Anti-VEGF Treatment for Diabetic Macular Oedema: Clinical and Laboratory Insights

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A thesis submitted to the University of Sydney in fulfillment of the requirements for the degree of Doctor of Philosophy

Save Sight Institute
Faculty of Medicine
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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Bobak Bahrami

May 2018

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

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AUTHOR ATTRIBUTION STATEMENT

This thesis is presented as a thesis by publication. All material presented in this thesis are reproduced from manuscripts identified at the start of each chapter that were drafted by the candidate unless otherwise stated. The candidate confirms that the most significant contribution to each manuscript included in this thesis is by him.

Under the guidance of Associate Professor Andrew Chang the candidate generated hypotheses, collected and graded the data, and performed analyses for the clinical trial that comprise the third through to the sixth chapters of this thesis. The candidate performed the systematic literature review comprising the second chapter of this thesis. Dr. Timothy Schlub supervised the analyses performed by the candidate and offered statistical advice regarding more refined analyses for the third chapter of this thesis. Dr. Kehui Luo provided additional statistical guidance in the fourth chapter of this thesis. Dr. Tunde Peto provided advice regarding and confirmed the grading of ultrawidefield imaging in chapter five.

Furthermore, for the seventh chapter of this thesis, the candidate designed, planned, executed and analysed all experiments under the guidance of Dr. Weiyong Shen and Prof. Mark Gillies.

In addition to the five published peer-reviewed publications and two manuscripts currently under external review, the candidate presented research for this thesis at two national and three international conferences.

SUPERVISOR'S STATEMENT

As the primary supervisor for the candidate and corresponding author on the manuscripts contained in this thesis, I agree with the author attribution statement and acknowledge that the candidate's contribution was significant to warrant inclusion in this thesis.

Andrew Chang

Clinical Associate Professor, Sydney Medical School

List of Abbreviations

BCVA	Best corrected visual acuity
BRB	Blood retinal barrier
CI	Confidence Interval
CMT	Central macular thickness
DMO	Diabetic macular oedema
DR	Diabetic retinopathy
DRIL	Disorganisation of the inner retinal layers
DRCR.net	Diabetic retinopathy clinical research network
ELM	External Limiting Membrane
ERM	Epiretinal Membrane
ETDRS	Early treatment of diabetic retinopathy study
FA	Fluorescein angiography
HbA1c	Glycated haemoglobin
IA	Imaging artifacts
IOL	Intraocular lens
IOP	Intraocular pressure
IRF	Intraretinal fluid
ISe	Inner segment ellipsoid
nAMD	Neovascular age-related macular degeneration
OCT	Optical coherence tomography
PDR	Proliferative diabetic retinopathy
PlGF	Placenta growth factor
PVD	Posterior vitreous detachment

PRP	Panretinal photocoagulation
RPE	Retinal pigment epithelium
SD	Standard deviation
SE	Standard error
SRF	Subretinal fluid
STZ	Streptozotocin
UWFA	Ultrawidefield fluorescein angiography
VEGF	Vascular endothelial growth factor
VMA	Vitreomacular adhesion
VMT	Vitreomacular traction

ABSTRACT

Background/Aim:

Diabetic retinopathy (DR) is a leading cause of vision impairment, characterised by vascular damage and neurodegeneration. Anti vascular endothelial growth factor (VEGF) drugs have revolutionised the management of the most common cause of vision impairment in DR, diabetic macular oedema (DMO). These drugs have been shown to restore vision in DMO and to induce regression of vascular changes in DR. Despite anti-VEGF therapy, a proportion of patients may have persistent DMO. The aim of the work detailed in this thesis is to investigate the effect of switching therapy between two anti-VEGF drugs for persistent DMO and to assess the potential effects of anti-VEGF drugs in modulating neurodegeneration in DR through production of neurotrophic factors.

Methods:

A prospective, single-arm, open-label clinical trial of patients with persistent DMO despite prior treatment with bevacizumab was conducted. Patients were switched in therapy to aflibercept and reviewed every 4 weeks for 48 weeks. Primary outcomes were change in best-corrected visual acuity (BCVA) and central macular thickness (CMT). Secondary functional and anatomical outcomes included microperimetry, quality of life, ultrawidefield photography and fluorescein angiography (FA). Diabetic conditions were simulated *in vitro* using ARPE-19 cell-line culture. Once conditions were established, production of neurotrophic factors was quantified using enzyme-linked immunosorbent assay

(ELISA) under normal and simulated diabetic conditions with and without the addition of anti-VEGF drugs.

Results:

There was a significant improvement in primary visual and anatomical outcomes. Segmentation and fixation artifacts on automated OCT calculations were increased in the presence of DMO. Peripheral ischaemia identified on FA was associated with a poorer baseline vision and greater vision gain. Microperimetry outcomes correlated with objective and subjective vision outcomes. There was downregulation of pigment epithelium derived factor (PEDF) expression in hypoxic states in the in vitro model compared to control. In the absence of hypoxia, the addition of anti-VEGF drugs all led to a significant downregulation of PEDF. Brain derived neurotrophic factor (BDNF) secretion was downregulated in high glucose states and upregulated in hypoxia. Placental growth factor (PlGF) was not detected as secreted by ARPE-19 as measured by ELISA.

Conclusions:

Intravitreal aflibercept was effective in improving anatomical and visual outcomes among patients with incomplete response to intravitreal bevacizumab. Peripheral ischaemia may be an important biomarker to response in patients with persistent DMO. Microperimetry may provide important information about subjective visual function not well assessed with visual acuity. Neurotrophic factor secretion may be effected by the diabetic state, having consequences for long-term vision outcomes.

Peer-Reviewed Publications

Chapter 1

- **Bahrami B**, Hong T, Gilles MC, Chang A. Anti-VEGF Therapy for Diabetic Eye Diseases. Asia Pac J Ophthalmol (Phila). 2017 Nov-Dec;6(6):535-545.

- **Bahrami B**, Zhu M, Hong T, Chang A. Diabetic macular oedema: pathophysiology, management challenges and treatment resistance. Diabetologia. 2016 Aug;59(8):1594-608.

- Chang AA, Hong T, Ewe SY, **Bahrami B**, Broadhead GK. The role of aflibercept in the management of diabetic macular edema. Drug Des Devel Ther. 2015 Aug 6;9:4389-96.

Chapter 2

- **Bahrami B**, Hong T, Zhu M, Schlub TE, Chang A. Switching therapy from bevacizumab to aflibercept for the management of persistent diabetic macular edema. Graefes Arch Clin Exp Ophthalmol. 2017 Jun;255(6):1133-1140.

Chapter 3

- **Bahrami B**, Ewe S, Hong T, Zhu M, Ong G, Luo K, Chang A. Influence of retinal pathology on the reliability of macular thickness measurement: a comparison between optical coherence tomography devices. Ophthalmic Surg Lasers Imaging Retina. 2017 Apr 1;48(4):319-325.

Manuscripts submitted and under review

Chapter 3

- **Bahrami B**, Hong T, Schlub TE, Chang A. Aflibercept for persistent diabetic macular edema: 48 week outcomes. Retina. *Under review Feb 21 2018*

Chapter 6

- **Bahrami B**, Nair R, Spooner K, Hong T, Chang A. Correlation of functional and morphological retinal impairment in patients with persistent diabetic macular edema. Graefe's Archive for Clinical and Experimental Ophthalmology. *Under review Apr 29 2018*

Conference Presentations and Published Abstracts

RANZCO 47th Annual Scientific Conference: Wellington, New Zealand, 2015

- **Bahrami B**, Ewe S, Hong T, et al. The Efficacy of Aflibercept in the Management of Treatment-Resistant Diabetic Macular Oedema. Clin Exp Ophthalmol. 2015 Oct; 43:16-33.
- Ewe SYP, **Bahrami B**, Zhu M, Hong T, Ong GJM, Chang A. A Comparison of Macular Thickness Measurements Across Three Different Spectral-Domain Optical Coherence Tomography (SD-OCT) Machines. Clin Exp Ophthalmol. 2015 Oct; 43 (S1):79-123.

RANZCO 48th Annual Scientific Conference: Melbourne, Australia, 2016

- **Bahrami B**, Nair R, Hong T, Chang A. Effect of aflibercept on diabetic retinopathy severity in patients with treatment-resistant diabetic macular oedema: 12 month outcomes. Clin Exp Ophthalmol. 2016 Nov; 44:80-140.
- Nair R, **Bahrami B**, Spooner K, Hong T, Chang A. Assessing changes in macula microperimetry among patients with treatment resistant diabetic macular oedema switched to intravitreal aflibercept over 12 months. Clin Exp Ophthalmol. 2016 Nov; 44 (S1):80-140.

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- **Bahrami B**, Zhu L, Zhang T, Zhu M, Chang A, Gillies MC, Shen W. Effects of anti-VEGF drugs on human retinal pigment epithelium under oxidative stress

Section 1: Background and Literature Review

Chapter 1: Anti-VEGF drugs for Diabetic Eye Diseases

The management of diabetic retinopathy has seen a paradigm shift through the identification of vascular endothelial growth factor (VEGF) as a key mediator of disease. The introduction of anti-VEGF drugs has subsequently revolutionised outcomes in this potentially blinding disease.

This chapter summarises the role of anti-VEGF drugs in the management of diabetic eye diseases, presenting long-term findings from key randomised clinical trials and discussing the future role of VEGF-targeted therapy. The concept of refractory disease is introduced which will be explored in more depth in Chapter 2.

The material presented in this chapter has been published in peer review literature as, and are reproduced from:

- **Bahrami B**, Hong T, Gilles MC, Chang A. Anti-VEGF Therapy for Diabetic Eye Diseases. Asia Pac J Ophthalmol (Phila). 2017 Nov-Dec;6(6):535-545.

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ABSTRACT

Diabetic retinopathy (DR) is a leading cause of vision impairment and blindness in the working-age population. The identification of vascular endothelial growth factor (VEGF) as a key mediator in the pathogenesis of DR has revolutionised the management of this vision-threatening disease. There is now strong evidence supporting intravitreal anti-VEGF therapy as first line in the management of sight-threatening diabetic macular oedema (DMO), as well as a growing body of evidence to support the use of anti-VEGF drugs for proliferative DR. This chapter summarises the role of VEGF in DR, the evidence for anti-VEGF therapy, safety considerations and, the future of anti-VEGF therapy for the management of DR.

BACKGROUND

Diabetic retinopathy (DR) is a leading cause of vision impairment, affecting 93 million people worldwide. [1] Of these, 28 million have vision-threatening DR. Vision loss in DR is most commonly due to diabetic macular odema (DMO), but may also be a consequence of complications of proliferative DR (PDR), such as vitreous hemorrhage from neovascularization, tractional retinal detachment or neovascular glaucoma.

An improved understanding of the complex pathophysiology of DR has identified vascular endothelial growth factor-A (VEGF) as a key mediator of the progression to advanced disease. [2, 3] Development of drugs which target VEGF have revolutionised the management approach in DMO and have an expanding growing role in the management of DR. These anti-VEGF drugs have been reported to be safe and effective through multiple clinical trials. Despite their efficacy, there are a proportion of patients who have an incomplete response to therapy. Future strategies to manage DR include alternate methods of blocking the VEGF pathway with increased efficacy and reduced number of treatments.

PATHOPHYSIOLOGY OF DIABETIC RETINOPATHY

The mechanisms resulting in the development and progression of DR are multifactorial, complex and incompletely understood. Whilst primarily thought of as a microvasculopathy, there is increasing evidence to suggest neuronal and glial dysfunction are consequences of DR independent of vascular damage. [4, 5] Consequently, the pathogenesis of DR should consider the interactions of neuronal, glial, and vascular cells as part of a neurovascular unit affected. [6]

Important systemic risk factors for DR include duration of diabetes, glycemic control, type of diabetes and hypertension. [1, 7, 8] Hyperglycemia is a key component in the development of DR and is thought to lead to alteration of biochemical pathways in the retina, resulting in inflammation and oxidative stress. [9-14] Cytokines such as interleukin (IL)-6, IL-1 beta, tissue necrosis factor-alpha and monocyte chemoattractant-1 are upregulated as part of this response, as are angiogenic factors such as angiopoetin-2, erythropoietin and VEGF. [14, 15] These secreted factors lead to blood-retinal barrier (BRB) breakdown and increased permeability of retinal vessels resulting in DMO and to neovascularization, the hallmark of PDR. These pathways will be reviewed in further depth in Chapter 2.

Of the cytokines and growth factors upregulated in DR, VEGF has been identified to play a critical role. There are five members of the VEGF family in humans: VEGF-A (commonly referred to as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). [16]

VEGF-A is a 45 kDa heparin-binding homodimeric glycoprotein and is secreted by glia, ganglion cells, endothelial cells, astrocytes and the retinal pigment epithelium (RPE). [17, 18] This factor has essential physiological roles in vascular development and important roles in neuronal survival. There are four main isoforms of VEGF-A that bind and activate the tyrosine kinase VEGF receptor (VEGFR)-1 and VEGFR-2, which are both mainly expressed on the cell surface of the vascular endothelium. VEGFR-2 is thought to be responsible for the pathological mitogenic and microvascular permeability effects of VEGF-A. [19, 20] Levels of intravitreal VEGF-A are strongly correlated with advancing DR and DMO. [2, 21]

The other members of the VEGF family have less important roles in vascular development but may play a role in DR. PlGF binds to VEGFR-1 and produces transphosphorylation of VEGFR-2, amplifying VEGF-A driven angiogenesis and BRB breakdown through VEGFR-2. [22] In vitro and in vivo studies support the role of PlGF in DR. [23] Exogenous PlGF added to human RPE culture and injected into rat eyes has been shown to impair outer BRB function. [24] PlGF knockout in an Akita mouse model of diabetes has been shown to prevent DR. [25] Higher vitreous levels of PlGF are found with increasing levels of retinal ischemia seen in advanced DR. [26]

There is limited evidence to suggest that VEGF-B, which also binds to VEGFR-1, is involved in the pathogenesis of DR. VEGF-B overexpression in mice *via* gene transfer resulted in increased choroidal and retinal neovascularization. [27] However, levels of VEGF-B in vitreous fluid of patients with PDR are not raised

compared to non-diabetic controls. [28] It has been reported that VEGF-B prevents hyperglycemia-induced retinal apoptosis. [29]

VEGF-C, which binds to both VEGFR-2 and VEGFR-3, has important roles in adult angiogenesis and lymphangiogenesis. VEGF-C expression is increased in diabetic retina and in vitro has been shown to potentiate the angiogenic effects of VEGF-A on VEGFR-2. [30] Blocking VEGF-A in the retina may lead to compensatory upregulation of VEGF-C, which will in turn compensate for reduced signaling through VEGFR-2. [31] Single nucleotide polymorphisms in the VEGF-C gene have been associated with presence of DR and DMO in white patients with diabetes, further enhancing the evidence that VEGF-C may influence the development and progression of DR. [32]

EVOLUTION OF THERAPIES FOR DIABETIC RETINOPATHY

Strategies for managing sight-threatening DR have evolved over the past four decades. Well-established therapies, such as laser photocoagulation and intravitreal corticosteroid therapy, may indirectly affect the VEGF pathway and signaling.

Laser photocoagulation

Retinal photocoagulation revolutionised the management of both PDR and DMO following landmark clinical trials in the 1970s and 1980s. The Diabetic Retinopathy Study (DRS) established that panretinal photocoagulation (PRP) could reduce the rates of severe vision loss in PDR by more than 50% over a period of two years. [33] The destruction of photoreceptors in areas of hypoxia and subsequent reduced oxygen consumption is believed to reduce the production of VEGF driving neovascularization. [34] Levels of intravitreal VEGF are reduced following PRP for PDR, supporting this hypothesis. [2]

The Early Treatment Diabetic Retinopathy Study (ETDRS) demonstrated focal/grid macular laser photocoagulation could reduce the rates of vision loss in in clinically significant DMO by half. [35] Whilst the mechanisms of focal laser are also unclear, it is hypothesised that laser interaction with the RPE alters the expression of cytokines such as pigment epithelium derived factor (PEDF), a counter-regulator of VEGF. [36-40]

These laser procedures are not without associated risks and side effects, including loss of the peripheral visual field, pain during the procedure, severe vision loss if the fovea is targeted and rupture of Bruch's membrane. [40] Alternate laser therapy for DMO is discussed in further depth in Chapter 2.

Corticosteroid therapy

Corticosteroids were the initial intravitreal pharmacotherapy studied for the management of DMO. [41, 42] These drugs inhibit the expression and action of cytokines, inhibit leukocyte recruitment and maintain the BRB through enhancement of endothelial cell tight junctions. [15, 43, 44] They may also modulate VEGF gene expression or modulate signaling downstream from the VEGFR-2. [45, 46]

Corticosteroids are less widely utilised for primary management of DMO due to their ocular side effect profiles, which includes raised intraocular pressure and accelerated cataract formation. [47, 48] However, they remain an important treatment modality for a disease that can be challenging to manage. The role of these drugs will be discussed in further depth in Chapter 2.

ANTI-VEGF DRUGS

The three most widely used anti-VEGF drugs are bevacizumab (Avastin, Genentech, San Francisco, CA, USA), ranibizumab (Lucentis, Genentech, San Francisco, CA, USA) and aflibercept (Eylea, Regeneron, Tarrytown, NY, USA).

Pegaptanib sodium (Macugen, Eyetech Pharmaceuticals, Cedar Knolls, NJ, USA) is an aptamer that selectively binds the VEGF-A 165 isoform and has some efficacy in the management of DMO and PDR. [49, 50] Use of pegaptanib in DR is not widespread due to access and availability of alternate and perhaps more effective anti-VEGF agents. These drugs are summarised in Table 1.1.

Bevacizumab is a 149 kDa, full-length monoclonal antibody to all isoforms of VEGF-A. This drug was developed for its anti-angiogenic effects in neoplastic disease and proved revolutionary as an adjunct to chemotherapy in prolonging survival in metastatic cancer. [51] It is not formulated for intravitreal use and consequently is most commonly prepared by compounding pharmacies.

Ranibizumab is a 48 kDa monoclonal antibody fragment that binds to all isoforms of VEGF-A. It lacks the IgG Fc segment that full-length antibodies have, and consequently, it has the lowest molecular weight of these three inhibitors. The smaller size of this drug provides a potential advantage in terms of retinal penetration. [52] The absence of an Fc segment avoids the theoretical interaction of ranibizumab with Fc receptors on immune cells, which could lead to cytotoxicity. [53]

Aflibercept is a 115 kDa fusion protein, combining the second binding domain of VEGFR-1 and the third binding domain of VEGFR-2. These are fused to the Fc segment of human IgG1 and the molecule acts as a decoy receptor, binding all isoforms of VEGF-A, VEGF-B and PlGF. [54] Aflibercept may also bind galectin-1, a protein that is physiologically expressed throughout the retina but upregulated in PDR. [55, 56] It has angiogenic effects and protein levels are elevated in eyes with PDR, with no correlation to VEGF-A levels. [56, 57]

Pharmacokinetics

Pharmacokinetics of intravitreal aflibercept (2.0 mg) have been compared to that of ranibizumab (0.5 mg) and bevacizumab (1.25 mg) in a study of 56 patients with neovascular age-related macular degeneration (nAMD). [58] Systemic exposure to aflibercept was higher than that of ranibizumab, with maximum serum concentration five- and seven-fold greater after the first dose and third doses respectively and minimum serum concentration 37- and 53-fold greater after the first and third doses, respectively. Additionally, there was accumulation of both aflibercept and bevacizumab after three intravitreal injections but not ranibizumab. Aflibercept was also the most potent of these three drugs in reducing plasma-free VEGF with levels undetectable from 3 hours post-dose to greater than 1 week post-dose. It is postulated that the Fc fragment present in both the bevacizumab and aflibercept molecules extends their serum half-life, accounting for these differences. [59] The clinical significance of this is, however, yet to be elucidated.

Drug clearance from the vitreous of the eye occurs across the retina through the choroidal circulation and through diffusion into the anterior chamber to exit via the trabecular meshwork. [60] There are no published reports about the intravitreal half-life of aflibercept in humans; however, there have been two rabbit models estimating this through immunoassay and radioisotope imaging techniques as between 4.5 days and 4.58 days. [61] Given the anatomic and biological differences between humans and rabbits, the half-life is expected to be longer in humans, suggested to be 9 days based on its intermediate molecular size between ranibizumab and bevacizumab. [60] Further mathematical modeling suggests that aflibercept is able to maintain significant intraocular binding activity up to 10–12 weeks after a single injection.

Table 1.1: Summary of different anti-VEGF drugs

Drug name	Structure	Mechanism of action	Molecular Size	Intravitreal half-life	US FDA Approved Indications
Pegaptanib (Macugen, EyeTech Pharmaceuticals)	Pegylated RNA aptamer	Binds VEGF-165 isoform of VEGF-A	50 kDa	10 days	nAMD
Bevacizumab (Avastin, Genentech)	Full length monoclonal antibody to VEGF-A	Binds all VEGF-A isoforms	149 kDa	7.0 days *	Metastatic colorectal cancer, non-small cell lung cancer, glioblastoma, metastatic renal cell carcinoma, cervical cancer, ovarian, fallopian tube or peritoneal cancer
Ranibizumab (Lucentis, Genentech)	Monoclonal antibody fragment to VEGF-A	Binds all VEGF-A isoforms	48 kDa	2.5 days *	nAMD, RVO, DMO, mCNV, DR
Aflibercept (Eylea, Regeneron)	Fusion protein of binding domains of VEGFR-1 and -2, contains Fc portion	Decoy receptor for all isoforms of VEGF-A, VEGF-B and PlGF	115 kDa	3.6 days *	nAMD, RVO, DMO

nAMD= neovascular age related macular degeneration, RVO= retinal vein occlusion, DMO= diabetic macular oedema, DR=diabetic retinopathy, mCNV=myopic choroidal neovascularisation

* from rabbit animal model data [62]

ANTI-VEGF THERAPY FOR DIABETIC MACULAR OEDEMA

Landmark clinical trials have demonstrated the efficacy of intravitreal bevacizumab, ranibizumab and aflibercept in the management of DMO. The results of these trials are summarised in Table 1.2.

Anti-VEGF vs. Laser

The Bevacizumab or Laser Treatment (BOLT) randomised trial compared the effect of 1.25mg bevacizumab to macular laser as a control over a two-year period. [63] Bevacizumab was given as three loading doses six weeks apart and subsequently on an as needed basis every six weeks. Laser was administered at baseline with retreatment every 16 weeks as needed. There was a significant improvement in vision and a non-significant reduction in central macular thickness (CMT) in the bevacizumab group at one and two years.

Ranibizumab has been evaluated against laser in the RISE/RIDE, RESTORE, READ-2, REVEAL and Diabetic Retinopathy Clinical Research network (DRCR.net) Protocol I studies. [64-68] All of these trials demonstrated superiority of ranibizumab to laser for vision gains and improvement in CMT after a 12 month period, which was maintained over 36 months in the RISE/RIDE, RESTORE and READ-2 studies and over 5 years for the Protocol I study. Additionally, the Protocol I study demonstrated no benefit of prompt laser as an adjunct to intravitreal ranibizumab therapy with some suggestion that this could in fact limit visual gain at 3 years. [69]

Aflibercept was assessed initially in the phase II DA VINCI study. Four different dosing regimens were compared and demonstrated no anatomical or visual difference between 4-weekly and 8-weekly administration of the drug, following a loading dose. [70] All aflibercept arms of the study were superior to laser only treatment. The phase III VISTA/VIVID studies with the efficacy similar between 4-weekly and 8-weekly dosing, a benefit, which was maintained over a three year, follow-up. [71]

Baseline glycemic control does not seem to have a bearing on response to therapy with anti-VEGF drugs as reported in the RISE/RIDE and VISTA/VIVID studies. [72, 73] This may contrast with laser therapy, which had poorer visual and anatomical outcomes with an increasing glycosylated hemoglobin level in the VISTA/VIVID studies. [73]

Dose Variation

The 0.3mg and 0.5mg arms of RISE/RIDE had similar vision and anatomical outcomes at 3 years of follow up. [64] The READ-3 study compared the effects of 0.5mg and 2.0mg ranibizumab administered monthly for six doses and subsequently on an as needed basis. [74] The mean visual gain after two years of therapy was greater in the 0.5mg arm than the 2.0mg arm (11.1 vs. 6.8 letters, $p=0.02$) with no anatomical difference noted. [74] The results of these studies suggest that peak of the dose-response curve for ranibizumab is at least at 0.5mg and perhaps even 0.3mg.

Similarly, the phase II DRCR.net Protocol H study demonstrated no meaningful anatomical or visual differences between 1.25mg and 2.5mg of bevacizumab administered as two doses six weeks apart. [75] These results were also validated in a randomised controlled trial, which also found no differences between 1.25mg and 2.5mg doses. [76] Response to bevacizumab in this study was more marked in treatment naïve patients compared to those with previous therapy, regardless of dose used. [76]

Anti-VEGF vs. corticosteroid

The DRCR.net Protocol I study compared combined 4mg intravitreal triamcinolone (Trivaris, Allergan, Inc., Irvine, CA) and laser to 0.5mg ranibizumab. [47] All intravitreal drugs were given as three loading doses four weeks apart and subsequently on an as needed basis every four weeks. After two years of treatment, visual improvement was significantly better in the ranibizumab arms (7±13 letter change, $p=0.01$ and 10±15 letter change, $p=0.0001$) but not the triamcinolone arm (0±21 letter change, $p>0.05$) compared to laser control. However, subgroup analyses of pseudophakic eyes showed similar improvements with ranibizumab groups as in the triamcinolone group (8±12 and 7±9 vs 8±9 letter change respectively) at one-year. Half of participants in the triamcinolone arm experienced a significant elevation in IOP compared with 9% and 11% in the ranibizumab and laser arms respectively. Additionally, 59% of participants in the in the triamcinolone arm required cataract surgery compared with 14% in both the ranibizumab and laser arms.

The BEVORDEX study compared bevacizumab to a slow-release intravitreal dexamethasone implant (DEX implant; Ozurdex, Allergan Inc., Irvine, CA). [48] Treatments were given on an as needed basis, every 4 weeks in the bevacizumab arm and every 16 weeks in the DEX implant arm. After two years of treatment, there was no significant difference in vision gain or reduction in CMT between the two groups. Eyes randomised to DEX implant required fewer injections in both the first and second year of treatment compared to bevacizumab (mean 2.8 vs. 9.1 injections in the first year, 2.2 vs. 4.8 in the second year). Subgroup analysis demonstrated similar visual outcomes for pseudophakic eyes randomised to DEX implant compared with bevacizumab, with worse outcome for phakic eyes. Topical ocular hypotensives to manage an intraocular pressure rise were required in 22% of patients in the DEX implant arm compared to none in the bevacizumab arm. [48]

Comparison of different anti-VEGF drugs

The DRCR.net Protocol T compared the safety and efficacy of 1.25mg bevacizumab, 0.3mg ranibizumab and 2.0mg aflibercept for DMO. [77] Drug was administered every four weeks unless vision was 20/20 or better, CMT was below threshold and there was no worsening or improvement in response to the past two injections. The two-year results from this study showed that there was no overall difference between the three agents in terms of visual outcome. [78] Both ranibizumab and aflibercept arms had improved reduction of the CMT compared to the bevacizumab arm. There was no significant difference in injection number over the two-year study period between the three groups.

Aflibercept had improved visual outcomes to both bevacizumab and ranibizumab at one-year follow up in patients with poorer baseline visual acuity (less than 69 letters), with no difference between bevacizumab and ranibizumab. Gains at one year were 18.9 letters with aflibercept, 11.8 letters with bevacizumab and 14.2 letters with ranibizumab ($p<0.001$ aflibercept vs. bevacizumab, $p=0.003$ aflibercept vs. ranibizumab, $p=0.21$ for ranibizumab vs. bevacizumab). However, the difference between aflibercept and bevacizumab was the only significant comparison at the two-year follow up point (mean 18.1 vs. 13.3 letter gain, $p=0.02$). Patients randomised to aflibercept were less likely to require rescue laser treatment than either of the other groups. [78]

Delaying treatment

Delaying anti-VEGF treatment in patients with DMO by more than 12 months appears to result in poorer long-term outcomes. Patients initially randomised to laser in the RESTORE trial who were switched to ranibizumab after 12 months had similar visual and anatomical outcomes at 36 months of follow up compared to patients initially receiving ranibizumab. [66] However, in the RISE/RIDE and VISTA/VIVID studies, patients who were initially randomised to laser treatment and received delayed anti-VEGF after 24 months did not achieve the same degree of vision gain as those initially randomised to the drug arms of those trials. [64, 71]

Table 1.2: Key randomised controlled trials of anti-VEGF drugs for the management of diabetic macular oedema

Study Name	Population size (study eyes)	Follow-up duration (months)	Inclusion Criteria	Treatment arms	Mean Vision Change (letters)	Mean CMT Change (µm)	Proportion of eyes with regression of DR
BOLT [63]	80	24	VA 20/40 to 20/320, CMT >270µm	Bevacizumab (1.25mg q6)	8.6±9.1	-146±171	31.4%
				Laser therapy	-0.5±10.6	-118±112	20%
RISE [64]	377	36	VA 20/40 to 20/320, CMT ≥275 µm	Ranibizumab (0.3mg q4)	11.0±12.9	-261.2±196.2	38.5%
				Ranibizumab (0.5mg q4)	14.2±12.8	-269.1±178.9	40.9%
				Sham *	4.3±14.9	-200.1±215.6	24.3%
RIDE [64]	382	36	VA 20/40 to 20/320, CMT ≥275µm	Ranibizumab (0.3mg q4)	11.4±16.3	-261.8±180.8	39.3%
				Ranibizumab (0.5mg q4)	10.6±12.9	-266.7±207.8	37.8%
				Sham *	4.7±13.3	-213.2 ±193.5	23.4%
RESTORE [66]	208	36	VA 20/32 to 20/160	Ranibizumab (0.5mg q4)	8.0±1.1 (SE)	-142.1	14.8%
				Ranibizumab (0.5mg q4) + Laser	6.7±1.1 (SE)	-145.9	28.3%
				Laser alone**	6.0±1.1 (SE)	-142.7	16.0%
READ-2 [67]	74	36	VA 20/40 to 20/320 CMT ≥250 µm	Ranibizumab (0.5mg q8 after two loading q4 doses)	10.3	-132	Not reported
				Ranibizumab (0.5mg q12) + Laser	8.9	-243	
				Laser alone ***	1.4	-163	
REVEAL [68]	396	12	VA 20/32 to 20/160	Ranibizumab (0.5mg q4)	6.6±7.7	-134.6	Not reported
				Ranibizumab (0.5mg q4) + Laser	6.4±10.7	-171.8	
				Laser alone	1.8±8.3	-57.2	

Protocol I [65]	235	60	VA 20/32 to 20/320	Ranibizumab (0.5q4) + Prompt laser	8±13	-167±168	Not reported
				Ranibizumab (0.5mg q4) + Deferred laser	10±13	-165±165	
DA VINCI [70]	176	12	VA 20/40 to 20/320 CMT ≥250	Aflibercept (0.5mg q4)	11.0	-165.4	40%
				Aflibercept (2mg q4)	13.1	-227.4	31%
				Aflibercept (2mg q8)	9.7	-187.8	64%
				Aflibercept (2mg PRN)	12.0	-180.3	32%
				Laser	-1.3	-58.4	12%
VISTA [71]	461	36	VA 20/40 to 20/320	Aflibercept (2mg q4)	10.5	-200.4	29.9%
				Aflibercept (2mg q8)	10.4	-190.1	34.4%
				Laser****	1.4	-109.8	20.1%
VIVID [71]	404	36	VA 20/40 to 20/320	Aflibercept (2mg q4)	10.3	-215.2	44.3%
				Aflibercept (2mg q8)	11.7	-202.8	47.8%
				Laser****	1.6	-122.6	17.4%
BEVORDEX [48]	68	24	VA 20/40 to 20/400 CMT >250μm	Bevacizumab (1.25mg q4 PRN)	9.6 (95% CI 6.9-12.3)	-122 ^	Not reported
				Dexamethasone (0.7mg q16 PRN)	6.9 (95% CI 2.7-11.1)	-187 ^	
Protocol T [78, 79]	609	12	VA 20/32 to 20/320	Aflibercept (2mg q4)	13.3±11.1	-169±138	24.8%
				Bevacizumab (1.25mg q4)	9.7±10.1	-101±121	22.1%
				Ranibizumab (0.3mg q4)	11.2±9.4	-147±134	31.0%

DR=diabetic retinopathy, VA=visual acuity, CMT=central macular thickness, q(x)=every (x) weeks, PRN=as required, SE=standard error, CI=confidence interval. * Sham patients eligible for switch to active therapy at month 24. **Laser group eligible for 0.5mg q4 ranibizumab from month 12. ***Laser group eligible for 0.5mg ranibizumab from month 6. **** Laser group eligible for 2mg aflibercept q8 from week 24. ^ 12 month anatomical outcome, 24 month thickness not quantitatively

ANTI-VEGF FOR REFRACTORY DMO

Despite the efficacy of anti-VEGF drugs for managing DMO, there are a proportion of patients who have an incompletely respond to therapy. From Protocol T, the rates of meeting failure criteria between weeks 24 and 1 year for aflibercept, bevacizumab and ranibizumab were 27%, 41% and 37% respectively. [77]

Incomplete response to therapy poses a clinical challenge, and several strategies have been proposed to manage these patients including switching to corticosteroid drugs, increasing dose of anti-VEGF drug, combination therapy and switching between anti-VEGF drugs.

