CLINICAL OUTCOMES OF RANIBIZUMAB TREATMENT IN DIABETIC EYE DISEASE

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A THESIS SUBMITTED FOR THE DEGREE OF

MD (RES)

2014

Declaration

I, Oliver Comyn, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

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Abstract

Background

The vascular endothelial growth factor (VEGF) inhibitor ranibizumab is emerging as an efficacious treatment for diabetic macular oedema. Large clinical trials have shown improvements in visual acuity and reduced central retinal thickness. Details of its effect on other retinal functional parameters are lacking. There is a concern that repeated ranibizumab treatment could exacerbate macular ischaemia or lead to global retinal dysfunction by inhibiting physiological isoforms of VEGF.

Outcomes of surgery for advanced proliferative retinopathy remain variable and post-operative complications including recurrent haemorrhage can limit visual recovery. VEGF is strongly implicated in the pathogenesis of advanced retinopathy, so VEGF inhibition prior to surgery may improve outcomes. Trials have failed to demonstrate a clear benefit for bevacizumab, so investigation of the licensed intraocular agent ranibizumab represents a logical next step.

Aims

To investigate the effects of ranibizumab and laser treatment in diabetic macular oedema on the following parameters: visual acuity, protan and tritan colour contrast sensitivity, 4° and 12° macular sensitivity by microperimetry, electrophysiological indices from pattern and full field electroretinograms. To report structural retinal changes following ranibizumab and laser treatment in terms of qualitative and quantitative optical coherence tomography outcomes, and to quantify macular ischaemia by fluorescein angiography.

To investigate the effect on visual acuity at three months post-surgery of ranibizumab pre-treatment in patients undergoing vitrectomy for advanced proliferative diabetic retinopathy.

Methods

Randomised clinical trial of intravitreal ranibizumab vs. laser in 36 subjects with centre-involving diabetic macular oedema **(**The LUCIDATE study).

Randomised clinical trial of pre-operative intravitreal ranibizumab vs. subconjunctival saline injection in 30 subjects undergoing vitrectomy-delamination for advanced proliferative diabetic retinopathy (The RaDiVit study).

Results

Thirty six subjects with diabetic macular oedema were recruited and 33 completed the trial. Ranibizumab treated subjects gained a mean of 6 letters compared with 0.9 letter loss for laser at 48 weeks. Retinal sensitivity improved in the central macular 4° and 12° in both groups but to a greater extent with ranibizumab. There was no evidence of worsening global retinal dysfunction by electroretinograms in either group. Retinal thickness decreased in both groups: there was a 132 μm reduction in central macular thickness with ranibizumab compared with 103 μm for laser. Fluorescein angiography showed no evidence of significantly increased macular ischaemia in either group.

Thirty subjects with advanced proliferative diabetic retinopathy were recruited, underwent surgery, and completed the study. At three months post-surgery, visual acuity in the ranibizumab group was 53 letters compared with 47 letters in the control group.

Conclusion

In diabetic macular oedema, there is evidence that ranibizumab leads to greater improvements in visual acuity and retinal sensitivity than laser, with a corresponding greater reduction in retinal thickness. There is no evidence that it worsens macular ischaemia or indices of global retinal electrophysiological function, but larger trials designed to address each of the outcomes investigated here would be required to confirm these findings.

In proliferative diabetic retinopathy, there is evidence from this small pilot study that ranibizumab treatment leads to better visual acuity at 3 months post-surgery. An appropriately powered trial would be required to confirm this.

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List of publications, abstracts and manuscripts in preparation

The following publications and abstracts have arisen in connection with this work:

Peer-reviewed papers

Comyn O, Heng LZ, Ikeji F, Bibi K, Hykin PG, Bainbridge JW, *et al.* Repeatability of Spectralis OCT Measurements of Macular Thickness and Volume in Diabetic Macular Edema. *Invest Ophthalmol Vis Sci* 2012; **53**:7754-7759.

Heng LZ, Comyn O, Peto T, Tadros C, Ng E, Sivaprasad S, *et al.* Diabetic retinopathy: pathogenesis, clinical grading, management and future developments. *Diabet Med* 2013; **30**:640-650.

Comyn O, Lightman SL, Hykin PG. Corticosteroid intravitreal implants vs. ranibizumab for the treatment of vitreoretinal disease. *Curr Opin Ophthalmol* 2013; **24**:248-254

Comyn O, Sivaprasad S, Peto T, Neveu MM, Holder GE, Xing W, *et al.* A randomized trial to assess functional and structural effects of ranibizumab versus laser in diabetic macular edema (The LUCIDATE study). *Am J Ophthalmol* 2014; **157**: 960-70)

Comyn O, Wickham L, Charteris D, Sullivan PM, Ezra E, Gregor Z, *et al.* A randomized controlled trial of RAnibizumab pretreatment in DIabetic VITrectomy (the RaDiVit pilot study). *In preparation.*

Abstracts

Comyn O, Ikeji F, Bibi K, Hykin PG, Bainbridge JW, Patel PJ. Repeatability of Spectralis OCT Retinal Thickness and Volume Measurements in Diabetic Macular Oedema. *Association for Research in Vision and Ophthalology (ARVO) Annual Meeting 2012.*

Comyn O, Peto T, Bunce C, Neveu MM, Holder GE, Patel PJ, *et al.* The LUCIDATE study: a randomized clinical trial to evaluate the long-term functional and anatomical effects of repeated ranibizumab therapy compared with laser in diabetic macular edema. *ARVO Annual Meeting 2013.*

Gohil R, Comyn O, Keane PA, Patel PJ, Bainbridge JW, Sivaprasad S, *et al.* Evaluation of choroidal thickness by enhanced depth imaging OCT in the LUCIDATE study: a randomised clinical trial to compare outcomes of ranibizumab with laser in diabetic macular oedema. *ARVO Annual Meeting 2013.*

Comyn O, Bainbridge JW, for the RaDiVit Study Group. A pilot randomized controlled trial of ranibizumab pre-treatment for diabetic vitrectomy (The RaDiVit Study). *ARVO Annual Meeting 2014.*

Comyn O, Gohil R, Patel PJ, Bainbridge JW, Hykin PG, Sivaprasad S. Correlation of retinal sensitivity with retinal thickness and morphological features of diabetic macular oedema. *ARVO Annual Meeting 2015 (submitted).*

Acknowledgements

I am extremely grateful for the support and inspiration provided by my two supervisors, Professor James Bainbridge and Mr Phil Hykin, whose superb clinical knowledge and experience helped consistently to keep me on the right track. Thank you for everything you have taught me.

The trials would not have been possible without funding support from Novartis and I also gratefully acknowledge support from the National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital and the UCL Institute of Ophthalmology; a grant from the Special Trustees of Moorfields Eye Hospital funded the cytokine analysis.

I am very grateful for contributions to this work from Tunde Peto and Tanya Mansour in the Moorfields Eye Hospital Reading Centre who graded photographs, angiograms and OCT scans in the trials. Thank you to Catey Bunce and Wen Xing for statistical support in the trials and general statistical advice for this thesis. Rishma Gohil is an enthusiastic medical student who rapidly learnt OCT image analysis and contributed greatly to the choroidal thickness study. Praveen Patel and Sobha Sivaprasad have always been on hand to provide advice, critique and inspiration, so I thank them and all the staff of the clinical trials unit at Moorfields Eye Hospital.

I would like to thank Robin Ali and all the members of the Molecular Therapy Group for welcoming me, providing ideas, support and friendship.

I am constantly grateful for the unending support of my parents, family and long-suffering friends. Most of all I would like to thank my wife Kate, who provided Dorset Cereals and so much more through a long and challenging process, and never lost hope.

1 Introduction

Diabetes mellitus will become more prevalent over the next century and therefore presents a significant public health problem. Diabetic eye disease will ultimately affect a considerable proportion of patients with diabetes and, if untreated, will cause sight loss from diabetic macular oedema and proliferative diabetic retinopathy. Research into treatments for these two main causes of loss of vision in diabetes is therefore of critical importance.

1.1 Diabetes mellitus and epidemiology

Diabetes mellitus is a chronic disorder of glucose metabolism characterised by a deficit in insulin production or lack of response to insulin. In type 1 diabetes, there is an absolute deficit in insulin production by the pancreas, whereas type 2 diabetes is a syndrome of insulin resistance associated with obesity, hypertension and hyperlipidaemia, termed "the metabolic syndrome". Other types of diabetes include gestational diabetes or steroid-induced diabetes. In common to all types of diabetes is sustained hyperglycaemia, which left untreated leads to complications caused by damage to the vascular and nervous systems.

The World Health Organisation reported on World Diabetes Day 2012 that an estimated 347 million people worldwide had diabetes (defined by fasting plasma glucose ≥ 7.0 mmol/l, or on medication for diabetes) and of these, 80% lived in low to middle income countries [1]. Numbers of people with diabetes are expected to increase enormously in the first third of the current century, fuelled in part by the accompanying rise in obesity and resultant increase in type 2 diabetes. Estimates for the prevalence of diabetes in 2000 stood at 2.8% with a predicted rise to 4.4% by 2030 [2]. The predicted future global burden is shown in Figure 1.

Exploring the likely age breakdown of those affected by diabetes shows that the largest increase will occur in the 45-64 age group in developing countries, shown in Figure 2, demonstrating that this disease will continue to affect working age populations and create a significant economic burden, particularly in those countries with the fewest resources to deal with this. In developed countries, an ageing population means that the largest increase will occur in the over 65s, so cost-effective interventions for the management of diabetes and its complications will be important for health services in these countries.

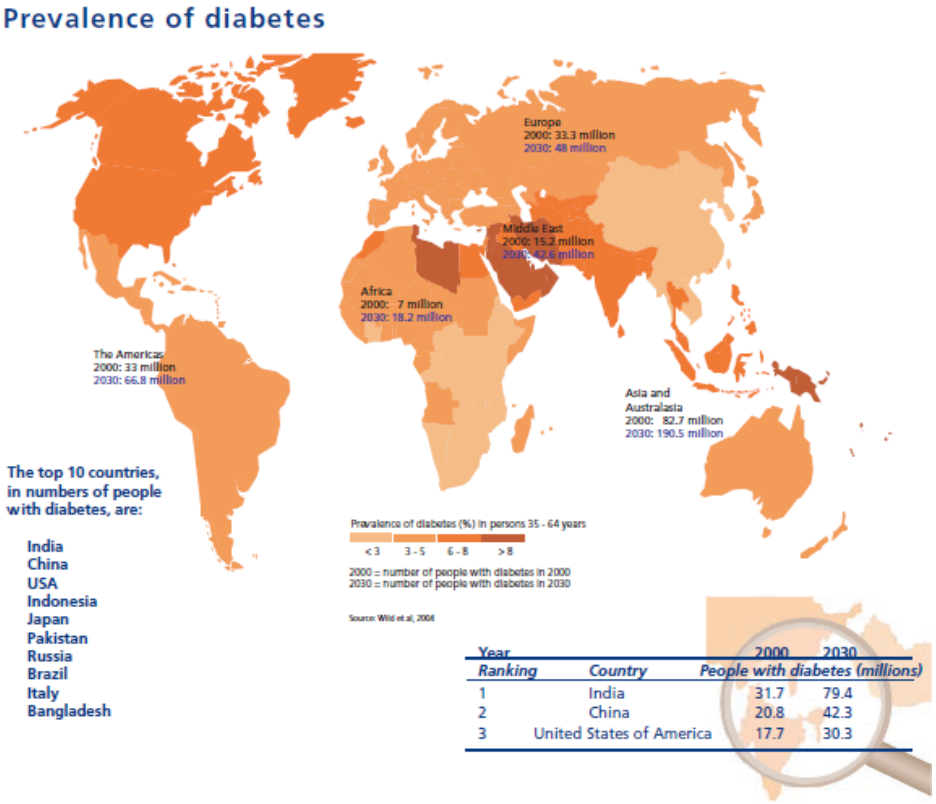


Figure 1 – World Health Organization data showing increased prevalence of diabetes by 2030 (data from Wild *et al*. [2]).

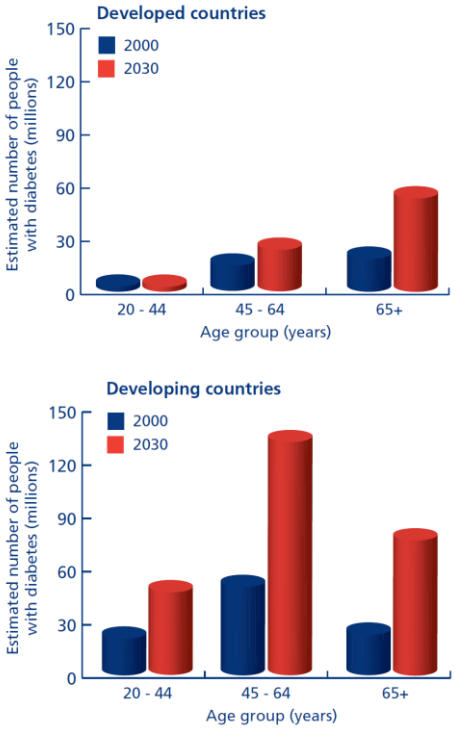


Figure 2 – Numbers projected to be affected by diabetes broken down by age and development level (from [2]).

In the United Kingdom (UK), the latest estimate for the number of people with diabetes is derived from data arising from General Practice as part of the Quality and Outcomes Framework. This was reported by Diabetes UK, the leading UK diabetes charity, as 2.9 million in October 2011 [3], giving an overall prevalence of 4.45%. The breakdown for different countries of the UK is shown in Table 1; note that this data is for the adult population and depends on general practitioners correctly recording the diabetic status of their patients and so is likely to be an underestimate.

Country	**Prevalence / %**	**Number of people**
England	5.5	2,455,937
Northern Ireland	3.8	72,693
Scotland	4.3	223,494
Wales	5.0	160,533

Table 1 – 2011 estimates for prevalence of diabetes in the adult population of the UK, taken from [3].

This high prevalence of diabetes in the UK means that approximately 10% of the NHS budget is spent on the care of persons with diabetes.

1.2 **Diabetic retinopathy**

The retina is the thin, transparent tissue lining the inner surface of the eye and is responsible for transducing light focussed by the optical structures of the eye into electrical signals that pass along the optic nerve to the brain. Histologically, the retina is traditionally divided into ten layers, shown in Figure 3, and receives a dual blood supply. The highly vascular choroid supplies the outer retina, consisting of the rod and cone photoreceptors, while the inner retina is supplied by capillaries arising from branches of the central retinal artery.

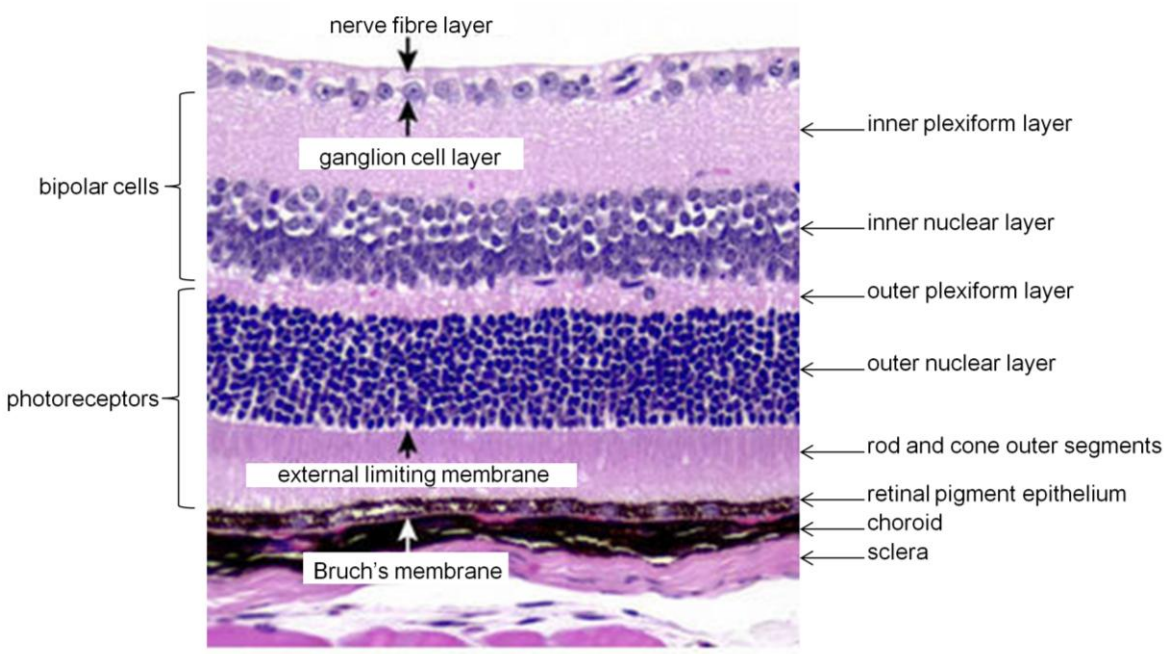


Figure 3 – Histology of the retina. Adapted from "histology-world.com".

The macula, found only in primates, is a specialised region of the retina containing xanthophyll pigment and is responsible for the fine, detailed vision required for reading and face recognition. It has a number of anatomical adaptations that allow it to perform this role: a dense concentration of photoreceptors and two or more layers of ganglion cells. Retinal capillaries are displaced from the specialised central area of the macula termed the fovea, with an avascular zone approximately 500 µm in diameter identifiable in normal subjects.

The microvascular disorder of the retina occurring in patients with diabetes and including features of vascular occlusion and leakage is termed diabetic retinopathy.

1.2.1 Non-proliferative diabetic retinopathy

The earliest features of diabetic retinopathy are the manifestations of damage to the retinal capillaries, including their supporting cells - the pericytes, and the capillary basement membrane. The first lesions visible clinically are typically microaneurysms: microscopic dilations of retinal capillaries that can be filled with blood. The capillary disease can lead to leakage of exudates from the vasculature and retinal haemorrhages. Capillary loss in areas of the retina results in ischaemia, visible clinically as cotton wool spots which are caused by impaired axoplasmic flow in the retinal nerve fibre layer.

Figure 4 shows the typical fundus appearance of this stage of the disease. In more severe forms of non-proliferative retinopathy, increasing tissue hypoxia and ischaemia leads to extensive retinal haemorrhages and changes in vascular calibre, notably venous dilatation from impaired autoregulation. Abnormal connections between the arterial and venous sides of the circulation termed intraretinal microvascular abnormalities (IRMA) develop. Further details on the pathogenesis of diabetic retinopathy are discussed in Section 1.3.

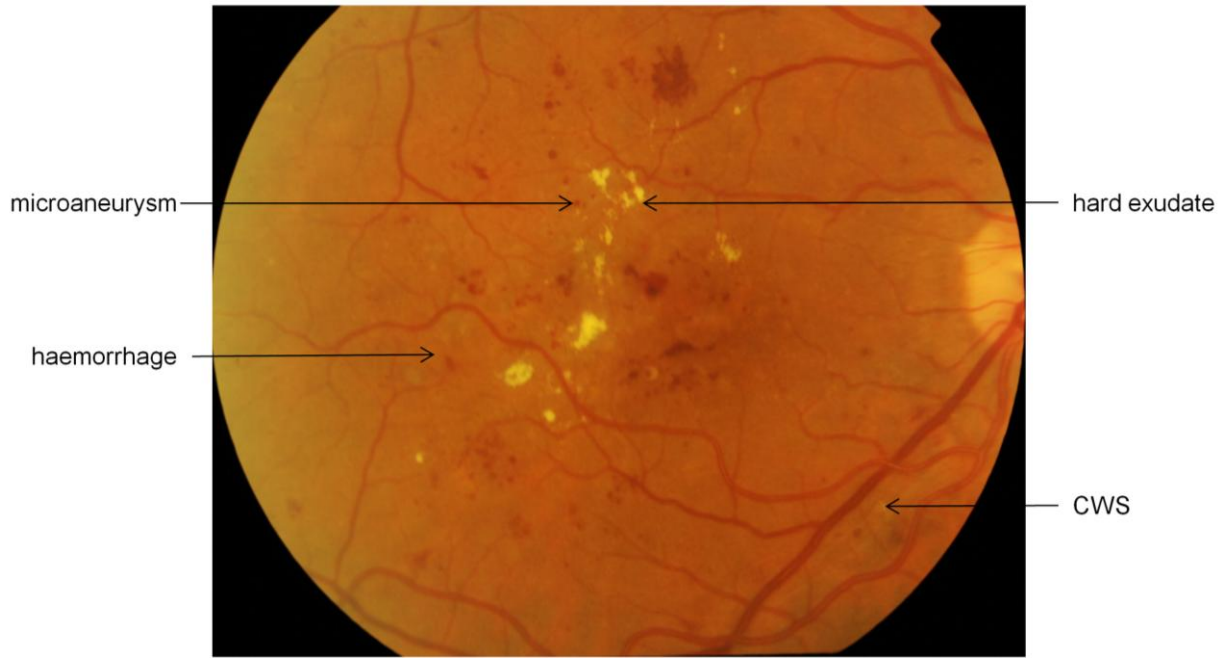


Figure 4 – Colour fundus photograph showing the changes of non-proliferative diabetic retinopathy. CWS – cotton wool spot.

1.2.2 ***Proliferative diabetic retinopathy***

Increasing tissue hypoxia and impaired blood flow leads to the release of angiogenic growth factors and the formation of new blood vessels at various sites in the eye, termed proliferative diabetic retinopathy. In the posterior segment of the eye, new blood vessels form typically at the optic disc (new vessels at the disc, NVD) or elsewhere in the retina (new vessels elsewhere, NVE), shown in Figure 5. Severe ischaemia leads to the diffusion of angiogenic factors to the anterior segment of the eye and the development of new vessels on the iris or in the anterior chamber drainage angle, NVI or NVA. Left untreated, this anterior segment neovascularisation can lead to neovascular glaucoma and loss of vision.

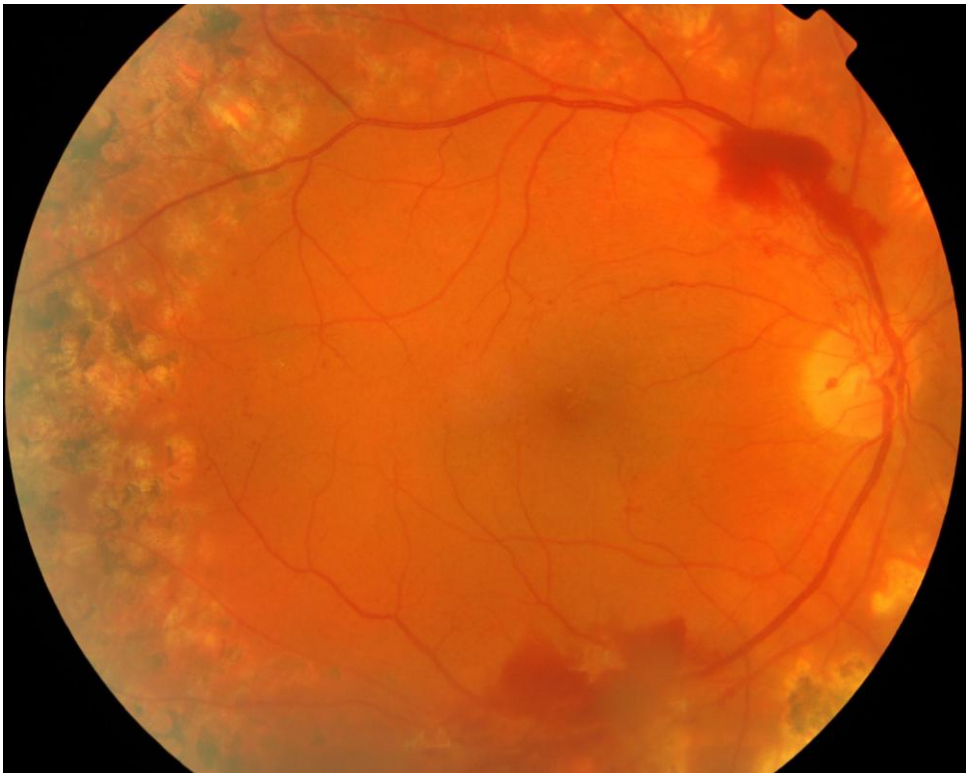


Figure 5 – Colour fundus photograph to show proliferative diabetic retinopathy. New vessels at the disc and new vessels elsewhere are visible, with preretinal haemorrhage at the superior and inferior arcades. Photocoagulation scars are present.

The vitreous gel of the eye provides a scaffold that can support the growth of abnormal new vessels from the retinal circulation. Traction on these vessels can lead to vitreous haemorrhage and uncontrolled fibrovascular proliferation can result in

tractional retinal detachment, shown in Figure 6. Breaks in the retina can occur and this is termed combined tractional-rhegmatogenous retinal detachment; ultimately vision can be lost. There is therefore the potential for considerable visual morbidity in patients with proliferative diabetic retinopathy.

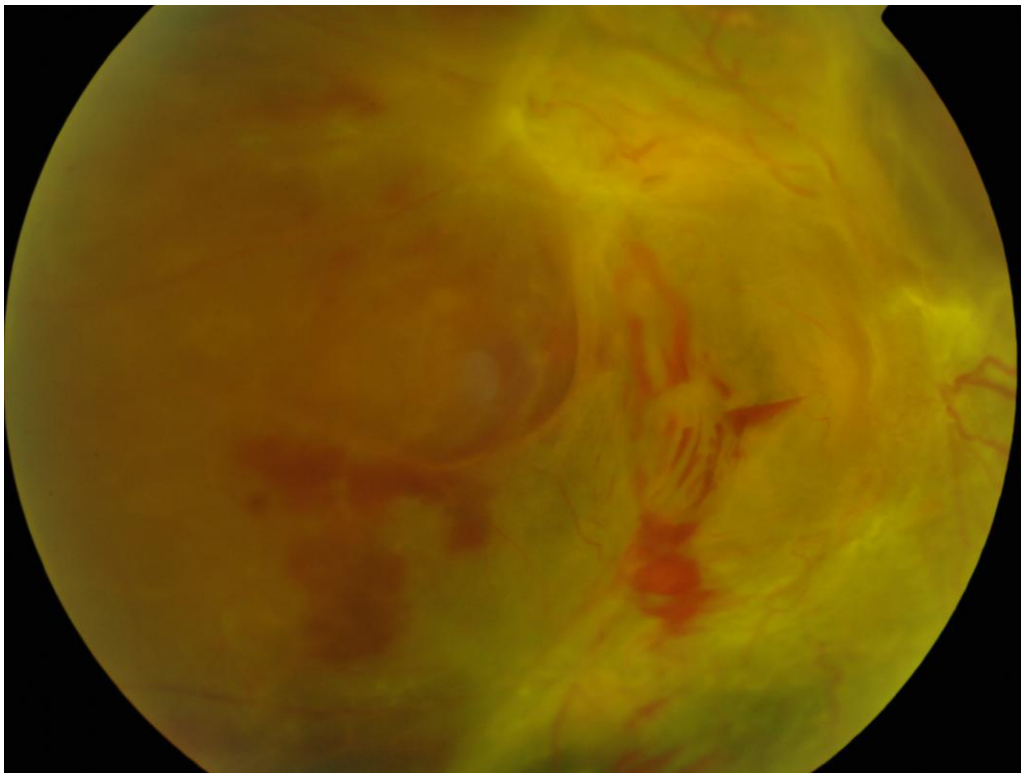


Figure 6 – Colour fundus photograph of a right eye with advanced fibrovascular proliferation and tractional retinal detachment involving the macula.

*1.2.3 **Diabetic maculopathy and diabetic macular oedema***

The microvascular changes of diabetic retinopathy as described above are frequently present in the macular area and this is termed diabetic maculopathy. Typical patterns seen include clusters of microaneurysms around the foveal avascular zone and exudate arranged in a circinate pattern around leaking microaneurysms.

Breakdown of the inner blood-retinal barrier leads to increased vascular permeability and the leakage of fluid into the retina; this is termed diabetic macular oedema (DMO). Clinical examination reveals retinal thickening. The Early Treatment Diabetic Retinopathy Study (ETDRS) provided three definitions of what they termed clinically significant macular oedema (CSMO), used as criteria for laser treatment in

the initial study [4], and shown diagrammatically in Figure 7. CSMO was defined as 1) any oedema occurring within 500 µm of the centre of the fovea (A); 2) oedema associated with exudates lying within 500 µm of the centre of the fovea (B); 3) oedema of area one disc diameter or greater, any part of which lies within one disc diameter of the foveal centre (C). Visual loss in DMO occurs because of disruption of the retinal architecture leading to neuronal dysfunction, but pre-existing pathological changes that are present before the clinically visible lesions probably also contribute.

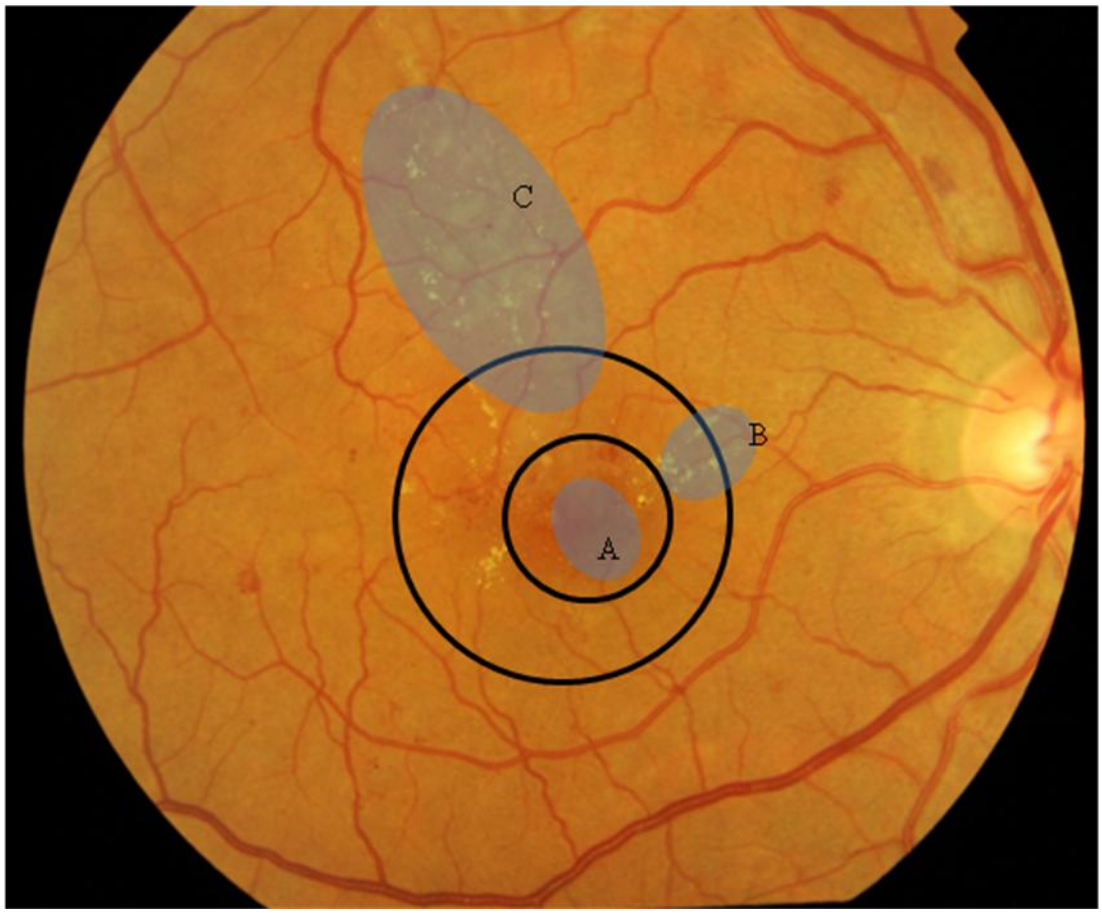


Figure 7 – Diagrammatic representation of CSMO as defined in the ETDRS. Inner circle shows 500 µm radius from fovea; outer circle shows radius of one disc diameter. A – oedema within 500 µm; B – exudates within 500 µm with associated thickening; C – oedema of greater than one disc diameter area, any part lying within one disc diameter of the fovea.

1.2.4 Epidemiology of diabetic retinopathy and maculopathy

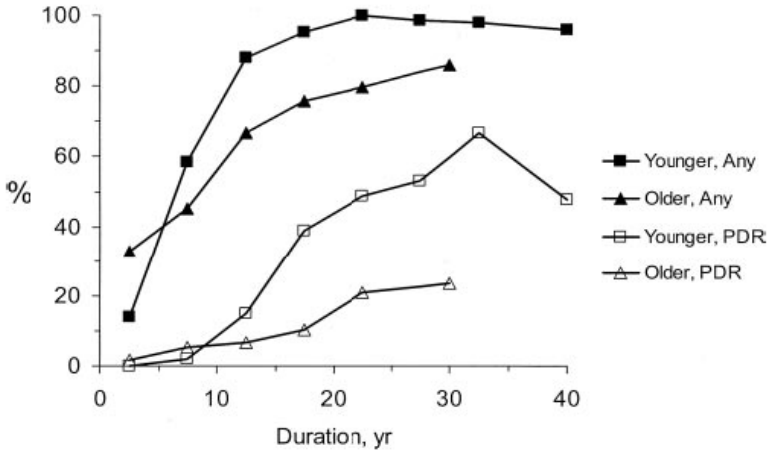
Population-based studies have reported the prevalence of diabetic retinopathy among diabetic patients and have further broken this down to report the prevalence of different grades of retinopathy and sight-threatening retinopathy necessitating referral to an ophthalmologist. The studies show that significant numbers of patients with diabetes have retinopathy and that this may present at an advanced stage.

The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) was designed to report the prevalence of retinopathy and identify risk factors for eye disease by studying patients with diabetes in southern Wisconsin. From 10,135 eligible patients, a sample of 2990 was studied, comprising a younger onset group taking insulin (approximately equivalent to type 1) and a group diagnosed over the age of 30 (approximately equivalent to type 2). Prevalence of retinopathy and of maculopathy in these two groups is shown in Table 2.

	Young onset (diagnosis before 30 years, taking insulin) n=1210 / %	**Older onset (diagnosis over 30 years of age) n=1780 /%**
Any retinopathy	71	50
Proliferative retinopathy	23	5
Diabetic Retinopathy Study high-risk characteristics for visual loss	10	2

Table 2 – Prevalence of retinopathy in the WESDR at baseline [5, 6] summarised in [7].

A major finding from the study was that the prevalence of retinopathy increased with increasing duration of diabetes. This applied to both the younger and older onset groups and to severity of retinopathy. Duration of disease was also found to be a risk factor for the prevalence of DMO, again in both younger and older onset groups, shown in Figure 8A and B.



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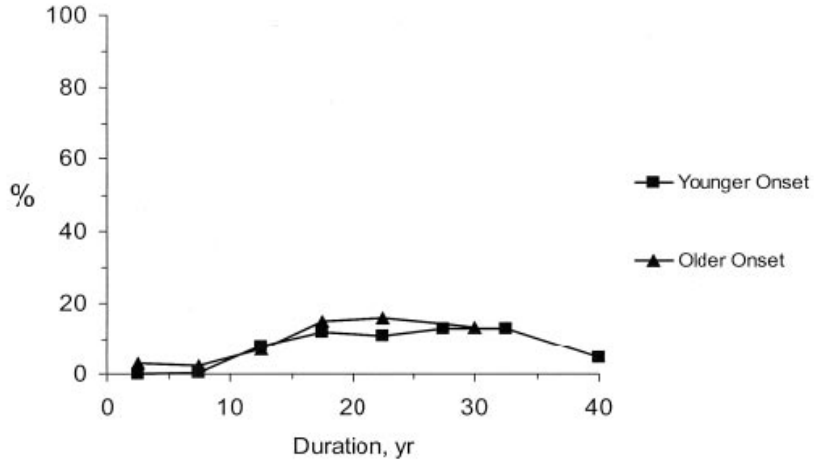

B

Figure 8 – Increase in prevalence of retinopathy and proliferative retinopathy (A) and clinically significant macular oedema (B) with increasing duration of diabetes in both groups from WESDR. Taken from [7].

In the UK, one of the earliest population-based studies arose from examining the population of Melton Mowbray in Leicestershire. This research examined the eyes of diabetic patients requiring insulin in 1987 and found a population prevalence of 10.9/1000 for any diabetes. Of the 113 patients examined, 50% had no evidence of retinopathy, but amongst the remaining patients 9% had evidence of proliferative or advanced diabetic eye disease and 8% had untreated maculopathy [8]. This study found that risk factors for severity of retinopathy were duration of diabetes, elevated blood pressure and age at examination.

The Liverpool Diabetic Eye Study reported the prevalence of different grades of diabetic retinopathy and maculopathy before the introduction of a retinopathy screening programme. The point prevalence of diabetes was 12.4/1000 and 33.6% of patients examined had retinopathy [9]. Of note, significant levels of retinopathy that could require treatment or maculopathy were present in 4.5% and 9.2% of patients respectively, with 13.4% of patients examined graded as having sight-threatening eye disease. At entry into the screening programme in Liverpool, diabetic retinopathy status was evaluated in 8062 patients with diabetes. Prevalence of diabetes overall was 1.8% and prevalence of different categories of retinopathy is shown in Table 3.

	Type 1 diabetes (%)	**Type 2 diabetes (%)**
Any retinopathy	45.7	25.3
Proliferative retinopathy	3.7	0.5
Sight-threatening disease	16.4	6.0

Table 3 – Prevalence of retinopathy at entry into screening in Liverpool, UK [10]

Screening for diabetic retinopathy and its impact are discussed further in Section 1.5.1. It is clear, however, from the findings of the Liverpool Diabetic Eye Disease Study and the Melton Mowbray study, that without a systematic programme to detect early diabetic eye disease, a significant number of patients will miss out on treatment for sight-threatening disease.

1.3 **Pathophysiology of diabetic retinopathy**

Studies into the epidemiology of diabetes demonstrated that duration of diabetes was a major risk factor for diabetic retinopathy. This important finding sheds light on the possible pathogenic mechanisms underlying the development of retinopathy. While previously it had been thought that hypertension co-existing with diabetes was responsible for the development of vascular abnormalities, it now became apparent that changes in the vasculature develop as a result of the cumulative effects of hyperglycaemia. The WESDR aimed to establish this link between hyperglycaemia and progression of retinopathy and showed that the relationship between increasing glycosylated haemoglobin level and retinopathy severity was present in both younger and older onset groups with diabetes, providing evidence that glycaemic level was more important than type of diabetes in the development of this complication [7, 11].

Although the initiating event in the development of diabetic retinopathy may be chronic hyperglycaemia, the exact pathophysiology is complex and controversies exist concerning the most important factors and events. Hyperglycaemia leads to the accumulation of advanced glycation end-products such as sorbitol and the activation of a number of interconnected biochemical mechanisms. Some of these are shown in Figure 9. Implicated biochemical pathways include oxidative stress, the polyol and hexosamine pathway, and the activation of protein kinase C isoforms.

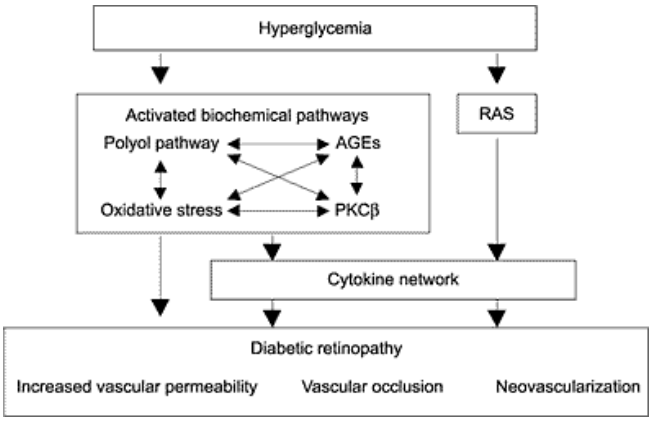

Figure 9 – Interaction between biochemical pathways in the pathophysiology of diabetic retinopathy. From [12].

The biochemical pathways activated by hyperglycaemia alter the cytokine profile in the retina, recruiting leukocytes as part of the inflammatory response, and in turn mediate damage to endothelial cells and pericytes, which leads to the visible clinical lesions of microvascular disease. Thickening of the basement membrane of capillaries in the inner and outer retina leads to a loss of their barrier function and leakage of plasma into the retina. However, the metabolic and biochemical changes that are present in the retina do not only affect blood vessels, but also affect the network of retinal neurons. Therefore, it is helpful to think of pathological changes in diabetic retinopathy as affecting the entire neurovascular unit, defined as the physical and biochemical relationship among the retinal neurons, glia and associated vasculature [13]. Disturbance in the retinal vasculature is likely to lead to neuronal dysfunction because of the high metabolic activity of the retina, which makes it susceptible to changes in the supply of oxygen [14, 15]. Oxygen sensing occurs through activation of transcription factors such as the hypoxia inducible factor (HIF) which in turn alters cytokine activation with further downstream effects. The dual blood supply of the retina means that changes to oxygen supply may affect the inner and outer retina differently. Photoreceptors receive a copious blood supply from the highly vascular choroid, but the inner retina containing second and third order neurons and glial cells has a comparatively sparse blood supply. Biochemical disruptions leading to vascular changes in the inner retina are therefore liable to affect inner retinal neurons. The resulting dysfunction may manifest as reduced vision and reduced indices of other tests which depend on inner retinal neuronal function such as colour vision and electrophysiology, discussed further in section 2.1.

Retinal structure and function is maintained in part by intact inner and outer blood-retinal barriers. The outer blood-retinal barrier is formed by the tight junctions of the *zonula occludens* of the retinal pigment epithelial cells, while the inner blood-retinal barrier is formed from the tight junctions of capillary endothelial cells. When vascular disease leads to the breakdown of this inner blood-retinal barrier, macular oedema occurs. The increase in permeability of retinal capillaries is mediated by an activated cytokine network and in particular a rise in vascular endothelial growth factor (VEGF), discussed further below. Protein kinase C (PKC) is an important

mediator of this aspect of the pathology: phosphorylation of isoforms such as PKC-β activated by VEGF result in breakdown of the tight junctions required to maintain vascular integrity, through endocytosis of occludin proteins [16]. PKC inhibitors such as ruboxistaurin have been investigated as possible therapies for macular oedema.

The prolonged biochemical insult of hyperglycaemia leads to vascular occlusion and progressive capillary loss. This results in impaired oxygen delivery and gives rise to the clinical situation of macular ischaemia. This manifests clinically as enlargement of the foveal avascular zone (FAZ) and loss of the perifoveal capillary network which can be seen on fluorescein angiography, for example in Figure 10. Visual acuity is likely to be reduced and other indices of retinal function similarly affected.

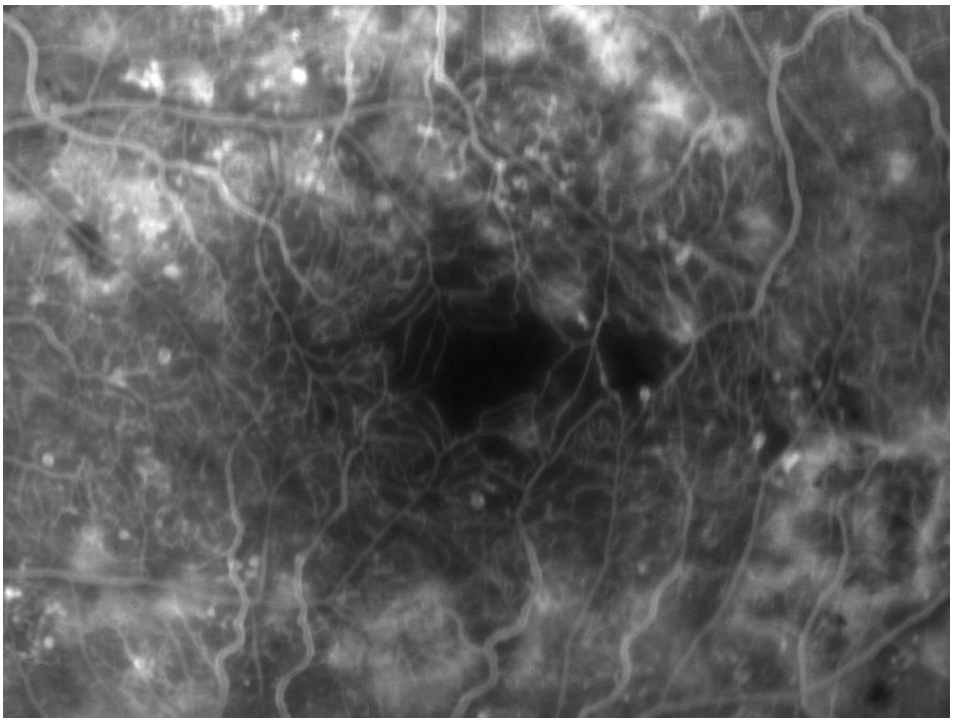

Figure 10 – Detail of macula from fundus fluorescein angiogram to show partial loss of perifoveal capillary network in patient with diabetic macular oedema and macular ischaemia. Foveal avascular zone size is 650 μm – upper limit of normal.

In parallel to the changes in blood-retinal barrier status that are seen in diabetic retinopathy, the accumulation of pro-angiogenic growth factors such as VEGF leads to the formation of new blood vessels which are abnormal in both structure and permeability. These are the hallmark of proliferative diabetic retinopathy.

In summary, although complex pathways and multifactorial biochemical changes are implicated in the pathogenesis of diabetic retinopathy, VEGF has emerged as a key cytokine released in response to hypoxia and ischaemia. It has been shown to be an activator of inflammatory pathways and some of the biochemical pathways noted above such as protein kinase C. Its biology and role in angiogenesis and increased vascular permeability is discussed below.

1.4 Vascular endothelial growth factor – a mediator of angiogenesis and vascular permeability

Vascular endothelial growth factor-A (VEGF-A, subsequently referred to as VEGF) is one member of a family of growth factor proteins including VEGF-B, -C, -D, -E and placental growth factor (PlGF). It is an angiogenic and vascular permeability factor, and because these processes underlie pathological changes in a number of ocular diseases including diabetic retinopathy, VEGF inhibition has become an important therapeutic modality in their treatment.

1.4.1 VEGF biology

The discovery of VEGF arose through the observation that angiogenesis was an inherently important factor in the growth of tumours. Michaelson proposed that a diffusible factor ("Factor X") in the eye was responsible for neovascularisation in diabetic eye disease [17], and in parallel investigations, Ashton and Cook at the Institute of Ophthalmology in London demonstrated that a diffusible factor could induce corneal neovascularisation [18]. It was subsequently shown that a diffusible factor was also implicated in angiogenesis in tumours. Initial candidate molecules for angiogenesis included epidermal growth factor, tumour necrosis factor and transforming growth factors α and β. These molecules are all important in the landscape of angiogenesis but were not able to induce endothelial cell proliferation alone. The basic and acidic fibroblast growth factors (FGF) were found to be capable of inducing angiogenesis through an action on endothelial cells, but importantly are not secreted by cells and so do not fit with observations of a diffusible factor.

The search for a diffusible factor led to the first identified function of the molecule now known as VEGF: as a vascular permeability factor, when it was demonstrated that ascites fluid collected from guinea pigs with liver tumours could induce vascular permeability [19]. Ferrara *et al.* discovered and sequenced a heparin binding growth factor that fulfilled the criteria for a soluble growth factor and was able to induce angiogenesis through mitogenic activity on endothelial cells [20]. They termed this

VEGF. At the same time, sequencing of the vascular permeability factor previously described revealed that this was the same molecule [21].

VEGF exerts its action through two main tyrosine kinase receptors: VEGFR-1 (flt-1), VEGFR-2 (KDR) and neuropilin co-receptors. Interaction with the different receptors conveys different functions in physiological and pathological angiogenesis, comprehensively reviewed in [22].

VEGF exists in different isoforms achieved by differential splicing of mRNA, shown in Figure 11. Different isoforms are referred to as $VEGF_{xxx}$, where xxx refers to the number of amino acids present in the protein, ranging from 121 to 206, with $VEGF_{165}$ being a particularly common form. Exons 1-4 are conserved between isoforms while variations in exons 5-8 affect the properties of the molecule. In particular, exon 8 controls endothelial cell mitogenic activitiy, illustrating that some of the activities of VEGF may be isoform dependent. A change in the distal splice acceptor site in exon 8 gives rise to new variants of the $VEGF_{xxx}$ molecules which have been termed $VEGF_{xxxb}$, and were first identified in a renal cell carcinoma line [23]. These may have different properties from the conventional $VEGF_{xxx}$ isoforms and may play a role in the maintenance of vascular physiology in health rather than disease.

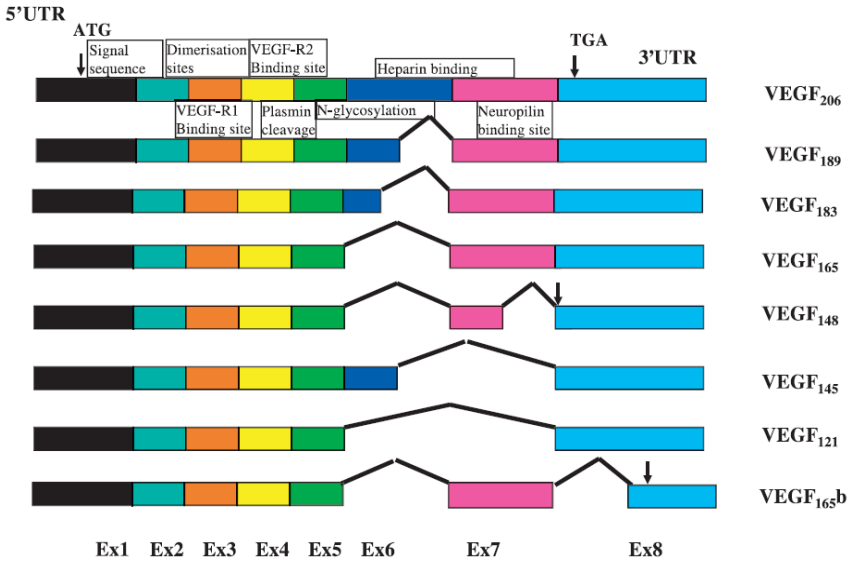

Figure 11 – Exon structure of different VEGF isoforms to show how differential splicing leads to their formation. UTR – untranslated region. From [24].

