activation demonstrated by increased GFAP staining (a potent marker of retinal Mc and astrocyte activation). Retinal glial activation is a sign of retinal inflammation. Activated astrocytes are visible in red-free images by their increased light scattering, but are not visible in colour photos.[205] Activated astrocytes (resident retinal macrophages) may also be visible as one of the causes of hyper-reflective spots in SD-OCT images.[214]

Secondary VDys may result from a large number of diseases, especially those with an inflammatory aetiology,[205, 212] and results from a significant increase in circulating ET-1, which constricts vessels resulting in reduced blood flow to the eye and the kidney.[215, 216] Circulating ET-1 has little effect on the retina as long as the blood-retina barrier is intact. If the blood-retina barrier is breached ET-1 has direct access to smooth muscle cells or pericytes, leading to vasoconstriction. Central serous chorioretinopathy (CSC) characterised by accumulation of subretinal fluid secondary to a defect in the outer blood-retina barrier, is thought to be more common in individuals with pVDys.[205] No barrier exists for the choroid, therefore increased ET-1 causes reduced choroidal blood flow. Diffusion of ET-1 from the choroid to the optic nerve head (ONH) is possible in view of a "physiological barrier defect" leading to reduced ONH blood flow.[205] Abnormal communication between choroid and retina via the ONH may predispose to serous detachment and oedema of the macular retina.[217]

Dietary intervention recommended for VDys is similar to that recommended for AMD and includes plenty of fresh fruit and vegetables, especially those rich in antioxidants such as anthocyanosides or flavonoids. Black currant anthocyanins have been shown to normalise levels of ET-1 in glaucoma patients. Cocoa and other foods rich in flavonols may improve endothelial function. Omega-3 fatty acids improve vascular regulation. Magnesium may reduce the vasoconstrictive effects or ET-1. Ginkgo biloba may protect mitochondrial inner membranes against oxidative damage, an area not reachable by antioxidant vitamins.[205]

Individuals with early AMD were found to have systemic and retinal vascular alterations. Chronic inflammation, implicated in the pathogenesis of AMD, is tightly linked to diseases associated with vascular endothelial dysfunction.[218, 219] The presence of numerous positive feedback (“vicious”) cycles has been noted in the development of AMD and these also appear to feature in the potential risk of migraine, Rph and VDys for AMD (fig. 1.3 and 1.5). Feigl *et al*. reported that the post-receptoral retinal cell layers, particularly the inner nuclear layer are located in a watershed zone between the retinal and choroidal blood supplies, making these layers preferentially vulnerable to ischaemia. The authors propose that retinal ischaemia could trigger RPE dysfunction leading to the cascade of changes associated with AMD.[220]

Figure 1.3 Possible role of migraine, Rph and VDys in AMD development



Abbreviations: FAZ: foveal avascular zone, HZ, homozygous, ET-1, endothelin-1, BM, Bruch's membrane.

1.4 Potential pathogenic mechanisms of AMD

1.4.1 Normal age-related changes

Normal changes associated with ageing may be observed in the outer retina, RPE, Bruch's membrane and choriocapillaris, which are difficult to differentiate from those seen in AMD. Changes include; a reduction in the number of RPE cells, increased RPE lipofuscin (containing A2E) and decreased RPE melanin pigment granules, increased thickness, lipid content and calcification of Bruch's membrane leading to reduced nutrient transport, incorrect cellular adhesion and apoptosis, extracellular deposits around Bruch's membrane leading to chronic inflammation (release of inflammatory cytokines, angiogenic factors and immune complexes and reduced choroid and choriocapillaris thickness.[221]

1.4.2 Oxidative stress

A growing body of evidence suggests that cumulative oxidative stress contributes to the pathophysiology of AMD.[222] The macula is particularly prone to oxidative stress for a number of reasons. Foveal metabolism is one of the highest in the body.[223] Foveal cones have 10 times the number of mitochondria per unit volume compared to rods, producing considerable levels of ROS.[224] Diurnal patterns of photoreceptor OS phagocytosis are known to generate ROS.[225, 226]

Phagocytosis leads to a life-long accumulation of lipofuscin, which among other fluorophores contains A2E.[227, 228] Abnormal lipofuscin accumulation leads to the development of drusen in early and intermediate stages of AMD.[222, 229] A2E is toxic to the RPE by stimulating the production of free radical and superoxide generation upon exposure to light.[230, 231]

Proteins, lipids and deoxyribonucleic acid (DNA) can undergo lipid peroxidation under constant exposure to light or oxidative stress.[222] Docosahexaenoic acid located in the OS disc membranes of PR is particularly prone to lipid peroxidation.[228]

Free radicals are chemical particles that contain one or more unpaired electrons causing these particles to become highly reactive. Free radicals can be derived from: (a) UV light, x-ray or gamma ray exposure, (b) reactions catalysed by metals, (c) pollutants in the air, (d) neutrophils and macrophages during inflammation, (e) by-products of the mitochondrial respiratory chain, and (f) ischaemia / reperfusion-mediated tissue injury.[232, 233] Free radicals are essential for the normal function of many bodily processes.[234, 235] If the levels of free radicals overwhelm the ability of the body to regulate them, resulting from insufficient antioxidant protection or excess free radical production, damage to the body may result. This is termed oxidative stress.[235]

The retina / RPE / choroid (R/RPE/C) tissue uses a complex array of antioxidant measures to combat the various sources of oxidative stress to which it is exposed.[228, 236, 237] Some components of the R/RPE/C antioxidant system diminish with age (e.g. melanin and melatonin,[238, 239] whereas others, such as glutathione S-transferase (GST) activity increase with age. [240] Other antioxidants, including macular pigment (MP), may exhibit a complex or non-linear relationship with age.[241-244] Pro-oxidants (e.g. lipofuscin) increase with age.[245, 246] These changes result in an age-related shift in R/RPE/C reduction / oxidation (redox) state in favour of pro-oxidant activity.[235] Chronic inflammation is associated with increased levels of free radicals and will shift redox balance further towards pro-oxidant activity.[247] (See appendix A2.1 for a summary of the R/RPE/C antioxidant system).

1.4.3 Lipofuscin accumulation

AMD is characterised by an accumulation of intracellular lipofuscin and extracellular drusen.[230, 248] The efficiency of the RPE to recycle products of phagocytosis is reduced by oxidative stress,[230, 249, 250] resulting in increased levels of undegradable, intracellular lipofuscin,[248] and higher amounts of incompletely degraded material outside the RPE as drusen.[251] RPE lipofuscin accumulation in AMD has been compared with lipofuscin-like deposits that occur with other neurodegenerative diseases, such as AD (β-amyloid or τ-protein), Huntington disease (huntingtin protein), Parkinson's disease (Lewy bodies) and amyotrophic lateral sclerosis (non-amyloid aggregates).[248, 252, 253]

Increased levels of RPE lipofuscin result from lipid peroxidation secondary to increased oxidative stress,[248] and lipofuscin may itself induce oxidative damage in the RPE and surrounding tissues,[108] therefore it is feasible that a self-perpetuating positive-feedback cycle may result with oxidative stress increasing RPE lipofuscin and *vice versa*. The development of drusen is likely to be influenced by genetic predisposition or environmental stressors, which would explain the absence of drusen in some elderly patients.[254]

1.4.4 Inflammation

Early / intermediate AMD is associated with activation of resident immune cells in the retina (RPE, Mc, microglia and macrophages) and choroid (peri-capillary macrophages and giant cells).[222, 255] The complement system forms part of the non-adaptive, innate immune system, although it can be recruited by the adaptive immune system. The complement system consists of a group of three primitive enzymatic cascades (classical, lectin and alternative), with a shared final pathway forming the membrane attack complex (MAC) leading to increased capillary permeability, cell lysis and inflammation by cytokine release.[222, 229, 248, 256]

In the eye, the complement system is continually activated at low levels and is regulated by intraocular complement regulatory proteins (CD35, CD46 and CFH).[257] Complement factor

H is a natural inhibitor of complement component 3 (C3) convertase and impedes activation of the alternative complement pathway.[229]

Oxidative stress in the RPE can activate the complement pathway.[258] In the presence of detrimental complement gene polymorphisms including; CFH, complement factor B (CFB), complement component 2 (C2) and complement component 1 (C1),[229] this may lead to a failure to modulate complement response leading to the formation of MAC and other pro-inflammatory responses related to AMD progression.[222] Complement factor H polymorphisms account for approximately 50% of AMD cases in the United States. This finding supports the involvement of inflammation and complement activation in AMD pathogenesis.[256]

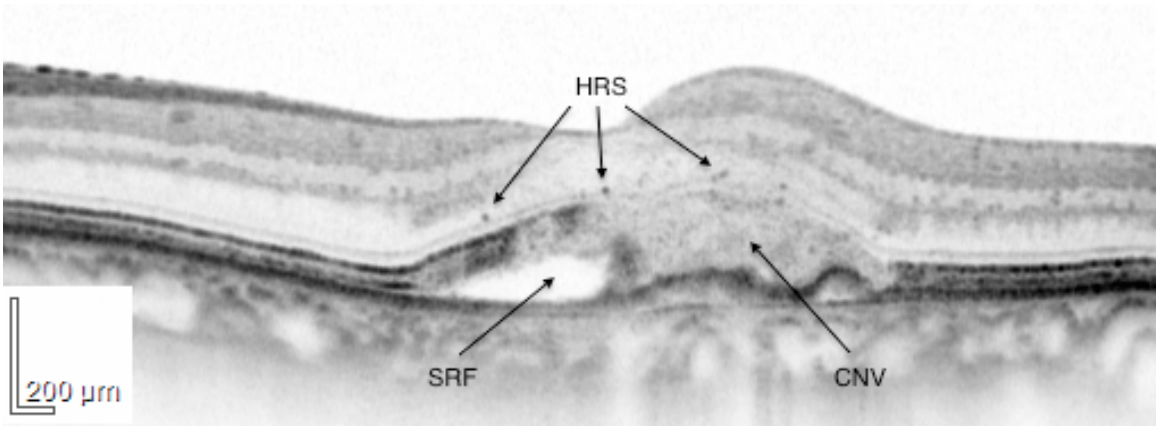
The inner and outer blood-retinal barriers contribute to the retinal status as an immune privileged site.[259, 260] The privileged status is preserved by local active mechanisms with an immuno-modulatory role that suppress responses to antigens.[259, 261] Abnormal chronic inflammation is thought to contribute to AMD development and progression.[257, 259] The source of this abnormality may include the loss of immune privilege due to increased capillary leakage and the presence of detrimental complement polymorphisms.

Para-inflammation represents an intermediate stage between basal and robust levels of inflammation, indicating an attempt by the affected tissue to maintain homeostasis. Para-inflammation is associated with increased expression of the anti-inflammatory cytokine, interleukin-10 (IL-10) and shifts in the polarity of the macrophage population from the pro-inflammatory (M1) to the anti-inflammatory (M2) subtype.[221] Drusen contain plasma proteins that are up-regulated during an inflammatory response.[57] Patients with Dercum's disease, as well as those with obesity were also found to exhibit a low-grade inflammatory response associated with an increased density of macrophages.[262] (See fig. 1.4).

1.4.5 Mitochondrial function

Mitochondria contribute to the ageing process by their accumulation of mitochondrial DNA (mtDNA) mutations and net production of ROS.[252] The level of mtDNA damage in RPE tissue preceding significant AMD changes was greater than that found for normal ageing, suggesting a role of mtDNA damage in AMD pathophysiology.[263] Mitochondrial dysfunction due to mtDNA lesions is evident as severe disruptions to mitochondrial cristae structure and a decrease in the number of mitochondria in donor RPE tissue from AMD cases.[264] Potential mechanisms causing increased mtDNA damage associated with AMD include; oxidative stress from ROS, reduced mtDNA repair and decreased mitochondrial autophagy. Autophagy is the mechanism for lysosomal elimination of damaged cellular components.[263]

Figure 1.4 Suspected lipid-laden macrophages indicated by hyper-reflective spots in a case of confirmed nAMD using spectral domain OCT (SD-OCT)



HRS: hyper-reflective spots (hyper-reflective spots appear hypo-reflective in reverse-contrast), SRF: subretinal fluid, CNV: choroidal neovascularisation. Heidelberg Spectralis SD-OCT image in reverse-contrast. (© Everett, 2014).

1.4.6 Lipid-related factors

Apolipoprotein E (ApoE) is a multifunctional glycoprotein constituent of very low-density lipoprotein (VLDL) involved in cholesterol transportation by facilitating the binding of lipoproteins to LDL receptors.[256] In the eye, ApoE immunoreactivity is associated with RPE, photoreceptor OS, Bruch membrane, Mc and the retinal ganglion cell layer, and is a ubiquitous component of all types of drusen.[265]

Apolipoprotein E exists in three major allelic variants (ε2, ε3 and ε4). ε2 is associated with increased risk of AMD, whereas ε4 may confer some protection by suppressing expression of VEGF and the chemokine (C-C motif) ligand 2 (CCL2).[256, 266] Apolipoprotein E polymorphism is associated with circulating CRP, a marker for infection and inflammation. CRP levels were higher for ε2 variations and lower for ε4 variations, although this was not considered to be related to inflammation.[267] The high-density lipoprotein pathway is also associated with AMD, with variants in the hepatic triglyceride lipase (LIPC) gene conferring a protective effect against intermediate and large drusen as well as advanced AMD.[256, 268]

1.4.7 Müller cell dysfunction

Müller cell dysfunction leads to a reduction in their supportive and protective role in the retina and may lead to or exacerbate changes resulting in AMD.[269, 270] (See section 1.2 for the main Mc functions). Müller cells overlying and immediately adjacent to drusen showed increased immunoreactivity to GFAP and vimentin.[271] Amphibian studies have shown that targeted disruption of Mc metabolism led to dysmorphogenesis of photoreceptor OS membranes.[272] In murine studies Mc changes also preceded photoreceptor degeneration.[273]

Pseudocysts commonly associated with GA in humans may be caused by Mc degeneration.[274] Müller cell activation is associated with increased levels of retinal glutamate leading to retinal excitotoxicity.[269, 275, 276] Activation of Mc is also associated with a reduction in reduced L-glutathione (GSH) production, which reduces retinal protection against oxidative stress,[269, 276] and increased levels of proangiogenic factors and proinflammatory factors contributing to neovascular and inflammatory triggers.[270] Muller cells are sensitive to damage by light in animal studies,[277-279] and are susceptible to lipid peroxidation.[280]

1.4.8 Early AMD

Retinal pigment epithelial cells are phagocytically the most active cells in the body, phagocytosing up to 10% of PR OS length each day in a process termed heterophagy. Each RPE cell services 30-40 PR and is therefore an enormous challenge for the RPE endolysosomal system required to degrade ingested PR OS. Cell death of RPE cells puts additional strain on neighbouring RPE cells, leading to an age-related accumulation of lipofuscin in RPE cells, RPE dysfunction and possibly further cell death. The following is known about the mechanism involved in PR OS heterophagy; The integrin ITGAV-ITGB5 is needed to bind OS, MERTK triggers their ingestion, protein tyrosine kinase 2 links signals between ITGAV-ITGB5 and MERTK, MFGE8 regulates circadian rhythm of heterophagy (rods in the daytime and cones at night). An age-related decrease in ITGAV-ITGB5 leads to RPE lipofuscin accumulation and reduced retinal adhesion.[281] It is plausible that dysfunctional heterophagy is responsible for SDD observed subretinally.

Drusen are thought to develop as a result of lysosomal dysfunction leading to the accumulation of higher than age-normal levels of lipofuscin (containing oxidised low-density lipoproteins and lipid peroxidation end products) within RPE cells. Once formed, lipofuscin cannot be degraded by proteasomal or lysosomal enzymes, or become transported into the extracellular space. Lipofuscin accumulation and mitochondrial damage lead eventually to RPE degeneration. Reduced levels of autophagy (specifically macroautophagy), which involves the formation of autophagosomes (double membrane vesicles), which combine with lysosomes and degrade their contents with several acid hydrolases, is a consequence of age and has been implicated in AMD pathogenesis. Reactive oxygen species produced by light-stimulation of A2E were reported to have a detrimental effect on autophagosome creation.[282]

The RPE lysosomal component βA3/A1-crystallin has been found in human drusen material and may regulate autophagy and heterophagy. Nuc1 rats (a mutation associated with Mc activation and suppression of programmed cell death in the developing retina) with a spontaneous mutation in the Cryba1 gene (which codes for βA3/A1-crystallin) develop deposits between the basal lamina of the RPE and the inner collagenous layer of Bruch's

membrane during normal ageing. This is a possible mechanism for the development of drusen.[281] Drusen contain many pro-inflammatory factors (e.g. Apo-E, coagulation and acute phase proteins, IgG, complement components and activators), which in the presence of gene polymorphisms governing immune regulation (e.g. CFH) leads to chronic inflammation and oxidative stress. If the ocular redox state is insufficient to deal with this, development or progression of AMD may result.

Thompson *et al*. have proposed a novel mechanism for the growth and possibly the formation of sub-RPE drusenoid deposits. Cholesterol-containing lipid droplets, a few micrometers across located beneath the RPE in Bruch's membrane become coated in insoluble hydroxyapatite (HAP) (a form of calcium phosphate and the most abundant form of calcium in the human skeleton). These hollow HAP spherules then act as the initial binding site for more protein and lipids including; CFH, vitronectin and β-amyloid. Thousands of these microscopic HAP spherules (0.5-20 μm in diameter) were found in large drusen regardless of their retinal location (macular or peripheral retina). These HAP spherules were also found outside the sub-RPE deposits, ready to bind with proteins at the RPE-choroid interface. These calcium deposits were distinct in form and location from the well known age-related calcification of the elastin layer of Bruch's membrane.[283]

Microscopic hydroxyapatite crystals are also found in the joints of individuals with hydroxyapatite crystal deposition disease, characterised by chronic joint inflammation and joint pain.[284] Hydroxyapatite crystals have also been implicated in the aetiology of carpel tunnel syndrome.[285] If there is an association between ocular and joint HAP deposits this may, in part, explain the weak association between hand grip strength and AMD in men.[286]

Focal hypopigmentation can occur independently of drusen and represent either loss of RPE cells or reduced intracellular melanin granules. Focal hyperpigmentation represents changes at RPE level, due to either; increased melanin content and / or proliferation of RPE cells, or migration of pigment-containing cells (RPE, macrophages and Mc that have phagocytosed melanin) into the neurosensory retina.[56, 287] Melanin scavenges free radicals, works as a weak antioxidant (against iron and copper ions) and protects against iron ion-induced lipid peroxidation (although ability reduces with age). Melanin's redox cycle is regenerated with ascorbic acid (vitamin C). Melanin levels reduce significantly with age. Photo-degradation of melanosomes resulted in a loss of antioxidant properties, while their ability to deactivate cationic photosensitisers was preserved.[238]

Age-related macular degeneration has been associated with impaired iron metabolism leading to an accumulation of iron within the RPE. The RPE cells of individuals with AMD have approximately five times the concentration of total iron compared to age-matched

normal RPE cells. It is plausible that age-related changes in melanosomes may contribute to RPE dysfunction and AMD development.[238]

Decreased choriocapillaris density was observed in early AMD. Biesemeier *et al*. noted a 27% loss of choriocapillaris in areas of intact RPE in early AMD.[288] Choriocapillaris ghost vessels (acellular choroidal capillaries) were associated with basal linear deposits (diffuse drusen) and SDD.[70] Whitmore *et al*. reported a direct relationship between choriocapillaris ghost vessels indicating lower vascular density and overlying drusen, with the former likely to be the cause of the latter.[61]

1.4.9 Geographic atrophy

Death of RPE cells is accompanied by a loss of overlying PR, atrophy of the choriocapillaris and hypopigmentation. Geographic atrophy may occur after the collapse of PED, with drusen regression and in areas of hypo- and hyperpigmentation. It is assumed that RPE death is the driving force behind the progression of GA, but PR loss and choriocapillaris thinning have been observed outside the atrophic area (see previous section). The factors leading to RPE dysfunction and death were described in the previous section, but essentially are thought to be a consequence of excessive lipofuscin accumulation, hypoxia and oxidative stress, partly from photo-oxidation of A2E, causing an inflammatory response that overwhelms the RPE, causing eventually, apoptosis.

The death of RPE cells places an additional metabolic load on neighbouring RPE cells, which may also be dysfunctional and subsequently, result in their death also. This progressive loss of RPE cells in an outward, "slow-burn" manner is characteristic of the progression of GA. The amount of FAF (which represents the amount of lipofuscin) bordering the area of GA has been reported to correlate with the speed of progression of GA, with a greater amount of rim area focal hyperfluorescence being positively correlated with speed of progression.[84, 289]

It is plausible that cells with more lipofuscin would be under greater stress and more likely to become dysfunctional and apoptotic more quickly. Hwang *et al*. reported that the predictive value of increased FAF for progression of GA was little different to that expected by chance.[290] Hopkins *et al*. have expressed caution regarding the use of FAF as a non-invasive marker for GA progression because of the lack of confirmation of a causal relationship between RPE lipofuscin accumulation and AMD. The authors have recommended harmonisation of FAF terminology and image acquisition methods, as well as, the development of a disease database, a universal classification system and algorithms for the correct interpretation of FAF patterns.[291]

In contrast with nAMD, which is associated with inflammatory cells, GA has not been associated with a marked recruitment of leukocytes from the circulation (because the blood-retina barrier is preserved). Geographic atrophy has been associated with activation of cells already present in the retina including; microglia, Mc, RPE cells and occasional macrophages. Choroidal cells associated with GA include; peripheral immune cells mainly pericapillary macrophages, giant cells and mast cells.[73]

Different end stages of AMD exhibit different rates of loss of RPE and choriocapillaris. Choriocapillaris was thin but viable despite RPE loss in GA, whereas, a loss of choriocapillaris endothelial cells was observed beneath the intact RPE in cases of nAMD.[61]

1.4.10 Neovascular AMD

Neovascular AMD is characterised by defects in Bruch's membrane and / or the outer blood-retina barrier, while RPE cells are maintained. The direct pathological contact between RPE and vascular endothelial cells can enhance the pro-angiogenic potential of the endothelial cells to proliferate and migrate, similar to the process induced by hypoxia.[282] The two major pathways by which VEGF (VEGF-A) is produced and secreted in RPE cells are; in response to complement, and as a result of oxidative stress. If the complement system is not properly regulated (e.g. gene polymorphisms in complement regulators such as CFH), then activation of complement proteins can damage host tissue and recruit immune cells to the affected tissue.

Macrophages (resident microglia and migratory choroidal monocytes) are thought to be involved in nAMD pathogenesis, indicated by the presence of hyper-reflective spots on SD-OCT images of nAMD and by higher serum levels of CRP and the cytokine interleukin-6 (IL-6) in individuals with nAMD.[292] Infiltration of migratory macrophages signifies a break down of immune privilege, leading to a more extreme inflammatory response than the eye is equipped to deal with. Chronic inflammation or para-inflammation are features of several AMD risk factors; age, smoking and obesity. Oxidative stress results from a state of para-inflammation.[293] The effect of oxidative stress is additive to the effects of complement activation.[248]

In humans, reactive Mc are known to extend processes through gaps in Bruch's membrane, along which retinal neurons migrate out of the retina and into the choroid,[294] it is therefore feasible that activated Mc may be an additional source of VEGF and other pro-angiogenic factors leading to CNV in the choroid and RAP in the retina.[295] Reactive Mc may also contribute to retinal oedema[294] and reduced blood-retina barrier integrity,[276] other characteristics of nAMD.

Laser induction leading to fragmentation of Bruch's membrane in animal studies,[296, 297] including primate,[298, 299] is known to trigger the formation of CNV. The neovascular response to laser induction of Bruch's membrane was reported to be greater in the macular area, with little response from nasal or peripheral locations.[298] Invasion and secretion of VEGF by neutrophils was cited as a possible mechanism for laser-induced CNV.[297] Remodeled processes of reactive Mc that extend through gaps in Bruch's membrane in human subjects with AMD are another possible source of angiogenic factors.[294]

Defects in adhesion between RPE and Bruch's membrane due to lipid accumulation in Bruch's, with normal RPE junctions PR OS to RPE adhesion leads to early sub-RPE CNV. Reduced PR OS to RPE, or RPE to RPE adhesion secondary to inflammation (despite normal adhesion between RPE basement membrane and Bruch's) leads to early subretinal CNV. Simultaneous reduction in RPE to RPE epithelial binding and RPE to Bruch's adhesion may lead to either subretinal or sub-RPE CNV, which often progresses to a combined pattern of CNV. Small holes in Bruch's membrane and normal epithelial junctions and cellular attachments were not associated with the development of CNV.[300]

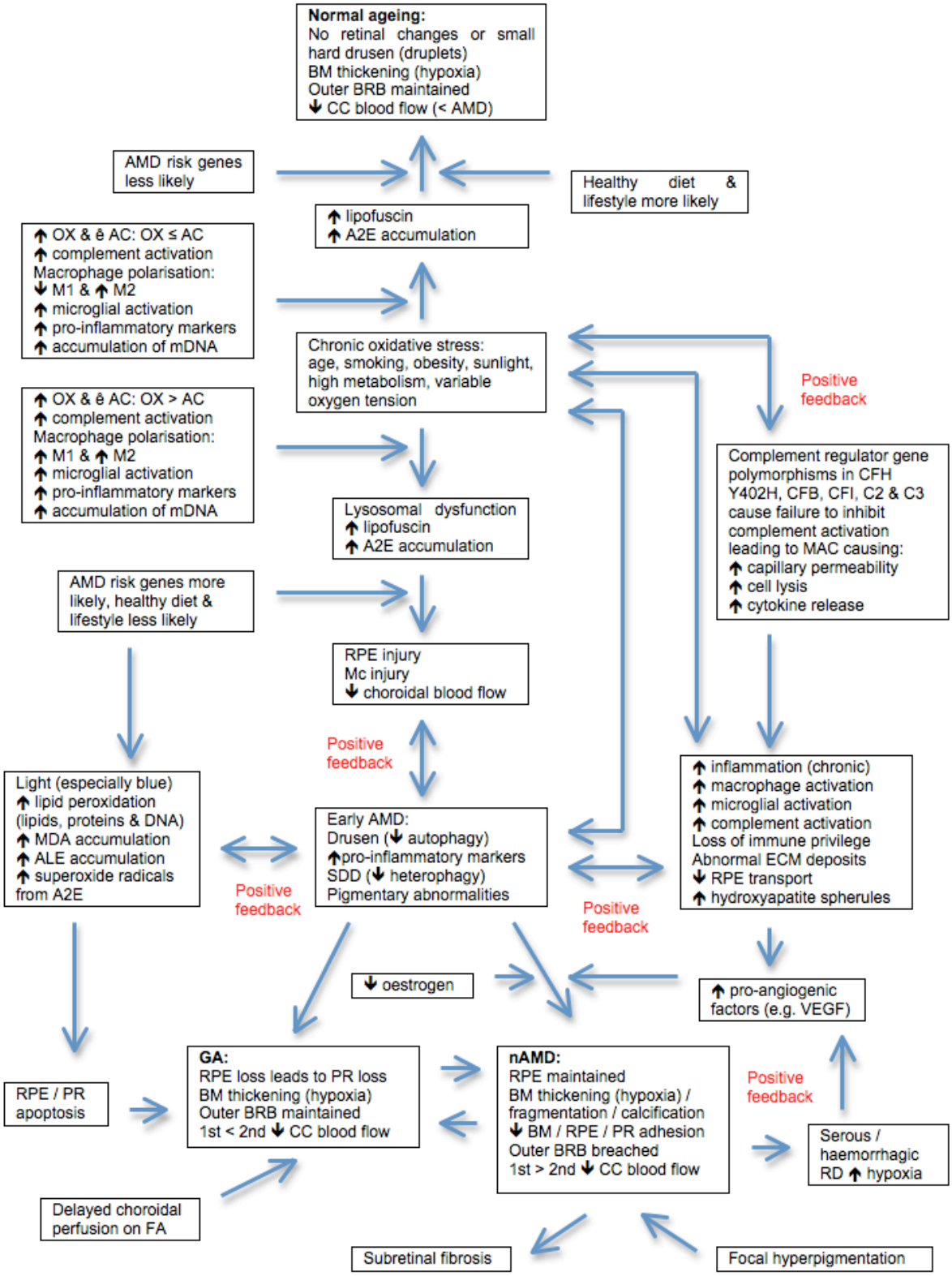
The link between pathological changes in the RPE and the development of choroidal new vessels is the result of a multifactorial interplay between oxidative stress, hypoxia and autophagy in nAMD pathogenesis. Positive feedback ("vicious") cycles represent mechanisms or processes that once started and allowed to reach a critical level may escalate beyond the ability of the eye to control them, leading eventually to changes associated with AMD. These mechanisms, including the presence of positive feedback cycles between them have been summarised in fig. 1.5. Further positive feedback cycles were illustrated in fig. 1.3.

Although a full explanation of the cause of AMD is lacking, current evidence suggests that AMD has a multifactorial aetiology, affected by ocular redox state, multiple genes as well as environmental factors. There is evidence that AMD stages may be selectively influenced by different environmental and genetic RF.

Smoking increases early AMD risk, whereas expression of the ABCA1 (HDL) gene is associated with lower risk of intermediate and large drusen. Late AMD is positively associated with detrimental complement and ARMS2 / HTRA1 gene polymorphisms, cardiovascular disease and raised cholesterol.

Past and current smoking and LIPC SNPs were associated with nAMD. ARMS2 / HTRA1 was associated with the size of the neovascular lesion. Obesity was associated with increased risk of conversion from large drusen to GA.[123, 124]

Figure 1.5 Summary of normal ageing and possible AMD pathogenesis



Abbreviations: A2E: N-retinylidene-N-retinylethanolamine, OX: oxidative stress, AC: antioxidant capacity, M1 & M2: pro- and anti-inflammatory macrophage subtypes, CFH, CFB and CFI: complement factors H, B and I, C2 and C3: complement components 2 and 3, mDNA: mitochondrial DNA, MAC: membrane attack complex, ECM: extracellular matrix, MDA: malondialdehyde, ALE: advanced lipid peroxidation end products, SDD: subretinal drusenoid deposits, BM: Bruch's membrane, CC: choriocapillaris, BRB: blood-retina barrier, 1st: primary, 2nd: secondary, FA: fluorescein angiography, RD: retinal detachment.
Major references used for this summary: Zarbin (2004),[108] Stefánsson *et al*. (2011),[301] Bhutto *et al*. (2012),[257] Ardeljan *et al*. (2013),[221] Shin *et al*. (2013),[256] Kaarniranta *et al*. (2013),[281] van Lookeren *et al*. (2014)[222] and Kaarniranta *et al*. (2015).[302]

The identification of positive feedback ("vicious") cycles between mechanisms provocative for AMD development may determine the progression to early and advanced stages of AMD, rather than changes associated with simple ageing. The effect of epigenetics must be considered as the genetic risk of progression to late AMD associated with CFH Y402H may be multiplicatively increased by non-genetic factors such as smoking.[303, 304]

1.5 Visual consequences of AMD

In order to understand the visual consequences of AMD it is important to comprehend how AMD affects the many different components that make up the visual experience. The following references were major sources of information for this section; Sunness,[305] Ivers et al,[306] Lovie-Kitchin and Feigl,[307] Hogg et al,[95] and Neelam et al.[308]

Table 1.12 The visual consequences of AMD

Visual acuity
Early AMD (hard / soft drusen plus pigmentary changes or soft indistinct drusen) was associated with a significant drop in LogMAR distance visual acuity (VA) of two or less letters, compared to individuals without AMD.[309] This result is not, however, clinically meaningful as a test for early AMD or reliable as test-retest variability (limit of agreement, LoA) with LogMAR testing is 1-2 lines of letters.[310] Late AMD was associated with a drop in VA of 6-8 lines depending on the absence or presence of central cataract, but only when late AMD was located in the centre or inner subfields of the WARMGS macular grid.[309] Geographic atrophy progresses slowly and VA gradually worsens over time. Visual acuity is a poor indicator of GA severity because it is unrelated to the extent of the atrophic area and the amount of functional deficit.[308] Visual acuity was found to be a poor predictor for conversion to nAMD.[311] Hogg *et al*. reported that most of the variation in VA in individuals with nAMD was associated with the following changes; fibrosis, atrophy, exudates and blood, with foveally-located fibrosis particularly affecting VA.[312] Visual acuity is a poor measure of AMD because it specifically assesses the fovea, whereas AMD is a condition that is characterised by foveal sparing, at least until the late stage of the disease.
Metamorphopsia
Early AMD may present as subtle distortion on the Amsler grid in the absence of spontaneously reported distortion being reported as a symptom, but Amsler distortion is not a reliable indicator of early AMD. Geographic atrophy may present as a loss of vision affecting the retinal location affected by the lesion, but not in every case. Neovascular AMD characteristically presents as a sudden loss of vision or sudden onset of, or increase in distortion, which is usually detectable on the Amsler grid. Individuals with nAMD were 3.65 times more likely to report metamorphopsia than those with dry AMD (it was not clear whether the authors are referring to GA and early AMD, or just GA) and was more likely to be centrally-located.[313] Metamorphopsia is also detectable by observing any straight line, e.g. a window frame.[314]
Visual fields
The results of studies examining early and late AMD with perimetry have produced mixed results. Several studies have detected parafoveal defects,[315-317] whereas another reported no significant difference compared to age-matched eyes.[318] Midena *et al*. reported that visual field sensitivity was not significantly decreased with hard drusen or fine pigmentary changes (this retinal appearance may now be classified as normal ageing changes rather than early AMD),[318] whereas, Tolentino *et al*. found a significant association between visual field sensitivity and area of RPE atrophy, but not with the area of drusen.[319]
Dark adaptation
The majority of studies have reported that AMD is associated with prolonged dark adaptation (DA), specifically; cone-mediated DA,[320-322] rod-mediated DA,[320, 322] and generally.[323, 324] One study found no significant difference in DA between individuals with AMD and those without.[325] This study used the Scotopic Sensitivity Tester-1 (SST-1) which uses full-field stimulation, therefore it is possible that macular dysfunction was masked by the influence of the peripheral retina. In monocular nAMD cases with fellow eyes exhibiting high-risk features for progression (large drusen, more than minimal drusen confluence and focal hyperpigmentation), prolonged, foveal photopic DA (cone PR) combined with colour matching abnormalities was the most effective predictor for subsequent development of nAMD, although neither measure alone was significantly predictive.[321] Ioannis *et al*. confirmed that DA was prolonged in eyes with nAMD and their fellow eyes with dry changes ranging from early AMD to GA.[326] Abnormal DA (> 45 min) after > 95% retinal bleach was also found in 90% of fellows eyes (with drusen only) of monocular cases of serous PED or RPE tear, compared to 60% of nAMD without PED, 60% of drusen only and 9% normal eyes. Dark adaptation was significantly longer for fellow eyes of monocular serous PED or RPE tear, but not for the other two groups, compared to normal eyes.[327] Overall, DA was significantly prolonged with AMD and in fellow eyes of unilateral nAMD or, serous PED or RPE tear, but was also prolonged in a proportion of normal eyes. Significantly prolonged DA may be a marker for RPE / PR separation, an indication of reduced cellular adhesion, a consequence of Bruch's membrane hydrophobicity due to lipid deposits or abnormal choroidal perfusion.[327] The significance of prolonged DA in normal eyes for progression to AMD requires more data from longitudinal studies, but may be a particular risk for the nAMD phenotype.

GRT
GRT may be used to differentiate macular or retinal disease, associated with longer recovery times, from neural or optic nerve disease in patients with reduced VA of unknown cause.[328, 329] When compared to other markers of retinal function such as static and dynamic contrast sensitivity, and mean central (10°) retinal sensitivity, GRT appeared to be the most sensitive indicator of retinal damage.[315] Equilibrium bleach GRT caused by the continuous exposure of light for a short period of time (usually between 5-s and 2-min depending on the study) is associated with longer GRT in individuals with AMD in the majority of studies (table 1.13). The results for photo-flash GRT, resulting from a very brief exposure to a high luminance light source are less consistent for AMD. The reason for this is likely to be that equilibrium bleach places a greater stress on the RPE whereas, photoflash bleach recovery is more greatly influenced by the cone-specific Mc visual cycle.[330] Sandberg *et al*. reported that longer equilibrium bleach GRT (RR 1.30, 95% CI 1.10 to 1.54) and the extent of visible macular abnormalities (RR 1.62, 95% CI 1.06 to 2.59) were independent RF for the development of nAMD in fellow eyes of those with nAMD.[126]
Spatial contrast sensitivity
Spatial contrast sensitivity (SCS) may be measured in clinical practice using the Pelli-Robson chart. Spatial contrast sensitivity function reduces with age over a wide range of luminances, for medium and high spatial frequencies, with peak sensitivity shifting towards low spatial frequencies.[331] Spatial contrast sensitivity, especially at high spatial frequencies was reduced in the presence of confluent drusen and, focal hyperpigmentation and atrophy of the RPE.[315] It was not considered that SCS has any predictive value for the future development of nAMD.[315, 332] Compared to normal individuals, those with AMD demonstrated a relative loss of SCS function at photopic and mesopic luminances compared to scotopic luminances, which was attributed to compromised adaptation.[333] Adaptation is thought to be mediated via blue light stimulation of intrinsically photosensitive retinal ganglion cells (ipRGC).[334-336] High spatial frequencies are reduced with age and with fixation away from the fovea and will therefore be affected by centrally-located macular lesions and eccentric fixation. Reduced SCS may reflect AMD progression, but is not predictive for future change in AMD. Reduced SCS may, however, reflect difficulties with day to day living, reading, facial recognition and driving that may be less apparent with VA testing.[308]
Temporal contrast sensitivity
Flicker sensitivity may be a particularly suitable test for individuals with AMD because temporal resolution is relatively unaffected by age or optical blur.[337, 338] The opponent (chromatic) and non-opponent (luminance) systems detect flicker at high and low temporal frequencies, respectively.[311] Functional changes in individuals with AMD may be detected earlier with flickering compared to static stimuli, as a consequence of neurovascular coupling.[339] Flickering lights induce parallel increases in neural activity and in retinal blood flow (by 30% in the case of monochromatic flicker).[340] Choroidal blood flow may be particularly reduced in AMD.[341] As a consequence of this, the metabolic demand imposed by flicker on neural tissue in the outer retina may not be matched by the choroidal circulation. It is also plausible that increased retinal circulation resulting from neurovascular coupling may further reduce choroidal blood flow, exacerbating the functional deficit in AMD.
Colour vision
Early AMD was associated with defective colour vision in most, but not all studies.[308] Colour defects associated with early AMD tended to be blue-yellow (tritan) defects, consistent with Kollner's rule that lesions at the level of the receptors or in the pre-retinal media, are more commonly associated with tritan defects. There is an enormous loss in the ability to recognise tritan optotypes in early AMD and this was related (Spearman's rank order correlation) to the severity of morphological retinal changes in AMD,[342] and colour contrast sensitivity along tritan, but not protan or deutan confusion lines worsened in individuals who developed late AMD over a 2-year follow-up period.[343] Abnormal colour matching (combined with slow DA) at detecting high-risk fellow eyes of monocular nAMD cases.[324] In AMD the S-cone system appears to be the most vulnerable to damage for several reasons: photoreceptor density is less for S-cones, compared to M- and L-cones, S-cone receptive fields do not overlap, S-cones are more sensitive to RPE changes as photopigment recycling requires a longer diffusion distance compared to M- and L- cones and are responsible for absorbing lower light frequencies that are more actinic, and therefore potentially more damaging than that absorbed by the two other cone types.[308]
Blindness and visual impairment
Age-related macular degeneration is the most frequent cause of severe, irreversible visual impairment in the developed world,[43] and the third most frequent cause of visual impairment and blindness, globally, after cataract and glaucoma (excluding refractive error).[344] In 2010, it was estimated that there were 32.4 million blind individuals (VA < 3/60) and 191 million vision-impaired individuals (VA < 6/18, ≥ 3/60). 2.1 million (95% uncertainty interval, UI 1.9 to 2.7) individuals were blind and 6.0 million (95% UI 5.2 to 8.1) were vision-impaired due to macular diseases. Between 1990 and 2010 the number of individuals who were blind or vision-impaired due to macular diseases increased by 36% and 81%, respectively, whereas, the global population increased by 30%.[345]

1.5.9 Consequences of visual loss

Early AMD may be visually asymptomatic or there may be reduced VA, distortion, loss of colour perception, prolonged dark DA and reduced sensitivity to high spatial frequencies. Geographic atrophy produces a paracentral scotoma initially in most cases that slowly enlarges to include foveal vision. There is no current medical treatment for GA. Vision loss and distortion associated with nAMD is sudden-onset and rapidly progressive, but is treatable and to a degree reversible in most cases with intravitreal VEGF inhibitors. Central visual loss may be associated with eccentric viewing, using a less or unaffected, para-

Table 1.13 The association between AMD and GRT

Condition or Environmental Factor	Reference	GRT Method	Effect on GRT	Notes
AMD	Chilaris *et al.* (1962)[346]	Equilibrium	Longer	Equilibrium bleach GRT was prolonged in 13 out of 15 studies examining subjects with AMD. Forsius *et al*. found no increase in GRT with AMD, except in one case with CNV, using the Keeler direct ophthalmoscope. This may be due to the long working distance (30 cm) and short glare duration (15 s) used by this study. Significant positive associations were found between equilibrium GRT and RPE pigment changes,[315, 317] and drusen number and confluence,[315] however, Smiddy *et al*. reported no association for drusen severity.[350] Longer GRT was an independent RF for the development of nAMD in fellow eyes of unilateral nAMD cases.[126, 353] The association between photo-flash GRT and AMD is controversial.
	Severin *et al.* (1963)[347]	Equilibrium	Longer (+fellow)	
	Forsius *et al*. (1963)[348]	Equilibrium	None	
	Glaser *et al*. (1977)[349]	Equilibrium	Longer	
	Smiddy *et al*. (1984)[350]	Equilibrium	None (drusen)	
	Brown *et al*. (1986)[320]	Equilibrium	Longer	
	Collins *et al*. (1989)[351]	Equilibrium	Longer	
	Wu *et al*. (1990)[352]	Equilibrium	Longer	
	Cheng *et al*. (1993)[317]	Equilibrium	Longer	
	Sandberg *et al*. (1995)[353]	Equilibrium	Longer (fellow)	
	Midena *et al*. (1997)[315]	Equilibrium	Longer	
	Phipps *et al*. (2003)[354]	Equilibrium	Longer	
	Binns *et al*. (2007)[355]	Equilibrium	Longer	
	Dhalla *et al*. (2007)[356]	Equilibrium	Longer	
	Dimitrov *et al*. (2011)[357]	Equilibrium	Longer	
	Schmitt *et al*. (2003)[358]	Photo-flash	None	
	Bartlett *et al*. (2004)[359]	Photo-flash	Longer	
	Wolffsohn *et al*. (2006)[360]	Photo-flash	None	
	Newsome *et al*. (2009)[361]	Photo-flash	Longer (GA & nAMD)	

foveal retinal location in an attempt to improve vision. Visual tasks require a complex combination of many ocular and cortical parameters. Tasks commonly affected by vision loss due to AMD have been listed below.

Table 1.14 Summary of the consequences of visual loss

Facial and expression recognition
Facial recognition is a complex resolution task involving sensory input from visual, non-visual and memory cues. Expression recognition may be considered to be a relatively less complex and more visual task. Facial and expression recognition were significantly associated with distance and reading VA, but not contrast sensitivity or colour vision.[362, 363] The importance of higher spatial frequencies for facial recognition has been reported,[364, 365] however the reduction in high spatial frequencies associated with AMD does not appear to affect facial recognition significantly.[364, 365]
Driving
Sengupta *et al*. reported that 80% of individuals with AMD (bilateral drusen, GA or nAMD, 47% had GA and 53% had central foveal scarring from nAMD in the better seeing eye, and VA ≤ 6/9.5 in both eyes or < 6/60 in one eye) continued to drive at 2-year follow-up. Twenty five percent of individuals with central vision loss due to AMD ceased driving completely. Driving cessation was significantly associated with reduced VA in the better seeing eye (OR 1.5, per 0.1 LogMAR reduction in VA; 95% confidence interval, CI 1.2 to 1.9) and reduced SCS (OR 1.4, for each 0.1 decrement in log SCS; 95% CI 1.1 to 1.7), although only VA was significantly associated with driving cessation in a multivariate model. The type of AMD (nAMD vs. non-nAMD was not associated with driving cessation.[366]
Reading difficulty
It has been reported that central visual loss associated with AMD, and to some extent cataract is associated with a relatively more detrimental effect on reading and other near tasks, compared to the more peripheral visual loss associated with chronic open-angle glaucoma which has greater impact on balance, walking and driving.[367] Ivers *et al.* reported that although a reduction in best corrected VA (per 2-line / 10-letter reduction) was significantly associated with difficulty reading the newspaper (OR 2.8, 95% CI 2.4 to 3.4), distance tasks were similarly affected by reduced best corrected VA; difficulty seeing a friend across the street (OR 3.1 95% CI 2.5 to 3.7), difficulty recognising detail on television (OR 2.5, 95% CI 2.1 to 2.9), and trouble driving at night (OR 1.9, 95% CI 1.6 to 2.4).[306] Reading is rated as "extremely important" for those with and without vision loss.[368] This is not surprising as reading encompasses much more than just reading for pleasure. Reading is also essential for many other essential daily tasks such as shopping, finances, cooking and navigation.[368]
Reduced mobility and falls
Falls are one of the major causes of mortality and morbidity in older adults. It was estimated that every year 30-40% of individuals over 65 years of age will fall at least once.[369] Central (OR 2.36, 95% CI 1.02 to 5.45) and peripheral (OR 1.42, 95% CI 1.06 to 1.91) field loss were independently associated with increased risk of falls.[370] Visual impairment adversely affects balance and the ability to avoid obstacles. Two thirds of individuals with AMD were reported to have balance and visuomotor deficits leading to an increased risk of falls.[371] Impaired VA (worse than or equal to 6/7.5) was associated with increased risk of falls, two or more falls in the past year (OR 2.02, 95% CI 1.13 to 3.63) in BDES.[372] In addition to reduced VA (OR 4.23, 95% CI 2.34 to 7.64), reduced near vision (OR 5.00, OR 95% CI 2.28 to 10.94) and reduced contrast sensitivity (OR 2.40, 95% CI 1.16 to 3.92) all in the better eye, and reduced binocular vision (OR 3.20, 95% CI 1.85 to 5.56) were significantly associated with increased chance of nursing home placement in a multivariate model in BDES.[372] Mobility on foot appeared more limited for those with significant peripheral field loss (e.g. glaucoma), whereas mobility in or on a vehicle may be more severely affected by central visual loss (e.g. AMD).
Depression and anxiety
The prevalence of depression (major and minor depression combined) among community-dwelling older American

individuals is approximately 12%.[373] Pooled results from 12 studies comparing rates of depression in individuals with and without visual impairment (various causes) revealed a two-fold increase in depression associated with visual impairment (OR 1.94, 95% CI 1.68 to 2.25).[374] Dawson *et al*. reviewed 16 studies examining depression and anxiety related to visual impairment specifically due to AMD. Prevalence estimates for depression associated with AMD ranged from 15.7-44% and was significantly higher than that for controls.[375] Two studies found that increasing AMD severity was associated with an increase in depressive symptoms,[376, 377] although one study reported no difference in depression between early and late AMD.[378] Depression rates were significantly greater than controls for nAMD, despite the condition being treatable.[379, 380]

The high prevalence of depression in AMD and benefits of behavioral treatment suggests that health professionals in primary care should offer referral to not only those requiring ophthalmological treatment, but also those that may benefit from other forms of support.

Charles Bonnet syndrome

The prevalence of visual hallucinations in individuals with visual impairment was estimated to be 0.5-40%,[381, 382] with complex hallucinations experienced by 11-15%.[383] A survey of Macular Society members (n = 1254) regarding CBS revealed the following types of hallucinations; patterns (63% of individuals), faces (39%), objects (39%), figures (36%) and animals (32%). Typical hallucinations short-lived, lasting either minutes (44%) or seconds (34%). When at their worst hallucinations occurred; monthly (21%), weekly (30%), daily (22%) or constantly (13%). At the onset of CBS symptoms, 38% reported that the hallucinations were "fear-inducing", reducing to 8% at the time of the questionnaire. Hallucinations related to CBS had an effect on the daily activities of 46% of respondents.[384]

Longitudinal studies have estimated that 28% of individuals with CBS recover at one year,[385] and that the average duration of CBS symptoms in 18 months,[386] however, in the Macular Society survey 75% of respondents reported that CBS symptoms continued for five years or more.[384]

Quality of life

The impact of AMD on QoL was associated with reading, watching television, driving and emotional well-being.[387] Compared to individuals with equivalent visual loss and no hallucinations, those with CBS also have reduced measures of QoL and functional ability.[388] Early AMD, despite the presence of good VA may be associated with near vision, night driving and glare-related difficulties.[389] Lamoureux *et al*. assessed 219 AMD cases using the IVI 28-item instrument with data fitted to the Rasch model (assumes that the probability of an individual selecting a response category for any item is a logistic function of the relative distance between the item level of difficulty and the individual's level of ability). The authors reported that IVI was able to discriminate between individuals with differing levels of visual impairment; mild (< 6/12 to 6/18), moderate (< 6/18 to 6/60) and severe (< 6/60), ANOVA;$F(2,216) = 23.4$, $p < 0.001$, with restriction of participation mean logit values of; 1.06, 0.11 and -0.73 for mild, moderate and severe visual impairment, respectively.[387]

It is clear that the visual loss associated with AMD leads to a dramatic alteration in an individual's lifestyle, requiring adaptation of daily tasks, resulting in visual hallucinations that may be prolonged and frightening in some cases, leading to increased risk of reduced social engagement and independence, all increasing the risk of isolation and depression. It was reported that the QoL experienced by individuals with AMD is equivalent to those suffering from conditions such as melanoma, bone marrow transplant and acquired immune deficiency syndrome (AIDS).[390] A holistic approach to support is essential for the individual to cope with the consequences of visual loss.

1.6 Macular pigment

Macular pigment (MP), macular carotenoid or macular xanthophyll is a collective term for the dietary carotenoids; L and Z which are selectively absorbed into the retina, particularly the foveal retina, at much higher concentrations than that found in other tissues. A third MP, meso-zeaxanthin (MZ) is though to be produced in the retina from L.

1.6.1 The Macular pigment spatial profile

The macula lutea is a yellowish region centred on the fovea with a diameter of 4.5 to 6 mm, or 2.5 disc diameters, responsible for the central 15° to 20° of vision.[391, 392] The MP spatial profile typically exhibits a central peak at the foveola with an approximately exponential decline, reducing 100-fold within a few millimeters (6° to 8° of eccentricity), where the level of MP becomes optically undetectable.[244, 393] Dramatic intersubject variation in the precise shape of the spatial profile has been reported.[394] MP is located in the fibres of Henle (cone axons) at the fovea and in the inner and outer plexiform layers parafoveally.[395, 396] In the periphery MP is associated with rod OS membranes.[397, 398] MP may also be present in cone OS.[399] Müller cells may act as a reservoir for MP (fig. 1.6).[400]

Figure 1.6 Foveal cross-section showing the yellow MP

The centrally peaked MPOD spatial profile seen in most healthy individuals, measured using resonance Raman spectroscopy (RRS). About 12% of the population were reported to have a central "dip" in their MPOD profile at 0.25° retinal eccentricity, which was associated with tobacco use and increasing age, and hypothesised to relate to a deficit of MZ.[401] Beirne confirmed that 12% of participants had a central "dip" at 0.25° in the spatial profile using HFP.[402]

Nieto *et al*. reported that a higher percentage of patients with AMD (42%) and those with a primary FH of AMD (37%) had a lower MPOD level at 0.17° compared to 0.5° retinal eccentricity. A central "dip" in MPOD was present in only 31% of normal cases.[403] Hogg *et al*. reported that deposition of MP at the central location of the MPOD spatial profile is greatly influenced genetics.[404] The combination of homozygous risk alleles at CFH and ARMS2 loci was associated with significantly lower MPOD at 0.5° and 1.0° retinal eccentricity, but not at 0.25°,[405] where the central "dip" would be expected.

1.6.2 The discovery of MP

Wald identified MP as members of the xanthophyll family in 1945. The first separation of the carotenoids from the macula was made by Bone *et al*. in 1985, whom established that MP was composed of two components: L and Z. Handelman *et al.* confirmed this in 1988. The poly-isoprenoids, L and Z are isomers (i.e. have the same chemical formula: C_{40} H_{56} O_2), but are not stereoisomers.[406] In 1993, it was established that retinal zeaxanthin is composed of two main stereoisomers: (3R,3'R)-zeaxanthin (Z) and (3R,3'S)-zeaxanthin (MZ) along with small amounts of (3S,3'S)-zeaxanthin and trace amounts of 3'-epileutin, lactucaxanthin, 3'-dehydrolutein and ε,ε-carotene-3,3'-dione (fig.1.7).[391, 407] The history of MP was reviewed by Davies and Morland. [408]

1.6.3 Dietary sources of MP

More than 700 naturally occurring carotenoids have been discovered,[409] of which up to 50 may be found in the Western diet.[410], 21 carotenoids (including 14 cis-isomers) are found in the serum, but only the following are variably found in significant quantities: α-carotene,

Figure 1.7 The structures of the three main components of MP

β-carotene, β-cryptoxanthin (all pre-cursors of vitamin A), lycopene, L, Z, canthaxanthin and astaxanthin.[411-413] Dietary sources of macular carotenoids with a very high (> 2 mg / 100 g) content of L include: kale, spinach, broccoli and yellow / green peppers, and of Z: orange / red peppers and Chinese Wolfberry.[414, 415] Dietary sources of MZ are more controversial but may include certain species of fish, shrimp and sea turtle, and eggs from countries such as Mexico, where hens are fed with MZ-enriched feed.[416-418]

1.6.4 Absorption of MP

The role of the stomach in the absorption of lipid-soluble carotenoids is to initiate their transfer from the food matrix to the lipid portion of the meal, by gastric mixing to form a lipid emulsion.[419] The lipid-carotenoid emulsion then enters the duodenum leading to a fat-induced secretion of bile acids from the gall bladder and lipases from the pancreas, resulting in the solubilisation of the carotenoids and dietary fat in the form of micelles.[419] Solubilisation is required for the micelles to enter the unstirred water layer surrounding the microvilli of the enterocytes.[420]

Once considered to be a purely passive process, enterocyte cytosol uptake of carotenoids is now known to involve active transport via several apical membrane protein transporters including: scavenger receptor class B member 1 (SR-BI or SRB1), cluster determinant 36 (CD36) and Niemann-Pick C1-Like 1 (NPC1L1).[421] Carotenoids and lipids are formed into chylomicrons in the Golgi apparatus of the enterocytes and released via the lymphatic system in to the bloodstream.[419]

1.6.5 Transport of MP

Chylomicrons in the bloodstream are rapidly degraded and transformed to chylomicron remnants by the lipoprotein lipase. Most carotenoid-bearing chylomicron remnants are stored in the liver, from where some are re-secreted into the bloodstream where as a consequence of their polar nature, xanthophylls are evenly distributed between HDL and LDL. L and Z are primarily carried by HDL.[422]

Extrahepatic tissues take up carotenoids released from lipoproteins, especially LDL. [420] L and Z are found in many mammalian tissues other than the liver: kidney, lung, pancreas, spleen, heart, thyroid, testes, prostate, breast (and breast milk, especially colostrum), ovary, and brain tissue, as well as skin, blood serum and adipose tissue.[169, 423-432] By far the highest concentration of MP is found in ocular tissue at the macula, however the three main macular xanthophylls (L, Z and MZ) and their by-products are also found in the retina, RPE / choroid, ciliary body and the lens.[433]

1.6.6 Storage of MP

The liver is the major storage site for carotenoids because of its large size and abundance of carotenoid binding proteins, including SR-BI.[419, 434] The large volume of adipose tissue in the body is also a major storage site for carotenoids.[419] In women, and with obesity (both genders), adipose tissue is considered to compete with the macula for L and Z in the serum.[164, 424, 435, 436]

The macula has the highest concentration of xanthophylls of any tissue, concentrating L and Z almost exclusively.[437] Central macular levels of L and Z are 1,000 to 10,000 times higher compared to serum levels, suggesting a mechanism resulting in selective absorption.[438-440] Evidence of macular storage of MP may be inferred from the maintenance of raised MPOD levels 70 to 80 days after supplementation had ceased.[441] The strong anchoring of xanthophylls by their polar hydroxyl groups is thought to enhance L and Z stability within cell membranes.[442] Gass hypothesised that the Müller cell cone (Mcc) may act as a storage site for macular xanthophylls (fig. 1.8).[400]

Macular pigment has been observed in epiretinal membranes and pseudo-operculae, both of which contain Mc.[443, 444]

1.6.7 Bioavailability of MP

The absorption and transport of MP is affected by a number of factors such as: the food matrix nature (food type or supplements), dietary fat (aids carotenoid solubilisation), phospholipids (crude lipid mixture > glyco > phospho > neutral), dietary fibre, carotenoid nature (free-form > esterified), and other factors such as inflammation and gender.[445-448] The highest levels of L and Z are found in selected leafy green vegetables, however MP

Figure 1.8 Drawing of the anatomy of the fovea centralis showing the Mcc

bioavailability has been reported to be higher for certain fruits (e.g. orange, kiwi and grapefruit) and egg yolk.[165, 449]

It is possible that when several carotenoids are consumed together, one carotenoid may have an inhibitory effect on the absorption, metabolism and transport of another. In some studies, supplementary β-carotene is suspected to competitively reduce absorption of L.[450-452] However when considering food-derived carotenoids, the biological significance of these interactions is controversial.[419] For a more detailed account of carotenoid absorption, transport and storage please refer to the following references.[419, 440, 453, 454]

1.6.8 Lutein and zeaxanthin retinal transport and capture

Retinal uptake and capture of L and Z is not fully understood. Snodderley *et al.* reported that retinal xanthophyll is concentrated in the inner part of the foveola and perifoveolar area, located in the cone photoreceptor axons, known as the Henle fibre layer (HFL) and the inner plexiform layer.[395] Gass hypothesised that as there is minimal nerve fibre layer in the foveolar region, it is probable that most of the xanthophyll is located within the Mc.[400]

High-density lipoprotein deficiency in Wisconsin HypoAlpha Mutant (WHAM) chicks was associated with a deficiency of L and Z in the tissues, especially the retina. High L diet increased the L content of some tissues via LDL and VLDL transport, but retinal L remained very low. This supported the primary role of HDL as the specific transporter of L and Z into the retina.[455]

Apolipoprotein E is involved in the efflux of lipids from the RPE into Bruch's membrane.[456] Apolipoprotein E, which is produced by Mc and the RPE is also known to have a role in lipid transportation and binding of lipoproteins to target sites within the central nervous system (CNS), and in targeted uptake of the lipoproteins carrying L and Z.[457] Therefore it is

plausible that the ApoE profile might influence the transport, capture and stabilisation of L and Z at the macula.[440] The ε4 allele of the ApoE gene has a higher affinity to bind HDL and may confer protection against the development of AMD.[266, 458]

It has been shown that interphotoreceptor retinoid binding protein (IRBP) thought to chaperone the exchange of 11-cis-retinal, 11-cis-retinol and all-trans-retinol between photoreceptor OS, the RPE and Mc, showed a similar affinity to bind carotenoids and to a lesser degree fatty acids. It was suggested that IRBP might have a role in binding L and Z in the interphotoreceptor matrix (IPM).[459]

Immunoreactive labeling for steroidogenic acute regulatory domain protein (StARD3) identified as a L-binding protein in the primate retina, was found especially strong for cone inner segments (IS) and their axons in the HFL and in all nuclear layers (outer, inner and ganglion cell layers). Labeling for StARD3 did not however co-localise with glutamine synthetase, a glial / Mc marker. [460] It has been noted that although ApoE is synthesised and secreted by Mc, receptors for ApoE are found on retinal ganglion cells.[461] The Pi isoform of glutathione S-transferase (GSTP1) is the xanthophyll-binding protein for Z and MZ, with a weaker affinity for L.[462] L and Z were also reported to bind to the non-specific xanthophyll-binding protein, tubulin.[463]

1.6.9 Macular pigment functions

The three main macular xanthophylls are isomers (L and Z) or stereoisomers (Z and MZ), however their ability to block blue light as well as their antioxidant and free radical scavenging performance are different (table 1.15). Further information about MP functions may be found in the following references.[408, 464, 465]

Table 1.15 Summary of L, Z and MZ retinal functions

Function	L	Z	MZ
Peak absorbance [439, 466]	445 to 452 nm*	451 to 463 nm*	463 nm
Approx. range [439, 466]	390 - 520 nm	390 - 530 nm	390 - 530 nm
Ratio of L, Z and MZ: Serum[467] Foveola (< 0.25 mm)[468] Fovea[467, 469, 470] Periphery (9 to 12 mm)[468] Whole retina[467]	 3 to 5 1 1 2 2	 1 2.4 1 to 2 1 1	 0 n/a 1 0 0.5
Primary function	Rod protection	Cone protection	Cone protection
Orientation to membrane [471-474]	Parallel and / or perpendicular	Perpendicular	Perpendicular
Primary attribute[471, 475] [476]	Better blue light filter than Z	Better lipid peroxidation than MZ	Better $O2^-$ scavenger than Z
Special feature[471, 477]	Dual orientation**		Pure antioxidant***
Binding protein[460, 462]	StARD3	GSTP1	GSTP1
Source[448, 478]	Diet	Diet	Retinal L****

* The small difference in peak absorption between L and Z is due to the interaction of the double bonds in the β-ionone rings(s) with the polyene chain.[479] Peak absorption depends on the medium in which the carotenoid is measured.[480] ** The ability to orientate parallel to and perpendicular to cellular membranes in unique to L, and probably relates to the entire ε ring to rotate with respect to the rigid, conjugated double bond chain of the molecule.[471] *** Pure antioxidant = little or no pro-oxidant behavior at high carotenoid concentration and high oxygen tension.[477] **** L is oxidised to MZ in the central retina via double-bond isomerisation, although whether this process is the sole source of retinal MZ has recently been disputed.[407, 418, 478, 481] $O2^-$: superoxide radical.

1.6.10 Macular pigment hypotheses

A number of theories have been proposed for how MP may benefit the visual system. These hypotheses are based on the assumption that greater levels of MP are beneficial. These are summarised in appendix A2.3. Figure 1.9 illustrates the specific blue light absorbance of MP. Two references were used as the main source of information for this section.[465, 482] Details of the Mc / neuroglial cell hypothesis, a new hypothesis for MP proposed by the author are given in section 4.3 of the final discussion of this thesis.

Figure 1.9 Foveal cross-section showing the absorption of blue light by MP

1.6.11 Macular pigment measurement (in vivo)

Methods of MPOD measurement are divided into subjective and objective techniques. Subjective (psychophysical) methods include: colour matching using a tristimulus colorimeter,[483] motion or flicker minimisation using either motion photometry,[484] or HFP.[485, 486] Objective techniques include: fundus reflectometry using spectral analysis of light reflected from the retina,[487, 488] one- or two-wavelength FAF (1-WFAF or 2-WFAF) which relies on the fluorescence of lipofuscin,[404, 489, 490] and Raman spectroscopy which relies on the small portion of light that is back-scattered from MP, at longer or shorter wavelengths (inelastic scattering) than the monochromatic light source.[491]

1.6.11.1 Heterochromatic flicker photometry

Heterochromatic flicker photometry is the most widespread method of MPOD assessment in clinical practice,[492, 493] and was the method used in this study, therefore this method will be discussed in greater detail.

Macular pigment optical density measurement by HFP is accomplished by the observation of a small, typically 1° diameter (retinal eccentricity 0.5°) circular stimulus that alternates between a test wavelength absorbed by MP (blue light, typically 460 nm) and a reference wavelength not absorbed by MP (green light, typically 540 nm). While observing the circular stimulus, the observer adjusts the intensity of the test wavelength to a null point indicated by minimal or no perceived flicker. At the null point the adjusted test wavelength and the reference wavelength are perceived as having equal or close to equal intensity, the ratio of test to reference wavelengths being dependent on the amount of MP. More blue light is required to achieve the null point with higher levels of MP. This process is then repeated for a peripheral target (typically 7° or 8°), where MP is minimal.[485]

Macular pigment optical density at the test wavelength is calculated from the equation

$$\log (I_c / I_p), \qquad \text{(Eq 1.1)}$$

where I_c = intensity of blue light for the central target and I_p = intensity of blue light for the peripheral target.

This results in a unit-free value for MPOD described as optical density units or density units, abbreviated to DU. Because MPOD is by definition unit-free, this author has followed the convention of many authors of recent publications not to add DU to any values of MPOD reported in this thesis.

