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The biochemical rationale for normobaric hyperoxia treatment of retinal disorders

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THE BIOCHEMICAL RATIONALE FOR NORMOBARIC HYPEROXIA TREATMENT OF RETINAL DISORDERS

by

CHRISTOPHER HSU

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Approved by

First Reader

Brigitte Ritter, Ph.D.
Assistant Professor of Biochemistry

Second Reader

Jorge G. Arroyo, M.D., M.P.H
Associate Professor of Ophthalmology
Harvard Medical School

DEDICATION

I would like to dedicate this work to my family.

THE BIOCHEMICAL RATIONALE FOR NORMOBARIC HYPEROXIA TREATMENT OF RETINAL DISORDERS

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ABSTRACT

Purpose: Ischemic retinopathies such as diabetic retinopathy (DR), retinal vein occlusions (RVO), and age-related macular degeneration (AMD) are ocular diseases caused by abnormal changes in the microvasculature that results in ischemia. This is often followed by a secondary phase characterized by pathological neovascularization and leakage of fluid, which contributes to a loss of visual acuity in affected patients. Anti-VEGF therapy, the current standard of treatment for ischemic retinopathies, is invasive, costly, and lacks a known treatment period. Supplemental oxygen provides the therapeutic potential of not only oxygenating hypoxic retinal cells, but also reducing the neovascularization and edema associated with many ischemic retinopathies through the downregulation of proangiogenic and pro-inflammatory cytokines.The objective of this study is to understand the biochemical underpinnings of treating ischemic retinopathies with hyperoxia. The elucidation of the effect hyperoxia on the molecular level may help guide the development of future studies regarding this novel treatment.

Methods: 68 undiluted vitreous samples were obtained during pars plana vitrectomy (PPV) and the concentration analysis of 34 proteins was analyzed using the Bio-Plex Pro Human Cancer Biomarker Assay. Vitreous samples were divided into three groups: (1) eyes of patients who underwent PPV for epiretinal membrane peeling (ERMP) and/or macular hole (MH) with no history of diabetes mellitus (non-DM group); (2)

eyes of patients who underwent PPV for ERMP and/or MH with a history of diabetes or nonproliferative diabetic retinopathy (DM group); (3) eyes of patients who underwent PPV for proliferative diabetic retinopathy (PDR group). Mann-Whitney *U* tests were performed to compare the biomarker concentrations between the three groups.

Results: Numerous growth factors and inflammatory cytokines were significantly upregulated between the non-DM and PDR groups - Angiopoietin-2, EGF, Endoglin, G-CSF, HB-EGF, HGF, PDGF, PIGF, sHER2/neu, TIE-2, VEGF-A, VEGF-D, IL-18, IL-6, IL-8, PECAM-1, sCD40L, SCF, sFASL, sIL-6Ra, TNF-α, Leptin, PAI-1, and uPA.
A literature search of these proteins revealed many to be directly activated by HIF-1 transcription factor, which is the "master switch" for genes transcribed during a hypoxic event.

Conclusion: The abundance of proangiogenic and pro-inflammatory factors in PDR that are also upregulated by HIF-1 demonstrate the potential for using hypoxia to treat PDR (and other ischemic retinopathies) through the reduction of HIF-1. This study also shows the wide variability in the expression levels of these proteins which helps provide a better understanding of their degree of involvement in the pathogenesis of ischemic retinopathies.

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LIST OF ABBREVIATIONS

AMD ..... Age-Related Macular Degeneration

BRB ..... Blood-Retinal Barrier

BRVO ..... Branch Retinal Vein Occlusion

CRVO ..... Central Retinal Vein Occlusion

DME ..... Diabetic Macular Edema

DR ..... Diabetic Retinopathy

EPO ..... Erythropoietin

ERMP ..... Epiretinal Membrane Peeling

HIF-1 ..... Hypoxia-Inducible Factor 1

MH ..... Macular Hole

NPDR ..... Non-Proliferative Diabetic Retinopathy

PDR ..... Proliferative Diabetic Retinopathy

PO_2 ..... Partial Pressure of Oxygen

PPV ..... Pars Plana Vitrectomy

RD ..... Retinal Detachment

ROS ..... Reactive Oxygen Species

RPE ..... Retinal Pigment Epithelium

RVO ..... Retinal Vein Occlusions

VEGF ..... Vascular Endothelial Growth Factor

VHL ..... Von Hippel-Lindau Disease

INTRODUCTION

The retina is considered the most metabolically active tissue in the human body due to the high energy cost of phototransduction, neurotransmitter exocytosis, and protein/ion transport by photoreceptors (Wong-Riley, 2010). Impairment of the retinal blood supply is associated with numerous retinopathies, and often adversely impacts retinal function and consequently, vision. In patients with retinal detachment (RD), hypoxia caused by the physical separation of photoreceptors from the underlying choroid leads to photoreceptor degeneration (Lewis et al., 2004). Although reattachment surgery can successfully repair the retina anatomically, vision acuity is not always restored due to the loss of photoreceptor structural integrity during the ischemic period (Put et al., 2014). Hypoxia also plays a causative role in diseases such as proliferative diabetic retinopathy (PDR), exudative (wet) age-related macular degeneration (AMD), and retinal vein occlusions (RVO) by upregulating expression of vascular endothelial growth factor (VEGF), a potent promoter of neovascularization and vascular leakage (Linsenmeier & Zhang, 2017). Anti-VEGF medications have revolutionized the treatment of these conditions in recent years due to their efficacy in reducing pathological angiogenesis and edema. However, the invasive nature, cost, and lack of a known treatment period of anti-VEGF therapeutics are a burden to patients and healthcare institutions (Zhao & Singh, 2018). More importantly, accumulating data suggest that an abundance of cytokines/chemokines other than VEGF contribute to the pathogenesis of ischemic retinopathies, which may explain why some patients do not respond to anti-VEGF

treatments (Bolinger & Antonetti, 2016). Therefore, there is a need to consider an alternative treatment that can be used adjunctively with anti-VEGF agents or by itself to improve the management of retinal diseases.

Supplemental oxygen, or hyperoxia, directly targets the hypoxic nature of retinal detachments and ischemic retinopathies by supplying the retina with a higher concentration of inspired oxygen. In addition, hyperoxia may diminish expression levels of numerous proangiogenic and pro-inflammatory factors by reducing the activation of hypoxia-inducible factor 1 (HIF-1) – the "master switch" of oxygen homeostasis (Arjamaa & Nikinmaa, 2006). Previous research has demonstrated the potential of hyperoxia in reducing photoreceptor death after a RD and decreasing macular thickness in patients with diabetic macular edema (DME); however, a deeper understanding of the effectiveness of hyperoxia has yet to be established (Mervin et al., 1999; Nguyen et al., 2004). This study investigates the biochemical rationale for the normobaric hyperoxia treatment of retinopathies in an ongoing pilot study conducted at the Beth Israel Deaconess Medical Center.

1. Significance of Oxygen for Retinal Function

A dysregulation of the oxygen supply to the retina often leads to a loss of retinal function due to the critical role that oxygen plays in supporting the high metabolic demand of the retina (Linsenmeier & Padnick–Silver, 2000). While low oxygen tissue levels can contribute to retinopathy, excessive amounts of oxygen lead to an increased amount of reactive oxygen species (ROS) and cellular damage (Blasiak et al., 2014). As a

result, maintenance of oxygen levels and a healthy vascular network are necessary to support optimal retinal function.

