Understanding the Molecular Mechanisms of Abnormal Ocular Angiogenesis-Diabetic Retinopathy

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HYDERABAD-500 046

INDIA

By

Shahna S.

Under the supervision of

Dr. Inderjeet Kaur

Kallam Anji Reddy Molecular Genetics Laboratory

Prof. Brien Holden Eye Research Centre

L V Prasad Eye Institute

Hyderabad- 500 034

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Dedicated to my family, Dr. Inderjeet and Dr. Subho

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ABSTRACT

Purpose

The major aim of the present study was to dissect the underlying molecular mechanisms of abnormal ocular angiogenesis in diabetic retinopathy by identifying the key players and their role in proliferative diabetic retinopathy progression using a combination of proteomics and cell culture -based approaches

Methods

The study was done according to the guidelines of Declaration of Helsinki and was approved by Institutional Review Board (IRB approval number: LEC 02-14-029), L V Prasad Eye institute, Hyderabad. The study cohort consisted of PDR (n=120) patients and non-diabetic controls (n=120) for vitreous proteome analysis. For serum analysis, blood samples were collected by venipuncture from PDR (n=38), NPDR (n=38) and NDM (n=38) subjects. Diabetic (n=3) and non- diabetic donor retinas (n=3) were also collected from cadaveric donors to validate the vitreous findings using immunostaining. All the samples were collected with prior informed consent. For global vitreous proteome analysis 100µL of samples collected from PDR (n=3), NPDR (n=3) and NDM (n=3) and subjected for in gel-digestion followed by LC-MS/MS using Q Exactive (Thermo Scientific) interfaced with nanoflow LC system (Easy nano-LC 1200, Thermo Scientific). The acquired raw data for vitreous humour samples were searched against the human proteome from UniProt database (release 2018.09 with 73099 entries) using the Andromeda search engine and MaxQuant (version 1.3.0.5). Differentially expressed proteins in diabetes and proliferative DR was identified and further analyzed based on their key functions and associated biological pathways by REACTOME

and KEGG pathway analysis. For targeted analysis of complements and microglial activation in PDR pathogenesis, a detailed investigation of the classical and alternative complement pathways was done for the C3, C4b, C1q, Factor Bb, CFH proteins in the vitreous and their levels were compared to serum obtained from systemic circulation by western blotting. Further, microglial infiltration and activation in the vitreous was evaluated by analyzing the levels of CD11b, MMP9, IL-10 and IL-8 in the vitreous samples by western blotting, Zymography and multiplex ELISA, respectively. Immunostaining was done in retinal tissues collected from diabetic and non-diabetic donors for complement C3, CFH and glial proteins CD11b and GFAP to validate the vitreous proteome data. The level of inflammation, ECM degradation and angiogenesis in vitreous samples were further evaluated by analyzing the levels of opticin by western blotting, sPECAM, VEGF, VEGFR2 and sVEGFR1 by multiplex ELISA. For studying neuro-glial interaction under diabetic stress, culture of mixed population of retinal cells comprising of microglia, astrocyte, Müller glia and neurons were generated from the retinal tissues of control donors. The characterized cells were exposed to hypoxia and high glucose, by giving $CoCl_2$ and D-glucose, respectively, for 24 hours. The cellular activity was measured by evaluating the shift in intracellular Ca^{2+} levels in a time dependent manner and compared with cells under no-stress using a pre-designed algorithm. Further, a correlation of Ca^{2+} activity with gene and protein expression for evaluating glial activations were performed by real time PCR and high-resolution protein imaging, respectively.

Results

Global vitreous proteome profiling

Proteomic analysis detected 1079 proteins including 27 novel vitreous proteins from the present study. 16 of these were reported for the first time and the rest 11 of them were found

reported in eye proteome database other than vitreous database. Protein comparison across different groups revealed fifteen proteins to be differentially expressed in diabetes compared to no-DM controls, which included 8-upregulated (FRZB, AGRL, APLP1, APLP2, IGFBP6, COL1A2, VCAN) and 7-downregulated proteins (GSTP1, IDH1, LDHA, VASN, LDHB, PRDX1, C6). Ten proteins were found to be differentially expressed in proliferative stage of the disease, which included 4 upregulated proteins (LRG1, APCS, IGHM, APCS) and 6 downregulated proteins (MYOC, TGFβ, VSAN, SOD3, ENPP2, PPT1). Pathway analysis of these differentially regulated proteins revealed that diabetes enhanced the upregulation of pathways such as extracellular matrix organization, degradation of extracellular matrix and regulation of Insulin growth factor by IGFBPs. While metabolic pathways such as glycolysis, pyruvate metabolism and pathways of detoxification of ROS were found to be downregulated in diabetic vitreous. Amyloid fiber formation was found to be the major pathway associated with the differentially expressed proteins of retinopathy compared to 'no-retinopathy' group with the presence of two of the up-regulated proteins (APCS, FGA). Along with upregulation of proteins involved in angiogenesis (LRG1), inflammation (APCS, FGA, PPT11) was also observed at retinopathy stage. Downregulation of detoxification of ROS was also found to be profound in the retinopathy vitreous.

Role of complement and microglial activation

The present study identified a significant localized increase in total C3 and its activated fragment C3bα' in the PDR vitreous (1.9±0.25 au, p=0.004, PDR=2.76±0.65 au, p=0.006) and not at the systemic level. The classical pathway proteins C1q and C4b were not found to be contributing in PDR pathogenesis both in the vitreous and serum level. Significant decrease in the level of Bb of factor B (PDR: 0.97±0.15 au, $p=0.03$, Controls: 1.89±0.38 au) along with

a significant increase in CFH (Control: 0.96±0.172 au, PDR: 3.68±0.66 au, $p=0.0004$) was observed in PDR vitreous. Western blotting of CD11b clearly identified microglial infiltration in the PDR vitreous. Along with this a significant increase in inflammatory markers such as sPECAM (PDR: 105.5±16.69 pg/mL, Control: 49.54±4.76pg/mL, $p=0.01$), pro-inflammatory marker IL-8 (PDR: 29.47±14.14 pg/mL, Control: 12.79±3.13pg/mL, $p>0.05$, N.S) and decrease in anti-inflammatory marker IL-10 (PDR: 0.57±0.09 pg/mL, Control: 2.24±0.42pg/mL, $p=0.001$) were observed. Immunostaining of complement C3 showed an increased deposition in the retinal layers of DM tissues with an obvious increase in the number of $CD11b^{+ve}$ cells. Likewise, CFH was found only in the DM tissue and it was found to be co-localized only with $CD11b^{+ve}$ cells. Increased gelatinolytic activity corresponding to MMP9 and simultaneous decrease in anti-angiogenic protein opticin ($p<0.05$) was seen in the PDR vitreous. The levels of VEGF, VEGFR2 and sVEGFR1 were increased significantly in the PDR vitreous.

Neuroglial interaction under diabetic stress

An increased level of Ca^{2+} modulation and spiking were observed in cells under stress. Population level classification based on Ca^{2+} suggested that hypoxia was the major contributor of glial activation over high glucose. In accordance with the differences in Ca^{2+} modulations observed between hypoxic and high glucose condition, there was an increased response of gene and protein expression under high glucose and hypoxia treated cells. Further protein expression analysis identified a significant increase in microglial activation (IBA-1) in cells under stress especially in hypoxic treatment.

Conclusions

The quantitative analysis of proteins based on their intensity categorized their involvement in diabetes and retinopathy complications. An active detoxification mechanism was found in NDM group, while in DM and retinopathy this clearance mechanism was found to be downregulated. Most importantly DM group showed a significant upregulation of ECM modifying proteins, whereas in retinopathy group angiogenic and inflammatory proteins were found to be upregulated. This suggested that diabetes induced ECM- reorganization, which further supported the neovascular progression in proliferative stages of the disease. A significant increase in total C3 level and its reactive C3bα' fragment in the PDR vitreous indicated its role in PDR pathogenesis. Increased level of CFH independent of systemic circulation, suggested it to be a localized phenomenon and could be a probable feedback mechanism to arrest formation of reactive C3bα'. Co-localization of C3 with $CD11b^{+ve}$ cells suggested that microglia could also contribute to complement deposition in the retina along with the other retinal cell types. Further co-localization of CFH with CD11b suggested a synthesis of CFH by activated microglia in diabetes. Extensive microglial activation and infiltration in PDR vitreous suggested its strong involvement in pathogenesis and also pointed towards a major source of CFH protein seen in the PDR vitreous. The decrease in the levels of anti-angiogenic proteins and increased MMP9 activity along with increased level of angiogenic proteins clearly proved that extensive ECM remodeling and angiogenesis occurred in the PDR vitreous and these further suggested a significant role of the microglia in this entire processes. Further *in vitro*- neuro-glial interaction identified an elevated Ca^{2+} level and microglial activation, suggesting a strong pathological involvement of these cells in PDR

pathogenesis over macroglial population. This strongly indicated that microglial cells were the major contributor of pathological alterations in the retina during DR.

1. INTRODUCTION

Diabetes and diabetes-induced complications are common threats to the developing world and affects all the age groups and economies worldwide. Diabetic retinopathy (DR) is one of the major devastating manifestations of diabetes and causes vision loss in the absence of timely intervention and treatment. The pathophysiology of DR is mainly driven by prolonged hyperglycemia and poor glycemic control along with various other risk factors such as dyslipidemia, hypertension, pregnancy and genetic predisposition (Jenkins, Joglekar, Hardikar *et al.* 2015, Miljanovic, Glynn, Nathan *et al.* 2004, West, Klein, Rodriguez *et al.* 2001, Whitehead, Wickremasinghe, Osborne *et al.* 2018). The complexity of DR encompasses the entire retinal functions, which include compromised neuronal activity, alteration in retinal vasculature that lead to gradual neurodegeneration, neuroinflammation and vascular complications (Duh, Sun and Stitt 2017). Clinically, DR is characterized by features that include formation of hard exudates, microaneurysm, neovessels formation, vitreous hemorrhage, fibrovascular proliferation and retinal detachment. Based on these visible retinal vascular changes, DR is classified into non-proliferative diabetic retinopathy (NPDR) along with sub-categories such as mild, moderate and severe and proliferative diabetic retinopathy (PDR) (Roy, Amin and Roy 2016, Wu, Fernandez-Loaiza, Sauma *et al.* 2013). One third of the global diabetes population were reported to have signs of DR and among them, another one third had vision threatening complications of DR (Lee, Wong and Sabanayagam 2015). In India, the number of diabetes and diabetes associated complications are relatively higher, with an estimated prevalence of 21.7% of DR in Indian adults affected with diabetes (Gadkari, Maskati and Nayak 2016). Hence, DR is one of the major causes of vision impairment in India.

Retina is the neurosensory layer, which is an extension of the central nervous system (CNS) tissue. Three major features make the retina highly vulnerable to any kind of noxious insults like diabetes. These includes- (a) the requirement of high energy for its survival compared to any other tissue with limited number of mitochondria, (b) requirement of more energy for the unmyelinated retinal neurons for their metabolic activity and (c) metabolic demands of the retina that are supported by limited vascular supply (Altmann and Schmidt 2018). Therefore, high glucose-induced hypoxic stress largely affects the retinal vasculature and contribute to a significant microvascular dysfunction. The therapies such as retinal laser photocoagulation to burn the neovessels, injection of intravitreal anti-VEGF (vascular endothelial growth factor) to neutralize the potent mitogenic cytokine VEGF and Pars Plana Vitrectomy (PPV) at the advanced disease stage are the current treatment modalities for this disease. These solely target the vascular changes in the retina and are usually beneficial for a limited time, while the disease continues to progress due to the underlying metabolic alterations, and cause irreparable damage to the retina (Bandello, Lattanzio, Zucchiatti *et al.* 2013). Moreover, an optimal level of VEGF is also required for normal neuronal survival, thus arresting VEGF could have detrimental impact on the retinal neurons (Nishijima, Ng, Zhong *et al.* 2007). Another important aspect of DR is the imbalance in the generation of reactive oxygen species (ROS) and its scavenging mechanism that creates an extensive oxidative damage to the tissue (Kowluru and Chan 2007). The elevated ROS are known to activate various metabolic pathways such as Advanced glycation end product (AGE) pathway, Protein Kinase-C (PKC) activation, polyol pathway and NF-kB activation leading to amplification of the inflammatory milieu in diabetes that results in vulnerable changes in the retina (Tarr, Kaul, Chopra *et al.* 2013). The treatment strategies using anti-oxidants have also been found to be efficacious in arresting DR or diabetes associated vascular complications (Johansen, Harris, Rychly *et al.*

2005, Mayer-Davis, Bell, Reboussin *et al.* 1998). Thus, it is crucial to understand the underlying biochemical changes in the retina specific to diabetic retinopathy complications, which might help to develop a targeted approach for treating this metabolic complication.

The functional outcome of a disease is critically dependent on the protein alteration that occurs before and during the disease, stage of diagnosis and timely and early intervention to prevent the progression. The current available modalities for the diagnosis of DR includes sophisticated and powerful imaging tests such as optical coherence tomography (OCT) and fundus fluorescein angiography (FFA). However, these can only detect the vascular changes in the retina and not the underlying neuro-inflammatory and neurodegenerative changes that are known to precede vascular changes (Xu and Chen 2016). Thus, it is important to study all such alterations and biomarkers from the affected tissues and biological materials of the patients for developing a better and efficient diagnostic test. Further, a deeper understanding of the underlying disease pathways and mechanisms would also provide newer targets for risk assessment that may finally aid in retarding the disease progression.

Vitreous humor (VH) is a transparent and hydrated gel, positioned in the posterior part of the eye, attached to the retina and one of the major reliable sources for exploring the retinal alterations. Unlike aqueous humor, there is no out flow and constant replenishment of this fluid (Gao, Fu and Hui 2015). Hence, the protein shed into the vitreous from a normal physiological or pathological state would be retained until it is surgically removed. Thus, studying the vitreous proteome would be an ideal choice to understand the underlying biochemical alterations occurring in the retina in DR pathogenesis. Several vitreous proteome studies were conducted in PDR to explore the underlying disease mechanism. The first vitreous proteome analysis in DR was done by Nakanishi *et al.* in 2002 that identified proteins such as complement C4, α1-antitrypsin, α2-HS glycoprotein and Pigment epithelium-derived

factor (PEDF) in PDR vitreous for their potential role in disease pathogenesis (Nakanishi, Koyama, Ikeda *et al.* 2002). This was followed by several proteome studies that explored the disease mechanisms in PDR and suggested the involvement of various proteins, such as apolipoproteins, complement proteins, zinc α-2 glycoproteins, serotransferrin, coagulation pathway proteins (Garcia-Ramirez, Canals, Hernandez *et al.* 2007, Wang, Feng, Hu *et al.* 2013), kallistatin, thioredoxin, von Willebrand factor (vWF), chromogranin (Kim, Kim, Yu *et al.* 2010, Kim, Kim, Kim *et al.* 2007), proteins of the complement and coagulation system, glutathione peroxidase 3, Immunoglobulins and cellular adhesion molecules (Hernandez, Garcia-Ramirez, Colome *et al.* 2013, Loukovaara, Nurkkala, Tamene *et al.* 2015) in disease pathogenesis. While many such studies have explored the disease mechanisms in DR by cataloguing these proteins, a large number of proteins are yet unidentified due to the underlying complexity of this metabolic disease and also due to the various approaches and study designs across these studies. Moreover, it is also important to discriminate the localized changes in the retina that are specific to the proliferative retinopathy versus the diabetes induced systemic alterations, as it may facilitate in understanding the disease mechanisms exclusive to DR. However, none of the previous have tried to addressed this crucial aspect.

One of the major findings provided by the previous vitreous proteome studies is the considerable involvement of immune pathways in PDR pathogenesis, especially by the identification of a greater number of complement pathway proteins in PDR vitreous (Gao, Chen, Timothy *et al.* 2008, Garcia-Ramirez *et al.* 2007, Loukovaara *et al.* 2015). The complement pathway is one of the major innate immune defense mechanisms of the retina and other than its role in maintaining retinal integrity, it is also involved in tissue morphogenesis during development (Hawksworth, Coulthard, Mantovani *et al.* 2018). In ocular angiogenic conditions such as age-related macular degeneration (AMD) and

Retinopathy of prematurity (ROP), the potential role of complements in promoting neovascularization is well documented (Geerlings, de Jong and den Hollander 2017, Rathi, Jalali, Patnaik *et al.* 2017). Later a knock-out of complement protein C3 in animal models of AMD, showed a reduction in the neovessels formation (Bora, Sohn, Cruz *et al.* 2005). Along with the proteomics findings, the two major significant evidences of complement involvement in PDR include the depositions of complement proteins C3d (Gerl, Bohl, Pitz *et al.* 2002) and downregulation of complement inhibitors such as CD59 and CD55 in the diabetic retina (Zhang, Gerhardinger and Lorenzi 2002). Additionally, the genetic association studies of complement pathway genes with DR progression have also revealed an association between polymorphisms in complement genes *C5*-rs17611, *CFH*-rs800292, *CFB*-rs1048709 and *C5* (rs2269067) with DR development in the Chinese population (Wang, Yang, Li *et al.* 2013, Xu, Yi, Yu *et al.* 2016, Yang, Wang, Ren *et al.* 2016). However, a genetic and functional validation of these variations across different populations, along with their protein expressions and pathological significance are yet to be observed. While most of these studies have suggested an important role of complement pathway in PDR pathogenesis, none of these have identified the key proteins involved in PDR pathogenesis and mechanism of complement activation in DR progression.

Going forward several studies have shown that microglial activation ameliorate tissue damage with a chronic inflammatory response under prolonged duration of diabetes (Ramirez, de Hoz, Salobrar-Garcia *et al.* 2017). Additionally, these studies have reported the activation of microglia at different stages of DR in human retinal tissues. Likewise, animal studies have provided evidence for the role of microglial activation in causing neuronal apoptosis in the diabetic retina (Zeng, Green and Tso 2008, Zeng, Ng and Ling 2000). Since microglia and

complement support the critical defense mechanism of the retina, a possible cross talk may be ongoing between them, which might further ameliorate disease progression.

Though clinical evaluation and therapeutic approaches for DR are solely dependent on its vascular manifestations, evidences from clinical studies clearly suggest that along with neovascularization, an unavoidable neurodegeneration further accelerates vision loss in DR (Garcia-Ramirez, Hernandez, Villarroel *et al.* 2009, Sohn, van Dijk, Jiao *et al.* 2016, Wolff, Bearse, Schneck *et al.* 2015, Zeng *et al.* 2008). This may indicate the involvement of a common mediator for neovascularization and neurodegeneration in DR. The retinal organization is very complex, which comprises of five different types of neurons, retinal glia and vascular cells. The functional integrity of retina rely on its neuro-vascular coupling function, which connects neurons and vasculature through the glial cells (Simo, Stitt and Gardner 2018). Thus, being a critical mediator of neuro-vascular coupling, activation of retinal glia including microglia, Müller glia and astrocytes are known to enhance the secretion of potent mitogenic cytokines and inflammatory molecules, which further corroborates the microvascular complication and inflammation in the retina (Gu, Xu, Zhang *et al.* 2019). The secondary messenger Calcium (Ca^{2+}) plays a critical role in neuro-glial and vaso-glial and glial-glial communications (Biesecker, Srienc, Shimoda *et al.* 2016). The abnormal Ca^{2+} level in the CNS tissues is also an indicator for neuronal damage and abnormal levels of Ca^{2+} is known to activate various signaling pathways which further activate apoptotic, angiogenic and neurodegenerative genes (Weber 2012). Since glial cells are involved in neurovascular coupling, it is also important to understand how the glial modulations in the diabetic retina impact the neuronal health and vascular modification in DR. Thus, studying the Ca^{2+} modulation in a system comprised of all retinal glial types and neurons may help us to understand the pathological alteration of glial cells and its contribution in neovascularization and neurodegeneration in DR.

Based on the existing literature, it is evident that DR being a metabolic disorder, the major challenge lies in the inability to target a particular mechanism or pathway in disease pathogenesis. Therefore, the present study was designed to understand the underlying molecular mechanisms in DR through a comprehensive proteomics and cellular biology-based approaches. This includes global proteome profiling, which further expands the understanding of key and novel proteins and pathways involved in DR progression. This is followed by a detailed targeted investigation to understand the key mechanisms of complement activation and mediators in DR progression including neovascularization and neurodegeneration. These approaches would help to identify potential biomarker(s) for disease diagnosis and therapeutic targets for arresting DR progression. The present study is an attempt to address the lacunae in the existing knowledge about the underlying pathophysiology of DR through three major aims:

1.1. Major Aims

1. To understand the proteome alterations in DR by global proteome profiling of vitreous humor from diabetic (PDR, diabetic but no-retinopathy) and non-diabetic subjects
2. Systematically investigate the complement pathway activation and identify the role of microglia in DR pathogenesis
3. Evaluate neuroglial interactions under diabetic condition using a primary human mixed retinal culture system

2. REVIEW OF LITERATURE

Diabetes is a group of serious metabolic diseases characterized by hyperglycemia due to impaired glucose metabolism in the body. It leads to long term complications and affects every parts of the body. It is one of the common diseases seen worldwide. Diabetes causes reduced life expectancy mainly due to the microvascular as well as macrovascular complications of this disease. As per the published reports in the year 2017, about 450 million people were affected with diabetes in worldwide in 2015 and this figure was expected to rise up to 642 million by 2040 (Ogurtsova, da Rocha Fernandes, Huang *et al.* 2017). Further it was also estimated that around one fifth of all adults with this metabolic disease live in Southeast Asia and in India alone, about 60 million people are suffering from diabetes (Raman, Ganesan, Pal *et al.* 2017). The International Diabetic Federation (IDF) estimated the prevalence of diabetes in Indian adults of 20 -79 years to be 8.8% and it will rise to 11.4% in 2045 (Figure 2.1).

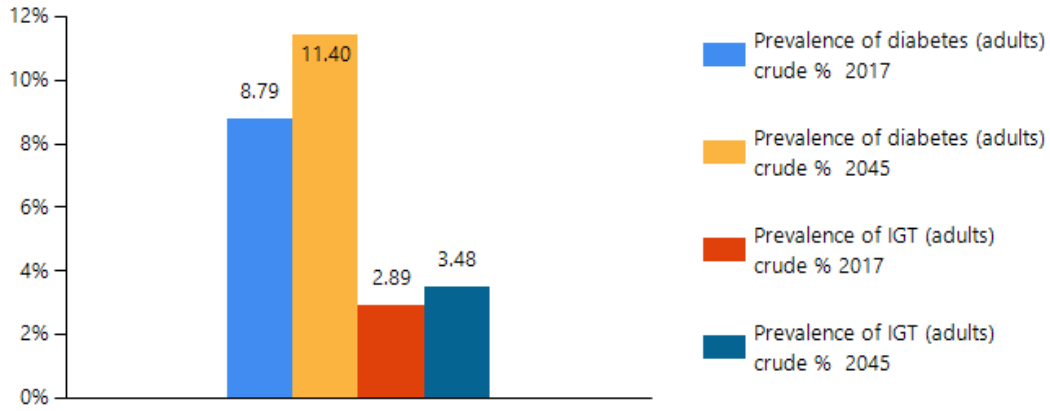


Figure 2.1: Prevalence of Diabetes and IGT (Impaired glucose tolerance) in Indian adults (20-79 years), 2017 and 2045, Adapted from http://reports.instantatlas.com/report/view/846e76122b5f476fa6ef09471965aedd/IND

2.1. Complications of Diabetes

The increased incidence of diabetes further increases the risk of complications associated with diabetes. Diabetes increases the rate of morbidity and mortality by enhancing the risk of many health problems such as cardiovascular diseases, stroke, diabetic foot ulcers, neuropathy, vascular complications such as nephropathy and other retinopathies.

2.2. Diabetic retinopathy

Diabetic Retinopathy (DR) is one of the major vision threatening retinal neurovascular complications of diabetes and causes irreversible vision loss if untreated. The pathophysiology of diabetic retinopathy is mainly driven by prolonged hyperglycemia due to poor glycemic control in patients with diabetes (Whitehead, Wickremasinghe, Osborne *et al.* 2018). The major characteristics features of DR are loss of pericyte, breakdown of blood retinal barrier, microaneurysms, dot blot hemorrhages, cotton wool spots, capillary occlusions, macular edema, neovascularization, which causes fibrovascular proliferations and retinal detachment, that ultimately lead to vision loss in patients (Gardner, Antonetti, Barber *et al.* 2002). Concurrent with the rise in diabetic prevalence globally, there is increase in number of diabetic retinal complications. According to a meta-analysis from 1990-2010 done by Leasher, *et al.* 2010, about 0.8 million people were blind and 3.7 million visually impaired globally due to DR and contributing further to an alarming increase of 27% blindness and 64% visual impairment since 1990 (Leasher, Bourne, Flaxman *et al.* 2016). One third of the people with diabetes develop some sort of retinopathies (Yau, Rogers, Kawasaki *et al.* 2012) and in India the incidents rate of DR was calculated as 9.2% (Raman *et al.* 2017). According to All India Ophthalmological Society's Diabetic Retinopathy Eye Screening Study in 2014, the prevalence of DR across the nation was

reported as 21.7% (Gadkari, Maskati and Nayak 2016). In addition to this nationwide study, many cross-sectional regional survey/studies have also been conducted in Indian population to estimate the prevalence rate of this sight threatening metabolic disease (Table 2.1).

Table 2.1: Prevalence of DR in different regions in India

Region/ study name	No. of diabetic subjects enrolled	Prevalence of DR (%)	References
Chennai, Tamil Nadu	1000	24	(Ramachandran, Snehalatha, Vijay *et al.* 1996)
Hyderabad, Andhra Pradesh Eye Disease Study (APEDES), Andhra Pradesh	2532	22.4	(Dandona, Dandona, Naduvilath *et al.* 1999)
Chennai, Tamil Nadu	448	7.3	(Rema, Deepa and Mohan 2000)
Palakkad, Kerala	5212	26.2	(Narendran, John, Raghuram *et al.* 2002)
Chennai, Chennai Urban Rural Epidemiology Study (CURES), Tamil Nadu	1382	17.6	(Rema, Premkumar, Anitha *et al.* 2005)
Theni, Tamil Nadu	2802	12.2	(Namperumalsamy, Kim, Vignesh *et al.* 2009)
Chennai, Shankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetic Study (SN-DREAMS III), Tamil Nadu	2730	10.3	(Raman, Ganesan, Pal *et al.* 2014)
Western India	168	33.9%	(Ramavat, Ramavat, Ghugare *et al.* 2013)

2.3. Classifications of Diabetic retinopathy:

Development of an efficient and adequate therapy for this complex eye disease requires a proper categorization and classification of the severity stage of this disease. The first standardized classification of DR was attempted in 1968, known as Airlie House Classification of DR, which described the impact of metabolic control, photocoagulation and pituitary ablation on DR (Goldberg and Jampol 1987). This classification did not cover most of the disease aspects, however, it still provided a valid base for the development of new classifications of DR. Diabetic retinopathy study (DRS) classification was generated from the modification of Airlie classification that included DR categorization based on a mutual comparison of 7 standard fundus stereo photographs of the different regions of patient's retina (1981). This was further modified by Early Treatment of Diabetic Retinopathy Study (ETDRS). This classification of disease is based on these variety of disease, ranging from no retinopathy to severe grade of retinopathy including vitreous hemorrhage and retinal detachment (Wu, Fernandez-Loaiza, Sauma *et al.* 2013). Additionally, ETDRS classified the vision threatening complication of DR, i.e. Diabetic Macular Edema (DME) as focal and diffuse based on the micro aneurysmal fluorescein leakage (1995). While it was considered to be a gold standard for DR classification, it was found to be complicated to follow in clinical examination point due to its extended categorization of the disease based on the fundus photography. In order to simplify this classification, International Clinical Disease Severity Scale for DR was generated in 2002 which combined the cumulative data obtained from the large-scale Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) and the ETDRS classification. In this system, DR has been classified broadly into five different stages

based on the apparent vascular manifestations during the disease progression (Wilkinson, Ferris, Klein *et al.* 2003). This consists of a stage of no apparent retinopathy where there are no abnormalities in the retina followed by the broad categorization of non-proliferative stage and proliferative stage of the disease as detailed below (Fong, Aiello, Ferris *et al.* 2004) (Figure 2.2).

1. Non- proliferative Diabetic Retinopathy (NPDR)
2. Proliferative Diabetic Retinopathy (PDR)

2.3.1. Non-proliferative Diabetic Retinopathy:

NPDR or the background retinopathy is an early stage of diabetic retinopathy. The high glucose level induce damage to the retinal capillaries and these tiny blood vessels develops small outpouchings known as microaneurysms. These microaneurysms eventually ruptures and form dot blot hemorrhages in the retina. The gradual progression of NPDR leads to the obstruction of the affected vessels and infarction of the retinal nerve fiber layer results in the development of cotton wool spots in the retina. The NPDR is mainly classified as:

i. Early or Mild NPDR: The patients in this category have a very low risk of progressing to PDR. The patients with mild NPDR present at least one microaneurysms in their fundus examination.

ii. Moderate NPDR: This category of patients presents with multiple microaneurysms, intraretinal hemorrhages or venous bleeding.

iii. Severe NPDR: This is the most advanced stage of NPDR where the patients present with cotton wool spots, venous bleeding and severe intraretinal microvascular

abnormalities. Within a year, 50 -75% patient of this class will progress to proliferative stage of the disease (Aiello 2003).

2.3.2. Proliferative Diabetic Retinopathy (PDR):

PDR is the advanced stage of DR, characterized by proliferation of new blood vessels in the retina due to the release of ischemia induced vaso proliferative molecules like vascular endothelial growth factor (VEGF). These vessels are known as neovessels which are fragile and leaky and cause hemorrhages (Stitt, Curtis, Chen *et al.* 2016). Most importantly, the neovessels eventually grow off the retina into the vitreous and make fibrovascular proliferations mainly consists of glial cell types. These fibrovascular proliferations pulls down the retina into the vitreous and cause retinal traction, retinal detachment and sudden vision loss (Roy, Amin and Roy 2016).

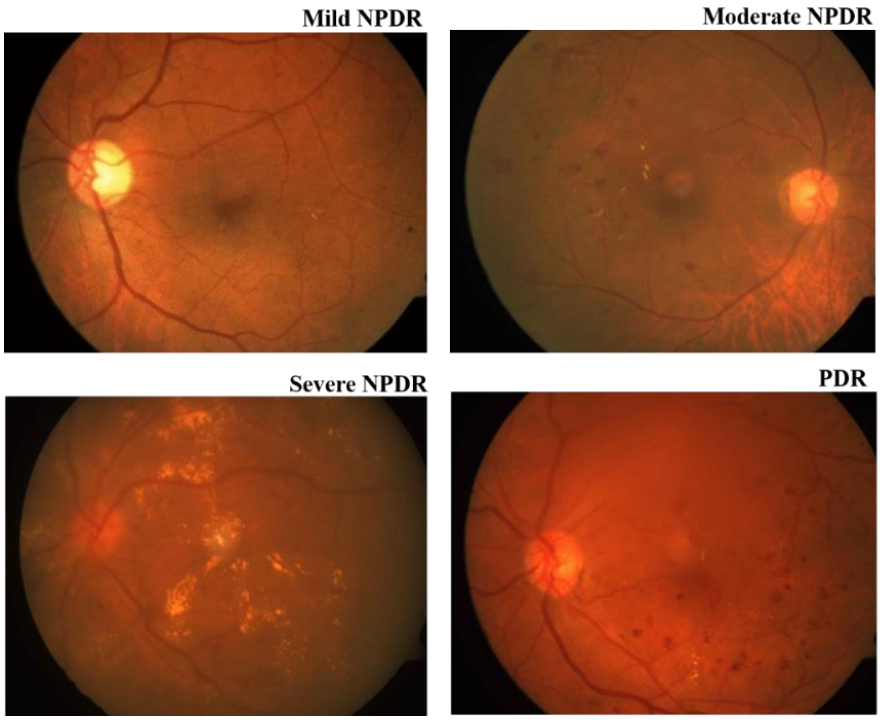


Figure 2.2: Fundus images of patient of mild, moderate, severe and PDR- obtained from patients of present study cohort

2.4. Complications of DR:

The deterioration of vision in DR varies depending on the complications caused by the severity/progression of the disease, including:

2.4.1. Vitreous hemorrhage

The neovessels formed in the retina due to diabetes is very fragile and starts bleeding into the vitreous cavity and cause vitreous hemorrhage. The minor hemorrhage appears as floaters in the vision, whereas the severe hemorrhages entirely block the vision due to accumulation of blood in the vitreous cavity. The vision loss due to vitreous hemorrhage is repairable and it clears within a few weeks and restore the vision if the retina is not damaged (El Annan and Carvounis 2014).

2.4.2. Tractional Retinal Detachment (TRD)

It is one of the common complications and reason for vision loss in the advanced stage of DR. The neovessels in the retina gradually leaks and form scar tissue, known as fibrovascular proliferations. This eventually starts contracting and pulling the neural retina into the vitreous cavity. The degree of vision loss in TRD depends on the loss of function of neurons due to the detachment. Also, TRD enhances inflammatory environment in retina (Cruz-Inigo, Acaba and Berrocal 2014).

2.4.3. Neovascular Glaucoma (NVG)

NVG is one of the major complications of DR and it is one the commonly seen secondary effect of PDR as well as in central retinal vein occlusion (CRVO). In NVG, the imbalance of the pro and anti-angiogenic molecules in the retina due to ischemia drives neovascularization in the anterior surface of the iris and iridocorneal angle of anterior

chamber. This induces obstruction of the aqueous outflow, and causes elevation of intra ocular pressure (IOP), which gradually lead to severe vision loss (Rodrigues, Abe, Zangalli *et al.* 2016).

2.4.4. Diabetic Macular Edema (DME)

DME is one of the major reasons for vision loss in DR, and it is independent of the stage of the disease. It can cause vision loss in all categories of DR, i.e. from NPDR to PDR. DME occurs due to the dysfunction of the Blood Retinal Barrier (BRB) due to diabetes. The inner and the outer BRB breakdown cause fluid leakage in to the retina. The leaked fluid along with the circulating proteins accumulate in the central area of vision, macula, and form hard exudate. This severely impair vison and cause catastrophic loss of vision if untreated (Wenick and Bressler 2012).

2.5. Risk factors involved in the development of diabetic retinopathy:

2.5.1 Longer duration of diabetes

Diabetic duration is one of the strongest risk factors for DR development. As per WESDR, there is 8% prevalence of any form of retinopathy with 3 years of diabetes duration and this prevalence gradually rise up to 25%, 60% and 80% at a duration of 5, 10 and 15 years of diabetes (Klein, Klein, Moss *et al.* 1984). The study done by Aiello *et al*, reported that more than 60% of the subjects after 20 years of diabetes develop any form of DR irrespective of their diabetic control (Aiello, Gardner, King *et al.* 1998). The SNDRIII study in 2014, has reported that a significant association of duration of diabetes with DR development in South Indian population. According to this, the prevalence of DR is 37.1% in those with more than 15 years of diabetes (Raman *et al.* 2014). A

nationwide study in Indian population published on 2016, reported an increase in DR prevalence with the increase in the duration of diabetes. According to this survey, the prevalence of DR in individuals who had DM less than 6 months is 9.23 % and a significant rise in their number with an increase in the DM duration i.e. 15.12 % and 35.12 % of DR prevalence in individuals between 6-months to 5-years and more than 5 years DM duration respectively (Gadkari *et al.* 2016).

2.5.2. Poor glycemic control

Poor glycemic control is known to enhance the risk of DR development. The controlled blood glucose levels were shown to reduce the frequency and severity of microvascular complications in DM patients (Diabetes, Complications Trial Research, Nathan *et al.* 1993). The statistics of Action to Control Cardiovascular Risk in Diabetes (ACCORD) eye study had revealed a 3.1% rise in DR development in patients of standard glycemic control treatment than those with an intensive glycemic control treatment at 4 years of DM (Group, Group, Chew *et al.* 2010). A study also reported 42% decrease in the risk of retinopathy progression in every 10% reduction in glycated hemoglobin level (HbA1c)-whereas every 10% increase in HbA1c enhanced the progression of retinopathy development to 64% (1995).

2.5.3. Hypertension

High blood sugar along with elevated blood pressure enhances the risk of cardiovascular and renal diseases (Turner, Millns, Neil *et al.* 1998). The United Kingdom Prospective Diabetes Study (UKPDS), in 2002 had shown the association of systolic blood pressure in diabetic patients with DR development. They identified diabetic patients with a systolic blood pressure ≥ 140mmHg were 2.8 times more prone to develop DR as compared to

diabetic patients with low systolic blood pressure <125 (Klein and Klein 2002). Also, the metanalysis done by Wat *et al* 2016 to identify the correlation of systemic risk factors and prevalence of DR reported a positive correlation of hypertension and DR prevalence (Wat, Wong and Wong 2016). On the contrary, the ACCORD (Action to Control Cardiovascular Risk in Diabetes) study in 2010 had reported the increased rate of DR in patients undergoing strict blood pressure treatment than that of standard blood pressure treatment (Group *et al.* 2010).

2.5.4. Dyslipidemia

Elevated lipid levels are known to induced endothelial dysfunction by reducing the bioavailability of nitric oxide, which further enhances the formation of hard exudates in the retina (Landmesser, Hornig and Drexler 2000). The analysis from ETDRS identified the patients having elevated serum cholesterol level are at a higher risk of developing retinal hard exudates. Further, the patients with longer period of hard exudates are at higher risk of losing visual acuity (Chew, Klein, Ferris *et al.* 1996). The evaluation of Diabetes Control and Complications Trial study (DCCT) in 2004 reported a significant association of serum lipid levels in the development of macular edema, hard exudates and other complications of DR (Miljanovic, Glynn, Nathan *et al.* 2004). A systemic review published by Rui, *et al* .2018, based on a meta-analysis reported that, lipid lowering agents reduce the risk of DR development and lower the risk of development of DME in patients with diabetes (Shi, Zhao, Wang *et al.* 2018).

2.5.5. Pregnancy

The women who are having gestational diabetes are at high risk of developing type 2-diabetes and thus chance to get DR in later part of their life (Jenkins, Joglekar, Hardikar

et al. 2015). Studies have shown a significant progression in DR in women with moderate to severe retinopathy than mild or no retinopathy at pregnancy (Temple, Aldridge, Sampson *et al.* 2001). Diabetes in Early Pregnancy study (DIEP) in 1995 have shown that 54.8% of the women with moderate or severe retinopathy progressed to the next stage of DR, whereas only 21.1% of the women with mild or no retinopathy showed progression in DR (Chew, Mills, Metzger *et al.* 1995). The effect of pre-gestational diabetes and DR development in Irish-Atlantic population had shown that a significant increase in the progression of DR in women during pregnancy (Egan, McVicker, Heerey *et al.* 2015). In a study done by North Indian population of pregnant women with NPDR and PDR during had identified worsening of the proliferative stage of the disease with fibrovascular proliferation during pregnancy, while NPDR patients were found to be stable during the entire period of gestation (Makwana, Takkar, Venkatesh *et al.* 2018).

2.5.6. Genetic predisposition

The genetic studies have performed on DR by various groups in multiple population have identified a large number of putative genes and genetic variants associated with this disease. The most widely studied genes for genetic association studies were vascular endothelial growth factor (*VEGF*), transcription factor (*TCF7L2*), erythropoietin (*EPO*), aldose reductase (*ALR*), genes in renin angiotensin system and receptors for advanced glycation end products (*RAGE*) genes (Abhary, Hewitt, Burdon *et al.* 2009, Awata, Kurihara, Takata *et al.* 2005, Luo, Zhao, Chen *et al.* 2013, Ramprasad, Radha, Mathias *et al.* 2007, Tong, Yang, Patel *et al.* 2008). But variation reported in these genes are not replicated in other populations, hence as of now there are no accepted candidate gene in DR (Simo-Servat, Hernandez and Simo 2013). But studies have shown that prevalence of

DR in associated with ethnicity, which provide an insight into the importance of genetic and environmental factors in the development of DR. For instance, a population based DR development study in Latinos identified a higher prevalence of DR development in Latinos compared to non-Hispanic whites and African Americans (West, Klein, Rodriguez *et al.* 2001). Studies have also shown that native American ancestry is one of the major risk factors for severe DR complications in Latino population (Gao, Gauderman, Marjoram *et al.* 2014). In Indian scenario, South Indian population is at higher risk for developing severe complication of diabetes including diabetic retinopathy compared to other parts of the country (Shah and Kanaya 2014). But there are very few studies have done in India to understand the genetic factors involved in the development of DR. One of reported polymorphism found to be associated with DR in South Indian cohort was rs2070600 (G > A) in exon 3 of *RAGE* (Balasubbu, Sundaresan, Rajendran *et al.* 2010). Also, the variant of Z-2 of *ALR2*, 18 of insulin like growth factor-1 (*IGF -1*) and AA genotype of inter cellular adhesion molecule -1 (*ICAM-1*) were also identified as a high-risk factor allele in Indian population (Kumaramanickavel, Sripriya, Ramprasad *et al.* 2003, Uthra, Raman, Mukesh *et al.* 2007, Vinita, Sripriya, Prathiba *et al.* 2012). A recent Genome Wide Association Study (GWAS) done in South Indian population had shown an association of *GRB2* on chromosome 17q25.1 (rs9896052) with Sight-Threatening Complication of DR (STDR) (Burdon, Fogarty, Shen *et al.* 2015).

2.6 Neurovascular unit of the retina

Retina and brain are the sensitive and critical neurovascular unit of the living systems. The term neurovascular unit describe the functional coupling between the glia, neurons and with the highly orchestrated vasculature in the CNS, which is critical for CNS homeostasis

(Hawkins and Davis 2005). The vertebrate retina consists of an array of ten layers comprised of neurons and glia, which is nourished by an orchestrated retinal vasculature. The retina consists of five types of neurons of precise functions such as photoreceptors, bipolar cells, horizontal cells, amacrine cells and retinal ganglion cells. All the neurons cell bodies are distributed in three cellular layers, where the synaptic connections are constrained (Nguyen-Ba-Charvet and Chedotal 2014). The outermost layer is the retinal pigment epithelium (RPE), which is followed by the photoreceptor layer consists of rods and cones. The cell bodies with nuclei of rods and cones reside in the outer nuclear layer (ONL). The outer limiting membrane (OLM) comprised of projection of retinal Müller glia, is present just below the ONL. The outer plexiform layer (OPL) consists of projection of rods and cones which end as rod spherules and cone pedicles respectively, where the dendrites of horizontal and bipolar cells in the inner nuclear layer (INL) make synaptic connections with the photoreceptors. The cell bodies of retinal Müller glia reside in the INL and their projections are across the retina i.e. from the OLM to the inner limiting membrane, thus Müller glia provides mechanical support to the retina. The bipolar cells axons and dendrites of the ganglion cells in the ganglion cell layer (GCL) and amacrine cells form synapsis in the inner plexiform layer (IPL). The axons of the retinal ganglion cells are in the retinal nerve fibre layer (RNFL) where it makes connections with the optic nerve head to the visual cortex in the brain. This is followed by the inner limiting membrane (ILM), which is the innermost layer of the retina and it mainly comprised of astrocytes and end feet of the Müller glia. Unlike that of other glial types, astrocyte mainly reside in the RNFL and ILM, where the vasculatures are intact. Another an important type of retinal glia, which is of mesodermal origin is retinal microglia and they mainly

located in the IPL and GCL (Chen, Yang and Kijlstra 2002). The schematic representation of the retina is given in the figure 2.3.

Retina is highly sophisticated and its specific organization contribute susceptibility to diabetes and to the development of DR. Three major structural conditions required for the retina for the normal functioning. First, highly organized and less dense blood vessels in the retina, second unmyelinated neuronal axons to prevent interference of light transmission due to the lipids in the myelin. The unmyelinated neurons require more energy to maintain their membrane potential and lastly the retina maintain comparatively low number of mitochondria due to the presence of light absorbing cytochrome particles in it. Thus, the requirement of high metabolic demand of retinal neurons with limited vascular supply and limited number of mitochondria upsurges the risk of retina to get affected in a metabolic stress conditions like diabetes (Altmann and Schmidt 2018).

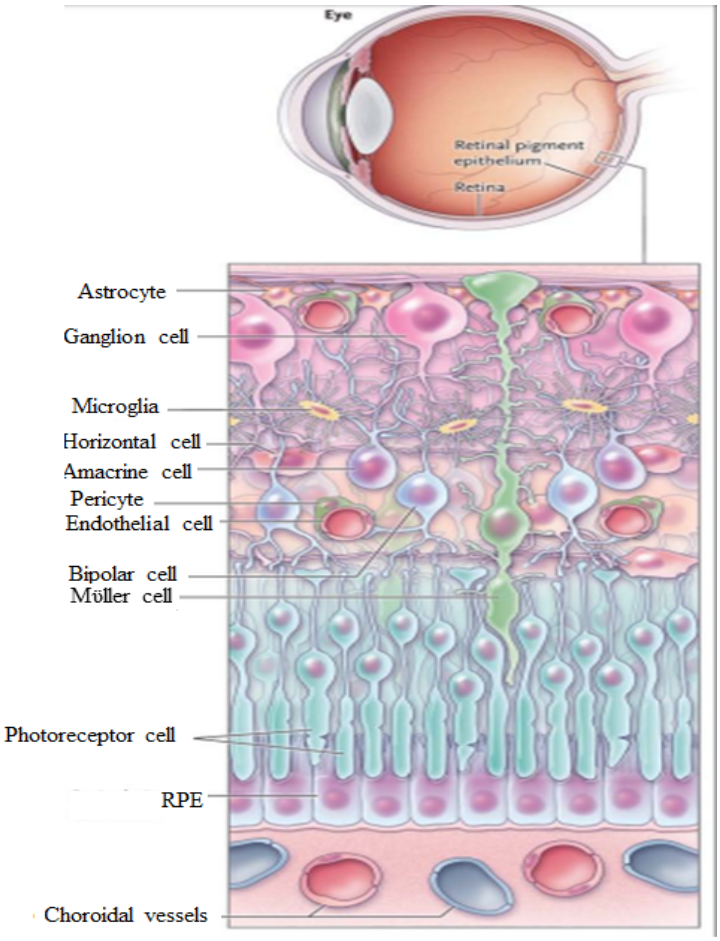


Figure 2.3: Schematic representation of neural retina, adapted from (Antonetti, Klein and Gardner 2012).

Retina consists of three major types of glial populations, such as astrocytes Müller glia and retinal microglia. Astrocytes and Müller glia are the retinal macroglial population, and it shares many common features due to its homogeneity in origin.

2.6.1 Astrocytes

The term astrocyte was coincided by Michael von Lenhossek in 1891, due to the stellate morphology of these cells in CNS (Parpura and Verkhratsky 2012). In retina, astrocytes reside in the innermost layer, i.e. in the nerve fiber layer and its presence and dispersal is

highly correlated to the blood vessels presence and distribution, thus it act as a scaffold for blood vessel development in the neuroretina (Vecino, Rodriguez, Ruzafa *et al.* 2016). In retina, astrocyte originate in the optic nerve head and it is considered that these astrocytes are migrated from the brain. These cells enter in to the retina along with the vasculatures migrating to the innermost layers of the retina (Stone and Dreher 1987). They wrap around the blood vessels as an insulator and perform a vital role in the formation of blood retina barrier. Also, the ion channels present in astrocytes play a crucial role in maintaining neuronal function such as synaptic transmission and plasticity and thus preserves the homeostatic balance (Olsen, Khakh, Skatchkov *et al.* 2015).

2.6.2 Müller glia

They are the largest population of glial cells in the retina and consist of around 10 million in numbers, consist of 90% of retinal glial population. They are neuroepithelial in origin and arise from multipotent retinal progenitor cells (RPCs) during development (Jadhav, Roesch and Cepko 2009). They span through the entire thickness of the retina i.e. from outer limiting membrane to inner limiting membrane and make functional connections with vitreous body, vasculatures and all the retinal neurons, especially, their end process extensions surround the cell bodies and dendrites of RGCs. Müller cells play a major role in ion homeostasis, glucose metabolism, neurotransmitter recycling and neuronal survival by providing neurotrophic factors (Newman and Reichenbach 1996, Sorrentino, Allkabes, Salsini *et al.* 2016). In addition to these, Müller cells express a wide variety of ion channels and transporters and they release various cytokines and growth factors depends on the retinal microenvironment. The branched end process of the Müller glia ensheath the blood capillaries, which act as a bridge for the metabolic exchange between vasculatures

and retinal neurons (Vecino *et al.* 2016). The neurotrophic factors released from the Müller glia ensure neuronal protection by controlling various aspects for the survival of neurons such as neuronal differentiation during development and synaptogenesis (de Melo Reis, Ventura, Schitine *et al.* 2008). Müller glia plays a critical role in preventing neurons from excitatory amino acids toxicity, most importantly from glutamate and γ-amino butyric acid (GABA). Müller cells take up the excitatory glutamate released by retinal neurons using glutamate aspartate transporter (GLAST) and recycle the glutamate to glutamine using glutamine synthetase and release back to neurons (Bringmann, Grosche, Pannicke *et al.* 2013, Poitry, Poitry-Yamate, Ueberfeld *et al.* 2000). Potassium spatial buffering for the survival of neurons by redistributing and normalizing potassium (K^+) ions is done by the Kir channels present in the Müller glia. This function of these cells prevent accumulation of K^+ ions in the retinal microenvironment and helps in neuronal survival (Orkand, Nicholls and Kuffler 1966).

