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**Diabetic Retinopathy: In Vitro and Clinical
Studies of Mechanisms and Pharmacological
Treatments**

Saker SAKER

Thesis submitted to the University of Nottingham in fulfilment for
the degree of Doctor of Philosophy

Ophthalmology

Supervisors: Mr Winfried Amoaku and Dr Elizabeth Stewart

Declaration

Except where acknowledged in the text, I declare that this thesis is my own work and is based on research that was undertaken by me in Academic Ophthalmology, under the supervision of Mr. Winfried M Amoaku and Dr Elizabeth A Stewart at the School of Medicine, University of Nottingham, UK.

Saker SAKER

Student ID No. 4149209

Date: 16.12.2015

Abstract

Diabetic retinopathy (DR) is the leading cause of visual impairment in the working-age population in developed countries and thus is one of the major ocular health problems worldwide. Diabetes seems to have paradoxical effects on the microvasculature in different tissues. The concentration of glucose in the cellular microenvironment determines the cells ability to proliferate, as well as its permeability, and react to different cytokines. The main cause of visual loss in diabetic eye disease is caused by an increase in microvascular endothelial permeability which leads to vessel leakage and fluid build-up, diabetic macular oedema (DMO). Endothelial cell permeability is influenced by multiple factors which have not been fully elucidated, particularly in human models. In addition, the gene and protein expression between retinal and choroidal endothelial cells, even in humans, has been shown to be affected differently by diabetes. Current treatments for DMO include steroid such as dexamethasone (DEX) and anti-vascular endothelial growth factor (VEGF) drugs (e.g. ranibizumab, Novartis). These target reducing vascular leakage in the macula once it has occurred, but do not attempt to treat the underlying pathology and evidence suggests treatment does not result in a completely dry macula in most cases. The ideal treatment for DMO should improve vision and improve morphological changes in the macula. With the introduction of microperimetry, we are able to determine macular sensitivity and to correlate it with a precise location of oedema. The aim of this project was to investigate, *in vitro*, the role of

hyperglycaemia in the pathogenesis of DR through effects on the proliferation, vascular permeability and/or alteration in adhesion molecule expression of the human ocular micro-vascular endothelial cells (EC). This was supplemented with clinical studies on pharmacological treatments for DMO.

The main conclusions made were that high glucose increased retinal EC (REC) permeability *in vitro* and that this increase was likely caused by a decrease in selective tight junction protein expression. The levels of the angiogenic stimulator VEGF-A in the REC were not altered by high glucose, despite increased expression of the related gene hypoxia inducible factor (HIF)-1a levels. DEX both reduced permeability and restored tight junction expression and a combination of DEX and ranibizumab reduced REC permeability more than either agent alone *in vitro*. Fixation stability is dependent on foveal cone function. Eyes with good visual acuity normally have excellent fixation stability. However, eyes with poor visual acuity may have reduced fixation. Clinically, this study has shown that DMO eyes with good fixation stability related to good central cone function, normally demonstrate good visual acuity. Where fixation stability is impaired, VA may be reduced. VA changes following treatment of DMO correlated better with fixation stability parameters than with morphological changes on OCT. Fixation stability may, therefore, be a new important parameter in quantifying and predict the functional impact of DMO, and may be, other macular disorders.

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Abbreviations

AJ	Adherens Junctions
BBB	Blood brain barrier
BRB	Blood Retinal Barrier
CEC	Choroidal Endothelial Cell
CNV	Choroidal Neovascularisation
DEX	Dexamethasone
DMO	Diabetic Macular Oedema
DR	Diabetic Retinopathy
EC	Endothelial Cell
IRMA	Intraretinal microvascular abnormalities
JAM	Junctional Adhesion Molecule
PDR	Proliferative Diabetic Retinopathy
REC	Retinal Endothelial Cell
RPE	Retinal Pigment Epithelium
TJ	Tight Junctions
NG	Normal Glucose
HG	High Glucose
FS	Fixation Stability
RS	Retinal Sensitivity

RVP	Retinal Vascular Permeability
(HIF)-1a	Hypoxia-inducible Factor 1-alpha
VEGF	Vascular Endothelial Growth Factor
OCT	Optical Coherence Tomography
MP	Microperimetry
MAIA	Macular Integrity Assessment

Chapter 1: General Introduction

1.1 Rationale and Aims

Diabetic retinopathy (DR) is an important cause of visual impairment and blindness amongst working-age groups in the developed world. As the incidence of diabetes mellitus (especially Type 2) is increasing in the western countries, it is anticipated that diabetic complications, particularly retinopathy, will become more prevalent in the future (Diamant et al., 2007). The search for pathogenic mechanisms that entirely explain the natural history of retinopathy and indicate the best therapeutic target is still unsuccessful. The incidence of DR correlates with poor glycaemic control and hyperlipidaemia (Ferris et al., 1996, Klein et al., 2002). The main retinal insults are vascular and neural; vessel occlusion, leukostasis, and alteration of the blood–retinal barrier tight junctions, result in increased vascular permeability. Neuronal damage may result from generation of free radicals, mitochondrial dysfunction, leading to neuronal swelling and apoptosis (Antonetti et al., 2012). Diabetic choroidopathy is a less well studied entity, and is thought to occur in the advanced stages of diabetic eye disease (Hidayat and Fine, 1985, Bischoff and Flower, 1985, Fryczkowski et al., 1989). Choroidopathy might trigger the development of retinopathy due to retinal tissue hypoxia and over-expression of VEGF, contributing to further retinal damage and macular oedema. As such, the retinal and choroidal vascular beds seem to be affected differently by diabetes. It is becoming increasingly clear that the functional and structural heterogeneity is present in the retinal and choroidal

circulations. Vascular endothelial cell phenotype is highly heterogenic and site-specific, particularly in the retinal and choroidal vascular bed (Yu et al., 2014). Diabetes and hyperglycaemia have obvious effects on intraocular vascular permeability, adhesion to leukocytes, as well as angiogenesis (Miyamoto and Ogura, 1999, Ciulla et al., 2003) . These alterations result in increased vascular leakage (increased permeability), vascular occlusions, ischaemia, and angiogenesis as occurs in proliferative retinopathy (Bhavsar and Tornambe, 2006, Bhavsar, 2006) . However, the exact mechanisms underlying these changes are not fully understood, and require further elucidation.

The main cause of visual loss is diabetic macular oedema (DMO), increase vascular permeability and breakdown of the blood-retinal barrier (BRB) leads to fluid build-up, damaging visual receptors. Experimental work aiming at a sufficient mechanistic explanation of retinopathy genesis is often carried out by using representative cells cultured in high glucose concentrations (Querques and Delle Noci, 2009, Simo et al., 2006, Saker et al., 2014a). Laser photocoagulation has been the mainstay of treatment for both DMO and PDR until recently when pharmacological treatments were introduced. The current treatments for DMO target reducing vascular leak in the macula once it has occurred, they do not attempt to treat the underlying pathology. These pharmacological treatments are aimed at antagonising vascular endothelial growth factor (VEGF) or non-VEGF inflammatory pathways, and include intravitreal injections of anti-VEGFs (ranibizumab, aflibercept or bevacizumab) or steroids (fluocinolone, dexamethasone (DEX) or

triamcinolone) as single therapies. The current treatments for DMO at reducing vascular leak in the macula once it has occurred, and aim for a dry macular subsequently. Similarly treatments of PDR aim at treating the neovascularisation when it occurs. No attempt is made at addressing the underlying pathology, although it is possible that some of these treatments may alter retinal function in other ways (prevention of neurodegeneration) in addition to reducing vascular leakage.

The aim of this research was to investigate, *in vitro*, the role of hyperglycaemia in the pathogenesis of DR through effects on the proliferation, vascular permeability and/or alteration in adhesion molecule expression of the human ocular micro-vascular endothelial cells (EC). This was also supplemented with clinical studies on pharmacological treatments for DMO.

1.2 Objectives

- To investigate *in vitro* the permeability of primary human retinal endothelial cells (REC) and human choroidal endothelial cells (CEC) under normal glucose (NG) and high glucose (HG) conditions.
- To investigate expression of key tight junction (TJ) molecules in REC and CEC in NG and HG.
- To investigate *in vitro* the proliferation and apoptosis of primary human REC under HG conditions.

- To investigate the effects of HG, and vascular endothelial growth factor (VEGF) in isolation or combination on EC permeability in vitro, and compare the amelioration by anti-VEGF therapy (ranibizumab) and DEX.
- To investigate the changes in REC TJs in high glucose medium and their modulation by DEX, ranibizumab, and combination of DEX and ranibizumab. To evaluate the ability of MAIA MP in detecting early changes in retinal sensitivity in eyes with DMO that are receiving intravitreal anti-VEGF therapies or steroids, and correlate them with morphological changes on OCT and visual acuity (LogMAR) at different time points.

1.3 Structure of the Eye

The eye is the highly specialised organ of visual perception with an extraordinarily intricate structure. It has 3 coats, the outer fibrous coat of cornea and sclera, the middle uvea, and the innermost layer of retina (Forrester et al., 2007). From anterior to posterior, the uvea is made up of the iris, ciliary body, and choroid, as a continuous layer. The choroid is very vascular, with a principal function of nourishing the outer third of the neuro-retina and retinal pigment epithelium (RPE). The retina consists of the neuro-retina and the RPE. The RPE is a monolayer of pigmented cells on which the neuro-retina lies. The retinal vasculature provides nourishment to the inner two-thirds of the neuro-retina, whilst the outer third of the neuro-retina and RPE receive their nourishment from the choroid.

Eyes receive light energy by specialized nerve cells, rods and cones, in the retina. The energy is converted into electric potentials which are relayed through the optic nerve and to the occipital cortex of the brain which processes the information to a conscious perception of vision.

1.3.1 The Retina

The retina is derived embryologically from the optic vesicle (neuro-ectoderm), with an outer wall that becomes the RPE, a potential sub-retinal space and an inner wall that becomes the neural retina. RPE is a continuous hexagonal monolayer of epithelial cells that extends anteriorly from the margins of the optic nerve to the ora serrate, where it is continuous with the pigmented layer of the ciliary body (Kolb, 1995b). The apices form microvilli

that envelop the photoreceptor outer segments. Near the apices adjacent RPE cells are joined by numerous TJ to form the outer blood-retinal barrier. The basement membrane of the RPE forms the inner layer of Bruch's membrane. Neural retina is a thin layer of transparent neural tissue, comprising photoreceptors, integrators (bipolar, horizontal, amacrine, ganglion cells), the output pathway (nerve fibre layer), and the support cells (Muller cells). Anteriorly the neural retina is continuous with the non-pigmented layer of the ciliary body (Kolb, 1995b). The macula is defined histologically by a multi-layered ganglion cell layer and approximates to a 5500µm oval centred on the fovea and bordered by the temporal arcades. The macula is further divided into perifovea (1500µm wide band defined by 6 layers of bipolar cells), parafovea (500µm wide band defined by 7-11 layers of bipolar cells) and fovea (1500µm diameter circular depression) (Kolb, 1995a).

1.3.2 The Choroid

The choroid extends from as far anteriorly as the ora serrata to the optic nerve posteriorly. It is comprised of four layers, from the inside to outside, they are termed the Bruch's membrane, choriocapillaris, stroma, and suprachoroid (Forrester, 2007).

Bruch's membrane is 2-4µm thick centrally, while it is only 1-2µm thick peripherally. Bruch's membrane consists of the basement membrane of the RPE, an inner collagenous zone, a middle elastic layer, an outer collagenous zone, and the basement membrane of the choriocapillaris (Forrester, 2007).

The stromal layer consists of loosely arranged collagen fibres, providing a framework for blood vessels, nerve fibres, and different types of cells. Macrophages, mast cells, plasma cells, and lymphocytes are present in the stroma, and the most commonly observed cells are melanocytes and fibroblasts (Forrester, 2007). The vascular architecture of the stromal layer is structured such that it may be sub-divided into 3 layers: the outer layer of large vessels, the middle layer of medium sized vessels and an innermost layer adjacent to Bruch's membrane composed of capillary vessels (choriocapillaris). The choriocapillaris EC are fenestrated on the side facing Bruch's membrane.

The suprachoroid is located between sclera and the large vessel layer of the choroidal stroma, is a transition zone between them. It consists of tightly packed collagen fibres from choroid and sclera. This network contains fibrocytes, melanocytes, ganglion cells, and nerve plexuses (Forrester, 2007).

1.4 Clinical Features of Diabetic Retinopathy

DR is diagnosed and monitored according to clinical appearance on fundoscopy, and fluorescein angiography. The initial changes called background DR do not endanger central vision. These include microaneurysms (the earliest observable clinical change), haemorrhages of different types, exudates, cotton wool spots (CWS), and retinal arteriolar and venous calibre changes (Simonsen, 1974) (Gelissen and Ziemssen, 2010). Subsequent changes of progressive ischaemia lead to the development of

proliferative DR (PDR) which is characterised by the development of abnormal new blood vessels originating from the retinal venules. Indeed, PDR may result in visual loss due to either spontaneous vitreous haemorrhage or retinal detachment induced by subsequent traction from connective tissue in the new vessels. Diabetic maculopathy may occur in association of both background and proliferative retinopathy. Diabetic maculopathy indicates the occurrence of diabetic changes in the macular area, and may be in the form of focal exudation, diffuse oedema, ischaemia or a mixed picture (Haritoglou et al., 2015, Manasseh et al., 2015, Gelissen and Ziemssen, 2010).

1.4.1 Micro-aneurysms and Haemorrhages

The occurrence of small red dots existed temporal to the foveal avascular zone (FAZ) is considered the earliest clinical signs in DR (Dobree, 1970). These red dots may represent either micro-aneurysms (less than 100 μm) or small haemorrhages resulting from localised ruptures of the retinal capillaries. Their density is highly correlated with the condensed retinal capillary system in that area. It has been reported that the risk of developing retinopathy increases according to the number of background red dots. However, a few red dots do not necessarily indicate a higher risk of developing DR than no lesions (Kohner and Sleightholm, 1986, Klein et al., 1989, Klein et al., 1995). According to Klein et al (1995), the relation between the progression of DR and the number of red dots is parallel, ranging from years to decades. Another study revealed that the location of some

vascular lesions in DR might frequently change, reflecting a consistent style in formation and resorption of these lesions (Bek and Kjaergaard, 2004).

1.4.2 Blood-Retinal Barrier Breakdown and Subsequent Retinal Oedema

Retinal exudates are lipoprotein precipitates resulting from plasma leakage from retinal blood vessels (Murata et al., 1992, Diezel and Willert, 1961). Exudates appear as sharply defined granular deposits in the inner retina. However, one lesion may enlarge and combine with other surrounding lesions, resulting in constellations of exudates, or may be absorbed totally or partially. The microvascular weakness may be localised as in micro-aneurysms, or diffuse along the microvasculature, or a combination of these. An increased plasma ultra-filtration is primarily attributable to the BRB breakdown. The latter is induced by capillary disruption and/or hyperperfusion. The disruption of the BRB can be elucidated by fluorescein angiography where intravenously injected dye is identified either as focal leakage areas or in diffuse patterns (Bresnick, 1983). Fluorescein leakage is attributed to a disturbance in the balance between osmotic and hydrostatic forces in the retinal vasculature resulting from the increased capillary leakage, which leads to abnormal accumulation of fluid and solutes in the retinal tissue (Stefansson et al., 1983). The dynamics of the detailed structural changes in the capillary walls and the subsequent formation of retinal oedema in diabetic patients have only been sparsely studied. Accordingly, leakage of fluorescein is still a frequently used sign of the gross processes leading to DMO.

Although exudates develop later than red dots (micro-aneurysms and haemorrhages), their distribution may be similar: usually temporal to the fovea. Furthermore, their density significantly decreases from the vascular arcades towards peripheral retina. Exudates and macular oedema are thought to have a detrimental effect on the neuronal tissue. Hence, the occurrence of these manifestations in the macular region is considered a widely used marker of a possible vision-threatening retinopathy, so-termed 'Diabetic Maculopathy'. The formation of exudates and/or retinal oedema either larger than one disk diameter and a part of this area is within one disk diameter from the fovea, or when these lesions develop within a half disk diameter from the fovea is called 'Clinically Significant Macular Oedema'.

1.4.3 Retinal Cotton Wool Spots

Retinal CWS are fluffy whitish lesions located in the superficial layers of the retina, and approximately one third to a half disk diameter in size. Retinal CWS are primarily attributed to focal disturbances in the axoplasmic transport of the retinal nerve fibre layers (McLeod et al., 1977). These abnormal events hold up of axoplasmic transport occur secondary to microinfarction of the retinal nerve fibre layer. As a result there is an accumulation of intracellular organelles in the nerves. These organelles are normally inversely transported from the terminal end of the axon in the lateral geniculate body (Diezel and Willert, 1961).The resulting swelling in the

affected retinal nerve fibres and the subsequent thickening of the inner retinal layers will diffuse light and mark the lesion with its characteristics.

Solitary CWS do not play a specific prognostic role in developing DR (ETDRS, 1991). In contrast, increased CWS may indicate progression of DR (ETDRS, 1991). CWS develop within days and regress over months according to the underlying stimulus (Kohner et al., 1969). The size of the lesion will gradually diminish and assume an irregular grainy pattern until it totally disappears.

1.4.4 Pathological Changes of Retinal Arterioles

It has been frequently demonstrated that increased vascular tortuosity and dilated retinal arterioles occur early in DR (Stefansson et al., 1983, Kristinsson et al., 1997, Kylstra et al., 1986). This is primarily due to the early hyper-perfusion in diabetes. Increased immunoreactivity to S-100 protein expressed by retinal perivascular glial cells is critical in areas with retinal capillary hyper-perfusion (Bek, 1997b). The arteriolar changes in diabetes may not play a specific prognostic or diagnostic role in DR since they may occur in other diabetic vascular complications such as end stage-renal failure, accelerated atherosclerosis and arterial hypertension (Ashton, 1953, Yamagishi et al., 2005). However, marked arteriolar involvement should be considered as a characteristic, late development in the diabetic process itself when assessing a super-added hypertensive complication in diabetes. (Ashton, 1953).

1.4.5 Capillary Occlusion

In the late stages of DR, retinal capillary occlusion may occur. This initially appears in mid-peripheral retina and progresses towards the peripheral retina (Shimizu and Muraoka, 1981). For no obvious reason, post-surgical retinal ischaemia in older diabetic patients often manifests as neovascularisation of the iris and the anterior chamber angle. Accordingly, increased intraocular pressure and subsequent neo-vascular glaucoma may be the initial presentation of retinal capillary occlusion. Histological studies have reported an integral role of glial cells in vascular occlusion since an in-growth of Muller cells into the occluded capillaries has been described (Bek, 1997a). This glial in growth occurs after the initial events of EC loss and thrombus formation in the retinal capillaries.

1.4.6 Intra-Retinal Micro-vascular Abnormalities (IRMA)

IRMAs were originally described as hyper-cellular dilated channels, some of which were believed to represent attempts at neovascularization (Bresnick et al., 1977). They are now attributed to disturbances in the distribution of the retinal blood flow (Imesch et al., 1997). These IRMA are thought to develop as a compensatory mechanism whereby dilated retinal vessels can bypass regions of occluded capillaries. More importantly, the formation of IRMA significantly indicates that DR has entered a pre-proliferative stage. These vessels are observed clinically as irregularly branching vessels usually connected to arterioles but may join with venules in the retina, and the lining EC may demonstrate fenestrations (Imesch et al., 1997).. IRMA are histologically characterised by a large diameter and an abnormally deep

location in the retina (Bek, 2002). IRMA have more of a burgundy colour as opposed to red, they are not commonly found around the disc, they usually occur adjacent to cotton wool spots, and may be associated with other signs such as 'omega' venous loops, venous reduplication and white lines which represent occluded arterioles.

1.4.7 Venous Manifestations

In the more advanced stages of DR, the retinal venules dilate. It is a commonly held belief that venous dilation may be adapted due to the increased retinal blood flow. Since retinal arterioles also dilate and lengthen, it may be quite difficult to differentiate between them and the adjoining venules. One other pronounced venous change is a segmental dilation of the retinal venules associated with the diameter becoming larger towards the retinal periphery in so-called venous beading or sausages. These abnormal characteristics indicate that retinopathy has entered a pre-proliferative stage (Bek, 2002). In addition, metabolic acidosis induced by capillary occlusion in the retinal periphery may result in similar venous changes, particularly a string of beads or sausages (Bek, 2002).

1.4.8 Retinal and Optic Disc Neovascularisation

As the peripheral ischaemic areas resulting from retinal capillary occlusion release different growth factors, a process of retinal and optic disc neovascularisations may occur. It has been shown that their growth pattern is strongly similar to that of foetal angiogenesis since the increasing number of metabolically active cells through retinal development is correlated with increasingly relative ischaemia. As a result, the proliferation of EC and the subsequent formation of new vessels is stimulated (Hughes et al., 2000). In addition, the micro-circulation is formed by the link between the vascular fronds resulting from this proliferation and the arteriolar counterparts. It has been shown that occluded capillaries in DR cannot be replaced by these new vessels in the mature retina (Martin et al., 2003). Hence, it is hypothesised that the occurrence of the newly formed vessels and their growth into the vitreous indicate that these vessels never involve any connection with related arterioles to maintain blood flow. Two feeder vessels are usually generated from the same area on the venule to induce a fan-shaped neovascularisation. Naturally, there may not be significant circulation of blood in these new vessels since there is no significant difference in the pressure between these feeder vessels. Neovascularisation may result in adverse effects. In particular, vitreous haemorrhage and tractional retinal detachment resulting from the shrinkage of connective tissue surrounding the pre-retinal new vessels may ultimately occur. Retinal photocoagulation can significantly mitigate neovascularisations yet it may not lead to complete disappearance of the new vessels (Bek and Kjaergaard, 2004). In the more

advanced stages of the development of neovascularisation, long thin feeder vessels with broader lumen could be observed feeding the irregularly branching advancing front (Bek and Kjaergaard, 2004).

1.5 Clinical Diagnosis in Diabetic Retinopathy

Other retinal vascular disorders may present with morphological lesions similar to those that occur in DR. Accordingly, the distribution patterns, the dynamics and the combination of various retinopathy lesions is crucial in any discussion about methods of diagnosis in DR.

A number of other diseases of the retinal vascular system may be characterised by both the peripheral and central features in the retina. According to (Tagawa et al., 1986), age might be a determinate factor regarding the differences in distribution patterns. Younger patients with type I diabetes mellitus might frequently develop PDR due to favourable conditions for neovascularisation as the posterior hyaloid membrane is intact, and serves as a scaffold for the neo-vascular growth. In contrast, older type II diabetic patients might predominantly suffer from diabetic maculopathy which may be primarily attributed to an age-related arteriolar down-regulation in the retina. However, other researchers have recently revealed that age does not play a determinant role in these differences. Instead, they suggest that the distribution patterns in DR may reflect the regional differences of vision-threatening complications between the peripheral and central retinal capillary perfusion (Shiraya et al., 2006).

It is almost commonplace to state that different morphological lesions related to DR concurrently develop in different areas of the retinal microcirculation. It has been demonstrated that hyper-perfusion might result in different lesions, typically localised temporally from the foveal area. Furthermore, these might also extend to the remaining part of the macular region (Dobree, 1970). Consequently, it was suggested that the progression of DR is primarily correlated with significant changes in the retinal micro-vasculature rather than that of larger arterioles. This hypothesis has been buttressed by the observation that DR lesions which occurred adjacent to the larger vascular arcades did not play a prognostic role in a potentially vision-threatening retinopathy. In other respects, the development of these lesions away from the main arcades towards either the macular region or the retinal periphery prognosticates further progression of the disease (Hove et al., 2006). However, DR lesions may resemble those of hypertensive retinopathy in terms of a distribution pattern around the larger retinal arterioles and the optic disc (Bek and Helgesen, 2001). This may demonstrate an integral role of blood flow and impaired auto-regulation in further development of DR (Kohner et al., 1995).

As far as the dynamics of DR lesions are concerned, it is often argued that capillary occlusion is the initial step towards the formation of both micro-aneurysms and dot haemorrhages (Chibber et al., 2007). However, it would seem that red dots initially presenting in DR might play a diagnostic role in older patients with type II diabetes mellitus. One other important interdependence between exudates already arranged in a circinate pattern

and associated red dots may reflect different responses to a localised vascular abnormality (Chibber et al., 2007).

Ischaemic diabetic maculopathy may present with retinal oedema and haemorrhages without exudates. Thus, it is quite reasonable to identify the typical capillary occlusion using fluorescein angiography in such conditions.

As suggested above, it would seem that visual impairment in DR may be attributed to various mechanisms correspondent to individual retinal lesions. It would seem, therefore, that the pathological correlates of these lesions are crucial for understanding how diabetic retinopathies result in visual dysfunction. The particular change, size and the location of the retinal region involved by the disease will determine symptomatology. In this regard, arterial and venous changes as well as micro-aneurysms do not directly result in visual dysfunction, whereas haemorrhage, exudates and neovascularisation lead to visual impairment due to the blockage of retinal photoreceptors. CWS may generate either local relative scotomas that regress partly or an arcuate scotoma may result from longer lasting lesions. More importantly, retinal ischaemia may result in irreversible visual impairment. In particular, localised defects in visual field in the ischaemic areas as may occur (Bek, 1992, Moller and Bek, 2003). In contrast, subclinical perturbations in the ERG may not be considered as remarkable changes (Pahor, 1998, Lovestam-Adrian et al., 2000).

Appropriate methods of treatment for DR should primarily prevent central as well as peripheral visual loss attributed to either diabetic maculopathy or

PDR. Retinal photocoagulation may adversely affect everyday activities as a result of changes in dark adaptation, contrast sensitivity and the peripheral visual field. Localised defects in visual field in the ischaemic areas as well as retinal damage in the laser-treated regions may occur (Bek, 1992, Moller and Bek, 2003).

1.6 Diabetic Macular Oedema and Retinal Vascular Permeability

DMO is a principal cause of visual impairment for persons with diabetes. Although DMO can occur at any stage throughout the progression of diabetes, including non-proliferative (NPDR) and proliferative (PDR) retinopathy, its prevalence is primarily attributable to advanced stages of diabetes (Resnikoff et al., 2004, Bhagat et al., 2009). Risk factors responsible for DMO include both hypertension and hyperglycaemia (Klein et al., 1998).

DMO mainly results from an increase in retinal vascular permeability (RVP) which, in turn, may occur either focally or diffusely in the retina. Poor visual acuity can be primarily attributed to increased retinal thickness (Kim et al., 2006). Nevertheless, many research teams have suggested that macular thickness is not the sole indicator of visual acuity (Davis et al., 2008). The EC of retinal vessels play a key role in the inner blood-retinal barrier (BRB), whereas the apical TJ of RPE contributes to the outer BRB. A marked increase in RVP is associated with advanced DR suggests that additional biochemical factors may contribute to visual loss (Krogsaa et al., 1981, Plehwe et al., 1989). The facilitated diffusion of circulating lipids and proteins

results in a considerable transport across the retinal endothelium into the interstitial fluid of the neuro-retina. This lipoprotein accumulation is largely responsible for lipid deposition and thickening of the macula (Marmor, 1999). Consequently, excessive RVP regulates neuro-retinal function and retinal fluid homeostasis, subsequently leading to visual acuity loss.

1.6.1 The Blood-Retinal Barrier

The BRB demonstrates its properties via regulating the exchange of water, metabolites, nutrients, proteins and neurotransmitters as well as the efflux of any toxic materials resulted from metabolism. In addition, it has a potential impact on restraining the entry of toxins, inflammatory cytokines, circulating immune cells and antibodies thus shielding the neural retina from the circulating blood. It was shown that all tissues in the rat except the brain had been stained using intravenously injected trypan blue (Cunha-Vaz, 2004). It was demonstrated that histamine injected intravenously in the rat could induce the vascular permeability in different ocular tissues except the retina (Ashton and Cunha-Vaz, 1965). This, in turn, could reflect a constant milieu of the BRB. Subsequent morphological studies by (Cunha-Vaz, 2004) showed that the BRB consists of two independently regulated components: the inner and the outer BRB. The former, provided by the endothelium of the retinal vessels, is held responsible for BRB functions in the inner retina, whilst the latter formed by the RPE for the outer retina (Cunha-Vaz, 2004). Choroidal endothelial cells (CEC) have fenestrations similar to EC elsewhere in the body, whereas the EC of the retina vasculature have no fenestrations and maintain virtually all the specific barrier milieu of the BRB. Moreover,

their surrounding pericytes, also known as Rouget cells, are more concentrated in the retina compared to the brain (Stewart and Tuor, 1994). Many aspects of the physiology of BRB are still not fully understood and, in most cases, based on logical deductions from animal and clinical studies in other natural barriers such as the blood-brain barrier (BBB). There are powerful connections between the extracellular fluid of the retina and the nearby vitreous, leading to a marked diffusion of solutes.

1.6.2 Molecular Biology of the Blood-Retinal Barrier

The main methods by which various molecules pass through endothelial and epithelial cell layers can be briefly classified into trans-cellular and paracellular routes. The former includes active transport (receptor-mediated uptake), passive diffusion, facilitated diffusion (channel-facilitated transport), endocytosis and fenestrations. Another method of trans-cellular flux, primarily used for the absorption of extracellular fluids (ECF) is known as pinocytosis. EC in the BBB and BRB, as well as RPE cells lack fenestrations. Moreover, they have less pinocytosis activity (Burns and Hartz, 1992). It has been shown that vascular endothelial growth factor (VEGF) may mediate the transport across choriocapillaris EC via interfering with their existing fenestrations. (Peters et al., 2007) supported this hypothesis when they reported that intravitreal injection of anti-VEGF agents in cynomolgus monkeys led to a significant decrease in these fenestrations. Based on this finding, it is possible to suggest a substantial method of treatment for DMO – VEGF inhibition (Peters et al., 2007). Since the choriocapillaris has fenestrations, the outer BRB formed by the RPE cells regulates functions in

the outer retina. An asymmetric distribution of proteins alongside the RPE monolayer to form apical TJ plays a key role in the proper function of this barrier (Rizzolo, 1997). The inner BRB is formed by the TJ between the retinal vascular EC. The control of paracellular permeability and polarity are primarily attributable to TJ. TJ proteins are sophisticated components with pivotal roles both in intercellular adhesion and in cell motility (Erickson et al., 2007). There have been significant steps forward regarding our understanding of properties of the TJ. These include marked progress in the composition of the apical junctional components and the distribution of cytoskeletal proteins which may be important in the development of both choriocapillaris and the neural retina (Rizzolo, 1997).

Water is actively transported out of the retina through the choriocapillaris, thereby preserving retinal attachment. Furthermore, lactate also actively moves from the RPE cells to the choroid (Philp et al., 1998) (Philp et al., 2003). Another important aspect of RPE function is that it has the ability to actively transport lipophilic toxins and drugs back to the main circulation through the P-glycoprotein presented in the BRB (Greenwood, 1992, Kennedy and Mangini, 2002, Maines et al., 2005).

The transfer of fluid between cells is primarily via the permeability of TJ. Under pathologic conditions, a considerable dysfunction in the cellular continuum and TJ's disassembly can ultimately result in increased vascular permeability.

1.6.3 Tight Junction Proteins

The control of paracellular permeability and polarity are primarily attributable to TJ. These junctions are correlated with an apparent fusion of the outer leaflet of the plasma membranes of adjacent cells (Anderson and Van Itallie, 1995) and can express sealing sites of the intercellular space. In addition, their protein composition plays an integral role in the molecular architecture. However, membrane lipids may participate in this role as well. It has been reported that TJ proteins include the tetra-helical molecules occludin (Furuse et al., 1993), claudins (Furuse et al., 1998a) and immunoglobulin-like cell adhesion molecule called junctional adhesion molecules (JAMs) (Martin-Padura et al., 1998).

The membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins found at distinct subcellular regions, such as the cytoplasmic surface of the plasma membrane (Dimitratos et al., 1999) including intercellular TJ proteins. These include zonula occludens (ZO)-1 as a cytoplasmic scaffolding protein (Stevenson et al., 1986) and ZO-2 (Gumbiner et al., 1991) and ZO-3 (Haskins et al., 1998) in relation to the small G-proteins rab3b (Weber et al., 1994) and rab 13 (Zahraoui et al., 1994) as well as cingulin (Citi et al., 1988), 7H6 (Zhong et al., 1993) and symplekin (Keon et al., 1996).

Whatever the experimental proof is, the restraint resulting from TJ to the paracellular diffusion of solutes and ions may be exerted by the ability of transmembrane TJ proteins to seal the interspace between nearby EC. This

could be clearly seen by the fact that TJs primarily concentrated in brain vessels stain intensely for occludin (Furuse et al., 1993). However, genetic ablation of occludin does not result in long term effects on TJ organisation or intercellular permeability (Schulzke et al., 2005). This might indicate a compensatory mechanism by other molecules (Saitou et al., 1998). Therefore, it is still unknown whether the effect of occludin is either independent or cooperative with other TJ including claudins and JAMs. In addition, several signalling receptors co-localise with TJ components (Mitic and Anderson, 1998). It has been demonstrated that changes in the organisation of both TJ and actin are correlated with EC permeability (Anderson and Van Itallie, 1995). Signalling roles and even nuclear localization may be attributed to cytoplasmic components of TJ (Gottardi et al., 1996, Muller et al., 1993). In this regard, a cytoplasmic component (ZO-1), which demonstrates nuclear staining in sub-confluent cultures, is activated alongside the outer tip of the villus in tissue sections in epithelial cells *in vitro* at sites of wounding (Gottardi et al., 1996). As such, it is thought that morphogenetic procedures leading to the formation of new vessels may be largely regulated by TJ components. However, this concept remains obscure at the present time, and may be worth to investigating in future research programmes.

Leukocyte extravasation *in vitro* (Martin-Padura et al., 1998) can be directed by JAMs, which is effective *in vivo* with respect to cytokine-induced meningitis (Del Maschio et al., 1999) as well as in a model of skin inflammation (Martin-Padura et al., 1998). Naturally, this effect is mainly

attributable to inhibition of leukocyte adhesion to JAMs located at inter-endothelial junctions and the blockade of their processes through endothelial junctions.

It has been shown that paracellular permeability can be reduced by transfection of JAM cDNA, which in turn promotes occludin localisation at intercellular junctions (G. Bazzoni, submitted). As a consequence, JAMs through its homotypic multimerisation, plays an integral role in the organisation of TJ.

The formation of strands at intercellular junctions, which have similar features to TJ, can be induced by transfection of claudins in specific cells. Interestingly, these cells no longer express TJ (Furuse et al., 1998b).

As suggested above, immunoglobulin-like cell adhesion molecule, JAM, is a small immunoglobulin that is localised at TJ in epithelial cells as well as the endothelium. It also plays a principal role in the organisation of TJ and regulates leukocyte extravasation through endothelial intercellular junctions both *in vitro* and *in vivo* (Mandell et al., 2004, Mandell et al., 2005).

Owing to the fact that claudins form a huge family of 18 homologous proteins related to various expressions, different types of claudins can be expressed on the surface of the same cell. Furthermore, they may interfere with homotypic and heterotypic methods (Furuse et al., 1999). It would seem that the different claudins substitute for each other as gene inactivation of the individual claudin family components do not produce deleterious effects. This

property, in turn, may effectively reveal their role in TJ in the endothelium. It has been concluded that soluble mediators can direct a vascular morphogenesis through their actions on either EC or perivascular cells (Hanahan, 1997).

1.6.4 Zonula Occludens Proteins

It has been frequently demonstrated that numerous junctions are held responsible for attachment of both epithelial and EC. They depend on the function of certain proteins associated with particular structural features, including the presence of one or more transmembrane components characterized by cytoplasmic and extracellular portions. Many research programmes across the world continue to investigate the mechanisms of creating a regulated intercellular seal (Jesaitis and Goodenough, 1994, Itoh et al., 1999). Some findings indicate that protein-protein interactions at the cytoplasmic matrix of such transmembrane junctional proteins regulate the extracellular function of the protein which accomplishes either homophilic or heterophilic binding to extracellular domains of junctional proteins of adjacent cells (Haskins et al., 1998).

Zonula occludens or *zonulae occludentes* (ZO) proteins, including ZO-1,-2 and-3, are localized at junctional regions. In addition, the expression of junctional plaque is primarily attributed to the function of these scaffolding proteins recruiting a preponderance of proteins to the cytoplasmic surface of the junction. ZO proteins have been considered as the central organisers of TJs (Erickson et al., 2007). However, these studies were re-evaluated since

ZO were found to modulate the cadherin-based adherent junctions (AJs) in TJ-deficient cells (Howarth et al., 1992). The principal role of ZO proteins at the junctional sites is to regulate claudin polymerisation in epithelial cells, a concept which was reported from the utilisation of a reverse genetic approach (Umeda et al., 2006, Fanning and Anderson, 2009): that AJ formation is a prerequisite for TJ development. Disruption of VE-cadherin leads to breakdown of intracellular adhesions and subsequent re-structuring of both TJ and AJ.

It has been reported that ZO proteins can interact with the trans-membrane proteins expression at TJ, such as occludin, claudins, junctional adhesion molecule (JAM) as well as coxsackievirus and adenovirus receptor (Cohen et al., 2001, Itoh et al., 1999). Their association with AJ establishes a considerable link between the trans-membrane components and the cytoskeleton (Fanning and Anderson, 2009). Furthermore, ZO proteins integrate with a significant series of cytoplasmic proteins, including signalling molecules, and adapters in order to modulate cell morphology and signalling (Balda and Matter, 2008, Bauer et al., 2010).

ZO proteins are classified as one of the members of membrane-associated guanylate kinase like proteins (MAGUK) as indicated previously. These are scaffolding proteins which regulate multi-molecular complexes at distinct subcellular regions, such as the cytoplasmic surface of the plasma membrane (Dimitratos et al., 1999, Gonzalez-Mariscal et al., 2000). In addition, it has been reported that it is possible to derive a highly supported

evolutionary model for crucial multi-domain families through analysing encoded protein domains. In this regard, at least four of the seven MAGUK subtypes were present in early metazoan lineages, such as Porifera (te Velthuis et al., 2007).

1.6.5 The Role of JAMs in Leukocyte adhesion and Trans-endothelial Migration

The interactions of JAMs have an impact on the permeability of endothelial-cell or epithelial-cell layers and are therefore an important component of inflammatory processes (Mandell et al., 2004, Mandell et al., 2005). The first evidence for a role of JAMs in inflammatory reactions came from studies using the blocking monoclonal antibody BV11, which is specific for mouse JAM-A. Their findings indicate that neutrophil recruitment in some mouse models of inflammation such as cytokine-induced meningitis is primarily reduced by BV11, alluding to the fact that this antibody inhibits monocyte transmigration *in vitro* (Del Maschio et al., 1999). Another important aspect to be considered is the interaction of JAM-A with the lymphocyte function-associated antigen 1 (LFA1) involved in trans-endothelial migration of leukocytes (Ostermann et al., 2002). Subsequent studies have shown that JAM-A deficient endothelial-cells are less effective in inducing the arrest and transmigration of monocytes (Zernecke et al., 2006). Similarly, JAM-C on EC mediates increased transmigration of lymphocytes. In turn, a soluble form of JAM-C and a monoclonal antibody specific for this protein have the potential to block lymphocyte transmigration across endothelial-cell monolayers (Johnson-Leger et al., 2002). In addition, it has been shown that JAM-C

plays an integral role in trans-endothelial migration of neutrophils, which can also be inhibited in a mouse model of acute inflammatory peritonitis using soluble JAM-C *in vitro* (Chavakis et al., 2004).

It has been reported that JAM-A interactions stabilise cellular junctions and decrease vascular permeability (Liu et al., 2000). However, studies of JAM-C-deficient micro-vascular EC revealed that vascular endothelial permeability can be primarily regulated by JAM-C by destabilising or competitive binding vascular endothelial-cadherin junctions (Orlova et al., 2006b). Accordingly, preferential expression of JAM-A or JAM-C in various vascular components may contribute to the specific properties of intercellular junctional compartments. The overexpression of JAM-A in the brain vascular system may contribute to the tightly sealed junctions in the blood-brain barrier. In contrast, JAM-C is highly expressed in inter-EC junctions in lymph nodes, reflecting the potential permeability of these junctions (Ozaki et al., 1999). Likewise, an up-regulation of JAM-C expression or apical redistribution of JAM-A may play a role in permeability as 'gate keepers' in inflammation and vascular pathology.

1.6.6 The Role of JAMs in Angiogenesis

The angiogenic process involves the migration, proliferation and differentiation of EC that results in the generation of new blood vessels. This is fundamental to the growth of tumours and collaterals during ischaemia. Angiogenesis is regulated by multiple growth factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Signalling crosstalk with angiogenic receptors is mediated by the integrins $\alpha_v\beta_5$ and $\alpha_v\beta_3$ expressed by ECs (Naik et al., 2003). JAM-A regulates angiogenesis based on interactions with the integrin $\alpha_v\beta_3$ in the absence of bFGF. However, its redistribution and subsequent dissociation of the JAM-A- $\alpha_v\beta_3$ -integrin complex occur after EC activation with bFGF. This, in turn, can initiate angiogenesis via angiogenic signals in order to transverse these receptors. One other important consideration is the role of JAM-A in bFGF-induced activation of extracellular signal-related kinase (Naik et al., 2003). Moreover, JAM-A is required for the JAM-A- $\alpha_v\beta_3$ -integrin-specific endothelial-cell migration on vitronectin (Naik and Naik, 2006). Thus, this protein is involved in angiogenesis through correcting the EC motility, directional movement and focal contact formation.

More recently, studies on JAM-C have shown that its blockade inhibits both tumour growth and angiogenesis (Lamagna et al., 2005a). In models of hypoxia-induced retinal neovascularisation, a JAM-A specific blocking monoclonal antibody reduced the angiogenic process (Orlova et al., 2006b, Lamagna et al., 2005a). The interactions between tumour cells and ECs that may contribute to the metastatic process are mediated by homophilic

interactions of JAM-C (Santoso et al., 2005). Notably, whether JAM-C functionally interacts with integrins or vascular growth factor receptors expressed on ECs is still a subject of intense investigation.

1.7 VEGF, Pro-inflammatory Cytokines and Pharmacologic Treatments in DMO

DMO is one of the primary causes of visual impairment in patients with DR (Congdon et al., 2003). PDR remains the leading cause of blindness among working age population, and DMO is more frequent in type II diabetes. Apart from vascular endothelial growth factor (VEGF), which plays an integral role in both DMO and PDR, several growth factors have been thought to be involved in the pathogenesis of PDR rather than DMO (Simo et al., 2006). Leukostasis, acellular capillaries and platelet aggregation constitute that main structural changes leading to the capillary drop out. Severe hypoxia attributed to capillary occlusion is the essential condition for the initiation of new vessels in PDR. Hypoxia upregulates the expression of angiogenic factors directly or through hypoxia-inducible factor. Apart from promoting EC proliferation, angiogenic factors also results in increased expression of integrins and proteinases. As a result of proliferating ECs, new capillary tubes will be formed, generating a network that circulates blood into the newly vascularises region (Simo et al., 2006). This new vascular network subsequently matures and undergoes remodelling to form a stable vascular network. However, these fragile new vessels are prone to bleeding. More importantly, they have the potential to grow towards the vitreous body in which they are ultimately anchored by means of fibro-vascular components,

leading to retinal detachment, which can be accompanied by catastrophic visual loss (Ferrara, 2004).

The main growth factors involved in PDR development are VEGF, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and angiopoietins. Recently, other factors such as stromal cell-derived factor 1 (SDF-1) and connective tissue growth factor (CTGF) have been involved in the pathogenesis of DR. Finally, pro-inflammatory cytokines also play an important role in DR pathogenesis. Among angiogenic factors, VEGF-A seems to be the most important. However, there is now ample evidence to suggest that the development of DR is a multifactorial complex process in which the balance between angiogenic and anti-angiogenic factors is crucial. VEGF-A, which we refer to as VEGF, is part of a family that includes VEGF-A to VEGF-E and placenta growth factor (PlGF) (Simo et al., 2006, Ferrara et al., 1992, Ferrara, 2004)

VEGF is a potent mitogen for micro- and macrovascular EC derived from arteries, veins and lymphatics, but it is devoid of consistent and appreciable mitogenic activity for other cell types (Ferrara, 2004).

Apart from physiological actions, VEGF has other effects that although triggered by pathological stimuli are very important. These include the capacity to promote the formation of collateral vessels, which is essential in the recovery from ischaemic events. In this regard, it is important to state that VEGF acts as a chemo-attractant to mobilize ECs from the bone marrow.

VEGF can be synthesized by numerous retinal cell types including RPE cells, pericytes, ECs, glial cells, choroidal fibroblasts, and ganglion cells (Simo et al., 2006). The human VEGF gene is organised in eight exons separated by seven introns and is localised in chromosome 6p21.3. By alternative exon splicing, four main VEGF isoforms can be generated having 121, 165, 189, and 206 amino acids, respectively (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆) (Ferrara, 2004). VEGF₁₆₅ is the most abundantly expressed VEGF-A isoform and has optimal characteristics of bioavailability combined with high biological potency (Leung et al., 1989). VEGF is known also as vascular permeability factor (VPF) due to its ability to induce vascular leakage. The main mechanism by which VEGF induces vascular leakage in the retina is by lowering the levels and activity of occludin and ZO-1, two critical proteins for the integrity of TJ, and/or by increasing vesicular transport (Simo et al., 2006).

The crucial role of VEGF in retinal neovascularisation and vascular permeability has led to the development of VEGF inhibitors in experimental studies for the treatment of PDR and DMO. There are many strategies for blocking VEGF action, but until recently only those based on blocking VEGF by means of antibodies or aptamers were used in clinical practice (Hernandez and Simo, 2007, Simo and Hernandez, 2008). In this regard, pegaptanib sodium (Macugen, Eyetech Pharmaceuticals/Pfizer), (ranibizumab, Novartis) and Aflibercept (Eylea, Bayer HealthCare) have been approved for the treatment of choroidal neovascularisation in wet age-related macular degeneration. Targeting VEGF has resulted in the use of

anti-VEGFs including pegaptanib, ranibizumab, aflibercept and bevacizumab in the treatment of DMO (Elman et al., 2011, Korobelnik et al., 2014, Diabetic Retinopathy Clinical Research et al., 2010). It has recently been shown that ranibizumab rapidly and sustainably improved vision, reduced the risk of further vision loss, and improved macular oedema in patients with DMO, with low rates of ocular and non-ocular harm (Nguyen et al., 2012). Similar reports have been published on the efficacy of aflibercept in the treatment of DMO (Korobelnik et al., 2014, Brown et al., 2015). It should be emphasized that, at present, anti-VEGF therapy is currently used by many ophthalmologists as a pre-treatment of vitrectomy for severe PDR and its usefulness as an adjunct to traditional laser treatment is being investigated (Protocol S). Nevertheless, in contrast to the rapid response of retinal neovascularisation, the response of DMO to anti-VEGF treatment is not prolonged and is subject of significant variability. It was previously suggested that DMO without a significant ischaemic event may not benefit from anti-VEGF therapy as much as PDR (Sang and D'Amore, 2008).

It is known that retinal vascular leakage in DMO is contributed to by vascular endothelial growth factor (VEGF) upregulation as well as non-VEGF dependent inflammatory pathways, so that chronic subclinical inflammation is important in the pathogenesis of DR (Johnson, 2009, Adamis, 2002). As inflammation is considered important in the pathogenesis of diabetic retinopathy, the potential use of anti-inflammatory agents such as glucocorticoids as treatment are being extensively (Bhavsar, 2006). Corticosteroids have been reported to have significant effects on REC

(Edelman et al., 2005, Jousseaume et al., 2002). Corticosteroids may reduce DMO by targeting the non-VEGF-dependent inflammatory pathways including blockage of the arachidonic acid pathway (reduction of prostaglandin synthesis), and inhibition of the release of pro-inflammatory mediators, including VEGF. This may be mediated through modulation of endothelial cells by regulating phosphorylation, organization, and content of tight junction proteins (Jousseaume et al., 2004).