1.1 Anatomy of the Retina

Located posterior to the vitreous cavity of the eye, the retina is a multilayered structure that converts incoming light into electrical signals that the brain can process (Figure 1). The major cellular components of the retina are the photoreceptors, retinal pigment epithelium (RPE) cells, interneuron cells, ganglion cells, and glial cells. The photoreceptor layer, which harbors the rods and cones, is responsible for the process of phototransduction (Figure 2). Rods exhibit a high sensitivity to light and mediate vision

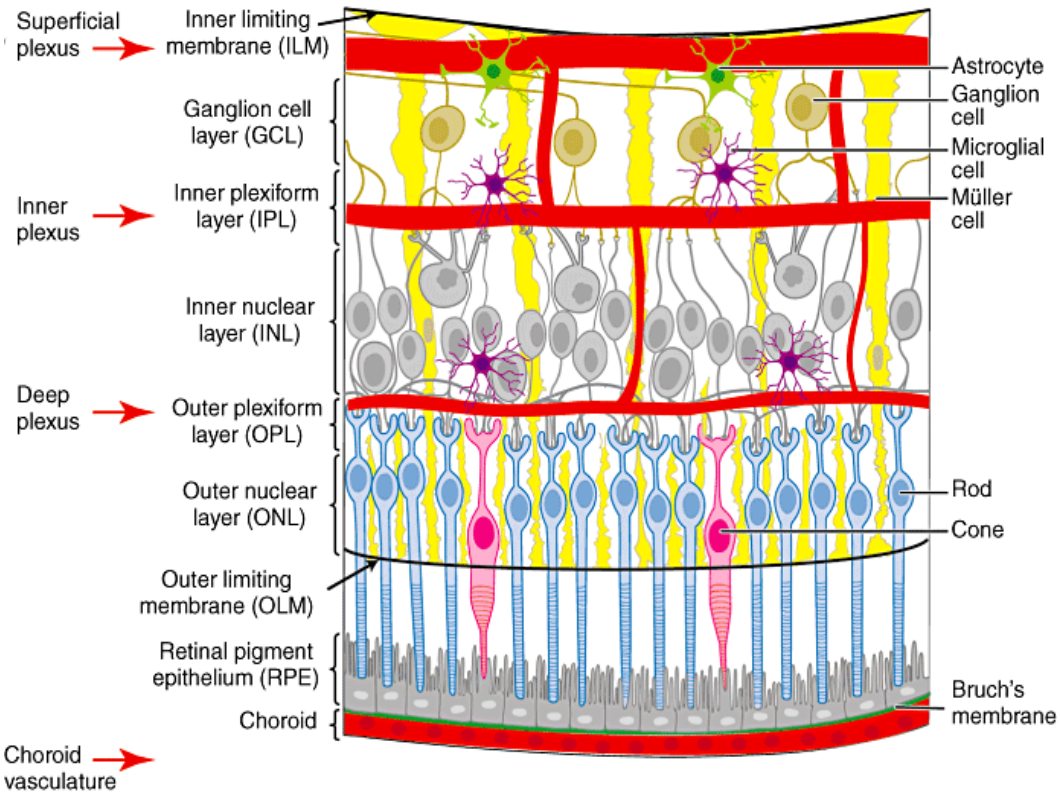

Figure 1 – Cells and Layers of the Retina. Illustrated above are the cellular components of the retina, organizational layers, and the retinal and choroidal circulations. Figure taken from Coorey et al., 2012.

in dim environments while cones have a comparatively low sensitivity to light and contribute to high acuity color vision. In the human retina, rods comprise 95% of all photoreceptors and dominate the peripheral retina while cones account for 5% and are concentrated within the fovea (Narayan et al., 2017). The four main structural regions of each photoreceptor are the synaptic terminal, cell body, mitochondria-containing inner segment, and an outer segment that contains visual pigment responsible for light absorption.

RPE cells are joined together by tight junctions and collectively form the outer blood-retina barrier (BRB), a physiological barrier that separates the choriocapillaris from the photoreceptors (Narayan et al., 2017). Interneurons, which include bipolar, amacrine, horizontal, and interplexiform cells, are located within the inner nuclear layer and transmit electrical signals emitted from the photoreceptors to the ganglion cells. The ganglion cells convey electrical information from the retina to the rest of the brain. Their axons congregate at the optic disk and exit the eye as the optic nerve.

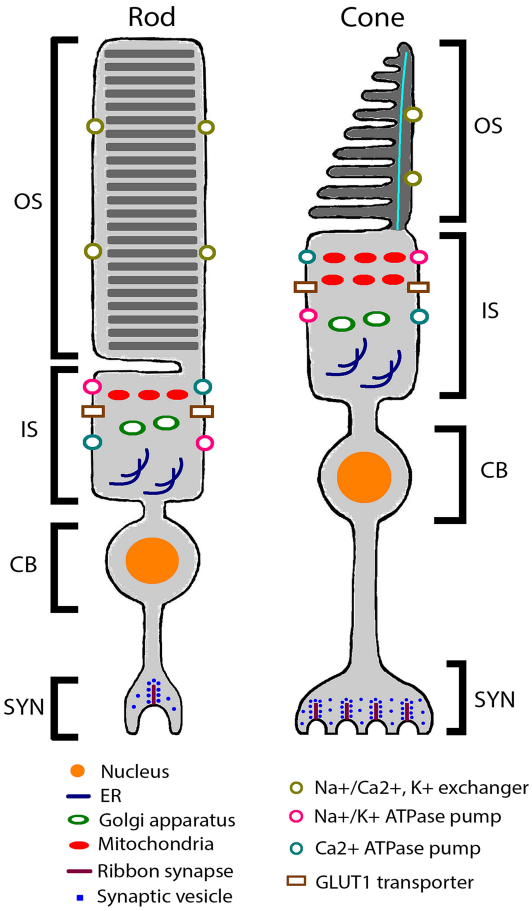

Figure 2 – Structural Anatomy of Photoreceptors. Rods and cones have four main structural regions: outer segment (OS), inner segment (IS), cell body (CB), and synaptic terminal (SYN). Figure taken from Narayan et al., 2017.

Finally, the four glial cell types – Muller cells, astrocytes, microglia, and oligodendrocytes – collectively function to maintain an optimal local environment (Joussen et al., 2007).

1.2 Retinal and Choroidal Circulation

The blood supply of the retina is provided by the retinal and choroidal vasculatures. The retinal circulation, which is derived from the central retinal artery, consists of three parallel yet interconnected vascular plexuses – the superficial, inner, and deep plexus (Figure 1). Together they vascularize the inner retina (Eshaq et al., 2014). On the other hand, the choroidal circulation is supplied by the long and short posterior ciliary arteries and forms a single layer of densely arranged fenestrated capillaries called the choriocapillaris. Located immediately behind the retina, the choroid supplies blood to the avascular outer retina (photoreceptors and RPE) (Eshaq et al., 2014). This unique dual blood supply circumvents the dilemma of blood vessels positioned directly anterior to the photoreceptor outer segments, which would interrupt light absorption (Narayan et al., 2017). However, the two circulations are non-overlapping, thus making the retina vulnerable to either retinal or choroidal ischemia.

The choroidal circulation is characterized by a high flow rate (estimated 1400ml/100g per minute) and low O_2 extraction (<1 vol %) (Linsenmeier & Padnick–Silver, 2000). The combination of these two factors generates a large enough O_2 gradient between the choroid and outer retina to sustain the high metabolic demand of the photoreceptor inner segments (Linsenmeier & Padnick–Silver, 2000). Changes in the partial pressure of oxygen (PO_2) of the choriocapillaris or the distance between the

choroid and photoreceptor inner segments alter the O_2 consumption of photoreceptors. For example, a study has shown that a 100μm retinal detachment lowers the average O_2 consumption of the outer retina to 34% of that in a healthy individual (Linsenmeier & Padnick–Silver, 2000). In contrast, the retinal circulation exhibits a low blood flow rate and high O_2 extraction (40-50 vol %) similar to that of the rest of the brain (Bill & Sperber, 1990). Here, a large O_2 flux such as that associated with the choroidal circulation is unnecessary due to the proximity of the retinal capillary plexuses to the inner retinal cells (Bill & Sperber, 1990).

The regulation of the two vasculatures also differ significantly. The retinal circulation is controlled by autoregulation and responds to changes in hydrostatic pressure and fluctuations in O_2, CO_2, pH, and temperature (P. A. Campochiaro, 2000). During hyperoxia, the retinal circulation theoretically vasoconstricts and maintains inner retinal PO_2 levels close to that at air breathing. However, hypercapnia superimposed on hyperoxia may reduce the autoregulatory constriction of retinal vessels (Yu et al., 2007). On the other hand, the choroidal circulation is not auto-regulated such that its regulation is independent of local metabolic factors (Bill & Sperber, 1990). Instead, it is strongly innervated by the autonomic nervous system and responds to systemic changes (P. A. Campochiaro, 2000). Under hyperoxia, the PO_2 of the choriocapillaris increases and enhances the O_2 flux to the outer retina (Linsenmeier & Zhang, 2017).

1.3 Energy Consumption of Photoreceptors

Neuronal activity is tightly coupled with energy metabolism, especially so with the metabolically demanding visual system. Retina sections stained with cytochrome c

oxidase, the terminal enzyme of the electron transport chain and a measure of oxidative capacity, reveal intense labeling throughout the inner segments of photoreceptors – an indication of mitochondrial abundance (Wong-Riley, 2010). ATP generated by the inner segments fuel major ATP-consuming activities within the photoreceptor, primarily the active transport of ions and glutamate release at synaptic terminals (Narayan et al., 2017).