1.4.2 ***VEGF in diabetic retinopathy***

Experimental evidence specific to diabetic retinopathy showed that VEGF activity in the eye reprises its role elsewhere in the body as an angiogenic and vascular permeability factor. Additional evidence for a neuroprotective action in the retina reinforces the link between neurons and vasculature in the concept of the neurovascular unit.

Aiello *et al.* first showed that VEGF was present in the ocular fluid of patients with neovascular disorders including diabetic retinopathy [25]. Concentrations of VEGF correlated with the severity of the ocular disease, with highest levels present in subjects with active proliferative diabetic retinopathy or active iris neovascularisation. In subjects who had undergone laser photocoagulation to reduce disease activity, VEGF levels were raised in the early phase and then lowered. This correlation between activity and VEGF level suggested that inhibition of VEGF might be a possible therapeutic modality. In a mouse model of ischaemia-induced retinal neovascularisation, neutralising VEGF using a chimeric receptor protein significantly reduced the area of neovascularisation compared with control eyes [26], and in non-human primates, inhibiting VEGF blocked the development of iris neovascularisation [27]. The observation that a state identical to diabetic retinopathy could be induced in the eyes of primates by injections of VEGF provided further evidence that VEGF is a causative factor in diabetic eye disease [28].

While neovascularisation is an important cause of visual loss in retinal vascular disease, macular oedema from breakdown of the inner blood retinal barrier leads to reduced vision in greater numbers of patients with diabetes, as shown by the epidemiological studies discussed above. VEGF as a vascular permeability factor is also implicated in the development of macular oedema. Immunohistochemical studies demonstrate the presence of VEGF in retinal capillaries affected by diabetes [29], and show that these capillaries have increased permeability. Qaum *et al.* demonstrated that it had a direct causative role in the induction of vascular permeability in diabetes by showing that messenger RNA levels for VEGF were elevated in rats with early diabetes and that this corresponded temporally to breakdown in the inner blood retinal barrier [30]. Furthermore, this increased

vascular permeability could be reversed in a dose-dependent fashion by systemic administration of a high affinity chimeric protein known as VEGF-Trap, comprised of soluble VEGF receptor and F_c proteins. This represents compelling evidence for the direct involvement of VEGF in blood-retinal barrier breakdown.

While these findings demonstrate the direct role of VEGF on the vasculature, both in terms of leading to neovascularisation and inducing vascular permeability, there is also evidence that VEGF acts on retinal neurons which adds a new dimension to the understanding of the pathological effects of its upregulation and possible consequences of inhibition. For example, *in vivo* work in the rat has shown that administering VEGF isoforms following an ischaemia-reperfusion injury leads to a reduced number of apoptotic cells in the retina [31]. In addition to a reduction in apoptosis, increased blood flow resulted and an importance in ischaemic preconditioning was demonstrated. Secondly, systemic neutralisation of VEGF by sFlt1 (soluble VEGF receptor expressed by adenoviral vectors) led to increased apoptosis of retinal neurons in the mouse [32].

1.4.3 ***Activation of VEGF pathways by hypoxia and ischaemia***

VEGF is of critical importance in physiological angiogenesis as VEGF receptor knockouts are lethal and even knockout of a single allele of the *Vegf* gene in mice is lethal embryonically. While physiological angiogenesis represents a large and important area of study, it is the activation of VEGF and its role in pathological angiogenesis, such as in cancerous tumours and ocular disease that is relevant to the current study. For example, in human glioblastoma cell lines, VEGF and its *flt* receptor are both upregulated and are present around areas of tumour necrosis [33]. Furthermore, demonstration that VEGF mRNA upregulation occurs in response to hypoxia in these tumours and in other cell lines [34] suggests that hypoxia sensing is an important component of the angiogenic response. Investigation into the effect of hypoxia on human retinal pigment epithelial cells demonstrated a similar response: these cells synthesise increased levels of mRNA for VEGF and release soluble forms of VEGF after a hypoxic stimulus [35].

The link between hypoxia in tumour tissue or in the retina and the increased expression of VEGF seen in these tissues is now understood to be mediated by transcription factors which are altered in the presence of hypoxia, termed hypoxia inducible factors (HIFs). HIF-1, for example has been shown to be responsible for increased transcription of VEGF in hypoxic conditions [36]. HIF-1α dissociates from the product of the Von Hippel Lindau tumour suppressor gene in conditions of hypoxia and is then able to bind to hypoxia responsive elements of nuclear DNA together with HIF-1β. This in turn leads to activation of a number of target genes including those connected with angiogenesis. While hypoxia is an important factor in activating the HIF pathway, inflammation also leads to downstream effects mediated by this mechanism. Therefore in diabetic retinopathy, where vascular disease leads to tissue hypoxia and the upregulation of inflammatory pathways also occurs, the HIF pathway may be central to the pathophysiology.

Greater understanding of the signalling pathway for hypoxia and the cytokines involved in this gives rise to the possibility of new therapeutic targets in diabetic retinopathy. For example, the mammalian Target Of Rapamycin protein (mTOR) is a tyrosine kinase that induces expression of the hypoxia inducible factor and hence VEGF further downstream, and so inhibition of this target could potentially reduce VEGF activity. Rapamycin (sirolimus) inhibits the mTOR binding complex and a Phase 1 study of 50 patients receiving sirolimus either subconjunctivally or by intravitreal injection revealed no significant safety concerns [37]. Further results from study of this drug are awaited, to better understand its possible role in the treatment of diabetic retinopathy.

*1.4.4 **Summary***

VEGF is the soluble mediator of angiogenesis and vascular permeability first proposed over sixty years ago. Oxygen sensing in the retina leads to its upregulation through changes in the structure of transcription factors which also activate other cytokines involved in the complex angiogenic pathway. VEGF is strongly implicated in the pathogenesis of diabetic retinopathy and so inhibition of its activity is a potential therapeutic target. Its role in health and disease is complex, meaning that

VEGF inhibition risks blocking important physiological functions. The evidence for VEGF as a neuroprotective factor and its possible role in maintaining a healthy circulation in the retina means that in macular ischaemia in particular, there is a theoretical risk that VEGF blockade could accelerate the disease process.

1.5 Treatment of diabetic eye disease

Successfully treating diabetic eye disease begins with prevention through appropriate systemic management of the patient. Early retinopathy changes may not cause visual loss and may be present for considerable time. Screening programmes are therefore necessary to identify the development of potentially sight-threatening retinopathy, which is frequently asymptomatic in its initial stages but requires prompt treatment if visual loss is to be prevented. Continued systemic control of diabetes remains important as eye disease is treated; retinopathy may be the first indicator of microvascular disease elsewhere in the body and may occur following sub-optimal control. Historic treatment of retinopathy included pituitary ablation [38] while the introduction of laser treatment for proliferative retinopathy and maculopathy was shown to prevent sight loss. Drugs delivered by the intraocular route include steroids and anti-VEGF agents; these address the molecular cause of the disease.

1.5.1 ***Screening***

There is now a well-established national programme in the UK to screen patients with diabetes over the age of 12 for retinopathy. The National Health Service (NHS) Diabetic Eye Screening Programme offers annual digital fundus photography to all patients with diabetes over the age of 12 years. Two images of 45° field are captured by the photographer for grading. The clinical grading of retinopathy used by the screening programme is based on the original ETDRS grading scheme and consists of mild and moderate non-proliferative diabetic retinopathy (previously termed background diabetic retinopathy), severe non-proliferative retinopathy (pre-proliferative diabetic retinopathy) and non-high-risk and high-risk proliferative diabetic retinopathy. If potentially sight-threatening retinopathy is identified, referral to a specialist eye unit for further assessment and treatment is organised to take place within a pre-specified time frame.

Clinical outcomes for screened patients

Patients with diabetes but without evidence of retinopathy are graded as R0. Those with retinopathy not meeting the criteria for referral to ophthalmology (“non-referable retinopathy”) are graded as R1 and both of these groups are invited to

return for annual photography. The main purpose of the screening programme is to identify those with the potential for sight-threatening diabetic retinopathy, which includes severe non-proliferative and proliferative retinopathy (grades R2, R3) with or without the additional presence of maculopathy (M1). These patients are either referred to a Hospital Eye Service, to a digital imaging surveillance clinic, or for clinical examination in a dedicated Slit Lamp Bio-microscopy Service [39].

Standards are in place to ensure the prompt assessment of patients identified as having sight-threatening retinopathy depending on the severity: in cases of active proliferative diabetic retinopathy (R3a), patients must be seen in an eye clinic within a maximum of 4 weeks and, if needed, treated by laser panretinal photocoagulation within another 2 weeks. In the presence of M1 (referable maculopathy), R2 (referable retinopathy) or treated proliferative diabetic retinopathy with no active lesions (R3s), an appointment within 13 weeks must be offered in the hospital eye service or a surveillance clinic., in order to confirm the diagnosis and instigate treatment if necessary.

1.5.2 Systemic control in the treatment of diabetic eye disease

There is persuasive evidence from large, well-designed randomised controlled trials that improved glycaemic control has beneficial effects on the development and progression of retinopathy in both Type 1 and Type 2 diabetes.

The Diabetes Control and Complications Trial (DCCT) established that intensive glycaemic control in Type 1 diabetes reduced the risk of development of retinopathy (primary prevention) and slowed its progression in a group with mild retinopathy at baseline (secondary prevention) [40]. A total of 1441 patients with no retinopathy or mild non-proliferative changes were randomised in two cohorts to either insulin pump therapy or regular insulin injection. In the primary prevention cohort, the adjusted mean risk of retinopathy was reduced by 76% with intensive therapy, although the two groups were similar for the first 36 months [40]. Intensive therapy in the secondary progression group reduced the risk of three-step progression by

54%, also reducing the risk of proliferative disease and the need for laser photocoagulation.

For patients with Type 2 diabetes, the UK Prospective Diabetes Study (UKPDS) showed that intensive diabetic control reduced the risk of needing retinal photocoagulation (relative risk ratio compared with conventional treatment = 0.71). Additionally, a smaller proportion of patients treated intensively had a two-step progression in retinopathy after 6 years of follow-up [41].

Observational studies following both of these trials showed that the beneficial effect of strict glycaemic control is maintained even if metabolic control subsequently deteriorates. In the Epidemiology of Diabetes Interventions and Complications (EDIC) study, which followed the Diabetes Control and Complications Trial cohort, there continued to be a significantly lower risk of retinopathy progression in the group previously receiving intensive control. Despite HbA_{1c} (glycosylated haemoglobin) levels converging, the risk of a 3-step progression in retinopathy was reduced by 75% after 4 years [42]. In the UKPDS, differences in HbA_{1c} levels had been lost after 1 year of extended follow-up, but after 10 years there was still a 24% risk reduction for microvascular disease in the sulphonylurea–insulin group [43]. This ‘metabolic memory’ phenomenon or ‘legacy effect’, where early optimal glycaemic control has long-term beneficial effects, may be related to reduced accumulation of advanced glycation end products that are formed during periods of hyperglycaemia and lead to vascular stiffness and dysfunction [44].

The UKPDS also examined the effect of intensive blood pressure control (< 150/85 mmHg) with captopril or atenolol on microvascular complications in 1148 hypertensive patients with Type 2 diabetes [45]. By a median of 7.5 years, there was a 34% reduction in risk of a two-step deterioration in retinopathy grade in the intensively treated group. All of the studies discussed used standard ETDRS photographs assessed by masked graders to determine retinopathy progression. In contrast to the legacy effect seen following intensive glycaemic control, there was not a persistent reduction in risk of microvascular disease when strict blood pressure control was not maintained after the end of the randomised study [46].

Angiotensin-converting enzyme inhibitors have also been shown to have a beneficial effect on retinopathy development in patients with Type 1 diabetes independently of their reduction in blood pressure. The EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus (EUCLID) study demonstrated a 50% reduction in risk of a one-step retinopathy progression in patients treated with lisinopril [47]. Retinopathy progression was a secondary endpoint in the Renin Angiotensin System Study (RASS), which demonstrated 65 and 70% reductions in risk of progression for normotensive patients with Type 1 diabetes treated with enalapril and losartan, respectively [48]. The Diabetic Retinopathy Candesartan Trials Programme (DIRECT) was specifically designed to investigate the effect of an angiotensin receptor blocker on the incidence and progression of retinopathy. In patients with Type 1 diabetes, candesartan reduced the incidence of retinopathy but did not significantly affect progression [49]. Similarly, in DIRECT-Protect 2, there was no reduction in progression of retinopathy in patients with Type 2 diabetes receiving candesartan, but regression was increased in this group by 34% [50].

Studies such as the Heart Protection Study have demonstrated conclusively that statins reduce the risk of cardiovascular events in patients with diabetes [51], so trials of statins specifically to look at their effect on retinopathy are lacking. However, evidence is emerging that the addition of a fibrate to pre-existing statin therapy does have a beneficial effect on diabetic retinopathy. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study examined need for laser retinopathy as a tertiary outcome in a trial of fenofibrate therapy to reduce cardiovascular disease; more patients in the control arm required one or more laser treatments [52]. The ACCORD-Eye study not only demonstrated reduced risk of retinopathy progression with intensive glycaemic control in patients with Type 2 diabetes, but also showed a 40% reduction in relative risk of retinopathy progression with the addition of fenofibrate to simvastatin [53].

Taken together, the extensive investigations into systemic glycaemic control in diabetes accompanied by good blood pressure and lipid management show that biochemical changes in the whole body can influence the degree and progression of diabetic retinopathy. This provides indirect clinical confirmation of laboratory

studies which reveal the multifactorial nature of the pathophysiology of diabetic retinopathy.

1.5.3 Laser treatment for diabetic retinopathy

Mild to moderate non-proliferative diabetic retinopathy is typically managed by observation. The development of sight-threatening lesions in the form of proliferative diabetic retinopathy or macular oedema is an indication for treatment; for 30 years laser photocoagulation of the retina was the mainstay of this and continues to have a role today.

1.5.3.1 ***Proliferative retinopathy***

The Diabetic Retinopathy Study (DRS) showed that performing panretinal laser photocoagulation was an effective treatment for patients with high risk proliferative retinopathy, as subjects who were randomised to peripheral scatter laser treatment had a 50% lower risk of severe visual loss after five years [54]. The DRS defined high risk characteristics for visual loss as moderate or severe new vessels on the disc or nearby; mild new vessels on the disc associated with haemorrhage, and new vessels elsewhere associated with haemorrhage. This study still informs treatment today, although the technique of laser has changed so that direct treatment to new vessels is no longer performed.

Laser photocoagulation exerts its effects by a number of mechanisms, reviewed in detail by Stefánsson [55]. The destruction of photoreceptors in ischaemic retina reduces peripheral retinal oxygen demand and rebalances the circulation to improve blood flow to the central retina. It is also probable that improved diffusion from the choroid through laser scars contributes to improved oxygenation of the retina and downstream effects on the vasculature that not only reduces the stimulus for neovascularisation but decreases macular oedema by reducing vessel permeability.

1.5.3.2 ***Diabetic macular oedema***

The ETDRS demonstrated that laser treatment for clinically significant macular oedema, as defined above, reduced the chance of moderate visual loss at 3 years by half, with 12% of treated eyes experiencing a drop in acuity of two lines compared to

24% of untreated eyes [4]. Fluorescein angiography can be used to guide laser treatment which is now performed in a modified manner to the original grid method described in the ETDRS. Focal laser is applied to focal leaks such as microaneurysms while a grid pattern is applied to diffuse leaks and areas of capillary non-perfusion that are not contiguous with the foveal avascular zone. Focal laser causes heating of the retinal pigment epithelium (RPE), activation of matrix metalloproteinases and may lead to gliosis and scarring. Closure of microaneurysms directly by laser is not required as the changes in the RPE may be sufficient to alter the retinal capillaries by improved oxygenation and autoregulation. Treatment should be applied with caution in macular ischaemia and should generally be performed at least 500-750 μm from the foveal centre [56, 57].

Recent evidence for the long term benefit of laser came from the extended follow up of patients in the Diabetic Retinopathy Clinical Research network's (DRCR.net) trial of triamcinolone versus laser for DMO [58]. In this study, laser-treated patients gained 5 letters (one line on the standard ETDRS chart) of visual acuity at three years from randomisation, although this analysis only included the subset of patients who continued participation in the trial [59].

Lasers used can be in the green-yellow range of wavelengths or in the infrared range when using the diode laser. Conventional laser treatment can cause scars in the retina and theoretically can lead to the development of a scotoma, so there has been recent interest in the micropulse diode laser as a means of limiting collateral damage. A randomised clinical trial investigating this showed that visual acuity outcomes were equivalent with micropulsed laser to conventional green laser, but visible retinal scars occurred less frequently [60]. The choice between green and yellow wavelengths for conventional laser has also been shown by the DRCR.net not to affect outcomes [61].

1.5.4 Medical agents for the treatment of diabetic macular oedema

The complicated pathogenesis of DMO, with interconnected biochemical pathways leading to the activation of a number of different cytokines presents numerous targets for pharmaceutical intervention. Results of trials for some of these, such as the protein kinase C inhibitor ruboxistaurin, have been disappointing, perhaps because of the multifactorial nature of the disease. Mixed evidence exists for the use of steroids, but a growing number of phase 3 trials have demonstrated the efficacy of anti-VEGF agents in this condition.

1.5.4.1 ***Glucocorticoids / steroids***

Steroids exert their effects through multiple pathways. They have a direct anti-inflammatory effect as they inhibit the formation of arachidonic acid by phospolipase-A2 and subsequently reduce activity in the cyclo-oxygenase and lipoxygenase pathways, but they also exert effects on numerous cytokines through intracellular action. They alter vascular permeability through effects on endothelial cell tight junctions and VEGF production. They are therefore attractive agents in theory for a disease like DMO with multiple biochemical pathways involved in the pathogenesis. However, they also cause cataract and raised intraocular pressure in a proportion of patients and this is their principle drawback. The steroids triamcinolone, dexamethasone and fluocinolone acetonide have been investigated as treatments for DMO, with the latter two now available as slow release intravitreal implants, recently reviewed [62].

The DRCR.net laser vs. triamcinolone study randomised participants to 1mg or 4mg triamcinolone or laser treatment, with the primary outcome at two years. Retreatment was performed if indicated every four months. Although the triamcinolone treated patients initially appeared to do better in terms of visual acuity at 4 months, there was no difference between treatment groups after one year. At the two-year primary endpoint laser was found to be superior to triamcinolone treatment with a gain of 1±17 letters in the laser group compared to 2 or 3 letter losses in the 1mg and 4mg triamcinolone groups respectively [58]. This difference was not solely attributable to the development of cataract.

The dexamethasone drug delivery system marketed as Ozurdex® (Allergan; Irvine, California, USA) consists of dexamethasone bound with a biodegradable copolymer of lactic and glycolic acids [63]. Injected through the pars plana of the ciliary body using a 22G injection system, it releases 700 µg of dexamethasone as the polymer degrades, so therapy can be repeated as required. Ozurdex is approved for macular oedema caused by central and branch retinal vein occlusion and for non-infectious uveitis of the posterior segment of the eye, but has also been evaluated for DMO; further studies are underway.

A subgroup analysis of a randomized trial of Ozurdex in multiple causes of macular edema showed in 171 patients with DMO that Ozurdex resulted in a statistically significant difference in the proportion of patients gaining two or more lines at 90 days (33% vs. 12% for untreated controls) [64]. A trial of 55 patients with DMO who had undergone vitrectomy showed statistically significant visual acuity increases of 6.0 and 3.0 letters at 3 and 6 months respectively [65]. To date no trials have been published reporting the longer term efficacy and safety outcomes following repeated administration. Two Allergan-sponsored, prospective trials are in progress to achieve a licence for this formulation of dexamethasone in DMO (NCT00168389, NCT00168337, www.clinictrials.gov).

Iluvien® (Alimera Sciences; Alpharetta, Georgia, USA) is an injectable intravitreal insert, delivered using a 25G injector system and designed to release fluocinolone acetonide for up to three years, which has been evaluated for the treatment of DMO at two doses: 0.2 µg/day and 0.5 µg/day [66]. It has received a marketing authorisation for this indication in a number of European Union member states including the United Kingdom and France.

The FAME study was a sham-controlled double-masked trial to investigate Iluvien in its two different dosages that recruited 951 subjects with persistent DMO. At the 24 month primary endpoint, 28% of patients receiving either dose of the implant had an improvement of 3 or more lines, compared to 16% with sham [67]. Visual acuity improvements were 4.4 or 5.4 ETDRS letters compared to 1.7 with sham. The rate of glaucoma filtration surgery was 8.1%. However, approaching 90% of phakic

patients required cataract surgery, which did not limit their benefit from the implant in terms of final visual acuity. Results from this study mean that Iluvien has recently been approved by the National Institute for Health and Care Excellence for the treatment of chronic DMO, only in pseudophakic patients.

Results of the studies of three agents discussed above show that a visual acuity gain of approximately one line can be achieved with steroid treatment. It appears that longer-acting implants are required for this as triamcinolone injection did not have long-term benefits over laser. The side effects of cataract and raised intraocular pressure make further surgical procedures likely during the course of a patient's disease if steroid treatment is used.

1.5.4.2 *Inhibitors of VEGF*

The importance of VEGF in the pathogenesis of ocular diseases where neovascularisation is a prominent feature led to the development of agents to inhibit VEGF action. These inhibitors were shown in animal models to reverse neovascularisation and blood-retinal barrier breakdown, suggesting a possible therapeutic use in diabetes against both PDR and DMO. Four VEGF inhibitors are currently commercially available and have been investigated in the treatment of diabetic eye disease.

Pegaptanib

Pegaptanib (Macugen®, Pfizer UK Ltd) is a nucleotide apatmer modified by pegylation, designed to bind to $VEGF_{165}$. There is limited evidence for its efficacy in DMO as the manufacturers chose not to continue the process to obtain a licence after the early phase trials.

A phase II study showed in 2005 that pegaptanib injections could improve visual acuity in subjects with DMO, as 34% of those treated with a 0.3mg injection gained at least 10 letters (two lines) at the 36 week primary endpoint [68]. A larger study randomising participants to 0.3mg or sham injection showed similar results, with 36.8% gaining two lines compared to 19.7% receiving sham injection. This study

also showed sustained effectiveness for pegaptanib with a mean gain of 6.1 letters at 2 years [69].

One further noteworthy point is that pegaptanib was the first anti-VEGF agent reported to induce regression of neovascularisation in diabetic retinopathy. In a small subgroup analysis of patients in the phase II trial with neovascularisation at trial entry, 8 of 13 were shown to have regression of new vessels following pegaptanib treatment [70]. This additional effect of anti-VEGF agents has been explored further in analyses of the larger trials of ranibizumab discussed below; a trial in progress is also evaluating ranibizumab for the primary treatment of proliferative retinopathy (DRCR.net protocol R study).

Bevacizumab

Bevacizumab (Avastin®; Genentech, South San Francisco, CA, USA) is a recombinant, humanised mouse monoclonal antibody directed at all isoforms of VEGF. It is an IgG antibody with a molecular weight of 149 kDa and is synthesised in a Chinese hamster ovary cell line expression system. It was initially demonstrated in tumour cell lines that application of this antibody could inhibit VEGF-mediated angiogenesis and tumour growth, leading to the possibility of its use as a therapeutic agent in the control of tumours and neovascular diseases [71]. The vitreous half-life of bevacizumab in rabbits was found to be 4.32 days, while in the aqueous and serum the half-life was 4.88 and 6.86 days respectively [72]. Maximum serum concentration of bevacizumab occurred 8 days after injection and levels remained above 1μg/ml for 29 days. Trans-retinal transport mediated by the F_c portion of the antibody results in the presence of bevacizumab in the systemic circulation.

A randomised controlled trial of repeated bevacizumab therapy compared with standard macular laser therapy for DMO (The BOLT study) suggested bevacizumab treatment to be superior. This trial recruited 80 subjects with centre-involving DMO and randomised them 1:1 to receive either bevacizumab injections six weekly, with three loading injections and then retreatment as required, or macular laser therapy every four months. Bevacizumab treatment resulted in a median gain of 8 ETDRS letters at one year, compared with a drop of a 0.5 letters with laser [73]. Central

macular thickness decreased in both groups and was significantly lower at 12 months than baseline. This decrease was greater in the bevacizumab treated group (-130 µm vs. -68 µm for laser) although this did not reach statistical significance. There was also limited evidence for a beneficial effect of bevacizumab on grade of diabetic retinopathy.

A secondary aim of the study was to investigate the effect of bevacizumab on macular ischaemia, given the theoretical concerns with VEGF inhibition in this condition. Subjects were only included if they did not have significant macular ischaemia at baseline as the greatest linear dimension (GLD) of the foveal avascular zone (FAZ) was required to be less than 1000 µm and perifoveal capillary dropout graded as less than "severe" by the reading centre. After four months of therapy with bevacizumab, there was no detectable increase in macular ischaemia. The FAZ GLD and FAZ area remained the same, with no change in the grade of perifoveal capillary loss in either group [74]. Two year data from the study showed that visual acuity improvements could be sustained with a median of 13 injections over 24 months; bevacizumab treated subjects gained a mean 8.6 letters with almost half gaining two lines [75]. No new safety concerns were identified over two years of the study.

Ranibizumab

Ranibizumab (Lucentis®; Genentech, South San Francisco, CA, USA and Novartis Pharma AG, Basel, Switzerland) is a recombinant, humanised monoclonal antibody fragment, F_{ab} (IgG1 kappa isotype) to all isoforms of VEGF. It is produced by an *Escherichia coli* expression system and has a molecular weight of 48 kDa. Affinity maturation studies carried out on the native Fab, V12, derived a variant termed Y0317 which has a 22-fold higher binding affinity to VEGF [76]. This protein differs by six amino acid residues from the parent antibody and was at least 30 times more potent at inhibiting mitogenic activity in human umbilical vein endothelial cells.

Pharmacokinetic studies for ranibizumab were carried out using the same rabbit model as bevacizumab and it was found to have a half life in the vitreous of 2.88 days [77]. No ranibizumab was detected in the serum or the fellow eye in this experiment. A study to investigate the pharmacokinetics of ranibizumab in cynomolgus monkeys found a half-life of 2.6 days in the vitreous following bilateral

intravitreal injection of 0.5mg [78]. Ranibizumab was detected in the serum at a concentration of 150ng/ml at 6 hours but declined rapidly thereafter. It was also detected in both neural retina and RPE, with a pharmacokinetic profile suggesting levels sufficient to inhibit VEGF activity from monthly dosing.

The clinical evidence base for ranibizumab is now considerably larger than for the previous two agents with a number of phase III trials reporting the effects in a total of thousands of patients; ranibizumab is now licensed in Europe and the USA for DMO. The pivotal trials on which recommendations for treatment are based are discussed below. In addition to the visual acuity improvements seen in the usual primary outcome measures, a number of other useful findings have emerged as part of exploratory outcome data.

The RESTORE study was a randomised, double masked, sham controlled trial that took place in multiple centres across Australia, Europe, Turkey and Canada, and was designed to compare ranibizumab treatment to laser. Patients with visual impairment due to DMO (n=345) were randomised to receive ranibizumab, ranibizumab plus laser or laser alone. The two ranibizumab-treated arms had improvements in acuity of 6.4 or 6.8 letters at 12 months compared to 0.9 letters with laser alone, following a regimen of three initial injections then treatment on an as-required basis. Approximately 23% of patients in the two ranibizumab arms had gained three lines (15 or more letters) of visual acuity compared with 8.2% in the laser arm [79]. In the extension phase of this study when all three groups received ranibizumab, visual acuity was maintained in year 2 with on average a further three or four ranibizumab injections for those previously treated with ranibizumab; previous laser-treated patients gained vision from the 12 month primary endpoint to month 24 (+5.4 letters) [80].

The DRCR.net protocol I study of laser, ranibizumab and triamcinolone included 854 eyes from patients with centre-involving DMO. In this randomised trial an eye could receive ranibizumab with either prompt or deferred laser, laser alone or triamcinolone with prompt laser treatment. “Prompt” in this trial meant around the time of initial treatment, and “deferred” prevented laser from occurring prior to 24

weeks. The two ranibizumab treated groups each gained a mean 9 letters by the one year primary outcome compared with 4 letters for triamcinolone and 3 letters for laser alone [81]. The difference was sustained at two years, shown in
Figure 12. In addition to the superior gain in terms of absolute number of letters, the two ranibizumab groups had the greatest chance of a 10 letter gain (around 40%) and the lowest chance of a 10 letter drop in visual acuity (around 5%). A planned subgroup analysis explored factors that might be associated with improved outcomes. In the ranibizumab groups, visual acuity at baseline was predictive of response as subjects with visual acuity of 65 letters or fewer gained on average more letters than those with better visual acuity at baseline (13 vs. 5 letter gain). This may be attributable to a "ceiling effect": as visual acuity has a theoretical maximum, subjects with better acuity at baseline have less scope to improve. Change in retinal thickness over the study was evaluated by optical coherence tomography (OCT) scanning. At one year, subjects treated with ranibizumab had a retinal thickness reduction of approximately 140 μm from baseline. Retinal thickness at trial entry was also predictive of response: subjects with greater central retinal thickness (≥400 μm) responded better (11 letters vs. 7 letters) to ranibizumab than those with thicknesses <400 μm.

A further finding from the exploratory analysis is worthy of note: triamcinolone with prompt laser treatment was as effective as ranibizumab for subjects who were pseudophakic at baseline. This result, achieved with a median of three injections of triamcinolone, suggests that this relatively cheap drug still has a role in the management of patients with DMO.

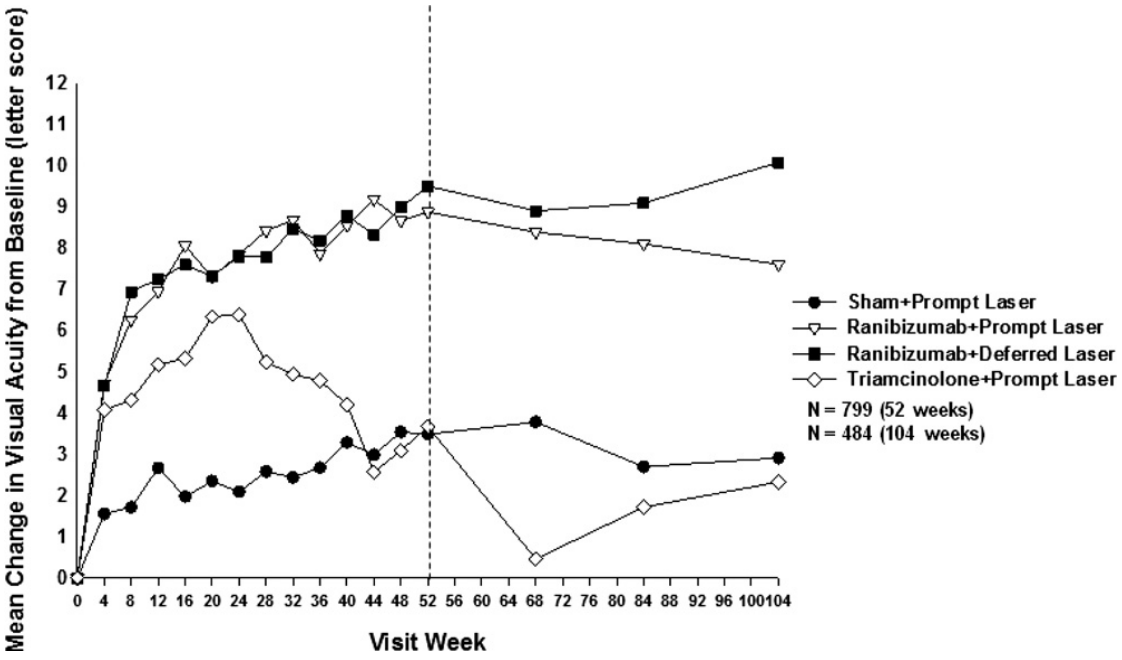

Figure 12 – Change in visual acuity in the DRCR.net protocol I study of ranibizumab, laser and triamcinolone in DMO. From [81].

The separately published two-year results of the trial are complicated by a protocol amendment that occurred subsequent to the publication of the one year primary endpoint meaning that all subjects could switch to receive ranibizumab if they desired. However, they confirmed that the one-year gains with ranibizumab were sustained and suggested a marginal benefit for the group with deferred laser [82]. The sustained visual acuity benefits were achieved with only a further 1-2 injections in the ranibizumab arms, but more subjects in the deferred laser group began to need at least one session of focal/grid laser (investigators judging this were masked to treatment allocation). Therefore, while it appears that performing laser at the outset of treatment confers no additional benefit, it is not possible to infer with certainty that it has no role at all. By three years, evaluating only subjects initially assigned to ranibizumab to provide details of long term effects, the benefit was again sustained with the difference between prompt and deferred laser groups becoming more apparent. If laser were deferred initially, the visual acuity gain at three years was 9.7 letters compared to 6.8 if laser were performed promptly [83]. The trial continues and five year results are awaited.

RISE and RIDE were two identical, randomised, sham-controlled trials of 0.3 mg and 0.5 mg ranibizumab in DMO that formed the manufacturer's application for a licence for the condition in the USA. In contrast to the DRCR.net protocol I study they did not use laser or triamcinolone as controls and they featured mandatory monthly dosing to the primary endpoint at 24 months. The trials showed that monthly dosing with ranibizumab was superior to sham treatment as between 30 and 40% (slight differences between trials and ranibizumab dose) gained three lines by two years; the mean visual acuity gain was 8.5 to 9.9 letters [84]. The visual acuity and OCT results from these two pivotal trials are shown for information in Figure 13.

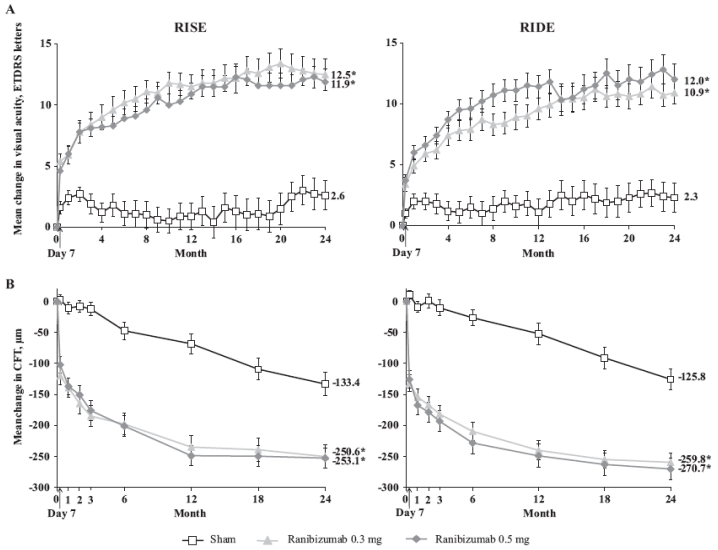

Figure 13 – Visual acuity and OCT results from RISE and RIDE, taken from [84]. CFT = central foveal thickness; * = P<0.0001 compared with sham.

Although not powered to detect a difference between the two doses of ranibizumab used, RISE and RIDE showed no additional benefit for 0.5 mg compared to 0.3 mg so this lower dose is that used in the USA for DMO, while in Europe 0.5 mg continues to be used. The RESOLVE study was an earlier randomised trial that investigated ranibizumab in DMO at these two doses but also allowed the dose to be doubled to 0.6 mg or 1.0 mg based on response; although relatively small numbers were involved it did not show significant differences between doses [85].

In summary, randomised trials of ranibizumab in DMO have demonstrated its superiority over laser. Subjects treated over two years can expect to gain on average between 8 and 10 letters; 30-40% of treated patients should gain two lines. This can

be achieved with injections on an as-required basis as subjects in the DRCR.net trial received approximately 7 injections in year 1 and 2-3 in year 2. Improvements in visual acuity are mirrored by reductions in retinal thickness. Subjects with worse visual acuity and increased macular thickness at baseline are likely to do better. Combining treatment with laser appears to confer no additional benefit and actually may reduce visual acuity gains. The optimal dose and retreatment strategy are not yet fully defined. Grade of diabetic retinopathy is likely to improve in a number of subjects treated with long-term ranibizumab as shown by sub-analyses in RESTORE, DRCR.net and RISE/RIDE trials.

Aflibercept

Aflibercept, formerly known as VEGF Trap-Eye and now marked as Eyelea® (Regeneron Pharmaceuticals, New York, USA) is a fusion protein consisting of the key binding domains of VEGF Receptors 1 and 2 fused with the F_c portion of IgG. It possesses a tight binding affinity for all VEGF-A isoforms and placental growth factor (PlGF).

The DA VINCI trial was a Phase II study that compared four dosing regimens of aflibercept with standard laser therapy in patients with centre involving DMO. At the six month primary endpoint, improvements in the aflibercept groups ranged from 8.5 to 11.4 letters compared to a 2.5 letter improvement in the laser group [86]. Two phase III trials of aflibercept in DMO are currently underway and it is likely that it will have a role in the management of DMO.

Differences between the anti-VEGF agents

Pegaptanib is specific to $VEGF_{165}$ while bevacizumab, ranibizumab and aflibercept bind all isoforms of VEGF. Compared with ranibizumab, bevacizumab is approximately three times larger and has different pharmacokinetic properties. It lasts longer in the vitreous and is present in higher concentrations in the peripheral circulation following intravitreal injection. It also appears to be present in the fellow eye of rabbits receiving a uniocular injection. Ranibizumab, in contrast, has a higher

binding affinity for VEGF and higher potency for inhibiting its action in *in vitro* studies. It is found in all layers of the retina after intravitreal injection. A study in rhesus monkeys to compare the retinal penetration of the F_{ab} form of the VEGF antibody with a full-length antibody showed that the rhuMab HER2 antibody (Herceptin, trastuzumab) did not penetrate further than the internal limiting membrane [87], suggesting that this may also be the case with bevacizumab. The differences in binding between the agents may explain differences in clinical efficacy and safety profile. Aflibercept is designed to have a high affinity for VEGF and has been marketed as being suitable for two-monthly dosing in age-related macular degeneration. Ongoing phase III trials will investigate whether this is the case in DMO, but head-to-head trials of all the agents to evaluate clinical efficacy and safety are desirable.

Safety of anti-VEGF agents

The randomised trials carried out investigating anti-VEGF agents in DMO have not identified any risks particular to the condition. Endophthalmitis occurs in approximately 1 in 1000 injections despite the use of povidone iodine pre-injection [88]. This will likely significantly worsen vision if it does occur. There has been concern in the ophthalmic community for some years about the theoretical risk of anti-VEGF agents entering the systemic circulation and increasing the risk of heart attack and stroke. Pharmacokinetic evidence presented above suggests that this risk is likely to be higher with bevacizumab and aflibercept than ranibizumab. A recent review of rates of stroke from the largest randomised controlled trials in age-related macular degeneration (AMD) provided some evidence of increased risk that was not statistically significant [89]. Subjects receiving bevacizumab in the Comparison of AMD Treatments Trials (CATT) did not have an increased risk of arterial thromboembolic events compared with ranibizumab treated subjects, but did have a higher rate of other serious systemic adverse events not known to be associated with systemic anti-VEGF therapy. However, these occurred in greater numbers in the as-required (*prn*) treatment group. A large, population-based nested case control study examining factors associated with heart attack and stroke in 91,000 patients with

retinal disease did not find that prior treatment with anti-VEGF agents was a risk factor [90].

1.5.5 Surgical treatment of diabetic retinopathy

1.5.5.1 *Vitrectomy for diabetic macular oedema*

Vitrectomy has been advocated for patients with reduced visual acuity due to DMO. Initially it was thought it may be effective in patients with diffuse DMO and without macular traction due to removal of the VEGF reservoir from the vitreous gel. However, it is likely only effective in patients with DMO and co-existent macular traction; when release of the latter during surgery allows the macula to resume a more normal contour which may in turn improve visual acuity. A review of patients in the USA undergoing such surgery found two factors predictive of visual improvement, namely eyes undergoing epiretinal membrane removal (i.e. with traction) and poor initial visual acuity [91], although the latter may have arisen due to a ceiling effect on visual acuity for those with a better level of vision at baseline. However, although the chance of a two-line improvement was 26%, there was a 22% risk of a two-line loss of visual acuity, so the risks and benefits are delicately balanced and surgery may not be in the patient's best interests.

1.5.5.2 *Surgical treatment for proliferative diabetic retinopathy*

As discussed above, the DRS showed that panretinal laser photocoagulation reduced the risk of visual loss in proliferative diabetic retinopathy. However, some patients with proliferative retinopathy may not receive timely treatment, while others may continue to progress without treatment, so that features of advanced diabetic eye disease such as fibrovascular proliferation, vitreous haemorrhage and tractional retinal detachment may develop. If left untreated, these conditions will lead to irreversible loss of vision, so in this situation surgery may be required to prevent this. The Diabetic Retinopathy Vitrectomy Study (DRVS) investigated the outcome of performing early vitrectomy for non-clearing vitreous haemorrhage and demonstrated for the first time an advantage in performing surgery promptly rather than deferring for a year [92]. Since then, advances in surgical technique and a

clearer understanding of the indications for surgery has led to improved outcomes, discussed further in Chapter 3.

The indications for performing vitrectomy surgery in proliferative diabetic retinopathy are: non-clearing vitreous haemorrhage; tractional retinal detachment threatening the macula; combined tractional-rhegmatogenous retinal detachment; and subhyaloid haemorrhage obscuring the macula [93]. The objectives of diabetic vitrectomy surgery are to clear the ocular media and relieve antero-posterior traction on the retina. Firstly, core vitrectomy is performed to clear vitreous haemorrhage and remove the vitreous gel. Dissection of fibrovascular membranes either by segmentation or delamination relieves traction and releases tractional retinal detachment. In some cases the tractional process leads to retinal breaks before surgery (combined tractional rhegmatogenous retinal detachment) although creating further breaks during the separation of delicate tissues is very common. Tamponnade agents are used to secure any retinal breaks, and laser is applied to the retina to prevent further neovascularisation [94]. One of the factors determining the likely success of diabetic vitrectomy is the status of the posterior vitreous in terms of its attachment to the retina. Fibrovascular attachments develop through proliferation of new vessels and associated fibrous tissue. This can result in focal or broad vitreoretinal attachments which require careful separation. While surgery for cases with a complete posterior vitreous detachment and hence no fibrovascular attachments may be technically straightforward, in cases where broad vitreoretinal adhesions are present the outcome may not be as successful. Pre-existing macular ischaemia may also compromise visual results. Pharmacological therapies such as anti-VEGF agents have been used as adjuncts to surgery and their role is also discussed further in Chapter 3.

1.6 **Problems and Aims**

The preceding chapter outlined the clinical problem of diabetic retinopathy and reviewed the pathophysiology and treatment of the two most common sight-threatening complications: diabetic macular oedema and proliferative diabetic retinopathy.

The work presented in this thesis attempts to address some of the clinical issues that remained unresolved, at the time of commencing the work, in these two clinical areas.

*1.6.1 **Diabetic macular oedema***

Modified ETDRS macular grid and focal laser therapy can stabilise vision and reduce the chance of moderate visual loss in subjects with diabetic macular oedema, and if presenting visual acuity is worse than 6/12, carries a 40% chance of a one-line gain in visual acuity [4] and a 20% chance of gaining two lines [58]. However, the RESOLVE study and READ-2 studies were phase 2 trials investigating ranibizumab that suggested the potential for significant visual improvement greater than that achieved with laser [85, 95]. Phase 3 trials were subsequently commenced to assess efficacy, but were not designed specifically to address certain concerns in the medical retina community about the safety of long-term ranibizumab therapy. Therefore the specific aims of the clinical trial presented in Chapter 2 were to address some of these clinical problems.

Problem 1: The effect of repeated anti-VEGF therapy in diabetic macular oedema

Diabetic maculopathy and retinopathy are diseases associated with focal and global retinal ischaemia and animal studies have shown that bevacizumab can lead to ultrastructural changes in the choriocapillaris of non-human primates [96] and increased capillary non-perfusion in rabbits [97]. It is therefore possible that repeated anti-VEGF therapy with the biologically similar agent ranibizumab could lead to changes in peripheral retinal structure via these choriocapillaris alterations and have

a resulting effect on peripheral retinal function. Follow-up from phase 2 studies was not of sufficient duration to exclude the possibility of long-term retinal dysfunction resulting from repeated ranibizumab use.

Aim 1: To investigate the effect on peripheral retinal function of repeated ranibizumab

Reports of electrophysiology following anti-VEGF treatment for DMO are lacking in the literature, with small case series only reporting effects of bevacizumab or ranibizumab on pattern and multifocal ERG (see Section 2.1.1.4). Repeated full-field electroretinography in this trial would allow the effect of ranibizumab on peripheral retinal photoreceptor and neuronal function to be investigated. This investigation should be performed at baseline and at one year to establish the longer term effects of repeated treatment.

Problem 2: Could anti-VEGF agents exacerbate pre-existing macular ischaemia?

Ischaemic diabetic maculopathy is a difficult disease to treat as permanent structural damage has occurred to the fine perifoveal capillary network responsible for the oxygen supply to the inner retina at the macula. The possibility that constitutively expressed physiological isoforms of VEGF exist that may be neuroprotective has been postulated [98]. This raises the possibility that pan-anti-VEGF inhibition by an agent such as ranibizumab could exacerbate macular ischaemia. The first commercially available anti-VEGF agent for intraocular use, pegaptanib, was a selective $VEGF_{165}$ inhibitor, and it was hoped that greater selectivity might obviate potential problems arising from pan anti-VEGF inhibition. Unfortunately clinical experience with this agent is limited in DMO so it is not possible to determine whether this is the case or not. One case report identified progression of disease in a patient with pre-existing ischaemia who received bevacizumab [99], but a report from a randomised trial of bevacizumab in DMO did not find an adverse effect on the foveal avascular zone [74].

Aim 2: To investigate the effect of ranibizumab on macular ischaemia

Carrying out serial fundus fluorescein angiography with grading of the foveal avascular zone size and area, with recording of perifoveal capillary loss, after the methods of Michaelides *et al.*, would address this question.

Problem 3: Visual acuity is only one measure of retinal function in DMO

The changes of diabetic retinopathy affect the entire neurovascular unit, so changes to neuronal function coincide with the vascular pathology that is the hallmark of the disease. Visual acuity tests the function of the retina at the fovea, but does not give information on the function of the surrounding macula. Measures of retinal function that to a greater extent rely on function of the entire macula such as colour vision, pattern electroretinograms and retinal sensitivity from microperimetry have been reported to be reduced in diabetic macular oedema (see Section 2.1). To date these have been assumed to be irreversible, as there is little evidence of changed function following laser treatment.

Aim 3: To investigate the effect of ranibizumab on different retinal functional modalities

Suitable tests to achieve this aim include colour contrast sensitivity, fundus-related microperimetry and pattern electroretinography.

Problem 4: How can clinical retreatment decisions be made based on retinal thickness in DMO?

Phase 3 trials of ranibizumab in DMO and other diseases either adopt a mandatory monthly retreatment schedule (e.g. RISE/RIDE [84]) or employ some form of algorithm based on clinical status to guide retreatment. Such algorithms typically include visual acuity as one measure, but also may employ automated measures of retinal thickness from OCT scans (e.g. DRCR.net protocol i trial [81]). When fixation is poor and OCT devices do not employ eye-tracking software, significant measurement variability can occur between scans and this has been demonstrated in age-related macular degeneration [100]. It is necessary to establish the test-retest variability, or repeatability, of newer spectral domain OCT devices as these are used with increasing frequency in clinic settings.

Aim 4: To determine the repeatability of retinal thickness and volume measurements using Spectralis SD-OCT

Recruiting patients for a clinical trial offers the opportunity to carry out repeated imaging studies, so with multiple scans taken at baseline and subsequently analysed, coefficients of repeatability for scans from this device can be calculated. This will be a first step to developing possible algorithms for guiding clinical practice.

Problem 5: The uncertain role of the choroid in the pathogenesis of DMO and diabetic retinopathy

The choroid supplies oxygen to the outer retina and changes in its thickness have been reported with increasing severity of diabetic retinopathy by several investigators [101-103]. Furthermore, animal studies have shown that permeability of the choroidal vasculature may be altered by VEGF inhibition [96]. Therefore it is plausible that ranibizumab treatment may lead to measurable changes in the choroid which could be relevant to understanding its mechanism of action in DMO.

Aim 5: To investigate the effect of ranibizumab treatment for DMO on choroidal thickness

Using the enhanced depth imaging mode of the Spectralis OCT device, the choroid can be imaged. A preliminary study of changes in choroidal thickness over the course of the trial may help determine whether there is an effect of VEGF inhibition on choroidal thickness, which has been claimed by other investigators [104].