2.6.3 Microglia

The concept of microglia was introduced by Pio del Rio-Hortega in 1919 (Sierra, de Castro, Del Rio-Hortega *et al.* 2016). Microglia are the immunocompetent cells of the CNS with proliferative, migratory and phagocytic activity. They are of mesodermal origin and derived from hematopoietic stem cells, they enter the retina through blood circulation while development and differentiated into ramified parenchymal microglia. In CNS, microglia are the key regulator of immune system response and through the extended ramified processes they constantly monitor the surrounding microenvironment (Davalos, Grutzendler, Yang *et al.* 2005). Microglia exists in two forms known as inactivated ramified and activated amoeboid microglia (Chen *et al.* 2002). In normal retina, they possess a small

ovoid shaped cell body with multiple ramified projections in the ganglion cell layer, inner plexiform layer and outer plexiform layer and these projections transiently contact neuronal synapsis to monitor their functional status. Up on activation they attain the amoeboid shape with a migratory activity and move to the site of injury (Hume, Perry and Gordon 1983, Wake, Moorhouse, Jinno *et al.* 2009). The microglia at the ramified stage have only a minimal expression of cell surface markers and low-level release of chemokines and cytokines. While in the resting stage, they constantly monitor the surrounding microenvironment and get activated under any stress, hence resting microglia can also be described as monitoring microglia (Lynch 2009). The activated amoeboid microglia are highly mobile and up on stimulus they express receptors for cellular activity and abundantly release chemokines and cytokines, which can be either beneficial or harmful to the affected tissue depending on the extent of microglial activation and tissue damage. Their complex physiology is relied on the secretory function and the receptor system present on their surface. They communicate with other macroglial cells and neurons through their large signaling system (Kettenmann, Hanisch, Noda *et al.* 2011). Microglia plays a critical role in neuronal development in CNS tissues by a process known as synaptic pruning. These are also essential for retinal blood vessel formation during development, neuronal survival and axonal growth. The loss of microglia in the retina leads to synaptic breakdown and thus affects the overall retinal function (Wang, Zhao, Zhang *et al.* 2016). Microglia express fractalkine receptors (CX3CR1) on their surface and the ligand for this receptor is largely present in the neurons (CX3CL1). This receptor-ligand interaction plays a crucial role in microglial-neuronal cross talk during synaptic pruning and the activation of microglial phagocytosis whenever there is an apoptotic cells

or signal that interferes with neuronal function under normal physiological condition (Paolicelli, Bisht and Tremblay 2014). Microglia are highly dynamic in nature and depends on their environmental milieu they either turned in to a M1 macrophage cells or M2 macrophage cells. M1 microglia is classically activated type microglia and it release pro-inflammatory cytokines upon activation such as TNF-α, IL-1 β, IL-6, IL-12, IL-23 and other chemotactic factors. Whereas, M2 phenotypes are alternatively activated microglia, and they express anti-inflammatory cytokines such as arginase-1, IL-10, IL-4, IL-13 and low level expression of 1L-12 and IL-23 (Figure 2.4) (Martinez, Helming and Gordon 2009).

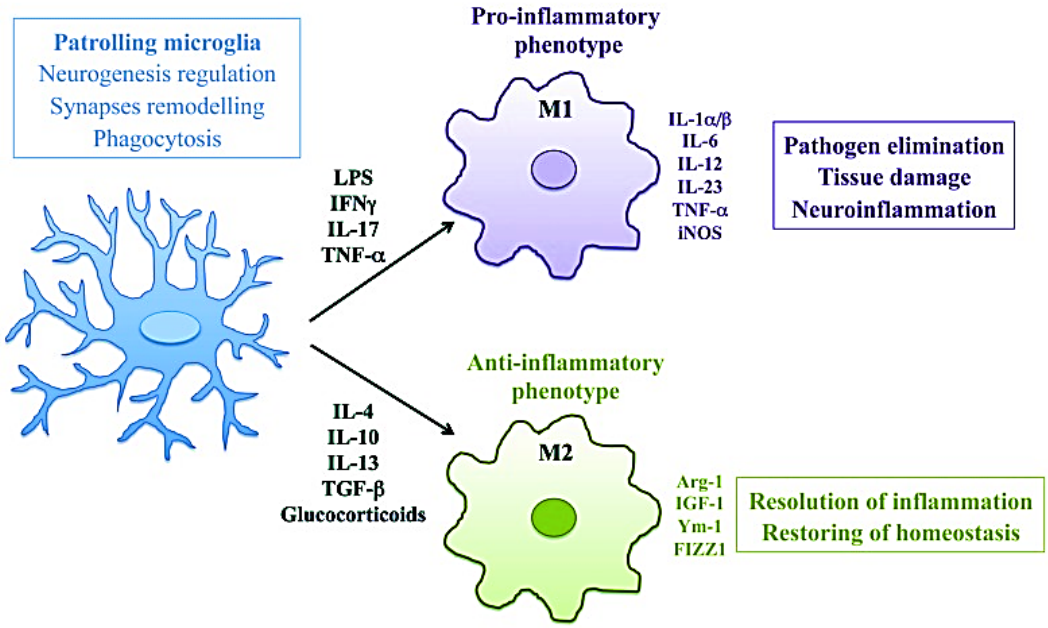


Figure 2.4: Microglial activation and polarization in resting and during neuroinflammation, Adapted from (Salvi, Sozio, Sozzani *et al.* 2017)

2.7 Effect of Diabetes on Neural retina and Neurovascular dysfunction

The co-ordinated and complex interactions and functional coupling between neurons, glia and vasculature in the retina are mandatory for the maintenance of retinal homeostasis and it is termed as neurovascular coupling. Vasculature provide nutrients to the neural retina depends on their metabolic demands and the major mediator which connects vasculature and neural circuitry are the glial cells due to its multifunctionality and close proximity with both the type of cells in the CNS (Gardner and Davila 2017). The complex metabolic disease like diabetes impairs these physiological functions and several alteration in retinal vascular activity have been reported in patients with either type-1 or type-2 diabetes even without any clinical feature of DR (Lott, Slocomb, Shivkumar *et al.* 2012, Pemp, Garhofer, Weigert *et al.* 2009). Diabetes cause impairment in neurovascular coupling, which was well characterized in diabetic retina using flickering light responses. Flickering light elevates the metabolic demands of the neuroretina, thus it enhances vascular activity by increasing the rate of vasoconstriction and vasodilation of retinal arterioles, termed as functional hyperemia (Michelson, Patzelt and Harazny 2002). These vascular activities are maintained by the glial cells by secreting various molecules such as intermediates of arachidonic acid such as prostaglandin E2 (PGE2) and epoxyeicosatrieonic acid (EET). It was also shown that diabetes cause abnormal decline of flickering response of the blood vessels prior to the onset of DR and that further, worsen with increasing stages of diabetic retinopathy (Lim, Ling, Ong *et al.* 2017, Mandecka, Dawczynski, Blum *et al.* 2007).

Diabetes not only cause alteration in vascular activity, but it also induces neuronal loss even at an early stage of the disease. The evidences such as loss of color and contrast

sensitivity in patients with diabetes and changes in retinal functions measures through electroretinogram pointing towards the significant damage in neural retina due to diabetes (Lecleire-Collet, Audo, Aout *et al.* 2011, Sakai, Tani, Shirasawa *et al.* 1995). The first quantitative report of neuronal apoptosis in diabetes was done by Barber *et al* in 1998, using streptozotocin (STZ) induced rat model of diabetes. They found that induction of diabetes caused significant reduction in the inner plexiform later and inner nuclear layer after 7.5 months of diabetes. In these rats, there was a 10% reduction in number of retinal ganglion cells compared with non-diabetic rats (Barber, Lieth, Khin *et al.* 1998). Chhablani *et al* in 2015, measured the level of neuronal damage in diabatic eyes of different stages of retinopathy using Spectral Domain Optical Coherence Tomography (SD-OCT). The study measured the retinal thickness in 13 PDR, 43 NPDR and 20 diabetic eyes and compared it with 66 eyes of age matched control subjects. The outcome of the study revealed a significant thinning of the retinal ganglion cell nuclei and dendrites in diabetic eyes ganglion cell inner plexiform layer (GCIPL) irrespective of the stages of DR and diabetes compared to normal subjects. Also, in RNFL, RGCs axons found to be reduce in thickness in all diabetic eyes. Most importantly, there was no significant difference in thinning of the retinal layers between diabetes groups with or without retinopathies. This clearly proved diabetes induce neuronal damage and onset of the damage is independent and prior to the visible vascular changes in DR (Chhablani, Sharma, Goud *et al.* 2015). The terminal deoxyribonucleotide transferase dUTP nick end labelling (TUNEL) in rat model of diabetes and SD-OCT studies in clinical cases of diabetes had shown similar findings such as the loss of retinal neurons cause thinning of retinal layers and it occurs in early course of diabetes prior to any other visible vascular changes.

Table 2.2: Experimental evidences for an early neuronal cell loss in DR

Method used	Types of neurons	Stage of DR	Disease model	Functional effect
SD-OCT	RGC	Mild, Moderate and Severe DR and no retinopathy	Human	Progressive loss of RGC diabetes without retinopathy to severe DR (Ng, Chiang, Tan *et al.* 2016)
Electroretinography (ERG)	Rods	2,4 and 12 weeks of diabetes	STZ induced diabetic rats	Reduced rods oscillatory potential after 2 days of diabetes (Phipps, Fletcher and Vingrys 2004)
ERG	RGC	2, 4, 6, 10-weeks of diabetes	STZ induced diabetic rats	Reduction in RGC, rod and cons function after 4 weeks of diabetes (Ly, Yee, Vessey *et al.* 2011)

2.8. Mediators of neurovascular damage in DR

Diabetes induced damage to the retina is mediated by multiple factors.

2.8.1. Inflammation

Inflammation is an unavoidable effect of diabetes and is found to be associated with many neuro-vascular and cardio-vascular abnormalities of diabetes. Inflammation trigger many vascular abnormalities by abnormally activating body's defense system. For instance, the Hoorn study on cardiovascular diseases, showed that inflammation increases the expression of soluble vascular cell adhesion molecule-1 (sVCAM-1) in type-2 diabetic

patients which further enhance the risk of atherosclerotic lesions (Jager, van Hinsbergh, Kostense *et al.* 2000). The association of diabetes induced inflammation and development of retinopathy was reported for PDR based on the comparison of the level of inflammatory and immune markers such as sICAM-1, sVCAM-1, IL6 and TNF-α in the serum and vitreous of PDR subjects compared with control subjects. The study had shown an elevated level of all the four analysed cytokines in the serum and vitreous samples of PDR. Most significantly they found a positive correlation of HbA1c with sVCAM-1 and TNF-α level at intraocular level and a TNF–α dependent increase of VCAM-1 in the vitreous humor (Adamiec-Mroczek and Oficjalska-Mlynczak 2008).

Inflammation causes endothelial dysfunction too. Hecke, *et al.* 2005, showed the association of inflammation with retinopathy in a population-based study consisting of 625 individuals between the ages of 50-74 years. The study included measuring the levels of inflammatory markers such as C-reactive protein (CRP), sICAM-1and sVCAM-1 and fundus image analysis to identify the retinal vascular abnormalities. A significant increase in the inflammatory markers in 13.6% was seen in the individuals those having retinopathy changes in the retina irrespective of diabetes. This clearly shows inflammation mediate vascular damages in retina (van Hecke, Dekker, Nijpels *et al.* 2005).

Diabetic retina shares many features of the inflammation, and these inflammatory responses cause neurovascular damage in the retina. Glial cell activation, elevated vascular permeability, leukostasis and the presence of inflammatory cytokines at elevated level are the major inflammatory responses noted in the diabetic retina. Hence, DR is considered as a chronic neuroinflammatory disease of the retina.

2.8.2. Leukostasis

Leukocyte adhesion is the direct of evidence inflammation in DR. Capillary occlusion due to the adherence of leukocytes in the vessels is one of the major inflammatory responses commonly seen in early phase of diabetes and this further induce vascular damage by releasing the cytotoxic products. The leukocyte adherence and effect on neovascularization was studied in alloxan induced diabetic rat retina. The retina sections from 2-, 5- and 9-months diabetic rats were analysed for the number of monocyte and granulocyte adherence in retina versus systemic circulation. Monocytes and granulocytes were significantly elevated after 2 months onset of diabetes and even in the peripheral retinal regions. These adhered leukocytes in the diabetic retina caused capillary occlusion at early phase of diabetes such as at 2 and 5 months. The capillary occlusion by leukocytes triggered endothelial swelling and disorganization of the capillaries, which further induced neovascularization in diabetic rats (Schroder, Palinski and Schmid-Schonbein 1991). The inhibition of leukocyte adhesion using inhibitors of CD18 and ICAM (intercellular Adhesion Molecule 1) resulted in reduced leukocyte adhesion and endothelial damage in streptozotocin induced rat model of diabetes (Joussen, Murata, Tsujikawa *et al.* 2001).

2.8.3. NF-κB activation

An important cause of neuroinflammation in DR is the enhanced activation of inducible transcriptional factor NF-κB. The phosphorylation of cytoplasmic IκB subunit by IκB kinase complex cause proteosomal degradation of IκB and translocation of released NF-κB subunit into the nucleus. This NF-κB act as a transcriptional factor to activate genes involved in inflammation, immune response, cellular survival, proliferation and apoptosis (Kern 2007). The activation of NF-κB in rat model of diabetes at different time interval

had shown that duration of diabetes enhances the expression of NF-ϰB and related cell death. The study used streptozotocin induced diabetic rats at 4, 8, 12 and 16 weeks. Apoptosis was studied by staining the retinal sections with TUNEL and identified no obvious apoptosis at 4 weeks, whereas the number of TUNEL cells gradually increased at 8 weeks and significantly elevated at 12 weeks of diabetes in the inner nuclear layers. And at 16 weeks of diabetes TUNEL staining was taken up by vascular endothelial cells and peripheral cells. A gradual increase of NF-ϰB was noted in diabetic retina from 4 weeks to 16 weeks of diabetes (Jiang, Chen, Yang *et al.* 2015). Further, NF-ϰB activation cause upregulation of inflammatory cytokines by activating various inflammatory and oxidative stress related pathways. IL-1β activation on rat retina after 2 months of diabetes identified a 2.5- fold upregulation compared to control. IL-1β injection caused a 2.5-fold increase of retinal cell death and microvascular lesions in the retina associated with increased oxidative stress by NOs release. Further, the activation of NF-ϰB was checked and found a significant increase of activated fragment of NF-ϰB in the retina of rat with IL-1β injection. This suggested that IL-1β induced apoptosis was mediated by oxidative stress and transcriptional activation of NF-ϰB (Kowluru and Odenbach 2004).

2.8.4. Inducible Nitric Oxide Synthase (iNOS)

iNOS is one of the key inflammatory mediators produced by various types of cells in retina under stress, which accelerate the inflammatory environment in the retina. iNOS has high affinity to protein-bound iron thus, it inhibits key enzymes which is having a catalytic iron centres of crucial pathways such as mitochondrial electron transport, citric acid cycle etc. Also, at higher concentration, iNOS directly interfere with DNA and cause strand breaks and fragmentation (Forstermann and Sessa 2012). The level of iNOS in

human diabetic retina were studied by immunolabelling retina with antibodies of iNOS and glial reactivity. While there was no iNOS staining in the control retina, the diabetic retina without any signs of retinopathy showed a significantly increased staining of iNOS in the elongated cellular processes in the retinal Müller glia. This suggested that retinal Müller glia synthesis iNOS at early phase of diabetic retinopathy and cause microvascular damage in the retina (Abu El-Asrar, Desmet, Meersschaert *et al.* 2001). Subsequently, iNOS knockout diabetic mice were also shown to reduce leukostasis, retinal capillary loss, and no loss of cells in GCL compared to the wild type diabetic mice (Zheng, Du, Miller *et al.* 2007). A study done by Leal *et al.* 2007, suggested the involvement of iNOS in retinal inflammation and Blood Retinal Barrier (BRB) breakdown. Diabetes were induced to iNOS knock out and normal mice and the leukostasis were evaluated in the retinal whole mount. This identified a significant increase in leukocyte adherence in wild type mice retinal capillaries compared to non-diabetic mice however, the iNOS knock out mice had a significant decrease of leukocyte adherence. BRB breakdown analysis by staining with Evan's blue further identified absence of vascular lesions in control mice. While, the level of ICAM, which is partly involved in the adhesion of leukocyte were comparable in between the control mice and iNOS knockout mice, the diabetic mice displayed a higher level of ICAM in the retina. Further diabetes caused a decreased in the level of the tight junctional proteins. These studies clearly established the role of iNOS in retinal inflammation by upregulating ICAM, leukostasis and BRB breakdown under diabetic condition (Leal, Manivannan, Hosoya *et al.* 2007).

2.8.5. Cytokines and Chemokines

Diabetes is known to elevate the cytokines and chemokines involved in the inflammation, which further deteriorate the diabetes induced damage. Compared to other Proliferative Vitreoretinopathies (PVR), PDR retina contain higher levels of inflammatory cytokines. The comparison of IL-8, monocyte chemotactic protein-1 (MCP-1), macrophage colony stimulating factor (M-CSF) level among the vitreous samples of PDR, PVR and control samples by ELISA had shown significantly elevated level of IL-8 and M-CSF in vitreous samples in the patients with PDR compared to controls (Elner, Elner, Jaffe *et al.* 1995). Caspase-1 is known as IL-1β converting enzyme, because of its role in generation of mature IL-1β. Caspase-1 and IL-1β levels were found to be upregulated in diabetic retina in experimentally induced diabetes. The mice were made diabetic by streptozotocin and after a week of diabetic injection, minocycline; an anti-inflammatory drug known to reduce caspase-1 activation was injected into diabetic mice. While diabetes induced a significant increase of caspase-1 and IL-1β and the minocycline treatment reduced their levels and the acellular capillary formation in the diabetic mice. Müller glial cells are known to secrete caspase-1 actively under diabetes and these cells undergo apoptosis under high glucose conditions. The retinal Müller glia were isolated and treated under high glucose condition in presence of minocycline to inhibit caspase-1 activity and IL-1β neutralizing antibody showed reduction in the secretion of caspase-1 and IL-1β by Müller cells. The IL-1β neutralization also reduced the level of casapase-3, and thereby inhibited the apoptotic process under high glucose (Vincent and Mohr 2007).

Apart from cytokines, chemokines also play a crucial role in diabetes mediated inflammation and retinal damage. CCl2 also known as MCP-1 is one of the strongest

chemokines for the recruitment of monocyte. The studies on DR has shown an increased level of CCL2 in the diabetic eye induced recruitment of monocytes/ macrophages which further caused vascular damage in the retina (Capeans, De Rojas, Lojo *et al.* 1998, Rangasamy, McGuire, Franco Nitta *et al.* 2014). The estimation of chemokines vitreous and serum samples of PDR, PVR and RD controls through ELISA identified elevation of chemokines such as MCP-1, CXCL10 and CXCR3 in the vitreous samples compared to the control samples (Abu El-Asrar, Struyf, Kangave *et al.* 2006)

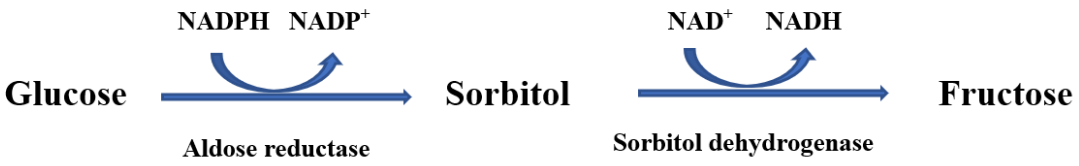
Table 2.3: List of cytokines associated with diabetic retinopathy progression

Cytokine/ chemokine tested	Methods and samples used	Upregulated cytokines in cases	Downregulated cytokines in cases	Effects of Dysregulated cytokines on DR	References
IL-6, IL-8, IL-1β, MCP1, VEGF, PEDF	ELISA. Vitreous	IL-6, IL-8, MCP-1, VEGF	PEDF	Inflammation, Neovascularization	(Murugeswari, Shukla, Rajendran et al. 2008)
sVEGFR1, VEGF, PEDF	ELISA using vitreous, HUVEC	sVEGFR1, VEGF	PEDF	Angiogenesis	(Matsunaga, Chikaraishi, Izuta et al. 2008)
VEGF, ICAM-1, IL-6, MCP-1, PEDF	ELISA, Vitreous	VEGF, ICAM-1, IL-6, and MCP-1	PEDF	Inflammation Increased retinal vascular permeability and the severity of DME	(Funatsu, Noma, Mimura et al. 2009)
IL-1β, TNF-α	ELISA, vitreous	IL-1β, TNF-α	NIL	Suggested macrophage mediated inflammation in PDR	(Demircan, Safran, Soylu et al. 2006)
TNF-α, IL-6, IL-1β, CRP	ELISA, serum	TNF-α, IL-6	NIL	Enhanced inflammation in diabetes and DR.	(Hernandez-Da Mota, Soto-Bahena, Viveros-Sandoval et al. 2015)
IL-1ß, IL-6, IFN-γ, IL-2, IL-4, IL-13, VEGF, placental growth factor (PGF)	ELISA, vitreous	IL-1ß, IFN-γ, VEGF, PGF	IL-13	Pro-inflammatory cytokines and angiogenetic factors are associated with inflammation and angiogenesis in PDR	(Tsai, Kuehn, Tsiampalis et al. 2018)

2.9. Major inflammatory pathways involved in DR progression

2.9.1. Polyol pathway

The polyol pathway of glucose metabolism activates when intracellular glucose levels are extremely high. Activation of this pathway induce the production of unusual sugar molecules such as sorbitol and fructose and this cause vascular and neuronal damage (Gabbay 1973). When the glucose concentration become excess, the hexokinases, that are involved in the conversion of glucose gets saturated. This results in the activation of aldose reductase enzyme, which convert glucose to sorbitol using NADPH (Nicotinamide Adenine Dinucleotide) as a co-factor and produce $NADP^+$. The produced sorbitol is then metabolized into fructose by the enzyme sorbitol dehydrogenase and reduce NAD^+ to NADH. The decrease in NADPH and generation of NADH leads to a rapid change in the cytoplasmic redox state. NADPH is required for the generation of antioxidant glutathione and its availability reduced in oxidatively stressed cells(Dunlop 2000). The major consequences of polyol pathway are the enhanced accumulation of glutamate in the retina, increased the level of cytosolic calcium, generation of AGE products and proinflammatory responses (Obrosova and Kador 2011).



The presence of AR enzyme in the retinal cells are indicative of excess glucose accumulation and activation of the polyol pathway. The immunohistochemical analysis of retina for aldose reductase had identified the localization of this enzyme in the cytoplasm of pericytes (Akagi, Kador, Kuwabara *et al.* 1983). Alvin *et al.* 2005 identified a significant reduction of pericyte

loss and vascular lesions in AR knock out ($AR^{-/-}$) diabetic mice. The effect of AR knockout on angiogenic protein VEGF was evaluated and found a significant reduction of VEGF staining in retinal glia and neurons in knockout compared to diabetic, thereby suggesting that AR deficiency repressed diabetes induced activation of VEGF gene of angiogenesis. Most importantly gliosis was found to be significantly reduced in $AR^{-/-}$diabetic mice. And the knockout mice developed a smaller number of neovessels compared to diabetic mice and lower level of cleaved caspase -3. Thereby, the study clearly showed the effect of the activation of polyol pathway in diabetic retina (Cheung, Fung, Lo *et al.* 2005).

2.9.2. Protein kinase C (PKC)– pathway

PKC, comprised of a group of multifunctional serine- threonine kinases that regulate the activity of other proteins in a living system. Till date, 12 known PKC isoforms are identified and broadly divided into three groups such as classical, novel and atypical. Among the three PKC isoforms classical isoforms including PKC-α, PKC-β/2, and PKC-δ, are known to be highly involved in the pathogenesis of diabetic complications (Giacco and Brownlee 2010). The activation of classical PKC is reliant on Ca^{2+} ions and phosphatidylserine and diacyl glycerol (DAG) greatly enhance classical PKC activation. The binding of secondary messengers to the regulatory domain of PKC at their plasma membrane side activate the signaling transducers which further induce the activation of various downstream signaling pathways involved in vascular damage and tissue injury in diabetic complications (Safi, Qvist, Kumar *et al.* 2014). Further analysis of the activation of PKC and associated vascular abnormalities in diabetic mice retina identified activated PKC isoforms such as PKC- β/2 and PKC-δ in mice after 3 months of diabetes. A significant increase in acellular capillaries and

increased pericyte loss by the PKC mediated activation of P38/MAPK pathway was observed in diabetic mice (Geraldes, Hiraoka-Yamamoto, Matsumoto *et al.* 2009). PKC activation induced BRB breakdown in diabetic mice was studied by Kim *et al.* The diabetic mice had increased vascular permeability and tight junctional proteins such as ZO-1, ZO-2 and occludin in the retina. Human retinal microvascular endothelial cells (HRMEC) when treated with AGE had a significant reduction of tight junctional proteins whereas PKC inhibition prevented the loss of tight junctional proteins. A decrease in vascular permeability of the cells with restoration of tight junctional proteins was seen upon treatment with PKC-δ inhibitors, suggested that the increase in vascular permeability was mediated by PKC activation. Further, PKC- δ translocations from cytosol to membrane were seen in the AGE treated cells, whereas the inhibition of PKC-δ prevented this translocation. These results indicated that hyperglycemia activates PKC-δ by translocating the subunit of PKC from cytosol to membrane, that further phosphorylates and activates downstream pathways involved in angiogenesis and tissue remodeling in DR (Kim, Kim, Jun *et al.* 2010).

2.9.3. Hexosamine synthesis pathway

The metabolism of glucose in the living organism is carried out mainly by the glycolysis. But under overt glucose conditions, hexosamine synthesis pathway gets activated to metabolize the glucose and resulting in the generation of complex molecules, such as proteoglycans, glycolipids and also cause glycosylation of proteins in post translational modifications (Kim, Nakayama and Nayak 2018). In hexosamine pathways, the fructose-6 phosphate formed in glycolysis, react with glutamine and form glucosamine-6 phosphate, by the enzyme glucosamine 6-phosphate aminotransferase (GFAT). Further, acetyl Co-A transfer acetyl

group to this compound and generates N-acetyl glucosamine 6-phosphate that further isomerizes to form N-acetyl glucosamine 1-phosphate. Next, UTP react with this product and generate the final product as UDP-N-acetyl glucosamine (UDP-GlcNAC), a substrate for O-linked glycosylation by the enzyme O-GlcNAc transferase (OGT)(Buse 2006). Increased O-glycosylation of proteins can make the cells insulin resistance by causing a defect in the glucose transporter, GLUT4 in the cells, that leads to cellular stress and damage (Cooksey, Hebert, Zhu *et al.* 1999).

2.9.4. Advanced Glycation End (AGE) Product accumulation

One of the major causes of diabetes induced changes are due to the accumulation of AGE in the tissues. AGEs are the non-enzymatically glycated and oxidized form of lipids, proteins or nucleic acid after a long-term exposure to glucose moiety, which create severe metabolic changes in the body. This glycation can affect any circulating proteins mainly serum albumin, insulin, hemoglobin etc. The AGE gradually cross link with the Extra Cellular Matrix (ECM) proteins or the receptors present in ECM, cause structural modification and create cellular stress. Also, AGE activate many inflammatory pathways which are associated with endothelial dysfunction, microvascular complications and neuronal damage (Negre-Salvayre, Salvayre, Auge *et al.* 2009). In retina, the receptors for AGE (RAGE) are mainly seen in the cell types such as endothelial cells, RPE, Müller glia, microglia and also retinal pericytes. Hence binding of RAGE- AGE mainly affects the functioning of these cells and activate many downstream signaling pathways such as NF-ƘB, MAPK, angiogenic pathways and so on, which eventually leads to the release of pro inflammatory cytokines such as TNF-α, MCP-1, IL-6, IL-1β,

angiogenic molecules like VEGF, PDGF etc. that have a negative impact on retinal functions (Kandarakis, Piperi, Topouzis *et al.* 2014).

The vitreous analysis of diabetic patients by had shown that the significant increase of AGE in diabetic vitreous compared to the age matched controls. Further, more collagen cross linkages were identified in the diabetic vitreous compared to that of no diabetes, indicating the changes in ECM protein due to AGE accumulation (Stitt, Moore, Sharkey *et al.* 1998). The effect of AGE inhibition and retinal vascular lesions were studied in mice model of DR. The mice were injected with STZ and pyridoxamine (PA), an inhibitor of AGE and ALE. Retinas were evaluated for the morphological changes associated with diabetes. The mice were developed acellular capillaries, a prime sign of DR vascular lesions after 29 weeks of diabetes, but these lesions were significantly lower in the diabetic mice treated with PA and were comparable to that of the non-diabetic mice. Further significant levels of ECM genes such as laminin, collagen IV, and fibronectin were identified in diabetic mice compared to non-diabetic. The immunofluorescence analysis identified significantly lowered deposition of AGE/ALE complex in the inner retinal layers in PA treated DM mice, suggesting that AGE/ALE accumulation in the retina due to diabetes caused a pathological alteration in ECM proteins (Stitt, Gardiner, Alderson *et al.* 2002). Curtis *et al.* 2011 identified increased accumulation of AGE/ALE in the retina, especially in Müller glia, GCL and INL, whereas the PA treatment significantly reduced the same. Further oxidative stress in the retina was found to be significantly upregulated especially in the regions of Müller glial processes, GCL and in OLM while PA treatment significantly reduced the expression of haemoxygenase-1 in the retina. Similar results were seen for GFAP staining in the astrocyte and in the Müller glia in DM group. Both the studies clearly showing diabetes induced AGE accumulation cause

oxidative damage, vascular permeability and gliosis, thus enhancing the pathogenesis of DR progression (Curtis, Hamilton, Yong *et al.* 2011).

2.9.5. Oxidative stress

The hypoxia/ischemia induced by diabetes play a key regulatory role in the development of diabetic complications. The reactive oxygen species (ROS) generation under normal cellular conditions get cleared off from the cells by an efficient scavenging system in the body. But an imbalance on ROS production and scavenging in the body cause an excessive accumulation of ROS or molecular oxygen which create oxidative stress in the system, which is often found to be associated with diabetic complications (Kowluru and Chan 2007). Diabetes induced oxidative stress occur through multiple mechanisms such as reduction of antioxidant glutathione, alteration in the in redox balance, impairment in the function of superoxide dismutase enzyme, autooxidation of glucose etc (Dunlop 2000, Wohaieb and Godin 1987, Zhao, Jin, Gao *et al.* 2018).

Hypoxia is a major trigger for the activation of multiple angiogenic and inflammatory pathways. Under hypoxic environment the accumulation of HIF-1 α (Hypoxia-Inducible Factor-1-alpha) activates the downstream VEGF gene, increased level of VEGF cause abnormal vascular sprouting and neovascularization. Hypoxia also activate various inflammatory signaling mechanisms in DR such as activation of PKC signaling cascade, JNK kinases and transcriptional activation of NF-κB, b-catenin/Wnt, p53 etc, which further lead to the expression of multiple genes involved in angiogenesis, release of inflammatory cytokines, chemokines, growth factor etc (Brownlee 2001). The activation of these pathways in turn, enhances the generation of ROS.

The mitochondrial damage due to oxidative stress cause impairment to the retinal cells. Studies have shown that under high glucose conditions, mitochondrial network in the retinal Müller glia cause a significant heterogeneity in the membrane potential and mitochondrial fractionation leading to enhanced release of cytochrome c (Tien, Zhang, Muto *et al.* 2017). Similar findings were also reported in STZ induced 8 months old diabetic rats, where enhanced cytosolic level of cytochrome c, BAX and caspase-3 expression were seen in diabetic retina as well as in retinal capillaries and in pericytes under high glucose exposure. Thus high glucose induced oxidative stress could cause mitochondrial damage and facilitate apoptosis of the cells through caspase-3 activation (Kowluru and Abbas 2003). An overall mechanism of oxidative stress and inflammatory pathway activation is given in the Figure 2.5.

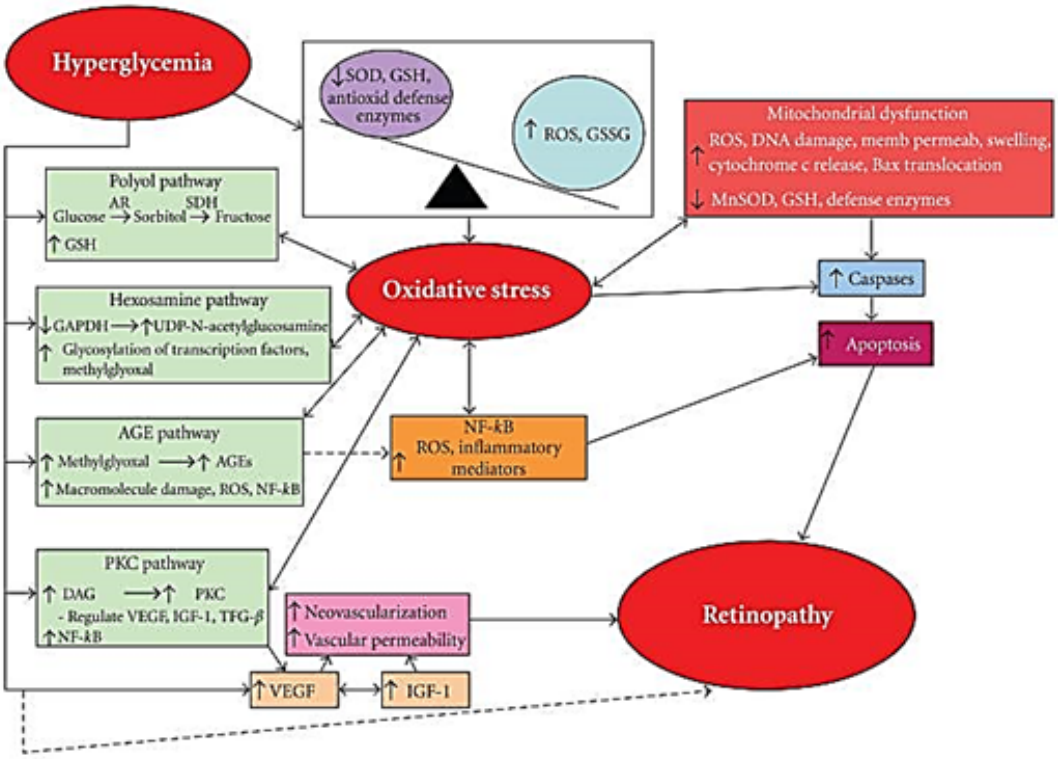


Figure 2.5: Mechanism of inflammation and oxidative stress in the pathogenesis of Diabetic retinopathy. Adapted from (Kowluru and Chan 2007)

2.10. Diabetic retinopathy proteome:

The analysis of a complete set of proteins in an organism with respect to a particular tissue at a specific condition and including its functions and interactions with other proteins define the term proteome. The proteome analysis can identify proteins in a complex mixture and reveal their association in a particular disease pathogenesis which further help to identify a potential therapeutic target for the disease. There have been several proteomic studies on diabetic retinopathy by various groups using different body fluids such as serum, retina and vitreous to identify the disease mechanism.

2.10.1 Serum/Plasma proteome in DR:

Serum and plasma are enriched with dynamic range of proteins and analysis of these fluids helps to understand the systemic variation of proteins according to a particular disease state. The advantage of serum/plasma proteomics is the easy access of the biological material at different stages of the disease without any invasive technique. This can help to understand variation in systemic protein profile of the patients from a non- proliferative stage of the disease to the advanced vision threatening phase of DR. Plasma proteins analysis from DR patients compared to the diabetic controls using 2-Dimensional Electrophoresis (2DE) followed by matrix assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-ToF-MS) identified 5 differentially expressed proteins. Of these, three proteins including retinol-binding protein 1, diphosphoinositol polyphosphate phosphorhydrolase 3-alpha and neuroglobin were found to be downregulated in DR while the other two including Hemoglobin subunit gamma 2 and CD-160 antigen were upregulated. A significant change in the expression of neuroglobin suggested for its potential role as a biomarker for DR

progression (Gopalakrishnan, Purushothaman and Bhaskar 2015). Liu *et al* in 2011, compared serum proteome between diabetic individuals with no diabetic retinopathy (No-DR), NPDR, PDR and healthy non -diabetic controls by 2D- DIGE followed by MALDI-TOF-TOF MS. This approach identified gradual increase of proteins such as beta 2-glycoprotein I (β(2)-GPI) from no-DR to NPDR and PDR as 1.54, 2.43, and 2.84 fold compared to that of healthy controls, suggestive of the involvement of β (2)-GPI in DR occurrence and progression (Liu, Hu, Wu *et al.* 2011). Another study by Chie, *et al.* 2012, compared plasma proteome between DR patients who developed DR after 5 years of diabetes and diabetic patients with no retinopathy after 10 years of diabetes. 77 plasma proteins were found to be involved in the inflammation and the coagulation pathway in DR. In addition to the previously suggested biomarkers of DR, the study also identified differential expression of proteins such as afamin and arginine N-methyltransferase 5 as a potential biomarkers for DR (Lu, Lin, Chou *et al.* 2013).

2.10.2 Proteome studies using diabetic retina

While serum proteomics identified some major proteins as biomarker for DR, the direct evidence and disease mechanism of DR can be understood only by studying the retinal tissue proteome. There are very limited number of studies who have attempted to understand the diabetic retinal proteome. Godfrey *et al* in 2006, compared the proteome of diabetic versus control retina using STZ induced and rat model of diabetes by 2DE, MALDI-MS and Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) based protein identification. Both the methods together, identified a total of 103 retinal proteins. The study identified unique expression of heat shock protein (Hsp) 70 subtype 1A (Hsp70.1A) and Hsp

70 subtype 8 (Hsp 70.8), which are known to cause cellular stress in the diabetic rat retina. Other than heat shock proteins, the study also identified platelet activating factor alpha 2 subunit which is known to have a role in vaso-obliteration in OIR model. The major upregulated proteins included beta catenin complex B, a major molecule involved in Wnt signaling, glyceraldehyde 3 phosphate, and a protein involved in the activation of AGE pathway. Dihydropyrimidase-related protein (DRP) type 2, a protein needed for neural circuitry development and Succinyl CoA ligase were the important downregulated proteins observed in the diabetic retina compared to the control (Quin, Len, Billson *et al.* 2007). Patrice *et al* performed a detailed investigation on retinal proteome at different time course of diabetes using SD rats after STZ injection using 2-dimensional difference gel electrophoresis (2D DIGE) followed by isobaric tags for relative and absolute quantification (ITRAQ) of protein identification. This study had identified overexpression of crystalline proteins in diabetic retina along with increased duration of diabetes, suggesting for the role of crystallins in vascular remodeling of diabetes (Fort, Freeman, Losiewicz *et al.* 2009). The study done by Gao *et al* 2009 intended for a comprehensive evaluation of the retinal proteome between STZ induced 2 months old diabetic mice versus their age matched controls by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A total of 1792 proteins were identified from the retinal lysates, of these, 55 and 10 retinal proteins with more than 2-fold increase and decrease respectively were found in the diabetic retina. These included proteins related to metabolic process, apoptotic pathways and oxidative phosphorylation. Further treatment of angiotensin II receptor antagonist candesartan in STZ rats identified a reversible expression of 23 of the proteins which were upregulated. These included mainly the proteins of lipid metabolism and

apoptotic proteins such as apoptosis inhibitor 5, dynamin-like 120 kDa protein and NADH-ubiquinone oxidoreductase 75 kDa subunit (Gao, Phipps, Bursell *et al.* 2009).

Another important aspect of DR is retina neurodegeneration. Sundstrom, *et al* in 2018, have analyzed the presence of brain neurodegenerative proteins in diabetic retina. The study has used diabetic and non-diabetic cadaveric retinas and analyzed the proteome by LC-MS/MS. In order to understand the alteration in the neuronal signaling system involved in diabetes, a detailed investigation was done for the neurotransmitter and other nervous system signaling molecules. This identified the presence of pathways such as unfolded protein response and neuroprotective role of thimet oligo peptidase 1 (THOP1) in Alzheimer's disease in control, whereas in diabetic retina, pathways involved in dopamine degradation and Parkinson's signaling were found to be enriched with glial activation. In addition to these pathways, diabetic retinas were also found to be enriched with proteins of the neuregulin signaling which is needed for neuroprotection under the inflammatory condition and amyloid processing pathway, an important signaling mechanism that enhances neurodegeneration in Alzheimer's disease. This shows a common link that exist between retina and brain neurodegenerative disease as well as it emphasizes neurodegeneration as an important event in the diabetic retina (Sundstrom, Hernandez, Weber *et al.* 2018).

2.10.3 Proteome analysis of vitreous humor:

Even though the studies have shown the usefulness of serum/plasma to identify the potential biomarkers for development of diabetic retinopathy, they cannot predict the molecular events occurring in the localized environment of retina. The infiltration of serum proteins occurs after the BRB breakdown, which may enhance the disease severity by causing an additive effect on the altered proteome of the diseased retina. Though, serum proteome cannot

replicate the actual molecular mechanism exclusive to the retina however, most of the studies on understanding retinal proteome used animal model for exploring the molecular mechanisms or the diabetic retina from cadaveric eye balls due to the lack of accessibility of retinal tissues from human donors. Alternatively, vitreous humor due to its close proximity to the retinal microenvironment can also serve as a tissue for to exploring molecular mechanisms of the disease pathogenesis in diabetic retinopathy.

2.11. Vitreous humor

Vitreous humor is an acellular, transparent, viscous, highly hydrated extracellular matrix structure and occupies the cavity behind the lens and is surrounded by and physically attached to the neural retina. It provides structural support to the eye throughout the life, act as a channel for the lens metabolic requirements, also it inhibits cellular growth and large macromolecules in order to maintain the transparency and light entry into the retina (Arciniegas and Amaya 1980).

2.11.1. Biochemistry of the vitreous humor

Vitreous is mainly composed of water (98-99%), remaining including mixture of proteins, sugars and salt. The most abundant protein present in the vitreous is collagen. The collagen fibrils are organized as a triple helix of 3-alpha chains in the vitreous and heterotypic collagens present in the vitreous includes collagen type II, type IX and hybrid of collagen V/XI, also a small amount of type VI collagen. Most abundant and 75% of the total collagen present in the vitreous is type II collagen(Seery and Davison 1991). Collagen V/XI are only the 10% of the total vitreous collagen and forms the central core of the major vitreous collagen fibrils. It is a heterotrimer comprised of α1 chain of collagen XI and α2 chain of collagen V and the

third chain nature is unknown. It is also a fibril forming collagen and expose N -propeptides. Type IX collagen is not a fibrillar form of collagen. It is composed of three different α-chains (α1, α2 and α3) and has a more complex structure compared to fibrillar type. Type VI collagen is present in vitreous in a minor amount. These fibrils are comprised of collagen VI monomers that are assembled into a tetramer with disulphide bond. Type VI collagen bind to type II collagen and hyaluronan in the vitreous and thus it contributes a significant role in the vitreous gel structure.

The other major non- collagenous part of vitreous are fibrillin and fibrillin containing microfibrils, glycosaminoglycans (GAG) which includes hyaluronan, chondroitin sulphate proteoglycans and heparan sulphate proteoglycans. The jelly nature of the vitreous humor is stabilized by the GAG. Among the various GAG in the vitreous the hyaluronic acid consists of 96.2 % of the total GAG and chondroitin and heparan sulphate are 3.5% and 0.3% respectively (Peng, Yu, Lin *et al.* 2018).

2.11.2. Anatomy of the vitreous humor

Anatomically vitreous humor is divided into central vitreous, the basal vitreous, vitreous cortex and vitreoretinal interface (Figure 2.6).

i. Central vitreous

The central vitreous consists of the main region of the vitreous body and remnants of hyaloid vascular system known as hyaloid channel or Cloquet canal is present in this region. The central region contains very low concentration of collage fibrils which is anteriorly inserted into the vitreous base and posteriorly into the vitreous cortex (Sebag 1987).

ii. Vitreous base

The vitreous base is a 3-D structure that overlaps with the ora serrata, i.e. the serrated junction between the ciliary body and the retina, thus form the anterior loop of the vitreous. Vitreous base occupies high density of collagen fibrils and they are oriented at right angles to the peripheral retina and inner surface of the ciliary body. The vitreous base is closely adhered to the retinal surface especially to the non-pigmented epithelium basement membrane and internal limiting lamina of the peripheral retina. The retinal component of the vitreous base tends to enlarge with age and lead to the development of irregular posterior base. This anatomical variation in vitreous base can cause retinal break in many retinal pathological conditions (Sebag 2015).

iii. Vitreous cortex

The vitreous cortex also known as hyaloid surface is a thin membranous outer zone of vitreous consists of 100-300 μm wide in size and it surrounds the central gel. It contains more concentration of collagen than the central vitreous, which make it to distinguish from the central vitreous. Vitreous cortex consists of anterior and posterior vitreous cortex. The anterior cortex is adjacent to the ciliary body, lens and the posterior chamber and lies anterior to the vitreous base. The posterior cortex extends to the posterior to the vitreous base and is adherent to the inner retinal surface. The basal vitreous and vitreous cortex contains low concentration of vitreous hyalocytes, hence these are not completely acellular vitreous (Bishop 2000).

iv. Vitreoretinal interface

The region in which vitreous interface with neighboring retina is termed as vitreoretinal interface, this interface consists of regions such as posterior vitreous cortex, inner limiting lamina of the retina and intervening ECM (Sebag 1992). Due to aging or in any vitreoretinal pathological conditions, the confirmation of the collagen fibrils get altered either due to enhanced cross linkage of the collagen peptides, cause redistribution of the collagen fibrils and make certain area devoid of collagen, resulting in liquefaction of the vitreous and weakening of the adhesion at the vitreoretinal interface (Le Goff and Bishop 2008).

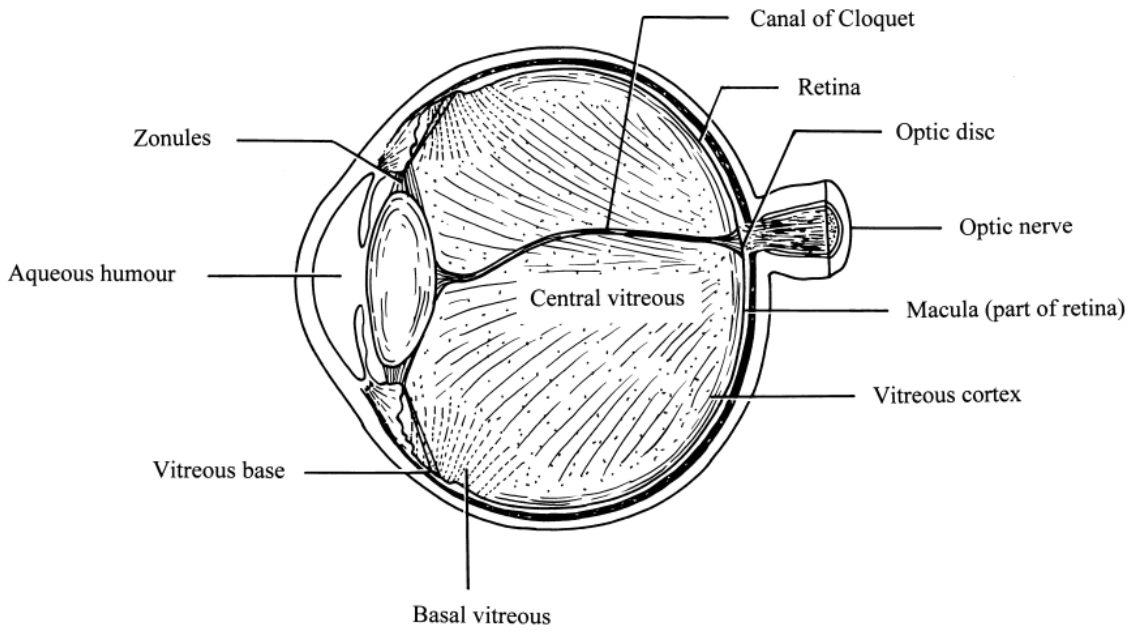


Figure 2.6: Anatomy of the vitreous humor. Adapted from (Bishop 2000)

2.11.3. Usefulness of vitreous for studying the disease pathology

Despite the major structural constituents, vitreous is also nourished with proteins, growth factors and so on. The average protein content of the human vitreous is calculated as 1200μg/mL and the most abundant proteins among them are albumin and immunoglobulins (Grus, Joachim and Pfeiffer 2007). Other than the proteins being trafficked in to the vitreous compartment during developmental stages, the local secretion of the proteins from the surrounding tissue, mainly from the retina also influence the vitreous protein content (Wu, Sauter, Johnson *et al.* 2004). Additionally, vitreous humor is devoid of blood vessels and the nourishment to the vitreous is provided by retinal vessels and the ciliary body. Thus, changes in the retina due to any disease pathology cause secretion/release of retinal proteins into the vitreous and it will retain there for a longer period of time due to the lack of a replenishing system unlike that of aqueous humor in the eye. Hence, the biochemical alteration in the retina due to a particular disease state affect the vitreous proteome also. Obtaining retinal tissue specimen for the gaining biochemical and molecular understanding of the disease pathology is not possible but vitreous can be obtained from the vitrectomy surgery as a part of treatment strategy in the end stage of disease like DR. These vitreous specimens reflect the exact pathological alteration happened in the retina and thus, studying the vitreous proteome allows to explore the events that are occurring in the retinal pathology (Murthy, Goel, Subbannayya *et al.* 2014, Wang, Feng, Hu *et al.* 2012).

2.11.4. Diabetic retinopathy – Vitreous Proteomic Studies

Identification of vitreous proteins in pathological and normal conditions were studied by many researchers using multiple strategies and techniques. Mass spectrometry is a method to

identify the proteins based on their mass to charge ratio is considerably used technique for vitreous proteome analysis. Even though mass spectrometry is the abundantly used method for protein identification, the complexity of the vitreous fluid contributed by the complex metabolism of diabetes is still not completely elucidated out.

