Diabetic Macular Oedema: The role of steroids and VEGF

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MD Thesis

University College London
Diabetic Macular Oedema: The role of steroids and VEGF

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

Zoe Ockrim
Abstract

Despite advances in controlling diabetes, diabetic macular oedema remains the leading cause of blind registration in the working population in England and Wales. The only proven effective treatment for diabetic macular oedema is laser photocoagulation. However this treatment has limited benefits since it reduces the chance of moderate visual loss by approximately 50% and is unlikely to improve visual acuity.

Intravitreal steroids have been used in the treatment for diabetic macular oedema. Initial pilot studies suggest it can decrease retinal thickening and increase visual acuity in the long-term. Vascular endothelial growth factor is thought to play a critical role during the pathogenesis of diabetic macular oedema. The mechanism of action of both steroids and vascular endothelial growth factor on permeability has still to be fully elucidated. The aims of this thesis were to establish a reliable model of retinal microvascular endothelial cells and to characterise cellular changes following exposure to corticosteroids or vascular endothelial growth factor. Separate clinical work was aimed at evaluating the benefits of steroid treatment alone or combined with pars plana vitrectomy as a treatment of diabetic macular oedema. We also aimed to identify any prognostic indicators for treatment by both examining the morphological features of diabetic macular oedema observed on optical coherence tomography and by assaying the vascular endothelial growth factor concentration in the ocular fluids of eyes with diabetic macular oedema.

Our results show that our retinal and brain microvascular endothelial cells were morphologically very similar; in particular with respect to the spatial localization of junctional proteins. Vascular endothelial growth factor led to an increase in the permeability and a decrease in the staining of the
junctional proteins. By using signal transduction inhibitors, we showed that vascular endothelial growth factor-induced permeability and vascular endothelial growth factor-induced zonula occludens-1 loss occurred via different pathways suggesting that zonula occludens-1 loss was unlikely to be the downstream effector of vascular endothelial growth factor-induced permeability. Hydrocortisone leads to a decrease in permeability and an increase in the junctional expression of a number of tight junctional proteins. Both hydrocortisone and triamcinolone were able to inhibit vascular endothelial growth factor but not lysophosphatidic acid induced permeability suggesting that steroids are able to counteract the effects of certain but all vasoactive compounds. Overall our results suggested that steroids and VEGF lead to opposing effects on microvascular endothelial cells.

Our randomized controlled trial showed that intravitreal triamcinolone was no more beneficial than laser photocoagulation for persistent diabetic macular oedema. A retrospective analysis of the morphological characteristics observed on Optical coherence tomography did not provide any characteristic that was prognostic of the outcome of intervention. Additionally, an exploratory case series of pars plana vitrectomy with 4 mg intravitreal triamcinolone was unable to show that combined treatment was of benefit in the long-term. Lastly the intraocular concentration of vascular endothelial growth factor was not predictive of the outcome of treatment.
Acknowledgements

Firstly I would like to thank my supervisors. Many thanks to Dr Patric Turowski for being my pillar of strength. He has guided me through my lab work, encouraged my ideas, shown patience at my mistakes and given direction to my thesis. It has been a steep learning curve for me and Patric has always shown faith even when I doubted myself.

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I am grateful to Sally Falk, clinical research co-ordinator, who proved to be a perfect personal assistant. She ensured that every patient and their case notes was in the right place at the right time. I am confident that the praise we received from the patients was due to her organisational and personal skills. My database was always up to date and on the countless occasions when asked her for additional information she produced it without hesitation.
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Finally, thank you to my family for keeping my sane.

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<tbody>
<tr>
<td>ACE</td>
<td>Angiotension converting enzyme</td>
</tr>
<tr>
<td>AMP</td>
<td>Abnormal macular profile</td>
</tr>
<tr>
<td>ANCHOR</td>
<td>Anti-VEGF antibody for the treatment of predominantly classic choroidal neovascularization in AMD</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
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<td>AJ</td>
<td>Adherens junctions</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycosylation end products</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCVA</td>
<td>Best corrected visual acuity</td>
</tr>
<tr>
<td>BMECs</td>
<td>Brain microvascular endothelial cells</td>
</tr>
<tr>
<td>BRB</td>
<td>Blood retinal barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cl-5</td>
<td>Claudin-5</td>
</tr>
<tr>
<td>CME</td>
<td>Cystoid macular oedema</td>
</tr>
<tr>
<td>CSMO</td>
<td>Clinically significant macular oedema</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes control and complication trial</td>
</tr>
<tr>
<td>DIRECT</td>
<td>Diabetic retinopathy candesartan trials</td>
</tr>
<tr>
<td>DNA</td>
<td>Did not attend</td>
</tr>
<tr>
<td>DMO</td>
<td>Diabetic macular oedema</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC-1</td>
<td>Extracellular domain-1</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>ETDRS</td>
<td>Early treatment of diabetic retinopathy study</td>
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<tr>
<td>EUCLID</td>
<td>EURODIAB controlled trial of Lisinopril in insulin-dependent diabetes mellitus</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAZ</td>
<td>Foveal avascular zone</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FIELD</td>
<td>Fenofibrate intervention and event lowering in diabetes</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FO</td>
<td>Fast oscillation</td>
</tr>
<tr>
<td>GC</td>
<td>Corticosteroids</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycosylated haemaglobin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks buffered saline solution</td>
</tr>
<tr>
<td>HC</td>
<td>Hydrocortisone</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HOPE</td>
<td>Heart outcomes protection evaluation study</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IRMAs</td>
<td>Intraretinal microvascular abnormalities</td>
</tr>
<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesional molecule</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>MARINA</td>
<td>Minimally classic/occult trial of the anti-VEGF antibody ranibizumab In the treatment of neovascular AMD</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>mmHG</td>
<td>Millimeters of mercury</td>
</tr>
<tr>
<td>MVL</td>
<td>Moderate visual loss</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced form of nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced form of nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NPDR</td>
<td>Non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>NSD</td>
<td>Neurosensory detachment</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>PAI</td>
<td>Fibrinolytic inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDR</td>
<td>Proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>PDS</td>
<td>Plasma derived serum</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PHT</td>
<td>Posterior hyaloid traction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PPV</td>
<td>Pars plana vitrectomy</td>
</tr>
<tr>
<td>PSCLO</td>
<td>Posterior subcapsular lens opacity</td>
</tr>
<tr>
<td>PVD</td>
<td>Posterior vitreous detachment</td>
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<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>PVR</td>
<td>Proliferative vitreoretinopathy</td>
</tr>
<tr>
<td>RMECs</td>
<td>Retinal microvascular endothelial cells</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate polyacrylamide</td>
</tr>
<tr>
<td>SVL</td>
<td>Severe visual loss</td>
</tr>
<tr>
<td>TA</td>
<td>Triamcinolone</td>
</tr>
<tr>
<td>TER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TLCK</td>
<td>Tosyl-lysine-choromethylketone</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom prospective diabetes study</td>
</tr>
<tr>
<td>VA</td>
<td>Visual acuity</td>
</tr>
<tr>
<td>VEC</td>
<td>Vascular endothelial cadherin / VE cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGF-R</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>vWF</td>
<td>Von willlebrands factor</td>
</tr>
<tr>
<td>WESDR</td>
<td>Wensconsin epidemiological study of diabetic retinopathy</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
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1. Introduction
1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disorder that is characterised by chronic hyperglycaemia, with disturbances in the metabolism of carbohydrate, fat and protein, secondary to an absolute or relative lack of the hormone insulin (World Health Organisation, 1985). It is estimated that approximately 1.3 million people (2%) have been diagnosed with diabetes in the UK (National Institute for Health and Clinical Excellence, 2008) and there are a further 1 million people undiagnosed in the community (British Medical Association, 2004).

The clinical diagnosis of DM is often prompted by symptoms such as increased thirst and urine volume, recurrent infections, unexplained weight loss and, in severe cases, drowsiness and coma. High levels of glycosuria are usually present. For symptomatic patients, a single random blood glucose estimation in excess of the 10 mmol/l for venous blood and 11.1 mmol/l for capillary blood is diagnostic (World Health Organisation, 1985). For asymptomatic individuals, at least one additional abnormal plasma or blood glucose result is required (for example a fasting glucose of above 7) or an abnormal oral glucose tolerance test is essential (World Health Organisation, 1985).

DM is classified as type 1, type 2 or gestational DM. Other types of DM such as maturity onset diabetes of the young, which is due to single gene defects, exist but are very rare (Vaxillaire and Froguel, 2006). Additionally, extensive damage to the pancreas for example following chronic pancreatitis or cystic fibrosis, may result in secondary DM.
Type 1 DM is characterized by the loss of insulin-producing beta cells of the islets of Langerhans in the pancreas, leading to a deficiency of insulin. The majority of type 1 DM is caused by T-cell-mediated autoimmune attack of the beta cell (Rother, 2007) but it may also be idiopathic.

Type 1 DM accounts for about 10% of DM cases in North America and Europe (2004). Obesity is generally not a feature. Sensitivity and responsiveness to insulin are usually normal, especially in the early stages. It traditionally affects children but not exclusively. The principal treatment is the administration of artificial insulin, without which, diabetic ketoacidosis often results in coma or death.

Type 2 DM is due to insulin resistance / sensitivity, combined with reduced insulin secretion which may become absolute. The defective responsiveness to insulin involves the insulin receptor, however the specific pathogenesis is unknown (Sesti et al., 2001). In the early stages the predominant abnormality is reduced insulin sensitivity, characterized by elevated levels of insulin in the blood. At this stage, hyperglycaemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver. As the disease progresses the therapeutic replacement of insulin often becomes necessary. Type 2 DM may go unnoticed for years because symptoms are typically mild or non-existent.

There are numerous theories as to the exact cause and mechanism of type 2 DM. Central obesity is known to be a predisposing factor. Abdominal fat produces a group of hormones called adipokines that may possibly impair glucose tolerance (Arner, 2005). Increasing physical activity and weight loss can restore insulin sensitivity. Other factors include aging and family history.
In the last decade, type 2 DM has increasingly begun to affect children and adolescents, likely in connection with the increased prevalence of childhood obesity (Rosenbloom A, 2003).

The preponderance of DM in some families suggests a familial pattern of inheritance however the genetics of type 2 DM are complex and not completely understood, but presumably this disease is related to multiple genes and most patients demonstrate 2 defects in glucose handling; insulin resistance and some degree of insulin deficiency.

Gestational diabetes resembles type 2 DM and occurs in about 2%–5% of all pregnancies and may improve or disappear after delivery. It is fully treatable but requires careful medical supervision throughout the pregnancy. However, about 20%–50% of affected women develop type 2 diabetes later in life.

Chronic hyperglycaemia results in increased morbidity and mortality from both macrovascular and microvascular complications. Diabetic complications account for at least five per cent of NHS costs (Currie et al., 1997). Macrovascular complications cause diseases such as coronary heart disease, stroke and peripheral vascular disease, while microvascular complications affect the kidney, eye and peripheral nervous system. Patients who show evidence of end-organ damage for example diabetic neuropathy or advanced diabetic retinopathy have a 1.76 times higher risk of mortality compared to uncomplicated DM cases (Swerdlow and Jones, 1996).

Throughout the world, the incidence of diabetes continues to rise. It is estimated that the incidence will double between 1997 and 2010 (Amos et
al., 1997). Risk factors for the development of type 2 diabetes include obesity, physical inactivity, ageing and genetic predisposition (Zimmet, 1999). In fact increasing urbanization and the introduction of a western diet has a significant impact on the rising incidence of DM.
1.2 Biochemical and cellular consequences of hyperglycaemia

Hyperglycaemia is known to be the primary pathogenic factor in the development of diabetic complications (1998a). However the mechanism by which hyperglycaemia induces diabetic complications remains unclear and several interrelated pathways have been implicated.

The retina is one of the few tissues, which does not require insulin to transport glucose into the cell. Instead, a glucose transporter (GLUT-1) facilitates glucose entry into retinal endothelial cells (Sone et al., 2000). As a consequence, hyperglycaemia leads to high intracellular glucose levels, resulting in an increased flux of glucose via the sorbitol pathway (Fig 1.1.1) (Crabbe and Goode, 1998). Aldose reductase is the first enzyme in this pathway and it has a low affinity for glucose. During normoglycaemia, metabolism of glucose by this pathway is a very small percentage of the total glucose use. In contrast, in a hyperglycaemic environment, increased intracellular glucose results in increased enzymatic concentration of sorbitol.

The reduction of glucose to sorbitol consumes NADPH. NADPH is normally required for regenerating glutathione and therefore a reduction in its concentration increases intracellular oxidative stress (Lee and Chung, 1999). It has also been proposed that oxidation of sorbitol by NAD+ increases the cytosolic NADH/NAD+ ratio, a pathway that eventually increases advanced glycosylation endproduct (AGE) precursors and protein kinase C (PKC) activation (Williamson et al., 1993). Studies of the inhibition of the sorbitol pathway in vivo have failed to prevent diabetic retinopathy although a positive effect on diabetic neuropathy has been observed (1990).
Figure 1.1: Schematic representation of the sorbitol pathway
Advanced glycation end products (AGEs) are found in increased amounts in diabetic retinal vessels (Stitt et al., 1997). They are formed when excess carbohydrates causes glycation of protein side-chains in a non-enzymatic way (Stitt et al., 1997). Intracellular proteins modified by AGE have altered functions (Giardino et al., 1994) and modified extracellular proteins interact abnormally with other matrix components. For example, AGE modification of collagen IV decreases endothelial cell adhesion (Haitoglou et al., 1992). Lastly modified plasma proteins, bind to AGE receptors and increase the production of reactive oxygen species (Tsuji and Sakurai, 1998).

Hyperglycaemia induces *de novo* synthesis of the second messenger diacylglycerol (DAG), which leads to selective activation of PKC isoenzymes in particular PKC-beta (Koya and King, 1998). PKC is also activated indirectly by increased activity through the sorbitol pathway (Keogh et al., 1997), as well as through ligation of AGE receptors (Portilla et al., 2000). Activation of PKC has many effects such as blood flow abnormalities in the retinal and renal circulations (Ishii et al., 1996), probably by depressing nitric oxide production (Craven et al., 1994); increasing VEGF production (Williams et al., 1997); vascular occlusion by increasing TGF-beta, collagen and fibronectin expression (Studer et al., 1993) and by inhibiting the expression of fibrinolytic inhibitor PAI-1 (Feener et al., 1996).

Shunting of excess intracellular glucose into the hexosamine pathway might also cause several manifestations of diabetic complications (Kolm-Litty et al., 1998). Activation of the hexosamine pathway leads to changes in the gene expression such as a reduction in the transcription of transforming growth factor alpha and beta and PAI-1 as well as alteration the function of some proteins for example PKC.
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Diabetes has also been found to induce the production of inflammatory mediators suggesting a role for anti-inflammatory agents such as steroids. The adhesion of leukocytes to the retinal vasculature is one of the earliest events in experimental diabetes (Miyamoto et al., 1999). Enhanced vascular permeability, endothelial cell damage, and capillary nonperfusion are some of the pathological consequences of diabetic retinal leukocyte adhesion (Miyamoto et al., 1999; Joussen et al., 2001).

When neutralizing anti-intercellular adhesion molecule-1 (ICAM-1) antibodies are administered to newly diabetic animals, retinal leukocyte adhesion is suppressed (Miyamoto et al., 1999; Joussen et al., 2001). In nondiabetic subjects, ICAM-1 is localized within the choriocapillaris. In contrast during diabetes, there is a significant elevation of ICAM-1 in both the choroidal vasculature and within retinal blood vessels (P < 0.05) (McLeod et al., 1995) and consequently elevated neutrophil levels in the choroid and retina are observed (McLeod et al., 1995). Furthermore, when experimentally induced diabetic animals are treated with anti-VEGF antibodies both retinal ICAM-1 mRNA and leukocyte adhesion are suppressed (Joussen et al., 2002).
1.3 Blood Retinal Barrier and vascular permeability

The retina is a transparent layer of neural tissue that functions to capture photons of light, convert the photochemical energy into electrical energy and to transmit this to the brain to be interpreted as visual images. Its function depends on intact communication of all cell types; neurons, microglia, Müller and pigment epithelium, and requires a highly defined environment in order to maintain its function. The retina has exceptionally high metabolic demands which are met by a blood supply which is well separated from the neuroretina by the blood-retinal barrier (BRB).

The BRB is formed at 2 levels, the inner and outer BRBs. The inner BRB is formed by the retinal vasculature while the outer BRB is composed of the retinal pigment epithelium (RPE).

The RPE is critical for normal function of the photoreceptors and control of fluid volume and ionic concentrations in the subretinal space. In diabetes, failure of the RPE to effectively move fluid out of the retina may contribute to the persistence and progression of macular oedema. Some studies of experimental diabetes have demonstrated that structural and functional alterations of the RPE precede changes in the retinal vasculature (Kirber et al., 1980; Vinores et al., 1989; Klein et al., 1980; Tso et al., 1980). Structural changes in the RPE include dilation of the smooth endoplasmic reticulum, thickening of the basal laminae of the RPE and Bruchs membrane, dilation and redundancy of basal membrane infoldings, formation of large vacuoles, and focal necrosis (Vinores et al., 1989; Grimes et al., 1984). Reduced barrier function has been demonstrated using tracers such as fluorescence or HRP (Kirber et al., 1980; Tso et al., 1980). Additionally, the choroid (McLeod and Lutty, 1994) from patients with diabetes demonstrated
angiopathic changes consisting of extensive capillary dropout, beaded capillaries, neovascularization, and Bruch's membrane degeneration.

Although these structural changes in the RPE and choriocapillaris of diabetics have been described (Vinores et al., 1990; Yanoff M, 2002), the retinal vasculature is the predominant site of leakage during diabetic retinopathy (Vinores et al., 1989).

The inner BRB is composed of the endothelium of the retinal capillary vessels (Cunha-Vaz et al., 1975; Foulds, 1990), which are adapted to this function by a lack of fenestrations and transport vesicles (Greenwood, 1992; Raviola, 1977) and by forming highly specialized junctions between the cells (Stewart and Tuor, 1994). The retinal capillary vessels comprise 2 plexi of blood vessels, the superficial and the deep layers. Distinctions exist among retinal vascular beds. The superficial vessels develop in the presence of a rich network of astrocytic processes that is largely absent in the deep plexus (Gariano et al., 1996) and certain agents differentially interfere with murine superficial and deeper retinal vessel growth (Dorrell et al., 2002).

The endothelium is surrounded and supported by pericytes and glial cells (astrocytes and Müller cells). In this respect it is structurally similar to the blood-brain barrier (BBB) which is also formed by microvascular endothelial cells, supported by pericytes and astrocytes. However the BBB is a more permeable barrier and has less pericytes per endothelial cell (Stewart and Tuor, 1994).

Pericytes are modified smooth muscle cells that regulate retinal vascular flow by dilating and contracting (Hirschi and D'Amore, 1996). Loss of
pericytes represents an early feature of DR and correlates with the formation of microaneurysms (Speiser et al., 1968a). Glial cells regulate retina metabolism and modulate the function of both neurons and blood vessels (Speiser et al., 1968b; Gardner et al., 1999).

Increased permeability may occur by two broad mechanisms. Transport of substances between cells is referred to a paracellular transport while transcellular transport refers to the movement of substances through the cell. Permeation through the paracellular space of endothelial cells is intuitively the main route. The relevance of this pathway has been underlined by numerous studies that imply inter-endothelial junction proteins in regulating vascular permeability in vitro and in vivo. Nevertheless, Hofman et al (Hofman et al., 2000) demonstrated an increase in retinal permeability for plasma proteins induced by VEGF is predominantly caused by an increase in active trans-endothelial transport via pinocytotic vesicles.

### 1.4 Endothelial cell-cell junctions

The inner BRB forms a physiological barrier and comprises a single layer of non-fenestrated endothelial cells. Cell-to-cell junctional complexes are composed of regulated protein structures including adherens junctions and tight junctions (fig 1.2).
1.4.1 Adherens junctions

Adherens junctions (AJ) are ubiquitously distributed along the vascular tree and are expressed in both blood and lymphatic vessels. They are composed of transmembrane proteins, predominantly cadherins and junction-associated proteins, predominantly catenins, that can also be found in the cytoplasm and nucleus (Ben-Ze'ev and Geiger, 1998).

1.4.1.1 VE Cadherin

Vascular endothelial cadherin (VEC) represents the major transmembrane component of AJ in capillary endothelial cells. It forms a dynamic complex with the catenins. When the cells are subconfluent or detaching and migrating, phosphorylated VEC is associated with p120 and beta-catenin. However when cells are confluent, VEC loses its tyrosine phosphorylation and associates also with gamma-catenin and the actin cytoskeleton (Breviario et al., 1995).

VEC is largely responsible for morphogenesis and maintenance of the vasculature by establishing cell-cell contact (Carmeliet et al., 1999). Mutant VEC genes are lethal in the early embryonic stage due to collapse of the vascular network and circulatory insufficiency (Carmeliet et al., 1999). Additionally, VEC has a role in the maintenance of the endothelial barrier illustrated by the increased permeability in response to anti-VEC antibodies injected in experimental animal models (Corada et al., 1999).

The extracellular domain of VEC is involved in endothelial cell-to-cell adhesion. It consists of five cadherin-like repeats that form a rigid, rodlike structure. Dimers between VEC molecules can either occur between
adjacent cells or opposing cells (Shapiro et al., 1995). Antibodies directed at the most distal extracellular domain (EC1), cause a decrease in the barrier function of endothelial cell monolayers \textit{in vitro} and blood vessels \textit{in vivo} (Corada et al., 2001).

\textbf{1.4.1.2 Catenins}

The catenins belong to family of proteins which contain a large number of repeated sequence motif, the armadillo repeat. The armadillo family includes alpha-catenin, beta-catenin, gamma-catenin (also known as plakoglobin) and p120. They form interactions with AJs and the actin cytoskeleton (Dejana et al., 2008). The role of many of these proteins is largely undetermined. They have been known to have a role in gene expression and cellular signaling, characterized by their ability to translocate to the nucleus (Ben-Ze'ev and Geiger, 1998;Cowin and Burke, 1996) as well as a role in paracellular permeability (Navarro et al., 1995).
Figure 1.2: Schematic diagram illustrating the proteins comprising the junctional complexes found between endothelial cells in the blood-retinal barrier.

JAM, junctional adhesion molecule; Cl-5, claudin-5; F-actin, filamentous actin; ZO-1, 2 and 3, zonula occludens 1, 2 and 3; VEC, vascular endothelial cadherin.
1.4.2 Tight junctions

Tight junctions (TJ) were first described in epithelial cells as the most apical components of the intercellular junctional complex (Cereijido M, 2001b) and were first described in epithelial cells. In endothelial cells, they are of similar architecture but are spatially intermixed with adherens junctions (Cereijido M, 2001b). On electron microscopy, they appear as regions where the plasma membranes from adjacent cells appear to fuse together and obliterate the intercellular space (Cereijido M, 2001a). This area completely encircles the cell and consists of anatomising strands of proteins that interact with proteins on adjacent cells (Staehelin, 1973). Like AJ these proteins are made up of intramembrane proteins (occludin (Furuse et al., 1993), claudin-5 (Morita et al., 1999) and junctional adhesion molecule (JAM)) and junction-associated proteins (ZO-1, ZO-2 and ZO-3 (p130) (Stevenson et al., 1989; Jesaitis and Goodenough, 1994; Haskins et al., 1998a), 7H6 (Haskins et al., 1998b) and cingulin (Stevenson et al., 1989), (fig 1.2).

Tight junctions perform a number of different functions, including cell-cell adhesion and in epithelial cells, establishing cell polarity. In addition, tissues that produce a tight barrier for example the BBB have an increased expression of TJ, suggesting their role in endothelial permeability. Permeability through the TJ may be altered either by changing the junctional constituents, by posttranslational modifications of junctional proteins (Wong, 1997; Antonetti et al., 1999), or by their interaction with the actin cytoskeleton.
1.4.2.1 Occludin

Occludin is a 55.9 – 65 kDa transmembrane protein with 4 membrane spanning loops, 2 extracellular loops and cytoplasmic carboxyl (-C) and amine (-N) termini. The carboxyl terminal normally interacts with ZO-1 (Furuse et al., 1993) but can also interact with ZO-2 and 3 (Haskins et al., 1998a).

Occludin is not required for structural integrity of TJ. In occludin -/- mice an overt disruption of vascular barrier is not observed (Saitou et al., 1998). Nevertheless, it plays a crucial role in permeability (Matter and Balda, 1999). Arterial endothelial cells express an 18-fold greater occludin content and have a much high solute barrier than their venous counterparts (Kevil et al., 1998). Additionally, pathological models of increased permeability for example experimental diabetes is associated with a reduction in occludin levels (Antonetti et al., 1998; Barber et al., 2000).

In MDCK cells, occludin lacking its first extracellular loop is still able to colocalise with ZO-1. In contrast, constructs lacking the second, or both, extracellular loops were absent from TJ and were found only on the basolateral cell surface, suggesting that the second extracellular domain is required for stable assembly of occludin in the TJ (Medina et al., 2000). Additionally the second extracellular loop has been implemented in the barrier function of the TJ. Transelectrical resistance is reduced in vitro by a synthetic peptide (OCC2) corresponding to the second extracellular domain of occludin (Wong and Gumbiner, 1997).
1.4.2.2 Claudin 5

Claudin 5 (Cl-5) is a member of a large family of proteins (Hiiragi 1998). Cl-5 has a specificity for endothelial cells (Morita et al., 1999), however recently, it has also been found in epithelial cells of the choroidal plexus and in the abdominal tract (Kiuchi-Saishin et al., 2002; Rahner et al., 2001).

Similar to occludin, Cl-5 has 4 transmembrane domains, 2 extracellular loops and cytoplasmic N and C termini (Morita et al., 1999). Site-directed mutations of particular amino acid residues in the first extracellular domain alter the properties of the TJ. In particular, mutation of Cys$^{64}$ leads to an increase in the flux of monosaccharides (Wen et al., 2004). The importance of Cl-5 in the barrier function of cells is further illustrated by the Cl-5 knockout mouse, where TJ are still formed, but the absence of Cl-5 leads to detection of smaller (HRP-conjugated streptavidin) but not larger molecules (albumin) across the blood brain barrier (Nitta et al., 2003).

1.4.2.3 Zonula Occludens-1

Zonula occludens-1 (ZO-1) was the first TJ protein to be identified (Stevenson et al., 1986). It is a 225 kDa protein that belongs to the membrane associated guanylate kinase family of proteins (MAGuK) that contain a region of homology to guanylate kinase (GK) but does not have any GK activity. It is found in TJ bound to occludin (Furuse et al., 1994) and with ZO-2 and ZO-3 (Jesaitis and Goodenough, 1994; Haskins et al., 1998a). However in cells without occludin, it also is found associated with AJ (Saitou et al., 1998) and should therefore not be considered a bona fide TJ marker.
ZO-1 serves many functions. It binds to the actin forming a scaffold with the cytoskeleton (Fanning et al., 1998) and it has an integral role in permeability. There is evidence that this involves small Rho GTPases (Wojciak-Stothard and Ridley, 2002). Activated RhoA disrupts TJ assembly by inducing Myosin light chain dependent actin stress fibers formation and initiating cytoskeleton retraction (Dudek and Garcia, 2001).

ZO-1 expression is decreased in conditions of increased permeability and increases with barrier tightness (Krause et al., 1991). Furthermore, the phosphorylation of ZO-1 leads to a decrease in barrier function (Takeda and Tsukita, 1995).
1.5 Vascular Endothelial Growth Factor as a vasoactive and angiogenic factor in the retinal vasculature

1.5.1 VEGF

Vascular endothelial growth factor (VEGF) is a family of homodimeric glycoproteins with potent vasoactive (Senger et al., 1983) and angiogenic potential (Leung et al., 1989). The VEGF family includes VEGF A, VEGF B, VEGF C, VEGF D, VEGF E, and placental growth factor (PIGF).

VEGF-A is the most studied isoform and usually referred to as VEGF. It plays a predominant role during embryonic development and in many pathological processes. It is a 36-45 kDa protein with an N-terminal signal sequence and a heparin binding domain. The human gene for VEGF A resides on chromosome 6p21.3 (Vincenti et al., 1996). VEGF exists as multiple isoforms resulting from alternative pre-mRNA splicing of eight exons (fig 1.3) The isoforms are named according to their amino acid content; for example VEGF_{121}, VEGF_{165} or VEGF_{189} and are generically referred to as VEGF_{xxx} (Houck et al., 1991;Ferrara, 2004). All currently described isoforms contain exons 1-5 and exon 8. Exons 6 and 7 code for a heparin-binding region and therefore the longer isoforms are matrix bound whilst the shorter forms are soluble (Plate and Warnke, 1997). VEGF 165 is the most abundant form in vivo and is partly bound and partly diffusible. VEGF_{121}, and VEGF_{145} and VEGF_{165} all induce proliferation of endothelial cells in vitro and angiogenesis in vivo (Poltorak et al., 1997;Park et al., 1993;Zhang et al., 1995). Most cell types produce several VEGF variants simultaneously. Usually the 121 and 165 forms are the predominant forms. In contrast, VEGF145 expression seems to be more restricted to reproductive organs (Poltorak et al., 1997).
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Longer extracellular matrix bound forms are released slowly, following exposure to heparin and heparinases, or more rapidly as bioactive fragments, through cleavage by plasmin or urokinase (Plouet et al., 1997).