1.6.11.2 Tinsley Macular Pigment Screener

The instrument used in this study was the Tinsley Macular Pigment Screener 1000 (MPS 1000, Tinsley Precision Instruments Ltd, Essex, UK), also known as M/POD in the UK and QuantifEYE in the USA. This instrument uses a novel method for setting flicker thresholds designed to be less demanding for naïve and elderly observers.[486] Rather than adjusting the blue light intensity to obtain the null point indicated by minimal or no flicker, the observer views the target for a series of blue / green light intensity ratios, while the flicker rate is gradually reduced from above the critical fusion frequency (CFF) and responds by pressing a button at the first appearance of flicker.

The intensity of the blue and green lights is reciprocally-yoked, so there is no overall change in luminance for each preset ratio.[486] The testing sequence continues for a series of blue / green luminance ratios until a V-shaped function is obtained for the central target (0.5° eccentricity), (fig. 1.10). The minimum of the V-shaped function corresponds to the equalisation of the blue and green luminance. This process is repeated for the peripheral target (an 8° target of 1.75° diameter, giving a minimum eccentricity of approximately 7°).[486]

Figure 1.10 Central and peripheral V-shaped functions from MPS 1000

With the MPS 1000 / 9000 screener MPOD is calculated from the equation

$$k \log (Lbc / Lbp), \qquad \text{(Eq 1.2)}$$

where Lbc and Lbp are the luminance of the blue light at the point of minimum flicker (i.e. at the minima of the V-shaped functions), for the central and peripheral targets respectively. A correction factor, k = 1.2 was added to account for three factors: (a) the overlap of the wavelength spectra of the green light with the MP absorbance spectrum, (b) the overlap of the spectra of the blue (465 nm) and green (530 nm) light-emitting diodes (LED) used and (c) maximum absorbance (λmax) of the MP, as defined by Wyszecki and Stiles in 1982 (fig. 1.11).[486, 494]

Figure 1.11 Macular pigment absorbance spectra from three different authors

1.6.12 Repeatability and reliability of HFP measurements

Other groups using the MPS 9000 (Tinsley Precision Instruments Ltd, Essex, UK), a later version of the HFP instrument used in this study, have assessed repeatability. Bartlett *et al.* calculated the coefficient of repeatability (CoR) for 40 participants by multiplying the SD of the mean difference between repeated measurements by two, reported 0.28 and 0.33 for repeatability and, 0.25 and 0.26 for reproducibility for two different operators.[499] Van der Veen *et al.* reported a mean test-retest variability of 0.0195 (SD 0.047) resulting in a lower CoR of 0.09, however only 11 participants were assessed.[486] Abell *et al*. reported a high

level of test-retest reliability for MPS 9000 MPOD measurements recorded one week apart, with an intra-class correlation coefficient (a composite measure of intra-observer and inter-observer variability) of 0.98 (95% CI 0.97 to 0.98) for right eyes and 0.99 (95% CI 0.99 to 0.99) for left eyes, for 201 participants. The SD of the mean difference between repeated measurements was not reported and therefore it was not possible to compare the CoR with that of Bartlett *et al*. and van der Veen *et al*.[500]

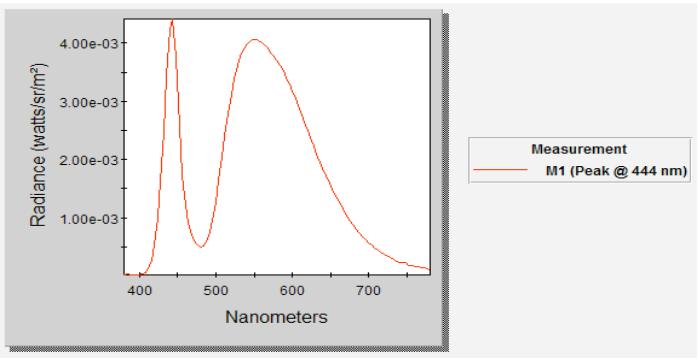
1.6.13 The neurophysiological mechanism of HFP

The neurophysiological substrate of heterochromatic flicker photometry has been identified as the phasic, magnocellular system of the primate visual pathway,[501] which under the conditions of fast flicker (> 15 Hz) and high luminance (> 1000 Trolands, abbreviated to Td) will favour contributions from medium and long wavelength cones,[486] while rods and short wavelength cones are strongly suppressed.[502]

1.6.13.1 Prevention of rod and S-cone intrusion

Rod and S-cone distribution is not constant across the retina, both receptors are present in greater numbers paracentrally and in the periphery, but are absent from the fovea.[503, 504] S-cone density (primate) was reported to exhibit a large within-group variation.[505] In an attempt to equalise spectral sensitivity at different eccentricities, rod and S-cone contribution to HFP measurements is reduced by using a flicker frequency above 12 Hz to 15 Hz and, depending on the HFP instrument, by a broad-spectrum, bright white background to enhance photopic vision and therefore rod suppression,[441, 486, 502] or a blue-coloured background designed to reduce both rod and S cone intrusion by spectral adaptation.[502, 506-508] The background illumination of the MPS-1000 instrument used in this study appeared white overall, but spectral analysis indicated a significant peak in the blue region (444 nm) of the visible spectrum (fig. 1.12).

Figure 1.12 Spectral radiance graph for MPS 1000 background illumination



Measurement obtained by the author during the MPS 1000 testing mode, using the PR-650 SpectraScan SpectraColorimeter (Photo Research Inc.). © Everett, 2014.

1.6.14 Factors affecting *in vivo* measurement of MPOD

Factors affecting the *in vivo* measurement of HFP MPOD can be related to instrument design (e.g. central target size, peripheral target location and fixed or variable flicker frequency), physiological (e.g. presence of cataract or IOL, macular thickness, pupil size, flicker and cone sensitivity differences and state of adaptation), instrument noise affecting repeatability and reproducibility, and ocular disease (e.g. changes in retinal structure or function, or disorders affecting MP uptake or transport). Factors known to affect *in vivo* MPOD measurements have been listed in appendix A2.4, together with how they are affected by age. Figure 1.13 shows MP present in an epiretinal membrane (ERM).

Figure 1.13 OCT and surgical photographical images of ERM containing MP

1.7 Glare recovery time

1.7.1 The visual cycles

In addition to the canonical (classical) retinoid visual cycle in which chromophore are recycled through the retinal pigment epithelium (RPE), two further visual cycles have been described. The cone-specific visual cycle and intrinsically photosensitive retinal ganglion cell (ipRGC) visual cycle. Each visual cycle has aspects that are important to the subjects discussed in this thesis and have therefore been summarised below.

1.7.1.1 Canonical (classical or Wald's) retinoid visual cycle

Photoreception takes place in the OS of rod and cone photoreceptors when a molecule of visual pigment absorbs a photon. Visual pigment is a G protein-coupled receptor consisting of a protein (opsin) covalently bonded to a vitamin A-derived chromophore (11-cis-retinal).[509] 11-cis-retinal undergoes a light-triggered isomerisation to all-trans-retinal, which in turn induces changes in the pigment producing its physiologically active state (metarhodopsin II).[510]

The activated visual pigment molecule triggers a transduction cascade resulting in the rapid closure of cyclic guanosine monophosphate (cGMP) gated cation channels in the OS membrane, photoreceptor hyperpolarisation leading to suppression of the circulating dark current and signalling of second-order neurons, and a reduction in the release of the neurotransmitter glutamate from its synapse.[509-511]

Efficient photopigment regeneration is essential for proper photoreceptor functioning.[510] The chromophore all-trans-retinal is reduced to all-trans-retinol (vitamin A) within rod and cone OS by a set of retinal dehydrogenases (RDH).[512] All-trans-retinol is then exported from the photoreceptor OS and chaperoned to the adjacent RPE by an interphotoreceptor retinoid-binding protein (IRBP), the identity and mechanism of which remains controversial.[513-516] At the RPE all-trans-retinol is converted to 11-cis-retinol and finally oxidised to 11-cis-retinal by another set of RDH.[510, 512] Unbound retinoids in the interphotoreceptor matrix (IPM) situated between the photoreceptor OS and RPE plasma membrane, are prone to degredation and are cytotoxic.[517] Transport of 11-cis-retinal back to the rod and cone OS completes the canonical retinoid visual cycle.[518]

Rods depend entirely on the output of 11-cis-retinal from adjacent RPE cells, whereas cones can use 11-cis-retinal from the RPE and 11-cis-retinol from adjacent Müller glial cells. This additional source of recycled photopigment is known as the cone-specific visual cycle.[519]

1.7.1.2 Cone-specific visual cycle

The maintenance of continuous, cone-mediated vision in bright daylight appears to be at odds with the rate of visual pigment recycling reported for the canonical visual cycle.[520-522] The first evidence supporting the involvement of retinal Müller glial cells in a non-RPE, cone-specific visual cycle came from the observation that cultured Mc derived from cone-rich chicken retinas were able to synthesise 11-cis-retinoids from all-trans-retinol in isolation, suggesting that they exhibit isomerase and retinyl ester synthase (RES) activity.[523]

Mata *et al.* proposed that Mc were responsible for chromophore recycling and subsequent supply of 11-cis-retinol to cone photoreceptors.[522] Cones are able to utilise 11-cis-retinol, whereas rods are not, restricting the use of Mc recycled chromophore to cones. [520] Muniz *et al.* confirmed that RES activity in chicken is an acyl coenzyme A (CoA): retinol O-acyltransferase (ARAT).[524]

Subsequent studies have demonstrated Mc-mediated, cone-specific visual cycles in animals with rod-rich retinas such as rodents and primates, including humans.[521, 525] The Mc to cone ratio in the primate fovea is 1:1, falling to 2:1 at an eccentricity of 30° [526-528]

suggesting a close relationship between Mc and cone function and increased foveal cone vulnerability associated with disorders leading to Mc pathology.[520, 526]

The additional source of 11-cis-retinal available to cones may explain in part how human cone circulating current is fully recovered after just 100 ms from a steady bleach of approximately 90% of photopigment, whereas rods take at least 20 minutes (min) to recover fully.[529] The continuous and open structure of the cone OS facilitates rapid phototransduction and metabolism, and allows for fast metabolite exchange between cones and IPM, affording a higher rate of photopigment recycling than the canonical route.[520, 522, 525, 530, 531] This may also contribute to the greater vulnerability of cones to lipid peroxidation secondary to oxidative stress and their subsequent need for increased antioxidant protection.[228, 532] (See A2.2 for a summary of Mc functions in additional to cone photopigment recycling).

1.7.1.3 Intrinsically photosensitive retinal ganglion cell visual cycle

While the detrimental effect of blue light exposure on retinal physiology has been widely reported,[250, 533] the benefits of retinal blue light exposure have received less attention in the literature.[534-539]

Melanopsin-containing ipRGC were first identified in 2002 as a third type of photoreceptor with an integral role in several non-visual functions.[540] ipRGC express the photopigment melanopsin (Opn4) diffusely along their dendrites and within the stroma.[541] In humans, the majority of non-visual function responses are maximally sensitive to blue light stimulation (circa 480 nm).[540-542]

It is currently believed that phototransduction in ipRGC is closely related to that found in invertebrate rhabdomeric photoreceptors,[543, 544] and that Opn4 activates a Gq class of G-proteins followed by stimulation by phospholipase C, which leads to the opening of cation-selective transient receptor potential channels (TRPC).[541] Unlike the canonical and cone-specific visual cycles which require complex cascades to regenerate photopigment after light-triggered isomerisation, it has been hypothesised that Opn4 functions as a bi-stable pigment, able to regenerate its own light-activated chromophore by absorbing a second wavelength of light at 587 nm, although this is controversial.[541, 545-547]

1.7.1.3.1 Intrinsically photosensitive retinal ganglion cell functions

The human retina contains approximately 3000 ipRGC, representing 0.2% of the total number of retinal ganglion cells.[548] Current evidence from rodent data suggests that there are five distinct subtypes of ipRGC (termed M1 to M5) differentiated by dendritic morphology and axonal projections within the inner plexiform layer.[540] Two of these ipRGC subtypes (M1 and M2, not to be confused with the macrophage anti-inflammatory

subtypes), form an overlapping mosaic (photoreceptive network) covering the whole retina, whereas a third subtype does not contribute to this mosaic.[549, 550] The photoreceptive network extends to within the macula region in human and macaque, with a small number of processes crossing the foveal pit.[540]

Intrinsically photosensitive retinal ganglion cells of the subtype M1 are predominantly responsible, with additional input from rods and cones, for circadian photoentrainment via the retinohypothalamic tract to the suprachiasmatic nuclei (SCN) and the intergeniculate leaflet (IGL), and the pupillary light reflex (PLR) via the olivary pretectal nuclei (OPN).[551-555] Melanopsin is also involved in the regulation of the sleep-wake cycle, temperature regulation, cognitive function and alertness, as a result of light-activated suppression of melatonin from the pineal gland and cortisol secretion via activation of the adrenocortical axis.[535-538, 556-558]

There is evidence from murine data that retrograde signalling from ipRGC influence the level of adaptation via dopaminergic A18 amacrine cells.[334-336] Retinal dopamine release varies diurnally in vertebrates, increasing during the day and decreasing at night.[559] Melatonin can acutely inhibit retinal dopamine release.[560]

Flickering lights are the most effective stimulant for dopamine release in the primate retina.[561] In mammals dopamine is believed to modulate the spatial extent of the horizontal cell (HC) syncytium by uncoupling HC gap junctions.[562] Dopamine agonists were found to suppress the retinal flicker response, however maximal hyperpolarisation of HC with a bright white light was able to partially restore the initially suppressed flickering response components.[562] Light aversion (photophobia) under non-pathological conditions is considered to be mediated by ipRGC, whereas pathological causes of photophobia are thought to be ipRGC-independent, modulated instead by rod and cone photoreceptors.[540]

Migraine is associated with increased photophobia in approximately 80% of cases.[563, 564] The mechanism underlying the deficiency of habituation in migraine, causing increased retinal light sensitivity has not been fully explained, but recent evidence suggests that it may result from thalamo-cortical dysrhythmia.[565, 566] Photophobia, especially that resulting from migraine is preferentially triggered by blue light exposure,[567-569] suggesting that ipRGC could be involved in this condition.

1.7.2 Glare recovery time (or test)

Glare recovery time, also known as photostress recovery time (PSRT) and in the older literature, the macular dazzling test (MDT) or nyctometry, refers to the time taken for retinal sensitivity (visual acuity (VA) or contrast sensitivity), to return to a predefined level after

being exposed to a controlled, intense centrally directed light source for a predetermined length of time.[308]

The concept of GRT was introduced by Bailliart in 1954.[570] This method involved dazzling the macula with an ordinary ophthalmoscope and recording the time taken for visual recovery.[571] Other methods of GRT assessment have included the use of infrared pupillometry,[572] Maxwellian view optical system,[573] electroretinography,[330] visually evoked responses,[574] scanning laser ophthalmoscopy,[575] nyctometry[576] automated perimetry,[328, 356] and light sources including; indirect ophthalmoscopes,[352] pen torches,[573] flood lighting,[577] and car headlights.[578]

Margrain and Thomson examined the causes of variability in GRT testing. Their conclusion was that the bleaching method was the primary source of GRT variability.[573] Compared to their laboratory GRT set-up, a Maxwellian view optical system designed to constantly bleach approximately 96% of cone photopigment, the ideal clinical method of GRT would be required to produce a retinal illuminance of 5.5 log Td for 30 seconds (s) or more.[573] The direct ophthalmoscope held close to the eye with a pupil size ≥ 2 mm to approximate Maxwellian viewing, for 30 s was able to fulfill this criterion. A random selection of ophthalmoscopes was found to bleach between 98% and 99.6% of cone photopigment under these conditions.[573]

Margrain *et al.* are currently working on a retinal densitometer designed to objectively measure rod and cone DA simultaneously. The densitometer accurately measures the change in colour of photopigments as they recover after the bleach (fig. 1.14). Early tests revealed a high ability to discriminate between AMD and non-AMD groups (T. Margrain, personal communication, December 10th, 2013).

Figure 1.14 Functional retinal imaging "map" produced by the retinal densitometer

Retinal image of early AMD Functional retinal image "map"

The source of illumination used for GRT testing produces an intentional, temporary central scotoma due to the higher than normal degree of photopigment bleaching, [579] and can therefore be considered as an, albeit rather unnatural, form of DA and a dynamic assessment of macular function.[329] The recovery of visual function after exposure to the light source used in GRT is believed to be largely the result of cone photopigment regeneration.[308, 349, 580] The kinetics of cone photopigment recovery may be different depending on the duration of light exposure used. Photo-flash sources result in a recovery time that is dependent on the percentage of photopigment bleached, whereas the recovery time associated with equilibrium (longer duration) exposure is not correlated with the percentage of photopigment bleached.[581]

Visual recovery after GRT was attributed to pigment regeneration via the canonical visual cycle,[580] however the more rapidly recovering, cone-specific visual cycle is likely to make a significant contribution to cone pigment regeneration after the bleach.[521, 522, 531] The relative contributions of the canonical and cone-specific visual cycles to visual recovery after retinal bleaching are unknown,[525] but it is likely that equilibrium bleaching will deplete local stores of 11-cis-retinal, placing a greater burden on the RPE for pigment regeneration in comparison to photo-flash bleaching.[330, 582] It is also important to consider, but difficult to quantify the contribution of neural adaptation mediated by ipRGC to the recovery time,[329, 583, 584] which may be expected to affect equilibrium bleach to a greater degree than photo-flash bleach.[585]

1.7.2.1 Glare recovery time in ocular disease and other factors

The association between AMD and GRT was discussed in section 1.5. Other factors known to affect GRT are summarised in appendix A2.5.

1.8 Study aims

The aims of this study were as follows:

To investigate the effect of the order of measurement (first eye vs. second eye) on MPOD values and the effect of ocular dominance on MPOD and GRT values. To investigate the association of the confirmed and putative AMD and ocular vascular perfusion (OVP) RF; age, gender, BMI, percentage body fat (%BF), iris colour, AMD FH, migraine, Rph and VDys, with MPOD and GRT. To investigate whether GRT is a suitable surrogate measure for MPOD. To investigate the inter- and intra-session repeatability for the GRT method used in this study (table 1.16).

Table 1.16 The study hypotheses

The order of eye measurement
Consensus among researchers is that in the absence of pathology there is generally good interocular agreement for MPOD measurements.[586, 587] Many studies investigating HFP MPOD values for both eyes have made sequential measurements from the right and then the left eye.[586, 588-590] While in many research studies means of multiple readings are taken from each eye, in clinical practice it is more likely that only a single measurement would be taken from each eye and that the right eye would precede the left eye measurement. The author has noted a trend for higher threshold values on visual field testing for the second eye tested compared to the first. Measurement of MPOD using HFP similarly involves the occlusion of the second eye during first eye testing and (unlike field testing) the exposure of the first eye to a prolonged high-luminance background light source. It is therefore feasible that if the second eye is measured immediately after the first, the MPOD results could be affected by the difference in conditions experienced by each eye if only a single measurement is taken. In this study the order of right and left eye measurements were randomised in an attempt to reduce bias (learning / fatiguing effect) due to sequential right then left eye measurement.
Ocular dominance
There is some weak evidence to suggest that dominant eyes receive a greater lifetime light exposure compared to non-dominant eyes and therefore may be at greater risk of developing AMD (see section 1.3.4.2). In order to assess whether ocular dominance affects HFP MPOD and GRT readings, it is first essential to exclude any learning or fatiguing effect due to the order of eye measurement. One study reported no significant association between MPOD and eye dominance, but the order of eye measurement was not reported.[591] Another study reported a bias towards longer GRT in the dominant eye, however the results are likely to have been biased by a learning effect.[329] In this study first eye (randomised right or left eye) measurements of MPOD and GRT were compared with ocular dominance in an attempt to reduce bias due to learning / fatiguing effects.
AMD RF
The confirmed or putative RF for AMD; age, gender, BMI, iris colour and FH of AMD were selected because of the ease of measurement in an optometric practice setting. Percentage body fat was calculated from BMI, age and gender. Age-related macular degeneration has been associated with reduced MP in some,[175, 592] but not all studies.[593, 594] No major protective effect of MPOD was seen with early AMD,[595] although higher dietary MP,[168] and supplemental MP in the presence of poor dietary intake of MP,[452] were associated with a lower risk of developing late AMD. The results from studies examining the association between MP and AMD RF have also been controversial to date; age (no significant association[166, 402] vs. age-related decline[596, 597] vs. peak in middle age range[598]), gender (no significant difference[166] vs. lower in females[599]), BMI (no significant association[591] vs. lower for higher BMI[166]), male %BF (lower for higher %BF[600]), female %BF (no significant difference[600] vs. lower for higher %BF[436]), iris colour (no significant difference[597] vs. lower for lighter iris colour[601]), FH of AMD (no significant difference[401] vs. higher[594] vs. lower[597] for FH of AMD). Age-related macular degeneration has been associated with longer GRT, although the association appears more consistent for equilibrium compared to photo-flash bleach methods.[308] The association between GRT and AMD RF is controversial; age (no significant association[352, 358] vs. longer GRT[571, 573]), gender (no significant association[361] vs. longer for females[602]), iris colour (no significant association[329]), AMD FH (non-significant trend towards longer GRT[603]). The author is unaware of any studies reporting the association of BMI or %BF with GRT.
Calculated %BF
Body mass index measurements are easily and non-invasively obtained in the clinical setting and generally correlate strongly with %BF (r = 0.60 to 0.82),[604] however BMI does not directly measure adiposity,[600] differentiate between adipose tissue mass and muscle tissue mass or allow for differences related to gender or age.[605] For the same BMI, women typically have approximately 10% higher body fat compared to men.[606, 607] In this study %BF was calculated from BMI, age and gender according to the (CUN-BAE) algorithm derived by Gomez-Ambrozi *et al*.[608] (See section 2.2). This %BF algorithm was selected over other %BF algorithms because it was based on a predominantly White European population.
Inclusion of OVP RF

Conditions that lead to reduced retinal vascular perfusion, e.g. reduced diastolic blood pressure and ocular ischaemic syndrome are associated with longer GRT.[609-612] Ocular ischaemic syndrome is associated with subfoveal choroidal thinning representing impaired choroidal circulation.[148] Choroidal perfusion and ischaemia (choroidal and arguably retinal) are inversely associated with AMD risk.[613-615] The OVP RF migraine, Rph and VDys were included in this study because they are also associated with reduced or unstable OVP and ischaemia.[196, 205, 616]

The OVP RF; migraine, Rph and VDys are not currently considered to be RF for AMD, however they are associated with reduced or dysfunctional retinal and choroidal blood flow,[617-619] and in the case of VDys signs of retinal inflammation.[205] (See section 1.3 and fig. 1.3). Frandsen *et al*. reported higher objectively-measured MPOD for individuals with migraine.[193] The author is unaware of any studies examining MPOD for individuals with Rph or VDys, or GRT with migraine, Rph or VDys.

GRT as a surrogate measure of MPOD

The idea to investigate the use of GRT as a surrogate measure for MPOD was suggested by one of the study supervisors (Dr Hannah Bartlett). Previous studies have shown a significant inverse correlation between 5-s bleach time GRT using a high blue light content glare source and MPOD.[620-623]

The decision to use the Keeler Specialist direct ophthalmoscope as the glare source was made by the author for two reasons. Firstly, the 30-second bleach method using this light source was reported to bleach in excess of 95% of retinal photopigment,[573] and it was hoped that this would improve the high level of inter-subject variation inherent with GRT measurements.[620, 624] Schmitt *et al*. and Bartlett *et al*. concluded that longer duration bleach than that afforded by photo-flash methods might lead to less variation in GRT results.[358, 359] A later study confirmed that equilibrium bleach GRT was considerably more repeatable than photo-flash bleach GRT when examining the same population. Coefficient of repeatability was 85 s for equilibrium bleach GRT and 184 s for photo-flash bleach GRT for 23 individuals of mixed gender, ranging in age from 21 to 70 years.[330]

Secondly, the direct ophthalmoscope and letter chart needed to measure GRT using the method described by this study would already be used by the vast majority of optometrists in practice, therefore negating any additional expenditure for specialised equipment. If GRT measured with the direct ophthalmoscope could be successfully used as a surrogate measure for MPOD, this would significantly reduce the financial pressure on optometrists to sell supplements to cover the cost of equipment used and clinic time required to measure MPOD.

GRT repeatability

The method of GRT used in this study (30-second equilibrium bleach with the direct ophthalmoscope held as close as possible to the subjects eye) was reported to be the best clinical technique in 2002,[573] however, the author is unaware of any repeatability studies for this technique. For this reason inter- and intra-session repeatability was included in this study. The learning effect or bias of using the same test chart letters compared to different letters for repeated measures of GRT was also assessed. Some optometrists use test charts with a fixed set of letters, whereas others use a computer-driven test chart that allows the letters to be changed to prevent patients from learning them.

Chapter 2 Macular pigment optical density study (Experiment 1)

2.1 Brief introduction

The aim of this practice-based, cross-sectional study was two-fold. (a) To investigate the effect of sequential versus randomised order of measurement on the interocular comparison of MPOD measurements and the effect of ocular dominance on MPOD measurements. (b) To investigate the relationship between MPOD with confirmed and putative AMD RF (age, gender, BMI, %BF, iris colour and AMD FH), and OVP RF (migraine, Rph and VDys). Risk factors were limited to those easily measureable in optometric practice. Background information about MPOD and its association with selected AMD RF was discussed in the introduction to this thesis (sections 1.3 and 1.6) and is summarised below in the brief introduction to this chapter.

Table 2.1 Summary of investigations (MPOD)

Interocular comparison	The interocular difference in MPOD has been examined in many studies. Consensus among researchers is that in the absence of pathology there is generally good interocular agreement.[586, 587] The author is unaware of any studies examining the effects of eye order (randomised; right vs. second eye and sequential; first vs. second eye) on HFP-derived MPOD measurements.
Ocular dominance	In MPOD research it is usual to obtain results from the right eye only. Right eye dominant outnumber left eye dominant cases by approximately 2-1. It is plausible that dominant eyes are exposed to greater lifetime retinal light levels compared to non-dominant eyes and therefore may be at greater risk of developing AMD. One study reported a trend towards higher MPOD for dominant eyes, but this did not reach significance.[591]
Age	Age is the strongest, established RF for AMD,[14, 41, 43, 625] whereas the role of MPOD in AMD risk has been described as putative.[626] It has been argued that a significant decline in MPOD level with age may result in higher levels of retinal oxidative stress and therefore lead to increased risk of AMD.[596, 597] The association between MPOD derived by psychophysical methods and age has remained controversial for many years.[439]
Gender	Gender has not been consistently reported to be a RF for AMD. The Beaver Dam Eye Study and BMES suggested that women might have a higher risk of developing AMD.[625, 627] The higher prevalence of late AMD in women compared to men has been explained in part by the larger number of women in the older age range. [1], [45] Higher body fat levels in women may lead to competition with retinal carotenoid uptake, resulting in lower female MPOD levels.[164]
BMI	Higher than normal BMI was associated with increased risk of both early and late AMD.[133, 628] Another large study found no association between BMI and AMD.[39] MPOD was found to be inversely associated with BMI-defined obesity in both genders,[436] or only male gender.[600] BMI may not accurately reflect adiposity level, represented by percentage of body fat (%BF) however.[604, 629] For any value of BMI, female %BF is higher than male %BF.[600]
%BF	Higher levels of abdominal fat (waist / hip ratio), but not BMI or %BF was associated with increased risk of AMD in men, whereas all three anthropometric measures were related to increased AMD risk in women.[630] %BF was inversely correlated with MPOD in both genders,[600] or only male gender.[432] In the present study %BF was estimated from BMI, age and gender using the CUN-BAE (Clinica Universidad de Navarra - Body Adiposity Estimator) algorithm.
Iris colour	Light iris colour was associated with significantly greater light transmission and reduced choroidal melanin compared to darker irides.[631, 632] Significantly more cases of AMD have been reported for individuals with light compared to dark irides,[633] however BDES found no association between iris colour and AMD incidence and progression,[634] but did report an association between lighter iris colour and the development of RPE pigmentary abnormalities (ARM).[635] The relationship between MPOD and iris colour is also controversial; Two studies have reported significantly lower MPOD associated with lighter iris colour,[601, 636] while another study found no significant association.[637]
FH of AMD	Family history of AMD is a confirmed RF for the development of AMD. The Blue Mountains Eye Study found that AMD FH was significantly associated with both early ARM (OR 2.17 95% CI 1.04 - 4.05) and late AMD (OR 3.92 95% CI 1.34 - 11.46). [117] Family history of AMD was associated with a higher risk of MPOD profile with a central depression at 10 min eccentricity.[403] Peak MPOD measurements were confirmed to be largely genetically determined.[404]
OVP RF	Vasospasm has been reported to play a central role in the pathogenesis of migraine, Rph and VDys.[194, 619, 638] Vasospasm was reported to affect choroidal and ciliary vessels more than retinal vessels.[619] Reduced choroidal blood flow was reported in individuals with non-nAMD using colour Doppler imaging,[639] laser Doppler flowmetry[640] and indocyanine green angiography.[641] Fellow eye risk for developing nAMD was inversely associated with choroidal perfusion.[642] Age-related macular degeneration RF including age, gender and iris colour, were associated with a reduction in choroidal blood flow.[643]
Migraine	Participants were classified as self-reported migraine or non-migraine sufferers. For simplicity in

	this practice-based study migraine sufferers were classified as with or without aura according to the third edition of the International Classification of Headache Disorders, ICHD-3, June 2013.[644] Migraine classification is considerably more complex than this and individuals with migraine can be classified with more than one migraine subtype. The reported presence or absence of light-trigger for migraine was not included in the headache classification cited above, but was added because higher levels of MP are associated with lower retinal blue light exposure. Therefore it was considered plausible that higher MPOD could reduce the light-trigger for migraine. A later study reported that MPOD measured objectively using FAF was significantly higher in participants with migraine, however the authors did not further classify individuals with migraine by subtype.[193]
Rph	Participants were classified as having Rph if they reported that their fingers turned white in cold temperatures, indicating a restriction of blood flow to the affected area. Raynaud's phenomenon may be described as primary or secondary.[194, 645] No distinction between types was made in this study, but all cases were aged over 30 years indicating that all were secondary Rph (sRph). In addition to the effect on the choroidal circulation, Rph is associated with a reduction in retinal capillary blood flow, which could result in ischaemia leading to retinal dysfunction.[646]
VDys	Vascular dysregulation may also be described as primary or secondary VDys (sVDys is associated with other, usually auto-immune disorders).[647, 648] No distinction was made between types in this study, but the age range would suggest that both types were represented. Primary VDys occurs more frequently in young, slim, adult females and is associated with a history of cold hands (and sometimes feet) unrelated to ambient temperature.[648] Individuals with pVDys have disturbed autoregulation, leading to instability in ocular blood flow leading to repeated, mild reperfusion injury and oxidative stress.[647]
Difficulty with HFP	It is assumed that participant difficulty with HFP increases with age. The author is unaware of any systematic analysis of the relationship between age, MPOD value and GRT with difficulty obtaining results using HFP methods to assess MPOD.

2.1.1 Research objectives

The aim of this research was to contribute to the body of knowledge that has been collected for the relationship between MPOD and the following AMD RF: age, gender, BMI, iris colour and AMD FH. In an attempt to make an original contribution to the literature, the association between MPOD and the following OVP RF: migraine, Rph and VDys, and the effect of eye order and calculated %BF on MPOD measurement were also investigated. There have been no previous studies of MPOD levels in the type of sample investigated here.

2.2 Materials and methods

2.2.1 Subjects

A priori sample size estimation

Calculating an *a priori* sample size estimation allows the recruitment of sufficient participants to reduce the risk of an underpowered (false-negative) result. There are four possible explanations for a non-significant result in a trial; the study was appropriately powered and the result was genuinely non-significant, the study was appropriately powered but the non-significant result occurred by chance (1 in 20 chance at $p = 0.05$), there was a significant difference, but the study was underpowered (sample size too small), or one or more aspects of the trial were biased in favour of the control group. There are ethical consequences of conducting underpowered studies.[649]

We calculated that a sample size of 150 will provide 80% power at $\alpha = 0.05$ for a moderate Pearson correlation of 0.2. An acceptable ratio of participants to predictor variables when using multiple regression analysis was reported to be between 10:1 and 40:1. The original incarnation of this study had seven predictor variables; age, gender, BMI, iris colour, AMD

FH, migraine and Rph. Percentage body fat and VDys were not included because it was anticipated that %BF and VDys would share a significant degree of variance with BMI and Rph, respectively. According to this the minimum acceptable number of participants would be 70. Recruitment of 150 participants provided a ratio of 21:1 and represented a good compromise between the limits reported above (10: 1 and 40:1).

Tabachnick and Fidell reported a formula for calculating the sample size required when undertaking multiple regression analysis, taking into account the number of independent variables and assuming a medium-sized relationship between the independent variables and the dependent variable ($\alpha = 0.05$ and $\beta = 0.20$).[650]

$$n \geq 50 + (7 \times m) = 99 \text{ participants,} \qquad \text{(Eq 2.1)}$$

Where n = sample size and m = 7 (number of independent variables).

Post hoc sample size estimation

Sample size for the comparison between two means for MPOD was calculated retrospectively from the data collected for age, gender, mixed-gender BMI, iris colour and AMD FH from other studies (table 2.2), assuming 80% power (1 - β) at the 5% significance level (table 2.3). Effect sizes (d) were obtained from the mean of at least two other studies (if available). Similarly sized studies with White participants were included preferentially. The sample sizes were corrected for unequal numbers in each group (i.e. allocation ratio, r = larger group number / smaller group number). Sample size estimation was not performed for calculated %BF because this was derived from the BMI, age and gender data.

The author is unaware of any studies examining the association between HFP-derived MPOD and difficulty with HFP, migraine, Rph and VDys. In this case the effect size may be determined by logical assertion and conjecture,[651] or by calculation. G*power statistical software was used to calculate the effect size (from the mean and SD from each of the MPOD groups, for each RF). The calculated effect size was then used to the calculate sample size using the formulae in table 2.3.

Table 2.2 Independent variable effect size for MPOD extracted from the literature

Independent variable	Study	Reference	n	Effect size (d)
Age	Nolan (2004) Neelam (2005) Lam (2005)	[600] [652] [653]	100 118 92	0.17 0.06 0.16 **Mean = 0.13**
Gender	Mellerio (2002) Nolan (2004) Iannaconne (2007)	[508] [600] [654]	124 100 183	0.12 0.02 0.03 **Mean = 0.06**
Mixed-gender BMI	Hammond (2002) Nolan (2004)	[436] [600]	400 100	0.05 0.12 (male) 0.17 (female) **Mean = 0.12**
Iris colour	Hammond (2000) Mellerio (2002) Ciulla (2004)	[599] [508] [655]	128 124 280	0.05 0.13 0.04 **Mean = 0.07**
AMD FH	Nolan (2007) FH early AMD FH GA FH nAMD	[597]	 41 55 79	 0.09 0.11 0.12 **Mean = 0.10**
Migraine / Rph / VDys	No previous studies			n/a

Table 2.3 *Post hoc* sample size estimates for the MPOD study

AMD RF data	Age ≤ 50 vs > 50 years	Gender male vs female	BMI ≤ 25 vs. > 25	Iris colour light vs dark	AMD FH FH vs no FH
Mean difference (MPOD) Standard deviation (S) Effect size (d)	0.06 0.17 0.13	0.01 0.17 0.06	0.02 0.16 0.12	0.04 0.16 0.07	0.01 0.17 0.10
n per group (2-sided) $16/(d/S)^2$ Power = 80%, α = 5% Assuming r = 1	27	128	29	84	46
Sample size (M)	54	256	58	168	92
Allocation ratio (r) Number in smaller group (M1) (1 / (1 + r)) x M Number in larger group (M2) (r / (1 + r)) x M	1.3 23 31	2.7 69 187	1.1 28 30	1.6 65 103	4.8 16 76
Corrected value for M1 (M1c) M1c = r + (1/2r x M) [656]	37	348	33	136	226
Corrected sample size (Mc) M1c + M2	68	535	63	239	302
OVP RF and miscellaneous (bold border) data	Migraine yes vs no	Rph yes vs no	VDys yes vs no	Ocular dominance D vs ND	Difficulty with HFP yes vs no
Mean difference (MPOD) Standard deviation (S) Effect size (d)	0.001 0.16 0.08*	0.002 0.15 0.14*	0.01 0.15 0.04*	0.07 0.17 0.18	0.02 0.17 0.06
n per group (2-sided) $16/(d/S)^2$ Power = 80%, α = 5% Assuming r = 1	64	19	225	14	128
Sample size (M)	128	38	450	28	257
Allocation ratio (r) Number in smaller group (M1) (1 / (1 + r)) x M Number in larger group (M2) (r / (1 + r)) x M	4.9 22 106	2.7 10 28	2.5 129 321	1 - -	4.1 50 207
Corrected value for M1 (M1c) M1c = r + (1/2r x M) [656]	318	54	565	-	531
Corrected sample size (Mc) M1c + M2	424	82	886	28	738

*Effect size calculated from MPOD values of mean and SD using G*Power assuming equal group size. Effect size convention: d = 0.2 small, d = 0.5, medium, d = 0.8 large. vs = versus. D: dominant eye, ND: non-dominant eye

2.2.2 Recruitment

This study was undertaken at the Bath Road practice of Norville Opticians in Cheltenham. The study required the recruitment of non-smokers aged 20 years and above with no eye disease. Data was collected for 150 participants over a 14-month period from the 4th of August 2010 to the 12th of October 2011, outside normal clinic hours. See appendix 4 for information sheets and poster.

Initially patients whom appeared to meet the requirements above were sent an invitation to participate with the reminder letter for their next routine eye examination. The reminders were computer generated based on the time since their last eye test. Over a period of one month 100 invitations were sent out. Response was very poor, with only one respondent, who was excluded as a smoker.

Posters and information sheets were displayed at four Cheltenham practices. Colleagues were emailed with information about the study and were invited to refer any suitable patients. The author presented a talk about MP at the Norville Opticians annual professional staff meeting, where recruitment information was disseminated to colleagues. Suitable patients were invited by the author to participate in the study during their routine eye examination. This proved to be the most effective method of recruitment.

2.2.3 Inclusion / exclusion criteria

Table 2.4 Inclusion / exclusion criteria for both MPOD and GRT studies

Inclusion criteria
Gender: all genders Age: ≥ 20 years BMI: ≥20 and <30 Corrected LogMAR visual acuity ≤ 0.1 (6/7.5 equivalent Snellen acuity) Healthy macula appearance No Amsler distortion Normal reported cholesterol levels Willingness and ability to give written, informed consent and willingness and ability to comply with the study requirements Participants with BMI ≥ 30 were examined as a separate group
Exclusion criteria
Age: <20 years BMI: <20 or ≥30 Corrected LogMAR visual acuity > 0.1 Amsler distortion Abnormal macular appearance or drusen History of macular disease Reported raised cholesterol level Current pregnancy Current smoker Alcohol consumption within two hours Diabetes Glaucoma Reported poor night vision Dietary absorption disorders (e.g. Crohn's) Current use, or use within six months, of medications that are known to affect macular function Inability to give informed consent Refusal to give written, informed consent and / or refusal to comply with the study requirements
Removal of a participant during the study

Pupils too small to allow macular view Inability to fixate ophthalmoscope light (e.g. eccentric fixation, nystagmus) Inability to provide results for any of the ophthalmic tests performed

2.2.4 Justification for inclusion / exclusion criteria for MPOD study

Table 2.5 Justification for inclusion / exclusion (MPOD study)

Factor	Justification for inclusion / exclusion
Age	Patients under 20 years of age were excluded because data collection was undertaken on a school / college day (Wednesday) and those under 16 years old would require a parent or guardian to be present. A separate MPOD study had been planned at Aston for teenagers. The lower age cut-off (< 20 years) was intended to reduce study non-attendance due to the higher fail to attend rates associated with the 16 to 19 year age group. At the time of the study protocol and ethics submission to Aston University, it was not certain whether an additional NHS ethics submission would have to be completed. We received advice that studies involving minors (those under the age of legal competence) would most likely require additional NHS ethics approval. Candidates were also advised that obtaining NHS ethics approval could take more than a year. This would have left insufficient time to complete the study within the original time frame. All patients aged 16 and under, and the majority of those aged under 20 years of age would have been NHS patients in full-time education. Excluding participants specifically because of their NHS status would have been unethical and an additional source of bias, therefore, in addition to the reasons given two paragraphs above the decision was made by the author to set the lower age limit for this study at 20 years. Suitable participants aged 20 years of age and above were included regardless of their NHS status. The author would argue if approached by a representative of the local NHS ethics committee, that their NHS status was not in any way related to their selection, therefore making additional NHS ethics approval unnecessary, after ethics approval from Aston University had been granted.
BMI	The lower limit of 20 Kg / m^2 was chosen to exclude participants with subnormal BMI due to athletic and weight loss programs and eating disorders. Obesity (BMI ≥ 30 Kg / m^2) has been reported to associate with lower levels of fat-soluble MP due to competitive absorption by adipose tissue.[164, 436]
VA	Although evidence for the acuity hypothesis for MPOD was not supportive,[657] reduced VA may indicate macular disease, which was associated with lower MPOD measurements in some,[596, 658] but not all studies.[593, 594, 655, 659] Eccentric fixation may lead to an underestimation of HFP-derived MPOD due to off-centre, central measurement.
Reported cholesterol status	Raised cholesterol may be related to lower serum HDL levels. Serum L levels were significantly associated with serum HDL, but not LDL.[660] Other sources have reported that L and Z are equally distributed between HDL and VLDL / LDL lipoprotein fractions.[454, 661] Participants were included if they reported that their doctor had advised that their total cholesterol level was currently normal, regardless of whether or not they were receiving any medical or dietary treatment for cholesterol. No time scale was defined for the term "currently". For this study total cholesterol was not defined in numerical terms because values are widely known to vary with factors such as age, gender and body weight, and the values defined as "normal" or "abnormal" may vary with the presence of concurrent medical conditions, the requirement for preventative therapy in high risk groups or genetic propensity for raised cholesterol. No participant asked for a numerical definition for normal or high total cholesterol. Participants unaware of their cholesterol status were recorded as "unknown". Therefore the included group contained participants with either "reported normal" or "unknown", rather than "confirmed normal" cholesterol levels. Reported raised cholesterol was significantly associated with lower MPOD levels in one study.[597]
Pregnancy	Pregnancy was associated with a 100% increase in serum L and a 50% increase in serum Z, which returned to normal levels by one-month *post-partum*.[662] Recorded as "yes", "no" or "unknown". Participants reporting "unknown" were included.
Smoking	Smoking is the most significant environmental RF for AMD development,[114] associated with two to three times AMD risk compared to non-smokers.[663] Smoking was associated with lower MPOD levels,[664] specifically at the central part of the MPOD spatial profile.[401]
Diabetes	Type 2 diabetics with or without retinopathy had reduced MPOD compared to non-diabetics.[665] Recorded as "yes", "no" or "unknown". Participants reporting "unknown" were included.
Intestinal malabsorption syndromes	Dietary absorption disorders such as Crohn's, ulcerative colitis, irritable bowel and participants with a history of bowel surgery were excluded. L and Z are taken up by mucosal cells, in the duodenum (first part of the small intestine) after bile-mediated emulsification into micelles.[440] Macular carotenoid absorption was enhanced by higher levels of co-consumed lipid and ascorbic acid,[666, 667] but may be reduced by β-carotene co-consumption.[450, 451, 668] Intestinal malabsorption syndromes such as coeliac and Crohn's disease are known to cause deficiencies in lipid-soluble nutrients and were associated with 37% lower MPOD compared to non-affected participants.[669] Recorded as "yes", "no" or "unknown". Participants reporting "unknown" were included.

2.2.5 Ethical approval / informed consent

This study was approved by the Aston University, Audiology / Optometry Research Ethics Committee (AOREC) on the 12th of May 2010. (Reference number AO2010.15 HB) and adhered to the tenets of the Declaration of Helsinki, (sixth revision, October 2008).[670] See appendix 5 for the confirmation of ethics clearance forms.

2.2.6 Instrumentation

MPOD measurements were obtained using the MPS 1000 screener, software version 0.42 (Tinsley Precision Instruments Ltd, Croydon, Essex). This method of MPOD testing was selected because it is easier for naïve and elderly subjects and was the least expensive method available when the study protocol was submitted.

LogMAR VA was assessed using a computer monitor running test chart software from Thompson Software Solutions (Test Chart 2000 Pro version 2.4.01). Test chart illuminance was 55 Lux (30 cm from screen) and consulting room ambient illumination was 82 Lux, measured in the position and direction of gaze of the participant (Sinometer LX1010BS Digital Lux Meter). Weight and height were measured using the WeightWatchers precision electronic scale, model 8965U and a stadiometer respectively.

Pupil size was measured to the nearest millimeter using a ruler with a millimeter scale in ambient room lighting.

2.2.7 Methods

2.2.7.1 Explanation for randomising the order of eye measurements for MPOD

In many studies examining the interocular difference in HFP-derived MPOD the order of right and left eye measurement is not reported. Several studies have examined the right eye first and then the left eye, a protocol that may result in a learning or fatiguing effect on the left eye result.[507, 586, 590] Heterochromatic flicker photometry is a psychophysical method characterised by a high level of background illumination and often performed, especially in a clinical setting, with no period of adaptation between successive measurements. Snodderley *et al*. reported that for female participants measured in the eye order right then left, mean MPOD was consistently and significantly lower in the left eye at two different visits separated by a week; $p < 0.001$ for visit 1 and $p < 0.05$ for visit 2.[507]

The protocol adopted by Liew *et al*. was to alternate the first eye measured for each subsequent twin pair tested (e.g. right then left for the first twin pair, left then right for the second twin pair etc.).[588]

In the present study the decision was made to randomise the order of right and left eye measurements in order to prevent any selection bias. A comparison could then be made at a single visit between right and left eye measurements, reducing the influence of any learning or fatigue effects that may have occurred as a consequence of the order that the eyes were measured.

2.2.7.2 Body mass index

Body mass index may be calculated by dividing participant height in metres by the square of their weight in kilograms. For this study participants were classified as normal weight (20 to 24.9), overweight (25 to 29.9) and obese (≥ 30) according to their BMI value.[671, 672] Other sources define a BMI value of 18.5 as the lower limit for normal weight individuals.[673] For the BMI and %BF comparisons, data were included for those participants with BMI values < 20 and ≥ 30. It is generally accepted that BMI increases with age.[674]

Body mass index was calculated fro the equation

$$\text{BMI (Kg / m}^2\text{)} = \text{weight (Kg)} / \text{height (m)}^2, \qquad \text{(Eq 2.2)}$$

2.2.7.3 Estimation of %BF from BMI, age and gender

Percentage body fat may be measured with skin calipers, bioelectrical impedance, hydrostatic weighing, dual-energy x-ray absorptiometry (DEXA) and air-displacement plethysmography. Measurement with skin calipers and bioelectrical impedance require the removal of clothing by the subject, whereas the latter three methods require expensive equipment. For these reasons an alternative method of estimating %BF was sought.

In the optometric practice setting percentage body fat may be estimated from BMI, and other factors such as age and gender by the use of one of several predictive algorithms.[607, 608, 675] The Clínica Universidad de Navarra - Body Adiposity Estimator (CUN-BAE) algorithm (see below) from Gomez-Ambrozi *et al*. was chosen for this study because their data was derived from a population of similar (Spanish) ethnicity.[608] Percentage body fat calculated using the CUN-BAE algorithm was highly correlated ($r = 0.89$, $p < 0.0001$) with actual %BF measured using air displacement plethysmography.[608]

Percentage body fat (CUN-BAE) was estimated according to the equation

$$\begin{aligned}\%BF = {} & -44.988 + (0.503 \times A) + (10.689 \times G) + (3.172 \times BMI) \\ & - (0.026 \times BMI^2) + (0.181 \times BMI \times G) - (0.02 \times BMI \times A) \\ & - (0.005 \times BMI^2 \times G) + (0.00021 \times BMI^2 \times A)\end{aligned} \qquad \text{(Eq 2.3)}$$

Where A = age (years), G = gender (male = 0, female = 1)

For this study participants were classified as normal weight (male ≤ 20%, female ≤ 30%), overweight (male 20.1 to 25%, female 30.1 to 35%) and obese (male > 25.1%, female > 35.1%) according to their CUN-BAE %BF value.[676]

2.2.7.4 LoA and 95% CI calculations and log10 back-transformation for Bland-Altman plots

Limits of agreement (LoA)

Bland and Altman reported that for a normal distribution, 95% of the differences (i.e. LoA) would lie between

$$d +/- 1.96s, \quad \text{(Eq 2.4)}$$

Where d and s are the mean difference and SD of the mean difference between the two data sets.[677]

Carstensen reported that using 2s, rather than 1.96s compensates partly for the omission of the 1/n term in the LoA equation and partly for ignoring the estimation of the variance.[678]

The correct multiplier for s used to derive the LoA is also dependent on the sample size

1.96s is appropriate if n > 668. < 2s, if n > 85 and 2.08s, if n = 30,[678] (Eq 2.5)

Where n = the sample size.

For this study 2s was used for n > 80, and 2.08s was used for n ≤ 30.

95% confidence intervals on each LoA

LoA are only estimates and therefore CI should be calculated and reported. The 95% CI on each LoA was calculated according to McAlinden *et al*.[679]

The CI on each LoA may be calculated according to

$$\text{LoA} +/- t_{0.05} \text{ for } (n-1)\text{df} \times \text{standard error}, \quad \text{(Eq 2.6)}$$

Where $t_{0.05}$ for (n-1)df represents the t distribution critical value (two-tailed) for α = 0.05, for n – 1 degrees of freedom. The t-values were retrieved from a t-distribution critical values table and the standard error (SE) was calculated from

$$SE = \sqrt{(3s^2 / n)}, \quad \text{(Eq 2.7)}$$

Where s = SD of the mean difference between the two data sets and n = number of data.

2.2.7.5 Iris colour

For this study participants were categorised according to six different iris colours (blue, grey, green, hazel, brown, black) as reported by Hammond *et al*,[636] by visual inspection by the author and confirmation by the participant. Any disagreement would be settled by a third party, by observation of the iris under natural daylight (if available at the time of the appointment) or normal room illumination if not. The designated iris colour was not disputed by any participant. No participants had black irides and so this category was removed, leaving five colour categories. Blue, grey and green irides were classed as light, and hazel and brown irides were classed as dark.

In order to increase group sizes for the investigation of MPOD and iris colour with gender, grey blue and green iris colours were classified as "light" and brown iris colours (hazel and brown) were classed as "dark" according to Murray *et al.* and Kirby *et al*.[401, 680]

2.2.7.6 Comparison of relative retinal illuminance for different iris colours (blue and brown) and pupil sizes.

The pupil area is 16 times less (50.27 mm / 3.14 mm = 16) for a 2 mm compared to an 8 mm pupil. This would equate to 16 times (93.8%) lower retinal illuminance for the 2 mm pupil, ignoring the effect of light transmission through the iris (table 2.10, results section).

It is accepted that some light will pass through the iris pigment epithelium. Watts reported that for humans, brown irides transmit 5.5% (SD 2.8%) and blue irides transmit 14% (SD 6.3%) of incident light.[681]

The following equations were used to calculate the data in table 2.10

$$\text{Pupil area calculated using } \pi.r^2, \qquad \text{(Eq 2.8)}$$

$$\text{Iris area calculated from } \pi.r^2 \text{ (iris) - } \pi.r^2 \text{ (pupil)}, \qquad \text{(Eq 2.9)}$$

Where r is the pupil or iris radius in mm.

Retinal illuminance is calculated from

$$T = LS \qquad \text{(Eq 2.10)}$$

Where L is the luminance of the stimulus in cd/m^2 and S is the pupil area in mm^2.

Object luminance being equal, relative retinal illuminance for different pupil sizes may be calculated for the effect of pupil size only, and for the combined effect of pupil size and blue and brown irides

Retinal illuminance (pupil only, excluding iris) = pupil area x 0.945, (Eq 2.11)

Retinal illuminance (blue iris) = ((iris area x 0.14) + pupil area) x 0.945, (Eq 2.12)

Retinal illuminance (brown iris) = ((iris area x 0.055) + pupil area) x 0.945, (Eq 2.13)

Assuming 5.5% loss due to Stiles-Crawford effect.

2.2.7.7 Group analysis of MPOD with age (the 50-year cut-off)

In order to perform an independent-samples t-test to compare MPOD scores with age, it was necessary to create one categorical independent variable (age) with two different levels (younger and older) from the continuous data. The statistical software (SPSS) provides a function termed "visual binning" which allows the user to divide continuous data into two or more discrete groups of approximately equal number of data. A second option would be to review the literature and select age ranges reported by other authors. Comparing HFP-derived MPOD and age, Murrey *et al*. reported MPOD for subjects under and over 60 years of age[680] and Demirel *et al*. reported MPOD for subjects under and over 50 years of age.[682]

Many visual parameters (e.g. contrast sensitivity and visual evoked potential latency) in normal populations exhibit a biphasic relationship with age, characterised by functional stability up to approximately 50 years of age, after which an abrupt age-related decline in function is observed.[683] This decline is thought to be caused by changes in the neural system rather than the effects of media opacification or pupil miosis.[684]

For the group analysis of MPOD with age in this study, participants were categorised as younger (≤ 50 years) and older (> 50 years) because it is feasible that the results obtained from HFP, a psychophysical method of testing, may also be affected by the decline of function in those over 50 years of age. In addition, selecting a 50-year rather than a 60-year cut-off created groups that were more equal in size.

2.2.7.8 Categorisation of ocular vascular risk factors

In this practice-based study the author did not have access to equipment required to measure ocular blood flow directly, therefore the conditions associated with vasospasm; migraine, Rph and VDys were selected as markers for abnormal ocular perfusion. These conditions were selected as they present with symptoms that are readily assessed in an Optometric practice setting.

Participants were asked whether or not they experienced migraines. Individuals reporting that they experienced migraines were also asked whether or not their migraines were associated with aura and whether or not they were light-triggered.

Participants were classified as individuals with Rph if they answered positively to the question; "Do your hands and / or feet go white or blue when they get cold?"

There is no criterion standard for the diagnosis of VDys.[205] Participants were classified as individuals with VDys if they answered positively to the question; "Do you get cold hands and / or feet whatever the temperature of environment?"

2.2.7.9 Measurement of ocular dominance

Ocular dominance was categorised using the Porta test variant of the Miles test described by Roth *et al*.[685] This test was selected as it is easy to perform and uses equipment available in all optometric consulting rooms. Participants were asked extend one arm and align their index finger on this arm with a single letter on the test chart six metres away, with both eyes open. The author alternately covered each of the participants' eyes with an occluder and the participants reported which eye when occluded caused the largest alignment change of the target. The dominant eye was recorded as the eye that when covered caused the largest change in alignment. If the change of alignment was judged to be equal for both eyes, the participant was classified as equi-dominant.

2.2.8 Procedure

Procedure for data collection

Subjects were pre-adapted to normal room illumination for 10 min, during which time the consent forms were read and signed.

Table 2.6 Procedure for first session data collection (MPOD)

Sequence	Procedure
1	Pre-measurement exclusion factors were reviewed.
2	LogMAR VA and distance fixation, pupil size measurements were recorded for both eyes. Gender, date of birth and iris colour were also recorded.
3	Maculae were examined with direct ophthalmoscope, through non-dilated pupils for visible signs of pathology.
4	Medication and nutritional supplements were recorded.
5	The order of eye measurement was determined by the pseudo-random method of coin toss.
6	Participants reporting a history of migraine or epilepsy were warned about the risk of light-triggered symptoms.
7	A single central and peripheral MPOD measurement was obtained for both eyes, under normal room illumination. The non-tested eye was occluded with an opaque eye patch. Distance glasses (non-tinted) were worn for MPOD measurement. If none were available or if contact lenses were worn these were removed and the equivalent distance prescription in a trial frame was substituted.
8	Participants' weight and height were measured. BMI was calculated from Eq 2.2.
9	Exclusion factors were reviewed after the measurements above were recorded.
10	Percentage body mass was calculated after data collection was complete, from the BMI, age and gender data using the Clinica Universidad de Navarra-Body Adipose Estimator (CUN-BAE) algorithm.[608]
11	Eye dominance data measured by finger pointing and alternate occlusion, a variation of the Miles test,[685] was collected retrospectively for 42 subjects, at their subsequent routine eye appointments.

2.2.8.1 Time scale for data collection

Data was collected from participants on days when no clinic was running, every Wednesday or every other Wednesday, depending on room usage in this single consulting room practice.

Data for the first session (MPOD and GRT measurements) were collected from the 4th of August 2010 to the 12th of October 2011.

The examination time required for each participant in the first session was approximately 50 min; therefore appointments were scheduled at one-hour intervals. This limited the number of participants seen to a maximum of 16 to 32 each month, depending on room availability.

Table 2.7 First session procedure for MPOD measurement (up to 23 min per subject)

Informed consent, exclusion criteria and explanation of MPOD procedure	MPOD 1st eye	MPOD 2nd eye	discussion of MPOD results and procedure for GRT
10 min	5 min	5 min	3 min

Glare recovery time was measured for each eye at a minimum of eight and 18 s after MPOD measurements for each eye (see table 3.6 in the following chapter).

2.2.9 Randomisation / masking

The order of eye measurement was decided by coin toss, heads = right eye first, tails = left eye first. A different coin was used each day to avoid bias to one side of the coin. No masking was used in this study.

2.2.10 Statistical analysis

Statistical analyses were performed using SPSS 22 statistical software (IBM Corporation). Data were examined for normality using histograms, normal Q-Q plots, Shapiro-Wilk tests and corrected Kolmogorov-Smirnov tests. Pearson product-moment correlation coefficient was calculated between MPOD and each of the independent variables. Student's t-test and one-way ANOVA were used to compare the within-group differences in the independent variables with MPOD. Non-parametric tests were used where normality was not demonstrated or where group sizes where smaller than 25. Significance testing was two-tailed unless otherwise stated.

2.2.11 Study design

The cross-sectional study design was deemed the most suitable for this practice-based project. This type of study design is rated low on the traditional hierarchy of evidence, according evidence-based medicine,[686] having strengths and weaknesses compared to other study designs. Strengths include: being quick and easy to conduct, data is only collected once, prevalence may be measured, multiple exposures may be studied and the design is good for descriptive analysis and hypothesis generation. Weaknesses include: the

inability to demonstrate cause and effect, the inability to measure incidence, associations may be difficult to interpret and this study design is susceptible to bias due to low response and misclassification due to recall bias.[687]

2.3 Results

Data from 100 White participants, naïve to previous MPOD measurement were included in this study. Mean MPOD for the first eye measured was 0.39 (SD 0.16). Mean age was 50.3 years (SD 10.4 years), ranging from 24.2 to 75.8 years. The number of male and female participants was 27 (27%) and 73 (73%), respectively. Unless otherwise stated, MPOD results are presented for the first eye measured, derived from 44 right eye and 56 left eye measurements.

To obtain a fuller understanding of the relationship with BMI and %BF, participants excluded for low (n = 4) and high (n = 12) BMI were re-included for these analyses only. These re-included participants had no additional reasons for exclusion other than reported raised cholesterol, which is associated with raised BMI. The mean age for the 116 White participants was 51.0 years (SD 11.0 years), ranging from 24.2 to 75.8 years. The number of male and female participants was 32 (28%) and 84 (72%), respectively. Expressed as a percentage of the population examined, normal weight participants represented 48.3%, median age 45.6 (IQR 12.1), overweight 41.4%, 51.9 (IQR 17.0) and obese 10.3%, 60.7 (IQR 20.0).

Ocular dominance was recorded retrospectively for 49 cases. Four equidominant cases were excluded. Data was missing for one eye in one case. Ocular dominance was confirmed for 30 right (68.2%) and 14 left (31.8%) eyes. Median MPOD values for dominant, non-dominant and equidominant eyes were; 0.41 (interquartile range, IQR 0.21), 0.46 (IQR 0.28) and 0.53 (IQR 0.20), respectively. Participants mean age was 50.0 years (SD 11.4 years), ranging from 24.2 to 75.8 years. The number of male and female participants was 14 (32%) and 30 (68%), respectively.

This section is limited to results that are significant, approaching significance or have not been reported previously. For a full summary of the results for this chapter please refer to appendix (A1.1). The "A" prefix indicates that the figure or table referred to may be found in the appendix.

2.3.1 Demographics for first eye MPOD measurements

Table 2.8 Summary of results for first eye MPOD

Variable	Subcategory		Number of data n	Mean MPOD Median MPOD	SD IQR
Age (years)	Full age range		100	0.39	0.16
	≤ 50		57	0.37	0.16
	> 50		43	0.43	0.17
Gender	Male		27	0.40	0.18
	Female		73	0.39	0.16
BMI (both genders)	Slim	< 20	4	0.46	0.12
(n = 116)	Normal	20 to < 25	52	0.40	0.24
	Over-weight	25 to < 30	48	0.41	0.23
	Obese	≥ 30	12	0.29	0.23
BMI (male)	Slim	< 20	0	-	-
(n = 32)	Normal	20 to < 25	7	0.41	0.39
	Over-weight	25 to < 30	20	0.39	0.26
	Obese	≥ 30	5	0.34	0.31
BMI (female)	Slim	< 20	4	0.46	0.12
(n = 84)	Normal	20 to < 25	45	0.38	0.24
	Over-weight	25 to < 30	28	0.42	0.20
	Obese	≥ 30	7	0.26	0.19
Male %BF (CUN-BAE)	Lean	≤ 20%	2	0.48	-
(n = 32)	Over-weight	> 20 to 25%	6	0.45	0.29
	Obese	> 25%	24	0.36	0.27
Female %BF (CUN-BAE)	Lean	≤ 30%	7	0.46	0.14
(n = 84)	Over-weight	> 30 to 35%	25	0.36	0.24
	Obese	> 35%	52	0.41	0.22
Iris colour	Grey		12	0.42	0.23
	Blue		33	0.41	0.22
	Green		15	0.36	0.28
	Hazel		16	0.37	0.24
	Brown		24	0.46	0.38
	Black		0	-	-
Reported AMD FH	First and second degree		17	0.36	0.22
	First degree only		11	0.36	0.24
	Second degree only		6	0.34	0.16
	None		82	0.41	0.27
	Unknown (adopted)		1	0.46	-
Reported migraine	Yes		17	0.36	0.22
	Light-triggered		6	0.43	0.31
	Non-light-triggered		11	0.31	0.20
	Aura		10	0.41	0.26
	No aura		7	0.31	0.20
	No migraine		83	0.41	0.26
Reported Rph	Yes		27	0.41	0.14
	No		72	0.39	0.17
	Unknown		1	0.12	-
Reported VDys	Yes		28	0.39	0.15
	No		69	0.39	0.17
	Unknown		3	0.46	0.21
Pupil size	≤ 3.9mm		40	0.38	0.17
	≥ 4mm		49	0.41	0.16
	Unknown		11	0.37	0.15
Difficulty with MPOD measurement	Yes		21	0.41	0.25
	No		79	0.41	0.24

Abbreviations. IQR: interquartile range, CUN-BAE: Clínica Universidad de Navarra - Body Adiposity Estimator. Median and IQR (in grey) are shown rather than mean and SD for groups with less than 25 cases, unless these groups were excluded from analysis. Non-parametric testing was used for the analysis of groups containing < 25 cases.[688]

Fifty of the 150 participants were excluded from this study. Please refer to tables A1 and A2 in Appendix A1 for a summary of the reasons for exclusion and frequency analysis of those excluded.

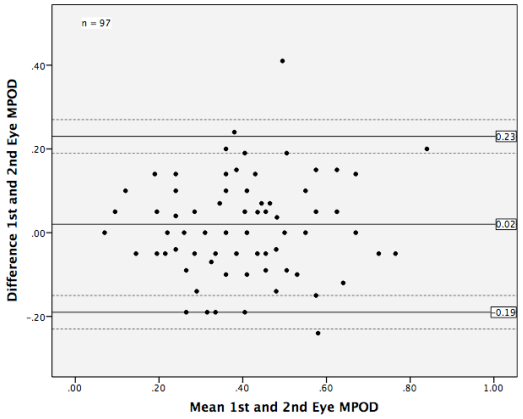
2.3.2 Interocular comparison of MPOD

2.3.2.1 First versus second eye sequential MPOD measurements

Data was missing for one eye in three cases. No significant difference between first and second eye MPOD was found, with ($p = 0.49$) or without ($p = 0.61$) a single outlier. The relationship between first and second eye MPOD was investigated using Pearson product-

moment correlation coefficient. Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity. There was a large, positive correlation between first and second eye MPOD measurements ($r = 0.79$, $n = 97$, $p < 0.001$), which remained significant after the removal of one outlier ($r = 0.81$, $n = 96$, $p < 0.001$). See fig. 2.1 for the Bland-Altman plot.

