An investigation of vitreous biomarkers associated with proliferative vitreoretinopathy development

By

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Abstract (200 words)

Introduction

During a rhegmatogenous detachment, all non-neuronal cell types undergo proliferation, resulting in PVR formation in 5-10% of cases. PVR remains the most common cause of surgical failure and poor visual prognosis[1,2]. Numerous surgical adjunctive agents to prevent post-operative PVR have been evaluated with limited success. Correct identification of pathogenesis and predictive biomarkers would identify therapeutic targets and allow the development of an accurate predictive model for PVR to inform management decisions.

Methods:

Design: (1), prospective observational clinical study evaluating potential predictive biomarkers of PVR on luminex cytokine analysis and metabolomic analysis of vitreous; (2), cell culture study of the fibrogenic potential of identified biomarkers on human RPE cells

Outcome measures: (1), development of PVR; (2), expression of collagen and epithelial-mesenchymal transition by RPE cells in vitro.

Results: IL-1 β and IL-7 predict PVR development. The metabolomic predictors of PVR such as 2-Hydroxyvalerate and 2-Phosphoglycerate are involved in amino acid and glutamate metabolism. IL-6, PDGF and VEGF did not have affect RPE cells fibrotic phenotype *in vitro*.

Conclusions: Cytokine biomarkers that predict PVR are inflammatory cytokines IL-1β and Il-7, with identified metabolomic changes consistent with these cytokine systems, therefore representing potential therapeutic targets. Inflammatory cytokines IL-6, VEGF and PDGF do

not directly act on RPE cells in vitro to cause fibrosis, highlighting the important role of other retinal cells and the immune system in PVR development.

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Abbreviations

ASCOT Adjunctive Steroid Combination in Ocular Trauma
AqH aqueous humour
BMI body mass index
BDGF brain-derived nerve factor
CCL22 chemokine C-C motif ligand 22
DAMPs damage-associated molecular patterns
dsDNA Double-stranded DNA
dNTPs deoxynucleoside triphosphates
EGF epidermal growth factor
ECM extracellular matrix
EMT epithelial-mesenchymal transition
FGF fibroblast growth factor
GMT glial-mesenchymal transition
Gp130 glycoprotein 130 kDa
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GCL ganglion cell layer
GABA γ–aminobutyric acid
IL interleukin
IFF inflammatory/fibrotic factors
ICAM-1 intercellular adhesion molecule-1
IS inner segment
IGF-1 insulin-like growth factor 1
ILM internal limiting membrane
IPL inner plexiform layer
JAK janus kinases
MH macular hole
MIF macrophage migration inhibitory factor
NGF nerve growth factor
NT3 neurotrophin 3
NMR nuclear magnetic resonance
NLRP3 NOD-like receptor family and pyrin-domain-containing-3
OPLS-DA orthogonal projection to latent structures discriminate analysis
OCT optical coherence tomography
ONL outer nuclear layer
OLM outer limiting membrane

OPL outer plexiform layer
OS outer segment
PCR polymerase chain reaction
PVR proliferative vitreoretinopathy
PVD posterior vitreous detachment
PDR proliferative diabetic retinopathy
PCA principal components analysis
PLS-DA partial least square discriminant analysis
PQN probabilistic quotient normalization
qPCR quantitative real time PCR
RGC Retinal ganglion cells
RPE retinal pigment epithelium
RRD rhegmatogenous retinal detachment
RIPK Receptor interacting protein kinase
STAT signal transducer and activator of transcription
SRF subretinal fluid
ssDNA single-stranded DNA
TRAIL tumour necrosis factor related apoptosis inducing ligand
tPA tissue plasminogen activator
TIMP tissue inhibitor of metalloproteinase
TGF-β2 transforming growth factor β2
TMSP trimethylsilyl 2,2,3,3-tetradeuteropropionic acid
TNF tumour necrosis factor
VCAM-1 vascular cell adhesion molecule-1

CHAPTER 1

INTRODUCTION

SECTION 1A

THE ANATOMY OF THE EYE

The eye can be viewed as having two principal anatomical segments: the anterior segment and the posterior segment. The anterior segment includes the cornea, iris, lens and the aqueous humour. The posterior segment is the back two-thirds of the eye that includes the vitreous gel, choroid, sclera, optic nerve and retina (refer to **Figure 1.1**). The posterior segment is bounded anteriorly by the lens and extends to the back of the eye. It is much larger than the anterior segment. Vitreoretinal disorders, such as proliferative vitreoretinopathy (PVR) primarily affect the posterior segment of the eye. This section will concentrate on describing the anatomy of the structures involved in PVR.

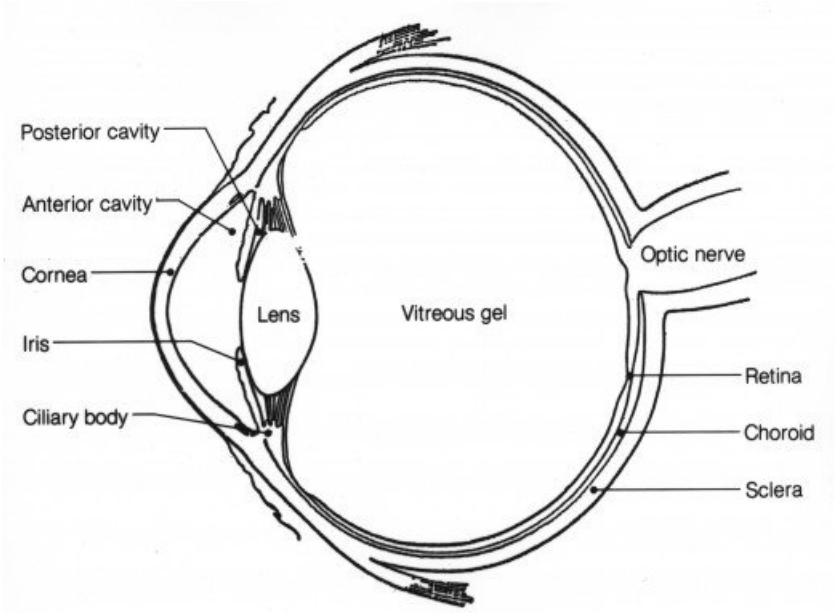

Figure 1.1 Cross-section of the human eyeball*. Taken from https://owlcation.com/stem/Anatomy-of-the-Eye-Human-Eye-Anatomy*

THE VITREOUS

(i) Anatomy

The vitreous cavity is a space defined within the eye between the lens and its zonular fibres anteriorly and the ciliary body, retina and optic disc posteriorly. In humans its volume is generally around 4ml. The volume can be greater in larger myopic eyes to as much as 10ml. The cavity is entirely occupied by vitreous gel. The vitreous gel is a virtually acellular viscous fluid with 99% water content. Its low molecular and cellular content is essential for the maintenance of transparency. The main constituents of the vitreous gel are hyaluronic acid and type 2 collagen fibrils. The cortical part of the vitreous gel has a higher content of these constituents compared with the less dense core gel. Centrally there is a tubular condensation called Cloquet's canal. This is a remnant of the primary vitreous, stretching between the lens anteriorly and the optic disc posteriorly.

There are gel condensations within its substance and along its boundaries. The boundary concentrations are called the anterior and posterior hyaloid membranes. The posterior hyaloid membrane is adherent to the internal limiting membrane (ILM) of the retina by the insertion of vitreous gel fibrils. The ILM has type IV collagen and constitutes the basement membrane of the Muller cells. During a posterior vitreous detachment (PVD) the plane of cleavage of the gel from the retina is between the ILM and the posterior hyaloid membrane. The process of PVD will be described in further detail in **Section 1B**.

(ii) The anatomical relevance of vitreous in vitreoretinal pathology

The vitreous has areas of strong adhesion to the surrounding structures. These adhesions form the basis of vitreoretinal pathology. The vitreous base is an annular zone of adhesion approximately 3 to 4mm wide that straddles the ora serrata. Its anterior border is the site of insertion of the anterior hyaloid membrane. The posterior border is the common site for retinal tear formation. Adhesion of the vitreous base to the retina and the pars plana is difficult to break, even with severe trauma.

Other areas of strong adhesion include Weigert's ligament, a circular zone of adhesion 8 to 9mm in diameter, between the gel and posterior lens capsule. It is the junction between the anterior hyaloid membrane and the expanded anterior portion of Cloquet's canal. The macroscopic anatomical landmarks are shown in **Figure 1.2**.

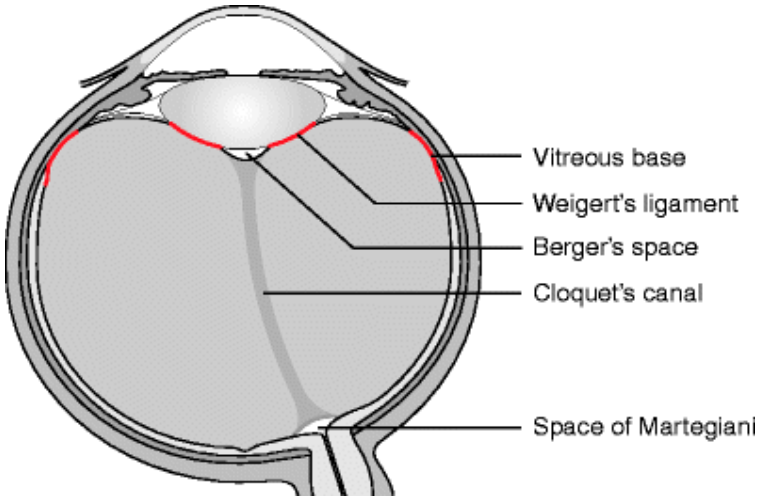

Figure 1.2. Anatomical landmarks in relation to vitreous humour*. Taken from https://entokey.com/anatomy-and-clinical-examination-of-the-eye/*

There is a ring of adhesion around the margin of the optic disc. During a PVD, gliotic tissue is avulsed from the edge of the optic nerve head to produce a Weiss ring that can be visible during biomicroscopy as an indicator of a PVD. An area of increased adhesion is also

present at the retina in the parafoveal region, which can result in macular hole (MH) formation, shown in **Figure 1.3**.

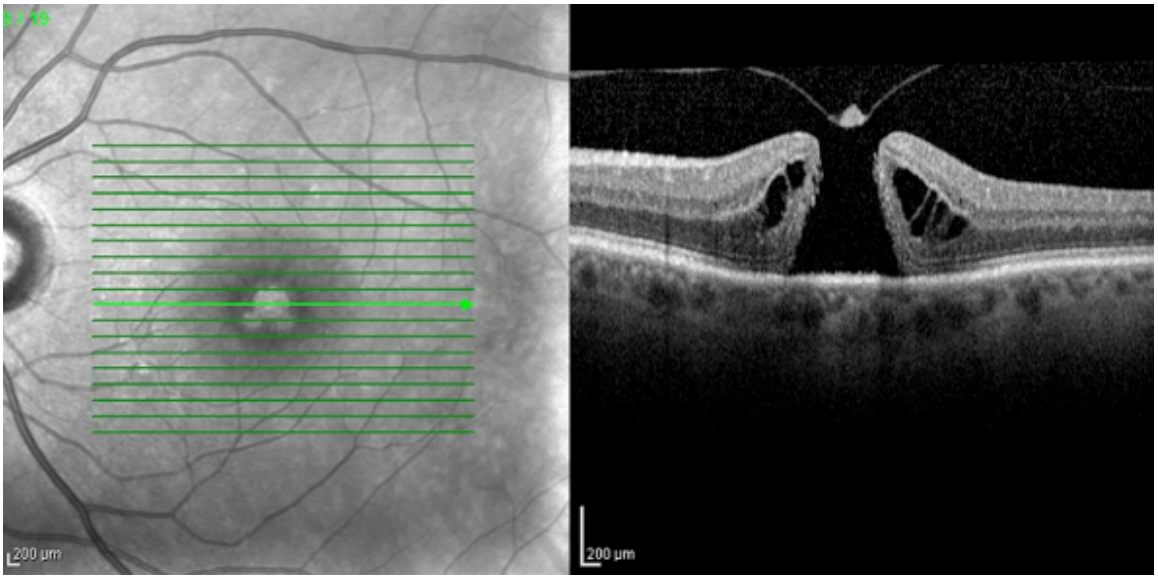

Figure 1.3. Optical coherence tomography showing a full thickness macular hole. *Taken from https://visionandsurgery.com/eye-conditions/macular-hole/*

Lattice degeneration are areas of retinal thinning and vascular sclerosis in the peripheral retina with overlying degenerative vitreous gel. The lesions are normally circumferential and are found in approximately 7% of normal eyes. They are frequently associated with tearing of the retina due to vitreoretinal adhesion. Some eyes have strong vitreoretinal adhesions along the course of the retinal veins. Paravascular adhesions may result in retinal tear formation.

(iii) Role of the vitreous

The vitreous plays a role in oculogenesis. After oculogenesis the vitreous has no well-substantiated function. The gel is not required to maintain the shape or structure of the

eye. An eye devoid of gel is not adversely affected under normal physiology; however, an eye without vitreous gel is at risk of nuclear sclerotic cataract and weaker lens zonules, which may result in lens dislocation. The exact mechanism of this development is unknown; although the vitreous is thought to reduce the oxidative stress on lens fibres and thus reduce cataractogenesis as the oxygen concentration remains low within the vitreous. The vitreous is involved in the development of sight-threatening conditions, including retinal detachment, due to the anatomical features described earlier.

(iv) Physiology of the vitreous

Molecules move around the vitreous gel due to diffusion and convection and by the effects of saccades on the fluid component of the gel. Molecules with an anionic charge (e.g. Chloride, lactate, ascorbate) move more easily in the gel compared to those with a cationic charge. Convection is estimated to account for 30% of movement of molecules in the vitreous because there is a pressure differential from the anterior ingress of aqueous from the ciliary body to the posterior egress of fluid through the retina by the retinal pigment epithelium (RPE) pump. The saccadic movement of the eyes also induces convection currents in the anterior vitreous that circulate around the vitreous base anteriorly because of the effect of the indentation of the lens into the vitreous cavity.

The viscosity of vitreous is approximately 5 to 2000 mPas (aqueous = 1 mPas). The vitreous is relatively hypoxic (pO_2 of 30 to 40 mmHg). The relatively small proportion (2 to 3%) of ocular blood supply to the retina from the central retinal artery has a profound effect on the vitreal PO_2. There are oxygen gradients in the vitreous, with higher PO_2 in

the posterior vitreous than the anterior. Ascorbate concentrations are high in the vitreous[3]. Ascorbate enters the eye through active transport from the blood by the ciliary epithelium. The concentration of ascorbate in the vitreous is maintained by a sodium-dependent ascorbate transporter (SLC23A2) in the pigmented layer of the ciliary epithelium[4]. The ascorbate may react with oxygen to reduce the PO_2, decreasing the exposure of the lens to oxygen. Exposure of the lens to oxygen promotes the progression of cataract formation. The lack of ascorbate in vitrectomised eyes means lower oxygen consumption causing increased oxygen levels and the rapid development of cataract[4].

THE RETINA

(i) Retinal anatomy

The retina is a light sensitive tissue that comprises neuronal and glial cells. Following light absorption, the visual cycle is activated. The visual cycle is a process by which light is converted into electrical signals in the rod cells, cone cells and photosensitive ganglion cells. The visual cycle is the biological conversion of a photon into an electrical signal in the retina, which is transmitted from the eye in the optic nerve. **Figure 1.4** shows a cross-section of an eye with a schematic enlargement of the retina.

The human retina is further subdivided into a central and peripheral retina. The central retina comprises the macula lutea, which contains lutein, a yellow pigment to filter out short wavelength light to prevent chromatic aberration. The macula is a region of the retina that provides 20 degrees of visual field. This is further subdivided into an area of

maximal cone density, the fovea centralis. The cone density is 15,000/mm^2 at the fovea, with 4,000 to 5,000mm^2 at the macula. This makes the macula an area of greater spatial resolving power than the peripheral retina. There are 6 million cones and 120 million rods in total within the retina. The macula is cone-dominated, whereas the peripheral retina is rod dominated.

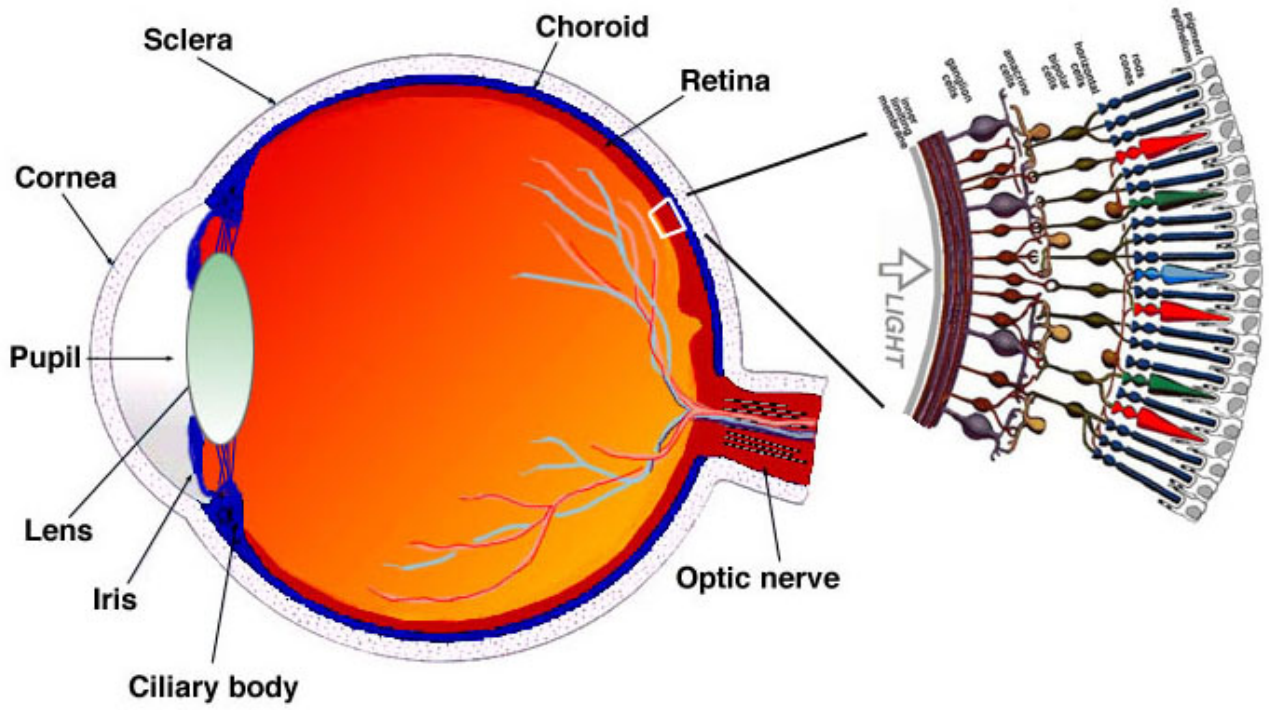

Figure 1.4. Schematic enlargement of the retinal layers. *Taken from* http://webvision.med.utah.edu/book/part-i-foundations/simple-anatomy-of-the-retina/

(ii) Morphological subdivision of the retina

The human retina consists of ten layers, shown in **Figure 1.5** that extends from the outer retina adjacent to the RPE to the inner retina.

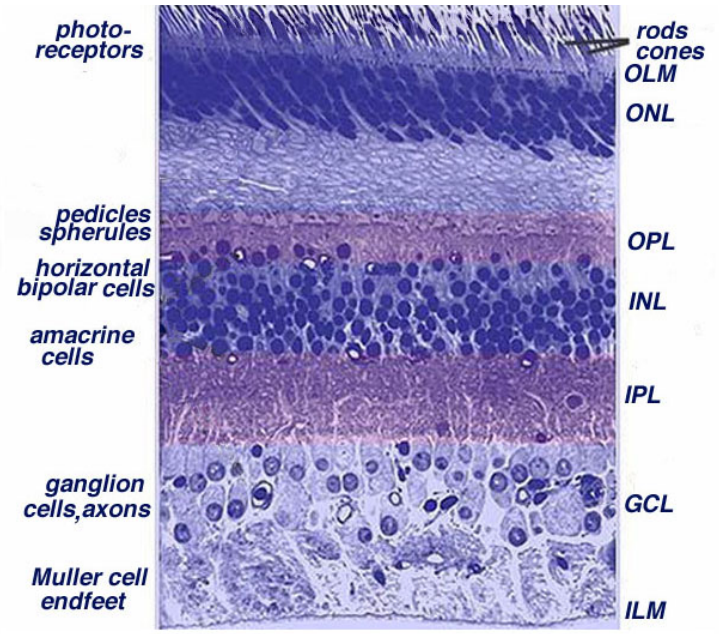

Figure 1.5. Toluidine-blue stained (0.5-1µm) histological cross-section of the human retina*. Taken from http://webvision.med.utah.edu/wp-content/uploads/2011/01/husect.jpeg*

Neuronal cells

(i) Photoreceptors

The outer retina comprises the light-sensitive rod and cone photoreceptors. Both types of cell contain outer segments (OS) that interact with the RPE layer. The OS contain discs composed of visual pigments (opsins) and are continually shed and processed by the RPE. The visual pigments are responsible for light absorption and initiation of the neuroelectrical impulse. Each OS is connected *via* a modified cilium to an inner segment (IS) that contains a high density of mitochondria. The OS and IS are separated from the cell body by the outer limiting membrane (OLM). The photoreceptor cell bodies are located in the outer nuclear layer (ONL). Processes extending from the photoreceptors whose cell bodies are in the ONL form synapses at the outer plexiform layer (OPL)[5].

(ii) Bipolar cells

Bipolar cells are second order neurons that transmit signals between photoreceptors and ganglion cells. The cell bodies are in the inner nuclear layer (INL). Single or multiple dendrites synapse with photoreceptors and horizontal cells. Their single axons synapse with ganglion or amacrine cells.

There are two types of bipolar cells, ON centre and OFF centre, which differ in function. Under scotopic conditions, release of the neurotransmitter, glutamate, from photoreceptors, maintains hyperpolarisation of ON centre bipolar cells and a depolarized state of OFF centre bipolar cells. This situation reverses under photopic conditions, with depolarisation of ON centre bipolar cells[6].

(iii) Interneurones

Horizontal cells, of which there are three types (HI, HII and HIII), are the interneurons of the INL. HI cells connect to all three cone subtypes (S, M and L cones), whereas HII cells connect to both rods and cones. HIII cells form connections to S cones only. Together with ON- and OFF-bipolar cells, horizontal cells have a role in retinal processing and lateral inhibition, which is achieved by release of mainly γ–aminobutyric acid (GABA)[7].

Amacrine cells are located in the INL and GCL, where they act as interneurons between bipolar cells and ganglion cells. The dendritic processes synapse within the inner

plexiform layer (IPL). There are approximately 40 subtypes of amacrine cells based on the stratification of dendrites in the IPL, shape and their neurotransmitters. These cells play a role in modulation of signals reaching ganglion cells[8].

The function of interplexiform cells is to communicate information between the two plexiform layers of the retina using either GABA or dopamine as a neurotransmitter[9].

Retinal ganglion cells

Retinal ganglion cells (RGCs) are located at the inner retina within the ganglion cell layer (GCL). These cells receive input from the photoreceptors *via* bipolar and amacrine cells. RGC axons form the optic nerve that projects to visual centres in the brain, forming synapses in the thalamus, hypothalamus and midbrain[10].

Glial cells

(i) Müller cells

Müller cells are glial cells arranged in a scaffold network that protect the underlying retinal architecture. Müller cell components at the inner retinal surface lie adjacent to the inner limiting membrane (ILM) in an expansion of 'endfeet'. Their outer limit terminates at the outer limiting membrane (OLM) of the retina, comprising tight junctions between these cells and photoreceptor inner segments[11].

Aside from their role in providing anatomical support, Müller cells may also be responsible for maintaining the avascular outer retina, involving water and pH homeostasis, production of vitreal collagens, stimulating glycogenolysis, and in the removal of extracellular K+ to the vitreous[12]. Müller cells also have an optical role in providing a low-scattering passage for light from the retinal surface to the photoreceptor cells[13].

(ii) Astrocytes

Astrocytes comprise the other type of retinal microglia and are so-named due to their characteristic star shape. Predominantly found in the nerve fibre layer, GCL, IPL and INL, astrocytes provide nutritional and metabolic support to the retinal ganglion cells[14]. As they are intimately associated with retinal blood vessels, astrocytes are thought to have a role in maintaining the blood-retinal barrier[15].

(iii) Microglia

Microglia are small glial cells distributed throughout the retina from the nerve fibre layer to the OPL. Possessing phagocytic activity, activation of microglia under physiological or pathological conditions can result in their release of a number of growth factors, including brain-derived nerve factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT3), in addition to the cytokines, tumour necrosis factor (TNF)-α and interleukin (IL)-6[16].

Retinal pigment epithelium

Located in the posterior part of the eye, the RPE comprises a monolayer of polarized cuboidal/columnar epithelial cells from the margins of the optic disc to the ora serrata. The RPE has a major role in the phototransduction cascade[17] and in the phagocytosis and renewal of photoreceptor outer segments[18]. The RPE and tight junctions selectively transports ions, fluid, nutrients, and metabolic waste products between the retina and the choriocapillaris[15]. By this action, it maintains the integrity of the outer retina by regulating the composition of the extracellular spaces that face the RPE apical and basolateral membranes[15].

SECTION 1B

THE DEFINITION OF PROLIFERATIVE VITREORETINOPATHY

Proliferative vitreoretinopathy (PVR) describes an accentuated retinal scarring process that occurs in 5-10% of rhegmatogenous retinal detachment (RRD) cases and is the main cause of surgical failure[19]. Penetrating or blunt trauma may also result in proliferative vitreoretinopathy. Clinically, PVR is characterised by the growth and contraction of cellular fibrotic membranes within the hyaloid and retina and occurs on both the inner and outer retinal surfaces. Contraction of these membranes causes foreshortening of the retina, leading to stretch holes or traction, which redetaches the retina. As the proliferation matures, the once compliant retinal tissue becomes rigid and immobile, making repair more difficult. Though the clinical manifestations of PVR are wide, they share a common underlying pathology and can be considered as the end-point of a number of intraocular processes associated with RRD and vitreous changes. It is distinct

from the fibrous proliferation associated with proliferative diabetic retinopathy (PDR)[20]. For example, fibronectin levels are higher in PVR compared to PDR membranes[21], with greater retinal and immune cell proliferation[22]. Eyes with either pre-existing or established PVR are at a higher risk of increased retinal inflammation and fibrosis after repeated vitreoretinal surgery[23,24].

Proliferative vitreoretinopathy has been referred to by various nomenclatures[25]. It was originally referred to as massive vitreous retraction, based on its ophthalmoscopic appearance (See **Figure 1.6**) and then massive periretinal proliferation, based on its histologic findings. Figure 1.6 shows tractional preretinal and subretinal membranes running across the macular region.

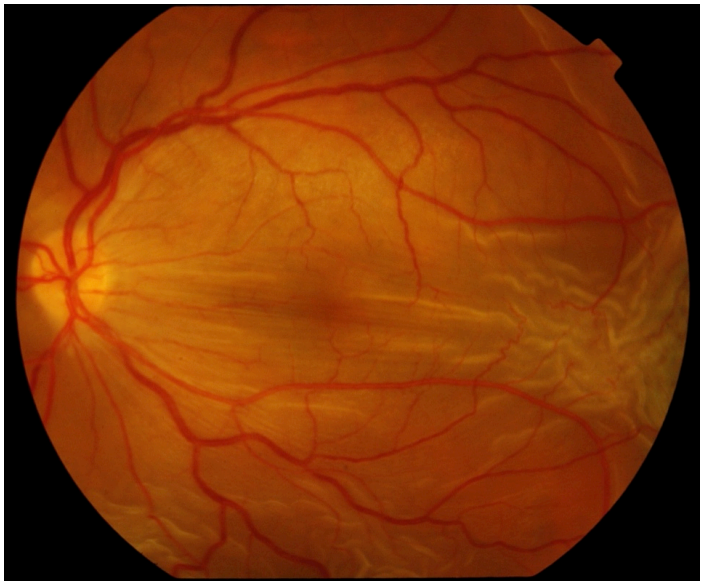

Figure 1.6. Appearance of PVR on biomicroscopy*. Taken from http://www.snec.com.sg/eye-conditions-and-treatments/common-eye-conditions-and-procedures/Pages/proliferative-vitreoretinopathy.aspx*

The 1983 Retina Society classification of PVR was amended in 1989 by the Silicone Oil Study Group2 to its current designation: proliferative, to denote proliferation of retinal

pigment epithelium cells, as well as fibrous metaplasia of cells derived from the RPE and glial cells; and vitreoretinopathy, to refer to the structures affected, namely the vitreous and retina.

SECTION 1C

PATHOGENESIS OF PROLIFERATIVE VITREORETINOPATHY

PVR development is characterised by a sequence of distinct cellular and trophic responses that are described in the sections set out below. Retinal ischaemia develops immediately after retinal detachment, followed by progressive photoreceptor apoptosis and contraction of fibrotic epiretinal membranes[26]. PVR retinal fibrosis is initiated by fibroblasts derived from RPE cells that undergo epithelial-mesenchymal transition (EMT) and begin collagen and extracellular matrix (ECM) deposition[27], orchestrated by a dysregulated panel of pro-inflammatory, chemotactic cytokines and mitogenic growth factors[26], which induce an exaggerated inflammatory reaction at sites of retinal tears and detachment[28]. The early identification of inflammatory/fibrotic factors (IFF) that predict the subsequent development of PVR and direct treatments aimed at impeding/inhibiting PVR development after retinal re-attachment surgery would constitute a significant clinical advance.

(i) Ischaemia

In the holangiotic human retina, the inner 2/3 and outer 1/3 of the retina are supplied by retinal vessels and diffusion through the RPE from choroid plexus vessels, respectively[29]. After retinal detachment, the inner retina remains perfused, but the outer retina

immediately becomes ischaemic with consequent blood-retinal barrier (BRB) breakdown[30,31]. Approximately 20% of photoreceptors die by necrosis, caspase-dependent apoptosis and necroptosis after 3d of retinal detachment and >50% by after 28 days[32,33] and the structural changes associated with macula-off retinal detachment exacerbate the ensuing reduced vision[34,35]. Receptor interacting protein kinase (RIPK1 and RIPK3) mediate the principal photoreceptor cell death signalling pathways when caspases, particularly caspase-8, are inhibited by the pan-caspase inhibitor Z-VAD after retinal detachment[36]. PVR pathogenesis involves ischaemic processes driving the up-regulation of angiogenic and inflammatory growth factors and cytokines[37]. Inflammation triggers ischaemia-induced angiogenesis and fibrogenesis. The severity of retinal detachment correlates with the extent of BRB breakdown and the presence of IFF[38-40].