It is important to note that photoreceptor energy consumption is also light-dependent (Figure 3). During darkness, cation channels in photoreceptor outer segments open and create a large inward gradient of ions known as the dark current (Ramsey & Arden, 2015). The partial depolarization resulting from the influx of cations propagates glutamate release from the synapses. In addition, Na^+K^+ ATPases located in the inner segment transmembrane actively pump out the excess Na^+ to maintain the dark current.

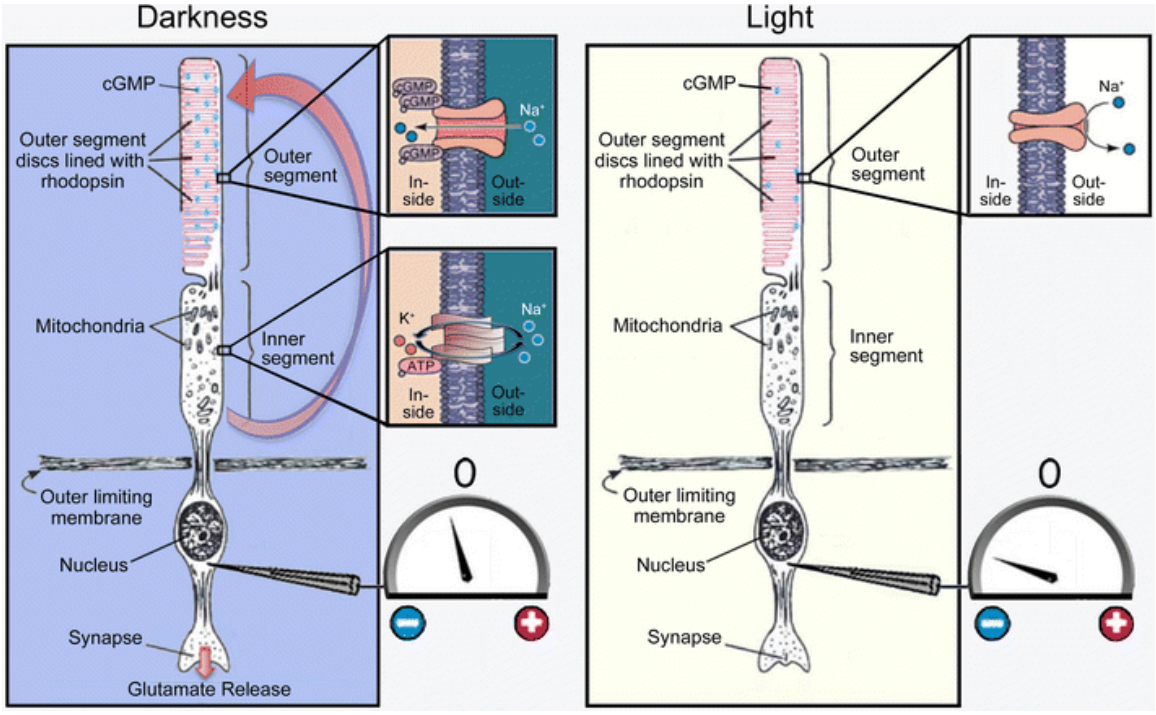

Figure 3 – Energy Consumption in Darkness and Illumination. The metabolic demand and oxygen consumption required to sustain the dark current and glutamate exocytosis is substantial. Figure taken from Ramsey & Arden, 2015.

This cycle of continuous neurotransmitter release and Na^+K^+ ATPase activation consumes large amounts of ATP produced in the inner segments. During illumination, photoreceptors engage in phototransduction, which converts photons into electrical signals through a signal transduction cascade and causes closure of cation channels in the outer segment. These processes also consume energy but are relatively small compared to that in the dark current (Ramsey & Arden, 2015). In the rods of mice, ATP expenditure is estimated to be $9*10^7$ ATP s^{-1} in darkness and only $2*10^7$ ATP s^{-1} under illumination (Wong-Riley, 2010). In fact, there is evidence that the dark current exacerbates hypoxia in ischemic retinal diseases due to its toll on the oxidative capacity of the receptor (Ramsey & Arden, 2015). Thus, sufficient blood flow and oxygen delivery through the retinal/choroidal circulations are critical for retinal function due to the high metabolic demand of the eye.

2. Rationale of Hyperoxia as a Potential Treatment

Hypoxia is implicated in the pathogenesis of numerous retinal diseases, although the mechanisms through which they manifest varies (Linsenmeier & Zhang, 2017). As a result, the treatment efficiency of supplemental oxygen could fluctuate depending on the type of retinal disorder and the method in which ischemia is induced. Previous research has sought to elucidate the role of hypoxia in different retinopathies – RD, RVO, AMD, PDR – and some clinical studies have investigated the potency of hyperoxia in improving functional and anatomical outcomes.

2.1 Oxygen and Diabetic Retinopathy

Diabetic retinopathy (DR) is the most frequent complication of diabetes and the leading cause of blindness in the developed world (Al-Shabrawey et al., 2015). There are two main groups of DR – non-proliferative diabetic retinopathy (NPDR) is characterized by increased vascular permeability, microaneurysms, and intraretinal hemorrhages while proliferative diabetic retinopathy (PDR) is marked by additional neovascularization (Figure 4) (Joussen et al., 2007). One of the initial abnormalities of DR is the decrease of retinal perfusion caused by a vasoconstriction of arteries (Semeraro et al., 2015). Retinal pericyte loss is another characteristic feature of DR that contributes to retinal ischemia by causing endothelial cell damage and capillary destabilization (Semeraro et al., 2015). The resulting hypoxic state induces biochemical alterations that include an upregulation of vascular endothelial growth factor (VEGF) and secretion of pro-inflammatory cytokines (Linsenmeier & Zhang, 2017). VEGF, in particular, is a critical player during hypoxia due to its potency in mediating retinal angiogenesis and the breakdown of the BRB (Arjamaa & Nikinmaa, 2006). Ultimately, the hyperpermeability of the abnormal blood vessels and disruption of the BRB could lead to an accumulation of intraretinal and subretinal fluid known as diabetic macular edema (DME), which is a major cause of vision regression among diabetic patients.

One pilot study treated diabetic patients suffering from chronic DME with three months of supplemental oxygen (4 L/min of O2), and observed reductions in macular thickness and improvements in visual acuity (Nguyen et al., 2004). In another study, diabetic subjects that were on 100% oxygen exhibited improvements in contrast sensitivity whereas normal subjects showed no significant changes (Harris et al., 1996).

In addition, Harris et al (1996) found that retinal blood flow was not diminished in diabetic subjects after hyperoxia, which indicates that diabetic patients may have an impaired autoregulatory capacity. Therefore, patients with DR may experience increased benefits from supplemental oxygen due to the lack of hyperoxia-induced vasoconstriction in the retinal circulation.

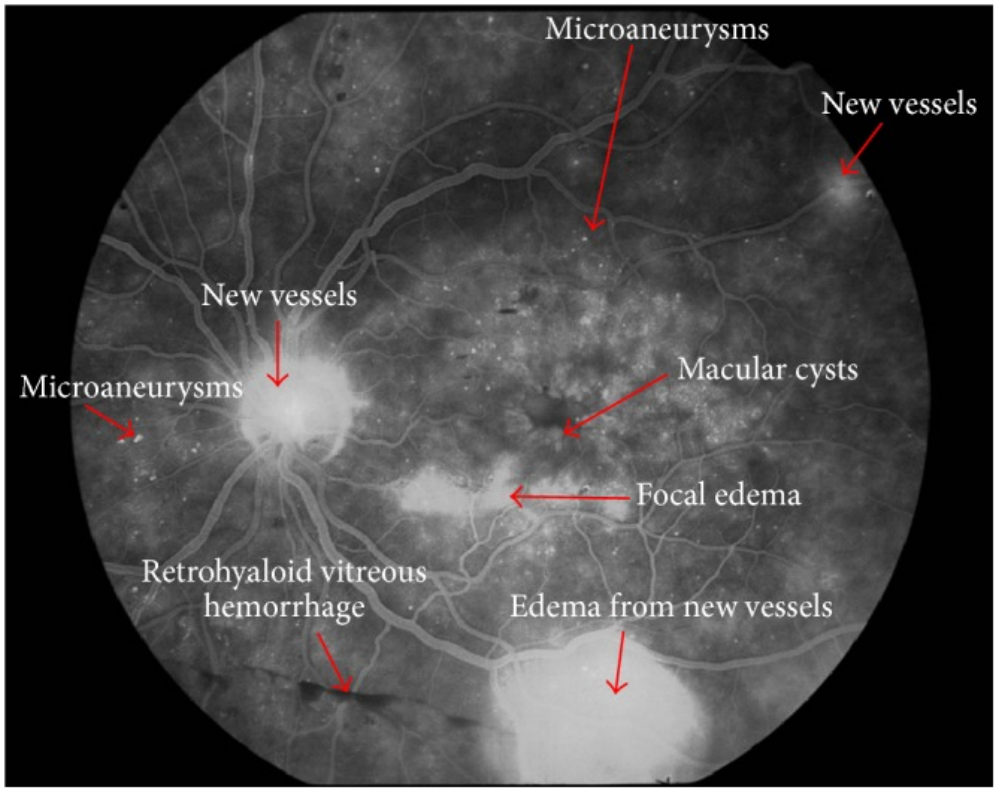

Figure 4 – Fluorescein Angiography of PDR Patient. The abnormal growth of new vessels and the resulting edema are indicated by the red arrows. Figure taken from Romero-Aroca et al., 2016.