The REEF study evaluated patients who had incomplete response to bevacizumab and switched these patients to 0.5mg ranibizumab. [80] The dose of ranibizumab was increased to 2.0mg if there was residual oedema or less than 10% improvement in CMT after three months of therapy. After switch 76% of participants had anatomical and visual improvement with 0.5mg ranibizumab. Of the remainder of the patients who subsequently had a dose increase (n=6) 50% had further anatomical improvement.

Other retrospective series have shown visual and CMT improvement in switching therapy from bevacizumab to ranibizumab for patients with incomplete response. [80-83] Similarly, switching from either bevacizumab or ranibizumab to aflibercept may have a benefit in improving CMT. [83-86]

EFFECT OF ANTI-VEGF ON THE SEVERITY OF DIABETIC RETINOPATHY

Trials of anti-VEGF drug for DMO have shown additional benefit in leading to an improvement in DR severity score (DRSS). DRSS is based on clinically observable signs such as hemorrhages, microaneurysms and intraretinal microvascular abnormalities. Reversal of these changes supports the importance of VEGF in the pathogenesis of the disease and suggests that reversibility of vascular damage may be possible.

The two-year outcomes of RISE/RIDE trials showed the cumulative probability of progression of DR was 11.2-11.5% in the ranibizumab arms compared to 33.8% in the sham treatment arm. [87] Median DRSS remained unchanged in the sham arm but improved by two steps in both ranibizumab treatment arms. Similarly, in the 148-week analysis of the VISTA/VIVID trials, 17.4-20.1% of patients receiving laser had an improved DRSS compared with 29.9-47.8% of patients receiving aflibercept when censoring for rescue treatment. [71]

There were greater improvements in DRSS in the ranibizumab and aflibercept arms in the Protocol T study, compared to the bevacizumab arm at 12 months. [79] This difference was not maintained at two years of follow up, however, improvements were associated with a higher number of intravitreal injections. DRSS was more markedly improved in the aflibercept arm compared to both ranibizumab and bevacizumab in patients with PDR. This may be explained by the effect of aflibercept on inhibiting galectin-1 as previously discussed. [56]

Anti-VEGF for PDR

Early studies observed that bevacizumab may be effective as an adjunct or an alternative to PRP for the regression of PDR. [88] Subsequently, two randomised clinical trials have reported the efficacy of anti-VEGF drugs to PRP for the management PDR, summarised in Table 1.3.

The DRCR.net Protocol S was a two-year study of patients with treatment-naïve, high-risk PDR randomised to receive either 0.5 mg ranibizumab as frequently as every four weeks or PRP completed in one to three visits. [89] Patients who had concurrent DMO were also recruited to this study. At two years follow-up, ranibizumab had non-inferior outcomes to PRP in terms of visual acuity. Additionally, there was less development of DMO, peripheral field loss and need for vitrectomy in the ranibizumab arm. This study is collecting efficacy and safety data for five years until 2018. Safety evaluation was challenging in these patients as half of the participants in the PRP group subsequently received ranibizumab for DMO so there was no control group without ranibizumab exposure. [90]

CLARITY was a randomised controlled trial comparing 2.0mg intravitreal aflibercept to PRP for the management of high- and low-risk PDR. [91] Patients with DMO were excluded to avoid confounding of visual outcomes and patients previously treated with PRP were included. Patients randomised to the aflibercept arm were given three loading injections four weeks apart followed by

injections every four weeks on an as needed basis. The patients who received aflibercept had improved vision outcomes at the 52-week primary outcome time point.. At the 52-week point, 30 patients in the PRP group (29%) had developed DMO compared to 12 patients (11%) of the aflibercept group. Total regression of neovascularization favored the aflibercept group, as did the total DR severity.

The cost of repeated anti-VEGF drug administration is an important consideration in the management of PDR, with PRP being a highly cost effective, once-off therapy. [92] The cost-benefit of such treatment may not be viable for ranibizumab for cases of PDR alone but may be acceptable for cases with concurrent DMO. [93] There are other important considerations such as the effect of withdrawing anti-VEGF therapy, compliance and potential complications in situations of missed follow-up with intravitreal injection.

Table 1.3: Summary of key randomised controlled trials assessing anti-VEGF drugs for the management of proliferative diabetic retinopathy (PDR).

Study Name	Population size (study eyes)	Follow-up duration (months)	Inclusion Criteria	Treatment arms	Mean Vision Change (letters)	Mean CMT Change (µm)	Regression of neovascularisation at last follow up
Protocol S [89]	394	24	VA ≥20/320	Ranibizumab (0.5mg q4 PRN)	2.8 (95% CI 0.4 to 5.2)	-47 (95% CI -61 to -33)	35%
				PRP	0.2 (95% CI -1.9 to 2.3)	-3 (95% CI -15 to 9)	30%
CLARITY [91]	232	12	VA ≥20/80	Aflibercept (2mg q4 PRN after three loading q4 doses)	1.3 (0.6 SE)	-8.9 (2.3 SE)	64%
				PRP	-2.9 (0.7 SE)	24.0 (5.5 SE)	34%

VA=visual acuity, CMT=central macular thickness, q(x)=every (x) weeks, PRN=as required, SE=standard error, PRP=panretinal photocoagulation, CI=confidence interval

ANTI-VEGF FOR COMPLICATIONS OF PROLIFERATIVE DIABETIC RETINOPATHY

Vitreous Hemorrhage

Intravitreal bevacizumab may assist in PDR complicated by vitreous hemorrhage (VH) by reducing the need for vitrectomy and reducing vitreous clear up time. Vitrectomy was required in 10% of eyes that had received bevacizumab compared to 40% of those who had been managed with observation for 12 weeks in a case-control study. [94] Vitreous clear up time was 11.9±9.5 weeks in the bevacizumab group compared with 18.1±12.7 weeks in the control group (p=0.02). Thirty-one of forty patients (77.5%) received only one injection in the follow up period.

Intravitreal ranibizumab (0.5mg) was compared with saline for the management of PDR related VH in the DRCR.net Protocol N. [95] Following three intravitreal injections four weeks apart, primary outcomes were assessed after 16 weeks in this randomised controlled trial of 261 eyes. There were lower rates of recurrent VH, improved visual acuity and PRP completion rates in the ranibizumab groups. However, rates of vitrectomy were not statistically different at 16 weeks between the ranibizumab (12%) and saline (17%) groups (4% difference, 95% CI -4-13%).

Intravitreal aflibercept is being compared to prompt vitrectomy and PRP for the management of VH in PDR in the DRCR.net Protocol AB (NCT02858076). The

longer-term data from this two-year study will provide important guidance for the role of medical and surgical management of this complication of PDR.

Surgical Adjuvant

Intravitreal bevacizumab administered as a surgical adjuvant has been shown to reduce postoperative VH, reduce surgical time, lower complications, and lead to improved post-operative visual acuity in patients with PDR undergoing vitrectomy. [96-98] Ranibizumab has also been studied for this indication in two smaller studies with similar benefits. [99, 100]

Timing of delivery of pre-operative anti-VEGF drug may be important in this indication. Too short an interval may be insufficient to induce regression of neovascularization to assist in surgery. [101] Too long an interval may lead to fibrovascular contraction and be associated with tractional retinal detachment. [102] Patients randomised to receive intravitreal bevacizumab 5-10 days prior to vitrectomy had less intraoperative complications and improved visual outcomes at six months follow up compared to those patients receiving bevacizumab 1-3 days prior to vitrectomy, suggesting a longer interval may be optimal. [103]

Neovascular Glaucoma

Neovascular glaucoma (NVG) can be a complication of PDR and is associated with increased levels of intraocular VEGF. [104] Intravitreal bevacizumab has been shown to lead to regression of neovascularization of the iris and angle and

assist in controlling IOP in multiple studies of NVG, though most of these studies included etiologies other than PDR. [105]

Bevacizumab delayed the need for glaucoma surgery in a retrospective, comparative case series of 163 eyes with NVG treated with and without intravitreal bevacizumab. [106] This effect was more marked in eyes that received PRP in addition to intravitreal bevacizumab, suggesting PRP may have additional therapeutic benefit in managing NVG. Supporting this, combination of bevacizumab with PRP led to a significant reduction in intraocular pressure and regression of neovascularization in a retrospective, consecutive case-control study of 23 patients receiving either combination bevacizumab and PRP or PRP alone as treatment of NVG. [107]

Aflibercept was shown to be effective in the causing regression of neovascularization of the iris and the angle in four cases of NVG, two of which were related to PDR. [108] The PDR patients required eight injections each over a 12-month follow-up period.

SAFETY CONSIDERATIONS

VEGF has extensive physiologic roles, both locally in the eye as well as systemically. [16, 109] Consequently, disruption of these pathways through pharmacological intervention may be of concern.

Systemic Adverse Events

There has been well-documented increased risk of hypertension, [110, 111] arterial and venous thromboembolism, [112, 113] cardiac ischemia, [114] bleeding, [111, 115] and delayed wound healing in studies of anti-VEGF drugs used systemically for cancer therapy. [116] However, multiple randomised clinical trials and meta-analyses have failed to demonstrate an increase in these systemic adverse events when anti-VEGF drugs are administered as intravitreal injection compared to placebo. [117, 118]

The reported safety profile of intravitreal anti-VEGF may be confounded by the exclusion of patients with prior history of stroke or myocardial infarction from these trials. This is especially relevant in patients with diabetes who have a higher risk of cardiovascular events than that of the general population. A meta-analysis of patients with a higher baseline stroke risk receiving ranibizumab demonstrated a higher stroke risk compared with placebo in patients receive therapy for neovascular age-related macular degeneration (nAMD) (OR 7.7; 95% CI, 1.2-177). [119]

More aggressive treatment with monthly dosing of anti-VEGF drug in DMO may be associated with increased systemic adverse events. [120] Patients receiving monthly aflibercept or 0.5mg ranibizumab for 24 months had increased risk of death compared to laser (OR 2.98; 95% CI, 1.44-6.14; P = .003) having cerebrovascular accident (OR, 2.33; 95% CI, 1.04-5.22; P = .04) or vascular death (OR, 2.51; 95% CI, 1.08-5.82; P = .03) in a meta-analysis of RISE/RIDE and VISTA/VIVID data. [120]

When comparing anti-VEGF drugs, the two-year outcomes of the Protocol T study found a higher rate of non-fatal stroke and vascular death in the ranibizumab arm compared to the aflibercept arm. [78] However, the rates of these cardiovascular adverse events (12%) were higher in this study than other studies of ranibizumab (3-9%), leading the authors to concede that this finding may represent a type II error. [78, 121, 122]

Ocular adverse events

Every intravitreal injection carries the risk of endophthalmitis. A meta-analysis of 16 trials of intravitreal therapy found 52 cases from a total of 105,536 injections, a rate of 0.049%. [123] Additionally, this study found that streptococcal isolates were much higher than with intraocular surgery, suggesting that minimizing oropharyngeal droplet transmission could be a strategy for reducing rates of endophthalmitis. [123] Though rates lower than this have been reported in institutional audits, [124, 125] when considering

treatment often requires repeated injection cumulative risk, per-patient rates over two years may approach 1%. [126]

As there is no commercial intravitreal preparation of bevacizumab, special consideration is required for the safety of this drug, as it needs to be prepared by compounding pharmacies. The quality of drug can vary between compounding pharmacies. [127] Contamination during compounding can have catastrophic consequences, with several reports of endophthalmitis from contaminated batches of drug. [128] Counterfeit preparations may be associated with poorer quality control and higher rates of endophthalmitis. [129] Inadequate purification of preparations can also lead to culture negative endophthalmitis. [130]

Rhegmatogenous retinal detachment (RD) is a rare complication of intravitreal injection and is thought to occur either as a result of direct trauma during the injection procedure or disruption of the vitreous gel triggering posterior vitreous detachment. The rate of RD was 5 in 35,942 injections (0.013%), all occurring between 2 and 6 days following injection, in a multicenter case series. [131]

Increased risk of raised intraocular pressure (IOP) has been reported in eyes receiving multiple anti-VEGF injections. [132, 133] Comparing treated to untreated eyes of patients with nAMD, there were greater odds (5.75 95% CI, 1.19-27.8; P = 0.03) of experiencing an elevation in IOP greater than 5 mmHg in those who had received 29 or more injections compared with 12 or fewer injections in one study. [132] The mechanisms by which these injections can

cause raised IOP are unclear but these findings highlight the importance of monitoring the IOP of patients receiving long-term intravitreal anti-VEGF drug.

There is concern that anti-VEGF therapy may have pro-fibrotic and exacerbating effects on PDR. Several studies have shown increased fibrosis in patients who had received intravitreal bevacizumab prior to vitrectomy for PDR. [134, 135] This is thought to be the result of an angio-fibrotic switch where there is a shift to increased expression of connective tissue growth factor with VEGF blockade. [135]

Due to the physiological role of VEGF as a neurotrophic factor, there are theoretical safety concerns for long-term use of these inhibitors. [136] Sustained neutralization of VEGF has been shown to cause retinal neurodegeneration [137] and signaling through VEGFR-2 is essential for Müller cell survival. [138]

Clinical evidence in humans is scant and hard to establish, given part of the pathology of DR is neurodegeneration. However, some clinical data suggests that anti-VEGF drugs may have positive effects on the neural retina. Restoration of foveal photoreceptors, demonstrated on optical coherence tomography, has been observed in patients following 12 months of treatment with ranibizumab. [139]

Worsening of macular ischemia due to blocking of the physiological effect of VEGF is another theoretical concern and potential contraindication with administration of anti-VEGF drug. [140] There was no difference in progression of macular ischemia in analysis of the BOLT study with administration of

bevacizumab compared to laser control. [141] Besides this study, there is a lack of data on progression of macular ischemia studies in randomised trials comparing anti-VEGF drugs to a sham or laser control.

FUTURE ANTI-VEGF STRATEGIES

Future anti-VEGF strategies aim to reduce the need for frequent intravitreal injection, thus reducing the burden of treatment as well as the cumulative risk for adverse events such as endophthalmitis and retinal detachment. They may provide a management option for treatment resistant patient.

Anti-VEGF antibodies and proteins

Brolucizumab is a single chain, antibody fragment with a molecular weight of 26kDa, almost half of that of ranibizumab. The theoretical advantages of a smaller molecular are better penetration of ocular tissues, faster clearance and lower systemic exposure. Phase I/II data showed that brolucizumab was non-inferior to ranibiuzmab for the management of nAMD with a possible longer treatment interval and an acceptable safety profile. [142] Phase III studies comparing this antibody to aflibercept for nAMD (NCT02434328 and NCT02307682) will complete data collection in 2017 and are expected to serve as the basis for future trials for managing DMO.

Similar to aflibercept, conbercept (Chengdu Kanghong Biotech Co., Ltd., Sichuan, China) is a fusion protein of the second binding domain of VEGFR-1 and the third and the fourth binding domains of VEGFR-2 fused to the Fc region of human IgG. A retrospective study of 51 patients with DMO treated with conbercept with or without macular grid laser, showed an improvement in both visual and anatomical outcomes after 12 months of therapy. [143] It is currently being

evaluated against macular laser photocoagulation for DMO in a randomised controlled trial (NCT02194634).

Designed ankyrin repeat proteins (DARPins) are small, non-immunoglobulin proteins that bind target proteins with high affinity and specificity. Abicipar pegol (Molecular Partners, Zurich, Switzerland; marketed by Allergan, Dublin, Ireland). A phase I/II study of this DARPin for DMO demonstrated maximal vision improvement at 12 weeks following injection, though the rates of ocular inflammation were high amongst these patients (61%). [144] This high rate of inflammation was thought to be secondary to impurities in the preparation and future studies of abicipar corrected this. An updated preparation of abicipar pegol is under investigation in phase III randomised controlled trials for AMD in the SEQUOIA (NCT02462486) and CEDAR (NCT02462928) studies. These studies are evaluating an extended treatment interval of 12 weeks and it is expected that future phase III studies for DMO would evaluate a similar protocol.

Gene Therapy

Gene therapy targeting the VEGF pathway has been evaluated in patients with nAMD. In a phase I study, nine patients had subretinal injections of a recombinant adeno-associated vector (rAAV) encoding soluble Flt-1 (sFlt-1). [145] The protein that is transduced is the soluble form of VEGFR-1. Consequently, VEGF-A, VEGF-B and PlGF are bound in much the same way as aflibercept. No major concerns were noted in this study and many patients did

not require rescue intravitreal anti-VEGF therapy. By targeting VEGF, this therapy may provide an effective means for treating DMO and PDR.

Alternate drug delivery methods

Controlled drug delivery systems may provide a solution to the frequency for which drugs need to be administered. Anti-VEGF loaded nanoparticles have been shown to reduced diabetes-induced vascular leakage in mice. [146] These drug delivery systems enhance the half-life of a drug but also increase solubility, protect it against oxidation. Other polymers have also been trialed in animal studies and may present a more efficient method of administering treatment. [6]

Mechanical devices such as a surgically implanted pump may provide a means of delivering up to 100 programmable doses of intravitreal drug. [147] One such subconjunctivally-implanted device was loaded with ranibizumab and well tolerated in a three-month pilot study of 11 patients with DMO. [147]

SUMMARY

The pathophysiology of DR is incompletely understood. However, clinical experience with anti-VEGF drugs has shown that it is a powerful mediator of disease. The introduction of pharmacological anti-VEGF therapy has revolutionised the management of DMO although managing persistent disease is a common challenge. Anti-VEGF drugs may play an important role in the management of advanced DR and PDR in the future. Future anti-VEGF therapies for DR may utilize advanced methods of drug delivery and newer drugs may target VEGF.

Chapter 2: Diabetic Macular Oedema: Pathophysiology, Management Challenges and Persistent Disease

The overall application of anti-VEGF drugs in diabetic eye diseases was summarised in the preceding chapter. In this chapter there is a focus on the pathophysiology of diabetic macular oedema as well as the evolution in the diagnosis, prognosis and management of this condition.

The theme of refractory disease is explored further with a systematic review of the literature and a qualitative presentation of clinical data regarding the definition and management of DMO persisting after anti-VEGF treatment.

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BACKGROUND

Diabetic macular oedema (DMO) is a leading cause of vision loss and the most common cause of vision loss in diabetic patients, affecting an estimated 21 million people worldwide in 2010. [1, 148] DMO typically affects adults of a working age, having not only direct costs to the healthcare system but also indirect costs incurred by productivity lost at work. [148, 149]

The prevalence of DMO varies amongst the diabetic population, affecting 14.3% and 5.6% of type 1 and type 2 diabetic patients respectively. Duration of diabetes is a risk factor, with 3.2% of those living with diabetes for less than 10 years affected compared to 20.0% of those having diabetes for greater than 20 years. The prevalence of DMO is also higher among those with poorer HbA1C, hypertension and serum cholesterol greater than 4.0 mmol/L. [1]

The diagnosis and management of DMO was standardised by the Early Treatment of Diabetic Retinopathy Study (ETDRS) report number 1. [35] Macular focal/grid laser photocoagulation was found to halve the rate of vision loss in DMO over three years. Macular laser therapy consequently remained the standard of care for DMO for over two decades.

In recent decades, the pathophysiological pathways involved in the development of DMO have been better identified. Consequently, new classes of therapies have been studied and developed in addition to macular laser, initially intravitreal corticosteroids and subsequently anti-vascular endothelial growth factor (VEGF) drugs. Furthermore, imaging advances with optical coherence tomography

(OCT) and fluorescein angiography (FA) has allowed for improved diagnosis, assessment of prognosis and monitoring response to therapy.

Despite these advances, there remain a proportion of patients who do not adequately respond to current standard of care pharmacological therapeutic options. This phenomenon may be termed treatment-resistant DMO, however, there is no consensus as to what constitutes treatment-resistant DMO.

Indicators commonly used to measure treatment effectiveness are duration, number and response to previous treatments in conjunction with vision, central macular thickness (CMT) and residual oedema presenting within or under the retina. Although clinical trial data guides the prevalence of treatment resistance with pharmacotherapy based on these criteria, these indicators have limitations. For example, improvement in visual acuity can be dependent on baseline visual acuity, that is, there is less vision to be regained in a patient with a better presenting visual acuity. CMT values differ depending on the OCT machine used as well as the presence of pathology. [150] This will be the focus of Chapter 4 of this thesis.

In the Diabetic Retinopathy Clinical Research network (DRCR.net) Protocol I study, treatment success was defined as visual acuity equivalent to 20/20 or a CMT less than 250 microns. Approximately 75% of patients were incomplete responders to this criteria following four loading doses of ranibizumab at 16 weeks of follow-up. Despite additional laser therapy being given to all arms in this study, 40-50% of participants in the ranibizumab and triamcinolone arms

were incomplete responders following 12 months of therapy. [151] These rates did not change significantly at two years of follow up suggesting that response is likely to be seen following 4-12 months of therapy. Similar rates of response to anti-VEGF drugs have been found in the DRCR.net Protocol T study. [77] As such, reasonable criteria for treatment resistance based on trial data would be at least 4-6 treatments with intravitreal pharmacotherapy with at least six months of follow up and significant residual oedema visualised on OCT.

Treatment-resistance likely reflects the complex pathophysiology of DMO. This Chapter summarises the pathophysiology of DMO, mechanisms of action for established therapies and reviews case series and trials for the management of treatment-resistant DMO.

METHODS

A systematic literature search was performed using MEDLINE (from 1966 to February 2016) and EMBASE (from 1950 to February 2016). Keywords used included; refractory, recalcitrant, treatment-resistant, switching, pathogenesis, VEGF, risk factors, genetics, steroid, laser, vitrectomy, optical coherence tomography, fluorescein angiography and diabetic macular oedema. The reference lists of cited papers were examined to find additional articles of relevance. Only papers published in English from peer-reviewed articles and original descriptions were included and there was no restrictions applied to study type.

PATHOPHYSIOLOGY

The pathophysiology of DMO is multifactorial and complex, involving mechanical and biochemical pathways triggered by hyperglycaemia. Better understanding of these pathways has led to the development of effective therapies, including laser photocoagulation, vitreoretinal surgery and systemic and ophthalmic pharmacotherapy.

The common pathway that leads to macular oedema in DMO as well as other exudative retinal conditions is breakdown of the blood-retinal barrier (BRB). [152] The BRB consists of the inner BRB and the outer BRB, which exist to maintain homeostasis in the neural tissue. The inner BRB is formed by tight junctions between retinal endothelial cells, the surrounding basal lamina, pericytes, astrocytes and microglia. The outer BRB is formed by the tight junctions between retinal pigment epithelium (RPE) cells. Impaired integrity of the BRB leads to leakage of plasma solutes into the interstitial spaces causing oedema through increased osmotic pressure. Fluid subsequently accumulates in different spaces within and underneath the retina as demonstrated in Figure 2.1.

Disruption of the BRB in diabetic retinopathy (DR) results from the release of inflammatory cytokines and growth factors released in states of chronic hyperglycaemia. Important implicated factors include VEGF-A, placenta growth factor (PlGF), interleukin (IL) -8, IL-6, IL-1 beta, tumour necrosis factor-alpha and matrix metalloproteinases. [152-154]

Hyperglycaemia mediated activation of several identified biochemical pathways promotes formation of these factors. These mechanisms include increased flux through the polyol pathway, activated protein kinase C (PKC) and the formation of advanced glycation end products (AGEs).

Aldose reductase utilises nicotinamide adenine dinucleotide phosphate (NADPH) to reduce excess glucose to sorbitol in the polyol pathway. Whilst some sorbitol is oxidised to fructose by sorbitol dehydrogenase through the use of nicotinamide adenine dinucleotide (NAD+), the majority remains unchanged. The consumption of NADPH in this pathway prevents the regeneration of glutathione and other free radical scavengers, increasing oxidative stress on the cell. [155]

Increased diacylglycerol is produced in hyperglycaemic states. This activates PKC, the beta isoform of which is found in high concentrations in the retina. [156] Activated PKC beta mediates retinal vascular permeability leading to hypoxia and upregulates VEGF signaling pathways further leading to BRB impairment. [3]

Hyperglycaemia also causes non-enzymatic glycation of plasma proteins and the basal lamina, which lead to the production of AGEs. Accumulation of AGEs in the vitreous causes cross-linking of collagen, leading to an abnormally adherent vitreo-retinal interface. [157] These mechanical forces contribute to DMO. AGEs also bind to AGE receptors in Müller cells causing upregulation of nuclear factor-κB, increasing transcription of inflammatory cytokines and VEGF. [158] BRB

function may be affected directly by AGE-mediated altering of transmembrane proteins such as integrins. [152]

The pathophysiology of DMO is a multifactorial process. Disease that is refractory to a particular approach to treatment may reflect a failure to recognise one or a combination of the pathways involved or upregulation of alternate growth factors as discussed further below. [153, 159]

Many authors have attempted to identify and treat treatment resistant DMO. These, including their criteria for defining treatment resistance are summarised in Table 2.1.

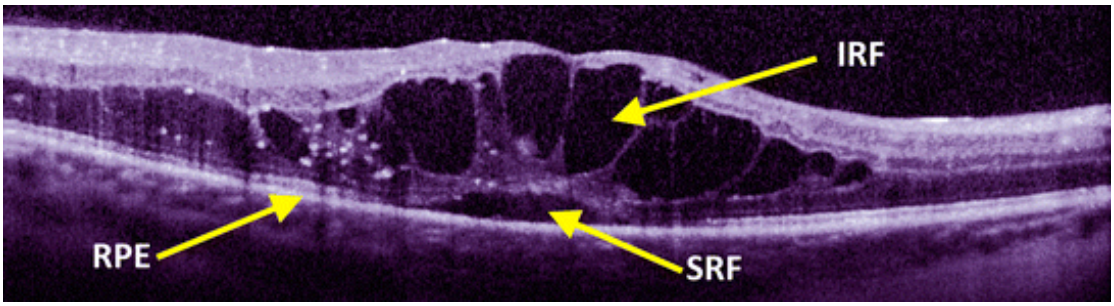

Figure 2.1: Optical coherence tomography (OCT) in a patient with diabetic macular oedema involving the fovea.

OCT provides an ultrastructural overview of the retina similar to a histological section. Note the presence of subretinal fluid (SRF) accumulating above the retinal pigment epithelium (RPE) and intraretinal fluid (IRF).

Table 2.1: Overview of studies of diabetic macular oedema resistant to therapy with anti-VEGF or steroid drugs

Author	Number of eyes	Definition of treatment-resistance	Inclusion/Exclusion Criteria	Design	Intervention	Mean follow-up	Outcome
Alshahrani 2016 [160]	26 (26 subjects)	At least 6 intravitreal injections of bevacizumab, ranibizumab or aflibercept over six months	CMT>350μm, no improvement of 2 or more lines on Snellen chart	Retrospective case series	Dexamethasone implant	6 months	Reduced CMT and improved BCVA at 1 and 3 months
Bansal 2015 [161]	67 (52 subjects)	At least 3 bevacizumab/ triamcinolone injections and at least 3 sessions focal/grid laser	CMT>300μm, intravitreal injection within last 3 months	Retrospective case series	Dexamethasone implant	24 weeks	Reduced CMT and improved BCVA, Maximal benefit at 6 weeks
Ciulla 2015 [81]	33 (22 subjects)	Mean 5.1 treatments with macular laser, intravitreal bevacizumab, triamcinolone, or dexamethasone implant	Persistent DMO for 6 months despite at least two prior treatments	Retrospective series	Intravitreal ranibizumab 0.3mg	48 weeks	Reduced CMT and improved BCVA
Dhoot 2015 [80]	43 (43 subjects)	Intravitreal bevacizumab, at least 2 prior injections 7 weeks apart within 1 year of baseline visit	CMT>300μm Intravitreal steroid/laser within 6 months	Prospective cohort study	Intravitreal ranibizumab 0.5mg for 3 injections If non-responsive then 2.0mg injections from month 3 onwards	12 months	Reduced CMT and improved BCVA, half of those swapped to higher dose ranibizumab responsive
Dutra Meideros 2014 [162]	58 (58 subjects)	Intravitreal anti-VEGF, triamcinolone, pars plana vitrectomy, macular laser	CMT>250μm No treatment in prior three months	Retrospective case series	Dexamethasone implant	6 months	Reduced CMT and improved BCVA,

Escobar 2015 [163]	40 * (76 eyes from 76 patients analysed, 40 of which were treatment-resistant)	Any two of: intravitreal injections (not specified), vitrectomy, laser photocoagulation	CMT>300 µm, treatment within past 3 months excluded, known steroid responders excluded	Prospective cohort study	Dexamethasone implant with PRN laser photocoagulation Retreatment with dexamethasone implant from 3 months onwards as per study protocol	6 months	Reduced CMT and improved BCVA,
Gutierrez-Benitez 2015 [164]	14 (14 subjects)	Intravitreal ranibizumab as monotherapy or in combination with other treatment	No decrease in CMT or stabilisation of BCVA following ranibizumab therapy	Retrospective case series	Dexamethasone implant	7.6 months	Improved CMT and BCVA
Hanhart 2015 [82]	8 (5 subjects)	Intravitreal bevacizumab, at least 2 prior injections	CMT>325 µm Previous steroid therapy	Retrospective case series	Intravitreal ranibizumab	541 days	Improved CMT, non-significant BCVA gain
Haritoglou 2006 [165]	51 (51 subjects)	Macular laser, intravitreal triamcinolone, or vitrectomy	No treatment in preceding 6 months	Prospective cohort study	Intravitreal bevacizumab, single dose	12 weeks	Improved CMT and BCVA
Jeon 2014 [166]	20 (20 subjects)	At least 3 monthly injections of intravitreal bevacizumab	No treatment in preceding 2 months	Prospective cohort study	Intravitreal triamcinolone	3 months	Improved CMT and BCVA, CMT gains not sustained at 3 months
Kim 2012 [167]	46 (41 subjects)	Macular laser, intravitreal triamcinolone or a combination of the two	CMT >250 µm, visual acuity >20/40	Retrospective case series	Vitrectomy, intravitreal triamcinolone and laser	3 years	Improved CMT and BCVA
Kim 2015 [168]	20 (20 subjects)	Two or more consecutive bevacizumab injections	CMT>300 µm or <150 µm reduction in CMT	Retrospective case series	Intravitreal triamcinolone	3 months	Improved CMT and BCVA, BCVA gains not sustained at 3 months

Lazic 2014 [169]	16 (15 subjects)	At least 3 monthly injections of intravitreal bevacizumab with or without previous laser	CMT>225 µm, DMO previously treated with steroid No treatment in preceding 1 month	Prospective cohort study	Dexamethasone implant	4 months	Improved CMT and BCVA, not sustained at 4 months follow up
Lim 2015 [84]	21 (19 subjects)	At least 3 prior injections of intravitreal bevacizumab or ranibizumab	No defined anatomical or visual criteria	Retrospective chart review	Intravitreal aflibercept	5 months	Improved CMT and BCVA
Maturi 2015 [170]	40 (30 subjects)	Intravitreal bevacizumab with or without macular laser	CMT >250 µm HbA1c>10 Known steroid response not responding to 2 topical meds 20/32 to 20/320 Anti VEGF in past 4 weeks, intravitreal steroid in past 8 weeks, laser in past 16 weeks	Prospective, single-masked, randomised, controlled trial	Intravitreal bevacizumab +/- dexamethasone implant	12 months	Improved CMT, no change in BCVA at 12 months
Ornek 2008 [171]	17 (16 subjects)	Macular laser and intravitreal triamcinolone	None reported	Prospective cohort study	Intravitreal bevacizumab	6 weeks	70% improved vision at 6 weeks
Rahimi 2015 [85]	50 (37 subjects)	At least 4 prior intravitreal injections with bevacizumab or ranibizumab	No reduction, incomplete resolution, or an increase in CMT from baseline	Retrospecive case series	Intravitreal aflibercept	4.6 months	Significant improvement in CMT, BCVA improvements non-significant

Totan 2015 [172]	30 (30 subjects)	Three prior injections with intravitreal bevacizumab	CMT >275 µm previous vitrectomy panretinal or grid laser photo-coagulation (within 3 months prior to investigation) previous steroid treatment	Prospective cohort study	Dexamethasone implant	6 months	Improved CMT and BCVA at 3 months, BCVA gains lost at 6 months
Wood 2015 [86]	14	Regular intravitreal ranibizumab and/or bevacizumab	Persistent retinal fluid	Prospective cohort study	Intravitreal aflibercept	1 month	Improved CMT, no change in BCVA
Yolcu 2014 [173]	25 (25 subjects)	Bevacizumab (at least 2 injections) and triamcinolone	CMT>500 µm, exclusion criteria laser within 6 months, vitreoretinal surgery, FAZ> 800 µm, hypertension, HbA1c>8% Only pseudophakic eyes	Prospective cohort study	Combination intravitreal bevacizumab and triamcinolone	12 months	Improved CMT and BCVA
Yuksel 2013 [174]	71 (59 subjects)	Macular laser with or without intravitreal/subtenon triamcinolone injection	CMT>300 µm Vitrectomy exclusion criteria	Retrospective case series	Intravitreal bevacizumab	9 months	Improved CMT and BCVA
Zhioua 2015 [175]	13 (12 subjects)	Monthly injections of intravitreal ranibizumab for 6 months	CMT>300 µm BCVA ≤ 20/40 HbA1c>8.5 Macular photocoagulation in preceding 12 months	Retrospective case series	Dexamethasone implant	9 months	Improved CMT and BCVA

BCVA= best-corrected visual acuity, CMT=central macular thickness, HbA1c=glycated haemoglobin

IMAGING IN DIABETIC MACULAR OEDEMA

The diagnosis and management of DMO is facilitated by multiple imaging techniques. Fundus FA visualises the retinal vasculature and identifies lesions of DR, areas of ischaemia demonstrated by capillary dropout and areas of impaired BRB function demonstrated by leakage of dye. It can aid in predicting the prognosis and response to treatment in DMO. An illustration of this is degree of capillary non-perfusion and macular ischaemia demonstrated with an enlarged foveal avascular zone.