The first vitreous proteome analysis in DR was done by Shimizu *et al* 2002. The study compared vitreous proteome between PDR with Macular Hole (MH) by 2D-PAGE followed by MALDI-TOF. 50 different proteins were identified comprised of 52 total proteins in the study. Of these, 35 proteins were specific for the vitreous humor and remaining were plasma proteins, such as albumin, apolipoprotein-A1, antitrypsin, immunoglobulins, complement C4 etc. The major finding was the identification of a strong neuronal protein Cystatin C in the vitreous and identification of anti-angiogenic protein PEDF in vitreous samples (Nakanishi, Koyama, Ikeda *et al.* 2002). Next, Ouchi *et al* (2005), compared vitreous profiling between DME from preproliferative retinopathy samples using 2D followed by liquid chromatography mass spectra (LC-MS/MS). This study identified a total of 29 proteins such as 14 proteins in DME and 15 proteins in non-DME group. Out of that 6- spot intensities were more in DME which were corresponding proteins to such as PEDF, Apolipoproteins A4 and A1(Apo A4 and Apo A1), plasma retinol binding proteins (PRBP), thyroid hormone receptor interacting protein-11 (Trp-11) and vitamin D-binding protein. The level of PEDF was quantitative estimated using ELISA and found a significant increase of this protein in DME compared to no DME and only 1 protein spot of ApoH was found exclusively in no-DME vitreous (Ouchi, West, Crabb *et al.* 2005). Gao *et al*, in 2008 used 1D SDS PAGE followed by nano LC/MS-MS and profiled proteins in PDR, no retinopathy diabetic vitreous with non-diabetic control samples. The study identified an overall 272 proteins from 17 vitreous samples and abundance

of 56 proteins were found in PDR compared to diabetic group. The biological pathway analysis further revealed 30 proteins of coagulation, complement and Kallikrein-kinin system. Proteins such as apolipoprotein A-IV, complement C4b and factor B were found to expressed abundantly in diabetic group and an increased tendency of this proteins were detected in PDR as well. The major finding of the study was the identification of intracellular proteins in diabetic groups which could be related to the intraocular hemorrhages in the diabetic conditions and release of erythrocyte components in to the vitreous and thus might have a role in the PDR pathogenesis (Gao, Chen, Timothy *et al.* 2008).

Schori *et al* 2018, have compared vitreous proteome of wet AMD, dry AMD, ERM and PDR by nano HPLC-MS/MS method of identification and quantified based on their intensities. They have identified a total of 1162 proteins and the total quantifiable proteins were 677. Hierarchical clustering of proteins groups recognized many common clusters of proteins between dry and wet AMD, whereas in PDR overall protein content were high and highly upregulated protein clusters were seen. Among differentially expressed 142 proteins in PDR 84 were upregulated and 58 were downregulated proteins. Hemoglobin subunit b (HBB), HEM2 and carbonic anhydrase 1 (CAH1) were the top three upregulated proteins in PDR. The pathway analysis done between each group to get the top enriched class of proteins identified enrichment of oxidative stress pathway proteins for dry AMD, focal adhesion protein pathways in neovascular AMD and enrichment of proteins associated with the complement and coagulation pathway in case of PDR (Schori, Trachsel, Grossmann *et al.* 2018). Studies were also done to understand the differences in proteome profile between PDR and DME. Such a study done by Hernandez *et al* 2013 have compared the proteome between DME with and without PDR in comparison to that of non- diabetic idiopathic macular hole

cases (IMH) by differential gel electrophoresis (DIGE) followed by MALDI. They identified 25 intravitreal proteins including β-crystallin S, clusterin and transthyretin that were found to be significantly downregulated and hemopexin was significantly upregulated in DME group compared with PDR and controls. In case of PDR, 6 differentially expressed proteins were observed compared with DME and IMH, including upregulated proteins such as β2-glycoprotein (ApoH), gelosin, vitamin D binding protein and downregulated proteins such as IRBP, Metalloproteinase inhibitor-2 and prostaglandin H2 D-isomerase. Even though the protein profiles revealed differences in PDR and DME group, both the groups had shown a similar changes in certain proteins such as upregulation of Complement C1q subunit c, complement C4A, FGβ- chain, FGγ- chain and glutathione peroxidase 3 compared with the control IMH group (Hernandez, Garcia-Ramirez, Colome *et al.* 2013). Another study done by Kim *et al* in 2007 profiled vitreous proteins of the PDR cases compared with the macular hole as controls using methods such as IS/2-DE/MALDI -MS, nano-LC-MALDI-MS/MS, and nano-LC-ESI-MS/MS. A total of 415 proteins were identified in PDR, and 346 proteins in controls. Most important proteins identified included hepatocyte growth factor activator, kallistatin, thioredoxin, Wnt inhibitory factor, secreted frizzled-related protein, von Willebrand factor (vWF) and chromogranin. These proteins play a major role in immune system process and response to stimulus (Kim, Kim, Kim *et al.* 2007). Further quantitative estimation of proteins in PDR and NPDR compared to control MH vitreous with Multiple Reaction Monitoring (MRM) method revealed increase in the expression of proteins such as thyroxine binding globulin, kallistatin, vWF, hepatocyte growth factor activator, and glyceraldehyde 3- phosphate dehydrogenase. (Kim, Kim, Yu *et al.* 2010).

Shitama *et al* 2008, compared vitreous proteome between PDR with vitreous proteome of patients of MH and fibro retinal membrane. This study identified a significant increase of number of proteins in the PDR vitreous with the fibrinogen-γ showing a 173-fold increase in PDR. Several other proteins such as α-1-B-glycoprotein, complement component C3 and vitamin D-binding protein also showed significant increase in PDR vitreous. Further comparison of NPDR and DME vitreous proteome with retinal detachment (RD) vitreous identified a significant upregulation of complement C3 in NPDR cases (Shitama, Hayashi, Noge *et al*. 2008).

The most extensive analysis of vitreous proteome in DR was performed by Loukovaara *et al* 2015 in a total of 138 vitreous samples. In addition to comparing the proteome between PDR and non-PDR, they also studied the change in proteome after treatment with anti-VEGF agents: bevacizumab and avastin. The study analyzed 74 PDR vitreous, 49 non-PDR vitreous, 5 PDR and 10 non-PDR vitreous after the treatment with anti-VEGF agent. A total of 2482 proteins were totally identified in the study from all the groups and majority of them were found to be extracellular or transmembrane proteins. Further analysis of the proteome using label free quantitation revealed a significant difference in 238 proteins between non-PDR and PDR groups of which 230 proteins were upregulated in PDR groups. These included proteins involved in inflammation, complement activation, blood coagulation, protease inhibitor, apolipoproteins, IgGs and cellular adhesion molecules. Further, the quantification of complement and coagulation proteins had shown a clear-cut increase of these proteins in the PDR vitreous compared to the controls. The comparison of proteome between PDR and post-VEGF PDR vitreous identified downregulation of 72 abundant proteins of cell adhesion, insulin signaling, cilia, ATPase activity, apolipoproteins, immune response,

crystallin, peptidase UBPS1, cellular transport and apoptotic proteins in PDR after anti-VEGF treatment (Loukovaara, Nurkkala, Tamene *et al.* 2015). Chen *et al* 2018, in order to understand the molecular mechanism of anti-VEGF treatment in PDR, compared vitreous proteome between PDR with and without anti-VEGF ranibizumab (IVR-intravitreal ranibizumab group). The study identified 654, 600 and 586 proteins respectively in PDR, IVR and non-DM group. Of these 72 proteins were differentially expressed between PDR cases compared to DM and 339 proteins differentially expressed in IVR groups compared to PDR. The Gene Ontology (GO) annotation of these 339 proteins identified in the IVR included major proteins in the innate immune response, complement activation, platelet degranulation, endocytosis, apoptosis, proteolysis and heme scavenging system, indicating thereby that anti VEGF therapy affects these signaling pathways in eye. Out of these 339 proteins, 66 proteins were found to be decrease and 42 proteins were found to be increase in its expression. Go annotation further revealed that the downregulated proteins are mainly the proteins of platelet degranulation and upregulated ones were involved in immune response. Thus, they clearly demonstrated that the anti-VEGF therapy not only reduce the VEGF level, but also acts on other important contributors of PDR such as platelet degranulation, apoptosis, inflammation and so on (Zou, Han, Zhao *et al.* 2018). Similar analysis of another anti-VEGF agent conbercept in the PDR vitreous revealed, the intravitreal anti-VEGF differentially regulated proteins of the immune response, platelet degranulation, inflammation and complement activation, supporting the previous vitreous proteome analysis (Zou, Zhao, Yu *et al.* 2018). The major vitreous proteome analysis done in DR vitreous studies are given in the Table 2.4.

Table 2.4: Major proteome studies done in DR vitreous

Strategy used	Case / Control	No. of proteins identified	Major upregulated proteins in cases	Major pathways in cases	Ref.
2D, MALDI-TOF	PDR=15, MH =15	Total-25	Acute phase reactant proteins, PEDF	Not defined	(Kim et al., 2006)
DIGE+ MALDI-MS TOF	PDR=8, MH=10	Total=11	ZAG, Apo A1, Apo H, fibrinogen A, and the complement factors C3, C4b, C9 and factor B	Complement classical pathway	(Garcia-Ramirez et al., 2007)
SDS-PAGE, nano-LC/MS/MS	PDR=7, DM+ No DR=4, NDM=6,	Total-252	Angiotensinogen, CFI, C3, CFB, serum albumin, APO A-II, APOC3, Transthyretin, CA- 1, HBA1, HBD A1BG, Vitamin D-binding protein, IGHG1 protein	kallikrein-kinin, coagulation, and complement systems	(Gao et al., 2008)
2D DIGE, MALDI-TOF MS, Western blot	PDR=10, Corneal transplant=10	Total-29	CA, DDAH, ACOT1, gamma-enolase, and MDH, tubulin alpha-1B chain, gamma-enolase	Not defined	(Wang et al., 2012)
(RP-HPLC)-ESI-MS/MS, Western blot	PDR=8, Corneal transplant=8	Total -96	angiopoietin-related protein 6, apolipoprotein A-I (Apo A-I), estrogen receptor alpha (ERα, ESR1), tubulin	Glycolysis/gluconeogenesis, complement and coagulation cascades, gap junction, and phagosome pathways.	(Wang et al., 2013a)
LC-MS/MS	PDR-5, MH=5	Total -57	Fibrinogen, prothrombinC4a, CFB, CFI, Histidine-rich glycoprotein, Isoform LMW of kininogen-1, Isoform HMW of kininogen-1	coagulation, complement and kallikrein-kinin systems	(Balaiya et al., 2017)
EASY-nLC II system, LC-MS/MS	PDR=9, IMH=9	Total-610, PDR-398. MH-546	APOF, CA1, catalase (CAT), collagen type I alpha 1 chain (COL1A1), C2, C4 binding protein alpha (C4BPA), CFD, CFHR3, CFH, FGA, paraoxonase 1 (PON1) and vitronectin (VTN)	complement and coagulation cascades	(Li et al., 2018)
DIGE, MALDI-MS TOF, Western blotting	PDR=4, MH=8	Not mentioned	Apo-A1, Apo-H upregulation in PDR	Not defined	(Simo et al., 2008)

Even though, several studies were done to catalogue the profile of proteins involved in PDR, a large number of proteins involved in disease pathogenesis are yet to be identified due to the underlying complexity of this metabolic disease. Also, since most of the studies used abundant protein depletion method to enrich the less abundant proteins, however, these approaches can cause the loss of low abundant but crucial proteins involved in disease pathogenesis. While most of the proteomic studies compared PDR with age matched non-diabetic controls, very few studies addressed the proteome difference between PDR and diabetic no retinopathy, which can clearly elucidate the diabetes induced change in the vitreous. Hence, we wanted to study the proteome profile of PDR, diabetic and non- diabetic vitreous samples with maximum number of protein identification, as that may provide the list of novel proteins or mechanisms involved in this complex metabolic disease. Further, the pathway analysis of observed proteins in previous studies identified upregulation of immune system pathways predominantly complement system pathway proteins in PDR vitreous. However, a detailed understanding the role of complement pathway in PDR pathogenesis is not done. Thus, in the present study, we decided to do a detailed analysis of role of immune system pathway in diabetic retinopathy progression.

2.9. Innate immune system of the retina

Retina is an immune privileged tissue in the body, and it is separated from the systemic attack of immune cells by the blood retinal barrier (BRB), comprising of inner BRB formed by vascular endothelial cells with tight junctions and outer BRB formed by RPE. This strong physical barrier protects the retina from the attack of any systemic immune cells. Also, BRB protects the retina by segregating retinal antigens from the systemic circulation and protects

the retina from T cell activation, known as immunological ignorance (Avichezer, Grajewski, Chan *et al.* 2003). Hence the immune response and consequent inflammatory response are mainly mediated by the localized innate immune responders in the retina.

Diabetes, causes an overall change in the body metabolism and most of the diabetic complications often show an association with hyper activation of innate immune cells. Diabetic retinopathy, widely accepted as a chronic neuroinflammatory disorder and the involvement of local immune regulators and immune defense system play a role in disease pathogenesis, where the exact role of these regulators and mediators are not well studied. Neurons and RPE are the major immune regulators of the retina, whereas the immune defense system comprised of microglia, perivascular macrophages and the complement system (Xu and Chen 2017). The cellular response in a retinal microenvironment is often under the monitoring of tissue immune defense system, which in turn gets activated under these conditions and enhance the release of cytokines or other molecules to restore the homeostasis (Chen and Xu 2015). However, prolonged condition of diabetes, excess activation of these immune system components enhances inflammation in the retina and can cause chronic neuroinflammatory damage.

2.12.1 Microglia and retinal defense mechanism

The phagocytic cells in the CNS, i.e. microglia are the key immune defense system and major source of cytokines and complement proteins (Walker, Kim and McGeer 1995). Multiple signals released from the retinal neurons activate microglia under varied conditions. The crosstalk of neurons and microglia plays a critical role in the regulation of neuroimmune response. CXC3CL1 (fractalkine), endocannabinoids, nerve growth factor and transforming

growth factor (TGF β) are some of the neuronal signaling molecules which activate microglia under varied conditions and controls immune related and migratory functions of microglia (De Simone, Ambrosini, Carnevale *et al.* 2007, Stella 2009, Zhang, Xu, Liu *et al.* 2012). Neural dysfunction in diabetic retinal environment can cause exaggerated microglial activation. The excessive activation of microglia followed by a retinal damage is known to create inflammatory environment in the retina as a part of its defense mechanism. Under altered retinal microenvironment, microglial cells undergo a phenotypic transition to an activated amoeboid shape, followed by proliferation and migration into the injured or damaged area. This morphological and functional transition of the microglia induce release of various cytokines like IL-6, IL-8, TNF-α etc, which further ameliorate tissue damage with a chronic inflammatory response under prolonged duration of insult (Ramirez, de Hoz, Salobrar-Garcia *et al.* 2017). Zeng *et al*, proposed an association between microglial activation and neuronal apoptosis in diabetic retina using STZ induced mice model of diabetes. In these mice, microglia were found to be hypertrophic after 1-month onset of diabetes and increased in number. At 4-6 weeks of diabetes, the number of hypertrophic microglia reached in its peak and were found to be localized in the GCL and IPL of the neuronal retina with an associated decrease in neural cell density. At 14-16 weeks of diabetes, microglia were found to be dispersed in the ONL with a considerable damage in photoreceptors. The results of this study clearly demonstrated that activated microglia under diabetes enhanced neuronal death in the inner and outer layers of the retina (Zeng, Ng and Ling 2000).

2.12.2 Complement system in retinal defense mechanism

The complement system is the body's natural defense system and is the major defense system present in the retina. Complement system activates through three different pathways, namely classical pathway, alternative pathway and lectin mediated pathway. The network of proteins in the complement system act as an immune modulator and plays an important role in modulation of immune and inflammatory responses (Yanai, Thanos and Connor 2012). There are almost 40 complement proteins and complement regulators are involved in the complement pathway **(Figure 2.7).** The trigger for the classical pathway is by the binding of an antigen with FC region of an antibody, whereas the alternative pathway is activated with the spontaneous hydrolysis of thioester bond in complement C3. The lectin pathway of complement needs the recognition of a lectin binding surface, which is mainly from a pathogen like microbes. All the three pathways converge in the central complement molecule C3 and activation leads to the formation of C5b-9-membrane attack complex (MAC) (Vignesh, Rawat, Sharma *et al.* 2017). The detailed mechanisms and the proteins involved in complement pathway are shown in the Figure 2. 7.

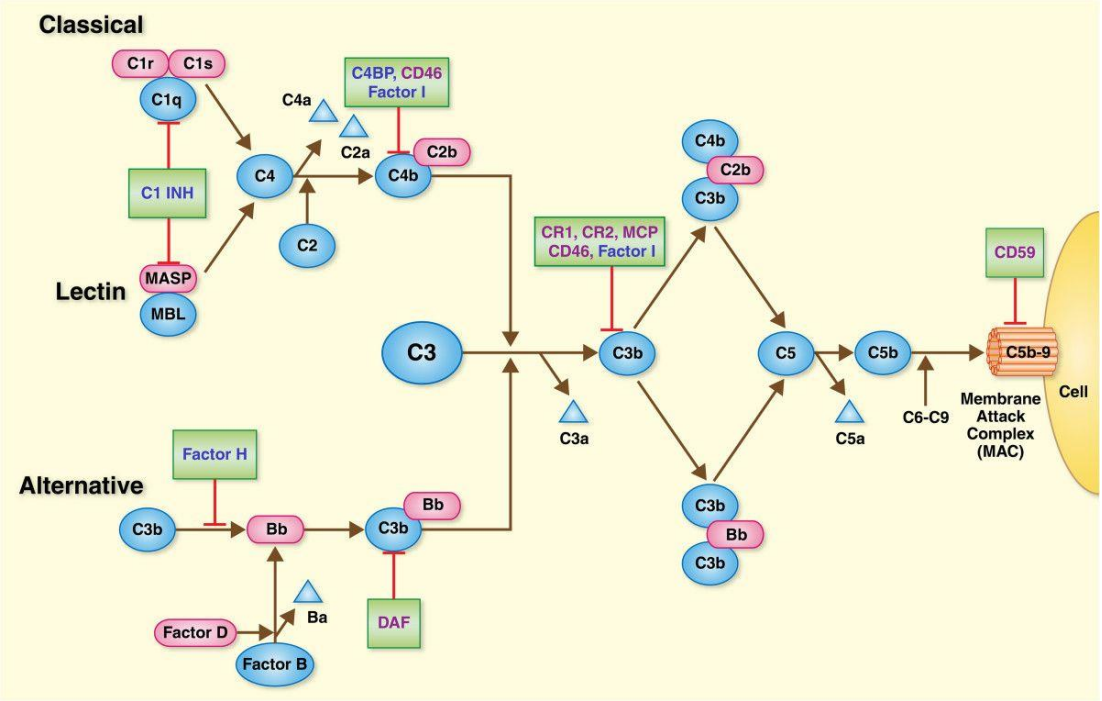


Figure 2.7: Schematic representation of complement pathway. Adapted from (Danobeitia, Djamali and Fernandez 2014)

Complement proteins in the body are mainly synthesized by the liver hepatocytes, whereas in the CNS tissue like retina, neurons and the glia form the source of complement proteins (Alexander, Anderson, Barnum *et al.* 2008). In eye, a low-level activation of the complement pathway is mandatory to retain the immune privileged state and is strictly under the control of complement regulatory proteins(Sohn, Kaplan, Suk *et al.* 2000). However, the activation of complement pathway is also dependent on the age. Chen *et al* in 2010 had demonstrated that the age dependent activation of this immune pathway in mice model. Neural retina samples taken from 3 months and 20 months old mice were subjected to microarray analysis. A significant increase of genes involved in stress response, glycoprotein synthesis and regulation of biological process were identified in adult mice. Among those, the genes of immune and

defense response such as complement activation, phagocytic pathway, chemotaxis and myeloid cell differentiation were found to be highly upregulated. Predominantly the classical pathway genes such as C1qa, C1qg, C3 and C4 and alternative pathway genes such as CFB and C3 were found to be highly upregulated in aged mice retina. Immunofluorescence in the mice retina found the deposition of the activated fragment of C3 in RPE/Bruch's membrane as well as in the ganglion cell layer to the IPL layer of the neuroretina in adult mice, whereas in young mice C3d was restricted in the RPE/Bruch's membrane. Most importantly, significant activation of microglia was observed in the adult mice, that suggested for an age depended activation of complement pathway is association with microglial activation (Chen, Muckersie, Forrester *et al.* 2010).In retinal degeneration the prolonged expression of complement proteins, predominantly C1s, C2, C4B, C4, and C3 were reported. The immunolocalization of the central complement C3 recognized microglia as the prime source of complement in retinal degeneration and these C3 expressing microglial cell were shown to be abundant in retinal vasculature and in the degenerative photoreceptor layers (Rutar, Natoli, Kozulin *et al.* 2011). Likewise, altered immune response and complement activation by microglia contributes significantly in the progression of neurodegenerative disease like Alzheimer's. (Cai, Hussain and Yan 2014).

The RPE and microglia are the key source of complement alternative and classical pathway of proteins. The effects of inflammatory cytokines on the complement activation were studied after treating RPE and microglia with inflammatory cytokines such as TNF-α, IFN-γ, and IL-6. This study identified an upregulation of C2 in RPE and C1, CFB and CFH in microglia after treatment with IFN-γ and down regulation of CFH in RPE and microglia by TNF-α. TNF-α was found to induce C3 gene expression in RPE and microglia, whereas IFN -γ

induced C3 gene expression only in microglia. This study clearly identified that the prime source of retinal complement proteins RPE and microglia and alterations in retinal microenvironment activate complement pathway (Luo, Chen and Xu 2011). The staining of complement proteins such as C1q, C3b and MAC in the inner retinal layers in glaucomatous eyes, suggested classical pathway of complement activation in glaucoma (Tezel, Yang, Luo *et al.* 2010). Howell *et al*, have also shown the upregulation of complement cascade at early stage in mice model of glaucoma (Howell, Macalinao, Sousa *et al.* 2011).

Over the past decade, the role of complement cascade in retinal pathology was extensively studied in age related macular degeneration. The analysis of the molecular composition of AMD drusen identified inflammatory components and further detailed investigation of these revealed several complement proteins such C3, C5b-9, CFB, and CFH to be the major constituents of these deposits (Anderson, Radeke, Gallo *et al.* 2010, Johnson, Leitner, Staples *et al.* 2001). The genetic investigation on role of complement genes in AMD pathogenesis also showed association of polymorphisms in complement genes such as (serpin family G member 1) SERPING1, C3, CFH and CFB with an increased risk of AMD (Cipriani, Leung, Plagnol *et al.* 2012). The tyrosine-402 > histidine-402 polymorphism of CFH gene (Y402H) was found to be strongly associated with AMD pathogenesis (Narayanan, Butani, Boyer *et al.* 2007).

Similarly, the aberrant activation of complement pathway was reported in premature babies affected by Retinopathy of Prematurity (ROP), characterized by abnormal retinal neovascular growth in the retina (Rathi, Jalali, Patnaik *et al.* 2017). A significant association of Single Nucleotide Polymorphisms (SNPs) in the CFH and CFB genes and complement activation was suggested based on the comparison of the level of activated fragments of complement

C3 among the vitreous of ROP babies and controls. The study clearly identified multiple activated fragments of C3 in ROP vitreous whereas in control vitreous only the intact C3 was present. A cytospin analysis of the vitreous further identified infiltration of CD68 positive phagocytic microglia in the ROP vitreous only. Subsequent invitro studies using microglial cell line revealed microglia to be the source of activated complement proteins in ROP. Thus, this study suggested that complement activation mediated by microglia plays a major role in abnormal neovascularization in premature babies (Rathi *et al.* 2017).

2.12.3 Dysregulated Complement pathway in diabetes and diabetic retinopathy

The excessive activation of complement pathway was shown in number of diabetes associated complications, that included cardiovascular diseases, diabetic nephropathy, diabetic neuropathy and so on (Falk, Sisson, Dalmasso *et al.* 1987, Kostner 2004, Rosoklija, Dwork, Younger *et al.* 2000). The analysis of complement activation at systemic level among insulin dependent and independent diabetes was performed by Morimoto *et al*, in 1988 by comparing the complement titer, activation product and complement inhibitors. The study identified increased expression of complement activation products such as C3 and C4 in diabetic patients, especially C3d, a cleaved product of C3 was found to be more in insulin dependent diabetes and ACH50, which is a measurement of alternative pathway of complement, in insulin independent groups compared to control subjects (Morimoto, Taniguchi, Yamashiro *et al.* 1988). Fujita *et al* in 2013, correlated the systemic complement activation with microvascular abnormalities in diabetic patient's eyes. The major complement proteins such as C3, C4 and Factor B and their activated products iC3b/C3 and Bb/Fb were found to be significantly elevated in the plasma samples of diabetic patients compared to controls.

Further, retinal examination identified retinopathy changes in patients with high level of systemic complement activation, suggesting a pathological role for complements in DR (Fujita, Hemmi, Kajiwara *et al*. 2013).

Very few genetic studies were also done to understand the complement genes in DR development. Xu *et al*, studied the association of polymorphisms in C5 gene in type-2 diabetes PDR patients. Of the total 4 SNPs screened in PDR, NPDR and diabetic control patients, a significant association in C5 rs2269067 polymorphism was observed with DR development. Further, analysis of this association with cytokine production identified a significant production of IL-6 in patients with C5 rs2269067 polymorphism. But they did not show how this C5 SNP is causing risk for DR development was not explained (Xu, Yi, Yu *et al*. 2016). A study done by Yang *et al*, also looked into the association of complement genes with susceptibility to DR development and identified only a marginal association between C5 SNP rs17611 with DR (Yang, Wang, Ren *et al*. 2016). Another genetic screening study done by Wang *et al*, have looked into the association of CFH and CFB gene with diabetic retinopathy development. This identified a significant association two SNPS such as rs1048709 of CFB and rs800292 of CFH with DR (Wang, Yang, Li *et al*. 2013). But the effect of the mutant genotypes on the respective protein expression and pathological significance is not yet uncovered. While various proteomic studies have independently have revealed an elevated deposition of complement protein in the diabetic retinal and vitreous specimens.

A study by Gerl *et al*, in 2002, identified an extensive deposition of C3d and C5b-9complex in the retinas of diabetic retinopathy in the choriocapillaries and Bruch's membrane which was absent in both control and diabetic retinas. The vitronectin, needed for the stable C5b-9 complex were also found in DR eyes and was localized within choroid and Bruch's membrane.

The absence of CRP and classical complement proteins in these eyes further suggested activation of alternative pathway of complement in DR (Gerl, Bohl, Pitz *et al.* 2002). Followed by this Zhang *et al*, found the membrane attack complex MAC staining were found to be localized in the small-medium sized vessel region in retinopathy eyes that was in strong contrast with negligible or punctiform staining of retinal vessels in controls. Further the level of glycosylphosphatidylinositol anchored (GPI), CD55 and CD59 were found to reduce significantly in diabetic retinas compared to control retinas but the level of transmembrane inhibitor of complement CD46 was found to be similar in all retinas (Zhang, Gerhardinger and Lorenzi 2002). A significant increase in the systemic level of glycosylated CD 59 (GCD59) was also noticed for diabetic patients suggesting thereby that GCD59 can act as a marker for complement mediated diabetic complications (Ghosh, Sahoo, Vaidya *et al.* 2013).

Other than retina, excessive deposition of complement C5b-9 complex was also noted in renal tissues of 60% of the diabetic patients, an and found to be associated with glycated inactivation of CD59 inhibitor (Qin, Goldfine, Krumrei *et al.* 2004). Complement mediated damage of the retinal pericytes under diabetes were shown in cultured retinal pericytes by treating with high glucose. The cytotoxicity measurement identified a significant damage of the high glucose treated retinal pericytes when they were exposed to sera containing complement proteins compared to the complement proteins depleted sera. Thus this study clearly shown a mechanism of the retinal vessel damage by pericyte loss due to complement activation under high glucose conditions (Li, Smith, Li *et al.* 2012). All these studies, thus markedly pointing towards the altered innate immunity in diabetic environment, however a clear understanding of the same is still not lacking subject to a comprehensive evaluation of the human biological material.

2.12.4 Role of complement in retinal neovascular complications

Beyond its role in retinal defense mechanism, studies have also shown the ability of complement in tissue remodeling and in morphogenetic process. In case of choroidal neovascularization, the deposition of C3, MAC complex and upregulation of angiogenic cytokines such as VEGF, TGF-β2 and BFGF (basic fibroblast growth factor) were found in the neovascular complex of laser induced CNV (choroidal neovascularization) mice. But C3 (C3-/-) knock out mice failed to develop CNV and the depletion of complement reduced the level of angiogenic factors in the retina, indicated complement enhanced neovascularization in pathological conditions (Bora, Sohn, Cruz *et al.* 2005). In another study, complement was shown to have a protective role in oxygen induced retinopathy (OIR) mice model by preventing abnormal neovascularization. In these mice, C3 depletion was found to enhance pathological retinal vascularization. Following this Sweiggard *et al* studied the role of alternative complement pathway in retinal neovascularization using Factor b knock out ($Fb^{-/}$) OIR mice. The knock out mice tend to develop more neovessels and delay in regression of neovessels. Also, the same study had shown hypoxia induced reduction of complement inhibitor CD55, enhanced neovessels clearance through the activation of alterative pathway of complement. But Fb depletion arrested this clearance process by preventing alternative pathway of complement activation (Sweigard, Yanai, Gaissert *et al.* 2014).

Various proteomic studies done in DR had clearly shown the presence of complement proteins and upregulation of complement and coagulation pathways in the vitreous samples of DR. Gerl *et al* showed the complement C3d deposition in choriocapillaries and also in age matched control retina also. Likewise, a recent study published by Chirco *et al* in 2015, had

shown deposition of complement and membrane attack complex as a mechanism during normal aging to maintain retinal integrity (Chirco, Tucker, Stone *et al.* 2016). In this scenario, it is not clear whether the complement deposition in diabetic retina is having specific contribution to abnormal neovascularization, or it is a mechanism to maintain the retinal integrity while aging. Hence a detailed investigation on DR and role of complement activation is needed as that might provide a novel therapeutic invention for DR treatment.

2.13 Role of neuroglial interactions in retinal homeostasis:

The term neuroglia was proposed by Rudolf Virchow in 1858 to describe the significant interaction of neurons and glia in the CNS (Parpura and Verkhratsky 2012a). The coupled action of neurons and glia is mandatory for the proper functional outcome in CNS.

2.13.1. Glial activation and neurovascular damage in diabetic retina

Glial population in the retina plays a critical role in retinal homeostatic balance and abnormal glial activations cause numerous irreversible degenerative changes in the retina. Since glial cells plays a critical role in neurovascular coupling, various studies have carried out by different groups to address diabetes induced changes in glial function and their role in neurovascular damage in the diabetic retina.

2.13.2 Role of macroglia in retinal damage in DR

Retinal astrocytes and Müller glia are the macroglial population in retina and they undergo enormous morphological and functional changes during the progression of DR. Astrocytes, a major cell type, involved in maintaining the integrity of BRB were found to be reduce in number during the initial phase of diabetes, a mechanism probably enhancing the leakage and

breakdown of BRB (Mogi and Horiuchi 2011). Barber *et al*, studied the effect of diabetes on glial cells morphology at the BRB and associated alterations in vascular endothelial cell tight junction proteins in 2 and 4 weeks of streptozotocin induced diabetic rats with age matched controls. Analysis of glial gibrillary acidic protein (GFAP), a major protein present in retinal astrocytes was evaluated at 2 weeks and 4 weeks of diabetes in the entire retina. At 2 weeks of diabetes, astrocytes undergo morphological alterations such as hypertrophy with enlarged cell bodies and multiple process with reduced expression of GFAP protein followed by the drastic loss of them at 4 weeks. On the contrary, GFAP expression found to be increased in the deeper layer of Müller glia at 2 and 4 weeks of diabetes. Müller cells are known to express GFAP, while undergoing gliosis along with its own cellular marker, Glutamine Synthetase (GS). This clearly indicated diabetes induce a differential pattern in GFAP expression in macroglial population, by an early phase of astrocyte loss and Müller gliosis. The analysis of the tight junction proteins occludin in these mice further revealed loss of this proteins in the outer and inner capillary layers compared with controls. This observation suggested that the loss of retinal astrocyte alters the tight junctional proteins in the inner retinal layers with an enhanced vascular permeability in diabetes (Barber, Antonetti and Gardner 2000). Later, a study evaluated the time course changes in astrocyte and tight junctional protein connexin 26 in retina in diabetic mice at 2, 4, 6 and 10 weeks of STZ injection. This study clearly suggested that diabetes induced HIF-1α activation in the retina caused reduction in astrocyte number and connexin-26 proteins at 6 weeks of diabetes. Further, these changes in retina caused a decline in neuronal function especially the RGC, that are in close proximity with astrocyte in retina.

The mechanism of phenotypic alteration in astrocyte and reduction in number during diabetes was studied by Shin *et al*. Astrocytes when grown under high glucose condition for a period of 5 days showed no change in apoptosis, but an increased proliferation rate and GFAP expression. Further, these had significant reduction in the network formation under high glucose condition. Additionally, the capillary morphogenesis by endothelial migration had also reduced drastically in the presence of these high glucose treated astrocytes. These cells were found to produce inflammatory cytokines such as TNFα and IL-1β and shown more anchored with ECM proteins, which caused inflammation with reduced migration of them for network formation (Shin, Huang, Gurel *et al*. 2014).

Alterations of Müller glia functions under any pathological condition also affects retinal neurons. One of the major functions of this macroglial population is to prevent neurons from the toxic effect of excitatory amino acids like glutamate which is mainly regulated GLAST transporter present in Müller glia. The altered microenvironment of diabetes affects GLAST transporter in Müller glia and causes glutamate excitotoxicity, which is lethal for retinal neurons (Ng, Zeng and Ling 2004). Elevated level of the excitatory amino acids such as glutamate and Gamma Amino Butyric Acid (GABA) along with an upregulation of VEGF was observed in the vitreous samples of patients with proliferative diabetic retinopathy (Ambati, Chalam, Chawla *et al*. 1997). The upregulation of these amino acids indicated the defects in the GLAST transporter systems. Müller glial cells isolated from the diabetic rats after 4 and 13 weeks of diabetes were evaluated for the efficiency of GLAST transporters electrophysiologically using patch-clamp method. This study identified the dysfunction of GLAST transporter after 4 weeks onset of diabetes and their activity reduced to 67% at 13 weeks of diabetes (Puro 2002).

A major contributing factor for glutamate excitotoxicity in diabetes is due to reduced functioning of glutamate synthetase enzyme, which is essential for recycling of glutamine as an essential neurotransmitter. Leith *et al* have demonstrated glial reactivity and glutamate excitotoxicity in streptozotocin induced diabetic rat model after 1 and 3 months of diabetes. The study revealed no change in glial reactivity after 1 month of diabetes, whereas a 5-fold increase of GFAP was observed at the beginning of 3 months of diabetes. The evaluation of association of glial reactivity and glutamate synthetase activity identified 65% reduction of glutamate to glutamine conversion in diabetic rats compared to controls and accumulation of glutamate in the diabetic retina. This suggested that diabetes cause glial reactivity which affect the enzymatic activity of glutamate synthetase and lead to glutamate excitotoxicity and retinal neuronal damage (Lieth, Barber, Xu *et al.* 1998).

Altered the membrane conductance of K^+ in Müller glia under diabetes also shown to induce toxicity in the retina. Kir4.1 is the most important K^+ inward channel present in the Müller glia. Hence, the effect of diabetes on Kir4.1 channel was analysed by immunostaining. A polarized arrangement of Kir4.1 channel on Müller cells was seen in control retinas, while an overall decrease of Kir4.1 protein and altered distribution of these protein in the Müller glia was noted for diabetic retinas found. Thus it was proposed that the alteration of K^+ shuttling due to altered microenvironment of diabetes caused swelling of the Müller glia, which could be one of reason for diabetic macular edema (Pannicke, Iandiev, Wurm *et al.* 2006).

VEGF is a potent angiogenic molecule and in the retina, it is mainly secreted by RPE, endothelial cells, astrocytes, RGC and Müller glia. The VEGF secreted by Müller glia is a potent contributor of neovascularization in all type retinopathy, which was well proved using

conditional Müller glial VEGF knock out mice models. The conditional deletion of VEGF in Müller glia caused a significant reduction of whole retinal and vitreous level of this protein compared to wild type mice, without any defects in choroidal and retinal vascular development. In order to understand the role of Müller glial derived VEGF in neovascularization, the mice were subjected for oxygen induced retinopathy (OIR). There was a significant reduction of neovascularization (40.3%) and vaso obliteration (29.6%) in knock out compared to wild type with OIR. This signifies Müller glia derived VEGF is a major contributor of neovascularization in abnormal ocular angiogenic conditions (Bai, Ma, Guo *et al.* 2009). Further to this the level of HIF1-α and VEGF were evaluated in these mice and found reduction of VEGF and upregulation of HIF1-α in knockout mice compared to control, which confirmed VEGF knock out did not alter the HIF-1 αaccumulation in diabetes. A 3-fold increase was also seen in retinal leukostasis in wild type diabetic mice but not in control and KO diabetic mice. The ICAM -1 and TNF-α levels were increased in diabetic mice but not in diabetic KO mice and non -diabetic controls. This signified Müller glia derived VEGF as a major contributing factor for retinal inflammation under diabetes by the transcriptional activation of NF-κB (Wang, Xu, Elliott *et al.* 2010).

2.13.3 Role of microglia in retinal damage in DR

Microglial activation is one of the major prominent features of DR and it is very rapid and often precedes before to macroglial activation and neurovascular changes. A systematic investigation on effect of diabetes on neurovascular changes in alloxan induced diabetic mice model demonstrated that diabetes primarily affects retinal microglial population. In these mice, decline in retinal b-wave/-a-wave were noticed in 3 months of diabetes without any

vascular changes. while there was no evidence of macroglial activation, they found microglia with phenotypic changes such as enlarged cell bodies, thick processes and shorter dendrite of activated amoeboid type in the inner retinal layers indicating that microglial activation is the prime event in diabetic eye and was the prime reason for retinal neuronal damage (Gaucher, Chiappore, Paques *et al.* 2007). The spatiotemporal characterization of microglia based on their density, morphology and laminar distribution 4 ,8 and 12 weeks in streptozotocin induced diabetic rats also identified slightly hypertrophied morphology of microglia at 4 weeks and more hypertrophic and amoeboid in 8 and 12 weeks of diabetic duration. However, in diabetic retina, the number of microglial cells were found to be increased gradually and at 12 weeks of diabetes their numbers became found to be significantly higher in the GCL and reduced in IPL. These observations along with microglial mRNA expression further suggested that in a diabetic milieu, microglia undergo a morphological alteration and distribution pattern without much change in the total density and these further contribute to disease progression in early diabetic state (Chen, Zhou, Gong *et al.* 2015).

The degree of activation of microglia at various stages of DR had shown to be varied in human diabetic retinopathy, i.e. from a minimal amoeboid shape change in the perivascular region in back ground retinopathy to heavily clustered amoeboid microglia in the neovessels of NFL in PDR (Zeng, Green and Tso 2008). Unlike that of the macroglial population, these defense system players in the retina get activated even with a minor change in retinal homeostasis and release pro and anti-inflammatory cytokines and generate an inflammatory response in the neural retina. The inflammatory response elicited by the microglia play a vital role in neuroinflammation in DR. Another study by Canataroglu *et al* identified predominance of different cellular components and inflammation in different retinopathies. They measured the

level of inflammatory cytokines IL-6 and IL-8 in the vitreous of PDR, proliferative vitreoretinopathy (PVR) and traumatic PVR. 85.7% of the PDR subjects and 100% of the traumatic PVR cases had significantly elevated level of IL-6 and IL-8 in the vitreous. Whereas in PVR the level of IL6 and IL8 were significantly elevated in 80% and 60% of the patients respectively. Cytological evaluation of the vitreous samples done to understand the cellular sources of the cytokines, revealed the predominance of 50% macrophages, 40% RPE, 7% lymphocyte and 3% neutrophils in PDR vitreous. In case of PVR the mean cellular composition was 60% RPE, 35% macrophages, 3% lymphocytes, and 2% neutrophils. Likewise, in case of traumatic PVR, neutrophils predomination was 88% and 4% predominance of RPE, macrophages and neutrophils were found. This clearly identified variable cell types to induce inflammation in retinopathies and that resident macrophage is the major source of inflammation in diabetic retinopathy (Canataroglu, Varinli, Ozcan *et al.* 2005).

In order to understand the exact mechanism of elevated inflammatory response, the diabetic rats were treated with minocycline, a drug which selectively inhibit M1 type microglial activation after 3 days of STZ injection. Quantification of TNF- α and IL1 –β after 2 weeks of treatment identified a reduction of the cytokines as 50% and 40% respectively in minocycline treated groups compared to untreated diabetic rat, which suggested microglia induce inflammation in diabetes. To further elucidate the mechanism of microglia activation, minocycline pretreated primary microglial cells were treated with pro inflammatory cytokines TNF- α, IL1–β and IL-6 invitro and checked the expression of Cyclo-Oxygenase-2 (COX-2), which is one of the key inflammatory cytokines involved in early pathologies of DR. The minocycline untreated microglia showed a significant 5- fold increase of COX2 after the treatment with TNF- α, IL1–β and IL-6. But the minocycline pretreatment reduced and

completely prevented the expression of COX2 in microglia treated with TNF-α. Thus, diabetes causes the activation of microglia at an early stage, which further induce the secretion of inflammatory cytokines, that exacerbates neurovascular cell death and progression of DR (Krady, Basu, Allen *et al*. 2005). The underlying mechanisms responsible for a diabetes induced microglial activation are not completely understood yet. Evidences from animal and human diabatic retinopathy studies have shown that diabetes induce accumulation of AGE in retinal capillaries (Schalkwijk, Ligtvoet, Twaalfhoven *et al*. 1999, Tang, Zhu, Lust *et al*. 2000). In order to elucidate the role of AGE in diabetes, Amadori-glycated albumin (AGA), a major type of AGE was evaluated in the retina. Both AGA and albumin were found to be increased 4.5 and 4- fold after 8 weeks of diabetes compared to control and tend to colocalize with microglia. Table 2.5. enlisting few of the studies of glial activation in DR.

Table 2.5: Evidences of glial activation in diabetic retinopathy

Types of glia	Disease stage and duration of DM	Changes noticed	Proteins changed	Ref
Astrocyte	4 weeks of diabetes in STZ diabatic rat	Decreased cell density for early BRB breakdown	Reduced GFAP	(Rungger-Brandle, Dosso and Leuenberger 2000)
Müller glia	Rat Müller glia treated with high glucose (HG) for 48 hrs.	Upregulation of VEGF sand downregulatio n of PEDF	Increased GFAP	(Mu, Zhang, Liu *et al.* 2009)
Müller glia, Astrocyte	STZ diabatic rat after 3 months	Glutamate excitotoxicity	Increased GFAP	(Lieth *et al.* 1998)
Microglia	AGE treated microglia	NF-kB activation and Inflammation	Increased TNF α secretion	(Wang, Yu, He *et al.* 2007)
Microglia	Human DR	Hypertrophic microglia	Increased expression of OX42 or IBA-1.	(Zeng *et al.* 2008)
Müller glia	6 months old STZ induced diabetic rats	Inflammation, gliosis	Upregulation of GFAP and acute phase proteins	(Gerhardinge r, Costa, Coulombe *et al.* 2005)
Microglia	2 weeks old STZ induced diabetic rats	Retinal inflammation	TNF α, IBA-1	(Ibrahim, El-Shishtawy, Pena *et al.* 2010)

2.13.4 Neuro-glia cross talk and neurodegeneration in diabetic retina

The functional architecture of the retina is dependent on the intimate partnership and cross talk between the neurons and glia. The proper alignment and activation of glial cells with respect to the neuronal activity and vice versa is mandatory for retinal homeostasis. Some of

the major highlight of this cross talk are involvement of glia in synaptogenesis, synaptic transmission of signals, removal of toxic biproducts of neurotransmission and protecting retina from the abnormal retinal microenvironment and so on. This cross communication between the cell types are mainly maintained by various soluble factors such as neurotransmitters, trophic factors, hormones, etc. secreted by either glial or neuronal cells (Gomes, Spohr, Martinez *et al.* 2001).

Similar to that of neuron- glia communication, the cross talk between glial cell types also cause important impact in the retina. For example, it was shown that, the cross talk between the glial cell types drive secretion of many inflammatory cytokines which are detrimental to the retinal microenvironment. The interplay between Müller and microglial population in retinal inflammation during diabetes was studied in diabetic mice model. The transgenic diabetic mice with Müller glial CD40 expression was found to induce cytokines such as TNF-α, IL-1β, ICAM-1, NOS2 also leukostasis and capillary degeneration in the retina. Further analysis revealed CD40 expressed Müller glia are not the source of retinal TNF-α and IL-1β in these mice (Portillo, Lopez Corcino, Miao *et al.* 2017). Various factors known to be involved in neuro-glial interaction is given in the table 2.6

Table 2.6. List of factors involved in neuro-glial communication in retina

Signaling molecules	Produce r cells	Effector cells	Functional outcome	Ref.
D-serine	Astrocyte	Post synaptic neuronal NMDA receptor	Activation glycine site of NMDA (N-methyl-D-aspartate) for synaptic plasticity	(Baranano, Ferris and Snyder 2001)
Fractalkine	Neurons	CX3CR1 on microglia	Modulation of microglial activation during normal and disease state	(Sheridan and Murphy 2013)
Glutamate	Neurons	Müller glia GLAST transporters	Synaptic neurotransmission and protection from glutamate excitotoxicity	(Lehre, Davanger and Danbolt 1997)
ATP	Neurons	Activation of purinergic receptors on glia	Proliferation, motility, survival and differentiation in response to neural impulse activity, synaptic transmission, response to nervous system injuiry, Intercellular signaling between astrocyte, oligodendrocyte, microglia	(Fields and Burnstock 2006)
ATP	Müller glia	Microglia	Regulation of microglial dynamic process motility	(Wang and Wong 2014)
Thrombospondin1, TGFβ1	Astrocyte	Neurons	Synaptogenesis	(Christopherson, Ullian, Stokes et al. 2005, Diniz, Almeida, Tortelli et al. 2012)
BDNF	Müller glia	RGC	Neurotrophic factor	(Seki, Tanaka, Sakai et al. 2005)

2.14. Ca^{2+} signaling

The second messenger calcium (Ca^{2+}), regulate many intracellular and extracellular signaling in all eukaryotic cells, which include synaptic processing, cell to cell communication, cellular adhesion and so on (Marambaud, Dreses-Werringloer and Vingtdeux 2009). Ca^{2+} undergo complex changes in their cytoplasmic concentration depends on the external stimuli (Verkhratsky and Kettenmann 1996). In neurons, Ca^{2+} act as a critical element which ties membrane excitability with biological functions of the cell due to highly developed extensive and intricate signaling pathways to couple the Ca^{2+} signal to their biochemical machinery(Bezprozvanny 2010). Neurons contain enormous Ca^{2+} dependent structures consisting of proteins involved in synapsis formation, Ca^{2+} dependent phosphatases, kinases, enzymes, transcription factors, etc. Hence, a wide variety of neuronal functions are extremely dependent and sensitive to the cellular Ca^{2+} concentrations, thereby a fine defect and dysregulation of Ca^{2+} signaling can cause destructive consequences to the neurons (Bezprozvanny 2010). Table 2.8. enlisted examples of consequences of Ca^{2+} imbalance in neurodegenerative diseases.

Table 2.7: Ca^{2+} dysregulations in neurodegenerative diseases

Disease condition	Associated change with respect to Ca^{2+}	Outcome	References
Alzheimer's disease	Increased Ca^{2+} induced activation of calpain proteases	Calpain mediated degradation of neuronal proteins	(Kurbatskaya, Phillips, Croft et al. 2016)
Glaucoma	Cleavage of Ca^{2+} dependent protein phosphatase calcineurin	RGC death	(Huang, Fileta, Dobberfuhl et al. 2005)
Diabetic neuropathy	Upregulation of T-type Ca^{2+} currents and enhanced Ca^{2+} entry into dorsal root ganglia neurons	Amplification of peripheral pain transmission by enhanced sensory neuron excitability	(Jagodic, Pathirathna, Nelson et al. 2007)
Prion disease	Altered endoplasmic reticulum (ER) homeostasis due to over expression of Ca^{2+} pump SERCA in neurons	Neuronal death	(Torres, Castillo, Armisen et al. 2010)
Amyotrophic lateral sclerosis	Chronic excitotoxicity mediated by Ca^{2+} permeable AMPA type glutamate receptors induced depletion of ER Ca2+ and mitochondrial Ca^{2+} overload	Motor neurons degeneration	(Grosskreutz, Van Den Bosch and Keller 2010)

2.14.1. Ca^{2+} in neuro-glial cross talk in retina

In retina, Ca^{2+} does a bidirectional signaling between neurons and glia and cross communication of these cells relay on the Ca^{2+} wave propagated from neurons to glia and vice

versa. Newman et al, have studied the impact of Ca^{2+} waves in neuronal and glial activity using isolated rat retina. The stimulation of end feet of retinal Müller glia or astrocyte somata by mechanical nudging triggered Ca^{2+} wave transmission from their end feet to the Müller glial process within the GCL and IPL. Following this, an increased or decreased firing rate of retinal neurons, indicated the modulation of excitatory or inhibitory neuronal activity by glial Ca^{2+} wave propagation (Newman and Zahs 1998). The light induced selective stimulation of neurons were found to induce change glial Ca^{2+} waves at various transient rate in isolated rat retinal cups. The glial Ca^{2+} propagation due to neuronal stimulation were found to be prohibited by the inhibition of ATP without change in neuronal Ca^{2+} transients, indicating that release of ATP from neurons is an important contributor to this bidirectional signaling by activating purinergic receptors in the glial cells (Newman 2005). In addition to neuro-glial communication, Ca^{2+} regulate neurovascular coupling by enhancing the release of glial vasodilating mediators. The stimulation of neurons by light excitation induced a spontaneous rise in Müller glial Ca^{2+} transients and dilation of the retinal capillaries near the Müller glial end feet. The stimulation of the Müller glia alone by electrical stimulation also caused a raise in Ca^{2+} transients and capillary dilation. This further indicated that capillary dilation acts highly in accordance with the glial activity depending on the neuronal metabolic demands (Biesecker, Srienc, Shimoda et al. 2016).