The use of alternative 3’ spicing in the exon 8 results in almost identical sister isoforms referred to as VEGF_{xxx} and VEGF_{xxxb} (Bates et al., 2002). VEGF_{xxxb} isoforms results from distal splicing of exon 8. The result is that the VEGF receptor binding and dimerization domain remain. However, the altered C-terminal domain is thought to act as a competitive inhibitor of VEGF_{165}. It binds to the receptor but does not stimulate its full tyrosine phosphorylation (Woolard et al., 2004). Therefore expression of these two isoform families, regulated by alternative splicing, could determine the balance between angiogenic and anti-angiogenic processes. In agreement VEGF_{165b} has been found to inhibit tumour growth (Rennel et al., 2008) and proliferative retinopathy (Konopatskaya et al., 2006). Recent studies also suggest that this family of isoforms forms a substantial proportion of the total VEGF, ranging from 1% in placental tissues (Bates et al., 2006) to over 95% in normal colon tissue (Varey et al., 2008). The proximal splicing switch appears to occur in a number of diseases associated with angiogenesis, including diabetic retinopathy (Perrin et al., 2005) and cancers (Varey et al., 2008).
Figure 1.3: Exon structure of VEGF A gene and the generation of splice variants. The VEGF gene consists of eight exons (top). VEGF isoforms result from alternative splicing of these exons. The isoforms are labelled according to the number of encoding amino acids. All currently described isoforms contain exons 1-5 and exon 8. The heparin-binding region is coded by exons 6 and 7. Alternative splicing in the exon 8 results in a sister family of isoforms referred to as VEGF\textsubscript{xxx} and VEGF\textsubscript{xxxb}. 
1.5.2 VEGF receptors

VEGF binds several transmembrane tyrosine kinase receptors. The receptors are structurally related to platelet-derived growth factor family of receptors, containing seven similar extracellular immunoglobulin domains, a single hydrophobic transmembrane domain and a conserved intracellular tyrosine kinase domain (Neufeld et al., 1999). There are three known receptors VEGF-R1, 2 and 3 (Neufeld et al., 1999) as well as neuropilin-1, which is a co-receptor for VEGF A, enhancing its binding with R2 (Soker et al., 1998) (fig 1.4).

VEGF-R1 was the first receptor identified for VEGF and is also a receptor for PlGF and VEGF-B. It is expressed on endothelium and on some non-endothelial cells including haematopoietic stem cells, macrophages and monocytes (Peters et al., 1993; Sawano et al., 2001). VEGFR-1 expression on vascular endothelial cells may act as a decoy receptor by inhibiting VEGF signalling. Inactivaton of the VEGFR-1 gene in mice leads to death in-utero due to failure of endothelial cells to organise into functional blood vessels (Fong et al., 1995). In these animals there is an abnormal increase in endothelial progenitor cells. Expression of altered VEGFR-1 in which the tyrosine kinase domain was deleted while the ligand-binding domain remained intact was found to be compatible with normal vascular development and angiogenesis in transgenic mice, suggesting that VEGFR-1 signalling is not required for normal development (Hiratsuka et al., 1998). Additionally pre-RNA slicing leads to the existence of a soluble form of VEGFR-1 without the transmembrane and intracellular domains and as a consequence is unable to mediate any signal transduction and therefore is likely to function as a decoy receptor which further suggests a negative role of VEGFR-1 in vivo (Kendall et al., 1996).
In contrast VEGFR-2 binds all VEGF isoforms. In endothelial cells, VEGFR2 localization may determine specific signaling events after VEGF stimulation. For example, VEGFR2 associated with cadherins at cell-cell junctions may facilitate the vascular permeability response (Weis et al., 2004b), whereas VEGFR2 associated with [alpha]v integrins ([alpha]v[beta]3 and [alpha]v[beta]5) at the cell-matrix interface may influence permeability as well as angiogenesis (Reynolds et al., 2002).

Finally, VEGF-R3 binds VEGF-C and VEGF-D only (Pajusola et al., 1992) (Finnerty et al., 1993).
**Figure 1.4:** Isoforms and receptors of the VEGF family. There are 3 signaling tyrosine kinase receptors of the VEGF family (VEGF-R1, VEGF-R2 and VEGF-R3) and 2 co-receptors, neuropilin -1 and -2. Different isoforms of the VEGF family selectively bind to the various receptors and co-receptors as indicated.
1.5.3 VEGF and pathology.

VEGF and its receptors are critical for normal embryologic development (Carmeliet et al., 1996) as well as the female reproductive cycle (Ferrara et al., 1998). VEGF receptors are found in other healthy adult organs such as the glomerulus of the kidney (Esser et al., 1998), the gastrointestinal mucosa (Shifren et al., 1994) and the retinal (Thieme et al., 1995). Previous studies (Carmeliet et al., 1996; Ferrara et al., 1996) have shown that targeted deletion of VEGF in mice leads to abnormal vascular development and embryonic lethality, emphasizing the critical role for VEGF in blood vessel development.

VEGF is the only angiogenic factor that also potently induces vascular leak. It has been implicated in many pathological processes, such as rheumatoid arthritis (Fava et al., 1994) and in most human cancers (Volm et al., 1997; Brown et al., 1995). Although VEGF-induced angiogenesis is often accompanied by a vascular permeability response, VEGF-induced-vascular leak is not required for angiogenesis (Eliceiri et al., 1999). Notably both angiogenesis and oedema are central features of diabetic eye disease.

Hypoxia induces VEGF transcription (Brooks et al., 1998). Hypoxia upregulates the transcription factor hypoxia-inducible factor (HIF)-1α (alpha), which binds to the VEGF-A promoter and induces the transcription of VEGF and VEGFR1 (Carmeliet et al., 1998; Li et al., 1996a). The role of HIF in diabetic retinopathy is not yet clear but it has been implicated in VEGF overexpression and hyperpermeability of the blood-retinal barrier in diabetes (Poulaki et al., 2002).
1.5.3.1 VEGF and DMO

VEGF levels are elevated in the vitreous of eyes with diabetic macular oedema (Funatsu et al., 2002; Patel et al., 2006c). Elevated VEGF mRNA is found in the ganglion cell layer as well as the inner and outer nuclear layers, and an increase in VEGF protein is found in the inner retina in close proximity to capillaries (Hammes et al., 1998). In a model of diabetic retinopathy, blood–retina barrier breakdown was both prevented and reversed by VEGF inhibition (Qaum et al., 2001).

The mechanisms underlying VEGF-induced permeability are unclear. VEGF increases permeability via multiple mechanisms, including leukocyte-mediated endothelial injury (Joussen et al., 2001) and dissolution of tight junctions (Antonetti et al., 1999). The amount and duration of VEGF exposure that are required for BRB breakdown may be less than that for neovascularisation (Tolentino et al., 1996; Tolentino et al., 2002). However neovascularisation is also characterised by vascular permeability and some investigators have speculated that vascular permeability is an antecedent and required step for neovascularisation to take place (Dvorak et al., 1991).

Furthermore, VEGF has been implicated in the disruption of the RPE and a reduction in the regulated removal of subretinal fluid. VEGF produces a significant drop in transepithelial electrical resistance in both RPE cell lines (ARPE-19) and primary porcine RPE cells (Ablonczy and Crosson, 2007). This response was only observed following apical administration and this and further work suggests that apically-oriented VEGF-R2 receptors are responsible for this effect (Ablonczy and Crosson, 2007).
1.6 Diabetes and the Retina

Diabetic microvascular complications cause damage to the retinal vasculature resulting in diabetic retinopathy (DR), which is the most common cause of blind registration in the working population in England and Wales (Bunce and Wormald, 2008).

The Diabetic Control and Complications Trial (DCCT) showed that DR tends to cluster in families (1997a). This and the differences in the frequency of disease in ethnic populations (Guillausseau et al., 1997) suggest that genetic disposition maybe important in DR. Additionally, there are distinct morphological manifestations of DR. Some subjects show maculopathy and others showing much more extensive retinal vascular disease, however, it is unclear whether these different morphologies represent distinct pathogenetic mechanisms. Imperatore undertook a genome-wide scan for susceptibility genes for DR (and nephropathy) in families using affected sib-pair linkage analysis. There were indications that elements on chromosomes 3 and 9 influenced both nephropathy and retinopathy, but no clear genomic region was designated for retinopathy alone (Imperatore et al., 1998).

DR can be separated into two different subtypes, diabetic maculopathy and diabetic retinopathy. Diabetic maculopathy occurs when the microvasculopathy affects the macula, threatens the central visual acuity and may be present at any level of retinopathy. Diabetic retinopathy arises when the microvasculopathy leads to visible changes in the rest of the retina. Diabetic retinopathy falls into two clinical stages: nonproliferative diabetic retinopathy and proliferative diabetic retinopathy.
In the Wisconsin Epidemiologic Study of Diabetic Retinopathy, 3.6% of type 1 diabetics had a visual acuity of 20/200 or less in the better eye and in type 2 diabetes, 1.6% had a VA of 20/200 or less (Moss et al., 1998).

Diabetic maculopathy (DMO) affects approx 29% of diabetic patients with disease duration of 20 years of more and accounts for 80% of blind registration due to diabetic retinopathy (McMeel et al., 1977; Patz et al., 1973). The incidence of DMO is directly proportional to the severity of diabetic retinopathy (Henricsson et al., 1999).
1.6.1 Non-proliferative and proliferative diabetic retinopathy

Prior to any clinically detectable changes in the retina, microvascular changes such as death of retinal pericytes (Kuwabara and Cogan, 1963) and basement membrane thickening occur and lead to an altered microvascular blood flow. Capillary closure is a predominant pathological feature associated with microaneurysms and becomes more evident as the disease progresses (Ashton, 1953). Endothelial adhesive properties are changed resulting in increased leukocyte and platelet adhesion and fibrinolysis (Miyamoto et al., 1998; Ceriello et al., 1993). Subtle alteration in vascular permeability are also observed.

Non-proliferative diabetic retinopathy (NPDR) occurs when the first clinically detectable lesions of DR are seen. These consist of microaneurysms, retinal haemorrhages, cotton wool spots, exudates and subsequently vascular changes such as beading, looping and intraretinal microvascular abnormalities (IRMAs).

In the natural course of DR, approximately 50% of patients with very severe NPDR progress to PDR within 1 year (1991b). Proliferative diabetic retinopathy (PDR) develops secondary to capillary closure. The resultant retinal ischaemia upregulates growth factors such as VEGF leading to neovascular proliferation on the surface of the retina. These represent an attempt to supply oxygenated blood to the hypoxic retina. However, these blood vessels are fragile and haemorrhage easily. The Diabetic Retinopathy Study demonstrated that without laser treatment, the 5 year risk of severe visual loss (SVL), (<5/200) was 50% (1976;1978). The 5 year risk of SVL following early pan retinal photocoagulation is 2-6% (1991b).
1.6.2 Diabetic Maculopathy

Arteriolar dilation and an increase in retinal blood flow is characteristic of early DR (Skovborg et al., 1969; Kohner et al., 1975). Hyperglycemia leads to an increase in retinal blood by altering perfusion pressure and due to a lack of compensatory autoregulation during diabetes (Sinclair et al., 1982). This is detected clinically (Kohner et al., 1975) as well as in experimental hyperglycemia (Atherton et al., 1980). With the relative rise of perfusion pressure, increased exudation into the retina especially through the diseased vessels in diabetics, explains the early exudative features of DR. The increased perfusion pressure may also contribute to the production of microaneurysms by exerting pressure on the vessel wall weakened by the loss of pericytes (Addison et al., 1970).

Vascular leakage may also occur in response to capillary closure. Hypoxia and thus macular ischaemia can result from a number of factors. Increased basement membrane thickening reduces the bore of affected vessels which results in an increase in resistance. Blood flow is also restricted by haematological abnormalities, including increased plasma viscosity due to increased fibrinogen levels (Lowe et al., 1980) and red cell aggregation (Peduzzi et al., 1984). Additionally leucocyte adhesion to the vascular endothelium and upregulation of adhesion molecules such as ICAM-1 play an important role in producing retinal hypoxia.

Macular ischaemia is characterised by enlargement of the foveal avascular zone (FAZ) and / or perifoveal capillary drop-out on fundus fluorescein angiography and is at present untreatable. The presence of macular ischaemia may have a negative effect on the visual outcome of treatment (Chung et al., 2008). It is associated with BRB breakdown and the formation
the macular oedema. Additionally, hypoxia itself has been shown to produce alterations in the TJ proteins (Witt et al., 2003) correlating with increased permeability.

Diabetic macular oedema (DMO) is characterised by thickening of the retina within 2 disc diameters of the fovea. The oedema is caused by an increase in permeability of the inner BRB and in decreased efflux of fluid, which leads to an accumulation of intraretinal fluid, primarily in the inner and outer plexiform layers.

Retinal thickening can be appreciated only with stereoscopic viewing of the retina. However other clinical signs such as microaneurysms and exudates act as monocular clues to its existence. Microaneurysms are thought to play a significant role by acting as sources for fluid and lipid transudation (chang S, 2002). Data from a study by Klein et. al. (Klein et al., 1995) showed that the increase in the number of retinal microaneurysms at the 4-year follow-up was associated with increased incidence of Clinically significant macular oedema (CSMO) at the 10-year follow-up. The United Kingdom Prospective Diabetes Study (UKPDS) reported similar findings (Kohner et al., 1999). However, whether the formation of the microaneurysms per se is the cause or, whether the microaneurysms are part of a morphological signal of another pathophysiological process is largely unknown.

DMO can be classified as focal and diffuse. Focal DMO is primarily caused by focal leakage from microaneurysms, dilated retinal capillaries or IRMAs. Clusters of microaneurysms may be seen in areas of circinate exudates with fluid and fundus fluorescein angiography confirms that the microaneurysms leak. Diffuse DMO is caused by generalised leakage from dilated capillaries throughout the posterior pole and is generally associated with capillary loss.
CSMO was defined by the Early Treatment of Diabetic Retinopathy Study (ETDRS) as DMO in which central visual acuity was threatened (table 1.1).

The preponderance of diabetic retinopathy varies between type 1 and type 2 diabetics. Patients with type 1 diabetics tend to have a higher incidence of PDR (10% in type 1 DM compared with 5% in Type 2 DM) (Zander, BJO, 2000), while patients with type 2 DM have a higher prevalence of maculopathy (53% type 2 DM compared with 42% type 1 DM) (Zander et al., 2000).

The best estimate of the natural history in eyes with DMO comes from the treatment group in the ETDRS in which focal/grid photocoagulation was deferred. This study found that the incidence of moderate visual loss (MVL), (defined as a loss of 15 letters on the ETDRS chart) if treatment was deferred was 8% at 1 year, 16% at 2 years, and 24% at 3 years in comparison to the incidence of MVL if treatment was given immediately of 5% at 1 year, 7% at 2 years, and 12% at 3 years, which corresponds to a 50% risk reduction (1985).
Table 1.1: Definition of clinically significant macular oedema (CSMO) as set out in the Early Treatment of Diabetic Retinopathy Study (ETDRS) (1985)

Thickening of the retina at or within 500 µm of the centre of the macula.

Hard exudates at or within 500 µm of the centre of the macula if associated with thickening of adjacent retina.

A zone or zones of retinal thickening 1 disc diameter or larger, any part of which is within 1 disc diameter of the centre of the macula.
1.7 **Current management of Diabetic Macular Oedema**

The morbidity associated with DMO can be reduced by primary prevention, regular screening, early detection and timely treatment.

1.7.1 **Primary prevention**

The DCCT examined the role of tight blood glucose control with routine management of type I diabetes. There was a 26% reduction in the risk of development and progression of DMO in the tight control group over 9 year of follow-up period. (1995). Following the DCCT, the expectation was generated that a similar improvement in the incidence of diabetic complications would occur in type II diabetes. The United Kingdom Prospective Diabetes Study (UKPDS) addressed the issue of tight glycaemic control in type II patients (Stratton et al., 2000). The UKPDS demonstrated at 10 years, a 17% reduction in the risk of progression of retinopathy, a 29% reduction in the need for laser treatment and a 16% reduction in the risk of legal blindness in the in the intensively treated group (1998b). It is interesting to note that both the DCCT and the UKPDS, took 10 years to show a difference between the treatment groups and this should be kept in mind for novel therapies.

Unfortunately, perfect glycaemic control remains elusive in many patients and there is a rise in glycated haemoglobin levels despite intensive treatment over a 10-year period. Type I diabetic patients appear to obtain greater benefit from tight glycaemic control than those with type II diabetes (McCormack and Greenhalgh, 2000), however this is at the risk of significant hypoglycaemic events (1991c).
Other risk factors associated with DMO include age, male gender, systolic blood pressure, proteinuria (Klein et al., 1984) and hyperlipidaemia (Chew et al., 1996).

Hypertension at baseline is associated with a 40% increase in the risk of DMO (Klein et al., 1998). In normal subjects, the retinal vasculature is autoregulated over widely varying levels of systemic perfusion pressure. However, in diabetic eyes autoregulation of the retinal circulation is impaired, leading to increased flow, which is proportional to the systemic blood pressure. Increased retinal blood flow is also found during, pregnancy, and autonomic neuropathy, all of which are associated with an increase in diabetic retinopathy. In contrast, conditions that reduce retinal blood flow such as carotid artery stenosis and elevated intraocular pressure appear to offer protection from diabetic retinopathy (Archer, 1999).

The UKPDS assessed the effect of blood pressure treatment on diabetic retinopathy. Patients with hypertension were randomised to either tight control of blood pressure (<150/85 mmHg) with a beta-blocker or an angiotensin converting enzyme inhibitor (with additional agents if required), or to less tight pressure control, (<180/105) avoiding the use of beta-blockers or ACE inhibitors. After 7 years follow-up, there was a 35% reduction in the progression of DR in the tight control group. At 9 years, there was 47% reduction in the risk of MVL and the need for photocoagulation was reduced by 35% in the tight control group (of which 78% was macular photocoagulation) (Adler et al., 2000). No benefit of the ACE inhibitor (captopril) over the beta-blocker (atenolol) was observed (Adler et al., 2000).

However, the benefit of ACE inhibitors in delaying the ocular complications of diabetes in addition to their blood pressure lowering effect is likely but less
well confirmed. The role of Renin-Angiotensin System (RAS) in the eye appears to be involved in regulation of blood flow (Ferrari-Dileo et al., 1996) and IOP (Constad et al., 1988; Giardina et al., 1990). Renin, angiotensiogen and ACE have been identified in human eyes along with their corresponding mRNA suggesting it is locally produced (Wagner et al., 1996). Additionally the levels are not related to circulating levels (Kohler et al., 1997; Danser et al., 1994). In diabetic rat, levels of ACE in retinal vessels are elevated compared with non-diabetic controls (Okada et al., 2001).

The EUCLID study, investigated the use of lisinopril, an ACE inhibitor in patients with type I diabetes, who were predominantly normotensive (1997b). At 2 years of follow-up, the ACE inhibitor group had a 50% reduction in the progression of retinopathy compared to placebo (Chaturvedi et al., 1998). However, the findings of this study were weakened owing to differences in initial and final glycated haemoglobin levels favouring lisinopril.

The Heart Outcomes Protection Evaluation Study (HOPE Study), which was a multicentre study of ramipril versus placebo of patients at high risk of cardiovascular disease (2000), has supported the specific effect of ACE inhibition. Although this study was not designed to show an effect on diabetic retinopathy, a reduction in need for laser treatment in the ramipril group was observed. Additionally the DIRECT studies, showed that candesartan reduced the incidence of DR by 25% compared to placebo. However there was no change in progression of retinopathy in those patients with established DR (Chaturvedi et al., 2008).

Data from the ETDRS (Chew et al., 1996) and more recent studies (Sen et al., 2002; Chowdhury et al., 2002) suggest that lipid lowering drugs may decrease the risk of hard exudate formation and associated vision loss in
patients with diabetic retinopathy. The mechanisms by which lipid-lowering agents might improve exudative diabetic retinopathy are not yet established. Basic research has suggested that oxidised LDL cholesterol is toxic to retinal endothelial cells, and that statins may improve endothelial function, platelet aggregation, and reduces growth factor-induced DNA synthesis.

More recently the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study (Keech et al., 2007) found a reduction in progression of established DMO in the fenofibrate group compared with the placebo group. The mechanism of action does not seem to be related to plasma concentrations of lipids.

Based on these and similar findings, guidelines on the optimal control for diabetics have been adopted. HbA1c should be 6.5% or less, blood pressure 140/80 or under and total cholesterol less than 4mmol/l or low-density lipoprotein (LDL) cholesterol level below 2.0 mmol/l (National Institute for Health and Clinical Excellence, 2008). However despite optimal blood glucose control, complications still occur and 20 years after the onset of diabetes, almost all patients with diabetes will have some degree of retinopathy. Even at the time of diagnosis of type 2 diabetes, about a quarter of patients have established background retinopathy (British Medical Association, 2004).
1.7.2 Regular screening and early detection

It has been shown that early detection of sight-threatening diabetic retinopathy and timely treatment halves the risk of MVL (1985). DR conforms well to the principles of screening; it is an important health problem with a recognisable presymptomatic state, the screening procedure is acceptable to the public, there is an appropriate treatment and it is cost effective.

In 2001, the National Service Framework Diabetes Standards were published for England and Wales (National Institute for Health and Clinical Excellence, 2008). These included the targets and standards for diabetic retinopathy screening, stating that by 2007, 100% of patients diagnosed with diabetes should be screened on an annual basis. The standards included that the screening test must be performed by digital photography, screening staff must be adequately trained and have a clinical lead and programme manager and there must be good links between the hospital and primary care.

The strength of screening is illustrated in Iceland. In 1980 Iceland started its screening program. All diabetics were screened and laser treatments carried out when needed. In 1980 the prevalence of legal blindness in the type 1 diabetes population was 2.4% (Danielsen et al., 1982) but by 1994 it had decreased to 0.5% for type 1 diabetes (Kristinsson et al., 1994b) and 1.6% for type 2 diabetes (Kristinsson et al., 1994a).
1.7.3 Current treatments

To date, conventional macular laser therapy is the only proven effective treatment for CSMO (1985). The principal aim of laser photocoagulation is to prevent visual loss. The ETDRS assessed the effect of laser photocoagulation versus observation and found that at 3 years the risk of MVL was reduced by 50% (from 24% to 12%) in the laser group. Visual acuity improved in only 3% of patients (1987). Therefore despite all these current interventions, visual loss due to DMO still occurs and in some patients even the most aggressive treatment cannot prevent vision loss (Frank, 2002). Therefore, there is a desperate need for further treatment options to reduce visual impairment due to DMO.

A number of pharmacological agents for DR and DMO are currently being investigated. These include PKC beta inhibitors (2007), angiotensin converting enzyme (ACE) inhibitors (Sjolie and Chaturvedi, 2002) and anti-VEGF agents (Chun et al., 2006).

1.7.3.1 Protein Kinase C inhibitors

As previously discussed (section 1.2), protein kinase C (PKC) has been shown to be involved in the pathogenesis of DMO. Ruboxistaurin, is an orally active PKC β inhibitor which was developed to reduce the permeability of the BRB (Jirousek et al., 1996). In the retina of diabetic animals, ruboxistaurin has been shown to decrease retinal PKC activity, normalize retinal blood flow (Jirousek et al., 1996), reduce Na+/K+ ATPase activity (Kowluru et al., 1998) and reduce VEGF-induced angiogenesis and VEGF-induced permeability (Aiello et al., 1997).
The Protein kinase C diabetic macular oedema study group (PKC-DMES) was designed to investigate the effect of ruboxistaurin on DMO (2007). This multicenter randomized controlled study assessed patients with oedema further than 300 µm from the center of the macula, without prior photocoagulation. Over 30 months, 686 patients received placebo or ruboxistaurin orally (4, 16, or 32 mg/day). The primary outcome was progression to sight-threatening DMO or application of focal/grid photocoagulation for DMO. There were no statistical differences between the groups. However secondary analysis show that 32 mg ruboxistaurin significantly reduced the occurrence of MVL and reduced the encroachment of CSMO to the centre of the macula in patients with moderate to severe NPDR (Aiello et al., 2006).

1.7.3.2 Anti-VEGF agents

As previously discussed VEGF has been implicated as an important factor in vascular permeability and DR. There are several different anti-VEGF drugs which have been used in the management of DMO, including pegaptanib (Macugen), bevacizumab (Avastin), and ranibizumab (Lucentis). Pegaptanib is an aptamer, which binds to and thus neutralises VEGF165, whereas ranibizumab, an antibody fragment and bevacizumab, a whole antibody, bind to all isoforms of VEGF.

VEGF undoubtedly plays a central role in the development of DR. However, apart from its pro-angiogenic role its pleiotropic functions range from inducing survival of endothelial cells to potent vasodilation (Ferrara and Gerber, 2001; Pandya et al., 2006). Consequently, although VEGF blockade may be useful in arresting DR, it is predicted to result in additional unwanted side effects. Indeed, hypertension (due to the increased vasoconstriction) and proteinuria (due to glomerular dysfunction) are the most frequent...
adverse effects of systemic VEGF blockade and are particularly worrying in the diabetic population (Simo and Hernandez, 2008).

Although systemic absorption is minimized by intravitreal injection, concern is raised as VEGF maintains fenestrations in the choriocapillaris (Peters et al., 2007) and is important for retinal ganglion cells survival (Nishijima et al., 2007). This adverse effects on the local oxygen supply in ischaemic tissue have to be considered.

Pegaptanib sodium
A multicentred randomized, controlled, phase II trial evaluated the efficacy of pegaptanib versus sham injection in patients with centre-involving DMO (Cunningham, Jr. et al., 2005). At 36 weeks, 0.3 mg pegaptanib led to an increase in median visual acuity (20/50 versus 20/63 (sham)) and the mean central retinal thickness decreased by 68 µm versus an increase of 4 µm with sham. 34% versus 10% of the study group experienced a gain visual acuity (10 or more ETDRS letters) and laser photocoagulation was necessary in fewer patients in the study group.

Bevacizumab and Ranibizumab
Initial pilot studies have shown that bevacizumab and ranibizumab reduce macular thickness improve visual acuity in the short-term in patients with DMO (Haritoglou et al., 2006; Arevalo et al., 2007; Chun et al., 2006; Nguyen et al., 2006). However all studies to date are non-comparative case studies in small numbers of patients.
1.7.3.3 Growth Hormone inhibitors

Prior to the publication of the ETDRS, pituitary ablation was a treatment option for PDR. Improvement in the degree of retinopathy was found to correlate with the postablation level of growth hormone (GH). Since then it has been established that Insulin-like growth factor (IGF-1) a downstream product of GH is elevated in the vitreous of patients with PDR (Grant et al., 1986) and that IGF-1 along with VEGF induce retinal neovascularizations. (Frystyk et al., 2003; Schultz and Grant, 1991). Experimental inhibition of the GH-IGF-1 axis in a model of acute proliferative retinopathy prevented new vessel formation, and small clinical trials indicate that the administration of the GH inhibitor, octreotide maybe beneficial.

A recent multicentre trial in United States, Canada, and Brazil, found that 30 mg octreotide in a long-acting release injection once per month lead to a delay in the progression of retinopathy but no effect was observed for visual acuity and progression of DMO (Grant M, 2008). The most frequent side effects being diarrhoea, development of cholelithiasis, and mild hypoglycemia. A similar European study however failed to confirm these results. When comparing serum IGF-1 it was suggested that the American-based study had better patient compliance and study monitoring than the European study.

1.7.4 Vitrectomy

The vitreous is the transparent gelatinous mass that fills the space between the lens and the retina. It is mostly composed of water (98%) with salts, sugars, collagen type 2 and 9, and glycosaminoglycans. The vitreous is loosely adherent to the anterior body of the retina, the macula and the optic
nerve. If the vitreous pulls away from the retina, as is more common with age, it is known as a vitreous detachment.

The vitreous has implicated in the production of DMO. Eyes with diabetic retinopathy are much more likely to develop macular oedema if the vitreous is still attached. A retrospective study by Nasrallah et al compared the prevalence of posterior vitreous detachment (PVD) in 125 eyes of elderly diabetic patients with and without CSMO. They observed a PVD in 20% of the CSMO group and a PVD in 55% of the non-CSMO group (Nasrallah et al., 1988). It is postulated that an attached posterior hyaloid may cause tangential macular traction leading to macular oedema; a theory that is supported by the fact that both spontaneous vitreous separation and vitrectomy can result in improvement of CSMO (Hikichi et al., 1997).

Vitrectomy may be useful in the absence of obvious traction (Tachi and Ogino, 1996; Ikeda et al., 1999; Ikeda et al., 2000; La Heij et al., 2001). Several explanations have been suggested as a mechanism of action in this instance, such as the vitreous may act as a potential reservoir of inflammatory molecules or growth factors such as vascular endothelial growth factor, which promotes vascular permeability (Aiello, 1997). Another possible explanation is that vitrectomy may improve oxygenation of the retina (Stefansson et al., 1990a).