Figure 2.1 Bland-Altman plot for first versus second eye MPOD

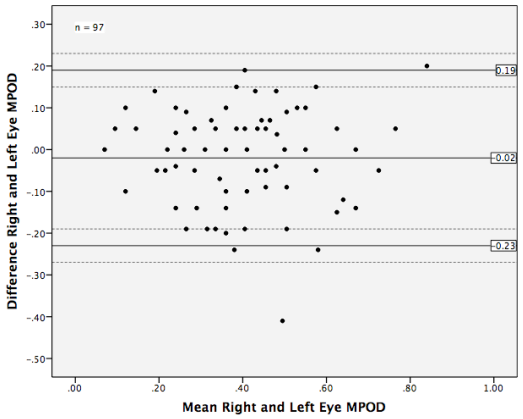


Mean (first and second eye MPOD) = 0.39 (SD 0.15).
Mean difference = 0.02 (SD 0.11).
Limit of agreement (n = 97) = 0.21 (95% CI 0.04).
After removing one outlier, LoA (n = 96) = 0.20 (95% CI 0.04).

2.3.2.2 Right versus left eye randomised MPOD measurements

Data was missing for one eye in three cases. No significant difference between right and left eye MPOD was found, with ($p = 0.47$) or without ($p = 0.58$) one outlier. There was a large, positive correlation between first and second eye MPOD measurements ($r = 0.79$, $n = 97$, $p < 0.001$), which remained significant after the removal of a single outlier ($r = 0.81$, $n = 96$, $p < 0.001$). See fig. 2.2 for the Bland-Altman plot.

Figure 2.2 Bland-Altman plot for right versus left eye MPOD



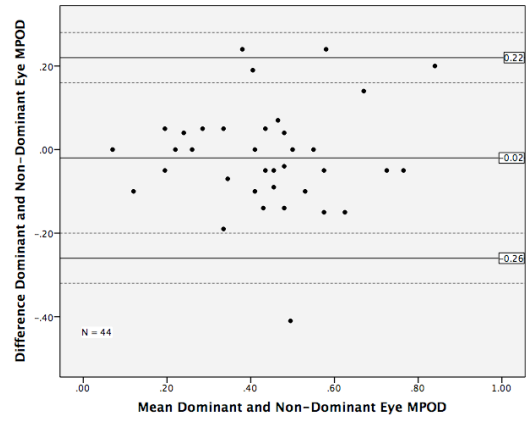
Mean (right and left eye MPOD) = 0.39 (SD 0.15).
Mean difference = -0.02 (SD 0.11).

Limit of agreement (n = 97) = 0.21 (95% CI 0.04).
After removing one outlier, LoA (n = 96) = 0.20 (95% CI 0.04).

2.3.2.3 Dominant versus non-dominant eye randomised MPOD measurements

No significant difference between dominant eye and non-dominant eye MPOD was found, with (p = 0.68) or without (p = 0.87) one outlier. There was a large, positive correlation between dominant and non-dominant eye MPOD measurements (r = 0.77, n = 44, p < 0.001), which remained significant after the removal of outliers (r = 0.82, n = 43, p < 0.001). See fig. 2.3 for the Bland-Altman plot.

Figure 2.3 Bland-Altman plot for dominant versus non-dominant eye MPOD

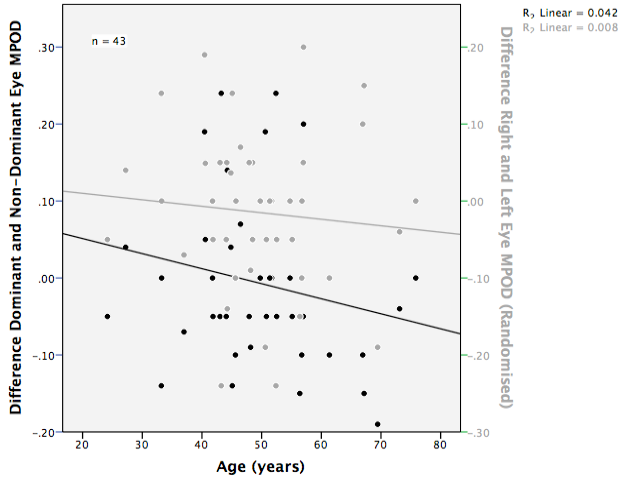


Mean (dominant and non-dominant eye MPOD) = 0.43 (SD 0.17).
Mean difference = -0.02 (SD 0.12).
Limit of agreement (n = 44) = 0.24 (95% CI 0.06).
After removing one outlier, LoA (n = 43) = 0.21 (95% CI 0.06).

2.3.2.4 The effect of age on the difference between dominant and non-dominant eye MPOD measurements

In an attempt to assess whether MPOD levels in the dominant eye were reduced compared to the non-dominant eye as a consequence of increased light exposure over the duration of life, the difference between dominant and non-dominant eye MPOD and the difference between right and left eye MPOD (randomised) for the same cases (n = 44) was plotted against age. Fig. 2.4. Pearson correlation was r = -0.32, r^2 = 10.4% p = 0.03 and r = -0.23, r^2 = 5.1%, p = 0.14 for the difference between dominant and non-dominant eye, and randomised right and left eye MPOD measurements, with age respectively. The larger correlation in the ocular dominance group was retained after the removal of one outlier (-0.41) from both groups (n = 43), however, the relationship was no longer significant; r = -0.21, r^2 = 4.2%, p = 0.19 and r = 0.09, r^2 = 0.8%, p = 0.57, respectively.

Figure 2.4 Difference between dominant and non-dominant eye MPOD with age



2.3.2.5 Calculation for sequential bias

Sequential bias was calculated by performing an independent samples t-test on the difference between sequential (first minus second eye) and randomised (right minus left eye) MPOD measurements. No significant bias was detected with or without outliers.

2.3.2.6 Calculation for bias due to ocular dominance

Bias due to ocular dominance was calculated by performing an independent samples t-test on the difference between eye dominance (dominant minus non-dominant eye) and randomised (right minus left eye) MPOD measurements. No significant bias was detected with or without outliers.

2.3.3 MPOD and AMD risk factors

The relationship between MPOD and the AMD RF was investigated using Pearson product-moment correlation coefficient. Preliminary analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity

No significant correlation with MPOD was found for age, male age, female age, Total BMI, male BMI, female BMI, male %BF, female %BF or pupil size, with or without outliers. Small correlations with MPOD were found for several variables, but the failure to reach significance despite a large sample size (in most cases) suggests that the level of confidence that these results are genuine is likely to be small.

Group analysis revealed significantly higher MPOD in the ≥ 50 to < 60 years age range.

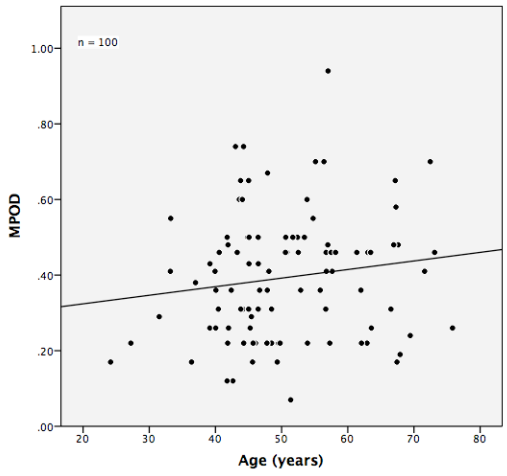
Table 2.9 Significant associations between MPOD and age

Variable		n	MPOD mean & SD (or Median and IQR shown in grey)	Statistic	P-value	Size effect (if significant)
Age 4 age groups (years)	< 45 ≥ 45 to < 50 ≥ 50 to > 60 ≥ 60	32 25 24 19	0.37 (0.24) 0.31 (0.20) 0.46 (0.13) 0.41 (0.24)	8.066*	**0.045**	-
Post-hoc tests Two follow-up Mann-Whitney U tests α level = 0.025	≥ 45 to < 50 ≥ 50 to > 60	25 24	0.31 (0.20) 0.46 (0.13)	U = 155.5 Z = -2.907**	**0.004**	0.291
	≥ 50 to > 60 ≥ 60	24 19	0.46 (0.13) 0.41 (0.24)	U = 166.5 Z = -1.516**	0.130	-

*Kruskal-Wallis test, ** Mann-Whitney U test. Size effect for Mann-Whitney U test: small = 0.1, medium = 0.3, large = 0.5.

No significant associations were observed for gender, total BMI, male BMI, female BMI, male %BF, female %BF, mixed-gender iris colour, male iris colour, female iris colour, AMD FH and pupil size, with or without outliers. Fig. 2.5 shows a scatter plot of MPOD and age. The possible outlier with an MPOD value of 0.94 was retained as this was within what would be considered the normal range of MPOD values. Removal of this case did not alter the direction or significance of the correlation between MPOD and age.

Figure 2.5 Scatter plot for MPOD and age



Pearson r = 0.14, r^2 = 2.1%, p = 0.15.

Pearson r (0.94 MPOD value case removed) = 0.13, r^2 = 1.7%, p = 0.20.

2.3.3.1 Results for the comparison of relative retinal illuminance for different iris colours and pupil sizes.

Table 2.10 shows the results for the estimated values for the difference in retinal illuminance for different iris colours (blue vs. brown) across a range of pupil sizes (2-8 mm), calculated from Eq 2.8 to Eq 2.13 in the methods section.

Table 2.10 Relative retinal illuminance for blue and brown irides with pupil size

	Pupil size (mm)						
	2	3	4	5	6	7	8
Pupil & Iris Area (mm^2)							
Pupil area	3.14	7.07	12.57	19.64	28.27	38.49	50.27
Iris area	109.96	106.03	100.53	93.46	84.82	74.61	62.83
Relative Retinal illuminance (Td)							
For pupil size only, excluding the effect of iris transmission	2.97	6.68	11.88	18.56	26.72	36.37	47.51
Difference in retinal illuminance for pupil size compared to 2 mm pupil size	x 1	x 2.25	x 4	x 6.25	x 9	x 12.25	x 16
For eyes with blue irides, including the effect of pupil transmission	17.52	20.70	25.27	30.92	37.94	46.24	55.91
For eyes with brown irides, including the effect of pupil transmission	8.68	12.19	17.10	23.42	31.13	40.25	50.77
Difference in retinal illuminance for iris colour (blue vs brown)	x 2.02 higher for blue eyes	x 1.70 higher for blue eyes	x 1.48 higher for blue eyes	x 1.32 higher for blue eyes	x 1.22 higher for blue eyes	x 1.15 higher for blue eyes	x 1.10 higher for blue eyes

Assumptions: Retinal illuminance was calculated using Eq 2.10 with a luminance value of 1 cd/m2. 100% transmission through the pupil, 14% through blue iris and 5.5% through brown iris. Iris light transmission is uniform across pupil and iris area. Pupil size was assumed to be equal for blue and brown irides. No light absorption by ocular media. Scleral transmission was ignored for this calculation, but is likely to be greater for those with blue irides. Pupil and iris are spherical. Iris diameter = 12mm. The reduction (5.5%) in retinal illuminance due to the Stiles-Crawford effect was equal for all pupil sizes.

2.3.3.2 MPOD and pupil size

A non-significant, small positive correlation was found between MPOD and undilated pupil size measured in ambient illumination. After the removal of two outliers and correction for age using partial correlation a small positive correlation was revealed ($r = 0.22$, $p = 0.049$).

2.3.3.3 Macular pigment optical density and OVP RF

Group analysis revealed no significant differences between MPOD and presence / absence of migraine, migraineous aura, light trigger for migraine, Rph and VDys, with or without outliers.

2.3.4 Difficulty with the HFP task

All 100 participants were naïve to previous MPOD measurement. Overall 21 (21%) of the participants, 6 male and 15 female, ranging in age from 31.5 to 72.5 years (median 53.9 years), experienced some difficulty with this method of MPOD measurement for one or both eyes. 21 subjects repeated the peripheral test and 4 subjects repeated the central test. The remaining 79 participants ranged in age from 24.2 to 75.8 years (median 47.9 years). Age estimated central results were obtained for 6 of the 21 subjects, who were unable to obtain a peripheral result after three attempts. Group analysis using the Mann-Whitney U-test for difficulty with MPOD measurement with MPOD ($p = 0.56$), age ($p = 0.38$) and GRT ($p = 0.25$) revealed no significant association with any variable.

2.3.5 Other interesting findings

Strabismus / eccentric fixation

Two participants had unilateral strabismus. MPOD measurements from the strabismus and non-strabismus eyes were 0.02, 0.07 and 0.22, 0.26, respectively.

Poor fixation of peripheral target

One participant was unable to fixate the peripheral target without gaze returning to the central target. MPOD measurement was 0.

Central floater

One participant had a large floater affecting central vision in one eye. MPOD values were 0.02 for the affected eye and 0.46 for the unaffected eye.

Coloboma

One participant presented with unilateral coloboma. MPOD values were 0.07 for the affected eye and 0.22 for the unaffected eye.

2.4 Discussion

The key results and how they compare to those of other studies are discussed below. Unless otherwise stated comparison was limited to studies using HFP with a central target eccentricity of 0.5° and a population consisting of White or predominantly White ethnicity.

A literature search was performed using Web of Science, Science Direct, PubMed Central (PMC) and Google Scholar for the following search terms: glare recovery and photostress recovery combined with interocular, ocular dominance, age, gender, body mass index, percentage body fat, iris colour, pupil size, AMD family history, migraine, Raynaud's and vascular dysregulation. Wildcard symbols were used to search for variations in spelling. Further references were retrieved from the papers revealed by the literature search.

2.4.1 Interocular comparison

The mean MPOD value for this 100% White, UK population was 0.38 (SD 0.17) and 0.40 (SD 0.16) for the right and left eyes respectively (n = 97, three monocular cases excluded). The mean difference in randomized MPOD measurements (-0.02 SD 0.11) was not significant with or without one outlier.

These results were higher than those reported by studies using the MPS 9000, a later version of the MPS 1000, with mean values ranging from 0.32 to 0.35,[499, 689, 690] but comparable with the range of mean MPOD values obtained by HFP with a peripheral target eccentricity of more than 6° (0.34 to 0.42).[507, 654, 691] Variation in mean values obtained from

the same MPOD measurement technique may be explained by differences in macular carotenoid dietary intake between studies and other population differences such as age or BMI.

The LoA (CI 95%) was 0.21 (0.20 with one outlier removed), suggesting that an interocular difference outside this value may be regarded as abnormal. The normal range of values reported by this study for the right eye (0.18 to 0.58) was slightly greater at the higher MPOD range than that reported by the MP Consensus Panel (0.20 to 0.50).[439] This may be reflection of population differences in macular carotenoid intake and method of MPOD measurement (single measurement compared to means of multiple measurements).

Kanis *et al*. reported a Pearson correlation of 0.93 ($p < 0.001$) and an intra-class correlation coefficient (for absolute agreement) of 0.91 ($p > 0.001$) for an objective method of MPOD measurement (Foveal Reflection Analyzer). The authors reported that an interocular difference in MPOD of 34% might indicate of pathology.[586] The slightly smaller normal range of MPOD reported by Kanis *et al*. is likely to be a consequence of differences in measurement methods between the two studies.

The present study used HFP (MPS 1000) for which same-eye repeatability (using the later version; MPS 9000, also known as QuantifEye in the USA) has been reported by three other studies for healthy subjects. de Kinkelder *et al*. reported the mean difference in MPOD for repeat measures of the right eye was -0.02, with LoA of 0.18 and a mean relative difference of 18.1%.[492] Bartlett *et al*. reported coefficients or repeatability for right eye data (or left eye if right eye was excluded) of 0.33 and 0.28, respectively for two operators, on average values derived from four sets of readings performed after an initial practice reading. Coefficient of repeatability was calculated by multiplying the SD of the mean differences by 1.96. No learning or fatigue effect was reported using ANOVA ($F = 1.463$, $p = 0.240$).[499] Loughman *et al*. assessed inter-session repeatability for the eye with the best VA (or the dominant eye if VA was equal for each eye). Values for the coefficient of repeatability were 0.18 (visit 1-visit 2), 0.21 (visit 2-visit 3) and 0.18 (visit 1-visit 3). No learning or fatigue effect between repeat measures of MPOD was detected, indicated by a non-significant result for Mauchley's test of sphericity.[690]

Berendschot and van Norren also used the FRS method of MPOD measurement, for which the mean within subject variation was reported to be 2.5% to 5% for their more recent setup which allows for the determination of the directional component of fundus reflectance.[487] Hammond *et al*. reported high levels of interocular correlation (using the intra-class correlation coefficient for absolute agreement), for HFP (instrument not reported) ($r = 0.76$, p not available) and 2-WFAF ($r = 0.96$, p not available).[692]

Although this study found that mean MPOD was slightly lower in the right eye, this difference was not significant. Seven other studies, including one of non-White ethnicity also reported lower or a trend toward lower HFP-derived MPOD values for the right eye.[500, 586, 590, 596, 597, 652, 693] Four studies (Murray *et al.* included Whites and Asians) reported higher values, or a trend towards higher values for the right eye,[507, 587, 637, 680] and three studies,[591] (two from the same twin study population),[588, 589] reported equal right and left MPOD values.

Interocular correlation was high; Pearson r = 0.79, p < 0.001 (without outlier; r = 0.81, p < 0.001). The level of interocular correlation was higher than that reported by Hagen *et al*., comparing a single value of MPOD for each eye using the MacuScope screener (Macuvision Europe Ltd., Solihull, UK). Pearson r = 0.43, p < 0.014 (visit 1), Pearson r = 0.43, p < 0.038 (visit 2) and Pearson r = 0.58, p < 0.003 (visit 3). Between visit variability demonstrated by the coefficient of variance for all three measurements was 36.1% for all the right eyes and 23% for all the left eyes.[590] The results obtained for the present study were similar to other studies comparing a single value of MPOD for each eye using MPS 9000. Abell *et al*. a high level of interocular agreement (coefficient not specified) of r = 0.893, p < 0.01.[500] Murray *et al*. used age-estimated centre-only readings, reporting a Pearson correlation for right and left eyes of r = 0.7, p< 0.001.[680]

The results from the present study also compared favourably to studies comparing the means of multiple MPOD values. Snodderly *et al*. reported a high level of interocular correlation of MPOD values based on five readings per target, measured at two separate visits using the macular densitometer (Macular Metrics Corp., Rehoboth, MA); Pearson r = 0.79 (visit 1) and r = 0.80 (visit 2), p values not available.[507] Beatty *et al*. reported good interocular agreement from the means of three to six readings of HFP-derived MPOD values using simple regression; r = 0.866, p < 0.001.[596] Iannaccone *et al*. used the macular densitometer with a modified protocol limited to three readings per MPOD measurement. The level of interocular correlation was high; Pearson correlation, r = 0.82, p < 0.0001.[654]

It was unexpected that the level of interocular correlation obtained by this study for naïve subjects and single central and peripheral readings would be comparable to that from studies comparing means of multiple MPOD readings. It is possible that this was a consequence of the stringent exclusion criteria imposed by this study, the randomised order of the right and left eye measurements, designed to limit the effect of learning and fatigue and the use of flicker detection rather than minimisation, designed to be easier for naïve subjects.

The results for interocular comparison from studies using objective methods of MPOD assessment (2-WFAF, FR and RRS) indicated a higher correlation compared to that from

HFP studies. Hammond *et al*. reported an interocular intra-class correlation (for absolute agreement) of r = 0.96 for MPOD measurements using 2-WFAF.[692] Liew *et al*. reported an intra-class correlation (for absolute agreement) for right and left eyes of 0.96 with 2-WFAF.[588] Liew *et al*. reported in a later study that intra-class correlations (for absolute agreement) ranging from 0.91 to 0.97 for interocular MPOD measurements using 2-WFAF measured at retinal eccentricities of 0° (fovea), half-degree, 1° and 2°.[589] Kanis *et al*. used the Foveal Reflection Analyzer, a method of FR used previously by Berendschot and van Norren,[694] to measure MPOD from both eyes. The authors (Kanis *et al*.) reported a significant linear relation between interocular measurements, Pearson r = 0.93, $p < 0.001$ and an intra-class correlation coefficient (for absolute agreement) of 0.91, $p < 0.001$.[586] Two studies using RRS have reported interocular comparisons for MPOD. Neelam *et al*. reported good interocular agreement in MPOD levels. The mean difference in Raman scores was 2.37 ± 324.94, with a maximum interocular difference of 804 (Wilcoxon signed rank test, p = 0.669).[652] Gellerman *et al*. reported that MPOD levels correlated well between right and left eyes in normal subjects aged from 21 to 84 years. The authors did not provide correlation coefficients for the interocular comparison.[695]

Objective methods were associated with higher levels of interocular correlation compared to HFP. This is likely to be the result of lower instrument bias associated with objective methods.

It is a testament to the changes made to the design of the MPS screener to simplify the HFP task for naïve and elderly participants. These results support the protocol for taking a single central and peripheral MPOD reading with the MPS screener, rather than multiple readings in the practice setting. If time is not limited it has been recommended that multiple readings should be taken.[690]

Non-pathological causes of uniocular reduction in MPOD revealed by this study included; eccentric fixation secondary to strabismus, poor fixation of the peripheral target, vitreous floaters affecting central vision and coloboma.

No significant interocular difference in MPOD was detected by this study. This is in agreement with the consensus drawn from the literature on this subject for healthy eyes, suggesting minimal interocular differences.[680] Although the two eyes are treated as independent for statistical purposes,[696] MPOD levels would be expected to be similar in healthy eyes as they share dietary, circulatory, environmental, systemic pathological and genetic factors that may influence MPOD levels.

If the interocular difference, whether significant or not significant, falls within the noise level for the instrument used to measure MPOD, the results should be viewed with caution. In

this scenario it would not be clear whether the interocular difference represented a genuine difference or was simply the consequence of instrument noise. Bartlett *et al*. assessed the later version of this instrument (MPS 9000) for two operators, reporting coefficients of repeatability of 0.28 and 0.33, respectively.[499] Ideally, the present study would have assessed the repeatability of MPOD measurements for this operator and population, however time constraints on data collection in this practice-based study prevented this.

The results suggest that it would be acceptable in practice to measure MPOD from one eye because in the absence of any pathological or physiological reason for low MPOD in either eye, the MPOD values should be similar.

2.4.2 Investigation of sequential bias

The mean MPOD values for the first and second eye tested sequentially (n = 97, three monocular cases excluded) were 0.40 (SD 0.17) and 0.38 (SD 0.16). The first eye measurement was higher than the second eye measurement. The mean difference in sequential MPOD measurements, +0.02, (SD 0.011) was not significant with or without one outlier however. The difference may be a consequence of learning or fatigue however the effect of second eye occlusion (about five min), during first eye testing could not be discounted.

Sequential bias was calculated by performing an independent t-test of the interocular difference in MPOD values measured randomly and sequentially. With randomized measurements as the reference, the mean difference between measurements was significant; -0.03 (95% CI -0.06 to 0.04), p = 0.03, eta^2 = 0.02 (small effect). Eta^2 represents the effect size calculated for the independent samples t-test. The value of eta^2 is classified as small (≤ 0.01), medium (≤ 0.06) and large (≥ 0.14). Eta^2 expressed as a percentage (multiplied by 100) indicated that in this case the effects of sequential bias explained 2% of the variance in MPOD. After the removal of one outlier, no significant bias due to sequential measurement of MPOD was found (p = 0.08).

Sequential and randomised MPOD measurements did not reveal any significant differences, however the bias between these two measurements was significant (and was close to significance after the removal of a single outlier from the MPOD data). These results suggest that the measurement of each eye in quick succession (no rest period between measurements) will contribute to the variability in MPOD measurements due to machine and operator bias. The LoA on HFP MPOD measurements is large, often larger than the change in MPOD that is expected after dietary or supplementary MP modification. Because HFP MPOD measurement is a psychophysical test it involving a bright background it would make sense to allow a resting period similar to that suggested between GRT measurements (e.g. 10 min) to allow cone recovery. This may reduce variability in

MPOD measurements, however it would make repeated measurements (recommended for HFP) less attractive in the clinical setting.

2.4.3 Investigation of bias due to ocular dominance

Approximately 67% of the general population exhibits right eye dominance.[154-156, 697] The non-dominant eye is closed to reduce glare when exposed to sunlight,[153] exposing the dominant eye to an increased level of sunlight exposure compared to the non-dominant eye. It may therefore be plausible that ocular dominance could represent a surrogate marker for ocular light exposure, with the dominant eye exhibiting signs of ocular light damage earlier the non-dominant eye.[153, 157]

Neelam *et al*. reported a trend for higher MPOD in the dominant eye (0.312) compared to the non-dominant eye (0.303), however the association did not reach significance, p = 0.234.[591] This study and the study reported in this thesis used the same method of categorising ocular dominance; a variation of the Miles test described by Roth *et al*.[685]

The relationship between ocular dominance and MPOD was investigated in order to establish whether the potential for increased light exposure could explain the lower MPOD values reported for the right eye (two thirds of which would show ocular dominance) in this and other studies.

The percentage of right eye dominant participants from the small sample (n = 46) used in this study was 63%, including four cases that appeared to exhibit equi-dominance. When the equi-dominant cases were excluded, the right eye dominant percentage increased to 69%. These results are similar to the findings from other studies of ocular dominance.

The mean MPOD values for dominant and non-dominant eyes (n = 44, after the removal of four equidominant cases and a further case for whom MPOD data were missing for one eye) were 0.42 (SD 0.18) and 0.44 (SD 0.17). The mean difference in MPOD measurements categorised by ocular dominance -0.02 (SD 0.12) was not significant with or without a single outlier.

The bias due to ocular dominance was calculated by performing an independent t-test on the difference between randomised right versus left eye MPOD measurements and dominant versus non-dominant eye MPOD measurements from the same sample. Randomised right and left eye measurements represented the reference for comparison with the ocular dominance data. This reference was not ideal because the sample contained a higher proportion of dominant right eyes (approximately two thirds). The perfect reference would include 50% right eye and 50% left eye dominant cases, randomised to exclude any effects of bias due to ocular dominance.

The mean difference between randomised right versus left eye and dominant versus non-dominant eye MPOD measurements was -0.01 (95% CI -0.06 to 0.04), p = 0.75. No significant interocular bias due to ocular dominance was found. The author is unaware of any studies examining the bias in HFP-derived MPOD due to sequential measurement and ocular dominance.

The difference between dominant and non-dominant eye MPOD was plotted against age in an attempt to reveal any evidence of progressive reduction in MPOD in the dominant eye compared to the non-dominant eye, that may relate to increased retinal light exposure to the dominant eye with age (see fig. 2.4). The Pearson correlation was small, negative and significant (r = -0.32, p = 0.03), although the removal of a single outlier resulted in a non-significant correlation (r = -0.21, p = 0.19). No significant correlation was found for the difference between randomised right and left eye MPOD measurements with age with or without a single outlier in the MPOD data. This association is interesting and warrants further study.

Demirel *et al*. reported significantly lower MPOD values in age-matched patients who had undergone cataract surgery (p = 0.039) and a significant inverse correlation (r = -0.66, p = 0.005) between HFP-derived MPOD and the postoperative period measured up to 10 years after cataract surgery. No correlation was demonstrated between MPOD and age. The authors excluded other factors reported to influence MPOD levels such as smoking status, ethnicity, iris colour, micronutrition supplementation and ocular disease.[682] Although it is tempting to associate the reduction in MPOD to increased retinal light exposure in patients who have undergone cataract extraction, the cross-sectional study design prevents any conclusion of cause and effect. It is also possible that these results were caused by other factors not assessed by the authors such as; altered PO_2 gradients due to the removal of one oxygen consumer (the lens), changes in long-term adaptation secondary to the increased luminance and altered chromatic balance of light incident on the retina (MPOD was measured psychophysically), altered inflammatory status secondary to the operation and post-operative changes in diet, lifestyle and exercise level due to the improvement in VA.

Nolan *et al*. reported no significant difference in HFP-derived MPOD measured one week before and after, and one year after cataract extraction and implantation with clear (UV-blocking) IOLs. Participants implanted with yellow-tinted (and UV-blocking) IOLs demonstrated a significant increase in MPOD one year after cataract extraction, but no difference one week before and after surgery.[698] The authors concluded that yellow-tinted IOLs that filter blue light are associated with raised levels of MPOD in the absence of any increase in serum L and Z. An alternative explanation for the authors' findings could be that

differences in long-term adaptation (designed to maintain colour constancy), have differentially affected the psychophysically derived MPOD results for the two IOL types.

Obana *et al*. reported objective measurements of MPOD (RRS) in patients who had undergone cataract extraction and implantation with either clear or yellow-tinted intraocular lenses (IOLs). Macular pigment optical density was assessed post-operatively on days; 1, 4, 7 and 14, and months; 1, 2, 3, 4, 6, 12, 18 and 24. No difference in MPOD was detected between patients with the clear or yellow-tinted IOLs up to six months after surgery, however, from one year onward, MPOD levels were significantly higher in the yellow-tinted IOL group compared to the clear IOL group. The authors concluded that the results from this longitudinal study supported observations that excessive light exposure was inversely associated with MPOD.[699]

Conversely Ciulla *et al*. reported no significant difference in HFP-MPOD before and at a mean of 8.1 weeks (SD 4.7 weeks) after cataract surgery,[700] and Jongenelen *et al*. found no significant relationship between retinal stray light and MPOD measured psychophysically, after correction for age and axial length.[701]

The studies of Nolan *et al*.[698] and Obana *et al*.[699] have highlighted two important points; 1) There was no significant difference in MPOD values before or one week after cataract extraction, for either clear or yellow-tinted IOLs. This supports the conclusion that MPOD values for the MPOD methods used by these authors (macular densitometer and RSS, respectively) are unaffected by clinically significant cataract. 2) The augmentation of MPOD that is purported to be associated with differential retinal light exposure is not evident until at least one year after cataract extraction.

The non-dominant eye is closed to reduce glare when exposed to sunlight,[153] and pterygia are more likely to develop in the dominant eye.[153, 157] Pterygia and pinguecula have been used as surrogate markers for prolonged sunlight exposure. Pterygia were associated with a two to three-fold increase risk of early and late AMD.[162] It is therefore plausible that the dominant eye is at a greater risk of developing AMD than the non-dominant eye and the trend for lower MPOD found in the dominant eye may indicate a higher level of chronic retinal light damage. The higher incidence of AMD after cataract extraction may be coincidental as cataract development is also associated with a higher level of chronic light damage.

While no significant difference was found between dominant and non-dominant eye MPOD and no bias was found compared to randomised eye MPOD measurements, the significant negative correlation with age for dominant eye minus non-dominant eye MPOD with age was interesting. This could suggest a relative decrease in dominant eye or increase in non-

dominant eye MPOD with age and may support the greater light exposure theory for the dominant eye theory. When eye order was randomised, right minus left eye MPOD did not show a significant correlation with age (fig. 2.4). These results warrant further research.

The corrected sample size required to detect a difference in MPOD of 0.07 assuming 80% power at 5% significance was estimated to be 28. This study had sufficient power to detect a significant difference.

2.4.4 Age

This study found no significant correlation for mixed gender MPOD and age. Group analysis revealed that MPOD was significantly higher in the ≥ 50 to < 60 years age range compared to the ≥ 45 to < 50 years age range.

A review of the literature examining the relationship between MPOD measured using HFP, with a 0.5° (or 0.48°) [596] central target and age, revealed 19 papers from White and predominantly White populations and three papers from non-White populations reporting no significant age association.[166, 393, 401, 402, 486, 502, 508, 588, 598, 601, 637, 654, 655, 680, 682, 693, 702-707]

Eleven papers for White or predominantly White and one paper for non-White populations indicated an age-related decline in MPOD.[394, 500, 591, 596, 597, 599, 600, 652, 653, 694, 706, 708] Results from seven of the 12 studies reporting an age-related decline were derived from the same predominantly White regional population, one of which returned no age association after adjustment for outliers and ethnicity.[394] The largest sample from the same population also reported no age association.[704]

The latter two studies by Nolan *et al*. used customized flicker frequencies. Eight out of the 12 studies reporting an age-related decline utilized fixed or pre-set flicker frequencies.[500, 591, 596, 597, 599, 600, 652, 694] A comparison between MPOD measurements of 121 healthy subjects using the Eyemet Maculometer (fixed flicker) and the Macular Metrics Densitometer (variable flicker) was conducted. A trend towards a negative correlation ($r = -0.12$, $p = 0.21$) between MPOD and age was found for the Maculometer, but no correlation ($r = 0.01$, $p = 0.89$) with age was reported for the Densitometer.[691]

The MPS 1000 instrument used in the present study allows the flicker frequency to be individually set for each participant as part of the initial flicker detection sequence prior to MPOD measurement. This instrument should immune to the effects of smaller pupil size and the age-related decline in flicker sensitivity. The bright background used by HFP to minimise rod and blue cone contribution is also likely to minimise any age-related differential in pupil size.

O'Brian *et al*. reported that HFP requires the target to be presented at or near the ideal frequency of flicker for each subject. If the frequency is too low fusion (reduced or absent flicker) never occurs, making the task difficult for naïve subjects. If the frequency is too high fusion occurs at a wide range of values and measurement error is high.[709]

In addition to the inability to customize the flicker frequency, the observation that the MPOD profile may broaden with age,[710] and that a peripheral target positioned at a less eccentric location may lead to an under-estimation of MPOD in the elderly,[468, 694] may all contribute to the explanation of the age-related decline reported in these studies.[691]

Population differences have been offered as one explanation for the controversy reported for the relationship between MPOD and age.[408] An alternative explanation was reported by Berendschot *et al.* who assessed MPOD using seven different methods in the same population. An age-related decline in MPOD was observed with HFP and one of five methods of FR, whereas no decline in MPOD with age was reported for the other four FR methods and 2-WFAF.[694]

Olmedilla-Alonso *et al*. reported results for HFP MPOD following multivariate regression controlling for age, gender, dietary and serum L and Z levels and serum HDL and LDL. They concluded that MPOD is significantly lower in older age range (45 to 65 years) compared to the younger age range (20 to 35 years), despite dietary intake and serum L and Z being higher. In the younger age range MPOD was influenced by serum L, whereas in the older age range the presence of serum L and Z in relation to circulating lipids (L and Z / cholesterol and triglycerides) was a determining factor.[706]

Multiple regression analysis was not performed due to the lack of significant findings in this study. Burke *et al.* found no difference in MPOD between age groups, after adjustment for BMI, dietary carotenoids or serum carotenoid concentrations,[166] and Nolan *et al*. reported that controlling for BMI reduced the correlation between MPOD and age for both genders.[600] Yu *et al*. reported a bivariate correlation between MPOD and age at 0.25° eccentricity for a non-White population ($r = -0.17$, $p = 0.01$, $r^2 = 0.03$).[693] Their multivariate analysis model, which also included BMI, gender, smoking status and light exposure returned a higher value for the variance shared between MPOD and age ($r^2 = 0.06$), indicating 6% shared variance between MPOD and age.

Abell *et al*. reported a significant decline in MPOD with age using the MPS 9000 ($r = 0.22$, p n/a), which increased slightly ($r = 0.27$, p n/a) after correction for gender, iris colour and smoking status. Despite the increase in correlation after correction for other factors, a shared variance of only 7% was achieved between MPOD and age.[500]

The present study found that mixed-gender MPOD was significantly higher (α level = 0.025) in the ≥ 50 to < 60 year age range, compared to the ≥ 45 to < 50 year age range (p = 0.004), although group sizes were small and this result may have occurred by chance. Results from five medium-sized studies (three White, two non-White, n = 71 to 280) and one large study (n = 5,581) have also suggested a trend towards higher MPOD levels in middle age compared to younger and older age groups.[166, 486, 598, 601, 653, 705]

This suggests that the relationship between MPOD and age may not be linear in these samples. Lima *et al*. have also suggested a peak in MPOD values at 50 years of age, using 2-WAF at retinal eccentricities of 0.5°, 1.0° and 2.0°.[244] The consequence of non-linearity combined with the very large inter-subject variation reported for MPOD,[497, 599, 601, 711, 712] may lead to ambiguity (under-estimation of the degree of correlation) in the results from correlation analysis and multiple regression based on linear correlation (see fig. 2.5).

Table 2.11 The relationship between MPOD and age for all *in vivo* methods

In vivo MPOD measurement	Association between MPOD and age					
	Positive ($p < 0.05$)	Trend towards positive ($p \geq 0.05$)	No association	Trend towards inverse ($p \geq 0.05$)	Inverse ($p < 0.05$)	Biphasic relationship
HFP target Eccentricity	-	-	-	-	-	6[166, 486, 598, 601, 653, 705]
10' arc	-	-	2[166, 598]	-	-	-
0.25°	-	-	3[394, 401, 402]	-	5[693, 705]	-
0.35°	1[506]	-	-	-	-	-
0.48°	-	-	-	-	1[596]	-
0.5°	1[713]	1[703]	25[166, 393, 394, 401, 402, 486, 500, 502, 508, 588, 598, 601, 637, 654, 655, 680, 682, 693, 702, 704, 705, 707, 714]	-	9[591, 597, 599, 600, 652, 694, 705, 706, 708]	-
0.75°	-	-	-	1[715]	-	-
1.0°	-	-	7[166, 394, 401, 402, 598, 693, 705]	1[653]	3[705]	-
1.75°	1[402]	-	8[394, 401, 693, 705]	-	-	-
2.0°	-	-	2[166, 598]	-	-	-
3.0°	-	-	1[394]	-	-	-
5.0°	-	-	1[394]	-	-	-
FAF	3[244, 588, 716]	1[489]	4[404, 694, 717, 718]	-	1[659]	1[244]
FR	1[593]	2[489, 710]	9[432, 487, 694, 703, 719, 720]	1[694]	2[717, 721]	-
RRS	-	-	-	-	7[652, 658, 695, 713, 715, 722, 723]	-
CM	2[724, 725]	-	-	1[483]	-	-
Total = 107	9	4	62	4	28	-

CM: colour matching. All results were included where studies reported MPOD for each eccentricity. All results were included where studies reported MPOD for more than one method of MPOD measurement. The number allocation was not scaled to correct for differences in sample size between studies.

In view of the controversy surrounding the relationship between MPOD and age, the author has compiled two tables. Table 2.11 shows the association between MPOD and age for the different *in vivo* methods of MPOD measurement. Two *ex vivo* studies were also found. These employed high performance liquid chromatography (HPLC) to assess MP levels

reported no significant association with age.[468, 726] A2.4 lists the factors affecting the association between HFP MPOD and age.

Overall the majority of studies (including all *in vivo* methods of measurement) reported no significant association between MPOD and age. The results from a proportion of the HFP MPOD studies that reported an inverse association may have been a due to equipment design (i.e. less eccentric peripheral target and fixed-flicker). Fundus autofluorescence and FR tended not to show a significant association with age, whereas all studies using RRS reported a significant, inverse association between MPOD and age.

It is reasonable to question whether the biphasic relationship between MPOD and age reported by six HFP studies,[166, 486, 598, 601, 653, 705] is a consequence of the design of the HFP instrumentation, or an age-related difference in response to the psychophysical methods used. To counter this argument, a biphasic relationship with the highest MPOD levels in the middle age range, was also demonstrated using 2-WFAF.[244] Further research would be sensible to confirm this relationship using objective methods of MPOD measurement.

Delori *et al*. reported that 2-WFAF and FR correlated highly with MPOD determined by HFP. After correction for differences in test field diameter, 2-WFAF MPOD was larger than HFP MPOD by approximately 0.23, especially at low values of MPOD. Fundus reflectance MPOD values were generally much lower than HFP MPOD, except at low values of MPOD.[489]

The results of Berendschot *et al*.[694] are interesting. An age-related decline in MPOD was not observed for all of objective methods, despite being found for HFP for the same population. Measurement of MPOD by HFP was performed with undilated pupils in the study reported by Berendschot *et al*. Pupil size reduces with age, but this study found no association between MPOD and pupil size. Measurement of MPOD using commercial methods such as the MPS 1000 used in the present study is performed under free-viewing conditions, where the effects of pupil size are not corrected. Caruso-Avery compared Maxwellian view (pupil size effects are excluded) vs. free-view measurement of MPOD for 30 experienced subjects with natural, undilated pupils, concluding that pupil size did not significantly affect MPOD measurements.[727]

Another factor not normally reported for HFP-measured MPOD is that a learning effect leading to higher measured values of MPOD may occur with experienced subjects. Ten days of perceptual learning was reported to increase critical flicker fusion threshold by 30% using the Densitometer.[728] It was reported that age leads to a decline in the stability of visual perceptual learning.[729] While this effect may bias MP longitudinal and supplement studies where multiple repeated measures of MPOD are requisite, it would not have

affected the present study in which single measurements were obtained form naïve participants.

Age summary

Age is the strongest, established RF for AMD. Advancing age is associated with an increase in AMD prevalence, incidence and progression.[14, 41, 43, 625] The three largest studies to date (n = 1,698 to 5,581) and the majority of bivariate analyses have suggested that there is no significant relationship between HFP-derived MPOD and age.[166, 393, 401, 486, 502, 508, 588, 598, 601, 637, 654, 655, 693, 702-705]

Results from this study revealed a trend for MPOD being highest in late middle age. Six other studies found that HFP-derived MPOD peaked in middle age.[166, 486, 598, 601, 653, 705] This biphasic relationship with age was also demonstrated using 2-WFAF.[244] This may be the result of improved eating habits leading to higher macular carotenoid consumption in middle age, combined with reduced food intake, lower macular carotenoid absorption and / or transport and the greater likelihood of MP depletion due to retinal damage in older subjects.[594, 598, 730, 731]

The possible lack of linearity in the association between MPOD and age suggests that group analysis is a more appropriate statistical tool than correlation. The controversy surrounding this relationship may be explained wholly or partly by a combination of population differences, age-related and gender-related differences in macular carotenoid dietary intake, absorption and retinal transport, questionable relationship linearity, changes in MPOD profile width with age and gender, and differences in instrument design.[408, 468, 497, 598, 599, 601, 691, 694, 710-712, 731]

The corrected sample size required to detect a difference in MPOD of 0.06 assuming 80% power at 5% significance was estimated to be 68. This study had sufficient power to detect a significant difference.

Cross-sectional MPOD studies should include sufficient numbers to allow group analysis between several age ranges, and should be corrected for macular carotenoid dietary intake and %BF in addition to other known co-variants. Many researchers have recommended further longitudinal studies to investigate the effects of MPOD over time.[506, 593, 594, 621, 626, 659, 716]

2.4.5 Gender

This study revealed a trend for higher male MPOD compared to female MPOD, but this was not significant. A review of the literature revealed 12 studies (including three non-White) reporting either significantly lower MPOD in females,[432, 508, 597, 599, 693, 732] or a non-significant

trend towards lower female MPOD.[486, 591, 601, 705, 733, 734] Five studies found either significantly higher MPOD in females,[704] or a trend towards higher female MPOD.[166, 394, 654, 718] Three studies reported no significant gender difference,[680] but male and female MPOD data were not available in two of the studies.[401, 500]

Van der Veen *et al.* reported from the largest MPOD study to date (unselected data from 48 USA Optometric practices using the MPS 9000, n = 5581), that the gender difference in MPOD was age-related, with male MPOD being higher up to the age of 59 and female MPOD exceeding male values in participants aged 60 years and over. The authors concluded that the higher MPOD values in females over 60 years of age resulted from their greater likelihood to have consumed L and Z supplements.[486] The third National Health and Nutrition Examination Survey (NHANES III) reported that in addition to increased supplement use in both genders from the time periods (1988 to 1994) to (2003 to 2006), females are consistently more likely to use general nutritional supplements than males.[735] Olmedilla-Alonso *et al*. reported that the literature concerning gender differences in dietary intake of L and Z is inconsistent.[706]

The observation that women may have lower MPOD levels, particularly in the lower age range compared to men,[486] or equal levels despite higher L and Z consumption,[599, 732] has been attributed to higher female adiposity. Adipose tissue is the major storage site for the lipid-soluble macular carotenoids, and it has been suggested that this may lead to competition with the retina for serum L and Z.[164, 424, 435] Olmedilla-Alonso *et al*. reported significantly lower HFP MPOD for women compared to men in the older age range (45 to 65 years), but not the total age range (20 to 65 years). Dietary intake of L and Z, and levels of circulating lipids (L and Z / cholesterol / triglycerides) were significantly higher in the older age range, but did not correlate with gender after controlling for other factors.[706]

Alterations in serum levels of L and Z have been reported for the different phases of menstruation,[736, 737] which in humans, appears to be a consequence of variable utilization of predominantly LDL cholesterol from the serum for luteal steroidogenesis in the *corpus luteum*.[738-740] Significant alterations in serum L and Z were also noted for pregnancy[662] and lactation. [428] These observations may suggest gender-specific, competitive differences between serum and adipose tissue for these carotenoids.[164]

The width of the MPOD spatial profile was found to increase as a response to MP supplementation.[689] Yu *et al.* also reported that the MPOD profile was wider for Chinese females.[693] If this trend is evident across ethnicities, this could explain some of the lower female MPOD values in studies using HFP method with a less eccentric peripheral reference. Gender differences in the variability of retinal thickness were reported, but

MPOD was found to be positively associated with foveal width, regardless of gender and ethnicity, but not retinal thickness.[394, 741]

The number of male participants in this study was less than half that for females. This was due to difficulties with male recruitment, an issue also reported by another study.[588]

Overall no significant difference in MPOD was reported for male and female gender when all *in vivo* methods of MPOD assessment were considered (table 2.12).

The HFP studies that reported lower MPOD for females may be explained by the use of older HFP equipment with smaller peripheral target eccentricity,[508, 597, 599, 727, 732, 733] wider female MPOD spatial profile,[716] which may be associated with age and / or increased supplement use, correction for the effect of other influencing factors[598] and a variable relationship for MPOD and age between genders.[598, 653] Only one study out of 12

Table 2.12 The relationship between MPOD and gender for all *in vivo* methods

In vivo MPOD measurement	Association between MPOD and gender (male vs female)					
	Positive female MPOD higher ($p < 0.05$)	Trend towards positive ($p \geq 0.05$)	No association	Trend towards inverse ($p \geq 0.05$)	Inverse female MPOD lower ($p < 0.05$)	Association variable with age
HFP target Eccentricity	-	-	-	-	-	3[598, 653]
10' arc	-	-	1[166]	-	1[598]	-
0.25°	-	-	11[394, 401, 653, 693, 705, 734]	-	4[598, 653, 705]	-
0.5°	-	-	17[166, 394, 401, 486, 500, 601, 652-654, 680, 705, 734]	1[733]	10[508, 597, 599, 65 693, 704, 706, 732] 2[653, 705]	-
1.0°	-	-	14[166, 394, 401, 598, 652, 653, 693, 705, 734]	-	-	-
1.75°	-	-	11[394, 401, 598, 693, 705, 734]	-	1[653]	-
2.0°	-	-	4[166, 653]	-	-	-
3.0°	-	-	1[394]	-		-
2-WFAF	-	-	4[489, 717, 718]	-	-	-
FR	-	-	3[489, 593, 717]	-	1[432]	-
RRS	-	-	3[658, 722, 723]	-	-	-
Total = 89	0	0	69	1	19	-

All results were included where studies reported MPOD for each eccentricity. All results were included where studies reported MPOD for more than one method of MPOD measurement. The number allocation was not scaled to correct for differences in sample size between studies.

comparing objectively measured MPOD for each gender reported a significant difference.[432]

The risk of advanced AMD is similar for men and women, however nAMD is more common in women than men. Increased AMD in women may be associated with a decline of oestrogen (antioxidant and anti-inflammatory agent) during the menopause.[302] Oestrogen deficiency also increases bone calcium turnover during the menopause. Increased free

calcium may act as a "seed" in the early development of macular drusen.[283] The author is unaware of any studies examining oestrogen levels and MPOD.

Gender summary

Gender is a weak and inconsistent RF for AMD. There is inconsistency in the literature regarding the association between gender and AMD prevalence.[1, 45, 627] This study revealed a non-significant trend for lower female MPOD. Despite the majority of cross-sectional studies reporting lower MPOD for females,[432, 486, 508, 597, 599, 601, 693, 705, 732-734] gender has not been consistently reported to be a RF for AMD. Beaver Dam Eye Study and BMES suggested that women might have a higher risk of developing AMD.[625, 627] The higher prevalence of late AMD in women compared to men, has been explained in part by the larger number of women in the older age range.[1, 45] Another recent study reported no gender differences in AMD risk.[9]

Gender differences in MPOD appear to associate with age and adiposity.[164, 424, 435, 486] HFP-derived gender differences may also relate to variations in both MP supplementation and the MPOD spatial profile width.[689, 693] In order to systematically assess the relationship between MPOD and gender, any study would need to be sufficiently powered to allow gender comparisons at a number of different, regularly spaced age groups, and correct for adiposity and dietary carotenoid intake, in addition to other known co-variables.

The corrected sample size required to detect a difference in MPOD of 0.01 assuming 80% power at 5% significance was estimated to be 535. This study did not have sufficient power to detect a significant difference. Objective methods of MPOD assessment (e.g. FAF or FR) would remove any uncertainty about the possible effect of gender on peripheral target measurements using on HFP-derived MPOD.

2.4.6 Body mass index

This study found no significant association between MPOD and mixed-gender or separate-gender BMI.

Ten other studies examined the association between mixed-gender BMI and HFP-derived MPOD. Three studies reported a significant correlation between MPOD and BMI,[401, 436, 597] and three reported no significant correlation.[166, 591, 601] Four predominantly White studies using group analysis revealed significantly or almost significantly (Burke *et al.*, p = 0.06), [742] lower MPOD with higher BMI; > 25, [394] > 27,[166] > 29,[436] and > 30,[704] whereas two non-White studies (South-Indian and Chinese) reported no significant difference between groups.[693, 734] These results may indicate ethnic differences in the relationship between MPOD and BMI.

The percentage of obese subjects is lower than that reported for the UK in 2002 (male 23% and female 25%), and estimated by projection for 2012 (35% for non-manual and 45% for manual social class).[743] Although the participants were drawn from a population thought to contain higher numbers of non-manual and more highly educated participants, factors known to be associated with lower levels of obesity,[744] selection bias (against those visibly obese) is likely to explain the low percentage of obesity reported by this study.

Overall studies examining White or predominantly White participants were controversial, although the majority reported a decrease in MPOD with higher BMI, with a tendency for this to affect males more than females. Three studies examining non-White participants; Chinese,[693] South Indian,[734] and Asian,[723] reported no significant association between MPOD and BMI at any eccentricity tested (table 2.13).

Two studies reported correlations for each gender,[436, 600] both reported significant inverse correlations for males, but only one of these studies was significant for females.[436] Broekmans et al. using spectral fundus reflectance also reported a significant correlation for

Table 2.13 The relationship between MPOD and BMI for all *in vivo* methods

In vivo MPOD measurement	Association between MPOD and BMI (lower vs higher)				
	Positive ($p < 0.05$)	Trend towards positive ($p \geq 0.05$)	No association	Trend towards inverse ($p \geq 0.05$)	Inverse ($p < 0.05$)
HFP target Eccentricity					
10' arc	-	-	1[742]	-	-
0.25°	-	-	2[693, 734]	-	1[401]
0.5° mixed	-	-	5[166, 591, 601, 693, 734]	1[742]	6[394, 401, 436, 597, 637, 704]
0.5° male	-	-	-	-	2[436, 600]
0.5° female	-	-	1[600]	-	1[436]
1.0°	-	-	2[693, 734]	-	2[401, 742]
1.75°	-	-	3[401, 693, 734]	-	-
2.0°	-	-	-	-	1[742]
2-WFAF	-	-	1[718]	-	-
2-WFAF 0.5°	-	-	1[716]	-	-
2-WFAF 2.0°	-	-	-	-	1[716]
FR male	-	-	-	-	1[432]
FR female	-	-	1[432]	-	-
RRS	-	-	1[723]	-	-
Mixed = 29	0	0	17	1	12
Male = 2	0	0	0	0	2
Female = 2	0	0	1	0	1

All results were included where studies reported MPOD for each eccentricity. All results were included where studies reported MPOD for more than one method of MPOD measurement. The number allocation was not scaled to correct for differences in sample size between studies.

males only.[432] Mares *et al.* reported a significant reduction in MPOD with increasing BMI in a female population.[637]

Body mass index summary

Higher BMI is a moderate and consistent RF for AMD. Body mass index is also positively correlated with age. The trends revealed by this study, although not significant were in

agreement with the majority of other White or predominantly White studies. MPOD was lower, especially in the obese groups for mixed-gender analysis and particularly in obese males for separate-gender analyses.[166, 394, 401, 436, 597, 600, 704] The lack of an association between MPOD and BMI in the two non-White studies examined may suggest ethnic differences in this relationship.[693, 734]

Discrepancies in MPOD levels between genders have been attributed to different amounts and location of body fat.[745-747] Ethnic differences may be associated with alterations in body fat acquisition and differing responses to metabolic syndrome.[745, 748-750] Whites were found to be more prone to dyslipidaemia (high cholesterol), whereas African Americans tended to express dysregulation of glucose metabolism.[751]

Lutein and Z are distributed equally between LDL and HDL in the plasma, with a progressive decrease in L and Z from light to dense LDL.[752, 753] HDL cholesterol was also reported to be important for the delivery of L to the retina.[754] The reduction in HDL associated with obesity predominantly seen in White ethnicity is consistent with the possibility of ethnicity-specific differences in transport and / or retinal capture of carotenoids.[755] The positive relationship between obesity and age is well known.[756, 757]

The association between BMI and MPOD is controversial, but consensus appears to support the trend revealed by this study, for lower MPOD in White or predominantly White populations associated with mid- to upper-range overweight or obese levels of BMI. An inverse association between MPOD and BMI was more likely for male participants.

The corrected sample size required to detect a difference in MPOD of 0.02, assuming 80% power at 5% significance was estimated to be 63. This number would be required for each gender. A trend towards lower MPOD with higher BMI was revealed by this study for both mixed-gender and separate-gender analyses. This study had sufficient power to detect a significant difference for mixed gender, but not separate gender associations.

Although BMI does not directly measure adiposity,[600] it is simple to measure in clinical practice. Interpretation of the resulting measurement is confounded by age, gender and ethnicity as well as the inability to distinguish adipose tissue form muscle mass.[605-607] Studies examining BMI as a RF for AMD or MPOD should correct for gender, age and ethnicity in addition to other known co-variables.

2.4.7 Percentage body fat

A trend was noted for lower MPOD in the obese group for males, but not females however the association was not significant.

Two studies reported mixed gender correlations for %BF and MPOD. Both reported a significant negative correlation.[600, 758] Two studies analysed genders separately. Nolan *et al.* compared MPOD with total %BF whereas Bovier *et al.* measured total %BF as well as arm, leg and trunk %BF and relative trunk fat (percentage of total body fat in the trunk region). These studies used dual-energy X-ray absorptiometry (DEXA) to measure %BF. Both studies reported a significant inverse correlation for men only. This agreed with the trend reported for this study.[600, 758]

Mixed-gender analysis of %BF was avoided in the present study because male and female %BF values are not comparable. Female %BF is approximately 10% higher than that of males, thus values categorised as normal for females would be classed as obese for males.

Two studies reported group analyses. Hammond *et al*. reported a significant difference for mixed gender MPOD values for %BF values < 27 compared with > 27. Male and female genders analyzed separately were reported to follow a similar trend.[436] When participants with a %BF value of < 27 were analyzed alone, no difference across the distribution was found. This suggested a non-linear relationship between MPOD and %BF with a drop-off in MPOD value only when %BF reached obese levels. Nolan *et al.* found that the relationship approached significance for male ($p = 0.06$) and female participants ($p = 0.053$) for %BF values < 25 compared to > 25.[600]

The trend most apparent from the literature was that of a reduction in MPOD, most evident in males, once %BF reached obese levels. Although this study did not reach significance for gender, the same trend was observed. The number of male participants was small and therefore caution should be exercised with any interpretation. Gupta *et al*. found no association between MPOD and waist / hip ratio or waist circumference for a South Indian population, after correction for age and gender, suggesting that there may be ethnic differences in the response of MPOD to obesity.[734]

After converting BMI values to %BF using the CUN-BAE algorithm it was apparent that considerably more participants were classified as obese. BMI and %BF percentages for normal, overweight and obese were as follows, male: BMI 21.9% / 62.5% / 15.6%, %BF 6.2% / 18.8% / 75.0%, and female: BMI 58.3% / 33.3% / 8.3%, %BF 8.3% / 29.8% / 61.9%. Gómez-Ambrosi *et al.* concluded that BMI measurement under-estimated the level of obesity (and cardiometabolic risk) compared to %BF measurement.[676]

The android fat distribution (centrally deposited adipose tissue e.g. abdominal) typically seen in men and the gynoid fat distribution (gluteo-femoral adipose tissue) characteristic of the female body shape become more pronounced during puberty.[746, 747] The time after menopause is associated with a transition to a more android adipose tissue distribution in

females.[759] In men, but not women abdominal obesity measured by waist / hip ratio or waist circumference was positively associated with AMD prevalence, however BMI was not associated with AMD prevalence in either gender.[630] L and Z concentrations were consistently higher in abdominal adipose tissue compared to that from gluteal and femoral locations.[760]

It has been suggested that adipose tissue generally, and abdominal adipose tissue specifically, may act as a "sink" for carotenoids,[164, 760] reducing the availability of serum carotenoids for retinal absorption, particularly in obese men. This may explain the results reported by van der Veen *et al*. that in the under 60 year age range male MPOD is higher than female (due to higher female total %BF), but above 60 years of age female MPOD is higher than male (due to higher levels of L and Z "locked" in male abdominal adipose tissue).[486, 758] Increased levels of obesity may also affect both L and Z transport and retinal capture, as a result of an associated reduction in HDL cholesterol.[129, 752, 753]

Studies in adults and children have shown an association between the hypo-functional seven-repeat allele (7R) of the dopamine-4 receptor gene (DRD4) and increased eating behaviour and / or obesity, especially in females.[761, 762] Silveira *et al*. reported that pre-school children from both genders, carrying the 7R allele had a less healthy pattern of habitual food intake compared to non-carriers.[762] A literature search revealed no previous studies examining the association between the hypo-functional 7R allele of the DRD4 gene and AMD. Migraine without aura (but not migraine with aura), was found to show a significant genetic association with DRD4.[763]

Percentage body fat summary

The non-significant trends in MPOD levels revealed by this study agreed with the other studies published, reporting significant negative correlations for mixed-gender %BF and significance for only male %BF in gender-separate comparisons.[600, 758] Studies performing group analysis revealed stable MPOD until obese levels of %BF were reached, when especially for males MPOD levels were seen to fall.[436, 600] This suggested that the relationship between MPOD and %BF might not be linear. MPOD levels appeared to be influenced by the amount (especially younger females) and location (especially older males) of body fat.[164, 760] Obesity was also associated with lower HDL levels, which may affect L and Z transport and retinal capture.[129, 752, 753]

The author did not have access to equipment to measure %BF directly. The use of calculated %BF derived from BMI, age and gender values using the CUN-BAE algorithm was simple to use in this practice setting and offered the opportunity to assess an estimate of %BF for each gender with MPOD.

Although the Northern European population investigated by the present study was similar ethnically to that from which the CUN-BAE algorithm was derived, it is recommended that the use of general predictive algorithms across different ethnic population groups should be avoided, without prior testing of their validity.[764] This was not possible for this practice-based study and therefore the values for estimated %BF should be treated with caution.

Sample size calculation was not performed for %BF as these values were calculated from BMI, age and gender rather than measured directly. Studies investigating associations with %BF should be corrected for age, gender and ethnicity in addition to other known co-variables. The lack of a linear relationship between MPOD and %BF would suggest that group analysis is preferable to correlation. In view of the inherent limitations of BMI measurements and if no direct method of %BF measurement is available, calculated %BF measurements might be used in preference to BMI measurements in Optometric research.[600, 605-607]

2.4.8 Iris colour

No significant association was found for mixed gender MPOD and iris colour analysed as five separate colour groups (grey, blue, green, hazel and brown) or when categorised as either "light" (grey, blue and green) or "dark" (hazel and brown).

The observation that White individuals have a higher risk of developing AMD than Black individuals has led researchers to question whether the higher levels of melanin found in the eyes of Black individuals is protective for AMD. Consensus is that the association between iris colour and AMD is weak at best. Berendschot *et al*. reported no significant difference in either MPOD or melanin optical density in eyes with or without AMD, or between various stages of AMD.[593]

Six studies compared HFP MPOD in light and dark irides, three reported significantly higher MPOD in darker irides,[508, 599, 680] one reported higher MPOD in darker irides (brown vs. not brown irides) in patients with RP,[765] and three (two from the same regional population) reported no significant difference between light and dark irides.[401, 402, 597] Seven studies assessed MPOD levels for participants divided into three iris colour groups, two reported a significant difference between groups 1 (blue or grey) and 3 (brown or black),[601, 636] two reported a non-significant trend for higher MPOD with darker irides, [654, 733] and three reported no significant difference. [394, 701, 713]

Abell *et al.* reported that following multivariate regression with correction for age, gender and smoking status, MPOD measurements for participants with green iris colour was significantly lower than MPOD recorded from participants with blue irides.[500]

Although a trend for higher MPOD with darker irides was noted for males and females, this was not significant for either gender. Two other studies have examined the effect iris colour separately by gender. Broekmans *et al.* using spectral fundus reflectance noted a trend for higher MPOD with darker irides in men, but the opposite trend for women. Numbers for some of the groups in this study were small however.[432] Mares *et al.* reported results from a large (n = 1698), female only study noted a trend for lower MPOD in darker irides.[637]

Pigmentation of the RPE is a change associated with early AMD and an indicator for future progression to late AMD. Levels of melanin are known to reduce with age and melanin may exhibit irreversible oxidation. It is plausible that increased retinal illumination associated with lighter pupils combined with reduced antioxidant capacity in older age may lead to earlier irreversible melanin pigment changes compared to those with darker pupils.

In this study a higher percentage females had light irides compared to males (63.0% versus 51.9%), but the difference was not significant. Comparing only blue and brown irides a larger gender difference, favouring females with blue irides compared to males was noted (65.8% versus 42.1%), but again this was not significant. Vingerling also reported that there were no significant gender differences in eye colour distribution.[766]

There is some plausible evidence supporting the potential for gender differences in iris colour distribution. Brown irides, or rather faces associated with brown irides make men appear significantly more trustworthy to women, but not *vice versa*,[767] and the blue-eyed phenotype may confer some adaptive advantage for women as this may be favoured by sexual selection.[768-770]

The variable distribution of iris colour between genders, brown more common for male participants and blue more common for female participants and the observation by two studies that female MPOD is lower for darker irides,[432], [637] may explain the controversy surrounding the results of the mixed-gender studies reported at the start of this section.

No significant association was found for pupil size and MPOD. The pupils of those with blue irides were reported to be larger in size in ambient illumination, and to contract less quickly and by a reduced degree than those with brown irides.[771-773] A trend for smaller median pupil size for brown (3.0 mm, IQR 2.0 mm) compared to blue irides (4.0 mm, IQR 1.0 mm) was found, although this was not significant. The difference in pupil size alone between brown and blue eyes would equate to 1.77 times higher retinal illuminance for blue eyes. Correction for iris colour and the difference in pupil size revealed retinal illuminance values 2.25 times higher in the lighter irides group, assuming the pupil size difference was maintained under MPOD testing.

An estimate of combined retinal illuminance (pupil and iris) was calculated for blue and brown eyes over a range of pupil sizes (2 mm to 8 mm). Retinal illuminance was higher for blue eyes compared to brown eyes for all pupil sizes. The percentage increase in retinal illuminance for blue compared to brown eyes increased significantly with smaller pupil size (10% greater for 8 mm and 102% greater for 2 mm pupils respectively for blue eyes compared to brown eyes). Comparing 8 mm to 2 mm pupils, retinal illuminance was estimated to be 3.2 times and 5.8 times greater for blue and brown eyes respectively. Pupil size was the main determinant of retinal illumination (see table 2.10).

HFP-derived MPOD has been reported to be higher in several different non-White populations (range of means 0.43 to 0.56) compared to most White populations.[653, 693, 705, 774-776] This has been attributed to the higher proportion of dark irides in non-White populations, however other factors such as higher dietary carotenoid intake may also be contributory.[705, 776]

Previous studies have combined iris colours into two groups (light: blue, grey, green and dark: hazel, brown, black),[401, 402, 597, 599] (light: blue, grey, light brown and dark: mid brown, dark brown),[508], three groups (group 1: blue or blue / grey, group 2: green / hazel, group 3: brown / black),[394, 601, 636, 654, 733] (group 1: blue / grey, group 2: green, group 3: brown / black),[701] and four groups (blue, green, light brown, dark brown).[637] Broekmans *et al.* used the more complex, 5-grade classification system designed for use in multicentre studies such as EDCCS. Iris colour was determined by a combination of iris colour (blue, grey, green, brown) modulated by the proportion of total iris area with brown or yellow pigment, compared to four standard iris photos [432, 777]

Variability in the classification of iris colour groups between studies may also account for some of the inconsistency in the results reported. Attempts have been made to standardise iris colour classification.[777, 778] The five iris colour, 3-group (1: blue and grey, 2: green and hazel, 3: brown) classification system examined by Muinos Diaz *et al*. had an inter-observer reliability of 0.79 (Cohen's kappa was used as this takes in to account chance agreement and is thus a more robust measure than simple percentage agreement), with an agreement of 89.6%.[778] This system is simple to implement and would appear appropriate for use in Optometric practice.

