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Citation: Hobby, A. E. (2021). An investigation of the effect of long-term anti-VEGF therapy on retinal function and structure in people with neovascular age-related macular degeneration. (Unpublished Doctoral thesis, City, University of London)

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An investigation of the effect of long-term anti-VEGF
therapy on retinal function and structure in people with
neovascular Age-Related Macular Degeneration

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A Thesis submitted for the degree of Doctor of Philosophy



Division of Optometry and Visual Science

School of Health Sciences

January 2021

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All data from this thesis are available on request. Please contact Angharad Hobby
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Acknowledgements

Firstly, I thank my supervisors, Alison Binns, Byki Huntjens, David Crabb and Omar Mahroo. Your support, dedication, patience and understanding through all the late nights and early mornings is so very appreciated and I could not have completed this thesis without your guidance. I must also give an additional special thanks to Byki for the ice cream and escapes to green spaces at the most difficult of times in this process.

I'm extremely grateful to the College of Optometrists for their financial and professional support, without which none of this work would have been possible. Equally so to the staff and research participants at each of my study sites; City, University of London, St Thomas' Hospital, and Moorfields Eye Hospital. You collectively (and sometimes individually) travelled hundreds of miles and gave up dozens of hours for the benefit of this research. Your contribution was invaluable.

I must also acknowledge the contribution of my friends to this PhD journey. My 'Crabb Lab' colleagues (who've gone by many names...), thank you for expanding the mind of this small-town girl and for so much laughter – the memories are incredible and I wouldn't have wanted anyone else alongside me. To Chelsey, my number one from day one. I don't have the words to express my admiration and gratitude for you and everything you do. Also, Steph and Helen, two more strong, brilliant women that continue to amaze me every time we connect. Thank you all for your kindness and support over the last few years and always.

To my work friends during write up, your intelligence and resilience amazes me. In particular; to Caroline – your kindness and level headed attitude is so refreshing and helpful. To Greg, being reunited professionally has been a dream – long may it continue. And to Sean and Sian, who've become such great and loyal friends in such a short space of time. I hope this goes a little way to thanking you all.

Last and by no means least, I thank my family. Dad, thank you for the Sunday morning rants and jokes. Mum, thank you for plying me with food and love before each return to London. To my siblings; Elinor, a confidant and virtual hug whenever I needed it, and Sam for the comic relief. To my Gran'ma, your zest for life was inspiring and your madness kept me going throughout, 'Insta-gran'. And finally, to my Gran and Bampy, I'm as proud of you as you say you are of me.

You will all never know how much your support, love, and friendship has meant to me.

Thank you so much for everything.

List of Abbreviations

AMD:	Age-related macular degeneration	NHS:	National Health Service
Amp:	Amplitude	NICE:	National Institute for Clinical Excellence
ANOVA:	Analysis of Variance	OCT:	Optical Coherence Tomography
ANCOVA:	Analysis of Covariance	OS:	Outer Segment
Anti-VEGF:	Anti-Vascular Endothelial Growth Factor	PRL:	Photoreceptor Layer
CI:	Confidence interval	RGC:	Retinal ganglion cell
CoR:	Coefficient of Repeatability	RNFL:	Retinal nerve fibre layer
CoV	Coefficient of Variance	RPE:	Retinal pigment epithelium
DTL:	Dawson Trick Litzcow	SD:	Standard deviation
ETDRS:	Early Treatment Diabetic Retinopathy Study	SD-OCT :	Spectral Domain OCT
FD-OCT:	Frequency Domain OCT	TC:	Transient Cone
GA:	Geographic Atrophy	td:	Trolands
GCC:	Ganglion Cell Complex	TRT:	Total Retinal Thickness
IQR:	Interquartile Range	TtP:	Time to Peak
LOCS II:	Lens Opacities Classification	VA:	Visual Acuity
nAMD:	neovascular Age-related macular Degeneration	VEGF:	Vascular Endothelial Growth Factor

Declaration

The work contained in this thesis was completed by the candidate, Angharad Elizabeth Hobby (AEH), under the supervision of Dr Alison Binns, Dr Byki Huntjens, Professor David Crabb, and Dr Omar Mahroo. It has not been submitted for any other degrees, either now or in the past. Where work contained within has previously been published, this has been stated in the text. All sources of information have been acknowledged and references have been given. The University Librarian of City, University of London is permitted to allow the thesis to be copied in whole or in part without further reference to the author. This permission only covers single copies made for study purposes, subject to normal conditions of acknowledgement.

Abstract

Anti-vascular endothelial growth factor drugs (anti-VEGF) are used in the treatment of retinal conditions including neovascular age-related macular degeneration (nAMD), and macular oedema associated with diabetic retinopathy or vein occlusions. Despite the fact that treatment often extends over many years, requiring repeated injections, little is known about the long-term effects on the healthy retina also exposed to the treatment. The aim of this research was to investigate the relationship between cumulative dose of anti-VEGF received and parameters of extramacular function and retinal layer thicknesses in people undergoing treatment for nAMD.

Chapter 2 presents a systematic review of the literature conducted to evaluate current evidence regarding the effect of anti-VEGF drugs on peripheral retinal function and structure of animals and humans. The review found that the majority of evidence was not supportive of a detrimental effect of treatment on retinal function or structure. Crucially, however, there were significant limitations in the evidence base available to date. Of particular note was the dearth of studies which evaluated the effect of multiple injections over a prolonged period of time in animals or humans (for example, only 22.5% of the assessed studies followed up the subjects/participants for longer than 9 months after the first anti-VEGF treatment). A few studies with longer follow up times did report a small effect of treatment on retinal function in humans, but sample sizes were small. It was concluded that there is a need for research evaluating the cumulative effect of multiple retreatments of anti-VEGF drugs on retinal structure and function in humans.

Chapters 3 and 4 report on aspects of electroretinogram (ERG) protocol development. In Chapter 3 the basic protocol for the PhD research was piloted, and the repeatability assessed. The final protocol consisted of a Transient Cone ERG (ISCEV standard; white 3 cd.s.m⁻² stimulus, 4 ms duration, 2 Hz, over a white background of 30 cd.m⁻²), a flicker ERG (41 Hz, red stimulus peak wavelength 635 nm, 12 ms duration, 41 Hz, mean luminance 30 cd.m⁻²), and a photopic negative response (PhNR) recorded to a series of stimulus intensities (0.11-3.35 cd.s.m⁻² red stimulus, peak wavelength 635 nm, 4 ms duration, 2 Hz, blue LED background, peak wavelength 465 nm, 158.8 scotopic cd.m⁻²). In Chapter 4, the repeatability of a desktop ERG recording system (Espion™, Diagnosys) was compared to that of a handheld system which uses skin electrodes instead of conjunctival electrodes (RETeval®, LKC). Reliability was found to be similar between the two. Amplitudes, but not times to peak, were substantially different in the handheld device. An electrode placement study aimed to determine the impact of the position of the skin sensor strip when recording ERGs using the handheld RETeval® device. Placement was important with respect to amplitudes – skin surface electrodes recorded lower amplitude values when placed further away from the manufacturer recommended position (2mm below the lower lid margin). However, time to peak was relatively unaffected by electrode position.

Chapter 5 presents a cross-sectional study which aimed to investigate the relationship between number of anti-VEGF injections received by people with nAMD and parameters of the full-field ERG and optical coherence tomography (OCT) layer thicknesses (adjacent to the macula). Thirty-two participants were recruited from St Thomas' Hospital. There was a tendency across all stimulus intensities for PhNR amplitude to reduce with increasing number of injections, a relationship that was statistically significant prior to Bonferroni correction at three intensities. Increasing number of injections was also associated with a reduced thickness of the nasal outer segment layer. Increasing time since first injection was significantly associated with reduced PhNR 0.11 cd.s.m^{-2} amplitude, and increased Transient Cone ERG b-wave time to peak, as well as reduced nasal nerve fibre layer thickness, photoreceptor outer segment layer thickness and total retinal thickness, and reduced temporal ganglion cell complex thickness, and outer segment thickness. A second study was conducted at a different test site using the RETeval® hand held device, with the aim of determining whether these ERG findings were consistent in a second set of participants ($n = 19$). There was a trend across all data towards reducing amplitudes and increasing implicit times with increasing number of injections. However, this was only statistically significant for the time to peak of the a-wave in the PhNR ERG protocol. The signal to noise ratio was small, however, and the lower than desired sample size means that this study was not powered to detect weak correlations.

To conclude, the cross-sectional analysis of participants undergoing anti-VEGF therapy for nAMD highlighted a possible relationship between number of injections and certain parameters of the full-field ERG and retinal layer thicknesses measured adjacent to the macula. Of particular note was the relationship between PhNR amplitude and number of injections, and the apparent thinning of the photoreceptor outer segments with increasing number of injections and increasing time since first injection. However, the effect size was small. A number of potential confounding variables have been identified, including the effect of the disease process itself on the outcome measures assessed. Furthermore, interpretation of these findings should be made with caution given the low sample size, which limited the ability to detect small and medium effect sizes. The results of this study suggest that it is unlikely that long term anti-VEGF treatment causes a large negative effect on retinal structure or function which may be reassuring to practitioners and patients.

Chapter 1. An Introduction to the Thesis

1.1. Introduction

This thesis describes a series of studies into the structural and functional changes in the peripheral retinae of persons suffering from neovascular age-related macular degeneration (nAMD) who are being treated with intravitreal injections of anti-Vascular Endothelial Growth Factor (anti-VEGF) drugs. After the age of 55 years, age-related macular degeneration (AMD) increases in prevalence with increasing maturation, and appears to show a higher prevalence in women and in people of European ethnic origin (Chakravathy et al., 2010; Klaver et al., 2004). It begins as early AMD before eventually progressing into intermediate AMD and then either geographic atrophy or nAMD (Ferris et al., 2013).

This chapter begins with an overview of the structure of the retina, before introducing the pathogenesis and clinical features of AMD. A discussion of VEGF and its role in the eye follows, along with an introduction to anti-VEGF drugs and evidence regarding their toxicity. The final sections describe the medical imaging and electrophysiological techniques used to examine changes in structure and function over time in participants affected by and treated for this disease. The aims and hypotheses of the work conducted in this PhD are outlined at the end of the chapter.

1.2. The Neurosensory Retina

Located between the vitreous humour and the choroid, the human retina is made up of 9 layers (Kolb, 2013; see Figures 1.1 and 1.2) and encompasses the entire area between the optic nerve head and the ora serrata – around 65% of the interior surface of the eye (Oyster, 2006). Where the retina reaches the ora serrata it persists with the ciliary body's epithelial layers (Remington, 2005; Kolb, 2011). At less than 0.5 mm thick the photoreceptors (rods and cones) lie outermost, nearest the retinal pigment epithelium and the retinal ganglion cells (RGCs), which are the output neurons of the retina, lie closest to vitreous humour, innermost. Light that enters the eye at the pupil traverses to the retinal surface and through the retinal thickness to the photoreceptors, and there begins the electrochemical process of phototransduction – a sequence that converts the light rays to electrical signals that travel back through the retina, down the optic nerve, and through the optic tracts to the lateral geniculate

nucleus and then via optic radiations to the primary visual cortex and beyond for interpretation (Remington, 2005; Forrester et al., 2016).

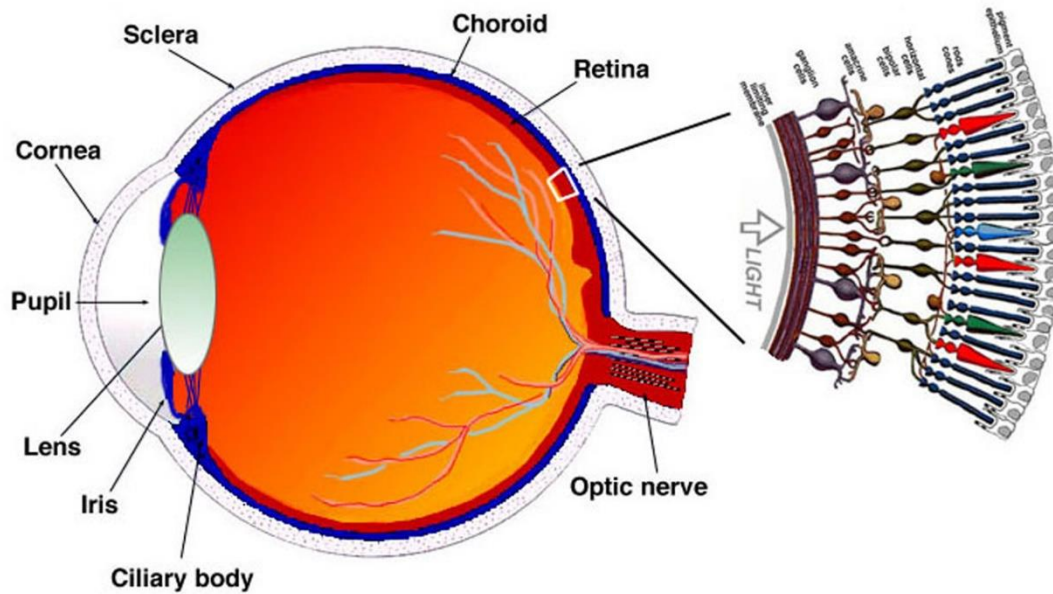


Figure 1.1. A cross sectional illustration of the human retina, illustrating the location of key ocular structures and a small schematic of the retinal anatomy (Kolb et al., 2003).

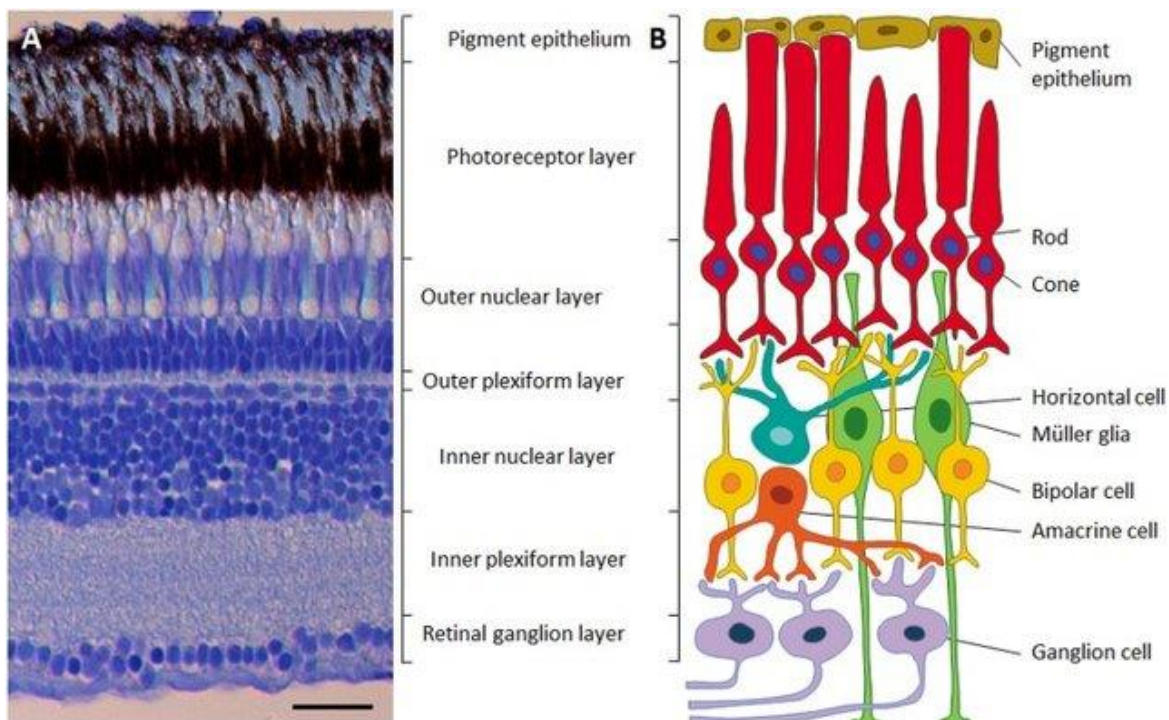


Figure 1.2. A cross sectional image electron microscopical and schematic image of the retinal layers (Gramage et al., 2014).

1.2.1. The Photoreceptor Layer

The photoreceptor layer consists of the neurons rods (~92 million) and cones (~4.6 million), in which phototransduction occurs (Curico et al., 1990). Whereas rods maximally operate in scotopic conditions (providing poorer visual acuity and less temporal sensitivity, but reduced light threshold in these conditions; Kolb, 2013), cones maximally operate under photopic conditions (Curico et al., 1990), providing colour vision as well as higher levels of visual acuity and better temporal sensitivity (Fu, 2010). Whilst the peak density of cone photoreceptors is at the fovea, rod density is maximal at approximately 18 degrees eccentricity (see Figure 1.3, data from Osterberg, 1935, reproduced by Kolb, 2013).

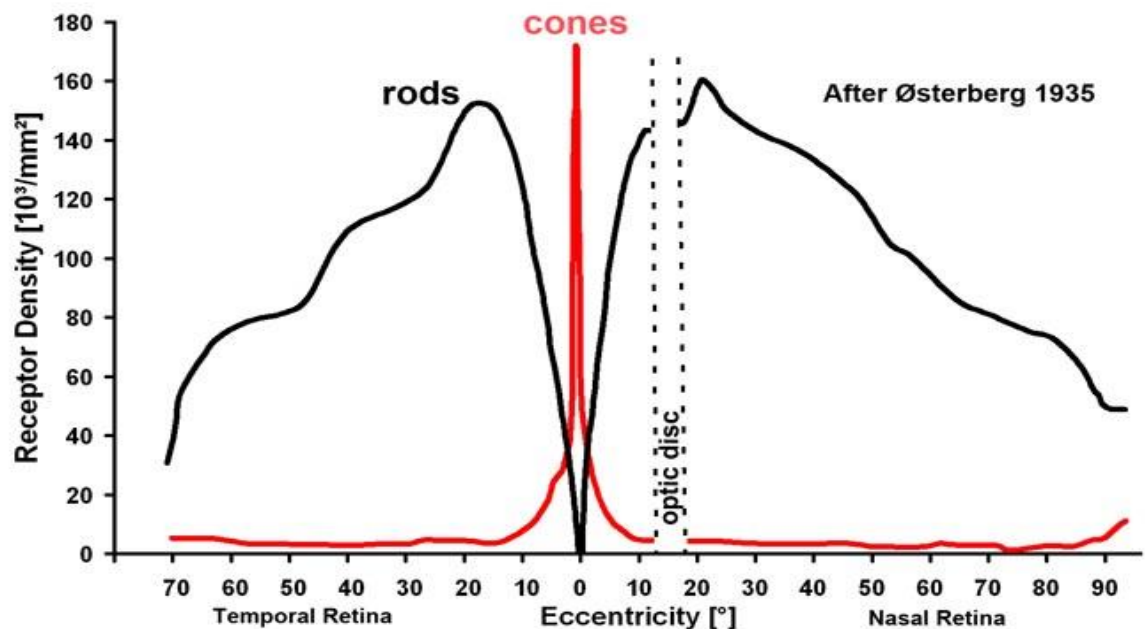


Figure 1.3. The distribution of rod and cone photoreceptors along the horizontal meridian (data from Osterberg, 1935 reproduced by Kolb, 2013).

The outer segments of rods and cones contain photopigments for phototransduction and a cilium connecting the segments is rich in mitochondria to fuel the process (Hoang et al., 2002). Photoreceptor axons extend to the outer plexiform layer where they form synaptic terminals (Mustafi et al., 2009). Rods are longer neurons and are significantly more prevalent in the retina in order to maximise light absorption (Masland, 2001).

1.2.2. The External Limiting Membrane (ELM)

Not thought of as a true membrane, the ELM is made of adhesions between inner sections of the photoreceptors and Müller cells, acts as a molecular sieve, and affords a separation between the subretinal space and the remaining neural retina (Bunt-Milam et al., 1985).

1.2.3. Bipolar cells

Bipolar cells (see Figure 1.2) transmit electrical signals from the photoreceptors to the RGCs. There are eleven different types of bipolar cells in the human retina, only one of which serves rod photoreceptors (Boycott and Wassle, 1991; Kolb et al., 1992). All bipolar cell bodies are located in the inner nuclear layer of the retina with their dendrites spreading into A) the outer plexiform layer to synapse with both horizontal cells and photoreceptors, B) the inner plexiform layer to synapse with RGCs – sometimes indirectly via amacrine cells. Bipolar cells either have ON-centres or OFF centres (centre polarising and centre depolarising, respectively) as shown by Nelson et al. (1978) in their studies with cats, and also thought to be consistent with the human retina (Kolb et al., 1992).

1.2.4. Horizontal cells

The outer plexiform layer residing horizontal cells (see Figure 1.2) consist of 3 types (HI, HII, and HIII), which laterally interconnect (Kolb et al., 1992; Kolb et al., 1994). It is primarily the cone photoreceptors that provide the horizontal cells with electrical signals (although HI cells also synapse with rod photoreceptors). These cells are thought to be involved with improving retinal spatial resolution, light/dark adaptation, and colour coding in the retina (Kolb, 2006).

1.2.5. Müller cells

Müller cells are a form of glial cell which span from the inner limiting membrane to the photoreceptors at the level of the inner segments (see Figure 1.2). Their function in the retina is to maintain potassium levels, influence the configuration of the inter-photoreceptor matrix, and provide glycogen for metabolism of carbohydrates (Newman, 1985). Additionally, Müller glia modify neovascularization, retinal

inflammation, and vascular leakage/lesions by producing a major angiogenic factor, vascular endothelial growth factor (VEGF or VEGF-A) (Le, 2017).

1.2.6. Amacrine cells

Amacrine cells (see Figure 1.2) have a cell body situated in the inner nuclear layer and provide lateral connections by synapsing with bipolar cells and RGCs in the inner plexiform layer. There are more than 25 types of amacrine cells in the human retina (Mariani, 1990; Kolb et al., 1992) and, although these are not fully understood, several are thought to have a role in the adjustment of the retinal responses to visual stimuli. The subtype AII, however, is well characterised. It is known to provide a link from the rod to cone bipolar cells, linking to the pathways of the cone RGCs (Kolb and Famiglietti, 1974).

1.2.7. Retinal Ganglion Cells and Retinal Nerve Fibre Layer

The retina's innermost layer comprises RGCs, which receive modified neurological signals from the bipolar cells and forward these to the optic nerve head in the form of action potentials along unmyelinated retinal surface axons. These axons form the retinal nerve fibre layer (RNFL), which is thickest at the optic nerve head rim. There are in excess of 18 types of RGC which have been identified in the primate retina (Kolb et al., 1992). Human RGCs are subdivided into parvo (P, or midget) and magno (M or parasol) types, which project to the parvocellular and magnocellular layers of the lateral geniculate nucleus (LGN), respectively (Leventhal et al., 1981; Perry and Cowey, 1981; 1984; Perry et al., 1984). Around 80% of primate retina RGCs are P (midget) cells and ~10% are M cells. The 1.2 million human RGCs synapse with the bipolar and amacrine cells (Quigley et al., 1982; Curcio and Allen, 1990; Wassle, 2004). Though around 50% of the RGCs are found in the macula, there aren't any at fovea due to lateral cell body displacement from the connecting cones (Curcio and Allen, 1990; Drasdo et al., 2007).

1.2.8. Retinal circulation

The retina has one of the highest per weight oxygen consumptions of the human body (Wong-Riley, 2010) and is incredibly metabolically active in order to maintain neuronal processing of ever-changing visual information (Sickel, 1972; Beatty et al., 2000;

Nowak, 2006). In order to do this efficiently, the retina has two circulatory systems. The retina's blood supply originates from the ophthalmic artery, with outer retinal layers being supplied by the choroidal circulation, via the short posterior ciliary arteries, whilst inner retinal layers are supplied by the central retinal artery. The central retinal artery reaches the eye via the optic nerve head (Hayreh, 1962), then dividing into superior and inferior branches, subsequently subdividing into temporal and nasal branches, and further again into the capillary networks known as the superficial network and deep network – supplying the retinal nerve fibre/ganglion cell layers and the inner nuclear layer respectively (Iwasaki and Inomata, 1986).

The choriocapillaris is the capillary network of the choroidal circulation. Unlike the inner retinal circulation, the capillaries are anastomosing and fenestrated, allowing transition of molecules into and out from the vessels (Snell and Lemp, 1998). The blood retinal barrier of the choroidal circulation is provided by the tight junctions of the retinal pigment epithelium. The choriocapillaris supplies such retinal components as the photoreceptor layer and the otherwise avascular macular region, around 500µm in diameter (Hendrickson, 2005. Oyster, 2006).

1.2.9. The Macula

The macula is the area of the retina where the RGC layer has a thickness greater than one cell in anatomical terms (Guedes et al., 2003). It has a diameter of 5-6 mm or ~20° and is located roughly 1mm inferior to the optic disc centre (Kolb, 2011; Oyster, 2006), and it is this description most often used to define the macula area in various clinical grading guidelines (Klein et al., 1991; Bird et al., 1995; AREDS, 2001a). The recent Beckman Initiative scale defined the macula as extending to two disc diameters from the macular centre (Ferris et al., 2013). The fovea (1.5mm diameter or ~5°) is located at the centre of this macular region (Remington, 2005; Kolb, 2011) and it is at the centre of this structure, the foveola (0.35 mm diameter or ~1°), that total retinal thickness is at its minimum (Polyak, 1949) and cone density is at its greatest in the healthy eye (Remington, 2005; Kolb, 2011). It is also here that maximum resolution is obtained in order to gain optimal visual acuity. The surrounding neuron dense parafoveal region subsequently joins with the perifoveal region which in turn amalgamates with the peripheral retina. The foveal pit does not contain the inner nuclear and ganglion cell layer's second and third order neurons (Kolb et al., 2020).

This is due to tightly packed cone photoreceptors and additionally the radial distribution of the circuitry to propel ganglion cells positioned at the outer part of the foveal slope to pass messages further along the visual pathway while the cone mosaic in the rod free foveal pit forms the foundation of human visual acuity. Three types of macular luteal pigment are present in the retina; lutein, zeaxanthin, and meso-zeaxanthin, largely located in the fovea within the layer of the fibres of Henle and the parafoveal inner nuclear layer (Bone et al., 1985; Trieschmann et al., 2008). These pigments have two important roles in retinal function – absorption of blue light in the inner retina, and scavenging of free radicals within photoreceptors (Kachik et al., 1997; Snodderly et al., 1984).

1.3. Associated Retinal Structures

1.3.1. The Retinal Pigment Epithelium

This single layer of 3-4 million hexagonal, uniformly arranged and densely packed cells is found between the photoreceptor layer and the choroid and becomes shorter and flatter as retinal eccentricity increases (Panda-Jones et al., 1996; Boulton and Dayhaw-Barker, 2001). The RPE plays a vital role in the optical, metabolic, physical and transformational functions of the visual process, including but not limited to stray light absorption to prevent light scatter within the eye, regeneration of visual pigment to 11-cis retinal after photopigment bleaching, and nutrient exchange with the choroid (Bok, 1993; Strauss, 2005). The RPE also contains light absorbing pigments, such as melanin, and antioxidants (for example, dismutase and catalase [Miceli et al., 1994; Simo et al., 2010]) which assist the carotenoids lutein and zeaxanthin present in Henle fibres of the macula in preventing photo-oxidative damage (Beatty et al., 2000; Beatty et al., 2001). Finally, the RPE produces maintenance factors to help to preserve retinal health, including anti-angiogenic pigment epithelial derived factor (PEDF) and pro-angiogenic vascular endothelial growth factor (VEGF) (Strauss, 2005; Simo et al., 2010; Frank et al., 1996; Witmer et al., 2003).

In animal models, destruction of the RPE results in atrophy of choroidal vessels, choroidal endothelial fenestration loss, and increased choroidal collagen deposition (Leonard et al., 1997; Korte et al., 1989). This is observed in humans where, as dry AMD becomes more advanced, RPE degeneration precedes choroidal vessel atrophy (Sarks et al., 1988; Sarks et al., 1976; McLeod et al., 2002; Young et al., 1987).

Lipofuscin pigment gathers with age in the RPE and correlations between this and subsequent cell apoptosis have been previously demonstrated (Dorey et al., 1989; Holz et al., 1999; Sutter, 2000). This accumulation of the pigment is thought to happen as a result of incomplete digestion of material during phagocytosis and the lipofuscin molecule itself leaves the RPE more vulnerable to oxidative damage (Margrain et al., 2004). The RPE also undergoes a decline in cell density and regularity with age (Panda-Jones et al., 1996). These changes can cause functional disruption with age, such as a slowing of the regeneration of visual pigment, and decline in the rate of dark adaptation (Bonilha, 2008).

1.3.2. Bruch's membrane

Bruch's membrane is a 5-layer structure between the choriocapillaris and the RPE. It is thin (approximately 2µm at birth) but thickens with age to approximately 4-6µm in the tenth decade (Ramrattan et al., 1994) and is made up of the basement membranes of the RPE and choriocapillaris, a central elastic layer, and both an inner and outer collagenous layer (Sumita, 1961; Del Priore et al., 2006; Booij et al., 2010). The structure of Bruch's membrane helps to strengthen the RPE layer and also serves as a molecular sieve during exchange between the outer retina and the choroidal circulation of nutrients, oxygen and biomolecules along with metabolic waste (Booij et al., 2010).

1.3.3. The Choroid

The choroid is located between the retina and sclera. The smallest and most dense capillaries within this layer are found within the choriocapillaris and the densest of these are beneath the macula (Fryczkowski and Sherman, 1988). The choroid's main function is to distribute metabolites and remove the waste products from the outer retina. This is a vital task and nowhere more so than the macula where no other vasculature is present to manage this. The lack of surplus vasculature in the macula area has been suggested to be a reason it is so susceptible to ageing changes (Penfold et al., 2001; Provis et al., 2005) compared to the dual supplied areas elsewhere in the retina. Age related changes to choroidal structures include increased spacing between capillaries, and a reduction in number and/or diameters of vessels (Ramrattan et al., 1994) and with this knowledge it is unsurprising to note that evidence of a reduction of

choroidal blood volume and flow has been found with increase in age (Grunwald et al., 1998).

1.4. Age-Related Macular Degeneration

1.4.1. Overview

Age-related Macular Degeneration (AMD) was responsible for approximately half of sight impairment and severe sight impairment registrations in England and Wales in 2012-2013 (Quartilho et al., 2016). In fact, it is thought that over 750,000 people in the UK will have AMD by the end of this decade (Minassian et al., 2011). Manifestations of AMD can eventually lead to loss of central vision and ultimately visual impairment. This can negatively affect an individual's quality of life along with abilities to read, write, recognise faces and drive (Taylor et al., 2016; Mitchell and Bradley, 2006). Affected persons can suffer from a loss of independence or income and even depression and anxiety (Rovner et al., 2002; Berman and Brodaty, 2006; Taylor et al., 2016).

The exact mechanism of AMD pathogenesis remains unknown though current evidence suggests it is a multi-factorial process. In brief, these pathogenic processes are thought to include age-related Bruch's membrane thickening and increased hydrophobicity (Spraul et al., 1996), oxidative stress (Beatty et al., 2000), lipofuscin formation (Beatty et al., 2000), chronic inflammation (Ambati and Fowler, 2012), hypoxia (Steffanson et al., 2011), and ischaemia (Feigl, 2007). Although the biophysical mechanism is uncertain, the functional visual loss caused by AMD can be traced back to loss of photoreceptor function as a secondary factor to either a neovascular or atrophic event. The following subsections summarise the mechanisms thought to be implicated in AMD onset. Greater detail of this can be reviewed in Stefánsson et al. (2011), Luthert (2011), Ambati et al. (2003) and Ambati and Fowler (2012).

1.4.2. Lipofuscin

Lipofuscin is a pigment which accumulates in RPE cells with increasing age (Wing et al., 1978). It is a lipid-protein aggregate containing toxic molecules such as N-retinylidene-N-retinylethanolamine (A2-E), and forms due to incomplete phagocytosis of shed photoreceptor outer segment discs by the RPE (Eldred and Lasky, 1993; Kennedy et al., 1995). Lipofuscin accumulation is thought to be a significant contributing factor to the pathogenesis of age-related macular degeneration and later, geographic atrophy (Holz

et al., 2001; Kennedy et al., 1995). A key piece of circumstantial evidence is the strong relationship between increasing age and both increased risk of AMD and increased accumulation of lipofuscin in the RPE (Beatty et al., 2000). It has also been shown that RPE cells of eyes with geographic atrophy are densely packed with lipofuscin (Sarks et al., 1988). This is supported by findings that the annular pattern of macular degeneration is well correlated with the area of greatest lipofuscin concentration (Weiter et al., 1988; Holtz et al., 2001).

There are two likely mechanisms by which lipofuscin may contribute to RPE damage in AMD, in addition to possible mechanical effects. Firstly, lipofuscin acts as a photosensitising agent, and so can propagate oxidative stress in the RPE (Boulton et al., 1993; Gaillard et al., 1995). Secondly, components such as A2-E also have a directly toxic effect on cell functions, including lysosomal activity (Holz et al., 1999). Holz et al. (2001) could not establish whether lipofuscin accumulation is a cause or an effect of RPE cell death, but the RPE loss with which it is associated certainly appears to occur prior to the ensuing disruption of the adjacent choriocapillaris and photoreceptors (Roth et al., 2004; Sunness, 1999).

1.4.3. Chronic inflammation

There is substantial evidence to support the hypothesis that an inflammatory response may contribute significantly to AMD pathogenesis (e.g. see Ambati and Fowler., 2012). For example, evidence has shown there to be inflammatory proteins within drusen (Anderson et al., 2002; Hageman et al., 2001; Johnson et al., 2001). Roth et al. (2004), Shankar et al. (2007) and Chakravathy et al. (2010) suggested that it was these drusen along with other age and AMD related retinal debris that caused the release of inflammatory markers. A genetic predisposition to development and progression of AMD is thought to be due to variants in the particular genes that influence immune responses, for example Complement Factor H (Sparrow et al., 2012, Donoso et al., 2010; Despret et al., 2006; Li et al., 2006), which may be responsible for almost half of AMD cases (Haddad et al., 2006). Moreover, fragments of inflammatory proteins have been shown to be associated with expression of vascular endothelial growth factors (VEGF) and as such could be a factor in the neovascularisation of the choroid that results in nAMD (Nozaki et al., 2006).

1.4.4. Oxidative stress

Due to the high levels of light exposure and concentrations of polyunsaturated fatty acids in photoreceptor outer segments the macula is at high risk of oxidative damage (Beatty et al., 2000, Roth et al., 2004). Many studies have explored links between oxidative stress and AMD (e.g. AREDS, 2001a; AREDS2, 2013; Weikel et al., 2012; Tan et al., 2008b; San Giovanni et al., 2007; Seddon et al., 2006; Roth et al., 2004; Zarbin, 2004, Beatty et al., 2000) and it is known that some of the constituents of drusen arise due to protein modifications from oxidative processes (Hollyfield et al., 2003; Crabb et al., 2002). Antioxidants have been thought to have a protective effect on macular tissue and may even act as an AMD prophylactic and/or slow the rate of disease progression when taken as high dose oral supplements (AREDS 2001a/b; AREDS2, 2013; Chew et al., 2014; Roth, et al., 2004). Conversely, a significantly increased risk of developing AMD is also found in persons who smoke (Velilla et al., 2013; Khandhadia and Lotery, 2010; Khan et al., 2006; Thornton et al., 2005), a factor known to increase oxidative stress.

1.4.5. Hypoxia and metabolism

The high oxygen demand of the macular area and its merely sufficient blood supply render it unsurprising that outer retinal hypoxia has been suggested to be a factor in the pathogenesis of AMD (Stefansson et al., 2011). It can be seen that age-related changes in the choriocapillaris are mirrored by increases in AMD prevalence (Sarks, 1976; Ramrattan et al., 1994; Klein et al., 1992). These age-related changes in the choriocapillaris vasculature have been presented as a causative factor in AMD onset (Sarks, 1976; Friedman et al., 1995; Ciulla et al., 1999; Friedman, 2008). Blood flow reduction in AMD has been shown to be associated with abnormalities in functional parameters, for example flicker electroretinogram (ERG) implicit times (Remulla et al., 1995). Functional changes resembling those seen in AMD have also been reported in studies where experimental hypoxia was induced in subjects (Feigl et al., 2007; Feigl et al., 2008). This provides circumstantial evidence to support a relationship between hypoxia and AMD onset.

It has been suggested that the vulnerability of the outer retina to hypoxia is due to the limited choroidal blood supply humans evolved to supply the macular area while

maintaining high acuity levels (Provis et al., 2005). In other words, under dark-adapted conditions, the oxygen supply provided by the choroidal circulation is barely sufficient to meet the high metabolic requirements of the photoreceptor dark current (Wang-Wirawan and Linsenmeier, 2003). Age and AMD-related reductions in choroidal blood flow (Grunwald et al., 1998; Ramrattan et al., 1994; Sarks, 1976) and thickening of Bruch's membrane (Guymer et al., 1999; Karwatowski et al., 1995; Ramrattan et al., 1994), are likely to place stress on this system. For example, thickening of Bruch's membrane and the presence of basal linear deposits associated with AMD (Curcio et al., 2005) result in a longer distance over which oxygen must travel to reach the outer retina from the choroid. This leads to a reduced diffusion flux (due to Fick's law), which may result in a reduced oxygen availability at the retina (Steffanson et al., 2011). This effect is likely to be exacerbated by the fact that the choroid is unable to respond to changes in local metabolic demand due to a lack of regulatory system in the otherwise sympathetically innervated structure (Delaey and van de Voorde, 2000). Ultimately, these factors are likely to impact the delivery of nutrients and oxygen to the macula leading to a stress response and possibly feeding into a subsequent inflammatory response (Provis et al., 2005).

Literature reviews have discussed the links between retinal hypoxia and early AMD and/or ageing (Stefansson et al., 2011; Feigl et al., 2007), and the chronic presence of hypoxia could stimulate expression of VEGF and subsequent neovascularisation (Witmer et al., 2003). This is supported by the finding of increased levels of VEGF in eyes with AMD, even at an early stage (Kliffen et al., 1997; Witmer et al., 2003). Whilst factors other than hypoxia can also result in increased VEGF expression, including inflammation and acidosis (Ferrara et al., 2003), the presence of hypoxia inducible factor, a precursor to VEGF production, in choroidal neovascular membranes (Inoue et al., 2007; Sheridan et al., 2009), is supportive of at least a contributory role of hypoxia in this process.

1.4.6. Clinical features of Age-related Macular Degeneration

According to the Beckman initiative, AMD can be categorised as early, intermediate, or late, with late AMD either taking the form of nAMD, and/or geographic atrophy (See Table 1.1, Ferris et al., 2013).

Classification of AMD	Definition (lesions assessed within 2 disc diameters of fovea in either eye)
No apparent aging changes	No drusen and No AMD pigmentary abnormalities*
Normal aging changes	Only drupelets (small drusen ≤ 63 μm) and No AMD pigmentary abnormalities*
Early AMD	Medium drusen > 63 μm and ≤ 125 μm and No AMD pigmentary abnormalities*
Intermediate AMD	Large drusen > 125 μm and/or Any AMD pigmentary abnormalities*
Late AMD	Neovascular AMD and/or Any geographic atrophy

AMD = age-related macular degeneration.
 *AMD pigmentary abnormalities = any definite hyper- or hypopigmentary abnormalities associated with medium or large drusen but not associated with known disease entities.

Table 1.1. The classification of AMD according to Ferris et al., 2013.

NICE guidelines for the classification of AMD are similar, but omit the ‘intermediate’ form of AMD in favour of early AMD at low, medium or high risk of progression, and include ‘indeterminate’ and ‘wet inactive’ stages of late AMD (NICE, 2018). Late AMD is responsible for the majority of sight impairment in AMD. The prevalence of geographic atrophy was expected to be half of that of nAMD in 2020 (Minassian et al., 2011), however it was responsible for 53% of registrations as sight impaired/severely sight impaired in 2011-2012 attributable to AMD, due to the currently untreatable nature of the atrophic form of the disease (Bunce et al., 2015).

1.4.7. Early/Intermediate Age-related Macular Degeneration

Early AMD is characterised by the presence of drusen of medium size ($> 63 \leq 125$ μm) within the macular area but no pigmentary changes, whereas intermediate AMD is characterised by drusen larger than 125 μm and/or AMD pigmentary changes in the macular area (Ferris et al., 2013). Treatment of these preliminary forms of AMD is

largely supportive and advisory as there is no medical intervention currently available to halt the progression of these conditions (Denniston and Murray, 2009). Patients may be referred to counselling sessions, be advised on lifestyle changes (such as dietary advice, high dose vitamin supplementation or smoking cessation), or be offered refractive aids to assist with low vision that can no longer be fully corrected by spectacles (Royal College of Ophthalmologists, 2013).

1.4.8. Geographic Atrophy

Geographic atrophy is a term used to describe advanced non-exudative AMD and refers to the degeneration of the retinal pigment epithelium (RPE) and photoreceptors in discrete regions (Ferris et al., 2013). As with early and intermediate AMD there is currently no medical intervention available to treat this condition and so the treatments available are of a supportive and educational nature (Denniston and Murray, 2009).

1.4.9. Neovascular Age-related Macular Degeneration

Neovascular AMD is caused by the growth of novel, unstable vessels from the choriocapillaris, through breaks in Bruch's membrane, to proliferate beneath the RPE or in the subretinal space. These vessels leak fluid and blood beneath the RPE, into the subretinal space, and into the retina itself, causing a rapid loss of vision if untreated (Tufail et al., 2010; Nozaki et al., 2006).

Suggested stimuli for choroidal neovascularisation include ischaemia and inflammatory factors, leading to an imbalance of inhibitory and stimulatory growth factor expression (e.g. Pigment Epithelial Derived Factor [PEDF] and VEGF). Evidence for the role of VEGF in this process is found in the presence of VEGF-A in CNV membranes, in the RPE of people with high risk AMD, and in the vitreous of people with nAMD (Witmer et al., 2003).

In 2008 the methods of treating nAMD changed when the National Institute for Health and Care Excellence (NICE) approved the use of ranibizumab for intraocular use (NICE Guidelines TA 155). Previously only photodynamic therapy (PDT) could be offered to these patients. PDT was specifically indicated for patients with a subtype of nAMD termed "classic" (where the choroidal neovascularisation manifests as an area of early,

discrete hyperfluorescence on fluorescein angiography, with increase in size in the later phases of the angiogram); other types of nAMD (“occult” or “minimally classic” choroidal neovascularisation) were not responsive to PDT. Though this PDT treatment slowed the progression of their sight loss, lost sight was not restored (TAP Study Group, 1999), and adverse effects on retinal health were reported (Tzekov et al., 2006). With the approval by NICE of anti-VEGF drug ranubizumab (Lucentis®), came the opportunity to offer a treatment to the patient which may be able to improve their visual function rather than purely limit the damage (Rosenfeld et al., 2006). Since the first approval of ocular anti-VEGF treatment more anti-VEGF drugs have now been approved for this use, for example aflibercept (‘Eylea®’; Regeneron) (Royal College of Ophthalmologists, 2013) and at time of writing anti-VEGF drugs are considered as first choice of treatment for nAMD. This is not surprising when you consider anti-VEGF has been shown to have greater treatment effectivity than its predecessor (PDT) and additionally, is also sometimes able to restore vision to patients, rather than merely prevent disease progression (Brown et al., 2006; 2009).

1.4.10. Age-related Macular Degeneration: A Summary

Early stages of AMD involve a complex interaction of factors including ageing changes in the choroid (Margolis and Spaide, 2009; Grunwald et al., 1998; Ramrattan et al., 1994; Sarks, 1976) and Bruch’s membrane (Green, 1999; Karwatowski et al., 1995; Ramrattan et al., 1994), oxidative stress (Beatty et al., 2001), local inflammation (Donoso et al., 2006; Donoso et al., 2010), reduction in choroidal perfusion (Friedman et al., 1995; Ciulla et al., 1999; Harris et al., 1999; Ciulla et al., 2002; Feigl, 2009), and lipofuscin and membranous deposition (Kennedy et al., 1995; Holz et al., 2001; Roth et al., 2004). Eventually these factors will cause progression from early AMD to the intermediate and later stages of AMD such as geographic atrophy in non-exudative (dry) AMD and choroidal neovascularisation in neovascular (wet) AMD and cause a dramatic reduction in central vision. It is possible that a patient may not progress to the neovascular stage of AMD and only experience the slower dry related changes, depending on how VEGF is expressed in the retina following the changes previously described (Witmer et al., 2003; Frank et al., 1996), however it is also possible for patients to develop nAMD in an eye with late stage dry AMD i.e. geographic atrophy (Sunness et al., 1999).

1.5. Vascular Endothelial Growth Factors and Anti-Vascular Endothelial Growth

Factor drugs

1.5.1. Vascular Endothelial Growth Factors

Production of new blood vessels (angiogenesis) is a vital process in human physiology throughout every stage of life. It is stimulated by angiogenic growth factors, including vascular endothelial growth factor (VEGF). VEGF is actually a protein family (VEGF-A, -B, -C, -D, and -E) that use tyrosine kinase receptors to action their tasks (VEGFR-1, -2, and -3) (Andreoli and Miller, 2007). There are multiple isoforms of VEGF-A, with VEGF₁₂₁ and VEGF₁₆₄₍₁₆₅₎ being the major isoforms expressed in the eye (Robinson and Stringer, 2001). Key functions of VEGF are promotion of mitosis of endothelial cells, leading to angiogenesis (Leung et al., 1989), and the induction of vascular permeability, to an extent 50,000 times greater than histamine (Senger et al., 1983; Senger et al., 1990). VEGF proteins have a role in many physiological processes such as menstruation and response to inflammatory or ischaemic disease (Hoeben et al., 2004), and are critical in embryonic development of vasculature, including in the eye (Gogat et al., 2004). Absence of VEGF expression by the RPE in developing eyes has been shown to cause abnormalities of the RPE cells, halt development of the choriocapillaris, and ultimately result in loss of retinal function and microphthalmia (Marneros et al., 2005, Saint- Geniez et al., 2006).

In addition to their role in embryonic development, VEGF proteins are also present in the vascularised areas of the healthy adult eye, i.e. the conjunctiva, retina, and the choroid-retinal epithelial complex (Kim et al., 1999; Blauwgeerss et al., 1999; Saint-Geniez et al., 2009; Famigiletti et al., 2003). Specifically, in the retina of the healthy eye, VEGF protein and mRNA have been located in the cone photoreceptors, the inner nuclear layer, amacrine cells, glial cells, Müller cells, RGCs and RPE cells (Kim et al., 1999; Blauwgeerss et al., 1999; Saint-Geniez et al., 2009; Famigiletti et al., 2003). Furthermore, VEGFR2 receptors (the main signalling receptor for VEGF) have been identified on structures including the capillary endothelium of the retina, choroid and ciliary body, Muller cells, and photoreceptor cells (Saint Geniez et al., 2008; Ford et al., 2011; Ford et al., 2012). The presence of VEGF proteins throughout the healthy adult eye suggests that the tasks they perform are vital in order to maintain the health of the retina and associated structures (Saint-Geniez et al., 2008; Famigllietti et al., 2003;

Kim et al., 1999; Marneros et al., 2005; Blaauwgeers et al., 1999; Saint-Geniez et al., 2009; Stone et al., 1995). One putative role of VEGF in the eye is in neuroprotection. It has been shown that apoptosis of retinal neurons as a result of ischaemia is substantially reduced in the presence of VEGF (Nishijima et al., 2007). VEGF is also thought to play an important role in ischaemic preconditioning, the process by which apoptosis occurs to a lesser extent if a short period of ischaemia precedes a long period of ischaemia. Nishijima et al. (2007) also demonstrated a loss of RGCs in normal adult mice upon chronic inhibition of VEGF-A function, whilst Saint-Geniez et al. (2008) reported a significant increase in cell apoptosis in inner and outer retinal layers upon the systematic neutralisation of VEGF in adult mice. The presence of VEGF receptors on retinal glia and neurons may provide a mechanism for this apparent neuroprotective effect (Shima, 2009). There is also strong evidence that VEGF generated by the RPE is vital for the maintenance of aspects of choriocapillaris health (Saint-Geniez et al., 2009). One such aspect is the maintenance of the fenestrations of the vessels of the choriocapillaris - a vital component in chorioretinal morphology that allows molecular transportation between the retina and choroid to maintain ocular health and function (Kim et al., 2020; Adamis et al., 1993; Esser et al., 1998). RPE cell damage and death, with a subsequent reduction in the production of VEGF, has been shown to be followed by choriocapillaris atrophy and fenestration loss (Korte et al., 1984; Leonard et al., 1997). Decreased VEGFA levels have been shown to result in a thickening of choroidal endothelial walls with fewer fenestrations, and have ultimately harmed the normal structure and function of the RPE (Ford et al., 2011).

Whilst angiogenesis is a natural and necessary process (Andreoli and Miller, 2007), the pathological upregulation of VEGF stimulates the disorganised growth of new blood vessels which, as a result of the increased vessel permeability induced by VEGF, are also leaky (Shima, 2009). This uncontrolled growth of retinal blood vessels is a significant factor in ocular neovascular diseases such as neovascular age-related macular degeneration (nAMD), diabetic retinopathy and retinal vein occlusion (Andreoli and Miller, 2007; Tufail et al., 2010; Nozaki et al., 2006; Ferrara, 2002). VEGF proteins, in particular VEGF₁₆₄ – known to be present in ocular structures - have also been shown to have pro-inflammatory properties, creating leukocyte-based inflammation (Melder et al., 1996; Miyamoto et al., 2000; Shima, 2009). This

inflammation is also likely to play an important role in the pathogenesis of nAMD. As previously mentioned, anti-VEGF therapy is currently the treatment of choice for nAMD (see Section 1.3.9).

1.5.2. Anti-Vascular Endothelial Growth Factors

Anti-VEGF molecules are recombinant, monoclonal antibodies, fragments of antibodies or fusion proteins containing components of VEGF receptors, that bind to receptor sites of VEGF molecules. Figure 1.4 illustrates the mechanism of action. Intraocular use of anti-VEGF, specifically the antibody fragment ranibizumab ('Lucentis®'; Novartis), was approved in 2006 and 2007 in the USA and Europe respectively (FDA, 2006; EMA, 2007) though non-fragment versions of the molecule (bevacizumab ['Avastin®'; Gentech Inc]) have been used in an off label capacity since 2005 (Rosenfeld et al., 2005).

Ranibizumab comprises a monoclonal antibody fragment effective against VEGF-A (Heier et al., 1996). It was first developed with the aim of improving the passage of the drug into the retina when compared to the bulkier antibody bevacizumab (Andreoli and Miller, 2007). After being intravitreally injected it binds to the receptor binding site of active VEGF-A, including that of the biologically active, cleaved form of this molecule, VEGF110 (Mitchell et al., 2010; Burgess et al., 2013). This binding prevents the VEGF-A molecule from binding with its receptors (VEGFR1/VEGFR2) on endothelial cell surfaces, in turn reducing vascular leakage, cell proliferation and angiogenesis. The effectivity of ranibizumab was documented in the findings of first the Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular AMD (MARINA) trial (MARINA, 2006) and the Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in AMD (ANCHOR) trial (Brown et al., 2009). Both these trials revealed the significantly improved best corrected visual acuity outcomes compared to previously documented information on nAMD treatment outcomes. Further details of the trial evidence are given in Section 1.5.3.

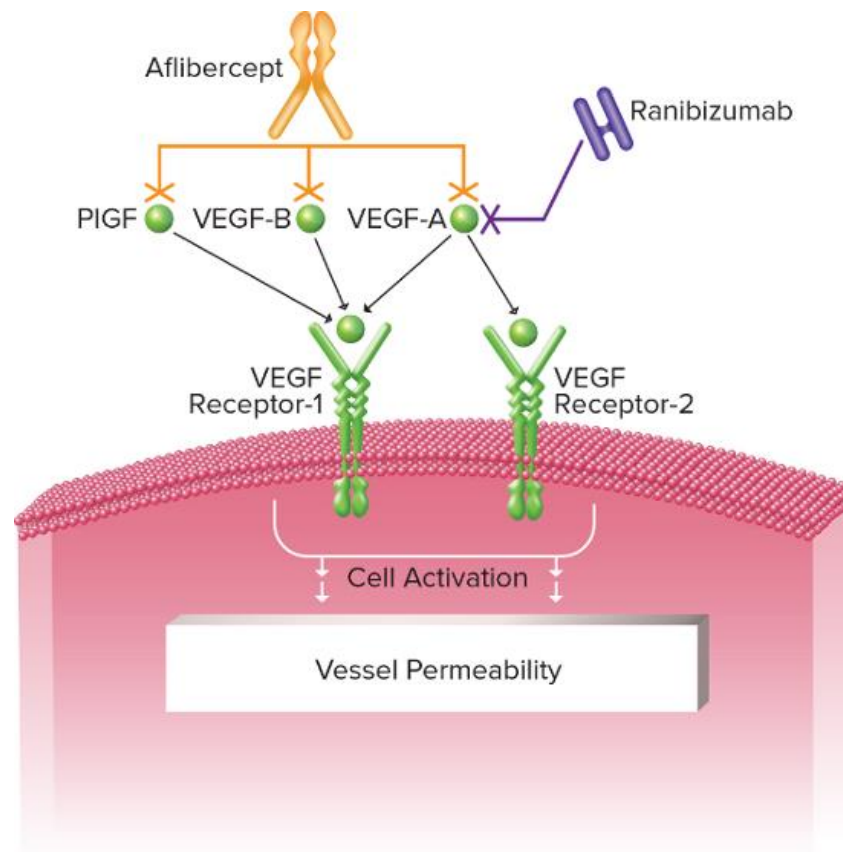


Figure 1.4. Diagram explaining the mechanisms of action of the clinically approved drugs ranibizumab and aflibercept. The single binding site of ranibizumab and multiple sites of aflibercept are shown preventing VEGF-A, VEGF-B and PIGF from binding with VEGF receptors and initializing angiogenesis. Taken from www.scienceofdme.org/treat, accessed September 2019.

Pegaptanib ('Macugen®'; Pfizer) and the off-label drug bevacizumab also act via this mechanism (Kim, 2007). Bevacizumab is a full-length humanized monoclonal antibody directed against all isoforms of VEGF-A, but was originally developed as an intravenous treatment for metastatic colorectal cancer (Hurwitz et al., 2004). Pegaptanib is a pegylated ribonucleic acid oligonucleotide ligand (aptamer) which binds to the receptor sites of the VEGF₁₆₄ isomer - thought to be the primary origin of eye disease related neovascularization (Gragoudas et al., 2004). Though bevacizumab has not been approved for 'off-label'/'unlicensed' use, studies such as the IVAN trial (Chakravarthy et al., 2012) have shown that it is just as safe and effective as approved ocular anti-VEGF agents while also being significantly more cost effective. For example, in the Comparison of AMD Treatments Trials (CATT) trial, similar clinical outcomes were obtained between groups receiving ranibizumab and bevacizumab (regardless of scheduled or pro re nata [PRN] treatment provision) over time (CATT Research Group,

2016; Martin et al., 2011). The parity in outcomes was retained in the 5-year follow-up data, although the visual gains in all groups made in the first two years of the study were not maintained through the final 3 years (Maguire et al., 2016). Thus, despite initial qualms the molecule may be too large to penetrate the retina – leading to the development of ranibizumab - bevacizumab has now been shown to still be able to penetrate the tissue and impede ocular VEGF activity (IVAN study investigators, 2012; Chakravarthy et al., 2013b; CATT Research Group, 2011; 2016). It is worth noting however, that a link has been proposed between treatment with bevacizumab and higher incidence of systemic adverse effects of a serious nature (Martin et al., 2012; Lanzetta et al., 2013), although this finding is not reflected in all studies (e.g. Maguire et al., 2016).

The more recently developed aflibercept (Eyelea™) is neither an antibody nor an antibody fragment. It is a type of recombinant fusion protein made up of human VEGF binding sections attached to a section of human IgG1 immunoglobulin (Khan et al., 2014). It has a different mechanism, acting as a decoy receptor for both VEGF-A and VEGF-B isoforms as well as placental growth factor (PGF) and thus stops VEGF binding to and activating its receptors with higher affinity than the previously mentioned anti-VEGF treatments (Khan et al., 2014; Frampton, 2012). As well as acting on several types of growth factors, aflibercept also has an 8-week dosing regimen which could help to reduce the logistical burden on health care services (Sarwar et al., 2016). This is due to fewer doses required to achieve equal efficacy to other anti-VEGF treatments (Schmidt-Erfurth et al., 2014; Trichonas and Kaiser, 2013; Heier et al., 2012; CATT Research Group, 2011; Dixon et al., 2009). There have been shown to be many other positive factors in usage in nAMD management such as retinal structural improvement (Hatz and Prünte, 2017; Lim et al., 2017), good long term outcomes (Eleftheriadou et al., 2017), and an improvement in visual-related quality of life in patients who had previously been non-responders to nAMD treatment with ranibizumab (Zhu et al., 2017).

1.5.3. Anti-VEGF drug regimens: evidence from clinical Trials

In section 1.5.2., a brief overview is given of the evidence supporting the effectiveness of anti-VEGF drugs in the treatment of nAMD. Here further consideration is given to the findings of the key trials evaluating effectiveness and optimal dosing regimen.

There have been several large clinical trials examining ranibizumab anti-VEGF regimens. The MARINA trial was a multicentre, randomized, double-masked, sham-controlled study. Seven hundred and sixteen patients were randomized to one of three groups; sham (n = 238), ranibizumab 0.3 mg (n = 238), or ranibizumab 0.5 mg (n = 240). The patients in this trial had the “occult” or “minimally classic” forms of nAMD (section 1.4.9). These same treatment modalities were examined in patients with “classic” nAMD that were recruited to the ANCHOR study, an international multicentre, double masked, randomized, active-treatment-controlled clinical trial. In this study patients at the study sites were randomized to either a group with verteporfin PDT plus monthly sham intraocular injection, or to sham verteporfin PDT plus intravitreal ranibizumab 0.3 mg or 0.5 mg injections, also issued monthly. The MARINA and ANCHOR studies showed 95% of patients treated with ranibizumab preserved visual acuity (i.e. lost less than 15 letters) whereas only 62% of sham or PDT participant groups did ($P < 0.01$) (Brown et al., 2006; Kaiser et al., 2006; Rosenfeld et al., 2006; Miller et al., 2005).

Results from the MARINA and ANCHOR clinical trials suggest a dose-response curve and that 0.5 mg ranibizumab produces greater efficacy in functional and anatomic outcomes than 0.3 mg ranibizumab (Brown et al., 2009; Rosenfeld et al., 2006).

Further to this, a study by Brown et al. (2013) investigated the effects of giving higher doses of ranibizumab to patients not responding to the approved 0.5 mg dosage as recommended by NICE (NICE TA 155, 2008). Eighty-seven participants who had previously shown insufficient improvement in retinal structure and function with the approved dosage were given three intravitreal injections of ranibizumab at a concentration of 2 mg and showed statistically significant improvements in retinal structure and function with this higher dose. However, this higher dose is not licensed for use and potential long term adverse effects of an increased dosage have not been evaluated.

Two main trials have investigated the relative effectiveness of bevacizumab and ranibizumab. The CATT clinical trial was a single-blind, multicentre, noninferiority trial, where randomly assigned patients (n = 1208) with neovascular AMD received intravitreal injections of either ranibizumab or bevacizumab pro re nata or with monthly treatment plans. The Inhibit VEGF in Age-related choroidal Neovascularisation (IVAN) clinical trial was a multicentre, masked, non-inferiority randomised trial that also compared results from ranibizumab (0.5 mg) or bevacizumab (1.25 mg). Six-hundred and ten adults (aged 50+ years) with as yet untreated active neovascular nAMD were recruited from 23 UK hospitals and randomly assigned to intravitreal injections of each respective drug type in monthly pro re nata regimens and reviewed monthly. Both trials reported similar efficacy of the two drugs (CATT Research Group et al., 2011; CATT Research Group et al., 2012; IVAN study investigators et al., 2012).

Trials have also been conducted to determine the optimal treatment regimen for these drugs. The HARBOR study was a randomized, double-masked, multicentre, active treatment-controlled trial where treatment naïve nAMD patients aged 50 years and over (n = 1098) were randomized to receive intravitreal injections of ranibizumab 0.5 mg or 2.0 mg. These interventions were issued either pro re nata (PRN) or monthly after 3 initial loading doses (issued once per month). Pro re nata treatment employs regular follow ups (around once a month) with retreatment when disease progression is seen (Spaide 2007; Lalwani et al., 2009; Boyer et al., 2009; Tano and Ohji, 2010; Dadgostar et al., 2009; Gupta et al., 2010). Two year follow up data showed similar mean best corrected visual acuity improvements in all groups, suggesting that a PRN dosing strategy may be used after the initial loading phase of treatment, reducing patient and health service burden whilst maintaining effectiveness (HARBOR study group, 2014). However, other major trials have suggested that PRN regimens fair less well over a prolonged follow up period (Maguire et al., 2016; Lalwanik et al., 2009) possibly due to the damaging effects of repeated retinal fluid accumulation and resolution. Furthermore, in some studies patients undergoing anti-VEGF therapy were shown to be at greater risk of serious adverse events such as stroke or myocardial infarction (Kemp et al., 2013; Chakravarthy et al., 2013). While there are also some studies that disagree with these associations (i.e. no increased risk of serious event or mortality with anti-VEGF exposure [Campbell et al., 2012; Curtis et al., 2010]) there has

been some indication that pro re nata treatment plans have been shown to have greater mortality rates than those undergoing monthly injections (for example, Chakravarthy et al., 2013). This interesting finding occurred irrespective of anti-VEGF drug type. More recent evidence suggests that improved outcomes may be obtained with a 'treat and extend' regimen, whereby an injection is provided on every patient visit, but the frequency of follow-up visits is determined by clinical observations, such as whether macular fluid accumulation is noted, or whether the macula remains dry (Augsburger et al., 2019). Intervals between treatments and follow ups are progressively prolonged in the absence of disease progression (Brown and Regillo, 2007; Chiang and Regillo, 2011), resulting in reduced treatment frequency and reduced appointment burden compared to a monthly treatment regimen.

Retreatment rates are of importance for a number of reasons. As well as the economic and infrastructural considerations mentioned above, the number of injections received is also relevant with respect to the risk of complications, as each injection carries with it a small but potentially devastating risk of endophthalmitis (Pilli et al., 2008). More recently, potential adverse effects of a less acute variety have been reported. Sadda et al. (2018) examined incidence of macular atrophy in 1095 eligible patients in the HARBOR study over 2 years and found that those undergoing monthly anti-VEGF therapy were at greater risk of developing geographic atrophy than those on a pro re nata treatment program. Similarly, the IVAN trial two year report showed an increase in risk of developing geographic atrophy with monthly compared with discontinuous treatment. This effect did not differ between bevacizumab and ranibizumab, suggesting that it relates to retreatment frequency rather than the type of anti-VEGF drug used (Chakravarthy et al., 2013). These findings provide some evidence of a dose-dependent relationship between anti-VEGF drug use and macular atrophy.

The final anti-VEGF drug currently available for the treatment of nAMD is aflibercept. The VIEW 1 and VIEW 2 Trials compared safety and efficacy of aflibercept to that of ranibizumab (Heier et al., 2012). These double-masked, multicenter, parallel-group, active-controlled, randomized trials issued 2419 patients with sub- or juxta-foveal choroidal neovascularization (CNV) lesions with either intravitreal aflibercept 0.5 mg (monthly), aflibercept 2 mg (monthly), aflibercept 2 mg (every 8 weeks after 3 initial monthly loading doses), or ranibizumab 0.5 mg monthly. The findings showed

aflibercept injected monthly or two monthly (after three monthly loading doses, initially) gave similar efficacy and safety as monthly doses of the already approved ranibizumab 0.5 mg treatment, suggesting that aflibercept issued every-2-months is a reasonable alternative treatment, with the added benefit that patients are less at risk from more frequent injections and medical retina clinics may be less overburdened.

The NICE guidelines for treatment of nAMD currently only approve the use of aflibercept (NICE TA 294, 2013) or ranibizumab (NICE TA 155, 2008) for the treatment of nAMD. In the treatment of nAMD with anti-VEGF drugs there is a loading phase (three injections on consecutive months) and a subsequent maintenance phase, usually following the PRN or treat and extend (Brown and Regillo, 2007; Chiang and Regillo, 2011) treatment regimens. Freund et al. (2015) used data from treat and extend studies (n = 11) and an algorithm to identify the ideal application of treat and extend treatment schedules, recommending extending intervals between treatments by two weeks at a time up to a maximum of 12 weeks.

1.5.4. Toxicity Data for anti-VEGF drugs

Studies have been performed to assess the toxicity of systemic and ocular anti-VEGF therapy. The risk of intraocular injection of anti-VEGF drugs causing a systemic adverse effect is dependent on whether the action is confined to the treated eye. There is evidence to suggest that treatment of one eye has an effect on the contralateral eye (Avery et al., 2006; Karaca et al., 2003; Hanhart et al., 2014; Acharya et al., 2013). In a study by Karaca et al. (2003) four patients with retinopathy of prematurity treated with bevacizumab were examined for adverse effects of the treatment, with findings indicating that bevacizumab had made its way into the systemic blood stream in concentrations high enough to affect vascular activity in the untreated eyes of the patients. This was supported by evidence from Avery et al. (2014), where 56 patients undergoing intravitreal anti-VEGF treatment for nAMD were assessed for their systemic exposure to the various anti-VEGF drugs used (ranibizumab 0.5 mg, bevacizumab 1.2 mg, or aflibercept 2.0 mg). All three anti-VEGF drugs were observed to move quickly into the bloodstream after intravitreal injections, but ranibizumab was noted to clear much more quickly than bevacizumab or aflibercept. The latter caused far greater systemic exposure and notably produced a clear decrease in plasma free VEGF. Though Avery et al. (2014) found ranibizumab to be cleared from the systemic

blood stream quickly, Acharya et al. (2013) examined contralateral untreated eyes of patients being treated for cystoid macular oedema and found that two of three patients with bilateral disease but unilateral ranibizumab (0.5 mg) treatment saw clinically significant structural and functional improvements in both the treated and untreated eyes. Similar observations were reported by Hanhart et al. (2014), where patients with bilateral diabetic macular oedema saw bilateral improvement in structure and function of both treated (bevacizumab 1.25 mg) and untreated eyes of patients. This evidence seems to indicate that systemic/contralateral eye effects of unilateral treatment are possible and should be taken into consideration in studies where fellow eyes of living subjects/participants are used as a control.

In accordance with the pharmacokinetic evidence of systemic circulation of intraocular anti-VEGF treatment (Avery et al., 2014), there is some evidence to show that intravitreal injection of anti-VEGF therapy puts patients at increased risk of serious adverse events such as ischemic stroke (e.g. Pratt et al., 2014; Tlemsani et al., 2016; Ranpura et al., 2010). However, others have reported that there is no significant increased risk of adverse events associated with systemic anti-VEGF therapy (e.g. Campbell et al., 2012, Manresa et al., 2015). In a meta-analysis, Bressler et al. (2012) found no evidence of an overall increased risk of cerebrovascular accident in five randomised controlled trials of ranibizumab for the treatment of nAMD. It may be that particular groups, such as those with a high baseline risk of stroke (Bressler et al., 2012) are at an increased risk of serious adverse events, especially if receiving bevacizumab (Soloman et al., 2014) or aflibercept (Beaumont et al., 2014; European Medicines Agency, 2018) rather than ranibizumab.

In addition to evaluate the safety of anti-VEGF therapy at a systemic level, the effect of treatment on specific retinal cell lines and cultures *in vitro* has also been widely investigated (e.g. Parisi et al., 2019; Chae et al., 2018; Schniels et al., 2013; Luthra et al., 2006; Ammar et al., 2013; Klettner et al., 2014; Malik et al., 2013; Saenz-de-Viteri et al., 2016; Spitzer et al., 2007; Januschowski et al., 2014). Overall, these cell-line studies have demonstrated little evidence of toxicity at a cellular level. The following paragraph provides an overview of the key studies.

Parisi et al. (2019) investigated the effects of ranibizumab, bevacizumab, and aflibercept on primary human retinal RPE *in vitro*, demonstrating no cytotoxic effects

of any of the agents, but a reduction in RPE cell proliferation after treatment with ranibizumab or bevacizumab. Chae et al. (2018) demonstrated that RPE senescent cells were similarly not affected by cytotoxicity after exposure to clinical levels of all three drugs. Schnichels et al. (2013) took a rat RGC line, human RPE cell line and a mouse photoreceptor line and used cell assays to assess whether the anti-VEGF drug aflibercept (0.125, 0.5, 2 mg) had any effect on cell morphology. Its effect was compared to more established drugs ranibizumab (0.125 mg) and bevacizumab (0.3125 mg). Their findings suggested that aflibercept does not induce apoptosis, cause reduced cell viability or lead to any change in cell morphology. Luthra et al. (2006) evaluated human RPE (ARPE-19), rat neurosensory retinal (R28), and human microvascular endothelial (HMVECad) cells in response to bevacizumab of 0.125 mg/mL, 0.25 mg/mL, 0.50 mg/mL, and 1 mg/mL concentrations. They found no significant differences in cell viabilities in the cells treated with bevacizumab, compared to controls. Ammar et al. (2013) investigated the effect of aflibercept (0, 0.125, 0.25, 0.5, and 1 mg/mL) and ranibizumab (0, 0.125, 0.25, 0.5, and 1 mg/mL) on ARPE-19 cells. Cell assays showed that both aflibercept and ranibizumab had no toxic effect on the ocular cells, even at the highest concentration tested (1 mg/mL). Klettner et al. (2014) investigated the effects of aflibercept (125 mg/mL, 500 mg/mL) on porcine RPE cells over 7 days and found no cytotoxicity. However, when compared to bevacizumab and ranibizumab, aflibercept and bevacizumab did impair the phagocytic and the wound healing ability of the RPE cells. Malik et al. (2013) compared the safety profiles of 0.5×, 1×, 2× and 10× the normal clinical concentrations of ranibizumab, bevacizumab, aflibercept and ziv-aflibercept on human RPE cells in an ARPE-19 culture. They found that cell viability decreased in the cells exposed to bevacizumab, aflibercept and ziv-aflibercept of 10x clinical concentrations, but not those exposed to lower concentrations. Bevacizumab and ziv-aflibercept also showed mild toxicity of mitochondria at clinically relevant doses, which may be evidence of the early stages of cell apoptosis even at lower doses of anti-VEGF therapy. Ranibizumab displayed no changes in cell viability at all concentrations. Saenz-de-Viteri et al. (2016) investigated the effect of single and repeated doses of ranibizumab (0.125 mg/ml), bevacizumab (0.25 mg/ml) and aflibercept (0.125, g/ml) on an adult human RPE cell line. There was no measurable toxic effect found after repeated or single anti-VEGF interventions under normal conditions and when the cells lines were placed under oxidative stress.

Spitzer et al. (2007) also used cell lines (human RPE, rat RGCs and porcine choroidal endothelial cells) to compare the anti-proliferative and cytotoxic properties of bevacizumab 0.1-0.3 mg/ml, pegaptanib 0.025-0.08 mg/ml and ranibizumab 0.04-0.125 mg/ml in another histological analysis. Again, no evidence of cytotoxicity was found and there was no significant difference between each of the drugs in terms of cell growth inhibition.

Although these studies suggest that anti-VEGF treatments have little toxic effect on the retina, the findings of these snap shot cell line studies are not necessarily directly applicable to the clinical situation, where patient exposure to anti-VEGF therapy is often long term. Also, investigating toxic effects on individual cell lines is very different to an evaluation of the effect of the drugs in the whole retina *in vivo*, where the physiological environment is very different. Thus, there is a significant need to review literature relating to animal and human studies of the ocular effects of anti-VEGF drugs, with a particular emphasis on those studies which have examined the effect of long-term treatment. A systematic review of this literature is presented in Chapter 2.

The following sections discuss the techniques which are used in this PhD to evaluate peripheral retinal structure and function in people receiving anti-VEGF therapy for nAMD.

1.6. The Electroretinogram

1.6.1. Overview

The electroretinogram (ERG) is an objective technique for recording electrical signals produced by the retina in response to visual stimulation. The ERG is just one of a range of ocular electrophysical tests, which also include the visual evoked potential (VEP; a measurement of evoked responses from the visual cortex in response to visual stimuli; Odom et al., 2010) and the electro-oculogram (EOG; an indirect measure of the standing potential of the eye, which is strongly influenced by the health of the RPE; Constable et al., 2017). There are also several types of ERG (for example, flash [full field or focal], patterned, multifocal), which employ different stimuli and testing conditions to assess different retinal layers and regions (Wolpert and Tsang, 2011), but all involve electrodes on or near the cornea and around the orbit in order to measure the response elicited from the retina (Weisinger et al., 1996). Different conditions and

stimuli generate different traces and waveforms, parameters which may be varied include adaptational status (photopic vs scotopic conditions) and stimulus characteristics such as duration, frequency, and wavelength. The patterned ERG records a response from the macular region to a reversing checkerboard stimulus (Bach et al., 2013). Multifocal ERGs are recorded using numerous (typically 61 or 103) hexagonal stimuli which are presented to the central retina flickering according to a pseudorandom 'M' sequence. The summed response of the retinal areas stimulated are recorded, and local retinal responses attributable to each hexagonal stimulus derived using a computer algorithm (Hood et al., 2012; Sutter and Tran, 1992). Further information for the interested reader can be found in reviews by Sutter (2000), Hood et al. (2002), and Harrison et al. (2006).

This thesis focusses on photopic flash ERG measurements (for reasons explained in Section 3.1) and thus so does this review. Full-field flash ERGs (ffERGs) are a widely used electrophysiological method of assessing the summed activity of the retina (McCulloch et al., 2015, Fishman, 2001). Whilst the ffERG illuminates the whole retina, the focal flash ERG illuminates and stimulates a small part of the retina, with an adapting surround preventing stimulation of more peripheral retinal locations (Miyake et al., 1998). As only stimulus area changes between the focal and full field response, these two types of ERG are analysed in the same fashion (Kondo et al., 2008a).

These traces consist of subcomponents, each of which is dominated by the activity of a different retinal component (see Figure 1.5). In brief, the subcomponents of the ERG recorded in response to a flash or series of flashing stimuli in photopic conditions may include: the a-wave – a negative peak generated by the photoreceptors and post receptor cone pathways (Brown and Wiesel., 1961; Brown et al., 1995; Heynen and van Norren, 1985; Knapp and Schiller, 1984; Bush and Sieving 1994), the b-wave – a positive peak following the a-wave, dominated by ON-bipolar cell activity, with influence from OFF-bipolars, and photoreceptors (Sieving, 1993; Sustar et al., 2006; Sieving et al., 1994; Evers and Gouras, 1986; Miller and Dowling, 1970), oscillatory potentials – wavelets seen on the rising edge of the b-wave, which have been attributed to amacrine cell activity (Asi and Perlman, 1992; Janaky et al., 1996; Wachtmeister, 1998; McCulloch et al., 2015; Rangaswamy et al., 2006), the d-wave – a waveform generated by the cone 'OFF' pathway, seen only in long duration ERGs

(Perlman, 2001; Stockton and Slaughter, 1989), the Photopic Negative Response (PhNR) – a negative waveform following the b-wave, generated by the spiking activity of RGCs, which may be separated into an -ON and -OFF component using a long duration flash (Viswanathan et al., 1999; Rangaswamy et al., 2007; Colotto et al., 2000; Viswanathan et al., 2001; Gotoh et al., 2004; Machida et al., 2008b; North et al., 2010; Horn et al., 2011; Kremers et al., 2012; Pageni et al., 2012; Preiser et al., 2013; Gotoh et al., 2004; Rangaswamy et al., 2004; Chen et al., 2006; Kizawa et al., 2006; Ueno et al., 2006a; Chen et al., 2008; Matsumoto et al., 2009; Matsumoto et al., 2011; Moon et al., 2011; Nakamura et al., 2011b; Wang et al., 2012; Morny et al., 2015; Viswanathan and Frishman, 1997), and the i-wave, which may precede or follow the PhNR in some flash conditions (Viswanathan et al., 1999; Viswanathan et al., 2001; Rangaswamy et al., 2007; Sustar et al., 2009; Machida, 2012).

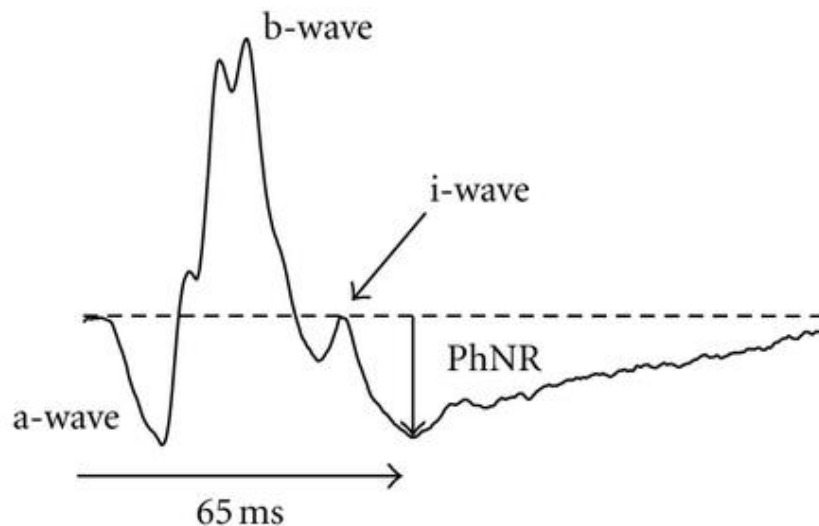


Figure 1.5. Example cone dominated ERG, recorded in response to a long duration stimulus, labelled with the a-wave, b-wave, i-wave, and PhNR wave components. Image adapted from Machida (2012).

Not all subcomponents of the flash ERG are seen in all waveforms produced, for example, the d-wave is only seen for long duration stimuli in photopic conditions. Generally speaking, the a-, b-, and PhNR subcomponents of the waveform are present in all photopic flash ERGs. When the stimulus is presented at a high temporal frequency (e.g. 30 Hz), the individual subcomponents are no longer visible, and a repeating periodic waveform (the flicker or steady state ERG) is seen (see Figure 1.6).

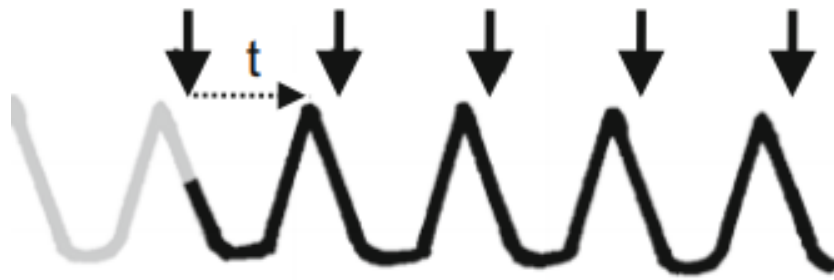


Figure 1.6. Example 30 Hz flicker ERG waveform produced in response to a stimulus presented at high temporal frequency, t = Time to Peak. Adapted from McCullough et al. (2015).

Stimulus durations are referred to as brief, short or long in relation to photoreceptor temporal integration time where a photoreceptor summates photons to produce a single response i.e. 100 ms for rods and 10-15 ms for cones (Kolloniatis and Luu, 2007; Warrant, 1999). Separate ON and OFF responses are elicited by long duration stimuli (>100 ms), whilst combined responses from the ON and OFF pathways are elicited by brief flashes (shorter than photoreceptor integration time [Sustar et al., 2006; Kondo et al., 2000; Sieving, 1993]).

Amplitude and time parameters are used to measure aspects of ERG waveforms. Amplitude is the displacement of the subcomponent peak or trough from the baseline measurement (or the preceding trough) at the specified timepoint in the waveform – often but not always the time to peak. Time to peak (sometimes instead referred to as ‘implicit time’) is measured from the stimulus onset to the peak or trough of the waveform subcomponent being assessed (Weisinger et al., 1996; Fishman, 2001). With increasing age, there is a trend towards elongation of time to peak measurements and decrease in response amplitude, regardless of presence or absence of retinal disease (Freund et al., 2011; Miyata et al., 2007; Birch and Anderson, 1992; Visawanathan et al., 2001; Birch and Fish, 1988; Weleber, 1981).

1.6.2. Recording ERGs

The International Society for Clinical Electrophysiology of Vision (ISCEV) has standardised basic clinical electrophysiological protocols for comparability, including the ERG (McCulloch et al., 2015), VEP (Odom et al., 2010), and EOG (Constable et al., 2017). These guidelines include recommendations regarding adaptation levels for the test, appropriate pupillary dilation, electrode types, recording equipment and stimulus parameters. However, they also accept that in certain research circumstances it can be appropriate to deviate from the guidelines, in order to tailor a test to assess the function of the individual retinal structures of specific interest.

Three types of electrode are required when recording ERGs; active, reference and ground. The ground electrode is usually placed on the ear or forehead of the participant, away from the eye. Corneal or bulbar conjunctival contact is paramount for the active electrode throughout testing in order to obtain optimal ERG amplitude measurements, and the associated reference electrode should be proximal to the eye (McCulloch et al., 2015). If the active and reference electrodes are both of the same type then the potential difference between the two will be zero, an ideal situation (Coupland, 2006). There is a notable drop in amplitude of ERG measurements when changing from contact lens electrodes (corneal contact) to Dawson-Trick-Litzkow (DTL) silver impregnated fibre electrode (bulbar conjunctival contact) (McCulloch et al., 1997). However, the contact lens electrode is uncomfortable, impairs vision and requires participants to have anaesthetic and so the DTL fibre electrode serves its designed purpose in overcoming these problems and is often used in its place as it provides a good quality signal (Dawson et al., 1979). Although not recommended for routine clinical use, periorbital skin surface electrodes are also used on occasion as active electrodes, at the expense of response amplitude, usually in a paediatric setting in which minimal invasiveness is a significant benefit (Noonan et al., 1973; Zhang et al., 1983; France, 1984; Esakowitz et al., 1993; Kriss, 1994; Bradshaw et al., 2004).

1.6.3. ERG component origins

In order to interpret electroretinographic findings it is necessary to understand the retinal sources that are summated to produce the ERG waveforms recorded (Wolpert and Tsang, 2011; Frishman, 2006; Granit, 1933). A number of methods have been used

to establish the origins of ERG components (Frishman, 2006). In humans, known presence of a disease affecting certain retinal neurons can help to identify the origins of retinal subcomponents through case and cohort studies (Viswanathan et al., 2001; Miyata et al., 2007; Kondo et al., 2008b). Additionally, analysis of change in waveforms when one or more stimulus parameters are altered can also give valuable information on retinal origins of subcomponents, especially valuable in humans where non-invasive techniques are required (Robson and Frishman, 1998). One of the ways of determining retinal origins of waveforms in experimental animals is current source-density analysis, where microelectrodes detect electrical signals and find the retinal layer whence they came (Karwoski and Xu, 1999; Heynen and van Norren, 1985; Xu and Karwoski, 1994; Xu and Karwoski, 1995; Xu and Karwoski, 1997; Baker et al., 1988). Another option in animals studies is the use of pharmacological agents to obstruct synapses between layers of the retina in order to determine the signal's origin (Luo and Frishman, 2011; Hood et al., 1999; Viswanathan et al., 1999).

The classic component analysis of the ERG was carried out by Granit (1933) as he observed the loss of various subcomponents of the ERG as a cat slipped deeper into anaesthesia. This was the first experiment which showed that the (in this case long

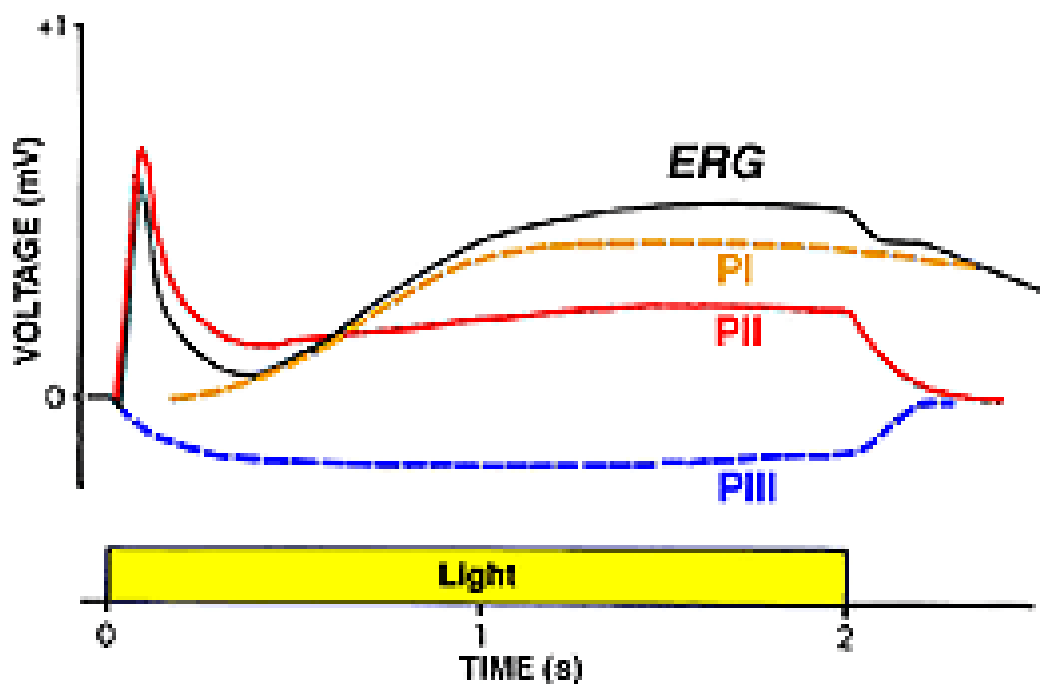


*Figure 1.7. The ERG of a cat in response to a 2 sec light stimulus. The components, P-I, P-II and P-III, have been isolated by deepening the state of anaesthesia (Granit, 1933).
Figure from Perlman et al. (2013).*

duration) flash ERG at any point in time is the composite of three processes occurring simultaneously but with different latencies in different layers of the retina. Granit named these processes PI, PII and PIII which all dominate the response at different time points giving rise to the a- (dominated by fast PIII), b- (dominated by PII) and c- waves (only recorded from the dark-adapted eye, formed from the sum of PI and slow PIII). During deepening anaesthesia, PI, a slow corneal-positive wave disappeared first, then the corneal-positive PII component which peaked quickly and then gradually dropped to an intermediate level during the light stimulus. The most resistance to anaesthesia was shown by the PIII component, a faster developing cornea-negative wave which remained as such for the duration of the stimulus. See Figure 1.7.

Whilst Granit's component analysis was a breakthrough in developing understanding of the origins of the ERG, the processes PI, PII and PIII are not accessible clinically and it is the subcomponents (a-wave, b-wave etc) which are widely evaluated. Hence, it is the retinal origins of these subcomponents which will now be discussed.

1.6.4. The a-wave

As can be seen in Figure 1.5, the a-wave is the first negative peak of the flash ERG. It is best observed in response to stimuli of over 10 cd.s.m^{-2} in dark adapted eyes – with a mixed rod and cone response being recorded when a white stimulus is used (McCulloch et al., 2015; Frishman, 2006). The a-wave is also recordable in the photopic ERG (McCulloch et al., 2015), which is the focus of this PhD.

The foremost edge of the fast PIII response component shapes the a-wave and both microelectrode based animal studies (Brown and Wiesel, 1961a; Brown and Wiesel, 1961b; Brown, 1968) and circulation modifying studies (Brown et al., 1965; Brown, 1968) suggest that the photoreceptors are the main origins of this subcomponent in photopic and scotopic ERGs. Pharmacological studies have agreed with the photoreceptoral origins of the photopic a-wave but also provided evidence of a cornea-negative post-receptoral contribution (Sillman et al., 1969; Bush and Sieving, 1994), identified as originating in cone-driven OFF bipolar cells (Robson et al., 2003). Bush and Sieving (1994) reported that the relative influence of the negative inner retinal contribution to the a-wave is lessened with increasing stimulus intensity, hence at low intensities the contribution of the outer retina to the a-wave is minimal. The cornea-positive

contribution from the depolarising bipolar cells does not influence the first 10 ms or so of the a-wave amplitude, but eventually the a-wave is shortened somewhat by the ascension of the bipolar cell dominated PII component (Bush and Sieving 1994; Sillman et al., 1969).

1.6.5. The b-wave

The b-wave is a positive wave (Figure 1.5) dominated by the PII response, occurring immediately after the a-wave and is recordable with low luminance stimuli due to its high sensitivity (Frishman et al., 1996). In scotopic conditions it occurs at around 40-60 ms and at around 25-35 ms in photopic conditions. The photopic b-wave principally arises from the ON and OFF bipolar cell activity (Sieving, 1993; Sustar et al., 2006). Pharmacological evidence suggests that ON-bipolar cells contribute a large, positive and sustained potential to the formation of the b-wave, which is curtailed in the photopic ERG by a cornea-negative potential attributable to OFF-bipolar cell or horizontal cell activity (Sieving et al., 1994; Evers and Gouras, 1986). It therefore appears that ON-bipolar cell activity is required to produce the leading edge of the photopic b-wave, but that the trailing edge is dependent on the activity of hyperpolarizing neurons.

The peak appears dominant in response to low luminance stimuli due to the absence of the a-wave's opposite reaction and is at its largest when the subject is undergoing ERGs in a dark-adapted environment with a bright flash stimulus used to elicit a response from both the rod and cone photoreceptors. Another feature particularly evident in the dark-adapted participant (though also visible in photopic responses at times) is a series of wavelets superimposed on the climbing edge of the b-wave called oscillatory potentials (OPs; Asi and Perlman, 1992; Janaky et al., 1996; Wachtmeister, 1998; McCulloch et al., 2015). These are thought to originate from the amacrine cells, and can be isolated from the b-wave using electronic filters (Wachtmeister, 1998; Rangaswamy et al., 2006).

1.6.6. The d-wave

The d-wave is a positive potential only present when the ON and OFF responses are separated by long duration light stimuli; brief flashes result in it being merged with the b-wave (Sieving, 1993; Sustar et al., 2006). The origin of the d-wave has been shown

through current source density analysis to be largely from the OFF bipolar cells with a small input from the photoreceptors (Xu and Karwoski, 1995; Sieving et al., 1994; Sieving, 1993). This finding was reinforced by the observation that the d-wave was reduced and/or eliminated where kynurenic acid was introduced to monkey retinas – as this compound is known to block responses from amacrine, RGC, and OFF bipolar cells – but the same effect didn't occur when inner retinal cells were blocked by the presence of N-methyl S-aspartate (Sieving, 1993).

1.6.7. The Photopic Negative Response (PhNR)

A slow occurring negative potential, the PhNR can be found after the b-wave, representing the combined ON and OFF responses of the RGCs in the brief flash photopic ERG (see Figure 1.5) and as two separate waves (one after the b-wave and one after the d-wave) where long duration flashes occur (representing the separated ON and OFF responses of the RGCs) (Viswanathan et al., 1999; Kondo et al., 2008a). The PhNR was first described in 1993 as a novel negative response elicited in response to brief red flash stimuli in photopic conditions (Spileers et al., 1993), though it wasn't named and described fully until some years later. Spileers et al. (1993) referred to their subcomponent discovery as the photopic threshold response and it was suspected that it was an equivalent to the scotopic threshold response (Sieving et al., 1986; Sieving and Nino, 1988; Wakabayashi and Sieving, 1988; Frishman et al., 1993). Viswanathan et al. (1999) delved in greater detail into the PhNR response, recording ERGs in response to red flashes on a blue rod-saturating background under various stimulus durations and luminance levels. By influencing the retinal response through pharmacological blockade or induction of glaucoma, to alter the response of the RGCs and their associated axons, they were able to show that these retinal components were the source of this subcomponent. For instance, elimination of the PhNR has been known to occur when macaque monkeys were given experimental glaucoma (Viswanathan et al., 1999; Rangaswamy et al., 2007), and a reduced PhNR has been reported in patients with naturally occurring primary open angle glaucoma and other RGC disease (Colotto et al., 2000; Viswanathan et al., 2001; Gotoh et al., 2004; Machida et al., 2008b; North et al., 2010; Horn et al., 2011; Kremers et al., 2012; Pangeri et al., 2012; Preiser et al., 2013; Gotoh et al., 2004; Rangaswamy et al., 2004; Chen et al., 2006; Kizawa et al., 2006; Ueno et al., 2006a; Chen et al., 2008; Matsumoto

et al., 2009; Matsumoto et al., 2011; Moon et al., 2011; Nakamura et al., 2011b; Wang et al., 2012; Morny et al., 2015). Further evidence for the origins of the PhNR are found in animal studies, where the PhNR is eliminated by the introduction of the sodium action potential blocking tetrodotoxin which prevents the action of both some of the amacrine and the RGCs (Viswanathan et al., 1999), and micro-electrode studies identifying the largest PhNR responses in and around the optic nerve head - though these also suggest glial involvement in the response (Viswanathan and Frishman, 1997).

Much like the other flash ERG subcomponents, the PhNR subcomponents are measured in terms of amplitude and implicit time (Fishman, 2001; Movassat, 2012). However, both the time to peak and amplitude factors of the PhNR are considered difficult to identify which can lead to inaccurate assessment. This is owing to several factors, including the small positive 'i-wave' that occurs within the same timeframe as the PhNR (Viswanathan et al., 1999; Viswanathan et al., 2001; Rangaswamy et al., 2007; Sustar et al., 2009; Machida, 2012) making it difficult to identify the most negative part of the PhNR trough. Furthermore, the PhNR subcomponent is also affected by the duration, luminance, and wavelength of both the flash stimulus and background (Viswanathan et al., 1999; Colotto et al., 2000; Viswanathan et al., 2001; Sustar et al., 2006; Rangaswamy et al., 2007; Mortlock et al., 2010; Binns et al., 2011) and factors such as subject age, electrode type, amplitude of other ERG subcomponents, and stimulated area of the retina (Viswanathan et al., 2001; Mortlock et al., 2010; Machida et al., 2011).

Until recently there were no guidelines or standard protocol for recording or analysing the PhNR. Indeed, when this PhD study began no such guidance was in existence. Previously, study teams have used numerous mixtures of ERG parameters and analysis techniques to optimise PhNR recordings (Viswanathan et al., 1999; Colotto et al., 2000; Drasdo et al., 2001; Rangaswamy et al., 2007; Binns et al., 2011). The three most common techniques for analysing the PhNR included;

1. Measuring from b-wave peak to PhNR trough (Binns et al., 2011; Drasdo et al., 2001)
2. Measuring from pre-stimulus baseline to trough of PhNR (Binns et al., 2011; Viswanathan et al., 2001 and 1999; Colotto et al., 2000)

3. Measuring from peak of b-wave *OR* pre-stimulus baseline to a fixed time point (Rangaswamy et al., 2007)

However, the International Society for Clinical Electrophysiology of Vision (ISCEV) published their first protocol standard for this in 2018 (Frishman et al., 2018). The protocol recommends that the background stimulus should be blue (450-485 nm) and 100 scotopic cd/m² (equivalent to 10 photopic cd/m²) and the flash stimulus should be red (630-660 nm) displayed for less than 5 ms and of a strength of 1.0-2.5 cd.s.m⁻². Frishman et al. (2018) also suggested PhNR analysis options in their ISCEV guidelines. The aforementioned methods of PhNR analysis are all referred to with the advice that using a specific timepoint may be an advantage in diseased retinæ where subcomponent troughs may be very difficult to locate. It also warns that peak to trough measurements may be influenced by factors affecting the b-wave origins and so may confound the results. The guidelines also emphasise the importance of age matched control data for comparison of this ERG parameter as the PhNR is known to be affected by participant age (as previously discussed), and of assessing test-retest repeatability, as variation of PhNR amplitudes has been shown to be greater than that of a- and b- waves (Joshi and Viswanathan, 2017; Tang et al., 2014; Binns et al., 2011; Mortlock et al., 2010).

In this study, a series of PhNR stimulus luminances were used to assess photopic retinal function. It is worth noting that in most photopic ERG parameters a decrease in time to peak and increase in amplitude are witnessed with increasing flash luminance until the point of saturation is reached (Fishman et al., 2001) – such is the case with the PhNR subcomponent (Viswanathan et al., 1999; Binns et al., 2011).

1.6.8. The ERG in AMD

Studies have demonstrated a lack of evidence of a measurable deficit in the ffERG in patients with early or intermediate AMD (Jackson et al., 2004; Holopigian et al., 1997; Sunness et al., 1985). However, patients with late stage AMD have shown abnormalities in a-wave and b-waves in some studies (Jackson et al., 2006a; Walter et al., 1999). The recording of localised responses in the multifocal ERG allow for more accurate monitoring of focal retinal dysfunction in AMD, with studies noting a decline in mfERG amplitudes in both scotopic (Chen et al., 2004; Feigl et al., 2005b; 2006) and

photopic conditions (Huang et al., 2000; Li et al., 2001; Chen et al., 2004). This localised response may also be elicited using focal ERGs with a stimulus subtending any small part of the retina. Responses in focal flash ERGs have been shown to be negatively affected (i.e. elongated time to peak measurements and reduced amplitude measurements of a- and b- wave parameters) by AMD in numerous studies (Seiple et al., 1986; Sandberg et al., 1993; Remulla et al., 1995; Sandberg et al., 1998; Falsini et al., 1999; Binns and Margrain 2007; Piccardi et al., 2009; Wood et al., 2011b). The degree of amplitude reduction seems to mirror the extent of AMD progression, particularly in flicker amplitude parameters of those with advanced AMD (Falsini et al., 1999).