2.2 Oxygen and Retinal Vein Occlusions

Retinal vein occlusions (RVO) involve an obstruction of venous return from the retinal circulation (Khayat et al., 2018). Classically, RVO is broken down into central

retinal vein occlusion (CRVO) if the obstruction occurs within the central retinal vein, or a branch retinal vein occlusion (BRVO) if any tributary is obstructed (Ip & Hendrick, 2018). After the thrombus has formed, retinal blood flow decreases and autoregulation increases intravenous hydrostatic pressure, causing damage to vascular endothelial cells and increasing vascular permeability (Khayat et al., 2018). This results in retinal hemorrhages and macular edema, both of which contribute to vision impairment. In addition, the increased intravenous hydrostatic pressure could decrease the pressure difference across capillary networks, causing capillary nonperfusion and retinal hypoxia (Khayat et al., 2018). The ischemic retina upregulates VEGF production prompting neovascularization, which frequently leads to numerous vision-threatening complications (Joussen et al., 2007). However, retinal oximetry and comparisons of oxygen saturation in RVO patients have demonstrated that ischemia is not associated with all patients with RVO for reasons that are not yet fully understood (Hardarson & Stefánsson, 2012). As such, risk of neovascularization is 35% in ischemic RVO patients compared with 10% in the non-ischemic counterparts (Ip & Hendrick, 2018).

Supplemental oxygen may be beneficial in treating RVO due to the reduced blood flow and increased VEGF production associated with the disease. There is, however, limited literature on the effects of hyperoxia on RVO. One study investigated the effects of hyperbaric hyperoxia on miniature pigs with experimental BRVO and found that the resulting choroidal O_2 gradient was high enough to supply O_2 to the ischemic inner retina (Pournaras et al.,1990).

2.3 Oxygen and Age-related Macular Degeneration

Age-related macular degeneration (AMD) involves the degeneration of the central retina and is classified into two clinical forms – non-exudative/dry AMD and exudative/wet AMD (Stefánsson et al., 2011). Dry AMD, which accounts for 85% of all cases, is characterized by drusen and loss of photoreceptors while the hallmark of wet AMD is choroidal neovascularization (Vadlapatla et al., 2013). Measurements of oxygen tension have demonstrated that choroidal blood circulation is diminished in both wet and dry AMD, and that the loss of choroidal perfusion is positively correlated with AMD severity (Metelitsina et al., 2008). In addition to the ischemic choroid, it has been suggested that drusen formation and thickening of the Bruch's membrane in dry AMD also impair the diffusion of O_2 from the choriocapillaris to the photoreceptors (Stefánsson et al., 2011). For wet AMD, hypoxia induces VEGF release from RPE cells, which causes choroidal neovascularization (and leakage of fluid) into the subretinal space through a break in Bruch's membrane (Peter A. Campochiaro, 2015). Hyperoxia may benefit AMD patients in two ways – it could increase diffusion of O_2 from the choroid to the photoreceptors and lower the production of VEGF and other proangiogenic factors to reduce choroidal neovascularization.

2.4 Oxygen and Retinal Detachment

RD is characterized by a separation of the retina from the underlying RPE, and the most common form of RD – rhegmatogenous retinal detachment – is caused by a tear in the retina that allows leakage of fluid from the vitreous cavity to the subretinal space (Put et al., 2014). The increased distance between the retina and the underlying choroidal blood supply reduces the flux of O_2 reaching the photoreceptors, which induces

photoreceptor apoptosis and subsequently, the loss of mitochondria in the inner segments and the degeneration of outer segments (Vadlapatla et al., 2013).

Mathematical models have suggested that supplemental oxygen increases choroidal oxygen tension and the O_2 flux to the inner segments to compensate for the poor diffusion during a RD (Linsenmeier & Padnick–Silver, 2000). In addition, feline and squirrel models with surgically-induced RD have demonstrated improved photoreceptor survivability after treatment with 70% oxygen (Lewis et al., 2004; Sakai, Lewis, Linberg, & Fisher, 2001). As a result, patients with RD could benefit from hyperoxia between diagnosis and the scheduled reattachment surgery to limit photoreceptor apoptosis (Mervin et al., 1999).

3. Biochemical Underpinnings of Hyperoxia Treatment

Retinal cells are sensitive to fluctuations in oxygen tension and employ intricate mechanisms to adjust to sudden deviations in PO_2. The hypoxia-inducible factor (HIF-1) is a transcriptional regulator that plays a central role in mediating the cellular changes following oxygen deprivation (Vadlapatla et al., 2013). Under hypoxic conditions, HIF-1 is activated and upregulates the expression of various genes, many of which contribute to the development and progression of retinal pathologies such as DR, AMD, and BRVO/CRVO (Linsenmeier & Zhang, 2017). Of the numerous HIF-1 downstream products, VEGF has garnered the most attention due to its role as a potent angiogenic and vascular permeabilization factor in many retinal disorders (Gao et al., 2017). Although VEGF antagonists have revolutionized the treatment of ocular diseases involving

neovascularization, there are other proangiogenic and pro-inflammatory factors activated by HIF-1 that are unaffected by VEGF inhibitors (Iwase et al., 2013). Since HIF-1 is activated by hypoxia, supplemental oxygen could reduce the expression of HIF-1 in patients with ischemic retinal disorders. By targeting this common denominator in hypoxia-induced events and reducing the transcription of its downstream effectors, there is a possibility of achieving greater efficacy than with VEGF inhibitor treatments alone.

3.1 Regulation of HIF-1

HIF-1 is a heterodimeric protein consisting of two subunits – HIF-1α and HIF-1β (Hong et al., 2004). During normal oxygen perfusion, HIF-1α is heavily hydroxylated and binds to the von Hippel-Lindau disease (VHL) protein, which targets it for ubiquitin-mediated proteasomal degradation (Figure 5)(Joussen et al., 2007). When cells are hypoxic, HIF-1α is not hydroxylated and freely dimerizes with the constitutively expressed HIF-1β, forming the HIF-1 complex (Joussen et al., 2007). The assembled HIF-1 transcription factor then translocates into the nucleus and activates an array of hypoxia-inducible genes responsible for regulating energy metabolism, angiogenesis, cell cycle, and apoptosis (Arjamaa & Nikinmaa, 2006). As a result, oxygen plays a critical role in regulating the function of HIF-1 through the post-translational modification of HIF-1α.

