Functional biomarkers of hypoxia in age-related macular degeneration.

A thesis submitted to Cardiff University for the degree of

Doctor of Philosophy

By

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Summary

Age-related macular degeneration (AMD) is predicted to affect 196 million people by 2020 (Wong et al. 2014). To date there is no clear pathogenesis for the condition however, hypoxia has been implicated (Stefánsson et al. 2011). Currently, treatment is only available for neovascular AMD. To develop treatments targeted for early AMD a better understanding of the pathogenesis is required. There is also a need for sensitive functional biomarkers to improve diagnosis and monitoring and to expedite the evaluation of therauptics in clinical trials.

The aim of this research was to investigate the hypothesis that hypoxia is involved in the pathogenesis of early AMD. Studies were carried out exploring the effect of transient systemic hypoxia (14% oxygen) and hyperoxia (60% oxygen) on scotopic thresholds and electroretinograms (ERGs) of participants with early AMD. It was hypothesised that the visual function of participants with AMD, but not age-matched controls, would improve during the hyperoxic episode and that hypoxia would have a greater detrimental effect on visual function in people with early AMD.

There were no significant differences in scotopic thresholds within each group when breathing 60% or 14% oxygen compared to medical air (21% oxygen). There were also no significant differences in full-field ERG parameters between gas conditions or groups, apart from the amplitude of the b-wave which was significantly reduced under hypoxia in the control group. The amplitude of the focal flicker ERG was significantly higher in the control group than the AMD group when breathing both 14% and 21%

oxygen. However, there were no significant differences in the parameters of the focal ERG within each group.

These findings suggest that hypoxia is not responsible for the elevation of scotopic thresholds reported in AMD. There is also no evidence that ERG changes are attributable to hypoxia. This thesis provides no evidence to support the role of hypoxia in the pathogenesis of early AMD.

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List of Abbreviations

AMD	Age-related Macular Degeneration
AREDS	Age-Related Eye Disease Study
ARM	Age-Related Maculopathy
ATP	adenosine triphosphate
CFH	Compliment factor H
COPD	Chronic obstructive pulmonary disorder
CRBP	Cellular retinol binding protein
CNV	Choroidal neovascular membrane
CS	Contrast sensitivity
DA	Dark adaptation
ERG	Electroretinogram
FA	Fluorescein angiography
FAF	Fundus autofluorescence
FDT	Frequency doubling perimetry
HIF	Hypoxia-inducible-factor
GA	Geographic atrophy
GPD	Guanosine diphsophate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate

HSP90	Heat shock protein 90
ICG	Indocyanine green angiography
IRBP	Interstatial retinaldehyde binding protein
JNK	Jun kinases
KYN	Kynurenic acid
logMAR	Logarithm of the minimum angle of resolution
LRAT	Lecithin retinol acyl transferase
MAC	membrane attack complex
mfERG	Multifocal electroretinogram
nAMD	Neovascular Age-related Macular Degeneration
NICE	National Institute for Clinical Excellence
NO	Nitric Oxide
O_2	Oxygen
OCT	Optical coherence tomography
OP	Oscillatory potential
PED	Pigment epithelial detachment
PEDF	Pigment Epithelial Derived Factor
PEST	Parameter estimation by sequential testing
PDA	Piperidine-dicarboxylic acid
PDE	Phosphodiesterase
PhNR	Photopic negative response
PR	Photoreceptor
QUEST	Quick estimate by sequential testing

RDH	All-trans retinol dehydrogenase
ROI	Reactive Oxygen Intermediates
RPE	Retinal Pigment Epithelium
SAP	Standard automated perimetry
SDF-1	Stromal cell derived factor 1
SITA	Swedish interactive threshold algorithm
SpO_2	Peripheral oxygen saturation
ST	Scotopic threshold
SWAP	Short wavelength automated perimetry
TSP-1	Thrombospondin-1
VA	Visual acuity
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular Endothelial Growth Factor
VEP	Visual evoked potential
VF	Visual field
ZEST	Zippy Estimate of Sequential Testing

1 Introduction

1.1 General introduction

Age-related macular degeneration (AMD) is a disease characterised by progressive central vision loss due to outer retinal degeneration in the macula. It is the leading cause of blindness among white populations in the USA in persons over the age of 40 (Congdon et al. 2004) and is predicted to affect 196 million people globally by 2020 (Wong et al. 2014). There are currently treatments available for one form of the late stage of AMD (neovascular AMD), but there are no treatments targeted at the early stage of the condition, or the other late stage (geographic atrophy). Treatments targeted at early age-related macular degeneration (early AMD) are appealing due to the preservation of many aspects of visual function at this stage. However, for these to be developed, a greater understanding of the pathogenesis of early AMD is required and functional biomarkers for the condition are necessary for clinical trials and for diagnosing and monitoring the condition.

Age-related macular degeneration is a multifactorial condition and hypoxia has been implicated in the early pathogenesis (Feigl 2009; Stefánsson et al. 2011). This thesis primarily aims to investigate the hypothesis that hypoxia is involved in the pathogenesis of early AMD and whether aspects of scotopic visual function can be used as functional biomarkers of hypoxia in early AMD.

Chapter one will provide an overview of the healthy retina, tissue oxygenation, hypoxia and AMD. Chapters two and three provide systematic reviews of literature exploring the effect of hypoxia on visual function and the role of hypoxia in the pathogenesis of AMD. Chapters four to seven are experimental chapters describing the effect of hyperoxia and hypoxia on scotopic visual function and Chapter 8 concludes the thesis by providing a general discussion and plans for future work.

1.2 Morphology and function of the retina and blood supply

This section will provide an overview of the healthy retina and a description of each retinal layer. The later sections will concentrate on the macula and the retinal blood supply.

1.2.1 General retinal structure

The retina is a highly specialised multifunctional tissue consisting of nine layers lying between the vitreous and the choroid of the posterior eye (Klob 2012). During phototransduction, the photoreceptors collect photons of light energy and transform them into electrical impulses to be interpreted by the visual cortex. Although each layer is specialised to perform a specific function, each is also intricately linked to and dependent on the others. Therefore successful retinal function is dependent on a normal physiology of all the layers. Figure 1-1 shows the retinal layers in more detail.

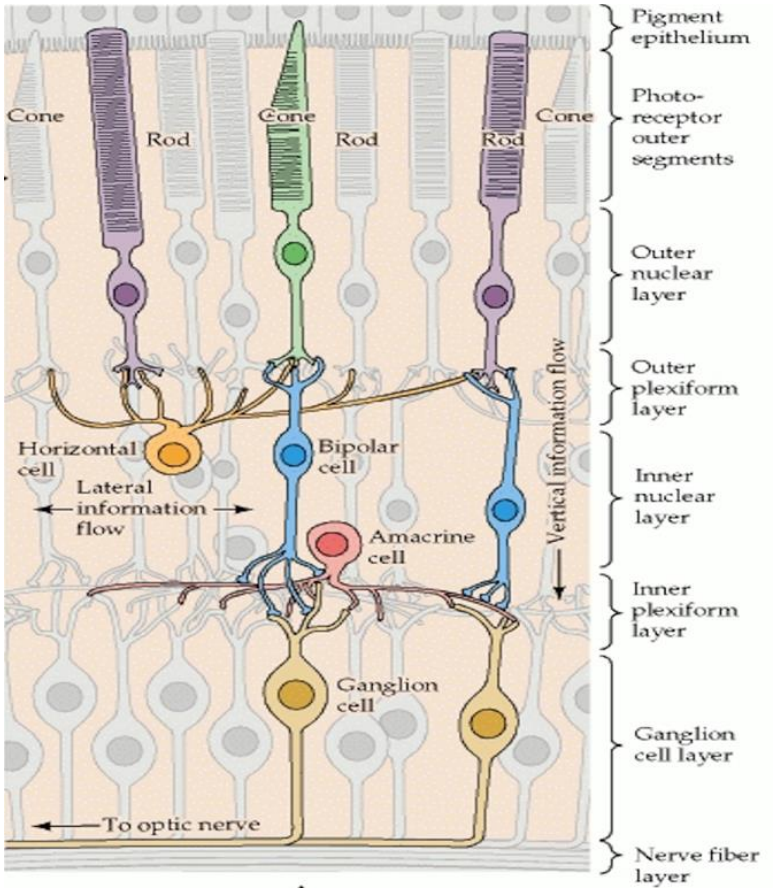


Figure 1-1 Diagram of the structure of the retina (Purves et al. 2001).

The following section will consider the retinal layers and associated structures in more detail, starting from Bruch's membrane and progressing to the inner layers.

1.2.2 Bruch's membrane

Situated between the choroid and the retinal pigment epithelium (RPE), Bruch's membrane is a 2-4 µm thick (Bhutto and Lutty 2012), five-layer structure. Acellular, it is rich in collagen and elastin with each layer containing ligands with three primary functions: to regulate the diffusion of molecules including nutrients and waste products

between the choroid and RPE, to act as a barrier for cellular migration between the two layers and to provide physical support for RPE cell adhesion (Booij et al. 2010). Bruch's membrane are known to be affected by many factors including age (Pauleikhoff et al. 1990; Hogan et al. 1967) and genetics (Piguet et al. 1993).

The structure of Bruch's membrane undergoes the following changes with age:

- A thickening of 135% in 10 decades (Bhutto et al. 2012).
- A reduced capacity for macromolecular permeability (Moore and Clover 2001), altering the metabolic exchange between the RPE and the choroid.
- An increase in collagen cross-linking which increases strength, but decreases elasticity and has a negative effect on permeability (Booij et al. 2010).
- Calcification which results in brittleness and breaks, facilitating neovascularisation (Spraul et al. 1999).
- The accumulation of advanced glycation end products (AGEs), which could lead to reduced lysosomal enzyme degradative capacity resulting in the accumulation of lipofuscin and RPE dysfunction (Glenn et al. 2009).
- The accumulation of lipid which is associated with the destruction of Bruch's membrane architecture and which may lead to photoreceptor dysfunction (Pauleikhoff et al. 1990).
- A decreased RPE adhesion to Bruch's membrane (Afshari and Fawcett 2009).
- The development of drusen. These are extracellular deposits formed by Bruch's membrane deposited between it and the RPE.

1.2.3 Retinal pigment epithelium.

Lying adjacent to Bruch's membrane on the basolateral side and extending apical villi to surround photoreceptor outer segments, the RPE is a pigmented, single layer of hexagonal cells. It forms a selectively permeable blood retinal barrier between the choroidal blood supply and the photoreceptors. High resistance occluding junctions between cells control retinal homeostasis by regulating the transepithelial movement of nutrients and waste to and from the photoreceptors. It also supports the photoreceptors in their functions in the following ways (see Boulton and Dayhaw-Barker 2001 for review):

- Phagocytosing the periodically shed discs of the receptors.
- Aiding in the regeneration of visual pigments.
- Absorbing scattered light in melanin granules.
- Storing and transporting retinoids.
- Synthesising some growth factors and enzymes.
- Providing a retinal defence against reactive oxygen intermediates (ROIs) though the high density of melanin pigment contained within cells.

Photoreceptor survival is intrinsically linked to the RPE functioning correctly. Poor disc phagocytosis, altered transepithial transport and changes in the secretion of growth factors can all lead to photoreceptor degeneration or proliferative disease (Marneros et al. 2005; Lutty et al. 1999; Jackson et al. 2002). The RPE and the photoreceptors can be considered together as a functional unit. Evidence to support this includes the synergistic

interaction of the tissues in the visual cycle, where the structure of the visual pigment retinal is altered by both the RPE and the photoreceptors. Also, the effect of genetic abnormalities, for example the photoreceptor gene ABCR leads to RPE degeneration, whilst abnormal RPE genes such as RPE65 affect the photoreceptors (Strauss 2005).

1.2.4 Photoreceptor cells

The retina contains over 92 million rod and 4.6 million cone photoreceptors (Curcio et al. 1990) enabling vision across a wide range of wavelengths, light levels and temporal frequencies. Cone photoreceptors provide a higher level of spatial and temporal resolution and allow colour vision, but are insensitive to low levels of light. Rods on the other hand give better sensitivity in low light levels, but poor spatial and temporal resolution and provide only monochromatic vision. The information sent to the visual cortex from phototransduction depends on the amount of light falling on the photoreceptor receptive field, the type of photopigment stimulated and the influence of other photoreceptors and contributing interneurons such as amacrine and horizontal cells.

Rods and cones are not present uniformly across the retina; cone density peaks at the fovea and decreases rapidly with eccentricity. In the peripheral retina the cone density is highest in the nasal region, being 40-45% higher than in the temporal retina (Curcio et al. 1990). Rods are absent from the fovea and rod density is greatest in an elliptical ring around 3-5mm (around 10-15 degrees) from the foveola (Jonas et al. 1992).

As seen in Figure 1-2, structurally both rods and cones have outer segments (cone outer segments have a shorter, conical structure), cell bodies containing the cell nucleus, inner segments containing mitochondria (cone inner segments are around 4 microns larger than the rods) and axon terminal which extends into the outer plexiform layer.

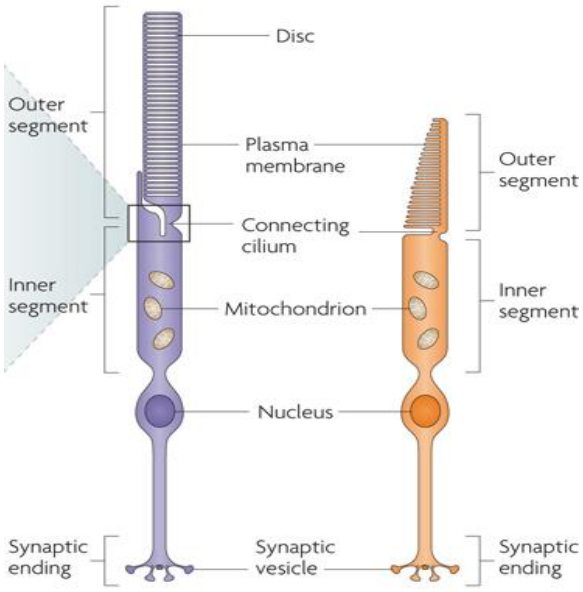


Figure 1-2. Diagram of the rod (left) and cone (right) photoreceptors (Wright et al. 2010).

Both rod and cone outer segments contain stacks of double folded disc membranes containing the visual pigment. This pigment consists of a protein (opsin) and a chromophore (retinal). Rods contain a single visual pigment, rhodopsin, and each cone contains one of three different types of pigment, each excited by a specific wavelength of light. The spectral sensitivity of S-cones peaks at approximately 440 nm, M-cones at 545

nm and L-cones peak at 565 nm. The differential responses of the cone types with their overlapping spectral sensitivity curves allows colour discrimination to occur.

In the dark, photoreceptors are depolarized and metabolically active, resulting in a continuous release of the neurotransmittor glutamate. This activity results in the energy demands of the rods being the greatest of any cell in the body (Arden et al. 2005). Phototransduction takes place in the outer segment of a photoreceptor. It is initiated by light, causing the photoisomerization of the chromophore 11-cis-retinal to all-trans-retinal (Palczewski and Baehr 2005). This triggers hyperpolarisation of the cell, resulting in less glutamate being released at the synapse with the bipolar and horizontal cells in the outer plexiform layer. After phototransduction has taken place, the photoreceptor cannot react to further light until the visual pigment has been regenerated. This regeneration process is refered to as the retinoid cycle, which is the recovery of visual pigment to a regenerated state. Detailed reviews of these processes can be read in Lamb and Pugh (2004) and Lamb and Pugh (2006).

1.2.5 Bipolar cells

Bipolar cells transmit information from photoreceptors to the retinal ganglion cells (RGCs). Their cell bodies are in the inner nuclear layer, with processes extending into both the inner and outer plexiform layers. There have been 11 types of bipolar cell found in the human retina, differentiated by their connection to rods (one type) or cones (ten types) (Kolb et al. 1992). Cone bipolar cells connect to a single cone, or sum information from

only few cones, whereas rod bipolar cells sum information from as many as 40-45 rods in the peripheral retina (Kolb et al. 1992).

Bipolar cells can also be characterised by their response to the signal received from the photoreceptors. ON bipolar cells are stimulated by the decrease in glutamate released by the photoreceptors at light onset and depolarise, sending a graded potential to nearby cells. Whereas OFF bipolar cells are inhibited (hyperpolarise) in response to reduced glutamate at light onset and no signal is sent.

1.2.6 Horizontal cells

Horizontal cells are involved in the lateral modification of the visual signal. Their cell bodies are present in the inner nuclear layer and their dendrites synapse with photoreceptors and bipolar cells. Three types of horizontal cell have been discovered (Kolb et al. 1992) and their main function is to create the centre surround organisation of the bipolar cell receptive field.

1.2.7 Amacrine cells

Amacrine cells are interneurons residing in the inner nuclear layer of the retina. They synapse with bipolar cells and retinal ganglion cells in the inner plexiform layer, serving to modify the retinal signal to the ganglion cells. To date, over 30 different types of amacrine cell have been discovered (Kunzevitzky et al. 2010). AII amacrine cells are the most common amacrine cell type (Demb and Singer 2012) and are unique as they are not

involved in lateral processing but in the vertical flow of information from rods to the inner retina. They integrate rod signals into the cone pathway, which is involved in the removal of noise and enhancing the rod signals under scotopic conditions.

1.2.8 Ganglion cells

Retinal ganglion cells have their cell bodies in the ganglion cell layer, with axons in the retinal nerve fibre layer that exit the eye via the optic nerve. In the primate retina classification is based on the area of terminal projection, giving three main catagories. Magnocellular ganglion cells have large cell bodies and dendritic axons that cover a large area up to 270μm^2 (Kolb et al. 1992). This cellular pathway is stimulated by movement but saturates when viewing targets at high contrast, so can only resolve coarse data (Derrington and Lennie 1984). Parvocellular cells have smaller dendritic trees and are found mainly in the fovea, where they have a 1:1:1 relationship with cone and bipolar cells (Kolb and Marshak 2003), resulting in excellent resolution and colour discrimination. Around 10% of RGCs are konicellular cells which process information regarding blue/yellow colour vision (Yücel et al. 2003). Not all RGCs are concerned with the processing of visual information. A ganglion cell type containing melanopsin comprises of 1-3% of the RGC population (Wässle 2004). These photosensitive ganglion cells have been shown to respond directly to light, sending information to the suprachisamic nucleus and the pretectum, influencing the circadian rhythm and the adaptation of pupil size to luminance (Wässle 2004; Berson 2003).

1.2.9 Specialisation of the macula

Grading scales consider the macular region to be 6mm in diameter, centred on the fovea and corresponding to 20^{o} visual angle (Davis et al. 2005; Bird et al. 1995). The area can be further divided into the foveola, fovela, parafoveal and perifovea. Figure 1-3 shows the densities of rods, cones and RGCs present in the macular region.

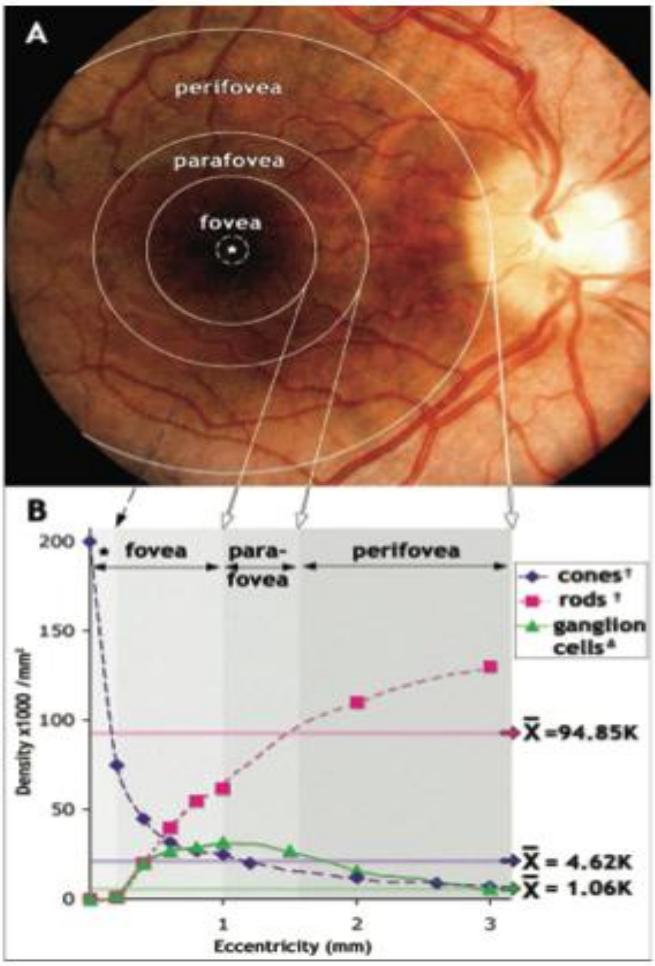


Figure 1-3. Panel A Regions of the macula; Panel B rod, cone and RGC densities across the macula (Provis et al. 2005).

The foveola is located at the centre of the fovea, it is 350µm in diameter and contains only cone and Müller cells (Nag and Wadhwa 2012). All retinal cell layers are present in the remaining area of the fovea which totals 0.8mm in diameter (Curcio et al. 2000). The parafovea, a rod dominated area, is where the retina is at its thickest due to the displacement of the foveal RGCs (Provis et al. 2005). The perifovea is at the periphery of the parafovea and forms the edge of the macula. Figure 1-4 shows the retinal thickness of the macula at different locations and the layers present.

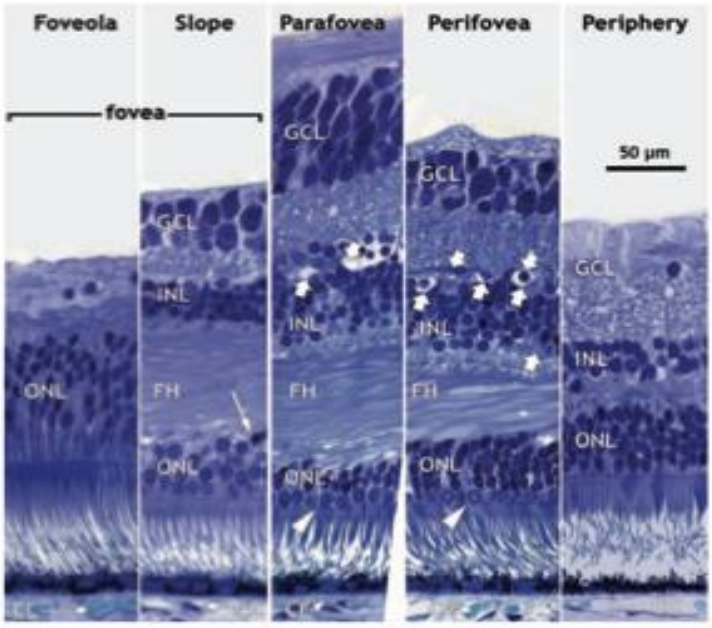


Figure 1-4. Photomicrographs showing the relative thickness of the adult human retina, and its constituent layers, at different locations (Provis et al. 2005). GCL- ganglion cell layer. INL- inner nuclear layer. ONL- outer nuclear layer. FH- fibres of Henle.

The macula contains the highest levels of macular pigment in the retina. Composed of lutein and zeaxanthin, macular pigment absorbs blue light (Pease et al. 1987). Beatty et al. (2000) discussed in their comprehensive review that macular pigment may limit oxidative damage by filtering blue light.

1.2.10 The retinal blood supply

Like all tissues, the retina requires a constant supply of oxygen (O_2). In fact, the levels of O_2 required by the retina have been reported to be higher than those required by the brain (Ames et al. 1992). This is due to the photoreceptors having the highest metabolic demand of any cell, in particular the rods, each of which requires 10^8 ATP s^{-1} in the dark (Okawa et al. 2008). With the stimulation of light, cones use more ATP than rods, however the retina has adapted to reduce this demand by having overall fewer cones in the retina and concentrating them where they are required. Another adaptation to facilitate the effective use of O_2 by the retina is the depolarization of photoreceptors in the dark, this means that in the light, retinal O_2 consumption rate actually decreases by around 36-68% (Wangsa-Wirawan and Linsenmeier 2003). This is mainly due to the drop in ATP required by the rods as the dark current is suppressed, meaning they require less than a quarter of the energy they require in the dark (Okawa et al. 2008).

The retina has a dual blood supply. The inner retinal layers are supplied by the retinal blood supply, and the photoreceptor layer is supplied by the choroidal circulation, which supplies around 90% of the O_2 to photoreceptors in the dark (Wangsa-Wirawan and

Linsenmier 2003). The retinal blood supply can be seen clinically as a sparse system that extends from the central retinal artery, entering the eye via the optic nerve. It then branches into four retinal arteries, which further branch into the superficial and deep vascular plexus.

The choroid is supplied by the ophthalmic artery via the short posterior ciliary arteries that supply the posterior choroid, and the long posterior ciliary arteries that supply the anterior choroid, iris and ciliary body. They pierce the globe around the optic nerve head, branching out to form three layers of the choroidal circulation (Lange and Bainbridge 2012a). The three vascular layers of the choroidal circulation include the outer Haller's layer composed of large blood vessels, the inner Sattler's layer of medium and small arteries and arterioles and the choriocapillaris (Nickla and Wallman 2010). The choriocapillaris is the capillary component of the choroidal vasculature which is a single, continuous layer lying adjacent to Bruch's membrane (Bhutto et al. 2012). It is thickest at the fovea at 10µm, thinning to 7µm in the periphery (Nickla et al. 2010). Venous drainage is from the four vortex veins (Hayreh 1990). The location of retinal and choroidal circulation can be seen in Figure 1-5.

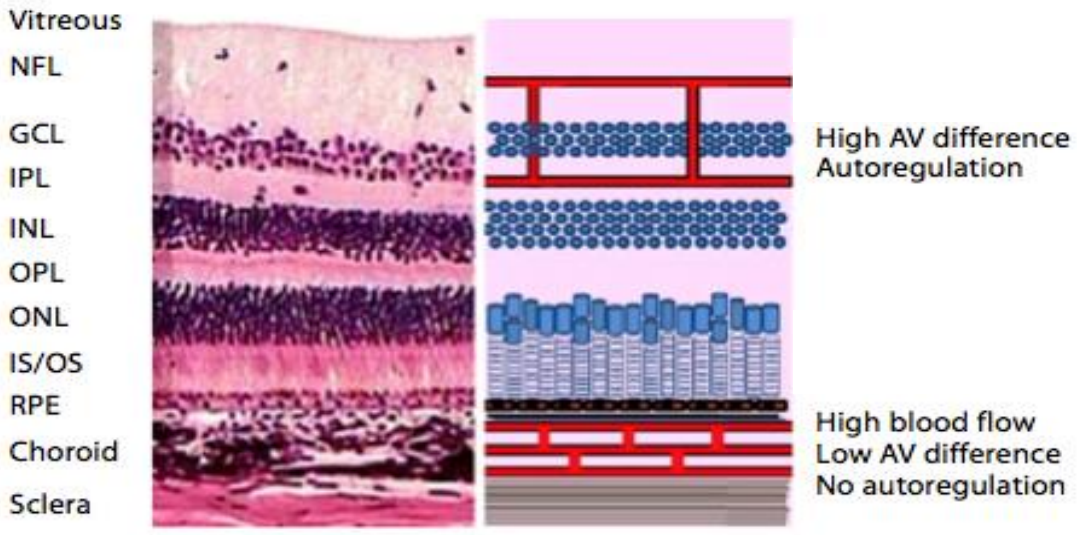


Figure 1-5. Histology and corresponding schematic diagram highlighting the vascular supply of the human retina (Lange et al. 2012a).

The choroidal and retinal blood supplies differ in their blood flow and velocities. The retinal supply has a low blood flow but good autoregulation and a high oxygen extraction ratio (8ml O_2 per 100ml) (Lange et al. 2012a). Its sparse vasculature is advantageous as it does not block light (Caprara and Grimm 2012), which would shadow the underlying photoreceptors. The choroid has poor autoregulation and a poor oxygen extraction ratio, however it is highly vascularised (Caprara et al. 2012) and the blood flow is 20 fold greater than the retinal supply (Lange et al. 2012a).

Despite the fact that the retina seems to be a well vascularised tissue, the O_2 supply has been found to be barely sufficient under dark-adapted conditions. The oxygen tension at the proximal side of the inner segments of the photoreceptors reaches 0mmHg in the dark (Wangsa-Wirawan and Linsenmeier 2003), meaning that the O_2 supplied is only just

sufficient to meet the metabolic needs of the photoreceptors. This can be seen in Figure 1-6.

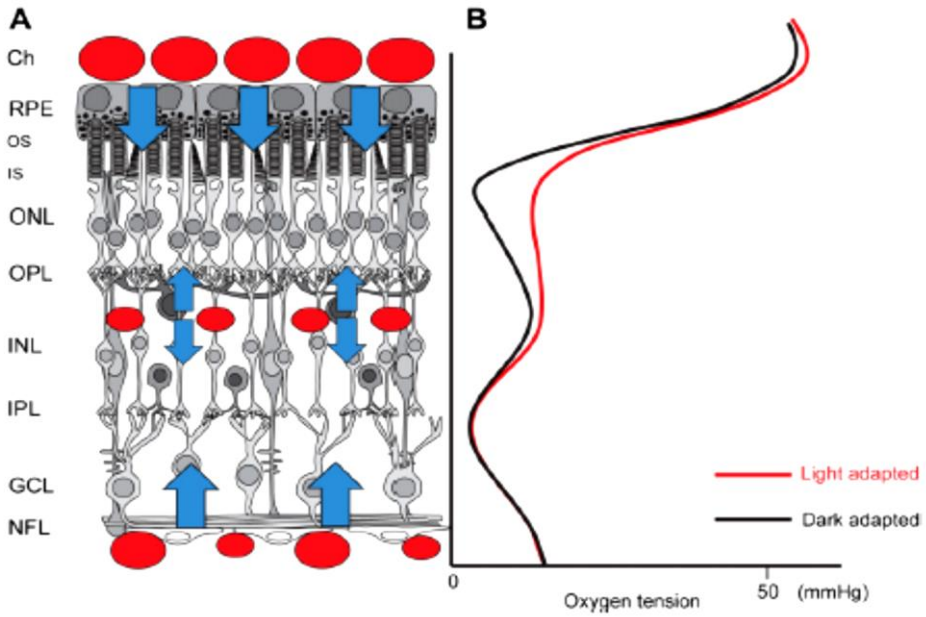


Figure 1-6. Schematic of oxygen distribution in the retina of the rat (Caprara et al. 2012).

Changes to the ocular blood flow, in particular, a reduction in blood flow which leads to hypoxia, has been implicated in a number of conditions including AMD (Ciulla et al. 2002; Friedman 1997), glaucoma (Harris et al. 1999; Zhang and Ma 2007) and diabetic retinopathy (Pemp and Schmetterer 2008).

The choroidal blood supply is not uniform throughout the retina and locally there are watershed zones within the choriocapillaris. This is because each arteriole behaves as an end arteriole with no anastomoses (Hayreh 1990), leaving tissue in this area between supplies and susceptible to the effects of a reduced blood supply. There has been a link

between watershed zones and neovascular AMD (nAMD) (Feigl 2009; Giovannini et al. 1994; Mendrinos et al. 2009), with Mendrinos and Pournaras (2009) finding choroidal neovascularisation occurring within the watershed zones of 88% of their subjects. There are a high number of watershed zones in the macula (Xu et al. 2010; Hayreh 1990) which has been found to be affected more by hypoxia then the peripheral retina (Feigl et al. 2008). Watershed zones also exist between the two blood supplies, with the inner nuclear layer lying between the choroidal and retinal supplies.

There is a difference between the peripheral and central inner retinal oxygenation, with the oxygenation saturation of blood in peripheral arterioles (94.7%) found to be less than that of macular vessels (99.7%) (Heitmar and Safeen, 2012). Within the macula itself there is also a dissimilarity in blood supply, with the parafovea having a dense inner retinal capillary supply and the fovea being an avascular zone, with a choroidal supply only (Yu et al. 2010).

Blood supply does not remain stable though out life, even in the absence of any disease there are changes with age. A review by Ehrlich et al. (2009) concluded that choroidal blood flow declines with age, possibly due to vascular constriction and vessel drop out. For example, Grunwald et al (1993) reported that there was a 20% decrease in velocity with age, whilst Grunwald et al (1998) found that foveolar blood flow decreases with age. This was linked to the decrease in diameter of the blood vessels and the decrease in density that occurs with ageing.

1.3 Tissue oxygenation and hypoxia

This section of the introduction will discuss tissue oxygenation. The importance of oxygenation homeostasis and regulation is discussed, along with the consequences of a lack of oxygenation, with particular emphasis given to the effects at a retinal level. This is significant as this thesis is concerned with the potential role of reduced retinal oxygenation in the pathogenesis of AMD.

To ensure that tissues have a constant supply of oxygen and a means of waste removal, aerobic organisms have developed a complex circulation system. This network is so extensive that nearly every cell in the body is within 100-200 µm of a capillary (Polverini 2002). A decrease in blood supply to a tissue leads to ischaemia. Common causes of ischaemia include narrowing (stenosis) or blockage of the arteries. This, eventually, will lead to hypoxia; an insufficient supply in oxygen to the tissue, leading to cell death as adenosine triphosphate (ATP) production is reduced. Hypoxia can also be caused by a reduction in oxygen carried in the blood. Peripheral oxygen saturation (SpO_2) describes the percentage of haemoglobin saturated with O_2 at any one time and should be 97-99% in a healthy individual. A decrease in SpO_2 can be the result of reduced pulmonary efficiency or when breathing air at high altitudes. At high altitudes the oxygen saturation of the air is reduced, for example, from 20.9% at sea level to 11.3% at 16000 ft (the height of Montblanc).

The development of the vascular network results from vasculogensis, with vessels forming from the aggregation and differentiation of endothelial precursor cells (angioblasts) (Zhang et al. 2007). This network is then expanded by angiogenesis; the growth of new blood vessels from existing ones. Both of these processes also occur postnatally via chemical regulation during reproduction and physiological wound healing, where the growth takes place over a short time span. However, new vessels can also grow pathologically, where the process can remain in place for months or years (Polverini 2002). Angiogenesis has been implicated in many systemic conditions including inflammatory conditions such as Crohn's disease, arthritis and psoriasis (Polverini 2002). New blood vessels formed during angiogenesis have an abnormal structure, lacking a basement membrane and pericytes (Zhang et al. 2007), therefore they are fragile and predisposed to leaking and haemorrhage.

Chemical upregulation leading to angiogenesis has been found to be stimulated by hypoxia (Yue and Tomanek 1999), and inflammation (Wagner et al. 2008). Many chemicals have been found to be involved in angiogenesis, and in healthy tissue there is a balance between growth factors (e.g. vascular endothelial growth factor (VEGF), angiopoietins and basic fibroblast growth factor) and growth inhibitors (e.g. pigment epithelium-derived factor (PEDF) and thrombospondin-1 (TSP-1)). It is the imbalance of these factors that leads to the growth of new vessels. These vascular growth regulators will be discussed in more detail in section 1.3.1 .

The regulation of O_2 is extremely important to the structural and functional integrity of healthy tissue. Both hypoxia and hyperoxia can lead to cell death, with hyperoxia leading to the creation of reactive oxygen intermediates (ROIs) and oxygen toxicity (Semenza 1999). Systemically there are oxygen-sensing mechanisms that detect changes in supply and, in the case of reduced O_2, start the process for metabolic adaptation or to restore the supply. This can be acheved by increasing the efficiency of energy producing pathways at a cellular level by switching to glycolysis (Michiels 2004) and promoting of the utilisation of glucose (Kaur et al. 2008; Michiels 2004) or by modifying the existing supply. For example vessel dilation (Lange et al. 2012a) and enabling the growth of new vessels from the surrounding vasculature (a form of angiogenesis referred to as neovascularisation).

Oxygen sensing pathways are controlled by molecular regulators, such as hypoxia-inducible-factor transcription factors (HIFs), and intracellular organelles, for example mitochondria (Giaccia et al. 2004). Hypoxia-inducible-factors consist of a hetero-dimer (a protein composed of two polypeptide chains differing in composition) with α and β subunits, and are a key transcriptional regulator involved in the process of restoring oxygen homeostasis. Although these pathways are crucial, they themselves can also have detrimental effects. The molecular regulators for example, play a role in the development of tumours by facilitating the adaptation of tumour cells to hypoxic conditions, enabling angiogenesis. Hypoxia-inducible-factor-1α has been found to be over expressed in the

majority of breast and colon cancer metastases (Semenza 1999). Hypoxia-inducible-factors will be discussed in more detail in section 1.3.1

Hypoxia also has effects at a retinal level. It can stimulate physiological processes such as retinal vessel growth in ocular development (Kim et al. 2012), but is also linked to the aetiology of retinal pathology including AMD (Feigl 2009) and diabetic retinopathy (Caprara et al. 2012). In these conditions a lack of oxygen due to changes in the existing vasculature leads to neovascularisation. These vessels are unstable and unregulated as demonstrated by the increased choroidal blood flow seen with changes in ocular perfusion pressure in eyes with nAMD, but not in healthy controls (Pournaras et al. 2006). This is thought to be due to the new vessels in the neovascular membrane being unable to increase their flow resistance.

The following section provides a detailed description of the physiological molecular mechanisms that regulate tissue oxygenation.

1.3.1 Molecular response to hypoxia

Hypoxia-inducible-factors regulate the expression of hundreds of genes, either directly or indirectly concerned with biochemical events. This includes the instigation of angiogenesis by up regulating VEGF (Ratcliffe 2007), and stimulating the transcription of erythropoietin, which is a regulator of red blood cell production (Michiels 2004). It also

plays an important role in the developing retinal vasculature (Sheridan et al. 2009; Semenza 1999).

There are 3 isoforms of HIF α subunits, with HIF-1α and HIF-2α being closely related, sharing 48% of amino acid sequencing (Ke and Costa 2006) and HIF-3α having a more distant relationship (Ratcliffe 2007). Little information is currently available on HIF-3α, but HIF-1α and HIF-2α have been researched extensively. Both HIF-1α and HIF-2α function to overcome the cellular effects of hypoxia, however whereas HIF-1α is expressed systemically, HIF-2α is mainly expressed in the lung (Ema et al. 1997). Although there is evidence to suggest that both HIF-1α and HIF-2α are involved in regulation of erythropoietin (Ratcliffe 2007) and VEGF (Lange et al. 2012b), HIF-1 α is the primary factor mediating the hypoxia response. HIF-1α is constantly being produced and degraded during normoxia, having a short half-life of 5-8 mins (Berra et al. 2001). Although it is expressed in all cells, its activation results in different responses, due to the differing cell types, other cell signals taking place, or the duration of the hypoxia (Semenza 1999). Degradation occurs via the ubiquitin/proteasome pathway, due to interaction with the von Hippel-Lindau (VHL) protein, this process is shown in Figure 1-7. Under hypoxic conditions however, this degradation does not occur and the protein concentration of HIF-1α increases with lowering O_2 concentrations (Yu et al. 1998; Semenza 2000). When this occurs HIF-1α relocates to the nucleus, where the α unit can dimerize with the β unit, activating HIF. There has also been some recent evidence to suggest that HIF -1α is continuously expressed and active in the retina, and not degraded, suggesting an ocular

neuroprotective role, which is independent of a hypoxic environment (Hughes et al. 2010).

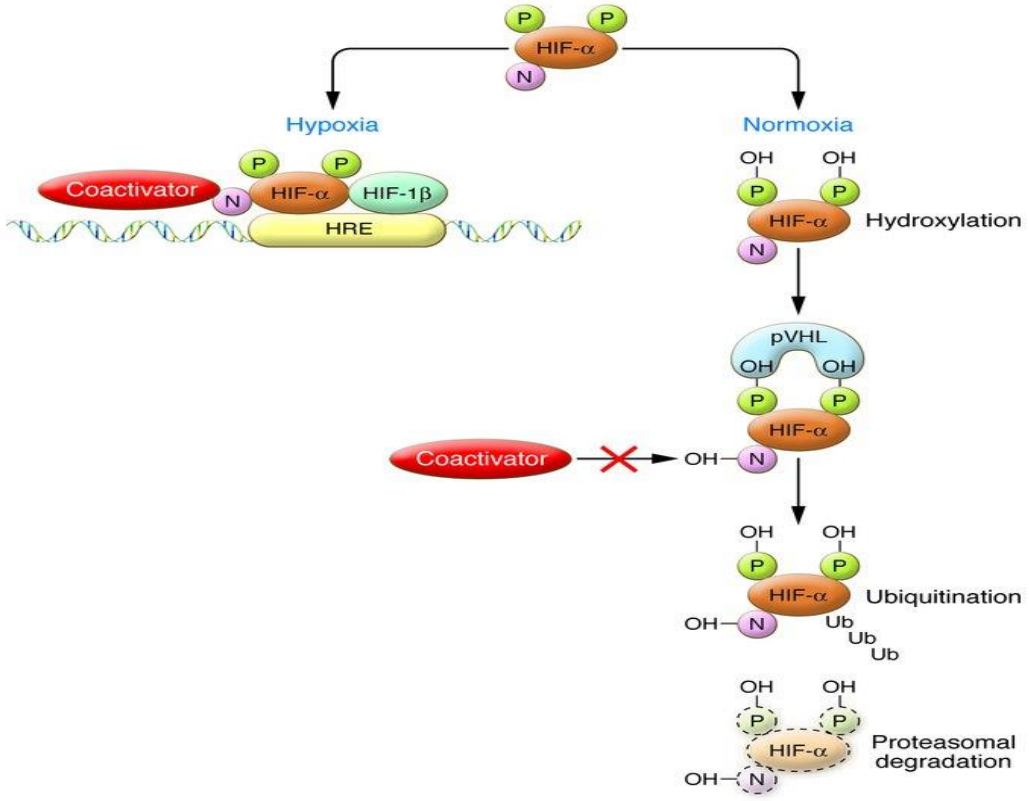


Figure 1-7. HIF activity under hypoxic and normoxic conditions (Ratcliffe 2007).

Ferrara and Henzel (1989) reported the discovery of a potent growth factor which was selective for vascular endothelial cells, which they named VEGF. This growth factor we now know as one of the key angiogenic growth factors with important physiological and pathological roles (Marneros et al. 2005; Alon et al. 1995; Nishijima et al. 2007). These include roles in cell migration, cell proliferation, vascular permeability and cell apoptosis (Penn et al. 2008). The VEGF family includes five isoforms of VEGF, (due to alternative

exon splicing and differing heparin and heparan-sulfate binding ability), placenta growth factor and snake venom VEGF. Although all VEGF isoforms are involved in tissue growth it is VEGF-A, of which there are nine subtypes, which has the greatest influence during neovascularisation. Of these subtypes VEGF 121 and VEGF 165 are the predominate forms (Neufeld et al. 1999). The regulation of VEGF gene expression is mediated by HIF and stimulated by hypoxia, UV and H_2O_2 (Grisanti and Tatar 2008). There are also other pathways of expression, with inflammatory cytokines, other growth factors and hormones all able to upregulate VEGF (Ferrara 2004).

These other pathways indicate that there is localised control over growth factor regulation, and suggest a role for inflammation in neovascularisation. This link to inflammation is an important one as inflammation has been associated with the development of choroidal neovascular membranes (CNV) in AMD (Campa et al. 2010). The complement cascade (part of the innate immune system) and its final membrane attack complex (MAC) have been associated with an increase in growth factors including VEGF and neovascularisation (Bora et al. 2005). Also the depletion of complement component 3 and MAC have been found to lead to a reduction in VEGF and the inhibition of CNV (Bora et al. 2006; Bora et al. 2005). Several inflammatory markers such as metalloproteinases and macrophages have also been associated with neovascularisation (Buschini et al. 2011; Bressler 2009). Metalloproteinases are secreted by the RPE and can increase the bioavailability of VEGF (Tatar et al. 2007).

Vascular endothelial growth factor has also been found to be a direct neuroprotectant against ischaemic damage, independent of an increased blood supply, both in the brain after ischaemic injury (Sun et al. 2003) and in the retina (Nishijima et al. 2007). Vascular Endothelial Growth Factor-A promoted the survival of neurones in the absence of an increase in blood flow in rats where transient ischaemic episodes had been created in the eye (Nishijima et al. 2007). The authors concluded that RGC viability was directly linked to VEGF, when it was depleted in the retina they found a dose dependant decrease in RGC numbers. This evidence of an important physiological role for VEGF in the healthy eye has obvious implications for the current treatment for nAMD that inhibits VEGF expression.

1.3.2 The intraretinal effect of hypoxia

With the blood supply, metabolic demand and structure of each retinal layer differing, the susceptibility to and effect of hypoxia is different throughout the retina.

The photoreceptor layer is highly susceptible to hypoxia due to its reliance on the choroid, which has poor autoregulation. Under normal conditions, an adequate supply of O_2 can be maintained. However, there are factors which can either increase the metabolic demand of the photoreceptors or decrease either the choroidal blood supply or oxygen saturation of the blood, leading to hypoxia. Some of these can be encountered on a regular basis such as changes in altitude (Arjamaa et al. 2009), with altitudes above 3000m causing chronic hypoxia (Feigl 2009), whilst flying in an aircraft can result in a

hypoxic environment due to reduced cabin pressures (Connolly and Hosking 2006; Feigl et al. 2008).

The inner retinal supply is able to increase blood flow to meet extra demand, with Brinchmann-Hansen et al. (1989) finding that human retinal arteries dilated by 9.4% in response to acute hypoxia. However, the choroidal response to changes in O_2 is poor (Wangsa-Wirawan and Linsenmeier, 2003; Lange et al. 2012a), although there is some capability to autoregulate (Polska et al. 2007; Luksch 2003). Polska et al (2007) found that a change in perfusion pressure led to changes in choroidal blood flow, which Luksch (2003) concluded was regulated by nitric oxide.

The bipolar cells and the inner nuclear layer may also be affected by hypoxia. These cells are metabolically active, containing large numbers of mitochondria, and therefore have a high oxygen demand (Caprara et al. 2012). The post receptoral hypothesis for AMD onset suggests that the cells located in the inner nuclear layer are affected by hypoxia due to this area being located in the watershed zone between the retinal and choroidal blood supplies. It has been suggested that this area is affected first by early AMD (Feigl 2007; Feigl 2009). Inner retinal cells, including RGCs can also be affected by a reduction in the inner retinal blood supply. For example, a reduced inner retinal circulation, and disruption to the auto regulation of the retinal supply have been implicated in glaucoma (He et al. 2011; Flammer et al. 2002).

1.4 Age-related macular degeneration

Age related macular degeneration is a progressive, degenerative disorder of the macula causing an exaggeration of the normal ageing process. Early AMD leads to two different advanced subtypes, nAMD and geographic atrophy (GA). Loss of vision tends to be more rapid in nAMD, but both subtypes eventually cause a loss of central vision. This chapter aims to provide the reader with a overview of the current understanding of AMD, including its grading, pathogenesis, effect on visual function, investigation techniques, and current treatments.

1.4.1 Prevalence

Age-related macular degeneration is the most common cause of legal blindness in developed countries (Roth et al. 2004; Seddon and Chen 2004), with 25-30 million people being affected worldwide (Schmiert al. 2006). This prevalence is set to increase along with the ageing population, to around 679,000 to 755,867 cases of late stage AMD by 2020 in the UK alone (Owen et al. 2012; Minassian et al. 2011). Age-related macular degeneration has personal consequences for individuals, being linked to falls (Cruess et al. 2008) and to an increased risk of depression (Casten et al. 2004; Berman and Brodaty 2006; Siaudvytyte et al. 2012). Brody et al. (2001) found that 32.5% of 151 people with AMD had a depressive disorder, a proportion comparable with life threatening conditions including cancer and cerebrovascular disease. People with AMD, even with mild vision loss (VA <6/12), have also been found to struggle with daily activities, including reading

and recognising faces (Hassell et al. 2006). There is also a wider socioeconomic effect, with the average annual societal cost (including direct vision related medical costs, direct non- vision- related costs and non-medical- related costs) per patient treated for bilateral nAMD estimated at €5300 in the United Kingdom (Cruess et al. 2008).

1.4.2 Clinical features

1.4.2.1 Early age-related macular degeneration

Under the International Classification and Grading System early AMD (also known as Age-Related Maculopathy or ARM) is commonly seen in patients over the age of 50 years (Bird et al. 1995). It is defined by the Beckman Initiative for Macular Research Classification Committee as the presence of medium drusen (>63 µm but ≤125 µm) without AMD pigmentary abnormalities (focal hyper or hypopigmentation) (Ferris et al. 2013).

Drusen are the most common manifestation of early AMD. They are deposits between the RPE and the inner collagenous zone of Bruch's membrane and are classified by their location, size and appearance (Booij et al. 2010). Hard drusen are small [less than 63µm (Ferris et al. 2013)], well defined, discrete yellow deposits and in themselves are not a risk factor for AMD (Bird et al. 1995). However they are associated, along with soft drusen, with local photoreceptor changes, as even small drusen can cause structural disruption. Also a large number of hard drusen are a risk factor the development of soft drusen and pigmentary abnormalities (Klein et al. 2002). As the drusen size increases, changes, such as photoreceptor displacement and an altered RPE eventually lead to

photoreceptor abnormalities (Johnson 2003). Clinically, soft drusen are paler than hard drusen, larger in size and have a sloping border with indistinct edges (see Figure 1-8). They constitute a risk factor for advanced AMD (Klein et al. 1997; Feigl 2007), with the risk of advanced AMD increasing with the size and confluence of soft drusen (Bird et al. 1995; Ferris et al. 2005).

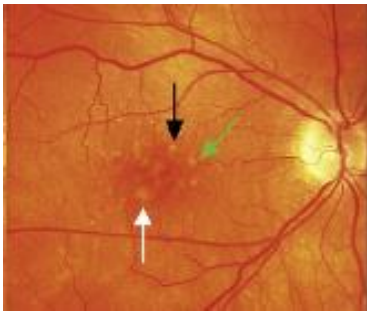


Figure 1-8 Retinal photograph of soft drusen. Black arrow shows drusen >63µm, green arrow shows drusen >125µm and white arrow shows drusen >250µm. (Miller 2013).

The theory of drusen formation, despite many studies, is still inconclusive, but the contents of drusen point towards inflammatory processes and oxidative stress (Booij et al. 2010). Although still unproven, the link to inflammatory and immune mediated responses comes from the presence of cells in drusen linked to immune processes. These include macrophages and fibroblasts (Booij et al. 2010) and cells linked to the acute phase of inflammation including amyloid P components and complement proteins (c5a) (Hageman et al. 2001; Rodrigues 2007). It is thought that the accumulation of these chemokines and cytokines initiates an inflammatory response which leads to a degenerative effect on RPE cells and adjacent photoreceptors, stimulating AMD

progression (Rodrigues 2007). This theory is reinforced by a genetic link between AMD and genes from the complement system such as complement factor H (Hageman et al. 2005). Many molecular components of drusen such as lipids and carbohydrates are thought to be phagocytosed waste material from shed photoreceptor outer segment discs (Grisanti et al. 2008). Booij (2010) found that drusen also contain proteins from the choroidal cells.

First described by Mimoun at al. (1990), reticular pseudodrusen (RPD) are collections of granular material situated in subretinal space between the RPE and the boundary of the inner and outer segments of the photoreceptors (Zweifel et al. 2010b) which can be classified as dot, ribbon or peripheral (Suzuki et al. 2014).

Reticular pseudodrusen can be viewed using fundus photography, blue channel fundus photography, OCT imaging, infra-red reflectance and FAF. It has been proposed that at least two different imaging techniques are used to confirm the presence of RPD (Sivaprasad et al. 2016; Ueda-Arakawa et al. 2013b), of which one should be OCT (Zweifel et al. 2010a), as each class has a different optimum detection method (Suzuki et al. 2014).

Although similarities exist in the composition of drusen and RPD, with both containing complement factor H, vitronectin and Apolipoprotein E (Rudolf et al. 2008; Hageman et al. 2001), they do not contain material derived from photoreceptors (Rudolf et al. 2008) suggesting that they differ in origin from soft drusen. Genetically, the incidence of RPD has been linked to both the CFH and the ARMS2 genes, which are also linked to AMD

(Joachim et al. 2014; Ueda-Arakawa et al. 2013a; Yoneyama et al. 2014). The link with inflammatory genes and the presence of inflammation mediators in RPD suggests a role of inflammation in their pathogenesis and it has been proposed by Sivaprasad et al. (2016) that there may be a link with parainflammation. There are also links with the development of RPD and hypoxia, with a reduced macular choroidal thickness found in eyes with RPD and an association of RPD with choroidal watershed zones (Alten et al. 2013).

Reticular pseudodrusen are thought to be a risk factor for early AMD, with Huisingh et al. (2016) finding that eyes that are classified by the AREDS scale as normal, but with the presence of RPD were 2.24 times more likely to develop early AMD than eyes without. There are also links with the progression to late stage AMD (Hogg et al. 2014; Joachim et al. 2014), and in particular GA (Finger et al. 2016; Finger et al. 2014).

1.4.2.2 Intermediate AMD

Intermediate AMD indicates a progression of the condition. Clinical signs include drusen larger than 125 microns and/ or changes the the RPE which result in hyper and hypopigmentation (Ferris et al. 2005).

1.4.2.3 Geographic atrophy

Geographic atrophy (GA) comprises 85-90% of advanced AMD cases (Bhutto et al. 2012). Usually bilateral, it may develop at different rates in each eye, causing a gradual deterioration of vision over months or even years, eventually leading to central scotomas

(Ambati et al. 2003). There is currently no treatment for GA (Ambati and Fowler 2012), and it is responsible for 20% of registrations of legal blindness due to AMD (Buschini et al. 2011).

Geographic atrophy is characterised by sharply delineated roughly round areas over 175µm in diameter of hypopigmentation or depigmentation (Bird et al. 1995), which can be associated with drusen. This represents an area of RPE atrophy, which is often associated with the atrophy of the underlying choriocapillaris. Ultimately, GA leads to photoreceptor death. Atrophy onset is associated with an increased visibility of underlying large choroidal vessels, and is often preceeded by regression of previously visible drusen (Ambati et al. 2003). Often developing in the parafoveal region, GA spares the fovea until the later stages of the disease (Ambati et al. 2003). Progression is slow compared to the neovascular form of the disease but GA can co-exist in eyes with nAMD (Bhutto et al. 2012).

1.4.2.4 Neovascular Age related macular degeneration.

Neovascular AMD results from the growth of new blood vessels from the choroid. Clinical features may include a green-grey lesion, haemorrhages and hard exudates from vessel leakage (Bird et al. 1995), leading to RPE detachments and scarring. Visual symptoms tend to be acute in onset with early manifestations of metamophopsia ultimately leading to a central scotoma (Blasiak et al. 2013). Choroidal neovascularisation has been sub classified into two groups, based on the presence of a sub-retinal (classic) or sub-RPE

(occult) neovascular membrane (Ambati et al. 2003). Classification is based on the presentation on fluorescein angiography. Classic CNV is described by a well-demarcated area of intense early hyperfluorescence (Bhutto et al. 2012) which progressively leaks in the late phases of the angiogram. Occult CNV is characterised by a poorly demarcated lesion appearing later in the fluorescein angiogram and leaking intensely.

A pigment epithelial detachment (PED) is the separation of the RPE from the inner collagenous layer of Bruch's membrane and is a clinical feature in in many chorio-retinal diseases including AMD. Symptoms may include metamophopsia and a reduction in central vision or it may be asymptomatic (Zayit-Soudry et al. 2007). Clinically, hyperopia may be present due to the raised retina, seen as a single, or multiple elevations accompanied by pigmentary changes.

Pigment epithelial detachment can have a direct link with hypoxia, with the RPE elevation increasing the distance oxygen travels from the choroid to the photoreceptors, thus decreasing the oxygen flux according to Fick's law of diffusion (Wangsa-Wirawan and Linsenmeier 2003; Stefánsson et al. 2011).

The progression and outcome of all types of PED are similar (Pauleikhoff et al. 2002). Treatment, where appropriate, involves photodynamic therapy (Zayit-Soudry et al. 2007) or anti VEGF injections (Lommatzsch 2010).

1.4.3 Grading and Classification Systems for AMD

There have been numerous different classifications and severity scales based on photographic images of the retina (Bird et al. 1995; The age-related eye disease study research group 2001; Sparrow et al. 1997; Davis et al. 2005; Ferris et al. 2013). In 1995 a collaboration of several groups formed the international ARM epidemiological study group. They published The International Classification and Grading System for Age-Related Macular Degeneration (Bird et al. 1995), a detection and grading scale designed to standardise the assessment of AMD for use in research. The Age-Related Eye Disease Study (AREDS) group also developed a grading scale for use in its studies and trials (The Age-related Eye Disease Study Research Group 2001), both of these scales were developed from the Wisconsin Age-Related Maculopathy Grading System. More recently, the Beckman Initiative for Macular Research AMD Classification Committee outlined a 5–step grading scale for AMD which ranged from 'no ageing changes', through 'normal ageing changes', 'early AMD', and 'intermediate AMD', to 'late AMD' (Ferris et al. 2013).

Classification of AMD status in this thesis is based on the simplified AREDS 5 point scale that has been widely used to grade AMD severity (Ferris et al. 2005). This grading scale takes into account the disease status for both eyes and is shown in Table 1-1. It is derived from longitudinal data obtained from the AREDS trial (Ferris et al. 2005), and an increased grade is strongly related to an increased 5 year risk of developing advanced AMD.

Five-Year Rates of Advanced AMD (In One or Both Eyes for Patients With Both Eyes at Risk)

	Patients Without Advanced AMD in Either Eye at Baseline*			Patients with Advanced AMD in One Eye at Baseline†		
Risk Factors	No. at Risk	No.	%	No. at Risk	No.	%
0	1466	6	0.4			
1	635	20	3.1			
2	455	55	11.8	149	22	14.8
3	328	85	25.9	178	63	354
4	317	150	47.3	273	145	53.1

Abbreviation: AMD, age-related macular degeneration.

*Assign 1 risk factor for each eye with large drusen.

Assign 1 risk factor for each eye with pigment abnormalities.

Assign 1 risk factor if neither eye has large drusen and both eyes have intermediate drusen (Table 1).

†Assign 2 risk factors for the eye that has neovascular AMD.

Assign 1 additional risk factor if the eye at risk has large drusen.

Assign 1 additional risk factor if the eye at risk has pigment abnormalities.

Table 1-1. Simplified AREDS scale (Ferris et al. 2005).

The aim of this PhD was to compare the effect of the different gas conditions on people with early AMD and healthy controls i.e. only a binary grading of normal or early AMD was required. A detailed grading of the severity of AMD related changes was not instrumental to the data analysis. It was therefore considered unnecessary to use a scale providing greater resolution such as the AREDS 9 point scale. The simplified scale also had the advantage of allowing a risk grade for a fellow eye, when the eye studied shows no/very early signs of AMD. Studies have shown that, in these cases, participants may have changes to choroidal perfusion and visual function in the better eye, which indicate that a subclinical stage of AMD is present although the fundus displays minimal features of AMD (Remulla et al. 1995; Chen et al. 1992). As the principal objective of the research in this thesis was to determine the role of retinal hypoxia in visual function in early AMD, it was essential to be able to grade these very early stages of the disease.