With development of ultra-widefield imaging, FA can be performed with visualisation of up to 200 degrees of the retina (Figure 2.2). Extensive ischaemia in the retinal periphery has been associated with recalcitrant disease and ultra-widefield FA (UWFA) may aid in the identification of DMO that is likely to be treatment-resistant. [176] UWFA outcomes will be the topic of Chapter 5 of this thesis.

Optical coherence tomography (OCT) utilises interference of light to produce high-resolution ultrastructural images of the macular region. These images produce cross-sectional visualisation of oedema, similar to a histological section (Figure 2.1). OCT technology has developed significantly in recent years. The resolution and acquisition speed of scanning has improved from early time domain OCT to current spectral domain OCT (SD-OCT). SD-OCT allows for improved axial resolution and imaging deeper structures including the choroid, which can be affected in DMO. [177] Coupled with eye tracking, newer machines

allow for reliable and reproducible quantifiable measurements of the central macular thickness (CMT). OCT biomarkers, such as subretinal fluid (SRF), disorganization of the inner retinal layers (DRIL), inner segment ellipsoid (ISe) band and external limiting membrane (ELM) integrity have all been correlated with visual acuity (Figure 2.3). [178-181] Serial imaging with OCT is critical in the management of DMO. Indeed, the ETDRS criteria for clinically significant macular oedema is becoming less relevant as OCT has established itself as the new reference standard for the diagnosis and monitoring of DMO. [182]

Recognising morphological biomarkers, such as those on OCT or FA, may become an important factor in predicting which patients are likely to have recalcitrant disease, guiding individual treatment regimens. [183] Future improvements in imaging, such as OCT angiography, which non-invasively visualises retinal capillary layers may play a role in the diagnosis, monitoring and management of DMO. [184]

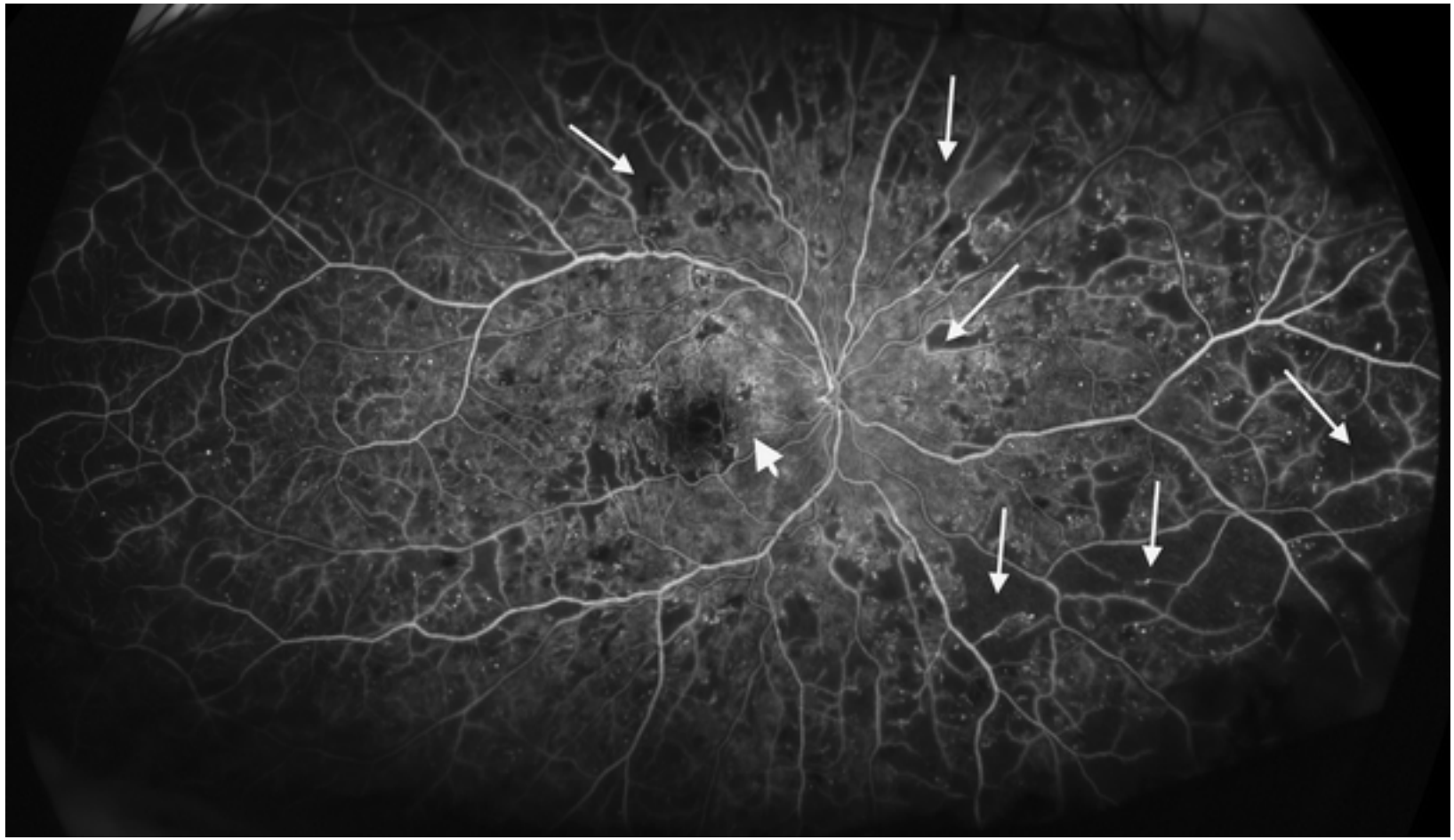

Figure 2.2: Ultra-widefield FA of a patient with severe non-proliferative diabetic retinopathy and DMO.

There is significant leakage of dye into the macula (arrowhead) and areas of capillary non-perfusion corresponding to ischaemic retina (arrows). The discrete dots seen throught the image correspond to microaneurysms.

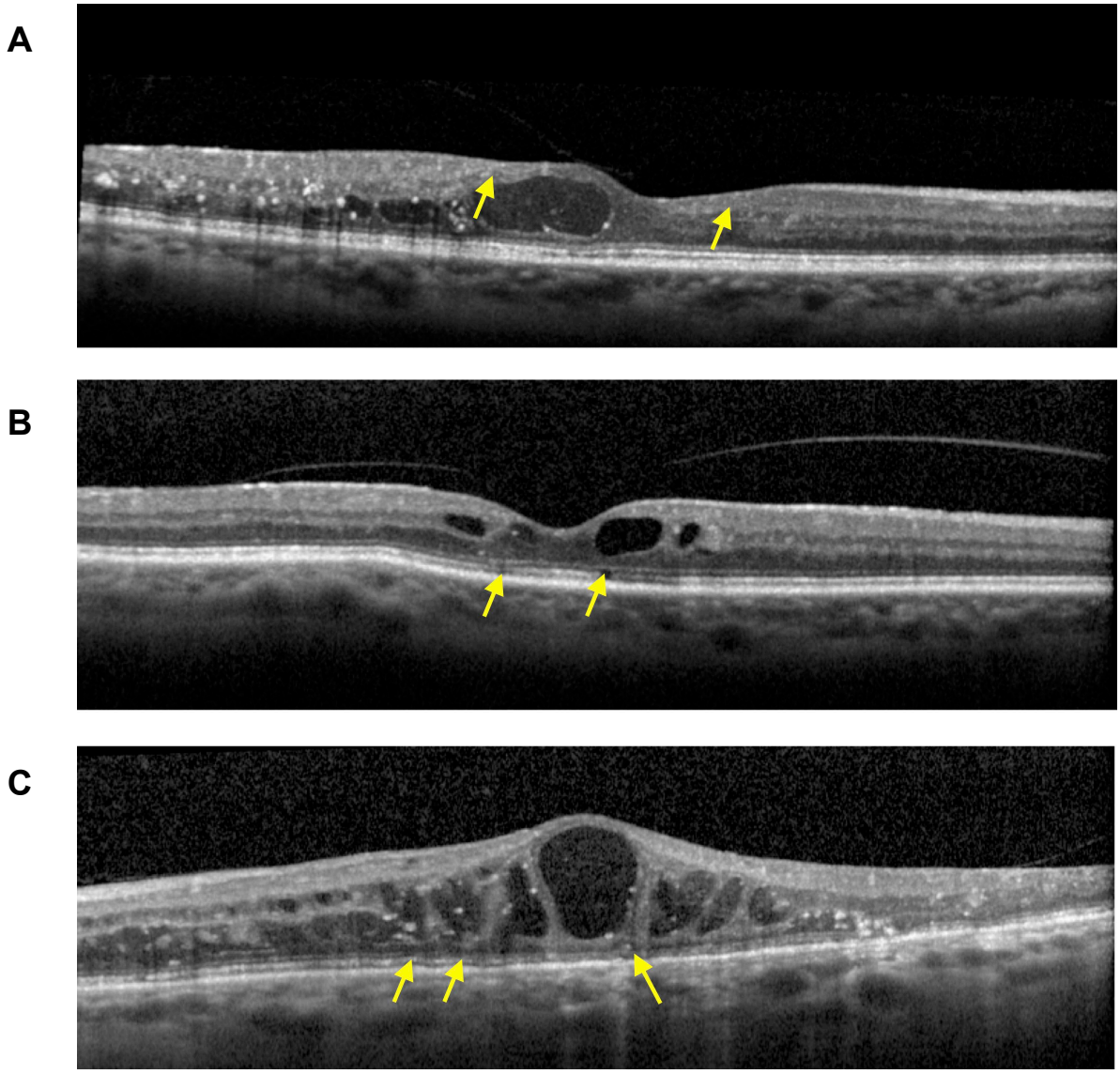

Figure 2.3: Optical coherence tomography biomarkers correlated with vision outcomes in diabetic macular oedema.

Illustrated here with arrows are (A) disorganization of the inner retinal layers (DRIL), (B) inner segment ellipsoid (ISe) band disruption and (C) external limiting membrane (ELM) disruption. Figure 2.1 illustrates subretinal fluid (SRF).

ENVIRONMENTAL AND GENETIC FACTORS

Hypertension, established cardiovascular disease, advanced DR and proliferative DR (PDR) has been associated with diffuse DMO in retrospective series. [185] Despite this correlation, blood glucose, blood pressure and lipid control found no benefit in modifying disease prognosis in DMO in the prospectively designed Action to Control Cardiovascular Risk in Diabetes (ACCORD) study of patients with type 2 diabetes. [186]

Recent meta-analysis of randomised clinical trials of lipid control showed no strong relationship between dyslipidaemia and DMO. [187] However, statins may have an independent effect on stabilising the BRB through reduction of retinal vascular inflammation rather than by their lipid lowering effect alone. [188]

Fenofibrate, which is thought to exert its therapeutic effect on DR through non-lipid biochemical pathways, has shown some promise in the management of DMO. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study demonstrated participants who received fenofibrate as opposed to placebo were less likely to require laser therapy for both proliferative DR as well as DMO. [189] In the ACCORD Eye Study, participants who used fenofibrate had reduction in the progression of DR compared with placebo, although this study demonstrated no benefit in the improvement of DMO. [190] The addition of fenofibrate and statins to the management of patients with treatment-resistant DMO may be a consideration for treating physicians.

Many clinical trials evaluating the treatment of DMO exclude patients with uncontrolled diabetes based on their glycated haemoglobin (HbA1c). This selection bias makes management of these patients especially challenging given the scant evidence to support the use of therapies. Additionally, these patients may represent a significant proportion of those with recalcitrant disease. While the role of systemic medical therapy for DMO is unclear, preventing the progression of retinopathy should be a clear goal in the management strategy of all diabetic patients, especially given the association between HbA1c and worsening retinal ischaemia. [191]

There are disparities in risk of developing DR amongst patients of different ethnic groups, even when environmental factors are corrected for. These differences may be explained by a genetic predisposition to disease. Polymorphisms in the VEGF gene have been shown to be associated with severity of DR and have been associated with an increased risk of development of DMO. [192, 193] Additionally, polymorphisms in the AKR1B1 gene that encodes for aldose reductase have been associated with DR development, irrespective of ethnic background. [194] Further studies examining genetic factors associated with DMO may lead to improved diagnosis and tailored treatments for this condition. These factors may also be determinants for response to treatment and contribute to treatment resistance.

LASER THERAPY

The ETDRS demonstrated that focal/grid argon laser photocoagulation of macular lesions led to a significant reduction in vision loss in eyes with "clinically significant macular oedema". [35] This was defined as retinal thickening within 500 μm of the macular center, hard exudates within 500 μm of the macular centre with adjacent retinal thickening and/or one or more disc diameters of retinal thickening, part of which is within one disc diameter of the macular centre. Figure 2.4 is a fundus photography illustrating an example of clinically significant DMO prior to and following ETDRS guided laser photocoagulation.

Whilst the rate of vision loss was halved in this study, visual improvements were modest or non-existent. Vision improvements are seen in approximately 15% of patients after 3 years of follow up. [195] By vision criteria, treatment-resistance with macular laser therapy reaches rates of 85%.

Macular laser photocoagulation improves DMO through a number of proposed mechanisms. Firstly, photoreceptors and RPE cells are destroyed via a photothermal mechanism, thus reducing oxygen consumption. This reduced oxygen consumption in the outer retina is postulated to increase oxygen flux from the choroid to the inner retina, thus leading to arteriolar constriction and decreased Starling forces driving oedema. [196] Secondly, photocoagulation induces changes to the RPE cells causing their proliferation and release of cytokines such as TGF-beta, which antagonise the effects of VEGF. [197] Finally, there can be direct ablation of microaneurysms contributing to oedema identified as leaking on FA.

Macular laser photocoagulation has the risk of causing several sight-threatening complications including scotoma, choroidal neovascularisation and subretinal fibrosis. [198] Given these risks and the availability of anti-VEGF drugs, macular focal/grid laser is now generally reserved for non-centre involving DMO. From the three year results of the DRCR.net Protocol I study, use of focal/grid laser may even lead to poorer long-term visual outcomes in patients with DMO when initiated at the same time as anti-VEGF therapy. [69]

Recent advances in technology have led to the development of lasers different wavelengths, lower energies, duration of pulses and pattern deliveries aiming to target pathology directly. This aims to reduce collateral damage to the retina and surrounding structures.

Diode lasers with a shorter pulse length are one such example, which are effective in the management of DMO. A twelve-month randomised controlled trial compared conventional argon laser with micropulse diode photocoagulation for the management of clinically significant DMO and found anatomical and functional outcomes to be similar. [199] The photothermal effect of these micropulse lasers is designed to be confined to the RPE and may spare complications associated with photoreceptor, ganglion cell loss and nerve fibre layer damage. These therapies may also be more comfortable for the patient as choroidal heating is less likely to occur. Whether micropulse laser can be effective as an adjunct to other therapies for DMO is yet to be investigated.

Future directions for laser photocoagulation include treatment of peripheral ischaemia identified on ultra-widefield FA. These ischaemic areas have been have been hypothesised to drive DMO in treatment-resistant cases and laser may have a future role in management of resistant patients. [176, 191]

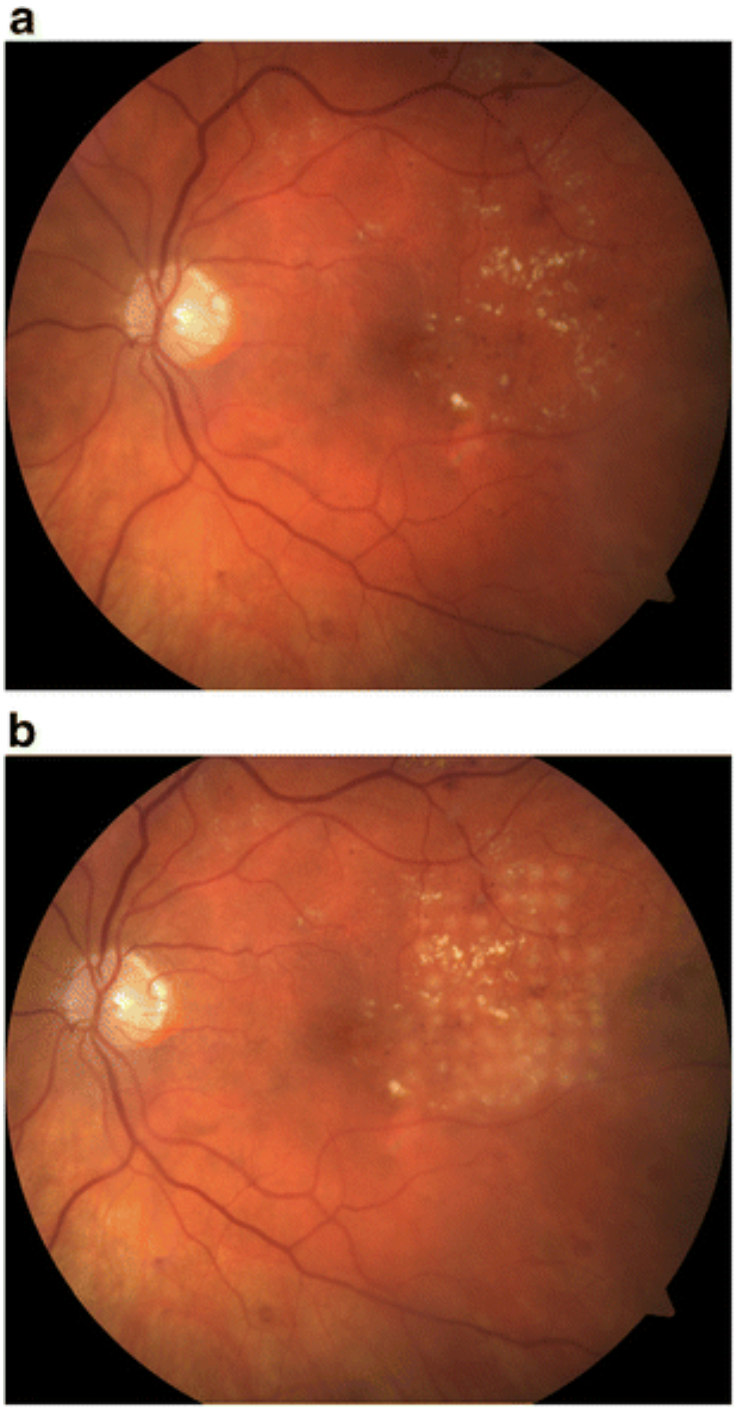

Figure 2.4: Fundus photograph demonstrating clinically significant macular oedema (a) before and (b) after ETDRS-guided laser photocoagulation.
Note the presence of hard exudates associated with retinal thickening within 500 μm of the macular centre, as well as other changes of diabetic retinopathy including retinal haemorrhages and microaneurysms

SURGICAL MANAGEMENT

The vitreous body is variably attached to the inner limiting membrane (ILM) of the neurosensory retina at macula. Over time this vitreoretinal adhesion can weaken and the vitreous pulls away from the macula. If this separation is complete, then it is termed a posterior vitreous detachment (PVD). If incomplete, remaining adhesions in a partial PVD can cause traction at the macula in a condition termed vitreomacular traction (VMT).

Vitreoretinal interface abnormalities such as these may be part of the pathophysiology of DMO and related to AGE mediated pathways as previously described. DMO associated with a thickened and taut posterior vitreous face and VMT has been shown to be responsive to vitrectomy. [200] In this surgical procedure, the vitreous body is surgically removed. Vitreomacular adhesion without traction has also been associated with DMO. [201] Vitrectomy causing a PVD has been shown to improve visual and anatomical outcomes in cases of non-tractional DMO, and enzymatic PVD may play a future role in the management of treatment-resistant DMO. [202] The ILM may act as a diffusion barrier to mediators such as VEGF, and peeling this as part of vitrectomy may also improve anatomical outcomes in treatment-resistant DMO. [203]

Furthermore, vitrectomy is postulated to improve DMO through increasing oxygen delivery to ischaemic areas via two mechanisms. [196] Firstly, the vitreous is replaced by less viscous aqueous humour, which increases the diffusion capacity of oxygen as well as other molecules as per Fick's law. Secondly, fluid currents within the vitreous cavity are more efficiently able to

transport oxygen from well-perfused areas to ischaemic retina. This concept of vitreoperfusion and microplasmin induced PVD increasing intravitreal oxygen levels have been demonstrated in animal models, supporting this theory. [204]

Vitrectomy may be considered in cases refractory to treatment with repeated pharmacotherapy and especially in cases where there is vitreomacular attachment or VMT. As monotherapy, vitrectomy is insufficient to manage DMO as it fails to address the continual production of growth factors and cytokines implicated in the pathogensis. A systematic review and meta-analysis of vitrectomy in eyes without epiretinal membranes or traction showed anatomical improvement in DMO at six months follow up. However, at 12 months this effect was lost and there was a suggestion that they could lead to inferior functional outcomes as compared with laser. [205]

Conversely, removing the vitreous may have implications complicating the management of DMO. The half life and intravitreal concentrations of injected intravitreal drugs is significantly reduced in vitrectomised eyes suggesting reduced efficacy of these treatments. [206] One study has demonstrated poorer visual acuity and CMT with anti-VEGF therapy in vitrectomised eyes. [207]

ANTI-INFLAMMATORY THERAPY

Inflammation plays a key role in the pathogenesis of DMO with increased expression of inflammatory mediators, leukocyte adhesion, complement activation, macrophage infiltration and acute phase proteins. [154, 208] Accumulation of leucocytes coincides with vascular dysfunction leading to breakdown of the BRB, premature cell injury and death and ischaemia. [209] Experimental data also shows that by blocking leucocyte adhesion, BRB breakdown and endothelial cell injury are prevented. [210]

Corticosteroids

Treatment of DMO with corticosteroids has been shown to reduce the vitreous levels of inflammatory cytokines and VEGF. [211] Glucocorticoids function by reducing inflammation and maintaining the BRB through increased expression of tight junction proteins. [212]

Three types of corticosteroids have been evaluated for DMO in clinical trials: triamcinolone (Kenalog-40 (Bristol-Myers Squibb, Princeton, NJ), Triescence (Alcon, Ft. Worth, TX), Trivaris (Allergan, Irvine, CA)), dexamethasone implant (Ozurdex, Allergan, Irvine, CA) and fluocinolone implant (Retisert, Bausch + Lomb, Rochester, NY and Iluvien, Alimera Science, Alpharetta, GA).

Macular laser has been compared with intravitreal triamcinolone for DMO in a prospective, randomised controlled trial of 84 eyes. [213] The triamcinolone group demonstrated a significant gain in vision at 2 years at the expense of

increased cataract formation and elevated intraocular pressure (IOP), both common adverse events in trials evaluating intravitreal steroids. [214] Similar results have been found in other studies comparing triamcinolone to laser. [215, 216]

In a randomised controlled trial of 171 eyes, 0.35mg and 0.7mg dexamethasone implants were evaluated for DMO. [217] Whilst there were significant reductions in CMT, the best-corrected visual acuity (BCVA) was unaffected. The Macular Edema: Assessment of Implantable Dexamethasone in Diabetes (MEAD) study subsequently demonstrated anatomical and BCVA improvement in 0.35mg and 0.7mg dexamethasone implants compared with a sham procedure in a larger study of 1048 eyes. [218]

Fluocinolone implants, which contain 0.59 mg of fluocinolone acetonide, release approximately 0.5 μg of drug per day for approximately 3 years. The surgically inserted Retisert implant was compared with sham injections combined with standard of care laser in a prospective randomised trial of 196 eyes. While this study showed improved visual outcomes for the fluocinolone arm, there were notable complications with surgical intervention required for cataract in 91% of phakic participants and ocular hypertension in 33.8% participants. [219] The Fluocinolone Acetonide for Macular Edema (FAME) study was a randomised trial of 953 eyes, which compared 0.2- or 0.5-μg/day Iluvien implants delivered via an intravitreal injection to sham injections. [220] After 24 months, eyes gained an average of 4.4 and 5.4 letters in the low and high dose fluocinolone groups respectively compared with 1.7 letters in the sham group. Additionally, this

preparation had lower rates of intervention for ocular hypertension and cataract than the trial of Retisert. Currently, Iluvien is the only preparation approved for use in DMO.

Corticosteroids have been compared to anti-VEGF drugs for the management of DMO as summarised in Chapter 1 in the DRCR.net Protocol I and BEVORDEX studies. [47, 48]

Given side effects of steroids, they are generally reserved as second line therapy after anti-VEGF drugs. Indeed, this is why switching therapy from steroid to anti-VEGF is scantly reported. [165, 171, 174] However, with the need for regular injections reduced, there can be an argument made for the use of these agents earlier in a treatment algorithm, especially in pseudophakic eyes. Caution and close monitoring are required to assess for elevated and uncontrolled IOP, which can lead to rapid visual loss.

Non-steroidal anti-inflammatory drugs

Despite the efficacy of steroid therapy for DMO, the response to topical non-steroidal anti-inflammatory drugs (NSAIDs) has not been demonstrated in clinical trials. [221] The DRCR.net Protocol R compared 0.1% nepafenac with placebo and demonstrated no anatomical or visual benefit after 12 months of therapy for non-centre involving DMO. [222]

An animal model has demonstrated the benefit of high dose systemic aspirin in the suppression of BRB breakdown but the translational dose in humans of 50mg/kg would result in severe side effects. [223]

Switching from anti-VEGF to Steroid

Higher intravitreal levels of inflammatory cytokines are associated with more severe DMO, suggesting that inflammation may play a greater role in treatment-resistant disease. [224] Chronic DMO may also be more likely to be driven by inflammatory cytokines. In the long-term follow up of the FAME study, eyes with DMO for longer than 3 years had superior visual outcomes to those with a shorter history. [220] Switching therapy from anti-VEGF agents, which may be more efficacious earlier in the natural history of DMO, to steroid therapy, follows this rationale.

Several studies have assessed the utility of a dexamethasone implant for DMO resistant to anti-VEGF therapy, as summarised in Table 2.1. These are mostly retrospective case series or prospective cohort studies, most of which demonstrated both anatomical and visual improvement at the end of follow up. [160-164, 169, 170, 172, 175] The only randomised controlled trial assessing dexamethasone for anti-VEGF resistant DMO found an anatomical benefit in combination with bevacizumab but demonstrated no change in BCVA at 12 months. [170] Switching therapy to intravitreal triamcinolone is also effective but the duration of action of this is limited compared to dexamethasone implants. [166, 168]

Anti-VEGF THERAPY

The identification of VEGF-A as a key growth factor in the pathogenesis of DMO has revolutionised the management and treatment outcomes of this condition as described in Chapter 1.

Switching between anti-VEGF agents

Though all anti-VEGF drugs block the action of VEGF, differences in size, structure, mechanism of action and half-lives result in different clinical effects as demonstrated in Protocol T.

Previous studies of treatment-resistant neovascular age-related macular degeneration (nAMD) have shown benefit in switching therapy from one anti-VEGF drug to another, prompting others to investigate this approach in DMO. [225] These studies, summarised in Table 2.1, are heterogeneous in their design, inclusion and exclusion criteria and follow up, making comparison difficult. However, there seems to be a universal anatomical improvement in switching from bevacizumab to ranibizumab [80-82] and there may be a benefit in switching from either of these drugs to aflibercept. [84-86] The benefits of switching most likely have to do with improved binding affinity with VEGF and blockade of PlGF with aflibercept and will be explored further in Chapter 3. [226]

Dose response

Compared to nAMD, the intravitreal concentration of VEGF in DMO may be higher, suggesting there may be a role for increased blockade of the VEGF pathway for treatment-resistant disease. [227]

In addition to the aforementioned REEF study in Chapter 1, the READ-3 study randomised participants with DMO to receive either 0.5mg or 2.0mg of ranibizumab. The six-month results demonstrated no benefit to the higher dose of therapy. [228] It is likely that the effects of higher doses of ranibizumab may be equivocal. Similarly, a head to head study of 1.25mg and 2.5mg doses of bevacizumab showed no differences in BCVA or CMT after six months of therapy. [76]

COMBINATION THERAPY

The combination of anti-VEGF agents and coritcosteroids may be more effective in certain patients with DMO who are difficult to control with anti-VEGF agents alone. In a three arm randomised trial, bevacizumab was compared with combination bevacizumab/triamcinolone and macular laser photocoagulation. After 16 weeks of follow-up, combination therapy for these patients provided a longer period of BCVA and CMT improvement. [229]

Several studies have explored the efficacy of combined laser and anti-VEGF therapy, with rescue laser forming part of the DRCR.net Protocol T study design. [77] Over the 2-year follow up of this study, 41% of aflibercept, 64% of bevacizumab and 52% of ranibizumab eyes received macula grid/focal laser in addition to intravitreal injection. [78] Though some smaller prospective studies have shown reduced need for intravitreal injections with combined laser therapy, larger trials such as RESTORE showed no benefit to combination laser and ranibizumab. [121, 230] A three-arm trial compared intravitreal bevacizumab, macular laser photocoagulation and a combination of the two. This demonstrated significant improvements in visual acuity and reduction in CMT in both bevacizumab treatment arms at one month though this was not maintained in the bevacizumab monotherapy arm at three and six months. This study was limited by only a single treatment being given and a six-month follow up. [230]

SUMMARY

Incomplete response to treatment for DMO has been described since the ETDRS report number 1 in 1985. Focal/grid macular laser as defined in this study has limitations for treating DMO and may result in poorer visual outcomes due to photoreceptor damage and other complications. OCT, ultra-widefield FA, new laser technology, vitrectomy and a range of pharmacotherapies are available as diagnostic and treatment tools for treating clinicians. Although corticosteroid and anti-VEGF drugs have been shown to be more effective than argon laser in clinical trials, suboptimal response continues to be observed in a subgroup of these patients. Resistance to pharmacotherapy may represent abnormalities in the vitreoretinal interface. Anti-VEGF resistance may represent a progression in the natural history of DMO with inflammation or alternative growth factors or cytokines increasingly contributing to the pathophysiology with time. The dynamic nature of DMO means that treatment modalities may need to be individualised throughout the course of treatment. Efficacy for therapies must be balanced with their risks. Trials evaluating treatment-resistant DMO are heterogeneous in design, follow-up, eligibility criteria and intervention. Therefore, formulating recommendations becomes challenging given the scarcity of strong scientific data. Nevertheless, from the current studies reviewed in this paper, patients who are refractory to one treatment may benefit from switching to a different agent or a combination therapy.