3.2 Vascular Endothelial Growth Factor

VEGF, a 48 kDa homodimeric glycoprotein, is an endothelial cell mitogen and angiogenic factor that is critical during developmental vasculogenesis to ensure sufficient

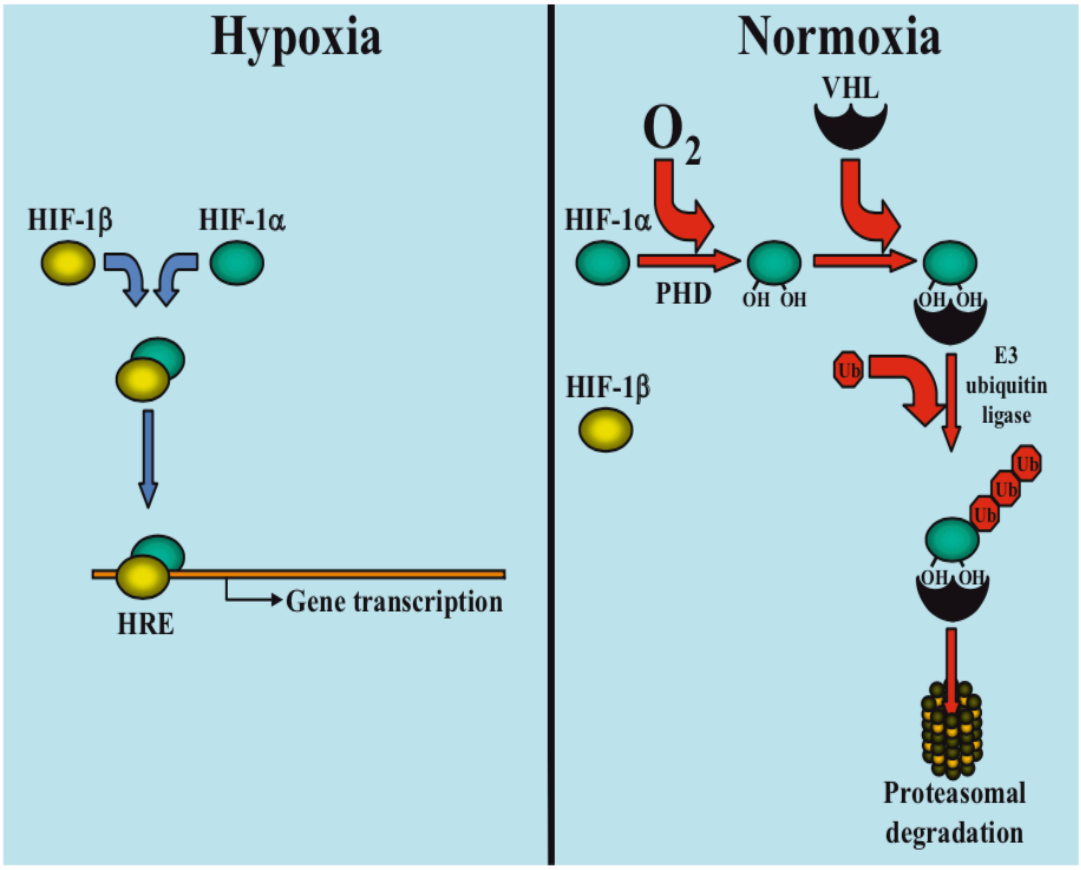

Figure 5 – HIF-1α Regulation is Oxygen Dependent. During normoxia, HIF-1α is hydroxylated and degraded by proteasomes, preventing the assembly of HIF-1 heterodimer. In hypoxia, HIF-1α is not hydroxylated and freely binds to HIF-1β forming HIF-1, which activates downstream effectors. Figure taken from Joussen et al., 2007.

retinal vessel architecture (Cheng et al., 2017). But in many retinal diseases, VEGF release promotes pathological angiogenesis that features permeable vessels susceptible to leakage of fluid (Campochiaro, 2015). Studies have demonstrated that VEGF expression is elevated in retinopathies involving ocular neovascularization, including PDR, wet AMD, and ischemic CRVO/BRVO (Joussen et al., 2007). The hemorrhaging and fluid leakage from these abnormal blood vessels distort vision and can eventually lead to the formation of scar tissue (Bolinger & Antonetti, 2016).

The approval of anti-VEGF treatments by the FDA in 2004 has been monumental in improving the prognosis of patients with neovascular retinopathies (Zhao & Singh, 2018). However, more studies are beginning to indicate that a significant number of patients with pathological angiogenesis do not respond to anti-VEGF treatments, which suggests the involvement of non-VEGF mediators in the pathogenesis of these retinal diseases (Bolinger & Antonetti, 2016).

3.3 Pro-inflammatory Cytokines

It is widely accepted that inflammation assumes an important role in retinopathies such as DR and AMD (Semeraro et al., 2015). In DR, the release of inflammatory molecules including ICAM-1, TNF-α, IL-1, and COX-2 contributes to the degeneration of retinal capillaries (Semeraro et al., 2015). Other pro-inflammatory cytokines such as IL-6 and IL-8 may also contribute to neovascularization and enhance leukocyte adhesion to the endothelium (Semeraro et al., 2015). Using rat models of DR, one research group observed that the onset of HIF-1α expression is closely correlated with the upregulation of IL-6, IL-1β, and TNF-α and suggest that the targeted inhibition of HIF-1α could lower the levels of these cytokines (Gao et al., 2017). Another study found that in HIF-1α knockout mice, ICAM-1 expression was significantly attenuated, while vascular leakage and neovascularization were reduced as well (Lin et al., 2011). These results not only indicate that inflammatory cytokines are potent mediators in the pathophysiology of many retinopathies, but also that HIF-1 inhibition may be a viable therapeutic option.

3.4 Other Mediators Involved in Ischemic Retinopathies

Erythropoietin (EPO) is usually produced in the kidneys and triggers the synthesis of red blood cells in the event of hypoxia (Arjamaa & Nikinmaa, 2006). However, in the event of acute retinal hypoxia, EPO is also expressed through a HIF-1 mediated mechanism and functions to induce retinal angiogenesis (Arjamaa & Nikinmaa, 2006). In fact, retinal EPO stimulates neovascularization in PDR independently of VEGF, which could explain cases of patients who do not respond to anti-VEGF treatment (Watanabe et al., 2005). Suppression of both EPO and VEGF also inhibited neovascularization to an extent greater than that achieved by each compound alone (Takagi et al., 2007).

In addition, apoptotic signaling events contribute to photoreceptor cell death following retinal ischemia, which can impair visual prognosis even after successful treatment (Linsenmeier & Padnick–Silver, 2000). In cases of RD, most photoreceptor apoptosis occurs 1 to 3 days post-detachment and is regulated by caspase activation (Yang et al., 2004). Similarly, caspase-dependent apoptosis of ganglion cells occurs soon after developing DR resulting in progressive impairment of visual acuity (Adamiec-Mroczek et al., 2015). In a recent study, Caspase-3 – an indicator of cellular apoptosis – was found to be elevated in retinal tissues in a rat model of DR (Gao et al., 2017). Furthermore, inhibition of retinal HIF-1α significantly reduced expression levels of Caspase-3, which strongly suggests the involvement of HIF-1α in the apoptotic pathway (Gao et al., 2017). As a result, hyperoxia treatment may also reduce retinal cell apoptosis by attenuating HIF-1 activation after ischemic insult.

Some other growth factors implicated in the pathogenesis of ischemic retinopathies include insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor

(bFGF), placental growth factor (PGF), and angiopoietin-2 (ANGPT2) (Iwase et al., 2013; Semeraro et al., 2015). Furthermore, chemokines such as monocyte chemoattractant protein-1 (MCP-1) and stromal cell-derived factor (SDF-1) stimulate the migration of leukocytes to endothelial cells, which further enhances fibrosis and

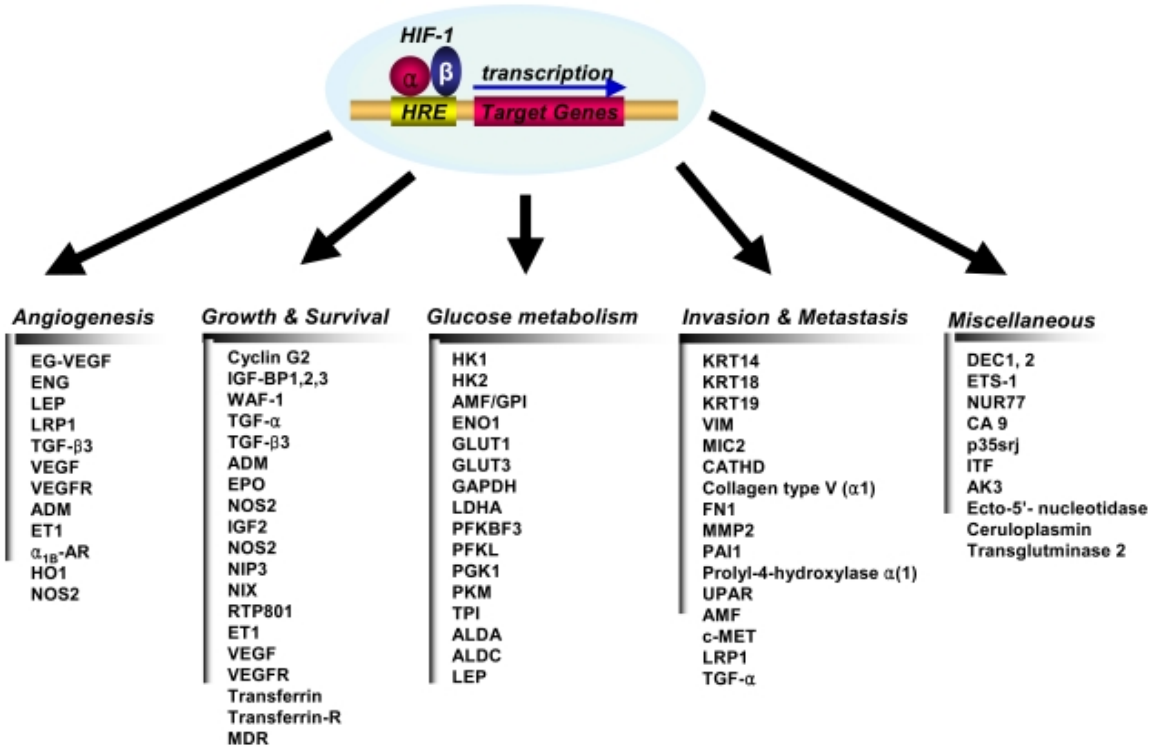

Figure 6 – Target Genes Transcriptionally Activated by HIF-1. Figure taken from Takagi et al., 2007.