In retina, the glial cells release gliotransmitters, vasoactive agents and thereby control the neuronal activity. For example, the ATP release from the glial cells cause hyperpolarization of the RGC and reduce the spike generation in RGCs. Also, the released ATP from the glia cause retinal arteriolar constriction and modulate the vascular tone. Additionally, the stimulated glial cells release metabolites needed for neurovascular coupling arachidonic metabolites such as

PGE3 (Prostaglandin E3), 20-HETE (20-Hydroxyeicosatetraenoic acid), EETs (Epoxyeicosatrienoic acids). Thus, glial cells play a major role in modulating neuronal activity and vascular blood flow in retina (Newman 2015). Hence, the alteration in glial functions affect retinal neuronal and vascular functions, and thus exploring glial alterations in diabetic milieu, is an indirect measurement of retinal neuronal degenerative changes.

2.14.1. Glial Ca^{2+} modulation under the conditions of pathological angiogenesis:

Ca^{2+} plays a major role in high glucose induced oxidative stress and activation of angiogenic genes in retinal Müller glia. The exposure of Müller glia under high glucose condition caused a significant elevation of HIF1α (Hypoxia inducible factor 1-alpha) and VEGF. But the treatment of cells with Ca^{2+} chelator BAPTA-AM (1,2-bis (*o*-aminophenoxy) ethane-N,N,N',N'-tetra acetic acid) significantly reduced the expression of these genes under high glucose. To elucidate the pathways through which Ca^{2+} mediated cellular responses, phosphorylation of CaMKII (calmodulin dependent protein kinase II) and CREB (cAMP response element-binding protein) were evaluated under normal glucose, high glucose, normal glucose with ionophore, and high glucose with BAPTA-AM. While pCaMKII (phosphorylated CaMKII) and pCREB (Phosphorylated CREB) were elevated in Müller glia treated with high glucose and ionophore containing normal glucose, the suppression of CREB and CaMKII was found to down regulate the protein and mRNA level of HIF1-α and VEGF. This show that high glucose activates the angiogenic genes in Müller glia via the Ca^{2+} activated CaMKII-CREB pathway (Li, Zhao, Wang et al. 2012). Various studies done showing altered Ca^{2+}under conditions of hypoxia and hyperglycemia are provided in the table 2.9.

Table 2.8: Ca^{2+} alteration studies done under the conditions of hypoxia/ high glucose

Study model	Type of Stress	Changes noticed in Ca^{2+} level	Suggested outcome	References
Primary human retinal Müller glia	Supply of bFGF	Enhancement in L-type Ca^{2+} current	Proliferation of Müller glia	(Puro and Mano 1991)
Retina from STZ induced diabetic rat	Diabetes	Inhibition of voltage dependent Ca^{2+} channels in microvasculature	Alteration in retinal perfusion	(Matsushita, Fukumoto, Kobayashi et al. 2010)
Astrocyte from rat hippocampus	Hypoxia and Hypoglycemia	Enhanced voltage-gated Ca^{2+} influx	Post ischemic reactive response	(Duffy and MacVicar 1996)
Microglia and neurons from rat retina	High glucose	Augmented Ca^{2+} response in neurons through voltage sensitive calcium channels, and in microglial through P2X receptor channels	Possibility of the release of neurotransmitters and inflammatory mediators found in diabetic retinas	(Pereira Tde, da Costa, Santiago et al. 2010)
STZ induced diabetic rat retina and isolated Müller glia	High glucose	Upregulation of the calcium sensor Calsenilin	May play a role in the progression of DR	(Chavira-Suarez, Sandoval, Quintero et al. 2012)
STZ induced diabetic rat retina	Diabetes	Enhanced level of glutamate receptor and Ca^{2+} binding proteins calbindin and paravalbumin in diabetic retina	Biochemical alterations in the diabetic retina	(Ng et al. 2004)

From the studies done so far, it is very evident that studying the Ca^{2+} signaling can help to derive the neuropathological damage in the CNS. However, all these studies used either cell lines, or isolated retinal cell, or retinal eye cups from the non- human origin. While they have provided a good idea about altered Ca^{2+} in the neurodegenerative diseases, these suffer from issues pertaining to the loss of the retinal microenvironment in the isolated cells due to the lack of other cell types needed for neurovascular coupling, modification already induced by transformed cell lines, and non- human origin. Further these studies never attempted studying Ca^{2+} signaling under diabetic stress using either diabetic retinal tissues or primary retinal cell cultures under diabetic stress and evaluated the difference in Ca^{2+} signaling under hypoxia and hyperglycemia in a system containing both retinal glia (microglia, astrocyte, Müller glia) and neurons.

Thus, the present study, tried to address some of the lacunae in diabetic retinopathy research to get a clear understanding of the underlying molecular mechanisms.

3. METHODOLOGY

The present study was carried out to understand the molecular mechanisms underlying abnormal angiogenesis in the pathogenesis of proliferative diabetic retinopathy. A comprehensive strategy comprising global vitreous proteome analysis followed by a detailed systematic investigation on the role of complement pathway and microglial activation and their cross talk in PDR pathogenesis was studied in details. 1D coupled with LC-MS/MS was carried out for human vitreous proteome profiling, complement pathway and microglial activation was studied in the vitreous samples by western blotting, ELISA and validated them by immunostaining using retinal tissues collected from diabetic and non-diabetic cadaveric donors. Other than protein profiling and investigation of complement and glial role in PDR pathogenesis based on vitreous protein analysis, an *in-vitro* neuro-glial culture system was developed from human cadaveric retina. This was used to understand the neuroglial interaction under diabeteic stress and also to validate the role of microglial involvement in neovascularization and neurodegeneration in DR using immunofluorescence, measurement of intracellular calcium level by live- cell imaging, gene and protein expression analysis by real time PCR and quantitative high -resolution protein imaging.

3.1 Enrolment of study participants

The study participants (including DM, PDR/NPDR/NDR and NDM) were recruited from Smt. Kannuri Santhamma Centre for vitreoretinal Diseases, L V Prasad Eye Institute, Hyderabad, India. The study was done according to the guidelines of Declaration of Helsinki and study was approved by Institutional Review Board (IRB approval number: LEC 02-14-029). The study participants included PDR patients and non- diabetic controls (NDM) for the vitreous proteome analysis. The non-diabetic controls were collected from the subjects while

undergoing surgeries for idiopathic macular hole (IMH) and retinal detachment (RD). For global proteome analysis vitreous samples were also collected from diabeteic but no-retinopathy (NDR) subjects while undergoing surgeries for IMH. Blood samples were taken from the PDR, NPDR patients and NDM controls for serum protein analysis. Diabetic and non-diabetic retinas were collected from the cadaveric donor eye balls and complete history including age, cause of death and diabetes status, etc were recorded based on the details provided by the family members. The overall strategy used for the study are provided in the figure 3.1.

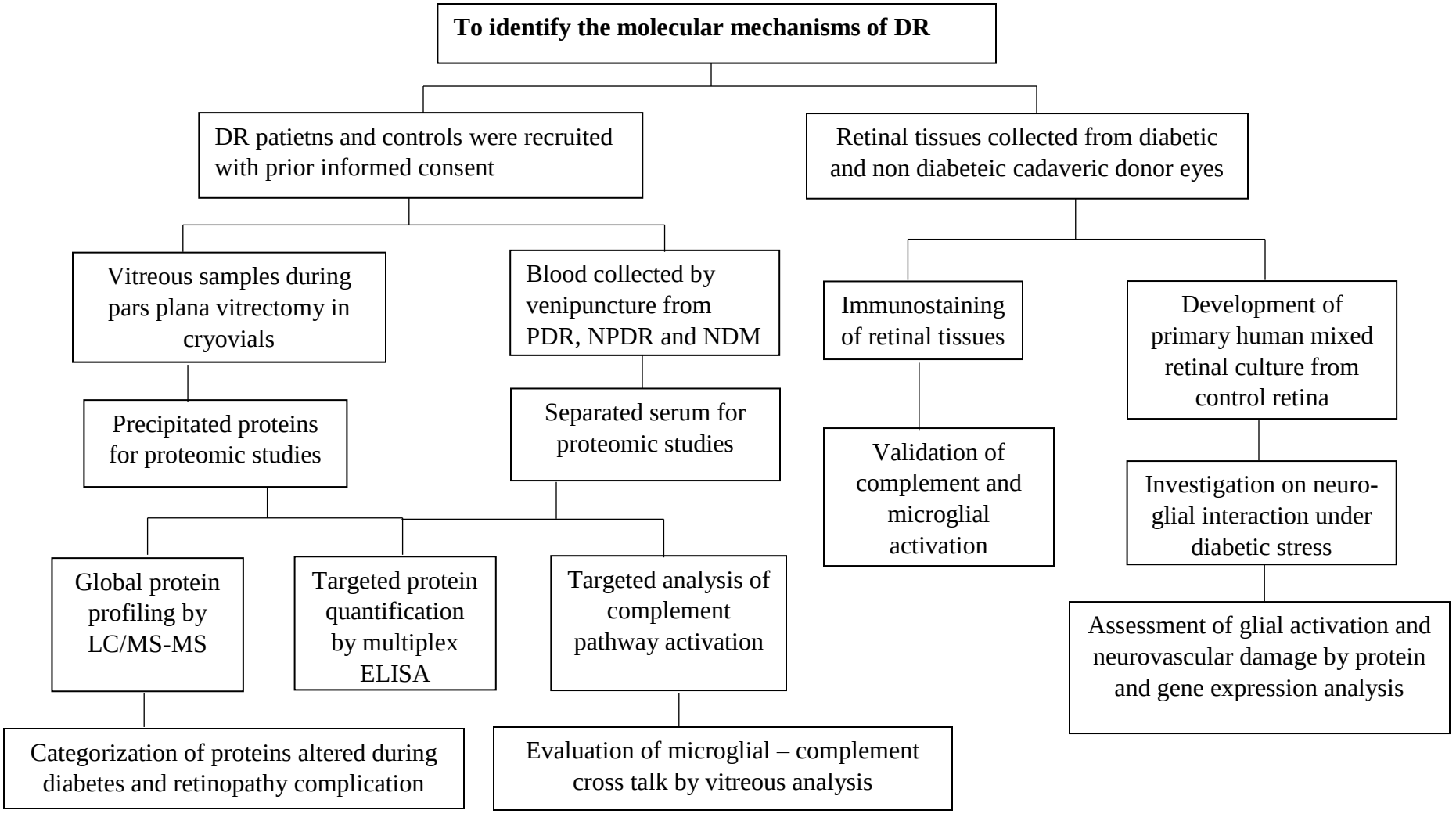


Figure 3.1. Strategies used for understanding the underlying mechanisms of Diabetic Retinopathy

3.2. Clinical evaluation of the study participants

All the participants included in the study underwent a detailed clinical examination by the ophthalmologist, that included a clear diabetic history, history of any other systemic disease, visual acuity test, dilated fundus examination by indirect ophthalmoscopy, fundus photography, Fundus fluorescein angiogram, optical coherence tomography and B-scan.

3.2.1. History

Detailed systemic and medical history of study participants was obtained from them and medical record that included:

- Dietary habits
- Life style (Smoking/Alcohol)
- Body max index (BMI)
- Diabetic history
- Treatment strategies (oral medicine/ insulin/ diet control)
- H/o gestational diabetes (Female)
- Hypertension
- Systemic diseases
- Previous ocular complications/surgeries
- H/o of anti VEGF treatment
- H/o Laser surgeries
- Best Corrected Visual Acuity (BCVA)
- Family history of diabetes

3.2.2. Visual acuity test

The ability of the eye to distinguish the fine details of an object from a specific distance was measured through visual acuity. Standardized logMAR chart or Snellen's chart was used to evaluate visual acuity. Snellen chart contains series of letters of different size arranged in rows and columns, whereas in logMAR chart, the letters are arranged in a logarithmic proportional change in letter size and spacing with same number of letters in each line. In both the method of visual acuity evaluation, patients were advised to sit 14-20 feet away from the chart and asked to read out the letters after covering one eye and continued until the patient was no longer accurately seeing the letters by asking to read smaller and smaller letters in the chart. The test was done in both the eyes of the pateints and visual acuity was calculated.

3.2.3. Indirect ophthalmoscopy

Indirect ophthalmoscopy was done in patient's eyes to examine the peripheral view of the retina, specifically to obtain a wide view of the fundus to understand changes in the retinal vasculature like beading and venous looping, neovascularization of the retina, retinal details such as intraretinal hemorrhages, microaneurysms, tractional or combined detachment and optic nerve head associated changes like neovascularization of the disc or disc pallor. Indirect ophthalmoscopy comprised of a small binocular lens with mirrors and a light source attached to a head band. Before the test, the patient's pupil was dilated using the drug tropicamide, a mydriatic agent and allowed for complete dilation for 30-45 minutes. Once the pupil was dilated, the fundus was examined using indirect ophthalmoscope.

3.2.4. Fundus photography

Fundus photography was done to record the changes in the retina using a fundus camera after dilating patient's eyes with tropicamide. The patients were advised to sit at the fundus camera by keeping their chin rest and forehead against the bar. The camera was focused and aligned to get the proper fundus view and photographs were taken at various angle and the images were recorded. Red free images were also obtained which helped analyze the vascular changes better.

3.2.5. Fundus fluorescein angiography (FFA)

FFA is a technique to understand retinal circulation in normal and diseased conditions. In FFA, a fluorescein dye - sodium fluorescein, which is an orange red crystalline hydrocarbon of molecular weight 376 Daltons in size is injected intravenously. This dye can pass through most of the body fluids and circulates in vasculature and tissues of retina and choroid where it can be visualized based on its fluorescence emission. The fluorescence will be localized into the vessels or capillaries if there is no damage. The leaked fluorescence indicated damaged vessels or any hemorrhage in the retina. The patient's eyes were dilated using tropicamide and sodium fluorescein were injected intravenously in the forearm. Fundus photographs were taken immediately after the dye injection by illuminating retina with a blue light of excitation wavelength at 465-490 nm. The emission of yellow green light was obtained at 520-530 nm and the resultant angiogram was obtained in black and white background.

3.2.6. Optical Coherence Tomography (OCT)

OCT is a method to visualize cross sections of the retinal architecture and the vitreoretinal interface. The resultant OCT image provide information about distinctive retinal layers with

an accurate measurement in their thickness in micron scale. The eyes were dilated using tropicamide and patients were asked to sit in front of the OCT camera by keeping the head rest on a support system to avoid any movements while scanning. The patients were asked to focus on the lens of the equipment at a small blinking target. The scanning was performed without direct contact with the eye and images were generated. The resultant images were analysed by the clinician for disease diagnosis.

3.2.7. B-scan ultrasound

B-scan ultrasound is a method to get information regarding the intraocular structures such as lens, vitreous and retina when direct visualization is impossible due to ocular conditions such as cataract, hemorrhage, edema, corneal opacities, etc. The images will be taken directly placing the probe on the patient's eyelid or placing the probe directly on the ocular surface after giving a topical anesthetic. Further, the probe was moved at different directions with directed ocular movements to get entire globe details. The two directional B-scan images were generated and analysed by the clinician.

3.3. Vitreous humour collection

Vitreous humour was collected from the study participants while undergoing pars plana vitrectomy as a part of routine management of the disease from the proliferative diabetic retinopathy patients (n=120), diabetic patients having no symptoms of retinopathy but diagnosed with idiopathic macular hole (n=10), Control vitreous humor samples were collected from the subjects while undergoing surgeries for RD and IMH (n=120). The complete clinical details were also documented for clinical correlation in the study proforma. The study proforma and informed consents are attached in the Annexure 1. The detailed

inclusion and exclusion criteria of the study participants are given in the table. The diagnosis of DR was based on the fundus photography and OCT report of the study patients.

Table 3.1: Inclusion and exclusion criteria for vitreous sample collection

With possible DR (Inclusion) (Patients samples) (n=120)	**Without possible DR (inclusion) (Control groups) (n=120)**	**Exclusion criteria**
• Vitreous hemorrhage • Diabetic TRD • Diabetic combined RD • PDR • NPDR patients undergoing cataract surgery	• ERM • RD • Pseudo hole/ Lamellar hole/ Macular hole • Valsalva retinopathy • IOL removal • PPV+PPL+/-SFIOL	• Endophthalmitis • History of trauma • History of uveitis • Hemorrhage

3.4. Blood sample collection and serum separation

For serum, 2 mL of blood samples were collected in a red vacutainer contains clot activator (BD Biosciences, USA) from PDR, NPDR and NDM subjects and separated the serum within 1 hour of sample collection by centrifugation at 1500g for 15 minutes. The samples were stored at -80˚C and thawed prior to the experiments.

3.5. Sample processing and protein precipitation

Vitreous humor samples were collected immediately after the surgery in a sterile 1.5 mL cryovial in ice cold condition. The samples were centrifuged at 14,000 g for 10 min at 4˚C to remove any cellular debris and the supernatant was aliquoted and stored at -80˚C till the next application. The total proteins in the vitreous samples were precipitated by mixing them with an equal volume of lysis buffer comprised of 150mM NaCl, 0.25 % Sodium deoxycholate,

1mM EDTA, 50mM Tris HCl (pH=7.4), 1% NP 40 and 0.05 % SDS for 30 minutes in ice cold conditions. 2.5 volumes of ice-cold acetone were added to it and mixed well by inverting the tubes and then incubated overnight at -80˚C. The precipitated proteins were collected by centrifugation at 14,000 g for 1 hour at 4˚C and dissolved the pellet in 50 µL 1x PBS containing protease inhibitor. The total protein in serum and vitreous samples were calculated by Bicinchoninic Acid (BCA) assay.

3.6. Bicinchoninic acid Assay

The BCA assay is used to quantify the total protein concentration in a given sample by colorimetric method. The principle of this assay is the ability of aromatic amino acids in the protein to reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^{1+}) in an alkaline solution, resulting in the purple color formation. The amount of colour developed can be measured colorimetrically at 595 nm absorbance, which will provide an estimate of protein concentration in the sample.

a) Protocol for BCA assay

i. 1mg/ml of BAS stock was prepared in 1xPBS.

ii. A serial dilution of nine BSA standards were prepared from the stock BSA.

iii. 10µL of protein standard and test proteins were pipetted into a 96 well plate in triplicate in their corresponding wells.

iv. BCA reagent was made freshly by mixing 50 parts of reagent A with 1part of reagent B (50:1).

v. 200µL of prepared BCA was added in the respective wells and incubated in dark at 37˚C with constant shaking.

vi. Absorbance was measured at 595 nm by colorimetrically using spectrophotometer.

b) Estimation of protein concentration

Standards and sample triplicates average optical densities (OD) were calculated after subtracting the blank OD value from them. A graph was plotted with OD on X-axis and standard concentrations on Y-axis. A linear regression plot with $r^2>0.09$ was taken into account for calculating the test sample concentration by plotting the test OD in the standard graph and corresponding concentrations in the Y-axis. An equal concentration of protein from the controls and patients were used for further experiments.

3.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A second step of normalization of the proteins was performed by subjecting an equal concentration of proteins estimated by BCA method to a 10% SDS -PAGE followed by staining the gel with Coomassie Brilliant Blue (CBB).

a. Casting the SDS-PAGE gel:

The resolving and stacking gels were prepared as per the composition given in the table. The resolving gel was poured in the space between the aligned glass plates and kept sufficient space for the stacking gel. Water saturated butanol was added on the top of the resolving gel to prevent oxidation and then it is left for 45 min to polymerize the gel. Butanol was removed and washed the gel surface with double distilled water (dd. H_2O) and poured the stacking gel. A multi well comb for protein loading was inserted into the stacking gel. Comb was carefully removed after the polymerization and washed the wells with dd. H_2O to remove the un polymerized residues of the gel.

b. Sample preparation

10 μg of sample was mixed with 4x gel loading buffer (Laemmli buffer) at a final concentration of 1X in an appropriate sample volume. (e.g. for 15 μL of vitreous sample added 5 μL of 4X loading buffer). The samples were denatured by boiling at 95°C for 10 minutes followed by snap chilling these prior to gel loading.

c. Sample loading

i. The gel was placed in an electrophoretic chamber and sufficient volume of 1X running buffer was added on to the top and bottom reservoirs of the electrophoretic apparatus (Thermo Scientific) by diluting 10X tris glycine SDS (10X TGS) (250 mM Tris, 1.92M glycine and 1% SDS) with 9 volumes of distilled water.

ii. The snap chilled samples were centrifuged and loaded into the respective wells along with a pre-stained protein ladder (Invitrogen).

iii. Electrophoresis was done at 75 V (8v/cm) until the dye front reached into the resolving gel and gradually increased to 100 V(15v/cm) until the loading dye reached the bottom of the resolving gel.

d. CBB staining of the gel

The amine groups in the proteins bind to sulfonic acid present in the CBB though ionic and Vander Wall's interactions, which helps to protein visualization in the gel.

i. The gel was removed carefully from the glass plates and washed once with dd.H_2O

ii. CBB stain was made by dissolving 0.025% CBB R-250 in 40% methanol, 10% acetic acid and filtered it using Whatman grade -1 paper and place d the gel on it with constant shaking on a platform shaker until gel achieved a uniform blue colour.

iii. Destained the gel by multiple washes with destaining solution consists of 30%methanol and 10% acetic acid until a visible protein band appeared in the transparent gel background.

Table 3.2: Composition for 10% SDS PAGE

Reagents	Resolving gel (10mL)	Stacking gel (2mL)
dd. H_2O	4	1.4
30% Acrylamide-Bis acrylamide	3.3	0.33
1.5 M Tris HCl (pH=8.8)	2.7	Nil
1M Tris HCl (pH=6.8)	Nil	0.25
10% SDS (w/v)	0.1	0.02
10% APS (w/v)	0.1	0.02
TEMED	0.0006	0.002

3.8. Mass spectrometry

Mass spectrometry is a powerful and sensitive analytical technique to identify the compound by measuring its ions mass to charge ratio (m/z). Mass spectrometry was used for the profiling of vitreous humor from study participants by separating the proteins in 10% SDS PAGE gel and subjected the excised gel pieces containing required size protein to trypsin digestion followed by mass spectrometry run and analysis. The detailed procedure used for in gel mass spectrometry was given below.

a. Staining and destaining the protein bands

i. 100μg of protein from controls and patients were loaded on to a 10% SDS PAGE followed by staining with CBB as explained in the section 3.6. The gel image was taken and marked the areas of excision in each lane as per the molecular weight. A sharp cut using a sterile scalpel was made in the marked lanes and excised the gel pieces out and cut it into 1 mm size cubes.

ii. The gel pieced were de-stained with 500 μL of freshly prepared solution of 70%50 mM Ammonium bi carbonate (NH_4HCO_3) with 30% Acetonitryl (ACN) and vortexed in a thermomixer for 23˚C for 30 minutes and this step was repeated until the gel pieces became completely devoid of CBB stain.

iii. The pieces were washed twice with 500 μL of 50mM $NH_4HCO_3$for 10 min at 22˚C in a thermomixer.

iv. The gel pieces were dehydrated with 500 μL of 100% ACN at 22˚C in a thermomixer for 10 minutes and repeated dehydrating step until the pieces became opaque white grain. The supernatant was removed and completely dried the gel pieces in a vacuum concentrator for 10 min.

b. In-gel trypsin digestion

i. 100μL of 10 mM Dithiothreitol (DTT) was added in each tube containing gel pieces and shaken for 5min at 23˚C and replenished the pieces with 10- 20 μL of DTT to completely soak the pieces and incubated the samples at 56˚C for 45 min in a thermomixer at 1400 rpm.

ii. Excess DTT was removed from the gel and 55 mM Iodoacetamide (IDA) as the same volume of DTT added to the samples and then incubated in dark at 23˚C in a thermomixer at 1400 rpm for 30 minutes.

iii. The gel pieces were washed and dehydrated with 500 µL of 50mM NH_4HCO_3 and 100% ACN as mentioned earlier and extracted proteins were vacuum concentrated for 10 min.

iv. 80-100 µL of 1X trypsin (15ng/ µL) was added to the peptide mix and kept the samples in incubation for 16 hours at 37˚C.

c. Extraction of peptides

i. Peptides were extracted by adding 500 µL of 5% formic acid in 30% ACN and incubated the sample for 1hr at 1400 rpm in thermomixer at 23˚C.

ii. Samples were sonicated for 5 min and the supernatant was collected in another Eppendorf. The gel pieces were dried in 200µL of 100% ACN and transferred this solution to the previously collected peptide extract. The samples were then vacuum concentrated for 8-10 hours and stored at -20˚C till further use.

d. Desalting of gel extracted peptides with C18 tips

i. The samples were thawed in room temperature for 10-15 minutes and added 20 µl of 0.1% TFA followed by sonication for 5 minutes.

ii. 5µg of protein passed through C18 tips were washed twice with 10 µL ACN and thrice with10 µl 0.1% TFA by pipetting up and down slowly.

iii. The samples were passed through the columns for 15-20 times without trapping any air bubbles and washed the tips twice with 20 µL of 0.1%TFA.

iv. The peptides were eluted with 2X 20 µL of 50 % ACN 1% FA by pipetting up and down 5 times and dried in vacuum concentrator and dissolved the peptides in 10µL of 2% formic acid.

v. The samples were vortexed for 10s and sonicated for 5 minutes and kept the samples for mass spec analysis using Q exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, USA).

e. Running the sample in mass spectrometer

Mass spectrometry (MS) analysis was performed using Q Exactive (Thermo Scientific) interfaced with nanoflow LC system (Easy nLC 1200, Thermo Scientific). Thermo Xcalibur MS data system software was used as the system controller. Trypsin-digested desalted peptides (10µL) were loaded onto PepMap RSLC C18 3 µm, 100 Å, 75 µm × 15 cm (Thermo Fisher Scientific) at a flow rate of 1 µL/min. Peptides were separated using 120 min linear gradient of the mobile phase [5% ACN containing 0.1% formic acid (buffer-A) and 95% ACN containing 0.1% formic acid (buffer-B)] at a flow rate of 300 nL/min. The data acquisition was performed in the electrospray ionization positive mode, with charge state + 2, capillary temperature 300 °C and spray voltage at 2.2 kV. Full scan MS spectra were acquired at a resolution 70,000 and scan range 400–1750 m/z. The top 10 most intense peaks containing doubly and higher charged states were selected for sequencing and fragmentation in the HCD (High Energy Collision-Induced Dissociation) mode at a scan resolution of 17500 with normalized collision energy set to 29%. Dynamic exclusion was activated to minimize repeated sequencing for the entire sequencing event. These experiments were done at the proteomics laboratory of Centre for Cellular and Molecular Biology, Hyderabad under the supervision of Dr Swasti Roychowdhary.

f. MS/MS data analysis

The acquired raw data for vitreous humour samples were searched against the human proteome from Uniprot database (release 2018.09 with 73099 entries) and a database of known contaminants using the Andromeda search engine and Maxquant (version 1.3.0.5). All MS/MS spectra from each sample were analysed using minimum one peptide for identification and 0.5% FDR (false discovery rate) on both peptide and protein level. Other search parameters included constant modification of cysteine by carbamidomethylation, enzyme specificity trypsin, Label-Free Quantification (LFQ) selected and match between runs with 2 min time window. iBAQ option was selected to compute abundance of the proteins.

g. Bioinformatic analysis

The identified protein lists from the samples were further analysed by various bioinformatics tool for proteome analysis. The proteins were compared with eye proteome database for identifying the novel vitreous ad eye proteins detected from the present study. Abundant vitreous proteins were evaluated in the vitreous samples by analysing the top 20 proteins based on their intensity and peptide detected. Gene Ontology (GO) was permed for the hierarchical classification of identified proteins based on their involvement in major GO-Slim biological process, GO-slim molecular function and GO-Slim cellular components using PANTHER-GO database for the complete set of proteins in each category. To identify the proteins shared between the groups and unique proteins present in each set, Venn diagram was generated using Gene Venn tool. The unique proteins and proteins shared only between diabetes group

and proteins shared only between no-retinopathy groups were evaluated based on the proteins and enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) and REACTOME pathway analysis through STRING bioinformatics tool. Label free quantitation was done to evaluate protein expression based on their intensity. Differential expression of proteins in diabetes compared to no-diabetic group and retinopathy compared to no-retinopathy groups were evaluated by calculating the mean the fold change. Further, significantly enriched KEGG and REACTOME pathways in diabetes and retinopathy groups were analysed from the differentially regulated proteins list. Heat map was generated using R statistical software. The overall work flow of LC-MS/MS is given in the flow chart (Figure 3.2).

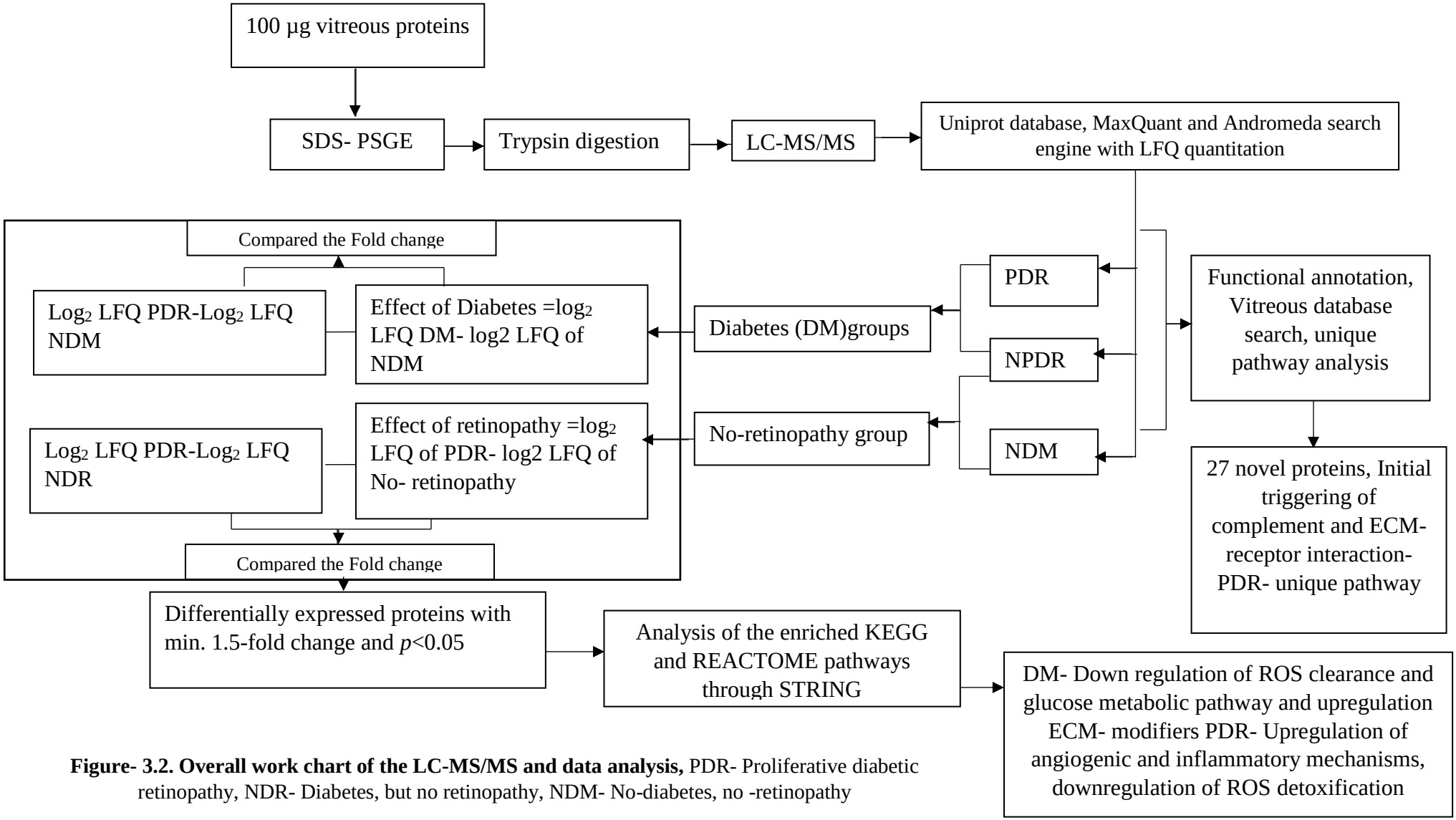


Figure- 3.2. Overall work chart of the LC-MS/MS and data analysis, PDR- Proliferative diabetic retinopathy, NDR- Diabetes, but no retinopathy, NDM- No-diabetes, no -retinopathy

3.9. Western blotting

Western blotting was performed in vitreous and serum samples for elaborating the role of complement pathway in PDR pathogenesis. Levels of total complement C3 and its fragments in systemic circulation were analyzed and compared between PDR (n=12), NPDR (n=12) and NDM (n=12) subjects. Further, complement activation in vitreous humor was studied by western blotting of C3 in vitreous samples collected from PDR (n=42) and NDM (n=42) subjects. Next, the type of complement pathway activated in PDR was evaluated by analyzing proteins of classical pathway (C1q: serum (PDR: n=8, NPD: n=8 and NDM: n=8), vitreous (PDR: n=17, NDM: n=17), C4b (vitreous, PDR: n=8, NDM:, n=8) and alternative pathway of complement (Factor Bb, Vitreous- PDR: n=22, NDM: n=22) and regulator of alternative complement pathway (CFH, Serum (PDR: n=12, NPDR: n=12, NDM, n=12), vitreous (PDR: n=31, NDM: n=31) using western blotting. The level of vitreous antiangiogenic protein, Opticin (vitreous-PDR: n=15, NDM: n=15) and inflammatory protein CRP (Vitreous, PDR: n=5, no-DM: n=5) was also evaluated by western blotting in vitreous

a. Sample preparation

Normalized protein samples were mixed with protein loading buffer as per the standardized concentration for antibody detection. (C3= 10 μg, C4β=20 μg, C1q=30 μg, CFH=30 μg, Factor Bb=25 μg, Opticin=15 μg, CRP=15 μg). Samples were run in reducing (C1q, CFH) and non-reducing (C3, CFB, C4 β, Opticin, CRP) conditions as given in the below sample preparation table. The samples were boiled at 95°C for 10 minutes followed by snap freeze on ice.

Table 3.3: Western blotting sample preparation under reducing and non- reducing gel conditions

Requirement	Reducing condition	Non- reducing condition
Protein	X μg	X μg
β-Mercapto ethanol	1μL	Nil
4X loading dye (125mM Tris HCl, pH- 6.8, Glycerol-50%, 4%SDS, 0.2%) (w/v orange G)	5 μL	5 μL
Milli Q	Make up to 20 μL	Make up to 20 μL

b. Gel electrophoresis and transfer of the proteins from the gel to Polyvinylidene Difluoride (PVDF) membrane

i. The gel was run as detailed in the section 3.6. The gel was carefully removed from the glass plates and washed it in 100 ml of distilled water and equilibrated it in transfer buffer containing 1.92 M glycine, 25mM Tris and 20% methanol for 15 min.

ii. The filter pads for the wet transfer was soaked in the buffer and removed the bubbles to allow the free transfer of proteins into the membrane.

iii. The PVDF (Immobilon®-FL transfer membrane, Millipore) and 2 pieces of 3 mm thick Whatman Grade 3 filter papers were cut according to the gel size.

iv. The PVDF membrane was activated by dipping it in 100% methanol for 20-30 seconds and rinsed it with dd.H_2O and placed it in transfer buffer along with Whatman filter papers for 3 minutes.

v. For protein transfer from polyacrylamide gel to the PVDF membrane a gel membrane sandwich was prepared in the order of –filter pads (2 No.)- Whatman membrane (1

No.)- gel- activated PVDF membrane- Whatman membrane (1 No)-filter pads (2 No.) from cathode to anode direction in the transfer blot module (XCell II™ Blot Module, Thermo Fisher scientific) and placed the cassette in the transfer system (XCell SureLock Mini-Cell, Thermo fisher Scientific) horizontally.

vi. The blot module was filled with 1X transfer buffer until the gel-sandwich was completely covered and outer chamber was filled with 650 mL of ice-cold deionized water to avoid heat generation while transfer. Transfer was done at a constant 25 V for 1 to 2 hours dependent on the molecular weight of the proteins to be detected.

c. Ponceau S staining

Ponceau S staining was done after every transfer to detect the efficiency of protein transfer to the PVDF membrane. Also, it was used as a normalization control for the western blotting experiments to confirm the equal protein loading across all the samples.

i. The membrane was removed after the transfer and washed with 100mL of deionized water and placed it in a clean tray containing Ponceau S solution (0.1% Ponceau S (w/v) in 5% acetic acid) for 5 -10 minutes with constant agitation.

ii. Removed the stain and images were captured for further comparison after de-staining the blot with deionized water until the dye completely washed away from the membrane.

d. Blocking and primary antibody incubation

i. For blocking, the membrane was incubated in commercially available Odyssey Blocking buffer (LICOR) for 2 hours at room temperature with constant shaking.

ii. PVDF membrane was placed carefully into a sealed sterile plastic bag and primary antibodies were diluted in blocking buffer (Table 3.4.) was added carefully into the membrane with the help of a pipette. The membrane was kept for incubation for overnight at 4°C with a constant shaking.

e. Washing

The blots were removed carefully from the plastic bag with the help of a pair of forceps and placed it in a cleaned plastic tray. The blot was washed twice with 1XPBS containing 0.2% Tween-20 followed by washing twice with 1X PBS.

f. Secondary antibody incubation and visualization of the protein band

i. The blots were incubated with Infra-red (IR) labelled secondary antibody in blocking buffer for an hour at room temperature with gentle shaking at dark. The blots were washed as mentioned above.

ii. The protein transfer side of the PVDF membrane was placed on the Licor fluorescent scanner and the bubbles were removed using a roller. The scan was done and the corresponding protein images were generated on to the computer screen attached to the system.

The antibodies used and their dilutions are given in the table 3.4. The following protocol was used for western blotting.

Table 3.4: Primary and secondary antibodies used for western blotting

Sl. No	Antibody	Dilutions (µl)	Company	Catalogue No.
1	Ms monoclonal to C3	1:300	Santa Cruz	sc-28294
2	Ms monoclonal to CFH	1:200	Santa Cruz	sc-166613
3	Ms monoclonal to C1q	1:1000	Abcam	ab71089
4	Rb monoclonal to CFB	1:500	Abcam	ab72658
5	Ms monoclonal to C4β	1:200	Santa Cruz	sc-74524
6	Rb polyclonal to CRP	1:1000	Abcam	ab 31156
7	Rb monoclonal to CD11b	1:200	Abcam	ab133357
8	Rb monoclonal antibody to Opticin	1:500	Abcam	ab170886
9	Anti- Rb 800cw	1:10000	Licor	926-32211
10	Anti-Ms 680 RD	1:10000	Licor	926-68070

3.10. Gelatin zymography

Zymography is a simple and powerful technique for the detection of proteolytic activity of the matrix metalloproteinase enzymes such as MMP2 and MMP9 based on its degrading capability of gelatin. In this method, a polyacrylamide gel is co-polymerized with gelatin substrate and allow the proteins to get separated on this gel matrix. Under denaturing and non-reducing conditions of electrophoresis, SDS maintains the inactive state of MMPs and proteins get separated out. In post electrophoresis processing, enzymatic activity of the MMPs retain due to the partial renaturation with Triton X-100 and subsequently gel will be incubated in calcium containing activation buffer. This induce the activation of MMPs and induce gelatin degradation leaving a cleared zone and that can be identified after staining of the gel. The detailed protocol is given below.

i. Glass plates were cleaned thoroughly with deionized water and assembled vertically it in the gel casting system.

ii. The resolving and stacking gels were prepared as per the composition given in the table 3.5.

iii. 10μg of vitreous protein was mixed with 4X sample preparation buffer at a final concentration of 1X and incubated at room temperature for 10 min and loaded the samples in gel at a constant voltage of 125V (starting current = 30 -40 mA/gel) until the dye reached bottom of the gel.

iv. The gel was removed from the cassette and placed it in a plastic tray containing 100mL of renaturing solution (2.5% v/v Triton X-100 in d. H_2O) and kept for incubation at room temperature for 30 min with gentle agitation and rinsed the gel with 300mL of deionized water.

v. 100mL of 1X developing buffer (0.05M tris HCl, pH- 7.8, 0.2M NaCl, 5mM $CaCl_2$, 0.02% Brij 35) was added on to the gel and incubated at room temperature for 30 min with gentle agitation and replaced the developing buffer with 100mL of fresh developing buffer and incubated at 37°C for 16 hours in a closed tray for MMPs activation.

vi. Decanted the developing buffer and added 100 mL of staining solution (0.05% CBB in 5% Methanol and 10% acetic acid) until the gel get a uniform blue staining.

vii. Gel was kept in destaining solution (5% methanol and 10% acetic acid in dH_2O) until the areas of gelatinolytic bands were visible as a clear sharp band over the blue background.

Table 3.5: Reagents required for 7.5% gelatin zymography preparation

Requirement	Resolving gel	Stacking gel
Deionized water	2mL	3.08 mL
30% Acrylamide-Bis acrylamide mix	2mL	0.67 mL
Gelatin (4mg/ml)	2 mL	Nil
1.5M Tris HCl (pH=8.8)	2 mL	Nil
1M Tris HCl (pH=6.8)	Nil	1.25 mL
10% SDS	80µL	50µL
10%APS	80µL	50 µL
TEMED	10 µL	10 µL

3.11. Retinal tissue collections

Diabetic (n=3) and control (n=3) retinal tissues were collected from cadaveric eye balls, Ramayamma International Eye Bank, LV Prasad Eye Institute, Hyderabad according to the tenets of declaration of Helsinki. The retina was used for immunostaining for validating vitreous proteome data. A brief systemic and medical history of the donor was obtained from the donor's form and consent to use this tissue for research purposes was obtained from the family.

3.12. Validation of complement activation using retinal tissues collected from diabetic and non-diabetic cadaveric eyes.

Immunohistochemistry was done for the complement proteins C3, CFH and also for the activated microglial markers CD11b and macroglial marker GFAP. In addition to this, CXCR4, a chemokine receptor for chemotactic activity were also evaluated in the retinas of diabetic and control subjects. Real time PCR was also done to check the expression of complement proteins and CXCR4 level in the retina of diabetic and control subjects.

3.12.1. Immunohistochemistry

Immunohistochemistry is a method to detect the protein expression in tissue using an antibody specific to the antigen. The visualization of the antigen can be either by chromogenic method or by fluorescent labelling. The detailed protocol followed for IHC is given below.

a. Tissue embedding and sectioning

For IHC, the retinal tissue was fixed overnight in 4% formalin and then placed in a mold containing liquid paraffin and allowed to cool to get an immobilized tissue embedded blocks. The tissue sections were made using microtome with a thickness of ~4-6μm in size and mounted the sections over charged slides and allowed the sections to adhere on the slide.

b. Deparaffinization and rehydration

The paraffin was must be removed from the tissue sections and rehydrated prior to antigen-antibody staining. Deparaffinization was done by placing the tissue sections at 70°C for 30 minutes in hot air oven followed by xylene wash. The sections were gradually rehydrated by treating them with 100% and 95% ethanol followed by wash with running tap water, distilled water and 1X PBS.

c. Antigen retrieval

The protein- cross links formed during formalin fixation can mask antigenic site for the antibody binding. In order to bind the antibody, the masked epitopes need to be unmasked by antigen retrieval method. Antigen retrieval was done by microwaving the sections thrice at full power (5min each) using Tris citrate buffer of pH 6.

d. Permeabilization and antibody staining

Permeabilization was done by incubating the sections in methanol for 30 min at -20˚C followed by washing thrice with 1X PBS. To avoid non -specific binding blocking was done using 2%BSA for 1 hour at room temperature. The sections were incubated with primary antibodies diluted in blocking buffer for overnight at 4˚C. Washing was done thrice with 1x PBS and incubated with appropriate fluorescent labelled secondary antibodies for 1hour at room temperature. The list of primary and secondary antibodies used and their respective dilutions are given in the table 3.6.

e. Mounting and visualization

The sections were washed with 1XPBS thrice and pat dried on the bed of blotting sheets. The mounting medium containing DAPI counter stain for nuclei were added on the sections and placed a coverslip over that without making air bubbles. The slides were sealed and visualized under fluorescent microscope (EVOS).

Table 3.6. List of primary and secondary antibodies used for immunostaining

Sl. No	Antibody	Dilutions (µl)	Company	Catalogue No.
1	Ms monoclonal to C3	1:50	Santa Cruz	sc-28294
2	Ms monoclonal to CFH	1:50	Santa Cruz	sc-166613
3	Rb monoclonal to CXCR4	1:50	Santa Cruz	sc-9036
4	Rb polyclonal to GFAP	1:500	Dako	Z0334
5	Rb monoclonal to CD11b	1:300	Abcam	ab133357
6	Goat anti Rb 488	1:300	Life Tech.	A-11008
7	Goat anti Ms 488	1:300	Life Tech	A-10680
8	Goat anti Ms 594	1:300	Life Tech.	A-11005
9	Goat anti Rb594	1:300	Life Tech.	A-11012

3.13. Multiplex ELISA

Multiplex ELISA based on the Luminex® xMAP®technology was used in the current study. It allows identification of multiple analytes from a single sample by using internally colour coded polystyrene microspheres with two fluorescent dyes. 100 discrete colour beads were generated by fine-tuning the relative volumes of these two fluorescent dyes. Each microsphere was coated with specific capture antibody and when the bead captures an analyte from the test sample, a second biotinylated antibody will be introduced followed by incubation with streptavidin -PE conjugate as a reporter molecule on the surface of each microsphere. Thus, each individual microsphere will be identified, and the concentration of the analytes will be quantified based on the fluorescent reporter signals by the digital signal processors.

3.13.1. Protocol for Multiplex ELISA

i. 6 proteins were selected based on its role in inflammation and angiogenesis in retinal neovascular conditions. The concentration on these 6 proteins were evaluated in vitreous of PDR cases (n=10) and controls (n=10) using multiplex bead immunoassay based on LUMINEX platform.

ii. Vitreous humor samples from PDR and control subjects were centrifuged at 1000g for 10 minutes for removed the debris. The supernatant was collected and diluted with assay buffer as 1:3 dilution. HANG2MAG-12K kit for the analytes such as sPECAM, VEGFR2, sVEGFR1and HCYTMAG-60K-PX30 panel for 1L-6, IL-10 were used.

iii. All the reagents were thawed at room temperature, 20-30 min prior to the assay. The antibody vials were sonicated for 30 seconds followed by vortexing for a minute. The

antibodies were light sensitive, hence always covered them to protect from the light exposure.

iv. 60 µL of antibodies from each vial were added to the mixing bottle and made up to 3mL volume with bead diluent and mixed thoroughly by vortexing.

v. Further, 1X wash buffer was made from the 10X stock solution by diluting it with deionized water.

vi. Standards were prepared by reconstituting them with 250µL of deionized water as per the instruction provided in the manual. 200 µL of 1 X assay buffer was added into the each well of the 96- well plate and incubated for 10 minutes at room temperature.

vii. Decanted the assay buffer from the plate using vacuum and added 25µL of assay standards and quality controls in to the respective wells. Further 25 µL of diluted test samples (vitreous humors) and 25 µL of targeted antibodies containing beads were added into their respective wells.

viii. After overnight incubation (16-20 hours) at 4˚C on constant shaking, gently removed the plate contents and washed the plate thrice with 1X wash buffer.

ix. 25 µL of detection antibodies were added into each of the well and incubated for 1hour at room temperature in a plate shaker under dark. Followed by this, 25 µL of reporter tag comprised of streptavidin-phycoerythrin were added on each of the wells and incubated in dark with gentle shaking for 30 min.

x. Further, the contents of the wells were emptied carefully and washed the plate thrice with wash buffer and 150 µL of sheath fluid was added on each of the wells. Plate was scanned under Luminex system with xPONENT® software and the generated results were exported in terms of median fluorescent intensity and calculated the

concentration of the analytes in the PDR and controls. The significance was calculated based on the t-test with a p value <0.05.

3.14. Generation of a model system for understanding neuron-glia interaction in diabetic retinopathy using human cadaveric retina

The mechanisms of neurodegeneration in diabetic retinopathy remain largely unknown mainly due to lack of techniques to develop and characterize the disease models that can simulate the inherent neuron-glia interactions in human retina. Specifically, establishment of a mixed retinal culture remains a challenge due to different conditions required for their optimal growth and differentiation. In order to study their functional interaction under hypoxia and high glucose conditions primary mixed retinal culture mimetic of retinal microenvironment was established using human retinal tissues.