1.7. Optical coherence tomography

Optical coherence tomography (OCT) is a form of non-invasive and cross-sectional imaging that exploits interferometry to generate 2-D and 3-D depictions of the retina (Drexler et al., 1999) and so lends itself ideally to medical and research purposes (see Figure 1.8). It is a relatively new technology first reported as a one dimensional “A-scan” (an intensity profile in the axial direction) by Fercher et al. (1988), with a cross-sectional two-dimensional B-scan (achieved by scanning in the transverse direction across the retina) later being described by Huang et al. in 1991. The pioneering human retinal OCT scans were performed by Swanson et al. (1993) and Fercher et al. (1993) and the use of the technology both clinically and for research purposes has grown exponentially ever since.

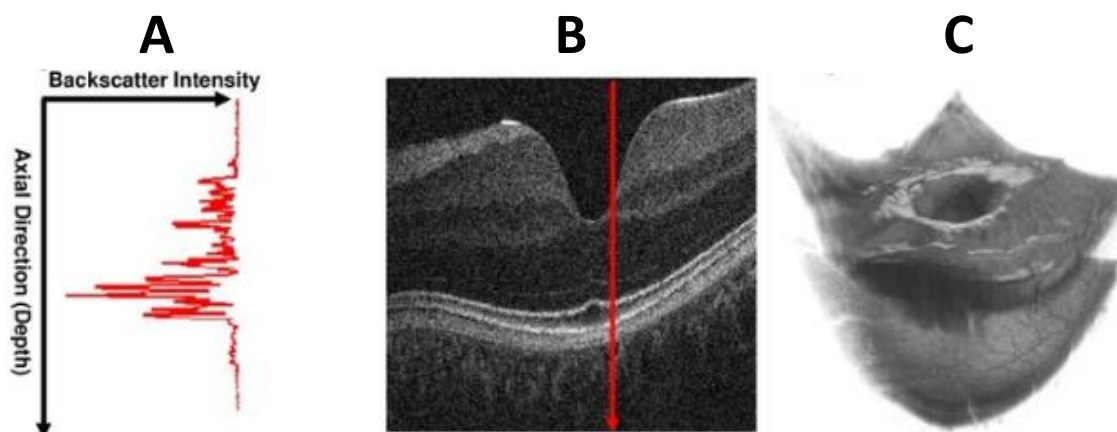


Figure 1.8. Examples of raster scans generated from retinal OCT imaging. A; a tissue depth-profile (an A-scan) is first developed from a single measurement, B; several of these in a line on the retina form a cross-sectional image (a b-scan), C; arrangement of multiple adjacent B-scans forms a 3D image (a C-scan, right, ‘en face’ when used in summation with other C-scans). Image adapted from Kraus et al., 2012.

In brief, the OCT technique is typically based on a Michelson interferometer with a low coherence, broad bandwidth light source, where a beam is split into two paths by a semi silvered mirror, with one beam entering the eye (the measurement beam), and one (the reference beam) that does not (Fujimoto et al., 2000). As the light of the measurement beam passes through the eye, it interacts with ocular structures. Some of the light is reflected back out of the eye by these structures, some is absorbed, and some is scattered. The back scattered measurement beam is recombined with the reference beam, and the signal interference between the two is used to generate an image. The theory is similar to echo sound delay in ultrasound but using optical light-based signals.

The OCT image can be characterised with respect to two types of resolution which can be entirely decoupled (Hitzenberger, 2003). Transverse (also known as lateral) resolution is defined by the magnitude of the focussed transverse spot of the optical beam. This determines the ability to resolve small retinal lesions, or even retinal neurons. It is determined in part by scan rate – a higher scan density providing higher transverse resolution - but is also limited by the optics of the imaging device and the eye. Integration of adaptive optics into the latest research devices aims to correct optical aberrations to enhance transverse resolution to a cellular level (Pircher and Zawadzki, 2017). Axial (also known as depth) resolution is the spatial resolution of the image through the depth of the retina. High axial resolution assists with viewing individual retinal layers. Axial resolution is determined by the coherence length of the light used (broad spectral bandwidth light has low coherence and provides better axial resolution), and the sampling properties of the signal at the detector. It is not affected by the optics of the imaging device, optical aberrations from the refractive surfaces from the eye nor the pupillary aperture (Aumann et al., 2019), which confers a distinct benefit on OCT imaging.

In a time-domain OCT (TD-OCT) there is a reference mirror as part of the system, the position of which can be adjusted to alter the pathlength of the reference beam to match the path length of the reflected measurement beam at different depths. In so doing, the intensity of light back scattered from structures at different depths in the tissue sample can be assessed. This process allows for the production of discrete data points displayed as A-scans comprising a series of measurements of reflectivity at

various retinal depths (although this depth is limited due to absorption and scattering of light [Huang et al., 1991]). If these A-scans are combined with other lateral/transverse scans they form a B-scan, a cross sectional image of the retina, and 3D C-scans can be obtained when combining with additional vertically displaced B scans. Time domain OCT scans are acquired at a rate of 400 A-scans per second, and provide an axial resolution of 8–10 μm (Sull et al., 2010). The low sampling rate limits the suitability of this technology for collecting detailed C-scans, as eye movements are likely to occur during the prolonged raster scanning process.

Advancements since 1991 include Frequency Domain OCT (FDOCT), which can be subdivided into Spectral Domain OCT (SD-OCT), and Swept Source OCT. These techniques have allowed the study of retinal structures and pathological features (such as drusen) in greater detail and with enhanced transverse and axial resolution (Drexler et al., 2004). FD-OCT allows scan rates of 26,000-100,000 A-scans/second to be achieved, with an axial resolution of 2–7 μm (Kashani et al., 2017). This is achieved by eliminating the movement of the reference mirror, and by using a broad-bandwidth light source with a charge-coupled device (CCD) camera and a spectrometer (for SD-OCT) or by replacing the light source with a light source which sweeps through a range of frequencies (swept source OCT) (Kashani et al., 2017). This eliminates the scan rate limitations in TD-OCT attributable to the rate of movement of the mirror, and enables the simultaneous measurement of the whole A-scan providing a much quicker (Drexler and Fujimoto, 2008; Povazay et al., 2007; Drexler, 2004; van Velthoven et al., 2007), as well as a more detailed and clinically valuable scan (Drexler, 2004; Schmidt-Erfurth et al., 2005; Drexler and Fujimoto, 2008). Following the simultaneous A-scan, the reflectivity profile is attained by using the interferometric information and then processed using Fourier transformation (van Velthoven et al., 2007). Swept-source OCT typically uses a light centred on a longer wavelength than SD-OCT (>1000 nm, compared to 800-900 nm). This longer wavelength enables greater tissue penetration. Furthermore, the latest generation of swept-source OCT provides a scan rate in excess of 100,000 A-scans/second, enabling an increased scan density and area (Drexler and Fujimoto, 2008; Ploner et al., 2016).

The retinal sections provided by OCT scans are similar to those provided by histological studies (see Figure 1.9), though advantageously are available *in vivo* (Huang et al.,

1991). The layers present on an OCT image do not directly correlate with the anatomic layers, boundaries displayed represent changes in the optical density of the retina which are not necessarily coincident with cellular boundaries (Cuenca et al., 2020; Spaide and Curcio, 2011; Srinivasan et al., 2008). For example, Spaide and Curcio (2011) attempted to determine the anatomical correlates of the four outer hyper-reflective bands of the OCT image by forming a scale model drawing of the outer retina based on histological studies published between 1900 and 2009. The boundaries of outer retinal tissue compartments on this model were compared with variations in intensity of OCT scans using longitudinal reflectance profiles to determine the region of origin. Results of this work showed high probability that SD-OCT bands have been correctly attributed to the ELM (the first, innermost hyper-reflective layer) and to the RPE (the fourth, outermost hyper-reflective layer). The second band (usually attributed to the inner and outer segment boundary of the photoreceptors), in fact associates with the ellipsoid section of the photoreceptor inner segments, whilst the third band is attributable to the wrapping of the cone outer segments in the apical microvilli of the RPE (the so-called 'contact cylinder').

The advances in SD-OCT such as higher resolution, speed and signal noise reduction (Drexler and Fujimoto, 2008; Povazay et al., 2007; Schmidt-Erfurth et al., 2005; Drexler, 2004; van Velthoven et al., 2007) has allowed clinicians to use more exact quantitative data in clinical management. The availability of technology such as this has resulted in treatment guidance using these structural quantitative factors to advise clinicians in their treatment of ocular conditions (Murakami and Yoshimura, 2013) such as diabetic macular oedema (NICE TA346, 2015). It is not surprising then that OCT technology is used increasingly in both primary and secondary ophthalmic care. The ability to precisely image the retina and pinpoint the source of any abnormality without invasive action is extremely advantageous to clinicians and this is utilised to diagnose, monitor and manage patients with various retinal conditions – including AMD (Regatieri et al., 2011). Indeed, more recently, there have been developments in algorithms allowing volumetric analysis of drusen automatically, facilitating disease progression monitoring within traditional medical retina clinics (Gregori et al., 2011; Yehoshua et al., 2011), and by artificial intelligence (De Fauw et al., 2018).

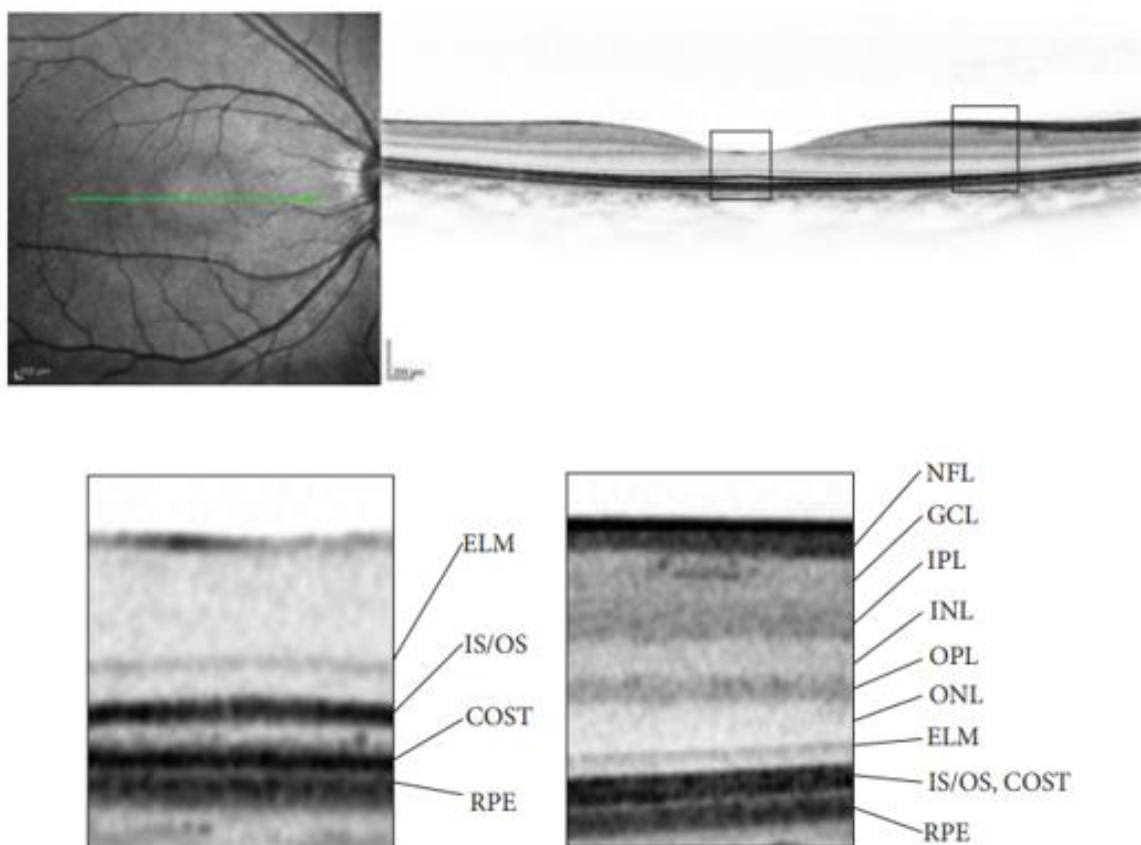


Figure 1.9. Two-part image showing a retinal photograph and the corresponding macular OCT b-scan. Top panel shows retinal photograph of a right eye (left) and a SD-OCT macular section corresponding with the green line on the adjoining fundus photograph. Bottom left shows a magnified segment of the foveal aspect of the b-scan and shows the external limiting membrane (ELM), inner and outer segment junction (IS/OS), and the cone outer segment tips (COST), the retinal pigment epithelium (RPE). Bottom right shows a magnified section of the parafoveal aspect of the b-scan and indicating; the retinal nerve fibre layer (RNFL), ganglion cell layer (GCL, also known as the ganglion cell complex, GCC), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and the outer nuclear layer (ONL). Image adapted from Murakami and Yoshimura, 2013.

1.7.1. Optical coherence tomography in AMD

Optical coherence tomography has become the imaging modality of choice in AMD because of the advantage it holds in detecting very small morphological changes of the retinal layers and subretinal space, in turn allowing precise measurement of structures and earlier detection of structural changes in retinal disease (Sayanagi et al., 2009; Khurana et al., 2009; Regatieri et al., 2011). Additionally, SD-OCT provides more detailed images of fluid collection in the intraretinal, subretinal, and subretinal pigment epithelium spaces than the previous time-domain technology, resulting in higher rates of detection of active nAMD (Regatieri et al., 2011).

Once diagnosed, some biomarkers in OCT scans have been suggested as possible predictors of treatment outcomes in nAMD (Schmidt-Erfurth and Waldstein, 2016; Phadikar et al., 2017). For example, the presence of subretinal fluid identified using OCT at the beginning of anti-VEGF therapy for nAMD has been known to be a good prognostic indicator of improvement in visual acuity over time (Simader et al., 2014; Waldstein et al., 2016). Intraretinal fluid has been noted to respond well to anti-VEGF treatments but if intraretinal cystic spaces are present at baseline, there is an association with poorer visual outcomes (Simader et al., 2014; Waldstein et al., 2016). In the late stages of nAMD RPE atrophy can be visualised on OCT by the appearance of choroidal structures otherwise not visible in the healthy retina – another negative biomarker for eyes as yet untreated for nAMD (Freund et al., 2010, Keane et al., 2012).

OCT scans have been shown to assist detection of nAMD before patients are even symptomatic of the disease, with sub-RPE fluid being the most common identifier in these cases (Padnick-Silver et al., 2012). In addition, statistical models have been produced that are able to distinguish control eyes from those affected by AMD (Farsiu et al., 2014) or calculate the statistical likelihood of conversion from early or intermediate AMD to nAMD by using quantitative measures of early retinal structural changes visible by OCT and fundoscopy (De Sisternes et al., 2014).

In addition to the clinical applications of OCT, there has been substantial use of the technology in a research environment to probe the mechanisms of AMD onset and progression. For example, there is evidence that macular retinal but not choroidal thickness is reduced in people with early AMD (Wood et al., 2011a), and from other

reports that localised thinning occurs to the photoreceptor layer overlying drusen (Kaluzny et al., 2009; Malamos et al., 2009; Schuman et al., 2009). Other studies have made use of SD-OCT technology to probe the morphology of subretinal drusenoid deposits associated with AMD (Zweifel et al., 2010; Spaide et al., 2018). It has also been shown to be possible to use OCT technology to monitor progression of AMD through changes in drusen volume (Gregori et al., 2011; Yehoshua et al., 2011).

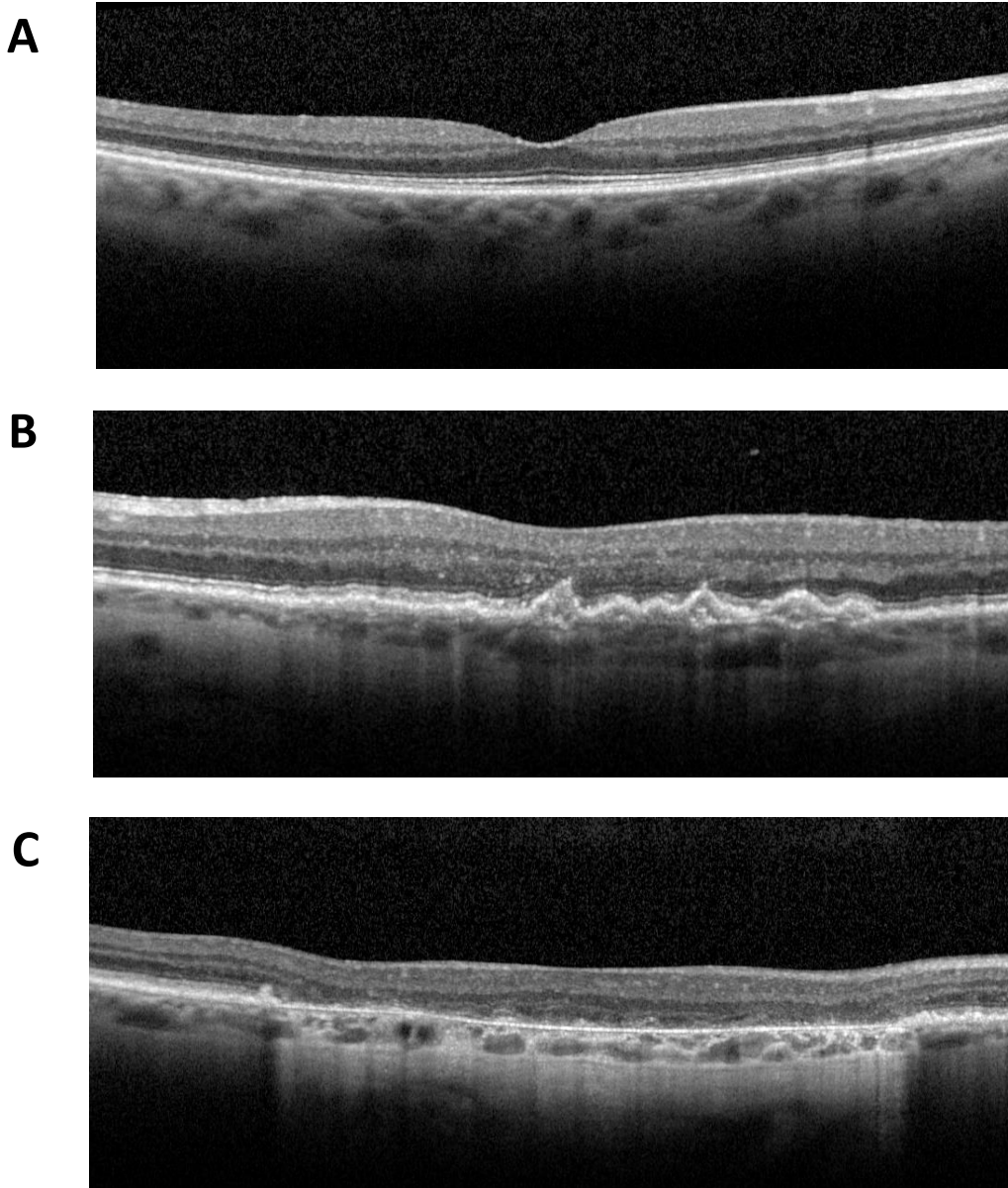


Figure 1.10. Examples of the presentation of various stages of AMD in OCT imaging. A, healthy macula; B, Intermediate AMD – localised elevations of the RPE indicate soft drusen at the macula; C, Late AMD – geographic atrophy of the heavily pigmented RPE has led to a window defect and subsequently an increased view of the choroid. These scans were obtained using a Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) at City, University of London.

1.8. Rationale and Aims

The function of VEGF-A in the adult eye is still under investigation. It has been suggested to have an important role in the maintenance of the adult choriocapillaris (Marneros et al., 2005; Blaauwgeers et al., 1999; Saint Geniez et al., 2009), supported by the continued production of VEGF-A by the RPE throughout life (Adamis et al., 1991; Saint-Geniez et al., 2006). VEGF is also produced by many cell types in the retina, including pericytes (Saint Geniez et al., 2008), retinal neurons (Famiglietti et al., 2003; Kim et al., 1999), Müller cells (Saint-Geniez et al., 2008; Famiglietti et al., 2003), and astrocytes (Saint-Geniez et al., 2008), suggesting a wider role in retinal health. The receptor, VEGFR2, is expressed by photoreceptor cells and Müller cells as well as by the retinal microvasculature (Saint-Geniez et al., 2008). There is also evidence that VEGF-A has a directly neuroprotective role in the retina. Nishjima et al. (2007) demonstrated a loss of RGCs in normal adult mice upon chronic inhibition of VEGF-A function, whilst Saint-Geniez et al. (2008) reported a significant increase in cell apoptosis in inner and outer retinal layers upon the systematic neutralisation of VEGF in adult mice.

There is, therefore, reason to believe that the long-term attenuation of VEGF-A activity could have a deleterious effect on the structure and function of the retina, manifesting in both the inner and outer retinal layers. In humans, it has also been reported that patients undergoing monthly anti-VEGF injections for nAMD are more likely to develop macular atrophy than individuals receiving fewer injections in a PRN type regimen. This suggests that there may be a dose-dependent increased risk of atrophy associated with repeated anti-VEGF use (Chakravarthy et al., 2013; Sadda et al., 2018). This gives reason to hypothesise that the thickness of retinal layers assessed using OCT may be impacted by repeated anti-VEGF injections.

The overarching aim of this PhD is to investigate the effect of anti-VEGF therapy on peripheral retinal structure and function.

The specific aims of this PhD are:

1. To conduct a systematic literature review to evaluate the current evidence base regarding the effect of anti-VEGF therapy on retinal integrity (Chapter 2).

2. To develop an ERG protocol for the evaluation of peripheral function, and to determine the repeatability of the protocol (Chapters 3 and 4).
3. To evaluate the relationship between number of anti-VEGF injections and time since first injection and parameters of the ERG and OCT (Chapter 5).

Chapter 2. How does anti-VEGF therapy affect the physiology and morphology of the peripheral retina? A Systematic Literature Review

2.1. Introduction

2.1.1. Vascular Endothelial Growth Factor

Production of new blood vessels (angiogenesis) is a vital process in human physiology throughout every stage of life. It is stimulated by vascular endothelial growth factor (VEGF), a family of proteins that promote mitosis of endothelial cells. VEGF proteins have a role in many anatomical processes such as menstruation and response to inflammatory or ischaemic disease (Hoeben et al., 2004). They are also present in the vascularised areas of the healthy adult eye, i.e. the conjunctiva, retina, and the choroid-retinal epithelial complex (Kim et al., 1999; Blauwgeerss et al., 1999; Saint-Geniez et al., 2009; Famigiletti et al., 2003). Specifically, in the retina of the healthy eye, VEGF protein and mRNA have been located in the cone photoreceptors, the inner nuclear layer, amacrine cells, glial cells, Müller cells, retinal ganglion cells (RGCs) and retinal pigment epithelial (RPE) cells (Kim et al., 1999; Blauwgeerss et al., 1999; Saint-Geniez et al., 2009; Famigiletti et al., 2003).

However, uncontrolled growth of retinal blood vessels is a significant factor in ocular neovascular diseases such as neovascular age-related macular degeneration (nAMD), diabetic retinopathy and retinal vein occlusion (Ferrara, 2002). It is the upregulation of VEGF that stimulates the growth of the new, leaky blood vessels in nAMD and other retinal vascular diseases (Andreoli and Miller, 2007; Tufail et al., 2010; Nozaki et al., 2006).

2.1.2. Anti-Vascular Endothelial Growth Factors (VEGF)

Anti-VEGF drugs are therapeutic drugs that act upon VEGF in ocular diseases and prevent stimulation of further abnormal vascular growth. There are several clinically used anti-VEGF drugs each with a slightly different action mechanism (see section 1.4).

In 2008 the methods of treating nAMD changed when the National Institute for Health and Care Excellence (NICE) approved the intraocular use of ranibizumab to treat the condition (NICE Guidelines TA155). In 2013, NICE also approved the intraocular use of aflibercept for treating nAMD (NICE Guidelines TA294). Anti-VEGF drugs are also approved for use in other retinal vascular disorders, including diabetic eye disease (e.g.

NICE Guidance TA274, 2013; TA436, 2015) and retinal vein occlusion (NICE Guidance TA283, 2013; TA305, 2014). Bevacizumab remains an off-label treatment option for ocular conditions. Given the high prevalence of these retinal conditions, many people undergo intraocular anti-VEGF treatment.

The treatment regimen for these drugs usually involves an initial loading phase of at least three injections at monthly intervals. Thereafter, patients often require multiple retreatments, for example on 'as required' or 'treat and extend' protocols. As such, patients are exposed to the drugs repeatedly, often over a period of years.

Section 1.5.4 of the introduction reviews evidence from cell line and *in vitro* studies regarding the toxicity of anti-VEGF drugs. Whilst the majority of cell-line studies suggest that anti-VEGF treatments have little toxic effect on the retina, the physiological environment is very different *in vivo*, and exposure to anti-VEGF is often long term in a clinical situation rather than a single episode. Thus, there is a need to review literature relating to animal and human studies of the ocular effects of anti-VEGF drugs, with a particular emphasis on those studies which have examined the effect of long-term treatment.

2.1.3. Rationale and objectives

VEGF proteins are produced by a number of ocular structures in the healthy eye (e.g. Müller cells and retinal neurons), and are required to maintain the normal functioning of the retina and associated structures (Saint-Geniez et al., 2008; Famiglietti et al., 2003). Because of this, it is possible that long term inhibition of these VEGFs may have a degenerative effect on the structure of the retina and its function. If the population of people aged over 60 years shows the expected increase in coming years (UN, 2004), so will the occurrence of nAMD and therefore the use of anti-VEGF treatment in this population. In addition, anti-VEGF treatments are now also being used to treat other conditions such as Retinopathy of Prematurity (ROP), Diabetic Retinopathy (DR) and Central/Branch Retinal Vein Occlusions (CRVO/BRVO) (National Institute for Health Research, NIHRIO (HSRIC) ID: 14932, 2018; NICE guidance TA274, 2013; TA436, 2015; TA283, 2013; TA305, 2014). These conditions can occur in patients of a much younger age and so treatment duration is likely to be longer, resulting in increased exposure.

Therefore, it is important for clinicians to understand the effects of long-term treatment on the eye.

In order to evaluate the effect of anti-VEGF drugs on the retina, it is necessary to separate the pathological effects of the disease being treated and the treatment effect of the drug on the disease from any potential deleterious effects of the drug on structure and function of the otherwise healthy retina. In animals, this can be achieved by including studies evaluating the effect of the drugs on the retinas of healthy animals, thereby excluding the potential confounding effect of ocular pathology. In humans, however, where studies of anti-VEGF drugs invariably recruit participants with pathology, it is necessary to include studies which include an evaluation of a retinal location minimally affected by the disease being treated. The peripheral retina is relatively unaffected by AMD (Jackson et al., 2004; Holopigian et al., 1997; Sunness et al., 1985), and functional and structural evaluation of the periphery in people undergoing anti-VEGF therapy for macular disease may provide useful information about the effect of the treatment on the healthy retina.

Therefore, the primary aim of this systematic review was to evaluate the evidence in the literature regarding the effect of anti-VEGF therapy on the physiology and morphology of the peripheral retina. Additionally, this review aimed to investigate the trends or gaps in knowledge presented in the current data. This is the first systematic literature review to evaluate evidence regarding potential toxicity of anti-VEGF therapy in the peripheral retina.

2.2. Methods

2.2.1. Search strategy

The electronic databases 'Ovid' and 'Web of Science' were searched over the time frame January 1950 to August 2019. The following keywords were used:

- 'Lucentis' OR 'Ranibizumab' OR 'anti-VEGF' OR 'Eylea' OR 'Aflibercept' OR 'Avastin' OR 'Macugen' OR 'bevacizumab' OR 'Pegaptanib' OR 'Vascular endothelial growth factor' OR 'anti Vascular Endothelial Growth Factor primary' OR 'anti Vascular Endothelial Growth Factor secondary' OR 'VEGF' AND

- 'ERG' OR 'Electroretinogram' OR 'Visual Function' OR 'Retinal Function' OR 'Peripheral Retina' OR 'OCT' OR 'Optical Coherence Tomography' OR 'Retinal Structure' OR 'Retinal Morphology' OR 'Retinal Physiology' AND
- 'Intra Ocular' OR 'Intraocular' OR 'Intravitreal' OR 'Intra vitreal'

Eligibility of the studies produced by this search was assessed independently by two researchers (AEH and DT). The lists of eligible studies produced by each individual were compared, and any discrepancies discussed. A third researcher resolved any disagreements (AMB). Full text copies of the eligible studies were obtained and a reference list search was performed for eligible studies not previously identified by the database search.

2.2.2. Inclusion and exclusion criteria

Studies were included if they evaluated in vivo or ex vivo peripheral retinal structural and/or functional changes with clinically available ocular anti-VEGF therapy in animals or humans, were written in English, and the full article was available. For the purposes of this review, the macula was defined as a circular area subtending 20 degrees visual angle (6mm diameter) centred on the fovea (Polyak, 1941). Any retinal areas located outside of the macula were considered to be 'peripheral'. Studies evaluating full-field retinal function were also included. Studies were excluded if only the full study was unavailable (for example, conference abstracts, letters to the editor, and protocol papers were excluded), if the study was unavailable in the English language, did not involve clinically available anti-VEGF treatment, or did not involve assessment of peripheral retinal structure and/or function. Studies examining retinal function using electrophysiology were deemed eligible if they used a full field flash electroretinogram (ERG), electro-oculogram (EOG), or a multi focal electroretinogram (mfERG) or pattern electroretinogram (PERG) that had a stimulus size encompassing the area outside the macula. PERGs and mfERGs of unspecified or macula only stimulus size and visual evoked potentials (VEPs) were excluded from this review as they do not provoke an electrical response dominated by the peripheral retina. Studies using OCT to assess retinal structure in humans were only included if the OCT scans extended radially beyond 3mm either side of the fovea (e.g. beyond the EDTRS field), as these would

include data from an extra macular area of the retina (Polyak, 1941). Of the OCT studies, only those data which were not dominated by macular changes were included. For example, a wide field scan which stretched beyond the macula was not included if it used a mean thickness parameter which averaged data across the whole of the scan. Studies solely investigating combination treatment therapies (i.e. anti-VEGF drugs injected intravitreally along with concurrent intravitreal steroids or laser treatment) were also excluded as it would not be possible to separate the differential effects of the multiple treatments. Additionally, studies which included people with retinal disease affecting extra-macular retinal function (e.g. glaucoma) were excluded as the disease process may have confounded results. Also, studies that looked at the development of postnatal vasculature or *in vitro* cell lines were excluded, only those that assessed the effect of anti-VEGF interventions in the adult eye were included.

2.2.3. Data collection

Data relevant to the study within eligible papers were compiled using a data extraction table. Details collected included; the aim of the study, types of outcome measured, follow up duration and type of disease affecting the participants (if any).

The data extraction table was summarised to form the Table of Included Studies (Appendix 1). Emphasis in the text of the results section is placed on studies with human participants, studies with the highest number of participants, and studies with the longest follow up periods or greatest number of intravitreal injections. Quality of studies was assessed through the evaluation of key sources of bias in the studies, based on questions identified in the CASP checklists (<https://casp-uk.net/casp-tools-checklists/>). The potential sources of bias are elaborated on in the discussion and also in the final column in the Table of Included Studies (see Appendix 1).

2.1. Results

2.1.1. Literature Search

The database searches yielded 10,346 responses. During the selection process the two authors (AEH and DT) independently agreed 6385 (80%) of the eligibility of the studies assessed. The two authors resolved any discrepancies by discussion. Additionally, 2 extra studies were identified by the prospective and retrospective searches of citation lists of key primary studies. The flow diagram in Figure 2.1 describes the study

selection process. Of the 7981 articles screened for eligibility (after excluding duplicates and any which clearly did not address the topic), full-text articles were assessed of 321, of which 75 were deemed to be eligible for inclusion. The main reasons for excluding articles at the full-text review stage were; studies were performed on eyes still undergoing vascular development or on cell lines, no assessment of peripheral retinal structure/function, the anti-VEGF agent not being clinically available, and the article solely discussing the anti-VEGF agent's effect on the control of retinal disease.

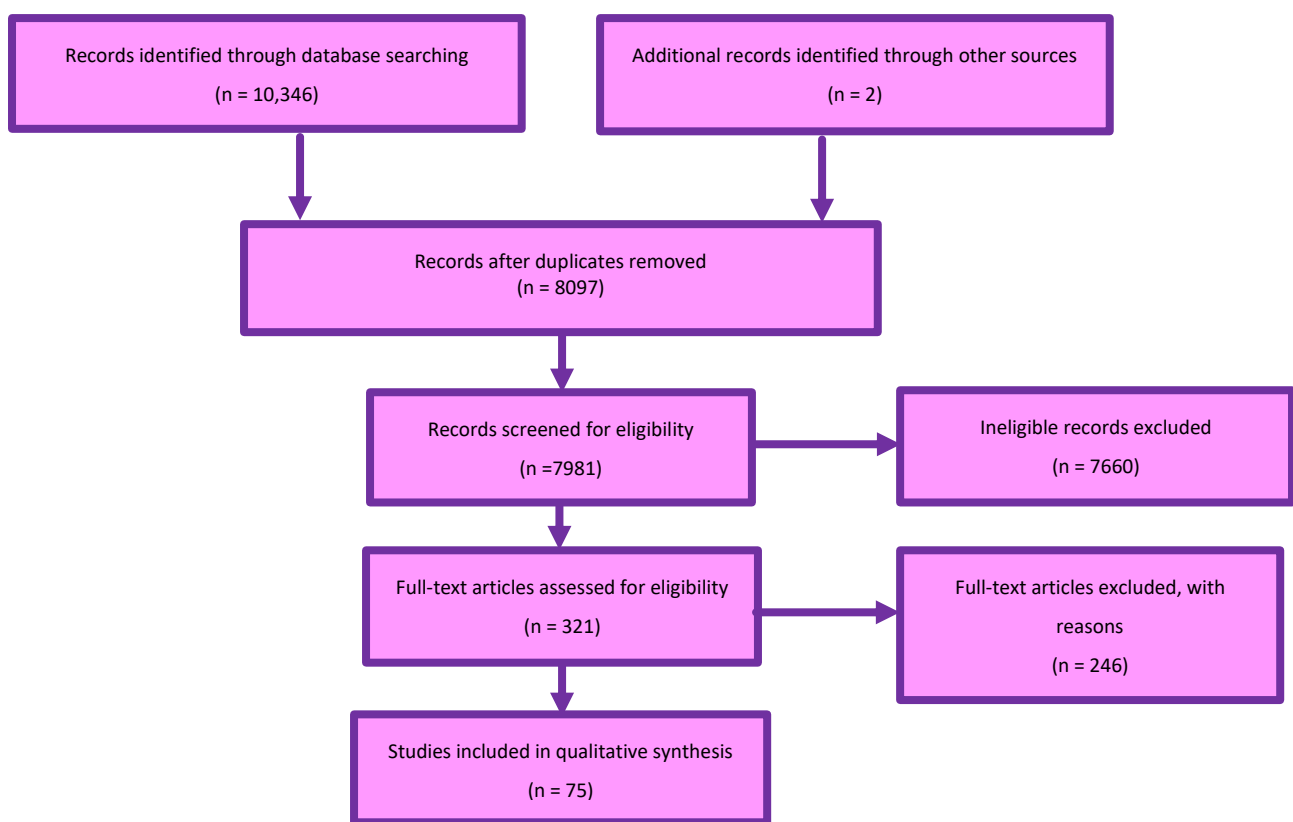


Figure 2.1. Flow diagram showing study selection process.

2.1.2. Study Subjects and Follow Up Length

The summary of included studies table can be found in Appendix 1. Thirty two of the included studies (43%) measured the effects of anti-VEGF therapy on animals, while 43 studies (57%) measured the effects of anti-VEGF therapy on human volunteers with macular pathology.

The follow up duration varied greatly between the eligible studies, ranging from immediately after first injection of the anti-VEGF agent (Luke et al., 2006 and Luke et al., 2009) to a full year or more after initiation of treatment (Shin et al., 2014; Ferrara et al., 2007; Wu et al., 2010, Demirel et al., 2015, Horsely et al., 2010, Jo et al., Parlak et al., 2014, Zeimmsen et al., 2008). The longest follow up period observed was 96 months (8 years) in the study by Valverde-Megías et a. (2019). The most common follow up length was greater than 3 months but less than or equal to 6 months (Figure 2.2). Nine of the eligible studies (12.7%) followed participants/subjects for between 9 and 12 months following the first exposure to intravitreal anti-VEGF therapy. Seven studies followed participants for longer than 12 months (9.9%), thus 77.5% of the studies only followed up the subjects for up to 9 months after the first treatment. This indicates that there is a lack of longitudinal data assessing the effects of repeated anti-VEGF treatments on the peripheral retina.

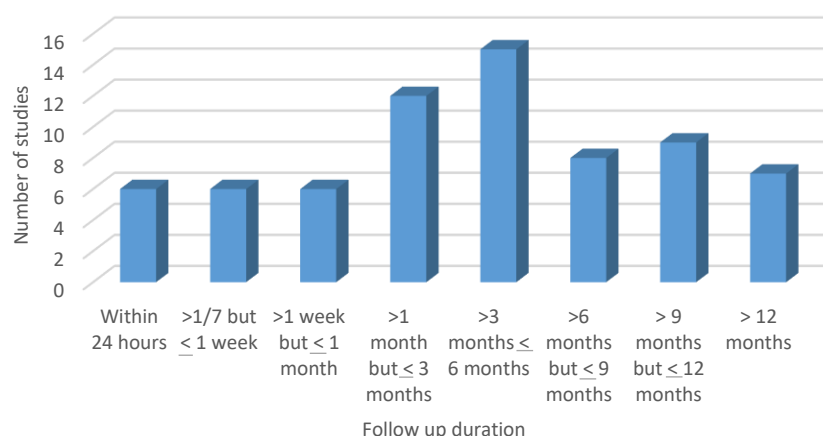


Figure 2.2. Histogram to show the distribution of mean follow up duration of eligible studies. It can be seen that the most common follow up time was 'greater than three months but less than 6 months'.

2.1.3. Study Design

All types of study design were eligible for inclusion in this systematic review. Figure 2.3 displays the proportion of studies of each type of design.

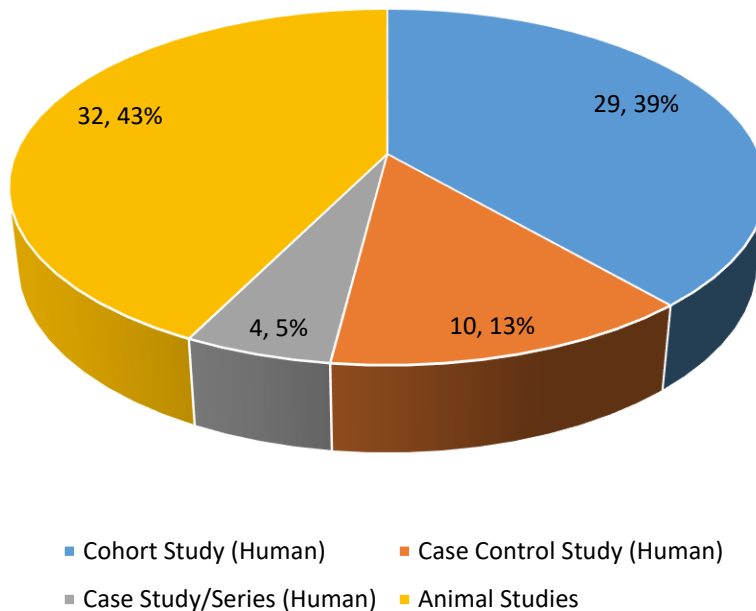


Figure 2.3. Pie chart displaying the distribution of study designs in all eligible studies (total n = 75).

It can be seen that, within the human studies, the most common study design was the cohort study (29; 39% of human studies). Due to the ethical considerations in human participants of providing the optimal treatment for disease, and of not treating healthy eyes unnecessarily, only one study (1%) was a randomised control trial, which would provide the strongest evidence (Comyn et al., 2014). However, this trial randomly assigned patients to either receive macular laser or ranibizumab i.e. there was no untreated control group.

2.1.4. The effect of anti-VEGF therapy on animal retinas in vivo and ex vivo

2.1.4.1. Assessments of visual function

Animal studies have provided useful evidence regarding the effect (or lack thereof) of treatment on retinal function, assessed using electroretinography. Of the 32 animal studies included, 16 (50%) performed assessments of visual function and all used electroretinogram (ERG) tests on their subjects in order to do this. Of these all 16 (100%) performed scotopic ERGs on the subjects, whilst 9 (56%) performed photopic ERGs in addition. Two studies found a significant effect of anti-VEGF therapy on scotopic retinal function (Sakurai et al., 2009, Myers et al., 2012), but the remaining studies found no significant effect. None of the nine animal studies that performed photopic ERGs found a significant difference in photopic retinal function following anti-VEGF treatment. However, there were significant variations in the length of the follow up period and the dosage of anti-VEGF treatment administered and so the results must be reviewed with caution. The following paragraphs summarise the findings of these studies, beginning with those relating to scotopic aspects of visual function.

Of the included studies which evaluated the scotopic ERG, 13 included an evaluation of the a-wave (Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Inan et al., 2007; Wu et al., 2010; Januschowski et al., 2014; Sakuri et al., 2009; Manzano et al., 2006; Heiduschka et al., 2008; Myers et al., 2012; Ramon et al., 2018; Zayit-Soudry et al., 2010; Zayit Soudry et al., 2011). This waveform is dominated by rod photoreceptor activity, especially at early times post flash and at high stimulus intensities, however the scotopic a-wave is also shaped by activity of the rod bipolar cells and third order neurons (Robson and Frishman 1996; 1999).

Luke et al. (2006; 2009; 2010) performed a series of studies evaluating scotopic a-wave amplitudes after healthy post mortem bovine eyes were treated with anti-VEGF drugs (bevacizumab 0.08 mg/ml, 0.5 mg/ml, 0.8 mg/ml; ranibizumab 2mg/ml; pegaptanib 0.006 mg/ml, 0.06 mg/ml, 0.2 mg/ml). In all three of these studies there was no significant reduction in a-wave amplitude post treatment, however all tests took place within 24 hours of the introduction of the anti-VEGF agents. Whilst Januschowski et al. (2014) did find a significant reduction of the a-wave amplitude immediately after exposure of healthy post-mortem bovine retinae to either 0.5 mg or 2 mg aflibercept,

it was not sustained to the completion of the washout period. Manzano et al. (2006) injected bevacizumab (one of 4 concentrations; 500 µg/0.1 mL, 1.0 mg/0.1 mL, 2.5 mg/0.1 mL, and 5.0 mg/0.2 mL) into one eye of 12 healthy rabbits, and an equivalent volume of saline solution in the contralateral control eye. Baseline scotopic ERGs were repeated 14 days after exposure and showed no significant change in a-wave amplitude. Inan et al. (2007) similarly reported no difference in scotopic ERG a-wave parameters between test and control eyes of healthy rabbits at 14 or 28 days after receiving 1.25 mg or 3 mg bevacizumab intravitreally in their right eyes, and an equivalent volume of saline in their left eyes.

Only one study reported a treatment effect on the scotopic a-wave which extended beyond the washout period. Sakurai et al. (2009) investigated the effects of 6.25 mg or 12.5 mg bevacizumab in monkey eyes (n = 2 per group, fellow eye control received the same volume of saline). At three days and one week after the injection there was a significant reduction in the a-wave amplitude of the scotopic bright flash ERG, and a delayed implicit time at one week in the eyes that had received the anti-VEGF intervention. These effects were only seen in the two monkeys receiving the higher dosage, suggesting that higher doses of bevacizumab may have a retinotoxic effect. However, no significant difference was found between the experimental and control eyes at 4 weeks, suggesting that any effect is transient.

Thirteen included studies assessed scotopic b-wave parameter measurements, giving an indication of retinal ON-bipolar cell function (Guo et al., 2010; De Oliveira Dias et al., 2014; Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Shahr et al., 2006; Inan et al., 2007; Wu et al., 2010; Heiduschka et al., 2008; Ramon et al., 2018; Zayit-Soudry et al., 2010; Zayit-Soudry et al., 2011; Sakurai et al., 2009). For example, Guo et al., (2010) performed scotopic ERGs on their control and active therapy healthy rat eyes and found that the b-wave amplitudes were very similar between groups 1 week after a single dose of bevacizumab (3.75 mg/ml). The same follow up time and singular dose protocol was employed by De Oliveira Dias et al. (2014). In their study of 18 healthy rabbits intravitreally injected with 25 mg/ml of ziv-aflibercept or 40 mg/ml of aflibercept, they observed no significant change in the b-wave amplitude. Similarly, Luke et al. (2006; 2009; 2010) reported no significant reduction in scotopic b-wave amplitude in post mortem healthy bovine retinas up to 24 hours after treatment with

bevacizumab (0.08 mg/ml, 0.5 mg/ml, 0.8 mg/ml) ranibizumab (2 mg/ml), or pegaptanib (0.006 mg/ml, 0.06 mg/ml, 0.2 mg/ml). Post mortem healthy bovine retinæ were also used by Januschowski et al. (2014), who recorded scotopic ERGs as a same day follow up test after single dose perfusion with 0.5 mg or 2 mg aflibercept. Although b-wave amplitude reductions were noted initially after treatment, these did not remain at the end of the washout period. Shahar et al. (2006) also tested scotopic b-wave amplitude after a single dose of anti-VEGF agent (bevacizumab, 0.1 mL/2.5 mg) in the test eyes of healthy albino rabbits, and 0.1 mL saline in the contralateral control eye. b-wave amplitude was not significantly different to that of control eyes after the single 2.5 mg dose injection and remained unaffected for the 4-week follow up period. Inan et al. (2007) also assessed scotopic b-wave amplitude over 4 weeks after single dose injections of 1.25 mg and 3 mg intravitreal bevacizumab in test eyes of healthy rabbits (and equivalent volume saline in the fellow control eyes). Once again, no significant change was found in b-wave amplitude over the follow up duration. Similarly, Manzano et al. (2006) injected 4 concentrations of bevacizumab (500 µg/0.1 mL, 1.0 mg/0.1 mL, 2.5 mg/0.1 mL, and 5.0 mg/0.2 mL) into the eyes of 12 healthy rabbits (3 rabbits each received one concentration, fellow eye saline control). Scotopic ERGs were performed at baseline and day 14 after exposure and showed no significant change in b-wave amplitude. Ramon et al. (2018) also found no evidence of permanent change to the scotopic ERGs of 12 healthy albino rabbits assessed up to 4 weeks post injection with 0.1 mL ziv-aflibercept. Whilst Heiduschka et al. (2008) did report an immediate reduction in all scotopic ERG parameters in mice receiving 0.025 mg bevacizumab (n = 17), the vehicle solution (n = 14), balanced saline solution (n = 10), and a sham treatment group (n = 3) also showed a similar reduction, and thus the effect cannot be attributed to the anti-VEGF drug itself. Furthermore, a gradual recovery was noted over the 25 day follow up period.

Two included studies examined the a/b ratio of the scotopic ERGs. De Oliveira Dias et al. (2014) used a single intravitreal exposure of 25 mg/ml of ziv-aflibercept or 40 mg/ml of aflibercept treatment in their study involving 18 healthy rabbits. No significant change in the a/b ratio was witnessed after 1 week. Inan et al. (2007) also found no significant change in a/b ratio in their healthy rabbit study (bevacizumab 1.25 mg and 3 mg single intravitreal injections, followed up over 28 days). The results

of these studies indicate that electrophysiological activity and therefore retinal function remained normal for 28 days following bevacizumab treatment.

This apparent lack of short-term effect on scotopic visual function after a single administration of the drug is not directly generalisable to clinical applications of anti-VEGF therapy which involve a minimum of 3 loading injections, and usually multiple additional treatments over a prolonged period of time. In the one animal study using the scotopic ERG that did employ a more comparable protocol, Zayit-Soudry et al. (2010) administered nine injections (bevacizumab 2.5 mg, or ranibizumab 1 mg) to the test eye of 12 healthy rabbits at 14 day intervals, whilst the fellow eye received a saline control. ERG responses for all parameters were reported to be similar in the experimental and control eyes throughout the follow up period.

Only two studies reported a significant treatment effect on the scotopic b-wave. Myers et al. (2012) studied the effect of pegaptanib (0.3 mg), ranibizumab (0.5 mg), bevacizumab (1.25 mg) and saline solution (control) intravitreal injection on groups of healthy rabbits (n = 48 rabbits, 12 in each of the four groups) using the ISCEV standard ERG protocol 8 weeks post treatment. The amplitude of the b-wave in the dark-adapted ERG with a dim light stimulus was significantly lower in all anti-VEGF groups than the control rabbits at the 8-week follow up. Sakurai et al. (2009) in their study of 6.25 mg or 12.5 mg bevacizumab in monkey eyes (n = 2 per group, fellow eye control received the same volume of saline) also found a reduction in amplitude and delayed timecourse of the scotopic rod response at the higher dose of bevacizumab, but this was not sustained to 4 weeks.

As with the assessed scotopic ERGs, the photopic responses recorded from animals showed no statistically significant reduction after treatment (e.g. Sakurai et al., 2009; Inan et al., 2007; Zayit-Soudry et al., 2010; Shahar et al., 2006; Heiduschka et al., 2008; Manzano et al., 2006). However, the applicability of these results to AMD treatment in humans was again limited by the number of injections provided, and the brevity of the follow up period.

Photoreceptor function was assessed by the examination of a-wave parameters in seven studies (Inan et al., 2007; Manzano et al., 2006; Myers et al., 2012; Ramon et al., 2018; Sakurai et al., 2009; Zayit-Soudry et al., 2010; Zayit-Soudry et al., 2011). As in the

scotopic ERG, the photopic a-wave is an indicator of photoreceptor function, influenced by more proximal retinal contributions (Bush and Sieving, 1994). Sakurai et al., (2009) injected a single eye of four healthy monkeys with a high dose of bevacizumab (6.25 mg or 12.5 mg) and an equivalent volume of saline into their contralateral eyes. No significant changes in photopic a-wave measurements occurred over the four-week follow up period. The same result was observed over the same follow up period by Inan et al. (2007), although here the single doses of intravitreal bevacizumab were much lower (1.25 mg or 3 mg) and healthy rabbits were examined. Healthy rabbits were also used by Zayit-Soudry et al. (2010). These subjects each received 9 injections of either ranibizumab (1 mg) or bevacizumab (2.5 mg) every two weeks for 18 weeks. Photopic a-wave parameters once again showed no significant difference from baseline measurements throughout the follow up period, which was in this case far more similar to the repeat anti-VEGF treatment regime patients are exposed to. In turn this suggests no detrimental effect of anti-VEGF therapy on cone photoreceptor function.

ON-bipolar cell function was assessed by nine studies through photopic b-wave parameter analysis (Inan et al., 2007; Manzano et al., 2006; Myers et al., 2012; Ramon et al., 2018; Sakurai et al., 2009; Zayit-Soudry et al., 2010; Zayit-Soudry et al., 2011; Shahr et al., 2006; Heiduschka et al., 2008). Shahr et al., (2006) reported no significant differences in photopic b-wave amplitude between the control and experimental eyes of 10 albino rabbits followed up for 4 weeks after a single bevacizumab injection (0.1 mL/2.5 mg). Inan et al. (2007) also assessed the effect of intravitreal bevacizumab (1.25 mg and 3 mg in this case) on healthy rabbit retinae over 28 days and found no statistically significant difference over the follow up period. The a/b wave ratio also showed no significant difference between baseline and follow up. A similar 4 week follow up was executed by Sakurai et al., (2009) but this time in the eyes of 4 healthy monkeys. The effects of a high dosage of bevacizumab (6.25 mg or 12.5 mg) in one eye and equivalent volume of saline in the contralateral eye were investigated and no significant change from baseline in photopic b-wave parameters was seen. Manzano et al. (2006) injected one eye of 12 healthy rabbits with 500 µg/0.1 mL, 1.0 mg/0.1 mL, 2.5 mg/0.1 mL, or 5.0 mg/0.2 mL of intravitreal bevacizumab. No significant change in photopic ERG parameters was found between baseline and

follow up (performed 14 days after anti-VEGF exposure). Myers et al. (2012) also studied the effect of pegaptanib (0.3 mg), ranibizumab (0.5 mg), bevacizumab (1.25 mg) and saline solution (control) intravitreal injection on groups of healthy rabbits using the ISCEV standard protocol 8 weeks post treatment. No significant difference was observed in any photopic ERG parameters after this time.

Photopic b-wave parameters were also assessed by Zayit-Soudry et al. (2010) over a much longer 18-week follow up period. Nine treatments of ranibizumab (1 mg) or bevacizumab (2.5 mg) were injected into 12 healthy rabbit eyes at 14 day intervals and again, no significant difference in the photopic b- wave amplitudes were seen. This suggests that, even after repeated anti-VEGF injections, ON-bipolar cell function is unaffected.

The only study using photopic ERG assessment on animal subjects that reported any negative effect of anti-VEGF was Heiduschka et al. (2008). After the injection of 0.025 mg bevacizumab in 17 healthy wild-type mice, immediate reductions in amplitudes of the transient and flicker ERG waveforms were observed, followed by a gradual recovery over 25 days. However, a similar effect was seen in mice receiving an injection of the vehicle solution (n =14), balanced saline solution (n = 10), or a sham treatment (n = 3). Hence, the effect is likely to be attributable to the intravitreal injection process rather than the drug itself.

Whilst the findings of these studies assessing visual function in animal subjects largely suggest that anti-VEGF treatment does not significantly impact on peripheral retinal function, many took place over a limited follow up period and/or after a single injection of the anti VEGF agent. This does not leave much time for toxic effects to manifest, nor provide information about the effects of long-term repeated treatments. Even in studies where longer follow-up periods have been employed no significant effect was reported, or where effects were noted these appear to have been transient. However, even those animal studies with longer follow up periods did not evaluate the kind of repeated treatment regime used in the management of diseases such as AMD. In addition, 10 of the studies assessing visual function in animal subjects did not include a control group for comparison in their assessments (de Oliveira Dias et al., 2014; Inan et al., 2007; Januschowski et al., 2014; Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Manzano et al., 2006; Shahar et al., 2006; Zayit-Soudry et al., 2010;

Zayit-Soudry et al., 2011) and a further two studies used the contralateral eyes of the same animal subjects as control eyes and so did not account for possible systemic effect (Guo et al., 2010; Sakurai et al., 2009). There was also some lack of clarity noted in the follow up assessments of the subjects (Guo et al., 2010) and in one case, the use of unequal numbers of subjects in groups which may have added bias (Heiduschka et al., 2008).

2.1.4.2. Assessment of peripheral retinal structure

Investigations using immunohistochemistry involve detecting antigen proteins in tissue cells using the knowledge that antibodies will bind to them. Immunohistochemical assessments are invasive and so are performed on animals or post mortem / enucleated human eyes. Histology is the study of microscopic tissue structure. Of the 32 animal studies, 24 (75%) performed immunohistochemical or histological analysis of retinal tissue. The majority found no detrimental effect of anti-VEGF treatment (Arraes et al., 2009; Bakri et al., 2006; Cheng et al., 2009; de Oliveira Dias et al., 2014; Guo et al., 2010; Iriyama et al., 2007; Januschowski et al., 2014; Kim et al., 2008; Lu et al., 2014; Manzano et al., 2006; Ozaki et al., 2000; Sari et al., 2006; Thaler et al., 2010; Zayit-Soudry et al., 2011).

There is evidence from 14 studies using light or electron microscopy that the structural integrity of the retina remains sound after exposure to anti-VEGF drugs (Arraes et al., 2009; Bakri et al., 2006; Cheng et al., 2009; de Oliveira Dias et al., 2014; Guo et al., 2010; Iriyama et al., 2007; Januschowski et al., 2014; Kim et al., 2008; Lu et al., 2014; Manzano et al., 2006; Ozaki et al., 2000; Sari et al., 2006; Thaler et al., 2010; Zayit-Soudry et al., 2011). For example, Arraes et al. (2009) performed a histological analysis of 30 eyes (15 healthy rabbits in 3 groups of 10 eyes) 90 days after injection with either 0.1 ml saline (sham group), 1.25 mg bevacizumab (group 1), or 2.5 mg bevacizumab (group 2). They found no differences in the neurosensory retina between bevacizumab and saline injected eyes. Bakri et al., (2006) injected 1.25 mg bevacizumab or 2.5 mg bevacizumab into the eyes of two groups of 4 healthy rabbits. After a 29 day follow up period only mild vacuolisation of the RGC layer and disruption of the outer segments of the photoreceptors was found. However, these were present in both the test eyes and the control eyes, which suggests that this issue was unrelated to the anti-VEGF drugs administered. Sari et al. (2006) looked at four groups of healthy

rabbits; group 1 received intravitreal injection of 1.25 mg bevacizumab in their right eye three times at 25 day intervals, the left eyes of group 1 rabbits served as untreated control (group 2), group 3 received 1.25 mg intravitreal bevacizumab in both eyes 3 times at 25 day intervals, and group 4 received no injections at all. Light microscopy demonstrated normal retinal histologic findings for all groups. De Oliveira Dias et al. (2014) injected 25 mg/ml of ziv-aflibercept or 40 mg/ml of aflibercept into the eyes of 18 healthy rabbits and found no major evidence of toxicity damage during follow ups with histological analysis and electron microscopy (1 and 7 days post intervention). Manzano et al. (2006) performed light microscopy on the eyes of 12 healthy rabbits divided into three groups receiving different doses of intravitreal bevacizumab (500 µg/0.1 mL, 1.0 mg/0.1 mL, 2.5 mg/0.1 mL or 5.0 mg/0.2 mL). After 14 days histological analysis was performed and showed no evidence of retinal toxicity. Similarly, landiev, et al. (2011) found no structural changes on light or electron microscopy and no gliosis or apoptosis in healthy porcine eyes injected with a single dose of 1.25 mg bevacizumab and followed up over two, three and seven days.

Findings by Romano et al. (2012) suggest there may be a significant effect of anti-VEGF therapy on the microscopic structure of the inner retina. In this study 20 healthy adult rats were studied, 10 were administered a single injection of 4µl bevacizumab in one eye, while the other 10 received three injections every seven days, and the fellow eyes of each group were injected with the same volume of saline as a control. Histological analysis showed bevacizumab reliably induced neuronal loss in these animals (severe loss of RGCs and damage of inner and outer nuclear layers) with a large decrease in RGC specific protein shown during western blot testing in the group receiving multiple bevacizumab injections. However, Thaler et al. (2010) found no significant reduction in RGC counts in healthy rat eyes or those with induced RGC damage, injected with anti-VEGF drugs (bevacizumab 25 mg/ml and 125 mg/ml, ranibizumab 10 mg/ml and 60 mg/ml and pegaptanib 3.3 mg/ml and 20 mg/ml).

The effect of anti-VEGF on the histology of the RPE and choriocapillaris has also been evaluated, with variable findings reported. Karawya et al. (2008), investigated the effect of a single 5 mg, or 0.1 mg bevacizumab injection on the retinae of 10 healthy rabbits one week after exposure. No substantial histological changes were found. However, ultra-structural changes such as significantly increased numbers of

mitochondria in the retinal pigment epithelium and prominent endoplasmic reticulum were noted. Arraes et al. (2009) performed histological analysis of 30 eyes (15 healthy rabbits, three groups of 10 eyes) 90 days after injection with either 0.1 ml saline (sham group), 1.25 mg bevacizumab (group 1), or 2.5 mg bevacizumab (group 2). They found no differences in the RPE of the eyes across the groups and also commented on a lack of significant difference between groups in terms of thickness of the retinal layers. Julien et al. (2014) introduced aflibercept (2 mg) and ranibizumab (0.5 mg) to healthy monkey eyes with a follow up time of seven days and noted that aflibercept was taken up by RPE and neuronal cells, whilst ranibizumab permeated via intercellular spaces. It was also found that eyes exposed to aflibercept showed haemolysis (and ultimately cell death) in the choriocapillaris, and eyes exposed to ranibizumab and aflibercept showed a reduction in area of the choriocapillaris with greater evidence of detrimental effect in the eyes exposed to aflibercept.

Other studies have reported on immunohistochemical evidence of toxicity. A number of these reported no significant evidence of retinal toxicity in animals exposed to anti-VEGF drugs. For example, Lu et al. (2014) reported no toxic effects on the neurosensory retina of bevacizumab injected into 72 diabetic rats (group 1 - 3µL saline, group 2 75µg bevacizumab, and group 3 (3µL (75µg) bevacizumab-chitosan nano particles), up to eight weeks after the injection. Januschowski et al. (2014) used aflibercept (0.5 mg or 2 mg) on retinal whole mount cultures of post mortem healthy bovine retinae for a 24 hour period. The subsequent TUNEL assay of the inner retina and photoreceptors showed there was no increase in apoptosis in the eyes receiving aflibercept compared to control eyes. Kaempf et al. (2008) found no evidence of toxicity of bevacizumab (0.25 mg/mL, 0.5 mg/mL, 1.25 mg/mL) in healthy porcine retinae when compared to controls. Following (up to 28 days) intravitreal injection of a single dose of bevacizumab (0-2.5 mg/mL) in an unspecified number of healthy mice, Kim et al. (2008) reported that the bevacizumab caused no decrease in the expression of retinal neurofilament, a vital aspect of the development and maintenance of retinal axons. Sari et al. (2006) also reported that there was no evidence to show that multiple doses of bevacizumab 1.25 mg (injected 3 times with a 25 day interval) in healthy rabbits caused any toxic effect on uveo-retinal tissue, any lipid peroxidation, oxidative reaction or apoptosis.

However, other studies have detected subtle signs of early cellular damage in different retinal layers using immunohistochemical techniques. For example, Cam et al. (2017) found no evidence of retinal abnormality on light microscopy in the rabbit groups exposed to anti-VEGF interventions (ranibizumab 0.25 mg, aflibercept 1 mg, and bevacizumab 0.3125 mg and 0.625 mg), after a three-week follow up. However, immunohistochemical analysis with TUNEL and caspase-3 showed significantly raised levels of cell apoptosis in the groups of healthy white rabbits exposed to anti-VEGF treatments. Ramon et al. (2018) had a longer follow up time of 4 weeks and also saw no histological evidence of retinal damage following exposure of their 22 healthy albino rabbit eyes to ziv-aflibercept (0.1 mL). However, there was noted to be a degree of retinal stress via the expression of GFAP in Müller cells of the treated eyes which was not present in the control eyes. At 14 and 28 day reviews, Inan et al. (2007) found intense apoptotic protein expression in the eyes of healthy rabbits exposed to bevacizumab (1.25 mg or 3.0 mg) but minimal expression of this in the control. Avci et al. (2009) evaluated groups of healthy rabbits over three months. Each group received bevacizumab (0.25, 0.625, 1.25, or 2.5 mg), or pegaptanib sodium (0.15, 0.3, or 0.6 mg) intravitreally injected once (with follow ups at 14 days or 3 months) or once every three months (follow up at 3 months). While routine light microscopy revealed no toxicity related adverse effects, a significant increase in apoptotic activity was found in the photoreceptors of the eyes that received either type of anti-VEGF therapy compared to the controls. Further increases in apoptotic activity were noted in the eyes with higher doses of bevacizumab in particular. Myers et al. (2012) also found immunohistochemical evidence to suggest that bipolar cell structures may be affected by anti-VEGF interventions. Healthy rabbit eyes injected with pegaptanib (0.3 mg) and ranibizumab (0.5 mg) were followed up after 9 weeks and found to have significantly reduced protein kinase C (PKC) labelled rod bipolar cells, whereas those injected with bevacizumab (1.25 mg) had normal PKC labelling.

These findings may suggest that although retinal function and structure (under light microscope) may appear unremarkable, the retinal ultrastructure may be negatively affected by anti-VEGF therapy as early as one month after first exposure.

Immunohistological markers of abnormality have also been reported in other associated structures. For example, a clinically significant increase in cell apoptosis and

decrease in cell viability was reported in RPE cell cultures from healthy white rabbits after 72 hours of exposure to anti-VEGF (aflibercept 0.5 mg/mL, bevacizumab 0.3125 mg/mL, and ranibizumab 0.125 mg/mL) by Sahiner et al. (2018). Kaempfer et al. (2008) noted that bevacizumab treated (0.25 mg/mL, 0.5 mg/mL, 1.25 mg/mL) healthy porcine retinæ showed enhanced SMA expression in retinal blood vessels (a sign of the loss of functional VEGF modulating smooth muscle cells in mature vessels). Zayit-Soudry et al. (2010) reported glial fibrillary acidic protein immunoreactivity in the retinal astrocytes of healthy rabbits receiving 9 bevacizumab (2.5 mg) or ranibizumab (1 mg) injections at 14 day intervals, with a total review time of 18 weeks. This is an indicator of early cell damage, although the structure of the retinæ appeared otherwise intact.

Retinal structure was also assessed by fundoscopy by Karawya et al. (2008) and De Oliveira Dias et al. (2014). Karawya et al. (2008) assessed the retinæ of 10 healthy pigmented rabbits treated with 5 mg or 0.1 mg bevacizumab (second eye injected with saline as control). At the second clinical examination one week later there were no significant changes noted during fundoscopic assessment. De Oliveira Dias et al. (2014) also assessed the retinæ of healthy rabbits (n = 18) but in this case after intravitreal injections of ziv-aflibercept (25 mg/ml) or aflibercept (40 mg/ml). Follow ups performed 24 hours and 7 days later found no significant changes during the fundoscopic assessment, agreeing with Karawya et al., (2008).

In summary, 3 studies (Romano et al., 2012; Karawya et al., 2008; Julien et al., 2014) suggest that anti-VEGF therapies may be having an effect on peripheral retinal structure apparent using microscopy, and 8 more (Inan et al., 2007, Kaempfer et al., 2008; Avci et al., 2009; Zayit-Soudry et al., 2010; Myers et al., 2012, Cam et al., 2017; Ramon et al., 2018; Sahiner et al., 2018) provide immunohistochemical evidence of structural changes taking place. However, although much of the immunohistochemical and histological evidence seems to suggest a lack of impact of anti-VEGF drugs on retinal structure, it is weakened by missing protocol details in the published reports (such as number of subjects [Ozaki et al., 2000]), uneven numbers within subject groups (Julien et al., 2014; Heiduschka et al., 2008) and the fact that the follow up periods are relatively short which leaves little time for toxicity effects to take hold. Furthermore, nine of the 24 studies assessing retinal structure in animals (38%) failed

to include a sham/saline control group (de Oliveira Dias et al., 2014; Inan et al., 2007; Iriyama et al., 2007; Januschowski et al., 2014; Manzano et al., 2006; Ozaki et al., 2000; Sahiner et al., 2018; Zayit-Soudry et al., 2010, Zayit-Soudry et al., 2011). This is important as there is evidence that the intravitreal injection process itself may have a detrimental effect on the retinal structure (Bakri et al., 2006). An additional six studies (25% of animal studies assessing retinal structure) used the animal subject's contralateral eye as a control group, neglecting the possibility of systemic effect of anti-VEGF injection (Bakri et al., 2006; Cheng et al., 2009; Guo et al., 2010; Karawya et al., 2008; Romano et al., 2012; Sakurai et al., 2009). Finally, as the studies do not specify what retinal location their samples are taken from it is not possible to ascertain if this evidence represents peripheral, central or a mixture of retinal locations.

2.1.5. The effect of anti-VEGF therapy on human participants

2.1.5.1. Assessments of visual function

Sixteen of the 43 included studies with human participants (37%) used electrophysiology to examine visual function of persons exposed to anti-VEGF therapy. Eight studies (19% of 43 human studies) evaluated the scotopic ERG (Macky and Mahgoub, 2012; Skaat et al., 2011; Comyn et al., 2014; Pedersen et al., 2010; Maturi et al., 2006; Zeimmsen et al., 2008; Holm et al., 2015; Stahl et al., 2009). Of these, two (25% of scotopic ERG studies) showed a significant deleterious effect on at least one subcomponent (Comyn et al., 2014; Pedersen et al., 2010). Thirteen studies (30% of 43 number of human studies) evaluated the photopic ERG (Comyn et al., 2014; Maturi et al., 2006; Holm et al., 2015; Macky and Mahgoub, 2012; Pedersen et al., 2010; Skaat et al., 2009; Almedia et al., 2015; Zeimmsen et al., 2008; de Oliveira Dias et al., 2015; Feigl et al., 2007; Moon et al., 2013; Terauchi et al., 2019; Yagura et al., 2016). Of these, six (46% of photopic ERG studies) showed a significant deleterious effect of anti-VEGF therapy on at least one subcomponent (Pedersen et al., 2010; Almedia et al., 2015; de Oliveira Dias et al., 2015; Feigl et al., 2007; Terauchi et al., 2019; Yagura et al., 2016).

Of those evaluating scotopic ERGs, Macky and Mahgoub (2012) found no statistically significant change in a- or b- wave amplitudes in 55 participants with choroidal neovascularisation secondary to AMD or myopia 6 weeks after receiving a single

bevacizumab (1.25 mg) injection each. Skaat et al., (2011) assessed the effects of bevacizumab (1.25 mg) on the scotopic retinal function of 12 patients with nAMD using the ISCEV standard protocol 1 month after treatment commenced. There was a pre- to post-injection increase in a-wave and b-wave amplitudes, which was statistically significantly higher in treated than in the untreated (fellow) control eyes. This difference indicated a significant improvement in peripheral retinal function, attributable to the treatment. Holm et al. (2015) performed ISCEV standard scotopic ERGs on 20 patients undergoing ranibizumab (0.5 mg) therapy for diabetic macular oedema. There was no change in rod function over the follow-up period (a maximum of four weeks after the last ranibizumab injection). Maturi et al. (2006) also performed scotopic full-field ERGs on five people with AMD at baseline and, after one (four patients) or two (one patient) treatments with intravitreal bevacizumab (1.25 mg) over a one or three month follow up period, found there to be no clinically significant change in scotopic ERG amplitude or implicit time in response to a dim or bright flash (mixed rod and cone response). However, the sample size in this study was prohibitively small. Stahl et al. (2009) also found no statistically significant difference between ERGs before and after bevacizumab (1.25 mg) therapy in 10 individuals with CNV attributable to a range of conditions. In this study, 2-3 bevacizumab injections were provided at monthly intervals, and follow-up continued until three weeks after the third injection. Zeimmsen et al. (2008) performed ISCEV standard scotopic ERGs, and a scotopic light response ERG on 20 consecutive cases of nAMD treated by a single injection of 1.25 mg bevacizumab, revealing no significant changes from baseline at the four month follow-up. These results provide interesting insight but should be viewed with some caution due to the short follow up time and small sample sizes of the studies.

Longer follow up times were employed by Pedersen et al. (2010; 6 months) and Comyn et al. (2014; 48 weeks). Pedersen et al. (2010) assessed a- and b-wave amplitudes and implicit times of scotopic ERGs in 26 patients commencing treatment with bevacizumab (1.25 mg) for nAMD. Each participant was given the first injection of bevacizumab at the beginning of the study with the remainder of the injections being given on an 'as required' basis thereafter. Over the six month study period the mean number of injections was 3 (range 1 – 4). The dim flash ERG did not change at all over

the follow up period, suggesting no retinotoxic effect of bevacizumab on purely rod responses. However, b-wave amplitude of the combined rod and cone white 2.0 log response was decreased and the a-wave implicit time of the combined rod and cone white 3.0 log response was increased from baseline to the 6 month follow up. Comyn et al. (2014) used ISCEV standard full field flash ERGs to assess the effects of ranibizumab (0.5 mg) treatment for diabetic macular oedema as part of a prospective, randomised single masked clinical trial. Intravitreal injections were provided to 22 participants at baseline, 4 weeks, and 8 weeks then every 4 weeks (as required). Over the 48-week follow up there was no significant change in the scotopic 0.01 ERG in the ranibizumab group. In the scotopic 11 (mixed rod and cone) ERG the mean amplitude of the a-wave decreased during follow up, although the implicit time stayed the same. The b-wave for this ERG protocol also maintained a consistent implicit time but decreased slightly in amplitude. None of the small changes witnessed were deemed significant clinically but there was at least some mild loss of function to note.

Most studies have similarly found no negative effect of anti-VEGF therapy on the photopic ERG in humans (Stahl et al., 2009; Maturi et al., 2009; Zeimmsen et al., 2008; Comyn et al., 2014; Marmor et al., 2009; Moon et al., 2013; Chabblani et al., 2015). Other human studies have actually reported an improvement in peripheral photopic retinal function (increased ERG amplitude and/or reduced time to peak) assessed after exposure to anti-VEGF therapy (Skaat et al., 2011; Macky and Mahgoub, 2012; Holm et al., 2015; Pedersen et al., 2010; de Oliveira Dias et al., 2015; 2007; Moon et al., 2013). However, there have also been human studies which have reported a significant detrimental effect of treatment on the photopic ERG (Pedersen et al., 2010; Feigl et al., 2007; Almeida et al., 2015; Terauchi et al., 2019; Yagura et al., 2016).

A number of studies with human participants examined photopic retinal function after a single dose of anti-VEGF treatment, often bevacizumab. For example, Macky and Mahgoub (2012) recorded photopic ERGs in 55 patients receiving a single initial injection of bevacizumab (1.25 mg) for CNV secondary to AMD or myopia. Photopic b-wave amplitude increased in both the injected and fellow eyes at the 1 and 6-week follow ups, indicating an improvement in function. The increased amplitude reported in the contralateral eye could be indicative of a systemic effect of the drug. Skaat et al. (2011) assessed the effects of a single bevacizumab injection on nAMD patients by

performing ISCEV standard full field flash photopic ERGs at 2 incremental light intensities 1 month after the treatment. The a-wave amplitudes increased significantly more in the treated group than the control group between the pre- and post- injection recordings. Mean b-wave amplitude was found to have increased in the treated group between the pre- and post- injection recordings but not to a significantly greater amount than in the control eyes. Again, these findings suggested an improvement in peripheral retinal function post treatment.

Chhablani et al. (2015) investigated the effect of a single dose of ziv-aflibercept (1.25 mg) on ISCEV standard photopic full field ERG measures and found no significant difference in any a- and b-wave parameters at a 30 day follow up. Yagura et al. (2016) examined the a-wave components of photopic ERGs in 19 patients receiving ranibizumab (0.5 mg) or aflibercept (2.0 mg). These ERGs were performed intraoperatively at three timepoints; before the intraocular injection of anti-VEGF (T1), just after the injection of anti-VEGF (T2), and after the performance of paracentesis to normalise IOP (T3). A delay in the a-wave time to peak was sustained to T3 but other seemingly affected components (b-wave and PhNR) returned to baseline measurements by T3 which may suggest that retinal neurons have different sensitivities and responses to anti-VEGF treatments. However, it must be considered that as the ERGs were recorded immediately after treatment, the responses are likely to reflect the effect of intravitreal injection itself rather than of anti-VEGF drug toxicity.

Other studies have employed a longer follow up period. For example, Comyn et al. (2014) performed photopic full field flash ERGs according to ISCEV standards (Marmor et al., 2009) in 22 patients undergoing ranibizumab (0.5 mg) treatment for diabetic macular oedema. No clinically significant differences were found in the photopic single flash ERG a- and b-wave amplitudes or implicit times over the 48-week follow up period. In contrast, Pedersen et al. (2010) performed a prospective pilot study over a 6 month duration. Twelve previously treatment naïve patients received between 1 and 4 bevacizumab (1.25 mg) treatments for nAMD during this time. Results showed an initial decrease in b-wave amplitude and increase in a-wave implicit time before subsequent recovery. This may suggest transient photoreceptor and/or ON-bipolar cell dysfunction but, given the lack of a control group and the relatively small sample size, the results must be interpreted with caution.

Flicker ERGs were also performed in several of the studies assessed (Comyn et al., 2014; Yasuda et al., 2015; Holm et al., 2015; Terauchi et al., 2019). No significant difference in retinal function was seen over time in the 30 Hz flicker ERG results in the study by Comyn et al. (2014; in which ranibizumab [0.5 mg] was given to 22 patients with diabetic macular oedema, followed up after 48 weeks, treated with 3 intravitreal injections and then as required). Yasuda et al. (2015) used the same flicker ERGs to examine 15 patients also being treated for macular oedema with ranibizumab (unspecified dosage) 1 month after treatment, with flicker implicit time decreasing following treatment, indicating an improvement in cone function. This same effect was reported by Holm et al. (2015), where 20 patients with diabetic macular oedema being treated with ranibizumab (0.5 mg) therapy underwent 30 Hz flicker ERGs and time to peak reduced significantly between baseline measurements and final follow-up (~3 months later). Only Terauchi et al. (2019) reported an increase in RETeval® flicker ERG implicit time after intravitreal anti-VEGF in patients (n = 79) with nAMD, DMO or retinal vein occlusion. Although maximum follow up was only 24 hours post injection, it was noted that time to peak measurements increased in the non-injected (control) eye as well as the treated eye. They suggested that the effect on the contralateral eye may indicate a systemic effect of the drug. The latter study, although the follow up period was brief, did benefit from a larger sample size than the other studies (Terauchi et al., 2019).

The PhNR is thought to reflect the function of the RGCs (Frishman, 2006; Viswanathan et al., 2018). Moon et al., (2013) found no statistically significant difference between PhNR amplitudes or PhNR/b-wave amplitude ratios before and after treatment with 1.25 mg bevacizumab in 32 patients with macular oedema followed for up to 22 months, 4 weeks after the third intravitreal injection.

Many studies reported no significant difference in photopic ERG waveforms holistically after anti-VEGF treatment. For example, Stahl et al. (2009) recorded full field photopic ERGs 3 weeks after 2-3 monthly intravitreal injections of bevacizumab (1.25 mg) had been delivered to 10 individuals with various retinal conditions. Zeimssen et al. (2008) performed ISCEV standard photopic ERGs on 20 patients after receiving a single injection of bevacizumab (1.25 mg) for treatment of nAMD. ERGs were re-measured four months after the baseline measurements. Maturi et al. (2006) investigated the

effects of 1-2 intravitreal injections of bevacizumab (1.25 mg) at 1-3 month follow-ups of five patients being treated for nAMD. All three of the above studies found no clinically significant reduction of photopic ERG amplitude or delay in implicit time after treatment (Stahl et al., 2009; Zeimmsen et al., 2008; Maturi et al., 2006).

Three included studies used other electrophysiological tests to assess retinal function (Zeimmsen et al., 2008; Feigl et al., 2007; Almedia et al., 2015). Zeimmsen et al. (2008) performed electrooculograms (EOGs) on 20 patients undergoing a single injection of bevacizumab (1.25 mg) for the treatment of nAMD. Re-measurements were taken four months after the baseline measurements revealing no significant changes. Feigl et al. (2007), used peripheral multifocal ERG (mfERG) to assess the neuroretinal function of three patients undergoing treatment for nAMD in an observational case report. It should be noted that one of the participants had chronic open angle glaucoma but that this was well controlled with topical medication. There was found to be a statistically significant reduction of peripheral mfERG amplitudes after three ranibizumab (0.3 mg) treatments. Almeida et al. (2015) also used peripheral mfERG to assess retinal function in 21 participants with nAMD receiving three doses of ranibizumab, and being followed up at 30, 60 and 90 days after the initial injection. The peripheral ring of the mfERG responses (24-45 degrees eccentricity) showed a significant reduction in the N1 waveform amplitude and a delay in implicit time at all follow up times, compared to those of a control group of 21 age-matched controls. The P1 amplitude was not affected in comparison to the controls but P1 implicit time was significantly delayed. This suggests that both the photoreceptors and bipolar cells of the patients in the study may have been negatively affected by the ranibizumab administered to combat their nAMD.

In summary, the majority of studies involving scotopic and/photopic ERGs in human participants suggest that anti-VEGF therapies may have no significant effect on the peripheral retina. There was even some evidence of an improvement in function post anti-VEGF treatment (Skaat et al., 2011; Macky and Mahgoub 2012; Yasuda et al., 2015; Holm et al., 2015). However, assessments in many of the studies have relatively short follow up times or are performed after just one exposure to anti-VEGF therapy which is very unlike the clinical recommendations for use of anti-VEGF treatment. Furthermore, untreated control groups were not used in these studies, either

randomised or otherwise. Many studies used the untreated eye as a form of control (e.g. Macky and Mahgoub, 2012; Skaat et al., 2011), however the validity of this approach may be restricted by systemic effects of intravitreal anti-VEGF injections (Avery et al., 2006; Karaca et al., 2003; Hanhart et al., 2014; Acharya et al., 2013).

There were, however, some indications of a negative effect on retinal function of the treatment. This included a reduction in a- and/or b-wave amplitude (Comyn et al., 2014; Pedersen et al., 2010; Yagura et al., 2016), and increased a-wave time to peak (Pedersen et al., 2010; Yagura et al., 2016) of scotopic and/or photopic transient ERG responses, an overall reduction in peripheral mfERG amplitudes after anti-VEGF therapy (Feigl et al., 2007; Almeida et al., 2015) and abnormal flicker responses (Terauchi et al., 2019). It is particularly relevant to note that, whilst the majority of studies reporting no negative effect of the treatment only administered a single intravitreal injection (for example, Macky and Mahgoub, 2012), participants in the studies reported by Comyn et al. (2014) and Pedersen et al. (2010) received several injections and were followed up for longer (48 weeks and 6 months, respectively). This may indicate a potential retinotoxic effect which requires a longer follow up and/or repeated treatments in order to manifest.

This review highlights the need for further work ascertaining the extent of any detrimental effect of anti-VEGF therapy on visual function in humans – particularly in the context of multiple and/or long term exposure. There was insufficient evidence to determine whether any dysfunction preferentially affected photopic or scotopic pathways.

2.1.5.2. Outcomes investigated in peripheral retinal structure in human participants and/or clinical studies

Eight of 43 eligible human studies (19%) assessed aspects of retinal blood flow following anti-VEGF therapy and a number of studies have reported that anti-VEGF therapy is associated with a reduction in vessel diameter (Papadoupoulou et al., 2009; Fontaine et al., 2011; Benatti et al., 2017; Kurt et al., 2018; Corvi et al., 2015; Lanzl et al., 2011; Terai et al., 2014; Barack et al., 2012). Papadoupoulou et al. (2009) conducted a prospective, consecutive, interventional case series to see if ranibizumab (0.5 mg) had an effect on retinal arteriolar diameter in 11 eyes of 11 patients having this

treatment for nAMD. Patients were followed up for four months and a significant decrease in retinal arteriolar diameter was observed after each ranibizumab injection was given, with the effect increasing with each injection. This suggested that ranibizumab may stimulate vasoconstriction, with a cumulative effect associated with multiple retreatments. Fontaine et al., (2011) also described a significant decrease in arteriolar diameter, although not in arterial blood flow, in 23 patients who had received 1.25 mg intravitreal bevacizumab on three occasions at five week intervals. Patients were followed up one week after the first injection, five weeks after the first injection, and five weeks after the third and final injection, with the vasoconstrictive effect persisting to the longest follow up period. Benatti et al. (2017) used spectral domain OCT (SD-OCT) to look at change in arterial dilation one month after first intravitreal ranibizumab (0.5 mg) injection for 15 eyes of 15 patients commencing treatment for DMO. They reported a significant constriction of retinal arterial blood vessels. Kurt et al. (2018) also found significantly constricted blood vessels using SD-OCT in the treated eyes compared to the control eyes of 62 participants receiving either ranibizumab (0.5 mg) or bevacizumab (1.25 mg) therapy. Corvi et al. (2015) performed dynamic and static retinal vessel analysis on 11 eyes of 11 patients one week and one month after they had been treated with ranibizumab (0.5 mg). During dynamic analysis it was found that vein dilation had increased, however during static analysis it was found that there was a vasoconstrictive effect on both arteries and veins. As a consequence of vasoconstriction, it has been hypothesised that use of anti-VEGF treatment could ultimately lead to ischaemia and therefore stimulate more VEGF production (Benatti et al., 2017).

However, such findings have not been universal. Lanzl et al. (2011) assessed the effect of flickering light on retinal vessels of 10 patients with nAMD who received a single dose of 1.25 mg bevacizumab. Over the three-month follow up period there was no change to mean arterial dilation or retinal dynamic vascular parameters. Terai et al. (2014) studied 30 eyes injected with 0.05 mL ranibizumab at four week intervals to treat diabetic macular oedema. There was no significant effect on vessel diameter, assessed by dynamic and static vessel allowance stats analysis, for up to three months after initiating treatment. Furthermore, Barack et al. (2012) described an increase in retinal blood flow velocity following vasoconstriction at a one month follow up

appointment following a single bevacizumab (1.25 mg) treatment. This study of 12 eyes of 12 patients also found that the increase in blood flow was most dramatic immediately after receiving anti-VEGF therapy. This suggests that compensatory mechanisms may allow blood flow to be increased to offset any vasoconstrictive effects, a finding consistent with Fontaine et al., (2011) who also described normal arterial blood flow, despite reduced vessel diameter following anti-VEGF therapy. Further investigation is required to fully characterise the relationship between changes in vessel calibre and blood flow following anti-VEGF therapy, and the timecourse and permanence of any effect, especially over the longer term.

Optical Coherence Tomography (OCT) is used in many hospital clinics to assess the retinal structure of patients receiving anti-VEGF treatment for nAMD or central macular oedema (as a condition in itself or secondary to another condition such as diabetes or retinal vein occlusion; see section 1.6). As a result, many of the studies describing examining anti-VEGF treatment include macular scan analysis. Only those data which did not directly relate to the macula were included in this review. Twenty of the eligible studies (47% of human participant studies) used OCT to assess retinal structure during anti-VEGF treatments. All of these studies involved human volunteers.

A number of studies used OCT to assess the impact of anti-VEGF therapy on peripapillary RNFL thickness (Enders et al., 2017; Kopic et al., 2017; Valverde-Megias et al., 2019; Zucchiatti et al., 2017; Shin et al., 2014; Horseley et al., 2010; Yun et al., 2016; Jo., 2016; Sobaci et al., 2013; Demirel et al., 2015; Entezari et al., 2014; El-Ashry et al., 2015; Yau., 2015; Parlak et al., 2014). Most of these studies failed to find a significant reduction in RNFL associated with the treatment. For example, Shin et al. (2014) assessed retinal nerve fibre layer thickness using OCT in 148 eyes of 135 patients who received either bevacizumab (1.25 mg), ranibizumab (0.5 mg) or a combination of these. Participants were followed up for a minimum of 12 months and injections were repeated as required (number not specified). They reported that multiple intravitreal injections of anti-VEGF did not lead to significant change in RNFL thickness in wet AMD, diabetic macular oedema, or retinal vein occlusion patients over the follow up period, compared to the untreated control group. Number of injections and IOP fluctuations were not correlated with change in RNFL thickness over time. Horsely et al. (2010) assessed the mean peripapillary RNFL thickness of 41 eyes of 37

patients receiving treatment for nAMD with pegaptanib, bevacizumab, and/or ranibizumab. After at least 12 months of follow up there was found to be no significant change in RNFL thickness. Jo et al. (2016) recorded average and four-quadrant RNFL thicknesses in an Optic Disc Cube scan (6mm x 6mm, Cirrus HD-OCT) in 20 eyes of 20 patients with AMD at baseline, six and 12 months after three monthly intravitreal injections of ranibizumab (concentration not given). There was found to be no significant change in average RNFL thickness. The areas that did reduce in thickness over follow up were those corresponding to the pathologic areas or those adjacent to these. This was suggestive of RNFL thinning attributable to the disease process rather than the treatment itself. Sobaci et al. (2013) assessed 35 eyes of 35 patients being treated for nAMD with bevacizumab (1.25 mg/ml) and 30 eyes of 30 patients being treated for nAMD with ranibizumab (0.5 mg/0.05 ml). They found no significant difference in the change in peripapillary RNFL between treated and untreated eyes over a follow up period of at least one year. Similarly, Demirel et al. (2015) found no significant difference in peripapillary RNFL mean total or nasal thickness of 29 eyes receiving intravitreal ranibizumab (unspecified quantity) compared to fellow untreated eyes, or to the 27 control eyes of healthy normal patients. Likewise, Entezari et al. (2014) found that there was no significant difference in mean thickness of the peripapillary RNFL layer pre- to post-treatment in 18 eyes of 18 patients, followed up at 12 and 24 weeks after two injections of bevacizumab (1.25 mg). El-Ashry et al. (2015) performed a before and after study of peripapillary RNFL thickness in 30 eyes of 30 patients receiving ranibizumab for nAMD. The RNFL thickness was not significantly different at one month after the third injection compared to pre-injection in any of the quadrants measured. Zucchiatti et al. (2017) found no change in RGC complex thickness or peripapillary RNFL thickness over 12 months of follow up (24 eyes), after three monthly loading doses of ranibizumab then treatment 'as required'. Yau et al. (2015) compared peripapillary RNFL thickness (mean total and quadrant) in 29 eyes of patients receiving treatment for unilateral nAMD to their fellow eyes with a diagnosis of non-exudative AMD (control group). The mean total RNFL thickness in the treated eyes was significantly greater than the non-treated eyes. The treated group had thicker RNFL temporally than the non-exudative group but all other locations were similar and no significant differences were found between any of the optic nerve head parameters of the two groups.

In contrast, other studies have reported effects on the peripapillary RNFL thickness post anti-VEGF treatment (Parlak et al., 2014; Enders et al., 2017; Valverde-Megias et al., 2019; Yang et al., 2017). Parlak et al. (2014) followed 22 patients receiving ranibizumab therapy for nAMD (three monthly injections followed by a treat and extend protocol; minimum follow up 12 months). Here, significant peripapillary RNFL thinning was found after the third month compared to baseline, but there was no statistically significant difference between the RNFL thickness of the study and control eyes. There was also no difference in RNFL thickness between eyes treated with more than and fewer than five intravitreal injections. Valverde-Megias et al. (2019) also witnessed a loss in RNFL thickness over the 96-month follow up of their 20 participants being treated with ranibizumab for nAMD (mean number of injections was 21). However, these effects were also seen in the contralateral control eyes as well as the treated eyes. The finding of a similar effect in treated and untreated eyes in both of these studies suggests either a systemic interocular effect of ranibizumab or some other confounding factor. For example, thinning changes are possibly attributable to ageing in the 96-month follow up (Leung et al., 2012).

Enders et al. (2017) measured peripapillary RNFL thickness using spectral domain OCT in 76 participants treated for nAMD with anti-VEGF treatment. Each participant had a minimum number of nine injections and two groups were examined, those who received anterior chamber paracentesis (ACP) and those who did not. Loss of RNFL was present in both groups, however it was significantly more marked in the group that did not have ACP to lower their IOP after anti-VEGF treatment. This suggests that anti-VEGF treatments could be causing some damage to the RNFL, worsened by the rise in IOP following each anti-VEGF injection.

Yang et al. (2017) reported outcomes from 50 participants with diabetic macular oedema, receiving intravitreal anti-VEGF therapy, and 90 controls with diabetic retinopathy only. The treated group showed a significant reduction in peripapillary RNFL thickness and retinal thickness between baseline and six months, whilst those in the control group remained stable. However, this was interestingly no longer significant when the study's index to compensate for macula oedema was applied. The authors surmise that the strong relationship between peripapillary RNFL thickness and retinal thickness may mean that measured changes in peripapillary RNFL thickness may

not represent any real loss or gain of RNFL. This suggests that caution should be applied when interpreting the results of other similar studies where a reduction in macular oedema may influence peripapillary thickness measurements.

In a multi-modal imaging study, Filek et al. (2019) reported a significant increase in cup volume and vertical cup to disc ratio measurements in 30 patients with diabetic macular oedema receiving anti-VEGF injections compared to 21 controls. In addition, the study also found a (non-significant) trend towards a deterioration in the peripheral visual field over time, consistent with the evidence of RGC loss.

Whilst the majority of OCT studies have evaluated RNFL thickness (or in two cases, retinal blood flow and/or vessels [Benatti et al., 2017; Kurt et al., 2018]), four examined other imaging parameters after anti-VEGF treatment (Yun et al., 2016; Kim et al., 2016; Sitniska et al., 2019; Filek et al., 2019). For example, Yun et al. (2016) assessed peripapillary choroidal thickness in 39 eyes of nAMD patients receiving 0.5 mg intravitreal ranibizumab for three months (then retreated as required). This study found that choroidal thickness was reduced in the temporal aspect of the disc (towards the macula) at three months post baseline and changes were maintained at six months. The superior, nasal and inferior choroidal peripapillary thickness did not show any significant changes after treatment. Thus, changes in the peripapillary choroidal thickness were limited to the macular area, and may reflect the effect of the drug on the AMD disease process.

Kim et al. (2016) looked at RPE atrophy via fundus imaging in 65 eyes of 62 participants. Their results showed an overall trend in progression of RPE atrophy in eyes with nAMD during anti-VEGF treatment with increasing severity correlated with an increase in number of anti-VEGF injections. This suggests that RPE atrophy could be exacerbated by anti-VEGF treatment. However, Sitniska et al. (2019) found that the extent of RPE atrophy was associated with duration of nAMD, not with that of anti-VEGF treatment, in 52 nAMD patients with no baseline RPE atrophy evaluated over three or more years using multimodal imaging.

There is very mixed evidence regarding the effect of anti-VEGF treatment on parameters of OCT imaging. Whilst there is a substantial body of evidence demonstrating a lack of effect on the peripapillary RNFL, other studies have suggested

a thinning (Enders et al., 2017; Entezari et al., 2014; Valverde-Megias et al., 2019; Yang et al., 2017; Yau et al., 2015; Parlak et al., 2014). However, this finding could be attributable to ageing (in those studies where a similar effect was seen in control eyes (i.e. Parlak et al., 2014; Valverde-Megias et al., 2019), the effect of the raised IOP associated with the treatment (Enders et al., 2017), resolution of macular oedema (Yang et al., 2017) or a toxic effect of the anti-VEGF therapy itself. There is also some evidence to suggest that RPE atrophy may progress as a function of anti-VEGF therapy (Kim et al., 2016), although this has been disputed (Sitniska et al., 2019). Further investigation is clearly required, employing an untreated control group and correcting for any oedema resolution.

2.2. Discussion

2.2.1. Trends Discovered

Evidence from the examined studies show very mixed results. Of studies that assessed peripheral visual function in animals after exposure to anti-VEGF, the majority reported no significant effect post treatment (Guo et al., 2010; De Oliveira Dias et al., 2014; Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Inan et al., 2007; Wu et al., 2010; Januschowski et al., 2014; Manzano et al., 2006; Heiduschka et al., 2008; Ramon et al., 2018; Zayit-Soudry et al., 2010; Zayit Soudry et al., 2011; Shahar et al., 2006). Only two studies reported a significant abnormality in scotopic ERG parameters up to one week (Sakurai et al., 2009) and 8 weeks (Myers et al., 2012) after exposure to a single high dose of anti-VEGF. The majority of immunohistochemical and histological studies also reported no detrimental effect of anti-VEGF treatment (Arraes et al., 2009; Bakri et al., 2006; Thaler et al., 2010; Kaempfer et al., 2008; Kim et al., 2008; Karawya, et al., 2008; Lu et al., 2014; De Oliveira Dias et al., 2014; Manzano et al., 2006; Sari et al., 2006; Cam et al., 2017; Januschowski et al., 2014; Landiev et al., 2011). However, some studies did find evidence of changes in retinal structure following treatment with anti-VEGF drugs, such as evidence of increased apoptotic activity in photoreceptors (Avci et al., 2009), severe loss of RGCs and damage of inner and outer nuclear layers (Romano et al., 2012), bipolar cell loss (Myers et al., 2012), retinal astrocyte damage (Zayit-Soudry et al., 2010), increased apoptosis and reduced viability of RPE cells (Sahiner et al., 2018), and damage to the choriocapillaris (Julien et al., 2014). This evidence of

retinal damage by anti-VEGF should be considered with caution. Various different animals were used in the studies and few of these were very similar in structure to the human retinae, weakening this evidence. The majority of studies also failed to evaluate a protocol comparable to the treatment strategy used in humans.

Evidence of human participant studies relating to visual function was also mixed. Largely, the evidence from these studies suggested there may be no effect of anti-VEGF on peripheral retinal function (Staal et al., 2009; Maturi et al., 2006; Stahl et al., 2009; Zeimmsen et al., 2008; Marmor et al., 2009; Moon et al., 2013; Chabblani et al., 2015; Holm et al., 2015). There was actually an improvement in peripheral retinal function reported post treatment by Skaat et al. (2011) and Macky and Mahgoub (2012). However, detrimental effects of anti-VEGF drugs on the scotopic and mixed rod and cone ERG a- and b- waves amplitudes parameters have been reported (Pedersen et al., 2010; Comyn et al., 2014), and on the subcomponents of the photopic flash ERG (Yagura et al., 2016; Pedersen et al., 2010), flicker ERG (Terauchi et al., 2019), and mfERG (Feigl, 2007; Almeida et al., 2015). There was no evidence of preferential loss of rod or cone function.

Regarding retinal structure, similar mixed evidence related to retinal blood vessel diameter dilation and constriction was found. A reduction in retinal arterial diameter immediately after anti-VEGF treatment was a common finding (Papadoupoulou et al., 2009; Fontaine et al., 2011; Benatti et al., 2017; Corvi et al., 2015; Kurt et al., 2018), with the effect reported to persist for follow up periods of up to 5 weeks (Fontaine et al., 2011). However, other studies reported no change in blood vessel parameters (Lanzl et al., 2011; Terai et al., 2014), or suggested that blood flow remained consistent (Fontaine et al., 2011), or even increased post treatment (Barack et al., 2012). It was suggested that compensatory mechanisms may be in place resulting in normal blood flow despite vasoconstriction (Barack et al., 2012). A similarly inconsistent picture was derived from studies evaluating OCT in patients undergoing anti-VEGF therapy. The majority of studies reported no significant effect of anti-VEGF therapy on peripapillary RNFL thickness (Sobaci et al., 2013; Shin et al., 2014; Demirel et al., 2015; Entezari et al., 2014; El-Ashry et al., 2015; Yau et al., 2015; Jo et al., 2016; Zucchiatti et al., 2017). In contrast, four studies provided evidence suggestive that anti-VEGF exposure resulted in thinning of the RNFL (Parlak et al., 2014; Enders et al., 2017; Valverde-

Megias et al., 2019; Yang et al., 2017). However, a similar effect was reported in both treated and fellow (control) eyes of two studies (Parlak et al., 2014; Valverde-Megias et al., 2019), suggesting that ageing or contralateral effect of the treatment may have been a factor, whilst another study found the effect to disappear when a correction factor was applied for resolution of macular oedema (Yang et al., 2017). Other OCT studies implicated anti VEGF therapy in the progression of RPE atrophy (Kim et al., 2016) and in changes to optic nerve head parameters (Filek et al., 2019).