Overall, including all *in vivo* studies, the majority reported significantly lower MPOD for lighter irides or a trend for the same. Objective studies were more likely to report no association between MPOD and iris colour (see table 2.14).

Iris colour summary

Iris colour is a weak and inconsistent RF for AMD. This study found no significant

association between MPOD and iris colour generally, or for light compared to dark irides for mixed or separate gender analyses.

The sample size was small for each group, especially for male gender. The observation of two other studies that revealed a trend for female MPOD to be lower for darker irides, opposing the relationship reported by most mixed gender studies may indicate gender-specificity in iris colour associations.[432, 637]

Table 2.14 The relationship between MPOD and iris colour for all *in vivo* methods

In vivo MPOD measurement	Association between MPOD and iris colour (dark vs light)				
	Positive ($p < 0.05$)	Trend towards positive ($p \geq 0.05$)	No association	Trend towards inverse ($p \geq 0.05$)	Inverse ($p < 0.05$)
HFP target Eccentricity					
0.25°	-	-	1[401]	2[394, 402]	-
0.5°	-	-	3[597, 701, 713]	5[394, 402, 654, 733, 776]	8[401, 500, 508, 599, 601, 636, 680, 765]
0.5° female	-	1[637]	-	2[394, 402]	-
1.0°	-	-	-	2[394, 402]	1[401]
1.75°	-	-	1[401]	1[394]	-
3.0°	-	-	-	1[394]	-
5.0°	-	-	-		-
2-WFAF 2.0°	-	-	1[489]	-	-
FR	-	-	-	-	-
2.0° mixed	-	-	1[489]	-	-
Male	-	-	-	-	1[432]
Female	-	1[432]	-	-	-
RRS	-	-	2[658, 713]	-	-
Total = 33	0	2	8	13	10

All results were included where studies reported MPOD for each eccentricity. All results were included where studies reported MPOD for more than one method of MPOD measurement. The number allocation was not scaled to correct for differences in sample size between studies.

Retinal illuminance estimates calculated for this study suggested that retinal illumination is predominantly governed by pupil size (ignoring absorption by the ocular media), and that increased differential light transmission between light and dark irides is positively related to smaller pupil size (see table 2.10). Pupil size measured in ambient illumination before MPOD testing was not significantly associated with HFP MPOD, after correction for age. Stringham *et al*. reported a small, albeit non-significant positive correlation between MPOD and natural pupil size.[622]

It has been reported that the tissue of lighter irides transmits about three times the amount of light compared to that of dark irides, due to differences in stromal absorption,[681] and this light transmission differential may be compounded by larger pupil size and slower constriction speed reported for light irides.[771, 773] Iris pigment epithelium and RPE melanin concentration is thought to be equal for different iris colour and race,[4, 779] however choroidal melanin levels are lower for light irides suggesting reduced retro-retinal antioxidant and free radical-quenching potential which may adversely affect the RPE.[6-8, 631, 632]

Studies examining iris colour as a RF for AMD or MPOD should correct for gender, pupil size, and light exposure in addition to other known co-variables. The association between iris colour and both AMD risk and MPOD level is controversial. Correction for the additional variables reported above may improve the validity of iris colour associations.

The corrected sample size required to detect a difference in MPOD of 0.04 assuming 80% power at 5% significance was estimated to be 239. This number would be required for each gender. Although a trend towards lower MPOD with lighter irides was revealed by this study, it lacked the power to detect a significant difference.

2.4.9 Family history of AMD

Liew *et al*. reported from a classical twin study (monozygotic, MZyg: 100% genetic similarity versus dizygotic, DZyg: average of 50% genetic similarity), that genetics are an important determinant of MPOD with heritability estimates of 67% for HFP and 85% for 2-WFAF.[588]

Twin studies have also firmly established the genetic predisposition to AMD,[780-782] with an estimated heritability of between 46% and 71%.[782] Unlike most complex traits, where <10% of genetic variance is explained by common variants,[783] AMD is unique in that relatively few allelic variants explain a large amount of the genetic risk.[784, 785] Risk variants at two major AMD susceptibility loci, CFH at 1q31 and ARMS2 / HTRA1 at 10q26 are thought to account for over 50% of AMD cases.[786-790]

A trend for lower MPOD was observed in cases of reported primary and secondary AMD FH compared to those with no reported AMD FH, however this was not significant. Group sizes for AMD FH were small.

Five studies have examined the relationship between confirmed or reported FH of AMD and MPOD measured at 0.5° eccentricity, in White or predominantly White populations. One study reported significantly lower MPOD in those with a confirmed AMD FH, which remained significant after controlling for confounding variables.[597] Two reported significantly higher MPOD measurements for those with AMD FH.[594, 704] Two reported no significant difference. Kirby *et al*. also reported no significant difference at 0.25°, 1.0° and 1.75° eccentricity.[401, 601] Nieto *et al.* reported that 42% of AMD, 37% of first-degree relatives of AMD and 31% of normals exhibited a central depression in their MPOD profile measureable at 0.17°, but not evident at 0.5° eccentricity.[403] Hogg *et al*. confirmed that the centrally located peak MPOD is highly heritable, whereas MPOD measured at a paracentral location determined by the width of the spatial profile at half peak was influenced more by environmental factors than genetics.[404]

The identification of the CFH gene on chromosome 1q31 as the first major AMD susceptibility gene resulted from the first ever genome-wide association study (GWAS) to successfully identify a risk variant for a complex disease by an undirected, genome-wide search.[788] The discovery of CFH and the subsequently identified modifiers CFB, C3 and CFI strongly suggested a major role of the alternative complement pathway and therefore inflammation in AMD pathogenesis.[786]

The alternative complement pathway is continuously activated ("tick-over") by spontaneous hydrolysis of the internal thioester bond in C3 to form C3(H_2O).[791, 792] The inhibitory regulators (CFH, CFI and CFB) are therefore required to prevent inappropriate over-activation and tissue damage.[792] Drusen contain almost all complement pathway proteins, including C3 and CFH, as well as other inflammatory mediators such as fibrinogen, vitronectin and CRP.[53, 793, 794] The presence of activation products in circulating blood suggests that AMD-related inflammation is not limited to the retina, but is systemic.[792] Serum levels of the inflammatory marker CRP are raised in those with, and at future risk of AMD.[795-797]

The modulated inflammatory response (immune privilege) normally exhibited by the macula may be bypassed and up-regulated (especially in those with pro-AMD complement gene polymorphisms) by a sufficiently strong systemic immune response.[798] This may induce a low grade, pro-inflammatory macrophage response and eventually subretinal neovascularisation.[292] It is interesting that significantly lower AMD incidence was observed for those taking long-term anti-inflammatory medication.[799, 800]

Although the Carotenoids in Age-Related Eye Disease Study (CAREDS) reported that for females, variations in 13 SNPs from 10 genes affecting carotenoid transport, uptake and metabolism accounted for 5.1% of the variability in MPOD.[801]

ApoE encodes Apolipoprotein E, a protein that has a central role in lipid transport and distribution in the central nervous system. The ε2 allele is associated with increased AMD risk whereas the ε4 allele confers reduced AMD risk.[802-805] Those with at least one ε4 allele were reported to have higher MPOD.[806]

ABCA4, also known as ABCR or STGD1 encodes a photoreceptor-specific ATP-binding cassette transporter of retinaldehyde. ABCA4 is defective in autosomal recessive forms of Stargardt disease, cone-rod dystrophy and RP.[807] MPOD at 0.2° and 0.5° was significantly lower in those with ABCA4 mutations, and was strongly influenced by disease stage (abnormality of foveal architecture). Serum levels of L, Z, but not β-carotene were also significantly lower in affected individuals.[808]

Healthy individuals with gene variants of β-carotene 15,15'-monooxygenase (BCMO1) that have "high" compared to "low" β-carotene conversion efficiency in the plasma, were significantly associated with higher levels and lower levels of MPOD measured using HFP. No significant difference in MPOD was demonstrated for different BCMO1 variants in individuals with AMD.[809]

It has been reported that genes associated with the HDL pathway (LIPC and ABCA1) are associated with early stages of AMD, whereas ARMS2 / HTRA1 and the complement pathway genes (CFH, C3 and C2) are associated with the advanced stages.[268] The association between high-risk CFH genotypes and early AMD was found to increase with age from an OR of 0.37 to 0.48 (< 55 years of age) to an OR of 1.87 to 2.80 (>75 years of age).[810]

A full dietary assessment was not completed, however, participants were asked about current use of MP and fish oil supplements. Only one participant reported current use of MP supplements, which precluded any meaningful analysis. Twenty one participants reported current use of fish oil supplements. A non-significant trend for higher median MPOD was revealed for the fish oil supplement group. The omega-3 fatty acid DHA not only associates with rhodopsin and L in rod OS membranes, it also is thought to assist in retinal absorption of MP.

Family history of AMD summary

Family history of AMD is a strong and consistent RF for AMD. The use of reported FH of AMD in this practice-based Optometric study was convenient as the data was easy to acquire and avoided the practical and ethical difficulties and cost involved in obtaining and analysing participant samples for AMD risk genes. The usefulness of associating AMD FH with MPOD is limited, for confirmed as well as for reported AMD FH because the two main genes associated with the highest risk of AMD (CFH and ARMS2) were not individually found to associate with lower MPOD. Individuals homozygous for risk alleles of both CFH and ARMS2, however, had significantly lower MPOD measured at 0.5° eccentricity.[405]

The lack of a significant association between MPOD and AMD FH found by this and other studies,[401, 601] may be explained by the observation that AMD risk genes influence them differently. AMD FH is associated with CFH genes,[405] whereas HFP-derived MPOD in females measured at 0.5° eccentricity, is associated with specific genes affecting xanthophyll binding, carotenoid cleavage, retinoid recycling, lipid and carotenoid transport and metabolism, HDL or cholesterol status, long-chain fatty acid synthesis or metabolism and the gene associated with Sjögren-Larrson syndrome.[801]

The higher MPOD associated with those with a FH of AMD may have resulted from their being more likely to receive advice about AMD prevention or supplement usage.[594, 704] The 0.5° target size used in this study may also have been too large to detect genetic differences in MPOD, found to be strongest at the spatial profile peak, within 0.17° (10') eccentricity.[403, 404]

A limitation of any study examining AMD family history, whether reported or confirmed, is the requirement to control for factors other than genetic that may have increased the risk of developing AMD in the affected relative. This study excluded smokers from taking part, but participants were not questioned about smoking or other risk-increasing lifestyle habits or environmental exposure of their AMD affected relatives.

The production of a commercial genetic test to predict those individuals from the general population that will develop AMD has been hampered by the challenge that remains in developing a unifying genetic susceptibility hypothesis.[785, 811] The Royal College of Ophthalmologists in the UK and the American Academy of Ophthalmology have concluded that routine genetic testing for AMD should be avoided until it can be shown from clinical trials that treatment would benefit those with specific disease-associated genotypes.[812, 813]

Development of the Macula Risk PGx test assessing 15 nucleotide polymorphisms in 12 AMD risk genes and the publication of two recent papers, have lead to an NHS technology alert suggesting that the components of supplements recommended to AMD patients at high risk of progression may be genetically tailored in the near future.[124, 814]

Awh *et al.* reported that patients with no CFH risk alleles and with one or two ARMS2 risk alleles derived maximum benefit from zinc-only supplementation. Conversely patients with one or two CFH risk alleles and no ARMS2 risk alleles derived maximum benefit from antioxidant-only supplementation. The outcome measure used in the study was the rate of progression from moderate to advanced AMD.[814] Feigle *et al*. reported that MPOD was significantly affected by specific BCMO1 gene variants (the level of β-carotene conversion efficiency was positively associated with MPOD), and that BCMO1 SNPs should be determined when assessing the effects of carotenoid supplementation.[809]

McKay *et al.* reported that five SNPs in the scavenger receptor class B, member 1 (SCARB1 or SR-BI) gene were significantly associated with serum L concentration and one SNP in SCARB1 was significantly associated with MPOD ($p < 0.01$). No evidence of a gender-specific interaction between serum L, MPOD or SCARB1 SNPs was found. This study performed multiple regression analysis with correction for age, BMI, gender, HDL, LDL, triglycerides, smoking and dietary L and Z levels.[815] The protein encoded by this

gene mediates cholesterol transfer to and from HDL.[816] It is possible that genetic variants within SCARB1 up- or down-regulate endothelial function or inflammatory pathways.[815]

Yanova-Doing *et al.* confirmed the association between BCMO1 and SCARB1 with baseline levels of serum L. The authors also reported four gene variants that were associated with MPOD response to supplementation with 18 mg L and 2.4 mg Z, for six months. Single nucleotide polymorphisms on RPE65 and FADS1 were positively correlated with MPOD level, whereas SNPs on ABCA1 and SCARB1 were negatively correlated with MPOD level after supplementation.[817]

Until targeted treatment for risk genes is proven effective the best advice for AMD prevention, especially for those at high genetic risk, and those with early AMD is modulation of environmental and lifestyle RF.[97, 799, 818] The second Age-Related Eye Disease Study (AREDS2) supplementation (with L and Z, but without β-carotene) may be offered to those with bilateral large drusen, or large drusen in one eye and advanced AMD in the other eye.[452, 819]

Many UK Ophthalmologists feel however, that in view of the high nutritional content of the Western diet, a large proportion of qualifying patients would not benefit from AREDS or AREDS2 supplements. The AREDS2 study confirmed that there was no benefit from L and Z supplementation to those who consumed ≥ 1.03 mg per day of L and Z in their diet. However, participants with a low dietary intake of L and Z at the start of the study (≤ 0.82 mg per day), who took the AREDS formulation with L and Z for the duration of the study, were 25% less likely to develop advanced AMD compared to participants with an equivalent dietary intake of L and Z who did not receive L and Z supplementation.[452, 820]

The corrected sample size required to detect a difference in MPOD of 0.01 assuming 80% power at 5% significance was estimated to be 302. Although a trend towards lower MPOD with AMD FH was revealed by this study, it lacked the power to detect a significant difference.

2.4.10 Migraine

A non-significant trend for lower MPOD for individuals reporting migraine was noted. Trends for higher MPOD for light-triggered and lower MPOD for non-light-triggered and non-aura migraine were noted. Numbers of migraine cases were small for all comparisons.

One study using an objective method of MPOD measurement (FAF) found significantly higher MPOD levels in migraineurs compared to controls.[193]

Cortical hyperexcitability / hyper-responsivity has been proposed as the cause of migraine symptoms,[821-823] however, gastric symptoms have been reported to originate from

peripheral dopamine receptors in the gut.[824, 825] It is plausible that peripheral dopamine receptors in the retina may contribute to light sensitivity in migraine, although this is not certain.[826-828]

The mechanism underlying migraineous aura is not considered to be retinal in origin, but due cortical spreading depression, which is an intense depolarisation of neuronal and glial membranes with loss of resistance and cessation of synaptic activity that spreads across the cortex at about three mm / min,[829] and an associated reduction in cerebral neurovascular coupling.[617]

Flicker has been implicated as a significant trigger for migraine.[830, 831] It is accepted that the perception of flicker is mediated through the geniculo-cortical pathway with a photopic spectral sensitivity which peaks at 560nm (yellow).[832] Photophobia and the flicker aversion response may involve a subcortical pathway derived from the population of Opn4-containing ipRGC with peak sensitivity at 480nm (blue), although this remains controversial.[826-828, 832]

Retinal adaptation is thought to be related to sustained activity from ipRGC to A18 dopaminergic amacrine cells which are likely to be responsible for dopaminergic signalling in steady illumination,[334] and HC coupling mediated by the direct effect of dopamine on HC gap junctions.[833] Dopamine levels are low interictally but increase during a migraine attack.[186, 834] Dopamine receptors are thought to be hypersensitive in migraineurs as a consequence of low dopamine in the interictal phase.[835-837]

Higher MPOD levels have been associated with reduced visual discomfort and fatigue, especially that derived from blue light,[567, 622, 838, 839] which may help to modulate symptoms of photophobia in migraineurs interictally, and in other conditions such as Meares-Irlen syndrome (MIS), also known as Visual Stress, characterised by an inability to adapt (habituate) to a steady, bright background.[840, 841] This symptom may be observed with dyslexia, but it is also present in the non-dyslexic population.

In light-triggered migraine however, the inability to habituate to flicker, rather than uniform brightness is of greater importance in triggering the ictal phase of migraine.[830, 842] Higher MPOD levels have been associated with increased visual performance, glare recovery and flicker sensitivity (critical flicker, or fusion frequency, CFF),[579, 621, 702, 843] whereas CFF for migraine sufferers with and without aura was significantly lower compared to controls.[831, 844]

MPOD reduces blue light exposure to cones located in the outer retina by approximately 40%,[845] but is less likely to affect ipRGC located in the inner retina. The geniculo-cortical

and ipRGC pathways are neither tightly coupled, nor operate in isolation,[334, 335, 846] therefore any intervention (e.g. MP) that modulates one of these pathways unilaterally, has the potential to correct in imbalance between these two pathways or conversely, trigger migraine symptoms by creating an imbalance, especially in those whose ability to habituate is already reduced or absent.[847-849]

Choroidal thinning is a feature of age and AMD. Inter-ictal migraine is associated with foveolar choroidal thinning and chronic ischaemia. In the ictal phase of migraine choroidal thickness was reported to be variously thinner or thicker than average, possibly suggesting ischaemia / reperfusion, which is associated with an inflammatory response. Reduced neurovascular coupling may lead to retinal ischaemia and the combination of retinal and choroidal ischaemia may induce a retinal watershed zone affecting bipolar cells in the inner nuclear and inner plexiform layers. Retinal ischaemia has the potential to activate Mc leading to further inflammatory changes and down-regulating antioxidant capacity. The effect of reduced retinal habituation (adaptation) may lead to larger pupil size and increased retinal illuminance under high luminance conditions and dopamine-related dysfunction in disc shedding and phagocytosis which have the potential to affect deposition of supra-RPE reticular pseudodrusen and sub-RPE drusen respectively (section 1.3 and fig. 1.3).

A legitimate criticism of this theory is that while migraine generally exhibits a reduction in frequency and severity with age, AMD risk clearly increases with age. The R/RPE/C antioxidant system (see A2.1) is more effective in younger individuals when the circulatory and inflammatory effects of migraine are at their greatest. There is evidence that the risk of developing early AMD associated with several CFH polymorphisms was actually lower than normal for individuals less than 55 years of age (OR 0.37 to 0.48), whereas the same CFH polymorphisms were associated with increased AMD risk for those aged over 75 years (OR 1.87 to 2.80).[810] It is of course plausible that choroidal thinning may remain after migraine headaches have ceased with age, and that any migraine-related retinal / choroidal damage at an early age may have an accumulative effect on AMD risk in older age.

Migraine summary

The author has suggested a plausible mechanism (fig. 1.3) for migraine to be considered as a RF for AMD. This study found a nonsignificant trend for lower MPOD with migraine. A positive significant association between objectively-measured MPOD and migraine was reported by Frandsen.[193] A trend for higher than normal HFP-derived MPOD in light-triggered migraine and lower than normal for individuals with migraine generally, and in non-light-triggered migraine and migraine without aura was revealed.

The relationship between MPOD and light trigger for migraine requires further investigation for two important reasons.

1 If this association is confirmed, it is important to establish whether the relationship is causal, i.e. does more MP lead to a greater risk of light-triggered migraine?

2 If a causal relationship between MPOD and the light trigger for migraine were confirmed, it would suggest that caution should be exercised when increasing MPOD levels in migraine sufferers because the risk of light-triggered migraine may be increased.

Dopamine is significantly involved in the pathophysiology of migraine, as well as in other conditions associated with light-sensitivity such as attention deficit hyperactivity disorder (ADHD).[186, 835, 850] Pharmacological downregulation of over-responsive dopamine receptors using dopamine agonists may be used for migraine prevention.[186, 836]

It is plausible that a reduction in light-triggered migraine may also be achieved by adopting a high dopamine diet; increasing intake of oily fish,[851] bananas[852] and anecdotally almonds, eggs and kale,[853, 854] whilst avoiding sugar and saturated fat.[855, 856] Exercise and obesity avoidance will also normalise dopamine levels,[857, 858] although the pattern of habitual food consumption, healthy or otherwise may be genetically determined.[762] This advice is remarkably similar to that recommended for AMD prevention.

Dietary (nutritional) therapy is an established treatment for phenylketonuria (PKU),[859-861] a rare metabolic disorder caused by a deficiency in the production of the hepatic enzyme phenylalanine hydroxylase (PAH), causing elevated levels of phenylalanine.[862] L-phenylalanine is converted to the amino acid L-tyrosine by PAH and tyrosine undergoes further enzymatic conversion to form dopamine.[862, 863] Patients with PKU with a dietary insufficiency of tyrosine will develop symptoms associated with low dopamine levels.[864, 865] Therefore there is precedence for dietary modification leading to increased levels of dopamine.

The corrected sample size required to detect a difference in MPOD of 0.001 assuming 80% power at 5% significance was estimated to be 424. This study lacked the power to detect a significant difference.

2.4.11 Raynaud's phenomenon

A non-significant trend for higher MPOD was noted for individuals reporting symptoms of Rph. The author is unaware of any previous studies examining the association between MPOD and Rph.

It is likely that individuals with both subtypes of Rph, primary and secondary were represented in this study. Under normal conditions primary Rph (pRph) is characterised by normal nail-fold capillaries, whereas sRph sufferers have tortuous and dilated capillaries

and areas of vessel dropout.[866] In Optometric practice nail-fold capillaries may be examined through an oil drop using a direct ophthalmoscope set at 40 diopters.[194]

Oxidative stress resulting from ROS secondary to ischaemia and reperfusion is involved in the pathogenesis of Rph.[194] N-acetylcysteine, a drug with antioxidant properties, primarily used as a mucolytic agent and Probucol, the cholesterol lowering and antioxidant drug, were both found to significantly lower frequency and severity of Rph episodes.[867, 868] Conversely, treatment with micronutrient antioxidants; selenium, β-carotene, vitamin C, vitamin E and methionine did not significantly improve Rph secondary to limited cutaneous systemic sclerosis.[869] While calcium channel blockers remain the most widely used drugs to treat Rph, other treatments are available and have been reviewed in detail by several authors.[194, 203, 204, 870]

The higher prevalence of Rph, like other vascular disorders such as migraine in women,[194] especially between menarche and menopause, suggests that hormones are involved with its pathogenesis.[871] Epidemiological studies have suggested that oestrogen is associated with Rph, possibly acting as a vasodilator associated with nitric oxide (NO) production and cytochrome P450 activity.[872, 873] In section 2.4.5 the association between lower oestrogen and increased AMD risk was discussed. The author is unaware of any studies associating Rph with MPOD or with increased AMD risk, despite a number of shared RF between Rph and AMD (table 1.11). A plausible theory for the involvement of Rph in AMD risk was proposed in the introduction to this thesis (fig. 1.3).

It is plausible that MPOD deposition in the retina may be adversely affected reduced or dysfunctional OVP, however retinal MP levels were reported to be relatively stable despite variations in dietary and serum L and Z, and saturable implying that above a certain MPOD level no more MP will be absorbed. The implication of these observations is that MPOD levels may not be affected by local variations in OVP.

Raynaud's phenomenon (pRph and sRph), like migraine, is associated with reduced retinal habituation and has the potential to lead to increased levels of ischaemia / reperfusion, oxidative stress and inflammation. These factors in the present of detrimental complement pathway gene polymorphisms have the potential activate changes that may result in AMD.

A higher prevalence of migraine in patients with primary Rph was also found by another study. This association was even greater for those with a family history of Rph.[874] Whole genome linkage analysis has identified three candidate genes for Rph; beta subunit of the muscle acetylcholine receptor and the serotonin 1B and 1E receptors.[875]

Raynaud's phenomenon summary

MPOD levels of patients with reported Rph were not significantly different from normal. The association between Rph and migraine suggests that they are part of a more widespread disorder of vascular tone with a genetic predisposition.[194, 876]

Oxidative stress is involved with Rph pathogenesis,[194] with catalase being reported as a reliable marker of the severity of oxidative stress in this condition.[877] Both ilaprost and probucol have been considered for the treatment of Rph.[877, 878] Both have antioxidant activity, and probucol was also found to down-regulate glial reactivity, induce ApoE production and improve HDL function,[879, 880] all of which may also benefit AMD risk and progression.

The corrected sample size required to detect a difference in MPOD of 0.002 assuming 80% power at 5% significance was estimated to be 82. This study had sufficient power to detect a significant difference.

2.4.12 Vascular dysregulation

No significant difference in MPOD was found for those participants with and without self-reported VDys. When calculated to three decimal places, median MPOD was 0.002 lower for individuals reporting symptoms of VDys compared to those without. The author is unaware of any previous studies examining the association between MPOD and VDys.

No distinction was made between primary vascular dysregulation (pVDys) and secondary vascular dysregulation (sVDys) in this study, but based on the age range of the sample it is likely that both types were represented.

Those with pVDys are more likely to suffer from migraine, but the two conditions are principally independent, however like migraine, pVDys may be associated with reduced neurovascular coupling in response to flickering light.[618] In this study 41% of participants with migraine also reported symptoms of VDys.

As a consequence of deficient autoregulation choroidal blood flow was reported to be higher than normal in subjects with pVDys, possibly in an attempt to maintain constant ocular temperature, despite reduced peripheral blood flow.[881, 882] However, in glaucoma patients with pVDys, choroidal blood flow was lower than those without pVDys.[205]

Glial activation of retinal astrocytes and Mc results from hypoxia in VDys. [883] The resultant changes in function and morphology of these cells leads to a reduction in antioxidant capacity by lowering GSH levels,[294, 884, 885] and can trigger an inflammatory response in this immune privileged tissue.[269, 798, 886] Macrophages are visible on OCT as hyper-reflective

spots in some patients with VDys, glaucoma, AMD, diabetes and vein occlusions.[205, 887, 888] Light-triggered glial activation is also a component of AMD pathophysiology.[278, 295, 889]

Like migraine, pVDys is associated with dysfunctional neurovascular coupling in response to flickering light. Vascular dysregulation is considered to affect ocular blood flow to a greater degree than Rph.[205] No studies were found comparing VDys with AMD, MPOD or GRT. Shared RF for VDys and AMD include; reduced choroidal blood flow, oxidative stress, inflammation, retinal glial activation, blood-retina barrier defects and lack of exercise.[205, 228, 257, 613, 614, 890, 891] The author has proposed a possible association between VDys and AMD risk (fig. 1.3).

In animal models exposed to retinal ischaemia / reperfusion injury, L was found to decrease Mc gliosis by inhibiting GFAP, thus exerting an anti-inflammatory effect by suppressing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and resultant production of pro-inflammatory markers interleukin-1 beta (IL-1β) and cyclooxygenase-2 (Cox-2) in Mc.[892-894] (See section 4.3).

Vascular dysregulation summary

The author has suggested a plausible mechanism (fig. 1.3) for Rph and VDys to be considered as RF for AMD. The OVP RF Rph and VDys may potentially be more important factors in AMD risk than migraine because they are more likely to persist until later in life than migraine. The alteration in choroidal circulation secondary to VDys did not significantly affect levels of MPOD. This analysis would have benefitted from a larger sample size, differentiation between pVDys and sVDys and controlling for age, gender and other factors thought to affect both variables.

Vascular dysregulation, especially sVDys, which is known to be associated with reduced choroidal blood flow, should be considered as a putative RF for AMD. Those with pVDys combined with harmful complement gene polymorphisms and / or reduced antioxidant capacity may also have increased risk of AMD.

Dietary advice designed to improve ocular circulation and reduce oxidative stress in VDys,[895] is likely to benefit those at risk of AMD with reduced choroidal circulation. Foods containing omega-3 (oily fish and linseed / flax seed), polyphenols (green tea, red wine, dark chocolate), lycopene (tomatoes) and anthocyanines (blueberries and bilberries) should be added to normal dietary recommendations for AMD. If the anti-inflammatory effects of L reported in animal studies can be replicated in humans, this may also be recommended in dietary or supplement form for those with VDys.

The corrected sample size required to detect a difference in MPOD of 0.01 assuming

80% power at 5% significance was estimated to be 886. This study did not have sufficient power to detect a significant difference.

2.4.13 Difficulty with HFP task

It was observed that older individuals and those with advanced stages of ocular disease experience more difficulty with the HFP task, especially with detection of flicker in the peripheral target.[507, 680] An inappropriate flicker rate for the HFP target was also reported to increase difficulty with the HFP task.[709] The author is unaware of any study comparing age, MPOD or GRT with participants experiencing difficulty with the HFP task and those without difficulty.

Crossland *et al*. reported no significant relationship between age and fixation stability.[896] The presence of reduced VA, and / or central scotoma secondary to ocular disease are likely reduce the ability to fixate a steady target. The likelihood of reduced VA and the presence of central scotoma increase with age. Reduced VA was associated with lower MPOD measurements in some,[596, 658] but not all studies.[593, 594, 655, 659]

The Baltimore Study of Ageing reported that distance VA was reduced with age in healthy individuals and those with ocular disease.[897] In the present study participants were excluded if LogMAR VA was less than 0.1, if any macular disease was visible with the direct ophthalmoscope or if they reported any history of macular disease.

Group analysis revealed no significant difference in MPOD values for participants who experienced some difficulty with HFP MPOD measurements requiring the measurements to be repeated, compared to those who found no difficulty with this technique. Twenty one (21%) of the 100 participants, six male and 15 female were required to repeat MPOD measurements. The median age of those experiencing difficulty was 53.9 years (IQR 25.5 years) compared to 47.9 years (IQR 11.8 years) for those without difficulty. A Mann-Whitney U test revealed no significant difference between the ages of the two groups.

To test whether there was an association between GRT and difficulty with HFP task, a Mann-Whitney U test was performed. Although those experiencing difficulty had longer GRT (41 s, IQR 28 s) compared to those without difficulty, (40 s, IQR 24 s) the difference was not significant.

This study did not include any participants with ocular disease. The results suggest that difficulty with the HFP task may be more greatly affected by ocular disease (which increases with age) than age.

The corrected sample size required to detect a difference in MPOD of 0.02 assuming 80% power at 5% significance was estimated to be 738. This study did not have sufficient power to detect a significant difference.

2.5 Chapter conclusion

This study examined the relationship between MPOD measured at 0.5° eccentricity using heterochromatic flicker photometry (HFP) and ocular dominance and difficulty with HFP task. The association between MPOD and six confirmed or putative RF for AMD; age, gender, BMI, %BF, iris colour and AMD FH, and three OVP RF; migraine, Rph and VDys was also investigated.

No significant differences were found between right and left eye, first and second eye or dominant and non-dominant eye MPOD, however, there was a significant bias between dominant minus non-dominant eye and right minus left eye MPOD (both with eye order measured randomly), although this did not survive the removal of a single outlier from the MPOD data. A small negative correlation was found for the difference between dominant and non-dominant eye MPOD, and age that warrants further study. No significant difference in MPOD, age or GRT was found between those experiencing and not experiencing difficulty with the HFP task. This result differs from the observations reported by other authors, who reported that difficulty with MPOD was associated with increasing age.

No significant differences in MPOD levels were found for any of the AMD RF examined, although the trends followed the results reported by the majority of other studies. Sample size calculations were based on MPOD differences reported from similar studies using HFP-derived MPOD values, or calculated using G*Power statistical software if previous data was not available.

It is evident that physiological conditions such as vitreomacular traction resulting in pseudo-operculae, strabismus, floaters and coloboma and a variety of pathological conditions will adversely affect MPOD measurements. Other causes of variation in MPOD with age are listed in appendix A2.4.

Spectral domain OCT macular scans may be used to exclude the majority of these conditions and should be included in any MPOD study. Objective measurement of the MPOD spatial profile would control against individual variability in MPOD measurement due to irregular MP deposition, inevitable if MPOD is only recorded for one eccentricity.

The greater question of whether a lack of MPOD is associated with an increased risk of AMD development or progression through early stages of AMD remains open. AREDS2

reported that MP supplementation reduced progression to advanced AMD, but only in participants with a low dietary intake of L and Z.[898]

It is a concern for those promoting the ocular protection theory for MP that there appears to be no significant association between MPOD and age. Previous studies finding an inverse correlation between HFP-MPOD and age may have been affected by instrument design characteristics that lead to an underestimation of MPOD in the elderly. Age is the most important RF for AMD development and progression and the protection theory ought to predict that MPOD levels would be lower for participants with AMD. The majority of studies to date have not reported a significant difference in MPOD levels between participants with early AMD and without AMD.[404]

A further concern is that repeatability (CoR) for this method of HFP MPOD measurement is greater than the expected increase in MPOD after improved dietary intake or supplementation of macular xanthophylls. Results from this study suggest that a period of rest between HFP MPOD measurements may be beneficial and has the potential to improve repeatability.

The relationship between MPOD and age may not be accurately described by linear statistical methods. The peak in MPOD level in the middle age range followed by a drop in MPOD in older participants reported by several studies and revealed as a trend in this study, would not be apparent using bivariate correlation.

There are arguments against the protection theory for MP. Werner and Beirne have independently concluded that their results do not support the protection theory for MP, due to the lack of an association with age.[506, 899]

The bias on repeated MPOD measurements, particularly with HFP, is considerably greater than the expected increase in MPOD after supplementation. Bartlett *et al*. reported a coefficient of repeatability for two users of 0.33 and 0.28, for the MPS 9000 screener,[499] whereas the mean increase in MPOD derived from 36 studies of healthy eyes and eyes with AMD, after MP supplementation was 0.16 (SD 0.34).[900] Excluding results from five studies examining patients with AMD, the present author has calculated a mean increase of only 0.08 (SD 0.05) for the remaining 31 studies of healthy eyes from the data reported in the previous paper. It is therefore difficult to be confident that a "low" value is really low, unless the test is repeated on another occasion (unlikely in a commercial clinical setting) or that any post-supplement increase in MPOD is genuinely caused by MP supplementation.

The sample size was corrected for unequal size groups (allocation ratio). Assuming 80% power (1 - β) at 5% significance level, the sample size was sufficiently large to be confident

that there was no significant difference for ocular dominance, age, BMI (mixed-gender), and Rph. This study was underpowered to detect a significant difference for difficulty with HFP task, gender, iris colour, AMD FH, migraine and VDys. Participant numbers were small, especially for migraine however, and so these results should be interpreted with caution.

The mean MPOD value for this healthy, White UK population was 0.38 (SD 0.17) and 0.40 (SD 0.16) for the right and left eyes respectively, with eye order randomised (n = 97, three monocular cases removed). The LoA (CI 95%) for this sample was 0.21, suggesting a normal range of first eye MPOD values from 0.17 to 0.59.

What is currently known:

1) There is no interocular difference in MPOD between healthy eyes.
2) Although controversial, the majority of studies using subjective and objective methods of MPOD measurement show no significant association with AMD RF.
3) In some HFP studies the age association may have been caused by instrument design.
5) Migraine is associated with higher objectively measured MPOD.
4) Difficulty with HFP measurement of MPOD is age-related.

What this study has found:

1) A possible bias between sequential interocular measurements of HFP MPOD.
2) The difference between dominant and non-dominant eye MPOD increases with age.
3) No association between HFP-derived MPOD and any of the AMD or OVP RF, although several of the comparisons lacked sufficient power.
4) Difficulty with this method of HFP-derived MPOD is unrelated to age, MPOD or GRT.

Chapter summary

This chapter examined two aspects of MPOD. The effect of sequential versus randomised measurement and ocular dominance on MPOD measurements and the relationship between MPOD and selected AMD and OVP RF. The next chapter will investigate four different aspects of GRT. An interocular comparison of GRT and the effect of ocular dominance on GRT, the relationship between GRT and selected AMD and OVP RF, the suitability of GRT as a surrogate measure for MPOD and GRT repeatability.

Chapter 3 Glare recovery time study (Experiment 2)

3.1 Brief introduction

The aim of this practice-based, cross-sectional study was four-fold. (a) To perform an interocular comparison for, and to investigate the effect of ocular dominance on this method of GRT. (b) To investigate the relationship between GRT and selected confirmed and putative AMD RF (age, gender, BMI, calculated %BF, iris colour and AMD FH), and OVP RF (migraine, Rph and VDys). Risk factors were limited to those easily measureable in optometric practice. (c) Assess the suitability of this method of GRT as a surrogate measure for MPOD. (d) To investigate intra-session and inter-session repeatability, and the effect of using the same vs. different test chart letters for repeated measures of GRT. Background information about GRT and its association with selected AMD RF was discussed in the introduction to this thesis (sections 1.5 and 1.7) and is summarised below in the brief introduction to this chapter.

Table 3.1 Summary of investigations (GRT)

Interocular comparison	A high level of interocular agreement is important if GRT has any value as a baseline measure prior to the onset or progression of AMD, where ocular involvement may not be symmetrical.
Ocular dominance	One study using a photo-flash method of GRT reported a bias towards longer GRT in the non-dominant eye.[329]
Age	Age is the strongest, established RF for AMD.[14, 41, 43, 625] The relationship between age and GRT has been reported to be controversial.[308]
Gender	Gender has not been consistently reported to be a RF for AMD. The Beaver Dam and BMES suggested that women might have a higher risk of developing AMD.[625, 627] The higher prevalence of late AMD in women compared to men, has been explained in part by the larger number of women in the older age range.[1, 45] The relationship between GRT and gender has not been consistent.[361, 602]
BMI	Higher than normal BMI was associated with increased risk of both early and late AMD.[133, 628] Another large study found no association between BMI and AMD.[39] No previous studies were found comparing GRT and BMI.
%BF	Higher levels of abdominal fat (waist / hip ratio), but not BMI or %BF was associated with increased risk of AMD in men, whereas all three anthropometric measures were related to increased AMD risk in women.[630] The author is unaware of any studies comparing GRT and %BF.
Iris colour	Light iris colour was associated with significantly greater light transmission and reduced choroidal melanin compared to darker irides.[631, 632] Significantly more cases of AMD have been reported for individuals with light compared to dark irides,[633] however BDES found no association between iris colour and AMD incidence and progression,[634] but did report an association between lighter iris colour and the development of RPE pigmentary abnormalities (ARM).[635] No association between GRT and iris colour was reported by one study.[329]
OVP	Vasospasm has been reported to play a central role in the pathogenesis of migraine, Rph and VDys.[194, 619, 638] Vasospasm was reported to affect choroidal and ciliary vessels more than retinal vessels.[619] Age-related macular degeneration RF including age, gender and iris colour, were associated with a reduction in choroidal blood flow.[643] The author is unaware of any studies examining the association between GRT and the following OVP RF.
Migraine	Participants were classified as self-reported migraine or non-migraine sufferers. Migraineurs were further divided into self-reported aura / non-aura and light-triggered or non light-triggered groups.
Rph	Raynaud's phenomenon is a cold-triggered, episodic vasospasm of the arteries in the extremities, causing pallor followed by cyanosis and / or redness of the fingers or toes.[203] Raynaud's phenomenon is classified as primary (pRph) when ideopathic, with age of onset < 30 years and as secondary (sRph) when caused by another condition (e.g. connective tissue disorders), with age of onset > 30 years.[194, 645] Prevalence is reported to vary from 3.4% to 20% for women and from 3% to 12.5% for men,[194, 203] with pRph accounting for 81% to 89% of cases.[901, 902] Raynaud's phenomenon was associated with a reduction in retinal capillary blood flow, which could result in ischaemia resulting in retinal dysfunction.[646]
VDys	Vascular dysregulation may be classified as primary or secondary. The exact prevalence of pVDys is unknown. Krauchi *et al*. reported from a Swiss population, that 31% of women and 7% of men complained of cold extremities,[903] however, Gasser *et al*. reported that only about 10% of women and 3% of men exhibit classic symptoms of pVDys.[207] Primary VDys, formerly known as vasospastic syndrome, cases have an inborn difference in their response of their vascular system to cold temperature, mechanical and physical stress.[205, 206] Primary VDys occurs more frequently in young, slim, adult females, with symptoms manifesting at puberty and reducing with age,[208] and is associated with a history of

	cold hands (and sometimes feet) unrelated to ambient temperature.[648] Sufferers also tend to have low blood pressure, especially at night.[209, 210] They exhibit less desire to drink due to the anti-dipsogenic effects of prostaglandin E_2 on the hypothalamus, secondary to slightly raised levels of ET-1.[211, 212] Sleep onset is often delayed and sleep interrupted, especially if the feet are cold.[213] Systemic drug sensitivity is abnormal with pVDys cases requiring a reduced dose of some drugs (beta-blockers and calcium channel blockers) and possibly higher doses of others (e.g. painkillers).[212] Individuals with pVDys have disturbed autoregulation, leading to instability in ocular blood flow leading to repeated, mild reperfusion injury and oxidative stress.[647] Secondary VDys (sVDys) may result from a large number of especially inflammatory and / or auto-immune diseases,[205, 212, 647, 648] and results from a significant increase in circulating ET-1, which constricts vessels resulting in reduced blood flow to both the eye and the kidney.[215, 216]
MPOD as a surrogate for GRT	The predictive effective of MPOD levels for AMD is controversial,[175, 904] A significant, inverse correlation was reported between GRT and MPOD by several authors,[622, 623, 905] however another study found no significant association between these variables.[906]
GRT repeatability	Intra-session and inter-session repeatability was assessed for this method of GRT. Two studies examining inter-session repeatability for equilibrium bleach GRT were located.[330, 907] A literature search revealed intra-session repeatability studies for photo-flash GRT only.[329, 359, 361]
Bias from same vs. different test chart letters	Bias in GRT measurement related to prior knowledge of the test chart letters was assessed by comparing repeat measures using the same letters vs. different letters.

3.1.1 Research objectives

The aim of this research was to contribute to the body of knowledge that has been collected for the relationship between GRT and the following AMD RF; age, gender, BMI, iris colour and AMD FH, and to assess the utility of GRT as a surrogate measure for MPOD measurement. GRT repeatability was assessed. In an attempt to make an original contribution to the literature, the association between GRT and the following AMD and OVP RF; BMI and calculated %BF, migraine, Rph and VDys on GRT measurement were also investigated. Intra-session and inter-session repeatability and interocular comparison have not been assessed for this method of GRT prior to this study. There have been no previous studies of GRT levels in this population.

3.2 Materials and methods

3.2.1 Subjects

For the *a priori* sample size estimation see section 2.2.1.

Post hoc sample size estimation

Sample size for the comparison between two means for GRT was calculated retrospectively from the data for age and gender from other studies collected (table 3.2), assuming 80% power (1 - β) at the 5% significance level (table 3.3). Effect sizes were obtained from the mean of at least two other studies. Similarly sized studies with White participants were included preferentially. The sample sizes were corrected for unequal numbers in each group (i.e. allocation ratio, r = larger group number / smaller group number).

No previous studies were found for the association between the method of equilibrium-bleach GRT used in this study and ocular dominance, mixed-gender BMI, iris colour, AMD FH, migraine, Rph, VDys and MPOD. In this case the effect size may be determined by logical assertion and conjecture,[651] or by calculation. G*power statistical software was used to calculate the effect size (from the mean and SD from each of the MPOD groups).

The calculated effect size was then used to the calculate sample size using the formulae in table 3.3. Sample size estimation was not performed for calculated %BF because this was derived from the BMI, age and gender data.

Table 3.2 Independent variable effect size for GRT extracted from the literature

Independent variable	Study / Bleach time	Ref.	n	Effect size (d)
Age	Malik (1971) / 30 s Collins (1989) / 10 s	[571] [577]	60 65	≤ 40: > 40 = 29.2 ≤ 55: > 55 = 27.6 **Mean = 28.4**
Gender	Malik (1971) / 30 s Torkelson (1941) 40 s	[571] [907]	60 150	3.3 9.0 **Mean = 6.2**

The author is unaware of any studies comparing equilibrium-bleach GRT with mixed-gender BMI, iris colour, AMD FH, migraine, Rph or VDys..

Table 3.3 *Post hoc* sample size estimates for the GRT study

AMD RF data	Age ≤ 50 vs > 50 years	Gender male vs female	BMI mixed-gender ≤ 25 vs > 25 Kg / m^2	Iris colour light vs dark	AMD FH FH vs no FH
Mean difference (GRT) s Standard deviation (S) s Effect size (d)	5.1 14.7 28.4	3.6 14.2 6.2	-0.07 0.17 0.39*	-0.01 0.16 0.03*	-0.06 0.16 0.38*
n per group (2-sided) $16/(d/S)^2$ Power = 80%, α = 5% Assuming r = 1	4	84	3	455	3
Sample size (M)	8	168	6	910	6
Allocation ratio (r) Number in smaller group (M1) (1 / (1 + r)) x M Number in larger group (M2) (r / (1 + r)) x M	1.47 3 5	2.62 46 122	1.09 3 3	1.61 349 561	4.80 1 5
Corrected value for M1 (M1c) M1c = r + (1/2r x M) [656]	6	220	4	734	19
Corrected sample size (Mc) M1c + M2	11	342	7	1,295	24
OVP RF and miscellaneous (bold border) data	Migraine yes vs no	Rph yes vs no	VDys yes vs no	Ocular dominance D vs ND	MPOD ≤ 3.8 vs > 3.8
Mean difference (GRT) s Standard deviation (S) s Effect size (d)	-0.02 0.16 0.11*	-0.01 0.17 0.06*	0.01 0.16 0.03*	-0.01 0.19 0.03*	0.01 0.16 0.03*
n per group (2-sided) $16/(d/S)^2$ Power = 80%, α = 5% Assuming r = 1	34	128	455	642	455
Sample size (M)	68	256	910	1,284	910
Allocation ratio (r) Number in smaller group (M1) (1 / (1 + r)) x M Number in larger group (M2) (r / (1 + r)) x M	5.27 11 57	2.72 69 187	2.37 270 640	1 - -	1.1 433 477
Corrected value for M1 (M1c) M1c = r + (1/2r x M) [656]	184	351	1,081	-	502
Corrected sample size (Mc) M1c + M2	241	538	1,721	1,284	979

* Effect size calculated from Log10 GRT values of mean and SD using G*Power assuming equal group size. Effect size convention: d = 0.2 small, d = 0.5, medium, d = 0.8 large. vs = versus. Previous GRT studies have not performed Log10 transformation, therefore in order to meaningfully compare these results with the data obtained for age and gender in this study, the mean and SD were transformed back to non-logarithmic values. Back-transformation of the SD from the Log10 GRT values produced an asymmetrical back-transformed range (BTR), therefore the mean of half of the values of the BTR were used as an estimate of the SD (s) for this calculation. D: dominant eye, ND: non-dominant eye.

The opportunity for data collection was limited to 1-2 days per fortnight, dependent on whether the consulting room was in use on the author's day off. This allowed for data to be collected from a maximum of between 16 and 32 participants each month.

3.2.2 Recruitment

This study was undertaken at the Bath Road practice of Norville Opticians in Cheltenham. The study required the recruitment of non-smokers aged 20 years and above with no eye disease. Data was collected for 150 participants over a 14-month period from the 4th of August 2010 to the 12th of October 2011, outside normal clinic hours. See appendix 4 for consent form, information sheets and practice poster.

Initially patients whom appeared to meet the inclusion requirements were sent an invitation to participate with the reminder letter for their next routine eye examination. The reminders were computer generated based on the time since their last eye test. Over a period of one month 100 invitations were sent out. Response was very poor, with only one respondent, who was excluded as a smoker. Posters and information sheets were displayed at four Cheltenham practices. Colleagues were emailed with information about the study and were invited to refer any suitable patients.

The author presented a talk about MP at the Norville Opticians annual professional staff meeting, where recruitment information was disseminated to colleagues. Suitable patients were invited by the author to participate in the study during their routine eye examination. This proved to be the most effective method of recruitment.

3.2.3 Inclusion / exclusion criteria for both MPOD and GRT studies. Please refer to section 2.2.3 in the previous chapter. Inclusion / exclusion Information relevant to GRT is listed below.

3.2.4 Justification for inclusion / exclusion criteria for GRT study

3.2.5 Ethical approval / informed consent

This study was approved by the Aston University, Audiology / Optometry Research Ethics Committee (AOREC) on the 12th of May 2010. (Reference number AO2010.15 HB) and adhered to the tenets of the Declaration of Helsinki, (sixth revision, October 2008).[670]

An ethics amendment was approved by AOREC on the 22nd of September 2011, for the collection of additional data for the GRT repeatability study. See appendix 5 for the confirmation of ethics clearance forms.

3.2.6 Instrumentation

GRT was assessed after a 30-second (s) macular bleach using the macular stop on the Keeler Specialist direct ophthalmoscope set to the highest intensity setting and held approximately two cm from the eye of the participant. The ophthalmoscope was fully

Table 3.4 Justification for inclusion / exclusion (GRT study)

VA	Visual acuity was inversely correlated with GRT,[353] however no relationship between VA and GRT was evident for eyes with LogMAR VA of 0.12 and better.[573] Eyes with LogMAR VA of worse than 0.1 were therefore excluded.
Macular disease	Glare recovery time is increased in many macular diseases such as AMD, CSC and CMO.[908-910]
Alcohol consumption	Alcohol was found to induce increased GRT that was dose-related. GRT values peaked one to two hours after alcohol consumption and fell to pre-drink levels after approximately six hr.[911] Participants were asked if they had consumed any alcohol that day.
Marijuana use	Marijuana was reported to produce a dose-related increase in GRT, evident for at least two hours after ingestion.[912] For ethical reasons participants were not asked about marijuana use.
Diabetes	Glare recovery time was prolonged in 55% of diabetics without retinopathy and if significantly increased, was predictive for those at a high risk of developing retinopathy.[624] Recorded as "yes", "no" or "unknown". Participants reporting "unknown" were included.
Glaucoma	Glare recovery time was significantly delayed in patients with chronic open angle glaucoma compared to normals ($p < 0.001$).[913] GRT measured at extrafoveal retinal locations was also increased in patients with primary open angle glaucoma.[914] Recorded as "yes", "no" or "unknown". Participants reporting "unknown" were included.
Poor night vision	Poor night vision may be caused by a variety of conditions such as chronic bowel disease,[308] RP,[915] subclinical vitamin A deficiency,[916] certain medications and disorders affecting photopigment regeneration, which could adversely affect GRT. Participants reporting this symptom were therefore excluded.
Medication known to affect macular function	Participants were excluded if they reported taking any of the following medications commonly known to affect macular function: The quinoline antimalarials; Chloroquine or Hydroxychloroquine, the phenothiazine-derived antipsychotics; Chlorpromazine or Thioridazine, the oestrogen receptor antagonist Tamoxifen and the acne medication Isotretinoin.[917-920] Recorded as "yes", "no" or "unknown". Participants reporting "unknown" were included.
Intestinal malabsorption disorders	Micronutrient deficiency (including vitamin A) was reported to be quite common in patients with Crohn's disease and other chronic gastrointestinal diseases. [921] Recorded as "yes", "no" or "unknown". Participants reporting "unknown" were included.

charged prior to each GRT measurement confirmed by the charge indicator on the charger base. The same rechargeable ophthalmoscope battery and bulb were used for all GRT measurements. The method of GRT assessment used in this study was selected because it utilised instrumentation readily available to most practicing optometrists, represented what is considered to be the most reliable method of photostress testing estimated to bleach more than 98% of visual pigment,[360, 573] and as a consequence of long duration of bleach and approximation of the requirements of a Maxwellian viewing system was relatively insensitive to pupil size differences.[573]

The 30 s bleach was timed using a GymBoss interval timer / stop watch (gymboss.com). The interval timer was set to count down from 30 s, after which the stopwatch started automatically. This timer may be worn on the sleeve of the examiner, allowing the count down to commence at the same time as the start of the GRT bleach. The stopwatch was stopped when the subject was able to read all five letters on the line above that recorded as their VA. See the previous chapter for details of the other instrumentation used in this study.

3.2.7 Methods

See sections 2.2.7 for details regarding the measurement of BMI, %BF, LoA and 95% CI on LoA, iris colour categorisation, reasons for the 50-year cut-off in age group analysis, categorisation of OVP RF and measurement of ocular dominance.

3.2.7.1 Manufacturer information for the spectral output of the direct ophthalmoscope bulb.

The 3.5 Volt halogen / xenon ophthalmoscope bulb used in this study was free from any manufacturer markings or engravings. A spare 3.5 Volt ophthalmoscope bulb purchased at the same time as that used in this study was marked with the identification code; 1011-P-5065. This is different from the 3.5 Volt halogen / xenon bulb used in the current incarnation of the Keeler Specialist ophthalmoscope (Keeler Ltd, Windsor, UK), which is identified by the marking; 1011-P-7034. Spectral emission data for the older bulb (5065) was obtained from the manufacturer after data collection had been completed.

Figure 3.1 Manufacturer spectral emission data for the direct ophthalmoscope

3.2.7.2 Back-transformation of the log10 transformed LoA

Back-transformation of the log10 transformed LoA such that they may be represented on the non-log10-transformed Bland-Altman plots was achieved using methods described by Euser *et al*.[922]

$$\text{Back-transformed LoA} = 2x(10^{a} - 1) / (10^{a} + 1) \qquad \text{(Eq 3.1)}$$

Where a = Log10 derived LoA value and x = mean difference non-log10 data

Back-transformation of the 95% CI on each LoA was not covered Euser *et al*., but may be achieved by substituting the value of the LoA with the upper and lower value of the CI for each LoA.

When constructing the Bland-Altman plots the relevant mean difference value must be added to the LoA and CI values.

3.2.8 Procedure

3.2.8.1 Procedure for data collection

Subjects were pre-adapted to normal room illumination for 10 min, during which time the consent forms were read and signed. Macular pigment optical density was measured prior

to GRT in the first session, but not in the second session.

Table 3.5 Procedure for first session data collection (GRT)

Sequence	Procedure
1	Pre-measurement exclusion factors were reviewed.
2	LogMAR VA and distance fixation, pupil size measurements were recorded for both eyes. Gender, date of birth and iris colour were also recorded.
3	Maculae were examined with direct ophthalmoscope, through non-dilated pupils for visible signs of pathology.
4	Medication and nutritional supplements were recorded.
5	The order of eye measurement was determined by the pseudo-random method of coin toss.
6	Participants reporting a history of migraine or epilepsy were warned about the risk of light-triggered symptoms.
7	A single central and peripheral MPOD measurement was obtained for both eyes, under normal room illumination. The non-tested eye was occluded with an opaque eye patch. Distance glasses (non-tinted) were worn for MPOD measurement. If none were available or if contact lenses were worn these were removed and the equivalent distance prescription in a trial frame was substituted.
8	The first set of GRT measurements were obtained from both eyes, maintaining the same eye order as for the MPOD measurements. Glare recovery time measurements were recorded approximately eight minutes after MPOD measurements for each eye.
9	Participants' weight and height were measured. BMI was calculated from the Eq 2.2.
10	Exclusion factors were reviewed after the measurements above were recorded.
11	Percentage body mass was calculated after data collection was complete, from the BMI, age and gender data using the Clinica Universidad de Navarra-Body Adipose Estimator (CUN-BAE) algorithm.[608]
12	Eye dominance data measured by finger pointing and alternate occlusion, a variation of the Miles test,[685] was collected retrospectively for 44 subjects, at their subsequent routine eye appointments.
13	The intra-session repeatability measurements of GRT were taken for both eyes, maintaining the same eye order, 10 minutes after the initial GRT measurements and approximately 18 min after MPOD measurements for each eye.

3.2.8.2 Time scale for data collection

As data collection during clinics was not possible, data was collected from participants on days when no clinic was running, every Wednesday or every other Wednesday, depending on whether the consulting room was free, in this single consulting room practice.

Data for the first session (MPOD and GRT measurements) were collected from the 4th of August 2010 to the 12th of October 2011. Data for the second session (GRT repeatability) were collected for 30 participants from the 31st of August 2011 to the 8th of November 2011. The difference in time between repeat measures of GRT ranged from two weeks to nine months. Repeat measures were performed within six weeks for 18 participants and between three and nine months for the remaining 12 participants.

Table 3.6 Time scale for MPOD and GRT procedure (up to one hour per subject)

Informed consent, exclusion criteria and explanation of MPOD procedure	MPOD 1st eye	MPOD 2nd eye	discussion of MPOD results and procedure for GRT	E1 GRT1 1st eye	E2 GRT1 2nd eye	Weight and height for BMI pupil size and iris colour	E1 GRT2 1st eye	E2 GRT2 2nd eye
10 min	5 min	5 min	3 min	1-4 min	1-4 min	10 min	1-4 min	1-4 min

Table 3.7 Second session procedure for GRT (up to 31 min per subject)

Adaptation to room	GRT1 1st eye	GRT1 2nd eye	Adaptation to room	GRT2 1st eye	GRT2 2nd eye
5 min	1-4 min	1-4 min	10 min	1-4 min	1-4 min

Eye order from session 1 was maintained in session 2

The examination time required for each participant in the first session was approximately 50 min; therefore appointments were scheduled at one-hour intervals. The examination time required for the second session performed on a different day to the first session was shorter, requiring approximately half an hour per participant. Subjects remained in the consulting room for the entire duration of each session and eye order was randomised by coin toss.

3.2.9 Randomisation / masking

See section 2.2.9

3.2.10

Statistical analyses were performed using SPSS 22 statistical software (IBM Corporation). Data were examined for normality using histograms, normal Q-Q plots, Shapiro-Wilk tests and corrected Kolmogorov-Smirnov tests. Non-parametric tests were used where normality was not demonstrated or where group size was smaller than 25 participants. A significant level of positive skew was demonstrated for the GRT data, therefore log10 transformation was used to create log-normal data allowing the use of Bland-Altman plots and Pearson correlations. Partial correlation was used to control for the effect of the other independent variables. Significance testing was two-tailed unless otherwise stated.

3.2.11 Study design

The cross-sectional study design was deemed the most suitable for this practice-based project. This type of study design is rated low on the traditional hierarchy of evidence, according evidence-based medicine,[686] having strengths and weaknesses compared to other study designs. Strengths include; quick and easy to conduct, data is only collected once, prevalence may be measured, multiple exposures may be studied and the design is good for descriptive analysis and hypothesis generation. Weaknesses include; inability to demonstrate cause and effect, inability to measure incidence, associations may be difficult to interpret and this study design is susceptible to bias due to low response and misclassification due to recall bias.[687]

3.3 Results

Data from 100 White participants were included in this study. Median GRT was 40 s (IQR 25 s). Mean age was 50.3 years (SD 10.4 years), ranging from 24.2 to 75.8 years. The number of male and female participants was 27 (27%) and 73 (73%), respectively. Unless otherwise stated, GRT results are presented for the second set of measurements from the first eye data (Eye 1, GRT2), derived from 44 right eye and 56 left eye measurements.

To obtain a fuller understanding of the relationship with BMI and %BF, participants excluded for low (n = 4) and high (n = 12) BMI were re-included for these analyses only.

These re-included participants had no additional reasons for exclusion other than reported raised cholesterol, which is associated with high BMI values. The mean age for these 116 White participants was 51.0 (SD 11.0), ranging from 24.2 to 75.8 years. The number of male and female participants was 32 (28%) and 84 (72%), respectively.

Ocular dominance was recorded retrospectively for 49 cases. Four equidominant cases were excluded. Data was missing for one eye in one case. Ocular dominance was confirmed for 30 right (68.2%) and 14 left (31.8%) eyes. Median GRT values for dominant, non-dominant and equidominant eyes were; 35.5 s (IQR 26.0 s), 34.5 s (IQR 26.0 s) and 50.0 s (IQR 48.0 s), respectively. Participants mean age was 50.0 years (SD 11.4 years), ranging from 24.2 to 75.8 years. The number of male and female participants was 14 (32%) and 30 (68%), respectively.

Intra-session repeatability measurements of GRT were taken from both eyes of 30 participants. Participants mean age was 49.1 years (SD 8.5 years), ranging from 36.4 to 71.6 years. The number of male and female participants was 9 (30%) and 21 (70%), respectively.

As a consequence of the large number of results generated for this chapter, the following section is limited to results that are significant, approaching significance or have not been reported previously. For a full summary of the demographics and reasons for exclusion for this chapter please refer to the appendix section A1. The "A" prefix indicates that the associated figure or table may be found in the appendix.

3.3.1 Demographics for first eye GRT

The first GRT results for both eyes, from the initial session (GRT1) were likely to have been adversely affected by MPOD testing performed eight min before. For this reason the following statistical analyses were performed using the second set of GRT values (GRT2) for the first eye measured 10 min after GRT1 and 18 min after MPOD testing.

The second GRT session (Rep. GRT) was conducted 2-5 weeks after the first session for 18 of the 30 participants and 3-12 months after the first session for the remaining 12 participants.

3.3.2 Summary of results

Table 3.8 Summary of results for GRT

Variable	Subcategory		Number of data (n)	Median GRT (s)	Interquartile range (IQR) (s)
Age (years)	Full age range		100	40	25
	≤ 50 years		57	35	20
	> 50 years		43	45	26
Gender	Male		27	41	25
	Female		73	39	23
BMI (both genders) (n = 116)	Slim	< 20	4	30	40
	Normal	20 to < 25	52	35	20
	Over-weight	25 to < 30	48	43	27
	Obese	≥ 30	12	34.5	23
BMI (male) (n = 32)	Slim	< 20	0	-	-
	Normal	20 to < 25	7	40	34
	Over-weight	25 to < 30	20	43	25
	Obese	≥ 30	5	32	17
BMI (female) (n = 84)	Slim	< 20	4	30	40
	Normal	20 to < 25	45	35	19
	Over-weight	25 to < 30	28	43	39
	Obese	≥ 30	7	35	25
Male %BF (CUN-BAE) (n = 32)	Lean	≤ 20%	2	44.5	-
	Over-weight	> 20 to 25%	6	34.5	14
	Obese	> 25%	24	43	25
Female %BF (CUN-BAE) (n = 84)	Lean	≤ 30%	7	31	15
	Over-weight	> 30 to 35%	25	35	20
	Obese	> 35%	52	41	26
Iris colour	Grey		12	38	18
	Blue		33	38	16
	Green		15	47	27
	Hazel		16	37	31
	Brown		24	37.5	26
	Black		0	-	-
Reported AMD FH	First and second degree		17	45	26
	First degree only		11	50	39
	Second degree only		6	43.5	23
	None		82	38.5	23
	Unknown (adopted)		1	20	-
Reported migraine	Yes		17	33	29
	Light-triggered		6	33	24
	Non-light-triggered		11	33	35
	Aura		10	32	18
	No aura		7	51	29
	No migraine		83	41	24
Reported Rph	Yes		27	41	26
	No		72	40	22
	Unknown		1	25	-
Reported VDys	Yes		28	40.5	26
	No		69	41	25
	Unknown		3	33	-
MPOD	≤ 0.39		48	38.5	23
	> 0.39		52	42.5	28
Pupil size	< 4 mm		40	40.5	28
	≥ 4 mm		49	40	25
	Unknown		11	38	23

Abbreviations. CUN-BAE: Clínica Universidad de Navarra - Body Adiposity Estimator.

Fifty of the 150 participants were excluded from this study. Monocular GRT results were obtained from one participant. Therefore the total number of participants included in the GRT study was 100 for the association with AMD and OVP RF, and 99 for the interocular comparison of GRT. Please refer to the appendix section A1 for a summary of the reasons for exclusion and frequency analysis for those excluded.

3.3.3 Interocular comparison of GRT

Table 3.9 Bivariate correlation between first and second eye log10 GRT

GRT	Number of data (n)	Age mean (SD) years	GRT median (IQR) s	Statistic Pearson r	Shared variance	P-value
1st Eye GRT	99	50.2 (10.4)	40 (25)			
2nd Eye GRT	99	50.2 (10.4)	36 (24)	0.76	57.2%	< 0.001
1st Eye GRT*	96	50.3 (10.3)	39.5 (25)			
2nd Eye GRT*	96	50.3 (10.3)	35.5 (22)	0.85	71.9%	< 0.001

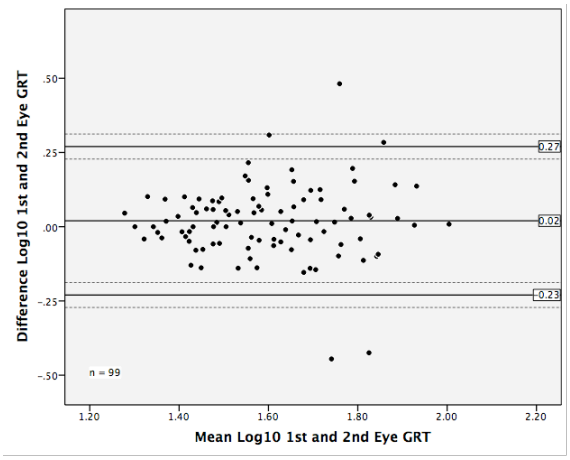
Abbreviations. IQR: interquartile range. Strength of correlation: r = 0.10 to 0.29 (small), r = 0.30 to 0.49 (medium), r = 0.50 to 1.0 (large). Probability values (p-values) < 0.05 are shown in bold. * excluding three outliers (ID: 31, 58 and 117).