1.4.4 Pathogenesis of age-related macular degeneration

The pathogenesis of AMD is a widely researched topic and current conclusions and reviews concentrate on three main theories:

- The oxidative theory
- The inflammation theory
- The hypoxia theory

This next section will discuss the evidence for each theory and the links between them.

1.4.4.1 The oxidative theory

Oxidation is a process referring to the removal of electrons from a chemical; it is an essential metabolic process to allow energy to be gained from carbohydrates, proteins and lipids. Reactive oxygen intermediates (ROI) are produced by enzymes involved in the oxidative process or as a result of photochemical reactions (Boulton et al. 2001b; Beatty et al. 2000). Reactive oxygen intermediates include free radicals, hydrogen peroxide and singlet oxygen; these molecules are unstable and claim electrons from surrounding stable molecules with the intention of achieving a stable state for themselves. This leaves the donor molecule unstable and accumulation of these molecules causes oxidative stress.

Reactive oxygen intermediates have the ability to damage many cell materials including carbohydrates, proteins, nucleic acids (Beatty et al. 2000) and mitochondria (Blasiak et al. 2013) leading to cellular degeneration. Oxidative stress has been linked to the ageing

process and is the suspected cause of many degenerative diseases including Parkinson's disease (Yildirim et al. 2011), Alzheimer's disease (Kaarniranta et al. 2011; Blasiak et al. 2013) and AMD (Beatty et al. 2000; Blasiak et al. 2013).

The retina is prone to oxidative stress with the daily exposure of 10^{12} to 10^{15} photons of light (Hunter et al. 2012) causing photochemical damage. Visible wavelengths cause tissue damage when absorbed by one of the several retinal chromophores including melanin, retinal and lipofuscin (Boulton et al. 1990; Chalam et al. 2011). Photochemical damage varies with wavelength; as the energy of a photon is inversely proportional to its wavelength, UV and blue light causes the most damage (Chalam et al. 2011). Although blue light damage has been proposed to be a risk factor for AMD, further research including a large scale clinical trial on blue light filters is required (Margrain et al. 2004).

The retina is prone to the creation of ROIs owing to its high oxygen tension due to the high blood flow in the underlying choiocapillaris, providing the oxygen necessary for ROI reactions to take place. Also photoreceptor outer segments contain large amounts of readily oxidised polyunsaturated fatty acids; around 50% of rod outer segments (Stone, Farnsworth and Dratz 1979). This is the highest concentration of any human tissue and can result in a chain reaction with the ability to produce large amounts of ROIs. Age also influences retinal oxidative stress, although protection from shorter wavelength light occurs with the ageing of the crystalline lens, there is an age related reduction in antioxidant protection and an accumulation of lipofuscin (Hunter et al. 2012; Wing et al. 1978).

Lipofuscin is a recognised hallmark of ageing, increasing with longevity. It is an aggregate of un-degradable polymers located in the lysosome of metabolically active cells throughout the body. Usually formed from the autophagic breakdown of cellular products, retinal lipofuscin differs in its composition with its major component being un-degradable end products from the phagocytosis of photoreceptor outer segments (Sparrow and Boulton 2005).

Although the exact components are still elusive, lipofuscin is known to be a chromophore, containing N-retinylidene-N-retinylethanolamine (A2E). Lipofuscin not only has the ability to compromise the host cell, but is also a ROI generator, particularly sensitive to short wavelength light (Różanowska et al. 1998).

Evidence of a link between oxidative stress and AMD comes from several different sources. A major association is between the reduction of retinal antioxidants with age and AMD. Melanin, a free radical scavenger, reduces with age, with the volume present in RPE cells reducing from 8% in the first two decades of life to 3.5% in the fourth to ninth decade (Feeney-Burns et al. 1990). This, along with the increase in the photoreactivity of melanosomes (Rózanowska et al. 2002) is thought to contribute to AMD development (Chalam et al. 2011). Smoking, the principal modifiable risk factor for AMD, is linked both to an increase in oxidative stress and a decrease in macular pigment (Alves-Rodrigues and Shao 2004; Fletcher and Chong 2008). Lipofuscin has also been linked to AMD, with a correlation between RPE lipofuscin content and photoreceptor death reported in early studies (Dorey et al. 1989). However, recent evidence suggests that the relationship

between lipofuscin accumulation and AMD may not be as strong as previously suggested (Rudolf et al. 2013; Hwang et al. 2006). Rudolf et al. (2013) found that although areas with advanced RPE abnormalities corresponded with the presence of lipofuscin, this may be due to vertically superimposed cells influencing autoflorecence rather than actual increases in lipofuscin. Furthermore, Hwang et al. (2006) found that lipofuscin accumulation was not a strong risk factor for the development of GA. There is also a possible link between UV radiation and inflammation in drusen formation (Chalam et al. 2011), which unites the oxidative and immunological theories.

1.4.4.2 The inflammation theory

Inflammation, the immune system's defensive response to harmful stimuli or injury, is an essential healing process to maintain homeostasis. However, inflammation is also linked to many disease processes including Alzheimers disease (Kaarniranta et al. 2011), Parkinson's disease, Huntington's disease (Buschini et al. 2011) and AMD (Penfold et al. 2001; Buschini et al. 2011), with the common response to various stimuli in all of these conditions being chronic uncontrolled inflammation. It is the autoimmune (Patel et al. 2005; Luthert 2011) and in particular the complement system (Bhutto et al. 2012), rather than acute inflammatory responses which are implicated in AMD (Buschini et al. 2011).

Chronic inflammation is a prolonged response to a stimulus, resulting in tissue damage. The complement system is an innate biochemical cascade that promotes inflammation triggering an amplification cascade which results in the activation of the cell-killing

membrane attack complex (MAC) (Bhutto et al. 2012). Macrophages are also involved in chronic inflammation, in both vasculogenesis and angiogenesis.

Along with other neurological tissues, the eye has long been thought of as immune privileged (Penfold et al. 2001), with anti-inflammatory and immunosuppressive molecules being expressed within the eye (Niederkorn 2003). The RPE is important in forming the blood retinal barrier and maintaining the immune privileged status. It has been postulated that changes in the RPE cells may disrupt this and lead to AMD development (Buschini et al. 2011). Evidence suggests though that central nervous system tissue is not completely isolated from the peripheral immune system (Carson et al. 2006).

In AMD the immune process has been implicated in drusen formation, with several immune molecules present in drusen including C3, C5 and C5b9 complement proteins vitronectin, amyloid P and apolipoprotein E (Kaarniranta et al. 2011; Buschini et al. 2011; Bhutto et al. 2012; Rodrigues 2007). Many constituents of drusen are also found in other immune related disease deposits, such as those found in Alzheimer's disease (Kaarniranta et al. 2011) and atherosclerosis (Rodrigues 2007). Geographic atrophy and nAMD are also thought to have an immunological component (Rodrigues 2007). Macrophages can secrete MMPs (Grisanti et al. 2008) and proteolytic enzymes which thin Bruch's membrane (Ambati et al. 2003), facilitating the migration of vascular endothelial cells and increasing the bioavailability of VEGF. Monocytes, from which macrophages originate have been found to be associated with nAMD (Chen et al. 2016). The complement

cascade has also been implicated in the formation of CNV (Rohrer et al. 2011; Rohrer et al. 2009) with MAC formation central in CNV development (Bora et al. 2007; Bora et al. 2005). The stimulus for the immune response has been postulated to be oxidative (Luthert 2011), possibly related to lipofuscin formation, as A2E is known to activate the complement system (Luthert 2011).

Genetic evidence strengthens the role of the immune system in AMD, with mutations in several genes regulating the immune response being linked to an increased risk of AMD. The single nucleotide polymorphic change at Y402H, in the complement factor H gene which regulates the complement immune system, has strong links with AMD (Edwards et al. 2005). Also, the deletion of complement factor H related genes CFHR1 and CFHR3 and variations in the complement genes C2, C3 and factor B have also been linked to AMD (Hughes et al. 2006; Seddon et al. 2013; Gold et al. 2006). Parainflammation has also been linked with sustained oxidative stress in ageing and AMD, linking the theories of the oxidative stress theory and inflammation (Chen and Xu 2015).

1.4.4.3 The hypoxia theory

Histologically, hypoxia has a strong link with AMD. Structural changes in early AMD such as an impairment in choroidal perfusion (Ciulla et al. 2002; Ciulla et al. 1999b; Grunwald et al. 2005), increased scleral rigidity (Friedman 2008) and a thickening of Bruch's membrane (Stefánsson et al. 2011; Pauleikhoff et al. 1992) could all result in retinal

hypoxia. The role of hypoxia in the pathogenesis of AMD is discussed in the systematic literature review in chapter 3.

1.4.5 Treatment

As understanding of the disease aetiology of AMD has improved, treatments have rapidly evolved. However they are still currently only available for the nAMD. Current treatments, for example anti VEGF therapies, are expensive and intensive with patients requiring retreatment monthly for the greatest improvement in VA (Rosenfeld et al. 2006).

1.4.5.1 Geographic atrophy and early age-related macular degeneration.

There are no current treatments for GA or early AMD, with the emphasis for these patients being the prevention of the development to nAMD by reducing oxidative stress (not smoking, wearing sunglasses) and taking antioxidant supplements (Age Related Eye Disease Study Research Group. 2001). However there are treatment strategies currently under investigation for GA including complement inhibition, for example POT-4 (Velez-Montoya et al. 2012), neuroprotection (Yehoshua et al. 2011) and increasing choroidal perfusion (Velez-Montoya et al. 2012). A phase III trial is currently underway investigating the role of the complement inhibitor Lampalizumab to reduce the progression of GA (Jack et al. 2016). In the phase II MAHALO trial Lampalizumab reduced disease progression by 20%, and in a subset of patients with the compliment factor I biomarker the disease progression was reduced by 44% (Gemenetzi and Lotery 2016).

1.4.5.2 Neovascular Age-related macular degeneration

There are currently several treatments with NICE approval for the treatment of nAMD including photodynamic therapy and anti-VEGF treatment. By neutralising VEGF-A, anti-VEGF treatment can control and cause regression of neovascularisation in AMD. There are four key pharmacological agents which have been used to treat nAMD; Ranibizumab (Lucentis), Bevacizumab (Avastin), Pegaptanib (Macugen), Aflibercept (Eylea). Ranibizumab and Afibercept are both licenced for use in nAMD by NICE (NICE 2008; NICE 2013). Their administration results in reduced new blood vessel formation by reducing cell proliferation, and it minimises vessel leakage. Ranibizumab treatment of neovascular AMD improves VA (Boyer et al. 2007; Kourlas and Abrams 2007) with a gain of 7.2 letters shown when injected with 0.5-mg Ranibizumab, compared with a decrease of 10.4 letters in a sham-injection group (Rosenfeld et al. 2006). Aflibercept (VEGF Trap-Eye) is a new VEGF inhibitor which has a 200 times greater affinity for binding VEGF than Ranibizumab (Chakravarthy et al. 2010a) and is comparable in terms of VA outcomes (Stewart 2012). It has a similar costing per injection compared to Ranibizumab, however as it remains in the vitreous for longer (Chakravarthy et al. 2010a), the overall cost of treatment may be less as Aflibercept is approved for use every 8 weeks (Stewart 2012).

1.5 Assessment of retinal structure and function in age-related macular degeneration.

This section of the thesis will give an overview of the assessment of AMD including a summary of clinical assessments used in practice. It will then concentrate on methods used in a research setting. Emphasis will be given to the psychophysical testing of scotopic thresholds and electrophysiology, which are the assessment methods used in this thesis. Finally, the chapter will discuss imaging techniques that may be used in the assessment of AMD.

1.5.1 Clinical tests of visual function in AMD

1.5.1.1 Visual acuity

Visual acuity (VA) remains the principal functional measure used to monitor AMD disease progression in clinical practice and it requires detection, resolution and recognition of a target. As detection and resolution are measurements of macular function, VA is often affected in late AMD, however, other aspects of retinal function are compromised before this change is seen (Lovie-Kitchin and Feigl 2005). The same size AMD lesions can result in varying losses of VA, (Neelam et al. 2009) making it an unreliable diagnostic tool, or indicator of disease progression. Depending on the size and position of the resulting scotoma, VA can still be good even when a subject is struggling, for example, with reading (Sarks et al. 1988). In 173 eyes diagnosed with late AMD it was found that 71.7% had a

reasonable recorded VA of 6/9-6/12 (Vinding 1990), and although Klein et al. (1995) found that late AMD did cause a reduction in VA of 7 lines, they also agreed that other measures of visual function may be more sensitive in detecting AMD.

1.5.1.2 Amsler chart

Self-monitoring is important for the timely detection of progression and successful treatment of nAMD (Trevino 2008). The Amsler grid (Amsler 1953) is given to patients at risk of developing nAMD for this purpose, and is designed to detect scotomas and metamorphopisa. Subtending 20^{o} at 30 cm, the chart uses a grid pattern composed of 1^{o} squares, with the patient asked to report any metamorphopsia (perceived distortions) or scotomas (gaps in the chart) whilst looking at a central fixation dot. However studies have suggested that patients are failing to notice these changes, with 77% of scotomas of 6^{o} diameter or less not being detected by Amsler testing (Schuchard 1993). These failings may be due to poor patient understanding of the test (Trevino 2008), poor or eccentric fixation (Loewenstein 2007) or due to a filling in effect (Crossland and Rubin 2007). Other self-monitoring methods, including Preferential Hyperacuity Perimetry (Crossland et al. 2007; Trevino 2008) and Macular Mapping (Trevino 2008) are currently under investigation.

1.5.1.3 Perimetry

Perimetry is a subjective technique that assesses the topographical sensitivity of the visual field. Accurate visual field assessment relies on central fixation, which is difficult for

patients with advanced AMD, however, certain perimetric techniques have been shown to be sensitive to AMD (Acton et al. 2012a).

Flicker perimetry measures the contrast threshold for a stimulus presented at a fixed temporal frequency. Flickering targets have been shown to be sensitive to GA progression and to monitoring AMD (Mayer et al. 1994; Phipps et al. 2004), being more sensitive than static perimetric targets (Phipps et al. 2004). They can also be used to predict progression from early to late AMD (Luu et al. 2012). Frequency doubling perimetry (FDT) measures the contrast sensitivity for a grating of fixed temporal frequency to assess the visual field. The short testing time and independence of results from pupil size makes this test attractive in AMD assessment. However, it has limited use in the functional evaluation of AMD due to its insensitivity in the detection of small AMD lesions (Sheu et al. 2002). It can, though, provide information about the size and depth of an area of sensitivity loss that correlates with reading speed in AMD (Anderson et al. 2011).

Short wavelength automated perimetry (SWAP) preferentially measures s-cone mediated thresholds. In the detection of AMD, VA has been found to better differentiate between people with AMD and those without than SWAP. Age-related macular degeneration lesions found with SWAP perimetry have been found to be greater in area and depth when compared to standard automated perimetry (Acton et al. 2012a). A correlation between lower SWAP sensitivity and soft drusen (Remky et al. 2001) has also been reported.

Microperimetry assesses the visual function of a specific area of the retina and correlates it with a fundus image, giving a comparison of structure and function. It can rapidly assess early vision loss in AMD (Dinc et al. 2008) and may be a useful tool in the detection and monitoring of AMD (Querques et al. 2008; Midena et al. 2007). A significant correlation has been found between reduced retinal sensitivity and drusen volume (Hartmann et al. 2011) and between field defects and a significant thinning of the outer segment associated with photoreceptor loss (Acton et al. 2012b).

1.5.1.4 Contrast sensitivity

Visual acuity measures the highest spatial frequency at which a high contrast target can be seen. An image, however, is made up of many different contrasts and spatial frequencies, so a contrast sensitivity assessment provides a comprehensive overview of an individual's spatial visual function. Contrast sensitivity is affected by many factors including light levels, eccentricity, hypoxia and age, reducing with normal ageing (Owsley 2011). Contrast sensitivity losses have been found to precede changes in VA in patients with early AMD (Stangos et al. 1995; Midena et al. 1997) GA, (Sunness et al. 1997) and with the increased sub retinal tissue volume seen in nAMD (Keane et al. 2010). It has been suggested that contrast sensitivity should be included as an outcome measure in the assessment of treatments for nAMD as it may show an improvement in visual function after treatment when VA does not (Monés and Rubin 2005; Rubin and Bressler 2002).

1.5.1.5 Colour vision

Tritan (blue-yellow) colour vision defects have been found in people with early and advanced AMD (Feigl et al. 2005b; Cheng and Vingrys 1993; Holz et al. 1995; Arden and Wolf, 2004; Dimitrov et al. 2011), which is possibly due to a reduction in S-cone sensitivity (Eisner et al. 1992). However this is not a universal finding in early AMD, possibly due to the changes being too subtle to respond to testing (Midena et al. 1997). A study by O'Neill-Biba et al. (2010) found that both tritan and red-green defects could be used as a sensitive measure of functional change in AMD.

1.5.2 Psychophysical Assessment of Visual Function in AMD

1.5.2.1 Introduction to psychophysics

Psychophysics is the study of the relationship between a stimulus and the sensation it evokes (Gescheider 1997). Based around threshold testing, it assumes that a sensation must exceed a critical threshold level to be perceived. The studies described in Chapters 4, 5 and 6 used a psychophysical assesement of scotopic visual function, therefore this section will give an overview of psychophysics.

The difference threshold is the difference that is required between two stimuli for them to be noticed as being different. Weber's law states that the difference threshold is linearly related to stimulus intensity (a higher stimulus intensity results in an increased difference threshold) (Gescheider 1997). The absolute threshold is the smallest amount of

stimulus energy required to produce a sensation, therefore at this point the stimulus is just detectable.

1.5.2.2 The psychometric function

A signal is not pure, but a mixture of signal and random noise. Therefore, when presented with a stimulus a participant must make the a decision if it is is just noise or if the stimulus is actually present. When the stimulus is weak, this is difficult and there are many errors that decrease as the stimulus signal increases. For this reason, thresholds are often defined in probabilistic terms (Neelam et al. 2009) and a psychometric function is used to determine the threshold (see Figure 1-9).

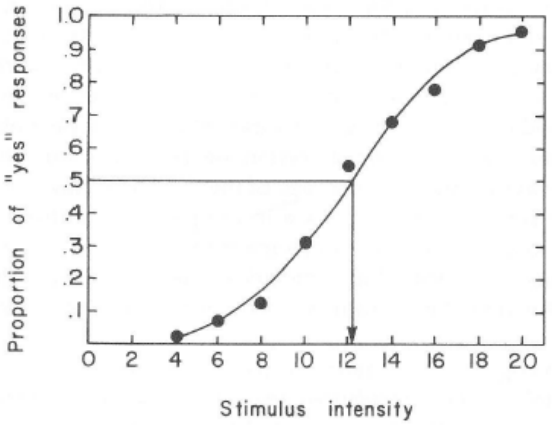


Figure 1-9. A typical psychometric function (Gescheider, 1997). Threshold is the stimulus intensity that would be detected 50% of the time.

The psychometric function plots the probability of detection at different stimulus intensities, indicating that when the same stimulus is presented repeatedly to an

observer at a particular intensity the response is variable, resulting in an ogive curve, rather than the consistent seen or not seen response that would be expected by a perfect observer. For example, in Figure 1-9 it can be seen that when a stimulus intensity of 12 is presented it will be seen 50% of the time by the observer. A stimulus towards the upper end of the y-axis is almost always detected, and the lower end represents a stimulus intensity that is almost never detected (Gescheider 1997).

There are two main techniques of gathering response data from a participant to plot a psychometric function, a yes/no or forced choice procedure. In the yes/no procedure for absolute thresholds the participant must judge in each trial if a stimulus is present or absent. A response is required on each trial and a cue is given to alert the participant to the trial. A proportion of these trials will be catch trials that contain no signal; if the participant responds positively to these trials they are recorded as false positives. A high percentage of false positives may indicate that the participant's confidence is high, or that there is a response bias.

The forced choice procedure is a sensitive method for assessing threshold which is uncontaminated by fluctuations in criterion (Gescheider 1997). In a two alternative forced choice procedure two intervals are presented, one of these intervals contains the stimulus and the other does not, the participant should then choose the interval when the stimulus was present. Even if the participant is unsure, they must give a response. This procedure eliminates response bias (Li et al. 2010), however it may still be affected by attention and memory. There may be any number of intervals presented, and with

increased intervals there is less likelihood that the participant guesses correctly by chance alone.

1.5.2.3 Classical psychophysical methods

The classical psychophysical methods were developed by Gustav Fechner in 1860 and consist of the method of constant stimuli, the method of limits and the method of adjustment. These techniques are limited by factors influencing participant responses, including the learning effect, response bias, participant confidence and fatigue. Therefore the assumption that the thresholds measured with these methods is a direct neural response is incorrect. There are also shortfalls of the testing procedure, including large amounts of wasted data if the stimuli presented are far from threshold and a lack of theoretical justification for aspects of the trials (Treutwein 1995). Adaptive procedures were developed to overcome some of these critiisms and will be discussed in the next section, with an empasis on the quick estimate by sequential testing (QUEST) procedure which was used to collect data for Chapters 4-6.

1.5.2.4 Adaptive psychophysical procedures

Unlike in classic psychophysical testing, when the stimuli chosen to present are known before testing, the stimuli used in adaptive procedures can be chosen dependent on the responses given on a previous trial/ trials, driven by an adaptive algorithm towards threshold (Leek 2001). This allows for the stimuli presented to be nearer the presumed

threshold. Adaptive procedures should take into account the following questions (Treutwein 1995):

1) When to change the testing level and where to place the trials on the physical stimulus scale?

2) When to finish the session?

(3) What is the final estimate of the threshold?

1.1.1.1.1 Theory of signal detection method

This method, proposed by Tanner & Swets (1954), is a forced choice method developed to remove the lack of control over the participant's decision criteria and to remove bias (Treutwein 1995) by conceptualizing what participants do when they are making a decision after being presented with a stimulus.

1.1.1.1.2 Maximum likelihood procedures

Maximum likelihood procedures calculate a new psychometric function after each trial based on information about the set of stimulus levels presented so far and the proportion of correct responses at each intensity level (Leek 2001). This means a statistical estimation of the participant's threshold can be calculated after each trial and used to decide on the next stimulus intensity to be presented. Prior knowledge is also used in these methods, with the method taking into account information about the expected shape of the psychometric function and an estimation of the participant's threshold (Watson and Pelli 1983). This information could be the result of preliminary trials before

testing, or by using a uniform prior probability density function (Treutwein 1995). The calculation of the final threshold estimate in maximum likelihood procedures differs with each procedure, but is commonly reached after a set number of trials has been completed, a step size interval has been attained or by using a dynamic stopping criterion (Treutwein 1995). In a study comparing the use of a set number of trials and a dynamic stopping criterion, it was found that there was little advantage in using a dynamic criterion (Anderson 2003)

There are several different maximum likelihood procedures including PEST (Parameter Estimation by Sequential Testing), ZEST (Zippy Estimate of Sequential Testing), SITA (Swedish Interactive Threshold Algorithm – which is a modified ZEST), YAAP and QUEST. QUEST, which is the method which was used to collect scotopic threshold data in Chapters 4-6, will be discussed in more detail in the next section.

1.1.1.1.3 QUEST

Published as a Bayesian adaptive psychometric method by Watson & Pelli (1983) based on the staircase method, the QUEST method assumes the participant's psychometric function follows a Weibull distribution and requires the input of prior knowledge regarding the expected threshold and variance (Watson et al. 1983). This means that the level of each presented stimulus is chosen based on the maximum likelihood of it being the threshold based on the prior knowledge and the responses from previous presentations. After a set number of trials has been completed, threshold (which is the

mode of the psychometric function) and the slope of the psychometric function are produced. The slope provides information on the variability of the participant's judgements, with high variability data resulting in a shallow slope (Neelam et al. 2009). The final threshold does not make use of the prior information, therefore is unbiased.

QUEST is based on the following assumptions (King-Smith et al. 1994):

- That the psychometric function has the same shape under all conditions, when expressed as a function of log intensity.
- That the participant's threshold does not vary from trial to trial.
- That individual trials are statistically independent.

Modified versions of QUEST (minimum variance and ZEST) were proposed by King-Smith et al. (1994), which use the mean of each psychometric function to calculate stimulus intensity and final threshold instead of the mode.

1.5.2.5 Limitations of adaptive psychophysical methods.

Overall adaptive psychophysical methods are seen as efficient and accurate (Treutwein 1995; King-Smith et al. 1994), however, there are limitations. Prior knowledge is required for most of the techniques, which require assumptions about the underlying slope of the psychometric function (Leek 2001). Although all techniques have been designed to be robust, there is still an element of participant variability. QUEST has taken this into account by including variability in the Weibull function, however this assumes a degree of intra-participant stability. Measurement bias, which is the result of repeated testing of a

threshold (the mean between these measurements and the actual threshold), is also present in adaptive methods (King-Smith et al. 1994). Finally, these techniques are also often computationally intensive (Leek 2001).

1.5.3 Dark adaptation and AMD

1.5.3.1 Introduction to dark adaptation and scotopic vision

The human visual system has the ability to operate over a 10 log unit range of luminance (Stockman et al. 2010; Lamb et al. 2004). The term 'dark adaptation' usually refers to the time taken for visual thresholds to reach a minimum ('absolute threshold') in the dark, after exposure to a light source that bleaches a substantial proportion of visual pigment. The time taken to reach absolute threshold in total darkness after a previous exposure to bright light is typically around 50 minutes, a similar duration to the time taken for the sun to set (Reuter 2011). However, in the era of electric lighting, we move between different lighting conditions quickly and so the long time taken for dark adaptation to occur can be problematic.

1.5.3.2 Factors affecting dark adaptation

There are several factors that influence the shape of the dark adaptation function and the final scotopic threshold. These include external factors, for example the brightness of the initial bleaching light, the size and position of the area of retina being tested and the wavelength of the stimulus (Fasih et al. 2010). Internal factors include pupil size, photochemical processes and neural adaption. Testing in the peripheral field results in a

dominant rod section, due to the increasing density of rods (Curcio et al. 1990). Testing with stimuli at shorter wavelengths, to which the rods are maximally sensitive (507nm), will also result in a more prominent rod portion (Fasih et al. 2010).

1.5.3.3 Ageing and dark adaptation

A common complaint of the elderly, even in the absence of ocular abnormalities, is difficulty seeing at lower luminances (Robertson and Yudkin 1944; Owsley, 2011; Jackson et al. 1999). Older adults typically have a loss of a log unit of scotopic sensitivity compared to younger adults (Jackson and Owsley 2000) which can result in an increase in falls (McMurdo and Gaskell 1991) or the avoidance of night driving (Ball et al. 1998). There is also a slowing of the rate of dark adaptation with increasing age, with it taking over 10 minutes longer for 70-year-olds to reach pre-bleach light sensitivity compared to those in their 20s (Jackson et al. 1999). Owsley (2011) and Jackson et al. (1999) suggest that it may be due to a slowing of the visual cycle with age, possibly due to a reduction in 11-cis-retinal at the photoreceptors. Figure 1-10 shows the increase in duration of dark adaptation and raised scotopic thresholds with ageing.

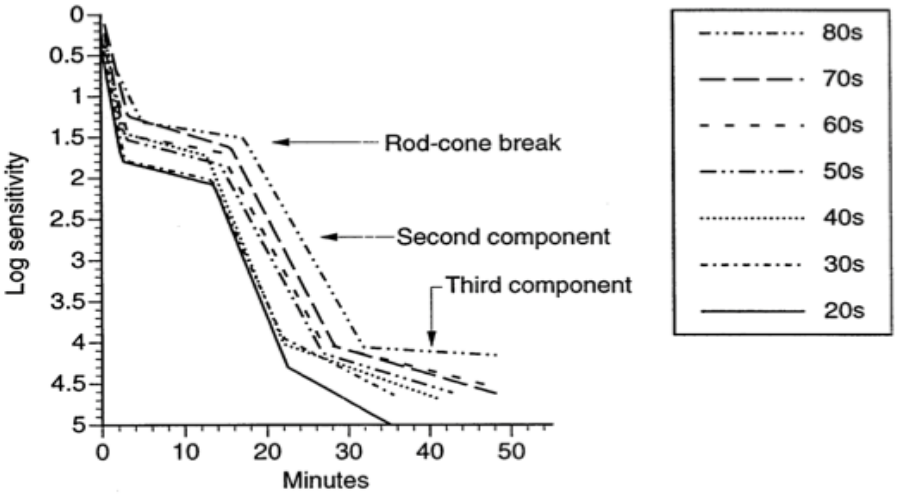


Figure 1-10. Dark adaptation as a function of decade of age (Jackson et al. 1999).

There are several possible contributors to the age-related loss of scotopic sensitivity, with senile miosis and the increased optical density of the ageing eye elevating scotopic threshold by about a 0.10–0.15 log units (Owsley 2011). But even taking these factors into account, there is still a reduction of between 0.39 to 0.50 log units sensitivity (Sturr et al. 1997). There must, therefore, be retinal factors contributing, which could include the age-related loss of photoreceptors (Curcio et al. 1993) and RGCs (Harman et al. 2000). Although the number of cones remains stable with ageing, the density of rods declines by 30% from 27 to 90 years old with the greatest loss in the parafoveal region (Curcio et al. 1993). The morphology of the remaining rods alters, with larger inner segments filling the spaces left behind (Curcio et al. 1993). Although this loss of rods would seem to be a good explanation for the loss in scotopic sensitivity, Jackson et al. (1998) found that the loss of scotopic sensitivity was not accentuated in the parafoveal region where rod loss is greatest. In addition, although the density of rods reduces with age, the quantity of

rhodopsin does not (van Kuijk et al. 1991). This suggests that the reduction in scotopic sensitivity with age may be due to other factors.

1.5.3.4 Dark adaptation and AMD

Patients with AMD often describe difficulties with vision under low luminance before changes under normal lighting conditions (Steinmetz et al. 1993; Owsley et al. 2006) and they may also complain of prolonged time to recover when exposed to bright light (Steinmetz et al. 1993). This is also seen clinically with a decrease in scotopic visual function seen when photopic function (Sunness et al. 1997; Neelam et al. 2009) and clinical status (Owsley et al. 2007) are normal. Therefore it is not surprising that dark adaptation has been found to be affected by AMD (Dimitrov et al. 2012; Dimitrov et al. 2011; Dimitrov et al. 2008; Owsley et al. 2001; Owsley et al. 2007; Steinmetz et al. 1993; Jackson et al. 2002; Sunness et al. 1997; Haimovici et al. 2002; Eisner et al. 1992; Eisner et al. 1991; Jackson et al. 2014b; Jackson et al. 2014a). Rod mediated parameters of dark adaptation have been suggested to be more susceptible to AMD than cone mediated functions (Owsley et al. 2007; Owsley et al. 2000; Owsley et al. 2001), corresponding to the histopathologic changes in AMD. Rod loss precedes cone loss in 75% of early and late AMD retinas (Jackson et al. 2002). Studies report changes in the dynamics of rod-mediated DA, including a prolonged time course of DA (Owsley et al. 2001; Steinmetz et al. 1993; Haimovici et al. 2002; Dimitrov et al. 2008; Eisner et al. 1991; Eisner et al. 1992; Dimitrov et al. 2011) and a delay in the rod-cone break (Owsley et al. 2001; Dimitrov et al. 2008). There is also an increase in the final scotopic threshold (Owsley et al. 2000;

Sunness et al. 1997; Steinmetz et al. 1993; Scholl et al. 2004). These changes are shown in the dark adaptation curves from a participant with no AMD, early AMD and intermediate AMD in Figure 1-11. The rate of rod recovery in DA was found by Dimitrov (2011) to be an indicator of functional loss in AMD that could be used for monitoring individuals at a high risk of developing advanced AMD. More recently, delayed rod- mediated dark adaptation has been found to be a functional biomarker for AMD (Owsley et al. 2016).

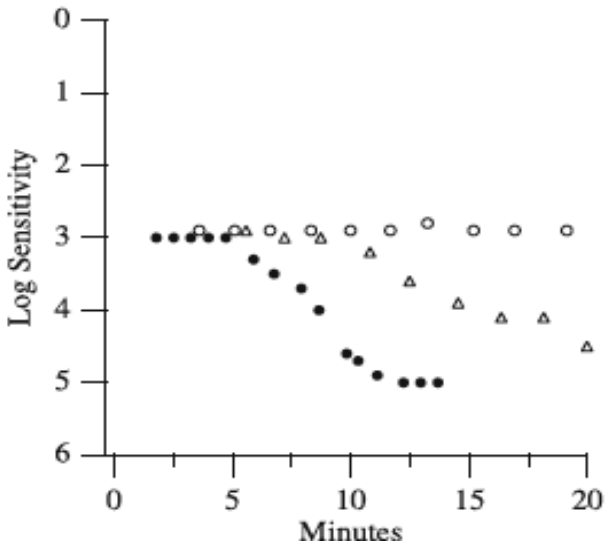


Figure 1-11. A Representative dark adaptation curves of a normal adult (closed circles), early AMD patient (open triangles), and intermediate AMD patient (open circles) (Jackson and Edwards. 2008).

The time course of cone parameters of DA have also been found to be affected by AMD (Gaffney et al. 2011; Phipps, 2003; Dimitrov et al. 2011; Dimitrov et al. 2008). Gaffney et al (2011) found that the time to rod cone break and time to cone recovery were highly diagnostic for early AMD at 12 degrees eccentricity, but final cone thresholds were

unaffected. This diagnostic potential is important clinically as cone recovery can be quantified in as little as 10 minutes (Gaffney et al. 2011), which is attractive to clinicians. This suggests that the kinetics of cone function are affected prior to steady state measures. If underlying changes to Bruch's membrane and the RPE are responsible for adaptation deficits, then cone adaptation may be expected to be affected regardless of the preferential loss of rods in AMD.

In addition to the assessment of scotopic thresholds, electrophysiology will also be used to assess visual function in this thesis and is discussed in detail in the next section. This section provides background information and an overview of the elements of the waveform which are to be analysed in chapter 7.

1.5.4 Electroretinography

Ocular electrophysiology provides an objective assessment of the integrity of the visual pathway from the eye to the brain. By recording the electrical signals produced by visual stimulation via the electroretinogram, electro-oculogram or visual evoked potential, the location and extent of the disruption in the visual pathway can be determined. These assessments measure evoked potentials that are transient electrical responses produced by neurones in response to a stimulus.

1.5.4.1 Electroretinogram

The electroretinogram (ERG) is the objective recording of the summed electrical activity produced by the retina in response to a visual stimulus. This can be measured under photopic or scotopic conditions and with a flash or pattern stimulus. Although the ERG is a summed response, different groups of cells become active at different times after the onset of a stimulus. Hence, each part of the waveform produced gives information about different cells within the retina. Recording the ERG requires three electrodes, an active on the cornea, reference on the skin at the outer canthus, or in the contralateral eye and earth on the forehead.

Since 1989 The International Society for Clinical Electrophysiology of Vision (ISCEV) has provided regularly reviewed standards for the recording parameters of full field clinical ERG techniques (McCulloch et al. 2014). The actual form of the wave produced depends on:

- state of adaptation
- stimulus wavelength
- stimulus intensity
- stimulus temporal frequency

Low temporal frequencies, shorter wavelengths, lower luminance stimuli and dark adaptation all help to isolate the rod response (Perlman 2001). The stimulus can also be patterned, global or focal to isolate different retinal locations.

1.5.4.2 Flash electroretinogram

Figure 1-12 shows the waveform produced in response to a single flash full field stimulus that records summed potentials from all the retinal cells. The resultant ERG has a repeatable waveform.

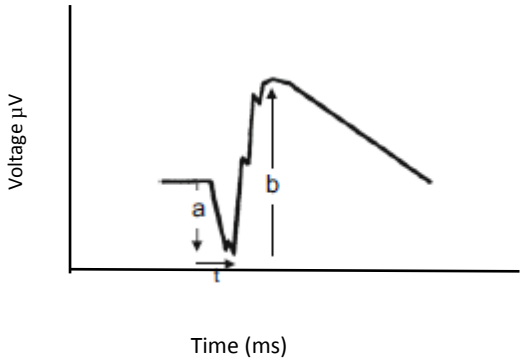


Figure 1-12. Flash electroretinogram waveform from a normal subject showing the amplitude of the a wave (a) and b-wave (b), and the implicit time of the a wave (t) (McCulloch et al. 2014).

In 1933, Granit determined that the scotopic transient ERG of the cat was attributable to the summation of 3 physiological processes, PI, PII and PIII (Granit 1933). These three processes were numbered depending on the order of disappearance under progressive anaesthesia. PI is a positive wave of long latency, PII is a reasonably fast positive wave and PIII, which develops faster than PI or PII, is a negative wave. PIII has been further divided into the 'fast' and 'slow' components that have also been attributed to different layers of the retina. However, these processes cannot be isolated in the standard ERG. Figure 1-13 shows that the three processes sum together to form the overall ERG

waveform that is shown in black. The clinically recordable peaks and troughs of the ERG are referred to as the sub-components. The following section will discuss certain sub-components of the ERG (those most relevant to this thesis), the recording parameters required to elicit them, and their proposed retinal origins.

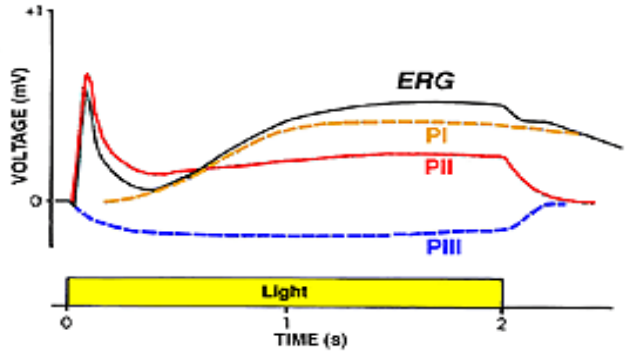


Figure 1-13. Granit's three processes of the ERG.

1.5.4.3 The a-wave

Appearing first in the waveform, the a-wave is a negative potential, and has been attributed to the leading edge of Granit's PIII component seen in Figure 1-13. The origin of this wave has been has been determined by intra-retinal electrode studies which found signals from the photoreceptors contributed to PIII (Brown et al. 1957). Current source density analysis suggests that the rod outer segments are the main source of the scotopic ERG a-wave, as the photocurrent they produce is of a similar sign and waveform to the currents that underlie the a-wave (Penn and Hagins 1969). Studies using the glutamate agonist sodium aspartate, which prevents the transmission of glutamate from the photoreceptors to bipolar cells, have found that the a-wave is not extinguished by its

administration which is also suggestive of a photreceptor source (Wakabayashi et al. 1988). In photopic conditions, the origins of the a-wave are more complicated and may involve postreceptoral activity from hyperpolarising bipolar cells or horizontal cells (Bush and Sieving 1994).

1.5.4.4 The b-wave

Evidence for b-wave origins come from single cell intracellular recordings from the inner nuclear layer. It is this layer and the bipolar cells within it which contribute to the positive b-wave (Brown et al. 1957; Brown and Wiesel 1961), which is attributed to Granit's process PII (Granit 1933). In particular, it is thought to be the ON bipolar cells that contribute to the b-wave. 2-amino-4-phosphonobutyrate, which abolishes the response of ON- bipolar cells to light has been found to diminish the b-wave (Tian and Slaughter 1995; Stockton and Slaughter 1989). Studies of the mud puppy retina have also suggested that there may be an involvement of the Müller cells (Miller and Dowling 1970), although studies have found that blocking the Müller cell activity using Barium ions does not reduce the b-wave amplitude (Lei and Perlman 1999).

1.5.4.5 The focal ERG

The focal ERG allows the identification of localised defects by using a uniform flickering or flashing stimulus with a desensitising surround to record responses from the central retina. When centred on the macula, the combination of a small stimulus and the suppression of signals from the peripheral retina by the surround allow a focal cone

driven response to be recorded. The flicker stimulus results in a sinusoidal waveform, and a flicker of greater than 30Hz will result in a cone driven response (McCulloch et al. 2014). A postreptoral contribution to the flicker ERG has also been proposed due to the effect of glutamate on the waveform (Kondo and Sieving 2001).

This recording is useful in conditions where localised functional changes occur such as in AMD and cone dystrophies, where small changes to the waveform may be swamped by the summed signals of the peripheral, healthy retina. However there are no international standards set for the recordings and good fixation is required (Berrow et al. 2010) which may make recording from individuals with nAMD and GA difficult.

1.5.4.6 The ERG in AMD

Different types of ERG recording have been used to investigate retinal function in AMD. Using the full field ERG to study peripheral visual function Sunness et al. (1985) concluded that peripheral visual function was not affected by AMD severity. Jackson et al. (2004) later agreed, finding no difference in the response onset or amplitude of the a-wave of the full field scotopic ERG, between participants with early and late AMD and age matched and younger controls. This is probably due to the full field ERG summing the overall retinal response, which would include peripheral rods not affected by macular degeneration and may mask macular abnormalities. In contrast, significant reductions in the a and b-wave amplitudes of the full field rod ERG and prolonged b-wave implicit times have been found in another study, suggesting that there is a global impairment in AMD,

possibly due to vascular changes (Walter et al. 1999). The differences between these studies can be explained in part by the small sample (n=7) of late stage AMD participants used in the Jackson et al. (2004) study, whereas there was no breakdown of disease classification of the 66 participants studied by Walter et al. (1999), which included both participants with nAMD and GA, which may have skewed results.

Using new multifocal ERG (mfERG) techniques which target the rod pathway, rod function has been found to be preferentially affected by AMD (Chen et al. 2004; Feigl et al. 2006). When assessing both cone and rod mfERGs, the rod mfERG response was found to be delayed in participants with early AMD, but there were no significant changes in the cone response (Feigl et al. 2005a). However, Li et al. (2001) did find changes in the foveal amplitude and latency of the cone dominated mfERG in participants with AMD and in asymptomatic eyes of participants with early AMD or AMD in the other eye, suggesting that the mfERG may be useful in diagnosing and monitoring at risk groups. Differences in the recordings have also been found between participants with nAMD and GA, with the response densities of N1 and P1 decreasing dramatically for the nAMD group compared to the participants with GA, suggesting that the mfERG may be used for AMD disease quantification (Huang et al. 2000). The mfERG may also be used for monitoring the effectiveness of treatments (Berrow et al. 2010).

The focal cone ERG has been found to have delayed implicit times in eyes with early AMD (Binns and Margrain 2007) and in the fellow eyes of participants with unilateral nAMD (Sandberg et al. 1998; Sandberg et al. 1993) suggesting that changes in the focal ERG are

present before changes in VA. A reduction in mean amplitude has been found in patients with GA, which was correlated with GA severity (Falsini et al. 1999). There is also evidence that focal ERG abnormalities could precede the morphological changes typical of more advanced disease (Falsini et al. 1999). Implicit time delays of 1ms have been found in eyes with a choroidal perfusion defect (Remulla et al. 1995), which may underlie hypoxia driven progression of AMD as discussed in section 2.2.5.

1.5.5 Imaging techniques

Age-related macular degeneration has been evaluated using a number of imaging techniques including stereo and digital fundus photographs, fluorescein angiography, optical coherence tomography and fundus autofluorescence. The following section will describe in more detail the imaging methods that will be used in this PhD.

1.5.5.1 Fundus photography

Fundus photography provides images for the assessment of the retina. Providing an objective record of the retina, they can be used for monitoring and diagnosis. Fundus photography can be used to document all clinical stages of AMD. Figure 1-14 is a retinal photograph showing nAMD and Figure 1-15 shows GA.

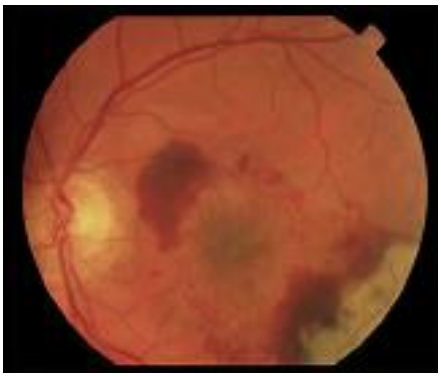


Figure 1-14. Retinal photograph showing nAMD (Miller 2013).

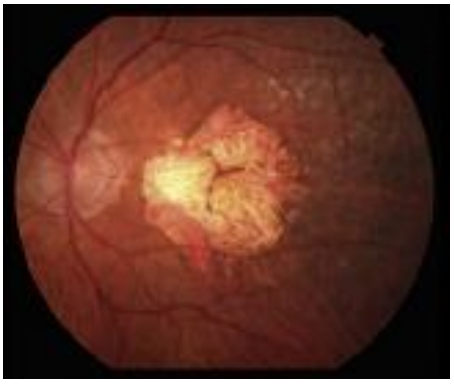


Figure 1-15. Retinal photo showing geographic atrophy (Miller 2013).

1.5.5.2 Optical coherence tomography

Optical coherence tomography (OCT) is a non-invasive in vivo imaging technology that uses low-coherence interferometry to produce cross-sectional images of the retina. Optical coherence tomography images showing a normal retina (Figure 1-16) and a retina with nAMD (Figure 1-17) are shown below. The image is based on optical scattering from internal tissue microstructures (Huang et al. 1991). In AMD the technique is used for diagnosis, monitoring and management (Regatieri et al. 2011).

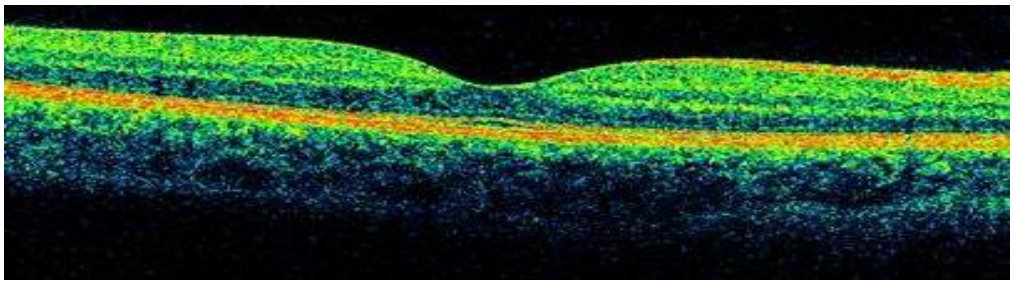


Figure 1-16. Optical coherence tomography of a normal retina

Http://www.illinoisretinainstitute.com/index.php?p=1_6_Normal-Retina (2012).

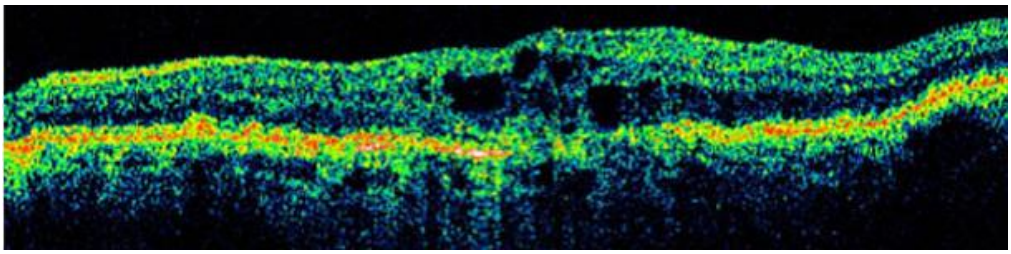


Figure 1-17. OCT image showing nAMD (Hageman 2016) avaliable at

http://webvision.med.utah.edu/imageswv/Hagerman.Fig36.jpg.

1.6 Rationale and Objectives of the PhD

The financial burden of AMD depends on the stage of AMD present, with considerable costs associated with the late stages of the disease (Schmier and Levine 2013). In 2010, hospital spending on Lucentis was the third highest of all drugs costing the NHS £129 million. When considering early AMD there are no direct non medical costs or indirect costs and changes to visual function have not yet taken place. Treating at the earlier stage of the disease is therefore appealing, but faces a number of challenges. These include the

requirement for clinical measurements for trial outcomes, a greater understanding of the earlier stages of the condition to develop treatments, methods of diagnosing the condition before clinical signs and symptoms are present.

The principal objective of the research presented in this thesis was to determine the role of retinal hypoxia in visual dysfunction in early AMD. This will help to clarify the role of hypoxia in AMD onset and will determine whether aspects of visual function may be assessed in early AMD as biomarkers for hypoxia in the disease process. This could ultimately lead to the development of clinical tests which are sensitive to desease progression and would hasten the development of new treatments targeted at the early stages of the disease. The specific aims were:

1) *To investigate if the dark adapted healthy retina is hypoxic by comparing scotopic thresholds under hyperoxic and normoxic gas conditions.*

   Hypothesis: the dark adapted retina is on a hypoxic knife edge, however it is hypothesised that in the normal population the oxygen supply meets its needs and therefore there will be no decrease in scotopic thresholds when breathing 60% oxygen, but that there will be an increase in scotopic thresholds when breathing 14% oxygen.

2) *To investigate if there is a topographical retinal effect of hyperoxia or hypoxia on scotopic thresholds.*

   Hypothesis: The maximum density of rods is at 12-18 degrees from the fovea (Curcio et al. 1990), and studies have found that hypoxia effects visual function

between 9-15 degrees (Feigl et al. 2011; Connolly and Hosking 2008b). Therefore it is hypothesised that hypoxia will affect the retina preferentially at 12 degrees.

3) *To investigate the effect of hyperoxia on scotopic threshold of participants with early AMD.*

   Hypothesis: As hypoxia has the ability to raise scotopic thresholds in healthy young participants, it could be that hypoxia also causes the rise in scotopic thresholds seen in patients with early AMD. If this is the case then temporarily reducing hypoxia in participants with early AMD, by the inhalation of 60% oxygen, should result in a decrease in scotopic thresholds.

4) *To investigate the effect of hypoxia on the scotopic thresholds of participants with early AMD*

   Hypothesis: A hypoxic episode will cause an increase in scotopic thresholds in both participants with early AMD and age-matched controls, but the effect will be greater in participants with early AMD.

5) *To investigate the effect of hypoxia on the amplitude and implicit time of the scotopic full field ERG a-wave and b-wave, and the amplitude and implicit time of the first harmonic of the flicker ERG.*

   Hypothesis: A hypoxic episode will result in a reduction in amplitude of the scotopic full field ERG a-wave and a reduction in amplitude and increase in implicit time of the b-wave in participants with early AMD and in the control group. These changes are expected to be greater in the group with early AMD. It is also

hypothesised that there will be a delay in the onset and a reduction in the amplitude of the first harmonic of the flicker ERG.

In order to develop a detailed understanding of the effect of hypoxia and visual function, and its potential influence on the pathogenesis of AMD, two systematic literature reviews were conducted:

1) A *systematic literaure review into the effect of hypoxia on visual function.*
2) *A systematic literature review into the role of hypoxia in early age-related macular degeneration.*

2 A review of literature regarding the effect of hypoxia on visual function and the role of hypoxia in the pathogenesis of Age-related macular degeneration.

This chapter summarises the findings from two literature reviews which were conducted on the effect of hypoxia on visual function and the role of hypoxia in the pathogenesis of AMD.

The first section of this chapter will present the findings of a literature review investigating the effect of hypoxia on visual function. This question has been of longstanding interest since the advent of aviation due to the reduction in atmospheric pressure with increases in altitude, resulting in a decrease in the oxygen concentration of inspired air. As early as 1939, hypoxia was shown to have an effect on many aspects of visual function (McFarland and Evans 1939). As this PhD aims to investigate the effect of hypoxia and hyperoxia on visual function in AMD, it is valuable to review current understanding of the impact of blood oxygenation in people without the condition.

The second section of this chapter will present the findings of a review conducted to investigate the relationship between hypoxia and AMD. The hypothesis that hypoxia has a role in the pathogenesis of AMD is based on the knowledge that the normal retina is on a

hypoxic knife-edge in the dark (Wangsa-Wirawan et al. 2003; Feigl et al. 2008). The theory suggests that, in early AMD, extra strain is placed upon an already critically limited outer retinal blood supply due to histological changes in the retina and associated structures, including the choriocapillaris and Bruch's membrane. The resultant hypoxia then causes changes at a cellular level, can upregulate growth factors and also cause apoptosis leading to the signs seen in nAMD and GA respectively (Ambati et al. 2003; Gerona et al. 2010; Guma et al. 2009). Current evidence suggests that there are three potential aetiologies for AMD (i.e. hypoxia, inflammation and oxidation; see section 1.4.4). However, there are possible overlaps between these putative mechanisms. For example, it has been proposed that inflammation may lead to hypoxia by increasing the metabolic demand of the retina, or that there may be changes in blood vessels secondary to inflammation (Arjamaa et al. 2009). There are also associations between hypoxia and genetics (Feigl 2009), and between the oxidative stress and hypoxia theories, with ROIs being created in hypoxic conditions (Kaur et al. 2008; Arjamaa et al. 2009).

The findings of both studies will be summarised at the end of the Chapter.

2.1 Search methods

A search of the Web of Science database was conducted using the search terms in Table 2-1. Hand searching of lists of references from included studies and literature reviews was also conducted.

Hypoxia	AND	Visual fields OR Colour vision OR Contrast sensitivity OR Dark adaptation OR Visual acuity OR Electroretinogram (search #1)
		Age-related macular degeneration OR AMD OR ARMD (search #2)

Table 2-1.Literature review search terms.

Studies published from January 1900- October 2015 were included. With respect to the first review, studies were excluded if they did not investigate an aspect of visual function under conditions of hypoxia. With respect to the second review, studies were excluded if they did not investigate a stage of AMD, or they did not relate hypoxia to the pathogenesis of AMD. Studies were also excluded from either review if they were not written in the English language or if they were a conference abstract only.

The abstracts of all potentially relevant studies were reviewed against the eligibility criteria, and full manuscripts were obtained for those which appeared to be eligible. Final eligibility was determined on assessment of the full manuscripts. In this review, when altitude/ simulated altitude conditions were given, equivalent oxygen concentration values were calculated using a hypoxia/altitude calculator, which can be accessed at http://www.altitude.org/air_pressure.php

2.1.1 Search results

For the first search into the effect of hypoxia on visual function a total of 42 articles were identified as meeting the eligibility criteria for inclusion (see Figure 2-1). Summary data of all included studies are presented in Appendix I. Studies were grouped into six catagories; visual acuity, visual fields, contrast sensitivity, colour vision, ERGs and dark adaptation. Of the included studies, 55% (23 studies) simulated different air conditions using a breathing mask, 20% (8 studies) used a hypobaric chamber and 26% (11 studies) ascended to altitude.

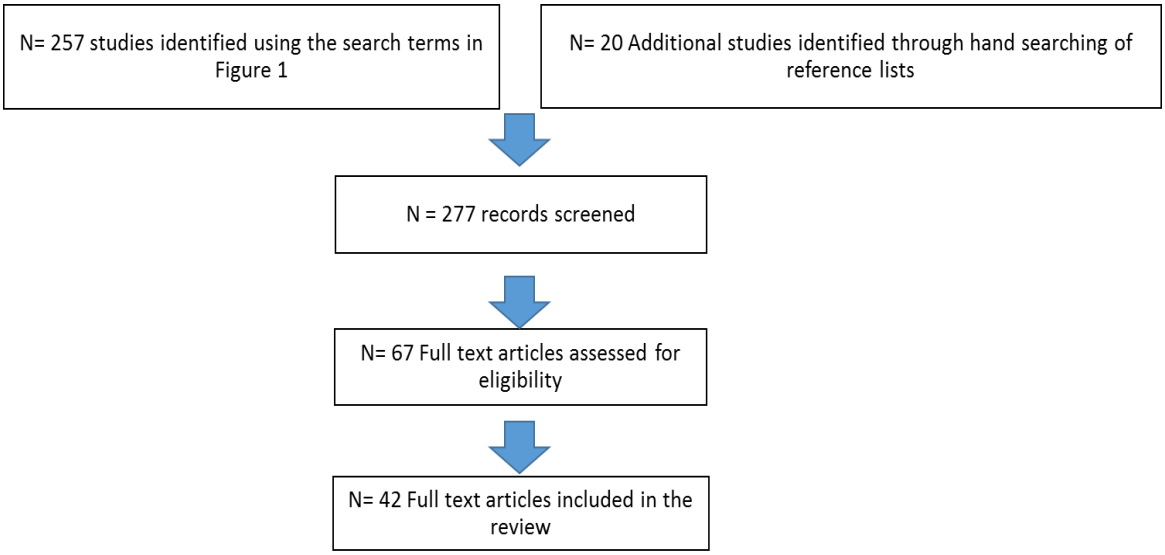


Figure 2-1. Flow chart showing results of the effect of hypoxia on visual function literature search.

For the second search into the role of hypoxia in the pathogenesis of AMD a total of 49 articles were identified as meeting the eligibility criteria for inclusion in the study (see

Figure 2-2). Summary data of all included studies are presented in appendix II. The studies were grouped into main sections relating to changes in specific morphological and functional features associated with hypoxia in AMD i.e. ocular vasculature, Bruch's membrane, growth factors and inhibitors and visual function. Of these studies 80% (39 studies) were on human eyes, 10% (5 studies) were on in vitro cells and 10% (5 studies) were on animal tissue.

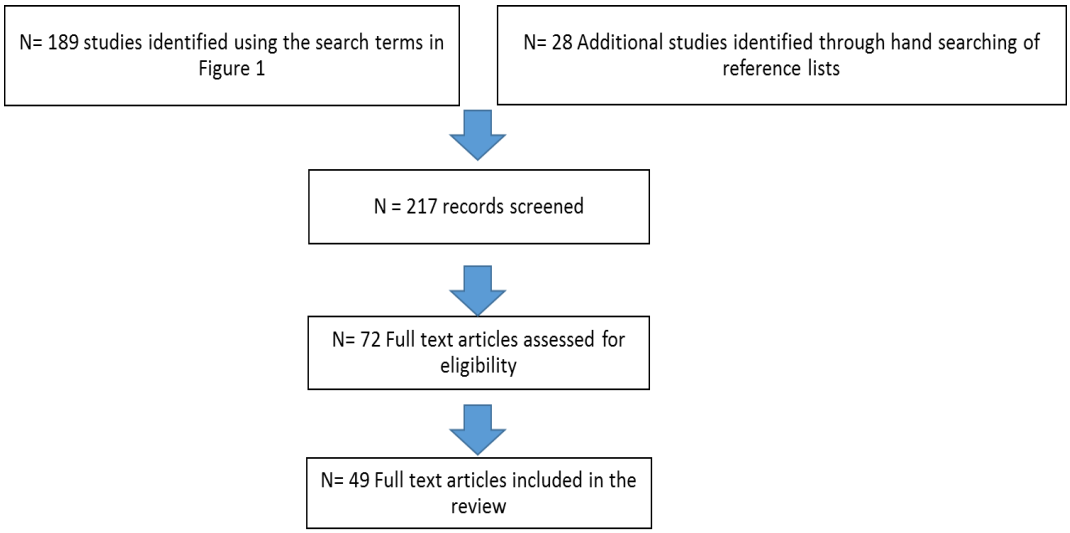


Figure 2-2. Flow chart showing results of the role of hypoxia in the pathogenesis of AMD literature search.