Section 2: Switching Therapy from Bevacizumab to Aflibercept in Persistent Diabetic Macular Oedema

Chapter 3: Aflibercept for the management of persistent diabetic macular oedema

As highlighted in the systematic review of the literature in the previous chapter, there is a relative paucity of prospective data regarding the management of persistent diabetic macular oedema.

This chapter presents the primary visual and anatomical outcomes from a clinical trial undertaken to assess the effect in a switch in therapy from bevacizumab to aflibercept for the management of persistent diabetic macular oedema.

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- **Bahrami B**, Hong T, Schlub TE, Chang A. Aflibercept for persistent diabetic macular edema: 48 week outcomes. Retina. *Under review Feb 21 2018*.

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RANZCO 48th Annual Scientific Conference: Melbourne, Australia 2016

Bahrami B, Nair R, Hong T, Chang A. Effect of aflibercept on diabetic retinopathy severity in patients with treatment-resistant diabetic macular oedema: 12 month outcomes. Clin Exp Ophthalmol. 2016 Nov; 44:80-140.

Association for Research in Vision and Ophthalmology Annual Meeting: Seattle, USA 2016

Bahrami B, Hong T, Zhu M, Chang A. The efficacy of aflibercept in the management of treatment-resistant diabetic macular edema: a 12-month prospective study. Invest. Ophthalmol. Vis. Sci. 2016; 57(12):2070.

Association for Research in Vision and Ophthalmology Annual Meeting: Baltimore, USA 2017

Bahrami B, Hong T, Zhu M, Chang A. Aflibercept for treatment-resistant DME: 48-week outcomes. Invest. Ophthalmol. Vis. Sci. 2017; 58(8):65.

ABSTRACT

Purpose: To evaluate functional and anatomical outcomes following a switch from intravitreal bevacizumab to aflibercept in patients with persistent diabetic macular oedema (DMO).

Methods: Prospective, single-arm, open-label clinical trial of patients with persistent DMO despite prior treatment with bevacizumab. Five loading doses of intravitreal aflibercept were administered every 4 weeks with subsequent injections administered every 8 weeks. Patients were reviewed every 4 weeks and best-corrected visual acuity (BCVA) and central macular thickness (CMT) were recorded. Primary outcome measures included change in CMT and BCVA at week 48 compared with baseline. Paired t-tests were used to assess change between baseline and follow-up visits.

Results: At baseline, 43 eyes from 43 patients were recruited with a median (interquartile range) of 12 (7-24) previous intravitreal anti-VEGF injections over a period of 18 (8-34) months. Mean ± standard deviation CMT reduced by 59 ± 114μm (p=0.002) and BCVA improved by 3.9 ± 7.0 letters (p=0.001) after 48 weeks in the 41 patients who completed the trial. BCVA improvements were more marked in patients who gained ≥ 5 letters following the first injection (8.9 ± 5.7 vs. 1.8 ± 6.5 letter gain at 48 weeks, p=0.002), a difference which remained significant following regression analysis with baseline BCVA. Vision gains and CMT reduction were similar in 9 fellow eyes eligible for inclusion being concurrently treated for DMO with bevacizumab.

Conclusion: Intravitreal aflibercept was effective in improving anatomical and visual outcomes among patients with an incomplete response to intravitreal bevacizumab with 48-weeks of follow-up. Patients with a good early response subsequent to switching had a better improvement in vision at 48-weeks.

BACKGROUND

Intravitreal anti-vascular endothelial growth factor (VEGF) drugs have revolutionised the management of diabetic macular oedema (DMO). Long-term clinical trial data has shown visual gain and anatomical improvements are maintained with use of these drugs. [63-65, 71, 78] Furthermore, the use of anti-VEGF drugs for DMO demonstrates a disease modifying effect and regression of diabetic retinopathy severity. [64, 71, 79]

Despite their marked benefit, a proportion of patients have persistent edema even with repeated anti-VEGF therapy. In the Protocol T study, 41% of patients in the bevacizumab arm met criteria for rescue laser after 24 weeks of treatment. [77] In a post-hoc analysis of the Protocol I study, 39.7% of eyes had a visual gain of less than 5 letters after 12 weeks of treatment. At the end of 156 weeks, this rate of suboptimal response was similar at 34.2%. [231]

Clinical trial data supporting the long-term use of anti-VEGF drugs rely mostly on treatment naïve patients or those who have had lengthy washout periods where treatment is ceased for a period of months prior to enrolment. [63-65, 71, 78, 232] Thus, those patients who have an incomplete response to therapy pose a management challenge. There is an increasing body of research evaluating strategies in managing patients with persistent DMO. These include higher doses of drug, switching between anti-VEGF agents as well as intravitreal steroids.

In this 48-week prospective cohort study, we evaluate the visual and anatomical outcomes in switching therapy from bevacizumab to aflibercept in patients with persistent DMO.

METHODS

Study Design

This study was a prospective, open label, single-armed, clinical trial of patients referred to a tertiary referral retinal clinic in Sydney, Australia. The trial was listed on the Australian and New Zealand Clinical Trials Registry (ACTRN12614001307695). Informed consent was obtained from all individual participants and the study was performed in accordance with the 1964 Declaration of Helsinki.

Study Participants

One eye from each patient was included in the study. Eligible participants were aged 18 or older, with DMO secondary to type 1 or type 2 diabetes mellitus, best corrected visual acuity (BCVA) between 34 and 85 ETDRS letters, retinal thickness greater than 300μm in the central 1mm ETDRS field on spectral domain OCT (SD-OCT) and at least 4 previous intravitreal injections of bevacizumab (2.5mg/0.1mL) in the 6 months prior to baseline examination. Exclusion criteria included prior intravitreal steroid therapy or vitrectomy surgery in the study eye within 3 months of baseline, cataract surgery or macular laser within 2 months of baseline, pregnancy, active proliferative diabetic retinopathy and uncontrolled diabetes mellitus (HbA1c≥12%). When both eyes were eligible for inclusion, the study eye was selected at the discretion of the primary investigator.

Study Protocol

All participants received 5 loading doses of intravitreal aflibercept (2.0mg/0.1mL) administered at 4-week intervals (week 0, week 4, week 8, week 12 and week 16). Further intravitreal aflibercept injections were then given at 8-week intervals (week 24, week 32 and week 40), as per product label indication, with a total follow-up of 48 weeks. Participants were reviewed at baseline, 1 week after the initial injection, and then every 4 weeks. At each visit ophthalmic examination was undertaken including BCVA assessed on an ETDRS chart, intraocular pressure (IOP) measured using Goldman applanation tonometry, and central macular thickness (CMT) measured with SD-OCT (Spectralis; Heidelberg Engineering, Heidelberg, Germany). In phakic eyes, nuclear, cortical and posterior subcapsular lens opacities were graded according to the Age Related Eye Diseases Study (AREDS) protocol. Fundus fluorescein angiography was performed at baseline to confirm the diagnosis of DMO and to exclude other causes of macular edema.

Retinal thickness was defined on OCT as the distance between the inner limiting membrane and Bruch's membrane. This distance was measured automatically with the inbuilt Heidelberg HRA/OCT software and checked manually to ensure correct segmentation. Segmentation lines were redefined manually if required. CMT values were calculated as the average retinal thickness in the central 1mm circle of the ETDRS grid. Progression scans utilising eye and landmark tracking were undertaken to ensure accurate measurement of the same anatomical location.

The morphology of DMO was analysed and classified on OCT as the presence of intraretinal fluid, subretinal fluid or both. The presence or absence of vitreomacular adhesion (VMA), defined as an elevation of the cortical vitreous above the retina surface in the perifoveal area without any changes in foveal contour or retinal morphology, was graded. The inner segment ellipsoid (ISe) band integrity was assessed in the central 1mm circle of the ETDRS grid with disruption as present or absent. The presence or absence of external limiting membrane (ELM) disruption within the central 1mm circle of the ETDRS grid was also graded. Disorganisation of the retinal inner layers (DRIL) affecting ≥50% of the 1-mm central retinal zone was graded as previously described [15].

All intravitreal injections were given according to a standardised procedure with strict aseptic technique. The eye was anesthetised using topical oxybuprocaine hydrochloride 0.4% and the conjunctiva was prepared with an antiseptic agent (povidone iodine 5% or chlorhexidine 0.1%). The intravitreal injection was delivered using a 30-gauge needle through the pars plana. Post-procedure topical antibiotic drops were not routinely administered.

Ocular and systemic adverse events were recorded. An increase in lens opacity grading of 2 or more AREDS levels in either nuclear, cortical, or posterior subcapsular cataract, IOP of 25 mmHg or more or a rise in IOP of 10 mmHg or more compared with baseline were considered an adverse event.

Additional examinations at baseline, 24 weeks and 48 weeks were lens status graded according to the Age Related Eye Diseases Study (AREDS) protocol and ultra-widefield (UWF) retinal imaging (Optos PLC, Dunfermline, UK).

An ETDRS 7 standard fields (7SF) template was overlaid on UWF imaging obtained and DR lesions were graded for severity and as predominantly peripheral or within the 7SF as previously described. [233, 234]

Statistical Analyses

All statistical tests were performed using IBM SPSS software (version 22; SPSS Inc, Chicago, Illinois, USA). Complete case analysis was performed with missing data excluded. Data was confirmed to be distributed normally using Shapiro-Wilk tests. Paired t-tests were used to compare differences in means of BCVA and CMT. Homogeneity of data was confirmed using Levene's test for all independent samples' t-tests, which were used to group patients by baseline vision less than or greater than or equal to 69 letters, baseline CMT less than or greater than or equal to 400μm and vision gain greater or equal to five letters at 4-weeks. In cases where variances were deemed unequal, adjustment was made to the analysis using the Welch-Satterthwaite method. Intrapatient BCVA and CMT variability was assessed using vision data from the fellow eye. Post-hoc analyses were performed comparing changes in BCVA and CMT in patients receiving bevacizumab treatment for DMO in their fellow eye with the study eye.

Multiple regression was performed to assess for an effect of outcomes with baseline vision, gender, age, duration and type of diabetes mellitus, glycated haemoglobin, duration and number of previous treatments for DMO, previous panretinal photocoagulation, morphology of DMO and lens status.

For all analyses, a p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Baseline Characteristics

Baseline characteristics of the patients are summarised in Table 3.1. Of patients screened, 9 had both eyes eligible as per inclusion criteria. There was no significant difference in the CMT (428 ± 84μm vs. 405 ± 91μm, p=0.42) or BCVA (70.0 ± 7.7 vs. 74.8 ± 8.2 letters, p=0.06) in the study eye compared to the fellow eye. Among the 43 patients recruited, 41 patients were included in the final analysis. One patient experienced a retinal detachment following the second injection and was withdrawn from the study. One other withdrew consent after the baseline visit and injection. Baseline mean ± standard deviation BCVA was 67.8 ± 10.3 letters, and baseline CMT was 417 ± 91μm. Completion of study visits is summarised in Table 3.2.

Table 3.1: Baseline characteristics of patient cohort

Characteristic	**Data**
Number of patients	43
Age (years), mean ± SD	62.9 ± 9.7
Male, n (%)	27 (62.7)
Right eyes, n (%)	21 (48.8)
Baseline BCVA (letters), mean ± SD	67.8 ± 10.3
Baseline CMT (μm), mean ± SD	417 ± 91
Pseudophakic eyes, n (%)	13 (30.2)
Type 1 Diabetics, n (%)	5 (11.6)
Duration of diabetes (years), mean ± SD	17.4 ± 10.6
HbA1c (%), mean ± SD	8.0 ± 1.7
Duration of anti-VEGF treatment (months), median (interquartile range)	18 (8-34)
Total number of anti-VEGF injections, median (interquartile range)	12 (7-24)
Interval between last bevacizumab and baseline aflibercept injection (days), mean ± SD	42.4 ± 13.1
Prior treatments in study eye Focal/grid macular photocoagulation, n (%) Panretinal photocoagulation, n (%) Vitrectomy, n (%) Triamcinolone, n (%)	 18 (41.9) 17 (39.5) 5 (11.6) 2 (4.6)

SD = standard deviation, BCVA= best corrected visual acuity, CMT= central macular thickness VEGF=vascular endothelial growth factor

Table 3.2: Summary of study visits and missed appointments

	Visit Week												
Patient ID	Week 1	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24	Week 28	Week 32	Week 36	Week 40	Week 44	Week 48
001										X			
002													
003													
004													
005													
006				X									
007												X	
008	X											X	
009		Withdrawn from study											
010													
011													
012													
013													
014						X		X		X	X	X	
015													
016													
017													
018						X						X	
019													
020													
021				Withdrawn from study									
022													
023													
024													
025													
026													
027												X	
028										X	X	X	X

029													
030						X							
031													
032													
033													
034													
035													
036													
037													
038						X							
039	X							X		X			
040													
041								X		X		X	
042								X					
043	X												

X =missed visit

Visual outcomes post switch to aflibercept

The changes in vision are summarised in Figure 3.1 with rates of changes further categorised by magnitude in Table 3.3. Mean ± standard deviation BCVA improved from 67.8 ± 10.3 letters to 71.7 ± 9.6 letters at 48 weeks (3.9 ± 7.0 letter gain, p=0.001). Vision gain was greater in patients with a poorer BCVA (less than 69 letters) at baseline (6.9 ± 7.4 vs. 1.7 ± 5.9 letter gain, p=0.02). Simple linear regression found a significant association between baseline BCVA and 48-week vision gain (standardised coefficient -0.44, p=0.004) with an R^2 = 0.19. Patients who gained 5 or more letters compared to those who gained less than 5 letters after the first injection at 4 weeks had superior vision gain at 48 weeks (8.9 ± 5.7 vs. 1.8 ± 6.5 letter gain, p=0.002). Multiple regression was performed to assess the effect of both baseline BCVA (standardised coefficient -0.31, p=0.04) and a 5 or greater letter gain at 4 weeks (standardised coefficient 0.37, p=0.01) on vision gain at 48-weeks with an R^2 = 0.31. Multiple regression did not identify a statistically significant effect of other variables on BCVA change at 48-weeks (Table 3.4).

Table 3.3: Vision and macular thickness changes 48 weeks after switching from bevacizumab to aflibercept

Characteristic	Data
BCVA change at 48 weeks	
≥10 letter gain, n (%)	10 (24)
5-9 letter gain, n (%)	7 (17)
<5 letters lost or gained, n (%)	19 (46)
5-9 letter loss, n (%)	4 (10)
≥10 letter loss, n (%)	1 (3)
CMT change at 48 weeks	
≥50µm reduction, n (%)	21 (51)
<50µm reduction or gain, n (%)	15 (37)
≥50µm gain, n (%)	5 (12)

BCVA=best corrected visual acuity, CMT=central macular thickness

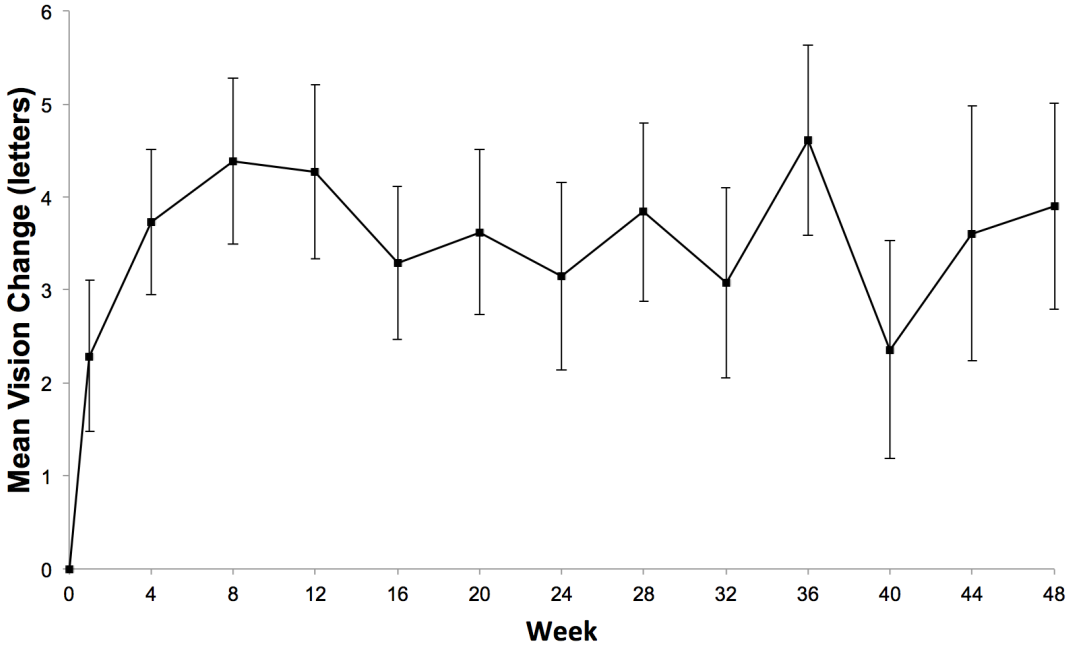


Figure 3.1: Graph demonstrating the change in mean best-corrected visual acuity (in letters) over 48 weeks compared with baseline values.
Error bars correspond to standard error of the mean.

Table 3.4: Multiple regression to assess effect of different factors on change in visual acuity

Parameter	Unstandardised coefficient	Standardised coefficient	P- value
Baseline vision	-0.453	-0.681	0.01
Age	-0.212	-0.277	0.24
Gender	-0.875	-0.055	0.78
Type of diabetes	0.388	0.200	0.927
Duration of diabetes	0.131	0.173	0.446
HbA1c	-1.526	-0.366	0.169
Previous PRP	-1.083	-0.075	0.733
Previous focal/grid macular photocoagulation	-2.396	-0.161	0.465
Number of prior intravitreal anti-VEGF injections	-0.216	-0.334	0.172
Duration of prior anti-VEGF therapy	0.043	0.151	0.543
Presence of subretinal fluid	-1.185	-0.060	0.763
Pseudophakia	-2.212	-0.148	0.504

PRP=panretinal photocoagulation, HbA1c=glycated haemoglobin

Anatomical and morphological outcomes post switch to aflibercept

Change in CMT is summarised in Figure 3.2 with further categorisation of change summarised in Table 3.3. Overall, mean CMT improved from 415 ± 92μm to 357 ± 108μm (-59 ± 114μm difference, p=0.002) at week 48. There was no difference in change in CMT for patients with a CMT less than or greater than or equal to 400μm at baseline (-87 ± 154μm vs. -35 ± 60μm, p = 0.19).

Morphological OCT findings at baseline and at the conclusion of the study are summarised in Table 3.4. Nine eyes had no SRF or intraretinal fluid present at 48 weeks of follow-up. There was a significantly greater reduction in CMT among these "dry" eyes (-135 ± 168μm vs. -36 ± 82μm, p = 0.02) but not a significant difference in vision gain (5.1 ± 6.5 vs. 3.3 ± 7.4 letter gain, p=0.50). Patients with SRF, ELM and ISe disruption and DRIL had worse BCVA at baseline and 48-weeks (Table 3.4). Multiple regression did not identify any a statistically significant effect of other variables on CMT change at 48-weeks (Table 3.5).

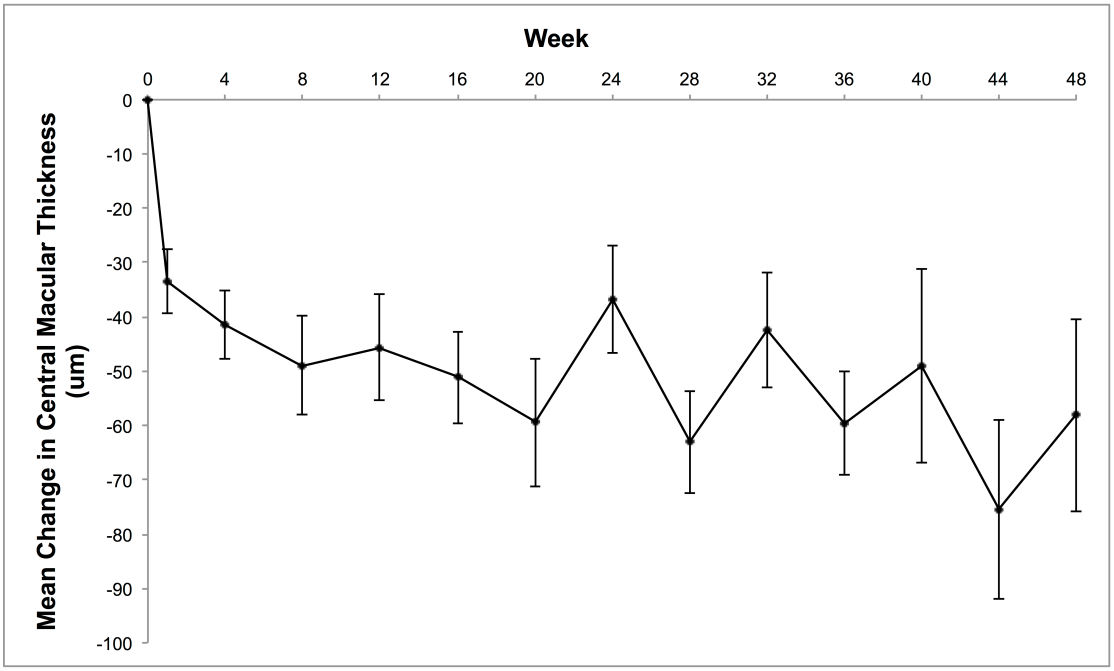


Figure 3.2: Graph demonstrating the change in mean central macular thickness (in micrometers) over 48 weeks compared with baseline.

Error bars correspond to standard error of the mean.

Table 3.4. Baseline anatomical features prior to switch from bevacizumab to aflibercept for diabetic macular oedema (DMO)

Characteristic	Baseline	Baseline BCVA with characteristic	Baseline BCVA without characteristic	Difference (p-value) *	48 weeks	48-week BCVA with characteristic	48-week BCVA without characteristic	Differenc (p-value)
DMO pattern								
Intraretinal fluid, n (%)	41 (100)	67.8 ± 10.3	N/A	N/A	32 (78)	69.7 ± 9.4	77.9 ± 8.1	**8.2 ± 3.5 (p=0.02)**
Subretinal fluid, n (%)	7 (17)	60.0 ± 8.1	69.4 ± 10.1	**9.4 ±4.1 (p=0.03)**	0 (0)	N/A	71.5 ± 9.7	N/A
VMA, n (%)	9 (22)	67.3 ± 10.9	70.1 ± 7.2	2.8 ±4.3 (p=0.52)	6 (15)	70.3 ± 9.9	71.7 ± 9.8	1.3 ± 4.3 (p=0.77)
ELM disruption, n (%)	29 (71)	59.7 ± 9.5	71.2 ± 8.8	**11.5 ± 3.1 (p<0.01)**	27 (66)	68.2 ± 8.6	77.8 ± 8.6	**9.6 ± 2.8 (p<0.01)**
ISe band disruption, n (%)	33 (80)	65.8 ± 10.2	75.1 ± 7.5	**9.4 ± 3.7 (p=0.01)**	38 (93)	71.2 ± 10.0	75.3 ± 1.5	**4.2 ± 1.8 (p=0.03)**
DRIL ≥50% in central 1mm, n (%)	33 (80)	66.2 ± 10.5	74.3 ± 6.7	**8.0 ± 3.0 (p=0.02)**	32 (78)	69.2 ± 9.0	79.7 ± 7.5	**10.5 ± 3.** **(p<0.01)**

VMA=vitreomacular adhesion, ELM=external limiting membrane, ISe=inner segment ellipsoid, DRIL= disorganisation of the inner retinal layers, BCVA=best-corrected visual acuity, *independent samples t-test

Table 3.5: Multiple regression to assess effect of different factors on change in central macular thickness

Parameter	Unstandardised coefficient	Standardised coefficient	P- value
Baseline CMT	-0.548	-0.428	0.063
Age	3.635	0.278	0.183
Gender	-89.553	-0.328	0.070
Type of diabetes	22.849	0.068	0.721
Duration of diabetes	-1.679	-0.130	0.526
HbA1c	13.582	0.191	0.349
Previous PRP	58.965	0.238	0.235
Previous focal/grid macular photocoagulation	0.067	0.000	0.999
Number of prior intravitreal anti-VEGF injections	2.340	0.212	0.411
Duration of prior anti-VEGF therapy	-2.475	-0.510	0.079
Presence of subretinal fluid	-21.938	-0.065	0.693
Pseudophakia	-41.851	-0.164	0.409

CMT=central macular thickness, PRP=panretinal photocoagulation, HbA1c=glycated haemoglobin

Fellow Eye Outcomes

Overall, BCVA remained stable over the study period (0.3 ± 6.1 letter change, p=0.73) in the fellow eye of the 41 patients in the final analysis. There was a small but statistically significant reduction in the CMT of the fellow eye from baseline to week 48 (-25 ± 46μm, p = 0.001).

Of the 41 patients, 16 were receiving concomitant treatment with monthly as needed bevacizumab for DMO in the fellow eye. When these patients were excluded from analysis, there was no significant difference in vision (0.1 ± 7.2 letter change, p=0.93) or CMT (-19 ± 45μm p=0.052) for the remaining 25 patients from baseline to week 48.

For the 9 patients whom initially had both eyes eligible for inclusion in the trial, there were similar vision (1.9 ± 5.4 vs. 1.0 ± 4.0 letter gain, p=0.70) and CMT (-57 ± 65μm vs. -55 ± 63μm, p = 0.95) outcomes between the eye that was switched to aflibercept and the fellow eye that remained on bevacizumab (paired t-tests). For treated fellow eyes, there were a greater number of injections administered (median 12 vs. 8 injections).

Diabetic Retinopathy Severity

Of the 41 patients who completed the trial, 20 had scatter panretinal photocoagulation (PRP) for proliferative DR (PDR). For the patients who did not have quiescent PDR, 1 had moderately severe non-proliferative DR (NDPR), 12 had moderate NPDR and 8 had mild NPDR. Of these patients, Diabetic

Retinopathy Severity Score (DRSS) improved by 1-step in 6 (28%), worsened by 1-step in 1 (5%) and remained unchanged in 14 (67%). An illustration of this is shown in Figure 3.3. Lesions were predominantly peripheral in 6 (28%) of these 21 eyes. BCVA (6.8 ± 6.2 vs. 3.7 ± 7.2 letter gain, p = 0.33) and CMT (-64 ± 99μm vs. -62 ± 119μm, p = 0.96) outcomes were similar in patients with regression of disease compared to those who remained stable or worsened.

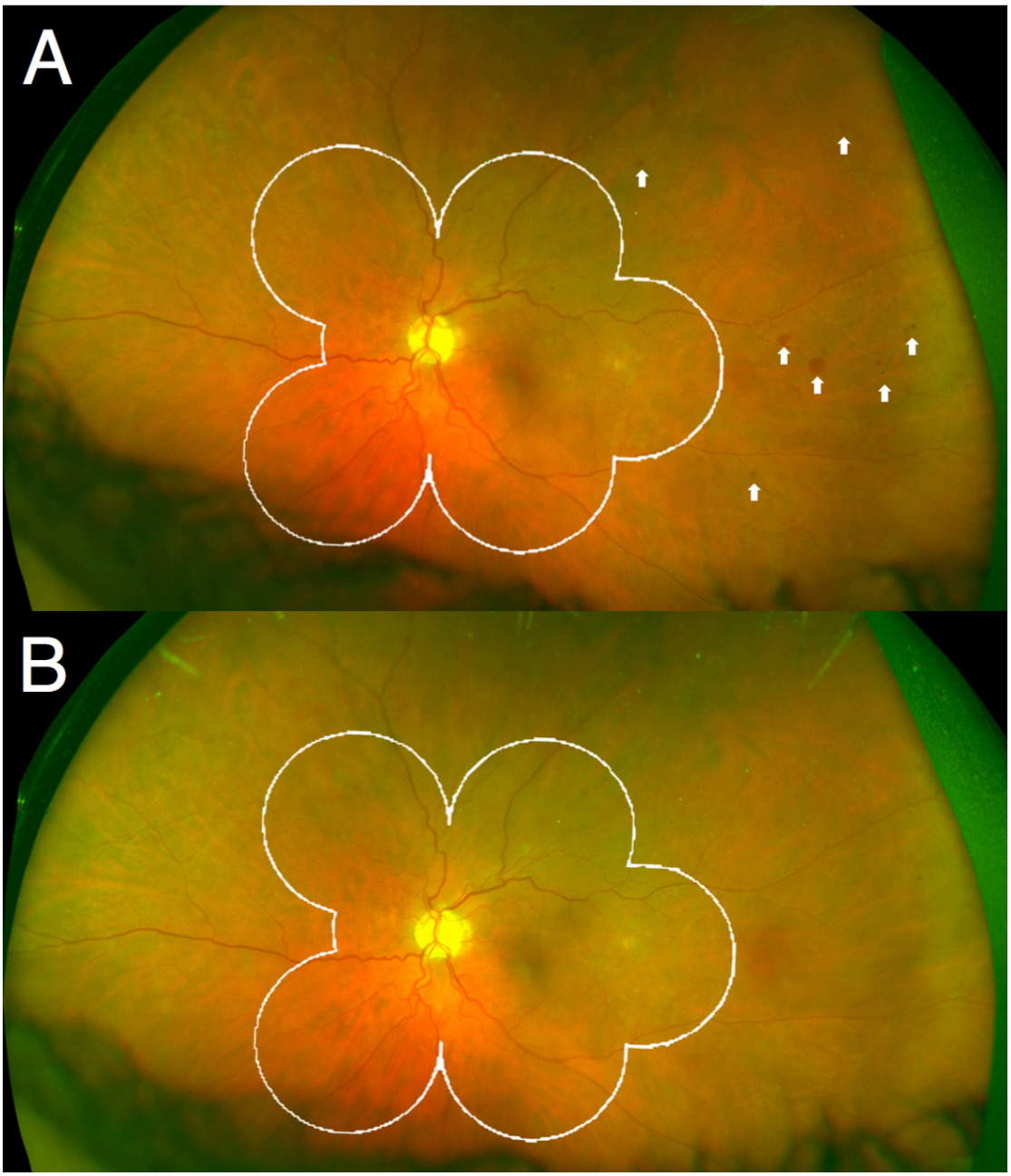


Figure 3.3: Ultra-widefield imaging at (A) baseline and (B) 48-weeks post switch to aflibercept therapy in a sample patient.

Standard ETDRS 7-field area is superimposed for reference. Arrows identify microaneurysms and hemorrhages.

Initial response to bevacizumab

Median duration (interquartile range) of treatment with bevacizumab prior to starting trial was 12 (7-24) injections over 18 (8-34) months. Vision data and CMT measurements were available for 39 and 30 patients respectively from referring practitioners. There was no significant improvement in vision (-1.0 ± 8.4 letters, p=0.48) or CMT (-22 ± 97 um, p=0.002) in these patients during this period.