3.14.1. Preparation of human mixed retinal cell cultures

Cadaveric eye balls of less than 65 years of age and enucleated and collected within 24 hours of death was received from Ramayamma International Eye Bank, LV Prasad Eye Institute, Hyderabad. In addition to this, retinal tissues were collected from patients of anterior staphyloma or patients from open globe injury after obtaining the informed consent. The study was strictly followed the tenets of declaration of Helsinki and also approved by institutional review board, LV Prasad Eye Institute. Eye balls were collected in a sterile glass chamber and incubated in 1X PBS containing 2X concentration of antibiotics (Penicillin and Streptomycin) for 15 minutes in a sterile laminar hood. A transverse cut was made on the posterior side of the eye ball using a sterile scalpel and separated the neural retina from choroid-RPE and vitreous humor and washed it in 1X PBS. The tissue was kept in a sterile petri-plate and finely chopped into small pieces using a fresh surgical blade. For getting a fine separation of the cells, the chopped tissue pieces

were treated with 0.25% of trypsin-EDTA for 15 min at 37˚C. Enzymatic activity was arrested by adding complete DMEM (10%FBS, 90% Dulbecco's Modified Eagle's Medium) and separated cells were collected by centrifugation at 1000g for 3 minutes. The undigested pieces of tissue were removed and cells were resuspended in complete DMEM (cDMEM) containing 1X antibiotics and GM-CSF (1ng/mL). The cells were seeded on a T-25 flask and incubated under standard tissue culture conditions consists of % CO_2 in a 37˚C incubator. The cells were kept undisturbed for 7 days followed by medium was changed every three intermittent days. The cells morphology was observed using inverted phase contrast microscope and images were taken and documented.

3.14.2. Passaging of the cells by trypsinization

Trypsinization was done to passage the cells when the cultures became 60-70% confluent. For trypsinization, complete medium was removed from the cells and washed with sterile 1X PBS. 1X Trypsin EDTA (0.25%) was added on to the cells and incubated for 2 -3 min at 37˚C to detach the cells from the culture dish followed by arresting the trypsin activity with cDMEM. Cells were collected by centrifugation at 1000g for 3 minutes and resuspended in 1mL cDMEM. The number of cells per mL were counted using hemocytometer. Once the cells become confluent, these were trypsinized and sub-passaged into multiples flasks for the next experiments and part of the cells were cryopreserved in medium containing 50% DMEM, 40% FBS and 10% DMSO and stored in liquid nitrogen.

3.14.3. Retinal cell characterization by Immunocytochemistry (ICC)

Immunocytochemistry is a method to characterize the cells using an antibody specific to the antigen present in the cells. A fluorescently labelled primary or secondary antibody by direct or indirect method help to visualize the cells under microscope. For ICC, the primary retinal cells (5000 cells/mL) were seeded on 18 mm diameter sterile glass coverslip and allowed to attain 70-80% confluency. The cells were fixed with 4% formaldehyde for 10 minutes and washed thrice with 1X PBS (pH=7.4) for a period of 5 minutes each. Further, permeabilization with 0.5% triton X-100 for 10 minutes and 1X PBS wash was done followed by blocking with 2% BSA for 1 hour at room temperature. The primary antibodies such as mouse anti-ionized calcium-binding adaptor molecule 1 (IBA1: microglia, 1:200, Abcam) rabbit anti-glial fibrillary acidic protein (GFAP; astrocytes; Dako), rabbit anti nestin (neuronal progenitor cells, 1:300, Millipore, rabbit anti glutamine synthetase (GS, Müller glia, 1:250, Abcam), rabbit anti β-III tubulin (neuronal marker, 1:300, Abcam) were diluted in blocking buffer and added to cells for overnight at 4°C followed by washing with 1X PBS thrice. The specific secondary antibodies such as Alexa flour 488 conjugated anti rabbit (1:300, Life Technologies), Alexa flour 594 conjugated anti rabbit (1:300, Life Technologies) and Alexa flour 594 conjugated anti mouse (1:300, Life Technologies) were diluted in blocking buffer and incubated with cells for 45 minutes at room temperature. The cells were washed thrice with 1X PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) in mounting medium (Slow fade gold antifade containing DAPI) (Life Technologies, Ref. S36939) and visualized under fluorescent microscope.

3.14.4. RNA isolation from the MRC cells

RNA was isolated form the cells using Trizol method of extraction. 1mL of Trizol (Thermo Fisher Scientific) was added to the cells and homogenized thoroughly using pipetting and

incubated 5-10 minutes room temperature (23˚C) for ensuring the complete dissociation of complex nucleoprotein complex. 200µL of chloroform was added and vortexed for 10s followed by incubation for 2 minutes at room temperature. For phase separation, the samples were centrifuged at 12,000 g for 15 minutes at 4˚C and collected the aqueous phase in a fresh tube. 500µL of isopropyl alcohol was added and allowed to precipitate the RNA for 10 minutes and pellets were collected by centrifugation at 12,000g for 10 minutes at 4˚C. Followed by washing was done with 75% ethanol at 7500 rpm for 5 min and air dried the pellet and suspended it in RNAase free water. Quantification was done using nanodrop and evaluated the purity by checking 260/280 ratio.

3.14.5. cDNA preparation

i. 800-1000 ng of total RNA was used for the cDNA preparation using verso cDNA synthesis kit (Thermo Fisher Scientific, cat no. AB1453A).

ii. Reaction mixture was prepared in ice cold condition as shown in the table 3.7.

Table 3.7. Reaction mixture for cDNA preparation (Reaction volume 20µL)

Sl. No	Reagent	Volume (µL)
1	5X cDNA synthesis buffer	4
2	dNTP mix	2
3	OligodT	1
4	RT enhancer	1
5	RT enzyme	1
6	RNA template	for 800-1000ng
7	Nuclease free H_2O	for 20µL

iii. After preparing the reaction mixture, cDNA was prepared by reverse transcription using the conditions such as 42° C for 30 min for cDNA synthesis for 1 cycle followed by inactivation at 95°C for 2 minutes.

3.14.6. Characterization of the cells by Polymerase chain reaction (PCR)

The cultured cells were further characterized based on PCR based gene expression. As similar to the immunofluorescence, RNA was extracted from the cells from P1 and P2 passages and converted in to cDNA as detailed in section 3.14.4. and 3.14.5. For PCR, cell specific primers such as *IBA-1* for microglia, *GS* and *CRALBP* for Müller glia, *ALDH1L*1 for astrocytes and *β-III tubulin* for neurons were used. To ensure cDNA conversion, a PCR for *β-actin* was done prior to the PCR for cells specific genes. Gene specific primers and

annealing conditions used for characterization was given in the table 3.8. The specificity of the primers was checked by primer BLAST.

Table 3.8: Primer sequence used for PCR-based characterization of the cells from P1 and P2 passage.

Gene	Forward	Reverse	Annealing Temp.
β Actin	TCTACAATGAGCTG CGTGTG	GGTGAGGATCT TCATGAGGT	58˚C
GS	ATGCTGGAGTCAAG ATTGCG	TCATTGAGAAG ACACGTGC G	60˚C
IBA-1	GACCTTAATGGAAA TGGCGATA	ATCTCTTGCCCA GCATCATC	58˚C
CRALBP	CTGGCAAAGTCAAG AAATCAC	TGTCCACCATCT TCCTGAG	58˚C
ALDH1L1	ATCTTTGCTGACTGT GACCT	GCACCTCTTCTA CCACTCTC	59 ˚C
βIII Tubulin	GCTCAGGGGCCTTT GGACATCTCTT	TTTTCACACTCC TTCCGCACCACA TC	60˚C

PCR reaction mixture of 25 µL was made using the gene specific primers and reagents as given in the table 3.9. 40 -50 ng of cDNA was added in each reaction and mixed by pipetting up and down and spun down. The PCR was carried out in a thermal cycler as per the conditions as shown in the Table 3.10.

Table 3.9: Reaction mixture for setting up PCR

Reagent	Working concentration	Volume (µL)
10X Taq Buffer with $MgCl_2$	1X	2.5
100mM dNTPS	2mM	2.5
Forward primer	5pm/ µL	1
Reverse primer	5pm/ µL	1
Taq enzyme (2U/ µL) (Cat No.)	1U/ µL	0.5
Autoclaved Milli Q H_2O	……	Made up to 25 µL reaction

Table 3.10: Program for amplification of genes using Thermal cycler

Step	Temperature (˚C)	Duration	No. of Cycles
Initial denaturation	95	5 minutes	1
Denaturation	95	1minute	35
Annealing	As provided in Table 8.	30s	
Elongation	72	30s	
Final extension	72	5 minutes	1

3.14.7. Stress induction:

Two major key culprits which cause damage to the retina under diabetic stress are hypoxia and high glucose. Hence hypoxia and high glucose stress were induced into the mixed retinal cells of earlier passages (P1 &P2) by chemical method

a. Hypoxia induction by $CoCl_2$ (Cobalt Chloride)

Hypoxia inducible factor 1 (HIF1) is a transcription factor involved in controlling alteration of cellular O_2 level. Under normoxic condition, HIF1-α synthesized undergo rapid hydroxylation at proline residue 564 and 402 by von Hippel-Lindau protein (pVHL) prolyl-

hydroxylase. But under hypoxic condition the activation of prolyl hydroxylase gets inhibited and resultant increase in HIF1-α stability and its accumulation in the cell nucleus and activate number of hypoxia sensitive genes. The metal ion cobalt inhibits the activation of this pVHL enzyme resultant the accumulation of HIF1-α in the cell nucleus, thus $CoCl_2$ act as chemical hypoxic agent. For chemical hypoxia a stock solution of 25mM $CoCl_2$ (Sigma, cat. No. C8661) was freshly made by dissolving it in DMEM and filtered through 0.2μm syringe filter. Cells were seeded on glass coverslip at a density of 5000 cells / well and allowed to attain 70% confluency. The cells were pretreated for 6 hours with serum free medium followed by 150μM of $CoCl_2$ for 24 hours. The cells devoid of $CoCl_2$ was used as a control.

b. High glucose by D-Glucose

High glucose stress was induced into the cells by treating them with D-glucose. The cells were seeded on a glass coverslip at a density of 5000 cells/mL and waited until they reach 70% confluency. Complete DMEM was removed and added serum deprived medium and incubated for 6 hours. In order to give high glucose stress 25Mm stock concentration of D-glucose was prepared by dissolving it in incomplete DMEM and filtered using 0.2- μm syringe filters under sterile conditions. A final concentration of 30 mM of D-glucose was added to the cells in serum deprived medium and allowed to stay for 24 hours under standard cell culture conditions. Serum deprived cells of similar duration was used as a control and cells with D-mannitol was used as an osmolarity control for the study.

13.14.8. Cell viability by Alamar blue method:

Alamar blue is a blue colour cell viability indicator dye, and it works based on the cellular metabolic reduction ability. The resazurin present in the dye act as an oxidation -reduction

indicator and produce a change in fluorescence based on the cell's metabolic activity. Metabolically active cells reduce resazurin to resorufin continuously and produce fluorescent pink to red colour which can be measured colorimetrically and it will be directly proportional to the cell's viability.

For viability, thousand cells /well were seeded on a 96 well plate and allow to attain 70-80% confluency. Hypoxia and high glucose stress were given to the cells as described in the section 13.12.4. Different concentrations of $CoCl_2$ (100μM,150μM, ,200μM and 250μM) and D-glucose (25mM, 30Mm and 40mM) were used for the cell viability detection. After 24hrs of drug treatment, 10μL of Alamar blue dye (Life Technologies, Cat no. DAL1025) was added and kept at 37°C in CO_2 incubator. Plane DMEM with Alamar blue was served as blank. After 3 hours of incubation absorbance was measured and subtracted the blank values from cells' absorbance value. The percentage of viability was calculated as

$$Percentage\ of\ cell\ viability = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

13.14.9. Time-lapse Ca^{2+}imaging of the cells

Cytosolic calcium plays a critical role in regulation of cellular activity and measurement of intracellular Ca^{2+} transients under hypoxic and high glucose conditions were done and compared with serum deprived control cells by live cell imaging. In order to perform the Ca^{2+} imaging, the cells were seeded and stress were given as explained in the section 3.12.4. After 24 hours of stress induction, the medium was removed and incubated the cells in high balanced salt solution (HBSS, Invitrogen, Life Technologies, Cat. No. 14025-076) for 15 minutes in CO_2 incubator. The lyophilized Ca^{2+} indicator dye, Flu -4-AM dye (Life

Technologies, Cat. No. F14201), were dissolved in 50µl of DMSO and vortexed and spun down under dark. HBSS were removed from the cells and replenished the cells with HBSS containing Flu -4-AM dye (1:750) and allowed to incubate for 30 minutes in CO_2 incubator. The cells were washed with HBSS three times of 5 minutes interval and coverslip containing cells were placed in a glass slide using a sterile-forceps. Replenished the cells with 200µL of HBSS and placed the cells for imaging in EVOS- fluorescent microscope, at excitation emission at 494_-506nm. Time lapse imaging was carried out for a duration of 10 minutes, by capturing images at every 10s intervals. The images were captured from multiple locations to cover the maximum number of populations of the cells. Every experiment was repeated in multiple coverslips and also in multiple biological replicates. The images acquired were analyzed with Matlab (The MathWorks, Natick, MA). An image segmentation algorithm, based on principal component analysis, was optimized for automated segmentation of cells present in MRC (Developed by IIT, Hyderabad). Using the algorithm, intracellular Ca^{2+} transients were measured by computing the maximum amplitude of Flu4-AM intensity and number of Ca^{2+}spike generated at control, hypoxic and high glucose conditions. *k*-means clustering was performed based on Ca^{2+} spike count and $Ca^{2+}{}_{max}$.

3.14.10. Quantitative Real Time PCR (qRT)

. RNA was extracted from control and treated cells and cDNA was prepared as given the section 3.14.4. and 3.14.5. qRT enables the detection and quantification of products generated during each cycle of PCR. SYBR Green chemistry was used to perform qRT, based on its ability to bind the minor groove of the double stranded (ds) DNA followed by the generation of a fluorescent signal. The intensity of florescence increases as per the production of ds DNA molecule and threshold of the signals were calculated. Reaction

mixture for qRT, was prepared by mixing 10μL of iTaq™ Universal SYBR® Green Supermix (BIO-RAD, Cat no. 38220090), 200nm of forward and reverse primers and 40-50ng of cDNA for a total reaction volume of 20 μL in MicroAmp™ optical 96 well reaction plate. The primer sequence used for qRT PCR is given in the table 3.11. The plate was tightly sealed with an optical adhesive sheet (Thermo Fisher Scientific. Cat. No. 4311971) and loaded on Applied Biosystems 7900 HT system as conditions mentioned in the table 9. The relative measure of the concentration of target gene (CT) was calculated by using software SDS 2.4 (Applied Biosystems). Gene expression was calculated from the CT values using the formula $2^{-\Delta\Delta CT}$. β actin was used as a normalization control for qRT PCR. Statistical analyses were performed using $2^{-\Delta\Delta CT} \pm$ SEM in three biological and three technical replicates.

Table 3.11: Primer sequences used for quantitative real time PCR

Gene	Forward primer	Reverse primer
VEGF - 165	ATCTTCAAGCCATCCTGTGT GC	CAAGGCCCACAGGGATTTT C
HIF1 a	CCAGCAGACTCAAATACAA GAACC	TGTATGTGGGTAGGAGAT GGAGAT
NERF2	AGTGGATCTGCCAACTACT C	CATCTACAAACGGGAATGT CTG
OXR1	CTGATGGTGATTAAAGACA GTG	CACTTAAAGACCTCAAACTC C
C3	TCACCGTCAACCACAAGCT GCTACC	TTTCATAGTAGGCTCGGAT CTTCCA
IL1 β	AGCTGATGGCCCTAAACAG A	GGAGATTCGTAGCTGGAT GC
Caspase-3	ACATGGCGTGTCATAAAAT ACC	CACAAAGCGACTGGATGAA C
BAX	TGCTTCAGGGTTTCATCCA G	GGCGGCAATCATCCTCTG
CXCR4	AGCATGACGGACAAGTACA GG	GATGAAGTCGGGAATAGTC AGC
Il-6	TTCGGTCCAGTTGCCTTCTC	GAGGTGAGTGGCTGTCTG TG
IL8	GACCACACTGCGCCAACAC	CTTCTCCACAACCCTCTGCA C
β Actin	TCTACAATGAGCTGCGTGT G	GGTGAGGATCTTCATGAG GT

13.14.11. Analysis of protein expression by large scale imaging

To understand the activation of microglial and macroglial population of cells under stress in the developed neuro-glial system, protein expression of two well -known markers of gliosis such as IBA-1 and GFAP were analysed. After the induction of hypoxic and high glucose stress, the cells were taken for immunofluorescence as explained in the section 3.12.4. A large-scale imaging of the sections was performed at 40X magnification using confocal microscope using 488 and 594 excitation-emission channels for visualization of the fluorescently labelled antibodies. In order to quantify the protein expression, 3-D scanning was done by acquiring Z-stacks of the sections. Further, a panorama was generated using mosaicking technique for a field of view (1.8mm X 1.8mm) containing 10X square sections with a diameter of 180 μm X180 μm with approximately 20% overlap of the sections. Further 100 sections of each slides were stitched together to obtain a large -scale spatial profiling by Leica LAS X software. Further, imaging was done using 63X oil immersion microscope, and Z-stack images along the Z- axis were captured (Total Z height = ~12 μm, Z- stack thickness between each slice = 0.5 μm). This provided a detailed morphology and protein expression of GFAP and IBA-1 under hypoxic, high glucose and control conditions. 3D surface plot for GFAP and IBA at three different conditions were generated based on the protein expression from the panorama images using Image J software and protein expressions were quantified.

The quantitative protein and Ca^{+2} images analysis was carried out using algorithms and other softwares at the IIT Hyderabad by Sarpras Swain under the supervision of Dr. Lopamudra Giri and Dr Soumya Jana.

4. RESULTS

Diabetic retinopathy is a vision threatening retinal neuro-vascular complication of diabetes. Based on earlier studies, we hypothesized that longer duration of diabetes, poor glycemic control, and genetic predisposition along with metabolic stress induced proteome alterations and complex interplay of glial cells with neurons and vascular cells may be crucial to DR pathogenesis. Hence, the present study was designed to analyze various aspects of DR in order to understand its development and key players involved in neovascularization and neurodegeneration in DR. The overall strategy involved proteomic profiling of the vitreous from diabetic and DR patients to identify the key proteins involved at different stages of DR progression followed by a detailed investigation on retinal defense mechanisms such as complement and microglia. Further, *in vitro* analysis of neuro-glial interaction and its impact on neurovascular changes in diabetic conditions were studied in a mixed retinal culture system.

4.1. Comparative proteomic profiling of proliferative diabetic retinopathy with diabetic and non-diabetic human vitreous samples

4.1.1. Details of the study cohort

Vitreous samples were obtained from patients while undergoing pars plana vitrectomy surgery with prior informed consent. For vitreous proteome profiling, the samples were collected from patients with proliferative stage of diabetic retinopathy (PDR; n=3), diabetic patients who did not develop any clinical features of retinopathy (NDR; n=3) and control samples from non-diabetic subjects without retinopathy (NDM; n=3). None of the patients had received any intraocular anti-VEGF injections as a part of their ocular complications prior to sample collection. Further details of these patients are provided in Table 4.1.

Table 4.1: Demographics of study subjects used for global proteome profiling

Categories of patients	Age in yrs (Mean ±SEM)	Gender	DM Type	No. of yrs. of diabetes	Indication for surgery
NDM (n=3)	58 ± 3.9	M	N/A	N/A	Retinal Detachment
NDR (n=3)	63 ± 5.1	M	Type 2	4 ± 1.5	Idiopathic macular hole
PDR (n=3)	62 ± 4.1	M	Type 2	19 ± 1	Tractional Retinal Detachment

4.1.2. Sample preparation and LC-MS/MS analysis

Lysed and acetone precipitated proteins were dissolved in 1X-PBS and total proteins in each of the sample was quantified by BCA assay. The mean concentration of the proteins were similar across the three groups (NDM=9.46±1.45 μg/μL, NDR=8.83±3.97 μg/μL, PDR=9.77±2.51 μg/μL). 100 μg of proteins from each patient was used for mass spectrometry. The samples were run in 10% SDS-PAGE under reducing conditions and stained with CBB and de-stained until the proteins bands were clearly visible. For in gel-digestion of the proteins, the resolved proteins in each lane was cut into four fractions of different molecular weight (Figure 4.1). The total amount of protein was similar for all the samples on the gel. The intensity of the band corresponding to albumin (fraction 2) was found to be slightly higher in PDR vitreous compared to NDR and NDM samples.

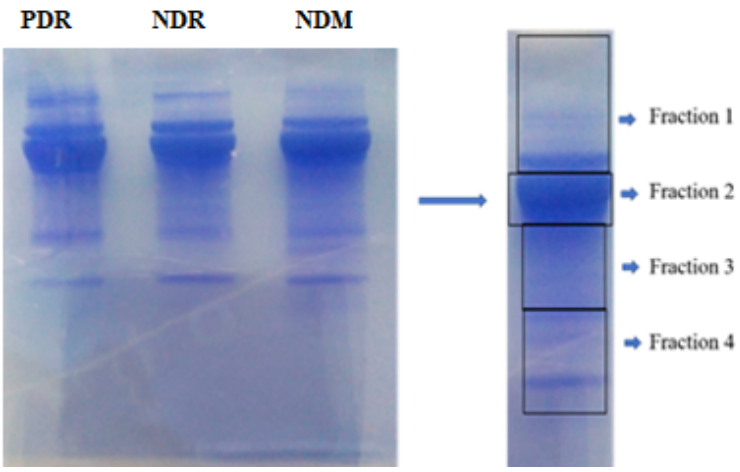


Figure 4.1. Representative CBB stained image of protein samples run in 10% SDS-PAGE. The fractions of similar molecular weight were taken from each of the sample lane for mass spectrometry

Trypsin digested and desalted peptides of each fractions were run separately in Q Exactive Quadrupole Orbitrap Mass Spectrometer (Thermoscientific) and the spectra obtained from each of the sample was searched using Andromeda search engine and MaxQuant against the Human proteome from UniProt database (Version 1.3.0.5). Based on the defined analysis parameters (minimum peptide identification for a protein as 2 with an FDR of 0.05% in protein and peptide levels) and following the removal of known contaminants,1079 proteins were identified in these vitreous samples. The total and the mean (Mean±SEM) number of proteins was higher in the NDM group followed by the other two groups as shown in Figures 4.2 and Figure 4.3.

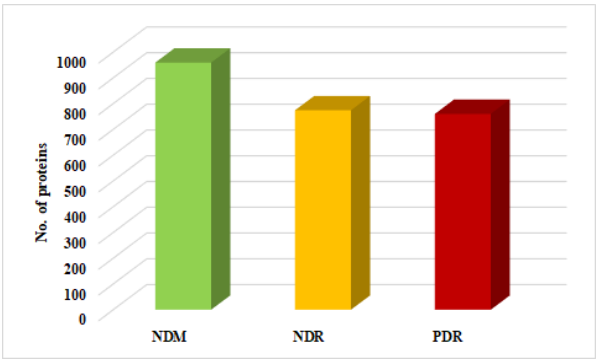


Figure 4.2. Total number of proteins identified across the three groups

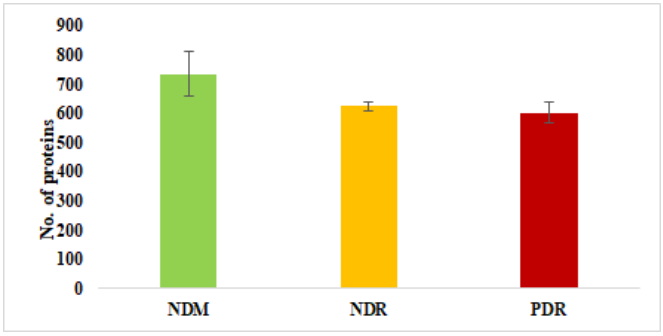


Figure 4.3. Mean number of proteins present in samples from each group

4.1.3. Comparison of the data with the published vitreous proteome database

The proteins identified in the present study were compared with the published vitreous proteome reports and eye databases, which included proteomes from all parts of eye including vitreous, aqueous humor, retina, RPE-choroid, optic nerve, lens, ciliary body, iris, aqueous humor, cornea, sclera and tear. It was observed that 938 proteins identified in the present study could be linked to the existing literature and databases, but 141 proteins were unique. In order to determine their novelty, the sequence coverage of these proteins were analysed and those proteins, which were not present in minimum three vitreous samples and had a low

sequence coverage of < 2% were excluded. Thus, 27 novel vitreous proteins were identified. Eleven of these proteins were found to be reported in eye proteome database (RPE-choroid, ciliary body, optic nerve, tear, sclera, iris, cornea, lens) and 16 proteins were not reported in any of the eye proteome database (Table 4.2 and Table 4.3)

Table 4.2: List of proteins identified in eye proteome database and their corresponding peptides and sequence coverage in the present cohort

Protein ID	Protein name and Gene ID	Mol wt (kDa)	Seq. Length	NDM (Peptides, n=3)	NDM Seq. coverage (%)	NDR (Peptides, n=3)	NDR, Seq. coverage (%)	PDR (peptides, n=3)	PDR, Seq. coverage (%)	Tissue identified in eye proteome
P01601	Ig kappa chain V-I region HK101	12.73	117	2±0.5	13.7	2±0.5	13.7	1.3±0.33	13.7	Tear
P01721	Ig lambda chain V-VI region AR	12.566	117	X	X	1.6±0.3	29.6	X	X	
P01772	Ig heavy chain V-III region KOL	13.074	117	3.6±0.33	34.2	X	X	2.3±0.88	33	Tear
P01780	Ig heavy chain V-III region JON	12.943	117	16.7±0.8	38.5	15±2.6	35.3	15±2.64	38.5	Tear
P08254	Stromelysin-1 (MMP3)	53.977	477	5±3.2	6	2.6±0.3	2.46	X	X	RPE -choroid
P23763	Vesicle-associated membrane protein 1 (VAMP1)	12.902	118	1.7±0.8	9	5.3±0.6	13.6	3±1.52	13.6	Retina, cornea, Iris, Ciliary body, optic nerve
P30508	HLA class I histocompatibility antigen, Cw-12 alpha chain (HLA-C)	40.885	366	5±1.1	9.9	6.6±0.3	18.8	X	X	Retina, cornea
P57053	Histone H2B type F-S (H2BFS)	13.944	126	10±5.5	32	7±1.1	23.3	5±2.08	17.7	Retina, RPE-choroid
P68431	Histone H3.1 (HIST1H3A)	15.404	136	6.6±4.17	16.2	4.3±1.2	16.4	3.6±1.66	13	Retina, cornea, Ciliary body, RPE-Choroid, lens
Q6UWF9	Protein FAM180A (FAM180A)	19.733	173	7±1.15	4.6	X	X	X	X	Sclera
Q95604	HLA class I histocompatibility antigen, Cw-17 alpha chain (HLA-C)	41.238	372	4.6±0.33	8.5	X	X	6.6±1.2	4.6	Retina

A detailed analysis was performed for the remaining16 proteins that were not reported in any of the eye proteome. The detailed comparisons of these proteins are given in the table 4.3.

Table 4.3: Novel vitreous proteins detected in the present study, the number of peptides identified and sequence coverage

Protein ID	Protein name and Gene ID	Mol wt (kDa)	Seq. length	NDM (Peptides, n=3)	NDM Seq. coverage (%)	NDR (Peptides, n=3)	NDR Seq. coverage (%)	PDR (peptides, n=3)	PDR Seq. coverage (%)
P01742	Ig heavy chain V-I region EU	12.659	117	4.3±1.3	25.3	3.3±0.33	22.2	4.33±1.51	25.3
P01709	Ig lambda chain V-II region MGC	12.382	118	12±2	16.4	13±1.73	17.8	13±2.1	16.4
P01704	Ig lambda chain V-II region TOG	12.597	120	4±1.52	13.3	7.6±2.72	13.3	4.33±1.8	13.3
K7ELM0	SEC14 like lipid binding 1 (SEC14L1)	16.721	143	11.3±1.2	7.7	13±1.73	7.7	16.33±1.4	7.7
A6NI72	Putative neutrophil cytosol factor 1B (NCF1B)	44.816	391	3±1.5	4.26	4±0.5	4.8	3±1.62	4.26
G5E9W9	GTPase IMAP family member 4 (GIMAP4)	39.033	343	2.6±1.66	7.86	1.3±0.33	7.7	3±2	8.5
Q9NZC2	Triggering receptor expressed on myeloid cells 2 (TREM2)	25.447	230	1.3±0.33	7.96	2±0.57	10.16	3±0.57	12.2
O00204	Sulfotransferase family cytosolic 2B member 1 (SLUT2B1)	41.307	365	3±0.57	5.2	X	X	4.6±0.33	5.2
Q96HR3	Mediator of RNA polymerase II transcription subunit 30 (MED30)	20.277	178	8.6±2.3	5.1	3.6±0.33	5.1	6.3±1.2	5.1
Q9BXD5	N-acetyl neuraminate lyase (NPL)	35.162	320	3.3±2.02	8.96	2.6±0.33	5.6	1.6±0.33	5.6
Q14587	Zinc finger protein 268 (ZNF268)	108.37	947	3.6±0.33	2.4	X	X	3.6±0.66	2.9
Q8IY82	Coiled-coil domain-containing protein lobo homolog (CCDC135)	103.5	874	6.6±0.88	4	X	X	6±1.5	3.3
H3BN98	40S ribosomal protein S15a (RSP15A)	27.168	237	2.3±1.33	8.43	1±0.57	2.3	2.3±1.3	8.4
I3L504	Eukaryotic translation initiation factor 5A-1 (EIF5A)	20.503	186	2.3±0.66	10.763	X	X	2.6±0.88	15.4
Q9BZF3	Oxysterol-binding protein-related protein 6 (OSBPL6)	106.3	934	2.3±0.33	2.13	X	X	2±0.57	1.5
P0DOY2	immunoglobulin lambda constant 2 (IGLC2)	11.293	106	63.6±2.3	89.93	63±7.2	89.9	63.3±2.47	89.9

Next, the significant molecular functions and biological processes were evaluated for the novel vitreous proteins along with their biological functions (Table 4.4).

Table 4.4: Significant molecular functions and biological process of the novel vitreous proteins identified from the present study

Proteins	Molecular functions
OSBPL6	Lipid binding
MMP3	Metallopeptidase activity
SULT2B1	Sulfotransferase activity
MED30	Transcription regulator activity
TREM2	Receptor activity
GIMAP4	GTPase activity
HIST1H3A, ZNF268	DNA binding
VAMP1	Auxiliary transport protein activity
OSBPL6	Transporter activity
Proteins	**Biological process**
SULT2B1, NPL	Metabolism
OSBPL6	Lipid metabolism
MMP3	Protein metabolism
NPL	Energy pathways
HIST1H3A, MED30, ZNF268	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
VAMP1, OSBPL6	Transport
GIMAP4, TREM2	Cell communication
GIMAP4, TREM2	Signal transduction

4.1.4. Relative abundance of proteins presents in each group

Abundance of proteins present in each group was calculated based on the intensity of each protein, which is a semiquantitative method for estimating protein abundance. Further, fractional distributions of these proteins in the vitreous samples were calculated based on the number of peptides present per protein in each category. This was done by taking the mean

percentage of number of total peptides for the 20 most abundant proteins identified from the present study vitreous proteome data. The Pie diagram (Figures 4.4a, 4.4b and 4.4c) provides summaries the relative amounts of highly abundant proteins present in PDR, NDR and NDM vitreous, respectively. A comparison of these proteins with NDR and NDM was done and is provided in table 4.5. The results identified albumin as the most abundant protein in the vitreous with relatively higher in PDR cases compared to NDR and NDM. The other abundant included Serotransferrin, Complement C3 (C3), Alpha-1 antitrypsin, Complement C4-B (C4-B), apolipoprotein A1 (Apo-A1), ceruloplasmin, hemopexin, FGA, apolipoprotein A-IV (Apo A-IV), Alpha -2 macroglobulin (A2M), Retinol binding protein 3 (RBP3), Ig -γ-1 chain C region, clusterin, α-1-antichymotrypsin, Antithrombin-III, apolipoprotein E, PEDF, transthyretin and complement factor B (CFB). There were no significant differences in the quantity of the abundant proteins between PDR, NDR and NDM.

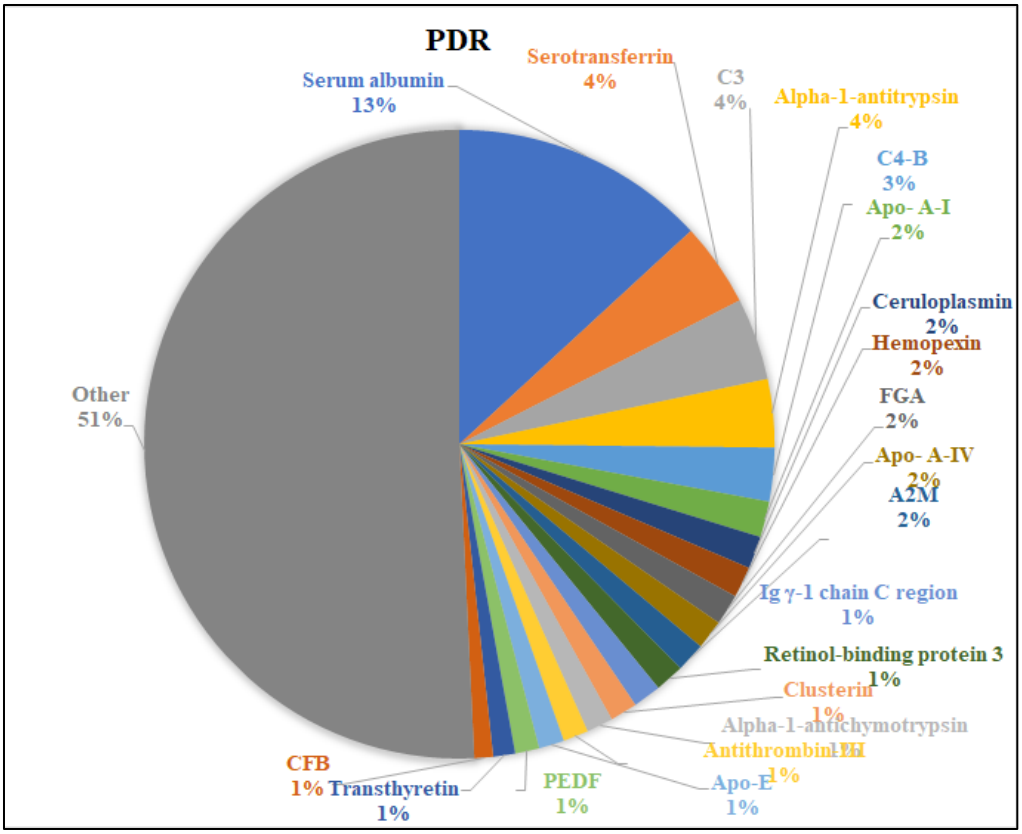


Figure 4.4a: Fractional distribution of abundant proteins present in PDR vitreous

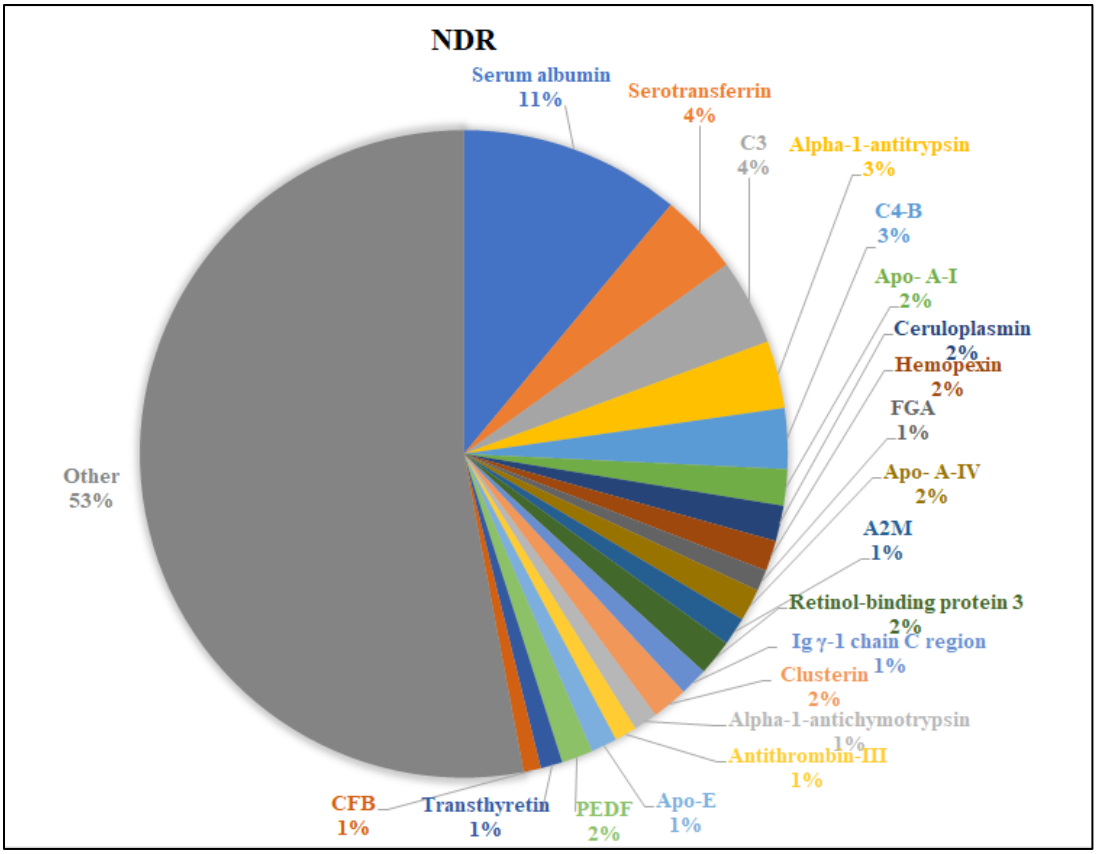


Figure 4.4b: Fractional distribution of abundant proteins present in NDR vitreous

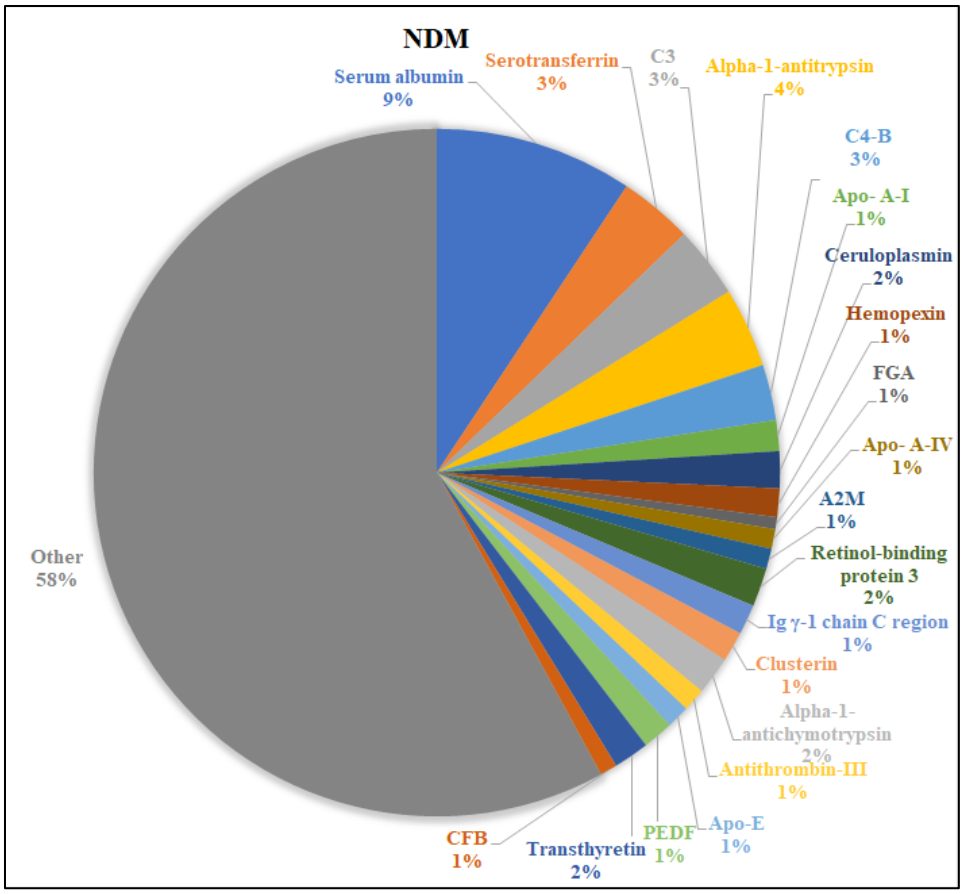


Figure 4.4c: Fractional distribution of abundant proteins present in NDM vitreous

Table 4.5. The percentage of abundant proteins detected in the vitreous of PDR (n=3), NDR (n=3) and NDM (n=3)

Protein names	% PDR	% NDR	% NDM
Serum albumin	13.13	11.02	9.28
Serotransferrin	4.3	4.03	3.45

Complement C3	4.22	4.29	3.36
Alpha-1-antitrypsin	3.51	3.37	3.74
Complement C4-B	2.75	3.02	2.6
Apolipoprotein A-I	1.84	1.81	1.44
Ceruloplasmin	1.66	1.77	1.73
Hemopexin	1.59	1.53	1.33
Fibrinogen alpha chain	1.57	1.06	0.56
Apolipoprotein A-IV	1.51	1.61	0.92
Alpha-2-macroglobulin	1.5	1.41	0.95
Retinol-binding protein 3	1.49	1.78	1.8
Ig gamma-1 chain C region	1.44	1.4	1.41
Clusterin	1.39	1.79	1.42
Alpha-1-antichymotrypsin	1.35	1.18	1.78
Antithrombin-III	1.3	1.11	1.08
Apolipoprotein E	1.29	1.305	1.08
Pigment epithelium-derived factor	1.23	1.56	1.43
Transthyretin	1.12	1.09	1.67
Complement factor B	0.96	0.86	0.8
Other	50.75	52.91	57.63

4.1.5. Gene-Ontology annotation of the proteins observed in the vitreous proteome

To get an initial understanding of the vitreous proteome and its functional overview in each group, Gene ontology (GO) was performed using PANTHER GO-database software (Version 13.1). This categorized proteins based on their role in biological processes, molecular functions and cellular compartments. In order to understand the distribution of proteins, percentages of proteins involved in each subcategory were calculated from total number of mapped proteins present in each group (Table 4.6). The data was further analysed as GO-slim functions based on binomial distribution after Bonferroni correction.

Table 4.6: Total and mapped protein IDs by GO- annotation in each category

	NDM	NDR	PDR
Total ID	960	774	760
Mapped ID	829	674	655

4.1.5.1. Identification of Biological process (GO-slim BP)

Overall, 34 significant GO-slim biological process were identified in the NDM, NDR and PDR groups. Almost 40% of the proteins fell in unclassified category under in each group (NDM: 307 (37%), NDR: 262 (38.8%) and PDR- 242 (36.9%)). For an optimal comparison, the significant biological process that involved >5% of the proteins and those below 5% of the proteins in each of these three group were analysed. As shown in the figure 4.5a, a large percentage of proteins were found to align with the biological process such as metabolic process (NDM: 33.7 %, NDR: 31.3%, PDR: 32.5%), signal transduction (NDM: 14.7%, NDR: 13.9%, PDR: 15.8%), cellular component organization or biogenesis (NDM:16.04%, NDR: 14.39%, PDR: 13.8%), immune system process (NDM: 10.37 %, NDR: 11.27%, PDR: 12.67%) and immune response (NDM: 8.56 %, NDR: 9.79%, PDR: 10.68%). The 5 sub-categories identified in biological processes with less than 5% included : response to biotic stimulus (NDM: 3.03%, NDR:3.7%, PDR:4.27%), complement activation (no-DM: 3.25%, DM: 3.7%, PDR: 4.12%), B- cell mediated immunity (NDM : 3.25%, NDR: 3.7%, PDR: 4.12%), cell recognition (NDM : 3.25%, NDR: 3.85%, PDR: 4.12%) and defense response to bacterium (NDM: 3.25%, NDR: 3.7%, PDR: 4.12%) (Figure 4.5b). The biological processes were largely identical among the study groups. But a considerable increase in percentage of the proteins were noted in the PDR group compared to NDR and NDM groups in some of

the sub-categories that included immune system process (percentage of increase-PDR vs NDR-1.39% and PDR vs NDM-2.29%, NDR vs NDM-0.9%) and immune response (percentage of increase-PDR Vs NDR-0.89% and PDR Vs NDM-2.12%, NDR Vs NDM-1.22%).

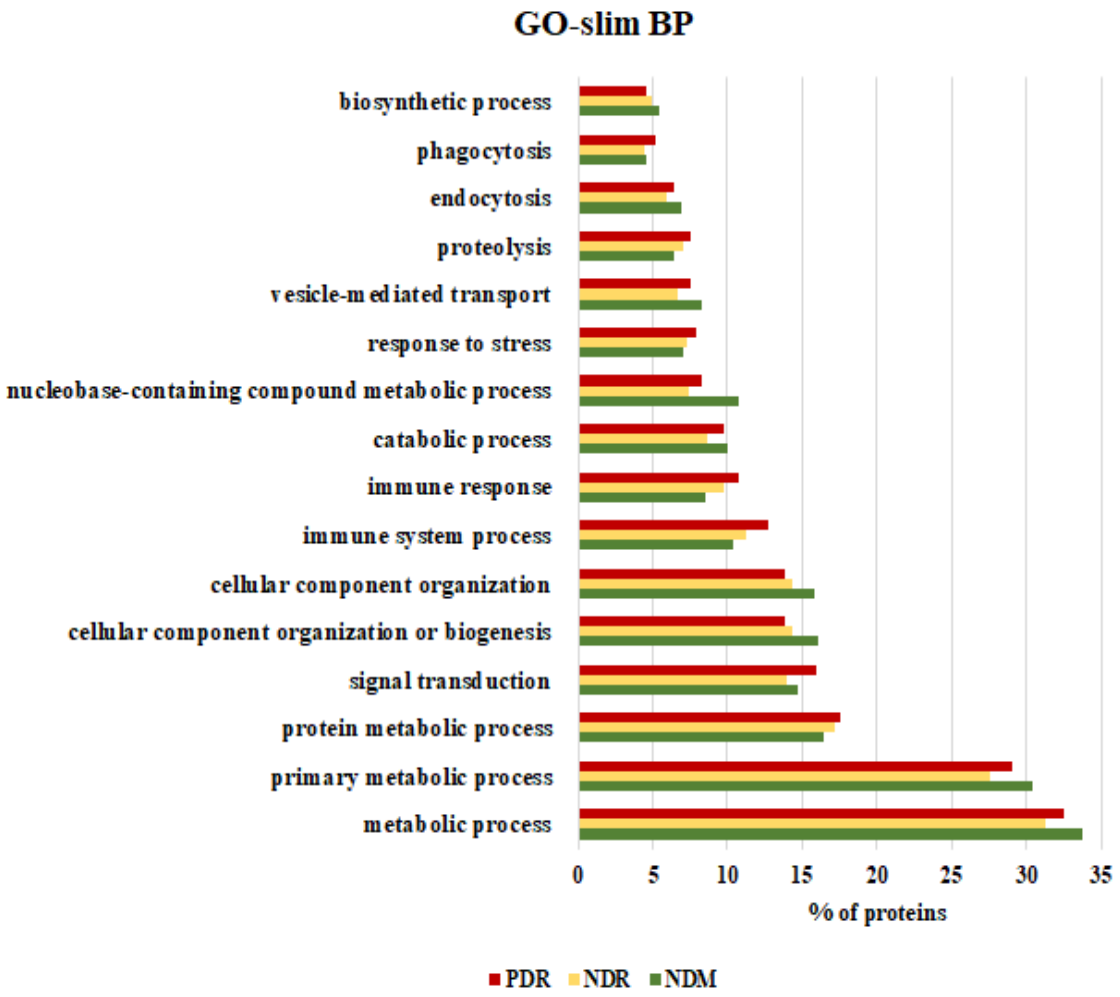


Figure 4.5a: GO-slim BP that involved more than 5% proteins from the three groups

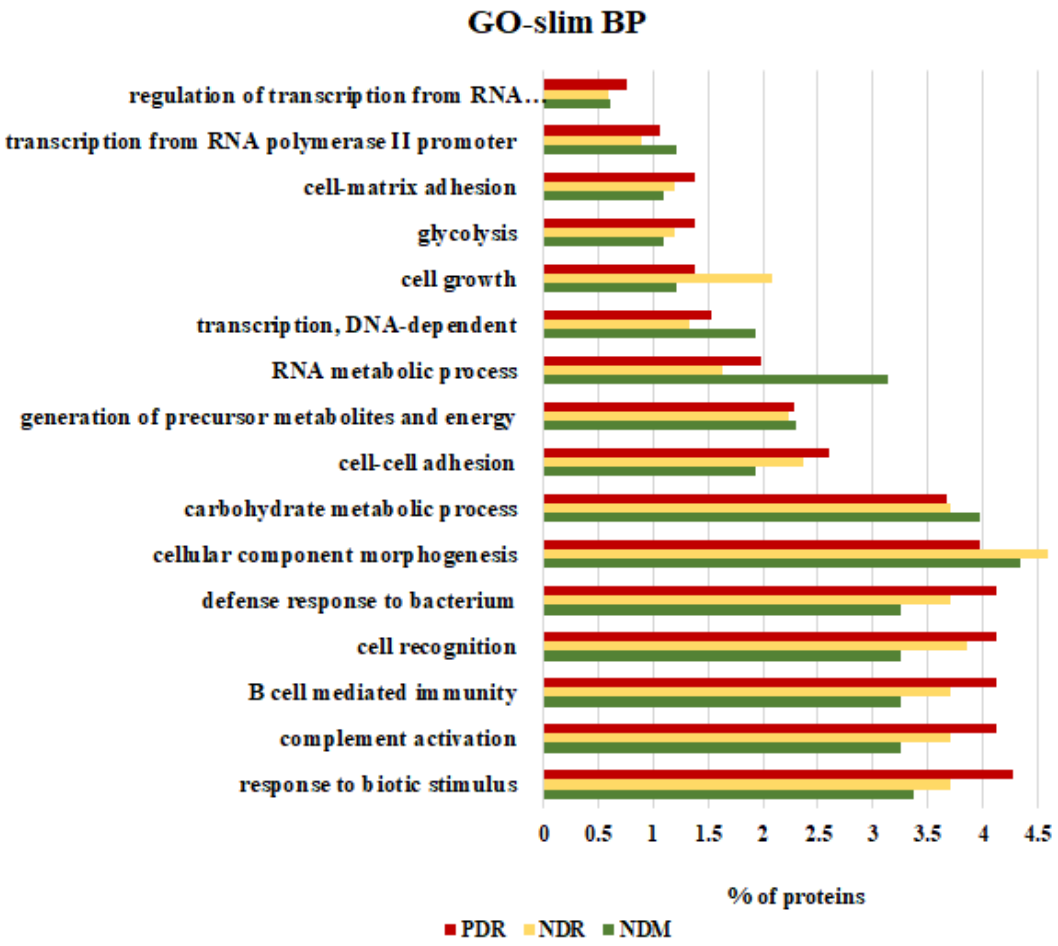


Figure 4.5b: GO-slim BP that involved less than 5% proteins from the three groups

4.1.5.2. Identification of Molecular function (GO-slim MF)

GO-slim molecular function identified 30 molecular functions in NDM group, 28 molecular functions in NDR group and 29 in PDR groups. Similar to biological process, a large number of proteins fell in the unclassified category (NDM: 380 (45.8%), NDR: 323 (47.92%), PDR: 315 (48.09%). For an optimal comparison, the significant molecular functions that involved >5% of the proteins and below 5% of the proteins in each group were analyzed. (Figure 4.6a and Figure 4.6b). The top 5 molecular functions identified were binding (NDM: 28.7%, NDR:

28.9%, PDR: 28.5%), catalytic activity (NDM: 28.46%, NDR: 27.15% PDR: 27.17%), protein binding (NDM: 20%, NDR: 21.9%, PDR: 21.06%), hydrolase activity (NDM: 15.9%, NDR: 16.02%, PDR:16.48%) and receptor binding (NDM: 8.8%, NDR: 10.9%, PDR: 10.38 %). The top 5 subcategories identified with <5% proteins included oxidoreductase activity (NDM: 4.9%, NDR: 4.1%, PDR: 4.1%), structural molecular activity (NDM: 4.9%, NDR: 3.2%, PDR: 3.6%), nucleic acid binding (NDM: 4.2%, NDR: 2%, PDR: 2.7%), calcium ion binding (NMD: 2.41%, NDR: 2.96%, PDR: 2.74 %), and antigen binding (NDM: 3.3%, NDR: 3.7%, PDR: 4.2%). There were no significant differences in the percentages of proteins between each sub category. However, within the molecular functions, the structural constituent of ribosome was found to be absent in the NDR group.