Several clinical trials continue to investigate the efficacy and safety of glucocorticoids, such as triamcinolone acetonide, fluocinolone acetonide and DEX for the treatment of diabetic macular oedema (Bhavsar, 2006, Flynn and Scott, 2005, Gillies et al., 2006, Kuppermann et al., 2007, Munir et al., 2005).

It has been frequently demonstrated that the vitreous levels of several inflammatory cytokines (including IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1) are increased in eyes with DMO and PDR (Yoshimura et al., 2009). In other studies, Funatsu et al (Funatsu et al., 2005, Funatsu et al., 2001) have reported significant elevations of VEGF, ICAM-1, IL-6, MCP-1, and pigment epithelium-derived factor, which correlated with retinal vascular permeability changes. In addition to this pathway, stimulation of 6-*N*-acetyl glucosaminyl-transferase in leukocyte can result in more modification of glycans on its surface thus inducing leukocyte rolling interactions to endothelial selectins (Chibber et al., 2007, Chibber et al., 2003).

Nehme and Edelman (Nehme and Edelman, 2008) have reported that hyperglycaemia, $\text{TNF}\alpha$, and IL-1 induce an inflammatory phenotype in human retinal pericytes, characterized by hypersecretion of inflammatory and angiogenic mediators. No such change was noted in the retinal microvascular ECs in that study. DEX at various potencies blocked hypersecretion of several proteins in pericytes (Nehme and Edelman, 2008). It was further suggested that corticosteroids may inhibit PLA2 and downstream products, for example, prostaglandins and leukotrienes, as well as inhibit adhesion signalling pathway components, including ICAM-1, IL-6, VEGF-A, and stromal cell-derived factor-1, which are involved in vascular permeability and inflammatory process (Kleinman et al., 2010). Different JAMs including JAM A, B, C are altered in diabetes and hyperglycaemia as discussed previously (Saker et al., 2014a). Furthermore, different capillary beds may respond differently to different stimulatory or inhibitory factors (Campochiaro, 2000) , and may differ in their basal and $\text{TNF}\alpha$ regulated expression of adhesion molecules and cytokines (Lehmann et al., 2000). This is supported by the observation that although DR is a microvascular disease whilst microangiopathy does not seem to play a major pathogenic role in the cerebral complications of diabetes, and the blood-brain barrier is not as susceptible to hyperglycaemia unlike the retinal microvasculature (Mooradian, 1997). The effects of DEX on retinal vascular endothelial cell permeability in high glucose media, and potential mechanisms including effects on TJ molecules, therefore, need further investigation.

Recent studies have reported that there are significant changes in the cytokine profile in DMO. The elevated cytokines include VEGF, MCP-1, IL-6, IL-8, interferon γ -induced protein-10 (Yoshimura et al., 2009, Roh et al., 2009, Funk et al., 2010). Klein et al (Klein et al., 2009) reported that sVCAM-1 and TNF α are associated with higher odds of developing more severe DR in patients with renal disease and PDR. Other evidence suggests that anti-VEGF treatments have no effect on the cytokines other than VEGF levels in intraocular fluids (Roh et al., 2009, Funk et al., 2010, Sohn et al., 2011).

The available evidence, therefore, implies that targeting VEGF alone or VEGF-independent pathways in isolation may not adequately block the increased permeability associated with diabetes and hyperglycaemia. A combination approach may be necessary in some cases. Steroids and anti-VEGF may have complementary and additive effects on retinal vascular permeability. This follows from the evidence of Nehme and Edelman (Nehme and Edelman, 2008) on the effect of DEX on REC and pericyte permeability, and the reports from Felinski (Felinski and Antonetti, 2005a, Felinski et al., 2008), in concert with the anti-VEGF trials. No current individual treatment modality in DMO results in a completely dry macula in most cases. This is understandable as there are multiple mechanisms including VEGF pathway, and the non-VEGF inflammation, which becomes more important in the chronic stages of DMO. Eyes with chronic DMO are more likely to benefit from intravitreal steroid therapy. A combination of anti-VEGF therapies with steroids will provide more optimal pharmacotherapy in DMO. An exploration

of the concept of combination pharmacotherapy in DMO therefore needs to be explored at the cellular level.

Chapter 2. Materials and Methods

2.1 Materials and Solutions

2.1.1 Retinal and Choroidal Endothelial Cell Isolation and Culture

Fibronectin (Sigma); EC complete medium (CM) EGM-2 MV bullet kit with hydrocortisone omitted (Lonza); EC serum free medium (SFM) EBM-2 Basal medium (Lonza) with 1x Antibiotic/antimycotic (Sigma); dynabeads CD31 (Life Technologies); 0.1% (w/v) collagenase, 0.1% (w/v) collagenase from *Clostridium histolyticum* (Sigma) in SFM; foetal calf serum (FCS; Sigma).

2.1.2 Functional Cell Assays

12 Well Cell Culture Cluster (flat bottom with lid, tissue culture treated, polystyrene) (Corning Incorporated, USA); Transwells, 0.4 μ m pore size HD (high pore density) in 12 well formats (Becton Dickinson, USA); Ranibizumab (Novartis); VEGF165 (R&D Systems).

2.1.3 Western Blotting

NuPAGE 12% Bis-Tris Gel (Invitrogen); NuPAGE MES SDS Running Buffer (20x) (Invitrogen); NuPAGE Transfer Buffer (20x) (Invitrogen); Tris Buffered Saline (TBS): 1 sachet of TBS pH 8.0 made up to 1 L with ddH₂O (Sigma); Tween 20 Molecular Biology Grade (Sigma); Bovine Serum Albumin (BSA; Protease free; Source Bioscience); BCIP (5-Bromo-4-chloro-3-indolyl phosphate) (Sigma).

<i>Antigen</i>	<i>Host</i>	<i>Supplier</i>	<i>Cat No.</i>	<i>Dilution (WB)</i>	<i>Dilution (IF)</i>	<i>Mwt (WB) or conjugation</i>
Occludin	Rabbit	Abcam	Ab31721	1:250		63kDa
Claudin 5	Rabbit	Abcam	Ab53765	1:500- 1:1000		23kDa
VE-Cadherin	Mouse	Abcam	Ab1956	1:50		130-140kDa
JAM-A/1	Mouse	Abcam	Ab17261	1:1000		33kDa
JAM-C/3	Rabbit	Abcam	Ab81331	1:1000		29kDa
Rabbit IgG	Donkey	Thermo Scientific	31345	1:2000		AP
Mouse IgM	Goat	Thermo Scientific	31322	1:2000		AP

Table 1. Primary antibodies list.

Primary antibodies list Host: animal raised in, supplier and cat no. and dilutions used for TJ protein expression analysis. WB: western blot and IF: immunofluorescence, mwt: observed or predicted molecular weight for target on WB or conjugation AP-alkaline phosphatase

2.1.4 Quantitative Polymerase Chain Reaction

β -Mercaptoethanol (β -ME; Sigma); 70% (v/v) ethanol (non-denatured, Sigma);

RNeasy Mini Kit (Qiagen): Buffer RLT; Buffer RW1; Buffer RPE; RNase-free water

QuantiTect Reverse Transcription Kit (Qiagen): gDNA wipe out Buffer;
 Quantiscript RT Buffer; RT Primer Mix; Quantiscript Reverse Transcriptase;
 RNase-free water; Universal PCR Master Mix TaqMan[®] (Applied
 Biosystems); TaqMan Assay: containing primer and probe mix.

<i>Gene Name</i>	<i>Assay ID</i>	<i>Accession Number</i>	<i>Exon Boundary</i>	<i>Amplicon Length</i>
GAPDH	4333764T	NM_002046.3	3	122
VEGFA	Hs00900055_m1	NM_001025366.2	3-4	59
HIF1A	Hs00153153_m1	NM_001243084.1	4-5	76
JAM-A (F11R)	Hs00375892_g1	NM_016946.4	9-10	65
JAM-C	Hs00230289_m1	NM_001205329.1	5-6	68
OCLN	Hs00170162_m1	NM_001205254.1	5-6	68
CLDN5	Hs00533949_s1	NM_001130861.1	1-1	82
PCDH12	Hs00170986_m1	NM_016580.3	1-2	70

Table 1. Details of the TaqMan[®] Gene Expression assay
 (probe sequences are proprietary)

2.2 Methods

2.2.1 Retinal and Choroidal Endothelial Cell Isolation and Culture

2.2.1.1 Fibronectin Coating Tissue Culture Dishes

Prior to microvascular EC isolation, 35 mm cell culture dishes were prepared by coating with 1 mL/dish of 5 µg/mL fibronectin in sPBS. The plates were incubated overnight at 37°C, 5% CO₂; excess liquid was removed before cell seeding.

2.2.1.2 Retinal and Choroidal Endothelial Cell Isolation and Culture

REC and CEC were isolated and cultured separately for each donor as previously described (Stewart et al., 2011). Human posterior segments were obtained from UK Transplant within 72 h of death. Corneas had previously been removed for transplantation and the posterior segments were stored at 4°C in sterile normal saline if consent for their use in research had been obtained. Donors were free of any known ocular disease and systemic infection. The project had the approval of the Manchester Eye Bank and the local research ethics committee (Nottingham Q1060301).

Dissection was conducted using instruments sterilized in 100% ethanol. Posterior segments were transferred to sterile Petri dishes and excess optic nerve was trimmed if necessary. The eyes were cut in 3 places in the sclera and the posterior segment flattened. The vitreous was removed and discarded and the retinal and choroidal tissue teased away, cut and stored in sPBS, samples from each pair of eyes were combined. The tissues were cut

into small pieces and centrifuged at 200 xg for 5 min. The pieces were incubated in collagenase I (0.1%) in SFM for 60 min at 37 degrees with agitation thoroughly every 10 min. After 60 min, the tissue was filtered through a sterile 40 µm filter into 100 µL FCS to neutralise the collagenase, and the eluate was centrifuged at 200 xg for 5 min. Cells were then re-suspended in 2.5 mL SFM and incubated with 35 µL of anti-CD31 coated Dynabeads (Invitrogen, UK) on a rocker for 15 min at speed 25. After the binding of the Dynabeads, the mixtures were washed 4 times with PBS using a magnet (Novagen, Magnetight separation stand) before being plated in CM.

2.2.1.3 Continued Cell Culture

Following isolation cells were cultured in CM on fibronectin coated 35 mm plates (2.2.1) at 37°C in a humidified atmosphere of 5% CO₂ and the medium was changed every 2/3 days until 80-90% confluent.

2.2.1.4 Sub-passage of Retinal and Choroidal Endothelial Cell

Cells were passaged when they were 80-90% confluent. Medium was aspirated and cells were rinsed with sPBS 3 times to ensure all traces of medium and serum had been removed. The cells were incubated in 1.5 mL TrypLE (Invitrogen, San Diego, USA) for 5mins at 37 to make cells detached from the bottom of the plate. Detached cells were collected and centrifuged, and then resuspended in 1.0 mL CM. 10 µm of the sample was taken for counting by haemocytometer. Cells were then seeded onto 6-well plates (5 x 10⁴ / well).

2.2.2 Functional Cell Assays

2.2.2.1 Proliferation Assay

Confluent EC were transferred into 6 wells of a 6 well cell culture cluster coated with fibronectin as above and maintained in NG medium. Once 60% confluent EC were washed once with sterile phosphate-buffered saline with antibiotics (sPBS+) and the culture medium was then changed to either NG medium or EGM2-MV with added glucose to give a final concentration of 25 mM glucose, HG for 72 h. Proliferation of REC and CEC were assessed using Cell-8 proliferation reagent (Sigma, Poole, UK). To determine the effect of DEX on proliferation, the day before the experiment, confluent REC were washed once with sterile PBS (sPBS) and stepped to 1% FBS-containing EBM-2 with antibiotics-antimycotics. The day of the experiment, the cells were incubated with cell culture-tested DEX (Sigma) at 1 μ M for 24 h. In selected experiments, 100 μ g/mL ranibizumab and/or 1000 pM VEGF was also added at this point. Medium was replaced with 1 mL working concentration of CCK8 reagent, incubated for 4 h at 37°C and the absorbance of 100 μ L each well was recorded at 650 nm using a microplate reader (Molecular Devices, Wokingham, UK) according to the manufacturer's instructions. EC proliferation was expressed as a percentage change relative to NG controls, examined in triplicate and repeated using REC from 7 different donors. Cell proliferation was expressed as a percentage increase relative to SRM controls. Each concentration was examined in triplicate and the experiment repeated using cells from at least 4 different donors.

2.2.2.2 Apoptosis Assay

Confluent EC were treated as section 2.2.2.1. After treatment EC were collected by centrifugation and the caspase-3 colorimetric assay (R&D Systems, Abingdon, UK) performed according to the manufacturer's instructions. In brief, EC were lysed by the addition of the Lysis Buffer (25 μ L per 1×10^6 cells), incubated on ice for 10 min and centrifuged at 10,000 xg for 60s. In a 96 well flat bottom micro-plate 50 μ L of cell lysate, 50 μ L of 2x Reaction Buffer 3 (with DTT) and 5 μ L of Caspase-3 colorimetric substrate was incubated for 1-2 hours at 37°C. Controls including no cell lysate and no substrate were also included. All plates were read on a micro-plate reader using a wavelength of 405 nm. The level of caspase-3 enzymatic activity in the cell lysate was directly proportional to the colour reaction.

2.2.2.3 In Vitro Vascular Endothelial Permeability

Human micro-vascular REC were seeded on 35 mm fibronectin coated culture dishes every other day. Confluent REC were allowed to adhere to the 0.4 μ m pore size HD (high pore density) in 12 well format membrane for three days considering both normo- and hyperglycaemic conditions. To determine the effect of HG on permeability, three days before the experiment, confluent ECs were washed once with PBS with antibiotics. Following this, the medium was changed to parallel media containing NG and HG for 3 days. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and the medium was changed every day throughout the 3 days. The experiments were repeated on 5 independent occasions, with different endothelial samples corresponding to RECs in normo- and

hyperglycaemic conditions. To determine the effect of DEX on permeability, the day before the experiment, confluent REC were washed once with sPBS and stepped to 1% FBS EBM-2. The day of the experiment, the cells were incubated with cell culture-tested DEX (Sigma-Aldrich, St. Louis, MO) at 1 μ M or 100 μ M for the indicated time 1 day and 3 days both in NG and HG conditions. In selected experiments, 100 μ g/mL ranibizumab and/or 1000 pM VEGF was also added at this point for 24 h.

This was assessed by the measurement of Evan's blue-labeled albumin across previously mentioned cultured cells. Confluent inserts were transferred to fresh 12-well cell culture clusters containing 2 mL Hank's balanced salt solution (HBSS) in the basolateral chambers. In the apical compartments, culture medium was replaced by 500 μ L of HBSS containing Evan's blue-labeled albumin (165 μ g/mL; molecular weight, 67 kDa), corresponding to different hyper and normo-glycaemic preparations. Both luminal (the top of an insert) and abluminal (the bottom of an insert) samples were measured at 20, 60, and 120 min, following treatment in normo- and hyperglycaemic conditions to determine the permeability of EC, using Kinetic Analysis in which reaction rates are calculated in milli OD per min (m OD/min) using a multi-scan plate reader.

2.2.3 Western Blotting

2.2.3.1 Sample Preparation and Electrophoresis

Cell medium was changed to parallel media (NG and HG) HG for three days. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and

the medium was changed every day. The expression of the selective TJ proteins (Occludin, Claudin 5, VE-Cadherin, JAM-A and JAM-C,) was determined with western blotting. The experiments were repeated on 3 independent occasions, with different endothelial samples corresponding to REC and CEC respectively in NG and HG conditions.

NuPAGE® Novex 12% Bis-Tris 1.0mm 12 well gel (Invitrogen) was rinsed with de-ionised water, and put in the apparatus (XCell II™ Blot Module, Invitrogen) after removing the comb from the gel. The NuPAGE® MES SDS Running Buffer (Invitrogen) was added to the tank. Samples were denatured at 95°C for 5 min before loading to the gel (20 µg protein per sample), and 10 µL of Full-Range Rainbow molecular weight marker (GE Healthcare Life Sciences, Buckinghamshire, UK) was loaded as well. The gel was then run at 200V for 40 min.

2.2.3.2 Transfer

Western blotting was performed using an X-Cell II Blot Module (Invitrogen, UK). Two pieces of Whatman filter paper (per gel), 4 or 5 blotting pads (Invitrogen, UK) and the nitrocellulose membrane were soaked in 1x transfer buffer (Invitrogen, UK); PVDF membrane (polyvinylidene difluoride, Millipore Immobilon-P Membrane, 0.45 µm) was pre-soaked for 2 min in 100% methanol. After electrophoresis SDS PAGE gels were washed for 2 min in 1x transfer buffer. The transfer apparatus was then assembled as per manufacturer instructions. The proteins were transferred at constant voltage of 30 V for 1-2 h.

2.2.3.3 Detection

After transfer, the membrane was blocked for 1 h at room temperature (or 4°C overnight) in 3% (w/v) bovine serum albumin (BSA) in TBST. The membrane was rinsed briefly in TBST then incubated with primary antibody at the appropriate concentration in 3% (w/v) BSA in TBST for 3 h at room temperature (or 4°C overnight). The membrane was rinsed in TBST then washed three times, for 5 min, in fresh changes of TBST. After washing, the membrane was incubated with secondary antibody in 3 % (w/v) BSA in TBST for 40 min at room temperature. The membrane was rinsed in TBST then washed for three times for 5 min in fresh changes of TBST. Membrane was then exposed to BCIP 8-12 mL for 30 min or more and monitored to ensure that the background did not become stained. After developing blots were washed with deionised water and then allowed to air dry and then scanned for image processing by Adobe Photoshop CS4 software.

2.2.4 Quantitative Polymerase Chain Reaction

2.2.4.1 Sample Collection

Nearly confluent cells were exposed to a mixture of Beta Mercaptoethanol (β -ME) and RLT Buffer 10 μ L per mL. A total of 700 mL was used for each well.

2.2.4.2 RNA Isolation

Following hyperglycaemic conditions, total RNA was extracted from the cells using RNeasy Mini RNA isolation kits (Qiagen, UK). Cell lysate was first homogenised by QiaShredder column (Qiagen, UK), in order to remove high molecular weight components. The QiaShredder column was centrifuged at 12000 rpm for 2 min, and equal volume of 70% ethanol was added into the filtrate. The samples were transferred to the RNeasy mini spin column and washed by Buffer RW1 and Buffer RPE. Finally, RNA was purified from the RNeasy Mini columns with 15 μ L RNase-free water. Each washing and elution step mentioned above was performed with stepwise centrifugation.

RNA was quantified using a spectrophotometer. Another fresh sample of RNase-free water was used to measure a blank. 1 μ L of each sample was placed on the machine to be analysed and stored.

2.2.4.3 Reverse Transcription of RNA

RNA samples were further processed for reverse transcription using the QuantiTect RT kit (Qiagen, UK). 2000 ng of purified RNA was incubated with genomic DNA Wipeout Buffer for 2 min at 42°C. The reverse-transcription mastermix, containing QuantiTect RT enzyme, QuantiTect RT Buffer and RT Primer Mix, was prepared and added to the template RNA. Samples were incubated at 42°C for 15 min, followed by incubating the samples at 95°C for 3 min. A 40 μ L final volume of cDNA was stored at -20°C for real-time PCR.

2.2.4.4 NanoDrop cDNA Quantification Technique

This is done at the post-genomic lab, in the department of pathology, Queen's Medical Centre, University of Nottingham, Nottingham. Specimens were kept in ice during transfer. The machine was first cleaned, initialised and blanked. Setting was confirmed for RNA40. 1 μ L of specimen was loaded for measurement. Results was labelled, saved and printed for record keeping purposes.

2.2.4.5 Quantitative Real-time Polymerase Chain Reaction Experiment Model

Real-time PCR was performed with Mx3005p real-time PCR instrument (Stratagene, La Jolla, CA). The qPCR experimental setup was carried out as detailed in the manufacturer's protocol (Applied Biosystems). Initially, template cDNA was diluted to 1:5 using nuclease-free water to perform the qPCR in triplicate. Each reaction was prepared to 20 μ L final reaction volume with 10 μ L of 2x gene expression master mix, 1 μ L of 20x assay (primers and probe mix; TaqMan; Applied Biosystems, Table 1), 5 μ L diluted cDNA, and 4 μ L nuclease-free water. Endogenous control was also run. Amplification conditions were set at 50°C for 2 min, 95°C for 10 min, 95°C for 15s and 60°C for 1 min. A total of 36 samples were analysed from 3 pairs of eyes in each group. Raw data were acquired (MxPro software, version 4.01; Stratagene) on the computer linked to the qPCR machine. The data were then analysed on a spreadsheet (Excel; Microsoft, Redmond, WA) using Independent samples t- test.

2.2.5 Statistical Analyses

Data were checked for normality before conducting the appropriate statistical test. All experiments were repeated at least three times and in each experiment, data were generated from multiple replicates. Independent samples t-test was used to compare data. For all analyses, P values below 0.05 were considered indicative of significant difference. Standard deviation was used to describe variability; results are represented as mean +/- standard deviation, except qPCR data which was expressed as standard error of the mean.

Chapter 3. The Effect of Hyperglycaemia on Permeability and Junctional Complex Molecule Expression

3.1 Introduction

Clinical evidence indicates that DMO results from breakdown of the inner BRB (Vitale et al., 1995) which is maintained by EC TJ complexes (Mitic and Anderson, 1998). TJ proteins include occludins, claudins, and JAMs, transmembrane molecules which interact with intracellular molecules including ZO and cingulin (Mitic and Anderson, 1998). Adherens junctions (AJ) are mediated by VE-Cadherin which promotes calcium dependent homophilic cell to cell contacts, and interact intracellularly with actin through catenins (Dejana et al., 2001) have reported that the principal TJ proteins found in REC are occludin and claudin-5. Phosphorylation of occludin and ZO-1 contribute to the dysregulation of EC paracellular permeability (Abbott et al., 2006, Thorin and Shreeve, 1998)

JAMs also signal to cytoskeleton associated proteins, as well as recruit cellular polarity proteins. As opposed to JAM-A, JAM-C increases paracellular EC permeability (Aurrand-Lions et al., 2001b, Orlova et al., 2006b). It is suggested that the preferential expression of different junctional molecules in different vascular beds may explain their specific physiologic functions (Aurrand-Lions et al., 2001b), for example the high expression of JAM-A in the blood-brain-barrier (Orlova et al., 2006b), the relative high expression of JAM-B in high endothelial venules (Aurrand-Lions et al.,

2001a), and JAM-A and C in the liver compared to its minimal expression of JAM-B (Geraud et al., 2012, Palmeri et al., 2000). Molecular crosstalk, substitution, or redundancy in the JAM system may also play a part in different tissues where all the different JAMs are expressed (Weber et al., 2007). JAM-C expression in REC (Daniele et al., 2007, Klaassen et al., 2009) and the RPE (Economopoulou et al., 2009) has been described. It is possible that preferential expression of different adhesion or TJ molecules (occludin, claudins, JAM-A, B and C) as well as AJ molecules (VE-Cadherin) and associated integrins in different vascular beds may contribute to their relative susceptibility to diabetes and hyperglycaemia. Different JAMs are reportedly altered in the retina in diabetes (Antonetti et al., 1998, Klaassen et al., 2009, Harhaj et al., 2006). It is also known that different capillary beds may respond differently to different stimulatory or inhibitory factors (Aird, 2003). In addition, the effects of JAMs are specific to cell type (Li et al., 2009). This is supported by the observation that although DR is a microvascular disease, microangiopathy does not seem to play a major pathogenic role in the cerebral complications of diabetes, and the blood-brain barrier is not as susceptible to hyperglycaemia as the retinal microvasculature (Mooradian, 1997).

High glucose concentrations induce phenotypic switch and modifies the intracellular signalling in REC. In examination of HG effects on vascular EC, one should also take into account that glucose concentrations higher than physiological levels lead to the accelerated formation of multiple biochemical species unusual in physiological conditions as well as the influence of

inflammation and ischaemia. There are contributory anatomical and biochemical changes that are interlinked in the pathogenesis of diabetic vasculopathy (Erusalimsky, 2009). Various choroidal abnormalities in diabetic eyes have been reported in several histopathological studies, including obstruction of the choriocapillaris, vascular remodelling, choroidal aneurysms, and choroidal neovascularization (McLeod and Luttly, 1994, Cao et al., 1998). Hidayat and Fine suggested that choroidal vasculopathy in diabetes may play a more prominent role in the pathogenesis of diabetic retinopathy than has previously been considered (Hidayat and Fine, 1985). In addition, Cao et al. suggested that the unexplained loss of visual function that may occur in diabetic subjects without retinopathy may be the result of diabetic choroidopathy (Cao et al., 1998). Recently, we proposed that an accurate understanding of choroidal damage is critically important for an accurate assessment of diabetic eye disease.

In this chapter, the expression of TJ and AJ proteins in REC and CEC with HG is investigated. Additionally, ocular microvascular trans-endothelial permeability is assessed in REC and CEC with HG conditions.

3.2 Results

3.2.1 Assessment of the Effect of High Glucose on Ocular Microvascular Trans-Endothelial Permeability

When the comparative paracellular permeability of REC and CEC was measured, the mean permeability of REC in NG was 0.063 ± 0.002 (Figure 3.1); when HG conditions were induced the mean permeability significantly

increased to 0.076 ± 0.007 ($p=0.008$). For CEC permeability significantly increased from 0.0315 ± 0.0006 in NG medium to 0.0328 ± 0.0005 ($p=0.017$) in HG medium (Figure 3.1). The relative increase in permeability in HG (compared to NG) was 20.6% for REC compared to 3.9% for CEC.

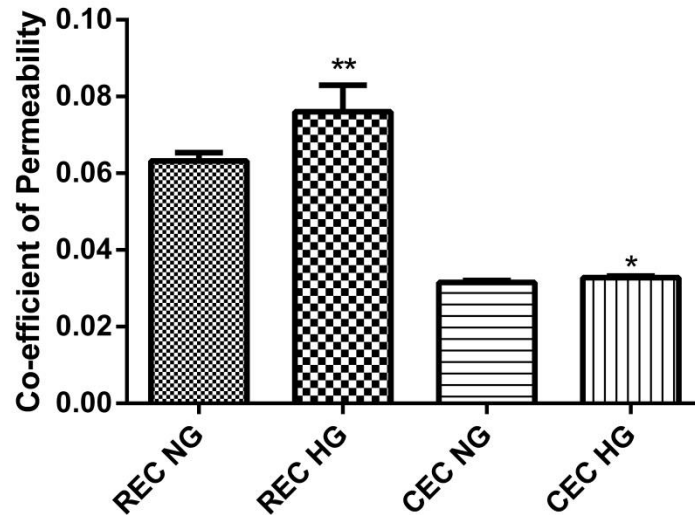


Figure 3.1. REC and CEC permeability in response to high glucose.

REC and CEC were exposed to HG (25mM glucose) for 72h then the trans-endothelial permeability assessed by measuring passage of Evan's blue albumin and calculating co-efficient of permeability, 5 different unmatched donor REC and CEC samples in NG and HG conditions were assessed, with three duplicates for each reading point (** $p<0.01$, * $p<0.05$).

3.2.2 Comparative protein expression profiling of selective tight junction and adheren junction proteins in REC and CEC

Previously, microarray analysis of mRNA from cultured RECs and CECs derived from 3 matched donors demonstrated that REC expressed higher levels of probesets for occludin (5.3 fold), claudin-5 (4.8 fold) compared with matched CEC (Saker et al., 2014a). There was no significant difference in the expression of probesets for JAM-A, JAM-C and VE-Cadherin between

matched REC and CEC. This was also reflected when the protein levels were assessed using western blotting which showed that CEC expressed lower levels of claudin-5 than REC ($p=0.0172$) and occludin (ns, $p=0.1366$), whereas the levels of JAM-A, JAM-C and VE-Cadherin were similar or slightly higher in CEC (Figure 3.2).

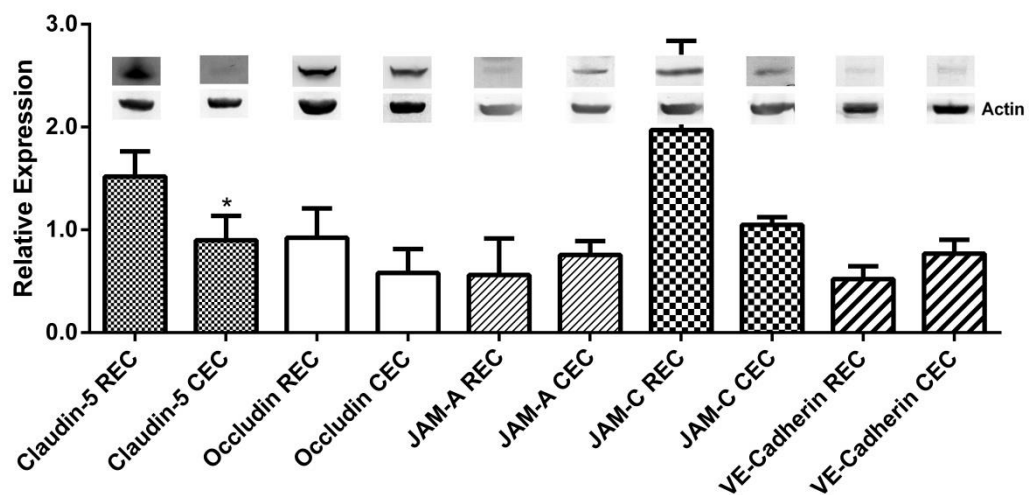


Figure 3.2. Differential endothelial protein expression profiling of junctional molecules.

Human REC and CEC lysates were collected and the expression of junctional proteins assessed using western blotting, expression in at least 5 unmatched donor REC and CEC samples was quantified using ImageJ relative to actin (lower band; ** $p<0.01$, * $p<0.05$).

3.2.3 Expression of tight junction and adherent junction proteins under hyperglycaemic conditions

When REC were exposed to HG conditions for 72h the expression of claudin-5 ($p=0.0423$), occludin ($p=0.0155$) and JAM-A ($p=0.0306$) was found to be reduced (Figure 3.3). The expression of VE-Cadherin and JAM-C were unchanged in REC under HG conditions. None of the junctional proteins in

CEC were significantly decreased by HG (Figure 3.3); however, JAM-C expression appeared to be consistently increased, although this increase was not statistically significant (ns, $p=0.0968$).

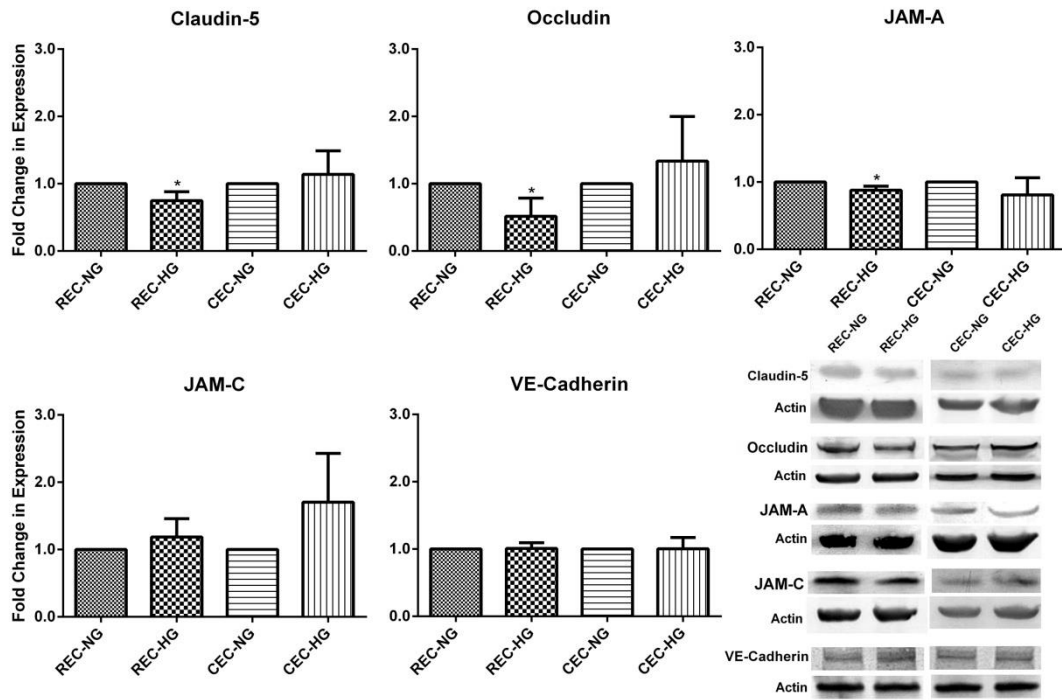


Figure 3.3. Comparative protein expression profiling of different junctional molecules with high glucose.

REC and CEC were exposed to HG (25mM glucose) for 72h then proteins collected and the expression of junctional proteins assessed using western blotting quantified with ImageJ and normalised against actin and matched NG samples. Expression changes in 5 unmatched donor REC and CEC samples were determined (** $p<0.01$, * $p<0.05$).

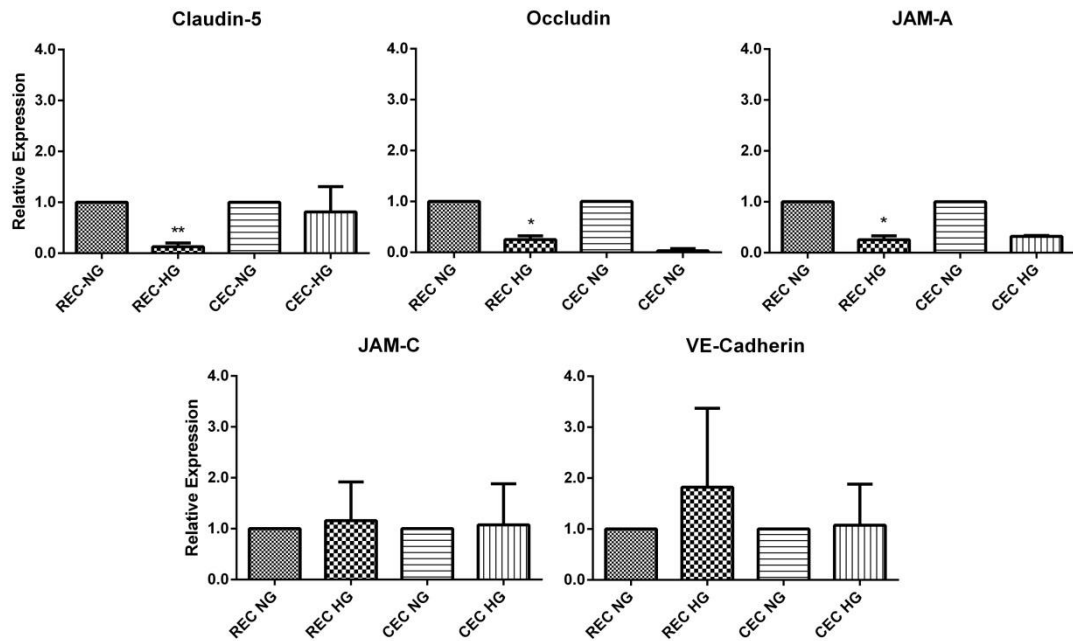


Figure 3.4. Comparative gene expression profiling of different junctional molecules with high glucose.

REC and CEC were exposed to HG (25mM glucose) for 72h then RNA extracted and the mRNA expression of junctional proteins assessed using qPCR. Expression changes in 3 replicates of 3 different unmatched donor REC and CEC samples were determined (** $p < 0.01$, * $p < 0.05$).

3.2.4 Gene expression changes in tight junction and adheren junction proteins with high glucose

When REC were exposed to HG conditions for 72 h there was a significant reduction in gene expression of claudin-5 ($p = 0.007$), occludin ($p = 0.031$) and JAM-A ($p = 0.037$; Figure 3.4). The expression of VE-Cadherin and JAM-C were unchanged in REC under HG conditions. None of the junctional proteins in CEC were significantly decreased by HG (Figure 3.4); however, occludin and JAM-A showed a (non-significant) trend towards decreasing gene expression.

3.3 Discussion

Clinical evidence indicates that DMO results from breakdown of the inner BRB (Vitale et al., 1995), and its incidence correlates with poor glycaemic control and hyperlipidaemia (Ferris et al., 1996, Klein et al., 2002). This study confirms that HG results in significant increase in paracellular permeability of REC in vitro. There is also a notable increase in permeability of CEC; however, the relative increase in permeability is higher in REC than CEC when exposed to the same degree of HG. This would support the clinical observation that vascular leakage due to diabetes affects the retinal vasculature more than that in the choroid (Vitale et al., 1995).

Previous studies have reported alterations in different JAMs and TJ in the retina in diabetes and hyperglycaemia (Antonetti et al., 1998, Harhaj et al., 2006, Klaassen et al., 2009). It is also known that different capillary beds may respond differently to different stimulatory or inhibitory factors (Campochiaro, 2000). In addition, the effects of JAMs are specific to cell type (Li et al., 2009). Our findings indicate that both REC and CEC express Claudin-5, occludin, JAM-A and C, and VE-Cadherin under NG conditions, although the expression of Claudin-5 and occludin is higher in REC, as shown by both our gene and protein expression evaluations. Similarly, HG seems to have a differential effect in altering the expression of studied junctional molecules in REC and CEC, as shown by the significant reduction in Claudin-5, occludin and JAM-A gene and protein expression in REC but non-significant changes in CEC.

To the best of my knowledge, this is the first study that compared junctional protein expression in human REC and CEC in NG and HG. Differences in the TJ molecule expression between the REC and CEC may reflect the physiological roles of the two different vascular beds, as well as explain some of the differential effects of diabetes on the vasculature, i.e. why diabetic vascular changes affect the retinal compared to the choroidal vasculature. Interactions in angiogenesis and vascular permeability alterations are most likely mediated by changes in TJ molecules in DR. This finding further confirms the phenotypic difference between REC and CEC in health and disease previously described (Aurrand-Lions et al., 2001a, Browning et al., 2005a, Browning et al., 2012, Stewart et al., 2011).

Recently, different groups have started to explore the use of JAM blockade as potential therapeutic targets in different diseases. (Naik et al., 2003) reported that antibody blockage of JAM-A resulted in disruption of angiogenesis in an in vitro model. (Goetsch et al., 2013) have shown that monoclonal antibodies against JAM-A can interfere with tumour proliferation in experimental models. (Lamagna et al., 2005b) and (Orlova et al., 2006a) independently have shown that a JAM-C specific blocking monoclonal antibody reduced the angiogenic process in models of hypoxia-induced retinal neovascularisation. Furthermore, JAM-C blockade inhibits both tumour growth and angiogenesis (Orlova et al., 2006a). (Hou et al., 2012) have reported upregulation of JAM-C in experimental CNV and that JAM-C antagonists suppressed CNV and decreased retinal blood vessel leakage in their model. Our finding of differential TJ expression between human REC

and CEC could inform and have implications on the future development of these clinical treatments of neovascular diseases in the eye. However, it is yet to be tested whether boosting JAM (particularly JAM-A) expression has any effects on reducing EC permeability in HG or diabetes.

This study is at variance with others that have shown increased VE-Cadherin expression with hyperglycaemia and diabetes (Davidson et al., 2000, Navaratna et al., 2007). (Klaassen et al., 2009) did not detect any gene expression of VE-Cadherin in control or streptozotocin rat retina. However, they described reduction in bovine EC expression of VE-Cadherin after 24 h VEGF exposure (Klaassen et al., 2009). It is possible that interactions between VE-Cadherin and the TJ molecules display some redundancy in intraocular EC as previously described by other investigators. (Taddei et al., 2008) described the upregulation of claudin-5 to be mediated indirectly by VE-Cadherin in murine EC lines in vitro.

This study did not evaluate the role of transcellular permeability in HG, and interactions with the paracellular permeability. Such correlations will be the subject of future studies. Furthermore, the roles of inflammation in retinal and choroidal capillary permeability, up-regulation of adhesion molecules which have a role in the progression of DR (Mohr, 2004), or other cell types e.g. pericytes in diabetic eye disease need further study. However, the significant strength of this study over others is that primary human REC and CEC were used as compared to bovine or murine EC, or streptozotocin diabetic rats (Taddei et al., 2008).

These findings show that human REC and CEC show different expression profiles for different TJ molecules in NG, and support the role of selected TJ molecules including occludin and claudin-5, JAM-A and C constituting a permeability barrier in intraocular micro-vascular EC, and their crucial role in the pathogenesis of diabetic eye disease. Exposure of human REC to HG for 72 h or longer results in significant reductions in the gene and protein expression profiling of occludin, claudin-5 and JAM-A as opposed to the slight alterations observed in CEC.

Chapter 4. High Glucose Effect on Proliferation and Apoptosis of Retinal Endothelial Cells

4.1 Introduction

Diabetes, generally leads to reduced wound healing capacity through reduced cellular infiltration, angiogenesis and extracellular matrix production (Altavilla et al., 2001). The concentration of glucose in the cellular microenvironment determines the cells ability to proliferate and react to different cytokines. Fibroblasts from chronic diabetic wounds have been shown to have reduced proliferation and response to growth factors even after several passages in culture (Hehenberger et al., 1998, Tchaikovsky et al., 2009). Similarly, there is reduction in the proliferation of Tenon's fibroblast derived from diabetics compared to non-diabetics when cultured in normal or increased glucose media (Browning et al., 2005a).

Angiogenesis, however, is a complex process and its role in diabetic disease appears contradictory, as increased vascularisation is seen in the pathogenesis of retinopathy, nephropathy and atherosclerosis, whereas reduced angiogenesis is the problem in the limbs and feet, as well as other tissues. (Costa and Soares, 2013). High glucose leads to endothelial dysfunction (Kolluru et al., 2012). Hyperglycaemia, in addition to inducing REC permeability (Saker et al., 2014b), generally leads to reduced cell proliferation (Laing et al., 2007), abnormal angiogenesis (Martin et al., 2003), increased EC apoptosis (Barber et al., 2011, Sheu et al., 2005, Ho et al., 2006, Ho et al., 2000, Leal et al., 2009, Kowluru et al., 2014). Some previous

studies have reported conflicting results on the effects of diabetes on EC proliferation. Morisaki et al (1999) (Morisaki et al., 1999) and Rymaszewski et al (1992) (Rymaszewski et al., 1992) reported increased proliferation of microvascular retinal EC (REC), and Sank et al (1994) (Sank et al., 1994) reported increased human umbilical vein EC (HUVEC) proliferation with high glucose. However, other studies have reported that glucose decreased proliferation in HUVEC (Curcio and Ceriello, 1992) human pulmonary artery EC (Liu et al., 2012) human dermal microvascular EC (Kamal et al., 1998), porcine aortic EC (Graier et al., 1995), and bovine REC (La Selva et al., 1996). Increased apoptosis of REC in diabetes and high glucose concentrations in vitro has been reported in several studies. EC death in diabetic human retina was previously reported by Mizutani et al (1996). It has also been reported that EC in experimental diabetes have a higher turnover rate (Mizutani et al., 1996, Sharma et al., 1985) which may result in early replicative senescence and irreversible cell cycle block (Mizutani et al., 1996, Sharma et al., 1985). Lorenzi et al (Lorenzi et al., 1985, Lorenzi et al., 1986) reported glucose toxicity to human EC in culture, DNA damage, and reduced replication and accelerated death. More recently, Varma et al (2005) (Varma et al., 2005) reported that high glucose concentrations (HPG) decreased HUVEC proliferation and survival through PI3K and Akt signalling, and that apoptosis only increased significantly at very high (40mM) glucose concentrations. These apparent conflicting results may be attributed to species variations as well as varying experimental conditions including cell passage used, degree and duration of glucose exposure and controls, and

other factors. However, the bulk of this previous work has been done with animal models or cell lines (Leal et al., 2009, Kowluru et al., 2014, Wright et al., 2010) or macrovascular EC (Sheu et al., 2005).

It has been suggested that decreased retinal blood flow in DR may increase hypoxia and in turn stimulate VEGF production and subsequent neovascularisation (Wright et al., 2010). Hypoxia is thought to stimulate VEGF production through the induction of hypoxia inducible factor (HIF), a major transcription factor in cellular response to hypoxia (Ziello et al., 2007, Krock et al., 2011, Yamakawa et al., 2003). However, HIF-1 α is potentially reduced through destabilisation in hyperglycaemia (Bento and Pereira, 2011, Wright et al., 2010).

The purpose of this *in vitro* study was to investigate the proliferation and apoptosis of primary human REC under HG conditions in vitro. Glucose concentrations lower than physiological (hypoglycaemia) were not evaluated in this study, but would be subject of future investigations.

In this chapter, it was investigated whether different levels of high glucose directly affected retinal endothelial cell (REC) proliferation and apoptosis in vitro, and whether REC HIF-1 α and downstream targets including VEGF-A were altered by high glucose levels. VEGF-A expression levels in REC were used to determine whether changes in HIF-1 α stability were translated into its transcriptional activity (transactivation).

4.2 Results

4.2.1 Retinal endothelial cell proliferation in high glucose conditions

Cultured REC were switched to culture media containing standard glucose (5 mM) or with glucose added to 25 or 40 mM glucose. After 3 days in high glucose the proliferation of REC reduced significantly by an average of 16.1% ($p=0.0017$) in 25 mM glucose and 31.4% ($p<0.0001$) for 40 mM glucose. After 7 days with high glucose REC proliferation was also reduced with both 25 mM (14.95%, $p=0.0042$) and 40 mM (14.1%, $p=0.0198$) glucose (Figure 4.1).

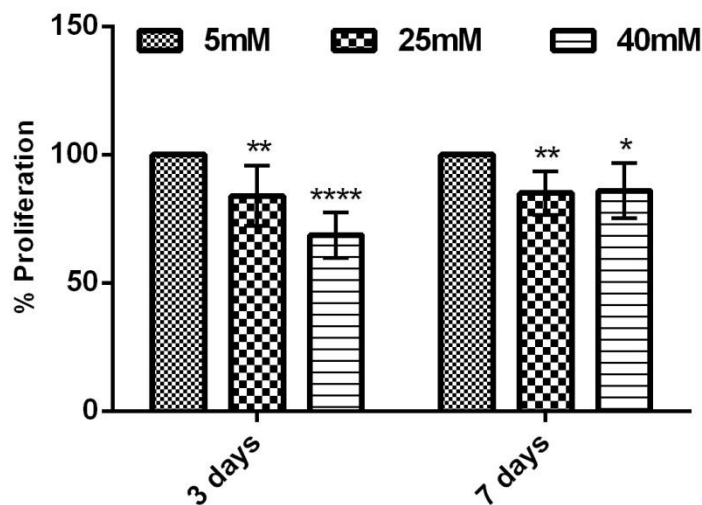


Figure 4.1. High glucose treatment decreased human REC proliferation over 3 and 7 days.

RECs were cultured in growth medium (5 mM), 25 mM or 40 mM glucose for 3 or 7 days before their proliferation was assessed using Cell-8 reagent. Bars show % proliferation compared to 5 mM $\pm$ SD ($n=8$; * $p<0.05$, ** $p<0.01$, **** $p<0.0001$).

4.2.2 Retinal endothelial cell apoptosis in high glucose conditions

RECs were cultured in media containing 5, 25 or 40 mM glucose for 3 days and the caspase-3 activity quantified. No significant change in caspase-3 activity was detected after 3 days with 25mM glucose concentration (ns, $p=0.0986$). However, caspase-3 activity (apoptosis) was significantly increased by $59.4\% \pm 7.8$ ($p<0.0001$) when the glucose concentration was increased to 40mM (Figure 4.2).