Visual outcomes at 48 months were similar among eyes demonstrating a good initial response to bevacizumab prior to switch (5 or more letter gain) compared to eyes with a poorer initial response (1.2 ± 4.9 vs. 4.1 ± 7.0 letter gain at 48 weeks, p=0.25).

Safety data

Adverse events encountered during the study are summarised in Table 3.6. Notable ocular adverse events included a rhegmatogenous retinal detachment. There was no progression of cataract severity or raised IOP in any of the study eyes and no patients required medical or surgical intervention for cataract or raised IOP.

Table 3.6. Ocular and systemic adverse events

Event	Frequency
Pneumonia	2 (4%)
Hypoglycemia	2 (4%)
Myocardial Infarction	1 (2%)
Transient Ischemic Attack	1 (2%)
Retinal detachment	1 (2%)
Spinal Fusion	1 (2%)
Bronchitis	1 (2%)
Cellulitis in leg	1 (2%)
Renal failure requiring dialysis	1 (2%)
Prostatitis	1 (2%)
Diabetic Ketoacidosis	1 (2%)

DISCUSSION

Persistent DMO following treatment with bevacizumab is a clinical management challenge. This trial provides prospective evidence that aflibercept may be effective in improving anatomical and vision outcomes in these patients over a 48-week period. Approximately 40% of patients gained one or more lines of vision and vision loss was rare.

There is limited data from prospective studies investigating a switch from bevacizumab to aflibercept. One prospective study of 14 eyes reported improvements in CMT but not in visual acuity when switching from bevacizumab or ranibizumab to aflibercept over a period of one-month. [86] Additionally, several retrospective studies have evaluated the effect of a switch in therapy from bevacizumab to aflibercept for persistent DMO with follow up periods ranging from one to five months. [83-85, 235] All of these have consistently demonstrated statistically significant reductions in CMT ranging from 60-124um. Significant improvement in visual acuity was observed in two of these studies with a mean gain of 0.05 and 0.06 logMAR, a similar gain to what was measured in this trial. [84, 235]

The effects seen in switching studies may relate to the differing structure and function of aflibercept compared with the monoclonal antibody bevacizumab. Aflibercept is a protein formed by the fusion of the binding domains from both VEGF receptor (VEGFR)-1 and VEGFR-2. The binding affinity of this drug to VEGF-A, the main member of the VEGF family responsible for the major pathological effects in DMO, is greater than that of bevacizumab. [226] Due to its

structure it additionally binds other members of the VEGF family, VEGF-B and placental growth factor (PlGF). [226] There are in vitro and in vivo evidence implicating elevated levels of PlGF in the pathogenesis of DR as well as DMO. [23] Aflibercept has also been shown to interact with galectin-1, a factor implicated in anti-VEGF-A refractory neoplasms. [56, 57] This protein may also play a role in the pathogenesis of DR. [56]

Response to anti-VEGF drugs may also be related to genetic variation in the VEGF-A gene. The CC genotype of the C-634G polymorphism of this gene has been associated with improved response to bevacizumab in the management of DMO, with patients significantly more likely to gain three or more lines in vision and have a greater than 50% reduction in their CMT. [236] It is possible that DMO in patients with certain polymorphisms in the VEGF-A gene may have disease increasingly driven by VEGF and will respond better to increased blockade of VEGF signaling pathways.

Both good and poor early response within the first 12 weeks of treatment has been correlated with 12-month to 3-year outcomes in other retrospective series and randomised trials treating DMO with bevacizumab, ranibizumab and the dexamethasone implant. [231, 237, 238] The data from this trial adds to this evidence and may aid clinicians in individualizing a management strategy.

Vision gains overall may have been limited due to a ceiling effect in some of these patients. This is reflected in patients with a worse baseline BCVA having greater gains at 48-weeks. Interestingly, the reduction in CMT in patients was not

different for those with a thicker CMT ≥400μm. This may suggest, an anatomical ceiling effect may not be as significant in patients with persistent DMO. Analyses based on baseline BCVA are also subject to confounding by regression to the mean, where those with a poorer baseline measurement would on average have a greater improvement in subsequent measurements and should be interpreted with this in mind.

Chronic macular edema may lead to irreversible damage to the neural retina, limiting visual gain as seen in the delayed anti-VEGF treatment arms of RISE/RIDE and VISTA/VIVID. [64, 71] The chronic nature of DMO in these patients may also explain why other commonly identified biomarkers for vision improvement, such as SRF, young age and duration of diabetes, may not be applicable as prognostic markers. [239] This includes OCT markers, such as SRF, DRIL, ISe band and ELM integrity. These markers have all been correlated with visual acuity, as was demonstrated in our study at baseline and at 48-weeks. [178-181] Presence of these factors at baseline was not associated with a worse outcome at 48-weeks, as other studies have shown. [240, 241] Additionally, we did not find a restoration of ISe band integrity, as others have suggested following treatment in treatment naïve patients treated with bevacizumab and ranibizumab. [139, 241]

Anatomically, the mean CMT reduction began to fluctuate at the point of treatment extension to 8 weeks. This finding is consistent with that of the VISTA/VIVID studies of mostly treatment naïve eyes. [71] These fluctuations are likely due to re-accumulation of fluid in a proportion of patients who may

require more frequent treatment to prevent the re-accumulation of DMO. Despite this, a significant reduction in CMT was maintained throughout all follow-up points and was maximal at 44 weeks.

These data also support previous reports of aflibercept promoting the regression of DR. [71, 79] This disease modifying effect in patients with prior anti-VEGF treatment was also observed in a post-hoc analysis of the VISTA/VIVID studies. [232] In that study, the effect of DR regression was similar in patients with DMO who were treatment naïve compared with those previously treated with anti-VEGF drugs.

Furthermore, the use of ultra-widefield imaging helped to accurately quantify the DRSS, 28% of which would have been otherwise missed by ETDRS 7SF imaging. This finding is consistent with a comparative study of where the number of hemorrhages and microaneurysms identified increased by 50% when using UWF images. [242] Whilst improvement in DRSS did not correlate with vision or CMT outcomes in our trial, peripheral retinal examination has important prognostic implications for progression of DR with peripheral lesions predicting risk of progression of DR. [233]

This study's strengths are the prospective design, standardised examinations inclusion criteria and treatment regime. Patients were treated intensively with bevacizumab prior to switch with no washout period to allow for re-accumulation of edema and exaggerate any treatment effect. This may be why no difference was seen in VISTA/VIVID patients with and without prior anti-VEGF

therapy, who had a washout period of three months prior to enrollment. [232] There was no additional macular laser administered, as was mandated in Protocol T, also removing another potential confounder for treatment effect. [77]. Finally, the dose of bevacizumab used prior to switch was higher than other trials for DMO (2.5mg vs. 1.25mg), which may be more effective for persistent DMO. [80]

This study is limited by the lack of a control group and a small sample size. There was no significant change in vision or CMT in a post-hoc analysis of untreated fellow eyes suggesting a treatment effect. Protocol I data suggests that patients with persistent DMO at 3 or 6 months post initiation of therapy would continue to improve if kept on the same therapy. [243] Supporting this, for fellow eyes continuing treatment with bevacizumab, there were similar vision and CMT outcomes in our trial. There was, however, a larger treatment burden in these fellow eyes, requiring a median of 12 injections compared to 8 in the eye receiving treatment with aflibercept.

Switching therapy from bevacizumab to aflibercept may be an effective strategy in the management of persistent, chronic DMO. The data from this prospective study shows that an early response to a switch in therapy may predict longer-term outcomes in this cohort of patients.

Chapter 4: The influence of retinal pathology on the reliability of macular thickness measurement: a comparison between optical coherence tomography devices

Optical coherence tomography has revolutionised the diagnosis and management of macular pathology. So ubiquitous have these devices become that outcomes from clinical trials use automated measurements generated by these devices as a reference standard.

The purpose of this chapter is to highlight the differences in measurements and readings provided by different OCT machines and to demonstrate the effect pathology has on repeatability and reliability. Patients enrolled in the clinical trial from this section of the thesis are compared with that of patients enrolled in a study of age-related macular degeneration as well as a sample of normal controls.

The results of this Chapter have important implications for the interpretation of anatomical outcomes from clinical trials.

The material presented in this chapter has been published and is reproduced from:

Bahrami B, Ewe S, Hong T, Zhu M, Ong G, Luo K, Chang A. Influence of retinal pathology on the reliability of macular thickness measurement: a comparison between optical coherence tomography devices. Ophthalmic Surg Lasers Imaging Retina. 2017 Apr 1;48(4):319-325

Furthermore, the data presented in this chapter has been published and presented at the following conference:

RANZCO 47th Annual Scientific Conference: Wellington, New Zealand, 2015
Ewe SYP, **Bahrami B**, Zhu M, Hong T, Ong GJM, Chang A. A Comparison of Macular Thickness Measurements Across Three Different Spectral-Domain Optical Coherence Tomography (SD-OCT) Machines. Clin Exp Ophthalmol. 2015 Oct; 43 (S1):79-123.

ABSTRACT

Purpose: To evaluate the repeatability, reliability and comparability of macular thickness measurements between three optical coherence tomography (OCT) machines in healthy eyes, eyes with diabetic macular oedema (DMO) and eyes with neovascular age-related macular degeneration (nAMD).

Methods: Twenty-three eyes with DMO, 26 eyes with nAMD, and 24 healthy eyes as controls were evaluated. Scans were performed on the swept-source Triton (Topcon) as well as the spectral-domain Cirrus (Zeiss) and Spectralis (Heidelberg) machines. Scans were evaluated for central macular thickness (CMT), presence of segmentation and fixation imaging artifacts (IA), re-scan reliability and agreement between machines and groups.

Results: Mean CMT was significantly different between all OCT machines in all groups ($p<0.01$ for all comparisons). Manually correcting IA did not alter these results. There was good scan repeatability among healthy and DMO eyes for each machine, but poor repeatability among the nAMD group with the Spectralis ($p=0.038$). IA was significantly increased in the presence of pathology.

Conclusion: There is poor agreement of CMT measurement between OCT machines in healthy eyes and those with DMO and nAMD. DMO and nAMD have a significant effect on the rate of IA in scans. Care is required when interpreting measurements from different OCT devices in clinical practice and research settings.

BACKGROUND

The development of optical coherence tomography (OCT) has revolutionised the diagnosis and management of retinal pathology. OCT is an increasingly accessible technology, with over 35 device manufacturers producing machines for commercial and research use. [244] These machines utilise different software and hardware to analyse obtained images. As a consequence of these variations, retinal thickness measurements obtained from different OCT machines are not comparable. Additionally, readings from individual machines may be unreliable due to image segmentation errors and artifacts. These differences are apparent in both healthy eyes as well as those with pathology. [150, 206, 245-254]

Reliable measurements of central macular thickness (CMT) are important in common vision threatening conditions such as neovascular age-related macular degeneration (nAMD) and diabetic macular oedema (DMO). This information can be indicative of disease activity and is an outcome measure in many clinical trials of these conditions. [122, 255-259] With the ubiquity of OCT, it is not uncommon for a patient to have multiple retinal scans performed by different practitioners on different machines at different times. Thus, interpreting quantitative data obtained from different machines poses a challenge.

In this study, we evaluate the reliability and comparability of CMT measurements and rates of imaging artifacts on two commonly used spectral-domain (SD) and a newer swept-source (SS)-OCT machine. We also evaluate the influence of DMO and nAMD on the OCT scan performance.

METHODS

Patients and volunteers from a single tertiary referral center were recruited for this cross-sectional study. Participants were divided into three groups: eyes with no previous ocular history used as control, eyes diagnosed with DMO and eyes diagnosed with nAMD. All study assessments were performed after obtaining informed consent from all participants and were conducted in accordance with the tenets of the Declaration of Helsinki.

Recruited patients had a best-corrected visual acuity (BCVA) greater than or equal to 55 ETDRS letters (Snellen equivalent 20/80) in the study eye and were able to fixate on machine-generated targets. Tropicamide 1% was used for pupillary dilation prior to posterior segment examination and scanning.

All OCT scans were performed by four technicians with previous clinical trial scanning experience. Scans were performed using the swept-source SS Triton DRI-OCT (Figure 4.1A, Topcon Corporation, Tokyo, Japan, software version 10.0), Spectralis SD-OCT (Figure 4.1B, Heidelberg Engineering, Heidelberg, Germany, software version 6.4.8.0) and Cirrus SD-OCT (Figure 4.1C, Carl Zeiss Meditec Inc., Dublin, California, USA, software version 6.0.2.81). For each participant, the same OCT operator performed all OCT scans on each of the machines on the same day. Two replicate scans were performed for each equivalent scanning protocol on all three OCT machines (Triton: 3D Macular Scan; Cirrus: Macular Cube 512x128; Spectralis: Dense Scan (49 lines)).

Figure 4.1: The three optical coherence tomography devices used in this study. A) Topcon Triton DRI OCT (image reproduced from https://bit.ly/2Hqlfty) , B) Heidelberg Spectralis OCT (image reproduced from https://bit.ly/2qTxRPE) and C) Zeiss Cirrus OCT (image reproduced from https://bit.ly/2FeBQuh)

Outcome measures were CMT, defined as the mean thickness from Bruch's membrane to the inner retinal border within the central 1 mm circle of the ETDRS grid, presence of imaging artifacts (IA), re-scan repeatability and agreement between machines and groups.

IA were classified as related to segmentation or fixation errors. Segmentation artifacts were due to inappropriate automated segmentation of retinal layers resulting in inaccurate retinal thickness measurements (Figure 4.2). Fixation artifacts were associated with inappropriate identification of the fovea (Figure 4.3). IA were subsequently corrected manually and analyses were redone.

Results were analyzed using IBM SPSS (version 21; SPSS Inc, Chicago, Illinois, USA). Paired t-tests were used to investigate differences between two repeated scans for reliability on each OCT machine, and between each pair of OCT machines for comparability. Mean measurements of the two replicate scans were used when evaluating the comparability between each pair of the three OCT machines. For the analyses presented, all eyes studied were considered independent from one another. A p value <0.05 was considered to be a significant difference.

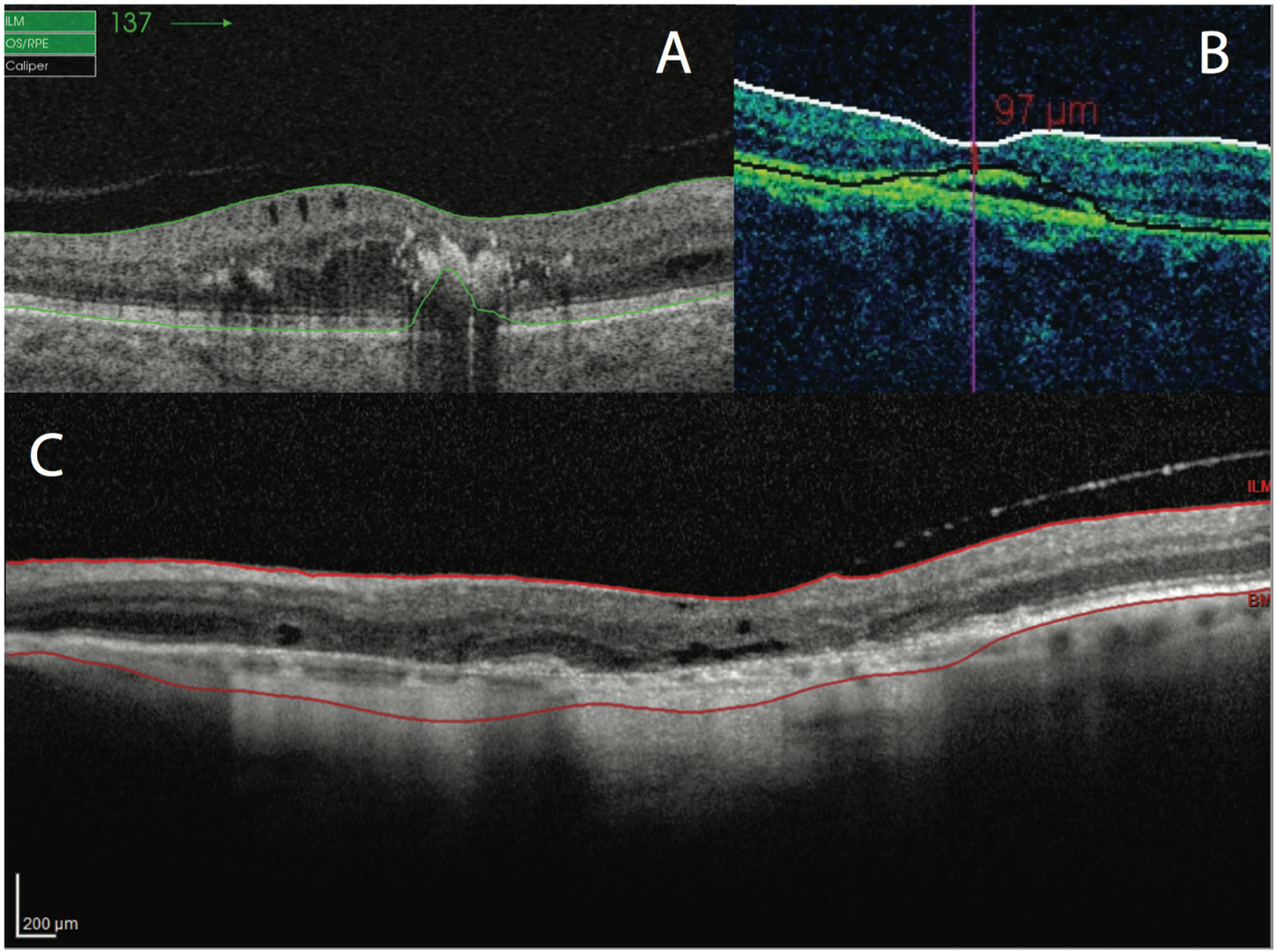


Figure 4.2: (A) Segmentation error with the Triton device due to retinal hard exudate in an eye with diabetic macular oedema. (B) Segmentation error with the Cirrus device due to pigment epithelium detachment in an eye with neovascular age-related macular degeneration (nAMD). (C) Segmentation error with the Spectralis device due to geographic atrophy in an eye with nAMD.

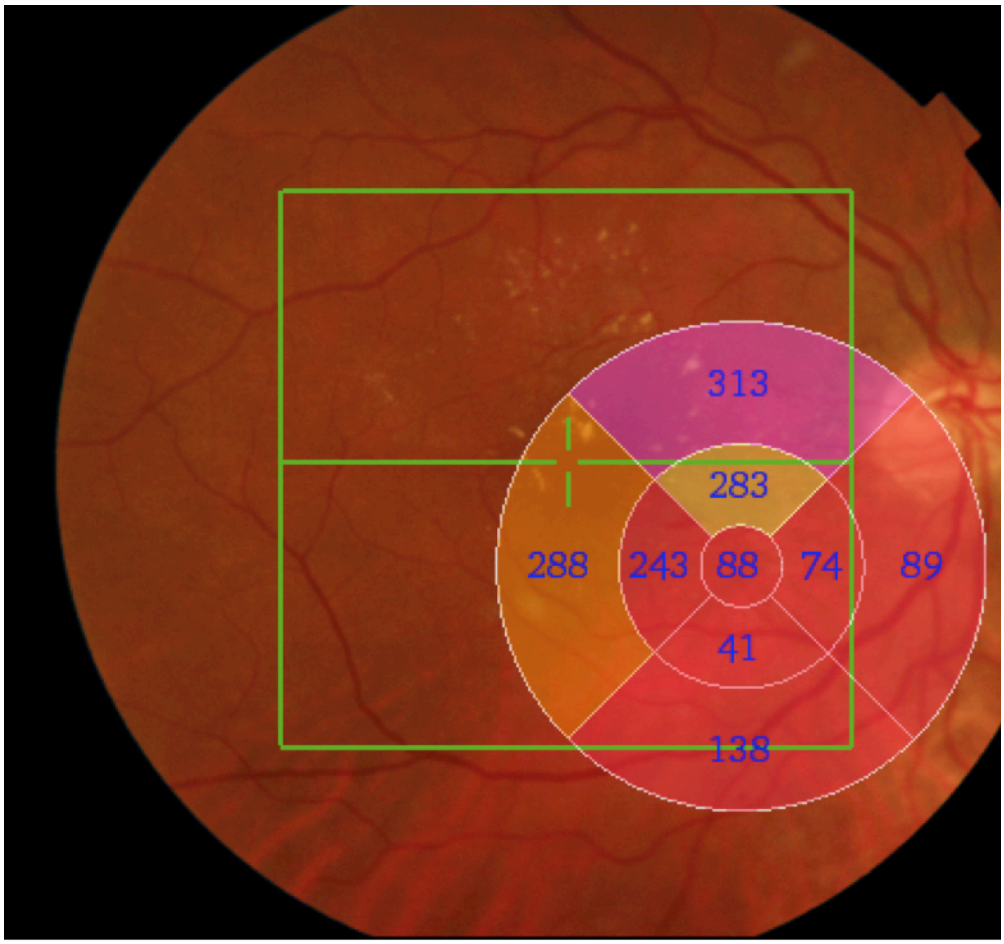


Figure 4.3: Fixation error on the Triton device resulting in incorrect ETD placement and calculation of central macular thickness.

RESULTS

Subjects

A total of 73 eyes from 45 participants were enrolled during the study period between June and August 2015. The control group consisted of 24 healthy eyes of 12 participants with no history of ocular disease, the nAMD group consisted of 26 eyes of 17 participants and the DMO group consisted of 23 eyes of 16 participants. Both the nAMD and DMO groups were undergoing treatment with anti-vascular endothelial growth factor (anti-VEGF) drugs. Baseline characteristics of the entire cohort are summarised in Table 4.1.

Table 4.1: Baseline characteristics of study participants

Characteristic	Control group	DMO group	nAMD group
Participants (n)	12	16	17
Eyes (n)	24	23	26
Female (n)	9	8	11
Age, mean years (SD)	42 (14.1)	62.1 (8.3)	83.5 (6.0)
BCVA, mean letters (SD)	55.8 (1.9)	47.4 (9.3)	42.2 (11.2)
Phakic (n)	24	20	11
Spectralis OCT			
CMT (µm), mean (SD)	265.0 (14.5)	337.7 (65.9)	286.3 (72.9)
Cirrus OCT			
CMT (µm), mean (SD)	245.9 (14.1)	319.8 (66.5)	229.6 (70.6)
Triton OCT			
CMT (µm), mean (SD)	231.5 (11.8)	287.4 (69.9)	213.2 (66.7)

DMO= diabetic macular oedema, nAMD= neovascular age-related macular degeneration, n = number, SD = standard deviation, OCT = optical coherence tomography, CMT = central macular thickness

Repeatability of Macular Scans

All test-retest repeated CMT measurements performed for the entire cohort demonstrated reliability in each machine with no significant differences between the first and second scans (Spectralis 3.0μm (95% CI -1.1 to 7.2μm, p=0.15), Cirrus -0.4μm (95% CI -2.8 to 2.0μm, p=0.74), Triton -0.4μm (95% CI -3.3 to 2.4μm, p=0.77)).

Subgroup analysis showed that the control and DMO groups maintained repeatability of scans across all 3 machines. The nAMD group, however, showed relatively poor repeatability, with a significant difference identified on CMT measurements obtained from the Spectralis (p=0.038), but not from the Triton or the Cirrus (Table 4.2).

Table 4.2: Mean difference between two consecutive scans stratified by subgroup before and after re-segmentation to correct for imaging artifacts.

		Before re-segmentation			After re-segmentation		
Subgroup	**OCT machine**	**Mean CMT (µm)**	**95% CI**	**P value**	**Mean CMT (µm)**	**95% CI**	**P value**
Control	***Spectralis***	-0.88	-4.7 to 2.9	0.64	0.79	-0.6 to 2.2	0.26
	Triton	-0.17	-1.1 to 0.8	0.72	-0.17	-1.1 to 0.8	0.72
	Cirrus	-2.38	-5.7 to 0.9	0.15	-1.29	-4.1 to 1.5	0.36
DMO	***Spectralis***	-1.83	-5.9 to 2.2	0.36	-3.26	-7.7 to 1.1	0.14
	Triton	-1.65	-6.6 to 3.3	0.49	0.61	-4.3 to 5.5	0.80
	Cirrus	0.13	-5.2 to 5.5	0.96	-0.74	-7.1 to 5.7	0.81
nAMD	***Spectralis***	10.96	0.7 to 21.2	**0.04**	-2.54	-7.8 to 2.7	0.33
	Triton	0.50	-4.9 to 5.9	0.85	3.65	-1.9 to 9.2	0.19
	Cirrus	0.88	-5.3 to 7.0	0.77	0.73	-2.2 to 3.7	0.62
Overall	***Spectralis***	3.04	-1.0 to7.1	0.15	-1.67	-3.9 to 0.6	0.15
	Triton	-0.39	-2.7 to 1.9	0.74	-0.40	-2.7 to 1.9	0.74
	Cirrus	-0.42	-3.2 to 2.3	0.77	1.41	-1.7 to 4.5	0.77

DMO= diabetic macular oedema, nAMD= neovascular age-related macular degeneration, n = number, OCT = optical coherence tomography, CMT = central macular thickness, CI=confidence interval

Imaging Artifacts (IA)

There was a low occurrence of IA in the control group (4.2%, 8.3% and 8.3% in Spectralis, Triton, Cirrus respectively). Higher rates of artifacts occurred in the DMO group (47.8%, 52.2% and 34.8% in Spectralis, Triton, Cirrus; $p<0.05$ compared to controls) and in the nAMD group (84.6%, 50%, 42.3% in Spectralis, Triton, Cirrus; $p<0.05$ compared to controls). When comparing machines, the incidence of IA in nAMD eyes was significantly higher on the Spectralis compared to Triton ($p=0.02$) and Cirrus ($p<0.01$). The prevalence of IA was otherwise similar in the control group and the DMO group across all 3 machines.

Poor reliability of repeated measures were associated with IA. This was supported by the overall scan-rescan reliability when manual re-segmentation corrected these errors (Table 4.2). The incidence of IA and the breakdown of pathological lesions found in the groups are summarised in Table 4.3.

Table 4.3: Incidence of artifacts by optical coherence tomography machine and subgroup.

		Total artifact, n (%)	Artifact type		P value*
OCT machine	**Subgroup**		***Segmentation***	***Fixation***	
Spectralis	***Control***	1/24 (4.2%)	0	1	
	DMO	11/23 (47.8%)	5	6	<0.01
	nAMD	22/26 (84.6%)	19	12	<0.01
Triton	***Control***	2/24 (8.3%	2	0	
	DMO	12/23 (52.2%)	5	7	<0.01
	nAMD	13/26 (50%)	10	3	<0.01
Cirrus	***Control***	2/24 (8.3%)	2	0	
	DMO	8/23 (34.8%)	3	2	0.04
	nAMD	11/26 (42.3%)	11	1	0.01

*P-value compared to control group within OCT machine type

DMO= diabetic macular oedema, nAMD= neovascular age-related macular degeneration, n = number, OCT = optical coherence tomography

Comparability between OCT Machines

Significant differences were seen in CMT across all three machines amongst the whole cohort as well as in each subgroup. The CMT mean measurements consistently measured highest in Spectralis, followed by the Triton and then the Cirrus, showing low agreement between machines ($p<0.05$ in all group comparisons) (Figure 4.4). These differences remained evident even after manual re-segmentation of scans to correct for IA.

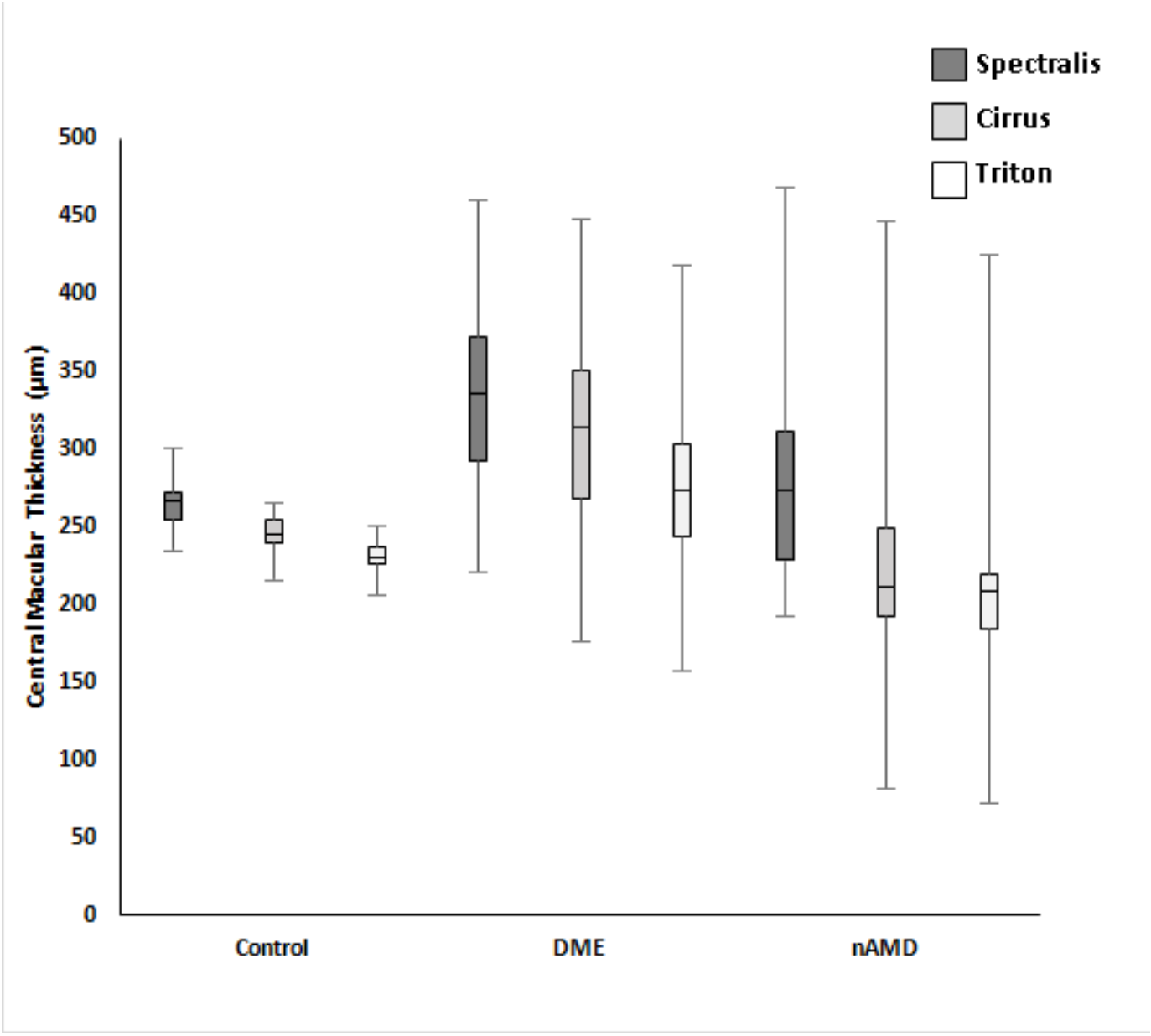


Figure 4.4: Average central macular thickness (CMT) for different groups and machines following manual resegmentation.

A consistent difference in measurements was found across all three optical coherence tomography machines, with the Spectralis device consistently recording the highest CMT values, followed by the Cirrus and the Triton devices, respectively, in all three groups.

DISCUSSION

In this study, there was good repeatability of OCT scans in both healthy eyes as well as those with nAMD and DMO. However, there was a significantly higher rate of IA in eyes with pathology, reducing the reliability of scans in these settings. Additionally, CMT measurements were not comparable between these three machines, with poor agreement of values in healthy eyes and those with pathology. Recorded CMT was highest in the Spectralis, followed by the Cirrus and then the Triton in all groups.

Similarly, previous studies have demonstrated good reliability and reproducibility of a number of SD-OCT machines in calculating CMT in healthy eyes, including the Spectralis and Cirrus. [150, 246, 247, 250, 252] Additionally, differences have been demonstrated between OCT machines in both healthy eyes as well as those with pathology. [150, 206, 245-247, 249-252, 254] However, these studies included eyes with only one form of pathology or mixed pathology with small sample sizes and no subgroups analyses. Only one study by Ho et al performed a subgroup analysis across differing pathology, corrected for IA and re-performed analyses finding a similar effect of pathology on IA. [245]

Outer retinal pathology such as subretinal fibrosis, drusen and geographic atrophy encountered in nAMD were more likely to result in segmentation IA. This was most evident in the Spectralis which generated differing automated segmentation on repeated scans in this group. Eyes with gross central macular thickening were more likely to have fixation IA, likely due to loss of detection of the normal foveal contour.