2.2 The effect of hypoxia on visual function.

2.2.1 The effect of hypoxia on visual acuity

Three studies have assessed the effect of hypoxia on VA. Willmann et al. (2010) found no change in VA when 14 healthy subjects ascended to 4559m, this was despite SpO_2

dropping to 69.35 +/4.36%. They discussed that this could be due to a range of autoregulatory mechanisms within the macular region to meet oxygen needs under exposure to hypoxia. A reduction in VA with both normobaric hypoxia (McFarland & Halperin 1940) and hypobaric hypoxia (Vecchi et al. 2014) has been observed. However, McFarland found that this was only significant under scotopic conditions whereas Vecchi et al. (2014) reported a reduction in VA under both photopic and scotopic conditions when participants were exposed to 13.44% O_2. It is interesting to note that this only became statistically significant after 45 minutes of breathing the reduced oxygen levels.

In conclusion, VA is relatively resistant to changes in hypoxia, but may be affected by increased durations of hypoxia or at lower light levels.

2.2.2 The effect of hypoxia on visual fields

Studies of the effects of hypoxia on visual field thresholds have produced variable results. Of the nine studies included in this review three studies have found no evidence that hypoxia affects visual fields (Feigl et al. 2011; Falk et al. 1991; Yap et al. 1995). Feigl et al. (2011) studied the sensitivity to static and flickering stimuli in seven locations (1°, 3°, 6°, 10°, 15°, 22° and 30° eccentricitiy) under photopic illumination in 14 normal subjects. Flickering stimuli are known to increase the metabolic demand of the retina and although, as expected, sensitivities were lower for flickering stimuli, the detection threshold of neither stimulus type was affected by breathing 12% oxygen. The effect of hypoxia on visual field sensitivity in subjects with glaucoma, in age matched normals and

in a group of younger subjects has also been investigated by measuring the central Humphrey 24-2 fields at ground level and at simulated 3000m altitude (15% oxygen) using a hypobaric chamber. Whilst older subjects had a lower sensitivity than younger subjects, altitude had no effect on these sensitivities in any participant group (Falk et al. 1991).

However, other studies have found hypoxia to have an effect on visual fields in photopic (Horng et al. 2008; Connolly et al. 2008b) and mesopic conditions (Feigl et al. 2011; Connolly and Hosking 2009b; Connolly 2011). A pilot study by Feigl et al. (2011) studying the effect of 12% O_2 on static perimetric thresholds in mesopic conditions, found that sensitivity was reduced at eccentricities of up to 15°. Connolly and Hosking (2008) concluded from their study that hypoxia affected photopic visual fields up to 9 degrees from fixation when breathing 14.1% O_2, with the effect reducing with further increases in eccentricity (out to 20 degrees). In comparison, some studies have found that the effect of hypoxia on photopic visual fields is greatest in the periphery, out to 30° (Horng et al. 2008). The conflicting results of these studies could be due to the differing background luminances. The relative influence of rod photoreceptors would have been lower in the study by Connolly and Hosking (2008), which employed a photopic luminance of 100 $cd.m^2$, compared to the 10 $cd.m^{-2}$ background luminance that was used in the study by Horng et al (2008). The effect of hypoxia in the peripheral retina could be due to the demands of the rods in the peripheral retina, as these contribute to vision up to 200$cd.m^2$, and are likely to be competing with cones for the limited oxygen supply

available (Horng et al. 2008). There is no evidence of a differential effect of hypoxia on photopic thresholds in the four field quadrants (Connolly and Hoskin 2008). Hypoxia has been found to reduce sensitivity to flickering visual field targets to a greater extent in mesopic than in photopic conditions (Connolly et al. 2009b; Connolly 2011; Feigl et al. 2011). This indicates that the outer retina may be operating under a state approaching hypoxia in mesopic conditions (Connolly et al. 2009b), possibly attributable to an increased rod and cone oxygen consumption under reduced light levels (Connolly 2011).

A study looking at gender effects of oxygenation levels on frequency doubling perimetry recorded photopic thresholds across the central 40 degrees of visual field (Connolly and Hoskin 2008). Thresholds were measured when participants were breathing 14.1%, 21%, and 100% O_2. Whilst participants showed a reduced performance under hypoxic conditions, in females alone, sensitivity was found to be enhanced in all retinal locations by the hyperoxic condition compared to normoxia (Connolly et al. 2008b). This study also observed an important effect of gas order, with prior hypoxia diminishing the benefit of subsequent oxygen, whilst if oxygen was presented first, then the impairment under hypoxia was not as great. However, the wash-out period between gas presentations in this study to prevent cross-contamination was only 2-3 minutes, which may have influenced these findings.

Demir et al. (2012) tested central 30 standard automatic perimetry (SAP) and short wavelength automated perimetry (SWAP) in people with chronic obstructive pulmonary disease (COPD) that were on long term oxygen therapy. Mean deviation, pattern standard

deviation and corrected pattern standard deviation were all statistically different between participants with COPD and healthy controls. However no detail was given regarding the extent and distribution of the visual field defects, or the arterial blood gas results. Therefore, although the study concluded that hypoxia in COPD seems to affect the retina and optic nerve, the level and range of participant hypoxia is unknown. It is also unknown what oxygen therapy the patients were receiving at the time.

In conclusion, the literature is somewhat inconclusive, but suggests that visual fields are affected by hypoxia under certain recording conditions. Under mesopic conditions there is some evidence to suggest that peripheral fields are relatively more affected, whilst under photopic conditions the central retina appears to be targeted by hypoxic effects. The impact of hypoxia on scotopic thresholds will be discussed in section 2.7, which discusses dark adaptation.

2.2.3 The effect of hypoxia on contrast sensitivity

Early animal studies recording single action potential activity from cat ganglion cells found that contrast sensitivity was preserved with hypoxemia (Enroth-Cugell et al. 1980). However, despite this evidence from animal studies, some human evidence suggests that hypoxia does reduce CS (Bridges and Kolder 1964; Nordmann and Roncin 1991; Connolly et al. 2009a; Connolly and Serle 2014). Bridges and Kolder (1964) found that breathing 10% oxygen was associated with a reduced sensitivity that continued post hypoxia for over 10 minutes. However three subjects were tested twice and the repeat data may

have influenced the results. At high altitudes (5400m), equating to 11% oxygen tension, a decrease in contrast sensitivity in the low and medium spatial frequencies was recorded in 12 healthy mountain climbers who ascended by foot (Nordmann et al. 1991). Although this will give a more realistic view of the effects of altitude, it does introduce other, external factors such as subject fatigue and changes in background lighting. More recently, CS has been reported to be affected by hypoxia in the mesopic range (Connolly et al. 2009a; Connolly 2010). Connolly and Barbur (2009) found that when measuring low contrast acuities, hypoxia (breathing 14.1% oxygen) necessitated 50% more contrast to maintain the acuity achievable when the subjects were breathing 100% oxygen. This was at both the fovea and at various eccentricities out to 5^{o} when testing in the mid-mesopic range.

In contrast, other studies have suggested that CS is resistant to hypoxia, with insignificant changes to visual capability down to an oxygen tension of 8% (Rossi et al. 1985; Kobrick et al. 1988; Yap et al. 1995; Connolly 2010). However Kobrick et al. (1988) only used 8 subjects who, over a 40 day period, had a reduction in oxygen tension to 8% in a decompression chamber. A high stimulus luminance was used, which is less likely to be affected by hypoxia and two of the participants did not complete the experiment due to altitude sickness. There are no participant details in the Rossi et al. (1985) study. Studying contrast sensitivity at both 18,000ft and 25,000ft (10.92% and 8.19% oxygen respectively) Pescosolido et. al. (2015) found that the reduction in CS was only significant at 25,000ft.

Connolly (2010) reported that foveal contrast sensitivity was resistant to changes in oxygenation when lighting conditions favoured cone function (photopic conditions).

Interestingly, Benedek et al. (2002) showed that short term hypoxia increased contrast sensitivity after measuring contrast sensitivity in the range of 0.5-4.8 cycles/degree in a hypobaric chamber at 5500m, equivalent to breathing 11% oxygen. He postulated that short hypoxic challenges (15 minutes) might enhance early visual processes, evidenced by a significant negative relationship between arterial oxygen saturation and contrast sensitivity values at low and medium spatial frequencies (0.5-4.8 c/deg). However there was no control of hypercapnia (high levels of CO_2 in the blood), which could have distorted the results. It was still concluded that chronic hypoxia would result in a visual loss.

Hypoxia has been postulated to be involved in the pathogenesis of diseases with vascular changes. The effect of breathing additional oxygen on the CS of patients with type 1 diabetes suggested that hypoxic tissue might result in the decline in contrast sensitivity seen in diabetes (Harris et al. 1996). When breathing 100% oxygen, the defects that had been seen at 12 and 18 cpd when breathing room air were improved, and at 12 cpd actually reached normal values.

In conclusion, contrast sensitivity, especially at low spatial frequencies, can be affected by hypoxia but is preserved at higher levels of illumination.

2.2.4 The effect of hypoxia on colour vision

From the 13 studies reviewed looking at the effect of hypoxia on colour vision, 11 concluded that colour vision was affected by hypoxia. Two studies found that hypoxia resulted in a protan type defect (Kobrick 1970; Bouquet et al. 2000) and two found a deutan type defect (Richalet et al. 1989; Richalet et al. 1998), although neither of these studies tested for a tritan defect. Seven studies reported a resultant tritan defect (Bouquet et al. 2000; Connolly et al. 2008; Karakucuk et al. 2004; Smith et al. 1976; Tekavcic-Pompe & Tekavcic, 2008; Willmann et al. 2010). A generalised loss of colour vision for both red-green and blue-yellow perception was found by two studies (Vingrys and Garner 1987; Connolly et al. 2014) and a small red-green defect was reported by Connolly et al. (2008).

Looking at the effect of oxygenation state and light levels on colour vision, both Connolly et al. (2008) and Connolly and Serle (2014) found that under mild hypoxia chromatic sensitivity decreased progressively with reducing luminance. This supports other results that the effect of hypoxia on colour vision is more pronounced under lower lighting levels (Kobrick 1970; Smith et al. 1976; Hovis 2012). Hovis (2012) studied the effects of hypoxia on colour vision in normals and those with red-green defects at ground level and 3780m (13.5% O_2) using three colour vision tests. The results suggested that mild hypoxia is equivalent to a slight reduction in luminance with respect to its effect on colour vision.

Smith et al. (1976) concluded that their tritan defect could be interpreted as evidence of the effect of hypoxia on the inner retina. Vingrys et al. (1987) also suggested an inner retinal effect of hypoxia. Discussing their colour vision results and the lack of an effect of hypoxia on a photostress test included in the same study they concluded that their generalised colour vision loss was similar to that found in optic neuritis- a condition affecting retinal ganglion cells. However Willman et al. (2010) and Connolly et al. (2008) proposed that the effects on photoreceptors are the cause of colour vision defects seen in hypoxic conditions. A tritan defect in hypoxic conditions was suggested by Willman et al. (2010) to be due the lower numbers of S cones in the retina. With only 5-10% of cones being S cones, if the maximum responses of all cone pathways were equal, the effect on the S cones would become detectable earlier than for the L and M cone pathways. Photoreceptor vulnerability was also suggested by Connolly et al. (2008), proposing that the S cones may have a higher metabolic demand than the other cone types.

However, not all studies have found hypoxia and altitude to have a significant effect on colour vision. Schmeisser et al. (1997) found no change in colour vision up to 4,200m (12% O_2) and Leid and Campagne (2001) studying up to 7,000m (9% O_2) agreed. Using the desaturated D15 test, Leid and Campagne (2001) demonstrated that in trained subjects, severe hypoxia due to altitude (on foot) does not lead to changes in colour perception. However in a group of nine of subjects, one already had a tritan defect and several suffered from severe altitude sickness that may have affected results and led to only two

subjects making it to an altitude of 7,000m. It should also be noted that the desaturated D15 has a weak sensitivity to red-green defects.

In conclusion, colour vision seems to be affected by hypoxia and studies have found this in the protan, deutan and tritan axes, particularly at lower light levels. Conclusions by two studies after analysis of ERG and the psychophysical findings were that the inner retina, in particular the RGCs, were sensitive to hypoxia and were responsible for this defect (Vingrys et al. 1987; Smith et al. 1976). The results of more recent studies (Connolly et al. 2008a; Willmann et al. 2010), suggest the outer retina is affected and that these changes could be influenced by the different proportions of photoreceptor types (Willmann et al. 2010) with the possibility that S cones have a greater metabolic demand (Connolly et al. 2008a), making them more susceptible to dysfunction in hypoxic conditions.

2.2.5 The effect of hypoxia on electrophysiology

Animal studies have shown that the b-wave of the ERG is affected by ischaemia (Block and Schwarz 1998; Kang Derwent and Linsenmeier 2000). More recently, studies on human subjects have used mfERG and flash ERG recordings to assess retinal function under hypoxic conditions and most have found changes to the waveform (Tinjust et al. 2002; Janáky et al. 2007; Klemp et al. 2007; Kofoed et al. 2009; Pavlidis et al. 2005; Feigl et al. 2007; Feigl et al. 2008; Schatz et al. 2014; Schatz et al. 2013).

Looking at the human photopic ERG, Tinjust et al. (2002), Janaky et al. (2007) and Brown, Hill, & Burke (1957) found that the a-wave amplitude and latency were not affected by hypoxia, suggesting that the PRs are spared from this effect. However, when recording scotopic ERGs, a significant decrease in the amplitude of the a-wave, but a preserved latency was found by two studies (Schatz et al. 2014; Schatz et al. 2013), suggesting a decrease in the quantity of cells that are responding, as opposed to a change in the functionality of the cells. A decrease in the photopic b-wave amplitude has also been reported in hypoxic conditions (Schmeisser et al. 1997; Tinjust et al. 2002; Schatz et al. 2014; Schatz et al. 2013). It is of interest that Schatz et al (2013 & 2014) reported that changes to the amplitude of the b-wave were only found with stimuli that affected both PR types; when rod only stimuli were used (0.01 cd.s/m^2), no significant changes were observed.

Decreases in the amplitudes of the oscillatory potentials (OPs) OP1, OP2 and OP3 with hypoxia have also been recorded (Janáky et al. 2007; Tinjust et al. 2002). Changes to the OPs indicate that the inner retina is affected by oxygenation changes as their origins are thought to represent activity from the amacrine and ganglion cells (Wachtmeister 1998). However, Schatz (2013 & 2014) found no changes in OPs. Further evidence that the inner retina is affected by hypoxia comes from the pattern ERG. A delayed N95 implicit time and depressed N95 amplitude in pattern ERG recordings has been seen in the case of mild, transient hypoxia (Kergoat et al. 2006). The origins of the N95 component of the PERG have been ascribed to the retinal ganglion cells (Heckenlively and Arden 2006).

There are differences between the findings of these studies. For example, Janaky (2007) did not report reduced b-wave amplitudes and found greater reductions in the OP amplitudes when compared to Tinjust et al. (2002). This may be explained by difference in recording protocols. Whereas Tinjust et al. (2002) averaged only photopic ERGs which did not involve dark adaptation, ERGs recorded by Janaky (2007) were involved recording ERGs after 20 mins dark adaptation allowing the assesment of rod, cone and OP functions separately.

Changes in the mfERG have been recorded, with the fovea being most sensitive to oxygenation changes (Klemp et al. 2007; Pavlidis et al. 2005; Feigl et al. 2007; Feigl et al. 2008). Klemp (2007) found that the P1 and N2 amplitudes were reduced by up to 38.5% and 33.0% respectively by hypoxia in photopic conditions, however the implicit times of the waveform components were unchanged. The oscillatory potentials were reduced, but this effect was not regional. The fast flicker and slow flash mfERG have both been found to be affected by hypoxia with a decreased N1 P1 amplitude indicating that ON and OFF bipolar cells were most affected (Feigl et al. 2007; Feigl et al. 2008). Figure 2-3 shows data collected by Feigl et al. (2007), showing reduced amplitude waveforms and reduced 3D scalar product response densities when the participant is hypoxic.

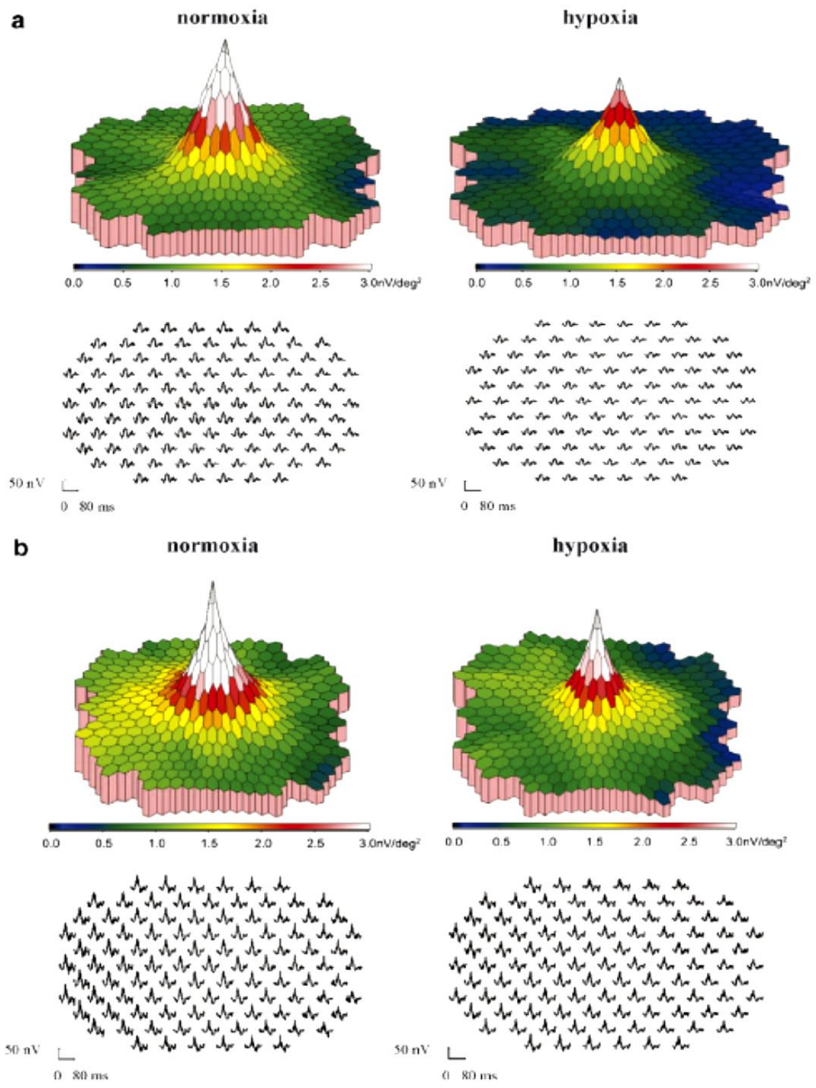


Figure 2-3. 3D plots of the scalar product response density (top) and trace arrays (below) for the fast flicker (a) and slow flash (b) paradigm before (left) and during hypoxia (right) for two representative participants (Feigl et al. 2007).

Kofoed et al. (2009) took a different approach to examining the effect of altitude, by taking residents living at 3600m and examining their mfERGs over 72 days after moving them to sea level, compared to eight healthy lowlanders. On day two, the highlanders

summed mfERG amplitude was 43.1% larger than the lowlanders' reference, 47.1% larger for P1 and 59.9% for P2. These differences increased over time, so that on day 72 they were 73.2%, 76.0% and 87.01% for the summed, P1 and P2 amplitudes respectively, showing a supernormal level with the relative hyperoxia. Implicit times were not significantly different between the two groups. These findings were despite a decrease in haemoglobin of 10.6% over the experiment in the highlander group, possibly due to adaptation of the retina to a different metabolic environment, and suggesting that these participants had a different type of retinal neuronal fitness.

The standing potential and the light rise in the electro-oculogram have also been shown to be affected when breathing 10% oxygen (Linsenmeier et al 1987). In this study, a subnormal light rise and an increase in the standing potential were found, suggesting either RPE or PR sensitivity to hypoxia. Linsenmeier (1990) later suggested that it was the PRs that were affected by hypoxia, based on a further study on cat retinas and a comparison with other electrophysiological papers at the time.

In conclusion, the ERG responses are sensitive to changes in oxygenation, with several components including the a-wave, b-wave and OPs affected by hypoxia. A preserved a-wave under photopic conditions suggests a resistance to hypoxia in cone PRs (Janáky et al. 2007), although there is evidence of a reduced a-wave amplitude in hypoxia under scotopic conditions (Schatz et al. 2014; Schatz et al. 2013), which may be due to the increased retinal metabolic demand in scotopic conditions. The mfERG shows changes with hypoxia, with a marked decrease in response density at the fovea. Several studies

have found reduced N1P1 amplitudes, suggesting that the bipolar cells are most affected by hypoxia although other sections of the waveform have also been affected.

2.2.6 The effect of hypoxia on dark adaptation.

The effect of hypoxia on dark adaptation has been recognised since the 1930s, with early studies finding that dark adapted thresholds were elevated in response to hypoxia, but that the time taken for DA to occur seemed not to be affected (McFarland et al. 1939; McDonald and Adler 1939; Wald and Harper 1942a). McFarland and Evans (1939) and McDonald and Adler (1939) both found an elevation of 0.40 log units in absolute threshold with hypoxia in both the rod and cone systems, whilst the rate of dark adaptation appeared unchanged. Data collected from 20 subjects in the McFarland and Evans (1939) study, showed that rod photoreceptors were more sensitive to the reduction in oxygen tension.

Figure 2-4 shows the mean adaptation curves of six subjects under reduced oxygen concentrations, ranging from 21% to 10.1% (McFarland & Forbes, 1940), and demonstrates raised dark adapted thresholds under hypoxic conditions. The data show that the thresholds for rod and cones were elevated, with the scotopic threshold ranging from 2.85 to 3.05 log microlamberts in the control series, to 3.50 to 3.85 log microlamberts when breathing 10.1% oxygen. It can also be seen that the curves are parallel with the control curves, showing the time course remained unaffected. Both McFarland and Evans (1939) and Mcfarland and Forbes (1940) found that, at the end of

the experiment when 100% oxygen was supplied to the subjects, the sensitivity increased rapidly. This led to the conclusion that the effect of hypoxia upon the dark adaptation function was not related to regeneration of visual pigment, as the recovery was too rapid, but instead to the neural retina and the central nervous system. This theory was also supported by the finding that reduced oxygen had a greater effect at lower light intensities (Hecht and Hendley 1946).

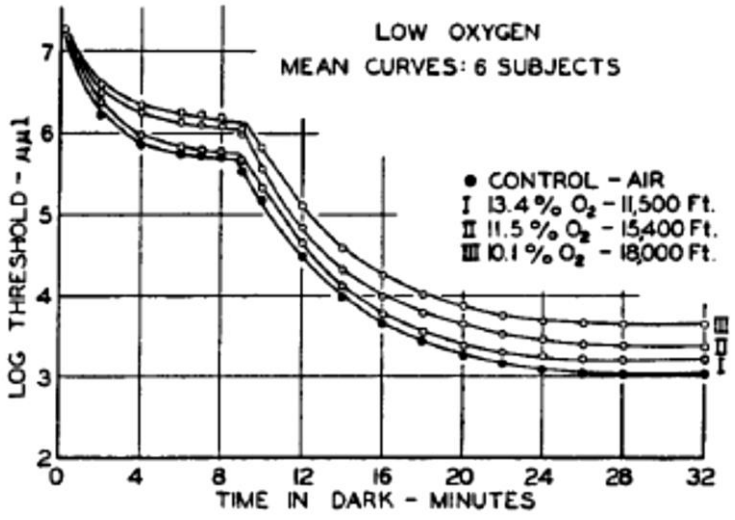


Figure 2-4. Mean dark adaptation curves for 6 subjects under various oxygen tensions. (McFarland and Forbes. 1940).

One recent study has found the time course of dark adaptation to be affected by hypoxia (Connolly et al. 2006). By studying dark adaptation using fixed stimulus intensities and measuring time as the dependent variable, recovery post bleach of both the cone and rod thresholds was recorded. As can be seen in Figure 2-5 the rod cone break was delayed and the rod section of the dark adaptation curve was shifted to the right when

participants were at simulated 4572m (12% oxygen) when compared to the dark adaptation curves obtained at ground level. However this study did not evaluate quantitative measures of adaptation, such as the time constants of rod and cone recovery.

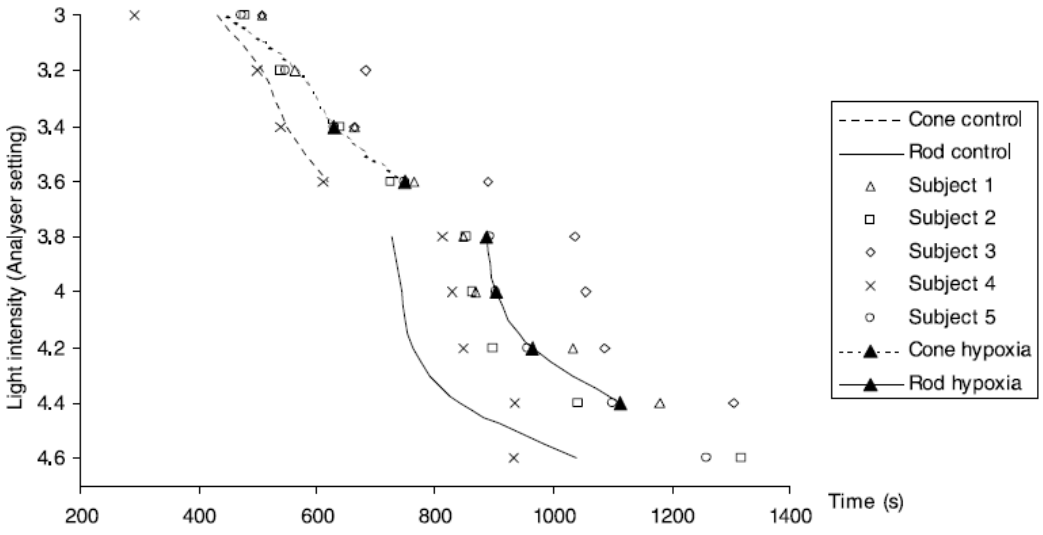


Figure 2-5. Effect of hypoxia when breathing air at 4572m on the rate of dark adaptation in 5 subjects showing detection time displacement relative to a control dark adaptation breathing air at ground level Connolly and Hosking (2006).

When comparing their results with the earlier findings of no change in the time course of the rod, cone or rod cone break portions of the curve, it seemed possible that the rod cone break in the earlier studies may have been missed by infrequent measurements of threshold. Connolly and Hosking (2006) discussed that if the rod cone break was inferred in the absence of data points, it could mean that the rod section of the curve may have

actually included data from the cone portion of the curve, masking the delay in the rod cone break and shifting the rod section to the right.

The effects of high altitude/ reducing oxygen concentration on thresholds have been found to be rapid and transient (Kobrick et al 1984; Wald & Harper, 1942). Wald and Harper (1942) found that the threshold adjusts rapidly (within 1-10 minutes) to hypoxia (8-11% oxygen), with exposures lasting five to six hours not translating to greater or more persistent changes. The results from Ernst and Krill (1971) indicated that the first four minutes of rod and cone adaptation were not affected by hypoxia, and concluded that this was due to this section relating to neural rather than photochemical processes. This hypothesis is in contrast to McFarland and Evans (1939), McFarland and Forbes (1940), McDonald and Adler (1939) and Hecht and Hendley (1946) who all concluded that the effect of hypoxia on dark adaptation was not related to photochemical processes but rather to neural processes. Kobrick et al. (1984), found that the major effect of hypoxia was in the first 10 minutes and suggested that the differences found could be due to the longer duration of hypoxia in their study.

Several studies have used hypoxia induced by disease to study the dark adaptation process. Thylefors et al. (2009) assessed dark adaptation in patients with respiratory insufficiency, all of which were on long-term oxygen therapy (for at least 4 months) when breathing their normal oxygen dose, and when they had been without it for at a mean of 4 hrs. They found no change in the dark adaptation function without oxygen, despite a reduced arterial blood oxygen saturation of 5.8%. They hypothesised that this could be

due to the lack of hypocapnia that would, at high altitudes in a normal population, cause vasoconstriction and reduce not only the concentration of oxygen but also its delivery. The subjects in their study were hypercapnic or normocapnic so may have larger blood vessel lumens, which would be further dilated during hypoxia, therefore preserving dark adaptation.

In conclusion, dark adaptation has been shown to be affected by hypoxia, with current evidence suggesting that it affects both final threshold and the rate of dark adaptation.

2.3 The role of hypoxia in the pathogenesis of AMD.

2.3.1 The relationship between changes to the ocular vasculature and AMD

The hypothesis that the aetiology of AMD is linked to changes in the ocular vasculature was first proposed when changes to the choroidal vasculature, including narrowed capillary lumens and a diminution or loss of cellularity of the capillaries were found in eyes with AMD (Kornzweig 1977). Later, a reduction in the perfusion of the choroid was found in participants with AMD (Prünte and Niesel 1988) and an increased scleral rigidity was found in eyes with AMD (Friedman et al. 1989).

The increase in scleral rigidity and vascular resistance has been further supported by other studies (Friedman et al. 1989; Pallikaris et al. 2006; Friedman et al. 1995). Increasing age is associated with an increase in ocular rigidity as well as the onset of

atherosclerosis and vascular lipid deposition and, therefore, a decrease in the compliance of the sclera and Bruch's membrane (Friedman 2008). This decrease in compliance reduces venous choroidal blood flow due to the vortex veins passing through the hardened sclera. This has been hypothesised to affect the transportation of metabolites and waste products from the RPE, leading to drusen formation and GA (Friedman 2008). According to this theory the resultant increase in choriocapillary perfusion pressure culminates in nAMD (Friedman 2004). This pathway of progression can be seen in Figure 2-6.

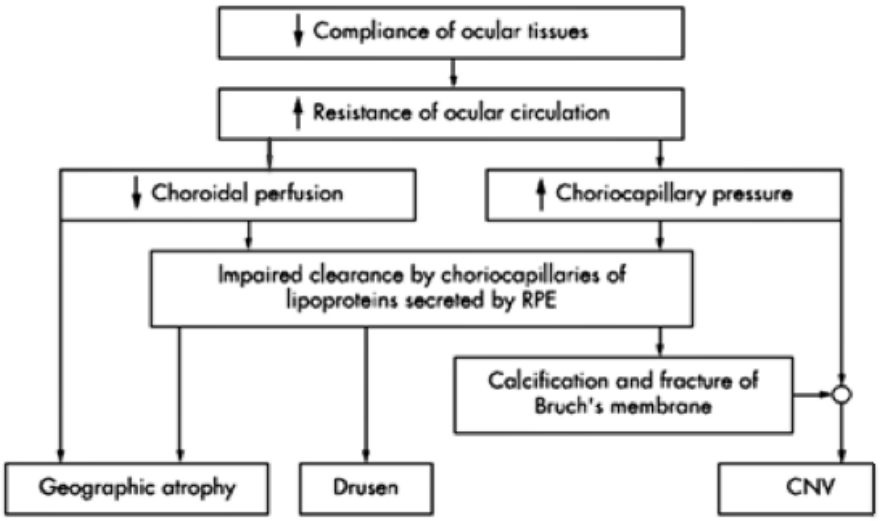


Figure 2-6. Schematic representation of the vascular model of the pathogenesis of age-related macular degeneration. (Friedman 2004).

However, not all studies have found ocular changes consistent with Friedman's vascular model. Pallikaris (2006) measured the ocular rigidity of three groups of participants; age matched controls, participants with GA and participants with nAMD. The findings

supported the vascular theory in nAMD, showing that average ocular rigidity measurements were significantly higher in participants with nAMD compared to controls and to participants with GA. However, there was no statistically significant difference between controls and participants with AMD when the two AMD groups were reviewed as one. It is important to note that Pallikaris (2006) did not take into account age and axial length, which have been linked to a reduction in choroidal perfusion, increasing the risk of nAMD (Böker et al. 1993). This could potentially confound the link between an increased ocular rigidity and nAMD. Also the nAMD group had been treated with PDT, which may have influenced the results. There were also differences in the study designs with Friedman (1989) using a direct manometric measurement and Pallikaris using an indirect technique. Friedman (1989) also did not distinguish between AMD types in his study; an over-representation of participants with nAMD could have influenced the results.

The vascular model is supported by studies finding a change in retrobulbar circulation. This is hypothesised to lead to a vascular impairment within the eye, altering the metabolic transport in AMD (Pallikaris et al. 2006; Friedman et al. 1995; Ciulla et al. 1999a; Hosal et al. 1998; Dimitrova et al. 2002). Friedman et al (1995), Hosal et al. (1998) and Dimitrova et al. (2002) all found that there were abnormalities in the retrobulbar circulation, particularly in the supply from the posterior ciliary artery when comparing participants with AMD to controls, but they did not investigate AMD subtypes. Ciulla et al (1999) studied age matched controls and participants with GA. It was concluded that participants with GA had a 26% decrease in mean end diastolic velocity in the nasal

posterior ciliary artery which was statistically significant. There was also a trend towards a lower peak systolic velocity in the nasal posterior ciliary artery and a lower end diastolic velocity in the temporal posterior ciliary artery. These findings are suggestive of a reduced flow and therefore perfusion in these vessels and the choroid in GA. Evidence has since suggested that the increasing blood pulsatility correlated with the severity seen in the retinal vessels of participants with AMD was due to a loss of compliance in the arterial vasculature (Sato et al. 2006).

With the advent of new imaging techniques, imaging of the choroidal circulation in vivo has improved. Laser Doppler flowimetry is based on the assessment of the Doppler shift of low-power laser light, which is scattered by moving red blood cells to provide a non-invasive, continuous measure of tissue blood velocity. It can therefore be used to assess the choroidal blood flow and volume and choroidal velocity can be calculated from these measurements. Indocyanine green angiography can also be used in the investigation of choroidal circulation. Changes in the choroidal circulation, which could lead to hypoxia, have been associated with AMD. Decreasing choroidal blood flow has been associated with ageing in normal participants (Grunwald et al.1998b), in participants with nAMD (Goldberg et al. 1998) and in participants with GA (Xu et al. 2010; Grunwaldet al. 1998a), which is possibly caused by a decrease in choroidal blood volume (Grunwald et al. 1998a). This suggests that an increasing risk of retinal ischaemia with age could be involved in the pathogenesis of AMD (Xu et al. 2010). Although no change in choroidal volume, flow or velocity was found in early AMD by Remsch (2000), choroidal filling defects were

associated with drusen in other studies (Staurenghi et al. 1992; Zhao et al. 1995). This is possibly due to an accumulation of hydrophobic material in Bruch's membrane with age. As Bruch's membrane should stretch to accommodate changes in blood volume (Curcio and Johnson 2013), this accumulation of material may alter its elasticity, resulting in localised obstruction. More recently a significant inverse relationship has been found between the total drusen area and choroidal blood volume and flow (Berenberg et al. 2012). As total drusen area has been associated with an increased risk of progression to advanced AMD (Solomon et al. 2009) these studies linking changes in blood flow with total drusen area suggest that ischaemia may be the reason for progression from early to late AMD (Berenberg et al. 2012). However, in the pathogenesis of drusen it is still unknown if ischaemia precedes drusen development (Bhutto et al. 2012) or if it is a consequence of drusen formation.

Choroidal changes such as a reduction in choroidal blood flow, volume and perfusion have been found to precede the development of nAMD (Metelitsina et al. 2008; Boltz et al. 2010; Grunwald et al. 2005; Üretmen et al. 2003; Mori et al. 2001). Pulsatile ocular blood flow and pulse amplitude were found to be lower in participants with nAMD compared to normal participants (Mori et al. 2001). However Chen (2001) and Remsch et al. (2000), comparing eyes with unilateral nAMD found that blood flow was higher in eyes with nAMD, possibly due to an autoregulative vascular response to a compromised metabolism. It was also found that pulsatile ocular blood flow (Chen et al. 2001) and perfusion (Remsch et al. 2000) were lower in eyes with disciform scarring. It could be that

these haemodynamic changes are the cause of nAMD and scarring or that haemodynamic changes could result from an increase in choroidal blood flow due to neovascularisation and a decrease in the requirement of circulation when there is disciform scaring. Therefore it is unknown if changes in circulation precede neovascularisation or GA or are a consequence of them. The differences between the findings of the Mori et al (2001) and Chen et al (2001) studies could be explained by differences in the stage of CNV being assessed; Mori et al (2001) did not classify the type of nAMD. It could be that the neovascular group had participants in that were not in the active stages of neovascularisation. There is also a possible effect of inter-individual variation such as age and blood pressure in the Mori et al (2001) study, which was controlled for by Chen et al (2001). Slow choroidal filling has also been found in early AMD (Pauleikhoff 1999), GA (Ciulla 2002; Prünte et al. 1988), nAMD (Chen et al. 1992; Giovannini et al. 1994; Zhao et al. 1995; Böker et al. 1993) and in the fellow eyes of participants with unilateral nAMD (Remulla et al. 1995).

Histological changes to the ocular vasculature have also been associated with AMD, including a loss of choroidal blood vessels with overlying RPE atrophy, with remaining vessels having a statistically significant restriction in lumen diameter (McLeod et al. 2002; Kornzweig 1977). A decrease in choriocapillary density and diameter with late AMD and basal laminar deposits was also found by Ramrattan et al. (1994). In contrast to the findings of Ramratten et al (1994), Spraul et al (1999) reported a loss of large choroidal vessels and an increase in the density of the choriocapillaris in the submacular area in

eyes with AMD compared to eyes without AMD. In the peripheral choroid an increase in the diameter of the large choroidal vessels has also been observed (Spraul et al. 1996). The differences between these studies could be explained by Ramrattan et al (1994) assessing eyes with GA and scarring, which are stages of AMD characterised by a loss of RPE, whilst Spraul et al. (1996) studied 21 eyes with nAMD and 19 with non-nAMD, few of which had GA.

Other abnormalities in blood vessels have been observed linking hypoxia and AMD. These include deep retinal vascular anomalous complexes, a form of neovascularisation which grows into the sub-retinal space which is associated with AMD and thought to be a response to hypoxia (Hartnett et al. 1996). Watershed zones, which are areas already associated with ischaemia have also been associated with nAMD (Mendrinos et al. 2009; Giovannini et al. 1994; Chen et al. 2001; Ross et al. 1998).

2.3.2 The relationship between changes to Bruch's Membrane and AMD

Bruch's membrane is known to thicken with ageing (Booij et al. 2010; Ramrattan et al. 1994), which could result in hypoxia at the photoreceptor level due to the increase in distance O_2 has to diffuse (Stefánsson et al. 2011). Links have been made between these ageing changes in Bruch's membrane and hypoxia in AMD. For example, a prolonged choroidal filling may be causally related to a diffuse thickening in Bruch's membrane (Pauleikhoff et al. 1990; Staurenghi et al. 1992; Piguet et al. 1992). Thickened Bruch's

membrane could also modulate the secretion of growth factors by RPE cells (Mousa et al. 1999) and may also prevent the VEGF secreted by the RPE from reaching the choriocapillaris, resulting in choroidal atrophy. The resulting accumulation of VEGF on the basal side of the RPE may also induce neovascularisation (Blaauwgeers et al. 1999). An increase in extracellular matrix components in cultured RPE cells which have undergone both hypoxia and reoxygenation has been found. Hypoxia and reoxygenation injuries are known to cause oxidative stress. An increase in the synthesis of extracellular matrix cells may contribute to their accumulation in Bruch's membrane as basal laminar deposits (Fuchshofer et al. 2009).

2.3.3 The relationship between hypoxia-led upregulation of growth factors and growth inhibitors, and AMD

The role of vascular changes leading to ischaemia in retinal neovascularisation was reported in 1948 by Michelson who described oxygen regulated angiogenesis in the retina, mediated by factor x; which was later identified as VEGF (Michelson 1948). The process of retinal neovascularisation is now well understood but the pathogenesis of choroidal neovascularisation is less clear (Campochiaro 2000; Das and McGuire 2003). The influence of pro-angiogenic cytokines, including VEGF, which are up-regulated by the production of HIFs in response to hypoxia, have been proposed to be central to the development of nAMD (Sheridan et al. 2009).

Hypoxia increases the levels of VEGF in the RPE (Young et al. 2005; Wu et al. 2007; Mousa et al. 1999; Aiello et al. 1995; Shima et al. 1995; Coassin et al. 2010; Blaauwgeers et al. 1999) and HIF-1a mRNA in the retina (Fuchshofer et al. 2009; Zhang et al. 2011; Inoue et al. 2007). The expression of VEGF by the RPE in normoxic conditions has been reported to be higher on the basal side, facing the choroid, which is suggestive of a role in choriocapillaris maintenance (Blaauwgeers et al. 1999). HIF -1 and HIF -2 have been found in choroidal neovascular membranes, mainly expressed in RPE cells, suggesting that RPE cells are hypoxic in AMD and that they are initiating a wound healing response (Sheridan et al. 2009).

Hypoxia-inducible factors not only upregulate VEGF, but also connective tissue growth factor which has been found to be expressed in RPE cells with hypoxia (Fuchshofer et al. 2009). This increases extracellular matrix components including fibronectin and collagen type IV in the RPE which may result in Bruch's membrane thickening in AMD (Fuchshofer et al. 2009).

The maintenance of the choroidal circulation is dependent on the balance of pro-angiogenic growth factors, such as VEGF, and anti-angiogenic factors, such as PEDF, which decreases in expression with age (Ogata et al. 2004). PEDF concentrations have been found to be significantly lower in RPE cells, RPE basal lamina, Bruch's membrane, and the choroidal stroma of eyes with nAMD when levels of VEGF were not different between aged controls and tissues with nAMD (Bhutto et al. 2006). These findings suggest that a

decrease in PEDF may disrupt the balance and be permissive for the formation of choroidal neovascularisation.

2.3.4 Hypoxia led cellular changes and AMD

Hypoxia not only causes changes in the expression of growth factors and inhibitors, but also affects the concentrations of other proteins and cell regulators involved in neovascularisation. Jun kinases (JNK) regulate cellular processes including cell proliferation and migration. They have also been found to be a critical factor in hypoxia-induced retinal VEGF production and in promoting hypoxia induced pathological angiogenesis, as seen in AMD (Guma et al. 2009). Protein 53 (P53), a tumour suppressor protein which regulates cell cycles and can cause RPE cell apoptosis and an increased expression of P53 via HIF-1, has been found as a response to hypoxia in RPE cells and in the retina (Gerona et al. 2010; Rosenbaum et al. 1998). Stromal cell derived factor 1 (SDF-1) recruits bone marrow derived cells to choroidal neovascular membranes, which can then differentiate into endothelial cells to participate in the neovascularisation process. Hypoxia induces SDF-1 expression in the retina (Lima e Silva et al. 2007), particularly in the RPE (Zhang et al. 2011). Vascular cell adhesion protein 1 (VCAM-1) mediates the adhesion of lymphocytes, monocytes and other cells to the vascular endothelium. It increases susceptibility to neovascularisation and its expression has been found to be high in the ischaemic retina, increasing further when both ischaemia and oxidative stress are present (Dong et al. 2011). Heat shock protein 90 (HSP90) is also thought to be a

regulator of VEGF expression as pre-treatment with Geldamycin, a HSP90 inhibitor, reduces $VEGF_{165}$ expression in RPE cells under hypoxia (Wu et al. 2007).

It has been shown that the RPE has the ability to synthesise haemoglobin, this may have consequences for hypoxia, as quantitative and qualitative defects in this synthesised haemoglobin may underlie the hypoxia that leads to AMD (Tezel et al. 2009).

2.3.5 The effect of hypoxia on visual function in AMD

There is functional evidence that hypoxia is part of the aetiology of AMD. Some visual functions such as dark adaptation (Owsley et al. 2000; Owsley et al. 2001), contrast sensitivity (Midena et al. 1997; Sunness et al. 1997), colour vision (Eisner et al. 1991) and flicker sensitivity are reduced in AMD. These same functions are also reduced in a comparable way in normal subjects under a hypoxic episode (Connolly 2010; McFarland and Forbes 1940; see chapter 2.2), suggesting that hypoxia could be the cause of the functional deficits seen in participants with AMD.

Areas in the retina with altered choroidal circulation have been associated with localised changes in visual function in participants with AMD. Areas of choroidal perfusion abnormality have been co-localised with discrete areas of raised scotopic threshold in participants with drusen (Chen et al. 1992). An association has also been found between a prolonged focal ERG implicit time and areas of delayed choroidal perfusion in the fellow eyes of participants with unilateral nAMD (Remulla et al. 1995).

2.4 Conclusions

In conclusion, the literature review into the effect of hypoxia on visual function found that all aspects of visual function are affected by hypoxia. Some functions, including visual fields and contrast sensitivity are preserved until lower oxygen concentrations/ higher altitudes are reached, whereas dark adaptation, colour vision and electrophysiological responses appear to be more sensitive indicators of hypoxia.

There is yet to be a consensus on which layer or cell type of the retina is most susceptible to hypoxia. Some studies conclude that the photoreceptors may be vulnerable due to the changes in visual function relating to the metabolic demand of the rods in mesopic studies (Connolly et al. 2009b; Connolly et al. 2008b; Connolly et al. 2008a), others that there is a preferential postreceptoral susceptibility (Feigl et al. 2007; Kang Derwent and Linsenmier 2000; Smith et al. 1976; Vingrys et al. 1987; Tinjust et al. 2002; Kergoat et al. 2006), and some that both are affected (Pavlidis et al. 2005). Kang Derwent and Linsenmeier (2000) suggested that the function of the photoreceptors may be preserved under hypoxemia as when oxidative metabolism is affected; they switch to glycolysis for energy production.

Considering the central and peripheral retina, there is some dispute as to the area most affected by hypoxia, dependant on the visual function being tested. Some studies conclude that the peripheral retina, out to 40-45° is more sensitive to changes in oxygenation (Ernest and Krill 1971; Horng et al. 2008; Connolly et al. 2008b), but Pavlidis

et al. (2005) and Klemp et al. (2007) suggested that it is the macula. This may depend on the function being assessed and the state of retinal adaptation.

All of the visual functions were affected by hypoxia more in scotopic and mesopic than photopic conditions. This can be explained by the increased metabolic activity of the retina in the dark.

There are a few points to note in the comparison of the data. Ernest and Krill (1971) and Vingrys and Garner (1987) cautioned against comparing studies conducted by the dilution of oxygen with those which altered the atmosphere, such as using a decompression chamber, due to the possible independent effect of barometric pressure. A review comparing hypobaric hypoxia (a low barometric pressure and low oxygen level) to normobaric hypoxia (normal barometric pressure and low oxygen levels) found that SpO2 was lower in hypobaric hypoxia for the same partial pressure of oxygen for short term exposure (Coppel et al. 2015). The control of the test environment could also have influenced results in some experiments (Bouquet et al. 2000; Schmeisser et al. 1997), which may make it difficult to compare climbing studies on foot with those conducted in the laboratory.

The possibility of carbon dioxide affecting results is something studies have aimed to control (Ernest et al. 1971; Vingrys et al. 1987). Hyperventilation has been found to raise visual thresholds short term by as much as 0.7- 1.0 log units (Wald et al. 1942a). This shows the potential impact of breathing patterns on results. More recent studies have

used subjects who are experienced in hypoxic environments, or provided training to minimise these effects (Leid et al. 2001; Pavlidis et al. 2005; Connolly 2011; Connolly et al. 2006).

The review into the role of hypoxia in the pathogenesis of AMD provided evidence that there is an altered blood supply to the outer retina in AMD. However, although the change in circulation has been shown in studies looking at the retrobulbar and the choroidal circulation, there is some question as to whether these changes lead to hypoxia (Campochiaro 2000). Thickening and deposition of Bruch's membrane in early AMD is hypothesised to exacerbate any hypoxia by increasing the distance over which oxygen must travel to reach the retina (Wangsa-Wirawan et al. 2003). Further research is required to conclusively determine whether hypoxia results from the reduced blood supply and from sub-retinal deposits in AMD (Harris et al. 1999).

Hypoxia is known to prevent the degradation of HIF, which leads to the up-regulation of growth factors including VEGF, stimulating neovascularisation in nAMD. A reduction in growth inhibitors, including PEDF, also influences the progression of neovascularisation (Bhutto et al. 2006). Hypoxia also influences the regulation of other proteins and cell regulators, which can also promote neovascularisation (Dong et al. 2011). There is new evidence that a reduction in the synthesis of haemoglobin by the RPE may also result in hypoxia in AMD (Tezel et al. 2009).

The visual deficits seen in AMD are also seen in normal participants during a hypoxic episode, suggesting that hypoxia could be the cause of these of deficits in AMD. This is evidenced by the co-localisation of changes in visual function with alterations in choroidal perfusion.

The evidence in the literature to date suggests that hypoxia may be involved in the pathogenesis of AMD, both in the early stages and in nAMD and GA. However, there is currently only circumstantial evidence to link the changes in visual function associated with AMD to the onset of hypoxia. This thesis aims to probe this relationship by investigating the effect of transient systemic hyperoxia and hypoxia on visual function in AMD.

3 Effect of respired oxygen concentration on SpO2 and scotopic thresholds in healthy controls

This chapter presents studies to investigate the effect of breathing 60% and 14% oxygen on scotopic thresholds in healthy controls, and the effect of the retinal location of the stimulus on the response to the hypoxia / hyperoxia. Studies also investigate the changes in SpO_2 levels over time when breathing 10% and 14% oxygen.

3.1 Effect of breathing 14% and 60% O_2 on scotopic thresholds and SpO_2

3.1.1 Background and Aims

Despite the presence of two vascular systems, the supply of O_2 to the retina is barely sufficient when dark adapted (Wangsa-Wirawan et al. 2003). The first aim of this preliminary pilot study was to investigate whether the dark adapted healthy retina is hypoxic by comparing scotopic thresholds under hyperoxic and normoxic gas conditions. If scotopic thresholds were reduced (i.e. increased sensitivity) under the hyperoxic (60% O_2) condition relative to normoxia (21% O_2), this would indicate that insufficient oxygen is reaching the retina under dark adapted conditions. It was hypothesised that the

inhalation of supplementary oxygen would have no effect on scotopic thresholds in dark adapted normals, as they are not expected to have hypoxic retinas.

The literature review in chapter 3 concluded that many aspects of normal visual function, including colour vision (Connolly et al. 2008a), dark adaptation (McFarland et al. 1940) and electrophysiology (Janáky et al. 2007) are affected by hypoxia. Hence, the second aim of this study was to determine if an episode of retinal hypoxia (breathing 14% O_2) would increase scotopic thresholds. It was hypothesised that scotopic thresholds would be elevated (i.e. reduced sensitivity) in this scenario. As the retina is on a hypoxic knife edge in the dark (Wangsa-Wirawan et al. 2003) a scotopic measure of visual function was selected on the hypothesis that it would be more affected than photopic visual function by outer retinal hypoxia . Dark adapted thresholds were therefore chosen as a measure of visual function in this study. They have also been found to be affected both by hypoxia in normals (as discussed in section 2.2.6) and by AMD (discussed in section 2.3.5)

Concentrations of 14% and 60% oxygen were chosen as the hypoxic and hyperoxic gas mixtures in this study. 14% oxygen has been used for several studies preciously and has been shown to safely and effectively reduce participants' SpO_2 (Connolly 2010; Connolly et al. 2009a). 60% oxygen has been used as a hyperoxic gas condition in a previous study of the effect of hyperoxia on visual function (e.g. Drasdo et al. 2002). In the studies reported in this thesis, participants were asked to breathe each gas condition for 5 minutes. This duration was chosen on the basis of preliminary pilot data (collected before this project started), which suggested that a 5 minute duration of hyperoxia in people

with AMD would be sufficient to effect a change in threshold. This also exceeded the 2 minutes allowed by Drasdo et al. (2002) before they commenced ERG data collection in the hyperoxic state in people with type II diabetes.

The effect of hypoxia on visual function has been predominantly assessed at single locations (Connolly 2011; McFarland et al. 1939; Karakucuk et al. 2004; Feigl et al. 2007). Hence, the third aim of this study was to determine if there is a topographical variation in the effect of hypoxia on scotopic thresholds in healthy controls. Three retinal locations 2^{o}, 7^{o} and 12^{o} were chosen. This area contains the fovea, which may be predisposed to hypoxia as a result of the absence of the inner retinal circulation, and also the area between 10-15 degrees which may be preferentially affected by hypoxia as this is where retinal rod density and therefore oxygen demand is highest (Jonas et al. 1992). The retinal area within 12 degrees is also the region of the retina which shows the greatest dysfunction in AMD. In future studies investigating the role of hypoxia in visual dysfunction in AMD, we wish to select the area most predisposed to hypoxia for psychophysical testing. This pilot study aims to identify this location.

3.1.2 Participants

Eighteen participants were recruited from the School of Optometry and Vision Sciences in Cardiff University and provided informed written consent before taking part. All procedures adhered to the tenets of the Declaration of Helsinki and the study was approved by the School of Optometry and Vision Sciences ethical committee.

Participants were excluded on the basis of: pregnancy; a history of asthma; chest disease; respiratory failure; chronic bronchitis; emphysema; an inability to walk more than 500 yards due to a shortness of breath; cardiac disease or previous chest surgery; if they were current smokers; a history of previous or current eye conditions known to affect retinal function; a VA less than 6/7.5 (0.1 logMAR).

3.1.3 Equipment

Stimuli were presented on a calibrated, gamma corrected (Methra, Vingrys and Badcock 1993) high-resolution computer monitor (Iiyama LS 902UT) driven by an 8-bit (nVIDIA Geforce 9) graphics board under software control (Matlab, Math-works, Cambridge, UK). A neutral density filter, with an optical density (OD) of 3.3 log units, was used to attenuate the luminance of the stimuli and allow the monitor to function within its linear luminance range. Scotopic thresholds were measured using a 3-down 1-up staircase procedure. Participants fixated on a cross in the centre of the screen, and indicated the perception of the stimulus using a keyboard. The stimulus was presented for 200msec, followed by a 600msec response window and then a randomly determined interstimulus delay of 0.9-2.4 secs, which generated, on average, a stimulus every 2.45 secs. Response to the stimulus within 600 msec reduced the luminance of the next presentation by 0.3 log units. If the response was outside this timeframe, or if there was no response, then the intensity of the following presentation was increased by 0.1 log units. Three locations were tested over 2 visits using targets stimulating the same total retinal area; a 2^{o} radius spot, a 7^{o} radius annulus (width 0.30^{o}) and a 12^{o} radius annulus (width 0.20^{o}). If the

computer detected that the lower end of the linear luminance range was being reached then it emitted a sound to indicate that a second neutral density filter (OD 1.5 log units) was required.

Normoxic, hyperoxic and hypoxic gas conditions were provided by having the participants wear a 60% ventimask (Intersurgical Ltd). The 60% ventimask mixes the contents of the gas cylinder in a 1:1 ratio with ambient air. Hence, a cylinder containing 100% oxygen delivered 60% oxygen, medical air provided 21% oxygen and a canister containing 8% oxygen provided 14% oxygen for inspiration. The participants continued to wear the same mask for all gas conditions, and were masked as to which cylinder was attached to the mask at any time. Throughout all experiments, SpO_2 was measured using a finger pulse oximeter (CMS 50E, Crucial Medical Systems). The pulse oximeter works by analysing the absorption characteristics of haemoglobin (Hb) and oxyhaemoglobin (HbO2) in near-infrared zones. The output of the device is SpO_2, pulse rate and the pulse waveform. The device can be seen in Figure 3-1.

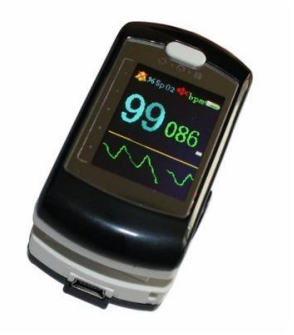


Figure 3-1. Crucial Medical Systems CMS 50E Pulse oximeter showing the output of the device.

3.1.4 Method

Preliminary assessment included history and symptoms, measurement of visual acuity, slit lamp biomicroscope assessment of the anterior chamber angle using Van Herick technique, dilated binocular indirect fundus examination, fundus photography and OCT (Topcon 3D OCT 1000).

The untested eye was patched and the eye being tested was dilated with 1% tropicamide (the eye with the best visual acuity or the right eye if both were equal). Refractive correction was worn if required. After an initial 30 minutes of dark adaptation, thresholds were measured at one of the retinal locations with the participant breathing one of the three gases. Each gas was inhaled for 5 minutes, followed by a 5 min washout period before the next gas was presented. Thresholds were recorded throughout. The participants returned for a second visit within 2 weeks of the first and data were collected from the remaining 2 retinal locations. A timeline of the data collection process is shown in Figure 3-2.

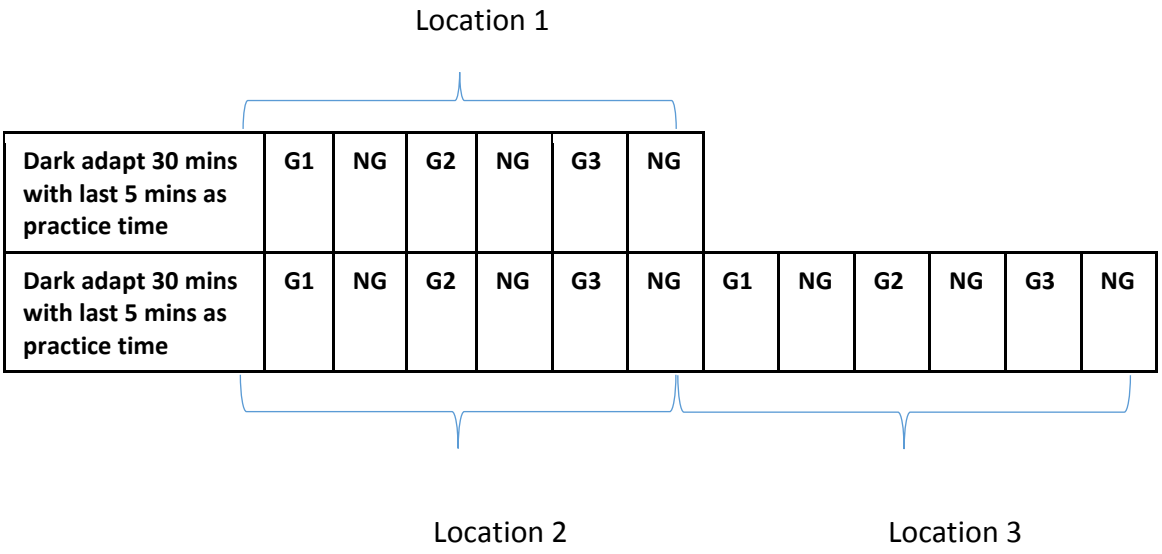


Figure 3-2. Timeline of experiments on visit 1 and 2. G1= gas 1, G2= gas 2, G3= gas 3. NG= no gas wash out period.

Both gas and location orders were randomised to minimise any impact from the learning effect or from fatigue. Results were exported straight into Microsoft Excel for analysis. SpO_2 readings were recorded every minute throughout. The experiment was stopped if the SpO_2 levels dropped below 85%. The participant set up can be seen in Figure 3-3.