### 1.7.5 Intravitreal Triamcinolone

The use of intravitreal corticosteroids (GCs) was first suggested over 30 years ago with dexamethasone as an adjunctive treatment for inflammation in endophthalmitis and triamcinolone acetonide as a possible treatment for proliferative vitreoretinopathy (PVR) (Stefansson et al., 1990b; Graham and
Peyman, 1974). Attention was directed towards triamcinolone acetonide (TA) because of its long duration of action owing to its relative insolubility and its lack of intraocular toxicity (Tano et al., 1980).

Like other GCs, TA suppresses inflammation and reduces extravasation from leaking blood vessels (Floman and Zor, 1977), however the complete understanding of its mechanism of action are not fully elucidated. Their anti-inflammatory activity appears to involve inhibition of the phospholipase A2 pathway and the production of prostaglandins and leukotrienes (Abelson MB, 1994). GCs also interfere with the release of inflammatory cell mediators such as ICAM-1 (Penfold et al., 2000) which promote leukocyte adhesion and extravasation (Penfold et al., 2002) and inhibit leukocyte chemotaxis (Abelson MB, 1994). Additionally GCs may have a positive response due to the downregulation of retinal VEGF expression (Nauck et al., 1998; Horiuchi and Weller, 1997).

Intravitreal TA has been shown experimentally to reduce BRB breakdown induced by pan retinal photocoagulation in rabbits eyes (Wilson et al., 1992). Initial pilot studies in humans show that a single 4 mg IVTA injection has a rapid effect in patients with diffuse DMO that have been both resistant to laser treatment and in those with no previous treatment (Patelli et al., 2005; Bonini-Filho et al., 2005; Cardillo et al., 2005) and suggest that intravitreal TA can improve the visual acuity by around 30% (Martidis et al., 2002) (Jonas et al., 2003).

A 4 mg intravitreal TA dose provides continual treatment for approximately 3 months before some eyes begin to have recurrent macular thickening (Beer et al., 2003). At 6 months, it has been shown that up to 90% of eyes may have recurrent macular thickening (Patelli et al., 2005; Bonini-Filho et al.,...
2005; Cardillo et al., 2005) and retreatment is as effective as the initial injection (Ozdemir et al., 2005b). However long-term evidence of efficacy and prospective data of intravitreal triamcinolone in the treatment of DMO is limited.

Recruiting for our trial began in 2002. Since this time, there have been numerous reports of the use of intravitreal TA for DMO. In particular there are three prospective randomised controlled trials using intravitreal TA for more than 6 months. Avitabile et al had the shortest follow up with a mean of only 9 months comparing eyes with DMO treated with intravitreal TA, macular laser or both. In this study, the groups receiving TA had better VA at all time points.

Similarly, in the study by Gillies et al. (Gillies et al., 2006), eyes with persistent DMO had a better visual outcome than the placebo group (subconjunctival saline) if they received 4 mg intravitreal TA at 2 years. Both groups received additional laser treatment if clinically indicated. The primary outcome measure of an improvement of ≥5 letters after 2 years was achieved in 19 of 34 (56%) eyes treated with intravitreal TA, compared with 9 of 35 (26%) eyes treated with the placebo (P = 0.006). It is significant to note that 15 of 28 (54%) treated versus 0 of 21 (0%) untreated eyes (P<0.0001) required cataract surgery.

In contrast, intravitreal TA did not have such positive results in the largest of these three trials (Beck et al., 2009;2008). Eight hundred forty study eyes of 693 subjects with DMO were entered into this study. Ar 2 and 3 years, laser photocoagulation proved more effective than either 1-mg or 4-mg doses of intravitreal TA for most patients with DMO.
1.8 Aims of thesis

1. To create and characterise an in vitro model of the blood-retinal barrier (and blood-brain barrier) by isolating microvascular endothelial cells from rat retinal (and cerebral) capillaries. Due to time limitations, the scope of this thesis was limited to isolated microvascular endothelial cells.

2. To investigate the effect of VEGF on the permeability across microvascular endothelial cells and on the spatial localisation of proteins that comprise the junctional complexes.

3. To investigate the effect of steroids on the permeability across microvascular endothelial cells and on the spatial localization of junction proteins.

4. To determine if repeated intravitreal injections of triamcinolone, improves BCVA at 1 year compared to conventional laser therapy and whether if this treatment has a role in the long-term management of patients with DMO.

5. To investigate the significance of the morphological characteristics of diabetic macular oedema as demonstrated by optical coherence tomography and to assess if they are predictive of outcome of the treatment.

6. To evaluate the safety and long term efficacy of combined vitrectomy and intraocular triamcinolone in patients with diabetic macular oedema without the need for re-injections.
7. To assay the concentration of VEGF in the ocular fluids in patients with DMO both before and after treatment for DMO and to assess if the concentration of vitreous VEGF is predictive of outcome of such treatment.
2. Methods
Chapter 2.1:
Laboratory Methods
All laboratory work was carried out by the study investigator under the supervision of Dr Patric Turowski and with help in basic methodology from other members of the laboratory (see acknowledgements).

### 2.1.1 Materials

All reagents used throughout were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK) with the exceptions mentioned below.

#### 2.1.1.1 Cell culture

All tissue culture plastic ware was purchased from Gibco Life Technologies Ltd (Paisley, UK). Polycarbonate transwell filters (6.5 mm diameter, 0.4 μm pore size) were obtained from Corning Costar Corporation (Corning, NY, USA).

Hanks’ buffered salt solution (HBSS) with and without Ca$^{2+}$ and Mg$^{2+}$, 10x HBSS with Ca$^{2+}$/Mg$^{2+}$, phosphate buffered saline (PBS), Hams’ F-10 medium with glutamax, penicillin-streptomycin and heat-inactivated European foetal calf serum (FCS) were all purchased from Gibco Life Technologies Ltd (Paisley, UK). Bovine serum albumin 0.5% and 22%, and bovine plasma-derived serum (PDS) were purchased from First Link. Collagenase /dispase was purchased form Roche and and collagen IV was purchased from BD biosciences.
2.1.1.2 Cell and Molecular Biology

Methanol was purchased from BDH Laboratory Supplies (Poole, UK) and sterile distilled water (dH₂O) was obtained from Braun (Melsungen, Germany). Bio-Rad Laboratories Ltd (Hemel Hempstead, Herts, UK) supplied Bradford reagent. 30% weight per volume (w/v) acrylamide, 0.8% w/v bisacrylamide stock solution and 10x sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer were both purchased from National Diagnostics (Atlanta, Georgia, USA). Molecular weight rainbow markers, enhanced chemiluminescence (ECL) hyperfilm, and calf intestinal alkaline phosphatase with buffer were purchased from Amersham Pharmacia Biotech International (Little Chalfont, UK). Protran® nitrocellulose membrane was obtained from Schleicher and Schuell (Dassel, Germany) and blotting paper was purchased from Whatman International Ltd (Maidstone, UK). Bovine serum albumin (BSA) used for Bradford assays was obtained from Pierce (Chester, UK). ECL lumi-light reagents were from Roche (Mannheim, Germany). BD Biosciences (Oxford, UK) supplied 26G syringes. Mowiol 4-88, for mounting cells, was purchased from calbiochem and recombinant rat VEGF was supplied by R&D systems.

2.1.2 Collagen coating of plasticware

Calf skin collagen type I (0.005 %) in HBSS was added to all cell culture plastic surfaces for coating for at least 1 hour at room temperature. The collagen solution was removed and the residual collagen was polymerised by placing in ammonia vapour for 15 to 30 min. Following this the plastic ware was extensively washed with HBSS to neutralize the pH.
Polyester filters were coated with human collagen IV (0.01 %) and fibronectin (0.005 %) for 2 hours at 37° C. They were washed in PBS before use.

### 2.1.3 Retinal endothelial cells

#### 2.1.3.1 Isolation of retinal endothelial cells

##### 2.1.3.1.1 Retrieval of eyes

All animals used for the cell cultures were maintained and treated in accordance with the ARVO resolution on the use of animals in research. Lewis rats, each weighing 150 – 170g were used for the isolation of tissues. The periorbital area of each freshly killed rat was cleaned with 70% ethanol. A medial and lateral cantholysis of each eye was performed. The insertion of the muscles to the globe was cut and the eye enucleated by cutting through the optic nerve. The whole eyes were immediately transferred to ice-cold working buffer (Ca\(^{2+}\)/Mg\(^{2+}\) - free HBSS, 10 mM Hepes, 100 i.u penicillin, 100µg/ml streptomycin, 0.5% Bovine serum albumin (BSA), pH 7.4)

##### 2.1.3.1.2 Isolation of the retina

Using a dissecting microscope under aseptic conditions, each eye was placed on a gauze swab soaked in working buffer. An incision was made just posterior to the limbus and using a pair of Vannas scissors each eye was hemisected and the anterior segment and vitreous removed. A 360° retinal detachment was created and the retina cut at its insertion to the optic nerve. The retinae were transferred to a fresh container of working buffer and kept in ice.
2.1.3.2 Digestion of the retina 1

The retinae were centrifuged for 5 minutes at 4°C at 600g and the supernantant removed. The pellet was resuspended in 5 ml of digest medium (Ca²⁺/Mg²⁺-free HBSS, 1mg/ml collagenase /dispase, 10mM Hepes, 20 units/ml DNAse, 0.147µg/ml Tosyl-lysine-choromethylketone (TLCK), 100 i.u. penicillin, 100µg/ml streptomycin.). The retinae were digested for 1 hour at 37°C, agitating every 15 minutes.

At the end of the one-hour digest, the sample was centrifuged for 5 minutes at 4°C at 600g, resuspended in 20 ml of 22% BSA and further centrifuged for 20 minutes at 4°C at 1000g. The supernatant was poured off and the tube inverted to drain off residual material. The pellet was resuspended in 1 ml of working buffer, transferred into a fresh container and topped up with 9 ml of working buffer and then centrifuged for 5 minutes at 4°C at 600g.

2.1.3.3 Digestion of the retina 2

The retinae were digested in a further 5 ml of digest medium for 1 hour at 37°C. Afterwards the tissue fragments were centrifuged for 5 minutes at 4°C at 600g and then resuspended into 1ml of working buffer. Capillary vessels were then further processed or cultured as detailed in paragraphs 2.1.5–2.1.7.
2.1.4 Brain endothelial cells

2.1.4.1 Isolation of brain endothelial cells

2.1.4.1.1 Retrieval of the Brains

The head and neck region of each freshly killed animal was sprayed with 10% ethanol. The head was removed from the body and any remaining vertebrae removed. A midline incision was made over the vertex of the skull and 2 lateral incisions opened the occipital bones so that the bone and dura matter could be peeled back and the brain exposed and removed. The brains were transferred in to a container of working buffer and kept on ice.

2.1.4.1.2 Isolation of the Cerebral cortex

Isolation of the cerebral cortex was performed under aseptic conditions. Each brain was placed on a sterile gauze swab soaked in working buffer. The cerebellum was removed and the hemispheres separated. The remaining meninges and the choroidal plexus were removed along with as much of the white matter as possible. The residual grey matter was transferred into a fresh container of working buffer and chopped into 1-2 mm$^3$ pieces with a scalpel.

2.1.4.2 Digestion of neural tissue 1

The neural tissue was centrifuged and digested in 15 ml of digest buffer as described for the retina. At the end of the first digestion, the tip of a Pasteur pipette was gently flamed to remove any sharp edges, and used to triturate the neural material. A second pipette was gently narrowed over the flame and used to further break up the neural tissue. The suspension was
centrifuged for 5 minutes at 4°C at 600g, and the pellet resuspended in 20 ml of 22% BSA. This was then centrifuged for 20 minutes at 4°C at 1000g. A myelin plug was formed on the top of the suspension and a pellet of microvessels at the bottom. The myelin plug was rolled away from the sides of the tube and disregarded. The supernatant poured off and kept. The tube was then inverted to drain off any residual material. The pellet was resuspended in 1 ml of working buffer, transferred into a fresh container and topped up with 9 ml of working buffer and centrifuged for 5 minutes at 4°C at 600g. The titration process was then repeated on the residual supernatant.

2.1.4.3 Digestion of neural tissue 2

The neural tissue was further digested in 5 ml of digest media for 2 - 3 hours at 37°C. As for the retina, the brain microvessels were centrifuged to remove the digest media and resuspended in working buffer.

2.1.5 Purification of microvessels

One problem encountered during the isolation and culture of primary endothelial cells is the growth of contaminating cells. 2 methods were used to reduce the overgrowth of vessels. The first was the use of a percoll density gradient and the second was the use of puromycin in the media to selectively kill non-endothelial cells.
2.1.5.1 Percoll density gradient

The gradient was pre-formed, in 10 ml sterile Du Pont centrifuge tubes. The tubes were coated with working buffer for 1 hour to prevent the microvessels sticking to the tube walls. The gradient was preformed by centrifuging 7 ml of percoll solution (50 ml percoll, 5 ml 10x HBSS with Ca\(^{2+}/Mg^{2+}\) and 45 ml 1x HBSS with Ca\(^{2+}/Mg^{2+}\)), in a Heraeus centrifuge for 1 hour at 4\(^{\circ}\)C at 25,000g.

The microvessels, resuspended in 1 ml of working buffer, were gently layered onto the preformed percoll density gradient and centrifuged for 20 minutes at 4\(^{\circ}\)C at 1000g. The capillary fragments from brain isolations were seen as hazy layer approximately two thirds down the tube. These were removed placed into a fresh tube and washed in working buffer. In the retinal extract the capillary fragments were more difficult to detect with the naked eye and therefore, a layer equidistant to the one seen in the brain isolates was taken. The washed microvessels pellets were resuspended onto growth media (Hams F-10 containing 16% bovine cell free plasma-derived serum (PDS), 2mM L-glutamine, 100 i.u. penicillin/100µg/ml streptomycin, 80µg/ml heparin (Grade I) and 75µg/ml endothelial cell growth supplement (ECGS)) ready for cell culture.

2.1.5.2 Puromycin

The use of puromycin to purify endothelial microvessels was first described by Perrière et al (Perriere et al., 2005). P-glycoprotein (P-gp) is a multidrug resistance-associated protein that is found on the plasma membrane of
endothelial cells in particular of the BBB and BRB (Beaulieu et al., 1997). Endothelial cells that express P-gp can survive treatments that target cells that lack P-gp (Jette et al., 1993). This characteristic can be used to select endothelial cells in culture as contaminating cells lacking P-gp are unable to survive (Chen et al., 1998).

Puromycin is an aminoacylnucleoside antibiotic produced by Streptomyces alboniger. Puromycin is cytotoxic to the cells lacking P-gp when it is metabolized in the cells into puromycin aminonucleoside, which goes on to produce reactive oxygen species, thus producing its toxicity (Zent et al., 1995; Gwinner et al., 1997).

Retinal and brain endothelial cells were seeding onto collagen coated tissue culture plates and incubated at 37°C in a 5% CO² / 95% air in humidified atmosphere. Cells were cultured in Hams F10 medium containing 16% bovine cell free plasma-derived serum (PDS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 80 µg/ml heparin (Grade I), 75 µg/ml endothelial cell growth supplement.

Following the 24 hour attachment period, the cell medium was changed and supplemented with 5 ng/ml puromycin. The medium containing puromycin was renewed every 24 hours for three days. Subsequently the cells were extensively washed and placed in normal growth medium.
2.1.6 Culture of retinal and brain endothelial cells

Cells were cultured / subcultured for up to 3 weeks at 37°C in 5% CO$_2$ and the growth medium was replaced every 2-3 days. During their growth, cultures were frequently inspected by inverse phase contrast microscopy to record their growth and morphology and to observe for an overgrowth of contaminating cells.

2.1.7 Trypsination and cell passage

After 7 - 8 days, cells were passaged. The cells were first washed with Ca$^{2+}$/Mg$^{2+}$-free phosphate buffered solution (PBS). 0.025% trysin in Ca$^{2+}$/Mg$^{2+}$-free PBS was added to the cells for 10-20 seconds and then aspirated. Under a microscope, the detachment of the cells could be observed. When 50% of the cells detached the cells were washed off with growth medium, triturated with a 5 ml pipette and plated onto collagen-coated plastic ware for experiments.
2.1.8 **Cell fixation**

2.1.8.1 **Methanol fixation**

The culture media was removed and immediately replaced with -20°C methanol for 5 minutes at room temperature. The methanol was diluted out and the dishes rinsed three times with phosphate–buffered saline (PBS).

2.1.8.2 **Formaldehyde / formalin fixation**

The culture media was removed and the cells immediately fixed with freshly prepared 3.7% formaldehyde in PBS for 15 minutes at room temperature. The cells were washed with PBS and the cells permeabilised by adding -20°C acetone (propanone) to the dish for 3 minutes. The acetone was then diluted out with PBS.

2.1.9 **Immunofluorescence**

Retinal and brain microvascular endothelial cells were passaged onto collagen coated dishes and grown to confluence. The cells were washed with PBS and fixed with either methanol or formaldehyde as described above. Samples were blocked with PBS, 0.5% BSA and 0.02% Azide (PBS/BSA/azide). The primary antibody at the desired dilution (table 2.1.1) was centrifuged at 6000g for 5 minutes to remove any particulates. This was added onto the monolayer of cells and incubated at 37°C for 1 hour.

Samples were again blocked with PBS/BSA/Azide and incubated at 37°C with the centrifuged secondary antibody at the desired dilution (table 2.1.1).
Hoechst stain (bis-benzamide) was added along with the secondary antibody. This stain binds double-stranded DNA and therefore permits visualisation of cell nuclei.

Cells were then washed with PBS/BSA/Azide, followed by PBS and then water and mounted in 10% Mowiol 4-88. Omission of the primary antibody served as a negative control. Cells were initially viewed with a fluorescent microscope (Axiophot) and thoroughly imaged on a confocal scanning microscope as detailed in the next paragraph.
Table 2.1.1: Details of primary antibodies used for immunofluorescence and immunoblotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host species</th>
<th>Clone</th>
<th>Dilutions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1 Occludin</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>1:50</td>
<td>Zymed</td>
</tr>
<tr>
<td>Cl-5 VEC</td>
<td>mouse</td>
<td>monoclonal</td>
<td>1:100</td>
<td>Zymed</td>
</tr>
<tr>
<td>a catenin</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>1:20</td>
<td>Santa cruz</td>
</tr>
<tr>
<td>b catenin</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>1:50</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>g catenin</td>
<td>mouse</td>
<td>polyclonal</td>
<td>1:50</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>P120</td>
<td>mouse</td>
<td>monoclonal</td>
<td>1:50</td>
<td>BD biosciences</td>
</tr>
</tbody>
</table>

ZO-1, zonular occludens-1; Cl-5, claudin-5; VEC, vascular endothelial cadherin / VE cadherin; a catenin, alpha catenin; b catenin, beta catenin; g catenin, gamma catenin.
### Table 2.1.2: Details of secondary antibodies and stains used for immunofluorescence and immunoblotting

#### Secondary antibodies for immunofluorescence

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host species</th>
<th>Clone</th>
<th>Dilutions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti rabbit FITC conjugated</td>
<td>goat</td>
<td>polyclonal</td>
<td>1:50</td>
<td>ICN/Cappel</td>
</tr>
<tr>
<td>anti rabbit rhodamine conjugated</td>
<td>goat</td>
<td>polyclonal</td>
<td>1:50</td>
<td>ICN/Cappel</td>
</tr>
<tr>
<td>anti goat FITC conjugated</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>1:50</td>
<td>ICN/Cappel</td>
</tr>
</tbody>
</table>

#### Secondary antibodies for immunoblotting

<table>
<thead>
<tr>
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<th>Host species</th>
<th>Clone</th>
<th>Dilutions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti rabbit HRP</td>
<td>goat</td>
<td>polyclonal</td>
<td>1:10,000</td>
<td>Southernbiotech</td>
</tr>
<tr>
<td>anti mouse HRP</td>
<td>goat</td>
<td>polyclonal</td>
<td>1:10,000</td>
<td>Imgenex</td>
</tr>
<tr>
<td>anti goat HRP</td>
<td>Rabbit</td>
<td>polyclonal</td>
<td>1:5,000</td>
<td>Southernbiotech</td>
</tr>
</tbody>
</table>

#### Secondary stains for immunofluorescence

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<th>Antigen</th>
<th>Dilutions</th>
<th>Source</th>
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</thead>
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<tr>
<td>Phalloidin FITC</td>
<td>1:50</td>
<td>Biotium</td>
</tr>
<tr>
<td>Phalloidin rhodamine</td>
<td>1:50</td>
<td>Biotium</td>
</tr>
<tr>
<td>Hoechst</td>
<td>1:100</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

FITC, Fluorescein isothiocyanate
2.2.10 Confocal laser scanning microscopy

Confocal laser scanning microscopy was carried out on a CLSM 510 (Zeiss, Welwyn Garden City) equipped with UV, argon and helium-neon lasers. Standard settings were used to visualize Hoechst, fluorescein and rhodamine staining. FITC and rhodamine were excited at 488 nm and 543 nm and visualized with 540 +/- 25 and 608 +/- 32 nm bandpass filter respectively, where the levels of interchannel cross talk were insignificant. The pinhole was set to 1 Airy unit. Images were acquired through a x40 or x60 oil immersion lens. Series of overlapping 0.8 µm sections spanning the entire cell thickness (ca. 4 µm) were recorded and projections generated as detailed in the figure legends.

2.1.11 Analysis of images

A change in the mean pixel intensity of the immunofluorescent images, captured by confocal, were quantified using ImageJ software package (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006.). Similarly this programme was used to quantify the change in the mean pixel intensity visualized on immunoblotting.
2.1.12 Transendothelial permeability assay

The passage of FITC-dextran through confluent cell monolayers was measured using transwell cell culture chambers (Polyester filter of 6.5 mm diameter, 0.4 um pore size, Costar). The wells were each coated with 150 µl collagen IV and fibronectin (both at 50 µg/ml) for 2 hours at 37°C. Brain endothelial cells were seeded at a density of 40 000 to 100 000 per well and grown until confluent.

The volume of the apical and basolateral chambers were 500 µl and 1500 µl respectively. 24 hours before experimentation, the medium (from both chambers) was diluted 1:2 with serum free medium. Each experiment included at least three wells per group. The principal findings were confirmed in at least two duplicate experiments.

Sodium fluorescence (FITC) of molecular weight 376 Da or fluorescein isothiocynate-labeled dextrans (FITC-dextran) of variable molecular weight were added to the top well (final concentration 1 mg/ml) and further incubated a 37°C. At desired time points 50 µl was sampled from the basal chamber and the volume replaced with 50µl of the serum free medium in order to maintain hydrostatic pressure throughout the experiment.

Each sample was diluted and then analysed in triplicate, along with reference samples from the apical chamber at time 0, were analysed on a plate reader spectrophotometer (Safire version 4.20) using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The permeability coefficient (Pe) was according to the following formula,

$$\frac{1}{P_{e_{cell}}} = \frac{1}{P_{e_{all}}} - \frac{1}{P_{e_{filter}}}$$
For calculating the Pe, slope of clearance versus time was determined by linear regression analysis and multiplied the surface area of the filter (0.65 cm$^2$). Finally, the reciprocal value of the permeability coefficient without cell layer ($P_{E_{filter}}$) is subtracted of the permeability coefficient of the experiment with cell layer ($P_{E_{all}}$) to gain the reciprocal value only for the cell layer.

### 2.1.13 Transendothelial electrical resistance

Transendothelial electrical resistance (TER) was measured through confluent monolayers using transwell cell culture chambers. Chambers were coated and seeded as above.

Subsequently changes in TER across the monolayers were monitored over a 2-week period using STX-2 chopstick electrodes connected to an EVOM epithelial voltohmmeter (World Precision Instruments, Herts, UK). Net TER values were calculated by subtracting the mean resistance determined for 2 filters in the absence of cells (prepared and treated as the experimental samples), from the value recorded for each monolayer grown on transwell cell culture chambers. Final resistance-area products (in ohms * cm$^2$) were obtained by multiplication with the effective growth area.

Each experiment included at least four wells per group. The principal findings were confirmed in at least two duplicate experiments. A resistance of over 100 (Ω *cm$^2$) was deemed significant.
2.1.14 Cell extraction

2.1.14.1 Total cells extracts

Cells were placed on ice and washed with ice cold PBS. Protein fractions were prepared from cultured cells by solubilisation in boiling SDS sample buffer (4% SDS, 20 mM tris buffer (pH 6.8), 20% glycerol, 0.005% bromophenol blue and 100 mM dithiothreitol (DTT)). Following lysis, the cells were scraped off the dishes using a rubber cell scraper and the cell lysates were syringed through a 26 G needle to shear genomic DNA, heated to 100°C for 5 minutes and then kept at -20°C until required.

2.1.14.2 Cell fractionation

Cells were placed on ice and washed with ice cold PBS. Subcellular protein fractions were prepared by solubilisation in lysis buffer (10 mM Hepes/sodium hydroxide (pH 7.5 containing 1 mM DTT, 1.5 mM Magnesium chloride, 5 mM EDTA, 100 nM calcyculin A, 1 mM pervanadate and a mix of protease inhibitors (E64, TPCK, pepstatin A, PMSF). Subsequently, the cells were scraped off the dishes using a rubber cell scraper and then lysed by syringing through an 18 G needle and a 23 G needle. Cells were inspected under a microscope to ensure completion of lysis (i.e. that nuclei had been released). Nuclei, polymerized cytoskeleton and large organelles were collected by centrifugation at 1000g for 5 minutes at 4°C. The supernatant was respun in an ultracentrifuge at 100 000g for 30 minutes at 4°C resulting in a high speed (membrane) pellet and a high speed (cytosolic) supernatant.
2.1.15 Protein concentration Determination (Bradford Assay)

The protein concentration of the cell extracts were determined by the method of Bradford (Bradford, 1976). Duplicate solutions containing 1µl, 2µl and 5µl aliquots were taken from each sample and each added to 995 µl of 1:50 Bradford reagent (Bio Rad protein assay). The absorption spectrum was measured at 595 nm and the protein concentration calculated by comparing to a standards curve produced from bovine serum albumin (BSA) standard.

2.1.16 Sodium Dodecylsulphate Polyacrylamide Gel electrophoresis (SDS-PAGE)

Tris-glycine slab gels were cast using the Biorad Mini-protein II system. All apparatus was thoroughly washed and rinsed before use. Separating gel mix contained 10% polyacrylamide, 375 mM Tris/Cl pH 8.8, 0.1 % SDS, 0.08 % APS and 0.0004 % TEMED. This was poured in between the gel plates and left to polymerise. A stacking gel (4.5% acrylamide, 125 mM Tris/Cl pH 6.8, 0.1% SDS, 0.15% APS and 0.002% TEMED) was cast on top.

50 µl of each sample was loaded per well. To visualize the migration of proteins and give an indication of the molecular weight of the detected proteins, 8 µl of markers were loaded into each gel along side samples. The standard molecular weight of the markers were 220, 97.4, 66, 46, 30, 21.5 and 14.3 kDa. Gels were typically run at 100 V for approximately 90 minutes or until the sample front had reached the end of the gel.
2.1.17 Electrotransfer

A Biorad wet-transfer gel tank was used to electrotransfer proteins to nitrocellulose membranes (Hybond ECL, Amersham). The components for the transfer were first equilibrated in the transfer buffer (25mM Tris, 192 mM glycine, 0.1 % SDS and 20% methanol). A stack of the components consisting of a pad, blotting paper, the gel, nitrocellulose membrane blotting paper and further pad were arranged and compressed to remove air bubbles. Electrotransfer of proteins was done at 12 V for approximately 75–90 minutes.

After transfer, the positions of the standard molecular weight proteins were located by soaking the membrane in 0.1% Ponceau red stain for 5 minutes. The blot was washed to remove the Ponceau Red and then blocked with TBS containing 0.1% Tween, 1% BSA and 0.2% triton X 100 for 1 hour at room temperature.

2.1.18 Immunoblotting

Proteins on blots were detected by probing the nitrocellulose membrane with primary antibodies diluted in TBS containing 1% BSA, 0.05% Tween-20 and Triton X 100 at room temperature for 1.5 hours (see Table 2.1.1). After extensive washing with TBS/BSA/Tween/Triton X100, the membrane was incubated for 45 minutes at room temperature with HRP-conjugated secondary antibodies of the appropriate species, diluted in PBS/BSA/Tween/Triton X100. The membrane was again extensively washed using PBS/BSA/Tween/Triton X100, then PBS/Tween/Triton X100 and finally with PBS alone.
Immunodecorated proteins were visualized by immersion of the blots into enhanced chemiluminescence detection reagent (ECL, Amersham, Bucks, UK) for 5 minutes. Membranes were then wrapped in transparent layer and exposed to light sensitive film (Hyperfilm, Amersham).

Membranes could be ‘stripped’ of bound antibodies and re-probed by incubating in stripping buffer (200 mM glycine / HCL ph 2., 1% v/v NP-40, 500 mM NaCL) for 20 minutes at room temperature. Membranes were then washed 3 times with Tween 20 /TBS, then reblocked and immunoblotted as before.

2.1.19 Statistical analysis

For all quantitative experiments, data was compared using a Student's t-test using a two-tailed P-value with a significance set at 0.05.
Chapter 2.2:
Clinical studies
2.2.1 Patient Enrolment

All studies were conducted in accordance with the ethical standards of the Declaration of Helsinki and were approved by the local Research and Ethics Committees at Moorfields Eye Hospital. Patients were recruited via the Medical Retinal Service at Moorfields Eye Hospital between February 2003 and June 2005. Written informed consent was obtained from all patients before details of medical and ophthalmic examinations were recorded.