(ii) Inflammation

Serum factors released into the vitreous, such as thrombin, stimulate the inflammatory phase of PVR development[41]. The development of PVR subretinal and epiretinal membranes is associated with vitreal accumulation of inflammatory cells[42] including a significant elevation CD163/CD206-expressing M2 macrophages[43-45]. Activated peritoneal macrophages injected into the vitreous of the rabbit trans-differentiate into fibroblast-like cells and initiate intra-retinal fibrosis similar to that seen in PVR[46]. Macrophages clear retinal debris, alter vitreal structure through matrix protein-proteolysis and secrete fibroblast growth factor (FGF) and transforming growth factor beta (TGFβ) which stimulate the accumulation and proliferation of fibroblast-like-cells within the incipient PVR epiretinal membranes[47,48]. T helper cells have both pro-fibrotic

and anti-fibrotic potential, demonstrated by the release of pro-fibrogenic cytokines such as interleukin-10 (IL-10), FGF2, platelet-derived growth factor (PDGF), TGFβ and vascular endothelial growth factor (VEGF)[49-51], as well as anti-fibrotic interferon-gamma (IF-γ), which inhibits collagen synthesis *in vitro*[52]. Vitreous cytokine changes in early PVR suggest the importance of T helper responses in early PVR, with T helper (TH) cells identified in vitreous and PVR membranes, with both TH1 and TH2 implicated, although in immunocompromised mice lacking antigen-specific T and B cell responses, intravitreal dispase still induces PVR[53,54].

(iii) Apoptosis

Apoptosis balances cell proliferation with cell loss and is mediated through either intrinsic or extrinsic signalling pathways initiated by intracellular death receptor-binding[55]. Apoptosis shares a number of PVR pathogenetic signalling pathways. For example, TGFβ up-regulates the survival of RPE cells, induces proliferation and down-regulates the death-inducing signalling molecule FasL, blocking T cell-mediated apoptosis[56,57]. Pro-apoptotic Fas and tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) are both up-regulated in the vitreous after retinal detachment and in established PVR, while TRAIL mRNA levels were significantly correlated with anti-apoptotic TGF-β2 titres, no correlation was found between TGF-β2 and Fas mRNA levels, although TUNEL measures of apoptosis did correlate with TGFβ levels[58]. Fas ligand receptor binding activates the extrinsic pathway of apoptosis in proliferating, but not in non-proliferating RPE cells[58,59]. The FasL/Fas system therefore has a probable role in removing excess RPE cells after retinal detachment and, may predispose to PVR when

defective[58]. Fas ligation also increases intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in non-ocular endothelial cells *in vitro*[60]. Soluble ICAM-1, soluble VCAM-1 and FasL and Fas are raised in the subretinal fluid (SRF) of RRD eyes with established PVR and in those that develop PVR later[61,62]. Levels of soluble forms of ICAM-1 and VCAM-1 are up-regulated at 7d, but not 28d after experimental retinal detachment in rats, consistent with their early role in recruiting immune cells[63]. Thus, vitreous levels of ICAM-1 and VCAM-1 are both associated with inflammation and may be up-regulated by apoptotic signalling in photoreceptors and RPE cells[64], but their inconsistent appearance in PVR makes both factors unlikely predictive PVR molecular biomarkers.

(iv) Cell migration and proliferation

After retinal detachment, PVR is initiated by TGFβ-activated RPE cells, which undergo EMT and form multi-layered dedifferentiated cell groups that migrate into the vitreous through breaks in the detached retina, with some evidence that Müller glia also undergo glial-mesenchymal transition (GMT) under the influence of TGFβ[65,66]. Fibroblasts in PVR membranes may therefore be derived from EMT-transformed RPE cells, GMT-transformed Müller glia and circulating fibrocytes[67]. Epiretinal membranes have an acellular collagenous core and layers of transformed and untransformed RPE cells, proliferating Müller glia, and IL-2 receptor$^+$ (IL-2R$^+$) T lymphocytes and macrophages[68,69]. Inflammatory growth factors and cytokines stimulate ECM formation, while plasma fibronectin induces the deposition of a fibroblast-derived collagen matrix and the production of locally synthesised fibronectin, thrombospondin and other proteoglycans[70] and the ensuing mature ECM regulates RPE and inflammatory cell migration[71]. RPE cells

respond to retinal detachment by proliferating and switching to an ECM and pro-fibrotic secretory phenotype[65,72]. Müller glia also proliferate and secrete ECM and pro-fibrotic and inflammatory mediators[73,74]. Annexin AII is a Ca2+-dependent phospholipid-binding protein that regulates RPE-phagocytosis of photoreceptor outer segments and is expressed in photoreceptor apoptosis[75], but also interacts with tPA to promote ECM degradation and is necessary for vitreal RPE cell migration in PVR[76,77]. Paracrine insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) stimulate tissue plasminogen activator (tPA) expression, which regulates ECM turnover by converting plasminogen to plasmin[78], activating pro-collagenase and initiating ECM degradation[79]. ECM degradation may release FGF-2 and TGFβ sequestered in the ECM, opposing further degradation and stimulating proliferation and ECM secretion[80].

(v) Wound healing and contraction

After retinal detachment, transformed cells in PVR membranes differentiate into myofibroblasts[65,66]. Alpha-smooth muscle actin intermediate filament synthesis is stimulated by IL-1 and contraction in myofibroblasts is mediated by Annexin A2 , exacerbating retinal detachment and releasing streams of RPE cells into the vitreous[81]. Such contractile activity measured by tissue culture assay reduces with both age and at longer times after initial diagnosis of retinal detachment, suggesting that activity is transient after retinal detachment but, nonetheless correlates with subsequent PVR development[82].

SECTION 1D

WHAT IS THE RELEVANCE OF PROLIFERATIVE VITREORETINOPATHY?

The primary cause of failed retinal detachment surgery is proliferative vitreoretinopathy (PVR). This is a sight-threatening condition affecting approximately 5 to 10% of cases. Despite advances in surgical techniques for detachment surgery, the incidence of re-detachment due to PVR is similar to that found in the early 1980s[83]. The presence of PVR both pre-operatively and post-operatively has an adverse effect not only on the surgical outcome but also on the final visual acuity achieved in successful cases[84-86]. PVR does not only occur as a complication in RD. It can develop in other ocular pathologies, such as penetrating globe trauma[87] or post-macular translocation surgery[88]. PVR management is currently restricted to surgery. Surgical reattachment success rates are reduced to 60-70% in eyes with PVR[89], compared to 85 to 90% in those without PVR[90,91]. Due to the unsatisfactory anatomical and functional success rates, new pharmacological techniques are under research based on the pathology of PVR but there is limited understanding into the biological processes and mediators involved in PVR development, limiting viable treatment options. A better understanding of PVR pathogenesis is necessary to develop new treatment strategies and as a result improve patient and surgical outcomes.

Pharmacological agents that have been proposed for the treatment of PVR have not been routinely used in clinical practice. Many of the drugs have shown promising results in animal studies but not the same drug efficacy in human studies. These pharmacological treatments have included anti-inflammatory, anti-proliferative, anti-neoplastic, anti-

growth factor and antioxidant agents, many of which have undesirable side effects. Therefore, ideally treatment should be reserved for those patients at high-risk of PVR. Identifying these patients poses a problem, despite previous research into preoperative clinical risk factors, intraocular biomarkers and genetic background. Patient identification would allow for earlier treatment before the development of advanced PVR, improving anatomical and functional outcomes. Early risk identification can be useful for surgical planning, possibly by using less traumatic surgical procedures to prevent inducing further reactive PVR.

The management and treatment of PVR create costs to the healthcare system. Patients that develop grade C (refer to **Section 1G** for grading of PVR) or above PVR post-operatively cost twice as much than a case without PVR due to a higher number of surgical procedures and more frequent and lengthy follow-up. Eyes with anterior PVR pose a greater burden to the patient and the healthcare system. The mean number of operations in an eye with grade C and anterior PVR is 1.7. Eyes with grade C PVR, but with no anterior involvement have a mean number of 1.3 operations.

PVR is an important area for further research in order to achieve more favourable and cost-effective results, diminishing the burden on the healthcare system and improving patient outcomes. Identifying those patients at high-risk of PVR who could benefit from current and future treatment strategies could reduce generalised exposure to harmful side effects and reduce costs. Future treatment approaches could involve targeting different signalling pathways involved in the disease process, requiring a greater

understanding and further investigation into the pathophysiology of PVR in order to achieve this.

SECTION 1E

CLASSIFICATION OF PROLIFERATIVE VITREORETINOPATHY

PVR was identified as an independent clinical entity in 1983 by the Retina Society Terminology Committee and a classification was created. Classification of PVR is based on the ophthalmoscopic appearance, the subjective evaluation of the amount of membrane contraction and its distribution[92]. The classification is not related to the evolution of PVR. PVR becomes clinically significant if it predisposes a RD case to surgical failure, causing a re-detachment or limiting visual recovery post-operatively. The initial classification subdivided PVR into four stages A, B, C and D from minimal to massive PVR (refer to ***Table 1.1***). However, this classification did not consider the location of the vitreoretinal traction and the magnitude of the contraction.

Retina Society Terminology Committee (1983)	
Grade	Clinical Signs
A (minimal)	vitreous haze and pigment clumps
B (moderate)	surface retinal wrinkling, rolled edges of the retinal, retinal stiffness and vessel tortuosity
C (marked) • C-1 • C-2 • C-3	full thickness fixed retinal folds in: • one quadrant • two quadrants • three quadrants
D (massive) • D-1 • D-2 • D-3	fixed retinal folds in four quadrants that result in: • a wide funnel shape; • a narrow funnel shape; • closed funnel without view of the optic disc

Table 1.1. Retina Society Terminology Committee (1983) classification of PVR[92].

In 1989 the Silicone Study Group introduced a new classification that included location and the type of contraction. Classification was further modified in 1991 according to the Silicone Study Group and also by other authors[93] (refer to ***Table 1.2***). This classification appears to be difficult to use in clinical practice and may not offer any aid in decision-making in relation to the treatment of the disease.

The Retina Society updated classification (Machemer et al., 1991)	
Grade and Type	Clinical Signs
A	vitreous haze, pigment clumps, pigment clusters on inferior retina
B	Wrinkling of inner retinal surface, retinal stiffness, vessel tortuosity, rolled and irregular edge of retinal break, decreased mobility of vitreous
CP (posterior) - Type: • Focal • Diffuse • Subretinal	Full-thickness retinal folds or subretinal strands posterior to equator (1-12 clock hours involvement) • Starfolds posterior to vitreous base; • Confluent starfolds posterior to vitreous base; optic disc may not be visible; • Proliferation under the retina; annular strand near disc; linear strands; motheaten-appearing sheets
CA (anterior) - Type: • Circumferential • Anterior	Full-thickness retinal folds or subretinal strands anterior to equator (1-12 clock hours involvement), anterior displacement, condensed vitreous strands • retina contraction inwards at the posterior edge of the vitreous base; with central displacement of the retina; peripheral retina stretched; posterior retina in radial folds; • anterior contraction on the retina at the vitreous base; ciliary body detachment and epiciliary membrane; iris retraction

Table 1.2. The Retina Society updated classification of PVR (1991)[93].

This classification may be too complex to be used in clinical practice and does not offer any indication for selecting the most appropriate management of the disease. Major drawbacks are found in its complexity and inability to be easily reproduced by different clinicians; therefore it has been rarely incorporated into the clinic.

SECTION 1F

CLINICAL RISK FACTORS OF PROLIFERATIVE VITREORETINOPATHY

Classification of PVR in clinical practice and preclinical research is based on the ophthalmoscopic appearance, the subjective evaluation of the amount of membrane contraction and its distribution[94]. The classification is not related to the evolution of PVR. PVR becomes clinically significant if it predisposes a RD case to surgical failure, causing a re-detachment or limits visual recovery post-operatively. This approach makes objective evaluation of the condition difficult and the most viable method of measuring the effect a therapeutic agent has on PVR is to measure its effect on the incidence of PVR related re-detachments[95], however this would not directly relate to the extent of fibrosis.

Considering the pathobiology of PVR, the majority of clinical factors associated with an accentuated healing response sufficient to cause PVR appear to include an inflammatory component or a release of RPE cells into the vitreous. Several studies have focused on the identification of a large number of overlapping risk factors and associations for the development of PVR, some of which are more consistent than others with the known underlying pathophysiology. These encompass the presence of a posterior vitreous

detachment; longer RD duration; greater physical extent of the detachment; associated vitreous haemorrhage; clinical signs of intraocular inflammation; a history of previous retinal or lens surgery; and increased retinal tear size, especially giant retinal tears. In cases of RRD presenting with pre-existing PVR[2,23,96,97], there is a significant risk of progression to further advanced PVR. Surgical factors associated with PVR development included extensive cryopexy and laser retinopexy; failure to close retinal breaks; peroperative scleral perforation; and peroperative vitreous haemorrhage[23,98,99]. This information can be used in surgical planning so that manoeuvres that risk intraocular haemorrhage or increase postoperative inflammation are avoided. Other associated factors included previous crystalline lens removal resulting in aphakia[100] or pseudophakia, the use of intravitreal air, the use of vitrectomy and a greater number of operations required to flatten the retina. The clinical risk factors associated with PVR are summarised in **Table 1.3**.

Author/year	AC activity	PVR**	Reduced VA	Greater area RD	Ocular haem[Y]	Increased Retinopexy	Larger retinal break	Lens operation	[Z]PVD	Choroidal detachment	Surgical factors
Conart *et al.* 2016	+										
Hoerster *et al.* 2013	+										
Koerner *et al.* 2012		+									
Ricker *et al.* 2012b	+										
Ricker *et al.* 2011a			+								
Ricker *et al.* 2011b			+								
Wickham *et al.* 2011		+		+	+[VH]						
Ricker *et al.* 2010			+								
Dieudonné *et al.* 2007					+[IO]						
Johansson *et al.* 2006			+			+[L]	+[ϕ]				
de la Rúa *et al.* 2005				+							
Dieudonné *et al.* 2004					+[IO]						
Hooymans *et al.* 2000		+						+[χ]			
Kon *et al.* 2000		+		+				+[D]			
Capeans *et al.* 1998									+[3A]		
Bonnet *et al.* 1996							+[δ]				
Duquesne *et al.* 1996		+					+[δ]				
Bonnet & Guenoun 1995						+[C]	+[δ]				
Cox *et al.* 1995		+					+[δ]	+[χD]			
Girard *et al.* 1994	+*	+		+			+[δ]			+	+[ζ]
Malbran *et al.* 1990	+*				+[PO]		+[δ]	+[D]	-[θ]		
Cowley *et al.* 1989		+				+[C]				+	+[ι]
Bonnet *et al.* 1988		+			+[VH]						
Bonnet 1984		+					+[ξ,κ]				+[Ч]

Table 1.3. Clinical risk factors associated with the development of Proliferative Vitreoretinopathy (PVR), Anterior chamber (AC), Visual acuity (VA), Posterior vitreous detachment (PVD), retinal detachment (RD)*AC cells present; **Any stage of PVR at time of diagnosis; [Y]Haemorrhage (haem), Vitreous haemorrhage [VH], Intraoperative haemorrhage [IO], postoperative haemorrhage [PO]; Laser retinopexy [L], cryopexy [C]; [ϕ]Retinal break greater than 1 disc diameter, [δ]giant retinal tear (break greater than 3 clock hours),[κ], [χ]pseudophakia, [D]aphakia; [A]Partial PVD associated with PVR, [θ]absence of PVD was associated with no PVR, [ι]use of intravitreal air injection, [ξ]use of vitrectomy, [Ч]greater number of operations required.

(a) *Serum and vitreous factors*

Pre-operative PVR

The association of post-operative PVR with the prior presence of PVR implies that the condition is progressive, even after initial retinal reattachment. A retrospective study of 1020 eyes with preoperative PVR found grade B to be a significant risk factor in the development of PVR postoperatively but not grade C1[23,100]. They hypothesised that grade B PVR represents an earlier form of PVR and has more potential for progression. Grade C1 PVR, a focal area of PVR that describes a star fold, may represent a different cellular response that results in a more localised area of proliferation that has spontaneously arrested; a non-evolutive form of the disease[96]. In a prospective study looking at pre-

operative PVR, grade C involving more than 1 clock hour was found to be a significant risk factor for post-operative proliferation[100].

Intra-ocular haemorrhage

Intraocular haemorrhage is an overt sign of BRB failure with direct leakage of fibrin and pro-inflammatory growth factors into the eye. Any form of intraocular haemorrhage occurring pre-operatively, peroperatively or post-operatively was associated with the generation of PVR. The association of vitreous haemorrhage with PVR could be related to the release of serum elements creating a rich pro-fibrotic environment[101]. The serum released into the vitreous as a component of the vitreous haemorrhage could elevate vitreous serum concentration above that induced by blood eye barrier breakdown alone. Serum constituents such as fibronectin induce cultured RPE cells to exert traction on collagen fibres[102]. This traction causes fixed retinal folds by the formation of subretinal bands on the surface of the retina. TGF-β, PDGF and FGF then induce RPE metaplasia, proliferation and migration, processes contributing to the wound-healing response characteristic of PVR.

(b) *Retinal pigment epithelial cell migration*

Duration of detachment

A long RD duration of over 3 months is a significant risk factor for PVR[103] and eyes with a longer RD duration tend to have more extensive detachments[104]. Therefore, the association between RD duration and PVR can be partly attributed to RD extent, but both remain independently associated. A longer duration of detachment allows more time for RPE cells to remain in contact with SRF and vitreous, allowing cells to migrate, proliferate

and produce ECM; resulting in contraction of epiretinal and subretinal membranes, fixed retinal folds and traction. The subretinal proliferative component in PVR can take the form of diffuse sheets of predominantly glial cells, which generally do not interfere with surgical reattachment, although they may limit the final visual result by inhibiting photoreceptor recovery by proliferation of RPE and Müller cells into the subretinal space shown in an experimental cat model [105,106], or can form taut bands of various configurations causing macular/retinal pucker and distortion that may require surgical intervention. [107]. Levels of VEGF are likely to be upregulated in longer duration and larger detachments. This could explain the association of these clinical features with the subsequent development of PVR[43].

Vitreous status

The separation of the vitreous from the retina depends on the adhesion between the internal limiting lamina of the retina and the posterior vitreous cortex. The presence of a partial PVD is associated with the development of PVR, possibly reflecting the juxtapositioning of migrating RPE cells with the posterior hyaloid face. The presence of an intact vitreous protects against PVR. It is possible that the intact vitreous body physically retards the migration of RPE cells, or directly inhibits the scarring response.

Tamponade agent

The use of air tamponade increases the risk of PVR, whereas other methods of tamponade, such as perfluoropropane or silicone oil does not. It has been postulated that the short-lived tamponade effect provided by air is not sufficient to ensure adequate wound healing, and this might lead to PVR[96]. Air tamponade is rarely used in a fashion to induce PVR. It was used in historical surgical techniques and is generally not used in modern surgery. However, PVR develops in cases where pneumatic retinopexy is used with expanding gas

tamponade. An expanding gas bubble causes retinal flattening by a combination of the surface tension of the bubble, preventing recruitment of fluid from the vitreous cavity through the retinal break, and the bubble squeezing subretinal fluid through the open break. As a result, a marked increase of flare and RPE cells in the vitreous body can be observed[108]. Subretinal fluid stimulates RPE cell migration and proliferation[109]. Subretinal fluid can increase the amount of RPE cell and mitogenic factor contact with the detached retina by transporting them to the surface.

Size of retinal break

Eyes with horseshoe tears are more likely to develop PVR than those with atrophic holes[110]. Cryotherapy is a stimulating factor for postoperative PVR in RDs due to horseshoe tears with curled posterior edges or retinal tears 180 degrees and over[111]. Traction exerted on a horseshoe tear may allow greater RPE cell movement through the break, inducing proliferation and migration. Larger retinal breaks are associated with the development of PVR. Large breaks have been classified as greater than 1 disc diameter in size[112] or a giant retinal tear (greater than 3 clock hours)[111,113]. This again could be due to a greater influx of RPE cells.

(c) ***Disruption of the blood-retinal barrier***

The signs of BRB breakdown due to inflammation include increased anterior chamber activity in which leucocytes and serum proteins can be seen in the aqueous humour. In the absence of inflammation the aqueous humour is optically clear. Levels of anterior chamber flare correlate strongly with aqueous protein levels and therefore the extent of BRB breakdown in RD, with higher flare values in patients who go on to develop PVR[114]. The existence of pre-operative or established PVR suggests that the cellular, extracellular and chemical elements required for wound healing are present in a pro-inflammatory

'soup'. A total protein level represents the sum of all detectable proteinaceous components in the vitreous. It provides information on the state of inflammation, breakdown of BRB and the severity of wound healing. Significantly higher protein levels, up to a fivefold increase, were found in the vitreous and subretinal fluid of eyes with pre-operative PVR compared to those without[115].

Extent of detachment

Detachments extending over 2 quadrants (6 clock hours) are more likely to develop PVR[96]. Larger detachments may be associated with greater BRB disruption through ischaemic injury resulting in a greater influx of serum components into the vitreous cavity[96]. Larger detachments are associated with PVR, supporting earlier scheduling of smaller macula-off RDs for surgery, to minimise the spread of the detachment and the risk of PVR[68]. Furthermore, disruption of the BRB is exacerbated by the requirement of extensive surgery for the management of large RDs. Larger detachments are associated with reduced visual acuity, as macula-off RDs with poor visual outcomes tend to cover a larger area than macula-on RDs, where vision is spared.

Retinopexy

Cryotherapy increases blood aqueous breakdown from the associated chorioretinal trauma, as well as promoting RPE migration. Cryotherapy causes a release of RPE cells throughout the ocular fluid, which is associated with alteration in the protein matrix seen on electron microscopy in animal models[116,117]. Cryotherapy induces a dose-dependent inflammation causing a breakdown of the BRB that allows the release of serum elements into the vitreous cavity demonstrated by computerised vitreous fluorophotometry in rabbit eyes[97,118]. TGF-β_2 is upregulated in the aqueous humour of diabetics, especially

after photocoagulation[119]. The incidence of PVR is significantly higher in RRD eyes managed with cryopexy than in eyes managed with laser retinopexy[111].

Lens status

The pathological mechanism of aphakia increasing the risk of PVR is unclear[100]. The posterior capsule has an important role in maintaining the BRB. Fluorescein angiography has found increased disruption of the BRB after intracapsular compared with extracapsular cataract extraction[120]. Capsular rupture in pseudophakic eyes also increases risk of anterior and posterior chamber communication[120]. It has been suggested that the posterior lens capsule may protect the anterior uvea, the site of active transport, from mechanical and physical irritation by the vitreous gel in phakic eyes[100]. It is possible that the intact lens provides a physical barrier to the transmission of inflammatory cytokines from the anterior chamber to the vitreous cavity and vice versa.

Miyake et al.[120] found that the outward active transport of fluorescein from the vitreous was reduced in aphakic compared with phakic eyes. More inflammation and disruption of the BRB may allow serum elements to enter from the anterior chamber and remain in the vitreous and promote the development of PVR. Other coexisting clinical factors are likely in aphakic eyes including; choroidal detachment, longer duration of detachment and cataract surgery that is complicated by vitreous loss.

Additional risk factors

Choroidal detachment is associated with BRB breakdown due to hypotony and inflammation that may stimulate the cellular processes implicated in PVR[96,97].

Excessive surgical procedures for extensive RD may induce the breakdown of the BRB, and may result in the induction of PVR[97,110]. Vitrectomy causes disruption of the BRB[121]. Mechanical stimulation, irrigation solution, fluctuations of intraocular pressure and

intraocular illumination can cause retinal damage and disruption of the BRB during vitrectomy surgery.

SECTION 1G

CANDIDATE BIOMARKERS FOR PREDICTING PVR AFTER RETINAL DETACHMENT

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(i) PVR inflammatory phase cytokines

IL-6

IL-6 is a multifunctional, pleiotropic cytokine that immune regulates, acute-phase inflammatory responses, haematopoiesis and inflammation[122]. IL-6 is produced by RPE, endothelial cells, fibroblasts, neutrophils, monocytes and macrophages in response to IL-1, IL-17 and TNF-α during systemic inflammation[122,123]. IL-6 is both pro- and anti-inflammatory in the eye and elsewhere[124-126], stimulating a paracrine and autocrine immune response by activating leukocytes and inducing the production of acute-phase proteins by hepatocytes[122]. IL-6 promotes T-cell proliferation, B-cell differentiation and survival, plasma-cell production of IgG, IgA and IgM and modulates metabolic, regenerative and intracellular signalling pathways[122,127]. IL-6 binds to an IL-6R, which also has a soluble form (sIL-6R) and then IL-6 bound to sIL-6R stimulates RPE cells proliferation *in vitro* and is necessary for subretinal scarring in a laser-induced choroidal neovascularisation mouse model[128,129]. sIL-6R forms as a complex with IL-6 that induces the formation with the signal transducing glycoprotein 130 kDa (gp130) on the surface

of cells that lack IL-6 and encourages the response of cells non-responsive to IL-6. Signal transduction of IL-6 involves the activation of janus kinases (JAK) family members. The activated kinases phosphorylate tyrosine residues on the cytoplasmic domain of the signalling receptor gp130, recruiting the signal transducer and activator of transcription (STAT) family. STAT1 and 3 is phosphorylated by JAK and forms heterodimers or homodimers. Dimerized STATs enter the nucleus and promote transcription of different genes, including genes of the acute phase response[129,130].

IL-6 induces the activation of JAK/STAT3 transcription factors that regulate proliferation of RPE, EMT and PVR progression. Blockade of the JAK1/STAT3 pathway and IL-6 deficiency may be promising strategies in the prevention and treatment of PVR.

IL-6 correlates with PVR severity and the production of MMP and tissue inhibitor of metalloproteinase (TIMP) expression, particularly MMP2 and TIMP1, indicating a role in fibrosis[131-136]. IL-6 can also stimulate corneal epithelial cells and stromal fibroblasts (and macrophages) to produce pro-fibrotic VEGF[131].

Like most inflammatory cytokines, IL-6 is present in sub-retinal fluid in high titres during retinal detachment and RRD repair[137,138], and their presence is correlated with the subsequent, development of post-operative PVR[28], as well as being elevated in the vitreous of patients with early PVR[53,139], and correlating with PVR severity[140], but, since subretinal and vitreous IL-6 levels significantly overlap between patients with uncomplicated retinal detachment and severe or future PVR, they have limited biomarker potential.

IL-1

IL-1α and IL-1β are the 2 major isoforms of IL-1, the former is biologically active, whereas the latter is activated by the inflammasome through capsase-1[141]. Once activated, both isoforms exert similar effects as potent pro-inflammatory cytokines that act as endogenous pyrogens[141]. They have diverse potentiating effects on cell proliferation and differentiation and regulate the function of immunocompetent cells, initiating and potentiating immune and inflammatory responses[141]. In animal models, IL-1 induces a proliferative response, generating PVR membranes in mouse eyes with pre-existing retinal holes[142]. An early response to retinal detachment is the infiltration into the sub-retinal space of IL-1β–secreting macrophages which may contribute to photoreceptor death through the NOD-like receptor family and pyrin-domain-containing-3 (NLRP3) protein inflammasome[143], as well as stimulating RPE cells to up-regulate inflammatory cytokines, including IL-6[144]. During necrosis induction, RIPK3 interacts with RIPK1 to form a pro-necrotic complex. Necrotic cells release endogenous molecules, termed as damage-associated molecular patterns (DAMPs), which in turn stimulate inflammasomes through NLRP3 in a RIPK3 dependent manner. DAMPs include hyaluronan-like proteins, heparin sulphate proteoglycan and biglycan[145]. Inflammasome activation has been found to be activated in animal and human eyes after RD, mediating photoreceptor cell loss. Increased levels of DAMPs have been reported in eyes with RD. Inflammation is an important pathological feature of necrosis. DAMPs released from necrotic cells enhance the inflammatory response and tissue injury, resulting in the development of PVR.

IL-1α and IL-1β are present in sub-retinal and vitreal fluid in cases of RRD and established PVR and are variably reported to be raised in PVR[53,133,139], whilst other studies suggest

that elevated IL-1α, but not IL-1β levels are associated with subsequent PVR risk[28,146]. Generic inflammatory cytokines are likely to be present in all eyes with retinal detachment irrespective of whether they subsequently develop PVR, and in the report suggesting IL-1α associated with subsequent PVR risk, there was extensive overlap between levels in patients who did and did not subsequently develop PVR[28], suggesting limited utility as a biomarker. However, when combined with other clinical and genetic markers, a single nucleotide polymorphism in IL-1 receptor antagonist was associated with PVR risk, supporting the role of IL-1 in PVR pathogenesis[147].