2.2.2. Limitations of included studies

A key limitation of the included studies was the limited duration of the follow up period and/or to the number of doses of the drugs administered. In humans, multiple intravitreal injections are required to treat conditions such as nAMD and diabetic eye disease (NICE Guidelines TA155; NICE Guidelines TA294; NICE Guidance TA274, 2013; TA436, 2015). Unless studies are designed to follow participants over the timecourse of a standard treatment protocol it is not possible to comment with confidence on any long-term effects, or on the effects of repeated treatment. In particular, many of the animal studies evaluated changes in retinal function and structure for only a brief period after the administration of a single dose of the drug in question (Arres et al., 2009; Bakri et al., 2006; Cheng et al., 2009; de Oliveira Dias et al., 2014; Guo et al., 2010; Inan et al., 2007; Iriyama et al., 2007; Julien et al., 2014; Kim et al., 2008; Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Myers et al., 2012; Ramon et al., 2018; Sakurai et al., 2009; Shahar et al., 2006). Seventy-seven percent of studies followed up participants for a maximum of nine months. Duration of follow up period also varied from patient to patient within several studies (Demirel et al., 2015; Horsely et al., 2010; Parlak et al., 2014; Shin et al., 2014;) which made it more difficult to make comparisons between participants.

It is also worth noting that in many of the studies no control group was used for comparison (de Oliveira Dias et al., 2014; Inan et al., 2007; Januschowski et al., 2014; Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Manzano et al., 2006; Shahar et al., 2006; Zayit-Soudry et al., 2010; Zayit-Soudry et al., 2011), or the contralateral eye of the subject was used for this purpose (Shin et al., 2014; Kurt et al., 2018; Valverde-Megias et al., 2019; Macky and Mahgoub, 2012; Skaat et al., 2011; Guo et al., 2010; Sakurai et al., 2009). The issue with the use of the contralateral eye is that there is

some evidence of a systemic circulation of anti-VEGF drugs administered through intravitreal injection, which may cause an interocular transfer of treatment effect (Avery et al., 2006; Karaca et al., 2003; Hanhart et al., 2014; Acharya et al., 2013). However, in human studies the use of a meaningful control group is challenging as it is unethical to randomly allocate individuals with a condition such as nAMD to a no treatment group when there is an accepted best practice treatment available. Furthermore, any intravitreal injection carries a risk of complications such as infection or central retinal artery non-perfusion (Jager et al., 2004), hence the use of a sham injection in human eyes is also non-ethical. The use of a fellow eye non-treated control in these circumstances is not ideal, but possibly an appropriate compromise solution. In contrast, in animal studies, it is possible to use a sham injection in control eyes (either of saline or the drug vehicle). This provides a useful means of discriminating between the effect of the drug itself and of the process of administration, as evidenced by Heiduschka et al. (2008). It is notable that only 11 (35%) of the animal studies used a control group of this type (three using the fellow eyes of treated animals, with the limitations described above).

Sample sizes were also limited in the included studies. The median sample size was 23 (range 1-148) in the human studies included. Given the number of potentially confounding factors introduced by the presence of disease, treatment effects on the disease itself, ageing effects, co-morbidities and the difficulty of employing a meaningful control group, well designed studies following large groups of individuals for a prolonged period of time after initiating treatment are required to allow for subtle treatment effects to become apparent above the noise.

In the vast majority of the histological studies the authors were nonspecific about where in the retina the sample cells were extracted from for assessment which gives the possibility that there may be some non-peripheral cells under scrutiny (e.g. Schnichels 2013; Spitzer et al., 2007; Julien et al., 2014; Iriyama et al., 2007). Furthermore, four of the animal studies performed did not perform baseline measurements before commencing treatments (Bakri et al., 2006; Kim et al., 2008; Cheng et al., 2009; Lu et al., 2014). This is an issue as it is then unknown if the subjects had any existing retinal abnormalities (e.g. due to age, injury or genetics) and so a comparison cannot be made to the post treatment data. Several of these also did not

state the age of the subjects when the experiment began (Bakri et al., 2006; Kim et al., 2008). Another potential confounding factor in animal studies was the variable degree of anti-VEGF dosage used. Some studies took care to match the anti-VEGF exposure to expected human dosage, but others varied considerably which may have limited the applicability to human outcomes. For example, high dose injections were used by (Avci et al., 2009; Manzano et al., 2006; Sakurai et al., 2009).

Some studies were unclear which standardised methods of ERG recording they have used when full field flash ERGs were delivered (for example, Chhablani et al., 2016, and de Oliveira Dias et al., 2015). Therefore, it was not always possible to tell which aspects of retinal function were assessed, limiting the extent of possible analysis.

Finally, for ethical reasons there was a clear lack of use of randomised controlled trials with human participants (only one, Comyn et al., 2014, is characterised as such, and in this study the comparison group was a macular laser treatment rather than a no treatment control). Though necessary, this does leave a gap in the evidence.

The considerable variability in the findings of the studies may be linked to the factors above, and to the wide range of dosage and follow up protocols between studies. There is a dearth of studies investigating the effect of cumulative dosage and long term exposure to anti-VEGF drugs.

2.3. Conclusions and Implications for Research and Practice

It is possible to see from this collection of studies that there is a lack of longitudinal data assessing the effect of anti-VEGF agents on the peripheral retina. Whilst the studies with short term follow ups generally seem to indicate a lack of retinal toxicity with this therapy, there are a few studies with longer follow ups (mostly in clinical environments e.g. Pedersen et al., 2010, Comyn et al., 2014) which do suggest that long term use of anti-VEGF therapy may cause retinotoxicity. These longitudinal data are required to evaluate the effects of repeated treatments over long periods of time, comparable to the treatment protocol for conditions managed with anti-VEGF therapy. In addition to longer follow up durations there is insufficient literature looking at multiple anti-VEGF injections and the cumulative effect of this on peripheral retinal function.

Chapter 3. Development and Repeatability Assessment of the Electroretinogram Protocol

3.1. Introduction

A number of the studies reviewed in Chapter 2 used electroretinograms (ERGs) to assess the effect of anti-VEGF therapy on the retina in animals or humans (Ramon et al., 2018; Terauchi et al., 2019; Yagura, et al., 2016; Guo et al., 2010; Lueke et al., 2006; Huang et al., 2012; Luke et al., 2006; Maturi et al., 2006; Macky and Mahgoub, 2012; Stahl et al., 2009; Heiduschka et al., 2008; Saint-Geniez et al., 2008; Moschos et al., 2007; Kurihara et al., 2012; Pedersen et al., 2010; Myers et al., 2012). Those studies that have assessed the retinal function of humans after anti-VEGF injections have reported mixed results. Most report no depletion in visual function assessed by ERGs, however, a reduction in scotopic a- and/or b-wave amplitude (Comyn et al., 2014; Pedersen et al., 2010), and increased a-wave time to peak (Pedersen et al., 2010) have been reported post treatment. Of the human studies that assessed retinal function with photopic ERGs, the bulk found that anti-VEGF therapies have no significant clinical effect upon the photopic ERG, or actually result in improved function (e.g. Maturi et al., 2006; Stahl et al., 2009; Skaat et al., 2011; Comyn et al., 2014; Moon et al., 2013; Ramon et al., 2018). Pedersen et al. (2010) observed an initial reduction in photopic b-wave amplitude, and increase in implicit time followed by a variable recovery over time (up to 6 months follow up). However, they failed to include a control group. Yagura et al. (2016) reported a significant change in full-field photopic ERG parameters immediately after treatment, but these were attributed to the effect of the intravitreal injection itself. Significant changes were also reported by studies evaluating the flicker ERG, followed up to 24 hours post injection (Terauchi et al., 2019), and the outer rings of the multifocal ERG (Feigl et al., 2007, Almeida et al., 2015). It is notable that, whilst the majority of studies reporting no significant effect of the treatment only administered a single intravitreal injection (for example, Macky and Mahgoub, 2012), participants in the studies finding a significant effect received several injections and were followed up for longer (48 weeks and 6 months, by Comyn et al. (2014) and Pedersen et al. (2010) respectively).

Thus, whilst there is some evidence that retinal function, assessed using the ERG, may be affected by long term anti-VEGF therapy, the evidence is equivocal and there is a need for more extensive human data to be collected, over prolonged follow up

periods, and after multiple injections. The aim of this PhD was to extend the knowledge base by studying a larger cohort of individuals who had received a range of doses of anti-VEGF drugs over a range of timeframes. To facilitate this, the strategy was to recruit participants attending the AMD clinics at St Thomas' Hospital on the day of their medical appointment, thereby avoiding the inconvenience of bringing individuals back on a separate occasion to participate in this study. Within the context of a busy hospital eye unit, this could only be achieved if the ERG protocol could be carried out within a timeframe which did not disrupt the flow of the clinic (a maximum of 20 minutes, and with a portable/semi-portable device). A balance had to be struck between thorough evaluation of retinal function and the time needed to complete the tests at each visit.

If it had been possible within the daily activities of the medical retina clinic, both photopic and scotopic ERG assessment would have been performed to provide a full picture of retinal function before, during and after anti-VEGF therapy. The inclusion of scotopic ERGs, recorded under dark adapted conditions, would be additionally beneficial because the peripheral retina of interest in this study is dominated by rod photoreceptors (Curcio et al., 1990; Curcio and Hendrickson, 1991; Osterberg, 1935), and because current human and animal ERG evidence does suggest that scotopic functional abnormalities may exist post exposure to anti-VEGF drugs (Sakurai et al., 2009; Pedersen et al., 2010; Myers et al., 2012; Comyn et al., 2014). However, Chapter 2 did not provide evidence of a selective loss of rod function in humans exposed to anti-VEGF therapy in the literature to date. Although it is also the case that rod dysfunction and loss is likely to precede cone dysfunction in AMD (Curcio et al., 1996; Owsley et al., 2000), this is not relevant to the choice of electrophysiological tests in this case as this study aims to evaluate the effect of the treatment on the relatively healthy areas of the retina, rather than on the AMD disease process itself. In order to achieve a complete evaluation of retinal function, a full ISCEV ERG test battery would ideally be performed, including the dark adapted ERGs (McCulloch et al., 2015). All of these scotopic ERGs require the participant to undergo a minimum of 20 minutes dark adaptation after exposure to light, which would be challenging from a practical viewpoint within the clinic schedule. Additionally, patients are typically exposed to high intensity white light during fundus imaging and subsequent funduscopy

examination by the ophthalmology team at the hospital prior to anti-VEGF injections, which may render the 20 minutes of recommended adaptation insufficient to achieve absolute threshold. This is particularly the case in people with AMD who are known to experience delayed rates of dark adaptation compared to healthy age-matched controls (e.g. Owsley et al., 2001; Owsley et al., 2007; Dimitrov et al., 2008). Thus, the task of achieving a steady state of dark adaptation alone would occupy the maximum time permitted per patient for data collection. The issuing of red dark-adaptation goggles to patients on arrival at the clinic was also an impractical option due to the frequency with which this would be interrupted for ophthalmic assessment. This would make monitoring for successful dark adaptation extremely difficult and results unreliable. Therefore, it was not feasible to perform scotopic testing for this PhD, and a photopic protocol was developed.

This chapter outlines i) the development of a photopic ERG protocol to assess cone photoreceptor function in people undergoing anti-VEGF therapy, and ii) an evaluation of the repeatability of this pilot protocol.

3.2. General Methods

3.2.1. Mydriatic Instillation

Before undertaking any ERG procedures, the pupils of all participants were dilated as recommended by the ISCEV Standards for Full Field Electroretinography (McCulloch et al., 2015). Maximal dilation was ensured by observing an absence of a pupillary reaction when tested with a pen torch to ensure a constant pupillary aperture, and maintain a constant retinal illuminance throughout the recordings. Maximal dilation also prevented contraction and dilation of the pupil in response to the flashing stimuli which might have increased biological noise levels on the recordings.

Before any mydriatic was instilled, all participants were assessed for the likelihood of development of angle closure glaucoma (ACG) as recommended by the College of Optometrists (2015). This involved measuring the intraocular pressure (IOP) and estimating anterior chamber depth of both eyes of the participant. IOP was measured using non-contact tonometry and anterior chamber depth was assessed using van Herick's technique (van Herick et al., 1969). Instillation of one drop of 0.5% tropicamide hydrochloride (Bausch & Lomb, Surrey, UK) into both eyes only proceeded

if the anterior chamber angle was open (van Herick grade 2-4) and IOPs were below 21mmHg (College of Optometrists, 2015). After instillation, a period of 20 minutes was allowed for the pupils to fully dilate. If full dilation had not been achieved in either eye an additional drop of 0.5% tropicamide hydrochloride was instilled into the eye in question. If the participant had dark irises 1% tropicamide hydrochloride was instilled in place of 0.5%.

3.2.2. Placement of Electrodes in ERG Recordings

Prior to electrode application, the area of skin was gently cleaned with Nuprep (D.O. Weaver & Co., Aurora, USA). This exfoliating gel removed debris such as dead skin cells, make-up and oils that may increase the resistance of the electrode-skin interface. Following this, skin electrodes were positioned according to the ISCEV guidelines (McCulloch et al., 2015). The earth electrode was placed in the mid-frontal position, roughly 5cm above the nasion. The reference electrodes were positioned on the notches at the temporal sides of the participant's supraorbital ridge, adjacent to the outer canthi (Figure 3.1). Each silver-silver chloride electrode (Viasys Healthcare Inc, Pennsylvania, USA) was attached using a 30mm length of Blenderm surgical tape (3M Health Care, St Paul, USA) with a small hole punched in the centre. Prior to application, the cup of each electrode was filled with TECA electrode conductivity gel (Viasys Healthcare Inc, Pennsylvania, USA) using a syringe. The electrode was then attached to the participant's skin using Blenderm Tape. The hole punched in the centre of the Blenderm tape allowed excess gel to escape during application and also permitted replenishment of the gel without removal of the electrode from the participant's skin if necessary.

Dawson-Trick-Litzkow (DTL) active electrodes were prepared (Unimed Electrode Supplies, Farnham, UK). Two 70 mm lengths of DTL fibre were cut and each wound around a metal hook enclosed in a retractable non-conductive case. The retracted casing was then allowed to relax, covering the end of the fibre and holding it in place. These DTL holders were attached to the patient's skin, adjacent to the reference electrodes temporal to each eye. The participant was then asked to look upwards, and the lower lid was gently pulled downwards as the DTL fibre was draped along the lower fornix of the eye.

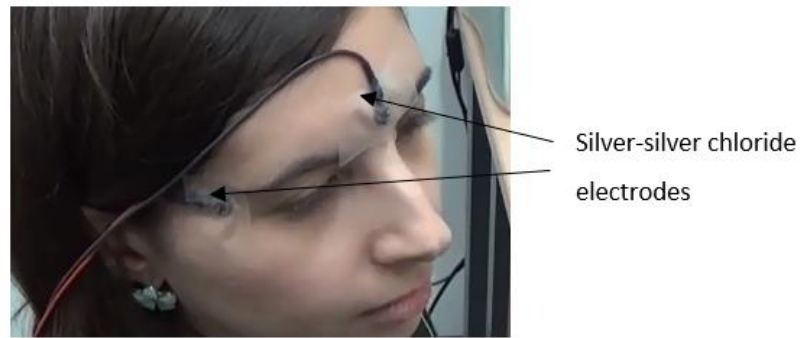


Figure 3.1. A participant being prepared to undergo ERG recordings.

All of the electrodes were attached to the amplifier and their resistance checked, to ensure that the impedance was $\leq 5 \text{ k}\Omega$ and approximately equal across all electrodes to reduce trace pollution by electrical artefacts. Where impedance of any electrode was $> 5 \text{ k}\Omega$ the electrode was detached from the patient, connections of electrical wires inspected, any areas of skin in contact with the electrode were recleaned with Nuprep gel, and fresh conductivity gel applied to the electrode before reapplication of the electrode to the patient in the relevant area with new Blenderm tape. In cases where impedance was $> 5 \text{ k}\Omega$ for DTL fibres, the current DTL fibre was disposed of and new fibre attached before replacement of the electrode with new Blenderm tape.

3.2.3. Equipment



Figure 3.2. Ganzfeld ColorDome stimulator and amplifier.

An Espion™ Ganzfeld Profiling System and ColorDome (Diagnosys, Cambridge) stimulator were used to record all ERGs (Figure 3.2). The ColorDome provides uniform stimulation of the visual field using LED or xenon flash illumination of a Ganzfeld bowl.

The system was calibrated by the manufacturer, and self-calibrated each day on initiating the device (Diagnosys LLC, 2020). Additional manual calibration checks were performed using the ILT 1700 Research Radiometer with SED033/Y/R luminance detector (model SED033, Able Instruments and Controls, Reading, UK).

Numerous parameters must be programmed into the computer system before recording takes place, these include; high and low pass frequency filters (Hz), number of responses to be averaged, the duration of time over which each response is recorded (ms), amplification of the recording system, and sensitivity of the artefact reject setting. Each of these parameters was varied between protocols according to the nature of the signal recorded. The bandpass filters were set from 0.3 to 500 Hz for all ERG recordings.

3.3. Development of Electroretinogram Protocol

3.3.1. Introduction

Since 1989, ERG protocols have been standardised by ISCEV so that ERGs can be performed all over the world and still be both reproducible and comparable (McCulloch et al., 2015, Marmor et al., 1989). Whilst this is very useful in the hospital and clinical environments, it is less useful in the research environment where a more flexible tailored approach may be beneficial. As such, ISCEV recommend that practitioners extend the test protocol where required to maximise the value of the ERG for clinical trials or further diagnosis. The pilot protocol therefore included the following mixture of standard and tailored ERGs;

- 1) A transient cone response (International Society for Clinical Electrophysiology of Vision Standards: (ISCEV) light adapted 3 ERG). This was included as it is a widely used clinical measure of retinal cone system function and will be familiar to clinicians (McCulloch et al., 2015).
- 2) Full field flicker ERG recorded at baseline and over time after bleaching 40%, 50% and 60% of cone photopigment with a red light of 500 cd.s.m⁻², 720 cd.s.m⁻², and 1000 cd.s.m⁻² intensity respectively, in order to assess recovery of cone function after a period of intense light adaptation. This is a measure of dynamic outer retinal function, and requires photoreceptor, RPE and choroidal integrity. This has been referred to in previous studies as the ERG photostress test or dynamic

cone ERG (Binns et al., 2005; Binns et al., 2007; Wood et al., 2011b), here we refer to the 'ERG photostress test'.

- 3) A photopic negative response (PhNR; brief red stimulus on rod saturating blue background at 6 increasing levels of luminance, ranging from 0.11–3.35 cd.s.m⁻²) to allow assessment of RGC function (Viswanathan et al., 1999; Viswanathan et al., 2001; Binns et al., 2011).

3.3.2. Aim

The aim of this section of the study was to evaluate the proposed protocol in order to determine its acceptability to participants and the optimal parameters (e.g. PhNR luminance levels, bleach intensity).

3.3.3. Methods

Participants

The study took place at the Division of Optometry and Visual Science at City, University of London. Local ethical approval was gained through the Optometry Proportionate Review panel 'Opt/PR/23' and written informed consent was obtained from all subjects, conforming to the tenets of the Declaration of Helsinki. Study data were collected from healthy volunteers aged 24 – 38 years between February 2015 and June 2015. To meet the inclusion criteria for the study, participants were required to be aged between 18 and 89 years of age and have a best corrected visual acuity of 6/12 or better in the test eye. They were also required to have only normal ageing changes in both eyes and no other pathology. Additionally, they were required to have sufficiently clear ocular media, adequate pupillary dilation and fixation in order to allow for good quality fundus imaging. Volunteers were excluded from the study if they possessed any of the following; history of photosensitive epilepsy, advanced AMD in either eye, any previous treatment for AMD, diabetic macular oedema, retinal vein occlusion and proliferative diabetic retinopathy, any concurrent ocular condition that could contribute to visual impairment, significant systemic disease known to affect visual function (such as Parkinson's disease, Alzheimer's disease or diabetes), any history of medication known to affect visual function (for example, tamoxifen or chloroquine), anterior chamber angles less than Van Herick grade 2.

Participant Pathway

After providing informed consent, participants underwent a structured history and symptom review and examination of the anterior and posterior eye using slit lamp biomicroscope and Volk lens, and IOP measurement using a non-contact tonometer (Pulsair IntelliPuff Non-Contact Tonometer [Keeler, UK]) to determine eligibility for inclusion. Best corrected visual acuity was assessed in each eye using an Early Treatment Diabetic Retinopathy Study (ETDRS) chart. In those volunteers who met the eligibility criteria, a topical mydriatic was instilled to each eye according to the protocol described in Section 3.2.1 and, thereafter, electrodes were applied according to Section 3.2.2. After maximal pupillary dilation had been achieved, the ERG recording protocol commenced using the equipment described in Section 3.2.3. The test order was as follows: 1) PhNR intensity-response series, 2) ISCEV photopic 3.0 ERG, 3) flicker ERG and photostress test. This photostress test was performed last as this was the most challenging test with respect to light exposure and participant concentration. Data were collected from both eyes simultaneously. At the end of the recording period, fundus photographs (using a TRC-NW6S Non-Mydriatic Retinal Camera [Topcon medical systems Inc., Tokyo]) and 30 x 35 Macular OCT images (using a Spectralis OCT [Heidelberg Engineering, Heidelberg, Germany]) were obtained.

Recording parameters

1) PhNR intensity-response series

The Photopic Negative Response ERG was recorded in response to a red LED flash (peak wavelength 635 nm) of 0.11 cd.s.m^{-2} presented for 4 ms (stimulus frequency 2 Hz) over a blue LED background (peak wavelength 465 nm) of $158.8 \text{ scotopic cd.m}^{-2}$, which was sufficient to saturate the rod photoreceptors (Aguilar and Stiles, 1954; i.e. $3.9 \log \text{ scot Td}$, assuming 8 mm pupil). Four sets of a series of 10 flashes were delivered, giving 40 responses in total. The artefact rejection level was set at $300 \mu\text{V}$. The timebase was 250 ms. This was then repeated with the red flash intensity at 0.21 cd.s.m^{-2} , 0.42 cd.s.m^{-2} , 0.84 cd.s.m^{-2} , 1.68 cd.s.m^{-2} and 3.35 cd.s.m^{-2} .

2) ISCEV photopic 3.0 ERG

The ISCEV photopic 3.0 ERG was recorded in response to a white LED flash of 3 cd.s.m^{-2} presented for 4 ms (stimulus frequency 2 Hz) over a white (6500 K) background of 30 (photopic) cd.m^{-2} . Four sets of a series of 15 flashes were delivered, giving 60 responses in total. The artefact rejection level was set at 300 μV . The timebase was 250 ms. If any of the 4 traces appeared to contain an artefact, additional traces were recorded to obtain, where possible, 4 superimposable traces.

3) Flicker ERG and photostress test.

The ERG photostress test involved the presentation of a cone photopigment bleach, generated by red (peak wavelength 635 nm) LED full field illumination of the Espion™ /ColorDome Ganzfeld bowl. The participant sat with their chin on the chinrest of the ColorDome System, looking at the fixation target within the Ganzfeld bowl for a period of 120 seconds in order to achieve this. Three intensities of bleaching illumination were used (500 cd.m^{-2} , 720 cd.m^{-2} and 1000 cd.m^{-2}). Assuming a pupil diameter of 8mm, this resulted in a retinal illumination of 4000 Td, 5760 Td and 8000 Td respectively (making no allowance for the Stiles Crawford Effect), providing a cone photopigment bleach of approximately 40, 50 and 60 % (see Equation 3-1; rearranged from equation 2a of Hollins and Alpern, 1973, by Paupoo et al. 2000, using parameters for cone regeneration described by Hollins and Alpern 1973). Mahroo and Lamb (2004) showed that, contrary to previous assumptions, cone photopigment regeneration does not follow first order kinetics, but appears to be “rate-limited”, with regeneration proceeding at an initial linear rate following a wide range of bleaching exposures. Calculating bleach levels is less straightforward, but pigment bleach after the exposures of the current study can be estimated from their Figure 9 (Mahroo and Lamb, 2004). The estimated bleach levels following 2 min exposure to 500 cd.m^{-2} , 720 cd.m^{-2} and 1000 cd.m^{-2} illumination, would be, using their formulation, 40, 53 and 67% respectively. Fifty ERGs were recorded on a timebase of 100 ms and averaged in response to a square wave flickering stimulus (red, peak wavelength 635 nm, presented for 12 ms at 41 Hz temporal frequency, mean luminance 30 cd/m^2 , present only while recording). One baseline trace

was obtained before the bleach, and the remainder at 10 second intervals for 5 minutes following bleach cessation.

$$B = \frac{I}{I + I_P} \left(1 - \exp \left(- \left(1 + \frac{I}{I_P} \right) \frac{t}{\tau_P} \right) \right),$$

Equation 3-1. Calculation of bleach provided by preadapting light prior to the photostress test; where I_P is the steady retinal illuminance that bleaches half the pigment, and τ_P is the time constant of pigment regeneration. For cones, Hollins and Alpern (1973) measured $I_P = 30,000$ Td, and $\tau_P = 105$ s.

3.3.4. Signal Analysis

The standards recommended by ISCEV for the measurement of amplitude and time to peak of transient and steady state ERGs have been adhered to in this study (McCulloch et al., 2015). Retinal responses were first averaged and zeroed to remove any DC component. The peaks of the a-wave, b-wave and PhNR wave were identified objectively in the ERG, defined by the largest positive or negative value within specific time windows; a-wave 5 - 30 ms, b-wave 20 - 50 ms, PhNR-wave 30 - 110 ms. All times to peak were measured from stimulus onset. Additionally, visual verification of this objective analysis was performed by the researcher. The amplitude of the a-wave was measured from the pre-stimulus baseline to the peak (negative peak or trough) of the a-wave, the b-wave was measured from the trough of the a-wave to the peak of the b-wave, the PhNR was measured from the pre-stimulus baseline to the trough of the PhNR wave (Mortlock et al., 2010).

The flicker ERG was Fourier analysed using the approach described by Stroud (1986). In brief, the ERGs were zeroed and drift removed (f). Times were converted to radians (x). Functions $f(x)\cos x$ and $f(x)\sin x$ were calculated. The means of these functions, multiplied by 2, gave the Fourier coefficients a_1 and b_1 . The amplitude of the first harmonic of the Flicker response was obtained by calculating $\sqrt{a_1^2 + b_1^2}$, whilst phase was given by $\text{ATAN } a_1/b_1$. The amplitude and time to peak of the first harmonic of the steady state ERG was assessed (as this is more robust to lighting conditions the participant is exposed to before ERG testing than the raw flicker response [McAnany and Noland, 2014]). The amplitudes of the first harmonic of the flicker ERG were normalised to the pre-bleach amplitude and plotted as a function of time after the

bleach for the ERG photostress test, and the recovery data were modelled using an exponential function (see Equation 3-2). Modelling was carried out using least squares fit paradigm in Microsoft Excel, and the time constant of regeneration was extracted for further analysis.

$$\text{Amplitude (t)} = a[1-B*\exp(-t/\tau)]$$

Equation 3-2. Recovery data modelling; where “t” is time after the photobleach in seconds, “a” is the pre-bleach amplitude, “B” is the proportion of photopigment bleached and “τ” is the time constant in seconds. “B” was constrained within the model to return a value of 0 or greater.

3.3.5. Results

Nine eligible participants between the ages of 24 and 38 were recruited (4 females and 5 males). The mean ($\pm$ SD) age was 30 ($\pm$ 5.32) years, the Median (IQR) age was 29 (25, 30) years.

Sample raw traces of the ISCEV standard photopic 3.0 ERG from the left eye of participant AB are shown in Figure 3.3 (grey traces), with the grand average shown in black. In this individual, clean uncontaminated final responses were obtained for all of the ERG protocols, although there were some traces contaminated with artefacts which were excluded from final averaged response to achieve this result. Figure 3.3 also shows the objectively identified location of the a- and b-waves. The ISCEV standard transient cone ERG was well tolerated by all participants.

Figure 3.4 shows sample PhNR responses from the right eye of the same individual (AB). Clear PhNR responses were obtained from this individual at all stimulus luminances. The PhNR responses can be seen to increase in amplitude with increasing stimulus intensity, levelling off at the highest stimulus luminance. The PhNR protocol was well tolerated by the majority of participants with only a few participants responding to higher intensity flashes with lid twitches (e.g. see Figure 3.5).

Figure 3.6 shows the results of the ERG photostress test from the left eye of participant AB. A reduction in ERG amplitude can be seen immediately following the cessation of the bleach at the 720 cd.s.m⁻² (50%) and 1000 cd.s.m⁻² (60%) bleach intensities, with the ERG amplitude recovering to the baseline level over the follow up

period of 5 minutes. However, this reduction in amplitude was only slight following the 50% (720 cd.s.m^{-2}) bleach intensity and it was completely absent at the 500 cd.s.m^{-2} (40%) bleach intensity. This is also shown graphically in Figure 3.7.

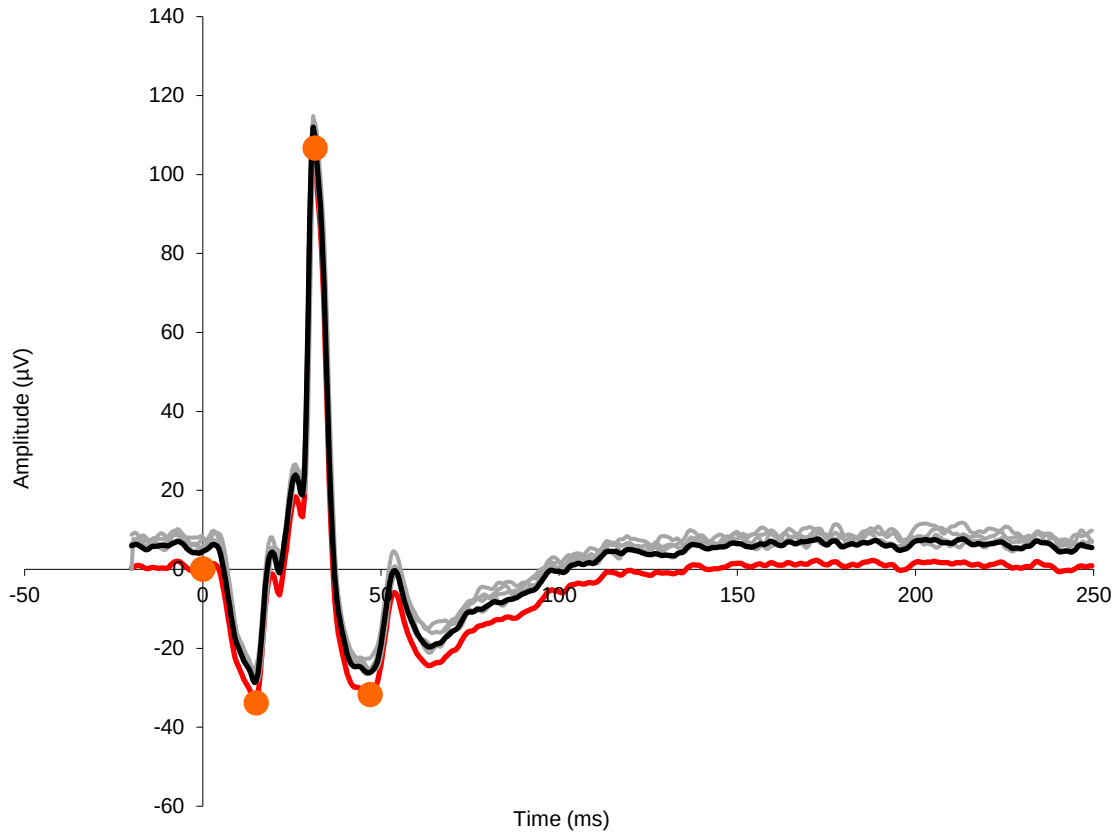


Figure 3.3. Averaged raw ERG traces from the right eye of participant AB during the ISCEV photopic 3.0 ERG. Four raw traces are shown in grey (each an average of $n = 15$ responses). The grand average of all traces ($n = 60$), is shown in black, and then again once zeroed in red. The orange markers indicate the objectively identified minima and maxima of the a- and b-waves subcomponents and the PhNR.

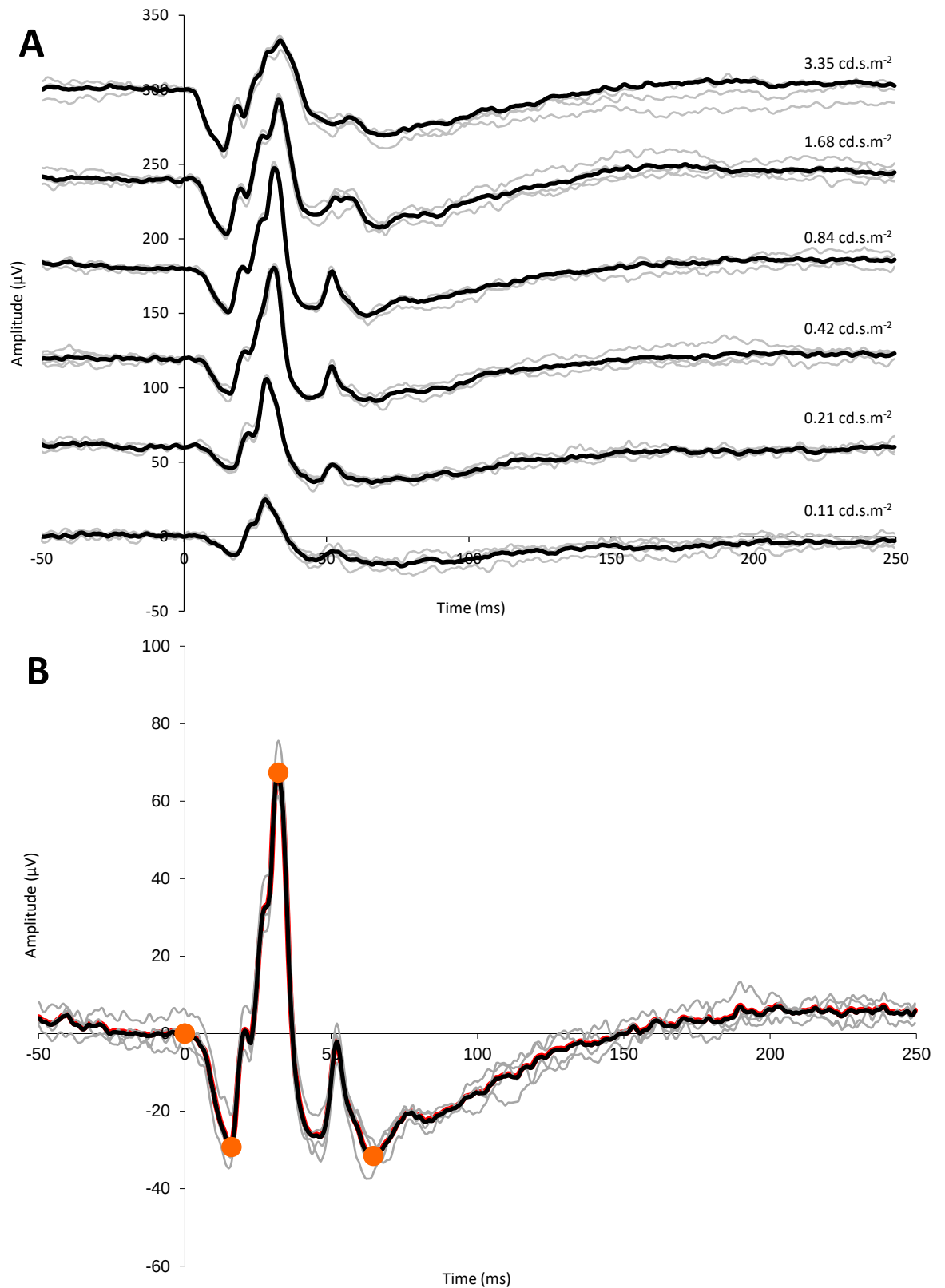


Figure 3.4. A, Raw ERG traces from the right eye of participant AB during the PhNR Intensity Response ERGs (grey lines, each representing 4 averaged responses, the grand average [$n = 40$] is shown in black). B, Grey lines represent the 4 averaged responses, the black line the grand average PhNR recorded to a stimulus of luminance 0.84 cd.s.m^{-2} , and the red line the response zeroed to correct for DC offset. Orange markers indicate the objectively identified minima and maxima of the pre-stimulus baseline, a-, b-, and PhNR waveforms.

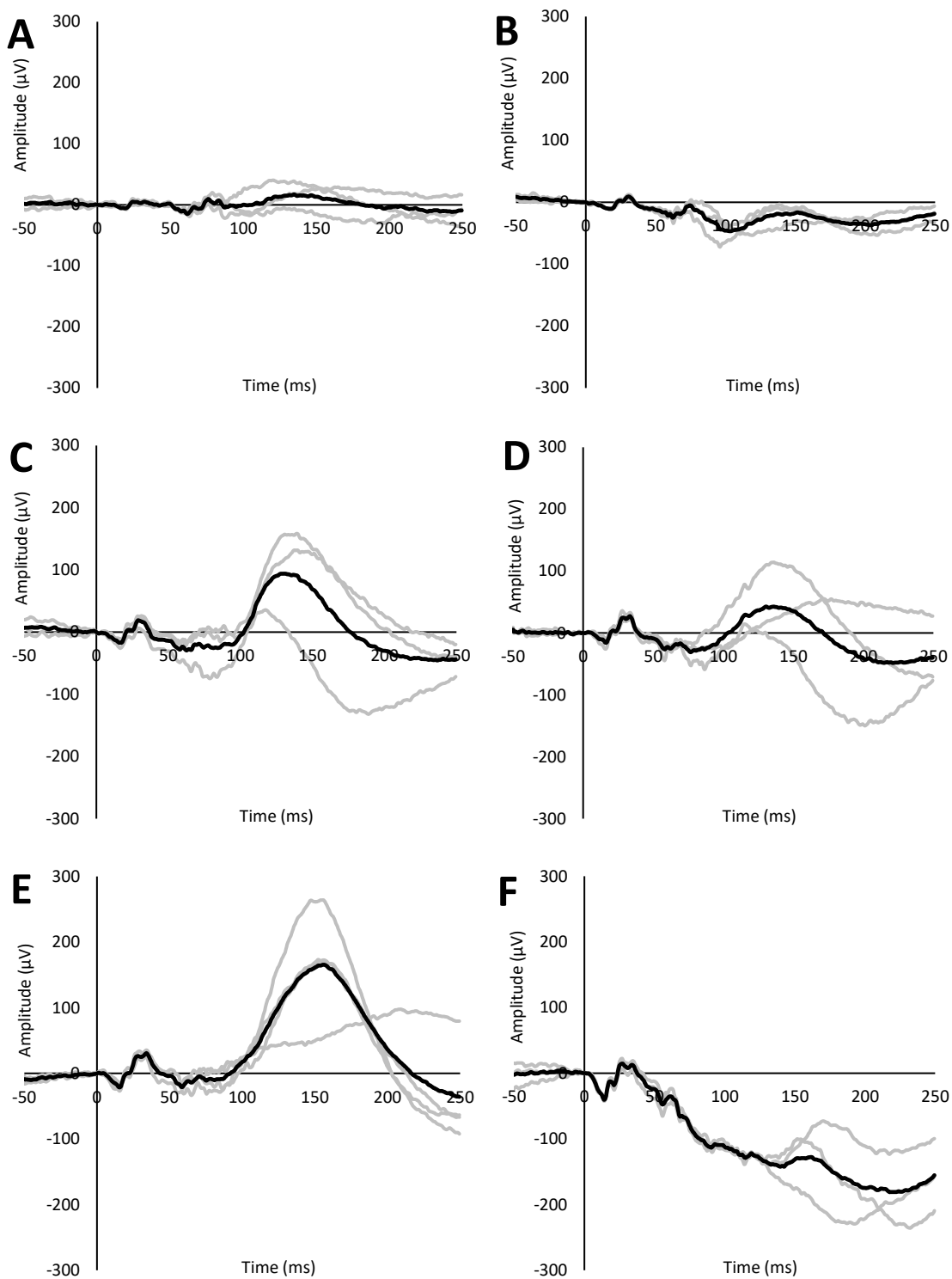
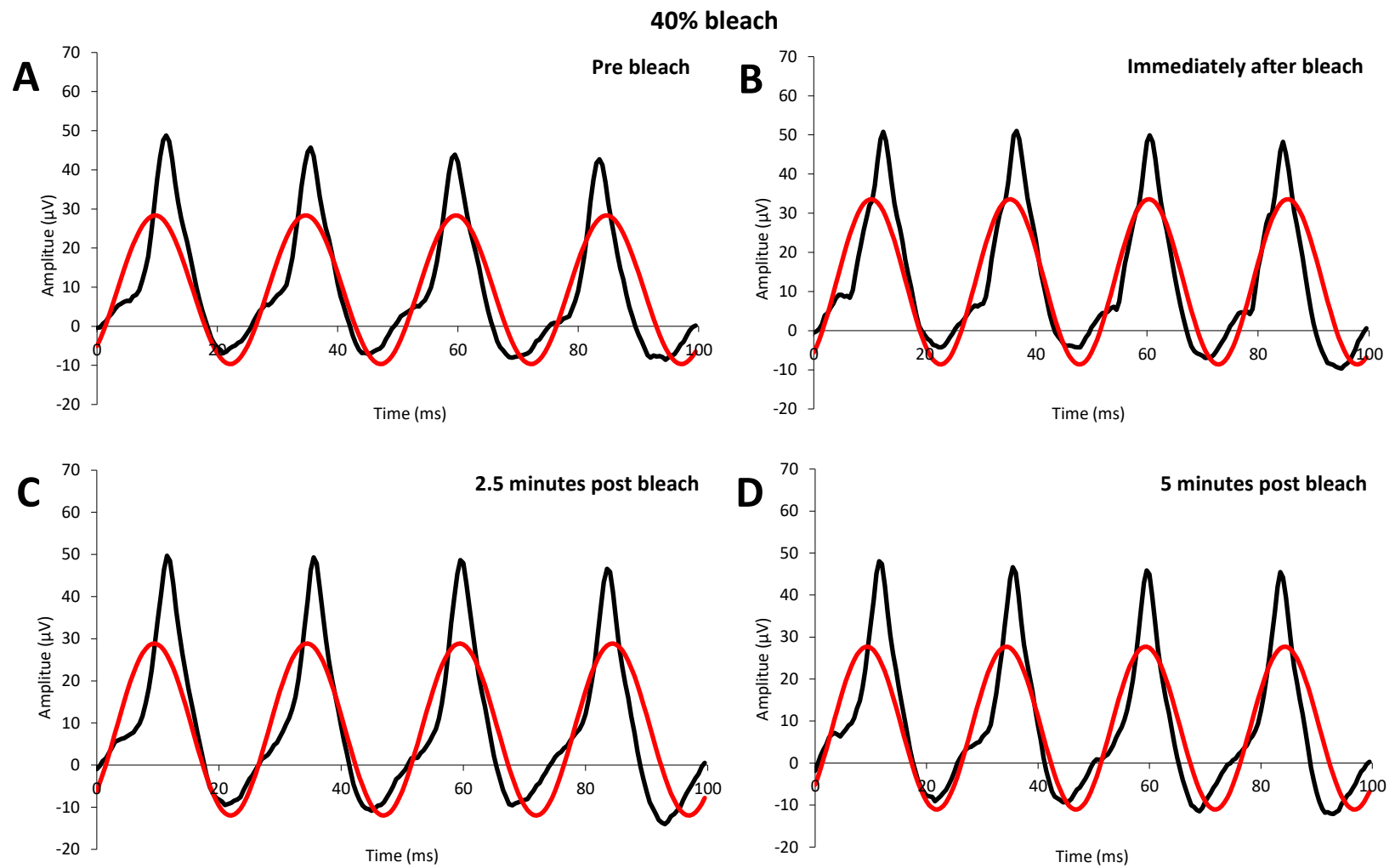
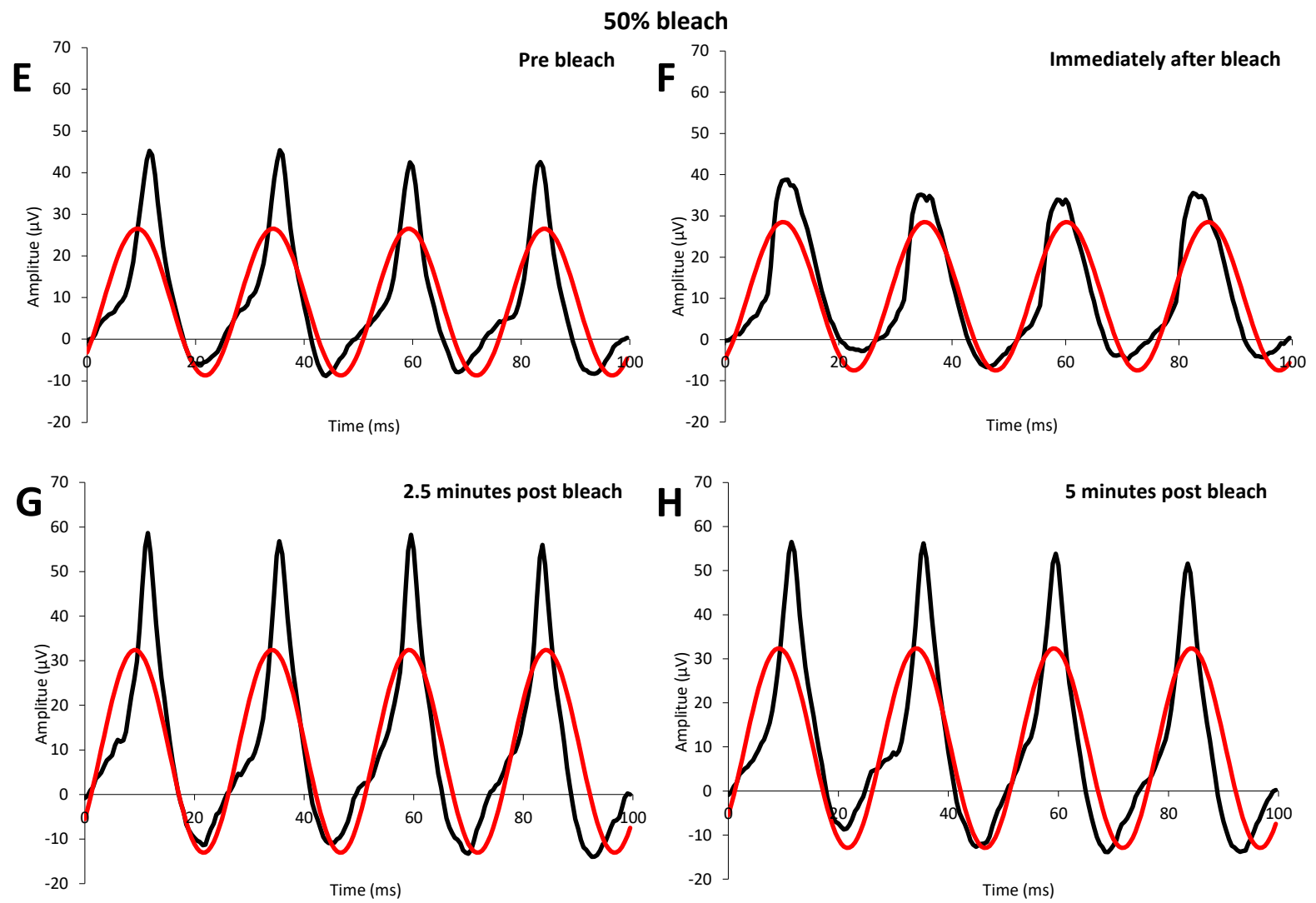


Figure 3.5. Raw PhNR ERG traces from LE of participant SH who suffered from lid twitches increasing in frequency as the intensity of the stimulus increased (grey lines, each representing 4 averaged responses, the grand average [$n = 40$] is shown in black). The latter part of each trace can be seen to be disrupted by this muscle movement. A) 0.11 cd.s.m^{-2} , B) 0.21 cd.s.m^{-2} , C) 0.42 cd.s.m^{-2} , D) 0.84 cd.s.m^{-2} , E) 1.68 cd.s.m^{-2} , F) 3.35 cd.s.m^{-2} . The lid twitches were highly repeatable and involuntary, and were not eliminated by repeated testing.





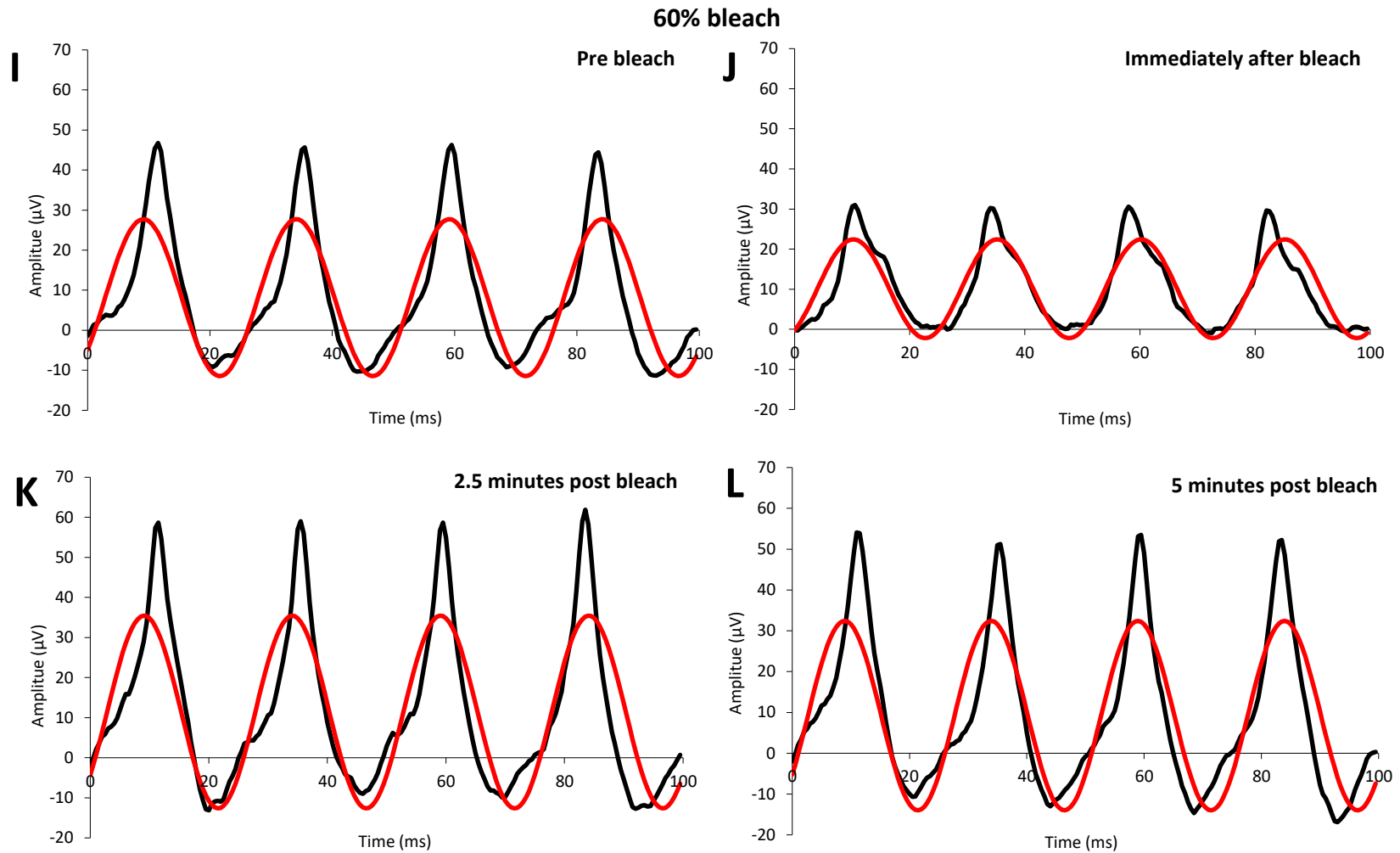


Figure 3.6. Raw 41 Hz flicker ERG traces from RE of participant AB before and following exposure to a bleaching light. Black lines represent the raw data ($n = 50$ responses averaged to a 41 Hz flickering stimulus), whilst the red line shows the first harmonic of the Fourier averaged responses. Light bleaching $\sim 40\%$ of cone photopigment (500 cd.s.m^{-2}): A, Pre bleach, B, immediately after bleach; C, 2.5 minutes post bleach; D, 5 minutes post bleach. Light bleaching $\sim 50\%$ of cone photopigment (720 cd.s.m^{-2}): E, Pre bleach; F, immediately after bleach; G, 2.5 minutes post bleach; H, 5 minutes post bleach. Light bleaching $\sim 60\%$ of cone photopigment (1000 cd.s.m^{-2}): I, Pre bleach; J, immediately after bleach; K, 2.5 minutes post bleach; L, 5 minutes post bleach.

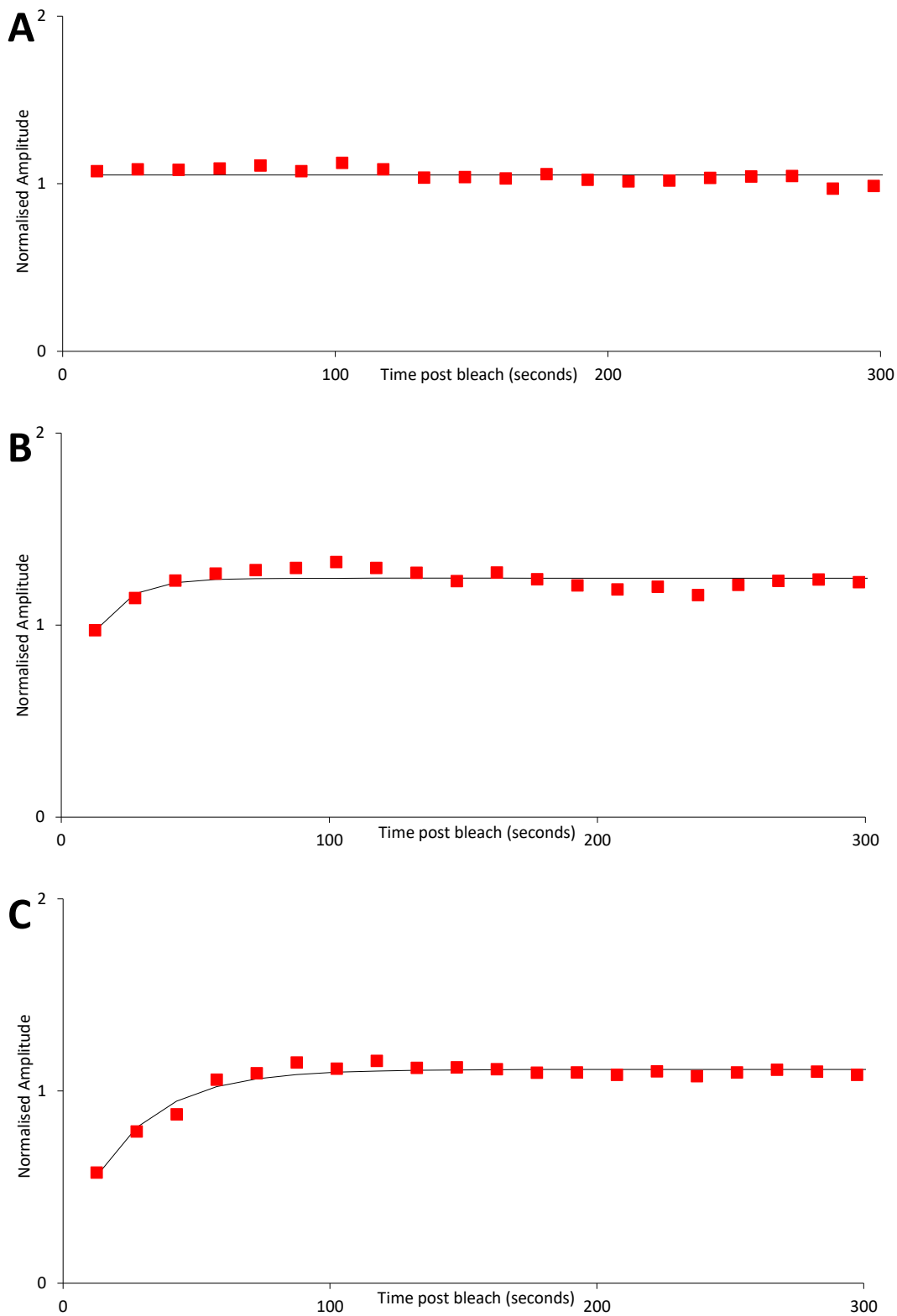


Figure 3.7. The recovery of amplitude of the flicker ERG first harmonic over time after exposure to a bleaching light during the ERG photostress test, where postbleach amplitudes were normalised to prebleach values. All data from the right eye of participant AB. A, 40% bleach (500 cd.s.m^{-2}); B, 50% (720 cd.s.m^{-2}) bleach; C, 60% (1000 cd.s.m^{-2}) bleach. Red dots indicate amplitude values, whilst the solid line is the best fitting exponential model (Equation 3-2).

The full ERG photostress test (at all three intensities) was only successfully recorded from two out of nine participants (22%). During the 40% bleach ERG only one volunteer (JH) produced the predicted reduction in ERG amplitude following the bleach in one of their eyes (RE). This suggests that other eyes tested did not properly receive the bleach intensity when it was delivered to them, or that the 40% bleach (500 cd.s.m^{-2}) was of an insufficient intensity to produce a measurable reduction in ERG amplitude. With increased intensity of 720 cd.s.m^{-2} (50% bleach) only 5 of the 16 eyes tested (31.25%) produced the predicted reduction in ERG amplitude immediately after the bleach. When the intensity of the bleach was increased to 1000 cd.s.m^{-2} (60%), one participant requested to withdraw from further testing as they felt too uncomfortable with the light intensity to continue. Of the remaining 16 eyes tested (from 8 participants) only 3 produced the predicted reduction in amplitude immediately after the bleach. The participants reported that the full field bleach at this intensity was too bright to tolerate, and tended to partially shut their eyes in response. This part of the protocol was also time consuming, with each bleach intensity requiring a minimum of 7 minutes for complete data collection.

Figure 3.8 shows the bleach recovery responses recorded unsuccessfully from participant AEH's left eye at the 3 different bleach levels, where the participant reported significant discomfort during the bleach. It can be seen that there was no reduction in ERG amplitude after the bleach at any intensity, suggesting that the individual was not able to keep their eyes open and as a result the bleach was not successfully delivered, or that the bleach intensity was insufficient to produce a reduction in amplitude.

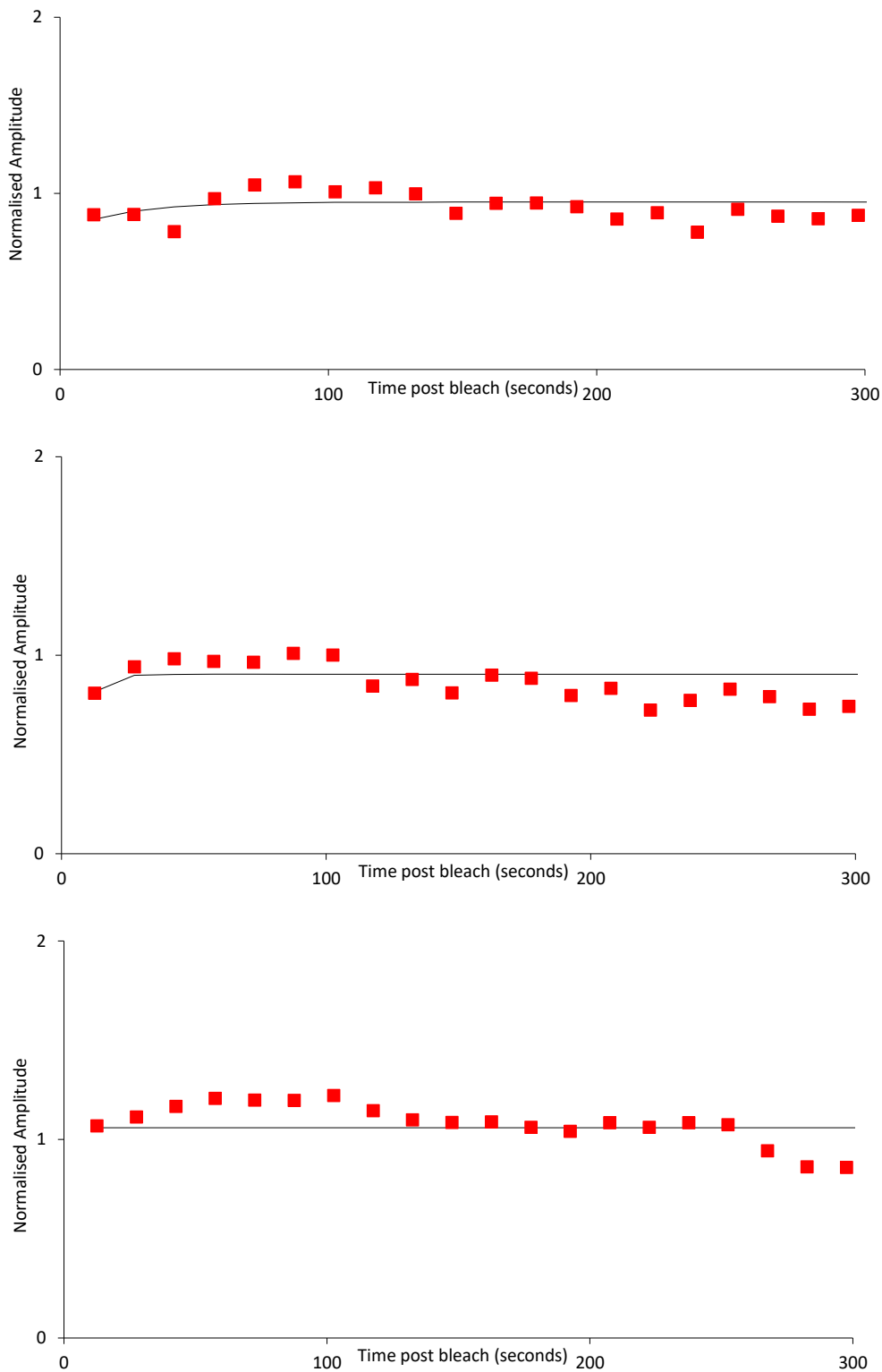


Figure 3.8. The recovery of amplitude of the flicker ERG first harmonic over time after exposure to a bleaching light during the ERG photostress test, where postbleach amplitudes were normalised to prebleach values. All data from the left eye of participant AEH for A) 40% (500 cd.s.m⁻²), B) 50% (720 cd.s.m⁻²) and C) 60% (1000 cd.s.m⁻²) bleach. All three show no reduction in flicker ERG amplitude after the bleach was delivered.

3.3.6. Discussion

The transient cone and PhNR protocol were both well tolerated by all participants. No issues arose with the recording of the ISCEV photopic 3.0 ERG. One volunteer struggled to control their blink reflex as the luminance increased to its highest levels in the PhNR ERG protocol (Figure 3.6). This resulted in many traces being rejected by the Espion™ device's bandpass filters and lengthened the test. Furthermore, the contamination of the traces by residual blink artefacts rendered this participant's data unsuitable for analysis. However, the remaining participants were able to produce usable data with some encouragement from the examiner, and re-recording of traces where necessary.

With respect to the ERG photostress test, there were substantial issues in this pilot study. In previous publications, following light adaptation the amplitude of the ERG was reduced, and returned to a pre-bleach level over a period of minutes (Binns et al., 2005; Binns et al., 2007). In contrast, across all bleach intensities in this study, ERG amplitude was not reduced in most patients by the adapting light. For example, only 3 of the 16 eyes tested (after one participant declined to undergo this test) showed a reduction in amplitude after the highest bleach intensity (60%). This indicates that an insufficient bleach was delivered to suppress the flicker response. The highest bleach intensity used (equivalent to ~60% cone bleach; Equation 3-1; Hollins and Alpern, 1973) was substantially lower than the ~86% used in previous publications (Binns et al., 2005; Binns et al., 2007). However, previous studies have employed only a focal bleach in order to assess macular recovery (Binns et al., 2005; 2007). The full-field and binocular nature of the bleach used in this study made it substantially harder to tolerate. The bleaching light was perceived to be very bright and uncomfortable by the participants and any increase in brightness of the adapting light above the 60% level would not have been tolerated. Indeed, six out of eight participants (75 %) reported significant discomfort in this study with the highest adapting light intensities, and were unable to keep their eyes fully open during the preliminary adaptation phase. It is likely that this reduced palpebral aperture is the explanation for the insufficient photopigment bleach delivered, rather than an insufficiently bright adapting light. The amplitude of the steady state 41 Hz ERG is largely determined by the activity of bipolar cells (Kondo and Sieving, 2002; Kondo and Sieving, 2001; Hare and Ton, 2002, Viswanathan et al., 2002), and its recovery post bleach has been shown to be closely

although non-linearly related to the regeneration of photopigment (Binns et al., 2005). Thus, it would be expected that a 60% depletion of photopigment would have a significant impact on the amplitude of the post bleach flicker response recorded, if the bleach were properly delivered to the retina. Furthermore, some participants (e.g. AB) did show a reduction in amplitude post bleach to the highest intensity adapting light, indicating that this was sufficient to reduce post bleach amplitude when effectively delivered. At the lowest bleach levels (40% photopigment bleach), enhanced comfort made it easier for participants to keep eyes open but the reduced intensity of the bleaching light meant that any reduction in amplitude attributable to the bleach fell within the measurement error of the test.

3.3.7. Conclusion

As the bleach recovery protocol was poorly tolerated by the majority of young volunteers, it is unlikely to be well tolerated by older patients being treated for nAMD. On this basis, the bleach recovery was removed from the final ERG protocol. However, the baseline flicker ERGs recorded as part of the bleach recovery protocol provided a measure of cone function which was quick to record and showed consistency between traces (see Figure 3.8). Additionally, the repeated stimulation of the cones in flicker ERGs puts them under metabolic stress and is likely to emphasise functional abnormality (Feigl et al., 2009). Therefore, a simple 41 Hz flicker ERG was retained in the final protocol. Although ISCEV Standards (McCulloch et al., 2015) recommend a 30 Hz flicker frequency, 41 Hz was chosen for this study for several reasons. The frequency of 41 Hz has successfully been used in previous studies (Binns and Margrain, 2005; Binns and Margrain, 2007). Both 30 Hz and 41 Hz flicker frequency are well above rod flicker detection frequency which ensures that the response will be cone dominated (Sharpe et al., 1989). Further to this, Seiple et al., (1989) investigated response amplitude as a function of temporal frequency in 6 normal participants and showed that the maximum response was achieved at 40 Hz, suggesting that a higher response amplitude may be obtained with the 41 Hz than the 30 Hz stimulus.

In contrast, the ISCEV transient cone and PhNR ERGs were recordable from the majority of individuals and will be used in the final protocol. As the duration of the protocol was significantly reduced by the elimination of the ERG photostress test, it was not necessary to limit the PhNR recording to one intensity, and the range of

intensity responses remained in the protocol. Further explanation for the rationale for retaining the full range of intensities is provided in the discussion in section 3.4.4.

3.4. Evaluation of Repeatability of Electroretinogram Protocol

3.4.1. Introduction

One aim of this PhD was to evaluate the effect of anti VEGF drugs on peripheral retinal function, evaluated using the ERG. Before using an ERG protocol in a clinical evaluation, however, it is first necessary to determine the inherent variability of the parameters assessed. This allows the determination of whether changes or differences in the ERG of people receiving the drug exceed the basic measurement error of the test. The aim of this section, therefore, was to assess the between session and between eye variability of the developed protocol in a group of healthy individuals.

Two amendments to the protocol described in section 3.3 were made at this stage. The first was that the ISCEV 3.0 photopic cone ERG and the 41 Hz flicker ERG were recorded using both a full-field and a focal stimulus. Subtraction of the focal response from the full-field signal allowed the macular contribution to the response to be eliminated when assessing peripheral retinal function. A second amendment was the exclusion of the bleach photostress test, to be replaced by a simple 41 Hz flicker ERG (rational explained in section 3.3.7).

Therefore, the final ERG protocol following the pilot study included the following tests.

1. A transient cone response (International Society for Clinical Electrophysiology of Vision Standards: (ISCEV) light adapted 3 ERG (McCulloch et al., 2015)). This was recorded both full-field, and from the central 20 degrees of the retina, as a focal response.
2. A 41 Hz Flicker ERG. This was recorded both full-field, and from the central 20 degrees of the retina, as a focal response.
3. A PhNR intensity response series (brief red stimulus on rod saturating blue background at six increasing levels of luminance, ranging from 0.11 – 3.35 cd.s.m⁻²). This was recorded from the full field only due to protocol time constraints.

3.4.2. Methods

Participants

Local Ethical approval was gained through the City, University of London Optometry Proportionate Review panel 'Opt/PR/23' and written informed consent was obtained from all subjects, conforming to the tenets of the Declaration of Helsinki. The study took place at the Division of Optometry and Visual Science at City, University of London and at St Thomas' Hospital, London. Inclusion and exclusion criteria were as described in section 3.2.3. Nine participants from the pilot study also took part in the repeatability study.

Participant Pathway

After providing informed consent, participants underwent a structured history and symptom review and examination of the anterior and posterior eye using slit lamp biomicroscope and Volk lens, and IOP measurement using a non-contact tonometer (Pulsair IntelliPuff Non-Contact Tonometer [Keeler, UK]) to determine eligibility for inclusion. Best corrected visual acuity was assessed in each eye using an Early Treatment Diabetic Retinopathy Study (ETDRS) chart. In those volunteers who met the eligibility criteria, a topical mydriatic was instilled to each eye according to the protocol described in Section 3.2.1 and, thereafter, electrodes were applied according to Section 3.2.2. After maximal pupillary dilation had been achieved, the ERG recording protocol commenced using the equipment described in Section 3.2.3. The test order was as follows: 1) PhNR intensity-response series, 2) ISCEV photopic 3.0 ERG (full field ERG), 3) ISCEV photopic 3.0 ERG (focal ERG), 4) 41 Hz flicker ERG (full field ERG), 5) 41 Hz flicker ERG (focal ERG). The PhNR intensity-response series test was performed first as this was the most challenging test with respect to participant concentration. Data were collected from both eyes simultaneously for full field tests. For focal responses the ERGs were performed on each eye separately. At the end of the recording period, IOP measurements were retaken, and fundus photographs (using a TRC-NW6S Non-Mydriatic Retinal Camera [Topcon medical systems Inc., Tokyo]) and 30 x 35 Macular OCT images (using a Spectralis OCT [Heidelberg Engineering, Heidelberg, Germany]) were obtained.

Equipment

The equipment used in the repeatability study has been described in Section 3.2.3. An additional piece of equipment was developed in order to record the focal ERGs by limiting the stimulus to the macular area of the eye. The focal response was achieved by placing a large wooden board in front of the Ganzfeld ColorDome (Figure 3.9). This 660 mm by 660 mm board featured a hole of 105 mm diameter cut into its centre and was painted gloss white. The retinal area of the hole subtended 20° visual angle when viewed from 300 mm, and the surround subtended 66°. The gloss white paint of the wooden board reflected the main room lighting in order to desensitise the peripheral retina and minimise effect of scattered light stimulating the peripheral retina. The mean luminance of the gloss board under standard room lighting was 5.87 cd.m⁻². The luminance and size of the surround were in accordance with previous studies reporting focal cone ERG protocols (Miyake et al., 1989; Kondo et al., 2008).

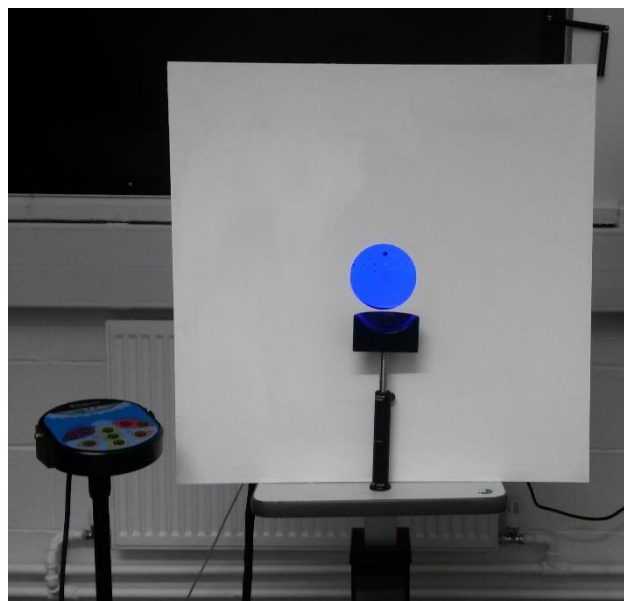


Figure 3.9. The focal stimulus equipment. The background illumination is coloured blue in order to clearly illustrate the central hole.

Electroretinogram Protocol

The specific recording parameters for the full-field ERGs are provided in Section 3.3.3. The only difference was for the 41 Hz flicker ERG, for which, in contrast to the ERG photostress test, 50 responses were averaged on each trace with four traces in total ($n = 200$ responses included in the grand averaged response). When focal responses were recorded, monocular testing was employed. The test eye was aligned with the

centre of the hole in the board, at a distance of 300 mm. The second eye was then tested. The test parameters and protocol for recording focal responses were identical to those for the full field ERG responses.

Signal Analysis and Statistical Analysis

Signal analysis and measurement was carried out according to the procedures outlined in Section 3.3.4 of the pilot protocol. In the two protocols in which focal ERG responses were recorded in addition to full field ERG responses (transient cone ERG and 41 Hz flicker ERG) the focal amplitude responses of each protocol were subtracted from the full field amplitude responses of the protocol at corresponding timepoints in the ERG waveform. The resulting new ERG amplitudes were plotted against the timepoints to form waveforms representing retinal function responses of the peripheral retina only. The peaks of the a-wave, b-wave and PhNR wave were identified objectively in this reconstructed ERG, defined by the largest positive or negative value within specific time windows; a-wave 5-30 ms, b-wave 20-50 ms, PhNR-wave 30-110 ms.

All statistical analysis was carried out in SPSS (versions 22-25; IBM), whilst graphs were produced in Microsoft Excel (Microsoft Office, Version 16). The means and standard deviations were determined for all parameters at each visit and a Bland and Altman analysis (Bland and Altman, 1986) performed to plot the agreement between the two sessions. This included plotting the differences between visits against the mean of the visits to enable visualisation of the spread of the data. According to the principles described by Bland and Altman (1986), the coefficient of repeatability (CoR) and between eye coefficient of agreement (CoA) were also calculated (see Equation 3-3). CoR and CoA were expressed both in absolute terms, and as a percentage of the mean value for the parameter, to facilitate comparison of variability of dissimilar parameters.

$$\text{CoR} = 1.96 \times \sqrt{(\Sigma(d^2 - d^1)^2 / (n-1))}$$

Equation 3-3. Coefficient of Repeatability; where d = the mean of the parameter in question and n = sample size.

A paired t-test was performed in order to assess if there was a statistically significant difference between the mean values measured at visits one and two. Bonferroni correction was applied to p-values to minimize the risk of type I errors i.e. by

multiplying p-values by the number of comparisons made (Bonferroni, 1936; Abdi, 2007).

3.4.3. Results

In total, 30 eligible participants between the ages of 21 and 60 were recruited, 21 females, 9 males. The mean (SD) participant age was 21 ± 9.67 years, median (IQR) 25 (24,30) years. Of these participants, 9 had already participated in the pilot study, and their pilot study data were used as visit 1 of the repeatability study. As the focal response was not included in the pilot study, these individuals did not participate in focal ERG recordings on both visits. One further participant also underwent their first repeatability study visit before the focal response protocol was finalised and so also did not participate in focal ERG recordings on both visits. In addition, three participants' focal 41 Hz flicker ERG responses were unreliable and so could not be considered for analysis. As a result, 67% of the total number of repeatability study participants ($n = 20$) provided reliable focal ERG data on two occasions for the transient cone response and 57% of participants ($n = 17$) provided reliable focal ERG data on two occasions for the 41 Hz flicker response.

Sample ERGs from the right and left eyes of one participant (JDB) are shown in Figures 3.10 to 3.15. Figure 3.10-3.11 present the raw full-field responses, before subtraction of the focal component. In this volunteer, clean and uncontaminated responses were obtained for all of the ERG protocols. The raw traces of the full field ISCEV transient cone ERG are shown in Figure 3.10. This protocol was well tolerated by 29 participants on all occasions. Data from one participant were excluded from analysis of this response due to contamination from a large repeatable eye movement. It can be seen from the raw traces alone that ISCEV transient cone ERG amplitude can vary considerably both inter-ocularly and between tests but time to peak does not appear to vary as much.

Figure 3.11 shows the full field 41 Hz flicker ERG traces from the same participant. Some individuals found this stimulus difficult to tolerate. ERGs were successfully recorded for 24 of 30 participants on this parameter (the remaining 6 participants were excluded from analysis due to their inability to tolerate the flicker stimulus and/or to subsequently provide an uncontaminated recording due to uncontrolled blink reflexes). Like the ISCEV transient cone ERG, it can be seen from the raw traces

alone that there are noticeable inter-ocular and inter-visit differences in 41 Hz flicker amplitude. Again, there does not look to be as much variance in the time to peak between eyes or visits.

The effect of the focal ERG subtraction is shown in Figures 3.12 and 3.13. It can be seen that the focal ERG was substantially smaller than the full-field response and the focal contribution to the full field ERG was minimal. In this participant, the b-wave of the focal response and the amplitude of the first harmonic of the focal flicker ERG were approximately 10% the magnitude of the full-field response.

Figure 3.14 shows sample traces from participant JDB to one stimulus intensity at visits one and two. The raw PhNR intensity response traces of this participant can be viewed in Figure 3.15. When amplitude was plotted as a function of stimulus luminance in Figure 3.16, it can be seen that the amplitude of the PhNR increased with increasing stimulus intensity until a saturation of the response amplitude at around $1.80 \text{ cd}\cdot\text{s}\cdot\text{m}^{-2}$. The PhNR was less well tolerated by volunteers than the other protocols. Several participants had trouble controlling their blink reflex with this PhNR intensity response ERG, particularly during the latter stages of the test where the flash intensity had increased to $0.84 \text{ cd}\cdot\text{s}\cdot\text{m}^{-2}$ and beyond. Often, this introduced artefacts in the traces, where the participants made a very repeatable eyelid twitch resulting in a drift in the trace which was not big enough to trigger the artefact rejection process. A sample of such a dataset from the pilot study is shown in Figure 3.6. This issue resulted in the PhNR data collection having to be abandoned with two participants after their blink reflexes produced such large, erroneous results that all traces were continuously rejected. In other participants, usable data were only collected at some intensities. Usable data were only collected at both visits for a maximum of 20 of a possible 30 participants at any given stimulus intensity. Complete usable data, showing no artefacts, at all intensities for both eyes and both visits were only obtained for 11 participants (37% of a possible 30).

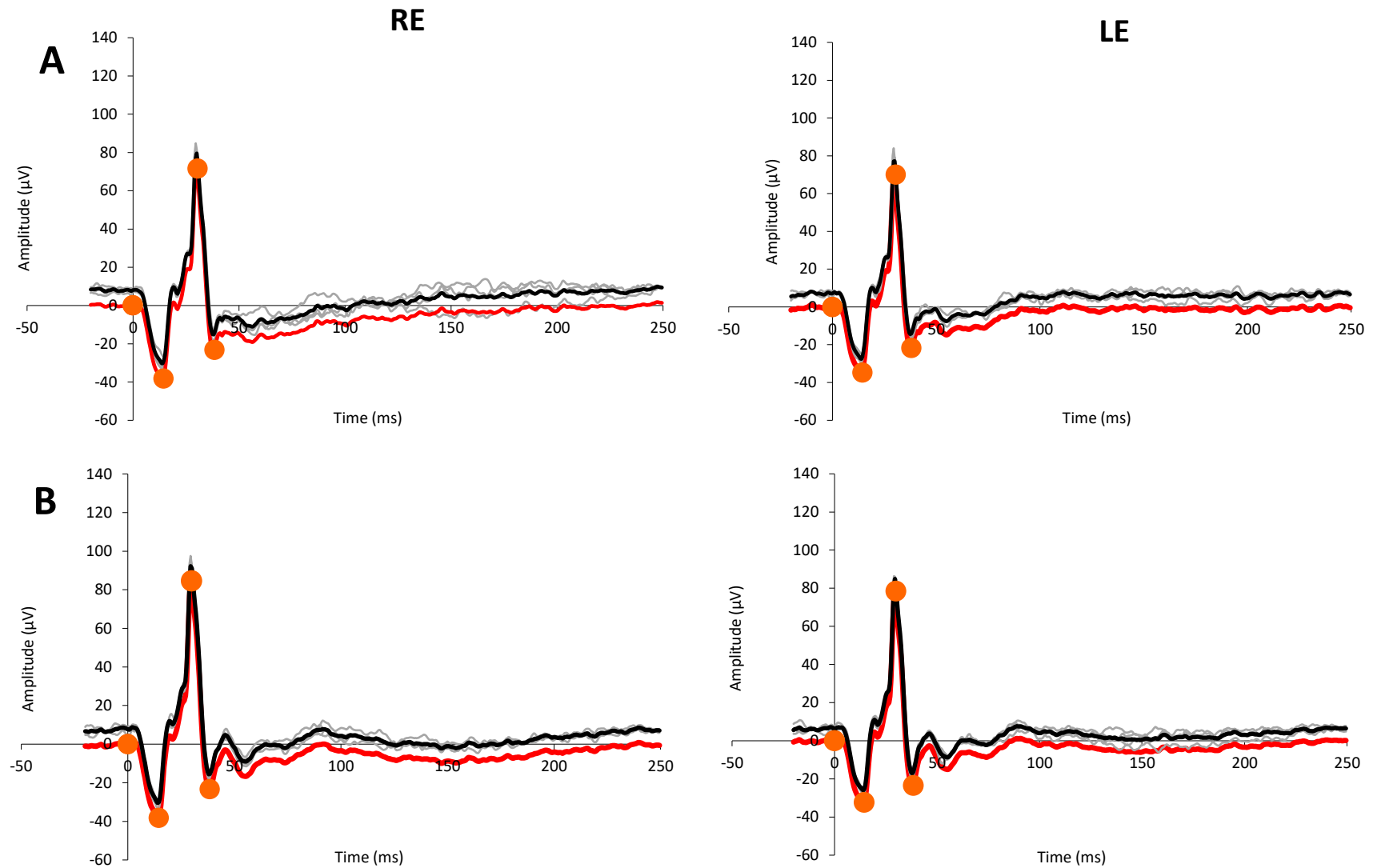


Figure 3.10. Averaged raw ERG traces of participant JDB during the ISCEV transient cone 3.0 ERG. On each graph, 4 raw traces are shown in grey (each an average of $n = 15$ responses). The grand average of all traces ($n = 60$), is shown in black, and the grand average zeroed in red. Maxima and minima of a-, b-, and PhNR wave subcomponents identified by orange dots. Right and left eyes on visit one (A) and visit two (B) are shown.

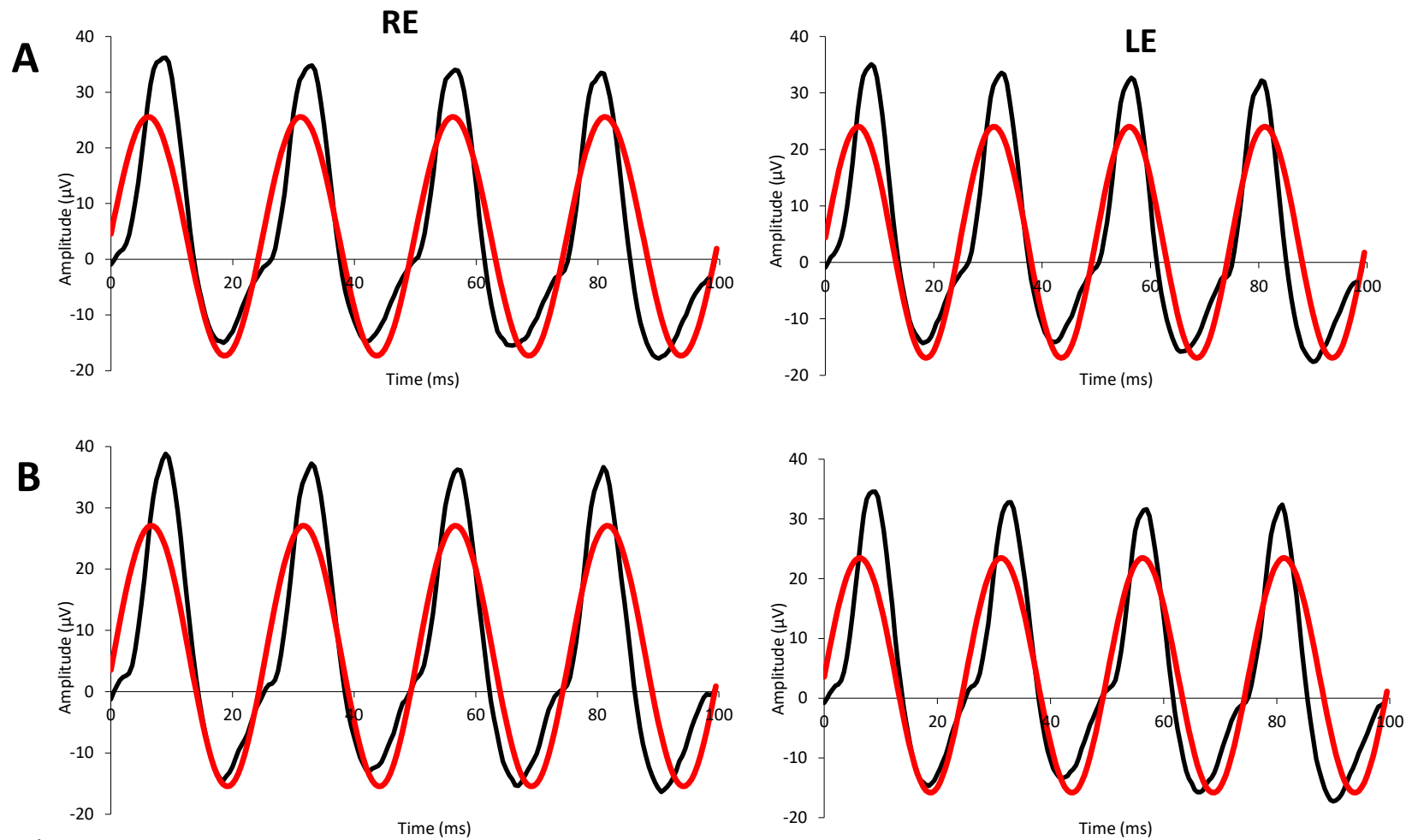


Figure 3.11. Grand average of raw 41 Hz flicker ERG traces (black) and first harmonic of averaged trace (red) from participant JDB; right and left eyes on visit one (A) and visit two (B) shown.

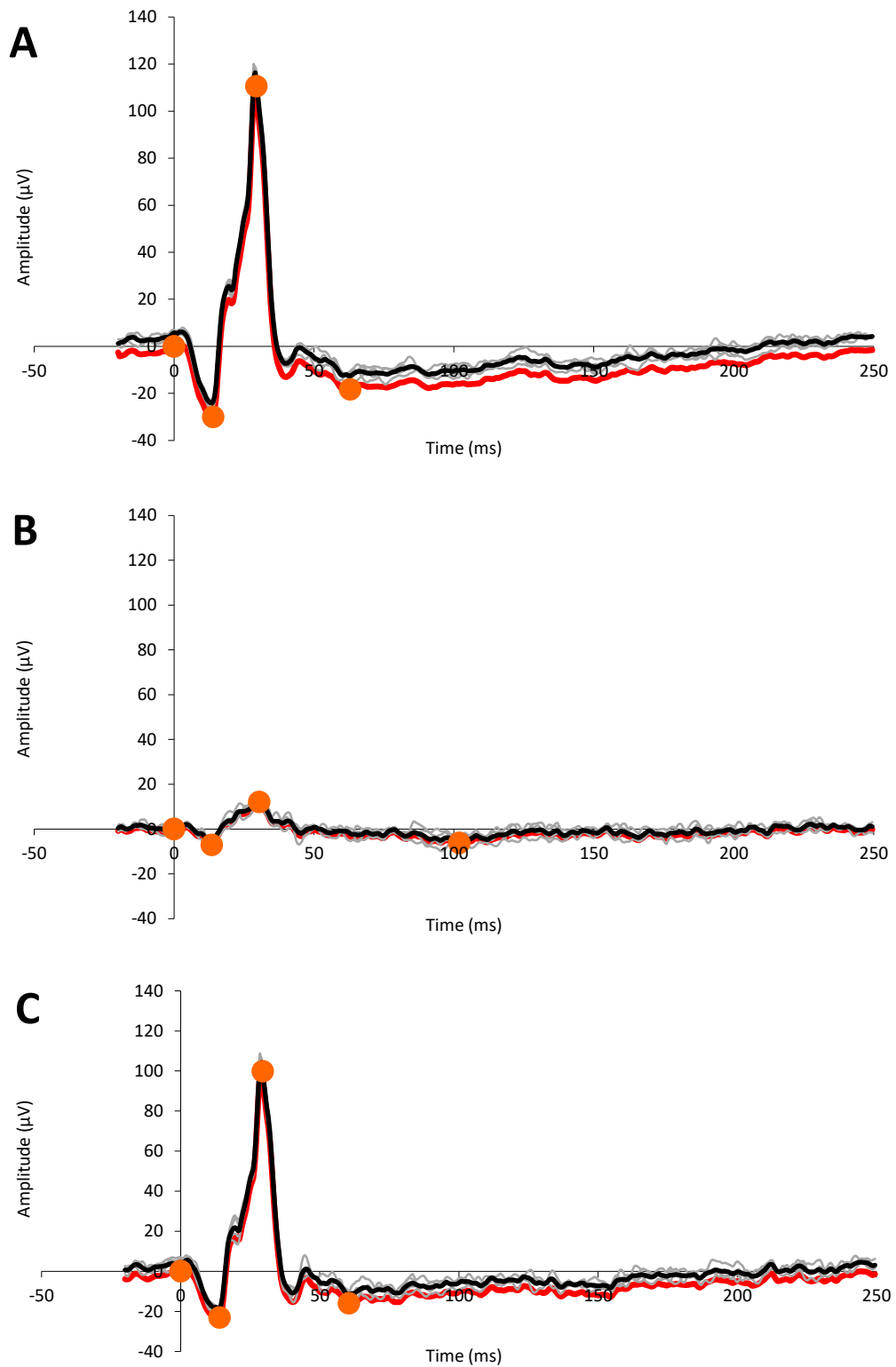


Figure 3.12. ISCEV transient 3 ERG traces from the RE of participant CG from visit 1. Panel A shows the full field ERG traces, panel B shows the focal ERG traces, panel C shows the peripheral retina only response (full field – focal). On each graph, 4 raw traces are shown in grey (each an average of $n = 15$ responses). The grand average of all traces ($n = 60$), is shown in black, and the grand average zeroed in red. Maxima and minima of a-, b-, and PhNR wave subcomponents identified by orange dots.

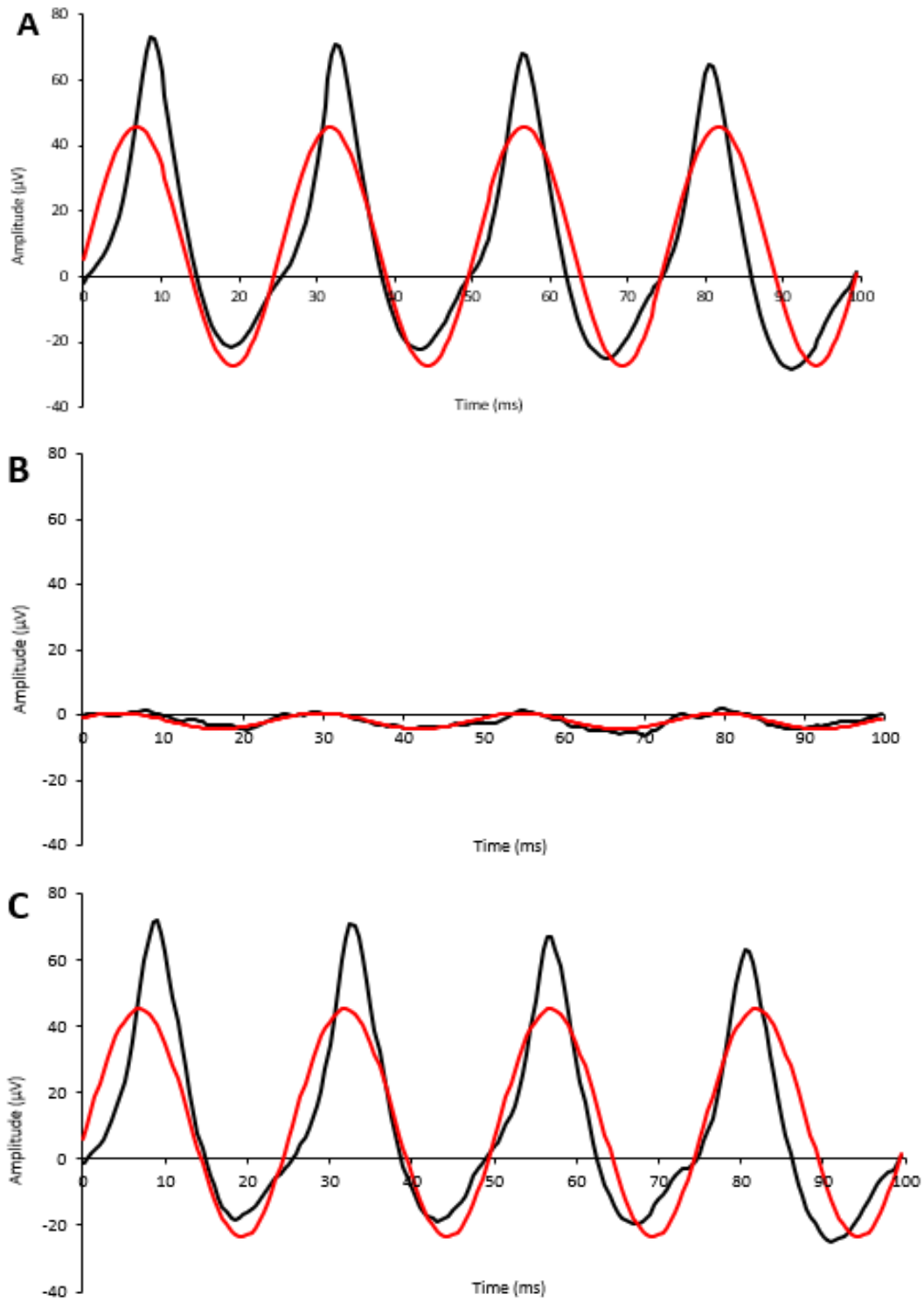


Figure 3.13. Raw (black) and first harmonic (red) flicker ERG traces from the RE of participant CG from visit 1. Panel A shows the full field ERG traces, panel B shows the focal ERG traces, panel C shows the peripheral retina only response (full field – focal).

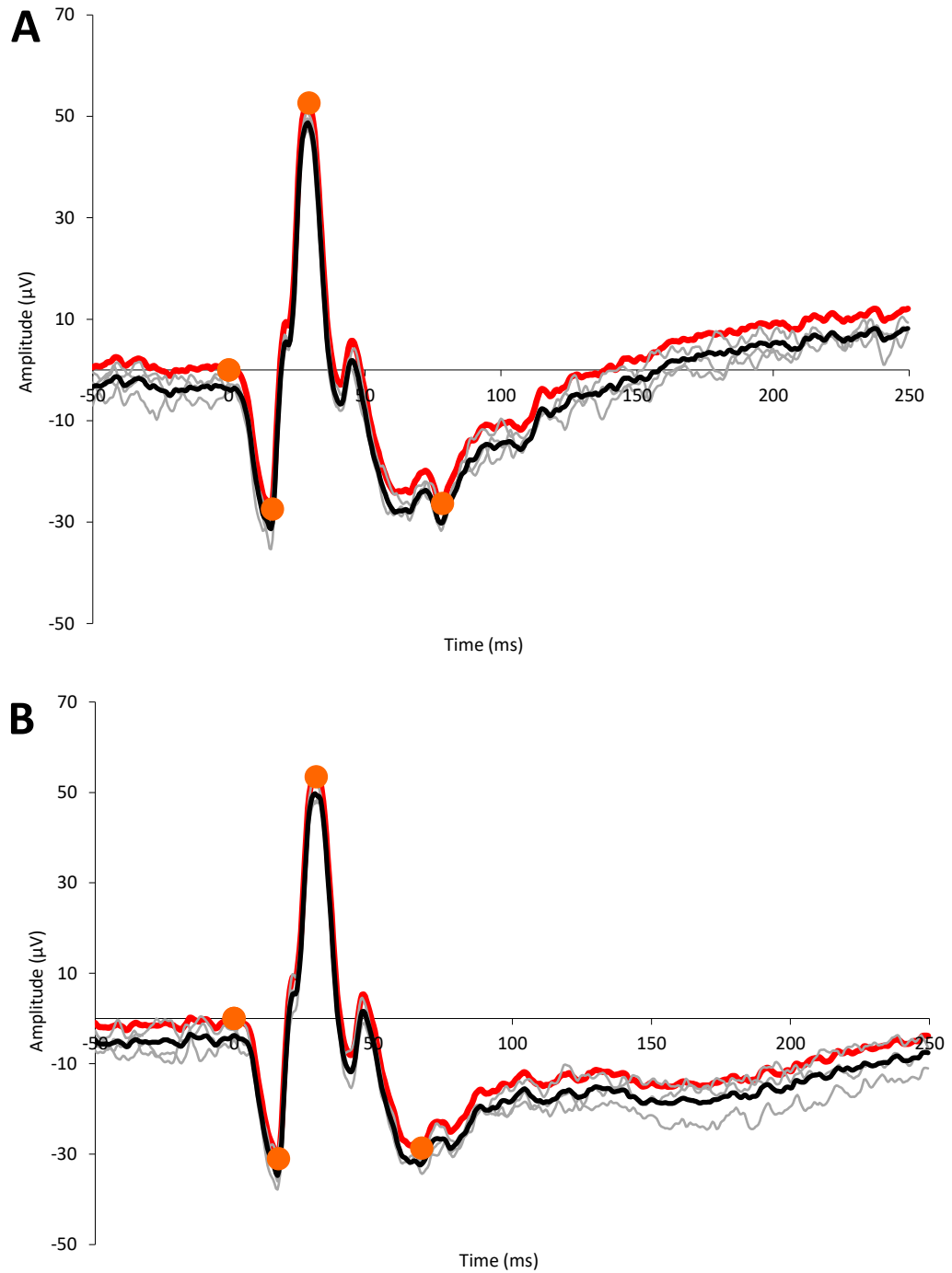


Figure 3.14. Raw average (black), and zeroed (red) PhNR ERG recorded to 0.84 cd.s.m^{-2} from the right eye of participant JDB on visit one (A) and visit two (B) (grey lines, each representing 4 averaged responses, the grand average [$n = 40$] is shown in black, the red line shows the trace after zeroing to remove DC offset). Maxima and minima of a-, b-, and PhNR wave subcomponents identified by orange dots.