angiogenesis (Semeraro et al., 2015). While there is still much to be uncovered about the involvement of proangiogenic and pro-inflammatory factors in the pathogenesis of retinal diseases, many are transcriptionally regulated by HIF-1 (Figure 6) (Takagi et al., 2007).

Instead of approaching the matter by implementing a combined cocktail of specific inhibitors to target these molecules, the inhibition of upstream HIF-1 may achieve the same effect with more convenience and efficacy. One study delivered a HIF-

1 inhibitor (Digoxin) intraocularly into a mouse model of choroidal neovascularization and found significantly reduced levels of HIF-1 target genes and a regression of ocular neovascularization (Iwase et al., 2013). Through the attenuation of HIF-1 and its associated effector genes, supplemental oxygen could offer a more cost-effective and non-invasive therapeutic avenue for treating ischemic retinal diseases.

Aims and Objectives:

The purpose of this study was to investigate the benefits of hyperoxia in treating ischemic retinopathies (RD, PDR, RVO, AMD) and to provide a biochemical justification of the treatment through the analysis of vitreous mediators in PDR patients. We hypothesized that through the enhanced delivery of oxygen to retinal cells and reduction of proangiogenic/pro-inflammatory factors, supplemental oxygen can improve the visual and anatomical outcomes of patients with retinal ischemia.

METHODS

This study was a combination of an ongoing pilot study of hyperoxia treatment and a retrospective analysis of vitreous mediators extracted from PDR patients. The aim is to evaluate the treatment of ischemic retinopathies with supplemental oxygen through the biochemical analysis of proangiogenic/pro-inflammatory factors in PDR patients. The following methods and data analysis details the collection and analysis of the vitreous samples obtained from patients who underwent vitrectomy, and was adapted from Kovacs et al., 2015.

Study Population

Undiluted vitreous samples were collected from 68 eyes of 68 patients who underwent pars plana virectomy (PPV) performed by Jorge Arroyo, MD, MPH, at the Beth Israel Deaconess Medical Center between November 2010 and September 2012. Patients who underwent PPV for epiretinal membrane peeling (ERMP)/Macular Hole (MH) or PDR were included in the analysis, while those who underwent surgery for other conditions were excluded. Clinical consent was obtained from each patient for sample collection.

Vitreous Collection, Storage, and Analysis

During PPV, undiluted vitreous and serum samples were extracted and collected from the patients with a biopsy technique detailed in Arroyo et al., 2005. In general, before turning on the infusion and initiating the vitrectomy, the vitreous cutter was used

to obtain a 0.5- to 1-ml undiluted vitreous sample. A 3-ml syringe on a three-way stopcock facilitated the manual aspiration of the vitreous specimen. Once an adequate amount of vitreous was obtained, the infusion was turned on and the stopcock was rotated to proceed with the vitrectomy. The undiluted vitreous specimens were then immediately placed into a cold storage freezer that was maintained at -80˚C. The samples were subsequently shipped overnight on dry ice to Schepens Eye Research Institute (Boston, MA, USA) for analysis with the Bio-Plex Pro Human Cancer Biomarker Assay.

The Bio-Plex Pro Human Cancer Biomarker Assay is a magnetic bead-based assay that accurately quantifies a mixture of 34 biomarkers involved in disease mechanisms including angiogenesis, metastasis, cell proliferation, cell adhesion/migration, apoptosis, and inflammation. The analyzed proteins consist of Angiopoietin-2, EGF, Endoglin, FGF, Follistatin, GCSF, HB-EGF, HGF, IGFBP, IL-6, IL-8, IL-18, Leptin, Osteopontin, PAI-1, PDGF, PECAM-1, PlGF, Prolactin, sCD40L, SCF, sEGFR, sFASL, sHER2/neu, sIL-6Ra, sVEGF-R1, sVEGF-R2, TGF-alpha, TIE-2, TNF-alpha, uPA, VEGFa, VEGFc, VEGFd. The concentrations of proteins were reported in picograms per millimeter.

To set up the assay, vitreous samples were thawed, the Bio-Plex machine was calibrated, and the lasers of the equipment were pre-warmed for 4 hours. The standards were reconstituted with 781µL standard diluent, and the controls were reconstituted with 250µL standard diluent. After vortexing the bottles for 5 seconds, they were iced for 30 minutes. During this 30-minute icing period, the 6.5-µm magnetic beads were prepared for the assay. The beads were vortexed for 30 seconds in a foil-covered vial, and then

5472µL Assay Buffer and 288µL beads were added to a 15-mL tube, and each well was filled with 50µL of this solution and shaken. After the 30-minute icing period, the samples were diluted with a 4-fold standard dilution series and 50µL standards, blanks, controls, and samples were added to each. The plate was then covered with adhesive plate sealer and foil and was shaken while incubating for 1 hour. Ten minutes prior to the completion of this incubation, the detection antibodies were vortexed for 5 seconds and 145µL detection Assay Buffer was added along with 2755µL Assay Buffer diluent. Once the 1-hour incubation was complete and the plate was washed three times, 25µL of this diluted detection antibody solution was added to each well and the plate was once again covered and shaken while incubating for 30 minutes.

The Stretavidin-PE (SA-PE) stock solution was prepared 10 minutes prior to the completion of this incubation period. The SA-PE solution was covered with foil and vortexed for 5 seconds prior to adding 60µL SA-PE as well as 5940µL Assay Buffer to a separate vial. After the incubation period, the plate was washed with antibodies three times and 50µL diluted, vortexed SA-PE solution was added to each well. The plate was once again incubated for 10 minutes then washed three times with antibodies. The magnetic beads were resuspended in 125µL Assay Buffer and added to each well of the plate, which was then shaken for 30 seconds. After removing the plate sealer, the Bio-Plex Machine then read the plate to record measurements for each of the 34 biomarkers.

All multiplex assay components, including the validation kits, calibration kits, and human Bio-Plex Pro Human Cancer Biomarker Assay Panels 1 and 2 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Human vitreous samples (50µL) were

run cleanly, and the results reflect the dilution factor of 1. Samples, standards, blank, and controls were run in duplicate on the multicytokine suspension array system (Bio-Plex; Bio-Rad Laboratories), as suggested by the manufacturer's protocol. Vortex-diluted (×1) magnetic coupled beads were first added to each well of the assay plate, followed by a plate washing (×2) with Uwash buffer (Bio-Rad Laboratories). Samples, standards, blank, and controls (50 μL) are added to each well according to the plate layout and incubated in the dark for an hour at room temperature with vigorous shaking. Between each incubation period, washing of the plate follows immediately (×3). Detection antibodies (×1) are added to the plate, with a 30-minute incubation period, followed by the addition of Streptavidin-PE (×1) for 10 minutes with shaking. After the final wash, the magnetic beads are resubmerged in assay, incubated, and shook for 30 seconds before the plate is analyzed via the Bio-Plex Manager Software (Bio-Plex; Bio-Rad Laboratories).