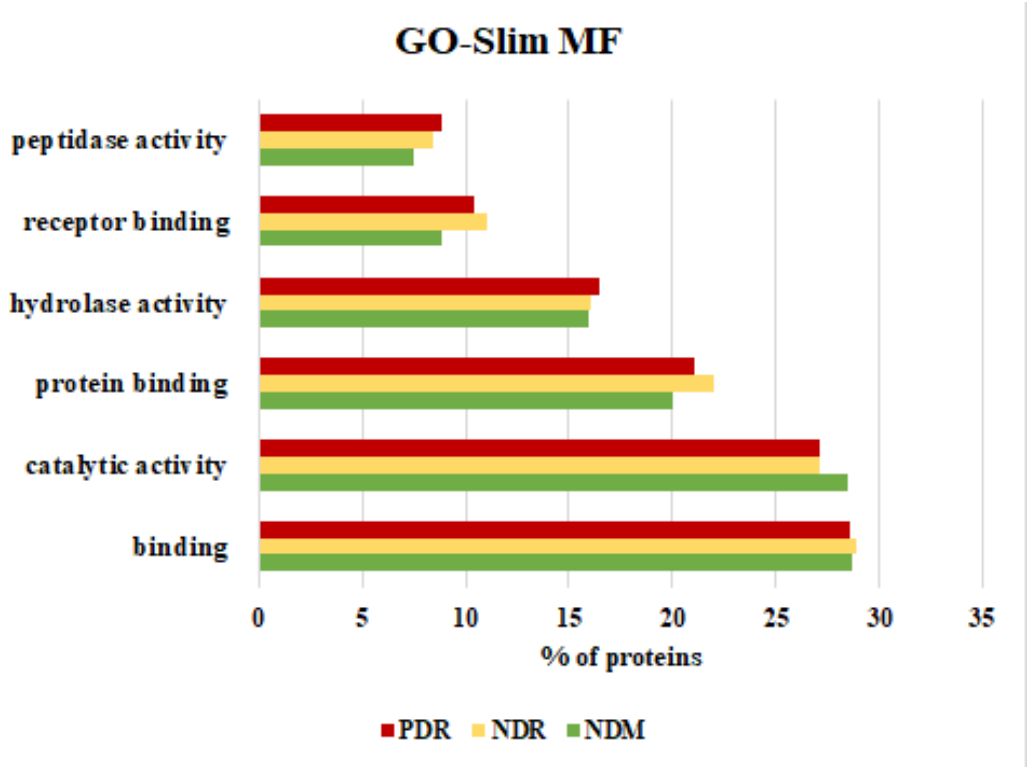


Figure 4.6a: GO-slim molecular functions, that involved > 5% proteins from the three groups

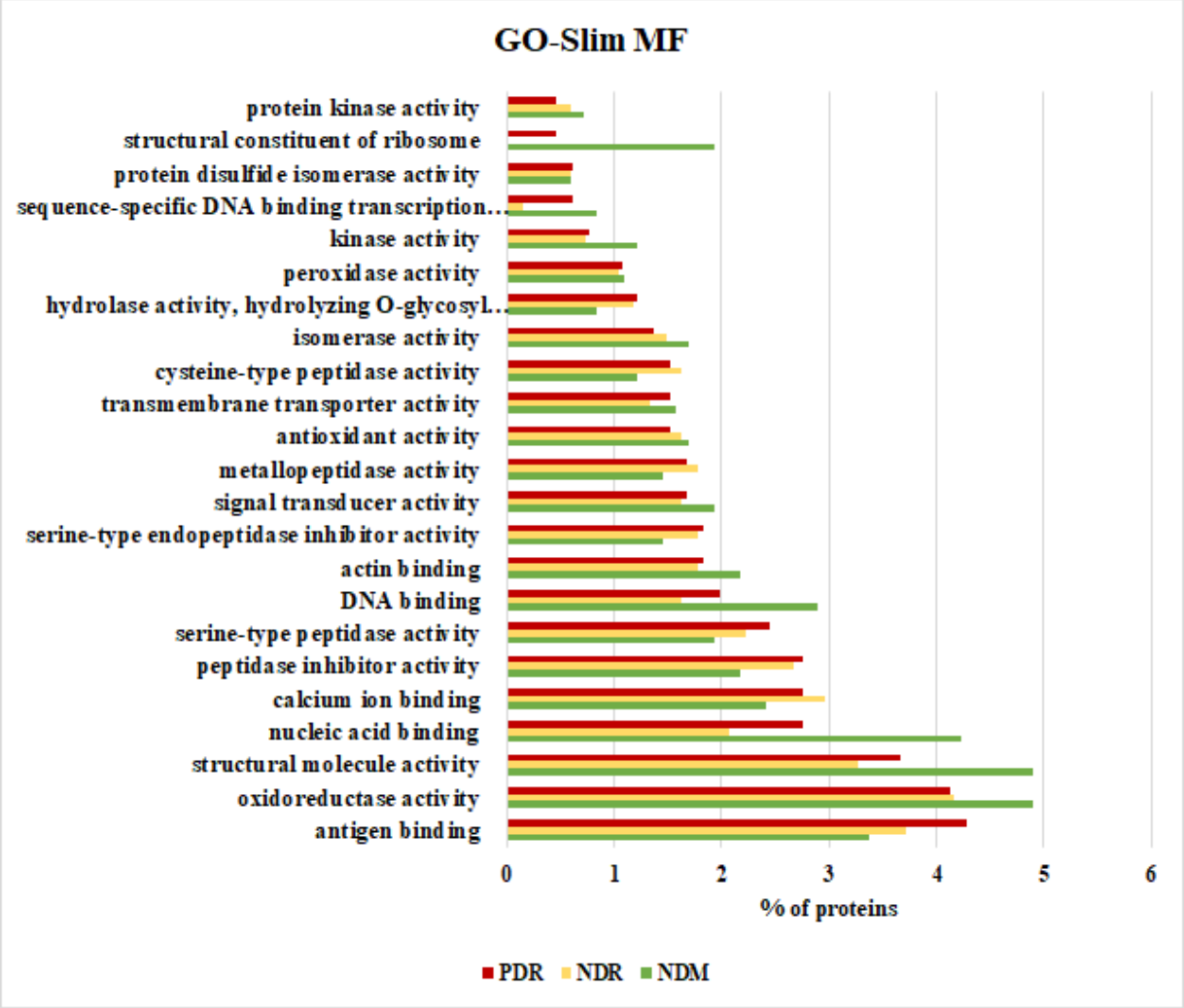


Figure 4.6b: GO-slim molecular functions, that involved < 5% proteins from the three groups

4.1.5.3. Identification of Cellular component (GO-slim CC)

A total of 20 different cellular components were identified in proteins of PDR and NDM, while 19 were identified in the NDR group (Figure 4.7). It was observed that 385 (46.4%), 329 (48.8%) and 307(46.9%) proteins could not be classified in NDM, NDR and PDR groups, respectively. A large proportion of the proteins were extracellular proteins that included

proteins in the extracellular region (NDM: 20.98%, NDR: 26.40% PDR: 27.06%), extracellular region part (NDM: 19.54%, NDR: 24.4% PDR: 25.22%) and extracellular space (NDM: 17.73%, NDR: 22.25% PDR: 22.62%). The percentages of extracellular proteins were found to be higher in PDR and NDR group compared to the controls. The proteins of the cytoplasmic part were found to be higher in NDM (10.61%) compared to NDR (6.37%) and PDR (7.95%). The proteins of the cytosol (NDM: 4.7%, NDR: 3.4% PDR: 3.9%), plasma membrane part proteins (NDM: 4.34%, NDR: 4.89%, PDR:5.19%), leaflet of membrane bilayer (NDM: 3.9%, NDR: 4.5% PDR: 4.89%) and extracellular matrix proteins (NDM: 2.65%, NDR: 3.56% PDR: 3.82%) were enriched in the three groups. Cytosolic large ribosomal subunit proteins were found only in NDM (1.2%) and PDR (0.3%) but not in NDR.

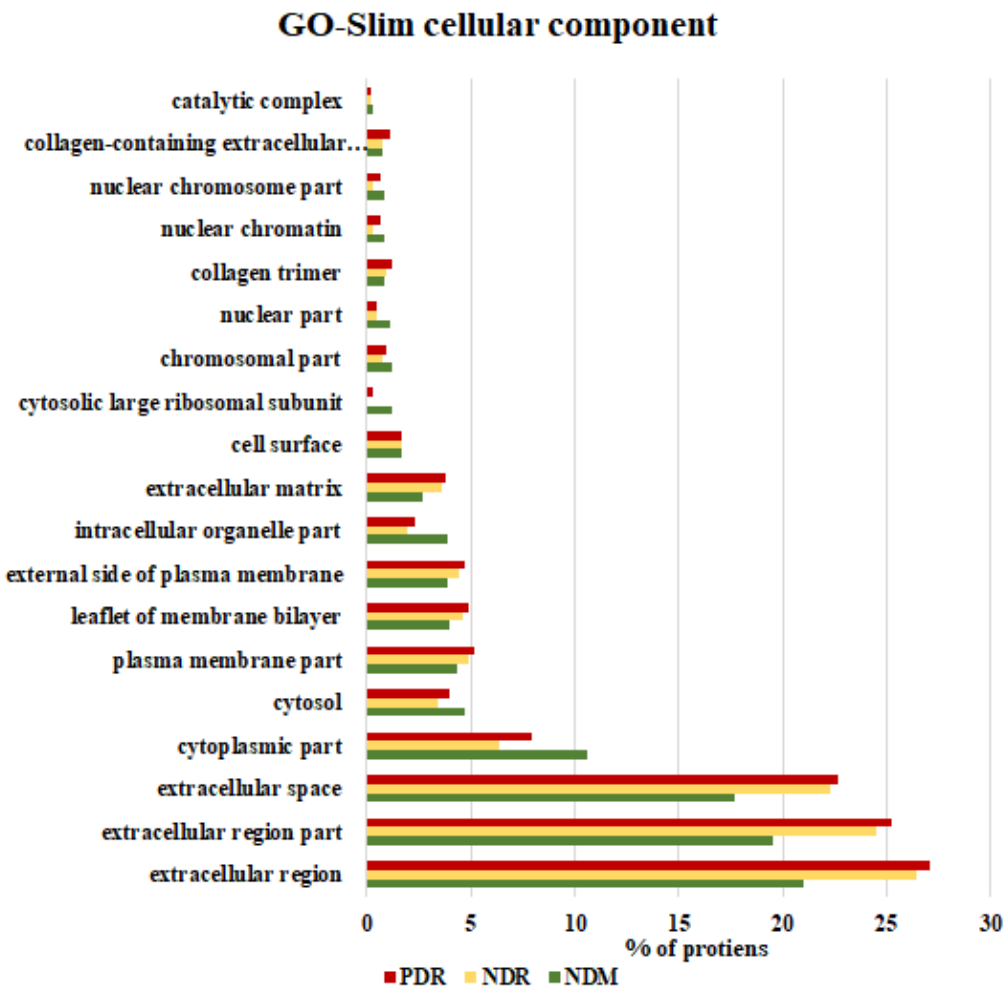


Figure 4.7: GO-Slim Cellular component of proteins present in all the three groups

4.1.6. Comparison of NDM, NDR and PDR vitreous proteome

To understand the common and unique proteins between NDM, NDR and PDR the list of proteins identified in each group were compared by generating a Venn diagram (Figure 4.8) using the Gene Venn software. A total of 618 proteins were commonly present in all the three groups.

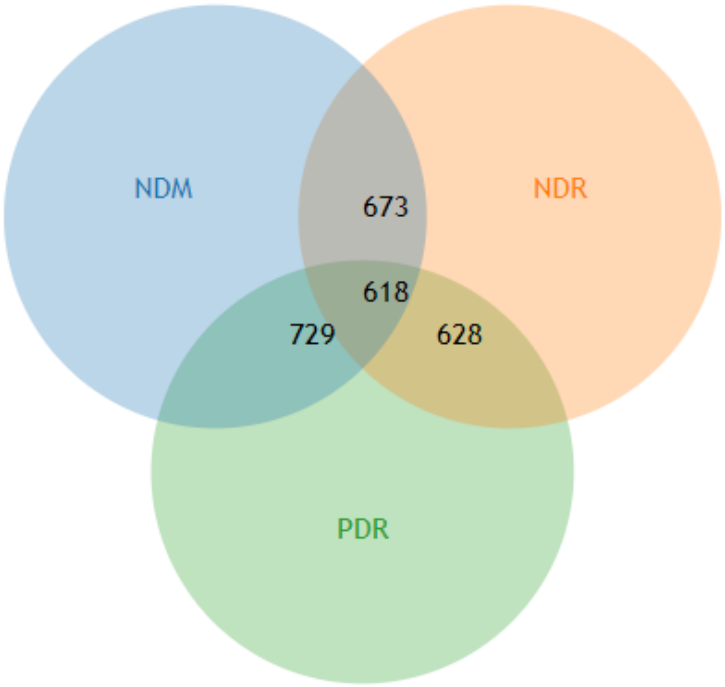


Figure 4.8. Venn diagram representation of proteins identified across the three groups

4.1.6.1. Analysis of unique proteins in NDM, NDR and PDR

There were 20, 90 and 175 unique proteins identified in the vitreous PDR, NDR and NDM, respectively. A detailed pathway analysis of these unique proteins was carried out using the STRING software. The REACTOME and KEGG pathways were analysed and compared between the groups to understand if any of the biological pathways were getting more activated in a study group. The KEGG and REACTOME pathways in PDR, NDR and NDM are represented in tables 4.7 and 4.8 respectively. Each of these pathways were analysed and compared between the groups to identify the similar proteins present in PDR, NDR and NDM. Only the phagosome pathway identified through KEGG was common between NDR and NDM, while rest of the pathways were found to be unique across the three groups.

Table 4.7: KEGG pathways identified based on unique proteins present in PDR, NDR and NDM

PDR	False discovery rate
Staphylococcus aureus infection	0.05
Complement and coagulation cascade	0.06
ECM-receptor interaction	0.08
NDR	**False discovery rate**
Cell adhesion molecules (CAMs)	7.00E-06
Lysosome	0.005
Type I diabetes mellitus	0.01
Allograft rejection	0.01
Graft-versus-host disease	0.01
Autoimmune thyroid disease	0.01
Viral myocarditis	0.01
Other glycan degradation	0.02
Phagosome	0.02
Antigen processing and presentation	0.02
Herpes simplex infection	0.04
NDM	**False discovery rate**
Ribosome	6.18E-09
Phagosome	1.20E-07
Endocytosis	8.23E-06
Cell adhesion molecules (CAMs)	0.0001
Leukocyte trans endothelial migration	0.0001
Salmonella infection	0.0001
Collecting duct acid secretion	0.0009
Epithelial cell signaling in Helicobacter pylori infection	0.0009
Glutathione metabolism	0.002
Regulation of actin cytoskeleton	0.002

Table 4.8: REACTOME pathways identified based on unique proteins present in PDR, NDR and NDM

PDR	False discovery rate
Initial triggering of complement pathway	0.002
Signaling by PDGF	0.03
ECM-proteoglycans	0.08
NDR	**False discovery rate**
Endosomal/Vacuolar pathway	0.003
Adherens junctions' interactions	0.003
Cell-Cell communication	0.01
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	0.01
Nectin/Necl trans heterodimerization	0.02
Extracellular matrix organization	0.04
Glycosaminoglycan metabolism	0.04
NDM	**False discovery rate**
Neutrophil degranulation	4.50E-21
Innate Immune System	7.28E-18
Immune System	2.97E-16
Signaling by ROBO receptors	2.04E-15
Axon guidance	1.27E-14
Seleno amino acid metabolism	2.33E-13
Eukaryotic Translation Elongation	1.32E-12
Peptide chain elongation	8.75E-12
Viral mRNA Translation	8.75E-12
Regulation of expression of SLITs and ROBOs	8.75E-12

4.1.6.2. Analysis of the common proteins shared only between diabetes (PDR and NDR) and non-retinopathy groups (NDM and NDR)

There were 54 common proteins in the non-retinopathy group (NDM and NDR) and 10 proteins in the diabetes group (PDR and NDR). This is despite the fact that no RECATOME or KEGG pathways were associated with the latter group. Interestingly, 4 REACTOME pathways were identified in the non-retinopathy group (Table 4.9).

Table 4.9: REACTOME pathways in the non-retinopathy group

REACTOME Pathway	False discovery rate
Innate Immune System	3.10E-06
Immune System	6.41E-06
Neutrophil degranulation	0.016
DNA Damage/Telomere Stress Induced Senescence	0.044

4.1.7. Quantitative analysis of protein expression through label free quantitation (LFQ) method

The understanding of differentially regulated proteins in each group is essential for disease characterization and for the identification of potential disease specific proteins. In order to achieve this, a proper understanding of diabetes induced alteration in the vitreous proteome is necessary. Hence, the mean fold change of protein expressions based on the normalized intensity values were calculated in DM group (PDR+NDR) compared to NDM group. Further, it was compared between PDR and non-retinopathy (NDR+NDM), PDR to NDR and PDR to NDM (Table 4.10).

4.1.7.1. Comparison of proteins among Diabetic Mellitus vs No Diabetic Mellitus (PDR+NDR Vs NDM)

There were 15 proteins that were found be differentially expressed in the DM group compared to NDM with a minimum fold change of 1.5 (p-value <0.05). This include 8 upregulated and 7 down regulated proteins. The levels of these proteins in in other combinations were calculated to understand if the expressions of these proteins were further getting altered at proliferative stages of the disease.

Table 4.10: Comparisons of the differentially regulated proteins across various phenotypes

Protein name	diabetes (PDR +NDR) Vs NDM	*p*-Value	PDR Vs NDM	*p*-Value	PDR Vs no retinopathy	*p*-Value	PDR Vs NDR	*p*-Value
FRZB	1.5 ± 0.27	0.02	1.28 ±0.26	0.009	0.45 ± 0.04	0.6	-0.38±0.63	0.6
AGRN	1.58 ± 0.45	0.02	1.34 ±0.59	0.09	0.43 ± 0.41	0.6	-0.48±0.21	0.5
APLP1	2.33 ± 0.79	0.04	2.5 ±0.99	0.04	1.42 ± 0.32	0.3	0.34±0.84	0.8
TIMP2	2.34 ± 1.2	0.05	2.07 ±0.8	0.2	0.76 ± 0.59	0.6	-0.55±0.05	0.5
IGFBP6	2.36 ± 1.19	0.04	2.14 ±0.93	0.2	0.86 ± 0.76	0.5	-0.43±0.47	0.6
APLP2	2.99 ± 0.83	0.04	2.29 ±1.38	0.08	0.44 ± 0.56	0.8	-1.41±0.82	0.4
COL1A2	3.01 ± 0.11	0.005	3.52 ±0.63	0.003	2.27 ± 0.54	0.07	1.02±0.77	0.4
VCAN	4.84 ± 1.29	0.006	5.63 ±0.91	0.02	3.6 ± 0.71	0.08	1.58±0.4	0.2
GSTP1	-2.87 ± 1.46	0.05	-2.5 ±0.68	0.2	0.74±0.44	0.5	-0.88 ± 0.86	0.6
IDH1	-2.79 ± 1.25	0.03	-2.23 ±1.49	0.2	1.13±0.85	0.2	-0.55 ± 0.79	0.7
LDHA	-2.66 ± 0.7	0.0002	-2.7 ±0.55	0.02	-0.07±0.14	0.7	-1.39 ± 0.18	0.2
VASN	-2.66 ± 0.36	0.01	-3.51 ±0.82	0.01	-1.71±0.84	0.06	-2.61 ± 0.57	0.01
LDHB	-2.64 ± 0.24	0.0002	-2.67 ±0.48	0.001	-0.06±0.39	0.9	-1.37 ± 0.25	0.2
PRDX1	-2.04 ± 1.12	0.04	-1.72 ±1.32	0.2	0.64±0.52	0.2	-0.54 ± 0.2	0.6
C6	-1.5 ± 0.35	0.02	-1.25 ±0.04	0.06	-0.44 ± 0.34	0.6	0.37±0.21	0.5

Data represented as Mean ±SEM

4.1.7.2. Upregulated proteins in DM vs NDM group

Eight proteins were found to be significantly upregulated in DM compared to NDM. The levels of these proteins showed a similar increase in PDR vs NDM group. This indicated that the expressions of these proteins are typically regulated by diabetes and there were no further changes during the proliferative stage of the disease (Figure 4.9).

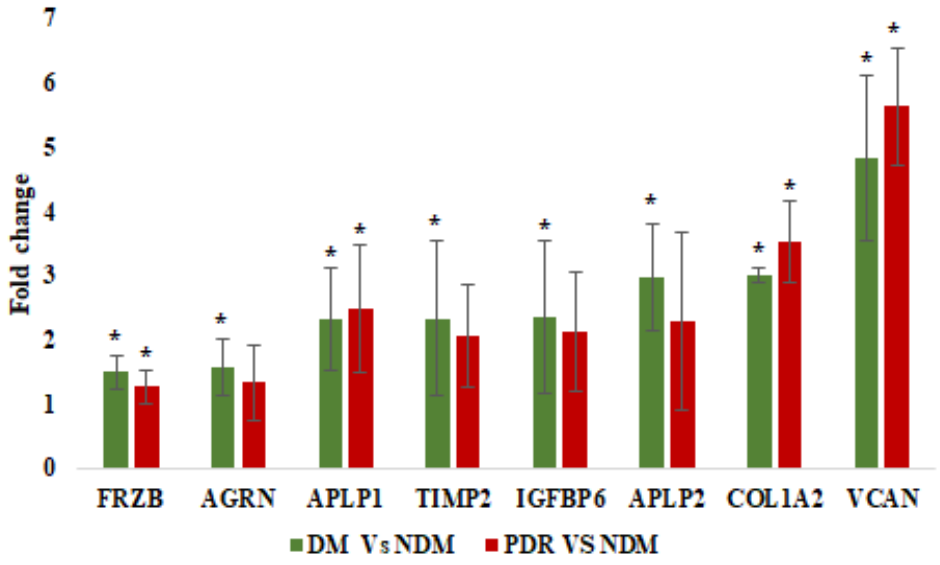


Figure 4.9: Significantly upregulated proteins in DMs vs NDM and PDR vs NDM group, *p<0.05

a. Major pathways associated with proteins upregulated in the DM vs no-DM groups

The KEGG and REACTOME pathway analysis was done to understand the major pathways getting affected during diabetes with respect to upregulated proteins. This identified only one significant KEGG pathway and 12 significantly enriched REACTOME pathways (Table 4.11). The KEGG pathway identified was ECM-receptor interaction, with an FDR value of 0.0016 with the proteins such as AGRN and COL1A2. Further, percentage of proteins

corresponding to each of the pathway from the total differentially regulated proteins were calculated. The top three pathways based on the number of proteins present in it was found to be associated with extracellular matrix organization, ECM proteoglycans and Regulation of IGF transport and uptake by IGFBPs as represented in the Figure 4.10.

Table 4.11: List of proteins present in different KEGG and REACTOME pathways of upregulated proteins in DM vs NDM group

KEGG Pathway	**Matching Proteins**
ECM-receptor interaction	AGRN, COL1A2
REACTOME pathway	**Matching proteins**
Extracellular matrix organization	AGRN, COL1A2, TIMP2, VCAN
ECM proteoglycans	AGRN, COL1A2, VCAN
Regulation of IGF transport and uptake by IGFBPs	APLP2, IGFBP6, VCAN
A tetra- saccharide linker sequence is required for GAG synthesis	AGRN, VCAN
Defective B4GALT7 causes EDS, progeroid type	AGRN, VCAN
Defective B3GAT3 causes JDSSDHD	AGRN, VCAN
Defective B3GALT6 causes EDSP2 and SEMDJL1	AGRN, VCAN
Non-integrin membrane-ECM interactions	AGRN, COL1A2
Integrin cell surface interactions	AGRN, COL1A2
Post-translational protein phosphorylation	APLP2, VCAN
Degradation of the extracellular matrix	COL1A2, TIMP2
Platelet activation, signaling and aggregation	APLP2, COL1A2

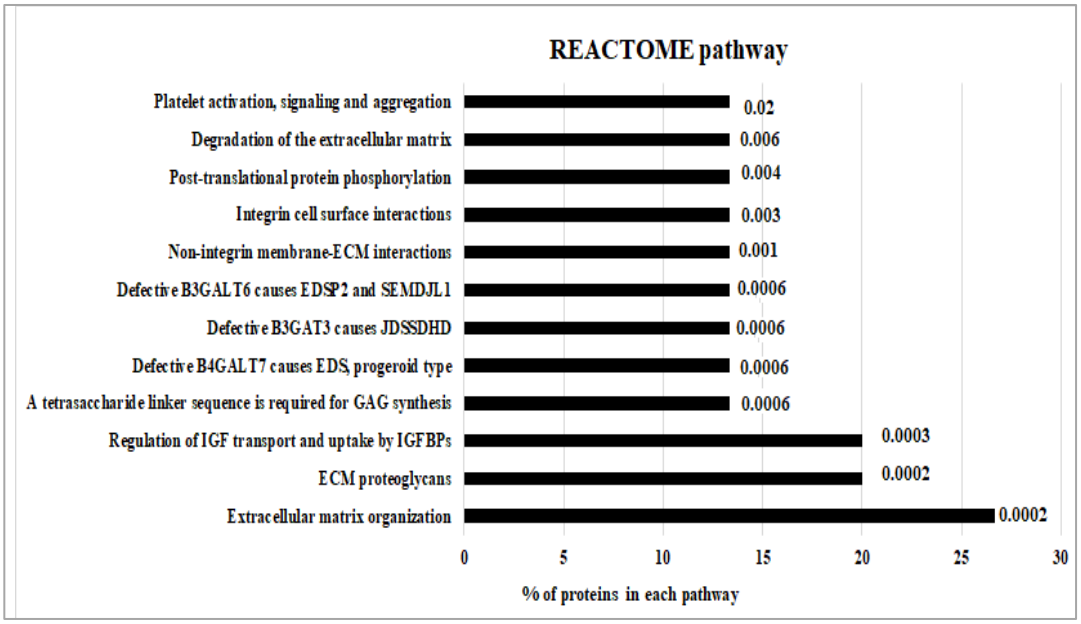


Figure 4.10: REACTOME pathway of upregulated protein in DM vs NDM group and corresponding FDR, The X- axis represent the percentage of proteins present in each of the pathway

4.1.7.3. Down regulated proteins in DM vs no-DM

7 proteins were found to be significantly down regulated in DM compared with NDM. These were majorly from the proteins involved in detoxification mechanism and glycolytic pathway proteins. The expressions of these proteins were similarly maintained in the DM vs no-DM and PDR vs NDM, which clearly proves diabetes is the key factor involved in these proteins downregulation and is not further affected by the proliferative changes occurring in the disease stage (Figure 4.11).

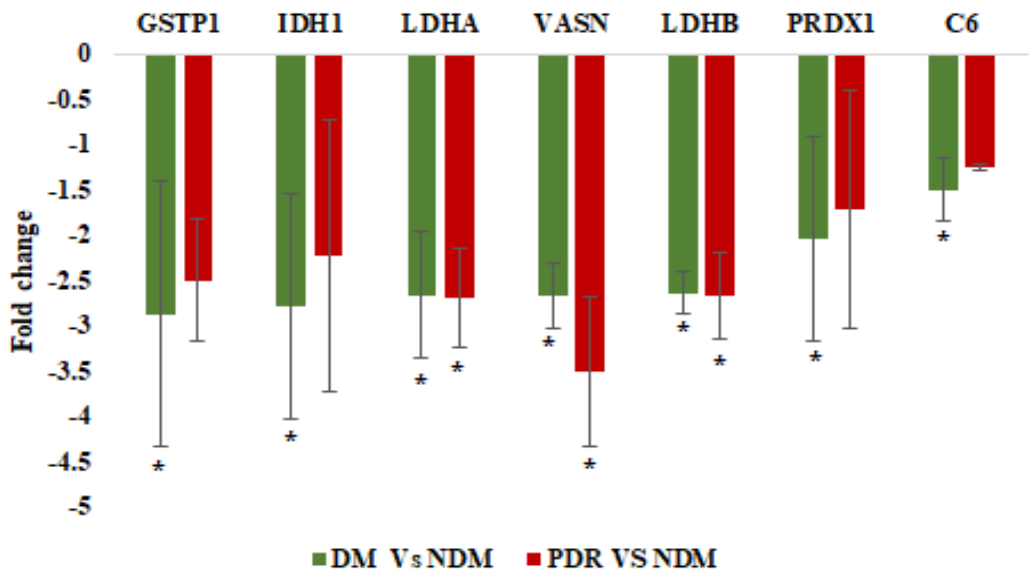


Figure 4.11: Significantly down-regulated proteins in DM vs NDM and PDR vs NDM group, *p<0.05

4.1.7.4. Major pathways associated with proteins down-regulated in the DM vs no-DM groups

There were 9 KEGG and 5 REACTOME pathways were found to be significantly enriched in the diabetes induced downregulated protein list (Table 4.12). Majority of the protiens were found to be present in metabolic pathways, which include metabolism of pyruvate, proponate and glycolytic pathways. The percentage of proteins in each pathway from the total differentially regulated proteins were calculated as shown in Figure 4.12 and Figure 4.13.

Table 4.12: List of proteins present in each of the KEGG and REACTOME pathways

KEGG Pathway	Matching proteins
Detoxification of Reactive Oxygen Species	GSTP1, PRDX1
Pyruvate metabolism	LDHA, LDHB
Metabolism	GSTP1, IDH1, LDHA, LDHB
Innate Immune System	C6, GSTP1, IDH1
Neutrophil degranulation	GSTP1, IDH1
REACTOME pathway	**Matching Proteins**
Glycolysis / Gluconeogenesis	LDHA, LDHB
Cysteine and methionine metabolism	LDHA, LDHB
Glutathione metabolism	GSTP1, IDH1
Pyruvate metabolism	LDHA, LDHB
Propanoate metabolism	LDHA, LDHB
Peroxisome	IDH1, PRDX1
Central carbon metabolism in cancer	IDH1, LDHA
Glucagon signaling pathway	LDHA, LDHB
Metabolic pathways	IDH1, LDHA, LDHB

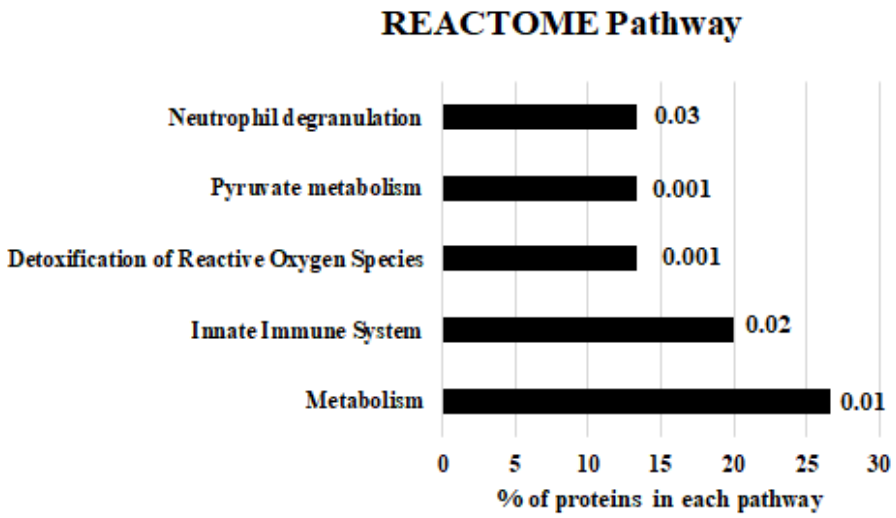


Figure 4.12: REACTOME pathway of down-regulated proteins in DM vs NDM group and corresponding FDR-The X- axis represent the percentage of proteins present in each of the pathway

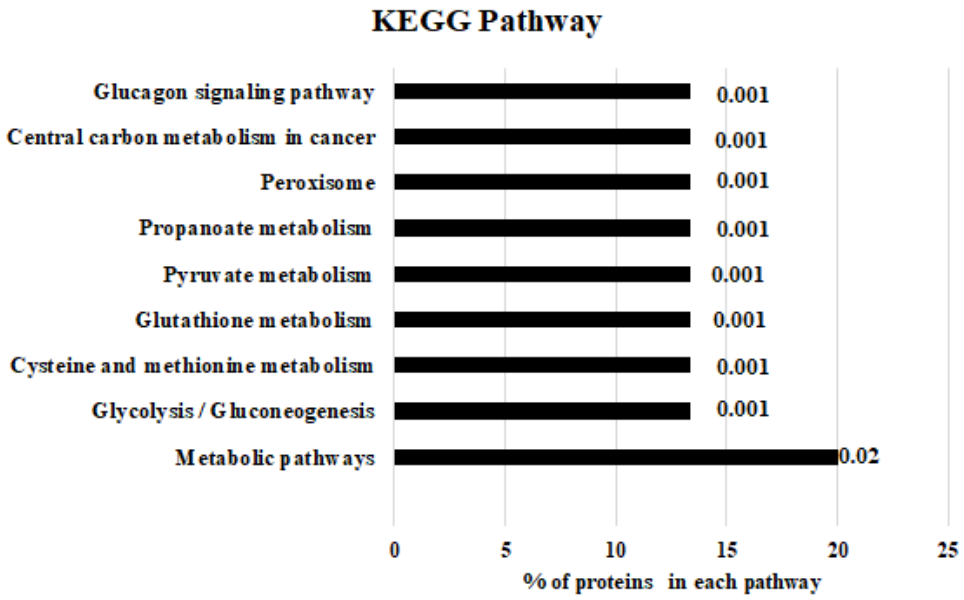


Figure 4.13: KEGG pathway of down-regulated proteins in DM vs NDM group and corresponding FDR- The X- axis represent the percentage of proteins present in each of the pathway

4.1.8. PDR vs No-retinopathy (PDR vs NDR+NDM)

Taking proteins that showed more than 1.5 folds change only, there were 10 proteins that were differentially regulated in retinopathy stage, which include 4 upregulated and 6 downregulated proteins. Level of these protein expression were also evaluated in PDR vs NDR, which further clearly identified a similar protein expression as seen retinopathy vs no-retinopathy group. This further confirmed the expression of these 10 proteins were typically dependent on the advanced stage of the disease. Most importantly, VASN expression was also found to be downregulated significantly in DM compared to no-DM as well as PDR vs NDM group. (Table 4.13).

Table 4.13: List of proteins found to be differentially regulated (based on log_2 fold change and *p*-value) in retinopathy stage and corresponding change in PDR vs NDR, PDR vs NDM and DM vs NDM are given

Gene names	PDR vs No retinopathy (NDR+NDM)	*p*-value	PDR vs NDR	*p*-value	PDR vs NDM	*p*-value	DM VS no DM (PDR+NDR)-(NDM)	*p*-value
FGA	2.52 ± 0.86	0.04	2.54 ± 1.5	0.1	2.5 ±1.04	0.07	1.23 ± 0.58	0.4
IGHM	1.88 ± 0.44	0.04	2.44 ± 0.39	0.06	1.31 ±0.26	0.06	0.09 ± 0.28	0.9
APCS	1.86 ± 0.53	0.04	1.62 ± 0.68	0.05	2.11 ±0.66	0.1	1.3 ± 0.95	0.2
LRG1	1.57 ± 0.59	0.02	1.95 ± 0.7	0.03	1.19 ±0.51	0.1	0.21 ± 0.3	0.8
SOD3	-1.75± 0.21	0.002	-1.66 ± 0.32	0.03	-1.85 ±0.24	0.004	-1.02 ± 0.23	0.2
VASN	-2.61 ± 0.57	0.01	-1.71 ± 0.84	0.06	-3.51 ±0.82	0.007	-2.66 ± 0.36	0.009
ENPP2	-1.52 ± 0.56	0.03	-1.38 ± 0.39	0.09	-1.42 ±1.04	0.1	-0.73 ± 0.48	0.3
TGFBI	-2.11 ± 0.13	0.03	-1.59 ± 0.87	0.1	-2.64 ±0.63	0.02	-1.84 ± 0.63	0.1
MYOC	-3 ± 0.56	0.03	-3.45 ± 0.49	0.01	-2.54 ±1.14	0.2	-0.81 ± 1.55	0.6
PPT1	-1.5 ± 0.46	0.05	-1.62 ± 0.21	0.07	-1.06 ±0.44	0.17	-0.25 ± 0.44	0.7

Data are represented as Mean±SEM

4.1.8.1. Pathway analysis of the differentially regulated proteins in PDR vitreous

4 up-regulated and 6 down-regulated proteins and their corresponding expression in PDR vs NDR are given in the Figure 4.14a and Figure 4.14b. The pathway analysis of the upregulated protein identified only one REACTOME pathway i.e. amyloid fiber formation with proteins such as FGA and APCS (FDR=0.04), while none of the pathways were found to be enriched in the downregulated protein list. While a significant upregulation in the angiogenic protein LRG1 and downregulation of detoxification protein SOD3 was observed at retinopathy group along with other proteins.

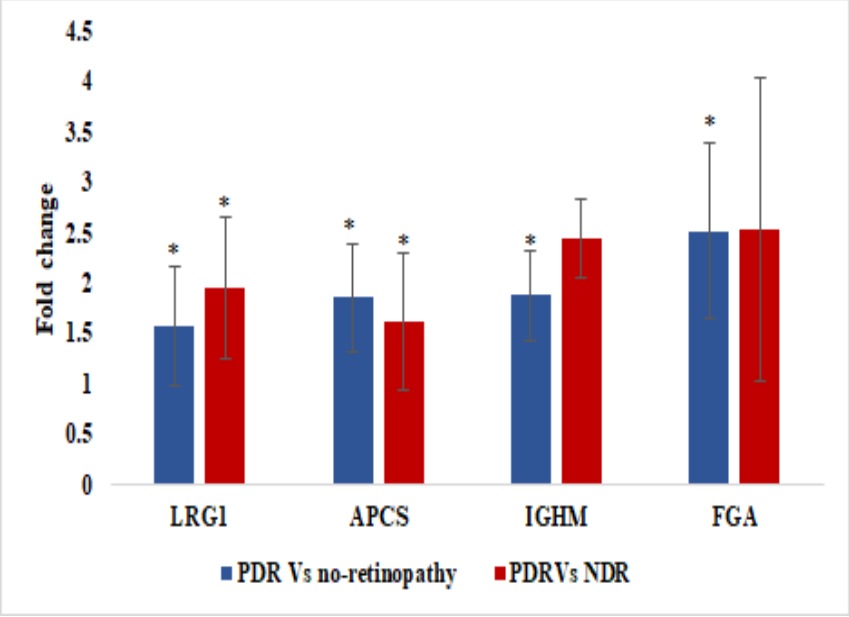


Figure 4.14a. Significantly up regulated proteins in PDRD vs no retinopathy groups, *p<0.05

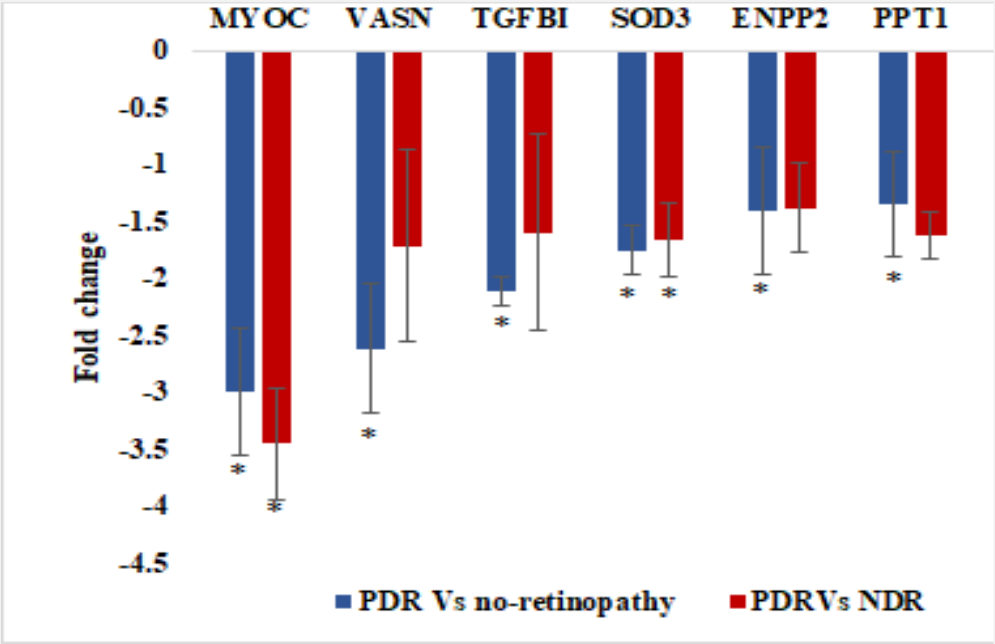


Figure 4.14b. Significantly down regulated proteins in PDRD vs no retinopathy groups, $*p<0.05$

4.1.9. Heat map representation of significant protein in diabetic and non-diabetic groups

A quantitative and qualitative comparative analysis of the significant proteins present in all the three groups were analysed by heat map (Figure 4.15). This further provide a better understanding about the altered proteome in diabetic (PDR, NDR) and non-diabetic group (NDM). For generating the heat map, proteins which were found to be significantly different in diabetes and retinopathy stages were taken and log 2 values of their intensities were used. Thus, total 25 proteins analysed and their profile is generated using R version 64.3.3.2. This shows a clear separation of proteins of three different groups and this further indicating difference of proteins in two disease state (i.e. diabetic and no diabetic stage).

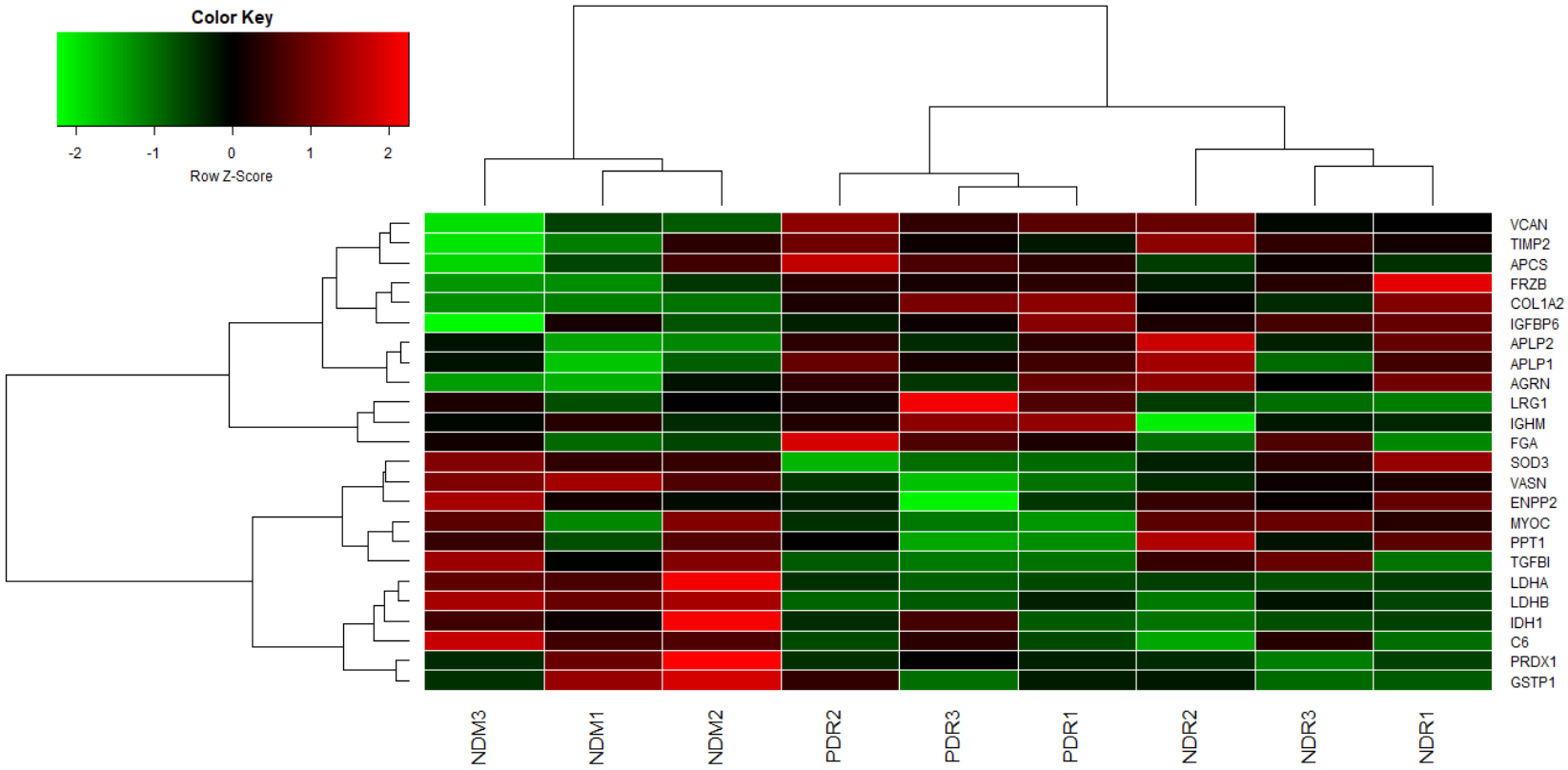


Figure 4.15: Heat map of significant proteins present in PDR, NDR and NDM group- Heat map was generated based on their log2 intensity values of each protein.

4.2. Systematic investigation on complement pathway activation and role of microglia in pathogenesis of Diabetic Retinopathy

Complement pathway and microglia are the major innate immune defense mediators of the retina. The pro-angiogenic role of complement pathway was reported in neovascular diseases such as AMD and ROP. Though several proteomic and immunostaining-based reports have shown the deposition of complement proteins in DR vitreous and retina, the mechanism of complement activation and the major mediators and key proteins involved in the disease pathogenesis are not clearly understood (Xu and Chen 2016). Similarly, the global proteome analysis in the present study also indicated an initial triggering of the complement pathway as a unique mechanism in PDR pathogenesis. Thus, it is important to understand if complement played a significant role in PDR progression and hence additional samples were analyzed by a targeted protein assay. Further, it was also shown that in DR there was an activation of microglial cells in the retina based on morphological transitions at early stages of the disease (Zeng, Green et al. 2008). Since, complement and microglia are the major defense mechanisms in the retina, it is important to understand the cross talk between these two systems, which may eventually promote the neovascular changes in PDR.