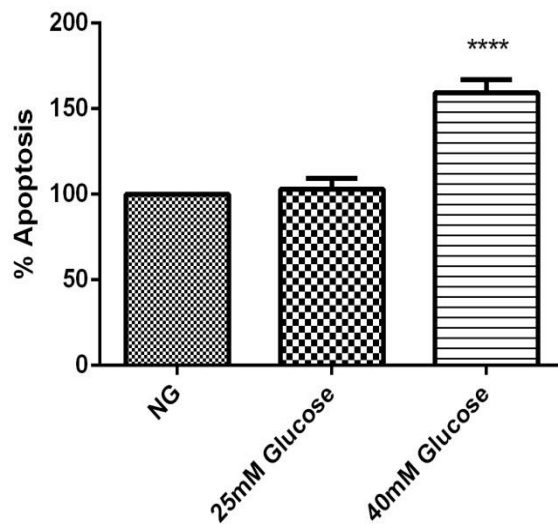


Figure 4.2. REC caspase-3 activity was noticeable at the much higher glucose concentration.

RECs were cultured in growth medium 5 mM, 25 mM or 40 mM glucose for 3 days before caspase-3 activity was assessed. Bars show % caspase-3 activity compared to 5 mM $\pm$ SD (n=8).

4.2.3 REC proliferation in 25mM Glucose and added VEGF

A parallel experiment investigated REC proliferation in 25mM glucose in the presence of VEGF-A. As previously, REC proliferation was reduced in 25mM glucose compared to 5mM. With the addition of VEGF-A to the medium containing 25mM glucose, REC proliferation was restored to near relative values in 5mM glucose although this was variable in some donors (ns, $p=0.8136$) (Figure 4.3).

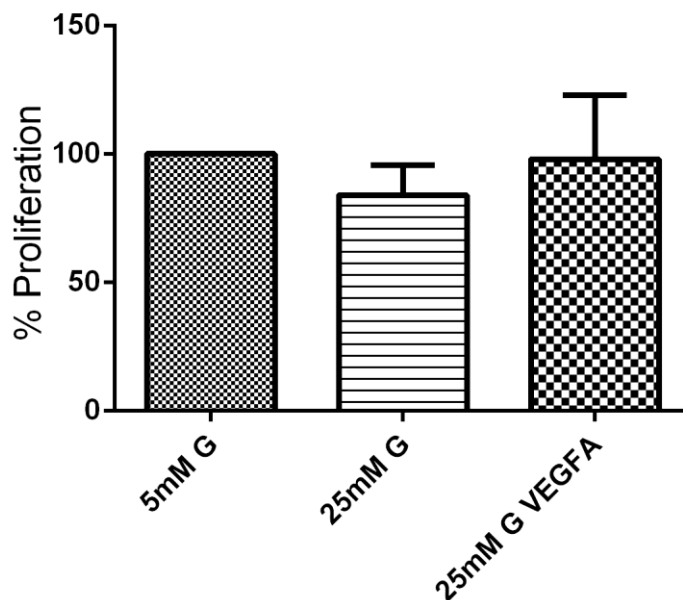


Figure 4.3. VEGF treatment restored human REC proliferation.

RECs were cultured in growth medium (5 mM), (25 mM) glucose + VEGF for 3 days before their proliferation was assessed using Cell-8 reagent. The medium in selected wells was replaced with SRM containing 1000 pM VEGF 165 and serially diluted to give the required final concentrations.

4.2.4 Expression of HIF-1 α and VEGF mRNA with 25mM glucose

Exposure of REC to 25mM glucose for 3 days induced a 2-fold increase in HIF-1 α mRNA expression ($p=0.0291$) compared to that in 5 mM. Despite the

increase in HIF-1 α , VEGF expression remained unchanged under similar conditions (ns, $p=0.7452$) (Figure 4.4).

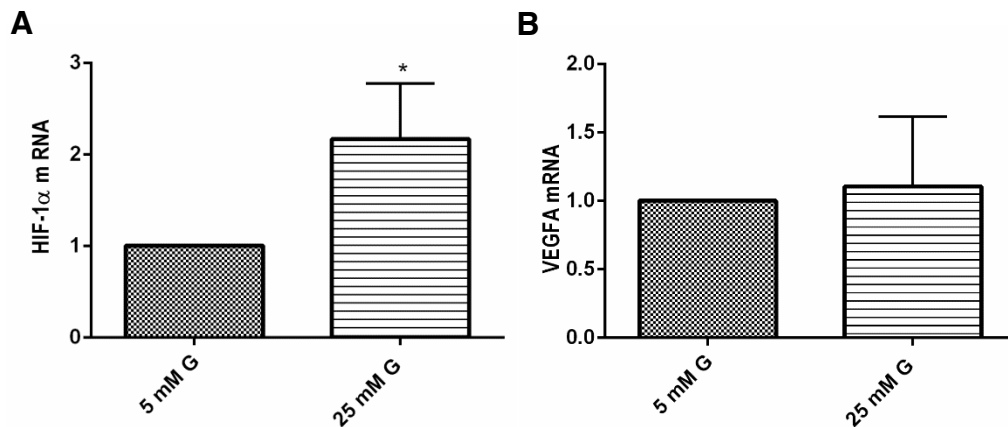


Figure 4.4. Effect of 25 mM glucose on Gene Expression Profiling of HIF-1a and VEGFA.

Gene expression was quantified using qPCR with after treatment with 25 mM for 72 h. Expression of HIF-1a was significantly increased with 25mM (figure 4A), whereas VEGFA expression remained unchanged (* $p<0.05$) (figure 4B).

4.3 Discussion

Diabetes and poor glycaemic control have complex and contradictory effects on angiogenesis which varies dependent on the tissue bed involved. In the retina increased angiogenesis presumably stimulated by hypoxia and increased growth factor production resulting from microvascular closure leads to the development of DR. However, it is thought that increased apoptosis also has a significant role in the development of DR (Jolly et al., 2005). Leal et al (2002) reported reduced proliferation and increased apoptosis with in the rat endothelial cell line TR-iBRB2 after 7 days high glucose; these cells (TR-iBRB2) are known to grow very rapidly under standard conditions compared to primary cells (Hosoya et al., 2001).

This study has shown for the first time that the high glucose levels have independent effects on REC proliferation and apoptosis. Whilst the reduction of EC proliferation is noted at 25mM and above, increased apoptosis is only significantly altered at the higher concentrations of high glucose. This implies that the response to clinical hyperglycaemia may be dependent on the different levels of high glucose, and that REC apoptosis is more important at higher degrees of hyperglycaemia compared to lower levels. This may partially explain why the retinal vasculature seems to show heterogeneous responses in the different diabetic patients, and the conflicting reports on EC growth in high glucose in the literature. It is suggested that increased apoptosis in REC that have an underlying reduced proliferation makes it impossible for the retinal vasculature to maintain normal turn-over required to maintain itself (Poulaki et al., 2002). This will result in retinal microvascular closure with subsequent increased hypoxia.

It is also important to point out that the reported effects of HG concentration are not the outcome of a single molecular event insult, but of a multitude of deleterious molecular interactions in HG conditions. These include the non-enzymatic glycation products, the AGE-proteins, the oxidant free radical species, among other components of the diabetic environment. Moreover, under insulin resistant conditions, as in Type 2 Diabetes mellitus, increased insulin concentrations and/or impaired insulin-signalling pathways may also contribute to endothelial dysfunction (Potenza et al., 2009). Elevated levels of VEGF-A would be subsequent to factors other than high glucose levels per se, including induced REC loss and subsequent ischaemia, and

contributed to by other cells in the retina. The possibility of increased production of VEGF-A by other intra-retinal cells in high glucose or hyperglycaemic conditions cannot be excluded in this model, and requires further investigation. Constitutive expression of HIF1a in normal retina from humans and rats has recently been reported (Hughes et al., 2010). It has also been previously reported that increased glucose did not influence the normoxic levels of HIF1a protein in REC (Poulaki et al., 2002), whilst others have suggested that HIF-1 α was potentially reduced by destabilisation in hyperglycaemia (Wright, McElhatten et al. 2010, Bento and Pereira 2011). The effect of glucose on HIF1a seems to be cell dependent (Lu et al., 2002, Catrina et al., 2004), as is the effect of hypoglycaemia (Williams et al., 2002). The constitutive expression of HIF1a by human REC in normal glucose media in vitro has been demonstrated in this model, which was significantly increased in high glucose conditions.

The findings from this study indicate that VEGF-A levels are not altered by high glucose in the REC, despite increased expression of HIF-1a levels. HIF-1a must therefore be acting through a non-transcriptional route. Notably, (Abcouwer, 2013) have reported elevated levels of HIF-1a in ERM from diabetic eyes, and (Limb et al., 1999) (Liu et al., 2012) in diabetic neovascular membranes, and rat retina in the presence of insulin (Poulaki et al., 2002). However, it has been suggested that hyperglycaemia alone did not influence HIF-1a protein in the rat retina or retinal pigment epithelial (RPE) cells in culture, although VEGF was upregulated (Poulaki et al., 2002). Our findings indicate that there is, as such, a 'disconnect' between

elevated HIF-1a and normal levels of VEGF-A expression in REC grown in high glucose levels. Hackenbeck et al (Hackenbeck et al., 2009) recently reported that HIF-1 or HIF-2 may induce cell cycle arrest. (Thangarajah et al., 2010) have recently confirmed that impaired wound healing in diabetics is because of poor vascularisation, and have shown that this impaired neovascularization results from a high glucose induced defect in transactivation of hypoxia-inducible factor-1a (HIF-1a), the transcription factor regulating vascular endothelial growth factor (VEGF) expression (Thangarajah et al., 2010). They attribute this to modification of the HIF 1a co-activator p300 by the glycolytic metabolite methylglyoxal (MGO), an advanced glycation end product (AGE) precursor (Thangarajah et al., 2010). A role of MGO in diabetes angiogenesis impairment in other vasculature has been described by some investigators recently (Kalapos, 1999, Yan et al., 2008, Giacco and Brownlee, 2010, Liu et al., 2012), which requires further exploration (Berner et al., 2012). Alternatively, Factor inhibiting HIF-1a (FIH) interaction may be modified by high glucose (Zarembek and Malech, 2005). However, (Catrina et al., 2004) have suggested that high glucose decreased hypoxia-induced stabilisation and function of HIF-1a in human dermal fibroblasts and human dermal microvascular endothelial cells in culture, although no effect was noted in normoxia. Diabetic foot ulcers expressed lower levels of HIF-1a compared to non-diabetic venous ulcer controls. Catrina et al, interestingly, (Catrina et al., 2004) observed a decreased transcriptional activation of HIF-1a as measured by reporter gene assays. The effects on HIF-1a in different ECs may therefore be different.

Further elucidation of the detailed mechanisms involved in reduced retinal endothelial cell proliferation, and increased apoptosis will open potential therapeutic avenues in modulating disease progression in preclinical or early diabetic retinopathy.

Although there is a common agreement that HG concentrations induce EC apoptosis (Zanetti et al., 2008), the circumstances that favour this process are duration of the HG (diabetes duration), via selective up-regulated caspase-1mRNA (Bucciarelli et al., 2009), and the presence of auto-antibodies in patients with DMO (Zimering and Pan, 2009). The intracellular consequences of EC apoptotic changes consist in DNA fragmentation, and mitochondrial dysfunction, manifest by alteration of membrane potential, release of cytochrome-c, and mitochondrial fragmentation, and these changes play a potential pathogenic role in mediating the risk of chronic DMO (Varma et al., 2005).

In conclusion, high glucose levels (of 25 and 40mM) significantly reduce REC proliferation to a similar extent. Apoptosis is noticeable at the much higher glucose concentrations. The reduced proliferation coupled with increased apoptosis will result in the REC inability to cope with replenishing lost cells with the resultant microvascular closure. Events subsequent to these will lead to clinical manifestations of DR. Identifying the detailed mechanisms involved in retinal endothelial cell dysfunction will allow therapeutic interventions.

Chapter 5. The Effect of Dexamethasone on Proliferation, Apoptosis and Permeability with Hyperglycaemia

5.1 Introduction

Inflammatory changes associated with DR, a common micro-vascular complication of diabetes (Ciulla et al., 2003), involve leukostasis (Miyamoto and Ogura, 1999), increased vaso-occlusion and micro-vascular permeability, and upregulation of different cytokines and intercellular adhesion molecules (Joussen et al., 2004, Yuuki et al., 2001). Indeed, acellular, non-perfused capillaries in DR corresponding to retinal ischaemia and DMO are likely to be attributable to accelerated death of both retinal capillary EC and pericytes (Hammes et al., 2002, Mizutani et al., 1996). The contribution of subclinical inflammation to this cell death is still a subject of intense investigation. Just as inflammation has been reported in the pathogenesis of DR, the potential use of anti-inflammatory agents such as glucocorticoids are being extensively studied (Bhavsar and Tornambe, 2006, Evans et al., 1988). In fact, the cytoplasmic glucocorticoid receptor (GR), which belongs to the nuclear receptor subfamily, plays an integral role in the biological actions of glucocorticoids. Accordingly, this receptor has the potential to positively or negatively regulate gene expression, depending on the response element sequence and promoter context (Evans, 1988). On the other hand, the GR can physically interact with different transcription factors such as activating protein (AP)-I, thus modulating gene expression

independently of glucocorticoid response elements (Necela and Cidlowski, 2004).

Several clinical trials are being conducted worldwide to investigate the efficacy and safety of glucocorticoids, such as triamcinolone acetonide, fluocinolone acetonide and DEX for the treatment of DMO (Bhavsar, 2006, Flynn and Scott, 2005, Gillies et al., 2006, Kuppermann et al., 2007, Munir et al., 2005). Nehme and Edelman (Nehme and Edelman, 2008) have reported that hyperglycaemia, $\text{TNF}\alpha$, and IL-1 induce an inflammatory phenotype in human retinal pericytes, characterized by hypersecretion of inflammatory and angiogenic mediators. No such change was noted in the retinal microvascular ECs in that study. Dexamethasone at various potencies blocked hypersecretion of several proteins in pericytes (Nehme and Edelman, 2008). It was further suggested that corticosteroids may inhibit downstream products, for example, prostaglandins and leukotrienes, as well as inhibit adhesion signalling pathway components, including ICAM-1, IL-6, VEGF-A, and stromal cell-derived factor-1, which are involved in vascular permeability and leucocytosis (Kleinman et al., 2010). Steroids including DEX may also inhibit TJ disassembly (Felinski and Antonetti, 2005a, Felinski et al., 2008). However, more studies are needed to gain a better understanding of the profile of inflammatory mediators and the cellular mechanisms underlying DEX actions.

In a previous chapter, the effects of high glucose media on apoptosis and proliferation of REC were described.

The purpose of this *in vitro* study was to investigate whether DEX can markedly alter retinal vascular leakage in human REC through changes in EC proliferation, apoptosis and permeability in order to identify its potential role both in hyperglycaemic and normo-glycaemic conditions.

5.2 Results

5.2.1 Effect of High Glucose and Dexamethasone on Retinal Endothelial Cell Proliferation

The addition of 25 mM glucose significantly decreased REC number compared to normal medium containing 5 mM glucose ($14.17\% \pm 3.519$, $p=0.0013$, Figure 5.1). DEX (100 μ M) reversed this effect and significantly increased REC numbers compared to 25 mM glucose ($18.51\% \pm 8.231$, $p=0.0411$). A slight cell protective effect was also seen in 5 mM glucose medium, although this was variable.

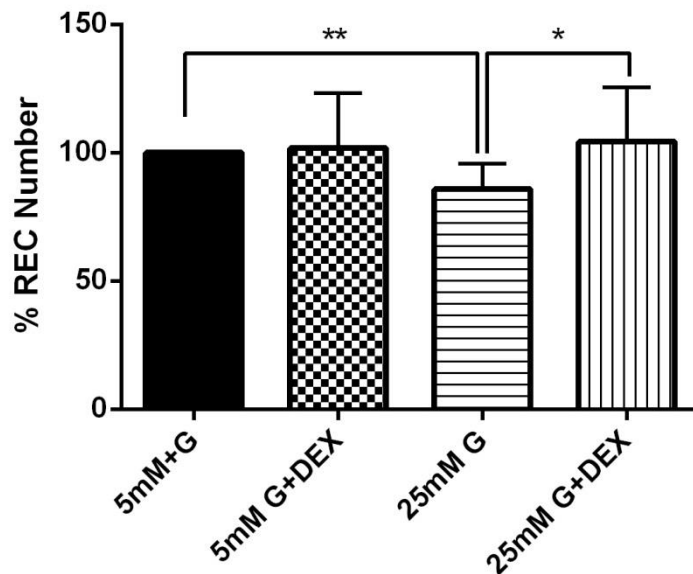


Figure 5.1. The effect of 25mM glucose and dexamethasone on REC numbers.

REC were incubated for 72 h in 5 mM or 25 mM glucose before being treated with 1 μ M DEX for 24 h then cell number quantified using Cell Counting Kit – 8 assay (* $p < 0.05$, ** $p < 0.005$).

5.2.2 Effect of High Glucose and Dexamethasone on Retinal Endothelial Cell Apoptosis

REC apoptosis as measured by Caspase-3 assay increased with the addition of 25mM glucose (ns, $p = 0.0986$, Figure 5.2). However, DEX (100 μ M) partially reversed this effect and significantly reduced REC apoptosis compared to 25 mM glucose without DEX ($p = 0.0052$, Figure 5.2). There was no significant increase of EC apoptosis in 5 mM glucose medium with (100 μ M) DEX, although this change was variable (Figure 5.2).

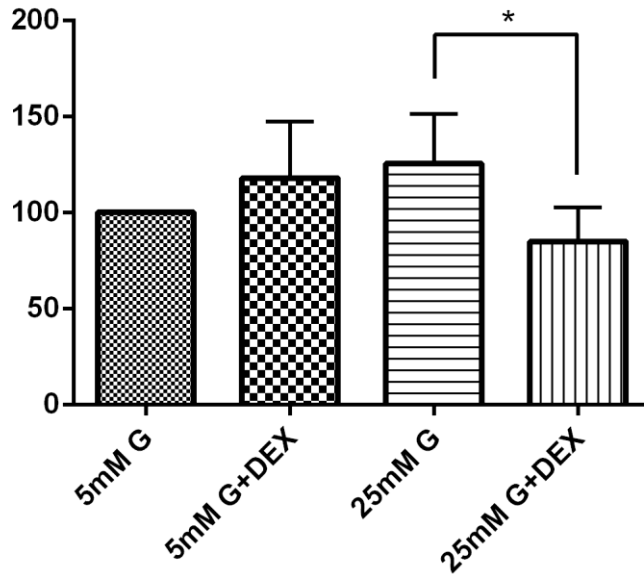


Figure 5.2. The effect of 25mM glucose and dexamethasone on REC apoptosis.

REC were incubated for 72 h in 5 mM or 25 mM glucose before being treated with 1 μ M DEX for 24 h then caspase-3 activity of apoptotic cells was recorded. The level of caspase-3 enzymatic activity in the cell lysate was directly proportional to the colour reaction. Data are expressed as mean $\pm$ SD based on four separate experiments (* p <0.05, ** p <0.005).

5.2.3 Effect of High Glucose and Dexamethasone on Retinal Endothelial Cell Permeability

The addition of 25 mM glucose significantly increased REC permeability compared to that in 5 mM glucose (p =0.0222, Figure 5.3A). When 100 μ M DEX was added for 24 (p =0.0197) or 72 h (p =0.0048) there was a significant decrease in REC permeability, amounting to a reversal of the effect of 25 mM glucose alone (Figure 5.3A). With 5 mM glucose, DEX showed a slight decrease in baseline permeability after 72 h (ns, p =0.2073). A lower concentration of DEX, 1 μ M (p =0.0312) still significantly decreased REC permeability in high glucose concentrations (Figure 5.3B).

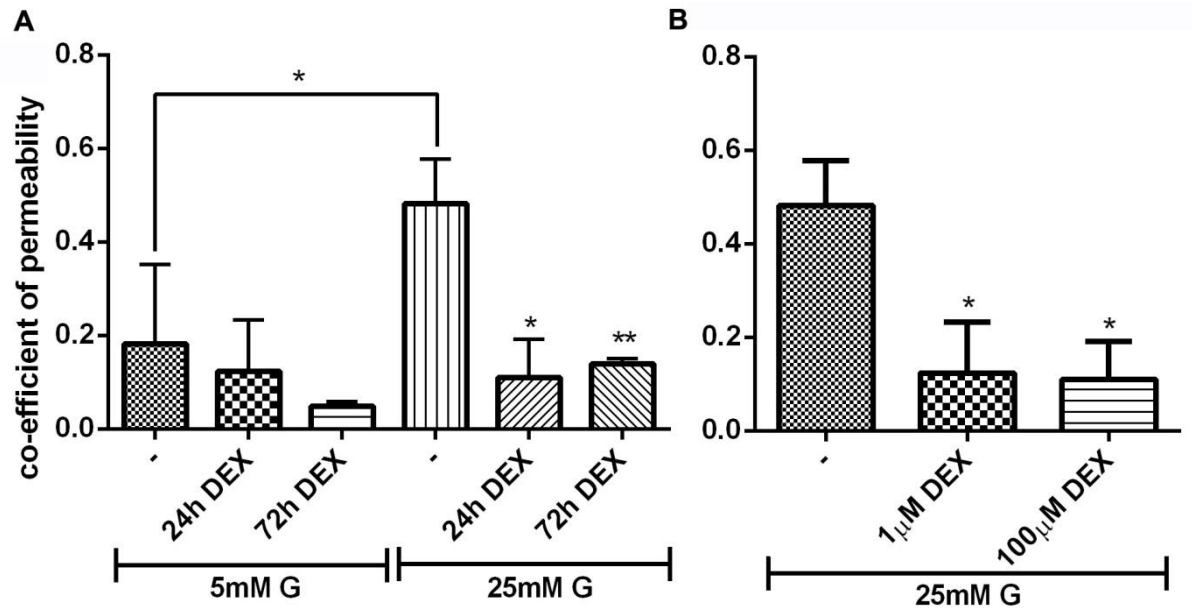


Figure 5.3. Effect of Dexamethasone on REC Permeability.

Trans-endothelial cell permeability was measured with 5 mM and 25 mM with and without 100 μ M DEX for 24 or 72 h (A) or with 1 μ M or 100 μ M DEX for 24 h. All time points and concentrations significantly decreased induced REC permeability (* p <0.05, ** p <0.005).

5.2.4 Effect of Dexamethasone on Gene Expression Profiling of Tight and Adherens Junction Components

The addition of 25mM glucose significantly decreased the mRNA expression of claudin-5, JAM-A, and occludin in REC (p <0.0001, Figure 5.4). Compared to 25mM glucose medium alone, the addition of (100 μ M) DEX significantly increased expression of claudin-5 (p =0.0042), and occludin (p =0.0018). JAM-A expression also increased slightly (ns, p =0.1556) compared to 25mM glucose alone, although this did not return to the 5mM glucose level as was seen with claudin-5 and occludin (Figure 5.4). Although 25mM glucose medium did not reduce VE-Cadherin expression, it appeared that (100 μ M) DEX may increase the expression of VE-Cadherin (ns, p =0.0624). JAM-C

expression was increased with 25m M glucose (ns, $p=0.0501$) but DEX did not change this significantly (Figure 5.4).

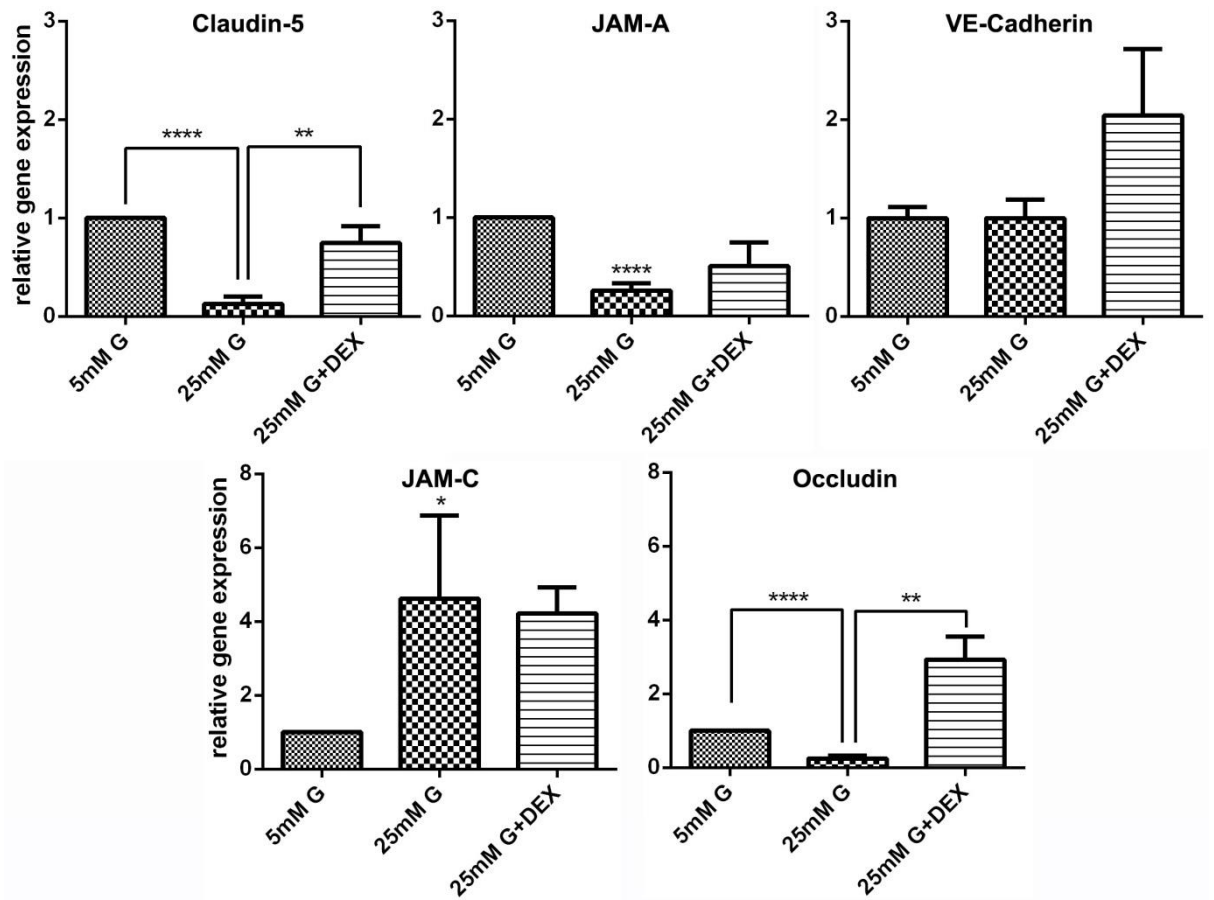


Figure 5.4. Effect of Dexamethasone on Gene Expression of Tight and Adherens Junction Complex.

Gene expression was quantified using qPCR with after treatment with glucose and 100 μ M DEX. Expression of claudin-5, JAM-A and occludin were reduced with 25mM and this effect was reversed with DEX treatment (* $p<0.05$, ** $p<0.005$, **** $p<0.0001$).

5.2.5 Effect of Dexamethasone and VEGF Combinations on Gene Expression Profiling of Tight and Adherens Junction Components

REC stimulated with 4.2 $\mu\text{L/mL}$ (1000pM diluted) VEGF 165 in 25 mM glucose medium demonstrated significant decrease in the mRNA expression of claudin-5 ($p=0.001$), JAM-A ($p=0.0192$), VE-Cadherin ($p=0.0004$) and occludin ($p=0.0002$). However, JAM-C expression markedly increased ($p=0.0015$). compared to the effect of diluted VEGF 165 on its own in 25 mM glucose medium, the combination of (1 μM) DEX and VEGF 165 significantly increased expression of claudin-5 ($p=0.0188$), JAM-A ($p=0.0051$) and occludin ($p=0.0043$). VE-Cadherin expression also increased slightly (ns, $p=0.0706$) similar to the effect of (1 μM) DEX on its own (ns, $p=0.0624$).

Just as the combination VEGF 165 and DEX did not significantly reduce the JAM-C expression (ns, $p=0.2321$), DEX alone did not alter this increase obtained with 25mM glucose and VEGF165 (Figure 5.5)

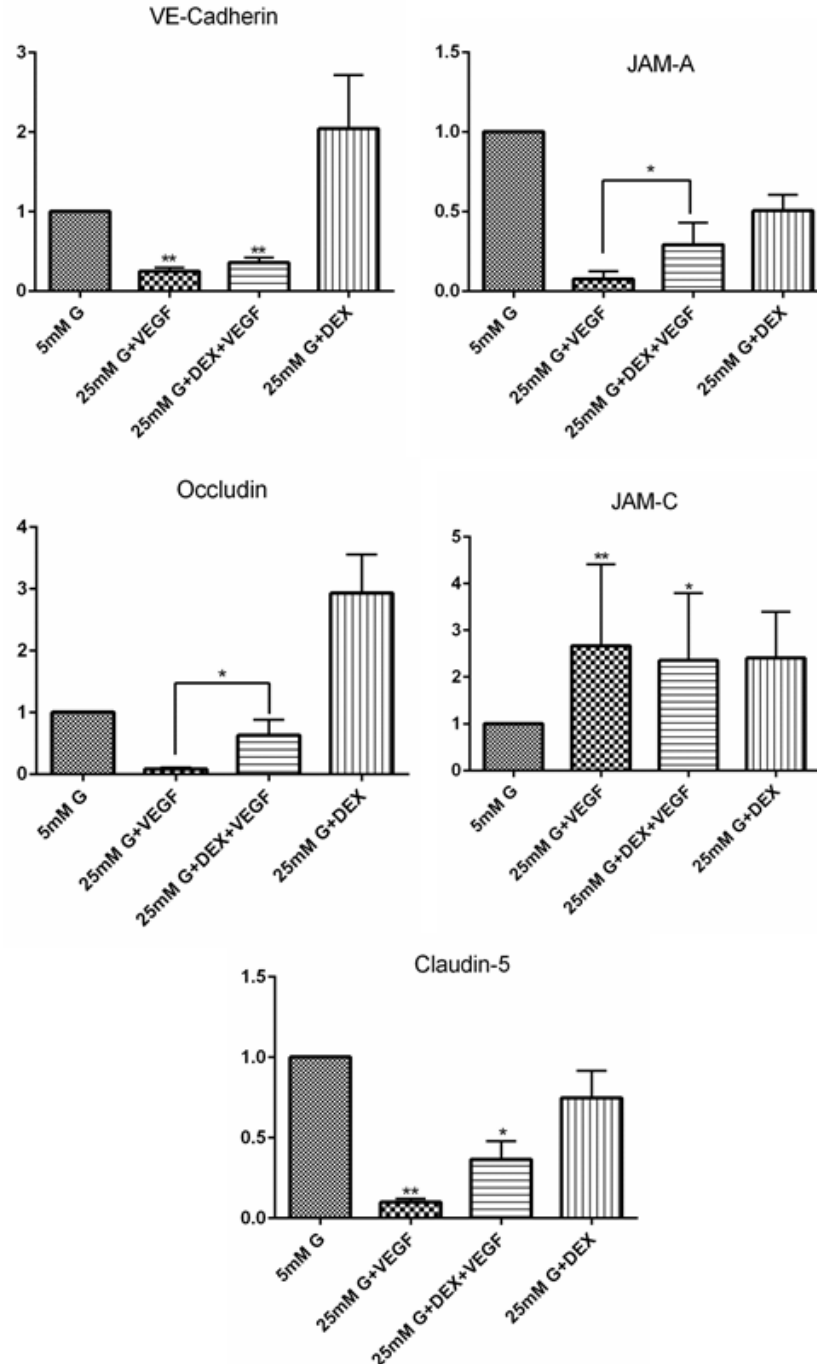


Figure 5.5. Effect of VEGF and DEX/VEGF Combinations on Gene Expression of Tight and Adherens Junction Complex.

Gene expression was quantified using qPCR with after treatment with glucose, 100μM DEX and 4.2uL/mL VEGF 165. Expression of claudin-5, JAM-A, VE- Cadherin and occludin were reduced with VEGF stimulated REC 25mM (*p<0.05, **p<0.005).

5.3 Discussion

This study has shown that there is significant reduction in REC numbers when cultured in 25 mM glucose. This agrees with previous reports of reduced REC proliferation (Lorenzi et al., 1985, Lorenzi et al., 1986), increased EC cell death and apoptosis (Mizutani et al., 1996, Liu et al., 2004) which were confirmed in an earlier chapter. This reduction is comparable to that previously shown by our group in human Tenon's capsule fibroblasts from diabetics and non-diabetics cultured in high glucose (Browning et al., 2005a), and in other EC elsewhere in the body (Eriksson et al., 2012, Calles-Escandon and Cipolla, 2001, Avogaro et al., 2008, Rask-Madsen and King, 2007). Potentially, reduced cell proliferation and increased apoptosis in the early stages of DR may increase retinal capillary occlusion leading to hypoxia, which in turn stimulates subsequent aberrant neovascularisation (Curtis et al., 2009, Wright et al., 2011) through increased VEGF production in the retina. This study has found that *in vitro* treatment of REC with DEX can reduce cell loss in media of high glucose (25 mM) concentrations. This may imply that DEX offers protection against REC loss in high glucose conditions, including clinical diabetic retinopathy.

Human REC show different trans-endothelial permeability profiles in normal and hyperglycaemic conditions (Saker et al., 2014a), where there is significant increase in REC permeability in HG. This is in keeping with previous reports (Ciulla et al., 2003, Miyamoto and Ogura, 1999, Mohr, 2004), although Nehme and Edelman (2008) were only able to show such changes in the retinal pericytes but not REC. VEGF-A is thought to be the

main factor responsible for increased retinal vascular permeability as upregulation of it results in phosphorylation of TJ proteins (occludin and ZO) and their disassembly (Harhaj et al., 2006, Antonetti et al., 1998). However, it has been suggested that VEGF modulation of ECs may not be an important mechanism of corticosteroid inhibition of vascular changes in high glucose as there was no effect on the secretion of inflammatory or angiogenic mediators in 25 mM glucose with or without DEX (Nehme and Edelman, 2008). This is supported by the increased in REC permeability and TJ alterations induced by VEGF165 in the presence of high glucose concentrations with or without DEX. Our study suggests that DEX may modulate REC permeability through a VEGF-independent pathway. Exposure of REC to DEX for 24 or 72 h results in significant reductions in the potential permeability of these cells. Our finding that the different doses of DEX restored most of the permeability profiles induced by high glucose indicates that the required therapeutic dose is low, and could have clinical implications for improvements in the development and delivery of anti-inflammatory therapies used to treat retinal diseases.

This study confirms that REC exposure to high glucose results in significant reduction in the gene expression of molecules important for TJ formation, including claudin-5, occludin and JAM-A. This study also found an increase in JAM-C in high glucose concentrations, which has been reported to induce permeability (Li et al., 2009). At the mRNA level, expression of claudin-5 and occludin were significantly increased by DEX in high glucose to levels that restored their expression to levels similar to those in normal glucose

medium. This change was associated with an increase in VE-Cadherin and JAM-A expression compared to high glucose alone, although these increases were not significant. However, the absence of any significant change in JAM-A or C expression with DEX in 25 mM glucose (compared to 25 mM glucose alone) indicates that DEX may restore permeability through specific pathways controlling TJ molecules. As such other treatments that modify TJ components other than claudin-5 and occludin may, in combination with DEX, produce additive effects in restoring EC permeability to the normal or near normal states.

Intraocular neovascularization is the key component in diabetic retinopathy and understanding of the molecular mechanisms involved in these diseases and targeting of the key molecular events could lead to novel therapy that may prevent new blood vessel formation and thus prevent blindness. It has been convincingly shown that VEGF is involved in the initiation as well as progression of neovascularization in diabetic retinopathy (Adamis et al., 1994, Shams and Ianchulev, 2006), which makes VEGF receptors and VEGF-induced signalling intermediates possible molecular targets to develop potential therapies (Gliki et al., 2002, Larrivee and Karsan, 2000, Guo et al., 1995). Indeed, various inhibitors such as anti-VEGF antibodies, e.g. ranibizumab (Novartis) and aflibercept (Eylea, Bayer), VEGF receptor inhibitors and receptor tyrosine kinase inhibitors, have been tested in preclinical studies and currently are at various stages of clinical trials. Moreover, emerging evidence suggests that many other angiogenic factors, besides VEGF, could be involved in the pathogenesis of neovascularisation

(Simo et al., 2002, Simo et al., 2006). Neovascularization requires migration and proliferation of EC including REC, but therapeutic interference in only one of these processes might not be enough to prevent this hallmark of PDR. Results of several studies based on *in vitro* or *in vivo* models indeed suggest that more than inhibition of VEGF (which is not the only factor controlling REC proliferation) is needed to suppress neovascularization (Aiello et al., 1995) especially for longer durations. Our finding that different doses of DEX *in vitro* (lower than the current therapeutic levels) were adequate in altering most of the permeability profiles could have clinical implications for the development and delivery of anti-inflammatory therapies used to treat retinal diseases.

Chapter 6. The Effect of Dexamethasone and Ranibizumab Combinations on Permeability and Proliferation with Hyperglycaemia

6.1 Introduction

DMO is responsible for significant visual impairment in diabetic patients (Klein et al., 1998, Bhagat et al., 2009). As previously described, the primary cause of DMO is fluid leakage resulting from increased vascular permeability through contributory anatomical and biochemical changes. These include endothelial cell death or dysfunction, pericyte loss or dysfunction, thickened basement membrane, loss or dysfunction of glial cells and loss/change of EC Glycocalyx (Ciulla et al., 2003, Mohr, 2004, Miyamoto and Ogura, 1999). The molecular changes include increased reactive oxygen species (ROS), pro-inflammatory changes: advanced glycation end products (AGEs), ICAM-1, Complement 5-9 deposition and cytokines, which result in increased paracellular permeability, tight junction disruption, and increased transcellular permeability (Bhavsar, 2006).

As described earlier, it is known that there are significant changes in the cytokine profile in DMO. The elevated cytokines include VEGF, MCP-1, IL-6, IL-8, IP-10 (Roh et al., 2009, Funk et al., 2010, Yoshimura et al., 2009, Sohn et al., 2011). Funatsu et al (Funatsu et al., 2005, Funatsu et al., 2002, Funatsu et al., 2001) have also previously reported increased levels of ICAM-1, IL-6, Angiotensin II, and VEGF in DR and DMO. Klein et al (Klein et al., 2009) reported that sVCAM-1 and TNFa are associated with higher odds

of developing more severe diabetic retinopathy in patients with renal disease and PDR. Other evidence suggests that anti-VEGF treatments have no effect on the cytokines other than VEGF levels in intraocular fluids (Sohn et al., 2011, Funk et al., 2010, Roh et al., 2009) .

The current treatments for DMO target reducing vascular leak in the macula once it has occurred; they do not attempt to treat the underlying pathology. These pharmacological treatments are aimed at antagonising vascular endothelial growth factor (VEGF) or non-VEGF inflammatory pathways, and include intravitreal injections of anti-VEGFs (ranibizumab, aflibercept or bevacizumab) or steroids (fluocinolone, dexamethasone or triamcinolone) as single therapies (Johnson, 2009, Jousseaume et al., 2004, Kaji et al., 2007).

The available evidence suggests that each individual treatment modality in DMO, targeting VEGF alone or VEGF independent pathways in isolation may not adequately block the increased permeability associated with diabetes and hyperglycaemia. As such monotherapy, with anti-VEGF drugs or steroid, does not result in a completely dry macula in most cases. The ideal treatment for DMO should improve vision and improve morphological changes in the macula (for example, reduce macular oedema) for a significant duration, reduced adverse events and be well tolerated by patients. A combination approach may be necessary as steroids and anti-VEGF may have complementary and additive effects on retinal vascular permeability. This follows from the evidence of (Nehme and Edelman, 2008) on the effect of dexamethasone on retinal endothelial cell and pericyte

permeability, and the reports from (Felinski and Antonetti, 2005a) and (Felinski et al., 2008), in concert with the anti-VEGF trials.

DMO results from breakdown of the inner blood retinal barrier (BRB) (Vitale et al., 1995) which is maintained by endothelial cell (EC) tight junctions (TJ) complexes (Mitic and Anderson, 1998). VEGF-A is thought to be the main factor responsible for increased retinal vascular permeability as upregulation of it results in phosphorylation of tight junction proteins (occludin and ZO) and their disassembly (Antonetti et al., 1998, Harhaj et al., 2006).

In the previous chapter, the effect of DEX on REC permeability was evaluated, where claudin-5 and occludin expression by REC in high glucose were significantly modulated by DEX.

This chapter evaluates the effect of DEX and ranibizumab (Lucentis, Novartis) combinations on REC permeability, proliferation and gene expression profiling with hyperglycaemia.

6.2 Results

6.2.1 Effect of Ranibizumab on Retinal Endothelial Cell Permeability in High Glucose

As shown in previous chapter, HG significantly increases REC permeability compared to normal glucose media ($p=0.0076$). This effect was reversed by the addition of 100 $\mu\text{g/mL}$ ranibizumab in serum-free medium for 72 h; the addition of ranibizumab significantly decreased REC permeability compared to HG alone ($p=0.0223$) (Figure 6.1).

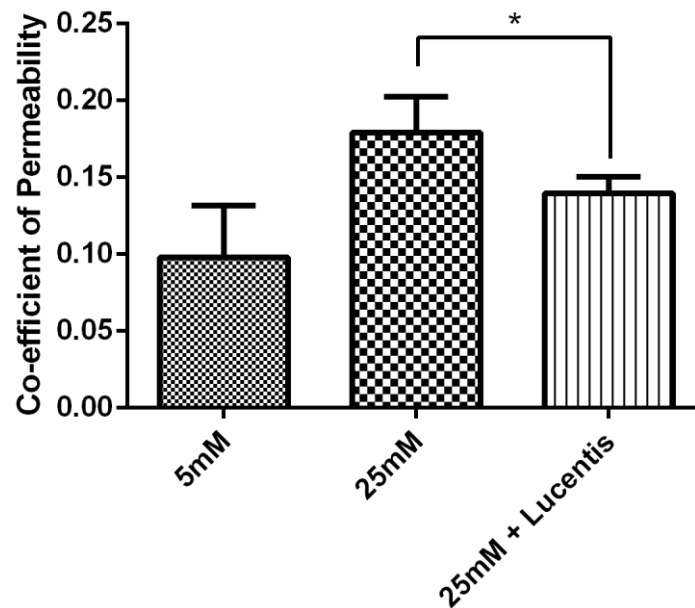


Figure 6.1. Effects of Ranibizumab on REC Permeability.

Transendothelial cell permeability was measured with 5mM and 25mM with and without 100 µg/mL ranibizumab for 72 h. Ranibizumab significantly decreased induced REC permeability (* $p < 0.05$, ** $p < 0.005$).

6.2.2 Effect of Dexamethasone and Ranibizumab Combinations on Retinal Endothelial Cell Permeability in Hyperglycaemia

It has been shown previously that HG significantly increased REC permeability. An increase in EC permeability with HG compared to NG was reproduced in this study. One concentration of ranibizumab (100µg/ml) was assessed for its potential to reverse this effect. This reduction in permeability was significant in HG conditions after 72 h ($p = 0.0142$) with DEX and 100 µg/mL ranibizumab combinations (Figure 6.2).

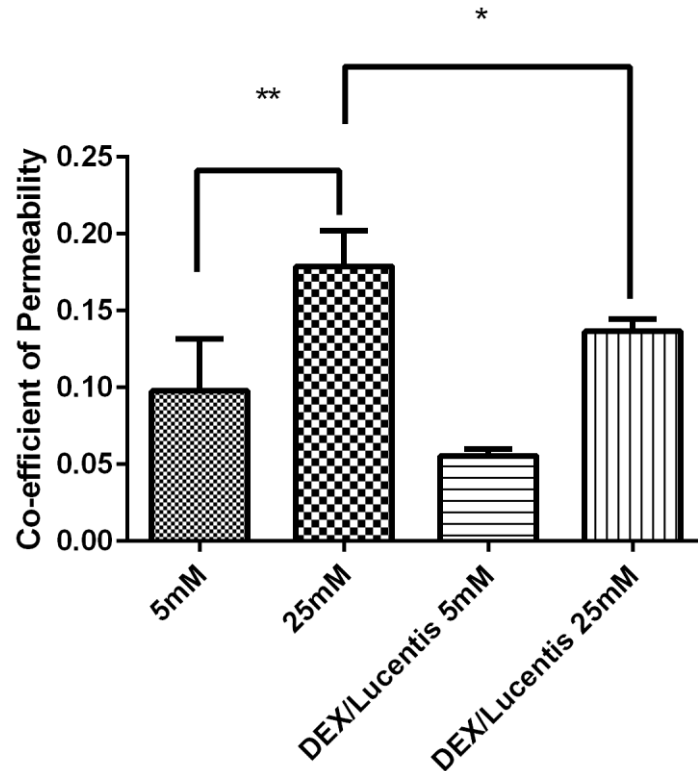


Figure 6.2. Effects of DEX/Ranibizumab Combinations on REC Permeability

Trans-EC permeability was measured with 5 mM and 25 mM with and without 1 μ M DEX and 100 μ g/mL Ranibizumab combinations for 72 h. All time points and concentrations significantly decreased induced REC permeability (* p <0.05, ** p <0.005).

6.2.3 Effect of Dexamethasone and Ranibizumab Combinations on Retinal Endothelial Cell Proliferation in Hyperglycaemia

The addition of 25 mM glucose significantly decreased REC numbers compared to normal medium containing 5 mM glucose (p =0.0016, Figure 6.3). DEX (1 μ M) in combination with (100 μ g/mL) ranibizumab reversed this effect and significantly increased REC proliferation rate compared to 25 mM glucose (p =0.0334).

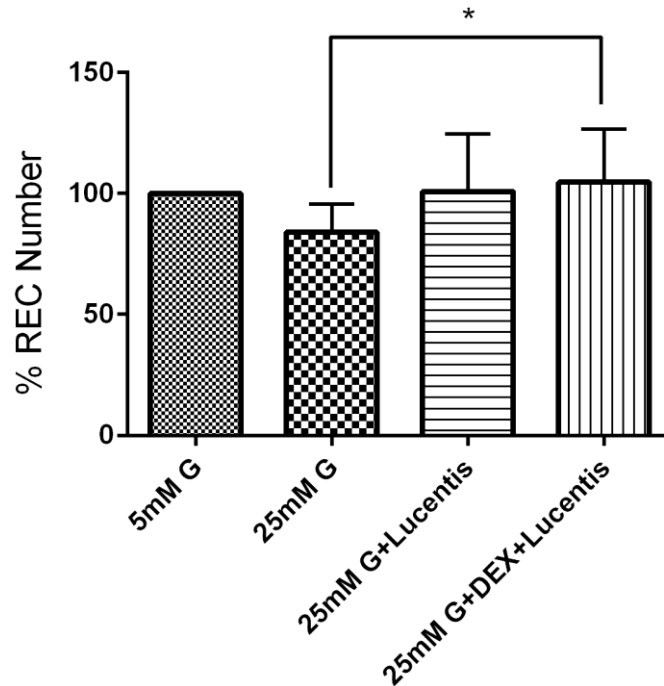


Figure 6.3. The Effect of 25 mM Glucose and DEX/Ranibizumab Combinations on REC Number

REC were incubated for 72h in 5mM or 25mM glucose before being treated with 1 μ M DEX and 100 μ g/mL Ranibizumab for 24 h then cell number quantified using Cell Counting Kit – 8 assay (* $p < 0.05$).