Limits of agreement (LoA) are only estimates and therefore 95% CI have been calculated for each LoA according to McAlinden *et al*.[679] (Methods section chapter 2)

Log10 values of GRT were used in the statistical analysis of repeatability because the GRT data were positively skewed. This allowed the generation of Bland-Altman plots. The log10 GRT data were transformed back to linear values (back-transformed) and presented on a Bland-Altman plot of the non-log10 data in order to facilitate their clinical interpretation according to Euser *et al*.[922] (See the methods section of this chapter).

No significant difference was found between consecutively measured first and second eye GRT from the second set of GRT measurements (GRT2). For the explanation of the value of the multiplier used to calculate the LoA, the calculation of the 95% CI on each LoA and the method of back-transforming the Log10-derived LoA to the non-Log10 data.

Figure 3.2 Bland-Altman plot showing interocular comparison for log10 GRT



From the log10 GRT data:

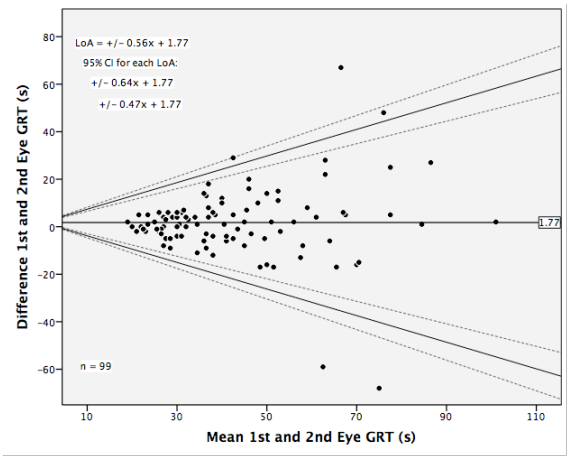
Mean first and second eye GRT = 1.59 (SD 0.16).

Difference between the means = 0.02 (SD 0.12).

LoA = 2 x 0.12 = 0.25 (95% CI 0.04).

After removing three obvious outliers LoA = 0.19 (95% CI 0.03).

Figure 3.3 Bland-Altman plot showing interocular comparison for GRT



From the non-log10 GRT data:
Mean first and second eye GRT = 42.7 s (SD 17.3 s).
Difference between the means = 1.8 s (SD 15.6).

$$\text{Back-transformed LoA} = 0.56x + 1.8 \qquad \text{(Eq 3.2)}$$

where x = mean of first and second eye GRT2 (s)

Limit of agreement for mean value of GRT of 42.7 s = 25.7 s (95% CI of 3.6 s).

Removal of three obvious outliers from the data above resulted in a log10 GRT LoA of 0.19 and a back-transformed LoA = 0.43x + 2.5, which for a mean GRT value of 41.9 s resulted in an LoA of 20.5 s (95% CI 2.8 s).

A Mann-Whitney U test performed on the non-log10 GRT data revealed no significant difference between the first and second eye GRT values, indicating no significant bias between the two GRT measurements.

3.3.4 Glare recovery time and ocular dominance

Table 3.10 Bivariate correlation between dominant and non-dominant eye log10 GRT

Log10 GRT	Number of data (n)	Age median (IQR) years	GRT median (IQR) s	Statistic Pearson r	Shared variance	P-value
Dominant eye	44	50.0 (11.4)	35.5 (26)			
Non-dominant eye	44	50.0 (11.4)	34.5 (26)	0.83	69.4%	< 0.001

Abbreviations. IQR: interquartile range.

A Mann-Whitney U-test was conducted to compare GRT for dominant and non-dominant eyes. Median scores were not significantly different for dominant eyes; 35.5 s (IQR 26 s) compared to non-dominant eyes, mean = 34.5 s (IQR 26 s), p = 0.76, two-tailed).

3.3.5 Glare recovery time and AMD risk factors

The relationship between GRT and the AMD RF was investigated using Pearson product-moment correlation coefficient. Preliminary analyses revealed a violation of the assumptions of normality, linearity and homoscedasticity. Kolmogorov-Smirnov (with Lilliefors significance correction) and Shapiro-Wilk tests for normality may reach significance for larger sample (n = 100), therefore scatter and Q-Q plots were also inspected. Non-parametric methods were used to assess the difference between groups. Log10 transformation was performed on the GRT data to allow the use of Pearson correlation and partial correlation corrected for age.

Table 3.11 Significant bivariate correlations for log10 GRT comparisons

Variable	No. of data n	Pearson r	Shared variance	P-value (2-tailed)
Age	100	0.329	10.8%	0.001
Female %BF	84	0.306	9.4%	0.005

The small positive, partial correlation between GRT and age was retained after controlling for BMI (r = 0.29, n = 100, p = 0.003). An inspection of the zero order correlation (r = 0.33) suggested that controlling for BMI had very little effect on the strength of the relationship between GRT and age. The small positive, Pearson correlation between GRT and female %BF was similarly age-related. After controlling for age female %BF (r = 0.11, n = 84, p = 0.31). The medium-sized, positive zero order correlation (r = 0.31) suggested that controlling for age had a significant effect on the strength of the relationship between GRT and female %BF.

Group analysis of the association between GRT and age, with age divided into two groups (≤ 50 years and > 50 years) was significant (p = 0.01).

Group analysis using the Kruskal-Wallis test between GRT and age, with age divided into four age ranges (< 45 years, ≥ 45 to < 50 years, ≥ 50 to < 60 years and ≥ 60 years), was significant (p = 0.03). Two follow-up Mann-Whitney U tests revealed a significant (at p = 0.025) difference between the ≥ 45 to < 50 year and the ≥ 60 year groups (p = 0.02), but not between the ≥ 45 to < 50 year and the ≥ 50 to < 60 year groups (p = 0.50).

Figure 3.4 Scatter plot for GRT and age

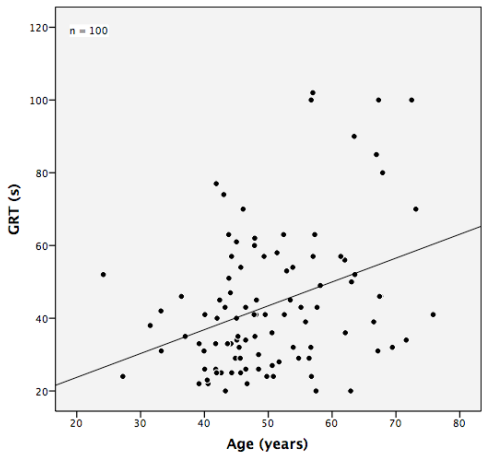
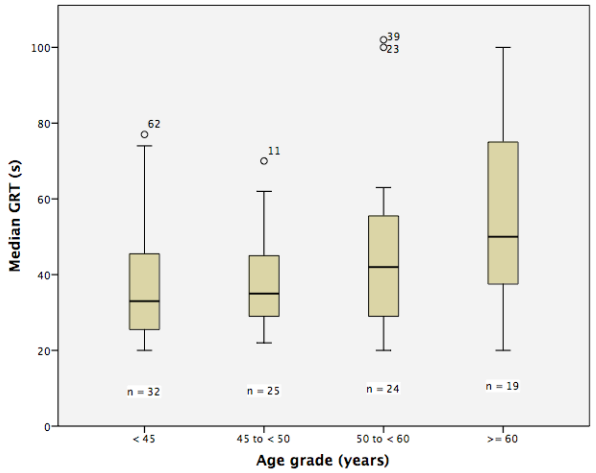


Figure 3.5 Box plot showing median GRT against age



3.3.6 Glare recovery time and OVP RF

No significant associations were found between GRT and migraine, Rph or VDys.

3.3.7 Glare recovery time as a surrogate test for MPOD

Table 3.12 Significant bivariate correlations between log10 GRT and MPOD

GRT	Number of data (n)	Pearson r	Shared variance	P-value (2-tailed)
MPOD (full age range)	100	0.218	4.8%	0.029
MPOD (> 0.39)	52	0.310	9.6%	0.025

Table 3.13 Significant partial correlation between log10 GRT and MPOD corrected for age

GRT	Number of data (n)	Partial correlation	Shared variance	P-value (2-tailed)
MPOD (> 0.39)	52	0.309	9.5%	0.027

Partial correlation was used to investigate the relationship between GRT and MPOD, controlling for age. There was a small, positive significant Pearson correlation between GRT and MPOD (full age range), and for above average MPOD values (> 0.39). After controlling for age a significant positive correlation was retained for above average MPOD values only, although the association with MPOD (full age range) approached significance

(p = 0.071). These results suggest that controlling for age had very little affect on the strength of the relationship between these two variables.

3.3.8 Glare recovery time repeatability studies

Table 3.14 Key for GRT abbreviations

GRT abbreviation	Explanation
First session	GRT performed 8-min (GRT1) and 18-min (GRT2) after HFP MPOD measurements
E1 GRT1 E2 GRT1 E1 GRT2 E2 GRT2	First GRT measurement for the first eye First GRT measurement for the second eye Second GRT measurement for the first eye (10 min after GRT1) Second GRT measurement for the second eye (10 min after GRT1)
Second session	Different day. GRT2 performed 10-min after GRT1, no prior MPOD measurements
Rep. E1 GRT1 Rep. E2 GRT1 Rep. E1 GRT2 Rep. E2 GRT2	Repeated E1 GRT1 Repeated E2 GRT1 Repeated E1 GRT2 Repeated E1 GRT2

Carstensen *et al*. reported that the correct factor required to derive the LoA is dependent on the number of data in the study. He advised that 2.08SD should be used if n = 30. In view of the small sample size 2.08SD was used to calculate the LoA in this repeatability study.[923]

Wilcoxon signed rank tests performed on the non-log10 transformed data revealed a significant difference in intra-session measurements of GRT from both eyes, in the first session. These GRT measurements were taken eight min (GRT1) and 18 min (GRT2) after MPOD testing. This level of bias would not be expected from repeated measures using the same method of GRT measurement and was likely to have been a consequence of prior MPOD testing affecting the first set of GRT (GRT1) values.[677]

Table 3.15 Significant results from GRT repeatability group analysis

GRT Intra-session	Number of data (n)	GRT median (IQR) s	Statistic	P-value p	Size effect
E1 GRT1 vs. E1 GRT2	100 100	45 (27) 40 (25)	Z = -5.573*	< 0.001	0.72 (large)
E2 GRT1 vs. E2 GRT2	99 99	40 (27) 36 (24)	Z = -5.901*	< 0.001	0.76 (large)
E2 GRT2 vs. Rep. E2 GRT2	30 30	33.5 (20) 40.5 (20)	Z = -2.608*	0.009	0.34 (medium)

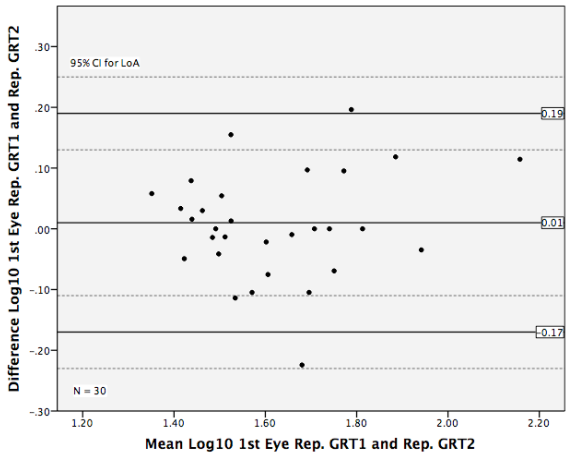
Abbreviations. IQR: interquartile range. *Wilcoxon signed rank test. Size effect: 0.1 (small), 0.3 (medium), 0.5 (large).

As a consequence of bias between first and second eye, GRT1 and GRT2 in the first GRT session due to prior MPOD testing, intra-session comparisons were made between first eye Rep. GRT1 and Rep. GRT2 in the second GRT session, and inter-session comparisons were made between first eye GRT2 in the first GRT session and first eye Rep. GRT2 in the second GRT session. Examination of the data using Wilcoxon signed rank tests revealed no significant difference between these groups.

3.3.8.1 Intra-session repeatability

No significant difference in GRT was found between repeat measures of the same eye recorded 10 min apart.

Figure 3.6 Bland-Altman plot for intra-session repeatability (Log10 data)



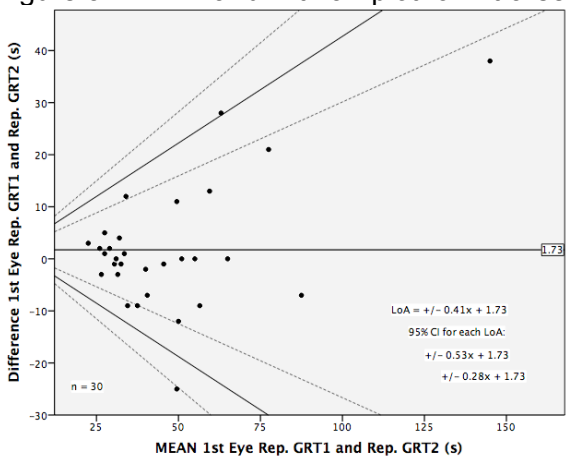
From the log10 GRT data:

Mean first eye GRT2 and Rep. GRT2 = 1.62 (SD 0.18).

Difference between the means = 0.01 (SD 0.09).

LoA = 2.08 x 0.09 = 0.18 (95% CI 0.06).

Figure 3.7 Bland-Altman plot for intra-session repeatability (LoA back-transformed)



From the non-log10 data:

Mean first and second eye GRT = 46.4 s (SD 24.6 s)

Difference between the means = 1.7 s (SD 12.01 s)

$$\text{Back-transformed LoA} = 0.41x + 1.7 \qquad \text{(Eq 3.3)}$$

Where x = mean rep. GRT1 and GRT2 (s)

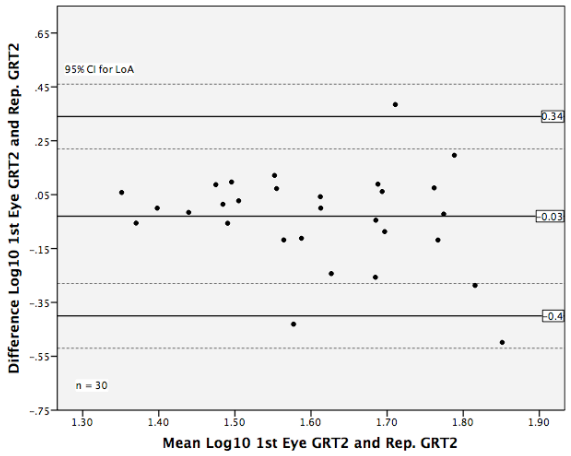
Coefficient of repeatability for mean value of GRT (46.4 s) = 20.8 s (95% CI 5.7 s).

A Wilcoxon signed rank test revealed no significant difference between repeat measures of the same eye, within one session. The size effect was negligible, indicating no bias between the two GRT measurements.

3.3.8.2 Inter-session repeatability

No significant difference in GRT was found between repeat measures of the same eye between sessions two weeks or more apart.

Figure 3.8 Bland-Altman plot for inter-session repeatability (Log10 data)



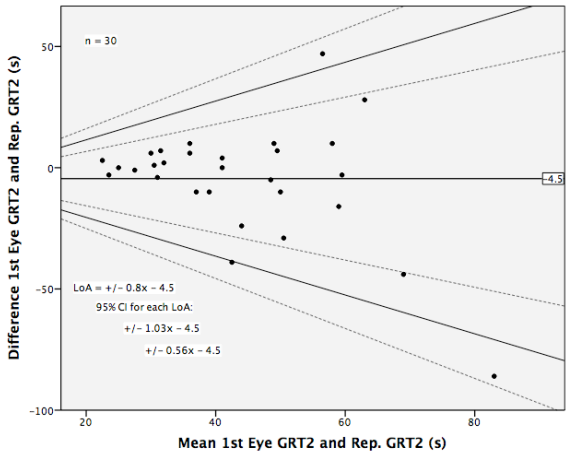
From the log10 data:

Mean first eye GRT2 and Rep. GRT2 = 1.60 (SD 0.14).

Difference between the means = -0.03 (SD 0.18).

LoA = 2.08 x 0.18 = 0.37 (95% CI 0.12).

Figure 3.9 Bland-Altman plot for inter-session repeatability (LoA back-transformed)



From the non-log10 data:

Mean first and second eye GRT = 43.2 s (SD 14.6 s)

Difference between the means = -4.5 s (SD 23.4 s)

Back-transformed LoA = 0.80x + -4.5 (Eq 3.4)

Where x = mean of GRT2 and rep. GRT2 (s)

Coefficient of repeatability for mean value of GRT (43.2 s) = 30.4 s (95% CI 10.6 s).

A Wilcoxon signed rank test revealed no significant difference between repeat measures of the same eye, within one session. The size effect was negligible, indicating no bias between the two GRT measurements.

Inspection of the back-transformed LoA for the intra-session measurements indicated a higher level of repeatability than inter-session measurements of GRT.

In view of the variation in time between repeat measures of GRT in the inter-session repeatability study (two weeks to 11 months), the data were re-analysed for participants in whom GRT was repeated within five weeks (n = 18).

From the log10 data:
Mean first eye GRT2 and Rep. GRT2 = 1.60 (SD 0.15).
Difference between the means = -0.04 (SD 0.16).
LoA = 2.08 x 0.16 = 0.33 (95% CI 0.14).

From the non-log10 data:
Mean first and second eye GRT = 42.6 s (SD 15.3 s)
Difference between the means = -6.1 s (SD 22.8 s)

Back-transformed LoA = 0.73x + -6.1 (Eq 3.5)

Where x = mean of GRT2 and rep. GRT2 (s)

Coefficient of repeatability for mean value of GRT (42.6 s) = 25.0 s (95% CI 11.0 s).

3.3.8.3 Analysis of bias due to learning effect of unchanged letters on test chart

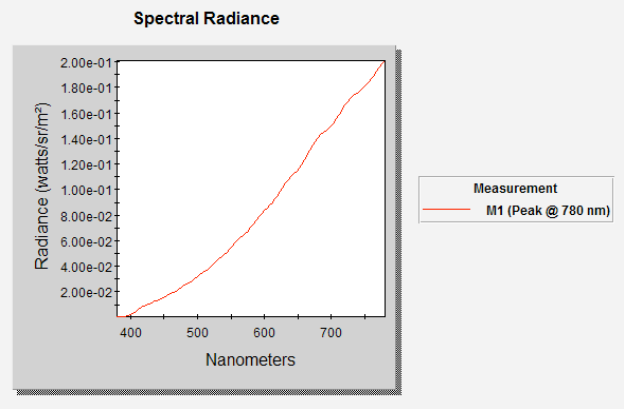
The same test chart letters were used as a target to assess first eye GRT1 and GRT2 in the second GRT session, whereas different letters were used for each GRT measurement for the second eye in the same session. In order to assess whether there was a learning effect from using the same letters for both GRT measurements for the first eye, an independent t-test was conducted for the mean difference between GRT1 and GRT2 for each eye. No significant difference was found between the variables, which indicated that using the same

letters for successive GRT measurements did not introduce any significant bias compared to changing the letters for successive GRT measurements.

3.3.9 Spectral analysis of the illumination sources used in this study

The spectral emission from the direct ophthalmoscope bulb (unmarked) used to determine GRT was measured. Four measurements were taken, all of which resembled the profile illustrated below (fig. 3.10). The emission spectrum shows very little blue light (450 - 495 nm) emission. This was comparable with spectral emission data (fig. 3.1) provided by the ophthalmoscope manufacturer (S. Church, personal communication, March 10, 2015).

Figure 3.10 Spectral emission measured for the Keeler Specialist direct ophthalmoscope



(© Everett, 2014).

Spectral measurements were also taken from the MPS 1000 screener background during the testing phase when background brightness was higher. Four results were recorded, all of which were similar. The results showed a significant peak at 444 nm.

An average of four measurements of total illuminance were taken from the direct ophthalmoscope and the MPS 1000 screener background during the testing phase using a light meter (Eurisem Technics EP628 Digital Lux Meter). The average illuminance measurements were surprisingly similar at 125 Lux for the direct ophthalmoscope and 109 Lux for the MPS 1000 screener background.

Margrain and Thomson reported that the retinal illuminance from a random selection of five direct ophthalmoscopes ranged from 6.18 to 6.86 log Td.[573]

Measurements in Lux may be converted to log Td using the formula

$$\text{Log Td} = \log (\text{measurement in Lux} / 0.0035) \qquad \text{(Eq 3.6)}$$

Where 1 Td = 0.0035 lm / m^2, Td = troland, lm = lumens

The ophthalmoscope used in this study was found to produce 4.55 log Td. Assuming a 5.5% reduction in retinal illuminance due to Stiles-Crawford effect,[330, 355] results in a value of 4.53 log Td retinal illuminance. This would equate to a photopigment bleach of approximately 50% at equilibrium.[581] Comparison between the GRT found by this study and that of the various methods used by Margrain and Thompson would suggest that a higher percentage bleach was obtained and that the light meter had underestimated the light output of the direct ophthalmoscope used in this study.[573]

In view of the lower measurement compared to Margrain and Thomson, the average illuminance of the ophthalmoscope was remeasured using a different light meter (Sinometer LX1010BS Digital Lux Meter). A similar average of 120 Lux was obtained at a distance of 2 cm. It was clear however that the circle of illumination from the ophthalmoscope did not cover the full area of the light sensor of either light meter. The ophthalmoscope light output was remeasured at a distance (11 cm) that ensured full light coverage of the lux meter sensor and the inverse square law was used to calculate the effective retinal illuminance at 2 cm (D. Thomson, personal communication, April 28, 2014).

Inverse square law

$$E_1 / E_2 = D_2^2 / D_1^2 \qquad \text{(Eq 3.7)}$$

Where E = illumination at centre, D = distance from light

The lux meter recorded 71 Lux at 11 cm, which after applying the inverse square law gave 2,239 Lux at 2 cm. After correction for Stiles-Crawford effect this is equal to 5.78 Log Td. This equates to a cone photopigment equilibrium bleach of approximately 95%. This is higher than the minimum 5.5 Log Td required for an ideal bleach and considerably closer in value to the 98% to 99.6% reported by Margrain and Thomson for a random selection of ophthalmoscopes.[573]

3.4 Discussion

The key results and how they compare to those of other studies are discussed below. Unless otherwise stated comparison was limited to studies using the direct ophthalmoscope as the source of illumination and populations consisting of White, or predominantly White ethnicity. In the absence of such studies, other methods of equilibrium bleach were considered before photo-flash bleach methods.

A literature search was performed using Web of Science, Science Direct, PubMed Central (PMC) and Google Scholar for the following search terms: glare recovery and photostress recovery combined with interocular, ocular dominance, age, gender, body mass index,

percentage body fat, iris colour, pupil size, AMD family history, migraine, Raynaud's and vascular dysregulation. Wildcard symbols were used to search for variations in spelling. Further references were retrieved from the papers revealed by the literature search.

3.4.1 Normal and abnormal GRT values

The median GRT for this 100% healthy White, UK population was 40.0 s (IQR 25 s). The mean age of this population was 50.3 years (SD 10.4 years), ranging from 24.2 years to 75.8 years.

Comparison of these results with those of Margrain and Thomson using the same method of GRT (mean GRT 50.2 s, SD 13 s, mean age 44.7 years, SD 15.3 years),[573] revealed lower values of GRT for the present study. This discrepancy did not appear to be age-related, but may be due to differences in retinal illumination secondary to ophthalmoscope bulb output or working distance, or population differences.

A of GRT greater than 68.4 s (42.7 s + 25.7 s) would be considered abnormal for this population. This compared well with the equivalent value of 76 s (50.2 s + (2 x 13 s)) derived from the data of Margrain and Thomson.[573]

3.4.2 Interocular comparison

The difference between the means for the two sets of raw GRT data was 1.8 s (SD 15.6 s). Interocular LoA for the mean value of GRT (42.7 s, SD 17.3 s) was 25.7 s, or 60.2% represented as a percentage of the mean GRT value. After the removal of three outliers from the GRT data, the LoA was reduced to 20.5 s, representing 48.9% of the mean GRT value. For other values of GRT, interocular LoA may be calculated using equation 3.2.

Consulting the back-transformed Bland-Altman plot (fig. 3.3) confirmed that the interocular LoA was positively correlated with the value of GRT. A Mann-Whitney U test revealed no significant difference in GRT between the first and second eye measurements of GRT, suggesting no bias or learning effect. Pearson correlation revealed a large positive inter-eye correlation between first and second eye measurements taken 18 min after MPOD testing in the first session.

The measurement of GRT from the first eye five minutes before GRT measurement in the second eye did not significantly affect the second eye measurements. For repeat measurements of the same eye 10 minutes were left between measurements to allow sufficient time for cone recovery.

Inspection of the Bland-Altman plot with back-transformed LoA indicated that although the interocular LoA increased with increasing GRT, generally the interocular agreement was

good with 72% of the subjects showing a between eye difference of 10 s or less, and 52% of subjects showing a between eye difference of five s or less.

Five of 99 (5%) participants had an interocular difference in GRT of ≥ 25 s, indicating a difference that would be considered abnormal. All were free from glare-related symptoms or ocular disease. No explanation found for the large interocular difference measured from these participants. The appearance of sporadic abnormal interocular differences in GRT in this healthy population would suggest repetition of abnormal measurements to confirm any difference.

Interocular comparison of GRT has been reported by seven studies. High levels of interocular correlation were reported by two studies (both photo-flash). Sloan *et al.* and Pratt *et al*. found an interocular correlation of r = 0.83, p n/a and r = 0.73, p n/a, respectively.[905, 924] Four studies (one equilibrium and three photo-flash bleach GRT) reported no significant interocular difference for right and left sequential GRT measurements.[361, 925-927] A learning or training effect was noted with sequential measurements in two studies (one equilibrium and one photo-flash), leading to lower GRT values for the second eye tested, although the differences were not significant.[924, 925]

Interocular comparison summary

Consensus suggests that there is good interocular agreement for GRT measurements taken from healthy subjects. No significant learning effect was found for this method of GRT testing despite the absence of a formal period of re-adaptation between measurements from each eye. Interocular differences greater than two standard deviations should be confirmed by repetition before any conclusion about the significance of their abnormality should be reached. Correlation measures the strength of the relationship between two variables, but not the agreement between them. For that reason LoA were calculated to measure the agreement between interocular GRT values.

3.4.3 Ocular dominance

A Mann-Whitney U test revealed no significant difference between dominant and non-dominant eye GRT.

One other study comparing GRT and ocular dominance was found. Loughman *et al.* reported that the non-dominant eye GRT (5.83 s, SD 1.72 s) was significantly longer than the dominant eye GRT (5.50 s, SD 1.70 s, p = 0.03).[329] The same method of categorising ocular dominance was used in both studies, a variation of the Miles test described by Roth *et al*.[685]

Disparity in the results may have caused by differences in GRT method and study design. In the study of Loughman *et al.* four sequential measurements were made, three from the dominant eye and a fourth from the non-dominant eye separated by two min intervals. GRT measurements were compared from the second dominant eye measurement and the first and only non-dominant eye measurement. The first dominant eye measurement (7.37 s, SD 3.20 s) was longer than the non-dominant eye measurement in their study.

The authors reported a significant learning effect producing shorter GRT with sequential measurement of the dominant eye. In the present study the order of the dominant and non-dominant eye GRT measurements was randomised, in order to reduce sequential bias or learning effects. It is accepted that the present study size was smaller (n = 44, compared to n = 100 for Loughman *et al*.). The learning effect for photo-flash methods may be reduced in clinical practice by increasing the adaptation period between sequential measurements of GRT to 5-10 min.[924, 925] This will of course increase the time required to obtain repeated measurements of GRT, making this approach less attractive in the clinical setting.

It is plausible that as the non-dominant eye tends to be closed under conditions of bright light or glare, the dominant eye may receive a greater lifetime exposure to light. It is however, difficult to measure this effect. Glare recovery time may be expected to be longer in dominant eyes if the increased retinal light exposure produced light-related retinal damage or increased Mc activation, adversely affecting the cone-specific visual cycle. Conversely, GRT may be expected to be shorter for dominant eyes if the increased light exposure had influenced retinal or cortical adaptation.

Ocular dominance summary

No significant difference in GRT was found for ocular dominance. Differences in GRT method and experiment design would explain the difference in results between this study and that of Loughman *et al.* In the previous chapter it was seen that a trend for lower MPOD in the dominant eye (0.40, SD 0.16) compared to the non-dominant eye (0.43, SD 0.17), although the difference did not reach significance (p = 0.56).

The corrected sample size required to detect a difference in MPOD of 0.6 s assuming 80% power at 5% significance was estimated to be 1,284. This study lacked sufficient power to detect a significant difference.

3.4.4 Age

This study of healthy subjects found a medium, significant positive correlation between GRT and age. The amount of shared variance between GRT and age was 10.8% (n = 100, p < 0.001). Partial correlation was used to control for BMI. A significant positive correlation was maintained (p < 0.001) after controlling for BMI, with a shared variance of 8.6%.

The degree of correlation was higher between GRT and age for those over 50 years of age ($r = 0.28$, $n = 43$) compared to those up to 50 years of age ($r = 0.08$, $n = 57$), although neither correlation reached significance, suggesting a trend for the progressive increase in GRT after the age of 50 years.

Group analysis between GRT and age, with age divided into two groups (≤ 50 years, $n = 57$ and > 50 years, $n = 43$) and four groups (< 45 years, $n = 32$, ≥ 45 to < 50 years, $n = 25$, ≥ 50 to < 60 years, $n = 24$ and > 60 years, $n = 19$), was significant ($p = 0.01$ and $p = 0.03$, respectively. *Post hoc* testing revealed a significant difference between ≥ 45 to < 50 compared to ≥ 60 years age groups. A trend was found for a steady increase in GRT with age for those up to middle age (approximately 50 to 60 years of age) and a steeper increase in GRT with age for those above the middle age range.

The correlation and group analysis data suggest that the relationship between GRT and age is biphasic rather than linear. The increase in GRT with age was greater for those aged over 50 years compared to that for those aged up to 50 years (see figs. 3.4 and 3.5).

All other studies including healthy subjects, examining the relationship between GRT assessed by equilibrium bleach, and age reported an increase in GRT with age.[330, 348, 571, 573, 574, 577, 928-931] Studies cited by other authors reporting no association with age included subjects with retinal disease.[349, 350, 352, 358]

The relationship between GRT assessed by photo-flash methods, and age is more controversial. Three studies reported a significant increase with age,[684, 926, 927] Bartlett *et al.* study reported a significant correlation for those aged 50 years and under only,[359] Newsome and Negreiro reported a significant correlation for those aged 55 years and over only,[361] Sloan *et al*. reported a normal curve relationship between GRT and age,[924] and Wood *et al*. reported a negative trend between GRT and age.[330]

Wood *et al*. examined the association between GRT and age for equilibrium and photo-flash methods. They concluded that equilibrium bleaching is likely to deplete local stores of 11-cis-retinal available to cone photoreceptors from Mc, placing a greater emphasis on the RPE for cone pigment regeneration, whereas in photo-flash methods 11-cis-retinal is likely to be available from Mc and the RPE for cone photopigment regeneration.[330] The variability in the association between photo-flash GRT and age may be a consequence of a differential contribution from these two sources of 11-cis-retinal for cone pigment regeneration.

Rushton and Henry compared the amount of cone pigment bleached and recovery time for equilibrium and photo-flash methods. They used a value of light energy for each method

calculated to result in a similar percentage of total photopigment bleach: 94% (equilibrium, 5.5 Log Td for 210 s) and 95% (three different exposure times for photo-flash, 7.5 x 10^6 Td s for 1.5 s, 0.75 s and 0.083 s). They reported that the equilibrium method bleached 94% of total cone photopigment. The photo-flash methods only bleached approximately 60% of total cone photopigment and full recovery was complete in about half the time required for the equilibrium method.[582] Differences in neural adaptation are also likely between equilibrium and photo-flash bleach methods.

Media opacification was not found to influence GRT.[581] Pupil size is recognised to reduce as a function of age.[932] No significant correlation was found in this study between GRT and pupil size. Sloan *et al.* reported no significant association between pupil size and GRT.[924] Three studies have reported that pupil mydriasis did not influence GRT,[909, 927, 933] however the effect of pupil miosis on GRT is controversial with one study (photo-flash GRT) reporting a significant reduction in GRT with pupil miosis,[927] and one study (photo-flash GRT) reporting no significant effect.[684] VA is known to deteriorate after the age of about 50 years,[684] however only subjects with good VA (0.1 LogMAR VA or better) were included in this study.

The biphasic or quadratic relationship between GRT and age in healthy subjects revealed by this study has also been reported by four other studies.[361, 577, 684, 928] Visual function and subject response variability were reported to decline from 50 years of age. [308, 934] Many visual parameters (e.g. age, CS and visually-evoked potential latency) in normal populations exhibit a biphasic relationship with age, characterised by functional stability up to approximately 50 years of age (up to 60 years for VA), after which an abrupt age-related decline in function is observed. It is thought that this decline in visual function is caused by changes in the neural system rather than the effects of media opacification or pupil miosis.[684]

Equilibrium bleach GRT places a greater stress on the RPE than photo-flash GRT. It is also possible that increased GRT in healthy elderly individuals is an indication of subclinical levels of RPE dysfunction.

Low levels of fruit and vegetable consumption is associated with increased risk of AMD,[168, 935] The negative correlation between GRT and MPOD reported by several researchers suggests that the age-related increase in GRT may be reduced in those with a poor diet, with the intervention of a healthy diet containing nutrients beneficial for retinal function and preservation.[620, 622, 623, 905] Richer *et al*. reported an improvement in GRT in AMD patients after supplementation with L for 12 months, which was significant for AREDS retinal stages 2 and 4, but not stage 3.[936]

Age summary

Age is the strongest, established RF for AMD. Advancing age is associated with an increase in AMD prevalence, incidence and progression.[14, 41, 43, 625] AMD was associated with longer GRT in the majority of studies (12 out of 14) reviewed by Neelam *et al*.[308] All of the equilibrium bleach methods reported longer GRT with AMD. The two studies showing no relationship used photo-flash bleaching methods.[358, 360]

The increase in GRT in healthy individuals with age is less controversial with equilibrium compared to photo-flash bleach methods. The relationship between equilibrium bleach-derived GRT and age is best described as biphasic or quadratic, increasing more steeply with age in healthy subjects, after the age of 50 to 60 years old. The age-related increase in GRT is likely to have a neural origin rather than being a consequence of media opacification or pupil miosis.

The corrected sample size required to detect a difference in GRT of 5.1 s assuming 80% power at 5% significance was estimated to be 11. This study had sufficient power to detect a significant difference.

3.4.5 Gender

No significant difference in GRT was found between genders.

Three early studies (all equilibrium) reported significantly longer GRT for females compared to males. Torkelson reported longer female GRT across the entire age range,[907] whereas Forsius *et al*. and Malik *et al*. reported a significant difference only for young to middle-aged subjects.[571, 602] Four more recent studies (two equilibrium and two photo-flash), revealed no significant difference in GRT with gender,[328, 361, 927, 937] although a trend for longer female GRT was noted by two of the studies.[328, 927]

In the previous chapter it was reported that female populations tend to have a greater proportion of blue irides than male populations, and that blue irides tend to associate with larger pupil size and slower pupil reactions to light. These factors were not significant, and did not influence the results in this study.

Females are known to have 10% higher %BF than males. This study revealed a significant correlation between female %BF and GRT, whereas no significant correlation was obtained for males. This correlation did not survive correction for age.

Females have a greater prevalence of migraine, Rph and VDys, conditions associated with reduced OVP, retinal ischaemia and dysfunctional adaptation and neurovascular coupling. No significant association was revealed between GRT and any of the OVP RF.

During the menopause (40-55 years of age) females experience a drop in oestrogen levels. Oestrogen is known to act as an antioxidant and anti-inflammatory agent.[302] It is possible that lower oestrogen levels, if not compensated by other antioxidant and anti-inflammatory pathways may increase retinal oxidative load and contribute to RPE dysfunction. If RPE function is compromised to a greater degree in females with lower oestrogen, this could explain longer GRT in females at or past the menopause. Inter-gender comparison of GRT for those under and over 40 years of age, with the exclusion of other factors known to affect GRT (see table A2.5) and measurement of serum oestrogen levels would allow the effect of oestrogen on GRT to be studied.

Gender summary

The increase in AMD risk associated with female gender is considered to be weak and inconsistent. No significant difference in GRT was noted for gender by this study, or any of the most recent studies. It is not clear why the significant gender differences reported by earlier studies were not replicated by later studies, despite similar methods of GRT assessment, although it is possible that the lack of correction for age may explain the earlier results. After correction for confounding factors such as age it is unlikely that a gender difference in GRT will be detected.

The corrected sample size required to detect a difference in GRT of 3.6 s assuming 80% power at 5% significance was estimated to be 342. This study lacked sufficient power to detect a significant difference.

3.4.6 Body mass index

The examination between BMI and %BF with GRT included subjects with BMI values < 20 and ≥ 30 (n = 116) excluded from the other comparisons.

No significant association was found for mixed- or separate-gender BMI with GRT, before or after correction for age. The author is unaware of any previous studies examining the association between GRT and BMI.

The percentage of obese subjects is lower than that reported for the UK in 2002 (male 23% and female 25%), and estimated by projection for 2012 (35% for non-manual and 45% for manual social class).[743] Although the participants were drawn from a population thought to contain higher numbers of non-manual and more highly educated participants, factors known to be associated with lower levels of obesity,[744] selection bias (against those visibly obese) is likely to explain the low percentage of obesity reported by this study.

Obesity is characterised by a dominance of M1 (classically-activated) macrophages over M2 (alternatively-activated) macrophages in insulin-dependent adipose tissue, leading to

increased inflammation and insulin resistance.[938] Increased M1 macrophage dominance is also a feature of AMD pathogenesis, but not normal age-related change, which is associated with M2 macrophage dominance (fig. 1.4).

Obesity is also associated with increased hypertension and dyslipidaemia. It is possible that hypertension is associated with increased AMD risk due to its affect on choroidal circulation and lipid deposition in Bruch's membrane.[39, 127, 939] Hogg *et al*. reported a significant association between nAMD and total, but not HDL cholesterol.[128]

It is therefore plausible that obesity may lead to inflammation-related RPE changes resulting in longer GRT, particularly in those with inflammation-related AMD risk gene polymorphisms. Positive associations were reported between BMI and plasma complement components (CFH, CFB and C3) and activation fragments (C3a and iC3b).[940]

Body mass index summary

Higher BMI is a moderate and consistent RF for AMD.[107, 109] Body mass index is also positively correlated with age. After correction for age, no significant correlation was found for mixed gender, male or female BMI with GRT.

The corrected (for unequal size groups) sample size required to detect a difference in GRT of 6.0 s assuming 80% power at 5% significance was estimated to be 7. This study had sufficient power to detect a significant difference.

Some subjects classed as non-obese based on their BMI value will actually be obese based on their %BF levels.[676] For this reason the results BMI were converted to %BF values.

3.4.7 Percentage body fat

The Clinica Universidad Navarra - Body Adiposity Estimator (CUN-BAE) algorithm estimates %BF by correcting BMI for age and gender.[608]

Female %BF was significantly and positively correlated with GRT, however, after controlling for age the correlation was not significant. The shared variance was 9.4% and 1.3% before and after controlling for age.

Group analysis for female %BF using the Kruskal-Wallis test revealed a significant increase in GRT between the three %BF groups (≤ 30%, > 30% to 35%, > 35%, representing normal, overweight and obese groups). Follow-up Mann-Whitney U tests ($\alpha = 0.025$) revealed significantly higher GRT for obese compared to normal weight subjects ($p = 0.02$) and a trend approaching significance for higher %BF in obese compared to overweight

subjects (p = 0.08). Although it is tempting to speculate whether increased retinal inflammation associated with obesity may lead to longer GRT, it is more likely that age differences between the three %BF groups are the cause the differences observed. The median ages for the three groups (normal, over-weight and obese) were 40.1 (IQR 9.5) years, 45.1 (IQR 7.1) years and 51.2 (IQR 15.6) years respectively. Male %BF was not significantly correlated with GRT. This may be a consequence of the smaller sample size for male %BF (n = 31).

It was clear that after converting the BMI values of participants to %BF values using the CUN-BAE algorithm, many more participants were classed as obese. BMI and %BF percentages for normal, overweight and obese were as follows, male: BMI 21.9% / 62.5% / 15.6%, %BF 6.2% / 18.8% / 75.0%, and female: BMI 58.3% / 33.3% / 8.3%, %BF 8.3% / 29.8% / 61.9%. Gómez-Ambrosi *et al.* concluded that BMI measurement under-estimated the level of obesity (and cardiometabolic risk) compared to %BF measurement.[676]

In the MPOD study (chapt.2) non-significant inverse trends were seen between MPOD and %BF that were stronger for males than females. It has been suggested that abdominal adipose tissue in males acted as a competitive storage site for serum-bound MP compared to gluteo-femoral adipose tissue in females.

The results for the association of GRT with %BF for each gender are contrary to the results from the MPOD study. Although the correlations were not significant (after correction for age), males had an inverse trend, whereas females exhibited a positive trend between GRT and %BF. For GRT, the difference in location or carotenoid absorbing properties of adipose tissue is not likely to influence the results. It is more likely that the total level of body fat (10% higher in females) explains the difference in the direction of the trends for each gender. Higher body may signify greater inflammation and therefore greater risk of RPE dysfunction leading to longer GRT. The possibility of a chance result would need to be excluded before this can be confirmed.

Percentage body fat summary

No significant correlation was found between GRT and male or female %BF, after correction for age. A positive correlation between GRT and %BF may be expected in view of the physiological changes thought to be associated with obesity (changes in the lipoprotein profile, and increased oxidative stress and inflammation),[941] the negative correlation between %BF and MPOD,[600, 758] and the negative correlation reported between GRT and MPOD.[622, 623, 905]

The author did not have access to equipment to measure %BF directly. The use of calculated %BF derived from BMI, age and gender values using the CUN-BAE algorithm

was simple to use in this practice setting and offered the opportunity to assess an estimate of %BF for each gender with MPOD.

Although the Northern European population investigated by the present study was similar ethnically to that from which the CUN-BAE algorithm was derived, it is recommended that the use of general predictive algorithms across different ethnic population groups should be avoided, without prior testing of their validity.[764] This was not possible for this practice-based study and therefore the values for estimated %BF should be treated with caution.

Sample size calculation was not performed for %BF as these values were calculated from BMI, rather than measured directly.

3.4.8 Iris colour

Group analysis for iris colour divided into five groups as recorded in this study (grey, blue, green, hazel and brown) and two groups (light: grey, blue and green, and dark: hazel and brown) according to Murray *et al.* and Kirby *et al*,[401, 680] revealed no significant associations.

In this study a higher percentage of females had lighter irides compared to males (63.0% versus 51.9%), but the difference was not significant. Reasons for this trend were discussed in the iris colour section in the previous chapter.

A non-significant trend for smaller mean pupil size for dark (3.0 mm, IQR 1.0 mm) compared to light irides (4.0 mm, IQR 2.0 mm) was revealed ($p = 0.21$). The mean ages of the two groups were similar at 49.1 years (SD 9.4 years) for dark and 52.3 years (SD 11.6 years) for light irides respectively.

Although the pupil size difference was not significant, the difference in pupil size alone between brown and blue eyes would equate to 1.77 times higher retinal illuminance for blue eyes. Correction for iris colour and the difference in pupil size revealed retinal illuminance values 2.25 times higher in the lighter irides group, assuming the pupil size difference was maintained under GRT testing. The pupils of those with blue irides were reported to be larger in size in ambient illumination, and constrict less quickly and by a reduced degree than those with brown irides.[771-773]

Equilibrium bleach GRT is strongly correlated with age (see the section on age above and therefore age must be considered when comparing GRT and iris colour. It would also be beneficial to correct for pupil size, whilst under GRT conditions, but this was not possible for the GRT method used in this practice-based study. Stringham *et al.* used an infrared camera to monitor pupil size under glare conditions in their lab-based study.[622] The close

proximity of the glare source to the participant, for the GRT method used in the present study would have precluded the use of this method of pupil size measurement.

Margrain and Thomson have demonstrated that quite large variations in retinal illuminance (5.5 log Td versus 6.0 log Td) produce only small differences (6%) in the amount of photopigment bleached when the retinal illuminance is high, whereas for lower levels of retinal illuminance (4.5 log Td versus 5.0 log Td) the difference in retinal bleach is much greater (27%).[573] The method of obtaining GRT used in this study is a high retinal illuminance method (approximately 6.2 log Td from the data of Margrain and Thomson), thought to bleach > 98% of retinal photopigment. Although this high level of bleach was chosen to improve repeatability (see repeatability section below), the very nature of this bleach method may negate the effect of any variation in retinal illuminance related to differences in iris colour. In order to assess the effect of differences in retinal illuminance due to iris colour differences using GRT, a lower percentage retinal bleach may be preferable.

A literature search revealed one other study where the effect of iris colour on GRT was examined.[329] This method used the MDD-2 device which is a high-intensity, photo-flash bleach method producing a peak irradiance at the cornea of 4.5 Watts / cm^2 for a duration of 0.2 ms, equivalent to approximately 5.2 Log Td (this author's estimation) retinal illuminance[361] Loughman *et al*. reported that there was no significant association between iris colour on GRT. The high level of retinal bleach achieved with this method may explain both the lack of any difference in GRT due to iris colour and the high levels of repeatability reported for this instrument compared to other photo-flash methods.[329, 361]

Melanin in the RPE may play a role in AMD development by biochemically protecting the neural retina against ROS.[7, 942] The effect of the age-related reduction of RPE melanin,[8, 633, 943, 944] possibly secondary to constant exposure to high levels of oxygen and light, may reduce the capacity for melanin to act as an antioxidant and may even lead to pro-oxidant behavior.[6, 945-949] Eumelanin, the main melanin pigment found in the pigment epithelia including the RPE, is less photoreactive than pheomelanin.[950, 951]

The relationship between iris colour and AMD is controversial. Hyman *et al.* reported an increased frequency of blue irides (OR 3.5, 95% CI 1.7 - 6.6) compared to brown irides among AMD cases compared to controls.[118] Other authors have also reported a significant association or a non-significant trend between light iris colour and AMD,[633, 766, 952, 953] The BMES concluded that blue iris colour was associated with an increased risk of both early and late AMD (OR 1.45 and 1.69 respectively), however 5-year, longitudinal results from the same group did not detect any significant relationship.[150, 954] The BDES did not find any association between iris colour and AMD incidence and progression,[634] but did report an

association between lighter iris colour and the development of RPE pigmentary abnormalities (ARM).[635]

Iris colour summary

Lighter iris colour is a weak and inconsistent RF for AMD. Iris colour is predominantly determined by the variation in the structure and composition of the melanosomes within the melanocytes of the iris stroma rather than melanin in the iris pigment epithelium. [779, 955, 956] Light iris colour is associated with significantly greater light transmission and reduced choroidal melanin compared to darker irides.[631, 632] RPE melanin was found to decrease by 50% over a lifetime.[81] Because melanin is able to act as an antioxidant this reduction may adversely affect the retinal / RPE / choroidal antioxidant system. Lifetime exposure to light is difficult to quantify.

No significant association was found between GRT and iris colour, however the nature of the GRT method used in this study may have prevented the detection of an association. A shorter duration, lower intensity bleach may produce a significant result for different iris colours. The effect of iris colour may be influenced by pupil size and gender. The differential light transmission between light and dark iris colours is greatly affected by pupil size, and by inference age (see table 2.10). Lower amounts of choroidal and RPE pigment associated with lighter iris colours may have an additive effect to any potential light-mediated tissue damage.

The corrected sample size required to detect a difference in GRT of 0.4 s assuming 80% power at 5% significance was estimated to be 1,295. This study lacked sufficient power to detect a significant difference.

2.4.9 Family history of AMD

Macular pigment optical density was assessed for participants with and without reported AMD FH in order to assess whether this test may be used as a possible marker for the future development of AMD.

A trend for longer GRT was found for those with a reported FH of AMD (45 s, IQR 26 s) compared to those without (38.5 s, IQR 23 s), however, the difference was not significant. The median ages of the two groups were similar; FH of AMD (49.5 years, IQR 18.3 years) and no FH of AMD (48.0 years, IQR 12.8 years).

A trend towards longer GRT for those with a positive reported FH of AMD was found by this and another study.[603] The lack of significance would preclude the use of this method of GRT as a surrogate test to predict AMD FH. For cases of early AMD a high percentage photoreceptor equilibrium bleach, likely to maximally stress the RPE would be the most

appropriate GRT test to perform,[330] however for those without evidence of AMD, where RPE function is likely to be normal a shorter duration, lower percentage photopigment bleach, placing a greater relative stress on retinal Mc may give more productive results.

Dimitrov *et al.* examined the utility of GRT measured with a modified technique proposed by Phipps *et al*.,[354] for the detection of early AMD. Glare recovery time after a 45-sec (> 95% photopigment) bleach using the detection of a centrally-fixated, 2° spot flickering at 5 Hz, for five diminishing contrast levels detected 71% of early AMD cases. The same study reported that VA alone detected only 7% of early AMD cases.[357]

Visual acuity is a poor marker for early AMD. The BDES recorded a two letter loss in LogMAR VA associated with early AMD,[309] however the one to two line variability of LogMAR VA testing would mask this subtle change.[957] Foveal cones are spared in early AMD, with photoreceptor loss being limited to parafoveal rods and cones.[958]

Rod recovery rate in DA using a 2° stimulus size at an eccentricity of 3.5° may be used to monitor those individuals at high risk of developing AMD due to FH or known genetic risk. [357] This method was able to detect functional loss in 87% of abnormal cases but it is difficult to perform in routine optometric practice. Testing rod function is an extremely arduous process, with an average difficulty score of 9.7, compared to a maximum score of 10.[357]

In a clinical practice setting it would be quicker and easier to measure time taken to reach the rod-cone break, the time at which rod photoreceptors become more sensitive than cones (approximately eight min). Time to rod-cone break and the rod intercept (the time at which visual sensitivity recovers to a criterion level of 0.005 cd / m^2) may be measured psychophysically using the AdaptDx dark adaptometer (MacuLogix, Hummelstown, PA, USA).[959]

Prolonged GRT in the fellow eye of those with unilateral nAMD was found to be an independent RF for nAMD development in the fellow eye.[126, 960] Evidence for GRT as a marker for future AMD development is sparse. Pratt *et al*. reported a non-significant trend for longer GRT measured by photo-flash bleaching method (Eger Macular Stressometer, EMS) in participants with a positive FH of AMD compared to those without.[603]

There are several mechanisms involved in AMD pathogenesis that may adversely influence GRT. AMD risk gene polymorphisms influencing inflammation and immunity (e.g. C2, C3, CFB, CFI) have the potential to disturb RPE and Mc photopigment recycling. Genes governing extracellular matrix and cell adhesion (e.g. ACE, COL8A1, TIMP3) may increase the risk of sub-retinal or sub-RPE fluid. Fluid separation is a recognised cause of increased

GRT (e.g. CSC). Genes controlling lipid / protein metabolism and transport (e.g. ABCA1, ABCA4, APOE, ELOVL4, LIPC) may influence levels of DHA and fat-soluble L in the rod (and possibly cone) OS membranes. Rhodopsin is known to co-localise with DHA and L in rod OS, and the absence of either or both will affect the efficiency of photopigment recycling. In the absence of DHA, DPA (docosapentaenoic acid) is substituted with a measureable reduction in function. Genes regulating angiogenesis (e.g. HTRA1, IL8, VEGFA) and cellular stress and toxicity (e.g. ABCA4, ACE, APOE) may also be up-regulated leading to down-regulation of normal cellular function.[122]

It is possible that the changes above are insufficient on their own to lead to a significant increase in GRT. Sufficient age and early retinal / RPE changes associated with AMD may also be required for GRT to be affected significantly. The lack of significance for the association between GRT and AMD FH, but the presence of significant association for fellow eyes of those with nAMD would appear to confirm this.

A full dietary assessment was not completed, however, participants were asked about current use of MP and fish oil supplements. Only one participant reported current use of MP supplements, which precluded any meaningful analysis. Twenty one participants reported current use of fish oil supplements. A non-significant trend for longer median GRT was revealed for the fish oil supplement group, which may be explained median age being more than 10-years older than the non-fish oil supplement group. The omega-3 fatty acid DHA not only associates with rhodopsin and L in rod OS membranes, it also is thought to assist in retinal absorption of MP. An age-matched comparison of GRT between groups would give more meaningful results.

Because of the close association between DHA and rhodopsin in rod OS membranes, absolute dietary reduction in omega-3 is likely to significantly increase GRT, however a relative dietary reduction may not significantly influence the results because humans have a limited ability to synthesise DHA from dietary alpha linolenic acid (ALA),[961] and are able to maintain retinal levels of DHA by very efficient recycling.[962]

The absence of benefit derived from dietary supplements prior to the onset of AMD may reflect retinal ability to recycle some of the nutrients required for normal function. Age-related macular degeneration is associated with retinal and RPE cell dysfunction, therefore nutrient recycling may be reduced or absence, signalling the time when a benefit from dietary supplements may be reached.

Family history of AMD summary

Family history of AMD is a strong and consistent RF for AMD. No significant difference in GRT was found for individuals with and without a primary or secondary FH of AMD. This

study and that of Pratt *et al*. reported a trend towards longer GRT with AMD FH.

Prolonged GRT associated with AMD may be related to multiple gene polymorphisms governing the following functions; inflammation and immunity, extracellular matrix and cell adhesion, lipid / protein metabolism and transport, angiogenesis and cellular stress and toxicity.

The corrected sample size required to detect a difference in GRT of 5.7 s assuming 80% power at 5% significance was estimated to be 24. This study had sufficient power to detect a significant difference.

3.4.10 Migraine

No significant associations were observed for individuals reporting migraine, light-trigger vs. no light-trigger or aura vs. no aura, compared to those without migraines. The author is unaware of any previous studies examining the effect of migraine on GRT. The results should be viewed with caution in view of the small sample size of the migraine groups.

It is plausible that the symptom described as light-induced amaurosis reported in cases of ocular ischaemic syndrome secondary to carotid occlusive disease,[612, 963-965] may actually represent longer GRT secondary to ocular vascular insufficiency.

Migraine is associated with a reduced ability to habituate to repetitive, stressful stimuli,[966] and (in the acute phase) reduced neurovascular coupling in response to flickering light.[617] Barbanti *et al*. postulated that migraine attacks are characterised by an ictal dopamine release in subjects with inter-ictal dopamine receptor hypersensitivity due to a chronic dopaminergic deficit synergistic to serotoninergic impairment.[186]

Plausible pathways that may lead to increased GRT in individuals with migraine include; lower or irregular retinal blood flow secondary to reduced neurovascular coupling, reduced or irregular choroidal blood flow secondary to vasospasm, which is associated with migraine and reduced adaptation secondary to the effects of reduced dopamine and hypersensitive dopamine receptors. This may affect adaptation directly by preventing ipRGC and A18 amacrine cell influence on light-related HC uncoupling, or indirectly by affecting the efficiency of OS phagocytosis by the RPE.

A summary of the mechanisms that may be associated with increased AMD with migraine, Rph and VDys are presented in fig.1.3.

Migraine summary

The author has proposed a plausible mechanism (fig. 1.3) for the consideration of migraine as an AMD RF. No significant difference in GRT was found for participants with a reported history of migraine compared to those who reported no migraines. The trend towards shorter GRT for those with and without light-trigger compared to non-migraineurs, and shorter GRT for those with aura, but longer GRT for those without aura, compared to non-migraineurs was interesting. This comparison should be repeated for a larger sample size. The minimal blue content in the GRT light source may have lead to a lack of significant association due to minimal ipRGC stimulation.

It is plausible that the generalised lack of dopamine (an antioxidant), reduced habituation (adaptation) in response to sustained retinal illumination and irregularities in vascular perfusion, leading to the possibility of increased retinal inflammation may contribute over time to AMD risk.

The corrected sample size required to detect a difference in GRT of 1.7 s assuming 80% power at 5% significance was estimated to be 241. This study lacked sufficient power to detect a significant difference.

3.4.11 Raynaud's phenomenon

No significant difference in GRT was found between individuals reporting a history of Rph and those reporting no Rph. No distinction between Rph subtypes was made in this study, but it was likely that pRph and sRph were represented.

Raynaud's phenomenon is associated with a reduced ability to habituate to repetitive, stressful stimuli.[967] Plausible pathways leading to longer GRT in individuals with Rph are similar to those reported for migraine (see previous section).

A literature search revealed no previous studies comparing GRT and Rph. It was speculated that GRT may be increased in participants with a reported history of Rph. Raynaud's phenomenon was associated with a reduction in retinal capillary blood flow, which could result in ischaemia leading to retinal dysfunction.[646] Oxidative stress is also involved with Rph pathogenesis.[194] These factors would suggest that Rph might be considered to be a plausible RF for AMD (fig. 1.3).

Raynaud's phenomenon summary

GRT was not significantly different for participants with a reported history of Rph compared to those without a history of Rph. Inclusion of pRph cases as a separate group may increase the possibility of finding a significant association with GRT. If ethically tenable, repeating the experiment for those with active symptoms of Rph produced by cold provocation may increase the chance of finding a significant result.

The corrected sample size required to detect a difference in GRT of 0.9 s assuming 80% power at 5% significance was estimated to be 538. This study lacked sufficient power to detect a significant difference.

3.4.12 Vascular dysregulation

Glare recovery time was not significantly different for individuals reporting a history of VDys compared to those without VDys. 78% of the VDys cases were female. No distinction between types was made in this study, but the age range would suggest both types were represented.

Primary VDys is associated with reduced neurovascular coupling in response to flickering light.[618] Primary VDys is associated with unstable blood flow.[205] This cyclic variation in OVP can lead to oxidative stress, secondary to reperfusion injury.[647] As a consequence of their young age, the majority of pVDys cases can cope with this level of oxidative stress and do not develop tissue damage.[205] In glaucoma, oxidative stress occurs mainly in the mitochondria of retinal ganglion cells and their axons. [205]

It is plausible that oxidative stress secondary pVDys could also affect mitochondria in other cell types implicated in AMD development, such as photoreceptors, Mc and the RPE, and may compound the effect of other AMD RF. Vascular dysregulation, especially sVDys is associated with a relatively constant reduction choroidal blood flow.[205, 212] Vascular dysregulation is often associated with low systemic blood pressure,[205] and pharmaceutical reduction in systemic blood pressure is associated with a significant increase in GRT.[610]

Vascular dysregulation, especially pVDys may be associated with retinal inflammation indicated by the presence of hyper-reflective spots (HRS) on OCT. These changes in addition to dysregulation of ocular perfusion, reported to be worse than that associated with Rph, suggest that VDys may represent a RF for AMD. Vascular dysregulation is significantly more common in females and thus may represent an AMD risk that is biased towards this gender.

Vascular dysregulation summary

The author has proposed a plausible mechanism (fig. 1.3) for the consideration of Rph and VDys as AMD RF. The OVP RF Rph and VDys may potentially be more important factors in AMD risk than migraine because they are more likely to persist until later in life than migraine. No significant difference in GRT was found for participants with a reported history of VDys compared to those with no history of VDys. The inclusion of participants with confirmed pVDys and sVDys as separate groups may lead to an improved association between VDys and GRT. If ethically tenable, assessing GRT after cold-provocation may

also increase the chance of finding a significant result for GRT. An investigation into the accumulative effect of VDys with other AMD RF on AMD risk would be worthwhile.

The development of OCT modules designed to image retinal and choroidal blood flow in real time opens up the possibility of correlating GRT with ocular blood flow, differentiating the effect on GRT of retinal and choroidal blood flow and allowing for the effects the ocular effects of "cold provocation" to be directly measured. Because the variation in ocular blood flow associated with all of the OVP RF examined in this study is likely to be dynamic and transient, dynamic assessment of ocular blood flow may reveal more than GRT measurement, which relies on a functional deficit to reveal an abnormal result.

The discovery of HRS in OCT images of patients with pVDys, indicating the presence of retinal inflammation (lipid-filled macrophages), offers direct evidence that this condition has an adverse effect on retinal health and could be associated with increased risk of AMD under conditions where the ability of the eye to down-regulate inflammation (e.g. CFH gene polymorphisms) are compromised. The ability of the eye to maintain immune privilege is reliant on its isolation from systemic circulatory inflammatory factors. Chronic ischaemia / reperfusion injury is likely to lead to a break down in the eye's immune privilege.

The author is unaware of any reports of HRS recorded for individuals with migraine or Rph, however, it is likely that they are also present in these conditions as the effects on ocular perfusion are similar for all three conditions. Migraine is also associated with low retinal dopamine, which may compromise adaptation and photoreceptor outer segment phagocytosis.

In addition to the small sample size, the lack of significance for the association between GRT and any of the OVP RF (migraine, Rph or VDys) examined in this study may be related to the method of GRT used (high percentage bleach equilibrium method). This method places maximum stress on the RPE and Mc, and results in longer GRT when these cells are dysfunctional. It is possible that even in the face of additional stress from OVP RF, the healthy retina and RPE are able to regenerate photopigment normally as a result of an interconnected and complex series of antioxidant and anti-inflammatory mechanisms (A2.1).

The corrected sample size required to detect a difference in GRT of 0.5 s assuming 80% power at 5% significance was estimated to be 1,721. This study lacked sufficient power to detect a significant difference.

3.4.13 Glare recovery time as a surrogate measure for MPOD

This study aimed to assess whether GRT using the direct ophthalmoscope, an instrument

owned by the majority of optometrists could be used as a surrogate test, or measure for MPOD measurement. When the analysis included the full age range, there was a significant positive Pearson correlation between GRT and MPOD ($p = 0.03$), however the correlation was not significant after controlling for age using partial correlation ($r = 0.18$, $n = 100$, $p = 0.07$). The direction of this correlation was positive across this large age range (24.2 to 75.8 years), unlike the negative correlation reported by four papers in the table below, which examined ages ranging from 18-41 years only.

The correlation between GRT and MPOD was assessed for those aged up to 50 and over 50 years of age separately in view of the age range (all < 50 years of age) investigated by other studies. A non-significant trend towards a positive correlation was observed for both age groups, larger in the younger age range. The results obtained by the methods used in this study do not support the hypothesis that GRT is shorter with higher levels of MPOD in the younger or older age range.

A significant positive correlation was found between GRT and participants with higher MPOD values (> 3.9), ($r = 0.31$, $p = 0.03$, $n = 44$), which survived correction for age ($r = 0.31$, $p = 0.03$). No significant difference in GRT was found for participants with lower MPOD values ≤ 3.9 ($n = 48$), with or without correction for age, although the direction of the trend was negative. These results are interesting and hint that the direction of the correlation between GRT and MPOD may be influenced by the level of MPOD, no or inverse correlation for low MPOD and positive for higher MPOD levels. A similar effect was seen in the AREDS2 study where MP supplementation was only significant if dietary intake of MP was poor.

This study found that the variation of GRT was biphasic with age. This study revealed a trend for a biphasic relationship for MPOD with age, and six others found that MPOD measurements were higher for those in the middle age range compared to those aged younger and older.[244, 497, 599, 601, 711, 712] This study and four others found a greater increase in GRT with age for those aged over 50 to 60 years of age, compared to younger subjects.[361, 577, 684, 928]

Several studies reported a significant negative correlation between GRT and MPOD, using photo-flash or short duration (5-s) equilibrium bleach methods.[620, 622, 623, 905] Stringham and Hammond reported a linear inverse relationship between GRT and increased MPOD levels measured at one, two, four and six months post supplementation with 10 mg L and 2 mg Z.[621] However, Nolan *et al*. reported no association between GRT and increased MPOD levels measured at three, 6-12 months post supplementation with 12 mg L and 1 mg Z in a placebo-controlled study.[968] It is noteworthy that none of these studies examined subjects aged over 50 years.

One study using a 30-s equilibrium bleach, found no association between GRT and MPOD.[906] The light sources used for retinal bleach by Loughman *et al.*, by Nolan *et al*. above and by this study (fig. 3.10), contained very little blue light component in their spectral emission, compared to those studies reporting a negative correlation between GRT and MPOD. At normal MPOD levels between 20% and 40% of blue light (at 460 nm) is absorbed at the macula, rising to as much as 90% blue light absorption for high MPOD levels.[969] The author accepts that differences in receptoral absorption and neural adaptation, as a consequence of the longer duration bleach may also have influenced these results (table 3.16).

Table 3.16 Summary of the literature for the association between GRT and MPOD

Source (Country of Study)	No. of Data (n)	Age Range (years)	GRT Light Source, Duration of Exposure and Blue Light Content (s)	Reported Direction of GRT vs. MPOD		
				Inverse	None	Positive
Stringham, 2007 (USA)[620]	36	18 - 41	Xenon arc, 5 s, high	✓		
Stringham, 2008 (USA)[621]	40	17 - 41	Xenon arc, 5 s, high	✓		
Loughman, 2010 (Ire)[906]	142	18 - 41	60 Watt bulb, 30 s, low		✓	
Stringham, 2011 (USA)[622]	26	23 - 50	White LED, 5 s, high	✓		
Nolan, 2011 (Ire)[968]	121	18 - 41	Tungsten lamp, 5 s, low		✓	
Hammond, 2013 (USA)[623]	150	20 - 40	Xenon arc, 5 s, high	✓		

Abbreviations: Ire, Ireland, LED, light emitting diode.