TGFβ

The TGFβ superfamily are important modulators of cell growth, matrix synthesis and apoptosis[80]. TGFβ opposes the actions of many pro-inflammatory cytokines and exists as $TGF\beta_1$ and $TGF\beta_2$ isoforms, with levels of $TGF\beta_2$ being predominant in the posterior segment of human eyes[148,149]. Both *in vitro* and *in vivo*, TGFβ isoforms regulate the synthesis and degradation of ECM, causing increased collagen accumulation and fibrosis[150]. TGFβ is secreted as part of a latent complex, cleaved into its active form by RPE cell-derived thrombospondin-1[151]. Activated TGFβ transforms RPE cells into type 1 collagen producing fibroblast-like cells and myofibroblast-like cells; actions that are dependent on a lack of normal cell-cell or cell-matrix interactions *in vitro*[152,153]. There are separate receptors (R) for $TGF\beta_1$ and $TGF\beta_2$, though many of these cross-react and $TGF\beta_2R$ co-localises with $TGF\beta_1$ and fibronectin expression in myofibroblastic RPE cells[154,155], though the relative roles of $TGF\beta_1$ and $TGF\beta_2$ in the fibrotic process of PVR have yet to be determined. $TGF\beta_2$ is found in high concentrations in the α-granules of

platelets and is also secreted by activated T lymphocytes and M2 macrophages, whose polarisation it also induces[45,150]. TGFβ_2 regulates TGFβR and downstream signalling molecule expression, as well as the transcription of genes that encode for pro-inflammatory growth factors and IL-1R and IL-6R[156,157]. TGFβ_2 can also induce the proliferation of fibroblasts at low concentrations by modulating autocrine PDGF secretion[158]. TGFβ_2 maintains the immunosuppressive status of aqueous humour (AqH) in mouse eyes afflicted with endotoxin-induced uveitis[125]. RPE cells secrete CTLA-2α, differentiating T cells into TGFβ-producing T_{reg} cells[159]. In patients with RRD caused by PVR, variably elevated levels of TGF-β_2 are recorded in aqueous and vitreous samples and excised PVR fibrous membranes[58,139,148,160,161], and single nucleotide polymorphisms in TGF$\beta_{1\&2}$ associate with PVR risk[147].

Since TGFβ isoforms regulate the synthesis and degradation of ECM proteins both *in vitro* and *in vivo*, causing increased collagen accumulation and fibrosis they are obvious candidates as PVR predictive biomarkers[150]. However, in conflicting data, some papers record no difference in vitreous and aqueous levels of TGFβ isoforms in retinal detachment patients who did or did not go on to develop PVR, whilst others recorded elevated levels in vitreous[162-164]. Nonetheless, levels of Decorin (a potent TGFβ antagonist and potential PVR treatment [165]) were higher in eyes with retinal detachment that did develop PVR supporting involvement of the Decorin-TGFβ axis in the pathogenesis of PVR[162], although variability in Decorin levels limits its utility as a biomarker to distinguish patients who will or will not go on to develop PVR.

(ii) **Chemokines**

Chemokines are small proteins that regulate the migration of leukocytes into sites of inflammation[166]. Chemokines are divided into two groups depending on their chemotactic activity and the arrangement of cysteine residues. CC chemokines attract monocytes, T lymphocytes, eosinophils and basophils. CXC chemokines recruit neutrophils and activated T lymphocytes[166]. Chemokine R are integral membrane proteins that specifically bind and respond to chemokines. For example, CCR2 is found on the surface of monocytes and binds monocyte chemo-attractant protein-1 (CCL-2), a chemokine that specifically mediates monocyte chemotaxis in experimental retinal detachment[166,167]. CCL2 levels are elevated in the vitreous of patients with PDR and in idiopathic epiretinal membranes[168]. Most chemokines tested for are elevated in the sub-retinal fluid of patients with primary RRD compared to vitreous from patients with macular hole[139,169-171]. One study found higher CCL2 levels in established PVR than in primary RRD, suggesting a late role in the disease process [172]. Zandi *et al* (2019) recorded elevated levels of a multiplicity of chemokines (CCL8, 15, 19, 22, 23, 26, 27 and CXCL6, 9, 10, 12) in cases of PVR compared to primary RRD without PVR but found that only levels of CCL19 associated with grade of PVR [139]. Ricker *et al* (2009, 2010 and 2012) found CCL17, 19, 22 and CXCL9 to predict the development of post-operative PVR and CCL19 also correlated with post-operative visual acuity[169-171,173], and Hoerster *et al* found aqueous CCL2 predicted the development of PVR[163].

CCL2 is produced locally by Müller glia and in cultured IL-1/TNF-α-stimulated $CCL2^{+}$ RPE cells, contributing to photoreceptor apoptosis after retinal detachment[174,175]. Many cell types (including human microglia and astrocytes) express CXCL8 in response to

inflammatory stimuli[176]. Müller glia resident in PVR membranes also express CXCL8 which chemo-attracts neutrophils and probably promotes gliosis[176,177]. CXCL9 and CXCL10 are specific for T lymphocytes[178,179]. CXCR3 and CXCL9R and CXCL10R are preferentially expressed on T lymphocytes mediating intra-ocular inflammation[180]. Cultured RPE cells produce CXCL9 and CXCL10 in response to TNF-α, IL-1β, and IFN-γ, which is inhibited by IFN-β[181]. Although absent from the vitreous in PVR, IFN-β may protect against retinal inflammation[181]. The CC chemokines CCL17, CCL18 and CCL22 mediate cell trafficking and activation of T lymphocytes[182-184]. CCL19 is crucial for the development of adaptive immunity, mediating migration of naïve, T_{reg} and natural killer T cells and B cells as well as macrophages within lymphoid tissue and stimulating macrophages and fibroblasts to secrete IL-8 and VEGF respectively[185,186].

During the development of PVR, locally generated chemo-attractive factors that both direct the migration and proliferation of RPE cells, fibrous astrocytes, fibroblasts and chemo-attract macrophages, lymphocytes and neutrophils are possible predictive PVR biomarkers[144,174,187-189]. However, levels of most of the above chemokines are raised in RRD irrespective of subsequent progression to PVR and levels overlap significantly between patients who do and do not develop PVR. The approach of Ricker *et al* (2012), who combined clinical predictors with levels of multiple cytokines including the presence of pre-existing PVR, CCL22 and IL-3 to improve predictive value may hold promise[170].

(iii) **Mitogenic growth factors**

PDGF and VEGF

PDGF and VEGF are closely related members of a superfamily of signalling molecules, with

a cysteine-knot structure formed by 8 cysteine residues[190]. Platelets are a major source of mitogenic factors, the activity of which is regulated by PDGF[191]. Intravitreal injected (*iviti*) PDGF into traumatised rabbit eyes causes severe PVR, as do *iviti* PDGF and platelets into traumatised pig eyes[192-194]. PDGF displays a wide spectrum of chemo-attractive and mitogenic activities for mesenchymal cells and glia[190]. Pro-angiogenic vascular endothelial growth factor (VEGF) is present in the developing PVR fibrotic membranes, as well as epiretinal and diabetic proliferative membranes. VEGF is synthesised and secreted by both retinal glia and RPE cells and levels may be raised in serum samples of patients with PVR, suggesting systemic levels confer disease susceptibility[195-197]. Levels average 2X higher in the sub-retinal fluid from eyes that go on to develop PVR compared to those that do not, although significant overlap between the vitreous VEGF levels in the two populations limits its utility as a biomarker in isolation[28]. RPE cells and retinal glia in epiretinal membranes express VEGF, PDGF and PDGFR and VEGFR[198-200], suggesting an important role in epiretinal membrane growth, although iviti bevacizumab (monoclonal antibody against VEGF) does not seem to prevent and may worsen further membrane development in eyes with advanced PVR[201,202]. PDGFα, FGF-2, TGFβ, IGF-1 and EGF are present in vitreous and SRF in PVR may promote RPE proliferation and fibrosis[28,203].

FGF-2

I*n vitro*, FGF-2 stimulates EMT production by RPE cells and is RPE-cell- but not Müller glia-protective (although it does stimulate migration of the latter cells)[199,204,205]. In conflicting reports, vitreal and sub-retinal fluid FGF-2 levels are raised in both PDR[206], established PVR[164,207,208], and elevated in vitreous but not aqueous or subretinal fluid of

RRD patients who subsequently develop PVR on follow up[28,163,164]. Thus, further evidence is required before FGF-2 is accepted as a predictive biomarker for PVR developing after retinal re-attachment surgery.

Adipokines

Adipokines are a group of trophic mediators, originally identified in adipose tissue but now known to be important in most inflammatory and immune responses and in wound healing in many tissues including the eye[209,210]. For example, in analyses of sub-retinal fluid sampled at the time of retinal re-attachment surgery for primary RRD, high leptin, adiponectin and cathepsin S levels and low TIMP-1 levels are associated with the development of postoperative PVR[211].

Leptin

Vitreal leptin levels are elevated in females and diabetics[212]. Mice defective in leptin and leptin-R have dysregulated immune and inflammatory responses and impaired wound healing[213]. High levels of serum leptin are associated with disease activity in Vogt-Koyanagi-Harada disease[214], highlighting a possible ocular inflammatory role. In a rabbit model, successful treatment of PVR was associated with reduced vitreous leptin levels[215]. SRF leptin levels correlate significantly with body mass index (BMI)[211], but there is no consistent association with PVR[211,216]. Obese patients are at increased risk of developing RRD [217], though this may be a mechanical effect and may or may not translate into a higher rate of PVR since the relationship between obesity and PVR remains unresolved.

Cathepsin S

The cysteine protease cathepsin S has a key role in antigen presentation [218] and is produced by RPE cells where it is crucial for photoreceptor cell maintenance by regulating rhodopsin lysosomal digestion[219,220]. Cathepsin S is also upregulated in detached neuroretina as early as 24 hours after detachment and levels of Cathepsin S are raised in the SRF of patients with retinal detachments that go on to develop PVR and correlate with the extent and duration of retinal detachment and this remains significant after correction for BMI[63,211]; however, overlap significant overlap between cathepsin S levels in patients who did and did not go on to develop PVR limits its utility as a biomarker in isolation.

TIMP and MMP

TIMP1 is a glycoprotein that inhibits MMP, a group of peptidases that degrade ECM and remodel collagen[221]. In addition, TIMP-1 promotes the proliferation of a wide range of cell types and may also have anti-apoptotic properties[222,223]. TIMP-1 regulates photoreceptor migration and expression is linked to retinal fibrosis[224] and angiogenesis[225-227]. RPE cells produce TIMP-1 both *in vitro* and in excised epiretinal and sub-retinal membranes[228,229]. Protease/protease inhibitor imbalance within the detached retina and adjacent vitreous may therefore contribute to PVR membrane formation.

A number of MMP isoforms are normally present in the vitreous[230]. MMP-2 is constitutively expressed in normal vitreous and probably regulates collagen turnover and the degradation of gelatin (denatured collagen) and a number of cytokines, including TGFβ[231,232]. Multiple hormones, cytokines and growth factors regulate MMP expression

and, in vitreal pathology such as diabetic retinopathy and retinal vein occlusion, increased expression is associated with VEGF expression[231,233]. MMP-12 is important for macrophage migration in murine retina and vitreous, but has not been detected in human vitreous[234]. Low levels of MMP3 are protective against experimental uveitis[235], while MMP9 levels correlate with the severity of wet AMD[236].

The most abundant protease inhibitor in human plasma in α1-anti-trypsin, which is consistently elevated in the vitreous of patients with PVR[237,238]. Vitreous MMP-1, -2, -3, -8, -9 and TIMP-1 levels correlate with PVR grade[232]. Vitreous MMP, TIMP-1 and α-1 anti-trypsin, are therefore all consistently elevated in patients with PVR and single nucleotide polymorphisms in MMP-2 associate with PVR risk[147]. In patients with retinal detachment, increased vitreous MMP-2 and -9 activity associates with subsequent postoperative PVR, with a negative predictive value (for low activity) of 100% for MMP-2 and 97% for MMP-9 (positive predictive values for high activity 16 and 19% respectively)[230].

Periostin

Periostin is a fibroblast-derived matricellular mitogenic protein that stimulates EMT in cancer cells, accelerates cutaneous wound healing by activating fibroblasts[239,240], and causes inflammatory chemotaxis of TH2 cells and M2 macrophages by inducing cytokine production[241,242]. In patients with PVR, vitreal periostin levels are elevated along with high periostin expression in PVR membranes[44] and is produced *in vitro* by RPE cells that undergo TGF-β_2-induced EMT[161]. These findings provide little support that periostin is likely to be useful as a predictive PVR molecular biomarker.

microRNA

Significant interest in the role of microRNA (miRNA), including exosomal miRNA[243], in systemic and ophthalmic disease, including diabetic retinopathy and age-related macular degeneration, has been reflected in an exponential increase in the number of publications in recent years[244]. A single study has evaluated miRNA as predictive biomarkers of PVR and found that miR-21, a profibrotic miRNA, was upregulated in the vitreous of eyes with PVR and was also upregulated *in vitro* by ARPE-19 cells after TGFβ-induced EMT, regulating migration and proliferation[245].

(iv) **Validation of molecular PVR biomarkers**

Predictive molecular biomarkers are agents present in tissues which forecast the risk of development of a specific pathology in which the biomarker may or may not persist [246]. The assessment of biomarker validity is critically dependent on reliability of the serial sampling technique and positive and negative predictive values. Serial consistency in harvesting SRF and vitreal fluid is difficult to achieve and can generate highly variable mean putative biomarker values and thus requires careful supervision and attention to detail. Serum samples would provide more reliable readings, but biomarker titres are likely to be significantly lower than those from retina, where factors are locally produced; consequently few serum-based studies have been reported [247]. In cases of retinal detachment which go on to the develop PVR, IFF molecules consistently present before PVR onset have potential PPV and those presenting in retinal detachment cases that do not develop PVR have NPV. One conundrum of screening potential biomarkers is that IFF feature in the retinal detachment condition irrespective of whether PVR ensues, probably

explaining why so few IFF have PPV status. Thus, factors other than IFF may constitute more plausible biomarker candidates. Factors with PPV that persist into the predicted disease state may also be used as putative prognostic biomarkers with a potential for targeting and monitoring anti-PVR treatments [248]. PPV/PNV rarely reach 100% and values are commonly much lower posing a problem in setting a threshold for assessing the status of biomarker rigour. Meaningful statistical estimates of PPV and NPV are dependent on the prevalence of PVR after RRD and as many studies use matched rather than consecutive cases, PPV and NPV cannot be meaningfully calculated. Therefore few studies claiming biomarker potential for particular IFF have evaluated their PPV/PNV. The most promising approach so far is in the combination of multiple clinical and laboratory biomarkers to improve the sensitivity and specificity of PVR prediction [147,170].

Section 1H

Posterior segment imaging

Optical coherence tomography (OCT) is a non-invasive imaging test. OCT uses light waves to take cross-sectional images of the retina, allowing visualisation of the retina's distinctive layers. It provides treatment guidance for various eye conditions. Measurements of vitreous signal intensity from OCT images in patients with uveitis have been used to objectively assess the inflammatory activity in patients with this disease. Current measures of disease activity are largely based on subjective clinical estimation, and are relatively insensitive, with poor discrimination and reliability. VIT/RPE-Relative Intensity was found to be significantly higher in uveitic eyes with known vitreous haze

than in uveitic eyes without haze or in healthy controls[249-253]. These results suggest that OCT-derived measurements of vitreous signal intensity may be useful to assess vitreous inflammation and may therefore provide a signal in patients with PVR. If validated, such measures may serve as an objective, quantitative disease activity endpoint. This would facilitate monitoring and treatment of patients with PVR or at risk of developing PVR in clinical practice.

Section 1I

THE IMPORTANCE OF PROLIFERATIVE VITREORETINOPATHY IN OCULAR TRAUMA

In trauma, PVR occurs in 27% of cases and is a major cause of poor visual and anatomical outcomes[254]. Its frequency, onset and outcome are strongly dependent on the nature of the trauma. The rate is as high as 70% in open globe injuries[87]. Ocular trauma is one of the leading causes of visual loss in the working age population[255,256]. It often affects young individuals and often causes permanent visual loss, with significant social and economic consequences[257]. In the military setting, approximately 10% of war injuries with major trauma have eye injuries that are bilateral in 15–25% of cases[258,259].

Therefore, appropriate management of these high-risk cases can improve functional outcome after injury and terminate the pathological processes involved in PVR development.

SECTION 1J

CURRENT AND EMERGING TREATMENTS OF PROLIFERATIVE VITREORETINOPATHY

(i) Pharmacologic agents

The post-operative incidence of PVR remains high, ranging from 4 to 34%. This is despite the evolution of surgical techniques to help improve primary surgical outcomes. This highlights the need for adjunctive pharmacological treatment as a preventative measure or to slow or halt the PVR progression.

PVR is a complex process similar to that seen in wound healing. Various cell types, cytokines and growth factors are involved in the processes seen in PVR development, which include inflammation, migration and proliferation. Pharmacological strategies have aimed at either modifying the inflammatory cascade or interfering with cellular proliferation. Drugs under investigation target one or more of the pathways involved in PVR development.

1. Anti-inflammatory

Corticosteroids were among the first agents tested in the prevention of PVR based on the hypothesis that PVR pathogenesis is due to inflammation. Corticosteroids have long been known to reduce intraocular inflammation and, depending on their concentration, to suppress cell proliferation.

Animal experimental studies showed benefit with intravitreal administration of triamcinolone. Hui *et al.*[260] induced PVR in a rabbit model by injecting activated macrophages into the vitreal cavity, followed by early treatment with 1 mg intravitreal triamcinolone. They found a 64.2% reduction in the RD rate. Giving methylprednisone periocularly to rabbits also showed a decrease in the incidence of PVR created by retinal tears/detachment, cryotherapy, and injection of PDGF, from 87% to 13%[261].

Their effectiveness in animal experiments has not translated to long-term success in humans. Human studies have failed to demonstrate the same beneficial effects in terms of reattachment rate, visual acuity, recurrence of PVR, or re- operation rate. It is possible that these drugs have not been applied at the proper time or they have not reached the appropriate concentration in the eye[262]. Koerner *et al.* [263] showed minimal difference in postoperative retinal fibrosis following systemic steroid administration 5 days after surgery, while others[260] have advocated earlier administration of steroids.

There is a huge variety in the route of steroid administration besides systemically, including intravitreal and subconjunctival application. Jonas *et al*.[264] reported on clinical outcome and complications in a case-control study in which they enrolled 16 patients who underwent pars plana vitrectomy for PVR and received an intravitreal injection of 10–20 mg crystalline triamcinolone acetonide at the end of surgery. The control group consisted of 144 patients. They reported a decrease in postoperative inflammation and pain; however the mean postoperative follow-up period was only 1.64 months. A double-blind clinical trial[30] was performed on 34 patients with RD listed for scleral buckling surgery. Patients were randomised between 0.5ml dexamethasone diphosphate and placebo

subconjunctival injections 5 to 6 hours preoperatively. Laser flare photometry measurements were taken at interval after surgery. Measurements were significantly lower in the treatment group one week postoperatively suggesting that steroid priming might be useful in reducing BRB breakdown and hence PVR. However, long-term outcomes were not reported on.

There are studies that have tested the efficacy of low doses of intravitreal triamcinolone after silicone oil tamponade[265-268]. The authors reported that it is safe and effective. However, the distribution and pharmacokinetics of triamcinolone and other anti-inflammatory drugs has been tested using spectrophotometry and triamcinolone has been reported to be insoluble in silicone oil[268,269]. Sediment of triamcinolone at the border of the silicone oil bubble may be harmful to retinal cells[268]. Further, it seems that relatively high intraocular levels of steroids would be required. In cell culture, only concentrations of dexamethasone over 200 mg/ml had an additive effect with 5-FU on the inhibition of human RPE proliferation[270].

Ozurdex is a biodegradable sustained-release dexamethasone implant, delivered in the vitreous body for conditions such as retinal vein occlusion. With the 0.7 mg Ozurdex implant, dexamethasone can reach plasma concentrations of 1.11 ng/ml. This concentration of dexamethasone can cause side effects such as raised intraocular pressure. Therefore 200 mg/ml is likely to cause problems.

Penetrating injuries of the eye are more likely to result in poor vision and PVR. The Adjunctive Steroid Combination in Ocular Trauma (ASCOT) trial[271], a randomised

controlled clinical trial has been developed to determine whether the addition of intravitreal triamcinolone at the time of surgery for penetrating eye trauma is effective in improving visual and surgical outcomes or not.

2. Antineoplastic / Antiproliferative

Cell proliferation is essential in the development of PVR, thus antineoplastic agents inhibiting cell cycle and cellular proliferation are being extensively explored and their intraocular toxicity levels are being established.

5-FU is one of the most tested compounds for the treatment of PVR. It acts on DNA synthesis by inhibiting thymidine formation.

Borhani *et al*. [272] evaluated a sustained-release bioerodible device in a rabbit model of PVR. 0.5 mg and 1 mg 5-FU showed significant efficacy in preventing PVR.

In humans, two recent randomized controlled trials compared 5-FU and placebo with low-molecular-weight heparin (LMWH), which reduces postoperative fibrin after vitrectomy by binding to fibronectin and growth factors[95,273]. They found a significant reduction in the incidence of postoperative PVR and in the reoperation rates in the treatment group[95].

In the other study[273], 641 patients presenting with primary RRD who were undergoing vitrectomy and gas tamponade. They found that adjuvant therapy with 5-FU and LMWH resulted in worse visual acuity, raising some toxicity concerns. A benefit was shown in subjects at higher risk of PVR, but not in an unselected population.

Daunorubicin is a topoisomerase inhibitor that inhibits DNA and RNA synthesis in a cell cycle-independent manner and has also been tested for PVR. The Daunomycin Study Group[274] was a multicenter, prospective, randomized, controlled clinical trial that assessed the efficacy and safety of daunorubicin during vitrectomy in eyes with PVR. Anatomical success rates were similar between the Daunorubicin and control groups, and there was no difference in best-corrected visual acuity.

Retinoic acid, in the form of 13-cis-RA (isotretinoin), has also been tested in several trials. While commonly used by dermatologists for the treatment of acne, isotretinoin was originally developed as an anti-proliferative agent for the treatment of basal cell carcinomas. In a prospective randomized trial of 58 eyes, those that received 20 mg daily had a lower rate of PVR-associated recurrent retinal detachment in eyes with primary RRD at high risk of developing PVR[275]. It also reduces rates of epiretinal membrane formation and has a beneficial effect on visual outcome[276].

3. Antigrowth factor / growth factor pathway inhibitors

With the increasing knowledge about the role that growth factors play in the pathogenesis of PVR, there has been a move towards blocking growth factors and their respective pathways as a means to halt progression of PVR.

Kinase inhibitors are being explored. Hypericin, for example, which is used as an antidepressant and antiretroviral, acts as an antiproliferative through inhibition of the protein kinase C pathway. Intravitreal injections of hypericin in a PVR rabbit model ameliorated the PVR outcome following a single injection of the drug[277]. Another more

recent study using a PVR ocular trauma model also showed a benefit for hypericin in decreasing PVR progression[278].

Tyrosine kinase inhibitors like herbimycin A have also been tested in a rabbit PVR model. They showed reduction in the number and severity of tractional retinal detachments when injected early; however, there was an initial drop in the electroretinogram B-wave amplitude following the injection, but that recovered subsequently[279].

TGF-β is another key player in the pathogenesis of this disease, given its role in extracellular matrix production, membrane contraction, and inflammation. Tranlisat, an inhibitor of TGF-β1 used as an anti-allergy drug, showed promising results in terms of reducing the severity of PVR following intravitreal injection in a rabbit model without apparent toxicity to the eye, thus future investigations might prove agents like tranlisat to be effective in treating PVR in affected patients[280].

In an attempt to improve success rates, the use of intravitreal pharmacologic agents[273-275,278-281] has been trialled. None of these agents are routinely used due to concerns of retinal toxicity and uncertain efficacy. Due to adverse effects of these drugs, only targeted treatment is appropriate.

(ii) Surgery

The mainstay of the management of PVR is surgical, with pars plana vitrectomy with membrane peeling being the primary procedure. Advances have been made in surgical techniques for the management of PVR, however recurrent detachment is common.

Different techniques are employed to dissect PVR membranes and bands to allow the retina to flatten, restoring normal anatomy. The aim of surgery is to treat retinal breaks, relieve tractional forces and stabilize the retina.

Early PVR can be managed with conventional surgery. More advanced cases require technically more challenging surgery, such as scleral buckling and retinectomy. Relaxing retinectomies of the shortened retina are required when complete relief of retinal traction has not been achieved after membrane removal. Typically silicone oil is used as a longer lasting tamponading agent. Perfluoropropane (C3F8) gas is an alternative option for less extensive disease. Overall anatomical success rate post-surgery is reported to be 60 to 90%[282,283]. However functional success (1.6 LogMAR vision) is only seen in 40 to 80%[282,283].

SECTION 1K

PROJECT DETAIL

(i) Project rationale and hypothesis

When the retina becomes separated from the retinal pigment epithelium under the conditions of a rhegmatogenous detachment, all non-neuronal cell types undergo proliferation and, consequently, have the potential for participating in the formation of scars or “membranes” characteristic of these diseases. When a cellular membrane grows on the vitreal surface of the retina, contraction causes retinal redetachment. PVR remains the most common (approximately 5%–10% of cases) cause of the failure of retinal reattachment surgery[1,2].

With advances in molecular biology and analytical technologies, the search for predictive biomarkers for PVR has received increasing attention. Correct identification of clinical risk factors and predictive biomarkers ultimately underpins the development of an accurate predictive model for PVR, which can be used to inform clinical decision-making. Several studies have identified a range of clinical and surgical risk factors for the development of PVR.

Although advances in the surgical management of PVR have improved chances for reattachment of the retina, the visual prognosis of patients remains poor, with only 11% to 25% of patients achieving visual acuity of 6/30[86]. Presumably, the decrease in visual acuity that results from retinal detachment and PVR results, in part, from the loss of photoreceptors because apoptosis is a clear consequence of detachment and can continue at low levels even after the retina is reattached[284]. Although numerous attempts have been made to reduce the incidence of PVR and subsequent membrane formation, to date no effective pharmacologic treatment has been found[283]. Until an effective preventive strategy with minimal adverse effects is identified, the use of pharmacological agents to prevent PVR is best targeted at high-risk patients, hence the need for predictive biomarkers.

A growing number of cytokines and growth factors have been implicated in PVR pathogenesis. Cytokines have regulatory functions in wound healing and induce the expression of several chemokines. These cytokines could behave as biomarkers, used as

prognostic factors in patients with primary retinal detachment to identify those at high-risk.

Multivariate logistic regression methods have been used to include certain biomarkers in various prediction models for postoperative PVR in previous studies. The biomarkers chemokine C-C motif ligand 22 (CCL22), interleukin-3 (IL-3), interleukin-6 (IL-6), macrophage migration inhibitory factor (MIF), intercellular adhesion molecule (ICAM-1), transforming growth factor β2 (TGF-β2) and total protein concentration were included in published prediction models[20,285].

I hypothesise that:

1) PVR is an inflammatory process. Predictive biomarkers for PVR will have an inflammatory role and will include TGF-B, IL-6 and FGF2. These will be particularly raised in high-risk cases and in retinal detachment compared to macular hole.

2) PVR is caused by RPE cell migration and proliferation. RPE cell proliferation should increase in the presence of key biomarkers involved in the development of PVR.

3) Key metabolities present in cell proliferation and inflammation will be raised in eyes that go on to develop PVR.

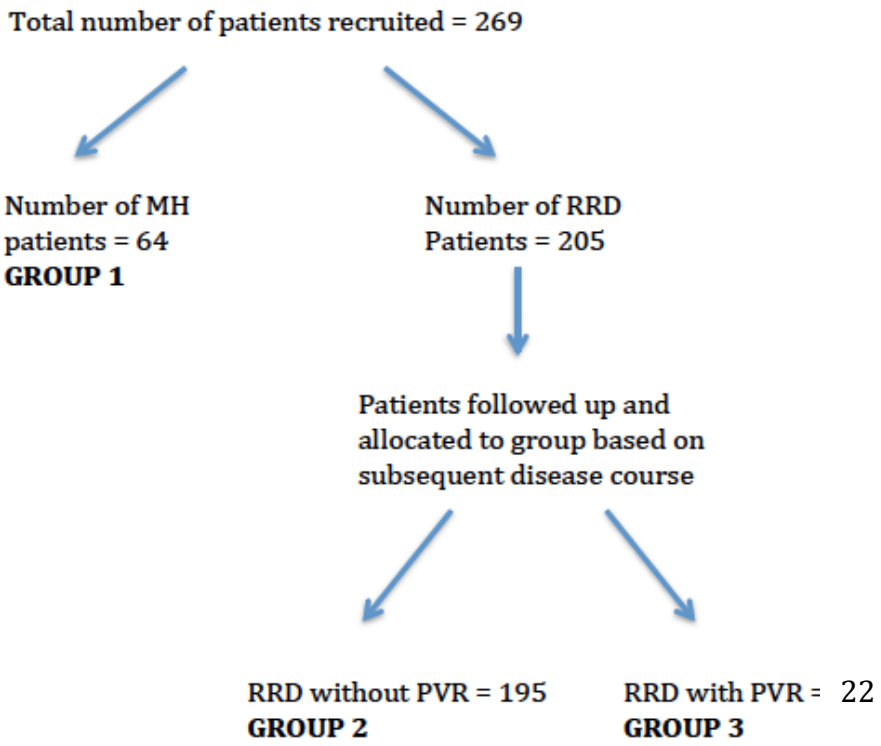
(ii) Aims and Objectives

Investigate the potential of a combined assessment of clinical risk factors with biomarker profiling for better prediction of PVR risk following RD surgery and identify potential future therapeutic targets.

1) Identify cytokine changes associated with RRD compared to MH
2) Identify metabolomic changes associated with RRD compared to MH
3) Identify cytokine changes associated with the development of PVR in RRD cases
4) Identify metabolomic changes associated with the development of PVR in RRD cases
5) Identify the effects of the observed predictive cytokine biomarkers of RRD and PVR on RPE cells in vitro

(iii) Experimental Plan

Vitrectomy samples from conditions where PVR does not occur (MH) and from patients with RRD who did not develop PVR will be compared to samples from patients with RRD who went on to develop PVR.



1) Multiplex (Luminex®) bead analyses will be used to quantify candidate cytokines in vitreous biopsy samples.

2) Objective measurement of vitreous inflammation using Optical Coherence Tomography will be performed in contrasting disease groups.

3) Human vitreous samples will be analysed by magnetic resonance spectroscopy to determine metabolomic profiles predictive of PVR development.

4) *In-vitro* validation with RPE cell culture will test the PVR inflammatory model and test / confirm conclusions made by the previous experimental work.

(iv) Vitreous humour sample collection

Patients were recruited from the tertiary referral vitreoretinal unit of the Birmingham and Midland Eye Centre. Ethical approval was obtained from the Solihull Research Ethics Committee (ethics number 12/WM/0330). These patients had to be capable of giving consent to participation. Patients excluded from sampling were those aged under 10 and those that previously had vitreo-retinal surgery (except for those with established PVR).