6.2.4 Effect of Dexamethasone and Ranibizumab Combinations on Gene Expression Profiling Of Tight Junction Components

HG conditions significantly decreased the mRNA expression of claudin-5 ($P=0.0067$), JAM-A ($p=0.0307$) and occludin ($p=0.0311$, Figure 6.4). Compared to HG the addition of 100 μ g/mL ranibizumab significantly increased expression of claudin-5 ($p=0.0042$), and occludin ($p=0.0018$). JAM-A expression also changed slightly (ns, $p=0.1556$) but this markedly increased with combination therapy (Figure 6.4). Although HG did not reduce VE-Cadherin expression, it appeared that DEX/Ranibizumab

combinations may increase the expression of VE-Cadherin (ns, $p=0.0624$). JAM-C expression was increased with HG. Ranibizumab on its own or in combinations with DEX did change this significantly compared to HG. However, HG with DEX/Ranibizumab combinations showed significantly higher JAM-C expression ($p=0.0015$) over the baseline NG (Figure 6.4).

The changes in REC TJs in high glucose medium and their modulation by DEX, ranibizumab, and combination of DEX and ranibizumab are summarised in Table 2.

	25mM G	25mM G+ DEX	25mM G+ Ranibizumab	25mM G+ DEX + Ranibizumab
<i>Claudin-5</i>	Decreased*	Increased*	Increased*	Increased *
<i>JAM-A</i>	Decreased*	No change	No change	Increased*
<i>VE-Cadherin</i>	No change	Increased	Increased	Increased
<i>JAM-C</i>	Increased*	No change	No change	Increased*
<i>Occludin</i>	Decreased*	Increased*	Increased*	Increased*

Table 2. Summary of Differential Endothelial Gene Expression

Summary of Differential Endothelial Gene Expression Profiling of Junctional Molecules achieved with DEX, Ranibizumab, and combinations of DEX and Ranibizumab in HG (25 mM glucose) ($p<0.05$).*

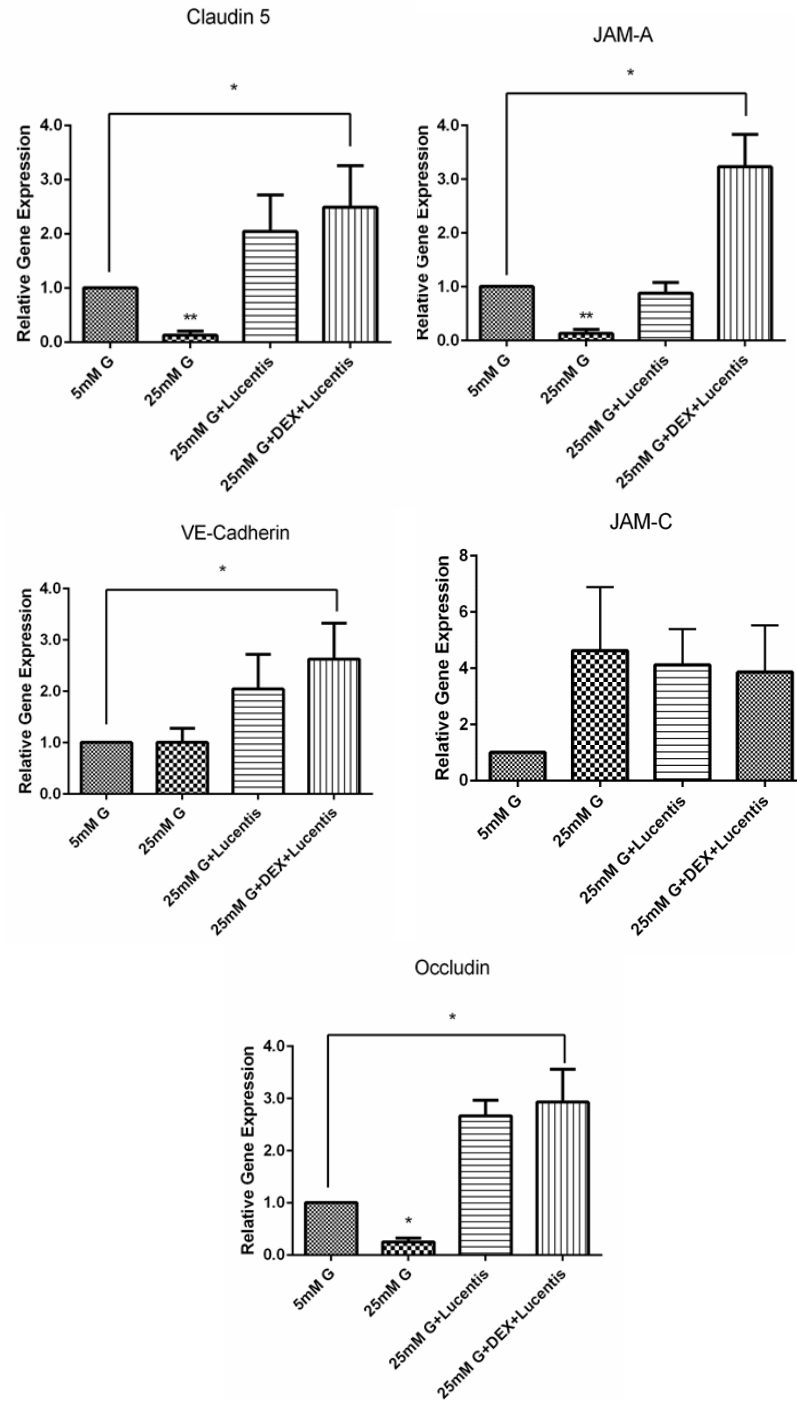


Figure 6.4. Effect of Dexamethasone and Ranibizumab Combinations on Gene Expression of Tight and Adheren Junction Proteins.

Gene expression was quantified using qPCR with after treatment with glucocse and 100 μ M DEX +100 μ g/ml Ranibizumab combinations. Expression of claudin-5, JAM-A and occludin were reduced with 25mM and this effect was reversed with Combinations treatment (* p <0.05, ** p <0.005).

6.3 Discussion

It has been reported that hyperglycaemia can stimulate both NF- κ B- and protein kinase C- dependent pathways thus upregulating endothelial adhesion molecules such as ICAM-1 (CD54) or E-Selectin (CD62E) (Omi et al., 2002, Morigi et al., 1998, Booth et al., 2002) vascular cell adhesion molecule-1: VCAM-1 (CD106) (Koch et al., 1995), and platelet endothelial cell adhesion molecule: PECAM (CD31), in ECs. ICAM-1 upregulation is thought to be VEGF dependent, whilst P- and E-Selectin are not (Lu et al., 1999). Vitreous levels of several inflammatory cytokines (including IL-6, IL-8, and MCP-1) are increased in eyes with diabetic macular oedema as well as PDR (Yoshimura et al., 2009). Funatsu et al (Funatsu et al., 2001, Funatsu and Yamashita, 2002, Funatsu et al., 2005) reported significant elevations of VEGF, ICAM-1, IL-6, MCP-1 and PEDF which correlated with retinal vascular permeability. In addition to this pathway, stimulation of 6-N-acetyl glucosaminyl-transferase in leukocyte can result in more modification of glycans on its surface thus inducing leukocyte rolling interactions to endothelial selectins (Chibber et al., 2000, Chibber et al., 2003, Chibber et al., 2007).

It has been frequently demonstrated that corticosteroids have significant effects on RECs (Felinski and Antonetti, 2005b, Felinski et al., 2008). It has been suggested that steroids may act by both suppressing inflammation and by directly affecting the ECs by regulating phosphorylation, organisation, and content of TJ proteins (Felinski and Antonetti, 2005a). Felinski et al (Felinski et al., 2008) have reported that glucocorticoid treatment of primary RECs

increases content of the TJ proteins occludin and claudin-5, in parallel with an increase in barrier properties of endothelial monolayers, therefore restoring endothelial para-cellular permeability. However, the exact mechanisms of action are poorly understood. Similarly, VEGF-A is thought to be the main factor responsible for increased retinal vascular permeability as upregulation of it results in phosphorylation of tight junction proteins (occludin and ZO) and their disassembly (Antonetti et al., 1998, Harhaj et al., 2006). A few trials have evaluated the combination of bevacizumab with triamcinolone (Ahmadieh et al., 2008, Soheilian et al., 2007, Soheilian et al., 2012, Mirshahi et al., 2010). Ahmadieh et al (Ahmadieh et al., 2008) evaluated bevacizumab alone in comparison to bevacizumab and triamcinolone, and determined that there was no further improvement with the combination compared to bevacizumab alone. Soheilian et al (Soheilian et al., 2012, Soheilian et al., 2007) compared combination of 1.25 mg bevacizumab and 2 mg triamcinolone with bevacizumab alone and laser photocoagulation alone. Bevacizumab alone improved BCVA at 36 months, compared to either the combination or laser alone group (Soheilian et al., 2007, Soheilian et al., 2012). Faghihi et al (Faghihi et al., 2008) compared intravitreal bevacizumab (1.25 mg) alone with combination of bevacizumab and focal laser, and determined at 6 months that both regimes had comparable vision improvement and that neither dosage was superior to the other. The outcomes reported by Faghihi et al (Faghihi et al., 2008) that combinations of bevacizumab and focal laser photocoagulation induced earlier recovery of VA compared to bevacizumab monotherapy although this was not confirmed

by Soheilian et al (Soheilian et al., 2009). Sheth et al (Sheth et al., 2011) did not show any difference in DME reduction or post injection VA in eyes treated with intravitreal triamcinolone vs. dexamethasone and bevacizumab. However, that's most likely because of the short duration of the dexamethasone used, as well as the small sample size (Sheth et al., 2011).

This study has shown that the combination of DEX and ranibizumab results in a higher reduction in REC permeability than either agent on its own, through modulation of REC TJ molecules. The findings confirmed that REC exposure to high glucose has resulted in significant reduction in the gene expression of molecules important for TJ formation, including JAM-A, claudin-5, and occludin. The further investigation of JAM-C, which has been reported to induce permeability, revealed non-significant increase in our study. However, the absence of any significant change in JAM-A or C expression with DEX in 25 mM glucose (compared to 25 mM glucose alone) indicates that DEX may restore permeability through specific pathways controlling TJ molecules. At the mRNA level, expression of claudin-5 and occludin were significantly increased by DEX in high glucose to levels that restored their expression to levels similar to those in normal glucose medium. Ranibizumab in high glucose medium increased the expression of claudin-5 and occludin. A combination of ranibizumab and DEX in high glucose medium increased claudin-5 and occludin levels, but in addition resulted in significant increases in JAM-A and VE-Cadherin levels compared to high glucose alone. No increase was noted in JAM-A or VE-Cadherin with either DEX or ranibizumab alone.

Clinically, therefore, it is possible that an initial combination of DEX and ranibizumab will allow a faster visual acuity rise to the plateau than achieved with ranibizumab on its own. This may induce a longer remission of DMO, and more permanently modulate the recurrence of DMO than after either agent on its own. The longer acting steroid, fluocinolone may also have a role in combination with anti-VEGF therapies. This is despite the side effect profile of steroids including cataracts, and increased intraocular pressure. Potentially, if such a combination works, only 1 or 2 dexamethasone implant injections will be required, but at the same time reducing the frequency of intravitreal anti-VEGF injections.

Chapter 7. Functional and morphological changes in DMO: baseline correlations, and changes with pharmacological treatments

7.1 Introduction

Several pharmacologic agents are now available for the treatment of DMO including anti-VEGF agents (Diabetic Retinopathy Clinical Research et al., 2010, Elman et al., 2011, Nguyen et al., 2012, Korobelnik et al., 2014, Mitchell et al., 2011, Massin et al., 2010) and corticosteroids (Kupfermann et al., 2007, Gillies et al., 2007, Diabetic Retinopathy Clinical Research et al., 2010, Elman et al., 2011, Korobelnik et al., 2014, Pearson et al., 2011, Campochiaro et al., 2011, Boyer et al., 2011). The introduction and frequency of these treatments has coincided with the introduction of less invasive investigating and monitoring systems including OCT (Yang et al., 2001, Sanchez-Tocino et al., 2002, Panozzo et al., 2003, Browning et al., 2004a, Vujosevic et al., 2011, Browning et al., 2004b) and fundus related perimetry, also known as microperimetry (MP) (Okada et al., 2006, Carpineto et al., 2007, Vujosevic et al., 2011, Vujosevic et al., 2008, Vujosevic et al., 2010, Alexander et al., 2012).

OCT is now well established in the evaluation of DMO, especially with the recent advances with spectral domain models (Hee et al., 1998, Strom et al., 2002, Sanchez-Tocino et al., 2002, Yang et al., 2001, Browning et al., 2004b, Soiberman et al., 2014, Diabetic Retinopathy Clinical Research Network Writing et al., 2014, Sun et al., 2014). It has contributed significantly to our

understanding of the anatomical changes of DMO and the intra-retinal damage and provides a valuable tool for the follow-up of macular oedema (Panozzo et al., 2003). Carpineto et al (Carpineto et al., 2007) studied the role of MP in evaluating fixation patterns and retinal sensitivity in patients who had clinically significant macular oedema with a diffuse pattern and type 2 diabetes mellitus. Furthermore, Okada et al (Okada et al., 2006) demonstrated a significant correlation between retinal sensitivity as measured by MP, visual acuity and foveal thickness as measured by OCT in eyes with DMO. Previous studies have also investigated the role of MP and fundus autofluorescence (FAF) in evaluating outcomes in DMO treated with laser photocoagulation and micro pulse laser photocoagulation (Vujosevic et al., 2010). More recently, Comyn et al (2014) (Comyn et al., 2014) reported functional and morphologic changes in eyes treated with ranibizumab or laser photocoagulation in the LUCIDATE Study. Similarly, Reznicek et al (2013) (Reznicek et al., 2013) and Kim et al (Kim et al., 2014) have reported functional outcomes of DMO eyes treated with anti-VEGF therapies. These available MP studies on DMO, however, used the first generation of MP technology (MP-1, Nidek Co; Japan) (Carpineto et al., 2007, Vujosevic et al., 2010) where the analysis of fixation stability was not studied as a main outcome of visual function affected by DMO. A more recent study by Kim et al (2014) (Kim et al., 2014) used the spectral OCT/SLO system (Optos OCT SLO, Optos, Scotland).

MP allows for the exact topographic correlation between fundus abnormalities and corresponding functional alterations by integration, with different methods, of differential light threshold (more commonly known as retinal sensitivity) and fundus imaging. The Nidek MP-1 is a mesopic test that requires pupil dilation or a 5–10 min dark light adaptation before starting the examination. The Optos system relied on pupil dilatation and 15 minute dark adaptation used a fixation target in the shape of a cross of 1° size and the MP was performed with Goldmann III size dot stimuli utilising 13 stimulus points and a four to two staircase strategy (Kim et al., 2014).

More recently, Centervue (Padova, Italy) developed a 3rd generation of MP systems, the Macular Integrity Assessment (MAIA). The greatest advantage of the MAIA is the ease of the operation since it does not require mydriasis; the much lower cost compared to other instruments is an added advantage. It utilizes the Scanning Laser Ophthalmoscope (SLO) technology to image the retina with higher resolution (of 1024 x 1024 pixels) and a more effective eye tracker to monitor the eye movement at 25 times per second improving reliability and usability for operators and patients (Vujosevic et al., 2010, Vujosevic et al., 2008, Morales et al., 2013). In addition, the MAIA is the only instrument that offers automatic estimates on PRL characteristics during early and late fixation (Morales et al., 2013).

This study aims to evaluate the ability of MAIA MP in detecting early changes in retinal function not only with the analysis of retinal sensitivity, but also with the analysis of fixation behaviour in eyes with DMO, and investigate their

correlation with central macular thickness measured with OCT and visual acuity (LogMAR) at baseline and in 3 months from commencing treatment intravitreal anti-VEGF therapies.

7.2 Methods

In a prospective, observational case series study, 23 consecutive patients with clinically significant DMO (Table 1A, 1B) presenting to the practice of WMA in the University Hospital, Queen's Medical Centre, Nottingham, were recruited into this study from August 2013 to July 2014. This study was approved by the institutional review body (National Research Ethics Service Committee ref: 12/EM/0116; R&D ref 12OY004). Full ophthalmological assessment was done. Informed consent was obtained from all the participants. Subjects received intravitreal injections of ranibizumab (Novartis UK, Frimley Park) 0.5mg in 0.5ml at 4 weekly intervals times 3, and then pro re nata as per NICE TA 237. By implication, all eyes had DMO with foveal thickness of 400 microns or more. Logarithm of the minimum angle of resolution (LogMAR) best-corrected visual acuity (BCVA) was evaluated by means of an Early Treatment of Diabetic Retinopathy Study chart (ETDRS). Foveal thickness was measured with the Topcon 2000 OCT (Topcon, Tokyo, Japan). Age, duration of diabetes, haemoglobin A1c (HbA1c) levels, duration of symptoms, history of previous laser photocoagulation, or vitrectomy were recorded. OCT examinations were analysed for vitreomacular adhesion/traction, and cystoid macular changes were quantified. All the

participants were assessed for visual function with the Macular Integrity Assessment MP (MAIA MP) instrument (CentreVue, Padua, Italy). Retina sensitivity (RS) and Fixation stability were assessed with MAIA MP over the central retina (10 degrees) projecting 37 light stimuli points with a, “4-2” projection strategy. The examinations were performed at baseline and months 3 after treatment. Main outcome measures were fixation characteristics and mean retinal sensitivities. The MAIA MP images were exported into the Topcon OCT system through the Topcon import-reference-image software for the manual morphological-functional correlation analysis. As the selected MP grid map measures light sensitivity in the central 3mm of the retina, only the standard internal retinal subfields (central, inner superior, inner nasal, inner inferior, inner temporal) as defined in the Age Related Eye Disease Study (AREDS) (Davis et al., 2005) were investigated. The outer OCT subfields were ignored in the current analysis.

The statistical analysis was performed with linear regression, Pearson’s product-moment correlation coefficient and two-tailed paired t test, without correcting for multiple comparisons, using GraphPad Prism 6. A *p* value less than 0.05 was considered statistically significant.

7.3 Results

Forty-two (42) eyes of 23 patients with mean age of 65 years-old (min = 25, max = 91) were recruited into this study. Patient characteristics including age, type and duration of diabetes, haemoglobin A1c (HbA1c) levels, duration of DMO symptoms, and history of previous laser photocoagulation, or vitrectomy are summarised in Table 3 and Table 4

The duration of DMO symptoms/presence varied from 14 to 60 (average of 30.09 months). LogMAR VAs and OCTs were performed at similar visits. At baseline, MP parameters of mean retinal threshold sensitivity (TS) (Figure 7.1) and fixation stability (FS) (Figure 7.2) demonstrated a wide range of values (TS: mean = 19.27dB, min = 0dB, max = 29.4dB) (FS: mean = 72.4%, min = 11%, max = 100%) at baseline.

At baseline, the LogMAR VAs ranged from 0.0 to 0.98. The central macular thickness measured with OCT (central subfield) ranged from 244 to 841 microns at baseline. VA measurements showed higher correlation with functional MP values of TS ($r = 0.617$) and FS ($r = 0.725$), than those found with central macular thickness measured with OCT ($r = 0.221$). These correlations between VA and the MP parameters are presented in Figure 7.3, Figure 7.4, Figure 7.5. The correlation between TS and FS was moderate ($r = 0.442$), whilst correlation with DMO duration was low as shown in Table 5.

The morphological changes of macular thickness, intraretinal cysts or subretinal fluid (SRF) on OCT decreased in all internal subfields at 3 months with treatment compared to baseline values. In particular the central subfield showed a statistically significant thickness reduction from $445.3 \pm 129.9 \mu$ at baseline to $337.4 \pm 107.4 \mu$ ($p = 0.01$). The other OCT internal subfields changes represented in Figure 7.6, also showed statistically significant reductions in thickness ($p < 0.05$). Changes in intraretinal cysts and SRF also showed significant reduction at 3 months compared to baseline ($p < 0.05$).

The LogMAR VA measurements demonstrated an overall improvement over the time (Figure 7.7); however, the changes were not statistically significant ($p = 0.12$). The TS and FS demonstrated only slight improvements in the minimum and maximum limits as shown in Figure 7.8 and Figure 7.9 respectively. The with time, which were not statistically significant (p values of 0.71 and 0.82 respectively). Table summarized the morphological and functional changes 3 months after treatment initiation.

The Pearson's product-moment correlation coefficient at 3 months after treatment commencement showed a higher, although moderate correlation between logMAR VA with both parametric parameters of TS ($r = 0.42$) and FS ($r = 0.58$), than the correlation shown with OCT values ($r < 0.25$), as demonstrated in Table.6

7.4 Discussion

The morphological changes and VA data in our cohort confirm the clinical efficacy of treatments of DMO with intravitreal injections of ranibizumab.

Okada et al (2006) reported that eyes with DMO had reduced sensitivity as measured with the Nidek MP1 compared to eyes with normal macula. Vujosevic-(Vujosevic et al., 2006) showed a correlation between VA and MP sensitivity in eyes with DMO. (Reznicek et al., 2013) reported significant correlations between CRT; the functional parameters of BCVA (in letters) and MP, at baseline and after 3 injections of ranibizumab. In the study by (Kim et al., 2014), there was no correlation between the retinal sensitivity measurements (MP and contrast sensitivity) with macular thickness measurements on OCT in eyes with DMO, although the equipment and examination strategies used were different. The integrity of the IS/OS junction (ellipsoid zone), and the outer retinal appearance on the OCT may provide adequate information. This may allow us to distinguish between thin macula which is scarred and which is due to resolution of oedema.

Retinal sensitivity on the Nidek MP1 improved at 12 weeks in eyes with DMO treated with ranibizumab or laser, and the improvement continued till week 48. No correlations with OCT measurements were, however, reported in that study.

Our analysis at baseline demonstrates that LogMAR VA may be more correlated to functional macular parameters of threshold sensitivity and fixation stability as measured with MP than morphological changes

measured with OCT. Similarly, there was better correlation between VA and macular sensitivity (TS and FS) at 3 months after treatment commencement.

Macular oedema associated with retinal vascular conditions may affect central vision such that significant reduction leads to the development of eccentric fixation. Generally, ophthalmologic examinations including OCT and MP are based on patient's ability to observe a fixation target, and adequately maintain that fixation during the examination process. If foveal function is affected, such examinations may not be accurate as fixation may not be optimally located over the foveal area, and therefore the central values reported by OCT, may represent eccentric values. The absence of significant change in functional measures of VA, and TS and FS at 3 months compared to baseline may imply that the functional loss in DMO especially of long duration may not be reversible in the short-term, as the neural disorganisation may not be reversible. This suggestion supports that of Vujosevic et al (Vujosevic et al., 2006), that MP may be useful in predicting functional outcomes in eyes DMO. The DMO in our patients had been present for long duration, and will support the suggested neural disruption. A larger cohort with recent onset of DMO would demonstrate better restoration of retinal sensitivity with treatment.

Although the present analysis suggests a stronger correlation between the MP functional parameters with LogMAR VA, a longer follow-up with a larger sample test is needed to confirm these findings.

The findings from this study demonstrate that patients with good visual acuity show highly stable fixation ($P1 > 90\%$) due to the intact integrity of cone photoreceptors which describes good foveal vision. Okada (Okada et al., 2006) have postulated that mean retinal sensitivities measured with MP are significantly correlated to visual acuity and foveal thickness. However, the results from this analysis suggest that fixation stability may be a new important parameter to describe functional macular function.

The lack of strong correlation between all the measured values, however, indicates that measurement of visual function with MP may be considered as an important tool to better diagnose retinal pathologies as part of multimodality imaging, responding to the need to combine morpho-functional information, and combining different images from different measurement technologies. Further studies are, however, needed to better understand the relationship between macular morphology and the different parameters of macular function.

The main limitations of this study are the small number of patients, chronicity of DMO, and short follow-up. A larger number of patients with recent onset of DMO, followed up for a longer period will provide further valuable information on the role of MP in evaluating outcomes in the treatment of diabetic macular oedema.

ID	Gender	Age (years)	Diabetes Type	Duration of Diabetes (months)	DMO Duration of Symptoms (months)	HbA1c (mmol/mol)	Eye	Right Eye VA (LogMAR)	Left Eye VA (LogMAR)
1	M	66	1	10	14	84	R,L	0.6	0.32
2	M	64	1	26	24	70	R,L	0.7	0.5
3	M	67	2	13	48	65	R,L	0.3	0.42
4	M	61	1	20	18	42	R,L	0.8	0.62
5	F	67	2	30	60	59	R,L	0.36	0.5
6	M	74	2	30	60	57	R,L	0.78	0.78
7	M	56	2	12	36	69	R,L	0.2	0.2
8	F	91	2	15	27	45	R,L	0.96	0.2
9	F	42	2	18	30	35	R,L	1.08	0.6
10	M	52	2	16	48	48	R,L	0.5	0.16
11	F	78	2	13	60	84	R	0.4	0.74
12	M	80	1	10	60	58	R,L	0.5	0.42
13	F	80	2	30	48	59	R,L	0.5	0.3
14	M	72	1	10	27	72	R,L	0.2	0
15	M	25	2	20	36	45	R	0.1	0.2
16	F	80	2	26	24	59	L	0.42	0.3
17	M	79	1	10	60	42	R,L	0.5	0.32
18	F	36	2	10	48	84	R,L	0.12	0.1
19	M	68	2	26	48	70	R,L	0.1	0.42
20	F	84	1	14	60	49	R,L	0.3	0.96
21	M	78	2	13	18	74	L	0.26	0.34
22	M	69	2	25	24	59	R,L	0.3	0.3
23	M	48	2	14	21	48	R	0.42	0.2

Table 3. Patient Data

Demographics	
No. of patients	23
Sex (female)	8 (34%)
Age (years)	65.12 (25 to 91) ^a
Systemic profile	
Duration of diabetes (months)	17.87 (10 to 30) [*]
HbA _{1c} (mmol/mol)	59.87 ± 14.34
Ocular profile	
DMO Duration of symptoms (months)	39.09 (14 to 60) ^a
Mean no. of previous macular laser treatments / vitrectomy	3 (1 to 4) ^a

Table 4. Baseline characteristics of participants with clinically significant DMO

DMO = diabetic macular oedema; HbA_{1c} = glycosylated haemoglobin; HbA_{1c} Levels 20.0 - 59.0 mmol/mol. Data are mean ± SD for normally distributed data or median (Interquartile range) for non-normally distributed continuous data (*); number (%) for categorical data.

^aNon - normally distributed continuous data.

	DMO t (months)	TS	FS
DMO t (months)	1		
TS	-0.153	1	
FS	-0.141	0.539	1

Table 5. Correlation table between DMO duration and MP parameters.

Pearson correlation coefficient table between duration of DMO and microperimetry parameters of TS and FS. t = time; FS=Fixation stability TS=Threshold sensitivity.

	CMT-OCT	VA	TS	FS
CMT-OCT	1			
VA	0.221	1.000		
TS	-0.090	-0.617	1.000	
FS	-0.218	-0.726	0.443	1.000

Table 6. Correlation table between CMT and OCT,

Pearson correlation coefficient table in baseline examinations between CMT-OCT LogMAR VA, and microperimetry parameters of TS and FS. CMT-OCT= Central Macular Thickness; FS=Fixation stability TS=Threshold sensitivity; VA=Visual acuity.

	Base-line	3 months post-op	Change	Correlation coefficient (r)	P Value	Statistical Significance (P < 0.05)
OCT-C (μ)	445.3 ± 129.9	337.4 ± 107.4	-108 ± 126	0.45	0.018	Yes
OCT-S (μ)	398.2 ± 72.8	333.8 ± 40.8	-64 ± 52	0.72	0.002	Yes
OCT-N (μ)	389.1 ± 75.6	337.5 ± 47.2	-52 ± 58	0.65	0.014	Yes
OCT-I (μ)	376.4 ± 70.8	319.5 ± 42.4	-57 ± 50	0.72	0.004	Yes
OCT-T (μ)	415.1 ± 92.3	337.3 ± 65.9	-78 ± 69	0.67	0.004	Yes
VA (LogMAR)	0.48 ± 0.2	0.41 ± 0.3	-0.07 ± 0.13	0.86	0.120	No
TS (dB)	20.2 ± 3.4	19.9 ± 3.5	0.32 ± 2.8	0.68	0.715	No
FS (%)	73.4 ± 27.0	74.4 ± 26.1	1 ± 14	0.86	0.820	No

Table 7. Changes in OCT, FS, TS, and VA.

Changes in optical coherence tomography (OCT) macular subfield thickness, FS, TS, and VA at 3 months. A two-tailed paired t test was used to determine statistically significant

changes (*P* values less than 0.05). VA= visual acuity; FS=fixation stability; TS= threshold sensitivity; OCT-C= central OCT subfield; OCT-S= inner superior OCT subfield; OCT-N= inner nasal OCT subfield; OCT-I= inner inferior OCT subfield; OCT-T= inner temporal OCT subfield.

	OCT-C	OCT-S	OCT-I	OCT-N	OCT-T	VA	TS	FS
OCT-C	1.000							
OCT-S	0.848	1.000						
OCT-I	0.873	0.802	1.000					
OCT-N	0.861	0.821	0.945	1.000				
OCT-T	0.827	0.888	0.799	0.758	1.000			
VA	-0.249	-0.193	-0.148	-0.054	-0.123	1.000		
TS	-0.218	-0.366	-0.134	-0.246	-0.303	-0.425	1.000	
FS	0.354	0.187	0.278	0.159	0.150	-0.584	0.247	1.000

Table 8. Cross correlation between VA, OCT and MP.

Pearson coefficient correlation at 3 months correlating morphological values (OCT) with LogMAR Visual Acuity, and functional parameters measured with microperimetry (TS=threshold sensitivity and FS= fixation stability).

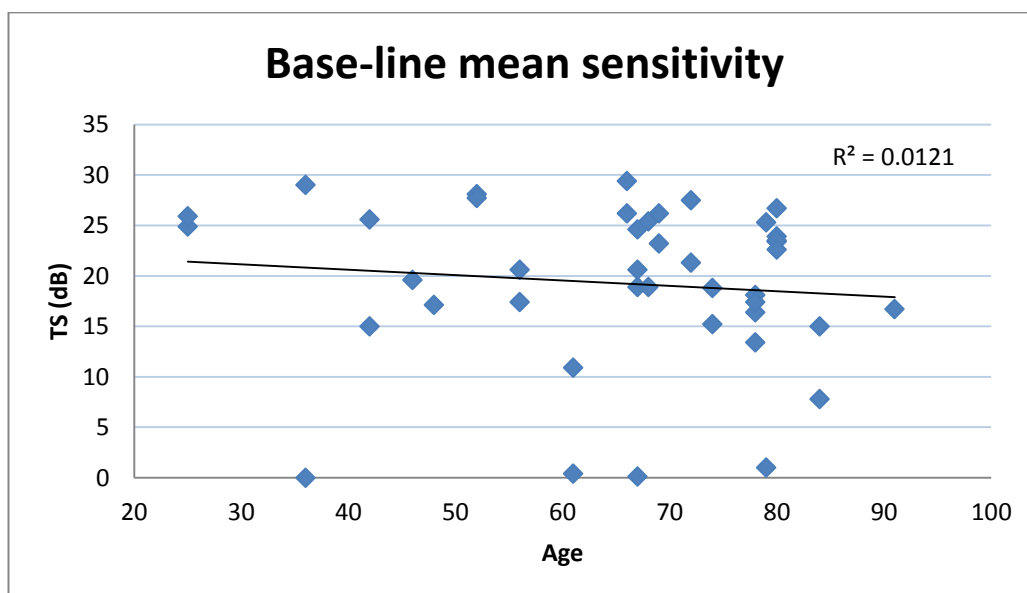


Figure 7.1. Distribution of baseline threshold sensitivity (TS) measured with microperimetry.

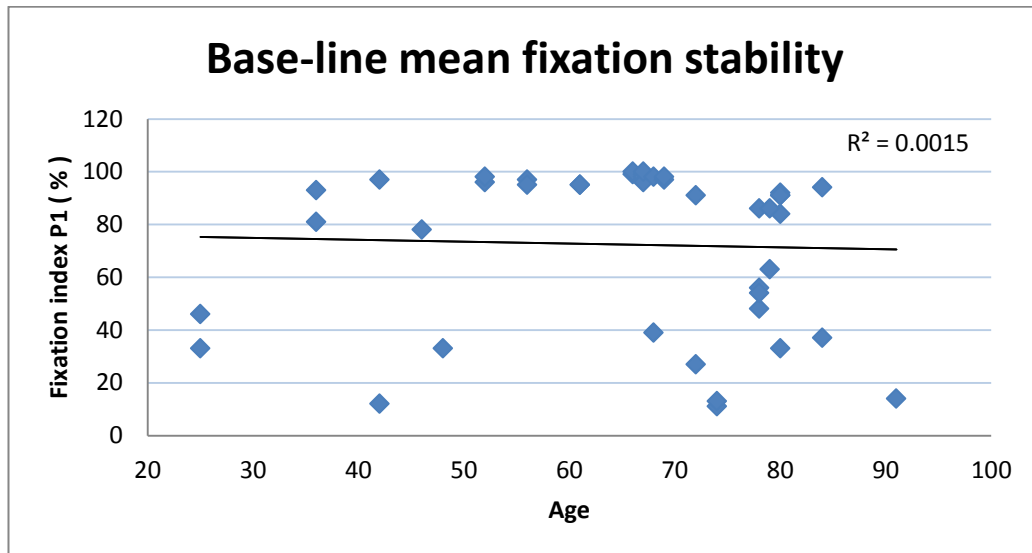


Figure 7.2. Distribution of baseline fixation stability (FS) measured with microperimetry.

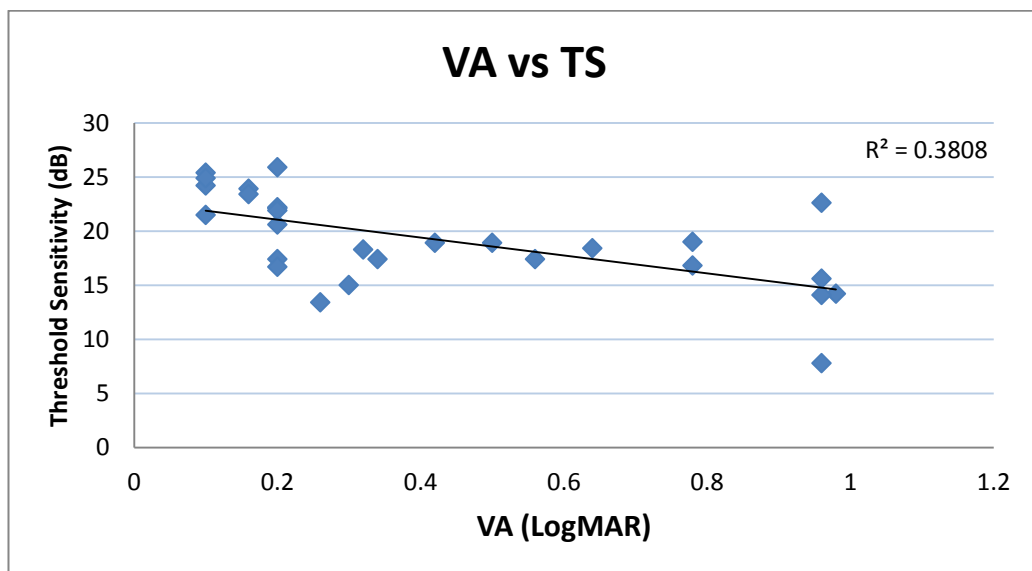


Figure 7.3. Linear regression analysis showing the relationship between LogMAR visual acuity (VA) and threshold sensitivity (TS) measured with microperimetry.

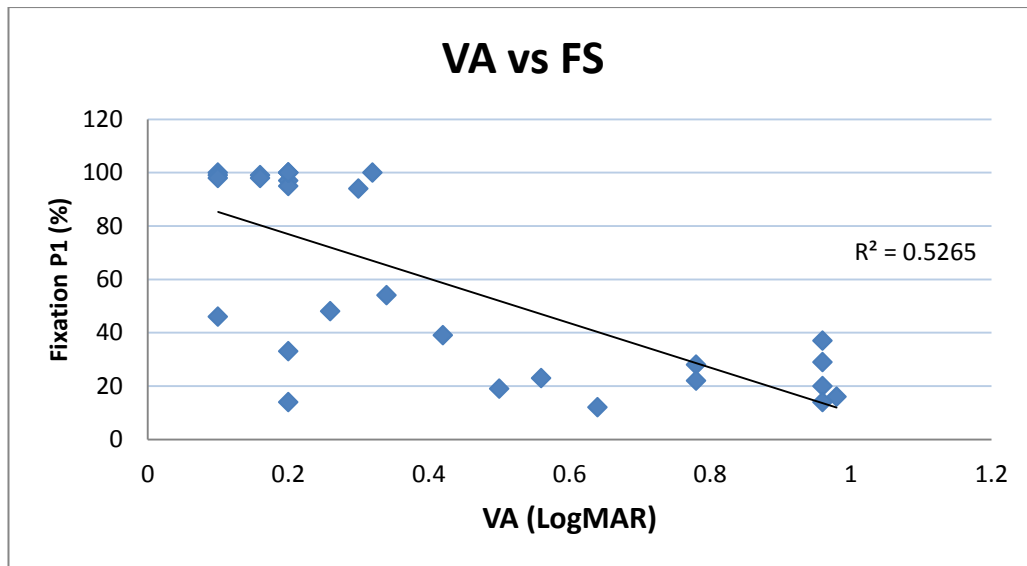


Figure 7.4. Linear regression analysis showing the relationship between LogMAR visual acuity (VA) and fixation stability (P1) measured with microperimetry.

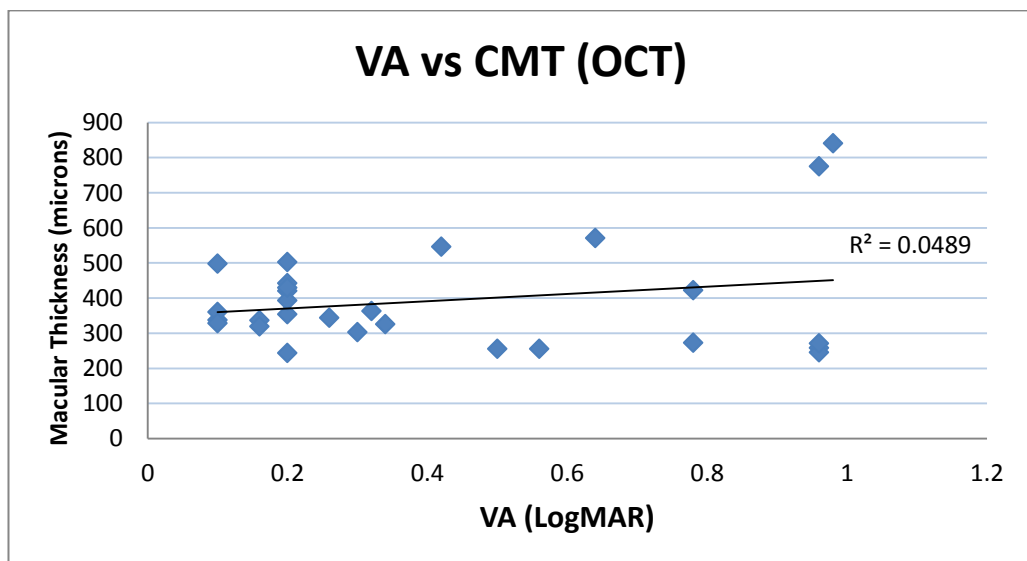


Figure 7.5. Linear regression analysis showing the relationship between visual acuity and central macular thickness measured with OCT.

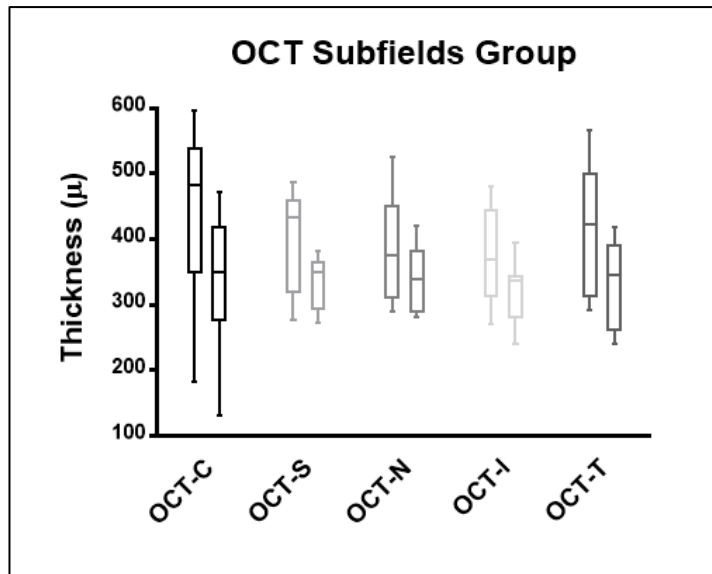


Figure 7.6. Macular thickness changes in the different internal subfields (Central, Superior, Nasal, Inferior and Temporal) in baseline and 3 months after treatment.

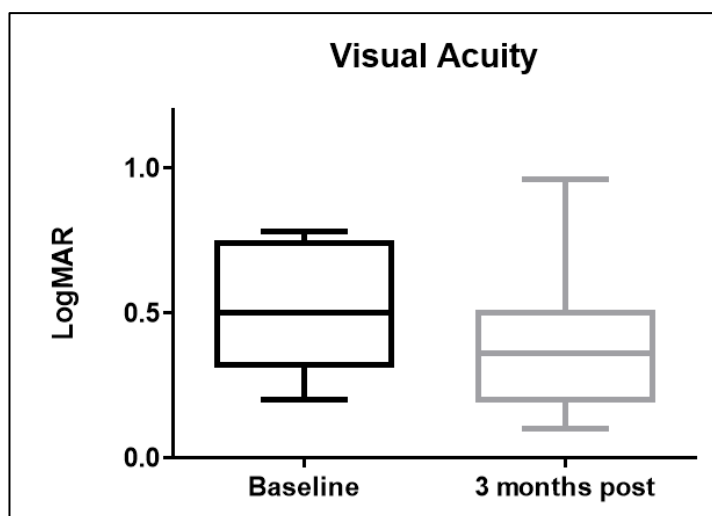


Figure 7.7. Whiskers box plot indicating variability of LogMAR visual acuity in baseline examinations and 3 months after treatment.

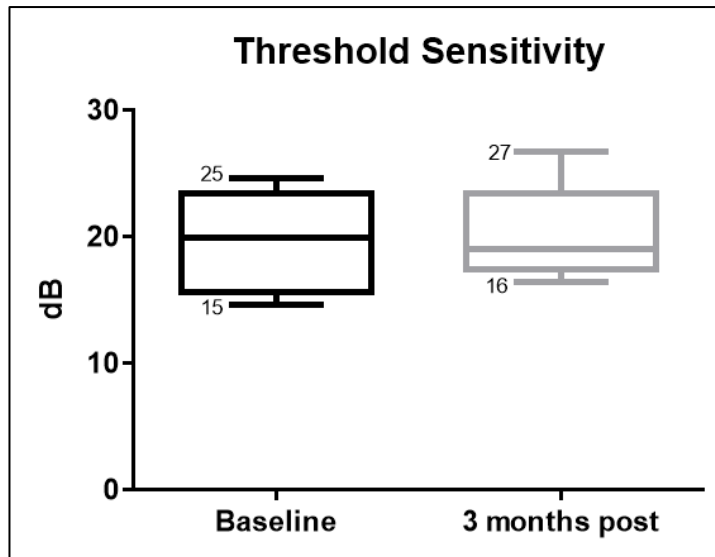


Figure 7.8. Whiskers box plot indicating variability of threshold sensitivity measured with microperimetry in baseline examinations and 3 months after treatment.

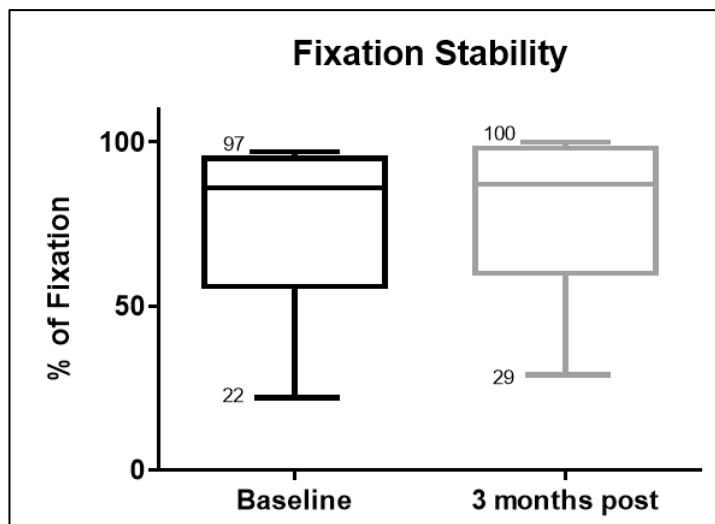


Figure 7.9. Whiskers box plot indicating variability of fixation stability in baseline examinations and 3 months after treatment.

Chapter 8. General Discussion

Diabetic retinopathy is an important cause of visual impairment and blindness amongst working-age groups in the developed world. As the world-wide incidence of diabetes mellitus (especially Type 2) increases, it is anticipated that its complications, particularly retinopathy, will become more prevalent in the future (Diamant et al., 2007). The incidence of DR correlates with poor glycaemic control and hyperlipidaemia (Ferris et al., 1996, Klein et al., 2002). DMO is responsible for significant visual impairment in diabetic patients (Klein et al., 1998, Bhagat et al., 2009). The primary cause of DMO is fluid leakage resulting from increased vascular permeability through contributory anatomical and biochemical changes including REC death or dysfunction, pericyte loss or dysfunction, thickened basement membrane, loss or dysfunction of retinal glial cells and loss/change of EC Glycocalyx (Miyamoto and Ogura, 1999, Ciulla et al., 2003, Mohr, 2004). The molecular changes have been reported to include increased reactive oxygen species (ROS), pro-inflammatory changes: advanced glycation end products (AGEs), ICAM-1, Complement 5-9 deposition and cytokines, which result in increased paracellular permeability, tight junction disruption, and increased transcellular permeability (Bhavsar, 2006, Bhavsar and Tornambe, 2006). Current treatments for DMO target reducing vascular leak in the macula once it has occurred, but do not attempt to treat the underlying pathology. These pharmacological treatments are aimed at antagonising vascular endothelial

growth factor (VEGF) or non-VEGF inflammatory pathways, and include intravitreal injections of anti-VEGFs (ranibizumab, aflibercept or bevacizumab) or steroids (fluocinolone, dexamethasone or triamcinolone) as single therapies (Joussen et al., 2004, Kaji et al., 2007, Johnson, 2009). The available evidence suggests that each individual treatment modality in DMO does not result in a completely dry macula in most cases. Further investigation of the mechanisms of these treatments on their own or in combination is therefore warranted.

Previous studies have reported alterations in different JAMs and TJ in the retina in diabetes and hyperglycaemia (Antonetti et al., 1998, Harhaj et al., 2006, Klaassen et al., 2009). It is also known that different capillary beds may respond differently to different stimulatory or inhibitory factors (Campochiaro, 2000). In addition, the expression and effects of JAMs are specific to cell type (Li et al., 2009). Our findings indicate that both REC and CEC express Claudin-5, occludin, JAM-A and C, and VE-Cadherin under NG conditions, although the expression of Claudin-5 and occludin is higher in REC, as shown by both our gene and protein expression evaluations. Similarly, HG seems to have a differential effect in altering the expression of the studied junctional molecules in REC and CEC, as shown by the significant reduction in Claudin-5, occludin and JAM-A gene and protein expression in REC but non-significant changes in CEC.