If both eyes were eligible for inclusion into the randomized controlled trial (RCT) or combined pars plana vitrectomy with intraocular triamcinolone (PPV + TA), the eye with the worst BCVA was included and the fellow eye followed standard treatment.

Study assessments are carried out by the study investigator. All information was stored in an anonymous fashion on an access database. Each patient was identified by their unit number and a unique study number.

2.2.2 Inclusion and exclusion criteria

Inclusion and exclusion criteria for all studies is shown in table 2.2.1. CSMO as defined by the ETDRS (1985) was diagnosed by slit lamp biomicroscopy and confirmed both angiographically and tomographically.

In addition any patient with a pre-existing PVD was excluded from the pars plana vitrectomy studies.
### Table 2.2.1: Study inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSMO persisting 4 or more months</td>
<td>Significant macular ischaemia (foveal avascular zone of greater than 1000μm or severe perifoveal capillary dropout as compared with ETDRS standard photograph) (1991a)</td>
</tr>
<tr>
<td>at least 1 prior laser treatment</td>
<td>baseline IOP greater than 23 mmHg</td>
</tr>
<tr>
<td>BCVA between 6/12 – 3/60</td>
<td>glaucoma</td>
</tr>
<tr>
<td>visual acuity in fellow eye ≥ 3/60.</td>
<td>co-existent retinal disease</td>
</tr>
<tr>
<td>duration visual loss &lt; 24 months</td>
<td>loss of visual acuity as a result of other causes</td>
</tr>
<tr>
<td></td>
<td>previous vitrectomy</td>
</tr>
<tr>
<td></td>
<td>intraocular surgery or laser within 3 months of study entry</td>
</tr>
<tr>
<td></td>
<td>previous inclusion in other clinical trials for DR</td>
</tr>
<tr>
<td></td>
<td>inability to return for follow up</td>
</tr>
<tr>
<td></td>
<td>inability to give informed consent</td>
</tr>
</tbody>
</table>

CSMO, clinically significant macular oedema; BCVA, best corrected visual acuity; ETDRS, early treatment of diabetic retinopathy study; IOP, intraocular pressure; DR, diabetic retinopathy
2.2.3 Sample size Calculation for RCT

The principal outcome measure was the proportion of patients who improved by 15 or more ETDRS letters at 12 months. Based on the original pilot studies, (Jonas et al., 2003; Martidis et al., 2002) the estimated frequency for this outcome measure in the TA arm would be approximately 20% and from the ETDRS the in laser arm approximately 3%. The study was powered at 80%, with a significance level of 0.05. Therefore for a ratio of study patients to control patients of 1:1, the calculated sample size was 41 patients in each arm. Adjusting for an estimated loss to follow up of 8% of eyes, we aimed to recruit 44 patients into each group.

2.2.4 Data collection

Best corrected visual acuity (BCVA) was measured by a trained optometrist, masked to treatment arm, using an ETDRS chart to record distance vision, a Bailey-Lovie chart at 25 cm to record near vision and a Pelli-Robson chart at 1 metre to record contrast sensitivity. Intraocular pressure (IOP) was measured using Goldman applanation tonometry. Fundoscopy was performed by slit-lamp biomicroscopy using a 66D lens. The degree of lens opacity was graded using the Wisconsin system for classifying cataracts (Klein BEK, 2007). Diabetic control was assessed by measuring Glycosylated Haemaglobin (HbA1c).

At 4, 8 and 12 months, follow up visits included BCVA, IOP and central retinal thickness.
2.2.5 Fundus Fluorescein Angiogram (FFA)

A FFA was performed at baseline to assess the degree of macular ischaemia and at 12 months to determine any change in the degree of macular ischaemia.

Macular ischaemia was graded based on the ETDRS grading system. The maximal linear diameter of the foveal avascular zone (FAZ) was measured using the measuring tool on the Topcon imaging system and amount of capillary drop out assessed against ETDRS standard photograph (fig 2.2.1).
**Figure 2.2.1:** Examples of levels of capillary drop out. Copy of Standard photograph 2 representing severe capillary dropout from ETDRS report 11 (1991a). Any patient considered to have equal or worse perifoveal dropout when compared to standard photo 2 was excluded from study (a). This patient was considered to have moderate capillary dropout and was therefore included in the study (b) while this patient was considered to have severe capillary dropout and was excluded from study (c).
2.2.6 Optical Coherence Tomography (OCT)

OCT was performed by a certified research technician, masked to the treatment arm. The macula was scanned using the six x 6mm radial line scan protocol on the Stratus OCT model 3000 scanner (Zeiss Humphrey Instruments, Dublin, CA). All computer generated measurements were visually inspected by the study investigator to exclude computerised artefacts. If no artifacts were observed, the foveal minimum was selected to represent the central macular thickness from the computer generated retinal thickness analysis program. In the case of computerised artifacts, the retinal thickness was calculated manually using the calipers available on the software. Macular volume, where possible, was calculated using the computer algorithm.

2.2.7 Randomisation for RCT

Patients were allocated to 4 mg of TA or further ETDRS laser photocoagulation using a centralised computer-generated randomisation program (based on the weighted coin method) in operation at the Clinical Trials Unit. Patients were retreated at 4 and 8 months if they had persistent CSMO on clinical examination with final review at 12 months.
2.2.8 Treatments

2.2.8.1 Intravitreal triamcinolone

Intravitreal TA was performed by the study investigator in a treatment in the outpatient clinic under sterile conditions. The eyes were prepared with 1% amethocaine followed by 5% povidone-iodine. A subconjunctival injection of 2% lignocaine was given using a 27-gauge needle, sufficient to raise a small bleb over the site of intravitreal injection. Then, using a sterile 27-gauge needle, 4 mg /0.1 ml of triamcinolone acetonide (Kenalog, Bristol Myers Squibb) was injected through the pars plana into the mid-vitreous without removing the vehicle. Following injection, indirect ophthalmoscopy was used to check the patency of the central retinal artery and if occluded a paracentesis was performed. Following the procedure 0.5% chloramphenicol was instilled and eyes were checked at the slit lamp, to look for triamcinolone crystals in the anterior chamber, to measure the IOP and to examine the fundus. Patients were discharged with 0.5% chloramphenicol four times a day for four days. A further post operative check was repeated after 1 week and 1 month.

2.2.8.2 Laser photocoagulation

Conventional macular laser was performed by the study investigator based to ETDRS guidelines (1985). A pre-treatment FFA was used to identify areas of leakage. Areas were treated with 100 micron argon green-only burns of 0.1s duration, with adequate power to obtain light grey burns in areas of leakage. Care was taken to avoid the foveal avascular zone (FAZ).
2.2.8.3 Pars plana vitrectomy with intraocular triamcinolone

All operations were performed by 2 surgeons. A standard 3-port pars plana vitrectomy was performed on all patients. The posterior hyaloid was detached from the posterior pole by using high suction power with the vitrectomy instrument at the optic disc. Further separation of the posterior hyaloid towards the peripheral retina was achieved with the vitrectomy instrument using a combination of suction and cutting. For patients with proliferative diabetic retinopathy the new vessels were delaminated with horizontal scissors and additional endolaser photocoagulation was applied. The internal limiting membrane was not peeled. At the end of surgery 4 mg /0.1 ml of intravitreal triamcinolone (Kenalog, Bristol Myers Squibb) was injected into the vitreous cavity via one of the vitrectomy ports. Patients were postured in the upright position for 8 hours. No further injections of triamcinolone were performed during follow up.

2.2.9 Collection of vitreous and aqueous samples

Undiluted vitreous fluid samples were harvested from the mid-vitreous at the start of vitrectomy. The aqueous fluid was acquired at the end of the operation before the injection of TA. Further aqueous samples were collected at 1 week and 4 months. All samples were frozen at -80°C until required.
2.2.10 VEGF Assay

The samples were defrosted and centrifuged to remove particulate material. VEGF levels were quantified by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Each assay was performed according to the manufacturer’s instructions.

In summary, the seven standards and one zero were run in duplicate along with the unknown samples which were run in triplicate. Where stated, plates were washed 3 times with the wash buffer supplied by the kit. All incubations were performed at room temperature.

50 μl of the standard solution and samples were added to the wells of a 96-well plate, which was pre-coated with an anti-human VEGF antibody and incubated for 1 hour at room temperature. After incubation, the plate was washed, and 100 μl biotinylated antibody was added to each well and incubated for 1 hour. After the second incubation, the plate was washed and 100 μl streptavidin-HRP reagent was added to each well and incubated for 30 minutes. The plate was then washed and 100 μl of TMB substrate solution was added to each well. The enzymatic colour reaction was allowed to occur in the dark at room temperature.

After 30 minutes, the reaction was stopped by adding 100 ul of stop solution to each well and the optical density was determined at 450 and 550 nm using an absorption spectrophotometer (Safire multiscan 12901300013, firmware V 2 00 03/02 Safire; XFLUOR4 version: V 4.20). Reading at dual wavelengths corrected for optical imperfections within the microtitre plate.
A standard curve was plotted from measurements made with the standard solution (from 0 to 1000 pg/ml). A curve-fitting statistical software package was used to plot a four parameter logistic curve fit and this formula was in turn used to interpolate the VEGF concentration in the unknown sample. The minimum detectable concentration was 14.7 pg/ml.

### 2.2.11 Grading of OCTs

This was a retrospective review of all baseline OCTs. 2 radial line scans were selected by the study investigator, from the original 6 scans captured at the baseline visit. Where possible the 2 scans were orientated at 90 degrees to each other.

Based on the classification system by Panozzo (Panozzo et al., 2004), the morphology of each OCT was classified by two independent masked graders. If there was any disagreement in categorization, a third investigator was asked reclassification the scans in question. The 2 OCT scans were graded for the present of the following morphological patterns; cystoid macular oedema (CME), neurosensory detachment (NSD), posterior hyaloid traction (PHT) and abnormal macular profile (AMP).

CME was defined as the localization of circular or ovoid areas of reduced reflectivity. (fig 2.2.2a,b). NSD was defined as a well delineated non-reflective intraretinal space adjacent to the hyper-reflective line of the pigment epithelium (fig 2.2.2b). PHT was defined as a continuous hyper-reflecting line over the inner retinal surface with at least one point of adhesion with the retina (fig 2.2.2a) and AMP was defined as the absence of the normal foveal depression (fig 2.2.2a,b).
Figure 2.2.2: OCT images illustrating morphological subtypes. CME and AMP (a) NSD with a normal macular profile (b). PHT, CME and AMP (c).

CME, cystoid macular oedema; AMP, abnormal macular profile; NSD, neurosensory detachment; AMP, abnormal macular profile.
2.2.12 Outcome measures

2.2.12.1 RCT

The primary outcome measure was the proportion of patients who improved by 15 or more ETDRS letters at 12 months. Secondary endpoints mean ETDRS letter score, mean central retinal thickness and mean macular volume at 12 months and adverse event reporting. Adverse event reporting included a change in IOP measurements, and a significant change in the size of the FAZ.

2.2.12.2 PPV + TA

The outcome measures were the change in visual acuity letter scores, median best corrected visual acuity (BCVA) and central macular thickness at 12 months and adverse event reporting. Adverse event reporting included a change in IOP measurements, and a significant change in the size of the FAZ.

2.2.12.3 Predictive value of OCTs

The outcome measure was determine the relationship between the OCT morphology and the baseline characteristics and the outcome of intervention.
2.2.12.4 Predictive value of VEGF

The outcome measures were the change in aqueous VEGF concentration before and after combined PPV with intravitreal TA and to assess whether the vitreous VEGF level was predictive of outcome at 4 and 12 months.

2.2.13 Statistical analysis

2.2.13.1 RCT

We analysed the data according to the intent to treat principle. However, data for 5 (5.7%) of the patients (4 of whom were on laser treatment) was not available on the primary outcome measure at one year. We did not consider it valid to carry forward data from earlier visits and thus adopted the available case analysis approach. Baseline characteristics of the patients were studied in order to assess the adequacy of randomisation. Fisher's exact test was used to assess the statistical significance of the difference in the proportion of patients in each group who improved by 15 or more ETDRS letters. Analysis of covariance was used to assess secondary outcome variables.

2.2.13.2 PPV + TA

Since this was an exploratory study on a small number of patients descriptive methods only were employed. Summary statistics (medians and
interquartile ranges) were computed, and box and whisker plots constructed to show the change in outcomes over time.

### 2.2.13.3 Predictive value of OCTs

The inter-grader and intra-grader reliability was calculated as a kappa value. The strength of agreement was based on table 2.2.2 as described by Landis and Koch (Landis and Koch, 1977). When there was a discrepancy in grading, a third observer was asked to grade the questionable OCTs. The OCT morphological subtypes were assessed to see if they had any influence over visual outcome using a 2-sample student t-test. $P < 0.05$ was considered statistically significant.

### 2.2.13.4 Predictive value of VEGF

Since this was an exploratory study on a small number of patients, descriptive methods only were employed. Summary statistics (medians and interquartile ranges) were computed and box and whisker plots constructed, to show the change in outcomes over time. Scatterplots and spearman rank sum correlations were used to assess relationships between VEGF levels and visual acuity at 4 and 12 months.
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<tr>
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Table 2.2.2  Strength of agreement based on Kappa values
3. Results
Chapter 3.1
Characterisation of primary retinal and brain microvascular endothelial cell cultures.
3.1.1 Introduction

Breakdown of the inner BRB (iBRB) has been proposed as one of the earliest retinal changes in diabetic retinopathy (Cunha-Vaz et al., 1975). However, the molecular mechanisms involved with disruption of the barrier are not completely understood. Both steroids and VEGF have been shown to modulate vascular permeability but their underlying mechanisms still await full characterisation.

The best index of physiological barrier function is the in vivo measurement of microvascular permeability. However this is a complex procedure requiring elaborate surgical techniques (Cunha-Vaz et al., 1975; Tornquist et al., 1990). In addition, in vivo quantification of permeability can be difficult and is prone to artefact such as from hydrostatic pressure. In contrast, in vitro methods allow the environment of cells to be precisely controlled and the molecular mechanisms in an individual cell type can be studied. Additionally they avoid the complexities of studies on living animals.

The aim of this thesis was to assess the effects of corticosteroids and VEGF on an in vitro model of the BRB and in particular the junctional proteins. Therefore an in vitro model of the BRB was required. An immortalised endothelial cell line generated from rat retinal microvascular endothelial cells was available (SV40 large T), however we were unable to detect any junctional proteins (data not shown). Therefore we sought to isolate primary cells from rat retinal capillary microvessels.
3.1.2 Rat retinal microvascular endothelial cells in vitro maintain endothelial cell characteristics

12 retinas were harvested from the eyes of 6 Lewis rats. Retinal microvascular endothelial cells (RMEC) were isolated from the retinas, by digesting the tissue with collagenase/dispase and DNAse, by physically separating cell types on a percoll gradient and by the selective growth of endothelial cells using puromycin which is toxic to contaminating cells that do not express P-glycoprotein.

At the end of the isolation period, small vessel fragments were identified on light microscopy (fig 3.1.1a,b). Single cells and small microvessel fragments of less than 10 µm diameter attached to the collagen-coated surface within 12 hours. After attachment, cells could be seen spreading out from the microvessels and cell division was noted by day 6.

RMEC grew to form elongated spindle-shaped cells, of average dimensions of 30 µm x 10 µm. Cells grew in a typical swirl-like configuration (fig 3.1.1c). Confluent monolayers exhibited characteristic density-dependent inhibition of growth. The isolated RMEC were 20% confluent by day 7. One isolation of RMEC from six animals yielded approximately 500 000 primary cells at day 14 and consisted of 96-98% pure endothelial cells. On day 7-8, the RMEC were passaged by trypsinisation, to separate the cells and encourage cell division.

Weibel-Palade bodies are secretory organelles which are highly specific to endothelial cells (Elgjo et al., 1975). One of their main constituent proteins is von Willebrand Factor (vWF). Its expression is considered to be a faithful marker of endothelial cells. Indirect immunocytochemistry of our RMEC cell
cultures with anti-vWF antibodies revealed staining of cigar-shaped organelles distributed throughout the cytoplasm of all RMEC (fig 3.1.1d). This distribution was reminiscent of Weibel-Palade bodies and similar to that reported for many other endothelial cells including HUVEC (Elgjo et al., 1975).
Fig 3.1.1: Retinal microvascular endothelial (RMEC) cell isolation. Phase contrast images of RMEC at 1 day (a,b) and at 1 week (c). RMEC at one week were fixed using formaldehyde and stained for von Willebrand factor (d).
Endothelial cells contain functional tight junctions (TJ) (Karnovsky, 1967) and we therefore characterized TJ in RMEC. The expression and distribution of occludin, zonula occludens -1 (ZO-1) and Claudin-5 (Cl-5) was analysed by indirect immunocytochemistry (see appendix for negative control staining). Staining of occludin was mostly restricted to junctional areas at cell-cell contacts (fig 3.1.2,a). ZO-1 was also primarily localised to the junctional areas of cells but additional diffuse intracellular localisation in the perinuclear cytoplasm and in the nucleus was detected (fig 3.1.2,b). Cl-5 staining delimited the cell boundary but diffuse staining was observed in the area of the main cell body of 1 -5 % of cells (fig 3.1.2,c-f). When assessed in more detail this diffuse stain appeared to be present mostly in apical sections of the preparations (fig 3.1.2,d,e) suggesting that membrane-inserted Cl-5 had not segregated to cell-cell contact areas in all cells. In addition vesicular Cl-5 staining was seen in most cells with increased density in the perinuclear area (fig 3.1.2,f). This vesicular Cl-5 staining was interpreted to represent newly synthesised protein present in endoplasmic reticulum, golgi or transport vesicles. All three proteins could be detected immediately after isolation but occludin and Cl-5 expression started to declined within 8 days of in vitro culturing (results not shown). RMEC cultured and passaged for 2-3 times over a period of 3-4 weeks were devoid of occludin or Cl-5 staining similarly that observed in immortalised cell lines (data not shown).

Because actin filaments play an important role in the organization of adherens junctions (AJ) and TJ, RMEC were analysed using rhodamine-phalloidin. Filamentous actin (F-actin) was identified as filaments throughout the whole cell with the main strands confined to the cortical / junctional area (fig 3.1.3).
Next, the distribution of AJ proteins, VE cadherin (VEC), and associated catenins, p120, alpha-, beta- and gamma-catenins, was analyzed by indirect immunocytochemistry (fig 3.1.4). All AJ proteins were localised in at the junctional area of RMEC. As expected, catenins showed some degree of non-junctional staining. Alpha-catenin showed strong cytoplasmic and nuclear staining, while weak nuclear staining was also observed for gamma-catenin.
Figure 3.1.2: Tight junction protein distribution in RMEC. Cells were grown to confluence and then fixed using methanol. Subsequently cells were stained for occludin (a), ZO-1 (b) and Cl-5 (c-f). The staining of occludin was mostly restricted to junctional areas at cell-cell contacts (a). ZO-1 was also primarily localised to the junctional areas of cells but additional diffuse intracellular localisation in the perinuclear cytoplasm and the nucleus was detected (b, arrow). Cl-5 staining delimited the cell boundary but diffuse staining was observed (c). Images are projections of all confocal sections spanning the entire cell. Shown in panels d, e, f are selected sections from the top (d), bottom (e) or middle (e) of the RMEC monolayer. Cells were stained for Cl-5 and confocal images were taken as 0.8µm slices through the cells. Diffuse stain appeared to be present mostly in apical sections of the preparations (d, arrow). In addition vesicular Cl-5 staining was seen in most cells with an increased density in the perinuclear area (f, arrowhead). ZO-1, Zonula occludens-1; Cl-5, claudin-5
Figure 3.1.3: F-actin distribution in RMEC. Cells were grown to confluence, fixed using 3.7% formaldehyde extracted using acetone and then stained using phalloidin-rhodamine. F-actin was identified as filaments throughout the whole cell with the main strands confined to the cortical / junctional area.

Image is a projection of all confocal sections spanning the entire cell.

F-actin, filamentous actin.
Figure 3.1.4: Adherens junction protein distribution in RMEC. Cells were grown to confluence and then fixed using 3.7% formaldehyde and permeabilised with acetone. Subsequently cells were stained for VEC (a), alpha-catenin (b) and beta-catenin (c), gamma-catenin (d) and p120 (e). All AJ proteins were located at the junction area of RMEC. All catenins showed some degree of non-junctional staining in particular alpha-catenin showed strong perinuclear staining (arrows), while gamma catenin showed weak nuclear staining (d, arrowheads). Images are projections of all confocal sections spanning the whole cell.

VEC, vascular endothelial cadherin / VE cadherin
3.1.3 Characterisation of junctional proteins by subcellular fractionation

Immunofluorence staining is notoriously prone to artefacts and quantification is difficult. Since we wanted to assess changes in the level of junction proteins, we further analysed them by subcellular fractionation and subsequent Western blotting.

RMEC were grown to confluence and then mechanically lysed in a buffer without detergents. Subsequently, lysates were fractionated by centrifugation. We recovered a low spin pellet containing mainly nuclei, large membrane fragments/compartments and F-actin and a high spin pellet containing the remaining membranes and cytoskeleton. The supernatant of the high spin pellet was considered to be the soluble cytosol. Protein extracts were quantified using a Bradford assay and equivalent amounts of cell extract were loaded for each lane.

VEC, Cl-5 and ZO-1 were primarily detected in the low and also high spin pellets (fig 3.1.5). This indicated that LSP was the main biochemical fraction to contain cell-cell junction structures. A small fraction of Cl-5 and ZO-1 was detected in the cytosol. Catenins were found in all fractions.
Figure 3.1.5: Localisation of junctional proteins in RMEC. RMEC were grown to confluence and then mechanically lysed in a buffer without detergents. Subsequently lysates were fractionated by centrifugation. This yielded a low spin pellet, a high spin pellet and a supernatant. All were analyzed along with whole cell lysates by Western blotting for the expression of junction proteins. Protein extracts were quantified using a Bradford assay and equal amounts of cell extract were loaded for each lane. Western blots show typical examples obtained for different proteins. VEC, Cl-5 and ZO-1 were primarily detected in the low and also high spin pellets. A small fraction of Cl-5 and ZO-1 was detected in the cytosol. Catenins were found in all fractions.

HSP, high spin pellet; LSP, low spin pellet; SN, supernatant after high spin; total, total cell lysates.
3.1.4 Isolation and characterization of brain MEC.

One disadvantage of using RMEC was the low yield of cells and this limited further experimentation. Based on the common assumption that the BRB and the BBB are similar in physiology and molecular composition, we also isolated brain microvascular endothelial cells (BMEC) from the rat BBB and then compared these with our in vitro RMEC.

BMEC were essentially isolated as detailed above for RMEC. At the end of the isolation period, small vessel fragments were identified by light microscopy. Single cells and small microvessel fragments of less than 10 µm diameter attached to the collagen-coated surface within 12 hours. After attachment, cells could be seen spreading out from the microvessels and cell division was noted by day 6 (fig 3.1.6a,b).

BMEC were indistinguishable from RMEC. They grew to form elongated spindle-shaped cells, of average dimensions of 30 µm x 10 µm. Cells grew in a typical swirl-like configuration (fig 3.1.6c). Confluent monolayers exhibited characteristic density-dependent inhibition of growth. The isolated BMEC were 95-100% confluent by day 7. One isolation of BMEC from six animals yielded approximately 1 x 10^6 primary cells at day 7 and consisted of 95% pure endothelial cells. On day 7 -8, the BMEC were passaged by trypsination at a dilution of 1:2.

Indirect immunocytochemistry of our BMEC cell cultures with anti-vWF antibodies revealed its staining in all cells (fig 3.1.6d). Staining was exclusively seen on cigar-shaped organelles which were distributed throughout the cytoplasm of BMEC.
**Figure 3.1.6: Brain microvascular endothelial cell isolation (BMEC).**
Phase contrast images of BMEC at 3 day (a,b) and at 1 week (c). BMEC at one week were fixed using formaldehyde and stained for von Willebrand factor (d).
The distribution of the TJ proteins occludin, ZO-1 and Cl-5, was analysed by indirect immunohistochemistry. Occludin was mostly restricted to junctional areas at cell-cell contacts (fig 3.1.7a). ZO-1 was also primarily localised to the junctional areas of cells but additional diffuse intracellular localisation in the perinuclear cytoplasm and the nucleus was detected (fig 3.1.7b). Similar to RMECs, Cl-5 staining delimited the cell boundary and diffuse staining was observed in 40-50% of cells. In addition vesicular Cl-5 staining was seen in some cells (fig 3.1.7c). All three proteins could be detected immediately after isolation but occludin and Cl-5 expression declined gradually after ca. day 8 of in vitro culturing (results not shown).

F-actin was identified as overlapping filaments throughout the whole cell. There was a concentration of the strands towards the cortical / junctional area of the cell (fig 3.1.8).

The distribution of AJ proteins, VEC, alpha and beta catenins, was analyzed by indirect immunocytochemistry (fig 3.1.9). All AJ proteins were mostly restricted to the junction area of BMEC. Beta catenin showed some perinuclear staining.
Figure 3.1.7: Tight junction protein distribution in BMEC. Cells were grown to confluence and then fixed using methanol. Subsequently cells were stained for occludin (a), ZO-1 (b) and Cl-5 (c). Occludin was mostly restricted to junctional areas at cell-cell contacts (a). ZO-1 was also primarily localised to the junctional areas of cells but additional diffuse intracellular localisation in the perinuclear cytoplasm and the nucleus was detected (b, arrows). Cl-5 staining delimited the cell boundary and diffuse staining was observed in 40-50% of cells. In addition vesicular Cl-5 staining was seen in some cells (c, arrowheads). Images are projections of all confocal sections spanning the entire cell. ZO-1, zonula occludens-1; Cl-5, claudin-5
**Figure 3.1.8: F-actin distribution in BMEC.** Cells were grown to confluence, fixed using 3.7% formaldehyde and then stained using phalloidin-rhodamine. F-actin was identified as overlapping filaments throughout the whole cell. Strands concentrated in cortical / junctional areas of the cell. Image is a projection of all confocal sections spanning the entire cell.

F-actin, filamentous actin.
Figure 3.1.9: Confocal images of adherens junction proteins in BMEC.

Cells were grown to confluence and then fixed using 3.7% formaldehyde and permeabilised with acetone. Subsequently cells were stained for VEC (a), alpha catenin (b) and beta catenin (c). All AJ proteins were mostly restricted to the junctional areas of BMEC. Beta catenin showed some perinuclear staining (c, arrows).

Images are full projections of confocal sections spanning the whole cell.

VEC, vascular endothelial cadherin / VE cadherin.
3.1.5 Permeability of MEC.

RMEC and BMEC form the main barrier in-vivo to solutes at the level of the BRB and the BBB respectively (Gardner et al., 1999). In order to correlate changes in the spatial localisation of the junctional proteins with changes in the permeability of MEC, we had to establish methods for testing permeability of our cells in vitro.

MEC were cultured on transwell cell culture chambers, which contain 2 wells. The base of the apical well comprises of a semi-permeable membrane onto which cells can be seeded to form a monolayer and subsequently the exchange of solutes or electrical charge across the monolayer can be measured. However the first challenge encountered was adherence of the cells to the membrane. Based on the results by Tilling et. al. (Tilling et al., 1998), we precoated the membrane with different extracellular matics and found that the combination of collagen IV and fibronectin allowed our cells to form a visually complete monolayer (results not shown).

In addition, the medium was supplemented with 550 nM hydrocortisone (HC) and 100 µM ascorbic acid in order to maintain adherence with the extracellular matrix. HC has been shown to increase in the barrier function of MEC (Antonetti et al., 2002). Ascorbic acid induces endothelial cells to synthesis their own basement membrane (Utoguchi et al., 1995).

Prior to experimentation we diluted our enhanced medium with serum- and hydrocortisone-free medium in order to strike the balance between unduly influencing the results of our experiments with growth factors and the risk of the MECs detaching from the filter.
Permeability was assessed either by measuring the transendothelial electrical resistance (TER) or transendothelial flux. TER depends on areas with the lowest electrical resistance between single cells (Madara, 1998). Transendothelial flux is the passage of detectable molecules for example FITC-conjugated dextrans through the monolayer which depends on the sum of transport across all cellular transport pathways.

### 3.1.5.1 Transendothelial electrical resistance of BMEC

BMEC were grown to confluence and then passaged onto transwell cell culture chambers at a density of 40 000 to 100 000 cells per well into culture medium containing 550 nM HC and 100 µM ascorbic acid until confluent. 24 hours prior to experimentation, the medium was diluted 1:2 with serum free medium containing HC only. Net transendothelial electrical resistance (TER) was measured at intervals until day 13. TER was calculated by subtracting the mean resistance determined from 2 filters in the absence of cells (table 3.1.1). The mean TER was 6.8 Ω·cm².