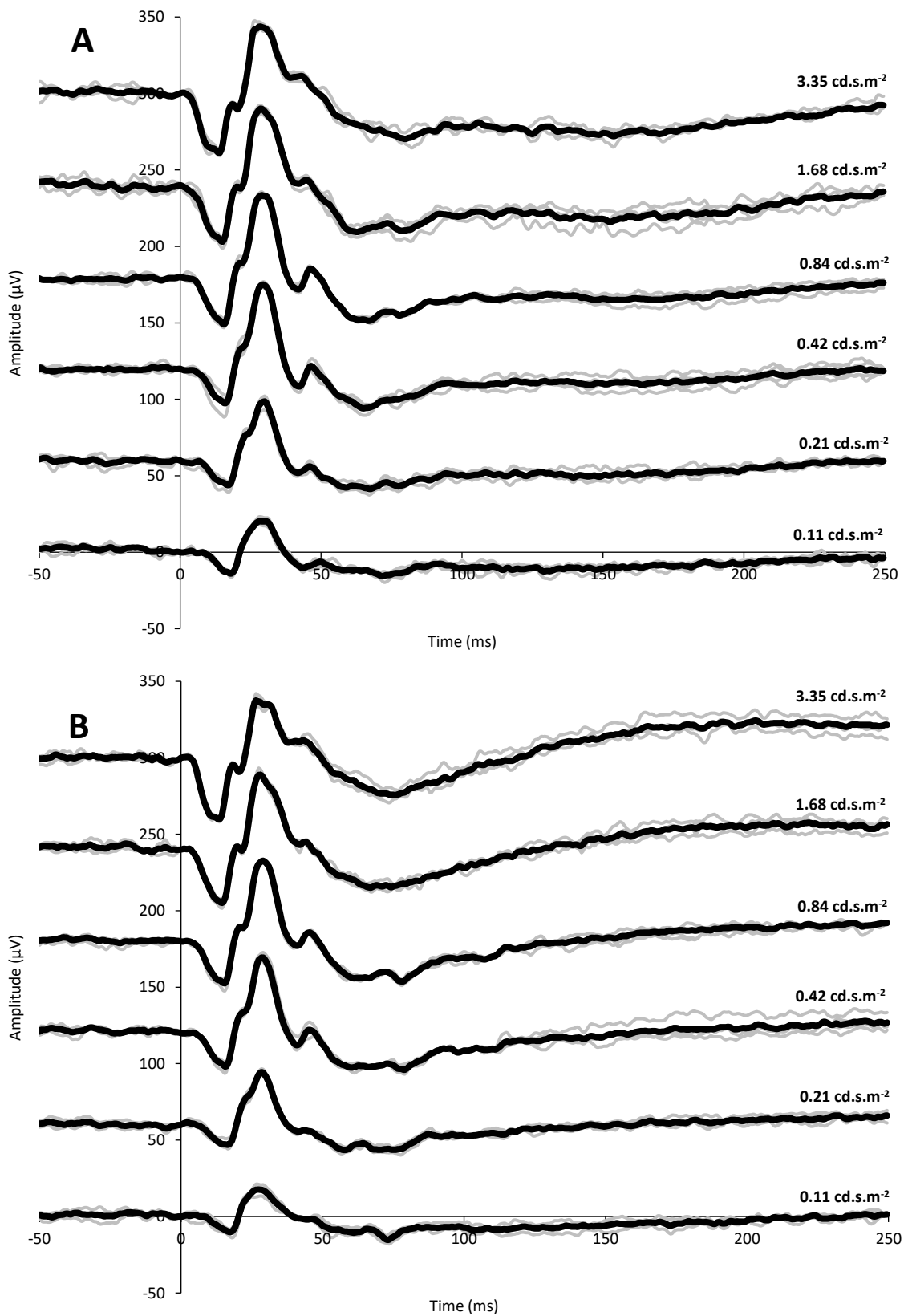


Figure 3.15. ERG traces from RE of participant JDB during PhNR Intensity Response ERGs on visit one (A) and visit two (B). Grey lines, each represent 4 averaged responses at each intensity, and the black line represents the grand average ($n = 40$) at each intensity.

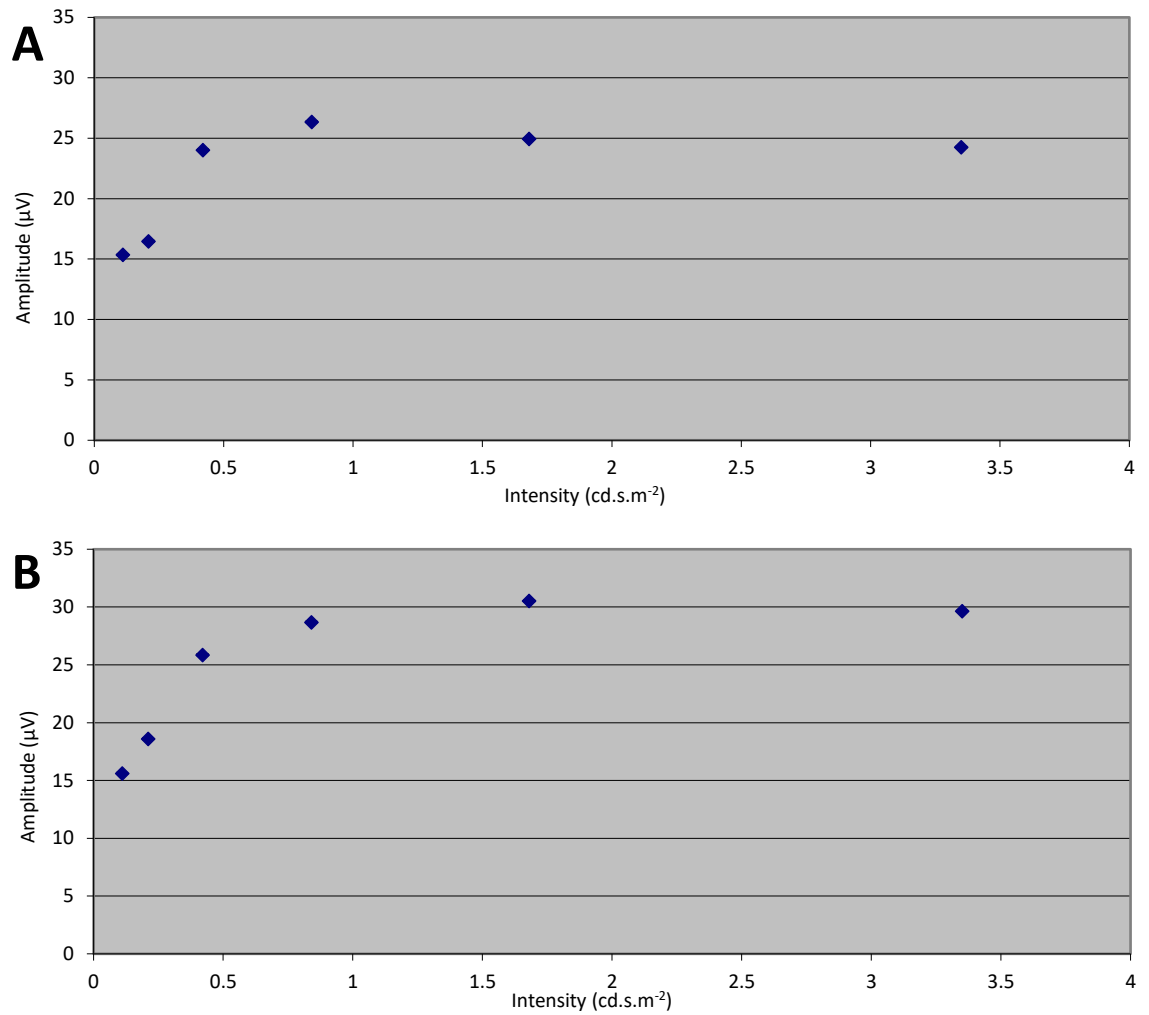


Figure 3.16. PhNR Intensity Response Series data from the right eye of participant JDB on visit one (A) and visit two (B). Blue diamonds show the mean amplitudes for each intensity.

Table 3.1 presents the mean data for each full-field parameter, along with the CoR, also expressed as a percentage of the group mean for the parameter. The expression of the CoR as a percentage allows a comparison of the repeatability of dissimilar variables, such as times to peak and amplitudes. When expressed as a percentage of the mean it can be seen that measures of time to peak are more repeatable than measures of amplitude in every case. The most repeatable parameter was the transient cone b-wave time to peak. The most repeatable PhNR parameter was time to peak of the response recorded to a stimulus of 0.84 cd.s.m⁻². Bland and Altman plots were produced to graphically represent the repeatability of the best-performing parameters (see Figures 3.17 and 3.18). Paired t-tests determined that there was no significant difference between visits for any parameter, except for PhNR time to peak

recorded in response to a stimulus of 0.84 cd.s.m^{-2} , which was slightly longer at visit 1 ($p = 0.03$). This statistical significance was not maintained after Bonferroni correction for multiple testing. This is mirrored in the fact that the bias line for all parameters on the Bland and Altman plots was close to zero.

Table 3.2 presents the CoA between eyes, CoA as a percentage of the mean, and p value data for the between eye analysis – performed on the visit one data. The parameters showing the highest agreement between eyes were the time to peak values. The two showing the highest level of concordance were a- and b-wave times to peak. Bland and Altman plots illustrate this in Figure 3.20. The parameters which differed most between eyes were the transient cone a-wave amplitude and 41 Hz Flicker ERG amplitude, illustrated by Bland and Altman plots in Figure 3.21. There was no significant difference between eyes for any parameter ($p > 0.05$).

A separate analysis was conducted, on the smaller subset of participants ($n = 20$) who provided both focal and full-field data, of the repeatability and between eye agreement of the peripheral only response (with focal contribution subtracted) (see Tables 3.3 and 3.4). It can be seen that, across the subset of participants from whom focal data were obtained, the peripheral only amplitude response was approximately 85 - 88% the magnitude of the full field response for the transient cone a-wave, 84 - 89% for the b-wave, and 89 - 94% for the first harmonic of the flicker response.

Between Visit Comparison			Visit 1	Visit 2	CoR		p	Bonferroni Corrected p	N
			Mean ($\pm$ SD)	Mean ($\pm$ SD)	Value	as % of mean			
Transient Cone	a-wave	Amp (μ V)	-32.42 ($\pm$ 10.67)	-31.41 ($\pm$ 7.77)	20.08	63**	0.60	1.00	29
		TtP (ms)	14.31 ($\pm$ 0.75)	14.34 ($\pm$ 0.57)	0.87	6	0.68	1.00	
	b-wave	Amp (μ V)	115.63 ($\pm$ 28.84)	114.15 ($\pm$ 23.88)	43.18	38	0.72	1.00	29
		TtP (ms)	30.69 ($\pm$ 1.10)	30.72 ($\pm$ 1.20)	0.94	3*	0.70	1.00	
PhNR	0.11 cd.s.m ⁻²	Amp (μ V)	-15.58 ($\pm$ 3.83)	-15.54 ($\pm$ 4.03)	6.05	39	0.96	1.00	20
		TtP (ms)	70.58 ($\pm$ 9.62)	71.48 ($\pm$ 5.91)	13.47	19	0.57	1.00	
	0.21 cd.s.m ⁻²	Amp (μ V)	-19.02 ($\pm$ 4.95)	-19.04 ($\pm$ 5.03)	6.21	33	0.98	1.00	17
		TtP (ms)	69.32 ($\pm$ 7.32)	69.56 ($\pm$ 6.17)	9.92	14***	0.85	1.00	
	0.42 cd.s.m ⁻²	Amp (μ V)	-26.17 ($\pm$ 6.96)	-26.33 ($\pm$ 6.46)	9.74	37	0.89	1.00	20
		TtP (ms)	69.93 ($\pm$ 10.07)	69.78 ($\pm$ 8.72)	20.04	29	0.95	1.00	
	0.84 cd.s.m ⁻²	Amp (μ V)	-29.63 ($\pm$ 7.38)	-28.35 ($\pm$ 6.51)	9.00	31	0.23	1.00	20
		TtP (ms)	72.03 ($\pm$ 11.48)	67.63 ($\pm$ 6.95)	16.67	24	0.03	0.54	
	1.68 cd.s.m ⁻²	Amp (μ V)	-30.78 ($\pm$ 7.37)	-28.66 ($\pm$ 5.43)	11.31	38	0.13	1.00	19
		TtP (ms)	71.74 ($\pm$ 8.35)	69.82 ($\pm$ 9.01)	21.21	30	0.45	1.00	
	3.35 cd.s.m ⁻²	Amp (μ V)	-29.64 ($\pm$ 8.57)	-29.37 ($\pm$ 9.94)	12.78	43****	0.86	1.00	17
		TtP (ms)	72.79 ($\pm$ 11.19)	69.15 ($\pm$ 10.16)	19.62	28	0.15	1.00	
Flicker		Amp (μ V)	39.37 ($\pm$ 16.44)	40.45 ($\pm$ 14.70)	22.44	56	0.65	1.00	24
		TtP (ms)	8.04 ($\pm$ 1.68)	8.23 ($\pm$ 1.54)	1.60	20	0.27	1.00	

Table 3.1. Displaying the mean ($\pm$ SD), Coefficient of Repeatability (CoR), the CoR expressed as a percentage of the mean, p value, and n tested for each ERG parameter in the between visit analysis. Most () and least (**) repeatable overall are indicated with asterisks. Most (***) and least (****) repeatable of the PhNR parameters are indicated with additional asterisks. Only one of the parameters was significantly different between visits, PhNR 0.84 cd.s.m⁻² TtP (p = 0.03). This did not reach significance after Bonferroni correction.*

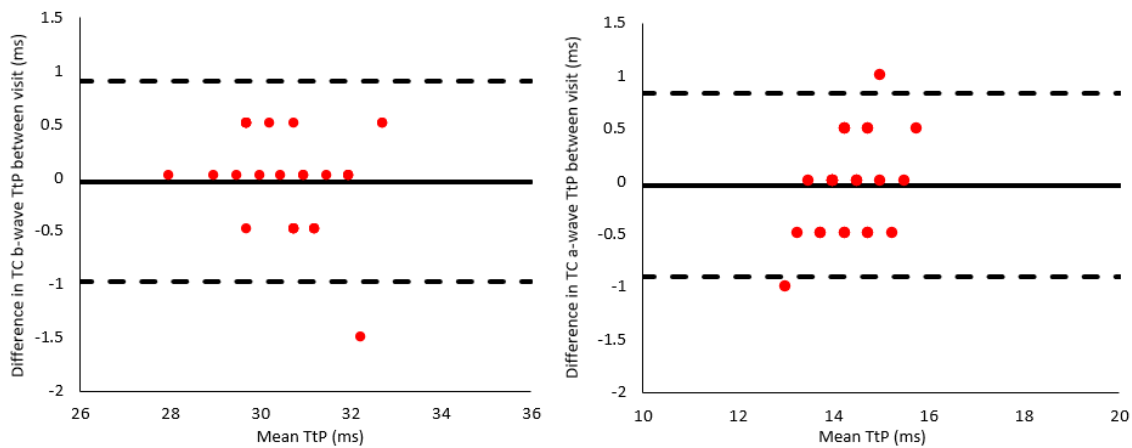


Figure 3.17. Bland and Altman plots showing the two most repeatable parameters for the between visit analysis; transient cone b-wave Time to Peak (left) and transient cone a-wave Time to Peak (right). The black dotted lines indicate the limits of agreement. The solid line represents the bias.

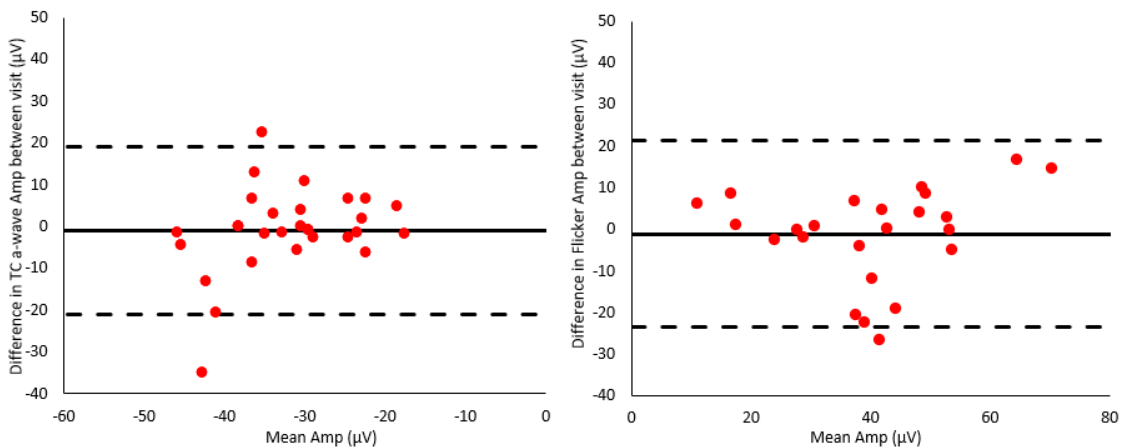


Figure 3.18. Bland and Altman plots showing the two least repeatable parameters for the between visit analysis; transient cone a-wave amplitude (left), and 41 Hz Flicker ERG amplitude(right). The black dotted lines indicate the limits of agreement. The solid line represents the bias.

Between Eye Comparison			RE	LE	CoA		p	Bonferroni Corrected p	n
			Mean ($\pm$ SD)	Mean ($\pm$ SD)	Value	as % of mean			
Transient Cone	a-wave	Amp (μ V)	-32.42 ($\pm$ 10.67)	-30.63 ($\pm$ 8.88)	16.89	54**	0.27	1.00	29
		TtP (ms)	14.31 ($\pm$ 0.75)	14.33 ($\pm$ 0.54)	0.96	7	0.85	1.00	
	b-wave	Amp (μ V)	115.63 ($\pm$ 28.84)	115.40 ($\pm$ 29.33)	37.27	32	0.95	1.00	29
		TtP (ms)	30.69 ($\pm$ 1.10)	30.60 ($\pm$ 1.09)	0.53	2*	0.10	1.00	
PhNR	0.11 cd.s.m ⁻²	Amp (μ V)	-17.01 ($\pm$ 4.24)	-15.35 ($\pm$ 3.07)	8.07	50****	0.12	1.00	17
		TtP (ms)	74.97 ($\pm$ 12.64)	68.91 ($\pm$ 11.17)	24.72	34	0.07	1.00	
	0.21 cd.s.m ⁻²	Amp (μ V)	-18.82 ($\pm$ 4.98)	-20.03 ($\pm$ 4.51)	4.97	26	0.05	0.90	20
		TtP (ms)	70.05 ($\pm$ 7.38)	68.40 ($\pm$ 8.56)	16.59	24	0.39	1.00	
	0.42 cd.s.m ⁻²	Amp (μ V)	0.39 ($\pm$ 6.76)	-23.89 ($\pm$ 6.43)	6.31	26	0.07	1.00	18
		TtP (ms)	73.14 ($\pm$ 11.25)	68.06 ($\pm$ 9.37)	24.86	35	0.11	1.00	
	0.84 cd.s.m ⁻²	Amp (μ V)	-28.19 ($\pm$ 7.01)	-27.59 ($\pm$ 8.27)	8.80	32	0.59	1.00	17
		TtP (ms)	71.26 ($\pm$ 8.97)	68.50 ($\pm$ 8.27)	16.89	24	0.20	1.00	
	1.68 cd.s.m ⁻²	Amp (μ V)	-29.68 ($\pm$ 6.52)	-29.88 ($\pm$ 7.25)	10.23	34	0.88	1.00	17
		TtP (ms)	70.79 ($\pm$ 7.80)	72.74 ($\pm$ 10.21)	18.75	26	0.42	1.00	
	3.35 cd.s.m ⁻²	Amp (μ V)	-27.97 ($\pm$ 6.65)	-27.03 ($\pm$ 7.10)	11.49	42	0.53	1.00	16
		TtP (ms)	73.13 ($\pm$ 7.94)	74.50 ($\pm$ 10.48)	11.68	16***	0.37	1.00	
Flicker		Amp (μ V)	39.37 ($\pm$ 16.44)	37.64 ($\pm$ 14.50)	15.34	40	0.29	1.00	24
		TtP (ms)	8.04 ($\pm$ 1.68)	8.04 ($\pm$ 1.61)	0.96	12	1.00	1.00	

Table 3.2. Displaying the mean ($\pm$ SD), Coefficient of Agreement (CoA), the CoA expressed as a percentage of the mean, p value, and n tested for each ERG parameter in the between eye comparison analysis. Most (*) and least (**) repeatable overall are indicated with asterisks. Most (***) and least (****) repeatable of the PhNR parameters are indicated with additional asterisks. None of the parameters for the right eye were significantly different to the left eye after Bonferroni correction.

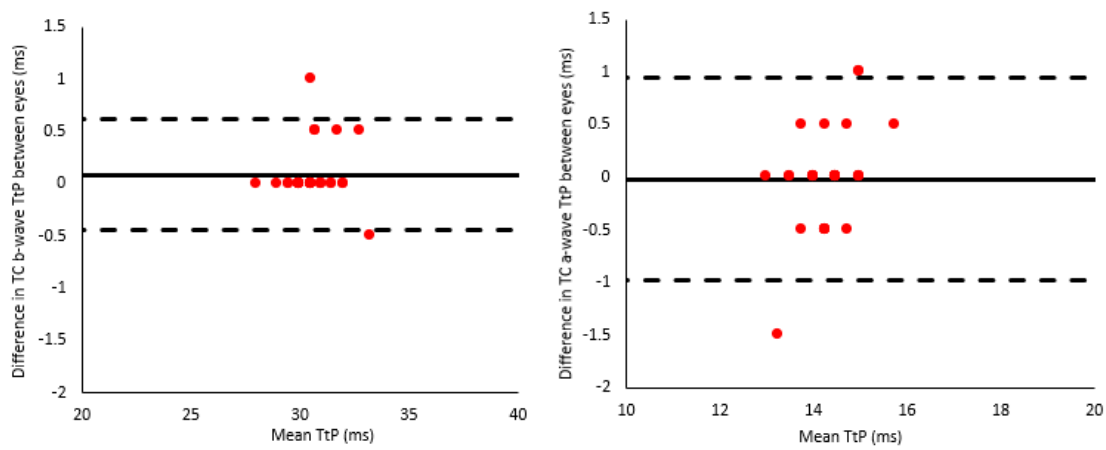


Figure 3.19. Bland and Altman plots showing the most consistent parameters for the between eye analysis; transient cone b-wave Time to Peak (left), and transient cone a-wave Time to Peak (right). The black dotted lines indicate the limits of agreement. The solid line represents the bias.

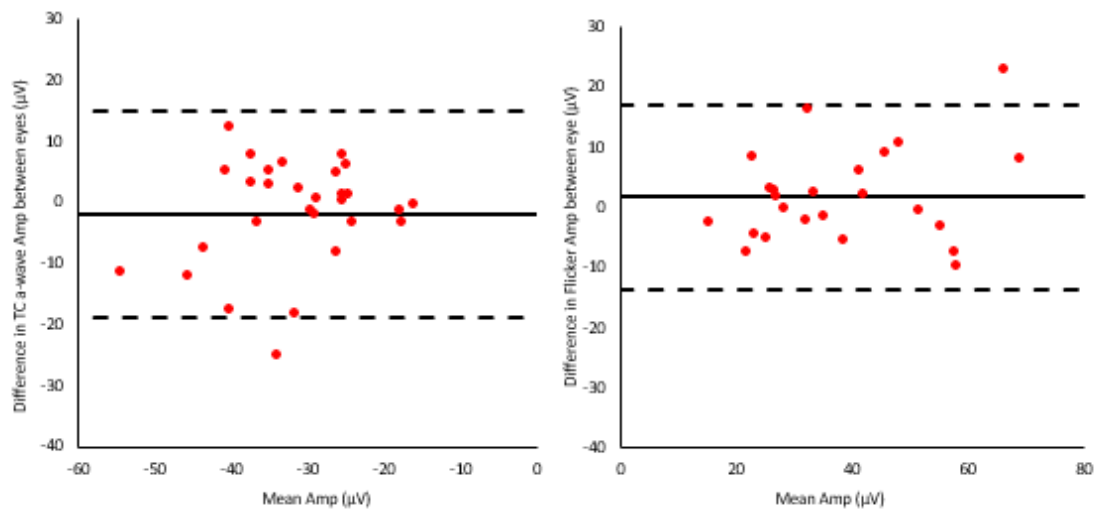


Figure 3.20. Bland and Altman plots showing the least consistent parameters for the between eye analysis; transient cone a-wave Amplitude (left), and 41 Hz Flicker Amplitude (right). The black dotted lines indicate the limits of agreement. The solid line represents the bias.

Between Eye Comparison			RE			LE			Peripheral Only Subtracted Response				
			Full Field Response Mean (SD)	Peripheral Only Subtracted Response Mean ($\pm$ SD)	% of full field response	Full Field Response Mean (SD)	Peripheral Only Subtracted Response Mean ($\pm$ SD)	% of full field response	CoA		p	Bonferroni Corrected p	n
									Value	as % of mean			
Transient Cone	a-wave	Amp (μ V)	-33.85 ($\pm$ 11.57)	-29.65 ($\pm$ 10.32)	88	-30.73 ($\pm$ 8.06)	-26.02 ($\pm$ 8.13)	85	19.44	70**	0.12	0.72	20
		TtP (ms)	14.35 ($\pm$ 0.67)	14.43 ($\pm$ 0.69)	101	14.35 ($\pm$ 0.59)	14.15 ($\pm$ 0.67)	99	1.67	12	0.17	1.00	20
	b-wave	Amp (μ V)	116.70 ($\pm$ 32.15)	103.70 ($\pm$ 27.95)	89	115.41 ($\pm$ 28.64)	97.30 ($\pm$ 27.59)	84	44.81	45	0.22	1.00	20
		TtP (ms)	30.53 ($\pm$ 1.08)	30.53 ($\pm$ 1.11)	100	30.40 ($\pm$ 0.98)	30.38 ($\pm$ 1.02)	100	0.46	2*	0.01	0.06	20
Flicker		Amp (μ V)	49.66 ($\pm$ 15.49)	44.22 ($\pm$ 16.09)	89	49.08 ($\pm$ 12.75)	44.41 ($\pm$ 13.83)	90	14.98	34	0.92	1.00	19
		TtP (ms)	8.44 ($\pm$ 1.65)	8.37 ($\pm$ 1.82)	99	8.47 ($\pm$ 1.48)	8.45 ($\pm$ 1.68)	100	0.67	8	0.33	1.00	19

Table 3.3. Peripheral Only Responses: Displaying the Mean ($\pm$ SD), Coefficient of Agreement (CoA), the CoA expressed as a percentage of the mean, p value, and n tested for each ERG parameter with the focal response subtracted in the between eye comparison analysis. Most () and least (**) agreeable overall are indicated with asterisks. Only one parameter was significantly different between eyes but this was not sustained after Bonferroni correction.*

Between Visit Comparison			Visit 1			Visit 2			Peripheral Only Subtracted Response				
									CoR		p	Bonferroni Corrected p	n
			Full Field Response Mean (SD)	Peripheral Only Subtracted Response Mean ($\pm$ SD)	% of full field response	Full Field Response Mean (SD)	Peripheral Only Subtracted Response Mean ($\pm$ SD)	% of full field response	Value	as % of mean			
Transient Cone	a-wave	Amp (μ V)	-33.85 ($\pm$ 11.57)	-29.65 ($\pm$ 10.32)	88	-31.15 ($\pm$ 8.30)	-27.22 ($\pm$ 7.91)	87	19.48	69**	0.29	1.00	20
		TtP (ms)	14.35 ($\pm$ 0.67)	14.43 ($\pm$ 0.69)	101	14.35 ($\pm$ 0.54)	14.38 ($\pm$ 0.65)	100	0.70	5	0.54	1.00	20
	b-wave	Amp (μ V)	117.21 ($\pm$ 31.67)	103.70 ($\pm$ 27.95)	88	112.16 ($\pm$ 24.29)	99.98 ($\pm$ 22.26)	89	37.76	37	0.40	1.00	20
		TtP (ms)	30.53 ($\pm$ 1.08)	30.53 ($\pm$ 1.11)	100	30.58 ($\pm$ 1.13)	30.53 ($\pm$ 1.13)	100	0.95	3*	1.00	1.00	20
41 Hz Flicker		Amp (μ V)	49.66 ($\pm$ 15.49)	46.95 ($\pm$ 14.61)	94	45.29 ($\pm$ 12.98)	42.68 ($\pm$ 12.57)	94	13.84	31	0.02*	0.12	17
		TtP (ms)	8.44 ($\pm$ 1.65)	8.65 ($\pm$ 1.68)	102	8.32 ($\pm$ 1.35)	8.62 ($\pm$ 1.51)	104	0.88	10	0.79	1.00	17

Table 3.4. Peripheral Only Responses: Displaying the mean ($\pm$ SD), Coefficient of Repeatability (CoR), the CoR expressed as a percentage of the mean, p value, and n tested for each ERG parameter with the focal response subtracted in the between visit comparison analysis. Most (*) and least (**) repeatable overall are indicated with asterisks. Only one parameter was significantly different between eyes but this did not reach significance after Bonferroni correction.

3.4.4. Discussion

Overall, repeatability data were collected from most of the 30 participants across the three ERG protocols. The transient cone ERG protocol was well tolerated by all participants, although one set of data was excluded due to an eye movement. Some individuals struggled to control their blink reflex in response to the 41 Hz flickering stimulus, resulting in contamination of ERG traces with biological artefacts. This meant that only 24 of the possible 30 participants contributed two sets of usable data in response to this stimulus.

When focal responses were subtracted from the transient and flicker responses, in the subset of 20 participants for whom these data were available, the amplitudes were reduced in magnitude by an average of 11%. The median (IQR) percentage reduction in amplitude was 11 (11,12), maximum and minimum reductions were 16% and 6%, respectively. A recent estimate using adaptive optics scanning laser ophthalmoscopy suggested that there is an average of 221,000 cones in a circular area of the retina centred on the fovea with a diameter of 14 degrees (i.e. somewhat smaller than the 20 degree diameter focal stimulus used in this study; Elsner et al. 2017). This would be approximately 3.5% of the total 6,400,000 cone photoreceptors estimated to populate the retina according to Osterberg et al. (1935). Figure 2c of Morny et al. (2019) suggests that the cumulative number of cones within an area of 314 deg^2 centred on the fovea (i.e. the area of the circular macular region with a diameter of 20 degrees) is approximately 500,000 (i.e. 7.8% the total cone population estimated by Osterberg et al. 1935). These figures are in line with the proportional contribution of the macular region to the full-field photopic ERG calculated in this study. It is clear that, even for the photopic system in which the density of cone photoreceptors is greatest in the central retina (Osterberg et al., 1935), the full-field response is dominated by the contribution of the extramacular retina.

PhNR data were collected at a majority of stimulus intensities in all but 2 of 30 participants. However, at each individual intensity usable data were only collected at both visits for a maximum of 20 individuals. Participants struggled in particular to control their eye movements and blink reflex after the stimulus intensity increased beyond 0.84 cd.s.m^{-2} (for example, only 17/30 provided data for the repeatability analysis at a stimulus intensity of 3.35 cd.s.m^{-2}). The brighter flashes resulted in

involuntary eyelid twitches which presented a repeatable but artefactual drift in the ERG traces. This may explain why the CoR was worst in the amplitude parameters of the PhNR intensity traces with flash intensities over 0.84 cd.s.m⁻². The two participants who could not control their blink reflex at any intensity had their tests abandoned at the point where they expressed discomfort or were producing such large artefacts with every flash that all responses were rejected by the artefact rejection system.

The PhNR response amplitude increased with intensity as expected, reaching a maximum at approximately 1.68 cd.s.m⁻² (see Figure 3.16). A similar saturation of the response amplitude has been reported by other studies at a similar stimulus intensity (Binns et al., 2011; Rangaswamy et al., 2007; Sustar et al., 2009; Viswanathan et al., 2001). Other researchers have similarly reported issues with blink artefacts/eyelid twitches when recording PhNR responses across a similar range of stimulus intensities (Sustar et al., 2011). Had it been possible to reliably obtain intensity response data across all intensities from the majority of participants, the data would have been modelled using the Naka Rushton function (Equation 3-4; Fulton and Hansen, 1988).

$$V = (V_{max} \cdot I^n) / (I^n + K^n)$$

Equation 3-4. Calculation of the Naka Rushton Function where V = response amplitude; Vmax = the maximum amplitude; I = stimulus intensity; K = the semi-saturation constant i.e. stimulus intensity at which the amplitude is half of Vmax; n = an exponent.

This is a hyperbolic curve which has been used to describe the increase in response amplitude of the ERG b-wave (Fulton and Hansen, 1988), d-wave (Sustar et al., 2008), and the PhNR (Binns et al., 2011) with increasing stimulus intensity. The parameters describing this model (Vmax, K, and n) are thought to reflect different aspects of retinal function and so may be affected differentially by disease (Fulton and Hansen, 1988). For example, a reduction in Vmax (maximal response amplitude) for the scotopic b-wave is thought to reflect loss of photoreceptors (Arden et al., 1983; Fulton and Hansen, 1988), while the parameter K (semi-saturation constant) is likely to be influenced by a reduced sensitivity of the photoreceptors (Hood et al., 1994). It may be expected that the parameters of the PhNR intensity-response function would similarly

reveal insights into different aspects of inner retinal function. Given that it was only possible to obtain a full intensity response function in both eyes on both occasions from 11 participants (37%), Naka Rushton modelling has not been used in this PhD. However, a loss of sensitivity of the RGCs, and a consequent change in the slope of the intensity response function (described by Naka Rushton parameters K and n), may result in the response being differentially affected at different stimulus intensities. We have, therefore, chosen to continue recording across the range of intensities throughout the other studies in this PhD in order to compare the effects of treatment on responses recorded at each intensity.

For pragmatic reasons, focal PhNR responses were not recorded (the burden of time and fatigue to the patient would be too great). Morny et al. (2015) recorded the PhNR under long duration stimulus conditions (in order to separate ON and OFF RGC contributions) from the full-field and the macular area (subtending 20 degrees of visual angle). In their control group, the PhNR ON response of the macular area was 24.3% of the magnitude of the full-field response. This suggests that allowance should be made for the macula contributing as much as a quarter to the total amplitude of the full-field PhNR response.

Measures of time to peak were consistently more repeatable (between eye as well as between session) than those of amplitudes across all ERG protocols. This was expected as other studies have also reported a higher variance amongst measures of amplitude than those of time to peak (Pescosolido et al., 2014; Fortune et al., 2004; Strain et al., 2001; Mortlock et al., 2010). The CoR may be considered to be the minimum change in each parameter required to exceed the measurement error. The CoR values are broadly of the same magnitude or lower than in previous human studies (Tang et al., 2014; Mortlock et al., 2010). For example, to a stimulus of 1.5 cd.s.m^{-2} , of similar wavelength properties to the PhNR stimulus used here, Mortlock et al. (2010) recorded a CoR of $20.75 \mu\text{V}$ and 36.67 ms for the PhNR baseline to trough measurement. This compares to a CoR of $11.31 \mu\text{V}$ and 21.21 ms for the PhNR amplitude and time to peak in our study when using a stimulus of 1.68 cd.s.m^{-2} .

In all parameters bar one it was found that there was no significant difference between the results of the two visits. Although a significant difference was found in the PhNR intensity response time to peak at 0.84 cd.s.m^{-2} , this did not reach

significance after Bonferroni correction. This indicates that there was no bias between visits e.g. due to learning effect.

3.4.5. Conclusion

In conclusion, the measures of time to peak were consistently more repeatable than those of amplitude. It was also noted that as the PhNR intensity response series caused some volunteers to struggle to control their blink reflexes, it would be preferable to perform this test first of all the protocols to prevent fatigue further impacting upon data quality. The data presented here will be of value in future longitudinal studies evaluating the change in ERG parameters over time during anti-VEGF treatment, where a change in any response beyond the CoR may be considered to be of significance. With respect to the cross-sectional study presented in Chapter 5 of this PhD, the interocular CoA will be valuable in determining a difference between eyes which may be considered to be beyond the inherent measurement error of the technique.

Chapter 4. The RETeval® System - a repeatability and agreement study

4.1. Introduction

Countless advances have been made in the field of electroretinography since Emile Dubois discovered the standing potential of the eye in 1849. Procedures have progressed from being invasive and experimental to minimally so and clinically applicable. In terms of full field flash ERGs, the gold standard stimulator is a Ganzfeld bowl (an evenly illuminated hemispherical bowl with a chin rest) to encompass the patient's full field of view (McCulloch, 2015). This is driven by an evoked potential delivery and monitoring system such as the Espion™ (Diagnosys LLC, Cambridge, UK), which is used in diagnostic and research environments worldwide. The Espion™ is versatile with respect to programming and may be used alongside multifocal, pattern or Ganzfeld stimulators. The ColorDome Ganzfeld stimulator (Diagnosys LLC, Cambridge, UK; see Figure 4.1) contains LEDs and a xenon flash unit, allowing for a range of coloured and achromatic, and long and short duration stimuli to be generated. The versatility of this type of system also extends to the type of electrodes one can use with it; Burian Allen contact lens (Burian and Allen, 1954), gold foil (Arden et al., 1979), Dawson Trick Litzkow (DTL) fibre (Dawson et al., 1979), and skin electrodes (Motokawa and Mita, 1942) are all compatible with this system. Despite these advantages there are drawbacks to desktop-based systems such as the Espion™/ColorDome system, for example limited portability complicates testing of less mobile patients, and the time required for measurements to be taken. On average, this includes 20-30 minutes adaptation for a full ISCEV standard full field flash protocol, in addition to time taken for patient dilation, electrode fitting, instruction, and obtaining the responses.



Figure 4.1. Images showing the two types of ERG stimulator device used in this study. A; ColorDome Ganzfeld stimulator by Diagnosys, Cambridge, UK. B; RETeval® ERG system by LKC Technologies, Inc., Gaithersburg, MD, USA.

The RETeval® (LKC Technologies, Inc., Gaithersburg, MD, USA) is a handheld device for recording the full-field electroretinogram (ERG), which aims to improve the ease of ERG recording in a general clinical or office environment (Liu et al., 2018). Data have been collected using the device in wide ranging conditions including diabetic retinopathy (Maa et al., 2016; Fukuo et al., 2016; Degirmenci et al., 2018), cataract (Miura et al., 2016), retinal vein occlusion (Yasuda et al., 2016; Miyata et al., 2018), and hereditary and acquired retinal disorders (Nakamura et al., 2016; Grace et al., 2017; Liu et al., 2018). The 'RETeval® Complete' is compliant with the ISCEV standards published in 2009 (Marmor et al., 2009; LKC Technologies, 2015). Equipped with a 60mm Ganzfeld dome and an infrared camera system (for monitoring the subject's gaze and pupil size) it is designed to record monocular full-field ERGs.

The RETeval® generates pre-stimulus and background lights by showing flashes at 283 Hz (perceived as continuous illumination), whilst stimuli comprise brief flashes of light (LKC Technologies, 2015). The visual stimuli are created by blue, green and red LEDs, often all presented together to form white light (maximum flash energy of 30 cd.sm² and duration 5 ms – although duration can be extended for pupil sizes smaller than 1.9 mm in order to achieve the required retinal illuminance). The device

contains a pupillometer which measures the pupil diameter throughout the protocol and constantly adjusts the strength of the flash according to Equation 4-1.

$$\text{Retinal illuminance (Troland [Td])} = (\text{pupil area in mm}^2) \times (\text{luminance in cd/m}^2)$$

Equation 4-1. The equation used to calculate retinal illuminance in Trolands.

This allows relatively constant retinal illuminance (as required by the ISCEV standard protocol [Marmor et al., 2009; McCulloch et al., 2015]) to be maintained regardless of the test subject's pupil size, and renders pupil dilation unnecessary. The RETeval® uses the assumption that the average dilated pupil diameter is 6 mm, with an area of 28.3 mm², when calculating the retinal illuminance of a stimulus or background in Td. For example, the light adapted 3.0 ERG has a flash luminance of 3 cd·s/m², the Td equivalent to this would be (3 cd·s/m² × 28.3 mm²) = 85 Td·s. Where pupil diameter exceeds or falls below 6 mm, the device adjusts the luminance to maintain a constant retinal illuminance.

However, there is evidence to suggest that the effective retinal illuminance delivered by the RETeval® system decreases for the largest pupil sizes (Kato et al., 2015; Kato et al., 2017). For example, Kato et al. (2015) evaluated the effect of pharmacological pupil dilation on the characteristics of the flicker ERG recorded using the RETeval® system in 10 healthy volunteers. The mean pupillary area gradually increased to become approximately 3.2 times greater post dilation than that at baseline. If no correction was made for pupil diameter, retinal illuminance would be expected to increase by a magnitude approximately equivalent to the increase in pupil area (according to Equation 4-1), with a consequent increase in response amplitude and reduction in implicit time until saturation. The authors reported no significant change in the amplitude of the fundamental component during pupillary dilation, however, implicit time became significantly longer for responses recorded beyond 15 minutes post dilation. This meant that a relatively constant retinal illuminance was maintained by the RETeval® until pupil diameter reached approximately 6.5mm (when pupil area was approximately 2.25x the mean area of the undilated pupil), at which point retinal illuminance appeared to be relatively reduced compared to the smaller pupil diameters.

A likely explanation for this is that Equation 4-1, and the calculation employed by the RETeval® to compensate for changes in pupil area, does not take account of the Stiles Crawford effect, whereby light rays entering the eye towards the periphery of the pupil have less effectiveness than those entering the centre of the pupil at stimulating the photopic visual system (Stiles and Crawford, 1933). As a result of this phenomenon, photopic illuminance calculated using trolands is an overestimate for large pupils. Hence, for a pupil of 8 mm diameter, the RETeval® calculates pupil area to be 50.3 mm², compared to the 28.3 mm² of the 'standard' pupil. Thus, it would reduce stimulus luminance by a magnitude of 1.78x in order to maintain a constant retinal illuminance according to Equation 4-1. However, according to Paupoo et al. (2000), retinal illuminance of the maximally dilated pupil should be considered to be 20 mm² when calculating retinal illuminance, to allow for the Stiles Crawford effect, suggesting that retinal illuminance should not be adjusted at all for pupil areas exceeding this.

Thus, the RETeval® is overcompensating for increased pupil diameter at the upper end of the range by correcting for the actual pupil area rather than the area corrected for the Stiles Crawford effect. This is unlikely to have a bearing on responses recorded using the natural pupil, but is likely to have a significant effect on implicit times of responses recorded using an artificially dilated pupil. Kato et al. (2015) also suggest that the distribution of the stimulus illumination across the retina may not remain constant for different pupil diameters, which may impact on the waveform recorded.

To minimise invasiveness, the RETeval® device is designed for use with manufacturer-specific skin surface 'sensor strip' electrodes. These consist of a single strip containing the earth, reference and active electrodes in one unit. However, it is possible to use other electrodes with the device if higher amplitude responses are required (LKC Technologies, 2018). The use of periorbital skin surface electrodes to record the electroretinogram has a long history, first described in 1942 (Motokawa and Mita, 1942), and reported in numerous publications since (for example, Noonan et al., 1973; Zhang et al., 1983; France, 1984; Esakowitz et al., 1993; Kriss, 1994; Bradshaw et al., 2004; Mortlock et al., 2010). Response amplitudes obtained with periorbital skin surface electrodes are substantially smaller than those recorded with corneal or conjunctival electrodes (France, 1984; Esakowitz et al., 1993; Kriss, 1994; Bradshaw et al., 2004; Mortlock et al., 2010), and electrode position and direction of patient's gaze

can have a significant effect on response amplitudes (Noonan et al., 1973; Zhang et al., 1983; Kriss, 1994). Accordingly, it has also been reported that responses obtained using the RETeval® Sensor Strip electrodes are lower in amplitude than those obtained using active electrodes which are in contact with the cornea or bulbar conjunctiva (Nakamura et al., 2016, Tang et al., 2018).

The disposable, single use RETeval® skin surface electrodes are provided in pairs with one specific to the right and one to the left eye. The manufacturer recommends that the nasal edge of the sensor strip should be aligned with the midline of the pupil and the superior edge of the strip should be placed 2 mm below the patient's lower lid margin (Figure 4.2). This type of electrode generally requires very little application time and was anecdotally reported by patients and participants to the researcher to be more comfortable than electrode types that make contact with the ocular surface. Although there is little to no literature to officially support this observation specifically in regards to ERGs, studies have shown that patients feel anxiety over the idea of similar procedures involving other medical devices (contact lenses) touching the anterior ocular surface (Hutchinson, 2001) and in eye care appointments in general (Margrain et al., 2003). This may be because there is a perceived risk of sight loss due to the discomfort caused by such procedures (Ettinger 1994; Shute 1996). However, some participants have also reported to the author a mild discomfort with the RETeval® skin surface electrodes when placed in the recommended position due to close proximity to the lower lid margin and eyelashes. Although the electrode is not touching the ocular surface, the proximity of the electrode to the lower lid margin could elicit an anxiety response (e.g. increased blinking rate) due to some kind of perceptive anticipation of a real, physical danger to the participant's eyes and sight (Edelman, 1992). This is discussed further in Section 4.3. There has been no systematic

evaluation of the effect of positioning of the Sensor Strip electrodes on response amplitude or waveform.



Figure 4.2. Participant wearing RETeval® specific electrodes.

The idea of an easily portable system, which does not require pupil dilation and uses minimally invasive electrodes, but which is able to provide standard ISCEV responses is an attractive one. However, limited repeatability data have been published on the RETeval® system (Wu et al., 2016; Asakawa et al., 2017; Tang et al., 2018; Liu et al., 2018). Both Wu (2016) and Tang et al. (2018) evaluated the between session repeatability of PhNR responses. Tang et al. (2018) reported a proportionally similar level of intersession repeatability for the PhNR protocol between the sensor skin electrodes (Coefficient of Repeatability [CoR] of $\pm 28\%$) and DTL fibre electrodes ($\pm 27\%$) used with the RETeval® system, despite the consistently lower amplitude of the former. Asakawa et al. (2017) and Liu et al. (2018) evaluated intrasession reproducibility of a range of ERG protocols using the RETeval® system by comparing two waveforms produced within one visit from the same participants and calculating intraclass correlation coefficients (ICC). Asakawa et al. (2017) reported ICC for the left eyes of all participants ($n = 50$) to be between 0.48 and 0.92 for different parameters (cone response - a-wave amplitude 0.71, a-wave implicit time 0.48, b-wave amplitude 0.87, b-wave implicit time 0.76; flicker response – amplitude 0.92, implicit time 0.91; rod response – amplitude 0.83, implicit time 0.77; maximal response - a-wave amplitude 0.72, a-wave implicit time 0.51, b-wave amplitude 0.92, b-wave implicit time 0.63; oscillatory potentials - amplitude 0.87, implicit time 0.85). This may be considered to range from a ‘moderate’ to ‘excellent’ degree of reliability (Koo and Li, 2016). Similarly, Liu et al. (2018) reported amplitudes measured by the RETeval® device have a high degree of reliability (average ICC 0.79, range 0.58-0.92) whilst implicit times had a moderate degree of reliability (average ICC 0.52, range 0.28-0.99).

However, between-session repeatability has not been reported for the photopic ISCEV equivalent tests conducted using the RETeval® system. This information is not only necessary to this PhD, which aims to employ both types of system in patient evaluation, but is also valuable to clinicians and researchers who may wish to incorporate the RETeval® in their practice.

Hence, the first aim of this chapter was to investigate the repeatability of the RETeval® system used with the skin sensor strip electrodes, and to compare this to the repeatability of the gold standard Espion™ system used with conjunctival DTL electrodes for recording ISCEV photopic responses. It was hypothesised that the absolute CoR would be higher for the desk-based Espion™ system, purely on the basis of the larger anticipated response amplitude. However, by determining the CoR as a percentage of the maximum response amplitude for each system, we aimed to determine whether the smaller response amplitudes of the RETeval® were associated with a proportionally greater level of response variability.

Published ISCEV standards recommend the recording of a 'light-adapted 30 Hz flicker ERG' as part of the photopic ERG protocol (McCulloch et al., 2015). The RETeval® system uses a non-standard 28.3 Hz stimulus for the ISCEV flicker protocol (although this falls within the acceptable range of 28-33 Hz outlined by the standard; both in Marmor et al., 2009, cited by the device manual, and in the more recent update of these standards published by McCulloch et al., 2015). In contrast, the flicker ERG used in the studies of this PhD which employed the desk-based ERG acquisition system (see Chapters 3 and 5) were based on an ERG photostress test protocol, which employed a stimulus with a temporal frequency of 41 Hz (Binns and Margrain, 2007). Hence, a secondary aim of this chapter was to investigate the effect of modest changes in stimulus frequency on the characteristics of the flicker response.

Given the special design of the RETeval® skin electrodes, incorporating active, reference and ground electrodes in a single strip, a final aim was to examine the effect of varying skin surface electrode position relative to the eye, on amplitudes and times to peak of standard light-adapted responses (designed to correspond to ISCEV standard stimuli [McCulloch et al., 2015]) recorded using the RETeval® stimulator.

4.2. An evaluation of the repeatability and agreement of the Espion™ and

RETeval® ERG systems

It is well documented that recorded ERG amplitude decreases with increasing electrode distance from the corneal apex (McCulloch et al., 1997; Mentzer et al., 2005; Kriss et al., 1994; Hamilton et al., 2015; Mortlock et al., 2010; Kurtenbach et al., 2017). As a result, we can hypothesise that the mean ERG amplitude will be reduced when using the RETeval® device with the skin sensor strip electrodes positioned below the lower lid margin compared to the data obtained from the Espion™/ColorDome system using the DTL electrode positioned inside the lower fornix. However, it is not clear whether this reduction in amplitude is also associated with an increased between-session and between-individual variability. This study evaluated the variability of data collected using the two systems, using comparable recording protocols. The data collected for this study were also used to address the second aim of this chapter, to evaluate the effect of stimulus frequency on the characteristics of the light adapted flicker response.

4.2.1. Aims

The specific aims of this study were to;

- assess the between session repeatability of the ERG signals recorded in response to full field flash stimuli presented in the portable RETeval® device using skin sensor strip electrodes and the standard desk top system, the Espion™ and ColorDome™, used in conjunction with DTL fibre electrodes.
- assess the agreement between ERG signals obtained using the same protocol on the RETeval® and Espion™/ColorDome systems to see if there is any clinically significant difference between responses obtained.
- examine the effect of adjusting flicker frequency on ERG signals obtained from both devices.

4.2.2. Methods

Participants

Ethical approval was gained through the City, University of London Optometry Proportionate Review Committee 'Opt/PR/23' and written informed consent was obtained from all subjects, conforming to the tenets of the Declaration of Helsinki. The study took place at the Division of Optometry and Visual Science at City, University of London. Participants were permitted to be included in the study if they were aged between 18 and 60 years, had a best corrected visual acuity of 6/12 or better in each eye, had no known ocular pathology and had sufficiently clear ocular media (Lens Opacities Classification System [LOCS] III grading scale of grade <2, Chylack et al., 1993), adequate fixation, and adequate pupillary dilation after instillation of one drop of 0.5% or 1.0% tropicamide to enable observation of the fundus and acquisition of high quality fundus photographs. Participants were excluded if they had any ocular pathology in either eye that could contribute to any sort of visual impairment, history of medications known to affect visual function (such as chloroquine or tamoxifen), history of photosensitive epilepsy, narrow anterior chamber angles, or systemic disease that is significant and known to affect visual function (such as Parkinson's disease, Alzheimer's disease or diabetes mellitus).

Participants were recruited internally at City, University of London via posters and word of mouth. Additionally, advertisement via Gumtree (www.gumtree.com) was utilised to reach a wider audience and recruit participants from outside the University. Both types of written advertisement included the contact details for the chief investigator by telephone and email, and participants were encouraged to approach the researcher if they would like to know more about the study. Following this, a telephone call was arranged to discuss the study in more detail and obtain contact details to send further study information to potentially eligible participants. Participants were sent the Participant Information Sheet so it would arrive at least 48 hours prior to commencement of their first participation appointment.

Outline of participant pathway

Participants attended for recordings on two occasions, the second occurring within a one-month period of the first. At the first visit, the investigator discussed the study

methodology with the participant, and gave them the opportunity to ask any questions before signing a consent form. This was followed by obtaining a structured history and symptoms. Participants then underwent a visual acuity assessment and examination of the anterior and posterior eyes to confirm ocular health and ability to participate - including slit lamp biomicroscope examination, crystalline lens evaluation, Van Herick assessment (van Herick et al., 1969), and measurement of intraocular pressure with a Pulsair IntelliPuff Non-Contact Tonometer (Keeler, UK). Any participant with an IOP exceeding 21 mmHg and/or and anterior chamber angle of < Van Herick grade 2 was excluded from the study at this stage due to the minor risk of inducing angle closure glaucoma (Pandit and Taylor, 2000). Provided a participant met all study criteria during these tests, ERG assessment then followed. Test order was not randomised as it was necessary to perform undilated tests first, before mydriatic drops were instilled.

After baseline tests were completed, RETeval® skin sensors were applied according to the manufacturer's guidelines (LKC Technologies, 2014). Nuprep gel was used to gently exfoliate the skin that would be in contact with the RETeval® device sensor strip skin surface electrodes. The electrode was then applied to the skin 2 mm below the lower lid margin with the nasal edge of the strip directly in line with the centre of the participant's pupil when their eye was in primary gaze position (Figure 4.2). Next, the electrode was attached to the RETeval® device by the cable provided and an automatic impedance/resistance check was performed by the internal software prior to the commencement of the ERG protocol. Any issues with high impedance/resistance were addressed prior to commencing stimulus presentation and ERG recording.

After completion of the RETeval® tests on the undilated eyes, tropicamide hydrochloride was instilled into each eye to induce mydriasis according to the protocol described in Section 3.2.3, and DTL fibre (active) and skin (reference and ground) electrodes applied for standard Ganzfeld ERG recording using the Espion™ system. Electrode application followed standard procedures (McCulloch et al., 2015) according to the methods described in Section 3.2.3. Once each electrode was attached to its relevant position on the amplifier of the Espion™, resistance was checked to ensure the impedance was ≤ 5 k Ω and roughly equal for all electrodes to reduce trace noise by additional artefacts, resistance exceeding 5 k Ω was managed as described in section 3.2.2. Electroretinograms were then obtained using the Espion™ device.

IOP measurements were retaken after Espion™/ColorDome ERG recording was completed. Following this, retinal photographs were taken using a TRC-NW6S Non-Mydriatic Retinal Camera (Topcon medical systems Inc., Tokyo), and 30 x 35 Macular OCT scans were performed using a Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany).

At the second visit, the protocol was identical, except for the omission of baseline tests and imaging (although IOP measurements were repeated). The subset of participants contributing to the study evaluating the effect of flicker frequency all consented to the extraction of data for this purpose on the occasion of their first visit and responses were recorded to additional temporal frequencies on the Espion™ system.

Electroretinogram Equipment and Protocols

All ERGs were recorded using either the Espion™ Ganzfeld Profiling System and ColorDome (Diagnosys, Cambridge) stimulator or the RETeval® device (see Figure 4.1). A full description of the Espion™ Ganzfeld Profiling System and ColorDome and its noise reduction strategies is provided in Section 3.2.3. The system was calibrated by the manufacturer, and self-calibrated each day on initiating the device (Diagnosis LLC, 2020). Additional manual calibration checks were performed using the ILT 1700 Research Radiometer with SED033/Y/R luminance detector (model SED033, Able Instruments and Controls, Reading, UK).

The RETeval® comprises a small Ganzfeld bowl mounted on a plastic handle and casing. The mouth of the Ganzfeld bowl is surrounded by rubber casing designed to fit closely to the participant's orbital rim when in use. Directly behind the Ganzfeld bowl at the rear of the machine is a power switch, joystick control, and a small computer screen. Here, various protocols may be selected for use and patient/participant specific data may be inputted. This screen is able to adapt to appropriate lighting conditions for the duration of dark-adapted protocol and displays ERG traces, averages and prompts or error messages during use. Beneath the Ganzfeld bowl on the device handle is a socket for the electrode connector to be fitted to prior to testing. There are two types of electrode connector wire provided with the device, one designed for use with the device specific skin surface electrodes, and another so that the device may be adapted for use with all other types of traditional ERG electrodes. When not in use the device is

stored in a USB connectable nest which supports the device upright and also provides the opportunity to link to a computer for removal of reports and raw data and for recharging of the device.

LKC technologies advise that noise reduction is imperative in non-flicker protocols due to the relatively low number of flashes involved (and therefore waveforms averaged) by the protocol. Skin preparation is advised to reduce impedance from electrode contact, and the following three methods are employed for detecting and managing excessive electrical noise during recording. The first of these is a disabling of the RETeval® electrodes, triggered if the impedance levels reach over 100 MΩ at any point during the test protocol (the so-called “electrode disconnect” function). The second method uses an algorithm to detect excessive noise, whereby the standard deviation (SD) of the electrical response between 48 Hz and 186 Hz is calculated (described by Wu et al., 2016). Responses are only accepted if this does not exceed 55 μV, as this is set as an internal requirement by the device (Wu et al., 2016). The electrical responses measured by the device are then amplified and digitized (resolution 24 bits, sampling rate 1.95 kHz; LKC Technologies, 2015). Additional flashes are automatically presented to replace the responses rejected by this artefact rejection system. Non-flicker tests for the RETeval® use a high-pass filter (0.3 Hz) to decrease electrode drift and offset. Additionally, a low pass filter (300 Hz) reduces the high frequency noise. The aforementioned filters are zero-phase filters, used to avoid shifts in waveform timing. Multiple flash measurements (total number is defined by the protocol) are averaged to enhance signal to noise ratio and a trimmed mean is used to lessen the effect of outliers, whereby the highest and lowest 25% of responses are excluded to calculate the interquartile mean (after eliminating artefactual responses whose amplitudes surpass 1 mV). Subsequently the waveform generated is managed using wavelet based denoising (Ahmadi and Rodrigo, 2013) where wavelets are tempered based on the signal to noise power between the post-stimulus (signal) and pre-stimulus (noise) parts of the waveform. The average peaks and troughs of waveforms are automatically calculated and displayed by the device across all waveforms collected during the measurement period.

During flicker protocols the background light is generated by providing short flashes of light which occur at 10 times the stimulus frequency. The built-in RETeval® flicker

protocols have a stimulus frequency of roughly 28.3 Hz. Wherever background illumination is required during these protocols a 283 Hz frequency is used (perceived as steady illumination as it is significantly above the human critical fusion frequency). These flicker protocols record on average between 5 and 15 seconds of data for each stimulus condition and use an internal precision metric to determine when to stop recording. The RETeval® was not able to be calibrated throughout the PhD using an external photopic light meter as it was only possible to run the ERG protocol with electrodes connected and an eye detected at the centre of the mouth of the ganzfeld bowl. If these conditions were not met the ERG protocol would not run and no stimulus would be presented. However, the RETeval® device calibrates itself using reflectance from the skin of the participants orbit at the beginning of each protocol and so regular self-calibration was achieved (LKC Technologies, 2015).

The pre-set ISCEV photopic ERG protocol of the RETeval® device was used, which is in accordance with the parameters outlined in the ISCEV full field flash ERG guidelines (McCulloch et al. 2015; LKC Technologies, 2015). Protocols performed on the RETeval® device were run twice on each eye of each participant and the averaged output of each of the runs were viewed superimposed upon each other by the clinician at the end of each part of the protocol for comparison. If any of the averaged traces showed significant artefacts the protocol was run an additional time to obtain two superimposable, artefact free traces for each test condition where possible. The bandpass filters were set from 0.3 to 500 Hz for all ERG recordings on the Espion™/ColorDome system.

The ERG protocols evaluated on the two devices included the following:

1. A transient cone response (equivalent to the International Society for Clinical Electrophysiology of Vision Standards: (ISCEV) light adapted 3 ERG [McCulloch et al., 2015]). This was recorded on both the RETeval® and Espion™/ColorDome systems.

The RETeval® protocol akin to the ISCEV standard transient cone 3 ERG is known as the “Light adapted 85 Td.s ERG”. A white (CIE Coordinates 0.33, 0.33) flash of 85 Td.s was presented at a temporal frequency of 2 Hz, against a white (CIE Coordinates 0.33, 0.33) background of luminance 848 Td. An averaged response to 30 flashes was recorded.

The time base for this protocol was 100 ms, with 20 ms of pre-stimulus baseline. The flash duration for this ISCEV protocol was 5 ms, except for in the case of small pupils where the device automatically increased stimulus duration to as long as 10 ms (in order to maintain a constant retinal illuminance). In cases where the pupil size was too small to achieve the required flash energy for the Troland protocol, the RETeval® device produced its maximum flash energy of 30 cd.s.m².

The Espion™ ISCEV photopic 3.0 ERG protocol was conducted in accordance with the ISCEV full field flash ERG standards (McCulloch et al., 2015). The photopic 3.0 flash ERG was recorded in response to a LED flash (white-6500K) of 3 cd.s.m⁻² presented for 4 ms at a temporal frequency of 2 Hz over a background adapting light of 30 cd.s.m⁻². Fifteen responses were averaged on each of 4 traces (a total of 60 responses for the protocol) and the artefact rejection level was set at 300 µV. The time base was 250 ms, and the pre-stimulus baseline was 20 ms. Where possible, if any of the 4 traces contained an artefact, additional responses were recorded to obtain 4 superimposable traces.

2. A PhNR ERG, red flash on blue rod suppressing background. This was recorded on both the RETeval® and Espion™/ColorDome systems.

The Photopic Negative Response ERG is not part of the core ISCEV standard recordings and so had customised parameters pre-set by LKC (LKC technologies, 2015), which were based on previously published optimal test conditions (Rangaswamy et al., 2007). A red (peak wavelength 635 nm, stimulus duration 4 ms) flash stimulus was presented, with a strength of 1.5 cd.s.m⁻², against a rod suppressing blue (peak wavelength 465 nm) background of 10 cd.m⁻². The timebase for this protocol was 120 ms, with 100 ms of pre-stimulus baseline recorded. One hundred responses were averaged at a temporal frequency of 2 Hz.

For the Espion™/ColorDome system the parameters of the Photopic Negative Response ERG were set to mirror the parameters of the PhNR protocol on the RETeval® device. This PhNR protocol was recorded in response to a red LED flash (peak wavelength 635 nm) of 1.5 cd.s.m⁻² pulse intensity presented for a period of 4 ms at frequency of 3.4 Hz over a blue LED background illumination (peak wavelength 465 nm) of luminance 10 cd.m⁻². Twenty-five responses were averaged on each of four

traces and the artefact rejection level was set at 300 μ V. The timebase was 250 ms, with a pre-stimulus baseline of 50 ms.

3. A flicker ERG. Recorded on both RETeval® and Espion™ systems to a stimulus of frequency 28.3 Hz and 30.0 Hz, respectively.

The RETeval® protocol akin to the ISCEV standard 30 Hz flicker ERG is known as the “Light adapted 85 Td.s flicker ERG”. A white (CIE Coordinates 0.33, 0.33) flash of 85 Td.s was presented at a temporal frequency of 28.3 Hz, against a white (CIE Coordinates 0.33, 0.33) background of luminance 848 Td. The number of flashes averaged ranged from 141-424 (depending on the number of traces rejected due to artefactual contamination). The time base for this protocol was 120 ms. The flash duration for this ISCEV protocol was 5 ms, except in the case of small pupils where the device automatically increased stimulus duration to as long as 10 ms (to maintain a constant retinal illuminance). Where the pupil size was too small to achieve the required flash energy for the Troland protocol, the RETeval® device produced its maximum flash energy of 30 cd.s.m².

The Espion™ ISCEV photopic 30 Hz flicker protocol was conducted in accordance with the ISCEV full field flash ERG standards (McCulloch et al., 2015). The photopic flicker ERG was recorded in response to a 30 Hz flickering white stimulus (6500K, 3 cd/m²), background luminance of 30 cd.s.m⁻². Twenty-five responses were averaged on each of eight traces, on a time base of 200 ms. The artefact rejection level was set to 300 μ V. Again, additional traces were recorded if, when viewed superimposed, any showed significant blink artefacts.

4. A 28 Hz and 41 Hz flicker ERG recorded solely on the Espion™ system (for n = 13 participating in the study evaluating the effect of stimulus frequency).

These additional flicker ERGs were performed at a rate of 28 Hz and 41 Hz with all other parameters equal to those of the 30 Hz flicker ERG.

Signal Analysis

For the RETeval® flash and flicker protocol, the ERG signals were processed on board the device. Noise reduction and filtering was carried out as described in the sub-section “Electroretinogram Equipment and Protocols” earlier in this section of the

chapter (4.2.2). The peaks of the a, b and PhNR waves were identified by the device in automatic processing. The a- and b- waves were identified as the maxima and minima of their respective peaks. The PhNR was measured both at 72 ms and at the minimum point between 55 ms and 100 ms. For the purposes of this study the latter measurement was used as this method was adopted in all other parts of this PhD thesis, and the protocol determined prior to the introduction of the PhNR ERG guidelines published by ISCEV (Frishman et al., 2018). The flicker ERG waveforms were analysed using the first harmonic of the electrical response (as this is more robust to lighting conditions the participant is exposed to before ERG testing than the raw flicker response [McAnany and Noland 2014]). The device extracts amplitude and implicit time measurements from the maximum of the first peak of the flicker first harmonic. The analysis of responses from the Espion™/ColorDome apparatus adhered to ISCEV standards for the measurement of amplitudes and times to peak of transient and steady state ERGs (McCulloch et al., 2015). Amplitude and time to peak of the a-wave, b-wave and PhNR were assessed. Time to peak measurements were calculated from stimulus onset. Peaks of the a-wave, b-wave and PhNR were defined by the largest value (most negative or positive, depending on the subcomponent) inside specific time windows; a-wave 70-110 ms, b-wave 100-140 ms, PhNR-wave 120-160 ms. The a-wave amplitude was measured from the pre-stimulus baseline to a-wave peak, the b-wave was measured from the a-wave peak to b-wave peak, and PhNR was measured from the pre-stimulus baseline to the most negative point on the PhNR trough. The flicker ERG was analysed by assessing the amplitude and time to first peak of the first harmonic of the averaged response, following zeroing to remove DC offsets and Fourier analysis.

Statistical Analysis

All statistical analysis was carried out in SPSS (versions 22-25; IBM), whilst graphs were produced in Microsoft Excel (Microsoft Office, Version 16). Formal sample size calculations were not performed for this part of the study. However, a sample of 21, tested twice, should be able to detect within subject standard deviation with a confidence interval of approximately 30% either side of the true value (Bland, 2010). The means and standard deviations at each visit were determined for all parameters which showed a normal distribution between participants. Medians and interquartile

ranges were calculated to summarise data which were not normally distributed. Bland and Altman analysis (Bland and Altman, 1986) was performed to summarise the between-session repeatability and the between technique agreement. This included plotting the differences between visits against the mean of the visits to enable visualisation of the spread of the data. According to the principles described by Bland and Altman (1986), the coefficient of repeatability (CoR) and limits of agreement (LoA) were also calculated (see Equations 4-2 and 4-3). CoR was also expressed as a percentage of the mean to enable comparison of the repeatability and agreement of parameters which may vary in absolute magnitude.

In order to determine whether one technique was more repeatable than another, it was necessary to calculate confidence intervals for the CoR. The standard error for CoR is described by Equation 4-4, and the confidence intervals were given by the standard error multiplied by 1.96. Where confidence intervals for the CoR expressed as a percentage of the mean did not overlap between parameters, the difference in repeatability was considered to be statistically significant. The coefficient of agreement (CoA) between the two devices at visit 1 was also calculated according to the same Bland and Altman procedure (where 'device 1' and 'device 2' replace 'visit 1' and 'visit 2' in the equations). The coefficient of variation for each parameter was calculated in order to evaluate the spread of data between individuals, according to Equation 4-5. A paired T-test was performed in order to assess if there was a statistically significant difference between the mean values measured at visits one and two, and between the mean data obtained using the two systems. Bonferroni correction was applied to p-values to minimize the risk of type I errors i.e. by multiplying p-values by the number of comparisons made (Bonferroni, 1936; Abdi, 2007).

A comparison of flicker ERG responses obtained in response to the 28 Hz, 30 Hz, and 41 Hz stimuli was conducted by repeated measures ANOVA analysis with post-hoc Bonferroni correction to identify any significant difference between the mean of the three sets of varying flicker frequency results.

$$\text{CoR} = 1.96 \times \text{SD}(v1-v2)$$

Equation 4-2. Coefficient of Repeatability; where v1 and v2 represent the value of the parameters for each individual at visits 1 and 2.

$$\text{LoA} = (d - 1.96 \times \text{CoR}) \text{ and } (d + 1.96 \times \text{CoR})$$

Equation 4-4. Limits of Agreement; where d = the mean difference in parameter between visits 1 and 2 across all participants, also known as the bias.

$$\text{Standard error (CoR)} = \sqrt{3 \text{ SD}^2/n}$$

Equation 4-3. Standard Error; where n = the sample size, and SD = the standard deviation of the differences between visits.

$$\text{CoV} = (\text{SD}/d) \times 100$$

Equation 4-5. Coefficient of Variation; where SD = the standard deviation, and d = the mean of the parameter in question.

4.2.3. Results

Data were collected from 23 healthy volunteers aged 23 – 53 years between April 2017 and December 2017. Of these, two decided to not return for their second data collection session (one due to unforeseen family circumstances, another declined to provide a reason). The final number of participants included in the analysis was 21 (13 females and eight males). The median (IQR) age was 32(26, 38) years. One participant (participant ID RR022) asked to cease the flicker ERG testing before full data had been collected according to the protocol as they found this too strenuous and uncomfortable. Additionally, one participant's second visit Espion™ PhNR ERG included a consistent eye movement resulting in its subsequent removal from analysis (participant ID RR023). As a result, a total of 20 participants completed the 30 Hz flicker protocol, and the between visit Espion™ PhNR ERG analysis. Thirteen participants elected to participate in the final aim to evaluate the effect of flicker frequency. All provided valid data.

Sample ERGs from the right and left eyes of one participant (RR010) are shown in Figure 4.3. The waveforms are seen to be broadly comparable, although differences in response filtering mean that high frequency components such as the oscillatory potentials are more defined on the Espion™ system transient responses. Clean and uncontaminated responses were obtained for all of the ERG protocols on both devices for this participant.

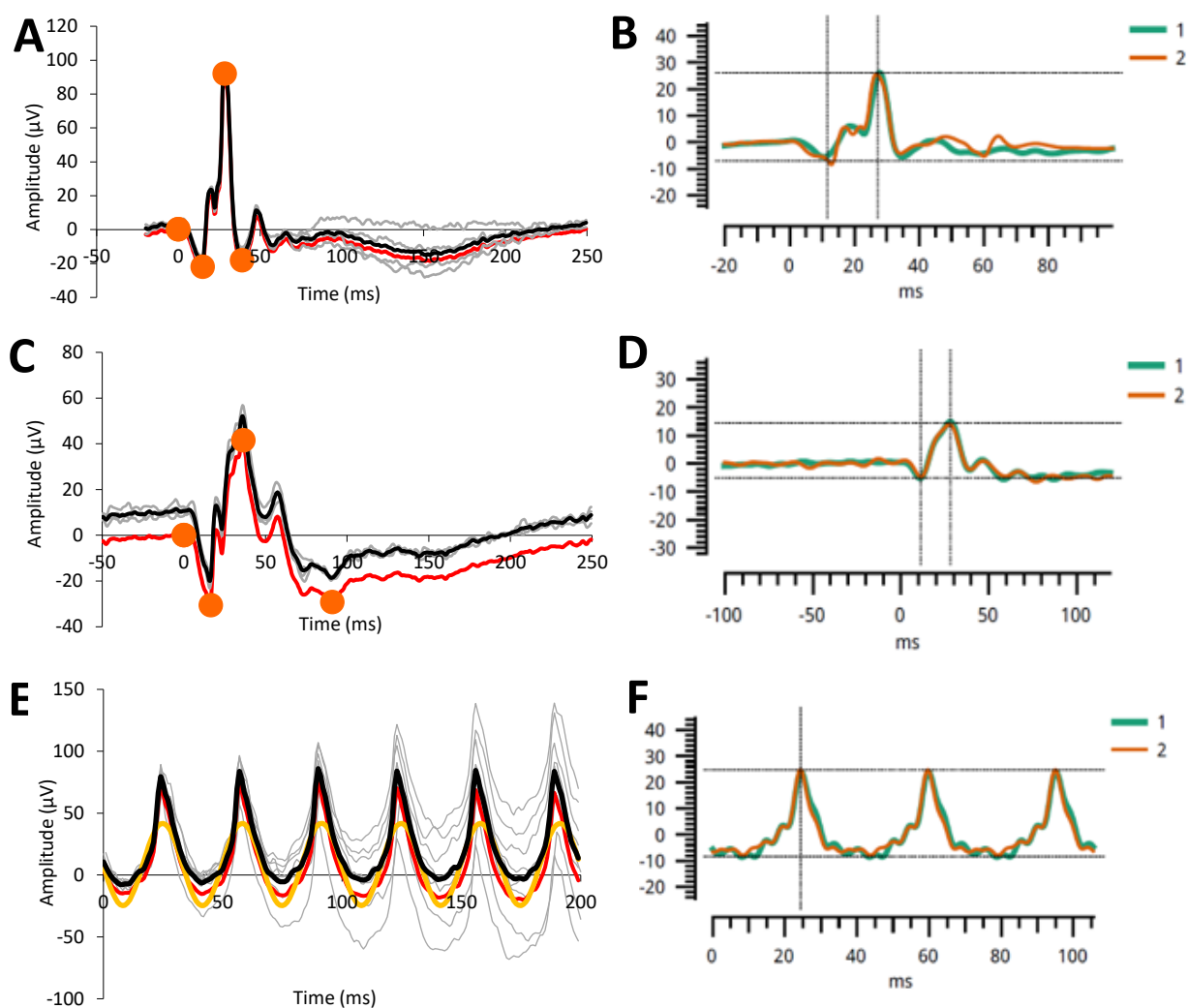


Figure 4.3. Sample data from one participant (RR010) recorded from the two devices. Panels A and B show ISCEV photopic 3.0 ERG, panels C and D show PhNR, and panels E and F show the 30 Hz flicker ERG responses. Data from the Espion™/ColorDome system are shown in panels on the left, and those from the RETeval® are shown on the right. In the traces from the Espion™/ColorDome system, raw data are shown in grey, the grand average of these data is shown in black, and this trace zeroed and corrected for drift is shown in red. In the case of the 30 Hz flicker ERG this data also displays the first harmonic of the Fourier analysed response in yellow. The orange spots indicate the locations identified as being maxima and minima when evaluating the parameters of the subcomponents. In the traces from the RETeval®, the two traces represent the two successive averaged responses, superimposed to demonstrate reproducibility.

Bland and Altman plots were generated to graphically represent the repeatability of the ERG parameters (see Figures 4.4 and 4.5). The Bland and Altman plots showed a mean difference between visits close to zero for all parameters, indicating a lack of bias (such as a learning effect). This was reflected in the lack of significant difference in parameters between visits (paired t-test, $p > 0.05$). However, there was some evidence that the bias within the data may not be consistent across the spectrum of times to peak and amplitudes, especially for the Espion™ system. For example, as the mean amplitude for the a-wave measurements increased, the difference between visits became more positive (crossing zero difference at $-22 \mu\text{V}$ indicating that above this point the difference was positive [i.e. higher on first visit] while for lower amplitudes than that point the second visit measured higher) (see Figure 4.5, B).

There was generally less scatter in between visit data for the implicit time than the amplitude measures. Tables 4.1 and 4.2 display the mean data for each parameter at each visit, along with the Coefficient of Repeatability (CoR), also expressed as a percentage of the group mean for the parameter – the lower the percentage value, the higher the repeatability. When expressed as a percentage of the mean it can be seen that the most repeatable parameter of the RETeval® device protocol was the 30 Hz flicker Time to First Peak (3%). The least repeatable was PhNR amplitude (80%). For the Espion™/ColorDome ERGs it can be seen that the most repeatable protocol parameter was the transient cone a-wave time to peak (4%), and the least the PhNR amplitude (38%). In all cases, the time to peak parameters were more repeatable than the amplitude parameters for both the RETeval® and Espion™/ColorDome devices.

In all but one parameter the protocol performed by the Espion™ device showed proportionally better repeatability (lower CoR as a proportion of the mean value). The exception to this was the 30 Hz flicker time to peak parameter, which was more repeatable when performed by the RETeval® device than by the Espion™ system (3% compared to 5% respectively). However, in all cases the confidence intervals for the CoR expressed as a percentage overlapped between devices.

Tables 4.1 and 4.2 also display the Coefficient of Variation data for the RETeval® and Espion™/ColorDome systems for visit 1 and 2 data. This is a measure of between subject variability, where a smaller value indicates a narrower spread of data. When observing the CoV analysis for all parameters, again it can be seen that there is more

between individual variability in the amplitude parameters than there is in those of time to peak. It can also be observed that there is a similar degree of variance in the results obtained from the two devices. The most variable RETeval® parameter was the PhNR amplitude (55% visit one, 47% visit two), and the least variable was the 28 Hz flicker time to peak (3% visit one, and 3% visit two). The most variable Espion™/ColorDome parameter was the Transient Cone a-wave amplitude (31% visit one, 23% visit two), and the least was the transient cone ERG b-wave time to peak (3% visit one, 3% visit two). For both devices, the variance remained similar in visits 1 and 2, with most parameters showing less than 5% change in CoV.

Figure 4.6 and Table 4.3 display data demonstrating the agreement between the two devices. Amplitudes of the ERG parameters were consistently smaller in the RETeval® than the Espion™ device. This was statistically significant for all measures even after Bonferroni correction, and is apparent in the bias line of the Bland and Altman plots in Figure 4.6. The flicker ERG time to peak and the transient cone a-wave time to peak were significantly longer for responses recorded using the Espion™ system compared to the RETeval®. This was particularly surprising for the a-wave of the transient cone response given the use of identical stimulus parameters between the two devices. Only b-wave time to peak and PhNR time to peak were not significantly different between devices. There is also some indication in these figures that the magnitude of difference between devices was not consistent across amplitude and time to peak values (Figure 4.6, parts B, C, and D). The zero-difference line was crossed at -21 μ V, 75 μ V, and 26 ms for the a-wave amplitude, b-wave amplitude and flicker time to first peak, respectively.

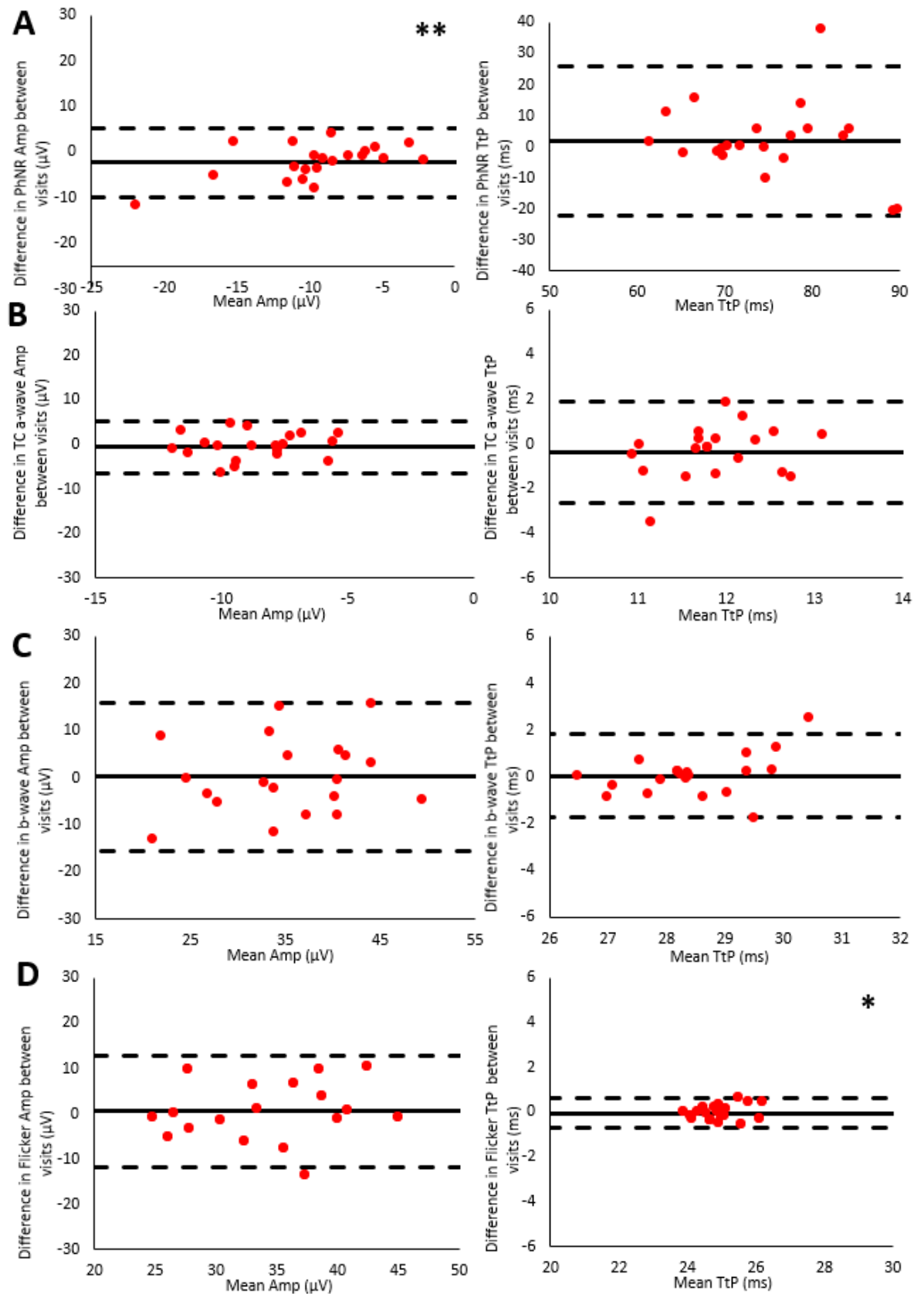


Figure 4.4. Bland and Altman plots for ERG parameters of the RETeval® device. Left; amplitude parameters, right; Time to Peak (TtP) parameters. A, PhNR; B, Transient Cone a-wave; C, Transient cone b-wave; D, 30 Hz Flicker. Most (*) and least (**) repeatable ERG parameters indicated with asterisks. Black dotted lines indicate the limits of agreement. The solid line represents the bias (mean difference between visits).

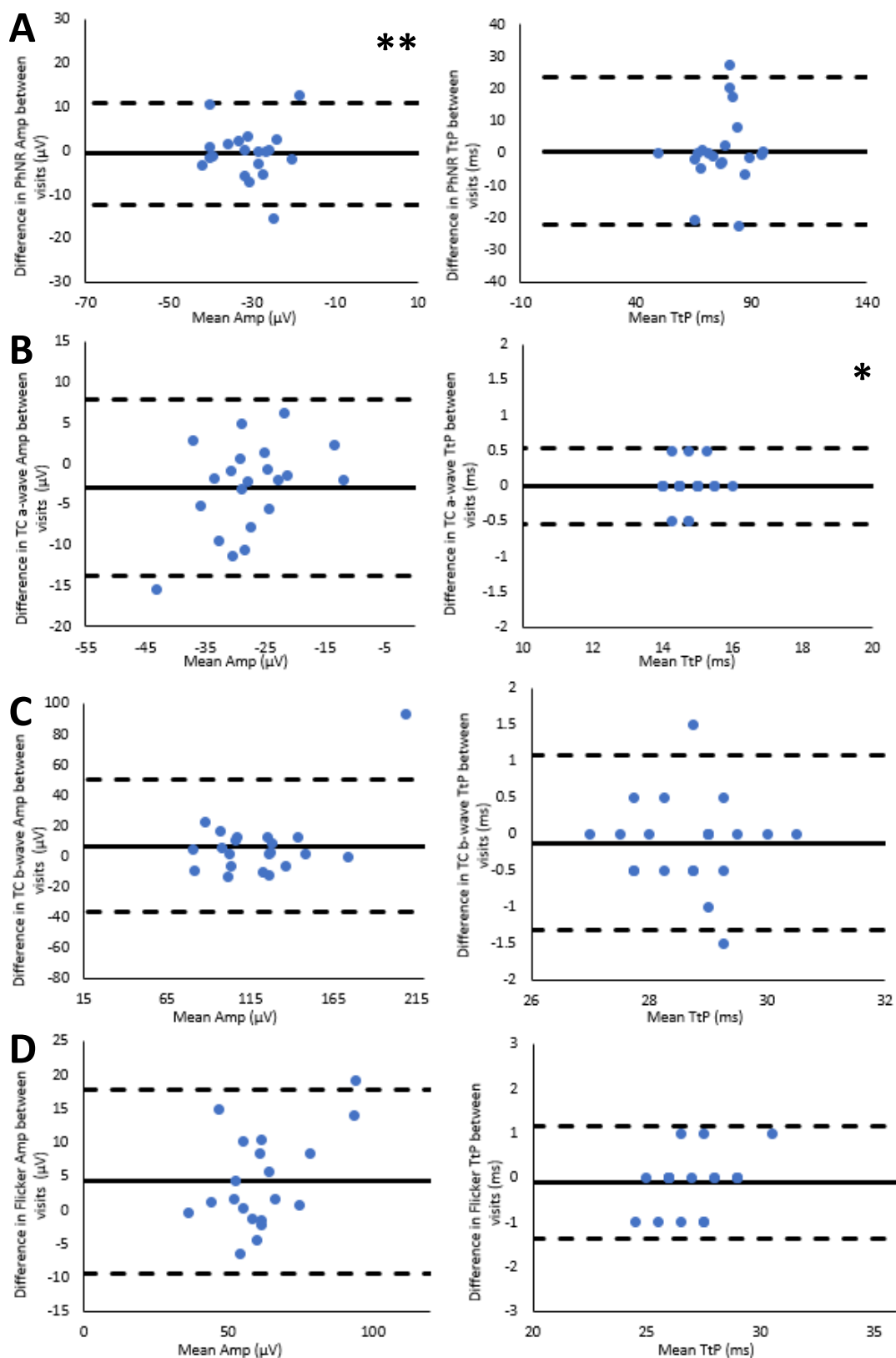


Figure 4.5. Bland and Altman plots for ERG parameters of the Espion™/ColorDome system. Time to peak (TtP) parameters are displayed to the left and Time to Peak parameters to the right. A, PhNR; B, Transient Cone a-wave; C, Transient cone b-wave; D, Flicker. The most* and least** repeatable ERG parameters are indicated with asterisks. The black dotted lines indicate the limits of agreement. The solid line represents the bias (mean difference between visits).

RETeval® ERG Parameter Between Visit Analysis	Transient Cone ERG				PhNR ERG		Flicker ERG	
	a-wave		b-wave		Amp	TtP	Amp	TtP
	Amp	TtP	Amp	TtP				
Mean (SD) Visit 1	-8.91 (± 2.58)	11.71 (± 0.94)	35.34 (± 9.35)	28.51 (± 1.30)	-10.55 (± 5.80)	75.82 (± 8.89)	33.05 (± 8.30)	24.93 (± 0.70)
Mean (SD) Visit 2	-8.38 (± 2.37)	12.09 (± 0.71)	35.19 (± 8.05)	28.47 (± 0.95)	-8.21 (± 3.82)	74.00 (± 11.11)	32.47 (± 8.01)	24.98 (± 0.63)
CoR (95% CI)	5.85 (1.41 - 10.29)	2.27 (0.55 - 3.99)	15.81 (3.81 - 27.82)	1.77 (0.43 - 3.12)	7.46 (1.93 - 12.99)	24.01 (6.22 - 41.79)	12.30 (2.96 - 21.64)	0.64 (0.16 - 1.13)
CoR as % (95% CI)	68 (16 - 119)	19 (5 - 34)	45 (11 - 79)	6 (2 - 11)	80 (21 - 138)	32 (8 - 56)	38 (9 - 66)	3 (1 - 5)
CoV V1 (%)	29	8	26	5	55	12	25	3
CoV V2 (%)	28	6	23	3	47	15	25	3
Paired T-test (p)	0.43	0.17	0.73	0.72	0.01	0.50	0.68	0.50
Bonferroni Corrected p	1.00	1.00	1.00	1.00	0.08	1.00	1.00	1.00

Table 4.1. The between visit and between subject repeatability data for the RETeval® system. No ERG parameters measured by this equipment were significantly different between visits after Bonferroni correction.

Espion™/ ColorDome ERG Between Visit Analysis	Transient Cone ERG				PhNR ERG		Flicker ERG	
	a-wave		b-wave		Amp	TtP	Amp	TtP
	Amp	TtP	Amp	TtP				
Mean (SD) Visit 1	-29.03 (± 8.84)	14.74 (± 0.56)	124.21 (± 37.96)	28.62 (± 0.89)	-31.25 (± 7.38)	77.15 (± 13.28)	63.62 (± 16.65)	27.00 (± 1.69)
Mean (SD) Visit 2	-26.12 (± 6.54)	14.74 (± 0.54)	117.29 (± 26.46)	28.74 (± 0.93)	-30.66 (± 7.56)	76.55 (± 11.77)	59.39 (± 13.28)	27.10 (± 1.45)
CoR (95% CI)	10.87 (2.82 - 18.93)	0.54 (0.14 - 0.93)	43.24 (11.21 - 75.27)	1.20 (0.31 - 2.08)	11.61 (2.80 - 20.42)	22.77 (5.49 - 40.06)	13.53 (3.26 - 23.79)	1.26 (0.30 to 2.21)
CoR as % (95% CI)	39 (10 - 69)	4 (1 - 6)	36 (9 - 62)	4 (1 - 7)	38 (9 - 66)	30 (7 - 52)	22 (5 - 39)	5 (1 - 8)
CoV V1 (%)	30.49	3.81	30.56	3.12	23.61	17.21	26.17	6.24
CoV V2 (%)	25.04	3.66	22.56	3.24	24.65	15.38	22.36	5.34
Paired T-test (p)	0.03	1.00	0.17	0.38	0.67	0.82	0.01	0.72
Bonferroni Corrected p	0.24	1.00	1.00	1.00	1.00	1.00	0.08	1.00

Table 4.2. Between visit and between subject repeatability for the Espion™/ColorDome system. No ERG parameters measured by this equipment were significantly different between visits after Bonferroni correction.

Between Device analysis	Transient Cone ERG				PhNR ERG		Flicker ERG	
	a-wave		b-wave		Amp	TtP	Amp	TtP
	Amp	TtP	Amp	TtP				
Mean (SD) RETeval®	-8.91 (± 2.51)	11.69 (± 0.92)	26.06 (± 5.87)	28.50 (± 1.27)	-10.55 (± 5.79)	75.82 (± 8.89)	33.36 (± 7.95)	24.89 (± 0.73)
Mean (SD) Espion™	-29.03 (± 8.84)	14.74 (± 0.56)	124.21 (± 37.96)	28.62 (± 0.89)	-30.86 (± 8.94)	77.93 (± 13.43)	63.62 (± 16.65)	27.00 (± 1.69)
CoA (95% CI)	16.21 (4.20 - 28.22)	2.37 (0.61 - 4.13)	66.34 (17.19 - 115.48)	1.91 (0.50 - 3.33)	19.89 (5.16 - 34.63)	28.70 (7.44 - 49.96)	28.17 (6.79 - 49.55)	3.02 (0.73 - 5.32)
CoA as % (95% CI)	83 (22 - 145)	17 (4 - 30)	88 (23 - 154)	7 (2 - 12)	98 (25 - 170)	37 (10 - 65)	57 (14 - 100)	12 (3 - 21)
Paired T-test (p)	<0.001*	<0.001*	<0.001*	0.57	<0.001*	0.52	<0.001*	<0.001*
Bonferroni Corrected p	<0.001*	<0.001*	<0.001*	1.00	<0.001*	1.00	<0.001*	<0.001*

Table 4.3. The between device agreement data for the RETeval® device and Espion™/ColorDome system. Parameters that were significantly different between visits are highlighted in with asterisks ().*

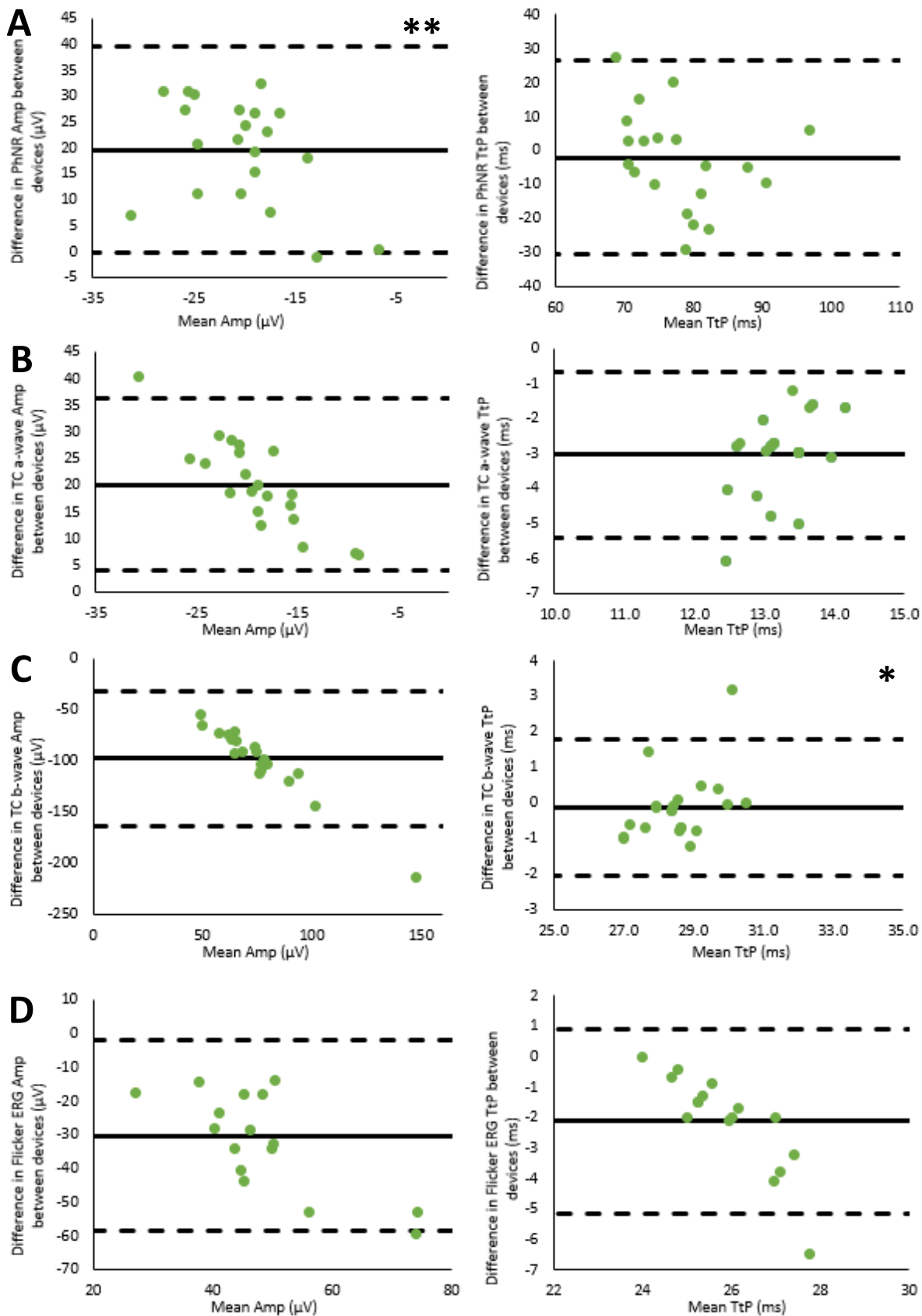


Figure 4.6. Bland and Altman plots assessing the agreement of the RETeval® and Espion™ devices. Plots for amplitude parameters are displayed to the left and Time to Peak parameters to the right. A PhNR, B Transient Cone a-wave, C Transient cone b-wave, D Flicker. The most* and least** agreeable are indicated with asterisks as shown. Black dotted lines indicate the limits of agreement. The solid line represents the bias (mean difference between visits).