4.2.1. Demographic details of the study cohort

A total of 240 subjects were enrolled for vitreous analysis that included clinically well-defined case of PDR (n=120) and ethnically matched non-DM controls (n=120) from patients with idiopathic macular hole and rhegmatogenous retinal detachment. The detailed demographics of these subjects are provided in table 4.14. There was no significant difference in the mean

ages of the patients and controls and females were slightly over-represented in the cases. The duration of diabetes was more than 15 years in among the cases.

Table 4.14. Demographic details for the cases and controls enrolled for vitreous humor analysis

Study subjects	Cases (n=120)	Controls (n=120)
Male (n, %)	52 (43.3%)	69 (57.5%)
Female (n, %)	68 (56.6%)	51 (42.5%)
Age (Mean±SEM)	54.6±0.93	55.8±0.71
Duration of DM in years (Mean±SEM)	15.59±0.73	Nil

For the analysis of complements in systemic circulation, a total 114 serum samples were obtained from PDR (n=38), NPDR (n=38) and no-DM controls (n=38) subjects. The demographics of the patients used for serum analysis are given in the table 4.15. There were no significant differences in the ages of the patients and the duration of diabetes between NPDR and PDR cases.

Table 4.15. Demographic details for the cases and controls used for serum analysis

Study subjects	PDR (n=38)	NPDR (n=38)	Controls (n=38)
Male (n, %)	23 (60.5%)	24 (63.1%)	22 (57.8%)
Female (n, %)	12 (39.4%)	14 (36.8%)	16 (42.1%)
Age (Mean±SEM)	53.86±1.61	59.83±1.32	65.8±1.03
Duration of DM in years (Mean±SEM)	15.05±0.9	12.88±1.4	Nil

4.2.2. Evaluation of complement pathway activation by analyzing the central complement protein C3

Complement component C3 activation is the central point where all the three pathways of the complement system converge. Proteolytic fragmentation of this central molecule is the major indicator for the activation of complement pathway and these fragments upon activation bind to the nearby tissues and enhance inflammatory process. Hence, the levels of C3 and its proteolytic fragments were evaluated in the vitreous and serum of PDR patients.

4.2.2.1. Evaluation of localized activation of complement C3 in retina by analysing vitreous humor biopsies

To evaluate complement activation in the retina, western blotting was done for C3 in vitreous biopsies obtained from PDR (n=42) and no-DM control (n=42) subjects under non-reducing condition using 15μg of total vitreous protein (Figure 4.16a). C3 abundance on the blot was measured by densitometry and compared between PDR and no-DM controls. Normalization of the total protein content for equal protein loading between cases and controls was based on Ponceau's staining of the blot. Based on equal protein loading, densities were analyzed for 38 PDR and 38 control samples. A significant increase in total C3 level (1.9±0.25, $p^*=0.004$) was observed in PDR subjects compared to no-DM controls (0.98±0.18) (Figure 4.16b).

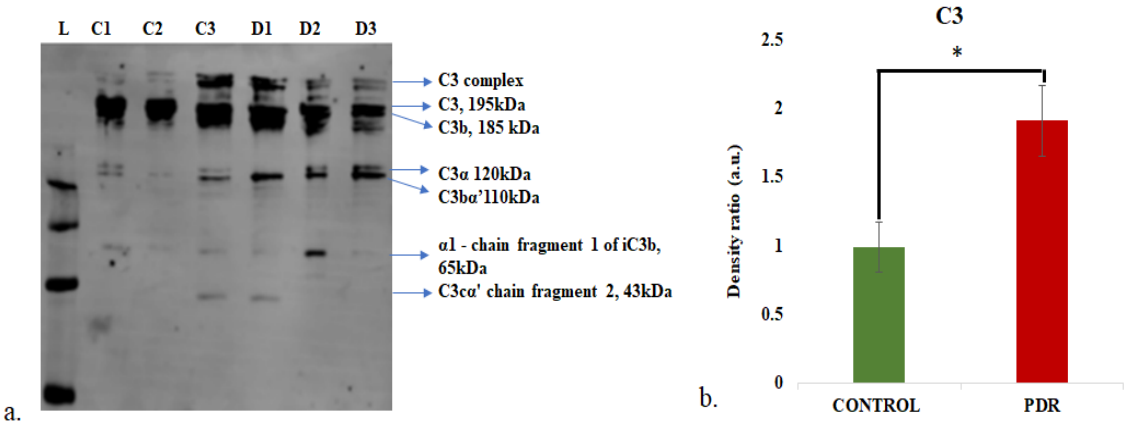


Figure 4.16: (a). Representative western blots of C3 in PDR and control vitreous, C=Control, D=PDR, (b). Mean band intensities of total C3 in PDR and control vitreous based on densitometry measurements (PDR, n=38 and Control, n=38), *p=0.004, Data represented as Mean ±SEM.

Further, C3 activation was evaluated by analysing the proteolytic fragments of C3. As shown in Figure 16a, activated fragments of C3 were found in both PDR and control vitreous. The major bands of C3 identified were intact C3 (195kDa), C3α (120kDa), C3bα' fragment (110 kDa), C3β (75kDa), α-1 fragment of iC3b (65kDa) and C3c α' fragment-2 (43kDa). Additionally, a band of more than 250kDa was also observed in all the samples, which might represent the C3 complex. The western blot did not identify a uniform pattern of C3 fragmentations either in PDR or controls. Hence, the fragmentation pattern of C3 in each of the samples were analysed and total number of fragments of similar molecular weight observed in PDR and controls were compared. The number of fragments identified in PDR and no-DM controls are represented in figure 4.17. Total number of each of the fragments in C3 were found to be similar between PDR and no-DM controls.

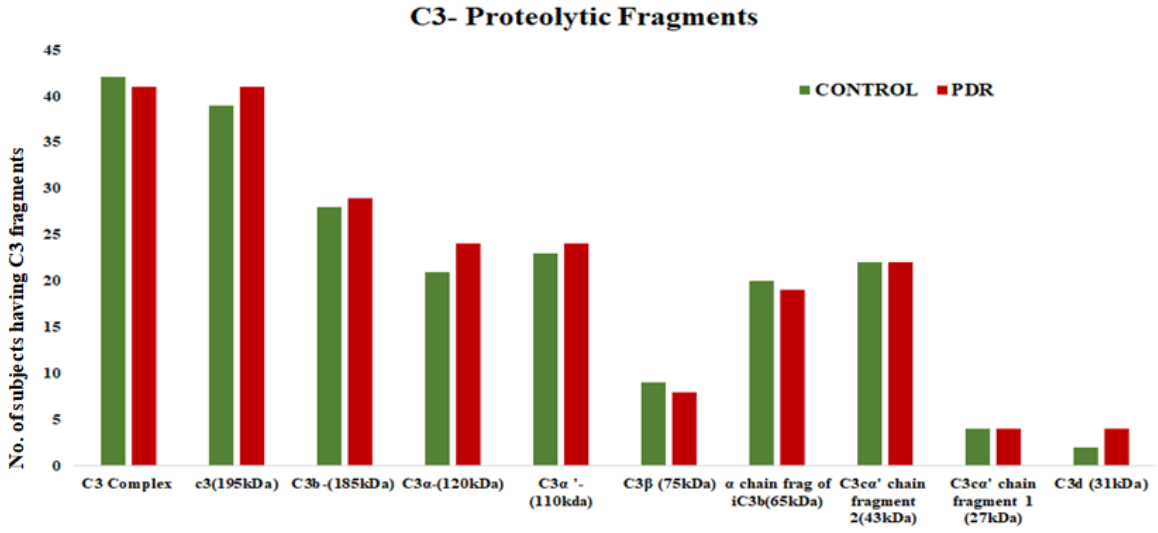


Figure 4.17: Comparison of total number of activated C3 fragments present in PDR (n=42) and control (n=42) vitreous. The X- axis represent the total number of fragments present in all the samples corresponding to the C3 fragments labelled in the Y-axis.

4.2.2.2. Densitometric measurements and comparison of activated C3 fragments between PDR and controls

Western blotting results clearly revealed activation of complement pathway in PDR and control vitreous based onC3 proteolytic fragments. Further analysis was done to understand the most significantly upregulated fragments of C3, based on the fact that the deposition of activated C3 fragments on tissue surface act as a signal for tissue inflammation. Thus, intensity of each of the activated C3 fragments present in minimum of 10 samples were analysed by densitometry and ratio of PDR vs controls were calculated between the samples with similar protein loading.

The activation of C3 generate the bigger 185kDa-C3b fragment by removing C3a of 10 kDa size from the intact C3 (195kDa). There was a slight increase in the level of intact C3 in PDR

vitreous (2.44±0.78a.u., n=*28*) compared to the no-DM controls (1.79±0.35a.u., n=28) (Figure 4.18a). The C3b is comprised of α' chain (110 kDa) and β chain (75kDa). The first activated product of C3 which drive complement pathway activation is C3b and a slight increase in its levels were noted in PDR (1.66±0.25a.u.., n=22) as compared to the control vitreous (1.06±0.16 a.u.., n=22) (Figure 4.18b).

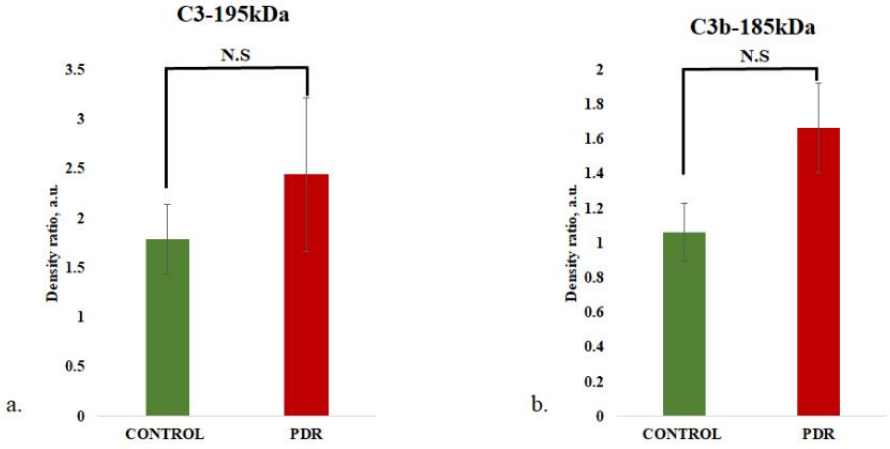


Figure 4.18. (a). Mean band intensities of intact C3 in PDR (n=28) vitreous compared to control (n=28)

(b). Mean band intensities of C3b, in PDR (n=22) vitreous compared to control vitreous (n=22) N.S.- not significant, $p>0.05$

Upon activation of C3, further fragmentation of C3b occurs and generate α-chain fragments. Two larger α-chain fragments such as C3α and C3bα' of molecular weight 120kDa and 110 kDa, respectively, were identified in the vitreous samples (Figure 4.19). The 120kDa fragment is part of intact C3 (195kDa), where the reactive thioester bond is internalized. The activation of C3 convertase cleaves 10kDa fragment of C3α and generate reactive C3bα' of 110kDa size, which is the part of active C3b. An increased-levels of both of these fragments in PDR vitreous was noted, particularly reactive C3bα' was found to be significantly increased in the

PDR vitreous (n=13, 2.76±0.65a.u.., *p*=0.006)* compared to no-DM controls (n=13, 0.73±0.17a.u..). The levels of C3α (120 kDa) was not significantly different between the controls (2.16 ±1.03a.u.. n=13) and PDR (3.22±1.02a.u.. n=13) cases (Figure 4.20a and Figure 4.20b).

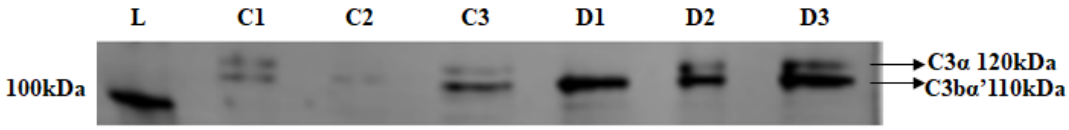


Figure 4.19: Representative western blots of C3α (120kDa) and C3-bα' (110 kDa) in PDR and no-DM vitreous, C: Control, D: PDR, L- protein ladder

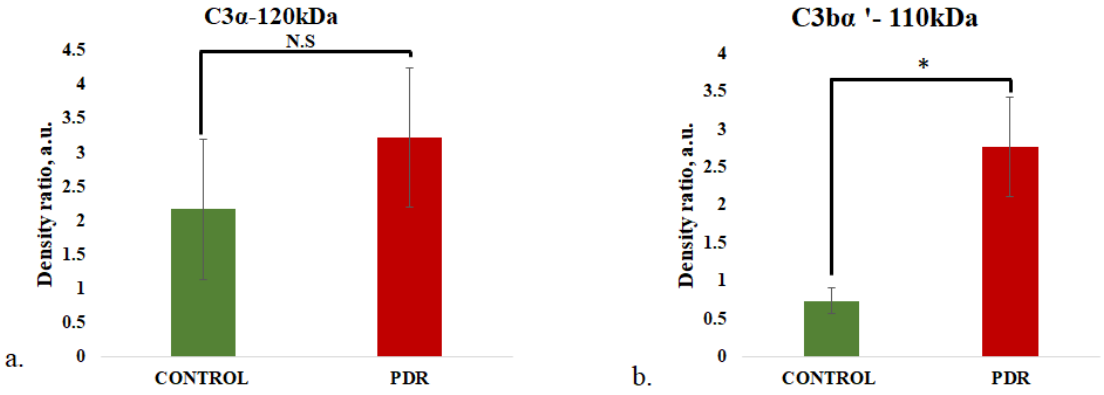


Figure 4.20: (a). Mean band intensities for C3α (120kDa) and (b). C3bα' (110 kDa) in PDR (n=13) compared to controls(n=13), N.S.- not significant

Next, the cleaved fragments of C3 of molecular weights 65kDa and 43kDa, respectively, were observed in PDR and control vitreous samples (Figure 4.21a and Figure 4.21b). These fragments corresponded to the C3α'1 chain of iC3b and C3cα fragment-2, which was generated by the fragmentation of C3b into iC3b during the deactivation step of active C3b.

The densitometry analysis identified an increase in the levels of these fragments in PDR vitreous compared to the controls, but these were not statistically significant (Figure 4.22a and Figure 4.22b). The C3α'1(65kDa: Control= 2.2±0.75 a.u., PDR =2.57±0.9 a.u., *p>0.05, N.S*, C3cα' fragment 2 (43kDa, Control=1.92±0.71 a.u., PDR= 4.8±1.7 a.u., *p>0.05, N.S*)

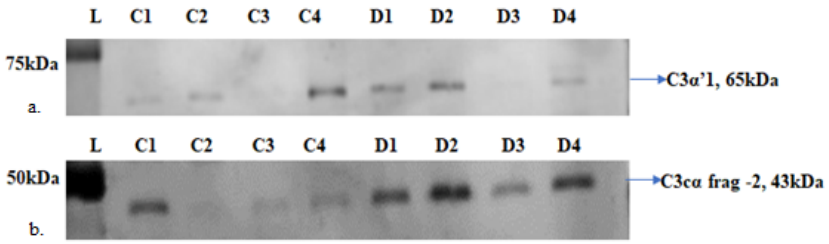


Figure 4.21: Representative western blots of (a). C3α'1 (65kDa) and (b). C3cα'fragment -2 (43 kDa) in PDR and no-DM vitreous, C: Control, D: PDR, L- protein ladder

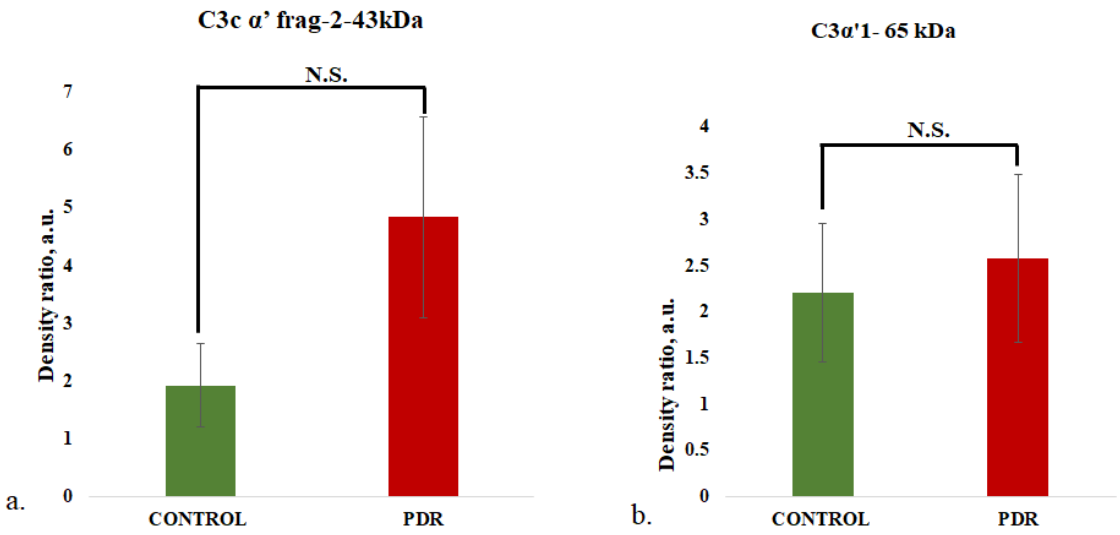


Figure 4. 22: (a). Mean band intensities for C3α'1 (65kDa) and (b). C3cα fragment 2 (43 kDa) in PDR and controls, N.S.- not significant

4.2.2.3. Systemic evaluation of complement C3 protein expression in serum

Total C3 and its activated fragments in systemic level was analysed in serum samples obtained from PDR, NPDR and no-DM controls using15μg of total proteins. Intensity of the bands were calculated through densitometry. The mean band intensity ratios of C3 levels in PDR and NPDR compared to no-DM and PDR vs NPDR was calculated. Western blotting revealed an increase in total C3 level in PDR and NPDR compared to controls (Figure 4.23a). The ratio of total C3 was calculated between PDR vs no-DM (1.69±0.58 a.u.*),* NPDR vs no-DM (1.38±0.24 a.u.) and PDR vs NPDR (1.19±0.23 a.u.) groups (Figure 4.23b). Thus, the total C3 levels were not significantly different across the groups. Unlike the vitreous, the C3 proteolytic fragmentation was found to be uniform across the serum samples of no-DM, NPDR and PDR subjects. Five major fragments of C3 (intact C3-195kDa, C3b-185kDa, C3β-75kDa) along with a band of ~90kDa size and C3 complex (250kDa) were observed in all the samples. The intensity of each of these bands were analysed further. But no significant differences were noted in any of these fragments between these groups.

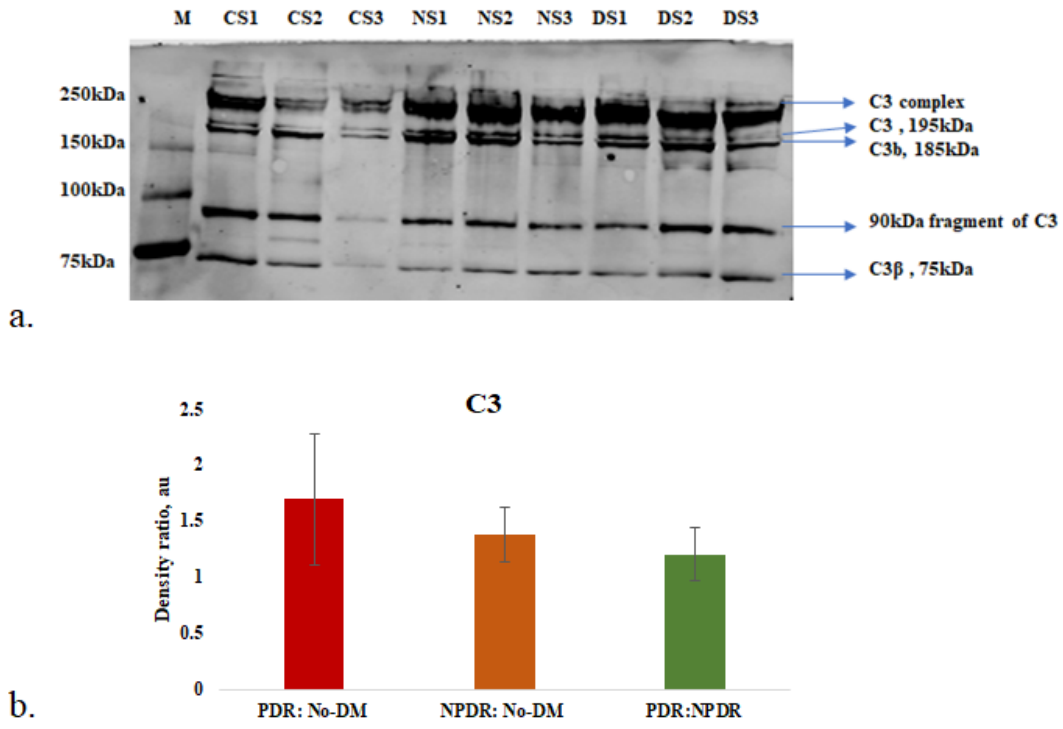


Figure 4.23. (a). Representative western blots of C3 in serum from PDR, NPDR and no-DM controls, and (b). Mean band intensities of total C3 in PDR, NPDR and no-DM controls
(PDR: n=12, NPDR: n=12, no-DM, n=12, $p>0.05$, Data represented as Mean ±SEM.

4.2.3. Evaluation of the role of classical and alternative pathway of complement activation in diabetic retinopathy

The activation of the complement pathway based on the levels of total C3 and its activated fragments revealed a significant increase of C3 in the vitreous of PDR as compared to controls. While the number of activated fragments of C3 were found to be similar across the PDR and controls, the intensities of these activated fragments were found to be increased in PDR vitreous. Additionally, a significantly increased level of reactive C3bα' fragment (110kDa) was seen in PDR cases. However, no significant change in total C3 and their activated fragments was observed in the serum of patients and controls. This suggested a

localized activation of complement pathway in PDR pathogenesis. Further, the relative contributions of classical and alternative pathway of complement activation in the pathogenesis of PDR were evaluated.

4.2.3.1. Role of the classical pathway of complement activation in PDR pathogenesis

The classical pathway of complement activation was evaluated in vitreous and serum samples by western blotting of complement proteins specific for classical pathway such as C1q and C4b. The classical pathway gets activated upon binding of antigen- antibody complex to the complement protein C1q. Further, this binding activates the complement component C4 and generate C4b and C4a. C4b is required for C3 convertase generation and continuation of classical pathway activation.

4.2.3.2. Evaluation of classical pathway of complement activation in vitreous humor of PDR and no-DM controls

C1q levels in vitreous of PDR and no-DM controls were assessed by western blotting using 30µg of total protein. As shown in the figure 4.24a, the level of C1q was not significantly different across PDR and no-DM controls. Densitometric quantification identified the mean band intensity of C1q fragment in PDR (1.81±0.44 a.u.) and it was not significantly different than the no-DM controls (1.32±0.38 a.u., Figure 4.24c). This suggested that the classical pathway may not have a significant role in complement activation in DR pathogenesis. This was further confirmed by analysing the levels of C4b (Figure 4.24b), which was not significantly different between PDR cases and controls (Control: 1.08±0.2 a.u., PDR: 1.39±0.4 a.u., $p>0.05$) (Figure 4.24d).

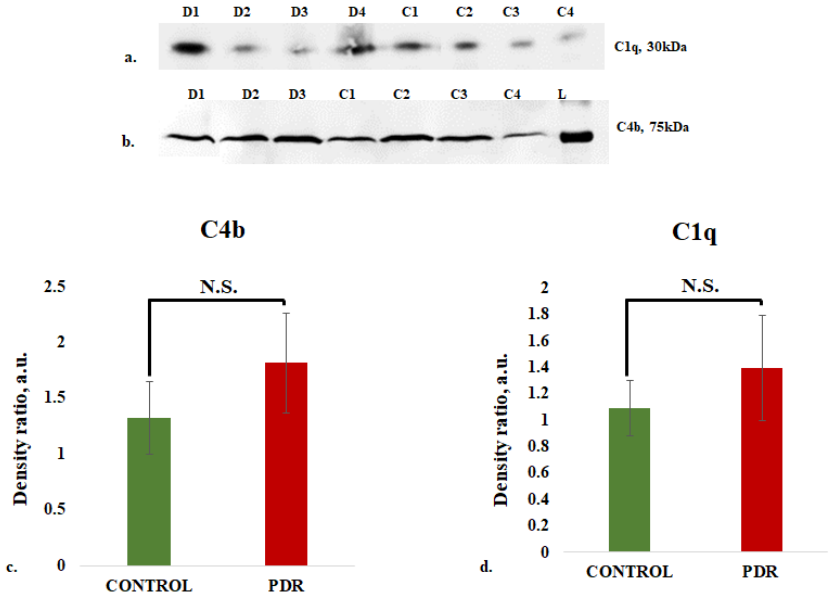


Figure 4.24: Representative western blots of (a) C1q in PDR and no-DM control vitreous, (b)Representative western blot of C4b in PDR and No-DM controls (c). Mean band intensity of C1q (30kDa) in PDR (n=17) and no-DM control (n=17) vitreous, (d). Mean band intensity of C4b in PDR (n=8), no-DM control (n=8) vitreous, N.S.- not significant

4.2.3.3. Evaluation of classical pathway of complement activation in systemic level

The systemic levels of C1q were assessed in serum samples of PDR, NPDR and no-DM controls. Similar to the levels of C1q observed in the vitreous, there was a slight upregulation of C1q in NPDR and PDR compared to the controls (Figure 4.25a and 4.25b). (PDR vs control: 1.12±0.43 a.u., *p>0.05, N.S*, NPDR vs Control: 1.43±0.46 a.u., *p>0.05, N.S,* PDR vs NPDR: 0.75±0.18 a.u., *p>0.05, N.S*).

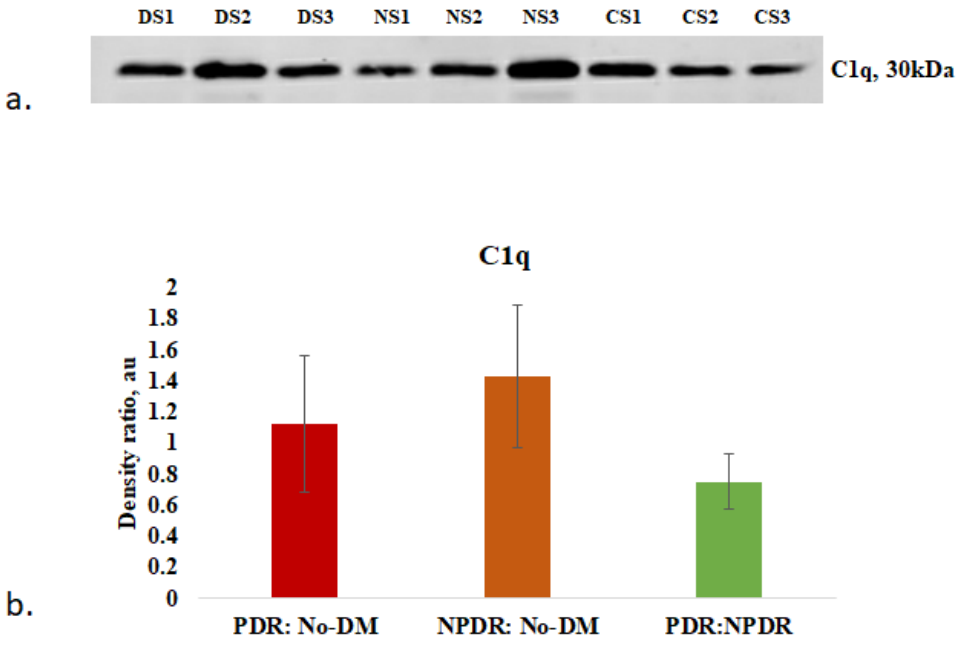


Figure 4.25(a). Representative western blots of C1q (30kDa) in PDR, NPDR and no-DM (b) Mean band intensities of C1q (30kDa) in PDR (n=8), NPDR (n=8) and no-DM control (n=8) serum, P>0.05 (N.S), DS: PDR, NS: NPDR and CS- Control

4.2.3.4. Role of alternative pathway of complement activation in PDR pathogenesis

As the classical pathway of complement did not contribute a significant role in complement pathway activation in PDR pathogenesis, the role of alternative pathway of complement was evaluated using complement factor B (CFB), which is a specific protein for alternative pathway of complement. It is a key component for the formation of C3bBb (C3 convertase) and proteolytic cleavage of CFB by Factor D generate Ba and Bb components in CFB. The Bb component interacts with C3b and form C3 convertase, which drives the alternate pathway.

Western blotting of factor Bb in vitreous identified a 63kDa fragment of factor Bb in vitreous samples (Figure 4.26a). Quantitative assessment of this fragment identified a significant decrease in the level of PDR vitreous compared to no-DM controls (PDR: 0.97±0.15a.u.,

n=22, Controls: 1.89±0.38 a.u., n=22, *p*=0.03;* Figure 4.26b). This suggested more bound form of Bb in the PDR vitreous, with a corresponding lower level of free Bb. This indicated the generation of additional C3 convertase in PDR vitreous that may activate the alternative pathway of complement.

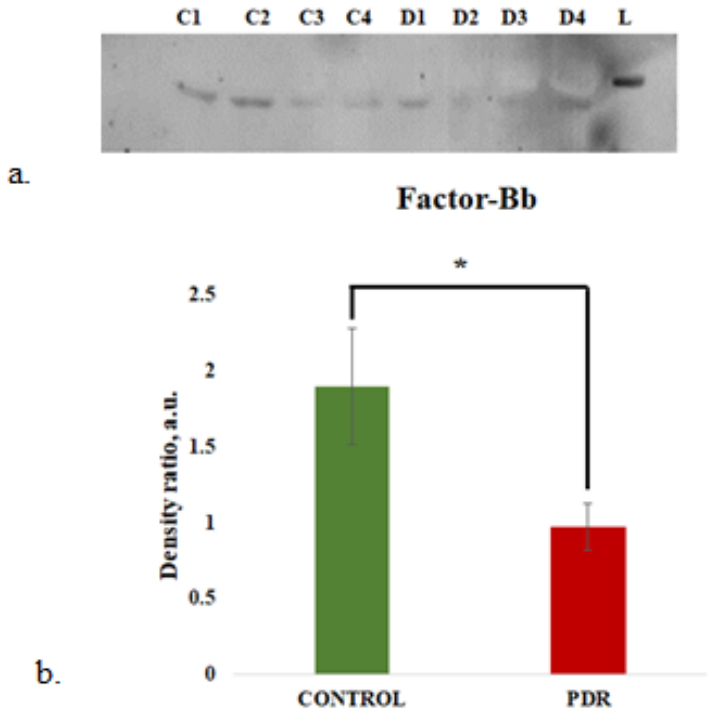


Figure 4.26 (a). Representative western blots of CFB in PDR and No-DM controls,(b) Mean band intensities of Bb of CFB in PDR and no-DM control vitreous, *p**=0.03 (D: PDR, C: controls, L- Protein ladder)

4.2.3.5. Evaluation of alternative pathway of complement regulation by complement factor-H (CFH) in DR

Decrease in the level of Factor Bb indicated more C3 convertase formation for activating alternative pathway of complement in PDR. CFH, being an important regulator of alternative

pathway of complement, regulates the alternative pathway at multiple stages using various co-factors. These were next assessed in PDR and control vitreous samples. Western blotting results identified a sharp 150kDa band of CFH in vitreous samples (Figure 4.27a). Next, quantification of CFH band intensity was assessed and this revealed a significant decrease in the level of CFH in the PDR vitreous as compared to the controls (Control: 0.96±0.172 a.u., PDR: 3.68±0.66 a.u., *p***=0.0004*; Figure 4.27b).

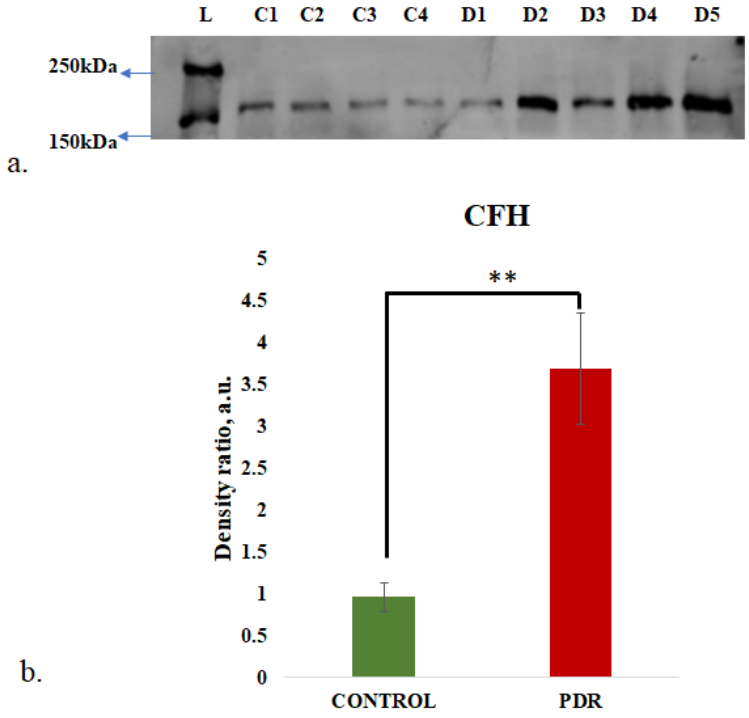


Figure 4.27(a). Representative western blot of CFH in PDR and No-DM controls, (b). Comparison of mean CFH band intensities in PDR (n=31), no-DM control (n=31) vitreous, *p***=0.0004 (D: PDR, C: controls, L-Protein ladder)

4.2.3.6. Further assessment if the increase in CFH level in Vitreous humor samples is contributed by serum infiltration

The level of CFH was quantified in the serum samples to assess whether increased levels of CFH is a localized effect or an additive effect from serum infiltration due to the breakage of blood retinal barrier during disease pathogenesis. The results identified a 150kDa band of CFH in the serum (Figure 4.28a), but unlike that of the vitreous, a decreased level of CFH was found in PDR compared to NPDR and controls (PDR vs control: 0.78±0.12 a.u., *p>0.05, N.S*, PDR vs NPDR*:* 0.66±0.07 a.u., *p>0.05, N.S,* NPDR vs control: 1.205±0.2 a.u., *p>0.05).* This confirmed that the increased level of CFH in PDR vitreous is not due to serum infiltration (Figure 4.28b) and could have been contributed by any of the retinal cell types.

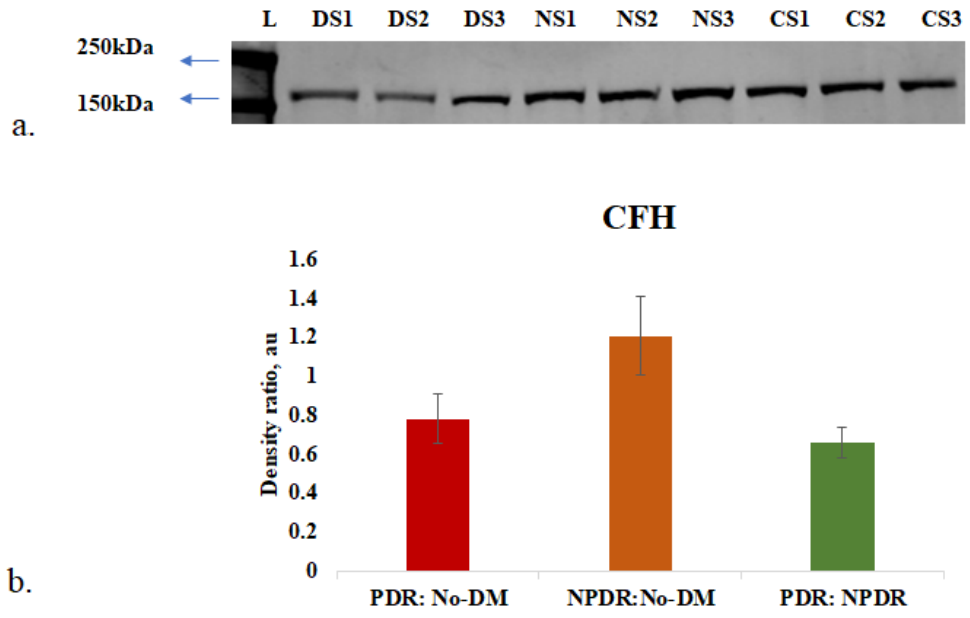


Figure 4.28. (a). Representative western blots of CFH (150kDa) in PDR, NPDR and no-DM control serum, (b) Mean band intensities of CFH (150kDa) in PDR (n=12), NPDR (n=12) and no-DM control (n=12) serum. CS: Control, DS: PDR, NS: NPDR and CS- Control

4.2.4. Validation of complement activation and CFH upregulation by immunohistochemistry using diabetic and non-diabetic cadaveric retinal tissues

Diabetic and non-diabetic donor retina tissues were collected from cadaveric eyes and immunohistochemistry was performed to validate the complement activation and Factor H upregulation in the retina. To understand if the glial activation contributes to complement pathway activation, a co-staining of glial fibrillary acidic protein (GFAP) for macroglial population and CD11b for activated microglial cells with CFH was performed in the sections obtained from the retina of diabetic and non-diabetic subjects.

4.2.4.1. Evaluation of complement C3 and its co-localization with GFAP in retina

Diabetic and non-diabetic retinal sections were stained with C3 and GFAP to evaluate C3 deposition on the retina and its cellular localization. The immunostaining results identified an intense staining of C3 in the all retinal layers of diabetic retina ranging from the outer nuclear to inner nuclear layers except RNFL layer, while a low-level of C3 expression was seen in control retina. The level of GFAP was also increased in the diabetic retinas as compared to non-diabetic retinas, suggesting gliotic changes that could have occurred during the course of diabetes. Most importantly, C3 was not found co-localizing with GFAP positive cells in the retinal sections, indicating C3 was not secreted by the macroglial population in the retina (Figure 4.29a and Figure 4.29b).

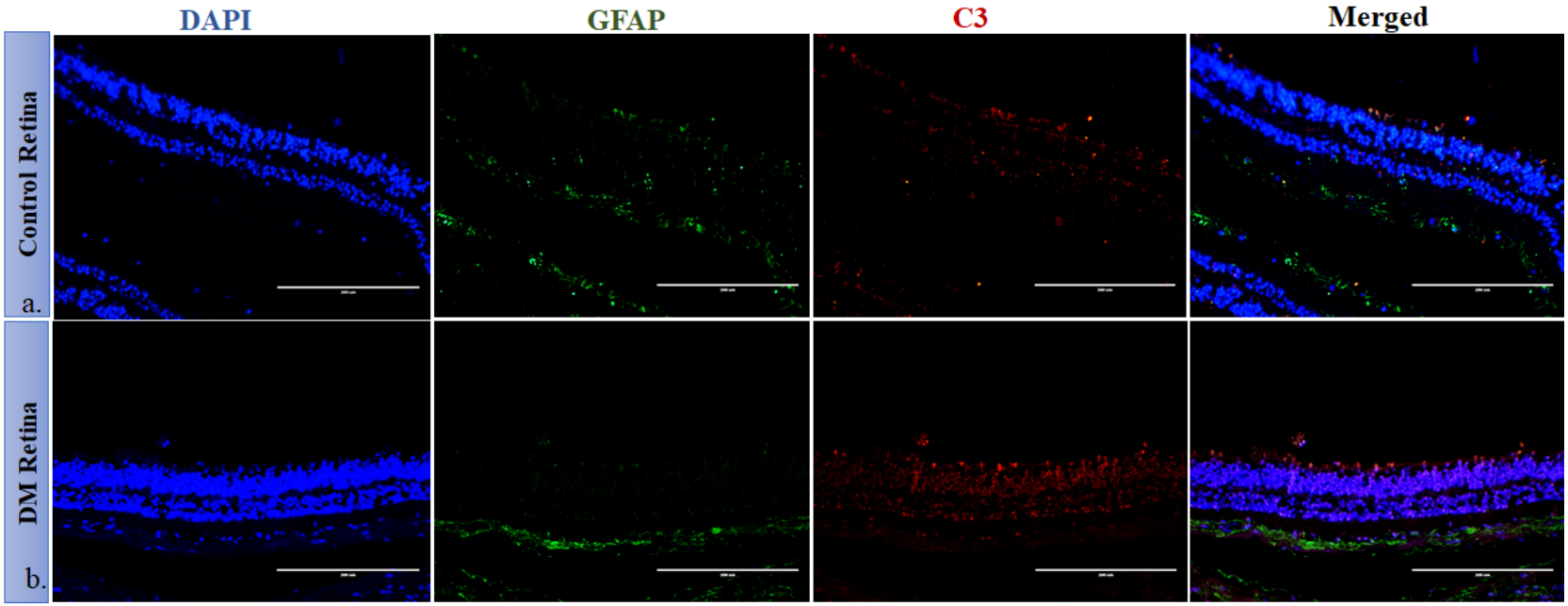


Figure 4.29: Representative immunostaining of retinal tissues collected from (a) Control and (b) Diabetic subjects using GFAP and C3 antibodies (Magnification= 20X, Scale bar=200µm)

4.2.4.2. To test if the complement C3 is contributed by microglial cells under diabetic conditions

CD11b is a known marker of activated microglia and also known as CR3, which is a receptor for complement C3. The co-localizations of C3 and CD11b were evaluated in the retinal sections. The immunostaining results identified an upregulation of CD11b and C3 in diabetic retina, while a relatively lower expression of both these proteins were observed in the non-diabetic retinas (Figure 4.30a and Figure 4.30b). While C3 deposition was observed throughout the retinal layers, including inner nuclear layers, a co-localization of C3 and CD11b was observed only in diabetic retina and not in non-diabetic retina, suggesting that other than the retinal pigment epithelium, microglial cells could also contribute to the increased C3 deposition in diabetic eyes.

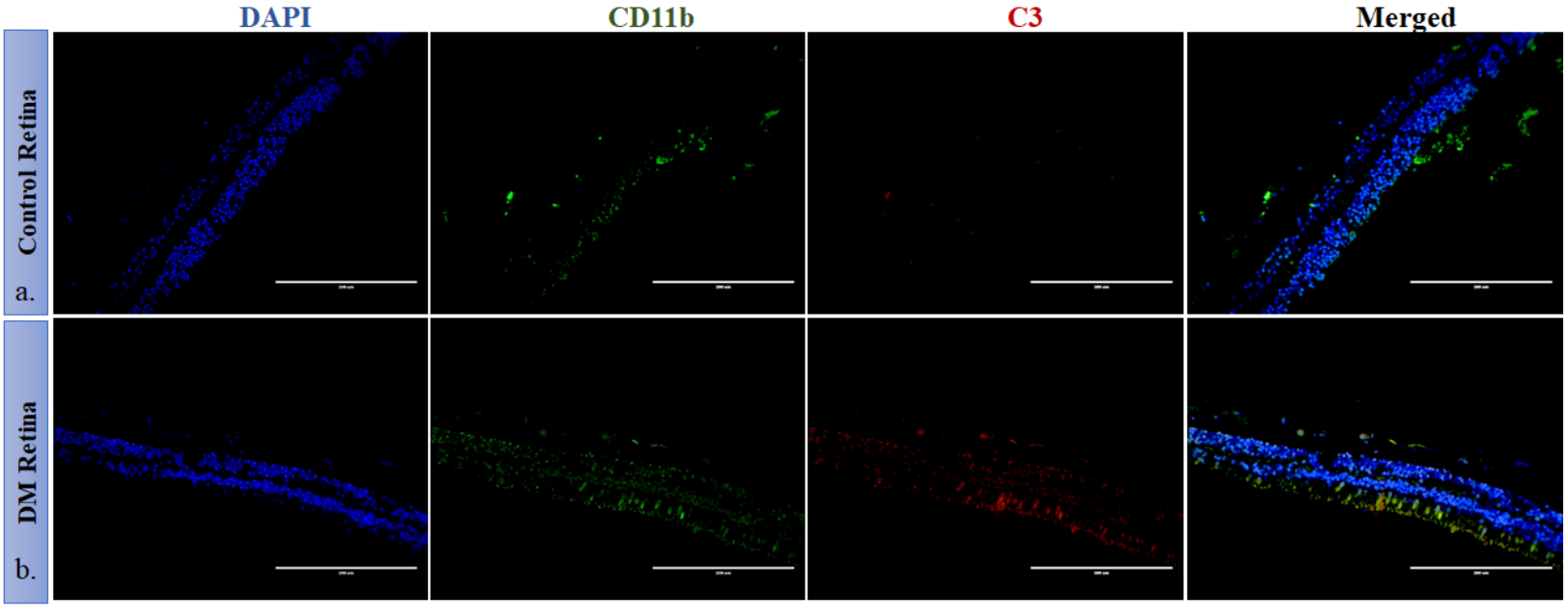


Figure 4.30: Immunostaining of C3 and CD11b in retinal tissues collected from (a) control and (b) diabetic retinas (magnification=20X, scale bar=200μm)

4.2.4.3. To evaluate if activated microglial cells contribute to CFH upregulation in the diabetic retina

Next, the upregulation of CFH by activated microglial cells was evaluated in donor retinal tissues. The number of CFH and $CD11b^{+ve}$ cells were very low in the non-diabetic retinas. On the contrary, greater number of $CD11b^{+ve}$ microglial population was found in the diabetic retinas in the inner nuclear layers and had an intense staining of CFH suggesting that the predominant source of CFH in diabetic retinas might be the activated microglial cells (Figure 4.31a and Figure 4.31b).

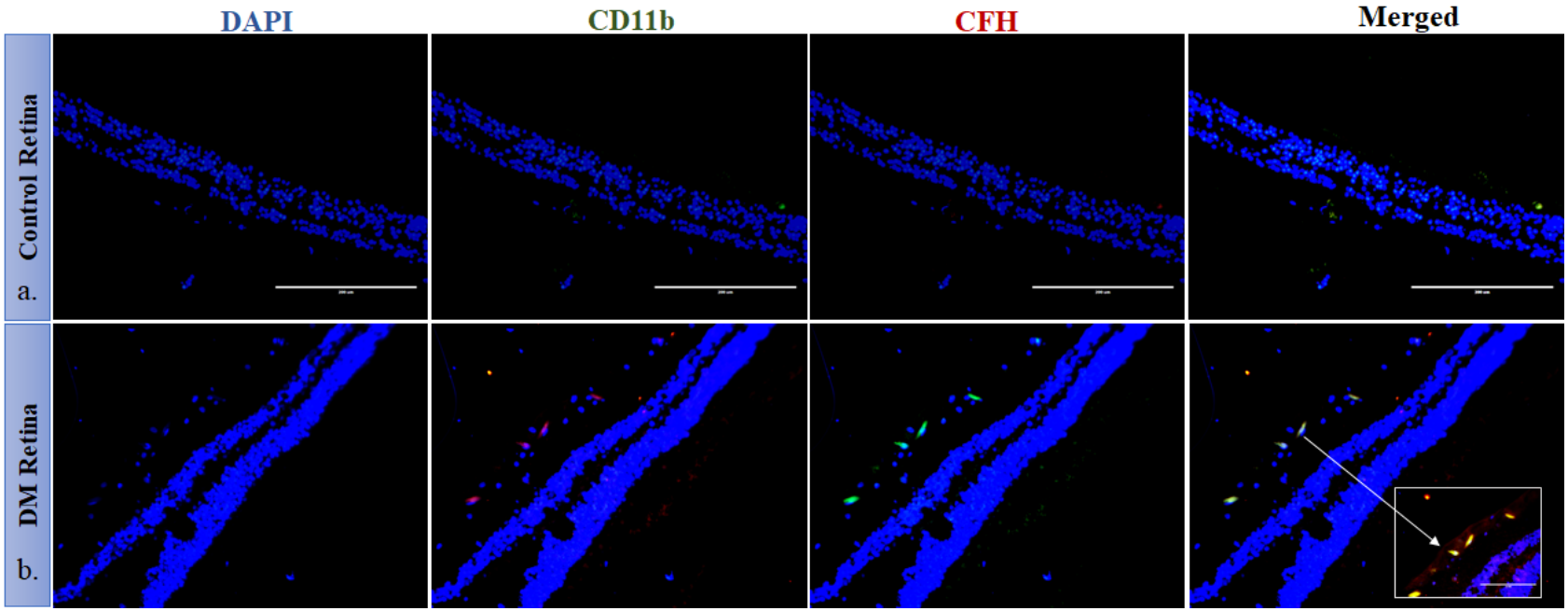


Figure 4.31: Immunostaining of CFH and CD11b in retinal tissues from (a) control and (b) diabetic retina (magnification=20X, scale bar=200µm**)**. Co-localized expression of CFH and CD11b is highlighted in enclosed box in panel b at 40X magnification

4.2.4.4. Evaluation of microglial activation by assessing the expression of CXCR4 in retina

The microglial activation and its migratory potential during diabetes was then assessed based on the level of CXCR4, a chemokine receptor that is largely present in activated microglial cells. The immunostaining of retinal tissues identified an intense staining and greater number of $CXCR4^+$ cells in the inner nuclear and outer plexiform layers of diabetic retina, while it was almost negligible in the control retinas (Figure 4.32). These results suggested an enhanced microglial activation and chemotaxis in diabetic eye.