To the best of my knowledge, this is the first study that has compared junctional protein expression in human REC and CEC in NG and HG.

Differences in the TJ molecule expression between the REC and CEC may reflect the physiological roles of the two different vascular beds, as well as explain some of the differential effects of diabetes on the vasculature, i.e. why diabetic vascular changes affect the retinal compared to the choroidal vasculature. Interactions in angiogenesis and vascular permeability alterations are most likely mediated by changes in TJ molecules in diabetic retinopathy. These findings further confirm the phenotypic differences between REC and CEC in health and disease previously described (Aurrand-Lions et al., 2001b, Browning et al., 2005b, Stewart et al., 2011). Recently, different groups have started to explore the use of JAM blockade as potential therapeutic targets in different diseases. Naik et al (2003) reported that antibody blockage of JAM-A resulted in disruption of angiogenesis in an in vitro model. Goetsch et al (2013) have shown that monoclonal antibodies against JAM-A can interfere with tumour proliferation in experimental models. Lamagna et al (2005a) and Orlova et al (2006b) have shown, independently, that a JAM-C specific blocking monoclonal antibody reduced the angiogenic process in models of hypoxia-induced retinal neovascularisation. The finding of differential TJ expression between human REC and CEC could inform and have implications on the future development of clinical treatments of neovascular diseases in the eye. However, it is yet to be tested whether boosting JAM (particularly JAM-A) expression has any effects on reducing EC permeability in HG or diabetes.

The findings from this study are at variance with others that have shown increased VE-Cadherin expression with hyperglycaemia and diabetes

(Davidson et al., 2000, Navaratna et al., 2007). Klaassen et al (Klaassen et al., 2009) did not detect any gene expression of VE-Cadherin in control or streptozotocin rat retina. However, they described reduction in bovine endothelial cell expression of VE-Cadherin after 24 h VEGF exposure (Klaassen et al., 2009). It is possible that interactions between VE-Cadherin and the TJ molecules display some redundancy in intraocular EC as previously described by other investigators (Taddei et al., 2008).

It has been shown that efficacy in choroidal vascular disease may not necessarily imply similar efficacy in retinal vascular disease or vice versa (Stewart et al., 2011). The results reported in this investigation, however, do not address the effects of the different drugs on the other intraocular structures, or the penetrability of the different layers/bioavailability in the eye, or toxicity to these other layers. Currently available anti-VEGF agents used in the treatment of intraocular vascular disease may have different efficacy profiles related to the particular intraocular vascular bed and/or disease (Stewart et al., 2011).

Our findings have shown that there is significant reduction in REC cell number by 25mM glucose. This reduction is comparable to that shown by our group in human Tenon's capsule fibroblasts from diabetics cultured in normal glucose medium and high glucose (Browning et al., 2005a), and in other EC elsewhere in the body (Eriksson et al., 2012, Calles-Escandon and Cipolla, 2001, Avogaro et al., 2008, Rask-Madsen and King, 2007). As such, diabetes or high glucose states result in a general reduction in cell

proliferation. Potentially, reduced endothelial cell proliferation and increased apoptosis in the early stages of diabetic retinopathy prevent the appropriate response to subsequent ischaemia and promote the later paradoxical abhorrent neovascularisation (Curtis et al., 2009, Wright et al., 2011). This study has found that *in vitro* treatment of REC with DEX can reverse REC loss.

The findings from this project have shown for the first time that high glucose levels have independent effects on REC proliferation and apoptosis. Whilst the reduction of EC proliferation is noted at 25mM and above, increased apoptosis seems to be significantly altered only at the higher concentrations of high glucose.

PDR is due to increased VEGF levels irrespective of the diabetic control, once the VEGF levels are high enough PDR will occur. A vicious cycle results as increased apoptosis leads to more capillary closure with more ischaemia and more VEGF production. Blood glucose levels determine vitreous glucose levels (Osuna et al, 2001; Krizova L et al, 2011). This implies that the response to clinical hyperglycaemia may be dependent on the different levels of high glucose, and that REC apoptosis is more important at higher degrees of hyperglycaemia compared to lower levels. The overall glucose control may determine which of these processes is more important. This is irrespective of what other effects fluctuations of glucose levels may have on these cells.

VEGF-A is thought to be the main factor responsible for increased retinal vascular permeability as upregulation of it results in phosphorylation of tight junction proteins and their disassembly (Harhaj et al., 2006, Antonetti et al., 1998). However, it has been suggested that VEGF modulation may not be an important mechanism of corticosteroid inhibition of angiogenesis or permeability (Nehme and Edelman, 2008). Our study suggests that DEX modulates REC permeability, at least in part, through a VEGF-independent pathway. Exposure of REC to DEX for 24 or 72h results in significant reductions in the potential permeability of these cells, associated with changes in REC TJ molecules. Furthermore, the effect of DEX on REC permeability is noticeable observed even at fairly low doses. This finding indicates that the low required therapeutic dose of DEX could have clinical implications for the development and delivery of anti-inflammatory therapies used to treat retinal diseases.

This study also found an increase in JAM-C, which has been reported to induce permeability (Li et al., 2009), in high glucose media. At the mRNA level, expression of claudin-5 and occludin were significantly increased by DEX in high glucose, compared to high glucose alone, in order to restore their expression to levels similar to those in normal glucose. This change, associated with an increase in VE-Cadherin expression, and no significant change in JAM-A or C expression indicates that DEX may restore permeability through specific pathways controlling TJ molecules.

The findings from this study also indicate that VEGF-A levels in REC are not altered directly by high glucose, despite increased expression of HIF-1a levels. HIF-1a must, therefore, be acting through a non-transcriptional route. Poulaki et al (2002) (Poulaki et al., 2002) have confirmed that acute intensive insulin therapy produces a transient worsening of diabetic blood-retinal barrier breakdown via an HIF-1alpha-mediated increase in retinal VEGF expression. However, it is suggested that hyperglycaemia alone did not influence HIF-1a protein in the rat retina or retinal pigment epithelial (RPE) cells in culture, although VEGF was upregulated (Poulaki et al., 2002). Our findings indicate that there is, as such, a 'disconnect' between elevated HIF-1a and normal levels of VEGF-A expression in REC cultured in high glucose levels. An alternative explanation may be that there might be an isoform of VEGF that was missed in our study as the antibody was against a more conserved part of the VEGF molecule and may vary across different isoforms and similarly the primer for PCR. Catrina et al (Catrina et al., 2004) have suggested that high glucose decreased hypoxia-induced stabilisation and function of HIF-1a in human dermal fibroblasts and human dermal microvascular endothelial cells in culture, although no effect was noted in normoxia. Interestingly, Catrina et al (Catrina et al., 2004) observed a decreased transcriptional activation of HIF-1a as measured by reporter gene assays. The effects on HIF-1a in different ECs may therefore be tissue dependent and different.

Anti-VEGF therapy with ranibizumab significantly reduced the increased permeability of REC induced by high glucose concentrations. However, the

reversal was incomplete, and did not return to levels identical to that in normal (5mM glucose) medium. The reduced permeability with ranibizumab was associated with increase in occludin, and claudin-5 levels compared to high glucose alone. Changes in JAM-A and VE-Cadherin were not significant.

The combination therapy of steroids and anti-VEGF may have complementary and additive effects on retinal vascular permeability. This follows from the evidence of Nehme and Edelman (Nehme and Edelman, 2008) on the effect of DEX on REC and pericyte permeability, and reports from Felinski (Felinski and Antonetti, 2005a), in concert with the anti-VEGF clinical trials. Furthermore, VE-Cadherin levels were more significantly altered by combination of DEX and ranibizumab than either agent on its own. This is in addition to the alterations of JAM-A and occludin induced by ranibizumab and DEX independently. The levels of JAM-C are also decreased by DEX and ranibizumab in high glucose concentrations, although the levels do not return to that in 5mM glucose.

It has been frequently demonstrated that corticosteroids have significant effects on RECs (Felinski and Antonetti, 2005b, Felinski et al., 2008). It has also been suggested that steroids may act by both suppressing inflammation and by directly affecting the ECs by regulating phosphorylation, organisation, and content of TJ proteins (Felinski and Antonetti, 2005a). Felinski et al (Felinski et al., 2008) have reported that glucocorticoid treatment of primary RECs increases content of the TJ proteins occludin and claudin-5, in parallel

with an increase in barrier properties of endothelial monolayers, therefore restoring endothelial para-cellular permeability.

The results from our laboratory show that a combination of DEX and ranibizumab reduced REC permeability more than either agent alone *in vitro* (Saker et al., 2014a). Clinically, it is possible that an initial combination of DEX and ranibizumab will allow a faster visual acuity rise to the plateau than achieved with ranibizumab on its own.

Over the past few years we have been frequently struck by the apparent disparity between the appearance of DMO and visual disturbances or symptoms in patients with DMO. MP findings point to the importance of factors not merely related to the thickness of the retina as prognosticators of macular function. Duration of oedema and alterations of retinal neural elements may be among them. The parameter “duration ” may have a relevant impact on the survival and/or functional reserve of macular cells undergoing the mechanical and toxic stress induced by oedema (Gardner et al., 2002).

Okada et al (2006) have postulated that mean retinal sensitivities measured with MP are significantly correlated to visual acuity and foveal thickness. However, the results from our analysis suggest that fixation stability may be a new important parameter to describe functional macular function.

Our findings could also contribute indirectly to the clarification of why DMO patients with similar oedema characteristics and similar diabetic control have different prognoses after therapeutic intervention.

8.1 Potential Future Directions

The further steps in this research theme would be to elucidate the detailed mechanisms involved in reduced retinal endothelial cell proliferation, and increased apoptosis. This would explain why the retinal vasculature seems to show heterogeneous responses in the different diabetic patients, and the conflicting reports on EC growth in high glucose in the literature.

If the role of transcellular permeability in HG, and interactions with the paracellular permeability were to be evaluated, it would open potential therapeutic avenues in modulating disease progression in preclinical or early diabetic retinopathy.

If the aspect of the project were to be expanded, it would be possible to understand the molecular redundancy in intraocular EC. This could be reflected by such interactions between VE-Cadherin and the TJ molecules as previously described by other investigators. (Taddei et al., 2008) described the upregulation of claudin-5 to be mediated indirectly by VE-Cadherin in murine EC lines in vitro.

It has been shown that DEX may offer protection against REC loss in high glucose conditions. However, the absence of any significant change in JAM-A or C expression with DEX in combination with 25 mM glucose (compared to 25 mM glucose alone) indicates that DEX may restore permeability through specific pathways controlling TJ molecules. Accordingly, other treatments that modify TJ components other than claudin-5 and occludin, in

combination with DEX, may produce additive effects in restoring EC permeability to the normal or near normal states.

Furthermore, the roles of inflammation in retinal and choroidal capillary permeability, upregulation of adhesion molecules which have a role in the progression of DR (Mohr, 2004), or other cell types e.g. pericytes in diabetic eye disease need further study. It has been shown that a combination of DEX/Ranibizumab reduced REC permeability more than either agent alone *in vitro* (Saker et al., 2014a). Further elucidation of the cellular mechanisms underlying the effects of different pharmacologic agents and their combination's interactions are also required.

Further investigation of the analysis of the oedematous macula would be interesting not only in DMO but also in other retinal disorders, and would incorporate a functional measure that may potentially supplement the predictive value of OCT and visual acuity. If MP technology were to be used in DMO, it would be possible to predict the functional outcome of treated DMO after interventions that seem equally effective in restoring normal foveal thickness. Prospective studies of larger samples are warranted to test the ability of MP technology to make a point-by-point prediction of functional outcome after various forms of intervention. We also need a long-term prospective study of diabetic eyes without oedema at baseline to document the morphologic and functional natural history of DMO.

8.2 Final Conclusions

The objectives set for this PhD were:

1. To investigate *in vitro* the permeability of primary human retinal endothelial cells (REC) and human choroidal endothelial cells (CEC) under normal glucose (NG) and high glucose (HG) conditions.

This was achieved in the initial experimental work undertaken; high glucose conditions significantly increased the permeability in both retinal and choroidal endothelial cells monolayers although the increase was higher in retinal endothelial cells. This may indicate differences in junctional molecule regulation of permeability in retinal compared to choroidal endothelial cells.

2. To investigate expression of key tight junction (TJ) molecules in REC and CEC in NG and HG. Human REC and CEC show different expression profiles for different TJ molecules in NG, and support the role of selected TJ molecules including occludin and claudin-5, JAM-A and C constituting a permeability barrier in intraocular micro-vascular EC, and their crucial role in the pathogenesis of diabetic eye disease. This also highlights that the increase in RVP is likely caused by alterations (could be increase or decrease, depending on the

particular TJ) in selective tight junction protein expression, leading to increased para-cellular permeability.

3. To investigate *in vitro* the proliferation and apoptosis of primary human REC under HG conditions. Previous studies have reported conflicting results on the effects of diabetes on EC proliferation. It was investigated whether different levels of high glucose directly affected retinal endothelial cell (REC) proliferation and apoptosis *in vitro*, and whether REC HIF-1 α and downstream targets including VEGF-A may be altered by high glucose levels. It has been shown that VEGF-A levels are not altered by high glucose in the REC, despite increased expression of HIF-1 α levels. HIF-1 α must therefore be acting through a non-transcriptional route.
4. To identify the effects of HG, and vascular endothelial growth factor (VEGF) in isolation or combination on EC permeability *in vitro*, and compare the amelioration by anti-VEGF therapy (ranibizumab [Ranibizumab, Novartis]) and DEX on the different adhesion and TJ molecules. The relative findings confirmed that REC exposure to high glucose has resulted in significant reduction in the gene expression of molecules important for TJ formation, including JAM-A, claudin-5, and occludin. The further investigation of JAM-C, which has been reported to induce permeability, revealed non-significant increase in our study. However, the absence of any significant change in JAM-A

or C expression with DEX in 25 mM glucose (compared to 25 mM glucose alone) indicates that DEX may restore permeability through specific pathways controlling TJ molecules. At the mRNA level, expression of claudin-5 and occludin were significantly increased by DEX in high glucose to levels that restored their expression to levels similar to those in normal glucose medium. Ranibizumab in high glucose medium increased the expression of claudin-5 and occludin. A combination of ranibizumab and DEX in high glucose medium increased claudin-5 and occludin levels, but in addition resulted in significant increases in JAM-A and VE-Cadherin levels compared to high glucose alone. No increase was noted in JAM-A or VE-Cadherin with either DEX or ranibizumab alone.

5. To evaluate the ability of MAIA MP in detecting early changes in retinal sensitivity in eyes with DMO that are receiving intravitreal anti-VEGF therapies or steroids, and correlate them with morphological changes on OCT and visual acuity (LogMAR) at different time points. In this regard, macular sensitivity and the fixation pattern in diabetic macular oedema, by using MAIA MP were quantified, and correlated with macular thickness determined by optical coherence tomography (OCT). This has also highlighted that patients with good visual acuity and intact central cone function demonstrate good fixation stability (higher than 90%), related to good foveal vision. More importantly, it has been concluded that fixation stability may be a new important

parameter to describe functional macular function. Although morphologic recovery on OCT may occur with pharmacologic therapies for DMO, functional recovery may be dependent on how much damage has occurred to the intraretinal circuit, which may be dependent on the duration of DMO. Measurement of visual function with MAIA may be considered as an important tool to better diagnose retinal pathologies as part of multimodality imaging, responding to the need to combine morpho-functional information, and combining different images from different measurement technologies

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Appendices - Publications



The effect of hyperglycaemia on permeability and the expression of junctional complex molecules in human retinal and choroidal endothelial cells

S. Saker¹, E.A. Stewart¹, A.C. Browning, C.L. Allen, W.M. Amoaku*

University of Nottingham, Academic Ophthalmology, Division of Clinical Neuroscience, B Floor, Eye and ENT Building, Queen's Medical Centre, Nottingham NG7 2UH, UK

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ABSTRACT

Diabetic retinopathy is the leading cause of preventable blindness in the working population and its prevalence continues to increase as the worldwide prevalence of diabetes grows. Diabetic choroidopathy is less well studied and occurs in the late stages of diabetic eye disease. The main cause of visual loss in diabetic eye disease is diabetic macular oedema caused by an increase in microvascular endothelial permeability. Endothelial cell permeability is influenced by multiple factors which have not been fully elucidated, particularly in human models. In addition, the gene and protein expression between retinal and choroidal endothelial cells, even in humans, has been shown to be heterogeneous. The aim of this project was to determine, *in vitro*, the effect of high glucose (25 mM) on human paracellular permeability in retinal and choroidal endothelial cells. The expression of selected tight junction molecules (Occludin, Claudin-5, JAM-A and JAM-C) and adheren junction (VE-Cadherin) molecules was also compared between retinal and choroidal endothelial cells and with high glucose. High glucose conditions significantly increased the permeability in both retinal and choroidal endothelial cells monolayers although the increase was higher in retinal endothelial cells. Under normal glucose culture conditions microarray analysis determined that occludin and claudin-5 gene expression was higher in retinal endothelial cells than choroidal endothelial cells, and western blotting indicated that claudin-5 protein expression was also higher in retinal endothelial cells whilst JAM-A, and C and VE-Cadherin levels were similar. In retinal endothelial cells exposed to high glucose claudin-5, occludin and JAM-A was found to be reduced, whereas the expression of VE-Cadherin and JAM-C was unchanged when evaluated with western blotting, immunofluorescence and qPCR. None of the proteins were significantly decreased by high glucose in choroidal endothelial cells. The increase in retinal endothelial cell permeability is likely caused by a decrease in selective tight junction protein expression, leading to increased paracellular permeability. This may indicate differences in junctional molecule regulation of permeability in retinal compared to choroidal endothelial cells.

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Abbreviations: JAM, junctional adhesion molecule; DR, diabetic retinopathy; EC, endothelial cell; BRB, blood retinal barrier; TJ, tight junctions; AJ, adherens junctions; REC, retinal endothelial cells; CEC, choroidal endothelial cells; CNV, choroidal neovascularisation; RPE, retinal pigment epithelium.

* Corresponding author. Tel.: +44 01159709796, +44 07940358923 (mobile); fax: +44 01159709963.

E-mail addresses: msxss1@nottingham.ac.uk (S. Saker), Elizabeth.stewart@nottingham.ac.uk (E.A. Stewart), Andrew.Browning@nuth.nhs.uk (A.C. Browning), Claire.allen@nottingham.ac.uk (C.L. Allen), wma@nottingham.ac.uk (W.M. Amoaku).

¹ Authors contributed equally to this publication.

1. Introduction

Diabetic retinopathy (DR) has been extensively studied over the years, and its incidence correlates with poor glycaemic control and hyperlipidaemia (Ferris et al., 1996; Klein et al., 2002). Diabetic choroidopathy is a less well studied entity, and is thought to occur in the advanced stages of diabetic eye disease (Bischoff and Flower, 1985; Fryczkowski et al., 1989; Hidayat and Fine, 1985; Ishibashi et al., 1999). Diabetes and hyperglycaemia have obvious effects on intraocular vascular endothelial cell (EC) permeability, adhesion to leukocytes, as well as proliferation (Ciulla et al., 2003; Miyamoto and Ogura, 1999; Mohr, 2004). These alterations result in

increased vascular leakage (increased permeability), vascular occlusions, ischaemia, and angiogenesis (Bhavsar, 2006; Bhavsar and Tornambe, 2006).

Clinical evidence indicates that diabetic macular oedema results from breakdown of the inner blood retinal barrier (BRB) (Vitale et al., 1995) which is maintained by EC tight junction (TJ) complexes (Mitic and Anderson, 1998). TJ proteins include occludins, claudins, and junctional adhesion molecules (JAMs), transmembrane molecules which interact with intracellular molecules including zonula occludens (ZO) and cingulin (Mitic and Anderson, 1998). Adherens junctions (AJ) are mediated by VE-Cadherin which promotes calcium dependent homophilic cell to cell contacts, and interact intracellularly with actin through catenins. Dejana et al. (2001) have reported that the principal TJ proteins found in retinal endothelial cells (REC) are occludin and claudin-5. Phosphorylation of occludin and ZO1 contribute to the dysregulation of EC paracellular permeability (Abbott et al., 2006; Thorin and Shreeve, 1998).

JAMs also signal to cytoskeleton associated proteins, as well as recruit cellular polarity proteins. As opposed to JAM-A, JAM-C increases paracellular EC permeability (Aurrand-Lions et al., 2001b; Orlova et al., 2006). It is suggested that the preferential expression of different junctional molecules in different vascular beds may explain their specific physiologic functions (Aurrand-Lions et al., 2001b), for example the high expression of JAM-A in the blood-brain-barrier (Orlova et al., 2006), the relative high expression of JAM B in high endothelial venules (Aurrand-Lions et al., 2001a; Palmeri et al., 2000), and JAM-A and C in the liver compared to its minimal expression of JAM-B (Geraud et al., 2012; Palmeri et al., 2000). Molecular crosstalk, substitution, or redundancy in the JAM system may also play a part in different tissues where all the different JAMs are expressed (Weber et al., 2007). JAM-C expression in retinal endothelial cells (REC) (Daniele et al., 2007; Klaassen et al., 2009) and the retinal pigment epithelium (RPE) (Economopoulou et al., 2009) has been described. It is possible that preferential expression of different adhesion or TJ molecules (occludin, claudins, JAM-A, B and C) as well as AJ molecules (VE-Cadherin) and associated integrins in different vascular beds may contribute to their relative susceptibility to diabetes and hyperglycaemia. Different JAMs are reportedly altered in the retina in diabetes (Antonetti et al., 1998; Harhaj et al., 2006; Klaassen et al., 2009). It is also known that different capillary beds may respond differently to different stimulatory or inhibitory factors (Aird, 2003). In addition, the effects of JAMs are specific to cell type (Li et al., 2009). This is supported by the observation that although diabetic retinopathy is a microvascular disease, microangiopathy does not seem to play a major pathogenic role in the cerebral complications of diabetes, and the blood-brain barrier is not as susceptible to hyperglycaemia as the retinal microvasculature (Mooradian, 1997).

The exact roles of these different molecules in diabetic eye disease are not fully understood. Although there are studies describing the adhesion and tight junction molecules in different intraocular vasculature (Daniele et al., 2007; Klaassen et al., 2009; Lamagna et al., 2005; McLeod et al., 1995; Penfold et al., 2002) and the RPE (Economopoulou et al., 2009) as well as experimental choroidal neovascularisation (CNV) (Hou et al., 2012), we are unaware of any studies comparing the expression of these important junctional molecules in human choroidal and retinal vascular EC and their relative expression in retinal and choroidal vasculature in diabetes. Furthermore, the role of JAM-C, in particular, in the retinal and choroidal EC permeability in diabetes (hyperglycaemia) requires elucidation. We have investigated the permeability of cell monolayers of human REC and choroidal endothelial cells (CEC) in normal glucose (NG) and high glucose conditions (HG) *in vitro*. We

used microarray analysis to determine the relative gene expression for selected junctional molecules in REC and CEC in NG, as well as compared the protein expression profiles of selective junctional proteins in REC and CEC in HG with western blots and qPCR.

2. Materials and methods

2.1. Endothelial cell culture

REC and CEC were isolated and cultured separately for each donor as previously described (Stewart et al., 2011). Paired eyes from each donor were used to make one primary REC line and one primary CEC line.

2.2. Microarray analysis

Microarray analysis of cellular mRNAs was performed as previously described (Browning et al., 2012). Affymetrix CEL files were imported into GeneSpring GX 11.0.1 and processed with the MAS5 algorithm. Data was then normalised with GC-RMA to provide expression values. To identify differentially expressed genes between cell groups, ANOVA was performed with Tukey-HSD post-hoc testing and Benjamini–Hochberg false discovery rate control. A difference in expression between probe sets with a corrected *p*-value of <0.05 and a fold change of greater than 2 in all samples from a particular tissue bed were considered to be statistically significant.

2.3. Trans-endothelial permeability assay

To assess transendothelial permeability of REC and CEC, cells were initially cultured on a 35 mm tissue culture dish coated for >2 h with 5 µg/mL fibronectin in EGM2-MV medium with hydrocortisone omitted and containing 5 mM glucose, classed as NG (Lonza, Tewkesbury, UK). Confluent EC were transferred into 6 wells of a 6 well cell culture cluster coated with fibronectin as above and maintained in NG medium. Once 80–90% confluent EC were removed and allowed to adhere to 6 × 0.4 µm pore size transwell inserts (Becton Dickinson, USA) in 12 well format. EC on transwell inserts were cultured to confluence in NG medium, before being washed once with sterile phosphate-buffered saline (PBS) with antibiotics and the culture medium was then changed to either NG medium, or EGM2-MV with added glucose to give a final concentration of 25 mM glucose (HG) for 72 h. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and the medium was changed every day throughout the 3 days. The permeability was assessed by the diffusion of Evan's blue-labelled albumin. Briefly, confluent inserts were transferred to fresh 12-well cell culture clusters containing 2 mL Hank's balanced salt solution (HBSS) in the basolateral chambers. In the apical compartments, culture medium was replaced by 500 µL of HBSS containing Evan's blue-labelled albumin (165 µg/mL; molecular weight, 67 kDa). Both luminal and abluminal sample absorbance was measured at 650 nm after 30, 60 and 120 min, using a microplate reader (THERMOMax). The coefficient of permeability was calculated as µL of luminal compartment volume from which the tracer is completely cleared. The experiments were repeated on 5 independent occasions, with 5 different unmatched donor REC and CEC samples in NG and HG conditions.

2.4. Western blotting

Confluent cells were allowed to attach in standard growth medium for 72 h and then the culture medium was changed to either NG or HG conditions (as in Section 2.2), for three days. Cultures

were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ and the medium was changed every day. Cell lysates were collected in 1× LDS Sample Buffer (Life Technologies) and western blotting performed as previously described (Stewart et al., 2011), primary antibodies Claudin-5, JAM-A, JAM-C, Occludin and VE-Cadherin (Abcam, Cambridge, UK) were used at the recommended dilutions. The experiments were repeated with samples from at least 5 donors. Protein expression was quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

2.5. Immunofluorescence

REC and CEC were cultured on 8 well glass chamber slides coated for >2 h with 5 µg/mL fibronectin in NG medium, once 80% confluent EC were washed once with sterile PBS with antibiotics and the culture medium was then changed to either NG medium, or HG for 72 h. Cells were washed with PBS before cells were fixed with 4% paraformaldehyde and permeabilised with 10% Triton-X-100 before blocking with 3% (w/v) bovine serum albumin (BSA) in PBS. Cells were stained with primary antibodies as used for western blotting, overnight, at 4 °C. Primary antibodies were visualised using the appropriate secondary antibody conjugated to tetramethyl rhodamine iso-thiocyanate (TRITC) at 1:400 for 1 h at room temperature. Slides were counterstained with 4',6-Diamidino-2-phenylindole (DAPI; 5 µg/mL; Santa Cruz, Germany) and FITC conjugated phalloidin before being examined on a fluorescence microscope (Olympus BX51) and imaged using CellF software (Olympus, UK). Each experiment was performed on 2 donors and comparative fluorescence was detected using the same settings on the imaging software.

2.6. Quantitative PCR

Quantitative real-time PCR was performed as previously described (Wei et al., 2013). In brief, total RNA was extracted and purified from REC and CEC in NG or HG conditions using an RNeasy Mini RNA kit (Qiagen) according to the manufacturer's protocol. RNA was quantified by using a Nanodrop and the quality was determined by agarose gel electrophoresis. RNA samples were further processed for reverse transcription using the QuantiTect RT kit (Qiagen). RT PCR was carried out with Roche Lightcycler 480. All samples were diluted 1:5 with nuclease-free water. A single 20 µL reaction mixture was prepared to each PCR optical tube, with 10 µL of 10 × Taqman gene expression master mix (Applied Biosystems, Foster City, CA), 1 µL of TaqMan® Gene expression assay (Applied Biosystems), 5 µL of diluted cDNA and 4 µL of nuclease-free water, were performed in optical tubes with optical caps on. Negative controls (non-template control and negative reverse transcriptase control) and positive controls (Universal Human Reference RNA) were also run on the PCR. Amplification conditions were set at 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. Each reaction was run in triplicate in a Stratagene MX-3005p Quantitative PCR instrument. A total of 12 samples were analysed from 3 different pairs of donor eyes in each treatment group.

2.7. Statistical analyses

All experiments were repeated at least three times with regards to gene expression profiling, and five times with regard to protein expression and permeability. In each experiment, data were generated from multiple replicates. Data were checked for normality before conducting the appropriate statistical test. Independent samples t-test was used to compare data. For all analyses, *p* values below 0.05 were considered indicative of significant

difference. Standard deviation was used to describe variability; results are represented as mean ± standard deviation.

3. Results

3.1. Assessment of the effect of high glucose on ocular microvascular trans-endothelial permeability

When the comparative paracellular permeability of REC and CEC was measured, the mean permeability of REC in NG was 0.063 ± 0.002 (Fig. 1A); when HG conditions were induced the mean permeability significantly increased to 0.076 ± 0.007 ($p = 0.008$). For CEC permeability significantly increased from 0.0315 ± 0.0006 in NG medium to 0.0328 ± 0.0005 ($p = 0.017$) in HG medium (Fig. 1B). The relative increase in permeability in HG (compared to NG) was 20.6% for REC compared to 3.9% for CEC.

3.2. Comparative protein expression profiling of selective tight junction and adheren junction proteins in REC and CEC

Microarray analysis of mRNA from cultured REC and CEC derived from 3 matched donors demonstrated that REC expressed higher levels of probe sets for occludin (5.3 fold), claudin-5 (4.8 fold) compared with matched CEC. There was no significant difference in the expression of probe sets for JAM-A, JAM-C and VE-Cadherin between matched REC and CEC. This was also reflected when the protein levels were assessed using western blotting which showed that CEC expressed lower levels of claudin-5 than REC ($p = 0.0172$) and occludin (ns, $p = 0.1043$), whereas the levels of JAM-A, JAM-C and VE-Cadherin were similar or slightly higher in CEC (Fig. 2).

3.3. Expression of tight junction and adheren junction proteins under high glucose conditions

When REC were exposed to HG conditions for 72 h immunofluorescent staining indicated that the cellular expression of claudin-5, occludin and JAM-A was visibly reduced (Fig. 3). Western blotting showed a statistically significant reduction in the expression of claudin-5 ($p = 0.0423$), occludin ($p = 0.0155$) and JAM-A ($p = 0.0306$) with HG (Fig. 4). The expression of VE-Cadherin and JAM-C were unchanged in REC under HG conditions. None of the junctional proteins in CEC were significantly decreased by HG (Fig. 4); however, JAM-C expression appeared to be consistently increased, although this increase was not statistically significant (ns, $p = 0.0968$).

3.4. Gene expression changes in tight junction and adheren junction proteins with high glucose

When REC were exposed to HG conditions for 72 h there was a significant reduction in gene expression of claudin-5 ($p = 0.007$), occludin ($p = 0.031$) and JAM-A ($p = 0.037$; Fig. 5). The expression of VE-Cadherin and JAM-C were unchanged in REC under HG conditions. None of the junctional proteins in CEC were significantly decreased by HG (Fig. 5); however, occludin and JAM-A showed a (non-significant) trend towards decreasing gene expression (Fig. 5).

4. Discussion

Clinical evidence indicates that diabetic macular oedema results from breakdown of the inner BRB (Vitale et al., 1995), and its incidence correlates with poor glycaemic control and hyperlipidaemia (Ferris et al., 1996; Klein et al., 2002). This study confirms that HG results in significant increase in paracellular

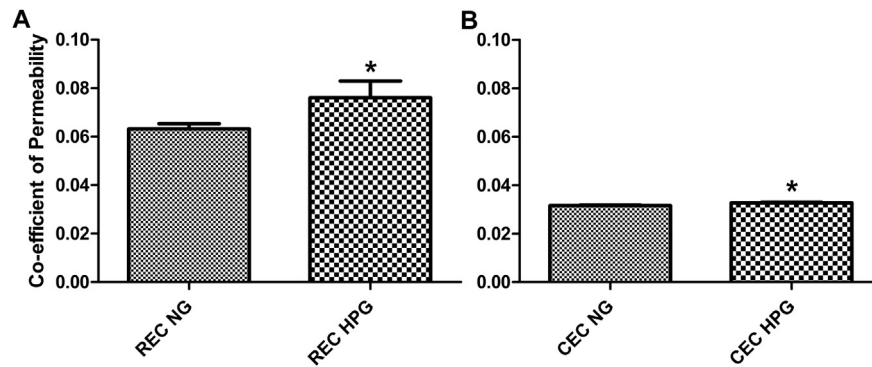


Fig. 1. REC and CEC permeability in response to high glucose. REC and CEC were exposed to HG (25 mM glucose) for 72 h then the trans-endothelial permeability assessed by measuring passage of Evan's blue albumin and calculating co-efficient of permeability, 5 different unmatched donor REC and CEC samples in NG and HG conditions were assessed, with three duplicates for each reading point (** $p < 0.01$, * $p < 0.05$).

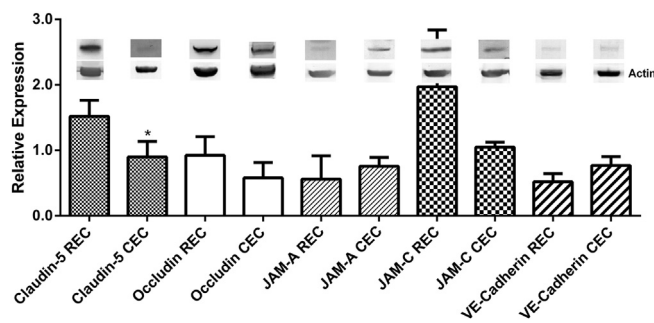


Fig. 2. Differential endothelial protein expression profiling of junctional molecules. Human REC and CEC lysates were collected and the expression of junctional proteins assessed using western blotting, expression in at least 5 unmatched donor REC and CEC samples was quantified using ImageJ relative to actin (lower band; ** $p < 0.01$, * $p < 0.05$).

permeability of REC *in vitro*. There is also a notable increase in permeability of CEC; however, the relative increase in permeability is higher in REC than CEC when exposed to the same degree of HG. This would support the clinical observation that vascular leakage

due to diabetes affects the retinal vasculature more than that in the choroid (Vitale et al., 1995).

Previous studies have reported alterations in different JAMs and TJ in the retina in diabetes and hyperglycaemia (Antonetti et al., 1998; Harhaj et al., 2006; Klaassen et al., 2009). It is also known that different capillary beds may respond differently to different stimulatory or inhibitory factors (Campochiaro, 2000). In addition, the effects of JAMs are specific to cell type (Li et al., 2009). Our findings indicate that both REC and CEC express Claudin-5, occludin, JAM-A and C, and VE-Cadherin under NG conditions, although the expression of Claudin-5 and occludin is higher in REC, as shown by both our gene and protein expression evaluations. Similarly, HG seems to have a differential effect in altering the expression of studied junctional molecules in REC and CEC, as shown by the significant reduction in Claudin-5, occludin and JAM-A gene and protein expression in REC but non-significant changes in CEC.

To our knowledge, this is the first study comparing junctional protein expression in human REC and CEC in NG and HG. Differences in the TJ molecule expression between the REC and CEC may reflect the physiological roles of the two different vascular beds, as well as explain some of the differential effects of diabetes on the

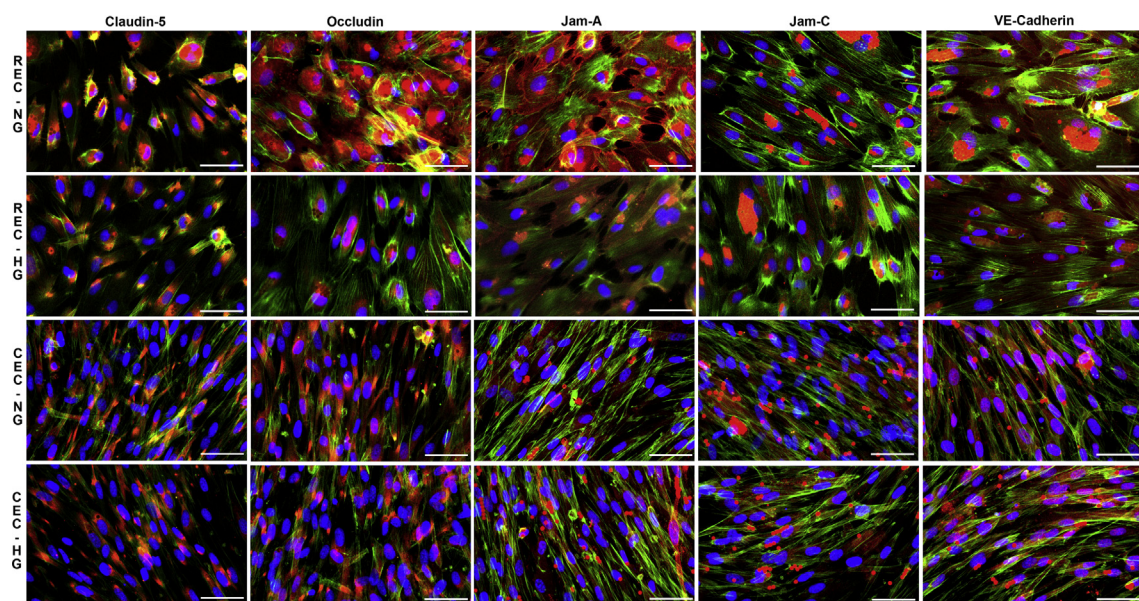


Fig. 3. Expression and localisation of junctional molecules with high glucose. Cultured REC and CEC were exposed to NG (5 mM) HG (25 mM) for 72 h then immunofluorescently stained with antibodies to junctional molecules (red) and counter stained with phalloidin (actin, green) and DAPI (nuclei, blue). In REC-HG claudin-5, occludin and JAM-A appeared to be visibly reduced when images were captured with consistent settings. Other junctional adhesion molecules and HG in CEC did not show a visible difference. Scale is 50µm.

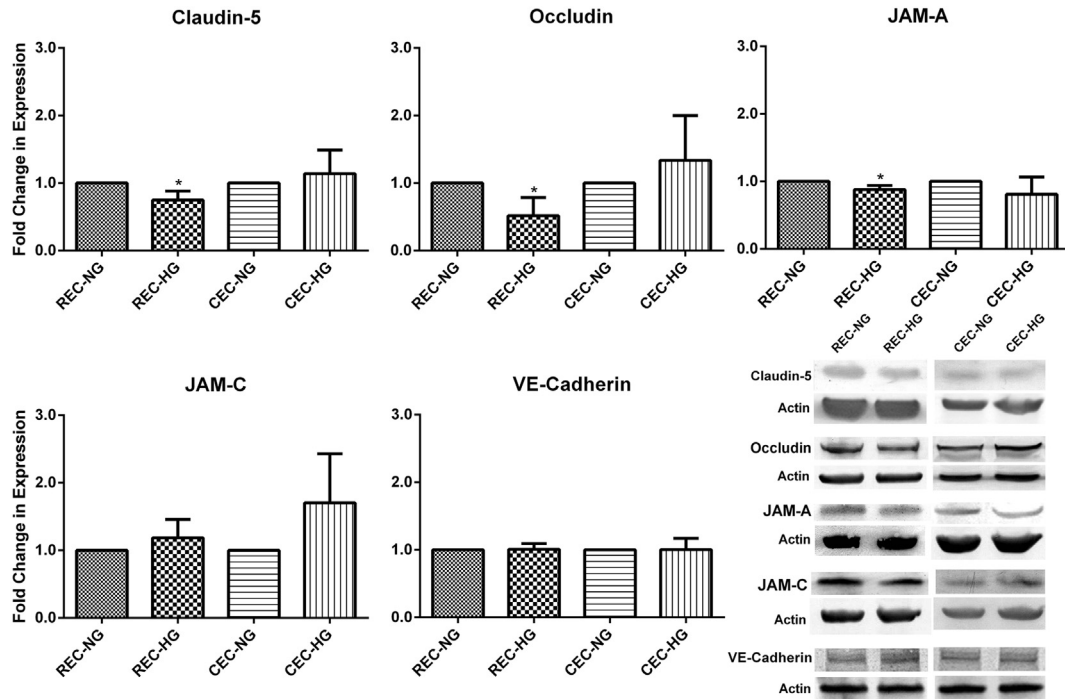


Fig. 4. Comparative protein expression profiling of different junctional molecules with high glucose. REC and CEC were exposed to HG (25 mM glucose) for 72 h then proteins collected and the expression of junctional proteins assessed using western blotting quantified with ImageJ and normalised against actin and matched NG samples. Expression changes in 5 unmatched donor REC and CEC samples were determined (** $p < 0.01$, * $p < 0.05$).

vasculature, i.e. why diabetic vascular changes affect the retinal compared to the choroidal vasculature. Interactions in angiogenesis and vascular permeability alterations are most likely mediated by changes in TJ molecules in diabetic retinopathy. Our finding further confirms the phenotypic difference between REC and CEC in health and disease previously described (Aurrand-Lions et al., 2001b; Browning et al., 2005, 2012; Stewart et al., 2011).

Recently, different groups have started to explore the use of JAM blockade as potential therapeutic targets in different diseases. Naik et al. (2003) reported that antibody blockage of JAM-A resulted in disruption of angiogenesis in an *in vitro* model. Goetsch et al. (2013) have shown that monoclonal antibodies against JAM-A can interfere with tumour proliferation in experimental models. Lamagna et al. (2005) and Orlova et al. (2006) independently have shown

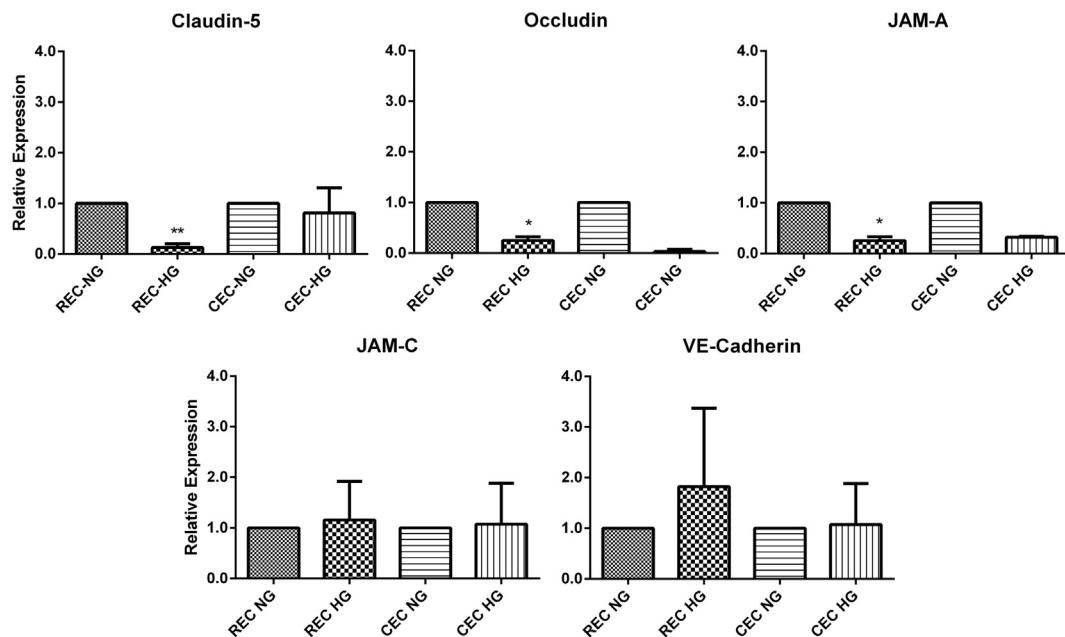


Fig. 5. Comparative gene expression profiling of different junctional molecules with high glucose. REC and CEC were exposed to HG (25 mM glucose) for 72 h then RNA extracted and the mRNA expression of junctional proteins assessed using qPCR. Expression changes in 3 replicates of 3 different unmatched donor REC and CEC samples were determined (** $p < 0.01$, * $p < 0.05$).

that a JAM-C specific blocking monoclonal antibody reduced the angiogenic process in models of hypoxia-induced retinal neovascularisation. Furthermore, JAM-C blockade inhibits both tumour growth and angiogenesis (Orlova et al., 2006). Hou et al. (2012) have reported upregulation of JAM-C in experimental CNV, and that JAM-C antagonists suppressed CNV and decreased retinal blood vessel leakage in their model. Our finding of differential TJ expression between human REC and CEC could inform and have implications on the future development of these clinical treatments of neovascular diseases in the eye. However, it is yet to be tested whether boosting JAM (particularly JAM-A) expression has any effects on reducing EC permeability in HG or diabetes.

Our study is at variance with others that have shown increased VE-Cadherin expression with hyperglycaemia and diabetes (Davidson et al., 2000; Navaratna et al., 2007). Klaassen et al. (2009) did not detect any gene expression of VE-Cadherin in control or streptozotocin rat retina. However, they described reduction in bovine endothelial cell expression of VE-Cadherin after 24 h VEGF exposure (Klaassen et al., 2009). It is possible that interactions between VE-Cadherin and the TJ molecules display some redundancy in intraocular EC as previously described by other investigators. Taddei et al. (2008) described the upregulation of claudin-5 to be mediated indirectly by VE-Cadherin in murine EC lines *in vitro*.

This study did not evaluate the role of transcellular permeability in HG, and interactions with the paracellular permeability. Such correlations will be the subject of future studies. Furthermore, the roles of inflammation in retinal and choroidal capillary permeability, up-regulation of adhesion molecules which have a role in the progression of DR (Mohr, 2004), or other cell types e.g. pericytes in diabetic eye disease need further study. However, the significant strength of our study over others is that we used primary human REC and CEC as compared to bovine or murine EC, or streptozotocin diabetic rats.

In conclusion, our findings show that human REC and CEC show different expression profiles for different TJ molecules in NG, and support the role of selected TJ molecules including occludin and claudin-5, JAM-A and C constituting a permeability barrier in intraocular micro-vascular EC, and their crucial role in the pathogenesis of diabetic eye disease. Exposure of human REC to HG for 72 h or longer results in significant reductions in the gene and protein expression of occludin, claudin-5 and JAM-A as opposed to the slight alterations observed in CEC.

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Preferred retinal locus profile during prolonged fixation attempts

Marco Ulises Morales, MEng,^{*} Saker Saker, MD,^{*} Rajnikant L. Mehta, MSc,[†]
Martin Rubinstein, FCOptom, PhD,[†] Winfried M. Amoaku, FRCS, FRCOphth, PhD[†]

ABSTRACT • RÉSUMÉ

Objective: The retinal area or location used during any fixation attempt defines the preferred retinal locus (PRL). It is presumed that during prolonged fixation attempts there may be various representative reference points within the PRL area. This study aims to clarify this presumption.

Design: Prospective, nonrandomized, observational case series.

Participants: Sixty-five eyes of 41 patients from the University of Nottingham, Queen's Medical Centre Retina and Low Vision Clinics.

Methods: A total of 65 eyes of 41 patients from the University of Nottingham, Queen's Medical Centre Retina and Low Vision Clinics were assessed for PRL using the Macular Integrity Assessment (MAIA) microperimetry equipment (CenterVue, Padova, Italy). The MAIA allows automatic calculation for 2 points named PRL_{initial} (PRL_i), which is calculated after the first 10 seconds of fixation, and PRL_{final} (PRL_f), which is calculated after the completion of all fixation attempts during the microperimetry test.

Results: Estimates of PRL_i and PRL_f were produced for all patients. Forty-six (71%) eyes were classified as having stable fixation; 40 of the 46 eyes (87%) had both PRLs location over the fovea centralis. Nineteen of 65 eyes (29%) were classified as having unstable or relatively unstable fixation; different PRL_i and PRL_f locations were found in 18 (95%) of the 19 eyes, including 13 (68%) with central geographic atrophy secondary to dry age-related macular degeneration. The mean rate of change was 5.3 units in fixation per unit change in distance in both PRL_i and PRL_f.