The lack of a negative correlation between GRT and MPOD is also likely to have been affected by the longer duration of the equilibrium bleach used in this study and that of Loughman *et al*. In humans short duration, high intensity photo-flash bleach was reported to bleach approximately half the amount of cone photopigment and lead to a GRT of about half of that compared to longer duration equilibrium bleach.[582]

Unlike photo-flash bleaches, Equilibrium bleaches are also likely to deplete local stores of 11-cis-retinol from Mc, placing a greater burden on the RPE for cone photopigment regeneration.[330] High percentage equilibrium bleach GRT is minimally affected by slight variations in the exposure time of the bleaching light source.[573] It is therefore likely that this method of GRT will be minimally affected by variations in blue light absorption due to variable levels of MP.

The author is unaware of any studies using a longer duration retinal bleach with a light source utilising a high spectral component of blue light. The contribution of neural adaptation to GRT may be different for photo-flash bleach compared to equilibrium bleach. Older papers have reported that neural adaptation accounts for the first 15 s of visual recovery after exposure to a bright light source, where after photoreceptor pigment regeneration takes over from neural factors.[924, 925] These papers were written before it was

established that the cone-specific, Mc visual cycle might be responsible for the earliest stage of visual recovery after bleach.[308, 349, 529, 580]

Contrary to the shorter duration bleach GRT required as a surrogate measure for MPOD, a longer duration, high percentage photopigment bleach GRT would be favoured as a marker for eye diseases such as AMD.[330, 357, 970]

Wood *et al.* found that GRT following equilibrium bleach was positively correlated with age, whereas photo-flash bleach was negatively correlated with age, after testing both GRT methods on the same population.[330] It is therefore essential to correct for age when comparing GRT and MPOD, as depending on the methods of assessment used, both variables may be biphasic with age.

From the scatter plots for GRT and MPOD with age it may be seen that both variables are characterised by high levels of intersubject variation. Other authors have noted this for GRT,[620, 624] and for MPOD.[497, 599, 601, 711, 712]

Previous studies examining the association between GRT and MPOD have only included participants aged 50 years or less. The logic behind this decision is based on the assumption that MPOD is stable with increasing age in healthy subjects, whereas GRT is known to increase significantly in subjects over the age of approximately 50 years. Glare recovery time particularly, is associated with increased variability with increasing age.[308, 330, 577] Older patients are more likely to have general or ocular pathology that that may confound one or both measurements. Age-related conditions such as AMD, type 2 diabetes and glaucoma are known to interact with both measurement principles, such that they no longer offer "optimum" conditions for measurement.

The effect of cataract on MPOD readings is dependent on the method of measurement. It would be expected that cataract would affect results from objective methods of MPOD measurement, as media opacification would modulate incident light directed onto and reflected light from the retina. Macular pigment optical density measurements based on HFP, however, appear to be immune to the effects of clinically significant cataract.[698] Wood *et al.* have demonstrated that the association between GRT and age is not consistent for different bleach methods, equilibrium versus photo-flash, which differ in the percentage of photopigment bleached.[330] It is also likely that the effect of cataract on GRT will differ depending on the method of GRT used. Margrain and Thomson reported that GRT resulting from higher percentage bleaches are less affected by a variation in exposure time than lower percentage bleaches.[573] This would suggest that cataract might affect the results from photo-flash bleach GRT to a greater degree than equilibrium bleach methods. Eye

disease is also associated with a greater level of intersubject variation in GRT compared to healthy cases.[624]

Glare recovery time as a surrogate measure for MPOD summary

The efficacy of a biological marker is inversely dependent upon the variability of the measurement within a given chronological age group.[932] Both GRT and MPOD are highly variable with age. It was also clear that equilibrium bleach GRT using a light source with a low blue light component did not produce a significant correlation with MPOD after correction for age and for the biphasic nature of both variables. Therefore GRT using the direct ophthalmoscope as the glare source was not found to be a suitable surrogate measure for MPOD.

A shorter duration, low-intensity bleach method (e.g. 5-s equilibrium bleach) using a blue light source with a peak absorbance matching that of MP (445 to 463 nm),[439, 466] would represent a better surrogate measure for MPOD. It may be possible to reduce the effects of intersubject variation and pathological differences in GRT by performing a second GRT test at least 10 min after the first measurement,[577] with a retinal luminance-matched light source with no blue light component. The ratio of these two GRT results (blue light and blue-free) may be represented as a single number in much the same way as HFP-derived MPOD measurements.

The measurement of MPOD in clinical (and especially commercial) optometric practice is associated with certain difficulties depending on the method of measurement selected. Subjective methods (e.g. HFP) are relatively inexpensive to purchase, but are conceptually difficult for some naïve and / or elderly patients, and may be time-consuming, particularly if means of multiple readings or readings from multiple eccentricities are required. Objective methods (e.g. 2-WFAF, RRS and FR) are quick to perform (although pupil dilation may be required) and provide additional information about the MPOD spatial profile, not available from a single eccentricity measurement using HFP. Objective methods are however more expensive to purchase and maintain, in the case of the 2-WFAF module added to the cost of OCT instrumentation, considerably more so.

A commercial model of MPOD measurement in clinical practice is based on generating revenue to off-set instrument and clinic time costs by selling MP supplements to patients with, or perceived to be at risk of developing AMD and / or patients with "low" MPOD readings. There is currently no evidence that MP supplements can prevent or delay the onset of AMD and only a specific subset of AMD patients (defined latterly by the AREDS2 study) may benefit from MP supplementation. The AREDS2 vitamin supplement formulation (which includes 10 mg of L and 2 mg of Z) lowered the risk of developing advanced AMD in patients aged between 50 and 85 years, with bilateral large drusen or large drusen in one

eye and advanced AMD in the fellow eye, whom also had a low dietary intake of MP (≤ 0.832 mg per day), by 25%.[452]

The idea behind this experiment was to assess whether GRT using the direct ophthalmoscope, an instrument already owned by most optometrists could be used to gauge MPOD levels in a practice setting. This would allow MPOD to be assessed rapidly and without the need to purchase expensive equipment. Supplement sales would not be required to fund this model of MPOD assessment.

The corrected sample size required to detect a difference in GRT of 0.4 s assuming 80% power at 5% significance was estimated to be 979. This study lacked sufficient power to detect a significant difference.

3.4.14 Glare recovery time repeatability

In the first session, GRT time was performed eight min and 18 min after completing MPOD measurements in both eyes. Prior MPOD testing appeared to significantly increase GRT eight min after MPOD testing, compared to 18 min after MPOD testing for both eyes ($p < 0.01$). This was considered to be an adaptive rather than a learning affect because it was not replicated in the second session for either eye, when MPOD testing was not performed prior to GRT measurements. For this reason GRT intra-session repeatability was assessed using the first eye data from the second session, and inter-session repeatability was assessed using the first eye data from the second set of GRT measurements from both sessions. Limits of agreement were calculated using the log transformed GRT data and then back-transformed in order to produce clinically meaningful values. Limits of agreement were calculated for repeated measures of GRT (i.e. coefficient of repeatability, CoR).

Intra-session repeatability

The difference between the means for the two sets of raw GRT data was 1.7 s (SD 12.1 s). Intra-session repeatability for the mean value of GRT (46.4 s, SD 24.6 s) was 20.8 s, or 45% represented as a percentage of the mean GRT value. For other values of GRT, intra-session repeatability may be calculated using equation 3.3.

It is clear from the back-transformed Bland-Altman plot that the repeatability will worsen (i.e. the LoA will increase in size) as the GRT value increases. A Wilcoxon signed rank test revealed no significant difference in intra-session GRT measurements of the first eye indicating no bias or learning effect. Overall intra-session repeatability was good with a high level of intra-session correlation ($r = 0.89$, $n = 30$, $p < 0.001$) and, 77% and 57% of subjects showing a repeatability of ≤ 10 s and ≤ five s respectively.

Two out of 30 (7%) normal participants had intra-session difference in repeated

measurements of GRT that was abnormal (greater than the value of the LoA). All were free from glare-related symptoms or ocular disease. No explanation found for the large interocular difference measured from these participants.

A Literature search did not reveal another study reporting intra-session repeatability for GRT derived from equilibrium bleach methods, however three studies were found that used the photo-flash method.

Bartlett *et al*. reported a significant intra-session difference between results taken one hour apart for one examiner (mean difference = 0.93 s, coefficient of repeatability, CoR = 4.98 s), but not the other (mean difference = 0.95 s, CoR = 6.19 s).[359] Newsome and Negreiro found no significant difference between right and left eye results repeated after five min.[361]

Loughman *et al*. reported that the third GRT measurement for the dominant eye, recorded two min after the second (ignoring the first measurement) was significantly shorter (mean difference = 0.39 s, CoR = 2.61 s). Although the 2-min interval between GRT measurements was determined to be sufficient in a pilot study, this may have contributed to the significant ($p < 0.001$) and progressive shortening of GRT for the three successive dominant eye measurements, although Mauchley's test of sphericity was not significant suggesting that this was not a learning effect.[329] Repeatability data for photo-flash GRT methods cannot be compared to the present study in view of the difference in method of GRT used.

Inter-session repeatability

The difference between the means for the two sets of raw GRT data was -4.5 s (SD 23.4 s). Inter-session repeatability for the mean value of GRT (43.2 s, SD 14.6 s) was 30.4 s, or 70% represented as a percentage of the mean GRT value. For other values of GRT, inter-session repeatability may be calculated using equation 3.4.

Three out of 30 (10%) normal participants had inter-session difference in repeated measurements of GRT that was abnormal (greater than the value of the LoA). All were free from glare-related symptoms or ocular disease. No pathological explanation was found for the large interocular difference measured from these participants.

The participants in this study were vetted for any causes of abnormal GRT known to the author (see table 3.4 and appendix A2.5). Participants were excluded if any reason for abnormal GRT was reported by the participant or detected by the author.

7-10% of participants were found to have differences in repeated measures of GRT, which were considered to be abnormal. These differences may have occurred by chance and

therefore the results should be repeated before the conclusion of abnormality is confirmed. Owsley *et al*. reported that 22% of normal subjects had abnormal DA time to rod-cone break measured psychophysically using the AdaptDx dark adaptometer.[959]

In the present study greater differences between repeated GRT measurements appeared to be associated with longer GRT measurements (and by association with age). The difficulty with this observation is that the age group of interest (the elderly for AMD risk) is also the age group that experiences the largest risk of abnormal results in normal participants. In the clinical setting the usefulness of a test would be reduced if a high percentage of abnormal results were obtained for normal patients.

Consulting the back-transformed Bland-Altman plot again confirmed that the repeatability was positively correlated with the value of GRT. A Wilcoxon signed rank test revealed no significant difference in intra-session GRT measurements of the first eye, indicating no bias or learning effect. Overall repeatability was not as good as intra-session, with a moderate level of inter-session correlation ($r = 0.41$, $n = 30$, $p = 0.02$) and, 73% and 40% of subjects showing a repeatability of ≤ 10 s and ≤ five s respectively.

Three other studies have examined inter-session repeatability, one using equilibrium bleach, one using photo-flash bleach and one comparing both methods of GRT.

Torkelson *et al*. reported a Pearson correlation of 0.77 (p not available) for repeat measures of GRT (40 s equilibrium bleach) separated by one week.[907] Elliot and Whitaker reported a test-retest correlation coefficient of 0.82 (p not available) and noted a small training effect leading to lower photo-flash GRT values two weeks after the initial readings.[684]

Wood *et al*. compared electroretinogram (ERG) GRT repeatability after four weeks for a two min equilibrium bleach and a 6.6 ms photo-flash bleach on the same population. Coefficient of repeatability (CoR), calculated from 1.96 x SD of the mean of the difference between the two sessions, were calculated as these give a better idea of agreement than correlation coefficients used by earlier GRT papers.[677] Inter-session CoR for GRT following a two min equilibrium bleach was found to be 85 s, whereas CoR following photo-flash bleach was significantly longer at 184 s. The authors concluded that on their population ($n = 23$) equilibrium bleach GRT was more repeatable than photo-flash bleach GRT.[330]

The lower level of inter-session correlation found by this study compared to the others, is likely to be a consequence of the difference in time between repeated measures of GRT for some of the subjects, resulting from the addition of the repeatability study close to completion of the data collection process. The second set of GRT data was collected 2-5

weeks after the initial set for 18 subjects, but 3-11 months after the initial GRT data for the remaining 12 subjects.

When the analysis was repeated for the 18 subjects retested within five weeks a higher level of inter-session correlation was achieved ($r = 0.58$, $p = 0.002$). The difference between the means for the two sets of raw GRT data was still larger than that for intra-session GRT; -6.1 s (SD 22.8 s). Inter-session repeatability for the mean value of GRT (42.6 s, SD 15.3 s) was 25.0 s, or 59% represented as a percentage of the mean GRT value. 83% and 33% of subjects showing a repeatability of ≤ 10 s and ≤ five s respectively.

Inter-session repeatability for this method (25-30 s) was worse than intra-session repeatability (20 s), and was associated with a greater mean difference between readings (6 s vs. 2 s) indicating a greater bias between inter-session GRT values. Ideally the mean difference should be zero, but this rarely happens in practice. If the mean difference is significantly different from zero then an assessment of repeatability is not possible.[677] Wilcoxon signed rank test revealed no significant difference between the means of intra- or inter-session repeated measures of GRT. The longer first GRT measurement for inter-session repeatability may have been due to HFP MPOD measurement 18 min before. The shorter second GRT may have been a training or learning effect.

Bias from using the same test chart letters

The same test chart letters were used for the first and second eye GRT measurements in first session. In the second session, the second eye GRT measurements (GRT1 and GRT2) were assessed using different test chart letters, whereas the first eye GRT measurements (GRT1 and GRT2) were assessed using the same set of test chart letters. To assess whether using the same letters lead to any learning effect in the first eye GRT measurements (GRT1 and GRT2), an independent t-test was performed on the difference between GRT1 and GRT2 for the first eye (same letters), and the difference between GRT1 and GRT2 for the second eye (different letters). The results revealed that using the same letters for the two first eye GRT measurements, taken 10 min apart did not significantly affect the results compared to changing the test chart letters between GRT1 and GRT2 for the second eye ($p = 0.12$). Optometrists should be able to perform repeated measurements of this method of GRT using the same test chart letters for each GRT measurement without fear of a learning effect in the results.

Glare recovery time repeatability summary

Intra-session repeatability of this method of GRT was high, however the high percentage (7-10%) of cases exhibiting an abnormal difference in repeated measures was a concern if this method was to be used in a clinical setting. Repetition of abnormal measurements would be sensible before any conclusion is made about the result. Inter-session GRT was

moderate, even after the removal of participants measured at a time interval of greater than five weeks.

Schmitt *et al*. and Bartlett *et al*. concluded that a longer duration bleach than that afforded by photo-flash methods may lead to less variation in GRT results.[358, 359] This was confirmed by Wood *et al*. who reported that equilibrium bleach GRT coefficient of repeatability (85 s) was less than half of that for photo-flash bleach GRT (185 s) after testing both GRT methods on the same population.[330]

It is possible that the low percentage of photopigment bleach may have contributed to the higher variability reported for one method of photo-flash GRT measurement (EMS).[359, 360] Higher levels of repeatability have been reported for a more recent photo-flash-based commercial GRT instrument (MDD-2).[329, 361]

An increase in recovery time for the second of two GRT performed sequentially on the same eye, in quick succession, may be a marker for eye disease compared to healthy eyes.[329, 361] Sufficient time should be allowed between successive bleaches to reduce the risk of any learning or adaptation effects.

3.5 Chapter conclusion

This study of healthy subjects found a significant, positive correlation between equilibrium bleach GRT and age, in agreement with all other equilibrium bleach studies of healthy populations. The relationship between photo-flash bleach GRT and age is controversial however. The association between GRT and age appeared to be biphasic, increasing more rapidly after 50 to 60 years of age. After correction for age no other AMD or OVP RF tested had a significant association with GRT.

Interocular comparison and intra-session repeatability of GRT were found to be good and inter-session repeatability was moderate, which didn't appear to be significantly affected by the lack of a constant time interval for repeated measures. The difference between the means was greater for inter-session GRT values. (although in practice this is never zero, it should be as low as possible in order to make a reasonable assessment of repeatability). Glare recovery time was not significantly affected by ocular dominance. This method of GRT was not a suitable surrogate measure for MPOD measurement due to the lack of blue light emission (expected for an instrument designed for ocular examination) and the high photopigment percentage, long duration bleach method.

Insufficient rest period between GRT measurements may lead to a learning effect between successive measurements, although this effect may be exploited in the investigation of

ocular disease. Many older papers report a long period of adaptation before measurements of GRT are made. Most modern papers report little or no period of adaptation.

The high level of intersubject variability, also noted by other studies, complicates interpretation of GRT results in the clinical setting and will challenge the adoption of any GRT method as a criterion standard. GRT may have greater use as baseline criteria for future change. It is clear that the duration, intensity and spectral emission of the GRT light source is likely to affect the association with AMD RF such as MPOD, AMD FH and iris colour and OVP RF such as migraine. This knowledge may be used to tailor the GRT characteristics to suit the variable under investigation.

Glare recovery time is regarded to be a measure cone photoreceptor recovery and is influenced by factors affecting classical (RPE) and cone-specific (Mc) photopigment recycling, adaptation (via ipRGC and dopamine-specific A11 amacrine cells) and cortical factors.

Glare recovery time testing with different coloured lights or even combinations of colours in centre / surround orientation may be used to isolate cone function (red centre, blue surround) and ipRGC (calculated from the difference between the cone condition above and a luminance matched blue target and a blue surround) function. Repeated GRT performed a short time after the initial GRT may aid the detection of ocular pathology. GRT measurements, like MPOD measurements show very high levels of inter-individual variation across the entire age range, making inter-individual comparison difficult. For this reason comparison between baseline and successive measurements would be more appropriate than comparison of the GRT value of an individual with a population mean GRT.

It is clear that mechanism underlying GRT is more complex than a simple differentiation between retinal and optic nerve disease. That GRT is affected by a reduction in OVP associated with ocular ischaemic syndrome was the inspiration to investigate OVP RF with the AMD RF and the development of the theory that migraine, Rph and VDys could contribute to AMD risk (fig. 1.3).

Glare recovery time may be longer in individuals with AMD, but it is by no means diagnostic for AMD. Prolongation of GRT due to ocular disease or medication may result from retinal / RPE tissue loss, degeneration or pigmentation (e.g. AMD, glaucoma, RP and quinoline antimalarials), retinal separation from the RPE (retinal detachment or CSC), reduced ocular perfusion (e.g. blood pressure reduction and reduced retinal perfusion pressure), medications that interfere with photopigment regeneration, and one of several causes of CMO (DMO, Irvine-Gass syndrome or CMO secondary to one of several medications listed

in appendix A2.5). Further information on GRT is also provided in section 1.7 of this introduction.

The usefulness of a GRT method (equilibrium) that places considerable strain on the RPE, for the assessment of AMD RF, before there is any RPE dysfunction may be limited. Although exhibiting reduced repeatability, photo-flash methods may be more appropriate to investigate subclinical RPE and Mc dysfunction.

Some patients with MIS report seeing centrally-located coloured blobs which track fixation, after viewing bright white backgrounds such as interactive white boards. The current explanation for these cases is cortical hyper-excitability, for which there is compelling evidence.[971, 972] The author has proposed a retinal explanation for MIS that may complement cortical hyper-excitability and represent an alternative explanation in some cases (see appendix 3).

The author is unaware of any studies examining GRT and / or AMD FH in cases of MIS, although DA was reported to be longer for dyslexia, and scotopic sensitivity syndrome, an older name for MIS (appendix A2.5). If GRT is found to be increased in MIS, this could represent a childhood marker for increased lipofuscin accumulation and AMD development. Further research is required, particularly to assess any relationship between GRT, MIS, AMD FH, AMD risk and MPOD non-responsivity.

Sample size calculations were based on MPOD differences reported from similar studies using HFP-derived MPOD values, or calculated using G*Power statistical software if previous data was not available. The sample size was corrected for unequal size groups (allocation ratio). Assuming 80% power (1 - β) at 5% significance level, the sample size was sufficiently large to be confident that there was no significant difference for age, BMI (mixed gender), and AMD FH. This study was underpowered to detect a significant difference for ocular dominance, gender, iris colour, migraine, Rph, VDys and MPOD.

What is currently known:

1) Interocular agreement in equilibrium-bleach GRT for healthy eyes is good.
2) Insufficient resting time between interocular GRT measurements may produce learning or fatiguing effects.
3) There is a significant increase in equilibrium-bleach GRT with age for healthy eyes, with some studies reporting a higher rate of increase after middle age.
4) Although few studies have examined GRT and other AMD RF. The majority of those studies reported no significant association. The author is not aware of any examining GRT and OVP RF.

5) Repeatability for GRT is moderate to high, with the equilibrium method reported to be higher than the photo-flash method.

What this study has found:

1) Confirmed the high level of interocular agreement for equilibrium-bleach GRT.
2) No difference between dominant and non-dominant eye GRT was found.
3) Equilibrium-bleach GRT was positively and significantly associated with age, and the higher rate of increase in GRT with age after middle age was confirmed.
4) No other AMD or OVP RF was associated with GRT after correcting for age.
5) Intra-session repeatability was good and inter-session repeatability was moderate.
6) No significant bias in repeated GRT measurements was found between using the same or different test chart letters for recording recovery after photostress.
7) This method of GRT was not found to be a surrogate measure of MPOD for the under 50 year or over 50-year age range.

Chapter summary

This chapter examined four different aspects of GRT. An interocular comparison for, and the effect of ocular dominance on GRT, the relationship between GRT and selected AMD and OVP RF, the suitability of GRT as a surrogate measure for MPOD and GRT repeatability. The next chapter will bring together the main outcomes from both experimental chapters and highlight the limitations and confounding variables associated with this study. Improvements in the study design will be discussed.

Chapter 4 Discussion

4.1 Main outcomes

No significant association was found between MPOD measured by HFP at 0.5° eccentricity and any of the AMD or OVP RF assessed by this study, for this healthy White, mixed-gender population. Interocular agreement was good with a non-significant trend towards sequential bias between first and second eye consecutive measurements after the removal of a single outlier from the MPOD data. MPOD was not significantly associated with ocular dominance, although an interesting trend for a greater reduction in MPOD for dominant eyes with age was noted. Difficulty with the HFP task was not significantly associated with age, MPOD or GRT.

Glare recovery time after a 30-s bleach using the direct ophthalmoscope was significantly and positively associated with age. No significant association was found for any other AMD or OVP RF examined in this study, after correction for age.

Interocular agreement and intra-session repeatability were good. Inter-session repeatability was moderate, requiring the remeasurement of abnormal values to confirm abnormal status. GRT was not significantly associated with ocular dominance. The LoA on interocular and repeated measures of GRT increased as a function of mean GRT (and by association, with age), requiring the use of a formula to determine abnormality. This would limit the use of this method of GRT in a clinical setting, particularly if participants were from a different population, requiring a population-specific algorithm to be calculated. This method of GRT was not found to be a good surrogate measure for MPOD.

Measurement of MPOD is likely to be a more reliable marker for factors affecting AMD risk compared to GRT, because it is relatively stable with age for each individual, although MPOD and GRT measurements exhibit considerable inter-subject variation. Measurements of MPOD obtained by HFP are less likely to be affected by functional retinal changes and media opacities because they are recorded as ratios of central and peripheral values (unless central only values are used with age-estimation software). Objective methods of MPOD are unaffected by retinal function but are affected pupil size and media opacification. Glare recovery time on the other hand is reliant on there being a functional retinal change to detect a significant finding and these are unlikely to be present in individuals with AMD RF before the age of 50 because AMD is rare before that age.

Because photo-flash GRT places less stress on the RPE, this method may be more suitable to detect subtle changes in GRT related to OVP or retinal ischaemia / Mc dysfunction compared to equilibrium methods, however photo-flash GRT has been reported to be less repeatable.

The lack of association between MPOD and GRT with all of the AMD and OVP RF (apart from GRT with age), suggests that either there is no association, the association was too small to be detected using these methods or the sample size was too small. This result could have occurred by chance, but this is unlikely.

4.2 New findings

4.2.1 Macular pigment optical density study

The potential for bias between sequential interocular MPOD measurements with no resting period between measurements. This may have ramifications for the recommendation to record means of multiple MPOD values from the same eye in a clinical setting, as it is likely that no resting period between measurements will be given.

The trend towards a reduction in MPOD measured from the dominant eye relative to the non-dominant eye with age has not been reported previously and warrants further investigation. This measurement may be a marker for ocular light exposure and has the potential to be predictive for future ocular disease.

This is the first study to examine the association between HFP-derived MPOD and migraine, and the association between any method of MPOD measurement and calculated %BF, Rph and VDys.

Older age was reported to be a factor in the difficulty experienced with the use of HFP.[507, 689, 973] This study revealed no statistically significant difference between the ages of naïve participants reporting no difficulty compared to those reporting difficulty. More than twice as many female participants reported difficulty compared to male participants. This may be a consequence of the gender ratio of the study population (73% female). There was a trend for longer glare recovery time for those reporting difficulty with the MPOD task, despite there being no significant difference in the mean age of the two groups.

The relative retinal illuminance for blue and brown irides was calculated for a range of pupil sizes was calculated (table 2.10). The observation that the difference in retinal illuminance for brown and blue iris colour is largely dependent on pupil size has not been reported before.

The author is unaware of any previous reports of reduced MPOD in a subject with coloboma. Results were derived from a single case and therefore analysis of further cases would be needed to confirm this association.

4.2.2 Glare recovery time study

This is the first study to log10 transform the GRT data to correct for positive skew and to present the repeatability and interocular comparison of the GRT results as Bland-Altman plots with back-transformed LoA and 95% CI.

This is the first study to examine the association between GRT and BMI, calculated %BF, migraine, Rph and VDys. The association between this method of GRT with iris colour and AMD FH has not been examined before.
No prior study has assessed the association between MPOD and this method of GRT.

No prior study has assessed interocular comparison and repeatability for this method of GRT.

The use of the same test chart letters for repeated measures of GRT did not bias the results compared to changing the letters. This suggests that optometrists with fixed-letter test charts can use the same letters for repeated GRT measurements.

The author believes that this is the first study to propose that the high level of percentage bleach during GRT, in addition to the previously reported lack of blue light content in the GRT light source, may explain the absence of a negative correlation between GRT and MPOD.

4.3 New theories generated by this study

One of the strengths of the cross-sectional study design is that it is good for the generation of new hypotheses.[687] The following theories were formulated during the course of this study. The Müller cell / neuroglial cell hypothesis for macular xanthophylls is described below, a plausible argument for the OVP RF migraine, Rph and VDys to be considered as AMD RF is presented in section 1.3 and fig. 1.3, and a plausible retinal theory for symptoms suggestive of MIS is described in appendix 3.

4.3.1 The Müller cell / neuroglial cell hypothesis for macular xanthophylls

This hypothesis suggests that MP preserves the functional capability of Mc, particularly those associated with cone photoreceptors in the fovea, and neuroglia in the brain by modulating glial activation (reactivity),[892, 893] and Mc de-differentiation caused by exogenous stressors (e.g. blue-light),[277-279] and endogenous stressors (e.g. oxidative stress).[974] Müller cell functions in addition to cone photopigment recycling were reviewed in appendix A2.2 of this thesis. Müller cells are crucial for the survival of photoreceptors,[273, 975, 976] and neurons.[977] Neuroglia also protect brain neurons.[978] Müller cells become activated in response to virtually all pathogenic stimuli,[269] and Mc activation is a feature of

many retinal disorders including; photic damage, retinal detachment, glaucoma, diabetic retinopathy and AMD.[270]

4.3.1.1 Glial reactivity in ocular and neurological disease

Neuroglial reactivity was reported as a pathogenic mechanism of AD and other neurodegenerative disorders.[978] Down-regulation of normal Mc function as a result of activation or de-differentiation causes many changes associated with AMD pathophysiology; reduced antioxidant production, neurotransmitter recycling, blood-retina barrier maintenance, neuroprotection and increased secretion of inflammatory and angiogenic factors. It is not certain whether Mc changes represent a primary or secondary factor in AMD pathogenesis. Photoreceptor degeneration followed Mc ablation with tamoxifen.[979] Müller cell activation (GFAP up-regulation) and photoreceptor degeneration were also reported after RPE ablation using tamoxifen.[980]

Macular telengiectasia type 2 is associated with a central depletion rather than a paracentral displacement of MP.[981] Müller cell loss or dysfunction is a critical component of MacTel type 2 and the area of MP depletion corresponds to the region of Mc loss.[982] Supplementation with L and Z resulted in an increase in MP in areas where MP was detected at baseline, but no increase where MP was absent.[983] This suggests that Mc may be involved in storage, trafficking and / or regeneration of MP,[981, 984] but this remains to be confirmed. In view of the ability for a subset of Mc to act as stem cells,[270] it would be beneficial to encourage the preservation of these cells for their potential as a target for therapeutic regeneration.[985]

Initial changes associated with AMD usually affect rods in the parafoveal retinal location.[958, 986] The spatial distribution of lipofuscin deposition generally matches that of rods.[246] Human data have shown that the retinal location where MP is most densely deposited (central fovea) is also the most resistant to degenerative change.[987, 988] These observations are cited by proponents of the protection hypothesis as evidence for the cone-protective role of MP.[987, 988]

4.3.1.2 Müller cells contain MP?

The majority of MP is located within cone axons in the Henle fibre layer at the foveola and within the inner plexiform layer at the edge of the foveal depression.[989] Müller cells, like cone axons, follow an outward-curving path in the foveal region.[395] An alternative theory proposed by Gass was that Mc could act as a reservoir for MP.[400] This theory was not favoured by Snodderly *et al*. in view of the similarity in appearance between MP and oil droplets occurring in cones, but not Mc of non-primate vertebrates.[395, 989] It was also reported that the antibody (N-62 StAR) for StARD3, the human retinal L-binding protein localised to cone inner segments and axons, but not with glutamine synthetase, a Mc

marker.[460] Gass *et al*. reported yellow-coloured scotoma caused by pseudo-operculae,[444] which are mainly composed of Mc and astrocytes.[990] Epiretinal membranes contain a variety cell types, including Mc, but not photoreceptors and their axons.[991] Epiretinal membranes were also found to contain MP.[443] Lutein and / or Z may be used as selective stains to improve visualisation of the ILM, which is composed of the end feet of Mc and astrocytes.[992, 993]

4.3.1.3 Müller cell dysfunction may increase the metabolic load on the RPE

The MP spatial profile peaks (in most normal cases) at the foveola and reduces exponentially to almost unmeasurable levels at 6-8° eccentricity.[244, 393] It is likely that this spatial profile affords the fovea greater protection from blue light than the parafovea. Early parafoveal involvement may also be a consequence of greater oxygen demand and higher photoreceptor to RPE cell ratio in the parafovea compared to the fovea. The mean ratio of photoreceptors to RPE cells in human eyes was higher at the macula than at the paramacula or periphery.[994] Data from humans and non-human primates indicated that the ratio of photoreceptors to RPE cells was lower at the fovea compared to the parafovea.[995] Photoreceptor and RPE cell numbers reduce with age, although the rate of loss with age is higher for RPE cells, leading to a greater ratio of photoreceptors to RPE cells with age.[994]

Data from primate studies has indicated that the parafovea, not the fovea has the highest oxygen demand in both light- and dark-adapted conditions and that oxygen consumption was greater at both locations by 16-36% when dark-adapted. This reflects the thinner retina, lack of inner retinal neurons and absence of retinal circulation at the fovea. Foveal oxygen is received almost entirely from the choroidal circulation under dark- and light-adaptation (with a minor contribution from the vitreous humor). Under light-adaptation the parafovea also receives 100% of its oxygen from the choroid, but when dark-adapted this changes to 90% from the choroid and 10% from the retinal circulation.[996, 997] The greater number of photoreceptors per RPE cell and greater oxygen demand reported for parafoveal compared to foveal retinal locations suggest a greater phagocytic load on the RPE paracentrally, leading to a greater accumulation of lipofuscin within the RPE and eventually the development of drusen in this region.[994] In Whites, photoreceptor loss significantly correlated ($p < 0.0001$) with lipofuscin in the adjacent RPE, but was, however, unrelated to age.[994] Photoreceptor degeneration and Mc activation were observed in human donor retinal tissue overlying hard and soft drusen.[271]

The central fovea of primates has a Mc to cone ratio of 1:1.[526] This changes to 2:1 at an eccentricity of approximately 30° from the fovea, reflecting lower cone numbers although each Mc will also serve numerous rods.[528, 998] Müller cells provide an additional source of all-trans retinol to cones but not rods, independent to that from the RPE.[521] Within cone OS this is oxidised to all-trans retinal.[525] This additional source of photopigment from Mc may

explain why cone recovery is considerably quicker than rods (ms vs. min) from a sustained bleach,[529] and may also contribute to the reduction in phagocytic load on the RPE at the fovea, supporting to the observation of greater parafoveal damage in AMD as well as in other retinal degenerations such as chloroquine / hydroxychloroquine retinopathy where cones appear to be spared in the early stages of the disease.

Because the secondary source of photopigment recycled via Mc is likely to relieve metabolic stress on the RPE, any situation where Mc are compromised would likely increase stress on the RPE. Although Mc are strikingly resistant to ischaemia, anoxia and hypoglycaemia compared to photoreceptors,[294] they become activated (reactive) or de-differentiated in response to pathogenic stimuli including blue-light exposure (in rats).[278, 279] Activation of Mc results in down-regulation of many essential functions including; glutamate recycling, GSH production, water transport and cone photopigment recycling.[269, 270]

4.3.1.4 Müller cell activation (reactivity) and down-regulation of activation by MP

Glial fibrillary acidic protein is the most sensitive, non-specific response to retinal disease and injury and is a recognised cellular marker for Mc activation.[270, 999] Measurements from the sera of patients with nAMD revealed up-regulation of GFAP.[1000] Murine models indicated that L has an inhibitory effect on GFAP expression in Mc.[893] Treatment with L also lowered levels of Mc gliosis in ischaemia / reperfusion injury and reduced nuclear levels of NF-κB, IL-1β and COX-2 after hypoxic injury in cultured murine Mc.[892] The NF-κB family of transcription factors has an essential role in inflammation and innate immunity.[1001] The association between the innate immune system and AMD is well known.[1002] The pro-inflammatory cytokine IL-1β is an important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis. Interleukin-1β was reported to significantly increase CNV in response to light damage, independent of the effect of VEGF in mouse and rat models.[1003] Cytochrome c oxidase (COX), also known as prostaglandin endoperoxide synthase (PTGS). Murine data suggested that COX-2 down-regulates VEGF expression in CNV.[1004]

Support for the beneficial effects of dietary or supplemental L and Z in the prevention of, or reduction in the progression of AMD has been demonstrated. The Blue Mountains Eye Study and EDCCS confirmed a lower risk of developing AMD in participants with the highest dietary intake of L and Z.[168, 1005] Results from AREDS2 revealed that L and Z supplementation reduced the rate of progression to advanced AMD in participants with a poor dietary intake of macular xanthophylls.[452]

Lutein and Z have been found in the brain of humans,[430, 1006] at levels that correspond to retinal levels of MP.[1006] Higher levels of MPOD were associated with improved cognitive function,[1007] and temporal processing speeds.[702, 843, 1008] The neural efficiency hypothesis

proposed by Renzi *et al*. states that L and Z located in the retina and in the brain improve neural function by enhancing gap junction communication. The authors cite Stahl *et al*. to support their hypothesis, however this group examined the effects of several non-vitamin A carotenoids including lycopene and canthaxanthin on gap junction communication, but not L and Z.[1009]

The Mc / neuroglial cell hypothesis may offer an alternative explanation for the beneficial effects of L and Z on brain function, as it is possible that these carotenoids also modulate neuroglial reactivity. The Mc / neuroglial cell and neural efficiency hypotheses may be complimentary if glial reactivity also alters gap junction communication between Mc / neuroglia and neurons. Glial cell dysregulation leading to impaired neural function was reported as a pathogenic mechanism in AD and other progressive neurodegenerative disorders.[1010]

Similarities have been drawn between AMD and AD pathogenesis.[253] A lower risk of AD mortality was associated with higher serum levels of L, Z and lycopene.[1011] Macular pigment optical density was more strongly related to measures of cognition (mini mental state examination score, visual-spatial and constructional abilities, language ability, attention and the total scale on the Repeatable Battery for the Assessment of Neuropsychological Status) in participants with established cognitive decline[1012]. Supplementation with L, Z and MZ resulted in improved contrast sensitivity in participants with AD, as well as in unaffected controls.[1013]

It is also plausible that the Mc / glial cell hypothesis may contribute to the explanation for the MP hypotheses relating to improved visual parameters (VA, CS, glare reduction) and GRT. Two double-masked, placebo-controlled studies reported a significant improvement in VA after supplementation with L alone or combined with antioxidants / nutrients.[936, 1014] Retinal oedema secondary to disturbed fluid transport may occur after Mc activation.[1015] It is plausible that MP may reduce glare associated with clinical and subclinical levels of retinal oedema by reducing Mc activation.

4.3.1.5 Müller cells as light guides and spectral filters

Guinea pig Müller cells were, in view of their radial orientation within the retina, reported to act as optical fibres transmitting light from the inner to outer retina with reduced distortion.[1016] Müller cells from the same animal were also reported to spectrally filter red and green light to cones and blue and purple light to rods.[1017] The blue-light-blocking properties of MP make it a possible candidate for involvement in this spectral filtering. At the fovea, the retina is thinner with the potential for increased outer retinal light exposure. Foveal Mc follow an outward arc with cone axons rather than the usual radial orientation seen in the non-foveal retina. It is therefore logical that MP would be denser in the foveal

region in the absence of radial Mc to guide and spectrally filter light to foveal cones and to afford greater protection to the exposed lateral aspect of Mc. It is no coincidence that blue cones are absent from the foveola where blue-blocking MP is at its most concentrated.

4.3.1.6 Glare recovery time shortened by MP

Glare recovery time, thought to be a measure of cone photoreceptor recovery was shorter for participants with higher levels of MP in four studies using a light source with a high blue-light content.[620-623] Lower light exposure resulting from the blue-blocking properties of MP was cited as an explanation for these findings.[622] It is also plausible that Mc photopigment recycling is better preserved in those with higher MPOD levels because MP reduces Mc activation, thus protecting Mc function. Three studies (including the present study) did not find an association between MPOD and GRT.[906, 968] These used light sources without a significant blue-light content. Two of these studies, the present study and that of Loughman *et al*.[906] used a longer glare source exposure time. It is possible that the longer exposure time resulted in complete depletion of Mc photopigment stores, thus negating any potential difference in GRT due to MP, as GRT would be more dependent on RPE recycling.[330] Nolan *et al*. used a shorter duration, minimal blue light glare source.[968] The lack of association between MPOD and GRT reported by this study may be a consequence minimal Mc activation due to the low blue-light content in the glare source.

Contrary to the damaging effect of blue light, pre-conditioning with red light (670 nm) was reported to ameliorate light-induced Mc specific markers for structure, retinal stress, metabolism and inflammation in rats, by enhancing the activity of COX, the rate-limiting enzyme involved in the mitochondrial respiratory electron transport chain that produces ATP.[1018]

4.3.1.7 Summary

Modulation of Mc and neuroglial reactivity and Mc de-differentiation by MP is offered as one explanation for the high levels of macular xanthophylls found in the eye and the brain. The effects of higher levels of MP on Mc and neuroglia would benefit individuals below reproductive age and therefore confer an evolutionary advantage to those individuals. The central peak of the MPOD spatial profile was found to be strongly heritable.[404] The Mc / neuroglial cell hypothesis may offer an alternative explanation to the neural efficiency hypothesis for the beneficial effects of high MP levels in the eye and L and Z in the brain.

The Mc / neuroglial cell hypothesis is a plausible optical and / or biological explanation for the association between MPOD, VA, CS, glare reduction and GRT reported by several studies.[482] If these affects can be replicated in humans (or non-human primates) this would suggest an anti-inflammatory, neuroprotective and visual function enhancing role, as well as a role in improving cognitive function for L, Z and possibly MZ achieved by modulation of

Mc and neuroglial cell activation. Visual function may also be enhanced by the optical fibre and spectral filtration properties of Mc.

4.4 Limitations

This practice-based study was limited by the small number of participants recruited (n = 150), the high number of excluded participants prior to data analysis (n = 50) and the long duration of time over which data was collected. Increasing the number of participants recruited to compensate for the high exclusion rate was discussed with the study supervisor, but the decision was made to adhere to the original number of recruits. The author collected data on days when there was no clinic running. This varied from one day a week to one day every two weeks. Data collection for each participant took one hour, limiting the number of participants examined to a maximum of eight to 16 every two weeks. Data collection was completed after 14 months, including the re-examination of 30 participants for the assessment of GRT inter-session repeatability.

Sample size estimates assuming 80% power, at 5% significance level were calculated from data derived from previous, similar studies or calculated using G*Power statistical software if no previous studies were available. This study was sufficiently powered to detect a true positive result for ocular dominance, age, BMI (mixed gender) and Rph in the MPOD study, and for age, BMI (mixed gender) and AMD FH in the GRT study. Data collection would have had to continue for several years to obtain sufficient data for some of the other variables. The time required to obtain sufficient data is a clear limitation of any sole-practitioner working full-time in a practice-based study.

The method of MPOD assessment used in this study only measured the level of MPOD at 0.5° eccentricity. The central "dip" at 0.25° eccentricity present in 12% of participants would not be detected using 0.5° eccentricity. The central part of the MPOD spatial profile was found to be irregularly-shaped and asymmetric. Measurement at a single point in the spatial profile may therefore increase intra-ocular and intra-subject MPOD measurement variability.

It is clear that a variety of physiological and pathological processes have the potential to adversely affect MPOD and GRT measurements. See sections 1.2 and 1.3 for further information. The use of SD-OCT would allow the exclusion of such cases and may lead to an improvement in the variability in both of these measurements.

All study participants were naïve to MPOD measurement and only a single measurement of MPOD was taken for each eye. Each MPOD measurement is derived from the results of several blue / green comparisons. The first eye MPOD measurements were used for data analysis.

This method of MPOD testing incorporates a pre-test flicker sensitivity routine prior to measurement to ensure consistency and each MPOD result is based on the minimum of a v-shaped function produced after presenting a series of blue-green luminance ratios.[486]

The first eye MPOD values were found to correlate well with the second eye values, r = 0.78 ($p < 0.01$) to 0.83 ($p < 0.01$), with and without outliers respectively.

This level of interocular correlation was comparable with other studies where MPOD values were based on multiple readings (r = 0.79 to 0.87),[507, 589, 596, 654] suggesting that it was reasonable to use the first MPOD values in this analysis. It is assumed that right and left eye MPOD measurements are the same for healthy participants, although interocular comparison is no substitute for repeatability, as right and left eye measurements are considered to be independent for the purpose of statistical analysis.[1019, 1020] Constraints on the examination time for each participant also meant that GRT values were based on a single result for each eye in all sessions.

Several of the variables were reported rather than confirmed. These variables were: FH of AMD, migraine, migraineous aura and light-trigger, Rph and VDys. For the purpose the inclusion / exclusion criteria participants were asked about the presence of normal cholesterol, dietary absorption disorders, pregnancy, diabetes, glaucoma and medication affecting macular function. These were recorded as "yes", "no" or "unknown". "Unknowns" were included as there was no evidence to exclude them. It is likely that a proportion of the "unknowns" would have been excluded if the answer to these questions had been known.

Poor fixation, variation in battery charge and difference in bulb type have been reported as limitations for the use of the direct ophthalmoscope for GRT measurement.[329, 924]

4.5 Confounding variables

Dietary analysis was not performed. Some studies, but not others reported a seasonal variation in diet.[1021-1023] It could be argued that seasonal variation may have affected the results of this study. Nolan *et al*. reported no statistically significant seasonal variation in MPOD[1024] The author is unaware of any studies examining the effect of seasonal variation on GRT.

Light intensity and physical activity are significantly higher in the summer and spring, whereas sedentary behavior and time spent in bed are significantly greater in the winter.[1022, 1023] Variation in daylight intensity could have influenced levels of long-term adaptation potentially affecting GRT and pupil size measurements. The seasonal variation in physical activity and weight are likely to have affected BMI and %BF measurements.

The results were not corrected for VA. In this study participants with LogMAR VA worse than 0.1 were excluded. The association between MPOD and VA is controversial. Several studies have reported a significant positive association between these variables,[906, 936, 1014, 1025] whereas several others found no significant association.[500, 657, 1026, 1027] Margrain and Thomson reported no significant association between VA and GRT for participants with a LogMAR VA of 0.12 or better.[573] Any effect of VA on MPOD and GRT was minimised by the exclusion of participants with a level of LogMAR VA worse than 0.1.

Refractive status and axial length were not recorded. No significant association was observed between MPOD and refractive status in healthy adults and Chinese, school-aged children.[591, 1028] Neelam *et al*. found no significant association between MPOD measurements using HFP and axial length.[591] Obana *et al*. reported a small, significant inverse association between FFS MPOD measurements and axial length.[723] Margrain and Thomson reported that GRT was not affected by the presence of myopia.[573] The effect of refractive status was considered to be minimal.

4.6 Improvements

It would have been beneficial to use the mean of at least three MPOD readings for each eye, however the time constraints on the duration of the examination prevented this. Three recent studies,[590] (two of which used the MPS 9000 screener),[500, 680] appear to have used single readings rather than means of multiple readings for their analysis. In clinical practice it is unlikely that more than one MPOD measurement per eye would be taken.

It would also have been beneficial to measure MPOD at multiple retinal eccentricities. There is evidence that genetic and environmental influence on retinal MP deposition varies with retinal eccentricity.[404] It is possible to modify the MPS screener with eccentric fixation targets in order that MPOD may be measured at retinal eccentricities greater than 0.5°, however, this would have significantly increased the examination time.[494]

Performing a repeatability study for MPOD would have been useful, in order to assess the level of instrument bias for the MPS 1000 for this operator and population. Unfortunately time constraints imposed by the practice setting of this study did not allow this.

The use of an objective method of MPOD measurement (FAF or FR) would have been preferable to HFP. These methods allow MPOD data to be collected for the entire MPOD spatial profile in a short period of time, however the higher cost of these methods precluded their use in this study. It is likely that participants with migraine would not have tolerated the level of flicker associated with MPOD measurement using FAF.

It was not anticipated that MPOD testing would adversely affect the first GRT values for each eye in the first session. Had this been anticipated, a longer time interval between MPOD testing, and GRT would have been planned. This would have increased the time taken to collect the data needed for this study.

During the photostress phase of GRT, fixation was monitored by direct observation of the fovea through the ophthalmoscope and therefore the effects of poor fixation were minimised. The use of a larger size stop during photostress would further minimise fixation errors, but would also increase rod photoreceptor bleach and possibly increased levels of lateral inhibition. The effect of ophthalmoscope battery charge variability was controlled by fully charging the battery, indicated by the absence of a flashing light on the charger base station between measurements for each participant. The same ophthalmoscope battery and bulb were used for all participants.

Glare recovery time endpoint was achieved when the participants had read all letters on the line above that representing their corrected VA according to Fingeret *et al*.[1029] Test chart letters were used as they are available to all optometrists in their practice. LogMAR letters were used rather than Snellen letters in view of their equal logarithmic progression of letter size.[573] The author noted differences in the letter reading speed and confidence of some participants, which may relate to personality type. Personality type was not assessed or corrected for in this study. In the laboratory setting the correct identification of the orientation of Gabor patches may be used as an alternative end point for GRT.[622] In Optometric practice this could be adapted to the identification of single letter Landolt C or Illiterate E orientation.

In this study iris colour was designated by mutual agreement between the author and each participant. It was planned that any difference in opinion would be settled by observation of the irides of the participant in natural daylight, by a third party. This was not required for any participants. The use of a standardised and validated iris grading system such as that described by Muinos Diaz *et al*. would allow for enhanced inter-study comparison.[778]

The addition of the assessment of habitual diet would have benefited this study. This may be assessed in Optometric practice-based research by the use of a food frequency questionnaire (FFQ) or a diet diary.[1030, 1031]

Pupil size was measured as an average over a few seconds by eye to the nearest millimeter using a ruler. In lab-based studies pupil size may be measured more accurately using an infrared pupillometer. Meeker *et al*. found that the median error in manual pupil size measurements (0.55 mm) was twice that of a pupillometer (0.23 mm).[1032] Portable infrared pupillometers are commercially available, but were not used in view of the cost.[1033]

The methods of MPOD and GRT measurement used in this study would have made simultaneous pupil size measurement very difficult.

Inter-session repeatability was adversely affected by the lack of a constant time interval between repeat measures for some of the participants. This would be remedied by examining all of the participants in block sessions, however this was not possible for a practitioner working full-time in a busy practice setting.

For variables dependent on participant reporting: FH of AMD, migraine, migraineous aura and light-trigger, Rph and VDys, confirmation could have been obtained. AMD FH may be confirmed by obtaining a signed letter from the treating Ophthalmologist for that relative, with their consent, although obtaining confirmation for deceased relatives would be more difficult. AMD FH may not be an accurate marker for future risk of AMD development however as the affected relative may have had increased exposure to high-risk environmental factors such as smoking or obesity, rather than a heritable genetic predisposition to develop AMD. The general practitioner may confirm the presence of migraine for each affected participant, with consent from the participant. Cold provocation testing may be used to confirm Rph and VDys. GRT testing during or just after cold provocation may be preferable.

The use of SD-OCT to exclude anomalous MPOD and GRT due to physiological and pathological conditions would have benefitted this study. This was not available at the location where the data was collected.

4.7 Future work

4.7.1 Objective measurement of MPOD and AMD / OVP RF

The use of an objective measure of MPOD such as 2W-FAF would allow not only a quicker and more accurate estimation of retinal MP levels, but would also allow examination of the MP spatial profile, known to be centrally deficient in certain AMD RF; age (0.25° eccentricity), current and previous smokers (0.25°), FH of AMD (0.17°, but not 0.5°) and AMD risk genes, CFH and ARMS2 (0.5° and 1.0°, but not 0.25°). The association between dominant and non-dominant eye MPOD difference and age may be investigated in greater detail. The Heidelberg Spectralis (Heidelberg Engineering Ltd, Hertfordshire, UK) HD-OCT 2W-FAF MPOD commercial module is currently being developed. The benefit of using this system is that retinal structure and thickness may also be examined by HD-OCT and physiological and pathological causes of anomalous MPOD values may be recorded. Heidelberg is developing an OCT angiography module for the Spectralis that will measure retinal and choroidal blood flow without the need for an injectable dye. This would be particularly useful for assessing ocular blood flow in individuals with AMD RF and those

with migraine, Rph and VDys. Ocular blood flow for the OVP RF could be assessed with the Spectralis OCT, with and without cold provocation.

4.7.2 Objective measurement of GRT and AMD / OVP RF

Glare recovery time, like MPOD is prone to a high degree of between and within subject variability and the lack of a criterion standard method of measurement. The retinal densitometer being developed at the Department of Optometry and Vision Sciences at Cardiff University, allows the objective measurement (by the change in photopigment colour after bleach) of rod and cone DA (T. Margrain, personal communication, December 10th, 2013). A range of different percentage bleaches could be implemented to assess the relative contribution of the Mc and RPE visual cycles, and to reduce the risk of missing subtle differences in GRT related to AMD / OVP RF due to the very high percentage bleach used in the present study. If the Cardiff University Densitometer is not available DA time to rod-cone break may be measured psychophysically in the clinical setting using the AdaptDx dark adaptometer.

4.7.3 The relationship between glial activation and MPOD

Increased GFAP is a recognised marker for glial and Mc activation. Serum levels of GFAP were significantly raised in individuals with intracranial haemorrhage in acute stroke.[1034, 1035] As retinal levels of MP are associated with cortical MP levels, serum GFAP may also be compared with objective MPOD measurements in order to investigate whether higher MPOD levels are associated with lower cortical glial reactivity. This study would be of interest to those studying AMD and neurodegenerative disorders such as AD. Comparing MPOD with Mc activation is more difficult *in vivo*. The antioxidant glutathione is released in the retina by Mc and HC. Light-induced activation of Mc leads to an increase in GFAP and a reduction in glutathione production. Levels of reduced GSH and total GSH were significantly and positively correlated with MPOD.[1036] By comparing MPOD with serum GFAP and GSH before and after photostress, as a marker for retinal levels of these compounds, it may be possible to associate MPOD with Mc reactivity.

For further studies associated with the retinal theory for MIS, see appendix A3.8.

4.8 Conclusions

This method of GRT was significantly associated with age, however no other AMD or OVP RF tested was significantly associated with MPOD or GRT. The relationships between MPOD and GRT with age revealed a trend towards being biphasic, therefore linear statistics may under-estimate the relationship between these variables. This may account for some of the controversy reported in the literature to date.

Interocular agreement for MPOD and GRT was good, and repeatability for GRT was good for intra-session and moderate for inter-session measurements. This method of GRT, using the direct ophthalmoscope as the source of light was not a suitable surrogate measure for MPOD. This was likely to be due to the minimal blue light content in the light source used and the high percentage level of the bleach. Increasing the percentage bleach level during GRT (for equilibrium versus photo-flash bleach) was reported to increase repeatability,[330] however this was achieved at the expense of sensitivity to detect subtle changes due to differences in iris colour or MPOD level, for which a lower percentage bleach would be preferable. GRT may be further enhanced by altering the colour of the light source and by performing repeated GRT after short time interval.

The methods used to measure MPOD and GRT in this study were easy and quick for the practitioner and were tolerated well by naïve participants. Interpretation of the results is more difficult in view of the very large level of inter-subject variation with age observed for both tests. An additional difficulty with HFP MPOD testing is that the improvement in MPOD expected after improved diet or MP supplementation is likely to be small, smaller than the limit of agreement (CoR) on repeat testing. Therefore any improvement in MPOD would be difficult to distinguish from instrument noise and a patient with "low" MPOD is still likely to have "low" MPOD after supplementation. These factors have adversely affected the widespread adoption of either test in clinical practice and have prevented the development of "criterion standard" tests.

One commercial model for MPOD testing used by many optometrists in clinical practice is profitable because it is linked to the sale of MP supplements. AREDS 1 and 2 have shown that antioxidant supplements are not beneficial for AMD prevention or at early stages of AMD development. Macular pigment supplements were only found to benefit AMD patients qualifying for AREDS supplements, with poor dietary intake of MP. Dietary assessment is not generally performed with MPOD testing in clinical practice, but perhaps it should be to gauge whether improved diet or dietary supplements are likely to benefit the patient. Even so approximately 10% of those with lower MPOD may be "non-responders" to intervention with increased MP.

Ideally optometrists could include MPOD (full spatial profile in preference to 0.5° eccentricity) testing as part of a dedicated routine for those at risk of developing AMD or with early AMD signs, much in the same way that we screen for glaucoma. The measurement of MPOD which is regarded as a putative rather than a confirmed RF for AMD, alone as a screening test for AMD risk is likely to be as much use as measuring IOP alone as a screening test for glaucoma. The difficulty with this plan is that despite the increasing year on year cost of AMD management and treatment, there is uncertainty within the NHS about whether any form of screening for the presence of AMD is cost effective

(although annual screening from 60 years of age is likely to be most beneficial).[1037] It is therefore unlikely that the NHS would contribute to the high cost of obtaining and maintaining equipment such as OCT and the extra clinic time that would be required for additional testing.

This may of course change in the near future if treatments for "dry" AMD prove to be successful. Fundus autofluorescence using the OCT is likely to be the measurement of choice for monitoring change before and during treatment.

The recommendation to perform repeated measurements of MPOD and GRT in a clinical setting would improve repeatability and limit false positive results, however the addition of a period of pre-adaptation (both are psychophysical tests) and a 5-10 minute period of rest between measurements may limit the use of these tests in routine clinical practice.

Rather than comparing the MPOD and GRT results with a population mean and attempting to interpret whether the results are normal or abnormal, it may be more appropriate to use these methods to record baseline data to compare with future measurements, in order to detect changes over time. Although there has been some success in associating MPOD with certain AMD RF (BMI, %BF, iris colour and AMD genetic risk, but not age - the greatest AMD RF), the association between this method of GRT and AMD RF appears to be limited to age.

Final literature review	17.05.15
Thesis word count	78,303

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Appendix 1

A1.1 Macular pigment optical density study results

Table A1 Reasons for exclusion (MPOD and GRT studies)

Participant ID	Reason for exclusion		
	Primary	Secondary	Tertiary
5	BMI ≥ 30	High cholesterol	Poor night vision
8	BMI ≥ 30	St John's wort	Poor night vision
9	High cholesterol		
10	Irritable bowel syndrome		
11	Failed to attend	Reassessment declined	No data collected
14	BMI ≥ 30		
16	BMI ≥ 30	High cholesterol	Macular drusen (LE)
17	St John's wort		
18	Smoker		
19	BMI < 20		
20	BMI ≥ 30		
24	Coeliac disease		
25	BMI < 20		
26	BMI ≥ 30		
29	Macular drusen (RE)	Poor night vision	
30	VA below 0.1 LogMAR		
32	BMI < 20		
36	Unable to complete GRT	Photophobic (migraine)	
39	Refused some tests		
41	Macular drusen (BE)		
43	BMI ≥ 30		
45	Tamoxifen		
51	BMI ≥ 30		
54	Macular drusen (BE)		
57	Poor night vision	Dull foveolar reflexes	
66	Macular drusen (RE)	Amblyopia (LE)	
67	Irritable bowel syndrome	Foveal view poor	
73	Too unwell to participate	Reassessment declined	
76	Diverticulitis / colostomy		
77	BMI < 20		
81	BMI ≥ 30		
86	Cetirizine		
91	BMI ≥ 30	High cholesterol	
92	BMI ≥ 30		
98	BMI ≥ 30		
103	High cholesterol		
105	Colitis		
106	BMI ≥ 30	Cholesterol level n/a	
108	High cholesterol		
110	Irritable bowel syndrome		
111	Malingering		
115	BMI ≥ 30		
123	BMI ≥ 30		
125	Smoker		
126	High cholesterol	Poor night vision	
127	Refused some tests	Poor night vision	
131	BMI ≥ 30		
140	Refused all tests	High cholesterol	
142	Poor fixation (BE)		
147	High cholesterol	History of solar burn	Poor night vision
Total	50	16	4

Table A2 Frequency analysis of primary reason for exclusion (MPOD and GRT)

Primary reason for exclusion	Number of data (n)	Percentage
BMI ≥ 30	16	32
High cholesterol	5	10
BMI < 20	4	8
Macular drusen	4	8
Smoker (reported as non-smoker during recruitment)	2	4
Poor fixation	1	2
Poor night vision	1	2
Incomplete data collection		
- Refused all tests except MPOD after giving consent	2	4
- Refused all tests after giving consent	1	2
- Unable to complete GRT, reassessment declined	1	2
- Malingering	1	2
- Unable to assess due to illness, reassessment declined	1	2
- Failed to attend, reassessment declined	1	2
Digestion / absorption disorders		
- Irritable bowel syndrome	2	4
- Diverticulitis / colostomy	1	2
- Colitis	1	2
- Coeliac disease	1	2
- Bowel disorder (unknown)	1	2
VA below 0.1 LogMAR	1	2
Medication or supplement affecting macular function		
- Tamoxifen	1	2
- Cetirizine	1	2
- St John's wort	1	2
Total	50	100

Table A3 Tests for normality

	Kolmogorov-Smirnov (with correction)			Shapiro-Wilk		
Variable	Statistic	df	P-value	Statistic	df	P-value
Eye 1 MPOD	0.097	100	**0.021**	0.964	100	**0.008**
Eye 2 MPOD	0.067	97	0.200	0.982	97	0.209

Probability values (p-values) < 0.05 are shown in bold. Significance with normality testing is not unusual for large sample size (≥ 100). Sample size in this ace was n = 100.

Table A4 Summary of interocular comparison results for MPOD

Interocular comparison	**Eye tested**	**Eye tested**
Right versus left (randomized)	**Right**	**Left**
Mean and SD (n = 97)	0.379 (0.166)	0.396 (0.161)
Independent t-test (right vs. left eye MPOD) Outlier removed	t(-0.723), p = 0.471 t(-0.548), p = 0.584	
Mean absolute difference: mean (right - left)	-0.017 (0.107)	
Mean relative difference: mean ((right - left) / right)	-0.114 (0.394)	Right MPOD lower than left
Correlation (Pearson 2-tailed) Outlier removed	r = 0.786, **p < 0.001** r = 0.814, **p < 0.001**	61.8% shared variance
LoA	2(0107) = 0.214	
LoA (outlier removed)	0.199	
First versus second (sequential)	**First**	**Second**
Mean and SD (n = 97)	0.395 (0.165)	0.379 (0.162)
Independent t-test (1st vs. 2nd eye MPOD) Outlier removed	t(0.692), p = 0.490 t(0.517), p = 0.606	
Mean absolute difference: mean (1st - 2nd)	0.016 (0.107)	
Mean relative difference: mean ((1st - 2nd) / 1st)	0.011 (0.312)	First MPOD higher than second
Correlation (Pearson 2-tailed) Outlier removed	r = 0.786, **p < 0.001** r = 0.812, **p < 0.001**	61.8% shared variance
LoA	2(0.107) = 0.214	
LoA (outlier removed)	0.199	
Dominant versus non-dominant (randomized)	**Dominant**	**Non-dominant**
Mean and SD (n = 44)	0.422 (0.180)	0.437 (0.174)
Independent t-test (dom vs. non-dom eye MPOD) Outlier removed	t(-0.410), p = 0.683 t(-0.166), p = 0.869	
Mean absolute difference: mean (dom - non-dom)	-0.016 (0.120)	
Mean relative difference: mean ((dom - non-dom) / dom	-0.103 (0.375)	Dominant MPOD lower than non-dominant
Correlation (Pearson 2-tailed) Outlier removed	r = 0.769, **p < 0.001** r = 0.846, **p < 0.001**	59.1% shared variance
LoA	2(0.120) = 0.240	
LoA (outlier removed)	0.210	
Bias calculations:		
Randomised vs sequential (n = 194) Outlier removed (n = 192)	t(-2.161), **p = 0.032** t(-1.739), p = 0.084	
Randomised vs ocular dominance (n = 88) Outlier removed (n = 86)	t(-0.325), p = 0.746 t(-0.354), p = 0.724	

Probability values (p-values) < 0.05 are shown in bold. Three monocular cases were removed from the interocular comparison data (first / second eye and right / left eye). Ocular dominance data was missing for 51 cases. One uniocular and four equidominant cases were removed. Calculations with and without one outlier.

Table A5 The difference between dominant and non-dominant eye MPOD with age

Variable	Pearson r	Shared variance	n	P-value (2-tailed)
Difference between dominant and non-dominant eye MPOD	-0.323	10.4%	44	**0.033**
Difference between dominant and non-dominant eye MPOD (outlier removed)	-0.206	4.2%	43	0.185
Difference between right and left eye MPOD (randomised)	-0.226	5.1%	44	0.140
Difference between right and left eye MPOD (randomised) (outlier removed)	-0.090	0.8%	43	0.568

Probability values (p-values) < 0.05 are shown in bold. One outlier (-0.41) was removed from both groups.

Table A6 Bivariate correlations for first eye MPOD comparisons

Variable	Pearson r	Shared variance	n	P-value (2-tailed)
Age	0.144	2.1%	100	0.154
Age ≤ 50 years	0.085	0.7%	57	0.528
Age > 50 years	-0.127	1.6%	43	0.415
Mixed gender BMI	-0.103	1.1%	116	0.277
Male BMI	-0.202	4.1%	32	0.267
Female BMI	-0.081	0.7%	84	0.466
Male %BF	-0.191	3.6%	32	0.295
Female %BF	-0.031	0.1%	84	0.776
Pupil size	0.051	0.3%	89	0.634

Abbreviations. n: number of data. Correlation size: small r = 0.10 to 0.29, medium r = 0.30 to 0.49, large r = 0.50 to 1.0.
11 cases missing from pupil data.