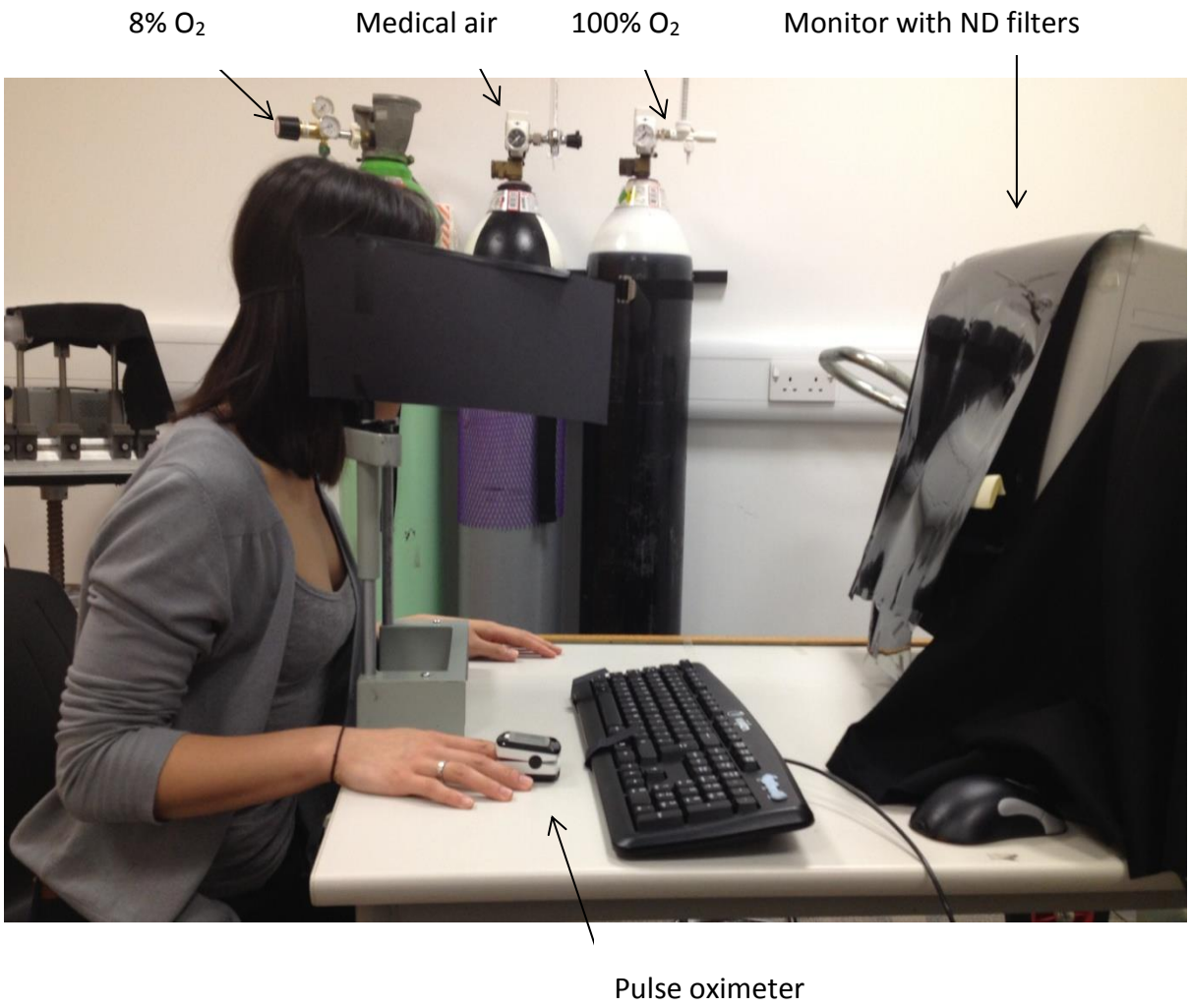


Figure 3-3. Participant set up.

3.1.5 Data analysis

SpO_2 and scotopic threshold data were tested for normality with a Shapiro-Wilkes test. The relationship between scotopic thresholds and SpO_2 was analysed with a repeated measures ANOVA test or with the non-parametric alternative Friedman test.

3.1.6 Results

Eighteen healthy individuals were recruited, between the ages of 20-48 years with a mean age of 24.7yrs +/- 7.0 SD. There was a gender split of 67% females and 33% males. Full datasets were collected for all three gases at all three locations for all participants.

Figure 3-4 Shows the SpO_2 data for a typical particiapant (RM) at the fovea over a visit. The gas order for this participant was 14% (5-10 minutes), 21% (15- 20 minutes) and 60% 25-30 minutes). It can be seen that for this individual there is a reduction in SpO2 to 96% in this individual when breathing the hypoxic gas, but SPO2 was constant at 99% for both normoxic and hyperoxic conditions.

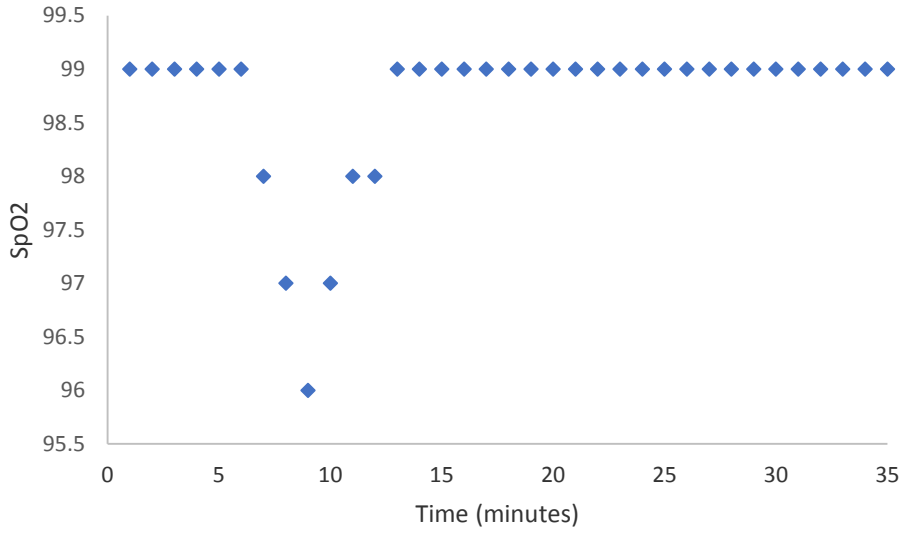


Figure 3-4. SpO2 data for participant RM during scotopic threshold foveal data collection.

Figure 3-5 shows the group averaged SpO_2 per minute for each of the three gases. It can be seen that breathing 60% oxygen resulted in a slight increase in SpO_2 that was greatest in the 4th minute with an increase of 0.46% compared to medical air. The graph also shows a reduction in SpO_2 with hypoxia, the greatest drop is in the 5th minute with a drop of 2.98% compared to medical air. Beyond the first minute, 95% confidence intervals for the 3 gases are not overlapping, indicating a significant difference in SpO_2 between gas conditions.

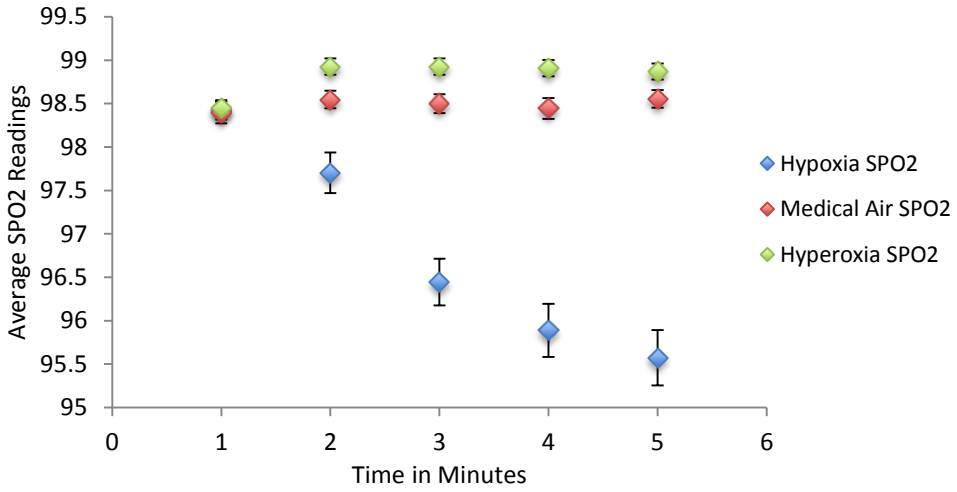


Figure 3-5. Group averaged SpO_2 for each minute after commencing inhalation, with 95% confidence intervals.

This significance was confimed with Friedmans analysis on the full 5 minute grouped data for all three gas conditions ($p<0.001$). Post hoc analysis using a Wilcoxon signed-rank tests with Bonferroni correction applied ($p<0.017$) was also run and confirmed that there was a statistical difference between SpO2 readings for 14% and 21% ($p=0.001$), 14% and 60%

(p= 0.001) and 21% and 60% (p=0.001). The SpO2 readings for the 14% O2 show no plateau within the 5 minutes, whilst a ceiling effect for the hyperoxia gas is evident. This is due to the SpO2 already averaging at near saturation (98.5%) with medical air.

Figure 3-6 to Figure 3-8 show the threshold data recording which took place over 30 minutes for a typical participant (TC) at 2, 7 and 12 degrees. Each data point is shown (blue diamonds) along with the gas order. It can be seen that there is no obvious change in threshold with either hyperoxic (60% O_2 at 5-10 minutes) or hypoxic (14% O_2 at 15-20 minutes) gas conditions compared to medical air (21% O_2 at 25-30 minutes) at any of the locations. There was no apparent trend in threshold vs. gas condition for any individual dataset.

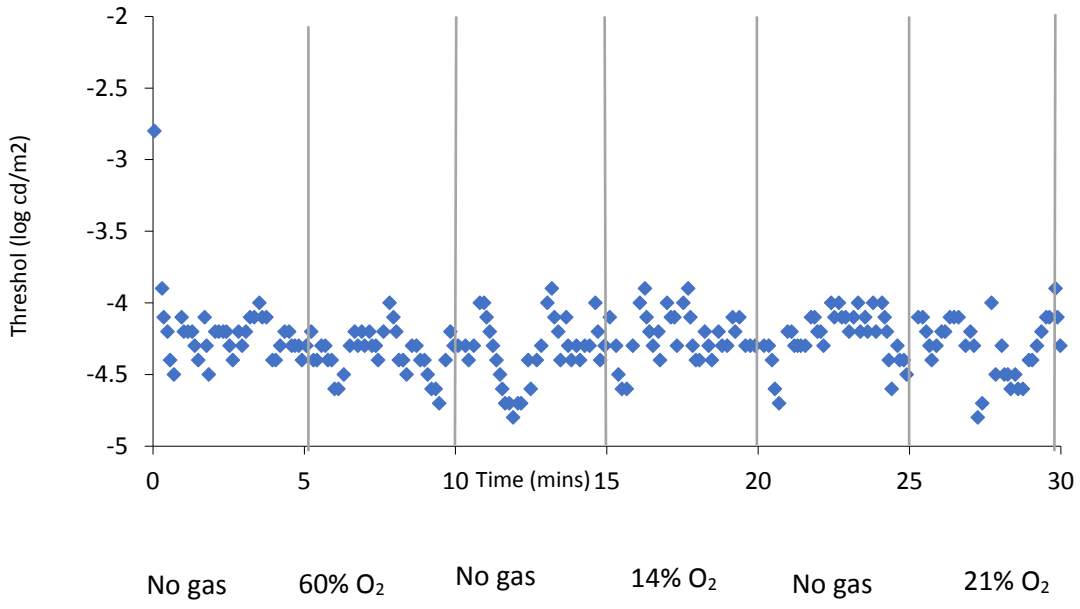


Figure 3-6. Plot of threshold as a function of time for a typical participant (TC) at 2 degrees.

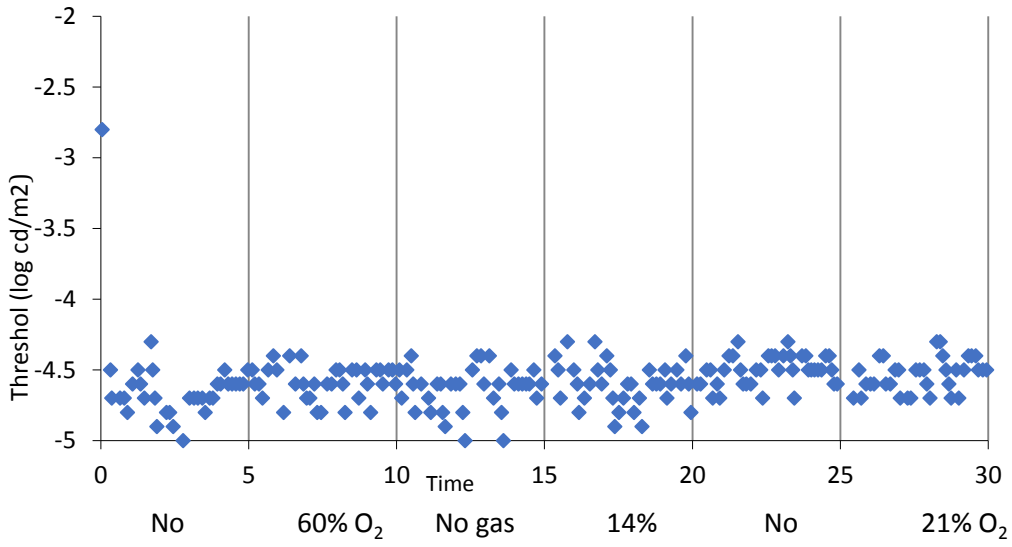


Figure 3-7. Plot of threshold as a function of time for a typical participant (TC) at 7 degrees.

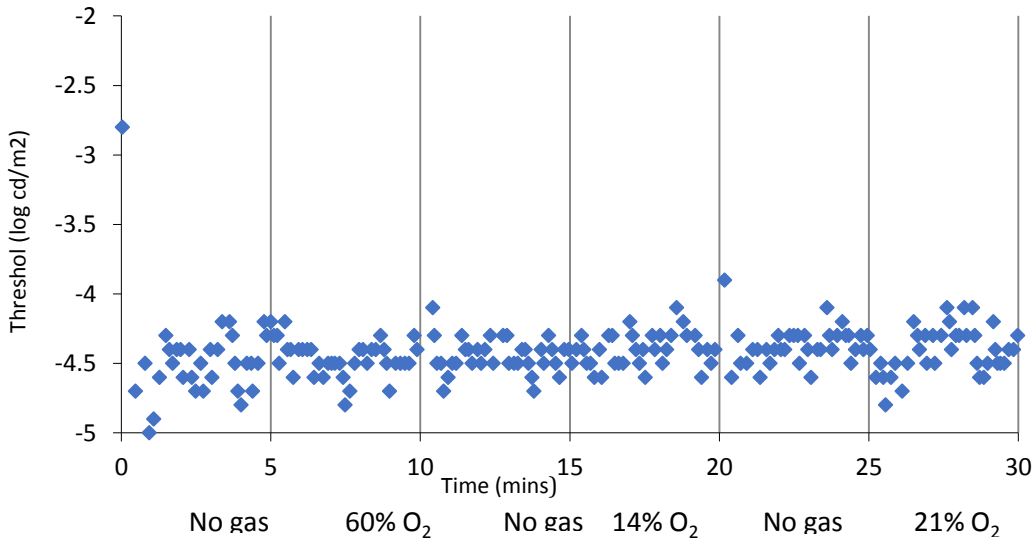


Figure 3-8. Plot of threshold as a function of time for a typical participant (TC) at 12 degrees.

Figure 3-9, Figure 3-10 and Figure 3-11 show the group averaged mean final minute threshold for all three gases at each location. The final minute thresholds (i.e the thresholds obtained in the 5th minute of inhalation of each gas) were chosen as this is the interval when SpO_2 was most affected by the 14% oxygen concentration (see Figure 4-7). The figures show that, overall, thresholds were highest at the fovea (Figure 4-9). This difference was statistically significant for each of the three gas conditions when compared to the other two locations (ANOVA – 14% p= 0.001, 21% p= 0.001, 60% =0.001). At location 12 degrees (Figure 4-11) there was the least variability in threshold recordings with smaller confidence intervals with all three gases compared to the other two locations. There was a slight trend for hypoxia to raise thresholds at all three locations. However, there was no significant difference in scotopic threshold with oxygen concentration at any of the three locations (ANOVA – fovea p= 0.673, 7 degrees p= 0.606, 12 degrees p= 0.135).

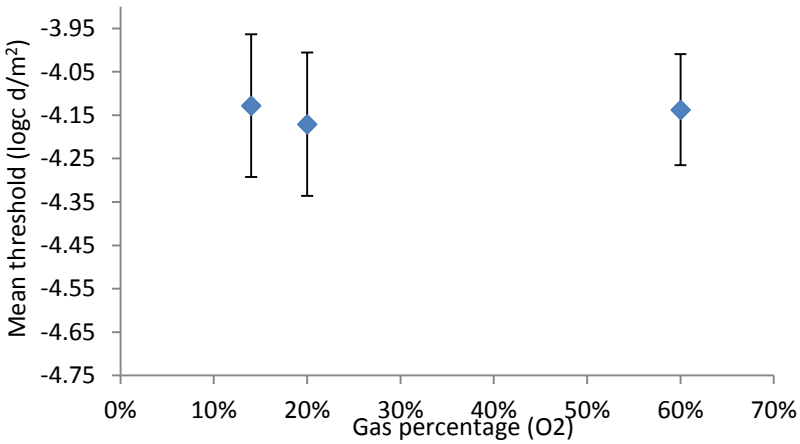


Figure 3-9. Group averaged final minute threshold for all three gases at 2 degrees eccentricity for the fovea (error bars show 95% confidence intervals for the mean).

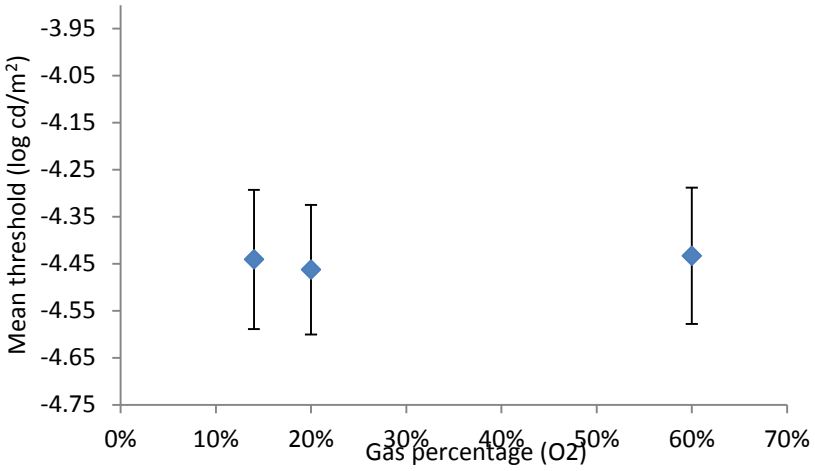


Figure 3-10. Group averaged final minute threshold averages for all the gases at 7 degrees eccentricity (error bars show 95% confidence intervals for the mean).

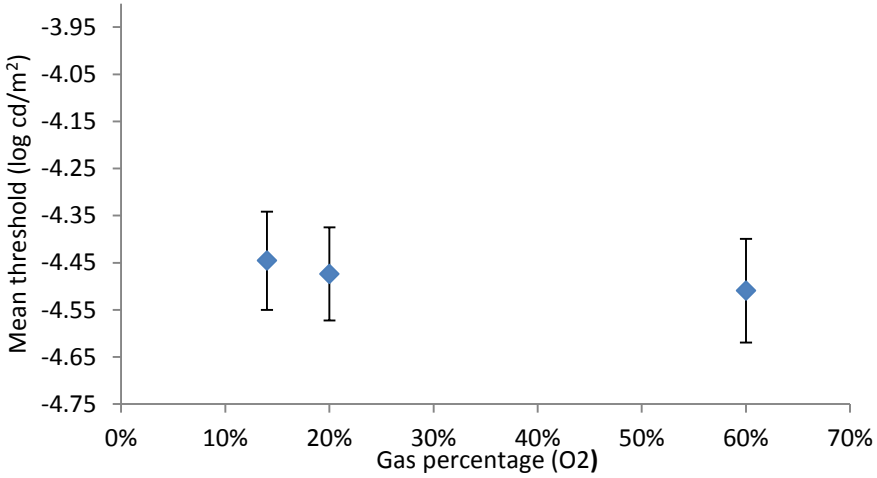


Figure 3-11. Group averaged final minute threshold averages for all three gases at 12 degrees eccentricity (error bars show 95% confidence intervals for the mean).

Whilst there was no significant difference in group averaged thresholds recorded under different gas conditions, it is also useful to look at individual data points to see whether

there is a relationship between SpO_2 and threshold. This analysis removes the potential confounding effect of inter individual variability in the physiological response to breathing the hypoxic/hyperoxic gas. Figure 3-12, Figure 3-13 and Figure 3-14 show the mean threshold for each minute for each individual plotted against SpO_2 at all three locations. The R^2 values for all three graphs indicate that the spread in threshold data cannot be explained by the participants' SpO_2. A maximum of 3% of the variation in threshold is explained by SpO2 (at the 7 degrees location).

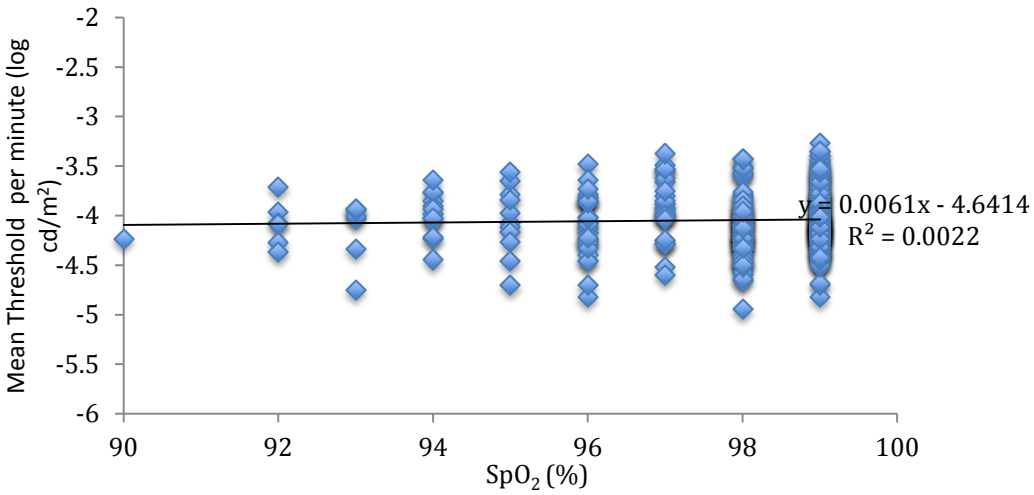


Figure 3-12. Mean threshold for each minute plotted against SpO2 for all 18 participants at 2 degrees eccentricity.

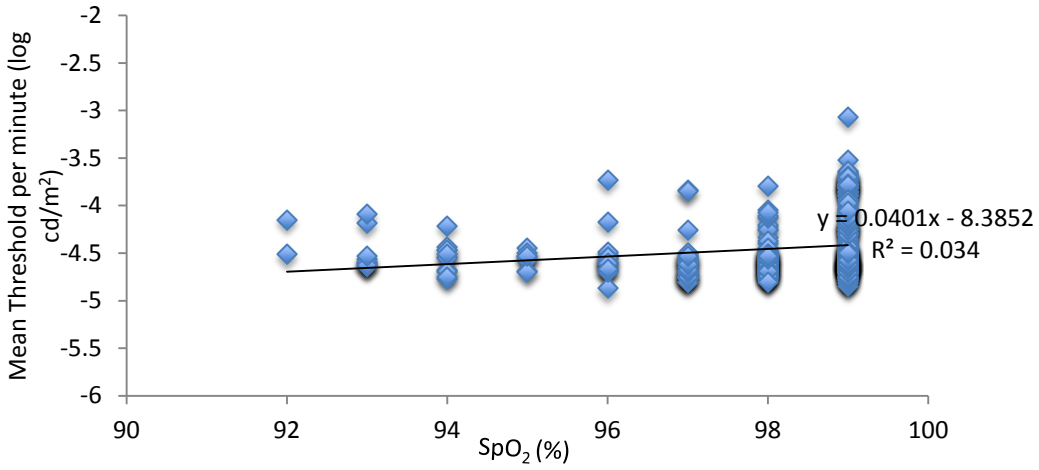


Figure 3-13. Mean threshold for each minute plotted against SpO2 for all 18 participants at 7 degrees eccentricity.

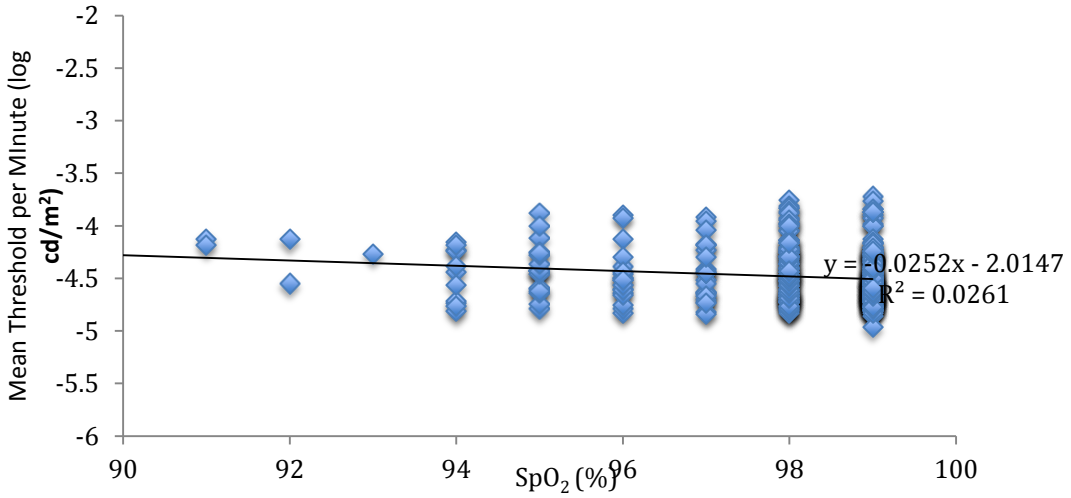


Figure 3-14. Mean threshold for each minute plotted against SpO2 for all 18 participants at 12 degrees eccentricity.

Figure 3-15 shows the relationship between the change in SpO_2 between 21% and 14% oxygen inhalation for the final minute and the change in final minute mean threshold between the two gas conditions at the fovea. Data points above the x axis indicate participants whose SpO_2 was reduced when 14% oxygen was inhaled,this encompassed all except two participants, one of whose SpO_2 marginally increased when 14% oxygen was inhaled and one of whom showed no change. Of the 16 participants who showed a reduced SpO_2 when 14% oxygen was inhaled, 8 fall to the left of the Y-axis, indicating that their thresholds increased as SpO_2 decreased, whilst 8 fell to the right of the Y-axis, suggesting that their thresholds were reduced by a reduction in SpO_2. In other words, the graph shows an even split of thresholds increasing and decreasing in the hypoxic condition, this trend was also seen at 7 degrees and at 12 degrees.

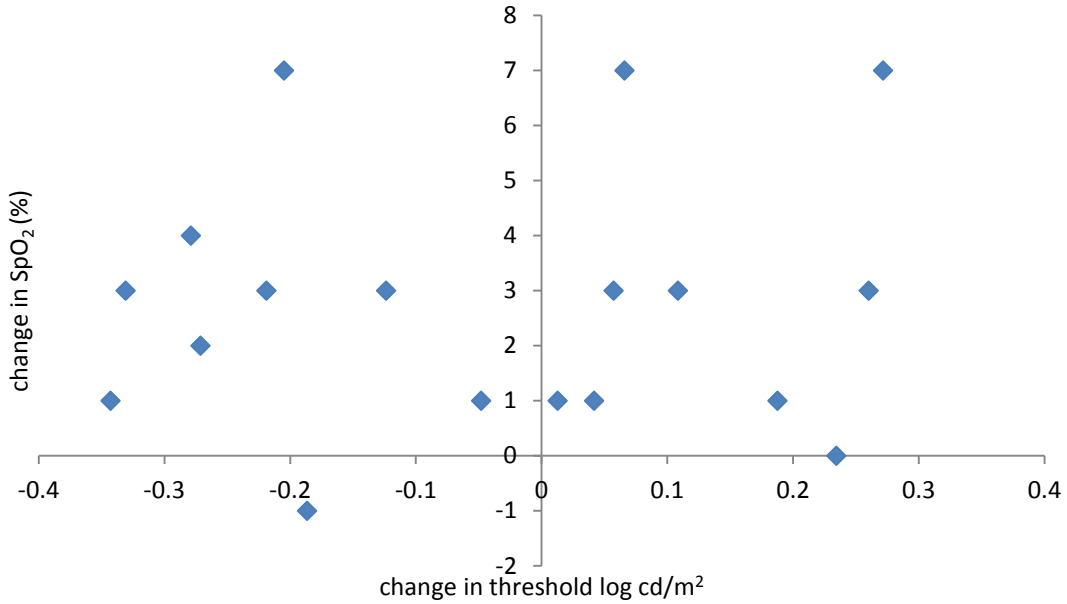


Figure 3-15. Relationship between change in final minute threshold data 21%-14% and change in final minute SpO2 21%-14% at the fovea.

3.1.7 Conclusion

This study showed that in this population there was no significant difference in thresholds when participants were inhaling 14%, 21% or 60% oxygen at 0 degrees, 7 degrees eccentricity or 12 degrees eccentricity. The SpO_2 data displayed in Figure 4-5 show that breathing 60% oxygen raised peripheral oxygen saturation minimally. On average, over the 5 minutes, SpO_2 readings were raised by 0.33% to around 99%. This could be explained by a ceiling effect, as participants' SpO_2 was on average 98.5% when breathing medical air, leaving little room for 60% oxygen to raise it further. These readings are consistent with the literature, with hyperoxia raising thresholds to 99.1% in 2 studies (Connolly et al. 2006; Connolly et al. 2008b) and to 100% in another (Klemp et al. 2007). It is not, therefore, surprising that the hyperoxic condition had no effect on thresholds. However, based on the evidence in the literature that visual function is affected by hypoxia (Connolly et al. 2006; Connolly 2011; Connolly et al. 2008b; Feigl et al. 2008; Feigl et al. 2007; Feigl et al. 2011; Connolly et al. 2008a), it was more surprising that thresholds were unaffected by breathing 14% O_2. An overall trend for thresholds to be raised by hypoxia was seen in all retinal locations, but this was not statistically significant, even at the 12 degrees location where confidence intervals were narrowest.

Published literature has shown that visual function is affected by hypoxia (see Chapter 2 for review). Colour vision (Connolly 2011; Hovis 2012), visual fields (Connolly 2011), dark adaptation (Ernest and Krill 1971; Connolly et al. 2006) and contrast sensitivity (Connolly 2011) have all been reported to be altered by hypoxia. Scotopic thresholds have also

been reported to be raised under conditions of induced hypoxia (Ernest et al. 1971; McFarland et al. 1939; Wald et al. 1942a; McFarland et al. 1940) which is in contrast to the results found in this study. There are several differences between these study designs and the design used here. Firstly, 10% oxygen was used in these studies, which would be expected to reduce SpO_2 to a greater extent than the 14% used in this experiment. These studies also used a longer duration hypoxic episode than that used here, with McFarland and Evans (1939) and Wald and Harper (1942) using a duration of 30 minutes or more. The 5 minute duration used in this study was less than any found in the literature, with most using a duration of a minimum of 15 minutes (Connolly et al. 2009b; Connolly et al. 2009a) or until a steady state of hypoxia was reached (Feigl et al. 2008; Feigl et al. 2011) which, when breathing 12% oxygen was found to take 2-3 minutes (Feigl et al. 2011).

Hypoxia induced by breathing 14% oxygen in this study decreased SpO_2 by, on average, 1.68% (to around 95.5% over the 5 minutes). However, SpO_2 decreased progressively with time breathing 14% oxygen and the greatest effect on the participants' oxygen saturation was in the fifth minute. It is unknown if, at this point, the participant had reached a hypoxic plateau or if the continued inhalation of 14% oxygen would have had further effect. Literature suggests that our participants had not reached a minimum SpO_2 after 5 minutes, with other studies reporting SpO_2 dropping to between 85.7% to 90.6% when breathing 12-14.1% oxygen (Connolly et al. 2006; Connolly 2011; Connolly et al. 2008b; Feigl et al. 2008; Feigl et al. 2007; Feigl et al. 2011; Connolly et al. 2008a). Two studies suggested an SpO_2 of 92% to indicate that significant hypoxia had been reached (Feigl et

al. 2011; Feigl et al. 2008), which was not achieved here. Even in a study where 15.2% oxygen was inhaled, an average SpO_2 of 92.8% was obtained (Connolly 2011), which is less than the lowest mean SpO_2 in this experiment of 95.6%.

A change in threshold with 60% oxygen was not expected to be seen in this population of young, healthy adults. Although the retina is on a hypoxic knife-edge in scotopic conditions, it is expected that there is enough oxygen present to meet demands. However, there is evidence that certain aspects of visual function, including threshold sensitivity to frequency doubling stimuli, low contrast acuity in mid mesopic conditions and dark adaptation parameters, are enhanced when in hyperoxic conditions at sea level, suggesting that the increased metabolic demand at reduced light levels may lead to a local retinal hypoxia sufficient to affect the performance of particular tasks (Connolly et al. 2009a; Connolly et al. 2009b; Connolly et al. 2006). Connolly and Hosking (2006) found that hyperoxia did not affect final scotopic thresholds or cone adaptation but did hasten the timecourse of rod dark adaptation. These effects would not be seen in this study, as the dynamics of the dark adaptation function were not investigated.

Few studies have looked at the topography of visual function under hypoxic conditions, of those that have, the data is conflicting. Feigl (2011) found all eccentricities between 1-15 degrees to be affected by hypoxia under mesopic conditions. Ernst and Krill (1971) studying dark adaptation found that peripheral rods tested at 45 degrees were affected more than central rods at 5 degrees. In contrast, mfERGs collected by Klemp (2007) suggested that the central retina was more sensitive to hypoxia. Connolly (2008) also

found that, when tested with FDT, the central 5-10 degrees from fixation were affected by hypoxia - although the fovea was unaffected. This diversity of results may indicate that hypoxia affects different aspects of visual function in a different fashion, which may reflect the different pathways governing different visual function thresholds. This study found no significant difference between the retinal locations tested, although there was some suggestion from individual datasets that the 12 degree location was more sensitive to changes in oxygenation, with a trend towards raised thresholds with 14% oxygen.

The main limitation of this study was the possible lack of steady state hypoxic conditions during the measurement of scotopic thresholds, either caused by the short duration of the hypoxic episode or the relativley high oxygen concentration. Two further studies were undertaken to address the questions generated by these results. Specifically:

- What duration of inhalation of 14% oxygen is required to reach equilibrium?
- If SpO_2 is reduced further, is there evidence of a significant elevation in scotopic thresholds and if so, which retinal location is affected most?

3.2 Investigation of the time course of changes to SpO_2 during inhalation of 14% oxygen.

3.2.1 Background and aims

The results of the previous study showed that SpO_2 declined with time over a five-minute period of inhaling 14% oxygen but did not plateau. The aim of this experiment was to monitor the time course of changes to SpO_2 when breathing 14% oxygen over 15 minutes in healthy control participants. It was hypothesised that SpO_2 readings will continue to decline past the 5 minute period, but plateau within 15 minutes. Based on the literature, 15 minutes was believed to be sufficent to produce steady state hypoxia (Connolly et al. 2009b; Connolly et al. 2009a).

A second aim was to compare the timecourse in older and younger individuals. As neither group had respiratory problems, such as asthma, it was hypothesised that there would be no difference between the SpO_2 readings of the two groups when breathing 14% oxygen. The results of this study were required to determine the time required for a change in oxygenation level to have maximal effect on SpO_2 .

3.2.2 Participants

Nine healthy control participants were recruited from the School of Optometry and Vision Sciences in Cardiff University, between the ages of 24-71. Inclusion and exclusion criteria were as described in section 3.1.2. Participants were divided into two groups; a younger

group age 24-36 years old and an older group age range 58-71yrs old. All procedures adhered to the tenets of the Declaration of Helsinki. The study was approved by the School of Optometry and Vision Sciences ethical committee.

3.2.3 Method

Participants were seated for the duration of the experiment and were advised to report any symptoms of hypoxia including dizziness. A finger pulse oximeter was used to measure the peripheral oxygen saturation data (see section 3.1.3). Baseline SpO_2 data were collected in normoxic conditions for 5 minutes, with the participant breathing room air. Then, using 8% oxygen delivered though a 60% venti mask (see section 3.1.3), the participant breathed 14% oxygen for 15 minutes. SpO_2 data were collected at 1 minute intervals in the hypoxic condition for a minimum of 15 minutes. The experiment was stopped if the SpO_2 levels dropped below 85%, or if symptoms such as dizziness or confusion were reported. After cessation of inhalation of 14% O_2, SpO_2 readings were taken until the participant's SpO_2 readings had returned to a normal level.

3.2.4 Results

The mean age for group 1 was 27.8 +/- 4.50 years and the mean age for group 2 was 66.5 +/- 5 years. Full 15 minute datasets were collected for all participants.

The group mean SpO_2 levels with confidence intervals are shown in Figure 3-16. It shows that the mean SpO_2 when no gas was inhaled for group 1 was 99.0% and for group 2 was

97.8%. This dropped to 97.0% for group 1 when breathing 14% oxygen and 93.6% for group 2. The graph shows that mean SpO_2 reached a minimum at around 8 minutes after commencing breathing the hypoxic gas for group 1. For group 2 the mean SpO_2 stabilised after 8 minutes and reached a minimum after 11 minutes of breathing 14% oxygen. For both groups the data plateaued after this point. The SpO_2 readings returned to within a normal level within 1 minute for group 1 and 5 minutes for group 2. The confidence intervals show that the data were less variable for group 1. It also demonstrates that there was a significant difference between groups as the confidence intervals do not overlap. The SpO_2 was significantly lower for group 2 at baseline, and was affected more by hypoxia than for group 1, as evidenced by the greater drop in SpO_2 value when breathing 14% oxygen.

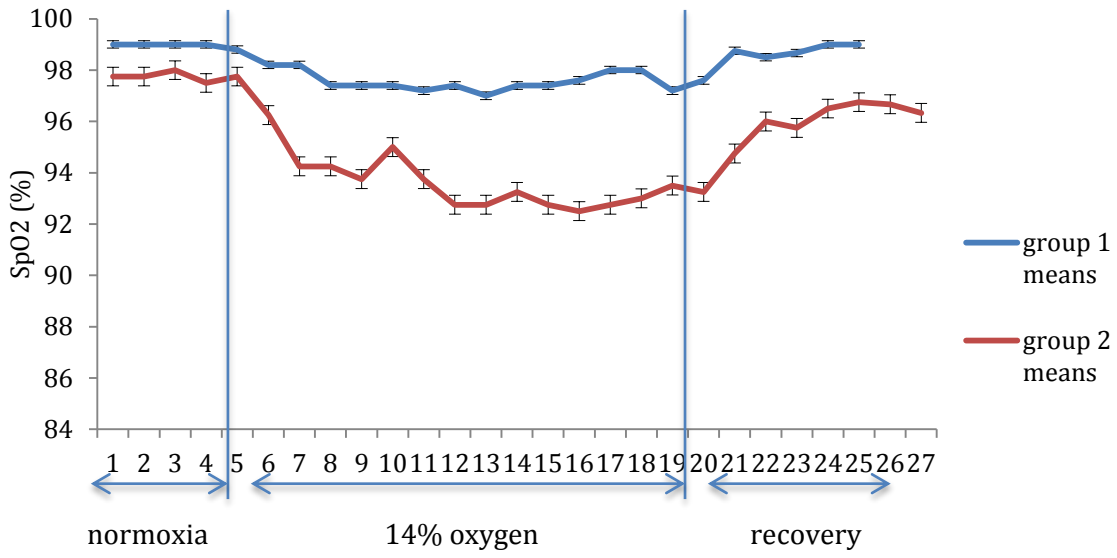


Figure 3-16. Group average data from younger (group 1) and older (group 2) participants, showing confidence interval bars.

3.2.5 Conclusions

The results from this study show that a period of longer than 5 minutes is required for the inhalation of 14% gas to result in the maximum effect on SpO_2 readings. The younger group's mean SpO_2 fell at a faster rate, taking 8 minutes compared to 11 minutes for group 2 to reach a minimum.

The results from this study appear to contradict earlier findings that suggested no difference in the SpO_2 readings between older and younger populations. Feigl (2008) found that when breathing 12% oxygen the SaO_2 levels decreased significantly for both groups, but that there was no difference between the two groups with the younger group dropping to 90% +/-2% and the older group dropping to 91% +/- 0.4%. However, the mean age of the older population used in their study was 55 years old, substantially younger than the older population used in this study (mean age 66.5 years old). It should also be noted that the sample size in our study was small and hence the comparisons between Feigl's study and this one should be interpreted with caution.

The reduction in SpO_2 measured in this study is less than that observed by other investigators, where drops to between 85.7% to 90.6 were found when breathing 12-14.1% oxygen for a 15 minute period (Connolly et al. 2006; Connolly 2011; Connolly et al. 2008b; Feigl et al. 2008; Feigl et al. 2007; Feigl et al. 2011; Connolly et al. 2008a). Comparing the study designs, Connolly (2006) used a hypobaric chamber to simulate an altitude of 15,000 ft. This method of reducing oxygen may not be comparable with the

methods used in this study. Also, an altitude of 15,000 ft is equivalent to an oxygen concentration of less than 12%, therefore SpO_2 would be expected to drop to a lower level than in this experiment. Feigl (2008) and Feigl (2007) also used oxygenation concentrations lower than 14%, using 12% oxygen. Furthermore, different mask types were used in previous studies. For example, Connolly et al (2008) used a Royal Air Force Aircrew Respirator MK5, consisting of an oxygen mask and headpiece that covered the head, neck and shoulders. Connolly and Hosking (2008) and Connolly (2011) both used masks that were suspended from a cloth helmet that ensured an adequate facial seal. Feigl (2008), Feigl (2011) used non-rebreathing masks and Feigl (2007) used a mouthpiece and nose clip. However, although the gas delivery system used in this study was different, participants were checked for the correct fitting of the mask in this study and fitting was adjusted if required, hence the gas delivered should have been broadly as expected.

To summarise, the results of this study indicate that a period of 15 minutes adaption to a change in oxygen concentration is enough to allow SpO_2 to reach a plateau and result in a steady respiratory state. However an inhaled oxygen level of <14% may be necessary to achieve the SpO_2 levels reported in the literature.

3.3 The effect of breathing 10% oxygen on scotopic thresholds in a group of normal participants

3.3.1 Background

Our preliminary findings suggested that there was no significant change in the scotopic thresholds of healthy individuals when breathing 14% oxygen for 5 minutes, compared to medical air (see section 3.1). These findings are contrary to the literature, which suggests that scotopic thresholds are increased by acute hypoxia (McFarland et al. 1939; McDonald et al. 1939; Wald et al. 1942a; McFarland et al. 1940). A follow up experiment suggested that 14% oxygen needed to be breathed for 15 minutes for the reduction in oxygenation to reduce SpO_2 levels to a stable minimum (see section 3.2). However, even when a stable SpO_2 was achieved, it did not drop to the levels described in previous studies. On this basis, it was hypothesised that the lack of a significant effect of hypoxia on thresholds reported in Chapter 5 may be attributable to an insufficient intensity and duration of hypoxic episode.

Therefore, the hypothesis of this follow-on study was that scotopic thresholds would be increased if the hypoxic episode was intensified by breathing 10% oxygen for 15 minutes. The aim of this study was to determine (i) whether an increased level of hypoxia would result in elevated scotopic thresholds, (ii) whether this effect is consistent across the three locations tested and (iii) to determine the gas flow rate required to achieve an

equilibrium SpO_2 of between 85-90%. The findings of this study were intended to guide further investigations looking at dark-adapted visual function in people with AMD.

3.3.2 Participants

Six participants were recruited from staff at the School of Optometry and Vision Sciences at Cardiff University. Exclusion and inclusion critera were as detailed in section 3.1.2. The participants provided informed written consent before participation, and all procedures adhered to the tenets of the Declaration of Helsinki. The study was approved by the School of Optometry and Vision Sciences ethical committee.

3.3.3 Method

Initially all participants underwent a trial of 15 minutes breathing 10% oxygen [delivered from a cylinder of 8% oxygen, via a non-rebreathing mask (Intersurgical)]. Lights remained on at this stage, and the participant was encouraged to report any feelings of dizziness or discomfort. The participant was seated throughout in a chair with arm rests, and SpO_2 was recorded continuously. The aim of this preliminary experiment was to exclude potential participants who became faint when undergoing a hypoxic episode. Any participants who reported symptoms of dizziness, nausea, or breathlessness were excluded from further study.

To dilate the pupils 1.0% tropicamide was then instilled into the eye being tested (the eye with the better VA or the right eye if both eyes were equal). At this point, lights were

extinguished and dark adaptation took place for 30 minutes. One of 3 targets (located at 2 degrees, 7 degrees or 12 degrees eccentricity), was chosen at random for the first trial. The psychophysical procedure used to determine threshold was as described in section 3.1.4.

After 30 minutes of dark adaptation, a 5 minute training session took place where thresholds were recorded continuously at location 1, but no gas was delivered. Gas 1 was then turned on and the participant breathed the first of two randomly assigned gases; medical air or 10% oxygen. Medical air was delivered at a flow rate of 15 L/min, whilst the 8% oxygen was delivered at the variable flow rate determined in the preliminary experiment. Participants were masked to which gas they are receiving at any time.

For the next 15 minutes, the measurement of scotopic thresholds continued at the first retinal location. After the 15 minutes, gas 1 was turned off and the participant continued to respond to the program during a 5 minute 'washout' period (pilot data suggested that 5 minutes were sufficient to eliminate any residual effect of the previous gas on thresholds). Thereafter, gas 2 was turned on and scotopic thresholds were measured for the next 15 minutes. A timeline for the experiment is shown in Figure 3-17.

dark adaptation 30 mins	Practice 5 mins	Testing gas 1 15 min	Wash out 5 mins	Testing gas 2 15 mins

Figure 3-17. Timeline of experiment. Gases 1 and 2 were medical air and 10% O_2, with the gas order randomly selected.

Participants' peripheral haemoglobin oxygen saturation (SpO2) was closely monitored throughout the experiment using a pulse oximeter with finger probe to ensure that the oxygen saturation did not fall below 85%. The experiment was to be terminated if SpO2 fell below this level, or if symptoms such as dizziness or confusion were reported. The participant was monitored (and asked to remain seated) until SpO2 rose back to baseline at the end of the experiment. At visit one the experiment was run once, testing one location. At the second visit, the remaining locations were tested, with the experiment running twice with a rest in of at least 10 minutes between.

3.3.4 Results

Five participants were recruited for this study. The mean age of the participants was 33 years (range 25-48 years) and the group contained 60% females. The required flow rate to achieve an SpO_2 of 85-90% was 13 l/min for the hypoxic gas for all apart from one participant; participant AB required the maximum flow rate of 15 l/min to show any reduction in SpO_2. One participant, AW, was excluded due to dizziness during the preliminary 10% oxygen trial. Full sets of data were collected for all remaining participants for all locations, apart from participant TC at location 24 degrees, where there were no threshold data for the final 4 minutes as the SpO_2 dropped to the cut-off point and the mask was removed. The participant felt well and their SpO_2 continued to be monitored and returned to a value of 99%.

Figure 3-18 shows the group averaged SpO_2 per minute for both gases. It can be seen that, when inhaling medical air, the SpO_2 was stable, with the mean at 98.2%. The small error bars indicate that the inter-individual variability was low. When inhaling 10% oxygen it can be seen that the highest SpO_2 reading was in the first minute (97%). Thereafter, SpO_2 dropped steadily within the first 5 minutes, reaching a plateau before dropping again. The lowest SpO_2 (88%) was seen in the 11th minute. The confidence intervals indicate that there was more variability between participants when compared to the medical air data. The difference in SpO_2 was significant at all time points between the two gases after the first minute with the confidence intervals not overlapping at any time.

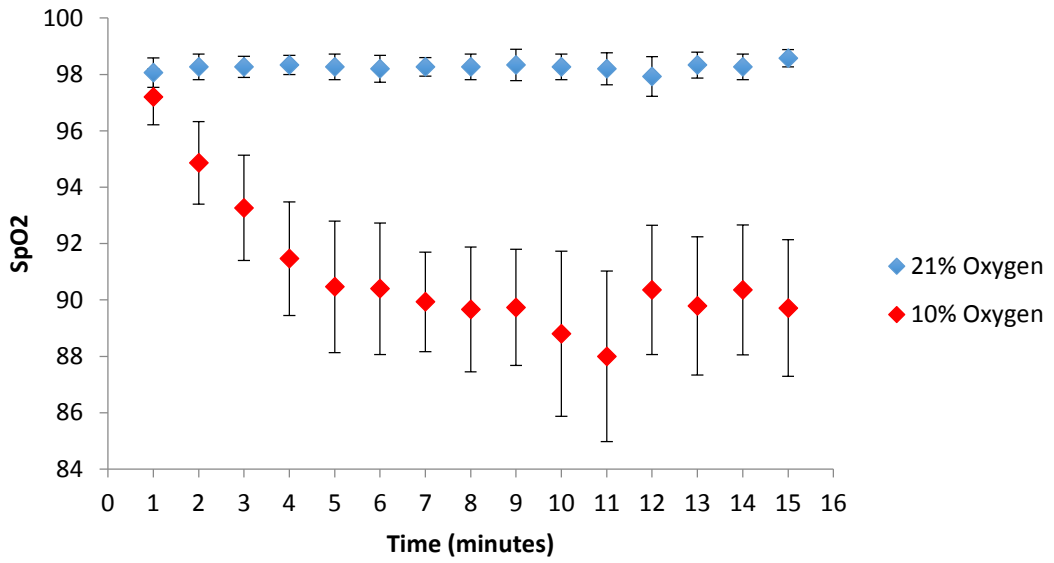


Figure 3-18. Group averaged SpO_2 for each minute of gas inhalation with 95% confidence intervals.

Figure 3-19, Figure 3-20 and Figure 3-21 show the thresholds recorded for each experiment for a typical participant at all three locations. It can be seen in Figure 3-20 (relating to a retinal location at 7 degrees eccentricity) that, where the hypoxic gas was supplied between 5-20 minutes, the thresholds appear to be raised compared to when medical air was supplied between 25-40 minutes. No obvious change in threshold is apparent with the hypoxic gas for the other two retinal locations.

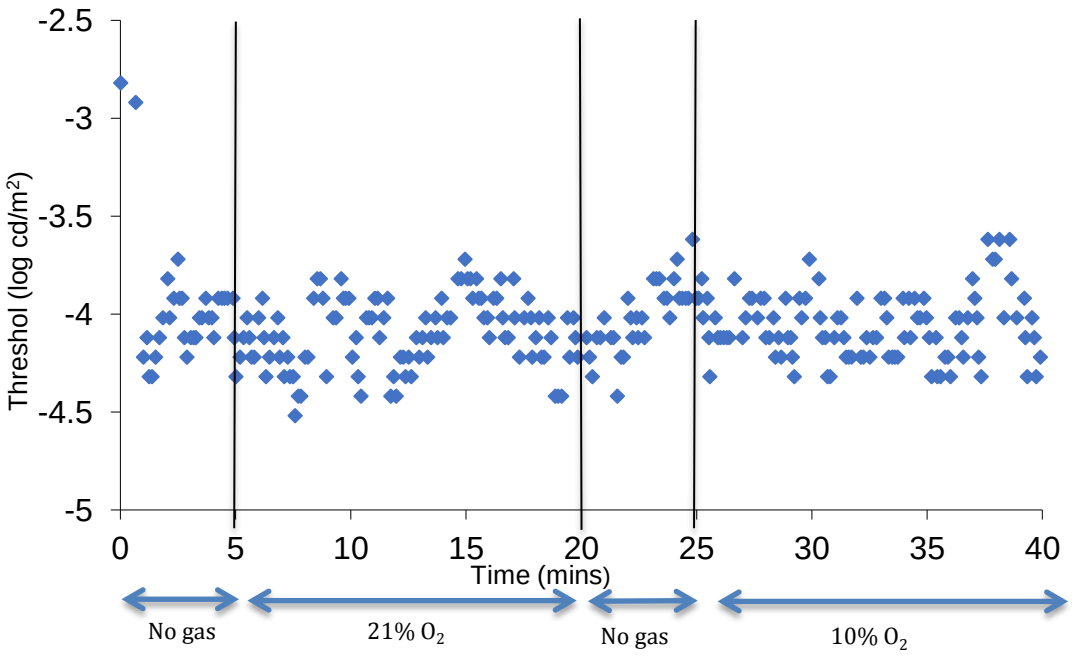


Figure 3-19. Plot of threshold as a function of time for a typical participant (TM) at 2 degrees.

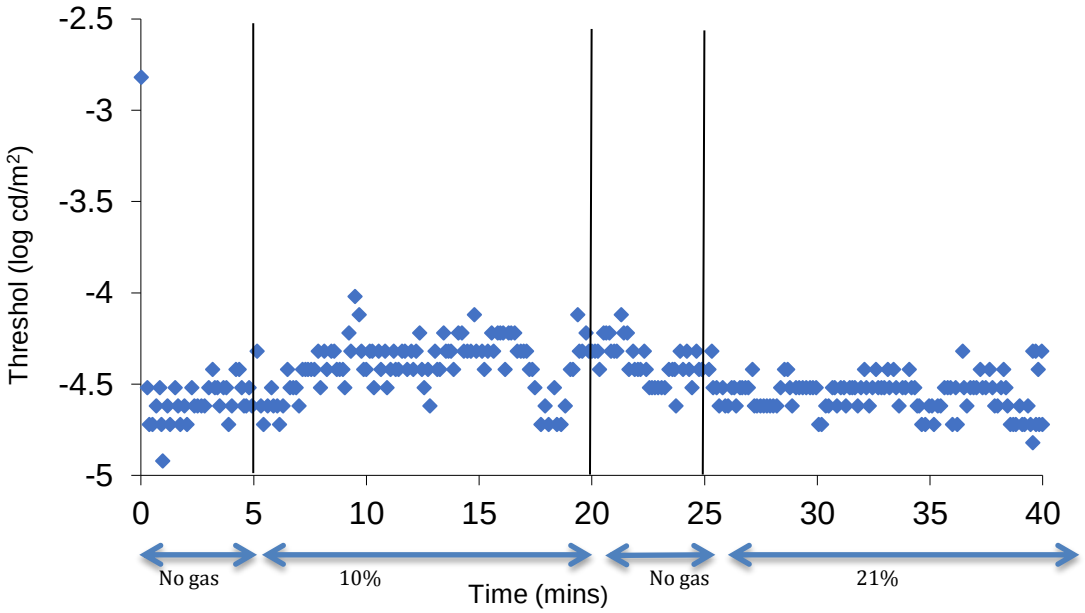


Figure 3-20. Plot of threshold as a function of time for a typical participant (TM) at 7 degrees.

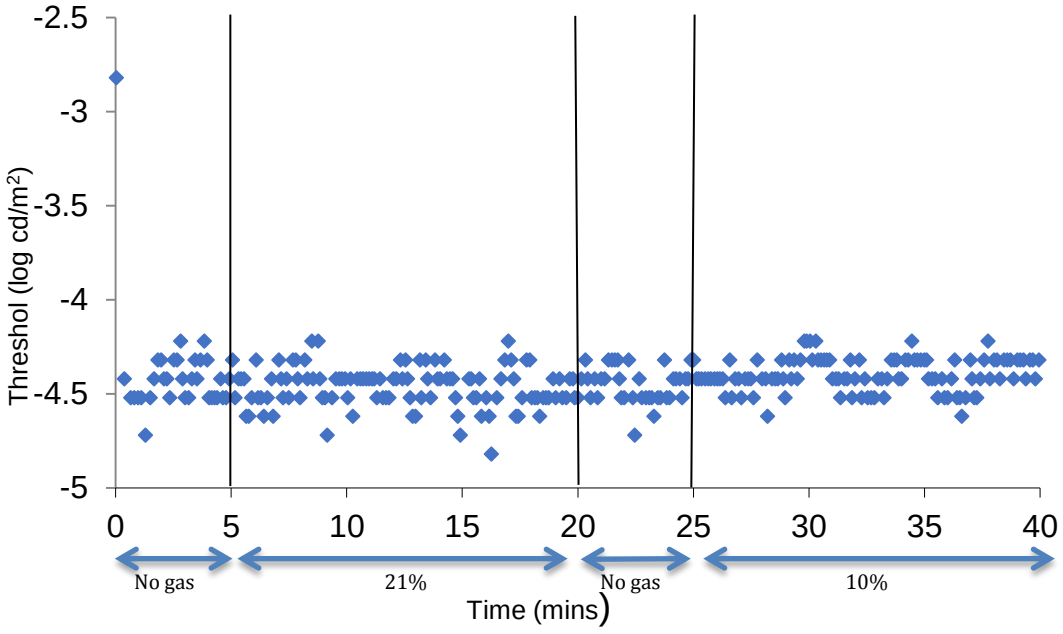


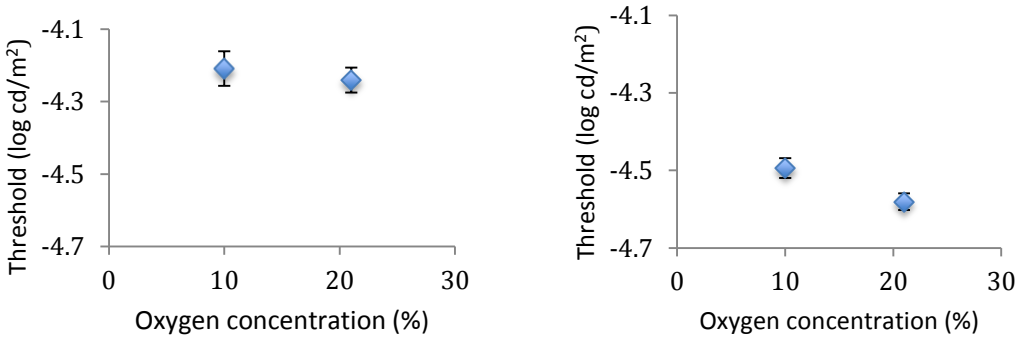
Figure 3-21. Plot of threshold as a function of time for a typical participant (TM) at 12 degrees.

Table 3-1 shows the mean threshold obtained over the last 5 minutes for each gas for each participant. The final 5 minutes was chosen as this time was when SpO_2 readings had stablised. For participant TC the mean threshold was calculated between minutes 30-35, due to the mask being removed during the final 5 minutes. Looking at this individual data it can be seen that inhaling 10% oxygen resulted in a significant rise in scotopic thresholds when compared to inhaling medical air in five instances. At 2 degrees it was significant for AN and MD, at 7 degrees it was significant for TM and 12 degrees it was significant for participants TC and TM.