### 3.1.5.2 Transendothelial permeability assay

BMEC were grown to confluence and passaged onto transwell cell culture chambers at a density of 40 000 to 100 000 cells per well into culture medium containing 550 nM hydrocortisone and 100 µM ascorbic acid until confluent. 24 hours prior to experimentation, the medium was diluted 1:2 with serum free serum free medium containing HC only. Transendothelial permeability was measured by calculating the clearance of fluorescein isothiocyanate (FITC) or FITC-conjugated with 40kDa dextran from the apical to the basolateral aspect of the membrane. The clearance of FITC / FITC-dextran was measured on a plate reader spectrophotometer and the
The permeability coefficient (Pe) was calculated (fig 3.1.10). The Pe for FITC was $2.16 \times 10^{-3} \pm 5.63 \times 10^{-5}$ cm/min and for 40 kDa FITC-dextran, $2.32 \times 10^{-4} \pm 1.83 \times 10^{-5}$ cm/min.
**Table 3.1.1:** Mean transendothelial electrical resistance measured from BMEC over time. Values are a mean over 4 wells. Control wells which consisted of

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TER, trans-endothelial electrical resistance

**Figure 3.1.10:** The flux of FITC and 40kDA FITC-dextran with time. BMEC were grown to confluence on transwell chambers. FITC or FITC-conjugated dextran (40kDA) was added to the apical chamber. The clearance of FITC (a) or FITC-dextran (b) was measured at intervals by spectrophotometry. The diffusive flux (Pe) for FITC was $2.16 \times 10^{-3} \pm 5.63 \times 10^{-5}$ cm/min and for 40 kDa FITC-dextran, $2.32 \times 10^{-4} \pm 1.83 \times 10^{-5}$ cm/min.

FITC, fluorescein isothiocyanate.
3.1.6 Discussion

3.1.6.1 Isolation of RMEC

In order to investigate the effects of steroids and VEGF on the barrier properties and on the junctional proteins of the BRB / BBB an in-vitro model of the blood-retinal barrier (BRB) was required. Isolation of primary cultures of microvascular endothelial cells has been established in numerous animal models most commonly from bovine and rat retinae and each has been shown to form a barrier to tracer molecules (Harhaj et al., 2006; Oshitari et al., 2006). Recently the bovine spongiform encephalopathy has presented a serious social problem (Giles, 2001) and therefore our initial study was to create a reproducible model of the BRB and we chose the rat model. One advantage of using the rat species is that future work in this lab may involve in vivo work on established models of experimental induced diabetic rats which could easily be compared to our in vitro model.

We describe a method to isolate retinal (and brain) endothelial cells adapted from the method of Abbott et al. (Abbott et al., 1992). Our isolated retinal microvascular endothelial cells (RMEC) grew in a typical swirls and exhibited density-dependent inhibition consistent with their endothelial cell lineage. The cells also expressed Weibel-Palade bodies in the cytoplasm of the cell and CI-5 around the cell periphery, both important endothelial cells markers.

The isolation of RMEC was easily accomplished but the maintenance of these populations with time was more difficult. A major problem associated with primary cultures is the difficulty in achieving 100% purity. In order to minimise the growth of other cell types, the microvessels isolation and culture procedure, in particular the use of the percoll gradient, was designed...
to restrict the number of non-endothelial cells plated out. The growth of contaminating cells in cultures is inhibited firstly by selecting a medium containing plasma-derived serum to promote the growth of endothelium and not contaminants such as pericytes (Greenwood, 1992) and secondly by using the puromycin (Perriere et al., 2005). By combining the above techniques a culture purity of 96 -98% and 95% was obtained for the RMEC and BMEC respectively.

### 3.1.6.2 Spatial localisation of TJ and AJ

We characterized the TJ in RMEC by determining the distribution of Cl-5, occludin, and ZO-1. Staining for all three proteins was mostly restricted to junctional areas at cell-cell contacts and was similar to previous studies (Antonetti et al., 2002; Deissler et al., 2008). However, we also observed diffuse cytoplasmic staining for Cl-5 as well as vesicular cytoplasmic staining. The diffuse stain appears to be present mostly in the apical sections of the preparations, suggesting that membrane-inserted Cl-5 had not segregated to cell-cell contact areas in all cells. In addition vesicular Cl-5 staining was seen in most cells and interpreted to represent newly synthesised protein present in endoplasmic reticulum, golgi body or transport vesicles, which is supported by the increased density of these vesicles in the perinuclear area. It is possible that they are due to artefact, however the antibody was centrifuged to remove particulates and this staining pattern was not observed with our negative control (results not shown) and therefore we conclude that they are real observations. Although neither of these cytoplasmic stains have been previously reported, they can be observed in MEC images of other researchers (Weidenfeller et al., 2005; Calabria et al., 2006).
ZO-1 was also primarily localised to the junction areas of cells, but additional diffuse intracellular localisation in the perinuclear cytoplasm and the nucleus was detected. Membrane-associated proteins including ZO-1 are known for their role in intracellular signalling and the cellular location is related to the cell activity. During remodelling of cell-cell contacts, ZO-1 can be detected in the nucleus (Gottardi et al., 1996).

Similarly we observed the junctional staining for the AJ proteins, VEC, alpha-, beta- and gamma-catenin and p120, with some additional nuclear/perinuclear staining for alpha- and gamma-catenin, which again concurs with previous observations (Deissler et al., 2008; Russ et al., 1998; Herrenknecht et al., 1991; Hegland et al., 1999; Romero et al., 2003). Like ZO-1, the membrane-associated proteins of the AJ also have a role in intracellular signalling (Novak and Dedhar, 1999) and therefore the cytoplasmic and nuclear locations are linked to the cellular activity.

We further analysed ZO-1, Cl-5, VEC, alpha and beta-catenin by Western blotting. We combined this with subcellular fractionation in order to corroborate our immunocytochemistry data. Detergent-free cell lysates were centrifuged twice, initially at a low speed and then the supernatant was centrifuged a second time at a high speed. We predicted that junction proteins would be found in either or both pellets. Indeed the majority of the junctional proteins were found in the low spin fraction and the remainder in the high spin fraction. This suggested that junction proteins were highly insoluble and primarily associated with bulky membrane and cytoskeletal components.

The soluble portion should contain only proteins from the cell cytoplasm. ZO-1, alpha- and beta-catenin are junctional-associated proteins and in
accordance detected to varying degrees the soluble portion, corresponding to the cytoplasmic / perinuclear staining observed on immunohistochemistry. Unexpectedly traces of Cl-5 were also detected in the soluble cytosolic fraction. Cl-5 is a known transmembrane protein and should only been found in association with membranes. In contrast VEC, the other transmembrane protein analysed biochemically, was exclusively restricted to membrane pellets demonstrating that cross-contamination of fractions did not occur. Cl-5 may associate with very small membrane compartments which were not separated from the cytosol by our centrifugation. As discussed above we also noted speckled cytoplasmic immunostaining and assumed that this was vesicular.

### 3.1.6.3 Comparison of RMEC / BMEC

We were able to passage the cells and increase the amount of material but passaging the cells also increased the amount of contaminating cells and over the course of time the cells showed a decline in the expression of various junctional proteins especially Cl-5 and occludin.

This limited the amount of material we were able to generate and it was difficult to consistently produce enough tissue to look at permeability. However it is recognized that endothelial cells of the cerebral and retinal capillaries have similar characteristics. Both form a non-fenestrated, single layer that possess TJ and AJ with a similar composition of proteins (Harhaj and Antonetti, 2004; Russ et al., 1998; Ueno, 2007). Both sets of endothelial cells are surrounded and supported by pericytes and glial cells (Farrell et al., 1987; Martin et al., 2000; Newman and Reichenbach, 1996; Brightman et al., 1983), are rich in mitochondria and possess specific carrier-mediated transport proteins for example GLUT1 and GLUT3 (Badr et al., 1999).
Additionally autoregulation of blood flow is a property of both the inner retinal and cerebral circulations (LASSEN, 1964; Bill and Sperber, 1990) and they both undergo similar changes in response to aging, diabetes and hypertension (see review Patton et. al. (Patton et al., 2005).

Therefore based on the assumption that the BRB and the BBB are similar in physiology and molecular composition, and that undoubtedly more cells could be generated from the BBB we also isolated brain microvascular endothelial cells (BMEC) and compared the endothelial cultures from the two tissues.

Our results showed that the two tissues models were very similar, in particular terms of the spatial localisation of the junction proteins. There were some subtle differences in terms of the catenins. In RMEC alpha-catenin showed some perinuclear staining however this was not evident in the BMEC. Similarly beta-catenin was isolated to the junctional area for RMEC but in BMEC there was some additional perinuclear staining. As previously discussed, not only are these junctional proteins but they are also nuclear signalling proteins and therefore their expression depends on the cellular activity and therefore we predict that the difference observed between the cell types reflect differing intracellular signalling.

3.1.6.4 Permeability of MEC

Our monolayer had a tendency to detach from the filter and therefore out initial challenge was to grow the cells on the membrane. Detachment from the filter could be limited (although not entirely eliminated) if we grew the cells on an extracellular matrix of collagen IV and fibronectin. In addition, we seeded cells of low passage (P1) and enhanced the medium with
hydrocortisone and ascorbic acid to encourage the cells to remain attached to the filter. Previous investigators have found a TER with bovine endothelial cells between 11.7 – 170 ohm cm$^2$ (Wang et al., 2001; Tretiach et al., 2004; Guo et al., 2008; Gardner et al., 1997; Yaccino et al., 1997; Gillies et al., 1995). Most notably the studies of higher resistance are either unpassaged cells or cells of low passage (P1 or 2). In comparison, cultured retinal pigment epithelial cells have a TER of around 50 – 100 ohm cm$^2$ (Zech et al., 1998; Ablonczy and Crosson, 2007) however TER of around 500 ohm cm$^2$ for RPE cells can be achieved after culture for 1 month (Geisen et al., 2006).

We used cells of low passage, however our TER was still very small suggesting that our cells were unable to form a good barrier. Due to the problem of growing and maintaining our MEC on the filters, measuring the TER in the long-term was not an option. However we demonstrated that our cells formed a good barrier to the passage of FITC / FITC-dextran.

TER depends on areas with the lowest electrical resistance between single cells (Madara, 1998). Before experimentation, our filters were inspected microscopically and any wells with visible gaps were discarded, however our low TERs suggest that microscopic gaps were present, either as occult gaps, at the edges of the monolayer or due to the presence of a contaminating cell. In contrast, transendothelial permeability is the passage of molecules through the monolayer which depends on the sum of transport across all junctional pathways and therefore would be assumed to be much more forgiving towards gaps.

### 3.1.6.5 Conclusion

Our *in-vitro* culture of the RMEC and BMEC were morphologically very similar and demonstrate a phenotype that suggests aspects of the BRB are maintained (e.g. TJ and AJ expression, barrier to FITC-dextran). We also
conclude that BMEC are a reasonable model system to dissect aspects of the BRB. Unfortunately due to the time limitation, we were unable to repeat the studies of permeability on RMEC.
Chapter 3.2
The effects of VEGF on the barrier function of blood-retinal and blood-brain microvascular endothelial cells.
3.2.1 Introduction

Apart from its angiogenic activity, VEGF displays potent vasoactivity. It induces increased vessel permeability (Senger et al., 1983) and induces microvascular barrier breakdown in a number of in vitro systems (Antonetti et al., 1999; Lakshminarayanan et al., 2000; Behzadian et al., 2003). However, the mechanism by which VEGF induces vascular permeability remains unclear. Activation by VEGF of its receptors leads to a multifaceted activation of downstream signalling pathways some of which are proposed to play an important role inducing microvascular permeability, presumably through changes in the endothelial cell-to-cell junctions. Antonetti et al. show that VEGF treatment leads to a down regulation in the junctional expression of occludin in bovine retinal endothelial cells in vitro and this correlates to a decrease in barrier function (Antonetti et al., 1998).

We used our RMEC and BMEC cultures to further characterise the role of VEGF at blood-retinal barrier (BRB) and blood-brain barrier (BBB).
3.2.2 VEGF induces permeability of BMECs

In order to investigate the effect of VEGF on the permeability of our endothelial cell monolayer, BMEC were grown to confluence and then passaged onto transwell cell culture chambers at a density of 40 000 to 100 000 cells per well. They were cultured until confluent and two days prior to experimentation the medium was diluted 1:2 with serum-free medium with hydrocortisone (HC). Due to the difficulties of maintaining a complete monolayer on the membrane, there were considerable variations between the monolayers and therefore we used each individual filter as its own control.

1 mg/ml 40kDa FITC-dextran was added to the apical chamber and the transendothelial permeability was measured by determining its flux from the apical to the basolateral chamber over 180 minutes. Then 50 ng/ml VEGF was added to the apical well and the change in transendothelial permeability of the 40kDa FITC-dextran was determined over the next 180 minutes.

VEGF lead to a significant increase in permeability of the BMEC monolayer. Increased permeability was observed as early as 30 minutes after the addition of VEGF and persisted for at least three hours (fig 3.2.1).
Figure 3.2.1: VEGF induces enhanced flux across BMEC. (a) BMEC were grown to confluence on transwell chambers. 1mg/ml 40kDa FITC dextran was added to the apical chamber. 50 ng/ml VEGF was added to the apical chamber at 180 minutes (arrow) and the change in the permeability of the monolayer was assessed by measuring the clearance of FITC-dextran (40 kDa). 30 minutes following the addition of VEGF, an increase in the clearance of FITC- dextran was observed (error bars = standard deviation). (b) Overall there was nearly a 2 fold (x1.8) increase in permeability with following the addition of VEGF.

FITC, fluorescein isothiocyanate; VEGF, vascular endothelial growth factor.
3.2.3 VEGF leads to an alteration in the spatial localisation of junctional proteins

Paracellular permeability has been proposed to be influenced by the level and localisation of junctional proteins. In order to investigate if VEGF leads to changes in the spatial localization of junctional proteins, RMEC were grown to confluence and passaged onto the centre of collagen coated plastic and cultured in primary medium. Cells were serum starved for 24 hours and then treated with 50ng VEGF for up to 24 hours, fixed and then the junctional proteins were identified by immunohistochemistry.

Firstly, we verified that RMEC responded to VEGF under these conditions. Cells were stained for filamentous action (F-actin) using phallodin. Changes in the spatial localisation of F-actin occurred very rapidly. Indicative of growth factor activation and in agreement with published date we observed stress fibre formation in response to VEGF (Cullen et al., 2000). This was seen as early as 5 minutes and the response was transient since by 60 minutes, F-actin distribution returned to the pretreatment appearance (fig 3.2.2).
Figure 3.2.2: Confocal images showing the spatial localization of F-actin in RMEC following treatment with VEGF. RMEC were treated with 50 ng/ml VEGF for up to 60 minutes and then fixed with methanol. Subsequently cells were stained for F-actin. The addition of VEGF led to a rapid increase in the stress fibre formation as early as 5 min. By 60 minutes the stress fibre formation was nearly back to control levels. Images are full projections of confocal sections spanning the whole cell.

F-actin, filamentous actin; VEGF, vascular endothelial growth factor.
Next, we analysed the distribution of junctional proteins following VEGF treatment and observed both early and late changes of junction protein in response to VEGF. After 1 hour, VEGF led to a reduction in the amount of junctional staining for ZO-1, alpha-catenin and gamma-catenin (fig 3.2.3-4). In contrast the spatial localisation of beta-catenin was unaffected by VEGF (fig 3.2.4a). The reduction in the junctional staining of ZO-1 and alpha-catenin was transient and by 24 hours the junctional staining for these proteins resembled untreated cell (fig 3.2.3), however gamma-catenin loss extended over the whole 24 hour observation period (fig .3.2.4b).

In contrast to the relatively rapid changes seen for the junction-associated proteins, changes to the transmembrane proteins in response to VEGF were much slower to occur. Occludin and VEC levels were unaffected within the first hour of treatment but significantly reduced after 24 h. Cl-5 showed an early response at 1 hour with a reduction of the cytoplasmic speckles, indicating that Cl-5 synthesis and export was inhibited (fig 3.2.5a). However, a strong reduction in the staining at the cell boundary took 24 h to manifest (fig 3.2.5b,c).
Figure 3.2.3: Confocal images showing the spatial localization of ZO-1 and alpha-catenin in RMEC following treatment with VEGF. Cells were grown to confluence, starved for 24 hours, treated with 50 ng/ml VEGF for up to 24 hours and then fixed with methanol (ZO-1) or 3.7% formaldehyde (alpha-catenin). Subsequently cells were stained for ZO-1 (a) or alpha-catenin (b). The addition of VEGF leads to a reduction in the junction expression of ZO-1 and alpha-catenin at 1 hour. By 24 hours the staining patterns for both ZO-1 and alpha-catenin reverted to control levels. Images are full projections of confocal sections spanning the whole cell.

RMEC, retinal microvascular endothelial cells; ZO-1, zonula occludens-1; VEGF, vascular endothelial growth factor.
Figure 3.2.4: Confocal images showing the spatial localization of beta and gamma catenin in RMEC following treatment with VEGF. Cells were grown to confluence, starved for 24 hours, treated with 50 ng/ml VEGF for up to 24 hours and then fixed with 3.7% formaldehyde. Subsequently cells were stained for beta-catenin (a) and gamma-catenin (b). After 1 hour, VEGF led to a reduction in the amount of junctional staining for gamma-catenin which extended over the whole 24 hours observation period. In contrast the staining for beta-catenin was not affected by VEGF treatment. Images are full projections of confocal sections spanning the whole cell.

RMEC, retinal microvascular endothelial cells; VEGF, vascular endothelial growth factor.
Figure 3.2.5: Confocal images showing the spatial localization of the transmembrane proteins in RMEC following treatment with VEGF. Cells were grown to confluence, starved for 24 hours, treated with 50 ng/ml VEGF for up to 24 hours and then fixed with methanol (Cl-5 and occludin) or 3.7% formaldehyde (VEC). Subsequently cells were stained for Cl-5 (a), occludin (b) or VEC (c). The addition of VEGF leads to a reduction of junctional expression of all three transmembrane proteins at 24 hours. Images are full projections of confocal sections spanning the whole cell. RMEC, retinal microvascular endothelial cells; VEGF, vascular endothelial growth factor; Cl-5, claudin-5; VEC, vascular endothelial cadherin / VE cadherin.
3.2.4 VEGF leads to a 30% reduction in ZO-1

As shown above, we observed a decrease in junctional ZO-1 staining in response to VEGF (fig 3.2.3 and 3.2.6) that coincided with increased permeability (fig 3.2.1). This has been noted before: For instance increased expression of ZO-1 is observed in parallel with increased electrical resistance in cultured rat brain capillary endothelial cells (Krause et al., 1991). Additionally, ZO-1 levels are reduced in the presence of elevated glucose concentrations (Gardner, 1995).

Taken together this suggested that ZO-1 may act as a critical down-stream effector of VEGF signalling possibly directly involved in barrier breakdown and in permeability. To test the hypothesis we tried to correlate VEGF-induced ZO-1 loss to VEGF-induced permeability more closely through the signalling pathways used.

Changes in detection of immunofluorescent staining may reflect actual reduction in protein levels but may also be due to reduced epitope accessibility. Western blot is usually considered more reliable to detect actual protein levels. Therefore to confirm that loss of ZO-1 immunostaining represented actual protein loss, RMEC were grown to confluence on collagen coated dishes and then serum starved for 24 hours. Cells were treated with 50ng/ml VEGF for 1 hour, lysates were prepared and analysed by Western blot for the expression of ZO-1.

Following 1 hour treatment with VEGF, there was a clear reduction of ZO-1 protein (fig 3.2.6a). Indeed quantification of three independent experiments revealed that reduction after one hour was 34%, n=3, P=0.04 (fig 3.2.6b).
Importantly, the reduction of ZO-1 observed by immunocytochemistry was very similar (30%, n=4, P=0.008) (fig 3.2.6d).

From these results, we concluded that we were able to obtain similar results if we analysed the change in intensity of ZO-1 stain, by immunocytochemistry or by biochemistry. Analysis of the cells by immunocytochemistry took much less material and therefore we compared effect of inhibitors of the signal transduction pathway to immunocytochemistry only.
Figure 3.2.6: Quantification of VEGF-induced ZO-1 loss. RMEC were grown to confluence and treated with 50ng/ml VEGF for 1 hour. Changes in ZO-1 were visualized by both Western blotting (a) or immunohistochemistry (c). Quantification of VEGF-induced ZO-1 loss showed a 30% reduction in the mean intensity of ZO-1 in both instances. n=3, P=0.04 (b), n=4, P=0.008(d).

VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1.
3.2.5 VEGF-induced loss of ZO-1 via PKC, PI3K AND ENOS

We next wanted to test whether VEGF-induced ZO-1 loss was a critical downstream endpoint for VEGF-induced permeability. Consequently, we compared the intracellular signalling pathway of VEGF-induced ZO-1 loss with the described pathway for VEGF-induced permeability.

VEGF binds several transmembrane tyrosine kinase receptors (VEGF-R) which induce intracellular signals and endothelial permeability (fig 3.2.7). VEGF-R2 activates membrane-associated kinases such as Src and phosphatidylinositol 3-kinases (PI3K) (Waltenberger et al., 1994; Guo et al., 1995). These along with Src kinase has been implicated in VEGF-induced permeability (Suarez and Ballmer-Hofer, 2001) (fig 3.2.7). In the following experiment we have used PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine) as a selective inhibitor or Src-family tyrosine kinases (Hanke et al., 1996) and wortmannin and LY294002 to inhibit PI3K (Cross et al., 1995; Vlahos et al., 1994).

A variety of other signalling components may also contribute to vascular leak such as endothelial nitric oxide synthase (eNOS) and protein kinase C (PKC) (Wu et al., 1999) (Radomski et al., 1990). We utilised L-NAME (NG-nitro-L-arginine methyl ester) and overnight treatment with phorbol ester PMA to prevent activation of eNOS and PKC respectively.

Increases in intracellular calcium have been shown to be induced by VEGF in vivo (Wu et al., 1999) and as in vitro (Brock et al., 1991) to increase permeability. We used BAPTA-AM (1,2 bis-(o-aminophenoxy) ethane tetraacetoxyethyl ester) to chelate Ca^{2+} (Harrison and Bers, 1987).
Figure 3.2.7: Schematic diagram illustrating the signal transduction pathway of VEGF-induced permeability.
The actin cytoskeleton is known to interact with junctional proteins and VEGF led to an increase in actin stress fibre formation (fig 3.2.2) and this may regulate permeability (Sun et al., 2006). We used cytochalazin D to disrupt actin filaments and inhibit actin polymerization (Mortensen and Larsson, 2003).

A summary of the inhibitors of the signal transduction pathway are shown in table 3.2.1.

Confluent RMECs were serum starved and then pretreated with PMA (160 nM, 18hr), wortmannin (300 nM, 1hr), LY294002 (15 µM, 1hr), PP2 (20 µM, 30min), BAPTA-AM (20 µM, 1hr), cytochalazin D (2 µM, 30min), or L-NAME (1 mM, 1hr). Cells were then treated with 50 ng/ml VEGF for 1 hour, fixed with methanol and stained for ZO-1. Staining intensity was quantified using the image analysis programme, ImageJ.

In untreated cells, VEGF led to the 30% reduction in the ZO-1 described above. Our data showed that inhibitors of PKC, PI3K and eNOS prevented the VEGF-induced ZO-1 loss (fig 3.2.8). In contrast, pre-treatment with PP2, cytochalazin D or BAPTA-AM was mostly ineffective in preventing VEGF-induced ZO-1 loss suggesting that Src protein kinases, the actin cytoskeleton and Ca2+ transients were not involved in ZO-1 downregulation.
Table 3.2.1: Summary of the action of the inhibitors of signal transduction pathway used to investigate VEGF-induced ZO-1 loss

<table>
<thead>
<tr>
<th>Name of Inhibitor</th>
<th>action on signal transduction pathway</th>
<th>abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2</td>
<td>inhibits Src-family tyrosine kinases</td>
<td>Src</td>
</tr>
<tr>
<td>wortmanin</td>
<td>inhibits phosphatidylinositol 3-kinase</td>
<td>PI3K</td>
</tr>
<tr>
<td>LY294002</td>
<td>inhibits phosphatidylinositol 3-kinase</td>
<td>PI3K</td>
</tr>
<tr>
<td>L-NAME</td>
<td>inhibits nitric oxide synthase (but only eNOS is expressed)</td>
<td>eNOS</td>
</tr>
<tr>
<td>PMA</td>
<td>stimulates and downregulates protein kinase C following prolonged exposure</td>
<td>PKC</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>calcium chelator</td>
<td>Ca2+</td>
</tr>
<tr>
<td>cytochalazin D</td>
<td>prevents actin polymerization</td>
<td>actin</td>
</tr>
</tbody>
</table>
Diabetic Macular Oedema: The role of steroids and VEGF

Figure 3.2.8: VEGF-induced ZO-1 loss was blocked by PMA, wortmannin and LY294002 and L-NAME. Confluent RMECs were pretreated with PMA (160nM, 18hr), wortmannin (300nM, 1hr), LY294002 (15 µM, 1hr), PP2 (20µM, 30min), BAPTA-AM (20µM, 1hr), cytochalazin D (2µM, 30min), and L-NAME (1mM, 1hr) and then stimulated with 50 ng/ml VEGF for 1 hour. RMEC were fixed and stained for ZO-1. In untreated cells, VEGF led to the expected 30% reduction in the ZO-1 stain. Pretreatment with PMA, wortmannin, LY294002, and L-NAME all led to a complete inhibition of VEGF-induced ZO-1 loss. In contrast, pre-treatment with PP2, cytochalazin D or BAPTA-AM was mostly ineffective in preventing VEGF-induced ZO-1 loss Results show the median value of 3 experiments plus interquartile range.
3.2.6 Discussion

VEGF led to an increase in permeability which was discernible within 30 min of treatment. Such a relatively rapid response has also been noted by other investigators (Bates et al., 1999; Esser et al., 1998; Feng et al., 1996). In addition Behzadian et. al. (Behzadian et al., 2003), found that VEGF induced a rapid and transient phase of permeability (1-2hr) followed by a delayed and sustained phase (6-24hr). Our data did not give any clear indication of a biphasic response to VEGF. However, our analyses were undoubtedly inappropriate to monitor such changes: flux rates at many more closely spaced time points would be required. Continuous measurements of hydraulic conductivity (Lakshminarayanan et al., 2000) or impedance (Nitz et al., 2003) would be more appropriate to resolve time-resolved changes of VEGF-induced permeability.

Significantly, we observed two clearly distinguishable phases of junctional rearrangements in response to VEGF, which may illustrate a biphasic response of endothelial cells. We found an initial rapid but transient loss in the junction-associated proteins, followed by a delayed but sustained loss in the transmembrane junction proteins. The membrane-associated proteins form reversible bounds with the transmembrane proteins, allowing signalling between the transmembrane proteins and the rest of the cell especially the nucleus. Therefore it makes sense that the loss of junction-associated proteins occurred rapidly. In contrast, the transmembrane proteins are hydrophobic proteins that form an integral part of the cell membrane and protein loss may require the turnover of the membrane and hence occurs more slowly.
To our knowledge, this is the first report of the VEGF-induced changes of alpha- and gamma-catenin. We showed a rapid but transient change in both of these proteins. There was no change in the spatial localisation of p120 or beta-catenin in accordance with previous results (Wong et al., 2000). One report describes a transient increase in cytosolic beta catenin but no correlative change in the junctional-associated pool is shown (Behzadian et al., 2003).

VEGF treatment also led to a reduction of ZO-1 within 1 hour. This was observed by immunocytochemistry and by Western blotting, clearly indicating that total intracellular levels of this protein were reduced which concomitantly affected its junction association. By 24 hours the staining pattern of ZO-1 on immunohistochemistry reverted to the appearance of untreated cells. Other investigators have looked at VEGF-induced ZO-1 loss and did not find a significant change in the level of its expression on Western blotting. However all of these investigators looked at ZO-1 after 24 hours and this may account for the difference (Brankin et al., 2005; Wang et al., 2001; Deissler et al., 2008).

Furthermore, we found changes in the spatial localisation of occludin and VEC that have also been noted by previous investigators (Behzadian et al., 2003; Wang et al., 2001; Antonetti et al., 1998; Nakamura et al., 2008; Esser et al., 1998). We also observed a significant reduction in Cl-5 after 24 hours of VEGF treatment. The loss of Cl-5 may well contribute to enhanced endothelial permeability as suggested by results obtained with Cl-5 knock out mice (Nitta et al., 2003). Rodewald noted that VEGF induced a reduction in Cl-5 staining in the ovaries (Rodewald et al., 2007). However a study by Deissler et al. found no change in Cl-5 expression in bovine retinal endothelial cell following VEGF treatment for 24 hours (Deissler et al., 2008). Clearly further investigations are required to explain these discrepancies.
We have shown that VEGF leads to rapid increase in permeability (within 30 minutes) and is associated with a reduction in the junctional localisation of ZO-1. Previous investigators have shown that during hypoxia there is reduction in the localization and expression of ZO-1 and hypoxia–induced ZO-1 loss is attenuated by neutralising antibodies to VEGF, making it a possible critical downstream-effector of VEGF-induced permeability (Fischer et al., 2002). In order to test this theory, we compared the signal transduction pathway of VEGF-induced ZO-1 loss to the established of the signal transduction pathway for VEGF-induced permeability.