Table A7 Group analysis for first eye MPOD with AMD and OVP RF

Variable		n	MPOD mean & SD (or Median & IQR shown in grey)	Statistic	P-value	Size effect (if significant)
Age 2 age groups (years)	≤ 50 > 50	57 43	0.37 (0.16) 0.43 (0.17)	-1.957*	0.053	-
Age 4 age groups (years)	< 45 ≥ 45 to < 50 ≥ 50 to < 60 ≥ 60	32 25 24 19	0.37 (0.24) 0.31 (0.20) 0.46 (0.13) 0.41 (0.24)	8.066	**0.045**	-
Post-hoc tests Two follow-up Mann-Whitney U tests α level = 0.025	≥ 45 to < 50 ≥ 50 to < 60 ≥ 50 to < 60 ≥ 60	25 24 24 19	0.31 (0.20) 0.46 (0.13) 0.46 (0.13) 0.41 (0.24)	U = 155.5 Z = -2.907*** U = 166.5 Z = -1.516***	**0.004** 0.130	0.291 -
Gender	Male Female	27 73	0.40 (0.18) 0.39 (0.16)	0.375*	0.709	-
BMI (both genders) (n = 116)	< 20 20 to < 25 25 to < 30 ≥ 30	4 52 48 12	0.46 (0.12) 0.40 (0.24) 0.41 (0.23) 0.29 (0.23)	5.075**	0.166	-
BMI (male) (n = 32)	< 20 20 to < 25 25 to < 30 ≥ 30	0 7 20 5	- 0.41 (0.39) 0.39 (0.26) 0.34 (0.31)	1.221**	0.543	-
BMI (female) (n = 84)	< 20 20 to < 25 25 to < 30 ≥ 30	4 45 28 7	0.46 (0.12) 0.38 (0.24) 0.42 (0.20) 0.26 (0.19)	4.206**	0.240	-
Male %BF (CUN-BAE) (n = 32)	≤ 20% > 20 to 25% > 25%	2 6 24	0.48 (-) 0.45 (0.29) 0.36 (0.27)	0.565**	0.754	-
Female %BF (CUN-BAE) (n = 84)	≤ 30% > 30 to 35% > 35%	7 25 52	0.46 (0.14) 0.36 (0.24) 0.41 (0.22)	1.481**	0.477	-
Iris colour 5 iris colour groups Mixed-gender	Grey Blue Green Hazel Brown	12 33 15 16 24	0.42 (0.23) 0.41 (0.22) 0.36 (0.28) 0.37 (0.24) 0.46 (0.38)	2.297**	0.681	-
Iris colour (mixed-gender) 2 iris colour groups	Light Dark	60 40	0.38 (0.14) 0.42 (0.20)	-1.067*	0.290	-
Iris colour (male) 2 iris colour groups	Light Dark	14 13	0.39 (0.23) 0.41 (0.37)	U = 83.0 Z = -0.389***	0.697	-
Iris colour (female) 2 iris colour groups	Light Dark	46 27	0.37 (0.13) 0.43 (0.20)	-1.620*	0.110	-
AMD FH 2-group	Yes No	17 82	0.36 (0.18) 0.41 (0.27)	U = 690.5 Z = -0.061***	0.952	-
AMD FH 3-group	1st degree 2nd degree No	11 6 82	0.36 (0.24) 0.34 (0.16) 0.41 (0.27)	0.004**	0.998	-
Migraine	Yes No	17 83	0.36 (0.22) 0.41 (0.26)	U = 701.5 Z = -0.037***	0.971	-
Light-trigger	Yes No No migraine	6 11 83	0.43 (0.31) 0.31 (0.20) 0.41 (0.26)	1.287**	0.525	-
Aura	Yes No No migraine	10 7 83	0.41 (0.26) 0.31 (0.20) 0.41 (0.26)	0.413**	0.813	-
Rph	Yes No	27 72	0.41 (0.14) 0.39 (0.17)	0.442*	0.660	-
VDys	Yes No	28 69	0.39 (0.15) 0.39 (0.17)	-0.056*	0.955	-
Pupil size	< 4mm ≥ 4mm	40 49	0.38 (0.17) 0.41 (0.16)	-0.604*	0.547	-

Abbreviations. n: number of data, IQR: interquartile range, CUN-BAE: Clínica Universidad de Navarra - Body Adiposity Estimator. Non-parametric testing was used for the analysis of groups containing < 25 cases.[688] Missing cases: AMD FH (1), Rph (1), VDys (3), pupil size (11). * Independent samples t-test (2-tailed). ** Kruskal-Wallis test. *** Mann-Whitney U test. Size effect for Mann-Whitney U test: small = 0.1, medium = 0.3, large = 0.5.

Table A8 Association between pupil size and blue and brown iris colour

Variable		n	Age (years) median (IQR)	Pupil size (mm) median (IQR)	Statistic	P-value	Size effect
Pupil size (mm)	Blue irides Brown irides	30 21	48.0 (9.4) 49.3 (20.0)	4.0 (2.0) 3.0 (1.0)	U = 252.5 Z = -1.260*	0.208	-

Abbreviations. n: number of data, IQR: interquartile range. * Mann-Whitney U test. Pupil size data was missing for six cases.

Table A9 Association between iris colour (light and dark) and gender

Gender		Iris colour		Total
		Light	Dark	
Male	Count	14	13	27
	% within gender	51.9%	48.1%	100%
	% within light or dark irides	23.3%	32.5%	27%
	% of total	14%	13%	27%
Female	Count	46	27	73
	% within gender	63.0%	37.0%	100%
	% within light or dark irides	76.7%	67.5%	73%
	% of total	46%	27%	73%
Total	Count	60	40	100
	% within gender	60%	40%	100%
	% within light or dark irides	100%	100%	100%
	% of total	60%	40%	100%

0 cells have expected count < 5. Pearson Chi-square test with continuity correction = 0.611, p = 0.434.

Table A10 Association between iris colour (blue and brown) and gender

Gender		Iris colour		Total
		Blue	Brown	
Male	Count	8	11	19
	% within gender	42.1%	57.9%	100%
	% within light or dark irides	24.2%	45.8%	33.3%
	% of total	14	19.3%	33.3%
Female	Count	25	13	38
	% within gender	65.8%	34.2%	100%
	% within light or dark irides	75.8%	54.2%	66.7%
	% of total	43.9%	22.8%	66.7%
Total	Count	33	24	57
	% within gender	57.9%	42.1%	100%
	% within light or dark irides	100%	100%	100%
	% of total	57.9%	42.1%	100%

0 cells have expected count < 5. Pearson Chi-square test with continuity correction = 2.024, p = 0.155.

Table A11 Association between MPOD and fish oil supplement use

Variable		n	Age median (IQR) years	MPOD Median (IQR)	Statistic	P-value	Size effect
Reported fish oil supplement use	Yes	21	57.6 (18.9)	0.46 (0.24)	U = 689.0		
	No	79	46.5 (11.7)	0.38 (0.26)	Z = -1.193*	0.233	-

Abbreviations. n: number of data. * Mann-Whitney U test.

Table A12 Difficulty with HFP MPOD measurement with MPOD, age and GRT

Variable		n	MPOD Median (IQR)	Statistic	P-value	Size effect
Difficulty with MPOD measurement with MPOD	Yes	21	0.41 (0.25)	U = 823.5		
	No	79	0.41 (0.24)	Z = -0.051*	0.959	-
With age (years)	Yes	21	53.9 (25.5)	U = 726.0		
	No	79	47.9 (11.8)	Z = -0.876*	0.381	-
With GRT (s)	Yes	21	41 (28)	U = 694.0		
	No	79	40 (24)	Z = -1.147*	0.251	-

Abbreviations. n: number of data. * Mann-Whitney U test.

Table A13 Additional interesting findings from MPOD study

ID	Condition	Eye	MPOD	Possible explanation
3	Central floater LE	Right	0.46	Central target obscured
		Left	0.02	
145	Bilateral central floaters	Right	0.17	Central target obscured
		Left	0.12	
12	Strabismus RE	Right	0.02	Eccentric fixation
		Left	0.22	
83	Strabismus LE	Right	0.26	Eccentric fixation
		Left	0.07	
142	Poor fixation	Right	0.07	Unable to keep peripheral target fixated?
		Left	0.00	
22	Macular drusen RE	Right	0.17	Reduced visual function RE
		Left	0.22	
29	Macular drusen RE	Right	0.48	Visual function not affected?
		Left	0.48	
54	Macular drusen RE	Right	0.17	Reduced visual function RE
		Left	0.31	
105	Colitis	Right	0.07	Reduced carotenoid absorption?
		Left	0.17	
131	Coloboma RE	Right	0.07	Reduced visual function RE
		Left	0.22	

A1.2 Glare recovery time study results

Table A14 Tests for normality

	Kolmogorov-Smirnov (with correction)			Shapiro-Wilk		
Variable	Statistic	df	P-value	Statistic	df	P-value
First eye GRT1	0.146	100	**< 0.001**	0.820	100	**< 0.001**
Second eye GRT1	0.161	99	**< 0.001**	0.882	99	**< 0.001**
First eye GRT2	0.133	100	**< 0.001**	0.881	100	**< 0.001**
Second eye GRT2	0.138	99	**< 0.001**	0.882	99	**< 0.001**
First eye RepGRT1	0.205	30	**0.002**	0.702	30	**< 0.001**
Second eye RepGRT1	0.200	30	**0.003**	0.876	30	**0.002**
First eye RepGRT2	0.148	30	0.093	0.821	30	**< 0.001**
Second eye RepGRT2	0.217	30	**0.001**	0.802	30	**< 0.001**

Probability values (p-values) < 0.05 are shown in bold. Data was missing for one case for second eye GRT (ID 2). Glare recovery time data was positively skewed (> 1), therefore non-parametric tests were used for AMD and OVP RF analysis.

Table A15 Bivariate correlations for log10 GRT interocular comparisons

Variable	Pearson r	Shared variance	n (per group)	P-value (2-tailed)
First vs. second eye	0.756	57.2%	99	**< 0.001**
First vs. second eye*	0.848	71.9%	96	**< 0.001**
Dominant vs. non-dominant eye	0.833	69.4%	44	**< 0.001**

Abbreviations. n: number of data. Probability values (p-values) < 0.05 are shown in bold. *Three outliers removed from the GRT data (ID; 31, 58 and 117). Correlation size: small r = 0.10 to 0.29, medium r = 0.30 to 0.49, large r = 0.50 to 1.0.

Table A16 Group analysis for GRT interocular comparison

GRT	Number of data (n)	Age mean (SD) years	GRT median (IQR) s	Statistic	P-value	Size effect
1st Eye GRT 2nd Eye GRT	99 99	50.2 (10.4) 50.2 (10.4)	40 (25) 36 (24)	U = 4621.0 Z = -0.694**	0.488	-
1st Eye GRT* 2nd Eye GRT*	96 96	50.3 (10.3) 50.3 (10.3)	39.5 (25) 35.5 (22)	U = 4297.5 Z - -0.807**	0.420	-

Abbreviations. IQR: interquartile range. *Three outliers removed from the GRT data, **Mann-Whitney U test.

Table A17 Group analysis for GRT and ocular dominance

Ocular dominance	Number of data (n)	Age mean (SD) years	GRT median (IQR) s	Statistic	P-value	Size effect
Dominant eye Non-dominant eye	44 44	50.0 (11.4) 50.0 (11.4)	35.5 (26) 34.5 (26)	U = 932.0 Z = -0.301*	0.764	-

Abbreviations. IQR: interquartile range. *Mann-Whitney U test.

Table A18 Bivariate correlations for log10 GRT comparisons

Variable	No. of data n	Pearson r	Shared variance	P-value (2-tailed)
Age	100	0.329	10.8%	**0.001**
Age (≤ 50 years)	57	0.083	0.7%	0.541
Age (> 50 years)	43	0.275	7.6%	0.074
Total BMI	116	0.120	1.4%	0.199
Male BMI	32	-0.156	2.4%	0.395
Female BMI	84	0.178	3.4%	0.105
Male %BF	32	-0.060	0.4%	0.746
Female %BF	84	0.306	9.4%	**0.005**
Pupil size	89	0.029	0.1%	0.788

Correlation size: small r = 0.10 to 0.29, medium r = 0.30 to 0.49, large r = 0.50 to 1.0. Probability values (p-values) < 0.05 are shown in bold. For the correlation with BMI and %BF only, 16 additional cases excluded for high (n = 12) or low (n = 4) BMI were added to the data. Other than reported raised cholesterol, which is associated with obesity, these cases had no addition reasons for exclusion. Data was missing in 11 cases for pupil size.

Table A19 Partial correlations for log10 GRT corrected for age

Variable	Number of data (n)	Partial correlation	Shared variance	P-value (2-tailed)
Age (controlled for BMI (20 to < 30)	100	0.294	8.6%	**0.003**
Total BMI	116	0.017	< 0.1%	0.861
Male BMI	32	-0.213	4.5%	0.249
Female BMI	84	0.093	0.9%	0.404
Male %BF	32	-0.184	3.4%	0.321
Female %BF	84	0.114	1.3%	0.307
Pupil size	89	0.179	3.2%	0.096

Table A20 Group analysis for first eye GRT with AMD and OVP RF

Variable		n	Age mean (SD) or median (IQR) years	GRT median (IQR) s	Statistic	P-value p	Size effect (if significant)
Age 2 age groups (years)	≤ 50 > 50	57 43	43.0 (5.2) 60.1 (7.0)	35 (20) 45 (26)	U = 869 Z = -2.483*	**0.013**	0.25 (small)
Age 4 age groups (years)	< 45 ≥ 45 to < 50 ≥ 50 to < 60 ≥ 60	32 25 24 19	41.8 (4.4) 46.5 (2.6) 54.9 (4.5) 67.2 (6.4)	33 (21) 35 (21) 42 (27) 50 (44)	8.907**	**0.031**	-
Post-hoc tests Two follow-up Mann-Whitney U tests α level = 0.025	≥ 45 to < 50 ≥ 50 to < 60	25 24	46.5 (2.6) 54.9 (4.5)	35 (21) 42 (27)	U = 266.0 Z = -0.681*	0.496	-
	≥ 45 to < 50 ≥ 60	25 19	46.5 (2.6) 67.2 (6.4)	35 (21) 50 (44)	U = 141.0 Z = -2.288*	**0.022**	0.23 (small)
Gender	Male Female	27 73	55.2 (10.5) 48.5 (9.9)	41 (25) 39 (23)	U = 834.5 Z = -1.173*	0.241	-
BMI (both genders) (n = 116)	< 20 20 to < 25 25 to < 30 ≥ 30	4 52 48 12	43.5 (42.2) 45.6 (11.4) 51.9 (17) 60.7 (20.0)	30 (40) 35 (20) 43 (27) 34.5 (23)	4.870**	0.182	-
BMI (male) (n = 32)	< 20 20 to < 25 25 to < 30 ≥ 30	0 7 20 5	- 47.9 (23.8) 54.6 (18.5) 60.0 (16.3)	- 40 (34) 43 (25) 32 (17)	1.760**	0.415	-
BMI (female) (n = 84)	< 20 20 to < 25 25 to < 30 ≥ 30	4 45 28 7	43.5 (42.2) 45.4 (11.4) 48.8 (13.0) 60.3 (22.1)	30 (40) 35 (19) 43 (39) 35 (25)	4.460**	0.216	-
Male %BF (CUN-BAE) (n = 32)	≤ 20% > 20 to 25% > 25%	2 6 24	46.1 (-) 44.6 (4.2) 61.2 (16.2)	44.5 (-) 34.5 (14) 43 (25)	1.847**	0.397	-
Female %BF (CUN-BAE) (n = 84)	≤ 30% > 30 to 35% > 35%	7 25 52	40.1 (9.5) 45.1 (7.1) 51.2 (15.6)	31 (15) 35 (20) 41 (26)	7.484**	**0.024**	-
Post-hoc tests Two follow-up Mann-Whitney U tests α level = 0.025	≤ 30% > 35%	7 52	40.1 (9.5) 51.2 (15.6)	31 (15) 41 (26)	U = 83.0 Z = -2.322*	**0.020**	0.23 (small)
	> 30 to 35% > 35%	25 52	45.1 (7.1) 51.2 (15.6)	35 (20) 41 (26)	U = 489.5 Z = -1.747*	0.081	-
Iris colour Five colour groups Mixed-gender	Grey Blue Green Hazel Brown	12 33 15 16 24	50.7 (8.7) 47.9 (10.5) 44.9 (19) 46.9 (16.4) 51.6 (20.9)	38 (18) 38 (16) 47 (27) 37 (31) 37.5 (26)	2.506**	0.644	-
Iris colour*** Two colour groups	Light Dark	60 40	49.1 (9.4) 52.3 (11.6)	40.5 (21) 37 (26)	U = 1140.0 Z = -0.422*	0.673	-
AMD FH 2-group	Yes No	17 82	49.5 (18.3) 48.0 (12.8)	45 (26) 38.5 (23)	U = 540.5 Z = -1.453*	0.146	-
AMD FH 3-group	1st degree 2nd degree No	11 6 82	57.5 (13.5) 43.1 (5.9) 48.0 (12.8)	50 (39) 43.5 (23) 38.5 (23)	2.435**	0.296	-
Migraine	Yes No	17 83	45.3 (13.2) 48.5 (13.4)	33 (29) 41 (24)	U = 636.5 Z = -0.633*	0.526	-
Light-trigger	Yes No No migraine	6 11 83	44.7 (11.2) 45.4 (16.3) 48.5 (13.4)	33 (24) 33 (35) 41 (24)	0.520**	0.771	-
Aura	Yes No No migraine	10 7 83	46.0 (13.6) 45.0 (9.3) 48.5 (13.4)	32 (18) 51 (29) 41 (24)	3.003**	0.223	-
Rph	Yes No	27 72	54.0 (9.6) 49.1 (10.5)	41 (26) 40 (22)	U = 9.595 Z = -0.098*	0.922	-
VDys	Yes No	28 69	50.8 (9.2) 50.4 (11.1)	40.5 (26) 41 (25)	U = 958.5 Z = -0.060*	0.952	-
MPOD	≤ 0.39 > 0.39	48 52	48.7 (10.9) 51.9 (9.8)	38.5 (23) 42.5 (28)	U = 1085.5 Z = -1.122*	0.262	-
Pupil size	< 4mm ≥ 4mm	40 49	53.9 (10.0) 48.0 (9.2)	40.5 (28) 40 (25)	U = 922.5 Z = -0.474	0.635	-

Abbreviations. n: number of data, IQR: interquartile range. *Mann-Whitney U test, **Kruskal-Wallis test, ***Iris colour divided into light (grey, blue and green irides) and dark (hazel and brown irides).

Table A21 Association between GRT and fish oil supplement use

Variable		n	Age median (IQR) years	GRT Median (IQR) s	Statistic	P-value	Size effect
Reported fish oil supplement use	Yes No	21 79	57.6 (18.9) 46.5 (11.7)	43.0 (25) 39.0 (23)	U = 672.5 Z = -1.329*	0.184	-

Abbreviations. IQR: interquartile range. *Mann-Whitney U test.

Table A22 Bivariate correlation between log10 GRT and MPOD

Variable	Number of data (n)	Pearson r	Shared variance	P-value (2-tailed)
MPOD (full age range)	100	0.218	4.8%	**0.029**
MPOD (≤ 50 years)	57	0.236	5.6%	0.078
MPOD (> 50 years)	43	0.117	1.4%	0.455
MPOD (≤ 0.39)	48	-0.064	0.4%	0.666
MPOD (> 0.39)	52	0.310	9.6%	**0.025**

Table A23 Partial correlation between log10 GRT and MPOD corrected for age

Variable	Number of data (n)	Partial correlation	Shared variance	P-value (2-tailed)
MPOD (full age range)	100	0.183	3.3%	0.071
MPOD (≤ 50 years)	57	0.221	4.9%	0.102
MPOD (> 50 years)	43	0.170	2.9%	0.283
MPOD (≤ 0.39)	48	-0.069	0.5%	0.645
MPOD (> 0.39)	52	0.309	9.5%	**0.027**

Table A24 Group analysis for GRT intra- and inter-session repeatability

GRT Intra-session	Number of data (n)	GRT median (IQR) s	Statistic	P-value p	Size effect (if significant)
E1 GRT1 vs. E1 GRT2	100 100	45 (27) 40 (25)	Z = -5.573*	**< 0.001**	0.72 (large)
E2 GRT1 vs. E2 GRT2	99 99	40 (27) 36 (24)	Z = -5.901*	**< 0.001**	0.76 (large)
Rep. E1 GRT1 vs. Rep. E1 GRT2	30 30	37 (25) 41.5 (26)	Z = -0.381*	0.703	-
Rep. E2 GRT1 vs. Rep. E2 GRT2	30 30	36.5 (26) 40.5 (20)	Z = -1.052*	0.293	-
Inter-session					
E1 GRT1 vs. Rep. E1 GRT1	30 30	48 (28) 37 (25)	Z = -1.549*	0.121	-
E2 GRT1 vs. Rep. E2 GRT1	30 30	40 (19) 36.5 (26)	Z = -0.473*	0.636	-
E1 GRT2 vs. Rep. E1 GRT2	30 30	37.5 (16) 41.5 (26)	Z = -0.536*	0.592	-
E2 GRT2 vs. Rep. E2 GRT2	30 30	33.5 (20) 40.5 (20)	Z = -2.608*	**0.009**	0.34 (medium)

Abbreviations. IQR: interquartile range. *Wilcoxon signed rank test. Size effect: 0.1 (small), 0.3 (medium), 0.5 (large). Data was missing for one case for the second eye data in the first session.

Table A25 Correlation for intra- and inter-session repeatability

Variable Intra-session	Number of data (n)	Pearson r	Shared variance	P-value (2-tailed)
Log10 Rep. E1 GRT1	30			
Log10 Rep. E1 GRT2	30	0.89	79.2%	**< 0.001**
Inter-session				
Log10 E1 GRT2	30			
Log10 Rep. E1 GRT2	30	0.41	16.8%	**0.024**
Inter-session (repeated within 5-weeks only)				
Log10 E1 GRT2	18			
Log10 Rep. E1 GRT2	18	0.58	33.4%	**0.011**

Table A26 Bias on repeat measures of GRT using same vs. different test chart letters

Variable	n	Age mean (SD) years	GRT mean (SD) s	Statistic	P-value	Size effect
Same letters (1st eye rep. GRT1-GRT2) Different letters (2nd eye rep. GRT1-GRT2)	30 30	49.1 (8.5) 49.1 (8.5)	1.7 (12.0) -2.6 (9.4)	1.567*	0.123	-

Abbreviations. n: number of data. *independent-samples t-test.

Appendix 2

A2.1 Summary of the retina / RPE / choroid antioxidant system

Antioxidant Enzyme	Main target	Location	Co-factor	Recycled by
CuZn-SOD [228]	O_2^-	All tissue [236]	Cu, Zn Melatonin [1038] CoQ10 [1039]	-
Mn-SOD [228]	O_2^-	RPE mt [236]	Mn	-
GPx [228, 1040]	Lipid hydroperoxides	All tissue [228]	CoQ10 [1039] Se [1041]	-
GR [1040]	GSSG recycling	All tissue [1040]	-	-
CAT [228]	H_2O_2	All tissue [228]	Fe [228] CoQ10 [1039]	-
MsrA [1042]	Free radical scavenging Neuroprotection	All tissue [1042]	α-crystallins [1042]	-
GSTP1 [1043, 1044]	Oxidative stress Nitric oxide scavenging	All tissue, abundant in foveal IPL / OPL & PR IS ER [1044]	Flavonoids [1043]	-
(HO)-1 [1043, 1045]	Oxidative stress ⇓ Ischaemia/reperfusion	RPE [1045, 1046]	Flavonoids [1043] Anthocyanin Astaxanthin [1047]	-
NQO1 [1043]	Oxidative stress	All tissue [1048]	Flavonoids Astaxanthin [1047]	-
Non-enzyme				
GSH [1040]	Oxidative stress Lipid peroxidation	PR OS [228] All tissue [1040]	Melatonin [1038] GST pi [1049] ALA [1050]	GR [1040] ALA [1051]
Melanin [5, 238, 1052]	Free radical scavenging Weak antioxidant Lipid peroxidation Bind cations (e.g. FE, Cu, Zn) Visible and infra-red light absorption	RPE / choroid [5]	Fe [236] Zn [1053]	Vit C [238] Photo-degradation reduces antioxidant capability [238]
Metallothionein [228, 232, 1054, 1055]	Free radical scavenging Bind cations (e.g. Fe, Zn, Cu, Cd) Neuroprotection Oxidative stress	All tissues [1056]	Zn [1057]	Se catalyst [232]
Dopamine [188-191]	Antioxidant Neuroprotectact Protect PR from light damage Excess dopamine may cause lipid peroxidation	All tissues?	-	Light triggers dopamine release. Dopamine and melatonin are mutually antagonistic [192]
Melatonin [1038, 1058, 1059]	Lipid peroxidation Free radical scavenging	All tissues [1060]	-	Light blocks melatonin release. Oxidation may be irreversible [239]
TRX1 [1061, 1062]	Oxidative stress Cellular redox	All tissues [1062]	CFH [1062]	TRXR1 [1062]
Oestrogen [302]	Antioxidant Anti-inflammatory Regulates AMD signalling pathways	All tissues [302]	-	Oestrogen receptors ERα & ERβ bind with and inhibit NF-κB signalling, reducing inflammatory IL-6 production [302]
Dietary				
Vitamin A [228] (Retinol, plus pro-vitamin A)	Lipid peroxidation Oxidative stress	PR OS [1063] All tissue [228]	Vit E [1064]	Retinol dehydrogenase [512]
Vitamin C [1065, 1066] (Ascorbate)	Free radical scavenging Ultra-violet (UV) light absorption in the cornea and anterior chamber	All tissue [1066]	Almond skin Flavonoids [1067]	ALA [1051] GSH [1068]
Vitamin E [228] (Tocopherols and tocotrienols)	Free radical scavenging Lipid peroxidation	All tissue [228, 1069]	Se [1063] Almond skin Flavonoids [1067]	ALA [1051] Vit C [1070] GSH [1070] CoQ10 [1071]
L, Z and MZ	Free radical scavenging	PR OS [397, 399]	Vit E [1073]	Vit C [1074]

MP [250, 475, 482, 892, 1072]	Singlet O_2 quenching Lipid peroxidation Neuroprotection ⇓ Ischaemia/reperfusion Blue light absorption	HFL [395] PF IPL & OPL [395] Mc [400, 443]		
Flavonoids [1067, 1075]	Oxidative stress Free radical scavenging Inhibit nitric oxide Inhibit O2- ⇑ enzymes Chelate trace elements Boost ocular blood flow ⇓ Ischaemia/reperfusion Neuroprotection	All tissue [1075] May deplete from tissue rapidly	-	Replenished from dietary sources
ALA / DHLA [1050, 1051] (Thioctic acid)	Oxidative stress Free radical scavenging Bind cations	All tissue [1051]	NAD(P)H [1076]	Self-recycling?
CoQ10 [1071, 1077] (Ubiquinone)	Oxidative stress ROS scavenging	All tissue [1071]	-	ALA [1078]
Taurine [1079]	Oxidative stress Neuroprotection ⇓ Glutamate toxicity ⇓ Inflammation	All tissues [1080, 1081]	Fe [1082] Vit B6 [1083]	Also synthesised from methionine with CDO [1081, 1083]

SOD: superoxide dismutase, GPx: glutathione peroxidase, GR: glutathione reductase, CAT: catalase, MsrA: methionine sulfoxide reductase, GSTP1: glutathione S-transferase pi isoform, (HO)-1: heme oxygenase, NQO1: NAD(P)H:quinone oxidoreductase 1, NAD(P)H: nicotinamide adenine dinucleotide phosphate, TRX1: thioredoxin, NF-κB: nuclear factor kappa B, IL-6: interleukin-6, GSH: glutathione, (Bio) Flavonoids: polyphenolic molecules derived from the outer surfaces of grapes, berries, tealeaves and some barks. Ocular function depends on the flavonoid examined, L: lutein, Z: zeaxanthin, MZ: meso-zeaxanthin, ALA: α-lipoic acid, DHLA: dihydrolipoic acid (reduced form of ALA), CoQ10: co-enzyme Q10. O_2^-: Superoxide anion, GSSG: Glutathione disulphide (oxidised form of glutathione), H_2O_2: hydrogen peroxide, ROS: reactive oxygen species. IPL: inner plexiform layer, OPL: outer plexiform layer, PR IS ER: photoreceptor inner segment ellipsoid region, PR OS: photoreceptor outer segments, HFL: Henle fibre layer, PF IPL: parafoveal inner plexiform layer. RPE mt: RPE mitochondria. Cu: copper, Zn: zinc, Mn: manganese, Se: selenium, Fe: iron, Cd: cadmium, TRX1: thioredoxin reductase, CDO: cysteine dioxygenase.

A2.2 Müller cell functions in addition to photopigment recycling

Physiological Process	Müller Cell Function Protein / Peptide	Notes
Metabolic support and nutrition of neurons	Delivery of lactate / pyruvate lactate dehydrogenase	Müller cells supply retinal cells with nutrients required for their oxidative metabolism. In murine models Mc destruction causes retinal dysplasia, photoreceptor apoptosis and eventually retinal degeneration and RPE proliferation.[1084]
	Storage of glycogen and glycogenolysis glycogen phosphorylase	Müller cells are strikingly resistant to ischaemia, hypoxia and hypoglycemia. Short periods of glucose deficiency and ischaemia may be compensated by glycogen deposits stored in Mc.[1085]
Water, potassium (K^+) and carbon dioxide (CO_2) homeostasis	Dehydration of the inner retina AQP-4 channel	Water accumulates in the retina as a by-product of the oxidative synthesis of ATP, from retinal blood vessels and as a result of intraocular pressure. Excess water is removed from Mc via AQP-4 channels to the retinal blood vessels. The subretinal space is dehydrated by the RPE (AQP-1).[294]
	Transcellular spatial buffering of K^+ currents Kir4.1 channel	Müller cells buffer extracellular levels of K^+ resulting from neuronal activity, controlling bi-directional movement of these ions through Kir-4.1 channels in the Mc plasma membrane.[294] Blue light exposure leads to a reduction in Kir4.1 protein in the whole retina and reduced K^+ conductance in rat Mc.[278] Dysregulation of K^+ homeostasis causes neuronal hyper-excitability and glutamate toxicity. Human data indicated that Kir currents were significantly lower in Mc extracted *post mortem* from subjects aged over 50 years compared to younger subjects, whereas calcium currents were approximately five times higher in older subjects over 55 years of age.[1086] Co-localisation of Kir4.1 and AQP-4 channels in the Mc plasma membrane may explain the retinal oedema associated with pathological alteration in Kir4.1 expression (e.g. inflammation).[1015]
	Carbon dioxide buffering carbonic anhydrase	Retinal neurons (especially photoreceptors in the dark) release high levels of CO_2, leading to a rapid acidification of Mc. Acidification, if not buffered might lead to modulation of glutamate uptake, acid-base transport and gap-junction coupling by Mc.[1087]
Neurotransmitter recycling	Glutamate GLAST	Glutamate is continuously released by photoreceptor terminals in the dark, but suppressed by light. ON-bipolar cells release glutamate in the light, whereas OFF-bipolars release glutamate in darkness.[276] Clearance of synaptic glutamate by Mc is necessary for normal functioning of excitatory synapses and to prevent neurotoxicity.[1088]
	GABA GAT-3	Gamma-aminobutyric acid is the main inhibitory neurotransmitter in the vertebrate retina. In the outer retina of mammals, GABA uptake is almost exclusively performed by Mc, whereas Mc and amacrine cells are responsible for GABA uptake in the inner retina.[276]
	Glutamine	Glutamate transported in to Mc is amidated to glutamine by the

	SN 1, SN 2	enzyme glutamine synthetase. Glutamine is released from Mc and taken up by neurons where it is used to synthesise glutamate and GABA.[276] If glutamate synthetase is pharmacologically blocked in Mc, bipolar and ganglion cells become deficient of free glutamate and functional blindness results within two minutes.[1088]
Release of other neuroactive substances	Storage and release of D-serine ATP	These neuroactive substances are stored or released depending on momentary neuronal activity and / or metabolic state. D-serine and glutamate control the excitability of neurons and are involved in photopigment recycling as they express CRALBP. Adenosine triphosphate transports chemical energy within cells for metabolism.[294]
Formation and maintenance of the blood-retina barrier	Müller cells support inner blood-retinal barrier integrity VEGF PEDF TGF-β Matrix metalloproteinases	Under normoxic conditions Mc secrete factors that decrease barrier function such as PEDF, which down-regulates expression of VEGF. Retinal hypoxia, inflammation and glucose-deprivation are associated with increased VEGF secretion from Mc, leading to increased vascular permeability. Müller cells also produce matrix metalloproteinases which impair barrier function of retinal endothelial cells.[1015] Ischaemia / reperfusion causes increased glutamate release from neurons and Mc activation leading to reduced K^+ buffering and glutamate uptake. Excess extracellular glutamate causes retinal cell damage. Müller cells are resistant to injury resulting from insufficient vascular perfusion, unlike other retinal neurons, especially photoreceptors.[294] Impairment of Mc glutamate metabolism (down-regulation of glutamine synthetase) was found to reduce the integrity of the blood-retina barrier.[276]
Regulation of retinal blood flow	Müller cells mediate neurovascular coupling Purinergic, P2Y receptors	Retinal glia, including Mc respond to neuronal activity by modulating retinal blood flow.[1089] Retinal glia communicate with their neighbours via increases in intracellular calcium in the form of a calcium wave propagated through gap junctions or by releasing ATP.[1090] In rat Mc, steady light causes the generation of calcium waves, the frequency of which is increased by flickering light.[1091] Calcium responses in Mc are mediated by metabotropic purinergic receptors and are triggered by ATP released from amacrine and ganglion cells. Vessel pericyte constriction and relaxation are stimulated by the release of ATP and adenosine, respectively.[1092] Studies conducted on pigeon and rat retinae reported that Mc become activated (indicated by increased levels of GFAP) when choroidal blood flow is altered or reduced.[1093, 1094]
Protection against oxidative stress	Free radical scavenging GSH	Müller cells synthesise GSH from glutamate, cysteine and glycine.[885] Glutathione levels in Mc decrease dramatically during hypoxia and hypoglycemia. Reduced GSH due to ischaemia may increase intraretinal oxygen free radicals. Glutathione levels in Mc were significantly lower with age, in guinea pigs,[1095] and under pathological conditions including retinal light injury, ischaemia and inflammation.[276] Extracellular cystine used to synthesise glutathione is transported in to Mc via the cystine-glutamate antiporter, which transports glutamine out of Mc in to the extracellular space. Excessive extracellular glutamate secondary to oxidative stress may inhibit the cystine-glutamate antiporter and lead to lower levels of GSH.[276]
Neuroprotection	Secretion of various factors involved in neuroprotection BDNF, CNTF, bFGF, IGF-1, NGF, neurotrophins-3 and -4, GDNF, LIF, PEDF, Bcl-2	Under pathological conditions Mc are able to protect photoreceptors and retinal neurons from cell death by the secretion of various factors; neurotrophic and growth factors, and cytokines. Retinal light injury is associated with up-regulation of the following factors; CNTF, bFGF, NGF, neurotrophin-3 and Bcl-2.[270]
	Phagocytosis of potentially harmful substances and particles (endogenous and exogenous)	Müller cells phagocytose debris from dead neurons and pigment from the RPE, foreign material such as copper particles and latex beads.[294]
Inflammation	Recruitment of inflammatory cells Ccl2 chemokine	Expression of Ccl2 by Mc promotes the infiltration of monocytes / microglia, contributing to the neuroinflammatory response and photoreceptor death following retinal light damage in the rat.[1096] Treatment with L was observed to inhibit up-regulation of GFAP (reduce Mc gliosis) and minimise deterioration of b-wave / a-wave ratio and oscillatory potentials in a murine model of retinal ischaemia / reperfusion injury, suggesting an anti-inflammatory role for L.[892]
Cell membrane integrity	Delivery of DHA to photoreceptors	Müller cells incorporate DHA into phospholipids which are channeled to photoreceptors.[1097] Müller cell membrane lipids, like those of photoreceptor OS exhibit an age-related susceptibility to lipid peroxidation secondary to oxidative stress.[280]
Circadian protection of photoreceptors	Neuroprotectants bFGF adenosine Antioxidants vitamin C	Photoreceptors use 3-4 times more oxygen when light-adapted and 6-8 times more oxygen when dark-adapted than other neurons in the central nervous system. Oxygen is supplied to photoreceptors via the choriocapillaris, which is not significantly regulated in response to oxygen requirement. The decrease in oxygen consumption from dark- to light-adapted states leads to an increase in retinal oxygen

	vitamin E glutathione	tension during day light hours. Photoreceptors are able survive circadian periods of hyperoxia because of retinal neuroprotective and antioxidant agents produced by Mc (e.g. bFGF and glutathione).[270]
Ammonia metabolism	Neutralisation of excess ammonia Glutamine synthetase	Glutamine synthetase found in Mc is the only enzyme available in the retina for ammonia detoxification. Glutamine synthetase activity is regulated by the availability of glutamate and ammonia and is up-regulated by pathology associated with raised ammonia levels (e.g. liver failure). Chronic exposure to high ammonia concentration may cause metabolic overload of Mc leading to retinal damage (hepatic retinopathy).[294]
Ionotropic receptors	Extracellular pH regulation $GABA_A$ receptor	Human Müller cells contain $GABA_A$ receptors (chloride channels). Stimulation with GABA evokes a fast, transient and a sustained current, both inward, leading to Mc depolarisation. These receptors are also permeable to bicarbonate, therefore they may be involved in extracellular pH regulation.[1092]
	Removal of extracellular calcium $P2X_7$ purinergic receptor	In human Müller cells extracellular ATP leads to the opening of $P2X_7$ receptors (calcium channels) causing calcium influx in to Mc, calcium release from intracellular stores and activation of big potassium (BK) channels, leading to cell depolarisation. Depolarisation is counter regulated by hyperpolarisation due to potassium influx through BK channels, which in turn increases the driving force for calcium influx. The light-induced decrease in extracellular calcium will down-regulate $P2X_7$ receptors.[1092]
Protection against apoptosis	Release of factors ApoE α2-macroglobulin	Müller cells protect photoreceptors and other retinal neurons from apoptosis by the release of ApoE and α2-macroglobulin.[270]
De-differentiation to pluripotent retinal progenitor / stem cells	Response to pathological stimuli	In response to pathological stimuli Mc are able to de-differentiate to cells exhibiting properties of pluripotent retinal progenitor or stem cells; proliferation, migration and transdifferentiation (to neurons and photoreceptors). De-differentiation of Mc adversely affects many normal functions including; glycosis, glutamate synthetase recycling, carbon dioxide siphoning, visual pigment recycling, potassium siphoning and water clearance. This contributes to inner retinal oedema, neuronal hyper-excitability and glutamate toxicity.[270]
Retinal Development	Scaffold for retinal cell orientation	From early stages of development immature Mc are important for the histotypic organisation of the developing retina. They provide orientation support for young neurons and their neurites. Reactive Mc in humans with AMD extend processes through gaps in Bruch's membrane, along which retinal neurons migrate out of the retina and into the choroid.[294]
Mechanoresponsivity	Müller cells respond to mechanical stress ERK c-Fos bFGF	Fifteen minutes after stretch, Mc showed activation of ERK. Subscription factor c-Fos and bFGF were upgegulated after one and three hours, respectively. Vimentin and GFAP levels remained unchanged three hours after stretch.[1098]
Storage site for macular xanthophylls?	Müller cells may store L and Z	Gass suggested that Mc may act as a reservoir for L and Z.[400] Macular hole is often accompanied by ERM containing MP.[443] Lutein and Z are used to stain the ILM (Mc end feet) during surgery.[992, 993] Müller cells were also reported to concentrate canthaxanthin, a xanthophyll structurally similar to Z.[1099]
Image transfer from inner to outer retina	Müller cells act as optical fibres and spectral filters	Müller cells are radially orientated, span the entire retinal thickness, have an extended funnel shape and a higher refractive index than surrounding tissue. Franze *et al*. demonstrated that guinea pig Mc can act as minimal distortion, low-loss optical fibres, transmitting an image through the retina.[1016] Labin *et al*. reported that guinea pig Mc spectrally filter and concentrate green and red light onto cones, whereas blue and purple light is leaked onto nearby rods.[1017]

AQP-4: aquaporin-4, ATP: adenosine 5'-triphosphate, AQP-1: aquaporin-1, Kir4.1: inwardly-rectifying potassium 4.1, GLAST: glutamate / aspartate transporter, GABA: Gamma-aminobutyric acid, GAT-3: GABA transporter subtype 3, SN 1: system N glutamine transporter 1 (aka SNAT3), SN 2: system N glutamine transporter 2 (aka SNAT5), CRALBP: cellular retinaldehyde-binding protein, BDNF: brain-derived neurotrophic factor, CNTF: ciliary neurotrophic factor, bFGF: basic fibroblast growth factor, IGF-1: insulin-like growth factor 1, NGF: nerve growth factor, GDNF: glial cell line-derived neurotrophic factor, LIF: leukemia inhibitory factor, Bcl-2: B cell lymphoma oncogene protein-2, Ccl2: C-C motif ligand 2. ERK: extracellular signal-related kinase, c-Fos: subscription factor c-Fos, Adapted from Bringmann *et al*. (2006) with permission.

A2.3 Summary of the current macular pigment hypotheses

MP Theory	Reference	Summary of hypothesis
Protection hypothesis	Kirschfeld (1982)[1100] Snodderly *et al*. (1984a)[989] Snodderly (1995)[1101] Tate *et al*. (1995)[226] Sedden *et al.* (2005)[782] Kim et al. (2008)[1102] Youssef *et al*. (2011)[250] Mares *et al*. (2011)[1103] Chew *et al*. (2013)[452]	Based on the assumption that oxidative damage is a significant factor in the pathogenesis of retinal disease. Macular pigment protects the retina from actinic blue light damage,[250] with a retinal distribution corresponding to the pattern of intraretinal damage caused by argon laser blue (488 nm) photocoagulation damage of the macula.[989] Macular pigment also acts as an antioxidant, scavenging and quenching free radicals produced by endogenously-triggered (e.g. phagocytosis)[226] and exogenously-triggered (e.g. blue light stimulated release of ROS from fluorophores such as A2E)[1102] processes. Recently AREDS2 reported that the risk of progression to advanced AMD was reduced by 18% for participants taking AREDS supplements plus L and Z compared to those taking AREDS supplements plus beta carotene. Dietary analysis also revealed 25% lower risk of progression to advanced AMD for those with a low L and Z diet before the study, taking L and Z supplements.[452] There is no evidence that L and Z supplementation lowers the risk of AMD development. Opponents of this theory have argued that AMD is primarily a genetic disease, explaining up to 71% of the variance.[782] Despite this, lifestyle alterations including healthy diet, not smoking and exercise were associated with a 71% lower risk of developing AMD.[1103]
Acuity hypothesis	Schultze (1866)[1104] Wooten *et al*. (2002)[711] Olmedilla *et al*. (2003)[1014] Richer *et al*. (2004)[936] Engles *et al*. (2007)[657] Loughman *et al*. (2010)[906]	Schultze theorised that MP might improve VA by reducing short-wavelength light prior to absorption by the photoreceptors.[711] Approximately 75% of MP is optimally located in the inner retina close to where short-wavelength light is focused (i.e. anterior to the photoreceptors). Engles *et al.* measured gap acuity and vernier acuity on white (included short-wavelength light) and yellow (excluded short-wavelength light) backgrounds. Macular pigment optical density did not correlate with either acuity measurement or either background. Their data did not support the acuity hypothesis.[657] Two double-masked, placebo-controlled studies reported a significant improvement in VA after supplementation with L alone or combined with antioxidants / nutrients.[936, 1014] Loughman *et al.* reported a significant association between VA and MPOD at all eccentricities measured (0.25, 0.5, 1, 1.75 and 3°).[906] Although these results are not in agreement with those of Engles *et al*., it is possible that MP improves VA via a mechanism unrelated to the reduction of chromatic aberration.
Glare hypothesis	Stringham *et al*. (2007)[620] Ham *et al*. (1976)[1105] Stringham *et al*. (2003)[567] Wenzel *et al*. (2006)[1106] Stringham *et al*. (2008)[621] Snodderly *et al*. (2010)[838] Stringham *et al*. (2011)[622] Hammond & Elliott (2013)[482]	Stringham and Hammond proposed the glare hypothesis in 2007. MP was strongly related to improvements in disability glare and GRT, and blue light filtering was reported to be the primary mechanism by which MP improved both outcomes (figure 1.9).[620-622] Low MP levels in subjects with AMD was offered as part of the explanation for their prolonged GRT.[482] There is also evidence that higher levels of MP may be associated with reduced photophobia.[567, 838] The photophobia response is greater for blue light compared to green or red light,[1106] The action spectrum for photophobia after correction for MP and ocular media absorption, was found to approximate the threshold for retinal damage in Rhesus monkeys,[1105] and the action spectrum for lipofuscin photoreactivity.[482] Stringham and Hammond commented that it is unlikely that the mechanisms governing MP deposition evolved to protect the retina from actinic damage (i.e. protection hypothesis), as most damage would occur after the reproductive period. Factors affecting visual performance, however, affect survivability and therefore may be selected in the next generation.[620]
Visibility hypothesis	Wooten *et al*. (2002)[711] Bartlett *et al*. (2010)[1107] Hammond *et al*. (2012)[1108] Hammond & Elliott (2013)[482]	Proposed by Wooten and Hammond in 2002. An object located far in the distance may be obscured by wavelength-dependent scattering of light in the atmosphere, making the object appear less visible against its surroundings. Atmospheric scattering is greatest for blue light. This theory suggests that MP will screen blue-dominant atmospheric scatter leading to greater visibility.[482, 711] Wooten and Hammond calculated that the visual range (furthest distance at which the target can be seen) may be 30% greater for those with very high (1.0) compared to those with very low (0.0) MPOD levels.[711] Simulating an increase in MPOD of 0.50, using an oil-based carotenoid solution placed in front of the eye, resulted in an average contrast threshold improvement of 25%.[1108] The real-world improvement in visibility is likely to be less significant, as the increase in MPOD after supplementation is usually ≤ 0.1 for healthy and diseased eyes.[1107] It was calculated that an increase in MPOD of this size would lead to a 5% increase in visual range.[711]
Contrast enhancement	Walls *et al*. (1933)[1109] Wolffsohn *et al*. (2000)[1110] Hammond & Elliott (2013)[482] Hammond *et al*. (2013)[623]	Walls and Judd proposed that yellow filters enhance contrast in 1933.[1109] The visibility of a yellow target on a blue background was improved by observation through a yellow lens.[1110] Retinex (retina and cortex) theory of colour vision suggests that any improvement in edge contrast will improve edge detection by the combination of lateral inhibition in the retina and simple cells in the visual cortex.[482] MPOD was reported to correlate positively and significantly with chromatic

		contrast. Pearson Product-Moment correlations were significant ($p < 0.001$) at all eccentricities examined; 0.25, 0.5, 1, and 2°.[623]
Mesopic acuity	Kvansakul *et al*. (2006)[1111] Anstis (2002)[1112] Pérez *et al*. (2003)[1113] Hwang *et al*. (2013)[1114]	Kvansakul *et al*. proposed that filtering of blue light by MP might selectively reduce rod signals under mesopic conditions, improving visual performance by increasing the relative contribution from cones.[1111] Mesopic vision describes the range of light intensity (10 cd m^{-2} to 0.001 cd m^{-2}) over which both rod and cone photoreceptors contribute to vision.[1114] The transition from cone (photopic) to rod (scotopic) mediated vision is associated with a Purkinje shift in peak sensitivity from 555 nm (yellow-green) to 505 nm (blue green).[1112] The use of a yellow filter improved brightness and contrast perception under mesopic conditions, in healthy subjects.[1113] Six months supplementation with L (10 mg) significantly improved high mesopic (1 cd m^{-2} background) contrast acuity thresholds. However, no significant association was observed between mesopic contrast acuity and MPOD.[1111] Nolan et al. reported no significant improvement in mesopic contrast sensitivity after 12 months supplementation with L (12 mg) and Z (1 mg).[968]
Neural efficiency hypothesis	Renzi *et al*. (2010)[843] Renzi *et al*. (2014)[1012] Stahl and Sies (2001)[1115] Johnson *et al*. (2008)[1007] Johnson (2012)[1006] Vishwanathan (2013)[430]	Macular pigment improves neural efficiency in three ways: reducing random neural signals unrelated to sensory stimuli (neural noise), improving processing speed and reducing the cortical area required for a cognitive task. Renzi *et al*. suggested a possible mechanism whereby carotenoids enhance gap junction communication between glia and neurons,[843] however the study cited (Stahl and Sies, 2001),[1115] investigated several non-vitamin A carotenoids listed in the footnotes to this table, but not L and Z. Retinal levels of L and Z were significantly correlated with levels in the cerebellum, pons, frontal cortex and occipital cortex.[430, 1006] Lutein alone, or in combination with DHA was related to improved cognitive function in the elderly, although confirmation of a causal relationship requires longitudinal studies.[1006, 1007]
Compensation hypothesis	Werner *et al*. (2000)[506] Werner *et al*. (1993)[1116] Beirne (2013)[899]	Proposed by Werner *et al*. in 2000.[506] There is little age-related change in the appearance of an achromatic stimulus, despite a reduction in short-wavelength light incident on the retina due to changes in the ocular media.[1116] The underlying neural mechanism is considered to involve a type of multiplicative scaling of receptor sensitivity, in proportion to long-term quantal catch (i.e. the light absorbed by the three cone types).[506] Thresholds for S-, M- and L-cone mechanisms increase linearly in the central retina with age. The sensitivity difference between 0° and 8° retinal eccentricity for the S-cone mechanism, but not the M- and L-cone mechanisms, was significantly related to peak MPOD, but was unrelated to observer age.[506] The results are consistent with the hypothesis that long-term changes in S-cones or their post-receptoral pathways, compensate for the loss of stimulation due to the presence of MP at the fovea (compensation hypothesis). Beirne reported no significant relation between MPOD and the rate of change of foveal vs. extrafoveal (12° eccentricity) acuity with increasing age.[899] The lack of age dependency in these studies does not support the (protection) hypothesis that higher MPOD protects S-cone mediated visual function in the aging eye.

In view of the strong correlation between carotenoid consumption and a lower risk of several types of cancer, Stahl and Sies investigated the effects of various carotenoids on gap junctional communication on the human Caucasian foetal foreskin fibroblast (HFFF2) cell line. Gap junctional communication significantly increased when HFFF2 cells were exposed to; 0.1 µM retinoic acid, 0.1 µM lycopene, 1.0 µM or 50 µM acyclo-retinoic acid, 1.0 µM 4-oxo-retinoic acid. Three apo-cleavage products of canthaxanthin and 0.1 µM acyclo-retinoic acids did not produce significant results. The data indicated that for retinoic acid, the presence of four conjugated double bonds in the side chain provided optimal activity. Increasing the number of double bonds decreased activity, whereas a lower number of double bonds lead to inactive compounds.[1115] The structural difference between retinoic acid and lycopene suggests that their stimulatory effects are unrelated. Lycopene and canthaxanthin have 11 and 13 conjugated double bonds respectively. Zeaxanthin and MZ also have 11 conjugated double bonds, whereas L only has 10. This suggests that Z and MZ may have a greater effect on gap junctional communication than L, although this remains to be demonstrated.

A2.4 Factors affecting *in vivo* MPOD measurements

Factor	Effect of age	Effect on MPOD	Explanation
Optical			
Media opacities (central)	Increase	Lower	Inverse relationship between MPOD and lens density.[1117] Spatial media changes are not uniform and individual variation in lens homogeneity increases with age.[689]
Media opacities (para-central)	Increase	Higher?	
Cataract extraction	Increase	None	No difference in MPOD was reported before and between one and eight weeks after cataract surgery.[698, 700]
Clear vs blue-blocking IOL	-	Equivocal	MPOD was significantly higher one year after cataract surgery for subjects with UV and blue-blocking IOL, in the absence of any serum changes in L and Z. MPOD for subjects with IOL that blocked UV only remained unchanged.[698] A reduction in MPOD six months to two years after cataract surgery was found to be greater for UV-blocking compared to UV and blue-blocking IOL.[699]
UV-blocking C/L	-	Higher	Wearing UV-blocking (no blue light absorption) contact lenses for five years was associated with a significant increase in MPOD compared to contact lenses with no UV-blocking.[1118]
Pupil size	Decrease	Lower?	Pupil size decreases linearly with age and is smaller with increasing age regardless of luminance level.[932] Pupil size is subject to a high degree of intersubject variation.[932] MPOD results for a Maxwellian set-up and a free-viewing set-up were highly correlated.[733] The present study found a small, significant, positive correlation between MPOD and pupil size after correction for age. Stringham et al. reported a trend towards a positive correlation.[622]
Biometric			
Gender	None	Equivocal (lower for females?)	The association between MPOD and gender is controversial. Approximately two thirds of studies examining HFP MPOD and gender reported lower MPOD,[432, 508, 597, 599, 693, 732] or a trend towards lower MPOD,[486, 591, 601, 705, 733, 734] for females. Gender differences in MPOD associate with age and adiposity,[164, 424, 435, 486] and differences in MP supplement use and MPOD spatial profile width.[689, 693]
BMI	Increase	Equivocal (BMI is related to gender and age)	It is generally accepted that BMI increases with age.[674] The relationship between MPOD and BMI is controversial.[166, 401, 436, 591, 597, 601] Participants of male gender are more likely to exhibit lower MPOD with higher BMI.[600]
Male %BF	Increase	Lower	Female %BF is approximately 10% higher than male %BF for the same BMI.[606, 607] Female MPOD may be lower than male because of higher female %BF at the younger age range. Male MPOD may be reduced in the older age range due to increased abdominal adipose tissue, which competes with retinal MP.[486, 758]
Female %BF	Increase	None	

Physiological Strabismus / eccentric fixation	-	Lower	If the central target is not coincident with the foveola, the central MP value will be underestimated, resulting in lower MPOD.
Floaters (central) Floaters (para-central)	Increase Increase	Lower Higher	Snodderly *et al.* and this study observed that floaters affect MPOD measurement.[507]
PVD	Increase	Lower?	Operculae associated with vitreomacular separation have been reported to cast a yellow shadow on the retina beneath.[444] Lutein and Z are known to stain ILM tissue.[992, 993] Sharifzadeh *et al*. reported a central loss of MP following PVD in a healthy subject.[1119]
Macular thickness Foveal thickness	Decrease? None	Equivocal Equivocal	Stratus OCT foveal thickness (FRT) was 182 µm (SD 23 µm) and macular thickness (MRT) was 212 µm (SD 20 µm).[1120] Foveal thickness is reported to remain relatively constant with age, however the change in macular thickness with age is controversial.[1121-1124] Macular thickness was reported to reduce by 0.26 µm to 0.46 µm per year.[1121] The relationship between MPOD and macular thickness (FRT and MRT) is also controversial. Two studies reported a significant, positive association.[589, 1125] Three studies reported no association.[394, 500, 586]
Pathological AMD	Increase	Equivocal	Comparison of (peripheral) MP between healthy donor eyes and donor eyes with AMD using HPLC, suggested lower MP levels in eyes with AMD, unrelated to the destructive effects of AMD or age-related decline.[175] In vivo studies of MPOD have been equivocal. Several studies reported lower MPOD for those with or at risk of AMD,[592, 596, 722, 904, 1126] several other studies reported no significant association.[593, 594, 655, 716, 1127] Two longitudinal studies reported no protective effect of MP against AMD progression,[452, 595] although AREDS2 did reveal a benefit of taking MP supplements in those with a poor dietary intake of L and Z.[452]
Diabetes	Increase	Lower	Lower MPOD was found for those with diabetes, with or without retinopathy,[665, 680] and a significant inverse correlation between MPOD and HbA1c was observed.[665] Topographic mapping revealed that MPOD is displaced by intraretinal cysts in DMO.[1128] Diabetes is associated with retinal Mc abnormality.[1129]
CSC	Equivocal	Lower	Central retinal thickness is reduced in chronic CSC.[1130] Average MPOD measured within 0.5° of the fovea was significantly lower in Japanese eyes with chronic CSC and their fellow eyes, and was independent of retinal thickness.[1131]
RP / other retinal degenerations	Increase	Lower	Macular pigment optical density was positively related to macular thickness in RP, Usher syndrome and choroideraemia.[765, 1132, 1133] Patients with ABCA4-RD had significantly lower MPOD at 0.2° and 0.5° eccentricity but not at 1° or 2° eccentricity.[808]
Glaucoma	Increase	Equivocal	Igras *et al*. reported that MPOD was significantly lower in glaucoma patients.[1134] Kanis *et al.* and Obana *et al.* reported no significant association between MPOD and glaucoma.[723, 1135]
MacTel types 1,2 and 3	Increase	Lower (especially MacTel type 2)	MPOD was lower for MacTel types 1, 2 and 3. MacTel type 2 was significantly lower than types 1 and 3.[1136] MacTel type 2 is characterised by a central depletion of MPOD.[981] Müller cell loss or dysfunction is a critical component of MacTel type 2, and the area of MP depletion was found to correspond to the region of Mc loss.[982]
Sjögren-Larrson syndrome (SLS)	Decrease	Lower	Patients with SLS lack the central peak associated with the typical MPOD profile.[1137] The presence of cystic changes in the majority of SLS cases may suggest that Mc are involved in the retinal changes associated with SLS.[457]
VMT and Macular hole	Increase	Lower?	Vitreomacular traction may lead to cystic changes, or if PVD occurs, a loss of ILM tissue in the form of an operculum.[443, 1138] These processes may result in lower

			levels of central MPOD by lateral displacement and by tissue loss respectively.
ERM Figure 1.13	Increase	Equivocal	Epiretinal membrane may occur in isolation or may be associated with VMT and / or macular holes.[443] ERM may appear yellow due to the presence of MP.[443] The effect of ERM on HFP-derived MPOD will depend on the location of the ERM. If the ERM is foveal MPOD may be higher than expected. If the ERM is located at a more peripheral location lower MPOD may result.
Cystic fibrosis	Decrease	Lower	MPOD was lower at all eccentricities compared to controls, although no central depletion was observed. Despite severely low serum and retinal MP levels, visual function was surprisingly good.[1139]
Albinism	None	Lower	Low mean levels of MPOD were reported for individuals with albinism using objective (mixed sample of tyrosine-positive and tyrosine-negative oculocutaneous, and ocular),[1140] and a single case with oculocutaneous,[1141] and subjective (samples included oculocutaneous only) methods of MPOD measurement.[408, 1142] Individuals with albinism exhibit variable degrees of amblyopia and nystagmus that may make subjective MPOD measurement difficult. Whether low melanin levels affect MPOD deposition is unknown. Ocular effects of albinism include an absence of the foveal pit due to the presence of persistent inner retinal layers over the fovea.[1143]
Alzheimer disease	Increase	Lower	Individuals with Alzheimer disease had statistically lower MPOD (2W-FAF), serum L and Z, VA and SCS, and a higher occurrence of AMD compared to controls.[1144] Supplementation with L, Z and MZ resulted in significantly higher MPOD, serum L, Z, and MZ, and SCS (1.2, 2.4, 9.6 and 15.2 cpd for the Alzheimer group and 1.2 and 2.4 cpd for the non-Alzheimer control group), in those with and without Alzheimer disease compared to those on placebo. Supplementation with MP did not affect cognitive function in the Alzheimer group or the non-Alzheimer control group.[1013]
Personal Factors Smoking	Peaks in early middle age but is lower in the elderly	Lower	Hammond *et al*. reported significantly lower MPOD (HFP) for smokers compared to controls matched for age and other confounders.[664] Current and past smokers had statistically lower HFP MPOD compared to never smokers.[597] Significantly lower MPOD in smokers was confirmed using an objective MPOD method (FAF).[1145] A central "dip" in MPOD at 0.25° retinal eccentricity was observed to be significantly more common in older individuals and current cigarette smokers.[401] Central macular thickness was similar for smokers and non-smokers, suggesting that low MPOD is a consequence of nicotine toxicity rather microstructural changes at the fovea.[1145]
Psychophysical VA	Decrease	Equivocal	Visual acuity was lower with age for healthy subjects and those with ocular disease.[897] The relationship between MPOD and VA is controversial.[593, 594, 596, 655, 658, 659]
Flicker sensitivity	Decrease	Lower?	Flicker sensitivity with HFP (not to be confused with CFF) is greater for the central, compared to the peripheral target location.[507] Flicker sensitivity is reduced with age, after correction for differences in retinal illuminance.[337, 1146] O'brian *et al*. reported that failure to set an appropriate flicker rate for HFP led to difficulty with the HFP task and higher levels of measurement error.[709]
S-cone sensitivity	Increase (S-cone Threshold)	Equivocal	Werner reported that the sensitivity difference between 0° and 8° retinal eccentricity for the S-cone mechanism, but not the M- and L-cone mechanisms, was significantly related to peak MPOD, but unrelated to observer age.[506] Beirne confirmed that under conditions of S-cone isolation HFP MPOD was not significantly related to age and reported that the rate at which foveal acuity changed compared to acuity at 12° with increasing age, was not significantly related to MPOD levels.[899] Neither study supported the hypothesis that higher MPOD levels protect S-cone visual function with age (i.e. protection hypothesis).
GRT	Increase	Equivocal	All studies of healthy subjects reported a positive

		(dependent on the blue content of the light source and duration of glare)	correlation between equilibrium bleach GRT and age.[330, 348, 571, 573, 574, 577, 928-931] The relationship between photo-flash bleach GRT and age is more controversial.[330, 359, 361, 684, 924, 926, 927] Four studies reported a significant negative correlation between GRT and MPOD.[620, 622, 623, 905] Loughman *et al.* and the present study (which corrected for the biphasic nature of both variables with age) found no significant association.[906]
Psychological Difficulty with HFP task	Equivocal	None	It was observed that older individuals and those with advanced stages of ocular disease experience more difficulty with the HFP task, especially with detection of flicker in the peripheral target.[507, 680] An inappropriate flicker rate for the HFP target was also reported to increase difficulty with the HFP task.[709] The present study found no significant difference in MPOD, age or GRT between healthy subjects experiencing difficulty and no difficulty with the HFP task.
Poor fixation	None	None	Crossland *et al.* reported no significant relationship between age and fixation stability.[896]
Training effect	Increase?	Equivocal (dependent on the type of HFP equipment is used)	Seitz *et al*. reported that CFF thresholds measured using chromatic flicker (MP densitometer) increased by an average of 30% after one hour of perceptual learning each day for nine days.[728] MPOD results are more likely to be affected by using fixed-flicker instrumentation rather than variable flicker instrumentation.
Visual attention	Decrease	Equivocal	Visual attention declines with age.[1147] Increased attention may reduce receptive field size.[1148, 1149] smaller receptive field size favours spatial resolution and larger field size favours temporal resolution.[1150, 1151] Perceptual learning may increase temporal resolution (CFF).
Statistical High intersubject variation	None	Equivocal	A high level of inter-individual variation across the entire age range has been reported for MPOD measurements,[497, 599, 601, 711, 712] High degrees of inter-individual variation also exist for factors leading to variation in HFP MPOD: media opacification,[1152] foveal thickness,[1120, 1153] pupil size,[932, 1154-1156] M- and L- cone photopigment λ_{max} and M- / L- cone ratio.[506] High levels of inter-subject variation increase the chance of a sporadic positive or inverse correlation.
Biphasic relationship	-	Equivocal	Six studies examining the relationship between HFP MPOD and age have reported levels which peak in middle age.[166, 486, 598, 601, 653, 705] Correlation analysis, which assumes a linear relationship, may not be appropriate in this case. The age range will influence bivariate correlation between MPOD and age.
Correction for other factors	-	Equivocal	Nolan *et al.* reported a significant inverse correlation between MPOD and age, however after removal of two outliers and correction for ethnicity no significant correlation was reported.[394] Where possible correction for other influencing factors should be made.
Correlation vs group analysis	-	Equivocal	Neelam *et al.* reported a trend towards a small negative correlation between MPOD and age ($r = -0.181$, $p = 0.063$), however group analysis revealed no significant difference in MPOD between subjects aged < 55 years and those aged ≥ 55 years ($p = 0.188$).[652]
Instrument design Age-estimation (MPS)	-	Equivocal	Age-estimated central MPOD accounts for 80% to 84% of actual MPOD.[680, 689] Higher Intersubject variation in MPOD may occur in the older age range.
IOL setting (MPS)	Increase	Equivocal	IOL setting assumes equal lens transmission characteristics compared to a 20 year old.[1157] Actual IOL light transmission may vary considerably from this value.[1158]
Central target size	-	Equivocal	Commercial HFP instruments use a 1° central target. This target size has the highest test-retest reliability.[507] Central "dip" affecting 12% of subjects is associated with age and smoking, will not be detected with this target size.[401] MPOD derived from the 1° target size will be variably influenced by environmental and genetic factors,[404] which vary with age.[735, 810]

Peripheral target location	-	Lower	MP is optically undetectable at 6° to 8° eccentricity.[393] Spatial profile width is greater for higher MPOD levels,[393] for females,[693] and increases with age,[721] and MP supplementation.[689] Greater profile width may under-estimate MPOD due to higher peripheral MP values, especially for instruments with less eccentric peripheral targets.
Fixed flicker	-	Lower?	Flicker detection is reduced with age, after correction for variations in retinal illuminance.[337, 1146] Loane *et al*. reported that the use of fixed flicker was associated with a greater number of subjects unable to obtain an MPOD result.[691]
Different HFP instruments	-	See above	Comparison of the Eyemet Maculometer (5.5° peripheral target location, preset-flicker) and the Macular Metrics Densitometer (7° peripheral target location, optimal flicker set for each subject), on the same population revealed a trend towards a decline in MPOD with age for the Maculometer (r = -0.21), but no correlation with age for the Densitometer (r = 0.01). The Maculometer also underestimated MP in subjects with higher MPOD levels[691]
HFP / objective measurement	-	Equivocal	Eight studies have compared the relationship (positive, inverse or none) between MPOD and age using different methods of measurement.
Delori *et al*.[489] Wüstemeyer *et al*.[717] Bernstein *et al*.[715] Liew *et al*.[588] Neelam *et al*.[652] Berendschot *et al.*[694] Van der Kraats *et al*.[703] Hogg *et al.*[713]			2-WFAF (positive), FR (positive) 2-WFAF (none), FR (inverse) HFP (trend for inverse), RRS (inverse) HFP (none), 2-WFAF (positive) HFP (inverse), RRS (inverse) HFP (inverse), 1 x FR (inverse) 4 x FR (none), 2-WFAF (none) HFP (trend for positive), FR (none) HFP 0.5° (positive), RRS (inverse) HFP spatial profile, 0.17°, 0.5°, 1.0°, 2.5° (none)
Genetic APOE ABCA4 BCMO1 CFH ARMS2 C3 C2 Factor B gene (BF)	Equivocal	Equivocal	The association between high-risk CFH genotypes and AMD was reported to increase with age.[810] It is plausible that the association between genes linked to variation in with MPOD levels (APOE, ABCA4 and BCMO1) will also vary with age. The effects of nutritional supplementation may also be influenced by genetics.[809, 814] No association was reported for the individual AMD risk genes (CFH, ARMS2, C3, C2 and BF) and MPOD. The combination of homozygous risk alleles at CFH and ARMS2 loci was associated with significantly lower MPOD at 0.5° and 1.0° retinal eccentricity.[405]
Dietary factors Dietary intake of MP	Equivocal	Equivocal	The association between dietary intake of MP with age and with gender are controversial.[706] Dietary intake of MP varies from 0.8 mg to 4.0 mg per day, depending on the population and measurement method.[1159] Beatty *et al*. reviewed the association between dietary intake of MP and MPOD. Three out of five studies reported a positive correlation and two studies reported no relationship.[1159] Johnson *et al*. reported significantly higher MPOD at four weeks, but not eight weeks after starting a L (spinach) and Z (corn) fortified diet.[164] Graydon *et al*. reported no difference in MPOD after eight weeks intake of spinach powder.[1160] Retinal MP levels change at a slower rate than serum MP levels in response to altered MP dietary intake and will be influenced by factors affecting digestion, absorption, transport and retinal capture.[1159]
MP supplements	Increase?	Increase (but the increment was small)	Macular pigment supplements are more likely to be taken by older patients,[486] especially those with early signs of AMD or a FH of AMD. This is despite evidence that antioxidant supplements do not prevent AMD development,[1161] or reduce progression in early AMD.[1162] AREDS2 reported reduced progression to advanced AMD for those taking MP supplements, but only if dietary intake of MP was low.[452, 898] NHANESIII reported that general supplement use has increased in both genders over the last 25 years and that females are more likely to take supplements than males.[735] Sabour-Pickett *et al.* reviewed the association between MP supplement use and MPOD for 34 studies. A mean MP supplement intake of 14.7 mg/day of L and 2.5 mg/day of Z (approximately 4 to 20 times normal dietary intake

			of MP), over a mean period of 20 weeks resulted in a small mean increase in MPOD of 0.08 (range 0 to 0.2.).[900] Bartlett *et al.* have also commented on the small degree of increment in MPOD after MP supplementation.[1107]
Intestinal malabsorption	Increase?	Lower (Coeliac and Crohn's)	Physiological changes with age are restricted to altered absorption of calcium, and perhaps zinc and magnesium. Achlorhydria (low or absent gastric acid) can lead to impaired absorption of vitamin B12, folic acid and calcium.[1163] Coeliac and Crohn's disease are known to cause deficiencies in lipid-soluble nutrients. Subjects with a history malabsorption had 37% lower MPOD (using RRS) compared to controls with no history of malabsorption ($P < 0.001$). No evidence of early AMD was observed in subjects with or without malabsorption.[669]

MPS: Macular Pigment Screener, PVD: posterior vitreous detachment, ABCA4-RD: ABCA4-associated retinal degenerations, VMT: vitreomacular traction. NHANESIII: the third National Health and Nutrition Examination Survey, SCS: spatial contrast sensitivity, cpd: cycles per degree. Factors affecting MPOD that were unlikely to alter significantly with age (e.g. refractive status, axial length and iris colour) or are unpredictable with age (e.g. ocular light exposure and exercise) were excluded from this table.