Variances in CMT measured may be partly explained by different hardware. The Triton utilizes SS-OCT to obtain images, compared with the Cirrus and Spectralis which both use SD-OCT. The differences in technology between these systems are reviewed elsewhere. [260]

Additionally, each of these machines utilizes different software to analyze obtained images. Identification of the outer retinal border differs between machines as a line above, through, or below the retinal pigment epithelium (RPE) thus affecting the CMT measurement. [252] This can also lead to differing rates of IA, especially in nAMD, which affects the Bruch's-RPE complex. [261] Scale calibration may also be an issue with differing standards for what defines 1μm on a scan. This can be critical as prognosis, classification and treatment guidelines for a wide range of retinal pathology may depend on these measurements. [255, 262] Fovea finding algorithms, as well as eye tracking software also differs, affecting rates of fixation IA. [263]

Updates in software may reduce IA but make comparisons between different versions unreliable. An industry standard for measurement formulas and calibrated segmentation algorithms across all available OCT devices would help address these issues. [245]

There may be a role for a conversion formula to allow for better comparability of macular thickness measurements across different OCT for different pathologies, as has been described by others. [264] Indeed, a conversion formula was utilised

in the Diabetic Retinopathy Clinical Research network Protocol T study to compare the time domain and SD-OCT devices used in the study. [255] However, these formulae would need to be verified with each update in imaging software to ensure accuracy and validity.

The strengths of this study are that all scans for each patient were formed on the same day, eliminating the effects of temporal changes on pathology. The data was collected prospectively with a defined protocol and evaluates the new Triton SS-OCT, which has not been studied for these purposes.

The pathologies evaluated in this study are limited to nAMD and DMO. A more complete comparison, including vitreomacular interface pathology, high myopia, retinal degenerations as well as segmentation of individual retinal layers, would be valuable. Whilst including patients able to fixate on a target minimised the effects of fixation errors, this may be a source of selection bias, limiting the application of these findings. Furthermore, controls were not age-matched and there was no sample size calculated for this study.

These study findings validate the presence of inter-device measurement variability and how reliability can be further affected by retinal pathology. Consequently, care should be exercised when interpreting measurements from different OCT devices in clinical practice and research settings. Investigators should be aware of the high rates of IA that occur in DMO and nAMD and consider manual correction of these errors in order to accurately report quantitative measurements. Further in-depth studies are required to evaluate

the reliability of OCT machines when performing scans in participants with poorer vision and other ocular and retinal pathology.

Chapter 5: Ultrawidefield fluorescein angiography predictors of response to switch to aflibercept in persistent diabetic macular oedema

Identification of morphological biomarkers are important for prognosis and can guide management of DMO. As discussed in Chapter 3, OCT biomarkers correlate well with vision and ultrawidefield imaging of the retina allows for more accurate grading of severity of diabetic retinopathy.

This chapter explores the utility and significance of ultrawidefield fluorescein angiography in these patients with persistent DMO. Peripheral ischaemia is thought to be an important pathological process in the development and progression of DMO and may be associated with persistent DMO.

ABSTRACT

Purpose

To explore the effect of peripheral ischaemia identified on ultrawidefield fluorescein angiography (UWFA) as a biomarker of response to switch in therapy from bevacizumab to aflibercept in persistent diabetic macular oedema (DMO).

Methods

Prospective, non-controlled clinical trial of 38 eyes from 38 patients with persistent DMO despite previous treatment with bevacizumab. All patients were switched to therapy with aflibercept following a loading dose protocol and followed up for 48 weeks. UWFA images were obtained on all patients at baseline and at conclusion of the trial. UWFA images were graded for non-perfusion and used to calculate an overall ischemic index (II) and a macular ischaemic index (MII). II was compared with the primary visual and central macular thickness (CMT) outcomes as well as OCT biomarkers including inner segment ellipsoid band disruption, external limiting membrane disruption, morphology of DMO and disoragnisation of the inner retinal layers. Paired and independent samples t-tests and Fisher's exact tests were used to assess change and associations.

Results

There was a significant overall improvement in vision (4.0 ± 7.2 letter gain, p=0.002) and CMT (-60 ± 111μm difference, p=0.002) in the 38 patients with UWFA data. Patients with an II greater than or equal to 50% at baseline had a poorer baseline visual acuity (60.1 ± 10.2 vs. 70.7 ± 9.0 letters, p=0.005) and a

worse MII (6.9 ± 25 vs 56 ± 52%, p<0.001). These patients gained significantly more letters of vision at 48 weeks (8.3 ± 9.3 vs. 2.6 ± 5.9 letters, p=0.03). At 48 weeks, there was no significant difference in absolute visual acuity in patients with an II greater than or equal to 50% compared to those with an index less than 50% (68.4 ± 6.0 vs. 73.3 ± 9.6 letters, p=0.16). There was no correlation between II with CMT or any OCT biomarker.

Conclusion

Patients with persistent DMO previously treated with bevacizumab with a worse ischemic index had poorer baseline visual acuity, potentially due to worse macular ischaemia. Despite this, these patients had a greater visual gain with similar final visual outcomes compared to those without marked peripheral ischaemia subsequent to switching to aflibercept.

BACKGROUND

Ocular imaging is a cornerstone of diagnosis and management of diabetic macular oedema (DMO). Historically, fluorescein angiography (FA) has played a pivotal role in qualifying vascular leakage and guiding therapy for DMO. [35] In the past two decades, optical coherence tomography (OCT) has revolutionised diagnosis of DMO, as well as led to the identification of prognostic biomarkers such as distribution of oedema, disruption to the inner segment ellipsoid (ISe) band and external limiting membrane (ELM), and disorganisation of the inner retinal layers (DRIL). [178-181]

More recently, ultrawidefield (UW) imaging enabled documentation of peripheral lesions, leading to more detailed description of disease severity and prognosis of progression. [233, 242] When combined with FA, ultrawidefield fluorescein angiography (UWFA) has been shown to identify 3.9 times more nonperfusion, 1.9 times more neovascularization, and 1.1 times more retinal pathology as compared with standard 7-fields photography. [234]

Peripheral ischaemia may be quantified through the calculation of an ischaemic index (II). [265] Such an index gives a ratio of non-perfused to perfused retina and has been shown to be associated with the prevalence of DMO in a retrospective study of 122 eyes (OR 3.75, 95%CI 1.26-11.13, $p<0.02$). [191] Furthermore, DMO that is recalcitrant to macular photocoagulation was found to be associated with a worse II in a retrospective study of 148 eyes with persistent DMO. [176] These patients also required a greater number of treatments with macular laser photocoagulation and had a lesser reduction in the CMT.

It is unclear what prognostic information can be gained from UWFA for patients with DMO being treated with anti-VEGF drugs. Herein we report the prognostic value of UWFA from a prospective clinical trial where patients with persistent DMO were switched from bevacizumab to aflibercept.

METHODS

Participants

Full inclusion and exclusion criteria for the patients enrolled in this trial were reported in Chapter 3.

Image Acquisition

UWFA images were acquired using the Optos 200TX (Optos Plc, Dunfermline, Scotland). An intravenous bolus of 5mL of 10%w/v fluorescein was given and images were obtained in the transit phase (up to 45 seconds) arteriovenous phase (1 to 2 minutes) and during recirculation (up to 10 minutes). A single best image from the arteriovenous phase of the study eye was selected for grading.

Calculation of ischaemic index

The ischaemic index was calculated using the concentric rings method previously described. [266] Briefly, UWFA images for each patient at baseline and 48 weeks were overlaid with the template of seven concentric rings (Figure 5.1) as supplied in the supplement to the publication by Nicholson et al. [266] Using ImageJ software (NIH, Bethesda, Maryland), the template was resized and repositioned for each image such that the innermost ring was equal in size to the optic disc and the central point of the template was placed at the fovea. Each of these seven rings was divided into 12 equal segments subtending an angle of 30 degrees at the fovea. Each segment was graded as perfused, non-perfused or non-gradable if more than half of the segment consisted of one of the three. Grading was validated by an independent external grader. Areas with scatter

laser were deemed not gradable and were excluded from the calculation of the index.

The II was calculated from the grading by multiplying each segment by the total disc area represented and then dividing non-perfused retina by the total gradable area. The macular ischaemic index (MII) was calculated from the 12 sectors comprising the innermost ring of the template.

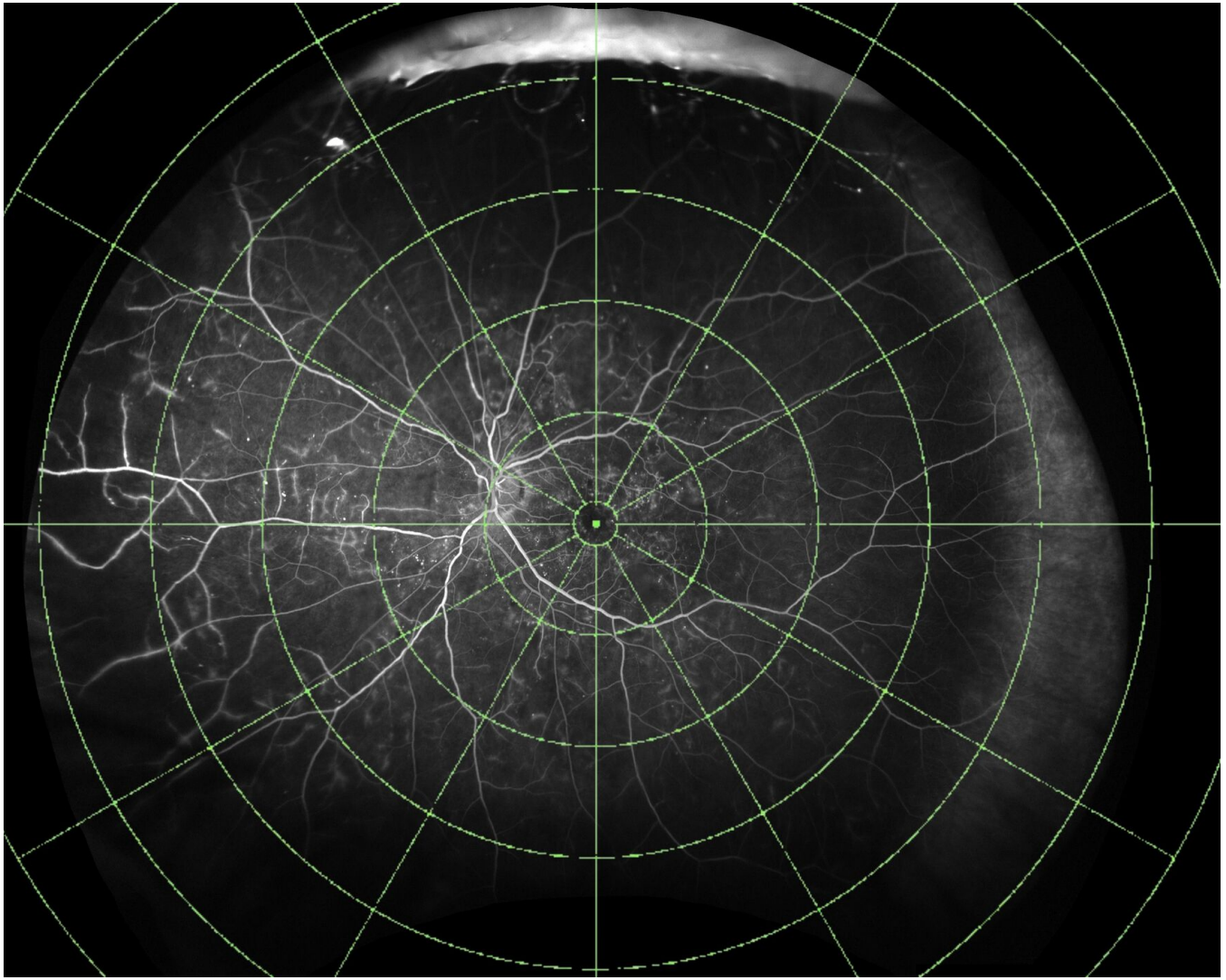


Figure 5.1: Ultrawidefield fluoresecein angiography with concentric rings overlay.

The size of the centre-most ring is calibrated to the size of the optic nerve and repositioned at the fovea. Each sector is then graded and calculated in relation to disc area.

Optical Coherence Tomography Grading

Images were graded for morphology of DMO (intraretinal fluid and/or subretinal fluid), presence or absence of disorganization of the inner retinal layers (DRIL), inner segment ellipsoid (ISe) band disruption, external limiting membrane (ELM) disruption in a 1mm area centred around the fovea as described in Chapter 3.

Statistical Analysis

All statistical tests were performed and figures produced using IBM SPSS software (version 22; SPSS Inc, Chicago, Illinois, USA). Patients were grouped by baseline II greater than or less than or equal to 50%. Data was confirmed to be distributed normally using Shapiro-Wilk tests. Homogeneity of data was confirmed using Levene's test for all independent samples' t-tests. Adjustment was made to the analysis using the Welch-Satterthwaite method for data that was not homogenous. Fisher's exact test was used to analyse categorical data when sample sizes in groups were small. Pearson's correlation coefficient was calculated for correlation analyses. For all analyses, a p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Of the 43 patients recruited, one withdrew consent after the baseline visit, one was withdrawn due to a retinal detachment in the study eye after the second injection, three did not have UWFA performed at baseline and six did not have UWFA performed at 48-weeks.

BCVA improved by a mean ± standard deviation of 4.0 ± 7.2 letters (p=0.002) and CMT reduced by 60 ± 111μm (p=0.002) in the 38 patients with UWFA data at baseline over the 48-week study period.

There was no significant change in mean II (24.9 ± 32.5% to 22.7 ± 29.7%, p=0.36) or MII (8.1 ± 23.5% to 7.9 ± 19.7%, p=0.88) from baseline to 48-weeks. There was correlation between II and MII at baseline (r=0.66, p<0.001) and at 48-weeks (r=0.57, p<0.001).

Patients with an II greater than 50% (n=9) at baseline had a poorer baseline visual acuity (60.1 ± 10.2 vs. 70.7 ± 9.0 letters, p=0.005; Figure 5.2a) and a worse MII (6.9 ± 25% vs 56 ± 52%, p<0.001). These patients gained significantly more letters of vision at 48 weeks (8.3 ± 9.3 vs. 2.6 ± 5.9 letters, p=0.03). At 48 weeks, there was no significant difference in absolute visual acuity in patients with an II greater than 50% compared to those with an index less than or equal to 50% (68.4 ± 6.0 vs. 73.3 ± 9.6 letters, p=0.16; Figure 5.2b).

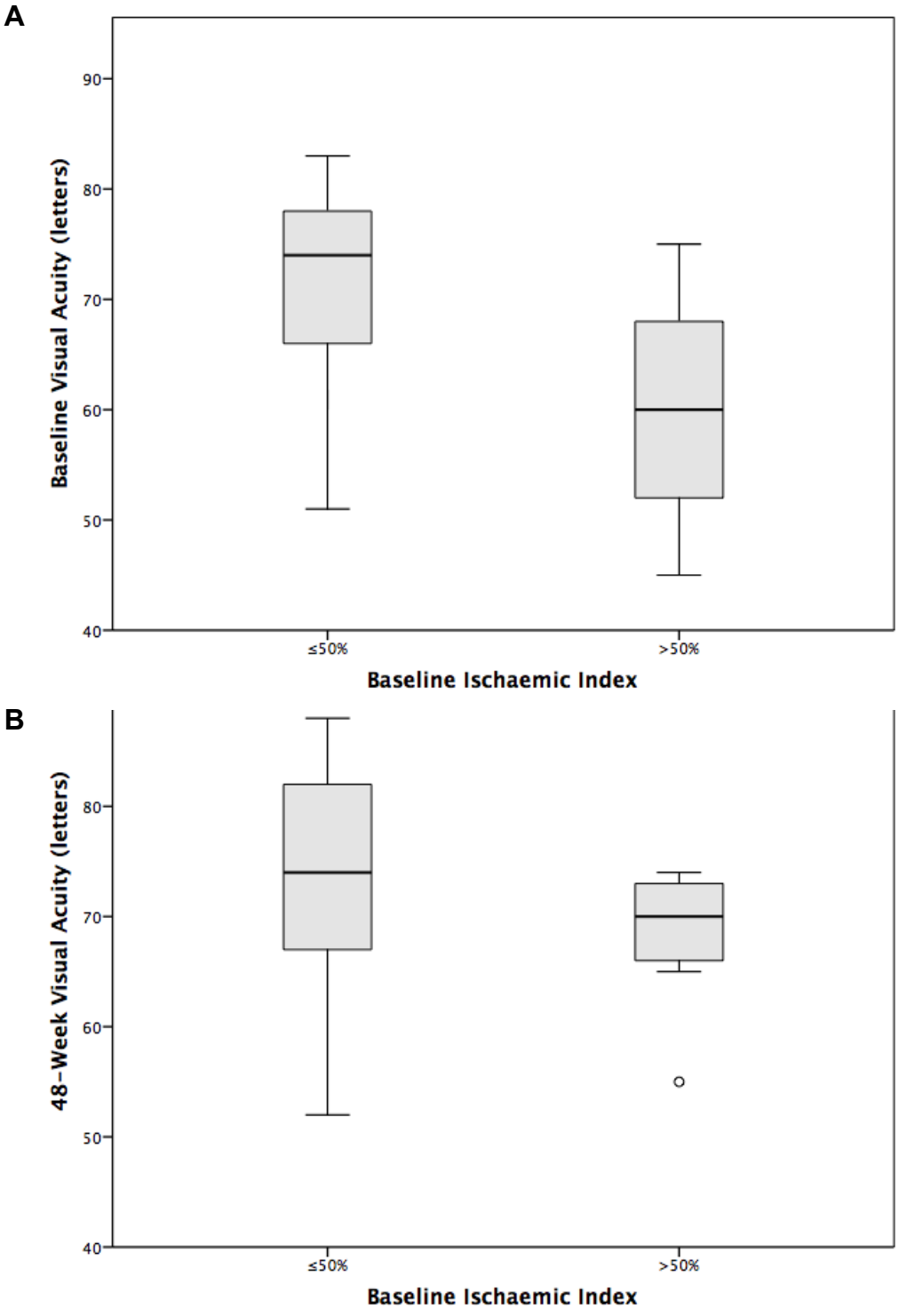


Figure 5.2. Boxplot showing (a) baseline and (b) 48-week visual acuities grouped by baseline ischaemic index

There was no difference in CMT at baseline or 48-weeks for patients with an II greater than 50% at baseline compared with those less than or equal to 50% (429 ± 61μm vs. 412 ± 101μm, p=0.63 and 395 ± 112μm vs. 343 ± 107μm p=0.22, respectively; Figure 5.3). There was no difference in change in CMT for patients with an II greater than 50% (-34 ± 73μm vs. -68 ± 120μm, p=0.42).

There was no significant correlation between II and HbA1c (r=0.16, p=0.40), duration of diabetes (r=0.25, p=0.13), or number of previous anti-VEGF injections for DMO (r=-0.31, p=0.06).

There was no correlation between II or MII and presence of subretinal fluid, disorganization of the inner retinal layers, external limiting membrane or inner segment ellipsoid band disruption (data not shown).

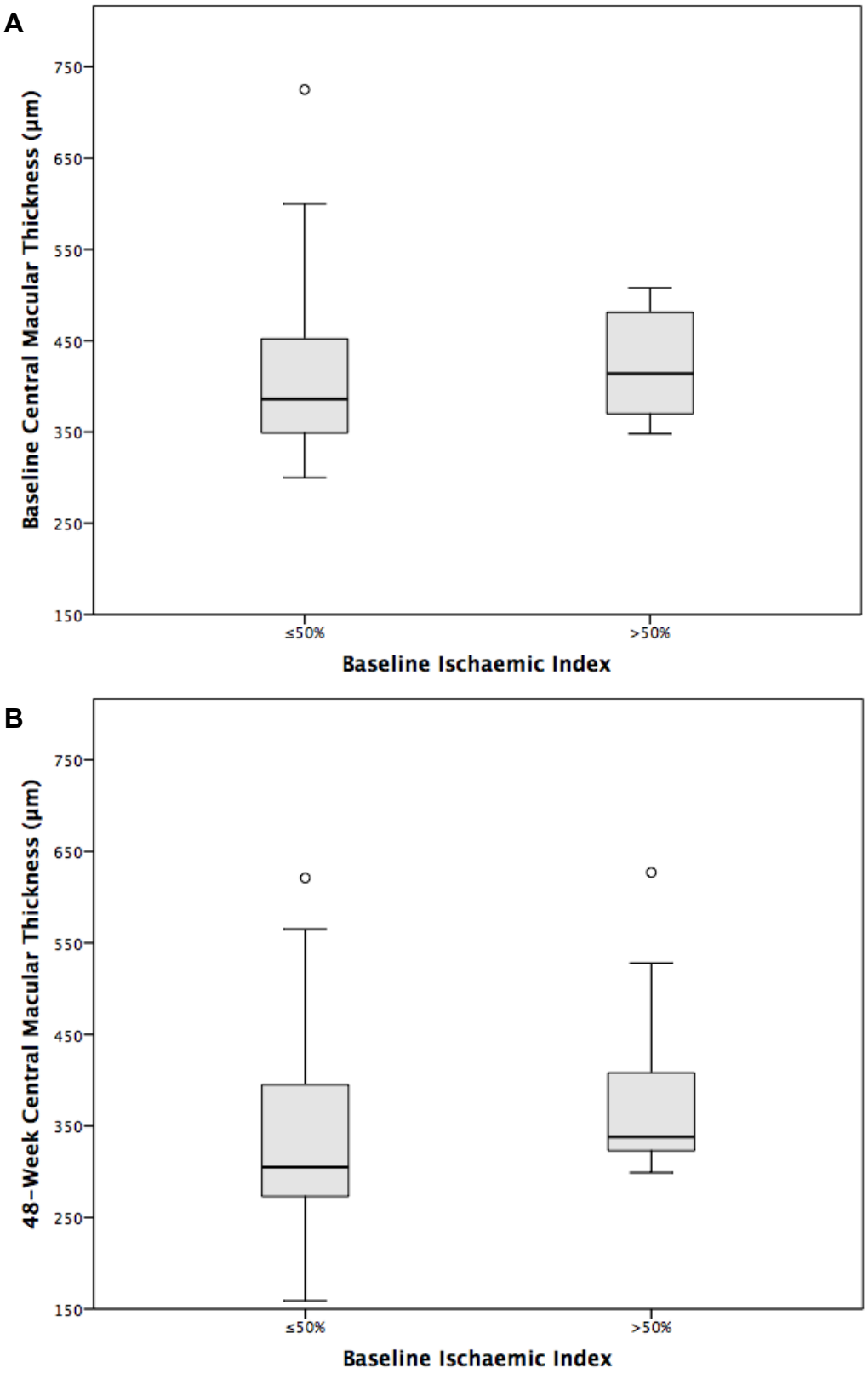


Figure 5.3. Boxplot showing (a) baseline and (b) 48-week central macular thickness grouped by baseline ischaemic index

DISCUSSION

Among patients with persistent DMO and significant prior treatment with bevacizumab, a worse baseline II was associated with a poorer baseline visual acuity. However, these patients gained more vision and had a similar final visual acuity to those with a lower II when therapy was switched to aflibercept.

Visual acuity is dependent on the health of the macula, both in the available blood supply as well as the integrity of the various cells involved in phototransduction. In this study, visual acuity was not associated with a thicker CMT but with worse macular ischaemia. Recent studies utilising OCT angiography have identified a negative correlation between macular capillary density and visual acuity. [267] OCT biomarkers such as ISe band and ELM disruption, DRIL and presence of subretinal fluid are all associated with a poorer visual acuity. [178-181] Whilst ischaemia may be postulated to explain these structural abnormalities, presence of these factors did not correlate with macular ischaemia in this study.

There are several potential explanations for a greater gain in vision for the patients with a higher baseline II. Firstly, the starting visual acuity was significantly lower, meaning that there was more potential for vision gain. Secondly, there may be a “ceiling effect”, to the amount of vision that can be gained in this cohort of patients with persistent DMO. Finally, ischaemia and hypoxia are strongly implicated in the pathogenesis of DMO. Thus areas of untreated retinal non-perfusion may stimulate the production of mediators such as VEGF-A and placental growth factor (PlGF) that contribute to the formation

and persistence of DMO. These factors may be more effectively inhibited by aflibercept, leading to improved outcomes in these patients. [226]

It has been hypothesised that scatter photocoagulation to areas of peripheral ischaemia may help in the management of DMO. Complete resolution of macular edema following panretinal photocoagulation was demonstrated in a case series of 17 eyes with florid proliferative DR and DMO. [268] Worsening of DMO was reduced over a period of six months in a clinical trial of 52 patients randomised to a single dose of bevacizumab either with or without targeted photocoagulation. [269] Reduced levels of VEGF in the eye following panretinal photocoagulation (PRP) may be responsible for this effect. [2] However, PRP is also known to exacerbate macular oedema likely through transient increases in inflammatory cytokines and VEGF. [270, 271]

Most recently, monotherapy with ranibizumab was shown to have similar outcomes to combination therapy with ranibizumab and targeted laser photocoagulation in a three year, randomised trial of 40 eyes from 29 patients with DMO and significant peripheral ischaemia. [272] There were no differences in treatment burden or visual or anatomical outcomes. The authors suggested areas of non-perfusion may represent dead rather than stressed tissue and thus do not contribute to increased production of factors driving DMO. This is similar to the outcomes presented in the RELATE study, where patients with branch and central retinal vein occlusion who were randomised to intravitreal ranibizumab therapy with targeted scatter laser had similar vision and anatomical outcomes to those treated with ranibizumab monotherapy. [273]

Contrary to Wessel et al, we did not find any correlation between diabetes control and II, nor did we find a relationship between duration of diabetes and II. [191] However, there was borderline significant negative correlation between number of previous injections and II. This may be due to a disease modifying effect of anti-VEGF drugs slowing the progression of retinal non-perfusion as was demonstrated in patients treated with ranibizumab in a retrospective analysis of the RISE/RIDE studies. [274] Whilst UWFA was not used in the quantification of this, it may explain the lack of change in II from baseline to 48 weeks in the participants of our study who were treated with 8 injections of aflibercept during the study period. Other authors have presented data suggesting a reversal of areas of non-perfusion. [275] Ischaemic index was reduced in a pilot study of nine eyes with DMO treated with the dexamethasone implant over a 12-week period. [276]

The strengths of this study are in the prospective and standardised nature of data collection in a clinical trial setting. The trial participants received the per protocol treatment during the study period and had retinal imaging performed at standardised time points. The methodology for grading ischaemia has been previously validated.

There are inherent limitations in the data and analyses performed due to lack of a control arm as well as a relatively small sample size from a single centre. Furthermore, correction for peripheral distortion and introducing validated,

reliable computer based segmentation for the calculation of peripheral ischaemia may yield more accurate results in the future. [277, 278]

The exploratory analyses from this study demonstrate that significant peripheral ischaemia may correlate with poorer visual acuity and a greater capacity for vision improvement in patients with persistent DMO switched to aflibercept. Additionally, there appears to be no clear association between degree of peripheral ischaemia and severity of macular oedema, suggesting that other factors may be involved in macular thickening in DMO. Future directions for these findings are to assess the effect of peripheral ischaemia as a biomarker of treatment response to anti-VEGF drugs in treatment naïve eyes as well as those treated with other modalities such as corticosteroids that target different pathological pathways.

Chapter 6: Correlation of functional and morphological retinal impairment in patients with persistent diabetic macular oedema

In previous chapters, objective anatomical and visual outcomes of a switch in therapy from bevacizumab to aflibercept have been analysed and discussed. One of the challenges and limitations of clinical trials in ocular pathology is the quantification of functional outcomes following interventions.

The purpose of this chapter is to explore both subjective and objective functional outcomes from these patients and to explore associations with morphological findings.

The material presented in this chapter is under peer-review for publication and is reproduced from:

Bahrami B, Nair R, Spooner K, Hong T, Chang A. Correlation of functional and morphological retinal impairment in patients with persistent diabetic macular edema. Graefe's Archive for Clinical and Experimental Ophthalmology *Under review April 29th 2018*

Additionally, preliminary data from this chapter was presented at the RANZCO 48th Annual Scientific Conference: Melbourne, Australia, 2016 as:

Nair R, **Bahrami B**, Spooner K, Hong T, Chang A. Assessing changes in macula microperimetry among patients with treatment resistant diabetic macular oedema switched to intravitreal aflibercept over 12 months. Clin Exp Ophthalmol. 2016 Nov; 44 (S1):80-140.

ABSTRACT

Purpose

To evaluate subjective and objective functional outcomes in patients with persistent diabetic macular oedema (DMO) switched from bevacizumab to aflibercept and to correlate these with retinal morphological abnormalities.

Methods

Prospective clinical trial of 43 eyes from 43 patients with persistent DMO. All patients were switched from bevacizumab to aflibercept with a loading dose protocol and were followed up for 48 weeks. Microperimetry (MAIA, Centervue, Padova, Italy) was performed at baseline and 48 weeks using a 4-2-1 strategy for the central 10 degrees of vision. Bivariate correlation analyses were calculated using Pearson's correlation coefficient for vision and central macular thickness (CMT). Independent samples t-tests were used to compare OCT biomarkers to macular sensitivity. The National Eye Institute Visual Functioning Questionnaire 25 (VFQ-25) was used to assess vision-related quality of life at baseline, 24- and 48 weeks. Changes in composite and subscale score were assessed using paired t-tests. The relationship between composite questionnaire scores and BCVA, CMT and macular sensitivity were assessed using Pearson's correlation coefficient.

Results

There was an improvement in BCVA (3.7 ± 7.2 letters, p=0.002) and CMT (58 ± 112μm reduction, p=0.002) in these patients over the 48-week study period. Average threshold sensitivity and BCVA correlated at baseline and 48-weeks

(r=0.72, p<0.001 and r=0.62, p<0.001 respectively). There was negative correlation between average threshold and CMT at baseline and 48-weeks (r=-0.35, p=0.02 and r=-0.37, p=0.03 respectively). Average threshold was poorer in the presence of subretinal fluid, inner segment ellipsoid band and external limiting membrane disruption at baseline and 48-weeks and for disorganization of the inner retinal layers at 48-weeks. There was no significant difference between baseline and 24-week and 48-week VFQ composite scores (0.7 ± 11.0, p=0.72 and -0.2 ± 9.1, p=0.94 respectively). VFQ composite scores did not correlate with BCVA or CMT. VFQ composite score correlated with average 6-degree macular sensitivity threshold at baseline (r=0.36, p=0.05) and 48 weeks (r=0.56, p=0.006).

Conclusion

There is good correlation between macular sensitivity and visual acuity in patients with persistent DMO. Sensitivity thresholds correlated with the presence of OCT biomarkers and negatively with CMT. Microperimetry may correlate with vision related quality of life and function in patients with persistent DMO.

BACKGROUND

There has been a paradigm shift in the management of diabetic macular edema (DMO) through the introduction of intravitreal anti-vascular endothelial growth factor-A (VEGF) drugs. As with most studies in ophthalmic research, assessment of functional change in these trials is largely limited to the measurement of visual acuity. Whilst this is an easily measured and useful objective outcome, other functional aspects of vision may be of value when assessing response to treatments.

Vision related quality of life assessments are one subjective measure of functional change and have been reported in landmark trials of anti-VEGF for the management of DMO such as RISE/RIDE and VISTA/VIVID. [259, 279] Other objective measurements generally not reported in these larger trials include contrast sensitivity, electroretinography, reading speed and microperimetry. [280] Such objective functional measures may be of increasing importance in diabetic retinopathy where neuronal and retinal sensitivity changes can predate vascular changes such as microaneurysms and haemorrhages that can be detected on clinical examination. [281, 282] However, these may be limited by access to devices, expense, and the time consuming and invasive nature of some of these tests.