Our data showed that PKC, PI3K, eNOS were involved in mediating the VEGF-induced ZO-1 loss. In contrast, inhibition of calcium transients, the actin cytoskeleton or Src protein kinases did not prevent VEGF-induced loss of ZO-1. All three signalling components have been unambiguously implicated in mediating endothelial permeability. In particular Src protein kinase has a clearly demonstrated role in mediating VEGF-induced endothelial permeability both in vitro and in vivo (Suarez and Ballmer-Hofer, 2001; Weis et al., 2004a). We therefore conclude that the level of intracellular (and junctional) ZO-1 is not a critical mediator of VEGF vasoactivity in BMEC or RMEC. Of course formal proof would require studying the permeability response to VEGF in the absence or presence of the same pharmacological inhibitors. Such studies have not yet been performed.
Chapter 3.3

The effects of steroids on the barrier function of blood-retinal and blood-brain microvascular endothelial cells.
3.3.1 Introduction

Breakdown of the blood-retinal barrier and the resultant vascular permeability remains one of the first observable alterations in diabetic retinopathy and strongly correlates with vision loss.

Initial pilot studies suggest that steroids such as intravitreal triamcinolone reduce the amount of DMO (Jonas et al., 2003; Martidis et al., 2002), however the mechanism of action that leads to the reduction in oedema remains poorly understood. They may act by suppressing inflammation and thus reducing microvascular endothelial permeability or they may have a direct role in the organization of cell-to-cell junctional complexes (which may in turn affect barrier function).

The aim of this particular study was to assess the role of corticosteroids, namely hydrocortisone and triamcinolone on the in vitro model of the blood-retinal barrier (BRB) and or the blood-brain barrier (BBB) as described in chapter 1.
3.3.2 **Glucocorticoids led to an improvement of MEC barrier function to low and high molecular weight solutes**

To compare the effect of steroids on the barrier function of MEC, the flux of FITC or FITC-conjugated protein across monolayers of BMEC was assessed. BMEC were grown to confluence and then passaged onto transwell cell culture chambers at a density of 40,000 to 100,000 cells per well. Cells were grown to confluence and two days prior to experimentation the medium was diluted 1:2 with serum free medium (plus or minus hydrocortisone). The transendothelial permeability was then measured by measuring the flux of FITC (389 Da), 40kDa FITC-coupled dextran or 70kDa FITC-coupled dextran.

In the presence of hydrocortisone, the cells formed a much tighter barrier to FITC (fig 3.3.1a). This barrier effect was enhanced when repeated with 40 kDa FITC-dextran. Indeed calculation of the diffusive flux revealed that hydrocortisone improved the barrier to function around two-fold for FITC and around a hundred-fold for 40 kDa FITC-dextran, indicating that hydrocortisone affected barrier in a size-dependent manner.

Similar experiments were performed to compare the effects of hydrocortisone (HC) and triamcinolone (TA) using 70kDa FITC. Both compounds enhanced barrier function of BMEC. In fact barrier improvement by TA was even greater than that achieved by HC (fig 3.3.1b).

When we assessed the vasoactive responsiveness of BMEC using the vasoactive compounds VEGF and lysophosphatidic acid (LPA) as inducers of permeability, we realized that both HC and TA were able to protect BMEC against VEGF-induced permeability but not against LPA induced
permeability (fig 3.2.2), suggesting that glucorticoids may be able avoid barrier breakdown secondary to VEGF but they may be unable to inhibit other types of vasoactive stimulation.
Figure 3.3.1: Comparison of diffusive flux of FITC and FITC-conjugated dextran in the presence and absence of steroids. Primary cerebral microvascular endothelial cells (BMEC) were isolated and grown to confluence and passaged onto transwell cell culture chambers. Cells were grown to confluence and 2 days prior to experimentation the medium was diluted 1:2 with serum free medium (plus or minus 550nM hydrocortisone). The transendothelial permeability was then measured by measuring the flux of FITC (389 Da) or 40kDa FITC-coupled dextran (a). In the presence of hydrocortisone, the cells formed a much greater barrier to FITC (left). The effect was even greater when repeated with FITC-dextran (right). When similar experiments were performed using a similar concentration of triamcinolone we also observed greater barrier function than that achieved with hydrocortisone. Results show a change in the permeability of BMEC to 70kDa FITC-dextran (b). P< 0.005.

FITC, fluorescein isothiocyanate; HC, hydrocortisone; TA, triamcinolone
Figure 3.3.2: Effect of steroids at reducing permeability secondary to the vascoactive compounds, VEGF and lysophosphatidic acid (LPA). Primary cerebral microvascular endothelial cells (BMEC) were isolated and grown in normal growth medium or medium supplemented with 550 nM hydrocortisone or 550 nM triamcinolone. Cells were passaged onto permeable transwell cell culture chambers and then exposed to 50ng /ml VEGF or 5 µM LPA for 1 hour. The permeability of 70 kDa FITC dextran measured over 4 h. HC and TA were able to prevent VEGF-induced permeability but were unable to prevent LPA induced permeability. 

Note: Within the time limitations of this thesis this experiment could only be independently performed twice. Therefore statistical analysis cannot be provided.

VAC, vasoactive compounds; HC, hydrocortisone, TA, triamcinolone; VEGF, vascular endothelial growth factor; LPA, lysophosphatidic acid.
3.3.3 The presence of hydrocortisone led to enhancement in the spatial localization of the junctional proteins

To compare the effect of steroid on the spatial localisation of junctional proteins, BMEC were cultured in the presence or absence of 550 nM hydrocortisone for up to 4 weeks. Cells were then fixed and stained to detect changes in the organisation of the junctional proteins.

Four-week BMEC cultures in the absence of hydrocortisone contained little or no Cl-5 or occludin (fig 3.2.3a,b). In clear contrast, the expression and junctional localisation of Cl-5 and occludin was preserved when cells were cultured with HC (fig 3.2.3d,e). Similarly in the presence of HC, junctional ZO-1 was also significantly enhanced (fig 3.2.3c,f).

In contrast we found minimal change in the expression of the AJ proteins (fig 3.2.4). The only change in the AJ proteins observed was that the junctional staining for VEC was more discretely concentrated in junctional areas suggesting a qualitative improvement of AJ. In contrast beta-catenin staining was unchanged by the presence of hydrocortisone.
Figure 3.3.3: Confocal images of TJ protein in the presence and absence of hydrocortisone. BMEC were grown in culture medium in the presence of absence or 550 µM hydrocortisone: –HC or +HC for 4 weeks and then fixed with methanol. Subsequently cells were stained for Cl-5, occludin and ZO-1. In the absence of HC many of the cells expressed little or no Cl-5 or occludin. In contrast, the expression and junctional localisation of Cl-5 and occludin was preserved in cells cultured + HC. Similarly in the presence of HC, junctional ZO-1 was significantly enhanced. Images are full projections of confocal sections spanning the whole cell.

TJ, tight junctions; HC, hydrocortisone; Cl-5, claudin-5; ZO-1, zonula occludens -1
**Figure 3.2.4: Confocal images of AJ proteins in the presence and absence of hydrocortisone.** BMEC were grown in the absence or presence of 550 nM hydrocortisone for 4 weeks and then fixed using 3.7% formaldehyde. Subsequently cells were stained for VEC or beta catenin. There was little change in the expression of either VEC and beta catenin although for VEC the staining was more discretely concentrated in junctional areas suggesting a qualitative improvement of AJ.

Images are full projections of confocal sections spanning the whole cell.
3.3.4 Discussion

In our study, we showed that glucocorticoids led to increased barrier function and enhanced tight junction protein expression. TA enhanced barrier function more effectively than HC. Additionally both HC and TA inhibited VEGF- but not LPA-induced permeability.

It has been known for a long time that glucocorticoids can increase the barrier properties endothelial cells (INGRAHAM et al., 1952; Long et al., 1966). In most of the Transwell experiments we used an HC-supplemented medium to allow the formation of a suitable barrier. However, 48 hours prior to experimentation we diluted down the medium 1:2 thus reducing the effect of the remaining HC on the control cells. Additionally our results show a significant change in the barrier function of MEC in response to fresh HC and therefore we were confident that the residual HC did not exert a significant effect on our MEC.

Hydrocortisone is a naturally occurring glucocorticoid. The physiological range of hydrocortisone is 70 – 550 nM (Karlson P and Doenecke, 1994). We chose a dose to mimic the physiological range and has been used by other investigators. In contrast, triamcinolone is a synthetic steroid which has been engineered to reduce the mineralocorticoids (salt-retaining) activity and thus its side-effect profile. In injectable form, it is licensed for intra-articular / intramuscular use. It has gained popularity for intravitreal injection as it forms a depot in the eye which remains in the vitreous for up to 3 months (Beer et al., 2003). Additionally, its safety has been well documented (Martidis et al., 2002; McCuen et al., 1981).
At the same dose, TA was more effective at increasing the barrier function of the cells to 70 KDa dextran, which suggests that it might enhance the barrier function \textit{in vivo}, even in the presence of high endogenous HC. Other have also shown the increased barrier function of MECs following treatment with HC (Hoheisel et al., 1998; Antonetti et al., 2002) but we believe that our report is the first to illustrate a beneficial effect of TA on the \textit{in vitro} barrier function of MEC.

Our BMEC showed an increase in the junctional staining of predominately TJ proteins following treatment with HC. These results corroborate findings of others in BMEC (Weidenfeller et al., 2005; Calabria et al., 2006; Romero et al., 2003). Additionally Antonetti and co-workers showed that 103 nM HC increases occludin and ZO-1 staining at the cell border of bovine retinal endothelial cells (Antonetti et al., 2002).

Edelman et al (Edelman et al., 2005) showed that corticosteroids inhibit VEGF-induced vascular leakage in a rabbit model of BRB breakdown. Our results confirmed that whilst HC and TA clearly attenuated the vasoactive response of VEGF they were both unable to inhibit LPA-induced permeability. It is possible that our system does not reflect the true ratio of physiological doses of all drugs. However we used LPA at 5 µM which is within the physiological range (Baker et al., 2001). Lysophosphatidic acid (LPA) is generated by platelets during blood coagulation for example following a cerebral vascular occlusion. Endothelial cells cultured \textit{in vitro} express LPA receptors and its stimulation leads to strong activation of Rho and Rho kinase (Hu et al., 2001). Consequently increases in permeability by actin stress fiber formation, contraction of endothelial cells and intercellular gap formation have been described (Hu et al., 2001). We have not observed any gap formation between BMEC or RMEC following VEGF treatment suggesting that this may be a prerogative of LPA. Clearly and intuitively the
mechanism of vasoactive stimulation is entirely dependent on the stimulus used and therefore it is not surprising that GC did not have a universal effect in preserving endothelial barriers.

It is likely that our results demonstrate that corticosteroids exert their effect via a specific intracellular pathway such as the downregulation of matrix metalloproteinases (MMPs) (Lohmann et al., 2004). These enzymes play an important role in the degradation of occludin (Lohmann et al., 2004).

Taken together, the opposing effects of VEGF and hydrocortisone on permeability and on TJ assembly provide compelling evidence that VEGF and hydrocortisone exert their effects at least in part, through specific changes in the junctional proteins. Our results also suggest that glucocorticoid may constitute a useful therapeutic option in treating diseases that have an underlying VEGF-induced vasoactive complication.
Chapter 3.4
Randomised controlled trial of intravitreal triamcinolone versus laser photocoagulation for diabetic macular oedema
3.4.1 Introduction

Diabetes Mellitus is the commonest cause of blindness in the working population in England and Wales with over 80% due to diabetic maculopathy (Evans J, 1995; McMeel et al., 1977). The prevalence of diabetic maculopathy is directly related to the duration of diabetes, with approximately 30% of diabetic patients affected after 25-30 years of disease (Klein et al., 1984). As the incidence of diabetes increases, the rate of diabetic complications including diabetic macular oedema (DMO) and visual loss are set to increase (Amos et al., 1997).

The only proven effective treatment for DMO is laser photocoagulation (1985). However this treatment only reduces the chance of moderate visual loss by 50% and is unlikely to improve visual acuity. As a result, better treatments are being sought.

Initial pilot studies using intravitreal triamcinolone (TA) suggest that it can improve visual acuity in patients with DMO in approximately 30% in the short-term (Jonas et al., 2003; Martidis et al., 2002). However long-term evidence of efficacy from prospective data is limited. The aim of this study was to determine if repeated TA improves ETDRS best corrected visual acuity (BCVA) letter score at 1 year compared to conventional laser therapy and whether this treatment has a role to play in the long-term care of patients with DMO.
3.4.2 Patient enrollment

A total of 88 eyes from 88 patients were included, of which 43 eyes were randomised to receive TA and 45 eyes to receive laser photocoagulation. One-year data were available for 83 patients (94%). 5 patients did not complete a follow-up. 2 patients died, 1 patient moved away from the area, 1 patient was unable to travel to the hospital and 1 patient (who received TA) refused follow up (fig 3.4.1).

3.4.3 Baseline characteristics

The baseline characteristics of the patients in each group are shown in Tables 3.4.1 and 3.4.2, which show that the groups were comparable with regards to all characteristics assessed.
**Figure 3.4.1:** Consort figure for randomised controlled trial of intravitreal triamcinolone versus laser photocoagulation for diabetic macular oedema.
### Table 3.4.1: Baseline characteristics of eyes in randomized controlled trial of triamcinolone versus laser treatment.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Triamcinolone (N=43)</th>
<th>Laser (N=45)</th>
<th>n*</th>
<th>Number (%)</th>
<th>n*</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>male</td>
<td>28 (65.1)</td>
<td>32 (71.1)</td>
<td>65</td>
<td>28 (65.1)</td>
<td>32 (71.1)</td>
<td></td>
</tr>
<tr>
<td>left eye</td>
<td>22 (51.2)</td>
<td>29 (64.4)</td>
<td>51</td>
<td>22 (51.2)</td>
<td>29 (64.4)</td>
<td></td>
</tr>
<tr>
<td>type II diabetes</td>
<td>43 (100.0)</td>
<td>44 (97.8)</td>
<td>100</td>
<td>43 (100.0)</td>
<td>44 (97.8)</td>
<td></td>
</tr>
<tr>
<td>western european</td>
<td>17 (39.5)</td>
<td>22 (48.9)</td>
<td>39</td>
<td>17 (39.5)</td>
<td>22 (48.9)</td>
<td></td>
</tr>
<tr>
<td>PDR</td>
<td>8 (19.5)</td>
<td>8 (17.8)</td>
<td>19</td>
<td>8 (19.5)</td>
<td>8 (17.8)</td>
<td></td>
</tr>
<tr>
<td>hypertensive</td>
<td>40 (93.0)</td>
<td>36 (80)</td>
<td>93</td>
<td>40 (93.0)</td>
<td>36 (80)</td>
<td></td>
</tr>
<tr>
<td>current smoker</td>
<td>1 (2.4)</td>
<td>4 (8.9)</td>
<td>2.4</td>
<td>1 (2.4)</td>
<td>4 (8.9)</td>
<td></td>
</tr>
<tr>
<td>PVD</td>
<td>6 (15)</td>
<td>8 (17.8)</td>
<td>15</td>
<td>6 (15)</td>
<td>8 (17.8)</td>
<td></td>
</tr>
<tr>
<td>pseudophakia</td>
<td>8 (18.6)</td>
<td>6 (13.3)</td>
<td>18</td>
<td>8 (18.6)</td>
<td>6 (13.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Mean (SD)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>age</td>
<td>62.3 (7.5)</td>
<td>64.8 (10.1)</td>
<td>62</td>
<td>62.3 (7.5)</td>
<td>64.8 (10.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Median (IQR)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>previous laser</td>
<td>3 (2-4)</td>
<td>2 (2-4)</td>
<td>2</td>
<td>3 (2-4)</td>
<td>2 (2-4)</td>
<td></td>
</tr>
<tr>
<td>duration diabetes (yrs)</td>
<td>15 (10-21)</td>
<td>13 (8-20)</td>
<td>15</td>
<td>15 (10-21)</td>
<td>13 (8-20)</td>
<td></td>
</tr>
<tr>
<td>duration CSMO (mths)</td>
<td>27 (15-48)</td>
<td>23.5 (12-31)</td>
<td>27</td>
<td>27 (15-48)</td>
<td>23.5 (12-31)</td>
<td></td>
</tr>
<tr>
<td>duration VL (mths)</td>
<td>12.5 (10-22)</td>
<td>18.5 (9-24)</td>
<td>12</td>
<td>12.5 (10-22)</td>
<td>18.5 (9-24)</td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>7.8 (7-8.7)</td>
<td>7 (6.5-8.1)</td>
<td>7</td>
<td>7.8 (7-8.7)</td>
<td>7 (6.5-8.1)</td>
<td></td>
</tr>
</tbody>
</table>

* n = number of cases data missing on particular characteristic.
PDR, proliferative diabetic retinopathy; PVD, posterior vitreous detachment; IQR, interquartile range; CSMO, clinically significant macular oedema; mths, months; HbA1c, glycosylated haemoglobin; VL, visual loss.
Table 3.4.2: Baseline characteristic of eyes in triamcinolone and laser groups.

<table>
<thead>
<tr>
<th>Characteristic (mean)</th>
<th>Triamcinolone (SD*)</th>
<th>Laser (SD*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>distance visual acuity</td>
<td>54.6 (13.3)</td>
<td>53.0 (14.2)</td>
</tr>
<tr>
<td>near visual acuity</td>
<td>0.83 (0.33)</td>
<td>0.82 (0.30)</td>
</tr>
<tr>
<td>contrast sensitivity</td>
<td>1.01 (0.32)</td>
<td>1.05 (0.29)</td>
</tr>
<tr>
<td>retinal thickness (µm)</td>
<td>413.4 (127.8)</td>
<td>410.4 (134.1)</td>
</tr>
<tr>
<td>macular volume (mm$^3$)</td>
<td>9.74 (2.41)</td>
<td>9.42 (2.02)</td>
</tr>
<tr>
<td>IOP (mmHg)</td>
<td>15.9 (2.7)</td>
<td>15.5 (3.1)</td>
</tr>
<tr>
<td>Size FAZ</td>
<td>732.0 (182.5)</td>
<td>667.4 (213.6)</td>
</tr>
</tbody>
</table>

SD, standard deviation; IOP, intraocular pressure; FAZ, foveal avascular zone
81 injections were performed over the 12 months (mean = 1.9 treatments per eye). In the same period, 73 laser treatments were performed (mean = 1.6 treatments per eye).

An outline of the injection profile for the patients undergoing intravitreal TA is summarised in table 3.4.3. 10 of 43 patients received all 3 injections. 22 injections (17%) were not performed due to a resolution of the CSMO on clinical examination. 6 injections (5%) were not performed as treatment was refused (2 treatments) or the patient failed to attend (4 treatments). 18 injections (14%) were not performed due to protocol exclusions which included: (i) A rise in the IOP (13 treatments (10%)); (ii) dense cataracts obscuring the fundal view (2 patients); (iii) medically unfit. 1 patient (2 treatments) was deemed unfit for further injections following a myocardial infarction just prior to the 2nd injection and 1 patient had a sterile endophthalmitis following the 2nd injection.
Table 3.4.3: Treatment profile for patients randomised to triamcinolone

<table>
<thead>
<tr>
<th>Reason for missed treatment</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>resolution of CSMO</td>
<td>22</td>
</tr>
<tr>
<td>refused treatment / DNA</td>
<td>6</td>
</tr>
<tr>
<td>protocol exclusion – (e.g. raised IOP, endophthalmitis, no fundal view)</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>46 / 129</td>
</tr>
</tbody>
</table>

CSMO, clinically significant macular oedema; DNA, did not attend; IOP, intraocular pressure.
3.4.4 Visual acuity

A change in the visual acuity letter score at 12 months is presented in fig 3.4.2. Improvement in 15 or more ETDRS letters was seen in 2 of 42 patients in the TA group (4.8%) and in 5 of 41 (12.2%) patients in the laser group (P = 0.265).

At baseline the mean ETDRS letter scores were 54.6 in the triamcinolone group and 53.0 in the laser group. At 12 months they were 54.4 and 54.7 respectively and analysis of covariance indicated that there was no evidence of a difference between the 2 therapies (P = 0.44) (table 3.4.4).

Similarly we found little evidence of any differences in the mean near acuity (Bailey-Lovie scores) or contrast sensitivity (Pelli-Robson scores).
Figure 3.4.2: Change in the ETDRS letter score in triamcinolone and laser eyes at 12 months.

ETDRS, early treatment of diabetic retinopathy study.
Table 3.4.4: Comparison of mean visual acuity scores (distance & near visual acuity and contrast sensitivity) for study and control eyes (standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>Triamcinolone</th>
<th>Laser</th>
<th>P value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ETDRS letter scores</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>54.6 (13.3)</td>
<td>53.0 (14.2)</td>
<td>P = 0.44</td>
</tr>
<tr>
<td>4 months</td>
<td>55.9 (14.0)</td>
<td>54.7 (18.7)</td>
<td></td>
</tr>
<tr>
<td>8 months</td>
<td>53.5 (12.3)</td>
<td>53.4 (19.7)</td>
<td></td>
</tr>
<tr>
<td>12 months</td>
<td>54.5 (13.6)</td>
<td>54.7 (17.7)</td>
<td></td>
</tr>
<tr>
<td><strong>Bailey-Lovie scores</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>0.83 (0.33)</td>
<td>0.83 (0.30)</td>
<td>P = 0.64</td>
</tr>
<tr>
<td>4 months</td>
<td>0.78 (0.29)</td>
<td>0.80 (0.30)</td>
<td></td>
</tr>
<tr>
<td>8 months</td>
<td>0.84 (0.34)</td>
<td>0.74 (0.30)</td>
<td></td>
</tr>
<tr>
<td>12 months</td>
<td>0.87 (0.33)</td>
<td>0.83 (0.29)</td>
<td></td>
</tr>
<tr>
<td><strong>Pelli-Robson scores</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>1.01 (0.32)</td>
<td>1.04 (0.29)</td>
<td>P = 0.95</td>
</tr>
<tr>
<td>12 months</td>
<td>0.96 (0.36)</td>
<td>0.93 (0.36)</td>
<td></td>
</tr>
</tbody>
</table>

ETDRS, early treatment diabetic retinopathy study; ANOVA, one-way analysis of variance
3.4.5 Retinal thickness and macular volume

At baseline the mean retinal thickness (RT) was 413.4 µm in the study group and 410.4 µm in the comparison group (fig 3.4.3). At 4 months the mean RT had fallen to 325.6 µm in the study group and 374.2 µm in the comparison group. At 12 months the mean RT was 322.1 µm in study group and the laser group had fallen to 346.7 µm. Even the largest difference observed (i.e. at 4 months) was not statistically significant in these data (P = 0.19) and analysis by ANOVA revealed no evidence of a significant difference (P = 0.738).

At baseline the mean macular volume was 9.74 mm$^3$ in the triamcinolone group and 9.42 mm$^3$ in the laser group (fig 3.4.4). At 4 months the mean macular volume had fallen to 8.45 mm$^3$ in the triamcinolone group and 9.08 mm$^3$ in the laser group. At 12 months the mean macular volume was 8.41 mm$^3$ in triamcinolone group and the laser group had reduced to 8.66 mm$^3$. Again analysis by ANOVA revealed no significant difference (P = 0.65).
Figure 3.4.3: Change in the mean retinal thickness in study and control eyes with time
Figure 3.4.4: Change in the mean macular volume in study and control eyes with time.
3.4.6 Adverse Events

We observed one case of sterile endophthalmitis. (1 case in 78 injections, 1.3 % of injections, 2.3% of patients). The patient presented at the 1 week review, following the third intravitreal injection, with a drop in visual acuity. The eye was white, with +1 of cells in the anterior chamber, and a hazy vitreous. A vitreous biopsy was negative and despite intravitreal antibiotics there was little change in the appearance of the vitreous 12 months after antibiotics administration.

There were 3 cases in 2 eyes of triamcinolone crystals in the anterior chamber. All cases were detected immediately following injection and settled spontaneously within 2 weeks of injection. The IOP remained within normal limits.

There was little change in the mean size of the foveal avascular zone either group from baseline to 12 months (study = 758 to 759 µm, control = 682 to 762 µm).

3.4.6.1 Intraocular pressure

The most common adverse event was a rise in intraocular pressure (table 3.5.5). There was a rise in the mean intraocular pressure in the study group from 15.9 mmHg to 20.3 mmHg at 4 months. Furthermore, 22 out of 43 patients required ocular antihypertensives at some point during follow up. However all rises in intraocular pressure were easily managed with one or more ocular antihypertensives and further intervention such as vitrectomy to
remove the TA crystals or glaucoma filtration procedures were unnecessary. There were no cases of glaucoma following TA.
### Table 3.4.5. Changes in the mean intraocular pressure (IOP) in study and control groups. (range)

<table>
<thead>
<tr>
<th>IOP measurements (mmHg)</th>
<th>Triamcinolone</th>
<th>Laser</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>baseline</td>
<td>15.9 (8-20)</td>
<td>15.5 (10-21)</td>
<td>0.5</td>
</tr>
<tr>
<td>4 months</td>
<td>20.3 (12-32)</td>
<td>15.0 (10-23)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>8 months</td>
<td>20.1 (12-48)</td>
<td>15.0 (8-22)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>12 months</td>
<td>18.2 (12-26)</td>
<td>14.6 (11-21)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

IOP, intraocular pressure
3.4.6.2 Cataract progression

The only statistically significant change was in the degree of posterior subcapsular lens opacity (PSCLO) in the TA group compared with the laser group (fig 3.4.5). The degree of lens PSCLO changed in the TA eyes from a mean of 1.1 to 1.9, while the laser eyes changed for a mean of 1.1 to 1.3.

2 eyes in the TA group and 1 eye in the laser group underwent additional cataract surgery during the follow up for visually significant lens opacity.
Figure 3.4.5: Mean change in the degree of lens opacity from baseline to 12 months in triamcinolone and laser eyes.

PSCLO, posterior subcapsular lens opacity.
3.4.7 Discussion

Our study directly compared intravitreal TA injection with argon laser photocoagulation for patients with persistent CSMO. TA is unlikely to gain acceptance in clinical practice unless it confers a significant advantage over conventional laser photocoagulation and consequently the primary endpoint was the proportion of patient in each group with improved visual acuity. The sample size calculations were based on a 17% difference which was calculated from the original 2 pilot studies.

This study did not achieve the primary endpoint of an improvement of 15 ETDRS letters at 12 months in the TA compared to the laser group. Preliminary studies had shown an improvement in VA and a reduction in central retinal thickness in CSMO (Jonas et al., 2003; Martidis et al., 2002). However these studies were typically uncontrolled. There was a small reduction in macular thickness in the short-term following TA in this study, although this was not statistically significant from the laser group. It is possible that our study was underpowered to show a significant difference. This seems unlikely as no difference was noted from the controls at any time point with respect to visual acuity and retinal thickness. In addition the visual outcome was better in the control group than in the TA group.

The Diabetic Retinopathy Clinical Research Network (DRCRN) evaluated the efficacy of 1mg and 4 mg doses of preservative free TA with laser photocoagulation (2008). This was a large multi-centred randomised controlled trial of similar design to our own, comparing 1 mg and 4 mg TA with macular laser photocoagulation with a primary outcome measure was at 2 years. The only difference in the baseline characteristics of this study compared to our study was a better baseline VA. This probably reflects the
fact that 40% of patients had no prior intervention for DMO and therefore it is likely that they had a shorter duration of CSMO. Both at 1 and 2 years there was no significant differences in visual acuity among the groups.

The DRCRN suggested that TA had a better outcome over the natural history of DMO. The best estimate of the natural history in eyes with DMO comes from the treatment group in the ETDRS in which focal/grid photocoagulation was deferred. The incidence of MVL,(defined as a loss of 15 letters on the ETDRS chart ) if treatment was deferred was 8% at 1 year. Our study was not powered to look at the risk of MVL however the incidence of MVL in the control group was comparable to the ETDRS (7%) whereas the incidence of MVL was in the study group was 12% (5 of 42 eyes). This suggests that the results of TA are worse than the natural history for DMO, however our patients had persistent DMO and therefore are not directly comparable. Furthermore, there was no change in the size of foveal avascular zone was seen form baseline to 12 months in the TA group. In particular the sudden increase in IOP associated with injection did not increase non perfusion of the macular and therefore it is unlikely that TA is worse than the natural history of DMO. However further studies are required to further investigate the effects on MVL.

Gillies and co-workers published a study suggesting benefits of triamcinolone for visual acuity after 2 years (Gillies et al., 2006). This was a prospective randomised study in which the primary endpoint was a 5 letter gain compared to 15 letter gain in this study. Furthermore 50% of their patients had cataract surgery in the 2nd year of study and these 2 factors may explain, at least in part, the different study outcomes. Interestingly, this study showed slight progression of posterior subcapsular cataract in the TA group. Although this change was statistically significant, we did not feel that
it was clinically significant there was no reduction in the retinal thickness in the patients with an increase in lens opacity.

Similarly, Avitabile and colleagues found a positive effect on both visual acuity and central retinal thickness in eyes treated with TA and TA combined with laser (Avitabile et al., 2005b). Despite the effect of the TA regressing at 6 months and some patients requiring a second injection, the results of TA were in marked contrast to laser. Eyes treated with laser alone had no change in either visual acuity or central retinal thickness.