The effect of flicker frequency on stimulus waveform

Additional 41 Hz and 28 Hz data were collected using both systems from thirteen participants in order to compare the effect of stimulus frequency on response characteristics. Sample data are shown in Figure 4.7. There is a clear difference in phase between the responses from these participants, as well as a lower response amplitude for the 41 Hz response. Mean responses from all thirteen participants are shown in Figure 4.8, where a similar pattern can be observed.

When a repeated measures ANOVA analysis with post hoc Bonferroni testing was performed, first harmonic amplitude was significantly higher for the 28 Hz than the 41 Hz protocol ($p = <0.01$), and time to first peak was significantly shorter for the 30 than the 28 Hz protocol (see Table 4.4).

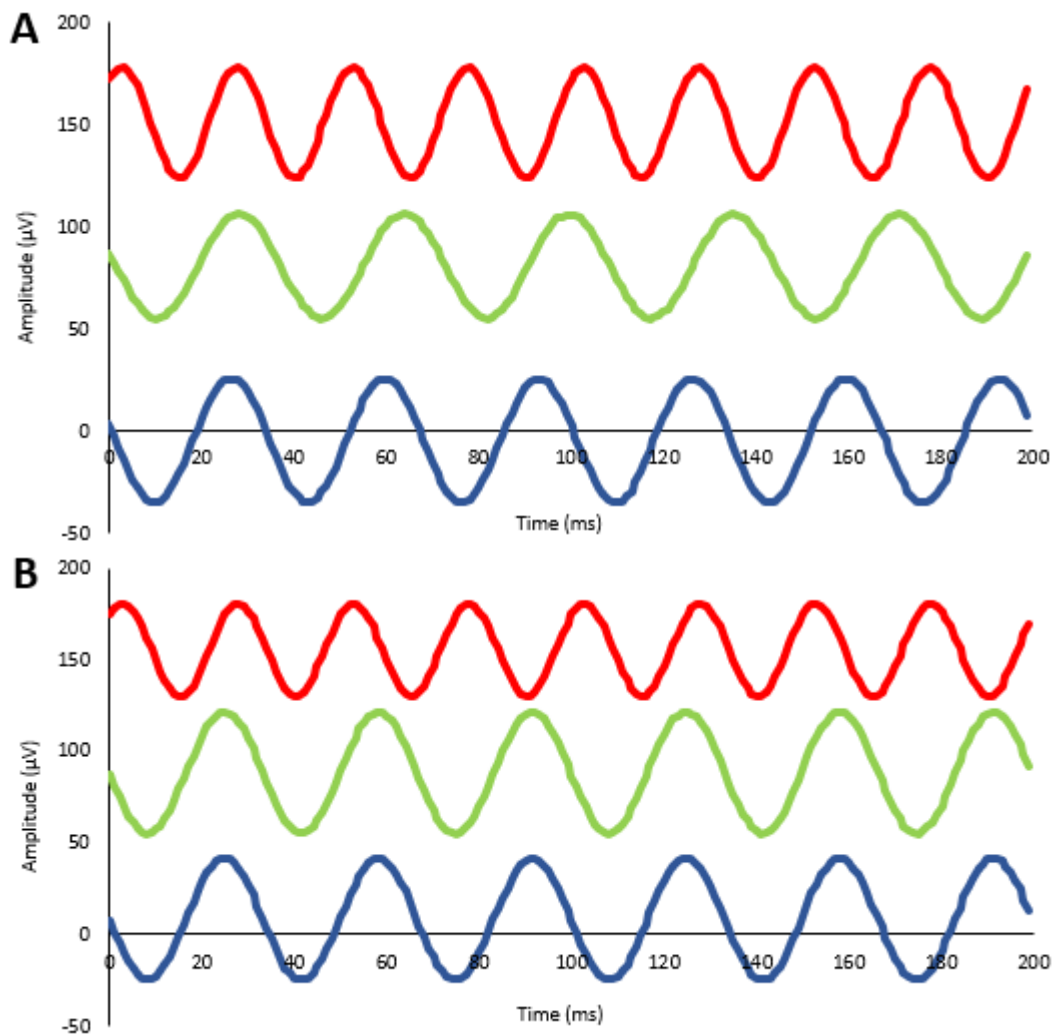


Figure 4.7. Averaged first harmonic waveforms for participants RR003 (A) and RR013 (B) recorded in response to; 41 Hz (red trace), 30 Hz (green trace), and 28 Hz (blue trace) stimuli, using the Espion™/ColorDome system.

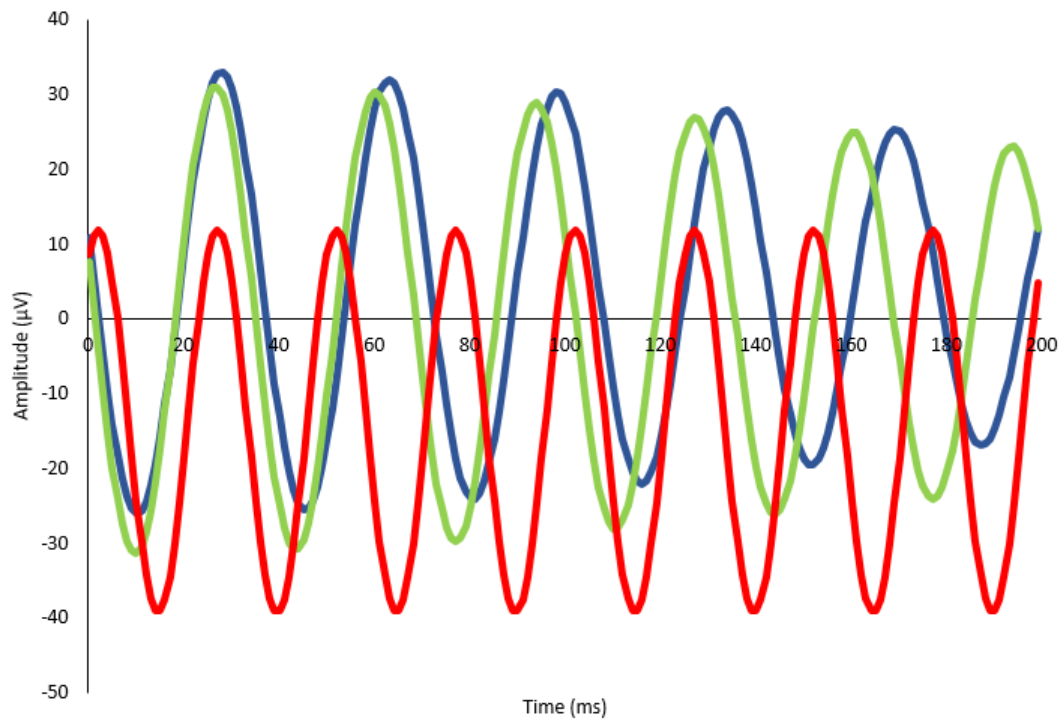


Figure 4.8. Group-averaged first harmonic waveforms for 13 participants recorded in response to 41 Hz (red trace); 30 Hz (green trace), and 28 Hz (blue trace) stimuli, using the Espion™/ColorDome system. Traces have been superimposed to facilitate comparison.

	Frequency (Hz)	Mean (SD)	Confidence Intervals (95%)	CI (as % of mean)	Repeated measures ANOVA with Bonferroni correction (p)		
					28 Hz	30 Hz	41 Hz
Amplitude of First Peak	28	60.39 (± 15.21)	46.07 – 74.71	76 – 124		0.55	<0.001*
	30	64.32 (± 14.29)	50.86 – 77.78	79 – 121	0.55		0.09
	41	53.86 (± 10.63)	43.85 – 63.86	81 – 119	<0.001*	0.09	
Time to First Peak	28	27.77 (± 1.30)	26.54 – 28.99	96 – 104		0.02*	1.00
	30	26.77 (± 1.48)	25.38 – 28.16	95 – 105	0.02*		0.26
	41	27.00 (± 1.48)	25.78 – 28.22	96 – 105	1.00	0.26	

Table 4.4. Table showing mean amplitudes and times to first peak of waveforms recorded in response to 28, 30 and 41 Hz frequencies. Repeated measures ANOVA with Bonferroni correction demonstrated significant output differences between some frequencies (highlighted with an asterisk).

4.2.4. Discussion

This is the first study to compare the repeatability of desktop and hand held ERG recording devices for the ISCEV photopic ERG protocols. Overall, all the ERG protocols in this study were well tolerated by participants. Measures of timing were consistently more repeatable than those of amplitudes on both devices. Although responses were significantly higher in amplitude when recorded using the Espion™ device (with DTL electrode), between session repeatability was only marginally higher for the Espion™ system as a proportion of mean response magnitude, and confidence intervals for the percentage CoR were overlapping between techniques.

The CoR is a value which describes the between session change which may be considered to fall within the measurement variability of a technique. In other words, 95% of people will provide a value for a parameter within the CoR of the first visit at their second visit, in the absence of pathology (Bland and Altman, 1986). Hence, the CoR may be considered to be the minimum change in each parameter over time required to exceed the measurement error. For example, in the RETeval® b-wave time to peak parameter a change > 1.77 ms would be considered clinically significant, whilst for b-wave amplitude, a change >15.81 μ V would imply clinical significance. Whether repeatability is considered to be 'good enough' depends on the magnitude of the change expected in the parameter as a result of the condition in question.

The CoR and CoV values for the photopic ERGs recorded using the Espion™ and RETeval® systems were broadly comparable to those reported in previous human studies (Wu et al., 2016; Tang et al., 2014; Mortlock et al., 2010) and in animal studies (Fortune et al., 2004). Wu et al. (2016) reported a CoR of 2.0 μ V for the PhNR response amplitude recorded using the RETeval®, whilst we report a value of 7.46 μ V. However, the % CoR in this study was somewhat higher at 79.5% than the 28% reported by Tang et al., using the RETeval® with sensor strips. With respect to the desktop (Espion) ERGs, Jeffrey et al. (2014) reported a repeatability coefficient of 0.29 for the 30 Hz flicker ERG amplitudes tested in their patients with X-linked retinoschisis, compared to 13.53 μ V reported in this study.

There were no significant differences between visits for amplitude and time to peak measurements for the RETeval® device or the Espion™/ColorDome system. This indicates that there was no systematic bias between visits e.g. due to learning effect.

In general, there was very little difference in CoV of most results between visits ($\leq 5\%$), suggesting that the variability of the results was also fairly consistent between visits.

The finding that time to peak was less variable than amplitude for all measures was expected on the basis of current literature (Pescosolido et al., 2014; Fortune et al., 2004; Strain et al., 2001; Mortlock et al., 2010). For example, Mortlock et al., (2010) reported less variability in time to peak than amplitude parameters in a study of 31 healthy participants using a photopic ERG protocol. The potential clinical significance of this was demonstrated in the study by Pescosolido et al. (2014), who assessed time to peak and amplitude parameters of flicker ERGs from 17 patients (34 eyes) with no retinal pathology and 12 patients (23 eyes) with various types of retinopathy. These were then related to the pathological state of the retina and showed that amplitude parameters were reduced in the patients with retinal pathology but also that there was a significant degree of variability and overlap between values from patients with and without retinal pathology. In contrast, time to peak measurements showed little variability between individuals and no overlap between disease states. The current study demonstrated that, due to lower levels of variability between visits and between subjects, time to peak measurements may provide a more accurate diagnostic measure of certain pathology. That is, as a result of the low measurement variability, even modest changes in time to peak parameters, such as an increase in time to peak in participants with diabetic retinopathy (Sato et al., 1994), may be indicative of pathology. However, this is not true in all cases as PhNR time to peak measurements have been shown to be insensitive to retinal changes induced by glaucoma (Drasdo et al., 2001; Viswanathan et al., 2001; Colotto et al., 2000), and were very variable in the current study.

In all parameters except transient cone b-wave time to peak and PhNR time to peak, it was found that there was a significant difference between the parameters obtained by the RETeval® device and the Espion™/Ganzfeld System. Smaller amplitudes with the RETeval® device were expected due to the use of skin electrodes. Studies have shown smaller amplitude measurements with skin electrodes when compared with conjunctival/corneal surface electrodes (Beresovsky et al., 2009; Kriss, 1994; Coupland and Janaky, 1989). For example, Beresovsky et al. (2009) examined participants with skin surface, microfibre, and contact lens electrodes used with the same protocol.

When comparing the amplitude measurements of the photopic ERGs a 40% reduction in ERG amplitude was observed for the skin surface electrode when compared with the results from the microfibre electrode. This is comparable to the significant reduction in amplitude measurements between the RETeval® device and the Espion™ device in this study (for example, a 63% reduction in mean b-wave amplitude when measured with the RETeval® skin surface electrodes, compared to the measurements obtained with the DTL fibre and Espion™ device). However, it is also important to acknowledge that the magnitude of the difference in amplitude between the Espion™ and RETeval® data was not consistent across amplitude values. This suggests that it is not appropriate to apply a simple correction factor to equate the two.

An interesting finding shows us that the flicker ERG time to first peak and a-wave time to peak differed significantly between devices, although b-wave and PhNR time to peak did not. There is conflicting evidence regarding this in previous studies (Beresovsky et al., 2009; Kondo and Ueno, 2004; McCulloch et al., 1998). Beresovsky et al. (2009) found that the time to peak measurements were significantly shorter for skin active electrodes than microfibre and contact lens electrodes (for rod and maximal responses). This is also mirrored in this study with time to peak values all shorter for the RETeval® than those recorded on the Espion™/ColorDome system. However, McCulloch et al. (1998) found no significant difference in time to peak measurements measured with Burian Allen, DTL, gold foil, C-glide, HKL loop, or skin electrodes. It is also possible that differences in times to peak occur due to differences in the response acquisition or measurement between devices. For example, the methods by which peaks are identified by respective systems (i.e. the use of a different algorithm for identification of waveform maxima and minima by each device) may have had an impact. Another possibility would be the response filtering of the two devices. In particular, a low pass filter of 500 Hz was used by the Espion, whilst one of 300 Hz was employed by the RETeval®. This is apparent in the greater prominence of high frequency oscillatory potentials in the transient responses recorded using the Espion, and may also have had an effect on the timing of the peaks. It is also possible that subtle differences in the stimulus characteristics may cause this difference in timing. Kondo and Ueno (2004) show that the waveforms of flicker ERG are affected by the stimulus being sinusoidal, sawtooth or square wave.

Despite the differences in waveform between devices, the CoR and CoV data actually showed similar levels of between session and between subject variability between the two systems for the majority of ERG parameters, and confidence intervals were overlapping. In other words, the variability is similar on a proportional level, so both should be able to detect the same percentage reduction in amplitude or change in time to peak. This means that the RETeval® device could be considered a viable option for clinical monitoring of disease. However, whilst in healthy subjects the results show a similar level of repeatability and variability between devices, given the substantially lower amplitude of responses obtained using the RETeval® device, in the case of a reduction in amplitude caused by pathology it is likely that the signal to noise ratio could become too low for signals to be measured.

The second part of the study found a significantly smaller mean response amplitude for the 41 Hz flicker frequency than the 28 Hz. This is surprising as evidence from studies by Seiple et al. (1989) and Falsini et al. (2007) both indicate that peak amplitude for the cone flicker response occurs at around 40 Hz. The 41 Hz flicker frequency was used in the main study of this PhD project because it allows a more rapid averaging of multiple responses, rapidly improving signal to noise ratio. Published literature suggests that the origins and amplitudes are similar to those of the 30 Hz response (Falsini et al., 2000; Kondo and Seiving, 2001). One possible explanation for the disagreement with published data is that participants struggled to maintain a wide open lid aperture during the 41 Hz flicker test due to discomfort with the flicker frequency (anecdotally reported by several individuals). The 28 and 30 Hz responses did not differ significantly in amplitude, but did have significantly different times to first peak (as might be expected on the basis of a change in temporal frequency). The 30 Hz stimulus is the standard ISCEV recommendation. Whilst 28.3 Hz (employed by the RETeval® device) does fall within the range of accepted ISCEV frequencies for the flicker ERG (McCulloch et al., 2015, Marmor et al., 2009), the significant difference found between the time to first peak parameters of the 30 Hz and 28 Hz flicker ERG suggests that the timing of responses is not directly comparable between systems using the different frequencies.

In summary, repeatability and variability between individuals was shown to be similar for the two devices, despite significantly smaller amplitudes measured by the RETeval®

compared to the Espion™/ColorDome System (for example, a 63% reduction in b-wave amplitude measurements performed by the RETeval® compared to those performed with the Espion™/ColorDome system). This suggests that while both devices may be used to detect the same kind of change in disease state, the waveform produced by each will be somewhat different between the devices. Implicit times were shown to be more reliable parameters in both devices. Additionally, even subtle changes in the frequency of the flicker ERG were shown to affect the waveform produced. This suggests that while various flicker frequencies may be effectively used to measure retinal function, they may not be directly comparable.

4.3. The effect of varying skin surface electrode position on electroretinogram responses recorded using a handheld stimulating and recording system

Although the manufacturers of the RETeval® device recommend positioning the skin sensors at a fixed 2 mm below the lid margin to balance patient comfort with optimal ERG parameter readings, patients often express a level of discomfort which is alleviated when the sensor is moved to a position around the infraorbital rim. Discussion with staff at St Thomas' hospital indicated that this preferred position is sometimes used during recording.

This study was (partly) presented at BCOVS 2016, Coleraine, Northern Ireland (Paper Presentation), ARVO 2016, Seattle, WA (Poster Presentation), City, UoL Doctoral Research Conference 2016, London, UK (Poster Presentation). This study was also published: Hobby, A. et al. 2018. Effect of varying skin surface electrode position on electroretinogram responses recorded using a handheld stimulating and recording system. *Documenta Ophthalmologica* 137(2), pp. 79-86. (10.1007/s10633-018-9652-z). AEH was responsible for collecting and analysing the data and writing the manuscript.

4.3.1. Aims

The aim of this study was to determine the effect of two different electrode positions on amplitudes and peak times of standard photopic responses (corresponding to ISCEV standard stimuli) recorded using the RETeval® stimulator.

4.3.2. Methods

Participants

As part of a larger study, adult twins were recruited from the TwinsUK cohort based at St Thomas' Hospital London (Groups 1 and 2), and a smaller group of volunteer adult participants was recruited from amongst the clinical and research staff at St Thomas' hospital London (Group 3). All participants underwent recording of photopic flicker ERGs using the handheld RETeval® stimulator. All were healthy normal participants with no known retinal or systemic disease affecting retinal function. Participants were excluded if they were taking medication known to affect retinal function (such as hydroxychloroquine) or if they suffered from photosensitive epilepsy.

Protocol

Ethical approval was gained through the National Research Ethics Service (NRES) Committee London – Harrow (11/LO/2029), and written informed consent was obtained from all subjects, conforming to the tenets of the Declaration of Helsinki. Stimuli were delivered through natural pupils, and strengths were automatically adjusted by the device according to pupil diameter to correspond to international standard stimuli. The ISCEV standard specifies the delivery of 3.0 photopic cd.s.m^{-2} white stimuli in the presence of a 30 cd.m^{-2} white background. The device delivers 85 Td.s stimuli on a background of 850 Td (which would correspond to the ISCEV standard strength stimulus delivered through a 6 mm diameter pupil). Flicker stimuli were delivered at 28.3 Hz.

In the first 48 consecutively recruited participants (Group 1), skin electrodes were placed in a “comfortable” position, ranging from near the lid margin to approximately 2 cm below the lid margin. In the next 200 consecutive participants (Group 2), electrodes were positioned in the manufacturer recommended location (Figure 4.9).

As ERG parameters from both twins of a given pair are highly correlated (Bhatti et al., 2017), only one twin from each pair was included for subsequent analysis. As ERG parameters vary with age (Neveu et al., 2011; Bhatti et al., 2017), the two groups were age-matched by ensuring the same numbers of participants in each age decade. Figure 4.10 demonstrates the participant pathway through the study.

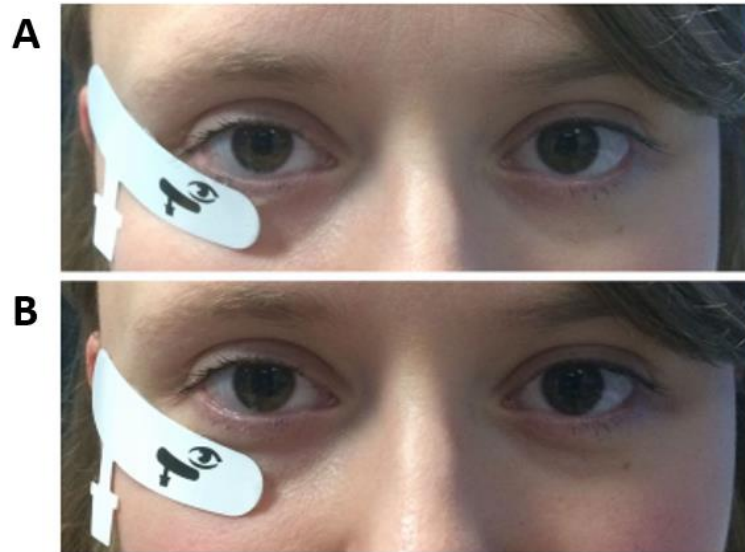


Figure 4.9. Two different electrode positions in a study participant. The skin sensor electrodes are placed 2 mm below the lid margin (manufacturer's recommended position, A), and in an inferior position (10–20 mm below lid margin, B).

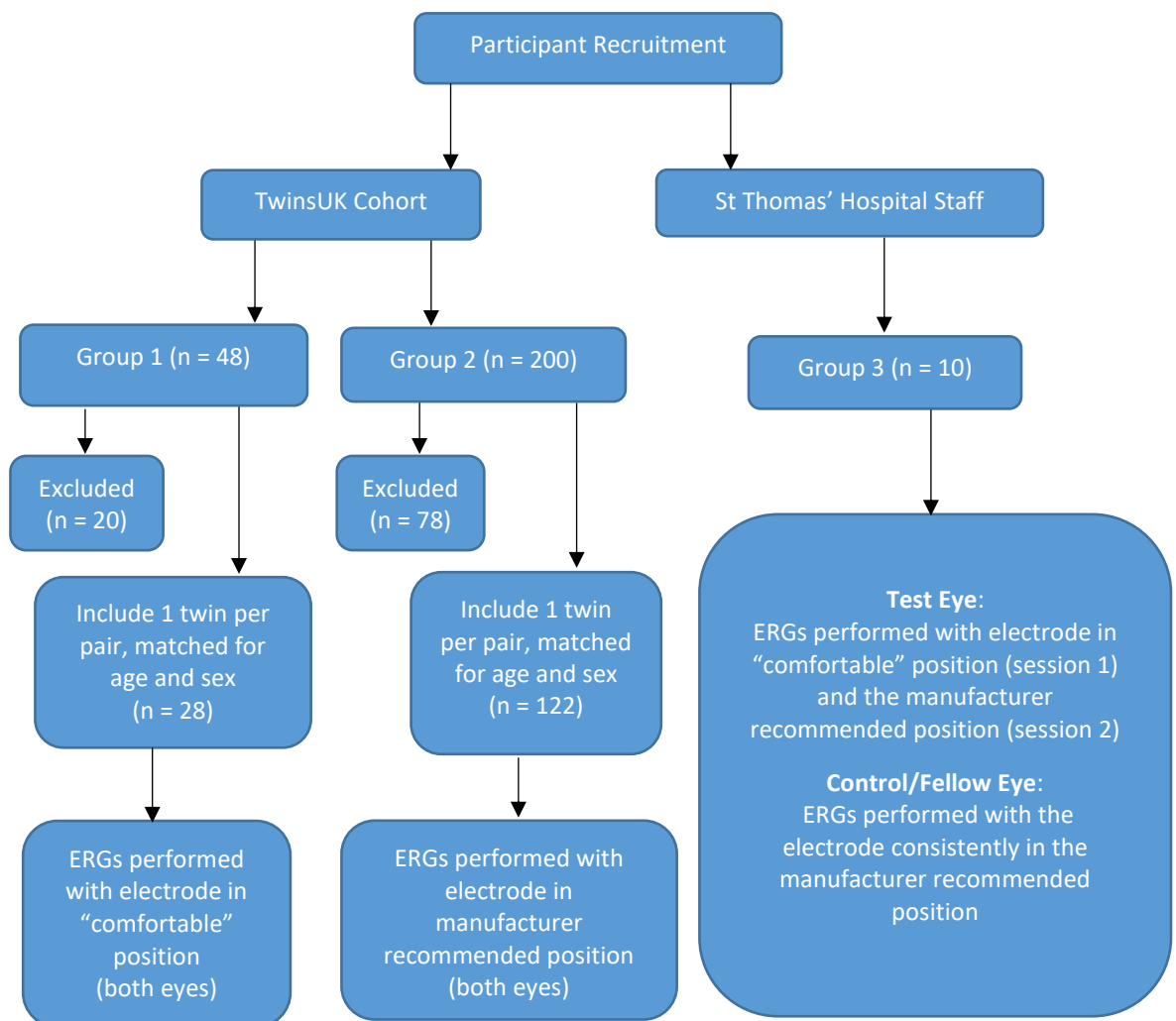


Figure 4.10 Participant pathway during study.

In a third group of healthy participants ($n = 10$), photopic flash (85 Td.s, 2 Hz) and flicker ERGs (as above) were recorded in two consecutive recording sessions: in one session, electrodes were placed in the recommended position on both eyes; in the other session, the electrode was placed 15 mm below the lid margin in the test eye, but remained in the recommended position (2 mm below the lid margin) in the fellow eye.

Statistical Analysis

The distribution of ERG data were evaluated for normality using the test, and found to be non-normally distributed, hence non-parametric tests were used. A Mann-Whitney U-test was used to determine whether differences in average data between Groups 1 and 2 reached statistical significance. Right eye and left eye data were analysed separately to allow for the fact that data were always collected from the right eye first, which may have introduced a confounding effect of adaptation. In the third group of healthy participants, ERG parameters from the test eye were normalised to those of the control eye to control for any adaptational changes between the right eye and left eye data collection. Following this, the resulting normalised parameters were compared in a between sessions analysis using a Wilcoxon signed rank test.

4.3.3. Results

Inter-group comparison

In the first group, 48 consecutive twin participants underwent recordings. Including only one twin per pair yielded 28 participants, mean (SD) age 60 (10) years; all were female (reflecting the demographics of the TwinsUK cohort, which is overwhelmingly female). In the second group of 200 consecutive participants, including only one twin per pair gave 122 participants, mean (SD) age 47 (16.8) years; 15% were male. The mean ages were significantly different ($p < 0.001$). The cohorts were then age and sex-matched by first removing the males from the second group, and then including the same numbers of participants per decade as in the first group (by excluding any excess participants in each decade in the second group in chronological order of recruitment). The mean (SD) age of the remaining 28 participants in the second group was now 61 (9.4), which was not significantly different from Group 1 ($p = 0.81$).

The flicker ERG amplitude was found to be significantly lower in the “comfortable” (Group 1) than the “recommended” (Group 2) position. Time to first peak did not, however, differ significantly between groups (see Table 4.5, and Figure 4.11).

		Group 1 (“comfortable” electrode position)		Group 2 (recommended electrode position)		Mann Whitney (p)
		Mean ($\pm$ SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Flicker ERG	Amp (μ V)	21.00 ($\pm$ 12.00)	18.60 (10.70, 28.70)	30.60 ($\pm$ 9.70)	31.7 (23.30, 37.90)	0.002*
	TtP (ms)	25.90 ($\pm$ 1.40)	25.5 (25.00, 26.60)	25.90 ($\pm$ 0.90)	26.00 (25.2, 26.70)	0.54

Table 4.5. Comparison of average values for flicker Amplitudes and Time to Peak parameters between the two age-matched groups. Significant values highlighted with an asterisk ().*

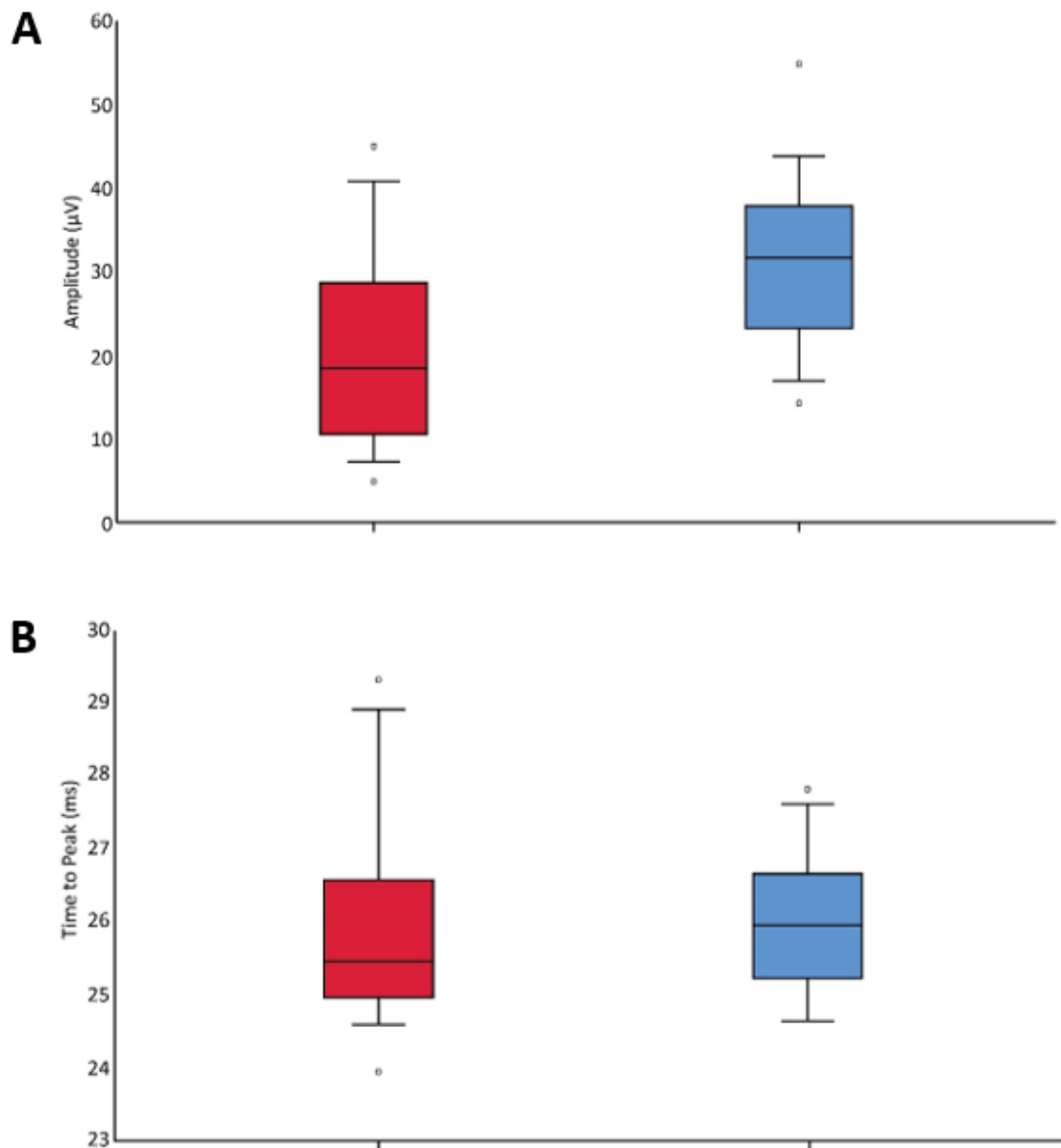


Figure 4.11. Boxplots comparing the distribution of flicker ERG parameters between participants in Groups 1 and 2. In Group 1 (red), the electrode was in a comfortable position (up to 20 mm from the lid margin); in Group 2 (blue), the electrode was placed in the recommended position of 2 mm below the lid margin. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted. Amplitudes are plotted in A and times to peak in B.

Intra-subject comparison

In the second part of the study, 10 healthy volunteers (4 male, 6 female; age range 23 to 45 years; mean (SD) 31 (7) years; median 34 years) underwent consecutive recordings. Sample recordings from one participant are shown in Figure 4.12: the waveforms were similar in both recording positions, but the response was substantially larger in the lid margin position. Green and orange lines show averages of consecutive runs of stimulus presentations, showing good intra-session reproducibility. Figure 4.13 shows median values for amplitudes and peak times for the group (test eye normalised to fellow eye as described in section 4.3.2). Again, amplitudes were significantly lower when the electrode was positioned at 15 mm, compared with 2 mm, from the lid margin, but peak times were similar; p values are given in the figure legend.

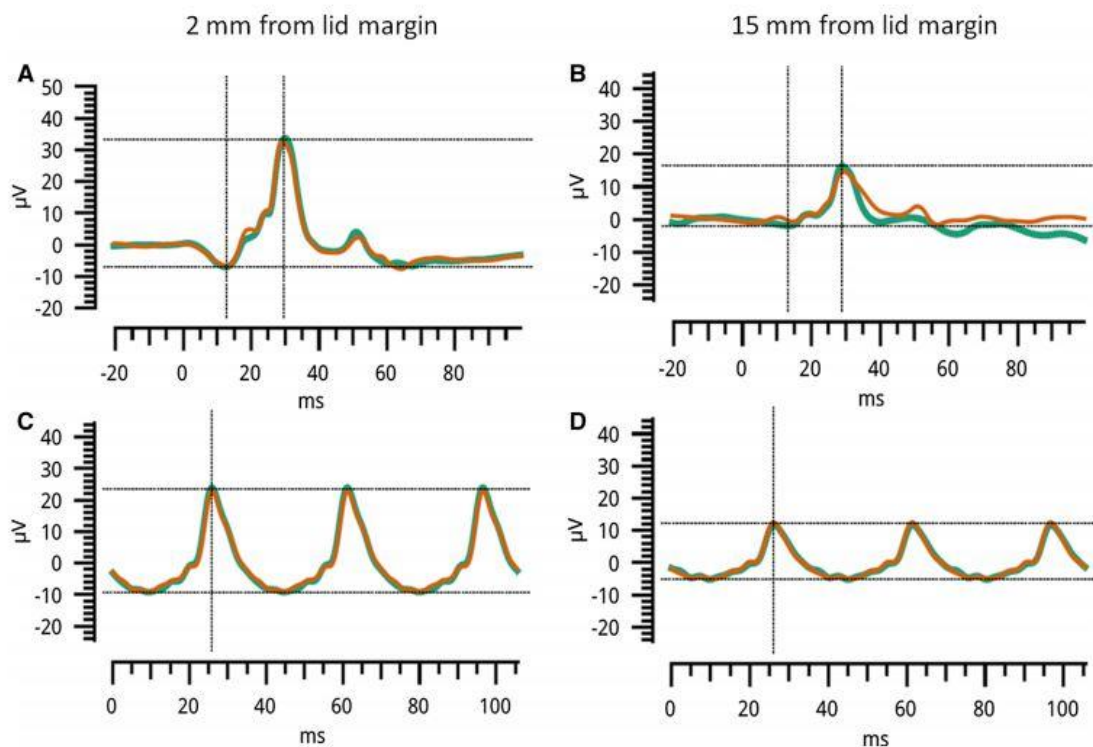
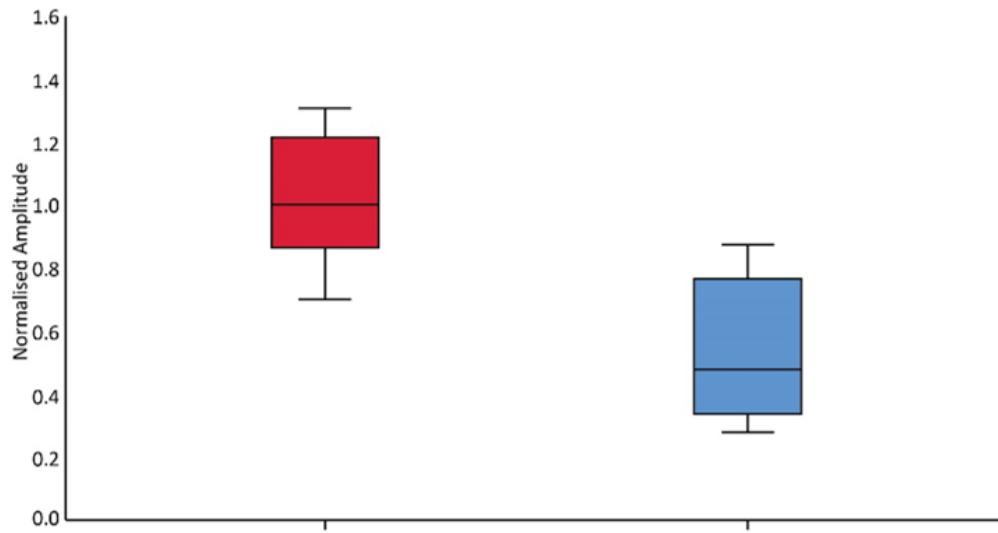
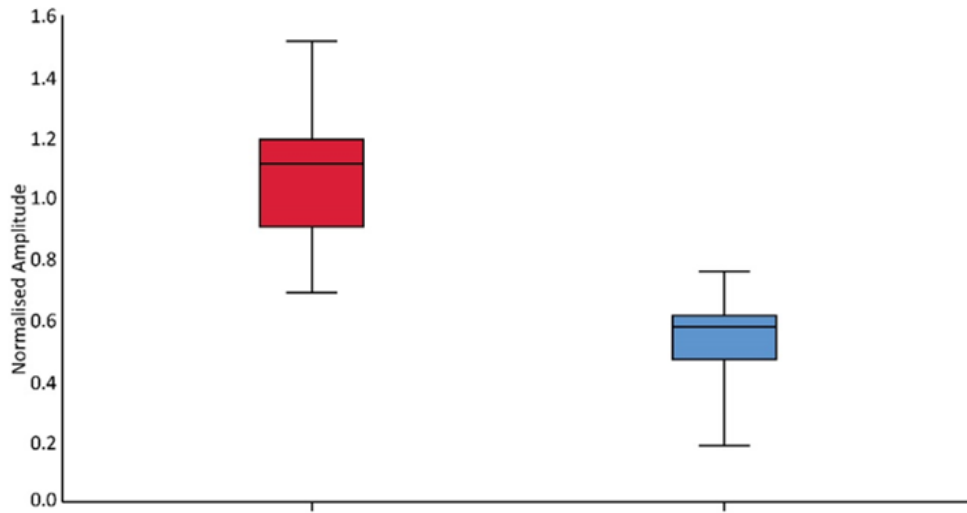
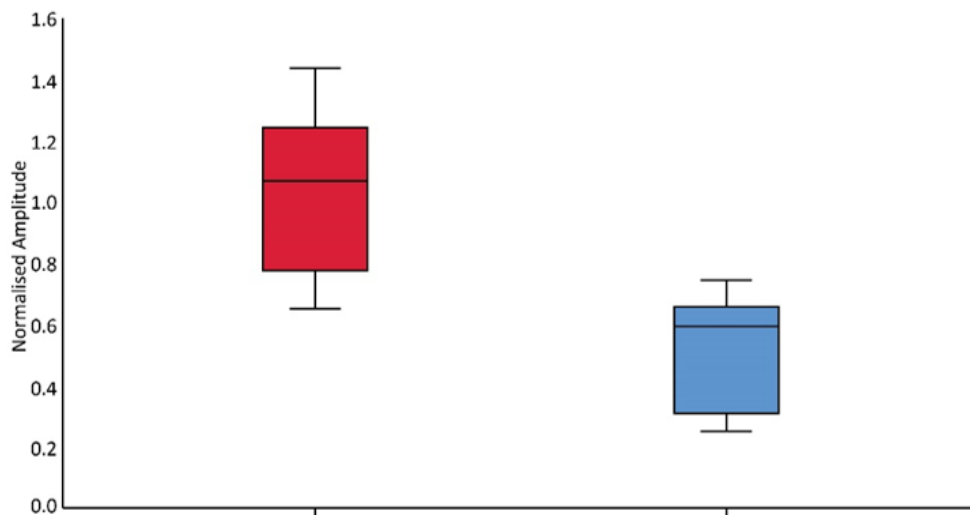


Figure 4.12. Sample traces from one participant.

A and B; Averaged light-adapted ERGs to a flash obtained from right and left eyes, respectively. In the right eye, the skin electrode was placed 2 mm from the lid margin. In the left eye, the electrode was 15 mm below the lid margin.

C and D; Light-adapted responses to the flicker stimulus obtained with electrodes placed as in A, and B, respectively. Green and orange traces represent averaged recordings obtained from consecutive runs of stimulus presentations.

A**B****C**

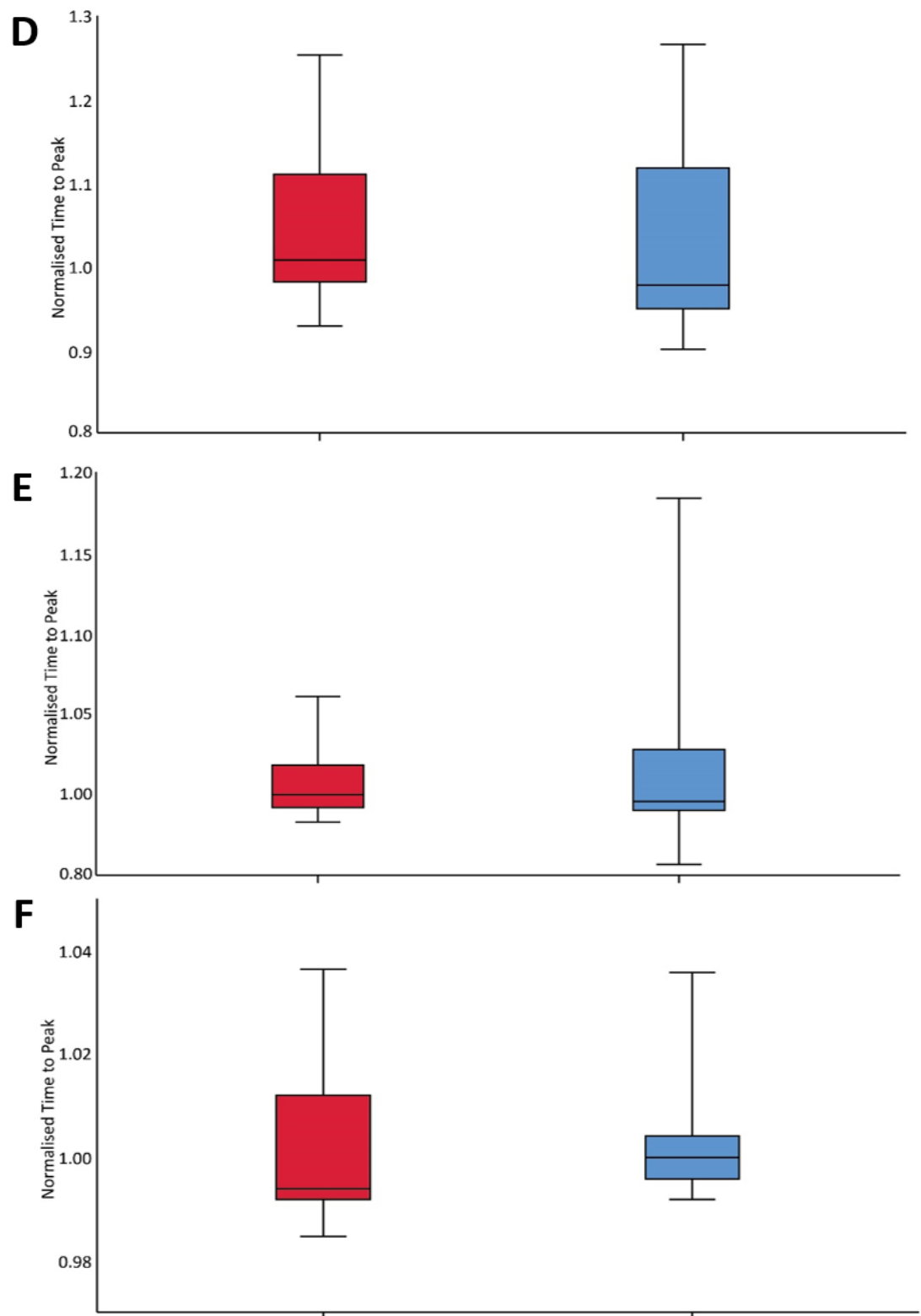


Figure 4.13: ERG parameters for different electrode positions (red = 2mm from lid margin, blue = 10-20mm from lid margin) in participants in the third group ($n = 10$; data normalised to control eye). Boxplots showing a-wave (A), b-wave (B) and flicker (C) ERG amplitudes, and a-wave (D), b-wave (E), and flicker (F) ERG peak times: differences were not statistically significant ($p = 0.85, 0.57$ and 0.56 , respectively). Boxes show median and upper and lower quartiles; whiskers extend to maximum and minimum values. Note that the y-axis scales differ in the lower panels and have been expanded considerably in F, where the actual range between maximum and minimum values is less than 0.1.

4.3.4. Discussion

This study investigated the effect on ERG responses of moving skin electrodes further inferiorly from the lid margin, both by comparing photopic flicker ERGs in two large groups of participants (with the two groups differing by electrode position), and by recording photopic flash and flicker ERGs in a small group in whom recordings were performed using two different positions in consecutive sessions. The findings from both investigations were consistent: placing the electrode in a more inferior position resulted in a significant reduction of response amplitudes (by approximately 30-50%) although peak times appeared to be unaffected. This finding emphasises the importance of consistent skin electrode positioning, particularly when amplitudes are being analysed.

It has previously been shown that recording electrode type and distance from the cornea significantly affect ERG recordings (France, 1984; Esakowitz et al., 1993; Kriss, 1994; Bradshaw et al., 2004; Mortlock et al., 2010); the further the electrode is from the cornea, the smaller the recorded response (Mortlock et al., 2010). Skin surface electrodes have been shown to be more variable than electrodes in direct contact with the ocular surface, although reproducible responses can be obtained (France, 1984; Bradshaw et al., 2004).

The findings of the present study suggest that skin recording electrode positioning is less critical with respect to analysis of peak time parameters. Nevertheless, the electrode must be positioned sufficiently close to the eye to elicit an ERG in which the peaks and troughs are discernible, in order to measure peak times. In this study, all healthy subjects produced a measurable flicker ERG, even when the electrode was positioned at 15 mm from the lid margin. However, in patients with subnormal responses in retinal disease, this can be more challenging. In some patients with reduced cone system responses measured using conventional ERG recording systems, it has not been possible to accurately determine peak times using the RETeval® system as amplitudes are too low to distinguish response components (Nakamura et al., 2016).

One limitation of the present study was that electrode placement in Group 1 was not consistent, and was determined more by participant “comfort”, whilst positioning in Group 2 was consistent. Also, the participants were not randomised to different

electrode positions, but the position was determined by whether they were in the first or second phase of subject recruitment. However, recordings from the third group of participants (in whom electrode placements were consistent and both positions were used in the same subject) were consistent with the findings from the first two groups. Also, potential time-dependent adaptational effects (the right eye was recorded from first in all subjects) were controlled for, in the first two groups, by comparing right eyes and left eyes separately, and, in the third group, by normalising the test eye to the control eye. Even when right and left eyes were compared separately in the first two groups, the results were found to be similar (amplitudes differed significantly between the two positions, but peak times did not).

Although multiple parameters were compared in the intra-subject comparison, a correction for multiple testing was not deemed necessary (Armstrong, 2014). The comparisons were predetermined and essentially explored differences in only two types of parameter, namely amplitudes and peak times. The finding that amplitudes were significantly different, and peak times were not, was observed consistently across all parameter comparisons, strongly supporting the validity of the conclusions of this study.

Considering the results of this aspect of the study it was decided to use the manufacturer's recommended electrode positioning for future work in this PhD. The reasons for this were two-fold; firstly, in order to maximise repeatability, and secondly to produce the largest possible amplitude response for each of the ERG protocols. Although comfort is a significant factor for participants and patients the duration of the protocol being performed in this study is significantly shorter than investigative procedures in hospital environments as only photopic ERGs are being performed. Thus, the electrodes are only in contact with the participant for a maximum of 10 minutes, and discomfort is less of an issue,

4.4. Conclusions

The RETeval® and Espion™/ColorDome system showed similar repeatability and variation between individuals. Electrode positioning is very important in obtaining repeatable ERG results. Maintaining the guidelines suggested by the manufacturer is recommended although, should the need arise, downward displacement of skin electrodes will still provide accurate time to first peak ERG measurements and can be

utilised where necessary. As shown in previous studies, smaller amplitudes were recorded using the skin electrodes of the RETeval® compared to the DTL fibre electrodes of Espion™/ColorDome System suggesting that both devices may be used to detect change in disease state but subcomponents of the waveforms produced may differ by device. As in previous studies, time to first peak measurements were more reliable parameters in both devices. Modest changes in flicker ERG frequency can affect the amplitude and time to first peak of the produced waveform, suggesting each of these frequencies may be used to measure retinal function but that results using different frequency of stimulation are not directly comparable.

Chapter 5. The Effect of the Number of Intravitreal anti-Vascular Endothelial Growth Factor Injections on the Structure and Function of the Peripheral Retina

5.1.1. Introduction

The progression of nAMD can be reduced by repeated ocular anti-VEGF injections (see Section 1.4; Brown et al., 2006; Rosenfeld et al., 2006). However, VEGF is produced by the healthy adult eye as well as being upregulated in the diseased state (Adamis et al., 1991; Kim et al., 1999; Famiglietti et al., 2003; Saint-Geniez et al., 2006, Saint-Geniez et al., 2008). The anti-VEGF drugs are non-specific and so target all VEGF regardless of whether or not it is within the physiologically normal range. This introduces the possibility that anti-VEGF drugs may have an adverse effect on the function and structure of the normal eye.

The literature investigating this hypothesis has been reviewed in detail in Chapter 2 of this PhD, but a brief summary of the salient points follows. The majority of animal studies reported no evidence of functional (Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Inan et al., 2007; Zayit-Soudry et al., 2010; Shahar et al., 2006; Heiduschka et al., 2008; Manzano et al., 2006) or structural (Arraes et al., 2009; Bakri et al., 2006; Thaler et al., 2010; Kaempfer et al., 2008; Kim et al., 2008; Karawya, et al., 2008; Lu et al., 2014; De Oliveira Dias et al., 2014; Manzano et al., 2006; Sari et al., 2006; Cam et al., 2017; Januschowski et al., 2014; Iandiev et al., 2011) abnormality in the retina after application of anti-VEGF drugs. However, there is some evidence from animal and *in vitro* studies to suggest that both outer and inner retinal function may be affected by this treatment, although this may show some recovery over time (Saint-Geniez et al., 2008; Nishijima et al., 2007; Sakurai et al., 2009; Myers et al., 2012). Histological and histochemical studies have also reported some evidence of structural changes following exposure to anti-VEGF drugs, such as increased apoptotic activity in photoreceptors (Avci et al., 2009), severe loss of RGCs and damage of inner and outer nuclear layers (Romano et al., 2012), bipolar cell loss (Myers et al., 2012), retinal astrocyte damage (Zayit-Soudry, et al., 2010), increased apoptosis and reduced viability of RPE cells (Sahiner et al., 2018), and damage to the choriocapillaris (Julien et al., 2014).

In humans, most studies found no negative effect of anti-VEGF therapy on peripheral retinal function (Staal et al., 2009; Maturi et al., 2006; Stahl et al., 2009; Zeimmsen et al., 2008; Marmor et al., 2009; Skaat et al., 2011; Macky and Mahgoub, 2012; Moon et al., 2013; Chabblani et al., 2015; Holm et al., 2015). However, reductions in scotopic rod and cone ERG amplitudes and delayed times to peak have been reported (Pedersen et al., 2010; Comyn et al., 2014), and abnormalities of the subcomponents of the photopic flash ERG (Yagura et al., 2016; Pedersen et al., 2010), flicker ERG (Terauchi et al., 2019), and mfERG (Feigl, 2007; Almeida et al., 2015). With respect to structural findings, the majority of studies reported no significant effect of anti-VEGF therapy on peripapillary RNFL thickness (Sobaci et al., 2013; Shin et al., 2014; Demirel et al., 2015; Entezari et al., 2014; El-Ashry et al., 2015; Yau et al., 2015; Jo et al., 2016; Zucchiatti et al., 2017). In contrast, four studies provided evidence suggestive that anti-VEGF exposure resulted in thinning of the RNFL (Parlak et al., 2014; Enders et al., 2017; Valverde-Megias et al., 2019; Yang et al., 2017), although a similar effect was seen in the control eyes of two of these studies (Parlak et al., 2014; Valverde-Megias et al., 2019), and the effect in one study was attributed to the resolution of macular oedema (Yang et al., 2017). There is also some evidence to suggest that RPE atrophy may progress as a function of anti-VEGF therapy (Kim et al., 2016), although this has been disputed (Sitnilska et al., 2019), and there have been reports of abnormal retinal blood vessel diameter and/or blood flow following anti-VEGF therapy in humans (Papadoupulou et al., 2009; Fontaine et al., 2011; Benatti et al., 2017; Corvi et al., 2015; Kurt et al., 2018).

The literature review performed in Chapter 2 highlighted numerous limitations in the studies, leaving unknowns such as missing baseline measurements (Bakri et al., 2006; Kim et al., 2008; Cheng et al., 2009; Lu et al., 2014), non-disclosure of subject age (Bakri et al., 2006; Kim et al., 2008), using histological analysis but failing to state which location retinal samples were taken from (Schnichels 2013; Spitzer et al., 2007; Julien et al., 2014; Iriyama 2007), and failing to give full details of ERG recording protocols (Luke et al., 2009; Macky and Mahgoub, 2012; Martinez-Castellanos et al., 2013; Myers et al., 2012; Pai et al., 2007; Pedersen et al., 2010; Sakurai et al., 2009; Stahl et al., 2009; Tokunga et al., 2014; Tolentino et al., 2004). There were also high levels of variation in follow up duration, with most studies only using a very short follow up

period (Demirel et al., 2015; Ferrara et al., 2007; Horsely et al., 2010; Parlak et al., 2014; Shin et al., 2014; Ahn et al., 2013), and/or following up after only a single dose of treatment (Arres et al., 2009; Bakri et al., 2006; Cheng et al., 2009; de Oliveira Dias et al., 2014; Guo et al., 2010; Inan et al., 2007; Iriyama et al., 2007; Julien et al., 2014; Kim et al., 2008; Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Myers et al., 2012; Ramon et al., 2018; Sakurai et al., 2009; Shahar et al., 2006). Many other studies were limited by the lack of a control group (de Oliveira Dias et al., 2014; Inan et al., 2007; Januschowski et al., 2014; Luke et al., 2006; Luke et al., 2009; Luke et al., 2010; Manzano et al., 2006; Shahar et al., 2006; Zayit-Soudry et al., 2010; Zayit-Soudry et al., 2011).

There is also a distinct gap in the literature with regards to examining the photopic negative response (PhNR) in people undergoing anti-VEGF therapy. The PhNR is an ERG component which has been attributed to the activity of RGCs (see Section 1.5.7; Colotto et al., 2000; Machida et al., 2004; Viswanathan et al., 1999; Viswanathan et al., 2000; Viswanathan et al., 2001; Rangaswamy et al., 2007). There is evidence to suggest that RGCs may be at risk of damage by anti-VEGF therapy. For example, in an animal model of ischaemia-reperfusion injury in rats, Nishijima et al., (2007) reported that VEGF provided a neuroprotective effect on retinal neurons, whilst chronic VEGF inhibition in adult animals led to a significant loss of RGCs. In humans, Beck et al. (2016) noted a significant reduction in RGC layer thickness in treated eyes compared to untreated control eyes of patients with nAMD receiving anti-VEGF therapy over an average follow up period of 45 months. This was measured within the macular area (the outer ring of the EDTRS grid) but is indicative of an effect of treatment on RGCs. However, only two studies (Yagura et al., 2016; Moon et al., 2013) have been performed on humans to monitor the PhNR after anti-VEGF treatment. Whilst these studies found no significant difference between any PhNR parameter before/after anti-VEGF therapy, ERGs were only recorded for up to 4 weeks after a course of three anti-VEGF injections. The impact of a more prolonged course of treatment on this subcomponent has not been evaluated.

A key area of concern in the current literature was the lack of any exploration of the relationship between anti-VEGF dose and effect on retinal structure and function. The studies presented in this chapter aimed to address this limitation.

5.1.2. Aims

The overall objective of the studies in this chapter was to determine whether there was a relationship between the cumulative dose of anti-VEGF therapy received for nAMD and parameters of retinal function and structure. Specifically:

- 1) To evaluate differences in retinal structure and peripheral retinal function between eyes in patients receiving anti-VEGF therapy in one eye only for nAMD.
- 2) To determine the relationship between number of anti-VEGF injections/ time since first injection and peripheral retinal function assessed using the full-field ERG.
- 3) To determine the relationship between number of anti-VEGF injections/ time since first injection and retinal layer thickness measured at the edge of the macula using the OCT.

These aims were addressed across two studies. The first took place at St Thomas' Hospital London (Section 5.2), and the second at Moorfields Eye Hospital London (Section 5.3). Aims 1 and 2 were addressed by both studies, whilst aim 3 was addressed by study 1 only (Section 5.2).

5.2. Study 1: A cross-sectional study of the effect of anti-VEGF dose on full-field ERG parameters recorded using a desktop ERG system

This project was (partly) presented at EURETINA 2018, Vienna, Austria (Paper Presentation), ARVO 2018, Honolulu, HI (Poster Presentation), BCOVS 2017, Plymouth, England (Paper Presentation), City, UoL Doctoral Research Conference 2017, London, UK (Paper Presentation) Optometry Tomorrow 2017, Birmingham, England (Poster Presentation).

5.2.1. Methods

Participants

Ethical approval was gained through the National Research Ethics Service (NRES) Committee London – Harrow (11/LO/2029), and written informed consent was obtained from all subjects, conforming to the tenets of the Declaration of Helsinki. Eligible patients attending the medical retina clinics at St Thomas' Hospital London for anti-VEGF treatment of nAMD between June 2015 and July 2017 were recruited for this study. The participant pathway is outlined in Figure 5.1

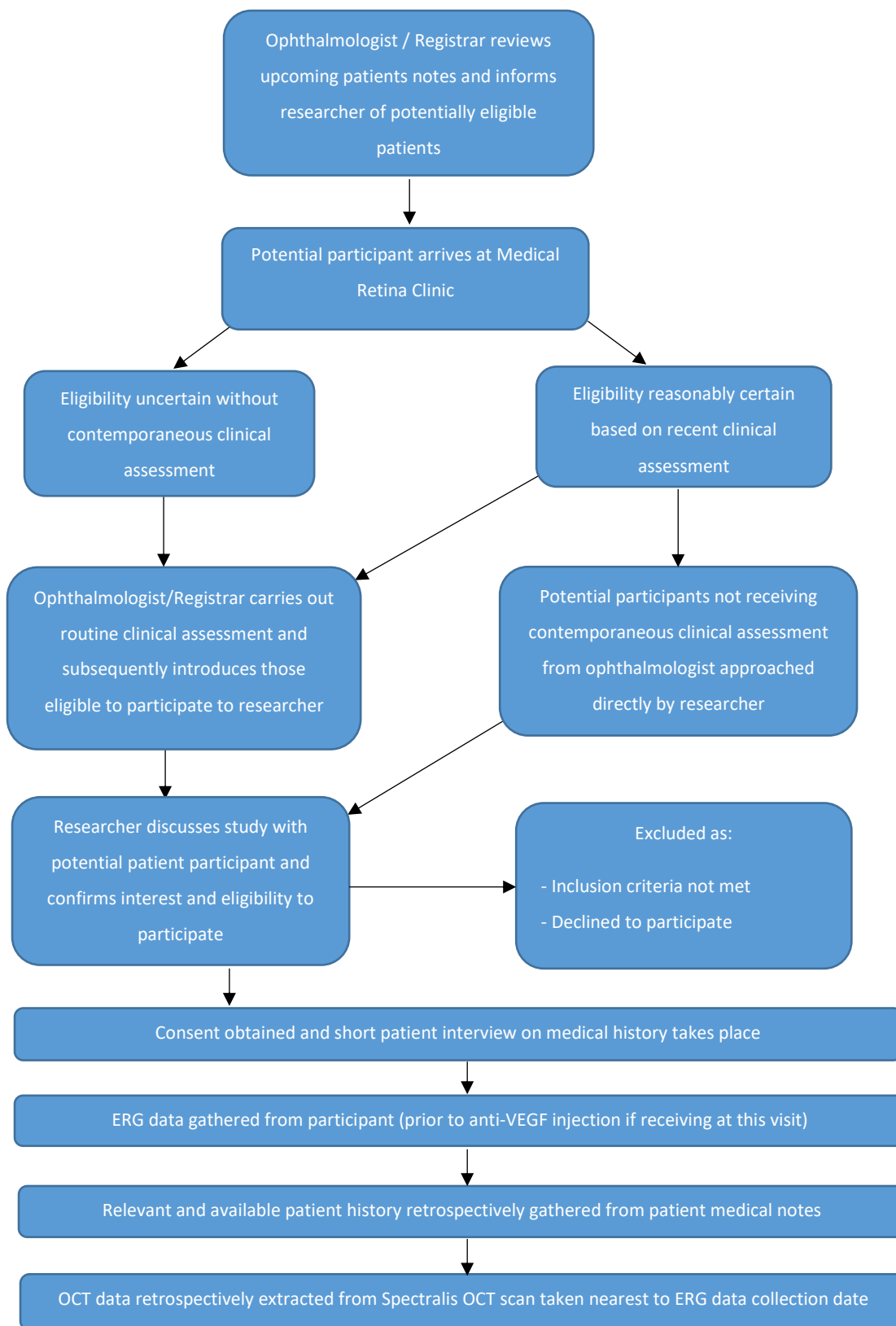


Figure 5.1. Recruitment pathway for patient participants within the Medical Retina clinic at St Thomas' Hospital, London.

Potentially eligible participants were identified by the screening of medical records by an ophthalmologist working within the medical retina clinic at the start of the clinical session. All potential participants were receiving or about to commence anti-VEGF treatment for nAMD. Exclusion criteria included suffering from any other eye condition that may affect retinal health (such as, but not limited to, glaucoma or retinal detachment), a diagnosis of diabetes, a diagnosis of photosensitive epilepsy, and long-term use of drugs known to affect retinal function such as tamoxifen or chloroquine. Individuals who had been identified as potentially meeting the inclusion criteria for the study were greeted by the researcher at the earliest available opportunity after their arrival at the clinic, and a brief discussion was held to determine interest in participation and whether eligibility criteria were met. Once eligibility was established, an information sheet on the study was provided and consent obtained. Participants took part in a short interview to obtain relevant ocular and general information including previous ocular history, general health, medications, allergies, and the date of their last OCT scan and consultation with the relevant consultant ophthalmologist or registrar based in the medical retina clinic. This last question was asked as it quickly became apparent that not all patients attended the medical retina clinic for scans and consultation on the same day that they received anti-VEGF treatment. Relevant information was also extracted from participant's medical notes where available. This information included; visual acuity, number of injections received to date, type(s) of injection received, date of birth, notes on ocular/general health, and the date anti-VEGF treatment began.

Optical Coherence Tomography

Participants received an OCT scan as part of their assessment within the medical retina clinic. Images were obtained on a Spectralis OCT (Heidelberg Engineering Inc) by a clinical medical imaging specialist working within the medical retina clinic at St Thomas' Hospital. In taking these, the specialist followed the manufacturers guidelines (Heidelberg Engineering, 2014). However, due to protocol established at the hospital, keratometry values for each individual patient were not taken as recommended. Instead, this parameter was maintained at 7.7 mm for each participant – the standard setting of the Spectralis OCT when in acquisition mode. As stated in the previous subsection, OCT scans may be performed on the day of anti-VEGF injections or some days

prior in anticipation of necessary treatment, which would then be booked for another occasion upon exiting the clinic.

Prior to commencement of this study it was agreed with imaging technicians at the hospital that patients would all receive a Spectralis OCT scan as part of their clinical care. It was originally proposed by the researcher that participants receive two OCT scans as part of the clinical and research pathway for this study; a wide field macular OCT scan, and an OCT scan of the optic nerve head. This was proposed in order to ascertain retinal structure data from areas as far away from the macular area as possible, and into the peripheral retina. However, the imaging technicians declined the inclusion of an optic nerve head OCT scan as it was felt this would impinge too greatly on the efficiency of the busy medical retina clinics. However, it was agreed that the standard macular scan used by the medical retina clinic could be adapted to facilitate data collection for this study. The scan would be focused on the macular area but extend as widely as the Spectralis OCT would allow so that retinal structure data might be extracted at a distance of 3 mm from the fovea for this study and no extra inconvenience would be incurred to any patients of the clinic. The parameters were requested as follows; 30 ° x 25 °, 61 B-scan (high definition), taken in primary gaze only, one scan per eye.

Electroretinography

All participants were dilated as part of their standard clinical assessment within the medical retina clinic (Tropicamide 1%, and Phenylephrine 2.5%), ensuring the dilation requirement recommended by the ISCEV Standards for Full Field Electroretinography (McCulloch et al., 2015) was met. Pupil reflexes were assessed before commencing recording to ensure that dilation was complete. Full field responses were obtained from both eyes of the participant simultaneously regardless of unilateral or bilateral disease. Focal responses were recorded monocularly to ensure the eye was correctly aligned with the stimulus.

Electrodes were applied according to the protocol in Section 3.2.2. The Espion™/ColorDome stimulator system (Diagnosys, Cambridge) was used to obtain all evoked potentials (see Section 3.2.3). The system was calibrated by the manufacturer on an annual basis, and self-calibrated each day on initiating the device (Diagnosis LLC,

2020). Additional manual calibration checks were performed using the ILT 1700 Research Radiometer with SED033/Y/R luminance detector (model SED033, Able Instruments and Controls, Reading, UK). The bandpass filters were set from 0.3 to 500 Hz for all ERG recordings, and artefact rejection levels were set at 300 μ V for all three protocols. Repeated traces were superimposed to check for consistency. For all ERG types, if any trace appeared to contain an eye movement or blink artefact, additional traces were recorded to obtain, where possible, superimposable traces and the affected trace was excluded from the averaged response.

The following photopic ERG protocols were applied to all participants, in accordance with the protocols evaluated in Chapter 3:

Photopic Negative Response

The Photopic Negative Response (PhNR) ERG was recorded in response to a red LED flash (peak wavelength 635 nm) of 0.11 cd.s.m^{-2} presented for 4 ms (stimulus frequency 2 Hz) over a blue LED background (peak wavelength 465 nm) of 158.8 (scotopic) cd.m^{-2} , which was sufficient to saturate the rod photoreceptors (Aguilar and Stiles, 1954; i.e. 3.8 log scot Td, assuming 7 mm pupil but making no allowance for the Stiles Crawford effect, which is minimal in scotopic conditions). Four traces were obtained for each run of each step of the protocol. Ten sweeps were averaged to obtain each trace (forty responses per run in total) with a 7 second break between each trace attempt. The post-stimulus recording timebase was 250 ms and there was a pre-stimulus baseline recording timebase of 50 ms. This was then repeated with the red flash stimulus intensity of 0.21 cd.s.m^{-2} , 0.42 cd.s.m^{-2} , 0.84 cd.s.m^{-2} , 1.64 cd.s.m^{-2} and 3.35 cd.s.m^{-2} .

ISCEV standard Transient Cone Response ERG

The ISCEV standard transient cone response ERG was recorded in response to a white LED flash of 3 cd.s.m^{-2} presented for 4 ms (stimulus frequency 2 Hz) over a white (6500 K) background of 30 cd.m^{-2} . Sixty responses were averaged over 4 traces (20 runs per trace). The timebase was 250 ms. This response was recorded twice per participant, once with the participant placing with their chin on the chin rest of the Espion™/ColorDome in order to record a full field response, and once with the participant placed 300 mm from the Espion™/ColorDome stimulator with the focal

stimulator board placed immediately in front of the stimulator (as described in Section 3.4.2). Focal responses were recorded from each eye separately, with the test eye aligned with the centre of the focal target.

41 Hz Flicker ERG

The flicker ERG was recorded in response to a 41 Hz flickering stimulus (red, peak wavelength 635 nm, 12 ms duration, square wave, mean luminance 30 cd.m⁻²) which appeared against no background light. A time base of 100 ms was used, 50 responses were averaged on each trace with 4 traces in total. This response was recorded twice per participant, once with the participant placing with their chin on the chin rest of the Espion™/ColorDome in order to record a full field response, and once with the participant placed 300 mm from the Espion™/ColorDome stimulator with the focal stimulator board placed immediately in front of the Espion™/ColorDome stimulator (as described in Section 3.4.2). Focal responses were recorded from each eye separately, with the test eye aligned with the centre of the focal target.

5.2.2. Analysis

ERG Signal analysis

ERG signal analysis was as described in Section 3.4.2. The focal ERGs were processed in the same manner as their corresponding full field ERG counterparts. The averaged focal ERG responses were then subtracted from their corresponding full field ERG counterparts in Microsoft Excel (Microsoft Office, Version 16) in order to produce a waveform derived solely from the peripheral retina. Once this waveform had been produced, the maxima and minima of the waveform subcomponents were then identified as outlined previously and analysed in the same fashion.

OCT analysis

OCT data were extracted by measuring relevant layers of the retinae of both eyes of the participants with the callipers installed on the Spectralis system software. Layer thicknesses were measured at defined points on a single horizontal OCT scan through the fovea. The scans had been acquired prospectively in EDI (“enhanced depth imaging”) mode to permit measurement of choroidal thickness. Unfortunately, this meant that the automated segmentation algorithm could not be applied (this is not

applicable in EDI mode) and so manual calliper measurements were performed. The layers were chosen (Table 5.1) both for their relevance to the present study and for reliability of measurement due to consistently identifiable boundaries. Using the ETDRS grid as a guide (ETDRS Research Group, 1985; 1991), measurements were taken at 3 mm nasally and 3mm temporally to the fovea (i.e. at the edge of the area subtended by the macula, ETDRS Research Group, 1985; 1991). The measurements taken in the temporal position were of the ganglion cell complex (GCC), photoreceptor layer (PRL), outer segment (OS), choroid, and total retinal thickness (TRT) see Figure 5.2 and Table 5.1 (identification of layers was based on the approach described by Staurenghi et al., 2014). This was repeated in the nasal position where, in addition, the retinal nerve fibre layer (RNFL) was also measured (Staurenghi et al., 2014). Measurements were not obtained in the superior and inferior positions due to the location of retinal blood vessels in this area, which resulted in distorted measurements of retinal layer thickness.

OCT Layer	Inner Boundary	Outer Boundary
Total retinal thickness	Inner limiting membrane*	Inner border of RPE/Bruch membrane complex*
Nerve fibre layer* (nasal to fovea only)	Inner limiting membrane*	Outer border of nerve fibre layer*
Ganglion cell complex	Inner limiting membrane*	Outer border of inner plexiform layer*
Photoreceptor layer	Outer border of outer plexiform layer*	Inner border of ellipsoid zone*
Outer segments	Outer border of ellipsoid zone*	Inner border of RPE/Bruch membrane complex*
Choroid	Outer border of RPE/Bruch membrane complex*	Choroid-sclera junction*

*Table 5.1. Table defining boundaries of layers used in present study. *Asterisk highlights those structures defined by the International Nomenclature for OCT Meeting Consensus Normal OCT Terminology (Staurenghi et al., 2014).*

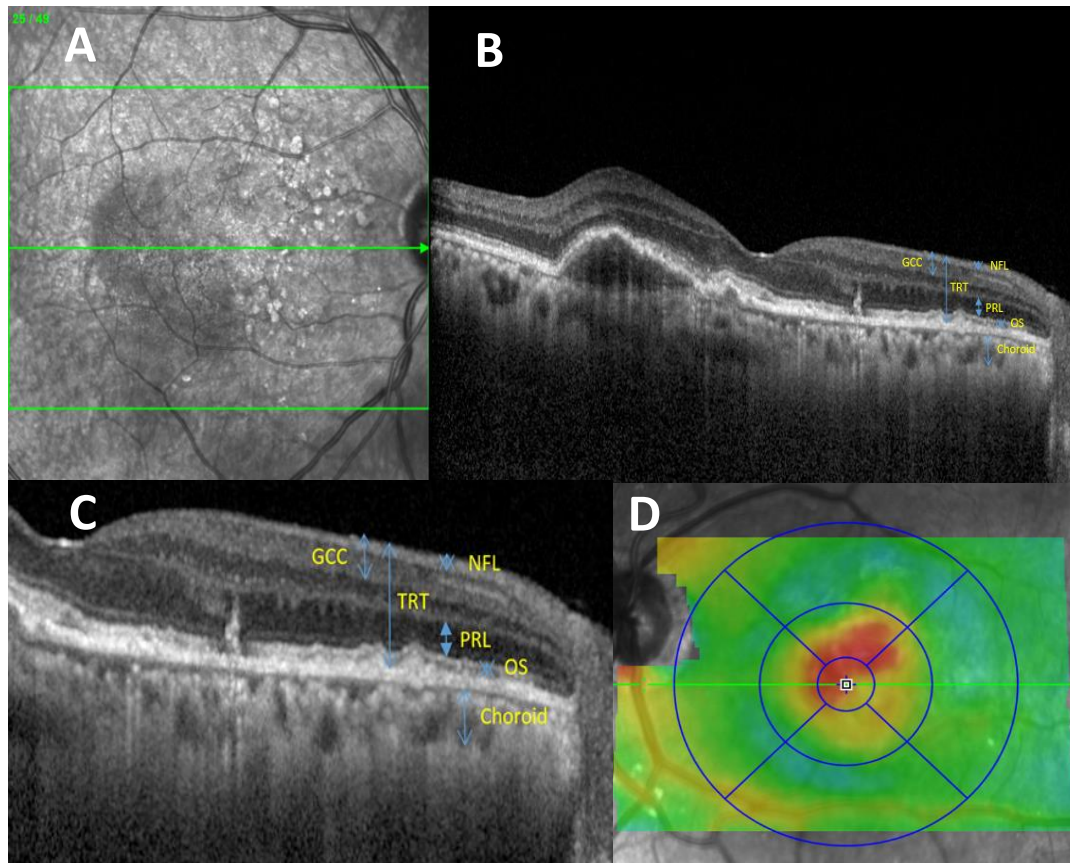


Figure 5.2. Example OCT section indicating the calliper measurements taken at 3mm nasally from the fovea. Images detail the retinal location (A) of the displayed cross-section (B), a magnified view of the retinal segments measured (C), and the ETDRS grid overlayed on an example retinal image (D). The retinal nerve fibre layer (RNFL) is only measured in the nasal retina due to its immeasurably low thickness in the temporal retina. Other retinal sections measured were the ganglion cell complex (GCC), photoreceptor layer (PRL), outer segment (OS), choroid, and total retinal thickness (TRT). Images A, B, and C are taken from an OCT scan of the right eye of participant MC0010. Image D is taken from an OCT scan of the left eye of participant GG0029.

Statistical analysis

All statistical analysis was carried out in SPSS (versions 22-25; IBM), whilst graphs were produced in Microsoft Excel (Microsoft Office, Version 16). ERG analysis was conducted on full-field data as well as data with the focal response subtracted.

The magnitude of any effect of anti-VEGF use on OCT/ERG parameters is unknown due to the limitations of current evidence (see Chapter 2), so accurate sample size calculations were not possible prior to commencing this study. However, it has been reported that one third of the distribution of correlation coefficients across 380 meta-analytic studies exceeded 0.30 (Hemphill, 2003). A sample size of 85 would be required to detect a correlation coefficient of 0.3, with a power of 80%, at an alpha (two tailed) of 0.05 (<http://www.sample-size.net/correlation-sample-size/>). Posthoc sample size considerations are presented in the discussion (Section 5.2.4).

In order to determine whether there were any significant between eye (treated vs untreated eyes) differences in ERG and OCT parameters in people with unilateral nAMD (aim 1), data were tested for normality (Shapiro-Wilk test) and a paired t-test was conducted for normally distributed data, whilst a Wilcoxon Signed-Rank test was used to analyse non-normally distributed data. In order to meet aims 2 and 3, to evaluate the relationship between the number of anti-VEGF injections and each ERG and OCT parameter, and the time since the occurrence of the first anti-VEGF injection and each ERG and OCT parameter, data from one eye of each participant were included (in bilateral cases the participant's right eye). Data from neophyte (previously untreated) patients were included to ascertain examples of retinal structure and function in participants with nAMD diseased retinae before the influence of anti-VEGF treatments. A Pearson's correlation analysis was used for normally distributed data, whilst Spearman's correlation analysis was performed on non-normally distributed data. Any of the data showing a statistically significant correlation with treatment dose/duration and meeting the necessary assumptions underwent a linear regression analysis to explore further the nature of the relationship between the functional/structural parameter and number of anti-VEGF injections/time since first anti-VEGF injection. Assumptions tested prior to conducting regression analysis were

normality of distribution of errors, the linearity of the relationship between the predictor and dependent variables, and homoscedasticity i.e. ensuring that errors had constant variance across all levels of the predicted value. All of the regression analyses performed met these assumptions. Linear regression analyses were not performed on those data showing significant correlations but did not meet these assumptions.

A correlation analysis was also performed to determine the relationship between age and number of injections received and between total number of injections and time since first injection. The former was conducted in order to determine whether any relationship between number of injections and each ERG and OCT parameter might be confounded by an age-effect (i.e. older participants having received a greater number of injections). Participants were then assigned a group number relating to the number of anti-VEGF injections they had received since being diagnosed with nAMD; Group 1 – 1 to 5 injections, Group 2 – 6 to 10 injections, Group 3 – 11 to 20 injections, Group 4 - 21 to 50 injections, Group 5 – new patients, yet to receive anti VEGF treatment and so having received zero injections. For normally distributed data, a one-way ANOVA test was conducted, whilst the non-parametric Kruskal-Wallis test was performed on non-normally distributed data to look for any significant differences in ERG parameters between injection groups. Bonferroni correction was applied to p-values to minimize the risk of type I errors i.e. by multiplying p-values by the number of comparisons made (Bonferroni, 1936; Abdi, 2007).

5.2.3. Results

The total number of participants assessed was 32. Median (interquartile range [IQR]) age of the participants was 83 (74, 86) years. The eldest participant was 93 years old and the youngest was 59. Median (IQR) number of prior anti-VEGF injections was 10 (3, 18). The minimum number of injections was 0 and the maximum was 44. The median (IQR) time since first injection was 18 (4, 29) months, with a range of 0-49 months. Twelve participants had received ranibizumab (Lucentis®) injections, of these 7 had converted to aflibercept (Eylea®) injections during the course of their treatment. Twenty participants had received solely aflibercept treatment and 27 participants had received aflibercept at some point during their treatment. Only one participant had received any bevacizumab (Avastin®) injections (participant MW had received two of these, though the date of treatment was unclear). Of the participants, 16 were male

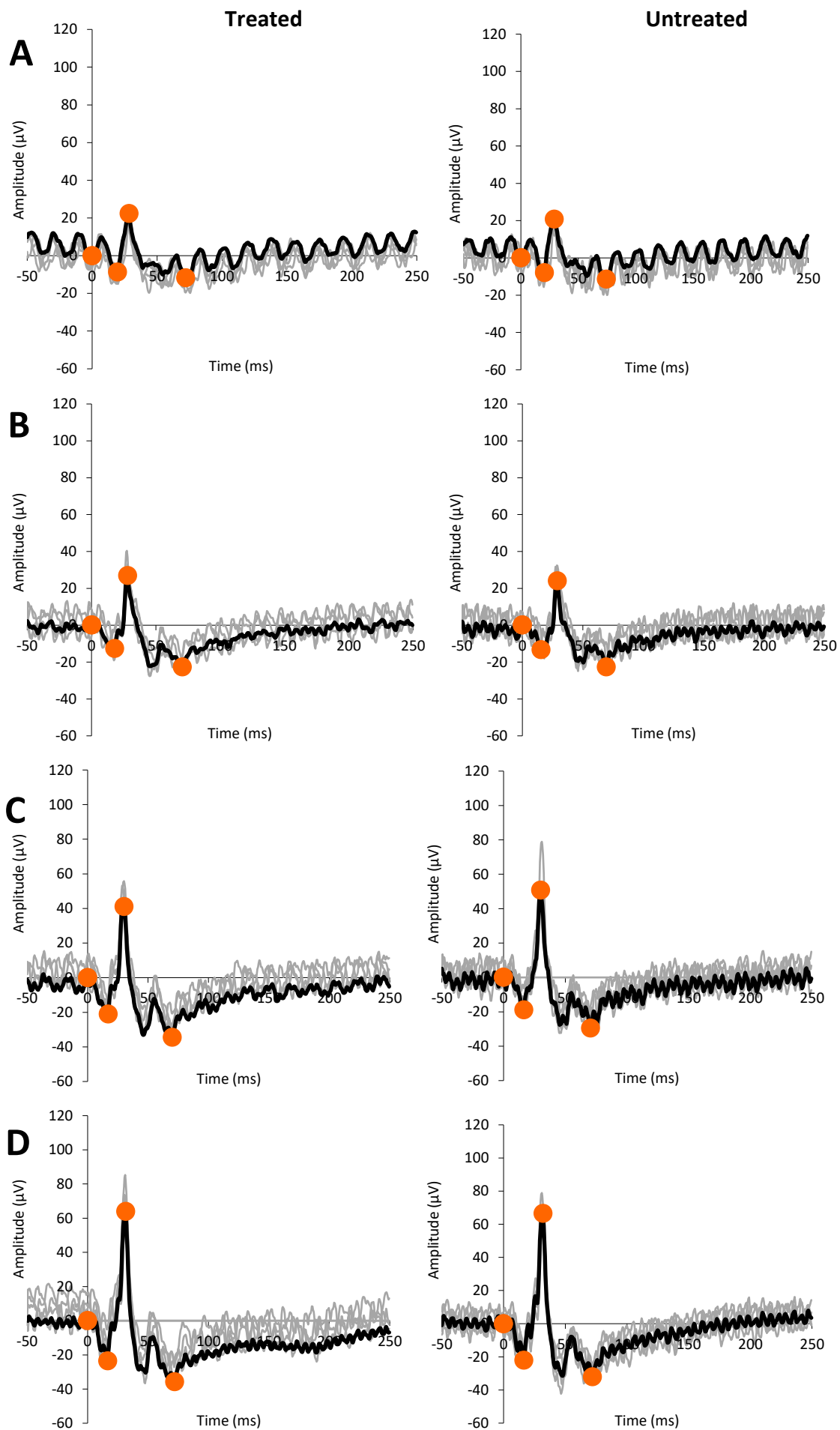
and 16 were female. The visual acuity of the participants ranged from 6/5 to 6/60 in eyes being treated for nAMD and 6/5 to hand movements in eyes not being treated for nAMD. This information is summarised in Appendix 2.

5.2.3.1. ERG data Analysis

Interocular analysis

Fourteen participants had unilateral nAMD, and provided data for the between eye analysis. Median (IQR) age of the participants was 86 (78, 88) years. The eldest participant was 92 years old and the youngest was 63. Median (IQR) number of prior anti-VEGF injections was 14 (10, 23). The minimum number of injections was 3 and the maximum was 44. The median (IQR) time since first injection was 21 (6, 36) months, with a range of 2-49 months.

All participants with unilateral AMD provided usable ERG data for all full-field protocols. Sample data from one participant are shown in Figure 5.3. Grand average ERGs from all participants with unilateral AMD can be viewed in Figure 5.4. It can be seen that there was no clear difference in ERG waveform between eyes. Some traces were contaminated by eye movements in the later part of the response. Where the early components were unaffected, the data were kept in the analysis.



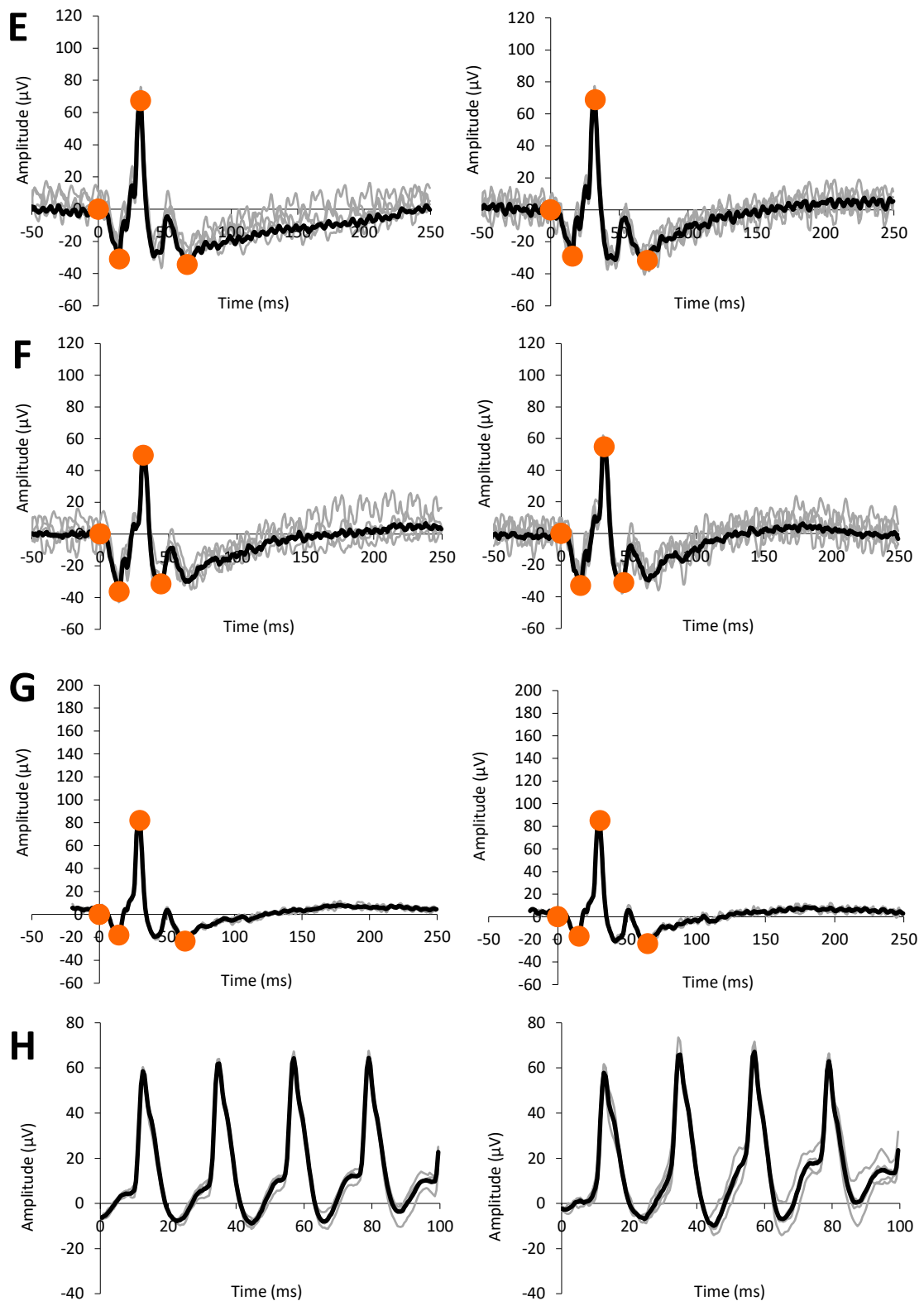
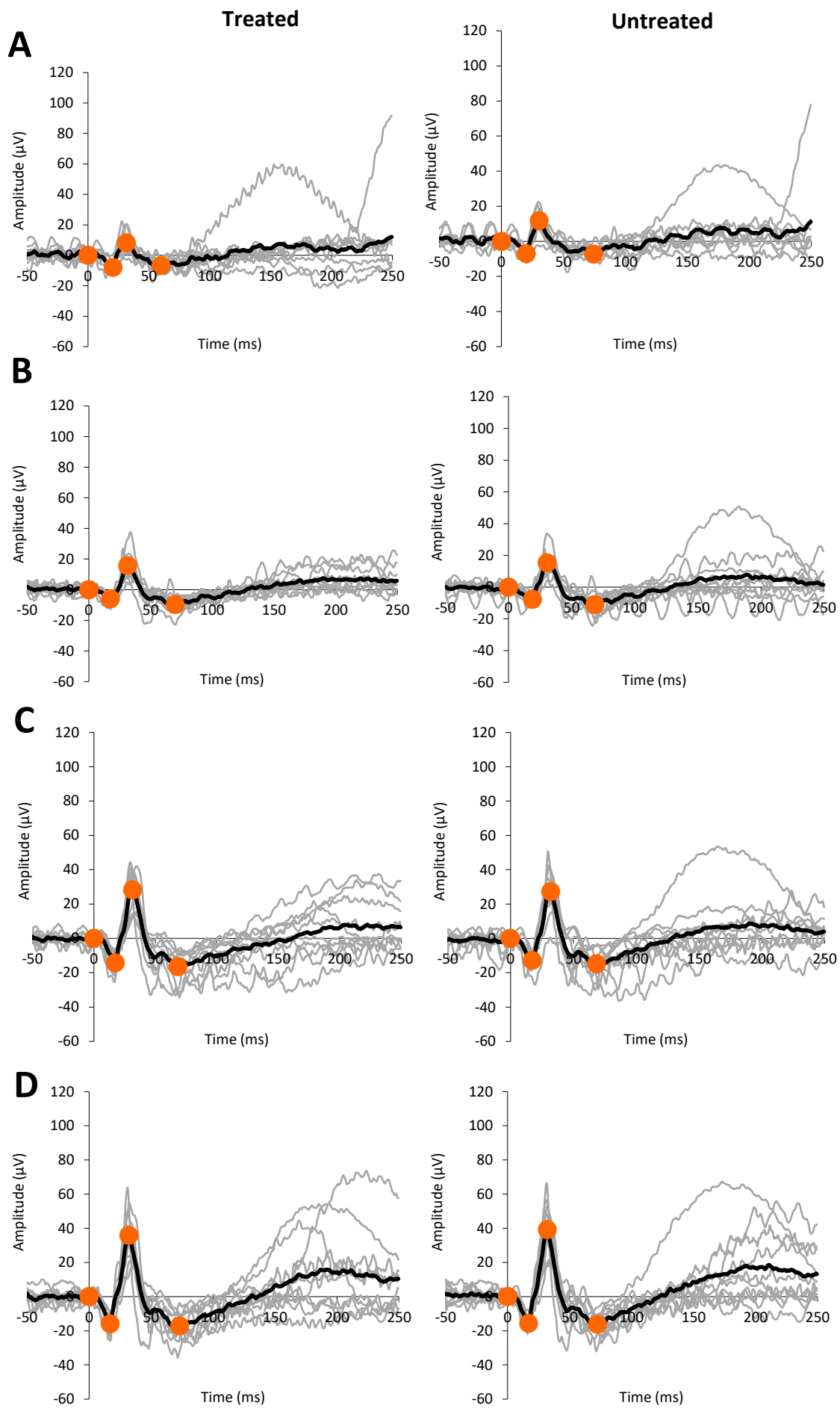


Figure 5.3. Example traces from one participant (GG0029) in the between eye analysis - Left, Treated eyes; Right, Untreated eyes. Individual traces shown in grey, grand average of all traces shown in black. Maxima and minima of a -, b -, and PhNR wave subcomponents identified by orange dots. A, PhNR 0.11 cd.s.m^{-2} ERG; B, PhNR 0.21 cd.s.m^{-2} ERG; C, PhNR 0.42 cd.s.m^{-2} ERG, D, PhNR 0.84 cd.s.m^{-2} ERG; E, PhNR 1.68 cd.s.m^{-2} ERG; F, PhNR 3.35 cd.s.m^{-2} ERG; G, Transient cone response ERG; H, 41 Hz flicker ERG. Traces of lower stimulus frequencies (A and B) were unfortunately often subject to mains interference which occurred at the study site due to unavoidable issues with electrical shielding in test areas and resulted in some contamination of traces.



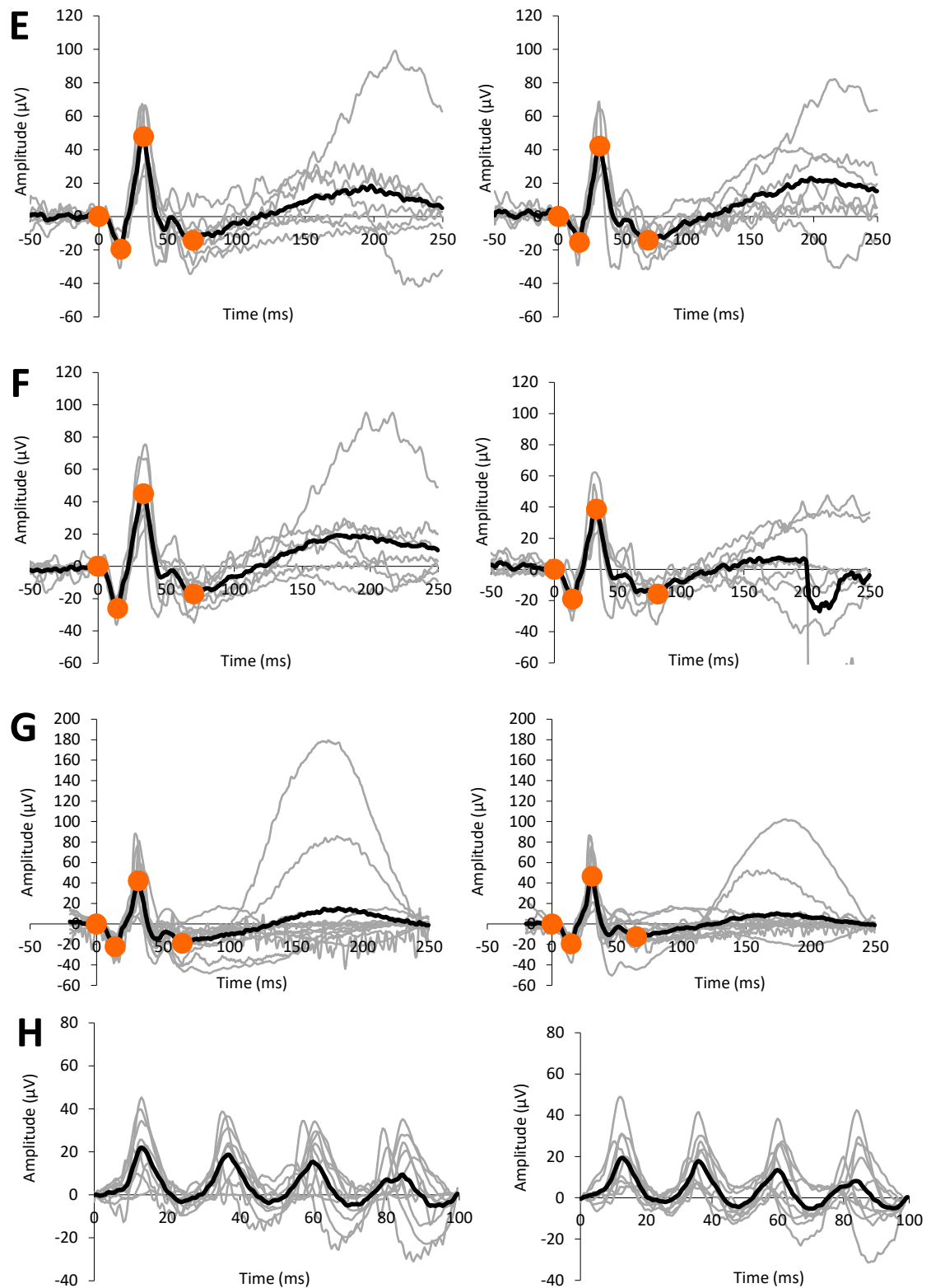


Figure 5.4. Grand average traces from all participants in the between eye analysis - Left, Treated eyes; Right, Untreated eyes. Averaged traces from individual participants are shown in grey, grand average of all participants in inter-ocular analysis shown in black. Maxima and minima of a-, b-, and PhNR wave subcomponents identified by orange dots. A, PhNR 0.11 cd.s.m⁻² ERG; B, PhNR 0.21 cd.s.m⁻² ERG; C, PhNR 0.42 cd.s.m⁻² ERG; D, PhNR 0.84 cd.s.m⁻² ERG; E, PhNR 1.68 cd.s.m⁻² ERG; F, PhNR 3.35 cd.s.m⁻² ERG; G, Transient cone response ERG; H, 41 Hz flicker ERG.

Box plots describe the distribution of data in the inter-ocular analysis (Figures 5.5-5.8). Only the 0.42 cd.s.m⁻² PhNR data are presented here for simplicity, but a similar spread of data was seen for all luminance levels. It can be seen that amplitude data had a larger range and greater variance than the time to peak measurements. There was no clear trend towards a reduced amplitude or prolonged time to peak in the treated vs. untreated eyes. The data were non-normally distributed and so a Wilcoxon signed rank test was performed to compare treated and untreated eyes. This showed that there was no significant difference between the treated and untreated eyes for any ERG parameter tested after Bonferroni correction of p-values ($p > 0.05$), see Table 5.2, although the flicker ERG first harmonic time to peak was significantly longer in the treated eyes prior to Bonferroni correction. Focal responses were obtained from 10 of the 14 participants with unilateral nAMD. Subtraction of the focal responses from the full-field transient and flicker ERGs did not impact on the majority of results (see Table 5.3). The only notable change was that there was found to no longer be a significant difference between treated and untreated eyes for the flicker Time to Peak parameter in the peripheral only data prior to Bonferroni correction ($p = 0.28$).