Conclusions: The representative points during prolonged fixation attempts may vary at different stages of fixation. This is reflected in the characteristics of fixation stability of the patients and presents a possible association with main pathology responsible for low vision.

Objet : Le secteur ou l'emplacement de la rétine utilisé pendant une tentative de fixation définit le lieu préféré de la rétine (LPR). L'on présume que, pendant les tentatives prolongées de fixation, il peut y avoir divers points de référence dans l'aire du LRP. L'étude a pour objet de clarifier cette présomption.

Nature : Étude prospective et comparative d'observations.

Participants et méthodes : En tout, 65 yeux de 41 patients de l'Université de Nottingham et des cliniques de la rétine et de la malvoyance du Centre médical Queens ont fait l'objet d'une évaluation du LRP avec un équipement micropérimétrique d'évaluation de l'intégrité maculaire (MAIA) (Centervue, Padova, Italie). L'évaluation MAIA permet le calcul automatique de deux points appelés LPR_{initial} (LPR_i) calculé après les 10 premières secondes de fixation et LPR_{final} (LPR_f) calculé après l'achèvement de toutes les tentatives de fixation pendant le test de micropérimétrie.

Résultats : Les estimations du LPR_i et du LPR_f ont été effectuées pour tous les patients. Quarante-six (71%) ont été qualifiés comme ayant une fixation stable; 40 des 46 (87%) avaient l'emplacement des deux LRP directement sur la fovéa. Dix-neuf des 65 yeux étaient classifiés comme ayant une fixation instable ou relativement instable; divers emplacements de LPR_i et de LPR_f ont été trouvés dans 18 (95%) des 19, y compris 13 yeux (68%) avec une atrophie géographique centrale secondaire à une DMLA sèche. Le taux moyen de changement était de 5,3 unités de fixation par unité de changement de distance des deux, soient LPR_i et LPR_f.

Conclusions : Les points représentatifs pendant les tentatives de fixation prolongée peuvent varier aux différentes étapes de fixation. Cela se reflète dans la stabilité des caractéristiques de fixation des patients et présente une possibilité d'association avec la pathologie principale responsable de la malvoyance.

Patients with visual loss resulting from foveal involvement of disease tend to adopt eccentric viewing by using a preferred retinal locus (PRL).¹ Characteristics of PRLs are still not fully defined or understood. Current reports suggest that PRLs characteristics change depending on type of macular disease, levels

of illumination, and tasks being undertaken.^{2–6} A new generation of instruments known as microperimeters are helping vision specialists to better understand retinal functions such as threshold sensitivity (TS) to light stimuli and fixation characteristics. The literature contains reports on the use of microperimeter in

From the ^{*}Division of Ophthalmology and Visual Sciences; and [†]The National Institute for Health Research Design Service for the East Midlands, University of Nottingham, Queen's Medical Centre, Nottingham, United Kingdom.

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Correspondence to Marco Ulises Morales, MEng, Division of Ophthalmology and Visual Sciences, University of Nottingham, Queen's Medical Centre, B Floor, East Block, Derby Road, Nottingham, NG72UH, Nottinghamshire, United Kingdom; msxmum@nottingham.ac.uk.

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assessing TS, fixation location, and fixation stability (FS).^{7–12}

The graphic fixation pattern produced with microperimetry represents the PRL. The resulting PRL describes patient's fixation characteristics in terms of location (central or eccentric) and stability (stable, relatively stable, or unstable).¹³ In addition, the PRL graphic representation provides information on size of PRL span density, as well as PRL span orientation. All microperimeters offer data on PRL characteristics as detailed earlier; however, characteristics such as PRL span during the course of fixation attempt deserve further study. The Macular Integrity Assessment (MAIA) microperimeter (CenterVue, Padova, Italy) is the only instrument that offers automatic estimates on PRL characteristics during early and late fixation. In the first 10 seconds of the examination, the MAIA microperimeter performs a fixation test in which a central point of reference of the average retinal position is calculated and labeled as PRL_i (PRL_i); subsequently, the projection of the preselected stimuli grid map begins. On completion of the test, the centre of the mass (barycentre) of the total fixation points is calculated and defined as PRL_f (PRL_f).

This study aims to analyze the location of both PRLs (initial and final) and their correlation with FS and VA in a group of patients with different pathologies who attended Retina and Low Vision Clinics.

METHODS

We recruited 41 patients (16 male and 25 female, $n = 65$ eyes), 26 (40%) male eyes and 39 (60%) female eyes (mean age 70 years, SD 13.82), attending the Retina and Low Vision Clinics of the Queens Medical Centre with varying retinal pathologies affecting the central vision such as dry age-related macular degeneration (AMD), neovascular (wet) AMD, non-AMD Choroidal neovascularization, anterior uveitis with macular edema, chorioretinitis, diabetic macular edema, and branch retinal vein occlusion with macular edema. All of the participants were assessed for visual function with the MAIA microperimetry instrument (CenterVue), after written or oral informed consent was obtained as approved by the National Research Ethics Service committee. Inclusion criteria for the study were adults older than 21 years with macular diseases as listed earlier and with a visual acuity (VA) from 0.0 to 1.6 logMAR. There were no limitations in terms of race, sex, or ethnicity. Excluded from the study were subjects with moderate-to-severe cataract (over grade 2 LOCS) or with other causes of opaque optical media, and patients who were uncooperative or unable to use the MAIA after instruction.

Microperimetry technology, also known as fundus-related perimetry,⁸ combines retinal imaging, retinal

sensitivity, and fixation pattern tests. Microperimetry with eye tracker has been used for more than a decade in the assessment of macular pathologies to precisely quantify macular sensitivity and FS.⁷ The light TS test is simultaneously performed with the fixation test superimposing the results over the surface of the retinal image, with the resultant excellent correlation of anatomic and functional outcomes. The light TS test is a subjective examination similar to the Standard Automatic Perimeter where light stimuli are shown at different light intensities, stimulating different areas of the retina several times according to selectable grid patterns. During the test, the patients are asked to fixate on the target displayed by the instrument. With the aid of eye-tracker algorithms based on retinal landmarks, microperimetry assures the reprojection of the light stimuli in precise anatomical loci; the retina position is sampled several times per second comparing the real-time image with the one acquired at the beginning of the test. The differential position is plotted over the initial retinal image. At the end of the test, a 2-dimensional point cloud pattern is shown, representing the retina location during the fixation attempt also known as the PRL.

In this study, the MAIA microperimeter (CenterVue, Padova, Italy) was used. The equipment uses a scanning laser ophthalmoscope to image the retina and projects a Goldman III size stimulus directly on the retina by means of a white LED at different light intensities,¹⁴ with a 4-2 full threshold projection as standard strategy. The maximum and minimum light intensity projected is 1000 and 0.25 asb corresponding to 0 and 36 dB of the retinal TS, respectively. The eye-tracker speed is 25 Hz.

All patients underwent the MAIA microperimetry standard examination consisting of the projection of 37 Goldman III size stimuli points with a presentation time of 200 milliseconds covering the central 10° of vision. Each eye is tested separately by occluding the other eye. The test contains 1 central stimuli and 3 concentric rings with 12 evenly distributed stimuli separated at 1°, 3°, and 5° from the centre. During the whole test the patients are asked to steadily gaze at the centre of the 1° red circle fixation target. The MAIA examination starts with 10 seconds of fixation test with no stimuli projection to define the initial PRL (PRL_i). Once the PRL_i is estimated, the projected stimuli grid map is automatically centred on the PRL_i and the randomized stimuli projection starts. At the end of the examination, the final PRL (PRL_f) is calculated as the barycentre of the plotted 2-dimensional point cloud pattern created during the fixation attempt, and the TS outcomes are presented. The automatic estimates of FS (stable, relatively unstable, unstable) are also produced as shown in Figure 1.

Classification of FS is represented by the indices P1 and P2, which are defined as the percentage of fixation points falling inside a circle of 1° and 2° radii, respectively, centred on the PRL_f. Eyes with P1 greater than 75% are

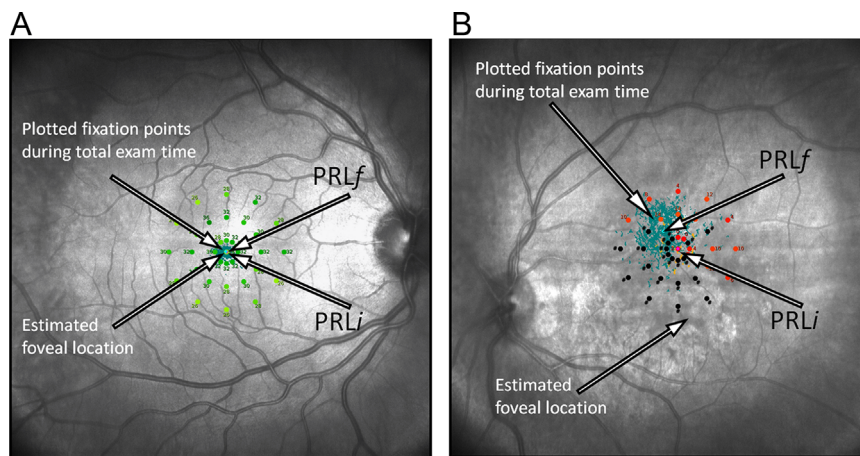


Fig. 1—Microperimetry examination outcomes. A, Normal patient showing PRL_{initial} (PRL_i) and PRL_{final} (PRL_f) in the same anatomical position with a mean threshold sensitivity (TS) = 29.8 dB and stable fixation with fixation points in P1 and P2 more than 95%. B, Patient with GA and eccentric preferred retinal locus (PRL); PRL_i and PRL_f in different loci, mean TS = 2.9 dB and unstable fixation with P1 = 23%, P2 = 70% of fixation points. In both cases, the microperimetry MAIAstd grid is centred on the PRL_i. MAIA, Macular Integrity Assessment.

classified as stable. If P1 is less than 75% and P2 is more than 75%, fixation is classified as relatively unstable. If both P1 and P2 are less than 75%, the pattern is described as representing unstable fixation.¹³

The patient characteristics are summarized using percentages, means, and standard deviations. Association in the FS and VA were all evaluated by using linear regression. All statistical analyses were performed using SPSS16 (IBM), and the significance level was set at 5% (2 sided).

RESULTS

A total of 41 patients (N = 65 eyes) completed the microperimetry test within an average time of 6 minutes.

Estimates of PRL_i and PRL_f were calculated by the MAIA instrument after the first 10 seconds and at the end of the test, respectively. Forty-two eyes (65%) of the studied population were diagnosed with dry or wet AMD, and 75% of these subjects showed a different locus of PRL_i and PRL_f.

Out of the total study population (N = 65), 44 eyes (68%) demonstrated almost identical locations for PRL_i and PRL_f, although with a wide range of average TS values (mean 22.5, min 9.4, max 29.9 dB; Fig. 2). Forty eyes (62%) had both PRLs located on the fovea (mean VA: 0.19 ± 0.26 logMAR; mean TS: 23.46 ± 5.28 dB; Fig. 3A), whereas 4 eyes (6%) were located eccentrically (mean VA: 0.79 ± 0.55 logMAR; mean TS: 13 ± 3.36 dB; Fig. 3B). All 44 eyes with the same location of PRL_i and

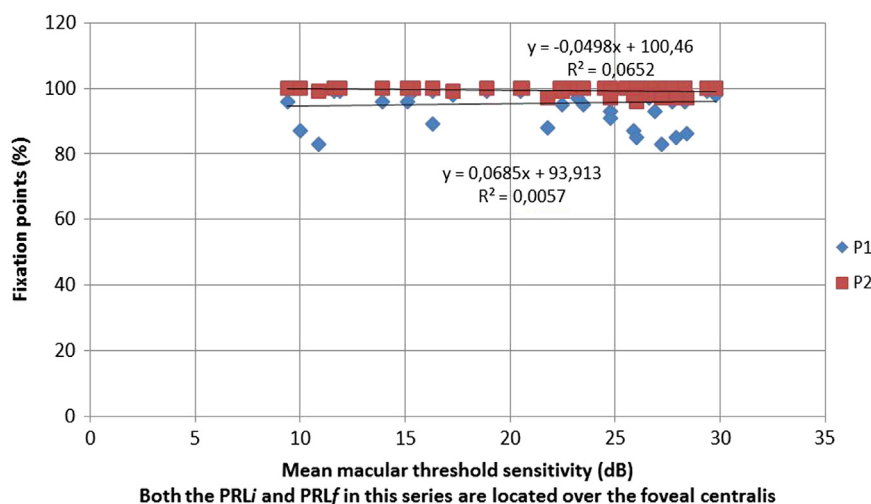


Fig. 2—Eyes (44) with both PRL_{initial} (PRL_i) and PRL_{final} (PRL_f) located over the central macula demonstrated good fixation stability (P1 and P2 > 75%) despite the wide range of threshold sensitivity values (min 9.4 dB, max 29.8 dB). PRL, preferred retinal locus.

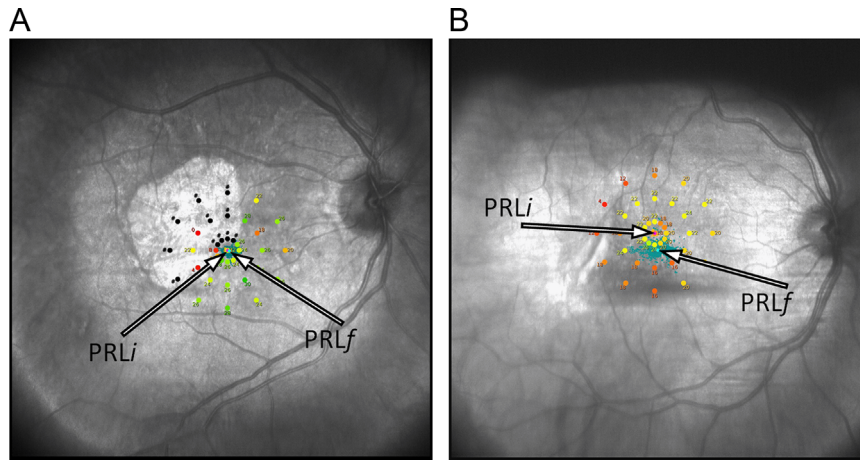


Fig. 3—Two patients classified as having stable fixation with different preferred retinal locus (PRL) characteristics. A, Patient with GA with both PRL_i and PRL_f in the same eccentric (inferior) loci with very stable fixation (P1 = 99%, P2 = 100%), low mean threshold sensitivity (TS) and visual acuity (VA) due to the geographic atrophy, TS = 15.4 dB, VA = 0.7 logMAR. B, Patient with wet age-related macular degeneration having PRL_i located over the foveal area, whereas the PRL_f is located inferiorly about 1° with P1 = 86%, P2 = 99%, threshold sensitivity = 19.5 dB, and VA = 0.1 logMAR.

PRL_f (100%) were classified as having stable fixation (P1 and P2 > 75%).

Out of the total study population (N = 65), 20 eyes (31%) demonstrated different locations for PRL_i and PRL_f (mean Δ PRL: $2.27 \pm 1.43^\circ$; mean VA: 0.75 ± 0.11 logMAR; mean TS: 12.06 ± 6.46 dB). Eighteen of these 20 eyes (90%) were classified as having unstable or relatively unstable fixation (Fig. 4).

Patients classified as having an unstable and relatively unstable fixation demonstrated that as the distance among PRL_i and PRL_f increases, VA (Fig. 5A), TS (Fig. 5B), and FS (Fig. 5C) tend to decrease. However, the mean TS and VA may have a wide range of values when both PRLs are located in the same position showing a stable fixation.

The VA shows a tendency to decrease when the distance between the PRL_i and the fovea centralis increases (Fig. 6A), as well as when the FS (Fig. 6B) and the TS (Fig. 6C) decrease. The greater the distance between PRL_i and PRL_f, the lower the FS measure. However, there may be cases where the FS is low, even when the distance between both PRLs is not large, particularly in cases where the PRL is located away from the fovea centralis (Fig. 7).

DISCUSSION

Previous studies have characterized PRLs in eyes with subnormal vision. Of special interest are the temporal

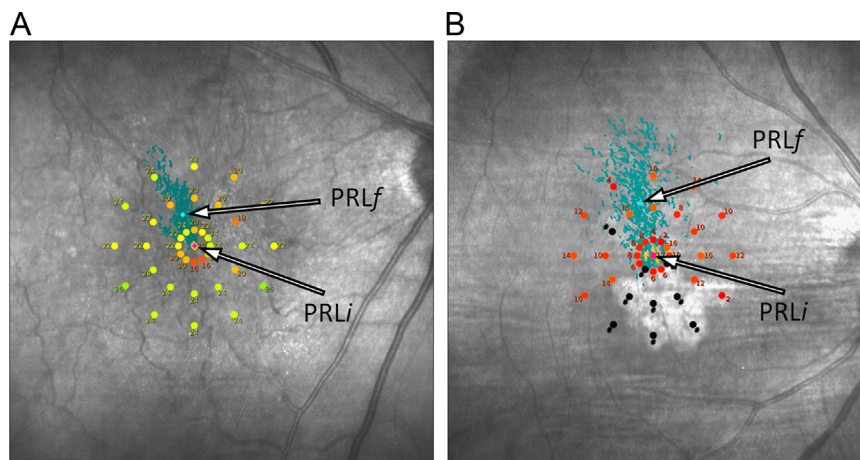


Fig. 4—Patients with different location of PRL_i and PRL_f. A, Patient diagnosed with wet age-related macular degeneration (AMD) and ERM with separation distance of 2° between the preferred retinal loci (PRLs), visual acuity (VA) = 0.3 logMAR, mean threshold sensitivity = 21.9 dB, and a relatively unstable fixation (P1 = 40%, P2 = 84%). B, Patient diagnosed with dry AMD; PRL_i located adjacent to the geographic atrophy and PRL_f separated 3° from PRL_i, VA = 0.66 logMAR, mean threshold sensitivity = 7.4 dB, and an unstable fixation (P1 = 22%, P2 = 51%).

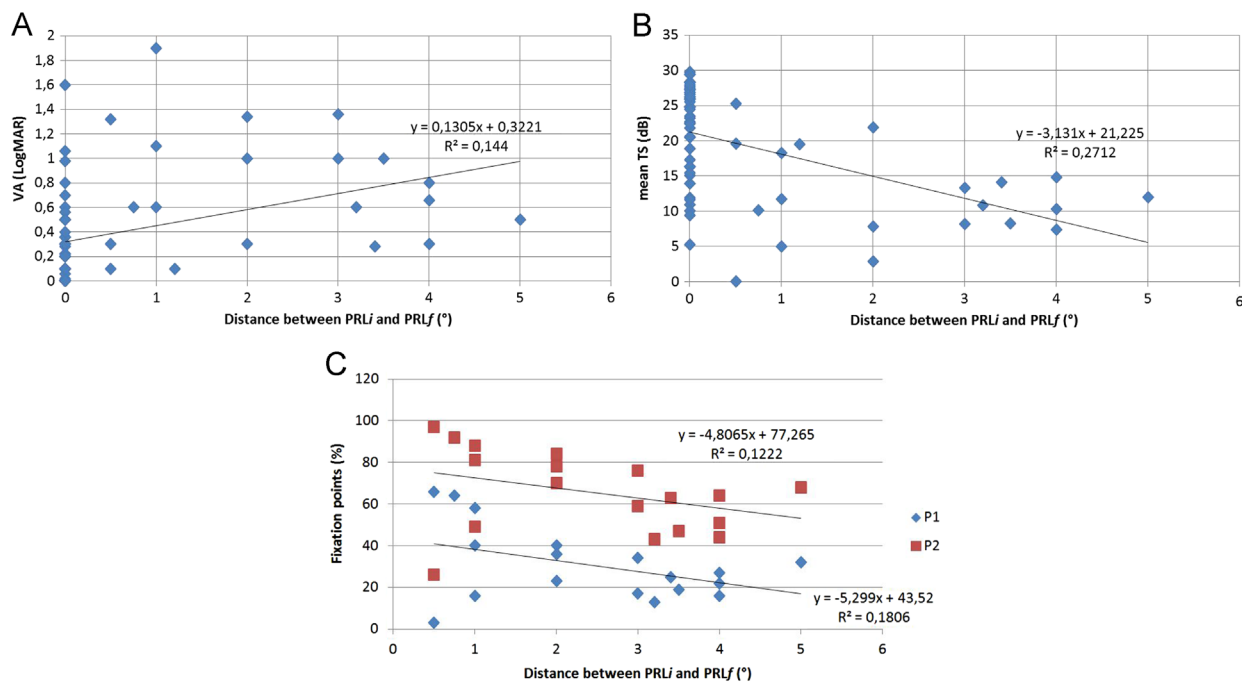


Fig. 5—Graphic shows that when the distance between PRL_i and PRL_f is minimal, visual acuity (VA) (A) and threshold sensitivity (TS) (B) may have a wide range of values, whereas they tend to decrease, as well as fixation stability (C), when the distance between both preferred retinal loci (PRLs) increases.

characteristics of PRLs during same or consecutive fixation attempts. Different authors have stated the importance of the analysis of FS and fixation location in patients with eccentric PRL as essential predictors during the evaluation of the effects of treatment.^{10,15} The analysis of PRL_i and PRL_f combines both FS and location, and may be useful information to complement prognosis data for visual outcomes in the treatment of pathologies affecting the central vision. In the assessment of retinal function, the anatomical location of PRL_i and PRL_f may be important because these fixation characteristics may reflect the possible association with oculomotor functions that ultimately dictate the span and direction of the PRL and FS.

The MAIA instrument by design allows identification of locations for the PRL at different stages of fixation (PRL_i = 10 seconds, PRL_f > 5 minutes) under same testing circumstances. To the best of our knowledge, this is the first study to characterize changes in PRLs during the course of a prolonged fixation attempt using microperimetry.

Eyes with both PRL_i and PRL_f close to each other are noted to have stable fixation located mostly over the fovea centralis. However, patients with eccentric PRL may also have both the PRL_i and PRL_f in the same anatomical location showing eccentric but stable fixation. Eyes with PRL_i and PRL_f far from each other are noted to have, as a rule, unstable fixation mostly located on the peripheral retina. Interestingly, TS may not be related to fixation characteristics particularly in cases of stable fixation. These characteristics may be important in eccentric fixation training.

The location of PRL being immediately adjacent to the geographic atrophy (GA) had been previously reported.¹⁶ This is supported by our finding that at least 1 of the PRLs in all cases of cGA (except 1) was located immediately adjacent to the GA. However, a large number (71%) of the eyes with cGA presented unstable or relatively unstable fixation with different PRL_i and PRL_f loci, although some (29%) shared the same eccentric PRL_i and PRL_f loci showing stable fixation.

Previously, PRLs were reported as “multiple” because they changed characteristics according to the type of macular disease, levels of illumination, and activity in which the patient is engaged.^{1–6,17} Lei and Schuchard² found cases with 2 different PRLs depending on the brightness of objects used in visual tasks. Similarly, Safran et al.³ and Deruaz et al.⁵ found multiple PRLs during the reading process; in both studies, 2 well-defined PRLs were located in different retinal zones. Conversely, our study has demonstrated cases with 2 different loci of high density of fixation points, within the same PRL span, in 2 different times of the same examination.

Previous studies have demonstrated that VA can vary depending on the FS and location.¹⁰ Our results show how VA tends to decrease as the distance between both PRL_i and PRL_f increase; however, further studies are needed to fully correlate the behaviour of both of the PRLs and VA during different stages of the pathology, treatment, and rehabilitation process.

It is important to note that the studies of the PRL during the fixation attempt with microperimetry have

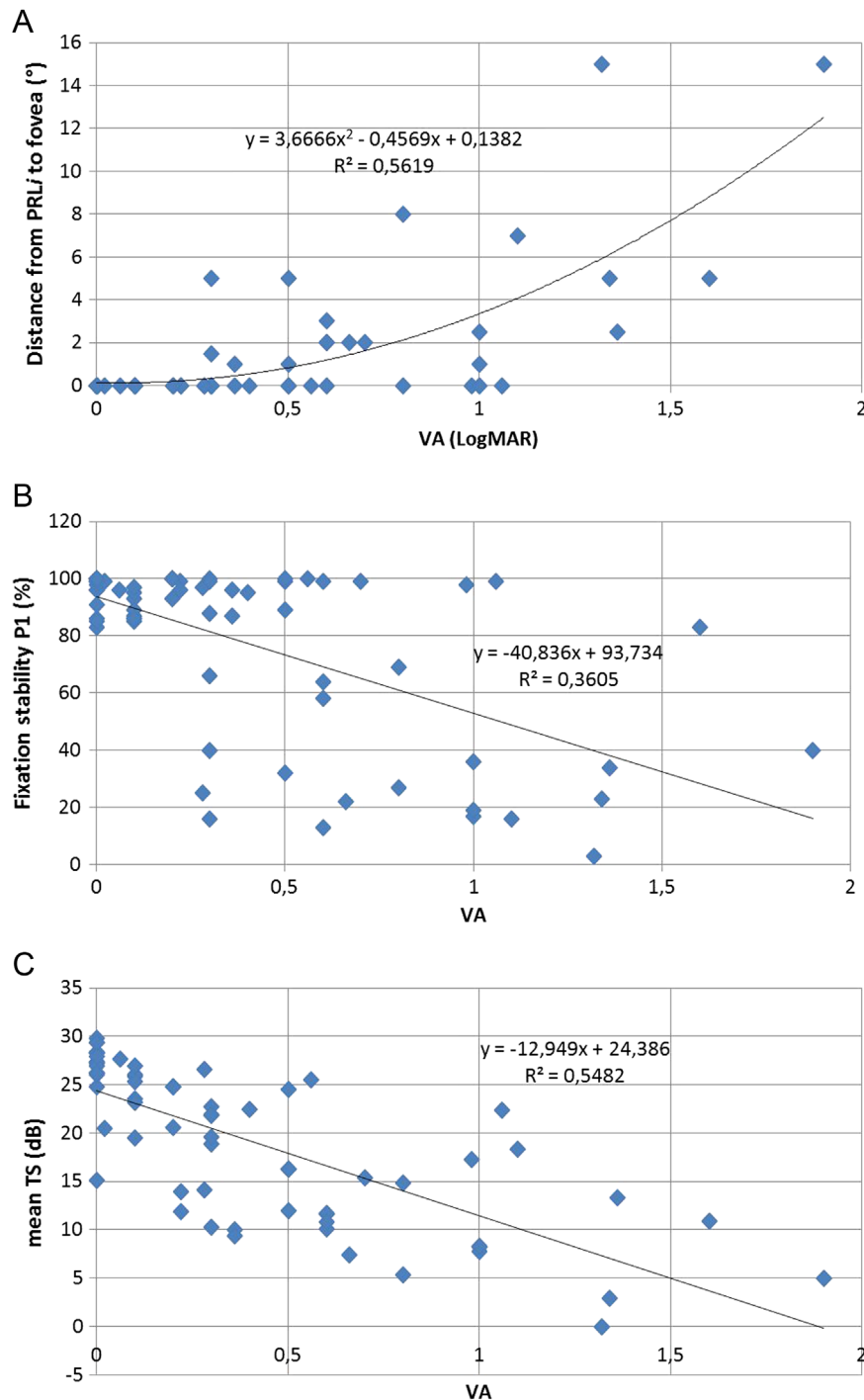


Fig. 6—Visual acuity (VA) shows a tendency to decrease when the distance between the PRL_i and the fovea centralis is increased (A), and also when fixation stability (B) and threshold sensitivity (TS) (C) decrease.

been monocular; thus, the fixation characteristics in stereoscopic conditions remain to be explored.

The PRL is the graphic representation of all the retinal area used during a fixation attempt by patients with compromised central vision. Characteristics of fixation, stability, and location appear to be dependent on the pathology and affect VA. The importance of defining more than 1 reference point within the whole PRL area (PRL_i and PRL_f) is to give eye care practitioners a better

understanding of the dynamic of the visual function. The representative points, PRL_i and PRL_f, may vary at different stages of fixation during prolonged fixation attempts. The PRLs are important and need to be considered during retinal functional assessment.

This study suggests that when both PRL_i and PRL_f are located in the same position, fixation is stable. Conversely, a greater distance between the PRL_i and PRL_f may be a defining parameter of unstable fixation. The superim;

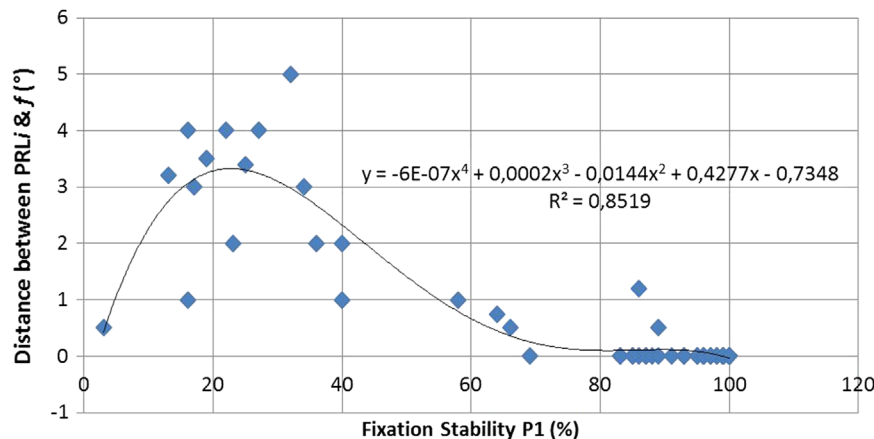


Fig. 7—Graph shows that when there is no distance between the PRL_i and PRL_f, the fixation is stable (P1 > 75%), and normally, the greater the distance between PRL_i and PRL_f, the lower the fixation stability, although our study showed 1 case with small preferred retinal loci (PRLs) distance and unstable fixation (P1 < 10%).

position of clinical features and microperimetric characteristics such as retinal sensitivity, FS, and fixation location may facilitate the determination of the differential impact of macular disorders and their corresponding VA. This could be relevant as prognostic factors for treatment and rehabilitation purposes.

Disclosure: M.U.M. has received consulting fees from and has stock options in CenterVue (Padova, Italy). The other authors have no proprietary or commercial interest in any materials discussed in this article.

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Preferred retinal locus profile during prolonged fixation attempts

Marco Ulises Morales, MEng,^{*} Saker Saker, MD,^{*} Rajnikant L. Mehta, MSc,[†]
Martin Rubinstein, FCOptom, PhD,[†] Winfried M. Amoaku, FRCS, FRCOphth, PhD[†]

ABSTRACT • RÉSUMÉ

Objective: The retinal area or location used during any fixation attempt defines the preferred retinal locus (PRL). It is presumed that during prolonged fixation attempts there may be various representative reference points within the PRL area. This study aims to clarify this presumption.

Design: Prospective, nonrandomized, observational case series.

Participants: Sixty-five eyes of 41 patients from the University of Nottingham, Queen's Medical Centre Retina and Low Vision Clinics.

Methods: A total of 65 eyes of 41 patients from the University of Nottingham, Queen's Medical Centre Retina and Low Vision Clinics were assessed for PRL using the Macular Integrity Assessment (MAIA) microperimetry equipment (CenterVue, Padova, Italy). The MAIA allows automatic calculation for 2 points named PRL_{initial} (PRL_i), which is calculated after the first 10 seconds of fixation, and PRL_{final} (PRL_f), which is calculated after the completion of all fixation attempts during the microperimetry test.

Results: Estimates of PRL_i and PRL_f were produced for all patients. Forty-six (71%) eyes were classified as having stable fixation; 40 of the 46 eyes (87%) had both PRLs location over the fovea centralis. Nineteen of 65 eyes (29%) were classified as having unstable or relatively unstable fixation; different PRL_i and PRL_f locations were found in 18 (95%) of the 19 eyes, including 13 (68%) with central geographic atrophy secondary to dry age-related macular degeneration. The mean rate of change was 5.3 units in fixation per unit change in distance in both PRL_i and PRL_f.

Conclusions: The representative points during prolonged fixation attempts may vary at different stages of fixation. This is reflected in the characteristics of fixation stability of the patients and presents a possible association with main pathology responsible for low vision.

Objet : Le secteur ou l'emplacement de la rétine utilisé pendant une tentative de fixation définit le lieu préféré de la rétine (LPR). L'on présume que, pendant les tentatives prolongées de fixation, il peut y avoir divers points de référence dans l'aire du LRP. L'étude a pour objet de clarifier cette présomption.

Nature : Étude prospective et comparative d'observations.

Participants et méthodes : En tout, 65 yeux de 41 patients de l'Université de Nottingham et des cliniques de la rétine et de la malvoyance du Centre médical Queens ont fait l'objet d'une évaluation du LRP avec un équipement micropérimétrique d'évaluation de l'intégrité maculaire (MAIA) (Centervue, Padova, Italie). L'évaluation MAIA permet le calcul automatique de deux points appelés LPR_{initial} (LPR_i) calculé après les 10 premières secondes de fixation et LPR_{final} (LPR_f) calculé après l'achèvement de toutes les tentatives de fixation pendant le test de micropérimétrie.

Résultats : Les estimations du LPR_i et du LPR_f ont été effectuées pour tous les patients. Quarante-six (71%) ont été qualifiés comme ayant une fixation stable; 40 des 46 (87%) avaient l'emplacement des deux LRP directement sur la fovéa. Dix-neuf des 65 yeux étaient classifiés comme ayant une fixation instable ou relativement instable; divers emplacements de LPR_i et de LPR_f ont été trouvés dans 18 (95%) des 19, y compris 13 yeux (68%) avec une atrophie géographique centrale secondaire à une DMLA sèche. Le taux moyen de changement était de 5,3 unités de fixation par unité de changement de distance des deux, soient LPR_i et LPR_f.

Conclusions : Les points représentatifs pendant les tentatives de fixation prolongée peuvent varier aux différentes étapes de fixation. Cela se reflète dans la stabilité des caractéristiques de fixation des patients et présente une possibilité d'association avec la pathologie principale responsable de la malvoyance.

Patients with visual loss resulting from foveal involvement of disease tend to adopt eccentric viewing by using a preferred retinal locus (PRL).¹ Characteristics of PRLs are still not fully defined or understood. Current reports suggest that PRLs characteristics change depending on type of macular disease, levels

of illumination, and tasks being undertaken.^{2–6} A new generation of instruments known as microperimeters are helping vision specialists to better understand retinal functions such as threshold sensitivity (TS) to light stimuli and fixation characteristics. The literature contains reports on the use of microperimeter in

From the ^{*}Division of Ophthalmology and Visual Sciences; and [†]The National Institute for Health Research Design Service for the East Midlands, University of Nottingham, Queen's Medical Centre, Nottingham, United Kingdom.

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Correspondence to Marco Ulises Morales, MEng, Division of Ophthalmology and Visual Sciences, University of Nottingham, Queen's Medical Centre, B Floor, East Block, Derby Road, Nottingham, NG72UH, Nottinghamshire, United Kingdom; msxmum@nottingham.ac.uk.

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assessing TS, fixation location, and fixation stability (FS).^{7–12}

The graphic fixation pattern produced with microperimetry represents the PRL. The resulting PRL describes patient's fixation characteristics in terms of location (central or eccentric) and stability (stable, relatively stable, or unstable).¹³ In addition, the PRL graphic representation provides information on size of PRL span density, as well as PRL span orientation. All microperimeters offer data on PRL characteristics as detailed earlier; however, characteristics such as PRL span during the course of fixation attempt deserve further study. The Macular Integrity Assessment (MAIA) microperimeter (CenterVue, Padova, Italy) is the only instrument that offers automatic estimates on PRL characteristics during early and late fixation. In the first 10 seconds of the examination, the MAIA microperimeter performs a fixation test in which a central point of reference of the average retinal position is calculated and labeled as PRL_i (PRL_i); subsequently, the projection of the preselected stimuli grid map begins. On completion of the test, the centre of the mass (barycentre) of the total fixation points is calculated and defined as PRL_f (PRL_f).

This study aims to analyze the location of both PRLs (initial and final) and their correlation with FS and VA in a group of patients with different pathologies who attended Retina and Low Vision Clinics.

METHODS

We recruited 41 patients (16 male and 25 female, $n = 65$ eyes), 26 (40%) male eyes and 39 (60%) female eyes (mean age 70 years, SD 13.82), attending the Retina and Low Vision Clinics of the Queens Medical Centre with varying retinal pathologies affecting the central vision such as dry age-related macular degeneration (AMD), neovascular (wet) AMD, non-AMD Choroidal neovascularization, anterior uveitis with macular edema, chorioretinitis, diabetic macular edema, and branch retinal vein occlusion with macular edema. All of the participants were assessed for visual function with the MAIA microperimetry instrument (CenterVue), after written or oral informed consent was obtained as approved by the National Research Ethics Service committee. Inclusion criteria for the study were adults older than 21 years with macular diseases as listed earlier and with a visual acuity (VA) from 0.0 to 1.6 logMAR. There were no limitations in terms of race, sex, or ethnicity. Excluded from the study were subjects with moderate-to-severe cataract (over grade 2 LOCS) or with other causes of opaque optical media, and patients who were uncooperative or unable to use the MAIA after instruction.

Microperimetry technology, also known as fundus-related perimetry,⁸ combines retinal imaging, retinal

sensitivity, and fixation pattern tests. Microperimetry with eye tracker has been used for more than a decade in the assessment of macular pathologies to precisely quantify macular sensitivity and FS.⁷ The light TS test is simultaneously performed with the fixation test superimposing the results over the surface of the retinal image, with the resultant excellent correlation of anatomic and functional outcomes. The light TS test is a subjective examination similar to the Standard Automatic Perimeter where light stimuli are shown at different light intensities, stimulating different areas of the retina several times according to selectable grid patterns. During the test, the patients are asked to fixate on the target displayed by the instrument. With the aid of eye-tracker algorithms based on retinal landmarks, microperimetry assures the reprojection of the light stimuli in precise anatomical loci; the retina position is sampled several times per second comparing the real-time image with the one acquired at the beginning of the test. The differential position is plotted over the initial retinal image. At the end of the test, a 2-dimensional point cloud pattern is shown, representing the retina location during the fixation attempt also known as the PRL.

In this study, the MAIA microperimeter (CenterVue, Padova, Italy) was used. The equipment uses a scanning laser ophthalmoscope to image the retina and projects a Goldman III size stimulus directly on the retina by means of a white LED at different light intensities,¹⁴ with a 4-2 full threshold projection as standard strategy. The maximum and minimum light intensity projected is 1000 and 0.25 asb corresponding to 0 and 36 dB of the retinal TS, respectively. The eye-tracker speed is 25 Hz.

All patients underwent the MAIA microperimetry standard examination consisting of the projection of 37 Goldman III size stimuli points with a presentation time of 200 milliseconds covering the central 10° of vision. Each eye is tested separately by occluding the other eye. The test contains 1 central stimuli and 3 concentric rings with 12 evenly distributed stimuli separated at 1°, 3°, and 5° from the centre. During the whole test the patients are asked to steadily gaze at the centre of the 1° red circle fixation target. The MAIA examination starts with 10 seconds of fixation test with no stimuli projection to define the initial PRL (PRL_i). Once the PRL_i is estimated, the projected stimuli grid map is automatically centred on the PRL_i and the randomized stimuli projection starts. At the end of the examination, the final PRL (PRL_f) is calculated as the barycentre of the plotted 2-dimensional point cloud pattern created during the fixation attempt, and the TS outcomes are presented. The automatic estimates of FS (stable, relatively unstable, unstable) are also produced as shown in Figure 1.

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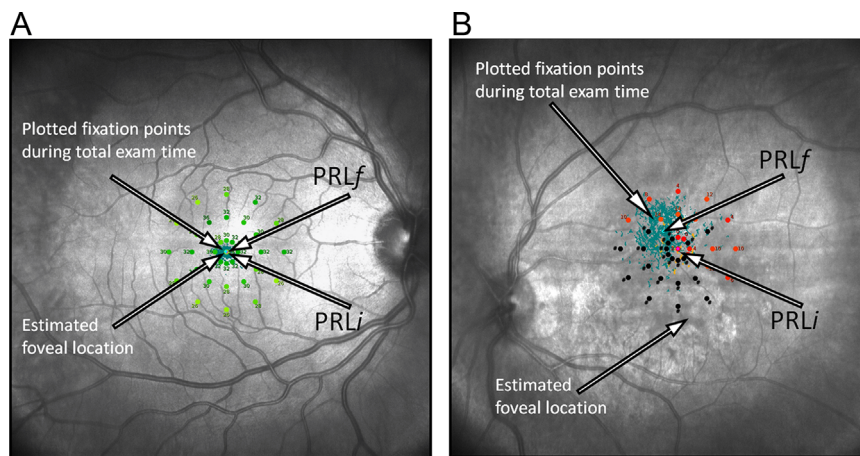


Fig. 1—Microperimetry examination outcomes. A, Normal patient showing PRL_{initial} (PRL_i) and PRL_{final} (PRL_f) in the same anatomical position with a mean threshold sensitivity (TS) = 29.8 dB and stable fixation with fixation points in P1 and P2 more than 95%. B, Patient with GA and eccentric preferred retinal locus (PRL); PRL_i and PRL_f in different loci, mean TS = 2.9 dB and unstable fixation with P1 = 23%, P2 = 70% of fixation points. In both cases, the microperimetry MAIAstd grid is centred on the PRL_i. MAIA, Macular Integrity Assessment.

classified as stable. If P1 is less than 75% and P2 is more than 75%, fixation is classified as relatively unstable. If both P1 and P2 are less than 75%, the pattern is described as representing unstable fixation.¹³

The patient characteristics are summarized using percentages, means, and standard deviations. Association in the FS and VA were all evaluated by using linear regression. All statistical analyses were performed using SPSS16 (IBM), and the significance level was set at 5% (2 sided).

RESULTS

A total of 41 patients (N = 65 eyes) completed the microperimetry test within an average time of 6 minutes.

Estimates of PRL_i and PRL_f were calculated by the MAIA instrument after the first 10 seconds and at the end of the test, respectively. Forty-two eyes (65%) of the studied population were diagnosed with dry or wet AMD, and 75% of these subjects showed a different locus of PRL_i and PRL_f.

Out of the total study population (N = 65), 44 eyes (68%) demonstrated almost identical locations for PRL_i and PRL_f, although with a wide range of average TS values (mean 22.5, min 9.4, max 29.9 dB; Fig. 2). Forty eyes (62%) had both PRLs located on the fovea (mean VA: 0.19 ± 0.26 logMAR; mean TS: 23.46 ± 5.28 dB; Fig. 3A), whereas 4 eyes (6%) were located eccentrically (mean VA: 0.79 ± 0.55 logMAR; mean TS: 13 ± 3.36 dB; Fig. 3B). All 44 eyes with the same location of PRL_i and

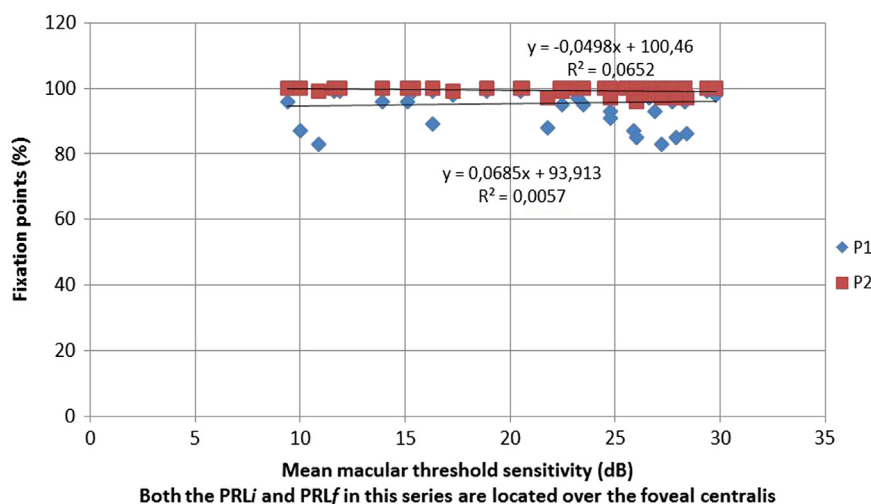


Fig. 2—Eyes (44) with both PRL_{initial} (PRL_i) and PRL_{final} (PRL_f) located over the central macula demonstrated good fixation stability (P1 and P2 > 75%) despite the wide range of threshold sensitivity values (min 9.4 dB, max 29.8 dB). PRL, preferred retinal locus.

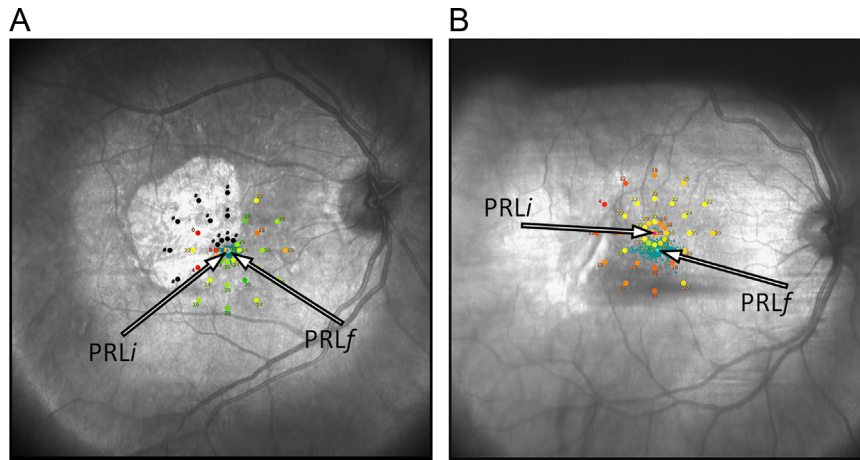


Fig. 3—Two patients classified as having stable fixation with different preferred retinal locus (PRL) characteristics. A, Patient with GA with both PRL_i and PRL_f in the same eccentric (inferior) loci with very stable fixation (P1 = 99%, P2 = 100%), low mean threshold sensitivity (TS) and visual acuity (VA) due to the geographic atrophy, TS = 15.4 dB, VA = 0.7 logMAR. B, Patient with wet age-related macular degeneration having PRL_i located over the foveal area, whereas the PRL_f is located inferiorly about 1° with P1 = 86%, P2 = 99%, threshold sensitivity = 19.5 dB, and VA = 0.1 logMAR.

PRL_f (100%) were classified as having stable fixation (P1 and P2 > 75%).

Out of the total study population (N = 65), 20 eyes (31%) demonstrated different locations for PRL_i and PRL_f (mean Δ PRL: $2.27 \pm 1.43^\circ$; mean VA: 0.75 ± 0.11 logMAR; mean TS: 12.06 ± 6.46 dB). Eighteen of these 20 eyes (90%) were classified as having unstable or relatively unstable fixation (Fig. 4).

Patients classified as having an unstable and relatively unstable fixation demonstrated that as the distance among PRL_i and PRL_f increases, VA (Fig. 5A), TS (Fig. 5B), and FS (Fig. 5C) tend to decrease. However, the mean TS and VA may have a wide range of values when both PRLs are located in the same position showing a stable fixation.

The VA shows a tendency to decrease when the distance between the PRL_i and the fovea centralis increases (Fig. 6A), as well as when the FS (Fig. 6B) and the TS (Fig. 6C) decrease. The greater the distance between PRL_i and PRL_f, the lower the FS measure. However, there may be cases where the FS is low, even when the distance between both PRLs is not large, particularly in cases where the PRL is located away from the fovea centralis (Fig. 7).