1.6.2 Advanced proliferative diabetic retinopathy

For advanced proliferative diabetic retinopathy, vitrectomy surgery can lead to good visual results, but is technically very challenging and outcomes can be compromised by recurrent post-operative haemorrhage. In this clinical situation, an agent that could facilitate safer and effective surgery and prevent post-operative haemorrhage could lead to better vision for patients. Trials that have investigated bevacizumab have suffered from methodological flaws and have chosen a variety of endpoints.

Chapter 3 presents a clinical trial designed to address the main clinical problems facing surgeons carrying out vitrectomy for advanced PDR and avoid some of the methodological problems seen in earlier trials with bevacizumab.

Problem 1: Patients after surgery may still have poor vision

Longitudinal data from cohorts of patients undergoing vitrectomy for advanced PDR show that a significant number do not regain functional vision in the operated eye [93]. This may be due to recurrent post-operative vitreous cavity haemorrhage, advanced retinal ischaemia or recurrent detachment. There is interest therefore in optimising surgery to improve vision for patients.

Aim 1: To investigate the effect on visual acuity of a pre-operative injection of ranibizumab in patients undergoing vitrectomy for advanced proliferative retinopathy

Choosing visual acuity as the primary outcome measure for the trial is appropriate because it is likely to correlate best with the patient's functional outcome and satisfaction.

Problem 2: Recurrent bleeding can occur both during and after vitrectomy surgery in this condition.

A number of studies have attempted to quantify intra-operative haemorrhage during diabetic vitrectomy using both qualitative and quantitative methods with varying results [105, 106]. There is not convincing evidence to support the routine use of pre-operative bevacizumab or ranibizumab in this condition, so further study to determine a potential benefit for patients is required.

Aim 2: To investigate intra-operative haemorrhage and post-operative haemorrhage

As a secondary outcome measure in the clinical trial, the degree of intraoperative haemorrhage as reported by the surgeon and as measured by quantitative techniques will be reported.

Problem 3: Exacerbation of tractional retinal detachment by pre-operative use of anti-VEGF agents

Investigators have reported new tractional retinal detachments and exacerbation of existing tractional pathology in subjects undergoing pre-operative bevacizumab injection [107, 108]. Again, methodology for identifying this progression and masking of surgeons reporting it has been variable. It is therefore unclear whether this represents a genuine effect of bevacizumab or natural progression of disease.

Aim 3: To investigate the effect of ranibizumab on progression of tractional retinal detachment

Ultrasound evaluation provides an objective means of measuring tractional retinal detachment that can be undertaken even in the presence of significant media opacity. With appropriate masking of the sonographer, measurements before and after administration of ranibizumab will help establish whether the contraction of fibrovascular membranes leads to new pathology.

Problem 4: The pathophysiology of proliferative retinopathy involves many cytokines in addition to VEGF

Diabetic retinopathy includes vascular leakage, occlusion and angiogenesis in addition to chronic inflammation. Numerous cytokines have been found to be upregulated in diabetic retinopathy, with VEGF arguably the most important of these (see Section 3.1.3). There are few opportunities to study the vitreous in these patients and the effect of ranibizumab has not been studied. It is therefore hypothesized that ranibizumab treatment may lead to measurable changes in the cytokine profile.

Aim 4: To record the level of VEGF and related cytokines in patients undergoing vitrectomy for advanced PDR

Sampling of vitreous and plasma and subjecting this to cytometric bead array allows the simultaneous quantification of many vitreous cytokines. This preliminary investigation may highlight cytokines worthy of further study.

2 The LUCIDATE* Study

This chapter outlines the backgrounds, methods, results and discussion of the LUCIDATE study: a randomised controlled trial exploring the detailed functional and structural effects of repeated ranibizumab therapy on diabetic macular oedema.

*LUCIDATE – LUCentis In Diabetic macular oedema – A Treatment Evaluation

2.1 **Background**

As discussed in chapter 1, diabetic macular oedema (DMO) is an important cause of visual loss and treatments until recently have been limited to laser with some evidence for the efficacy of steroids. Now, there is good evidence for the effectiveness of anti-VEGF treatments in this condition from large, well-designed randomised controlled trials. These phase 3 trials, typically involving hundreds of patients, are limited in the number of investigations that can be carried out on individual subjects. They generally take visual acuity as a primary endpoint, often with central retinal thickness from optical coherence tomography (OCT) imaging as a secondary endpoint. To date, there is little knowledge of the results that can be expected from other tests of visual function such as microperimetry, colour contrast sensitivity and electrophysiology following anti-VEGF therapy. The advent of spectral domain OCT imaging means that morphological features of DMO can be identified and changes to these features following treatment can be assessed. Macular ischaemia remains a theoretical concern with anti-VEGF agents so fluorescein angiography continues to be a useful modality for evaluating this. A trial to evaluate the functional and structural effects of ranibizumab in DMO may provide useful information and the answers to some of these questions.

2.1.1 ***Functional tests to evaluate diabetic macular oedema***

In contrast to tests that evaluate retinal anatomy and structure, tests of function are generally more time consuming, require greater patient co-operation and may be subject to more test-retest variability. Aside from widely used visual acuity testing, which is very strongly influenced by foveal function, they are not routinely employed in clinical trials or standard clinical practice. However, in the absence of an unambiguous structural biomarker which is known to correlate well with retinal function, these functional tests may be useful in establishing the effects of treatments. Having accurate and reproducible measures is therefore important for the diagnosis and monitoring of macular disease and for assessing responses to treatments in clinical trials.

2.1.1.1 *Visual acuity testing*

High contrast visual acuity is typically chosen as the primary endpoint for the large clinical trials of treatments for DMO discussed in Chapter 1. Several charts are used in routine clinical practice to assess visual acuity. Although used very commonly, the Snellen chart, created by Hermann Snellen in 1862 has a number of disadvantages that make it less suitable for clinical trials work. This chart utilises black letters, termed "optotypes", on a white background for maximal contrast. Each optotype is arranged on a 5x5 grid subtending 5 minutes of arc, with gaps in the letter structure required for discrimination subtending a visual angle of 1 minute of arc. However, there are different numbers of letters on each line and the spacing is not regular. Thus the chart is poor for accurate assessment of the lower end of the visual acuity spectrum and does not provide a continuous analysis of acuity. In 1976, Ian Bailey and Jan Lovie published their eponymously named chart featuring a new layout of 5 letters per row with spacing between letters and rows equivalent to the letter size. This was created to eliminate the crowding effect and standardise the number of errors that could be made on each line. The character size change is a logarithmic progression of letter sizes and the chart output is given as the logarithm of the minimum angle of resolution (LogMAR).

The advantages of the Bailey-Lovie LogMAR chart led to the development of standardised visual acuity charts for clinical trials use. Designed for use in the Early Treatment Diabetic Retinopathy Study, these "ETDRS" charts consist of three separate sets of Sloan letters based on the LogMAR chart characteristics [109]. Different charts are provided for right and left eyes and a third chart can be used during refraction prior to testing best-corrected acuity. These charts are now widely used in clinical trials and visual acuity is typically reported as a letter score from 0 to approximately 95.

2.1.1.2 *Colour vision testing*

Colour vision defects are an important cause of reduced visual function and have occupational implications for patients with diabetes. Reports of reduced colour vision in diabetes date from the 1970s.

The Farnsworth-Munsell 100-Hue test has been used for several decades to document colour vision defects and was employed in the ETDRS [110]. The test consists of arranging 85 numbered caps of equal contrast and saturation in a regular colour series. Following the method proposed by Kinnear [111], the square root of the error score generated by the test is used to normalise the results. Only 8.1% of subjects in the ETDRS had a SQRT 100-Hue score <7.5 (normal), and all of these had a visual acuity better than 20/40. Almost half of the patients investigated in this ETDRS sub-study had hue discrimination scores worse than the 95th percentile of a group of normal subjects. These findings suggest that even with relatively preserved visual acuity, colour vision may be impaired. Clinically significant macular oedema was reported as being associated with a worse hue discrimination score. However, this study also reports that even in eyes with no detectable macular oedema, over one third of eyes had abnormal hue discrimination. This suggests that neuronal changes are present even before the characteristic microvascular changes of diabetic retinopathy are visible and the concept of the "neurovascular unit" of the retina as an important concept in the pathogenesis of diabetic retinopathy is recalled. The defect in colour vision most commonly occurring in diabetic retinopathy has been reported to be in the tritan, or blue-yellow axis of colour discrimination [112]. A follow up report from the ETDRS population further classifies the colour vision defects seen in this group of patients [113], confirming that the commonest specific defect seen, in 26% of eyes studied, was a defect in discrimination in the blue-yellow axis. The severity of this was associated with increasing age and severity of macular oedema. 10% of eyes had a generalised reduction in hue discrimination with no specific axis. Therefore evidence suggests that colour vision defects are present early in diabetic macular disease and are very common.

Alternative methods for investigating colour contrast sensitivity using automated, screen based techniques have been explored. Arden *et al.* described in 1988 a system to generate gratings on a computer display and use these to establish thresholds of colour contrast sensitivity [114]. This system demonstrated that ophthalmologists using blue wavelengths of argon laser for retinal treatment developed reduced colour contrast thresholds [115]. This computer-linked technique evolved into the ChromaTest, which projects digital optotypes of different colour contrast, but equal

size and luminance, over the central 6.5° of the retina to measure protan and tritan sensitivities [116]. The Sussex Gratings Machine test is a cathode-ray tube based colour vision test that displays sinusoidal gratings along different colour confusion axes on a standard cathode ray tube television monitor. It has been used to investigate impairment in colour contrast thresholds in diabetic retinopathy [117, 118] and has corroborated previous findings of impairment in the tritan axis in this disease. It is known that accelerated lens ageing occurs in diabetes and this offers an alternative explanation for decreased tritan contrast sensitivity. Tregear *et al.* showed that for subjects with diabetes but no retinopathy, the reduced tritan sensitivity could be explained by correcting for increased yellowing of the lens [117]. However, even after this correction was made, subjects with background retinopathy were shown to have reduction in tritan sensitivity even in the absence of macular oedema. Furthermore, the presence of ischaemia as defined by clinical examination was shown to reduce red/green (protan) sensitivity as well as the expected reduction in tritan sensitivity.

The finding of reduced colour sensitivity preceding the development of significant diabetic eye disease has led investigators to explore the possibility of using colour vision testing as a screening tool for diabetic retinopathy [116, 118]. Detecting a functional impairment before structural disease has occurred could be advantageous in allowing patients access to clinical examination and treatment at an earlier stage. Ong *et al.* showed that the automated cathode ray tube technique employed by the Sussex Gratings Machine could yield a 94% sensitivity and 95% specificity for detecting sight-threatening diabetic retinopathy by measuring tritan contrast sensitivity [118]. The ChromaTest was investigated in a pilot study of subjects with type 2 diabetes and either untreated non-proliferative retinopathy or untreated clinically significant macular oedema [116]. Of subjects with non-proliferative retinopathy, 30% had tritan thresholds greater than normal and this figure in subjects with CSMO was 71%. However, this did not reach a high enough level of sensitivity or specificity from this preliminary study to justify its use as a screening tool. A recent systematic review of colour vision testing for screening for diabetic eye disease concluded that there was insufficient evidence to justify its inclusion in screening programmes at present [119].

2.1.1.3 *Microperimetry*

Conventional visual field examination, or perimetry, generally is unable to compensate for fixation that is eccentric, or that may be unstable; this limits its usefulness in macular disease where central scotomata may be present compromising the patient's ability to minimise eye movements during examination. It is therefore desirable to have a method of performing perimetry that allows stimuli to be projected onto known retinal locations with a greater degree of confidence, in order to evaluate more accurately retinal sensitivity in macular disease. This technique is termed fundus-related perimetry, or microperimetry.

Development of microperimetry

Early methods for carrying out this test utilised a scanning laser ophthalmoscope (SLO) connected to a monitor to enable visualisation of the retina while targets were presented [120]. Techniques evolved to include a computer on which the operator could select retinal targets to test, and which allowed some compensation for eye movements during testing [121], but the procedure remained relatively time-consuming and was not generally available in a clinical setting. The Nidek MP1 (Nidek technologies, Padua, Italy) was the first commercially available device that allowed automation of this type of examination, and has been validated against the pre-existing SLO-based techniques that had been in use for many years [122]. This machine projects a fixation target and stimuli on a liquid crystal display while the fundus is imaged in infra-red, under standard illumination conditions. The automatic eye tracker frequently monitors and corrects for eye movements so that the stimulus is projected with accuracy onto the known retinal location; allowing the matching of locational sensitivity to areas of visualised pathology [123].

Effect of DMO on retinal sensitivity

The automated nature of the Nidek MP1 device and the relative ease of performing examinations have led to a number of studies reporting retinal sensitivity in DMO, with attempts being made to correlate findings with structural changes present in the macula. In a study comparing normal subjects with 32 eyes of subjects with DMO,

MP1-determined retinal sensitivity was shown to be significantly lower (2.0dB and 2.8dB in the central 2° and 10° respectively, compared with 15.0dB and 14.8dB) when DMO was present [124]. This study also demonstrated correlations between retinal sensitivity and both visual acuity and foveal thickness. However, only a single thickness measurement from the central fovea was taken from OCT, so this finding has questionable significance. Further criticisms of this study include the omission of demographic data regarding the subjects and the lack of age-matched controls [125], although the magnitude of the disparity between sensitivity in normals and in those with DMO suggest that a genuine difference does exist.

Vujosevic and co-workers conducted a more detailed study to examine correlation between visual acuity, retinal thickness and retinal sensitivity. In subjects with CSMO, there was a significant correlation between retinal thickness in the five central OCT subfields and retinal sensitivity ($r=-0.48$, $p<0.0001$), but this correlation was not seen in subjects without macular oedema or with oedema that did not meet the criteria for CSMO [126]. This study utilised a customised 45-stimulus radial grid covering the central 12° of the macula; from this can be determined the microperimetry test loci that lie in corresponding OCT subfields. They demonstrated that in the presence of diabetes central macular sensitivity was lower than normal even in eyes with no macular oedema (11.9 ±3.4dB), and when macular oedema was present sensitivity was reduced further (4.7 ±3.5dB in the group with CSMO).

In a study evaluating 20 eyes with capillary non-perfusion as a result of diabetic retinopathy, MP1-determined retinal sensitivity was significantly reduced in areas of non-perfusion compared with immediately adjacent retina [127]. Average retinal sensitivity ranged from 0 to 1.7dB in non-perfused areas, whereas in perfused retina, sensitivities increased up to 13.3dB for some patients (overall mean not presented). This suggests that retinal non-perfusion identified by fluorescein angiography merits further exploration as a correlate of retinal function.

Retinal sensitivity changes reported by microperimetry following treatment for DMO

There are a number of reports describing the effect of treatments for DMO on retinal function as determined by microperimetry using the Nidek MP1. However, many of

these studies are uncontrolled, so results reporting changes in sensitivity should be interpreted with caution given the learning effect associated with the machine and the inherent test-retest variability present. Mean macular sensitivity has been shown to have a coefficient of repeatability (CR) of 1.81dB, but higher in the central 10° (2.13dB), with a CR for individual point sensitivity of 5.56dB [128].

Two uncontrolled studies report a modest increase in mean retinal sensitivity following intravitreal triamcinolone injection for DMO. Twenty eyes treated with 8mg triamcinolone had an improvement in mean macular sensitivity of 1.69dB at 3 months [129], while 11 eyes that received 4mg in a separate study showed a slight increase in retinal sensitivity of 3.23dB [130]. Without a control group it is difficult to ascertain whether this represents a true clinical change or is attributable to a learning effect.

A randomised trial has reported the effect of two different methods of laser treatment on retinal sensitivity in DMO. Subjects were randomised to either standard modified ETDRS macular laser or sub-threshold micropulse diode laser (MPDL). While visual acuity was stable in both groups, retinal sensitivity decreased in the group receiving standard ETDRS laser by 1.69 dB in the central 12° of the macula, but improved by 0.87 dB in the same area after MPDL. This change was accompanied by no increase in fundus autofluorescence with MPDL but evidence of increased autofluorescence with ETDRS laser, suggesting the newer technique may be less damaging to the retinal pigment epithelium [131]. A novel laser with even shorter pulse duration in the nanosecond range has shown promise in DMO. Retinal rejuvenation therapy (2RT) selectively targets individual RPE cells to ensure sufficient numbers of cells survive to prevent accompanying photoreceptor death. A pilot study in 28 eyes suggested the potential for improved visual acuity and reduced central macular thickness with this technique; further investigations are awaited [132].

Only one small prospective study reports the effect of an anti-VEGF agent on retinal sensitivity. A series of 26 eyes receiving three bevacizumab injections for DMO showed an improvement in retinal sensitivity of around 6 dB following three injections [133]. The investigators report parallel improvements in visual acuity and

retinal thickness, and therefore suggest that these changes may correlate with each other. A further study reports correlations between visual acuity and morphological features of DMO on OCT in subjects who had received a series of three ranibizumab injections. However, there is no mention in the published paper of the actual microperimetry results in the study [134].

Little can be therefore concluded about the long term functional effects of treatments on retinal sensitivity measured in this manner. Conventional laser appears to decrease retinal sensitivity as shown in the one well-designed randomised, masked trial, but it is not yet known with any certainty what the effect on sensitivity of medical agents for treating diabetic macular oedema might be.

2.1.1.4 *Electrophysiology*

Pattern electroretinogram

The pattern electroretinogram (PERG) is an electrophysiological recording that is used to investigate function of the macula. It is produced by a subject observing a reversing stimulus, such as a checkerboard, which retains a constant luminance throughout the reversal. Because there is no change in overall mean luminance, the response originates only from the area stimulated by the reversing pattern, which typically covers a 10-16° field. The main components of the PERG are a positive deflection at approximately 50 ms termed the P50 and a negative deflection at approximately 95 ms, the N95 component. The precise cellular origin of the P50 is unknown, but it arises partly in retinal ganglion cells and is driven by macular photoreceptors, so represents a true test of macular function [135, 136]. The typical PERG waveform is shown in Figure 14. Although the PERG is commonly used in a clinical setting to help distinguish between optic nerve and retinal disease, it has also been used a research tool and has found to be abnormal in diabetic retinopathy.

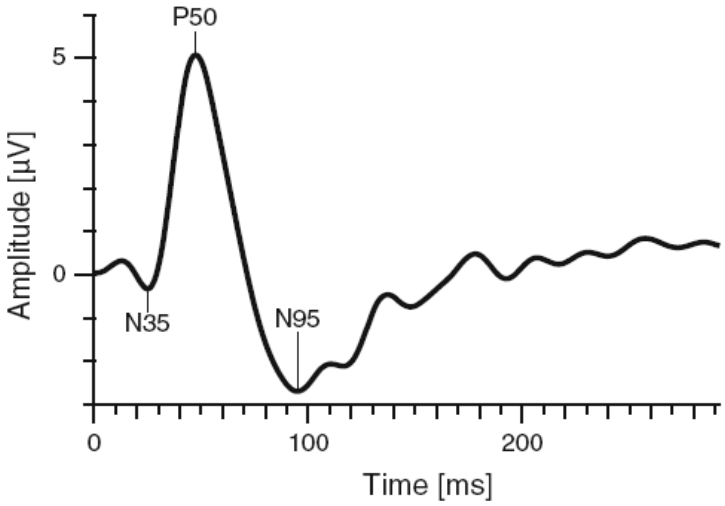

Figure 14 – The typical PERG waveform to illustrate the major components described above. A small early negative deflection is also recognised (N35). From [136].

Arden *et al.* recorded the PERG in subjects with normal visual acuity and varying grades of diabetic retinopathy. They showed that the amplitude of the PERG decreased depending on the severity of the retinal disease. Subjects with only a few microaneurysms present typically had normal PERG amplitudes, but by the time significant ischaemia was present as shown by capillary dropout on fluorescein angiograms, the PERG P50 amplitude had reduced by over 50% [137]. Coupland *et al.* also found that when there was no photographic evidence of diabetic retinopathy there was no PERG abnormality, but when retinal changes appeared the amplitude reduced [138]. Jenkins *et al.* provide supporting evidence for P50 amplitudes no different from normals in subjects with minimal diabetic retinopathy changes [139]. However, a different research group employed a slight variation in technique by using sinusoidal gratings instead of a checkerboard; this suggested that PERG changes were present even when no retinopathy was present [140]. Evidence therefore supports the conclusion that PERG amplitude reduces in diabetes when visible retinopathy is present.

These studies provide evidence of early retinal dysfunction in diabetic retinopathy and maculopathy, even in the absence of reduced visual acuity. The appearance of functional changes like PERG P50 amplitude reduction when retinal signs are

minimal provides further evidence for the effect of diabetes on the retinal neuronal network; suggesting again that it is not only a vascular disease but one affecting the whole neurovascular unit.

Effects of treatment for DMO on the pattern electroretinogram

Laser was shown in the ETDRS to reduce risk of visual loss when performed for CSMO, so is at best able to preserve vision with acuity improvements less likely. However, it has been shown that after macular photocoagulation for DMO there is a reduction in the PERG amplitude, reflecting the contribution of the paramacular neurons, located in the region where laser is performed, to the PERG waveform [141]. Although this study reported a decrease in the order of 40%, it only studied six eyes from three patients and did follow these patients for any period of time, so it possible that there may have been recovery of function later or that it could have worsened. As this study pre-dated the widespread availability of OCT it is not possible to know to what extent structural changes in the macula accompanied this functional change. However, evidence therefore exists for worsening retinal function even with preserved visual acuity.

Two longitudinal, uncontrolled studies report an increase in the PERG P50 amplitude after injections of triamcinolone and bevacizumab. In 40 eyes that received a single triamcinolone injection, visual acuity improved and there was an increase in P50 amplitude from 1.5 μv to 2.1 μv after six months [142]. Again, there is no correlation made with OCT. Patients in this study had relatively poor visual acuities at baseline (worse than 6/60 on average) so it is possible that fixation may have been impaired which may have reduced the PERG response. It is therefore possible that improved amplitudes at six months simply reflect better fixation as acuity improves. Similar results were reported by the same group after a single bevacizumab injection to 35 treatment-naïve eyes. In this study P50 amplitude increased from 1.4 to 2.2 μv at 6 months [143]. The same caveats apply about the lack of a control group and the possibility of PERG improvement being driven by improved acuity and hence fixation. It is also surprising given what is known about the pharmacokinetics of bevacizumab and the retreatment schedules used for anti-VEGF agents in clinical trials that a single injection could have an effect for six months.

In summary, the PERG may be an early indicator of neuronal dysfunction in diabetic retinopathy. As it evaluates the response of the macula, it may be employed usefully as a tool for evaluating the functional effects of treatment. There is some evidence that P50 amplitude may be reduced after laser treatment and increased after either steroid or anti-VEGF therapy, but randomised trials are lacking and there is little long-term follow-up data.

Full-field electroretinogram

The full-field electroretinogram (ERG) records the massed responses of the rods and cones to retinal stimulation. It is obtained using a Ganzfeld bowl which is designed to provide uniform illumination to the entire visual field. In scotopic conditions it measures the rod responses, and after light adaptation it is able to measure cone responses by using a background illumination to saturate rod responses. The 30 Hz flicker ERG is specific to cone responses as this flicker frequency is beyond the temporal resolution of rods. A typical ERG waveform consists of an a wave, which is generated by hyperpolarisation of the photoreceptors, and a b wave arising in ON-bipolar cells [135]. A standard set of ERGs consists of a number of rod- and cone-specific responses obtained in lighting conditions specified by the International Society for the Clinical Electrophysiology of Vision (ISCEV) [144]. Evaluation of ERG recordings comprises measurement of the amplitudes and implicit times of a and b waves. Wave amplitude generally reflects the functional integrity of the retina and is reduced when loss of function has occurred. Increased implicit times imply that retinal dysfunction is present. Oscillatory potentials are a further component of the electroretinogram that have been studied in diabetes. They are small oscillations superimposed on the ascending limb of the b wave, the source of which may be amacrine cells, although other investigators have reported contributions from photoreceptors and ON-bipolar cells [145].

In diabetes, the changes in the electroretinogram may correlate with disease severity and duration. Similarly to the findings from studies of the PERG in diabetes, subjects with early diabetes and no retinopathy may have no alteration in the ERG. For example, Uccioli *et al.* showed that subjects with less than six months' duration of

disease did not have a reduction in the electroretinogram [146]. Interestingly, other researchers have reported a bimodal distribution in amplitudes of ERG b waves in subjects with either no retinopathy or minimal disease: while one group of subjects had decreased amplitudes another group had an increase in magnitude [139]. No obvious cause for this in terms of correlation with age or duration of disease was found.

Full-field ERG represents the massed response of retinal neurons and so it is unsurprising that amplitude declines following panretinal photocoagulation, reflecting the loss of functional retina from laser burns aiming to destroy photoreceptors. This has been observed for many years [147]. Recently, investigators have evaluated the impact of ranibizumab treatment combined with PRP on electroretinograms in high risk proliferative retinopathy [148]. In this randomised study, subjects in the PRP + ranibizumab group could receive retreatment with ranibizumab if new vessels persisted; at the study endpoint this group had fewer laser burns and less decline in retinal function based on rod and cone response b wave amplitudes. There was a small increase in b wave implicit time, but no difference between groups. Increases to the implicit time of the b wave represent evidence of retinal dysfunction, so changes seen after a particular treatment may represent evidence of increasing ischaemia. Evaluating the full-field electroretinogram in a study where ranibizumab is given in the absence of significant retinal laser yields the opportunity to evaluate further its long term effects on generalised retinal function.

Multifocal electroretinogram

The multifocal ERG (mfERG) provides a means of evaluating localised retinal function. To record a mfERG the subject views an array of either 61 or 103 hexagons on a display screen, while fixating centrally, so that these hexagons fall on the posterior pole of the eye and stimulate the macula together with mid-peripheral retina out to 20-25°. The hexagons flicker in a pseudo-random sequence and a continuous ERG is recorded. Mathematical analysis then allows the extraction of an individual ERG from each hexagonal component. A typical mfERG is shown in Figure 15. The

individual components of the wave are similar to the typical waveform of the full field ERG but as they represent a mathematical construction they cannot be thought of as analogous. A standard for recording mfERGs has been published by ISCEV [149].

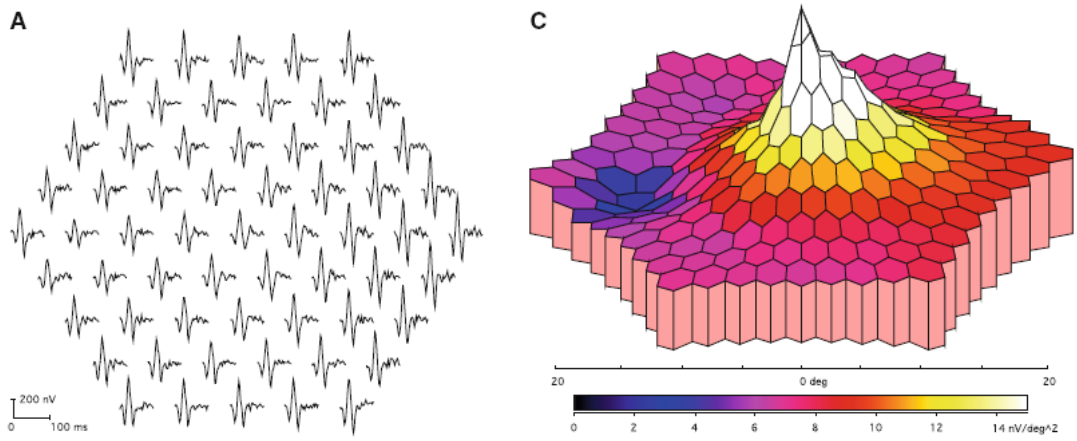

Figure 15 – Multifocal ERG recording from a normal subject. (A) shows the individual extracted ERGs from each of the 61 hexagonal stimuli. (C) shows this in graphical form. This recording is from a left eye as the decreased sensitivity from the blind spot nasal to the macula can be identified. From ISCEV standard [149].

Yamamoto *et al.* investigated the mfERG in subjects with diabetic macular oedema and found that when cystoid oedema was present, central responses were decreased. Additionally, they established that foveal thickness measured by OCT correlated to an extent with mfERG response amplitude [150]. Conflicting results for mfERG amplitude change following triamcinolone injection for DMO have been reported. A study of 15 patients receiving triamcinolone found an improvement in visual acuity together with increased mfERG amplitudes in the central and peripheral hexagons with no accompanying change in implicit time [151]. However, a similar study of 24 patients with DMO treated with triamcinolone failed to replicate this finding and did not detect a difference in mfERG amplitudes despite visual acuity improvement [152]. While it is possible that irreversible retinal dysfunction may be present in subjects with longstanding DMO, it is also possible that the signal to noise ratio of mfERG is too low to record changes in retinal function related to resolution of macular oedema. It may be that visual acuity improvements result from a highly localised improvement in function at the fovea that is not reflected in the mfERG

measurement. Multifocal ERG remains a research tool rather than a core diagnostic tool in electrophysiology and may be of some use in the evaluation of DMO.

2.1.2 Structural tests to evaluate diabetic macular oedema

Non-invasive imaging techniques have now become widespread in the management of DMO. Colour photography and fluorescein angiography have been in use for decades, but the decrease in cost and subsequent increase in availability of OCT scanning has meant that research into this modality has attracted a great deal of attention.

2.1.2.1 *Optical coherence tomography*

First described by David Huang at the Massachusetts Institute of Technology in 1991 [153], and developed for clinical use by Carmen Puliafito and James Fujimoto [154], OCT scanning allows the generation of cross-sectional images of the retina rapidly and non-invasively. It achieves this by interferometry and involves comparing the time delay of light reflected from the retina with a reference beam and analysing the interference patterns between the two beams; this has been extensively described in the literature [155]. OCT became commercially available in the 1990s with devices produced by Zeiss and now many different devices are available. OCT images are conventionally displayed as a cross-sectional B-scan image derived from combining several thousand axial or "A" scans (see Figure 17 below for example). Recently, interest has developed in combining several B-scans into an *en face* image (or C-scan).

The older, time-domain (TD) OCT machines obtained images by way of a movable mirror that scanned the imaging beam across the retina and acquired 400 A-scans per second. The Zeiss OCT1 had an axial resolution of around 15 µm, improved by about 7 µm in later devices such as the Zeiss Stratus OCT [156]. Spectral-domain (SD) OCT devices use a spectrometer to measure light reflectance across the spectral range so do not require a moving mirror, and are much faster than TD OCT; typically they are able to obtain 20-40,000 A-scans per second with a higher resolution of 4-7 µm axially. Examples include the Zeiss Cirrus device and the Spectralis OCT

(Heidelberg Engineering GmbH; Heidelberg, Germany), shown in Figure 16, which also incorporates eye tracking software and improved signal-to-noise ratio with the ability to superimpose multiple images from the same retinal location (see also section 2.1.2.2).

Figure 16 – The Heidelberg Spectralis OCT system (Image courtesy of Heidelberg Engineering GmbH, Germany).

OCT in diabetic macular oedema

Quantitative and qualitative approaches to measuring and characterising the OCT features of DMO have been adopted. Hee *et al.* demonstrated the viability of using OCT to image the retina of patients with DMO [157] and showed increased foveal thickness when macular oedema was present. They mapped the macular region using the nine ETDRS subfields, which has now become an accepted standard for OCT of the macula and thickness in the central subfield has been used as an outcome measure in clinical trials. Otani *et al.* described three different morphological features of DMO visualised on TD OCT: sponge-like retinal swelling, cystoid oedema and serous retinal detachment; they also identified the presence of hard exudates as hyper-reflective foci [158]. Both these early reports also found a negative correlation between central retinal thickness on OCT and visual acuity in this condition.

Increased OCT resolution has led to better identification of the OCT correlates of the anatomical retinal layers using newer SD OCT devices. Following on from the early descriptions of the patterns seen in DMO, it has now become easier to link the pathophysiological changes of blood-retina barrier breakdown with the accumulation of fluid in the layers of the retina, with typical appearances from a SD OCT image in DMO shown in Figure 17.

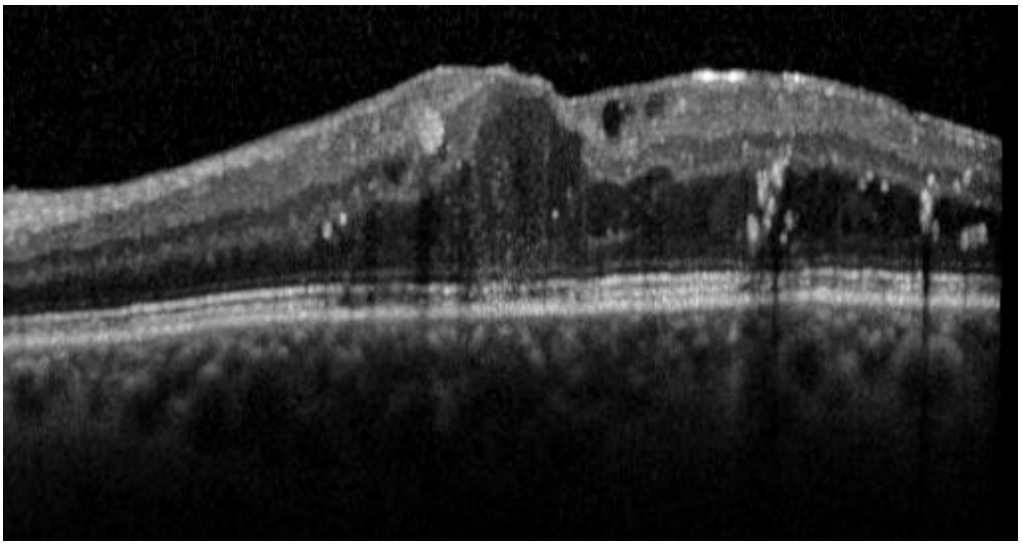

Figure 17 – OCT scan from patient with diabetic macular oedema, to show retinal thickening, fluid in inner and outer retina, and hyperreflective foci.

Angiographic appearances of macular oedema include petaloid and honeycomb patterns, as fluid accumulates in the fibre layer of Henle and the inner retina respectively. However, the appearance of apparent cysts on OCT imaging is misleading, as electron micrographic studies have shown that the fluid present in the retina occupies interconnected spaces, and the walls of these spaces represent stretched neuronal elements as retinal layers become separated by fluid accumulation. The cross-sectional area of retinal tissue crossing these spaces correlates with visual acuity in DMO [159].

Deak *et al.* correlated retinal sensitivity from microperimetry with morphological features on OCT scans in DMO to try and establish the link between observed OCT features and their functional effect. They found that large outer nuclear layer cysts and serous retinal detachment had the greatest effect on retinal sensitivity (-3.86 and

-3.65 dB), but retinal swelling alone did not have a detectable effect on sensitivity [160].

Investigating the longitudinal effect of treatment for DMO on some of the morphological changes present on OCT affords the opportunity to identify those which may be useful as markers of a good response to treatment, and which may signify irreversible retinal damage and hence a likely poor response to treatment.

Choroidal thickness evaluation by OCT

Limitations to the resolution of OCT at increasing retinal depth previously meant that even deeper structures such as the choroid were very difficult to image. Spaide described the technique of enhanced depth imaging (EDI), where pushing a conventional Spectralis OCT device closer to the subject's eye to obtain an inverted image allowed the choroid to be imaged. The device's eye tracking function and ability to average the signal from up to 100 images (automatic real time, ART) meant that high resolution images of the choroid could be obtained [161]. Now, conventional SD OCT devices incorporate an EDI setting that obviates the need to invert the image manually and is optimised for imaging the choroid. Choroidal thickness has been studied in healthy subjects and it is now widely accepted that it is influenced by axial length and refractive error; increasing in thickness in longer, myopic eyes and also decreasing in thickness with age [162]. Across the macula, the choroid is thinnest nasal to the fovea and does not correspond with the typical retinal thickness map seen in normal subjects.

The role of the choroid in the pathophysiology of diabetic retinopathy and DMO is not well understood, but the high oxygen requirements of the photoreceptors and the supply of these requirements by choroidal blood flow makes it a valid target for investigation. Indeed, reduced choroidal thickness has been reported in subjects with both type 1 and type 2 diabetes [101, 163], and there is a growing body of evidence that this may be related to severity of retinopathy or disease duration. It is possible that changes in choroidal thickness affect oxygen delivery to photoreceptors and may influence the production of VEGF and hence development of macular oedema.

Querques *et al*. investigated 63 subjects with diabetes with and without retinopathy and CSMO and also found decreased choroidal thickness in all diabetic subjects compared to normal controls. Although their results demonstrated a trend towards decreasing choroidal thickness with increased retinopathy severity and presence of macular oedema, they did not reach statistical significance [164]. Regatieri *et al.* in a similar study found no reduction in thickness if only non-proliferative retinopathy was present, but for subjects with macular oedema or treated proliferative disease the choroid was thinner [102]. A large study from Vujosevic *et al*. provides further evidence that increasing severity of retinopathy is associated with increased choroidal thinning [103].

The relative novelty of choroidal thickness measurement has meant that a standardised technique for obtaining measurements across the macula has not been defined, making comparison between studies difficult. Furthermore, the above studies, which have found decreased choroidal thickness in diabetes with an apparent progression as retinopathy worsens, have been cross-sectional in design. A prospective study to investigate the effect on choroidal thickness of treatment for DMO may contribute to understanding the role of the choroid in this condition.

2.1.2.2 ***Repeatability of OCT measures of macular oedema***

Measurement variation is an important concept in subjective, functional tests where patient performance can impact upon results such that there may be a difference between outcomes when a test is repeated even when no clinical change can have occurred. Measurement variation also occurs with tests that are not dependent on subjective responses, such as OCT scanning. In this situation, patient factors such as head position or ocular fixation may affect the computer-driven algorithms used to generate retinal thickness measurements and introduce measurement variability. Inclusion criteria for clinical trials and retreatment protocols in trials of intravitreal agents for macular disease frequently incorporate a quantitative OCT parameter such as central macular thickness to drive retreatment. Studying repeatability allows true clinical change to be distinguished from naturally occurring measurement variability. Clinicians can then define a threshold to recognise when a condition has changed,

which can subsequently be used in clinical trials and clinical practice to determine the need for further treatment or to identify a therapeutic response.

Repeatability of OCT retinal thickness measurements has been studied in DMO using time domain devices, with estimates for the coefficient of repeatability for retinal thickness in the central subfield of 21 μm and 38 μm from different investigators [165, 166]. Studies comparing time domain with spectral domain devices have failed to demonstrate a statistically significant improvement in coefficient of repeatability for the newer devices evaluating retinal thickness in DMO [167, 168]. The Spectralis SD OCT device has the capability to track eye movement and can automatically place follow-up scans in the same retinal location potentially leading to highly repeatable measurements of retinal thickness. However, this device had not been used to evaluate the repeatability of retinal thickness measurements in DMO when this programme of investigation commenced.

2.1.2.3 *Colour photography for diabetic retinopathy grading*

The gold standard for grading of diabetic retinopathy was established by the ETDRS as seven-field stereoscopic fundus photography with classification based on a modification to the Airlie House criteria for grading retinopathy [169]. Careful examination of the features of retinopathy that were predictive for progression led to the development of a numerical scale for the classification of retinopathy and macular oedema [170]. This scale ranges from level 10 (no retinopathy present) to level 85 (advanced PDR with macular detachment) with stages between representing different severities of non-proliferative and proliferative retinopathy. Seven-field stereoscopic grading is particularly time-consuming and uncomfortable for patients, so investigators have sought other methods to grade retinopathy based on fewer images. Evaluation of agreement in grading when either two or four fields were used showed high levels of agreement for four field photography across most grades of retinopathy [171]. More recently it has been shown that ophthalmic examination by an experienced clinician compares favourably with the gold standard, and even with two field photography (which is used in the UK diabetic screening programme) grading shows good agreement with seven field imaging [172]. Using an established Reading Centre to grade retinopathy provides another potential clinical trial outcome.

2.1.2.4 *Fundus fluorescein angiography*

Imaging the vasculature of the posterior pole of the eye using cameras and appropriate filters following a bolus intravenous injection of either 10% or 20% sodium fluorescein is termed fundus fluorescein angiography (FFA). The ETDRS established the value of FFA in the diagnosis and management of DMO and defined grades of capillary loss indicative of macular ischaemia with reference to standard photographs [173]. This study also described methodology for grading the foveal avascular zone and suggested an upper limit of normal for FAZ diameter of 1000 µm. However, inter-grader repeatability for this measure was low, typically with kappa values of 0.4 to 0.5, indicating only moderate agreement.

As discussed in Chapter 1, macular ischaemia remains a theoretical concern following anti-VEGF administration. Although the results from the BOLT study at 4 months and 12 months did not show any evidence of worsening macular perfusion following either bevacizumab or laser treatment [73, 74], there has not been a systematic evaluation of the effects of ranibizumab on these parameters. In the DRCR.net protocol I trial for example, fluorescein angiography was only obtained at the investigator's discretion, so there was limited opportunity to assess for progression of macular ischaemia. A prospective clinical trial evaluating the effect of ranibizumab on macular ischaemia may yield useful data.

2.1.3 Structure-function correlation in diabetic macular oedema

Imaging techniques employed in patients with DMO allow retinal structure to be evaluated. These currently include:

- OCT
    - Retinal thickness
    - Retinal morphology
- Colour fundus imaging
- Fluorescein angiography
    - Size of foveal avascular zone
    - Degree of perifoveal capillary loss

Tests evaluating the function of macula include visual acuity and microperimetry to measure retinal sensitivity. Correlation between these different modalities of investigation attempts to measure the effect of a structural change in the retina on the function of the retina in that area.

In DMO, the link between a structural change and a corresponding functional alteration has not been well demonstrated. It has been established in early studies of OCT in DMO that retinal thickness correlates negatively with visual acuity. For example, the DRCR.net evaluated OCT and visual acuity results from a large randomised trial to estimate the size of the effect of increased retinal thickness on acuity. They reported a 4.4 letter change in visual acuity for every 100 μm change in retinal thickness, but recognised that a variety of OCT results could be associated with the same visual acuity [174].

Studies of morphological changes and their effect on retinal function have suggested that different patterns of oedema have different effects on retinal function (e.g. Deak *et al.* investigating microperimetry and OCT morphology [160]). Pelosini *et al.* have shown in DMO that a better correlate of visual acuity is the cross sectional area of retinal tissue bridging across apparent cystic spaces visible on OCT [159]. New techniques of simultaneous microperimetry and OCT are becoming available that may make it easier to correlate these findings: Charbel Issa *et al.* describe the use of such a technique to correlate microperimetry findings with OCT in macular telangiectasia, for example [175].

The disadvantages of the functional tests used to evaluate the macula include their time-consuming nature, test-retest variability, and in the case of electrophysiology low signal to noise ratios. Structural imaging studies by contrast are rapidly acquired and subject to less test-retest variability. Therefore the demonstration of reliable structure-function correlation would allow structural tests to provide information about the function of the retina and may allow the identification of patients more likely to respond to treatment.

2.1.4 Aims and objectives of the study

At the time of commencing this study, ranibizumab had not received a licence for the treatment of DMO but phase III clinical trials with this aim were in progress. Standard treatment for DMO in the UK was laser photocoagulation.

As ranibizumab inhibits all isoforms of VEGF, there was concern that repeated use may lead to alterations in normal retinal physiology that might manifest in a number of ways, including reduced colour vision, reduced macular sensitivity and reduced electrophysiological indices.

Spectral domain OCT technology was also not widely available at the start of the study and changes in retinal structure seen with this imaging technique had not been reported.

Primary objectives

- To investigate the effect of ranibizumab on colour contrast sensitivity changes in DMO
- To investigate the effect of ranibizumab on macular sensitivity by microperimetry
- To investigate the effect of ranibizumab on the pattern and full-field electroretinogram
- To investigate structural changes seen on SD-OCT scans following ranibizumab treatment

Secondary objectives

- To establish the repeatability of retinal thickness and volume measurements in DMO using Spectralis SD-OCT
- To investigate the effect of ranibizumab on choroidal thickness in DMO
- To investigate the correlation between retinal sensitivity and retinal thickness in DMO

2.2 **Methods**

The LUCIDATE study (LUCentis In Diabetic macular oedema – A Treatment Evaluation) was a randomised clinical trial designed to compare the detailed structural and functional effects of repeated pan anti-VEGF inhibition with ranibizumab with the effects of standard macular laser therapy after 48 weeks of treatment.

2.2.1 ***Design, approval and participants***

This trial was a single centre, parallel group, phase IIb exploratory trial with imbalanced randomisation (2:1 ranibizumab:laser) and investigator masking conducted at Moorfields Eye Hospital.

The study conformed to the Declaration of Helsinki and prospective approval was obtained from the Central London Research Ethics Committee 4 of the UK National Research Ethics Service. The trial was registered at www.clinicaltrials.gov (NCT01223612). A clinical trial authorisation was obtained from the Medicines and Healthcare Products Regulatory Agency (MHRA) before recruitment commenced.

Adults aged 18 or over with centre-involving DMO in at least one eye were identified from Medical Retina clinics in the hospital. Informed consent was obtained from all participants before screening commenced but after a full explanation of the nature and possible risks of participation in the study.

2.2.2 ***Patient eligibility***

The following criteria were used to guide patient enrolment:

Inclusion criteria (ocular criteria apply to study eye)

1. Patients of either sex aged 18 years or over able to give informed consent throughout the study
2. Diagnosis of diabetes mellitus (type 1 or type 2)
3. Best corrected visual acuity between 55 and 79 ETDRS letters at 1 meter (Snellen equivalent $\geq$ 6/24 and $\leq$ 6/9)
4. Clinically apparent centre-involving DMO with OCT central subfield thickness $\geq$300 μm on Spectralis OCT

5. Sufficient pupillary dilation and media clarity for adequate fundus imaging
6. Intraocular pressure (IOP) <30 mmHg.
7. Visual acuity in fellow eye ≥ 2/60
8. Fellow eye has received no anti-VEGF treatment within the past 3 months and no expectation of such treatment during the study
9. Ability to return for study visits for 48 weeks of follow up

Exclusion criteria (ocular criteria were applied to study eye only)

1. Macular ischaemia (foveal avascular zone (FAZ) greatest linear diameter > 1000 µm or severe perifoveal capillary loss on fluorescein angiography).
2. Macular oedema from a cause other than diabetes e.g. post cataract surgery or related to vitreoretinal interface abnormalities.
3. Presence of an ocular condition such that visual acuity would not improve from resolution of macular oedema (e.g. permanent foveal pigmentary or atrophic changes, dense plaques of exudate or non-retinal conditions, such as amblyopia).
4. Presence of an ocular condition (other than diabetes) that might affect macular oedema or alter visual acuity during the course of the study (e.g. retinal vein occlusion, ocular inflammatory disease, neovascular glaucoma)
5. Substantial cataract likely to be responsible for at least three lines of visual loss (i.e. to 6/12 or worse in the absence of other pathology)
6. Any treatment for DMO in the past 3 months including macular laser treatment, injection of corticosteroid or anti-VEGF agents.
7. Panretinal photocoagulation (PRP) performed within 3 months prior to randomisation or anticipated in the next 6 months.
8. Active proliferative diabetic retinopathy in the study eye.
9. A condition that would prevent study participation, in the investigator's opinion.
10. Haemoglobin A_{1c} > 11.0 %
11. A past medical history of chronic renal failure that requires either dialysis or kidney transplant
12. Blood pressure >170/100 mmHg (i.e. systolic above 170 or diastolic above 100)
13. Arterio-thrombotic event within 6 months prior to randomisation including: myocardial infarction, acute congestive heart failure or other cardiac event, stroke, transient ischaemic attack

14. Major surgery planned during the trial period or within 28 days prior to randomisation.
15. Administration of another investigational drug within 30 days of randomisation.
16. Prior treatment with systemic anti-VEGF in 3 months prior to randomisation.
17. Pregnant or lactating women or women intending to become pregnant within the study period including 3 months after study cessation.
18. Intraocular surgery or major extraocular surgery within 3 months prior to randomisation or anticipated within next 6 months.
19. Aphakia.
20. Uncontrolled glaucoma.
21. Infective external ocular disease e.g. conjunctivitis, chalazion, or severe blepharitis
22. Allergy to fluorescein or ranibizumab.
23. Fertile male unwilling to use contraception for the duration of the study.

2.2.3 ***Sample size***

The study was a planned exploratory analysis and hence no power calculation was performed; 36 subjects were deemed a suitable number by the trial statisticians to allow an assessment of normality of the exploratory outcome data and conduct a descriptive analysis. The number was also chosen based on the resources available to conduct the study.

2.2.4 ***Randomisation***

One eye per participant was included in the study. If both eyes were eligible, the eye with worse visual acuity was included. Subjects were randomised 2:1 to receive ranibizumab or laser, using random permuted blocks of varying sizes, with the randomisation list generated by a computer. The allocation sequence was held by the trial statistician and concealed from the researcher enrolling and assessing participants. The final two subjects were randomised simultaneously.

*2.2.5 **Interventions in the trial***

Subjects in the ranibizumab arm received intravitreal injections of ranibizumab (Lucentis®, 0.5 mg in 0.05 ml solution for injection, Novartis Pharmaceuticals UK Ltd) at baseline, 4 weeks and 8 weeks then four weekly as required according to pre-defined retreatment criteria. Retreatment occurred if BCVA was reduced by ≥5 letters from maximum acuity or if OCT central subfield thickness was > 300 μm. Subjects in the laser arm received modified ETDRS focal/grid laser at baseline and then every 12 weeks as required guided by fluorescein angiography. Laser was performed using a green wavelength with 50-60 μm spot size, >500 μm from the edge of the FAZ with the aim of causing mild blanching of the retinal pigment epithelium. Focal laser was applied to untreated focal leaks and grid laser applied to areas of diffuse leakage and areas of non-perfusion that were not contiguous with the FAZ.