The most significant difference between this study and our own is the duration of macular oedema. Our aim was to include patients with reversible macular oedema, however in reality our study population included patients with a long duration of macular oedema (median duration 27 months, IQR 15-48 months). In comparison, the duration of macular oedema was 6-12 months (mean 9 months) for the study by Avitabile and colleagues (Avitabile et al., 2005b). Additionally the inclusion criteria for this trial were a visual acuity between 6/12 to 3/60. Patients were excluded if they had severe macular ischaemia but patients with a moderate amount of ischaemia were included. The estimate of macular ischaemia was made by the study investigators and not by independent graders. Therefore this study was biased towards patients with chronic macular oedema and included patients with quite advanced ischaemia. Although the population of patients selected for this trial is likely representative of patients that undergo intravitreal TA in clinical practice and suggests that patients with DMO should be treated early before irreversible changes occur. Perhaps if we had excluded patients with chronic macular oedema, we would have found a significant improvement in visual acuity.
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The study was designed to give repeated intravitreal injections to determine whether prolonged intravitreal TA improves visual acuity long-term compared to a single injection which may have only a transient effect. It is notable that 46/129 injections were not performed (28 of the 46 were the third injection). It could be postulated that the significant number of 3rd injections not performed may have adversely effect on outcome; if for instance, the first 2 injections had led to a significant improvement in visual acuity and reduction in central macular thickness. However, this is unlikely as at no study time-point there was a significant improvement in visual acuity or a decrease in central macular thickness or macular volume. It may be possible to have given final injections by raising the intraocular pressure threshold to exclude fewer patients. However, by choosing a threshold of 30 mmHg, there were no serious cases of persistent raised intraocular pressure requiring filtration surgery.

Additionally, based on the recommended guideline for macular laser, retreatment was performed every 4 months. A 4 mg intravitreal TA dose provides continual treatment for approximately 3 months before some eyes begin to have recurrent macular thickening (Beer et al., 2003). Perhaps, if we had chosen a retreatment time of 3 month, more patients would have reached our primary outcome measure.

At 1 month, a reduction in retinal thickness was detected (results not shown), which concurs with the results for initial pilot studies. Based on these results and the results of other investigators, TA maybe utilised as an adjunctive therapy to first reduce retinal thickness before applying macular laser photocoagulation. This combined treatment may prove more effective at reducing the risk of moderate visual loss associated with DMO and the DRCRN is currently recruiting for patients in a trial to assess the efficacy of this treatment.
Similarly, intravitreal TA may have a role in treating DMO (in the absence of significant ischaemia) in patients where laser treatment had failed. Additionally, it may have a role in macular oedema associated with other disease processes such as branch retinal vein occlusions and pseudophakic macular oedema.

In conclusion, this study was relatively small, with limited follow up. It directly compared TA to conventional macular laser therapy, which is the only recognised standard treatment for CSMO. However, the patient population tended to have chronic CSMO. There were few side effects with only one case of sterile endophthalmitis and no cases of retinal detachment or glaucoma. However, neither TA nor laser treatment led to an improvement in visual acuity nor a reduction in central retinal thickness at any time point. Therefore we did not find evidence to support the use of triamcinolone as a routine treatment for chronic CSMO. Since other studies suggest a role for intravitreal triamcinolone early in the treatment for CSMO aggressive treatment for CSMO should be undertaken as early as possible in order to avoid permanent structural changes.
Chapter 3.5

Do the morphological characteristics demonstrated by optical coherence tomography predict the outcome of treatment for persistent diabetic macular oedema?
3.5.1 Introduction

Optical coherence tomography (OCT) is a quick, non-invasive and reproducible method of producing high-resolution, cross-sectional images of the retina (Massin et al., 2001). It is based on the principle of low-coherence interferometry, which measures the time of flight delay of light reflected from ocular structures (Baumal, 1999) analogous to the ultrasound B-scan (Hee et al., 1998). Low-coherence infrared light is produced by a continuous-wave, superluminescent diode source, which is coupled into a fibre optic Michelson interferometer (Baumal, 1999). The echo delay and intensity of reflected light from internal tissue structures can be measured to produce 2- and 3D-images of the retina (Drexler et al., 2003).

A number of studies have been undertaken to correlate OCT images with histology. It is well established that the internal boundary corresponds to the internal limiting membrane and is well defined because of the contrast between the non-reflective vitreous and the highly reflective retina, while the external boundary represents the internal edge of the retinal pigment epithelium. However, there appears to be some debate between the relationship between OCT banding and specific retinal layers. While Toth et al. consider that the retinal morphology obtained by OCT correlates with those obtained by light microscopy, Chauhan & Marshall argue that OCT bands do not represent specific retinal layers (Toth et al., 1997; Chauhan and Marshall, 1999). Ultra-high resolution OCT images (not yet commercially available) have now been compared with histology with a high degree of concordance (Drexler et al., 2003).

However the retinal thickness measured by OCT has been shown to correlate with the thickness of retina in histological specimens (Chauhan and
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Marshall, 1999) and although these data do not replace visual acuity (VA), it is a useful adjunct and studies shown that retinal thickness broadly correlates with VA (Hee et al., 1998; Otani and Kishi, 2007; Yang et al., 2001). Recently a classification of different morphological OCT appearances in DME has been reported but their clinical significance remains unclear (Panozzo et al., 2004). This study aimed to describe the various morphologic OCT appearances seen in DME, to correlate them with the baseline visual acuity and to determine if any morphological subtype was predictive of the response to intervention.
3.5.2 Baseline characteristics and visual acuity

88 eyes of 88 patients were included in the original randomised controlled study. 87 (99%) baseline OCTs were available for analysis of OCT morphologic characteristics. Only one radial line scan was available for grading in 1 eye (1.1%).

Baseline demographics included 37 right eyes, a male to female ratio of 2:1, a mean (SD) patient age of 63.7 (+/-9.0) years and a mean duration of diabetes of 15.5 (+/-9.9) years. Prior focal laser photocoagulation had been performed a mean 3.1(+/-1.5) times. 14 eyes (11.5%) were pseudophakic.

An average of 1.7 treatments (laser or TA) were performed during follow up. The mean visual acuity (SD) was 53.8 letters (+/-13.7) at baseline and 55.3 letters (+/-14.5) at 12 months. Improvement of ≥15 ETDRS letters occurred in 7 (8%) patients and a decline of ≥15 ETDRS letters occurred in 7 (8%) patients.
3.5.3 Morphological characteristics

Eyes were graded according the presence or absence of cystoid macular oedema (CME), neurosensory detachment (NSD), posterior hyaloid traction (PHT) and abnormal macular profile (AMP). 2 OCT scan were ungradable with respect to CME and 1 was ungradable for NSD. There was a moderate to good degree of interobserver and intraobserver agreement (table 3.5.1).

The most frequent characteristic was the presence of CME (85%) (fig 3.5.1) 1 eye (1%) had an increased retinal thickness without any of the 4 key subtypes. 68 of 74 eyes (91.9%) with CME had other features. 20 of 21 eyes with NSD had CME but only 8 of the 21 eyes with NSD had PHT. PHT was always seen in association with CME. NSD and traction were never seen concurrently without other features. Only 1 eye had a NSD with an apparently normal overlying retina.

A subset of eyes with DME had NSD (21/87, 24%), which was only correlated with PHT in 9/24 eyes, had a shorter natural history of DME (19 vs 37 months, P=0.005) and of reduced visual acuity (14 vs 19 months, P=0.05) than eyes without NSD (table 3.5.2).
Table 3.5.1: Interobserver and intraobserver agreement between graders

<table>
<thead>
<tr>
<th>Morphological subtype</th>
<th>Kappa value</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Interobserver</td>
<td>Intraobserver</td>
<td></td>
</tr>
<tr>
<td>CME</td>
<td>0.447</td>
<td>0.392</td>
<td></td>
</tr>
<tr>
<td>NSD</td>
<td>0.638</td>
<td>0.718</td>
<td></td>
</tr>
<tr>
<td>PHT</td>
<td>0.713</td>
<td>0.598</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>0.546</td>
<td>0.738</td>
<td></td>
</tr>
</tbody>
</table>

CME, cystoid macular oedema; NSD, neurosensory detachment; PHT, posterior hyaloidal traction, AMP, abnormal macular profile.

Figure 3.5.1: Venn diagram illustrating the number of eyes that fall into each morphological subtype (percentage).

CME = cystoid macular oedema, NSD = neurosensory detachment; PHT, posterior hyaloidal traction, AMP, abnormal macular profile.
Table 3.5.2: Comparison of baseline characteristics in each of the morphological subtypes.

<table>
<thead>
<tr>
<th>Morphological subtype</th>
<th>n</th>
<th>CSMO (mths)</th>
<th>Visual loss (mths)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CME</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>75</td>
<td>28.2</td>
<td>29.7</td>
<td>0.09</td>
</tr>
<tr>
<td>absent</td>
<td>10</td>
<td>33.6</td>
<td>16.1</td>
<td></td>
</tr>
<tr>
<td>NSD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>21</td>
<td>19</td>
<td>13.6</td>
<td>0.003</td>
</tr>
<tr>
<td>absent</td>
<td>65</td>
<td>37.3</td>
<td>19.3</td>
<td>0.05</td>
</tr>
<tr>
<td>PHT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>8</td>
<td>26.5</td>
<td>15.9</td>
<td>0.2</td>
</tr>
<tr>
<td>absent</td>
<td>59</td>
<td>35.4</td>
<td>21.5</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>66</td>
<td>30.8</td>
<td>20.6</td>
<td>0.4</td>
</tr>
<tr>
<td>absent</td>
<td>21</td>
<td>37.8</td>
<td>17.5</td>
<td></td>
</tr>
</tbody>
</table>

CSMO, clinically significant macular oedema; mths, months; CME, cystoid macular oedema; NSD, neurosensory detachment; PHT, posterior hyaloidal traction; AMP, abnormal macular profile.
3.5.4 Comparison of visual acuity and retinal thickness for each of the four morphological subtypes.

The presence of a NSD, PHT or AMP did not correlate with the BCVA, (fig 3.5.2, top). In contrast, eyes with CME had a statistically significantly lower BCVA than eyes without CME (CME, 53 letters; no CME, 61 letters, P=0.05).

Similarly, eyes with CME had a statistically significant increase in retinal thickness compared to eyes without CME (CME, 418µm; no CME, 354µm; P=0.04), as did eyes with an abnormal macular profile. (abnormal profile, 436µm; normal profile 314µm; P=0.0005) (fig 2, bottom). There was no difference in the central retinal thickness in eyes with or without traction or a NSD.
Figure 3.5.2: Differences in the baseline BCVA (top) and retinal thickness (bottom) with respect to the different morphological subtypes. BCVA, best corrected visual acuity; ETDRS, early treatment diabetic retinopathy study; CME, cystoid macular oedema; NSD, neurosensory detachment, PHT, Posterior hyaloidal traction; AMP, abnormal macular profile.
3.5.5 Change in mean visual acuity and retinal thickness from baseline to 12 months for each of the four OCT characteristics.

None of the morphological subtypes was predictive of the change in VA nor retinal thickness from baseline to 12 months following treatment (laser or triamcinolone) (figure 3.5.3). Additionally, none of the morphological subtypes was predictive of final VA for laser and triamcinolone alone (results not shown).
Figure 3.5.3: Comparison of the mean change in BCVA (top) and retinal thickness (RT) (bottom) from baseline to 12 months for each of the four morphological subtypes distinguished by OCT. BCVA, best corrected visual acuity; ETDRS, early treatment of diabetic retinopathy study; CME, cystoid macular oedema; NSD, neurosensory detachment; CRT, central retinal thickness; PHT, posterior hyaloidal traction; AMP, abnormal macular profile.
3.5.6 Discussion

Fundus fluorescein angiogram is clinically the most widely available and useful clinical test to assess vascular hyperpermeability. It permits study of the circulation of the retina and choroid in normal and diseased states (Jaffe et al., 1981). In recent years, the availability of optical coherence tomography (OCT) has changed the clinical management of many eye conditions.

Optical coherence tomography is a non-invasive device that obtains cross-sectional, high-resolution images of the retina that provides repeatable values of retinal thickness (Huang et al., 1991). Additionally, images can also be used to determine microstructural features and investigators have debated whether this is can be correlated with histological features. During DMO, fluid first accumulates within the Muller cells, which may correspond to the OCT appearance of sponge-like swelling, namely increased retinal thickness in the absence of cystic spaces. Later, with cellular death, extracellular fluid arises in the inner and outer plexiform layers and this has been correlated to the cystic spaces observed on OCT.

Previous reports have evaluated the incidence of various patterns of DME on OCT; diffuse retinal thickening (DRT) that is increased retinal thickness with areas of reduced intraretinal reflectivity, CME, NSD and PHT (Otani and Kishi, 2007; Panozzo et al., 2003; Brasil et al., 2007; Ozdemir et al., 2005a; Catier et al., 2005). In our study, the incidence of DRT correlated with the absence of CME, and therefore we did not report this as a separate subtype. In addition, we also evaluated our OCTs with respect to the presence or absence of normal concave macular profile.
The presence of each subtype has been reported at 40 -85% for CME (Brasil et al., 2007;Otani and Kishi, 2007;Yamamoto et al., 2001;Panozzo et al., 2003;Kim et al., 2006), 15 – 37% for NSD (Otani and Kishi, 2007;Panozzo et al., 2003;Ozdemir et al., 2005a;Brasil et al., 2007;Catier et al., 2005) and 16 – 28% for PHT (Brasil et al., 2007;Kim et al., 2006). Despite being reported separately each subtype may also occur concurrently. Our data were collected using an OCT 3000 which is a scanner of higher resolution than that used to generate some of the earlier data. This may partially explain our high prevalence of all 3 subtypes. Additionally, the study investigator selected 2 perpendicular line scans to try the capture the maximum amount of pathology for each eye.

There are obvious limitations of this study. The most obvious is that this is a small retrospective case series with limited follow up. The patients were randomized to one of 2 interventions – laser or intravitreal triamcinolone and here we report the 2 treatments as one larger group. When analysed as 2 separate groups, there were no differences but the number of patients in each was small. Finally, our patients had persistent DMO despite previous laser treatment and this chronicity of the disease undoubtedly has an influence on the impact of treatment. Therefore, it would be interesting the repeat this study on eyes with CME prior to initial treatment.

NSD is seen on OCT as a triangular or dome shaped non-reflective intraretinal space with its greatest height at the central foveal. It is seen adjacent to the hyper-reflective line of the pigment epithelium, with a well-defined inner border and is of similar appearance during other disease processes such as branch retinal vein occlusion or following retinal detachment repair (Yamaguchi et al., 2006;Schocket et al., 2006). It is exclusively observed topographically and not clinically or with FFA. As a result, it has only recently been described. Therefore the significance of a
NSD is not yet apparent. We observed that NSD was present when the macular oedema was of shorter duration than in eyes without NSD. These data correspond to that of Gaucher et al., who reported that an NSD occurs in 20% of cases when the overlying neuroretina is of a normal thickness (Gaucher et al., 2008). They and others found no relationship between NSD and VA (Catier et al., 2005; Massin et al., 2001) as it was in our study. Otani et al observed that during the treatment of DMO, the NSD transiently enlarged in 3 eyes and developed transiently in another 3 eyes and they interpreted this as the movement of fluid from the retina to the subretinal space during the process of macular oedema absorption (Otani and Kishi, 2000). Another study describes it later in the disease evolution (Soliman et al., 2008).

The relevance of NSD is yet to be determined. More information, perhaps in the form of a large longitudinal study, is required to determine the significance of this feature and to understand if it truly represents the efflux of fluid through the RPE. Additionally it maybe associated with a drop in visual acuity and therefore the patient presents to the ophthalmologist sooner than if it were not to form.

In contrast, the presence of CME was associated with a worse VA which was comparable to that reported by Kim et al (Kim et al., 2006). Due to distortion of the normal anatomy and the loss of the foveal pit it becomes increasingly difficult to determine the exact position of the CME and to assess the thickness of the surrounding retina. Therefore, it was difficult to conclude from OCT if there was intraretinal or extracellular oedema and reflects our lack of true understanding about exact correlation between the morphological characteristics on OCT and the histological appearance.
With more recent advances in OCT technology the resolution of the images has increased from 10 µm to 3 µm and it will be interesting to observe if high resolution OCT improves correlations with histopathological changes. Perhaps, then we will be able to identify morphological patterns that identify intracellular oedema and extracellular oedema that is reversible from irreversible changes.

It has been suggested that CME and PHT are predictive of a poor outcome following intervention (Brasil et al., 2007). In contrast, the presence of a NSD does not necessarily correlate with the visual outcome following laser or intravitreal TA (Brasil et al., 2007;Gaucher et al., 2008). However, its presence predicted a poorer visual outcome following vitrectomy and inner limiting membrane peel (Shah et al., 2006) and following laser for branch vein occlusion (Ohashi et al., 2004). In our study, none of the OCT subtypes were predictive of the outcome of intervention.

In conclusion, CME was associated with poor baseline but not final visual acuity suggesting that CME cases should be treated. Some patients with DME develop a NSD, apparently unrelated to traction, with a more rapid onset of visual loss and should be considered for early treatment.
Chapter 3.6

Combined vitrectomy and intraocular triamcinolone for diabetic macular oedema
3.6.1 Introduction

Initial pilot studies using intravitreal triamcinolone (TA) suggested it can improve visual acuity (VA) in patients with diabetic macular oedema (DMO) in the short-term (Massin et al., 2004; Jonas and Sofker, 2001; Martidis et al., 2002). However repeated injections are often necessary due to a recurrence of the macular oedema. The vitreous has implicated in the production of DMO. Eyes with diabetic retinopathy are much more likely to develop macular oedema if the vitreous is still attached (Nasrallah et al., 1988). It is postulated that an attached posterior hyaloid may cause tangential macular traction leading to macular oedema. Additionally vitrectomy may be useful in the absence of obvious traction (Tachi and Ogino, 1996; Ikeda et al., 1999; Ikeda et al., 2000; La Heij et al., 2001).

By combining these two novel therapies there may be some benefit from approaching the disease process using two different strategies. The aim of this exploratory study was to evaluate the safety and long-term efficacy of combined PPV and intraocular TA in patients with persistent DMO and if this intervention reduced the need for reinjections of TA.
3.6.2 Baseline characteristics

This study was conducted in accordance with the ethical standards of the Declaration of Helsinki and were approved by the local Research and Ethics Committees at Moorfields Eye Hospital. Patients were recruited via the Medical Retinal Service at Moorfields Eye Hospital between February 2003 and May 2004. Written informed consent was obtained from all patients before details of medical and ophthalmic examinations were recorded.

16 patients were eligible for the study. 3 patients declined entry into the study and 1 patient was excluded from the study due to minimal DMO. A total of 12 eyes from 12 patients were recruited for this study. All the patients underwent a standard 20 G 3 port PPV with 4 mg in 0.1 ml TA injected into the vitreous cavity at the end of the operation. All patients completed all the follow up visits. The baseline characteristics of the patients are shown in the table 3.6.1.
Table 3.6.1: Baseline characteristics of patients undergoing PPV with TA

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>male</td>
<td>9 (75 %)</td>
</tr>
<tr>
<td>left eye</td>
<td>4 (33 %)</td>
</tr>
<tr>
<td>type 2 diabetes</td>
<td>9 (75 %)</td>
</tr>
<tr>
<td>systemic hypertensive</td>
<td>10 (83 %)</td>
</tr>
<tr>
<td>pseudophakia</td>
<td>1 ( 8 %)</td>
</tr>
<tr>
<td>proliferative diabetic retinopathy</td>
<td>7 (58 %)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (IQR)</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>61.5 (52.5-66.5)</td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>7.1 (6.6-7.6)</td>
<td></td>
</tr>
<tr>
<td>duration diabetes (years)</td>
<td>15.5 (13.5-21)</td>
<td>1</td>
</tr>
<tr>
<td>duration of oedema (months)</td>
<td>49 (24-54)</td>
<td></td>
</tr>
<tr>
<td>duration of visual loss (months)</td>
<td>24.5 (20.5-40.5)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number previous macular laser treatments</th>
<th>Number (%)</th>
<th>n* = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2 (17 %)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3 (25 %)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1 (8 %)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2 (17 %)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>distance visual acuity (ETDRS letters)</td>
<td>53.5 (50-58.5)</td>
</tr>
<tr>
<td>central macular thickness (µm)</td>
<td>409.5 (312.5-508)</td>
</tr>
<tr>
<td>IOP (mmHg)</td>
<td>16 (15.5-18)</td>
</tr>
</tbody>
</table>

* n = number of cases data missing on particular characteristic.
PPV, pars plana vitrectomy; TA, triamcinolone; IQR, interquartile range; HbA1c, glycosylated haemaglobin; ETDRS, early treatment of diabetic macular oedema; IOP, intraocular pressure, mmHg, millimeters mercury.
3.6.3 Distance visual acuity

4 of 12 eyes showed an improvement of 10 or more ETDRS letters (2 lines Snellen acuity) at four months and 2 of 12 eyes showed an improvement of 10 or more letters at 12 months (fig 3.6.1). Both patients were relatively young (24 and 35 yrs), had type 1 diabetes with treated proliferative diabetic retinopathy. 4 of 12 eyes lost 10 or more letters at 12 months.

The median letter score at baseline was 53.5 letters (fig 3.6.2). The median letter score was 59.5 letters at four months and 51 letters at twelve months.

3.6.4 Median central retinal thickness

The median retinal thickness was 409.5 µm at baseline (fig 3.6.3). The retinal thickness had fallen to 289.5 µm at 4 months and 387 µm at 12 months.
Figure 3.6.1: Changes in the BCVA letter scores following combined vitrectomy with triamcinolone.

BCVA, visual acuity; ETDRS, early treatment of diabetic retinopathy study.
Figure 3.6.2: Change in the median BCVA letter scores over time in patients undergoing combined vitrectomy with triamcinolone. Central line illustrates the median value, the boxes illustrate the interquartile range and the extremes illustrate the minimum and maximum values.

ETDRS, early treatment of diabetic retinopathy study.
Figure 3.6.3: Change in median retinal thickness over time in patients undergoing combined vitrectomy with triamcinolone. Central line illustrates the median value, the boxes illustrate the interquartile range and the extremes illustrate the minimum and maximum values.
3.6.5 Safety

One patient was noted to have an entry site break at the time of surgery which required laser retinopexy and a C₃F₈ gas tamponade. A second patient had a pneumatic retinopexy for a small superior retinal detachment. The retinae in both patients remained attached throughout follow up. One patient had a vitreous haemorrhage immediately following surgery which cleared spontaneously within 4 weeks.

A change in the median intraocular pressure (IOP) is shown in fig 3.6.4. 4 of 12 eyes required topical antihypertensive treatment because of an increased IOP of more than 30 mmHg following the combination therapy. All rises in IOP were transient and ocular antihypertensives were stopped within 1 month of the surgery.

There was an overall increase in the amount of nuclear sclerotic lens opacity from baseline to 12 months (baseline, 0.5; 12 months, 1.875) and two patients required a cataract extraction during the 12 month follow up period. Finally, there was no change in the size of the foveal avascular zone from baseline (baseline median 643 µm; 12 months, median 652 µm).
**Figure 3.6.4:** Change in median intraocular pressure over time in patients undergoing combined vitrectomy with triamcinolone. Central line illustrates the median value, the boxes illustrate the interquartile range and the extremes illustrate the minimum and maximum values.

mmHg, millimeters of mercury.
3.6.6. Discussion

The results of this exploratory study indicated that there was a short term improvement in visual acuity and a reduction in macular thickness following PPV and intraocular TA for patients with persistent DMO. However the long-term improvement in visual acuity (12 months) was limited to a small number of patients.

Vitrectomy for eyes without clinically visible traction is reported to be useful in eyes with DMO (Tachi and Ogino, 1996;Ikeda et al., 1999;Ikeda et al., 2000;La Heij et al., 2001). Following vitrectomy, oxygen transport to retinal area and the clearance of VEGF and other cytokines improved (Funatsu et al., 2003;Stefansson et al., 1990a). However the anatomical and functional recovery in eyes without clinically visible traction can be often be slow and unpredictable (Patel et al., 2006b;Patel et al., 2006a;Massin et al., 2003;La Heij et al., 2001). Additionally we sought to assess the VEGF-lowering effect of vitrectomy with corticosteroids.

Although our study was not designed to assess the short-term effects of PPV with TA, an OCT was performed at 1 week and 1 months which showed that there was an immediate reduction in retinal thickness in most patients (results not shown) suggesting that there was a predictable effect of PPV with the addition of TA.

There were no serious long-term consequences of our intervention; however 4 eyes had a reduction in VA at 12 months. Although there was no change in retinal thickness or in the dimensions of the FAZ in these patients, we speculate that the deterioration was due to continuing diabetic maculopathy; both DMO and occult macular ischaemia and associated neuronal cell death.
Furthermore, the 2 eyes that required cataract extractions did not have an improvement in the BVCA compared to baseline following the additional surgery.

This study was designed with a single intraocular injection of triamcinolone in order to test the hypothesis that PPV may reduce the need for repeat injections. Our results suggest that a short-term improvement in visual acuity and reduction in retinal thickness can be achieved from the combination therapy but in most patients there is a recurrence of the DMO and regression in the visual improvement achieved after 4 months suggesting that further intervention is necessary. However, we speculate that further injections would add little benefit to these patients. The vitreous acts as a relative barrier to the anterior movement of growth factors and drugs and therefore in the absence of the vitreous the half life of TA is reduced.

Furthermore one of the main weaknesses of this trial is the chronicity of the macular oedema. We tried to include patient with a short disease duration but in reality the patients we actually recruited had a chronic persistent DMO persist. Most of the patients had had at least previous macular laser treatments.

This study was small and with limited follow-up. The study population were patients with persistent macular oedema despite at least 1 prior laser photocoagulation. However many of our patients had many previous macular laser treatments and a long duration of macular oedema and visual loss. Interestingly, the 2 patients who responded well to surgery at 12 months were younger and had type I diabetes. It is possible that this exploratory treatment could have a beneficial effect at 12 months for patients...
at an earlier stage of their macular disease; for example younger patients, with better visual acuities or with disease duration less than 6 months and possibly reversible macular changes.

In this study we found that combining pars plana vitrectomy with intraocular triamcinolone has a transient beneficial effect on improving VA and reducing macular oedema. However, recurrence of macular thickening is still a main problem after the combination therapy.
Chapter 3.7

The intraocular concentration of VEGF before and after combined vitrectomy and intraocular triamcinolone for diabetic macular oedema.
3.7.1 Introduction

Vascular endothelial growth factor (VEGF) is a vasopermeability agent found in eyes with diabetic macular oedema (DMO) as well as active diabetic retinopathy (Funatsu et al., 2006; Aiello et al., 1994). Elevated VEGF concentrations in the eye are correlated with progression of diabetic retinopathy following vitrectomy (PPV) (Funatsu et al., 2005). It is unknown if elevated concentrations can predict the outcome of treatment for DMO.

This study assayed VEGF concentrations in the eye before and after combined vitrectomy and intravitreal triamcinolone to assess whether pre-operative vitreous VEGF concentrations were predictive of functional and anatomical outcomes.
3.7.2 Baseline characteristics

12 eyes of 11 patients were enrolled in the study. The baseline characteristics are summarised in table 3.7.1. The median age was 58 years, (IQR 42.5–64.5), the median duration of diabetes was 15 years, (IQR 13.5–20) and the median duration of macular oedema was 48 months, (IQR 23-52).
### Table 3.7.1: Baseline characteristics of patients undergoing combined vitrectomy with triamcinolone

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median</th>
<th>IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>58</td>
<td>42.5, 64.5</td>
</tr>
<tr>
<td>duration diabetes (yrs)</td>
<td>15</td>
<td>13.5, 20</td>
</tr>
<tr>
<td>duration DMO (mths)</td>
<td>48</td>
<td>23, 52</td>
</tr>
<tr>
<td>HbA1c</td>
<td>7.3</td>
<td>6.6, 7.6</td>
</tr>
<tr>
<td>size FAZ</td>
<td>624</td>
<td>440, 766</td>
</tr>
<tr>
<td>BCVA</td>
<td>53.5</td>
<td>(45, 58.5)</td>
</tr>
<tr>
<td>retinal thickness</td>
<td>506.5</td>
<td>(364, 527.5)</td>
</tr>
</tbody>
</table>

IQR, interquartile range; HbA1c, glycosylated haemoglobin; DMO, diabetic macular oedema; FAZ, Foveal avascular zone; BCVA, best corrected visual acuity.
3.7.3 Outcome of vitreous surgery

At four months, the visual acuity in 3 eyes had improved by ≥10 ETDRS letters. At twelve months, 2 eyes had improved by ≥10 ETDRS, while 4 eyes had deteriorated by ≥10 ETDRS (fig 3.7.1, top). The median BCVA was 53.5, 57.5 and 50 letters at baseline, four and twelve months respectively.

The median central retinal thickness was 506.5, 354 and 387 µm at baseline, four and twelve months respectively (fig 3.7.1, bottom). 2 patients underwent cataract extraction at 8 months for visually significant lens opacities.