DISCUSSION

Previous studies have characterized PRLs in eyes with subnormal vision. Of special interest are the temporal

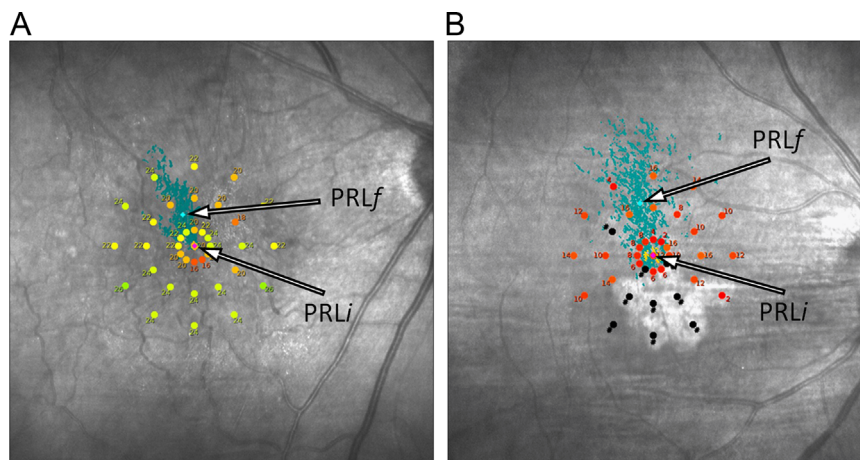


Fig. 4—Patients with different location of PRL_i and PRL_f. A, Patient diagnosed with wet age-related macular degeneration (AMD) and ERM with separation distance of 2° between the preferred retinal loci (PRLs), visual acuity (VA) = 0.3 logMAR, mean threshold sensitivity = 21.9 dB, and a relatively unstable fixation (P1 = 40%, P2 = 84%). B, Patient diagnosed with dry AMD; PRL_i located adjacent to the geographic atrophy and PRL_f separated 3° from PRL_i, VA = 0.66 logMAR, mean threshold sensitivity = 7.4 dB, and an unstable fixation (P1 = 22%, P2 = 51%).

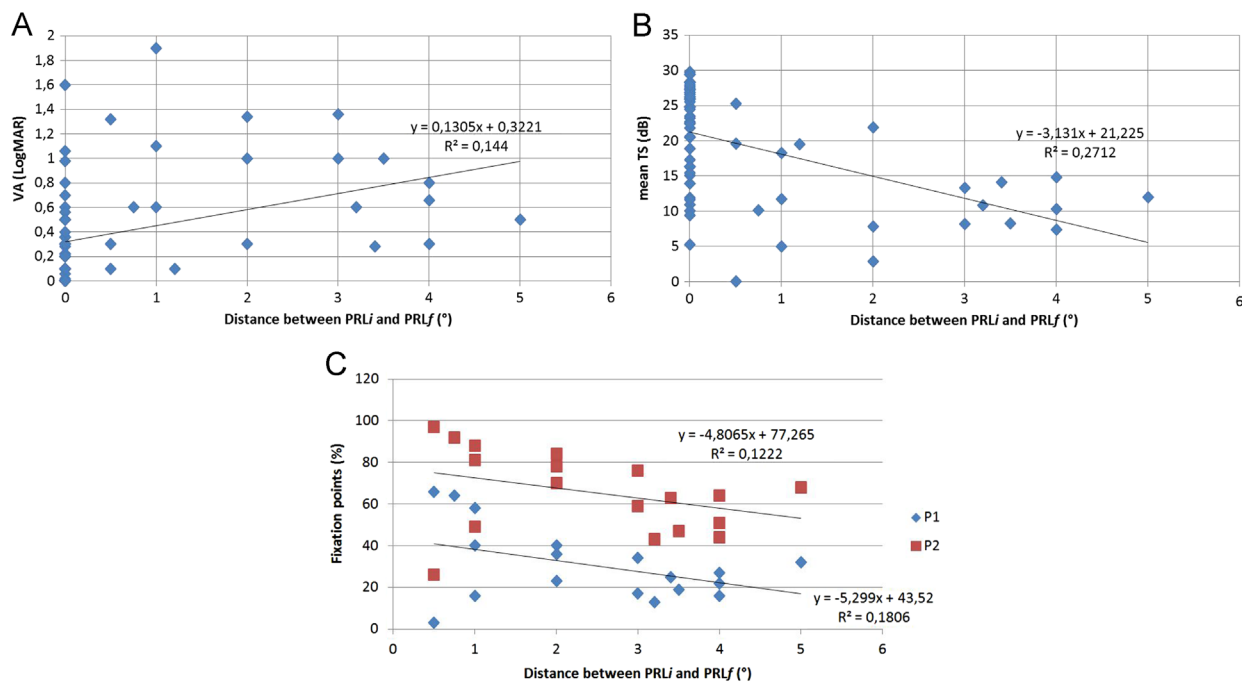


Fig. 5—Graphic shows that when the distance between PRL_i and PRL_f is minimal, visual acuity (VA) (A) and threshold sensitivity (TS) (B) may have a wide range of values, whereas they tend to decrease, as well as fixation stability (C), when the distance between both preferred retinal loci (PRLs) increases.

characteristics of PRLs during same or consecutive fixation attempts. Different authors have stated the importance of the analysis of FS and fixation location in patients with eccentric PRL as essential predictors during the evaluation of the effects of treatment.^{10,15} The analysis of PRL_i and PRL_f combines both FS and location, and may be useful information to complement prognosis data for visual outcomes in the treatment of pathologies affecting the central vision. In the assessment of retinal function, the anatomical location of PRL_i and PRL_f may be important because these fixation characteristics may reflect the possible association with oculomotor functions that ultimately dictate the span and direction of the PRL and FS.

The MAIA instrument by design allows identification of locations for the PRL at different stages of fixation (PRL_i = 10 seconds, PRL_f > 5 minutes) under same testing circumstances. To the best of our knowledge, this is the first study to characterize changes in PRLs during the course of a prolonged fixation attempt using microperimetry.

Eyes with both PRL_i and PRL_f close to each other are noted to have stable fixation located mostly over the fovea centralis. However, patients with eccentric PRL may also have both the PRL_i and PRL_f in the same anatomical location showing eccentric but stable fixation. Eyes with PRL_i and PRL_f far from each other are noted to have, as a rule, unstable fixation mostly located on the peripheral retina. Interestingly, TS may not be related to fixation characteristics particularly in cases of stable fixation. These characteristics may be important in eccentric fixation training.

The location of PRL being immediately adjacent to the geographic atrophy (GA) had been previously reported.¹⁶ This is supported by our finding that at least 1 of the PRLs in all cases of cGA (except 1) was located immediately adjacent to the GA. However, a large number (71%) of the eyes with cGA presented unstable or relatively unstable fixation with different PRL_i and PRL_f loci, although some (29%) shared the same eccentric PRL_i and PRL_f loci showing stable fixation.

Previously, PRLs were reported as “multiple” because they changed characteristics according to the type of macular disease, levels of illumination, and activity in which the patient is engaged.^{1–6,17} Lei and Schuchard² found cases with 2 different PRLs depending on the brightness of objects used in visual tasks. Similarly, Safran et al.³ and Deruaz et al.⁵ found multiple PRLs during the reading process; in both studies, 2 well-defined PRLs were located in different retinal zones. Conversely, our study has demonstrated cases with 2 different loci of high density of fixation points, within the same PRL span, in 2 different times of the same examination.

Previous studies have demonstrated that VA can vary depending on the FS and location.¹⁰ Our results show how VA tends to decrease as the distance between both PRL_i and PRL_f increase; however, further studies are needed to fully correlate the behaviour of both of the PRLs and VA during different stages of the pathology, treatment, and rehabilitation process.

It is important to note that the studies of the PRL during the fixation attempt with microperimetry have

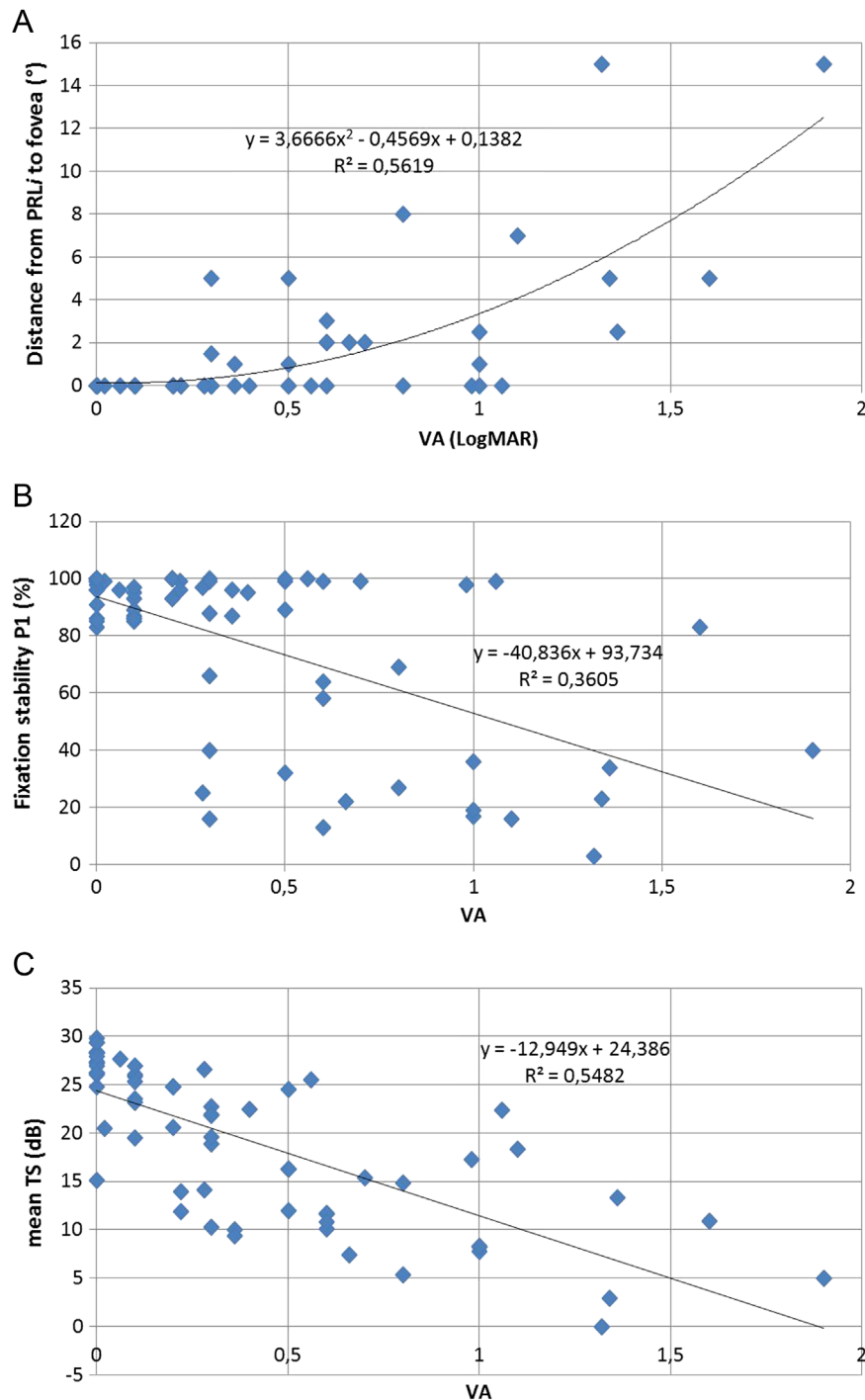


Fig. 6—Visual acuity (VA) shows a tendency to decrease when the distance between the PRL_i and the fovea centralis is increased (A), and also when fixation stability (B) and threshold sensitivity (TS) (C) decrease.

been monocular; thus, the fixation characteristics in stereoscopic conditions remain to be explored.

The PRL is the graphic representation of all the retinal area used during a fixation attempt by patients with compromised central vision. Characteristics of fixation, stability, and location appear to be dependent on the pathology and affect VA. The importance of defining more than 1 reference point within the whole PRL area (PRL_i and PRL_f) is to give eye care practitioners a better

understanding of the dynamic of the visual function. The representative points, PRL_i and PRL_f, may vary at different stages of fixation during prolonged fixation attempts. The PRLs are important and need to be considered during retinal functional assessment.

This study suggests that when both PRL_i and PRL_f are located in the same position, fixation is stable. Conversely, a greater distance between the PRL_i and PRL_f may be a defining parameter of unstable fixation. The superim;

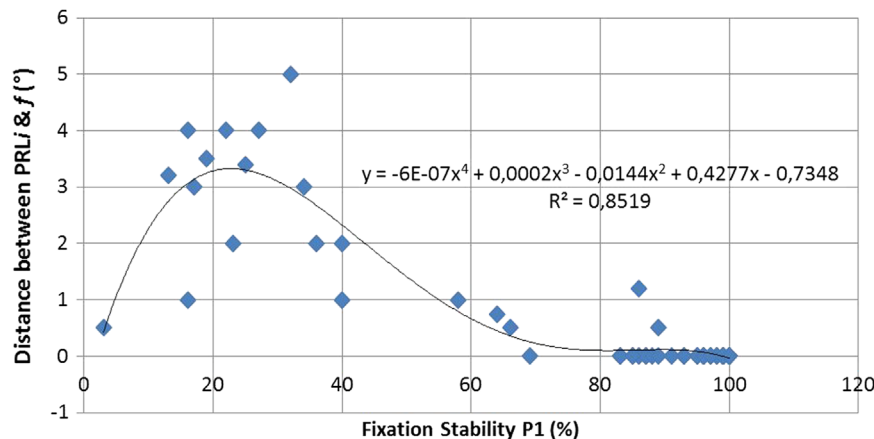


Fig. 7—Graph shows that when there is no distance between the PRL_{initial} (PRL_i) and PRL_{final} (PRL_f), the fixation is stable (P1 > 75%), and normally, the greater the distance between PRL_i and PRL_f, the lower the fixation stability, although our study showed 1 case with small preferred retinal loci (PRLs) distance and unstable fixation (P1 < 10%).

position of clinical features and microperimetric characteristics such as retinal sensitivity, FS, and fixation location may facilitate the determination of the differential impact of macular disorders and their corresponding VA. This could be relevant as prognostic factors for treatment and rehabilitation purposes.

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A review of therapies for diabetic macular oedema and rationale for combination therapy

WMK Amoaku, S Saker and EA Stewart

Abstract

Diabetic macular oedema (DMO) is responsible for significant visual impairment in diabetic patients. The primary cause of DMO is fluid leakage resulting from increased vascular permeability through contributory anatomical and biochemical changes. These include endothelial cell (EC) death or dysfunction, pericyte loss or dysfunction, thickened basement membrane, loss or dysfunction of glial cells, and loss/change of EC Glycocalyx. The molecular changes include increased reactive oxygen species, pro-inflammatory changes: advanced glycation end products, intracellular adhesion molecule-1, Complement 5–9 deposition and cytokines, which result in increased paracellular permeability, tight junction disruption, and increased transcellular permeability. Laser photocoagulation has been the mainstay of treatment until recently when pharmacological treatments were introduced. The current treatments for DMO target reducing vascular leak in the macula once it has occurred, they do not attempt to treat the underlying pathology. These pharmacological treatments are aimed at antagonising vascular endothelial growth factor (VEGF) or non-VEGF inflammatory pathways, and include intravitreal injections of anti-VEGFs (ranibizumab, aflibercept or bevacizumab) or steroids (fluocinolone, dexamethasone or triamcinolone) as single therapies. The available evidence suggests that each individual treatment modality in DMO does not result in a completely dry macula in most cases. The ideal treatment for DMO should improve vision and improve morphological changes in the macular (eg, reduce macular oedema) for a significant duration, reduced adverse events, reduced treatment burden and costs, and be well tolerated by patients. This review evaluates the individual treatments available as

monotherapies, and discusses the rationale and potential for combination therapy in DMO. A comprehensive review of clinical trials related to DMO and their outcomes was completed. Where phase III randomised control trials were available, these were referenced, if not available, phase II trials have been included.

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Introduction

In 2002, it was reported that diabetes affected 220 million people worldwide,¹ and anticipated that the prevalence of diabetes will double within the next 10 years.² More recent estimates indicate that the prevalence of diabetes in adults (aged 20–79 years) worldwide was 382 million people in 2012, and that this would likely increase to 592 million in 2035.³

Diabetic retinopathy (DR) has been extensively studied over the years, and its incidence correlates with poor glycaemic control and hyperlipidaemia.^{4,5} Diabetic choroidopathy is a less well-studied entity, and is thought to occur in the advanced stages of diabetic eye disease.^{6–9} As such, the retinal and choroidal vascular beds seem to be affected differently by diabetes. Diabetes and hyperglycaemia have obvious effects on intraocular vascular endothelial cell (EC) permeability, adhesion to leukocytes, as well as angiogenesis.^{10–12} These alterations result in increased vascular leakage (increased permeability), vascular occlusions, ischaemia, and angiogenesis.^{13,14} However, the exact mechanisms underlying these changes are not fully understood, and require further elucidation.

Academic Ophthalmology,
Division of Clinical
Neuroscience, University of
Nottingham, Queen's
Medical Centre,
Nottingham, UK

Correspondence:
WMK Amoaku, Academic
Ophthalmology, Division of
Clinical Neuroscience,
University of Nottingham,
Queen's Medical Centre,
B Floor, Eye and ENT
Building, Nottingham, NG7
2UH, UK
Tel: +44 (0)1159709796;
Fax: +44 (0)1159709963.
E-mail: winfried.amoaku@
nottingham.ac.uk

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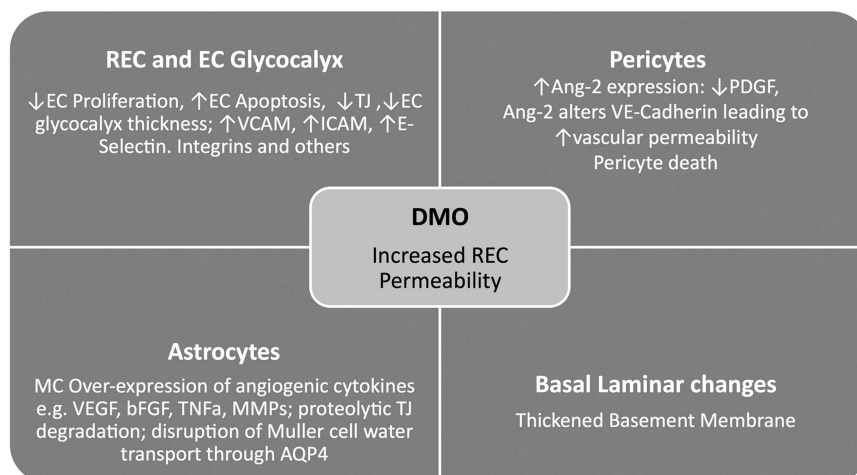


Figure 1 Pathogenesis of diabetic vasculopathy. DMO, diabetic macular oedema; Ang-2, Angiopoietin-2; EC, endothelial cell; ICAM, intercellular adhesion molecule; IP, interferon-induced protein; MMP, metallic metalloproteinase; VEGF, vascular endothelial growth factor; VE, vascular endothelial; PDGF, platelet-derived growth factor; TJ, tight junction, bFGF, basic fibroblast growth factor.

Diabetic macular oedema (DMO) is responsible for significant visual impairment in diabetic patients.^{1,2,15,16} In the retina, leakage is due to increased permeability that occurs at the retinal 'neurovascular' unit, which consists of single layer of tightly adherent ECs, basal lamina, surrounding pericytes, astrocytes, and microglia leading to increased EC trans- or paracellular permeability, as summarised in the recent review by Klaassen *et al.*¹⁷ There are contributory anatomical and biochemical changes that are interlinked (Figure 1). The anatomic changes include EC damage—death or dysfunction, pericyte loss or dysfunction, thickened basement membrane, loss or dysfunction of glial cells, and loss/change of EC Glycocalyx. The molecular changes include increased reactive oxygen species, pro-inflammatory changes: advanced glycation end products, intracellular adhesion molecule-1 (ICAM-1), Complement 5–9 (C5–9) deposition, cytokines, which result in increased paracellular permeability—small molecules and water: tight junction (TJ) disruption—and increased transcellular permeability—large molecules and water: caveolar transport, aquaporins, plasmalemmal vesicle-associated protein. The increased intraretinal fluid leads to progressive retinal dysfunction (see Klaassen *et al.*¹⁷ review). The contributions of the choroidal vasculature to the clinical disease of DR are less well understood, but again will be largely contributed to by the choroidal EC (CEC) alterations.^{6–9} It is known that retinal vascular leakage in DMO is contributed to by vascular endothelial growth factor (VEGF) upregulation as well as non-VEGF-dependent inflammatory pathways, so that chronic subclinical inflammation is important in the pathogenesis of DR.^{18–28} An early event in the pathogenesis of diabetic vasculopathy is leukocyte adherence to retinal vascular

endothelium, resulting in EC death, vascular leakage, and capillary closure¹⁸ (Figure 2).

The current treatments for DMO at reducing vascular leak in the macula once it has occurred, and aim for a dry macula subsequently. No attempt is made at addressing the underlying pathology, although it is possible that some of these treatments may alter retinal function in other ways (such as neurodegeneration) in addition to reducing vascular leakage.

This review evaluates the individual treatments available, and discusses the rationale and potential for combination therapy in DMO.

Laser photocoagulation

Laser photocoagulation was the recommended treatment for DMO for several years.^{29,30} The exact mechanisms of action of laser photocoagulation in reducing oedema in DMO are unknown. Plausible mechanisms include destruction of high-oxygen consuming photoreceptors, increased oxygenation of the retina through diffusion from the choroid, restoration of new retinal pigment epithelium (RPE) barrier, production of cytokines including transforming growth factor- β and pigment epithelium-derived factor from the stimulated RPE as discussed in the review by Bhagat *et al.*¹⁶ In the early treatment diabetic retinopathy study (ETDRS), DMO eyes treated with laser had lower rates of visual loss compared with control group (12% *vs* 24% at 3 years). This benefit was only noticeable in eyes with clinically significant DMO.^{29,30} In eyes with diffuse DMO, response to grid laser photocoagulation was of limited benefit, with only 15% showing a visual improvement, 24% developing visual deterioration, and 61% unchanged.³¹ The average

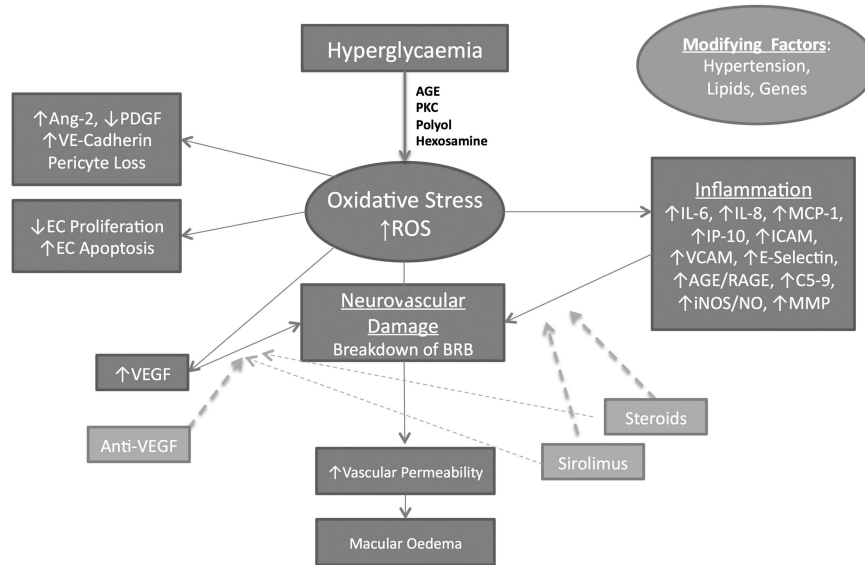


Figure 2 Pathophysiology and treatment mechanisms in diabetic macular oedema (DMO). AGE, advanced glycation products; Ang-2, Angiopoietin-2; EC, endothelial cell; C, complement; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, monocyte chemotactic protein; IP, interferon-induced protein; MMP, metallic metalloproteinase; NO, nitric oxide; NOS, nitric oxide synthase; RAGE, receptor for advanced glycation products; VEGF, vascular endothelial growth factor; VE, vascular endothelial; ROS, reactive oxygen species; PDGF, platelet-derived growth factor; PKC, protein kinase C; BRB, blood–retinal barrier.

best corrected visual acuity (BCVA) change in laser-treated eyes in the diabetic retinopathy clinical research network (DRCRnet) and RESTORE (ranibizumab monotherapy or combined with laser *vs* laser monotherapy for DMO) studies were +2.7 to +3.2 letters at 12 months, and the fovea remained thickened in a large proportion of the laser-treated eyes. Although effective in some cases of DMO, ETDRS protocol photocoagulation may require placement of burns close to the centre of the macula. Over time, laser burns may develop into areas of progressive RPE and neuroretinal atrophy that become larger than the original laser spot size and encroach upon fixation, or subretinal membranes may occur.^{32,33} Photocoagulation for DMO may be associated with loss of central vision, central scotomas, and decreased colour vision. In an attempt to reduce these adverse effects, many retinal specialists now treat with burns that are lighter and less intense than originally specified in the ETDRS (modified-ETDRS technique).³⁴ In the alternative approach of mild macular grid laser, mild, widely spaced burns are applied throughout the macula, avoiding the foveal region. By design, some burns could be placed in clinically normal retina if the entire retina was not abnormally thickened, including areas within the macula that are relatively distant from the area of thickening.³⁴ As such, laser photocoagulation is not advised in eyes where the leakage is close to the fovea and when the oedema is centre involving.

Subthreshold laser photocoagulation has recently been suggested as a better alternative in the treatment of DMO

as the collateral damage to the retina-choroid complex is limited.^{16,35} That is because subthreshold laser does not destroy the RPE on account of the much shorter duration. The role of subthreshold laser therapy in DMO has yet to be widely taken up, and requires further evaluation.

Pharmacologic treatments

Several pharmacologic agents are now available for the treatment of DMO including anti-VEGF agents^{36–41} and corticosteroids.^{36,42–48} These treatments are summarised in Table 1. These treatments are particularly useful in eyes with centre-involving DMO. Targeting VEGF has resulted in the use of anti-VEGFs, including pegaptanib, ranibizumab, aflibercept, and bevacizumab, in the treatment of DMO.^{36–41,43} Several clinical studies have investigated the efficacy of steroids, such as triamcinolone, fluocinolone, and dexamethasone, in the treatment of DMO.^{13,42,46–51} Corticosteroids may reduce DMO by targeting the non-VEGF-dependent inflammatory pathways including blockage of the arachidonic acid pathway (reduction of prostaglandin synthesis), and inhibition of the release of pro-inflammatory mediators, including VEGF. This may be through modulation of EC TJ molecules.¹⁹

Ranibizumab

In the RISE and RIDE Study of two multicentre double-blind controlled trials, of intravitreal ranibizumab

Table 1 Summary of outcomes of treatment with DMO with different agents

Pharmacological treatment/study (study type)	Study groups	Size	VA outcomes	OCT outcomes	Adverse events
Aflibercept VIVID and VISTA (RCT, multicentre, masked, phase III, randomised)	1:1:1, aflibercept 2 mg every 4 weeks (2q4) <i>vs</i> 8 weeks after 5 initial monthly doses <i>vs</i> macular laser photocoagulation (control group; baseline laser plus sham injections every visit)	872 Eyes of type 1 and DM	Mean BCVA gains of 12.51 and 10.71 <i>vs</i> 0.21 in the 2q4 and 2q8 groups <i>vs</i> the laser group at 12 m. Gain of 151 of 41.6 and 31.1% <i>vs</i> 7.8% in VISTA, and 32.4 and 33.3% <i>vs</i> 9.1% in VIVID at 12 m	Mean CRT reduction of –185.9 and –183.1 u <i>vs</i> –73.3 u in VISTA, and –195.0 and –192.4 <i>vs</i> –66.2 u in VIVID at 12 m	Event rates (ocular and non-ocular, and SAEs) were similar in the treatment the groups
Ranibizumab (Ran) RISE and RIDE (RCT, masked, multicentre, phase III)	Ran 0.3 and 0.5 mg <i>vs</i> sham injection control with laser rescue	377 Eyes	Gain of > 151 in 33.6% of 0.3 mg Ran, 45.7% of 0.5 mg Ran treated <i>vs</i> 12.3% in laser control group at 24 m	CRT reduction of 250.6 u in 0.3 mg Ran, 253.1 u in the 0.5 mg Ran, and –133.4 u in the laser control	Slight increase in deaths and APTC events among the 0.5 mg monthly treated group <i>cf</i> controls and the 0.3 mg group
Ran RESOLVE (RCT, double masked, multicentre, phase II)	2 Doses Ran (0.3 and 0.5 mg) <i>vs</i> sham control	151 Patients	Gain of 10.31 (11.81 in 0.3 mg group and 8.81 in 0.5 mg) Ran compared with –1.41 in control at 12 m	CRT reduction by 200.7 u in the 0.3 mg Ran, 187.6 u in the 0.5 mg <i>vs</i> 48.4 u in the control group	
Ran RESTORE (RCT, double masked, multicentre, phase III)	Laser controlled	354 Patients	BCVA score ≥ 151 improvement in 22.6% and BCVA letter score level ≥ 73 (6/12) in 53% in the Ran-treated eyes, 22.9 and 44.9% Ran plus laser <i>vs</i> 8.2% and 23.6%, respectively, in the laser-only group	CRT reduction by 118.7 u with Ran and 128.3 u with Ran plus laser compared with –63.3 u in the laser-treated group	
Ran, Tri DRCRN ^{et} 43 (RCT, multicentre, masked, controlled phase III)	4 Arms: 0.5 mg Ran plus prompt laser, 0.5 mg Ran plus deferred laser (≥ 24 weeks), 4 mg Tri plus prompt laser, or sham injection of Ran plus prompt laser Follow-up: 1–2 years	854 Eyes of 691 patients	Gain of 101 in 50%, and 151 in 30% of Ran and prompt laser eyes at 12 m compared with 47% and 28% in Ran and deferred laser, 33% and 21% in tri and laser, and 28% and 15% in the laser groups, respectively. Mean BCVA gain of 9.01 in the Ran +prompt laser, or Ran+deferred laser at 12 m, <i>cf</i> 41 in Tri plus laser, and +31 in the sham injection +prompt laser control group. At 2 years, outcomes are +7, +9, +2, and +31, and a gain of > 101 occurred in 44%, 49%, 41%, and 36%, respectively, at 2 years. In Tri group, pseudophakia gave VA outcomes similar to Ran group. Results maintained at 24 and 36 m with deferred laser doing better	CRT improvements mirrored VA changes. CRT reduction of –102 u with prompt laser, –131 u in Ran with prompt laser, –137 u in Ran with delayed laser and –127 u with triamcinolone with prompt laser at 6 m. CRT reduction at 2 years was –138 u, –141 u, –150 u, and –107 u, respectively	Progression of cataracts and IOP elevation significant in the Tri-treated group

Table 1. (Continued)

Pharmacological treatment/study (study type)	Study groups	Size	VA outcomes	OCT outcomes	Adverse events
Bevacizumab (Bev) BOLT Study (RCT)	2 Arms, Bev vs macular laser; 12 m	80 Eyes	VA gain of +8.61 in Bev group compared with -0.51 in the laser control group. > 101 gain in 49% in Bev vs 7% in control, and > 151 loss in 32% control group of to 4% Bev	CRT reduction of -146 u in the Bev group of -118 u in the laser group	
Bev Hong Kong Study ⁵⁶ (RCT)	2 Doses of Bev (1.25 and 2.5 mg Bev), 6 m	52 Eyes	VA gain of +5.51 in the 1.25 mg group, and 6.51 in the 2.5 mg group at	CMT reduction of 96 u in the 1.25 mg group of 74 u in the 2.5 mg group	
Bev Iran Study ¹³⁷ (RCT, masked)	2 Arms of Bev 1.25 mg with or without laser	80 Eyes of 40 patients	Mean change of BCVA of 0.138 LogMAR in the Bev group compared with 0.179 in the combination group. No difference in outcomes between the 2 groups	CMT reduction of -39 u in both groups	
Pegaptanib (Peg) ⁵³ (RTC, phase 2)	4 Arms: 0.3, 1, and 3.0 mg Peg 6 weekly vs sham control	172 Eyes of 172 patients	VA gain of +4.71 in the 0.3 and 1 mg, and +1.11 in 3.0 mg Peg groups vs -0.41 gain in controls at 36 weeks	CRT reduction of -68.0, -22.7, and -5.3 u in the 0.3, 1.0, and 3.0 mg Peg, respectively, vs +3.7 u in controls	
Peg ⁵⁴ (Multicentre, Phase II/III, RCT)	2 Arms: 0.3 mg Peg 6 weekly vs placebo controlled	260 Eyes of 260 patients	VA gain of +5.21 in 0.3 mg Peg group of 1.21 in controls at 12 m, and +6.11 and +1.31, respectively, at 24 m	CMT decrease of ≥25% in 31.7% vs 23.7%, and ≥50% in 14.6% and 11.9%, respectively, in the Peg and control groups at 12 m and ≥25%: 40.4% vs 44.6%, and ≥50%: 19.2% vs 26.1%, respectively, at 24 m	
Dexamethasone MEAD Study (2 RCTs with identical protocols)	3 Arms: 0.7 mg, 0.35 mg Dexamethasone implants and sham treated (1:1:1), followed up for 3 years	1048 Eyes of 1048 patients	VA gain of ≥151 was 22.2%, 18.4% and 12.0% in 0.7 mg, 0.35 mg, and control groups, respectively	Mean reduction of CRT of -111.6 u, -107.9 u, and -41.9 u, respectively, in 0.7 mg, 0.35 mg Dex, and sham controls, respectively	Cataract 67.9%, 64.1%, and 20.4%, respectively. IOP rise requiring treatment occurred in 41.5%, 37.6% and 9.1% of the 0.7 mg, 0.35 mg Dex, and sham-treated controls. IOP increases were controlled with eye drops; 2 in the 0.7 mg and 1 in the 0.35 mg Dex groups required trabeculectomy

Table 1. (Continued)

Pharmacological treatment/study (study type)	Study groups	Size	VA outcomes	OCT outcomes	Adverse events
Fluocinolone FAME Study (Multicentre, RCT)	3 Arms placebo-controlled: Fluocinolone implant 0.2 µg/day, 0.5 µg/day, and control groups (2:2:1)	956 Eyes of 956 patients	VA gain of +4.4, +5.4, and +1.7 in the 0.2 µg, 0.5 µg, and control groups, respectively, at 24 m. ≥151 gain in patients 29.0%, 29.0%, and 16.0%, respectively, in the 0.2 µg, 0.5 µg and control, groups at 24 m	CRT reduction of -167.8, -177.1, and -111.3 u in the 0.2 µg, 0.5 µg and control groups, respectively, at 24 m	Cataracts in 81.7% in 0.2 µg group cf 50.4% controls. Cataract surgery performed in 80.0% and 27.3%, respectively. IOP rise >30 mmHg in 18% of 0.2 µg Fluoro group cf 4.3% in controls. IOP lowering medication required in 38.4 and 14.1%, trabeculectomy required in 4.8% of 0.2 µg and 0.5% of controls
Triamcinolone (Tri) DRCR Network Protocol (Multicentre, RCT)	3 Arms: 1 mg Tri repeated 3.5X, 4 mg, Tri repeated 3.1X, and macular laser (2.9X) over 2 years	840 Eyes of 693 patients	BCVA gain of -21, -31, and +11 for the 1 mg, 4 mg Tri and laser groups, respectively, at 2 years. 101 gain occurred in 25, 28, and 31%. 101 loss occurred in 26, 28 and 19%	Reduction in CMT was -86, -77, and -139 u in the 1 mg Tri, 4 mg Tri, and laser groups, respectively, at 2 years	Progression of cataracts and elevated IOP esp in the 4 mg occurred in the Tri groups

Abbreviations: Ran, ranibizumab; Bev, bevacizumab; Peg, pegaptanib; Tri, Triamcinolone; VA, visual acuity; BCVA, best corrected visual acuity; CRT, central retinal thickness; CMT, Central Macular Thickness; IOP, intraocular pressure; APTC, Antiplatelet Trialists' Collaboration.

(0.3 and 0.5 mg; Lucentis, Genentech, South San Francisco, CA, USA/Novartis, Basel, Switzerland) was compared with laser photocoagulation, 45.7% in the ranibizumab group gained >15-letter improvement in visual acuity (VA) at 24 months compared with 12.3% in the laser-treated group.³⁹ There was a signal for increased deaths and acute thrombotic events (as per the Antiplatelet Trialists' Collaboration criteria) in patients treated with the monthly 0.5 mg ranibizumab compared with the laser-treated group. However, these differences were not statistically significant, and have not been repeated in related studies. In RESOLVE, a 12-month, multicentre, sham-controlled, double-masked study of eyes with centre involving DMO (age 18 years, type 1 or 2 diabetes, central retinal thickness (CRT) 300 µm), patients received 3 monthly injections of ranibizumab followed by rescue laser or prn ranibizumab.³⁷ BCVA improved from baseline by a mean of 10.3 ± 9.1 letters with ranibizumab and declined by 1.4 ± 14.2 letters with sham ($P < 0.0001$). A mean reduction of CRT of 194.2 ± 135.1 µm with ranibizumab and 48.4 ± 153.4 µm with sham ($P < 0.0001$) was achieved. Similarly, the RESTORE Study³⁸ demonstrated superiority of the 0.5 mg ranibizumab monotherapy group or in combination with laser photocoagulation compared laser alone, with a mean of seven injections over 12 months. There was a reduction in CRT on optical coherence tomography (OCT) by -118.7 u with ranibizumab compared with -63.3 u in the laser-treated group.³⁸ In the DRCR.net Study (Protocol I), 50% of eyes treated with intravitreal ranibizumab showed >10-letter improvement at 24 weeks compared with baseline; 30% gained >15 letters.⁴³ Results were similar whether laser photocoagulation was prompt or deferred for >24 weeks. The results were maintained at 24 and 36 months, although eyes with deferred laser doing better in the longer term (2.9 letters better at 36 months).^{36,43,52} These VA changes were associated with parallel reductions in CRT as measured with OCT.³⁶

Aflibercept

The VIVID and VISTA Studies are two parallel studies that compared the efficacy and safety of aflibercept (Eylea, Bayer) given every 4 or 8 weeks after an initial monthly dosing for 5 months with focal laser photocoagulation at 52 weeks.⁴¹ The results showed that there was significant superiority of aflibercept in anatomical and functional end points when compared with laser. The efficacy of the 8 weekly dosing was similar that of 4 weekly dosing. The mean BCVA improvement at week 52 was 12.5 and 10.7 letters in the 4 weekly and 8 weekly treated groups, respectively, compared with 0.2 letters in the laser photocoagulation group of VISTA. In the VIVID Group, the corresponding improvements were

10.5, 10.7, and 1.2 letters, respectively.⁴¹ The mean reductions in CRT were 185.9 and 183.1 compared with 73.3 μm ($P < 0.0001$) in VISTA and 195.0 and 192.4 compared with 66.2 μm in VIVID. The proportion of eyes gaining 15 letters or more were 41.6% and 31.1% compared with 7.8% in VISTA, and 32.4% and 33.3% compared with 9.1% in VIVID ($P < 0.0001$).⁴¹

Pegaptanib

Two different randomised controlled studies evaluated the efficacy of pegaptanib (Macugen, Eyetech Pharma, New York, NY, USA/Pfizer, New York, NY, USA) in DMO. In the Cunningham study (a phase II study), the 0.3 and 1 mg injection groups had a better visual outcome with a gain of +4.7 letters at 6 months.⁵³ In another study (Phase II/III), Sultan *et al*⁵⁴ compared outcomes with 0.3 mg pegaptanib with sham. The BCVA at 2 years was significantly better in the pegaptanib-treated group (gain of +6.1 letters) compared with sham (+1.3 letters). However, there was no difference in proportions of eyes gaining 10 letters or more (38.3% pegaptanib *vs* 30.0% sham).⁵⁴ Pegaptanib has not received marketing authorisation for the treatment of DMO in the European Union.

Bevacizumab

Bevacizumab (Avastin, Roche/Genentech), which is unlicensed for any intraocular indication, has also been used in the treatment of DMO. The BOLT study reported a 10-letter improvement in BCVA at 24 months for eyes treated with bevacizumab compared with 7% in the multiple focal laser photocoagulation group.⁵⁵ Similarly, there was an 8.6-letter improvement in the bevacizumab-treated group compared with -0.5 letters in the laser group. More significantly, there was visual loss of more than 15 letters or more in the laser compared with the bevacizumab group.⁵⁵ Lam *et al*⁵⁶ compared two different doses of bevacizumab (1.25 and 2.5 mg) in DMO and found no difference in outcomes at 6 months.

Triamcinolone

Triamcinolone reduces macular oedema (in DMO), although the visual outcome is limited, and there is significant association with elevated intraocular pressure (IOP) and cataract formation.^{57–59} In addition, injections needed to be repeated at variable intervals. There was no evidence of long-term benefits of intravitreal injection of triamcinolone as treatment for DMO.^{58,59} The commonly available Kenalog (Squibb) preparation of triamcinolone is unlicensed for intraocular administration. The preservative-free preparations of triamcinolone are not

freely available. Results from the Gillies *et al*⁶⁰ study in Australia were not very different from those in the DRCNnet studies.

Fluocinolone

Fluocinolone 0.59 μg implant (Retisert, Bausch and Lomb, Rochester, NY, USA) resulted in an overall VA improvement of ≥ 3 lines (compared with sham injection/standard of care (SOC)) in 16.8% of implanted eyes at 6 months ($P = 0.0012$), in 16.4% at 1 year ($P = 0.1191$), in 31.8% at 2 years ($P = 0.0016$; SOC, 9.3%); and in 31.1% at 3 years ($P = 0.1566$; SOC, 20.0%). However, there was a significant increase in cataracts and IOP.⁴⁶

With a different design of implant, fluocinolone acetonide implant (Iluvien, Alimera Sciences, Alpharetta, GA, USA) was compared with sham injection (with laser rescue) in patients with DMO in order to evaluate the safety and efficacy of an intravitreal insert of fluocinolone acetonide for the treatment of DMO. Two doses of 0.2 and 0.5 μg of fluocinolone implant were compared with the sham implant group for 36 months. The primary outcome was the difference in the percentage of patients with BCVA improvement by ≥ 15 letters from baseline at month 24 between the treatment was 28.7 and 28.6 in the low- and high-dose insert groups, respectively, compared with 16.2 in the sham group ($P = 0.002$). The mean improvement in BCVA letter score between baseline and month 24 was 4.4 and 5.4 in the 0.2 and 0.5 μg groups.⁴⁷ There was a significant reduction in macular oedema. Further analysis suggested that fluocinolone was more effective in eyes with long-standing DMO, where chronic inflammation was more important than the VEGF-mediated oedema. The fluocinolone implant lasts up to 2 years. However, there was a significant increase in the incidence of IOP elevation and cataracts.⁴⁷

Dexamethasone

In a prospective multicentre open label, 26-week study of 55 eyes to evaluate the safety and efficacy of dexamethasone intravitreal implant (Ozurdex, Allergan, Irvine, TX, USA) 0.7 mg in the treatment of DMO in vitrectomized eyes, there was a significant improvement in BCVA by 6.0 and 3.0 letters at 8 and 26 weeks, respectively, from baseline, and vascular leakage as shown by a reduction from baseline CRT (403 μm) of a mean -156 μm at week 8 ($P < 0.001$) and -39 μm (-65, -13 μm) at week 26 ($P = 0.004$). At week 8, 30.4% of patients had gained ≥ 10 letters in BVCA.⁴⁴ In their most recent report, Boyer *et al*⁴⁵ have evaluated the 3-year outcomes of treatment of DMO, and suggest that the dexamethasone implant was efficacious in the treatment of DMO over the medium term. The mean number of

treatments given over 3 years was 4.1, 4.4, and 3.3 with dexamethasone implant 0.7 mg, dexamethasone implant 0.35 mg, and sham, respectively. The percentage of patients with ≥ 15 -letter improvement in BCVA from baseline at study end was greater with dexamethasone implant 0.7 mg (22.2%) and dexamethasone implant 0.35 mg (18.4%) than sham (12.0%; $P < 0.018$). However, treatments needed to be given more frequently than 6 monthly, probably at 3–4 months intervals, to maintain a dry macula and retain good visual function.⁴⁵ The cataract-related adverse event rates in phakic eyes were 67.9%, 64.1%, and 20.4% in the dexamethasone implants of 0.7 mg, 0.35 mg, and sham groups, respectively. IOP rises associated with the dexamethasone implant were usually controlled with medication.

Gillies *et al*⁶⁰ recently reported that dexamethasone implant (Ozurdex, Allergan) was as efficacious as bevacizumab in reducing DMO, although progressive lens opacities may result in visual loss in the Ozurdex-treated group compared with those treated with bevacizumab.

Potential treatments

Angiopoietin (Ang)-2 blockage

Ang-2 is another cytokine with inflammatory and angiogenic properties that is expressed by EC. Ang-2 levels are regulated at the level of transcription by different cytokines including VEGF, as well as hypoxia and high-glucose levels,^{61–64} and stored in Weibel–Palade bodies before rapid release.⁶⁵ It is elevated in eyes with clinically significant DMO^{66–68} as well as proliferative DR (PDR),^{69,70} and is thought to induce loss of vascular endothelial (VE)-cadherin through phosphorylation.⁷¹ Ang-2 may act by exerting a permissive role for supplementing the action of other pro-inflammatory cytokines⁷² through sensitisation of ECs to tumour necrosis factor (TNF)- α and subsequent expression of ICAM-1. Similarly, in the presence of VEGF, Ang-2 increases vascular permeability.⁶¹ Recently, You *et al*⁷³ have reported that pan-retinal laser photocoagulation reduced the serum levels of Ang-2 and VEGF in diabetic patients with PDR compared with the significantly elevated pre-treatment levels. Another study⁷⁴ showed that the changes in serum VEGF and Ang-2 following laser photocoagulation were not significant. It has been suggested that benfotiamine, a vitamin B1 derivative may block three major pathways of hyperglycaemia damage and prevent experimental DR.⁷⁵ Yao *et al*⁶⁴ have suggested that high glucose increased Ang-2 transcription through methoxyglyoxal modifications. Benfotiamine has also been reported to have anti-inflammatory effects.^{76,77} Ang-2 is not expressed by pericytes; pericytes express

Ang-1 (and not Ang-2), which promote EC survival signalling, maintenance of endothelial barrier and vascular maturation and quiescence^{78–81} and reduces VEGF-stimulated leukocyte adhesion to ECs.⁸² Taurine has been reported to inhibit high glucose-induced apoptosis in retinal pericytes through increased Tie-2 expression and downregulation of Ang-2.⁸³

Sirolimus

Sirolimus (rapamycin), a macrolide antibiotic, has immunosuppressive and anti-proliferative activities through its inhibition of the mammalian target of rapamycin (mTOR).⁸⁴ Sirolimus, through its inhibition of mTOR, inhibits VEGF expression and signalling, and downregulates HIF-1A.^{85–90} In addition, sirolimus has been shown to inhibit VEGF-induced microvascular permeability, and reduces protein kinase C (PKC) activity,^{85–91} as well as reduces the expression of inflammatory cytokines.^{92,93} Recently, a phase 1 study of subconjunctival and intravitreal sirolimus in DMO was reported by Dugel *et al*.⁹⁴ No non-ocular adverse events were noted, and the systemic exposure to sirolimus was low. There were no serious ocular adverse events reported. A median BCVA improvement of 4.0 letters was noted at day 14 and maintained for 90 days, associated with a reduction in the CRT of -33 u at day 14 and -68.3 u at day 90. Similarly, Krishnadev *et al*⁹⁵ in their phase I/II study reported sirolimus to be safe when administered subconjunctivally, although the treatment effect was variable. Progress in the development of sirolimus formulations for intraocular delivery (DE-109, Santen Pharma, Osaka, Japan) is encouraging.