A standard three-port pars plana vitrectomy was set up. A vitreous sample was obtained with the vitreous cutter before the infusion was started. A minimum volume of 0.5ml was taken in each patient. The undiluted vitreous sample taken at the beginning of surgery was centrifuged at 10,000rpm for 10 minutes and stored at -80°C until analysis (refer to the sample collection protocol in **appendix V**). Vitreous samples were defrosted whilst being kept on ice. Vitreous samples were then centrifuged at 4°C at 10,000rpm for 15 minutes.

CHAPTER 2

ASSESSMENT OF HUMAN VITREOUS INFLAMMATORY BIOMARKERS

SECTION 2A

(i) Introduction and rationale

A major component of PVR progression is an exaggerated inflammatory reaction to retinal tears and detachment. Development of PVR involves a dynamic multistep and multifactorial cellular process with components of the response orchestrated by various growth factors and cytokines. Secreted factors in the vitreous cavity are associated with the pathological processes that define the disease. These trophic factors serve as signals between neighbouring cells, triggering important pathological processes including cell proliferation, inflammation, immunity, migration, fibrosis, tissue repair and angiogenesis. Since multiple pro-inflammatory, chemotactic and mitogenic growth factors and cytokines are involved in the development of PVR, a predictive biomarker profile that reflects disease progression might be expected.

With advances in molecular biology, imaging and analytical technologies, the search for predictive biomarkers has received increasing attention. An improved understanding of the pathogenesis and intraocular environment of the PVR eye is crucial for better management of patients, so that correct identification of these predictive biomarkers can be used to inform clinical decision-making. The ability to predict the likelihood of patients developing fibrotic membranes after retinal damage is important when planning surgery and could potentially allow the targeted use of anti-fibrotic agents. Future anti-

proliferative treatments can target specific cytokines and growth factors involved in the fibro-proliferative response, to break the cycle of PVR development.

Since a multitude of cytokines are thought to be involved in the disease process, it is necessary to examine a large cytokine/growth factor profile in patients in order to select appropriate sets of indicative markers. In previous studies, only a limited number of biomarkers have been measured in vitreous, subretinal fluid or aqueous samples. However, muliplex-bead-based immunoassays are now established that allow the identification of many molecules in a single small sample volume.

The use of OCT for direct visualization and quantitative analysis of vitreous inflammatory cells in patients with uveitis has been described. OCT-derived measurements of vitreous signal intensity may thus also be useful as an outcome measure in patients with PVR, which we believe, is essentially also an inflammatory disease. OCT can be performed easily with high reproducibility and repeatability. This could provide practitioners with a more objective measurement of inflammation and could be used as a prognostic tool for PVR development and also allow surgeons to monitor response to treatment.
This will enable determination of specific patterns of cytokines, chemokines and growth factors associated predisposition or risk of developing post-operative PVR. This study is the first, to our knowledge, to prospectively examine vitreous in those eyes with RRD that later go on to develop post-operative PVR. Previously quantitative analysis of inflammation by OCT has only been performed in uveitic eyes, with nothing yet published on PVR cases.

(ii) Hypotheses

(A) Levels of inflammatory cytokines are higher in those patients with retinal damage that go on to develop PVR than in those that do not.

(B) Vitreous signal intensity measured by OCT and reflecting enhanced numbers of inflammatory cells is raised in those patients with retinal damage who develop PVR.

(iii) Aims

Identify and measure, using multiplex bead analysis (Luminex® Bio-Plex Pro Human Cytokine 27-plex Cytokine Detection kit, BioRad, Hercules, CA), the angiogenic and inflammatory cytokines present in the vitreous of eyes that later develop post-operative PVR and compare these levels to those in patients with (i) retinal detachment that do not develop PVR and (ii) macular holes.

Having identified inflammatory markers present in PVR vitreous samples, this study will use OCT to quantify the extent of vitreous inflammatory activity and correlate it with disease progression. The aims of the OCT study are to (i) demonstrate a difference between vitrectomised and non-vitrectomised eyes as a positive control and (ii) demonstrate a difference in vitreous inflammation between MH and established PVR.

SECTION 2B

MULTIPLEXED IMMUNOASSAYS WITH FLUORESCENT MICROSPHERES (LUMINEX®)

(i) Principles of immunoassays

The luminex assay has become an important tool in cytokine detection and quantification because of its capacity to measure multiple different cytokines simultaneously in a single run of the assay with small sample size requirements. This enables patterns of numerous cytokines to be examined, providing a more inclusive and comprehensive depiction of disease than measurement of individual cytokines.

Luminex assays enable fast and accurate measurements of cytokines by utilizing hundreds of specially prepared micrometre-scale plastic beads, microspheres, which are internally dyed with a graded mixture of red or infrared fluorescent dyes. Varying the degree to which the beads are internally dyed creates hundreds of different fluorescent profiles that can be individually interrogated and classified in a single sample.

Excitation of each of the unique microspheres by a laser, using modified flow cytometry-based instruments, causes them to emit light at different wavelengths. Microspheres of a single identity are conjugated on their surface with a specific capture antibody for a desired cytokine.

The biological sample of interest (vitreous) is then combined with a mixture of microsphere sets. Each set is specific for a unique cytokine and incubated in a 96-well plate.

Using a variety of microsphere sets combined within a single assay, it is possible to carry out up to 100 tests in a single well of a 96-well plate. The biological sample-microsphere mixture is then washed, and a cocktail of detection antibodies specific for the desired cytokines is added. These detection antibodies are also conjugated with a reporter dye, which provides the microsphere with an additional distinct fluorescent emission signature when it binds the cytokine of interest. Thus, it is possible to differentiate various cytokines in a given sample based on the colour of the internally dyed beads themselves, while quantifying their relative abundance via measurement of the intensity of fluorescence of the reporter dye-conjugated detection antibody.

After another round of washing, the microsphere-cytokine-reporter compound is analysed as a single-bead suspension through a flow chamber equipped with excitation lasers and electronic detection apparatus that measures the intensity of fluorescence. When a microsphere passes through the detection chamber, a red laser excites both the red and the infra-red internal dyes inherent to each particular bead set, which allows the proper classification of the bead to 1 of the 100 microsphere sets. A green laser then excites any reporter dye associated with the binding of the cytokine of interest. The amount of reporter bound to the microsphere is dependent on the concentration of the particular cytokine in the vitreous. The emitted light from the dyes is measured and used

for quantitative analysis of each microsphere-cytokine-reporter conjugate. This is depicted in **Figure 2.1**.

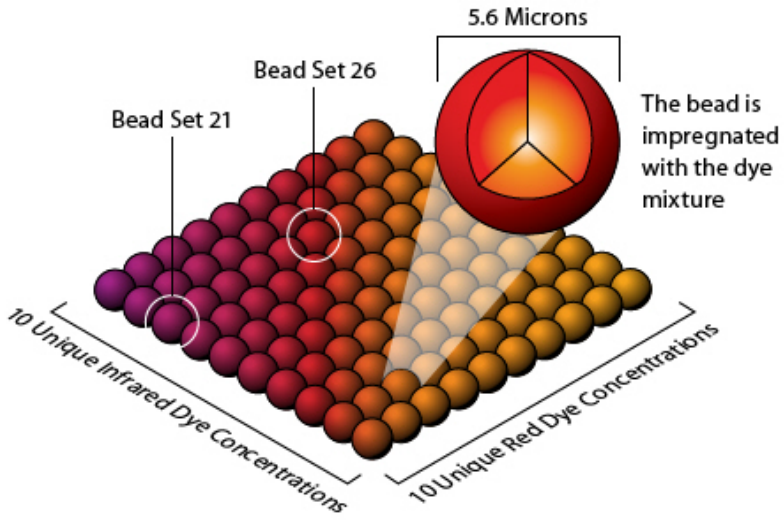


Figure 2.1. Principle of bead-based SPA by Luminex®. *Each Luminex® microbead is impregnated with a unique combination of two fluorophores emitting light in the red and infrared spectrum. The unique fluorescence signal of each bead allows multiplexing using up to 100 beads in one single reaction. Taken from Luminex® Inc.*

In the platform by Luminex® each bead set is impregnated with a unique mixture of two fluorophores which are both excited by a red laser at 635 nm. The emitted light can be detected at wavelengths of 660 nm (red) and 730 nm (infrared) using a dedicated footprint flow cytometer (Luminex®100/200TM). By measuring the composition of the emission up to 100 different bead sets with their coated HLA can be individually identified. The detection of HLA-specific antibodies is achieved by using a secondary

antibody conjugated with the reporter fluorophore R-phycoerythrin that is excited by a green laser (532 nm) and detected at 576 nm wavelength.

(ii) Methodology - Patients and samples

Vitreous samples were collected as described in **Section 1K**. The MH group acts as a control. We compare patients who go on to develop PVR with those who do not. The number of samples used in: GROUP 1 (stage 3 or 4 MH surgery) = 45; GROUP 2 (RRD surgery) = 71 and GROUP 3 (PVR developing after previous vitreo-retinal surgery) = 22.

(iii) Specific Luminex protocol for cytokines

Multiplexed immunoassays with fluorescent microspheres were used to measure multiple molecules in the vitreous samples; the Luminex® Bio-Plex Pro Human Cytokine 27-plex Cytokine Detection kit (BioRad, Hercules, CA. Cat #: M500KCAF0Y) was used according to the manufacturer's instructions. The molecules were cytokines IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN, TNF-α; the chemokines CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10; and the growth factors FGF, VEGF, G-CSF, GM-CSF and PDGF. The plate layout is shown in **Figure 2.2**.

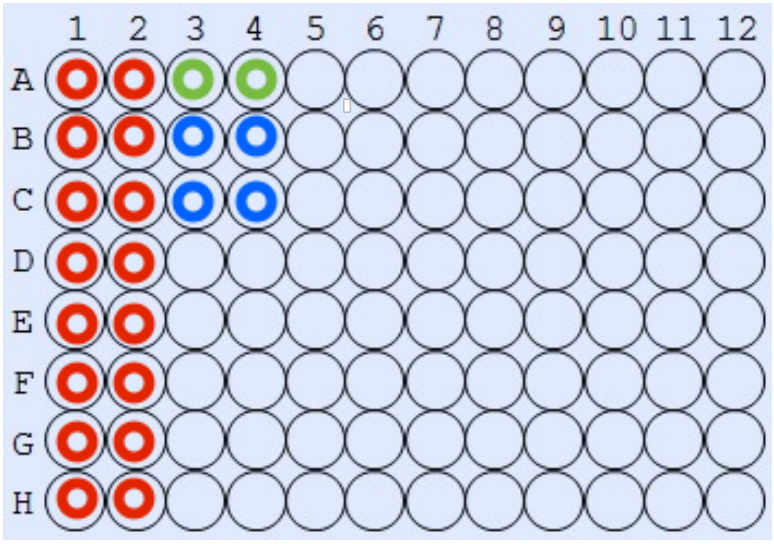


Figure 2.2. 96-well plate preparation for Luminex®*. Red = standard; green = blank; blue = controls, rest = vitreous samples.*

All reagents were allowed to warm to room temperature before use. Vitreous samples were diluted 1:4, 1:2, 1:1, and serial 1:4 dilutions of cytokine standards were prepared using manufacturer-supplied diluent (Bio-Plex Human Serum Diluent; Bio-Rad). Quadruplicate samples for which sample diluent was substituted for vitreous were used to determine background fluorescence. A vacuum filtration system was used for the wash steps. Cytokine standards or diluted vitreous samples were added to wells of a 96-well plate containing cytokine detection beads, which were coated with anti-cytokine antibodies, and incubated for 30 minutes. All incubations were carried out at room temperature with the 96-well plate sealed and placed on an orbital shaker (300rpm). The plate was then washed, streptavidin-phycoerythrin detection reagent was added and the plate was incubated for 10 minutes. The beads were then washed, resuspended in 125uL

wash buffer and shaken for 30 seconds at 1100. The plate was then read by a flow cytometry-based instrument (Bio-Plex Array Reader; Bio-Rad). Mean fluorescence intensity for each well was determined. Analysis software converted fluorescence readings to cytokine concentration by use of a calibration curve derived from a five-parameter logistic fit of fluorescence readings of the cytokine standards. Data were acquired on a validated and calibrated Bio-Plex 200 system (BioRad) and analysed with Bio-Plex Manager 6.0 software (BioRad).

(iv) Statistical comparisons of microarray analysis

Cytokine, chemokine and growth factor levels between different disease groups were analysed using both univariate and multivariable approaches. Analysis was performed on three disease groups: RD, PVR, and MH, with two comparisons made between two groups: (i) RRD (a combination of the RD and PVR groups) vs. MH, and (ii) RD vs. PVR. All out of range values are excluded from the analyses.

For each comparison of disease groups, the association between each candidate factor and the disease group was assessed through univariate logistic regression models. In each model disease group was the binary outcome and each candidate factor was included as the single model covariate. These models produced odds ratios that can be interpreted as the increase in odds of being in a disease group due to a unit increase in the value of the candidate factor. For example, in the RRD vs MH models, the MH group is set as the reference group and an odds ratio greater than 1 indicates that increasing the value of the candidate factor by 1 increases the odds of being in the RRD group. Conversely, an odds

ratio of less than 1 means that increasing the value of the candidate factor by 1 decreases the odds of being in the RRD group. All odds ratios are estimated with corresponding 95% confidence intervals and p-values. Tables of candidate factors generating p values less than 0.05 from the univariate models are produced for comparison with the multivariable model output.

Due to the exploratory nature of these analyses, the large list of candidate factors was reduced through stepwise model selection by AIC. Model selection was performed using forward, backward, and a combination of both approaches to compare the set of candidate factors retained in the models. Each candidate factor is either added or removed from the initial model, depending on search direction, and is retained in the model if the AIC is reduced. This stepwise selection approach is used for identifying potential predictors in future modelling work. The final multivariable model selected using a stepwise search in 'both' directions is evaluated and tables of the candidate factors generating p values less than 0.05 from the multivariable model are produced for comparison with the output from the univariate models.

All analyses were performed using R v3.4.2.

(v) Results

Table 2.1 below shows the mean concentration of measured cytokines in each group.

Table 2.1. Mean concentration of cytokines in RD, MH and PVR.

Cytokine	Mean concentration in pg/mL		
	RD	PVR	MH
IL1b	6.510	7.837	2.631
IL1ra	60.280	234.641	30.920
IL2	3.242	6.086	2.643
IL4	0.410	11.845	0.227
IL5	0.665	0.267	0.407
IL6	226.690	830.949	16.891
IL7	13.576	26.876	13.396
IL8	34.258	89.226	7.646
IL9	23.890	1.356	76.438
IL10	12.451	91.543	3.358
IL12	8.721	79.674	1.328
IL13	1.650	6.968	0.661
IL15	15.136	9.374	11.545
IL17	56.029	95.658	11.452
Eotaxin	2.290	32.034	1.1438
FGF	15.584	102.361	7.011
G-CSF	201.581	2775.533	3.169
GM-CSF	154.065	196.977	151.905
IFN-g	325.075	579.933	374.522
IP-10	866.503	520.116	27.108
MCP-1	365791.412	4270.783	533.409
MIP-1a	1.125	11.117	0
PDGF	5.693	29.566	3.409
MIP-1b	42.682	46.111	11.660
RANTES	9.631	792.888	5.391
TNF-a	10.862	115.521	2.948
VEGF	160.559	896.371	18.299

Univariate regression analysis was performed for each measured cytokine for the comparisons RRD vs MH (**Table 2.2**) and RRD without PVR development vs subsequent development of PVR (**Table 2.3**).

***Table 2.2. Univariate analyses of RRD vs MH*:**

Factor	Number of Observations	Lower	Upper	Odds Ratio	95% Confidence Interval	*p*-value
IL1b	138	0	1.89	1.002	(0.914,1.099)	0.962
IL1ra*	**138**	**22.83**	**58.197**	**1.996**	**(1.224,3.257)**	**0.006**
IL2	138	1.83	5.25	1	(0.921,1.086)	0.995
IL4	138	0.03	0.93	1.328	(0.895,1.969)	0.159
IL5	138	0	2.3	1.012	(0.919,1.114)	0.806
IL6	**138**	**24.715**	**106.138**	**5.567**	**(1.94,15.971)**	**0.001**
IL7*	**138**	**14.39**	**23.157**	**1.261**	**(1.01,1.574)**	**0.041**
IL8*	**138**	**8.725**	**24.262**	**5.74**	**(2.453,13.43)**	**<0.001**
IL9*	**138**	**2.71**	**12.81**	**1.771**	**(1.155,2.715)**	**0.009**
IL10	138	9.12	23.38	1.045	(0.882,1.238)	0.611
IL12p70	138	7.96	27.195	1.139	(0.904,1.435)	0.271
IL13	138	0	3.107	1.148	(0.907,1.452)	0.250
IL15	138	22.055	48.53	0.984	(0.819,1.182)	0.862
IL17	138	0	8.725	1.008	(0.973,1.045)	0.649
Eotaxin	138	3.9	14.918	1.206	(0.904,1.607)	0.203
FGFbasic	138	45.865	92.38	1.016	(0.827,1.249)	0.880
GCSF	138	0	42.023	1.402	(0.913,2.152)	0.122
GMCSF	138	145.435	209.34	0.96	(0.702,1.313)	0.798
IFNg	**138**	**2.71**	**70.86**	**1.246**	**(1.017,1.526)**	**0.034**
IP10*	**138**	**54.77**	**144.43**	**2.841**	**(1.517,5.319)**	**0.001**
MCP1MCAF	**138**	**319.665**	**663.25**	**1.341**	**(1,1.797)**	**0.050**
MIP1a	**138**	**0.75**	**1.93**	**2.001**	**(1.237,3.237)**	**0.005**
PDGFbb	138	0	10.36	1.508	(0.997,2.281)	0.052
MIP1b*	**138**	**14.13**	**25.488**	**7.378**	**(3.131,17.383)**	**<0.001**
RANTES	138	0	5.41	1.008	(0.914,1.112)	0.868
TNFa	138	3.89	15.44	1.087	(0.932,1.269)	0.288
VEGF	138	33.215	85.567	1.137	(0.957,1.351)	0.146

MH group is used as the reference group. Therefore, an odds ratio of greater than 1 means that the cytokine measurement is higher in RRD group. An odds ratio of less than 1 means that the cytokine measurement is higher in the MH group.

Stepwise selection was used to build a multivariable regression model of potential predictive factors to differentiate between RRD and MH. Candidate factors were retained using AIC. The output from the multivariable regression analysis is summarised in **Table 2.4**. for retained factors with p values for association <0.05:

Stepwise approach		
Forwards	***Backwards***	***Both***
IL1β MIP1b IP10 RANTES IL1ra IL9 IL7 IL15 IL13 IL2 IL8 IL5	IL1ra IL2 IL7 IL8 IL9 IL13 IL17 FGFbasic IP10 MCP1MCAF MIP1b RANTES TNFa	MIP1b IP10 RANTES IL1ra IL9 IL7 IL15 IL13 IL2 IL8 IL5

The cytokines highlighted with an asterix in **Table 2.2** are those that have a statistically significant odds ratio > 1 in all four analyses of RRD vs. MH: the univariate regression models and each of the three models based on stepwise selection. Those cytokines are found to be IL1ra, IL7, IL8, IL9, IP10 and MIP1b.

***Table 2.3. Univariate analyses of RRD vs PVR*:**

Factor	Number of Observations	Lower	Upper	Odds Ratio	95% Confidence Interval	*p*-value
IL1b*	**93**	**0**	**3.33**	**0.541**	**(0.318,0.921)**	**0.024**
IL1ra	93	39.26	65.55	0.788	(0.621,1.001)	0.051
IL2	93	2.13	5.98	0.972	(0.842,1.121)	0.694
IL4	**93**	**0**	**1.31**	**0.564**	**(0.321,0.992)**	**0.047**
IL5	93	0	5.07	1.089	(0.791,1.5)	0.602
IL6	**93**	**58.85**	**211.53**	**0.889**	**(0.812,0.972)**	**0.010**
IL7*	**93**	**15.24**	**26.72**	**0.735**	**(0.559,0.965)**	**0.027**
IL8	**93**	**18.54**	**33**	**0.719**	**(0.561,0.922)**	**0.009**
IL9	93	6.82	16.68	0.795	(0.545,1.16)	0.235
IL10	93	11.08	25.18	0.904	(0.72,1.136)	0.387
IL12p70	93	9.46	33.31	0.897	(0.678,1.188)	0.449
IL13	93	0.74	3.84	0.845	(0.666,1.071)	0.164
IL15	93	22.98	58.18	1.072	(0.732,1.569)	0.722
IL17	93	0	8.2	0.895	(0.731,1.096)	0.283
Eotaxin	**93**	**4.78**	**19.83**	**0.501**	**(0.304,0.828)**	**0.007**
FGFbasic	93	46.72	92.38	0.697	(0.48,1.011)	0.057
GCSF	93	6.34	59.92	0.872	(0.745,1.021)	0.089
GMCSF	93	144.3	208.93	0.687	(0.454,1.04)	0.076
IFNg	**93**	**14.71**	**119.98**	**0.739**	**(0.598,0.913)**	**0.005**
IP10	93	99.39	198.36	0.996	(0.985,1.008)	0.521
MCP1MCAF	93	404.86	833.79	0.811	(0.625,1.051)	0.114
MIP1a	93	0.98	1.93	0.86	(0.711,1.042)	0.124
PDGFbb	**93**	**0**	**12.43**	**0.524**	**(0.351,0.783)**	**0.002**
MIP1b	**93**	**19.2**	**33.54**	**0.703**	**(0.513,0.963)**	**0.028**
RANTES	93	0	3.64	0.892	(0.759,1.047)	0.163
TNFa	93	6.16	29.33	0.762	(0.532,1.093)	0.139
VEGF	93	36.33	96.65	0.968	(0.923,1.014)	0.169

PVR group is used as the reference group. Therefore, an odds ratio of greater than 1 means that the cytokine measurement is higher in RRD group. An odds ratio of less than 1 means that the cytokine measurement is higher in the PVR group.

Stepwise selection was used to build a multivariable regression model of potential predictive factors to differentiate between RRD and PVR. Candidate factors were retained using AIC. The output from the multivariable regression analysis is summarised in **Table 2.4** for retained factors with p values for association <0.05:

Stepwise approach		
Forwards	***Backwards***	***Both***
IL1β IL6 PDGFbb IL5 IL7 GMCSF	IL1β IL1ra IL4 IL7 IL8 IL9 IL10 IL12p70 IL17 Eotaxin GCSF IP10 MCP1MCAF MIP1b TNFa VEGF	IL1β PDGFbb IL5 IL7 GMCSF IL13

The cytokines highlighted with an asterix in **Table 2.3** are those that have a statistically significant odds ratio > 1 in all four analyses of RD vs. PVR: the univariate regression models and each of the three models based on stepwise selection. Those cytokines are found to be IL1β and IL7.

Results are presented in **Table 2.4** for the cytokines that were statistically significant in the univariate analyses and, of those selected by stepwise selection in both directions, the

cytokines that were statistically significant for the multivariable analysis. Results for both the RRD vs. MH and RRD vs. PVR comparisons are presented.

	Univariable			Multivariable	
Factor	**RRD vs MH**	**RRD vs PVR**		**RRD vs MH**	**RRD vs PVR**
IL-1β					
IL-1ra					
IL-2					
IL-4					
IL-5					
IL-6					
IL-7					
IL-8					
IL-9					
IL-10					
IL-12p70					
IL-13					
IL-15					
IL-17					
Eotaxin					
Basic FGF					
G-CSF					
GM-CSF					

IFN-γ					
IP10					
MCP-1					
MIP-1α					
PDGF-bb					
MIP-1β					
RANTES					
TNF-α					
VEGF					

Table 2.4. Summary of factors selected by stepwise selection.

The results from the multiple statistical analyses overlapped but gave strikingly different results with different methodologies. We therefore examined the correlation between levels of the different cytokines analysed, as the regression analyses input the cytokine levels as independent predictor variables and it is likely that the assumption of independence is violated (**Figure 2.3**). It is therefore also likely that the multiple cytokines found to be associated with RRD and PVR in the regression analysis are in fact correlated with each other. For instance IL7 strongly separates between RRD, MH and PVR in all univariate and multivariate comparisons, but is also strongly correlated with IL-9, -10, -11, -12 and -15 as well as PDGF and VEGF. This could explain the variable or absent inclusion of these factors in the regression models, as they do not independently associate with the different disease categories (and the strong association of IL-7 with RRD and PVR therefore masks their associations).

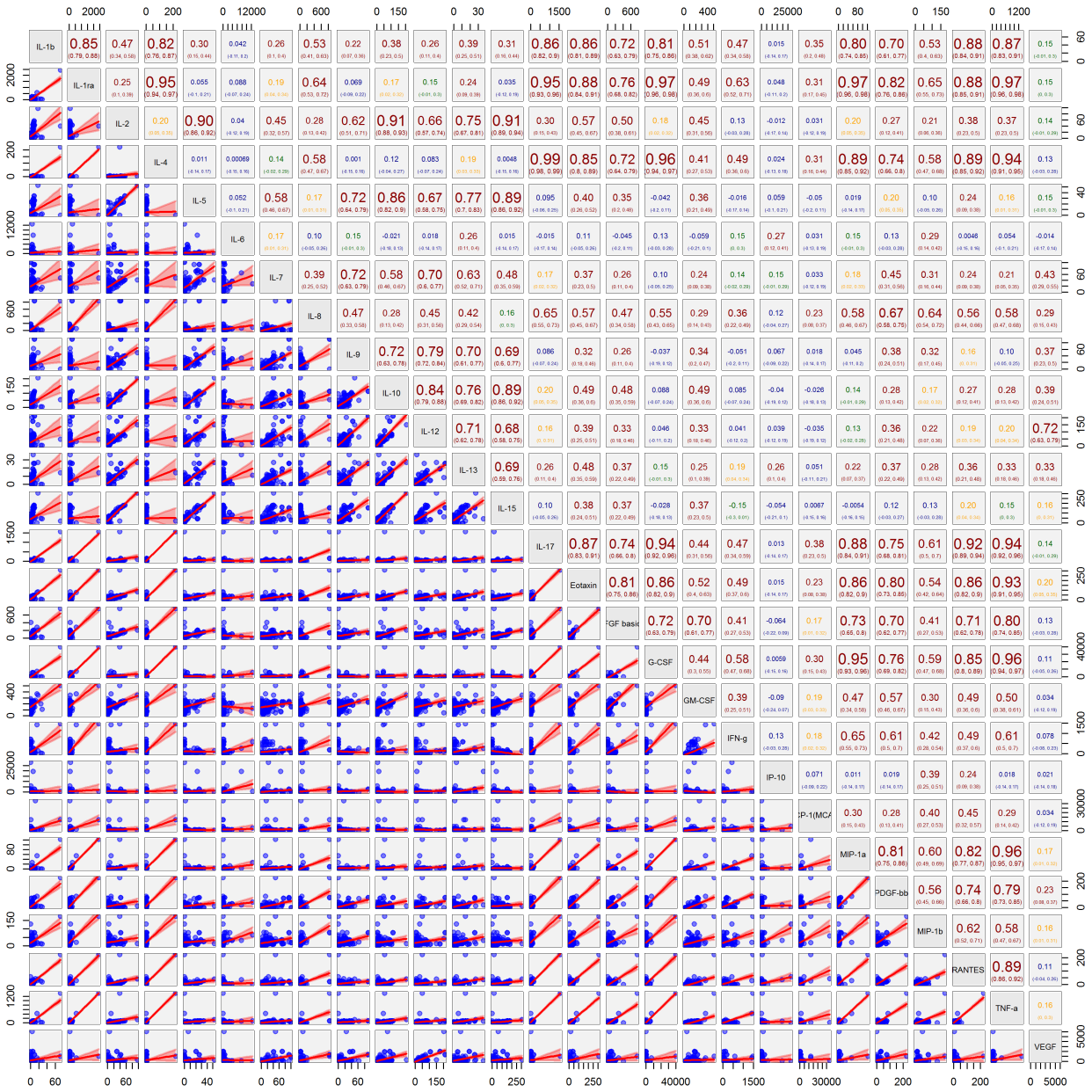


Figure 2.3. ***Pairwise correlations between cytokines***. The numerical values give the correlation coefficient and 95% CI for the correlation between that pair of cytokines, e.g. IL2 and IL5 have a correlation coefficient of 0.90 with 95% CI (0.86, 0.92). The colour and size of the number corresponds to the p-value of the correlation test. Red values are significant at the 1% level; orange values are significant at the 5% level; green values at the 10% level and blue values correspond to cytokine pairs that are not correlated with

each other (p-value >0.1). Overall the plot shows a high degree of correlation between the cytokines.

(vi) Results summary

The cytokines IL-1β and IL-7 were found to be significantly higher in the PVR group compared to the RD group. IL-1β is a pro-inflammatory cytokine and induces cell proliferation and differentiation. Raised levels of IL-1β have been reported in sub-retinal fluid and the vitreous in cases of PVR. IL-7 induces RPE gene expression and secretion of both MCP-1 and IL-8 in a time- and dose-dependent manner. Furthermore, IL-7 potentiates the production of MCP-1 and IL-8 from IL-1 and TNF-treated RPE cells, functioning as a pro-inflammatory peptide. IL-7 may synergize with IL-1 and TNF to induce chemotaxis of neutrophils, lymphocytes, and mononuclear phagocytes to sites of acute and chronic retinal inflammation. In addition IL-7 may co-stimulate B-lymphocytes with IL-1 to increase their ICAM-1 expression that enhances lymphocyte trafficking at the BRB and down regulates TGF-β that might otherwise suppress local inflammation and has been found in chronic retinal diseases. Therefore, IL-7 in conjunction with IL-1 and other local cytokines, may promote specific types of lymphocytes that infiltrate retinal tissue, such as CD4+ T lymphocytes and IL-2 receptor positive T lymphocytes[42]. Finally, IL-7 has been shown to have trophic effects on neural cells as well as increasing the number of astrocytes and microglia which may be important in retinal tissue integrity and responds to inflammation. This is in agreement with a study that found IL-7 to be raised in cadaveric vitreous of eyes with PVR[133].

IL-7 was also found to be significantly higher in the RD group compared to the MH group, as RD is thought to be a more inflammatory condition. Cytokines IL1ra, IL8, IL9, IP10 and MIP1b were also found to be higher in the RD group.