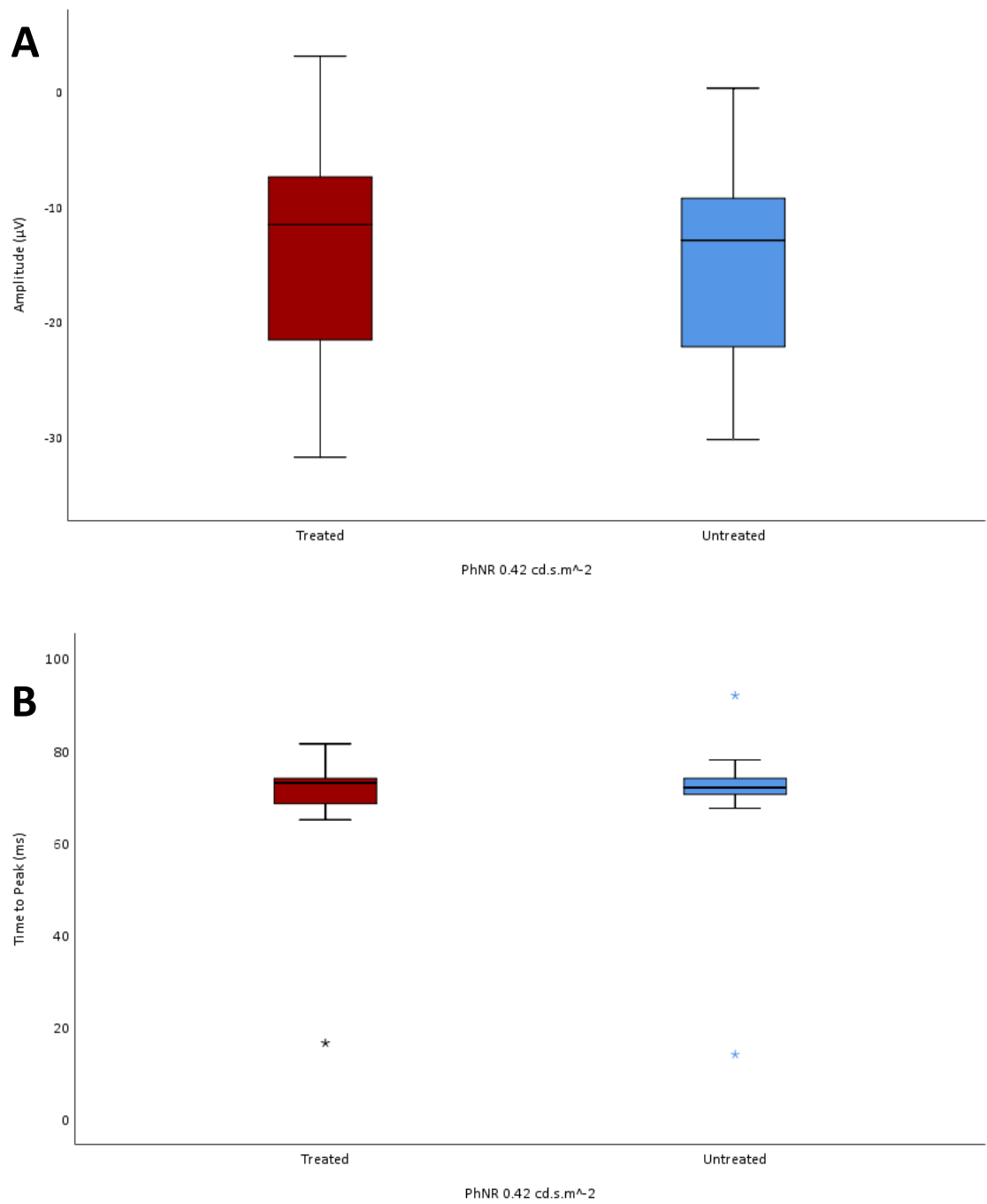


Figure 5.5. Box plots comparing the distribution of the PhNR 0.42 cd.s.m^{-2} data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted. Amplitudes are plotted in A and Time to Peak Values in B.

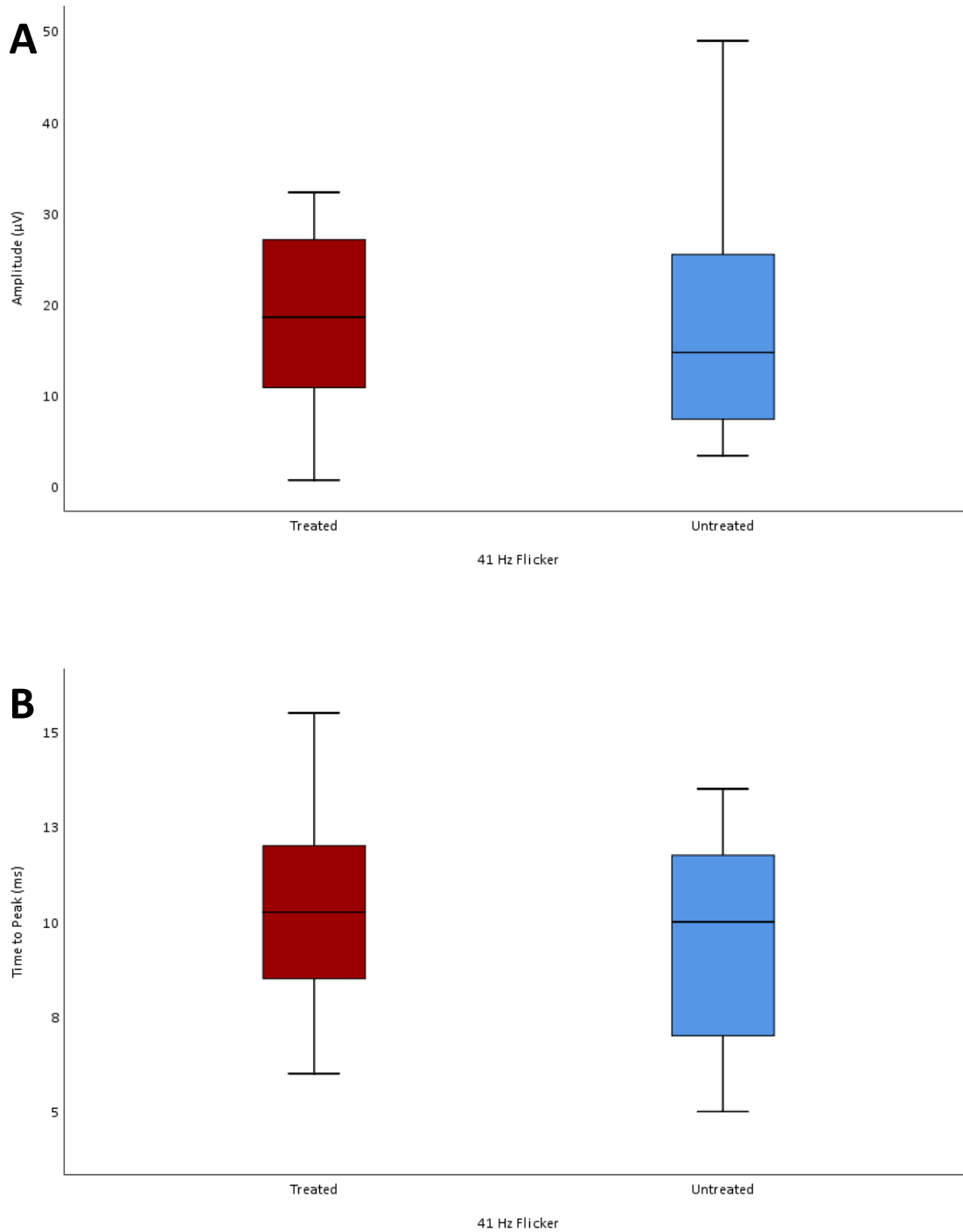


Figure 5.6. Box plots comparing the distribution of the full field 41 Hz flicker ERG data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values. Amplitudes are plotted in A and Time to Peak Values in B.

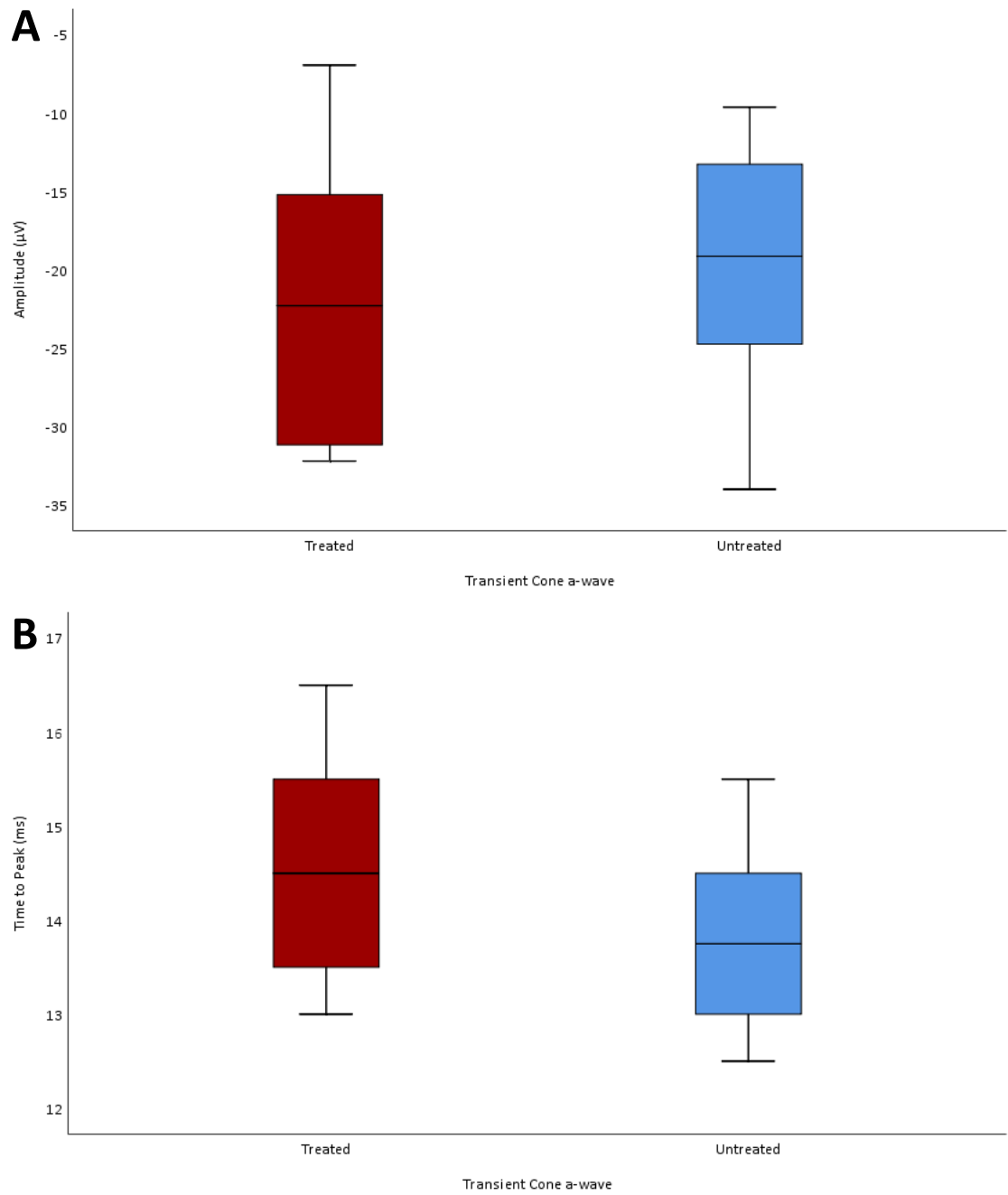


Figure 5.7. Box plots comparing the distribution of the full field Transient Cone a-wave data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values. Amplitudes are plotted in A and Time to Peak Values in B.

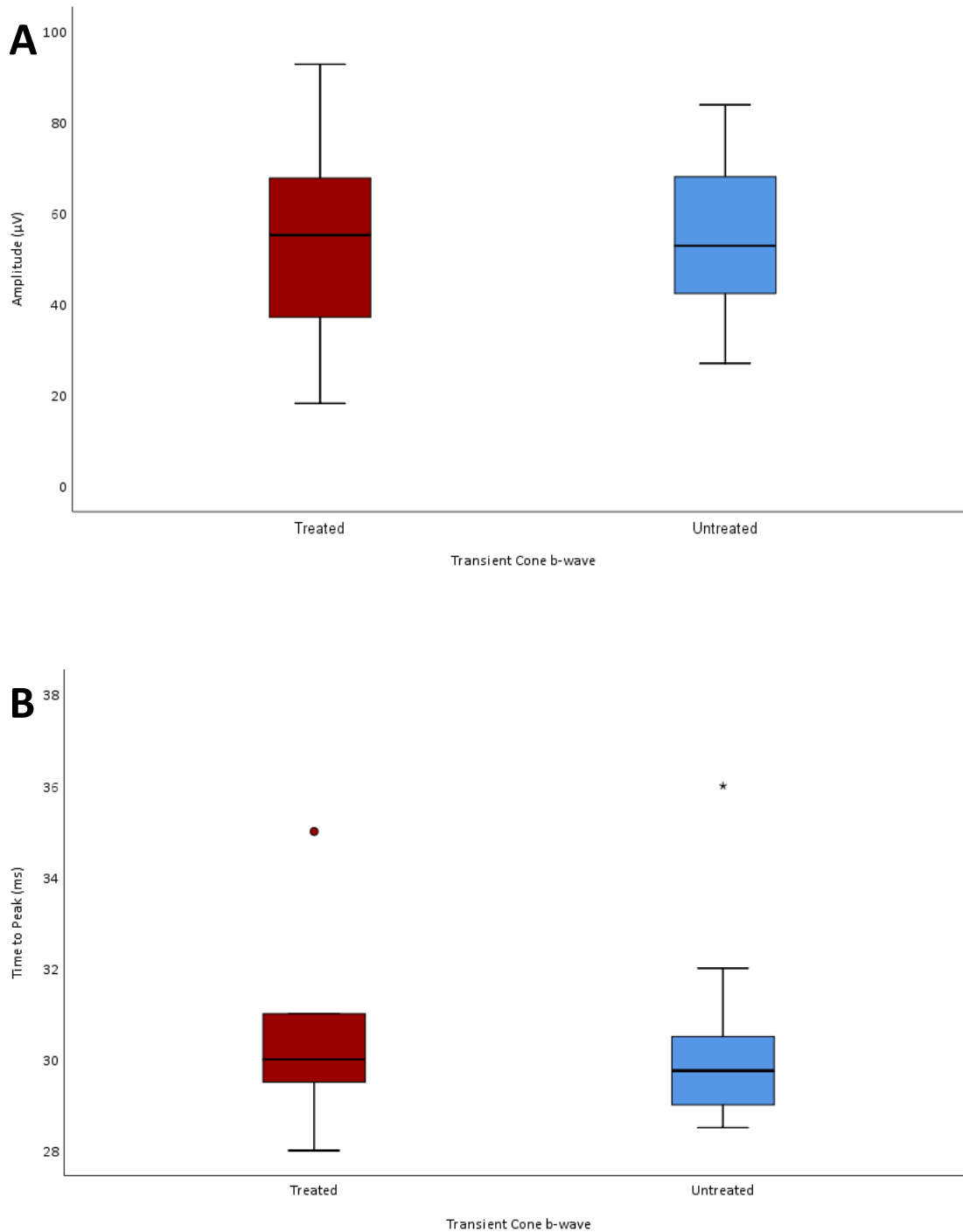


Figure 5.8. Box plots comparing the distribution of the full field Transient Cone b-wave data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted. Amplitudes are plotted in A and Time to Peak Values in B.

ERG Parameter			Median (IQR)		p	Bonferroni Corrected Value
			Treated	Not Treated		
PhNR	0.11 cd.s.m ⁻²	Amp (μV)	-11.70 (-6.84, -15.86)	-10.39 (-7.78, 12.65)	0.31	1.0
		TtP (ms)	66.50 (59.50, 76.00)	73.50 (66.50, 75.00)	0.16	1.0
	0.21 cd.s.m ⁻²	Amp (μV)	-14.79 (-10.03, -18.33)	-10.17 (-10.07, -18.24)	0.86	1.0
		TtP (ms)	68.00 (66.00, 69.75)	68.00 (65.25, 74.25)	0.89	1.0
	0.42 cd.s.m ⁻²	Amp (μV)	-13.16 (-11.15, -26.09)	-16.82 (-11.99, -26.23)	0.65	1.0
		TtP (ms)	70.00 (66.25, 75.13)	69.25 (64.13, 76.38)	0.80	1.0
	0.86 cd.s.m ⁻²	Amp (μV)	-18.66 (-13.19, -24.34)	-15.67 (-12.49, -22.86)	0.64	1.0
		TtP (ms)	70.25 (62.50, 73.50)	72.25 (63.63, 78.13)	0.40	1.0
	1.64 cd.s.m ⁻²	Amp (μV)	-16.70 (-7.16, -28.51)	-17.18 (-11.96, -26.69)	0.50	1.0
		TtP (ms)	68.00 (65.25, 73.75)	71.50 (69.75, 79.75)	0.60	1.0
	3.35 cd.s.m ⁻²	Amp (μV)	-15.44 (-10.94, -28.07)	-17.32 (-12.47, -27.65)	0.78	1.0
		TtP (ms)	75.75 (69.25, 80.75)	73.50 (66.75, 79.88)	0.83	1.0
Transient Cone	a-wave	Amp (μV)	-23.59 (-18.62, -29.50)	-18.93 (-16.03, -24.67)	0.06	1.0
		TtP (ms)	14.75 (14.00, 15.75)	14.50 (14.50, 15.00)	0.75	1.0
	b-wave	Amp (μV)	70.95 (54.59, 87.24)	67.32 (55.10, 84.24)	0.98	1.0
		TtP (ms)	30.75 (30.13, 32.00)	31.00 (30.00, 31.50)	0.81	1.0
41 Hz Flicker		Amp (μV)	18.55 (12.97, 26.54)	14.68 (7.54, 24.82)	0.64	1.0
		TtP (ms)	10.25 (8.75, 12.00)	10.00 (7.00, 11.63)	0.04*	0.32

Table 5.2. Median, IQR and Wilcoxon Signed Rank Test Values for interocular comparison of full field ERG parameters. ERG parameters that were found to have significant differences between treated and untreated eyes are highlighted with an asterisk (). There were no significant differences after Bonferroni correction.*

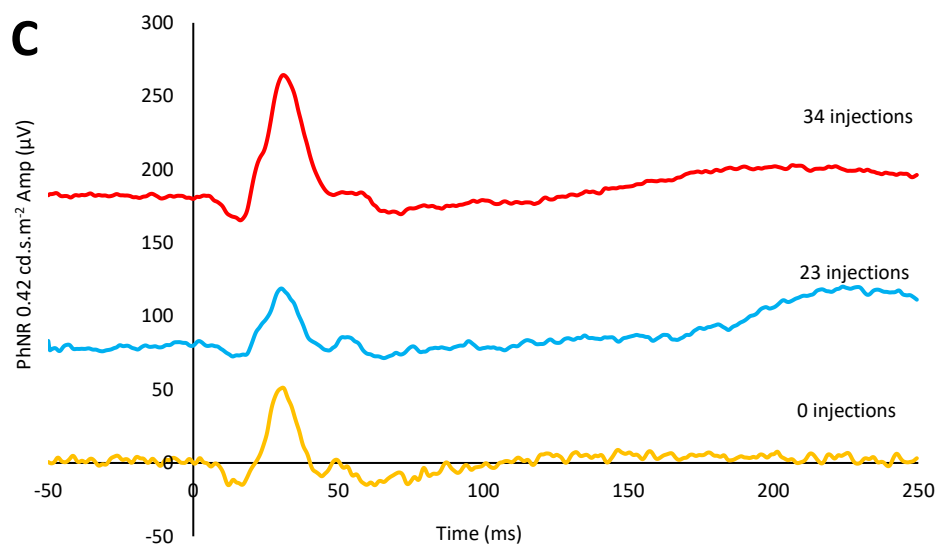
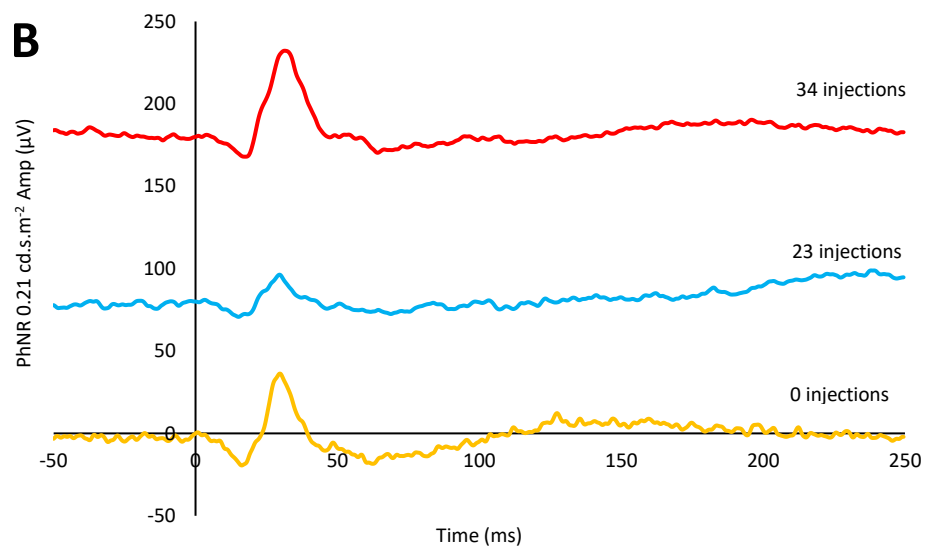
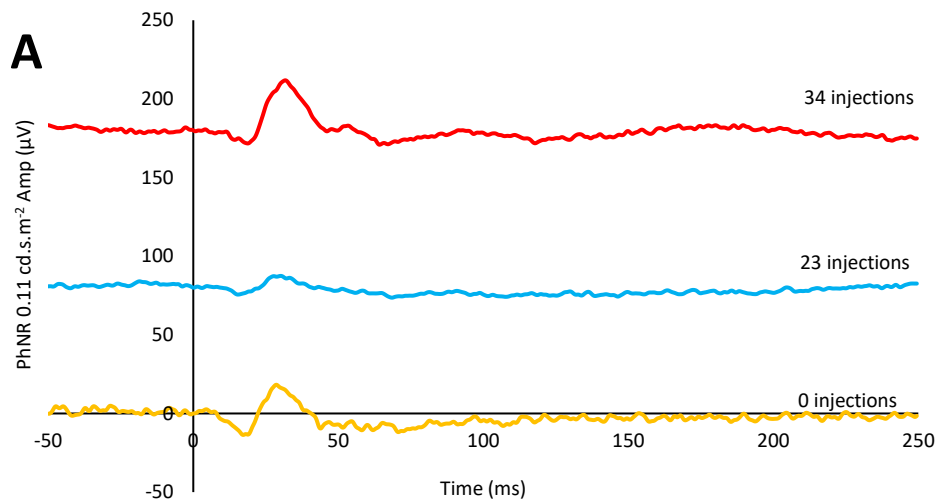
ERG Parameter			Full Field ERG Data				Peripheral Only Subtracted Response								
			Median (IQR)		Mean (± SD)		Median (IQR)		Mean (± SD)		% mean of full field response		p	n	Bonferroni Corrected Value
			Treated	Not Treated	Treated	Not Treated	Treated	Not Treated	Treated	Not Treated	Treated	Not Treated			
Transient Cone	a-wave	Amp (μV)	-23.59 (-33.31, -15.63)	-21.26 (-26.09, -16.46)	-24.69 (± 9.45)	-21.43 (± 8.06)	-21.27 (-31.50, -16.27)	-19.11 (-25.55, -12.59)	-24.59 (± 11.12)	-20.09 (± 7.95)	100	94	0.17	10	1.00
		TtP (ms)	14.25 (14.00, 15.75)	14.75 (14.13, 15.75)	14.65 (± 1.12)	15.05 (± 1.04)	14.00 (13.50, 15.75)	14.75 (14.00, 15.00)	14.40 (± 1.34)	14.80 (± 1.08)	98	98	0.72	10	1.00
	b-wave	Amp (μV)	70.95 (57.46, 90.18)	74.68 (59.22, 91.78)	75.60 (± 23.25)	72.83 (± 24.19)	64.47 (50.18, 90.84)	69.97 (48.69, 81.91)	70.83 (± 22.60)	66.24 (± 22.17)	94	91	0.51	10	1.00
		TtP (ms)	31.25 (30.13, 32.00)	31.25 (30.00, 31.50)	31.25 (± 1.45)	31.25 (± 1.82)	31.25 (30.13, 32.00)	31.00 (29.50, 31.50)	31.20 (± 1.35)	31.40 (± 3.01)	100	100	0.22	10	1.00
	PhNR	Amp (μV)	-24.47 (-31.09, -17.97)	-19.60 (-23.87, -16.57)	-26.32 (± 11.97)	-22.66 (± 11.65)	-25.63 (-40.56, -17.88)	-19.64 (-29.13, -16.05)	-27.44 (± 12.19)	-24.12 (± 12.08)	104	106	0.48	8	1.00
		TtP (ms)	65.25 (64.50, 66.63)	69.75 (62.50, 77.25)	65.50 (± 9.32)	69.19 (± 11.49)	55.50 (44.25, 66.50)	66.00 (45.38, 74.00)	57.00 (± 13.42)	62.19 (± 15.58)	87	90	0.31	8	1.00
41 Hz Flicker		Amp (μV)	20.66 (16.31, 27.07)	15.43 (12.07, 23.19)	20.51 (± 7.57)	20.26 (± 13.00)	20.26 (14.19, 26.38)	14.52 (10.07, 22.16)	19.29 (± 7.91)	19.08 (± 12.99)	94	94	0.87	7	1.00
		TtP (ms)	9.00 (7.75, 10.25)	9.00 (6.50, 10.75)	9.07 (± 2.03)	8.71 (± 2.67)	9.50 (8.25, 10.50)	9.00 (6.50, 10.75)	9.36 (± 1.99)	8.71 (± 2.67)	103	100	0.28	7	1.00

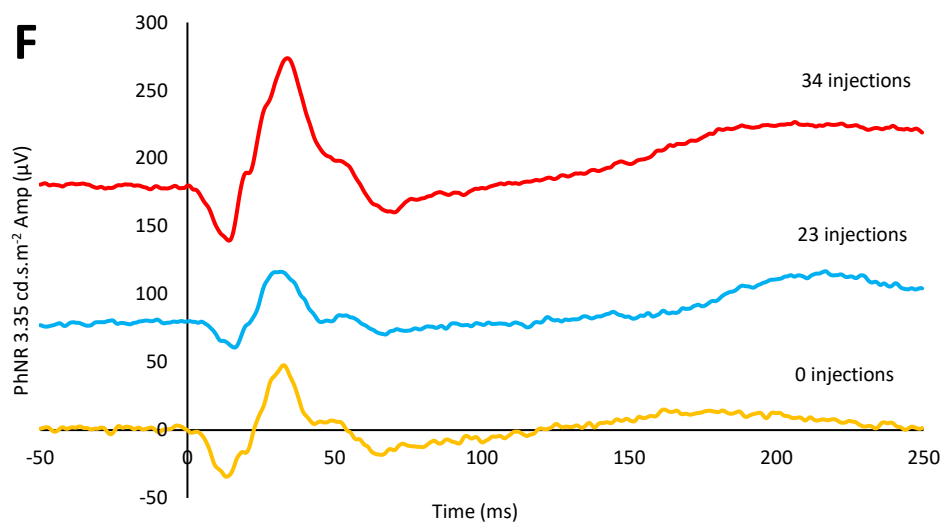
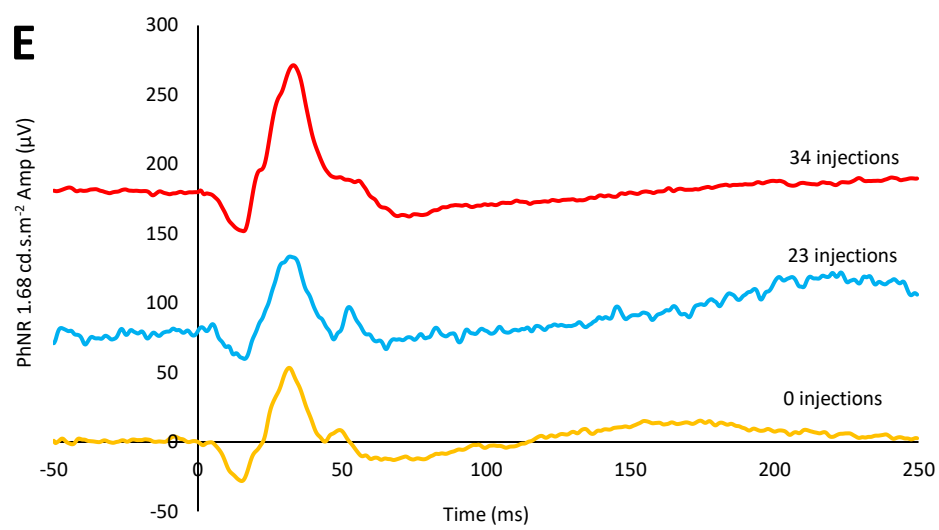
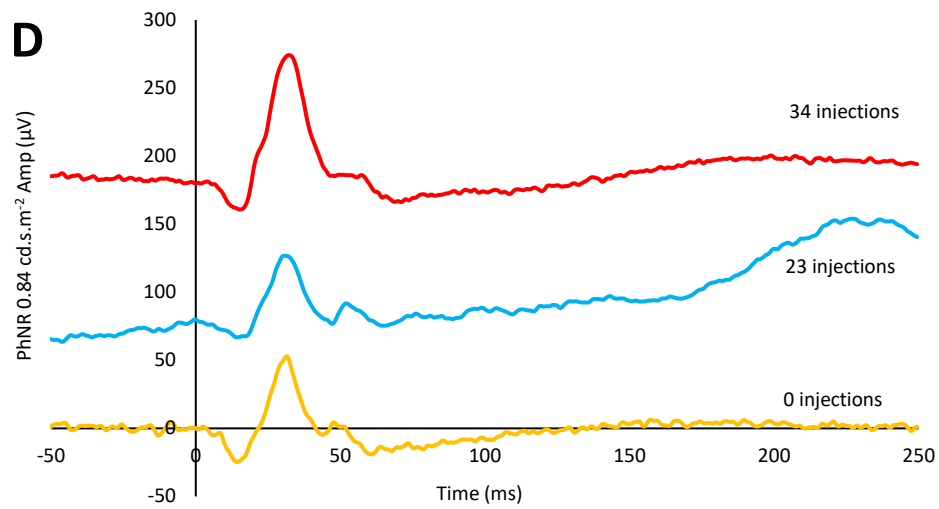
Table 5.3. Median (IQR), Mean (SD), and Wilcoxon Signed Rank Test Values for interocular comparison of peripheral ERG parameters. There were no significant differences in ERG parameters between treated and untreated eyes.

Between participant analysis

Data from one eye (either the eye with nAMD, or the right eye in the case of bilateral disease) of all 32 participants were then evaluated to determine the relationship between the number of injections received and each ERG parameter. Data were collected from 32 individuals including $n = 2$ prior to their first injection. This included the treated eyes of the 14 participants with unilateral nAMD who were included in the interocular analysis described in Section 5.2.4. Sample data from three participants having received various numbers of injections are shown in Figure 5.9.

It can be seen in these sample data that the PhNR response from the participant with the fewest injections appears to be slightly higher in amplitude than the other two responses, across the waveform. However, the individual having received the highest number of injections shows an apparently higher amplitude response to the transient cone ERG.





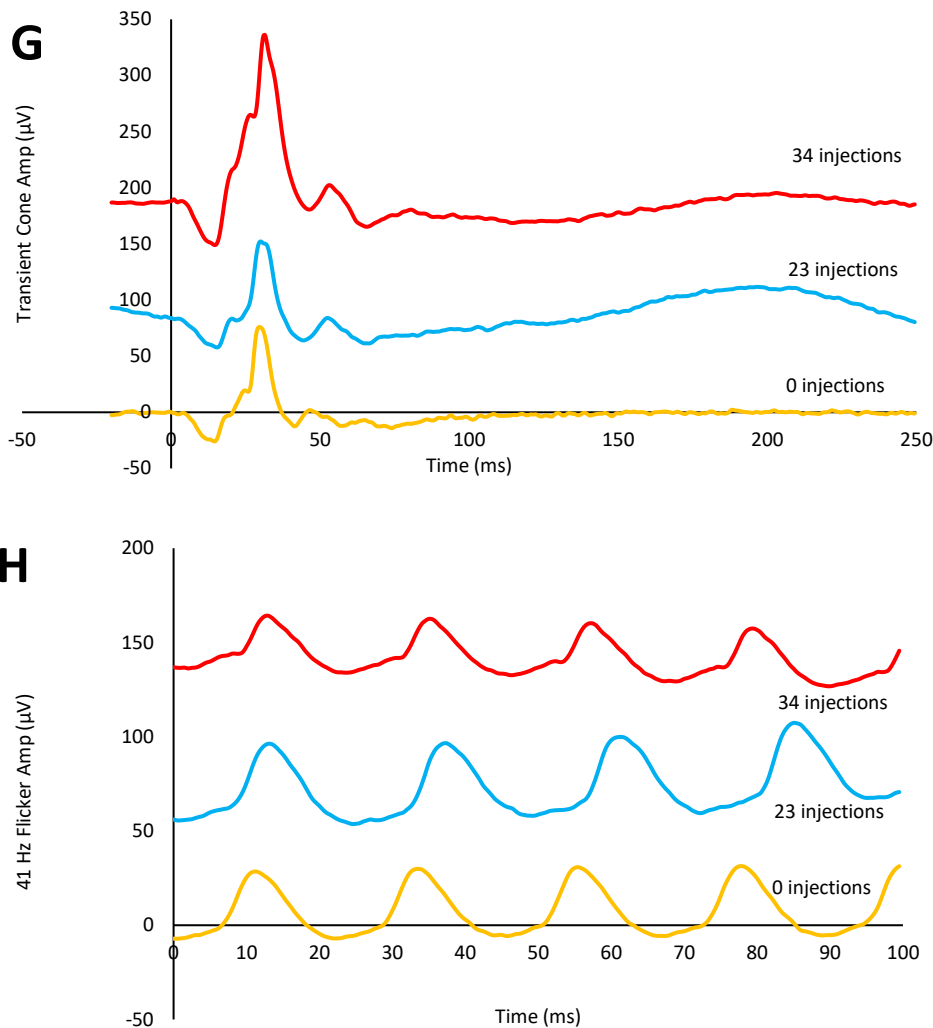


Figure 5.9. Sample traces of the waveforms produced from the three ERG protocols for three participants who received different numbers of anti-VEGF injections; Participants included CW (0 injections, orange), NP (23 injections, blue) and ST (34 injections, red). A, PhNR 0.11 cd.s.m^{-2} ERG; B, PhNR 0.21 cd.s.m^{-2} ERG; C, PhNR 0.42 cd.s.m^{-2} ERG, D, PhNR 0.84 cd.s.m^{-2} ERG; E, PhNR 1.68 cd.s.m^{-2} ERG; F, PhNR 3.35 cd.s.m^{-2} ERG; G, Transient cone response ERG; H, 41 Hz flicker ERG.

To further investigate the relationship between number of injections and ERG parameters, an assessment of the age of participants vs number of injections was first carried out to rule out the possibility of a relationship between older age and more anti-VEGF injections. If present, such a relationship could have a confounding effect, given the known impact of increasing age on ERG parameters (Gerth, 2002; Kergoat et al., 2001; Weleber, 1981). However, there was no significant correlation between participant age and the number of injections they had received (Spearman's; $p = 0.29$, $\rho = 0.19$, $\text{CI} = -0.17, 0.52$; Figure 5.10).

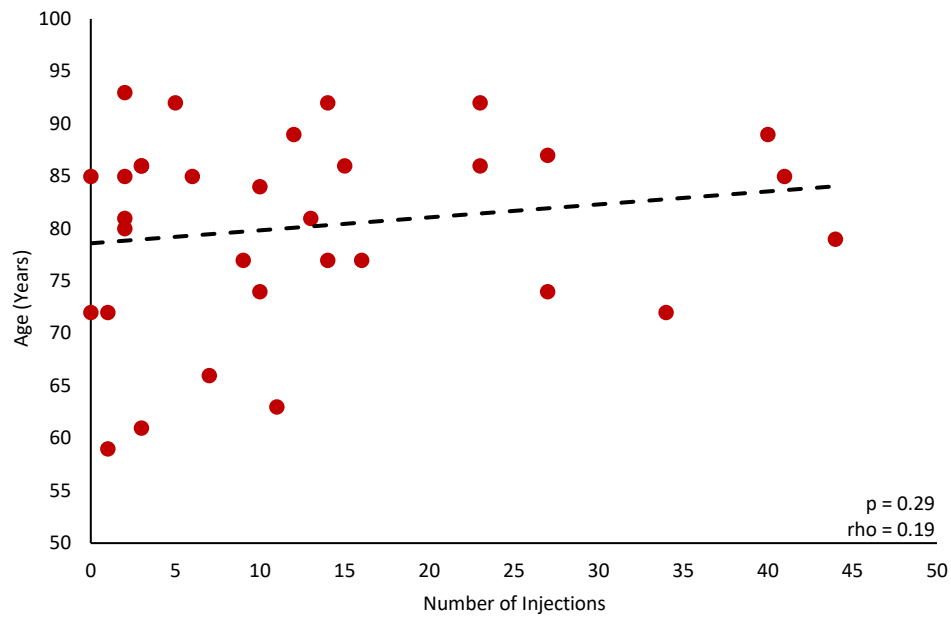


Figure 5.10. Scatter plot showing the age of participants (years) against the number of anti-VEGF injections received. The results of the Spearman's correlation analysis are also shown. There was no significant relationship.

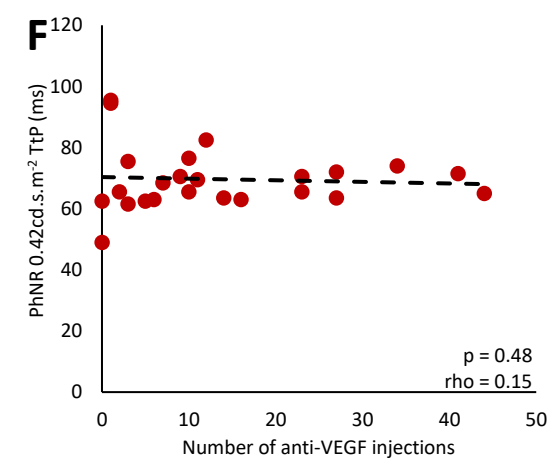
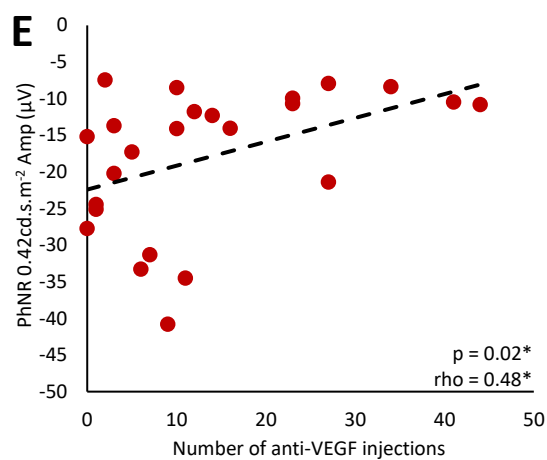
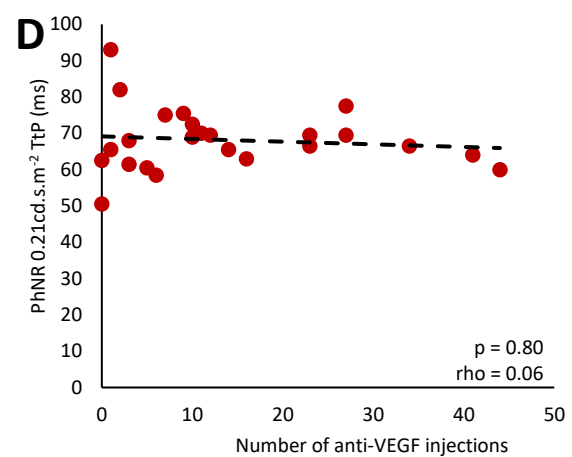
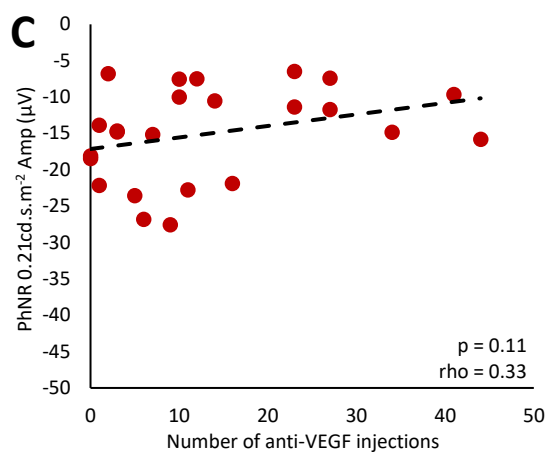
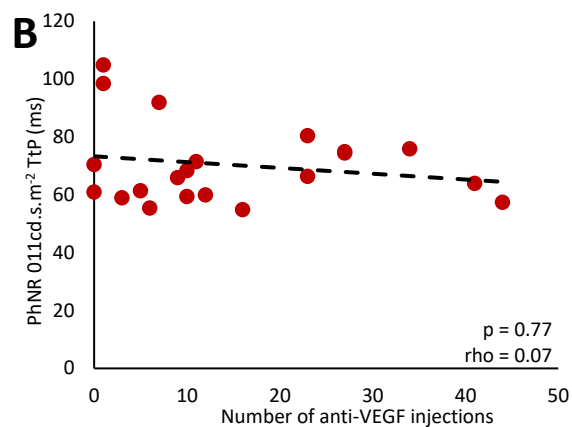
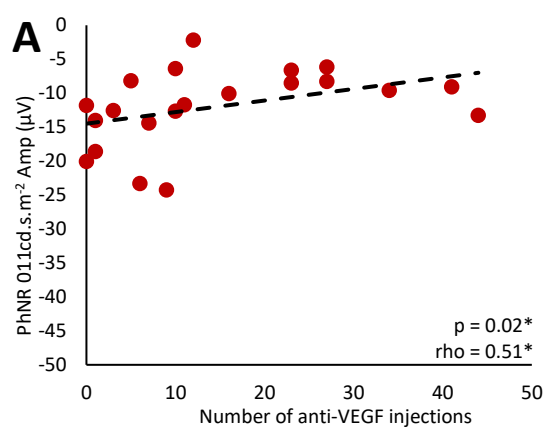
Scatter plots were then constructed (see Figures 5.11 and 5.12), and correlation analysis carried out to investigate the relationship between ERG parameters and number of anti-VEGF injections. Data were not normally distributed, so the non-parametric Spearman's correlation analysis was used to evaluate the relationship between number of injections and ERG response. There was no statistically significant correlation between total number of anti-VEGF injections received and the transient cone and 41 Hz flicker ERG parameters ($p > 0.05$, Figure 5.12; Table 5.4). However, the following ERG parameters did show a significant correlation with total number of anti-VEGF injections, PhNR 0.11 cd.s.m⁻² amplitude ($p = 0.02$, $\rho = 0.51$, CI = 0.14 - 0.76), PhNR 0.42 cd.s.m⁻² amplitude ($p = 0.02$, $\rho = 0.48$, CI = 0.07 - 0.76), PhNR 3.35 cd.s.m⁻² amplitude ($p = 0.03$, $\rho = 0.48$, CI = 0.09 - 0.76). After Bonferroni correction, these values became insignificant given the large number of comparisons conducted. However, it is notable that the trend towards reduced amplitude with increasing number of injections was consistent across the PhNR responses to different stimulus intensities. This is visually apparent in the scatter plots in Figure 5.11.

Focal data were obtained from 27 of the between participant dataset. In the five cases where focal ERG data were not obtained reasons included; time constraints ($n = 2$), participant unable/unwilling to comply with this part of the protocol ($n = 2$), and equipment failure ($n = 1$). The lack of a significant correlation for the 41 Hz flicker ERG

and ISCEV transient cone ERG remained when the focal contribution from the macula was subtracted to leave only the peripheral retinal response. Due to time constraints in the medical retina clinic it was not possible to take focal data for PhNR tests so peripheral data were not available. However, when the PhNR of the ISCEV transient cone ERG waveform (with macular contribution subtracted), was assessed there was no relationship between number of injections and PhNR amplitude or time to peak ($p>0.05$, see Figure 5.13, and Table 5.5).

Data from the parameters showing significant correlations with the number of anti-VEGF injections issued subsequently underwent linear regression analysis (see Table 5.6, and Figure 5.14). Linear regression assumptions were not met and therefore subsequent analyses were not performed for the following parameters which had significant correlations with number of anti-VEGF injections; 0.11 cd.s.m^{-2} PhNR amplitude, 3.35 cd.s.m^{-2} PhNR amplitude. In the case of the significant correlation between 0.11 cd.s.m^{-2} PhNR amplitude and number of anti-VEGF injections there was greater variance at the lower predicted values and so the regression assumption of homoscedasticity was not met. In the case of the significant correlation between 3.35 cd.s.m^{-2} PhNR amplitude and number of anti-VEGF injections the regression assumptions were not met due to a non-normal distribution of residuals. Regression model assessing the relationship between total number of anti-VEGF injections and 0.42 cd.s.m^{-2} PhNR amplitude statistically significantly predicted the PhNR outcome variables ($p = 0.03$). It was also established that $\sim 19\%$ of the variance in 0.42 cd.s.m^{-2} PhNR was due to the number of anti-VEGF injections.

As the data were non-normally distributed, the non-parametric Kruskal-Wallis test was carried out to compare ERG parameters between groups receiving different numbers of injections. There was no statistically significant difference in ERG parameter between any of the groups ($p>0.05$).



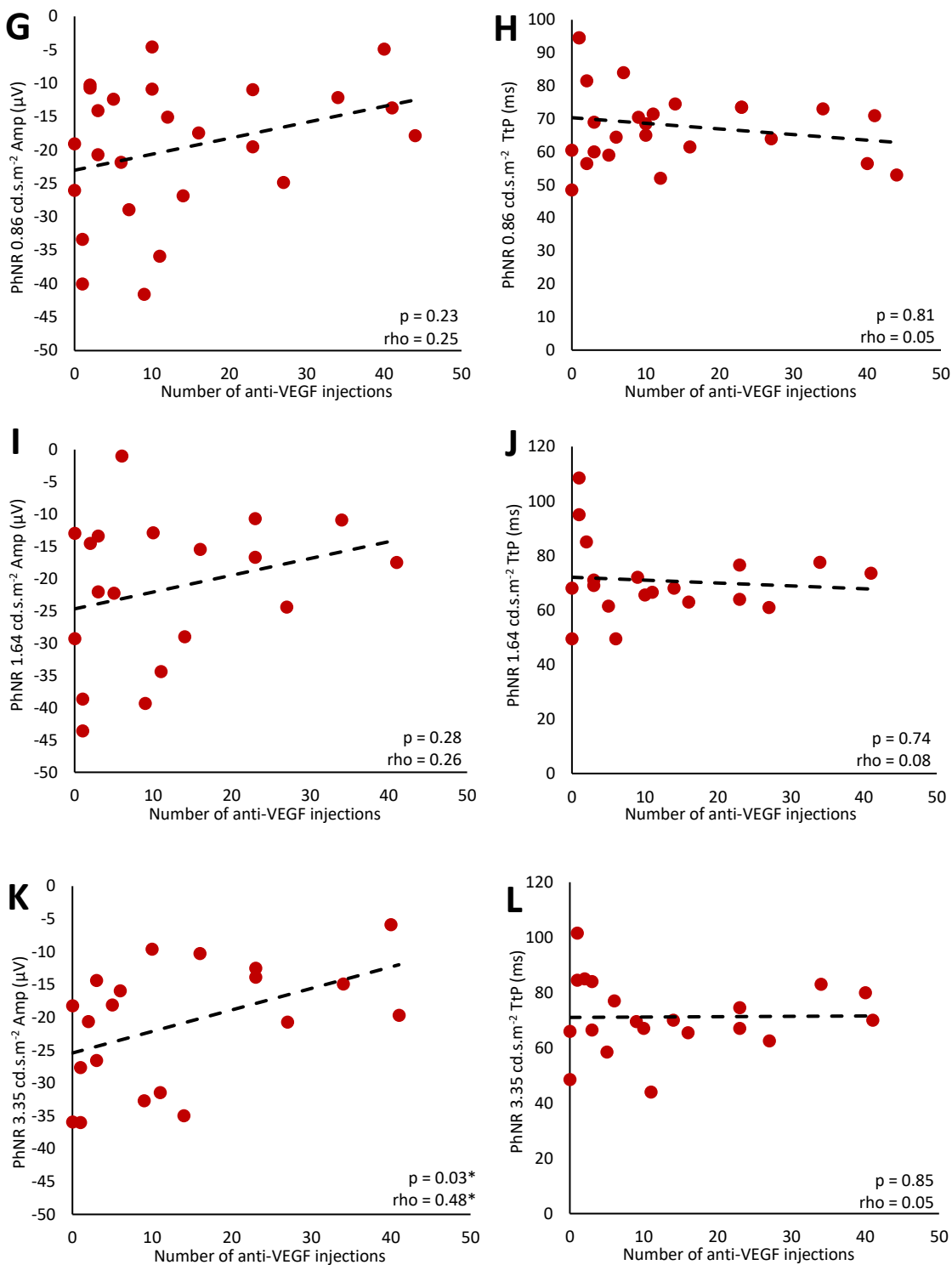


Figure 5.11. Scatter plots showing relationship between PhNR ERG parameters and number of injections. A, PhNR 0.11 cd.s.m⁻² Amplitude (Amp); B, PhNR 0.11 cd.s.m⁻² Time to Peak (TtP); C, PhNR 0.21 cd.s.m⁻² Amp; D, PhNR 0.21 cd.s.m⁻² TtP; E, PhNR 0.42 cd.s.m⁻² Amp; F, PhNR 0.42 cd.s.m⁻² TtP; G, PhNR 0.86 cd.s.m⁻² Amp; H, PhNR 0.86 cd.s.m⁻² TtP; I, PhNR 1.64 cd.s.m⁻² Amp; J, PhNR 1.64 cd.s.m⁻² TtP; K, PhNR 3.35 cd.s.m⁻² Amp; L, PhNR 3.35 cd.s.m⁻² TtP. The results of the Spearman's correlation analysis are also shown. Asterisks indicate a statistically significant correlation prior to Bonferroni correction (p<0.05).

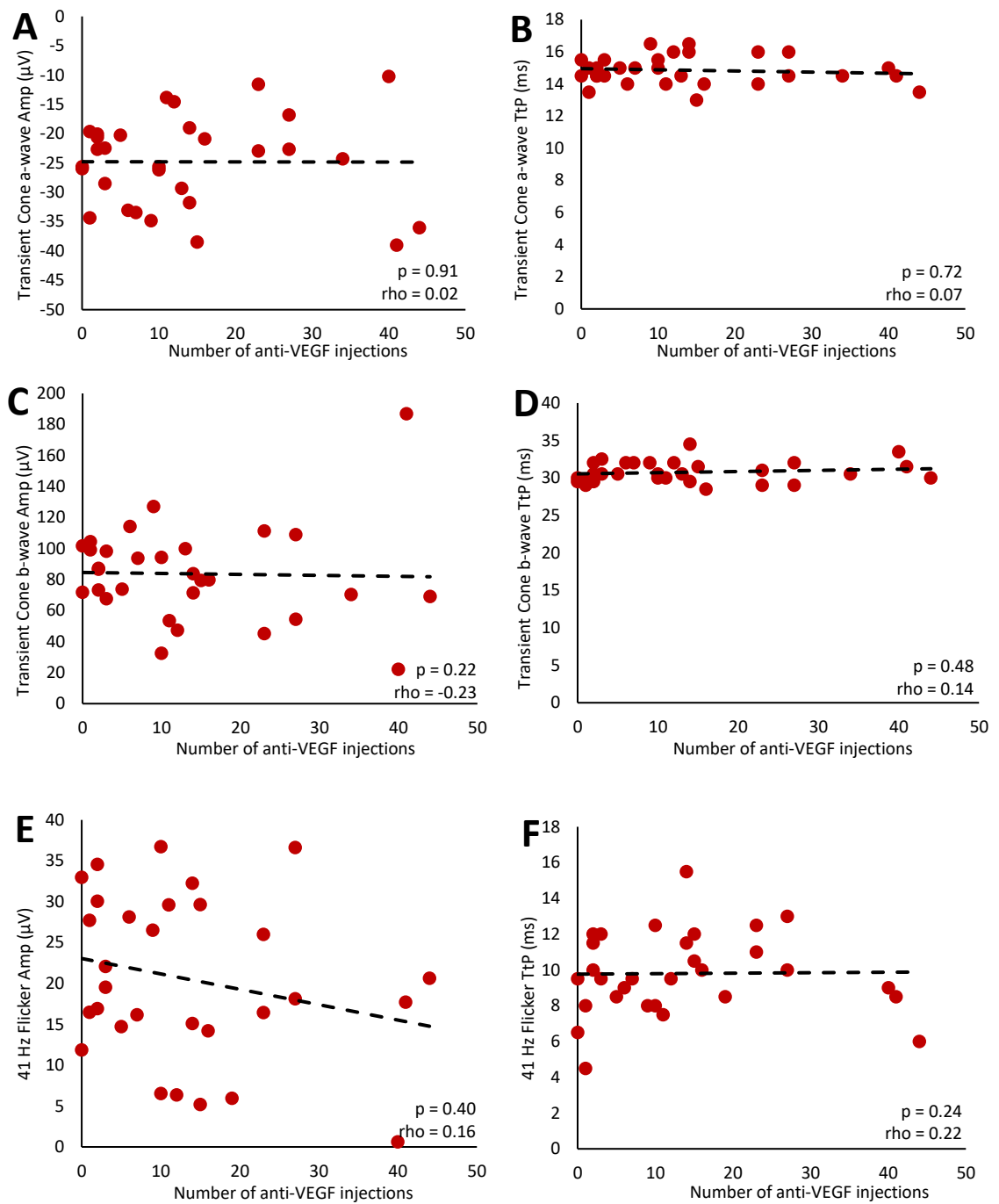


Figure 5.12. Scatter plots showing relationship between Transient Cone and 41 Hz flicker ERG parameters and total number of injections. A, Transient cone a-wave Amplitude (Amp); B, Transient cone a-wave Time to Peak (TtP); C Transient cone b-wave Amp; D Transient cone b-wave TtP; E 41 Hz Flicker Amp; F 41 Hz flicker TtP. The results of the Spearman's correlation analysis are also shown. There was no significant correlation between number of anti-VEGF injections and any ERG parameters in this part of the dataset.

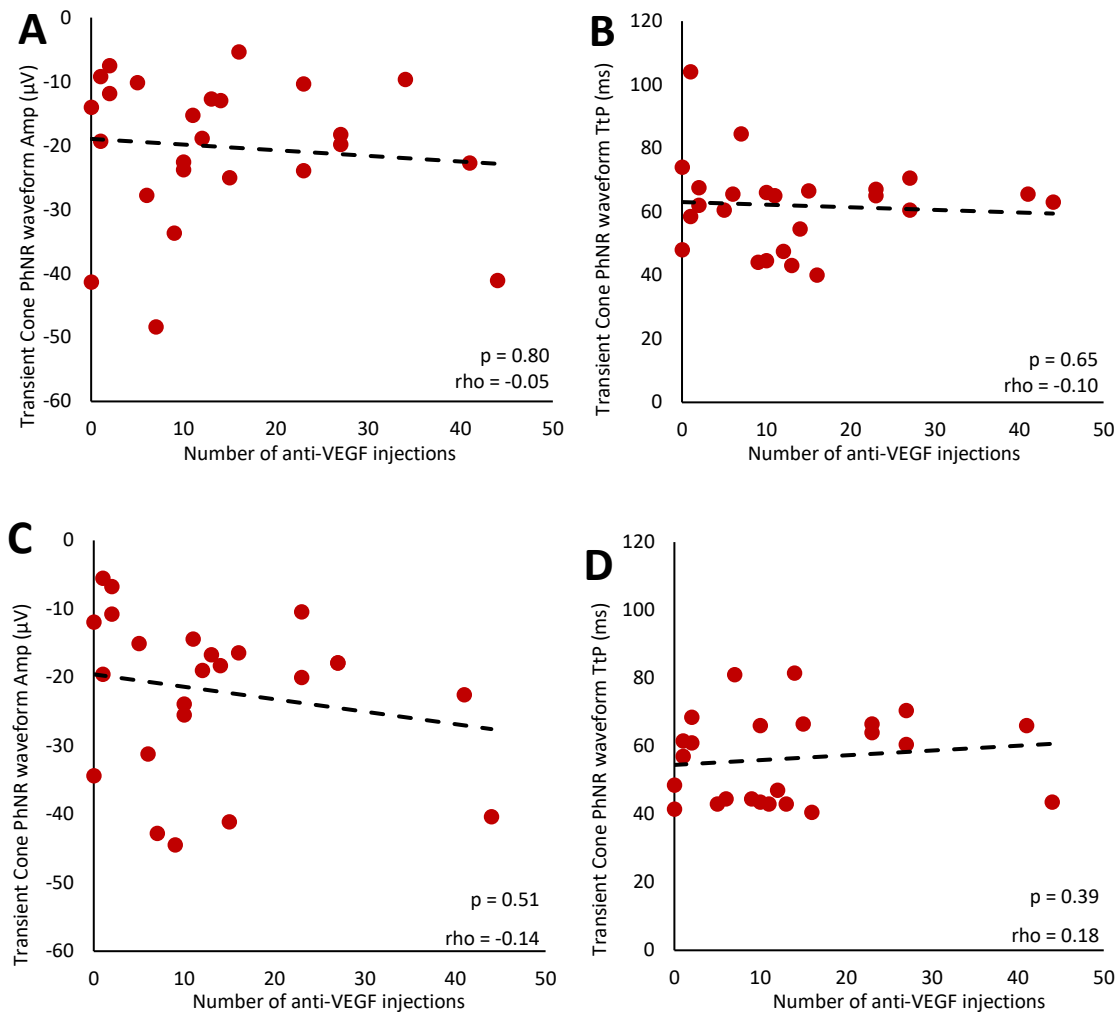


Figure 5.13. Scatter plots illustrating the correlation between total number of anti-VEGF injections and the PhNR waveform elicited within the Transient Cone ERG protocol. A, Transient Cone PhNR waveform Amplitude (Amp) -full field data; B, Transient Cone PhNR waveform Time to Peak (TtP) -full field data; C, Transient Cone PhNR waveform Amp - peripheral field data; D, Transient Cone PhNR waveform TtP - peripheral field data. The results of the Spearman's correlation analysis are also shown. There was no significant correlation between number of anti-VEGF injections and any ERG parameters in this part of the dataset.

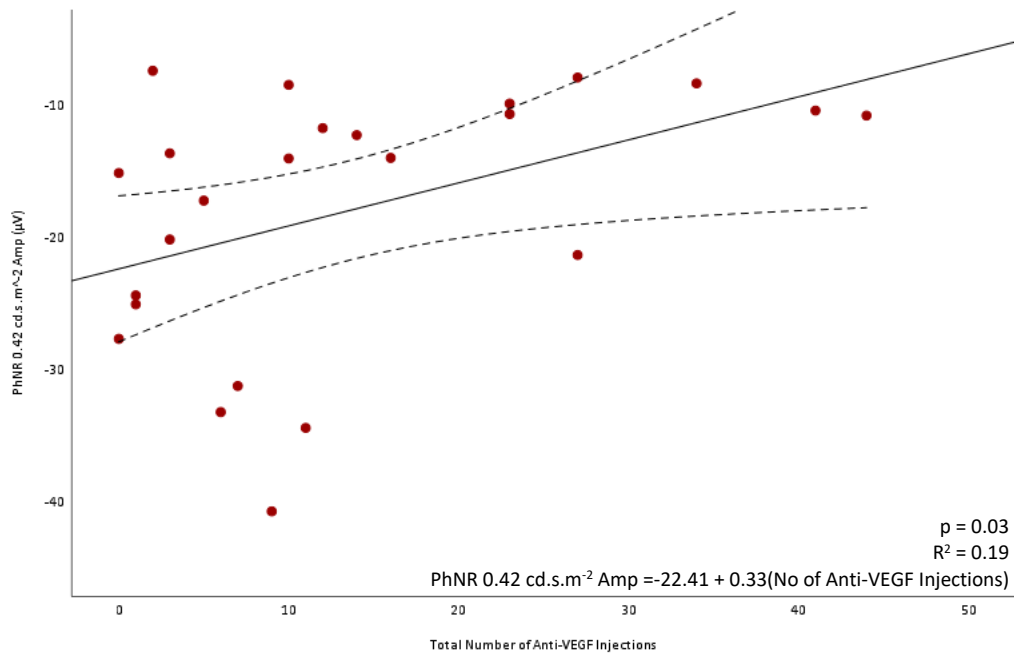


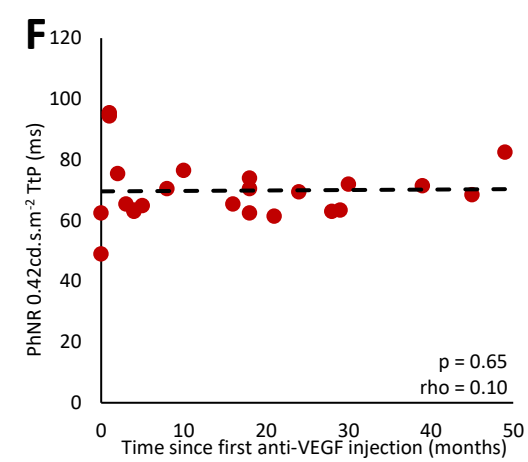
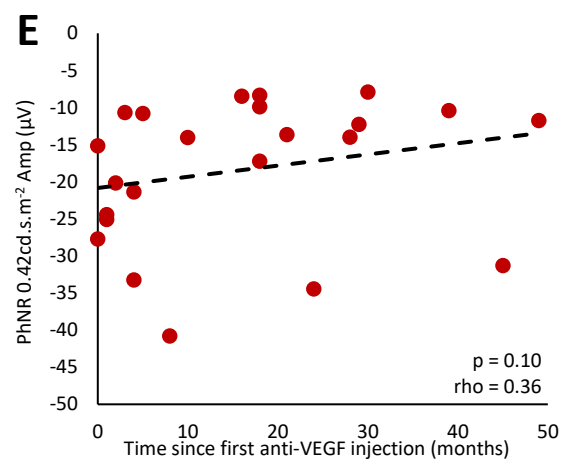
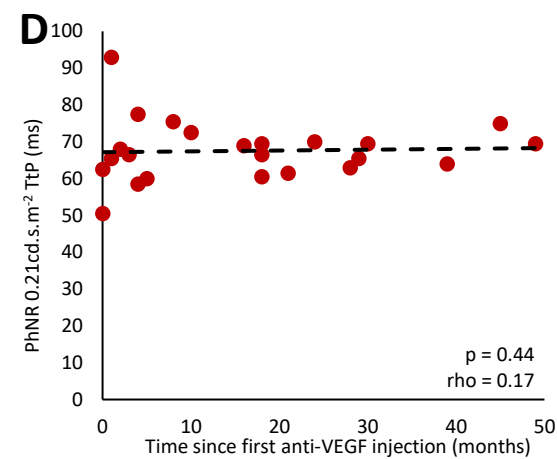
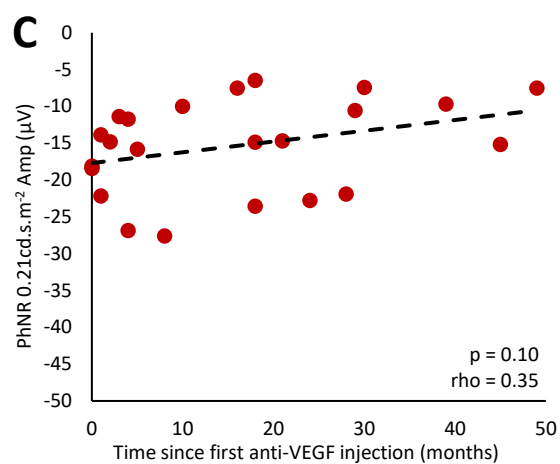
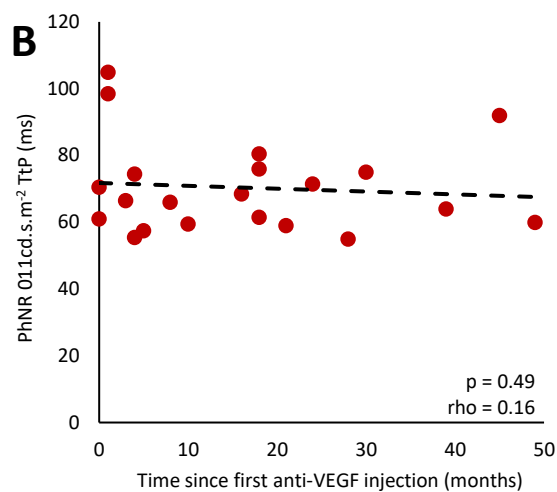
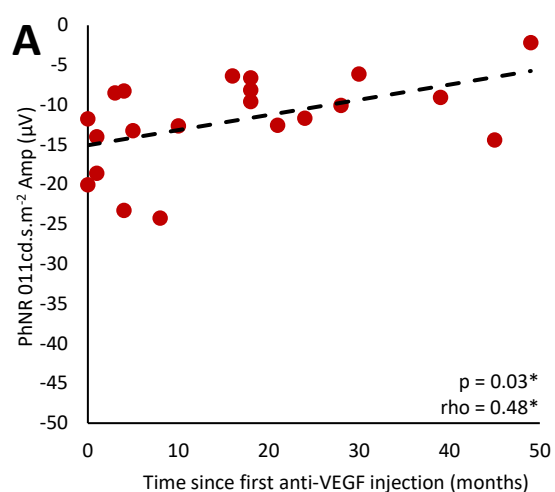
Figure 5.14. Scatter plot describing the linear regression of the relationship between full field PhNR 0.42 cd.s.m⁻² ERG Amp and total number of anti-VEGF Injections received. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

Non-parametric Spearman's correlation analysis was also used to evaluate the relationship between time since first anti-VEGF injection and ERG response (see scatter plots in Figures 5.15 and 5.16). There was no significant relationship between participant age and time since first anti-VEGF injection ($p = 0.08$, $\rho = 0.32$, $CI = -0.05 - 0.61$). There was no statistically significant association between time since number of injections and the majority of ERG parameters ($p > 0.05$, Table 5.4). However, three ERG parameters did have significant correlations with time elapsed since commencing anti-VEGF treatment; PhNR 0.11 cd.s.m⁻² amplitude ($p = 0.03$, $\rho = 0.48$, $CI = 0.02 - 0.78$), transient cone ERG b-wave Time to Peak ($p = <0.01$, $\rho = 0.57$, $CI = 0.27 - 0.78$), and 41 Hz flicker ERG amplitude ($p = 0.01$, $\rho = 0.46$, $CI = -0.72 - -0.09$). Transient cone b-wave time to peak remained significantly correlated after Bonferroni correction, suggesting a prolongation of time to peak associated with increasing time since the first injection (i.e. a longer total treatment duration). Although not statistically significant, there was a trend towards reducing PhNR amplitude with increasing time since first injection across all stimulus intensities.

Non-parametric Spearman's correlation analysis was also used to evaluate the relationship between time since first anti-VEGF injection and ERG responses in the peripheral only response, where the focal contribution of the macula had been subtracted. Once again, the transient cone ERG b-wave time to peak was shown to have a significant correlation ($p = <0.01$, $\rho = 0.64$, $CI = 0.36 - 0.80$, Table 5.5) with time since first injection, which remained after Bonferroni correction. This reinforced the suggestion of a prolongation of time to peak with increasing time since the first anti-VEGF injection. The peripheral 41 Hz flicker ERG amplitude was also significantly negatively correlated with the time since first injection ($p = 0.04$, $\rho = -0.42$, $CI = -0.71 - -0.03$), suggesting a reducing amplitude was associated with increasing time since first injection.

Data from the parameters showing significant correlations with the time since first anti-VEGF injection subsequently underwent linear regression analysis (see Table 5.6, and Figures 5.17 – 5.21). In the full field ERG responses, regression models assessing the relationship between time since first anti-VEGF injection and 0.11 cd.s.m^{-2} PhNR amplitude, transient cone b-wave time to peak, and 41 Hz flicker amplitude were shown to statistically significantly predict the respective PhNR outcome variables ($p = 0.02$, $p = 0.002$, and 0.004 , respectively). It was also established that ~25% of the variance in 0.11 cd.s.m^{-2} PhNR amplitude, ~29% of the variance in transient cone b-wave time to peak, and ~27% of the 41 Hz flicker amplitude were attributable to the time elapsed since first anti-VEGF injection. In the peripheral only ERG responses where focal macular responses were subtracted from full field responses, regression models assessing the relationship between time since first anti-VEGF injection and 41 Hz flicker ERG amplitude, and transient cone b-wave TtP were shown to statistically significantly predict their respective ERG outcome variables ($p = 0.03$, and $p = 0.003$ respectively). It was also established that ~18% of the variance in the 41 Hz flicker ERG amplitude and ~31% of the variance in transient cone b-wave TtP were due to the time elapsed since first anti-VEGF injection.

A Spearman's correlation analysis suggested that there is a strong relationship between number of injections received and time since first injection ($\rho = 0.51$, $p = 0.004$, $CI = 0.16 - 0.79$) i.e. the two independent variables assessed in this study.



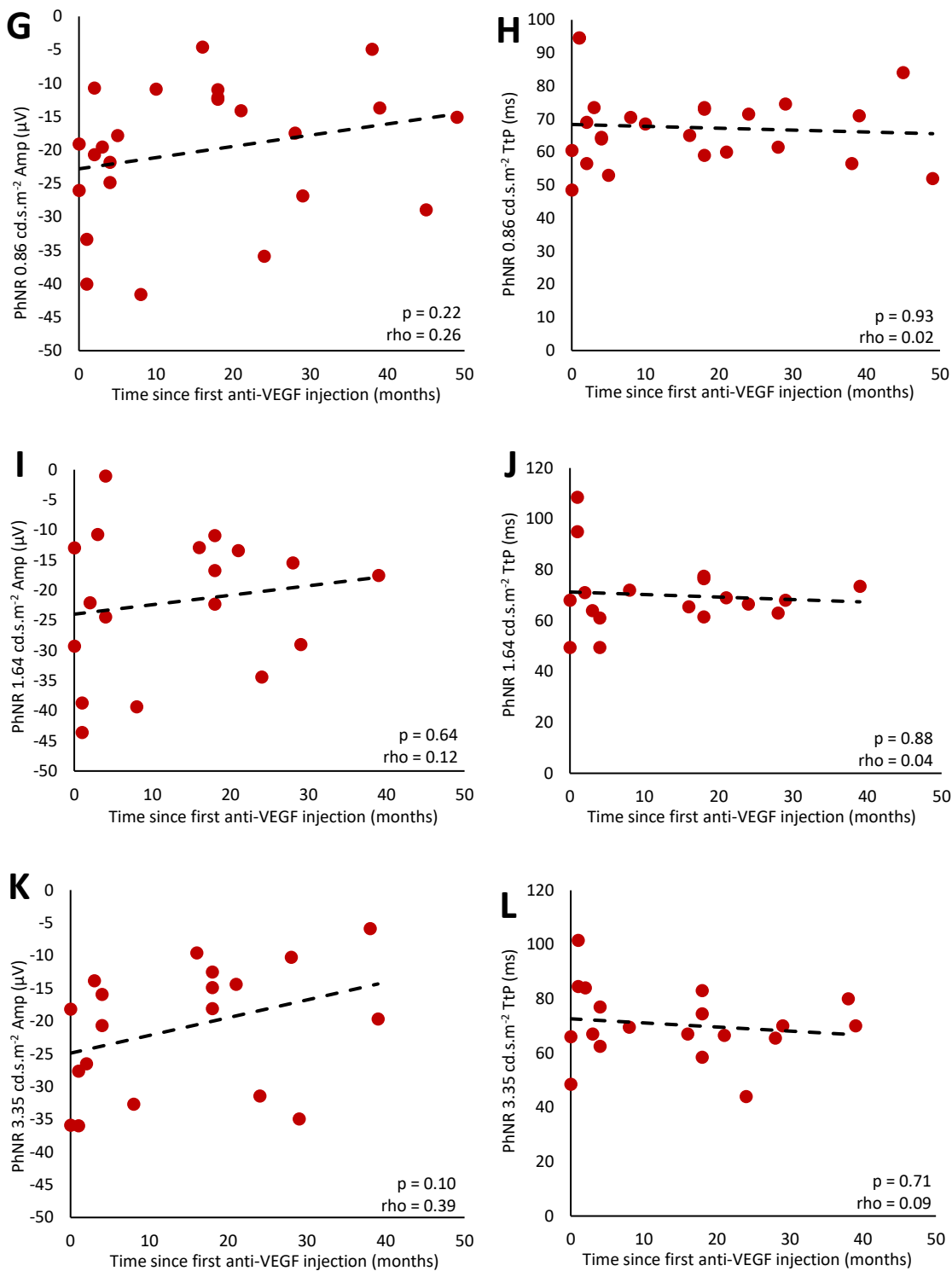


Figure 5.15 Scatter plots illustrating the correlation between PhNR parameters and time since first anti-VEGF injection. A, PhNR 0.11 cd.s.m⁻² Amplitude (Amp); B, PhNR 0.11 cd.s.m⁻² Time to Peak (TtP); C, PhNR 0.21 cd.s.m⁻² Amp; D, PhNR 0.21 cd.s.m⁻² TtP; E, PhNR 0.42 cd.s.m⁻² Amp; F, PhNR 0.42 cd.s.m⁻² TtP; G, PhNR 0.86 cd.s.m⁻² Amp; H, PhNR 0.86 cd.s.m⁻² TtP; I, PhNR 1.64 cd.s.m⁻² Amp; J, PhNR 1.64 cd.s.m⁻² TtP; K, PhNR 3.35 cd.s.m⁻² Amp; L, PhNR 3.35 cd.s.m⁻² TtP. Asterisks indicate a statistically significant correlation ($p < 0.05$).

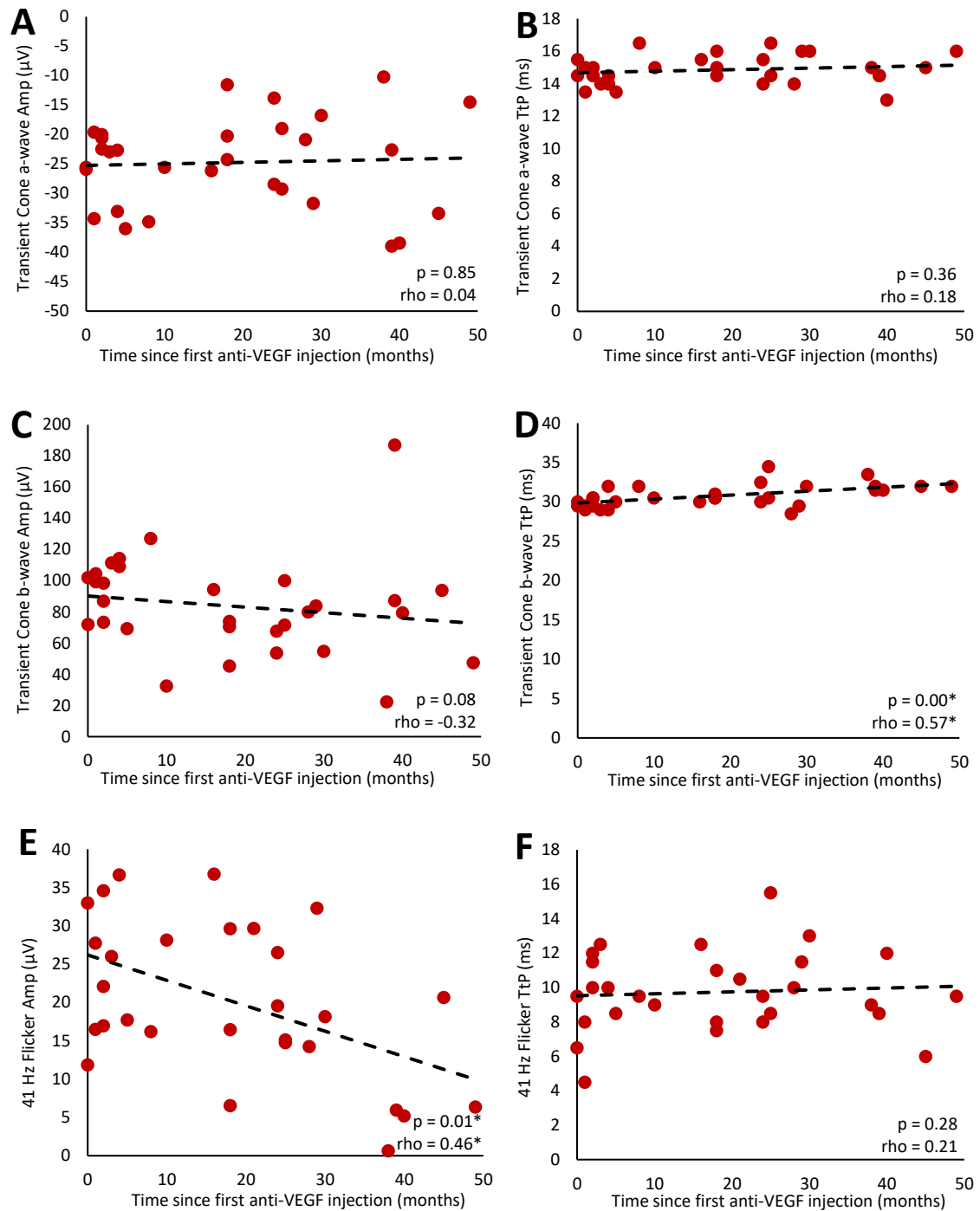


Figure 5.16 Scatter plots illustrating the correlation between Transient Cone and 41 Hz Flicker ERG parameters and time since first anti-VEGF injection.

A, Transient cone a-wave Amplitude (Amp); B, Transient cone a-wave Time to Peak (TtP); C Transient cone b-wave Amp; D Transient cone b-wave TtP; E 41 Hz flicker Amp; F 41 Hz flicker TtP. Asterisks (*) indicate a statistically significant correlation ($p < 0.05$).

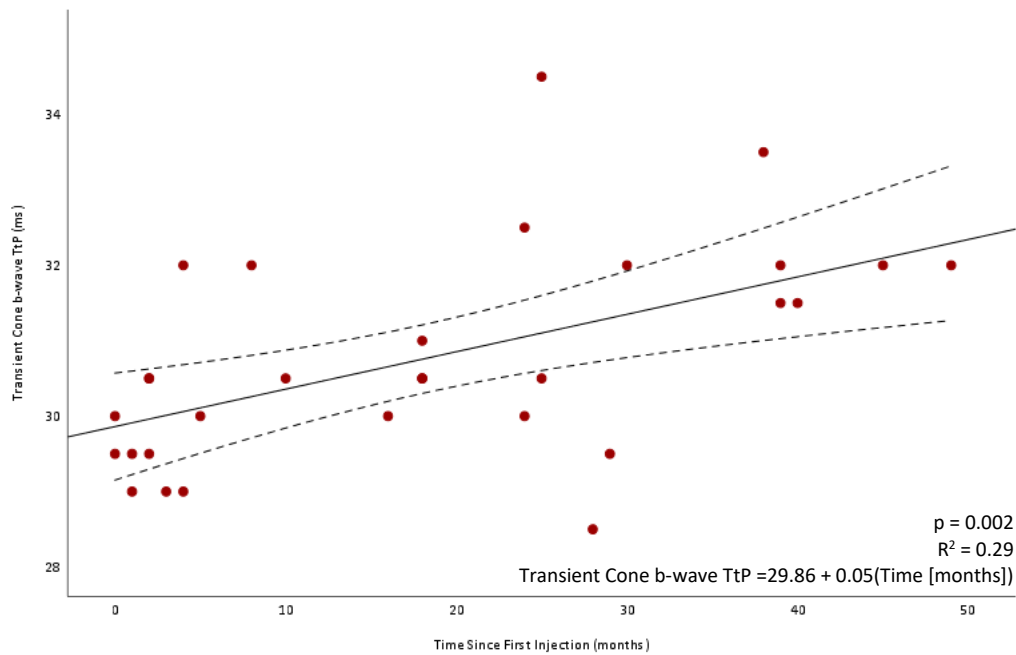


Figure 5.17. Scatter plot describing the linear regression of the relationship between Full Field Transient Cone b-wave ERG TtP and Time since first Injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

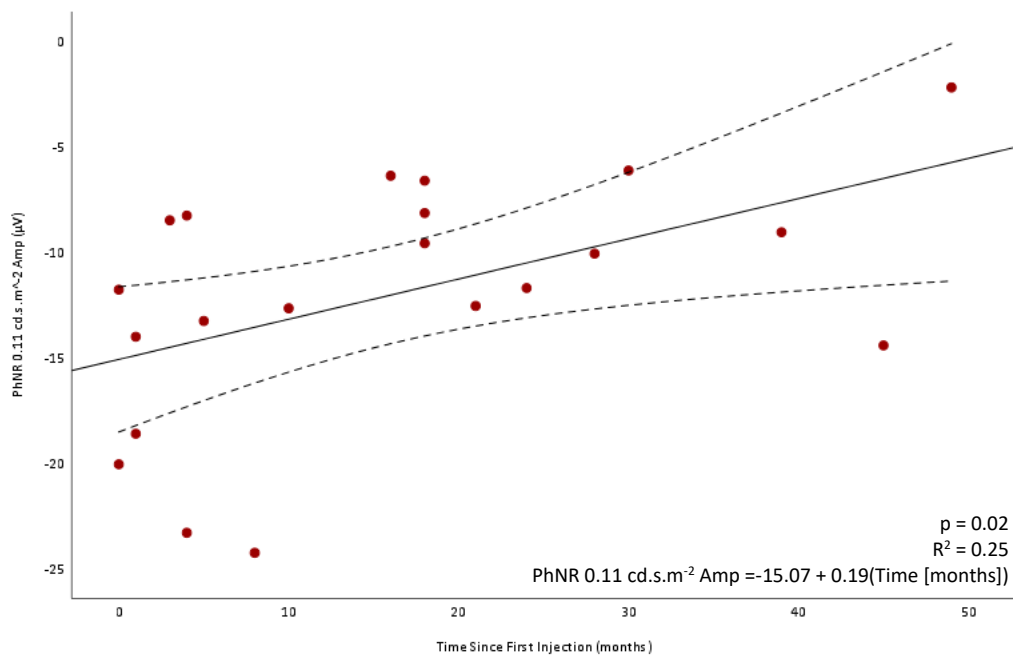


Figure 5.18. Scatter plot describing the linear regression of the relationship between full field PhNR 0.11 cd.s.m⁻² ERG amp and time since first injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

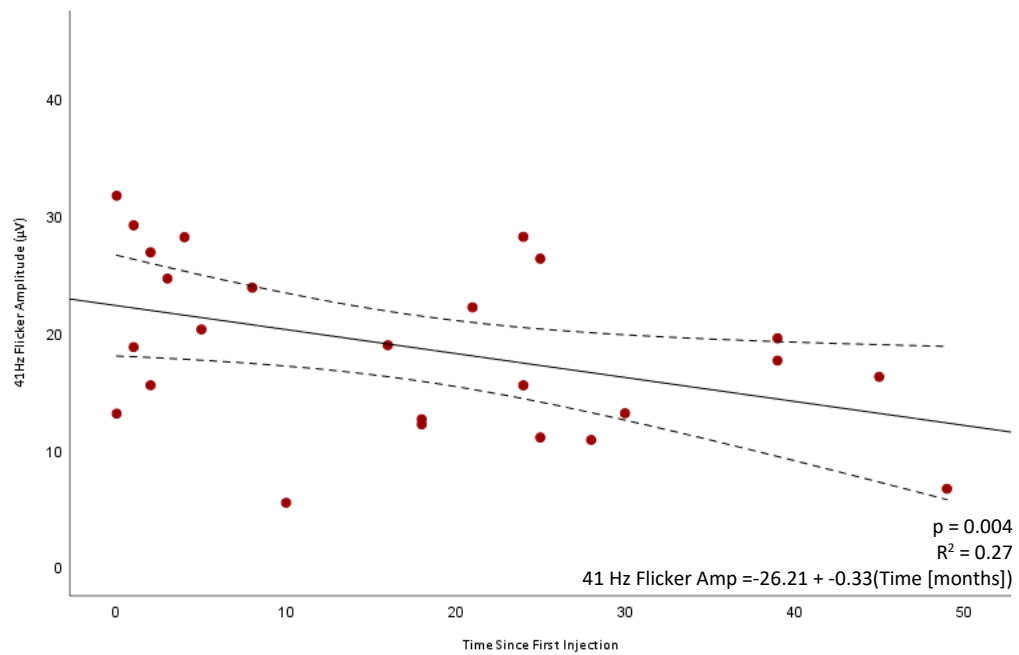


Figure 5.19. Scatter plot describing the linear regression of the relationship between 41 Hz flicker amp of the full field ERG and time since first Injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

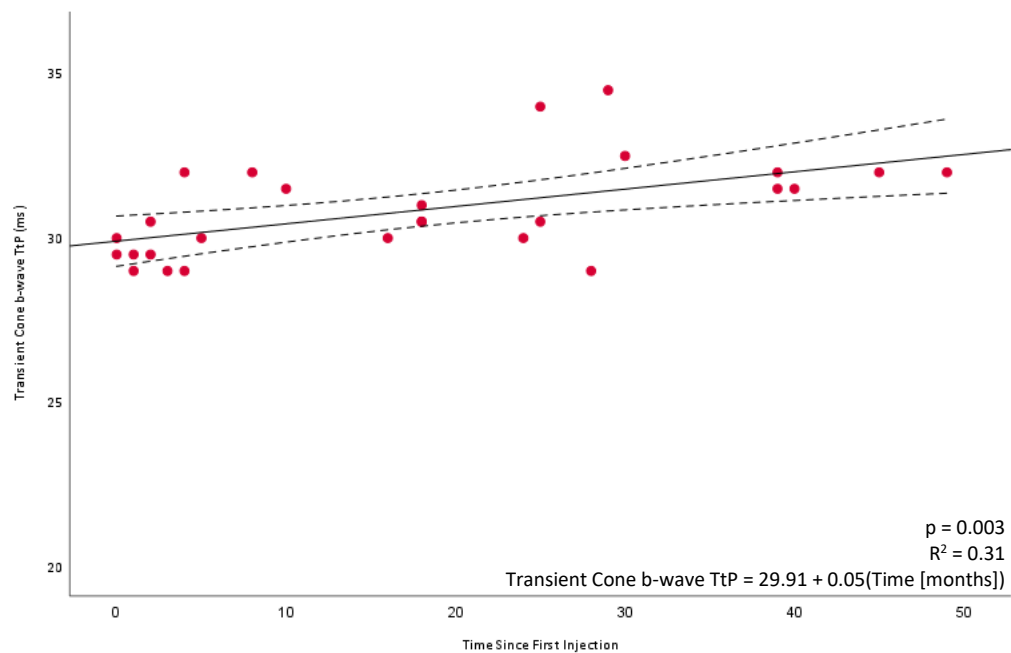


Figure 5.20. Scatter plot describing the linear regression of the relationship between transient cone b-wave TtP of the peripheral only ERG and time since first Injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

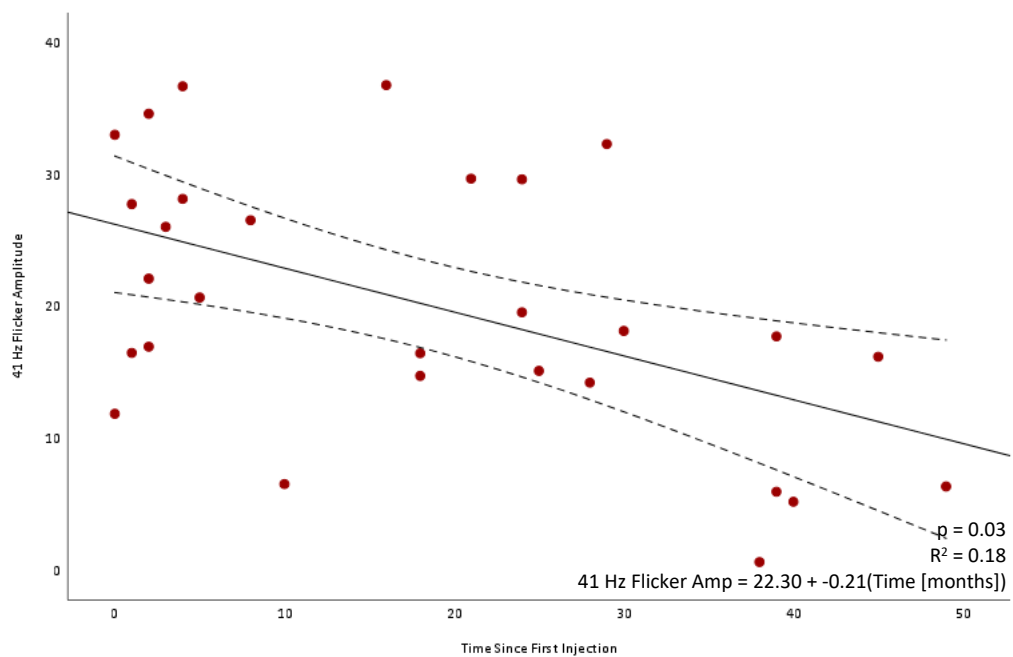


Figure 5.21. Scatter plot describing the linear regression of the relationship between 41 Hz flicker amp of the peripheral only ERG and time since first Injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

ERG Parameter			Median (IQR)	Spearman's Correlation									
				Number of anti-VEGF injections					Time since participants first anti-VEGF injection				
				Data Included (n)	p	Bonferroni corrected value	rho	Confidence Intervals	Data Included (n)	p	Bonferroni corrected value	rho	Confidence Intervals
PhNR	0.11 cd.s.m ⁻²	Amp (μV)	-10.07 (-9.37, -16.00)	21	0.02*	0.31	0.51*	0.14 – 0.76	21	0.03*	0.52	0.48*	0.02 – 0.78
		TtP (ms)	66.00 (59.25, 74.75)	21	0.77	1.00	0.07	-0.37 – 0.52	21	0.49	1.00	0.16	-0.57 – 0.33
	0.21 cd.s.m ⁻²	Amp (μV)	-18.12 (-13.29, -20.94)	24	0.11	1.00	0.33	-0.06 – 0.61	23	0.10	1.00	0.35	-0.04 – 0.65
		TtP (ms)	67.25 (62.88, 70.63)	24	0.80	1.00	0.06	-0.44 – 0.51	23	0.44	1.00	0.17	-0.32 – 0.59
	0.42 cd.s.m ⁻²	Amp (μV)	-17.25 (-14.28, -25.90)	24	0.02*	0.31	0.48*	0.07 – 0.76	23	0.10	1.00	0.36	-0.05 – 0.69
		TtP (ms)	67.00 (63.38, 72.50)	24	0.48	1.00	0.15	-0.35 – 0.60	23	0.65	1.00	0.10	-0.41 – 0.58
	0.84 cd.s.m ⁻²	Amp (μV)	-19.50 (-15.97, -28.43)	25	0.23	1.00	0.25	-0.13 – 0.58	24	0.22	1.00	0.26	-0.14 – 0.61
		TtP (ms)	68.50 (60.00, 73.50)	25	0.81	1.00	0.05	-0.52 – 0.42	24	0.93	1.00	0.02	-0.46 - 0.52
	1.68 cd.s.m ⁻²	Amp (μV)	-17.51 (-15.07, -28.96)	19	0.28	1.00	0.26	-0.22 – 0.66	18	0.64	1.00	0.12	-0.37 – 0.55
		TtP (ms)	68.00 (63.50, 75.00)	19	0.74	1.00	0.08	-0.61 – 0.44	18	0.88	1.00	0.04	-0.47 – 0.56
	3.35 cd.s.m ⁻²	Amp (μV)	-18.23 (-16.08, -26.28)	20	0.03*	0.58	0.48*	0.09 – 0.76	19	0.10	1.00	0.39	-0.11 – 0.77
		TtP (ms)	69.75 (65.88, 80.75)	20	0.85	1.00	0.05	-0.55 – 0.52	19	0.71	1.00	0.09	-0.58 – 0.42
Transient Cone	a-wave	Amp (μV)	-24.95 (-20.71, -29.24)	30	0.91	1.00	0.02	-0.40 - 0.41	30	0.85	1.00	0.04	-0.37 – 0.40
		TtP (ms)	14.75 (14.50, 15.50)	30	0.72	1.00	0.07	-0.40 – 0.30	30	0.36	1.00	0.18	-0.21 – 0.51
	b-wave	Amp (μV)	99.23 (77.23, 115.21)	30	0.22	1.00	-0.23	-0.57 – 0.15	30	0.08	1.00	-0.32	-0.61 – 0.05
		TtP (ms)	30.50 (29.63, 32.00)	30	0.48	1.00	0.14	-0.26 – 0.49	30	<0.001*	0.02*	0.57*	0.27 – 0.78
41 Hz Flicker		Amp (μV)	18.13 (14.47, 27.93)	30	0.40	1.00	0.16	-0.52 – 0.22	29	0.01*	0.18	0.46*	-0.72 - -0.09
		TtP (ms)	9.50 (8.25, 11.50)	30	0.24	1.00	0.22	-0.21 – 0.58	29	0.28	1.00	0.21	-0.16 – 0.54

Table 5.4. Association between total number of anti-VEGF injections and time since first injection and Full Field ERG parameters. Asterisks (*) indicate significant correlation (p<0.05).

ERG Parameter			Median (IQR)	Spearman's Correlation									
				Number of anti-VEGF injections					Time since participants first anti-VEGF injection				
				Data Included (n)	p	Bonferroni corrected value	rho	Confidence Intervals	Data Included (n)	p	Bonferroni corrected value	rho	Confidence Intervals
Transient Cone	a-wave	Amp (μV)	-21.16 (-29.39, -18.22)	27	0.39	1.00	-0.17	-0.51 – 0.22	27	0.50	1.00	-0.14	-0.52 – 0.26
		TtP (ms)	14.50 (13.50, 15.50)	27	0.85	1.00	-0.04	-0.37 – 0.29	27	0.59	1.00	0.11	-0.31 – 0.47
	b-wave	Amp (μV)	81.72 (60.70, 93.30)	27	0.85	1.00	-0.04	-0.40 – 0.38	27	0.37	1.00	-0.18	-0.50 – 0.20
		TtP (ms)	30.50 (29.75, 32.00)	27	0.63	1.00	0.09	-0.30 – 0.50	27	< 0.001*	<0.008	0.64*	0.36 – 0.80
	PhNR	Amp (μV)	-18.67 (-26.93, -14.92)	24	0.51	1.00	-0.14	-0.54 – 0.32	24	0.63	1.00	-0.10	-0.55 – 0.37
		TtP (ms)	58.75 (43.50, 66.13)	24	0.39	1.00	0.18	-0.22 - 0.56	24	0.14	1.00	0.31	-0.13 – 0.66
41 Hz Flicker		Amp (μV)	18.84 (12.98, 25.04)	25	0.19	1.00	-0.27	-0.59 – 0.19	25	0.04*	0.32	-0.42*	-0.71 - -0.03
		TtP (ms)	9.50 (8.00, 10.63)	25	0.18	1.00	0.27	-0.21 – 0.67	25	0.25	1.00	0.24	-0.14 – 0.58

Table 5.5. Association between total number of anti-VEGF injections and time since first injection and ERG parameters generated by the peripheral retina only. Asterisks () indicate significant correlation ($p < 0.05$).*

Test Data Type	Parameter (Dependent Variable)	N° Anti-VEGF or Time (Independent Variable)	Model Summary			ANOVA	Coefficients						Regression Equation
			R²	Adjusted R²	R² as %	Regression (p)	Constant	Confidence Intervals		N° Anti VEGF OR Time (months)	Confidence Intervals		
								Lower Bound	Upper Bound		Lower Bound	Upper Bound	
Peripheral Only ERG	Transient Cone b-wave TtP	Time	0.31	0.28	31	0.003*	29.91	29.14	30.67	0.05	0.02	0.09	Transient Cone b-wave TtP = 29.91 + 0.05(Time [months]) *
	41 Hz Flicker Amp	Time	0.18	0.15	18	0.03*	22.30	17.99	26.61	-0.21	-0.39	-0.02	41 Hz Flicker Amp = 22.30 + -0.21(Time [months]) *
Full Field ERG	PhNR 0.11 cd.s.m ⁻² Amp	Time	0.25	0.22	25	0.02*	-15.07	-18.51	-11.64	0.19	0.03	0.35	0.11 cd.s.m ⁻² Amp =-15.07 + 0.19(Time [months]) *
	PhNR 0.42 cd.s.m ⁻² Amp	N° Anti-VEGF	0.19	0.16	19	0.03*	-22.41	-27.94	-16.88	0.33	0.03	0.62	0.42 cd.s.m ⁻² Amp =-22.41 + 0.33(N° of Anti-VEGF Injections) *
	Transient Cone b-wave TtP	Time	0.29	0.27	29	0.002*	29.86	29.15	30.57	0.05	0.02	0.08	Transient Cone b-wave TtP =29.86 + 0.05(Time [months]) *
	41 Hz Flicker Amp	Time	0.27	0.24	27	0.004*	26.21	21.04	31.37	-0.33	-0.55	-0.12	41 Hz Flicker Amp = 26.21 + -0.33(Time [months]) *

Table 5.6. Linear Regression analysis of statistically significant correlations in full field ERGs and peripheral only ERGs with the macular response subtracted. Asterisks () indicate significant relationships (p<0.05).*

5.2.3.2. Analysis of OCT data

Interocular Analysis

Due to permissions restrictions at the study site it was not possible to extract sample OCT images for publication. However, Table 5.7 shows sample manual segmentation values from the treated and untreated eyes of three patients who had received different numbers of anti-VEGF injections. Box plots in Figures 5.22 - 5.27 show the distribution of data in treated and untreated eyes for each OCT parameter. There was no clear trend towards one eye having reduced retinal layer thickness compared to the other eye. The data were non-normally distributed and so a Wilcoxon signed rank test was performed to compare data from the treated and untreated eyes of the 14 participants with unilateral nAMD (see Table 5.8). This showed that there was no significant difference between the treated and untreated eyes for any OCT parameter tested after Bonferroni correction. However, the outer segments of the treated eyes were significantly thicker in treated eyes compared to untreated eyes prior to Bonferroni correction ($p = 0.03$).