*2.2.6 **Masking***

Visual acuity assessors, OCT technicians, photographers and electrophysiology technicians were masked to treatment allocation, as were fluorescein angiogram, fundus photograph and OCT graders, and reporting electrophysiologists. Subjects were not masked to the treatment they were receiving; to do so would have entailed designing sham injection and sham laser procedures.

*2.2.7 **Follow-up visits and investigations undertaken***

All subjects attended at baseline, 12, 24, 36 and 48 weeks. Subjects in the ranibizumab group also attended at four-weekly intervals between these visits for assessment and retreatment if required. At baseline, 12, 24 and 48 weeks subjects underwent detailed functional and structural evaluation of the retina using a number of investigation modalities.

2.2.7.1 ***Best-corrected ETDRS visual acuity***

This was measured using ETDRS charts in standard lighting conditions. Subjects underwent refraction at baseline, 12, 24 and 48 weeks and at intervening visits the

most recent subjective refraction was used. Refraction was carried out by a certified trial optometrist using ETDRS chart "R" at 4m. Visual acuity was measured in the study eye, then the fellow eye. Standard instructions were given to subjects to read the letters on the chart one by one and to guess any letters they were unsure of until no further letters could be seen. Visual acuity scores were calculated for 1m by adding 30 letters to the 4m score if more than 20 letters were read. If fewer than 20 letters were read at 4m, +0.75DS was added to the trial frame and the first six lines of the chart were read at 1m, with this score added to the 4m score. ETDRS chart "1" was used for right eyes and chart "2" for left eyes.

2.2.7.2 *Colour contrast sensitivity*

Colour contrast sensitivity (CCS) was evaluated using the ChromaTest, (City University, London), described in detail by Arden and more recently by Wong *et al.* [114, 116]. The test was regularly calibrated by technicians in the electrophysiology department. The subject is seated 1.5m from a computer monitor on which is displayed a coloured letter against an isoluminant background. When the subject correctly identifies a letter, the colour difference between the next letter and the background is halved (doubled after an incorrect guess). In this way, a threshold value for CCS is reached. The "diabetes" protocol of the test was used which measures CCS thresholds in the protan and tritan axes.

2.2.7.3 *Microperimetry*

Subjects underwent microperimetry in mesopic conditions in the study eye only using the Nidek MP1 device (Nidek technologies, Padua, Italy). After pupil dilation and a ten minute period of partial dark adaptation, subjects were initially given brief training on the operation of the machine. Standard settings were used with background luminance set at 4 apostilbs. The microperimetry protocol used a customised radial grid based consisting of 45 stimuli covering the central 12° of the macula; with the inner stimuli 1° apart and the outer stimuli 2° apart, shown in Figure 18. The stimulus size used was the Goldman III (26 min arc or 0.4 degrees) with a projection time of 200ms (white stimulus, white background). The starting light attenuation was set to 10dB and a 4-2-1 double staircase strategy, as

recommended by Vujosevic et al. to reduce testing time and possible fatigue was used [126].

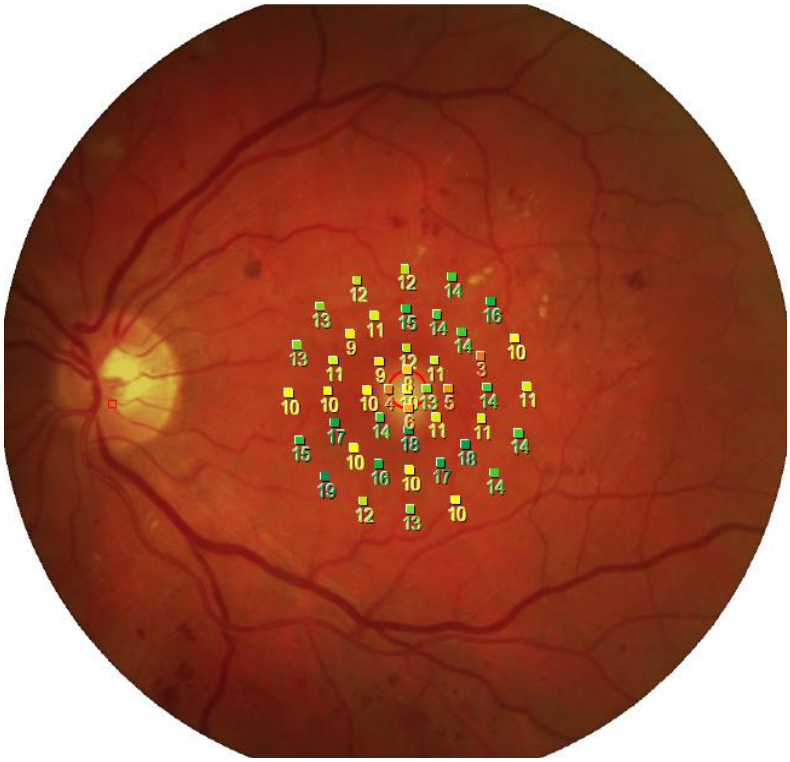

Figure 18 – Customised radial 45-point grid used for microperimetry; shown overlaid on a colour photograph of the fundus of one trial subject.

Mean values for the central 4° and 12° of the macula were entered into the trial database.

2.2.7.4 ***Electrophysiology***

Electrophysiological testing (rod and cone specific full-field electroretinogram (ERG), pattern ERG (PERG) and multifocal ERG (mfERG)) were performed according to the International Society for Clinical Electrophysiology of Vision (ISCEV) standards. Data were recorded from both study eye and fellow eye. The key elements of the investigation are detailed below.

The PERG was obtained before pupil dilation in photopic conditions with appropriate refractive correction in place. The subject observed an alternating checkerboard on the display screen with check size 43’ of arc (0.72^{o}) and a reversal rate of 2.2 Hz. Full details are specified in the ISCEV standard [136]. Amplitudes of the P50 and N95 component, and the P50 peak time were recorded. Full-field ERGs

were obtained using a Ganzfeld bowl stimulus after pupil dilation and full dark adaptation. Full details are specified in the ISCEV standard [144]; stimuli were used to record peak times and amplitudes of the dim flash rod response (b wave) and maximal rod response (a and b wave). After light adaptation the 30 Hz flicker response (amplitude and implicit time) and maximal photopic response (a and b wave amplitudes and peak times) were recorded. Multifocal ERGs were recorded using the Roland Consult system with a 61 hexagon pattern after the full field ERGs following the ISCEV standard [149]. Again, refractive correction was used where applicable and only data from the study eye was recorded.

2.2.7.5 *Optical coherence tomography scans*

Spectral domain optical coherence tomography (SD OCT) scanning was performed by technicians experienced in clinical trials work using the Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany). Standard preset scan settings were used for all scans, with the following scan sets recorded:

1. "Fast" volume scan; 25 horizontal lines, 20°, 512x496, Automatic Real Time (ART) 9, "High Speed" mode.
2. "Dense" volume scan; 49 horizontal lines, 20°, 512x496, ART 16, "High Speed" mode.
3. Single horizontal line scan; 768 A-scans, ART 100, "High Speed" mode.
4. Single horizontal line scan; Enhanced Depth Imaging (EDI) mode, 768 A-scans, ART 100, "High Speed" mode.

All scans were centred on the fovea. Each baseline scan was used as a reference for follow-up scans, to make use of the "TruTrack" software of the Spectralis device, which uses a reference beam to image the eye and allows follow-up scans to be taken at the same location as previous scans.

Additionally, three further "fast" volume scans were taken at baseline for evaluation of repeatability of retinal thickness and volume measurements, described below in section 2.2.10.2. Data for thickness and volume were obtained from the Heidelberg Eye Explorer (HEYEX) software (version 1.7.0.0, ©2011 Heidelberg Engineering GmbH, Heidelberg, Germany) and entered into an Excel spreadsheet without formally correcting for retinal boundary detection error. The "fast" volume scans were used for retinal thickness and volume measurements. "Dense" volume scans

were evaluated by the reading centre for grading of morphological features of DMO, with the high quality single line scan also used for evaluating foveal location and vitreomacular interface abnormalities. The scans were examined for the presence or absence of the following features in the inner or outer retina:

- Cysts
- Cystoid features
- Hyperreflective foci

Additionally, the integrity of the lines thought to represent the external limiting membrane (ELM) and inner segment-outer segment (IS-OS) junction (ellipsoid layer) was evaluated. Grading of all of these features was reported in the central subfield and in the inner four paracentral subfields. The foveal depression was graded as normal or abnormal, and the presence of vitreoretinal interface abnormalities (vitreomacular traction (VMT), epiretinal membrane (ERM), macular hole) was noted. Figure 19 shows two typical scans to illustrate the appearance of the features graded.

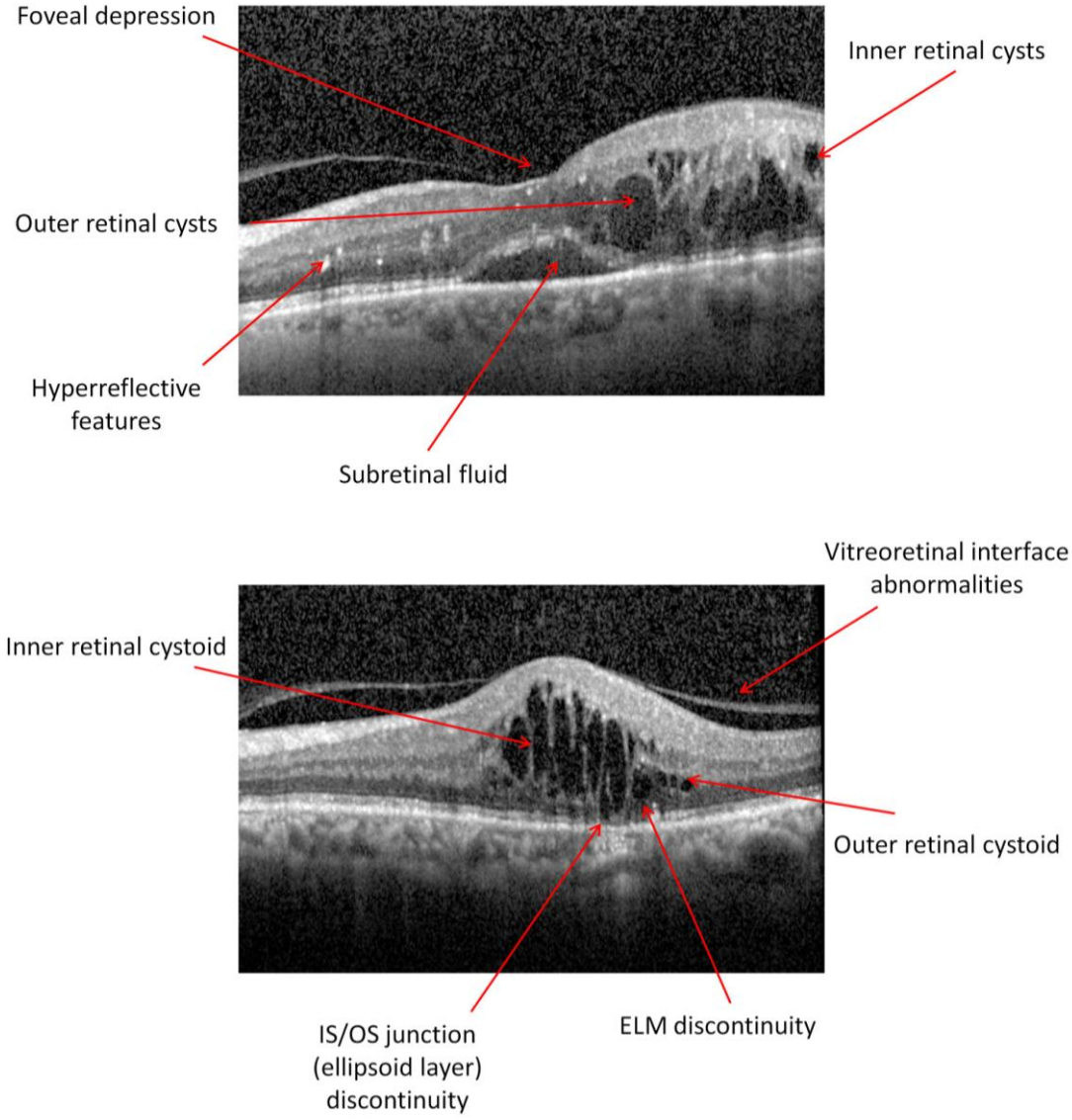

Figure 19 – Two representative OCT scans from subjects with DMO to illustrate the typical appearance of morphological features that were identified by graders in the reading centre.

2.2.7.6 ***Colour photography***

At baseline and trial exit (48 weeks) four-field standard ETDRS colour photographs were taken for reading centre assessment of grade of diabetic retinopathy, shown in Figure 20. The four fields were each obtained using a 30° field of view and are defined as follows:

Field 1 – Centred on disc, with the temporal border on the macula,

Field 2 – Centred on the macula, the nasal border over the centre of the disc

Field 3 – Superior to the macula, the inferior border level with the superior edge of the disc

Field 4 – Inferior to the macula, the superior border level with the inferior edge of the disc

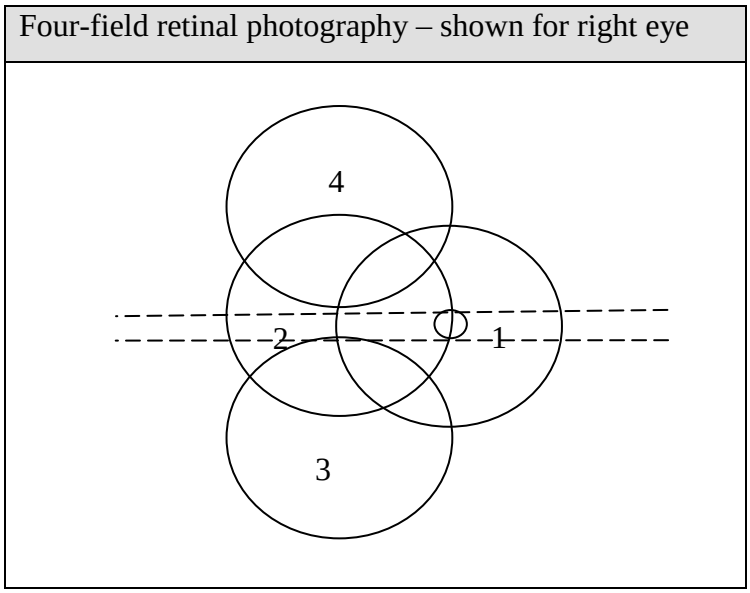

Figure 20 – Diagram of fields used for standardised ETDRS retinal photography.

Subjects were assigned a single numerical ETDRS grade at baseline and 48 weeks on the basis of the grading of the four images, in accordance with the standardised ETDRS grading system [170].

2.2.7.7 *Fundus Fluorescein Angiograms*

Fundus fluorescein angiography (FFA) was performed at the four main time points of the trial. After intravenous cannulation, subjects received a bolus injection of 5 ml 20% sodium fluorescein. A series of frames centred on the macula were obtained during the transit phase. During the mid-phase of the angiogram, peripheral shots were obtained before returning to the macula to obtain late frames. Images were examined by masked reading centre graders to record area and greatest linear dimension (GLD) of the foveal avascular zone (FAZ) and grade of perifoveal capillary loss (PFCL) in four quadrants. Defining the FAZ was carried out manually on the Topcon Imagenet software and automatically generated measurements for GLD and area were taken from this. Figure 21 shows an early phase frame from an angiogram to illustrate the FAZ and measurement of GLD.

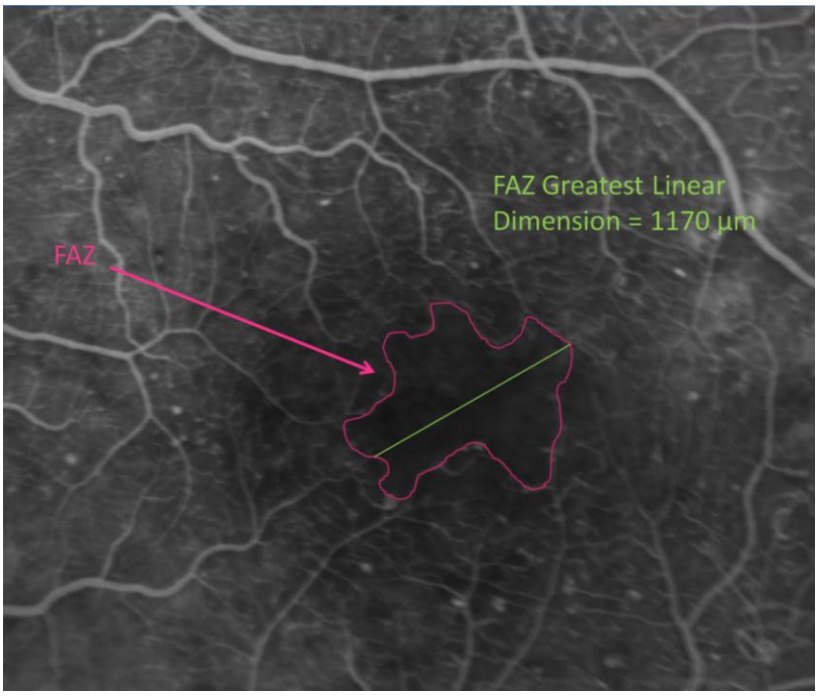

Figure 21 – Frame from a typical fundus fluorescein angiogram to illustrate the outlining and measurement of the foveal avascular zone (FAZ). Note – measurement indicative of method only.

Perifoveal capillary loss was graded in four quadrants around the macula (superior, inferior, nasal, temporal) with reference to ETDRS standard photographs and a numerical grade from 0 (absent) to 4 (severe) was assigned to each quadrant [173].

The masked grading of photographs both for retinopathy grade and foveal avascular zone measurements was carried out by a trained and certified senior diabetic retinopathy grader from the Reading Centre with 100% adjudication by the clinician in charge of the centre. This was in accordance with the protocol for another recent clinical trial [74]. All eyes with change and 10% of eyes without change were also discussed with an independent masked clinician before grade agreement. Ten percent of eyes were re-graded independently of the adjudication and verification process by the same senior grader and the intra-grader reliability was substantial (kappa of 0.76).

2.2.7.8 *Other investigations*

At baseline and at 48 weeks, subjects underwent HbA_{1c} testing to evaluate long-term diabetic control. Blood pressure was recorded at baseline, 12, 24 and 48 weeks.

Cataract grade was evaluated at baseline and 48 weeks using an ordinal scale from 0 to 3. Intraocular pressure was checked at every visit.

2.2.8 ***Outcomes***

The study had a number of prospectively defined exploratory structural and functional outcomes, derived from the comprehensive investigation set described above and reported at baseline, 12, 24 and 48 weeks:

Functional measures

1. Best-corrected ETDRS visual acuity
2. Colour contrast sensitivity: protan and tritan thresholds
3. Microperimetric retinal sensitivity: mean 4° and 12° sensitivity
4. Electrophysiological parameters: PERG P50 amplitude and implicit time, N95 amplitude; full field ERG rod and cone a and b wave amplitudes and implicit times, mfERG distribution of amplitudes

Structural measures

1. OCT quantitative parameters: macular thickness and volume in nine ETDRS subfields
2. OCT qualitative changes: presence of features of DMO in inner and outer retina (cysts, cystoid oedema and hyperreflective foci); neurosensory retina changes (ELM interruptions, photoreceptor IS-OS junction abnormalities); vitreomacular interface abnormalities (ERM, VMT, macular or lamellar hole).
3. Fluorescein angiography: greatest linear diameter (GLD) and area of foveal avascular zone (FAZ); degree of perifoveal capillary loss (PFCL)
4. Colour fundus photography: ETDRS grade of diabetic retinopathy

2.2.9 ***Statistical plan***

Baseline characteristics of the patients were compared by treatment status to assess the adequacy of the randomisation by the trial statistician. Summary measures for the baseline characteristics were mean and standard deviation for continuous (approximate) normally distributed variables; median and interquartile ranges for non-normally distributed variables; and frequencies and percentages for categorical

variables. Normality was assessed by the trial statistician by visual assessment of histograms to look for overt kurtosis or skewness of data. Since this was an exploratory trial, no intent-to-treat analysis was conducted. An available case analysis was conducted (as per a planned statistical analysis agreed upon prior to analysing the data). Adverse events were reported for the whole cohort. Summary statistics were computed for each outcome by treatment group. Analysis of covariance was used to compare continuous outcomes between treatment groups with baseline values of the outcome as a covariate when differences between groups at baseline were present. Any statistical tests conducted used a 2-sided P-value of 0.05; P-values were not formally adjusted for multiple testing. Data analysis was performed using STATA statistical software (version 12, StataCorp LP) and GraphPad Prism (version 6, GraphPad Software Inc., La Jolla, CA, USA).

2.2.10 ***Post-hoc exploratory investigations***

Additional post-hoc exploratory investigations included the evaluation of choroidal thickness by OCT enhanced depth imaging, correlation of retinal function and structure by examining microperimetry and OCT results and a study to evaluate the repeatability of retinal thickness and volume measurements using Spectralis OCT.

2.2.10.1 ***Longitudinal evaluation of choroidal thickness by enhanced depth imaging***

Horizontal line scans taken using the enhanced depth imaging (EDI) mode to visualise the choroid were evaluated by a grader masked to treatment allocation. The automated Spectralis segmentation algorithm defines the inner retinal boundary at the location of the inner limiting membrane (ILM) and the outer retinal boundary at Bruch's membrane (BM). The automated segmentation line from the ILM was manually aligned with the outer choroidal boundary, shown in Figure 22a, leaving the segmentation line for BM in place. The outer choroidal boundary was defined visually as the location where details of choroidal vasculature were no longer visible and a heterogeneous opacity, thought to represent the inner sclera boundary, was seen. This distance, from BM to outer choroidal boundary was taken to represent choroidal thickness. Thickness was measured at the fovea and at 500 µm intervals across the macula nasal and temporal to this, taking the automated measurement incorporated within the HEYEX software, shown in Figure 22b. Measurements were

taken from scans at baseline, 48 weeks and additionally at 12 weeks to look for an early effect of treatment. Data was analysed using GraphPad Prism.

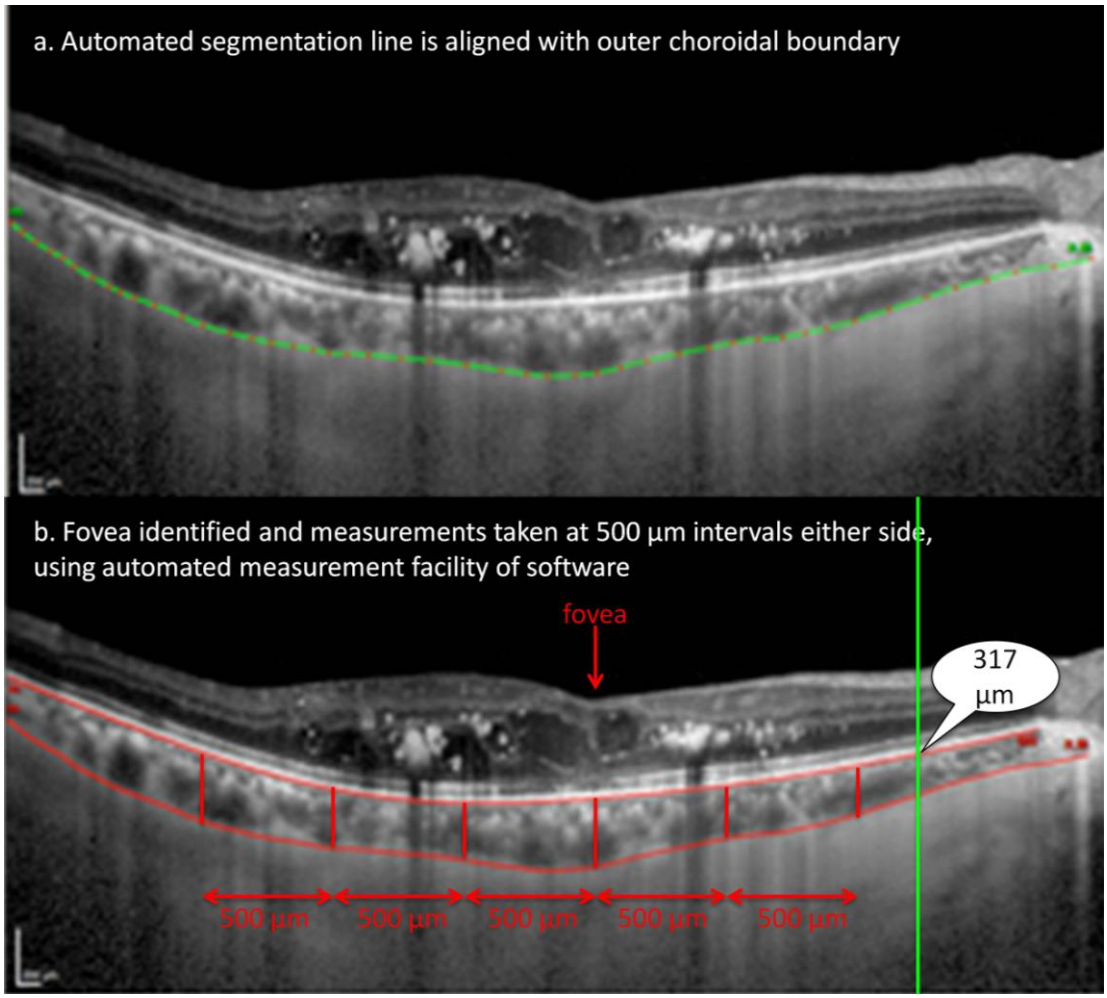

Figure 22 – Method of obtaining choroidal thickness measurements from EDI scans.

2.2.10.2 *Structure-function correlation*

Data were explored to investigate possible correlation between visual acuity and retinal thickness and then between retinal sensitivity and retinal thickness. The method for correlating microperimetry results with OCT results is described below.

Microperimetry results for each point tested were entered into an Excel spreadsheet and from this, mean sensitivity in an area of retina corresponding to each of the nine ETDRS subfields could be calculated. This was done by overlaying the ETDRS grid on the microperimetry grid and determining which test points were located in each subfield, shown in Figure 23. The microperimetry grid can be considered to consist of a central point plus four concentric circles at radii of 1°, 2°, 4° and 6°, while the

ETDRS circular subfields are defined by circles of radii 500 µm, 1500 µm and 3000 µm. The fovea has been reported to be located at approximately 15° from the disc [176] or alternatively at approximately 5mm from the disc [177], giving a conversion factor of approximately 1/3 mm per degree. Thus points forming the 2° circle lie at 0.66 mm radius and test retinal locations lying outside the central subfield. The points forming the 6° circle lie at a radius of approximately 2 mm and hence are outside the 1500 µm circle marking the inner subfields.

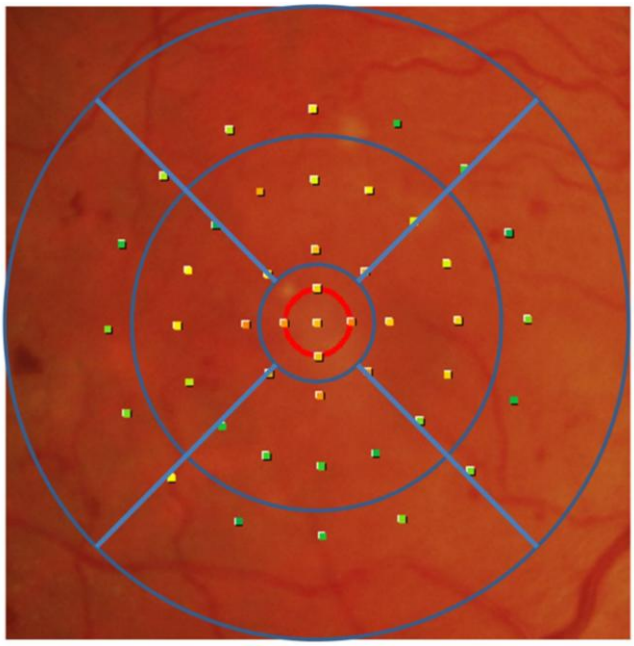

Figure 23 – Diagram to show overlay of ETDRS grid (blue), with 1mm, 3mm and 6mm diameter circles, on microperimetry test grid. Red circle shows microperimetry fixation target of 1° radius.

The arithmetic mean of all points lying in a given subfield, including those lying on the radial lines forming the boundaries, was taken to represent the mean sensitivity in that subfield. For OCT results, the single retinal thickness reading from each subfield on the ETDRS map displayed on the HEYEX software was taken as mean retinal thickness in that subfield without correcting for retinal boundary detection error (see section 2.2.10.3).

Scatter plots for each of the nine subfields using microperimetry and OCT results at baseline were created using GraphPad software to explore correlation between retinal thickness and sensitivity for all subjects at baseline. Further graphs were then created to show change in retinal sensitivity and change in retinal thickness. Correlation was

checked using Pearson's r statistic and if a significant correlation existed in the two groups, linear regression was then used to look for differences between groups to explore a possible treatment effect.

2.2.10.3 *Repeatability of macular thickness measurements in OCT study*

OCT images were used to evaluate the repeatability of retinal thickness and volume measurements. In addition to images from patients screened for this study, further images from patients with DMO undergoing screening for another clinical trial, which were obtained using the same OCT scanning protocol, were also included. For this study, subjects underwent four consecutive "fast" volume scans using the settings described above at a single sitting. Subjects sat back from the machine between each scan. The first scan was set as "reference" and the subsequent three scans were obtained in "follow-up" mode.

Retinal thickness and volume measurements for the nine ETDRS subfields were obtained from the HEYEX software and transferred to an Excel spreadsheet for calculation of coefficients of repeatability using methods described by Bland and Altman [178]. Specifically, in each subfield the variance of the four measurements for every patient was calculated initially in order to obtain S_w, the within subject standard deviation, from the square root of the average of the variances across all *n* subjects. Coefficient of repeatability (CR) was then calculated by 1.96 x √(2x average variance). The width of the 95% confidence interval for CR was then obtained by 1.96 x $\frac{Sw}{\sqrt{2n\,(m-1)}}$ where *n*=number of subjects and *m*=number of times the test was performed[179].

Scans were manually evaluated for the presence of inner or outer retinal boundary detection error. For the purposes of this study, significant automated boundary detection error was defined as the misplacement of either of the ILM or BM boundaries continuously over a section of scanned retina of 1mm or greater. This was deemed to have occurred when the automated line clearly and unambiguously deviated from the hyper-reflective interface representing Bruch's membrane, or deviated from the clearly visualised inner retinal boundary, e.g. by following hyper-reflective interfaces anterior to the retina. Examples of this are shown in Figure 24.

Scans with no significant boundary detection error were included in a sub-analysis to establish whether the CR improves when this type of error is absent.

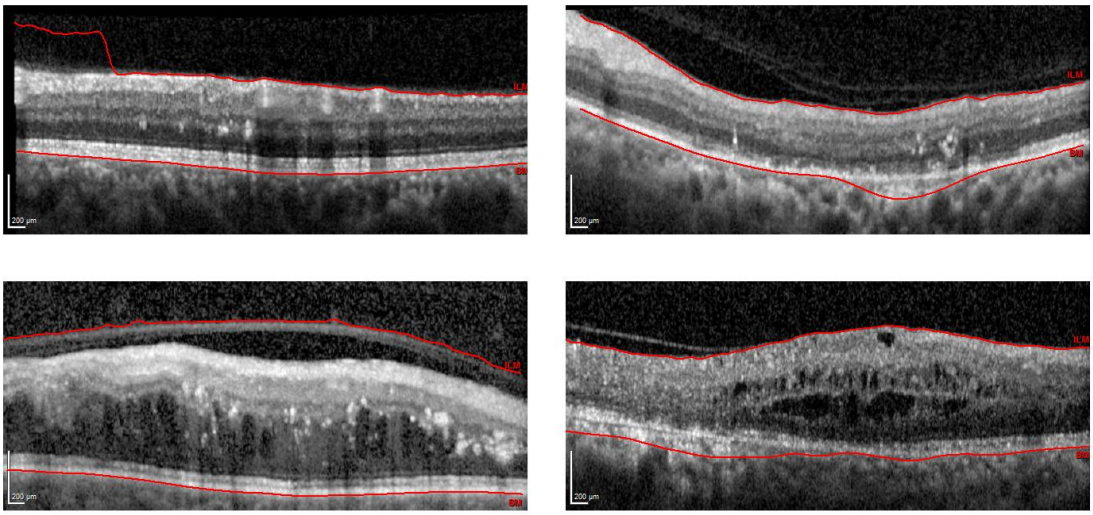

Figure 24 – Examples of boundary detection error. Top and bottom left: misplacement of ILM boundary. Top and bottom right – misplacement of BM boundary.

2.3 **Results of the LUCIDATE study**

2.3.1 ***Recruitment***

Recruitment for the study commenced in November 2010 after full approval was gained from the Medicines and Healthcare products Regulatory Agency, the National Research Ethics Service and the Moorfields Research and Development department. Recruitment was completed in July 2011 and the final follow-up visit was in July 2012.

In total 87 potential patients received the participant information sheet (PIS). Of these, 47 were invited for screening. Pre-screening was undertaken using existing clinical records to identify patients who would not meet the study criteria; patients were only invited for trial screening if they could potentially meet the study criteria. Table 4 summarises reasons for not screening 40 potential patients who had received the PIS. All these patients continued to receive standard NHS clinical care.

Reason	**Number**
Clearly would not meet inclusion/exclusion criteria • Macular ischaemia • Cataract/media opacity • Recent laser • Participating in another trial • Previous vitrectomy	11 • 5 • 2 • 2 • 1 • 1
Potentially suitable but study full before could be contacted	3
Declined • Unwell • Unable to travel • Unable to have time off work • Concerned about risk of injection • Needed both eyes treating • No reason given	15 • 1 • 3 • 2 • 3 • 1 • 4
Unable to contact/failed to return calls	11

Table 4 – LUCIDATE prescreening: reasons why potential participants who had received the participant information sheet were not invited for screening.

From the 47 potential participants screened, inclusion and exclusion criteria were met by 37 patients; reasons for screen failure are shown in
Table 5.

Reason	**Number**
Macular ischaemia	2
Active proliferative retinopathy	2
Visual acuity too good	5 *
HbA_1C too high	1
Fluorescein allergy	1
Ocular infection	1

Table 5 – Reasons for screen failure in LUCIDATE study. * - one patient failed screening twice, having been rescreened when vision had subjectively dropped. The total is greater than 10 because some patients had multiple reasons, e.g. proliferative retinopathy and poor visual acuity.

*2.3.2 **Patient disposition and demographics***

The 37 subjects who met the inclusion and exclusion criteria were randomised in accordance with the study protocol; 25 were randomised to ranibizumab and 12 to laser, in accordance with the planned imbalanced randomisation described in the trial protocol. Of these, 36 received treatment: one subject in the ranibizumab arm withdrew immediately after randomisation as he was not willing to comply with the contraceptive requirements of the study. The 48 week study period was completed by 22 (88%) patients in the ranibizumab arm and 11 (92%) in the laser arm. Participant flow through the study, as recommended by the statement of the Consolidated Standards of Reporting Trials (CONSORT) group, is shown in Figure 25.

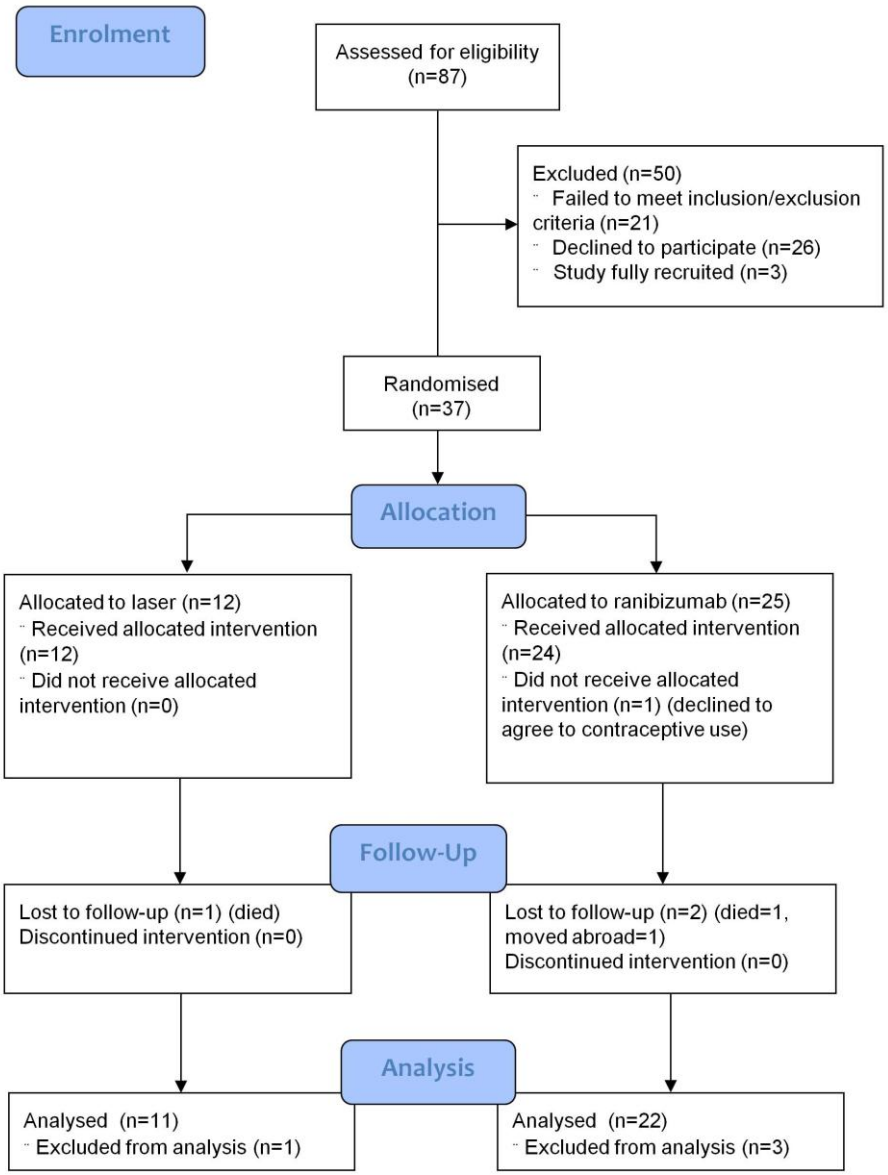

Figure 25 – CONSORT style diagram to show participant flow in the LUCIDATE study.

There were therefore 33 participants who completed the study. This comprised the analysis set for exploratory outcome measures in accordance with the pre-defined statistical analysis plan. Adverse events are reported for all 36 participants who received treatment. The baseline characteristics of study participants comprising the analysis set, including demographic information and details of their systemic condition are shown in Table 6; details of their ocular condition are shown in Table 7. The subjects who did not complete the study were comparable in terms of

demographic profile and systemic condition to those who completed the follow-up period.

	Ranibizumab group	**Laser group**
Number	22	11
Sex (female)	7 (31.8%)	5 (45.5%)
Age (years)	64.9 (58.4-71.0)*	67.4 (62.8-74.6)*
Ethnicity: White Black Asian Other	 15 (68.2%) 0 (0%) 5 (22.7%) 2 (9.1%)	 7 (63.6%) 1 (9.1%) 2 (18.2%) 1 (9.1%)
Type of diabetes 1 2	 4 (18.2%) 18 (81.8%)	 0 (0%) 11 (100%)
Duration of diabetes (years)	18.5 (10-26)*	18 (14-25)*
Systolic BP	130	135
Diastolic BP	75	71
HbA1C	7.93 (1.31)	7.25 (0.92)

Table 6 – Baseline characteristics of participants who completed the LUCIDATE study. Data are shown as mean (SD) or number (%) except * - median (interquartile range).

	Ranibizumab group	**Laser group**
Study eye (left/right)	17/5	5/6
Duration of DME (months)	21 (14, 27)*	32 (15, 60)*
Mean number of previous macular laser treatments	3 (1, 4) *	4 (3, 5)*
Cataract grade 0 1 2 3 IOL	 6 (27.3%) 12 (54.5%) 0 (0%) 0 (0%) 4 (18.2%)	 1 (9.1%) 5 (45.5%) 1 (9.1%) 0 (0%) 4 (36.4%)

Table 7 – Baseline ocular characteristics of participants who completed the study. Data are shown as mean (SD) or number (%) except * - Median (Interquartile range).

*2.3.3 **Treatments given***

All subjects in the ranibizumab arm received injections of ranibizumab at baseline, 4 and 8 weeks. All subjects in the laser arm received macular laser therapy at baseline. A summary of the total number of treatments given at each time point and the mean cumulative number of treatments given is provided in Table 8. Ranibizumab treated

subjects received a mean of 9 injections and laser treated subjects had a mean of 2.6 sessions of macular laser over the course of the trial.

	Ranibizumab group		**Laser group**	
	Number of treatments given at each time point	Mean cumulative number of treatments	Number of treatments given at each time point	Mean cumulative number of treatments
Baseline	22	1	11	1
4 weeks	22	2		
8	22	3		
12	16	3.7	9	1.8
16	17	4.5		
20	16	5.2		
24	13	5.8	4	2.2
28	14	6.5		
32	15	7.1		
36	15	7.8	5	2.6
40	12	8.3		
44	13	9.0		
48	N/A	9.0	N/A	2.6

Table 8 – Total number of treatments at each time point and mean cumulative number of treatments in the two groups.

One subject in each arm underwent cataract surgery in the study eye during the trial. At exit from the trial, there was no significant change in either group in systemic diabetes control or blood pressure.

*2.3.4 **Functional outcome data***

Results of macular function tests to demonstrate efficacy of ranibizumab and laser treatment are presented at baseline, 12, 24 and 48 weeks for the 33 subjects who completed the study. The data are summarised in Table 9.

2.3.4.1 ***Visual acuity***

Mean (SD) BCVA at baseline in the ranibizumab group was 70.4 (4.9) letters and 63.8 (5.7) letters in the laser group. This improved to 76.4 (8.5) letters in the ranibizumab group at 48 weeks but decreased to 62.9 (10.6) letters in the laser group (p=0.083 ANCOVA). This represented a 6 letter gain for ranibizumab versus a loss of 0.9 letters for laser. Box plots to show this data are displayed in Figure 26.

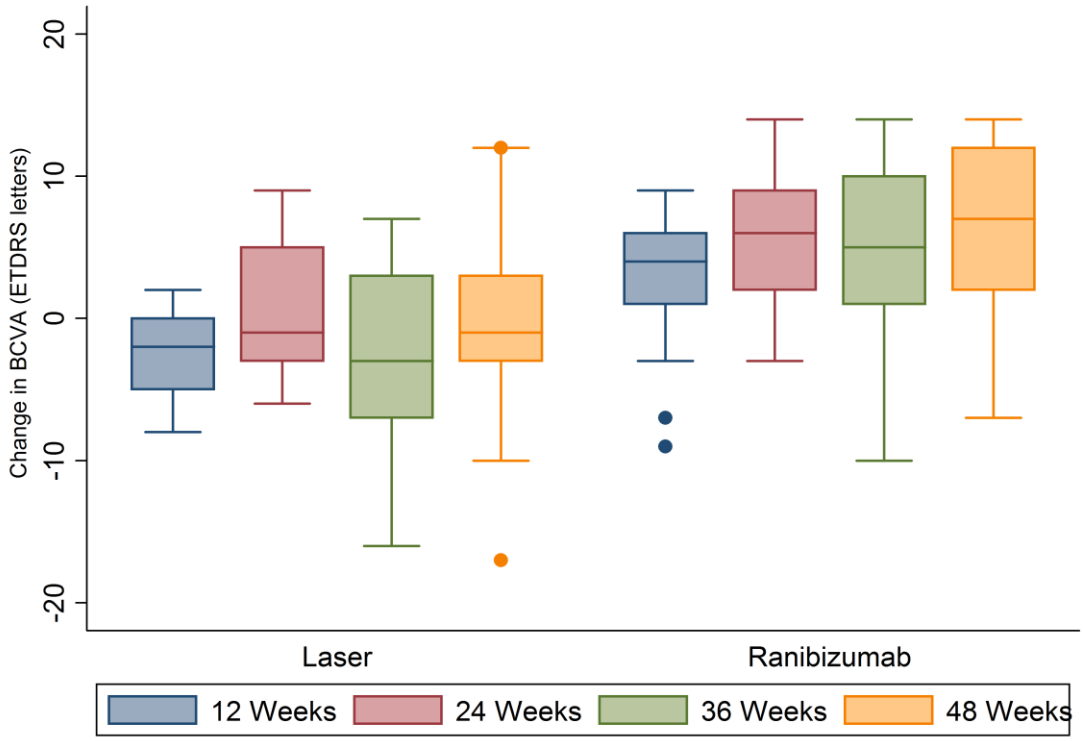

Figure 26 – Box plots of visual acuity data from the LUCIDATE study to show change in BCVA from baseline at four follow-ups.

2.3.4.2 *Microperimetry*

Retinal sensitivity in the central 4° improved from 10.8 (3.7) to 14.0 (4.2) dB in the ranibizumab group and from 10.2 (3.8) to 12.1 (3.4) dB in the laser group (p=0.19). Sensitivity in the central 12° improved from 13.3 (2.7) to 15.7 (2.8) dB in the ranibizumab treated subjects and for laser treated subjects the improvement was from 13.4 (2.5) to 14.5 (2.0) dB (p=0.12), shown in Figure 27.

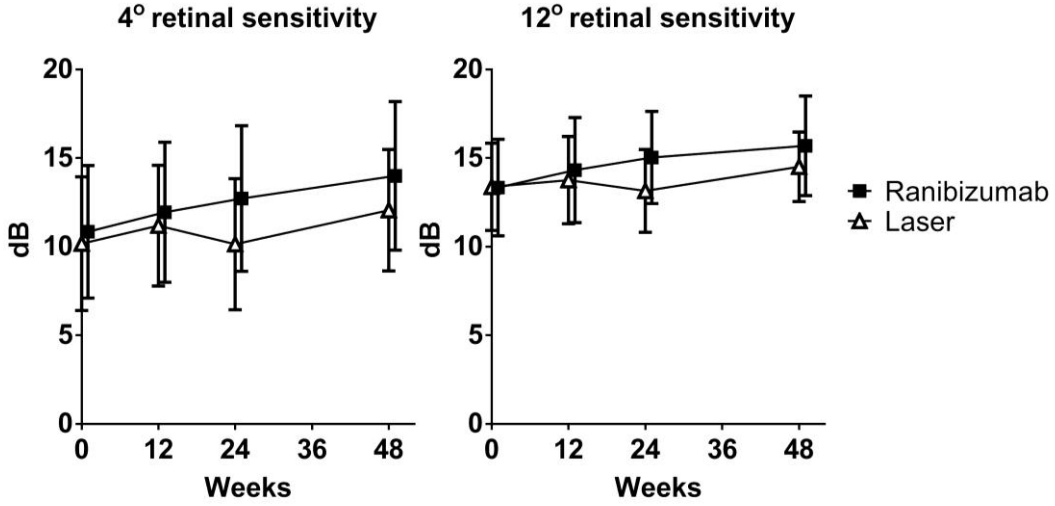

Figure 27 – Retinal sensitivity results from MP1 microperimetry for the two groups in the LUCIDATE study. Results shown as mean ± SD.

2.3.4.3 *Colour contrast sensitivity*

Colour contrast sensitivity in the protan axis improved for ranibizumab treated subjects from 21.4 (22.5) % to 18.0 (16.9) % but worsened in the group receiving laser from 22.9 (22.8) % to 31.0 (35.0) %. Tritan sensitivity also improved for ranibizumab treated subjects from 80.7 (29.6) % to 69.9 (34.5) %. There was improvement, to a lesser degree, in laser treated subjects from 88.9 (20.7) % to 85.8 (25.0) %. Results are shown in Figure 28; it can be seen that there is little evidence of a difference between the two groups at 48 weeks.

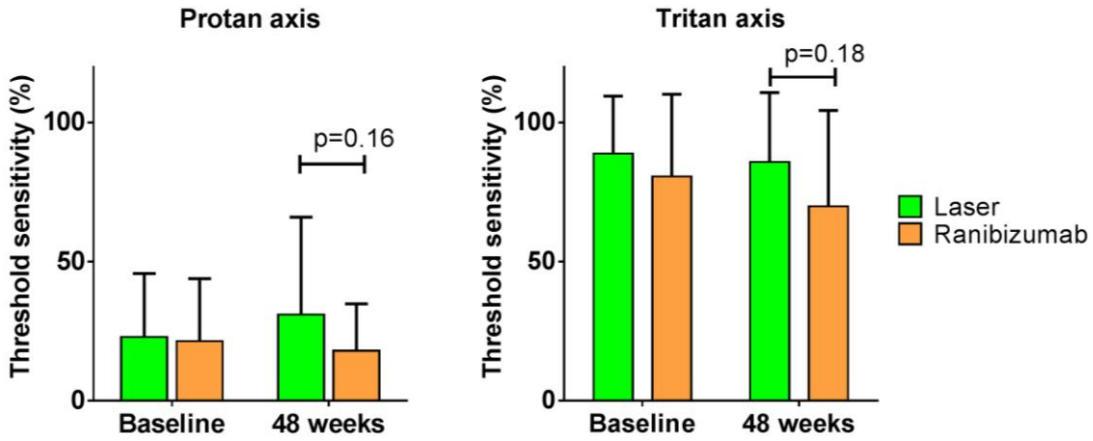

Figure 28 – Colour contrast sensitivity results for the two treatment groups. Bars show mean + SD. P values shown for two-tailed unpaired t test.