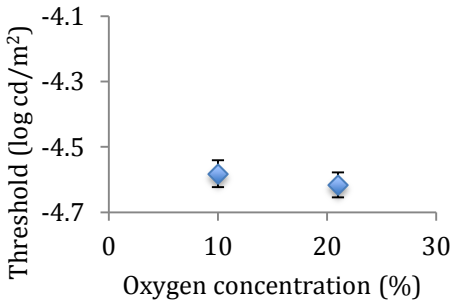
	Location 2 degrees		Location 7 degrees		Location 12 degrees	
Participant	10% O2	Medical air	10% O2	Medical air	10% O2	Medical air
AB	-4.35 ± 0.05	-4.27± 0.05	-4.41± 0.03	-4.49± 0.04	-4.50± 0.05	-4.47± 0.04
AN	**-3.90± 0.08**	**-4.21± 0.06**	-4.62± 0.06	-4.63 ± 0.03	-4.44 ± 0.03	-4.54 ± 0.04
TC	-4.46 ± 0.10	-4.47± 0.07	-4.58± 0.04	-4.65± 0.06	**-4.48± 0.04**	**-4.58± 0.03**
TM	-4.06± 0.08	-4.06± 0.06	**-4.38± 0.06**	**-4.58± 0.04**	**-4.40± 0.03**	**-4.49± 0.04**
MD	**-4.06 ± 0.06**	**-4.21 ± 0.07**	-4.51 ± 0.06	-4.56 ± 0.05	- 5.09± 0.05	-5.10 ± 0.06

Table 3-1. Mean threshold for the last 5 minutes of breathing each gas with 95% confidence intervals. Text in bold indicates no overlapping of the confidence intervals between the scotopic threshold between breathing 10% and 21% oxygen.

The group averaged thresholds for the final 5 minutes of inhaling each of the gases at all three locations are shown in Figure 3-22. At all locations, mean threshold was highest for the hypoxic gas, however this failed to reach significance at 2 degrees or at 12 degrees, where the confidence intervals are overlapping (paired t-test p= 0.320 and Wilcoxon signed rank p= 0.136, for 2 and 12 degrees respectively). Threshold was elevated to a greater extent by the hypoxic episode at 7 degrees, with non-overlapping confidence intervals, but this also failed to reach statistical significance (paired t-test; p= 0.062).



(a) (b)



(c)

Figure 3-22. Group averaged thresholds for both gases (a) at 2 degrees, (b) 7 degrees and (c) 12 degrees (c). The error bars show 95% confidence intervals for the mean.

Whilst SpO_2 was reduced by breathing 10% oxygen in all individuals, there was variability in the magnitude of the effect. Therefore, further analysis was carried out to look at the relationship between SpO_2 and threshold. Thresholds were plotted against SpO_2 in a linear regression analysis. The individual threshold data plotted against SpO_2 can be seen in for all three locations in Figure 3-23, Figure 3-24 and Figure 3-25. These graphs include the data from all 5 participants for every minute of gas inhalation. The low R^2 value indicate that the variability in the threshold data were not well explained by SpO_2 at any location.

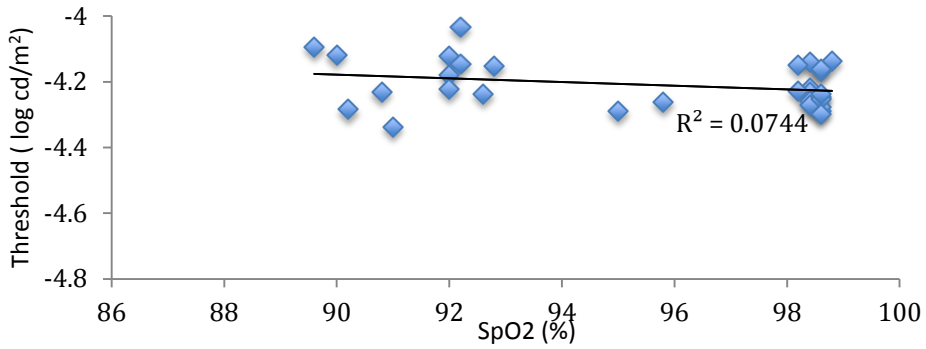


Figure 3-23. Mean threshold for each minute plotted against SpO_2 for all 5 participants at 2 degrees.

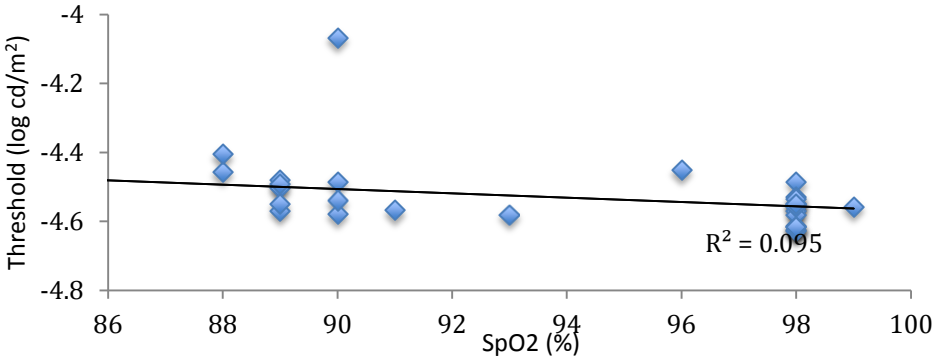


Figure 3-24. Mean threshold for each minute plotted against SpO_2 for all 5 participants at 7 degrees.

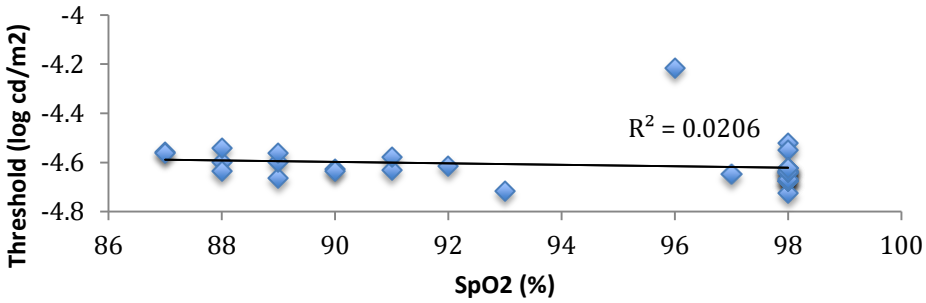


Figure 3-25. Mean threshold for each minute plotted against SpO_2 for all 5 participants at 12 degrees.

3.3.5 Conclusion

Inhaling 10% oxygen for 15 minutes resulted in a decrease in SpO_2 for all participants and an increase in mean thresholds at all locations, which approached statistical signficance at 7 degrees. The effect of breathing 10% oxygen on SpO_2 was more substantial in the first 5 minutes then levelled out. The SpO_2 dropped to its lowest reading in the 11^{th} minute with a mean value of 88% recorded. The SpO_2 was significantly different at all time points for 10% oxygen, when compared to medical air.

The literature has shown that scotopic thresholds are elevated by hypoxia (McFarland et al. 1939; McDonald et al. 1939; Wald et al. 1942a; McFarland et al. 1940). However, the mean change in threshold observed here was relatively small, just 0.078, 0.082 and 0.054 log units for the 2, 7 and 12 degree locations respectively.

A few studies have looked at the topography of visual function in hypoxic conditions (Feigl et al. 2011; Ernest et al. 1971; Klemp et al. 2007; Connolly et al. 2008b). However, this is the first study to compare the effect of hypoxia on scotopic thresholds at different locations across the macula. The 7-degree location was found in this study to be most affected by hypoxia, which is consistent with the literature evaluating other types of visual function. Connolly (2008) studying frequency doubling contrast sensitivity, found that the retina was preferentially affected by hypoxia at 9 degrees and that the fovea was unaffected. Ernst and Krill (1971), studied the effect of hypoxia on dark adaptation at

locations up to 5 degrees and also 45 degrees. They found that rod dark adaptation was affected preferentially at 45 degrees compared to 5 degrees.

The findings of slightly raised thresholds while breathing 10% O_2 in this study suggest that our prevous negative findings were due to the very modest effect of 14% O_2 on SpO_2. In this study, hypoxia seems to affect the retina most at 7 degrees, it suggests that the retina maybe more susceptible to the effects of hypoxia at this location when compared to the other locations.

3.3.6 Summary

The studies presented in this chapter indicate that:

i) An acute episode of hyperoxia has little effect on SpO_2 or scotopic thresholds in healthy controls (see section 4.1). This may be attributable in part to a ceiling effect, whereby SpO_2 was approaching 100% in the normoxic condition. It also supports our hypothesis that the dark adapted retina is not hypoxic in healthy individuals.

ii) Breathing 14% oxygen for 5 minutes was insufficient to induce a hypoxic level in controls of sufficient magnitude to affect scotopic thresholds (see section 4.2). Observing the effect of breathing 14% oxygen on SpO_2, it can be seen that more than 8 minutes were required for SpO_2 to reach a steady state. An 8 minute period was therefore selected as the minimum duration of breathing air types before data were collected in further studies in this thesis.

iii) Breathing 10% oxygen for 15 minutes induced a level of hypoxia in controls which was sufficient to cause an elevation in scotopic thresholds. The decrease in scotopic sensitivity approached statistical significance at an eccentricity of 7 degrees, indicating that this region may be most sensitive to hypoxic insult. This location was therefore used for the remaining experimental work in this thesis. The 10% oxygen condition was not tolerated by all individuals, even in the healthy control group, and hence was not employed in the older cohort with AMD for ethical reasons.

The next chapter of this thesis will study the effect of breathing 60% oxygen on the scotopic thresholds of participants with early AMD. It is hypothesised that breathing 60% oxygen will decrease scotopic thresholds in participants with early AMD due to the presence of a local retinal hypoxia. In the control group no difference in scotopic thresholds is expected when breathing 60% oxygen.

4 The effect of breathing 60% oxygen on scotopic thresholds in people with AMD

4.1 Background and aims

Delayed dark adaptation and raised scotopic thresholds are known to occur with ageing, with a 0.1 log unit of elevation of scotopic threshold occurring per decade (Jackson et al. 2000; Owsley et al. 2001). Therefore, it is not surprising that difficulty seeing in the dark is a common complaint of the elderly, even when no pathology exists (Owsley 2011; Steinmetz et al. 1993). Elevated scotopic thresholds and delays in dark adaptation are observed to a greater extent in people with early AMD (Owsley et al. 2000; Steinmetz et al. 1993; Brown et al. 1986; Scholl et al. 2004; Dimitrov et al. 2008; Owsley et al. 2007). For example, Owsley et al. (2000) reported that the dark-adapted mean field sensitivity was significantly lower (by 6.7 dB) in people with AMD than a group of age-matched controls.

A slowing of dark adaptation and raised scotopic thresholds have also been observed in young healthy participants, when transient hypoxia is induced (Connolly et al. 2006; McFarland et al. 1939), as discussed in chapter 2. Hence it is possible that hypoxia may contribute to raised thresholds in people with early AMD due to changes in the choroidal

circulation, a thickened Bruch's membrane and changes at a cellular level, including altered expression of growth regulators (see chapter 2; Mousa et al. 1999; Schlingemann 2004; Stefánsson et al. 2011; Giovannini et al. 1994; Pauleikhoff et al. 1990). If this is the case then temporarily reducing hypoxia in participants with early AMD, by the inhalation of 60% oxygen, should result in a decrease in scotopic thresholds i.e. a shift towards normal sensitivity.

This study aimed to investigate this hypothesis by measuring scotopic thresholds in people with and without early AMD, whilst they breathe medical air (21% oxygen) and a hyperoxic gas mix (60% oxygen). It was expected that during the period of increased oxygen delivery, thresholds would decrease in people with AMD, whilst remaining stable in the control group. Such a finding would strengthen the case for a role of hypoxia in the pathogenesis of AMD.

4.2 General Methods

4.2.1 Participants

Participants were recruited to the study from patients attending the University Hospital Wales Ophthalmology outpatients' clinic and Cardiff University Eye Clinic, or directly from local optometrists in the Cardiff area between 01 April 2013 and 13 August 2015. For recruitment from the University Hospital Wales Ophthalmology outpatients' clinic, TC

attended the nAMD treatment clinic and, along with the clinic consultant, reviewed the records of patients for eligibility.

If a patient was thought to meet the inclusion and exclusion criteria, then they were approached in the clinic by TC and the project was discussed. If the patient was still considered as meeting the criteria and was agreeable to be contacted then contact details were gathered. Potential participants were then contacted by telephone at a later date to check that they were still interested in participating in the study and, if so, then the participant information sheet was sent out. Individuals were then contacted after 10 days, and asked if they had read the information sheet. During all conversations the potential participant was encouraged to ask any questions. If they were still eligible and if they were happy to proceed then then an appointment was made for them to attend the School of Optometry and Vision Sciences where the PIS was reviewed once more before written, informed consent was obtained to the study. The recruitment procedure from Cardiff University Eye Clinic involved identifying records of patients who appeared to meet the eligibility criteria as age matched controls or who had a record of early or nAMD and who had consented to being contacted for research purposes. These patients were then contacted and invited to take part using the same method as participants from the University Hospital Wales Ophthalmology outpatients' clinic. Although information packs were sent to several local optometrists, no participants were recruited via this stream.

Inclusion criteria for all participants were: age over 55 years, corrected visual acuity of 6/12 or better, and a refractive error of < 6.00 DS in the most powerful meridian.

Exclusion criteria included: lung conditions (such as emphysema), systemic health conditions known to affect vision, taking medication known to affect visual function, significant cataract [above grade 2 on the LOCS III scale (Chylack et al. 1989)], ocular disease (other than AMD), repeatable visual field defect (a cluster of 3 or more points within the central 24 degrees of field), intraocular pressures above 21mmHg or narrow anterior chamber angles (Van Herick grades 0 and 1). Participants with a fundus appearance graded by TC as having early AMD in one or both eyes i.e. 1-4 points on the simplified AREDS 5 point grading scale, (Ferris et al. 2005) which was discussed in section 1.4.3 were assigned to the AMD group. This group also contained patients who were being treated for neovascular AMD in the non-test eye who had 0-2 risk points in the test eye (i.e. who had a total grade of 2-4 points on the simplified AREDS scale). The control group comprised participants with normal retinal ageing in both eyes and no sign of AMD (grade 0 of the AREDS 5 point scale). The study eye was the eye with the best visual acuity (control group), or the eye with early AMD (AMD group).

In the case of bilateral early AMD, the eye with better visual acuity was chosen. In both groups, in the case of equal acuities, the right eye was used as the study eye.

For grading purposes digital colour fundus photographs were displayed on a screen and the risk factors assigned according to the scale for each eye. If grading was ambiguous then a second grader, TM or AB was consulted for a second opinion.

All procedures adhered to the tenets of the declaration of Helsinki. The study was approved by South East Wales Research Ethics Committee.

4.2.2 Sample size calculation

The Bland and Altman nomogram was used to calculate the number of participants that should be recruited to each group. The nomogram is a graphical method of calculating sample sizes when the power, significance level and the standardised difference are set for a study. The power of a study is described as the probability that it will detect a real difference (of a set magnitude) as statistically significant, with a power of 80-90% commonly required (Bland and Altman 1999). The equation for calculating the standardised difference for continuous data is shown in equation 4-1.

Standardised difference = δ/s

Equation 4-1 Standardised difference equation (Bland and Altman. 1999), where δ = clinically relevant difference and s = standard deviation.

As this is the first study of its type, there are no data available regarding the size of the effect on scotopic thresholds and ERG responses when people with AMD breathe a hypoxic or hyperoxic gas. However, threshold data and standard deviations have been reported from healthy controls under conditions of normoxia, hyperoxia and hypoxia for a centrally located stimulus under mesopic conditions (Connolly and Hosking. 2009b). These previously published data suggest that a study powered to detect a standardised

difference of 0.73 would identify an effect at least as big as that which is seen in healthy controls under a hypoxic condition. For the data collection in Chapters 4, 5 and 6 to allow detection of a standardized difference of 0.73 using a power level of 80% and a statistical significance of 0.05, a sample size of N=30 per group was aimed to be recruited per study.

4.2.3 Baseline Measurements

Patient history and baseline measurements were completed at the start of the visit after consent was obtained. They included distance corrected visual acuity (ETDRS LogMAR chart), intraocular pressures (Topcon CT 80 tonometer), D-15 saturated colour vision test, central 24-2 suprathreshold field examination using a Humphrey field analyser and slit lamp biomicroscope assessment of the anterior chamber angle using the Van Herick grading technique. If the participant met all of the inclusion criteria then 1% tropicamide was instilled into both eyes. Dilated binocular indirect ophthalmoscopy examination and retinal photographs were obtained for all participants (Topcon 3D OCT-1000) in order to allow fundus grading, along with OCT images. Fundus grading was carried out by assessment of the retinal photographs and binocular indirect ophthalmoscopy examination according to the AREDS simplified scale (Ferris et al. 2005) by TC.

4.2.4 Equipment

All stimuli for the psychophysical studies were presented on a gamma corrected high-resolution monitor (Liyama LS902UT) driven by an 8-bit (nVIDIA Geforce 9) graphics board under the control of MATLAB. Scotopic thresholds were measured using the QUEST

Bayesian adaptive procedure (Watson et al. 1983) written with Psychophysics Toolbox extensions (Brainard, Pelli and Robson 2002). This psychometric measure determines final threshold using the maximum likelihood approach (Watson et al. 1983) as discussed in Section 1.5.2. The outputs of the programme were scotopic threshold and standard deviation. Gases were delivered via a 60% ventimask (intersurgical). A pulse oximeter (Crucial Medical Systems CMS 50E) was used to monitor the peripheral blood oxygen saturation (SpO_2) in all experiments. See section 3.1.3 for details.

4.3 Pilot Study

A pilot study was conducted prior to commencing recruitment for the main part of the study, in order to evaluate the feasibility of the protocol.

4.3.1 Methods

Baseline tests were carried out, as outlined in section 4.2.3. A break of 10 minutes was then provided, during which time the study eye was patched and room lighting was dimmed. Following this, a 40-minute period of dark adaptation took place, with the test eye still patched and no room lighting. The psychophysical procedures used to measure scotopic thresholds are outlined in section 4.2.4. At the end of the dark adaptation period, the participant undertook a practice session that consisted of a shortened version (20 trials) of the QUEST psychophysical procedure. The first of two randomly assigned

gases, was then delivered to the participant providing either 21% or 60% oxygen for inhalation. The participant was masked to which gas was being inhaled.

The first gas was inspired for 10 minutes and, within the last 5 minutes, scotopic threshold was determined using the QUEST 40 trial paradigm. There then followed a 5-minute wash out period where no gas was given. After the wash out period, the second of the two gases was turned on for 10 minutes and the scotopic threshold was recorded again within the final 5 minutes. Threshold recording was conducted in the final 5 minutes in order to ensure that an SpO_2 plateau had been reached by the 8-10th minute where the paradigm converged on the threshold. The SpO_2 of the participant was monitored throughout the duration of the experiment, and recorded every minute. A timeline of the experiment is shown in Figure 4-1.

dark adaption 50 mins	20 trials practice 5 mins	Start gas 1 5 mins	Record thresholds 5 mins	Wash out 5 mins	Start gas 2 5 mins	Record thresholds 5 mins

Figure 4-1. Experimental timeline. Note that gas 1 and gas 2 were assigned randomly.

To ward against hyperventilation, participants in all studies involving breathing air though a mask were coached on maintaining a regular breathing pattern. If it was felt by the examiner that the participant's breathing pattern had changed then this was discussed wtih the participant. The participant was also distracted from thinking about breathing though the mask with conversation during any adaptation period.

4.3.2 Results

Four individuals (three male) participated in the pilot study, with a mean age of 75 years (range 72-77 years). Three participants were classified as having early AMD (001JW, 002MD, 003RC), and one (000RE) was an age similar control. Figure 4-2 displays the mean SpO_2 readings per minute under both gas conditions. The graph shows that there was little variation over time in SpO_2 readings when breathing 60% O_2 or 21% O_2, where the average readings all fell within a 1.25% range. It can be seen that the 95% confidence intervals do not overlap between 5-9 minutes, suggesting a significant difference in SpO_2 values during this time.

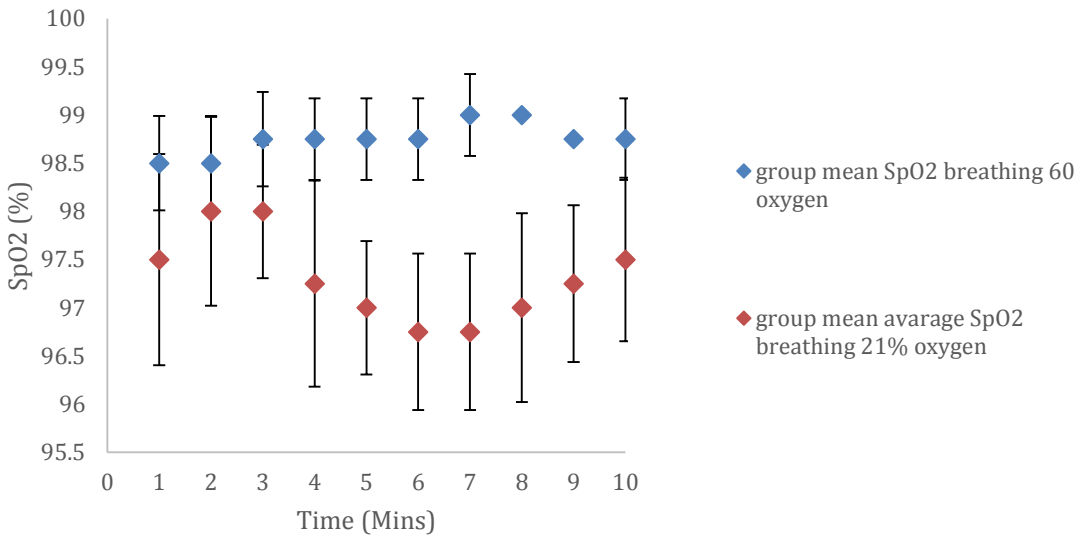


Figure 4-2. Mean SpO_2 per minute with 95% confidence intervals breathing 60% and 21% oxygen.

Sample scotopic threshold data obtained using the QUEST procedure under conditions of 60% and 21% oxygen are displayed for the age similar control and one participant

classified as having early AMD in Figure 4-3. The solid line in each plot indicates the threshold, whilst the dotted lines show the QUEST standard deviation. Each data point represents one trial presentation. The plots show that the QUEST algorithm converged on the participant's scotopic threshold after 40 trials.

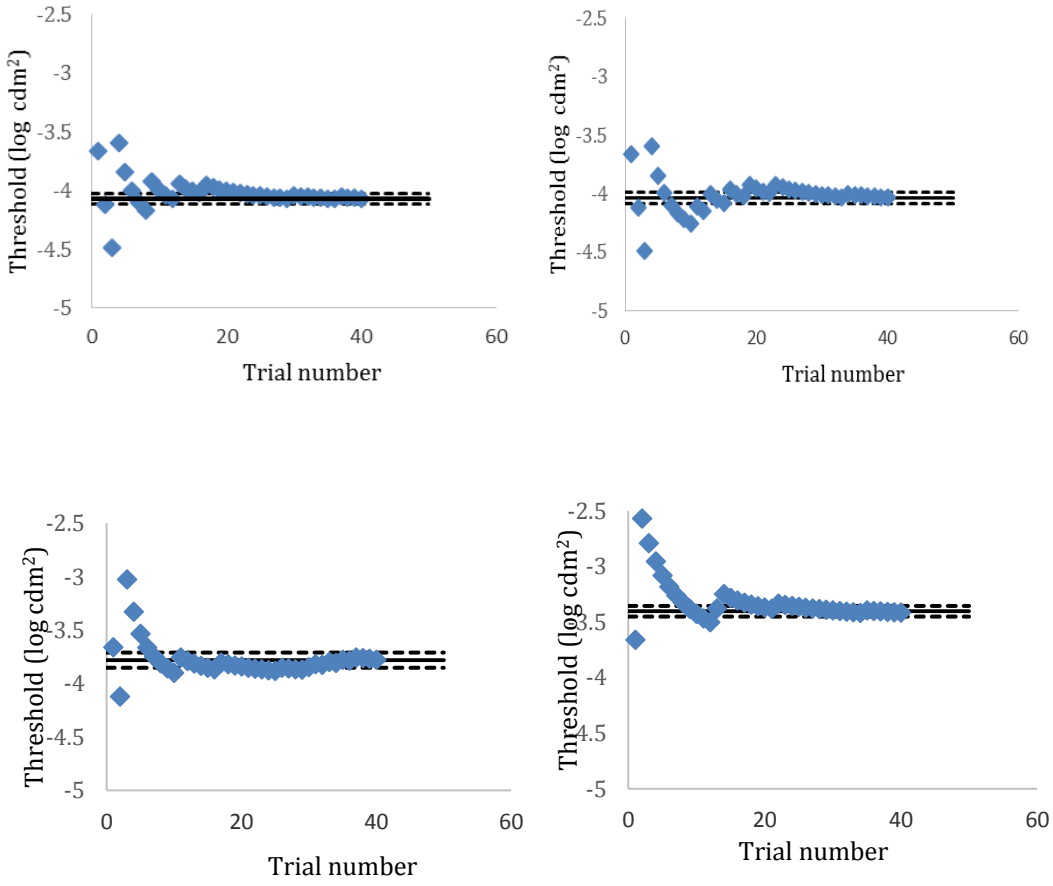


Figure 4-3. Sample scotopic threshold data from control participant 000RE (top row) and an individual with early AMD, participant 001JW (bottom row). The left column shows thresholds for 21% oxygen, the right column for 60% oxygen.

Table 4-1 summarises the scotopic thresholds of all four participants with confidence intervals whilst breathing each gas type. The table shows that the differences in the scotopic thresholds between breathing 21% and 60% O_2 were not significant for any participant as the confidence intervals for thresholds overlapped for all participants between the two gas conditions. However, it is of interest that the scotopic thresholds for all three participants with early AMD were lowest after breathing the second gas (see bold results in Table 2), regardless of whether this was 60% or 21% oxygen.

Participant reference	Threshold log cdm^{-2} +/- 95% CI with 21% oxygen	Threshold log cdm^{-2} +/- 95% CI with 60% oxygen
000RE (control)	-3.11 +/- 0.21	**-3.36 +/- 0.12**
001JW (AMD)	**-4.01 +/- 0.10**	-3.90 +/- 0.14
002MD (AMD)	**-3.32 +/- 0.11**	-3.22 +/- 0.35
003RC (AMD)	-3.81 +/- 0.11	**-3.85 +/- 0.11**

Table 4-1. Scotopic thresholds and 95% confidence intervals of all participants when breathing 21% and 60% oxygen. The bold threshold indicates the first presented gas.

4.3.3 Discussion

The 95% confidence intervals overlapping between scotopic thresholds when breathing 21% and 60% oxygen for all participants suggests that there were no significant differences in threshold between the two gas conditions for any of the participants

included in the pilot. This was contrary to our hypothesis that individuals with early AMD would show a reduction in threshold when breathing 60% oxygen. However, as can be seen from the results, the scotopic thresholds in all three participants with early AMD were non-significantly higher with the first gas presented, regardless of whether it was the 21% or 60% oxygen. One explanation for this trend could be that the participants were still not fully dark adapted when the measurement of scotopic thresholds began, which may have led to an elevated scotopic threshold on the first trial, with the first gas. Dark adaptation takes approximately 50 minutes to complete, and the 50 minutes of reduced/no lighting and eye patching should have been sufficient to reach absolute threshold. However, just before dark adaptation commenced, fundus photography and VOLK retinal examination were conducted. These procedures use a bright light that would have resulted in significant photopigment bleaching. Furthermore, people with AMD are known to have prolonged kinetics of dark adaptation compared to age-matched controls (Dimitrov et al. 2011; Owsley et al. 2007). Therefore, the 50 minutes of dark adaptation that preceded the measurement of scotopic threshold in this protocol may not have been sufficient. Consequently, it was decided that for all further studies these fundus examination procedures would be undertaken after scotopic threshold recording was complete and the dark adaptation period would be increased to one hour.

Although all participants undertook a shortened trial procedure as a training exercise, these findings could also be explained by a learning effect, or increasing confidence as the participant progressed though the repeated trials. To reduce the impact of the learning

effect, a new training protocol was implemented based on the pilot study data for all future data collection. From this point, all studies included a minimum of two practice trials, each consisting of 20 stimulus presentations. The main study only commenced when the thresholds obtained in two consecutive practice trials were within 1 standard deviation of each other. When necessary, a third training session was initiated. This ensured that for future data collection the participant understood the task. The results of these practice sessions were also used to estimate the final threshold, which was entered into the main MATLAB QUEST procedure.

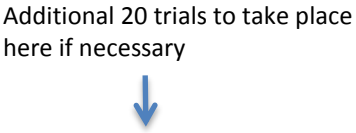
4.4 Main study

4.4.1 Methods

Twelve participants with early AMD and 11 age matched healthy controls were recruited to the main study. The participant selection criteria, baseline measurements, pupillary dilation and equipment used have been described in section 4.2. Procedures involving bright lights (i.e. VOLK examination and fundus photography) were undertaken at the end of the experiment.

Following the initial work up, the participant had a 15-minute rest period, during which room lighting was reduced and the test eye was patched, then a further period of 45 minutes of dark adaption with no room lighting and the test eye still patched. The protocol for the data collection was as described in section 4.3.1 for the pilot study. In

this study, before the main data collection took place the participants undertook 2 trial practice sessions and proceeded to the main trial if the thresholds of both trials were within 1 standard deviation of one another. If this was not the case then a third trial was undertaken, if this threshold was not within 1 standard deviation of the first two trials then the data collection was stopped at this point. The timeline of the experiment is shown in Figure 4-4.



Dark adaption	20 trials practice	20 trials practice	Start gas 1	Record thresholds	Wash out	Start gas 2	Record thresholds
60 mins	5 mins	5 mins	5 mins	5 mins	5 mins	5 mins	5 mins

Figure 4-4. Timeline showing the protocol of the main study investigating the effect of inhaling 60% oxygen on scotopic thresholds. Note that gas 1 and gas 2 were randomly assigned for each participant as 60% oxygen and 21% oxygen (medical air).

4.4.2 Data Analysis

The scripts driving the QUEST procedure used in this study were written by Watson and Pelli (1983) and implemented within the Matlab Psychophysics Tool box. Although the scripts came from a trustworthy source, it was decided to check the threshold data produced by the QUEST procedure independently. Hence, the data points for each participant were also fitted with a Weibull function (Equation 4-2) on a least squares basis using the solver function in Excel to determine threshold independently.

$$F(x,\alpha,\beta) = 1-e^{-(x/\beta)\alpha}$$

Where α = scale parameter, β = shape parameter, x = luminance.

Equation 4-2. Weibull cumulative distribution function.

This results in a psychometric function and the 50% point on this graph should be comparable to the threshold given by the QUEST procedure. The psychometric function produced by this analysis and the corresponding QUEST outcomes were compared to identify any systematic differences in these approaches by conducting a Bland and Altman type analysis (Bland and Altman 1999).

In both the AMD and the control groups, the distribution of scotopic thresholds and the SpO_2 readings when breathing 60% oxygen and 21% oxygen were tested for normality using the Shapiro-Wilkes test. For within group comparisons where the distribution of the data was normal, paired t-tests were used. The Wilcoxon signed rank test was used if the data were not normally distributed. When comparing the data between groups the independent samples t test was used if the data were normally distributed, or the Mann Whitney test was used for data that were not normally distributed.

4.4.3 Results

Twenty-three participants took part in the study, 11 controls and 12 participants with early AMD. 50% of participants with AMD were female, and the group had a mean age of

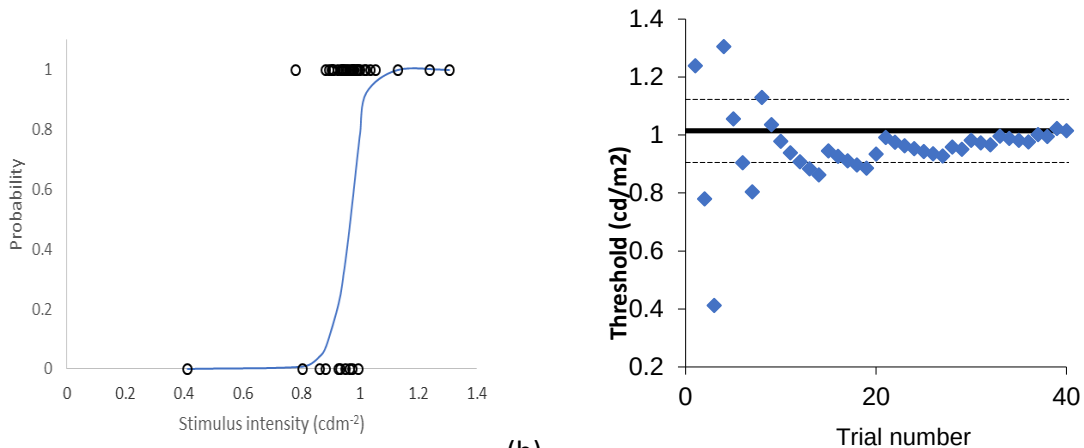
73.9 years +/- 6.2SD. The control group comprised 36% females, and had a mean age of 69.0 years +/- 5.5SD. Table 4-2 shows the characteristics of the early AMD group.

Participant number	Age	Gender	AMD status RE[+]	AMD status LE[+]	Combined status[*]	VA RE	VA LE
001JW	75	M	0	2- nAMD	2	-0.20	-0.08
002MD	77	F	2- nAMD	1- Large drusen	3	-0.20	-0.20
003RC	77	M	2- nAMD	1- large drusen	3	0.70	0.00
004JB	69	F	1- large drusen	2- aAMD	3	0.28	0.50
005AD	71	M	2- nAMD	0	2	0.12	-0.16
006DW	67	F	2- nAMD	1- large drusen	3	-0.14	0.12
008ST	86	F	2- nAMD	0	2	0.54	0.18
009MR	66	M	1- large drusen	2- nAMD	3	-0.60	-0.20
015AP	83	F	1- pigment changes	1- pigment changes	2	-0.20	-0.18
018UH	78	F	1- large drusen	2- nAMD	3	0.0	-0.10
019LP	70	M	2- nAMD	0	2	0.2	-0.14
021RJ	68	M	2- nAMD	1- large drusen	3	1.0	-0.14

Table 4-2. Characteristics of participants with early AMD; [+] the number given to the status of each eye reflects the number of risk points assigned according to the AREDS simplifed scale; * combined status reflects the AREDS simplified scale .

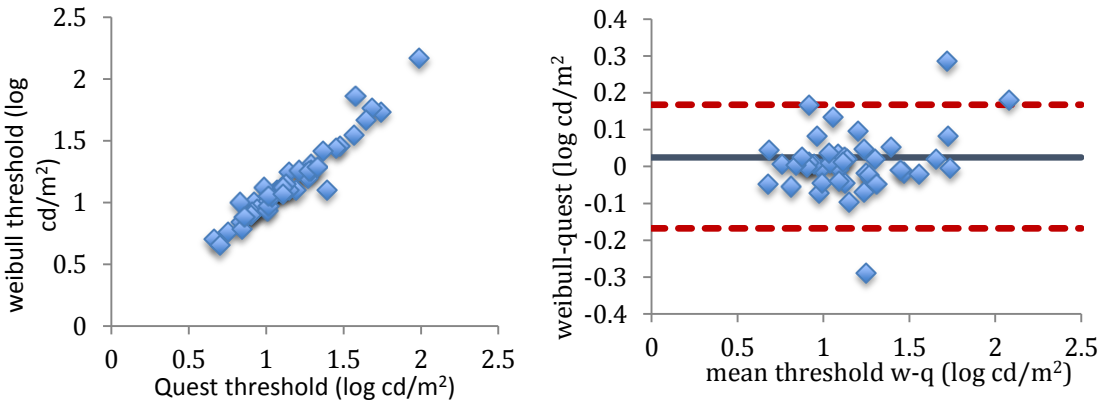
4.4.4 Validation of Quest results

The psychometric function produced by the Weibull analysis and the corresponding QUEST outcome are given below in Figure 4-5 for participant 015AP when breathing 60% oxygen. The points at 1 on the probabiity scale were seen points, and those at 0 were unseen. The scotopic threshold given by QUEST was 1.01 log cd/m^2 and the 50% threshold produced by the psychometric function was 1 log cd/m^2. Figure 4-6a indicates a strong positive correlation between the QUEST and Weibull scotopic thresholds across participants and Figure 4-6b shows that the limits of agreement between threshold techniques were small. Overall, the Weibull analysis verified that the QUEST procedure was reliable in assessing scotopic thresholds.



(a) (b)

Figure 4-5. Scotopic threshold results for participant 015ap by (a) psychometric function and (b) quest.



(a)

Figure 4-6. (a) Thresholds obtained using the QUEST technique plotted against the Weibull function results and (b) Bland and Altman type agreement graph plotting mean threshold (QUEST and Weibull) against the difference between QUEST and Weibull thresholds for each individual. The solid blue line indicates the mean difference between techniques, with the red lines denoting the 95% limits of agreement i.e. mean difference between techniques+/- 1.96* the SD of the differences.

4.4.5 SpO_2 results

Figure 4-7 shows the mean SpO_2 levels per minute for all participants. The lack of overlap in the confidence intervals suggests that there was a statistically significant difference in SpO_2 between the two gas conditions throughout the 5 minutes. Statistical analysis of the final 5 minutes of SpO_2 values confirmed that the difference in mean SpO_2 readings between breathing 60% and 21% oxygen over the 10 minutes was significant in people with early AMD (Wilcoxon signed rank $p< 0.001$) and in the control group (Wilcoxon signed rank $p= 0.00$). However, the absolute difference in mean SpO_2 between the two gas conditions was small, reaching a maximum of ~2% at 7 minutes. As the accuracy of

the pulse oximeter is +/- 2% the findings should be interpreted with caution. It can also be seen that there was more between-subject variability when participants were breathing 21%, as indicated by the larger confidence intervals.

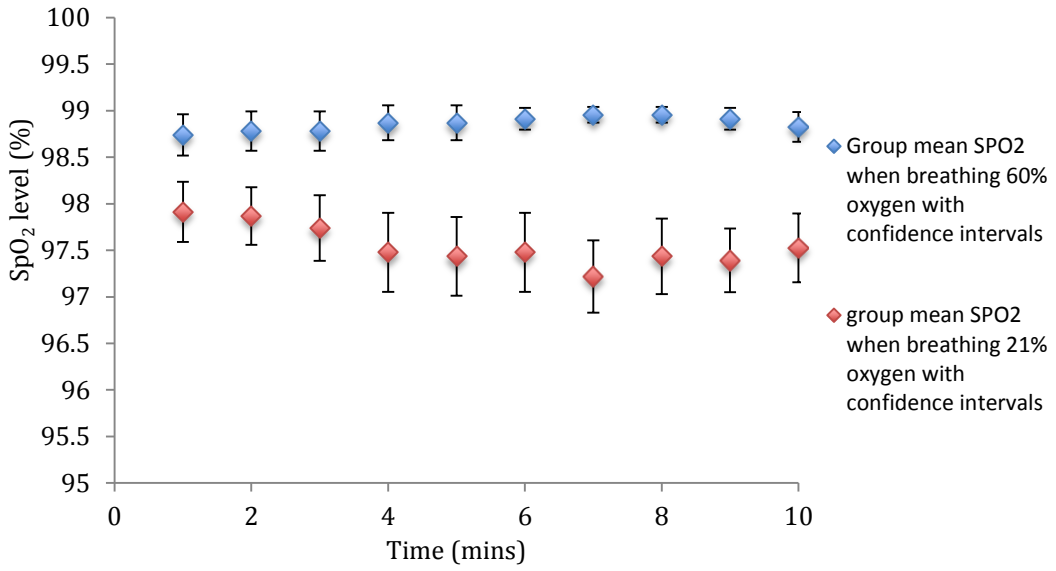


Figure 4-7. Mean SpO_2 per minute with 95% confidence intervals for all participants, breathing 60% and 21% oxygen.

A further observation which can be drawn from Figure 5-7 is that the group mean SpO_2 actually varied very little over the 10 minutes for either gas condition. When breathing 21% oxygen the range of average SpO_2 was between 97.2- 97.9%. When breathing 60%, the range of average SpO_2 was between 98.7-99.0%. There was no significant difference in SpO_2 between the early AMD group and the control group when breathing 60% oxygen (Mann-Whitney p= 0.144). There was, however, a statistically significant difference in SpO_2 between the two groups when breathing 21% oxygen (Mann-Whitney p=0.006).

4.4.6 Scotopic Threshold results

Scotopic threshold data were obtained for all 23 participants, under both gas conditions. As an example, the output of the QUEST procedure is shown in Figure 4-8 for the control participant 017DG alongside the SpO_2 readings per minute over the 10 minutes of gas inhalation.

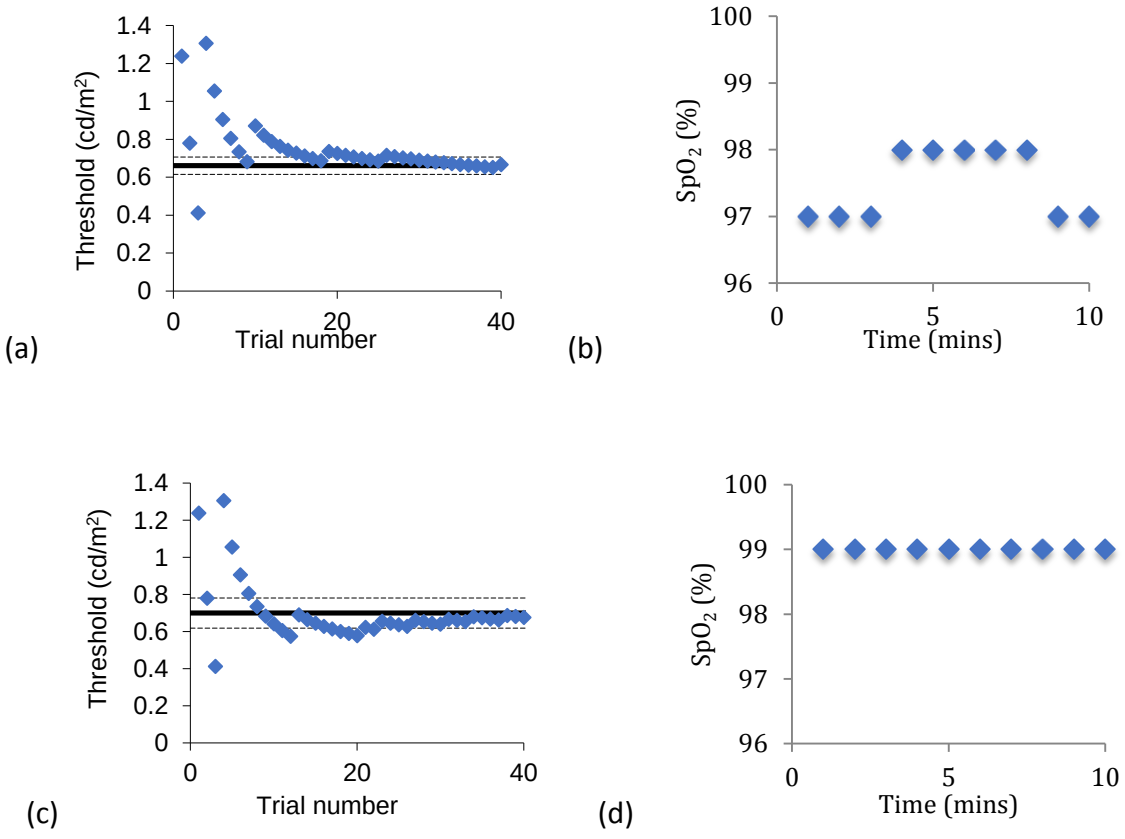


Figure 4-8. Scotopic thresholds with standard deviations and SpO_2 recordings over a full trial when breathing 21% oxygen (top panels) and breathing 60% oxygen (bottom panels) for control participant 017DG.

Figure 4-9 shows the group mean thresholds for each participant group, under each gas condition, with confidence intervals. The overlapping 95% confidence intervals suggest that there was no significant difference in scotopic thresholds when comparing breathing 60% oxygen and 21% oxygen in either group. This was supported by the statistical analysis which demonstrated no significant difference between the scotopic thresholds for the AMD group when breathing 60% and 21% oxygen (-3.89 log cd/m^2 ± 0.30 SD vs. -3.89 log cd/m^2 ± 0.31 SD; paired t-test p= 0.976), or the control group (-4.01 log cd/m^2 ± 0.27 SD vs. -3.95 log cd/m^2 ± 0.38 SD; paired t test p= 0.249).

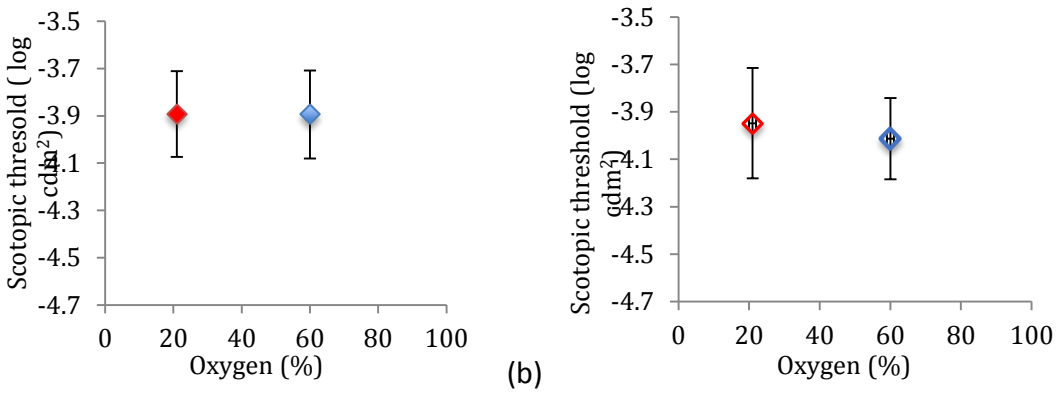


Figure 4-9. Group mean scotopic thresholds with 95% confidence intervals for 21% and 60% oxygen conditions for (a) the AMD group and for (b) the control group.

There were also no differences between the groups when breathing 60% oxygen (independent t-test p= 0.308) or 21% oxygen (independent t-test p=0.388). When the differences in scotopic threshold between gas conditions were calculated (threshold with 21% oxygen subtracted from threshold with 60% oxygen for each individual), there was

also no statistical difference in the results between the AMD and control groups (Mann-Whitney p= 0.413).

Due to a lack of any evidence of a difference between gas conditions in the AMD group findings (-3.89 log cd/m2 ± 0.30 SD vs. -3.89 log cd/m2 ± 0.31 SD), and the evidence of a ceiling effect on the participants' SpO_2 it was decided that data collection for this study would cease, and the following studies would investigate the effect of breathing a hypoxic gas on scotopic thresholds and ERG parameters.

4.4.7 Order effects

To minimise the impact of any order effects, the randomisation of the gas order was balanced such that the same number of individuals received each gas type first. However, there was no statistically significant difference in the scotopic thresholds between the first and second gases (paired t test p= 0.872).

4.5 Discussion

This is the first study to investigate the effect of breathing 60% oxygen on scotopic thresholds in people with AMD. According to these results, there was no significant difference in scotopic thresholds between gas conditions. The results for the control group agree with the literature, where hyperoxia was not found to alter final scotopic thresholds (Connolly et al. 2006; Kent 1966).

There was no statistically significant difference between the scotopic thresholds recorded in the two groups when breathing 21% oxygen. This is in agreement with Jackson et al. (1998), where participants with early AMD (less than 5 small drusen to 1 large drusen and/or hyerpigmentation) showed no statistically signficant difference in scotopic threshold when compared to age-matched controls. However, the results are in contrast to previous work which has found that scotopic threshold is higher in participants with AMD when compared to age-matched controls (Owsley et al. 2000; Owsley et al. 2007; Chen et al. 2004; Steinmetz et al. 1993; Brown et al. 1986; Owsley et al. 2001). There are several explanations that may explain this. Firstly, our group of participants with early AMD included individuals with early macular changes. All participants who presented with soft drusen and/or focal pigmentary changes in the absence of signs of advanced AMD in the test eye were included (simplified AREDS grades 1-4), as were individuals who presented with nAMD in the non test-eye, even if the test eye showed minimal drusen or pigmentary changes (AREDS grade 2-4). This is because there is strong evidence to suggest that the presence of advanced AMD in one eye increases the 5-year risk of developing advanced AMD in the other eye, even if the other eye has no other clinical signs present (Ferris et al. 2005). This suggests that in these people the macula of the apparently healthy eye is likely to have macular changes, even if these are not clinically visible. As one aim of this study was to see if functional biomarkers of hypoxia could be used to diagnose and monitor patients at risk of developing AMD before clinical signs are present, these participants were included. The inclusion of early stage disease results in the profile of clinical signs in the AMD group in this study differing to the groups found in

the other studies. This can be seen from the comparison table of studies investigating the effect of early AMD on scotopic threshold (see Table 4-3). Notably, participants with late stage AMD were included by Brown et al. (1986), Owsley (2000) and Owsley et al. (2007) which may have influenced their results. Further analysis of the data of Owsley et al. (2007) comparing only the participant group with early AMD to the control group (by calculating the 95% confidence interval), demonstrated no statistically significant difference in scotopic thresholds between the two groups.

Study	Early AMD participant details	Period of dark adaptation used	Results
Steinmetz et al. 1993	N=12 all with discrete and/or confluent drusen.	45 minutes	Dark adapted foveal threshold ranged from 1-24dB above normal values.
Owsley et al. 2007	N=83 all with AMD classified by stages 1-12 of the AREDS (2005) scale. 12 had CNV and 5 with GA.	60 minutes after bleach	Significant ($p<0.0001$) impairment in rod sensitivity
Owsley et al. 2001	N=20 with < 5 small drusen or ≥ 1 large drusen, focal hyperpigmentation or both.	30 minutes	Reduced scotopic sensitivity in AMD group by 0.4 log units when compared to normals
Owsley et al. 2000	N=71 with < 5 small drusen or ≥ 1 large drusen, focal hyperpigmentation or both. 9 late AMD	≥ 40 minutes	Mean field dark-adapted sensitivity was statistically lower by 6.7dB in AMD than in normal subjects ($p=0.006$).
Chen et al. 2004	N=24 drusen and hyper/hypo pigmentation.	40 minutes	Mean scotopic sensitivity of participants with early AMD was 2.73dB lower than in the control group. This was statistically significant ($p < 0.05$)
Brown et al. 1986	N=4. N=3 non- exudative AMD or drusen and n=1 with confluent drusen and haemorrhages.	20 minutes	Participants with early AMD showed decreases in cone and rod sensitivity (0.5-1.5 log units)

Table 4-3. Comparison table of studies into the effect of early AMD on scotopic thresholds.

There were also differences in the study designs, with all of the studies using a shorter duration of dark adaptation than the 60 minutes used in this study. As discussed in section 1.5.3, AMD results in a prolongation of dark adaptation (Owsley et al. 2001; Steinmetz et al. 1993; Haimovici et al. 2002; Dimitrov et al. 2008; Dimitrov et al. 2011; Eisner et al. 1992; Eisner et al. 1991). The order effect demonstrated in the earlier pilot data suggested that the 50 minutes of dark adaptation originally used for the recording of scotopic thresholds was not long enough for participants with early AMD to reach a final scotopic threshold. If the period of dark adaptation used by previous studies was similarly too short to allow absolute scotopic threshold to be reached in people with AMD, then it is possible that an artificial elevation of the scotopic thresholds would have been present in this participant group. Some studies even applied a photopigment bleach before the period of dark adaptation (Steinmetz et al. 1993, Owsley et al. 2007), after which it can take around 60 minutes for absolute threshold to be reached (Lamb et al. 2004; Thomas and Lamb 1999). This would lead to a greater difference in final thresholds between the two groups. This may be a further explanation for the previously reported elevation of scotopic thresholds in people with early AMD. As no order effect was found in the main study reported in this chapter after the hour of dark adaptation, one may conclude that this duration of dark adaptation gave both participant groups time to reach their final scotopic threshold.

One potential limitation of the study design was the use of a fixed termination rather than a dynamic termination in the QUEST procedure. The dynamic criterion continues the

procedure until the threshold can be estimated with a particular degree of confidence, however this can result in some participants not finishing the trials (Madigan and Williams 1987). Fixed termination after 40 presentations was used due to time constraints as each data set required completion between minutes 8 and 10 due to the dual requirements of gas adaptation and safety guidelines. Studies by Anderson (2003) and Madigan and Williams (1987) comparing dynamic and fixed stopping criterion in Bayesian procedures concluded that there was little evidence to recommend the use of the dynamic over a fixed criterion.

The finding that thresholds were unaffected in people with early AMD by a transient period of systemic hyperoxia may be explained by the high levels of peripheral oxygen saturation at baseline. The results indicated that participants in neither group were systemically hypoxic i.e. both groups showed a SpO_2 close to 100% at baseline. Therefore SpO_2 was close to saturation in both groups, and an increase in SpO_2 by breathing 60% oxygen was limited by the ceiling effect to only 1-2%. Therefore, the results from this study do not disprove the hypothesis that there is local retinal hypoxia in the participants with AMD i.e. caused by an impaired delivery of systemically circulating oxygen to the outer retina, but rather demonstrates that these participants do not suffer from a systemic reduction in blood oxygen saturation that can be reversed by breathing 60% oxygen. Furthermore, given the limited sample size, it is possible that the null findings may be attributable to a Type II error.

To overcome the limitations of this study, in further studies 14% oxygen was inhaled to create mild acute systemic hypoxia. Scotopic thresholds and dark adapted ERGs were recorded when inhaling 14% oxygen and medial air to test the hypothesis that, when breathing 14% oxygen, scotopic thresholds would increase in both groups but that the increase would be greater in the group with early AMD due to the compounding effect of the systemic hypoxic episode on a locally hypoxic retina.

5 The effect of breathing 14% oxygen on scotopic thresholds in people with early AMD

5.1 Introduction

The aim of chapter 5 was to study the effect of breathing 60% oxygen on scotopic thresholds of participants with early AMD. The hypothesis stated that if hypoxia was the cause of the rise in scotopic thresholds seen in early AMD, then the inhalation of 60% oxygen could temporarily decrease the scotopic thresholds in these participants. However, no statistically significant differences in scotopic thresholds were recorded between the normoxic and hyperoxic conditions. As discussed in section 4.5, it was hypothesised that this lack of effect was due to the lack of systemic hypoxia in these participants. The SpO_2 was close to saturation under control conditions and thus it was not possible to further increase the SpO_2 due to a ceiling effect. Therefore, this chapter presents the results of a modified study to investigate the effect of mild systemic hypoxia on the scotopic thresholds of participants with early AMD.

It was aimed to test the hypothesis that all participants (control and AMD) would show a decrease in scotopic sensitivity when experiencing a mild hypoxic episode (as discussed in sections 2.2.6 and 2.3.5). However, it was also hypothesised that, due to the local retinal

hypoxia that may be present in participants with early AMD, these changes would be greater in participants with early AMD.

5.2 General methods

5.2.1 Participants

Sixteen participants with early AMD, and 20 controls were recruited to take part in this study. The recruitment procedure, inclusion and exclusion criteria and baseline measurements followed the same methods as set out in section 4.2. As in the main study of chapter 4, procedures involving bright lights (e.g. VOLK fundal examination and fundus photography) were carried out after the scotopic threshold experiment to minimise photopigment bleaching effects.

5.2.2 Equipment

The apparatus and psychophysical methods for scotopic threshold measurement were as described in sections 3.1.3 and 4.4.1.

5.2.3 Methods

The methods for scotopic threshold data collection were as described in section 4.4.1 except the 60% oxygen condition was substituted with a hypoxic 14% oxygen mixture. To summarise, following 60 minutes of dark adaptation and 2-3 practice QUEST sessions (20 trials each), participants inhaled either medical air or 14% oxygen. Data collection then followed the timeline summarised in in Figure 5-1.

Additional 20 trials to take place hereif necessary

dark adaption 60 mins	20 trials practice 5 mins	20 trials practice 5 mins	Start gas 1 5 mins	Record thresholds 5 mins	Wash out 5 mins	Start gas 2 5 mins	Record thresholds 5 mins

Figure 5-1. Timeline showing the protocol of the study investigating the effect of inhaling 14% oxygen on scotopic thresholds. Gases 1 and 2 were randomly assigned as either medical air (21% oxygen) or a hypoxic gas (14% oxygen).

5.2.4 Data analysis

In both the AMD and the control groups, the distribution of scotopic thresholds and the SpO_2 readings when breathing 14% oxygen and 21% oxygen were tested for normality using the Shapiro-Wilkes test. For within group comparisons where the distribution of the data was normal, paired t-tests were used. The Wilcoxon signed rank test was used if the data were not normally distributed. When comparing the data between groups the independent samples t-test was used if the data were normally distributed, or the Mann Whitney test was used for data that were not normally distributed.

5.3 Results

Thirty-six participants took part in the study, 20 controls and 16 participants with early AMD. The AMD group contained 6 females and the control group 8 females. There was no significant age difference between the AMD group (mean age 72.9years +/- 4.9SD) and

the control group (mean age 68.3 years +/- 5.4SD). Data were not collected from one participant due to the participant's SpO_2 dropping below 85%. The participant felt fine and their SpO_2 returned to a normal level once the 14% oxygen was turned off. Therefore, data were collected from 35 participants. Table 5-1 shows the characteristics of all participants who sat for the studies in Chapters 5 and 6.

Participant number	Age	Gender	AMD status test eye[+]	AMD status fellow eye[+]	Combined status[*]	VA test eye (LogMAR)	VA fellow eye (LogMAR)	Group
001	77	M	1	2	3	0.00	0.02	AMD
002	78	M	2	2	4	0.20	0.82	AMD
003	61	M	0	0	0	0.00	0.00	CONTROL
004	74	M	1	2	3	0.08	0.52	AMD
005	76	M	1	2	3	-0.06	0.00	AMD
006	70	F	0	0	0	0.00	-0.02	CONTROL
007	69	F	1	2	3	0.02	0.48	AMD
008	82	M	1	2	3	0.24	0.30	AMD
009	65	M	0	0	0	0.02	0.06	CONTROL
010	68	M	0	0	0	0.06	0.10	CONTROL
011	69	M	1	2	3	0.02	1.40	AMD
012	73	M	0	0	0	-0.08	0.02	CONTROL
013	66	F	0	0	0	-0.16	0.24	CONTROL
014	71	M	0	2	2	0.00	0.20	AMD
015	78	F	1	2	3	0.00	0.00	AMD
016	77	F	0	0	0	0.10	0.10	CONTROL
017	72	F	1	1	2	0.00	0.06	AMD
018	72	M	1	0	1	0.08	-0.10	CONTROL
019	82	M	0	0	0	0.10	0.06	CONTROL
020	68	F	0	0	0	0.06	0.30	CONTROL
021	67	M	1	2	3	0.02	0.00	AMD
022	66	F	0	0	0	0.1	0.2	CONTROL
023	66	F	0	0	0	-0.16	-0.10	CONTROL
024	69	F	1	2	3	0.20	1.00	AMD
025	67	M	0	0	0	-0.10	0.00	CONTROL
026	74	M	1	0	1	0.00	-0.08	AMD
027	66	M	0	0	0	-0.20	0.00	CONTROL

028	62	M	0	0	0	-0.08	0.10	CONTROL
029	70	M	0	0	0	-0.18	0.00	CONTROL
030	61	F	0	0	0	-0.16	0.02	CONTROL
031	70	F	0	0	0	0.10	0.00	CONTROL
032	68	F	1	2	3	-0.01	0.10	AMD
033	61	M	0	0	0	0.08	0.08	CONTROL
034	79	F	1	1	2	0.10	0.14	AMD
035	64	M	0	2	2	-0.10	0.02	AMD

Table 5-1. Characteristics of all participants; [+] the number given to the status of each eye reflects the number of risk points assigned according to the AREDS simplifed scale; * combined status reflects the AREDS simplified scale score.