Combination therapy

Scientific basis

The available evidence suggests that each individual treatment modality in DMO does not result in a completely dry macula in most cases. Furthermore, it takes a while to achieve a dry macula in most cases, requiring frequent treatments. The ideal treatment for DMO should improve vision and improve morphological changes in the macular (eg, reduce macular oedema) for a significant duration, reduced adverse events, reduce treatment burden and costs, and be well tolerated by patients.

It is known that retinal vascular leakage in DMO is contributed to by VEGF upregulation and non-VEGF-dependent inflammatory pathways, which may be chronic and subclinical.^{18–28} Accumulating evidence also suggests that chronic subclinical inflammation has an important role in the pathogenesis of DR. An early event

in the pathogenesis of diabetic vasculopathy is leukocyte adherence to retinal vascular endothelium, resulting in EC death, vascular leakage, and capillary closure.¹⁸

Clinical evidence indicates that DMO results from breakdown of the inner blood–retinal barrier,⁹⁶ which is maintained by EC TJ complexes.⁹⁷ TJ proteins include occludins, claudins, and junctional adhesion molecules (JAMs), transmembrane molecules, which interact with intracellular molecules including zonula occludens (ZO) and cingulin. Adherens junctions are formed by VE-Cadherin, which promotes calcium-dependent homophilic cell to cell contacts, and interact intracellularly with actin through catenins. Dejana *et al*⁹⁸ have reported that the principal TJ proteins found in retinal ECs (RECs) are occludin and claudin-5. Phosphorylation of occludin and ZO1 contribute to the dysregulation of EC paracellular permeability.⁹⁹ It has been suggested that the hyper-permeability induced by diabetes and hyperglycaemia is VEGF mediated.^{100,101} VEGF-A is thought to be the main factor responsible for increased retinal vascular permeability as upregulation of it results in phosphorylation of TJ proteins (occludin and ZO) and their disassembly.^{102,103} Exposure of RECs to hyperglycaemia has also been reported to result in increased MMP-9 levels, which lead to active proteolytic degradation of EC TJs especially occludin.¹⁰⁴ Mukarami *et al*¹⁰⁵ and Felinski *et al*¹⁰¹ have attempted to elucidate the mechanisms of VEGF enhancement of vascular permeability. Other factors including TNF α and interleukin (IL)-1B,¹⁰⁶ nuclear factor- κ B (NF- κ B) receptor activator,^{21,22} hepatocyte growth factor (HGF),²³ and the accumulation of advanced glycation end products²⁰ are known to induce vascular leakage independent of VEGF-A. Adhesion molecules such as CD44^{24,25} may also contribute significantly to microvascular occlusions and angiogenesis.

JAMs also signal to cytoskeleton-associated proteins, as well as recruiting cellular polarity proteins. The preferential expression of different TJ and adherens junction molecules in different vascular beds may explain their specific physiologic functions, for example, the high expression of JAM-A in the blood–brain barrier (blood–retinal barrier),¹⁰⁷ and the relative high expression of JAM-A in the liver compared with its minimal expression of JAM-B and C, substitution or redundancy.¹⁰⁸ JAM-C is highly expressed in inter-EC junctions in lymph nodes.¹⁰⁹ Different JAMs including JAM-A, -B, -C are altered in diabetes and hyperglycaemia.¹¹⁰ Furthermore, different capillary beds may respond differently to different stimulatory or inhibitory factors.¹¹¹ Similarly, they may differ in their basal and TNF α -regulated expression of adhesion molecules and cytokines.¹¹² This is supported by the observation that although DR is a microvascular disease microangiopathy does not seem to have a major

pathogenic role in the cerebral complications of diabetes and the blood–brain barrier is not as susceptible to hyperglycaemia as the retinal microvasculature.¹¹³

It has been reported that hyperglycaemia can stimulate both NF- κ B- and PKC-dependent pathways thus upregulating endothelial adhesion molecules, such as ICAM-1 (CD54) or E-Selectin (CD62E),^{114–116} vascular cell adhesion molecule-1 (CD106),¹¹⁷ and platelet endothelial cell adhesion molecule (CD31), in ECs. ICAM-1 upregulation is thought to be VEGF dependent, whereas P- and E-Selectin are not.¹¹⁸ Vitreous levels of several inflammatory cytokines (including IL-6, IL-8, and monocyte chemotactic protein (MCP)-1) are increased in eyes with DMO and PDR.¹¹⁹ In other studies, Funatsu *et al*^{120–122} have reported significant elevations of VEGF, ICAM-1, IL-6, MCP-1, and pigment epithelium-derived factor, which correlated with retinal vascular permeability changes. In addition to this pathway, stimulation of 6-N-acetyl glucosaminyl-transferase in leukocyte can result in more modification of glycans on its surface thus inducing leukocyte rolling interactions to endothelial selectins.^{123–125}

Nehme and Edelman¹²⁶ have reported that hyperglycaemia, TNF α , and IL-1 induce an inflammatory phenotype in human retinal pericytes, characterized by hypersecretion of inflammatory and angiogenic mediators. No such change was noted in the retinal microvascular ECs in this study. Dexamethasone at various potencies blocked hypersecretion of several proteins in pericytes.¹²⁶ It was further suggested that corticosteroids may inhibit PLA2 and downstream products, for example, prostaglandins and leukotrienes, as well as inhibit adhesion signalling pathway components, including ICAM-1, IL-6, VEGF-A, and stromal cell-derived factor-1, which are involved in vascular permeability and leucocytosis (inflammation).¹²⁷ Steroids may also inhibit TJ disassembly. Further elucidation of the cellular mechanisms underlying increased permeability in diabetes and hyperglycaemia, and the effects of different pharmacologic agents and their interactions is required.

Corticosteroids have significant effects on RECs.^{101,128} It has been suggested that steroids may act by both suppressing inflammation and by directly affecting the ECs by regulating phosphorylation, organisation, and content of TJ proteins.¹⁰¹ Felinski *et al*¹²⁸ have reported that glucocorticoid treatment of primary RECs increases content of the TJ proteins occludin and claudin-5, in parallel with an increase in barrier properties of endothelial monolayers, therefore restoring endothelial paracellular permeability. However, the exact mechanisms of action are poorly understood.

Microarray analysis studies in our laboratory have determined that occludin and claudin-5 gene expression

was higher in RECs than CECs under normal glucose culture conditions.¹¹⁰ We have further determined that there are differences in HGF expression between RECs and CECs, as well as c-Met (the HGF receptor). Specifically, CEC secrete HGF at higher levels than REC.¹²⁹ These results would support the concept that CEC and REC behave differently under hyperglycaemia, and may explain, at least in part, some of the clinical differences observed in the frequencies/prevalences of DR and choroidopathy.^{6–9} Western blotting indicated that claudin-5 protein expression was also higher in RECs, whereas JAM-A and C and VE-Cadherin levels were similar.¹¹⁰ High-glucose conditions (equivalent to hyperglycaemia) significantly increased the permeability in both REC and CEC monolayers, although the increase was higher for RECs.¹¹⁰ In RECs exposed to high-glucose claudin-5, occludin, and JAM-A were found to be reduced, and JAM-C increased, whereas the expression of VE-Cadherin was relatively unchanged when evaluated with western blotting, immunofluorescence, and qPCR.

Recent studies have reported that there are significant changes in the cytokine profile in DMO. The elevated cytokines include VEGF, MCP-1, IL-6, IL-8, interferon γ -induced protein-10.^{119,130–132} Funatsu *et al*^{120–122} have also previously reported increased levels of ICAM-1, IL-6, Angiotensin II, and VEGF in DR and DMO. Klein *et al*¹³³ reported that sVCAM-1 and TNF α are associated with higher odds of developing more severe DR in patients with renal disease and PDR. Other evidence suggests that anti-VEGF treatments have no effect on the cytokines other than VEGF levels in intraocular fluids.^{130–132}

The available evidence therefore implies that targeting VEGF alone or VEGF-independent pathways in isolation may not adequately block the increased permeability associated with diabetes and hyperglycaemia. A combination approach may be necessary in some cases. Steroids and anti-VEGF may have complementary and additive effects on retinal vascular permeability. This follows from the evidence of Nehme and Edelman¹²⁶ on the effect of dexamethasone on REC and pericyte permeability, and the reports from Felinski,^{101,128} in concert with the anti-VEGF trials. The concept of combination pharmacotherapy in DMO has been given further impetus by preliminary results from our laboratory, which show that a combination of dexamethasone and ranibizumab reduced REC permeability more than either agent alone *in vitro*.¹¹⁰

Clinical evidence

A few trials have evaluated the combination of bevacizumab with triamcinolone.^{134–137} Ahmadi *et al*¹³⁴ evaluated bevacizumab alone in comparison to bevacizumab and triamcinolone, and determined that

there was no further improvement with the combination compared with bevacizumab alone. Soheilian *et al*^{135,136} compared combination of 1.25 mg bevacizumab and 2 mg triamcinolone with bevacizumab alone and laser photocoagulation alone. Bevacizumab alone improved BCVA at 36 months, compared with either the combination or the laser alone group.^{135,136} Faghihi *et al*¹³⁷ compared intravitreal bevacizumab (1.25 mg) alone with combination of bevacizumab and focal laser, and determined at 6 months that both regimes had comparable vision improvement and that neither dosage was superior to the other. The outcomes reported by Faghihi *et al*¹³⁷ that combinations of bevacizumab and focal laser photocoagulation induced earlier recovery of VA compared with bevacizumab monotherapy, although this was not confirmed by Soheilian *et al*.¹³⁸ Sheth *et al*¹³⁹ did not find any difference in DMO reduction or post injection VA in eyes treated with intravitreal triamcinolone *vs* dexamethasone and bevacizumab. However, that is most likely because of the short duration of the dexamethasone used, as well as the small sample size.¹³⁹ This concept has been given further impetus by preliminary results from our laboratory, which show that a combination of dexamethasone and ranibizumab reduced REC permeability more than either agent alone *in vitro*.¹¹⁰ In the DRCRNet Study,^{43,52} intravitreal 0.5-mg ranibizumab or 4-mg triamcinolone combined with focal/grid laser was compared with focal/grid laser alone for treatment of DMO. At 24 months, the mean change in the VA letter score from baseline was 3.7 letters greater (95% CI: –0.4 to +7.7) in the ranibizumab plus prompt laser group, 5.8 letters greater in the ranibizumab plus deferred laser group (95% CI: +1.9 to +9.8) and 1.5 letters worse in the triamcinolone plus prompt laser group (95% CI: –5.5 to +2.4).³⁶ These VA changes were associated with parallel reductions in CRT as measured with OCT.³⁶ Combination of dexamethasone implant with laser photocoagulation has been reported to result in more reduction in macular oedema and greater BCVA improvement up to 9 months compared with either agent on its own.¹⁴⁰ No significant difference was noted at 12 months in the number of eyes that gained 10 letters or more, however.

The way forward

It is possible that an initial combination of dexamethasone and anti-VEGF therapy (ranibizumab or aflibercept) will allow a faster VA rise to the plateau than achieved with anti-VEGF monotherapy, may induce a longer remission of DMO, and more permanently modulate the recurrence of DMO than after either agent on its own. This is important as complete dryness of the macular is only achieved in about 50–75% of eyes treated with anti-VEGF

monotherapy. Furthermore, such a combination will reduce potential systemic adverse events as may be associated with frequent anti-VEGF therapies. As the effect of the dexamethasone implant only lasts 3–6 months, any associated IOP rise is short-lived. The longer-acting steroid, fluocinolone may also have a role in combination with anti-VEGF therapies especially in eyes that are pseudophakic. This follows from the fact that fluocinolone is more effective in eyes with longer duration of DMO than those with more recent onset. This is despite the side-effect profile of steroids including cataracts, and increased IOP. Potentially, if such a combination works, only one or two dexamethasone implant injections will be required, but at the same time reducing the frequency of intravitreal anti-VEGF injections.

Macular laser photocoagulation (focal) applied after treatment with anti-VEGF and/or dexamethasone implant (deferred laser) allows lower energy laser applications thus reducing collateral damage. This, as seen in the DRCRnet studies, gives better outcomes and results in reduction of the number of intravitreal injections required, with increased BCVA gain (2.9 letters better at month 36).⁵²

In the future, mTOR inhibition by subconjunctival or intravitreal sirolimus, or inhibition of Ang-2 alongside anti-VEG therapies may also more result in significant benefit in DR. For the anti-Ang-2 drug, the mode of delivery will, however, depend on the particular properties.

Similarly, other drugs that may interfere with any of the mechanisms in the development of DR, for example, benfotiamine are worth exploring as adjuncts to other therapies in DMO.

Fovista is an anti-platelet-derived growth factor agent currently under development as an intravitreal agent for the treatment of neovascular age-related macular degeneration in combination of anti-VEGF drugs.¹⁴¹ At the present time, it is not envisaged that it will have a role in the treatment of DMO, until it is shown to be unlikely to induce more pericyte loss in the diabetic retina. Furthermore, systemic platelet-derived growth factor-B was not found to be significantly upregulated in patients with DMO when compared with normal individuals and the significant upregulation in neovascular age-related macular degeneration.¹⁴²

Further elucidation of the cellular mechanisms underlying increased permeability in diabetes and hyperglycaemia, and the effects of different pharmacologic agents and their interactions are required. Similarly, cellular and physiological changes in the choroidal vasculature in diabetes require further investigation.

Systemic control of hyperglycaemia (Hb A1C), blood pressure and serum lipids, as well as the addition

fenofibrates and/or statins in diabetics cannot be over-emphasised as these have a significant effect on the fluid leakage in the macula.^{143–146}

Conclusion

This review has evaluated the individual treatments available for DMO, and discussed the rationale and potential for combination therapy in DMO. Systemic factors including blood sugar, blood pressure, and lipids cannot be over-emphasised.

The ideal treatment for DMO should improve vision and morphological changes in the macular (eg, reduce macular oedema) for a significant duration, reduce adverse events, reduce treatment burden and costs, and be well tolerated by patients. Laser photocoagulation is of limited use in centre-involving DMO, and requires augmentation in eyes where the foveal centre is threatened. No current individual treatment modality in DMO results in a completely dry macula in most cases. This is understandable as there are multiple mechanisms including VEGF pathway, and the non-VEGF inflammation, which becomes more important in the chronic stages of DMO. Eyes with chronic DMO are more likely to benefit from intravitreal steroid therapy. A combination of anti-VEGF therapies with steroids will provide more optimal pharmacotherapy, and may be supplemented with macular laser photocoagulation as necessary in individual cases. Newer agents in the future will further enhance efficacy of our treatments for DMO.

Conflict of interest

WMK Amoaku has provided consultancy services to Alcon, Allergan, Bayer, Novartis and Thrombogenics. He has received travel grants from Allergan, Bayer and Novartis, and honoraria for lectures from Allergan, Novartis. He has participated in clinical trials for which his institution has received funding from Allergan, Novartis and Pfizer. His institution has further received research grants from Allergan and Novartis for non-clinical studies, and CentreVue (Italy) for clinical studies. The remaining authors declare no conflict of interest.

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Research article

Dexamethasone reverses the effects of high glucose on human retinal endothelial cell permeability and proliferation *in vitro*E.A. Stewart¹, S. Saker¹, W.M. Amoaku^{*}

University of Nottingham, Academic Ophthalmology, Division of Clinical Neuroscience, B Floor, Eye and ENT Building, Queen's Medical Centre, Nottingham, NG7 2UH, UK

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ABSTRACT

Diabetic macular oedema (DMO), a leading cause of preventable visual loss in the working population, is caused by an increase in microvascular endothelial cell permeability, and its prevalence is on the increase in parallel with the rising worldwide prevalence of diabetes. It is known that retinal vascular leakage in DMO is contributed to by VEGF upregulation as well as non-VEGF dependent inflammatory pathways, and the potential use of anti-inflammatory agents such as the glucocorticoids, including dexamethasone are being extensively studied. However, the mechanisms of action of dexamethasone in DMO reduction are not fully understood. Using human primary retinal endothelial cells (REC) the *in vitro* effect of dexamethasone in modulating the proliferation, permeability and gene expression of key tight and adheren junction components, and the expression of angiopoietins (Ang) 1 and 2 in high (25 mM) glucose conditions were investigated. High glucose decreased REC proliferation, an effect that was reversed by dexamethasone. High glucose conditions significantly increased REC permeability and decreased claudin-5, occludin and JAM-A gene expression; dexamethasone was effective in partially reversing these changes, restoring EC permeability to the normal or near normal state. High glucose levels resulted in reduction of Ang1 secretion, although Ang2 levels were consistently high. DEX increased Ang1 and decreased Ang2, indicating that the balance of Ang1/Ang2 may be important in determining functional changes in REC under high glucose conditions.

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1. Introduction

Diabetic macular oedema (DMO), central retinal (macular) thickening due to the accumulation of fluid is the most common cause of visual loss resulting from diabetic retinopathy (DR), a microvascular complication (Ciulla et al., 2003; Klein et al., 1998; Resnikoff et al., 2004). The incidence of DR correlates with poor glycaemic control and hyperlipidaemia, and aggravated by uncontrolled systemic hypertension, renal disease and smoking (Bhagat et al., 2009; Bhavsar, 2006; Bhavsar and Tornambe, 2006; Kiire et al., 2013). DMO results from breakdown of the inner blood-retinal barrier which is maintained by retinal endothelial cell

(REC) tight junction (TJ) complexes (Vitale et al., 1995), resulting in an increase in microvascular endothelial permeability. Hyperglycaemia results in the generation of reactive oxygen species (ROS) through activation of the protein kinase C (PKC) isoforms, over-activity of the hexosamine pathway, increased flux of glucose and other sugars through the polyol pathway, increased intracellular formation of advanced glycation products (AGEs) and increased expression of the receptor for AGEs (RAGE) (Brownlee, 2005). Other cellular effects of hyperglycaemia damage include the activation of the renin-angiotensin pathway (Anderson et al., 1993; Gilbert et al., 2000; Wright and Dodson, 2010), and peroxisome proliferator-activated receptor gamma (PPAR- γ) receptor (Muranaka et al., 2006). AGEs induce blood-retina breakdown in normal rats (Stitt et al., 2000), and AGE inhibition has been reported to reduce retinal vascular permeability in experimental diabetes (Stitt et al., 2002). It is known that retinal vascular leakage in DMO is contributed to by VEGF upregulation as well as non-VEGF dependent inflammatory pathways with several investigators reporting increased aqueous levels of IL-6, IL-8, IP-10, and MCP-1 in addition to VEGF in eyes with DMO (Koch et al., 1995; Lu et al., 1999; Roh

Abbreviations: Diabetic macular oedema, DMO; diabetic retinopathy, DR; tight junction, TJ; Junctional Adhesion Molecule, JAM; endothelial cell, EC; adherens junctions, AJ; retinal endothelial cells, REC.

^{*} Corresponding author.

E-mail addresses: E.stewart@nottingham.ac.uk (E.A. Stewart), msxs1@nottingham.ac.uk (S. Saker), wma@nottingham.ac.uk (W.M. Amoaku).

¹ These authors contributed equally to this publication.

et al., 2009; Yoshimura et al., 2009). Inflammatory changes associated with DR, involve leukostasis (Miyamoto and Ogura, 1999), with resulting increased vaso-occlusion and micro-vascular permeability, and upregulation of various cytokines (including TNF- α , and IL-6) (Jousseaume et al., 2004; Yuuki et al., 2001), and intercellular adhesion molecules including ICAM-1 (CD54), E-Selectin (CD62E), vascular cell adhesion molecule 1 (VCAM-1; CD106) and platelet endothelial cell adhesion molecule (PECAM; CD31) (Booth et al., 2002; Koch et al., 1995; Morigi et al., 1998; Omi et al., 2002). ICAM-1 upregulation is thought to be VEGF dependent, whilst P- and E-Selectin are not (Lu et al., 1999). It is also known that the accumulation of AGEs induce vascular leakage independent of VEGF-A, and that inhibition of AGE-mediated inflammation and oxidative stress with a soluble form of the AGE receptor (RAGE) led to significant reduction in leukostasis and break down of the blood–retinal barrier in diabetes (Kaji et al., 2007).

Non-perfused capillaries in DR corresponding to retinal ischaemia and DMO are likely attributable to accelerated death of both retinal capillary endothelial cells (EC) and pericytes (Hammes et al., 2002; Mizutani et al., 1996), as well as leukostasis (Miyamoto and Ogura, 1999). Nehme and Edelman (2008) have reported that hyperglycaemia, TNF- α , and IL-1 β induce an inflammatory phenotype human retinal pericytes, characterized by hypersecretion of inflammatory and angiogenic mediators. No such change was noted in the microvascular EC in that study. The contribution of subclinical inflammation to this microvascular cell death is still a subject of intense investigation. As inflammation is considered important in the pathogenesis of DR, the potential use of anti-inflammatory agents such as glucocorticoids as treatment are being extensively investigated (Bhavsar, 2006; Jousseaume et al., 2002). Corticosteroids have been reported to have significant effects on REC (Edelman et al., 2005). It has been suggested that steroids may act by both suppressing inflammation and by directly regulating phosphorylation, organization, and content of TJ proteins of the EC. Felinski et al. (2008) have reported that glucocorticoid treatment of primary REC increases the content of the TJ proteins occludin and claudin-5, in parallel with an increase in barrier properties of EC monolayers, leading to restoration of endothelial paracellular permeability. There is enough evidence that REC permeability is directly altered by hyperglycaemia (Felinski and Antonetti, 2005; Saker et al., 2014), and that the effects of hyperglycaemia, and therefore that of dexamethasone (DEX), are not solely on retinal pericytes as suggested by the earlier report of Nehme and Edelman (2008). Several clinical trials continue to demonstrate the efficacy and safety of glucocorticoids, such as triamcinolone acetonide, fluocinolone acetonide and DEX for the treatment of DMO (Bhavsar, 2006; Flynn and Scott, 2005; Gillies et al., 2006; Kupfermann et al., 2007; Munir et al., 2005). This is supported by recent reports on the reduction of oedema associated with decreased levels of inflammatory cytokines in DMO eyes treated with intravitreal triamcinolone (Funk et al., 2010; Sohn et al., 2011), and that increased levels of the same cytokines were observed in association with recurrences of DMO treated with intravitreal bevacizumab (Roh et al., 2009). We have previously demonstrated in our *in vitro* studies that high glucose conditions significantly altered REC micro-vascular permeability, and that this increase is likely caused by a decrease in selective TJ protein expression leading to increased paracellular permeability (Saker et al., 2014).

It is also known that the effects of VEGF on EC permeability are linked to angiopoietins (Thurston et al., 2000; Yancopoulos et al., 2000). Angiopoietins (Ang) 1 and 2 are cytokines that regulate vascular function through their receptor Tie2 on EC (Davis et al., 1996; Maisonnier et al., 1997). It is also known that Ang2 (Benest et al., 2013) and Tie2 (Koehne et al., 2000) are expressed by EC and increased in response to inflammation and hypoxia, while

Ang1 expression is often associated with mural cells and glia in induction and maintenance of the blood-retinal and blood-brain barriers. However, the autocrine secretion of Ang1 by EC *in vitro* and its relevance to modulation by DEX are unknown. Ang1–Tie2 signalling enhances EC barrier through VE-Cadherin (Gamble et al., 2000), and reduces inflammation-induced EC permeability (Baffert et al., 2006), whilst Ang2 leads to destabilisation (Eklund and Saharinen, 2013) and is reported to be increased in high glucose and diabetic vascular dysfunction (Yao et al., 2007). Alterations in the Ang1–Ang2 ratio may significantly influence EC permeability (You et al., 2014).

The purpose of this *in vitro* study was to investigate whether DEX can reverse the effects of high glucose (25 mM) conditions on the REC proliferation and permeability and the expression of key TJ, adheren junction (AJ) components and Ang1 and Ang2.

2. Methods

2.1. Retinal endothelial cell culture

Research was undertaken with the approval of the local research ethics committee (Nottingham Q1060301). Human posterior segments were free of any known ocular disease were obtained from the Manchester Eye Bank. In total 17 donors were used, 7 female, 10 male with ages ranging from 48 to 92 (mean $\pm$ SD = 74 $\pm$ 13.45). REC were isolated and cultured separately for each donor as previously described (Stewart et al., 2011). In brief, retinal membranes were removed by dissection and tissue was treated with 0.1% (w/v) collagenase (from *Clostridium histolyticum*) for 1 h at 37 °C. REC were then isolated using anti-CD31 Dynabeads (Life Technologies). REC were initially cultured on a 35 mm tissue culture dish coated for >2 h with 5 μ g/mL fibronectin (Sigma) in EGM2-MV medium with hydrocortisone omitted and containing 5 mM glucose, (CM; Lonza, Tewkesbury, UK). Confluent REC were transferred once into 6 wells of a 6 well cell culture cluster pre-coated with 5 μ g/mL fibronectin to expand before assays performed.

2.2. Trans-endothelial permeability assay

Once 80–90% confluent on the 6 well plates REC were removed and allowed to adhere to 6 $\times$ 0.4 μ m pore size transwell inserts (Becton Dickinson, USA) in 12 well format. REC on transwell inserts were cultured to confluence, before being washed once with sPBS and divided into 2 groups, treated for 48 h with either CM (5 mM glucose) or CM (25 mM), with medium changed every 24 h. After 48 h REC were washed once with sPBS and stepped to SRM for 24 h with the appropriate glucose concentration. Cells were then incubated DEX at 1 μ M or 100 μ M for 24 or 72 h. Permeability was assessed by the diffusion of Evan's blue-labeled albumin as previously described (Saker et al., 2014). Briefly, confluent inserts were transferred to fresh 12-well cell culture clusters containing 2 mL Hank's balanced salt solution (HBSS) in the basolateral chambers. In the apical compartments, culture medium was replaced by 500 μ L of HBSS containing Evan's blue-labeled albumin (165 μ g/mL; molecular weight, 67 kDa). Both luminal and abluminal sample absorbance was measured at 650 nm, using a microplate reader (THERMOMax). The co-efficient of permeability was calculated as μ L of luminal compartment volume from which the tracer is completely cleared. The experiments were repeated in duplicate wells on 5 independent occasions, with 5 different donor REC samples.

2.3. Proliferation assay

Proliferation of REC was assessed using Cell Counting Kit –8

(CCK8, Sigma). For each 6 well plate of REC from the same donor, once 70% confluent cells were washed once with sterile phosphate buffered saline (sPBS), divided into 2 groups and treated for 48 h with either CM (5 mM glucose) or CM (25 mM), with medium changed every 24 h. Each group of REC were then stepped to 1% foetal bovine serum (FBS)-containing EBM-2 (SRM), keeping the glucose level consistent (5 or 25 mM) for a further 24 h. REC were then incubated with SRM containing a consistent glucose concentration and 1 μ M cell culture-tested DEX (Sigma) for 24 h. Medium was then replaced with 1 mL working concentration of CCK8 reagent and incubated 37 °C, the absorbance of 100 μ L each well was read after for 4 h at 650 nm using a Thermamax microplate reader (Molecular Devices) according to the manufacturer's instructions. Cell number was expressed as a percentage change relative to parallel cultured, donor matched 5 mM controls, examined in triplicate and this assay repeated using REC from 9 different donors.

2.4. Quantitative real-time PCR

REC in 6 well plates were cultured and treated with 5 or 25 mM glucose plus 1 μ M DEX with the same regimen as the proliferation studies. Total RNA was extracted from using an RNeasy Mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. The lysate was homogenized using a QIAshredder spin column (Qiagen), and centrifuged for 2 min at full speed. An equal volume of 70% ethanol was added to the homogenized lysate and transferred to an RNeasy spin column. Buffer RW1 and Buffer RPE were applied to wash the spin column membrane. Finally, total RNA was eluted with 30 μ L RNase-free water, before quantification using a Nanodrop spectrophotometer. cDNA synthesis was performed in a 40 μ L final volume containing 2000 ng total RNA using the QuantiTect Reverse Transcript kit (Qiagen). PCR reactions were prepared in nuclease free optical tubes with 10 μ L of 10 $\times$ TaqMan gene expression master mix (Life Technologies), 1 μ L of the appropriate TaqMan Gene expression assay (CLDN5, hs00533949; JAM-A[F11R], hs00375892; JAM-C; hs00230289; OCLN, hs00170162; CDH5[VE-Cadherin], hs00901465; Thermofisher scientific), 5 μ L of diluted cDNA (1:5 in nuclease free water) and 4 μ L of nuclease-free water. Each reaction was run in triplicate on a Stratagene MX3000p qPCR instrument. Analysis of PCR results, including efficiency and CT calculations, were analysed using the Real Time PCR Miner algorithm (Zhao and Fernald, 2005). All values were normalised to readings of the HPRT1 (hs02800695,

Thermofisher scientific) endogenous reference gene and cells in 5 mM glucose. For each of the 4 treatments triplicate reactions were analysed and the experiment repeated with cells from 3 pairs of donor eyes.

2.5. ELISA

REC were treated with 5 mM or 25 mM then 1 μ M DEX as for qPCR, with medium collected every 24 h. Ang1 and Ang2 ELISA (R&D Systems) were performed as per manufacturer's instructions. ELISA results were corrected for serum-derived baseline growth medium content (Ang1, 573 pg/mL; Ang2, 403 pg/mL) or SRM (Ang1, 30 pg/mL; Ang2, 41 pg/mL) as appropriate.

2.6. Statistical analysis

All experiments were repeated with REC from at least three donors. In each experiment, data were generated from multiple replicates. Independent samples (unpaired, two tailed) t-test was used to compare data. For all analyses, P values below 0.05 were considered indicative of significant difference. For permeability and proliferation assays standard deviation (SD) was used to describe variability; for qPCR standard error of the mean (SEM) was used.

3. Results

3.1. Effect of high glucose and dexamethasone on retinal endothelial cell permeability

The addition of 25 mM glucose significantly increased REC permeability ($p = 0.0222$, Fig. 2A). When 1 μ M DEX was added for 24 ($p = 0.0111$) or 72 h ($p = 0.0004$) there was a significant decrease in REC permeability, amounting to a reversal of the effect of 25 mM glucose (Fig. 2A). With 5 mM glucose DEX showed a slight decrease in baseline permeability after 72 h (ns, $p = 0.2073$). Both 1 μ M ($p = 0.0027$) and 100 μ M ($p = 0.0011$) DEX significantly decreased REC permeability (Fig. 2B).

3.2. Effect of high glucose and dexamethasone on retinal endothelial cell proliferation

High (25 mM) glucose significantly decreased REC number compared to normal medium containing 5 mM glucose

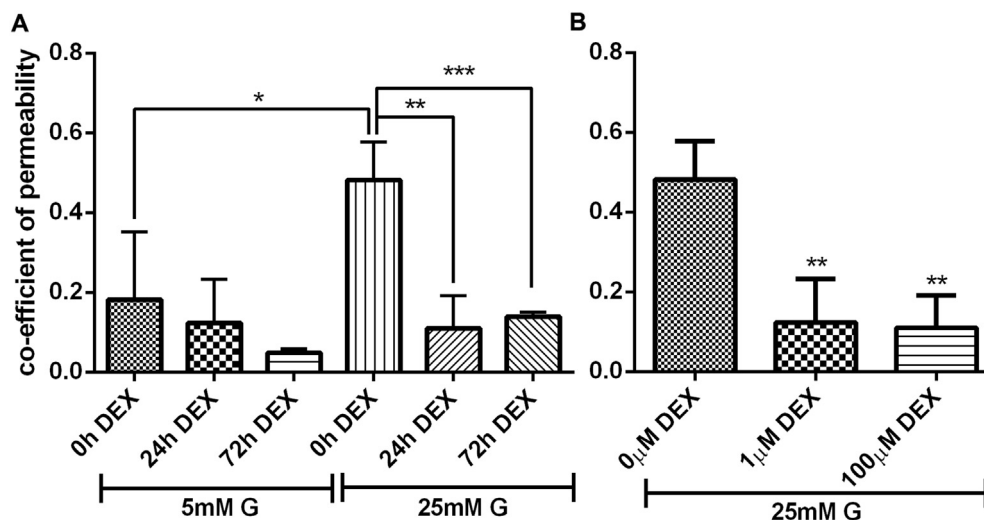


Fig. 1. Dexamethasone reduced baseline REC permeability and decreased glucose-induced hyperpermeability. Transendothelial cell permeability was measured (A) with 5 mM and 25 mM with and without DEX for 24 or 72 h and (B) with 25 mM glucose and 1 μ M or 100 μ M DEX for 24 h ($n = 5$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

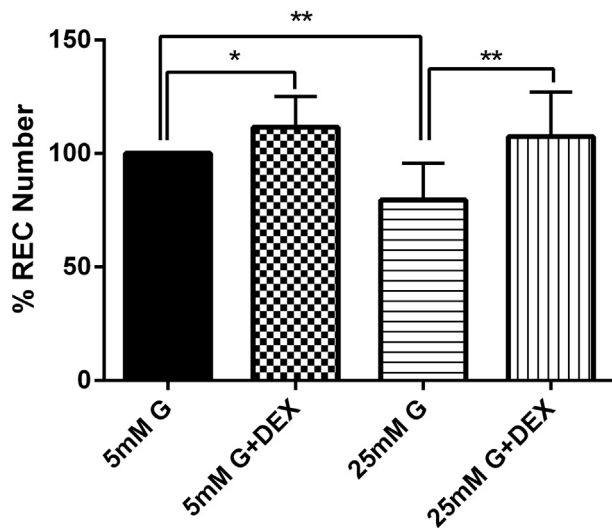


Fig. 2. Dexamethasone recovered glucose-induced reduction in REC number. REC were incubated for 48 h in 5 mM or 25 mM glucose before being treated with 1 μ M DEX for 24 h then cell number quantified using Cell Counting Kit –8 assay ($n = 9$, * $p < 0.05$, ** $p < 0.005$).

(20.48 ± 5.7 , $p = 0.0030$, Fig. 1). The addition of 1 μ M DEX reversed this effect and significantly increased REC numbers compared to 25 mM glucose (27.96 ± 8.988 , $p = 0.0077$). A slight but significant cell protective mitogenic effect was also seen in 5 mM glucose medium (11.52 ± 4.7 , $p = 0.0305$).

3.3. Effect of dexamethasone on gene expression profiling of tight junction components

Expression of claudin-5 ($P < 0.0001$), JAM-A ($p < 0.0001$) and occludin ($p < 0.0001$) was significantly decreased with 25 mM glucose (Fig. 3). The addition of DEX significantly increased expression of claudin-5 ($p = 0.0042$), and occludin ($p = 0.0018$); JAM-A expression also increased slightly (ns, $p = 0.1556$) although this did not return to the NG baseline level as was seen with claudin-5 and occludin (Fig. 3). JAM-C expression was increased with 25 mM glucose ($p = 0.0498$); DEX did not alter this change significantly. Similarly, although VE-Cadherin was not significantly changed in high glucose media, there appeared to an increase in expression in high glucose and DEX, although this was variable (ns, $p = 0.0624$).

3.4. Effect of high glucose and dexamethasone on Ang1 and Ang2 secretion

Secretion of Ang1 and Ang2 protein by REC was quantified using ELISA and corrected for baseline Ang1/Ang2 content in the media (Cm or SRM). Over a 72 h period REC in 5 mM glucose produced significantly higher Ang1: 303 pg–1367 pg (Mean $\pm$ SD; 792 ± 517) compared to REC in 25 mM glucose: 23 pg–289 pg (195 ± 116 ; $p = 0.0218$; Fig. 4A). After 24 h of 1 μ M DEX treatment this trend was reversed ($p = 0.0205$; Fig. 4B). REC produced consistently high levels of Ang2 in both 5 mM ($19,455 \pm 760$) and 25 mM ($19,102 \pm 812$; Fig. 4C). After 24 h of 1 μ M DEX treatment Ang2 production was significantly decreased compared to baseline

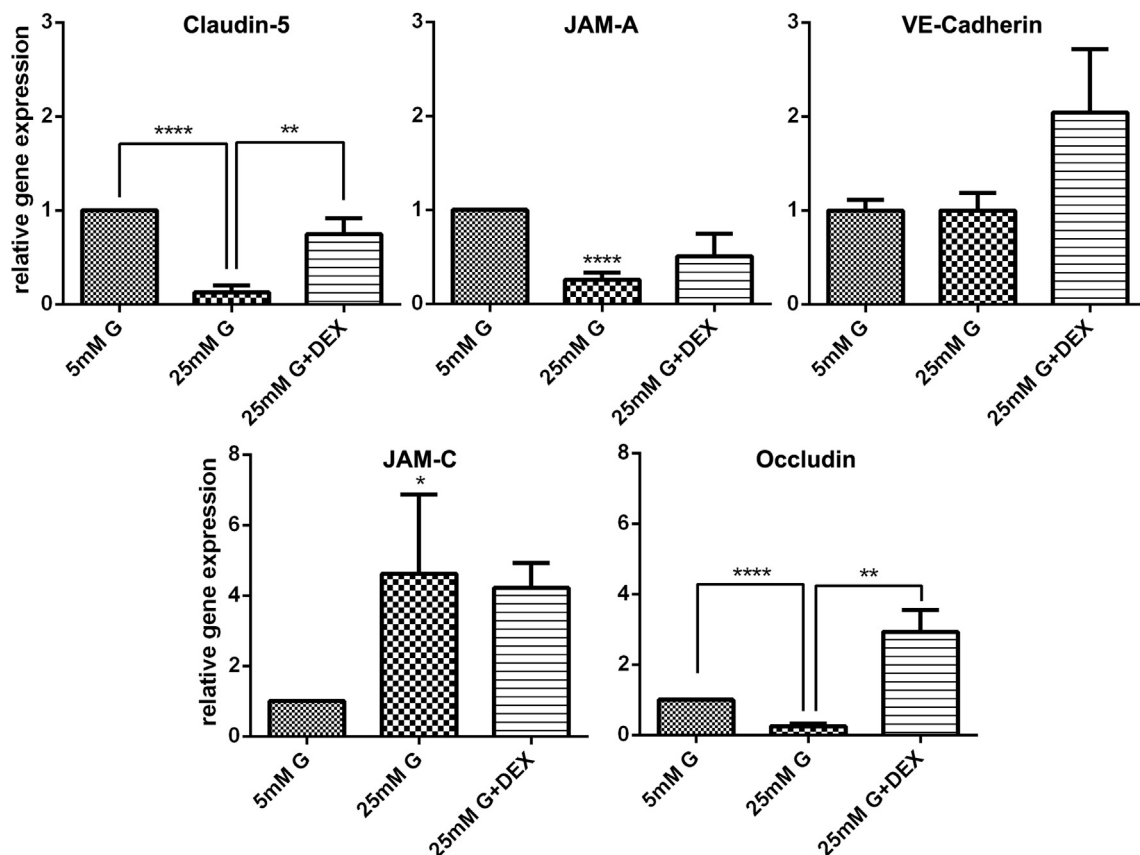


Fig. 3. Effect of high glucose and dexamethasone on gene expression of tight and adheren junction proteins. Gene expression after treatment with glucose and DEX was quantified relative to HPRT1 and normalised against donor matched parallel culture in 5 mM glucose using qPCR. Expression of claudin-5, JAM-A and occludin were reduced with 25 mM; this effect was reversed with DEX treatment ($n = 3$, * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$).

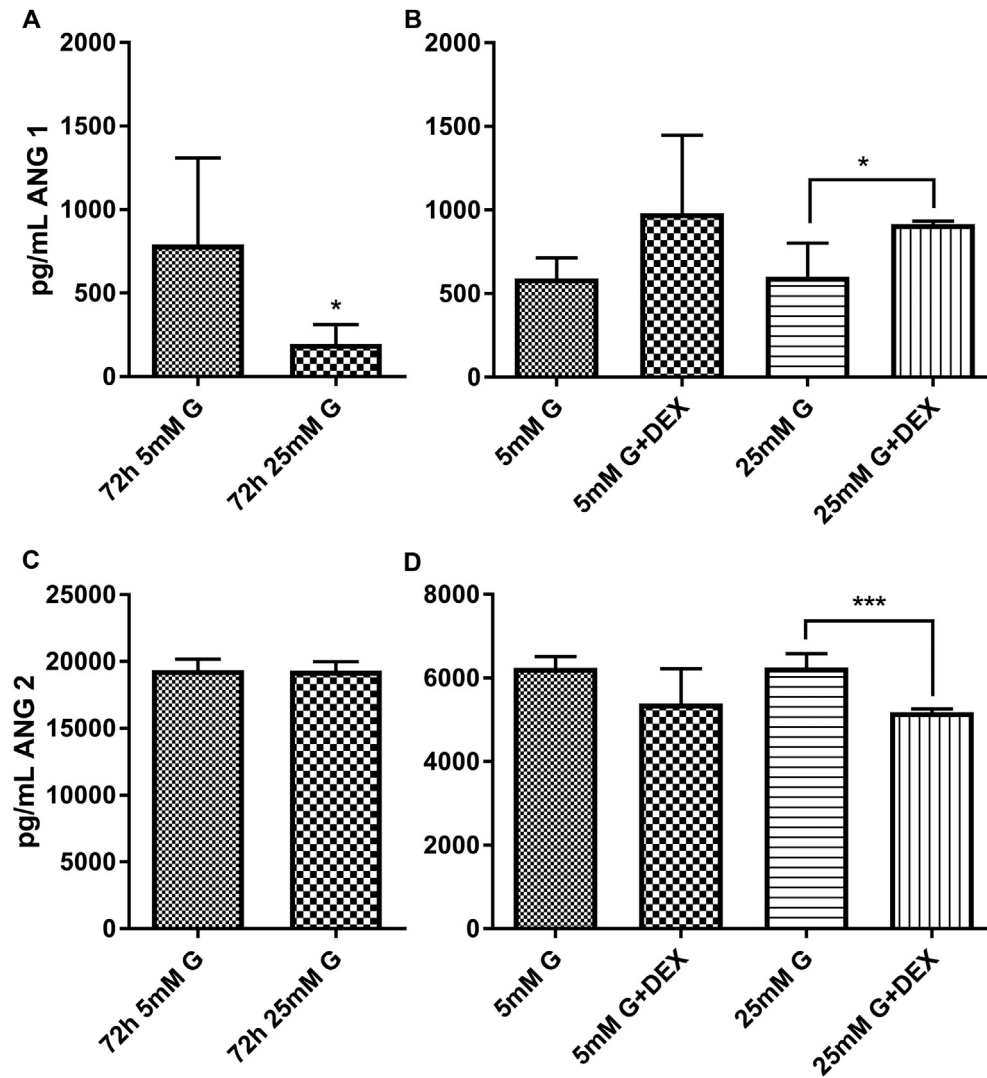


Fig. 4. Secretion of Ang1/Ang2 proteins by REC. A) Quantity (pg) of Ang1 secreted by REC over 72 h by cells in 5 or 25 mM glucose, B) DEX (24 h) significantly increased production of Ang1 in cells pre-treated for 72 h in 25 mM glucose, C) Ang2 levels were high in 5 and 25 mM conditions over 72 h, D) DEX treatment reduced Ang2 secretion. (n = 5, *p < 0.05, **p < 0.005, ***p < 0.001).

(p = 0.0008; Fig. 4D).

4. Discussion

Human REC show different transendothelial permeability profiles in normal and hyperglycaemic conditions (Saker et al., 2014), where there is significant increase in REC permeability in high glucose. This is in keeping with previous reports (Ciulla et al., 2003; Miyamoto and Ogura, 1999; Mohr, 2004), although Nehme and Edelman (2008) were only able to show such changes in the retinal pericytes but not REC in their *in vitro* model. VEGF-A is thought to be the main factor responsible for increased retinal vascular permeability as upregulation of it results in phosphorylation of TJ proteins (occludin and ZO-1) and their disassembly (Antonetti et al., 1998; Harhaj et al., 2006). However, it has been suggested that VEGF modulation of ECs may not be an important mechanism of corticosteroid inhibition of vascular changes in high glucose as there was no effect on the secretion of inflammatory or angiogenic mediators in 25 mM glucose with or without DEX (Nehme and Edelman, 2008). Contrary to the report of Nehme and Edelman (2008), this study has shown that exposure of REC to DEX

for 24 or 72 h results in significant reductions in the permeability of REC induced by high glucose levels. This study has shown that there is significant reduction in REC cell numbers by 25 mM glucose. This agrees with previous reports of reduced REC proliferation (Lorenzi et al., 1985, 1986), and increased EC cell death and apoptosis (Liu et al., 2004; Mizutani et al., 1996). This reduction is comparable to that shown by our group in human Tenon's capsule fibroblasts in diabetics and non-diabetics cultured in high glucose (Browning et al., 2005), and in other EC elsewhere in the body (Avogaro et al., 2008; Calles-Escandon and Cipolla, 2001; Eriksson et al., 2012; Rask-Madsen and King, 2007). Potentially, reduced cell proliferation and increased apoptosis in the early stages of DR may lead to retinal capillary closure, resulting in increased hypoxia which in turn stimulates subsequent aberrant neovascularisation (Curtis et al., 2009; Wright et al., 2011). This study has found that *in vitro* treatment of REC with DEX can reduce cell loss in media of high glucose (25 mM) concentrations. This may imply that DEX offers protection against REC loss in high glucose conditions and may allow enhanced endothelial repair in the diabetic retina.

For the first time, Ang1 expression and secretion by REC has been demonstrated. This expression and secretion is significantly

reduced by high glucose concentration, which is also associated with high Ang2 levels. Ohashi et al. (2004) did not find any changes in retinal expression of Ang1 and Tie2 mRNA in STZ diabetic eyes up to 6 months, whilst Ang2 mRNA was increased compared to that in non-diabetic eyes. In the study by You et al. (2014), the median serum level of Ang-1 was significantly higher in the patients with non-proliferative diabetic retinopathy (NPDR) compared to the patients with no diabetic retinopathy (NDR) and those with proliferative diabetic retinopathy (PDR), whilst the serum level of Ang2 was highest in the PDR group. Ang1/Ang2 ratio was lowest in the serum of patients with PDR. (You et al., 2014) This study supports previous reports which indicate that modulation of Ang1 and Ang2 are important in determining REC permeability (Harhaj and Antonetti, 2004; Jousen et al., 2001). Alterations (decrease) of Ang1/Ang2 ratios are associated with increased permeability, and an increase of this ratio by DEX, which translates into changes in selected TJ molecules, reduce REC permeability in high glucose media.

The reversal of increased REC permeability in diabetes and high glucose levels is, therefore, partly due to alterations in Ang1/Ang 2 ratio, as well as alteration in different TJ molecules. The role of Ang2 blockage as treatment for clinical diabetic macular oedema needs exploration. As permeability and DMO have such an impact on visual loss in DR this could have clinical implications for improvements in the development of anti-inflammatory and other therapies used to treat retinal diseases.

5. Conclusions

This study confirms that REC exposure to high glucose results in significant reduction in the gene expression of molecules important for TJ formation, including claudin-5, occludin and JAM-A, and that there is alteration of Ang1/Ang2 signalling. This study also found an increase in JAM-C, which has been reported to induce permeability (Li et al., 2009). At the mRNA level, expression of claudin-5 and occludin were significantly increased by DEX in high glucose to levels that restored their expression to levels similar to those in normal glucose medium. This change was associated with an increase in VE-Cadherin, and JAM-A expression compared to high glucose alone, although these increases were not significant. The reversal of Ang1/Ang2 ratio by DEX in high glucose supports a role for increase VE-Cadherin in the reversal of the permeability increase. However, the absence of any significant change in JAM-A or C expression with DEX in 25 mM glucose (compared to 25 mM glucose alone) indicates that DEX may restore permeability through specific pathways controlling TJ molecules. As such other treatments that modify TJ components other than claudin-5 and occludin (or further enhance these) may, in combination with DEX, produce additive effects in restoring EC permeability to the normal or near normal states.

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