	Ranibizumab group (n=22 all time points)	Laser group (n=11 all time points)
BCVA letter score		
0 weeks	70.4±4.9	63.8±5.7
12 weeks	73.1±6.2	60.8±5.6
24 weeks	75.7±7.7	64.4±7.5
36 weeks	75.3±8.9	61.3±9.0
48 weeks	76.4±8.5	62.9±10.6
Protan threshold sensitivity / %		
0 weeks	21.4±22.5	22.9±22.8
12 weeks	18.2±15.7	32.5±35.4
24 weeks	16.4±15.9	18.0±22.6
48 weeks	18.0±16.9	31.0±35.0
Tritan threshold sensitivity / %		
0 weeks	80.7±29.6	88.9±20.7
12 weeks	71.0±33.5	92.4±13.6
24 weeks	71.8±34.8	84.7±24.2
48 weeks	69.9±34.5	85.8+25.0
Microperimetry 4° sensitivity / dB		
0 weeks	10.8 ±3.7	10.2 ±3.8
12 weeks	11.9 ±3.9	11.2 ±3.4
24 weeks	12.7 ±4.1	10.1 ±3.7
48 weeks	14.0 ±4.2	12.1 ±3.4
Microperimetry 12° sensitivity / dB		
0 weeks	13.3 ±2.7	13.4 ±2.5
12 weeks	14.3 ±3.0	13.8 ±2.5
24 weeks	15.0 ±2.6	13.1 ±2.3
48 weeks	15.7 ±2.8	14.5 ±2.0

Table 9 – Summary of the results of functional investigations for subjects in the LUCIDATE study.

2.3.4.4 *Pattern electroretinogram*

The amplitudes of the major waveforms of the PERG, the P50 and N95, are shown in Table 10, together with the peak time for the P50 component. Where technical difficulties such as eye closure or excessive blinking during the test occurred, the results for that subject at that visit are not included. In cases where the PERG was undetectable despite a technically satisfactory examination, indicating severe macular dysfunction, the data are not included in group means as the amplitude and peak time of an undetectable wave should not be considered as zero. Laser treated subjects experienced a decline of 0.13 μv (10.5%) in the P50 component from baseline to 48 weeks, while ranibizumab treated subjects showed minor improvement (0.04 μv; 2.9%). These results are shown in Figure 29. There were only minor variations in the P50 peak time from visit to visit and there was no discernible difference between the two groups. Results for the amplitude of the N95 component mirrored those for the P50, with a decline of 0.21 μv (10.5%) for laser and a decline of 0.04 μv (1.8%) for ranibizumab.

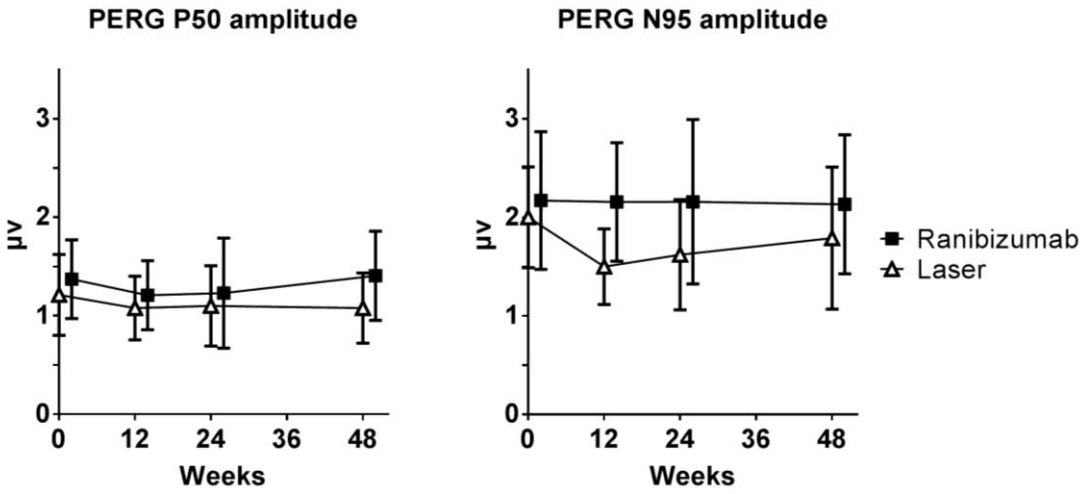

Figure 29 – Results of PERG in two treatment groups showing change in amplitude of P50 and N95 waveforms; data shown as mean ± SD.

	Ranibizumab				Laser			
	No. analysed	**P50 amplitude/μv**	**P50 peak time/ms**	**N95 amplitude/ μv**	**No. analysed**	**P50 amplitude/μv**	**P50 peak time/ms**	**N95 amplitude/ μv**
0 weeks	17	1.37 ±0.40	54.6 ±5.6	2.17 ±0.70	8	1.21 ±0.41	55.8 ±3.8	2.00 ±0.51
12 weeks	14	1.21 ±0.35	54.2 ±3.9	2.16 ±0.60	9	1.08 ±0.32	53.7 ±5.2	1.50 ±0.38
24 weeks	17	1.23 ±0.56	53.9 ±4.2	2.16 ±0.83	10	1.10 ±0.41	51.9 ±4.3	1.62 ±0.56
48 weeks	18	1.41 ±0.45	54.8 ±3.7	2.13 ±0.71	9	1.08 ±0.36	53.5 ±3.7	1.79 ±0.72

Table 10 – Amplitudes and peak times of the major waves of the PERG for ranibizumab and laser treated subjects. Data are presented as mean ± SD in μv and ms as appropriate.

2.3.4.5 *Multifocal electroretinogram*

Descriptive results of the mfERG are presented, as there were several technically poor recordings and mfERG has a high signal-to-noise ratio making quantitative analysis unreliable. Furthermore, as the waveform for each hexagon represents a computer-generated extraction of data from a larger ERG signal, it does not truly represent the actual ERG amplitude from that section of the retina. The results were evaluated in terms of the presence or absence of central and peripheral macular dysfunction, with reference to laboratory normal data, by an electrophysiologist experienced in the interpretation of mfERG.

All subjects at baseline had moderate to severely reduced central responses, defined as an amplitude reduction of 70% or greater, indicating evidence of moderate to severe central macular dysfunction. Mild to moderate peripheral macular dysfunction (25-70% reduction in amplitude) was present in 7 (64%) subjects from the laser group at baseline compared with 8 (36%) in the ranibizumab group.

By 48 weeks in the ranibizumab treated group, 7 (36%) had a mild to moderate increase in central function, 3 (14%) had worsened function and 12 (55%) remained the same. In the laser treated group, 2 (18%) had improved function, 3 (27%) worsened and 6 (55%) had no noticeable change. The degree of peripheral dysfunction remained similar in both groups.

An example of a multifocal ERG result demonstrating improvement in a ranibizumab treated subject is shown overleaf in Figure 30.

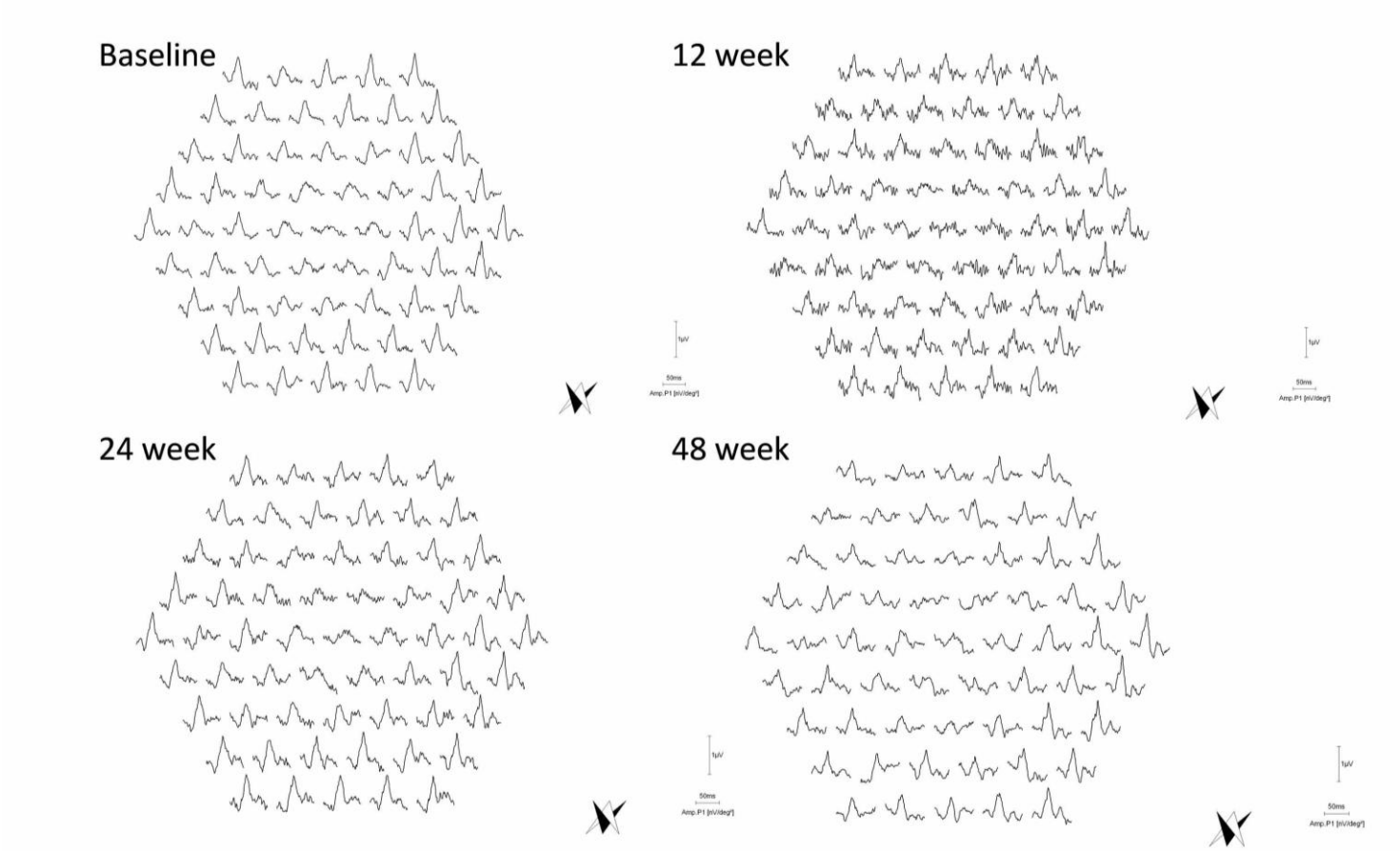

Figure 30 – Example of multifocal ERG from a ranibizumab treated patient. By 48 weeks, the amplitude of the central hexagon has increased. Paramacular and peripheral responses remain relatively unaltered.

2.3.5 Structural imaging studies

Details of the effects on retinal structure of the two treatments were obtained from OCT scans, colour photographs and fluorescein angiograms.

2.3.5.1 *Optical coherence tomography*

Retinal thickness and volume measurements were derived from the "fast" volume scan protocol comprising 25 individual line scans. Grading of morphological features was performed on the "dense" volume scans.

Retinal thickness and volume

Retinal thickness in the central subfield decreased in both groups over the course of the study. Ranibizumab treated subjects showed a decrease in thickness from 455 (79) µm at baseline to 324 (78) µm at 48 weeks. Laser treated subjects decreased from 488 (96) µm to 385 (98) µm. The graph in Figure 31 shows this reduction of 132 µm for ranibizumab compared to the 103 µm reduction for laser (p=0.06 at 48 weeks), but also illustrates the rapid reduction in retinal thickness for ranibizumab treated subjects which is evident at 12 weeks, compared to the slower reduction in thickness with laser.

Results from the other eight ETDRS subfields were similar, with reductions evident throughout the macula in both groups. The thickness data for all subfields are shown in Table 11 together with total macular volume.

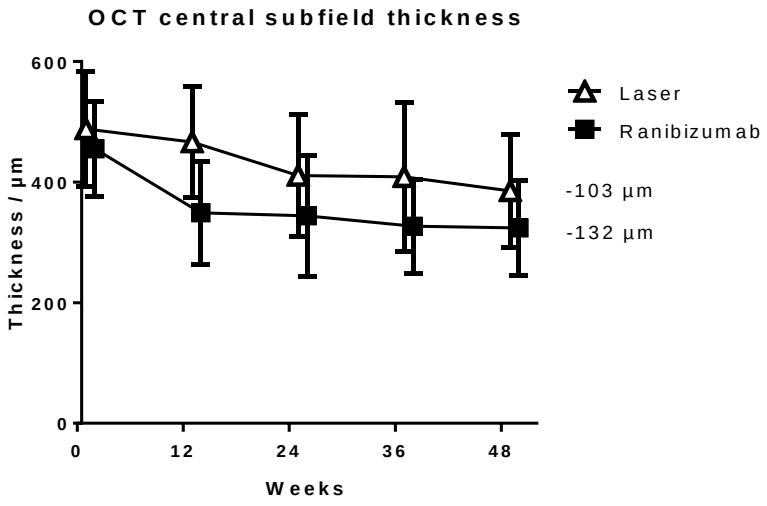

Figure 31 – Reduction in OCT central subfield thickness for two groups in the study; results shown as mean ± SD.

		Ranibizumab			Laser			P-value 48 weeks
		0 weeks	48 weeks	Change	0 weeks	48 weeks	Change	
Centre		455.4 ±78.7	323.9 ±78.1	-131.5 ±98.0	488.2 ±96.3	385.3 ±98.2	-102.9 ±88.4	0.06
Inner	**Superior**	458.7 ±84.2	369.7 ±61.0	-89.0 ±63.6	448.8 ±96.6	390.0 ±58.0	-58.8 ±67.3	0.37
	Nasal	424.7 ±56.6	356.4 ±31.9	-68.3 ±53.1	440.1 ±105.2	400.0 ±78.0	-40.1 ±46.5	0.03
	Inferior	413.9 ±64.1	339.3 ±26.5	-74.6 ±55.3	426.6 ±49.8	385.8 ±39.6	-40.8 ±37.2	0.0003
	Temporal	455.5 ±75.8	349.0 ±49.3	-106.5 ±68.6	450.2 ±70.3	384.9 ±40.6	-65.3 ±65.1	0.05
Outer	**Superior**	366.3 ±86.0	319.8 ±45.6	-46.5 ±48.0	360.5 ±73.4	336.7 ±51.3	-23.8 ±31.1	0.34
	Nasal	365.1 ±62.9	322.7 ±27.3	-42.5 ±46.4	370.0 ±59.5	358.5 ±53.5	-11.5 ±29.9	0.02
	Inferior	334.7 ±70.5	290.9 ±26.6	-43.8 ±49.5	331.5 ±42.2	319.9 ±37.5	-12.4 ±17.5	0.02
	Temporal	363.3 ±76.8	303.5 ±37.0	-59.8 ±46.3	369.3 ±104.4	340.3 ±74.4	-29.0 ±48.5	0.06
Total macular volume		10.53 ±1.62	8.96 ±0.81	-1.57 ±0.98	10.47 ±1.69	9.77 ±1.15	-0.69 ±0.82	0.03

Table 11 – Thickness (µm) in the nine ETDRS subfields and total macular volume (mm^3) from OCT scans for the two treatment groups. Data shown as mean ±SD. P-values for two-tailed t-test (unpaired), without correcting for multiple comparisons.

Morphology

The results of the grading to show the prevalence of the morphological features of DMO identified by masked reading centre graders is shown for the two groups in Table 12. Trends towards decreased prevalence of some of these features were identified in both groups, but clear differences between the groups were not apparent.

Feature			**0 weeks**		**48 weeks**		**Fisher's exact test P**
			Ranibizumab	**Laser**	**Ranibizumab**	**Laser**	
Central subfield	Subretinal fluid		22.7	18.2	4.5	18.2	0.25
	Inner retina	Cysts	22.7	18.2	27.3	27.3	1.00
		Cystoid	81.8	90.9	45.5	72.7	0.27
		HRF	81.8	90.9	77.3	63.6	0.68
	Outer retina	Cysts	27.2	9.1	27.3	9.1	0.39
		Cystoid	90.9	81.8	59.1	54.5	1.00
		HRF	77.3	81.8	63.6	45.5	0.70
Inner subfields	Inner retina	Cysts	54.5	54.5	68.2	45.5	0.44
		Cystoid	81.8	90.9	54.5	72.7	0.25
		HRF	95.5	100	100	100	1.00
	Outer retina	Cysts	40.9	45.5	50	54.5	0.71
		Cystoid	90.9	100	77.3	72.7	1.00
		HRF	95.5	100	95.5	100	1.00
Abnormal foveal depression			100	100	59.1	90.9	0.11
Interrupted ELM			68.2	63.6	54.5	100	0.01
Interrupted IS-OS junction			86.4	72.7	77.3	100	0.14

Table 12 – Prevalence (%) of morphological features of DMO in the two groups; n=22 for ranibizumab and n=11 for laser.

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The prevalence of subretinal fluid in the central subfield decreased in the ranibizumab arm but not the laser arm by 48 weeks. There was no clear evidence of a treatment effect in either group on retinal cysts in the inner or outer retina either in the central subfield or the four surrounding (inner) subfields. The prevalence of cystoid oedema did appear to decrease with both treatments. All subjects had an abnormal foveal depression at baseline. In the laser group, 9% of subjects (1/11) had a normal foveal depression by 48 weeks but in the ranibizumab group this was 40% (9/22 subjects). The two groups were comparable at baseline in terms of the prevalence of interruptions in the lines representing the external limiting membrane or inner segment-outer segment (IS/OS) junction (ellipsoid layer), but at 48 weeks the ranibizumab group showed a significantly lower prevalence of interrupted ELM compared with laser (P=0.01, Fisher's exact test). The prevalence of interrupted

IS/OS junction in the laser group increased while the ranibizumab group decreased (P=0.14).

Evaluation of vitreomacular interface abnormalities revealed very little change in either group over the course of the study. In the ranibizumab group, 5 subjects (22.7%) had an epiretinal membrane at baseline compared to 4 subjects in the laser group (36.4%). At 48 weeks there was no significant difference in these figures. One subject in the ranibizumab group had an incomplete PVD identified at baseline, which persisted until 48 weeks. No subjects had lamellar hole, macular hole or vitreomacular traction at any time point.

2.3.5.2 *Colour fundus photography*

Four-field color photographs were graded at baseline and 48 weeks to obtain the numerical ETDRS grade of diabetic retinopathy. One subject in the laser group worsened by one grade, 6 remained the same and 4 improved by one grade. Two of the ranibizumab group worsened by one grade; 1 by three grades and 10 remained the same. Seven improved by one grade and 2 by 2 grades (Table 4Table 13).

		Early Treatment Diabetic Retinopathy Study grade					
		35	**43**	**47**	**53**	**61**	**65**
Ranibizumab	0 weeks	1 (4.5)	9 (41.0)	5 (22.7)	6 (27.3)	1 (4.5)	0 (0)
	48 weeks	2 (9.1)	12 (54.5)	5 (22.7)	1 (4.5)	1 (4.5)	1 (4.5)
Laser	0 weeks	0 (0)	7 (63.6)	2 (18.2)	2 (18.2)	0 (0)	0 (0)
	48 weeks	2 (18.2)	5 (45.5)	3 (27.3)	1 (9.1)	0 (0)	0 (0)

Table 13 – Grade of diabetic retinopathy in the two groups at baseline and 48 weeks from masked reading center grading of colour fundus photographs.

Representative examples of colour fundus photographs from subjects in the study, presented together with OCT images and microperimetry data from baseline and 48 weeks, are shown in Figure 32 (ranibizumab) and Figure 33 (laser).

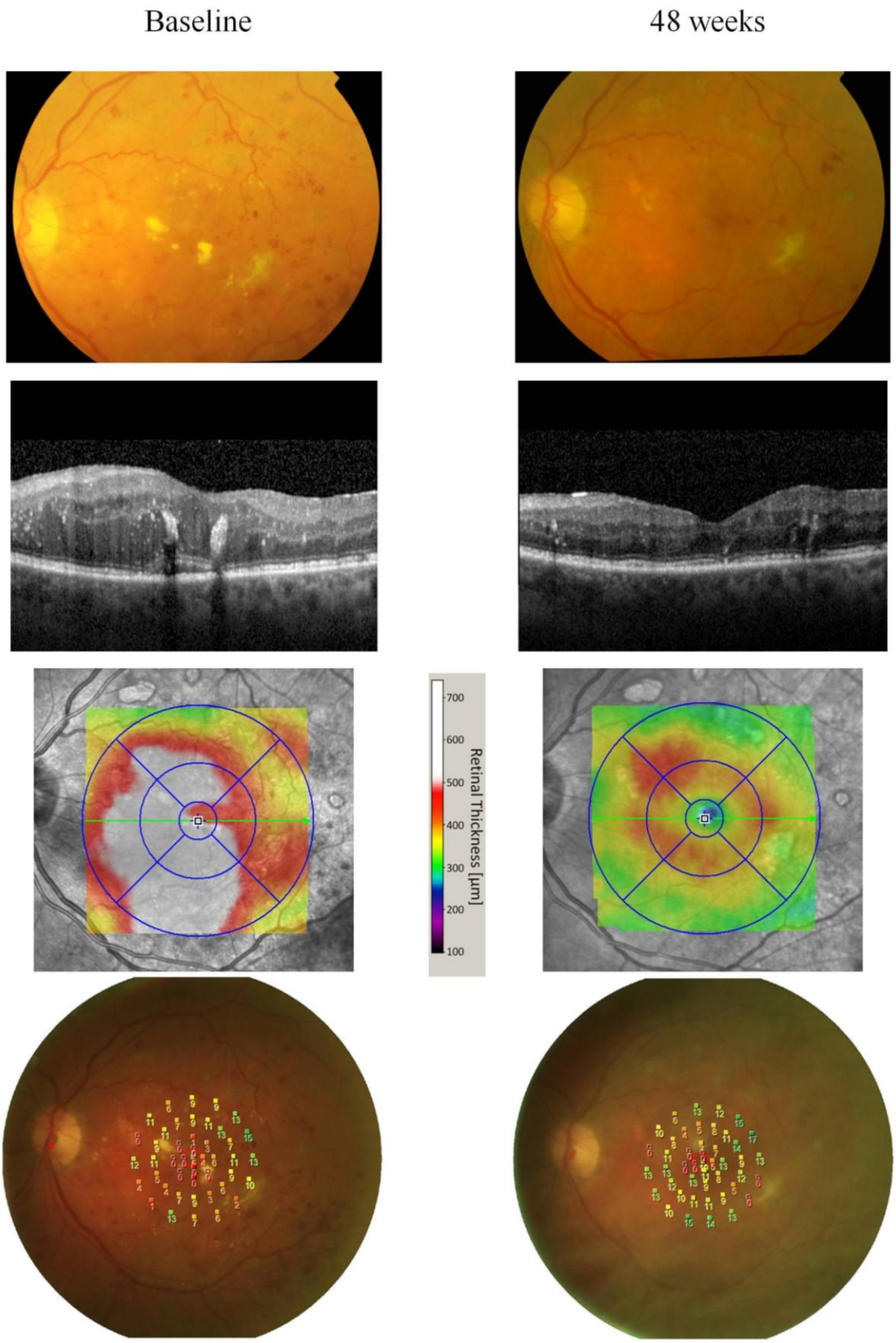


Figure 32 – Colour fundus photographs, OCT image, retinal thickness maps and microperimetry results from ranibizumab treated subject in the LUCIDATE study at baseline and 48 weeks.

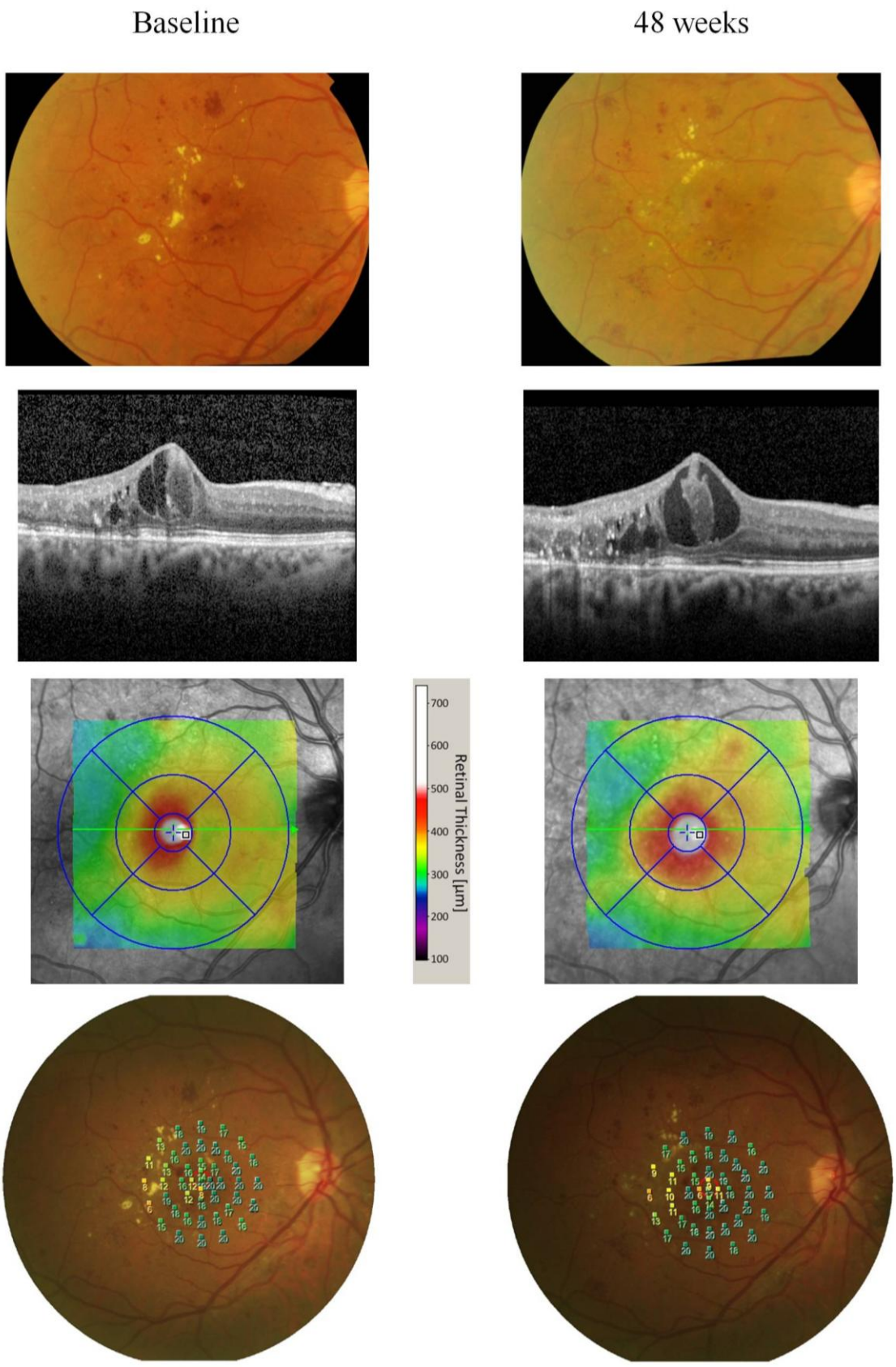


Figure 33 – Colour fundus photographs, OCT image, retinal thickness maps and microperimetry results from laser treated subject in the LUCIDATE study at baseline and 48 weeks.

2.3.5.3 *Fluorescein angiography*

All subjects had a baseline foveal avascular zone (FAZ) greatest linear dimension (GLD) less than 1000 μm on fluorescein angiography. One subject in the ranibizumab group had an angiogram that was deemed ungradable at baseline and was excluded from analysis. In both groups there was a small increase in FAZ area from baseline to 48 weeks (ranibizumab: 0.255 (0.102) mm^2 to 0.321 (0.111) mm^2; laser: 0.346 (0.163) mm^2 to 0.432 (0.192) mm^2). Although this suggests that macular ischaemia has progressed during the study, there was no evidence of a difference between the two groups to suggest a treatment effect (P=0.476, analysis of covariance).

Perifoveal capillary loss (PFCL) was graded by the reading centre in four quadrants from 0 (none present) to 4 (severe loss). A total score from 0-16 was obtained by adding the scores in the four quadrants. The ungradable angiogram in the ranibizumab group was similarly excluded from this analysis as was a further angiogram in the laser group where capillary loss could not be accurately graded. At baseline in the laser group, median (IQR) PFCL score was 9.5 (8.25-10.75) and in the ranibizumab group was 10 (9-11). By 48 weeks, this had worsened by at least one grade in 15/40 (37.5%) of quadrants graded in the laser group and in 24/84 (28.6%) of quadrants in the ranibizumab group. However, this represented a change in score to 11 (10-12) for laser and 10 (9-12) for ranibizumab. The changes in grade for each quadrant were added to give a single score per subject and there was no difference in score between the two groups (P=0.65, Rank-sum test).

*2.3.6 **Safety data***

2.3.6.1 *Full field electroretinography*

The comprehensive data set from the full field ERGs to evaluate rod and cone system function is shown in Table 14. Technically unsatisfactory recordings, e.g. those compromised by blink artifact, were excluded from this analysis.

			Ranibizumab		Laser	
		Time point	**Amplitude**	**Peak time**	**Amplitude**	**Peak time**
Rod (DA 0.01)	b-wave	0 weeks	140.5 ±69.5	105.5 ±9.5	120.9 ±41.2	109.3 ±10.6
		12 weeks	124.2 ±68.1	103.4 ±12.5	124.1 ±41.9	110.1 ±11.5
		24 weeks	135.8 ±81.1	103.9 ±11.0	132.7 ±48.8	112.0 ±11.3
		48 weeks	144.7 ±81.7	104.3 ±10.7	121.4 ±44.6	109.5 ±9.1
Bright flash (DA 11.0)	a-wave	0 weeks	248.6 ±76.5	14.4 ± 1.0	257.0 ±64.3	17.0 ±3.2
		12 weeks	235.8 ±88.8	14.5 ±1.3	245.0 ±71.5	17.6 ±3.2
		24 weeks	233.8 ±83.6	14.3 ±1.2	233.6 ±55.9	17.0 ±4.5
		48 weeks	227.5 ±76.5	14.3 ±1.3	249.5 ±68.1	16.2 ±2.6
	b-wave	0 weeks	386.6 ±125.1	53.0 ±5.4	365.0 ±53.5	56.5 ±6.2
		12 weeks	354.5 ±127.6	53.3 ±5.0	354.5 ±62.3	56.1 ±4.8
		24 weeks	354.5 ±122.7	52.3 ±4.5	364.5 ±87.5	56.2 ±4.9
		48 weeks	367.8 ±113.2	53.4 ±5.5	365.9 ±61.0	53.8 ±3.6
Cone flicker (LA 30Hz)	30 Hz	0 weeks	86.0 ±32.2	30.9 ±3.5	69.1 ±24.5	33.5 ±5.0
		12 weeks	76.1 ±23.4	30.4 ±3.1	62.7 ±19.9	33.5 ±4.9
		24 weeks	77.9 ±33.0	30.4 ±3.1	65.4 ±21.2	33.0 ±4.3
		48 weeks	79.2 ±28.1	31.6 ±3.4	62.3 ±20.2	33.0 ±4.2
Photopic single flah (LA 2.0)	a-wave	0 weeks	32.3 ±10.4	15.0 ±1.1	31.8 ±8.7	16.2 ±1.6
		12 weeks	28.3 ±7.8	15.5 ±3.0	26.4 ±8.7	16.2 ±1.9
		24 weeks	27.9 ±11.0	15.1 ±1.2	30.0 ±8.4	16.8 ±4.1
		48 weeks	29.3 ±9.9	15.4 ±1.2	29.5 ±7.6	16.3 ±1.5
	b-wave	0 weeks	114.8 ±42.5	32.4 ±2.2	88.2 ±27.9	35.0 ±4.1
		12 weeks	100.8 ±32.7	32.8 ±4.3	81.8 ±27.8	35.2 ±4.5
		24 weeks	98.5 ±43.3	32.4 ±2.3	89.1 ±26.1	35.3 ±7.0
		48 weeks	105.5 ±37.3	33.0 ±2.5	91.0 ± 19.7	34.6 ±3.8

Table 14 – Amplitudes and implicit times of the major ERG waveforms, shown in µv or ms respectively, presented as mean ± SD.

Rod system function was evaluated by the dark-adapted ERGs, comprising the dim flash ERG (DA 0.01) and the bright flash scotopic ERG (DA 11). There was no change identified in the dim flash ERG b-wave in either group over 48 weeks. For the brighter flash ERG (DA 11), the mean a-wave amplitude decreased in the ranibizumab group from 249 µv to 228 µv with no change in peak time, while the b-wave amplitude decreased from 387 µv to 368 µv, again with no change in peak time. There was no similar change in the laser group; shown in Figure 34. None of the changes were clinically significant in any patient.

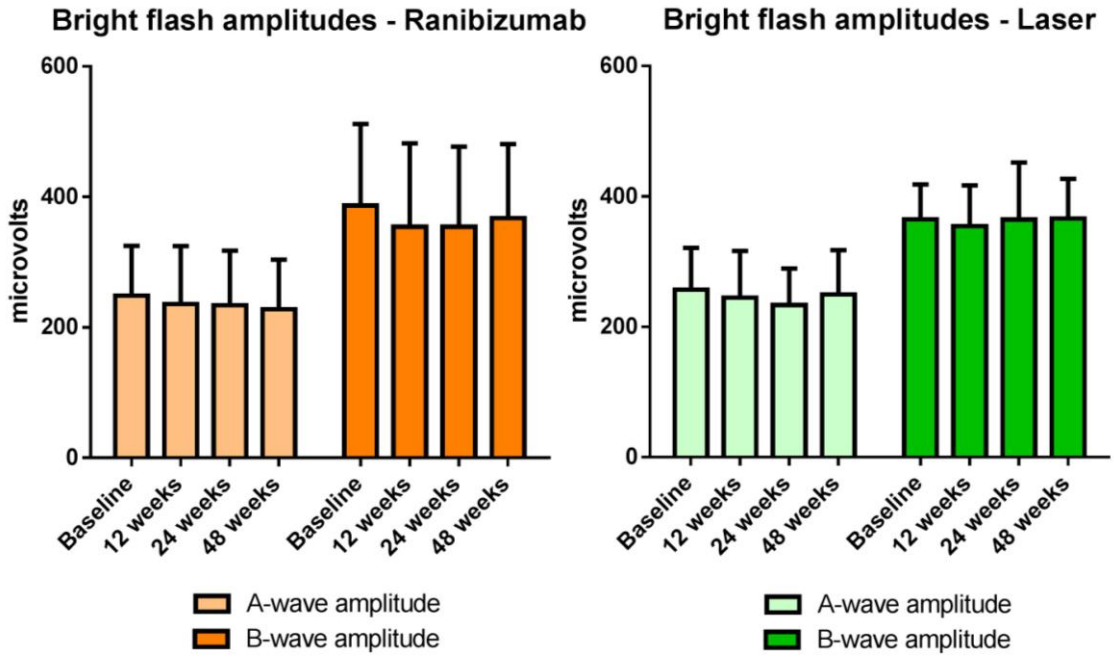


Figure 34 – Amplitudes of a- and b-waves from bright flash scotopic (DA 11) electroretinogram for ranibizumab and laser treated subjects in the LUCIDATE study.

Evaluation of the cone system by the light-adapted ERGs revealed no significant changes. Minor variability occurred in the amplitude of the 30 Hz flicker ERG (LA 30 Hz), but there was no change in peak time exceeding 1 ms in the group overall. Similarly, the photopic flash ERG (LA 2.0) did not reveal any significant change in function in either group. There was no clinically significant change in implicit time for any of the waveforms measured.

2.3.6.2 *Adverse events and withdrawals*

Ocular and non-ocular adverse events occurred in both treatment groups and are reported in Table 15. There were no cases of endophthalmitis in either treatment arm. Ocular adverse events occurred more frequently in the ranibizumab arm (19 vs. 1) and were related to the injection procedure itself. There were more non-ocular adverse events in the ranibizumab arm (44 vs. 17), although the frequencies of the most common of these were similar in the two arms. No serious adverse events were related to the study drug or injection procedure. There were 2 deaths reported during

the study (1 per treatment arm), neither of which related to the study drug. One subject in the ranibizumab arm died from advanced hepatocellular carcinoma which likely predated his entry into the trial as he was at that point under investigation for unexplained thrombocytopaenia. One subject in the laser arm died from acute renal failure precipitated by gastroenteritis while on an overseas trip. One patient in the ranibizumab arm withdrew after 20 weeks as he was moving overseas. There were no suspected unexpected serious adverse reactions (SUSARs).

N (%)	Ranibizumab (n=24)	Laser (n=12)
Ocular:		
Blepharitis		1 (8)
Conjunctivitis	1 (4)	
Corneal abrasion	3 (13)	
Eye pain/discomfort	4 (17)	
Posterior vitreous detachment	1 (4)	
Raised intraocular pressure	1 (4)	
Rash (periocular)	1 (4)	
Subconjunctival haemorrhage	6 (25)	
Visual disturbance	2 (8)	
Non-ocular:		
Abdominal pain/diarrhoea	1 (4)	
Acute renal failure	1 (4)	1 (8)
Anaemia	1 (4)	
Exertional dyspnoea	1 (4)	
Fall/collapse	1 (4)	1 (8)
Headache	4 (17)	1 (8)
Hepatocellular carcinoma	1 (4)	
Intermittent claudication	1 (4)	
Musculoskeletal pain/injury	7 (29)	3 (25)
Retinal detachment non-study eye	1 (4)	
Skin infection/swelling/rash	7 (29)	2 (17)
Toothache	1 (4)	
Upper respiratory tract infection	12 (50)	5 (42)
Urinary tract infection	2 (8)	2 (17)
Viral illness	3 (13)	1 (8)
Vitamin B deficiency		1 (8)
Death	1 (4)	1 (8)

Table 15 – Adverse events in the study, reported as number (%) of participants experiencing event.

2.3.7 ***Longitudinal evaluation of choroidal thickness by enhanced depth imaging***

The 33 subjects who completed 48 weeks follow-up also comprised the analysis set for the choroidal thickness study. At baseline, before treatment, mean (SD) choroidal thickness at the fovea was greater than for any other location measured across the macular at 285 (68) µm. The choroid was thicker in the temporal region than in the nasal region: the mean of five measurements from 0.5 to 2.5 mm temporally was 263 µm compared to 236 µm for the equivalent five measurements nasally. The profile of choroidal thickness for all subjects at baseline is shown in Figure 35.

There was no significant change in choroidal thickness in either treatment group over the course of the study at any location in the macula. Table 16 shows the measurements for choroidal thickness at the subfoveal location, and for mean choroidal thickness, obtained by taking a mean of the 12 measurements across the macula from 2.5 mm nasally to 3.0 mm temporally at 500 µm intervals and including the subfoveal measurement. Retinal thickness measurements from the central subfield are shown for comparison. The results for the two treatment groups for every location measured across the macula are shown in Figure 36A and B.

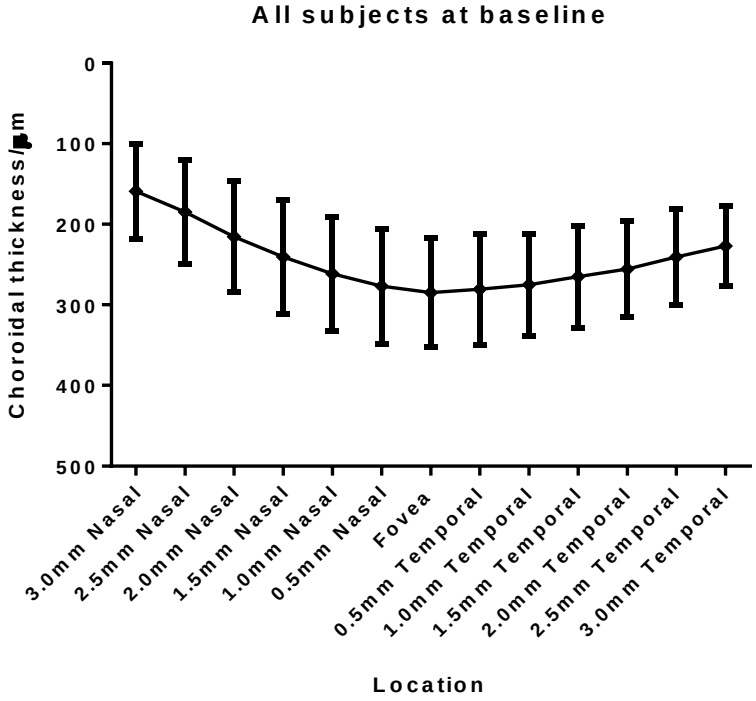
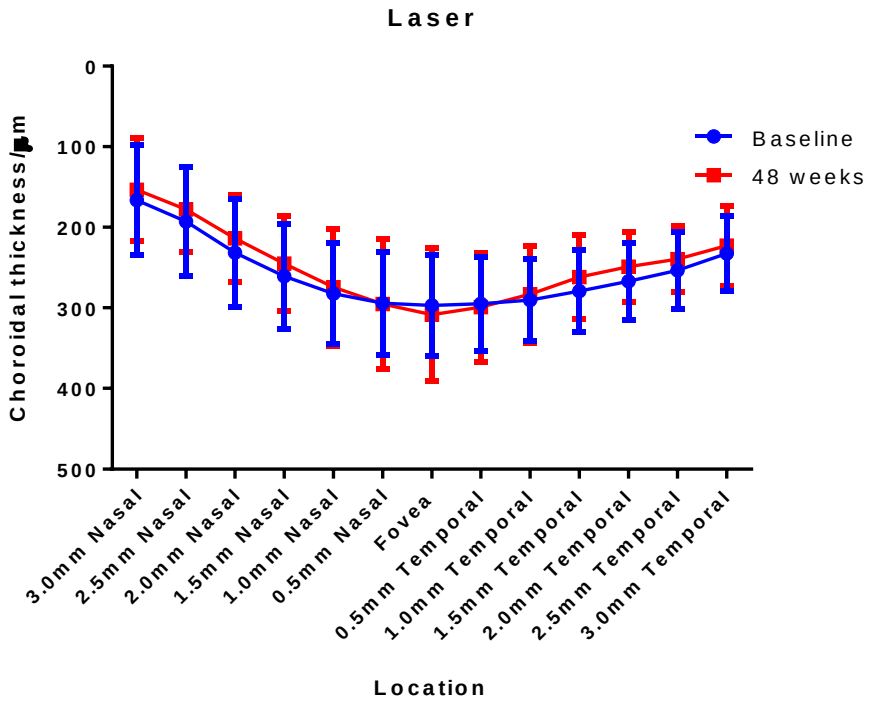


Figure 35 – Choroidal thickness across the macula (shown for a left eye) for all subjects at baseline. Inverted y-axis to demonstrate choroidal thickness profile in usual OCT orientation.

	Ranbizumab		**Laser**	
	Baseline	48 weeks	Baseline	48 weeks
Subfoveal Choroidal Thickness	278.5 ±62.7	278.7 ±81.8	297.0 ±70.7	308.2 ±75.2
Mean Choroidal Thickness	243.5 ±58.8	245.5 ±58.3	264.6 ±51.8	255.7 ±52.9
OCT central subfield thickness (retina)	455.4 ±78.7	323.9 ±78.1	488.2 ±96.3	385.3 ±98.2

Table 16 – Choroidal thickness measurements in µm for two treatment groups at baseline and 48 weeks. Retinal thickness measurements from OCT scans are shown for comparison.



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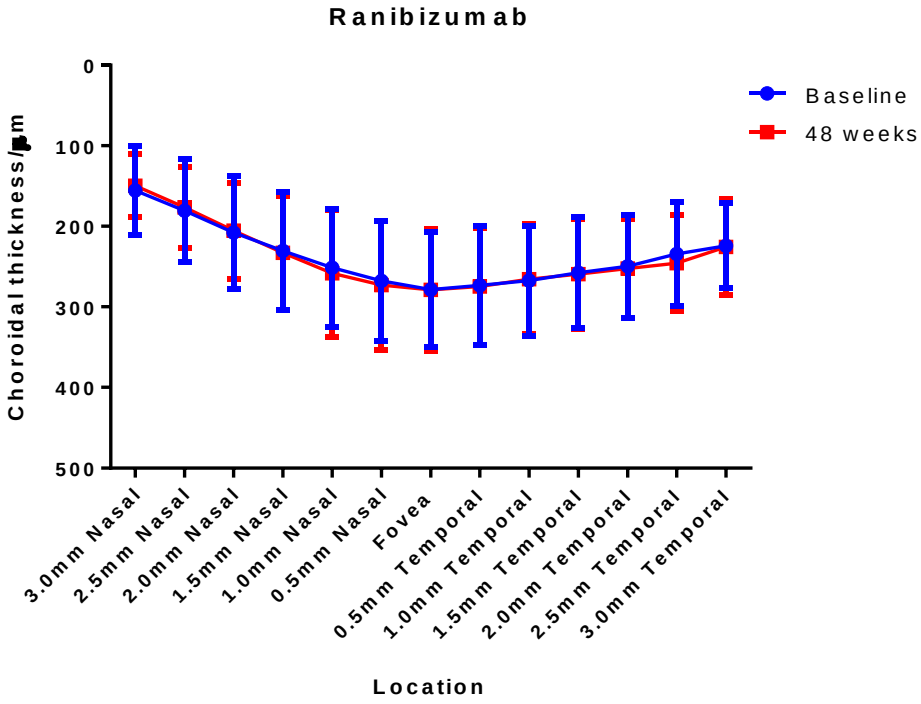
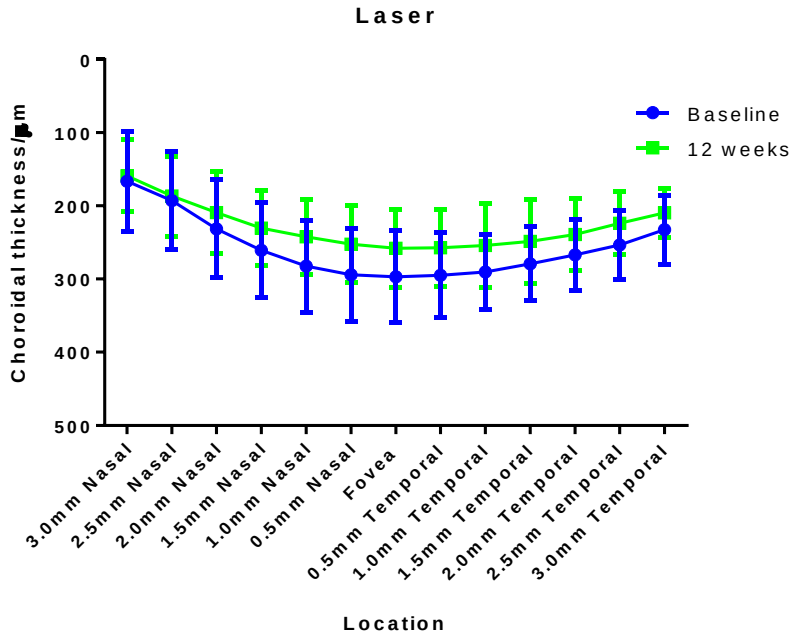


Figure 36A and B – Graphs to show choroidal thickness at baseline and 48 weeks in two treatment groups – laser (A) or ranibizumab (B). Graphs orientated to show choroidal profile for left eye in usual OCT view.

To investigate whether there was an early effect on choroidal thickness of either treatment, the scans taken at 12 weeks were analysed in the same way. All subjects at

this time point had either received three consecutive ranibizumab injections or macular laser. These results are shown in Figure 37A and B. There was no significant difference in either the laser or ranibizumab group between choroidal thickness at baseline and 12 weeks (multiple t-tests).



A

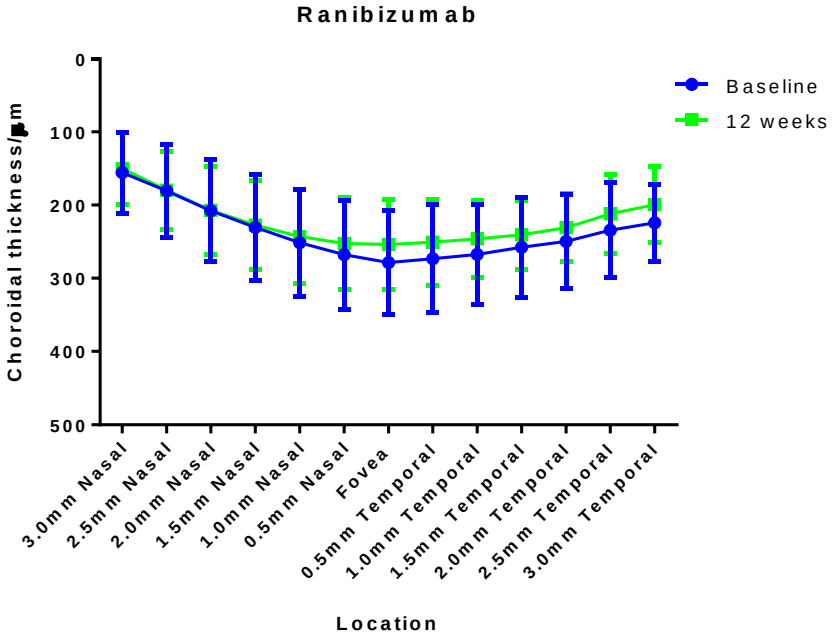


Figure 37A and B – Graphs to show choroidal thickness at baseline and 12 weeks in two treatment groups – laser (A) or ranibizumab (B). Graphs orientated to show choroidal profile for left eye in usual OCT view.