OCT Parameter		Participant (N° anti-VEGF injections in affected eye)					
		CW (0)		NP (23)		ST (34)	
		Treated	Untreated	Treated	Untreated	Treated	Untreated
Nasal	RNFL	67	72	77	75	62	70
	GCC	104	127	124	124	116	124
	PRL	70	70	75	73	50	65
	OS	39	26	28	39	15	39
	Choroid	70	70	126	113	50	46
	TRT	263	264	143	274	235	276
Temporal	GCC	86	86	83	77	98	65
	PRL	62	62	68	76	39	60
	OS	40	32	45	37	36	39
	Choroid	77	67	155	52	119	116
	TRT	248	227	246	237	225	179

Table 5.7. Sample OCT parameters for the affected and unaffected/treated and untreated eyes of three participants receiving different numbers of anti-VEGF injections. Participants included CW (0 injections), NP (23 injections) and ST (34 injections).

OCT Parameter		Median (IQR)		p	Bonferroni Corrected Value
		Treated	Not Treated		
Nasal	RNFL	68.00 (65.00, 73.75)	71.50 (59.75, 75.00)	0.64	1.00
	GCC	114.50 (108.75, 124.00)	122.00 (109.25, 127.00)	0.42	1.00
	PRL	58.50 (52.00, 66.50)	60.50 (50.00, 65.00)	0.97	1.00
	OS	34.00 (31.00, 39.00)	27.50 (21.50, 34.00)	0.03*	0.28
	Choroid	119.00 (89.00, 189.00)	115.00 (49.25, 129.00)	0.29	1.00
	TRT	264.50 (256.00, 275.50)	250.50 (244.25, 261.00)	0.17	1.00
Temporal	GCC	77.50 (75.00, 82.25)	75.00 (73.00, 77.75)	0.47	1.00
	PRL	69.50 (61.25, 72.75)	62.00 (54.75, 67.75)	0.13	1.00
	OS	37.00 (34.00, 41.00)	34.00 (29.00, 37.00)	0.06	0.65
	Choroid	181.00 (122.00, 217.00)	161.50 (122.75, 194.75)	0.89	1.00
	TRT	230.00 (217.00, 237.00)	218.50 (204.75, 231.50)	0.25	1.00

Table 5.8. Median, IQR and Wilcoxon Signed Rank Test Values for interocular comparison of OCT Parameters. Parameters that were found to have significant differences between treated and untreated eyes are highlighted with an asterisk (). There were no significant differences after Bonferroni correction.*

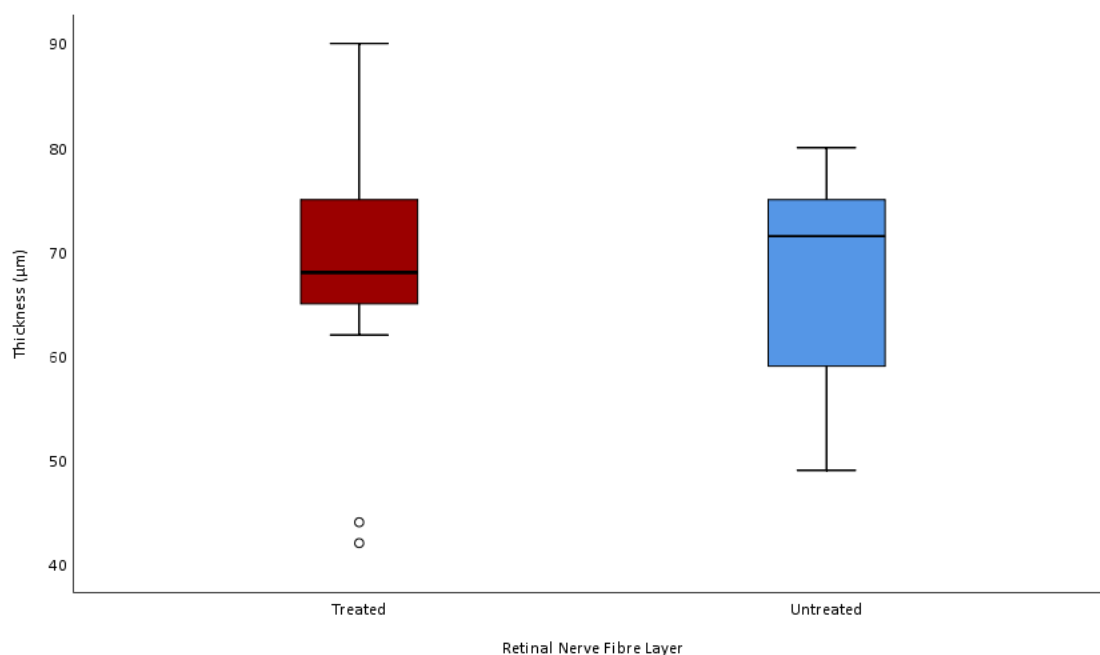


Figure 5.22. Box plots comparing the distribution of the nasal Retinal Nerve Fibre Layer OCT data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted.

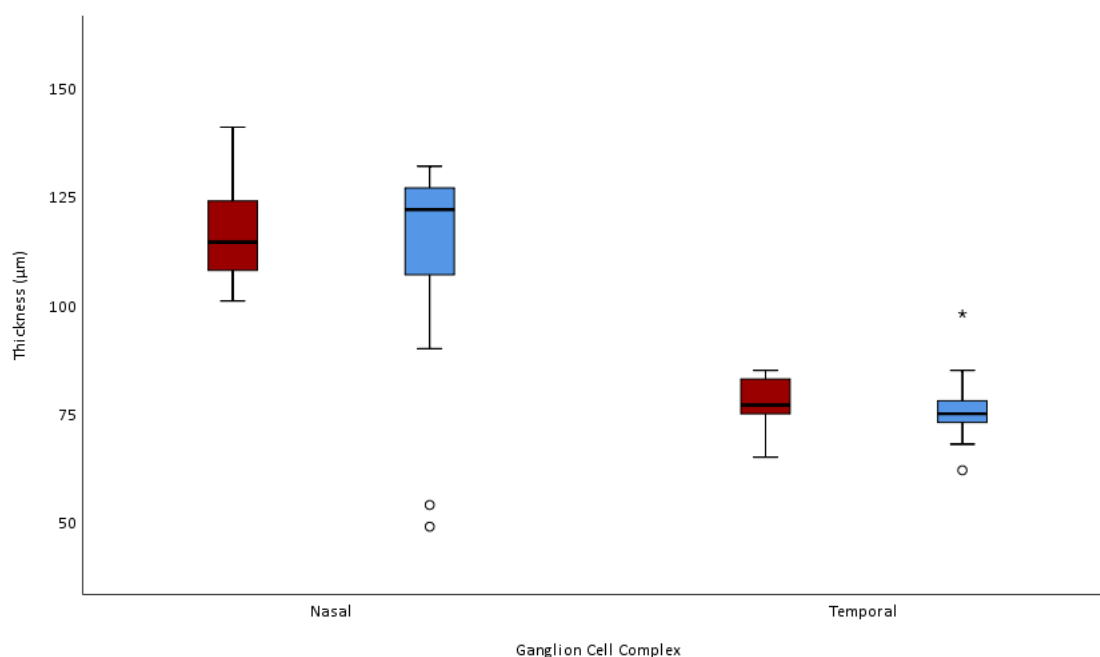


Figure 5.23. Box plots comparing the distribution of the nasal and temporal Ganglion Cell Complex OCT data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted.

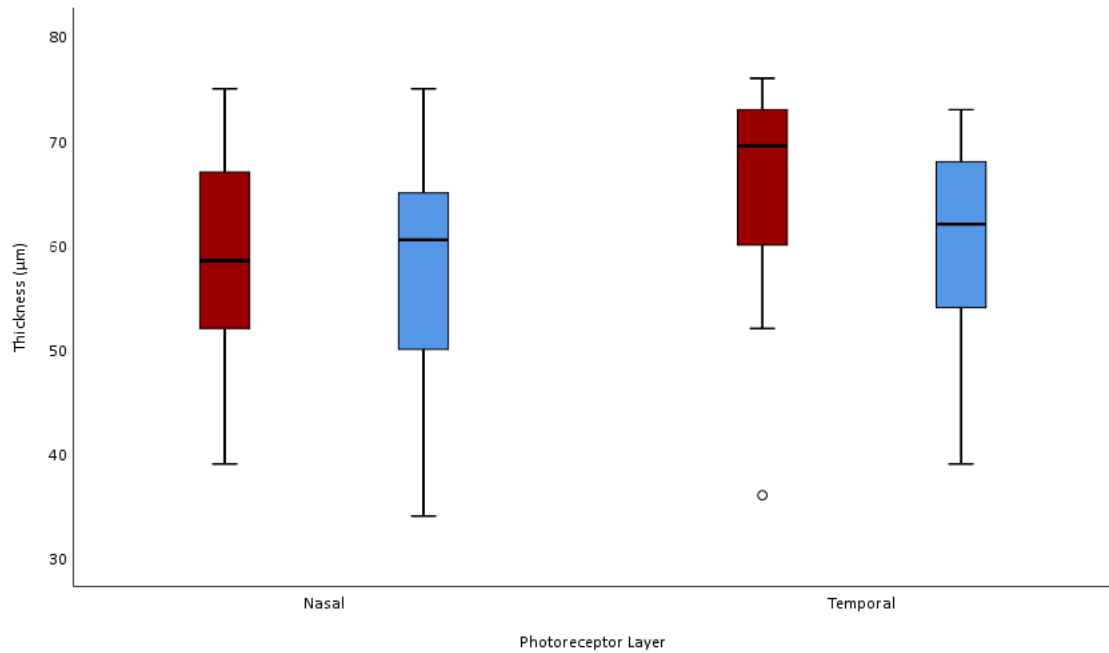


Figure 5.24. Box plots comparing the distribution of the nasal and temporal Photoreceptor Layer OCT data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted.

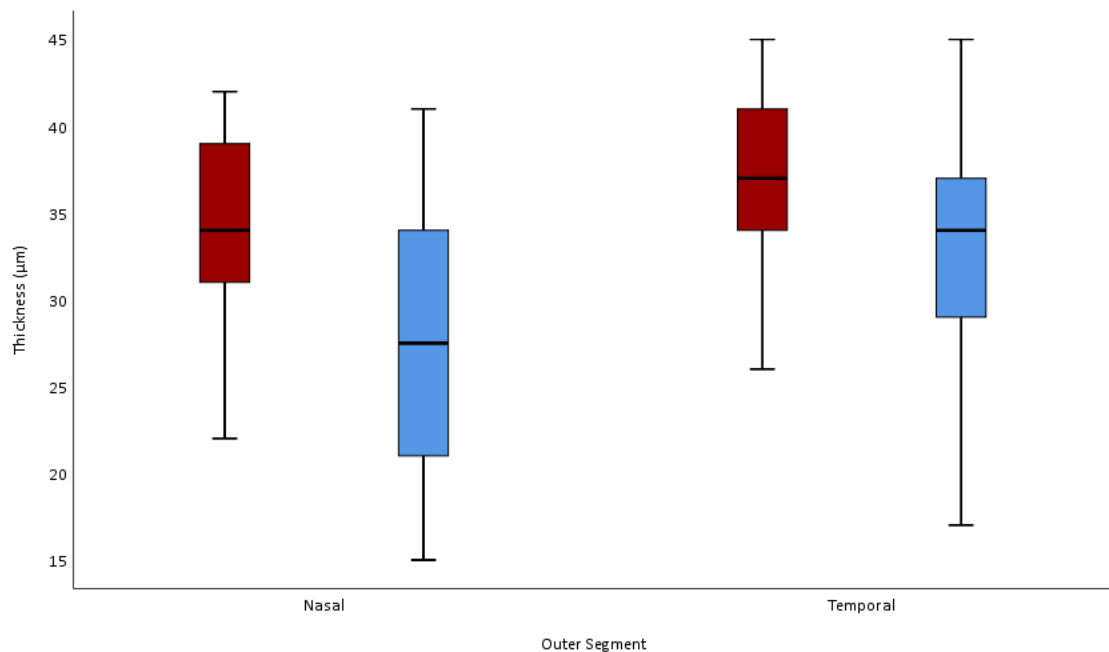


Figure 5.25. Box plots comparing the distribution of the nasal and temporal OS OCT data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values.

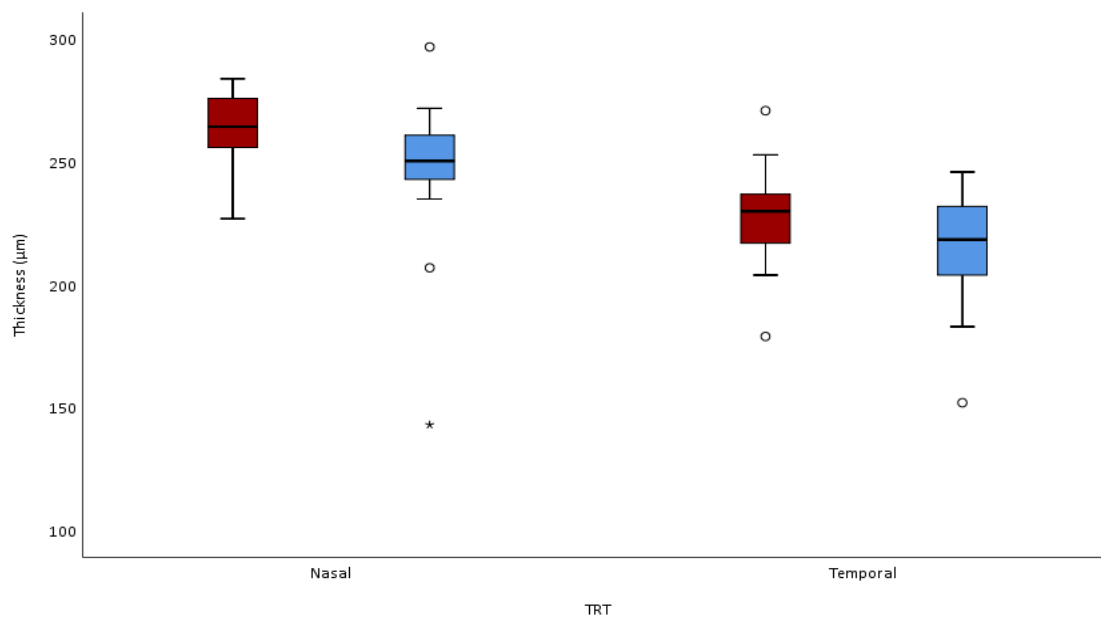


Figure 5.26. Box plots comparing the distribution of the nasal and temporal choroidal OCT data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted.

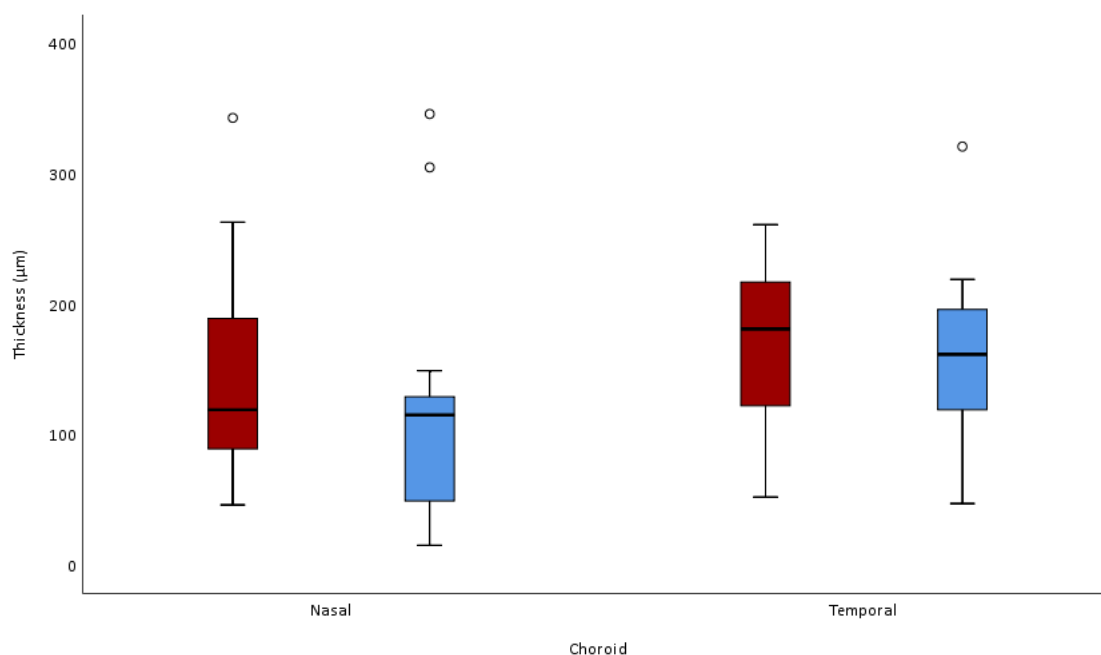


Figure 5.27. Box plots comparing the distribution of the nasal and temporal TRT OCT data of the treated and untreated eyes. Data from treated eyes is shown in red and untreated eyes in blue. Median, upper and lower quartiles (limits of box) are shown. Whiskers extend to maximum and minimum values (excluding outliers). High potential outliers are indicated by circles beyond the whiskers plotted. High extreme values are indicated by asterisks outside the whiskers plotted.

Between Participant Analysis

Table 5.7 displays sample segmentation data for three participants that had received different numbers of injections. Spearman's correlation analysis was used to evaluate the relationship between OCT parameters and number of anti-VEGF injections (Figures 5.28, 5.29). In the majority of cases there was no statistically significant association with the OCT parameters (Table 5.9). However, one parameter had a significant correlation with total number of anti-VEGF injections – nasal outer segment layer thickness ($p = 0.01$, $\rho = -0.50$, $CI = -0.76 - -0.12$). The negative correlation was indicative of a thinning of the photoreceptor outer segments with increasing number of injections. This p-value did not remain significant after Bonferroni correction. Data from this parameter subsequently underwent linear regression analysis (see Table 5.10, and Figure 5.30). In the regression model the number of injections received was shown to statistically significantly predict nasal outer segment thickness ($p = 0.02$). It was also established that ~18% of the variance in nasal outer segment thickness was attributable to the number of anti-VEGF injections received.

In the correlation analysis between OCT data and time since first injection (see Figures 5.31 and 5.32) several parameters showed significant correlations; nasal nerve fibre layer thickness ($p = 0.02$, $\rho = -0.45$, $CI = -0.69 - -0.11$), nasal photoreceptor outer segment layer thickness ($p = 0.02$, $\rho = -0.45$, $CI = -0.74 - -0.06$), nasal total retinal thickness ($p = 0.02$, $\rho = -0.44$, $CI = -0.71 - -0.06$), temporal ganglion cell complex thickness ($p = 0.02$, $\rho = -0.45$, $CI = -0.70 - -0.12$), temporal outer segment thickness ($p = 0.02$, $\rho = -0.46$, $CI = -0.73 - -0.09$). All of these were negative correlations, suggesting a thinning of the layers with increasing time since first injection. None of these p-values were <0.05 after Bonferroni correction. Data from the parameters showing significant correlations with the number of anti-VEGF injections issued subsequently underwent linear regression analysis (see Table 5.10, and Figures 5.33 – 5.37). Regression models assessing the relationship between time since first injection and nasal RNFL thickness, nasal OS thickness, temporal GCC thickness and temporal OS thickness were shown to statistically significantly predict the respective OCT outcome variables ($p = 0.01$, $p = 0.04$, $p = 0.005$, and $p = 0.04$ respectively). Time since first anti-VEGF injection was responsible for ~23% of nasal RNFL thickness variance, ~16% of nasal OS thickness variance, ~26% of temporal GCC thickness variance, and ~16% of

temporal OS thickness variance. Time since first injection was unable to statistically significantly predict the outcome variable in the nasal total retinal thickness model ($p = 0.17$, $R^2 = 0.07$).

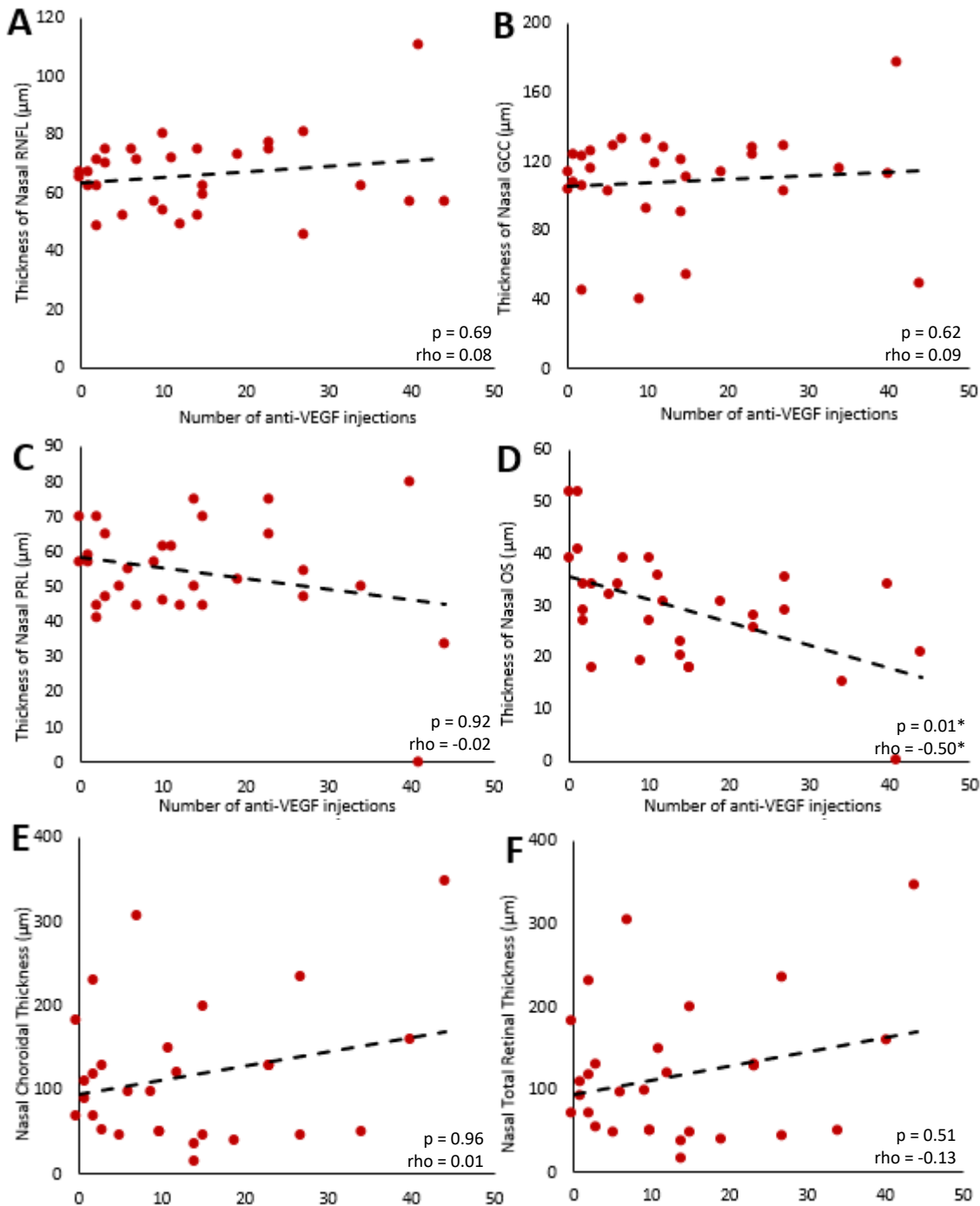


Figure 5.28. Scatter plots showing the correlation between nasal OCT parameters and total number of anti-VEGF injections. The thicknesses relate to: A, Retinal Nerve Fibre Layer (RNFL); B, Ganglion Cell Complex (GCC); C Photoreceptor Layer (PRL); D, Outer Segment (OS); E, Choroid; F, Total Retinal Thickness. The results of the Spearman's correlation analysis are also shown. Significant correlations are marked with an asterisk (*).

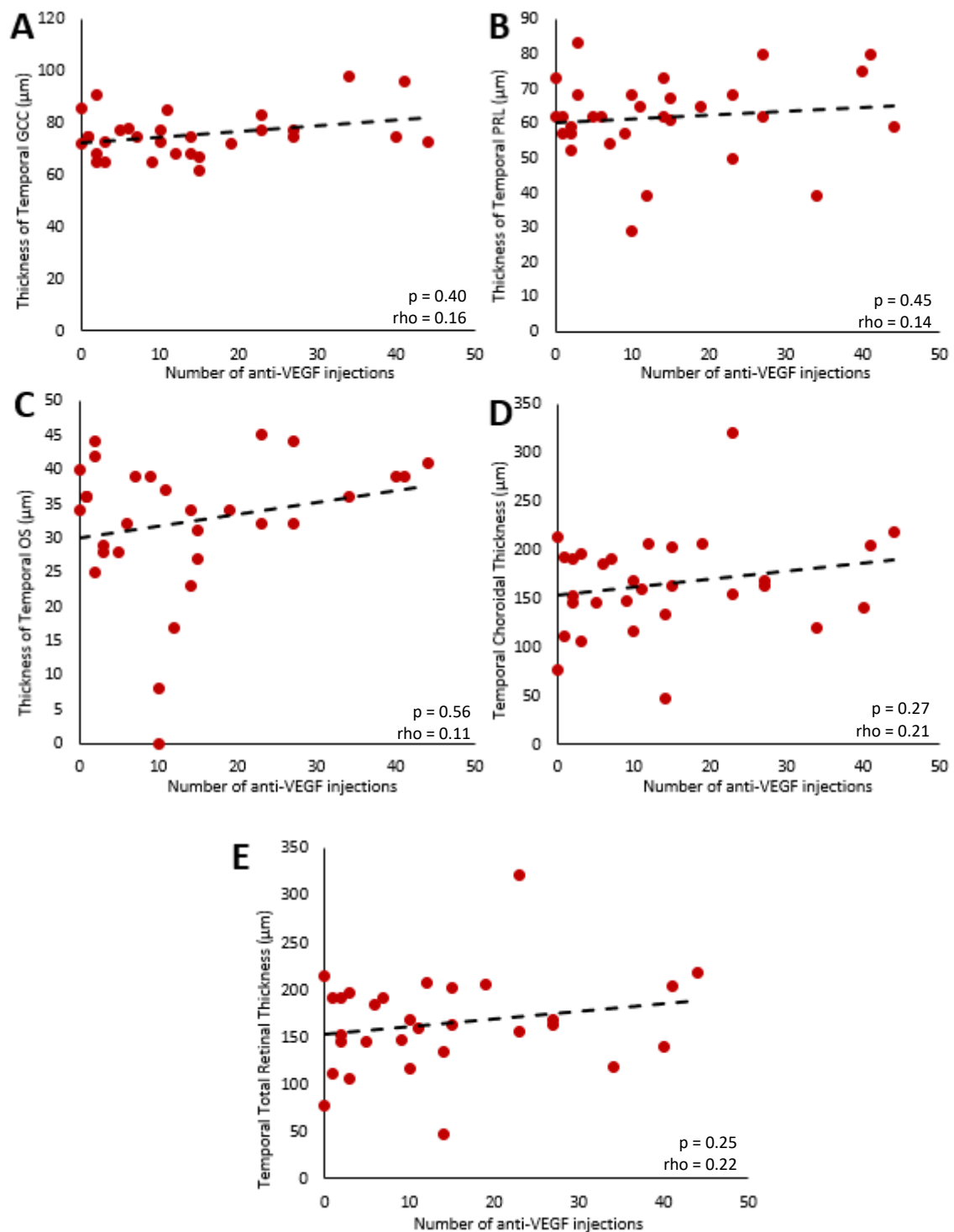


Figure 5.29. Scatter plots showing the correlation between temporal OCT parameters and total number of anti-VEGF injections. The thicknesses relate to: A, Ganglion Cell Complex (GCC); B, Photoreceptor Layer (PRL); C, Outer Segment (OS); D, Choroid; E, Total Retinal Thickness. The results of the Spearman's correlation analysis are also shown. None of the correlations were statistically significant ($p > 0.05$).

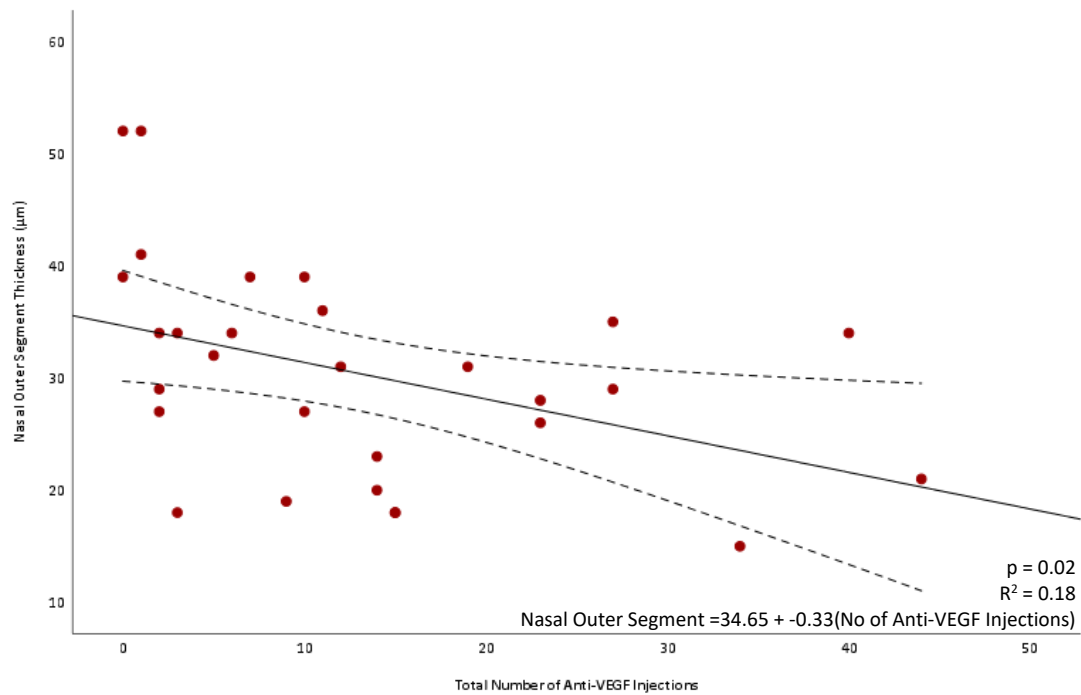


Figure 5.30. Scatter plot describing the linear regression of the relationship between nasal OS thickness and the total number of anti-VEGF injections. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

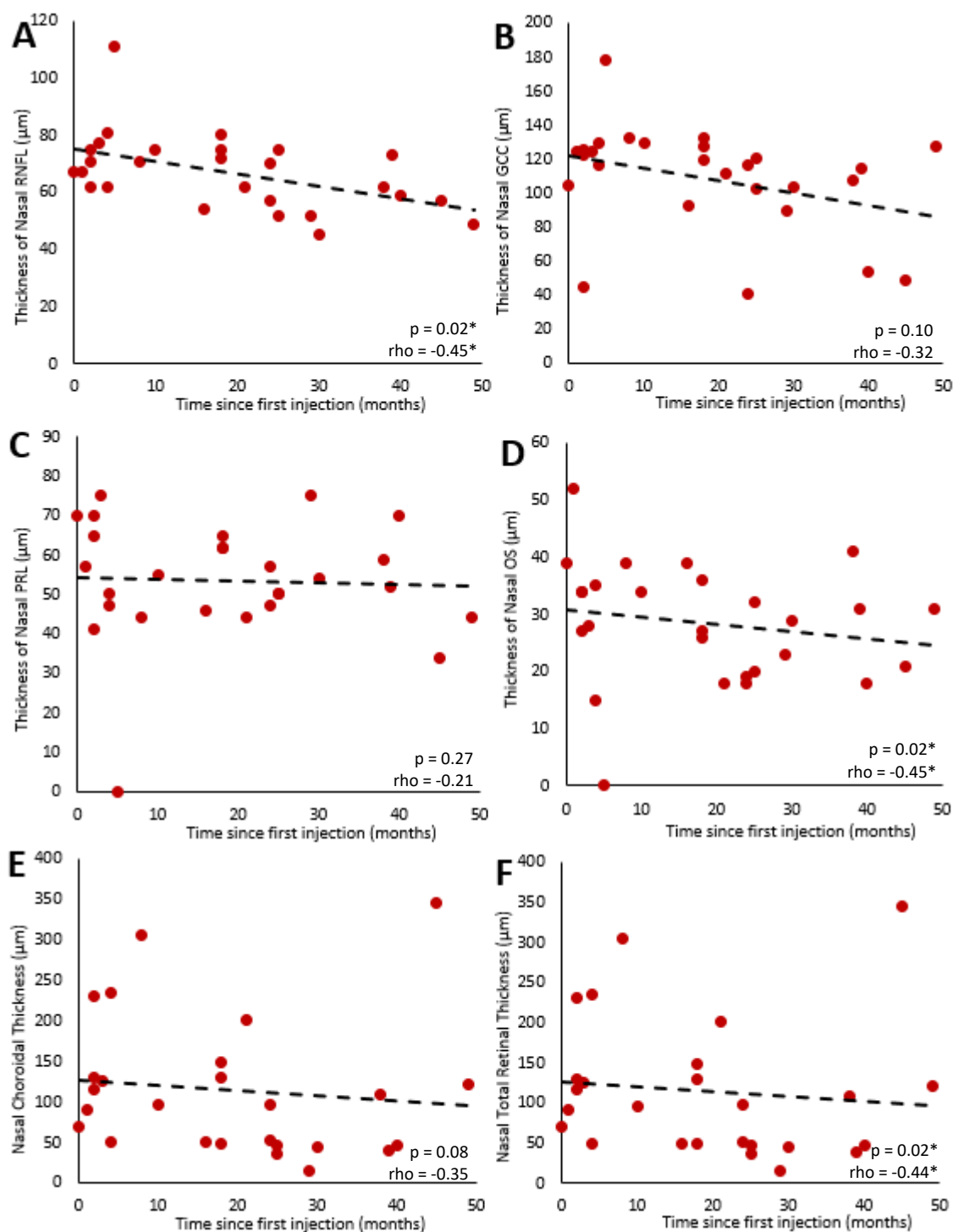


Figure 5.31. Scatter plots showing the correlation between nasal OCT parameters and time since first anti-VEGF injection. The thicknesses relate to: A, Retinal Nerve Fibre Layer (RNFL); B, Ganglion Cell Complex (GCC); C, Photoreceptor Layer (PRL); D, Outer Segment (OS); E, Choroid; F, Total Retinal Thickness. The results of the Spearman's correlation analysis are also shown. Significant correlations are marked with an asterisk (*).

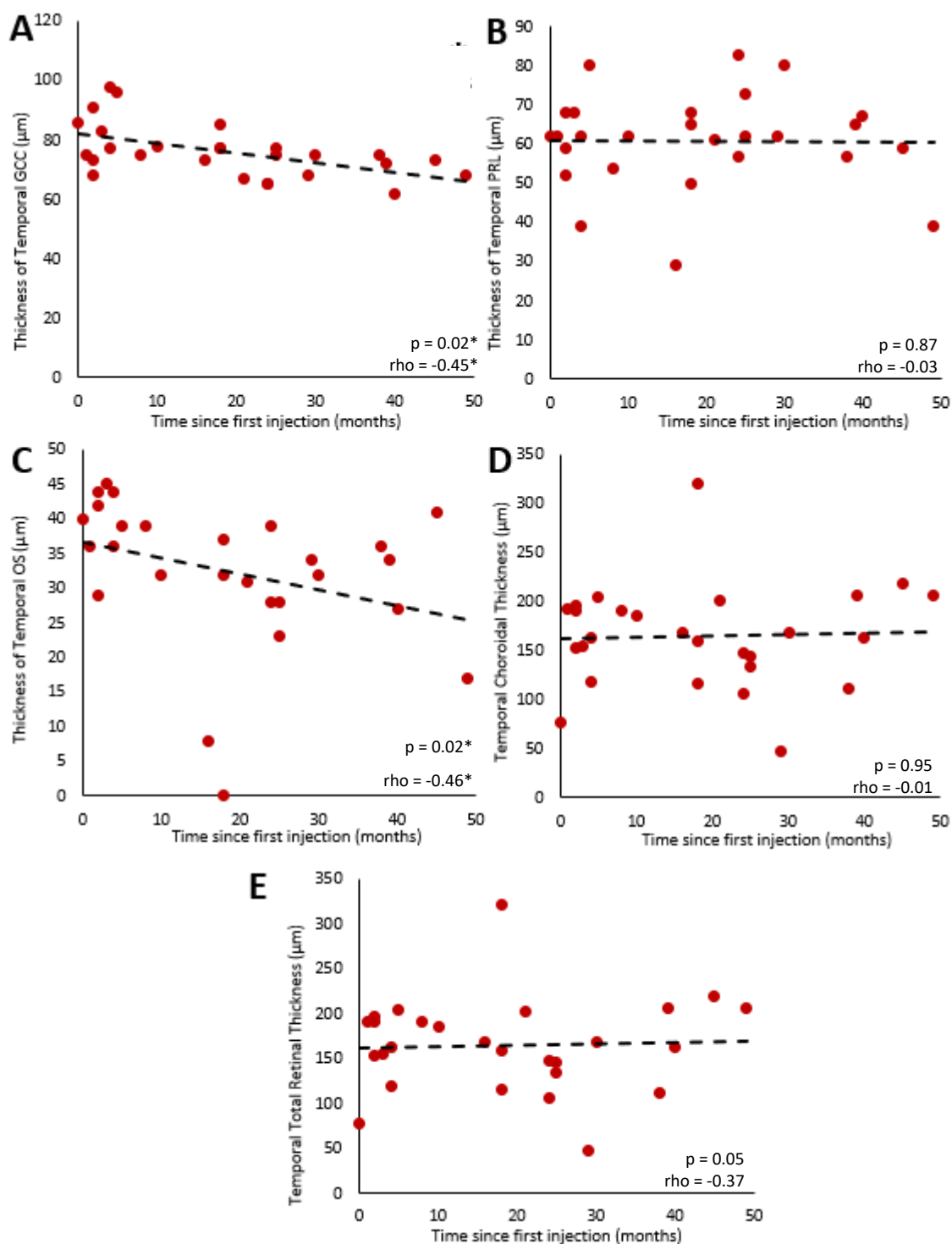


Figure 5.32. Scatter plots showing the correlation between temporal OCT parameters and time since first anti-VEGF injection. The thicknesses relate to: A, Ganglion Cell Complex (GCC); B, Photoreceptor Layer (PRL); C, Outer Segment (OS); D, Choroid; E, Total Retinal Thickness. The results of the Spearman's correlation analysis are also shown. Significant correlations are marked with a single asterisk (*).

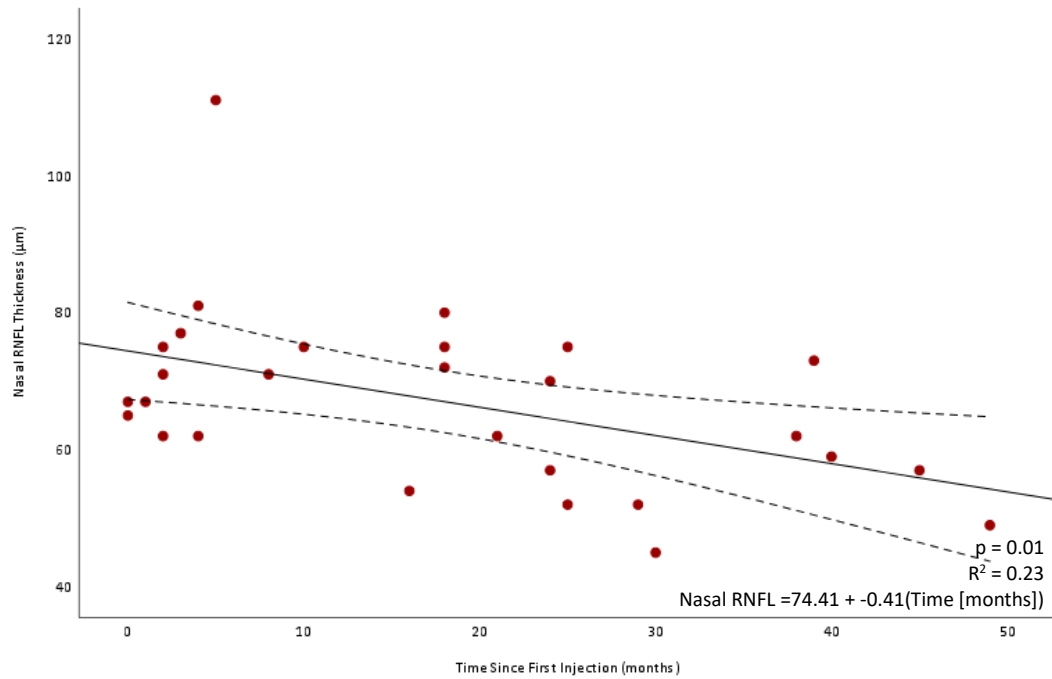


Figure 5.33. Scatter plot describing the linear regression of the relationship between nasal RNFL thickness and the time since first anti-VEGF injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

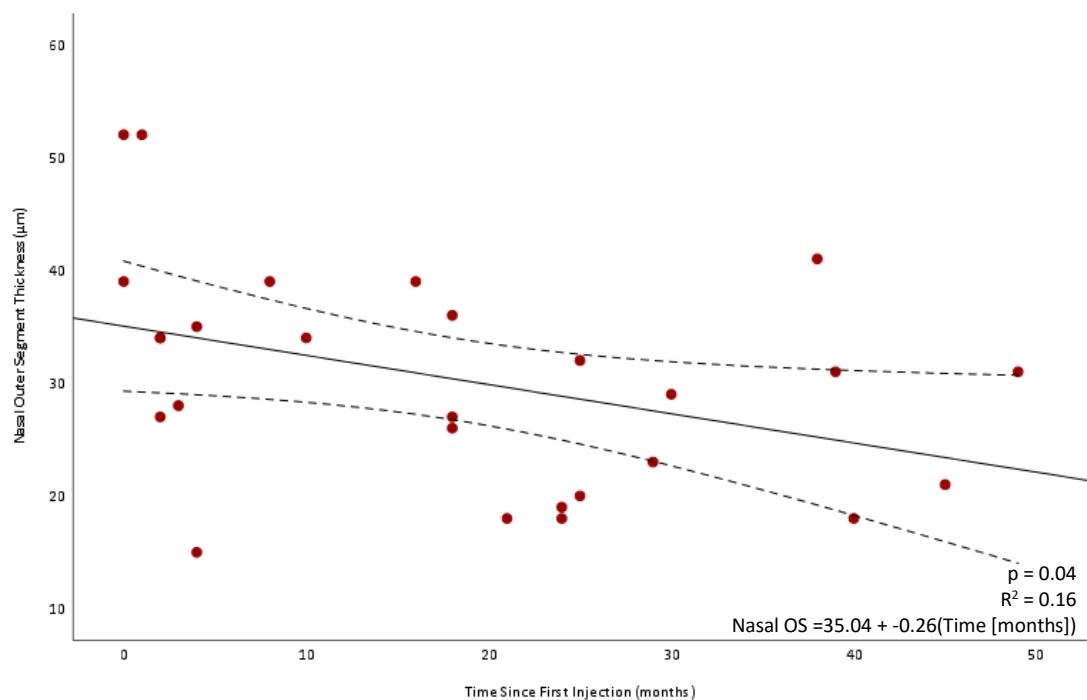


Figure 5.34. Scatter plot describing the linear regression of the relationship between nasal OS thickness and the time since first anti-VEGF injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

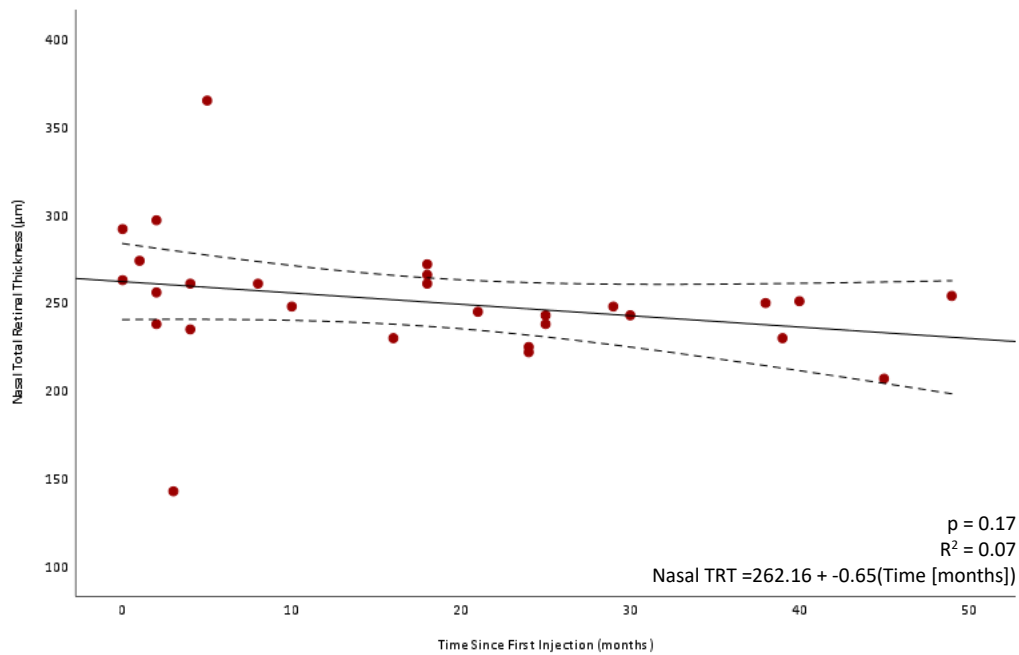


Figure 5.35. Scatter plot describing the linear regression of the relationship between nasal total retinal thickness and time since first anti-VEGF injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

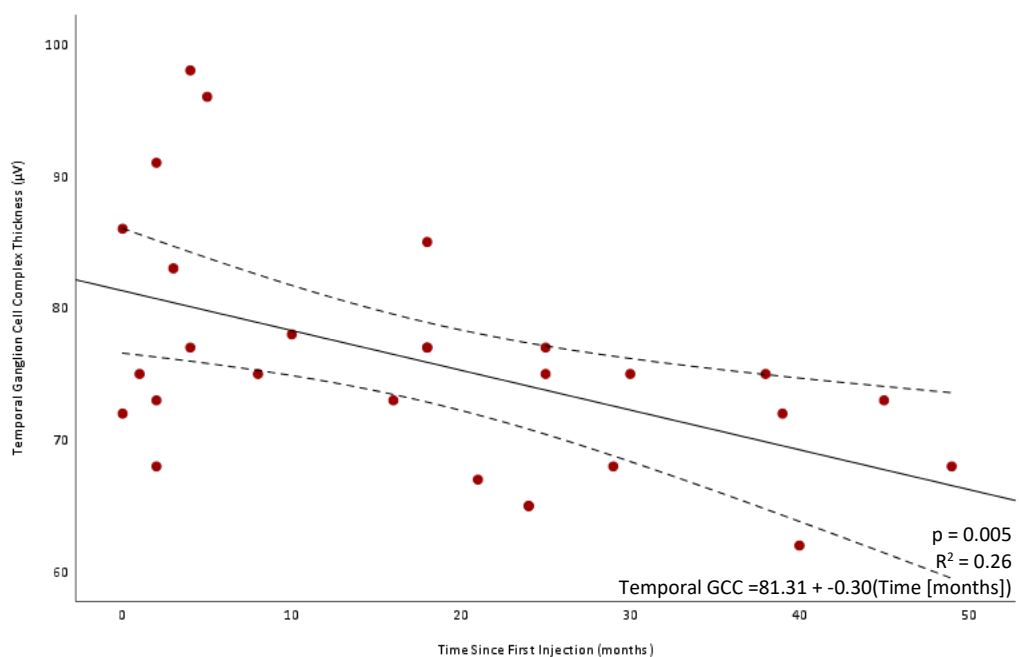


Figure 5.36. Scatter plot describing the linear regression of the relationship between temporal GCC thickness and time since first anti-VEGF injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

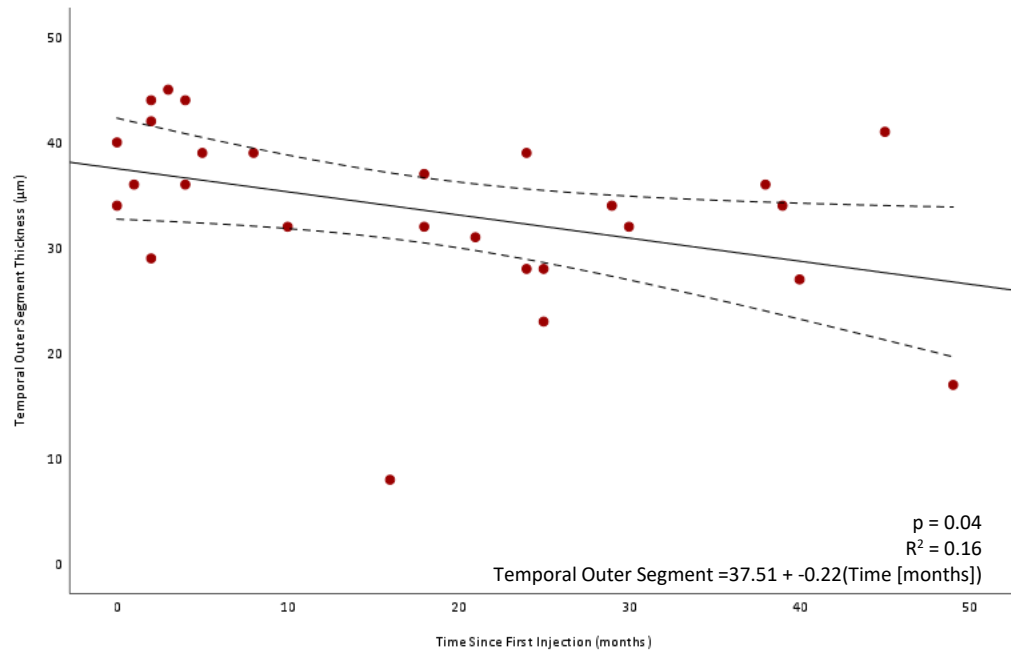


Figure 5.37. Scatter plot describing the linear regression of the relationship between temporal OS thickness and time since first anti-VEGF injection. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

OCT Parameter		Median (IQR)	Spearman's Correlation									
			Number of anti-VEGF injections					Time since participants first anti-VEGF injection				
			Data Included (n)	p	Bonferroni corrected value	Correlation Coefficient (rho)	Confidence Intervals	Data Included (n)	p	Bonferroni corrected value	Correlation Coefficient (rho)	Confidence Intervals
Nasal	RNFL	66 (57, 75)	30	0.69	1.00	0.08	-0.28 – 0.47	29	0.02*	0.22	-0.45*	-0.69 – -0.11
	GCC	115 (103, 125)	30	0.62	1.00	0.09	-0.24 – 0.46	29	0.10	1.00	-0.32	-0.65 – 0.08
	Photoreceptors	55 (47, 65)	29	0.92	1.00	-0.02	-0.44 – 0.38	28	0.27	1.00	-0.21	-0.57 – 0.23
	Outer Segment	31 (23, 35)	29	0.01*	0.11	-0.50*	-0.76 – -0.12	28	0.02*	0.22	-0.45*	-0.74 – -0.06
	Choroid	97 (50, 149)	29	0.96	1.00	0.01	-0.39 – 0.41	28	0.08	1.00	-0.35	-0.68 – 0.11
	Total Retinal Thickness	249 (236, 263)	30	0.51	1.00	-0.13	-0.51 – 0.28	29	0.02*	0.22	-0.44*	-0.71 – -0.06
Temporal	GCC	75 (69, 77)	30	0.40	1.00	0.16	-0.24 – 0.51	29	0.02*	0.22	-0.45*	-0.70 – -0.12
	Photoreceptors	62 (57, 68)	30	0.45	1.00	0.14	-0.24 – 0.51	29	0.87	1.00	-0.03	-0.41 – 0.33
	Outer Segment	34 (29, 39)	29	0.56	1.00	0.11	-0.26 – 0.46	28	0.02*	0.22	-0.46*	-0.73 – -0.09
	Choroid	163 (141, 195)	30	0.27	1.00	0.21	-0.22 – 0.57	29	0.95	1.00	-0.01	-0.46 – 0.43
	Total Retinal Thickness	221 (208, 235)	30	0.25	1.00	0.22	-0.18 – 0.55	29	0.05	1.00	-0.37	-0.68 – 0.04

Table 5.9. The OCT parameter correlation analysis with total number of anti-VEGF injections and time since first anti-VEGF injection. Significant correlations are highlighted with an asterisk (). After Bonferroni correction, no OCT parameters were significantly correlated with the time since first injection nor with total number of injections.*

Test Data Type	Parameter (Dependent Variable)	N° Anti-VEGF or Time (Independent Variable)	Model Summary			ANOVA	Coefficients						Regression Equation
			R ²	Adjusted R ²	R ² as %	Regression (p)	Constant	Confidence Intervals		N° Anti VEGF OR Time (months)	Confidence Intervals		
								Lower Bound	Upper Bound		Lower Bound	Upper Bound	
OCT	Nasal Outer Segment	N° Anti-VEGF	0.18	0.15	18	0.02*	34.65	29.71	39.58	-0.33	-0.61	-0.05	Nasal Outer Segment =34.65 + -0.33(N° of Anti-VEGF Injections) *
	Nasal RNFL	Time	0.23	0.20	23	0.01*	74.41	67.32	81.50	-0.41	-0.72	-0.11	Nasal RNFL =74.41 + -0.41(Time [months]) *
	Nasal OS	Time	0.16	0.13	16	0.04*	35.04	29.28	40.81	-0.26	-0.50	-0.01	Nasal OS =35.04 + -0.26(Time [months]) *
	Nasal TRT	Time	0.07	0.04	7	0.17	262.16	240.56	283.77	-0.65	-1.58	0.29	Nasal TRT =262.16 + -0.65(Time [months])
	Temporal GCC	Time	0.26	0.23	26	0.005*	81.31	76.58	86.04	-0.30	-0.51	-0.10	Temporal GCC =81.31 + -0.30(Time [months]) *
	Temporal Outer Segment	Time	0.16	0.13	16	0.04*	37.51	32.71	42.30	-0.22	-0.43	-0.01	Temporal Outer Segment =37.51 + -0.22(Time [months]) *

Table 5.10. Linear Regression analysis of statistically significant correlations in OCT parameters. Asterisks () indicate significant relationships (p<0.05).*

5.2.4. Discussion

The findings of this study suggest that an increase in number of anti-VEGF injections may be associated with a reduction in PhNR amplitude. Although this association was not found to be significant after Bonferroni testing, it was consistent across PhNR intensities, and the strength of the correlation was moderate to high (Hemphill, 2003). It was noted that confidence intervals for rho were wide and extended close to zero. Bonferroni adjustment is known to be a highly conservative measure which may increase the risk of type II errors (Perneger, 1998), and it appears likely that this consistent trend in PhNR amplitudes was not due to chance alone. A key point to note, however, is that while these results may indicate a relationship between the number of injections and PhNR amplitudes, they do not prove a causal relationship. Other possible explanations are discussed below. Linear regression analysis yielded coefficients of 0.33 for the two significant PhNR parameters, suggesting that for each injection the amplitude of the PhNR may be reduced by 0.33 μ V (CI = 0.03 – 0.62, and 0.02 – 0.63), although these values should be interpreted with caution given the wide confidence intervals. The R^2 parameters of the two statistically significant linear regression models were 0.19 and 0.22, indicating that up to 22% of the variance in PhNR amplitude was explained by the number of injections received. Due to the phenomenon of ‘shrinkage’, adjusted R^2 values were consistently lower, indicating that when applied to the entire population a smaller percentage of the variance would be expected to be explained by the number of injections. Other ERG parameters relating to the ISCEV Transient Cone and 41 Hz flicker ERGs remained largely unaffected by the number of injections.

Although many ERG parameters showed no correlation with time since first injection, the PhNR 0.11 cd.s.m⁻² time to peak ($p = 0.03$, $\rho = 0.48$), and transient cone ERG b-wave time to peak ($p = <0.01$, $\rho = 0.57$) ERG parameters did so, with transient cone ERG b-wave time to peak remaining significant after Bonferroni correction. Linear regression analysis suggested that an increase in total duration of treatment by 1 month was associated with a decrease in amplitude of 0.19 μ V (CI = 0.03 – 0.35) for the PhNR 0.11 cd.s.m⁻² ERG ($R^2 = 0.25$), and an increase of 0.05ms (CI = 0.02 – 0.08) for the transient cone ERG b-wave time to peak ($R^2 = 0.29$), although again this should be interpreted with caution given the wide confidence intervals).

Although PhNRs recorded to other stimulus intensities were not significantly correlated with time since first injection (as they were with number of injections received), they did show a trend towards reducing amplitude with increasing total duration of treatment. This is not surprising as there was a strong correlation between number of injections and time since first injection, as treatment tends to be repeated on multiple occasions after initiation. Given this strong relationship between total duration and cumulative dose of treatment, it was interesting that the significantly affected functional parameters differed between time since first injection and total number of injections received. The finding that PhNR parameters did not show as strong a relationship with time since first injection may suggest that cumulative dose is a more important factor than duration of treatment should it be the case that RGC damage is caused by anti-VEGF treatment.

The PhNR is a negative deflection of the ERG, first reported in 1998, which is elicited under photopic conditions (Viswanathan et al., 1998). The origins of the PhNR are believed to lie primarily with the spiking activity of RGCs and their axons, although contributions from amacrine cells, glial cells and astrocytes are also possible (see Section 1.5, Colotto et al., 2000; Machida et al., 2004; Viswanathan et al., 1999; Viswanathan et al., 2000; Viswanathan et al., 2001; Rangaswamy et al., 2007). The finding of a reduction in PhNR amplitude with increasing numbers of injections therefore suggests a possibility of damage to the RGCs that may be occurring with repeated anti-VEGF injections and/or as time progresses after initial treatment with intravitreal anti-VEGF. This was consistent with the OCT findings of a reduction in RGC layer and RNFL thickness associated with an increased time since first anti-VEGF injection, although there was no correlation between ganglion cell layer thickness or RNFL thickness and total number of injections.

The possibility of anti-VEGF injection resulting in abnormal PhNR parameters was also raised by Yagura et al. (2016), who recorded photopic ERGs from n =19 human participants before the injection of ranibizumab or aflibercept (T1), just after the injection (T2), and after the performance of paracentesis to normalise IOP (T3). There was a reduction in PhNR amplitude at T2, although this returned to normal by T3. In the other prior human study, Moon et al. (2013) found no significant effects of

treatment on the PhNR. However, ERGs in this case were only recorded for up to 4 weeks after a course of 3 anti-VEGF injections.

Animal studies have largely reported no histological changes to the inner retina after exposure to anti-VEGF treatment. For example, Cheng et al. (2009) found no effect of bevacizumab on rat RGCs after a 6 month follow up from both single and repeated anti-VEGF injections. Schnichels et al. (2013) also looked at rat RGCs and found no neurotoxic effect of aflibercept, bevacizumab, and ranibizumab. Spitzer et al., (2007) performed a similar analysis on rat RGCs with several anti-VEGF factors (bevacizumab, pegaptanib, ranibizumab) and also found no cytotoxic effect. Thaler et al. (2010) found no significant reduction in RGC counts in healthy rat eyes or those with induced RGC damage, injected with anti-VEGF drugs. However, there is evidence to suggest that RGCs may not escape completely unscathed. Nishijima (2007), for example, reported that chronic inhibition of VEGF-A function in normal adult rats led to a significant loss of RGCs, whilst Romano et al., (2012) reported the presence of neuronal damage including severe RGC loss in 20 healthy rats injected with bevacizumab, with a large decrease in RGC specific protein shown during western blot testing in a subgroup receiving multiple bevacizumab injections. It is notable that none of the studies evaluating the histological effects of anti-VEGF on the rat retina monitored for longer than 6 months after initiating treatment. In contrast, participants in the current study had been receiving anti-VEGF therapy for as long as 4 years.

Although it is possible that the reduction in PhNR amplitude with increasing number of anti-VEGF injections is attributable to the cytotoxic effect discussed above, there are other possible explanations which should be considered. One is that the macular contribution to the full-field PhNR confounded the results. Retinal ganglion cells are predominant in the macula, with a reduced density in the peripheral retina (Curcio and Allen, 1990; Watson, 2004). It has, correspondingly, been shown that the PhNR response density declines from approximately $\sim 0.1 \mu\text{V}/\text{deg}$ in the macular region to $0.003 \mu\text{V}/\text{deg}$ at 45° from the fovea (Morny et al., 2019). Hence, the full-field ERG may have a disproportionately high contribution from RGCs with receptive fields located in the central retina, which may mean that the reduced amplitude of the full-field PhNR associated with increasing number of injections was actually due to a disease-related worsening of macular function. However, Morny et al. (2019) also reported that the

contribution from each RGC to the PhNR was three times greater in the peripheral retina than the central retina, which is likely to reduce the relative contribution from the macula (Morny et al., 2019, Figure 2). According to data in this publication (Figure 2a), despite the reduced density of RGCs in the peripheral retina, approximately two thirds of the PhNR response amplitude is attributable to the extramacular retina. In a separate study by Morny et al. (2015) in which focal (20 degree diameter) and full field long duration PhNR responses were recorded from controls and people with autosomal dominant optic atrophy, in their control group the PhNR ON response of the macular area was 24.3% of the magnitude of the full-field response, suggesting that approximately 75% of the full-field response is attributable to contributions from beyond the macula. Hence, whilst the macular contribution is certainly present, the majority of the response still originates from the extramacular region. Unfortunately, it was not logistically possible to obtain focal macular PhNR responses from the participants to the red on blue stimuli to subtract from the full-field response in order to assess the effect of number of injections on the isolated peripheral retinal response. Whilst an isolated peripheral retinal response was obtained from the ISCEV transient cone ERG protocol, which showed no significant relationship between number of injections and PhNR parameters, this protocol is not ideal for the examination of RGC function. The ideal protocol for eliciting a PhNR waveform includes a red flash on a blue background as the optimal stimulus (Sustar et al., 2009; Rangaswamy et al., 2007). Thus, the white flash on a white background used to record the transient cone ERG may not have provided a strong enough stimulus to the RGCs to demonstrate the association between number of injections and PhNR response amplitude.

Another possible explanation for the association between reducing PhNR amplitude and increasing number of injections is that the AMD disease process may have been affecting the RGC function of the peripheral retina. It is logical that, if this were the case, those who had more advanced disease i.e. had received more injections, may have had a greater disease-related impairment of peripheral retinal function. Age-related macular degeneration is considered to predominantly affect the macular region, as this is where the majority of clinical features are reported (Davis et al., 2005). Hence, in this PhD an assumption has been made that by evaluating predominantly peripheral retinal function we reduce the risk of conflating the effects

of disease progression and prolonged exposure to anti-VEGF drugs on retinal structure and function. However, there is evidence to suggest that the peripheral retina is not completely free from the effects of the AMD disease process. For example, some studies including patients with advanced AMD have reported reduced amplitude full-field ERG responses compared to age-matched controls (Walter et al., 1999). It has also been reported that drusen are present in the mid and far periphery of most eyes with AMD (OPERA group et al., 2017; Lengyel et al., 2015). However, given that we know the initial loci of disease related damage in AMD are the outer retina and RPE (Ambati and Fowler, 2012; Ambati et al., 2003), if it were the case that the progressive reduction in PhNR amplitude seen with increasing number of injections were attributable to AMD disease progression, it would be logical to expect that the parameters of the a- and b- wave waveforms would be affected to a similar if not greater extent to the PhNR. For example, a number of studies have shown a-wave and b-wave parameters elicited from focal ERGs are abnormal in early, dry and nAMD (Sandberg et al., 1993; Remulla et al., 1995; Sandberg et al., 1998; Terasaki et al., 2002; Binns and Margrain et al., 2007). Hence, it seems unlikely that a preferential reduction of the PhNR such as that reported here can be attributed to the disease process alone. As treatment for choroidal neovascularization commences immediately upon diagnosis, it was not possible to collect data from a large group of people with nAMD not receiving treatment in order to investigate the effects of the disease on the peripheral retina.

The finding that there was no significant difference in PhNR characteristics between the treated and untreated eyes of participants with unilateral nAMD was surprising, especially in light of the strong correlation between number of injections and PhNR amplitude in the between participant analysis. However, despite the wide use of a contralateral untreated eye as a control in studies of anti VEGF toxicity (Beck et al., 2016; Shin et al., 2014; Kurt et al., 2018; Valverde-Megias et al., 2019; Macky and Mahgoub, 2012; Skaat et al., 2011; Guo et al., 2010; Sakurai et al., 2009) there is evidence to suggest that there may be an inter-ocular transfer of the anti-VEGF therapy which may confound this assumption of the fellow eye being unaffected by the treatment (Avery et al., 2006; Karaca et al., 2003; Hanhart et al., 2014; Acharya et al., 2013). For example, Acharya et al. (2013) examined contralateral untreated eyes of

patients being treated for cystoid macular oedema and found that two of three patients with bilateral disease but unilateral ranibizumab (0.5 mg) treatment experienced structural and functional improvements in both the treated and untreated eyes. Furthermore, serious systemic adverse effects in some trials occurred more often in patients who had received intravitreal anti-VEGF (CATT research group et al., 2011; IVAN research group et al., 2012, Chakravarthy et al., 2013), although other studies have suggested that systemic effects of the drugs are minimal, when pre-existing risk factors are controlled for (Bressler et al., 2012; Campbell et al., 2012; Manresa et al., 2015; Sawada et al., 2008). The presence of a systemic effect of the drug would also suggest that an interocular transfer of the effect was possible. This evidence suggests that, while the fellow untreated eye may be the only practical control where ethical reasons prevent people with nAMD being left untreated, interpretation of interocular analysis should be approached with caution. A further substantial limitation with respect to the interocular analysis was the limited sample size. The limitations section of this discussion considers further the impact this would have on the minimum detectable difference between groups.

The lack of significant correlation between the majority of a-wave and b-wave parameters and number of injections or duration of treatment suggests that the function of cone photoreceptors and ON-bipolar cells may be unaffected by repeated anti-VEGF injections. There was a significant association between transient cone b-wave time to peak and time since first injection. However, given the lack of association with number of injections it is possible that this is attributable to progressive disease related effects on the outer retina rather than the cumulative effect of the treatment. This is supported by other studies such as Moon et al. (2013) where photopic b-wave amplitude remained unaffected by anti-VEGF injections in 32 participants who received 3 anti-VEGF injections and were followed up 4 months later, and Comyn et al. (2014) in which no significant changes were seen in the photopic ERGs of 48 patients receiving ranibizumab for diabetic macular oedema, followed up for an average of 48 weeks. Similarly, none of the 28 studies reporting photopic ERG results from animals exposed to anti-VEGF drugs reported a significant effect. However, there were several human studies which did report an effect of anti-VEGF treatment on the photopic ERG. Pedersen et al. (2010) showed an initial, transient decrease in b-wave amplitude and

increase in a-wave implicit time in 12 patients with nAMD treated with bevacizumab and followed over a 6 month period. This may suggest transient photoreceptor and/or ON-bipolar cell dysfunction but, given the lack of a control group and the relatively small sample size, the results must be interpreted with caution. For the reasons described in Section 3.1, only photopic ERGS were recorded on this cohort. However, there is some animal (Sakurai et al., 2009, Myers et al., 2012) and human (Comyn et al., 2014; Pedersen et al., 2010) evidence to suggest that scotopic ERG abnormalities may exist in individuals exposed to anti-VEGF drugs. For example, the literature review in Chapter 2 reports several human studies which found a reduction in a-wave amplitude or increased implicit time of the scotopic ERG to be associated with anti-VEGF treatment (Comyn et al., 2014; Pedersen et al., 2010). Hence, designing an experimental protocol where it is possible to obtain scotopic ERG results would be valuable in future.

When considering the OCT data analysis, only the thickness of the photoreceptor outer segment layer in the nasal retina showed a significant (and strong) negative correlation with number of injections. Both the temporal and the nasal photoreceptor outer segment layer thicknesses were also negatively correlated with the duration of treatment. This suggests a relationship between an increasing number of anti-VEGF injections or duration of treatment and the structure of the photoreceptor outer segments. Linear regression analysis yielded B values ranging between -0.33 (relating to thickness changes associated with number of anti-VEGF injections [CI = -0.61 - -0.05]), -0.26 and -0.22 (relating to thickness changes associated with time since first injection in the nasal and temporal retina, respectively [CI = -0.50 - -0.11, and -0.43 - -0.01, respectively]), suggesting that as these independent variables increase by one injection or one month of treatment, the thickness of the outer segment may decrease by up to 0.33 μ m (although the width of the confidence intervals should be considered here). The R^2 parameter of the linear regression analysis ranged from 0.18 (relating to thickness changes associated with number of anti-VEGF injections) to 0.16 (relating to thickness changes associated with time since first injection), indicating that ~16-18% of the variance in outer segment layer thickness in this study population is explained by these treatment variables.

This finding is novel, and to date no other studies have directly evaluated the structure of the photoreceptor layer in humans post anti-VEGF treatment in this location at the edge of the macula. There is some evidence from animal studies to suggest an increased level of photoreceptor apoptosis in rabbit eyes that received bevacizumab and pegaptanib when compared to controls (Avci et al., 2009), whilst Inan et al (2007) reported disruption of mitochondria in the photoreceptor inner segments in rabbit eyes which had received 1.25 mg and 3 mg ranibizumab. However, the majority of animal studies reviewed in Chapter 2 reported no evidence of structural damage to the outer retina (e.g. list Arraes et al., 2009; Bakri et al., 2006; Cheng et al., 2009; de Oliveira Dias et al., 2014; Guo et al., 2010; Iriyama et al., 2007; Januschowski et al., 2014; Kim et al., 2008; Lu et al., 2014; Manzano et al., 2006; Ozaki et al., 2000; Sari et al., 2006; Thaler et al., 2010; Zayit-Soudry et al., 2011). Although our findings suggest a relationship between treatment and photoreceptor outer segment damage, caution should be applied in interpreting these results. It is possible, as we discuss later, that in this location at the edge of the macula some of the thinning effects seen relate to atrophy caused by the AMD disease process itself (especially given the known differential damage caused by AMD to the outer retina; Ambati and Fowler, 2012; Ambati et al., 2003).

The finding that the nasal and temporal photoreceptor outer segment thickness was significantly associated with the anti-VEGF treatment was not reflected in abnormal ERG a-wave parameters. This is perhaps not surprising. The ERG data were collected from the whole of the peripheral retina, whilst the OCT thickness measurements were obtained from the very edge of the macular region. Again, it is possible that the AMD disease mechanism may have influenced the OCT parameters in this region, as well as the drug regimen. A measurement from the more extreme periphery might have provided a more valuable measure of the effect of the anti-VEGF therapy on retinal structure, but was not possible to obtain within the clinic within the current patient pathway.

A greater number of OCT layers appeared to be reduced in thickness in association with a longer overall duration of treatment, rather than the actual dose received. This indicates that, as the duration of anti-VEGF therapy increases, the thickness of retinal components decreases. This may be due to the treatment itself or to progressive

disease related atrophy. Interestingly, the nerve fibre layer (nasally) and the GCC complex (temporally) are two of the areas showing reduction in retinal thickness. Linear regression analysis yielded B values of -0.41 (nasal RNFL [CI = -0.72 - -0.11]), and -0.30 (temporal GCC [CI = -0.51 - -0.10]), suggesting that as time since first injection increases by one month the thickness of these components may decrease by 0.41 and 0.30 μm , respectively. However, confidence intervals were wide and this value should be interpreted with caution. The R^2 parameter of the linear regression analysis ranged from 0.23 (nasal RNFL) to 0.26 (temporal GCC), indicating that ~23% and ~26% of the variance in these retinal thickness measurements is attributable to time elapsed since first anti-VEGF injection. Once again, confidence intervals were wide for rho and so caution should be exercised when interpreting the strength of these correlations. This supports the finding of a reduced PhNR amplitude with increasing number of anti-VEGF injections and/or time since treatment commenced. However, given the stronger relationship with time since first injection than total number of injections received, the possibility that this effect is due to anti-VEGF resolving intraretinal fluid at the areas measured for OCT analysis (3 mm nasally and temporally of the fovea), or due to disease related atrophic changes to the inner retina should be considered as a possibility. This is especially true as the location evaluated was on the very edge of the macular region targeted by AMD.

Previous OCT evidence has largely failed to find an association between retinal thickness measurements and anti-VEGF treatment in humans. The majority of previous studies have evaluated peripapillary RNFL thickness after anti-VEGF treatment (Enders et al., 2017; Kopic et al., 2017; Valverde-Megias et al., 2019; Zucchiatti et al., 2017; Shin et al., 2014; Horseley et al., 2010; Yun et al., 2016; Jo., 2016; Sobaci et al., 2013; Demirel et al., 2015; Entezari., 2014; El-Ashry et al., 2015; Yau et al., 2015; Parlak et al., 2014). A few of these studies did report a reduction in RNFL thickness associated with treatment (Parlak et al., 2014; Enders et al., 2017; Kopic et al., 2017; Valverde-Megias et al., 2019; Yang et al., 2017). Of these, the findings of Parlak et al. (2014) and Valverde-Megias et al. (2019) may be attributable to factors other than the anti-VEGF treatment itself, as the control group also showed a reduction in RNFL thickness. However, Enders et al. (2017) found a significant reduction in RNFL thickness in 76 participants treated for nAMD with anti-VEGF treatment, which was more marked in

the group that did not have ACP to lower their IOP after anti-VEGF treatment. This suggests that anti-VEGF treatments could be causing some damage to the RNFL, worsened by the rise in IOP following each anti-VEGF injection. Yang et al. (2017) reported a significant difference in peripapillary RNFL thickness and retinal thickness between baseline and 6 months in 50 patients receiving anti-VEGF for diabetic maculopathy, whilst that in the control group remained stable. However, this was interestingly no longer significant when the study's index to compensate for macula oedema was applied. Beck et al. (2016) also reported a significant decrease in RNFL and RGC layer thickness after repeated anti-VEGF injections (n = 34, with a mean number of 31.5 injections), this time measured in the outer ring of the ETDRS grid. The macular location of their assessment increases the possibility that this effect is a combination of disease and treatment effects. Filek et al. (2019) found significant increases in cup volume and vertical cup-to-disc ratio increased significantly over 24 months in 30 patients with diabetic macular oedema receiving anti-VEGF therapy compared to 21 controls. This supports the findings in this chapter that those individuals who have received repeated anti-VEGF injections may be predisposed to loss of inner retinal components. One interesting point made by Yang et al. (2017) was that peripapillary RNFL thickness is strongly affected by peripapillary retinal thickness as a whole. As such, measured changes in peripapillary RNFL thickness may not represent any real loss or gain of RGCs or their axons.

There is a variety of ways in which the anti-VEGF therapy may theoretically affect the RGCs and their axons. One possibility is that it doesn't relate to the drug itself but to the process of injection. For example, Atchison et al. (2017) found that there are spikes in IOP after each injection. This repeated rise in IOP could cause damage to RGCs over time. A review by Bracha et al. (2017) explored several factors relating to intraocular anti-VEGF injections and IOP and found that while there is currently no conclusive indication of damage to the RNFL by repeated intra-ocular injections there is an increased chance of developing chronic ocular hypertension. However, the finding in some animal studies that RGC cell damage and loss occurred after injection with an anti-VEGF agent, but not after injection with a saline or vehicle control suggests that the drug itself may also have a cytotoxic effect on these cells (Romano et al., 2012). For example, there is evidence of increased apoptotic activity in the retinas of animals

exposed to anti-VEGF drugs (Inan et al., 2002; Cam et al., 2017), and the withdrawal of a neuroprotective effect of the VEGF which may lead to increased vulnerability of cells to damage (Nishijima et al., 2007). Further research is required to determine whether changes to the inner retina may be dose dependent and additional studies probing the effects of anti-VEGF therapy on outer retinal structure are also warranted.

Limitations

A key limitation of this study is that, with all of these findings, it is possible that results may have been confounded by the presence of nAMD itself. Participants who may have received more anti-VEGF injections or had received them for a longer time period may have done so in order to control a more aggressive case of nAMD. Therefore, it is also possible that any statistically significant findings may have been caused by a decline in retinal structure and/or function as a result of progressive neovascular AMD.

A second key limitation was the restricted sample size. Despite reassurance from the researcher and some clinic staff, an overall reluctance to participate from patients was observed. Various reasons were given for this such as, “I just don’t feel like doing that today”, “I don’t want the light to harm my eyes”, “I don’t like the sound of anything else touching my eyes”, and “I have things to do, I can’t stay”. Undergoing an intravitreal injection can elicit significant anxiety, and so it is understandable that a number of patients declined any additional tests that were not essential to their clinical care. There was also a reluctance from clinical staff at the hospital to lend support to the study as they feared this impinged on their clinical time and space, which unfortunately influenced some of the potential participants’ decisions to consent to the study. Limitations existed on clinical time and space as the medical retina clinic remained very busy throughout the data collection period. The researcher attended medical retina clinics on three different days of the week to try to minimise the diminishing returns effect of the same participants returning every 4-8 weeks. This was somewhat successful. Additionally, the volume of patients requiring exclusion from potential participation in the study due to comorbidities such as glaucoma, pan-retinal photocoagulation, diabetes or other systemic disorders was unanticipated.

As a result of these factors, the desired sample size described in the method (Section 5.2.2) was not achieved. With respect to the interocular analysis, the final sample size

was 14. Using the nomogram described by Martin Bland to determine the sample size required for a comparison of means, the standardised difference detectable would be 1.4 (at an alpha level of 0.05, power 80%) (Bland, 2011). The minimum detectable difference in mean depends on the standard deviation of the outcome measure. As an example, using the standard deviation of the PhNR amplitude for the stimulus intensities giving the biggest effect in the between participant analysis (stimulus intensities 0.11 [$\pm$ 4.48], 0.42 [$\pm$ 9.68] and 3.35 [$\pm$ 9.05] cd.s.m⁻²; standard deviation calculated from study eye data i.e. SD = 5.7 μ V, 9.7 μ V and 9.4 μ V respectively), this equates to a minimum detectable difference for amplitude of 8.0, 13.6, and 13.2 μ V for the three respective stimulus intensities. Hence, we would have been unable to reliably detect a difference smaller than this between treated and untreated eyes.

With respect to the between participant analysis, the sample size achieved was sufficient to detect a correlation coefficient of 0.50, with 80% power, at an alpha (two-tailed) of 0.05 (<http://www.sample-size.net/correlation-sample-size/>). Thus, the final sample size of this study may have had insufficient power to detect small correlations between parameters and anti-VEGF dose. It should also be noted that ERG parameters (especially amplitudes) are subject to high rates of inter-subject variability (Fortune et al., 2004; Mortlock et al., 2010), which could mask a small treatment effect. Due to the small sample size, no attempt was made to perform a sub-analysis according to the type of anti-VEGF drug received. Given the small sample size, and the expected variability in ERG parameters between participants, confidence intervals on the rho parameter of the correlation analysis and on B of the linear regression analysis were wide, and the strength of the correlation should be interpreted with caution.

In addition to recruitment difficulties at the hospital location, OCT scans for the study had to be performed by clinic staff rather than the researcher for clinical governance reasons. During this process, clinical imaging staff at the hospital did not compensate for axial length by inputting either axial length or keratometry measurements (as recommended for the Spectralis OCT device). This resulted in no compensation for ocular magnification on OCT scan images which has been shown to have a significant effect on lateral retinal measurements (Ctori et al., 2015; Terry et al., 2016; Bennet et al., 2018; Odell et al., 2011). Differences in retinal magnification between individuals will mean that the actual image area covered by the ETDRS grid will have varied

between participants in this study, which may have introduced a confounding factor into the analysis (Terry et al., 2016; Bennett et al., 2018; Odell et al., 2011). However, although it may have introduced some additional variance into the analysis, as this study presents an analysis of mean results this is unlikely to have presented a source of systematic error.

OCT analysis with calliper measurements may have presented an additional source of measurement error. By measuring at only two retinal locations, increased variability will have been introduced due to focal changes in thickness e.g. due to the presence of drusen or localised atrophy which happen to occur at the measurement site.

Additionally, such manual measurements are prone to observer error and bias (Ctori and Huntjens, 2015). Future studies may choose to employ automated segmentation software to minimise errors of this type.

There is also a possibility that, although technically defined as ‘extra-macular’, the areas at the edge of the ETDRS grid may still be affected by retinal changes caused by nAMD. For example, Curcio et al. (1996; 1998) performed post-mortem fundoscopic and histological assessments of human donor eyes and found evidence of photoreceptor degeneration in eyes with AMD. Although Curcio et al. (1996) reported that photoreceptor loss in non-exudative donor eyes was largely confined to the parafoveal region, and that photoreceptor topography beyond 3 mm (~10 degrees) eccentricity was near normal, eyes with choroidal neovascularisation showed much wider areas of photoreceptor loss and disruption (especially of the rods), topographically related to areas of scarring and RPE loss. This could be responsible for the thinning of the photoreceptor outer segment layer reported in this study.

Measurements of retinal thickness at this location may also be influenced by changes in subretinal fluid attributable to the anti-VEGF treatment and/or progression of nAMD. Ageing changes are also likely to impact on RGC density (Curcio and Drucker; 1993), and other retinal thickness measurements (Chua et al., 2019). However, in this cohort no significant relationship was found between number of injections or time since first injection and increasing age, so ageing changes are not likely to explain the findings.

Additional variability may have been introduced by the failure to assess participants for congenital colour vision defects prior to participation (although no participants self-

reported congenital colour vision deficiency). Colour vision deficiencies may have had an effect on responses to chromatic stimuli (i.e. particularly in the PhNR assessment). Again, however, this is unlikely to have presented a source of systematic error.

Another area for consideration is that a type I error may have occurred due to the multiple analyses. Bonferroni correction was used to minimise the risk of a type I error, however the original p-values are also presented for consideration in light of the published increase in risk of a type II error when this adjustment is made (Perneger, 1998).

Lastly, this study protocol unfortunately did not include scotopic ERGs due to time constraints and available facilities at the hospital study site. This may be useful in future research as there has been some evidence of an effect on scotopic b-wave parameters of anti-VEGF injections in humans (Pedersen et al., 2010 Comyn et al., 2014) suggesting the function of rod-bipolar cells may be negatively impacted at least initially.

5.2.5. Conclusion

To conclude, this study found an intriguing relationship between the number of anti-VEGF injections received and the amplitude of the PhNR response as well as the retinal architecture using OCT. However, the sample size was small. To investigate this further, an additional study was undertaken to examine the PhNR and ISCEV standard photopic flash and flicker ERGs in a different cohort of participants undergoing anti-VEGF therapy for nAMD (see Section 5.3)

5.3. Study 2: A cross-sectional study of the effect of anti-VEGF dose on full-field ERG parameters recorded using the RETeval® handheld system (at Moorfields Eye Hospital)

5.3.1. Aims and rationale

This follow-up study aimed to determine whether the results of the exploratory study described in Section 5.2 could be replicated in a second sample of participants recruited from a different hospital. Specifically, the aim was to determine whether ERG parameters were associated with number of injections. The rationale for this was to provide a different sample and examine the data in the same fashion a second time to see whether results were consistent with study one.

In this follow-up study it was decided to use the RETeval® hand-held ERG recording system to obtain PhNR and photopic ERG data. This system can operate successfully with or without pupil dilation and only one skin electrode is attached to the patient below each eye, reducing the preparation time and increasing comfort (see Sections 4.2.4 and 4.3.4 for details). The modified protocol included only one PhNR stimulus intensity and the RETeval® system's inbuilt ISCEV standard photopic flash and flicker ERGs. On this basis, the time requirement for data collection for each participant was 15 minutes. It was anticipated that this might facilitate more rapid collection of data, enabling a larger sample to be collected within a shorter time-frame than study 1.

5.3.2. Methods

Participants

Ethical approval was gained through the National Research Ethics Service (NRES) Committee London – Harrow (11/LO/2029), and written informed consent was obtained from all subjects, conforming to the tenets of the Declaration of Helsinki. Patients attending the medical retina clinics at Moorfields Eye Hospital, London for anti-VEGF treatment of nAMD were recruited for this study between March and September 2017 (inclusive). The participant recruitment and data collection pathway are outlined in Figure 5.38. The participants were identified by the lead consultant ophthalmologist working within a weekly medical retina clinic, through screening of the patients' medical records. A preliminary discussion with the researcher confirmed

eligibility. The inclusion criterion was a diagnosis of nAMD, being treated with intravitreal injections of any anti-VEGF drug. Exclusion criteria were identical to study 1 (Section 5.2). Participants were also asked if they had ever been told they suffered from any form of colour vision deficiency. Once eligibility was established, an information sheet on the study was provided and consent obtained. Relevant clinical details such as number of injections received was achieved through retrospective data collection from participants' clinical notes by the main researcher with the assistance and supervision of the supervising ophthalmologist (OM).

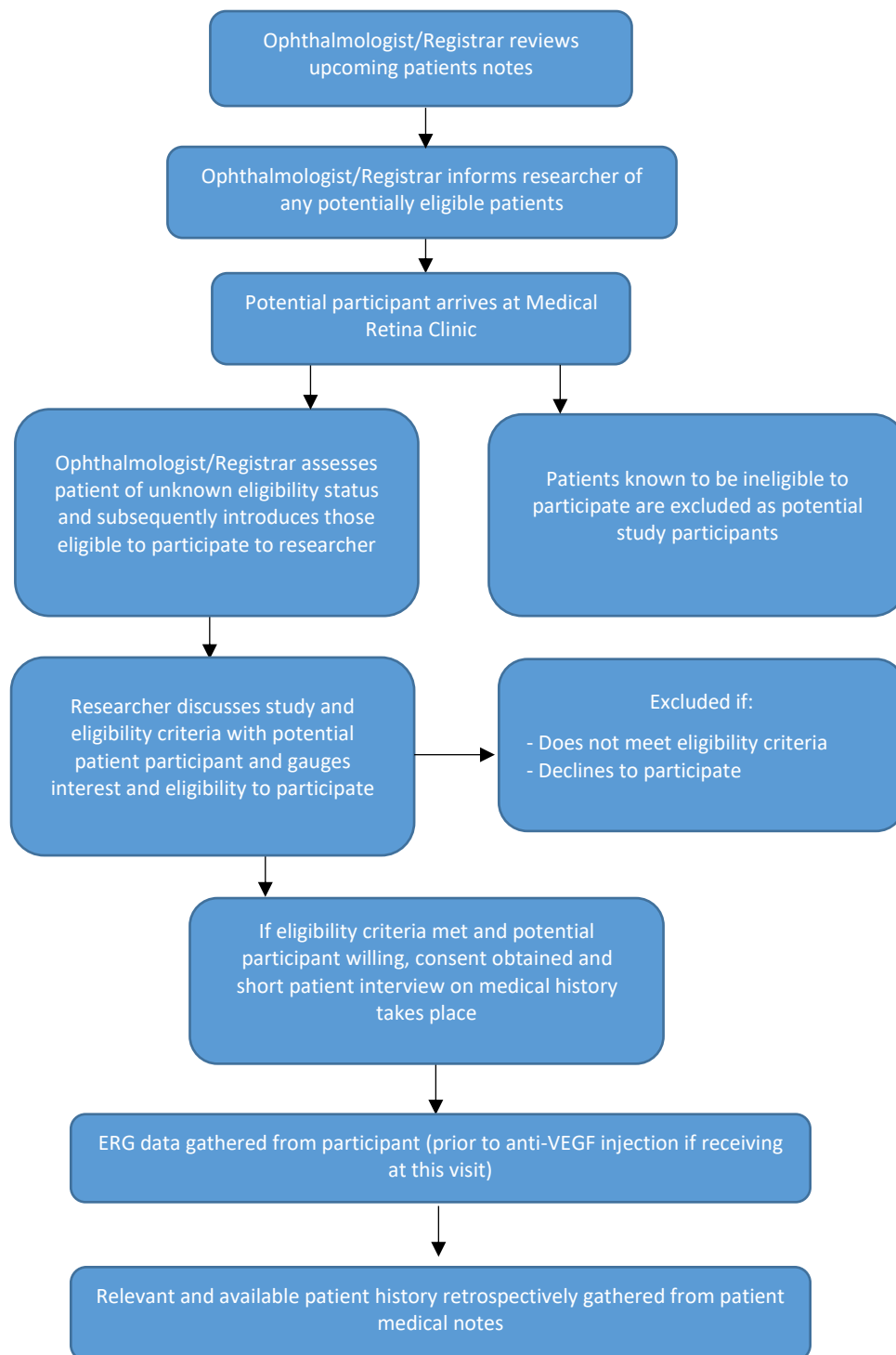


Figure 5.38. Recruitment pathway for patient participants within the Medical Retina clinic at Moorfields Eye Hospital, London.

Electroretinogram Equipment and Protocols

All ERGs were recorded using the RETeval® hand held ERG recording device (LKC Technologies). Details of the device are provided in Section 4.1. Skin sensor electrodes were applied beneath the lower lid of each eye, 2 mm below the lower lid margin (as recommended by the manufacturer and further described in Section 4.2.2). The RETeval® device does not require pupil dilation, as the intensity of the flashes is modified to maintain a constant retinal illuminance, however most participants were dilated as part of their routine clinical visit.

The ERG protocols evaluated using the RETeval® device included the following:

1. A transient cone response (equivalent to the International Society for Clinical Electrophysiology of Vision Standards: (ISCEV) light adapted 3 ERG [McCulloch et al., 2015])
2. A PhNR response, in which a red flash (1.5 cd.s.m^{-2}) flash stimulus was presented, on a blue rod suppressing background
3. A 30Hz flicker ERG (equivalent to the International Society for Clinical Electrophysiology of Vision Standards: (ISCEV) light-adapted 30 Hz flicker ERG (McCulloch et al., 2015))

Details of these protocols and the signal analysis were as described in Section 4.2.2.

Statistical Analysis

Statistical analysis was conducted in the same way as in study 1 (see Section 5.2.1) with one exception. Data were not available in regards to this study cohort's time since commencing treatment and thus this aspect of the analysis was not performed.

5.3.3. Results

Data were collected from 25 individuals; the right eye was analysed in cases of bilateral nAMD. Although 25 patients were examined, only 20 produced reliable recordings, and another was excluded because of incomplete injection data leaving $n = 19$. Of these 19 participants 2 declined to provide transient cone or 30 Hz flicker ERG data and one participant provided particularly noisy transient cone ERG data, resulting in exclusion of those data. This left a sample size of $n = 16$ for the transient cone ERG, and $n = 17$ for the 30 Hz flicker ERG. Of the 19 participants 8 were male and 11 were female.

Median (IQR) age of the participants was 81 (71, 87). The eldest participant was 96 and the youngest was 61. Median (IQR) number of prior anti-VEGF injections was 6 (2, 14). The minimum number of injections was 1 and the maximum was 60. Ten participants solely received aflibercept (Eylea®), 2 solely received ranibizumab (Lucentis®). The number of participants converting from ranibizumab to aflibercept was 6, and history of type of anti-VEGF therapy was unavailable for one participant. The visual acuity of the participants ranged from 6/6 to 1/60 in eyes being treated for nAMD and 6/5 to hand movements in eyes not being treated for nAMD. This information is summarised in Appendix 2.

Interocular analysis

Seven participants had unilateral nAMD and were suitable for this analysis. Median (IQR) age of the participants was 78 (65, 83) years. The eldest participant was 90 years old and the youngest was 62. Median (IQR) number of prior anti-VEGF injections was 6 (5, 12). The minimum number of injections was 3 and the maximum was 60. Sample data from the treated and untreated eyes of one participant are shown in Figure 5.39. It can be seen that there was no obvious difference in the amplitude and time to peak values of the treated and untreated eyes in the case of each parameter. Again, data were not normally distributed, hence the non-parametric Wilcoxon Signed Rank test was performed to determine whether there were any significant differences in average ERG parameters between treated and untreated eyes (see Table 5.11). No parameters were found to be significantly difference between eyes.

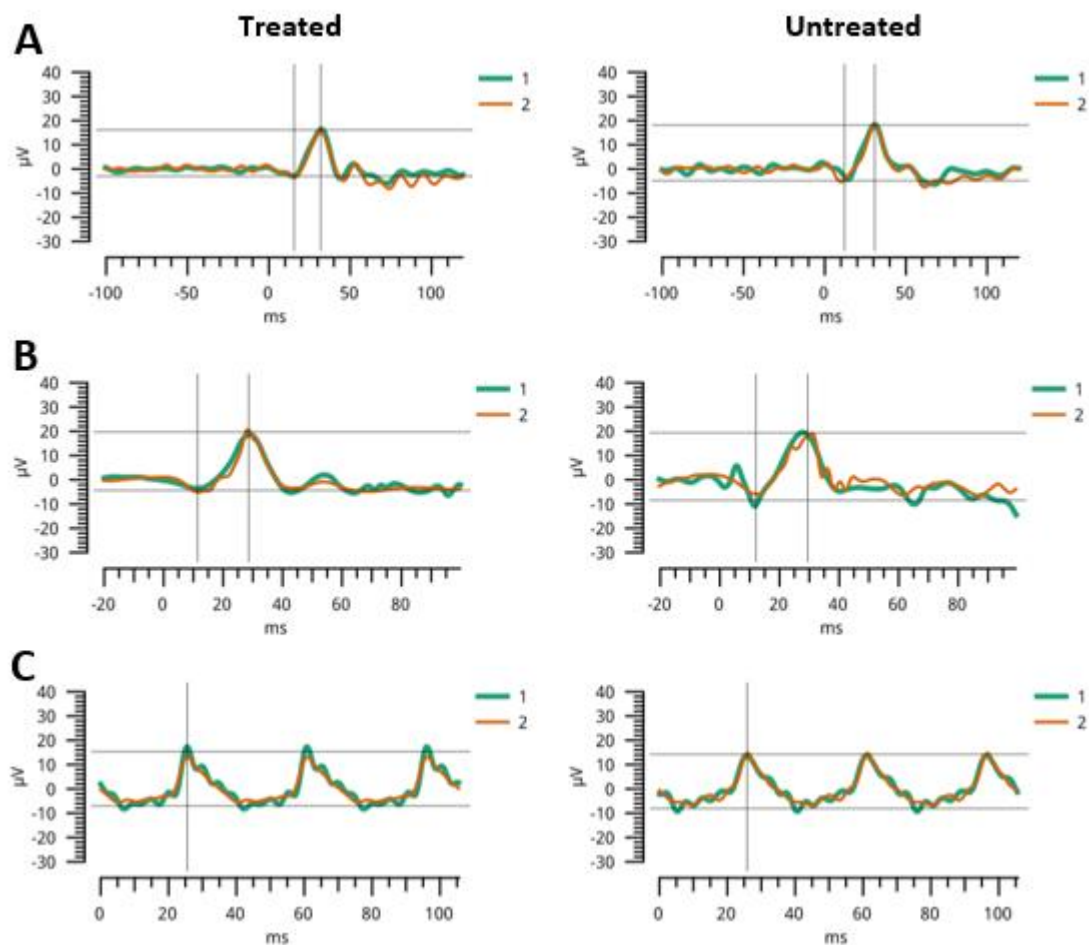


Figure 5.39. Sample RETeval® ERG data from the treated and untreated eyes of participant MEH40. This participant had received 6 anti-VEGF injections at point of assessment. A, PhNR ERG waveforms; B, ISCEV Transient cone 3 ERG waveforms; C, 30 Hz flicker ERG waveforms. Anti-VEGF treated eye data shown to the left, untreated eye data shown to the right. The protocol was run twice and the averaged traces for the first (1) and second (2) results are identified in green and orange respectively.

ERG Parameter			Median (IQR)		p	Bonferroni corrected value
			Treated	Not Treated		
PhNR	a-wave	Amp (μV)	-3.80 (-4.55, -2.90)	-4.60 (-5.80, -3.85)	0.23	1.00
		TtP (ms)	13.10 (12.95, 14.55)	13.10 (12.85, 14.95)	0.74	1.00
	b-wave	Amp (μV)	16.90 (15.65, 24.15)	21.00 (19.35, 22.25)	0.50	1.00
		TtP (ms)	30.00 (29.60, 31.60)	30.50 (28.85, 30.90)	0.06	0.76
	PhNR	Amp (μV)	-3.30 (-4.15, -2.85)	-4.90 (-5.85, -3.40)	0.24	1.00
		TtP (ms)	73.10 (71.70, 78.25)	70.40 (69.05, 74.55)	0.18	1.00
Transient Cone	a-wave	Amp (μV)	-6.10 (-7.80, -4.40)	-7.15 (-7.78, -5.03)	0.89	1.00
		TtP (ms)	12.05 (11.53, 13.78)	12.90 (12.30, 14.10)	0.46	1.00
	b-wave	Amp (μV)	22.85 (19.43, 29.28)	29.75 (28.28, 30.85)	0.17	1.00
		TtP (ms)	29.50 (28.85, 30.60)	29.50 (28.98, 30.40)	0.67	1.00
30 Hz Flicker		Amp (μV)	19.05 (14.75, 22.90)	22.10 (20.83, 22.70)	0.92	1.00
		TtP (ms)	25.70 (25.53, 27.75)	26.30 (25.43, 27.33)	0.60	1.00

Table 5.11. Results for the Wilcoxon Signed Rank Test comparing ERG parameters of the treated and untreated eyes of 7 patients using the RETeval® protocol. Parameters where significant difference between treated and non-treated eyes was observed are highlighted with an asterisk ().*

Between participant analysis

Again, an assessment of age vs number of anti-VEGF injections was carried out prior to the between participant analysis. There was no significant correlation between participant age and the number of injections they had received ($p = 0.32$, $\rho = 0.24$, $CI = -0.28 - 0.62$, see Figure 5.40).