IL-8 is an inflammatory and angiogenic mediator that is produced by endothelial and glial cells in retinae with ischaemic angiogenesis. The major effector functions of IL-8 are activation and recruitment of neutrophils to the site of infection or injury. IP-10 induces migration of activated T lymphocytes and natural killer cells. IP-10 has been found to be raised in subretinal fluid samples obtained from patients who underwent scleral buckling surgery for primary rhegmatogenous RD[286]. IL-9 is secreted by CD4+ helper cells. It acts as a regulator of a variety of hematopoietic cells. This cytokine stimulates cell proliferation and prevents apoptosis. MIP-1b is crucial for immune responses towards infection and inflammation. They activate human granulocytes that can lead to acute neutrophilic inflammation. They also induce the synthesis and release of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α from fibroblasts and macrophages.

The increased levels of inflammatory and angiogenic factors in the vitreous of RD and PVR eyes are probably related to the breakdown of the BRB and the expression of inflammatory and angiogenic factors by cells within the vitreous, such as macrophages, monocytes, glial cells and retinal pigment epithelial cells.

Chemokines and angiogenic factors are expressed by hypoxic retina. Therefore, levels of these factors are expected to be raised in RD and even more so in PVR vitreous. The retina is not expected to be hypoxic in MH eyes.

The results show strong evidence for an angiogenic and inflammatory component in the development of PVR.

SECTION 2C

OBJECTIVE MEASUREMENT OF VITREOUS INFLAMMATION USING OPTICAL COHERENCE TOMOGRAPHY

(i) Principles of Optical Coherence Tomography

OCT is an established non-invasive imaging technique[287], able to provide high-resolution, three-dimensional cross-sectional images of ocular tissue, and is frequently used for the diagnosis and follow-up of various retinal diseases. This technique uses light to capture micrometre-resolution, allowing imaging of disease morphology. OCT is based on low-coherence interferometry, using near-infrared light. The use of long wavelength allows light to penetrate into the scattering medium of the ocular tissue where it is reflected back and detected.

Low-coherence interferometry is a non-contact optical sensing technology. OCT works by splitting a beam of low-coherence light into a reference arm and a sampling arm. The sample beam is reflected off the structures in the eye and is recombined with the reference beam using a Michelson interferometer as shown in **Figure 2.4**. The reflected optical data from each single scan point is interpreted by the interferometer as an interference pattern and recorded as a depth profile (A-Scan). By scanning the probe in a linear fashion across the sample, a cross-section (B-scan) is obtained. 3D volumetric

images can be generated by combining multiple cross-sections[288].

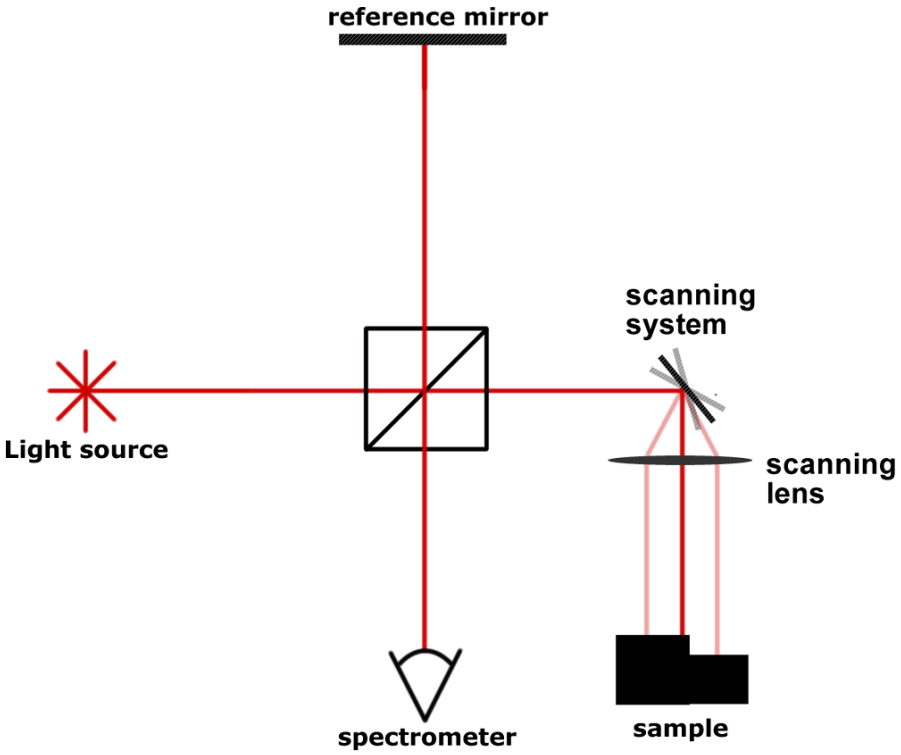


Figure 2.4. Schematic illustration of a low-coherence interferometry optical setup. *Taken from http://opticalengineering.spiedigitallibrary.org/article.aspx?articleid=1891707*

OCT is routinely used for detection and monitoring of conditions that compromise the retina. **Figure 2.5** shows an OCT scan through a healthy macula, showing clearly distinguishable reflective layers corresponding to the anatomical layers of the retina.

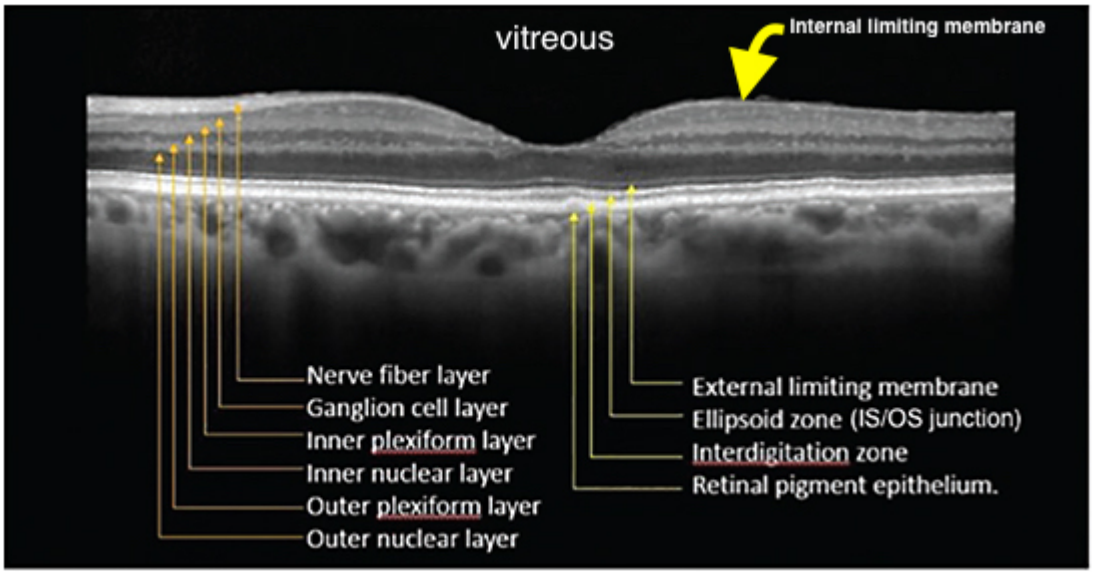


Figure 2.5. Spectral-domain optical coherence tomography cross-section of a healthy macula.
Taken and modified from http://www.ophthalmologymanagement.com.

Recently, quantitative assessment of OCT images is being investigated as an objective marker for clinical care of both posterior and anterior chamber disease. Tissue layers at varying depths and optical characteristics produce differing reflective intensities. Accordingly, OCT technology has been adapted for imaging the anterior chamber of the eye where it provides a resolution fine enough to visualise anterior chamber structures[289,290] and even individual inflammatory cells within the eye[291]. For example, degrees of anterior segment uveitic inflammation have been quantified with anterior segment OCT, showing a strong correlation with clinical grading scores[292]. Vitreous signal intensity from OCT images has also been able to objectively quantify the inflammatory activity in patients with uveitis[249]. Advances in these imaging techniques can help us to derive an objective measurement of inflammation, particularly for inflammatory conditions such as PVR.

(ii) Methodology

OCT image sets from two different patient groups were collected prospectively from the Birmingham Midland Eye Centre. Group 1 had stage 3 and 4 macula holes. Group 2 had RRD that went on to develop PVR. Those patients in group 1 with pre-vitrectomy and post-vitrectomy scans were included. Patients in group 2 with OCT images pre-vitrectomy surgery were included. Age, gender and diagnosis were recorded from information found on electronic medical records (Medisoft). Ethics approval for data collection and analysis were obtained from a UK National Health Service research ethics committee, and the research adhered to the tenets of the Declaration of Helsinki.

For each eye, OCT image sets were obtained using a spectral domain OCT system (Spectralis OCT, Heidelberg Engineering, Germany). Volume scans centered on the fovea were obtained with the point of maximum sensitivity on the vitreous side. Raw OCT data were exported from the Heidelberg Spectralis OCT system and imported into a software called "OCTOR". This software allowed for manual annotation and segmentation of an OCT image. In each case, three B-scans passing through the foveal central subfield (i.e., the 1 mm in diameter central subfield of the ETDRS grid) were manually segmented. These boundaries consisted of: 1) "vitreous top", the uppermost extent of the vitreous space as visualized on OCT, 2) internal limiting membrane (ILM), the inner boundary of the neurosensory retina, 3) retinal pigment epithelium (RPE)-inner, the inner boundary of the RPE, and 4) RPE-outer, the outer boundary of the RPE. The "vitreous space" (VIT) was defined as the space lying between the vitreous top and the ILM. The "RPE space" (RPE)

was defined as the space lying between the inner and outer boundaries of the RPE. These boundaries are shown in **Figure 1**.

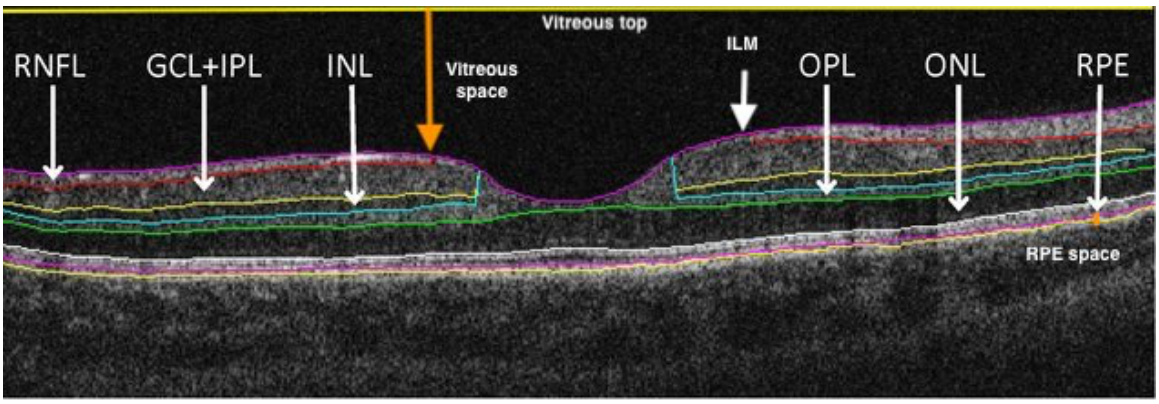


Figure 2.6. Illustration representing the layers for OCT segmentation. *Taken and adapted from https://www.researchgate.net/publication/283543538_The_Effect_of_Axial_Length_on_the_Thickness_of_Intraretinal_Layers_of_the_Macula.*

The intensity of the pixels within the annotated boundaries was measured and a mean intensity value generated. Mean intensity values were calculated for the vitreous space (termed "VIT-Absolute Intensity") and the RPE space (termed "RPE-Absolute Intensity") in the foveal central subfield. Intensity values for the vitreous and RPE spaces were then expressed as a ratio (termed "VIT/RPE-Relative Intensity"). All segmentation was performed by a single OCT grader (TM), masked to all clinical information at the time of grading. To assess reproducibility, the OCT image sets were also segmented by a second masked OCT grader (RC).

(iii) Statistical Analysis

For comparison of mean OCT intensity values between groups, the Wilcoxon related data test was used. For correlation of clinical vitreous haze scores with mean OCT intensity values, non-parametric Spearman testing was used. Multivariable regression was then used to model the effects of clinical characteristics on mean OCT intensity values. P values < 0.05 were considered statistically significant.

(iv) Results

(a) MH pre- and post-vitrectomy data

To validate the assessment of vitreous inflammation and examine the effects of vitrectomy on vitreous inflammation measured by OCT we compared measurements of vitreous/RPE intensity on pre- and post-vitrectomy OCT scans from patients with MH.

Table 2.5 Non-Parametric correlations between MH pre- and post-vitrectomy OCT data

	Correlations Spearman's rho (N = 12 in each case)							
		Vitreous intensity measurements in MH patients before surgery	**Intensity between the inner and outer boundaries of the RPE before surgery**	**Vitreous and RPE space intensity ratio before surgery**	**Vitreous intensity measurements in MH patients after surgery**	**Intensity between the inner and outer boundaries of the RPE after surgery**	**Vitreous and RPE space intensity ratio after surgery**	**Patient age**
Vitreous intensity measurements in MH patients before surgery	**Correlation Coefficient**	1	0.434	0.909**	-0.308	0.231	-0.266	0.330
	Sig. (2-tailed)	.	0.159	0	0.331	0.471	0.404	0.294

Intensity between the inner and outer boundaries of the RPE before surgery	**Correlation Coefficient**	0.434	1	0.175	0.287	0.531	0.294	0.091
	Sig. (2-tailed)	0.159	.	0.587	0.366	0.075	0.354	0.778
Vitreous and RPE space intensity ratio before surgery	**Correlation Coefficient**	0.909**	0.175	1	-0.273	0.084	-0.203	0.390
	Sig. (2-tailed)	0	0.587	.	0.391	0.795	0.527	0.210
Vitreous intensity measurements in MH patients after surgery	**Correlation Coefficient**	-0.308	0.287	-0.273	1	0.266	0.986**	0.141
	Sig. (2-tailed)	0.331	0.366	0.391	.	0.404	0	0.663
Intensity between the inner and outer boundaries of the RPE after surgery	**Correlation Coefficient**	0.231	0.531	0.084	0.266	1	0.203	-0.176
	Sig. (2-tailed)	0.471	0.075	0.795	0.404	.	0.527	0.585
Vitreous and RPE space intensity ratio after surgery	**Correlation Coefficient**	-0.266	0.294	-0.203	0.986**	0.203	1	0.200
	Sig. (2-tailed)	0.404	0.354	0.527	0	0.527	.	0.532

There was no evidence of any association between vitreous/RPE intensity ratio before and after vitrectomy (p=0.527) or age (p=0.210 before vitrectomy and 0.532 after vitrectomy) or gender (0.373 before vitrectomy and 0.727 after vitrectomy).

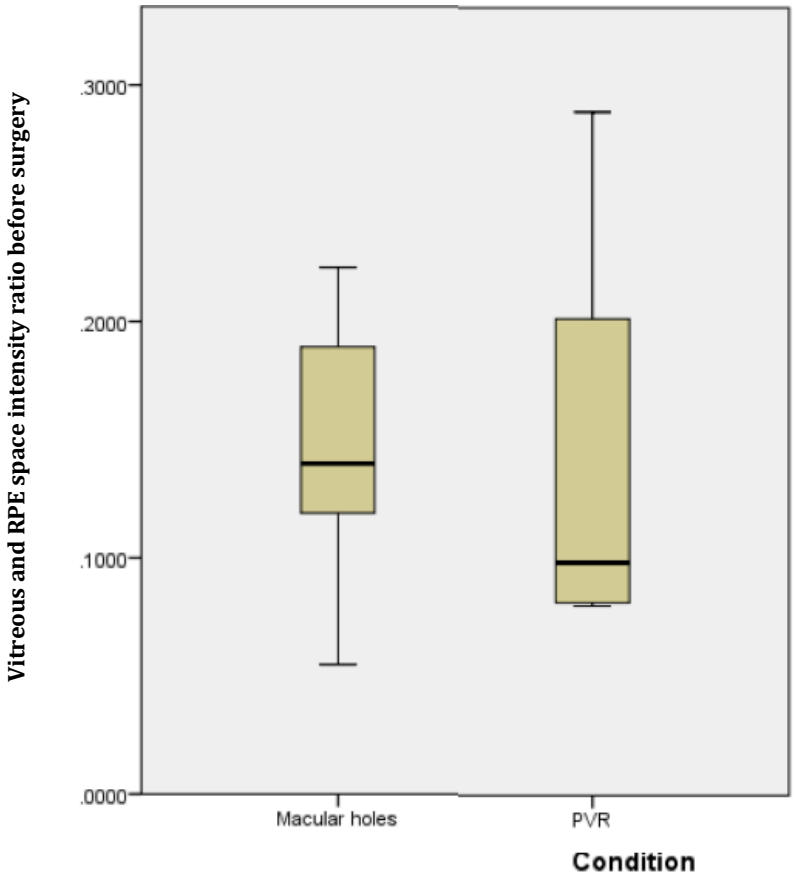
Comparisons between pre-operative and post-operative values were performed using the Wilcoxon related data test (**Table 2.5**), demonstrating that vitreous intensity and vitreous/RPE ratio were lower after than before vitrectomy.

Table 2.6 Pre-operative and post-operative comparison of MH group OCT data.

Comparison	**P value**	**Mean**	**95% Confidence interval**
Pre VIT against Post VIT	0.034	0.089 0.060	
Pre RPE against Post RPE	0.003	0.586 0.715	
Pre VIT/RPE against Post VIT/RPE	0.01	1.062 0.084	

(b) MH versus PVR at the time of surgery pre-operative data

Figure 2.7 Box Plot of Pre-VIT over RPE relative intensity for groups MH and PVR.



Condition		Pre_VIT_over_RPE_relative_intensity
MH	**Mean**	0.150
	N	12
	Std. Deviation	0.050
	Median	0.140
PVR	**Mean**	0.142
	N	4
	Std. Deviation	0.100
	Median	0.099

Table 2.7 Pre-operative OCT data for MH and PVR.

There is weak evidence to suggest a difference between MH and PVR pre-operative vitreous density (P = 0.52). There is weak evidence that MH and PVR pre-operative RPE intensity differ (P = 1.00).

(v) Results Summary

There was no evidence of any difference in vitreous/RPE ratio between MH and PVR patients before vitrectomy. Vitrectomy clearly reduces vitreous reflectivity on OCT, but there does not seem to be a detectable signal otherwise related to PVR, as the pre-op intensity did not differ between PVR and the MH patients.

SECTION 2D

Discussion and interim conclusion

Multiple cytokines found to be associated with RRD and PVR in the regression analysis are in fact linked. For instance IL-7 strongly separates between RRD, MH and PVR in all univariate and multivariate comparisons, but is also strongly correlated with IL-9, -10, -11, -12 and -15 as well as PDGF and VEGF, which could explain the variable or absent inclusion of these factors in the regression models, as they do not independently associate with the different disease categories.

The evidence presented shows that numerous cytokines, growth factors and chemokines have been implicated in contributing to the development of PVR and a panel of biomarkers rather than a single biomarker is likely to mark the cascade of events that occur during the development of PVR.

The inflammatory cell content of developing PVR subretinal and epiretinal membranes is high. Vitreous haze is a valuable sign of inflammation. Although OCT does not provide the exact composition of the vitreous compartment, higher vitreous signal intensity will be indicative of increased scattering of light and therefore increased density of particles within the space. Conversely, lower vitreous signal intensity will reflect a relatively clearer vitreous composition with fewer suspended particles.

Vitrectomy reduces vitreous reflectivity (VIT/RPE-Relative Intensity) on OCT. This can be seen when comparing the pre-operative and post-operative MH group. Although, this is of limited clinical significance, it functions as a positive control demonstrating that our analysis assesses vitreous composition.

OCT derived objective indices could detect early signs of vitreous inflammatory cells in PVR that are not visible on biomicroscopy. The use of quantitative imaging in PVR could also measure response to treatment or progression of the condition at a tissue level.

These results show that VIT/RPE-Relative Intensity has not been useful as an objective measure of disease activity in patients with established PVR. Future work carried out prospectively would provide us with a larger amount of data, as OCTs are not routinely

carried out on detachment cases. Pre-operative OCT on patients with RRD may show a stronger signal if active vitreous inflammation is present at that point.

CHAPTER 3

METABOLOMIC ANALYSIS OF HUMAN VITREOUS

SECTION 3A

Metabolomics experimental work

(i) Introduction and rationale

Metabolomics is used to identify endogenous changes in molecular metabolites within biological systems that result from changes in gene expression and protein activity. The eye is an anatomically discrete, immune-privileged, site making metabolomic analysis of ocular fluids/tissues possible in ocular disease. Metabolic products from inflammatory processes accumulate in the vitreous humour making this an ideal structure for sample collection and analysis.

Metabolomic analysis provides a systematic view of the development of PVR at the metabolite level. Inflammation and cell proliferation are key processes involved in the development of PVR and the identification of related key metabolites may provide us with future biomarkers for PVR diagnosis and treatment. There is no effective treatment for PVR. So it is important clarify the molecular mechanisms involved to help provide new insight into disease aetiology and identify new therapeutic targets for novel drugs for the treatment of PVR. This will allow the related biological pathways of RRD and PVR to be better elucidated.

Metabolomics has been used to differentiate ocular inflammatory diseases[293,294]. The

investigation of eye disease using such approaches has been reported but these publications are limited in scope and number. Nevertheless, significant changes in a number of metabolites in rabbit eye aqueous humour[295] and in rat lens[296] has been seen after UV-B exposure, and a rise in lactate in vitreous humour has been observed in a rabbit model of ocular hypertension[297]. A small human study comparing RD and PVR eyes concluded that markers of cellular proliferation and inflammation, such as uric acid involved in purine metabolism and D-glucuronolactone involved in ascorbate and aldarate metabolism, were raised in both groups [298]. Other biomarkers, such as L-carnitine and ascorbate, were down-regulated in the vitreous of RD and PVR eyes when compared to vitreous from healthy donor eyes[298]. There were also biomarker differences between the two patient groups, such as urea and cyromazine[298].

(ii) Hypotheses

1. Molecular markers of cellular proliferation and inflammation are raised in RRD and PVR.
2. These rises are more marked in eyes with PVR when compared to RRD due to the exaggerated cellular response characteristic of this condition.
3. Other markers are high in control eyes and down regulated in RRD and PVR eyes, such as those that down regulate inflammatory and fibrotic processes.

(iii) Aims

To differentiate the metabolites in vitreous samples from RRD and PVR patients, in

addition to identifying biomarkers of the pathological changes underpinning each condition.

(iv) Principles of high-resolution nuclear magnetic resonance spectroscopy

Metabolomics is a powerful means of exploring the complex molecular interactions that impact on disease[299]. Metabolomics is able to identify and measure the activity and flux of endogenous metabolites in cells, tissues and other bio-fluids with the recent technical advances in nuclear magnetic resonance (NMR) and mass spectrometry[294]. Metabolites include the products and intermediates of metabolism of carbohydrates, peptides and lipids, which may be altered in disease. It is based on the concept that the metabolic properties of tissues predispose to or are altered by disease processes, and these changes are reflected in characteristic molecular patterns (a metabolomic profile). Pattern recognition methods (principal component and partial least-squares analysis) allow the NMR data to be reduced and analysed quantitatively to provide pattern recognition maps that can assist in disease classification[300].

NMR spectra from bio-fluids are highly complex, containing signals from hundreds of metabolites that represent many key biochemical pathways[299]. These changed metabolites may be potential biomarkers for disease diagnosis and treatment. Metabolomics profiles reveal the consequences of changes in both gene and protein expression[301]. Many diseases are complex polygenic conditions, but the genetic component does not account for all of the risk of disease. It may be most appropriate to view gene expression and metabolism as inseparably linked when trying to understand

human diseases. Metabolomic analysis systematically illustrates the instantaneous pathological or physiological changes in an organism[294]. Recently, research using metabolomic approaches to analyse disease related biomarkers has attracted a great deal of interest[302-309].

Metabolomics combines either high-field nuclear magnetic resonance or mass spectrometry of bio-fluids with pattern recognition analysis (principal components analysis) to generate a dataset. High-field NMR minimises the electronic noise in the detection system, thus maximising signal from a small volume of sample[310]. Prior to analysis, proteins are removed by filtration during sample preparation as they can interfere with the quality of the spectrum derived from low-molecular-weight metabolites. Removal enhances the quality of the derived NMR spectrum[311].

It is suggested that bioinformatic analysis of the data can be translated into a clinical test that could help develop future therapy[312].

(v) Methodology

Patients:

A total of 66 vitreous humor samples were obtained from patients undergoing pars plana vitrectomy for macular hole (n = 21), retinal detachment who did not subsequently develop PVR (n = 31) and RRD who subsequently developed PVR (n = 14) as described in **Section 1K**. None of these patients were on any topical or systemic corticosteroid therapy

or immunosuppressive therapy at the time of vitrectomy. MH eyes act as a control, as no or minimal cellular proliferation and inflammation are apparent in this condition.

NMR sample preparation:

45uL of spun vitreous from the Eppendorf tubes was placed into individual champagne vials and mixed with 15uL of 4x NMR buffer. The final diluted sample contained D2O (10%) NaCl (150 mM), trimethylsilyl 2,2,3,3-tetradeuteropropionic acid (TMSP) (0.5mM), and sodium phosphate (20 mM) pH 7.4. The vials were then stored at -80°C.

Metabolomic analysis:

Samples were thawed and a standard volume (35ul) was transferred using an Anachem sampling-handling instrument to 1.7mm ID NMR tubes. These were capped and wiped clean before loading into the spectrometer via the Bruker Samplejet sample-handling robot.

One-dimensional 1H spectra were acquired at 298 °K with 128 scans using a standard Bruker NOESY 1D pulse sequence with pre-saturation water suppression Bruker DRX 600MHz NMR spectrometer equipped with a cryoprobe. Each sample was automatically shimmed prior to acquisition. Chemical shifts were calibrated with respect to the chemical shift position of the TMSP resonance.

Pre-processing of the spectra was done using NMRLab. Spectra were phased and baselines corrected. Spectra were segmented into 0.006 ppm (~2.5 Hz) chemical shift 'bins' between 0.2 and 10.0 ppm. Bins between spectral region 4.5 and 5.0 ppm were

excluded to eliminate residual water signals due to variation in water suppression efficiency. The spectra were overlaid and normalized using probabilistic quotient normalization (PQN), to account for differences in overall spectral intensities. They were then compiled into a matrix, with each row representing an individual sample. The columns were mean centered before multivariate analysis.

(VI) Statistical analysis

Multivariate statistical tools including unsupervised principal components analysis (PCA) of the pre-processed spectra and partial least square discriminant analysis (PLS-DA) were performed using PLS_Toolbox (Version 4.1; Eigenvector Research, Manson, WA) for MATLAB (version 7.5; The MathWorks, Cambridge, UK) and the software package SIMCA (Version 8, Umetrics AB, Ume°a, Sweden).

PCA reduces the dimensionality of data and summarizes the interrelation between multiple NMR spectra using Pareto-scaled data. This required calculation of new variables that are weighted linear combinations of the original chemical shift bins.

PLS-DA is a validated supervised analysis technique that was used to facilitate the detection of metabolite profiles consistently present in the vitreous and to evaluate separation between groups based on known clinical diagnosis. The PLS-DA model was cross-validated using Venetian blinds, a method which re-assigns randomly selected blocks of data to the PLS-DA model to determine the model's accuracy. Provisional assignment of the major peaks in the spectra was done using Chenomx NMR Suite version 4 (Chenomx, Edmonton, Alberta, Canada) in conjunction published literature. Chenomx is

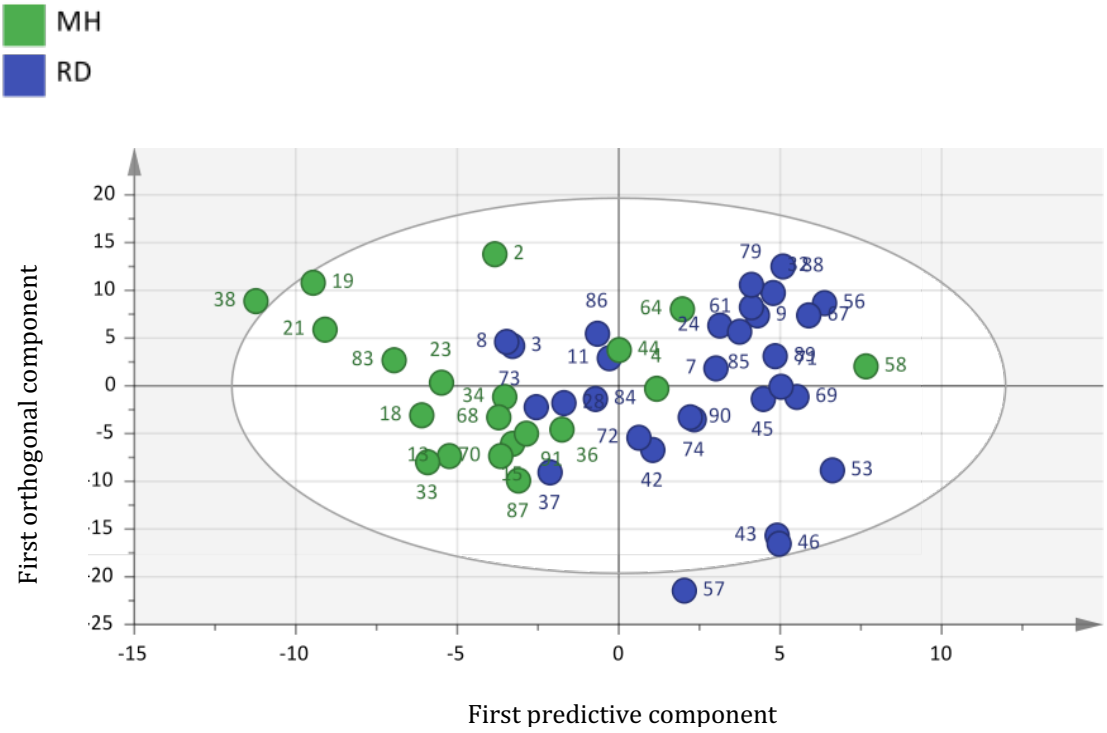
a NMR spectral analysis program for metabolic profiling, providing a library of compounds present in biofluid. Comparison of peak heights of selected metabolites was made using an unpaired Student T test.