Data were further examined to explore the possible correlation of choroidal thickness with retinal thickness and with visual acuity. At baseline, there was no correlation between choroidal thickness at the fovea and either retinal centre point thickness or central subfield thickness. Figure 38 shows results for retinal thickness in the central subfield and subfoveal choroidal thickness.

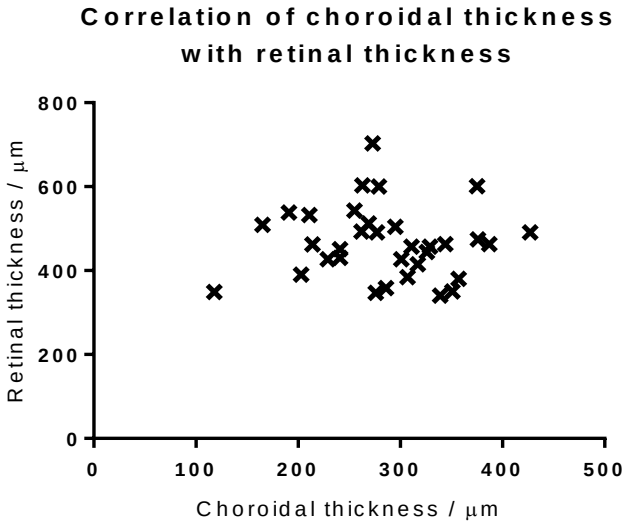


Figure 38 – Subfoveal choroidal thickness vs. retinal central subfield thickness (Pearson r = -0.03, P=0.86).

However, there was a weak positive correlation between choroidal thickness and visual acuity that did not reach statistical significance (Pearson r = 0.31, P=0.08), shown in Figure 39.

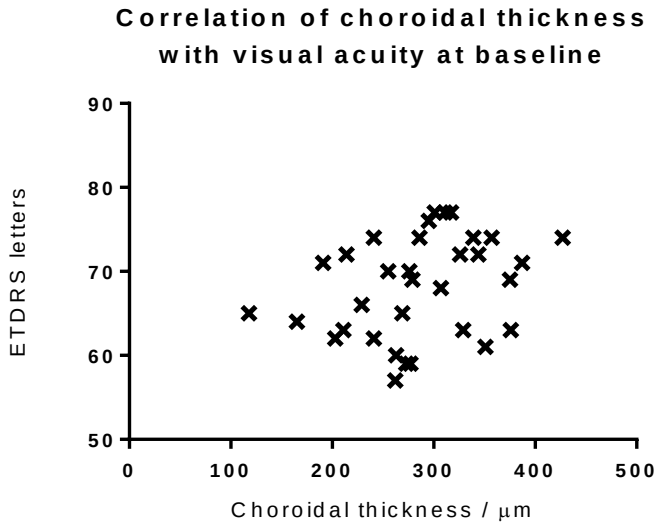


Figure 39 – Subfoveal choroidal thickness vs. ETDRS visual acuity for all subjects at baseline.

*2.3.8 **Structure-function correlation studies***

Data were analysed for the 33 patients who completed 48 weeks of follow up in the main study. Visual acuity at baseline showed a trend towards a significant negative correlation with retinal thickness (Pearson r = -0.3017, P = 0.088), shown in Figure 40. Applying linear regression to these results, the slope of the line gives a decrease of 2.1 ETDRS letters for every 100 µm gain in central subfield thickness.

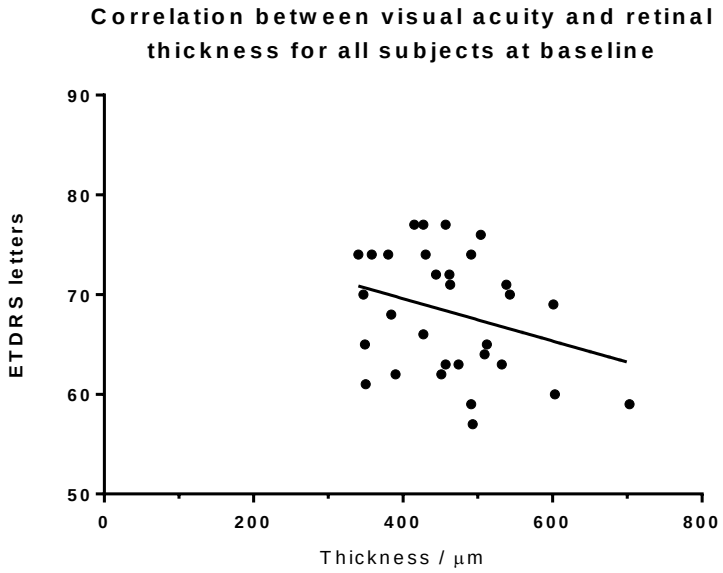


Figure 40 – Scatter plot to show ETDRS visual acuity vs. central subfield thickness for all subjects at baseline.

At 48 weeks, change in retinal thickness was plotted against change in visual acuity for two treatment groups. There was a significant correlation present for ranibizumab treated subjects (r = -0.65, P = 0.001) but not for laser (r = 0.025, P = 0.9). Linear regression for ranibizumab treated subjects only showed that a change of 4.1 letters was associated with a retinal thickness change of 100 µm, shown in Figure 41.

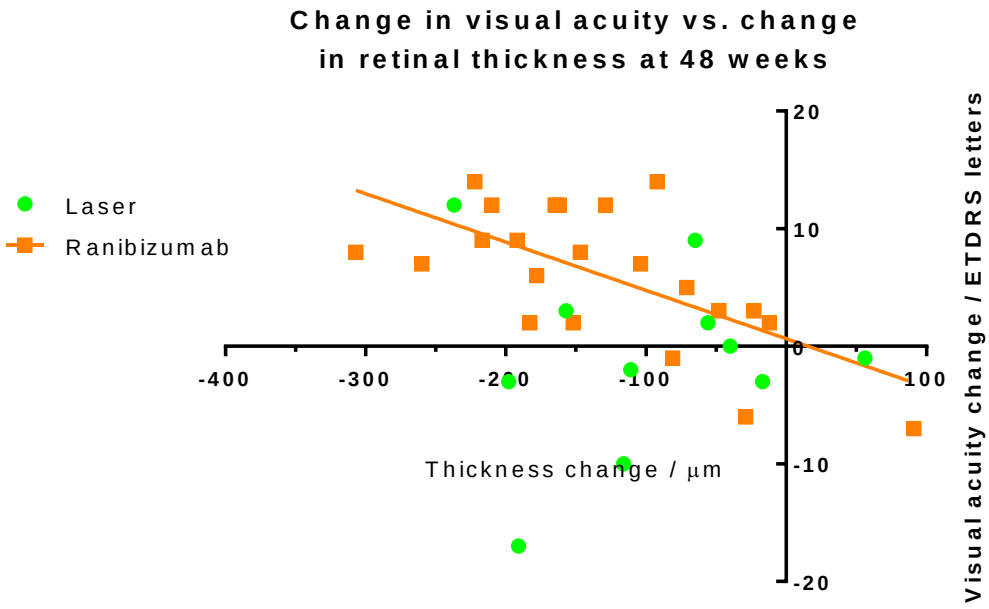


Figure 41 – Scatter plot to show correlation between change in retinal thickness and change in visual acuity for two treatment groups at 48 weeks. Linear regression shown for ranibizumab.

To explore whether there was better correlation between retinal thickness and an objective measure of retinal sensitivity, results for retinal thickness in the nine ETDRS subfields were correlated with microperimetry results in the same nine subfields. At baseline, a negative correlation was present: as retinal thickness increased, sensitivity decreased. Results for the nine ETDRS subfields are shown in Figure 42.

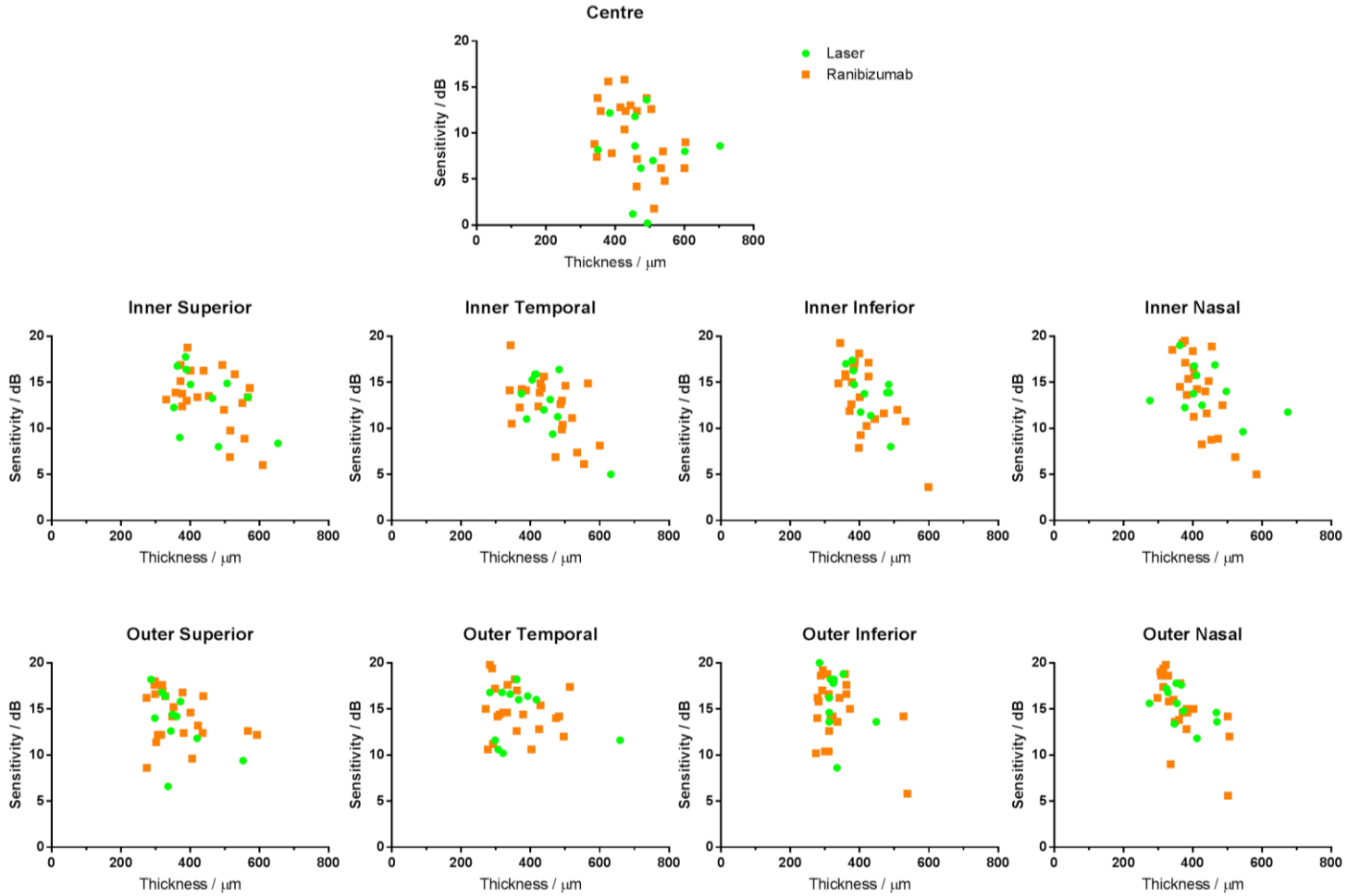


Figure 42 – Scatter plots to show correlation between retinal sensitivity and retinal thickness for all subjects at baseline, grouped by treatment allocation.

Because all nine subfields showed similar results and no difference between subjects allocated to laser or ranibizumab was evident, all points were combined to estimate the overall effect of thickness on sensitivity, shown in Figure 43. There was a statistically significant correlation between retinal thickness and retinal sensitivity overall ($r = -0.5216$, $P<0.0001$). The results of linear regression gave a slope of -0.02233 ± 0.002127, i.e. each micron increase in retinal thickness is associated with a loss of approximately 0.02 dB sensitivity. Alternatively, 1 dB change in retinal sensitivity is associated with a change in thickness of approximately 45 µm.

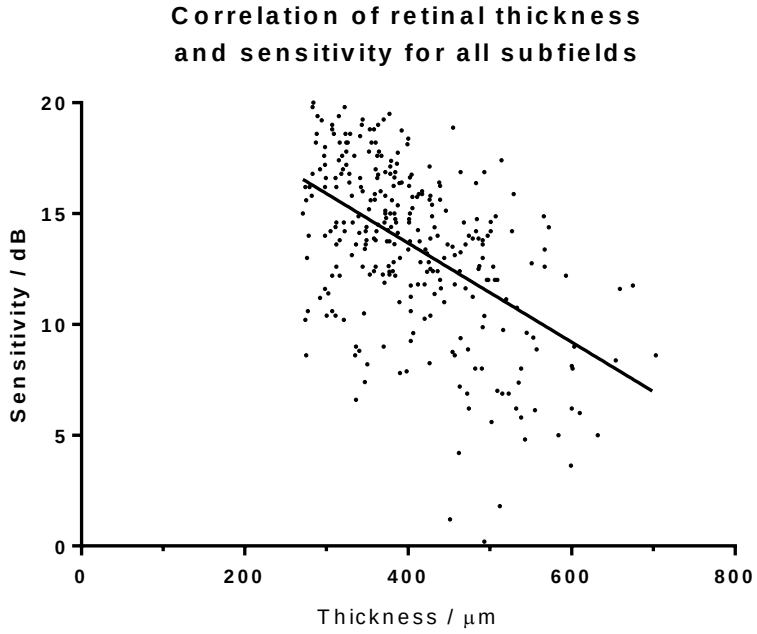


Figure 43 – Graph to show correlation of retinal thickness with retinal sensitivity for all subjects at baseline (n=33) in all ETDRS subfields.

Confirmatory evidence of this finding is shown by longitudinal evaluation of retinal sensitivity change and retinal thickness change, shown by scatter plots in Figure 44. Each of the nine subfields appears to show a similar negative correlation, so values for all nine subfields were combined to estimate the effect of thickness change on sensitivity, shown in Figure 45.

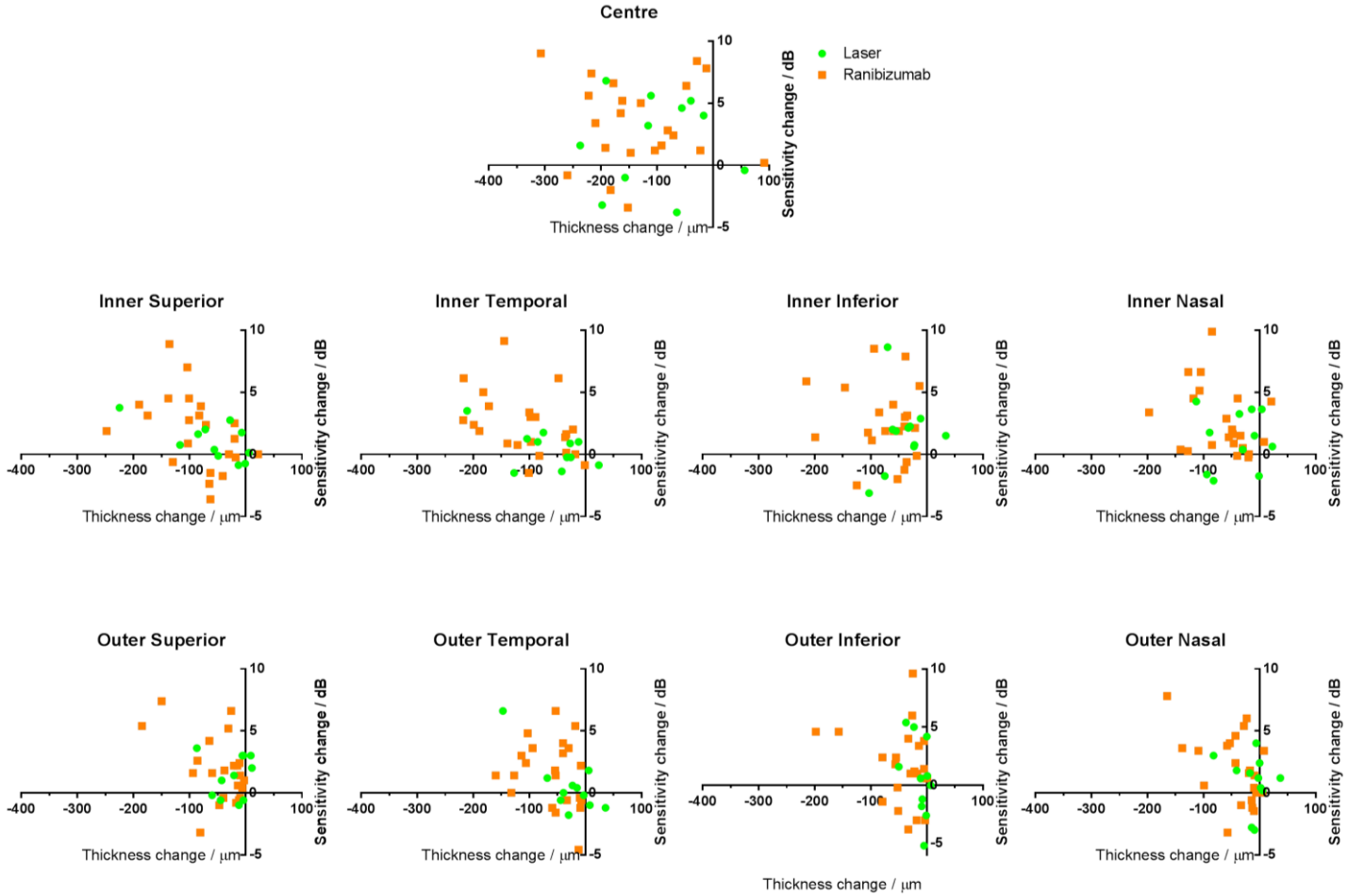


Figure 44 – Scatter plots to show change in retinal thickness from baseline to week 48 versus change in retinal sensitivity over the same period, plotted for nine ETDRS subfields

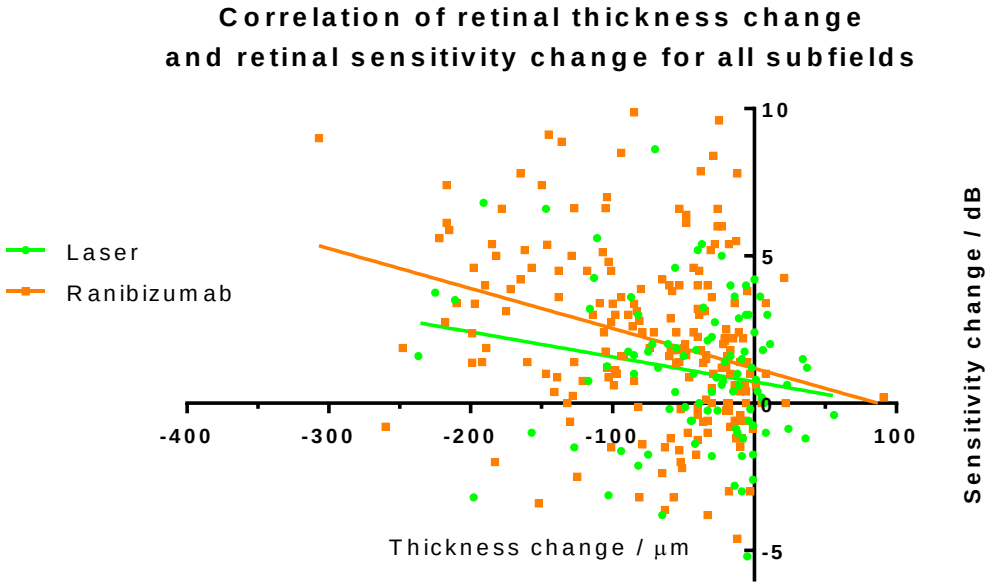


Figure 45 – Graph to show correlation between retinal thickness change and retinal sensitivity change for nine ETDRS subfields for laser and ranibizumab treated subjects.

For both treatment groups there was a significant correlation between change in retinal thickness and change in sensitivity (laser: r = -0.1990, P=0.0483; ranibizumab: r = -0.3074, P<0.0001). Linear regression showed that a 1 dB change in sensitivity was associated with a change in thickness of 118 µm for laser and 74 µm for ranibizumab, but there was no significant difference between the slopes for the two groups (P=0.35). Calculating a combined slope for all data points gives a change of retinal thickness of 82 µm for 1 dB change in sensitivity.

2.3.9 ***Repeatability of OCT measures of macular thickness and volume***

In addition to the 37 patients recruited to the LUCIDATE study, 13 patients who had been screened for LUCIDATE or another clinical trial investigating treatment of DMO with very similar inclusion/exclusion criteria were included in this sub-study to give a total of 50 subjects, of which 18 were female. The mean age was 61.7 years (range 30-82). 31 subjects were Caucasian, 12 Asian-Indian, 3 Afro-Caribbean and 4 from other ethnic groups. Mean ETDRS best-corrected visual acuity was 67 letters (range 47-82).

2.3.9.1 ***Automated measures of macular thickness and volume***

The mean thickness and volume values for each macular subfield with repeated measurements are summarised in Table 17 and Table 18.

Measurement		**1**		**2**		**3**		**4**	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Centre		476.7	102.4	476.5	459.5	476.8	102.7	476.6	103.2
Inner	Superior	456.9	94.4	455.2	90.8	455.3	90.7	455.1	90.9
	Temporal	455.7	87.3	454.6	85.6	454.6	86.2	454.9	85.7
	Inferior	435.2	419.5	434.5	416.5	434.0	71.6	434.5	71.8
	Nasal	441.5	84.0	440.7	82.8	441.1	83.2	440.8	83.6
Outer	Superior	371.2	83.7	372.2	84.2	371.8	83.9	372.6	85.1
	Temporal	368.6	83.8	368.1	81.9	368.2	82.3	368.6	82.4
	Inferior	348.8	66.9	349.5	66.4	348.8	67.3	349.4	66.9
	Nasal	374.3	61.0	373.7	59.4	372.9	60.1	373.8	59.9
Centre point thickness		473.6	125.2	475.0	469.5	475.2	125.8	473.9	126.1

Table 17 – Repeated measurements from nine OCT subfields and centre point thickness, in µm.

Measurement		1		2		3		4	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Centre		0.37	0.08	0.37	0.08	0.37	0.08	0.37	0.08
Inner	Superior	0.72	0.15	0.71	0.14	0.72	0.14	0.72	0.14
	Temporal	0.72	0.14	0.71	0.13	0.71	0.13	0.71	0.13
	Inferior	0.68	0.11	0.68	0.11	0.68	0.11	0.68	0.11
	Nasal	0.69	0.13	0.69	0.13	0.69	0.13	0.69	0.13
Outer	Superior	1.93	0.45	1.93	0.45	1.94	0.45	1.94	0.46
	Temporal	1.88	0.43	1.88	0.42	1.88	0.42	1.88	0.42
	Inferior	1.81	0.36	1.81	0.36	1.81	0.36	1.81	0.36
	Nasal	1.92	0.34	1.92	0.33	1.92	0.33	1.92	0.34
Total macular volume		10.73	1.73	10.72	1.69	10.72	1.69	10.73	1.70

Table 18 – Repeated macular volume measurements from nine OCT subfields and overall total macular volume, in mm^3.

For retinal thickness and volume in each subfield, for centre point thickness and for total macular volume, mean values for each patient were plotted against standard deviation to demonstrate no correlation between variability and the magnitude of the measurement. Results for central subfield thickness are shown in Figure 46; plots for other subfields similarly demonstrated no relationship.

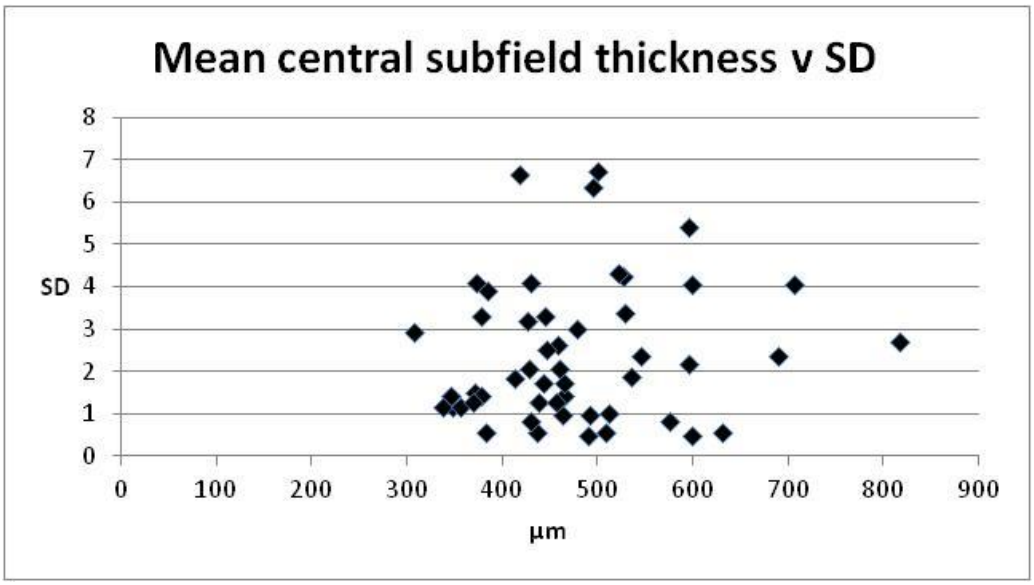


Figure 46 – Plot of central subfield thickness against standard deviation to demonstrate lack of correlation between size of measurement and degree of variability.

The coefficient of repeatability (CR) for retinal thickness in the central subfield was 8.03 µm (95% confidence interval 7.70 – 8.35 µm). CR in the other subfields ranged

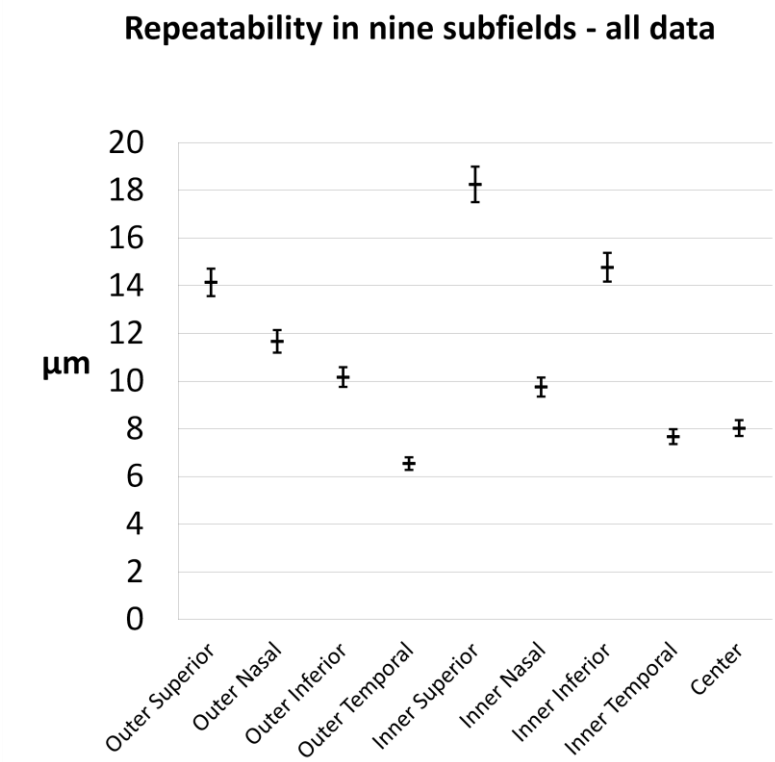
from 6.54 µm to 18.25 µm; results shown in Table 19. CR for macular volume was 0.08 mm^3 or lower for all subfields and 0.21 mm^3 for total macular volume.

Subfield		**Thickness / µm**		**Volume / mm^3**	
		CR	Confidence interval	CR	Confidence interval
Centre		8.03	7.70 – 8.35	0.01	0.01 – 0.01
Inner	Superior	18.25	17.51 – 19.00	0.03	0.03 – 0.03
	Temporal	7.67	7.36 – 7.98	0.01	0.01 – 0.01
	Inferior	14.77	14.16 – 15.37	0.02	0.02 – 0.02
	Nasal	9.75	9.35 – 10.15	0.02	0.02 – 0.02
Outer	Superior	14.14	13.56 – 14.71	0.08	0.08 – 0.09
	Temporal	6.54	6.27 – 6.81	0.04	0.04 – 0.05
	Inferior	10.16	9.75 – 10.58	0.05	0.05 – 0.05
	Nasal	11.67	11.19 – 12.14	0.07	0.06 – 0.07
Centre point		21.62	20.74 – 22.51		
Total macular volume				0.21	0.20 – 0.22

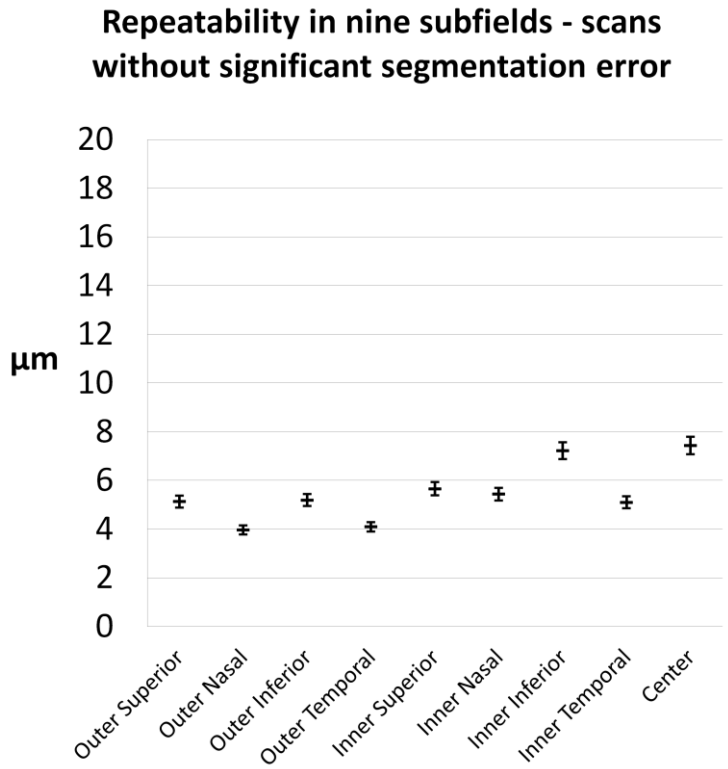
Table 19 – Coefficients of repeatability for macular thickness and volume in the nine ETDRS subfields, for centre point thickness and total macular volume.

2.3.9.2 *Scans with retinal boundary detection error*

Significant automated retinal boundary detection error as described above was present in 13 (26%) subjects. For the remaining 37 subjects without significant segmentation error, the CR was now 7.44 µm (95% confidence interval 7.09 – 7.79 µm) in the central subfield. Estimates for CR in the remaining eight subfields ranged from 3.97 µm (outer nasal) to 7.23 µm (inner inferior), demonstrating that the exclusion of scans with significant segmentation error reduced the CR and hence improved the repeatability of retinal thickness measures. Results for all subfields before and after the exclusion of scans with boundary detection error are shown in Figure 47A and B.



A



B

Figure 47A and B – Repeatability of retinal thickness measures before (A) and after (B) scans with significant boundary detection error were excluded.

2.3.9.3 *Prevalence of media opacity and vitreomacular interface abnormality*

Mild to moderate cataract was present in 7/13 (53.8%) of subjects with significant boundary detection error and in 21/37 (56.8%) of those without (p=1.00, Fisher's exact test). Similar numbers in both groups were pseudophakic (3/13, (23.1%) vs. 8/37, (21.6%), p=1.00). More subjects who had boundary detection error were found to have vitreomacular interface abnormalities (epiretinal membrane, vitreomacular traction, or thickened posterior hyaloid membranes) than those without boundary detection error (8/13 (61.5%) vs. 14/37 (37.8%), p=0.197).

2.4 Discussion

The LUCIDATE study was an exploratory single-masked randomised clinical trial, designed to compare the effect of ranibizumab with laser on a number of parameters evaluating retinal function and structure.

The study provided some evidence, but not statistically significant differences, for a marginal benefit of ranibizumab on visual acuity, retinal sensitivity, colour contrast sensitivity and retinal thickness. No new safety concerns have been reported, and in particular there was no evidence of an increase in macular ischaemia with ranibizumab and no worsening of global retinal dysfunction.

Post-hoc analyses showed no difference in choroidal thickness with either treatment for DMO, and a modest correlation between microperimetric retinal sensitivity and retinal thickness. The repeatability of OCT measures of macular thickness and volume using Spectralis SD-OCT have been reported.

2.4.1 Functional outcome data

Across different modalities, there is evidence that ranibizumab may lead to improved function compared with laser, but statistical significance was not reached.

2.4.1.1 *Visual acuity*

The visual acuity improvement of 6 ETDRS letters in the ranibizumab group is in the same order of magnitude as the improvement seen in larger trials such as the RESTORE study, or the DRCR.net protocol I trial. In RESTORE, the group treated with ranibizumab alone gained a mean ± SD of 6.8 ± 8.3 letters at one year and in the DRCR.net study the visual acuity gain was 9 ± 12 letters [79, 81]. This suggests that in terms of visual acuity response, the cohort in this study is comparable with the wider population of patients with DMO. In this study the lower limit to visual acuity for inclusion was 55 letters (approximately 6/24), compared to 39 (6/48) and 24 (6/96) letters respectively in the two larger trials. There is some evidence from the DRCR.net that participants with a lower visual acuity at baseline gain more letters with ranibizumab treatment although end up with a worse final acuity [180]. This may in part be due to a “ceiling effect” where patients with better acuity at baseline

have less potential for improvement. Given the better mean visual acuity at baseline in this study (70 letters compared with 63 in the DRCR.net study), it is perhaps unsurprising that a gain of only 6 letters was seen on average. The decline of 0.9 letters in the laser arm is comparable to the decline of 0.5 letters reported in the BOLT study carried out at the same institution on a similar cohort of patients [73].

The greatest limitation to interpretation of data from the study is that despite a valid randomisation method, subjects in the ranibizumab group had a mean BCVA 7 letters greater than the laser group at entry to the trial (70.4 vs 63.8). Stratified randomisation could have prevented this difference at baseline. This may have meant that the subjects in the two groups were not comparable in terms of disease status. Comparison of the baseline characteristics shows that there were no subjects with type 1 diabetes in the laser group and the duration of macular oedema was longer. The difference in baseline characteristics, including visual acuity, is a limitation to the study as the two groups may have had different severity of disease and subsequently different responses to the investigations undertaken.

Subjects with good visual acuity who did not meet the criteria for retreatment at the penultimate study visit (44 weeks) may have experienced a drop in visual acuity prior to the exit assessments. One subject who was treated at 40 weeks experienced a drop in visual acuity from 74 letters at 44 weeks to 59 letters at exit, and met the criteria for further ranibizumab treatment were the trial to have continued. Subjects treated with ranibizumab gained on average 8.9 letters from baseline if maximum acuity at any time point after baseline is considered.

2.4.1.2 *Microperimetry*

As discussed in the introduction to this chapter, microperimetry has been used to characterise decreased retinal sensitivity in DMO and to evaluate the effects of treatment, but no previous study has reported the effects of ranibizumab on retinal sensitivity in this condition.

The mean retinal sensitivity in the present study improved in the central 4° of the macula by 3.2 dB with ranibizumab and 1.9 dB with laser. Some improvement may

be attributable to a learning effect, but a trend towards greater functional improvement with ranibizumab compared to laser treatment appears to exist. Smaller improvements in retinal function are seen in the central 12° of the macula; this measure includes a greater amount of retina unaffected by central oedema.

A randomised trial comparing standard ETDRS laser with micropulsed diode laser carried out microperimetry at a number of time points over one year and showed a decline in sensitivity of 0.72 and 1.69 dB in the central 4° and 12° respectively following ETDRS laser and a small improvement with diode laser (0.74 and 0.87 dB at one year) [131]. Although the baseline characteristics of subjects in the two studies appear comparable, subjects treated with ETDRS laser in this study actually had improved sensitivity, to a greater degree than for micropulsed diode laser in the Italian study. Ranibizumab treated subjects had an even greater improvement. This finding suggests that ETDRS laser is safe and is not leading to loss of retinal function by this modality, although it remains fairly ineffective in terms of visual acuity change.

2.4.1.3 *Colour contrast sensitivity*

The presence of colour vision defects in diabetic retinopathy and their association with macular disease has been well documented. In this study as expected, subjects at baseline had raised colour discrimination thresholds in protan and tritan axes. Following treatment with ranibizumab, an improvement in the thresholds is apparent in both axes. After laser treatment, protan axis thresholds have increased. Defects in this axis have been reported to be associated with ischaemia [117]. Tritan axis thresholds have improved, but to a lesser degree than with ranibizumab. It is possible that a learning effect is present with this test, but the 12 week interval between testing makes this unlikely. The evidence from this study suggests that this type of functional defect in DMO may have an element of reversibility.

2.4.1.4 *Pattern and multifocal electroretinograms*

In keeping with the improved visual acuity, retinal sensitivity and colour contrast sensitivity for ranibizumab compared with laser, PERG P50 amplitude remains

preserved in the ranibizumab group while there is an approximate 10% decline for laser. At intermediate time points, the P50 amplitude in the ranibizumab group declined slightly, but this did not correlate with visual acuity or retinal sensitivity changes. PERG amplitudes have previously been reported to decrease following laser treatment in diabetic macular oedema [141], and although there are other reports of the amplitude increasing after injection of either triamcinolone or bevacizumab [142, 143], in this longer study with repeated ranibizumab injections and a control group for comparison, no evidence of a significant increase in amplitude has been demonstrated.

The central amplitudes of the mfERG improve in a greater proportion of ranibizumab treated subjects than laser, and fewer treated with ranibizumab show deterioration in central function. These changes in the central hexagon may represent alterations in macular or foveal function that are not large enough to drive an increase in the PERG amplitude which is recorded over a larger area of retina. The high signal to noise ratio of the mfERG coupled with high test-retest variability owing to patient fatigue and fixation instability makes the results from this test difficult to interpret reliably and hence quantitative analysis of mfERG results has not been carried out.

2.4.2 Structural imaging studies

Both groups in the study showed evidence of resolution of structural abnormalities of DMO on OCT, but there was also evidence of progression of ischaemia in both arms of the study. Ranibizumab treated subjects had better improvements in OCT retinal thickness and decreased prevalence of certain morphological features.

2.4.2.1 ***OCT***

OCT imaging enables rapid non-invasive interrogation of macular morphology and thickness and is well established as an essential imaging modality in the assessment of the patient with macular disease. The quantitative OCT findings in this study of a rapid (i.e. present by 12 weeks) and sustained decrease in central subfield thickness following ranibizumab treatment are in keeping with other trials of this agent in DMO. Additionally, laser treated subjects exhibited decreased retinal thickness, but to a lesser extent, and this difference approached statistical significance. Our OCT findings are in keeping with decreased retinal thickness shown in other studies. In

other subfields, thickness had also deteriorated in both groups, but always more in the ranibizumab group, and correspondingly, total macular volume was also reduced to a greater extent with ranibizumab. It is possible that the presence of boundary detection error on OCT may have had an impact on the results (see section 2.4.5). Thickness reductions in the inner subfields were always greater than in the corresponding outer subfield and greatest of all in the central subfield itself. This likely reflects the centre-involving nature of the macular oedema and the greater retinal thickness in these inner five subfields at baseline.

Evaluation of morphology by masked Reading Centre graders revealed that all subjects had an abnormal foveal contour at baseline and that there was a high prevalence (80-90%) of cystoid changes in the inner and outer retina in both groups, present in the central subfield and surrounding four subfields. Cysts are less common; their lower prevalence in inner retina in the central subfield reflects the retinal anatomy in this location which is dominated by outer retinal structures. Hyperreflective foci are very common, occurring in approximately 80-100% of scans graded. By 48 weeks, the prevalence of most of these features has decreased and just under half of the ranibizumab group have a normal foveal contour, while 90% of those treated with laser remain abnormal in this respect. However, there is no discernible benefit seen from ranibizumab in terms of a reduction in the prevalence of the features such as cysts, cystoid changes or hyperreflective foci when compared with laser.

The improved resolution of SD OCT has allowed the visualisation of high reflectivity lines in the outer retina. These lines are in the region of the photoreceptor inner segment-outer segment junction (termed the ellipsoid layer) and the external limiting membrane (ELM). However, it is likely that the axial resolution of SD-OCT is still insufficient to visualise these extremely thin features. These are shown in Figure 48, from Heidelberg, which illustrates the relationship believed to exist between OCT images and the layers of the retina defined histologically (see Figure 3, section 1.2).

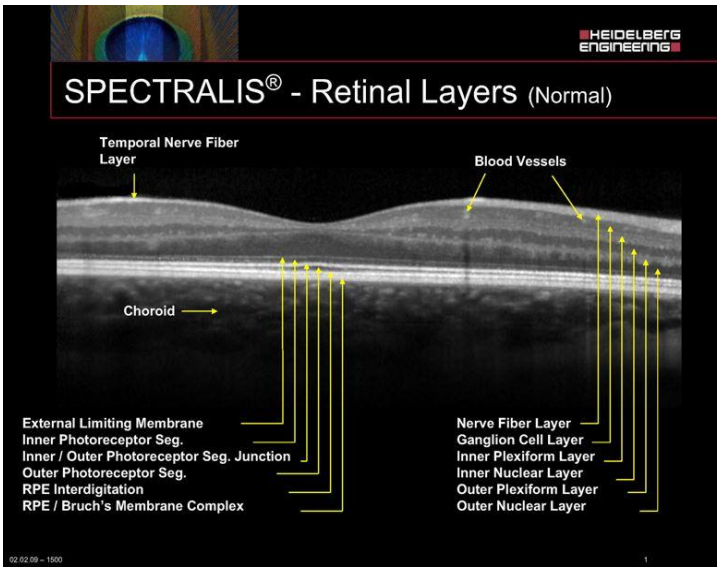


Figure 48 – Diagram to show the relationship between the recognised histological layers of the retina and the appearance of hypo- and hyper-reflective bands on a cross-sectional OCT scan (courtesy of Heidelberg Engineering, downloaded from http://eyewiki.aao.org/Optical_Coherence_Tomography).

In this trial, grading examined the integrity of the IS-OS junction and the ELM on OCT scans. Approximately two-thirds of subjects at baseline had visible discontinuity (interruptions) of the line thought to coincide with the ELM and more had interruptions of the IS-OS junction. By 48 weeks, all of the laser subjects now had interruptions present in both lines but the percentage with interruptions following ranibizumab treatment had decreased. This may be evidence of a treatment effect, but it is also possible that decreased retinal thickness overall in the ranibizumab group compared with laser has led to increased visibility of these lines and that in situations where extensive amounts of fluid remain, masking of deeper retinal structures occurs.

Since this trial was designed, a number of reports have linked integrity of the lines with altered visual acuity or retinal sensitivity. Maheshwary *et al.* showed that in DMO an increasing area of disruption of the IS-OS layer was associated with poorer visual acuity [181]. Correlating IS-OS disruption with retinal sensitivity from microperimetry, Landa *et al.* showed in wet and dry AMD that disruption correlated inversely with sensitivity and to a lesser extent with visual acuity [182]. The ELM has also been investigated in this setting, and Ito *et al.* found that integrity of this line correlated with visual acuity in addition to the IS-OS junction line [183]. These

findings merit further exploration and a further exploratory analysis of this data set could involve correlating interruptions in these outer retinal layers with retinal sensitivity from microperimetry results and examining the change longitudinally.

2.4.2.2 *Colour fundus photography*

In this small exploratory study, numbers do not allow a clear assessment of a treatment effect from ranibizumab or laser on the progression of diabetic retinopathy. In both groups, the trend was for grade of retinopathy to remain the same or improve slightly. Evaluation of the results from larger trials has shown that ranibizumab treatment is more likely than sham to result in an improvement of 2 or 3 grades or greater in diabetic retinopathy over two years [184]. Interestingly though, even with mandatory monthly dosing of ranibizumab for two years in this study, there were still approximately 11% of participants experiencing a progression in retinopathy. This suggests that while VEGF inhibition may be important for retinopathy progression overall, other factors may also be contributing.

2.4.2.3 *Fluorescein angiography*

Evidence from masked grading of fluorescein angiograms in this study suggests that in both groups there was a slight progression of macular ischaemia, based on enlargement of the foveal avascular zone area and a small increase in the overall grade of perifoveal capillary loss. A verification protocol between graders in the Reading Centre helped ensure that gradings were reproducible; only one angiogram was excluded from FAZ area grading at both time points. The finding of progression of ischaemia over one year is in contrast to the findings in the BOLT study where there was no increase in FAZ size following 12 months of laser or bevacizumab treatment [73]. There was no difference in enlargement between the two groups at 48 weeks so this is unlikely to represent a treatment effect of ranibizumab, rather it is more likely that this group of patients were different from the group enrolled in the BOLT study in terms of severity of disease and rate of progression. Further study of macular ischaemia following ranibizumab therapy may be warranted.

2.4.3 Safety data and adverse events

Safety outcomes in this small exploratory study included the data from full field electroretinograms representing global retinal function, the fluorescein angiography data discussed above and the reporting of adverse events.

2.4.3.1 *Full field electroretinograms*

Electroretinography data assessed global function of the rod and cone systems in the retina, and was reported by an experienced electrophysiologist who was masked to treatment allocation.

The electroretinography data do not suggest that deterioration in global retinal function has occurred in either group. The only index seen to change was the scotopic bright flash ERG in the ranibizumab group, which exhibited decreased a and b wave amplitude but no change in peak time. Therefore it is possible that localised loss of function has occurred, but there is no evidence of generalised dysfunction. The absence of any change in the cone system is reassuring and suggests that this may be a chance finding. A paired t-test for bright flash a-wave amplitudes in the ranibizumab group (n=20 for reliable recordings at baseline and 48 weeks) gives P=0.02, but for the b-wave amplitudes P=0.2, suggesting this may be statistical noise. A statistically significant deterioration in both wave amplitudes would be needed for evidence of a genuine effect. Other factors such as pupil dilation can affect electrophysiological results. Further investigation would require serial electroretinography on a similar number of patients undergoing ranibizumab treatment over a period of time.

2.4.3.2 *Adverse events*

The pattern of adverse events seen during the trial reflects those reported in larger trials of ranibizumab for both DMO and age-related macular degeneration. Ocular adverse events occurred more commonly in the ranibizumab arm and were related to the injection procedure. Non-ocular adverse events also occurred more frequently with ranibizumab treatment, with upper respiratory tract and urinary tract infections occurring commonly. This may in part be due to reporting bias; as ranibizumab subjects were seen every four weeks compared to 12 weekly for laser, there were more opportunities for them to report adverse events.

A recent consecutive case series to establish the incidence of endophthalmitis following intravitreal injection found a rate of 0.035% following injection in an office setting (i.e. not in the operating theatre) comparable to that used in this study [185]. It is therefore reassuring that in this study of approximately 200 intravitreal injections there were no cases of endophthalmitis. One death occurred in the laser arm, and the other serious adverse events that did occur were not felt to be due to the investigational medicinal product (ranibizumab). The death in the ranibizumab arm was due to advanced hepatocellular carcinoma. This subject was under investigation for thrombocytopaenia at trial entry; this is a feature of the condition and has been proposed in conjunction with α-foetoprotein levels as a screening test for this carcinoma. It is therefore highly likely that the tumour was present in its early stages at recruitment. Another subject in the ranibizumab arm had a number of hospital admissions meeting the definition for serious adverse events arising from an initial incident: a fall leading to a pelvic fracture. This was treated conservatively, but during recovery from this she developed recurrent urinary tract infections and anaemia which necessitated hospital admission. She developed nephrotic syndrome which was investigated by renal biopsy; this showed characteristic features of diabetic nephropathy rather than nephropathy as a result of anti-VEGF toxicity which has different pathological features (personal communication from Dr Yakoub, Consultant Nephrologist, Royal London Hospital).

All serious adverse events were reported to the trial sponsor in accordance with trial regulations and to Novartis following a contract agreed with them prior to commencing the trial.

2.4.4 ***Choroidal thickness***

The possible role of the choroid in the pathophysiology of diabetic retinopathy and the development of readily available imaging techniques to investigate its structure have led to several studies reporting changes in choroidal thickness in the presence of diabetic retinopathy. Different investigators have reported choroidal thinning in the presence of diabetic retinopathy, with some finding that more severe forms of retinopathy and the presence of macular oedema are associated with more thinning

[101-103, 163, 164]. Recently, a cross-sectional study has reported a further reduction in choroidal thickness following anti-VEGF therapy with bevacizumab [104]. This study retrospectively analysed patients who had received bevacizumab in one eye and laser in another. Although the authors state that patients were included if the two eyes had the same level of retinopathy, this raises the question of why the treating physician selected one eye for bevacizumab treatment. It is not possible to exclude the possibility that eyes treated with bevacizumab were different from those receiving laser.

Results from this exploratory, post-hoc analysis of a randomised clinical trial do not show evidence of a change in choroidal thickness over 48 weeks with either laser or ranibizumab treatment. As discussed above, unstratified randomisation meant that subjects in the laser group had on average worse visual acuity and slightly longer duration of DMO at baseline. Despite this, choroidal thickness in both groups was highly comparable at baseline and at 48 weeks. Both groups did, however, show some evidence of an early reduction in choroidal thickness at 12 weeks. The significance of this finding is unclear and it warrants further study.