Data Analysis

Samples were categorized into three groups: (1) eyes of patients who underwent PPV for ERMP and/or MH with no history of diabetes mellitus (non-DM group, n=29); (2) eyes of patients who underwent PPV for ERMP and/or MH with a history of diabetes or nonproliferative diabetic retinopathy (DM group, n=10); (3) eyes of patients who underwent PPV for proliferative diabetic retinopathy (PDR group, n=29). The control for this study is represented by the non-DM group since ischemia is assumed not to be present in patients without diabetes who have an epiretinal membrane or macular hole (Joussen et al., 2007). Mann-Whitney *U* tests

were performed to compare individual biomarker concentrations between the non-DM and DM group, DM and PDR group, and the non-DM and PDR groups. A 2-tailed *P* value of less than 0.05 (or 95% confidence interval) was considered statistically significant. Mann-Whitney *U* tests were chosen for the analysis because for each of the three comparisons, two independent and non-parametric groups were assessed for differences in biomarker concentrations. All data collection and analysis were performed with Microsoft Excel 2017.

RESULTS

Angiopoietin-2, EGF, Endoglin, FGF, G-CSF, HB-EGF, HGF, IGFBP, PDGF, PIGF, sEGFR, sHER2/neu, sVEGFR-1, sVEGFR-2, TGFα, TIE-2, VEGF-A, VEGF-C, and VEGF-D were labeled as "growth factors" due to properties that stimulate cellular growth, proliferation, and differentiation. IL-18, IL-6, IL-8, PECAM-1, sCD40L, SCF, sFASL, sIL-6Ra, and TNF-α were labeled as "inflammatory cytokines" based on their ability to promote vascular permeability changes, leukocyte recruitment, and the release of inflammatory mediators. Remaining biomarkers – Follistatin, Leptin, Osteopontin, PAI-1, Prolactin, and uPA – were categorized as "others" since their known function does not implicate them as either a growth factor or inflammatory cytokine

Assembled in Table 1 is a list of the vitreous biomarkers and their respective mean concentrations (pg/mL) found in each of the three categories – non-DM, DM, and PDR groups. Table 2 displays Mann-Whitney *U* test *P* values for comparative analysis of vitreous mediators between the non-DM and DM group (1 and 2), DM and PDR group (2 and 3), and the non-DM and PDR group (1 and 3). Comparison of vitreous proteins between the non-DM and DM cohort revealed elevated Endoglin, G-CSF, PIGF, VEGF-D, IL-6, and sCD40L. Between the DM and PDR group, several biomarkers were also enhanced, including PIGF, VEGF-A, IL-8, and Leptin. Comparison of the PDR to the non-DM group yielded elevated concentrations among numerous (1) growth factors: Angiopoietin-2, EGF, Endoglin, G-CSF, HB-EGF, HGF, PDGF, PIGF, sHER2/neu, TIE-2, VEGF-A and VEGF-D, (2) inflammatory cytokines: IL-18, IL-6, IL-8, PECAM-1,

Table 1. Mean Concentration (pg/mL) of Biomarkers Across non-DM, DM, and PDR patients. Shaded in gray are biomarkers regulated by HIF-1.

Biomarker Type	Biomarker	Non-DM	DM	PDR
Growth Factor	Angiopoietin-2	128.4	260.7	1389.7
	EGF	2.5	3.3	3.2
	Endoglin	16.8	35.9	42.4
	FGF-Basic	73.7	73.2	67.2
	G-CSF	15.2	110.9	29.8
	HB-EGF	1.1	3.2	2.9
	HGF	5399.4	5070.1	8711.7
	IGFBP-1	409.8	713.7	583.9
	PDGF	23.2	25.6	31.9
	PIGF	3.5	13.9	42.7
	sEGFR	2043.5	2938.5	2429.1
	sHER2/neu	289.1	485.3	466.7
	sVEGFR-1	1113.3	1145.8	573.5
	sVEGFR-2	2039.0	1383.6	2222.2
	TGFα	2.4	3.2	2.8
	TIE-2	1748.7	1654.7	1499.7
	VEGF-A	36.0	260.8	518.8
	VEGF-C	73.4	100.9	96.4
	VEGF-D	58.8	87.9	90.8
Inflammatory Cytokines	IL-18	7.6	11.7	39.4
	IL-6	17.3	579.7	170.3
	IL-8	5.7	56.4	34.3
	PECAM-1	376.3	414.1	486.8
	sCD40L	6.9	19.4	18.2
	SCF	46.2	61.2	58.2
	sFASL	10.3	18.1	19.0
	sIL-6Ra	277.1	541.1	605.4
	TNF-α	2.2	3.7	4.6
Other Biomarkers	Follistatin	101.1	210.7	134.5
	Leptin	280.3	348.2	1285.8
	Osteopontin	39881.3	30186.1	33348.5
	PAI-1	2878.5	6657.2	13731.2
	Prolactin	460.0	457.5	470.9
	uPA	135.8	284.2	275.4

Table 2. Mann-Whitney *U* Test *P* Values for Biomarker Comparison. Shown below are the *P* values for comparing biomarker concentrations between different sampling groups. 1= non-DM, 2=DM, 3=PDR. Statistical significance denoted by bolded numbers ($P<0.05$). Shaded in gray are biomarkers regulated by HIF-1.

Biomarker Type	Biomarker	1 and 2	2 and 3	1 and 3
Growth Factor	Angiopoietin-2	.166	.223	**.000**
	EGF	.180	.777	**.009**
	Endoglin	**.013**	.676	**.000**
	FGF-Basic	.850	.832	.811
	G-CSF	**.005**	.167	**.043**
	HB-EGF	.052	.620	**.000**
	HGF	.668	.052	**.003**
	IGFBP-1	.973	.167	.069
	PDGF	.460	.490	**.011**
	PIGF	**.003**	**.007**	**.000**
	sEGFR	.571	.777	.114
	sHER2/neu	.440	.860	**.003**
	sVEGFR-1	.605	.254	**.007**
	sVEGFR-2	.850	.887	.714
	TGFα	.855	.851	.673
	TIE-2	.295	.915	.064
	VEGF-A	.133	**.013**	**.000**
	VEGF-C	.304	.939	.063
	VEGF-D	**.021**	.958	**.001**
Inflammatory Cytokines	IL-18	.483	.080	**.002**
	IL-6	**.007**	.620	**.000**
	IL-8	.137	**.037**	**.000**
	PECAM-1	.345	.416	**.001**
	sCD40L	**.004**	.983	**.000**
	SCF	.571	.915	**.048**
	sFASL	.073	.750	**.038**
	sIL-6Ra	.061	.645	**.001**
	TNF-α	.085	.366	**.001**
Other Biomarkers	Follistatin	.154	.167	.170
	Leptin	.525	**.026**	**.000**
	Osteopontin	.172	.937	**.038**
	PAI-1	.255	.073	**.000**
	Prolactin	.797	.887	1.000
	uPA	.096	.524	**.002**

sCD40L, SCF, sFASL, sIL-6Ra, and TNF-α, and (3) other biomarkers: Leptin, PAI-1, and uPA (Table 1). Markedly lower concentrations for sVEGFR-1 and Osteopontin were observed for comparisons between PDR and non-DM (Table 1).

Vitreous Biomarkers that are HIF-1 Effector Genes

The shaded biomarkers in Table 1 and 2 represent genes that are directly upregulated by HIF-1 during hypoxia based on current literature (Gao et al., 2017; Peet, Kittipassorn, Wood, Chidlow, & Casson, 2017; Takagi et al., 2007; Vadlapatla et al., 2013). These include Angiopoietin-2, EGF, Endoglin, IGFBP-1, PDGF, PIGF, sVEGF-R1, sVEGF-R2, TGF-α, VEGF-A, VEGF-C, VEGF-D, IL-6, IL-8, SCF, TNF-α, Leptin, PAI-1. Between the non-DM and DM group, only Endoglin, PIGF, VEGF-D, and IL-6 were significantly elevated as HIF-1 target effectors. Comparisons of the non-DM to PDR group show that all HIF-1 target effectors mentioned above had statistically significant changes other than IGFBP-1, sVEGF-R2, TGF-α, and VEGF-C.

Figure 7 represents the percentage change in concentrations of HIF-1 inducible vitreous biomarkers between non-DM patients and PDR patients. The included biomarkers also demonstrated statistically significant change on Table 2. Of these biomarkers, all exhibited a positive percentage change with the exception of SVEGFR-1, which had a 48% decrease in concentration between the non-DM group and PDR group. In addition, numerous vitreous mediators had levels that increased multifold, with Angiopoietin-2, Endoglin, PIGF, VEGF-A, IL-6, IL-8, TGF-α, Leptin, and PAI-1

displaying a percentage change greater than 100%. VEGF-A displayed the greatest change with a 1340% increase in concentration.