Microperimetry allows the retinal sensitivity of a patient to be mapped onto an image of the fundus using a scanning laser ophthalmoscope. Thus, it allows for topographical correlation between function of the central retina and pathology present. Macular sensitivity quantified with microperimetry has been shown to

correlate with visual acuity as well as macular thickness measurements in DMO. [283] Furthermore, OCT biomarkers such as inner segment ellipsoid band disruption have been shown to overlap with focal scotomas and impaired macular sensitivity in DMO as well as other conditions such as age-related macular degeneration (AMD) and retinitis pigmentosa. [284-286] Whilst data correlating microperimetry findings and vision related quality of life in DMO are lacking, a relationship has been found in patients with retinitis pigmentosa. [287] This suggests information gained from microperimetry may explain subjectively reported visual function that is discordant with DMO observed on clinical examination.

In this study we present secondary functional outcomes from a cohort of patients prospectively switched from bevacizumab to aflibercept for the management of persistent DMO and correlate these with primary visual and structural outcomes.

METHODS

Study Design

The methods, inclusion and exclusion criteria for this open-label, non-randomised clinical trial have been previously described in Chapter 3.

Microperimetry

Microperimetry was performed at baseline and 48-weeks using the MAcular Integrity Assessment (Figure 6.1, MAIA; Centervue, Padova, Italy) microperimeter. This device uses a scanning laser ophthalmoscope (SLO) to obtain a retinal image and track eye movements during measurement. A pre-programmed method was used to obtain sensitivity measures ("Expert Examination"), which used a 4-2-1 staircase strategy on a grid consisting of three concentric circles of 2, 6 and 10 degrees in diameter. Background luminance was 4 asb, the size of the stimulus used was Goldman III, presentation time was 200ms and the dynamic range of stimuli ranged from 0 to 36 decibels (dB). A total of 37 points were assessed using this method and the output of the examination reports the sensitivity of each of these points with the image obtained by the SLO (Figure 6.2). The follow-up examinations were tracked such that the same anatomical points were assessed at the baseline visit and the 48-week time point.

Outcomes analysed included the threshold of the central foveal point, average threshold of all 37 points tested and the average threshold of the central 13 points corresponding with the central two-degree field of vision. This area

corresponds to the central ETDRS ring which CMT and OCT biomarkers were assessed.

Figure 6.1: The MAIA microperimeter

(CenterVue, Padova, Italy; image reproduced from https://bit.ly/2Hs8COA)

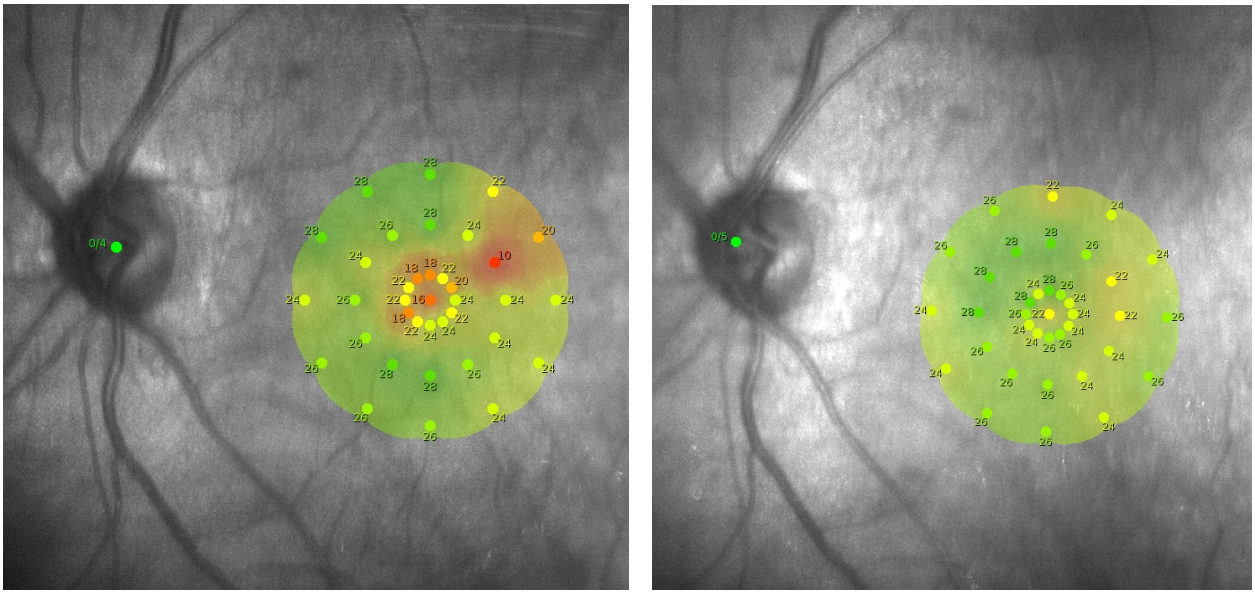


Figure 6.2: Tracked microperimetry output from the MAIA for a sample patient at baseline (left) and 48-weeks (right).

The numbers represent the threshold sensitivity for each point in decibels. A topographical map is made ranging from colours red to green to corresponds with the sensitivity in different areas within the macula. The three concentric rings represent an angle of 2, 6 and 10 degrees from fixation.

Self-reported Vision Function and Quality of Life

The National Eye Institute Visual Functioning Questionnaire-25 (VFQ-25) was distributed and collected from patients at baseline, 24- and 48-week time points. This vision related quality of life questionnaire assesses general health, general vision, ocular pain, near vision activities, distance vision activities, social functioning, vision-specific role difficulties, vision-specific mental health, dependency due to vision, driving, peripheral vision and color vision. The results from this questionnaire were converted to a composite score, which includes all subscales except general health, and analysed in accordance with published guidelines. [288]

Statistical Analyses

Statistical analyses were performed and figures created using IBM SPSS software (version 22; SPSS Inc, Chicago, Illinois, USA). Normal distribution of data was confirmed using Shapiro-Wilk tests. Paired t-tests were used to compare differences in means of BCVA, CMT, composite and subscale scores in the VFQ-25, and change in sensitivity measurements obtained with microperimetry. Independent samples' t-tests were used to analyse microperimetry outcomes grouping by presence of OCT biomarkers and grouping patients as good responders (gain of 1 line of vision or greater) in analysis of VFQ-25 data. Data was tested for homogeneity using Levene's test and if variances were unequal, adjustment was made to the analysis using the Welch-Satterthwaite method. Bivariate Pearson's correlation coefficient was calculated to assess relationships

between continuous variables. For all analyses, a p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Microperimetry Outcomes

MAIA microperimetry was completed by 43 patients at baseline and 36 patients at 48-weeks. Baseline values, change and correlation with BCVA and CMT are summarised in Table 6.1. Average threshold values were lower for those patients who had received grid/focal laser in the past at baseline (19.6 ± 4.3 vs. 22.3 ± 3.2, p=0.02) but not at 48-weeks (20.6 ± 3.1 vs. 22.6 ± 3.8, p=0.11). There was no correlation between average threshold at baseline and duration of diabetes (r=0.09, p=0.58), HbA1c (r=-0.20, p=0.27) or number of previous anti-VEGF injections (r=-0.04, p=0.80). The associations between OCT biomarkers with VA and central six-degree threshold are summarised in Table 6.2.

Table 6.1: Microperimetry outcomes and correlation with best-corrected visual acuity and central macular thickness at baseline and 48-weeks

Microperimetry Characteristic	**Baseline value (dB), mean ± SD (n=43)**	**Correlation of baseline value with BCVA at baseline**		**Change at 48-weeks (dB) (n=36)**		**Correlation of 48-week value with BCVA at 48-weeks**		**Correlation of change in value with change in BCVA at 48-weeks**	
	Mean ± SD	Pearson's correlation coefficient	p-value	Mean ± SD	p-value	Pearson's correlation coefficient	p-value	Perason's correlation coefficient	p-value
Average threshold	21.2 ± 3.9	0.61	**<0.001**	0.08 ± 1.9	0.79	0.62	**<0.001**	-0.02	0.90
Centre 2 degree threshold	20.2 ± 4.2	0.63	**<0.001**	0.62 ± 2.7	0.18	0.59	**<0.001**	-0.16	0.35
Centre point threshold	19.5 ± 6.3	0.41	**0.01**	0.81 ± 4.2	0.26	0.40	**0.02**	-0.004	0.98

Microperimetry Characteristic	**Correlation of baseline value with CMT at baseline**		**Correlation of 48-week value with CMT at 48-weeks**		**Correlation of change in value with change in CMT at 48-weeks**	
	Pearson's correlation coefficient	p-value	Pearson's correlation coefficient	p-value	Pearson's correlation coefficient	p-value
Average threshold	-0.35	**0.02**	-0.37	**0.03**	0.05	0.76
Centre 2 degree threshold	-0.16	0.35	-0.43	**0.01**	0.07	0.67
Centre point threshold	-0.24	0.17	-0.42	**0.01**	-0.17	0.34

Table 6.2: Differences in visual acuity and central two-degree average threshold in the presence of OCT biomarkers

Characteristic	Number of patients at baseline (n=43)	Baseline BCVA (letters) with characteristic	Baseline BCVA (letters) without characteristic	Difference (p-value) *	Centre two-degree threshold (dB) with characteristic	Centre two-degree threshold (dB) without characteristic	Difference (p-value) *
Intraretinal fluid, n (%)	43 (100)	67.8 ± 10.1	N/A	N/A	20.2 ± 4.2	N/A	N/A
Subretinal fluid, n (%)	7 (16)	60.0 ± 8.1	69.2 ± 9.8	**9.2 ± 4.0 (p=0.02)**	15.0 ± 4.4	21.0 ± 3.6	**6.0 ± 1.8 (p=0.002)**
ELM disruption, n (%)	29 (66)	60.7 ± 9.2	71.2 ± 8.8	**10.5 ± 2.9 (p=0.001)**	17.7 ± 4.2	21.1 ± 3.9	**3.4 ± 1.5 (p=0.03)**
ISe band disruption, n (%)	35 (81)	65.8 ±9.9	76.3 ± 6.0	**10.4 ± 3.7 (p=0.007)**	19.2 ± 4.1	23.7 ± 2.6	**4.5 ± 1.5 (p=0.006)**
DRIL, n (%)	35 (81)	66.3 ± 10.2	74.3 ± 6.7	**8.0 ± 3.8 (p=0.04)**	19.8 ± 4.1	21.6 ± 4.6	1.8 ± 1.7 (p=0.30)

Characteristic	Number of patients at 48-weeks (n=41)	48-week BCVA (letters) with characteristic	48-week BCVA (letters) without characteristic	Difference (p-value) *	Centre two-degree threshold (dB) with characteristic	Centre two-degree threshold (dB) without characteristic	Difference (p-value) *
Intraretinal fluid, n (%)	32 (78)	69.7 ± 9.4	77.9 ± 8.1	**8.2 ± 3.5 (p=0.02)**	19.9 ± 4.2	24.1 ± 5.3	**4.2 ± 1.8 (p=0.03)**
Subretinal fluid, n (%)	0 (0)	N/A	71.5 ± 9.7	N/A	N/A	20.8 ± 4.7	N/A
ELM disruption, n (%)	27 (66)	68.2 ± 8.6	77.8 ±8.6	**9.6 ± 2.8 (p=0.002)**	19.3 ± 4.4	23.9 ± 3.9	**4.6 ± 1.5 (p=0.005)**
ISe band disruption, n (%)	38 (93)	71.2 ± 10.0	75.3 ± 1.5	**4.2 ± 5.8 (p=0.03)**	20.3 ± 4.6	26.3 ± 2.5	**6.0 ± 2.7 (p=0.04)**
DRIL, n (%)	32 (78)	69.2 ± 9.0	79.7 ± 7.5	**10.5 ± 3.3 (p=0.003)**	19.6 ± 4.7	25.2 ± 1.1	**5.6 ± 1.0 (p<0.001)**

CMT=central macular thickness, VMA=vitreomacular adhesion, ELM=external limiting membrane, BCVA=best-corrected visual acuity, DRIL=disorganization of inner retinal layers *independent samples t-test

Vision Function Questionnaire Outcomes

The VFQ-25 was completed by 37, 33 and 24 patients at baseline, 24- and 48-weeks respectively. Changes in composite and subscale scores at 24- and 48-weeks are summarised in Table 6.3 for patients with complete data at these time points. There was no significant difference in any subscale or composite score at any time point. Patients classified as good responders (gain of greater than or equal to five letters) did not have a greater change in composite score at 24- (3.5 ± 12.4 vs. -3.9 ± 8.8, p=0.07) or 48-weeks (2.1 ± 8.2 vs. -0.9 ± 9.7, p=0.48). Patients in whom the study eye was the better-seeing eye had similar composite scores to those in which the study eye was the worse-seeing eye at 24- (-0.5 ± 10.6 vs. -1.6 ± 13.3, p=0.82) and 48-weeks (-0.7 ± 8.7 vs. 8.6 ± 11.9, p=0.18). There was correlation between visual acuity change and change in mental health (r=0.48, p=0.007) and dependence (r=0.54, p=0.002) subscales at 24-weeks and dependence (r=0.57, p=0.005) and driving subscales (r=0.73, p=0.001) at 48-weeks. No other significant correlations between change in BCVA and composite score or subscales were noted.

Table 6.3: Changes in Visual Function Questionnaire-25 scores during the study period

VFQ-25 Subscale	**Baseline score, mean ± SD (n=37)**	**Change at 24-weeks (n=33)**		**Change at 48-weeks (n=24)**	
		Mean ± SD	p-value	Mean ± SD	p-value
Composite Score	86.1 ± 12.9	0.7 ± 11.0	0.729	-0.2 ± 9.1	0.935
Subscales					
General Health	53.4 ± 25.8	-1.3 ± 16.6	0.662	0.9 ± 11.5	0.715
General Vision	71.9 ± 12.9	-3.3 ± 21.5	0.403	-4.6 ± 21.3	0.329
Ocular Pain	88.9 ± 16.3	1.7 ± 18.8	0.631	-1.1 ± 14.4	0.715
Near Activities	79.7 ± 19.2	1.7 ± 19.0	0.635	0.8 ± 16.8	0.835
Distance Activities	86.0 ± 16.8	2.2 ± 22.7	0.596	-0.4 ± 16.6	0.916
Social Functioning	92.9 ± 14.3	-1.3 ± 12.0	0.573	2.3 ± 16.2	0.518
Mental Health	82.4 ± 18.0	-0.8 ± 17.8	0.800	1.1 ± 14.9	0.724
Role Difficulties	82.4 ± 22.9	2.5 ± 28.1	0.630	-2.8 ± 17.6	0.459
Dependence	95.0 ± 13.7	0.6 ± 22.1	0.891	0.4 ± 13.5	0.896
Driving	82.8 ± 30.1	-2.9 ± 9.1	0.119	-0.9 ± 7.8	0.630
Color Vision	88.4 ± 30.7	-4.7 ± 25.7	0.311	-7.3 ± 29.0	0.231
Peripheral Vision	79.3 ± 32.5	-4.7 ± 30.7	0.395	-8.0 ± 37.3	0.294

VFQ-25=Visual Function Questionnaire-25, SD=standard deviation. P-values reflect paired t-test results.

Relationship between Microperimetry and VFQ-25

Average threshold correlated with VFQ-25 composite score at baseline (r=0.38, p=0.02) but did not reach statistical significance at 48-weeks (r=0.40, p=0.06). Centre six-degree threshold correlated with VFQ-25 composite score at baseline (r=0.36, p=0.046) and 48-weeks (r=0.56, p=0.01). Foveal threshold correlated at baseline (r=0.37, p=0.04) but not 48-weeks (r=0.28, p=0.21).

DISCUSSION

Functional vision is only partially represented through visual acuity measurements. Consequently, patients with a similar clinical examination and morphology of disease may report different degrees of visual impairment. Reporting functional outcomes other than visual acuity that are quantifiable are important in assessing the effects of treatments we administer.

In this clinical trial, patients with recalcitrant DMO who had received significant prior treatment were switched in therapy to aflibercept. Whilst we demonstrated statistically significant visual acuity gains, these did not correlate with a subjective overall improvement in reported vision-related quality of life (VR-QoL).

This lack of improvement may reflect the chronic nature of the disease in these patients and is concordant with other studies reporting VR-QoL in DMO. There was no significant change in any of the VFQ-25 subscales or composite score following 12 months of therapy in a study of 100 patients with persistent DMO who were randomised to either fixed or as needed dexamethasone implant. [289] Similarly, a study of 20 consecutive patients receiving bevacizumab for persistent DMO following vitrectomy showed no change in VFQ-25 scores at three months, althought this did show a brief improvement in the mental health subscale following the 1-month review. [290]

Subscale correlations between change in vision and mental health and dependency subscales at 24-weeks and dependency and driving subscales at 48-

weeks reported in our data may be disproportionately affected by an outlier which was evident when data was plotted (data not shown).

Whilst there was no significant correlation between vision outcome and VR-QoL, there was significant correlation between threshold sensitivities and VFQ-25 composite scores at baseline and 48 weeks. These results suggest microperimetry may help to quantify functional vision that impacts VR-QoL not well assessed with visual acuity in DMO.

Microperimetry average threshold directly correlated with absolute values of BCVA and inversely with CMT. This has been consistently documented in DMO. [283, 291] However, despite an overall significant improvement in BCVA and CMT, there was no significant change in overall average threshold, central six-degree threshold or foveal point threshold from baseline to 48-weeks. These findings are consistent with a study of 28 patients with DMO who were treated with micropulse laser and had microperimetry performed at baseline and three months. The investigators reported a significant improvement in both BCVA and CMT over this time period but no significant change in threshold sensitivity. [292]

There were further structural correlations with the average threshold in the central six degrees of vision, an area corresponding to the central 1mm area graded on OCT. All of the parameters evaluated (presence of SRF, ISe band disruption, ELM disruption and DRIL) had a significant effect on visual acuity.

Subretinal fluid, or neurosensory detachment of the retina, had the largest impact on threshold sensitivity. Accumulation of fluid in this space may effect both the oxygenation of the photoreceptor layer as well as impairing the elimination of metabolites, thus decreasing sensitivity. This was also shown to have a significant impact on retinal sensitivity in DMO in a cross sectional study of 26 eyes. [293]

The ISe band is thought to correspond to the junction of the inner and outer segments of the photoreceptors. Disruption of this layer has been strongly associated with poorer visual acuity in DMO. [181] Furthermore, a study utilizing a device capable of combined perimetry and OCT demonstrated point disruptions in the ISe band corresponded with areas of lower retinal sensitivity in DMO. [284] The ELM has a close relationship with the ISe band and may correspond to the cell bodies of photoreceptors and similar disruptions in this layer have been associated with poorer sensitivity in DMO. [294]

DRIL has shown strong association with visual acuity in DMO. [295, 296] This biomarker is thought to correspond to anatomical disruption in inner retinal cells such as bipolar, amacrine or horizontal cells, thus affecting phototransduction. [178] Whilst DRIL was not associated with poorer retinal sensitivity at baseline, there were strong associations at the 48-week follow up. Data regarding DRIL and microperimetry is lacking and the associations observed here warrant further investigation in future studies.

Patients who had received prior grid or focal laser for DMO had a poorer average threshold that was significant at baseline but not 48-weeks. The effect may not have been statistically significant at 48-weeks due to the sample size.
Whilst grid laser has consistently been shown to preserve vision in DMO, this therapy may come at the expense of loss of central visual function. Grid laser has been shown to impair central ten-degree thresholds with both argon green and krypton red laser in 64 eyes of 32 patients with DMO. [297] Furthermore, whilst this effect was again confirmed with green laser, micropulse laser was shown to improve overall average thresholds in a randomised clinical study of patients with DMO. [298]

The strengths of this study are prospectively collected data from a homogenous group of patients in a standardised manner. No other studies have evaluated correlation between subjective and objective functional outcomes in DMO.

This study is limited by the small sample size and poor response rate in the VFQ-25. Only 25 patients completed the VFQ-25 upon completing the trial and a more complete data set would have added weight to the results, especially changes in composite and subscale scores. The lack of a control group means conclusions regarding functional effects of a switch in therapy are limited. Furthermore, this analysis is limited as structure and function were assessed on separate devices and anatomical correlation was estimated manually. Devices that can reliably correlate retinal structure and function may be able to validate the findings in this study. [284]

Future clinical studies should aim to incorporate other functional measures such as microperimetry to assess the effects of treatment. This test is a relatively quick, non-invasive and objective way to measure functional outcome that may correlate with VR-QoL as we have shown here.

Section 3: In vitro effects of anti-VEGF drugs on human retinal pigment epithelium

Chapter 7: Effects of anti-VEGF drugs on human retinal pigment epithelium under high glucose and oxidative stress

The findings reported in Chapter 3 demonstrated the effect that anti-VEGF drugs have on the regression of vascular lesions typical of DR. However, as highlighted in Chapter 1, neurodegeneration is a feature of DR and may be influenced by blockade of VEGF. Clinical data regarding the effect of anti-VEGF drugs on neurodegeneration in the retina is conflicting.

The retinal pigment epithelium is known to secrete a large range of neurotrophic factors important to the survival of neurons and potentially affecting neurodegeneration. The purpose of this chapter is to investigate the effects of glucose, hypoxia and anti-VEGF drugs on the production of neurotrophic factors by the retinal pigment epithelium in an *in vitro* model of DR.

Preliminary findings from this chapter are to be presented at the Association for Research in Vision and Ophthalmology Annual Meeting in Honolulu, USA, April-May 2018 as:

Bahrami B, Zhu L, Zhang T, Zhu M, Chang A, Gillies MC, Shen W. Effects of anti-VEGF drugs on human retinal pigment epithelium under oxidative stress.

ABSTRACT

Background: Retinal pigment epithelium (RPE) is known to secrete factors important in retinal homeostasis. How this secretome changes in diabetic eyes treated with anti-vascular endothelial growth factor (VEGF) drugs is unclear.

Methods: Diabetic conditions were simulated *in vitro* using ARPE-19 cell-line culture, with high glucose (25mM) culture media and chemically induced oxidative stress using cobalt chloride. Stress was assessed using cell viability assays as well as Western blots and enzyme-linked immunosorbent assay (ELISA) for production of HIF-1a and VEGF-A. Once conditions were established, production of neurotrophic factors was quantified using ELISA under stress with and without the addition of anti-VEGF drugs. Changes were analysed with one-way ANOVA.

Results: Hypoxia induced downregulation of pigment epithelium derived factor (PEDF) expression. Under normoxia, the addition of bevacizumab, ranibizumab and aflibercept all led to a significant downregulation of PEDF. Glucose concentration had no effect on secretion of PEDF. Brain derived neurotrophic factor (BDNF) secretion was downregulated in high glucose states and was upregulated in hypoxia. Placental growth factor (PlGF) secretion by ARPE-19 was undetectable by ELISA.

Conclusions: Neurotrophic factor secretion may be effected by hypoxia, high glucose or anti-VEGF drugs. This variation under different conditions may influence neuron and photoreceptor survival in the diabetic state and has

potential clinical implications for preservation of vision in patients receiving anti-VEGF therapy. Further studies are warranted to determine the impacts of a diabetic state and these drugs on multiple neuroprotective pathways in diabetes.

BACKGROUND

The identification of vascular endothelial growth factor-A (VEGF) as a key factor in the pathogenesis of diabetic retinopathy (DR) has revolutionised the approach to managing this blinding disease. There is now a large body of evidence to support the use of these drugs for the management of diabetic macular oedema (DMO) as well as a growing body of evidence for their use in other ocular diabetic complications such as proliferative DR and neovascular glaucoma. [299]

DR is characterised, and graded in clinical severity, by the presence of vascular lesions on clinical examination. [300] Anti-VEGF drugs have been shown to have a disease modifying effect on DR severity by clearing these lesions when used for longer periods. [299]

In addition to these vascular changes, there is increasing evidence to suggest that neurodegeneration occurs in parallel to and may precede vascular damage in the pathogenesis of DR. [301] Reduced thickness of the retinal ganglion cell (RGC) layer *in vivo* as well as a reduced ganglion cell density post mortem was reported in a study of streptozotocin-induced diabetic mice. [281] Patients with diabetes had a thinner ganglion cell layer as measured on optical coherence tomography (OCT) in a case-control study. [302] Furthermore, ganglion cell layer thickness had a negative correlation with the duration of diabetes ($r=-0.53$, $p<0.01$) in these patients.

The effect of anti-VEGF drugs on modulation of this neurodegeneration is unclear. It is established that VEGF-A has important developmental and

physiological roles in the retina. [109] Inhibition of VEGF-A increased both inner nuclear layer and RGC apoptosis in a study of streptozotocin-induced diabetic rats. Anti-VEGF therapy reduced signaling through the phosphorylated Akt pathway, which is key for the neurotrophic effects of this growth factor. [303]

Clinical evidence has shown that retinal nerve fibre layer thickness may be reduced in eyes treated with anti-VEGF from a meta-analysis of trials of patients with neovascular age related macular degeneration (nAMD) though the strength of the evidence and findings from included trials was variable. [304]

In contrast to these findings, ranibizumab has been shown to be protective in an ischaemia-reperfusion model of rats. [305] Restoration of the inner segment ellipsoid band and external limiting membrane has been observed in a clinical study of DMO treated with ranibizumab, suggesting that anti-VEGF therapy may actually protect or restore photoreceptors. [139] The mechanisms for this are unclear and are yet to be validated in other studies.

The retinal pigment epithelium (RPE) is known to secrete a large range of neurotrophic factors important in the survival of neurons. [306] In this study, we assess the effects of high glucose and hypoxia, both of which are critical for the development of DR, on key factors that may be secreted by the RPE. Additionally, we assess the effect of clinical doses of bevacizumab, ranibizumab and aflibercept on the secretion of these factors.

METHODS

ARPE-19 Cell Culture

Human ARPE-19 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA, cat #11885-084) media containing 1g/L glucose in T25 cell culture flasks until confluent. Supplementation to this media included 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were incubated at 37^{o}C, 5% CO_2.

Once confluent, cells were seeded at a density of 7.5 x 10^3 cells per well in 96-well plates for cell viability and ELISA-based supernatant experiments or at a density of 3 x 10^4 cells per well in 24-well plates for Western blot experiments.

Stress Media

Culture media was replaced with stress media once cells had reached 85-90% confluence in either 24- or 96-well plates. To reduce the background levels of factors detected in the supernatant, a lower concentration of FBS was utilised. All stress media was supplemented with 1% FBS, 1% insulin-transferrin-selenium-ethanolamine (ITS-X, Gibco, cat #51500056), 100 U/mL penicillin and 100 U/mL streptomycin and was prepared fresh on the day of experiments. Cells were cultured in stress media for a treatment period of 24 hours for all experiments.

The concentration of control/low glucose was 1g/L and high glucose was 4.5g/L (Gibco, cat #11995-065) in DMEM. Hypoxia was induced chemically through the addition of cobalt chloride (CoCl2; Sigma, St. Louis, MO, USA). CoCl2 mimics the

hypoxic state through induction of hypoxia inducible factor 1- alpha (HIF-1a) activity in many mammalian cell lines including ARPE-19. [307, 308] HIF-1a is considered the master regulator of hypoxia and mediates homeostatic response to low oxygen states. [309]

AlamarBlue cell viability assay

Conditions to establish cell viability were either low glucose (LG) or high glucose (HG) with the addition of 0μM, 50μM, 100μM, 200μM, 400μM or 600μM CoCl2 for a total of 12 groups. LG without CoCl2 was used as the reference control group.

Cell viability was measured using a resazurin assay (alamarBlue, Invitrogen, Frederick, MD, USA). In this assay, mitochondria of viable cells are able to reduce resazurin, which is blue and non-fluorescent, to resorufin, which is pink and highly fluorescent. [310] Following 24 hours of treatment in 96-well plates, 15μL of alamarBlue was added to each well. Fluorescence measurements were performed following 120 minutes of incubation using a Tecan Safire2 fluorescence multi-well plate reader (Tecan, Männedorf, Switzerland).

Western blot analysis for HIF-1α

Treatment groups to quantify changes in HIF-1α were either low glucose (LG) or high glucose (HG) with the addition of 0μM, 100μM, 200μM or 400μM CoCl2 for a total of 8 groups. LG without CoCl2 was used as the reference control group.

Following 24 hours of treatment in 24-well plates, culture media was removed and cells rinsed three times with phosphate buffered saline. Cells were lysed and protein extracted with the addition of RIPA buffer (Sigma; cat #R0278) combined with protease inhibitor (Roche, Mannheim, Germany; cat #04 693 124 001). The lysed cells were centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatant was collected and protein concentration was determined using a bicinchoninic acid (BCA) assay (QuantiPro BCA assay kit, Sigma; cat #QPBCA). A standard amount of protein was mixed with NuPAGE loading dye and reducing buffer (Invitrogen; cat #NP0007) and heated for 10 min at 70°C. This was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA).

After blocking with 5% bovine serum albumin for one hour, the membrane was incubated with HIF-1α antibody (1:500, Novus Biologicals, Littleton, CO, USA; cat #NB-100-449) overnight at 4°C followed by incubation with secondary antibodies conjugated with horseradish peroxidise for two hours at room temperature.

Protein bands were visualised after extensive washing with enhanced chemiluminescence substrate (Bio-Rad, Hercules, CA, USA; cat #1705060) using the G:Box BioImaging system (Syngene, Cambridge, UK). Results were quantified using the GeneTools image analysis package (Syngene, software version 3.07). Protein expression was normalised to α/β tubulin (1:2000; Cell Signaling #2148).

Measurement of VEGF-A using Enzyme Linked Immunosorbent Assay

Treatment groups to quantify VEGF-A secretion were either LG or HG with the addition of 0μM, 100μM, 200μM or 400μM CoCl2 for a total of 8 groups. LG without CoCl2 was used as the reference control group.

Following 24 hours of treatment in 96-well plates, culture media was collected and centrifuged at 1400rpm for 1 minute. The supernatant was subsequently analysed using enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions to detect and quantify VEGF-A (Invitrogen; cat #KHG0111).

Anti-VEGF drug treatment and measurements of BDNF, PEDF and PlGF in conditioned media using Enzyme Linked Immunosorbent Assay

Based on the results of the above, a concentration of CoCl2 was chosen that would lead to the increased expression of HIF-1a and VEGF-A without a major reduction in cell viability. Dose appropriate concentrations of bevacizumab (1.25mg/4mL), ranibizumab (0.5mg/4mL) and aflibercept (2.0mg/4mL) were added to media based on an assumed volume of the human vitreous at 4mL as previously described. [311] Treatment conditions were media with LG or HG with or without CoCl2, with or without each of the anti-VEGF drugs for a total of 12 treatment groups.

ELISAs were conducted to detect pigment epithelium derived factor (PEDF; R&D Systems, Minneapolis, MN USA, cat #DY1177-05), brain derived neurotrophic factor (BDNF; R&D Systems, cat #DBD00) and placental growth factor (PlGF; R&D Systems, cat #DPG00) as per the manufacturer's instructions on the supernatant of treated cells.

Statistical analysis

For each ELISA, a standard curve was generated and concentrations of factors were interpolated from samples from this. All statistical analyses were performed and figures created using GraphPad Prism software (Version 7, San Diego, CA, USA). Differences between groups were calculated using a one-way ANOVA with a post hoc correction using Bonferroni's multiple comparisons test. Values presented in figures are reported as the mean ± standard error of the mean. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Effects of hypoxia and high glucose on the viability of RPE

The addition of $CoCl_2$ did not have a quantifiable effect on the viability of RPE below concentrations of 200μM (Figure 7.1). Mean viability of cells reduced to 90% of control with both LG ($p=0.009$) and HG ($p=0.02$) in the presence of 200μM $CoCl_2$. Glucose concentration did not have a significant effect on cell viability between the LG and HG groups.