From the loading plots of orthogonal projection to latent structures discriminate analysis (OPLS-DA), various metabolites could be identified as being responsible for the separation between clinical groups, and were therefore viewed as differentiating metabolites. Potential markers of interest were extracted from the combining S- and VIP-plots that were constructed from the OPLS analysis, and markers were chosen based on their contribution to the variation and correlation within the data set. With the completion of the OPLS-DA analysis, pathway analysis was performed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca). The pathway was constructed based on the potential candidates extracted from OPLS-DA analysis.

(vii) Results

To examine the metabolic changes associated with retinal detachment and the subsequent development of PVR we compared the NMR spectra of vitreous samples from patients with macula hole with samples from patients with retinal detachment who did not develop PVR and then we compared samples from patients with retinal detachment who did and did not go on to develop PVR.

Figure 3.1 Score plot of MH vs RD



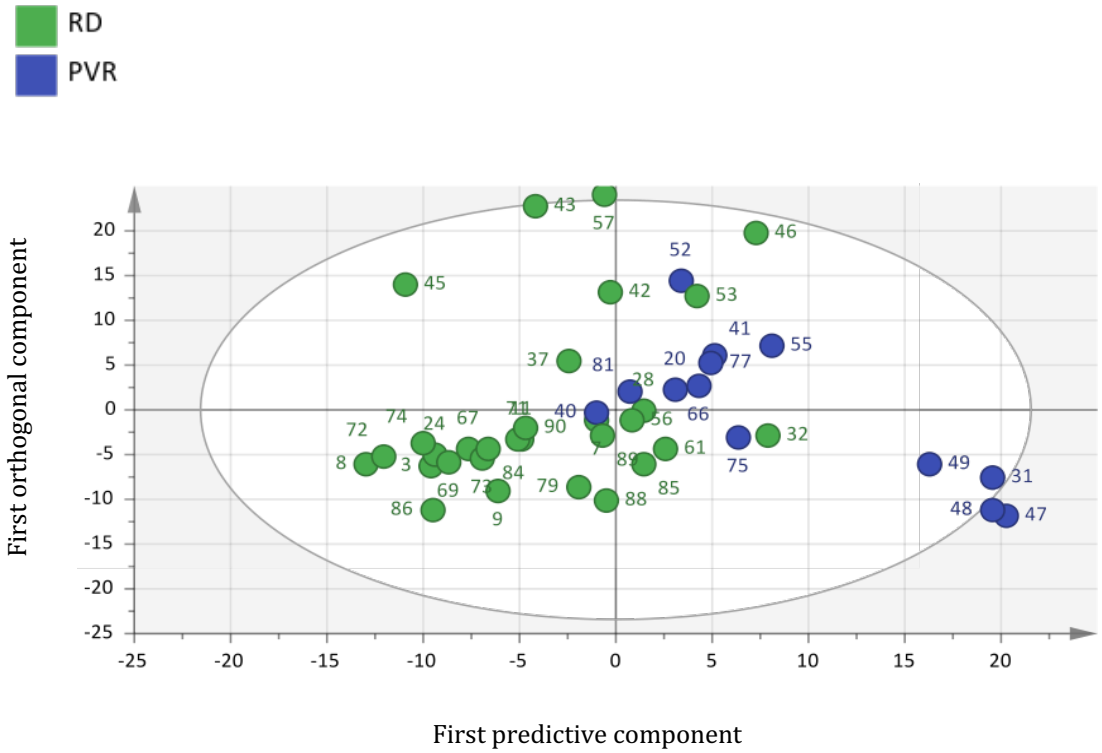
After pre-processing of the NMR spectra to align, normalize and bin them we performed a multivariate analysis on all the NMR bins using Orthogonal Partial Least Square Discriminant Analysis (OPLSDA) to compare the metabolic fingerprints of various groups of patients. The points plotted in Figure 3.1 and 3.2 relate to tube position in the NMR analysis. We had 91 vitreous samples and we acquired a spectrum for each. We acquired good spectra for all, with no protein breakthrough from the filters

Figure 3.1 shows a scatter plot of the two major latent variables in the data from MH and RD patients. Latent variables in PLSDA are the equivalent of principle components in a PCA analysis. Whereas PCA includes all the data points, PLSDA selects data that correlate and give the maximum difference between the classes being compared and so

these sets of data are the latent variables, which are inferred to be within the data by the PLSDA modelling.

While the graph suggests some separation of these groups cross-validation, in which multiple models are built leaving out 1/7th of the data, suggested this was not significantly different (p=0.58). Hotelling's T^2 is multivariate generalisation of Student's t-distribution. It provides a tolerance region for the data in a two-dimensional score plot. R^2 measures how well the model fits the data. **Figure 3.1** has an R2Xo value of 0.208. A large R^2 value close to 1 suggests good modelling. R2x(1) is 0.0773. R2x(1) is the R^2 value for the data along the axis that separates the classes. R2xo is the R^2 in the orthogonal direction, the axis that describes the variation within the data. This is useful to see how much variability there is in the data.

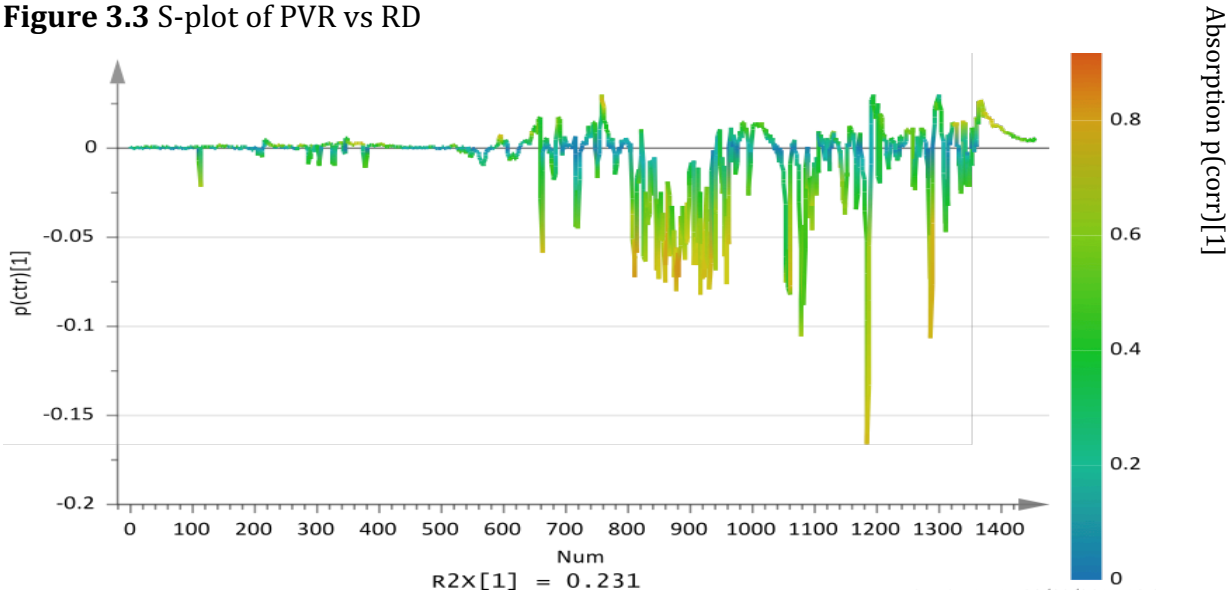
Figure 3.2 Score plot of RD vs PVR



We repeated this OPLSDA analysis in **Figure 3.2** but this time comparing PVR and RD. **Figure 3.2** shows a scatter plot of the two major latent variables in the data from PVR and RD patients. While there were fewer samples in these groups than in the previous analysis a separation was seen and cross validation gave p=0.039, suggesting there was a robust and reproducible difference between these two clinical groups. Vitreous samples 31 and 49 came from patients that had a retinal detachment secondary to blunt trauma. Samples 48 and 47 had retinal detachments with no unusual characteristics. **Figure 3.2** has an R2Xo

value of 0.272. R2x(1) is 0.231.

Figure 3.3 S-plot of PVR vs RD



In further analysis of these data we produced an S-plot (**Figure 3.3**), which indicates the regions of the NMR spectra which were discriminating between PVR (negative) and RD (positive) in the principle component analysis (**Figure 3.2**). The left *y-axis* represents p(ctr)[1], the covariance between a variable and the classification score. It indicates if an increase or decrease of a variable is correlated to the classification score. The right *y-axis* shows p(corr)[1], the correlation coefficient between a variable and the classification score (i.e. the normalized covariance). It gives a linear indication of the strength of the correlation. As the correlation is independent of the intensity of the variable, it will be a better measure for the reliability of the variable in the classification process. The *red colour* stands for the highest absolute value of the correlation coefficient. Strongly discriminating variables have a large intensity and large reliability. The x-axis represents the chemical shift. From this, together with a listing of the VIP scores (Variable Importance) of the NMR bins, we used the Human Metabolome Database (www.hmdb.ca) and NMR spectral analysis from Chenomx Inc Version no. 8.2 (Chenomx, Edmonton,

Alberta, Canada) to provisionally identify metabolities which may be discriminating between these patient groups.

Table 3.1. SIMCA model of MH versus RD.

Metabolite	Probability	Average/mM (MH)	Average/mM (RD)	Std. dev. (MH)	Std. dev. (RD)	Fold change
Alanine	0.234	0.065	0.078	0.021	0.033	1.192
***Creatinine**	**0.023**	**0.049**	**0.033**	**0.019**	**0.018**	**0.673**
Formate	0.236	0.051	0.043	0.018	0.018	0.845
Glucose	0.451	1.823	1.674	0.538	0.546	0.918
Glutamine	0.334	0.280	0.312	0.089	0.091	1.115
Lactate	0.394	1.656	1.887	0.582	0.832	1.140
***Methanol**	**0.020**	**0.088**	**0.139**	**0.028**	**0.071**	**1.578**
Pyruvate	0.585	0.047	0.066	0.030	0.093	1.409

Table 3.1 was derived from a model of MH versus RD on SIMCA. The only differences between these two groups was the level of creatinine and methanol. Creatine was found to be higher in the MH group and methanol was higher in the RD group.

Table 3.2 was derived from a model of PVR versus RD on SIMCA. The most significant metabolities are highlighted with an asterix; those being 2-hydroxyvalerate, 2-

phosphoglycerate, alanine, alloisoleucine, glutamine, histidine, methanol, urea, valine and myo-inositol. All these metabolites are higher in the PVR group.

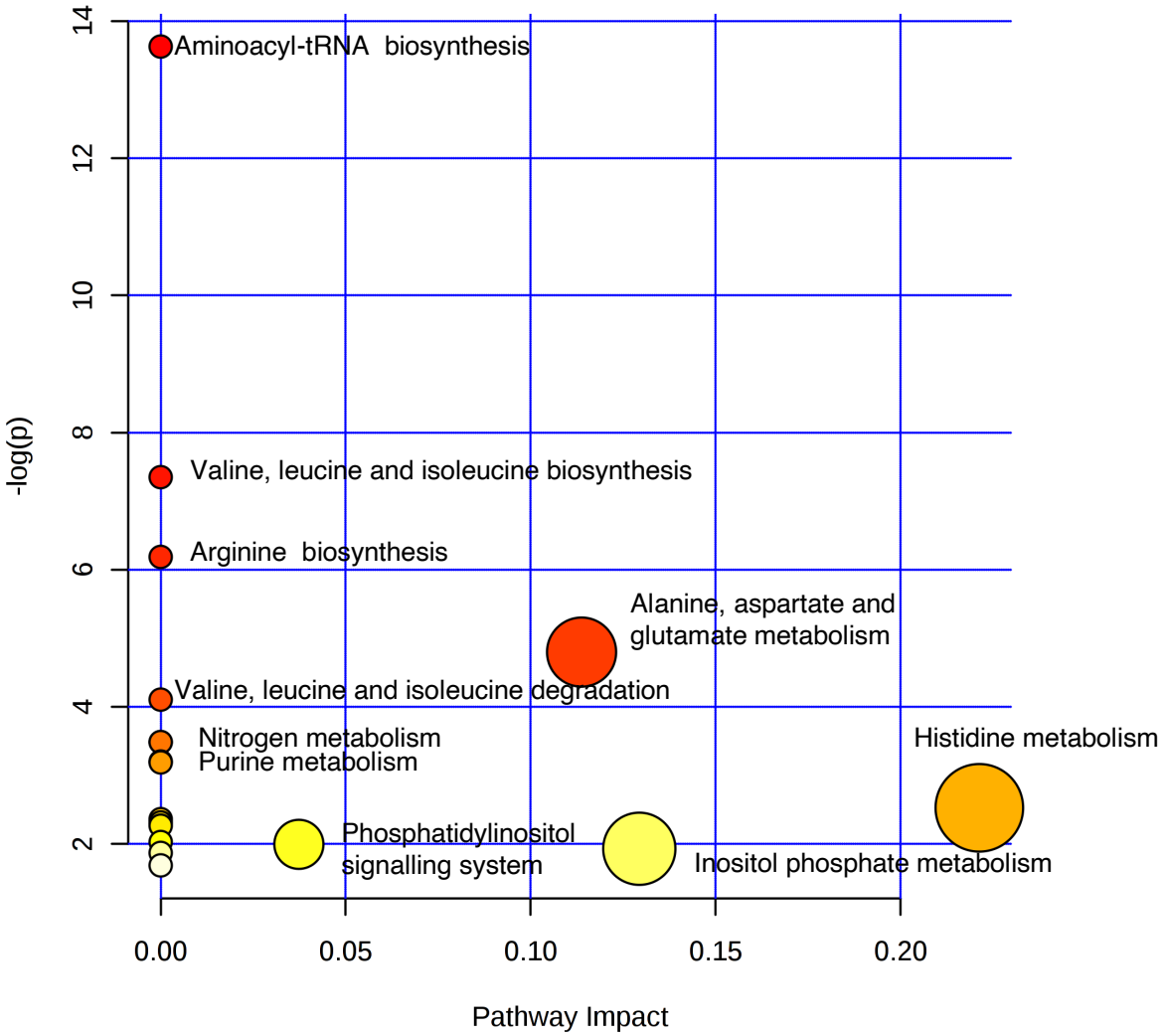
Table 3.2. SIMCA model of PVR versus RD.

Metabolite	**P value**	**Average/mM (RD)**	**Average/mM (PVR)**	**Std. dev. (RD)**	**Std. dev. (PVR)**	**Fold change**
2-Hydroxybutyrate	0.285	0.036	0.046	0.018	0.025	1.276
***2-Hydroxyvalerate**	**0.051**	**0.030**	**0.165**	**0.019**	**0.215**	**5.471**
***2-Phosphoglycerate**	**0.017**	**0.260**	**1.975**	**0.124**	**1.939**	**7.593**
4-Aminobutyrate		0.027				-3721.8
Acetaminophen	0.806	0.013	0.010	0.012	0.001	0.819
Acetate	0.772	4.153	3.607	4.705	5.678	0.869
***Alanine**	**0.003**	**0.080**	**0.158**	**0.045**	**0.113**	**1.971**
***Alloisoleucine**	**0.009**	**0.015**	**0.104**	**0.011**	**0.112**	**6.845**
Citrate	0.704	1.119	0.959	1.013	0.848	0.857
Creatinine	0.092	0.037	0.057	0.021	0.051	1.565
Ethanol	0.653	0.714	0.140	1.687	0.092	0.196
Formate	0.555	0.043	0.046	0.016	0.014	1.084
Gluconate		0.101		0.065		-977.778
Glucose	0.114	1.765	2.343	0.976	0.977	1.327
***Glutamine**	**0.002**	**0.294**	**0.847**	**0.101**	**0.766**	**2.882**
***Histidine**	**0.029**	**0.017**	**0.105**	**0.006**	**0.143**	**6.351**
Isopropanol		0.0256	0.004	0.067		0.135
Lactate	0.717	1.786	1.905	0.868	0.779	1.066
***Methanol**	**0.009**	**0.115**	**0.229**	**0.071**	**0.198**	**1.992**
Phenylalanine	0.105	0.019	0.034	0.011	0.003	1.763
Pyruvate	0.880	0.071	0.077	0.086	0.041	1.079
S-Sulfocysteine	0.252	0.063	0.516	0.001	0.586	8.142

Succinate	0.135	0.013	0.052	0.013	0.130	3.938
Tyrosine	0.960	0.027	0.026	0.017	0.017	0.975
***Urea**	**0.024**	**0.365**	**0.785**	**0.188**	**0.292**	**2.148**
***Valine**	**0.019**	**0.061**	**0.116**	**0.031**	**0.061**	**1.892**
***myo-Inositol**	**0.006**	**0.060**	**0.094**	**0.019**	**0.016**	**1.560**

We then entered the list of major discriminating metabolites into the Metaboanalyst software which generated the pathway plot shown in **Figure 3.4**. **Figure 3.4** shows all matched pathways according to the p values from the pathway enrichment analysis and pathway impact values from the pathway topology analysis. Each circle represents a different pathway; circle size and colour shade are based on the pathway impact and p-value respectively. Colours vary from yellow to red, red being the most significant. The *x*-axis represents the pathway impact value computed from pathway topological analysis, and the *y*-axis is the -log of the *P*-value obtained from pathway enrichment analysis. The pathways that were most significantly changed are characterised by both a high -log(*p*) value and high impact value (top right region).

Figure 3.4 Pathway analysis



Amino acid metabolism dominates the difference with alanine and aspartate featuring, but Aminoacyl-tRNA biosynthesis has a high impact and importance (-log(p)). Other pathways such as histidine and nitrogen also featured which, overall suggests amino acid degradation pathways may be different in the two groups of patients.

(viii) Results summary

There is strong evidence that metabolites 2-hydroxyvalerate, 2-phosphoglycerate, alanine, alloisoleucine, glutamine, histidine, methanol, urea, valine and myo-inositol are higher in the PVR group. These metabolites are involved in amino acid metabolism and glutamate metabolism.

SECTION 3B

Discussion and interim conclusion

Aminio acid metabolism is increased in association with PVR which may relate to an increase in IL-7 and IL-1β (refer to Section 2B(v)) associated with photoreceptor apoptosis and point to an excitotoxic mechanism of death.

Aminoacyl tRNA synthetases catalyze the ligation of amino acids to their cognate tRNAs. Their catalytic activities determine the genetic code, making it essential for protein synthesis and cell viability. Aminoacyl tRNA synthetases act as regulators and signalling molecules in various immune diseases, such as autoimmune diseases, infectious diseases, and tumour immunity[313]. Aminoacyl-tRNA biosynthesis is highly upregulated in cancer metabolism[314], perhaps because of the increased demand for protein synthesis in cancer. Proliferation of fibrocellular membranes in PVR would have a similar effect on protein synthesis.

Glutamate causes migration and proliferation of RPE and glial cells, key features of PVR development. Glutamate is an excitatory neurotransmitter in the retina, which after its

release from neurons is cleared from the extracellular environment via uptake by Muller cells subsequently transforming into glutamine. Glutamate levels increase secondary to ischaemia and cell death. Retinal detachment induces rapid, localized alterations in the glutamatergic system of the neural retina that are consistent with a massive efflux of neuronal glutamate in Muller cells and concomitant alterations in glutamate metabolism[315]. An acute efflux of neuronal glutamate in detached retina could contribute to excitotoxicity and to the initiation of structural alterations and changes in gene expression[316,317]. Glutamate antagonists are being considered as a potential candidate in the treatment of PVR[318].

The metabolites detected are involved in the glycolysis pathway. Many cells use aerobic glycolysis during rapid proliferation, which suggests it may play a fundamental role in supporting cell growth during the development of PVR.

Figure 3.5 Glycolysis pathway taken from https://www.metabridge.org/

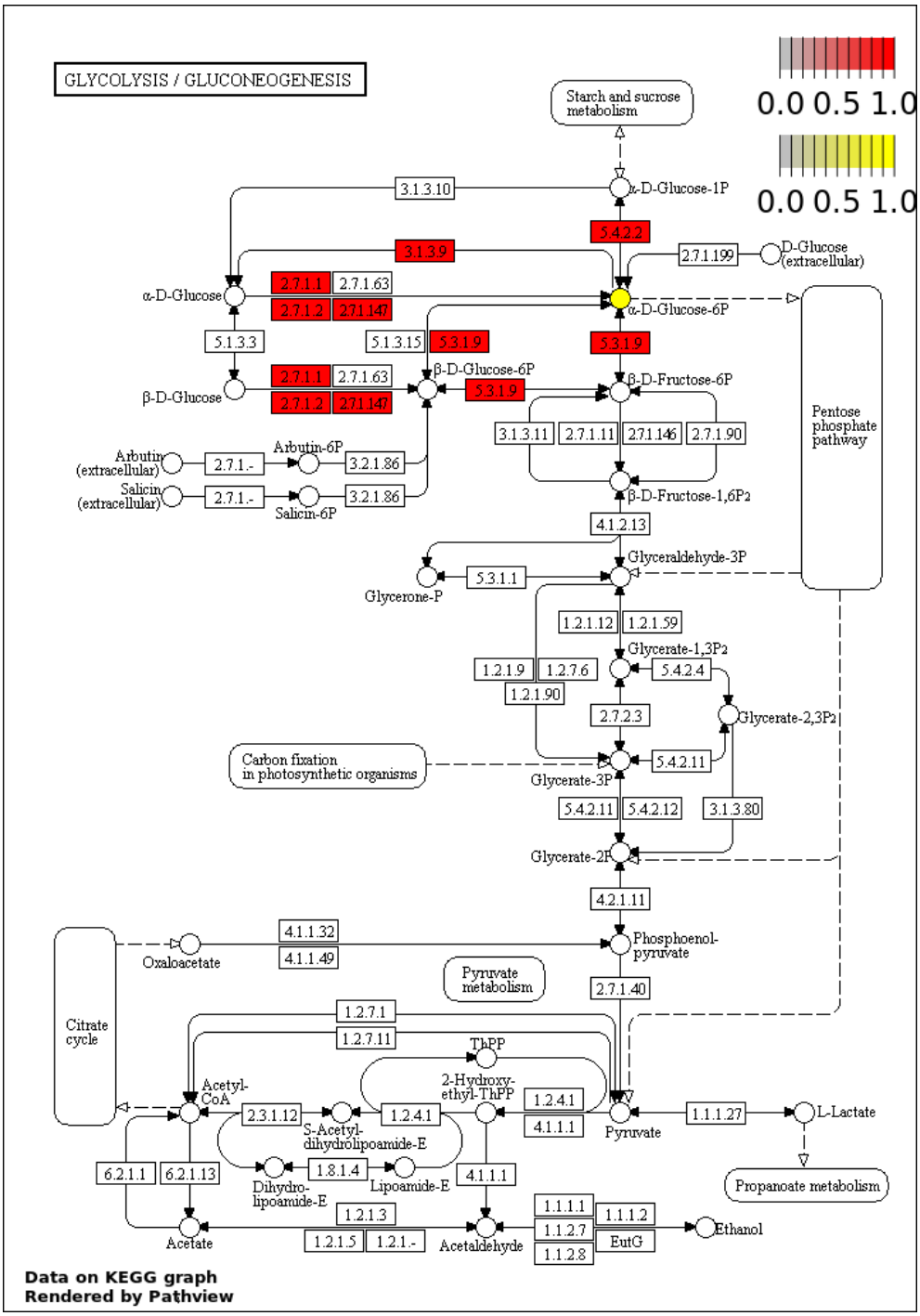
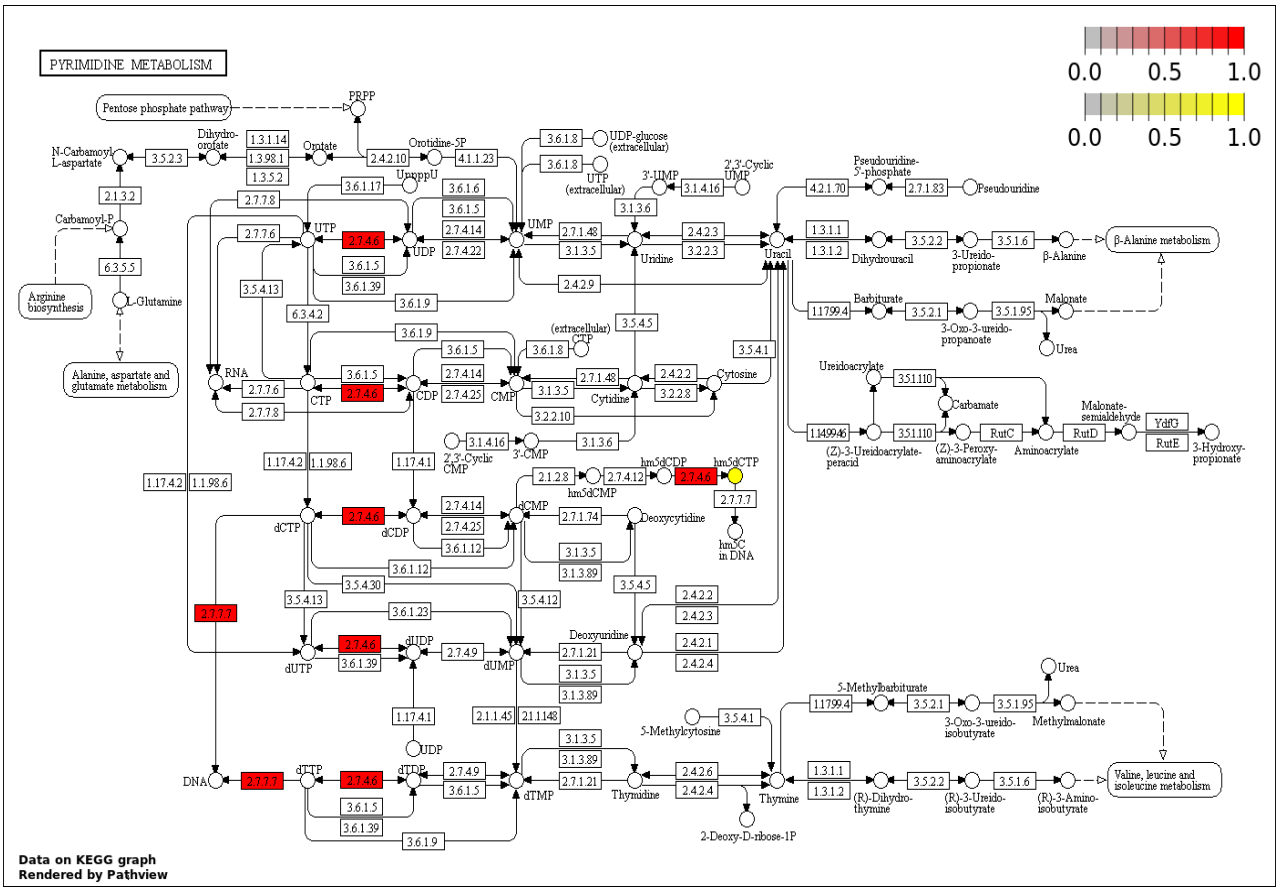


Figure 3.6 Pyrimidine metabolism taken from https://www.metabridge.org/



Pyrimidine metabolism is increased in PVR likely due to increased protein synthesis and cellular metabolism. The numbers in the pathway represent different proteins within a program defined gene list. The matching proteins are highlighted in red.

CHAPTER 4

IN-VITRO VALIDATION OF INFLAMMATORY MODEL

SECTION 4A

(i) Introduction

PVR formation is associated with a sequence of underlying inflammatory and fibrotic changes. Serum factors released into the vitreous stimulate the inflammatory phase of PVR development[41]. PVR retinal fibrosis is initiated by fibroblasts derived from RPE cells that undergo EMT and begin collagen and ECM deposition[27]. EMT and fibrosis is associated with vitreal accumulation of inflammatory cells[42] Pro-fibrogenic cytokines such as IL-6, PDGF and VEGF have been shown to induce collagen synthesis *in vitro*[52]. Plasma fibronectin induces the deposition of a fibroblast-derived collagen matrix.

After retinal detachment, transformed cells in PVR membranes differentiate into myofibroblasts[65,66]. Alpha-smooth muscle actin intermediate filament synthesis induces contraction and thus retinal redetachment. Vimentin is a major cytoskeletal component of mesenchymal cells and therefore a marker of EMT. Markers of EMT would be expected to be higher in PVR eyes as compared to eyes that do not develop PVR.

The cytokines selected for analysis were based on preliminary results from luminex analysis of the vitreous and not the final results.

(ii) Hypothesis

We hypothesise that IL-6, VEGF and PDGF would induce EMT and collagen expression in RPE cells in vitro. We also predict that with increasing concentrations of IL-6, VEGF and PDGF would cause increasing amount of cell proliferation and extracellular matrix formation.

(iii) Aims

RPE cell proliferation is a major component of PVR formation. The aim is to evaluate the effects of IL-6, VEGF and PDGF on EMT and fibrosis of human RPE cells in a cell culture system. Levels of cellular proliferation and EMT were quantified by cell counting and polymerase chain reaction (PCR) for EMT and fibrosis-associated mRNA. The concentrations used are based on levels found in the vitreous samples tested during the Luminex experimental work (Chapter 2, Section 2B).

SECTION 4B

RETINAL PIGMENT EPITHELIAL CELL CULTURE AND POLYMERASE CHAIN REACTION

(i) Culture of ARPE cell line

Media.

Ready prepared DMEM:F12 medium from Sigma Lab was supplemented with 1ml of 200mM glutamine and 10ml of heat-inactivated foetal bovine serum to make 100ml of complete medium. This was stored in an incubator at 37°C.

Growing primary cell cultures to a confluent monolayer, then growing subcultures.

A 10ml pipette was used to pipette 20ml into a universal container and 5ml into a 25cm^2 flask. An ampoule of ARPE-19 cells taken from the nitrogen store was placed into a small-lidded beaker of warm water. ARPE-19 cells were purchased from the American Type Culture Collection (ATCC CRL-2302). In the lab, the ampoule was dried and wiped with a tissue soaked in alcohol. Using a pipette the contents of the ampoule were emptied into the universal container containing the warm medium. This was mixed by gently pipetting up and down. This was spun at 640g for 10 minutes to pellet the cells. The supernatant was removed and1 ml of medium added from the 25cm^2 flask. This was mixed and transferred back to the flask. The flask was labelled with the cell type, passage number and date. ARPE-19 cells were incubated at 37°C in a humidified environment containing 5% CO_2.