While retinal thickness correlates with visual acuity and also with retinal sensitivity, (see section 2.4.5), there was no evidence of correlation between choroidal thickness and retinal thickness and only weak evidence of a correlation with visual acuity. This correlation with visual acuity may be a chance association, rather than causative: if choroidal thickness is linked to increased diabetic retinopathy severity this in itself may be associated with worse visual acuity. Further studies of the role of the choroid in diabetic retinopathy and effects of treatments for DMO on choroidal thickness are needed.

2.4.5 ***Structure function correlation***

In this study, there was not a statistically significant correlation between retinal thickness in the central subfield at baseline and visual acuity, as the P value was 0.08. Larger studies have shown that centre-point thickness measured on TD OCT correlates with visual acuity, with an effect size reported of 4.4 letters for every 100 µm change in thickness [174]. Examining visual acuity and retinal thickness change

did reveal a statistically significant correlation at 48 weeks, but only in the ranibizumab group, with a similar effect size (4.1 letters) found to that reported previously. The Bonferroni correction for multiple comparisons has not been applied to this exploratory data and further investigation of the strength of correlation is needed. In the laser group, the results were variable, with a number of subjects having decreased visual acuity despite reduced central subfield thickness. This result could be indicative of retinal atrophy occurring.

Results from correlating microperimetry with OCT show that a statistically significant correlation is present between retinal sensitivity and retinal thickness. In this trial, ranibizumab treatment resulted in decreased retinal thickness at 48 weeks in all subfields on OCT scanning. Identifying this correlation suggests that across the whole macula reducing retinal thickness improves sensitivity. This may have beneficial functional effects for patients; it would be interesting to examine reading speed, contrast sensitivity and functional measures other than the fovea-dominated measure of high contrast sensitivity. A previous report investigating the relationship between retinal sensitivity and thickness in 11 subjects found a change of 0.05 dB for every 1 µm increase in thickness over 280 µm, but with a wide confidence interval [186]. This relationship is in the same order of magnitude as that found in the current study.

Although statistical analysis shows a significant correlation is present, inspection of the scatter plot shows that there is still a wide range of retinal sensitivity associated with a particular thickness, so results should be interpreted with caution. While correlation between thickness and sensitivity may be important, other morphological changes present on OCT, such as interruptions in the ELM and IS-OS junction lines may have measurable effects on point sensitivity. Further study to identify those structures present on OCT that are indicative of functional loss should be undertaken. This may in turn have predictive value in terms of response to ranibizumab or other treatments.

2.4.6 ***Repeatability study***

Change in OCT based macular thickness measurement is used as one of the criteria for retreatment in eyes with DMO both in clinical practice and in clinical trials [73, 81, 84, 85]. While laser treatment is typically performed based on the detection of clinically significant macular oedema on slit-lamp examination, the advent of pharmacological treatments for DMO means that quantitative measures are now employed to guide enrolment and retreatment decisions; an approach followed in this trial. It is therefore important to establish the repeatability of OCT-derived macular thickness measurements, as a knowledge of repeatability would better allow physicians to identify true clinical change from measurement variability.

Spectral domain (SD) OCT technology has resulted in faster image acquisition time and higher resolution images compared to older time domain (TD) technology. The Spectralis OCT combines SD OCT technology with eye tracking and line scan averaging to improve the signal-to-noise ratio, potentially enhancing the ability of segmentation algorithms to detect the true inner and outer retinal boundaries. Data presented from this exploratory study represents the first report evaluating the repeatability of retinal thickness and volume measurements from the Spectralis OCT in eyes with DMO. Previous studies of repeatability in macular disease have yielded poorer repeatability estimates (higher CR values) than the current study (see Table 20). Evaluation of repeatability in neovascular age-related macular degeneration using Zeiss Stratus TD OCT has estimated CR for the central macular subfield at 67μm, representing 23% of the total macular thickness [100]. The Diabetic Retinopathy Clinical Research Network (DRCR.net) study examining subjects with DME using the Zeiss OCT-3 machine reported the half-width of the 95% confidence interval for change (equivalent to the Bland Altman CR) to be 38μm[166].

Evaluation of SD OCT devices has yielded similar results. The Cirrus SD OCT was found to have a coefficient of repeatability of 42.4 μm in the central subfield for subjects with neovascular AMD [187], which improved to 26.1 μm once scans with segmentation error had been removed. In DME, no significant difference was found between TD and SD devices in two separate studies [167, 168]. Although this study was not designed to compare the SPECTRALIS™ OCT directly with a TD device,

the estimates obtained for CR are an order of magnitude better than those previously reported in TD devices and in the SD devices that have been used in comparative studies.

Study	**Disease**	**Sample Size (eyes)**	**Device**	**Central (A1) Subfield Coefficient of Repeatability**
Massin et al. (2001) [165]	DME	10	TD (Humphrey OCT)	<21 µm*
Krzystolik et al. for the DRCR.net [166]	DME	212†	TD (Zeiss OCT3)	38 µm
Patel et al. [100]	nvAMD	51	TD (Stratus OCT)	67 µm
Forooghian et al. [167]	DME	33	TD (Stratus OCT) SD (Cirrus HD-OCT)	17.9 µm 19.0 µm
Domalpally et al. [168]	DME	63	TD (Stratus OCT) SD (Topcon 3D OCT 1000)	27.4 µm 20.1 µm
Parravano et al. [187]	nvAMD	49	SD (Cirrus HD-OCT)	42.4 µm

Table 20 – Summary of previous studies reporting coefficients of repeatability in macular disease using both TD and SD OCT devices. All TD studies used the 6 radial line scan protocol (6mm length) (termed "Fast Macular Thickness Map" by Zeiss) and SD studies used the 128 horizontal line protocol (512 A scans per line; 6mm x 6mm). *exact figure not reported. †107 patients.

Retinal boundary segmentation error has been shown to have an impact on the repeatability of retinal thickness measurements in neovascular AMD [100]. In this sub-study, coefficients of repeatability improved when scans affected by significant retinal boundary detection error were excluded. This suggests that when interpreting OCT scans, caution should be exercised in using automated retinal thickness values if segmentation error has occurred. Although a 1 mm length of scan was chosen to identify misplacement of retinal boundaries, there is no accepted definition for segmentation error and defining this differently could have led to different results. It was more common to find vitreomacular interface abnormalities in subjects whose scans were affected by segmentation error, and this may be an important cause for failure of the OCT algorithm to correctly place retinal boundaries.

Strengths of this repeatability study include the sample size and number of repeated measurements which ensure that the study is powered to estimate S_w to within 11% of the true population value [179]. Choosing to run this study in parallel to recruitment for a clinical trial is a potential weakness, however, as subjects may be more carefully selected and less indicative of the range of pathology present in general clinics.

Diurnal variation has been proposed as a cause for variability in macular thickness measurements. Studies utilising TD OCT devices have demonstrated diurnal variation in macular thickness in subjects with DMO, with retinal thickness decreasing throughout the day. Estimates for this change range from 13 μm [188] to 49 μm [189] in the central subfield. This has previously been of limited clinical relevance as the magnitude of this change is similar to the inherent test-retest variability of TD OCT devices reported in Table 20. This study with the Spectralis OCT found a smaller CR than the estimated diurnal variation previously reported. This suggests that this phenomenon may be clinically important given the greater precision of measurements taken with this device.

In summary, this study shows that using the Spectralis OCT, it is possible to obtain retinal scans with excellent intra-session repeatability in eyes with DMO. The results suggest that a retinal thickness change of greater than 8 microns in the central 1mm subfield is more indicative of clinical change rather than measurement variability. The results of this study may be used to design retreatment criteria in clinical trials. In clinical practice, the results can be used to distinguish true clinical change from measurement variability.

2.5 **Conclusions**

The aim of the LUCIDATE study was to evaluate the effect of ranibizumab on parameters of retinal function in order to establish whether there was evidence of worsening macular ischaemia or global retinal dysfunction, compared with laser treatment. Secondary objectives were to investigate the effect of ranibizumab and laser treatment on choroidal thickness in DMO, to investigate the correlation of

microperimetric retinal sensitivity with retinal thickness, and to study the repeatability of retinal thickness and volume measurements with Spectralis OCT in this condition.

The study has demonstrated in the modalities of visual acuity, colour contrast sensitivity, microperimetric retinal sensitivity and pattern electroretinogram that there is some evidence of benefit from ranibizumab compared to laser, but results did not reach statistical significance.

For grading of macular ischaemia from fluorescein angiography and evaluation of global retinal dysfunction from full-field electroretinograms, there is no evidence that ranibizumab treatment leads to a worsening of these parameters.

Exploratory post-hoc analyses report no change in choroidal thickness following ranibizumab or laser treatment in this condition, in contrast to some recent reports suggesting an effect of anti-VEGF agents on this parameter.

The Spectralis OCT device has been shown to yield highly repeatable measures of retinal thickness and volume in DMO, which improve when scans with retinal boundary detection error are excluded.

3 The RaDiVit* Study

This chapter reports a randomised controlled trial designed to investigate the impact of ranibizumab pre-treatment on diabetic vitrectomy surgery.

*RaDiVit – Ranibizumab in Diabetic Vitrectomy

3.1 **Background**

Treatment for proliferative retinopathy is directed at reducing or removing the molecular signals that lead to the growth of new vessels: neovascularisation. This is typically achieved by panretinal laser photocoagulation but uncontrolled proliferative disease may require surgery to clear fibrovascular tissue and prevent the sequelae of vitreous haemorrhage and tractional retinal detachment. When the typical results of surgery for retinopathy are examined it is seen that significant numbers of patients have poor outcomes and complications. As discussed in Chapter 1, vascular endothelial growth factor (VEGF) is strongly implicated in the pathogenesis of proliferative retinopathy: injecting VEGF into primate eyes causes retinopathy [28], levels of VEGF correlate with severity of retinopathy [25] and blocking VEGF leads to regression of iris neovascularisation in the primate eye [27]. VEGF blockade might therefore have a role as a treatment modality to improve the results of surgery for proliferative diabetic retinopathy.

3.1.1 ***Outcomes of surgery for proliferative diabetic retinopathy***

The Diabetic Retinopathy Vitrectomy Study established the benefit of early vitrectomy for vitreous haemorrhage that had persisted for at least one month. In the early surgery group, 25% had visual acuity of 10/20 (equivalent to 6/12) or better at 2 year follow-up, compared to 15% in the group where surgery was deferred for a year [92]. A further investigation by the same study group showed that surgery was beneficial for patients with severe active fibrovascular proliferation, with 6/12 visual acuity achieved by 44% of the group receiving early vitrectomy compared to 28% managed by observation and laser, with vitrectomy only performed for severe complications [190]. These findings in the 1980s continue to drive surgical decision making for advanced proliferative diabetic retinopathy, as shown by a prospective study of 174 vitrectomy procedures in diabetic patients at a single institution [93]. This study showed that the most common indication for surgery (43% of eyes) was non-clearing vitreous haemorrhage, with the next most common (32.8%) tractional retinal detachment (TRD) involving the macula. Results were good in terms of visual

acuity, with approximately 29% of eyes achieving 6/15 or better visual acuity, which is comparable with the results of the DRVS. The proportion of patients with very poor visual outcomes also appears to have reduced: only 1.8% of eyes in the Moorfields study had no light perception at final follow-up while in the DRVS this could be as high as 25% (although differences in laser management at the time of the earlier study make direct comparison difficult). Visual improvement following diabetic vitrectomy can be expected in approximately 75% of eyes treated with only 9% worsening significantly.

One of the most common complications that can occur following diabetic vitrectomy is post-operative vitreous cavity haemorrhage (POVCH), with the Moorfields study reporting an incidence of 22%, although other studies have reported a range of 5-80% (see [191]). This can occur in the early post-operative period or late (2-6 months) and if persistent can result in the need for further surgery. By impairing the view of the retina, it also makes further treatment of diabetic retinopathy difficult and can lead to raised intraocular pressure as the haemorrhage is reabsorbed. It occurs through a variety of mechanisms including clot breakdown and entry site neovascularisation. The additional visual disability and necessity for further surgical procedures are inconvenient for patients and physicians. Any intervention that could reduce the incidence of this complication would therefore be welcomed.

3.1.2 ***Anti-vascular endothelial growth factor agents as adjuncts to diabetic vitrectomy surgery***

Post-operative vitreous cavity haemorrhage may occur because of persistent neovascularisation, e.g. at surgical entry sites, but also on the retina; and advanced fibrovascular proliferation makes surgery difficult and is a recognised predictor of poor visual outcome [94]. An intervention to inhibit neovascularisation and reduce vascularity of fibrovascular membranes could therefore reduce post-operative complications and make surgery technically easier. Pre-operative therapy with anti-VEGF agents potentially would have this beneficial effect on new vessel proliferation through anti-angiogenic action and so warrants investigation to

establish whether it is a useful therapeutic strategy both to facilitate safe surgery and reduce the incidence of POVCH.

Numerous case reports and retrospective series have reported the effects of bevacizumab pre-treatment on diabetic vitrectomy surgery leading to the exploration of its impact in clinical trials. Because there has been no clear consensus on the clinical question requiring an answer, the trials have significant methodological differences and heterogeneous outcome measures, making direct comparison between them difficult. Ranibizumab has been little used apart from a single report in a randomised study investigating its impact on intra-operative haemorrhage.

3.1.2.1 ***Bevacizumab***

Case reports and small series investigating bevacizumab use illustrate the potential benefit of the drug but also highlight a safety concern. Chen and Park reported the first use of pre-operative bevacizumab in a patient undergoing diabetic vitrectomy, noticing a regression of vascular tissue anterior to the retina with an apparent switch to fibrosis facilitating surgery after a single injection [192]. In 18 eyes with PDR undergoing diabetic vitrectomy pre-operative bevacizumab "improved the ease of surgery" as dissection of fibrovascular membranes with regressed neovascularisation was technically easier; but no effect on visual acuity was found [193].

The rapid induction of fibrosis by regression of new vessels in fibrovascular membranes as a result of bevacizumab therapy could potentially exacerbate pre-existing TRD and may worsen the clinical situation. Ishikawa *et al.* reported that in eyes that had received bevacizumab with a longer interval between injection and surgery, strong fibrosis and adhesions could be present that did not occur in cases where the interval between injection and surgery had been shorter [108]. In 11/211 eyes that had received bevacizumab prior to diabetic vitrectomy in another study, tractional retinal detachment (TRD) developed or existing TRD progressed [107]. However, conclusions from these uncontrolled, retrospective studies should be interpreted with caution and do not provide definitive evidence that pre-treatment with bevacizumab worsens outcomes.

A number of randomised controlled trials have reported the outcomes of pre-operative bevacizumab, but differences in methodology make direct comparison between them difficult. Two early trials report a potentially beneficial effect on duration and difficulty of surgery:

Rizzo *et al.* randomised 22 patients with either tractional or combined tractional-rhegmatogenous retinal detachments to bevacizumab one week before surgery or no intervention [194]. Their main outcome measure was "feasibility of surgery" with visual acuity and anatomical outcomes at 6 months secondary outcomes. They showed that in patients who had received bevacizumab, surgery was typically shorter at 57 vs. 83 minutes, with fewer tool exchanges (27 vs. 53) and a tendency to carry out blunt membrane dissection more frequently than sharp dissection. There were no differences in visual acuity or retinal reattachment rate. This study was unmasked, does not describe the generation of the random number sequence used, and does not report methods of allocation concealment so it is not possible to exclude bias. These investigators did, however, recognise the potential impact of case complexity on their results, so they compared surgical outcome between the groups using a previously reported complexity score [195].

Modarres *et al.* report a randomised controlled trial of 40 patients receiving intravitreal bevacizumab 3-5 days prior to surgery, again with surgical metrics and visual acuity as outcome measures [196]. In this study, surgeons were masked to treatment allocation but the method of randomisation is not reported by the investigators. A total of 22 patients received bevacizumab and 18 formed the control group. There was no difference in visual acuity between the groups at 3 months, but all seven post-operative vitreous cavity haemorrhages occurred in the group that had not received bevacizumab. The investigators also report a shorter duration of surgery (62 vs. 95.5 minutes) and fewer uses of endodiathermy or the backflush cannula as surrogate measures of reduced intraoperative bleeding.

A further study by di Lauro *et al.* of 72 eyes randomised to sham or bevacizumab either 7 or 20 days before surgery adds to the evidence that surgery may be technically easier with pre-operative bevacizumab: surgical time, endodiathermy, intraoperative bleeding and iatrogenic breaks were all reduced in the bevacizumab group [105]. The investigators also report a reduced incidence of POVCH in the

bevacizumab groups, but lack of surgeon masking to treatment allocation again introduces the possibility of bias.

The randomised controlled trial least at risk of bias investigating bevacizumab for this indication was carried out by Ahmadieh *et al.* who randomised 68 eyes undergoing diabetic vitrectomy surgery to either intravitreal bevacizumab or sham [197]. This study includes both patient and investigator masking, uses a valid method of randomisation (random permuted block method) and reports on the outcomes of all participants. Some subjects experienced clearing of vitreous haemorrhage between injection and planned date of surgery, obviating the need for vitrectomy; but in the group that did undergo surgery, the investigators report a statistically significant improvement in visual acuity and reduction in POVCH for those that had received bevacizumab.

A systematic review by Zhao *et al.* of intravitreal bevacizumab in diabetic vitrectomy has attempted to bring together the disparate outcome measures used in the trials described above and carry out a meta-analysis [198]. Based on the results of six randomised trials and one non-randomised study, the meta-analysis showed a modest beneficial effect for bevacizumab on surgical parameters: intraoperative bleeding, frequency of endodiathermy, number of iatrogenic retinal tears and mean surgical time all favoured bevacizumab administration. Post-operatively, blood resorption time and recurrent vitreous haemorrhage were reduced and visual acuity was slightly better with bevacizumab. However, these results need to be interpreted with caution as not all studies were included in every comparison due to differences in reporting outcomes, and numbers involved in some comparisons were small.

A Cochrane review has analysed the impact of bevacizumab pre-treatment on post-operative vitreous cavity haemorrhage [191]. This review only included four trials [105, 194, 196, 197] and found a number of methodological flaws leading to risk of bias in all but Ahmadieh's trial report, and did not conduct a meta-analysis due to these methodological issues. The review does comment however, that three trials report reduced incidence of POVCH.

3.1.2.2 ***Ranibizumab***

Ranibizumab has not so far been evaluated for use in diabetic vitrectomy, except for one small randomised study that investigated intra-operative bleeding following an injection of the drug one week previously [199]. The study included 19 patients and collected a fluid sample from the vitrectomy surgical cassette which was then subjected to haemocytometry. The overall cell counts ranged in number over several orders of magnitude but a small reduction in intraoperative haemorrhage was reported with prior ranibizumab. The methodology used in this study was previously described by the same investigators in an investigation into the effect of bevacizumab on this outcome, which also showed a small reduction in erythrocyte count in the surgical fluid of patients in the treatment group [106]. The studies did not evaluate any clinical outcomes such as visual acuity or POVCH.

3.1.3 ***Cytokines in the vitreous of patients with advanced proliferative diabetic retinopathy***

Chapter 1 described the importance of VEGF in the pathogenesis of diabetic retinopathy, including how hypoxia sensing leads to the transcription of a number of genes and a cascade of cytokine production including VEGF and pro-inflammatory cytokines. Levels of VEGF are elevated in diabetic vitreous, but with complex pathways implicated in the pathophysiology of retinopathy, it is likely that other cytokines will have altered levels and be involved in the generation of neovascularisation and increase in vascular permeability. The cytokine profile in diabetic vitreous might then be altered by anti-VEGF therapy in ways not limited to a reduction in VEGF itself.

3.1.3.1 ***Cytokine changes in proliferative diabetic retinopathy***

The cytokines interleukin (IL) 6 and IL-8 are associated with macrophage and neutrophil activation, form part of the inflammatory cascade and have been repeatedly shown to be elevated in diabetic retinopathy. Yuuki *et al.* showed that elevated levels of both cytokines were found in the vitreous from diabetic subjects with proliferative retinopathy compared with non-diabetic controls [200] and other

investigators have corroborated this finding. Nakamura showed elevated levels of IL-6 in conjunction with increased levels of advanced glycation end products (AGEs) [201] and Petrovic *et al.* showed elevated levels of IL-8 in the vitreous from 71 eyes with proliferative retinopathy [202]. Arjamaa *et al.* hypothesised that as IL-6 is able to induce VEGF expression, its levels may be related to upregulation of HIF-1α following hypoxia sensing. They found that IL-6 levels correlated with degree of neovascular activity and also with IL-8 levels, but did not find an alteration in HIF-1α expression [203].

The chemokines monocyte chemoattractant protein-1 (MCP-1) and interferon-γ inducible protein of 10 KDa (IP-10) are involved in attracting monocytes and T-cells respectively to sites of inflammation. Abu El-Asrar *et al.* found that vitreous levels of these two signalling molecules were elevated in proliferative diabetic retinopathy [204]. These mediators have been shown to play a role in angiogenesis and fibrosis and results from the investigations described above suggest that local production is responsible for elevated levels as a corresponding rise in serum levels of cytokines has not been demonstrated.

Cytometric bead analysis allows the evaluation of concentrations of a number of cytokines simultaneously. Lange *et al.* investigated cytokine concentrations using this technique in diabetic vitreous in conjunction with measuring vitreous oxygen tension, and found elevated levels of IL-6 and IL-8 in addition to IP-10 and VEGF, confirming some of the findings described above [205]. They also found raised levels of the cellular proliferation cytokine Flt-3 ligand and the anti-inflammatory T helper cell 2 cytokine IL-9, suggesting that a delicate balance between pro- and anti-inflammatory messengers exists in this condition.

In summary, more than one group of investigators have shown elevated levels of IL-6, IL-8, IP-10 and MCP-1 in proliferative diabetic retinopathy. These cytokines are linked to angiogenesis and fibrosis but how they are regulated with respect to hypoxia sensing and the signals leading from ischaemia to their up-regulation have not been fully defined.

3.1.3.2 *Effects of anti-VEGF treatment on the cytokine profile in proliferative diabetic retinopathy*

Bevacizumab has been preferred to ranibizumab in investigations to date that have investigated the cytokine profile in diabetic retinopathy after VEGF inhibition, but a number of studies have shown that it reduces the concentration of VEGF in the vitreous at different time intervals following injection. For example, Ma *et al*. showed that VEGF was elevated in diabetic vitreous but the concentration was significantly reduced after bevacizumab injection [206]. This reduction in concentration was apparent even in subjects who had received bevacizumab a mean of 34 days before sampling, but VEGF levels were not as low following treatment as in subjects without neovascular pathology. Additionally, plasma levels of VEGF were significantly reduced following bevacizumab administration.

While reduced VEGF levels following bevacizumab injection have been confirmed by the findings of other investigators, there is limited and sometimes conflicting information regarding changes to other vitreous cytokines following VEGF inhibition. Arimura *et al.* found reduced VEGF and SDF(stromal cell derived factor)-1α after bevacizumab injection but no change in other cytokines, particular IL-6 and 8 [207]. Jeon and colleagues did find significantly elevated levels of IL-6 and 8 one day after bevacizumab therapy [208], but it is possible that the injection procedure itself could raise the levels of these pro-inflammatory cytokines in the short term as part of a wound healing response. Connective tissue growth factor (CTGF) levels increased after bevacizumab injection and correlated with increased retinal fibrosis detected clinically in one investigation [209], but although Sohn *et al.* also observed a clinical increase in fibrosis, they did not find a significant change in CTGF levels [210].

Evidence therefore validates the expected action of bevacizumab in substantially reducing levels of VEGF in the vitreous of subjects with diabetic retinopathy, but no convincing picture emerges of its effects on other pro- and anti-angiogenic or inflammatory cytokines. Ranibizumab has not been investigated in this setting, so new data may add to the understanding of the pathophysiology of diabetic retinopathy and the wider effects of VEGF inhibition in this condition.

*3.1.4 **Aims and objectives of the study***

Trials that have evaluated anti-VEGF agents have been of variable quality and many suffer from methodological flaws in the method of randomisation or masking. Most have investigated bevacizumab owing to its reduced price compared with ranibizumab or pegaptanib, but one study of intraoperative haemorrhage investigated ranibizumab. Different outcome measures have been chosen; typically either surgical metrics such as duration or ease of surgery, or incidence of post-operative haemorrhage. Reviews of the trials carried out have suggested that there may be a benefit for pre-operative bevacizumab on these outcomes. However, amongst the vitreoretinal surgical community there is still lack of agreement over whether anti-VEGF agents are beneficial in this condition.

The aims of the trial reported in Chapter 3 are:

- To investigate the effect of ranibizumab pre-treatment on visual acuity at three months following vitrectomy surgery for advanced proliferative retinopathy
- To investigate the effect of ranibizumab on intra-operative and post-operative vitreous haemorrhage
- To investigate the degree to which ranibizumab facilitates vitreoretinal surgery in this condition
- To investigate the safety of giving ranibizumab prior to surgery in terms of its effect on pre-existing tractional retinal detachment
- To investigate changes in the vitreous cytokine profile following ranibizumab treatment.

A trial to provide answers to these clinical questions is likely to require large numbers of patients, so a pilot study to determine the feasibility of such a trial and carry out a power calculation to estimate the numbers required is therefore an appropriate first step.

Previous trials conducted in this area have sometimes reported the results from a single surgeon and in other cases multiple surgeons have been used. While surgical skill remains a parameter that is difficult to quantify, the disadvantage of a single-

surgeon trial is that results may not be generalisable to the wider surgical community. Therefore this trial will feature multiple surgeons, but through randomisation and masking will attempt to address this potential confounding factor.

3.2 **Methods**

The RaDiVit study (Ranibizumab in Diabetic Vitrectomy) was a pilot study to investigate the impact of ranibizumab pre-treatment on vitrectomy surgery for the complications of proliferative diabetic retinopathy.

As a pilot study, there was no formal hypothesis to be tested, but the null hypothesis for a definitive study would be that ranibizumab pre-treatment has no effect on visual acuity when given prior to vitreoretinal surgery for advanced proliferative diabetic retinopathy.

3.2.1 ***Design, approval and participants***

This trial was a single centre, parallel group; phase II pilot study with 1:1 randomisation, featuring masking of patients, surgeons and investigators, conducted at Moorfields Eye Hospital, a tertiary referral centre for vitreoretinal surgery.

The study conformed to the Declaration of Helsinki and prospective approval was sought from the Central London Research Ethics Committee 1 of the UK National Research Ethics Service prior to commencing recruitment. The trial was registered at www.clinicaltrials.gov (NCT01306981). A clinical trial authorisation was obtained from the MHRA before recruitment commenced.

Adults aged 18 or over with complications of proliferative diabetic retinopathy requiring vitrectomy surgery with anticipated delamination of fibrovascular complexes were identified from vitreoretinal clinics at Moorfields Eye Hospital at the time they were listed for surgery. Subjects were required to have fibrovascular tissue proliferating from the retina in more than one location, as it is in this subset of patients that troublesome intra-operative and post-operative bleeding can occur. Subjects with a vitreous haemorrhage arising from a presumed single point of traction are likely to have excellent outcomes from surgery and do not represent a group of patients where anti-VEGF use would typically be considered.

3.2.2 ***Patient eligibility***

The following criteria were used to guide patient enrolment:

Inclusion criteria

1. Patients of either sex aged 18 years or over able to give informed consent throughout the study and return for study visits up to 12 weeks following surgery
2. Diagnosis of diabetes mellitus (type 1 or type 2)
3. Proliferative diabetic retinopathy in the study eye, with complications of this requiring vitrectomy surgery with anticipated delamination of pre-retinal fibrovascular complexes

Exclusion criteria (ocular criteria applied to study eye only)

1. Vitreous haemorrhage presumed to be caused by vitreous traction on a single, focal point of vitreoretinal attachment
2. Cataract that, in the opinion of the investigators, would be significant enough to impair the view during surgery; or planned combined cataract and vitrectomy
3. Previous vitrectomy on study eye
4. Uncontrolled glaucoma
5. Active or suspected ocular or periocular infections.
6. Active severe intraocular inflammation
7. Vision in fellow eye 3/60 or worse
8. Hypersensitivity to the active substance or to any of the excipients.
9. History of stroke, peripheral vascular disease, angina or myocardial infarction within six months prior to randomisation
10. Systemic anti-VEGF or pro-VEGF treatment within 3 months prior to randomisation
11. Pregnancy or lactation
12. Fertile male or female unwilling to use contraception

3.2.3 ***Sample size***

A sample size of 30 was deemed adequate to determine the feasibility and enable calculation of sample size for a subsequent definitive trial. Descriptive statistics only are presented for this pilot randomised clinical trial.

3.2.4 ***Randomisation***

One eye per participant was included in the study to guard against the impact of adverse events from exposure of both eyes to the investigational medicinal product. If both eyes were eligible, the eye with the clinical priority for surgery was included. Randomisation was stratified according to anticipated surgical complexity score as described by Castellarin *et al*. [195]. One point is scored for each quadrant of fibrovascular proliferation (FVP), one point for FVP both anterior and posterior to the equator, one point for tractional retinal detachment, one point for rhegmatogenous detachment and a final point for absence of posterior vitreous detachment. The score was determined pre-operatively based on the ultrasound findings. Scores of 5 and below were deemed low complexity; 6 and above high complexity, based on the finding of a mean complexity score of 5.5 by Rizzo *et al*. [194]. Subjects were randomised 1:1 to the ranibizumab group or control group, using random permuted blocks of varying sizes, with two separate randomisation lists generated by a statistician. The allocation sequence was held by the trial statistician and concealed from the researcher enrolling and assessing participants.

3.2.5 ***Masking***

The patients, investigators and operating surgeons were all masked to treatment allocation. Trial injections were administered by an unmasked investigator experienced in the delivery of intravitreal injections who had no further contact with the trial subject.

3.2.6 ***Intervention***

Subjects received their allocated intervention at 7 days ±1 day prior to surgery. Subjects in the ranibizumab arm received an intravitreal injection of ranibizumab (Lucentis®, 0.5 mg in 0.05 ml solution for injection, Novartis Pharmaceuticals UK Ltd), while subjects in the control arm received a subconjunctival injection of sodium chloride, 0.05ml of 0.9% solution for injection. Subjects in both groups were prepared identically with topical anaesthetic and povidone iodine; they received topical levofloxacin immediately before and after the injection and four times daily for four days after. Injections were administered using a 1ml syringe and 30-gauge

needle by the unmasked investigator.

3.2.7 ***Surgery***

Pars plana vitrectomy was carried out by a number of consultant vitreoretinal surgeons experienced in diabetic vitrectomy surgery, using 20-gauge instruments. A sample of venous blood and up to 1 ml of undiluted vitreous was obtained at the beginning of surgery. All surgeons used a similar technique of core vitrectomy followed by en-bloc delamination of fibrovascular membranes, control of bleeding by infusion pressure and endodiathermy. Retinal breaks were treated with endolaser and a suitable tamponnade agent was selected by the surgeon. All patients received additional endolaser panretinal photocoagulation. Vitrectomy duration was recorded from initial light pipe insertion to final removal after completion of laser. Additionally recorded were the number of backflush cannula applications, endodiathermy usage, anterior and posterior iatrogenic retinal breaks, intraoperative bleeding score (0-none; 1-mild, stopped by bottle elevation; 2-moderate, forming clots or persistent; 3-severe, covering half of posterior pole) and surgeon-derived complexity score as described above (from Castellarin *et al*.) to allow validation of the ultrasound derived complexity score.

3.2.8 ***Follow-up visits and investigations***

All subjects attended at baseline, 1 week later for surgery, then 6 and 12 weeks post-operatively.

At all visits subjects underwent refracted best-corrected Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity and a full ophthalmic examination including relative afferent pupillary defect (RAPD) check and intraocular pressure (IOP) measurement. At baseline and on the day of surgery subjects underwent standardised ophthalmic ultrasound by one of two operators masked to treatment allocation. Scans were obtained using an Acuson Sequoia 512 scanner with a 14 MHz linear probe (Siemens Medical Solutions USA; Mountain View, CA, USA). Ultrasound was used to determine the extent and location of fibrovascular proliferation, the presence of tractional or rhegmatogenous retinal detachment with measurement of height and base dimensions where appropriate. At baseline and 12

weeks post-operatively, blood pressure and HbA_{1c} were recorded and subjects underwent colour fundus photography and fluorescein angiography when this was not precluded by vitreous haemorrhage. ETDRS visual acuity, fundus photography and fluorescein angiography were carried according to the standardised protocols described in Chapter 2.

3.2.9 ***Treatment of vitreous and plasma samples***

Vitreous samples collected at the commencement of vitrectomy surgery were immediately placed into EDTA-coated Eppendorf tubes and kept on ice. At the earliest opportunity, the vitreous sample was centrifuged at 400g (2200 RPM) for 5 minutes at 4°C and divided into 110 µl aliquots in further EDTA-coated Eppendorf tubes and frozen at -80°C. Venous blood samples were collected using a standard EDTA-coated Vacutainer tube and immediately placed on ice. Blood was spun at 1500g (5000 RPM) for 10 minutes at 4°C and divided into 200 µl aliquots in plain Eppendorf tubes before being frozen at -80°C.

In addition to the 30 pairs of samples obtained during the trial a further 7 pairs of vitreous and plasma samples from non-diabetic control subjects were available for analysis. These had been obtained previously when the subjects had undergone vitrectomy for other indications (e.g. macular hole, epiretinal membrane). Samples had been treated in a similar way to the present samples.

3.2.10 ***Multiplex cytokine analysis***

Vitreous and plasma samples were subjected to multiplex bead analysis for detection of cytokines as described by Groer *et al.* [211]. Samples were analysed using a Milliplex 39 cytokine kit (catalogue number MPXHCYTO60-(KPMX39), Millipore UK Ltd, Oxford, UK) testing for the following cytokines: endothelial growth factor (EGF), eotaxin, fibroblast growth factor 2 (FGF-2), Flt-3 ligand (Flt-3L), fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), growth-related oncogene (GRO), interferon (IFN)-α2, IFN-γ, interleukin (IL)-1α, IL-1β, IL-1Rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IFN-inducible protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage-derived

cytokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-1β, sCD40L, sIL-2Rα, transforming growth factor (TGF)-α, tumour necrosis factor (TNF)-α, TNF-β, and VEGF.

Samples were tested at the laboratories of Millipore U.K. (Oxford, U.K.) using a Luminex analyser as described by Lange *et al.* [205]. Cytokine concentrations in this method were determined by comparing to standard curves prepared on the same plate and run in parallel to quality control samples. Cytokines not meeting the calibration standard were excluded from the analysis and samples where the coefficient of variation (CV) between samples exceeded 30% were also excluded.

3.2.11 ***Investigation of intraoperative bleeding***

Quantification of intraoperative haemorrhage was undertaken by recording a subjective score from the operating surgeon in addition to microscopic evaluation of surgical irrigation fluid. The contents of the vitrectomy surgical cassette at the end of surgery were collected and subjected to haemocytometry. This cassette collects aspirated fluid during surgery and contains irrigation fluid with blood cells released from intraoperative haemorrhage. Additionally it contains the vitreous contents with any pre-existing haemorrhage removed during the initial vitrectomy.

The total volume of fluid was calculated by mass, assuming 1g=1ml. After thorough agitation, a sample of fluid was placed on the Neubauer improved counting chamber (Figure 49) and examined under a microscope. Cell counts were obtained using a standard technique, where the mean of four counts of 0.1 µl was used, unless the cell density was very high, when four counts from the 0.004 µl grid were used.

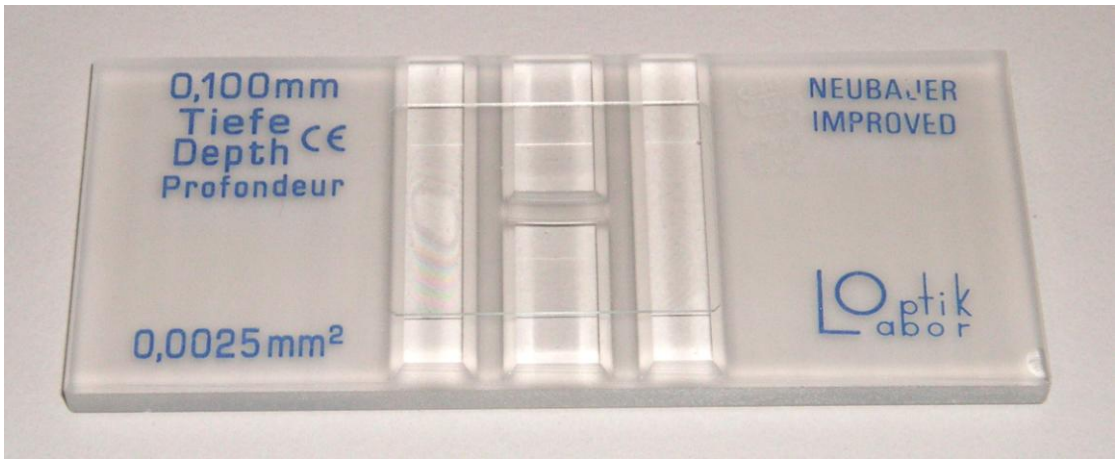


Figure 49- Neubauer improved counting chamber (from: Wikimedia commons).

3.2.12 ***Outcomes***

The following outcomes were defined prospectively:

Primary outcome measure:

ETDRS best-corrected visual acuity at 12 weeks following surgery

Secondary outcome measures:

1. Ease of performing vitrectomy surgery (subjective score by surgeon)
2. Intra-operative bleeding (subjective score by surgeon and objective analysis of collected surgical fluid by haemocytometry)
3. Incidence of post-operative vitreous haemorrhage
4. Extent of retinal neovascularisation (from masked grading of colour fundus photographs)
5. Height, transverse and longitudinal dimensions of tractional retinal detachment, measured by ultrasound
6. Extent of macular perfusion (from masked grading of fluorescein angiograms)
7. Vitreous and plasma levels of VEGF and related cytokines

3.2.13 ***Statistical methods***

This was a pilot randomised controlled trial, so no formal sample size calculation was conducted. Thirty patients were deemed sufficient by the group of trial investigators to explore feasibility and gather information needed to determine the size of a subsequent definitive trial.

Baseline characteristics of the patients were compared by treatment groups by the trial statisticians to assess the adequacy of the randomisation. Summary measures for the baseline characteristics were means and standard deviations (or median and interquartile range (IQR)) for continuous (or non-normally distributed continuous/ordinal variables); and frequencies and percentages for categorical variables. The trial statisticians assessed for normality by inspection of histograms. Summary statistics were computed for each outcome by treatment group. STATA statistical software (version 12, StataCorp LP) was used for data analysis. Although randomisation was stratified by complexity there was a single high complexity patient and so results are presented for the group in total.

Laboratory findings were analysed using GraphPad Prism (version 6.02, GraphPad Software, Inc.). The Kruskal Wallis test with adjustment of P values for multiple comparisons (three groups) was used to compare average cytokine values by treatment group in an exploratory analysis of laboratory findings.

3.3 **Results**

3.3.1 ***Recruitment***

A clinical trial authorisation confirming approval for the study was obtained from the MHRA on 11/03/2011; a favourable ethical approval was received from the Central London Research Ethics Committee (REC) 1 on 22/03/11 and local research and development approval was given on 30/03/11.

Fifty-nine potential patients were approached and given the patient information sheet (PIS); 21 of these declined to attend screening and 6 became ineligible due to changes in their condition. Thirty-two patients attended screening, two of whom did not meet the inclusion criteria (one pregnant, one no longer required surgery).

Thirty eyes of 30 subjects were enrolled in the study between August 2011 and December 2012, with follow-up completed in March 2013. Fifteen subjects were randomised to ranibizumab, 15 to control. All patients had been previously managed for proliferative retinopathy by panretinal photocoagulation prior to listing for surgery.

3.3.2 ***Patient disposition and demographics***

All subjects received their allocated intervention. Demographic and non-ocular characteristics of the trial participants were comparable between groups and are shown in Table 21.

	Control group	**Ranibizumab group**
Number	15	15
Female sex	9 (60%)	3 (20%)
Age (years)	48.7 ±18	57.1 ±14
Ethnicity: White or white British Black or black British Asian or Asian British Other Not recorded	 8 (53.3%) 2 (13.3%) 2 (13.3%) 3 (20.0%) 0	 8 (53.3%) 2 (13.3%) 3 (20.0%) 1 (6.7%) 1 (6.7%)
Type of diabetes 1 2	 6 (40%) 9 (60%)	 4 (26.7%) 11 (73.3%)
Duration of diabetes (years)	21 (15, 28)	19 (10, 23)
Systolic BP	132 ±22	128 ±22
Diastolic BP	75 ±9	78 ±11
HbA_{1c}	9.3 ±1.8 (n=13)	8.2 ±1.1 (n=14)

Table 21 – Patient demographics and non-ocular baseline characteristics. Results are shown as mean ± standard deviation for normally distributed data; median (IQR) if non-normally distributed continuous data; number (%) for categorical data. BP – blood pressure; HbA_{1c} – glycosylated haemoglobin.

The ocular characteristics of study participants are shown in Table 22; again the two groups are comparable in terms of degree of visual loss, vitreous haemorrhage grade and cataract.

	Control (n=15)	**Ranibizumab (n=15)**
ETDRS letter score	32.3 ±19 (0-62; CF=1, HM=1)	28.5 ±26 (0-72; HM=2, PL=1)
RAPD present	3 (20%)	4 (27%)
Rubeosis present	0	0
Anterior chamber inflammation grade (0-4)	0	0
Intraocular pressure (mmHg)	16.5 ±4	16.8 ±3
Lens status		
Pseudophakic	3 (20.0%)	5 (33.3%)
Cataract grade 0	4 (26.7%)	4 (26.7%)
1	6 (40.0%)	5 (33.3%)
2	1 (6.7%)	1 (6.7%)
3	1 (6.7%)	0 (0%)
4	0 (0%)	0 (0%)
Vitreous haemorrhage score		
0 (no haemorrhage)	3 (20.0%)	6 (40.0%)
1 (minor haemorrhage)	8 (53.3%)	5 (33.3%)
2 (disc and large vessels visible)	2 (13.3%)	1 (6.7%)
3 (no fundus view)	2 (13.3%)	3 (20.0%)

Table 22 – Ocular baseline characteristics of the two groups. Results are shown as mean ± standard deviation for normally distributed data; number (%) for categorical data. ETDRS – Early Treatment Diabetic Retinopathy Study; RAPD – relative afferent pupillary defect.

All subjects attended as planned one week after screening for surgery. The median interval between trial intervention and surgery was 7 days (range 6-8 days).

*3.3.3 **Losses to follow up and withdrawals***

No subject withdrew from the trial prior to the primary endpoint at 12 weeks. One subject was temporarily lost to follow-up and attended the final trial visit 50 weeks after surgery. His data were included in the final analysis. All other trial exit assessments were conducted within 4 weeks of the 12 week time point.

*3.3.4 **Primary outcome***

Mean (SD) visual acuity at 12 weeks post-operatively in the ranibizumab treated group was 52.6 (21) letters compared with 46.7 (25) letters in the control group. This

represents an improvement of 24 (27) letters for ranibizumab and 14 (31) letters for control. Visual acuity results are summarised in Table 23. No clinically significant visual acuity change occurred between trial intervention and surgery.

	Baseline	**Day of surgery**	**6 weeks**	**12 weeks**
Control	32.3±19.3	30.3±17.6	46.4±23.2	46.7±25.4
Ranibizumab	28.5±26.5	29.1±27.8	51.5±22.1	52.6±21.0

Table 23 – ETDRS visual acuity at different time points throughout the trial shown as mean ±SD.

3.3.5 Secondary outcomes

3.3.5.1 *Ease of performing vitrectomy surgery*

The majority of cases in both groups were performed under local anaesthetic (sub-Tenon's block). The median (IQR) duration of vitrectomy was 51 (38, 82) minutes in the control group and 63 (42, 87) minutes in the ranibizumab group. Further surgical metrics are listed in Table 24. Similar numbers of endodiathermy and backflush cannula applications occurred in both groups and there were similar numbers of iatrogenic breaks in the two groups. The overall surgical difficulty score was rated as 2 for more patients in the ranibizumab group and 3 for more patients in the control group. Twelve patients in each group required tamponnade agents to treat retinal breaks and/or proliferative vitreoretinopathy (PVR). In most cases, this was sulfur hexafluoride gas, although one subject in each group required perfluorocarbon and two subjects in the ranibizumab group required silicone oil.

	Control	Ranibizumab
Anaesthetic LA (number): GA (number:	 10 5	 12 3
Duration of vitrectomy in minutes (median (IQR)):	51 (38, 82)	63 (42, 87)
Intraoperative bleeding score: 0 1 2 3	 2 8 5 0	 3 10 2 0
Mean number of endodiathermy applications:	1.5	1.1
Mean number of backflush cannula applications:	2.8	2.5
Total number of iatrogenic breaks (all patients) Anterior: Posterior:	 6 9	 3 9
Overall surgical difficulty score: 0 (very easy) 1 (routine) 2 (moderate) 3 (complex, challenging) 4 (extremely difficult)	 1 2 3 9 0	 1 1 6 6 1
Surgeon-determined pre-op complexity score (0-8):	Median 4	Median 5
Tamponnade agent used: SF6 (number): C3F8: Silicone oil:	 11 1 0	 9 1 2

Table 24 – Surgical parameters for two groups. LA – local anesthetic; GA – general anesthetic; SF6 – sulfur hexafluoride; C3F8 – perfluorocarbon.

3.3.5.2 *Intraoperative haemorrhage*

More patients in the ranibizumab group had mild intraoperative bleeding (10 vs. 8) and fewer had moderate bleeding (2 vs. 5). Fluid was successfully collected from the vitrectomy cassette in 29/30 cases. In one case in the control group, a loose connection in the surgical tubing prevented the collection of fluid. Volumes of fluid collected varied considerably, as did concentration of red blood cells. The estimated total red blood cell counts obtained by multiplying the number of cells counted on

the haemocytometer by the total fluid volume are shown in Table 25. Data were not assumed to be normally distributed.

	Control n=14	**Ranibizumab n=15**
Lowest	1.15×10^6	2.72×10^6
Highest	3.65×10^8	5.20×10^8
Median	6.55×10^7	2.91×10^7
Interquartile range	8.44×10^7	8.31×10^7

Table 25 – Red blood cell counts from surgical fluid, shown as total number of red blood cells in the collected fluid.

There was no significant difference in red blood cell count in the operative fluid between the two groups (Mann Whitney U test p=0.37).

3.3.5.3 *Incidence of post-operative vitreous haemorrhage*

In both groups, there were minor variations in degree of vitreous haemorrhage between baseline and surgery. At 6 weeks post-op, there were two subjects in each arm with vitreous haemorrhage but these were both severe in the control group, compared to mild and moderate in the ranibizumab group. By 12 weeks no subject in the ranibizumab group had any vitreous haemorrhage while two persisted in the control group, summarised in Table 26.

	Baseline	**Surgery visit**	**6 weeks post-op**	**12 weeks post-op**
Control				
Grade: 0	3	4	13	13
1	8	7	0	1
2	2	4	0	1
3	2	0	2	0
Ranibizumab				
Grade: 0	6	5	13	15
1	5	6	1	0
2	1	1	1	0
3	3	3	0	0

Table 26 – Numbers of subjects with different grades of vitreous haemorrhage at different trial time points. For explanation of grading, see Table 22.

3.3.5.4 *Extent of retinal neovascularisation*

At baseline, colour photographs where grading was possible showed proliferative diabetic retinopathy in all subjects. By 12 weeks, four subjects in the ranibizumab group and one in the control group were graded as non-proliferative retinopathy. The remainder still had evidence of proliferative retinopathy but the modal ETDRS grade for both groups was 61 (mild PDR), indicating that surgery achieved the intended aim of treating advanced proliferative disease. Results are shown in Table 27

ETDRS Grade		**Baseline**		**12 weeks**	
		Ranibizumab	Control	Ranibizumab	Control
NPDR	20				
	35				
	43				
	47			2	
	53			2	1
PDR	61	5	1	7	11
	65			3	1
	71	2	2	1	
	75		1		
	81	2	2		
	85	2	6		
	90	3	2		

Table 27 – Numbers of subjects with different grades of diabetic retinopathy at baseline and 12 weeks. At baseline, 14 sets of photos were gradable in each group. At 12 weeks, 15 sets in the ranibizumab group and 13 in the control group were gradable.

Example images of the extent of retinal neovascularisation and presence of advanced proliferative retinopathy before and after surgery are shown in Figure 50.