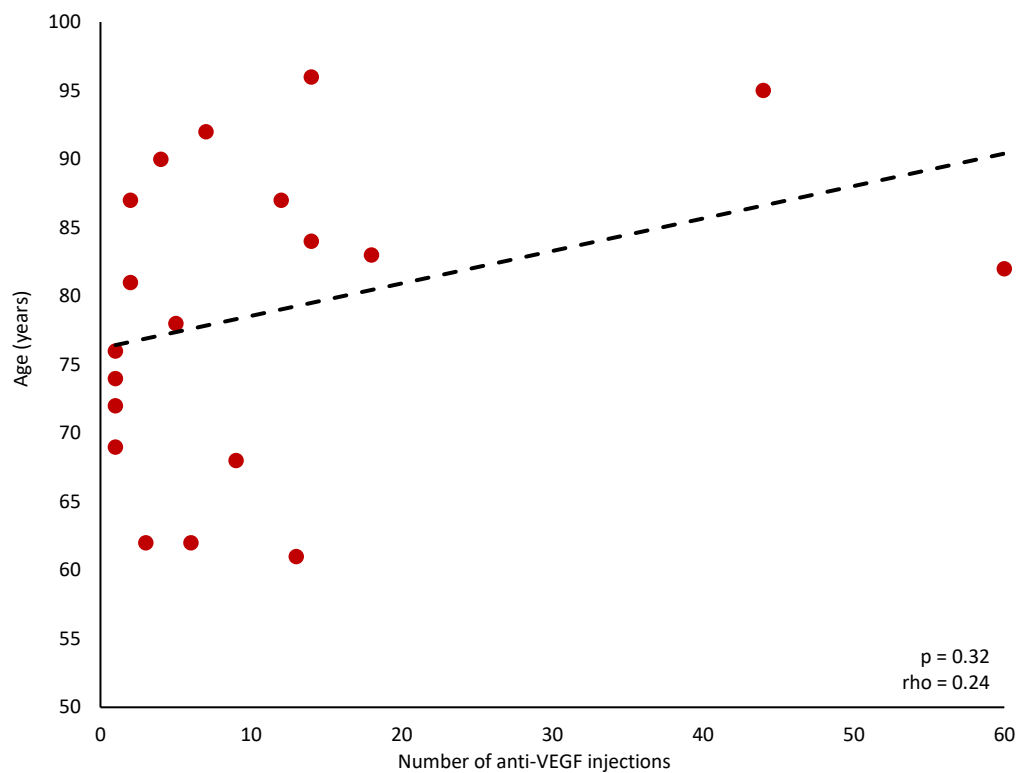
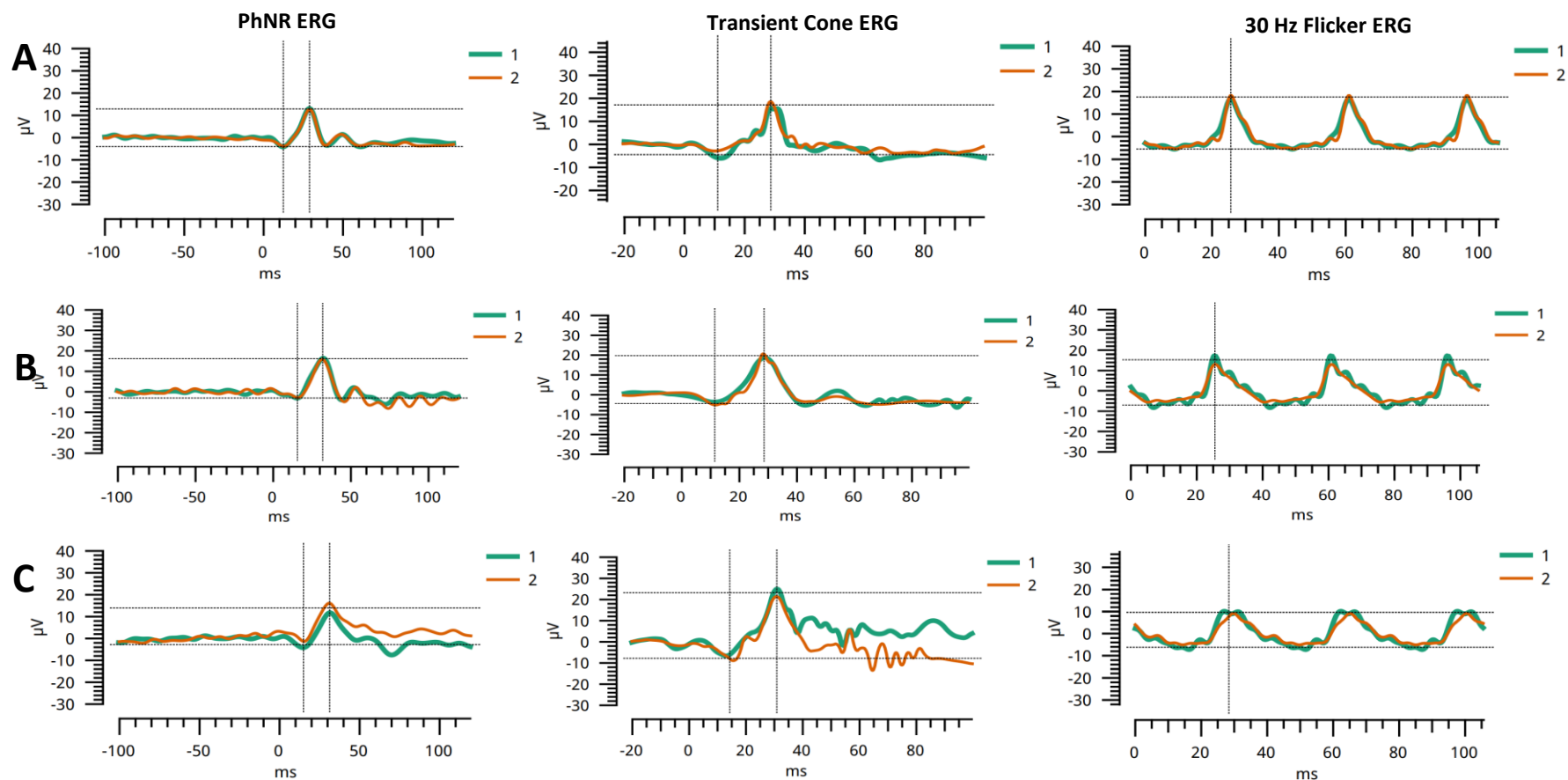


Figure 5.40. Scatter plot showing relationship between participant age and number of anti-VEGF injections in study 2. Results of the Spearman's correlation analysis are also shown. No significant correlation between number of anti-VEGF injections and participant age was observed.

Figure 5.41 shows ERG responses from participants receiving various numbers of anti-VEGF injections. A clear response is shown for all three protocols in these sample data. However, note that the PhNR response is minimal and dips only marginally below baseline. Little discernible difference is observed between responses despite the different numbers of anti-VEGF injections received.

Scatter plots were constructed to investigate the relationship between each ERG parameter and the total number of injections received (Figures 5.42 and 5.43). Two participants are excluded from the transient cone and 30 Hz flicker ERG results as they elected not to undergo transient cone or flicker ERG tests and another produced data that proved too noisy to analyse in these protocols (though the PhNR data produced was usable). There was a trend across all data towards reducing amplitudes and increasing time to peak values with increasing number of injections. However, this was only statistically significant for the time to peak of the a-wave in the PhNR ERG protocol ($p = 0.01$, $\rho = 0.587$, $CI = 0.13 - 1.05$; see Table 5.12), and this did not retain significance after Bonferroni correction. Data from this parameter subsequently underwent linear regression analysis (see Figure 5.44). The regression model assessing the relationship between total number of anti-VEGF injections and PhNR a-wave time to peak was shown to not statistically significantly predict the outcome variable ($p = 0.21$, $R^2 = 0.09$).



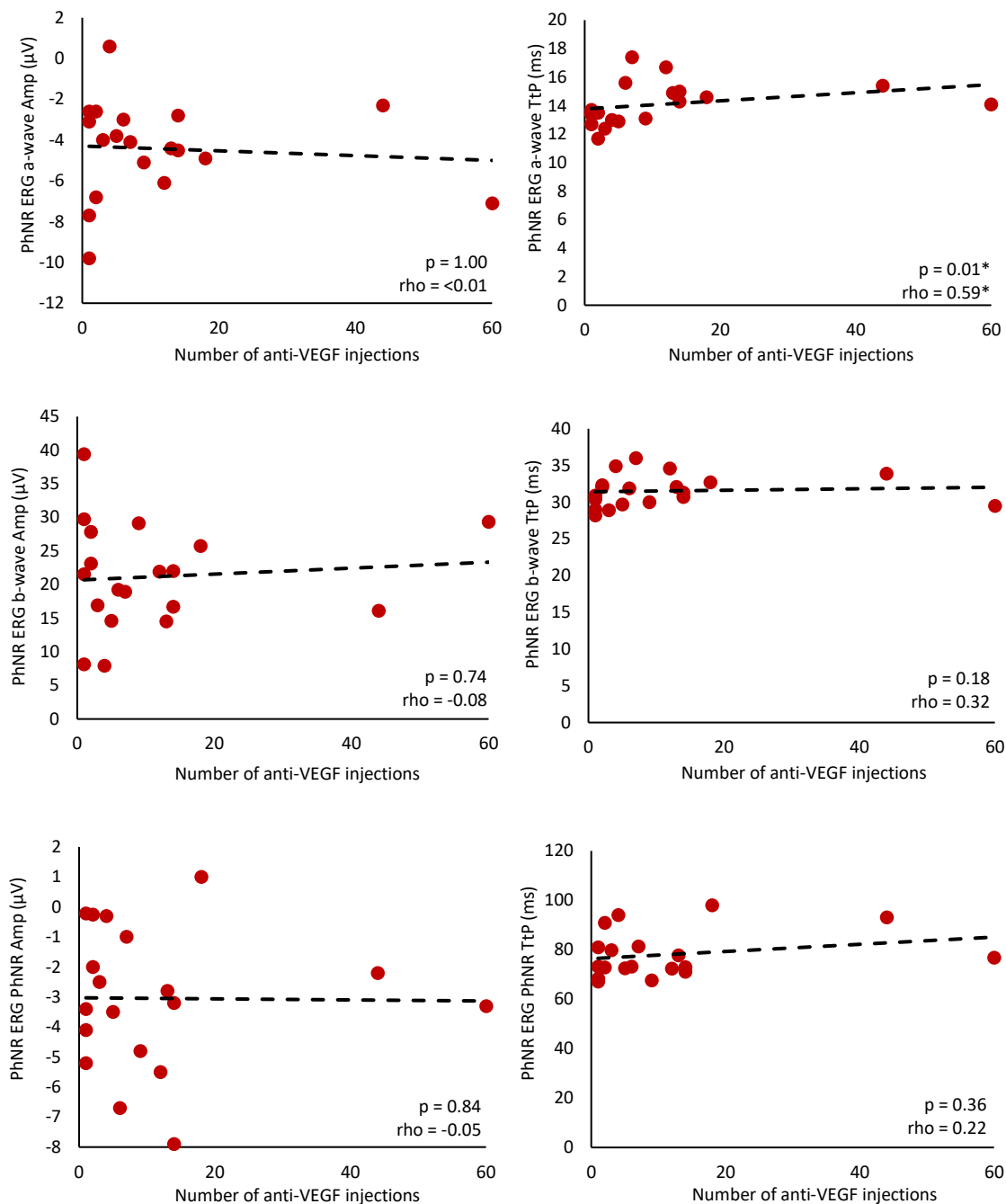


Figure 5.42. Scatter plots showing relationship between RETeval® PhNR ERG parameters and number of injections. A, PhNR a-wave amplitude; B, PhNR a-wave time to peak; C, PhNR b-wave amplitude; D, PhNR b-wave time to peak; E, PhNR amplitude; F, PhNR time to peak. Results of Spearman's correlation analysis are also shown. Relationships showing a statistically significant correlation are indicated with an asterisk ().*

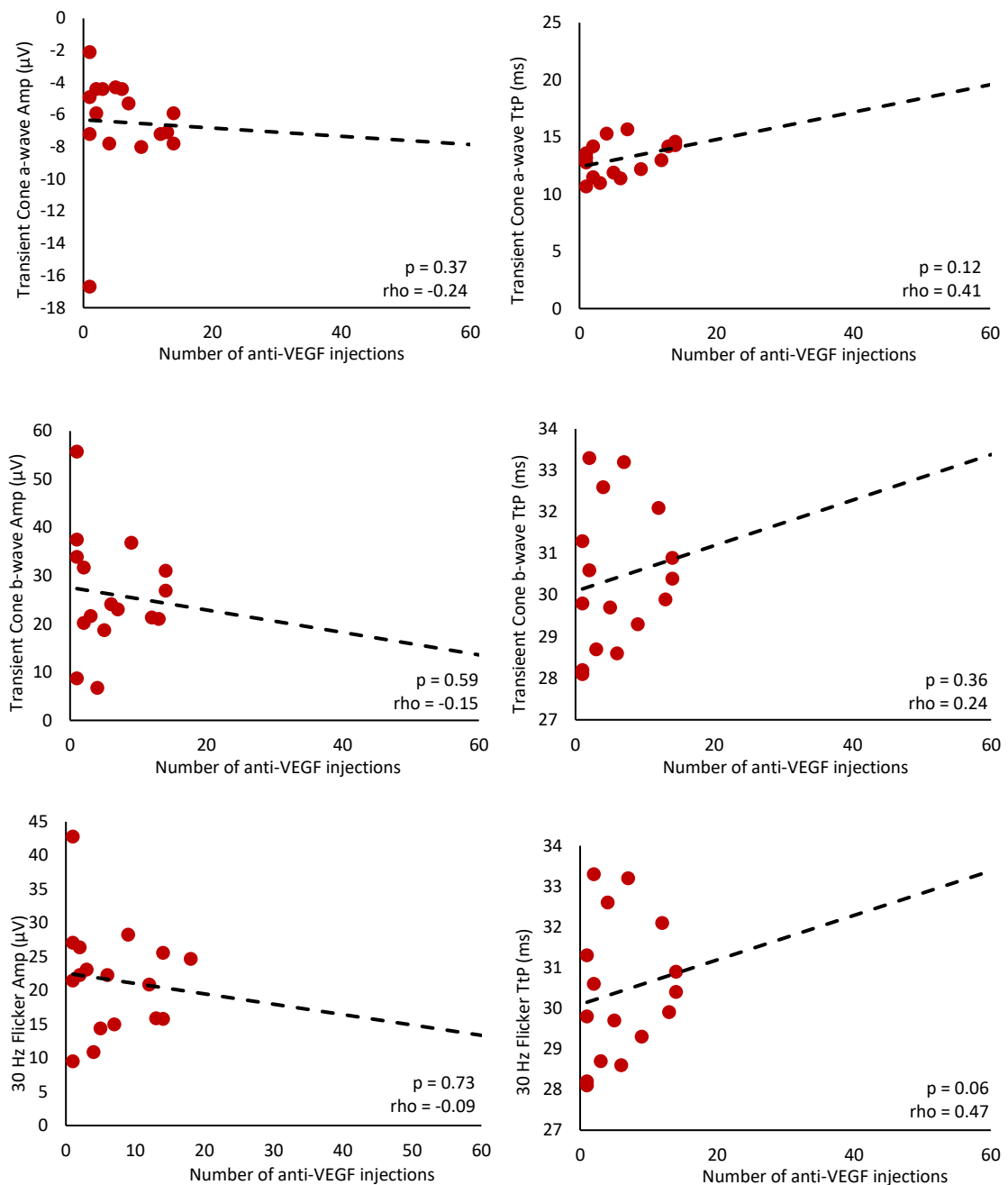


Figure 5.43. Scatter plots showing relationship between RETeval® Transient Cone and 30 Hz flicker ERG parameters and number of injections. A, Transient cone a-wave Amplitude; B, Transient cone a-wave Time to Peak; C, Transient cone b-wave Amplitude; D, Transient cone b-wave Time to Peak; E, 30 Hz flicker amplitude; F, 30 Hz flicker time to peak. Results of Spearman's correlation analysis are also shown. There was no significant correlation between number of anti-VEGF injections and any ERG parameters in this part of the dataset.

ERG Parameter			Median (IQR)	Spearman's Correlation				
				Number of anti-VEGF injections				
				Data Included (n)	p	Bonferroni corrected value	Correlation Coefficient (rho)	Confidence Intervals
PhNR	a-wave	Amp (μV)	-4.10 (-2.90, -5.60)	19	1.00	1.00	<0.01	-0.58 – 0.51
		TtP (ms)	13.70 (13.05, 14.95)	19	0.01*	0.12	0.59*	0.28 – 0.72
	b-wave	Amp (μV)	21.50 (16.40, 26.75)	19	0.74	1.00	-0.08	-0.56 – 0.51
		TtP (ms)	31.30 (29.85, 32.50)	19	0.18	1.00	0.32	-0.20 – 0.67
	PhNR	Amp (μV)	-3.20 (-1.50, -4.45)	19	0.84	1.00	-0.05	-0.55 – 0.42
		TtP (ms)	73.10 (72.35, 81.10)	19	0.36	1.00	0.22	-0.31 – 0.65
Transient Cone	a-wave	Amp (μV)	-5.90 (-4.40, -7.35)	16	0.37	1.00	-0.24	-0.70 – 0.28
		TtP (ms)	13.10 (11.80, 14.23)	16	0.12	1.00	0.41	-0.01 – 0.73
	b-wave	Amp (μV)	23.55 (20.80, 32.25)	16	0.59	1.00	-0.15	-0.68 – 0.51
		TtP (ms)	30.15 (29.15, 31.50)	16	0.36	1.00	0.24	-0.24 – 0.66
30 Hz Flicker		Amp (μV)	22.30 (15.80, 25.60)	17	0.73	1.00	-0.09	-0.61 – 0.44
		TtP (ms)	27.40 (25.50, 28.40)	17	0.06	0.72	0.47	-0.07 – 0.81

Table 5.12. Statistics describing the results of the Spearman's correlation analysis between total number of anti-VEGF injections and RETeval® ERG parameters. Statistically significant correlations are marked with an asterisk ()*

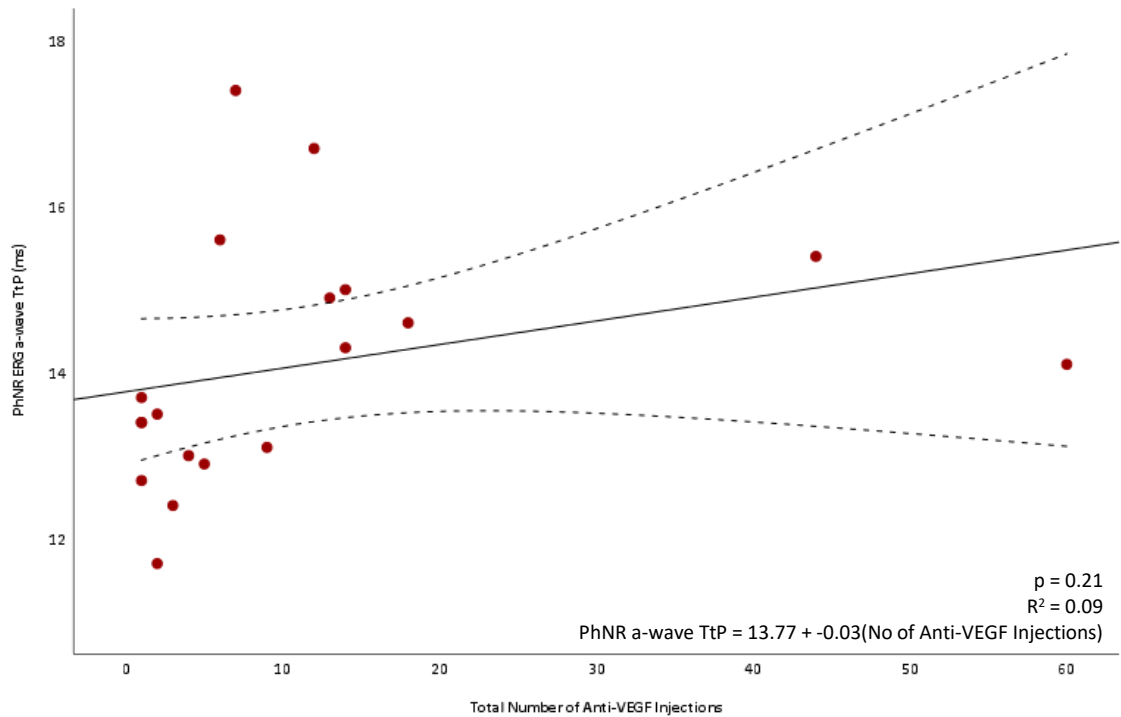


Figure 5.44. Scatter plot describing the linear regression of the relationship between PhNR a-wave TtP and Total Number of anti-VEGF injections. The regression line is indicated by the solid black line, 95% confidence intervals of the regression line are denoted by dashed black lines.

5.3.4. Discussion

Results from this second study show only one ERG parameter to have any correlation with number of anti-VEGF injections. This was a strong correlation (PhNR a-wave time to peak, $p = 0.01$, $\rho = 0.59$, $CI = 0.28 - 0.72$), although significance did not remain after Bonferroni correction, and confidence intervals for ρ were wide and later, linear regression analysis was shown not to statistically significantly predict the outcome. The data provided by the second study did not show a correlation between PhNR amplitude or any flicker or transient cone ERG parameters and number of injections. In this study there was also a statistically significant difference noted between treated and untreated eyes for three ERG parameters; PhNR b-wave time to peak, PhNR amplitude, transient cone b-wave amplitude. Again, this did not remain statistically significant after Bonferroni correction. The finding that the PhNR amplitude differed significantly between eyes for the intraocular comparison was somewhat supportive of previously discussed evidence that RGC function may be adversely affected by increasing cumulative dose of anti-VEGF intravitreal injections. However, there is some evidence to suggest that treatment effects are also present in

fellow untreated eyes of patients undergoing unioocular nAMD treatment (Avery et al., 2006; Karaca et al., 2003; Hanhart et al., 2014; Acharya et al., 2013). It may, therefore, also be possible that the difference between eyes is more indicative of the effect of disease progression on retinal function in the eye with nAMD, rather than a disease toxicity effect.

The use of the RETeval® device in this second study aimed to increase the sample size. However, 5 of the 25 participants recruited produced no usable data above the level of background noise using this device. As discussed in Section 4.3, the RETeval® produces responses of a smaller amplitude as a result of the skin placement of the active electrode. This is apparent in the median amplitude of the PhNR in study 2, which was substantially smaller than the median amplitude of the PhNR recorded to an equivalent stimulus intensity in study 1. The data in chapter 4 demonstrated that this difference in amplitude between the RETeval® and a desktop device was statistically significant. This is likely to have had a deleterious effect on the signal to noise ratio of the recorded responses. It is possible that the smaller than desired sample size, in combination with this increased level of measurement noise, may have masked subtle trends in the data. Other groups have similarly reported that the RETeval® device should be used with caution to assess the PhNR in cases of attenuated responses or elevated noise levels, due to the small response amplitude (Tang et al., 2018).

5.3.5. Limitations

Unfortunately, it was not possible to achieve the sample size originally foreseen for this aspect of the study. Although the RETeval® device and the associated electrodes provide a shorter total testing time, the process of obtaining consent, preparation and ERG recordings still takes around 15 minutes in total. This additional time required meant that due to a high volume of patients, time pressures in the medical retina clinic limited how many patients could be approached as potential participants, whilst clinic logistics meant that space was not always available for data collection. For the same reasons, additional widefield OCT scans were not able to be recorded for these participants. In addition, the data gathered from this study cannot be added to that from study 1 as the methods for recording these ERGs are not interchangeable (as previously discussed in Chapter 4, Section 4.2.4). The sample size of the interocular analysis ($n = 7$) would only enable the detection of a standardised difference of 1.7 (at

$p < 0.05$, power 80%, according to the Bland and Altman sample size nomogram, Bland, 2015). For the between participant analysis, the sample size was sufficient to detect a correlation coefficient of 0.60, with 80% power, at an alpha (two-tailed) of 0.05 (<http://www.sample-size.net/correlation-sample-size/>). As in study 1, the small sample size has resulted in wide confidence intervals for the significant parameter, making interpretation of the magnitude of rho difficult. The linear regression analysis similarly showed wide confidence intervals. As in study 1, the possibility of the influence of the participant's nAMD itself is also a possible confounding factor.

However, despite these limitations, there was some indication of a possible effect of treatment on the ERG, including a significant difference in PhNR amplitude between treated and untreated eyes of patients with unilateral nAMD.

5.4. Conclusion

Significant associations between number of anti-VEGF injections and time since first anti-VEGF injection and some ERG and OCT parameters were found in both studies, in particular those relating to RGCs. This was consistent with some of the evidence in Chapter 2, such as the reported detrimental effects of anti-VEGF therapy on cones (Kurihara et al., 2012) and RGCs (Nishijima et al., 2007) in animal studies. However, there were also many ERG and OCT parameters that showed no statistically significant correlations with anti-VEGF interventions, again consistent with the majority of studies in Chapter 2. Thus, this study concludes that there may possibly be a decline in some aspects of pan retinal photopic retinal function and structure with increasing number of injections and/or time since the first anti-VEGF injection, but, that there is insufficient power in the data collected to be certain of this. Future work is required on a larger sample of participants to further investigate these findings. Given the substantial levels of between participant variability in ERG response amplitudes (Fortune et al., 2004; Mortlock et al., 2010), a study investigating the changes in ERG parameters over time may be more sensitive to subtle changes.

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Chapter 6. An Overview of Findings and Future Work

6.1. Overview of findings

The aim of this work was to assess peripheral retinal structural and functional changes in persons being treated for nAMD. Thus far there is little evidence examining long term use of this type of intervention despite its continuing approval for use in retinal conditions affecting younger patients and an ageing population, therefore potentially leading to longer term usage. The existing literature provides insufficient knowledge to either support or reject the hypothesis that outer and inner retinal function and structure is affected by the treatment. This thesis identified a gap in the literature surrounding both retinal structure and function in nAMD (for example, the observation of insufficient literature examining the effects of multiple anti-VEGF injections and the cumulative effect of this on peripheral retinal structure and function, see sections 2.2 and 2.3), and subsequently addressed these factors – adding to the existing knowledge base. The knowledge base was also enhanced by work evaluating the repeatability of responses recorded using a new hand held full-field ERG recording device, and their agreement with ERGs recorded using a conventional desk top based system. The findings of the studies may prove helpful to clinicians in monitoring retinal function during anti-VEGF treatment and for other retinal function monitoring purposes.

Chapter 2 provided a systematic literature review of the evidence concerning peripheral retinal structure and function in animals and humans receiving anti-VEGF drugs. Most studies found that anti-VEGF therapy does not detrimentally affect retinal function (e.g. Maturi et al., 2006; Stahl et al., 2009; Skaat et al., 2011; Inan et al., 2007; Guo et al., 2010; Lueke et al., 2006; Zayit-Soudry et al., 2010; Zayit-Soudry., 2011; Shahar et al., 2006; Luke et al., 2009). However, there was some evidence that parts of the retina are affected by this treatment – such as reduction in flicker ERG parameters (Terauchi et al., 2019), reduced response in the outer parts of the multifocal ERG waveforms (Almedia et al., 2015; Feigl et al., 2007), and a- and/or b-wave amplitude and prolongation of time to peak values of these parameters in several studies (Heiduschka et al., 2008; Comyn et al., 2014; Pedersen et al., 2010). On some occasions, the reduction in ERG response was observed to recover over time after treatment, at variable rates (Heiduschka et al., 2008; Pedersen et al., 2010). It has been noted that in some cases similar patterns of reduction and recovery have been seen in

sham injection and anti-VEGF treatment groups – suggesting the method of intervention rather than the action of anti-VEGF therapy could be responsible for this finding (Heiduschka et al., 2008). Yagura et al (2016) suggested that these transient effects could be due to intravitreal injection rather than the introduced drugs themselves, for example by causing a transient rise in IOP. Another important finding of this review was the abundance of studies with only a short follow up time and/or single dose treatments. On a related note, the majority of studies reporting no significant effect in function belonged in this collection (e.g. Macky and Mahgoub, 2012). Those studies that followed up for longer periods after several injections were those which noted potential retinotoxic effect – suggesting the manifestation of this effect over time and/or after repeated treatments (Pedersen et al., 2010; Comyn et al., 2014).

Structurally, the evidence was also mixed, with some indications of a reduction in the RNFL thickness on OCT (Parlak et al., 2014; Enders et al., 2017; Kopic et al., 2017; Valverde-Megias et al., 2019; Yang et al., 2017; Beck et al., 2016), although numerous studies found no effect (Zucchiatti et al., 2017; Shin et al., 2014; Horseley et al., 2010; Sobaci et al; 2013; Demirel et al., 2015; Entezari., 2014; El-Ashry et al., 2015). The suggestion that the method of delivery rather than the mechanism of action of the anti-VEGF therapy is to blame for this observed effect was provided as a possible explanation for this aspect of the evidence also (Enders et al., 2017). It was also suggested that the resolution of macular oedema and consequent reduction in retinal thickness may be confounding the reported results (Yang et al., 2017). Whilst most studies of structural changes in humans evaluated the inner retinal layers, the effect of treatment on RPE atrophy progression has also been investigated, with mixed findings (Kim et al., 2016 found increasing severity correlated with increasing number of injections, whilst Sitnilska et al., 2019, attributed severity of atrophy to duration of AMD rather than treatment effect). The review of histology of retinal cells using light microscopy has suggested very little adverse structural change as a result of anti-VEGF treatment (Kim et al., 2008; Lu et al., 2014; Avci et al., 2009). However, immunohistochemical investigation with electron microscopy has shown evidence of cell damage in mitochondria, and cell apoptosis (Myers et al., 2012; Inan et al., 2007; Julien et al., 2014, Romano et al., 2012). These changes, amongst other retinal

structural interruptions (Karawya et al., 2008; Kaempf et al., 2008; Cam et al., 2017; Ramon et al., 2018; Sahiner et al., 2018) suggest potential progression of damage with prolonged anti-VEGF treatment.

This review highlighted the key finding of a lack of studies with long term follow up periods or evaluating the effect of repeated treatment and the subsequent importance of the development of these in future work, particularly in light of the studies reporting reduction in retinal structure/function over longer follow up periods.

In **Chapter 3** the protocol for the main studies in this work were developed. Good tolerance of the 41 Hz flicker and ISCEV standard transient cone ERGs was found, although some participants struggled with the PhNR intensity response ERG, particularly after the stimulus intensity increased beyond 0.84 cd.s.m^{-2} . After the preliminary pilot study it was necessary to exclude the bleach recovery ERG from the protocol, as most participants were unable to tolerate the full-field bleaching light at intensities high enough to bleach a substantial proportion of photopigment. The flicker ERG recorded to a stimulus of 41 Hz temporal frequency was retained as part of the protocol.

Repeatability was also explored by recording from a number of participants on two separate visits. As previously reported in many studies, measures of time to peak were consistently more repeatable than those of amplitude (Pescosolido et al., 2014; Fortune et al., 2004; Strain et al., 2001). Values of CoR in this protocol development chapter were largely of the same or lower magnitude than those found in previous human studies (Tang et al., 2014; Mortlock et al., 2010).

There was no significant difference between the results of the two visits in all but one parameter, indicating no bias between visits due to factors such as learning effect. The sole between-visit significant difference was found in the PhNR time to peak at 0.84 cd.s.m^{-2} (though this was not sustained after Bonferroni correction). There was also a general trend towards an increase in CoR expressed as a percentage of the mean value as stimulus luminance increased above 0.84 cd.s.m^{-2} . This increased variability is likely to be explained by the struggle to control blink reflexes at higher stimulus intensities. There was also no significant difference in between eye comparisons after Bonferroni correction was applied.

Chapter 4 explored the repeatability of and variation between the gold-standard desktop Espion ERG recording system and the newly developed handheld RETeval® ERG technology. The two devices displayed similar between participant variation and between session repeatability. Again, time to peak parameters were found to be more repeatable than amplitude measures, showing less between session variability on both devices. Reduced amplitudes were recorded using the skin electrodes of the RETeval® compared to the DTL fibre electrodes of Espion™/ColorDome System, as has been reported in other literature comparing ERG results with various electrode type (France, 1984; Esakowitz, Kriss, Shawkat, 1993; Kriss, 1994; Bradshaw, Hansen, Fulton, 2004; Mortlock et al., 2010). It was less expected to find that there were also significant differences between devices in the majority of response timings, despite comparable recording protocols. This was attributed to differences in response filtering, measurement algorithms, or stimulus waveform. This implies that both devices are valuable in disease detection but subcomponent waveform measurements may differ between devices – suggesting that either device may be used for this purpose but they should not be used interchangeably.

The study also reported modest but statistically significant changes in the waveform generated by flickering stimuli when frequency was varied between 28, 30 and 41 Hz. This is important to note as it suggests the 28 Hz frequency used in the RETeval® protocol is not directly comparable with the 30 Hz protocol recommended by ISCEV (McCullough et al., 2015). Similar waveforms are produced but device type must be kept consistent in order to make comparisons between tests or visits as the devices cannot be used interchangeably.

Additionally, the cruciality of electrode positioning in obtaining repeatable ERG results was shown by the second study of this chapter. Manufacturer guidelines are recommended to be followed for electrode placement in order to minimise between-subject and between-session variability in ERG amplitudes. However, inferior displacement of skin electrodes provided accurate time to first peak ERG measurements and can be utilised where needed – such as for patients who are either unable or unwilling to participate in ERG recordings with recommended electrode placement. Given the difference in response amplitude with electrode position, it is

important that consistency is used in electrode placement within subject when monitoring changes in the ERG over time.

Chapter 5 used the ERG protocol developed in Chapter 3 along with central retinal OCT scans to assess structural and functional changes in the retina in persons undergoing anti-VEGF therapy. A key finding was the significant correlation across stimulus intensities between reduced PhNR amplitude and increasing number of injections, whilst other ERG parameters relating to the ISCEV transient cone and 41 Hz flicker ERGs remained largely unaffected by the number of injections. Whilst the correlation between PhNR amplitude and number of injections was consistent across a range of stimulus intensities, it is important to note that correlation does not mean causation. Candidate explanations explored for this relationship, in addition to the possibility of anti-VEGF toxicity of RGCs, included the contribution of the macular region affected by AMD to the PhNR response (approximately 25% of the response is driven by the central retina according to Morny et al., 2015), and that the AMD disease process may have been affecting the RGC function of the peripheral retina (for example, it has been reported that drusen are present in the mid and far periphery of most eyes with AMD [OPERA group et al., 2017; Lengyel et al., 2015]). However, it was intriguing to note that the PhNR seemed to be differentially affected, given the known outer retinal locus of early AMD pathology (Ambati and Fowler, 2012; Ambati et al., 2003). It was also interesting to note that there was no significance difference in PhNR amplitude between eyes in people receiving unilateral treatment for the majority of ERG parameters. However, despite the wide use of a contralateral untreated eye as a control in studies of anti VEGF toxicity (Beck et al., 2016; Shin et al., 2014; Kurt et al., 2018; Valverde-Megias et al., 2019; Macky and Mahgoub, 2012; Skaat et al., 2011; Guo et al., 2010; Sakurai et al., 2009) there is evidence to suggest that there may be an inter-ocular transfer of the drug which may confound the assumption of the fellow eye being unaffected by the treatment (Avery et al., 2006; Karaca et al., 2003; Hanhart et al., 2014; Acharya et al., 2013).

When considering the OCT data analysis, only the thickness of the photoreceptor outer segment layer in the nasal retina showed a significant (and strong) negative correlation with number of injections. Both the temporal and the nasal photoreceptor outer segment layer thicknesses were also negatively correlated with the duration of

treatment. This suggested that an increasing number of anti-VEGF injections or duration of treatment may result in atrophy of the photoreceptor outer segments. This finding is novel, and to date no other studies have directly evaluated the structure of the photoreceptor layer in humans post anti-VEGF treatment in this location. There is some evidence from animal studies to suggest photoreceptor disruption after anti-VEGF treatment (Inan et al., 2007; Avci et al., 2009), and trials of anti-VEGF therapy have reported evidence of a dose-dependent relationship between anti-VEGF drug use and macular atrophy (Chakravarthy et al., 2011; Sadda et al., 2018). However, the majority of animal studies reviewed in Chapter 2 reported no evidence of structural damage to the outer retina (e.g. list Arraes et al., 2009; Bakri et al., 2006; Cheng et al., 2009; de Oliveira Dias et al., 2014; Guo et al., 2010; Iriyama et al., 2007; Januschowski et al., 2014; Kim et al., 2008; Lu et al., 2014; Manzano et al., 2006; Ozaki et al., 2000; Sari et al., 2006; Thaler et al., 2010; Zayit-Soudry et al., 2011). Although our findings suggest a relationship between treatment and photoreceptor outer segment damage, it is possible that in this location at the edge of the macula some of the thinning effects seen relate to atrophy caused by the AMD disease process itself.

A greater number of OCT layers appeared to be reduced in thickness in association with a longer overall duration of treatment, rather than the actual dose received. This may be due to the treatment itself or to progressive disease related atrophy. The RNFL (nasally) and the GCC complex (temporally) are two of the areas showing reduction in retinal thickness. These findings were consistent with some of the previous literature of negative effects of anti-VEGF on RGCs (Nishijima et al., 2007; Parlak et al., 2014; Enders et al., 2017; Kopic et al., 2017; Valverde-Megias et al., 2019; Yang et al., 2017; Beck et al., 2016), and with the observed relationship between PhNR amplitude and dose of anti-VEGF received. However, previous OCT evidence has largely failed to find an association between retinal thickness measurements and anti-VEGF treatment in humans. Again, it must be considered that confounding factors such as proximity of retinal disease features to OCT measures may have influenced these results, including the possible effect of treatment on subclinical outer retinal fluid. Future work is required on a larger sample of participants, using a wider field OCT assessing a point further from the macula to further investigate this interesting finding.

Whilst it was not possible to replicate the PhNR findings in a second group of participants tested using the RETeval® device, the small signal may have masked any subtle effect. Other groups have reported that the RETeval® device should be used with caution to assess the PhNR in cases of attenuated responses or elevated noise levels, due to the low response amplitude (Tang et al., 2018).

A key limitation to note with both studies in this chapter was that the sample size fell short of the level desired based on the preliminary calculations. Post hoc calculations presented in the discussion of Chapter 5 indicate that only a large effect size could be reliably detected with the final sample recruited. Furthermore, given the small sample size, confidence intervals for rho of the correlation analysis and B of the linear regression analysis were wide, and it was not possible to determine the magnitude of any effect with precision. This is discussed in detail in the discussion sections of chapter 5 (sections 5.2.4 and 5.3.5).

6.2. Conclusions and Clinical significance

Overall, the findings of this PhD provided some evidence of a dose-dependent relationship between increasing exposure to anti-VEGF drugs and RGC dysfunction assessed using the PhNR of the full-field ERG, as well as thinning of retinal layers assessed using OCT, especially the photoreceptor outer segments. This was not consistent across all analyses and across the two cross-sectional studies. This may be attributable, in part, to the sample size of this study, which was powered to detect only a large treatment effect. Therefore, whilst the results suggest that long term anti-VEGF treatment does not cause a large negative effect on retinal structure or function, it does not eliminate the possibility of a smaller effect. This may be of concern to certain patient groups. For example, patients with a prior diagnosis of glaucoma who are about to embark on long term anti-VEGF treatment may wish to be aware of any risk of increased loss of RGC function, and patients who begin treatment at a younger age may also wish to be aware of the risk of cumulative damage over time due to their potentially longer treatment duration. Further work is needed to investigate smaller effect sizes, preferably using a longitudinal study design to reduce the impact of inter-individual variation in parameters. The possibility of an effect of disease progression on the outcome parameters also cannot be excluded.

6.3. Ideas for Further Work

Recruitment of a larger cohort of individuals to a longitudinal evaluation is needed to investigate this further. Ideally this would be done before participants start any anti-VEGF treatment, and with a robust method of data collection to ensure transparency. Subtraction of the macular component from all ERGs, and the evaluation of a location further from the macula in the OCT analysis, along with automated segmentation of images would further enhance a future study.

In order to improve the sample size and to increase statistical power, it would be desirable to recruit from a range of hospital sites. Furthermore, by building these tests into the natural running order of the medical retina clinic recruitment may be further enhanced. Given the findings of a low response amplitude using the RETeval® in Chapter 5, and the similar findings of other groups to suggest that the skin sensor electrodes produce too small a PhNR signal for recording from this patient cohort (Tang et al., 2018), it would be recommended to use DTL fibre electrodes or a similar conjunctival electrode with the RETeval® device in further study in order to optimise the ERG signal whilst maintaining device portability. An additional step for further studies would be to attempt to introduce an evaluation of scotopic function into the battery of tests. It may be necessary given the logistic limitations of the medical retina clinic for this to take place on a separate occasion, perhaps on a sub group of individuals willing to attend an additional appointment.

It is useful to give some consideration to the sample size which would be required for this proposed longitudinal study. Cohen's d is a measure of effect size between two means. It is also known as the standardised mean difference, and describes the difference in two means (e.g. within a group between timepoints, or between two groups), divided by the standard deviation of the sample. It has been suggested that, as a rule of thumb, a study should be powered to detect an effect size of 0.4 (Brysbaert, 2019). In a repeated measures study, an effect size of 0.4 (a small to medium effect; Sawilowsky, 2009) would mean that two thirds of participants showed the effect (in this case a significant change in ERG amplitude or OCT layer thickness attributable to anti-VEGF therapy). For a simple repeated measures study, using a paired t -test, this would require a sample of one group of 52 participants (to achieve $p < 0.05$, with a power of 80%; Brysbaert, 2019). However, it would be desirable to

include a control group to exclude the possibility that the changes in these structural and functional parameters were caused by confounding factors such as ageing or disease progression. We have already discussed the potential limitations of using the untreated eye of people with unilateral disease as a control group (i.e. the possibility of interocular transfer of the treatment effect). If the control group chosen was a group of individuals with advanced dry AMD, a sample of two groups with 100 participants per group would be required to perform a between groups t-test sufficient to detect an effect size of 0.4 (80% power, $p=0.05$).

Using the data presented chapter 5 to estimate standard deviation, it is possible to determine the smallest difference in mean which would be detectable using this approach. If we look at some of the parameters which showed a significant association with treatment dose or duration, for the PhNR recorded to a stimulus of 0.42 cd.s.m^{-2} ($SD = 9.7 \mu\text{V}$), the smallest detectable difference between groups would be $3.9 \mu\text{V}$, for the 3.35 cd.s.m^{-2} PhNR ($SD = 9.4 \mu\text{V}$) the smallest detectable difference would be $3.8 \mu\text{V}$, for the OCT nasal photoreceptor outer segment thickness ($SD = 9.45 \mu\text{m}$) the smallest detectable difference would be $3.8 \mu\text{m}$. These values indicate the difference in absolute PhNR amplitude or OCT thickness between groups which would be detectable. However, in the longitudinal study, the amplitude/time to peak/thickness would be normalised to the baseline measure for all parameters and it would be this normalised value which would be compared between groups. The SD of this normalised data would differ from the SD within groups quoted above, and it is not possible to derive from the cross-sectional data presented in this thesis a precise indication of the magnitude of parameter change which would correspond with an effect size of 0.4.

Some studies evaluated in Chapter 2 suggested the possibility that the intravitreal delivery of anti-VEGF therapy may be the source of the damage to retinal structures and function, rather than the drug itself. For example, this may cause a spike in IOP which causes retinal damage (de Vries et al., 2020). There was also a notable lack of immunohistochemical analysis in human eyes. It may be valuable for further work to take place to explore the effect of anti-VEGF therapy on cadaver eyes of deceased patients who had undergone anti-VEGF therapy in order to collect immunohistochemical data and compare this to the results found in animal studies.

Additionally, it would be valuable for this same immunohistochemical analysis to be performed on cadaver eyes of deceased patients who had received intravitreal injections of other agents. This would allow assessment of whether detrimental effects to the retina are present in all eyes with this mechanism of delivery OR if they are present in only eyes undergoing anti-VEGF therapy, as this may provide information on whether it is the drug or the delivery mechanism that is causing a detrimental effect. Finally, living patients undergoing intravitreal treatments of various drug types in ophthalmology clinics could be assessed for retinal functional and structural changes throughout their treatment – again, to assess whether it is the drug or the mechanism of delivery that may be causing reductions in ERG parameters and detrimental effects on retinal structure.

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Appendix 1 – Data extraction table for the Systematic Literature Review in Chapter 2

Study (Lead Author, Year)	Study Design	Objectives	Methods	Key Outcome Measures	Key Results	Key Sources of Bias
Almeida, 2015	Case Control Study	The evaluation of retinal morphology and function of patients with advanced AMD.	21 eyes with nAMD injected with 3 doses of ranibizumab (strength of dose not stated). Assessed at baseline, 30 days, 60 days and 90 days after injection. Compared to an age-matched control group (n = 21 eyes).	Multifocal ERG (45°) N1 and P1 parameters.	Peripheral area showed a significant reduction in the N1 amplitude at all follow up days, compared to controls. N1 implicit time was delayed at day 30 only. P1 amplitude was not significantly different to controls but P1 implicit time was significantly delayed at days 30 and 60.	Nil of note.
Arraes, 2009	Animal Study	To evaluate bevacizumab toxicity in retina and RPE in pigmented rabbit eyes by means of histological studies	Thirty eyes of fifteen rabbits were distributed into three groups: sham group (S), received a 0.1 ml BSS intravitreal injection (10 eyes);	Histology (retinal layer thickness and cell morphology)	No histological differences in neurosensorial retina or in retinal pigmented epithelium were found and layer thickness did not differ significantly between	No randomisation.

			group 1, received a 1.25 mg (0.1 ml) bevacizumab intravitreal injection (10 eyes); and group 2, received a 2.5 mg (0.1 ml) bevacizumab intravitreal injection (10 eyes). After 90 days, rabbits were sacrificed and a histological examination performed.		balanced saline solution-injected eyes and bevacizumab-injected eyes.	
Avci, 2009	Animal study	To evaluate quantitatively the apoptotic activity after intravitreal injections of pegaptanib sodium and bevacizumab in the rabbit retina.	48 rabbits were assigned to 11 study groups (n = 4 in each group) and one control group (n = 2) for sham control and one saline-injection control group (n = 2). Bevacizumab (0.25, 0.625, 1.25, and 2.5 mg) and pegaptanib sodium (0.15, 0.3, and	Histology: hematoxylin and eosin (H&E) staining, caspase-3 and -9 staining, and TUNEL assay	No toxicity related adverse effects were observed by clinical and routine light microscopic assessment. Both drugs caused a significant increase in apoptotic activity in rabbit photoreceptor cells compared to controls. Bevacizumab, but not pegaptanib, caused	No randomisation.

			0.6 mg) were injected intravitreally. The eyes were enucleated at day 14, and 3 months after a single injection or at 3 months, with 1 injection in each of the 3 months.		increasing apoptotic activity at higher doses.	
Bakri, 2006	Animal study	To describe histologic changes after intravitreal bevacizumab in a pigmented rabbit.	8 healthy male rabbits. 4 rabbits received single injection 1.25mg intravitreal bevacizumab, 4 rabbits received single injection of 2.5mg intravitreal bevacizumab, non-injected eyes of all 8 eyes acted as control. 29 day follow up	Light microscopy of enucleated eyes.	Control and experimental eyes showed mild vacuolisation and disruption of photoreceptor outer segments. Optic nerve, retina and retinal pigment epithelium (RPE) otherwise normal. Conclusion: no evidence of toxicity.	Control eyes part of same animals.
Barack, 2012	Cohort Study	To evaluate the effects of intravitreal bevacizumab on retinal blood flow velocity.	12 eyes of 12 patients with CNV. Received bevacizumab (1.25mg). Followed up for 1	Retinal blood flow velocity	Increased retinal blood flow velocity after vasoconstriction. Increase in blood flow was most dramatic immediately after	No control group.

			month. Mean age 57.3 ± 20.3 years.		receiving anti-VEGF therapy	
Benatti, 2017	Cohort Study	To investigate the short-term effects of intravitreal ranibizumab on retinal vessel functionality in patients with diabetic macular oedema (DMO) by Dynamic Vessel Analyzer (DVA)	Participants with DME enrolled Complete ophthalmic evaluation, including optical coherence tomography with dynamic and static vessel analysis, using the DVA before (baseline), 1 week and 1 month after intravitreal ranibizumab. DMO participants compared with diabetic retinopathy (DR) without DMO subjects, and with	BP, heart rate, distance VA, IOP, slit-lamp biomicroscopy, fundoscopy, SD-OCT with automated CMT measurements 6x6 mm area	Significant decrease in mean arterial dilation in DMO eyes 1 week after injection. Retinal vascular dilation during flicker stimulation was reduced in participants with diabetic eye disease compared to control subjects. We also demonstrated a significant reduction in arterial vessels at static analysis in DMO patients compared to DR patients	Nil of note.

			normal non diabetic age-matched participants		without DMO and controls. Suggests intravitreal ranibizumab could cause vasoconstriction of retinal arteries, and ergo possible ischaemia. Query due to the effects of VEGF being partly because of the release of vasodilating nitric oxide.	
Cheng, 2009	Animal study	To examine the cytotoxicity of single and repeated anti-VEGF therapy to retinal ganglion cells (RGC) (<i>in vivo</i> and <i>in vitro</i>)	18 Rats. 6 had single injection of bevacizumab (125g in 5L), followed up after 1 month. 6 had a single injection, followed up at 6 months. 6 had multiple injections, followed up at 6 months. All left eyes received bevacizumab, right eyes received saline as control.	Histological analysis of cell viability using MTT assay	There was no significant effect on the number of RGCs or on the PC12 cells with either single or multiple applications of bevacizumab. Study authors believe this shows anti-VEGF drugs such as bevacizumab pose no threat to RGCs.	Control eyes part of same animal subject. No randomisation.

			Neuronal model PC12 cells were cultured in various concentrations of bevacizumab (0, 1, 2, 5, and 10 mg/mL) to model RGC exposure.			
Chhablani, 2016	Cohort Study	A safety evaluation of a single dose of ziv-aflibercept (1.25mg) to treat choroidal neovascularization secondary to AMD.	12 patients' eyes with choroidal neovascularization secondary to AMD received a single intravitreal injection of ziv-aflibercept each. Followed up at day 30.	Full field ERGs according to ISCEV guidelines (not specified).	No significant difference in implicit times, a- and b-wave amplitudes, and b/a ratios	No control group.
Comyn, 2014	Cohort Study	To compare the functional and structural effects of ranibizumab versus against those of macular laser therapy.	Subjects with centre-involving diabetic macular oedema were randomized to 3 loading doses of ranibizumab (0.5mg) then retreatment every 4 weeks as required (n=22); or macular laser	ISCEV standard photopic and scotopic ffERGs.	Scotopic: No change detected in the scotopic 0.01 ERG over follow up. Scotopic 11 ERG: mean amplitude of the a-wave and b-wave decreased but implicit times stayed the same. None of the small changes witnessed were	No control group.

			therapy. Follow up period 48 weeks. Mean age 64.9 years.		clinically significant. Photopic: No clinically significant change in parameters over the follow up period.	
Corvi, 2015	Cohort Study	To investigate the effects of intravitreal ranibizumab on retinal vessel functionality in retinal vein occlusion (RVO) patients.	11 eyes of 11 patients treated with intravitreal ranibizumab (0.5mg). Follow up 1 week and 1 month later.	Dynamic and static retinal vessel analysis.	Intravitreal ranibizumab increased vein dilation (dynamic analysis) and had a vasoconstrictive effect on both arteries and veins (static analysis).	No control group.

de Oliveira Dias, 2014	Animal study	To examine the safety of intravitreal (IVT) ziv-aflibercept on retinal disease in rabbits.	18 rabbits intravitreally injected with 25 mg/mL ziv-aflibercept or 40 mg/mL aflibercept. Examinations were performed at baseline, 24 hours or 7 days.	Funduscopy, scotopic ffERG (scotopic rod response and maximum response), OCT, light microscopy, and transmission electron microscopy.	All eyes showed normal funduscopy and OCT results (scan location not specified). No statistically significant change in b wave amplitude of a/b ratio was found. Histology and TEM showed no major signs of toxicity.	No control group.
de Oliveira Dias, 2015	Case study	To describe the results from a patient who received a single dose of ziv-aflibercept to their right eye	One patient with nAMD received a single ziv-aflibercept (1.75mg) dose in their right eye (patient had previously had 8 bevacizumab injections to RE). Followed up for 30 days.	Full field ERGs, Fundoscopy.	Full-field ERG showed an increase in rod and cone amplitudes 1 month after the injection.	Short follow up. No control.

Demirel, 2015	Case Control Study	To examine the effects of intravitreal ranibizumab injections on RNFL thickness in patients with nAMD.	29 eyes of 29 patients who had been given 10 or more intravitreal injections of ranibizumab. Fellow eyes and 27 healthy eyes used as the control group. >12 months follow up.	Mean total and nasal RNFL thickness assessed using OCT cube centred on the ONH.	No significant difference in mean total or nasal RNFL thicknesses in treated and fellow eyes or healthy control eyes. No correlations between number of injections and mean total and nasal RNFL thickness.	No randomising.
El-Ashry, 2015	Cohort Study	To evaluate the effect of repeated intravitreal ranibizumab injections for nAMD on the RNFL thickness	30 eyes of 30 patients with nAMD. Three monthly ranibizumab injections. Followed up for 1 month after third injection. Age range between 59 and 93 years (mean 77.6).	Mean RNFL thickness and mean RNFL thickness per quadrant in peripapillary OCT scan	The pre- and postinjection values of the mean RNFL thickness and mean RNFL thickness per quadrant were not statistically significant.	No control group.
Enders, 2017	Cohort study	To analyse peripapillary RNFL change in thickness after long-term intravitreal antiVEGF. Patients	76 participants treated for nAMD pro re nata (min n° injections = 9). RNF thickness change measured. Two groups;	SD-OCT	Participants without ACP, mean RNFL loss was significantly higher than participants with regular ACP. Participants without ACP, RNFL thickness loss	No control group. No randomising.

		with regular anterior chamber paracentesis (ACP) prior to intravitreal injections (IVIs) were compared to those without ACP.	n = 32 without ACP, n = 44 with regular ACP		was higher compared to IVI-naive fellow eyes ($p = 0.005$), whereas in ACP patients, no difference was detected ($p = 0.5$).	
Entezari, 2014	Cohort Study	To evaluate the effect of two intravitreal bevacizumab injections on peripapillary RNFL thickness in patients with nAMD	18 eyes of 18 patients receiving 2 intravitreal bevacizumab 6 weeks apart to treat nAMD. Followed up at 12 and 24 weeks post injection.	Mean peripapillary RNFL thickness assessed using OCT.	Temporary decrease of RNFL thickness after both injections but no significant change was detected in long term analysis.	No control group.
Feigl, 2007	Case Control Study	To evaluate neuroretinal function and anatomical outcomes in patients after three ranibizumab treatments.	3 patients with nAMD receiving ranibizumab (0.3mg) therapy. 20 healthy controls. Follow ups happened after each of the three monthly treatments.	mfERG N1P1 response density amplitudes and P1 implicit times	Statistically significant reduction of peripheral mfERG amplitudes after 3 ranibizumab treatments of 0.3mg.	Nil of note.

Ferrara, 2007	Case Study	An investigation into the change in visual acuity (VA) and retinal appearance after bevacizumab treatment.	Six eyes of 5 patients with central retinal vein occlusion (CRVO), mean age 69.9, received a bevacizumab injection (1.25mg/0.5ml), with retreatment as required. Follow up range 7-15 months, mean 12 months.	Fundoscopy and fundus photography.	Statistically significant decrease in optic nerve head swelling, venous tortuosity and diameter. Most changes within 1 month of the first injection. No vessels seen at the optic nerve head.	Two eyes of same patient used in one instance (confounding statistics). No control group.
Filek, 2019	Case Control Study	To evaluate long-term structural/functional changes that happen to the optic nerve and retina of participants with diabetic macula oedema being treated by ranibizumab	Thirty patients with clinically significant DMO requiring anti-VEGF injections underwent pre-injection baseline, 6, 12, and 24 month follow-up tests. Also, 21 control participants not requiring anti-VEGF followed up in the same manner.	OCT, BCVA, Visual Fields, Wide Field Fluorescein Angiogram, Stereo fundus photography	Cup volume and vertical cup-to-disc ratio increased significantly ($p<0.0014$) over the study period. Trend of deterioration of peripheral visual field at 12 and 24 months though not significantly.	Study only has controls with ocular disease, no age-matched healthy controls.

Fontaine, 2011	Cohort Study	To examine the effect of bevacizumab on retinal circulation.	18 in final analysis, mean age 71.8 ± 7.9 years. Received intravitreal bevacizumab (1.25mg/0.05ml), three injections given at 5 week intervals. A laser blood flowmeter was used at baseline, then at 1 and 5 weeks after first injection, and 5 weeks after third injection.	Retinal blood flow velocity and arteriolar diameter.	Found to be a significant decrease in arteriolar diameter but no significant arterial blood flow change.	No control group.
Guo, 2010	Animal Study	To evaluate the effects of an anti-rat vascular endothelial growth factor antibody (ARVA) and bevacizumab on retinal Müller glial cells (RMGCs) <i>in vivo</i> and <i>in vitro</i> .	20 Rats. Bevacizumab (3.75mg/ml) injected into the right eye, saline injected into the left eye as control. <i>In vitro</i> study involved bevacizumab (0.1, 0.25, and 1 mg/ml) on Müller cells. Follow up max 72	Scotopic ERG b wave amplitudes. RMGC viability; histopathologic changes and glial fibrillary acidic protein expression of RMGCs.	The study found that the b-wave amplitudes of the experimental and control eyes were very similar. No retinal toxicity was found in the treated or control eyes.	Follow up information unclear. Contralateral eyes from same animal subjects used.

			hours <i>in vitro</i> . 1 week <i>in vivo</i> . Ages of rats were 6 weeks in one group and 3-5 days in the other (it is unclear which is which).			
Heiduschka, 2008	Animal study	To examine whether retinal function is affected by an intravitreal injection of bevacizumab.	Wild-type mice. 10 BSS injected, 14 vehicle injected, 17 bevacizumab (1 µl, concentration 0.025 25mg per 1ml), 3 sham. Followed up 1, 4, 12 and 25 days afterwards.	Amplitudes and implicit times of scotopic ffERG a- and b-waves, OPs and photopic ERG b-waves, and 30-Hz flicker	Initial reduction in amplitude of all parameters followed by a recovery over time. Generally least change in sham treated animals. Photopic ERG parameters showed less recovery over time. Implicit times were not affected. In all four groups, a similar behaviour of the ERG parameters was seen.	Unequal subject numbers in groups. No randomising.

Holm, 2015	Cohort Study	To evaluate the influence of ranibizumab on ERG and OCT parameters in diabetic eyes with macular oedema.	N=20 with diabetic macular oedema (mean age 65.7 ± 9.8 years,) underwent ERG and OCT testing at baseline, 4 weeks after the first ranibizumab (0.5mg) injection, (just before the second injection), and 4 weeks after the third (and last) injection with ranibizumab.	ISCEV standard photopic and scotopic ffERGs	Cone implicit time with 30-Hz flicker reduced significantly between baseline and final follow-up. Rod function did not change significantly over follow-up period. No evidence of peripheral retinal toxicity – actually evidence of improvement in flicker ERG.	No control group
Horsely, 2010	Cohort Study	To investigate changes in RNFL measurements in nAMD patients treated with intravitreal pegaptanib, bevacizumab, and/or ranibizumab.	Forty-one eyes of 37 consecutive patients (mean age 79.2 ± 8.7 years) who underwent treatment with pegaptanib, bevacizumab, and/or ranibizumab for nAMD followed by OCT RNFL thickness assessment were	Mean RNFL thickness assessed through peripapillary OCT scan	Long-term treatment with anti-VEGF agents did not lead to significant changes in RNFL thickness in AMD patients undergoing anti-VEGF therapy.	No randomisation. No control group.

			studied. Patients were included if they had > 10 total anti-VEGF injections, RNFL measurements prior to the first injection, and at least 12 months of follow-up. Patients were divided into 3 groups depending on which anti-VEGF agent(s) they received.			
Iandiev, 2011	Animal study	To evaluate the effects of intravitreal bevacizumab on the retina.	7 young adult pigs injected with single injection of bevacizumab (1.25mg). Followed up over 2, 3 and 7 day periods. Groups split by follow up length: 2 day follow up = 4 pigs, 3 day follow up = 1 pig, 7 day follow up = 2 pigs	Histology, light microscopy. Immunostained against gliosis- and apoptosis-related proteins.	No structural changes seen on light microscopy. No evidence of retinal toxicity found nor any significant increase in apoptotic death.	No control group.

Inan, 2007	Animal study	To evaluate the preclinical safety of intravitreal bevacizumab.	Four groups of 6 rabbits (28 total). Group 1 and 2 received bevacizumab (1.25mg) and group 3 and 4 received bevacizumab (3.00mg). Groups 1 and 3 were terminated at 14 days, groups 2 and 4 were terminated at 28 days.	ISCEV standard scotopic ffERGs a- and b-wave amplitudes and interocular amplitude ratios. Histology.	Histology: damage to mitochondria was found in the inner segments of photoreceptors. ERG: No statistically significant change in the parameters during the follow up in any group.	No control group.
Iriyama, 2007	Animal study	To examine the effect of anti-rat VEGF antibody and bevacizumab on rat retina in vivo and in vitro, focussed on RGCs.	In vivo, after intravitreal injections of bevacizumab, anti-rat VEGF antibody, and control-IgG, viable RGCs were visualised by retro labelling with Fluo-gold and enumerated to examine the toxicity. In vitro, rat RGCs were incubated with VEGF, bevacizumab, anti-rat VEGF antibody, or	Histological analysis – number of viable RGCs.	No significant difference in vivo in the number of viable RGCs between groups after 7 days (in vivo), 3 days (in vitro).	No control group or randomisation.

			control-IgG for three days.			
Januschowski, 2014	Animal study	To evaluate the short-term toxic effects of VEGF Trap-Eye on retinal function during and after direct exposure	ERGs were recorded from isolated bovine retinas at baseline and after perfusion with 0.5mg or 2mg VEGF Trap-Eye, followed by a washout period of 100min. Retinal whole mount cultures exposed for 24hr to VEGF Trap-Eye then underwent histological assessment.	Scotopic ff ERG percentage a- and b-wave reduction at the end of the washout compared with baseline. Histology: TUNEL assay to assess the amount of apoptotic cells.	No significant reduction in the a- or b-wave amplitude for 0.5mg and 2mg VEGF Trap-Eye at the end of the washout phase. There were significant reductions in ERG parameters immediately after exposure to VEGF trap eye, but these were not sustained to the end of the washout period. There was no evidence of increased apoptosis in eyes receiving VEGF Trap-Eye on histological testing in the inner retina or photoreceptors.	No control group.
Jo et al, 2016	Cohort Study	To determine the effects of intravitreal	20 eyes of 20 patients with AMD received	OCT: average, and four-quadrant RNFL	Repeated ranibizumab injections had no	No control groups.

		anti-VEGF on retinal nerve fibre layer thickness (RNFL) in patients with AMD.	three intravitreal injections of ranibizumab (concentration not given) at monthly intervals from the diagnosis date. Follow ups 6 and 12 months after intravitreal injection.	thicknesses measured in Optic Disc Cube 200 × 200 scanning program (Cirrus HD-OCT, Carl Zeiss Meditec)	significant effect on average RNFL thickness. A significant decrease in RNFL thickness was seen over time in the pathological areas or segments adjacent to these. Suggests any reductions due to the disease rather than the treatment.	
Julien, 2014	Animal study	To explore the effects of intravitreal ranibizumab and aflibercept on retinal and choroidal tissue.	Monkey eyes, aged 3-8 years. Four injected with 0.5mg ranibizumab, 4 with 2mg aflibercept and 2 controls. Followed up for 1 and 7 days.	Diameter of choriocapillaris (CC) and the areas of CC with free haemoglobin, CC fenestrations and endothelial thickness	Increased haemolysis in the choriocapillaris and cell death. Aflibercept was uptaken by the retinal pigment epithelium and neuronal cells while ranibizumab permeated via cellular spaces.	Unequal sample groups. No randomising.
Kaempf, 2008	Animal Study	To evaluate the toxicity of bevacizumab in the adult mammalian	Porcine neural retinas, or retina-RPE-choroid were cultured for 3 days. Bevacizumab was	Histological and immunohistological analysis for the expression of glial	No toxic effects on RGCs or photoreceptors were observed at any concentration of	Short follow up time.

		neurosensory retina in culture	added to the culture (n=6 retinas per group) (0.25 mg/mL, 0.5 mg/mL, 1.25 mg/mL). Retina-RPE-choroid complex and neural retinas cultured without bevacizumab were used as controls for 3 days.	fibrillary acidic protein (GFAP), vimentin, glutamine synthetase, rhodopsin, smooth muscle actin (SMA), and apoptosis.	bevacizumab. Similar levels of apoptotic cells were observed in bevacizumab-treated cultures and controls. Significantly enhanced SMA expression in retina blood vessels was observed in retinas cultured in the presence of bevacizumab, which is an indication of the loss of functional VEGF modulating smooth muscle cells in mature vessels.	
Karawya, 2008	Animal study	To investigate the effect of bevacizumab on permeability of the choriocapillaris and choroidal structural changes	Retinae of 10 pigmented rabbits treated with a single injection of 5mg or 0.1mg bevacizumab. Second eye of each rabbit injected with saline as control. Clinical examination	FFA assessment of choroidal fluorescence, retinal vasculature, and presence of abnormal dye leak. Qualitative analysis of histological appearance	FFA showed decrease in background choriocapillaris fluorescence in 7 eyes injected with bevacizumab. No change in retinal vasculature, or abnormal dye leak. No appreciable histological	Controls were contralateral eye of same animal subject.

			after 1 week, Histological examination after this.		changes, although ultra- structural changes were present (RPE cells showed significant increase in numbers of mitochondria and prominent endoplasmic reticulum). No significant fundoscopic changes.	
Kim, 2016	Cohort Study	To investigate the quantitative changes of RPE atrophy during a 24-month follow-up period of anti-VEGF treatment for nAMD	65 eyes of 62 participants with treatment naïve nAMD received intravitreal anti-VEGF and followed up for 24 months. Minimum of 3 anti-VEGF injections given then issued pro re nata. RPE atrophy looked for via fundus imaging.	Fundus photography, OCT, Fundus autofluorescence were evaluated for RPE atrophy.	There's progression of RPE atrophy progresses in eyes with nAMD during anti-VEGF treatment. RPE atrophy after treatment more likely in participants with higher numbers of anti-VEGF injections and larger areas of RPE atrophy at month 4 (after loading doses). and larger numbers of anti-VEGF injections were associated with an increased risk of	Both eyes of some participants used (confounding statistics).

					progression of RPE atrophy the following treatment.	
Kim, 2008	Animal study	Ascertain whether bevacizumab is neurotoxic in the retina.	Healthy mice (number not stated). Single dose of bevacizumab intravitreally injected at concentrations from 0-2.5mg/ml. Saline injected in the eyes of the control group. Follow up periods of 1 day, 7 days and 28 days after injection.	1. Histological analysis. 2. TUNEL assay. 3. Cell viability assay. 4. Immunohistochemistry.	1. Bevacizumab eyes had no inflammatory cells in the vitreous, retina or choroid. Retinal thickness the same in experimental and control eyes. 2. No increase in TUNEL positive cells in any eyes (no apoptotic cell death) 3. Viability of retinal cells unaffected up to concentrations of 1.25mg/ml but 20% decrease found at 2.5mg/ml bevacizumab (compared to control). 4. No decrease in retinal	Nil of note.

					neurofilament expression. Conclusion: bevacizumab non-toxic to retinal neurones.	
Kopic, 2017	Case Control Study	To discover if administering bevacizumab to patients with POAG leads to additional reduction of RNFL thickness	60 participants all treated with bevacizumab in groups; AMD and POAG (group 1), and DMO and POAG (group 2). Control group made up of the fellow untreated eye of each participant. RNFL thickness at the ONH was measured.	OCT	Decrease of RNFL in both groups of patients, statistically more significant decrease in RNFL thickness in the second group.	Control group is present but control is fellow eye – could be confounded by systemic effect? Lack of age-matched healthy control group.
Kurt, 2018	Cohort study	To assess the effects of a single dose of intravitreal ranibizumab or bevacizumab on the	62 total participants; n = 32 received ranibizumab, n = 30 received bevacizumab. Non injected eyes used	OCT, fundus fluorescein angiography. Measures included the central retinal artery equivalent, central	Ranibizumab and bevacizumab caused significant blood vessel constriction.	Contralateral eyes used as control – possibly confounded by systemic effect.

		retinal vessel size in eyes with DMO.	as respective group control eyes.	retinal vein equivalent, and artery-to vein ratio.		
Lanzl, 2011	Case Control Study	Investigated the effect of flickering light on retinal vessels before and after bevacizumab injections.	10 Patients with nAMD, median age 77 years assessed, before and 3 months after a single Bevacizumab (1.25mg). Compared to 10 healthy older controls.	Speed and magnitude of retinal vessel response to flickering light.	After the flicker, mean arterial dilation was not different pre and post treatment in nAMD or in controls. Pre and post treatment AMD groups were slower to maximally dilate than controls. In the post-treatment AMD group arterial constriction after stimulation happened more slowly and retinal veins showed faster maximal dilatory response than in the control group.	Nil of note.

Lu, 2014	Animal study	To investigate; 1) effects of bevacizumab on pathological morphology of retina, 2) the expression of VEGF protein and VEGF mRNA.	72 Diabetic rats. 24 per group. Group 1 received saline, 2 bevacizumab (75µg), and 3 bevacizumab-chitosan nano particles (75µg). Follow ups at 1 week post injection, 4 weeks after injection and 8 weeks after injection).	Immunohistochemistry	No toxic effect on the eyes.	Nil of note.
Luke, 2006	Animal Study	To study the short term toxic effects of bevacizumab on retinal function	Bovine eyes (number not given). Bevacizumab was injected at three different concentrations; 0.08mg/ml, 0.25mg/ml, 0.8mg/ml. All performed on same day for ERGs.	Scotopic ffERG a and b wave amplitudes	No reduction in a or b-wave amplitudes	No control group.
Luke, 2009	Animal Study	To investigate the effects of ranibizumab on retinal function.	Post mortem bovine retinas (number unclear) were treated with 0.2mg/ml	Scotopic ERG a and b-wave amplitude.	No significant reduction in a or b wave amplitude after ranibizumab treatment.	No control group.

			ranibizumab. ERGs were performed.			
Luke, 2010	Animal Study	To evaluate the short-term toxic effects of pegaptanib sodium on retinal function.	Post mortem bovine retinas (number unclear) were treated with pegaptanib 0.006, 0.06, and 0.2 mg/ml. ERGs were performed.	Scotopic ERG a and b-wave amplitude	No significant reductions of or b-wave amplitudes during application of pegaptanib or during washout period	No control group.
Macky and Mahgoub, 2012	Cohort Study	To assess functioning of retina and optic nerve after exposure to bevacizumab in people with CNV.	55 patients with CNV underwent treatment with bevacizumab (1.25mg). Single injection. Follow up at 1 week and 6 weeks following injection.	ISCEV standard scotopic and photopic ffERG	Only statistically significant change was an improvement in photopic b-wave amplitude at 1 and 6 weeks in injected and fellow eyes.	No control group.
Manzano, 2006	Animal study	To evaluate the retinal toxicity of varying doses of bevacizumab when injected	Each of 4 concentrations of bevacizumab (500 µg/0.1 mL, 1.0 mg/0.1 mL, 2.5 mg/0.1 mL, and 5.0 mg/0.2 mL) was	Scotopic and photopic ffERG. Histological examination.	No significant (>30% reduction) change in ERG parameters after treatment. One eye injected with the highest concentration showed a	No control group.

		intravitreally in rabbits.	injected into one eye of 3 rabbits (12 rabbits total). ERGs recorded at baseline and day 14. Enucleated eyes were prepared for histologic evaluation of retinal toxicity (light microscopy).		decrease of 11% but one eye of the control group showed a decrease of 13%. No retinal toxicity was found in any eyes in histological examination.	
Maturi, 2006	Case Study	To investigate electrophysiological effects of bevacizumab treatment of nAMD.	5 nAMD patients followed up over 1 month (4 patients, each received 1 injection) and 3 months (1 patient who received one additional bevacizumab injection) periods while receiving bevacizumab (1.25mg) treatment. Mean age 79.7 years.	ISCEV standard scotopic and photopic ffERG	No clinically significant reduction in ERG amplitude or implicit time over follow up period.	No control group.
Moon, 2013	Cohort Study	To investigate the potential of OCT and photopic negative	32 patients with macular oedema. Received injections of	Photopic ff ERGs. White stimulus (2.4 cd.s.m ⁻²) on white background	Large pre and post treatment PhNR was associated with a better	No control group.

		response (PhNR) to predict visual outcome after intravitreal bevacizumab in patients with macular oedema secondary to CRVO.	bevacizumab (1.25mg). Followed up four weeks after third injection (up to 22 months later, 4 weeks after the third intravitreal injection).	(31.9cd.s. ^{m-2}). Baseline to trough PhNR amplitude assessed.	pre and post treatment visual acuity. No statistically significant difference between PhNR amplitudes or PhNR/b-wave amplitude ratios before and after treatment.	
Myers, 2012	Animal study	To investigate changes in retinal function and morphology after high-dose injection of three commonly used VEGF inhibitors.	48 6 month old rabbits were assessed in 4 groups of 12. One group received saline, the second received bevacizumab (1.25mg), the third ranibizumab (0.5mg) and the fourth pegaptanib (0.3mg). They were followed up for 8 weeks (ERG) to 9 weeks (histochemistry).	Immunohistochemistry, Scotopic ERG, Photopic Flicker ERG	Eyes injected with pegaptanib and ranibizumab had significantly less labelling of Protein Kinase C (PKC) labelled rod bipolar cells than controls, whereas those injected with bevacizumab had normal PKC labelling. Scotopic ERG: amplitude of the b-wave in the dark adapted ERG with a dim light stimulus was significantly lower in all anti VEGF	No randomising.

					groups than the control (saline injected) rabbits at 8 week follow up. Photopic flicker ERG: a-wave amplitude and implicit time no significant difference was found between the groups.	
Ozaki, 2000	Animal Study	An exploration of signalling pathways involved in retinal neovascularisation using 3 selective kinase inhibitors with different sensitivity profiles.	Mice (number of subjects is unclear) had ischaemic retinopathy induced and were treated with selective kinase inhibitors PTK787 and PKC412. They were followed up for 5 days.	Immunohistochemistry	No toxic effect on the eyes, mature retinal vessels were not adversely affected by PTK787 and PKC412.	No control group.
Papadopoulou, 2009	Cohort Study	To study the effect of ranibizumab on the arteriolar diameter of retinal blood vessels.	11 eyes of 11 patients with neovascular age-related macular degeneration (AMD). Each treated with ranibizumab (0.5mg).	Retinal arteriolar diameter.	Significant decrease in retinal arteriolar diameter was observed after each ranibizumab injection was given. Diameters decreased in size more and	No control group.

			Followed up for 4 months. Median age 76.		more with each injection suggesting that ranibizumab may stimulate vasoconstriction.	
Parlak, 2014	Cohort Study	To evaluate the long-term effect of intravitreal ranibizumab on the RNFL in nAMD.	22 patients diagnosed with unilateral treatment naive neovascular AMD received 3 monthly intravitreal ranibizumab injections, and were re-injected according to “treat and extend” protocol (average number of injections 4.7, range 3 – 11). Follow up of 12 months, minimum.	OCT: Peripapillary RNFL thickness	Significant RNFL thinning after the third month, no significant difference between treated and untreated eyes. No difference in RNFL thickness between eyes treated with more or less than 5 intravitreal injections. No statistically significant difference in RNFL thickness between study and control eyes over the follow up period but significant thinning occurred in both groups	No control group.

					compared to baseline values.	
Pedersen, 2010	Cohort Study	To assess retinal function after treatment with bevacizumab	26 eyes of 26 treatment naïve patients with nAMD. Bevacizumab (1.25mg) intravitreally injected. Follow up over 1 week, 6 weeks, 3 months and 6 months. Mean age 77.7 years. All patients given first injection, after this given on an 'as required' basis. Mean injections given: 3 (range, 1–4) injections given per patient at 6 months.	ISCEV standard Photopic and Scotopic ffERGs	Decrease in cone photoreceptor function at 3 months, then normalized at 6 months (compared to baseline). The rod ERG (dim white, 22.5 log) and the combined rod-cone response white 0.5 log, did not change during follow-up. Two of the 3 combined rod-cone responses showed changes from baseline to 6 months: b-wave amplitude of the white 2.0 log response was statistically decreased and	No control group.

					the a-wave implicit time of the white 3.0 log response was increased.	
Ramon, 2018	Animal Study	Evaluation of toxicity of ziv-aflibercept.	22 albino rabbits intravitreally injected with 0.1 mL of ziv-aflibercept solution (test eye) and 0.1-mL saline (control eye). Twelve rabbits had scotopic ERGs recorded 3 days, 1 week, 2 weeks and 4 weeks after injection. VEPs were recorded after the 4 week ERG follow-up. The remaining 10 had penetration of ziv-aflibercept into the retina during follow-up tested. Glial fibrillary acidic protein (GFAP)	ERGs, VEPs, Immuno-histochemistry, histology	ERGs showed no signs of permanent functional damage for any ERG parameter measured. VEPs of both the experimental and control eyes were of normal pattern and amplitude. Histology showed no signs of structural damage. Expression of GFAP was increased in Müller cells of the experimental eyes but not the control eyes which suggests mild retinal stress. Retinal penetration of ziv-aflibercept was observed 1 day post injection, strengthened over the	Nil of note.

			immunocytochemistry and retinal histology were performed after the termination of the follow-up period.		following 7 days and was then not detected at 14 days post injection. neovascularization and macular oedema.	
Romano, 2012	Animal study	To investigate the effect of repeated intravitreal injections of bevacizumab on retinal layers in rats.	20 adult rats. N=10 received a single injections of 4µl bevacizumab in the right eye, n=10 received 3 injections with a 7 day time interval. Fellow eye received same volume of balanced salt solution as a control.	Histological and immunohistochemical analysis and TUNEL assay were performed.	Bevacizumab consistently induced neuronal loss after multiple (but not single) treatments. Severe loss of RGCs and damage of inner and outer nuclear layers seen. Multiple injection of vehicle alone caused slight injury. Dramatic decrease in RGC specific protein (Brn3a) seen in western blot after multiple (but not single) treatment)	Control was fellow eye – possible systemic confounding effect.
Sahiner, 2018	Animal Study	To assess the effects of anti-VEGF drugs on retinal pigment	Enucleated eyes of white rabbits provided RPE cell cultures to be treated with aflibercept	Immuno - histochemistry	Aflibercept treated cells had decreased viability and increase in apoptosis compared to the control	No control group.

		epithelium cell culture.	(0.5 mg/mL), bevacizumab (0.3125 mg/mL), and ranibizumab (0.125 mg/mL). After 72 hours the following were performed; viability, apoptosis, proliferation, and senescence of the cells were evaluated in control and drug-treated cultures at the end of 72 hours		group, the ranibizumab group and the bevacizumab group. A statistically significant increase in cell apoptosis and decrease in viability were also in the latter two groups compared to the control group. There was no significant difference in cell proliferation and senescence between the groups.	
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Sakurai, 2009	Animal Study	To assess the ocular complications occurring after high dose bevacizumab.	Four monkeys used in total. Two groups tested, one received a single injection of 6.25mg bevacizumab and one received a single injection of 12.5mg bevacizumab. RE experimental eye, LE control eye. Follow up 28 days (3 days, 1 week, 4 weeks)	Photopic and scotopic (white-flash and rod response) ffERGs. Fundus photography (FP), fluorescein angiography (FA), and indocyanine green angiography (ICGA), Optical coherence tomography (OCT).	No significant differences in the ERG responses between the experimental and control eyes at any follow up in eyes treated with 6.25mg. No significant differences in light-adapted photopic cone responses after intravitreal injection of 6.25 or 12.5mg. Significant reduction in amplitude and increase in implicit time of the scotopic rod response and scotopic bright flash ERG a-wave at 3 days and 1 week in eyes treated with 12.5mg. No significant differences between experimental and control eyes at 4 weeks.	Control is contralateral eye – possible systemic effect.
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					No pathologic changes were observed by FP, FA, ICGA, OCT, and light microscopy in the eyes injected with either of the two bevacizumab doses.	
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Sari, 2006	Animal Study	To evaluate the effects of repeated 1.25 mg intravitreal bevacizumab injections using histologic and biochemical analyses.	24 rabbits were randomly assigned to groups. Group 1 (n =12 eyes): right eye received intravitreal injection of 1.25 mg bevacizumab 3 times with an interval of 25 days. Group 2 (n= 12 eyes): left eyes of the rabbits in Group 1 served as untreated control. Group 3 (n= 6 eyes): right and left eyes of each rabbit received an intravitreal injection of vehicle solution injected 3 times with an interval of 25 days. Group 4 (n= 6 eyes): no injection was made in this group.	Histological and biochemical examination (caspase 3 and 8 activity – apoptosis; glutathione content, catalase activity, and malondialdehyde content – antioxidant capacity and lipid peroxidation)	Light microscopy demonstrated normal retinal histologic findings for all groups. The activities of caspase 3 and 8 enzymes were the lowest in group 1, and malondialdehyde content was also significantly lower than Group 4. There was no evidence that a repeated dose of bevacizumab caused a toxic effect on uveoretinal tissue. Biochemically, it also did not cause any apoptosis, oxidative reaction, or lipid peroxidation.	Nil of note.
Shahar, 2006	Animal Study	To evaluate toxicity of bevacizumab and	Albino rabbits. Bevacizumab (2.5mg)	Photopic and Scotopic fERGs intensity	There was no significant difference in the Vmax of	No control group.

		its penetration into the retina.	intravitreally injected, single injection. Followed up at 3 hrs, 3 days, 1 week, 2 weeks, 4 weeks.	response function fitted with a Michaelis-Menten function	the b wave and the log semi-saturation time constant at all the different time intervals for scotopic or photopic conditions.	
Shin, 2014	Case Control Study	To investigate the effect of multiple intravitreal injections of anti-VEGF on the RNFL	148 eyes of 135 patients with AMD, diabetes mellitus retinopathy (DMR), or retinal vein occlusion (RVO). Intravitreal injections of either 0.125mg/0.05ml bevacizumab or 0.5mg/0.05ml ranibizumab. At least 12 months follow up. Patients received repeated injections (non specified number). Age-matched AMD, DMR, and RVO without	Peripapillary RNFL thickness measured using OCT	Multiple intravitreal injections of anti-VEGF did not lead to significant change in RNFL thickness in wet AMD, DMR, and RVO patients when compared to untreated control groups. Number of injections and IOP fluctuations were not correlated with change in RNFL thickness over time.	Two eyes from same patients used (confounding statistics).

			anti-VEGF injection formed the control group (183 patients).			
Sitnilska, 2019	Cohort Study	The aim of this study was to evaluate risk factors for the development of retinal pigment epithelium (RPE) atrophy in patients with neovascular age-related macular degeneration (nAMD).	Recruited 52 nAMD patients who did not have any RPE atrophy at baseline and were naïve to anti-VEGF treatment. Given 9 or more anti-VEGF injections over 3 or more years.	Multimodal imaging	Atrophy of the RPE was associated with duration of nAMD but not with anti-VEGF treatment.	No control group.
Skaat, 2011	Cohort Study	To assess the effect of bevacizumab on retinal function	12 patients with nAMD received single dose of bevacizumab (1.25mg)	ISCEV standard Photopic and scotopic ffERG.	Pre- to post injection increases in a-wave amplitudes were	No control group.

			therapy and were followed up for 1 month. Mean age 72 ± 11.5 years.		significantly higher in the treated than in the control eyes in both the scotopic and photopic ERGs. The pre- to post- injection increase in b-wave amplitude was significantly greater for treated than untreated eyes for the scotopic ERGs only.	
Sobaci, 2013	Cohort Study	To discover the effect of several ranibizumab or bevacizumab injections on RNFL and intraocular pressure (IOP)	35 eyes of 35 patients being treated with bevacizumab (1.25mg/ml). 30 eyes of 30 patients being treated with ranibizumab (0.5mg/0.05ml). All patients had nAMD. 1 year follow up (minimum).	Peripapillary RNFL layer thickness measured using OCT	Change in RNFL thickness was not statistically different between treated and untreated eyes.	No control group.

Stahl, 2009	Case Study	To evaluate retinal photoreceptor function after several bevacizumab injections.	10 patients with various retinal diseases requiring treatment with bevacizumab (1.25mg). Mean age of participants was 72. Followed up 3 weeks after the third injection. 2-3 injections occurred monthly.	Scotopic ERGs	No clinically significant difference between ERGs before and after bevacizumab therapy	No control group.
Teraï, 2014	Cohort Study	To examine the effect(s) intravitreal ranibizumab on retinal vessel diameter in patients with DMO.	30 eyes with DMO were injected with 0.05 mL ranibizumab at 4 week intervals and followed up for 3 months.	Dynamic and static retinal vessel allowance	No significant effect on retinal vessel diameter.	No control group.
Terauchi, 2019	Cohort study	To evaluate the retinal function before and soon after an intravitreal injection of an anti-vascular endothelial	Seventy-nine eyes of 79 patients treated with intravitreal anti-VEGF for nAMD, DMO, or retinal vein occlusion with macular oedema were studied. ERGs	RETeval™ 28 Hz flicker ERGs. Significance of changes in the time to peak and amplitudes was determined by t tests.	Time to peak wasn't significantly different between P1 and P2 but it was significantly longer at P3. Time to peak was significantly longer at P2	No control group. Not randomised.

		growth factor (anti-VEGF) agents.	performed on test and control eyes before anti-VEGF, within 2 hours post-injection, and 2 to 24 hours post-injection. Participants grouped by disease or by the injected agent and compared.		and P3 in the non-injected control eyes. Suggests intravitreal anti-VEGF injection may increase time to peak in the injected eye and the non-injected eye fellow eye soon after the intravitreal injection.	
Thaler, 2010	Animal Study	To evaluate the effects of intravitreal anti-VEGF in rat eyes with healthy RGCs and others with N-methyl-D-aspartate (NMDA)-induced RGC damage	Bevacizumab (25 mg/ml and 125 mg/ml.), ranibizumab (10 mg/ml and 60 mg/ml and pegaptanib (3.3 mg/ml and 20 mg/ml) were intravitreally injected each at a commercially available and higher concentration. Vehicles were injected as controls. In a different group, anti-VEGF was injected after NMDA	RGC counts. Electron microscopy histological examination.	RGC counts in healthy rat eyes were not significantly changed from those of the control animals after the administration of both low and high concentrations of bevacizumab, ranibizumab or pegaptanib. Even at higher doses, bevacizumab and ranibizumab showed no toxic effects on RGC in vivo in either untreated rats or in the NMDA-	Nil of note.

			treatment. Follow up 1 week or 2 months after injection.		induced RGC damage model.	
Valverde-Megias, 2019	Cohort Study	To investigate long-term effect of intravitreal ranibizumab administered for nAMD on RNFL thickness when used following a pro re nata regimen.	20 eyes of 20 participants with nAMD were recruited. Contralateral eyes of nine of these patients were included as controls and had no nAMD. Follow up period of up to 96 months. Mean number of injections was 21 (SD: 2.8)	Circumpapillary RNFL OCT.	Loss of RNFL observed, greatest in the superior temporal, then inferior and nasal sectors. No statistically significant difference in loss between injected eyes and control eyes. Equal decrease suggests no long-term effects of ranibizumab on the RNFL.	Contralateral eye as control – possible systemic effect.
Wu et al, 2010	Animal Study	To test effects and systemic exposure level after one or several bevacizumab injections	New born rabbit eyes. Eight days after injection, the serum concentration of bevacizumab (1.25mg) was determined. Morphologic and	One hour of dark adaptation followed by a white flash (3cd.s.m-2 luminance) on a dark background.	There was found to be no significant difference between experimental and control eyes. This indicated that bevacizumab does not cause a significant reduction in retinal	Nil of note.

			functional changes were evaluated over 12 months after bevacizumab injection.		function up to a year after treatment	
Yagura, 2016	Cohort Study	Electroretinographic evaluations of retinal function before, just after, and after intravitreal injections	19 eyes of 19 patients having intravitreal anti-VEGF treatment recruited. Underwent intraoperative photopic electroretinograms (ERGs) recorded before an intravitreal injection, just after the injection, and following aspiration of anterior chamber fluid.	Intraoperative photopic ERGs, noted monitoring of PhNR, a-wave, b-wave, and oscillatory potential components of the waveform. <i>BUT</i> also says follows protocol of Miyake et al (1991) and in this only flicker ERGs were examined. A little unclear on methods.	Mean amplitudes of the b-wave, PhNR, and oscillatory potentials (OPs) were significantly different at first follow up but not at second. Mean time to peak of the a-wave and oscillatory potentials at the first follow up, and the a-wave and the oscillatory potential (2) at the second follow up were significantly longer than baseline. Just after the intravitreal injection	No control group.

					retinal function was reduced and IOP elevated. Responses of individual ERG components were different. Suggests retinal neurons have different sensitivities to intravitreal injections.	
Yang, 2017	Case Control Study	Investigated the relationship between the peripapillary RNFL and peripapillary retinal thickness in patients with DMO.	50 eyes with non-proliferative diabetic retinopathy and DMO receiving intravitreal anti-VEGF injection (group I), and 90 eyes without DMO (group II). The peripapillary RNFL thickness, peripapillary retinal thickness, and a new RNFL index using a modelled relationship between the two parameters evaluated with SD-OCT,	OCT	In group I, the peripapillary RNFL and retinal thickness significantly decreased between baseline and 6 months ($p<0.001$). But in group II, both the parameters remained stable at the 6-month follow-up. Analysis with the new RNFL index to adjust for retinal oedema showed no significant change between baseline and 6 months in either group. Peripapillary RNFL	Not randomised. Unequal group numbers.

			Measurements taken at baseline and followed up after 6-months.		thickness is strongly affected by peripapillary retinal thickness so measured changes in peripapillary RNFL thickness may not represent any real loss or gain of RNFL. Considering this the developed RNFL index (which corrects for macula oedema), could help assess true changes in peripapillary RNFL thickness in patients with DMO.	
Yasuda, 2015	Cohort Study	To compare flicker ERG the amplitudes and implicit times before and after intravitreal ranibizumab injection in eyes affected by CRVO.	15 patients with macular oedema secondary to CRVO received intravitreal ranibizumab (concentration/dosage not given). Follow up 1 month.	Flicker ffERGs with RetEVAL™ system.	1 month after treatment the flicker ERG implicit time was significantly shorter than at baseline.	No control group.

Yau, 2015	Cohort Study	To compare the peripapillary RNFL thickness in dry versus nAMD eyes treated chronically with intravitreal injections of anti-VEGF	29 px with unilateral nAMD underwent at least 12 prior intravitreal anti-VEGF injections over a minimum of 2 years. The fellow eye with nonexudative (dry) AMD with no prior treatment served as the control group. SD OCT was performed on all pairs of eyes.	OCT optic nerve head parameters and peripapillary RNFL thickness (mean total and quadrant)	Mean total RNFL in the treated eyes (95.0 significantly greater than the nontreated fellow eyes. The temporal RNFL was greater in the treated group, whereas all other locations were similar. No significant differences were found between the 2 groups in any ONH parameters. There was no evidence of a deleterious effect of chronic treatment on RNFL.	Contralateral eye served as control group – systemic confounding?
Yun, 2016	Case Control Study	The purpose of this study was to investigate peripapillary choroidal thickness in eyes with neovascular age-related macular	39 eyes of 39 patients being treated for nAMD with ranibizumab were recruited. 39 eye of 39 age-matched controls were also recruited. The patients were treated with 0.5 mg IVR	SD-OCT	Mean peripapillary and sub-foveal baseline choroidal thickness of the eyes with neovascular AMD did not differ from the controls. Sub-foveal choroidal thickness decreased at 3 and 6	Nil of note.

		degeneration (AMD) and to assess whether peripapillary CT is affected by intravitreal injection of ranibizumab (IVR) in eyes with neovascular AMD	monthly for 3 months and repeated as needed. Peripapillary (superior, nasal, inferior and temporal areas) and sub-foveal Choroidal Thickness was measured in SD-OCT images at baseline, 3 months and 6 months was measured at four locations.		months after ranibizumab treatment. Mean peripapillary choroidal thickness did not show significant changes at 3 or 6 months Only temporal peripapillary choroidal thickness decreased from baseline to 3 months and was sustained at 6 months. Thus changes in peripapillary choroidal thickness after intravitreal ranibizumab were limited to the macular area.	
Zayit-Soudry, 2010	Animal Study	To assess the long-term retinal toxicity of repeated injections of ranibizumab and bevacizumab in rabbits.	12 rabbits injected with bevacizumab (2.5mg) or ranibizumab (1mg). Nine consecutive injections were administered at 14 day intervals. Followed up for 18 weeks.	Histology	No structural retinal damage from the anti-VEGF therapies was discovered but glial fibrillary acidic protein immunoreactivity was found in all of the rabbit subjects. This was	No control group.

					discovered to have come from retinal astrocytes and not in any Muller cells	
Zayit-Soudry, 2011	Animal Study	To study the long-term effects of a single intravitreal dose of bevacizumab	Bevacizumab (25mg/1ml) was injected into the right eyes of 12 rabbits at either 11, 18 or 25 days of age. Volume of bevacizumab changed according to subjects' individual ages and weights. 10 weeks follow up.	Histology	No rise of fibrillary acidic protein in the Muller cells of the animal retinae and no structural retinal damage in the control eyes or in the experimental eyes. During this study it was also noted that the network of capillary branches had reduced	No control group.
Zeimmsen, 2008	Cohort Study	To screen the safety monitoring of bevacizumab (Avastin) treatment by assessing	20 consecutive cases with a single injection of 1.25 mg bevacizumab for nAMD were monitored intensively.	Functional field score (Goldmann visual fields). ISCEV photopic and scotopic ffERGs. Scotopic ERG luminance	Abnormal photopic ERG seen at baseline compared to controls. No change in any ERG parameters (including Naka Rushton)	No control group.

		retinal function in two subgroups of patients	In n=10, EOG and ERGs were collected at baseline and 4 months. In n=10, visual fields were repeated after 3 and 12 months.	response function (modelled using Naka Rushton). EOG Arden ratio.	at 4 month follow up. No progression of visual field defects was seen within the follow-up of 1 year. Performance in EOG testing was affected by restricted fixation stability, but no deterioration seen at 4-month follow up.	
Zucchiatti, 2017	Cohort Study	To analyze the changes in GCC and peripapillary RNFL thickness, in central macular thickness and choroidal thickness on SD-OCT in patients with nAMD treated with intravitreal ranibizumab.	24 eyes of consecutive patients with untreated neovascular age-related macular degeneration were recruited. They received loading doses of 3 monthly intravitreal ranibizumab, and were subsequently treated pro re nata for 12 months.	OCT Primary outcome: changes in ganglion cell complex and retinal nerve fiber layer at the end of follow-up. Secondary outcomes all macula focussed.	GCC and peripapillary RNFL thickness showed no statistically significant changes over 12 months. Macular changes in thickness were present, all decreased over follow up period.	No control group.

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Appendix 2 – Demographic data available for participants in Chapter 5

		n			Age			Visual Acuity Range of Assessed Eyes	Anti-VEGF injections											
		Sex		N° of injections					Time Since first injection			Anti-VEGF Treatment Received								
All	M	F	Median	IQR	Range	Median	IQR	Range	Median	IQR	Range	Bevacizumab	Aflibercept <i>only</i>	Lucentis <i>only</i>	Converted from Lucentis to Aflibercept	Unknown				
Study 1	Between Participant Analysis	32	16	16	83	74, 86	59 - 93	6/5 – 6/60		10	3, 18	0 - 44	18	4, 29	0 - 49	1	20	5	7	0
	Inter-ocular Analysis	14	10	4	86	78, 88	63 - 92	Treated Eyes	Untreated Eyes	14	10, 23	3, 44	21	6, 36	2, 49	0	6	2	6	0
								6/5 ⁻¹ – 6/38	6/5 – 6/18 ⁻¹											
Study 2	Between Participant Analysis	19	8	11	81	71, 87	61 - 96	6/6 – 1/60		6	2, 14	1, 60	Data not available for this study			0	10	2	6	1
	Inter-ocular Analysis	12	7	5	77	69, 85	62 - 95	Treated Eyes	Untreated Eyes	4	1, 7	1 - 60				0	8	1	3	0
								6/6 - 3/60	6/5 - CF											

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Appendix 3 – Copies of published data from the work within the thesis

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