5.3.1 SpO_2 Results

The SpO_2 for both groups fell when breathing 14% oxygen. This reached a plateau for the control group after 7 minutes and the AMD group after 8 minutes. There was a significant difference in SpO_2 between the groups for the final 5 minutes when breathing 21% oxygen (Mann-Whitney p=0.01) and when breathing 14% oxygen (Mann-Whitney p=<0.0001). In both cases the control group had higher SpO_2 values compared to the early AMD group (97.1 vs 96.8% 21% O_2; 92.7% vs 91.4% 14% O_2).

All individuals showed a reduction in SpO_2 when breathing the hypoxic gas compared to medical air. Figure 5-2(a) shows the mean SpO_2 over 5 minutes for the AMD group when breathing 14% and 21% oxygen, the decrease in SpO_2 was statistically significant (Wilcoxon signed rank p=<0.0001). Figure 5-2(b) shows the mean SpO_2 over 10 minutes when breathing 14% and 21% oxygen for the control group. In this group breathing 14% oxygen also resulted in a significant drop in SpO_2 (Wilcoxon signed rank p<0.0001).

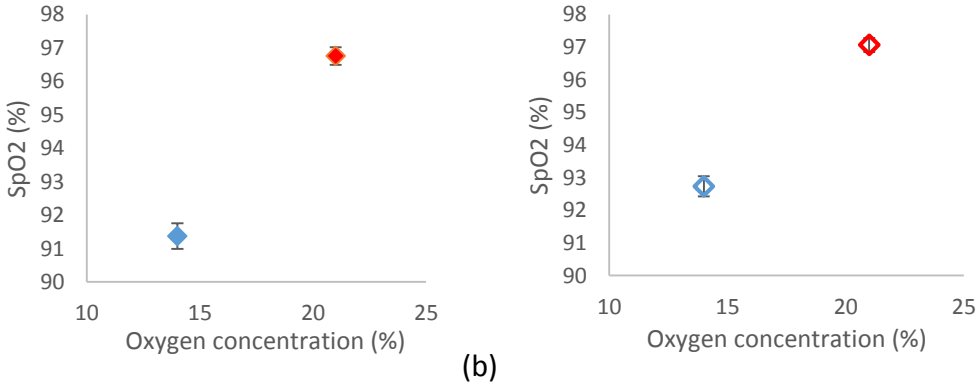


Figure 5-2. Mean group SpO_2 readings with confidence intervals for the final 5 minutes of breathing gas for (a) AMD group and (b) control group.

5.3.2 Scotopic threshold results

Scotopic threshold data were analysed for 33 out of 35 participants for both visits, as one participant (015) did not proceed to the main trial after failing the requirement for the scotopic threshold from each of the practice trials to be within one standard deviation of each other. The data for one control participant (022) had to be removed from analysis due an abnormal OCT indicating a pre-existing ocular condition.

An example of the psychophysical data is given in Figure 5-3, which shows a sample output of the QUEST procedure alongside the SpO_2 readings per minute for both gas types for participant 011. It can be seen that, for this individual, SpO_2 levels dropped by more than 5% when the hypoxic gas was provided. The scotopic threshold for this person with AMD was very similar under the two gas conditions.

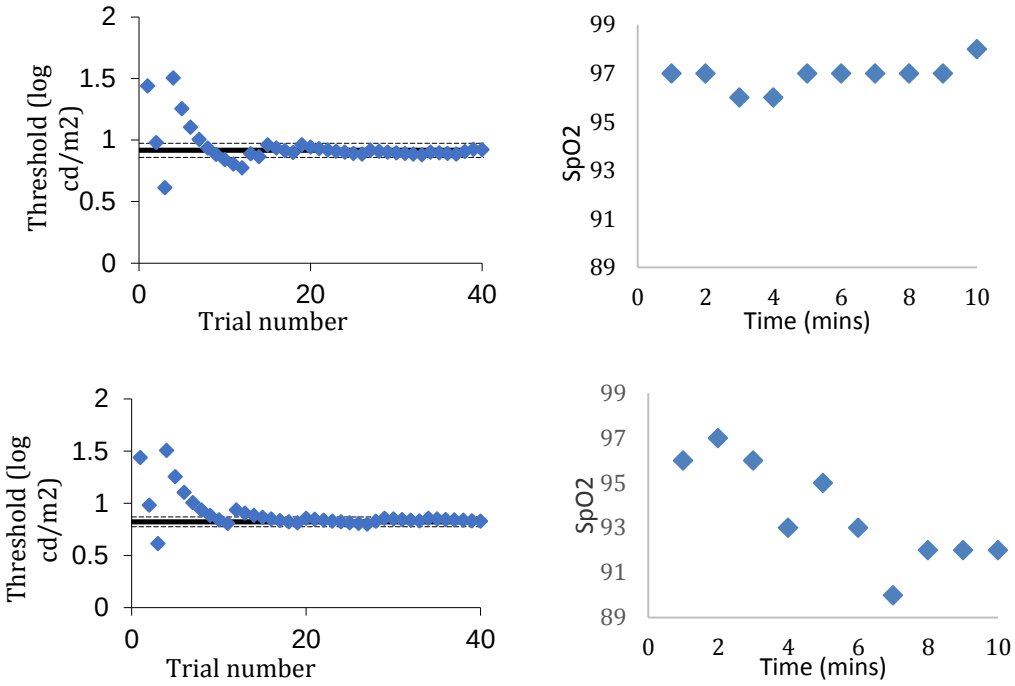


Figure 5-3. Scotopic thresholds with standard deviations and SpO_2 recordings over a full trial when breathing 21% oxygen (top panels) and breathing 14% oxygen (bottom panels) for early AMD participant 011.

The differences in threshold for each individual when breathing 14% were compared with breathing 21% oxygen and are shown in Figure 5-4. Points above the line represent an increase in threshold (decrease in sensitivity) when breathing 14% oxygen, and points below the line represent a decrease in threshold (increase in sensitivity) when breathing 14% oxygen. The difference for all but one participant within the control group was less than 0.15 log cd/m^2, whilst only 3 participants with AMD showed a change in threshold greater than this with the change in gas type. 44% (n=8) controls showed an increase in threshold when breathing 14% oxygen, whilst 60% (n=9) participants with AMD showed an increase in threshold when breathing 14% oxygen.

These plots indicate that there is no evidence of an effect of breathing the hypoxic gas on scotopic thresholds.

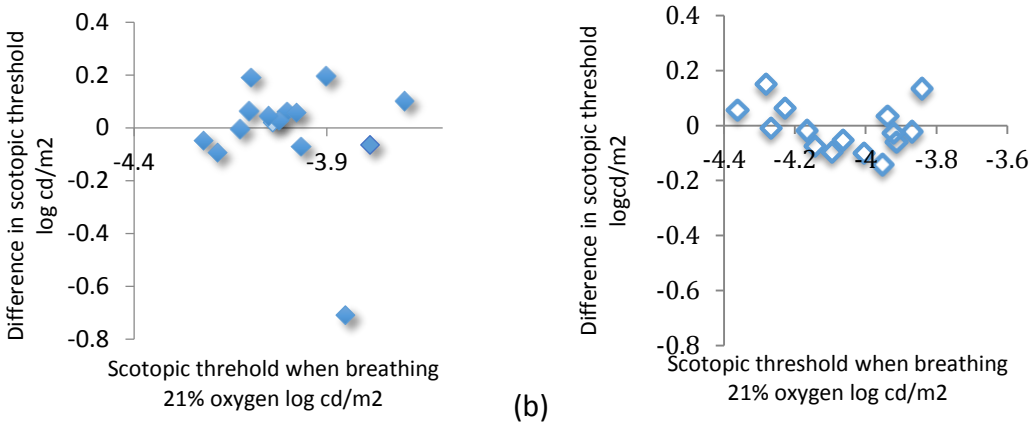


Figure 5-4. Change in threshold when breathing 14% oxygen compared to breathing 21% oxygen for (a) the AMD group and (b) the control group.

There was no significant difference in mean scotopic thresholds when breathing 14% oxygen compared to when breathing 21% oxygen within the early AMD (paired t-test p=0.781) or the control group (paired t-test p= 0.950); this can be seen in Figure 5-5, where the confidence intervals are clearly overlapping. There was also no significant difference in the change in thresholds between groups when values were compared under 14% and 21% oxygen conditions (Mann-Whitney AMD differences mean = -0.015, control differences mean = -0.001; p= 0.509).

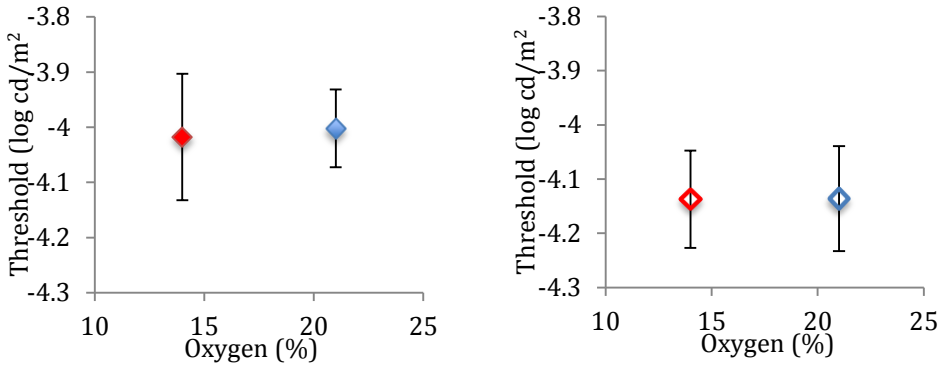


Figure 5-5. Group mean scotopic thresholds with 95% confidence intervals for 14% and 21% oxygen conditions for the AMD group (left panel) and for the control group (right panel).

There was, however, a statistically significant difference in mean scotopic threshold between the two groups when breathing 21% oxygen (independent t-test p=0.049) but not when breathing 14% oxygen (independent t test p= 0.130), with the AMD group showing a significantly higher threshold than the control group under normoxic conditions.

5.3.3 Order effects

Randomisation of the gas order was such that 16 participants received 14% oxygen first and 17 received 21% oxygen first. There was no effect of the order of presented gas (pair samples t-test p=0.196).

5.4 Discussion

This is the first study to investigate the effect of breathing 14% oxygen on scotopic thresholds in people with early AMD. The first hypothesis was that all participants (AMD and control) would show an increase in scotopic thresholds in hypoxic conditions. The results show that there was no statistically significant increase in

scotopic thresholds in either group when breathing 14% oxygen when compared to 21% oxygen. This finding is in contrast to previous studies which have suggested that hypoxia raises scotopic thresholds in normal participants (McFarland et al. 1940; McDonald et al. 1939; Wald and Harper 1942b; Kobrick, L et al. 1984; Ernest et al. 1971; Hecht et al. 1946). Table 5-2 details other studies that have examined the effect of hypoxia on the scotopic thresholds of healthy controls. It can be seen that in all studies the oxygen concentration supplied to create a hypoxic episode was lower than that used in this study, and the duration of breathing the air was longer. Whilst preliminary studies presented in this thesis have found that SpO_2 stabilises after 8-11mins of breathing air with an altered oxygen concentration (see section 3.2), there could be a compounding effect of an increased duration of inhalation and a lower level of oxygen inhaled in previous studies. It is also worth noting that all the previous studies had small participant numbers.

There was a statistically significant difference in scotopic thresholds between the groups when breathing 21% oxygen ($p=0.049$) suggesting that under normoxic conditions scotopic thresholds are reduced in people with early AMD. This finding is in agreement with the literature (Owsley et al. 2000; Owsley et al. 2007; Scholl et al. 2004) but not in agreement with Jackson et al. (1998) or the results from the investigation in Section 5, into the effect of breathing 60% oxygen and scotopic thresholds. The reason for this disparity is unclear as there were no significant outliers for any group which affected the statistical significance and there were no fundamental differences in the methods or equipment used between chapters 5 and 6. Whilst 9 control participants and 6 participants with early AMD sat for both studies,

there was a larger sample size for this study, compared to the study described in Chapter 5. It is also worth noting that the difference in the recorded mean threshold when breathing 21% for both the AMD and control group was not significant between studies, demonstrated by overlapping confidence intervals.

Study	Participant details	Period of dark adaptation used	Period of hypoxia	Percentage oxygen used	Results	Other details
McFarland and Forbes (1932)	N=6	32 mins	30 mins	13.3/11.4/ 10/7.3	Raised ST with reducing oxygen	No stats analysis Pressure constant Full DA curve
McDonald and Adler (1939)	N=1	60 mins	20 mins	10.4	ST rise of 0.6 log units	No stats analysis Normal pressure
Wald and Harper (1942)	N=4	uknown	30 mins	8-11	ST rise of 0.35 log units	Rise and fall in thresholds noted- related to breathing patterns
Kobrick (1984)	N=12	None- testing continued for 20 mins after bleach	16 days		Sig impairment of ST to green stimulus	Participants studied at altitude Full DA curve
Ernest and Krill (1971)	N=3	30mins	15mins	10	Increase in ST more in peripheral retina and had a greater effect on cones when compared to rods	Stats conducted between rod and cones and not rods and control.
Hecht and Hendley (1946)	N=8	Unknown	Unknown	9-16	Increase in threshold of 0.11 log microlamberts when breathing 14.9% oxygen	Studied contrast discrimination

Table 5-2. Comparison table of studies into the effect of a hypoxic episode on scotopic thresholds in normal participants.

The second hypothesis of this study was that the scotopic threshold would be selectively reduced in participants with early AMD in hypoxic conditions, due to the local retinal and choroidal changes that are present in early AMD. The results did not support this hypothesis i.e. there was no significant difference in the change in threshold between the two groups. This suggests that the retinas of people with AMD are, like age-matched controls, insensitive to hypoxia.

The absence of a significant finding could be explained by the lack of a stable reduction in oxygen at a retinal level for a duration long enough to alter the scotopic thresholds of patients with early AMD. It could be that short periods of hypoxia are insufficient to induce localized retinal hypoxia to an extent which will affect retinal function. It is also noteworthy that a greater proportion of individuals with AMD showed an increase in threshold under the hypoxic condition than the proportion in the control group showing a similar change. It could be that certain individuals with AMD are more susceptible to localised changes to retinal oxygenation.

A limitation of this study was that the effects of breathing reduced levels of oxygen were measured by changes in SpO_2, which is a systemic measure. It may be that it takes longer to create localised retinal hypoxia than it does to induce systemic changes. A further limitation of this study was that, for pragmatic reasons, it was not possible to achieve the sample size of n=30 per group which was the aim, based on the sample size calculations (see section 4.2.2). This introduces the possibility of a type II error in the negative findings of this study. However, the findings of this study may also suggest that hypoxia is less implicated in visual dysfunction in early AMD than hypothesized. This will be further explored by evaluating a different aspect of

visual function. The ERG has been shown to be affected by hypoxia (as discussed in chapter 2) and by AMD (as discussed in chapter 2). The next chapter of this thesis will investigate the effect of breathing 14% oxygen on global and focal ERG parameters. It is hypothesised that, whilst the ERGs for both the early AMD and the control groups will be affected by hypoxia, due to the altered choroidal and retinal structure in early AMD the effect will be greater in the early AMD group.

6 The effect of breathing 14% oxygen on the electroretinogram of participants with AMD.

6.1 Background and aims

As discussed in section 1.5.4, electroretinography is an objective technique, which is able to detect and localise subtle changes in retinal function. Section 1.5.4.6 concluded that both the amplitude and implicit times of focal ERGs are affected by early and intermediate AMD (Sandberg et al. 1993; Sandberg et al. 1998; Binns et al. 2007; Falsini et al. 1999). Significantly reduced full field a and b-wave amplitudes have also been found in participants with nAMD and GA (Walter et al. 1999). Implicit time delays of 1ms to the ERG have also been found in the fellow eye of participants with unilateral nAMD, which were associated with areas of choroidal perfusion defects (Remulla et al. 1995). This finding of a choroidal perfusion defect in the high-risk fellow eyes of participants with unilateral nAMD suggests that an altered retinal circulation is present before clinical signs of early AMD, and may predict the onset of nAMD. Furthermore, the study suggests that this circulatory abnormality can be detected with ERG recording. The ERG has also been found to be affected in healthy participants placed under a hypoxic episode, including significantly reduced OP and b-wave amplitudes (Schmeisser et al. 1997; Tinjust et al. 2002; Janáky et al. 2007). As changes to the ERG are also seen during hypoxia as discussed in section 2.2.5, including reductions in both the a and b-wave amplitude (Schatz et al. 2014), it could

be that hypoxia is the cause of the changes in the ERG seen in AMD. This evidence not only supports the potential role of hypoxia in AMD, but also suggests that the ERG may be a sensitive tool for the investigation of ischaemia and hypoxia in AMD.

This study aimed to investigate the effect of hypoxia on the focal flicker ERG and full-field scotopic ERG in participants with early AMD. Both ERGs were recorded under scotopic conditions to maximise the dark current and retinal oxygen demands. The focal flicker response was employed to probe dark-adapted macular cone function under different states of oxygenation, whilst the full-field rod response assessed pan-retinal rod photoreceptor function. The rationale for the inclusion of the focal ERG was that the macula is the primary locus of AMD related pathogenesis, and so is most likely to show signs of retinal hypoxia at an early stage. A cone response was chosen for the focal test as rods are highly directionally sensitive, and it is not possible to record a focal rod response within a short timeframe without the use of a desensitising surround (Binns and Margrain 2006). This would have resulted in a state of light adaptation which would minimise the oxygen demand of the tested retinal area. The full-field rod response was also included on the basis that rod photoreceptor loss precedes cone loss in early AMD (Jackson et al. 2002). It was also of interest to include a full-field response to determine whether there were any indicators of a global state of retinal hypoxia in participants with AMD. Full field testing also has the advantage that the measured response is larger than that seen in focal responses, which may decrease the effect of noise in a group of older participants with pathology.

It was hypothesised that breathing 14% oxygen would result in a reduction in amplitude and delay in the implicit time of the first harmonic of the focal flicker ERG both in participants with early AMD and in the control group. A reduction in amplitude of the scotopic full field ERG a-wave and a reduction in amplitude and increase in implicit time of the b-wave in participants with early AMD and in the control group was also proposed. It was further hypothesised that the effects of the transient systemic hypoxia on both ERGs would be greater in the group with early AMD, due to the local retinal hypoxia which is hypothesised to be associated with the disease onset.

6.2 General ERG recording methods

A silver-silver chloride electrode, filled with an electrolyte gel (TECA) was used as a ground electrode. This was positioned in the mid-frontal forehead position, after skin was prepared using an abrasive gel (Nuprep, Weaver and Company), and affixed with surgical tape (Blenderm). DTL fibre electrodes (Unimed electrode supplies) were positioned in the lower fornix of the test eye as an active electrode and the contralateral eye as a reference. Surgical tape was applied first to any area where the DTL electrode may have contact with the skin. The electrode set up can be seen in Figure 6-1.

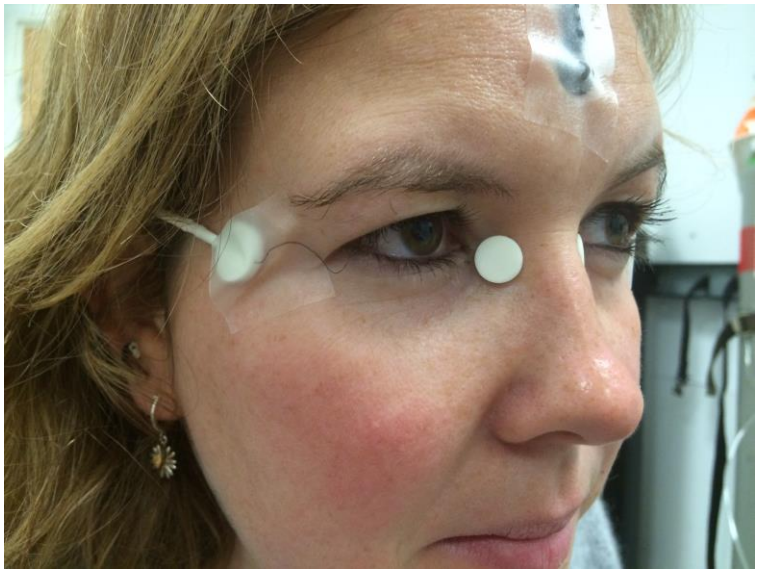


Figure 6-1. Participant set up for ERG recording.

All ERGs were recorded under dark adapted conditions. In the pilot study, a period of 35 minutes of dark adaptation was employed for the healthy controls, and 50 minutes for the participants with early AMD (reflecting the anticipated longer time required to reach absolute threshold in those with the condition (Owsley et al. 2007)). For the main study all participants underwent a total of 60 minutes dark adaptation to ensure that all individuals were at or close to absolute threshold. Following dark adaptation, the contralateral eye was occluded and ERGs recorded on the Medelec Synergy Evoked Potential Monitoring System (Oxford Instruments PLC, Old Woking, Surrey, UK) using a miniature Ganzfeld light emitting diode (LED) stimulator (CH electronics, Kent, UK). All ERGs were averaged and bandpass filtered from 1-100Hz. Artefact reject settings were applied to all traces to avoid any trace contamination by blinking or eye movements, for focal ERGs these were set to 50µV and for full field testing this was set to 500µV.

For the recording of focal ERGs a focal (20° diameter) red stimulus (peak wavelength 666nm) was used, with an average luminance of 40 cd/m^2, flickering with a square wave profile at a temporal frequency of 41Hz on a 50ms time base. A red stimulus was chosen to minimise light adaptation. For the recording of full field ERGs, traces were recorded to a blue 0.5Hz full field stimulus (0.00125cd.s.m^{-2}) stimulus averaged on a 200ms time base.

The miniature Ganzfeld (LED) stimulator consists of a 5cm diameter tube which houses a collection of LEDs that can be used as a full field stimulus (when held close to the eye) or as a focal stimulus when viewed at a distance (in this study, held at 13.5 cm to stimulate a region subtending 20 degrees at the retina). A peripheral surround may be used to desensitise the non-stimulated retina, in order to minimise the effect of scattered light on the focal response (Binns et al. 2007). In this study, however, we wished to maximise the state of dark adaptation and the peripheral surround was not employed. The equipment can be seen in Figure 6-2.

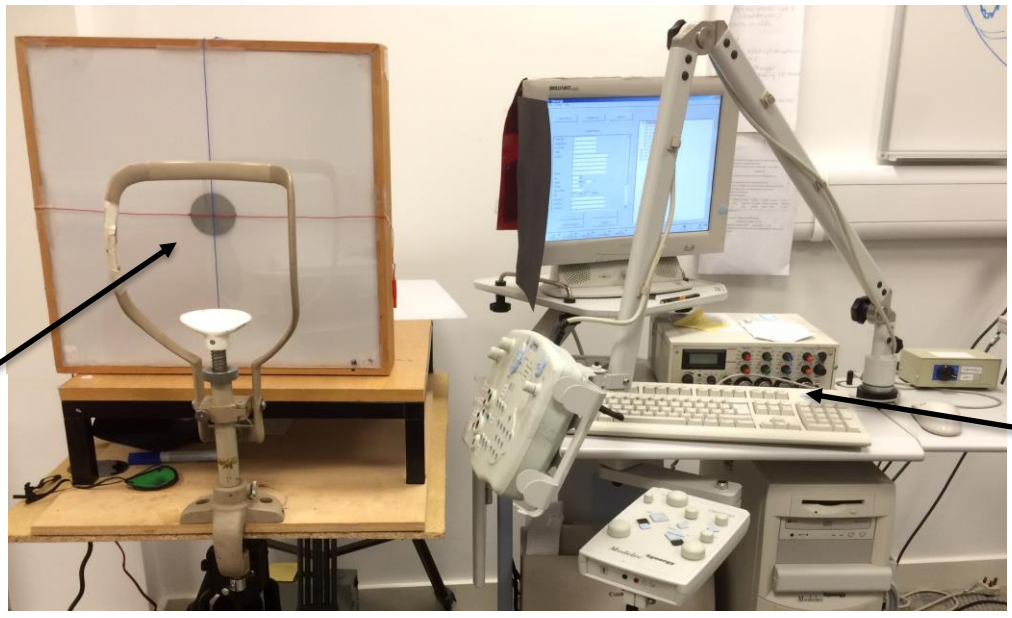


Figure 6-2. Equipment used for ERG recording.

6.3 ERG signal analysis

Data collected for all participants were exported to Excel Microsoft 2010 for Fourier analysis. For the focal ERG data the amplitude and implicit time of the first harmonic were analysed. In order to reduce high frequency noise, harmonics higher than the first twelve (i.e. above 60Hz) were removed from the full field data (preliminary analysis indicated that the signal contribution to harmonics above this frequency was negligible). The parameters chosen for full field analysis were the amplitude of the a-wave (measured from from baseline), and the b-wave (measured from the a-wave trough to the peak of the b-wave), and the implicit time of the b-wave. The a-wave and the b-wave were objectively identified within fixed time windows (the minimum amplitude between 0-6ms for the a-wave and the maximum amplitude between 180-200ms for the b-wave).

6.4 Pilot study optimising stimulus parameters for the ERG protocol

A pilot study was carried out to determine the optimum stimulus parameters to be used in the main study. As hypoxia in AMD is hypothesised to be greatest under dark adapted conditions, the optimal protocol for this study would employ the lowest possible stimulus luminance. The pilot study therefore had 3 aims:

- To establish the dimmest focal stimulus that could elicit recordable traces.
- To determine if any light adaptation occurred over a two minute period when a red 41Hz square wave flickering focal stimulus at the maximum luminance was used (average luminance 40 cd/m^2).

- To determine if any light adaptation occurred over a one minute period when a blue 0.5 Hz full field stimulus (0.01cd.s.m^2) was used to stimulate rod photoreceptors. 0.01cd.s.m^2 was chosen as it corresponds to the ISCEV standard for the dark adapted rod 0.01 ERG recording (McCulloch et al. 2014).

To establish the optimum stimulus parameters, three studies were designed to address these aims, which are described below.

6.4.1 Methods

Participants for this pilot study were recruited from the School of Optometry and Vision Sciences, Cardiff University and from the Medical Retina clinics at the University Hospital Wales. Distance corrected visual acuity (ETDRS LogMAR chart) and intraocular pressures were measured (Topcon CT 80 tonometer) and participants were excluded if they had a VA less than 6/7.5 (0.1 logMAR) or an IOP >21mmHg (average of three readings). Retinal photographs were taken to assign participants to one of three groups, younger normals (ages 26-37 years), older normals (ages 59-72 years) or participants with early AMD. One percent tropicamide was then installed into the eye used for recording, this was the right eye or, in the case of the participants with AMD, it was the eye diagnosed with early AMD. All procedures adhered to the tenets of the declaration of Helsinki. The study was approved by South East Wales Research Ethics Committee.

The ERGs were recorded according to the method described in section 6.2.

6.4.1.1 Stimulus intensity study

This study aimed to determine the lowest stimulus luminance that would elicit recordable traces in both normal participants and participants with early AMD. Neutral density filters (0.3, 0.6 and 0.9 log units) were used to attenuate the focal stimulus luminance to 20 cd/m^2, 10 cd/m^2 and 5 cd/m^2, respectively. 200 sweeps were averaged at each luminance level, starting with the lowest intensity stimulus. Responses were Fourier analysed to obtain the amplitude of the first harmonic.

6.4.1.2 Focal cone ERG stimulus adaptation study

The aim of this study was to see if any light adaptation occurred when a focal (20°) red stimulus at the maximum time-averaged luminance of 40 cd/m^2, flickering with a temporal frequency of 41Hz, was used for recording. Traces were averaged in sets of 25 sweeps, on a 50ms time base, continuously for a period of 2 minutes, with every third trace Fourier analysed to obtain the amplitude of the first harmonic. On average, this resulted in 33 traces (range 28-36) being obtained for each participant. A comparison was made between the amplitude of the first two and the final two responses analysed, on the basis that a significant difference in amplitude of the first harmonic would indicate that light adaptation had occurred during the recording period. Statistical significance was tested using a paired t test or Wilcoxon signed rank test if the data were not normally distributed.

6.4.1.3 Full-field rod ERG stimulus adaptation study

To record a full field response, the participant held the miniature Ganzfeld (LED) stimulator as close to the eye as possible. Three single responses were averaged on a 200ms time base to a full field blue 0.5 Hz stimulus (0.01cd.s.m^2). The stimulus was then viewed continuously for 1 minute, after which a second set of 3 responses was

averaged. For some participants additional data were collected with neutral density filters attenuating the light to luminances of 0.0025 cd.s.m^2 (6 participants) and 0.000625 cd.s.m^2 (4 participants). All traces were then Fourier analysed and the amplitude of the b-wave evaluated. To investigate if there was a statistical significance between the amplitude of the b-wave after 1 minute a paired t-test (or Wilcoxon signed rank test if the data was not normally distributed) was run.

6.4.2 Results

Participants were chosen to represent a diverse range of ages and included healthy controls and individuals with early AMD. Eleven participants took part, and their details can be seen in Table 6-1. Datasets were recorded for all participants for the stimulus intensity study and the focal cone ERG stimulus adaptation study, except for participant AB. Data sets were collected for all participants for the full field ERG stimulus adaptation study, except for CJ. Four participants also participated in the collection of full-field adaptation data at lower stimulus intensities. The participants were split into three sets which included 5 younger normals (mean age 29.4 years), three older normals (mean age 63.67) and three participants graded as 0-2 in the test eye on the simplified severity AREDS scale (Ferris et al. 2005) (mean age 72 years).

Participant	Age	Disease status	Studies participated in.
CJ	26	No Disease	1,2
VN	27	No Disease	1,2,3
GR	27	No Disease	1,2,3
LR	30	No Disease	1,2,3
AB	37	No Disease	3,4
RN	60	No Disease	1,2,3
RE	72	No Disease	1,2,3,4,5
SH	59	No Disease	1,2,3,4
LP	70	Early AMD	1,2,3,4,5
JB	69	Early AMD	1,2,3,4,5
MD	77	Early AMD	1,2,3,4,5

Table 6-1. Participant details and the studies they participated in. study 1= Stimulus intensity study, 2= focal stimulus adaptation study, 3= full field stimulus adaptation study, 4= full field stimulus adaptation study to 0.0025cd.s.m^2 5= full field stimulus adaptation study to 0.000625cd.s.m^2.

6.4.2.1 Stimulus intensity study

In all control participants apart from RE, data were reliably collected at all stimulus luminances. As expected, the amplitude of the traces reduced as stimulus luminance was reduced (see sample data from control LR in Figure 6-3, top panel). For participant RE (a member of the older control group), the lower luminances did not elicit a response distinguishable from noise levels (see sample data from RE in Figure 6-3, lower panel). For the participants with early AMD, clear responses were not obtained at any stimulus luminance for any participant, apart from JB, who showed a clear response at 40 cd/m2 and 20 cd/m2.

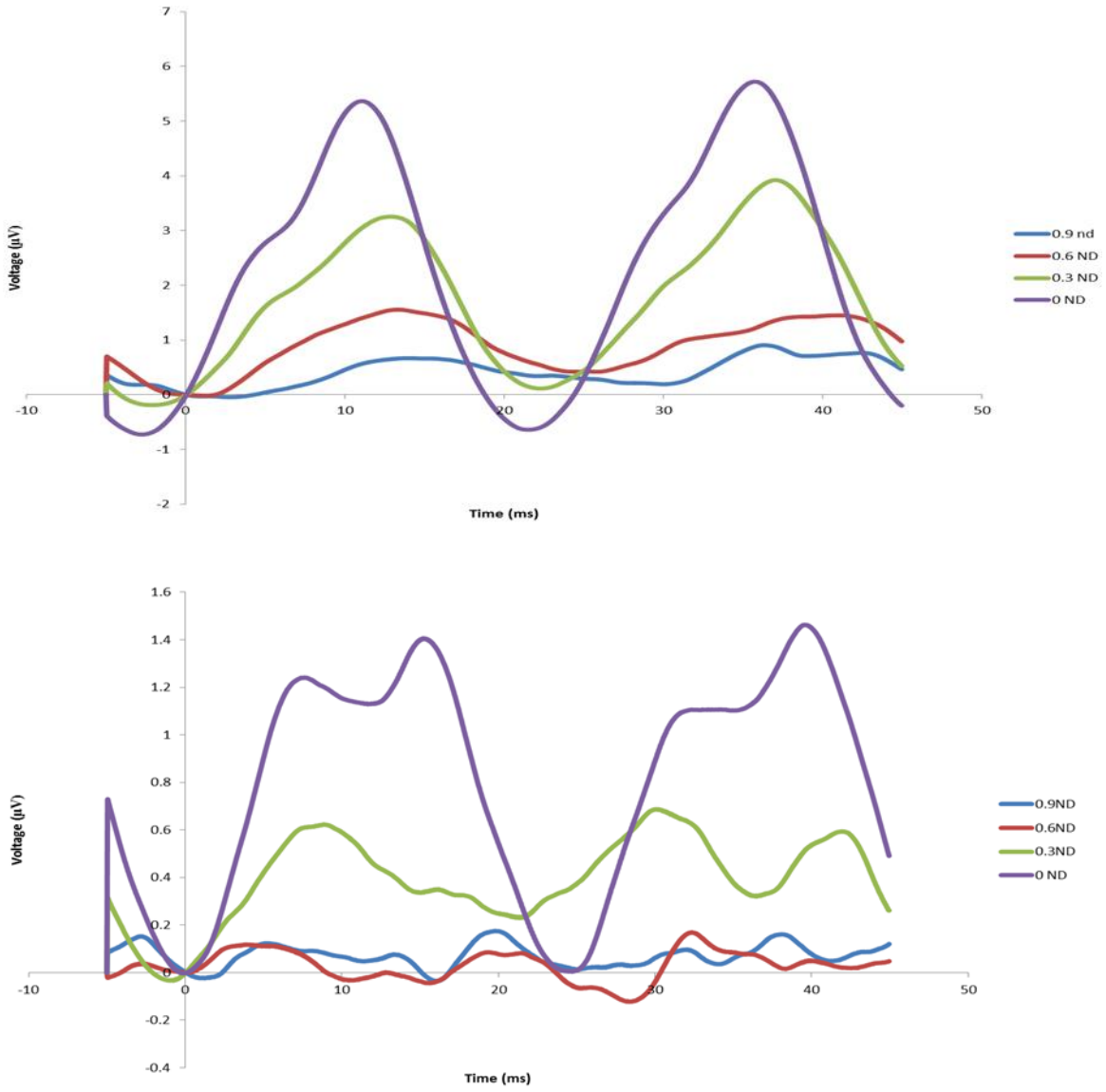


Figure 6-3. Results from focal cone stimulus intensity study from participant LR (top panel) and participant RE (bottom panel). The traces shown are each the average of 800 responses, before Fourier analysis.

Figure 6-4 shows the mean averaged amplitude of the first harmonic from all participants at all four luminances. It can be seen that there is an increase in the amplitude of the first harmonic with increasing luminance of the stimulus. Increasing CI bars indicate that the intersubject variability is greatest for the highest luminance.

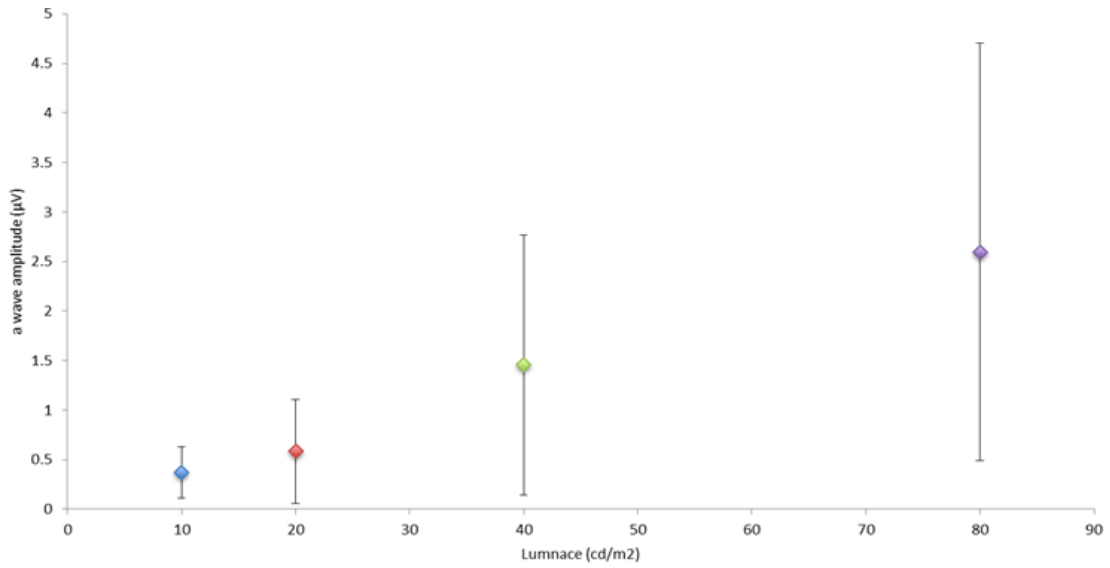


Figure 6-4. Mean amplitude of the first harmonic recorded at different luminance's with 95% confidence intervals.

6.4.2.2 Stimulus adaptation study focal cone ERG

Figure 6-5 shows every third trace for sample participant RN (older control group) over a period of 2 minutes. An average time of approximately 2 seconds elapsed between the beginning of each of these recordings. There was no clear systematic reduction in the response amplitude with increasing time after stimulus onset. This was the case for all participants where a clear response was obtained before Fourier analysis. However, the signals from all three participants with AMD were too small to produce a response above the level of background noise when only 25 responses were averaged.

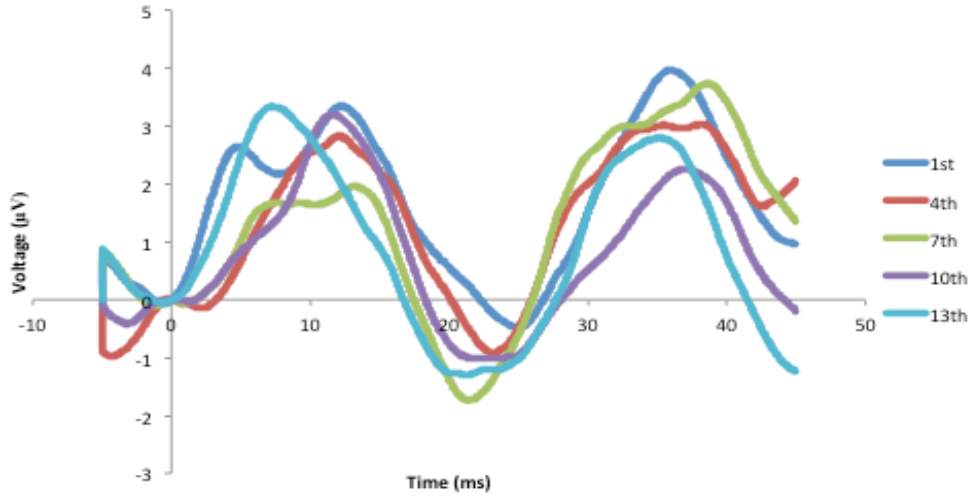


Figure 6-5. Results from focal cone stimulus adaptation study for participant RN.

For the group mean data, the results from the AMD group were not included in the analysis due to the data being contaminated with noise. The group mean amplitudes of the first harmonic for the first and final two waves with confidence intervals can be seen in Figure 6-6. There was no statistically significant difference between the amplitudes of the first and last traces (p=0.647) suggesting that there is no light adaptation to a stimulus averaging 40 cd/m^2 over a two minute period.

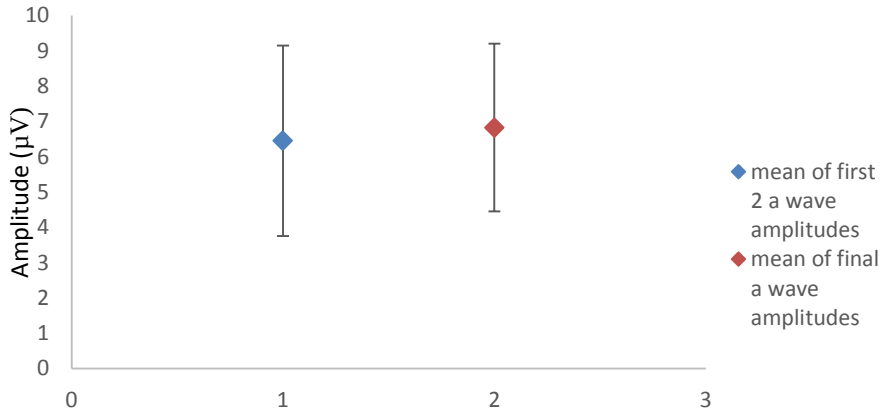


Figure 6-6. Group mean amplitude of the 1st harmonic at 0 minute (blue diamonds) and 2 minute (red diamonds) with confidence intervals.

6.4.2.3 Stimulus adaptation study full field rod ERG

Table 6-1 shows which data was collected for each participant for this study. Figure 6-7 shows the raw traces for participant RE when the luminance was attenuated to 0.000625 cd.s.m^2.

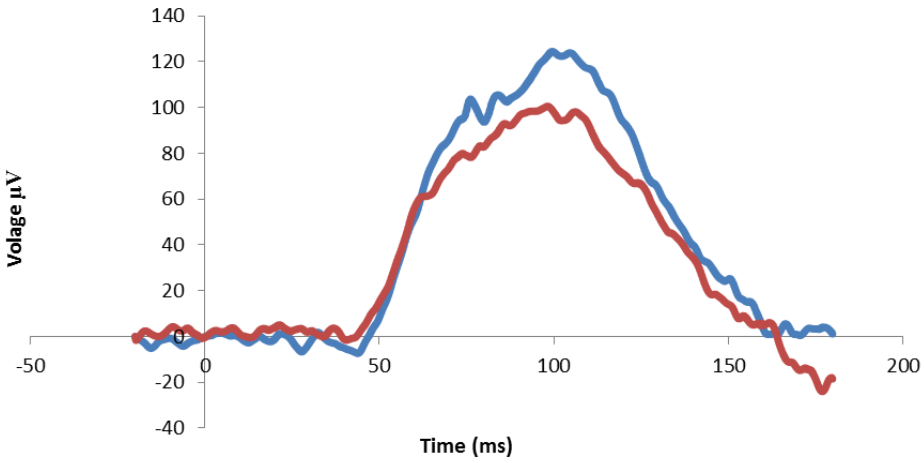


Figure 6-7. Recording before blue light adaptation (blue trace) and after 1 minute blue light adaptation (red trace) for participant RE with a stimulus luminance of 0.000625 cd.s.m^{-2}.

After Fourier smoothing had taken place, and b-wave amplitudes evaluated, the group mean of the b-wave amplitudes for each luminance before and after the 1 minute adaptation period were plotted and can be seen in Figure 6-8. In every case, the mean amplitude was lower after adaptation. However, there is an overlap of the confidence intervals for the first and last b-wave amplitudes for all luminances, suggesting that that there is no significant effect of light adaptation on ERG amplitude after viewing the stimulus for one minute at any luminance. This was confirmed with statistical analysis (paired t-tests, luminance 0.01 cd.s.m^2 p=0.139, luminance 0.0025 cd.s.m^2 p=0.946, luminance 0.000625 cd.s.m^2 p=0.180).

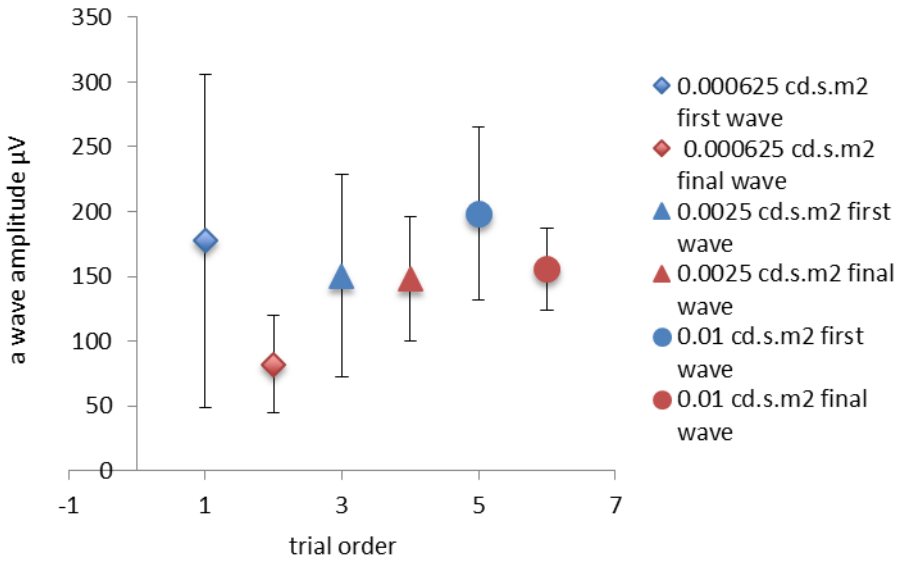


Figure 6-8. Mean group b-wave amplitudes with confidence intervals.

6.4.3 Pilot discussion

This pilot study aimed to determine the optimum stimulus parameters for a study into the effect of hypoxia on ERG parameters in participants with early AMD. The purpose of this optimisation was to identify the stimulus luminance that would allow clear responses to be recorded from all participants, whilst minimising the state of light adaptation.

The first pilot study suggested that a stimulus luminance averaging 40cd/m^2 is required to reliably elicit a focal flicker response from all individuals above the level of noise. Clear responses were not obtained from all participants at the lower luminances. Also, as signal sizes are reduced with age and with retinal disease a higher luminance stimulus will increase the chance of a recordable signal being obtained in all participants.

The results of the second study suggest that there was little light adaptation to the focal stimulus, even when viewing the highest available stimulus luminance of 40 cd/m^2. This finding is important for two reasons. Firstly, the effect of hypoxia in AMD is thought to be greatest under conditions of dark adaptation, hence ongoing light adaptation of the retina is undesirable in this study of dark-adapted cone function; secondly, when comparing responses before and after gas inhalation, it is vital that adaptation effects are not influencing responses. The red stimulus was used in order to minimise the effect of light adaptation as rod photoreceptors are relatively insensitive to red light.

For the full field testing, although there was no statistically significant effect of light adaptation on the ERG amplitude at any stimulus luminance there was a trend for the amplitude of the b-wave to be smaller after viewing the stimulus for 1 minute. Therefore to minimise the effect of light adaptation, for the main study the test stimulus used for full field ERG recording was $0.00125cd.s.m^{-2}$, an intensity which fell between the two lower stimulus levels.

6.5 Main Study

The aims and hypotheses of this study are described in section 6.1. Dark–adapted cone function (focal flicker ERG) and rod function (full-field rod ERG) were assessed in participants with AMD and age matched controls when breathing medical air and 14% O_2.

6.5.1 Methods

The main study followed the basic methods set out in section 6.2. All participants had previously taken part in the scotopic thresholds 14% oxygen study described in Chapter 5, and the inclusion and exclusion criteria are as previously described (see section 4.2.1). Baseline measurements were recorded as described in section 4.2.3. Participants that had been seen for the psychophysical testing within the past 4 weeks continued straight to the main ERG study data collection after consent and patient history were taken, without repeating baseline tests. Once 60 minutes of dark adaptation were complete, participants undertook up to 10 minutes of practice ERG recording, then the data collection followed the timeline set out in Figure 6-9. To summarise, the first of two randomly assigned gases (either air containing 14% oxygen or medical air containing 21% oxygen) was turned on and, after 8 minutes, the participant undertook 7 minutes of ERG recordings. In this period, the data collection for both the focal and the full field recordings took place. Focal ERG traces were recorded to a red (peak wavelength 660nm) stimulus of 20° diameter, with an average luminance of 40 cd/m^2, at a temporal frequency of 41Hz (50% duty cycle), averaged in blocks of 25 responses on a 50ms time base. Full field ERGs were recorded to a blue (peak wavelength 469nm) stimulus of 0.005cd.s.m^{-2}, duration 5ms, at a temporal frequency of 0.5Hz, averaged in blocks of 5 responses on a 200ms time base. The amount of data collected depended on the participant, but on average 20 blocks of focal data and 10 blocks of full field data per gas were collected. There was then a 5 minute washout period, before the second gas was turned on and the process repeated. Throughout the study the participant's SpO_2 was recorded at 1 minute

intervals using a finger pulse oximeter (CMS-50E) and the study was terminated if the participant's SpO_2 fell below 85%. Details of the 60% ventimask used for the gas inhalation and the finger pulse oximeter are provided in section 3.1.3.

Dark adaptation	Practice	Breathing gas 1	Recording of ERGs	Washout period	Breathing gas 2	Recording of ERGs
60 mins	10 mins	8 mins	7 mins	5 mins	8 mins	7 mins

Figure 6-9. Timeline showing the protocol of the study investigating the effect of inhaling 14% oxygen on ERG parameters.

6.5.2 ERG signal analysis

For all participants the raw traces were exported to an Excel spreadsheet for analysis. All of the individual traces (an average of 25 responses for the focal data and an average of 5 responses for the full field) were then grouped by the stimulus presented and the air type breathed and were then plotted on 4 graphs (14% focal, 14% full field, 21% focal, 21% full field). The data were then made anonymous and reviewed by eye by the author. Traces which were contaminated by noise or were outliers were removed. The remaining data were then averaged for each condition, with confidence intervals. The mean traces with confidence intervals, as well as the individual component traces for each individual participant were then reviewed by supervisor AB, who was masked to the participant group and air type breathed. Data that were still considered unreliable were removed from any further analysis. 'Unreliable' data was identified as that where the component traces were not reproducible, suggesting contamination by extraneous noise such as eye movements. Data containing an

obvious blink artefact or traces showing a gradient associated with a slow eye movement were also excluded.

Fourier analysis and the objective evaluation of the amplitude and implicit time of the first harmonic of the flicker ERG, and the a- and b-waves of the full-field ERG was carried out as described in Section 6.3

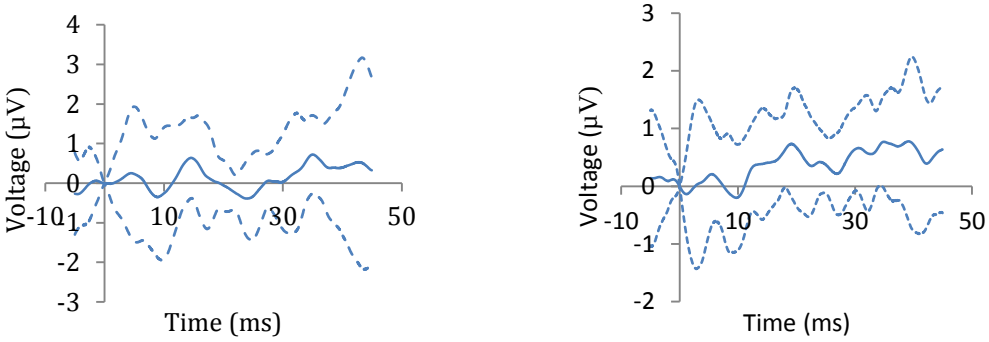
6.5.3 Data analysis

Comparisons were made for each parameter between gas types within each group, and between groups for each gas type. Data were tested for normality using the Shapiro-Wilkes test. For within group comparisons where the distribution of the data was normal, paired t-tests were used. The Wilcoxon signed rank test was used if the data were not normally distributed. When comparing the data between groups the independent samples t-test was used if the data were normally distributed, or the Mann Whitney test for data that were not normally distributed. Data were tested for order effects by running independent samples t tests if the data were normally distributed or the Mann Whitney test for data that were not normally distributed.

6.5.4 Results

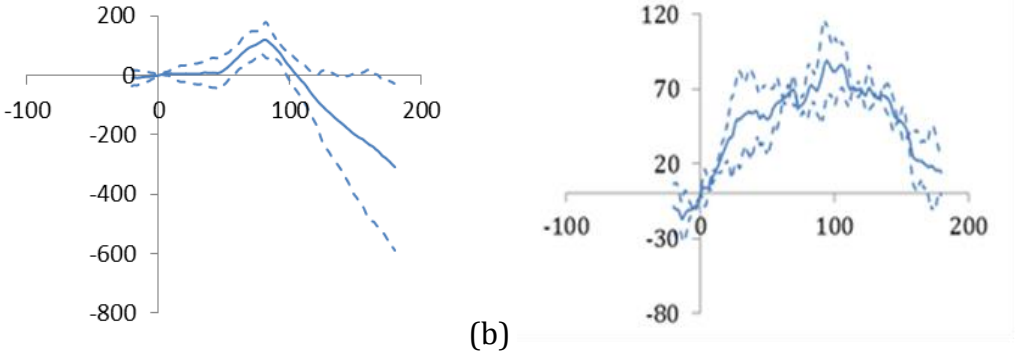
Datasets were collected from all of the 35 participants who participated in the investigation into the effect of breathing 14% oxygen on scotopic thresholds in AMD (see chapter 5). Their details are shown in Table 5-1. Retinal imaging was repeated and no participant showed a change in disease status between the two visits.

The visual evaluation of the data by TC and AB resulted in the datasets for 4 participants being removed for the full field analysis (participants 009, 010, 017 and 026). The datasets for 7 participants were also removed from the analysis of the focal ERG (participants 002, 012, 015, 021, 024, 034, and 035). Examples of this excluded data are shown below in Figure 6-10 and in Figure 6-11.



(a) (b)

Figure 6-10. Examples of excluded focal ERG datasets. Mean of focal data with confidence intervals after the removal of contaminated data for participant (a) 034EF when breathing 21% oxygen and (b) 021MR when breathing 14% oxygen.



(a) (b)

Figure 6-11. Examples of excluded full-field datasets. Mean of full field data with confidence intervals after the removal of contaminated data for participant (a) 009DG when breathing 21% oxygen and (b) 010RE when breathing 21% oxygen.

There were no order effects for any of the parameters measured [focal amplitude (paired t-test p=0.389) and implicit time (paired t-test p=0.349) of the first harmonic,

full field amplitude of the a-wave (Wilcoxon signed rank p= 0.739), amplitude (Wilcoxon signed rank p=0.814) and implicit time (paired t-test p= 0.950) of the b-wave]

6.5.4.1 SpO_2 results

Figure 6-12 shows the mean SpO_2 readings for each group under each gas condition. It is apparent that the peripheral blood oxygenation dropped by a significant amount for both groups (Wilcoxon signed rank, early AMD group p<0.001, control group<0.001) when breathing 14% oxygen. The SpO_2 values were significantly different between the two groups when breathing 14% gas (Mann Whitney p= 0.031), but there was no significant difference when breathing 21% oxygen (Mann Whitney p= 0.174). Both groups reached a plateau by the ninth minute.

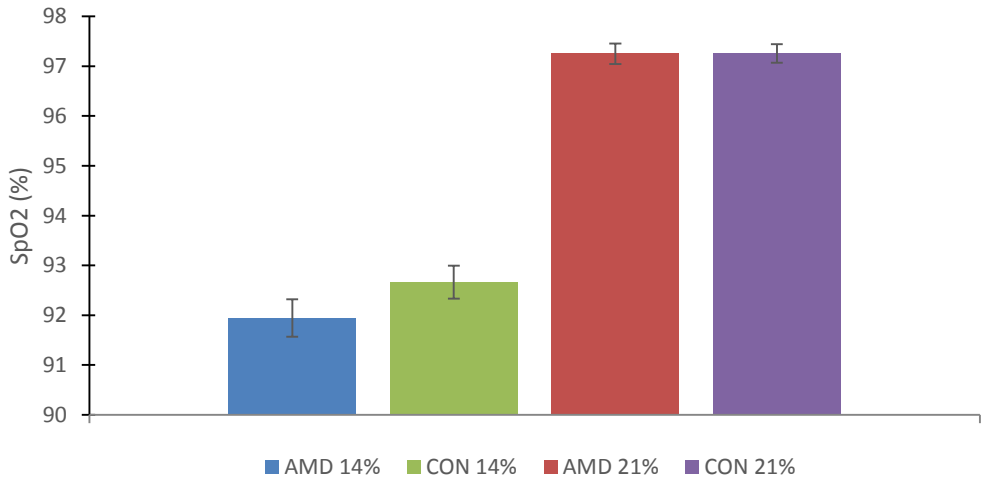


Figure 6-12. Mean SpO_2 for the AMD group and the control group when breathing 14% and 21% oxygen with confidence intervals.

6.5.4.2 Focal ERG results

Figure 6-13 shows sample data collected for participant 022 when breathing 21% oxygen, in order to demonstrate the data analysis stream. Traces which fell substantially outside the confidence intervals of the mean (dashed black) were

treated as outliers and excluded (red traces). Figure 6-14 shows the new average trace with outlying data excluded, and the Fourier analysis of the response.

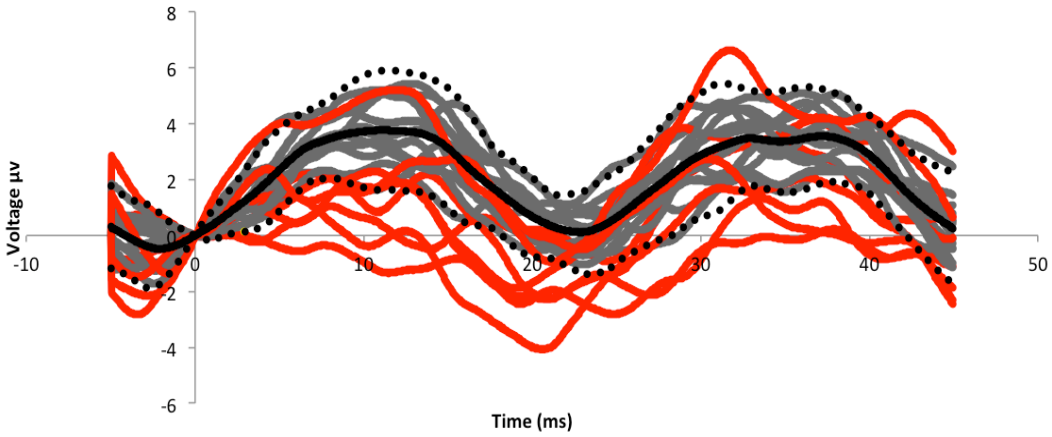


Figure 6-13. Data for the focal ERG recording for participant 022 when breathing 21% oxygen. Mean trace (black) with confidence intervals (black dotted). Rejected traces shown in red.