The cells were initially grown in a 25ml^2 flask then passaged into a 75ml^2 flask. The cells were checked under the microscope to ensure that a complete monolayer had formed on the base of the flask. The used medium was removed from the flask using a pipette and replaced with the same volume of PBS. The flask was given a gentle swirl to rinse the cells and then the PBS was removed with the same pipette. 0.5ml of defrosted trypsin-EDTA was added to the 25cm^2 flask. The top on the flask was replaced. The flask was rocked carefully to ensure that there was a thin layer of trypsin-EDTA at the base of the flask. The flask was placed in an incubator for 2 to 15 minutes. The cells were checked under the microscope at 5 minutes to see if the cells had dislodged. If not, they were left to incubate for longer or more trypsin was added if necessary. 20ml of medium was added to the

25cm^2 flask, mixed gently and 5ml was removed and placed in a 75cm^2 flask. A further 7ml of medium was added to the 75cm^2 flask. The cells were grown in a 75ml^2-sized flask until they were 70-80% confluent. The confluency of the cells were checked under the microscope by comparing the space occupied by the cells in the flask with the unoccupied space. Cells were maintained with complete medium containing 10% FCS at 37°C. The medium was changed twice a week.

The cells were passaged into a large 175cm^2 flask once the cells were approximately 80% confluent. This was performed using the same technique detailed above, but this time with using 1ml of trypsin-EDTA. After the cells were trypsinised, 48ml of medium was added to the 75cm^2 flask, mixed gently and 12ml was place in a 175cm^2 flask. A further 18ml of medium was added to the 175cm^2 flask. The medium was changed twice a week. Cells were grown to 80% confluence. Cells were trypsinised using 2mL of trypsin-EDTA. A volume of medium was added to the flask to make a cell count of 0.4 x 10^6 to 0.5 x 10^6/ml.

2ml of trypsinised cells were added to each well, a total of 21 wells on four 6-well plates. The cells were incubated overnight. The cells were then washed with PBS and incubated in serum free medium. Serum free medium was made up with 100 mL of DMEM and 1 mL of 200 mM of glutamine. The plates were treated with the cytokines of interest as illustrated on the plate maps at 6 different concentrations. Three wells were not treated and were used as the controls.

Figure 4.1. Example plate maps for IL-6.

Plate A

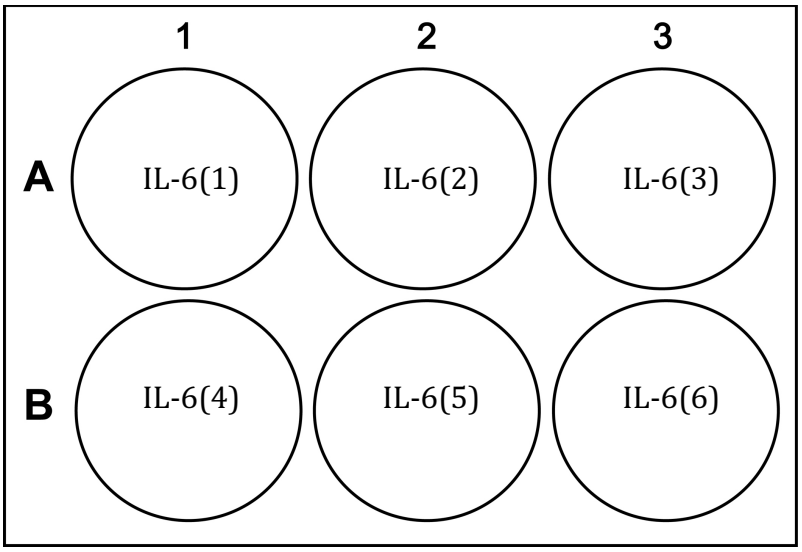
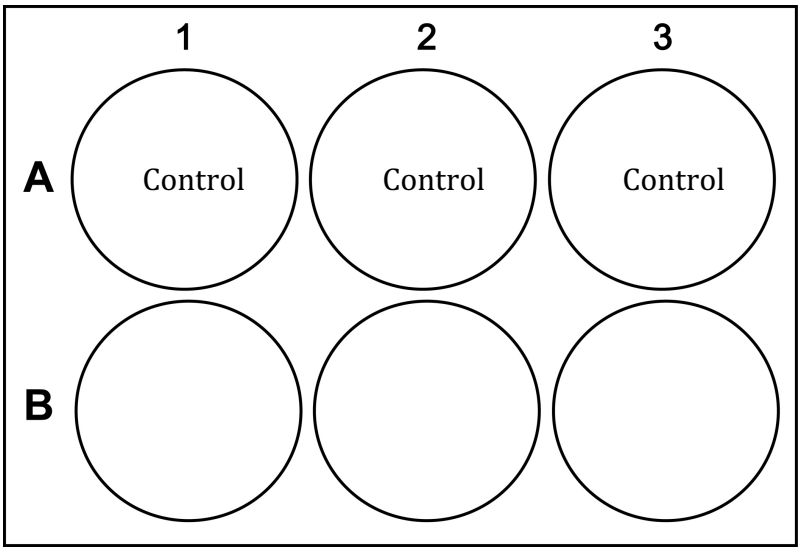


Plate B



The concentration of cytokines added to each well is shown in **Table 4.1**. These concentrations were based on the Luminex data (refer to Section 2Bv).

Cytokine	**Concentration pg/ml**

	1	2	3	4	5	6
IL-6	2	5	10	20	40	80
VEGF	100	200	400	800	1000	1600
PDGF-bb	100	200	400	800	1000	1600

Table 4.1. Cytokine concentrations added to cell culture medium.

(ii) Cell harvesting

The spent medium was removed from each well using a pipette. The same volume of PBS was added to each well and gently swirled. The PBS was then removed. 0.5ml trypsin EDTA was added to each well ensuring that the base of each well was covered by a thin layer of trypsin-EDTA. The plate was placed in an incubator for 2-15 mins. Cells were inspected under the microscope to ensure that they had dislodged. 2ml of medium was added to each well.

(iii) Counting cells

After the cells were harvested, the plate was gently swirled to ensure the cells were evenly distributed. Before the cells settled, 100 µL of cell suspension was removed and placed in an Eppendorf tube. 100 µL of trypan blue was added and gently mixed. 100 µL of Trypan Blue-treated cell suspension was applied to the hemocytometer. Both chambers were loaded under the coverslip, allowing the cell suspension to be drawn out by capillary action. The hemocytometer was placed under the microscope with focus on the grid lines

using a 10X objective. The live, unstained cells were counted in 5 squares using a hand tally counter.

Total viable cells

Total non-viable squares

Percentage of viable cells

Average no. of cells per square

Dilution factor (Final vol/vol of cells)

Concentration (Viable cells/mL)

Average no. of cells per sq. x dilution factor x 10^4

Counts were performed at Day 1 to 5. Cell cultures were repeated 3 times and performed in triplicate in each repeat.

(iv) Principles of PCR

The principle of PCR is to amplify a specific region of DNA and is based on the mechanism of DNA replication that occurs in vivo. Double-stranded DNA (dsDNA) is denatured to single-stranded DNA (ssDNA) and duplicated, with repetition of this process throughout the course of the reaction. There are three steps to a PCR reaction as follows:

1. Denaturation – during this stage dsDNA melts to form ssDNA.

2. Annealing –annealing of primers to ssDNA takes place. Oligonucleotides used as primers usually consist of relatively short sequences (15-25 nucleotides) complementary to recognition sites in the target DNA. Adequate binding results in the formation of strong

ionic bonds between the ssDNA template and primer, allowing the polymerase to attach and begin copying the template in the next step.

3. Elongation - during this step a heat-stable DNA polymerase synthesises a new DNA strand complementary to the DNA template strand resulting in a duplication of the starting target material. This occurs in the presence of MgCl2 by the addition of deoxynucleoside triphosphates (dNTPs) in the 5' to 3' direction. With extension of the primers by a few bases, this strengthens the ionic bonds to the template, reducing the chance of unbinding.

At the end of each cycle, the newly synthesised DNA strands serve as the template in the next cycle. Since both strands are duplicated during PCR, there is an exponential increase of the number of copies of the gene.

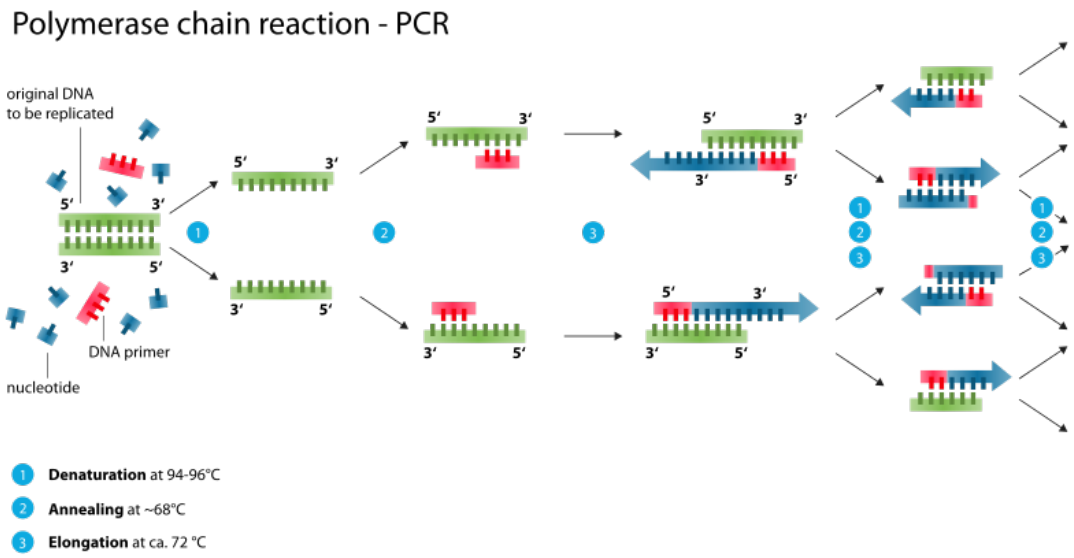


Figure 4.2. Schematic drawing of the PCR cycle showing the key steps: (1) Denaturation (2) Annealing (3) Elongation.
Taken from https://en.wikipedia.org/wiki/Polymerase_chain_reaction

(v) Methodology of quantitative real time PCR (qPCR)

RNA preparation

1. The cells were trypsinised in each well of the plates by adding 1ml of trypsin after removal of medium and washing with PBS.
2. 1ml of PBS was added to each well and the suspension was placed into labelled falcon tubes and centrifuged at 800rpm for 5 minutes at 10°C.
3. The supernatant was removed and cells were resuspended in 500µL of PBS.
4. This was centrifuged again at 10,000rpm for 1 minute.
5. The supernatant was removed.

Total RNA was extracted from ARPE-19 cells using the Qiagen RNeasy mini kit according to the manufacturer's instructions (Qiagen) as detailed below.

6. 350 µL Buffer RLT lysis buffer was added to the cell pellet and spun at 13,300rpm for 2 minutes in Qia shredder tubes.
7. 350 µL of 70% ethanol was added and mixed well by pipetting.
8. The sample was transferred to an RNeasy mini spin column that was placed in a 2mL collection tube. The lid was closed and the tube centrifuged for 15 seconds at 8000rpm. The flow-through was discarded.
9. 700 µL of buffer RW1 was added to the spin column. This was centrifuged for 15 seconds at 8000rpm. The flow-through was discarded.
10. 500 µL buffer RPE was added to the spin column. This was centrifuged for 15 seconds at 8000rpm. The flow-through was discarded.

11. 500 µL buffer RPE was added to the spin column. This was centrifuged for 2 minutes at 8000rpm.
12. The RNeasy spin column was placed in a new 2mL collection tube and centrifuged at full speed for 1 minute to dry the membrane.
13. The RNeasy spin column was placed in a new 1.5mL collection tube. 35 µL RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at 8000rpm to elute the RNA.
14. The spin column was removed and the collection tube was stored at -80°C.

The concentration and purity of RNA was measured by spectrophotometry using a NanoDrop (Thermo Scientific, USA). Purity was determined by the ratio of sample absorbance at 260 and 280nm. A ratio of approximately 2.0 indicated good quality RNA with much lower values indicating the possibility of protein, phenol or other contaminants.

Protocol for Quantitative real time PCR analysis

qPCR reactions were carried out using SYBR green mastermix (Applied Biosystems, Warrington, UK) in 25ul reactions using the Bio-rad iQ5 multicolor realtime PCR detection system (Bio-rad). Forward and reverse primers were optimised using a seven point standard curve and are listed in **Table 4.2**. Values obtained for the genes of interest were normalised to the human reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (HK-SY-hu-1200; Primerdesign UK). All qPCR experiments were performed in triplicate.

IL-6 forward	5’- ACTCACCTCTTCAGAACGAATTG-3’
IL-6 reverse	5’- CCATCTTTGGAAGGTTCAGGTTG -3’
VEGFA forward	5’- AGGGCAGAATCATCACGAAGT -3’
VEGFA reverse	5’- AGGGTCTCGATTGGATGGCA -3’
PDGFB forward	5’- CTCGATCCGCTCCTTTGATGA -3’
PDGFB reverse	5’- CGTTGGTGCGGTCTATGAG -3’
Fibronectin forward	5’- CGGTGGCTGTCAGTCAAAG -3’
Fibronectin reverse	5’- AAACCTCGGCTTCCTCCATAA -3’
Alpha-sma forward	5’- CCGACCGAATGCAGAAGGA -3’
Alpha-sma reverse	5’- ACAGAGTATTTGCGCTCCGAA -3’
Laminin forward	5’- TGACTTTCAAGACATTCCGTCC -3’
Laminin reverse	5’- AGGCGAAGTATCTATACACACCC -3’
Vimentin forward	5’- TGTCCAAATCGATGTGGATGTTTC -3’
Vimentin reverse	5’- TTGTACCATTCTTCTGCCTCCTG -3’
Collagen I forward	5’- GAGGGCCAAGACGAAGACATC -3’
Collagen I Reverse	5’- CAGATCACGTCATCGCACAAC -3’
GAPDH	Pre-optimised from Primerdesign product code: HK-SY-hu-1200

Table 4.2. Oligonucleotides used for qPCR analysis of fibrotic gene expression.

(vi) Results

ARPE-19 Cell Cultures

This experiment examined the effect of IL-6, VEGF and PDGF on EMT. It examines the potential to cause EMT in an *in vitro* culture system. Serum-free ARPE-19 cell cultures expressed low levels of mRNA for connective tissue molecules laminin (Figure 4.3), fibronectin (Figure 4.4) and collagen type I (Figure 4.5).

Cell culture effects of IL-6

In comparison to serum free cultures, cultures were supplemented with IL-6. ARPE-19 cells were treated with IL-6 in the range of 2 pg/ml to 80 pg/ml for 5 days. The addition of IL-6 did not induce upregulation of laminin ($P = 0.99$), fibronectin ($P = 0.977$), collagen I ($P = 0.995$), vimentin ($P = 0.983$) and alpha-SMA ($P = 0.939$).

Cell culture effects of PDGF

ARPE-19 cells were treated with PDGF in the range of 100 pg/ml to 1600 pg/ml for 5 days. The addition of PDGF did not induce upregulation of laminin ($P = 0.972$), fibronectin ($P = 0.97$), collagen I ($P = 0.589$), vimentin ($P = 0.857$) and alpha-SMA ($P = 0.983$).

Cell culture effects of VEGF

ARPE-19 cells were treated with VEGF in the range of 100 pg/ml to 1600 pg/ml for 5 days. The addition of PDGF did not induce upregulation of laminin ($P = 0.992$), fibronectin ($P = 0.94$), collagen I ($P = 0.647$), vimentin ($P = 0.85$) and alpha-SMA ($P = 0.927$).

Consistent with not having undergone EMT and not being in a pro-fibrotic state, cell cultures expressed low levels of vimentin and alpha-SMA in treated and untreated groups (Figure 4.6 and 4.7).

Figure 4.3 mRNA levels of laminin in ARPE-19 cell cultures

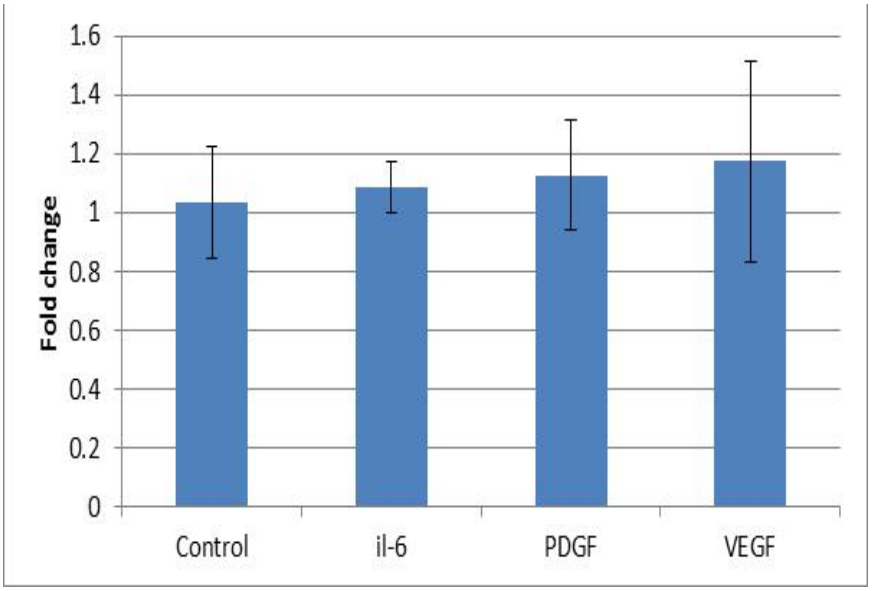


Figure 4.4 mRNA levels of fibronectin in ARPE-19 cell cultures

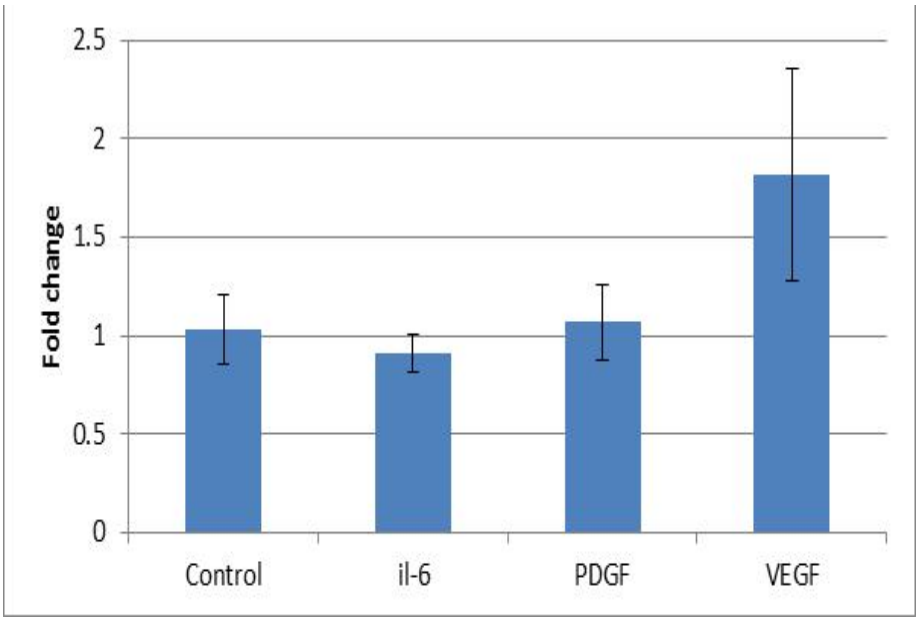


Figure 4.5 mRNA levels of collagen type I in ARPE-19 cell cultures

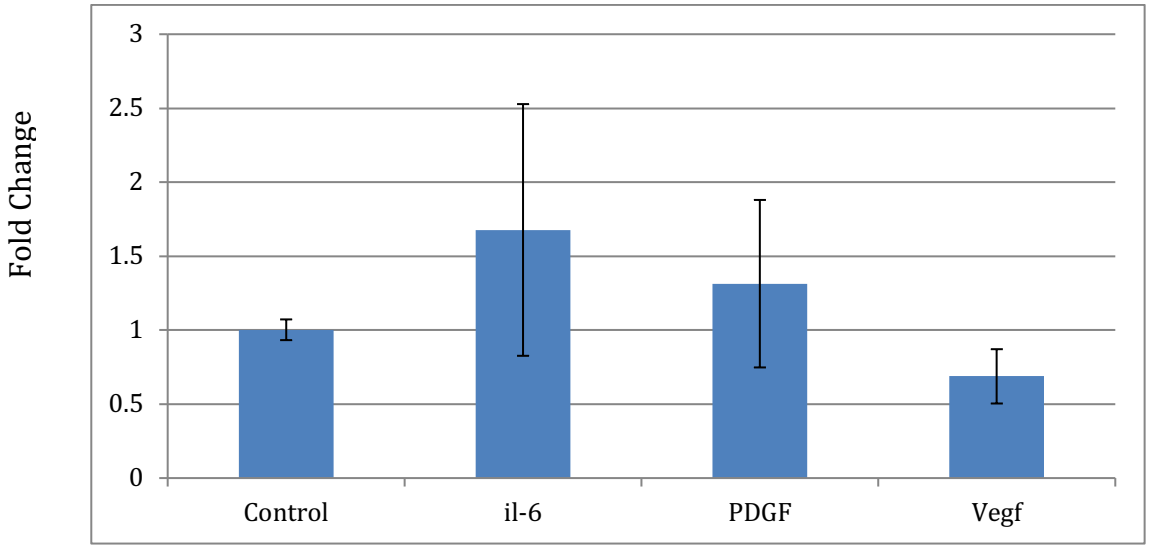


Figure 4.6 mRNA levels of vimentin in ARPE-19 cell cultures

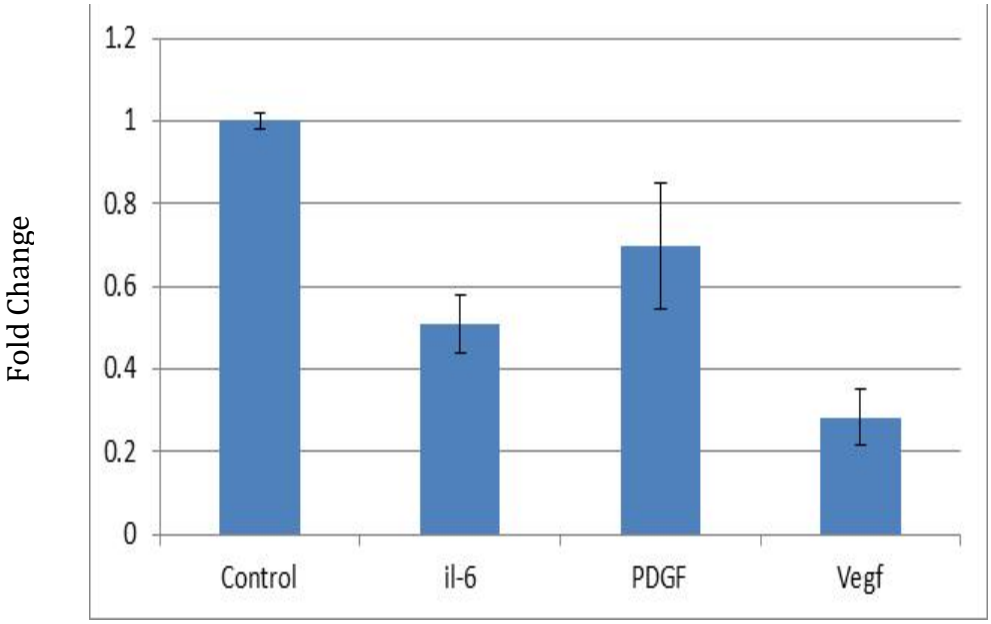
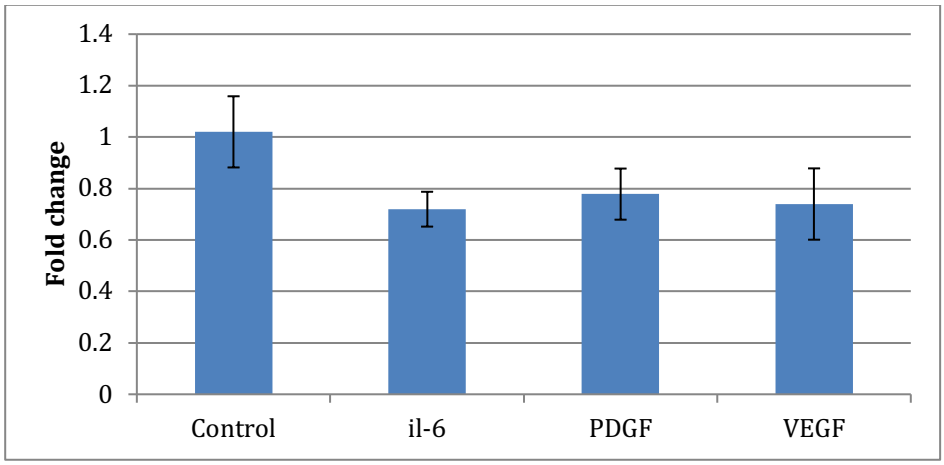


Figure 4.7 mRNA levels of alpha-sma in ARPE-19 cell cultures



(vii) Results summary

There is no direct effect of IL-6, PDGF and VEGF on ARPE-19 cells. Markers of EMT are not induced with the presence of these cytokines.

SECTION 4C

Discussion and interim conclusion

In vitro IL-6, PDGF and VEGF do not cause EMT and fibrosis. These results suggest that IL-6, PDGF and VEGF do play in the development of PVR by direct effects on untransformed RPE cells. It may be that a study using immune cells such as macrophages would or a study using combinations of cytokines, more analogous to the situation *in vivo* would demonstrate an effect. Unlike cells *in vivo*, cultured cells are devoid of their native microenvironment. RPE cells *in vitro* may have altered differentiation and behaviour, with culture conditions and media components having an effect on their viability. Genetic instability of continuous cell lines, with consequent variation in their properties between passages is an additional limitation on the use of RPE cell cultures. However, the absence

of a reliable causative relationship from a single cytokine on a single cell type is perhaps not surprising given that PVR is a complex and multifactorial condition.

Chapter 5

Final Conclusion and future directions

Retinal detachment induces cell migration and proliferation as well as the production of extracellular matrix proteins, which in turn lead to the development and contraction of vitreal, preretinal and subretinal membranes, both hallmarks of PVR. PVR occurs in up to 10% of rhegmatogenous retinal detachment cases and is the major cause of poor functional outcomes after primarily successful RD surgery.

PVR continues to remain a challenge for vitreoretinal surgeons. Combined assessment of clinical and surgical risk factors may aid in better prediction of PVR risk following RD surgery. Awareness of these factors in the development of PVR may allow for a more careful planning of surgery to minimise risk. Clinical classifications of PVR may need revision, as the current classifications are based entirely on anatomical descriptions at a single time point and do not take into account the biological activity and the pathways involved in PVR development.

Pathways involved in the development of PVR could provide insight into future treatment targets. Results of adjunctive treatment aimed at improving anatomical and visual outcomes have been disappointing. An inadequate understanding of PVR development may have contributed to this. PVR formation is a chain of events involving many cytokines and growth factors, which are interlinked. The elucidation of pathways important in the pathogenesis of PVR and the increased understanding of its pathobiology has led to the identification of new potential targets to prevent it or to be used in adjunctive prophylaxis.

Previous studies found associations between PVR and significantly increased concentrations of certain pro-inflammatory cytokines and growth factors in the vitreous[28,319,320]. Cell-signalling mediators, such as cytokines and chemokines are involved in the regulation of inflammatory processes, wound healing and scar formation. In eyes with retinal detachment, levels of a variety of cytokines and growth factors in the vitreous including IL1ra, IL7, IL8, IL9, IP10 and MIP1b. IL1β and IL7 were raised in eyes that went on to develop PVR. These cytokines could be used as biomarkers to determine the risk of PVR development. However, the wide error bars suggest that no single cytokine can be used as a biomarker in isolation Further work may involve cell culture work on ARPE with cytokines IL1β and IL7 and perform PCR to measure markers of EMT activity. A better method of developing an *in-vitro* model could possibly be by using a co-culture system of RPE and immune cells or by performing animal work. An animal model would permit extensive functional studies.

The metabolites that were dominant in PVR vitreous are mainly involved in amino acid metabolism and glutamate metabolism. Glutamate is an important excitatory neurotransmitter in the retina. After its release from neurons it is transported into Müller cells, where it is converted by glutamine synthetase into glutamine, which is no longer neuroactive[321]. Since glutamate is toxic in excess amounts, glutamine synthetase may function as a neuroprotectant[322]. Glutamine synthetase in the retina is localized almost exclusively in Müller cells. The uptake of glutamate by Müller cells is strongly influenced by the activity of glutamine synthetase[321].

IL-1β which induces rod and cone photoreceptor death, expresses glutamate ionotropic receptors such as AMPA and NMDA receptors[323,324]. Glutamate excitotoxicity has been demonstrated to be a major threat for photoreceptor survival[325]. IL-1 β has been shown to modify glutamate homeostasis by altering glutamate uptake and glutamine synthesis by Muller cells in animal models of ischaemic retinopathies[326] and by increasing intracellular levels of cysteine and extracellular levels of glutamate in hypoxic-ischaemic brain damage[327]. This suggests that glutamate mediates ischaemic and hypoxic injury to the retina.

Avoidance or management of PVR could be further investigated by administering a compound capable of reducing glutamate-induced and amino acid induced retinal cell migration and proliferation.

M1 and M2 macrophages have distinct metabolic profiles. M2 macrophage activation stimulates amino acid utilisation arginine, proline, alanine, aspartate and glutamine in particular[328]. In contrast, IL-7 and IL-1β fit with M1 macrophages[329]. So, there are features that point to both M1 and M2 activation in PVR.