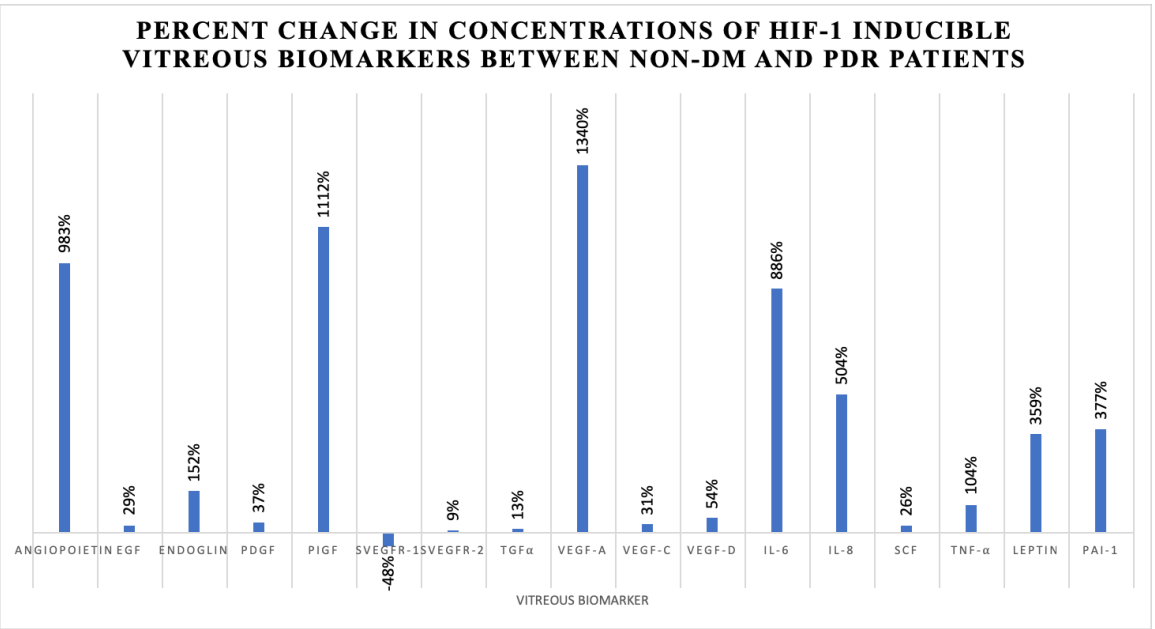

Figure 7 – Percent Change in Concentrations of HIF-1 Inducible Vitreous Biomarkers Between Non-DM and PDR patients.

DISCUSSION

An Evaluation of PDR Biomarkers

The results of this study provide a snapshot of the numerous proangiogenic and pro-inflammatory factors that are expressed in PDR (Table 1). In addition, the biomarkers tested in this assay are representative of a wide-range of disease mechanisms, including angiogenesis, metastasis, cell proliferation, cell adhesion/migration, apoptosis, and inflammation, which underscores the multidimensional pathophysiology of ischemic retinopathies such as PDR. The exponential increase of certain proteins from the non-DM (control) to DM group, and from the non-DM to PDR group indicates the significant role that they play in diabetes and PDR. Some of these mediators are well-validated in the current literature, such as VEGF, Angiopoietin-2, and PDGF, while others have been less documented.

Leptin is most recognized as an anti-obesity hormone that is synthesized in response to high intracellular triglycerides. Interestingly, leptin seems to also participate in the development of PDR since in the current study, leptin levels were shown to have increased by 359% when comparing between the non-DM and PDR groups (Figure 7). One study showed that in a mouse model of ischemia-induced retinopathy, overexpression of leptin stimulated neovascularization (Suganami et al., 2004). Although the exact mechanism for leptin production has yet to be defined, it is known to be a target effector of HIF-1 (Vadlapatla et al., 2013).

Another notable finding was PIGF, placental growth factor, which was the only protein that exhibited statistically significant changes across the three comparisons made

in Table 2. PIGF is a homolog of VEGF and interacts with VEGF's receptor – VEGF-R1- to enact angiogenic and mitogenic effects (Ziche et al., 1997). PIGF levels were also found to be enhanced in vitreous samples of PDR patients and correlated with the expression of VEGF (Mitamura et al., 2002). This current study also demonstrates that PIGF is upregulated in diabetics without PDR due to the statistically significant difference in PIGF concentration between the DM and PDR groups (Table 2). Knockout of PIGF in a diabetic mouse strain has been shown to prevent the development of PDR and also reduce the expression of HIF1 and VEGF (Huang et al., 2015). As a result, PIGF's synergy with VEGF as well as its strong expression in both diabetes (without PDR) and PDR highlights its importance as a target for inhibition.

Implications for Hyperoxia Treatment

The upregulation of a myriad of growth factors and inflammatory cytokines in PDR suggests that inhibiting multiple proteins could achieve more efficacy in attenuating the disease than blocking just one – as is the case with anti-VEGF treatments. Although the vitreous biomarkers of other retinopathies such as AMD and CRVO/BRVO were not analyzed in the study, it could be assumed that many of the same proteins are also involved due to the ischemic nature of these diseases.

The potential benefit of hyperoxia for patients with ischemic retinopathies (including RD, AMD, PDR, and RVO) lies upon its ability to deliver a higher concentration of oxygen to the photoreceptors and to limit the expression of various proangiogenic/pro-inflammatory factors. The latter is based on the concept that since

HIF-1 is the master regulator of many of the proteins involved in the pathogenesis of ischemic retinopathies and is activated by hypoxia, the usage of supplemental oxygen could reduce its activation and thus, downregulate the expression of pathological cytokines.

This study demonstrated a surge in the concentrations of proangiogenic and pro-inflammatory cytokines when comparing between vitreous samples of non-DM and PDR patients (Table 2). More importantly, a great proportion of these factors is regulated by HIF-1. An analysis of the biomarkers that are both significantly elevated in PDR and upregulated by HIF-1 shows the extent of gene expression that can be induced during retinal ischemia (Figure 7).

As expected, VEGF exhibited the greatest change in concentration (1340% increase) from non-DM to PDR eyes, which highlights the reason anti-VEGF therapeutics are still the standard of treatment for PDR-induced neovascularization and edema (Figure 7). However, figure 7 also shows other growth factors with similar levels of heightened expression in PDR. These include Angiopoietin-2 (983% increase) and PIGF (1112% increase), both of which are known to promote angiogenesis and vascular permeability (Khalaf et al., 2017). A reduction of HIF-1 through hyperoxia could lower the expression of these growth factors in addition to VEGF.

Inflammatory cytokines including IL-6, IL-8, and TNF-α are also found to be substantially upregulated in PDR with increases in concentration of 886%, 504%, and 104%, respectively, when compared to levels in non-DR patients. Il-6 and Il-8 are potent angiogenic mediators while TNF-α is implicated in increased BRB permeability

(Bolinger & Antonetti, 2016; Gao et al., 2017). Although the role of inflammation in inducing neovascularization is widely accepted, there are very few treatment options available that target inflammatory molecules in ischemic retinopathies (Zhang et al., 2011). Supplemental oxygen may be a great candidate for diminishing the expression of pro-inflammatory cytokines and limiting their pathological effect by deactivating HIF-1.

Limitations

Certain limitations in the present study should be discussed to improve future research. One limitation is the biomarker assay, which only screens for 34 growth factors and cytokines. A greater selection of biomarkers could reveal additional findings about the pathogenesis of PDR. In addition, since the goal of the experiment is to justify the treatment of hyperoxia with patients diagnosed with different ischemic retinopathies (RD, AMD, RVO, PDR), vitreous samples representative of non-PDR patients should be collected such that comparative analysis of mediator concentrations could be made. Lastly, although a sufficient number of eyes were analyzed, they were not evenly distributed across the three cohorts, which could have skewed some results. As a result, future prospective studies should include a relatively even number of vitreous samples that spans across a spectrum of ischemic retinal diseases. Other assays could also be included to widen the pool of cytokines analyzed.

Conclusion

In conclusion, the analysis of growth factors and pro-inflammatory cytokines in PDR patients revealed many noteworthy findings about the underlying molecules responsible for the pathogenesis of ischemic retinopathies. Furthermore, the involvement of numerous HIF-1 inducible genes helps elucidate the biomolecular mechanism in which supplemental oxygen can be beneficial to patients with PDR, RD, RVO, or AMD. Future studies will be necessary to confirm the extent in which hyperoxia can attenuate the disease progression of ischemic retinopathies.

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