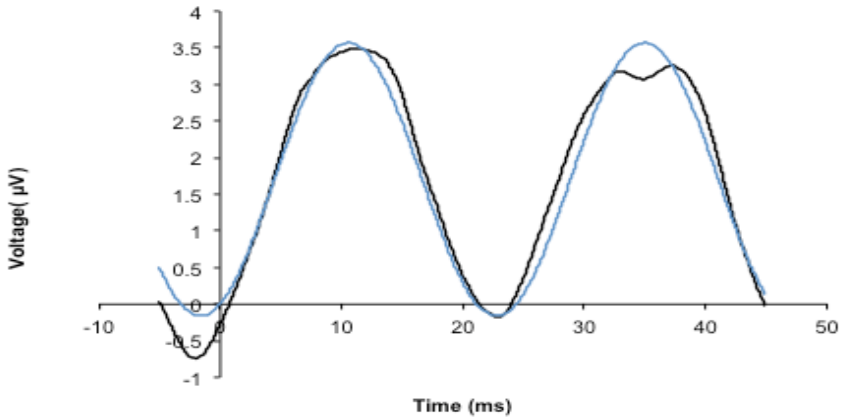


Figure 6-14. Mean (black trace) and Fourier analysed data (blue trace) for the focal ERG recording for participant 022 when breathing 21% oxygen.

The mean amplitude and implicit time of the first harmonic data are presented in Table 6-2. There was no statistically significant difference in the amplitude of the first harmonic when breathing 14% oxygen, when compared to breathing 21% oxygen within either the AMD group or the control group (paired t-tests AMD p= 0.592;

Control p= 0.990). However, there was a statistically significant difference in amplitude between groups when breathing 14% oxygen (independent t-test p= 0.006) and 21% oxygen (independent t-test p= 0.002), in both cases the control group had a greater mean amplitude than the early AMD group. Statistical testing showed that there was no significant difference in the implicit times of the first harmonic either within groups when comparing gas types (AMD group p=0.457, control group p= 0.349) or between groups when breathing 14% oxygen (Mann-Whitney p= 0.145) or when breathing 21% oxygen (Mann-Whitney p=0.668).

The differences between the amplitude and implicit time of the first harmonic when breathing 14% oxygen compared to 21% oxygen were calculated within each group and compared between the AMD and control groups (this can be seen in Figure 6-15). There were no significant differences in the change in amplitudes (independent t-test p=0.692) or implicit time (Mann-Whitney p=0.095) between gas conditions in the two groups.

	First harmonic amplitude	First harmonic implicit time
AMD group 14% mean +/- CI	0.82 +/- 0.29	-0.37 +/- 0.61
AMD group 21% mean +/- CI	0.65 +/- 0.23	-0.12 +/- 0.71
AMD group mean difference +/- CI	-0.10 +/- 0.23	0.31 +/- 0.73
Control group 14% mean +/- CI	1.25 +/- 0.18	-0.02 +/- 0.59
Control group 21% mean +/- CI	1.24 +/- 0.18	-0.36 +/- 0.55
Control group mean difference +/- CI	0.04 +/- 0.23	-0.30 +/- 0.55

Table 6-2. Focal mean amplitude and implicit time of the first harmonic for AMD and control groups with 95% confidence intervals.

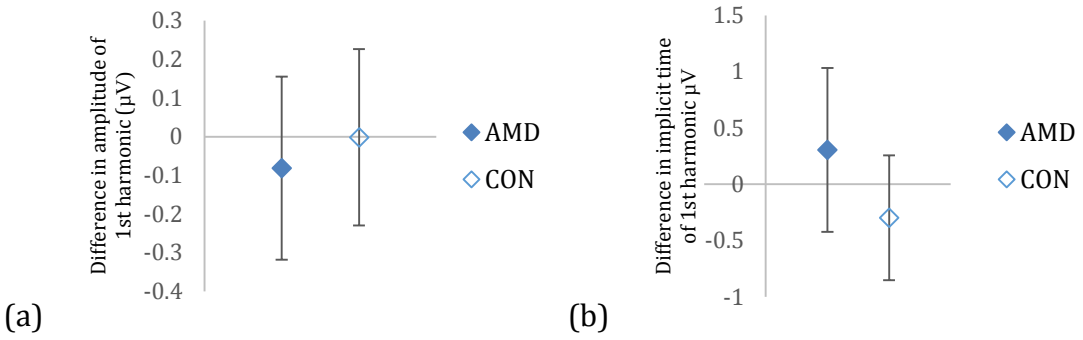


Figure 6-15. Mean difference in (a) amplitude of the first harmonic and (b) implicit time of the first harmonic between gas conditions for each group with 95% confidence intervals.

The change in SpO_2 when each participant was breathing 14% oxygen was calculated for each individual and compared with the change in ERG parameter, these results are displayed graphically in Figure 6-16. Whilst all participants became hypoxic when breathing the 14% oxygen, there was no association between the change in SpO_2 and any parameter for either group for both the focal parameters tested.

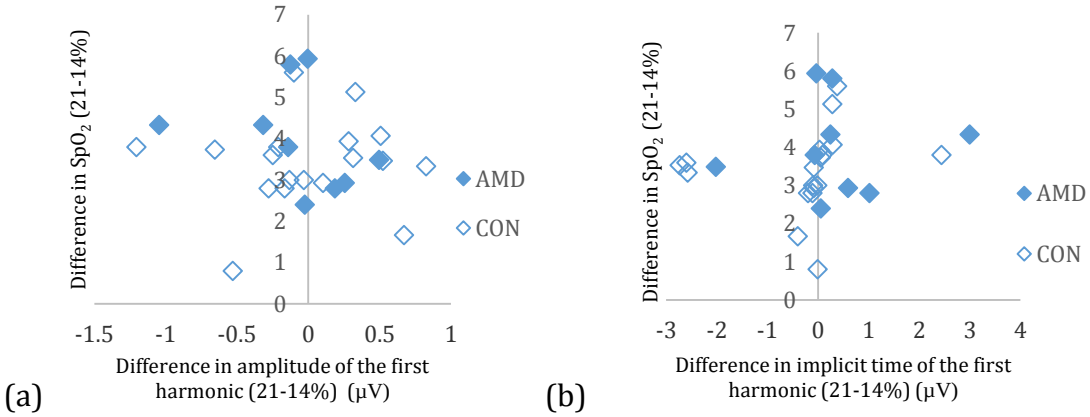


Figure 6-16. Change in SpO_2 compared to change in (a) amplitude of the first harmonic and (b) implicit time of the first harmonic.

6.5.4.3 Full field ERG results

Figure 6-17 shows an example of the full-field data collected for participant 028 when breathing 14% oxygen. The mean trace can be seen in black, with confidence intervals

in dashed black. Traces that were rejected from analysis are shown in red. Figure 6-18 shows the mean of the data after the rejection of contaminated traces, and the trace after Fourier removal of high frequency noise.

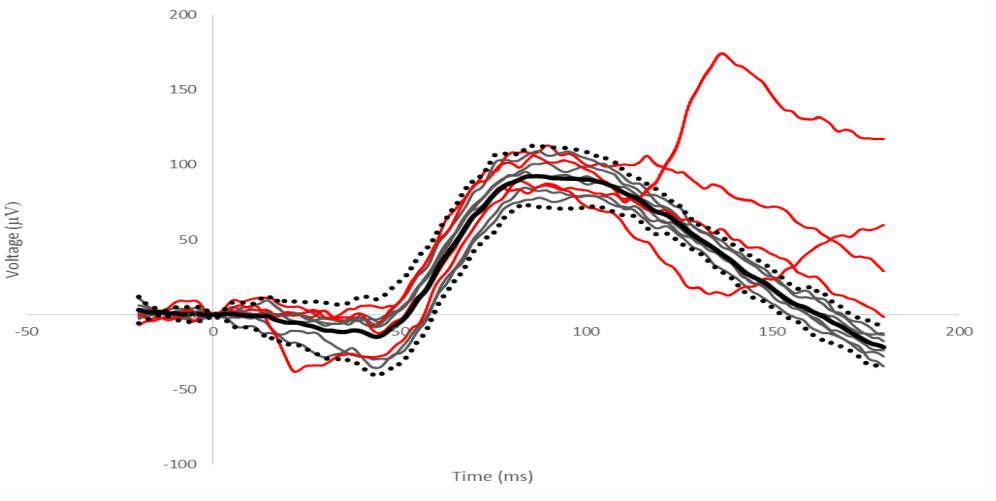


Figure 6-17. Data for the full field ERG recording for participant 028 when breathing 14% oxygen. Mean trace (black) with confidence intervals (black dotted). Rejected traces shown in red.

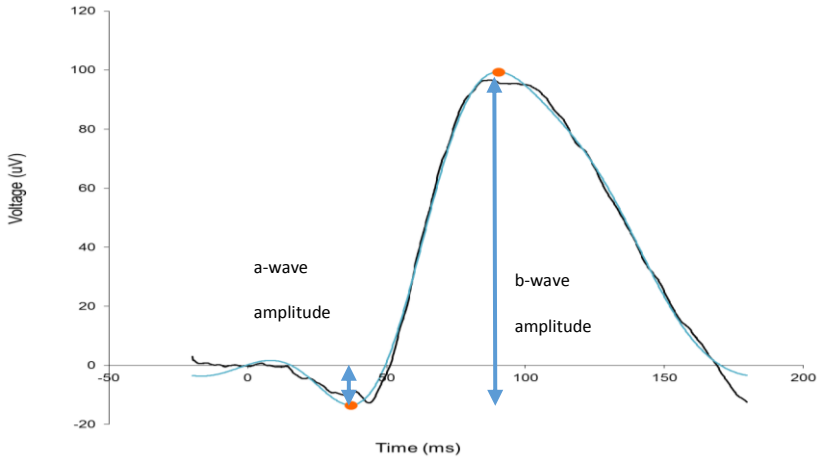


Figure 6-18. Mean (black trace) and Fourier analysed data (blue trace) for participant 028 when breathing 14% oxygen. The red dots represent the objectively determined

locations of the a-wave and b-waves. The arrows represent the amplitudes of the a- and b-waves.

The mean amplitudes of both the a-wave and the b-waves and the implicit times for the b-wave for both groups are presented in Table 6-3. Statistical analysis of the amplitude of the a-wave showed that there was no significant difference either within the groups when comparing the effect of breathing 14% oxygen to breathing 21% oxygen (Wilcoxon signed rank AMD group p= 0.594, paired t-test control group p= 0.222) or between the AMD and control groups when breathing 14% oxygen (Mann-Whitney p= 0.161) or when breathing 21% oxygen (independent t-test p= 0.155).

There was also no significant difference in the amplitude of the b-wave between the groups when breathing 14% oxygen (Mann-Whitney p= 0.830) or when breathing 21% oxygen (Mann-Whitney p= 0.421). Within groups, there was no significant difference in the amplitude of the b-wave when breathing 14% compared to breathing 21% oxygen in the AMD group (Wilcoxon signed rank p= 0.272). There was, however a statistically significant decrease in the amplitude of the b-wave when breathing 14% oxygen when compared to breathing 21% oxygen within the control group (Wilcoxon signed rank p= 0.028). There was no significant difference in the implicit time of the b-wave when breathing 14% compared to breathing 21% oxygen either within the groups (paired t-test AMD group p= 0.925, Wilcoxon signed rank control group p= 0.570) or between groups when breathing 14% oxygen (independent t-test p= 0.764) or when breathing 21% oxygen (Mann-Whitney p= 0.625).

	a-wave amplitude	b-wave amplitude	b-wave implicit time
AMD group 14% mean +/- CI	-3.63 +/- 3.12	80.26 +/- 19.62	95.14 +/- 5.16
AMD group 21% mean +/- CI	-4.46 +/- 1.78	84.05 +/- 18.02	94.91 +/- 3.54
AMD group mean difference +/- CI	-0.83 +/- 3.43	3.79 +/- 14.92	-0.21 +/- 4.49
Control group 14% mean +/- CI	-6.07 +/- 1.79	73.69 +/- 11.93	96.09 +/- 3.65
Control group 21% mean +/- CI	-7.63 +/- 2.73	89.62 +/- 14.65	96.42 +/- 4.80
Control group mean difference +/- CI	-1.57+/- 2.34	15.93+/- 11.25	0.33 +/- 3.53

Table 6-3. Full field mean amplitude of the a-wave for the AMD and control groups with confidence intervals.

Figure 6-19 and Figure 6-20 show graphs comparing the mean differences in all the parameters between gas conditions. There were no significant differences in the change in amplitude of the a-wave (Mann-Whitney p=0.356) or the change in amplitude (independent t-test p=0.211) or implicit time (independent t-test p=0.850) of the b-wave between gas conditions in the two groups.

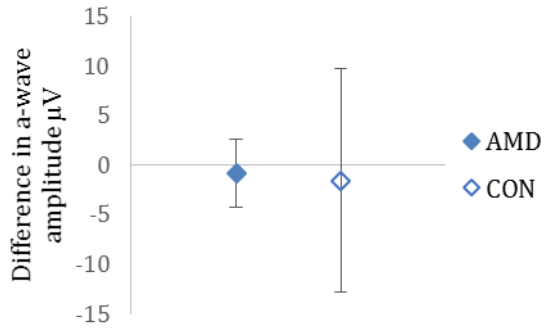


Figure 6-19. Mean difference in a-wave amplitude when breathing 14% oxygen for AMD and control groups with 95% confidence intervals.

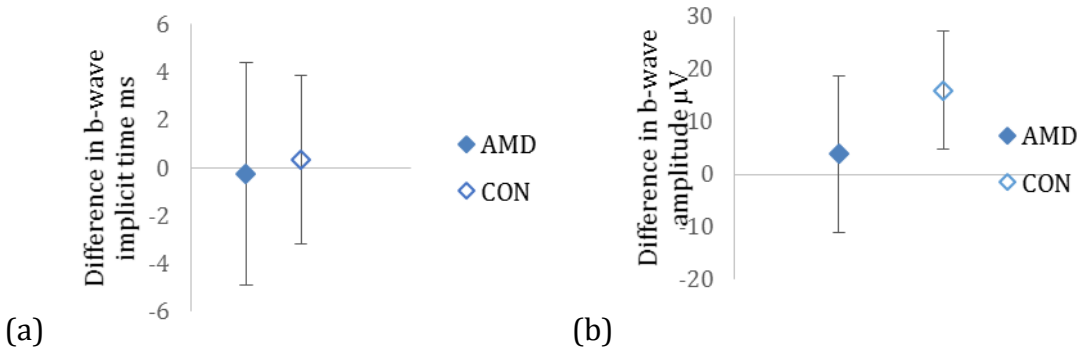


Figure 6-20. Mean difference in (a) b-wave implicit time and (b) b-wave amplitude between gas conditions in the two groups, with 95% confidence intervals.

No relationship was seen between the individual change in SpO_2 and the change in any of the ERG parameters between gas conditions, this can be seen in Figure 6-21.

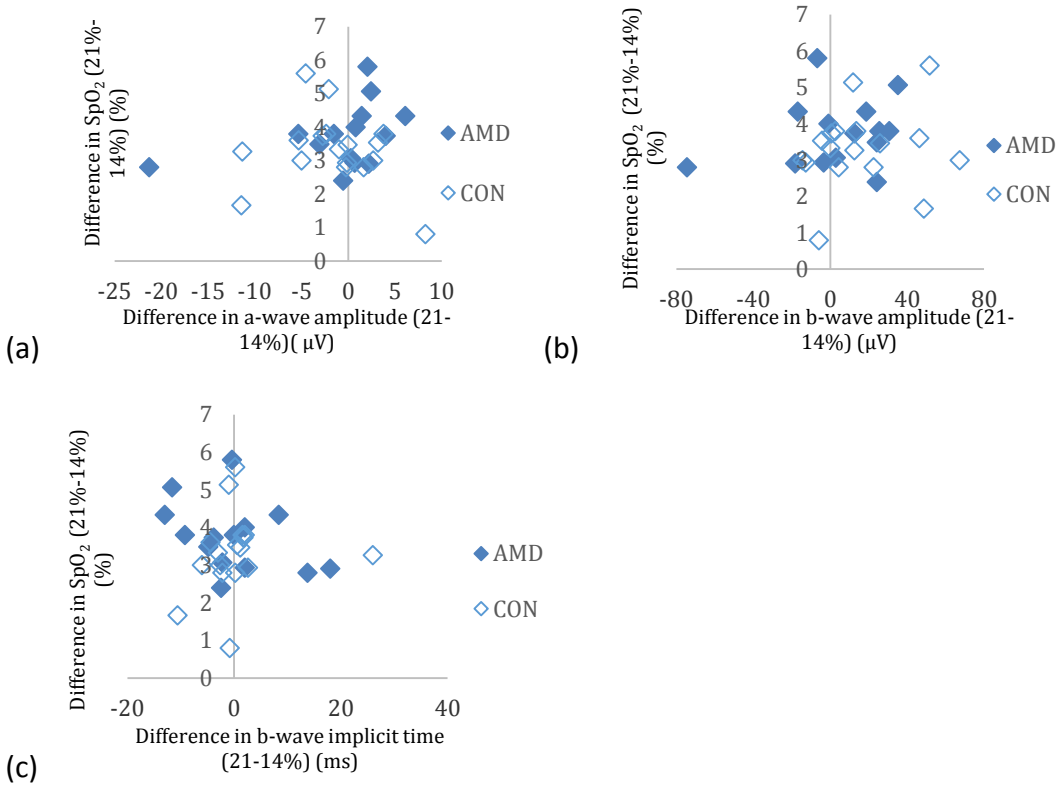


Figure 6-21. Change in SpO_2 compared to the change in (a) a-wave amplitude, (b) b-wave amplitude and (c) b-wave implicit time.

6.5.5 Discussion.

This study is the first to investigate the effect of breathing 14% oxygen on rod and cone scotopic ERG parameters in participants with early AMD. However, previous studies have shown that certain parameters of both the focal (Binns et al. 2007; Falsini et al. 1999) and full field ERG (Jackson et al. 2006; Walter et al. 1999) are affected by AMD when compared with control participants.

In this study, the amplitude of the first harmonic was reduced in AMD participants in comparison to control participants when breathing 21% and 14% oxygen, but there was no difference in the implicit time of the first harmonic. This is in agreement with Falsini et al. (1999) who studied 25 participants with bilateral GA, and reported a reduction in the amplitude of the focal ERG with no phase changes. Our findings are in contrast to those of Remulla et al. (1995) and Sandberg et al. (1993) who found a preserved amplitude, but delayed implicit time in the fellow eye of participants who had unilateral nAMD. Binns and Margrain (2007) studying the effect of early AMD on the focal flicker ERG, likewise found an increase in implicit time in participants with early AMD, but also measured a decrease in amplitude similar to that reported here. One key difference in methodology is that the protocol reported here was unique in involving participants being dark adapted before recording the flicker response at the minimum luminance possible (with no adapting surround employed). Whilst the flicker ERG is by nature a cone response, the characteristics of the cone signal may be different under these conditions e.g. due to the increased metabolic demand presented by the maintenance of the rod dark current under dark adapted conditions (Wangsa-Wirawan et al. 2003). We can be confident that the focal ERG recordings in

this study were predominantly focal, despite the lack of an adapting surround, due to the use of a red stimulus which is not prone to scatter by the optical media.

Although there are no studies comparing the effect of breathing 14% oxygen on focal ERG parameters between participants with early AMD and controls, it follows that if the amplitude of the first harmonic is reduced when breathing 21% in participants with early AMD when compared to controls, that it would also be reduced between the groups when breathing 14%, as found in this study.

The results from this study are in agreement with the literature regarding the effect of AMD on full field ERGs (Jackson et al. 2004; Sunness et al. 1985). Jackson et al. (2004) found that early AMD did not alter the amplitude of the a-wave and Sunness et al. (1985) concluded that the full field ERG b-wave was not affected by either early or late AMD. Walter et al. (1999) concluded that AMD resulted in reduced a-wave and b-wave amplitudes, however, no early AMD participants were included in that study, with all being graded as having either nAMD or GA.

The results from the control group in this study are in agreement with the literature, in that the amplitudes but not the implicit times of the b-wave are affected by hypoxia (Schatz et al. 2014; Schatz et al. 2013). However these same studies also recorded a significant decrease in the a-wave amplitude, which was not repeated in this study. This discrepancy may be explained by the fact that the reduction in a-wave amplitude in a hypoxic condition was only reported in the literature when brighter stimuli were used (3 cd.s/m^2 and 10 cd.s/m^2), compared to the dim flash employed in this study (0.00125cd.s/m^2).

Crucially, our results show that the change in ERG parameters when hypoxia is induced is not significantly greater in the AMD group than the control group for either the focal or the full-field response. Indeed, there were no significant differences between gas conditions for any of the parameters within either group, apart from the amplitude of the b-wave which was significantly reduced by the hypoxic episode for the control group. This finding was not in agreement with our hypothesis that stated that there would be a difference in the ERG parameters between gas conditions in both groups, and that the difference would be greater in the group with early AMD.

The validity of recording full field ERG parameters to assess changes due to AMD has been challenged, due to the possibility of any focal rod and cone changes being obscured by the summed response from the peripheral rods (Curcio et al. 1990) which are relatively unaffected by AMD. This was reflected in our study by the lack of a significant difference in full-field ERG parameters between groups. However, our rationale for including this ERG in the protocol was to determine whether there was evidence of global retinal hypoxia in people with AMD. Our findings suggested that this was not the case. Similarly, the use of a flickering stimulus to evaluate dark-adapted central retinal cone function might be contested in this study, given our hypothesis that any hypoxia will be maximal in the dark adapted eye. However, a rapidly repeating stimulus presented to the dark adapted eye might be expected to exacerbate hypoxia by further raising metabolic activity (Feigl et al. 2011), and it has the added benefit of allowing the substantial averaging of data within a limited timeframe. This maximises the signal to noise ratio of the response, which was vital in this study where the hypoxic conditions could only be applied for a limited time. The

pilot study also ensured that the luminance of the flickering stimulus was not sufficient to induce progressive retinal light adaptation.

There are several limitations to this study. The first was the use of inexperienced participants for ERG recording. The amplitude of the waveform recorded to focal ERG parameters is small and subject to contamination by noise and therefore requires excellent compliance from the participant. Although training was conducted before data collection took place, due to time restrictions this was for 10 minutes only. The use of experienced participants may have resulted in more reliable data collection.

Another limitation was the time constraint in the protocol due to safety when breathing 14% oxygen (i.e. this could not continue for a prolonged period). This led to a relatively short time window in which to average data, resulting in the collection of more variable data. It also meant that traces could not be reviewed until after data collection was complete. Ideally, review of the data at the time of collection would have enabled the removal of traces contaminated by eye movements, and the recording of replacements, allowing the collection of higher quality data. The combination of both these limitations resulted in more individual traces being rejected at the time of analysis. This particularly affected the focal results as even after Fourier analysis the data from 7 participants were excluded from analysis due to contamination by noise.

A further limitation was that, for pragmatic reasons, the sample size included in this study did not meet the original aim of n=30 per group (section 4.2.2). This increases the possibility of a type II error i.e. missing a small difference between groups which would become apparent if the sample size had been larger.

Finally, although the SpO_2 values in both groups decreased significantly during the hypoxic episode, it could not be proven that in this study this resulted in a significant decrease in oxygenation at a retinal level. Therefore, although a systemic hypoxic episode was created, it may be that this did not result in a local retinal hypoxia.

To summarise, these limitations notwithstanding, it may be concluded from the findings of this study that there was no evidence of focal or global retinal hypoxia in the participants used in this study, when compared to healthy age-similar controls. The next chapter of this thesis will discuss the findings of chapters 4, 5 and 6 and consider the future of this research area.

7 General discussion and future directions.

7.1 Discussion

Age-related macular degeneration is the leading cause of blindness in the devloped world (Congdon et al. 2004), and currently there is treatment available for less than 10% of those affected by the condition. A greater understanding of the pathogenesis is required to facilitate the development of new treatments at all stages of the disease, particularly in the early stages of the condition. An enhanced understanding of the disease mechanism would also help to develop improved tests for the early diagnosis and sensitive monitoring of AMD. The primary aim of this theisis was to gain a greater understanding of the role of hypoxia in the pathogenesis of early AMD, and to assess whether measures of dark adapted visual function could be used as functional biomarkers of disease-related hypoxia.

Two reviews of current literature in Chapter 2 were carried out as part of this PhD, which aimed to investigate the effect of hypoxia on visual function, and to review the evidence that hypoxia plays a role in the pathogenesis of AMD. These reviews indicated that there is substantial evidence that a transient period of induced hypoxia affects various aspects of visual function, including scotopic thresholds and ERG parameters in normal participants. In people with AMD a reduction in scotopic sensitivity and changes to ERG parameters are also seen, which are comparable to

those reported under a hypoxic episode in normals. However, direct evidence that this visual dysfunction in AMD is attributable to hypoxia is absent. The experimental work reported in this thesis aimed to probe the relationship between hypoxia and scotopic visual function in AMD by recording scotopic thresholds and ERGs during transient periods of induced hypoxia and hyperoxia.

In order to evaluate the effect of periods of transient hyperoxia and hypoxia on visual function in people with AMD, it was first necessary to evaluate the effect of these conditions in healthy controls. The studies carried out in Chapter 3 of this thesis looked at the effect of breathing 14% and 60% oxygen on the scotopic thresholds of normal participants for 5 minutes compared to a control medical air condition (21%). It was hypothesised that breathing 14% oxygen would result in an increase in scotopic thresholds, but that breathing 60% oxygen would not alter thresholds significantly. The study concluded that there were no significant differences in thresholds when inhaling 14%, 21% or 60% oxygen at any of the three retinal locations tested. This finding was unexpected, given the published evidence that a transient period of systemic hypoxia causes an elevation in dark adapted thresholds (McFarland et al. 1939; McDonald et al. 1939; Wald et al. 1942b). However, it appeared from examination of the data that SpO_2 was still decreasing after 5 minutes. This would suggest that a steady state of blood oxygenation had not been reached, and therefore that hypoxia was not at a level which would result in altered visual function. A follow up study found that a minimum of 8 minutes were required to reach this steady state plateau in SpO_2 when breathing 14% oxygen.

A further aim of the preliminary studies was to determine whether a particular macular region was likely to be more susceptible to the effects of hypoxia. When the intensity of the hypoxic episode was increased (by breathing 10% oxygen), the stimulus presented at 7 degrees eccentricity showed the greatest deficit in scotopic visual function, indicating a possible increased vulnerability to the oxygen deficiency at the location. This result is in agreement with Feigl et al. 2011 and Connolly et al. 2008b who found that visual function in the mesopic range is reduced between 1-15 degrees and at 9 degrees, respectively. As the fovea is free from rods (Nag et al. 2012) it is not suprising that it was not as sensitive to hypoxia in the dark as the area at 7 degrees. Retinal ganglion cell density peaks at 1.5-7 degrees from the fovea (Dacey and Petersen 1992) and these cells have also been found to have a high energy requirement (Wong-Riley 2010). It could be that the combined high density of RGCs and rods at this location increases its sensitivity to hypoxia.

These pilot studies provided important information regarding i) the necessary duration of the hypoxic/hyperoxic episode and ii) the optimal location to test for retinal hypoxia. This information informed the development of an optimised protocol, which was then applied to a wider cohort of people with AMD and age-matched controls in order to test the hypothesis that temporarily reducing the level of retinal hyopxia by breathing 60% oxygen would result in a decrease in scotopic thresholds in people with AMD (see chapter 4). Interestingly, there was no reduction in threshold in either control or AMD group associated with the transient hyperoxic condition. However, the results of this study indicated that participants with early AMD were not systemically hypoxic under control conditions (their SpO_2 levels were close to 100% at

baseline), therefore there appeared to be a ceiling effect at play whereby inhalation of 60% could not increase participants' SpO_2 significantly beyond the baseline level. It is therefore not surprising that the systemic hyperoxia did not affect the scotopic thresholds of pariticipants. Also, an increase in SpO_2 will not necessary result in a reduction in local retinal hypoxia as changes in the choroid and Bruch's membrane would still impede transportation of metabolites to the photoreceptors (Goldberg et al. 1998; Staurenghi et al. 1992).

The remaining experimental studies in this PhD, therefore, approached the problem from a different angle, testing the hypothesis that a transient *hypo*xic (rather than *hyper*oxic) event would have a greater effect on visual function in people with AMD than in healthy controls.

Surprisingly, as reported in chapter 5, there was no significant increase in scotopic thresholds in either controls or people with early AMD when breathing 14% oxygen compared to medical air. There is evidence in the literature that a hypoxic episode results in a tempory increase in scotopic thresholds in control participants (McFarland et al. 1939; McDonald et al. 1939; Wald et al. 1942b). This discrepency may be due to the hypoxic conditions employed in this PhD, as previous studies used both a lower concentration of oxygen and an increased duration of hypoxia. The effect of a hypoxic episode on the scotopic visual function of participants with early AMD has not been studied before, however it would be expected that participants with AMD would be preferentially suseptable to the effects of a hypoxic episode due to changes in the structure of the choroid and Bruch's membrane in AMD (Goldberg et al. 1998; Xu et

al. 2010) resulting in a possible localised retinal hypoxia already present in these participants.

Chapter 6 assessed another aspect of scotopic visual function; the ERG. Specifically the latency and amplitude of the first harmonic of the focal ERG and the amplitude of the a-wave and amplitude and latency of the b-wave of the full field ERG. It was hypothesised that the effect of a hypoxic episode would be to increase the implicit time and reduce the amplitude of the first harmonic of the focal ERG and to cause a decrease in the amplitude of the a-wave and an increase in the implicit time and decrease in the amplitude of the b-wave of the full field ERG in both participant groups. This effect was expected in to be greater in participants with early AMD.

Whilst the amplitude of the first harmonic was reduced in the group of particiapnts with early AMD, no other parameter of the focal or full field ERG was affected by early AMD, this was in line with the literature for the full field ERG (Sunness et al. 1985; Jackson et al. 2004). With respect to the focal ERG results, the findings were in agreement with Falsini et al. (1999) and Binns and Margrain (2007) who also found a amplitude of the first harmonic to be reduced in AMD, however some studies have also found a delay in the implicit time of the first harmonic (Binns et al. 2007; Remulla et al. 1995; Sandberg et al. 1993), which was not evident in this study. There was also no effect of breathing 14% oxygen on any of the tested parameters in participants with early AMD and within the age-matched controls, the only parameter to be affected was the amplitude of the b-wave which was significantly reduced in the control group.

The combined findings of chapters 4-6 provide no supporting evidence for the role of hypoxia in the pathogenesis of early AMD. This finding could be due to the profile of the participants recruited to the study. None of the participants in these studies were current smokers. People who smoke are at an increased risk of tissue hypoxia (Jensen et al. 1991) and therefore may have been more affected by systemic hypoxia. Therefore the participants in this study could require a longer duration and/or greater reduction in SpO_2 to elicit a retinal hypoxic effect. It may also be that the role of hypoxia in AMD is not as influential at the early stages of AMD seen in the participants in this study. The presence of drusen has been associated with choroidal filling defects (Staurenghi et al. 1992; Zhao et al. 1995). Some participants in this study had no age-related changes in the test eye, and therefore may not have been affected by hypoxia at this stage in the disease process. However, the overall findings of this PhD suggest that hypoxia is implicated to a lesser degree in the pathogenesis of AMD than had been hypothesised.

One of the main limitations of all of the studies included in this thesis is the sample size, which may not have been adequate to detect a small difference between groups or gas conditions. The sample size calculation described in section 4.2.2 was based on a study which looked at the effect of breathing different gas types on mesopic thresholds in healthy controls (Connolly and Hosking 2009b). These data are of limited relevance to the research presented in this thesis, which relates to the effect of the gas inhalation on scotopic visual function in people with early AMD. Hence, the prior sample size calculation which suggested that a sample of 30 would be required per group was not regarded as reliable. In practice, the main constraint on participant

numbers was the difficulty encountered in recruiting volunteers who satisfied the inclusion criteria, particularly given the high prevalence of co-morbidities such as diabetes in individuals of this age-group. The exclusion of participants who were currently smokers was also a constraint. The sample size achieved for the main studies in this thesis was actually n=12 (early AMD group) and n=11 (age matched healthy controls) for Chapter 4, and n=15 (early AMD group) and n=20 (age matched healthy controls) (Chapters 5 and 6). As such the studies would only have been able to detect a relatively large standardised difference of about 0.97 with 80% power at the 5% level. This means that, whilst there was no evidence of any effect of either gas type on visual function in the participants with AMD, there is the possibility that the apparent lack of significant findings is actually attributable to the studies being underpowered. The data presented in this thesis may prove to be valuable to any future researchers wishing to carry out further work in this field, as they provide data which will allow for more appropriate sample size calculations to be conducted for future studies.

A further limitation of this research was that there were no reliability studies carried out on the clinical measures assessed. As scotopic threshold measurements and the electroretinogram can be affected by extraneous factors such as electrical and neural noise, an estimate of reliability would have been valuable in determining that a true difference was not masked by poor test reliability. As the psychophysical and electrophysical methods used for data collection were unique to this study, there are no reliability studies from other sources. However two studies have evaluated the between session repeatability of measures of dark adapted visual function (Patryas et al. 2013; Kiser et al. 2006). Patryas et al. (2013) studied the repeatability of the dark

adaptation curve with data collected from stimuli presented on a Cathode ray tube screen. They concluded that cathode ray tube based dark adaptometry was highly repeatable with a coefficient of repeatability of 0.35 log cd.m-2 for the final threshold. Kiser et al. (2006) assessed the reliability of dilated dark adaptometry, dark adapted perimetry and dark adapted full field flash threshold testing in participants who were registered as having severe vision loss and in participants with normal vision as assessed with VA. They reported that over a period of three or more visits all of these tests were repeatable, with a coefficient of repeatability of +/-12.5 dB for the control group using full field flash testing and concluded that these tests could be used for monitoring outcomes in clinical trials.

However, the data collection within the studies contained in this thesis was conducted within a single session, therefore a between session repeatability measure may not have been directly applicable. Perhaps more relevant is the within subject reproducibility of scotopic ERG measurements by Gillan (2012) who recorded scotopic flash ERGs in 6 participants over 10 recordings. They showed that there was variation in amplitude of the b-wave both within (coefficient of variation varied from 0.02-0.41 µV for individual participants) and between participants (mean b-wave amplitudes ranged from 56.15-207.07µV for dilated recordings), and cautioned that the biological variation needs to be considered in both clinical and research settings. It is possible that this variability may have masked a small effect of gas type in the current study. However, the difference in methodology between these previous studies and our own means that such a conclusion should be treated with caution.

The studies contained in this thesis tested the hypothesis that there would be a greater effect of systemic hypoxia / hyperoxia on visual function in people with early AMD than healthy controls. It would have been interesting to include further subanalysis of the data based on other structural parameters, for example the presence of RPD and choroidal thickness, however this fell outside the remit of the present study. Furthermore, although an OCT scan was run on both eyes of every participant at all visits, the imaging system used employed a laser of wavelength 850nm unlike longer wavelength OCTs (1040nm and above), this is unable to reliably image the full thickness of the choroid in all participants (Unterhuber et al. 2005). Hence, a reliable measurement of choroidal thickness was not available for analysis.

A further limitation of this study was the lack of a second grader for assigning all participants into age-matched control or AMD groups. A second grader was only consulted when TC considered the grading to be ambiguous. As can be seen in Table 5-1 all apart from 3 participants had been assigned a grade of 2 in the fellow eye, these participants were undergoing treatment for nAMD and were therefore assigned to the AMD participant group. These individuals had already been classified as having unilateral nAMD by the Medical Retina team at UHW. In the 3 cases that required grading it was clear that they had at least one druse of larger than 125um or RPE changes and hence clearly had early AMD. However, a second, masked grader for grading participant images for those who had been classified as age-matched controls would have potentially improved grading reliability. Although grading could have been improved by using a scale with more resolution, this was not considered

necessary as the scale was only used to classify participants as normal or as having early AMD in the test eye.

In summary the main findings of this research indicate that:

- That hypoxia is not responsible for the elevation of scotopic thresholds reported in early AMD.
- That hypoxia is not responsible for the changes in ERG parameters found in early AMD.
- That scotopic thresholds and scotopic ERGs cannot be used as functional biomarkers of hypoxia in early AMD.
- The findings of this thesis do not support the role of hypoxia in the pathogenesis of early AMD, however the possibility cannot be excluded that a type II error was introduced due to the limited sample size.
- The data presented in this thesis may be valuable in providing the means of conducting accurate sample size calculations for future research.

7.2 Future directions

Further analysis on participant characteristics, including the presence of RPD and a measurement of choroidal thickness would have allowed for potential sub analysis on the data collected. For example, was there a difference in scotopic thresholds or ERG parameters between participants with or without RPD? Further studies, if undertaken using a similar protocol, could include a measurement of these parameters.

The findings of studies in this thesis concluded that ERG parameters were not affected by early AMD or by hypoxia. However there is evidence that the full field ERG is affected by late stage AMD (Walter et al. 1999; Binns et al. 2007) and that there is a reduction in the amplitude of the first harmonic (Falsini et al. 1999) and a delayed implicit time (Remulla et al. 1995; Sandberg et al. 1993; Binns et al. 2007) in the focal ERG with early and late AMD.

As the amplitude of the first harmonic was significantly affected by early AMD in this study, an extension of this work would be to measure this parameter on a selection of patients at different stages of the disease process, from early AMD to nAMD and GA. This research could lead to a better understanding of when the ERG is affected by AMD. Collecting data under a hypoxic episode from these individuals, as per the protocol in Chapter 6, could also determine if the role of hypoxia in the pathogenesis of AMD could occur at a later stage than that studied in this thesis.

A limitation of the studies in chapters 4-6 was the exclusion of participant groups which may be at a higher risk of systematic hypoxia. Tissue hypoxia is a result of cigarette smoking (Jensen et al. 1991) and smokers are also at an increased risk of AMD (Klein, Klein and Moss 1998; Evans 2001; Chakravarthy et al. 2010b). To assess the effect of hypoxia on the scotopic visual function of smokers, data would be collected as per the protocols in chapters 6 and 7, in the following 4 cohorts

- Participants with AMD- non smokers
- Participants with AMD- current smokers
- Age-matched control- non smokers

- Age-matched contol- current smokers

The hypothesis of this study would be that any effect of breathing 14% oxygen on the scotopic thresholds and ERG parameters would be greatest in the group containing participants with early AMD who are current smokers. If this was confirmed then it would strength to the role of hypoxia in the pathogenesis of AMD.

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9 Appendix I Table of included studies for the systematic literature review into the effect of hypoxia on visual function

Study	Participants	methods	results	conclusions
RGC sensitivity to mild systemic hypoxia. Kergoat 2006	* 20 healthy adults	* pERG conducted under 21% O2, carbogen or 12% o2	* amplitude and implicit time of P50 were not modified significantly with systemic hypoxia, they were depressed and delayed, respectively, for N95.	* Inner neuroretinal function remained unchanged during increased blood O2 and carbon dioxide levels known to alter retinal blood flow, but it was altered during decreased blood O2 levels * The generators of N95, namely the retinal ganglion cells, are particularly sensitive to transient, mild systemic hypoxia.
Spatial Contrast Sensitivity at Twilight: Luminance, Monocularity, and Oxygenation Connolly 2010	*6 men mean age 32. 6 women mean age 32.	*foveal cs at 7 spatial freq measured c 14.1% and 21% at luminances28.0 cd m- 2 v, and at 2.1 cd m -2 and ; 0.26 cd m- 2 . *then mesopic luminances tested again c 100% o2 & 14.1%	*cs- raised thresholds c reducing luminance. * More effect and reduced light & higher freq *respiratory changes= no effect *gender = no effct	*spatial contrast sensitivity exhibits greater resistance to respiratory disturbance than do foveal measures of low-contrast acuity, chromatic sensitivity or dynamic contrast sensitivity at comparable mesopic luminance *supports binocular summation
Low Contrast Acuity at Photopic and Mesopic Luminance Under Mild Hypoxia, Normoxia, and Hyperoxia conolly 2009	*6 men 6 women mean age 28.3 *non smokers	*binocular contrast acuity assessment test (landolt c) at fovea & locations up to 5* from fixation under 14.1%, air & 100% o2	* Statistically significant effects of breathing gas at 12 cd m- 2, 1 cd m-2, and 0.1 cd m-2 *At 12 cd m-2 the younger subjects in the this study show greater sensitivity than 'photopic standard'. Hypoxia elevated CAT relative to performance under hyperoxia	* Hypoxia caused the contrast thresholds to increase at all light levels, but particularly at mesopic luminance. Hyperoxia decreased contrast thresholds, but only at the lowest light level. * 14.1% req 26% more stimulus contrast at low photopic (12 cd m-2)

			*Relative to normoxia, low contrast acuity was impaired by hypoxia and enhanced by hyperoxia, both effects maximal at the fovea.	and upper mesopic (1 cd m-2) luminance to maintain the same level of VA. * At 0.1 cd m-2 hypoxia necessitates 50% more contrast to maintain acuity in the mid-mesopic range, relative to the sensitivity that is achievable with 100% o2.
Evaluation of visual field parameters in patients with chronic obstructive pulmonary disease Demir 2012	*29 healthy controls *38 COPD pxs *11 COPD current smokers	*Visual evoked potentials (VEPS), visual fields central 30-2 standard (SAP) & short wavelengtl (SWAP) *Bino testing but only 1 reliable field assessed	*No sig effect of tobacco and visual field parameters in COPD pxs. *optic nerve VEP P100 latency to be prolonged in COPD group. *SWAP and SAP mean deviation, pattern standard devition & corrected pattern st dev all stat different between COPD and controls.	*Hypoxia in COPD seems to effect retina and optic nerve
Color discrimination under chronic hypoxic conditions (simulated climb "Everest-Comex 97"). Bouquet 2000	*8 healthy males mean age 26.5	*24 pairs of identical or different coloured squares. Colour matching task. . Luminance & saturation were constant and set up to 127 units and 255 units. *pxs acclimatised to confinement (phase 1), res and acclimatisation to hypoxia (phase 2), bought to 8,848 m over a 31-day period (phase 3) Experiments performed in phase 1 and 3.	*Data collected in terms of errors *alterations in color discrimination increased slightly, but significantly, as altitude increased Independent of color, hypoxia led to significant alterations in color discrimination at altitudes of 8,000 m and 8,848 m. *For the entire duration of the experiment, I1 and 3 errors were found in the red and blue discrimination, respectively. No impairments were found in the green discrimination. *impairments in color discrimination only occurred at 8,000 and 8,848 m altitude in the blue discrimination.	*Although color discrimination was affected only slightly by our hypoxic experimental conditions, our results clearly evidence impairments of color discrimination in both the red and blue ranges that increased significantly as altitude increased. In contrast, no alteration of color discrimination was found in the green range. *red and green range results show the percentage of subjects showing alterations in color discrimination and the index of impairment increased significantly as altitude increased

Effect of Hypoxia on Simultaneous Visual Contrast. Bridges 1964	*7 healthy subjects age 19-35	Instrument provides gray test targets on back-grounds of variable luminance to induce contras phenomena, subjects asked to match the brightness of the R spot with that of the L spot. *This was done with 20%o2/ 10% o2 then 20% again	*During pre-hypoxia the threshold gradually decreases *During hypoxia and gradual increase in luminance is req to subjectively match R with L. *The posthypoxia period showed the greatest elevation of threshold. significant (p=0.02) when compared with hypoxia mean	* the difference in luminance between left and right gray spot can be used as a measure for the inhibitory effect occuring during hypoxia. *assumption- during hypoxia the subject has decreased sensitivity to luminance differences of the spots and their respective backgrounds. A luminance increase is req to maintain subjective brightness match of the two spots. An increase in the contrast threshold in conditions of lowered oxygen partial pressure has occurred.
Oxygenation State and Twilight Vision at 2438 m. Connolly 2011	*6 men mean age 30.6 6 women mean age 26.8	*3 experiments- bino contrast Acuity Assessment test (lanolt c, low contrast) stimuli at the fovea, 6 1.25°, 6 2.5°, and 6 5°, the mono FDT & bino CAD test. Experiments were conducted near sea level breathing 15.2% oxygen & 100% o2 *all carried out at at 0.1 cd m-2 and 12 cd m-2	* Oxygenation state was a statistically significant determinant of visual performance on all three visual parameters at mesopic, but not photopic luminance.	* The results indicate that twilight vision may be susceptible to conditions of altered oxygenation at upper-to-mid mesopic luminance
Mild hypoxia impairs chromatic sensitivity in the mesopic range. Connolly 2008	* 6 men mean age31.7 6 women mean age 26.1.	*CAD test was used to measure binocular and monocular R-G and Y-B chromatic sensitivity by using dynamic luminance contrast noise to isolate the use of color signals at 14.1% o2, 21%o2 (study 1) and then 14.1% and 100% o2 (study 2)	* Light level, number of viewing eyes, and oxygenation state were significant determinants of chromatic sensitivity.	* In the mesopic range, mild hypoxia impairs chromatic sensitivity progressively with reducing luminance. Binocular summation of chromatic signals is consistent and independent of the luminance channel
Color vision at very high altitude Leid 2001	*5 subjects over 2 expeditions both eyes- all seasoned climbers	*desaturated D15 used monocularly on first expedition (1997) yielded results at 5,300, 5,700, 6,450, and 7,000 m, on 2nd Data collected at 5,200 m	*10 eyes were tested at 6,500 m. Two tetartan abnormalities were observed.	*In the trained individuals, and after sufficient acclimation, severe hypoxia due to very high altitude and exacerbated by physical effort does not lead to major disturbances of colour perception detected by the desaturated D15 test.

***Oxygenation state and mesopic sensitivity to dynamic contrast stimuli. Connolly 2009**	*6 men 6 women mean ages 30 yrs	* Mono threshold sensitivity to frequency-doubled contrast stimuli assessed after bleach and dark adaptation, breathing 14.1% o2, 100% o2, and air viewing at background fields of 10 cd/m2 and 1 cd/m2.	*At low photopic luminance (10 cd/m2), sensitivity was marginally enhanced when breathing 100% oxygen. At mesopic luminance (1 cd/m2), sensitivity was consistently worse with hypoxia and greatest with o2 at all eccentricities and in all field quadrants,	* supports outer retinal (photoreceptor) susceptibility to hypoxia under twilight viewing
***Oxygenation and gender effects on photopic frequency-doubled contrast sensitivity. Connolly 2008**	*6 men 6 women mean ages 30 yrs *All 6/6 va c correction if req *	*mono threshold sensitivity measured breathing 14.1% o2, 100% o2, air and under hyperventilation to induce hypercapnia using frequency doubling perimetry (random gas order) 2 min washout	* The effect of hypoxia was statistically significant for the 'inner'data ($p = .001$) and almost statistical significance for the 'outer' data ($p = .051$). The apparent slight effect of hypocapnia to enhance sensitivity at the 'fovea' was not statistically significant. *For field quadrant anaylsis- a reduction in threshold sensitivity under mild hypoxia, no sig of hypercapnia. * greatest overall sensitivity during the experiments in which exposure to 100% oxygen immediately preceded exposure to hypoxia, and poorest sensitivity when hypoxia immediately preceded 100% oxygen,	* clear effect of exposure order to influence the overall sensitivities achieved with each breathing gas-prior hypoxia diminishes the benefit of subsequent oxygen & prior 100% oxygen mitigates the impairment of subsequent hypoxia * male sensitivity greater than female sensitivity over the nasal hemifield. * relative to normoxia, female sensitivity is slightly but consistently enhanced by 100% oxygen in all quadrants, male sensitivity is not. Hypocapnia appears to enhance male and female sensitivity on the nasal side of the field, but not the temporal, in comparison to the normoxic, normocapnic control exposures. * Mild hypoxia, equivalent to breathing air at only 3048 m, degrades temporal contrast sensitivity beyond the fovea under good viewing conditions

The effect of hypoxia on visual function. Psychophysical studies. Ernst 1971	*3 subjects 24-34 yrs old	*The fixation target was a projected circular re light which subtended one- third degree on the retina. *Test target sizes12' ½ deg, l° 3°, and 5° of arc on the subject's retina. Nasal field eccentricities at 5° or 45° from foveal fixation on the horizontal midline used. *The color of the test light was either white, yellow, red, or blue *measured under 10% o2	*mean percentage increase in the absolute visual threshold secondary to hypoxia greater at (45° eccentricity) than at (5° eccentricity) for all four (12', 1, 3, 5 deg) target sizes. *Red target threshold (cones) was affected by hypoxia to a greater extent than was the blue target threshold at 5 deg. *Hypoxia elevated cone seg of dark adaptation after 4 mins, rod-cone break time unaffected. During the early four minutes there was no change, but hypoxia elevated the later curve.	*hypoxia has a greater effect on peripheral rod thresholds than on central rod thresholds therefore hypoxia may cause a greater ischemia of the peripheral retina than of the central retina. *Red-light absolute thresholds thought to relate mostly to cone function at 5° retinal eccentricity, were affected more by hypoxia than blue-light absolute thresholds, thought to relate mostly to rod function in this area- why this is the case is not clear. *1st 4 mins of rod and cone adaption not effected by hypoxia- thought to be due to different processes- in early thought to be due to neural processes, later, photochemical processes
Effect of simulated altitude on the visual-fields of glaucoma patients and the elderly Falk 1991	*3 subject groups 6 glaucoma, 12 age matched normals, 6 normals under 36.	* Humprey central 24-2 in each 3 groups at ground level and 3000m. *1 eye – for glau eye with more clearly delineated depression. *hypobaric chamber used 50% tested ground then 3000m 50% 3000m then ground.	*Older subjects had lower sensitivity than younger- altitude had no effect on these sensitivities. *For glau group altitiude did not effect mean sensitivity for any type of point.	*No increase in regulations of the FAA medical standards req.
Local neuroretinal function during acute hypoxia in healthy older people. Feigl 2008	12 participants 2 groups, Younger group mean age 28+/- 4 Older group 55+/-5	mfERG slow flash to measure bipolar cells 50 degrees retinal field at 35 s cm (c rx if req) monocular c medical air and 12% o2	Effect of hypoxia on amplitude n1-p1 greater in older group at all locations. Effected p1 implicit times more than n1 (longer in older group) but no effect c o2 Amplitudes effected more centrally than peripherally c o2 in younger group	Central neuroretinal function was sig reduced during hypoxia at all ages Was a sig reduction amplitude and an increased implicit time in ops c age but not hypoxia but this was not the case in all areas Frequency of ops not effected c age therefore c age neurons can maintain temporal precision but req a greater stimulus energy to generate a response

			All locations effected by o2 in older group Age caused change in ops c age but not hypoxia	
Color vision changes in young subjects acutely exposed to 3,000 m altitude. **Karakucuk** **2004**	*Sixteen students, ages ranging between 14 and 17 yr,	*Farnsworth- Munsell 100-Hue (FM-100 Hue) test conducted at 1060 and 3000 m above sea level under photopic conditions	*Total number of errors (all 4 sectors together) was 132 at 1060 m and 194 at 3000 m out of 1344 possible correct. *significant increase in error numbers in sectors 1 and 3 indicated that the deterioration was significant in the blue-yellow range.	*moderate altitude can adversely affect the total number of errors in an acclimatized group of young people in an ideal photopic environment (3000 m) on FM-100 Hue testing
Hypobaric hypoxia reduces the amplitude of oscillatory potentials in the human ERG. **Janáky 2007**	*14 males mean age 39.7 yrs	* rod-(scotopic) ERGs, cone-(photopic) ERGs, maximal responses and oscillatory potentials (OPs)) and 30-Hz flicker ERGs were recorded *recorded after 20 mins dark adaptation, then during 15 min hypoxic experience equivalent to 5500m	* No significant changes in a or b waves * The OPs of the ERG, revealed a significant decrease in amplitude during hypoxic exposure. Both OP1 and OP2 amplitudes were significantly different. OP3 and OP4 not effected *From the initial mean ± SD value of 98.12 ± 0.83% it decreased to 82.75 ± 2.81%	* results suggest that the OPs are the most sensitive indicators of an acute, mild hypoxic challenge.

The effect of acute hypoxia and hyperoxia on the slow multifocal electroretinogram in healthy subjects. **Klemp 2007**	* 10 subjects	*Pupils were dilated to 7 mm. *Stimulus array contained 61 hexagons scaled by eccentricity (scaling factor 12.857) displayed at a frame rate of 75 Hz.	*Mean P1 amplitudes were significantly reduced during hypoxia compared with normoxia. *the amplitude reduction of first-order N2 decreased from 33.0% centrally to 18.3% at the highest eccentricity *hypoxia reduced the average first-order N1 amplitude by 9.5%. Hypoxia also reduced mfOP amplitudes, by 16.6% to 34.8%, but no effect of eccentricity. Hyperoxia had no significant effect on amplitude. * hypoxia nor hyperoxia had any effect on the latency of the P1 implicit times.	* with the mfERG, we have demonstrated profound regional variations in the sensitivity of the retina to hypoxia under photopic conditions in healthy subjects, the fovea being by far most sensitive and the amplitude reduction being prominent, while the implicit time is very nearly un-changed.
Multifocal electroretinography changes in the macula at high altitude: a report of three cases. **Pavlidis 2005**	*3 healthy climbers aged 31, 52 and 67 years.	*MF ERG 61 hexagonal elements displayed on a color monitor driven at a frame rate of 75 Hz. *Physiological parameters indicative of acclimatization were compared daily during the expedition at altitudes between 500 m and 5,05(m: hemoglobin, oxygen saturation,	*The central macula MF ERG responses were significantly reduced 1 week after high-alitude exposure, and had recovered by the follow-up examination performed during the following week	*MF ERG was found to be more susceptible to hypoxia-induced changes in the nonlinear dynamics of the eye than standard ERG. *High-altitude hypobaric hypoxia effects retinal function before clinical signs.
Mild systemic hypoxia and photopic visual field sensitivity. **Feigl 2011**	* 7 men 7 women mean age 20.9	*Medmont M700 Perimeter flicker perimetry used 61 locations concentrically arranged at 1°,3°,6°,10°,15°,22° and 30°. *Two static and two flicker tests were performed during normoxic and 12% o2, in a randomized order.	*Under photopic illumination, flicker and static visual field sensitivities at all eccentricities were not significantly different between hypoxia and normoxia conditions. The mean defect and pattern defect were not significantly different for either test between the two oxygenation conditions	*Preliminary visual field data from a small sample of healthy participants (n = 3) measured under mesopic illuminations suggests that perimetric sensitivity is significantly reduced at all eccentricities during hypoxia. *Although metabolic demand increased c flicker, under photopic conditions it is not altered by hypoxia

Visual Fields During Acute Exposure to a Simulated Altitude of 7620 m Horng 2008	*15 males mean age 31.4	*visual fields in 3 different zones central zone (0° – 10°), the moderate peripheral zone (10° – 20°), and the peripheral zone (20° – 30°)	*Sao2 % dropped to 64.0 6 5.4% within 3 min. *Mean visual sensitivity was significantly reduced by 7.2 6 1.6 dB. Furthermore, peripheral sensitivity was significantly more diminished than central sensitivity.	*Reduction in periphery may be due to rod demands as this testing tested rods and cones.
The effect of high- to low-altitude adaptation on the multifocal electroretinogram. Kofoed 2009	*8 healthy residents 3600 m above sea level were examined over 72 days after arriving at sea level. Control group of 8 healthy lowlanders.	*mfERG pupil dilation to a diameter of 7mm *An array of 103 eccentricity-scaled hexagons was displayed at a frame rate of 75 Hz.	*The highlanders' summed mfERG amplitude was 43.1% larger than the lowlander reference for N1, 47.1% for P1, and 59.9% for N2. On day 72, the differences between the two groups were increased to 73.2%, 76.0%, and 87.0% for the three amplitudes. *These observations were consistent throughout the stimulated field, when tested by eccentricity. *Implicit times were comparable between highlanders and lowlanders throughout the study.	*hematologic responses were observed in our study, with a decrease in hemoglobin (10.6%) erythrocytes(13.6%), and hematocrit (14.6%). *The nearly identical mfERG waveforms throughout study suggest the effect of relative hyperoxia and acclimatization was the same on all components of the mfERG. *Future studies should extend to 1 year and include baseline examinations, inc examinations of retinal structure.
Effects of hypoxemia on the a- and b-waves of the electroretinogram in the cat retina. Kang 2000	*Cats	*Responses to bright flashes of diffuse white light were recorded at 3-minute intervals during hypoxemic episodes lasting 15 minutes to 2 hours, flash intensity was varied over 6 log unit by means of neutral density filter. *To test for a cone contribution, responses were obtained to short-wavelength flashes and long-wavelength flashes	*During mild hypoxemia small changes in the a wave amplitude were detected but did not increase with further hypoxemia *The a-wave is more resistant to severe hypoxemia than the b-wave. This implies that photoreceptor transduction works almost normally during hypoxemia and that failure of inner retinal PO2 causes a decrease in the b wave (35%)	*Both a- and b-waves were resistant to hypoxemia, but the b-wave was more affected by low PaO2. Poss when the b-wave decreases, it is from a failure of the retinal circulation to maintain oxygenation of the inner retina. *It now seems necessary to postulate that when oxidative metabolism is affected, the photoreceptors switch more to glycolysis for energy production

Hyperoxia improves contrast sensitivity in early diabetic retinopathy Harris 1996	*12 insulin dependent dia (diag at least 5 yrs) (23 mean age) no or grade 1 dr. *12 normals (mean age 26)	*contrast sensitivity tested using CSV equiptment 3 6 12 18 cpd spatial freq tested, under normal conditions and breathing 100% o2 *also measured blood flow dynamics using scanning lazer ophthalmology	*diabetics had reduced sensitivity at 12 & 18 cpd c room air, when breathing o2 the reduction at 12 cpd was improved to normal values, there was improvement at 18 but less than 12.	*result suggests that some factor linked to retinal tissue hypoxia (or pseudohypoxia) causes the contrast sensitivity decline seen early type 1 diabetes * when healthy tissue is perfused with blood with an increased o2 content vasoconstriction ensures constant o2 delivery, in dia this constriction did not occur
Hypoxia, color vision deficiencies, and blood oxygen saturation. Hovis 2012	*All male, *Colour normal group- mean age 33.3. *Colour defective group mean age 37.6	*Cambridge colour test (CCT), Colour assessment and diagnosis (CAD) test and cone specific contrast test (CSCT, mono) performed at ground level and 3780m.	*CCT-no sig effect of altitude of sig interaction involving altitude *CAD- stat. sig altitude effects and interactions involving altitude, NCV group had increase in red-green threshold. Dichromats, larger change in blue-yellow compared to red-green. Anomalous trichromats increases in both *CSCT- no stat. sig altitude main effect involving altitude. *No relationship between results of CAD, CSCT or for grey or yellow background of CCT, but blue-yellow CAD effected by age (positive) time in chamber and SaO2 (neg)	*Any change in chromatic threshold at low photopic light levels at 3700 is likely to be small (10% increase) - consistent with the hypothesis that mild hypoxia is equivalent to a slight reduction in retinal illumination. *For CAD blue-yellow thresholds, for unexpected finding that a higher SaO2 resulting in a larger change in threshold poss accounted for by subects with lower SaO2 having lower blue-yellow thresholds relative to the ground condition.