There are limitations to this work. Vitreous samples were collected using a protocol to standardise methods. However, there are variables that are difficult to control, such as the timing of sample collection. This will be due to time variability between patient presentation to time of surgery. Samples were collected over a period of time; so, the length of storage will differ between samples. Freezing the vitreous samples over long periods of time may alter levels of some of their components potentially causing

decreases or increases in biomarker concentration. Among other factors, storage temperature and storage time are known to impact frozen samples. Thus, even in carefully collected and stored samples time alone can alter biomarker levels. Due to the nature of this work, sample volumes are small. Small samples can cause unreliable results. However, the experimental techniques used are designed to analyse such sample sizes.

This research provides a broad comparison of profiles of pro-inflammatory and pro-fibrotic cytokines in the vitreous as well as metabolomic changes, which should facilitate an improved differentiation of their relative importance with respect to the pathophysiological processes involved in the development of PVR. Identification of the particular cytokines involved can be of therapeutic as well as pathogenetic importance. This research will form the basis of future work into PVR and has highlighted areas that need to be answered.

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Appendix

(i) Patient information sheet

National Institute for Health Research
Surgical Reconstruction and Microbiology Centre

Study Title: Improving Patient Outcomes after Ocular Trauma

Patient Information Leaflet

IRAS number: 12/WM/0330 Date:

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The inside of the eye is lined by a light sensitive film called the retina which transmits information to the brain. The brain processes the retinal signals from both eyes which gives rise to sight. If the retina is damaged in any way, through traumatic injury or disease, this causes the retinal cells to die and the patient loses vision.

Most of the cavity of the eye is filled with a jelly called the vitreous humour. The treatment for lots of different retinal problems is an operation called a vitrectomy, where the vitreous jelly is removed. Normally, this vitreous is thrown away but sometimes it is kept and analysed to help with diagnosis and treatment.

Some patients have poor eyesight even after a vitrectomy. This is because they might get scarring inside the eye and on the surface of the retina: a condition known as proliferative vitreoretinopathy (PVR). In some patients, the light sensitive cells in the retina may also die.

The causes of PVR are not fully understood. Unfortunately, there is no effective way to prevent or treat PVR – except by doing a vitrectomy to remove the scar tissue. Similarly, there are no effective medicines or surgical procedures that prevent retinal cells from dying.

The purpose of this study is:

1) To find out what causes PVR, so we can find a way to prevent or treat it.
2) To find out if there is a test that we can do that will predict which patients are more likely to get PVR.
3) To find out how many of the cells in the retina die, see why they die and what we can do to stop them from dying.

The ultimate aim is to improve patient visual outcomes after injury to the eye and other retinal problems.

Why have I been invited?

You have been invited to take part in this study because you are about to undergo a vitrectomy for your eye problem.

Do I have to take part?

No, it is up to you to decide if you want to take part. We will describe the study and go through this information sheet with you, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part and give you a copy of this. You are free to withdraw from the study at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

If you decide to take part, we will give you the opportunity to discuss the research with us. After you have given your consent, we will collect some of the vitreous which is removed during your surgery, to analyse in the laboratory for our research. We will also collect urine and blood samples and may collect tears.
We will collect the vitreous removed during your vitrectomy, which would otherwise be thrown away. Sometimes, during a vitrectomy, the surgeon also has to remove small amounts of the retina or fluid from underneath the retina or inside the front of your eye – if that happens, we will collect all of these too. Taking a little fluid from inside the front of the eye (aqueous) is a fast and safe procedure that has a very low risk of damaging the eye, so we would like to take some of this fluid for the purpose of research, whether or not it is clinically necessary. It will not be painful, because of the anaesthetic we use for surgery. We will analyse these samples for molecules and proteins in the research laboratory and compare them to tear, urine and blood samples to see if the proteins we find are just in your eye.
After the procedure, you will not need any additional treatment or follow-up. We will review you in clinic as usual, but will take additional photographs of the back of the eye, and at 6 months we may carry out an electrical test to see how well the retina is working and a visual field test to see how well the eye is transmitting information to the brain.
If you have more than one operation, we ask you if we can collect any samples taken during these later procedures.

What will I have to do?

You don't need to do anything except agree to have samples taken at the time of surgery and to come to your routine appointments as planned. You will not have to make any extra visits to hospital, but it may be more convenient to attend a separate research clinic and if that is the case, we will give you that option and pay your travel expenses to attend.

When you come to clinic, we will use special cameras used in routine practice, to examine the retina and determine how much scarring and cell death there is. We will relate these test results to our laboratory analyses of your samples to try and work out what is causing PVR and whether we can predict this by any of the tests we have done.

Most of the clinic tests and treatments we will carry out are those that you would have had if you had not taken part. The key differences are:

1) We will collect information on an anonymised database so that we can analyse it at a later date, by comparing clinical results with our laboratory analyses of your samples.

2) At the clinic visit 6 months after surgery, as part of your normal care you may have special pictures taken of the back of your eye from time to time, using two sorts of camera, to look at the health of your retina. If you take part in the study, we will ensure these happen; they will take about 5 minutes each and involve a brief flash of light into each eye. The pictures produced are not ones that anyone can identify you from.

3) When you come to see us 6 months after the operation (this is often the last visit), we may perform electrical tests to see how well the retina is working and transmitting information to the brain. This test is called electroretinography, takes about half an hour to do, and are clinical diagnostic tests used in routine practice. The test involves some very thin wires being taped onto the skin around the eyes and a very thin thread (so thin that you can't feel it) running inside the bottom eyelid so we can record the electrical activity of your eyes whilst you are looking at either a pattern on a screen or another flashing light.

4) We will also ask you to do a visual field test, which involves looking at a screen and pressing a button when you see a light. This takes about 10 minutes.

When you have finished the study we will keep the information about your eyes and the results of the tests in a secure environment, but we will not need to access any personal information about you.

Expenses and payments

There should be no extra costs to you because you participate in this study, so we are not able to pay any expenses.

What are the possible disadvantages and risks of taking part?

There are no direct risks to the tests that we are doing. We will try and make the appointments for tests as convenient as possible.

There is a small chance that we might pick up an additional eye problem when we analyse the various clinical tests, that you didn't know about. If this happens, we will refer you to an appropriate specialist after talking to you and the Consultant in charge of your care, if that is what you want. Incidental findings like these may be good, as early treatment can be given; however, in a small number of cases the diagnosis of a co-incidental disease may have implications for future employment and insurance.

What are the possible benefits of taking part?

We cannot promise the study will help you, but the information we get from this study will help improve the care we give to people with retinal problems or eye injuries.

What if relevant new information becomes available?

If this same research is completed by another group, so our research became unnecessary, we would stop the study, but your normal care would not be affected.

What will happen if I don't want to carry on with the study?

If you want to withdraw from the study you can do so at any point. We would ask you whether we can continue to use your samples collected to the point of withdrawal. However, we will need keep your data.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (via switchboard on 0121-554-3801). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against the hospital, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Will my taking part in this study be kept confidential?

Yes.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the hospital where you are treated. They may also be looked at by representatives of regulatory authorities and by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant.

Your data will be collected and stored on paper and on an electronic database whilst you participate in the study. This is stored in the same way as your other medical records. You have the right to check the accuracy of your information and correct any errors.

What will happen to the results of the research study?

If possible we will publish the results in a scientific journal and present them at scientific meetings. Nothing would be published that could identify you.

Who is organising and funding the research?

The research is funded by the National Institute for Health Research (NIHR), the Ministry of Defence, University Hospitals Birmingham NHS Trust and the University of Birmingham through the NIHR Surgical Reconstruction and Microbiology Centre.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the Local Research Ethics Committee.

And finally ...

Thank you for taking the time to read this sheet and considering involvement in our research study.

Further information and contact details

The principle researcher is Maj Richard Blanch, who can be contacted in the following ways:

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For independent advice, please contact the Patient Advice Liaison Service:

Website: http://www.swbh.nhs.uk/patients-and-public/patient-advice-and-liaison-service

Email: pals@swbh.nhs.uk

Tel: 0121 5075836

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(ii) Study Protocol

TITLE
Improving Patient Outcomes after Ocular Trauma
NIHR SRMRC Research Theme 3: Regenerative Medicine
SPONSOR
University of Birmingham
Sponsorship reference no. RG_12-028

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PURPOSE

Eye injuries are a major cause of visual loss[1] and often cause disfigurement, major lifestyle changes and loss of career. Eye injuries can be divided into closed and open globe injuries, of which that latter is more severe. After open globe eye injuries, visual outcomes are poor due to retinal cell death, scarring and a failure of neuronal regeneration.

Little is known about the extent of retinal cell death after retinal injury, its mechanisms and potential strategies to reduce it.

Almost all open globe injuries require vitreo-retinal surgery. The main cause of poor outcomes after vitreo-retinal surgery for trauma or any other indication is proliferative vitreoretinopathy (PVR), a progressive intra-ocular scarring process, which occurs in 8.3% of eyes operated on for retinal detachment and 17% of eyes operated on after open globe injury[2,3].

PVR is characterised by the development of vitreal, epiretinal and subretinal fibrocellular membranes that place traction on the retina and cause tractional retinal detachment. There are currently no effective treatments to prevent the development of PVR and the only treatment is further surgery to remove the scar tissue and reattach the retina. The functional outcomes in patients who develop PVR are poor with up to 75% of patients having a visual outcome worse than 6/60 and over half of patients describing their (binocular) visual function as medium to bad[4]. There is thus a strong need for research in this area to improve our understanding of the pathogenesis of PVR and find new treatments.

Laboratory animal and clinical studies have investigated the cause of PVR, principally after rhegmatogenous retinal detachment. This occurs when the neurosensory layer of the retina detaches from the underlying retinal pigment epithelium following a tear in the retina (Greek: rhegma = torn, fissure)[5]. A number of recent studies have examined vitreous and subretinal fluid samples taken at the time of surgical repair for retinal detachments (excluding trauma patients) using multiplex beads, ELISA and RT-PCR and have found a series of alterations in cytokine levels which predict subsequent development of PVR including increased expression of interleukin-6, pigment epithelium-derived factor, macrophage migration inhibitory factor and a number of chemokine (C-C motif)[6,7,8,9,10].

We will investigate PVR and cell death in patients with trauma and other more defined retinal pathologies such as retinal detachment, diabetic retinopathy, uveitis, macular holes and floaters. This research aims to:

(1) Investigate the aetiology of proliferative vitreoretinopathy.
(2) Develop a biomarker screen to predict visual outcomes, complications and the potential for therapeutic interventions after vitreo-retinal surgery.
(3) Evaluate the extent and mechanisms of cell death after ocular injury.

We hypothesise that:

(1) Varying proteomic and metabolomic profiles will predict the development of proliferative vitreoretinopathy

(2) Patients with retinal detachment, trauma, diabetic retinopathy and uveitis will have more variable cytokine levels than patients with macula holes and floaters (who will have normal vitreous cytokine profiles) and this difference will explain the differing rates of PVR.

DESIGN

Prospective observational cohort study.

We will recruit patients due to undergo vitreo-retinal surgery for a variety of conditions including rhegmatogenous retinal detachment, macular hole, trauma, uveitis, diabetic retinopathy and established PVR. Patients will have baseline clinical examination, as part of routine clinical care. Patients will be followed up, also as part of standard care, at six months and then longer if clinically necessary. Most of the clinical investigations required are part of routine clinical care. Some non-invasive tests will be required in addition to routine care.

Ocular tissue samples and bodily fluid samples (blood and urine, with the option of collecting tears) will be collected at the time of surgery for laboratory analyses. The ocular tissue samples collected are all ones that would be routinely collected and discarded. Patients who undergo repeat vitreo-retinal surgery will have repeat samples taken and followed up for a further 6 months i.e. they will return to the start of the pathway shown in the summary diagram below.

RECRUITMENT

For elective cases, recruitment will take place in the outpatient department (clinic) and the inpatient ward at Birmingham & Midland Eye Centre. The patients will be approached by the operating surgeon or the doctor delivering care in clinic and will be consented for inclusion by either the study research nurses, the clinical research fellow or members of the direct clinical care team who are (1) involved in the study and (2) appropriately trained. For emergency cases, the same pathway as that for elective cases is preferred; however, in some cases, the patient will be seen in casualty or admitted and listed for surgery as an emergency. Such cases will be identified by liaising with the clinical and administrative staff who organise the operating lists and with the clinical teams involved. In these cases, it may not be possible to approach the patient in clinic and a member of the research team will approach the patient and seek consent.

INCLUSION CRITERIA

1) Patients undergoing vitreo-retinal surgery for:
    a. open globe injury.
    b. new rhegmatogenous retinal detachment.
    c. macular hole.
    d. diabetic eye disease requiring vitreo-retinal surgery.
    e. posterior uveitis requiring vitreo-retinal surgery.
    f. floaters requiring vitreo-retinal surgery for removal.
    g. established PVR after previous vitreo-retinal surgery.

h. Epiretinal membrane.

OR

Patients who have had vitreous samples tissue-banked in the previous 6 months and fell into one or more of the above groups.

2) Capable of giving consent to participation.

EXCLUSION CRITERIA

1) Previous vitreo-retinal surgery (except for group g. – established PVR)
2) Age under 10.

CLINICAL AND LABORATORY ASSESSMENTS

As part of routine clinical care, patients will usually be seen pre-operatively and post-operatively at 1 day, 2 weeks, 6 weeks and 6 months, with additional follow up as clinically required. No additional visits will be required for the study, but patients may be given the option to attend a separate research clinic. Patients may be seen in City Hospital or Queen Elizabeth Hospital Birmingham and will be followed up in the clinic in which they would otherwise have been seen.

Most of the clinical assessments are part of routine clinical care. At the 6 month visit after recruitment the patient will have the following assessments:

Visual acuity - part of routine clinical care. The assessments used will be standardised as logMAR. Where Snellen measurements have been taken outside of the study, these data will also be included.

Optical coherence tomography (OCT) – a non-invasive imaging modality, similar to fundus photography - that is part of routine care for most vitreo-retinal patients. Macular OCT and retinal nerve fibre layer (circle) OCT will be performed at every visit. Routine care would usually include macular OCT on more than one occasion, though possibly not at every visit, so there may be some additional OCT scans. Where this is available, they will be of the higher resolution spectral domain type. These will take no more than 5 minutes.

Fundus photography – part of routine clinical care for many patients, especially those who develop PVR. From a research point of view, it is essentially to allow documentation of PVR clinical grading. This will take no more than 5 minutes.

24:2 Humphrey visual field (HVF) – subjective testing of the subjects visual field - part of routine care for many patients, such as those with field defects or those suspected of glaucoma. Takes approximately 10 minutes.

In addition, on select patients (less than 50) at the discretion of the PI or CI patients may be requested to undergo:

Multifocal Electroretinography (mfERG) – non-invasive electrophysiology using skin and conjunctival surface electrodes while the patient views a visual stimulus. Not usually part of routine care. Takes 10-30 minutes.

Laboratory assessments will be performed on ocular tissue that would otherwise have been collected and discarded (1-2) or other bodily fluids that can be obtained in a minimally

invasive fashion (3-5). Aqueous may be collected as an additional procedure in cases where it would not routinely have been drained:

1) Intraocular fluid biopsies (inc. aqueous, vitreous, subretinal fluid suprachoroidal fluid)
2) Abscised retina
3) Blood
4) Urine
5) Tears

Vitreous is removed as part of a vitrectomy, which all study patients will have (see inclusion criteria). In some cases, a vitrectomy will be combined with cataract surgery, retinectomy and/or drainage of subretinal or suprachoroidal fluid – in such cases, the abscised retina and drained aqueous, subretinal and suprachoroidal fluid will also be collected.

Aqueous fluid is drained from the anterior chamber of the eye as part of cataract surgery during the first incision; before a viscoelastic substance is injected into the anterior chamber of the eye. It is also drained as part of many vitreo-retinal procedures to reduce the pressure within the eye. Where aqueous is taken as an additional procedure, it is taken using a fine needle through the cornea and is a fast, safe and minimally invasive procedure.

Intra-ocular fluid and tear samples will be collected and may be either placed in storage at -80 °C for later processing, or centrifuged at 10,000 rpm for 10 min to separate the cellular and aqueous components. The cellular component (pellet) will be cryoprotected in 50% fetal calf serum (FCS), 10% dimethyl sulfoxide (DMSO) and 40% RPMI-1640 medium and then snap frozen in liquid nitrogen or using cryo spray. The aqueous component will be separated into 1-200µl aliquots (depending on total volume – not more than 10 aliquots) and snap frozen in liquid nitrogen. All samples will be stored at –80 °C until metabolomic, cytokine, flow cytometric and mass spectroscopic analysis. Where silicone oil is present in the sample, this will be removed after centrifugation.

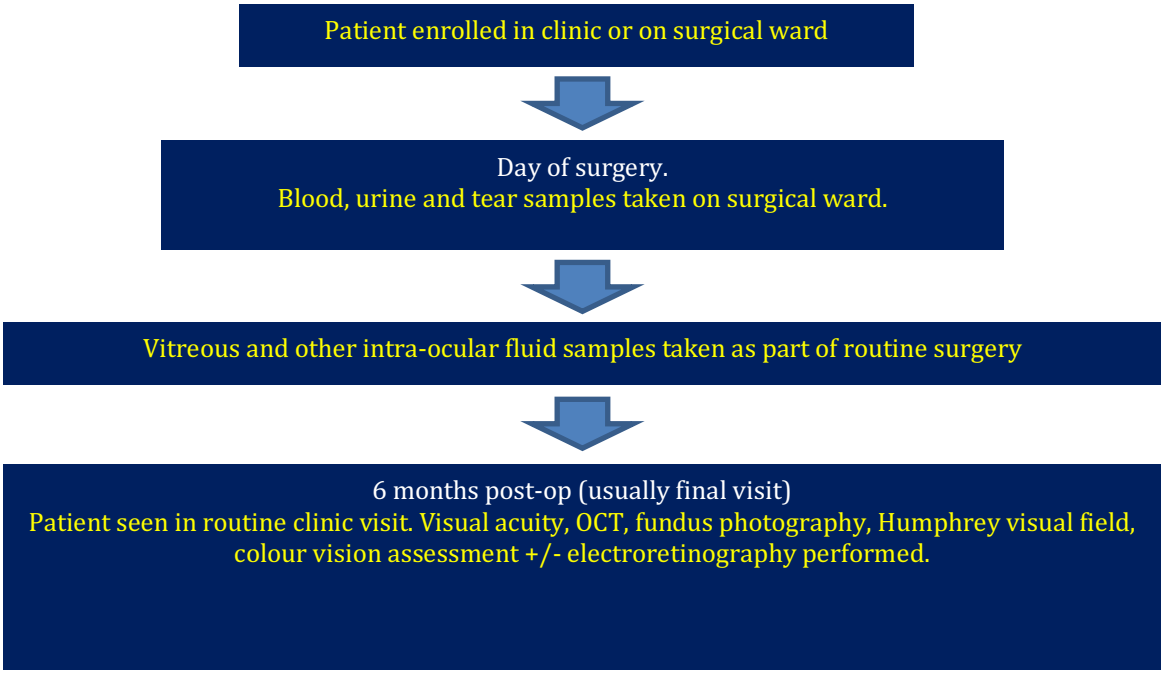
Retinal tissue will be immersed in RNA stabilising agent before being snap frozen in liquid nitrogen or using cryo spray, and stored at -80 °C for later genomic analyses.

Urine samples will be collected mid-flow into a sterile 20ml Universal and 5 x 0.5 ml aliquots will be centrifuged at 3000 rpm for 10 min, the cellular component (pellet) discarded and the aqueous component frozen at -80 °C as soon as possible (within 2 hours) until metabolomic, cytokine and mass spectroscopic analysis.

Blood will be collected into two EDTA (purple topped) and one heparinised (green-topped) 5ml vacutainer tubes. The EDTA tubes for DNA will be frozen at -20C until processed. The green-topped tube will be centrifuged at 3000 rpm for 10 mins and 5 x 0.5ml aliquots will be collected and stored at –80 °C until metabolomic and cytokine analysis.

All samples may be kept on ice or refrigerated at less than 5°c for up to 4 hours before processing.

Summary diagram:



Sampling grid:

Time point (months)		0 (surgery)	6
Clinical assessment and sampling	Vitreous sampling	✓	
	Aqueous sampling	✓	
	Blood sampling	✓	
	Tear sampling	optional	
	Urine sampling	✓	
	Visual acuity		✓
	OCT		✓
	Fundus photograph		✓
	HVF		✓
	mfERG		optional

CONSENT

Consent to participate will be taken by either the study research nurses, the clinical research fellow or members of the direct clinical care team who are (1) involved in the study and (2) appropriately trained.
Where children are recruited, this must be done by staff with specific experience in paediatric ophthalmology. Parents will be asked to sign the Parent Consent form, children aged 10-15 years will be asked to sign the Child Assent (10-15 years) form and children aged 16-17 years will be asked to sign a normal consent form.

RISKS

Blood and urine will be taken that are not part of routine clinical care. Phlebotomy may be associated with pain and bruising at the venepuncture site for days afterwards.
All other investigations required are non-invasive and all ocular tissue collected would be otherwise collected and discarded if the patient declined to participate.

BURDENS

Blood, urine and tears will be taken that are not part of routine clinical care. There will be some additional time associated with the additional investigations. No additional visits are required.

BENEFITS

1. Better prognostic advice to future patients after surgery.
2. An objective baseline to assess the need for and potential benefits of potential future treatments
3. Better understanding of the mechanisms of cell death and PVR in vitreo-retinal pathologies, to allow the development of novel therapies.

There are few direct benefits to the patients, as this is an observational study.

CONFIDENTIALITY

Patient identifiable data will be stored in paper form in a secure location in Birmingham Midland Eye Centre and electronically on a password-protected database accessible on NHS computers in Birmingham Midland Eye Centre.

WITHDRAWAL OF SUBJECTS

Subjects can chose to withdraw at any time by filling out a study withdrawal form. Any member of the research team will assist in this if required. On withdrawal, we will ask permission to keep any samples collected. If this is refused, any samples held will be destroyed. All data will be retained.

STATISTICS

Analysis will be by binary logistic regression. The binary measure will be presence or absence of PVR at 6 months. Due to the exploratory nature of the study, multiple comparisons will be performed. To reduce the possibility type 1 error, analysis will be in two stages and two different cohorts: (1), all proteomic and metabolomic data collected will be analysed to identify potential associations and develop specific hypotheses in patients with retinal detachment; (2), focussed analyses will be performed on a second cohort to test specific hypotheses developed in stage 1 in all groups of patients.

SAMPLE SIZE

All calculations were performed in G*power (v. 3.1.4, Kiel University, Germany).
Due to the exploratory nature of the study, multiple comparisons will be performed. To reduce the possibility type 1 error, analysis will be in two stages and two different cohorts:

1) All proteomic and metabolomic data collected will be analysed to identify potential associations and develop specific hypotheses in patients with retinal detachment.
2) Focussed analyses will be performed on a second cohort to test specific hypotheses developed in stage 1 in all groups of patients.

The main aim of this observational study is to investigate the aetiology of PVR. The most common cause of PVR is retinal detachment, after which it occurs in 8.3% of eyes[2]. The main way in which the aetiology of PVR will be studied is by cytokine analysis using luminex beads; a standard panel will examine 27 cytokines. In a recent paper looking at cytokine levels in subretinal fluid in patients with and without PVR after retinal detachment, the largest effect size was for the cytokine CCL19 for which levels of 144 pg/ml (std dev. 117) and 290 pg/ml (std dev.160) were found in the groups with and without PVR respectively[10]. A bonferroni correction for 27 comparisons reduces α from 0.05 to 0.0019 and with power (1-β) set to 0.8, 208 patients with retinal detachment would require to be recruited, of whom 16 would be expected to develop PVR. Without Bonferroni correction, 104 patients are required (of whom 8 are expected to develop PVR).
So for a two stage analysis, stage 1 with multiple comparisons requires 208 patients and stage 2 with fewer comparisons requires 104 patients giving a total of 312 patients with retinal detachment. A smaller effect size, such as that reported for IL-6 would require a total of 772 patients with retinal detachment.
Macular holes have a very low reported PVR rate; however, 27% develop epiretinal membranes[11], which share some features with PVR (a gliotic response). To detect differences between patients who do or do not develop epiretinal membranes, 46 to 110 patients would be required to have an 80% power to find a difference in levels of CCL19 or IL-6 respectively. Additionally the macular hole group provides a negative control i.e. if particular changes in the cytokine profile predict PVR in a proportion of patients with retinal detachment, these changes should not be present in the vitreous of patients having macular hole surgery. This is also the function of patients undergoing vitrectomy to treat floaters.
Trauma patients have a PVR rate of 17%[3], requiring 48 to 150 patients.
Data are not available to allow such calculations for uveitis patients, diabetic retinopathy patients or patients undergoing vitrectomy to treat floaters. There are fewer of these patients available than in the other groups and this data will be used for qualitative comparison with the other groups. Samples from patients who have established PVR will be obtained in 1 of 2 ways: (1), study patients who have repeat surgery for PVR; (2), patients with PVR after previous vitreo-retinal surgery who have not been previously enrolled. It is therefore difficult to estimate how many such patients we will recruit as a separate group and the number available will decrease with time after study commencement (as more patients are recruited at first surgery).
We aim to recruit patients to the different groups as follows:
Retinal detachment 450
Macular hole 100

Trauma	50	
Uveitis	50	
Diabetic retinopathy	50	
Floaterectomy		50
Established PVR	20	

DATA COLLECTION

Clinical data collected initially and any changes noted at every follow up visit:

All patients:

- Demographics (date of birth, gender, ethnicity, postcode)
- Date of assessment
- Visual acuity (logMAR and Pelli-Robson) - numeric
- RAPD (yes/no)
- IOP (mmHg)
- >6D myope (yes/no)
- AC activity (0, 1, 2, 3+)
- Lens status
- Symptom/injury onset (date)
- Medication
- Vitreous haemorrhage (yes/no)
- PVR on presentation (yes/no) + clinical grade + fundus photography documentation
- Macular status (oedema, pucker, hole, detached)
- Smoking status (never, ex, current) + Pack years & Duration smoke free
- Optical coherence tomography images of the retina and optic disc (date, cystic spaces (yes/no), macular volume, macular hole (yes/no), epiretinal membrane (yes/no), vitreomacular traction (yes/no), nerve fibre layer thickness)
- Humphrey visual fields (mean deviation, pattern standard deviation, reliable – yes/no)
- Where performed outside of protocol on clinical grounds, ultrasound and computer tomography scans (scleral rupture (yes/no), FB (0, 1, 2, 3, 4,5 >5), detachment (yes/no), choroidal haemorrhage (yes/no), vitreous haemorrhage (yes/no))

All patients having vitreo-retinal surgery:

- Date of surgery
- Surgery performed (ICD-10 codes) + any tamponade used and laser spot size, power and duration
- Clear cornea at time of surgery
- Grade of surgeon

Open globe injury patients:

- Classification (Birmingham Eye Trauma Terminology system[12])
- size of wound (mm)
- zone of injury (1, 2, 3)

- lens injury (yes/no)
- endophthalmitis (yes/no)
- Associated injuries (yes/no) + list
- Aetiology (e.g. occupational/leisure, sports/machine injury/road traffic collision)
- Systemic antiobiotics (yes/no)
- Primary repair – date, suture material, intravitreal antibiotics, topical antibiotics, surgery performed (ICD-10 codes), grade of surgeon.

Rhegmatogenous retinal detachment patients:

- Macular status (on/off)
- Clock hours detached
- No. breaks (1, 2, 3, 4, 5, >5)
- Size of largest break (clock hours <1, 1, 2, 3, >3)
- Posterior Vitreous Detachment (yes/no)

Diabetic retinopathy patients:

- Type of diabetes (1 or 2)
- Duration of diabetes
- Medical Treatment (inc. duration of insulin therapy if applicable)
- Previous Ophthalmic Treatment (laser, intravitreal injections, surgery)
- Grade of DR ($M_{0\text{-}2,}$ $R_{0\text{-}4}$)
- Blood pressure
- HbA_{1c}
- Haemoglobin, haematocrit
- Proteinuria
- Serum creatinine
- Cholesterol

Uveitis patients:

- Classification (anterior, intermediate, posterior, pan)
- Diagnosis
- Systemic immunosuppression in 1 year before surgery (yes/no)
- Pulsed systemic steroids at time of surgery (yes/no)
- Peri-ocular steroid injection (yes/no and time before surgery)

Macular hole patients:

- Stage (1a/b, 2, 3, 4)

Data collection will be by the research nurse, the clinical research fellow and other study investigators, initially on paper for entry into the database. The database has not yet been designed or built. Paper records will be stored in a secure location in Birmingham Midland Eye Centre.

Data collection and the future database will meet the obligations under the Data Protection Act 1998.

CONFLICT OF INTEREST

None is identified

DISCONTINUATION

No foreseeable circumstances would require discontinuation.

RESEARCH GOVERNANCE, MONITORING AND ETHICS & R&D APPROVAL

The study will be conducted in compliance with the Research Governance Framework for Health and Social Care and Good Clinical Practice (GCP).

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(iii) Patient consent form

IRAS Number: 12/WM/0330

PATIENT CONSENT FORM

Title of Project: **Improving patient outcomes after ocular trauma**

Investigator	Centre	Patient Identification Number
_____	☐☐☐☐	☐☐☐☐☐☐☐☐

Please initial box

1. I confirm that I have read and understand the Information Sheet dated 23 Nov 2015 (v4) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to the collection, storage and laboratory analysis (including genetic studies) of my eye tissue samples including vitreous, retina and fluid under the retina and inside the front of the eye where these are removed as part of routine surgery. ☐

4. I agree to the collection, storage and laboratory analysis of fluid from inside the front of my eye (aqueous) where this is not part of routine surgery. ☐

5. I agree to the collection, storage and laboratory analysis of blood, urine and tear samples. ☐

6. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

7. I consent to the collection, processing, reporting and storage within the UK of my personal and sensitive data (e.g. date of birth) for healthcare and/or medical research purposes in relation to this research. I understand that I will not be directly identifiable except to the study doctor and his/her study team. ☐

8. I agree to the storage of any of my samples that remain at the end of this project and their use in future ethically approved research, including genetic studies. ☐

9. I agree to take part in the above study. ☐

10. I agree to the use of my data or results which arise from their participation in this study and understand that those data or results will be limited to the use described in the information sheet. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person	Date	Signature