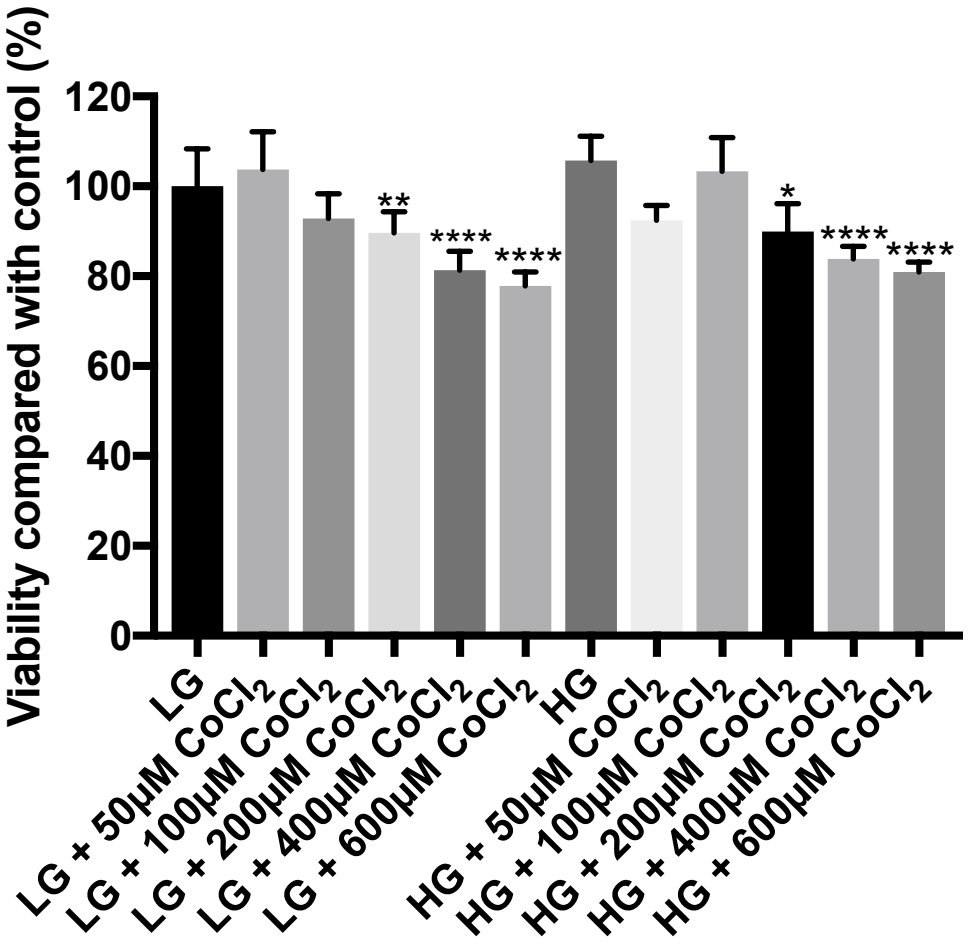
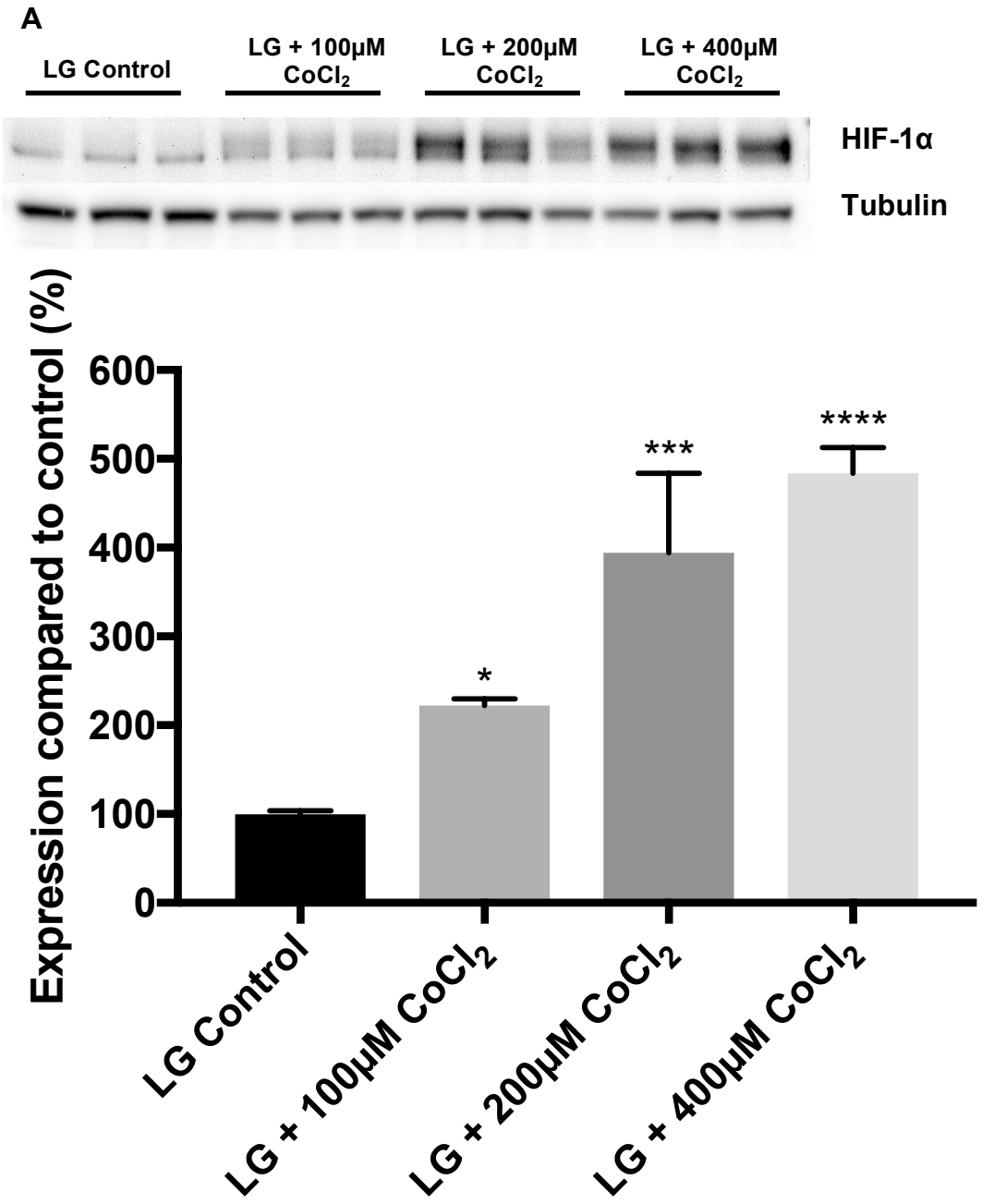


Figure 7.1: Effects of high glucose and hypoxia on ARPE-19 cell viability. Fluorescence readings by alamarBlue viability assays 24 hours after incubating cells in stress media. Values expressed as a percentage of low glucose control. LG=low glucose, HG=high glucose. *n* = 8 per group. * $p < 0.05$, ** $p<0.01$, **** $p<0.0001$

Effects of hypoxia and high glucose on the expression of HIF-1a and VEGF secretion in RPE

There was increased expression of HIF-1a for all concentrations of $CoCl_2$ in LG media (Figure 7.2A). Increased expression of HIF-1α was not apparent until concentrations of $CoCl_2$ were greater than 200μM in HG media (Figure 7.2B). Expression of HIF-1α was comparatively less in the HG media compared to the LG media. For example, there was 46% increased expression in HG media ($p=0.03$) compared with a 294% increase in LG media ($p=0.0002$) at 200μM $CoCl_2$.

There was increased secretion of VEGF-A for all concentrations of $CoCl_2$ in both LG and HG media (Figure 7.3).


A
LG Control
LG + 100μM CoCl2
LG + 200μM CoCl2
LG + 400μM CoCl2
HIF-1α
Tubulin
Expression compared to control (%)
600
500
400
300
200
100
0
*
***
****
LG Control
LG + 100μM CoCl2
LG + 200μM CoCl2
LG + 400μM CoCl2

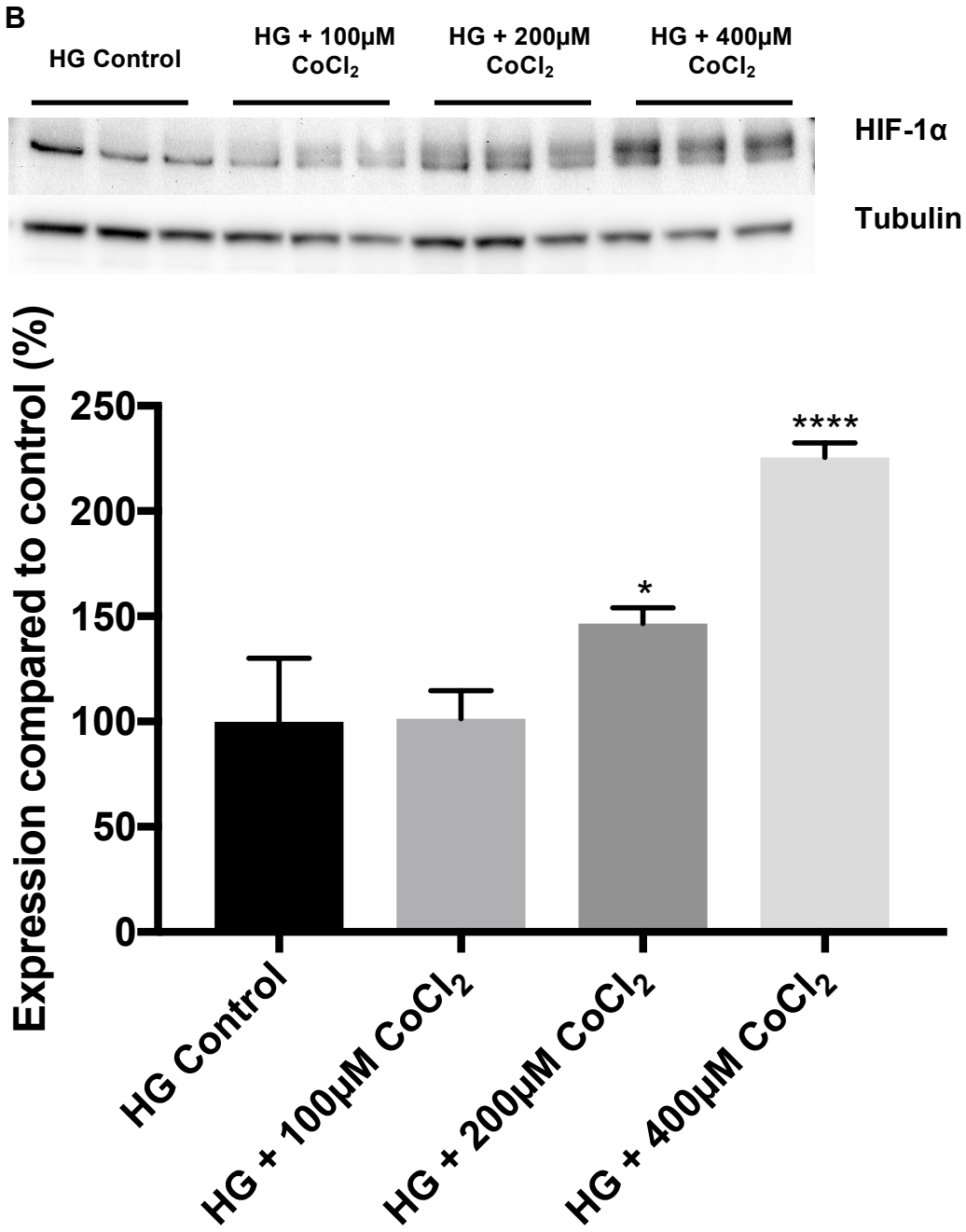


Figure 7.2: Effects of chemically-induced hypoxia on expression of HIF-1α. (A) low glucose (LG) groups and (B) high glucose (HG) groups. All comparisons to control group. * $p < 0.05$, *** $p<0.001$, **** $p<0.0001$. n = 3 per group.

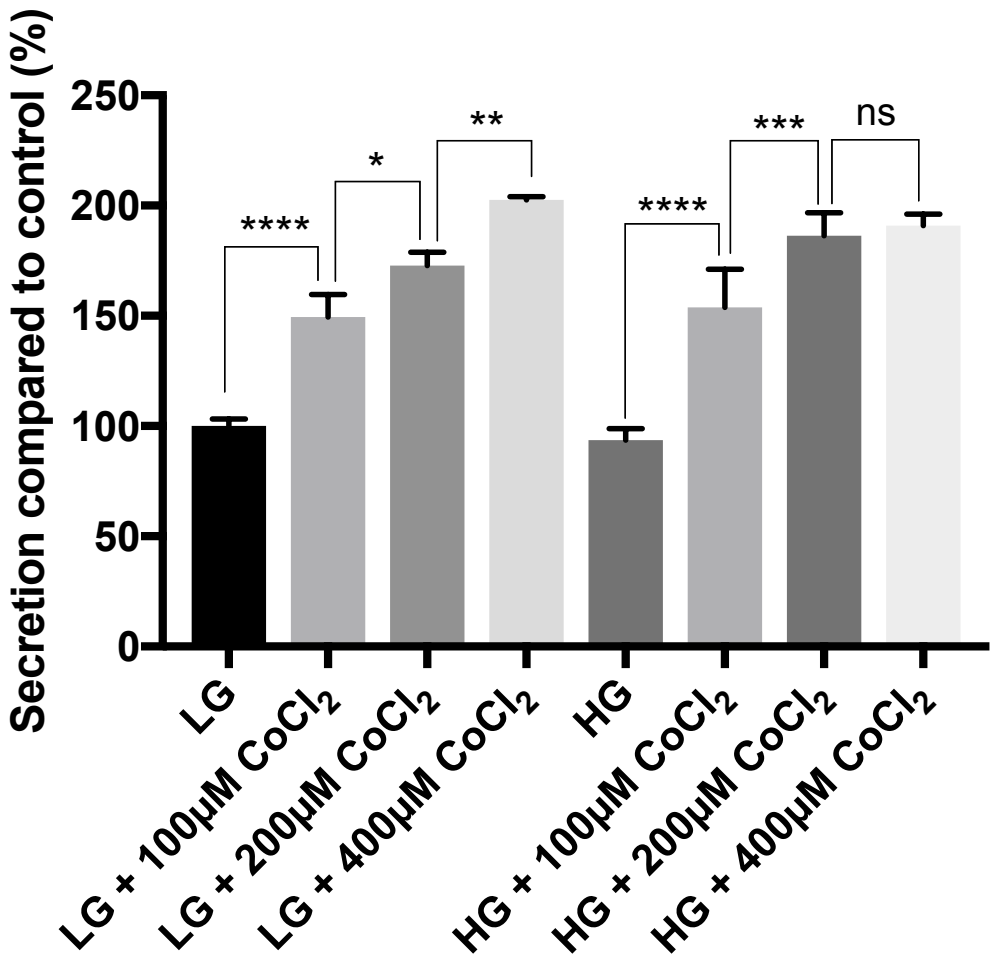


Figure 7.3: Effects of glucose and hypoxia on secretion of VEGF-A in ARPE-19. Results expressed as a percentage of control low glucose (LG) group. HG= high glucose, ns= not significant. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ n = 5 per group.

Effects of hypoxia, high glucose and anti-VEGF drugs on secretion of PEDF, BDNF and PlGF in RPE

Based on the results of the viability assay, HIF-1α and VEGF-A assays, a concentration of 200μM of $CoCl_2$ was used for all subsequent experiments.

PEDF secretion was significantly reduced with the addition of all three anti-VEGF drugs in the absence of hypoxia (Figure 7.4). There was no significant difference between the three drugs. There was a significant decrease in the secretion of PEDF in the presence of hypoxia in both LG and HG groups. There was no significant difference in secretion of PEDF with the addition of anti-VEGF drugs in the presence of hypoxia. There was no difference in secretion between the LG and HG groups in the presence or absence of hypoxia.

BDNF secretion was significantly increased in the presence of hypoxia in both the LG (Figure 7.5A) and HG (Figure 7.5B) groups. There was increased secretion of BDNF detected with aflibercept treatment in the absence of hypoxia in the LG group only (Figure 7.5A). There was an overall reduced secretion of BDNF in HG compared to the LG groups (Figure 7.5C). Levels of secreted BDNF were not significantly different compared to the LG groups when hypoxia was introduced to the HG groups (Figure 7.5C).

PlGF was not secreted by ARPE-19 using the ELISA assay.

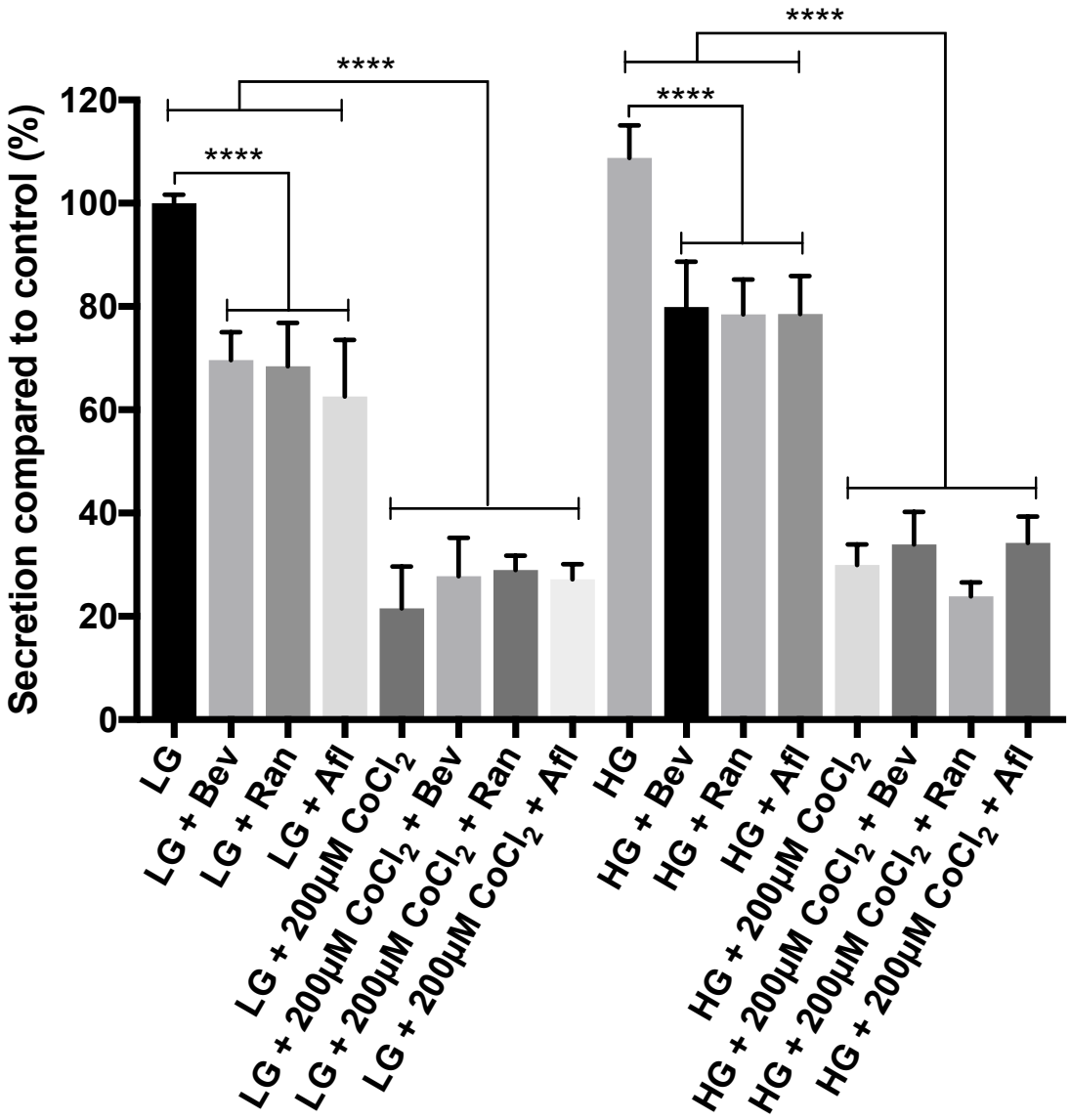


Figure 7.4. Effects of glucose and hypoxia on secretion of PEDF in ARPE-19. Results expressed as a percentage of control low glucose (LG) group. HG= high glucose, Bev=bevacizumab, Ran=ranibizumab, Afl=aflibercept. **** $p<0.0001$ n = 5 per group.

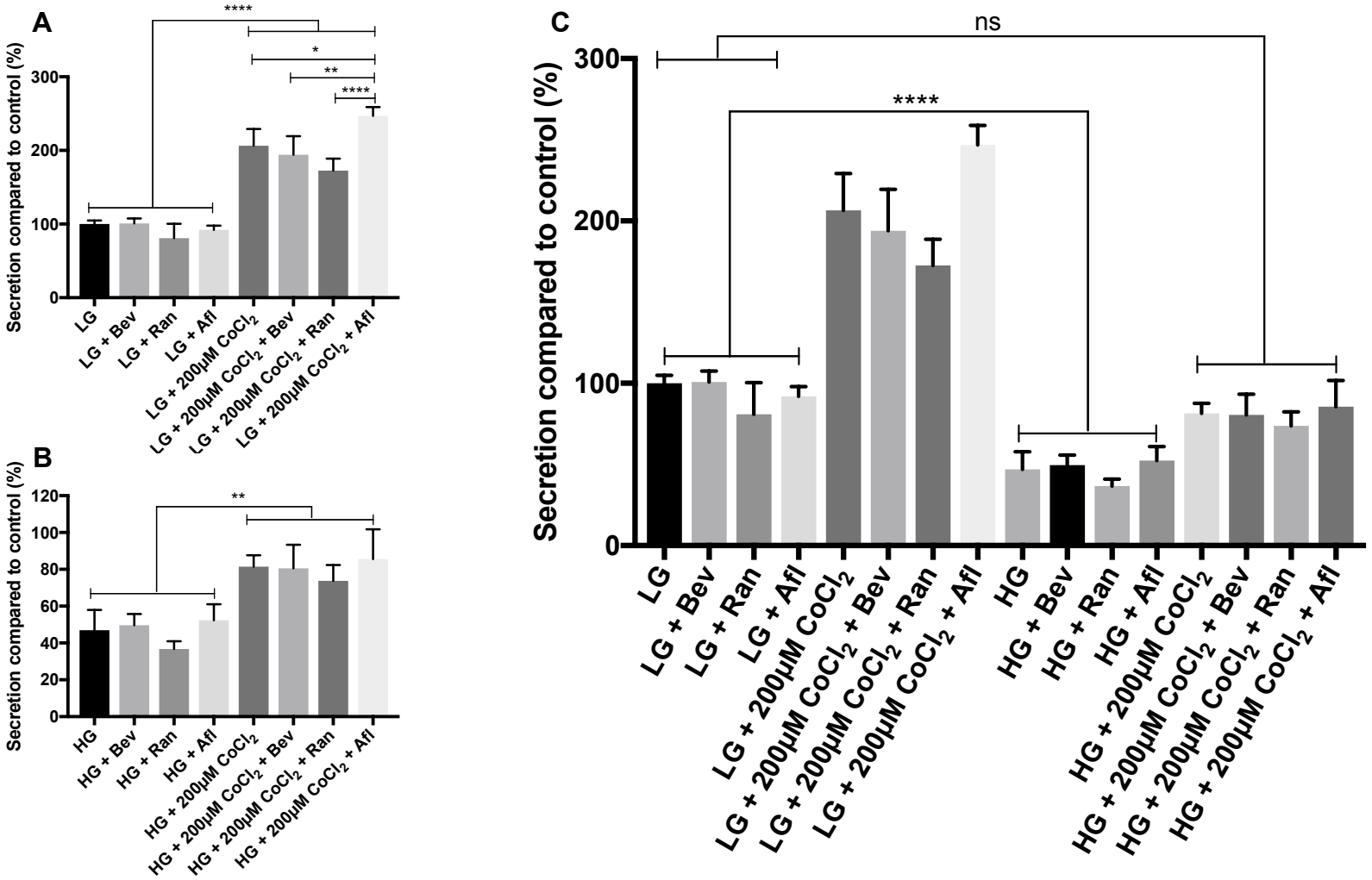


Figure 7.5. Effects of glucose and hypoxia on secretion of BDNF in ARPE-19.
Results expressed as a percentage of control low glucose (LG) group. (A) LG treatment groups, (B) high glucose (HG) treatment groups, (C) overall results. ns= not significant, Bev=bevacizumab, Ran=ranibizumab, Afl=aflibercept. * $p < 0.05$, ** $p<0.01$, **** $p<0.0001$ n = 5 per group.

DISCUSSION

Here we report that hypoxia and glucose affect the secretion of cytokines by the ARPE-19 in this *in vitro* study. Hypoxia appears to have a key effect on the secretion of these factors; whether it is upregulation of HIF-1α, VEGF-A and BDNF, or downregulation of PEDF. Glucose concentration appears to have less effect on the pro/anti-angiogenic factors but may influence the secretion of BDNF, an important neurotrophic factor. We also report that anti-VEGF drugs may have an influence on the secretion of PEDF in the absence of hypoxic stress.

There was no apparent difference on cell viability for different concentrations of glucose in the presence of hypoxia. Glucose appears to have a beneficial effect on the RPE *in vitro*. High glucose has been associated with increased proliferation of ARPE-19 as well as altering the morphology of these cells. [312] Additionally, higher glucose concentrations may increase the expression of tight junction proteins and increase the barrier function of these cells. [313]

We did not show a difference in VEGF-A secretion with an increased glucose concentration in ARPE-19 as others have shown. [312] In our study, the expression of HIF-1α under hypoxic conditions was relatively higher in LG media. This may suggest that cells cultured in a lower glucose concentration are more susceptible to hypoxic stress. However, this was not associated with a corresponding difference in secretion of VEGF-A between LG and HG groups.

Whilst the presence of hypoxic stress increased the secretion of BDNF, higher glucose concentration reduced secretion of this factor. BDNF is a neurotrophic factor that has been shown to have protective effects in photoreceptors and RGCs in several animal models. [314] These effects may be secondary to increasing glutamate uptake and upregulation of glutamine synthetase in Müller cells under hypoxic conditions. [315] BDNF is significantly decreased systemically in the serum of patients with proliferative DR compared to controls as well as patients with diabetes but without retinopathy. [316] Furthermore, BDNF is reduced in the serum and retina of STZ-induced diabetic rats. [316]

High glucose states represent an abundance of energy substrate and may consequently reflect a low stress environment. Consequently, this may lead to the secretion of lower levels of BDNF. Indeed, intraocular injection of glucose has been shown to protect the retina from ischaemic injury in rats and administration of topical and subconjunctival glucose has been associated with an improvement in contrast sensitivity in patients with primary open-angle glaucoma. [317, 318]

BDNF secretion was increased under hypoxic stress with the addition of aflibercept in low glucose settings only. Aflibercept, a fusion protein which combines the binding domains of VEGF receptor-1 and -2, differs in structure and function to both bevacizumab, a full-length monoclonal antibody to VEGF-A, and ranibizumab, an antibody fragment to VEGF-A. Aflibercept subsequently has the ability to inhibit not only VEGF-A but also VEGF-B and PlGF. Altered

autocrine signaling by the RPE as a consequence of inhibition of these other factors may explain these findings.

The addition of anti-VEGF drugs led to a reduction in secretion of PEDF in the absence of hypoxia. PEDF is an anti-angiogenic glycoprotein that is mainly produced by the RPE in the retina. PEDF appears to have strong associations with DMO. Vitreous levels of PEDF were significantly lower in patients with DMO than in diabetic patients without retinopathy and quantitatively were negatively correlated with retinal thickness in a case-control study. [21] Conversely, vitreous levels of VEGF-A were higher in patients with DMO and quantitatively positively correlated with retinal thickness. PEDF has counter-regulatory actions to that of VEGF-A and reduced secretion in hypoxic states demonstrated in our results is consistent with this. [319] Furthermore it has neuroprotective effects on retinal ganglion cells and photoreceptors in animal models. [320, 321]

VEGF-A is known to stimulate PEDF expression in RPE via VEGF receptor-1 in an autocrine manner. [322] Blocking this effect with anti-VEGF drugs will thus reduce expression and secretion of PEDF. The clinical significance of these findings may be a potential deleterious effect of anti-VEGF drugs in the absence of hypoxia. It also highlights a potential mechanism for neurodegeneration suggested to be a consequence of treatment with anti-VEGF treatment in nAMD, a condition not characterised by hypoxia.

There was no difference in the secretion of PEDF in low or high glucose groups in the presence of hypoxia with the addition of anti-VEGF drug. This is consistent

with a clinical study of patients with neovascular glaucoma in which aqueous levels of PEDF were found to remain constant following treatment with ranibizumab. [323] Our findings may represent the minimal secretion PEDF that has been inhibited by a hypoxic signal.

We were not able to demonstrate secretion of PlGF by ARPE-19 under any of the experimental conditions tested. This is consistent with other studies measuring PlGF with ELISA in both ARPE-19 and human primary RPE constitutively or under stress. [324, 325] However, this does contrast with a study that demonstrated that hypoxia not only increases expression of PlGF in ARPE-19 but alters RPE cell permeability. [24]

PlGF may have an important pathophysiological role in the development and progression of DR and DMO. [23] Additionally, blockade of this factor by aflibercept is cited as a key differentiating factor between the anti-VEGF drugs and as a reason to explain the differential results seen in head to head trials such as the DRCR.net Protocol T. [23, 78] Clarification of the source of this factor in the retina would be important.

There are obvious limitations in using an *in vitro* model and extrapolating these findings to a disease as complex as diabetes. The diabetic state consists of more than just hypoxia and hyperglycaemia. There are other growth factors, cytokines, and interactions between multiple cells types in play. The RPE is only one source of neurotrophic factors in the retina and microglia and Muller cells also secrete a range of these factors. [326] Nevertheless, the findings here will help generate

hypotheses in future animal and clinical studies and confirm their clinical significance.

Section 4: Implications of findings and future directions for research

As with the rest of the developed and developing world, the prevalence of diabetes in Australia is increasing. Consequently, there will be an increasing burden of care for the complications of diabetes such as diabetic retinopathy and diabetic macular oedema.

This thesis has addressed a number of key issues relating to persistent diabetic macular oedema, a management challenge with a poor evidence base. The data presented and published here has shown that there may be an overall anatomical and visual benefit in switching therapy from bevacizumab to aflibercept for these patients. This benefit is more apparent in individuals who have a poorer baseline visual acuity and also those who respond well after the first injection. The reasons for this early response warrant further research and may be related to the differences in the anti-VEGF drugs or genetic variations in responses to drug.

Further to this, we have identified morphological biomarkers in these patients that correlate well with vision but also may predict response to a switch in therapy. The devices for performing ultrawidefield imaging are becoming increasingly accessible and the severity of retinopathy and peripheral ischaemia will likely have increasing clinical significance in the management of diabetic retinopathy.

Functional vision is difficult to assess with visual acuity alone. This thesis showed that microperimetry might be an objective way to quantify this that correlates with subjectively reported outcomes. There is limited research

assessing a correlation between subjective and objective outcomes using microperimetry and validation of these findings in diabetic macular oedema as well in different pathologies is necessary.

Anatomical measurements that are generated automatically by OCT devices are used to assess response to therapy clinically at an individual level as well as an outcome for clinical trials. The findings from this thesis shows that automatically generated readings are both unreliable and inconsistent between different devices. These may also vary by different versions of the software used. This has important implications for the conduct and reporting of results from clinical trials.

The in vitro work highlighted signals which may be affected by the diabetic state and anti-VEGF drugs. This has important implications for our understanding to the neurodegeneration that is observed in diabetic retinopathy as well as how the treatments we apply to this condition may affect this process. These findings may also have applications to other retinal conditions which feature oxidative stress, such as retinal vein occlusions as well as those which are managed with anti-VEGF drugs such as neovascular age related macular degeneration. The results presented here are under further investigation analysing different neurotrophic as well as pro-fibrotic factors that may be implicated in diabetic retinopathy. Furthermore, these results are to be validated in a human primary cell culture model with a potential for further investigation in animal studies.

Further research is required into the identification of clinical and laboratory biomarkers to individualise pharmacotherapy and identify patients who may be poor and good responders to anti-VEGF therapy.

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