At baseline, the median concentration of VEGF in the vitreous (574.6 pg/ml, IQR 123.7, 678.2) was higher than in the aqueous (79.7 pg/ml, IQR 55.6, 172.5). At 1 week the median aqueous VEGF concentration had reduced to 60.9 pg/ml (43.5, 126.9, while at 4 months it had risen from baseline to 91.2 pg/ml (IQR, 75.6, 120)
Figure 3.7.1: Change in the BCVA letter scores (top) and central retinal thickness (bottom) following combined vitrectomy with intraocular triamcinolone. The central line illustrates the median value, the box illustrates the interquartile range and the extremes illustrate the minimum and maximum values.

BCVA, best corrected visual acuity; ETDRS, early treatment of diabetic retinopathy study
Figure 3.7.2: Change in the concentration of VEGF in the aqueous at baseline, 1 week and 4 months following combined PPV with intraocular triamcinolone. The central line illustrates the median value, the box illustrates the interquartile range and the extremes illustrate the minimum and maximum values.

conc, Concentration; VEGF, vascular endothelial growth factor.
3.7.4 Relationship between VEGF and visual acuity and central retinal thickness

There was little evidence of any association between the concentration of VEGF in the vitreous and the BCVA at 4 months or at 12 months (fig 4.3, top).

Similarly, there was little evidence of any association between the concentration of VEGF in the vitreous and the retinal thickness at 4 months or at 12 months, (fig 4.3, bottom)
Figure 3.7.3: The relationship between the concentration of VEGF in the vitreous and in the BCVA and retinal thickness at 4 months and 12 months. There was no strong relationship between the concentration of VEGF in the vitreous and the BCVA at 4 months, $\rho = 0.235$, $P=0.49$ (top left) and at 12 months, $\rho=0.48$, $P=0.13$ (top right) and in the central retinal thickness at 4 months, $\rho=-0.27$, $P=0.42$ (bottom left), and at 12 months, $\rho=-0.47$, $P=0.14$ (bottom right).

BCVA, best corrected visual acuity; ETDRS, early treatment of diabetic retinopathy study; VEGF, vascular endothelial growth factor; CRT, central retinal thickness.
3.7.5 Discussion

This study demonstrated that the VEGF concentration in the vitreous was elevated in eyes with DMO which was in agreement with previous studies (Patel et al., 2006c; Funatsu et al., 2006), although we did not include a control group and therefore were unable to show that our result was greater than non-diabetic eyes.

There was a modest reduction in the aqueous VEGF concentration one week following combined vitrectomy with TA. It is conceivable that triamcinolone accounts for this modest decline as TA into an non-vitrectomised eye leads to a marked reduction in vitreous VEGF concentration at 1 month (Brooks, Jr. et al., 2004a). Conversely elevated VEGF concentrations are maintained for 36 days following PPV in patients with PDR (Itakura et al., 2004).

We expected a greater reduction in aqueous VEGF concentration, although in order to avoid intraoperative complications, the aqueous was taken at the end of the operation. We speculate that the aqueous sample was diluted and therefore our baseline aqueous VEGF concentration artificially low.

We were unable to detect a relationship between vitreous VEGF concentration and outcome of combined surgery. All our patients had chronic macular oedema and it is possible visual acuity was limited by longstanding intraretinal anatomical and functional changes such as photoreceptor death and RPE dysfunction. If we had included patients with a better baseline visual acuity and of shorter duration of DMO, then the visual outcome may have been improved and an initial VEGF concentration may have been predictive of this.
It may also be that VEGF concentration is not related to the level of macular oedema at all. DMO and DR are very closely interrelated and therefore it maybe possible that the VEGF concentration only reflects the degree of overall ischaemia and therefore the level of DR as a whole.

At four months aqueous VEGF concentrations were increased. Previous studies suggest that increased vitreous VEGF concentrations correlate with severity of diabetic retinopathy (Funatsu et al., 2005) and actively leaking DMO (Funatsu et al., 2004;Funatsu et al., 2006;Funatsu et al., 2007) These results suggest that despite surgery, our patients continued to show active disease. Although this may have been expected due to chronic nature of DM and TA pharmacokinetics, it was anticipated that PPV would have arrested disease progression and reduce the need for reinjection of TA.

Following combined vitrectomy with intraocular triamcinolone for chronic DMO, elevated aqueous VEGF concentration recurred at 4 months demonstrating that PPV and TA together were unable to prevent disease activity and thus the need for reinjections for TA. Additionally baseline intravitreal VEGF concentrations were not predictive of surgical outcome.
4. Conclusions and Perspectives
4.1 VEGF and mechanisms of vascular hyperpermeability

Vascular permeability is the movement of fluids and molecules between the vascular and extravascular compartments. Normal basal vascular permeability refers to the molecular exchange in normal tissues and primarily takes place in the capillaries. The molecules exchanged consist largely of gases (O\textsubscript{2} and CO\textsubscript{2}), water, small molecules such as salts and sugars and only small amounts of plasma proteins. The extent of the process varies considerably between tissues and is largely a passive process, but can also be influenced by factors such as hydrostatic pressure and blood flow (Reed et al., 2001; Pietras et al., 2001). Hydrostatic pressure can be affected by arterial blood pressure, altered efflux of fluid through the lymphatic system, changes in the extracellular matrix, or by integrins in interstitial cells (Reed et al., 2001).

Vascular hyperpermeability is the increase in vascular permeability in response to a number of vascular permeabilising factors for example histamine and VEGF. Not only is the quantity of extravagated fluid greatly increased but its composition is also changed. The fluid is rich in proteins similar in composition to the plasma and is referred to as an exudate. The extravasated proteins include fibrinogen and fibrin, which allow the exudate to form a gel, trapping water and resulting in tissue swelling and oedema. When vascular hyperpermeability becomes chronic, it usually implies that the leaking blood vessels are abnormal for example through new blood vessels during neoplasia or ischaemia.

The route of solute flux across the vascular endothelium has been debated for some time. Transcellular flux is undoubtedly important, in particular under pathological conditions. Significantly more perfused horseradish
peroxidase or albumin is observed in intracellular vesicles in spontaneously diabetic rats (Vinores et al., 1993). Transcellular flux has also been attributed to the formation of caveolae and vesiculovacuolar organelles in response to VEGF and not through the formation of fenestrations (Feng et al., 1999; Dvorak and Feng, 2001; Hofman et al., 2000). However, the more common and intuitive theory of the passage of fluid and proteins through the BRB assumes the presence of a regulated paracellular route. In agreement, changes in the junctional proteins have been observed to correlate vascular leak (Antonetti et al., 1998; Davidson et al., 2000). A more direct indication arises from observation of mice lacking VEC or Cl-5 in which severe and selective vascular leak is observed, respectively (Corada et al., 1999; Nitta et al., 2003).

VEGF leads to an increase in permeability both in vivo and in vitro (Behzadian et al., 2003) and is thought to have a key role in the pathogenesis of DMO due to this vasoactivity. VEGF-induced vascular permeability is at least in part mediated via changes in the junctional complexes (Gavard and Gutkind, 2006). Indeed, addition of VEGF to our retinal endothelial cells in vitro led to a rapid increase in permeability within 30 minutes, which correlated with a reversible reduction of junction-associated proteins such as ZO-1, alpha-catenin and gamma-catenin. Prolonged exposure also induced a loss of Cl-5, occludin and VEC. Down-regulation of junctional proteins, in particular of occludin, VEC and ZO-1 in response to VEGF is in agreement with other reports (Antonetti et al., 1999; Behzadian et al., 2003; Esser et al., 1998; Wang et al., 2001; Nakamura et al., 2008). Taken together, these results underline that regulation of the paracellular space contributes to the endothelial vasoactive response. Accordingly, the neutralisation of VEGF function can reduce hyperpermeability in animal models of diabetes (Ideno et al., 2007; Ishida et al., 2003), again implying a direct role for VEGF in its pathogenesis. Additionally, intravitreal injections of VEGF into the primate eye, results in
vascular changes comparable to DR. Neovascularisation was observed in
the periphery and microaneurysms appeared on arterioles, while areas of
capillary nonperfusion, vessel dilation and tortuosity were seen on
angiography (Tolentino et al., 2002). Thus targeting VEGF to prevent
vascular endothelial permeability during DMO/DR appears like a promising
therapeutic strategy.

A great number of studies report that intravitreal VEGF levels are very high
in patients with proliferative diabetic retinopathy (Aiello et al.,
1994; Watanabe et al., 2005; Malik et al., 2005; Funatsu et al., 2004). Its
angiogenic growth factor activity is undoubtedly key in inducing the
neovascularisation seen in these patients. As outlined and discussed above
VEGF is also thought to play a prominent role in oedema formation during
DMO. In agreement, it was reported that patients with DMO have elevated
intraocular VEGF in the vitreous and aqueous (Funatsu et al., 2003; Patel et
al., 2006c). We found similarly elevated levels of VEGF in our study of 12
eyes with DMO although our median VEGF level was lower than the
previous 2 reports (575 vs 957 and 1125 pg/ml). Taken together, VEGF
levels in the eye are elevated during DMO but even higher during the
proliferative phase of the disease.

VEGF is upregulated by hypoxia, through the induction of hypoxic-inducible
factors (HIF). HIF translocates to the nucleus and upregulates the
transcription of VEGF mRNA in retinal pericytes, retinal endothelial cells and
RPE (Ozaki et al., 1999). Retinal ischaemia increases VEGF levels and
produces proliferative diabetic retinopathy (PDR) is a direct consequence.
As DMO and PDR are not independent entities, it is therefore conceivable
that the elevated VEGF in eyes with DMO is related to the degree of retinal
hypoxia and not to the degree of oedema / permeability. This would explain
why no relationship between the levels of VEGF in the ocular fluids and outcome of PPV with TA for DMO was detected in our trial.

However not all patients with an elevated VEGF level develop DMO. Maybe VEGF concentrations vary throughout different parts of the vitreous and perhaps VEGF gets trapped in mid vitreous or in some patients VEGF is unable to get through the inner limiting membrane at macula to invade retina and thus increase vascular permeability. The predilection of type 1 diabetic to develop proliferative diabetic retinopathy and of type 2 diabetics to develop DMO suggests a complex underlying disease pathology involving more than vascular hyperpermeability and elevated VEGF levels.
4.2 Mechanisms of diabetic macular oedema

Breakdown of the blood-retinal barrier occurs in many diseases and may lead to subretinal fluid or retinal oedema. The inner BRB (iBRB) is primarily affected in the diabetic eye and vascular hyperpermeability has been proposed as one of the earliest retinal changes in diabetes (Cunha-Vaz et al., 1975). Increased retinal thickness has been shown to coincide with increased leak from retinal blood vessels (Lobo et al., 2000) and correlates with vision loss in DR (Moss et al., 1998). Additionally, increased retinal vascular permeability is associated with increased permeability in the kidneys and increased urinary albumin excretion in type 2 diabetics (Knudsen et al., 2002).

In the normal healthy eye, retinal vascular hyperpermeability is compensated for by an efflux of fluid through the retinal pigment epithelium (RPE) and the redistribution of electrolytes and thus water via Müller cells (Bringmann et al., 2004). Although DM is primarily a retinal microvasculopathy, changes in morphology, permeability and electrophysiology of the RPE in experimentally diabetic animals have also been described. The RPE in spontaneously diabetic rats shows changes in the basal folding of the plasma membrane and increased permeability to horseradish peroxidase (Blair et al., 1984). Furthermore there is a loss of ionic autoregulation in RPE cells. Changes in the function of the RPE can be detected clinically as an alteration in the RPE-generated c-wave on electroretinography (MacGregor and Matschinsky, 1986) and a decrease in amplitude of the fast oscillation (FO) on electrooculogram (Schneck et al., 2008). The FO reflects the ability of the RPE to pump fluid and ions from the retina to the choroid and a decrease in the FO can be detected in diabetic patients prior to any visible retinopathy (Schneck et al., 2008). Consequently, DMO occurs when, in addition to
vascular leakage, the active transport mechanisms of the blood-retinal barriers are dysfunctional (Mori et al., 2002).

One of the first histological changes observed during diabetic retinopathy is the loss of capillary pericytes (COGAN et al., 1961). Hyperglycaemia followed abruptly by euglycaemia triggers has been shown to cause pericyte apoptosis (Li et al., 1996b). Loss of pericytes correlates with the formation microaneurysms, which are thin outpouchings from the retinal capillary wall (Yanoff M, 2002). Microaneurysms can lead to vascular leakage and retinal thickening and oedema.

Other histological changes in DR include capillary loss, caused by basement membrane thickening and occlusion of the vessel by leukocyte adhesion (Yanoff M, 2002). Capillary loss leads to macular and retinal ischaemia. Although there are no overt clinical manifestations of macular ischaemia, a featureless flat retina, white vessels, deep blot haemorrhages and cotton wool spots all suggest it. Fundus fluorescein angiography definitively diagnoses capillary loss as increased size of the foveal avascular zone and perifoveal capillary loss and is generally associated with retinal hyperpermeability. Experimental ischaemia of the rat retina evokes a biphasic water accumulation in the retina. During ischaemia the oedema is thought to be due to neuronal cells swelling (Uckermann et al., 2004) while during the reperfusion stage it is thought to be due to the glial cells swelling (Pannicke et al., 2004).

The retina is a compact tissue composed of neural and glial cells (Hogan, 1971). Glial cells occupy the interneuronal space and therefore in normal situations the extracellular space is virtually absent. Vascular leakage results in serum protein extravasation and osmotically driven water inflow
into the retinal tissue. The fluid enters the Müller cells causing intracellular swelling in the outer plexiform layer, initially a reversible process (Yanoff et al., 1984; Fine et al., 1981). However, excessive swelling of Müller cells results in their rupture and death to produce pockets of extracellular fluid and cell debris, which is seen clinically as cystoid macular oedema (Yanoff et al., 1984; Fine et al., 1981). Similarly swelling of ganglion cells bodies and their processes, precedes the loss of these cells (Duke-Elder and Dobree, 1967). With the advent of cell death, the visual loss becomes irreversible.

Apoptosis of retinal ganglion cells and inner nuclear layer cells are known to occur very early in the course of diabetes (Barber et al., 1998; Bu-El-Asrar et al., 2004). Although these changes are not detectable by ophthalmoscopy, a reduction in the oscillatory potentials seen in electrophysiological studies in patients with diabetic retinopathy indicates their loss (Simonsen SE, 1969). These changes often precede the onset of microvascular lesions (Parisi and Uccioli, 2001) and predict worsening of retinopathy better than clinical characteristics (Bresnick and Palta, 1987).

They are also critical for understanding why patients with DR lose vision, even if the macular oedema is treated. Clearly, visual loss in DMO is ultimately related to impaired neuronal function and not solely to vascular disease. At the cellular level, diabetes alters the function and structure of all retinal cell types.

In both our randomised controlled trial of intravitreal TA and laser and in our pilot study, we did not show a significant improvement in visual acuity in patients with persistent DMO at any time point. Even in patients who had a significant reduction in central retinal thickness in our RCT (both the TA and laser groups), there was no change in the mean visual acuity (56.6 vs 57.0
ETDRS letters, \( P=0.45 \). The cause of their visual loss must be more than simply DMO. Therefore we conclude that neuronal death or dysfunction was responsible for the lack of improvement in VA in this subset of patients.
4.3 Insights into the therapeutic mechanism of Corticosteroids

Corticosteroids (GCs) have been shown to reduce blood-retinal barrier breakdown (Wilson et al., 1992) and reduce DMO at least in the short-term (Martidis et al., 2002; Jonas and Sofker, 2001), however the mechanism of action of remains obscure. They have an established anti-inflammatory effect since they inhibit the release of arachidonic acid from cell membranes and hence the production of the inflammatory mediators, prostaglandins and leukotrienes. Additionally, glucorticoids down-regulate the inflammatory expression of endothelial adhesion molecules ICAM-1 and VCAM-1 (Penfold et al., 2000), as well as inhibit leukocyte-endothelial interactions in the retina of an experimentally-induced diabetic rat (Tamura et al., 2005).

GCs may also reduce macular oedema by their action on VEGF in vitro and in vivo. HC down-regulates the expression of the VEGF gene in human aortic vascular smooth muscle in culture (Nauck et al., 1998), while TA has been shown to reduce the secretion of VEGF by pigment epithelial cells during oxidative stress (Matsuda et al., 2005). In vivo, an intravitreal injection of TA reduces the vitreal level of VEGF in patients with diabetic retinopathy (Brooks, Jr. et al., 2004b) while dexamethasone reduces VEGF-induced BRB breakdown in an animal model (Edelman et al., 2005).

Furthermore, Uckermann et al (Uckermann et al., 2005) observed that TA had an inhibitory effect on the osmotic swelling of Müller glial cells in diabetic animals following transient ischemia, by altering the secretion of ions via Müller cell end feet into the blood and vitreous.
Antonetti et al (Antonetti et al., 2002) show that hydrocortisone has an effect on the tight junction proteins, with an increase in the occludin content and a decrease in occludin phosphorylation in bovine RMEC in vitro. Our study using rat brain microvascular endothelial cells confirmed this and showed that hydrocortisone preserved and increased junctional levels of occludin, CL5, VEC and ZO-1. In addition we observed a concurrent improvement of endothelial barrier, which was even more marked in the presence of TA. Thus steroids appeared to preserve adherens and tight junction complexes and enhance functionality of vascular barrier breakdown.

Our randomized controlled trial was designed to assess the long-term effects of TA on DMO with follow up visits every of months. In addition to the study protocol, an OCT was performed at 1 week and 1 month in all patients in the study group (results not shown). At 1 month (and 1 week) a reduction in the retinal thickness was observed, which agrees with previous short-term results suggesting that GCs may prove beneficial to control or even normalise the vascular dysregulation seen in DMO/DR.

However, DMO is a complex disease, and many factors have been shown to be involved in its formation. In addition to VEGF, PKC, the GH-IGF-1 and the RAS have all been shown to be involved. Over time, DMO leads to cells death which accounts for the irreversible nature of some visual loss. The role of GCs in the eye for DMO clearly has limitations. In our in vitro study, both hydrocortisone and TA were able to inhibit VEGF-induced permeability but neither steroid was able to inhibit the increase in permeability produced by lysophosphatidic acid (LPA). These results, suggest that steroids can normalise vascular endothelial cells in the short-term, but may not counteract all vasoactive stimuli.
We observed a reduction in retinal thickness in some patients at 1 months, but not all patients showed a complete resolution of DMO. Furthermore there was no significant reduction in retinal thickness following intravitreal TA at any study time-point and only 2 patients in our study group achieved our primary outcome measure of an improvement of 15 or more ETDRS letters. Many of the patient included in this trial had chronic DMO and it could be argued that if we had included patients at an earlier stage in the evolution of the disease then we would have seen an improvement in visual acuity. However, in our experience, TA is usually reserved for patients unresponsive to laser treatment and therefore our study population is likely to reflect the population of patients most widely treated with TA.

However, a reduction of retinal thickness even if short-lived may prove beneficial as an adjunctive treatment for DMO. Avitabile et al noted that TA and laser photocoagulation lead to a decrease in the number of eyes requiring reinjection (Avitabile et al., 2005b), while Shimura et al found that combination therapy lead to a lower intensity of laser spots and also prevents the decrease in central visual field sensitivity (Avitabile et al., 2005a) suggesting that a reduction in retinal thickness may increase the effect and decrease the side-effect profile of macula laser photocoagulation. Studies to assess the efficacy of intravitreal TA as an adjunctive therapy for DMO are currently underway (Diabetic retinopathy clinical research network).
4.4 Potential novel therapies for DMO including anti-VEGF therapies

Anti-VEGF drugs were first used in the eye for their anti-angiogenic effects in the treatment of age-related macular degeneration (AMD) (2002) and have been proven successful at delaying the progression and even at improving VA in patients with AMD (Rosenfeld et al., 2006). VEGF has also been implicated in the development of DMO and neutralisation of VEGF function can reduce hyperpermeability in animal models of diabetes (Ideno et al., 2007; Ishida et al., 2003). Thus targeting VEGF to prevent DMO with VEGF inhibitors such as bevacizumab provides new treatment strategies for DMO. Initial pilot studies have generally been optimistic that they can provide short-term stability or improvement in VA and retinal thickness in patients with DMO (Arevalo et al., 2007; Haitoglou et al., 1992). Again, these studies have been typically small groups of patients with relative short follow-up.

However, not all results have been so positive (Shimura et al., 2008; Paccola et al., 2008) and their application may be limited for example macular ischaemia has negative effect on the visual outcome (Chung et al., 2008). Our studies with intravitreal TA stress the importance of early intervention for DMO. Even in patients who had a response to treatment (as measured as a reduction in retinal thickness), there was no significant improvement in the visual acuity, suggesting that neuronal cell death had already occurred.

Pilot studies with anti-VEGF agents suggest that they may be beneficial in the treatment of DMO. At the present time there are no data from large multicentre randomised controlled trials using such agents and there remains unanswered concerns over anti-VEGF therapies such as its effect on blood pressure (due to the increased vasoconstriction) and renal function proteinuria (due to glomerular dysfunction) even from local intravitreal
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Injections (Simo and Hernandez, 2008; Zhu et al., 2007). At a local level, VEGF blockade may reduce the function of fenestrated vascular beds such as the choriocapillaris (Kamba et al., 2006; Baffert et al., 2006; Peters et al., 2007). VEGF offers neuroprotection to the retina and protects against ischaemia therefore local effects on the neuroretina of VEGF blockage should be closely observed (Nishijima et al., 2007).

Some neurons, such as motor neurons in the spinal cord, are strongly dependent upon VEGF for survival (Oosthuyse et al., 2001; Storkebaum et al., 2005; Wang et al., 2007). VEGF type 2 receptors have been identified on neurons, including retinal neurons, and it has been suggested that their activation may promote cell survival (Jin et al., 2000). A recent report suggested that VEGF antagonists may cause death of retinal ganglion cells (Nishijima et al., 2007).

VEGF and steroids had an opposing effect on our microvascular endothelial cell. Similarly, we showed that steroids were able to inhibit VEGF-induced permeability in vitro. Thus, if inhibition of VEGF shows a similar effect in vivo, they may prove no more efficacious than steroids at reducing retinal thickness and increasing visual acuity in patients with DMO.

Our results suggest that VEGF affects cellular processes which do not directly impinge on permeability (signalling to ZO-1 which does not correlate with permeability). Anti-VEGF treatments have been shown to have an effect on vascular tone and possible effects on the local oxygen supply an ischaemic tissue have to be considered (Ziemssen et al., 2008).

Furthermore Systemic absorption from intravitreal TA is not negligible (Ziemssen et al., 2008). VEGF is a growth factor that has been implicated in
many disease processes but it is important to remember that VEGF also has physiological functions. Data from the 2-year MARINA study shows no increase in the incidence of systemic adverse events associated with anti-VEGF therapy. However, in the pooled 1-year safety results from the MARINA and ANCHOR trials, a slightly higher incidence of myocardial infarction and stroke was noted with higher doses of ranibizumab (Rosenfeld et al., 2006). Patients with DM have a higher risk of cardio- and cerebrovascular disease, and therefore further prospective studies are needed to determine the long-term outcome including safety of anti-VEGF treatment for DMO.

VEGF is involved in many vascular responses including permeability, angiogenesis and migration of endothelial cells. VEGF signalling is clearly complex and interrelated. Identifying how VEGF brings about vasoactive changes including its intracellular signalling pathway are therefore important in order to identify more specific (down stream) targets in its signalling cascade.

By correlating signalling of VEGF to junctions on the one hand and to permeability on the other, we describe an approach which to our knowledge is novel. We provide evidence that it is suitable to identify whether a junctional molecule is likely to be a key mediators of VEGF-induced permeability. Potential targets identified by this method (with high correlation in signalling) can subsequently be verified by direct molecular approaches such as using small interfering RNA (siRNA). We believe that such vigorous testing is required to understand the effect of VEGF onto junctions and the development of more targeted and restricted drugs.
5. Future Work
5.1 Introduction

The aims of this thesis were to improve our understanding of the cellular events involved in DMO, and to assess novel therapies to reverse or limit the damage of this chronic disease. Our studies produced primarily negative results. Although these results provide invaluable information in DMO management, the ultimate aim of the ideal treatment for DMO has yet to be reached. This research has left many questions unanswered for future work and can only be seen as the start to discovering an effective treatment of DMO.

5.2 Laboratory work

Our experiments were limited by the relatively few retinal microvascular endothelial cells (RMEC) we were able to produce. In addition, they lost primary features rapidly in culture and could therefore not be propagated for extended times. Therefore we used brain microvascular endothelial cells (BMEC) to develop our experimental systems before repeated key experiments on RMEC. Due to the time constraints, MEC permeability was only performed on BMEC.

Growing the MEC on filters proved to be a challenge and as a result measurements of permeability were prone to artefact. As a consequence transendothelial electrical resistance across MEC monolayers did not provide any useful information. Additionally, we tried to fix and subsequently stain the MEC used to measure permeability but the attachment of the cells to the filter proved to fragile to survive the fixation process and therefore we were unable to perform immunofluorescence the same cells that showed changes in permeability.
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It is the intention of the laboratory to develop a repeatable method of growing MEC on filters and thus accurate methods of measuring permeability could be performed. It is anticipated that this would allow immunofluorescence of the cell cultures that show a change in permeability and it would also allow the experiments to be repeated on RMEC.

Furthermore, we would like to test the polarity of MEC. We have made some preliminary observations that VEGF application to the basolateral side of MEC was more effective in inducing permeability. This observation has already been reported in the literature (Wang et al., 2001) and suggest that MEC are functionally polarised. It is known that epithelial cells are polarised with receptors such as EGFR on only one surface. In addition TJ are formed more apically than AJ. However in endothelial cells TJ and AJ interdigitate and the polarity of retinal endothelial cells has not previously been explored. During the work of this thesis we attempted to test this hypothesis further, but, we realised that the cultures contained microscopic gaps and areas of TJ breakdown. Thus monolayers were unlikely to be homogenously polarised. Future work should concentrate on obtaining better cultures to persue this aim.

We would also like to continue the work with inhibitors of the signal transduction pathway. Until now we had to rely on work from the literature about VEGF-induced permeability and compare this against VEGF-induced ZO-1 loss. A better comparison is to compare VEGF-induced permeability with VEGF-induce TJ or AJ protein loss within our own cell systems.

Clearly, the aim here is to identify a specific intracellular signal transduction pathway that is critical for VEGF-induced permeability. Inhibitors of this
pathway could ultimately be utilised to prevent macular oedema without systemic complications which may limit its use.

The scope of the work of this thesis was limited to microvascular endothelial cells (MEC) taken in isolation. The BRB consists of many cells that function in tandem and therefore co-culture with other cell types such as pericytes may provide a better in-vitro model of the BRB.

Similarly some of our experimentations could be performed on an animal model. For example BRB permeability on diabetic and control rats can be measured using intravenous injection of fluorescent markers and analysed by digital image analysis of retinal sections. Additionally changes in the junctional proteins could be measured by immunofluorescence or immunoblotting retinal homogenates.

We assayed the amount of VEGF in the vitreous (and aqueous) of patients undergoing PPV. This consists of all isoforms of VEGF. It would be interesting to assay the individual isoforms of VEGF and in addition to look at the levels of other growth factors that have been implemented in the pathogenesis of DMO such as Insulin-like growth factors.

5.3 Clinical Work

We showed a negative effect from TA for the treatment of persistent DMO over 12 months. However, our work and work from previous trials have shown that intravitreal TA can reduce macular oedema in the short term. This effect may allow other therapies to be more effective in the long term;
for example the use of macular photocoagulation to a thinner macula than a macula with gross macular oedema.

In our study we recruited patients with persistent DMO. Other research tends to suggest that therapies used earlier in the evolution of DMO may offer better chance of improvement or stabilisation of VA. However, as we found very little change either the VA or retinal thickness of persistent DMO with TA alone, a second study using TA as a monotherapy for untreated DMO does not seem logical and a study of this nature is probably better conducted with anti-VEGF agents such a Bevacizumab.

The ideal treatment for DMO is one which targets the tissues that influence DMO but does has limited effects on the surrounding tissues. Additionally as DM and its complications is a chronic condition, it needs therapeutic agents that are easy to administer over the long-term. Furthermore, DR is an end organ disease and therefore the ideal treatment needs to prevents vascular changes rather that reverse them. Therefore an oral medication which would prevent other end organ damage (such as protein kinase C inhibition or perhaps fenofibrate ) or topical eye drop that can penetrate through to the posterior segment and provide vascular protection on a daily basis would be beneficial.

The evolution of DMO with OCT would provide invaluable information about DMO and would allow us to examine its relationship with both VA and ischaemia. A longitudinal long term study looking a the patterns of OCT in a cohort of patients attending diabetic retinal screening and then following the appropriate patients through referral to ophthalmology clinics could offer a better understanding of the evolution of the disease and any treatment effect.
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7. Appendix
7.1 **Negative controls of antibodies**

Microvascular endothelial cells (RMEC and BMEC) were grown to confluence and then fixed with methanol or formaldehyde and then stained with secondary antibodies and with hoescht. Images were generated using the same settings as the postively stained images (fig 7.1) (BMEC not shown).
Figure 7.1: **Negative controls of RMEC:** Retinal microvascular endothelial cells (RMEC) were grown to confluence and then fixed with methanol or formaldehyde and then stained with anti–goat FITC conjugated (a), anti-rabbit rhodamine conjugated (b), anti–mouse cy3 conjugated (c) and anti-rabbit FITC conjugated (d) and with hoescht. Images were generated using the same settings as the postively stained images.