Diurnal-variations of acute mountain-sickness, color-vision, and plasma-cortisol and acth at high-altitude. Richalet 1989	*8 healthy male lowland inhabitants. *mean age 31 *All normal cv	* 2 anomaloscopes- red-green axis. Chromotest- mono, using flickering mixed light *CV testing and AMS scores preformed every 2/24 between 8-20:00hrs. *Experiments performed and normoxia (N) and 4350m. Hypoxia days H1,H1, H2 H3.	*No sig difference in any parameter between any hypoxic day. *Between N and H days- sig difference for both tests. Decrease in sensitivity to green in favour of red was found. *In H both tests showed sig diurnal variations with highest scores in am compared to pm, also a sl increase at 18:00 and 20:00.	*both anomoloscopes are practice for field studies, to explore cv in red-green axis- it is a simple and objectable way to quantify AMS symptoms. *with hypoxia- both showed a decrease in sensitivity to green compared to red *Alterations in CV were clearer in am and values tended to reach normoxic levels during the day.
Contrast sensitivity as an early indicator of acute mountain sickness. Nordmann 1991.	*12 healthy subjects, age 29-41.	*bino contrast sensitivity at 7 freq from 1.5-40 cycles per degree. *tested at 5400m on arrival to base camp and also at base camp after trekking up to 8000m	*Decrease of cs seen constantly in all subjects after arrival at base camp *Impairment most evident in low and med spatial freq (-2.0 to -4.9db) Rtned to normal values after 36/24. *No changes from 5400m to 8000m. *1 subject suffered ams- these cs measurements didn't rtn to normal until 10mg pred administered.	*Cs measurements could give a quantitative evaluation of brain and retinal disturbances caused by hypoxia.
The contrast sensitivity of cat retinal ganglion cells at reduced oxygen tensions. Enroth 1980	* 31 adult cats- 19 for RGC recordings, 7 for retinal oxygen tension and 5 for both	*Extensive- see text	*Centre and surround of ganglion cells very resistant to hypoxia, maintaining a normal sensitivity until the arterial oxygen tension was 35mmHg or less.	*Retinal O2 tension well regulated over a wide range of arterial O2 tensions, corresponding well to the range over which ganglion sensitivity is normal *Ganglion cells resistant to hypoxa
Effect of hypoxia on FM 100-Hue test performance. Smith 1976	* 5 subjects (3 for 1670 lux, all 5 for 37 lux) *All normal colour vision	*Bino FM100 testing under 1670 lux and 37 lux each test lamination run twice- once breathing air and once with 10% O2. (starting gas varied)	*Under standard illumination 1670 lux hypoxia caused no increase in total error scores. *Under 37 lux 2 subjects showed tritan axis *Inter-observer variability emphasized with 37 lux.	*Stable oxyhemoglobin may not have been reached at beging of hypoxic testing as errors increased slightly on end run with hypoxic gas at 37 lux. *All errors showed along tritan axis. *considerable inter-observer variability in the errors on the tritan axis under

			*All subjects showed an increase in errors with 10% O2 t 37 lux.	reduced illumination, inc further by hypoxia. *attributes results of tritan defect to hypoxia being an insult to inner retina.
Color vision in the tritan axis is predominantly affected at high altitude. Tekavcic-Pompe 2009	*14 climbers- 1 female mean age 52.4. *All no cv defects.	* CV testing with Mollon-Reffin Minimalist tes at 300m (28 eyes) 1300m (28 eyes), 4000m (28 eyes), 5400m (8 eyes) and 300m 3 days later (24 eyes) and at300m 1 yr later (24 eyes) All tests performed under good conditions	* at 300 m, all eyes had normal color vision in all 3 axes. At 1,300 m, 100% of eyes showed normal color vision in the protan and deutan axes, while 25% showed minimally reduced discrimination in the tritan axis. At 4,000 m, 100% showed normal deutan axis, 4% minimally reduced protan axis, & 72% minimally reduced tritan axis discrimination. At 5,400 m 100% eyes tested showed normal protan & deutan axis discrimination, 75% showed minimally and 25% moderately reduced tritan axis discrimination. At 300 m 3 days after rtn, 100% showed normal deutan, 4% minimally reduced protan, & 38% minimally reduced tritan axis discrimination.	*Tritan colour axis predominantly affected at high altitude- but this is transient. *Changes in tritan axis discrimination correlated well with decreased O2 saturation.
Effects on colour discrimination during long term exposure to high altitudes on Mt Everest. Willmann 2010	* 2 healthy male subjects age 32 and 46.	*mono Cambridge color test after 30 mins dark adaptation, tests performed under mesopic conditions. * Control measurements were taken 1/52 prior to and 6/12 after the expedition. Measurements were taken in Kathmandu (1300 m) at 3450 m,	* With increasing altitude, colour discrimination thresholds were found to rise, predominantly for the tritan axes in both observers. Deutan (green) thresholds were minimally elevated at high altitude, whereas protan (red) was altered in one	* effect of chronic hypoxia on discrimination along the tritan axis suggests a selective vulnerability of S cone compared with L or M cone pathways, S cones may be particularly sensitive because of their relatively low

		4410 m, 5060 m, 5300 m, 6450 m, 7200m and 8000m over a period of 54 days	observer. Tritan colour discrimination thresholds decreased as a function of time spent at a given altitude.	abundance: only 5-10% of all cones are S cones. * Hypoxia decreases the maximum response rate of all cone pathways equally; however, as the number of S cones is limited, their absolute responses might become undetectable at earlier stages.
Dark adaptation during systemic hypoxia induced by chronic respiratory insufficiency. **Thylefors 2009**	*13 subjects- 6m men, 7 women mean age 68.7 yrs *All on long term O2 therapy for at least 4/12	*Dark adaptation measured in 3 visits, visits 1 and 3 with pxs normal O2 supplied, visit 2 with no O2 for 30mins to 8 hrs.	* No significant differences were observed between the three visits for the right eye, the left eye, or both eyes *Sig differences between visits 1 and 2 for Pao2, arterial o2 saturation and pulse oxymetry * dark adaptation was normal for their age and unchanged in all pxs * oxygen treatment made no difference, despite a significant difference of at least 5.8% in arterial blood oxygen saturation	*pxs had normal dark adaptation for their age. * The result may partly reflect the influence of PaCO2 on the lumen of choroidal & retinal vessels. At high altitudes, with hypocapnic vasoconstriction the o2 supply to the retina is further compromised, resulting in reduced dark adaptation. The authors hypothesize that respiratory insufficiency with hypercapnia or normocapnia will have larger choroidal and retinal vessel lumens, added to by further dilation of retinal vessels during hypoxia. The tentative net effect would be preserved dark adaptation
The effects of hypoxia on contrast sensitivity. **Rossi 1985**	Not reported	*Cs measured at 1.5,3,6,1,218 cycles per degree at 45 cd/m2 *measured pre, at and post 15,000ft altitude using a hypobaric chamber.	*No losses in cs which would be expected to produce sig performance related loss in visual capability. *Small individual differences in loss patterns for cs and spatial freq	

Effects of exposure to simulated altitudes on visual fields, contrast sensitivity, and dazzle recovery. **Yap 2005**	*12 males, age 19-30 *All no ocular/medical history *All vas 20/15	*Decompression chamber used to simulate altitudes sea level, 7000 ft, 12000 ft in random order. *At each altitude visual fields for 60 degrees, spatial CS (1.5,3,6,12 cpd), temporal CS (2,4,8,16,32 cps) (all re) and macular photostres test (le) performed.	*haemoglobin sat 99.5 at sea level, 96.8 at 7000ft, 91.2 at 12000ft. *no change in visual fields with altitude *No sig difference in spatial freq for any altitudes at 3,6,12, but sig difference at 1.5at 7000 (not relevant) *no sig difference in temp contrast sensitivity. **no sig difference in dazzle recovery.	* Results in line with other papers, in which va is uneffected up to 12000 ft, and fields to 26,240
Modification of color-vsiosn in the red green axis in acute and chronic hypoxia explored with a portable anomaloscope Rcihalet 1988	*8 subjects, mean age 35. *All normal cv	*chromotest conducted at sea level, in acute hypoxia after 2/7 at 4350 (h1), subacute hypoxi after 7/7 between 1000-4800m and 3/7 at 4800r (h2) and chronic hypoxia after 3/52 at 4800m. *2 measurements each eye.- result is mean	*Sig decrease in g sensitivity and increase in g were found in all hypoxic conditions. *No sig difference between hypoxic conditions *No correlation between age and variation in g,r or g/r values. *Variation in sensitivity related to arterial o2 saturation	*Decrease in green sensitivity related to hypoxia.
Operation Everest 2: Lack of an effect of extreme altitude on visual contrast sensitivity. Kobrick 1988.	*8 males, ages 21-31 yrs. * All no ocular/medical history.	*decompression chamber used to an altitude of 25000ft over 40/7 (all external factors controlle ie cold, dehydration). CS measured by Vistech test, testing 1.5,3,6,12,18 cpd daily	*Highest cs sensitivities for the midrange freq 3,6,12. *Altitude exposure had neither an overall;l effect on cs, nor separate effects within the respective spatial freq ranges. *Performances for individual subjects were all similar and had good continuity.	* CS was affected only slightly, if all, by the hypoxic conditions of this study. *All subjects were acclimatised which may have effect on results. *Small number of subjects

Short-term hypobaric hypoxia enhances visual contrast sensitivity. Benedek 2002	*14 healthy males *Mean age 32	*CS measured after 5,10,15 mins of hypoxia in hyperbaric chamber at 5500m *CS measured at 0.5,1.2,1.9,2.9,3.6,4.8,5.7,7.2,14.3 cpd *temporal cs also measured at 8hHz	*for both static and dynamic cs there were negative correlations with blood o2 saturation at low and med spatial freq *sig increased cs values in hypobaric hypoxic conditions	*early visual processes may be enhanced during a short hypoxic challenge, a marked inc was observed after only 5 mins of altitude exposure, which was sustained over the 15 mins. *it may be that mild transient hypoxia increases sensitivity, whereas prolonged choric leads to visual loss.
Respiratory effects upon the visual threshold Wald 1942	* Not noted	*Monocular dark adaptation with artificial pupils *anoxia of short and long duration (8-11%o2) *hyperventilation by breathing to beat of a metronome	*anoxia threshold rose within 1 to 10 minutes of anoxia to new levels 0.2 to 0.5 log unit above normal 0.35 ave. *On increasing the rate of breathing room air by 50 to 100 per cent the threshold fell, usually within 5 to 10 minutes, to about half its normal value *Due to variations in breathing patterns subjects yield characteristically different responses on sudden exposure to low oxygen tensions with breathing uncontrolled. The threshold may either rise or fall; and on release from anoxia it may rise, or fall to normal or subnormal levels.	*alkalosis induced by hyperventilation, can be abolished or reversed by adding carbon dioxide to the inspired mixtures. *The threshold adjusts to anoxia rapidly; exposures lasting 5 to 6 hours do not produce greater or more persistent changes than those of much shorter duration. *The speed of rod and cone dark adaptation appears to be entirely unaffected by anoxia
The effect of a moderate level of hypoxia on human color vision Vingrys 1987	* 2 subjects age 23 & 46 familiar with decompression.	*FM-100 hue, American optical HRR and Pickford-Nicolson anomaloscope use *Photostress *all tests conducted at sea level and 12000 ft	*no error on HRR plates *FM-100 hue change in error score of 75% with altitude- sl more red green errors *anomaloscope- cv depressed at 12000 ft- grerater loss for blue *No sig change in photostress recovery for altitude	*concluded rod and cones equally affected by mild-mod hypoxia. *Hypoxia effects inner retinal layer- prob ganglions

Effects of hypoxia and acetazolamide on color sensitivity zones in the visual field . Effects of hypoxia sensitivity and acetazolamide field on color zones in the visual Kobrick 1970	*24 males 18-30 yrs old c normal vision *randomised 2 groups acetazolamide or not	*red, green and blue stimulus, threshold determinations at 30 deg intervals *mono c natural pupil *sea level (20.93% O2) 13000ft (12.8%) 15000ft (11.8%) 17000ft (10.9%)	***** No sig effect of drugs *Effect of hypoxia was strong and cumulative- constricting zones of colour sensitivity in the visual field. (intense inverse relationship) *reduction in fields with longer exposure duration. *sensitivity reduced more for red than green or blue	* results match what was expected from purkinje law- selective desensitization to the perception of colour would occur under hypoxia with red affected 1st
The Effects of Variations in the Concentration of Oxygen and of Glucose on Dark Adaptation. McFarland and Forbes 1940	* Normal fasting subjects aged 25-37. *concentrations of oxygen changed by dilution with nitrogen or by inhaling oxygen from a cylinder.	*Low oxygen series in 13.3, 11.4, and 10.0 per cent oxygen corresponding to altitudes of 12,000, 16,000, and 19,000 feet, respectively (6 subjects). II. Control tests in air followed by tests during the inhalation of decreasing concentrations of oxygen going as low as 7.3 per cent oxygen in one case (5 subjects). *Other experiments with glucose	* In 10.1 per cent O, five of the six subjects were poorer in both the rod and cone sections of the curve varying from 0.15 to 0.40 of a log unit. *Dark adaptation curves elevated with increasing hypoxia- but all similar shaped and rate of adaptation unchanged. (rods and cones equally effected)	* extent of the impairment in light sensitivity under low oxygen might serve as a reliable objective test of one's ability to tolerate anoxia * In our opinion, the changes may be attributed directly to the effects on the nervous tissue of the visual mechanism and the brain rather than on the photochemical processes of the retina.
Anoxia and brightness discrimination Hecht 1946	* 7 men 1 women aged 17 and 25 years, apart from 1 aged 51.	*Brightness discrimination measured at 3 field intensities, approx. 1/1000, 1/100, and 1/10 millilambert, (actually 0.00085, 0.0102, and 0.12 millilambert), when breathing gas mixtures between 9 and 16 per cent oxygen corresponding to 7 altitudes (cones only) *Threshold after dark adaptation measured in 2 subjects.	*****The thresholds of night (rod) vision and day (cone) vision are equally affected by anoxia *brightness discrimination begins to deteriorate at fairly low altitudes. It is obvious at 8,000 feet, and becomes marked at 15,000 feet, where at low brightness, the contrast must be increased 100 per cent over the sea level value before it can be recognized.	*The impairment of brightness discrimination with increase in altitude is greater at higher altitudes than at lower. The impairment starts slowly and becomes increasingly rapid the higher the altitude. *Impairment of brightness discrimination varies inversely with the light intensity. It is most evident under the lowest light intensities studied * Since anoxia causes only a shift in log I it is shown that the photochemical receptor system cannot be affected. Instead the conversion of photochemical

				change into visual function is impaired in such a way that the conversion factor varies as the fourth power of the arterial oxygen saturation.
Alterations in dark adaptation under reduced oxygen tensions McFarland 1939.	* 20 subjects 2 female *aged 20-30 one was 42	* biophotometer measured threshold of light sensitivity at 15.7 per cent 02 (7,400 ft.), 13.7 per cent 02 (11,000 ft.) and 11.7 per cent 02 (15,000 ft.). *quincunx used with 5 lights of various luminances	*Dark adaptation curves elevated c reduced o2. 7400ft=0.10 log unit change, 11000ft 0.22, 15000=0.40. *	

10 Appendix II Systematic literature review into the role of hypoxia in the pathogenesis of age-related macular degeneration; Table of included studies.

study	*Participants*	*methods*	*results*	*conclusions*
Foveolar chorodial circulation and choroidal neovascularisation in age-related macular degeneration Metelitsina et al 2008	135 px c AMD but no cnv (193 eyes) Follow up yearly to 5 yrs 28 developed cnv during experiment	Longditudinal, observational, prospective study. Relative foveolar choroidal blood flow. (ChBflow), volume (ChBvol, velocity (ChBvel) vel calculation using laser Doppler flowmetry	Eyes in which cnv (19) developed had a 24% lower ChBvol & 20% less flow. No stat diff in vel between 2 groups. In 14 of 19 cnv eyes was yrly deceases in flow (3-54%) & ave yrly decease of 11.5% Vol decreased in these eyes yrly by 9.6% before formation of cnv. Eyes with no cnv increased flow 6.7% & vol 2.8%	Foveolar vol and flow were sig lower in eyes which developed cnv and the affected eyes chowed a marked chorodial circulatory decreases in the fovea- this was not observed in eyes which did not develop cnv. Therefore these changes preceed the formation of cnv. Eyes with lower flow at baseline are 3* higher risk of 3+ lines of visual loss than eyes with a higher circulatory parameters.
Choroidal blood flow & progression of age-related macular degeneration in the fellow eye in patients with unilateral choroidal neovascularisation Boltz et al 2010)	41 subjects with AMD (18 female) 26 had hypertension, 18 current smokers, 9 past smokers.	3 yr study, screening visit + 7 study visits every 6/12. BP, pulse rate (PR) hemodynamic parameters & IOPs measured.	Stage of ARM (higher value worse), subfoveal choroidal blood flow, fundus pulsation amplitude (all lower value worse) all stat sig influence on development CNV. Group which developed CNV-decline of hemodynamic values over time, non CNV group showed no changes to flow or FPA.	Lower the chorodial perfusion, higher the risk for CNV
Dark adaptation in age-related maculopathuy Brown et al (1986)	4 pxs c ARM 5 normals	0*/5*/10*/15*/ 25* target red or green lights flashing at 1Hz.	*Sig longer time constants for rods in ARM, but no diff in cone recovery time. *Deficiencies in final thresholds for rods and cones.	Time constants of rods portions of recovery curves. Depression of rod sensitivities out to 25*

The association between drusen extent and foveolar choroidal blood flow in age-related macular degeneration. Berenberg 2012.	*239 eyes of 157 pxs c amd but no neovasc	*ChBVel (measured) ChBVol (measured) and ChBFlow (calc) * no of drusen, individual druse area and total druse area meaused c combination of software and human readings, with good repeatability	*A significant inverse relationship between total drusen area and ChBVol was found. Larger average druse area sig associated with decreased ChBVol * Average druse area was also significantly inversely related to ChBVol * No of drusen did not effect circulatory measurements	* eyes with a larger extent of drusen, as measured by total drusen area & average druse area, tend to have decreased ChBVol & ChBFlow. * association between total drusen area & choroidal blood flow and vol was strong after age adjustment less strong for choroidal blood flow and volume. *Cannot conclude if decrease in choroidal blood flow causes the development of drusen or if the presence of drusen leads to decreased blood flow
Color Doppler Imaging Discloses Reduced Ocular Blood Flow Velocities in Nonexudative Age-related Macular Degeneration. Ciulla et al 1999	*25 normals mean age 70.2, 3 pxs c non-exudative amd mean age 73.	*Ophthalmic artery measured at 25mm behind globe. *short posterior ciliary arteries examined 10-20mm behind globe. *Central retinal artery examined 10mm behind globe. * Peak systolic velocity is the highest blood velocity achieved during systole, and it is calculated from the frequency of the peak in the Doppler-shifted waveform. End diastolic velocity refers to the lowest velocity occurring during diastole, and it is calculated for the frequency of the trough in the waveform.	* Post ciliary arteries in subjects with ARMD showed a consistent trend toward lower peak systolic velocity and end diastolic velocity. Especially in nasal posterior ciliary artery highly statistically significant almost sig for systolic. *Temp artery, nearly sig lowering diastolic, no sig change in systolic, no change in resistance. *No correlation between these changes and % macula area affected by macula changes. * In the ophthalmic artery, no affect AMD peak systolic velocity, end diastolic velocity, or resistance * Central retinal artery showed highly sig lower end diastolic	* A primary perfusion defect in choroid could account for some of the physiologic and pathologic changes in AMD. * Reduced blood flow in the nasal and temporal posterior ciliary arteries. Specifically, in subjects with AMD these changes, along with no change in resistance is consistent with reduced bulk flow in these ciliary vessels, suggesting that choroidal perfusion is abnormal in this form of AMD. * Was a perfusion deficit in the central retinal artery of the AMD group. Poss due to a generalised perfusion abnormality, or a

			velocity & the resistive index was higher in the ARMD group, no sig difference in systolic.	2ndary autoregulatory response to a primary change elsewhere.
Choroidal perfusion perturbations in non-neovascular age related macular degeneration. Ciulla et al 2002	*21 non-exudative AMD subjects, 21 normals	*Scanning laser ophthalmoscope indocyanine green (ICG) angiograms analysed by a new area dilution analysis technique. Four areas in the perifoveal region and two areas in the temporal peripapillary retina were evaluated by producing a graph of intensity of fluorescence of each area over time. The mean of the filling times and the heterogeneity of the filling times were assessed.	*Means of the filling times within the perifoveal regions and the hetereogeneity of the filling times between regions within the same eyes were significantly greater in the AMD patients. * The spread of 10% filling times was greater in AMD patients than in normal subjects for the four perifoveal regions.	* Objectively demonstrates delayed and heterogeneous filling of the choroid in patients with non-neovascular AMD with some perifoveal region specificity. *The haemodynamic heterogeneity is measured within each eye and not between subjects; consequently, this stat sig result does not originate from differences in AMD manifestations between subjects, but from increased haemo-dynamic heterogeneity between the analysed regions of the choroid in an AMD subject compared with a control subject. *These differences were most pronounced in the perifoveal regions
Hypothermia reduces secretion of vascular endothelial growth factor by cultured retinal pigment epithelial cells. Coassin et al 2010	Cell cultures	*ARPE-19 cells were grown in culture for up to 5 days under normoxic (20% O2) and hypoxic (1% O2) conditions at temperatures ranging from 27Cto40C. VEGF levels in the media were measured by ELISA and cell metabolic activity was	* ARPE-19 cell VEGF secretion was reduced by 38% at 34C compared 37C & ARPE-19 cell VEGF secretion was increased by 32% at 40C compared with 37C. Hypoxia increased ARPE-19 cell VEGF secretion by 84% at 37C. However, hypothermia decreased the hypoxia-induced increase of ARPE-	*Hypothermia decreases VEGF secretion and cellular metabolism in ARPE-19 cells in temperature-dependent fashion. Effects of hypothermia are cannot be obtained by pharmacological agents that slow cellular metabolism.

		measured by a fluorescent cell metabolic assay	19 cell VEGF secretion by 30%. Thiopental and nicotinamide were able to reduce RPE cell metabolism but not VEGF secretion.	*Hypothermia may have therapeutic effects by reducing the metabolic activity of the RPE and the energy req by the PRS, and by reducing VEGF secretion
Vascular cell-adhesion molecule-1 plays a central role in the proangiogenic effects of oxidative stress Dong et al 2011	*Mice deficient in superoxiude dismutase (which have inc oxidative stress) and compared with wild mice with ischemic retinopathy	See article- extensive	*The addition of excess oxidative stress to ischemia increased capillary non- perfusion which was blocked by VCAM-1 antibody. *Of six HIF-1–regulated genes examined, all except EPO showed a significant increase in expression when oxidative stress and ischemia were combined and the increase was blocked by treatment with anti- oxidants	*NV by oxidative stress is mediated primarily by factors other than VEGF. *Of six hypoxia-regulated genes tested, only VEGF was not up-regulated by the addition of excess oxidative stress to ischemia. *VCAM-1 plays an important role in this increased susceptibility to severe NV and neutralization of VCAM-1 may provide an adjunct to VEGF antagonists for treatment of retinal and choroidal NV
Quantifiying changes in RPE and choroidal vasculature in eyes with Age-related macular degeneration. McLeod et al 2002.	*6 eyes of 3 postmortem subjects. 2 with non-exudative AMD, 1 control with no AMD signs.	*The choroid, with the RPE was dissected from the sclera and incubated for APase activity. Pigment was partial bleached with H2O2. The choroid was then flat embedded and sectioned after image and morphometric analyses. Quantitative computer assisted morphometric analyses of all samples were compared.	* In normal eye 99-100% of RPE intact. * correlation between % surviving RPE in a given region with % vascular area decline with GA. *Regions of GA with complete RPE atrophy had substantial loss of choroidal BVs. * Significant linear relationship between the % of RPE covering an area and the density of choroidal bvs in that region.	*Vascular density decreased in the area of atrophy. *Areas of choroid devoid of RPE remained spome what well vascularised. *suggests that degeneration of the RPE is more advanced than the loss of choriocappilaris, but these results cant be used to draw conclusion about sequence in degeneration of 2 cell types.

			* remaining capillaries had stat sig severely constricted lumens smallest in areas of total RPE atrophy.	
Genetic and pharmacological inhibition of JNK ameliorates hypoxia-induced retinopathy through interference with VEGF expression. Guma et al 2009	Mice exposed to hyperoxia (75% oxygen) from postnatal day 7 to postnatal day 12 then in normoxia until wk 17. Control mice kept in ambient o2	*Mice killed at wk 17 and retinas dissected and stained *Quantification with analysis softwear. *Different assays used- see paper	*Hypoxia enhanced JNK activity and also created ROIs which can lead to JNK activation *JNK1 Does Not Regulate HIF-1 Expression. *These data show that hypoxia can induce VEGF transcription through activation of the JNK1-c-Jun pathway in addition to the well- established HIF-1 pathway.	*Intravitreal Injection of a JNK Inhibitor Prevents Retinal Neovascularization. * JNK1 is a critical factor in hypoxia-driven pathological angiogenesis in the retina. *JNK1 is critical for hypoxia-induced VEGF production. Retinal JNK-dependent VEGF expression occurs independently of HIF activation, and hypoxia stimulates JNK activity though ROS production
Postreceptoral adaptation abnormalities in early age-related maculopathy. Feigl et al 2006	*32 eyes of 32 participants were examined. 20 pxs in healthy control group and 12 participants in early ARM group	*Fast flicker mfERG stimulation- and slow flash mfERG paradigm total recording time of about 7 min per eye.	* Neither the fast flicker nor the slow flash mfERG paradigm itself discriminated significantly between two groups. *There were no significant differences in the N1P1 response densities between the 2 groups. *ARM pxs have a reduced ability to adapt under photopic conditions compared to controls.	*results give better insight into possible retinal mechanisms in early ARM- postreceptoral, hypothesis. * propose that the difference waveform component we extracted detects impaired fast adaptation abnormalities in the early course of ARM.-could reflect ischemic conditions that possibly trigger the genetic predisposition in ARM
Hypoxia/reoxygenation induces CTGF and PAI-1 in cultured human retinal	6 human donor eyes processed within 4 to 16 h after death. The	*RPE cells isolated and immunohistochemistry/ northern blot analysis/ western blot analysis performed	*There were no detectable cell varations in morphology at a microscopic level between cells	* Likely that hypoxic conditions are responsible for the observed CTGF expression in the underlying

pigment epithelium cells. Fuchshofer et al 2009	donors ranged in age between 18 and 71 years.		exposed to ambient air and cells maintained in hypoxic conditions. *Western blot analyses showed increased expression of CTGF protein after 12/ 24' 36 h of hypoxia compared to normoxia. * Cells kept for 12 h under hypoxic conditions showed an increase in extracellular matrix associated PAI-1 expression. *Following hypoxic conditions, there was an increase of HIF-1a mRNA compared to untreated control cells. *Hypoxia for 12 hrs followed by reoxygenation for 24 hrs increased mRNA expression of CTGF and increases the expression of fibronectin and collagen type IV.	RPE of choroidal neovascularization membranes. *Immunohistochemical studies demonstrated elevated extracellular matrix-associated PAI-1 staining after hypoxic conditions, supporting the involvement of HIF-1a in hypoxia-induced PAI- 1 gene expression in RPE cells. *Antioxidants had no influence on the hypoxia-mediated increase of CTGF and PAI-1, but did block the hypoxia/ reoxygenation-mediated up regulation. * Hypoxia & hypoxia/reoxygenation increased profibrinogenic factors and ECM components in cultured RPE cells. *Hypoxia and hypoxia/ reoxygenation might be involved in ECM accumulation in the RPE as seen in early AMD
Antioxidant N-Acetyl Cysteine Protects Retinal Pigmented Epithelial Cells from Long-Term Hypoxia Changes in Gene Expression. Gerona et al 2010	*6 eyes from 6 donor cows- RPE isolated using standard techniques for cellular dislocation and isolation.	*Total RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit. 8Exposed to 3% o2 for 72 hrs. *Controls without treatment were maintained in hypoxic and normoxic conditions.	*After 72 hrs hypoxia p53 expression levels increased, suggests p53 dependant apoptosis induced by long term hypoxia in the RPE. * no significant expression found in either hypoxia or NAC-treated cells for p53 effector gene bax,.	* p53 causes cell death by directly inducing mitochondrial permeability and apoptosis, independent of the transcriptional up-regulation of proapoptotic genes *Cell death produced by hypoxia in RPE may be mediated by

		*10mM NAC added to culture before induction of hypoxia and maintained during hypoxic stimulus.	* CASP8 mRNA showed a sig higher expression level in hypoxia- but not in NAC-treated cells.	initiator caspase 8, as values for CASP8 were also increased under hypoxia conditions and normal in NAC-treated cells.
Topographic variation of the choroidal watershed zone and its relationship to neovascularization in patients with age-related macular degeneration. Mendrinos 2009	*50 ICG video-retinograms of 50 pxs with wet AMD and watershed zones (retinl angiomatous proliferation inc) * 20 men/30 women,	*Videos reviewed	* 60% pxs had stellate wz- in these pxs the cnv occured In the centre of the wz *vertical wz 36% pxs- 3 subgroups (50%) had a vertical zone through the ONH with a portion extending into the fovea. In these cases, CNV occurred at the part extending into the fovea. In (33.3%) patients, the vertical zone coursed through the ONH with-out extending into the fovea In these cases CNV occurred at the temporal margin of the WZ and extended away from it towards the fovea. In 16.7% patients the vertical zone coursed through the fovea without involving the ONH- In these cases CNV occurred within the wz * An angle-shaped WZ coursing through the ONH and the fovea in 4% patients in both cases, CNV occurred within the WZ.	* Choroidal neovascularization occurred within the WZ in 44 of 50 (88%) patients (whenever it coursed through or extended into the fovea). * Even when the WZ did not involve the fovea, a portion of it always involved at least part of the macular region (i.e. either the temporal peripapillary choroid or the maculopapillary bundle).

Geldanamycin, a HSP90 inhibitor, attenuates the hypoxia-induced vascular endothelial growth factor expression in retinal pigment epithelium cells in vitro. Wu et al 2007	*A human RPE cell line was primarily isolated from a donor eye.	* For details on method for hypoxic treatment/heat shock treatment/assays and analysis see text	*1% O2 of hypoxic tension increased the VEGF gene expression 3.4-fold. *VEGF levels in hypoxia-conditioned media after 24 h are sig higher. *VEGF165, but not VEGF121 could be induced by HS treatment under both normoxia and hypoxia. *Hypoxia can stimulate RPE cell proliferation and prime cells to respond to exogenous growth factors. *HSP induction either facilitates the VEGF165 transcription or enhances transcript stability, promoting VEGF production. *First report that a pretreatment with GA can significantly suppress the VEGF gene expression in cultured RPE cells	*We only tested the short-term GA effect in this in vitro study; whether GA treatment for longer periods generates a deleterious side effect deserves to be further investigated. *Pretreatment with HSP90 inhibitor significantly attenuates the de novo VEGF production in RPE cells under hypoxia.
Association of Risk Factors for Choroidal Neovascularization in Age-Related Macular Degeneration With Decreased Foveolar Choroidal Circulation Grunwald et al 2010.	*273 study eyes of 204 AMD patients investigated. *No exudative AMD or other ocular pathology	*Laser Doppler flowmetry measurements of relative choroidal blood velocity, choroidal blood volume (ChBVol), and choroidal blood flow (ChBFlow) obtained. *Choroidal blood flow was calculated.	*A significant inverse correlation was observed between ChBVol and ChBFlow and age. *A significant inverse correlation was observed between spherical equivalent and ChBflow and ChBVol.	* AMD patients showed decreasing choroidal blood flow and volume, but not velocity, with increasing age are consistent with those of our previous study in normal subjects. * The presence of decreased choroidal blood volume and choroidal blood flow in the patients with history of SHTN suggests that an ischemic

				mechanism may be involved in the cause of this disease.
Choroidal filling in age-related macular degeneration: indocyanine green angiographic findings. Giovanni 1994	*145 AMD eyes	* abstract only	*a correspondence in 71% of cases between the site of the CNV and watershed areas and in 59% of cases between the CNV and areas of 'delayed choroidal filling'.	*These data support the hypothesis of a relationship between areas of presumed choroidal ischemia and evolution of AMD complicated with CNV. **Possibly, choroidal blood flow decreases may lead to imbalances in choroidal watershed vascular filling zones, the areas most prone to develop ischemia and hypoxia.
Reduced Foveolar Choroidal Blood Flow in Eyes with Increasing AMD Severity Grunwald 2005	* 26 control eyes of 17 normal subjects and 163 eyes with early AMD. *AMD study eyes were divided into three groups according to increasing risk for development of CNV	*Laser Doppler flowmetry was used to assess relative foveolar choroidal blood velocity (ChBVel), volume (ChBVol), and flow (ChBFlow).	*Mean ChBVel, ChBVol, and ChBFlow decreased with increased risk for CNV	*There is a systematic decrease in choroidal circulatory parameters with an increase in the severity of AMD features associated with risk for the development of CNV, suggesting a role for ischemia in the development of CNV.
Deep retinal vascular anomalous complexes in advanced age-related macular degeneration. Hartnett 1996	*11 patients (14 eyes) with AMD	*clinical and fluorescein angiography descriptions, infrared imaging and indocyanine green angiography	*Each study eye had a clearly defined anastomosis connecting the retinal circulation to a vascular complex in the deep retina *In seven eyes with RVACs, there were clinically recognizable retinovascular findings:	*These results indicate that retinovascular changes can be associated with nondisciform AMD. The authors speculate that neurodegenerative changes and hypoxia may lead to such changes

			intraretinal hemorrhages, telangiectasia, or microaneurysms	
Role of hypoxia and extracellular matrix-integrin binding in the modulation of angiogenic growth factors secretion by retinal pigmented epithelial cells. Mousa 1999	* Human RPE cells were cultured from post-mortem eyes	* Extensive- see paper- antibody blocking experiments	*Hypoxia Increases Levels of VEGF *FGF2 decreased with hypoxia *RPE cells under hypoxic conditions demonstrated time-dependent linear increase in VEGF in their media,	*Deposition of TSP1 and/or other ECM proteins in drusen or thickened Bruch's membrane in-patients with AMD could potentially modulate the secretion of angiogenic growth factors by RPE cells and could play a role in the development of choroidal neovascularization.
Synthesis and secretion of hemoglobin by retinal pigment epithelium. Tezel 2009	* 10 donor eyes	* Proteomic analysis	*Immunohistochemistry revealed the presence of haemoglobin within RPE and Bruch's membrane. The haemoglobin release rate was calculated to be 1.9 ・ 1.2 attomoles per cell per hour.	*Hemoglobin expression by human RPE brings a new perspective to the understanding of oxygen transport to the outer retina. Malfunction of RPE-hemoglobin production may underlie the pathophysiology of ocular diseases characterized by subfoveal hypoxia and VEGF upregulation,

Hypoxia specific SDF-1 expression by retinal pigment epithelium initiates bone marrow-derived cells to participate in Choroidal neovascularization in a laser-induced mouse model. Zhang 2011	* 1-mice 2- Human RPE cells cultured under chemical hypoxia	* 1-Lazer CNV induced then tissue processed for immunofluorescence * RNAi technique was used to knock down HIF-1α gene to observe the expression of HIF-1α and SDF-1 in hRPE cells	*2- hypoxia specific-HIF-1α influenced SDF-1 expression in hRPE cells.	* suggests that hypoxia-induced SDF-1 expression in RPE might be a critical initiator for recruitment of BMCs in CNV. SDF-1 might be another important factor in BMCs' differentiation into endothelial cells to participate in the CNV
Ocular rigidity in patients with age-related macular degeneration. Pallikaris 2006	* AMD group: 16 with neovascular and 16 with non neovascular AMD and 44 age-matched control patients	* Ocular rigidity measurement device used	* No statistically significant difference in ocular rigidity measurements between patients with AMD and control subjects. * When examined separately the two subgroups of patients with AMD, the average ocular rigidity measurements were higher in patients with neovascular AMD vs both control subjects and patients with non neovascular AMD	* increasing ocular rigidity could be an important component of the pathophysiologic cascade of neovascular AMD. It still remains to be elucidated whether hampered ocular rigidity is causative or reflects an event that results from the treatment or the changes that occur with aging and is accentuated in AMD
Pulsatile ocular blood flow study: decreases in exudative age related macular degeneration Mori 2001	* 10 patients with non-exudative AMD, 11 patients with exudative AMD, and 69 age matched controls.	* POBF, pulse amplitude (PA), systolic and diastolic blood pressures, intraocular pressure (IOP), refractive error, and axial length were compared	* No significant differences were found in age, systolic and diastolic blood pressures, IOP, or refractive error between patients with exudative and non- exudative AMD and control subjects. * In the patients with exudative AMD the POBF and PA were significantly lower than in the	*These data show that the POBF and PA exudative AMD pxs are lower than in the px with non-exudative AMD and normal subjects. Decreased choroidal blood flow may have a role in the development of choroidal neovascularisation in AMD

			patients with non- exudative AMD and control subjects	
Pulsatile ocular blood flow in asymmetric exudative age related macular degeneration. Chen 2001	* 37 patients with asymmetric exudative AMD	* pulsatile ocular blood flow measured in both eyes of each subject. * Adjusted for ocular perfusion pressure, IOPs, and pulse rate	* POBF was significantly higher in eyes with CNV than the contralateral eyes with drusen * Eyes with disciform scar had lower POBF than the contralateral eyes with drusen * There was no significant correlation between the POBF and the lesion size of the CNV	* The POBF in eyes with drusen was lower than their fellow eyes with CNV, but higher than their fellow eyes with disciform scar. This finding suggests that haemodynamic differences between fellow eyes in individuals are relevant to the development of CNV and the formation of disciform scar
Retinal haemodynamics in patients with age-related macular degeneration Sato 2006	* 25 eyes of 25 patients with AMD and nine eyes of nine age-matched control subjects	* retinal laser Doppler system measured pulsatility ratio (PR), the pulsatility index (PI), and the resistivity index (RI)	* PR, PI, and RI in the patients with AMD were each significantly higher than in the control group, and increased monotonically with increasing severity of AMD * no differences in mean blood velocity, arterial diameter, or blood flow rate among the groups	* This suggests that the increased blood flow pulsatility in the retinal arteries of the eyes with AMD is not due to increased distal vascular resistance, but instead is likely due to a loss of compliance in the arterial vasculature leading to the eye. * increasing vascular rigidity in the systemic arterial circulation is directly associated with an increasing severity of AMD.

Color Doppler Imaging of Choroidal Circulation in Patients with Asymmetric Age-Related Macular Degeneration Üretmen 2003	* 26 patients who had non-exudative AMD in the first eye and CNV secondary to AMD in the fellow eye	* Blood flow velocities, vessel pulsatilities and resistivities were measured from ophthalmic artery, nasal and temporal posterior ciliary arteries using colour Doppler imaging	* Systolic and diastolic velocities were lower in eyes with CNV for all vessels, except for the systolic velocity of the nasal posterior ciliary artery. * Pulsatility and resistivity indices were higher in eyes with CNV for all vessels.	* Impaired choroidal blood flow in AMD *choroidal blood flow is more impaired in eyes with AMD in CNV eyes than in fellow eyes.
Expression of hypoxia-inducible factor-1alpha and -2alpha in human choroidal neovascular membranes. Sheridan 2009	*12 surgically excised subfoveal CNV secondary to AMD (n=9) or punctate inner choroidopathy (PIC) were obtained during pars plana microsurgery. 26 Normal human eyes	* samples immunohistochemically probed with monoclonal antibodies against HIF−1α and −2α and compared to that for cell markers specific for vascular endothelial cells (CD34), macrophages (CD68), retinal pigment epithelial cells (RPE; panel cytokeratins/CK18) and VEGF. Following secondary antibody amplification, reactions were visualized with fast red chromogen	*Cellular immunoreactivity of membranes for HIF−2α was strong in eight out of nine AMD specimens but it was only weakly positive for HIF−1α in fivespecimens. In contrast, two out of three PIC specimens were weakly positive for HIF−1α but demonstrated no staining for HIF−2α. *Immunohistochemical analysis revealed areas within the CNV membranes that were predominantly immunopositive for CD68 and cytokeratin indicating the presence of RPE and/or macrophages and that these cells strongly co-localized with the presence of HIF and VEGF.	*The localization of HIF expression supports the concept that hypoxia is a major stimulus for the development of submacular wound healing and within this context CNV is but one component of this process.

The role of the p53 protein in the selective vulnerability of the inner retina to transient ischemia. Rosenbaum et al. 1998	*Sprague-Dawley rat.	*Transient retinal ischemia was induced using a high intraocular pressure (HIOP) model for 60 minutes. *Histopathologic outcome 7 days after ischemia. * analysis for evidence for apoptosis (TdT-dUTP terminal nick-end label [TUNEL] staining) and p53 protein expression performed at several points during reperfusion period. *Also wild-type mice, one homozygous and the other heterozygous for the p53 null gene, subjected to HIOP for 60 minutes, and histopathology 7 days later.	* marked thinning of inner retinal layers 7 days after ischemia. * p53 immunochemistry- elevated protein levels within the inner retina peaking 24 to 48 hours but was no longer present at 4 days after ischemia. *7 days subsequent to 60 minutes of ischemia in wild-type and transgenic mice, histopathologic evaluation demonstrated preservation of the retinal histoarchitecture in the heterozygous group compared with the wild-type or homozygous animals	* Supports the hypothesis that the delayed cell death that occurs after transient retinal ischemia is, in part, apoptotic. *Suggest a role for the p53 protein in the selective vulnerability of the inner retina to transient ischemia. p53 protein may be a target for future therapeutic agents.
Foveal electroretinograms and choroidal perfusion characteristics in fellow eyes of patients with unilateral neovascular age-related macular degeneration. Remula et al 1995	Fellow eyes of 67 patients with unilateral nAMD.	Fluorescein angiograms and foveal cone ERGs recorded in	*42% of the eyes had choroidal perfusion defect. ERG implicit times averaged 1 ms slower (p=0O0167) in eyes with abnormal choroidal perfusion delayed ERG implicit time with prolonged choroidal filling remained after controlling for age, acuity & drusen.	*there is prolinged choroidal filling in fellow eyes of nAMD.

Functional loss in age-related Bruch's membrane change with choroidal perfusion defect. Chen et al (1992)	* eyes which had delayed and prolonged choroidal filling, 6 controls	Scotopic thresholds measured with Humprey perimeter and compared with area of perfusion defect.	* In eyes without delayed choroidal perfusion, no discrete areas of increased ST. *In 7/8 eyes with prolonged choroidal filling, discrete areas of scotopic threshold elevation of up to 3.4 log units were recorded and these corresponded to regions of choroidal perfusion abnormality.	

11 Appendix III Matlab code for scotopic threshold program.

```
% Annulus THRESHOLD: YES/NO, QUEST
%
% This programme uses a YES/NO, QUEST driven staircase to determine
contrast
% thresholds for an annulus.
% The code is adapted from Gaboiumdemo, kbDemo and QuestDemo.
% Ver. 1: Tom Margrain on 28-11-12. This was a 2 IFC version.MattTom
QUEST
% results
% Issues: the frame refresh rate is 60Hz on the laptop but for some
reason
% it reduces significantly from time to time; I guess other programmes
% running the background are stealing resources. If this happens the
% stimulus will be flickering more slowly than expected. The main
problem
% seems to be for the first presentation, after that things are
generally
% better.
% Ver. 8 - Fully working 2 IFC programme. Only altered to Ver. 9
because 2
% IFC may be tricky for patients
% Ver. 9 - Here the programme is altered from 2 IFC to a Yes/No
% Ver. 10 - Now pushing the response key during the presentation
should
% work too + keep track of false positives + improve the 'feel' by
altering
% the timing of presentations
% Ver. 11 - implements a 'simple gamma correction' using code from the
online
% AdditiveBlendingForLinearSuperpositionTutorial. The important lines
of
% code that were not used before include
% PsychImaging('AddTask', 'General', 'EnablePseudoGrayOutput') - this
line
% lets us call up all screen output in the range 0-1 (0-100%) rather
than
% grey scale.
% PsychImaging('AddTask', 'FinalFormatting', 'DisplayColorCorrection',
'SimpleGamma');
% This line sets up simple gamma correction in the video output
'pipeline'
% PsychColorCorrection('SetEncodingGamma', win, gamma); So, all output
is
% gamma corrected automatically.
% This version also allows the 'blob' to occupy a 0-1 range rather
than
% 0-0.5 (line 130). It also changes the slightly misleading variable
```

```
% "contrast" to the more accurate "luminance" i.e. the output is
threshold
% luminance (lines 167, 173). The final output (threshold) is log
luminance
% as a fraction of the max screen luminance in cd/m2.
% Ver. 12 - This version is a modification of the 14Hz flicker code I
wrote
% for Claire. It produces an annulus and fixation cross. There is some
% redundant code. This programme is only to show how to do these
things it
% is not a calibrated programme to be used in experiments.
% Ver. 13 - the output (i.e. final threshold) is the log of the
normalised
% stimulus intensity without any adjustment for the number of filters
over
% the top.
% Ver. 14 - the print out to Excel now included a conversion back to
cdm-2,
% it should also print out the SD for the threshold but, I think i
have a
% mistake in here the SD is way too large!

% INSTRUCTIONS
% 1)This programme needs to write to an Excel file.
% 2)Tell subject the expt last 3.5 minutes (40 trials).
% 3)The task is to push any button when a flickering stimulus is seen.

% First, set up all the relevant parameters
trialsDesired = 40; % number of times a choise is offered
wrongRight={'wrong','right'}; % this is used at the end of each trail
to identify the response
stimFreq = 14; % temporal frequency of flickering gabor in Hz
screenMax = 122; % this it the measured maximum luminance of the
screen in cdm-2
KbName('UnifyKeyNames');
escapeKey = KbName('ESCAPE');
format compact
avgfps = [];
contrastPresent = [];
falsePositive = 0; % used to count false positive responses
%contrast = 1;
% PTB-3 correctly installed and functional? Abort otherwise.
AssertOpenGL;
Screen('Preference', 'SkipSyncTests',1) % this is not a good line to
have, only used because using 2 monitors
% This programme uses Psychophysics QUEST routines to determine
threshold
% to get these to work it is necessary to provide information about
the
% threshold we are looking for in particular an initial guess at the
% treshold and the standard deviation
% Provide our prior knowledge to QuestCreate, and receive the data
struct "q".
participant=[];
while isempty(participant)
    participant=input('Subjects name please: ', 's');
end
tGuess=[];
while isempty(tGuess)
    tGuess=input('Estimate threshold (e.g. -1): ');
end
```

```
tGuessSd=[];
while isempty(tGuessSd)
    tGuessSd=input('Estimate the standard deviation of your guess,
above, (e.g. 2): ');
end
pThreshold=0.82;
beta=3.5;delta=0.01;gamma=0.5;% These values may not be right for yes
/no
% beta controls the steepness of the psychometric function. Typically
3.5.
% delta is the fraction of trials on which the observer presses
blindly. Typically 0.01.
% gamma is the fraction of trials that will generate response 1 when
intensity==-inf.
q=QuestCreate(tGuess,tGuessSd,pThreshold,beta,delta,gamma);
q.normalizePdf=1; % This adds a few ms per call to QuestUpdate, but
otherwise the pdf will underflow after about 1000 trials.

% Use try and catch to rescue code from a crash
try

% Select screen with maximum id for output window:
screenid = max(Screen('Screens'));

% Open a fullscreen, onscreen window with gray background. Enable
32bpc
% floating point framebuffer via imaging pipeline on it, if this is
possible
% on your hardware while alpha-blending is enabled. Otherwise use a
16bpc
% precision framebuffer together with alpha-blending. We need alpha-
blending
% here to superimpose the gabor blob on the background. The programme
will
% abort if your graphics hardware is not capable of any of this.
PsychImaging('PrepareConfiguration');
%PsychImaging('AddTask', 'General', 'FloatingPoint16Bit');
PsychImaging('AddTask', 'General', 'FloatingPoint32BitIfPossible');
% Enable bitstealing aka PseudoGray shader: This line is needed so
that
% subsequent screen output is in the range 0-1 i.e. up to 100% of
screen output. No need to call grey levels.
% It also increase the number of grey scales that can be generated
PsychImaging('AddTask', 'General', 'EnablePseudoGrayOutput');
% Sets up the final video output pipeline to include gamma correction
PsychImaging('AddTask', 'FinalFormatting', 'DisplayColorCorrection',
'SimpleGamma');
% Finally open a window according to the specs given with above
% PsychImaging calls, clear it to a background color of 0.5 aka 50%
% luminance:
[win, winRect]=PsychImaging('OpenWindow',screenid, 0);
% This wait allow you to see the 'gamma correction' kick in! First its
not
% there, then,..
WaitSecs (0.1);
% OK, now apply the gamma correction to all outputs to the screen, so
% easy! The default value here is gamma = 2 but need to determine
exactly
% what it is for your screen
gamma = 1 / 2.0;
PsychColorCorrection('SetEncodingGamma', win, gamma);
```

```
WaitSecs (0.5);

% Enable alpha-blending, set it to a blend equation useable for linear
% superposition with alpha-weighted source. This allows to linearly
% superimpose gabor patches in the mathematically correct manner,
should
% they overlap. Alpha-weighted source means: The 'globalAlpha'
parameter in
% the 'DrawTextures' can be used to modulate the intensity of each
pixel of
% the drawn patch before it is superimposed to the framebuffer image,
ie.,
% it allows to specify a global per-patch contrast value:
Screen('BlendFunction', win, GL_SRC_ALPHA, GL_ONE);

% Query frame duration: We use it later on to time 'Flips' properly
for an
% animation with constant framerate:
ifi = Screen('GetFlipInterval', win);

% Create a gabor patch: This is 100x100 matrix that has periperal
values
% around zero and a central value of 1. The gray scale range of the
monitor
% is 1 at the centre of the gabor.
%[x,y] = meshgrid(-10:10, -10:10);
%whiteBlob = (exp(-((x/40).^2)-((y/40).^2)))

%Create an annulus
[x,y] = meshgrid(-263:263, -263:263);
    circle1 = 1 * (x.^2 + y.^2 <= (263)^2);
    circle2 = 1 * (x.^2 + y.^2 <= (253)^2);
    rawAnnulus = circle1-circle2;

% Create blob movie, first determine frame rate
rate = 1/ifi;

% Determine number of frames avaliable for a given stimulus frequency
framesAvaliable = rate/stimFreq;

% Determine the step size to get through 360 degrees (1 cycle) in the
% number of frames valiable
step = 360/framesAvaliable;

% Set up degrees, start a zero and increment in the loop according to
the
% step size
x=-180;

% Get the time at the very start to record the total experimental time
exptStart = Screen('Flip', win);

% Introduce experiment wait for signal to start
%Screen('FrameRect', win ,[0,128,0], [540,300,740,500],1);
Priority(2);% Uprate the priority for Matlab i.e. try to keep Windows
out!
Screen('DrawText', win, 'PUSH THE BUTTON WHEN YOU SEE THE RING', 350,
383, [1,1,1]);
```

```
Screen ('Flip', win);
WaitSecs (3);
Screen('HideCursorHelper', win);
Screen ('Flip', win); % Flipping removes the text from the screen

Screen('DrawLine', win, [1,1,1], 640, 212, 640, 812,8);
Screen('DrawLine', win, [1,1,1], 340, 512, 940, 512,8);
Screen ('Flip', win); % Flip the fixation cross onto the screen

% NOW WE CAN IMPLEMENT THE MAIN EXPT LOOP

for k=1:trialsDesired;

    % First pick up the threshold recommened by QUEST
    tTest=QuestQuantile(q); % Recommended by Pelli (1987).

    % Unlog tTest, this controls the luminance of the stimulus
    luminance = 10^tTest;

    % Generate the textures to be displayed during each frame, assume
60Hz so
    % going from 1 to 120 will be a 2s presentation
    for i=1:120;
        %Need to change contrast to a fraction
        annulus = rawAnnulus * (luminance);% * sind(x);
        gabortex(i)=Screen('MakeTexture', win, annulus, [], [], 2);
        x=x+step;
    end;

    % Wait a random period of time before giving the presentation
    randomdelay = rand*6;
    StartSecs = GetSecs;
    timeNow=0;
    while timeNow <randomdelay
        SecsNow = Getsecs;
        timeNow = (SecsNow - StartSecs);
        [ keyIsDown, timeSecs, keyCode ] = KbCheck; % this command
checks the keyboard for an input
        if keyIsDown % if a key is pushed this is what to do
            falsePositive = falsePositive+1;% counts the incorrect
responses
            Beeper(400)
            while KbCheck; end % this avoids KbCheck reporting
multiple events
        end
    end

    % Clear the screen and wait a bit
    %Screen ('Flip', win);
    %Screen('FrameRect', win, [200,200,200], [540,300,740,500]);
    %Screen('DrawLine', win, [1,1,1], 640, 100, 640, 700);
    %    Screen('DrawLine', win, [1,1,1], 340, 400, 940, 400);

    response = 0; % This flag holds a record of if the stimulus is
seen or not, 0=not seen
    count = 0; % This is needed to count the number of presentations
    vbl = Screen('Flip', win);
```

```
    tstart = vbl; % records the time the main presentation loop
started

    for i=1:120;
        Screen('DrawLine', win, [1,1,1], 640, 212, 640, 812,8);
        Screen('DrawLine', win, [1,1,1], 340, 512, 940, 512,8);
        %Screen('DrawText', win, 1,0 ,0, [200,200,0]);
        Screen('DrawTexture', win, gabortex(i),[],[],45);
        % Check the frame presentation time, does it always match the
        % frame referesh rate?
        vbl = Screen('Flip', win, vbl + ifi/2);
        %frametime(i)=vbl-lastTime;% puts frame time (ms) into an
array
        lastTime = vbl;
        count = count+1; %Will count up to 120
        % check for keyboard response during presentation
        [ keyIsDown, timeSecs, keyCode ] = KbCheck; % this command
checks the keyboard for an input
        if keyIsDown % if a key is pushed this is what to do
            response=1;% flags a correct response
            break % breaks out of the presentation loop
        end
    end;

    % Clear the screen of any residual gabor and get time stamp
        Screen('DrawLine', win, [1,1,1], 640, 212, 640, 812,8);
        Screen('DrawLine', win, [1,1,1], 340, 512, 940, 512,8);

    tend = Screen ('Flip', win); % records the time the presentation
stopped

    % Check frames per second for this trial, store result in array
avgfps
    avgfps(k) = count / (tend - tstart);

    % Now get the participants response
    ResponseSecs = GetSecs;% gets the time the stimulus was flipped
out

    % Wait for a response for three seconds, if in the 1st second =
correct
    while 1
        [ keyIsDown, timeSecs, keyCode ] = KbCheck; % this command
checks the keyboard for an input
        if keyIsDown % This bit of code is only executed if a button
is pushed
            fprintf('"%s" typed at time %.3f seconds\n',
KbName(keyCode), timeSecs - exptStart) %ResponseSecs);
            if (timeSecs - ResponseSecs)<1; % checks that response was
within 1s of stimulus offset
                response = 1; %this 'flag' means the response was
correct
                Beeper (1000) % make a 'beep' sound

            else
                response = 0; %this means the response was incorrect
(in this case too slow)
                Beeper (600)
                falsePositive = falsePositive+1;% counts the incorrect
responses
```

```
                break
            end

            %while KbCheck; end % this avoids KbCheck reporting
multiple events
            break
        end

        % Now, if no button push + long wait, time is up!
        SecsNow = GetSecs;
        timeSincePresentation = (SecsNow - ResponseSecs);
        if timeSincePresentation > 4; % If no response within 1s of
stimulus, its missed, get out of loop
            fprintf('stuck in loop') %ResponseSecs);
            break

        end % waiting for repsonse or time up

        %while KbCheck; end % this avoids KbCheck reporting multiple
events

    end

    % Print the results for this particular trial
    fprintf('Trial %3d at %5.2f is
%s\n',k,tTest,char(wrongRight(response+1)));
    LuminancePresent(k)=tTest;
    ResponseArray(k)=response; %this keeps track of right / wrong
responses
    LuminancePresentCdM2(k) = log10((10^tTest)*screenMax);

    % Now update the QUEST routines with the last threshold value
(this is all
    % in logs) and let it know if the participant responded correctly
(1) or
    % incorrectly (0)
    q=QuestUpdate(q,tTest,response); % Add the new datum (actual test
intensity and observer response) to the database.

    % Close all the windows that were opened for this presentation
    Screen ('Close');

    % Now go back for the next trial
end

% Display cursor again now its all over
Priority(0);% Resest normal priority
Screen('ShowCursorHelper', win);

% Ask Quest for the final estimate of threshold.
t=QuestMean(q);     % Recommended by Pelli (1989) and King-Smith et
al. (1994). Still our favorite.
sd=QuestSd(q);

fprintf('Final threshold estimate (mean±sd) is %.2f ± %.2f\n',t,sd);
fprintf('68 percent sure true mean is between %.2f and %.2f\n (mean
+/-SD)', (t+sd),(t-sd));

% Determine total experimental time and print this out
```

```
exptEnd = Screen('Flip', win);
exptTotal = exptEnd - exptStart

% Print out the frame per sec for each trial
%avgfps;

% Print out the number of false positives
falsePositive

% Close onscreen window, release all ressources:
Screen('CloseAll');

% The presentation rate has been a problem / variable so, these lines
are
% used to plot frame time.
figure(1)
k=1:trialsDesired;
plot (k,avgfps)
xlabel ('Trial')
ylabel ('Frames / sec')

% Plot the stimuli presented
figure(2)
k=1:trialsDesired;
plot (k,LuminancePresent, 'ob')
xlabel ('Trial')
ylabel ('Log luminance')

% Dump all the QUEST data to Excel
LuminancePresent = LuminancePresent(:); % This (:) changes the vector
from a row to a column
trialNumber = k(:);
today = now; % this is a weird number that represents the current date
and time, Excel understands!
name ={participant};
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', today,'Sheet1','B4');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', today,'Sheet1','B5');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', name,'Sheet1','B3');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', exptTotal,'Sheet1','F3');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', trialNumber,'Sheet1','A11');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', LuminancePresent,'Sheet1','B11');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', t,'Sheet1','F5');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', sd,'Sheet1','F6');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', falsePositive,'Sheet1','F7');

% Dump all the Luminance (cd/m2) data to Excel
% Convert QUEST thresholds to cdm-2 thresholds
LuminancePresentCdM2 = LuminancePresentCdM2(:);
luminanceThreshold = log10((10^t)*screenMax);
UpperSE=t+sd;
LowerSE=t-sd;
```

```
luminanceThresholdUpperSE = log10((10^UpperSE)*screenMax);
luminanceThresholdLowerSE = log10((10^LowerSE)*screenMax);

xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', today,'Cdm2','B4');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', today,'Cdm2','B5');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', name,'Cdm2','B3');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', exptTotal,'Cdm2','F3');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', trialNumber,'Cdm2','A11');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', LuminancePresentCdM2,'Cdm2','B11');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', luminanceThreshold,'Cdm2','F5');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', sd,'Cdm2','F6');
xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', falsePositive,'Cdm2','F7');
%xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', luminanceThreshold,'Sheet1','J5');
%xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', luminanceThresholdUpperSE,'Cdm2','J6');
%xlswrite('d:\Tamsin 2013 visit 1\QUEST annulus\QUEST
results\OriginalResults.xls', luminanceThresholdLowerSE,'Cdm2','J7');

% All done, phew!
catch % The following code is run if the experiment crashes
    Screen('CloseAll');
    rethrow(lasterror);
    psychrethrow(psychlasterror);
end
```