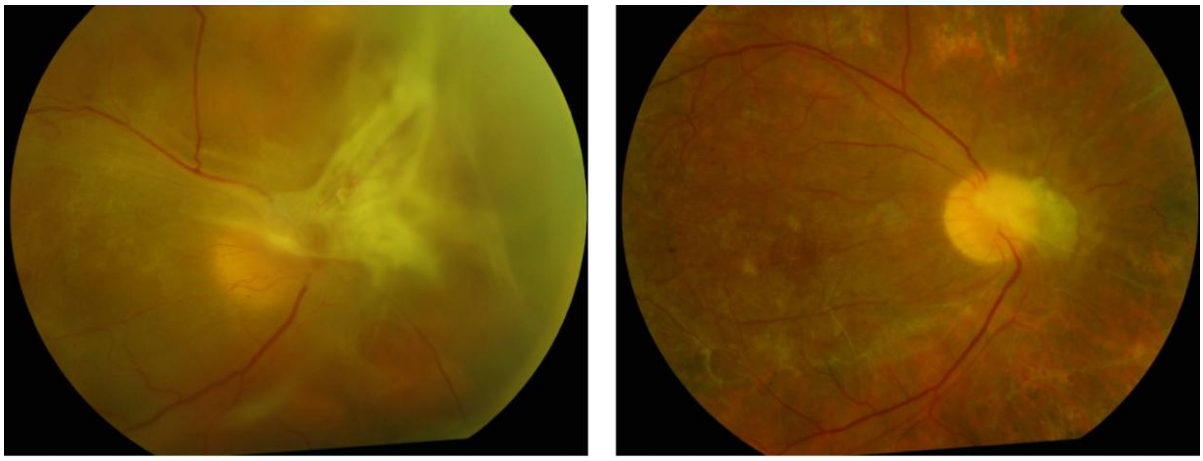


Figure 50 – Colour fundus photographs to show pre- and post-operative appearances of a subject with advanced proliferative diabetic retinopathy in the RaDiVit pilot trial. This subject was in the ranibizumab arm.

Area of retinal neovascularisation was graded in a step-wise fashion from 0 disc diameters (DD) to >30 DD when image quality allowed. In the ranibizumab group, 11/12 subjects graded at baseline had greater than 10 DD of proliferation; by 12 weeks 12/15 had fewer than 10 DD and of these, four had no visible proliferation. Similarly in the control group 11/13 at baseline had greater than 10 DD proliferation and at 12 weeks 13/13 had fewer than 10 DD; one had no visible proliferation.

3.3.5.5 *Extent of tractional retinal detachment*

The ultrasound appearance of a tractional retinal detachment in a patient with advanced proliferative retinopathy is shown in Figure 51. The results of ultrasound evaluation at baseline and on the day of surgery are shown in Table 28.

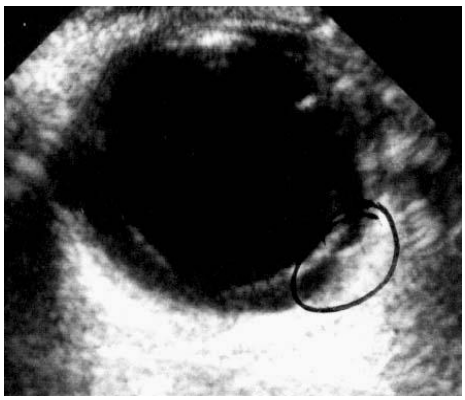


Figure 51 – B-scan ultrasound to show fibrovascular proliferation and tractional retinal detachment (circled) in advanced proliferative diabetic retinopathy. Image courtesy of Mr Paul Sullivan.

	Control		Ranibizumab	
	Baseline	1 week post injection	Baseline	1 week post injection
Quadrants of FVP: 1 2 3 4	 0 2 1 12	 0 2 0 13	 0 1 2 12	 0 2 2 11
FVP location Anterior: Posterior: Both:	 14 1	 15 0	 15 0	 13 2
Number with TRD present:	6 (40%)	7 (47%)	10 (67%)	11 (73%)
Number of TRDs involving macula:	5 (33%)	5 (33%)	5 (33%)	5 (33%)
Number with RRD:	1 (7%)	1 (7%)	1 (7%)	0 (0%)
Dimensions of TRD* Height (mm): Base (mm):	2.2±0.5 11.9±5.0 x 13.2±4.6	2.5±0.6 12.6±5.3 x 13.5±4.4	2.4±0.9 11.9±4.7 x 11.5±5.3	2.4±1.0 12.1±5.0 x 12.5 ±6.2
Dimensions of TRD Height (mm): Base (mm):	2.58±1.04 11.9±5.00 x 13.2±4.60 *	2.96±1.19 13.4±5.53 x 14.5±5.02	2.33±0.86 11.8±4.44 x 10.8±5.48	2.14±1.15 10.6±5.89 x 10.8 ±6.76
Extent of PVD: - None - Partial - Complete	 15	 15	 15	 15
Ultrasound derived complexity score (0-8)	Median 4	Median 4	Median 5	Median 5

Table 28 – Ultrasound findings for two groups at baseline and one week after study injection. FVP – fibrovascular proliferation; TRD – tractional retinal detachment; RRD – rhegmatogenous retinal detachment; PVD – posterior vitreous detachment. *Includes only those subjects with TRD present at baseline and 1 week where measurements could be obtained: n=5 control; n=9 ranibizumab.

No clear evidence of an increase in the number of quadrants of fibrovascular proliferation (FVP) was seen. Two subjects in the ranibizumab group had anterior FVP detected one week after injection. One subject in each group had a tractional

retinal detachment (TRD) identified on the day of surgery that had not been identified at baseline; in a further subject in the ranibizumab group ultrasound did not detect a retinal break (rhegmatogenous detachment) on the day of surgery that had been detected at baseline. The axial resolution of the ultrasound was limited by the calipers to ±0.2 mm. There was a small increase in height of TRD in the control group of 0.38 mm and in the ranibizumab group the decrease in TRD height was smaller than the limit of axial resolution. The accuracy of the transverse and longitudinal measurements of the base dimensions of the retinal detachment were limited by the resolution of the scanner to ±1.6 mm. No change in base dimensions exceeded this limit of resolution in either group. In all subjects the vitreous gel was attached to areas of TRD and vitreoretinal adhesions and detached elsewhere (partial PVD).

The median complexity score derived retrospectively by the operating surgeon in the two groups was the same as that obtained by ultrasound (see Table 24 and Table 28). However, scoring by the surgeon identified more TRDs and combined tractional-rhegmatogenous retinal detachments (TRRDs) than by ultrasound (control: 11 TRD of which 2 were TRRD; ranibizumab 13 TRD of which 6 were TRRD). This is likely due to the inability of ultrasound to detect small breaks in TRDs that are only visualised surgically.

3.3.5.6 *Extent of macular perfusion*

Fluorescein angiograms were only gradable at baseline in 3/15 subjects in the control group and 5/15 in the ranibizumab group. All showed moderate to severe perifoveal capillary loss. At 12 weeks, the mean (SD) FAZ greatest linear dimension (GLD) was 637 (236) µm in the control group (9 angiograms graded) and 765 (576) µm in the ranibizumab group (10 graded). FAZ area was 0.315 (0.147) mm^2 for control and 0.403 (0.562) mm^2 for ranibizumab. Median total score for perifoveal capillary loss was 14 in both groups, indicating scores of 3-4 (moderate to severe) in each of the four quadrants. Thus in both groups there was evidence of significant macular ischaemia but no evidence of a treatment effect.

3.3.5.7 *Vitreous and plasma levels of VEGF and related cytokines*

Vitreous samples were analysed on one 96-well plate and plasma samples analysed on a second 96-well plate between 2 replicates of quality control samples and following a calibration curve. Calibration data was fitted using a weighted ($1/y^2$) 5-parameter logistic curve fit of the mean Median Fluorescent Intensity (MFI) values.

One vitreous sample was too viscous to pipette and so was unable to be analysed. The quality control data for one cytokine (sCD40L) on the plate containing vitreous samples did not meet the required standard of accuracy and so this was excluded from the analysis of vitreous. The lower limit of quantification for the assay was reported as 3.2 pg/ml and the upper limit 10000 pg/ml except for sIL-2Rα where the lower limit was 16 pg/ml. For the purposes of statistical analysis where the cytokine concentration was below the limit of detection for the assay the concentration was taken as 1.6 pg/ml and where above the limit of detection was rounded down to 10000 pg/ml (following the method of Lange *et al.*). For sIL-2Rα the concentration was taken as 8 pg/ml when the result was below the detection limit of the assay.

Vitreous results

Twelve cytokines were not detected in the vitreous from any subject (IFN-γ, IL-1β, IL-2, IL-7, IL-9, IL-12(p40), IL-12(p70), IL-13, IL-17, sIL-2Rα, TNFα and TNFβ). Nine cytokines had significantly altered levels in the control group from this trial (representing diabetic subjects not treated with ranibizumab) compared with non-diabetic historical control samples. These are shown in Table 29. Seven showed increased levels in diabetes and of these, two: IL-1α and VEGF, were significantly reduced in the vitreous of subjects treated with ranibizumab compared to the diabetic subjects in the trial who received saline. Two further cytokines appeared to show decreased levels in diabetes, but Fractalkine was only detected in one sample from the current batch and IFN- α2 in three, compared to all of the non-diabetic samples so it is possible that differences in handling of the more recent batch of samples could have prevented detection of these cytokines.

Cytokine	Non-diabetic control (n=7)	RaDiVit saline control (n=14)	RaDiVit ranibizumab (n=15)	Kruskal-Wallis P-value
IL-1α	1.60 (0)	4.01 (8.64)	1.60 (0)	0.01 0.03*
IL-4	1.60 (0)	43.54 (27.77)	62.08 (56.79)	0.02 NS
IL-6	1.60 (0)	32.51 (74.42)	25.06 (12.45)	0.0007 NS
IL-8	3.82 (3.40)	43.68 (108.61)	46.15 (99.82)	0.0006 NS
IP-10	218.15 (102.90)	980.90 (808.69)	586.24 (653.32)	0.0001 NS
MCP-1	1446.60 (1988.81)	5318.61 (1759.68)	4625.98 (2801.65)	0.003 NS
VEGF	1.60 (0)	65.88 (505.66)	1.60 (0)	0.002 0.0001*
Fractalkine	58.99 (38.69)	1.60 (0)	1.60 (0)	<0.0001 NS
IFN-α2	20.95 (24.66)	1.60 (0)	1.60 (0)	0.0002 NS

Table 29 – Vitreous concentrations of cytokines (pg/ml) with levels significantly altered in subjects with diabetes. Two cytokines, IL-1α and VEGF, were reduced following ranibizumab treatment compared to levels in the control group. Data shown as median (interquartile range). P-values corrected for multiple comparisons. * - P-value for control vs. ranibizumab. See text for cytokine legend.

Further analysis showed a trend towards increased levels of EGF in diabetic subjects (P=0.08). MDC, although not significantly elevated in control diabetic samples compared to non-diabetics, was significantly reduced in ranibizumab treated samples (P=0.015) and Flt-3 ligand similarly showed a trend towards reduction by ranibizumab (P=0.06) despite no evidence of elevation in the control diabetic samples. The remaining cytokines did not have significantly different levels compared to non-diabetic controls.

Plasma results

In plasma, all 39 cytokines were detected in at least three samples. Six cytokines had altered levels in the diabetic control group compared with non-diabetic controls,

shown in Table 30. Only one of these, G-CSF, had increased levels in diabetes; the other five had reduced levels.

No cytokine had significantly altered levels in subjects in the ranibizumab group. VEGF levels were reduced in the ranibizumab treated group (29.04 (51.07) pg/ml vs. 65.41 (80.94) pg/ml) but this did not reach statistical significance, so an effect on systemic VEGF levels cannot be demonstrated or excluded.

Cytokine	Non-diabetic control (n=7)	Control (n=15)	Ranibizumab (n=15)	Kruskal-Wallis P-value
G-CSF	20.01 (16.97)	44.25 (23.10)	27.82 (22.25)	0.0087 NS
Eotaxin	1174.32 (120.08)	93.25 (77.08)	80.21 (53.62)	0.002 NS
Flt-3 ligand	62.99 (34.03)	1.60 (0)	1.60 (0)	0.0003 NS
GRO	1114.15 (544.69)	163.73 (319.82)	95.84 (55.47)	0.0194 NS
IL-13	4.78 (9.62)	1.60 (0)	1.60 (0)	0.04 NS
MCP-3	7.89 (17.30)	1.60 (0)	1.60 (0)	0.03 NS
sCD40L	5370.26 (1659.96)	473.32 (218.00)	366.44 (202.91)	0.003 NS

Table 30 – Plasma levels of cytokines with reduced concentration in the vitreous from subjects with diabetes. Values shown as median (IQR).

*3.3.6 **Safety***

There was one adverse event meeting the pre-defined criteria for an SAE in each group: in the control group, one subject was admitted to hospital with hypoglycaemia 10 weeks after surgery. In the ranibizumab group, one subject was admitted to hospital with raised intraocular pressure later on the day of surgery. Neither was believed to be related to study drug administration. Ocular and non-ocular adverse events are summarised in Table 31. There were more vitreous cavity haemorrhages in the control group than in the ranibizumab group, and more vitreous haemorrhages

in the non-study eye in the control group. More subjects in the control group had an upper respiratory tract infection.

	Control	**Ranibizumab**
Ocular adverse events		
Cataract	0	1
Eye pain	1	0
Eye redness	0	2
Raised intraocular pressure	0	1 (SAE)
Vitreous cavity haemorrhage	3 (6 episodes)	3 (4 episodes)
Vitreous haemorrhage in non-study eye	4 (5 episodes)	0
Non-ocular adverse events		
Hypoglycemia	1 (SAE)	0
Upper respiratory tract infection	5 (8 episodes)	1

Table 31 – Summary of ocular and non-ocular adverse events in the two groups during the RaDiVit study, shown as number of subjects experiencing adverse event. Where an adverse event occurred more than once in the same subject this is indicated. SAE – serious adverse event.

During surgery, there were fewer total iatrogenic breaks in the ranibizumab group than in the control group (12 vs. 15; see Table 24), and 10 vs. 12 required gas tamponnade. Two subjects in the ranibizumab group required silicone oil, however. No subject in either group developed recurrent rhegmatogenous retinal detachment during the study period.

3.4 Discussion

Ranibizumab pre-treatment in the RaDiVit study resulted in an additional gain of 10 letters compared with the control group at the three month primary endpoint. The results of this pilot randomised controlled trial suggest that using ranibizumab in this way prior to diabetic vitrectomy may be beneficial and warrants further exploration in a larger, powered study.

3.4.1 ***Design of the trial***

While previous randomised trials have used bevacizumab in this setting, this trial is the first to explore in detail the impact of ranibizumab in diabetic vitrectomy. Ranibizumab is the agent licensed for intraocular use in age-related macular degeneration and DMO, and has increased binding efficacy together with a shorter half life in the vitreous combined with bevacizumab. This means it may have a different safety profile and merits investigation. Reports of worsening tractional retinal detachment with bevacizumab therapy [107, 108] mean that safety concerns remain with the use of bevacizumab for this indication.

3.4.1.1 ***Endpoint selection***

Previous trials investigating bevacizumab have used a variety of primary endpoints that makes direct comparison between them difficult. Rizzo *et al.* chose "feasibility of surgery" [194], as did Modarres *et al.* [196] ("facilitation of the surgery and decrease of complications" *sic*). The primary outcome measure for Ahmadieh *et al.* was early post-operative vitreous haemorrhage [197]. In designing this pilot study, best corrected visual acuity was chosen as the single primary endpoint as it represents the outcome that is likely to be of most importance to patients; this outcome was reported at 3 months (12 weeks) post-operatively as this represents a time point when post-operative complications are likely to have settled, but post-vitrectomy cataract limiting vision should not have yet developed. If there was a difference in rates of post-operative vitreous haemorrhage at 3 months, this may be captured by a difference in visual acuity.

Duration of surgery and technical difficulty of operating are important surrogate endpoints that may have an impact on service delivery or post-operative

complication rate. Shorter operations may be safer for the patient in terms of anaesthetic risk, and are beneficial to hospitals needing to make the best use of limited operating theatre resources. However, as they may not affect the final visual outcome, they are appropriately included as secondary endpoints.

The first secondary outcome was prospectively defined as "Ease of performing vitrectomy surgery". This was assessed by vitrectomy duration and a surgical difficulty score reported by the operating surgeon, which is an unvalidated, subjective measure. The second of the secondary outcomes evaluated intra-operative haemorrhage by using a subjective score defined in a previous randomised trial [212], quantification of the use of instruments designed to control haemorrhage (backflush cannula and endodiathermy), and by laboratory evaluation of waste surgical fluid containing red blood cells as previously described by other investigators [106, 199]. There are two major limitations to this last technique: firstly, the surgical cassette will contain pre-existing haemorrhage aspirated at the start of the operation; and secondly, haemorrhage aspirated via the backflush cannula is not collected in the cassette but rather ends up in the collecting bag of the surgical drape. However, this fluid from the drape bag is likely to be further contaminated by conjunctival bleeding so does not represent a good measure of intraoperative haemorrhage arising solely from the vitreous cavity.

To our knowledge, no investigators have previously studied objectively the effect of anti-VEGF injection on the progression of tractional retinal detachment, although anecdotal reports where there had been a long interval between injection and surgery reported a progression of TRD in some cases (e.g. [108]). By using ocular ultrasound to measure the size of the TRD by a masked investigator, we were able to provide an objective determination of any change in TRD occurring following ranibizumab injection.

3.4.1.2 *Control group*

Given the clinical equipoise already stated, it was deemed unethical to give the investigational medicinal product to all subjects. Using the fellow eye as an internal control for subjects receiving ranibizumab would not provide useful comparison data as diabetic retinopathy is frequently of differing severity in the two eyes and it would

be unusual to find subjects who required this type of surgery in both eyes at the same time. It was therefore clear that a parallel-group, randomised design would provide the best methodology to assess the impact of ranibizumab administration.

Intravitreal injections carry a risk of intraocular infection (endophthalmitis), haemorrhage and retinal detachment, so injecting a placebo substance would not represent an ethical choice as a comparator. Furthermore, it is not believed that anti-VEGF injection for this condition exerts its effect by a physical mechanism on the fibrovascular tissues; another reason why placebo intravitreal injection is not required. Some trials of intravitreal injections have used sham injections in the control group, where the barrel of the syringe with no needle attached is pressed against the conjunctiva to simulate an injection. This is entirely safe, and unlikely to cause any side effects. However, the propensity of a genuine intravitreal injection to cause subconjunctival haemorrhage means that any patient with this finding one week later would by inference have received the active injection, leading to inadvertent investigator unmasking. Given the subjective nature of some of the assessments of surgical difficulty, it is important that both the operating surgeon and the investigator collecting the data remain masked to treatment allocation. We therefore chose to use subconjunctival injection of saline as the comparator procedure. This carries virtually zero risk of significant ocular adverse events, but because it carries a small chance of causing a subconjunctival haemorrhage, like intravitreal injection, it avoids the accidental unmasking of subjects that could occur with a sham injection. Another trial investigating ranibizumab for non-clearing vitreous haemorrhage in proliferative diabetic retinopathy received ethical approval to use this technique in their comparator arm (NCT01030770, www.clinicaltrials.gov).

3.4.1.3 ***Stratified randomisation***

Trials of small numbers of patients have an inherent risk of imbalance between the two groups being studied. This can occur despite the use of appropriate randomisation methods. It was felt that in this study there was a significant chance of imbalance between the two groups with respect to the complexity of cases, with the possibility of a misleading result arising if cases that were technically less difficult were clustered in one of the groups. The trial was therefore designed to use stratified

randomisation, with cases being classified as either low or high complexity based on the complexity score described by Grigorian *et al.* In their similar trial, Rizzo *et al.* found a mean complexity score of 5.5, so cases were deemed low complexity if they scored 1-5 (a score of 0 would make them ineligible for the trial) and high complexity for scores of 6-8. Because vitreous haemorrhage could impair the assessment of the fundus and hence make difficult the observation of features needed to derive a complexity score, we chose to use ultrasound findings for its determination. The final complexity score was also obtained retrospectively from the operating surgeon who had full knowledge of the status of the retina to validate the use of ultrasound in this setting.

3.4.2 ***Primary outcome results***

Subjects treated with ranibizumab one week prior to vitrectomy surgery in this study had better visual acuity at 12 weeks post-operatively than subjects receiving a control injection of subconjunctival saline. Vitrectomy surgery resulted in a substantial improvement in visual acuity in both groups, but to a greater extent in the ranibizumab treated group (approximately 5 ETDRS lines vs. 3 lines). This improved acuity is in keeping with visual acuity gains reported in a prospective study of diabetic vitrectomy surgery [93], but an additional beneficial effect of ranibizumab pre-treatment needs validating in a larger, powered, randomised controlled trial. It is highly unlikely that these results represent a negative effect from subconjunctival saline, as it is biologically implausible that a tiny injection outside the eye of a pharmacologically inactive substance could have any effect on visual acuity 12 weeks later.

At baseline, the two study groups were comparable and the lower visual acuity was in the ranibizumab group. There was no evidence of confounding factors that could influence the visual acuity outcome: similar numbers had type 1 and 2 diabetes; systemic control of diabetes and hypertension was similar; furthermore there were only small differences in numbers of pseudophakic patients and in cataract grade between the two groups.

*3.4.3 **Secondary outcome results***

If the difference in visual acuity between the two groups detected by the primary outcome measure represents a genuine difference, it is not possible to establish whether this is by a direct effect of ranibizumab treatment or because of a secondary effect, for example by reducing the rate of post-operative vitreous cavity haemorrhage or other complications. Exploration of the pre-defined secondary outcome measures may yield further information in this regard.

3.4.3.1 ***Ease of performing vitrectomy surgery and intraoperative haemorrhage***

In contrast to previous studies that have shown shorter surgical duration or fewer instrument exchanges when an anti-VEGF agent has been used as an adjunct prior to vitrectomy-delamination surgery, we found no evidence of a difference in the duration of vitrectomy between the two groups. The intraoperative bleeding score was skewed towards "mild" in both groups, but fewer in the ranibizumab group were scored as "moderate". This suggests that intraoperative haemorrhage in this trial did not present a major surgical problem. The difference is probably not clinically significant given that the number of uses of the backflush cannula and endodiathermy was very similar between groups. More surgeons rated the operation as "complex, challenging" in the control group but one case in the ranibizumab group was rated as "extremely difficult". This was due to the formation of strong vitreoretinal adhesions in a case of combined TRRD, where effective delamination is already very difficult. Most cases were rated as moderate to complex in difficulty and limited conclusions can be drawn from this previously unvalidated scale, especially given the inclusion of multiple surgeons in this study.

In contrast to the single previous report quantifying intraoperative haemorrhage in diabetic patients receiving ranibizumab pre-treatment [199], no difference was found in red blood cell count from operative fluid between the two groups. The technique of red blood cell counting using the haemocytometer is prone to error from a number of factors. The surgical cassette may not contain all of the intraoperative haemorrhage, red cell clumping or lysis could occur and errors in counting may also contribute to inaccuracy. A reliable and reproducible technique for quantification of intraoperative haemorrhage is still awaited; flow cytometry may be worthy of investigation.

3.4.3.2 *Incidence of post-operative vitreous haemorrhage*

Vitreous cavity haemorrhage occurred in three subjects in each group, although more frequently in the control group (6 vs. 4) and at the 12 week primary endpoint two subjects in the control group still had severe vitreous cavity haemorrhage obscuring fundus details whereas all ranibizumab treated subjects were free from haemorrhage. It still cannot be concluded whether anti-VEGF agents reduce the incidence of post-operative haemorrhage in this situation. The Cochrane review examining the effect of pre-operative bevacizumab on POVCH concluded that the results of only one study supported its use while methodological problems in the other studies prevented firm conclusions being drawn [191]. Because this study uses a different agent, it is unlikely that it could be included in an update to the review unless the terms of reference were changed.

3.4.3.3 *Extent of retinal neovascularisation and macular ischaemia*

Evidence from reading centre grading of colour photographs showed that following surgery, grade of diabetic retinopathy improved in both groups as fibrovascular tissue was removed and areas of proliferation were treated. By the trial exit, four subjects in the ranibizumab group and one in the control group were graded as non-proliferative retinopathy. Additionally, the area of retinal neovascularisation decreased substantially from enrolment to the primary endpoint in both groups, with the majority of subjects initially having greater than a 10 DD area of proliferation at the outset but less than 10 DD at exit. More subjects treated with ranibizumab had no evidence of neovascularisation at trial exit (4 vs. 1), consistent with the improved ETDRS retinopathy grade. The absence of neovascularisation in more subjects may be linked to the reduced incidence of haemorrhage in the ranibizumab group.

Very advanced diabetic eye disease meant that fluorescein angiograms were only graded in small numbers of patients at baseline. All subjects where angiograms were graded showed evidence of severe macular ischaemia: perifoveal capillary loss was graded as moderate to severe. Where angiograms were graded at exit, substantial variability of FAZ GLD and area was present in both groups, with no firm evidence of a difference. Again, all subjects had grades of perifoveal capillary loss suggestive of moderate to severe ischaemia. No conclusions can therefore be drawn about the

safety of ranibizumab in the setting of macular ischaemia from this small study with single dose administration.

3.4.3.4 *Extent of tractional retinal detachment and other ultrasound findings*

Ultrasound evaluation at enrolment and one week later on the day of surgery was chosen to allow detailed description of subjects with fibrovascular and tractional retinal pathology who had vitreous haemorrhage obscuring the fundus view, and to evaluate the impact of ranibizumab administration on the size and extent of tractional retinal detachment. Previous trials have excluded subjects where fundus details are not visible (e.g. [106]), which may lead subject selection to be biased towards those with more stable disease. In each group, the number with TRD increased by one between injection and surgery. This may represent new disease, but may be the result of detection of TRD not visualised on the first scan. Measurement of the height and base dimensions of the detachments did not reveal any evidence of an increase in TRD size and equally there was no evidence of increased fibrovascular proliferation.

A surgical complexity score has previously been described to allow objective comparison of difficulty between cases with different pathology and was used in this trial to stratify randomisation and achieve balanced groups. Other trials that have used this score, e.g. [194, 196], are not explicit about how this score is obtained; for the first time, ultrasound has been used to derive this complexity score pre-operatively. This ultrasound-derived complexity score correlates well with the score reported by the surgeon based on the findings during surgery. However, ultrasound failed to detect a number of small retinal breaks and so more subjects had combined TRRD identified at surgery. All subjects were graded as having partial posterior vitreous detachment (PVD) by ultrasound, as there were focal attachments of the gel to FVP and TRD, whereas surgeons graded PVD as absent when there was no detachment over these areas. Ultrasound grading in future studies should ensure that PVD is classified in the same way as during surgery.

3.4.3.5 *Vitreous and plasma cytokine levels*

Cytokine analysis confirmed that vitreous levels of VEGF were reduced by ranibizumab treatment one week previously. In addition, levels of IL-1α were also

reduced in the group treated with ranibizumab. This cytokine was detected in no non-diabetic samples, six untreated diabetic samples and two after ranibizumab treatment. It is implicated in inflammatory pathways and fibroblast activation so reduction in its concentration by ranibizumab, if confirmed by other studies, adds a new dimension to the understanding of the mechanisms by which VEGF inhibition exerts its effects. A change in the balance of intraocular cytokines from pro-angiogenic to pro-fibrotic has been postulated following VEGF inhibition, with raised levels of connective tissue growth factor detected after bevacizumab treatment and associated with increased fibrosis [209]. This has been termed the "angiofibrotic switch", and the discovery of reduced levels of another cytokine associated with fibrosis may provide another insight into this mechanism.

As discussed in section 3.1, IL-6, IL-8, IP-10 and MCP-1 have been found to be elevated in the vitreous of subjects with proliferative retinopathy and the present work adds to the evidence implicating these cytokines in this condition. In addition, we found raised levels of IL-4, a T-helper cell associated cytokine, in the diabetic samples but did not detect it in the non-diabetic controls. Other investigators analysing cytokines in diabetic vitreous have not found raised IL-4 [213-215], but have reported its presence in non-diabetic samples. It is therefore possible that degradation of the non-diabetic samples in this study has led to failure to detect this cytokine.

Two cytokines were reduced in the current set of samples, Fractalkine and IFN-α2, but these were only detected in a small number of samples so the significance of this finding is questionable. Lange *et al.* reported reduced IL-9 and Flt-3 ligand in their similar study; IL9 was not detected in any of the current samples and Flt-3 ligand levels were the same in the diabetic samples as in controls but with a trend towards reduction with ranibizumab. It is likely that some results may occur due to chance given the large number of cytokines being simultaneously tested. The relatively small patient numbers involved in the experiment mean that lack of statistical significance on testing should not be interpreted as proof of absence of a difference between two groups; it may be that difference too small to detect with this number of samples.

Plasma levels of VEGF in this study appeared reduced after a single administration of ranibizumab but this did not reach statistical significance. Other trials have reported reduced plasma levels of VEGF after repeated ranibizumab treatment [216], suggesting that further study of the pharmacokinetics of ranibizumab is warranted. In one small study of ten patients with DMO treated with different VEGF inhibitors, there was no change in plasma VEGF concentration after either ranibizumab or pegaptanib, however [217]. Cytokines with altered levels in the vitreous in this study did not have altered levels in plasma.

In summary, cytokine analysis in this small pilot study has confirmed elevated levels of cytokines previously implicated in the pathogenesis of proliferative diabetic retinopathy and has shown that ranibizumab treatment leads to a reduction in VEGF and IL-1α levels; the latter of these warrants further investigation and replication by other investigators.

*3.4.4 **Safety issues***

Ranibizumab administration prior to diabetic vitrectomy surgery appears to be safe, as no new safety issues have been identified in this small trial with only a single administration of ranibizumab. The SAEs that occurred were not related to study drug administration: raised intraocular pressure likely occurred as a consequence of intraocular gas tamponnade from surgery and a hypoglycaemic episode ten weeks after study enrolment was due to more proximate causes. The pattern of adverse events seen in both groups mirrors previous reports of adverse events seen in similar trials, with frequent occurrences of upper respiratory tract infections and mild ocular discomfort as a result of the injection procedure.

During surgery, the number of iatrogenic breaks was relatively low in both groups and similar numbers needed tamponnade with gas, although two subjects in the ranibizumab group required silicone oil. Thus no definite evidence of an adverse effect on the safety of surgery has been demonstrated.

3.4.5 ***Strengths and weaknesses of the study***

The double masked, randomised design of this study leads to a low risk of bias. Some of the assessments of surgery were subjective and so preserving surgeon masking adds to the validity of these assessments. Nevertheless, it is possible that the behavior of the tissues during surgery could give a clue to whether the subject had received ranibizumab. However, the use of a control injection that may cause a small, harmless, subconjunctival haemorrhage prevented accidental unmasking that could have occurred if only one group had received an active intervention. As this is the second trial to have used this technique for the control group, it is hoped that this will continue to be accepted by ethics committees.

Subjects included in the trial all had significant fibrovascular proliferation at the retina and so visual acuity improvement is unlikely to be driven by a group of low complexity patients likely to respond well to surgery without adjunctive treatment. The study included multiple surgeons, all experienced in diabetic vitrectomy surgery, so that any findings could not be attributed to an effect on one surgeon's technique only. However, including multiple surgeons introduces another variable that may make it more difficult to identify a small effect. The low number of subjects included in this pilot study and the relatively short follow-up mean that long term effects of this treatment cannot be evaluated. Longer follow-up after this type of surgery may increase the effect of confounding factors as post-vitrectomy cataract develops and changes to the status of diabetic retinopathy occur.

3.4.6 ***Power calculation and further work***

The results from this pilot study showed that mean (SD) visual acuity in the ranibizumab group at 12 months was 52.6 (21) compared to 46.7(25.4) for control. Assuming equal SD in the two groups and taking an average SD of 23.2 letters, a power calculation with an alpha of 0.05 yields the results shown in Table 32. Thus it can be seen that to detect a difference in visual acuity of the same magnitude (approximately 6 letters) found in this pilot study, a sample size of over 300 per group would be required.

	Power				
N per group	**99%**	**95%**	**90%**	**80%**	**50%**
100	14.14	11.89	10.69	9.24	6.47
150	11.52	9.69	8.72	7.53	5.27
200	9.97	8.39	7.54	6.52	4.56
300	8.13	6.84	6.15	5.32	3.72
400	7.04	5.92	5.32	4.60	3.22
500	6.30	5.30	4.76	4.12	2.88
1000	4.45	3.74	3.37	2.91	2.03

Table 32 – Power calculation from the RaDiVit study to show required sample size to detect differences of varying sizes with different powers (calculated using GraphPad StatMate version 2.00, GraphPad Software, Inc.)

It may be possible to refine this figure using a more sophisticated power calculation that takes into account the difference in visual acuity between groups and the slightly different SDs present, but nevertheless, given the rate of recruitment for this study, it would be difficult to perform at a single institution and would need a multi-centre, or possibly even multi-national set-up to ensure recruitment in a reasonable time frame.

3.5 **Conclusions**

The RaDiVit study was the first randomised controlled trial to investigate the use of pre-treatment with ranibizumab on the clinical outcomes of vitreoretinal surgery for advanced proliferative diabetic retinopathy. Its primary outcome measure was visual acuity at 12 weeks post-surgery, and a number of secondary outcome measures were evaluated.

The trial showed a potential beneficial effect for giving ranibizumab one week prior to vitrectomy for advanced PDR, as subjects in the treated group had better visual acuity at the primary endpoint compared to the control group. In this pilot study, statistical significance was not reached, and the wide range of visual acuity outcomes (and hence high standard deviation of the primary outcome result) means that no conclusion can yet be reached on the effect of ranibizumab in this condition.

There were fewer subjects with post-operative vitreous cavity haemorrhage in the ranibizumab group than the control group. No differences were seen in the degree of intra-operative haemorrhage, either by subjective (surgeon's bleeding score) or objective (haemocytometry) measures.

Ultrasound evaluation on the day of injection and one week later explored the effect of ranibizumab on existing vitreoretinal pathology. No objective evidence was found that ranibizumab either caused tractional retinal detachment or worsened existing detachment.

Investigation of vitreous cytokine levels has confirmed elevated levels of IL-1α, IL-6, IL-8, IP-10, MCP-1 and VEGF in subjects with diabetes. Two of these cytokines, IL-1α and VEGF were reduced following ranibizumab treatment. In plasma, there was a trend towards lower VEGF levels in subjects in the ranibizumab group but this did not reach statistical significance.

4 Conclusion

Vascular endothelial growth factor is now widely recognised as the most important cytokine in the complex pathways leading to physiological and pathological angiogenesis. Knockout of the *Vegf* gene is embryologically lethal, a multitude of *in vitro* studies place VEGF at the heart of pathways for vascular proliferation and permeability, but most importantly, clinical evidence shows that inhibition of VEGF is an effective treatment modality for neovascular diseases of the eye.

In this thesis, the results of two randomised clinical trials exploring the effects of VEGF inhibition with ranibizumab in the setting of diabetic eye disease have been presented.

This final chapter evaluates the strengths and weaknesses of the study designs, examines what conclusions, if any, can be drawn from the results, and discusses possible future work.

4.1 Trial design

The randomised clinical trial design remains the gold standard for comparing a new treatment against an existing treatment, or where there is no satisfactory existing treatment, against placebo. Therefore, for these two separate investigations, selecting a randomised trial design offered the best possibility of generating scientifically valid results.

4.1.1 The LUCIDATE study design

At the time of designing the protocol for the LUCIDATE study, the only licensed treatment available in the NHS for DMO was macular laser therapy. Ranibizumab was under investigation in large, phase 3 trials in this condition, but was awaiting a UK licence. The concept of intravitreal injections was daunting for patients, and carried risks of loss of sight that were much less likely with laser treatment. Therefore laser was selected for the control group in this study, to allow comparison of the new treatment with the existing standard treatment, and because it would be unethical to choose a placebo treatment when the ETDRS had shown laser to be superior to no treatment.

However, one significant failing in the study design was the inclusion of non-treatment naïve patients. Many of the recruited patients had already received laser as part of their standard care for DMO, so the ongoing effects of laser in these patients cannot be definitively excluded.

Recruitment for the study took just under nine months, with the first patient randomised on 11/11/10 and the final randomisation on 27/7/11. Although there was a high prevalence of DMO in the Medical Retina clinics, there were still considerable barriers to recruitment. Identifying 126 potentially eligible patients to randomise 37 subjects gave a ratio of 3.5 potential patients required to generate one randomisation. This means that a significant number of patients who could have benefited from the treatment were not included in the trial. Equally, those not included could have been different in some way from randomised subjects.

The investigations required to be undertaken by subjects at 0, 12, 24 and 48 weeks placed a significant burden on them and on the resources available. Carrying out all the tests required the patients to attend on two separate days and was time consuming. The 48 week end point was chosen as this represented the point four weeks after a 12th and final ranibizumab injection, which reflected the funding available. Other studies have typically chosen one year as a final outcome measure, with monthly rather than four-weekly retreatment. There are advantages in selecting a trial design that matches wider practice in order to improve the acceptability of results to the medical community. As it turned out, there were sufficient visits when retreatment with ranibizumab was not required to have had enough remaining to offer a 13th possible injection to those who required it and extend the trial to a full 52 weeks.

Justification for choice of investigations

All of the investigations undertaken were designed to offer new insights into the effects of ranibizumab on DMO. Visual acuity is the standard primary outcome measure in clinical trials for macular disease and OCT retinal thickness a common secondary outcome measure. Looking in more detail into OCT derived measures of retinal thickness and volume, together with morphological changes, increases the understanding of the effects of ranibizumab and laser across the whole macular area and in different layers of the retina. Microperimetry has been investigated in DMO and there are reports of the effects of laser and triamcinolone treatment. A thorough investigation of retinal sensitivity following ranibizumab treatment in this condition has not been reported previously. The same is true for colour contrast sensitivity: while reports of altered colour vision in DMO date from the 1970s, no previous investigation of anti-VEGF therapy has explored this functional measure. The potential adverse effects of pan anti-VEGF therapy on an ischaemic macula were a topic of interest when this trial was designed. This meant that significant macular ischaemia was a principal exclusion criterion that led to numerous potential patients not being recruited. Although the BOLT study reported no increase in FAZ dimensions or area following 12 months of bevacizumab therapy, serial fluorescein angiography was again performed with masked Reading Centre grading to look for an increase in macular ischaemia with ranibizumab. Similarly, global

electroretinography indices might have shown an overall decrease in retinal function after repeated anti-VEGF treatment.

The retreatment protocol in this study was designed to give maximum exposure to ranibizumab; this was to maximize the likelihood of a clinical response and to increase the chance of detecting any significant safety issues (although none were anticipated). There continues to be no consensus in the retina specialist community regarding the optimum treatment schedule for anti-VEGF therapy in diabetic eye disease, although certain themes are emerging. The DRCR.net protocol I trial gave an initial four ranibizumab injections at monthly intervals then retreated with two further injections if there was anything other than a complete resolution of oedema with good visual acuity. In the subsequent six months and in the following two year extension to the study, injections were given at investigator discretion when oedema continued to be present or there had been a drop in visual acuity. The RIDE and RISE licensing trials for ranibizumab gave monthly injections for two years. Although these latter two trials had the best visual acuity results, further work from the DRCR.net study shows that many subjects who received 7-8 injections in year 1 only need 2-3 in year 2 and 1-2 in year 3 to preserve visual acuity. Therefore it can be said with confidence that subjects in the LUCIDATE study were not under-treated.

4.1.2 ***The RaDiVit study design***

A pilot randomised trial was designed to investigate the impact of ranibizumab therapy prior to diabetic vitrectomy to inform a power calculation for a subsequent definitive clinical trial. Surgery is the only recognised intervention for this type of advanced proliferative retinopathy, and results of the Diabetic Vitrectomy Study demonstrated its superiority to observation. There is no recognised pre-operative treatment in this setting, so it was appropriate to compare ranibizumab with placebo, which for this trial was a harmless subconjunctival saline injection.

Bevacizumab has been extensively investigated in this setting but has not been widely adopted because of its unlicensed status and because of safety concerns

regarding exacerbation of tractional retinal detachment and pre-retinal fibrosis. Other trials have suffered from methodological flaws putting them at high risk of bias and have often reported single-surgeon outcomes, calling into question their generalisability. This pilot study chose to include multiple surgeons to avoid this problem, to be more representative of clinical practice, and because a definitive trial would likely require a multi-centre and hence multi-surgeon design. Further details of choices made in the design of the RaDiVit trial have been discussed in section 3.3.

4.2 Trial results and applicability

The trials showed evidence of benefit for ranibizumab over control in DMO and in surgery for advanced PDR but results were not generally statistically significant.

4.2.1 The LUCIDATE study

In the LUCIDATE study, detailed indices of retinal function were measured and retinal structure evaluated. The results showed the following trends at the 48 week endpoint:

- Better visual acuity with ranibizumab than laser
- Less reduction in colour contrast sensitivity with ranibizumab treatment compared with laser
- Improvement in retinal sensitivity measured by microperimetry with ranibizumab
- Less reduction in PERG P50 amplitude with ranibizumab
- Greater thickness reduction in retinal subfields with ranibizumab compared to laser

In several areas where there was potential concern that ranibizumab could exacerbate macular ischaemia or lead to generalised retinal electrophysiological dysfunction, there was no evidence that ranibizumab caused harm:

- No new serious adverse events were identified that could be attributed to ranibizumab
- There was no evidence of worsening of macular ischaemia with ranibizumab
- There was no evidence of global retinal dysfunction by full field electroretinography following ranibizumab treatment
- No evidence of a change in choroidal thickness with either ranibizumab or laser

The lack of statistical significance raises the possibility that these results have arisen by chance, or that the study was underpowered to detect the difference that exists between the two treatments.

Further study would be useful in light of the small reduction in rod function shown by the decreased bright flash a- and b-wave amplitude detected with ranibizumab therapy; again, the lack of power to detect a difference means that statistical noise is a possibility for this finding. The change detected was of small magnitude (10%) and did not reach statistical significance. It was not accompanied by a decrease in generalised cone function and in particular, the cone 30 Hz flicker wave, arguably the most sensitive of the electrophysiological tests, was not affected.

The study had an exploratory design and so a power calculation could not be performed as the magnitude of the effect was unknown when the study commenced. In addition to lack of power, the study did not employ stratified randomisation at baseline, resulting in two groups that had differences at baseline in visual acuity, type of diabetes and duration of DMO. This is a major failure of randomisation in this trial and raises the possibility that the two groups were not directly comparable at baseline.

Choroidal thickness and structure-function correlation are both areas of increased recent interest given the ready availability of higher resolution retinal imaging in the form of OCT. A post-hoc exploratory analysis in this study, which benefited from randomisation at baseline in contrast to many other published studies in this field, has not shown that choroidal thickness changes over 48 weeks of treatment with either ranibizumab or laser. This is an important negative finding in a rapidly evolving field and suggests that while the choroid may be important in the pathophysiology of diabetic retinopathy, choroidal vascular changes may not be VEGF dependent in the same way that retinal vascular changes are.

The study also explored the relationship between retinal thickness and retinal sensitivity. This showed that for ranibizumab treated patients, a decrease in retinal thickness coincided with increased retinal sensitivity. Further work in this area should explore the presence of structural abnormalities visible on OCT with their effect on retinal sensitivity.

Data from OCT scans at entry to the study were used to establish the repeatability of automated retinal thickness and volume measurements using Spectralis SD OCT in

DMO. This study showed that changes in thickness greater than 8 μm in the central subfield are likely to be indicative of clinical change rather than measurement variability. This may have implications for the design of clinical trials and making retreatment decisions in clinical practice in the future.

*4.2.2 **The RaDiVit study***

In the RaDiVit study, ranibizumab pre-treatment and sub-conjunctival saline in the control group led to improved visual acuity 12 weeks after diabetic vitrectomy. The results suggested a benefit for ranibizumab compared with saline, as the visual acuity gain was greater (24 letters for ranibizumab compared with 14 for control). A larger, powered trial that may require 300 subjects per arm would be needed to validate this finding in view of the large variability in outcome seen in terms of visual acuity in this study. It is therefore possible that the difference in visual acuity detected was driven by large changes in a small number of subjects that happened to occur in the ranibizumab group.

Other findings from the trial were:

- A trend towards a lower incidence of post-operative vitreous cavity haemorrhage in subjects treated with ranibizumab
- No evidence of new tractional retinal detachments or worsening of existing tractional retinal detachments with subjects treated with ranibizumab
- No evidence of a reduced surgical duration or reduced intraoperative haemorrhage with ranibizumab
- A change in the cytokine profile of the vitreous following ranibizumab injection, but no firm evidence of changes to plasma cytokines

Evaluation of cytokines in the vitreous and plasma of subjects in this trial has provided further evidence that the interleukins IL-6 and IL-8, together with IP-10 and MCP-1 are elevated in proliferative diabetic retinopathy. These cytokines are involved in the white blood cell recruitment as part of the inflammatory response, which plays a significant part in the pathophysiology of diabetic retinopathy. VEGF levels were reduced one week after ranibizumab administration as were levels of IL-1α, although in small numbers of subjects only. This cytokine is implicated also in

the activation of leukocytes in the inflammatory response and induces TNF-α release as well as activating cyclo-oxygenase pathways. It may therefore play a role in the induction of vascular permeability, and if it is activated downstream of VEGF, inhibitors of VEGF like ranibizumab could plausibly lead to reduced levels, but again validation in larger studies is required. The pharmacokinetics of ranibizumab are different from bevacizumab, and while the latter is readily detectable in the serum of rabbits following intravitreal injection, ranibizumab is not. Bevacizumab reduced serum VEGF to a greater degree than ranibizumab in subjects in the large IVAN trial for AMD and in this study, levels of plasma VEGF were not significantly different in subjects who had received a single dose of ranibizumab one week previously. Thus while concern remains about the possibility of arteriothromboembolic events as a results of systemic action of ranibizumab following intravitreal injection, this study has not shown reduced levels of VEGF systemically.

4.3 **VEGF inhibition in diabetic eye disease**

Ranibizumab is a monoclonal antibody fragment that binds all isoforms of VEGF-A. Differential splicing of messenger RNA gives rise to different isoforms of VEGF that exhibit different properties. To our knowledge, all of these are inhibited by the currently available VEGF inhibitors with the exception of pegaptanib, which selectively binds $VEGF_{165}$. If this were genuinely the most important VEGF isoform in the setting of pathological angiogenesis, it is surprising that trial results for this drug in AMD and DMO have not shown it to be more effective.

VEGF has been shown to be neuroprotective in the retina; for example it reduces retinal neuronal apoptosis as a result of ischaemia/reperfusion injury [31], and is released as part of ischaemic preconditioning. This raises the possibility that VEGF is a critical survival factor for neurons in ocular diseases characterised by retinal ischaemia, such as diabetic retinopathy, and that inhibiting VEGF by ranibizumab and other pharmacological agents could have deleterious effects on the retina. Detailed functional testing combined with evaluation of structures such as the perifoveal capillaries in the LUCIDATE study was carried out to explore a possible effect of repeated VEGF inhibition on the neurovascular unit. The results did not support a conclusion that ranibizumab led to significant reductions in retinal function. Subjects in the RaDiVit study, with more advanced diabetic retinopathy and more retinal ischaemia, still gained vision after surgery when ranibizumab pretreatment had been administered.

4.3.1.1 ***VEGFxxxb isoforms***

A further dimension to the understanding of VEGF biology has arisen with the discovery of new variants of the $VEGF_{xxx}$ molecules, such as $VEGF_{165b}$. This was first identified in a renal cell carcinoma line and arises through a change in the distal splice acceptor site in exon 8 [23]. This group of VEGFs activate VEGF receptors in a different way and hence may have different properties, being associated with physiological, rather than pathological angiogenesis [218].

Evidence that $VEGF_{165b}$ may inhibit angiogenesis and could have a cytoprotective effect by reducing the cellular insult following induced ischaemia [98] suggests that vascular homeostasis is maintained by a delicate balance between these isoforms. This could have implications for pan anti-VEGF inhibition as a treatment strategy: inhibiting isoforms of the molecule that are responsible for preserving healthy vasculature could have unwanted effects.

As greater understanding of the roles of different isoforms of VEGF in the landscape of angiogenesis and vascular homeostasis is reached, it may become apparent that pan anti-VEGF inhibition is an aggressive step to take given the delicate balance existing between isoforms. A switch in splice variants has been reported to occur in diabetic retinopathy [219], so therapeutic approaches in future could look at the molecular mechanisms behind this change in expression of VEGF isoforms that may underpin the development of pathological angiogenesis.

In the first instance, the development of an analytical test specific to different VEGF isoforms including $VEGF_{165b}$ would mean that vitreous and plasma samples from the RaDiVit study could be analysed in an isoform-dependent manner to shed further light on the effects of ranibizumab.

4.4 Future work

These trials both add to the existing literature on the place of anti-VEGF therapy in diabetic eye disease, and suggest a benefit for ranibizumab treatment compared to existing practice in two clinical scenarios. Further confirmatory work is needed.

Following the LUCIDATE study:

- Further analysis of morphological data from OCT scans and the correlation of structural changes with sensitivity changes should be carried out
- A powered study could be undertaken to validate the change in colour contrast sensitivity and retinal sensitivity measured by microperimetry with ranibizumab. This would only be of value if felt to be clinically relevant
- Larger studies examining the effect of anti-VEGF agents on the choroid should be undertaken to improve understanding of the role of the choroid in the pathogenesis of diabetic retinopathy

Following the RaDiVit study:

- A definitive, powered trial should be undertaken to test the hypothesis that ranibizumab pre-treatment leads to improved visual acuity at 12 weeks post-surgery for subjects with advanced proliferative diabetic retinopathy.

This is likely to present challenges in terms of recruiting the number of patients needed. Ongoing trials investigating the efficacy of ranibizumab as a treatment for proliferative retinopathy instead of panretinal laser may show that medical treatment leads to improved outcomes and reduction in the rates of development of advanced proliferative retinopathy. If this is the case, diabetic vitrectomy surgery could become a rarely performed procedure. At present though, despite screening and in some cases prompt panretinal photocoagulation, there remains a subset of patients who develop advanced retinopathy and are at risk of blindness without surgery. While this is the case, efforts should continue to optimise the results of that surgery in terms of visual outcome and post-operative complications.

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6 Appendix – publications arising

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