A2.5 GRT and its association with ocular and systemic disease, pupil size, medications, supplements and method of GRT measurement

Condition or Environmental Factor	Reference	GRT Method	Effect on GRT	Notes
AMD	See table 1.13			
Diabetic retinopathy	Zingerian *et al*. (1985)[624] Wu *et al*. (1990)[352] Brinchmann-Hansen *et al*. (1992)[576] Baptista *et al*. (2013)[1164] Schmitt *et al*. (2003)[358] Newsome *et al*. (2009)[361] Loughman *et al*. (2014)[1165]	Equilibrium Equilibrium Equilibrium Equilibrium Photo-flash Photo-flash Photo-flash	Longer (BGR) None (BGR) None (BGR) None (NDR) None (BGR) None (BGR) None (BGR)	Brinchmann-Hansen *et al*. reported significantly shorter and longer equilibrium GRT after seven years in diabetics with a cumulative mean HbA1 of below 10% and above 10% respectively. After seven years GRT had no predictive value for BGR status.[576] Retinal hypoxia for 25 min did not affect equilibrium GRT.[1166] Pro-inflammatory changes leading to prolonged retinal hypoxia and accumulative oxidative stress may cause diabetic retinopathy.[1167]
Cystoid macular oedema	Wu *et al*. (1990)[352] Severin (1980)[910] Newsome *et al*. (2009)[361]	Equilibrium Photo-flash Photo-flash	None (DMO) Longer (IGS) Longer (DMO)	Wu *et al*. reported longer GRT for DMO compared to normals, but this was not significant.
CSC	Forsius *et al*. (1963)[602] Ito *et al*. (1996)[575] Horiguchi *et al*. (1998)[914] Natsikos *et al*. (1980)[909] Verma *et al.*(1990)[1168]	Equilibrium Equilibrium Equilibrium Photo-flash Photo-flash	Longer Longer (inCSC) Longer (inCSC) None (exCSC) Longer Longer	Natsikos *et al*. reported that GRT was prolonged for a few weeks after the onset of symptoms, but returned to normal after six months. Verma also reported no prolongation of GRT in cases with healed CSC.
Retinal detachment	Krastel *et al*. (1980)[1169]	Equilibrium	Longer (inMac) None (noMac)	Krastel *et al*. reported GRT measurements from a mean of two weeks after reparative surgery for retinal detachment.
Retinitis pigmentosa / Stargardt disease	Sandberg *et al*. (1999)[1170]	Equilibrium	Longer (dRP)	Dark adaptation in RP using an equilibrium bleach source revealed abnormal rod adaptation. Cone adaptation prior to rod - cone break was normal.[915] Significantly longer GRT was observed for dominant RP with rhodopsin mutations.[1170]
Glaucoma	Harayama *et al*. (1981)[1171] Sherman *et al*. (1988)[913] Horiguchi *et al*. (1998)[914] Kamppeter *et al*. (2003)[1172] Baptista *et al*. (2013)[1164] Schmitt *et al*. (2003)[358]	Unknown Equilibrium Equilibrium Equilibrium Equilibrium Photo-flash	None (POAG) None (PACG) None (SG) None (OHT) Longer (COAG) Longer (inScot) None (exScot) Longer (COAG) None (POAG) None	The association between equilibrium GRT and glaucoma is unclear. Parisi *et al*. reported that visual evoked potential amplitudes recorded after equilibrium bleach were significantly longer for POAG, but not OHT compared to controls. The P100 latency was significantly greater for POAG and OHT compared to controls, suggesting reduced function of the outer retinal layers in these groups.[1173]

Cataract	Baptista *et al*. (2013)[1164] Elliott *et al*. (1991)[684] Schmitt *et al*. (2003)[358]	Equilibrium Photo-flash Photo-flash	None None None	Cataract did not significantly affect GRT. This result is expected provided a sufficient level of bleach is obtained by the GRT method.[1164]
MacTel type 2	Jindal *et al*. (2015)[1174]	-	-	Paracentral greying decreased in intensity on exposure to continuous light. After 15 min DA grey colouration returned. Possible photochemical reaction to chromophore released from abnormal Mc.[1174]
Müller cell gliosis / de-differentiation to progenitor or stem stems	Bringmann *et al*. (2009)[270]	-	-	De-differentiation of Mc to progenitor or stem cells in response to retinal stress, is associated with a functional uncoupling from neurons, leading to down-regulation of proteins involved in specific Mc functions, including photopigment recycling.[270]
Pupil size: Larger physiological pupil size Pharmacological mydriasis Pharmacological miosis	 Malik *et al*. (1971)[571] Severin *et al*. (1963)[1175] Elliott *et al*. (1991)[684] Henkind *et al*. (1967)[933] Gómez-Ulla *et al*. (1986)[927] Natsikos *et al*. (1980)[909] Gómez-Ulla *et al*. (1986)[927]	 Equilibrium Photo-flash Photo-flash Equilibrium Photo-flash Photo-flash Photo-flash	 None Longer None None None None Shorter	It is expected that equilibrium bleach GRT would be immune pupil size variation because pupils will have sufficient time to constrict and the accumulative nature of the bleach would minify the effect of variations in illuminance.[573] Photo-flash bleach GRT should be corrected for pupil size as retinal illuminance is likely to reflect the pre-constricted pupil size. This may explain the contrary results with age and larger intersubject variation reported for photo-flash compared to equilibrium methods reported by Wood *et al*.[330]
Yellow-tinted IOLs	Hammond *et al*. (2009)[1176] Hammond *et al.* (2010)[1177]	Equilibrium Equilibrium	None Shorter*	No significant difference in GRT was demonstrated between individuals with clear or yellow-tinted (blue light blocking) IOLs.[1176] *Hammond *et al*. reported a significantly shorter geographic mean difference in GRT for yellow-tinted compared to clear IOLs, however geographic means are normally reserved for percentages derived from values measured, rather than the values themselves.[1177]
Dyslexia The author is unaware of any studies examining the association between dyslexia or MIS with GRT	Stordy (1995)[1178] Stordy (2000)[1179] Greatrex *et al*. (2000)[1180]	- - -	- - -	Individuals with dyslexia exhibited significantly reduced DA compared to controls with no difference in dietary vitamin A dietary intake, measured using the Friedmann Visual Field Analyser 2 (Clement Clarke International, London, UK).[1178, 1179] Greatrex *et al.* reported no difference in DA for dyslexic individuals.[1180]
Scotopic sensitivity syndrome (now known as MIS)	Carroll *et al*. (1994)[1181]	-	-	Dark adaptation was reported to be abnormal in individuals with scotopic sensitivity syndrome.[1181]
Carotid occlusive disease Light-induced amaurosis is a symptom of ocular ischaemic syndrome, a major RF for stroke.[963]	Furlan *et al*. (1979)[964] Donnan *et al*. (1982)[1182] Ross Russell *et al*. (1983)[1183] Jacobs et al. (1985)[1184] Ross Russell *et al*. (1986)[1185] Katz et al. (1986)[611] Wiebers *et al*. (1989)[1186] Giroud *et al*. (1991)[1187] Roberts *et al*. (1992)[612] Blum *et al*. (1994)[965] Kaiboriboon *et al*. (2001)[963]	- - Equilibrium - Equilibrium Equilibrium - - Equilibrium - Unknown Unknown -	- - None - Longer Longer - - Longer - Longer Longer -	Several case reports have been published which describe unilateral or bilateral light-induced amaurosis, associated with unilateral or bilateral carotid occlusive disease. Symptoms were fully or partly resolved after treatment of carotid occlusion with surgery or medication.[611, 612, 963, 1186, 1187] The pathological mechanism was reported to involve delayed photopigment regeneration and retinal ischaemia secondary to reduced choroidal blood flow.[1186, 1187]
Ocular vascular perfusion	Lovasik *et al*. (1989)[609]	Equilibrium	Longer (lower RVPP) None (higher RVPP)	Lowering RVPP by scleral indentation resulted in a significant increase in GRT. The effect of increased RVPP resulting from body eversion, on GRT was variable, showing longer GRT for some

				individuals and shorter for others. These effects were independent of IOP, diastolic and systolic brachial and ophthalmic artery pressures.[609]
Low oxygen levels	Brinchmann-Hansen *et al*. al. (1989)[1166] Yap *et al*. (1995)[1188]	Equilibrium Equilibrium	None None	Altitudes of sea level, 8,000, 15,000 and 18,000 feet,[1166] and sea level, 7,000 and 12,000 feet,[1188] were simulated with a hypobaric chamber.
Drug-induced Blood pressure reduction:	Myhre *et al*. (1991)[610] The results from test and placebo groups were combined prior to analysis. Both groups exhibited a reduction in blood pressure, so it cannot be certain that this was the effect of the medication in the test group.	Equilibrium	Longer (mon) None (bin)	Myhre *et al*. reported that a medically-induced 5% reduction in mean brachial artery pressure was associated with a significant increase in monocular GRT. No significant difference was observed for binocular GRT. The same study reported that binocular GRT was inversely correlated with blood pressure. Monocular GRT exhibited a trend toward an inverse association.[610]
Drug-induced RPE pigment changes: Quinoline antimalarials Chloroquine Hydroxy-chloroquine	Carr *et al*. (1968)[1189] Hydroxychloroquine is associated with significantly less risk of maculopathy.	Equilibrium	Longer	Carr *et al*. reported significantly longer GRT in patients taking long-term chloroquine or one of its derivatives compared to normal subjects. Approximately half of the treated sample had macular changes consistent with chloroquine retinopathy (RPE pigment mottling eventually leading to bull's eye maculopathy).
Neuroleptic agent Melperone	Bergman *et al*. (1980)[1190]	Photo-flash	Longer	Melperone was reported to increase readaptation time (measured with optokinetic nystagmus, OKN response) maximal after 2-3 hours, depending on the dose lasting for seven hours after medicating.[1190]
Phenothiazine-derived antipsychotics Thioridazine and Chlorpromazine	Fornaro *et al*. (2002)[1191] Li *et al*. (2008)[1192] Richa *et al*. (2010)[1193]	- - -	- - -	Macular pigmentary changes can develop into a "salt and pepper" pattern which may affect DA.[1192, 1193] Retinal pigmentary changes may be the result of altered dopamine and melatonin activity.[1191]
Non-steroidal anti-inflammatory agents Indomethacin	Chiou (1999)[1194]	-	-	Dose-related retinal pigmentary changes, the level of which determine any DA abnormality.
Drug-induced CMO: Several drugs are thought to be associated with CMO and affect DA, but the author is unaware of any studies examining the association with GRT	Rosiglitazone Coluccellio (2005)[1195] Pioglitazone Oshitari *et al*. (2008)[1196] Paclitaxel Ham *et al*. (2012)[1197] Kuznetcova *et al*. (2012)[1198] Docetaxel Teitelbaum *et al*. (2003)[1199] Tamoxifen Makri *et al*. (2013)[919] Niacin (nicotinic acid) Courtney et al. (2014)[1200] Latanoprost Makri *et al*. (2013)[919] Adrenalin (epinephrine) Bozkurt *et al*. (2010)[1201]	- - - - - - - - -	- - - - - - - - -	Thiazolidinediones, oral antidiabetic agents rosiglitazone and pioglitazone were associated with CMO and decreased DA,[1202] however, it is not clear whether this association is biased by the underlying diabetes.[919] Taxane and niacin CMO are bilateral and angiographically silent.[1198] Tamoxifen maculopathy is bilateral and associated with CMO and yellow-white crystals in the superficial retina layers of the fovea and paramacula.[919] Latanoprost and adrenalin may increase CMO risk in cases with other CMO RF, such as posterior capsule rupture after cataract extraction.[919, 1201]
Drug-induced effects on DA: Fenretinide	Caruso *et al*. (1998)[1203]	-	-	Fenretinide resulted in a significant delay in the timing of the rod - cone break during DA.
ACU-4429	Kubota *et al*. (2012)[1204]	-	-	ACU-4429 caused a dose-related inhibition of the b-wave on electroretinograms and reduced DA.[1204]
Isotretinoin (11-	Radu *et al*. (2003)[1205]	-	-	Decreased night vision is a common

cis retinoic acid)	In a murine model of retinal degeneration, isotretinoin prevented the accumulation of A2E in the RPE.[1205]			adverse effect. Inhibition of 11-cis retinol dehydrogenase (enzyme that converts 11-cis retinol to 11-cis retinal) slows rhodopsin regeneration and chromophore recycling. Melanopsin regeneration may also be delayed.[1206]
Other drugs: Birth control medication	Heckenlively *et al*. (1978)[1207]	Equilibrium	Longer > 1 year None < 1 year	Subjects taking one of five different brands of birth control medication exhibited significantly longer GRT after 1-2 years, but not up to one year. These pills inhibit ovulation causing a physiological state of false pregnancy.[1207]
Benzodiazepine, Oxazepam	Bergman *et al*. (1979)[1208]	Photo-flash	Longer	Oxazepam was reported to increase readaptation time (measured with OKN response) maximal after two hours, lasting for five hours after medicating.[1208]
Supplements: MP (L and Z)	Stringham *et al*. (2007)[620] Stringham *et al*. (2008)[621] Loughman *et al*. (2010)[906] Stringham *et al*. (2011)[622] Nolan *et al*. (2011)[968] Hammond *et al*. (2013)[623]	Equilibrium Equilibrium Equilibrium Equilibrium Equilibrium Equilibrium	Shorter Shorter None Shorter None Shorter	All of the studies reporting an inverse association between MPOD and GRT used a short 5-second exposure using significant blue light content sources. The two studies reporting no association used low blue light content sources. All studies examined younger participants (< 50 years of age).
Omega-3 (DHA) Supplementation Human	Stordy (2000)	-	-	Dark adaptation was significantly improved in individuals with dyslexia after one month supplementation with 480 mg of daily DHA compared to controls.[1178]
Omega-3 (DHA) Dietary deficiency Primates	Jeffrey *et al*. (2009)[1209]	Photo-flash Photo-flash	Longer (saturation) None (subsaturation)	Omega-3 deficient monkeys showed a delay in rod recovery after a saturation level flash, but not a subsaturation level flash stimulus.[1210] Omega-3 deficiency affected rod, but not cone recovery measured with ERG after photostress.[1209]
	Neuringer *et al*. (2005)[1210]	Photo-flash Photo-flash	Longer (rods) None (cones)	
Recreational drugs: Alcohol (ethanol)	Adams *et al*. (1975)[911] Sekular *et al*. (1977)[1211] Adams *et al*. (1978)[912]	Equilibrium Equilibrium Equilibrium	Longer Longer Longer	Prolongation of GRT onset 30 min after consumption of a single dose of alcohol (0.75 ml / Kg), peaked at 1-2 hours and returned to pre-consumption values after six hours. Results were significant for a small target (10 min arc) but not for a larger target (100 min arc).[912] Pupil size was unaltered by alcohol.[911, 1211]
Tobacco	Sobaci *et al*. (2013a)[1212] Sobaci *et al*. (2013b)[1145]	Equilibrium Equilibrium	None None	Although GRT was not significantly different for chronic heavy smokers (≥ 1 box / day for ≥ 5 years), foveal threshold value (measured using automated perimetry) was statistically higher in smokers one min after GRT. The results suggested increased light adaptation as well as altered RPE function indicated by significant melanin pigment changes (measured by 1-WFAF) in chronic smokers. Chronic smokers had significantly larger pupils compared to non-smokers.[1145, 1212]
Marijuana	Adams *et al*. (1978)[912]	Equilibrium	Longer	Prolongation of GRT onset 40 min after smoking 15 mg of tetrahydrocannabinol, peaked at approx. one hour and returned to pre-ingestion values after five hours. Pupil size was significantly smaller 40 min after marijuana ingestion.[912]
Method-related factors: Higher illuminance of	Severin *et al*. (1963)[1175] Irikura *et al*. (1999)[1213]	Photo-flash Both	Longer Longer	Severin found a linear relationship between illuminance (range 86,080 to 242,100 lux) of the 150 ms glare

glare source				source and GRT.
Longer exposure to glare source	Irikura *et al.* (1999)[1213]	Both	Longer	For glare source exposure times of 0.1 to 1.6 s GRT increased with longer exposure time.
Brighter background luminance of target	Irikura *et al.* (1999)[1213]	Both	Shorter	For background luminance between 0.1 and 1.0 cd m^{-2} GRT was shorter for brighter backgrounds for all glare exposure times and glare source luminances examined.[1213]
Equilibrium vs photo-flash The percentages refer to the estimated amount of photopigment bleached by each method	Wood *et al*. (2011)[330]	Both	None	Wood *et al*. compared 84% equilibrium bleach with 98% photo-flash bleach. There was no significant difference in mean GRT between the two methods, although a trend for longer GRT with equilibrium was observed. Both methods deplete local stores of 11-cis retinal derived from Mc. Equilibrium bleach is likely put a greater stress on the RPE for cone pigment regeneration.[330]

Abbreviations. +fellow: results of GRT from affected eye and unaffected fellow eye of subjects with unilateral AMD, fellow: results of GRT from the fellow eye of subjects with unilateral AMD, BGR: background diabetic retinopathy, NDR: no diabetic retinopathy, IGS: cystoid macular oedema secondary to cataract extraction, presumed to be Irvine-Gass syndrome, inCSC: measurements taken within the area of retinal detachment, exCSC: measurements taken outside the area of retinal detachment, inMac: retinal detachment with macular involvement, noMac: retinal detachment with no macular involvement, dRP: dominant RP with rhodopsin mutations, POAG: primary open angle glaucoma, PACG: primary angle closure glaucoma, SG: secondary glaucoma, OHT: ocular hypertension, RVPP: retinal vascular perfusion pressure, mon: monocular, bin: binocular, inScot: measurements taken within the area of scotoma, exScot: measurements taken within the area of scotoma.

Drusen severity was defined by Smiddy *et al*. in their longitudinal study by the value of the composite score (higher score = more severe) from five drusen characteristics: size (graded from 1-4), number (1-3), distribution (1-3), demarcation (0-1) and degree of confluence (0-3).[350]

Appendix 3

A3.1 The retinal theory for Meares-Irlen syndrome

Retinal theory for Meares-Irlen syndrome (MIS), its treatment with diet and / or supplementation, its association with AMD and ocular vascular perfusion (OVP) RF and MIS as a childhood marker for retinal lipofuscin deposition and arguably increased AMD risk later in life.

This theory was formulated in response to four observations.

1) Light-induced amaurosis may result in cases of ocular ischaemic syndrome secondary to carotid occlusive disease.
2) Migraine, Rph and VDys are also associated with reduced or irregular ocular blood flow, which may induce retinal inflammation leading to functional retinal changes.
3) Migraine and other conditions associated with low dopamine levels are associated with symptoms described as MIS.
4) The author has encountered two patients on acne medication; one taking isotretinoin and one taking lymecycline. Both patients reported increased light sensitivity and coloured "blobs" when looking at bright white backgrounds, in the absence of any signs or symptoms suggestive of increased intracranial pressure. The symptoms disappeared in both cases after cessation of treatment.

A3.2 Meares-Irlen syndrome background

Symptoms of MIS, also described as Visual Stress, include movement, muddling and / or breaking up of words, patterns described as "worms", "rivers" or "waterfalls" affecting the print or the areas between the print, and blobs of colour moving across the page. Nausea, discomfort or pain may be experienced when observing white backgrounds such as books, computer screens and white boards. Signs of MIS include excessive blinking, yawning and frequently looking away from the bright surface on which the reading material is presented.[1214, 1215] Although individuals with dyslexia may experience symptoms suggestive of MIS and benefit from reduced background brightness, these conditions are not always associated. Dyslexia may be present without MIS and MIS may equally affect the non-dyslexic population.

The retinal theory for MIS was formulated after several patients presented to the author with symptoms that appeared to be persistent afterimages (multi-coloured blobs) affecting the central vision, during and after observing bright white backgrounds (e.g. interactive white boards). These afterimages were reduced or prevented by lowering the intensity of the white background or by wearing tinted spectacles.

A3.3 Current MIS theory

The current explanation for MIS is cortical hyper-excitability, for which there is compelling evidence.[971, 972] Neuroimaging has revealed significant differences between control and MIS groups for impulse response function (IRF) intensity in two brain regions (BA6 and postcentral gyrus), and the percentage of active voxels in four regions (BA6, postcentral gyrus, BA17 and BA19).[972] Brodmann area 17 (BA17) represents the primary visual cortex and BA19 represents the extrastriate visual cortex. Functional magnetic resonance imaging (fMRI) differences were found in these regions for those reporting visual distortions associated with migraine.[1216] The postcentral gyrus is an area in the somatosensory cortex, chosen to reflect the observation that visual stress may be more common in those scoring higher on the neuroticism scale, secondary to a neurological system more sensitive to external stimuli.[1217] BA6 represents the premotor and supplementary motor cortex, selected to test for hyperactivity in cortical areas other than the visual cortex.[972, 1217]

A3.4 Background to retinal theory for MIS

A3.4.1 The cone-specific / Müller cell visual cycle

Recent research has suggested that the maintenance of vision under photopic conditions is mediated by the cone-specific visual cycle,[520-522] and the recovery of visual function after

exposure to the light source used in GRT is believed to be largely the result of cone photopigment regeneration.[308, 349, 580] Cone photoreceptors are able to access a more rapid supply of recycled photopigment from retinal Müller cells (Mc) than is available via RPE recycling.[525] The additional source of 11-cis-retinal available to cones may explain in part how human cone circulating current is fully recovered after just 100 ms from a steady bleach of approximately 90% of photopigment, whereas rods take at least 20 min to recover fully.[529]

Many pathogenic stimuli, including light damage and oxidative stress cause Mc activation.[269, 278, 279] Müller cell activation results in a down-regulation of normal cellular function leading to neuronal hyper-excitability,[269] and is also likely to adversely affect GRT. Pathological change in Mc, represented by GFAP expression was prevented by L.[893]

Adolescents with symptoms of vascular dysregulation (VDys) may also report symptoms of MIS more regularly than those with no symptoms of VDys. Vascular dysregulation is associated with macrophage infiltration into the retina.[205] This inflammatory response is likely to be associated with activation of Mc and subsequent down-regulation of their contribution to cone visual pigment recycling.

It is plausible that the coloured blobs reported as a symptom of MIS are afterimages resulting from low-level equilibrium bleach due to the bright background. Wearing coloured filters would reduce the level of bleach, reducing GRT and alleviating the afterimage. Movement of print may be explained by a fractional delay in glare recovery combined with microsaccadic eye movement and ocular drift. Regional differences in GRT may explain the shimmering of print observed by some MIS sufferers.
It is possible that patients with MIS may lack or exhibit reduced learning or training effect, which is reported to result in shorter GRT after repeated exposure to sources of glare.[924, 925] Patients with MIS may even experience longer GRT after repeated GRT testing, similar to that exhibited by patients affected by ocular disease. An increase in recovery time for the second of two GRT performed sequentially on the same eye in quick succession, may be a marker for eye disease compared to healthy eyes.[329, 361] This effect could be readily tested provided that patients with MIS are able to tolerate the GRT procedure.

A3.4.2 Retinal adaptation

Multiplicative adaptation mechanisms include the pupil reflex and photopigment depletion, adjusting ocular sensitivity by scaling the input intensity by a multiplicative gain factor that decreases with increasing light intensity.[1218] There is evidence from human and murine data that retrograde signalling from ipRGC and possibly from the suprachiasmatic nucleus, may influence the level of adaptation via dopaminergic A18 amacrine cells.[334-336] A18 amacrine cells are thought to modulate AII (A2) amacrine cell and horizontal cell (HC) gap junctions.[335] In mammals dopamine is believed to modulate the spatial extent of the HC syncytium by uncoupling HC gap junctions, resulting in down-regulation of light and flicker sensitivity.[562]

Dopamine agonists were found to suppress the retinal flicker response, however maximal hyperpolarisation of HC with a bright white light was able to partially restore the initially suppressed flickering response components.[562] Retinal dopamine release varies diurnally in vertebrates, increasing during the day and in the light, and decreasing at night.[559, 833] Melatonin can acutely inhibit retinal dopamine release.[560] Flickering lights are the most effective stimulant for dopamine release in the primate retina.[561]

A3.4.3 Dopamine and MIS symptoms

Migraine appears to be more common among patients with MIS. Dopamine has been implicated in the pathophysiology of migraine,[186, 187, 834] and other disorders associated with increased light-sensitivity such as attention deficit hyperactivity disorder (ADHD).[850] Migraine and ADHD are associated with a lack of neural habituation, i.e. the inability to tune down sensitivity to external stimuli to comfortable levels.[565, 1219] Dopamine levels are low interictally but increase during a migraine attack.[186, 834] Dopamine receptors are thought to be hypersensitive in migraineurs as a consequence of low dopamine in the interictal

phase.[835-837] Photophobia affects 80% of migraineurs,[564] as well as approximately 50% of patients with mild traumatic brain injury.[540] Migraineurs and those with mild brain injury or stroke may benefit from precision tinting used to treat MIS.[1220-1222]

As well as light sensitivity migraineurs often report symptoms associated with increased dopamine levels such as premonitory yawning.[186] Increased yawning is also observed in those with MIS.[1214, 1215] Increased blink rate,[1214, 1215] another MIS symptom has been reported as a basic clinical marker for increased dopaminergic activity.[1223] Stringham et al. reported that greater discomfort was positively associated with increased pupil constriction under glare conditions.[622] Nerve signals associated with the pupil light response are relayed through the olivary pretectal nucleus (OPN).[553, 554] The OPN receives light-mediated input from ipRGC,[1224] which are modulated by dopaminergic A-18 amacrine cells.[334-336] This may indicate further evidence of hyperactivity in the ocular response to increased retinal or cortical dopamine levels.

A3.4.4 Strategies to increase dopamine levels

Increasing dopamine levels between migraine attacks may reduce interictal dopamine receptor hyper-excitability. This may be achieved by adopting a healthy diet, especially bananas[852] and lifestyle together with exercise and obesity reduction / smoking cessation.[855, 857, 858, 1225] In some cases the propensity to become obese may be genetically determined. An association has been reported between the hypo-functional seven-repeat allele (7R) of the dopamine-4 receptor gene (DRD4) and the consumption of a less healthy diet in adults, especially females and children.[761, 762]

The difficulty with any placebo-controlled experiment aiming to increase dopamine levels is that the placebo response is dopamine-mediated and is associated with a significant increase in dopamine levels.[1226, 1227]

A3.4.5 Meares-Irlen syndrome and pupil size

The author has noted that patients reporting MIS symptoms often have larger than normal pupils. The pupil reflex pathway is also mediated via ipRGC and involved in adaptation. The larger pupil size in affected patients may indicate a reduced retinal response to light and would lead to an increased level of retinal illuminance. Higher retinal light levels may cause or exacerbate Mc dysfunction.

Although cortical hyper-excitability / hyper-responsivity has been proposed as the cause of migraine symptoms,[821-823] gastric symptoms have been reported to originate from peripheral dopamine receptors in the gut,[824, 825] and it is plausible that peripheral dopamine receptors in the retina may contribute to light sensitivity in migraine, although this is not certain.[826-828]

A3.4.6 Photophobia

The photopigment contained in ipRGC is melanopsin (Opn4), which has a peak sensitivity of about 480 nm (blue).[540-542] It has been hypothesised that Opn4 functions as a bi-stable pigment, able to regenerate its own light-activated chromophore by absorbing a second wavelength of light at 587 nm (yellow), although this is controversial.[541, 545-547]

It is proposed that retinal dopamine receptor hyper-sensitivity, secondary to low dopamine levels, combined with an increase in retinal dopamine triggered by bright white backgrounds, combined with the retinal effects of reduced neurovascular coupling and reduced neural habituation will lead to a level of ipRGC-mediated adaptation that is incompatible with the actual level of illumination incident at the retina.

This may manifest as a delay in photopigment recycling, especially in the cones, compounding the effects of Müller cell activation and may contribute to the visual symptoms and photophobia reported by patients with MIS and related disorders.

Light aversion (photophobia or photo-allodynia) under non-pathological conditions is considered to be mediated by ipRGC, whereas, pathological causes of photophobia are

thought to be ipRGC-independent, modulated instead by rod and cone photoreceptors.[540] Migraineurs reporting pattern glare were more likely to choose a blue to green coloured filter for maximum comfort.[830]

Intrinsically photosensitive RGC are maximally sensitive to blue light and may regenerate oxidised Opn4 using yellow light, however these cells can also adapt.[1228] It is feasible that the filter providing maximum comfort is that selected with a colour or combination of colours that equalises the actual and expected levels of retinal illumination. This may be achieved by resolving any incongruity between the contribution of the retino-hypothalamic tract (ipRGC) and the retino-geniculo-cortical pathway (cones and rods). Whether this is achieved by colour-specific adaptation, stimulation or a mixture of these two actions is not known.

Conversely, the photophobia response is greater for blue light compared to green or red light.[1106] There is some evidence that higher levels of MP may be associated with reduced photophobia by selectively blocking blue light (i.e. operating as a yellow filter).[567, 838] Non-pathological photophobia is thought to mediated by ipRGC located in the inner retina.[540] Their retinal location makes it unlikely the MP will reduce blue light exposure however. It may be that MP is able to modulate the photophobia response in ways other than blue light reduction, such as the modulation of Mc activation by reducing light-induced oxidative stress in the retina. (See Mc hypothesis in the introduction to this thesis, section 1.3).

A3.5 Meares-Irlen syndrome and AMD risk factors

The author has noticed that many patients reporting MIS symptoms such as coloured "blobs" also presented with obesity, parents with a history of smoking, light irides or fussy eating habits leading to poor nutritional intake. The similarity with AMD RF was noted. MP was found to mitigate photophobia, improve visual performance and reduce visual fatigue when proofreading.[567, 839, 1224] It is plausible that increasing retinal levels of MP, by lifestyle and dietary modulation, especially for those selecting yellow or orange coloured filters could reduce or resolve MIS symptoms. It is not known whether there is an association between MP non-responsivity, central "dip" spatial profile (possibly secondary to MZ deficiency) and MIS. The central "dip" profile was more commonly observed for those with AMD and AMD FH.[403] If MIS is proven to be a marker for low retinal MP, MIS symptoms may also be an early-onset, predictive marker for increased risk of AMD in later life.

MIS and associated disorders such as ADHD, chronic fatigue and a subtype of dyslexia in which visual recognition is a primary deficit show anomalies in lipid metabolism, including low essential fatty acid status and decreased serum cholesterol.[1229] Genetic expression of the transporter molecule apolipoprotein B-100 (APOB) has been associated with abnormal lipid metabolism, and particularly with cholesterol levels. Cholesterol esters are important carriers of essential fatty acids (and possibly MP) into the retina.[455, 1229] A pilot study has shown that certain allelic variants of the APOB gene were more common in those diagnosed with MIS compared to those without MIS.[1229]

A3.6 Meares-Irlen syndrome and the potential for increased retinal lipofuscin deposition

Patients with migraine and those with a primary FH of migraine also appear more likely to report MIS symptoms and benefit from precision tints.[830, 1230] Migraine is associated with reduced or absent habituation to repetitive stimuli,[565, 566, 847-849] and a reduction in neurovascular coupling in response to flickering lights.[563, 617, 1231] Other conditions associated with reduced OVP such as Rph and VDys also exhibit these adaptation deficits,[194, 205, 618, 967] and are associated with migraine,[205] however the author is not aware of any studies examining their association with MIS.

It is plausible that the retina / RPE / choroid complex is less able to down-regulate metabolic activity in response to increased light levels if retinal adaptation is abnormal. Therefore patients with MIS may be more prone to the life-long accumulation of lipofuscin, particularly if this is associated with increased levels of Mc activity leading to activation of these cells. Visual cycle modulators were found to reduce RPE drusen accumulation in

murine models and were associated with reduced GA lesion growth and reduced incidence of CNV in one human study.[1232, 1233]

A3.7 Summary

The retinal theory of MIS combines two factors, which may occur secondary to retinal inflammation due to low dopamine or reduced OVP (ischaemia), acting alone or in combination.

1) Increased GRT due to Mc activation resulting in the perception of after images reported as coloured "blobs". Cortical glial activation may contribute to cortical hyper-excitability.

2) A state of adaptation that is incompatible with the level of retinal illumination, resulting from a light-induced increase in retinal dopamine and hypersensitive retinal (and / or cortical) dopamine receptors.

The retinal theory for MIS is complementary to the current cortical hyper-excitability theory for MIS. MP may modulate the symptoms reported in MIS. Macular pigment optical density is a marker for cortical L and Z levels.[430, 1006, 1012] In the absence of any cortical explanation, the retinal theory may provide an alternative explanation for MIS symptoms. Further research is necessary to confirm this theory.

In addition to offering an explanation for the light sensitivity symptoms reported for MIS, the retinal theory may also explain light sensitivity in other disorders associated with low dopamine levels, such as migraine and ADHD. This theory may also explain the association between MIS and migraine.

MIS may be treatable using diet and lifestyle modulation in cases where these factors are sub-optimal and modifiable.

A3.8 Further work relating to MIS

A3.8.1 Do individuals with symptoms of MIS have lower MPOD?

Higher levels of retinal MP were associated with shorter GRT, as well as reduced photophobia and glare.[620] Objective measurement of MPOD would be quicker and more informative (spatial profile data) than subjective MPOD testing, which in view of the bright background and flicker may not be tolerated. The objective method of choice would be 2-WFAF using the Spectralis HD-OCT, however the high level of flicker inherent in this method may also not be tolerated. Bernstein *et al*. assessed MPOD in infants and children using data derived from blue-light reflectance imaging with a digital video fundus camera (RetCam II or RetCam 3, Clarity Medical Systems inc., Pleasanton, CA).[1141] This method may be suitable for MIS sufferers but does involve ocular contact. Objective measurement of MPOD using the Visucam 200 (Carl Zeiss Meditec AG, Jena, Germany) takes a few seconds and is likely to be tolerated by those with MIS, however there are reservations about the suitability of this instrument for use in clinical and research settings.[1234, 1235] If MIS is associated with significantly lower MPOD, a double-masked, randomised controlled trial may establish whether MP supplementation could ameliorate MIS symptoms. It would be of interest to establish whether MIS symptoms modulated with a yellow or orange tint is associated with lower baseline MP (a yellow-coloured, blue-light-blocking filter) and a greater increase in MPOD after supplementation, compared to those modulated with other colour tints.

A3.8.2 Do individuals with symptoms of MIS have longer GRT?

Individuals with MIS report symptoms suggestive of after images after viewing bright white backgrounds. These symptoms are often ameliorated by reducing the brightness of the background. Subjecting a group of individuals that exhibit increased light sensitivity to equilibrium bleach GRT testing would be challenging. Better results may be obtained by photo-flash bleach techniques because of the difficulty in tolerating the longer exposure time required by the latter (MIS sufferers would close their eyes or look away). Recovery from photostress in normal individuals was reported to be shorter for brighter compared to

dimmer backgrounds, regardless of the brightness or exposure time of the glare source.[1213] A comparison of the recovery time between individuals with MIS to non-MIS controls for a range of background brightness may reveal differences in recovery time (longer GRT for MIS), particularly longer than normal GRT for higher luminance backgrounds.

The author has developed a screening test for glare related to white backgrounds by using reading matter illuminated overhead with a dimmable light. Illumination is decreased from the full brightness setting and then increased from a low illumination setting to gauge a comfortable level of white background brightness. Although this has not been tested experimentally, the author has noticed that individuals reporting coloured "blobs" on interactive whiteboards tend to prefer much dimmer white background brightness. This could be adapted to a portable unit to screen for MIS symptoms.

An alternate experiment would be to present a range of photostress luminances or exposure times to MIS and non-MIS controls and measure the recovery objectively using the densitometer developed by Margrain *et al*.

A3.8.3 Intrinsically Photosensitive RGC function in migraine, Rph, VDys and MIS

Intrinsically photosensitive RGC function may be directly assessed by comparing the sustained post-illumination pupil response to blue and blue-free light. Although this was designed to assess ipRGC function for glaucoma,[1236] it may also be used to detect ipRGC dysfunction in OVP RF and MIS. Multifunctional electroretinogram (mERG) may be used to detect delayed implicit time, which may indicate retinal ischaemia secondary to reduced choroidal perfusion.[220]

A3.8.4 Investigation of AMD risk genes and MIS

If it could be established that MIS is wholly or partly a retinal phenomenon, it would be of interest to investigate whether individuals with MIS symptoms as youths are at greater risk of developing AMD in later years. Genetic testing for risk genes associated with AMD and conditions known to cause increased retinal lipofuscin such as adult vitelliform and familial dominant drusen may be undertaken. If it was proven that MIS is associated with increased risk of AMD and / or other age-related maculopathy, individuals exhibiting symptoms suggestive of MIS would represent a "holy grail" in AMD research; a youth onset symptomatic marker for an age-related condition.

A3.8.5 How do Vista-Mesh lenses modulate MIS and migraine symptoms

Vista-Mesh spectacle lenses (Norville Group, Gloucester, UK) combine a very fine aperture (0.6 mm) mesh filter with a 90% light transmission factor brown contrast filter and a hard / multilayer anti-reflection coating. Anecdotal evidence suggests that these lenses reduce glare and improve comfort and visual performance in conditions such as MIS, migraine, stroke, keratoconus and glare related to white backgrounds and fluorescent lighting. Vista-Mesh lenses appear to be effective when used for conditions that also benefit from individually-selected coloured overlays and tints. How Vista-Mesh lenses, and indeed coloured overlays and tints improve visual performance is not fully understood.

Recent evidence from guinea pig Mc has revealed that they not only act as light guides, trafficking light from the inner to outer retina,[1016] they also spectrally filter red and green light to cones and, blue and purple light to rods.[1017] Activation of Mc is likely to reduce their light guiding and spectral filtering capability. It is possible that the polarising or collimating effect of the mesh filter in Vista-Mesh lenses could reduce retinal light scatter, improving light transmission through and spectral filtering within the retina. Masking would be difficult in a randomised controlled trial of Vista-Mesh lenses against control (tinted lenses without the mesh filter), because the mesh is just visible on close inspection. A double-masked lab-based study, where the examiner and participant are prevented from handling or inspecting the lenses, while performing speed of reading and glare sensitivity tests may be more appropriate.

Appendix 4

A4.1 Recruitment slip enclosed with patient reminders

Would you like to take part in a practice-based research study?

John Everett will be examining the relationship between the retinal nutrients, lutein and zeaxanthin, and the ability to recover from a bright light.
The relationship between these retinal nutrients and other factors such as age, gender, Body Mass Index (BMI), iris colour and family history of macular degeneration will also be examined.

It is free to participate and takes about 1 hour to complete the tests.

A4.2 Recruitment poster displayed in three Cheltenham practices

ARE YOU EATING ENOUGH

LEAFY GREEN VEG?

IT IS NOW POSSIBLE TO MEASURE THE LEVELS OF THE MACULAR PIGMENTS, **LUTEIN** AND **ZEAXANTHIN** IN THE RETINA

HIGH LEVELS OF THESE RETINAL NUTRIENTS, OBTAINED SOLELY FROM YOUR DIET, ARE ASSOCIATED WITH A LOWER RISK OF DEVELOPING AGE-RELATED MACULAR DEGENERATION (AMD)

WE ARE CURRENTLY PERFORMING A STUDY OF MACULAR PIGMENT LEVELS IN THE CHELTENHAM POPULATION

WE ARE OFFERING THIS TEST, AND SEVERAL OTHERS EXAMINING AMD RISK **FREE OF CHARGE** TO ANY ELIGIBLE PATIENT THAT IS HAPPY TO ATTEND A SINGLE, 60 MINUTE APPOINTMENT AT OUR NORVILLES, BATH ROAD PRACTICE

PLEASE ASK AT RECEPTION FOR FURTHER DETAILS AND ELIGIBILTY REQUIREMENTS

A4.3 Information sheet for potential participants

Information sheet for volunteers

Title of Project:
Glare Recovery Time as a surrogate test for, and the association other factors with, Central Macular Pigment Optical Density

Research Venue: Norville Opticians, 182 Bath Rd, Cheltenham, Glos, GL53 8HR

Name of Investigators: John Everett & Dr Hannah Bartlett

Invitation

You are 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.

EXPLANATION OF ANY POSSIBLE HAZARDS AND THE PROCEDURES TO BE USED

We are investigating ways of assessing the health of the eyes, in particular the retina. We will be measuring the levels of certain nutrients (lutein and zeaxanthin) in the retina, and comparing these measurements with how well the retina recovers from a bright light. This study involves one visit that lasts for about one hour.

During the visit various measurements will be taken. Most of the measurements will be familiar to you from your routine eye examination. You will undergo measurement of your visual acuity, your retinal appearance, and a grid chart to screen for distorted vision, your iris colour, your weight and height.

Two of the tests are slightly different from what you may have experienced during a normal eye examination. The first test measures the level of the retinal nutrients and simply involves looking at a small light target for a few seconds, while your chin is resting on a chin rest. The second test measures how long it takes for your eye to recover from a bright light and involves looking into a light source for a short period of time.

None of these tests will have any lasting effects after the examination. You will be able to drive to and from the examination. I would only ask that you refrain from drinking any alcohol on the day of the test, before you are examined, as this may affect some of the results.

These tests are not diagnostic and do not constitute a full eye examination. We will however inform you if any abnormality is found and we will discuss with you referral to an appropriate professional for that abnormality. You are perfectly free to ask any questions about any aspect of the study before deciding whether or not to take part. You are also free to leave the study at any time, without giving a reason.

You will be informed if any of the tests reveal results that do not fall within "normal" ranges. You will be given advice on what type of action, if any you need to take. We will also offer follow-up tests to track your progress if necessary.

Explanation of potential hazards

Some light-sensitive individuals may be affected by the bright and flickering lights, presented in the study. It is important that you tell us before you are tested, if you are sensitive in this way.

The retinal nutrients test involves being seated, while your chin is resting on a chin rest. Although this test is completed quickly (within 5 minutes), you will be able to take any breaks that you need. This is important if you suffer from any neck or back conditions.

The light recovery test involves looking into a safe, bright light for 30 seconds. You will experience an after-image when the light removed, which is temporary, lasting less than 1 minute in most cases.

EXPENSES AND PAYMENTS?

There are no expenses or payments for taking part in this study, however all examinations related to the study will be carried out without charge, and you will have access to a novel eye testing instrument, not normally available in a routine eye examination.

CONFIDENTIALITY OF INFORMATION

The confidentiality of personal information and the anonymity of all volunteers involved in this study will be preserved by storage of the data in a locked filing cabinet and the use of number codes instead of names. This locked cabinet will be accessible only to John Everett. Electronic copies of data sheets will be anonymous and held on a personal computer with password protection.

WHO HAS REVIEWED THE STUDY?

This study has been submitted for approval to Aston University's Ethics Committee.

CONTACT DETAILS OF THE INVESTIGATORS

John Everett everetdj@aston.ac.uk
Dr Hannah Bartlett H.E.Bartlett@aston.ac.uk

A4.4 Inclusion / exclusion sheet for participants

Retinal Nutrient Study

Which patients are needed?

If you are not sure whether these apply to you, please contact me and I will advise you.
Telephone Email everetdj@aston.ac.uk

Please note that some of these conditions will not be apparent until I can see you in person and examine you.

Patients eligible for this study (Inclusion criteria):

- Gender: Male and Female
- Age: 20 years or older
- BMI: 20-30
- Corrected visual acuity of 6/8 or better
- Healthy macular (back of the eye) appearance
- No Amsler (Grid chart) distortion
- Normal reported cholesterol levels
- Willingness and ability to give written, informed consent and willingness and Ability to comply with the study requirements
- A family history of macular degeneration.
- Taking multivitamins and / or supplements.

Patients not needed for this study (Exclusion criteria):

- Age: <20 years
- BMI: <20 or >30
- Corrected visual acuity less than 6/8
- Amsler distortion
- Abnormal macular appearance
- Current macular disease or history of macular disease
- Reported raised cholesterol level
- Current pregnancy
- Smokers
- Alcohol consumption within two hours
- Diabetics
- Glaucoma
- Poor night vision
- Dietary absorption disorders (e.g. Crohn's)
- Current use, or previous use of medications that are known to affect macular function
- Inability to give informed consent
- Refusal to give written, informed consent and / or refusal to comply with the study requirement.

Please turn over

Aston University

Information on this page has been removed for data protection purposes

Removal of a participant during the study:

- Inability to perform reliably on any of the ophthalmic tests performed
- Pupils too small to allow macular view, whilst performing glare recovery test
- Inability to fixate ophthalmoscope light (eccentric fixation, nystagmus)

If you are not sure if your medication affects macular function, please contact me with a list of the tablets that you take. Please note however, that if a tablet lists an eye side effect, it does not mean that every patient taking that tablet will experience that side effect. Discontinuing any medication without the consultation and agreement of your General Practitioner (GP) is not recommended.

Consent Form

Principal Investigator: John Everett, Norville Opticians, Cheltenham
Research Supervisor: Dr Hannah Bartlett, Aston University

Project Title: Evaluation of Glare Recovery Time as a surrogate for Central Macular Pigment Optical Density.

Invitation

You are 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 determine whether glare recovery time, using the direct ophthalmoscope macular stop, may be used as a surrogate test for central macular pigment optical density measurement. I want to analyse your results to assess whether glare recovery may be used as a cheaper and quicker surrogate, screening test for central macular pigment measurement. Your results will also be analysed to examine the relationship between central macular pigment optical density and your gender, age, BMI, iris colour, eye tested and the time of day (number of daylight hours). The relationship between family history of macular degeneration will also be compared with macular pigment levels.

Why have I been chosen?

You have been chosen by the practice computer as one of 600 consecutive patients, from a set date, due a routine reminder for your next eye examination.

What will happen to me if I take part?

By volunteering to take part you will be giving anybody in the research team consent to analyse your results. Other than the examination described above, you will not be required to carry out any further tasks.

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

There is a risk of breaching privacy and confidentiality in relation to the data that are collected. Keeping your data anonymous at all times will minimize this risk. As your optometrist, Mr. Everett will have access to your clinical records. He will be responsible for putting your results onto a database and maintaining your privacy and confidentiality. Dr Bartlett will only be given access to your data after your identity has been concealed.
Looking into the bright light for 30 seconds may be slightly uncomfortable.
There is a theoretical risk of triggering a migraine or an epileptic seizure in patients who already have these conditions, but have not yet been diagnosed. This effect has not been reported in any of the literature revealed by an extensive literature search.
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 at any time from the study. No sanctions will be taken against any patient who refuses to participate in or withdraws from the study.

Expenses and payments?

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

Will my taking part in this study be kept confidential?

Your data obtained during this study will be recorded such that your identity will remain obscured. Access to your data will be limited to Dr Bartlett and John Everett. Your data will be erased at the end of the study.
The patient records for those in any research study must be kept for 15 years after the study ends, and therefore your records will be marked accordingly. Privacy and confidentiality will be protected vigorously to the extent permissible by law. We cannot however guarantee privacy or confidentiality.
The maintenance of privacy and confidentiality does not protect the participant from divulging information about the study themselves, if questioned by other parties.

What will happen to the results of the research study?

We aim to publish the results of this study. Your data will be anonymised.

Who is organizing and funding the research?

John Everett is organizing and funding the study, with supervision from Dr Bartlett.

Who has reviewed the study?

This study 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 feel free to contact Dr Hannah Bartlett, (H.E.Bartlett@aston.ac.uk, (01212) 2044182).

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 this study has been conducted, then you should contact the Secretary of the University Research Ethics Committee on j.g.walter@aston.ac.uk or telephone 0121 204 4665.

Personal identification number for this study:

CONSENT FORM

Project Title: Evaluation of Glare Recovery Time as a surrogate marker for Macular Pigment Optical Density

Research venue: Norville Opticians, Bath Road, Cheltenham

Principal Investigator: John Everett
Project Supervisor: Dr Hannah Bartlett

		Please initial box
1	I confirm that I have read and understood the consent form and supporting information, 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 participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my Optometric care or legal rights being affected.	
3	I agree to take part in the above study.	

_____	_____	_____
Name of research participant	Date	Signature

_____	_____	_____
Name of person taking consent	Date	Signature

1 copy for research participant 1 copy for principal investigator

A4.6 Data collection sheet

Data collection sheet CMPOD / GRT study Date

Participant identification number for this study:

Excluders:

LogMAR VA LogMAR 0.1 or better	Y / N	Patient VA	R	L
		VA for GRT	R	L
Amsler distortion		Y / N		
Pupils too small to view macular		Y / N		
Healthy macular appearance		Y / N		
No nystagmus or eccentric fixation		Y / N		
Weight	Kg			
Height	Metres			
BMI	Kg / m^2 >20 / <30	Y / N		
Cholesterol reported normal		Y / N / U		
No dietary absorption disorders		Y / N / U		
Pregnancy		Y / N / U / N/A		
Smoker		Y / N		
Alcohol consumption within 2 hours		Y / N		
Diabetes		Y / N / U		
Glaucoma		Y / N / U		
Poor night vision		Y / N / U		
Medication affecting macular function		Y / N / U If Y, record overleaf		
Inability / refusal to give consent		Y / N		

Glare-triggered conditions:

Migraine / epilepsy	Y / N / U Warn if Y or U	
Migraine Aura / Light-Triggered	Raynaud's / Vascular Dysregulation	

Study data:

Gender	M / F / U / Not Disclosed			
Date of birth	/ /			
Age (decimal)	Years			
Iris colour	Grey / Blue / Green / Hazel / Brown			
Time of day	am / pm			
Number of daylight hours	Hours			
Coin toss	Heads = R	Tails = L		
Eye tested first	R / L			
CMPOD				
R			IOL	Y / N
L			IOL	Y / N
GRT	First values		Second values	
R	seconds		seconds	
L	seconds		seconds	
GRT Repeated	**DATE:**	**TIME:**	**RANDOM LETTERS**	
	First values		Second values	
R	seconds		seconds	
L	seconds		seconds	
MPOD Ease of use	No diff. / Diff. with central R or L / Diff. with peripheral R or L			

Family history of AMD	Y / N	1^{st} degree / 2^{nd} degree
Supplements / vitamins	Y / N	If Y, record overleaf

Key: Y=Yes, N=No, U=Unknown, N/A=Not Available

A4.7 Information summary for all study participants (included and excluded)
(To be sent to participants after thesis is accepted)

The association between MPOD and GRT with selected AMD and ocular vascular perfusion risk factors

Principal Investigator: John Everett, Norville Opticians, Cheltenham
Research Supervisor: Dr Hannah Bartlett and Dr Frank Eperjesi
Aston University

Study aims:

The study was designed to investigate the relationship between macular pigment optical density (MPOD) and glare recovery time (GRT) with the following age-related macular degeneration (AMD) risk factors: age, gender, body mass index (BMI), calculated percentage body fat (%BF), AMD family history and iris colour, and with the following ocular vascular perfusion (eye blood supply) risk factors: migraine, Raynaud's phenomenon and vascular dysregulation.

MPOD: This is a measure of the amount of a yellow pigment in your eye, derived from foods such as leafy green vegetables and eggs.

GRT: This is the time taken for the eye to recover after being dazzled by a bright light.

AMD: This is the most common cause of visual loss in the elderly.

Study results

None of the risk factors tested was related to MPOD.

Right eye MPOD was very similar to left eye MPOD.

GRT was found to increase significantly with age, but after correction for age none of the remaining risk factors tested was related to GRT.

When GRT was repeated for the same person the results were similar (i.e. the results showed good repeatability).

No relationship was found between MPOD and GRT, therefore this method of GRT was not a suitable surrogate test for MPOD testing.

Aston University

BCOVS
16-09-2011

Glare Recovery Time as a surrogate for Macular Pigment Optical Density

John Everett Department of Life and Health Sciences Aston University **everetdj@aston.ac.uk**

Introduction

Macular pigment (MP) consists of the dietary carotenoids; lutein and zeaxanthin, plus mesozeaxanthin, derived from retinal lutein (Fig.1). Low levels of MP have been associated with a higher incidence of age-related macular degeneration (AMD) and increased glare.

AMD is the most common cause of blindness in the Western world.

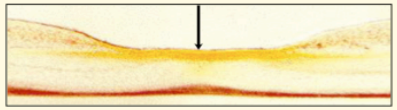


Fig. 1 Section of the human macula showing yellow MP. (Nolan 2010)

MP affects macula health by:

- Blocking blue light
- Eliminating free radicals
- Reducing inflammation
- Neuroprotection

Glare recovery time (GRT) is a diagnostic test used to differentiate macular disease from optic nerve disease.

Body mass index (BMI), iris colour, family history of AMD and supplement use, are thought to be associated with macular pigment optical density (MPOD), in addition to being confirmed or putative risk factors for AMD.

Aim of this study

- Is there a correlation between MPOD and GRT?
- Is there a correlation between MPOD and . . . ?
  - BMI, Iris colour, Age and gender
  - Right versus left eye
  - First versus second eye tested
  - Time of day (daylight hours)
  - Nutritional supplements
  - Family history of AMD
- Any correlation with migraine (light versus non-light triggered and presence versus absence of aura), Raynaud's and vascular dysregulation will also be assessed.

Method

130 healthy participants of the total 150 have been seen. 44 were excluded. MPOD was measured by heterochromatic flicker photometry, using the Tinsley M-POD Screener.

GRT was recorded after 30 seconds macular bleach using the Keeler direct ophthalmoscope macular stop. GRT mean 50.2 secs (SD 13 secs) with this method.[1] This is reported to bleach 96% of macular cones.[1] GRT inter and intra session repeatability will be tested.

Eye order was randomly allocated by coin toss.
BMI was calculated from participant weight and height.
Iris colour was confirmed by participant and examiner.

Results (Data collection is not complete)

MPOD (density units):

Eye 1	Mean 0.41	SD 0.16	n=86
Eye 2	Mean 0.40	SD 0.16	n=86
Right eye	Mean 0.40	SD 0.16	n=86
Left eye	Mean 0.41	SD 0.16	n=86

GRT (seconds):

			2nd GRT 10 minutes after 1st
Eye 1(1st)	Mean 51.7	SD 26.1	n=86
Eye 1 (2nd)	Mean 43.9	SD 19.2	n=86
Eye 2(1st)	Mean 48.8	SD 23.2	n=86
Eye 2(2nd)	Mean 42.8	SD 19.2	n=86

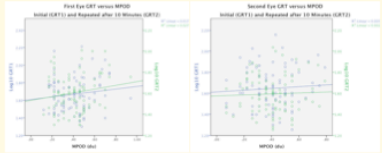
Paired-samples t-test (Reduction in GRT from eye 1 to eye 2)

GRT 1	t (82) = 1.59, p = 0.115 (2 tailed)	Not significant
GRT 2	t (82) = 0.95, p = 0.344 (2 tailed)	Not significant

Paired-samples t-test (Reduction in GRT from 1st to 2nd value)

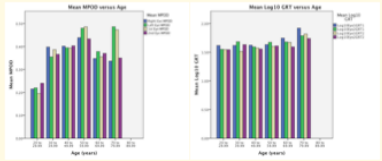
Eye 1	t (85) = 5.56, p < 0.0005 (2 tailed)	$Eta^2 = 0.27$
Eye 2	t (85) = 6.24, p < 0.0005 (2 tailed)	$Eta^2 = 0.31$

Wolffsohn et al. (2006) reported lower repeated GRT values after 10 minutes by 1.6 seconds (SD 5.2) using the Eger macular stressometer (p<0.05).[2]

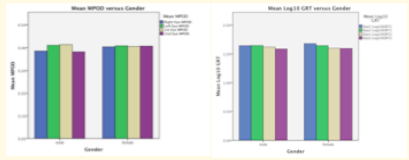


Pearson Correlation (Relationship between MPOD and GRT)

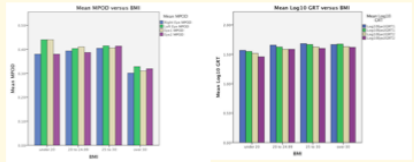
Eye 1 (GRT1)	r = 0.13, P = 0.235	Not significant
Eye 1 (GRT2)	r = 0.17, p = 0.130	Not significant



n = 81



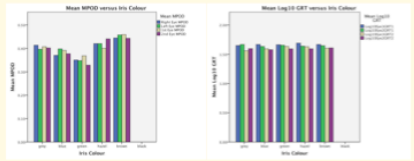
Mean MPOD



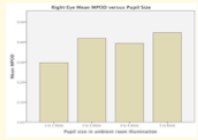
Mean MPOD (First Eye)

Independent-samples t-test (Reduction in MPOD with BMI)

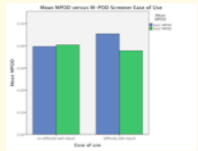
Normal / Overweight	t (84) = 0.12, p = 0.902 (2 tailed)	Not significant
Normal / Obese	t (57) = 2.03, p = 0.047 (2 tailed)	$Eta^2 = 0.06$

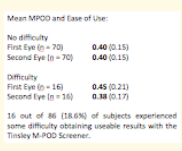


Mean MPOD (First Eye) Iris Colour:



Mean MPOD (Right Eye) Pupil size:





Mean MPOD and Ease of Use:

No difficulty
First Eye (n = 70) 0.40 (0.15)
Second Eye (n = 70) 0.40 (0.15)

Difficulty
First Eye (n = 16) 0.45 (0.21)
Second Eye (n = 16) 0.38 (0.17)

16 out of 86 (18.6%) of subjects experienced some difficulty obtaining useable results with the Tinsley M-POD Screener.

Case Reports

Two cases with Squint with eccentric fixation:

Case 12	R squint	R MPOD **0.02** L MPOD **0.22**
Case 83	L squint	R MPOD **0.26** L MPOD **0.07**

One case with reported long-term St John's Wort use:

Case 8	Eye 1 MPOD **0.41**	R GRT **80** secs, **86** secs
	Eye 2 MPOD **0.55**	L GRT **135** secs, **80** secs

One case with right optic disc coloboma:

Case 131	R MPOD **0.07**	Repeat R MPOD **0.12**
	L MPOD **0.22**	Repeat L MPOD **0.17**

This case is now undergoing nutritional supplementation (Nutrof Total) for two months.

One case with low MPOD after 12 months better diet:

Case 1	R MPOD **0.19**	GRT **58** secs, **80** secs
	L MPOD **0.24**	GRT **80** secs, **75** secs
1 year on:	R MPOD **0.26**	GRT **34** secs, **33** secs
	L MPOD **0.36**	GRT **30** secs, **29** secs

Conclusion

A strong correlation between MPOD and GRT would suggest that GRT could be considered as a surrogate test for MPOD, for healthy patients without eye disease in Optometric practice.

Stringham et al. (2011) controlling for iris pigmentation and pupil constriction reported a strong, negative correlation between MPOD and GRT ($p < 0.003$).[3] Loughman et al. (2010) however found no statistical relationship between MPOD and GRT.[4]

Initial analysis suggests a non-significant, weak, positive correlation between MPOD and GRT. Further statistical analysis, controlling for relevant variables that may be controlled in a practice setting will be performed when data collection is complete.

The spectral emission of the direct ophthalmoscope used in this study will be tested. Low blue light emission may contribute to the lack of significant, negative correlation between MPOD and GRT.[4]

References

1 Margrain and Thompson 2002 OPO 22:62-7
2 Wolffsohn et al 2006 BJO 90:432-434
3 Stringham et al 2011 IOVS (online ahead of print)
4 Loughman et al 2010 Vis Res 50:1249-1256

Study supervisors

Dr Hannah Bartlett PhD PGCertEd FAAO
Dr Frank Eperjesi PhD Dip Orth FAAO MHEA PGCertHE

Appendix 5

A5.1 Confirmation of ethics clearance for project AO2010.15 HB

Note that the title of the study has been altered.

Aston University
Life & Health Sciences

Response from AOREC

12th May 2010

Project title: ***Glare Recovery Time as a surrogate test for, and the association of other AMD risk factors with, Central Macular Pigment Optical Density***

Reference Number: AO2010.15 HB
Researchers: David John Everett, Dr Hannah Bartlett, Dr Frank Eperjesi

I am pleased to inform you that the Audiology / Optometry Research Ethics Committee has approved the above named project.

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

Yours sincerely

Aston University
Information on this page has been removed for data protection purposes

Chair AOREC

A5.2 Approval for the amendments to project AO2010.15 HB

Note that the title of the study has been altered.