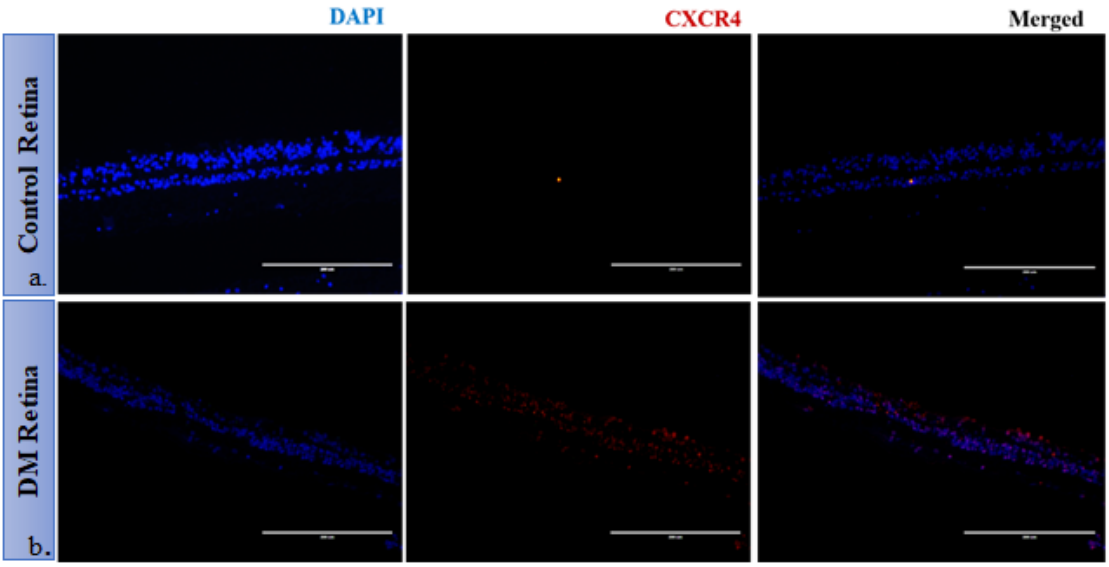


Figure 4.32: Immunostaining of CXCR4 in retinal tissues collected from (a) control and (b) diabetic retina (magnification=20X, scale bar=200μm)

4.2.5. Validation of microglial infiltration and activation in PDR vitreous

In order to validate the increased microglial activation and infiltration in the diabetic retina and vitreous cavity during proliferative stage of the disease, western blotting of the vitreous samples were done using the CD11b antibody. The results clearly identified a sharp band of CD11b in the vitreous humor samples of PDR subjects that was completely absent in the

non-diabetic samples, further confirming microglial infiltration into the vitreous cavity of PDR patients (Figure 4.33a). Further response to microglial activation in the retina was evaluated by analysing the MMP levels in vitreous based on the fact that microglia are the major source of gelatinolytic MMPs such as MMP9 and MMP2. Gelatin zymography was performed to evaluate the enzymatic activity of MMPs in the PDR and control vitreous. A clear gelatinolytic band of MMP9 of 82-85kDa was seen on zymography in the vitreous of both PDR and control though its intensity was much higher in the vitreous of PDR subjects (Figure 4.33b).

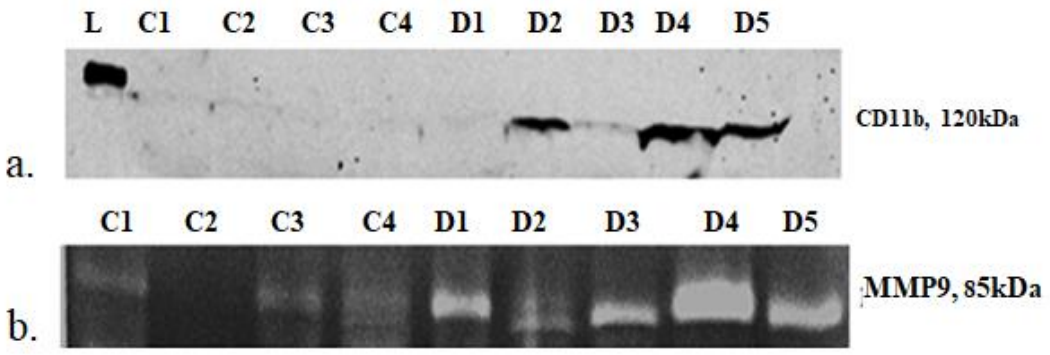


Figure 4.33: (a) Representative western blotting of CD11b in PDR (n=5) and control (n=4) vitreous. (b) Representative gelatin zymography of vitreous samples from PDR (n=10) and controls (n=10) (C-control, D- PDR)

4.2.6. Evaluation of inflammatory and angiogenic cytokines in the vitreous by multiplex ELISA

Multiplex ELISA was done for the quantitative estimation of various analytes such as sVEGFR1, VEGFR2, VEGF, sPECAM1, IL-10 and IL-8 in vitreous samples in a subset of PDR and controls. The sVEGFR1, VEGFR2 and VEGF are the key receptors and angiogenic

agents. VEGFR2 is involved in endothelial cell migration, whereas, sVEGFR1 is a truncated form of VEGFR1, where hypoxia enhances the upregulation of sVEGFR1. Quantification of these molecules through ELISA identified significant upregulation of sVEGFR1 in the vitreous of PDR (n=8) compared to the controls (control (n=8). Likewise, the levels of VEGFR2 and VEGF were significantly higher in PDR compared to the controls (Figure 4.34).

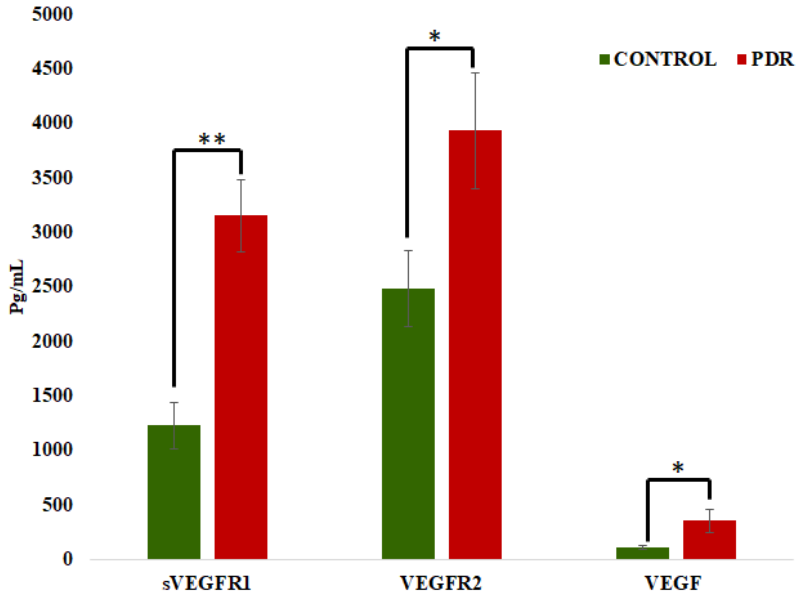


Figure 4.34: Quantitative estimation of sVEGFR1, VEGFR2 and VEGF in vitreous of PDR vs Control by multiplex ELISA, $**p=0.0002$, $*p<0.05$

Further, the level of inflammation in vitreous samples were analysed by quantitative estimation of the pro-inflammatory markers such as sPECAM, IL-8 and anti-inflammatory marker IL-10. The levels of sPECAM and IL-8 were found to be higher in PDR (n=8) vitreous compared to that of controls (n=8) (Figure 4.35a). In contrast, the level of anti-inflammatory cytokine

IL-10 was found to be significantly downregulated in PDR vitreous compared to controls (Figure 4.35b).

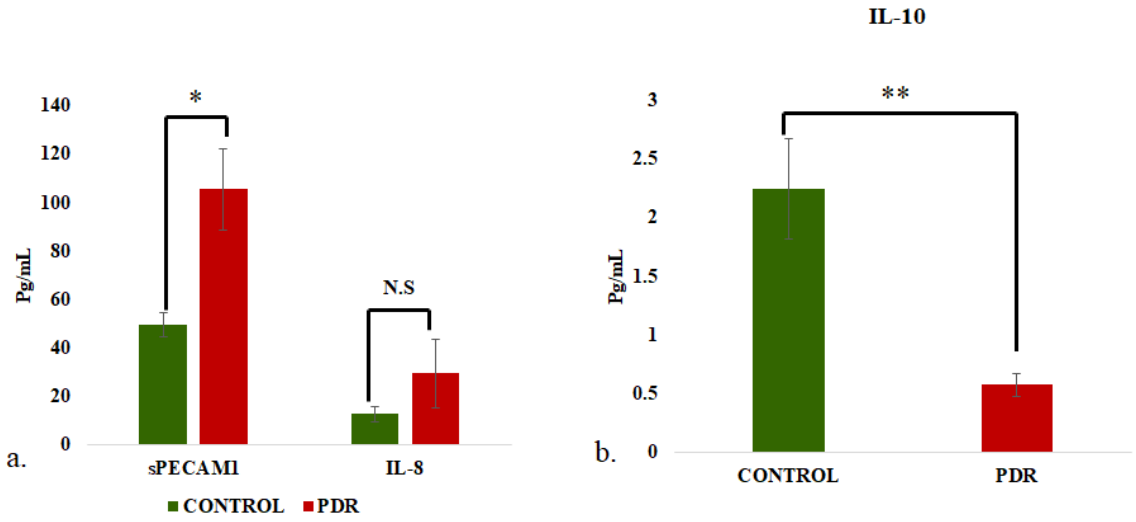


Figure 4.35: Quantitative estimation of (a). sPECAM, IL-8 and (b). IL-10 in vitreous of PDR vs Control by multiplex ELISA, *p<0.01, **p<0.001

4.3. To study the neuroglia interaction and modulation of calcium dynamics in primary human mixed retinal culture under hypoxia and high glucose conditions

In the present study, protein profiling identified significant involvement of glial cells in the pathogenesis of diabeteic retinopathy. Glial cells play a critical role in maintaining retinal homeostasis through neurovascular coupling. Recent studies have suggested that loss of retinal neurons precede the development of abnormal blood vessel growth in DR. Thus, changes in glial activity may have a damaging impact on the neuron survival in the initial phase of DR. An understanding of glial activation during the course of diabetes may offer a better understanding about the disease pathogenesis. However, studying the early events in DR would require a suitable model system that mimics the cellular composition of human retina

and response under diabetic conditions. Hence, the aim of the study was to understand glial activation and its interactions with retinal neurons under hypoxic and high glucose condition using a culture system comprising heterogenous populations of primary retinal cells of human origin, which can offer the measurements of real time responses under stress.

4.3.1 Establishment of primary mixed retinal cells from human retinal tissues

The single cell suspension of retinal cells, obtained from the retinas of cadaveric as well as from open globe injuiry cases were resuspended in complete DMEM and seeded into a 25mm^2 flask. The cells were found to be non-adherent on the culture dish on day 0 (zero). From day 1 (one) of culture, small colonies of cells started to adhere on the culture dish and attained round to ovoid shapes. From days 4 to 5, majority of the cells were seen adhered to the culture dish and attained a clear-cut cellular morphology. Apoptotic cells and cellular debris were removed with every change of medium. The number of apoptotic cells and floating cells reduced after 3-4 medium changes. The cells became 50-60% confluent by the days 12-14 and 80-90% confluent within 3-4 weeks in culture. The cells were heterogeneous in nature as evident from their morphology. Different cell types were found making connections among them (Figure 4.36a). Most importantly, these cells displayed both neuronal and glial morphology with a network of processes. The cells were able to grow even after trypsinization and a clear heterogenous population of the cells was evident until the 3rd passage (Figure 4.36b) after which they tend to become more homogenous and spindle shaped.

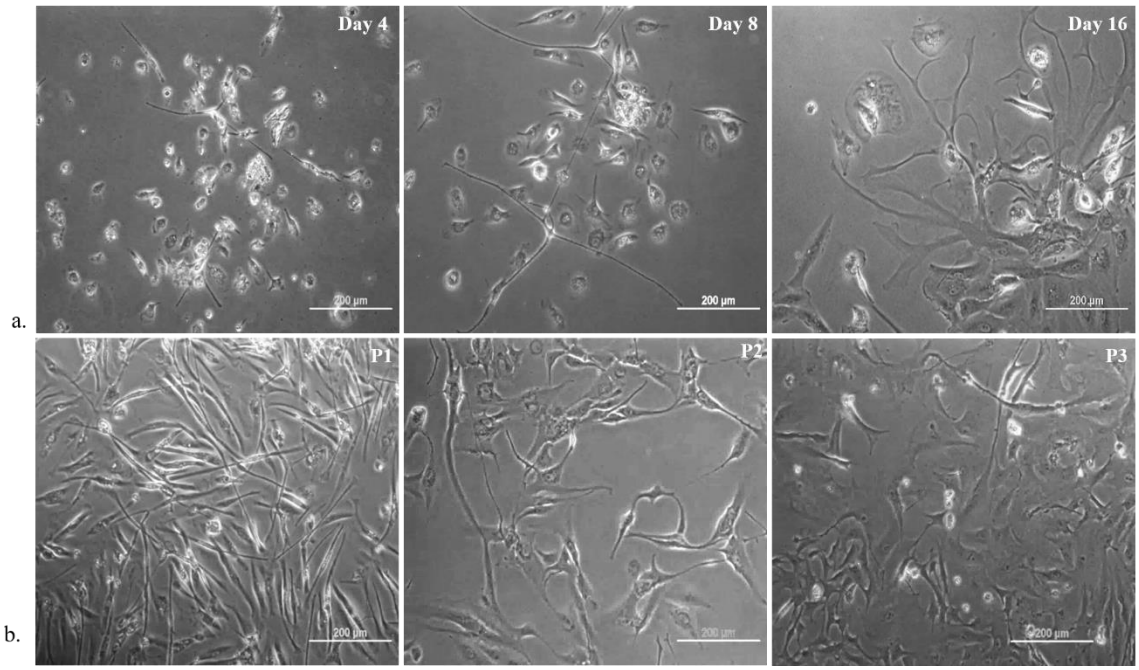


Figure 4.36: Representative phase contrast images of cells cultured from retina of human cadaveric/enucleated eyes. (a) Morphology of the cells after 4th, 8th and 16th days of culture at P0 stage. (b) Morphology of the cells in MRC at 1st, 2nd and 3rd passages (Magnification- 10X, scale bar - 200µm).

4.3.2 Characterization of the mixed retinal cultures

The cells were characterized based on immunofluorescence and gene expression. Cell specific proteins and genes for macroglial population, microglial cells, neurons and neuronal progenitors were used to characterize the cells in the mixed retinal cultures. Immunofluorescence of the cells in MRC clearly identified a heterogenous population of the cells in culture based on the positive staining for cell specific markers such as nestin for neuronal progenitors, glutamine synthetase (GS) for Müller glia, glial fibrillary acidic protein (GFAP) for astrocytes, IBA-1 for microglia and β-III tubulin for mature neurons. All the images displayed here were captured under 20x-magnification. As shown in the Figure 4.37, all the cells in the frame did not have positive staining for the specific markers used and clusters of other cell types were also evident based on the nuclear staining by DAPI which

further confirmed mixed population of cells in the established retinal culture system. In order to differentiate further the two cell types that tend to stay closer, IF was also performed with combinations of two different cells type specific markers such as β-III tubulin-GFAP, GS-GFAP and IBA-1- GS. This clearly confirmed the mixed phenotypes of the cells in this mixed culture system that were used for the subsequent analysis in this study (Figure 4.38). A clear interaction of different populations of cells confirmed the interactions between different cell types in the present culture system that closely resembles the human retina.

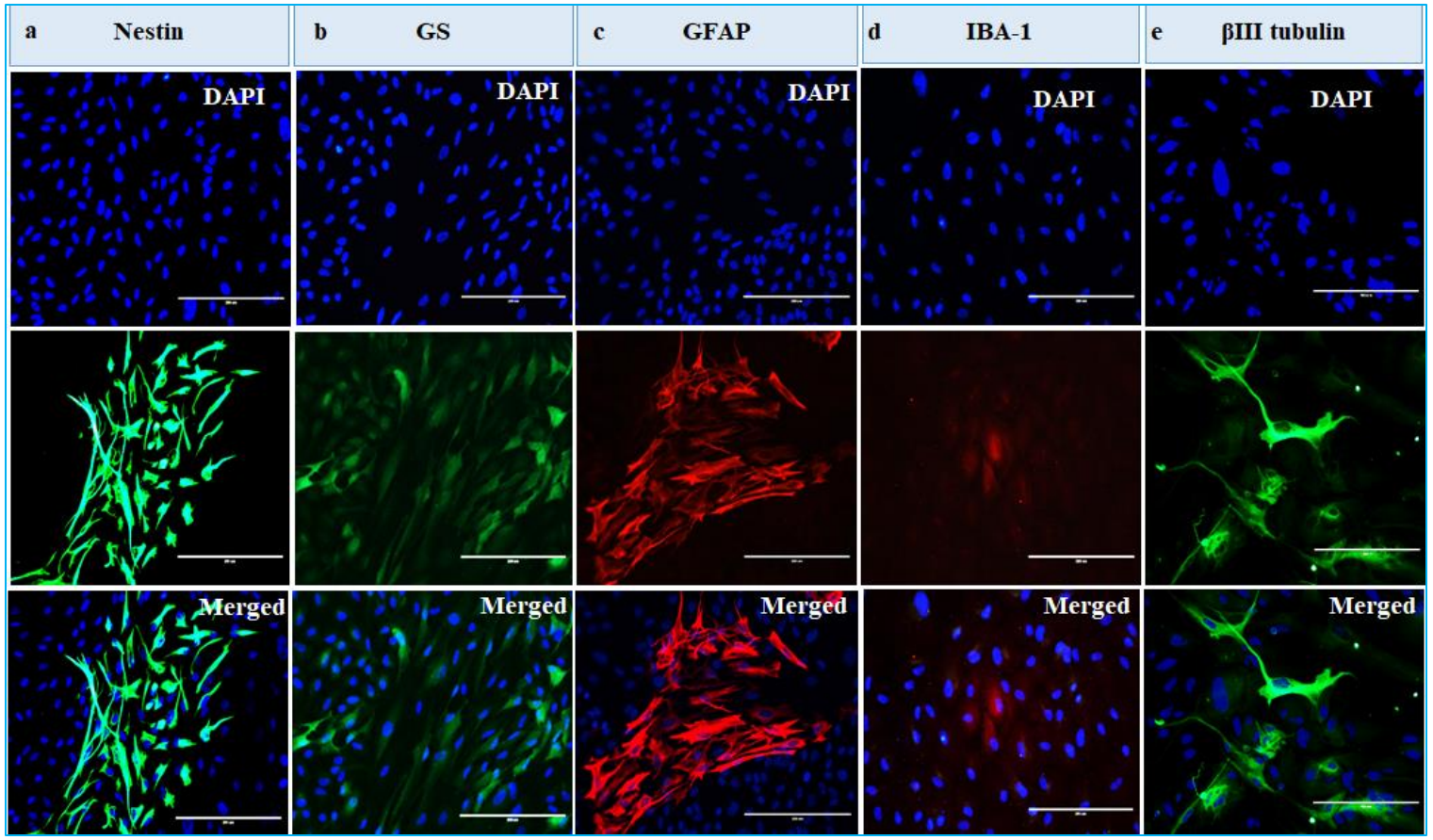


Figure 4.37. **Immunofluorescence based characterization of human primary mixed retinal cells**. (a) cells expressing neuronal progenitor marker; Nestin (b) cells expressing Müller glia marker; GS (c) cells expressing astrocytes marker; GFAP (d) cells expressing microglial cells marker; IBA1 (e) cells expressing Neuronal marker; βIII tubulin. The representative images clearly show the presence of neurons and all type of glial cells (Magnification- 20X, scale bar - 200μm)

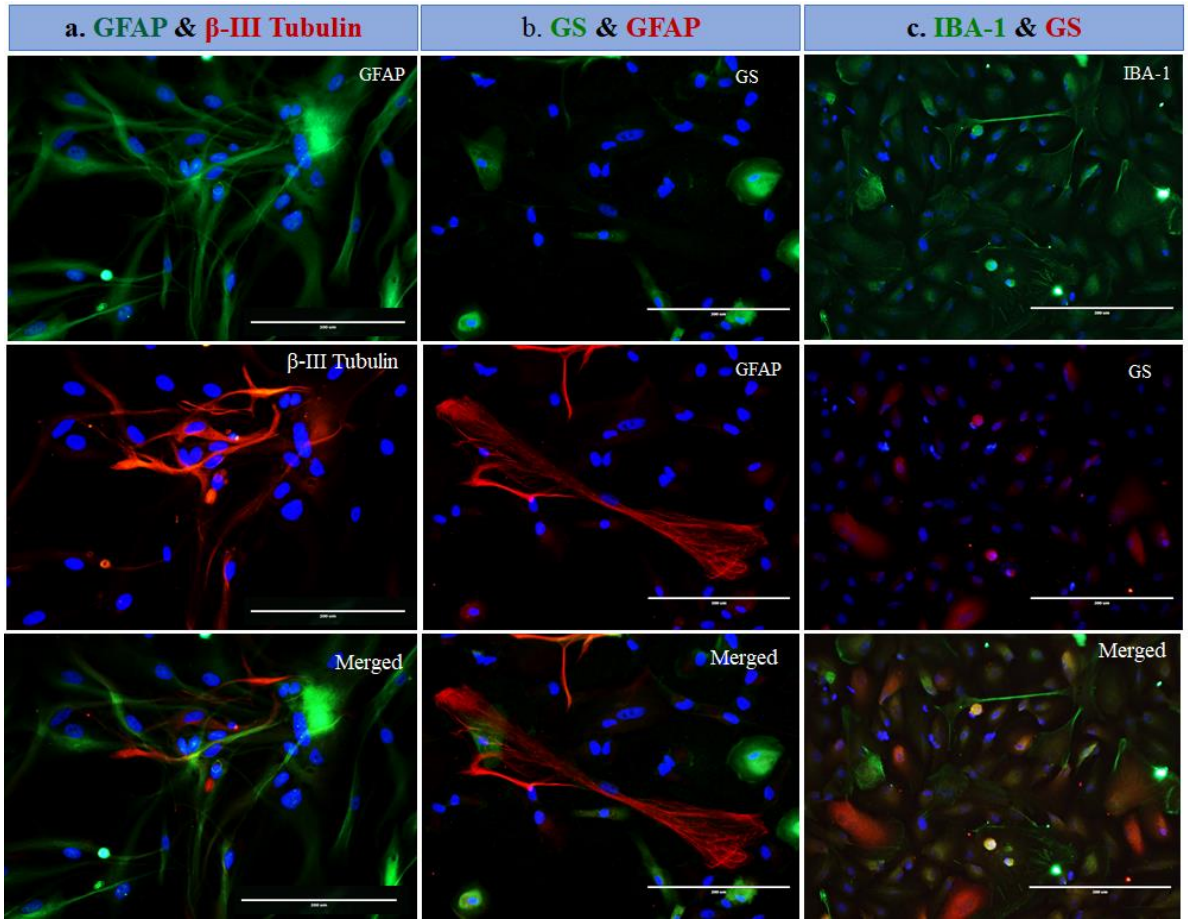


Figure 4.38. Representative images showing Immunofluorescence based characterization of different cell types in the mixed culture in the lab (a) cells expressing βIII tubulin and GFAP (b) GS and GFAP marker (c) cells expressing IBA-1 and GS. The representative images clearly display the cellular heterogeneity in the established mixed retinal culture system, (Magnification- 20X, scale bar - 200μm)

Likewise, PCR based characterization of the cells also confirmed the expression of genes specific to glial cells (GFAP- astrocyte, GS- Müller glia, IBA-1- Microglia), neural progenitor cells (nestin) and mature neurons in the dissociated retinal culture (βIII tubulin) as shown in the Figure 4.39.

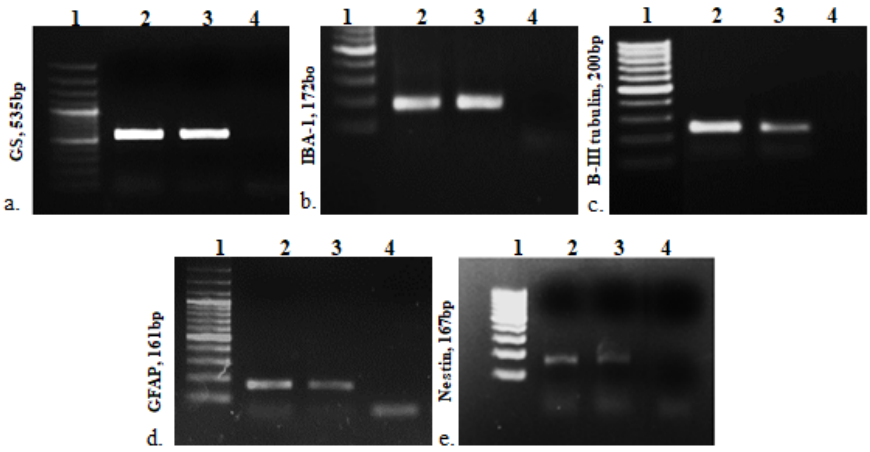


Figure 4.39: PCR based characterization for cell type specific markers; (a) GS, (b) IBA-1, (c) βIII tubulin, (d) GFAP and (e) Nestin, respectively. 1- represent molecular weight marker, 2- represents the cultured retinal cells and 3- represents retinal tissue as positive control, 4 represent PCR negative.

The cells were actively dividing until 4 (four) passages. Cells in passages 2 and 3 derived from three different donor sources were used to further study their interactions under the hypoxic and hyperglycemic stress conditions. The cellular characterization for ensuring the presence of all the four cell types were performed at every P1 and P2 passages using markers for Müller glia (GS and CRALBP), astrocytes (ALDH1L1), Microglia (IBA-1), neurons (βIII tubulin) prior to the induction of stress to these cells. As shown in figure 4.40, the PCR characterizations clearly identified expression of cell specific markers in the P1 and P2 passages of cells derived from three different donor retinal sources.

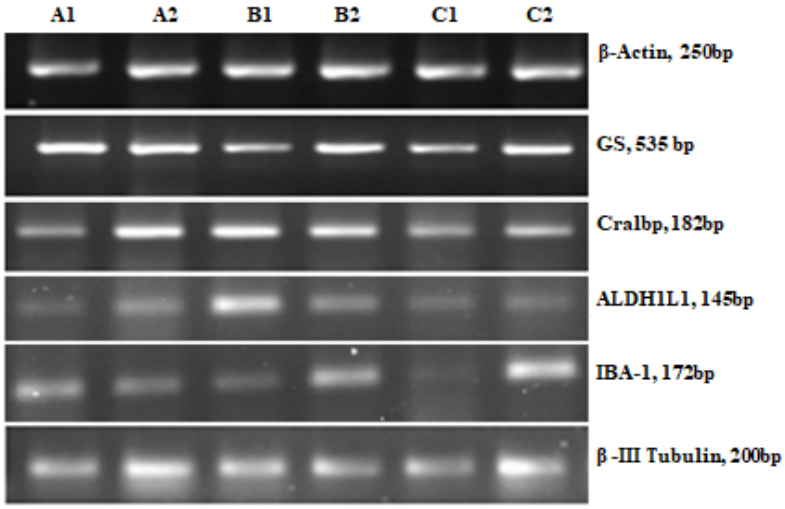


Figure 4.40: Gene expression of neuron and glial cell specific markers from three different retinal donors' tissue at P1 and P2 passages. (A, B and C represents primary cells cultured from three different retinal tissues and 1 and 2 represents the P1 and P2 passages, respectively)

4.3.3. Reproducibility of gene expression in MRC

The proposed mixed culture of primary retinal cells showed representation of both glial and neuronal cells of the retina. Further, real time PCR was done to understand the quantitative expression of each cell types in the mixed retinal cultures from retina donated by six donors after passage P1. For each experiment, gene expressions were evaluated in MRC by RT-PCR using GFAP as marker for astrocyte, GS for Müller glia, IBA1 for microglia and βIII-tubulin as a neuronal marker. Figure 4.41 represents the expressions of each cell type specific marker (ΔCt= Ct value of gene-Ct value of house -keeping gene) in six different donor cultures. The result showed that the percentage of each cell type derived from different eyes were not significantly different ($p > 0.05$), which confirmed approximately equal percentages of similar cell types in all the 6 cultured MRC system. Thus, there was good reproducibility of the culture method for generating mixed retinal cell culture population.

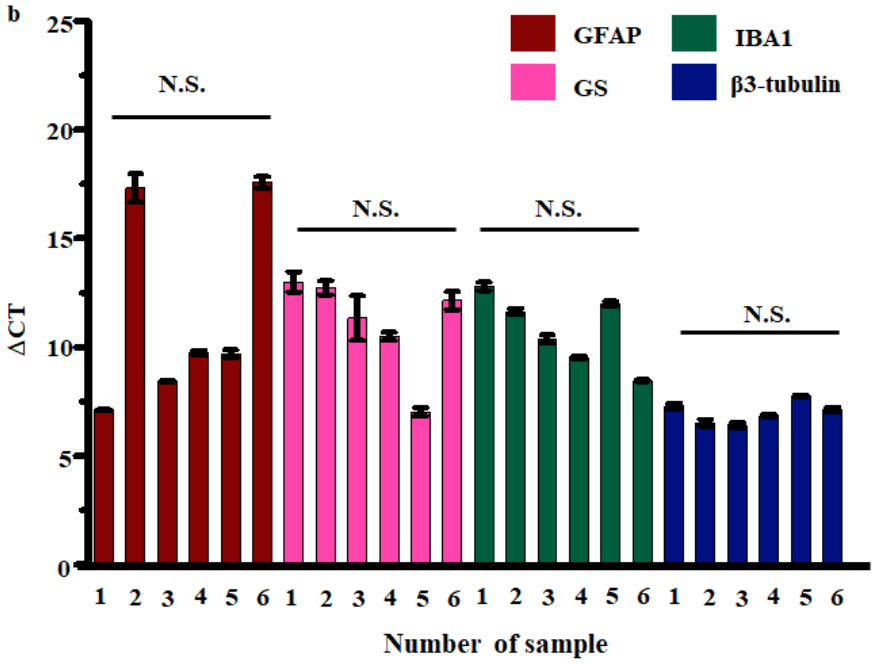


Figure 4.41. Analysis of reproducibility of cells in primary human mixed retinal cultures using quantitative real time PCR, Bar graph representing the subpopulations of four different cell types in MRC corresponding to samples from six donor retinas. Data is represented as Mean± SEM. Number 1-6 indicates six different donors.

4.3.4. Measurement of cell viability under hypoxia and high glucose conditions

In order to quantify the viability of cells after stress induction, the Alamar blue cell viability assay was performed. The serum deprived cells were exposed to different concentrations of $CoCl_2$ and D-glucose for a time period of 24 hours and the cell viability was measured at different concentrations of $CoCl_2$ (100, 150, 200 and 250 μM) for hypoxic and D-glucose (25, 30 and 40 mM) for high glucose stress and compared with the untreated cells in serum deprived medium. The cell viability in controls were always maintained as 100%. Each experiment was done with three biological and three technical replicates. As shown in Figure 6, hypoxia treatment induced a concentration dependent reduction in cell viability. The percentage of viable cells in 100, 150, 200 and 250 μm of $CoCl_2$ were 89.76±5.8 ($p>0.05$, N.S),

62.33±1.7 (*p*<0.0001*), 62.78±1.03 (*p*<0.0001*) and 60.98±0. 65 (*p*<0.0001*), respectively (Figure 4.42). A significant reduction in cell viability was observed at the concentration of 150, 200 and 250 μm of $CoCl_2$ though there was not much differences in cell viability between these concentrations. Based on these 150 μm $CoCl_2$ seemed to be an optimum concentration for hypoxia induction. Similar to that of hypoxia, a concentration dependent reduction in cell viability was observed in cells under high glucose concentration. The percentage of viable cells were found to be 84.07±8.74 (*p>0.05,* N.S), 71.5±4.06 (*p*<0.002*) and51.47±2.43 (*p*<0.0001*) at 25, 30 and 40mM D-glucose, respectively. A significant reduction in viable cells were found at concentrations of 30 and 40mM, where 40mM concentration was found to reduce half of the cell population. Therefore, 30mM of D-glucose was selected for high glucose induction in the mixed retinal cells and same concentration of D-mannitol was used as an osmolarity control (Figure 4.43).

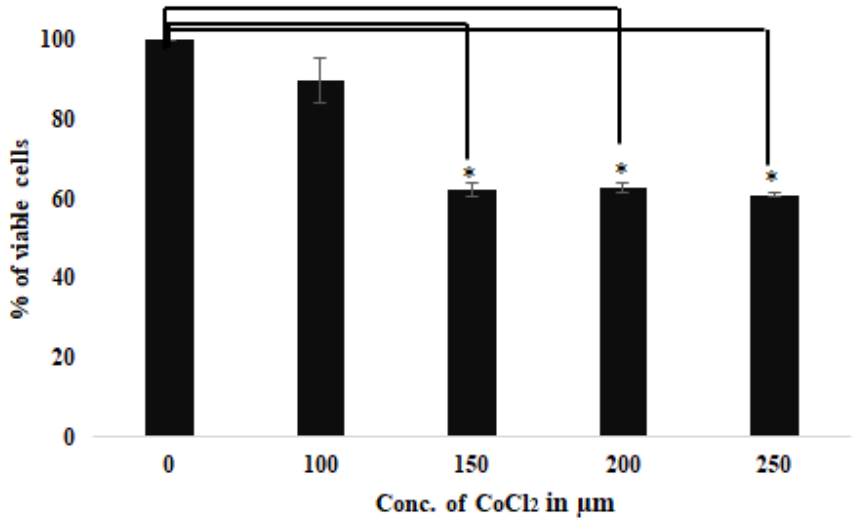


Figure 4.42: The mixed retinal cultures were treated with increasing concentration of $CoCl_2$ for a period of 24hrs. The viability was measured using Alamar blue based dose dependent cell viability assay. The data are represented as Mean ± SEM (n=3), **p*<0.0001.

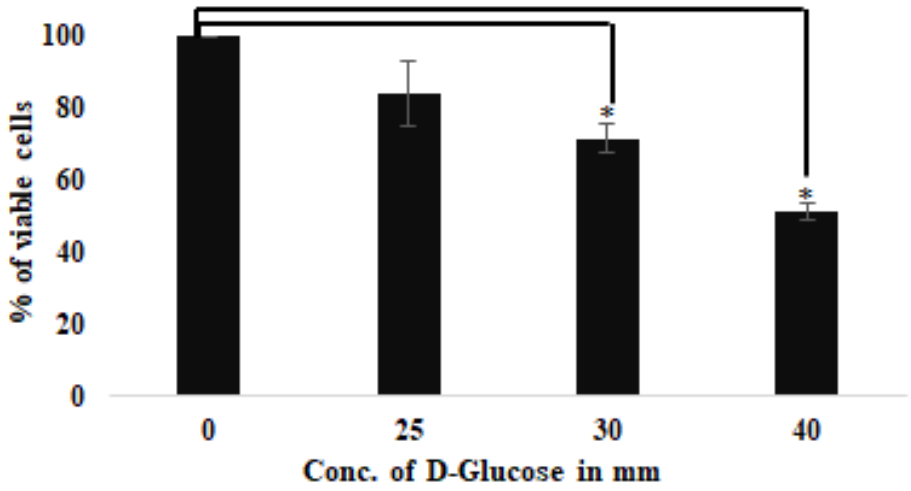


Figure 4.43: The mixed retinal cultures were treated with increasing concentration of D-glucose for a period of 24hrs. The viability was measured using Alamar blue based dose dependent cell viability assay (N=3). The data are represented as Mean ± SEM (n=3), *p<0.002.

4.3.5. Imaging of Ca^{2+}signaling in mixed retina population under hypoxic and high glucose conditions

Calcium is the secondary messenger and a major intrinsic signaling system present in the retinal cells. It plays a vital role in various biological functions. Glial Ca^{2+} is known to modulate neuronal activity and vice versa. Hence, studying intracellular Ca^{2+}signaling would be an ideal method to understand glial activity in the retina and its effect on neuronal cells under stress conditions in a system that closely mimic human retina. The levels of intracellular (cytosolic) Ca^{2+}in MRC were evaluated under hypoxic and high glucose condition and compared to basal cytosolic Ca^{2+}level in cells under no-stress condition by time lapse imaging after tagging with $Flu4^{am}$ Ca^{2+} indicator dye. First, the characterization of basal level of calcium in MRC was done under no stress and the corresponding time lapse imaging was done (Figure 4.44a). Followed by this, time-lapse imaging of intracellular $Ca^{2+}$for hypoxia and high glucose was performed and representative time lapse images are given in figures 4.44b and 4.44c. Further,

the time course of intracellular Ca^{2+} level in each of these conditions were analysed. The time lapse imaging clearly identified an increase in Ca^{2+}spiking in cells under stress compared to no stress conditions.The representative raw plots of cytosolic Ca^{2+} level in different cells are given in the Figure 4.44 (d) no stress (e) hypoxia (f) high glucose (n=9 cells). The analysis of Ca^{2+}spiking pattern under no stress, hypoxia and high glucose indicated that the intracellular Ca^{2+} oscillates at variable frequencies for different cells in MRC population. Most importantly, each of the cell in whole population did not show a shift in Ca^{2+} flux, which further could be due to a heterogenous population of cells in MRC.

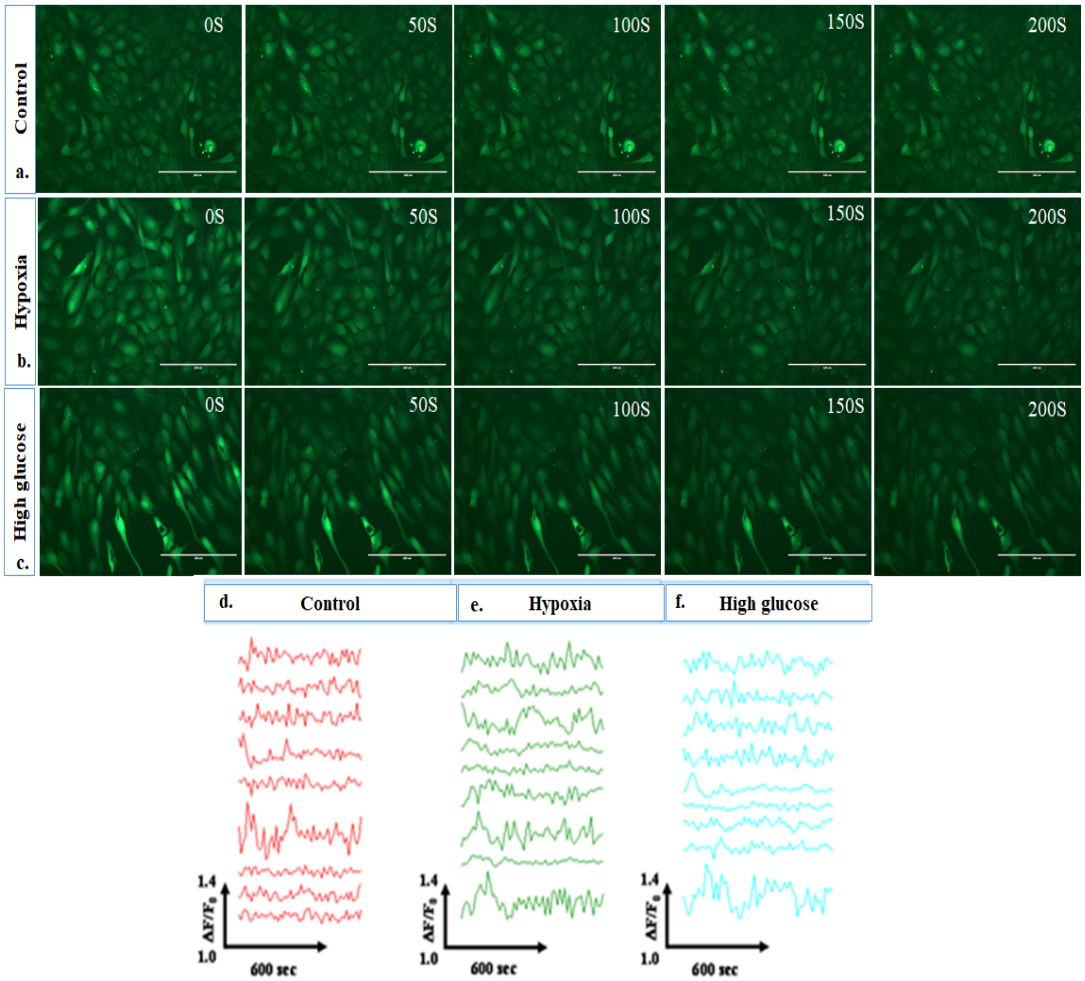


Figure 4.44. Fluorescence imaging of time course of cytosolic Ca^{2+} in human primary mixed retinal cells; representative time lapse images for (a) control (no stress) (b) hypoxia (150 μM $CoCl_2$) (c) hyperglycemia (30 mM D-glucose). Representative raw plots of time course of cytosolic Ca^{2+} under (d) no stress (e) hypoxia (f) high glucose (n=9).

In order to quantify the intracellular Ca^{2+} level spatial mapping of Fluo-4 intensity in each of the cells in MRC population was done. For prominent visualization of the Ca^{2+}spiking, heat map was generated in each of the cells in MRC population under control, hypoxic and high glucose condition. Thus, total of 150 cells were analysed in each condition (n=150). Representative spatial mapping of single cell in MRC population by heat map under no stress, hypoxia and high glucose are given in figures 4.45a, 4.45b and 4.45c. It was noted that not all the cells in a population showed Ca^{2+}spikes indicating that some cells had relatively less or no activity. Further, to observe Ca^{2+} spiking pattern and network activity in large MRC population, calcium spiking pattern in large number of cells (n= 150) were analysed by measuring the total number of Ca^{2+} spike and maximum Ca^{2+} amplitude (Ca_{max}) between no stress, hypoxia and high glucose condition. The corresponding raster plots were generated under each of the conditions (Figure 4.45d, Figure 4.45e and Figure 4.45f). The raster plots showed that there is an increase in Ca^{2+}spikecount in case of hypoxia and high glucose compared to no stress condition. The box plot showing the the comparison of Ca^{2+}spike count and $Ca^{2+}{}_{max}$ between no stress condition, hypoxia and high glucose are given in the Figure 4.45g and Figure 4.45h. The boxplot indicated that hypoxia induced a significant increase in Ca^{2+} spike count in MRC population ($p<0.05$). Similarly, high glucose was also found to induce significant increase in the Ca^{2+} spike count in MRC population ($p<0.05$). However, the number of calcium spikes were found to be higher in cells under hypoxia than high glucose ($p<0.05$).

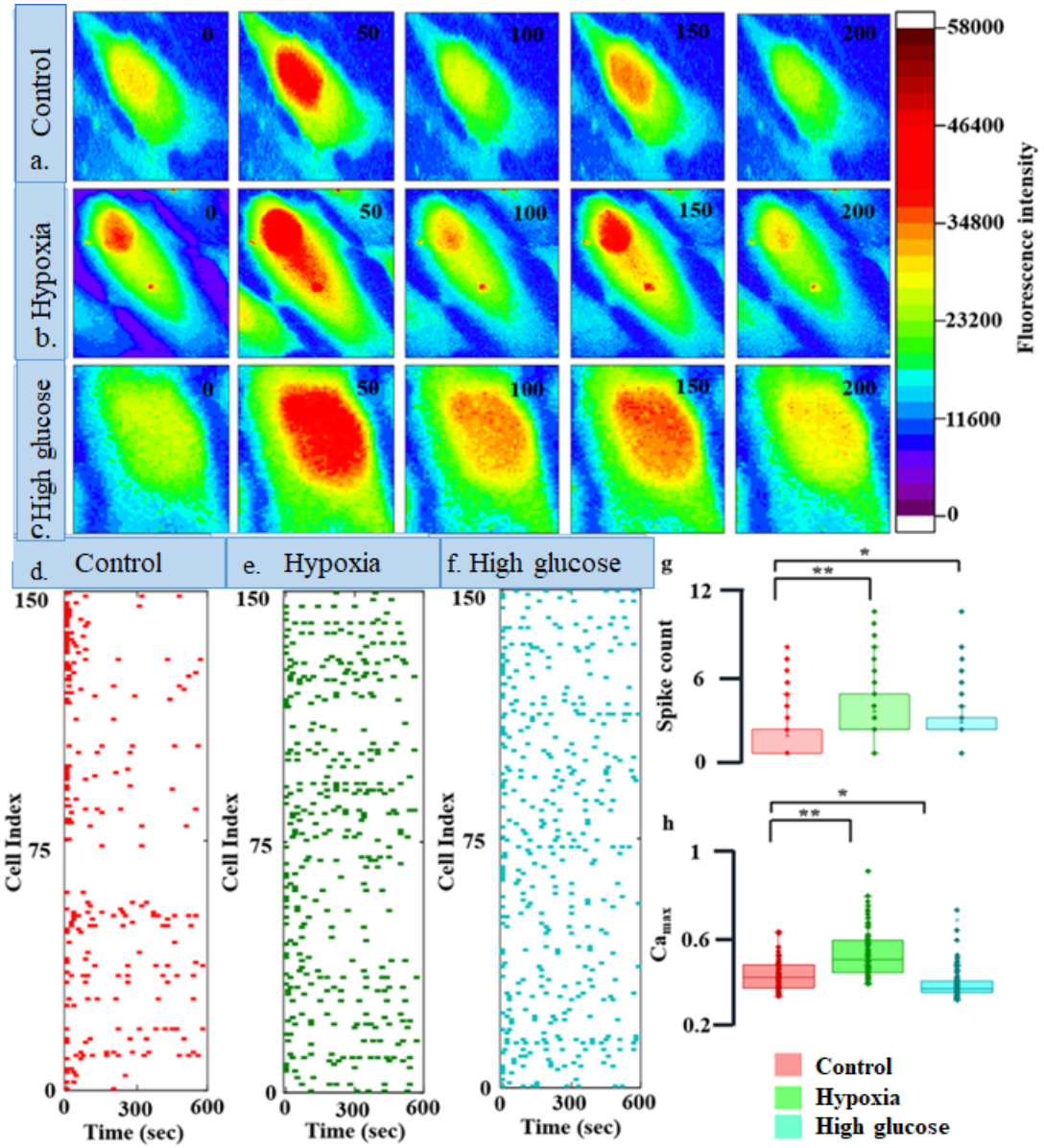


Figure 4.45: Representative spatial intensity mapping of Ca^{2+} flux in single cell present in MRC (a) control (b) hypoxia and (c) high glucose. Increasing fluorescence intensity is indicated using a standard pseudocolour heat map scale. Raster plot representing the network activity in MRC (d) control, (e) hypoxia and (f) high glucose, (n=150) (g) Comparison of Ca^{2+} spike count between no stress, hypoxia and hyperglycemia (h) Comparison of $Ca^{2+}{}_{max}$ between no stress, hypoxia and high glucose *p<0.05

4.3.6. Quantitative gene expression analysis by Real Time PCR

In order to understand the correlation of Ca^{2+} spiking and gene expression level, quantitative real time PCR (qRT) was done. Cells exposed to 150μM $CoCl_2$ and 30mM of D-glucose in serum deprived medium to induce hypoxia and high glucose conditions were harvested after 24 hours and RNA was extracted. Real time PCR was performed for3 sets of heterogeneous cell cultures derived from 3 different retina sources. The expression of representative genes from hypoxia signaling (*HIF1-a, NERF2,* and *OXR1*), inflammation (*IL-1β, IL-6, IL-8* and *C3*), angiogenesis (*CXCR4* and *VEGF*) and apoptosis (*BAX* and *Caspase 3*) were measured. All the experiments were done in three biological and technical replicates.

The expression of *HIF1-a* was found to be significantly upregulated by 2.28±0.37 ($p^*<0.05$) folds under hypoxia and 2.02±0.5 folds under high glucose ($p^*<0.05$). Likewise, the genes involved in oxidative stress response such as *OXR1* and *NERF2* were upregulated under both the conditions (hypoxia: 2.56±0.53 ($p^*<0.05$) and 2.59±0.44 ($p^*<0.05$); HG: 1.75±0.07 ($p>0.05$, *N.S*) and 1.21±0.023, $p^*>0.05$) respectively (Figure 4. 46).

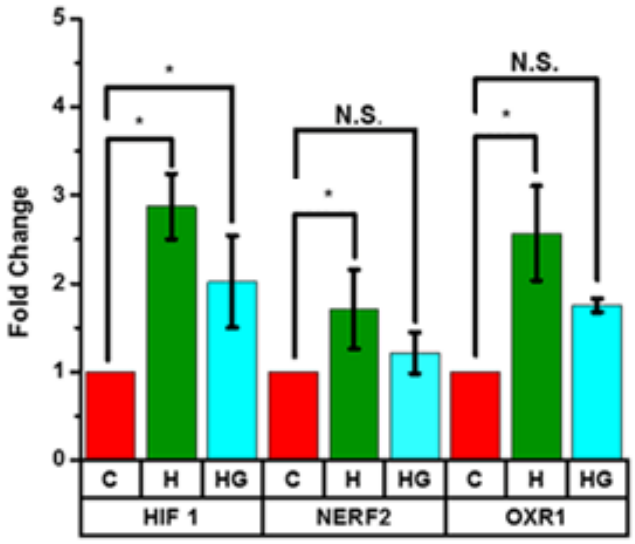


Figure 4.46. Quantitative gene expression analysis of genes involved in oxidative stress
**p*<0.05, N.S: not significant

The expressions of genes involved in angiogenesis such as *VEGF*, *CXCR4* and *C3* were increased in hypoxia 3.48±0.8 (*p*<0.05*), 6.89±1.02; (*p*<0.05*), 1.53±0.05 (*p>0.05, N.S*), respectively and in HG the expression of these genes were found to be present with a fold increase of 1.53±0.105; (*p>0.05, N.S*), 3.46±0.205; (*p*<0.05*) and 1.24±0.36 (*p>0.05, N.S*) ,respectively (Figure 4.47).

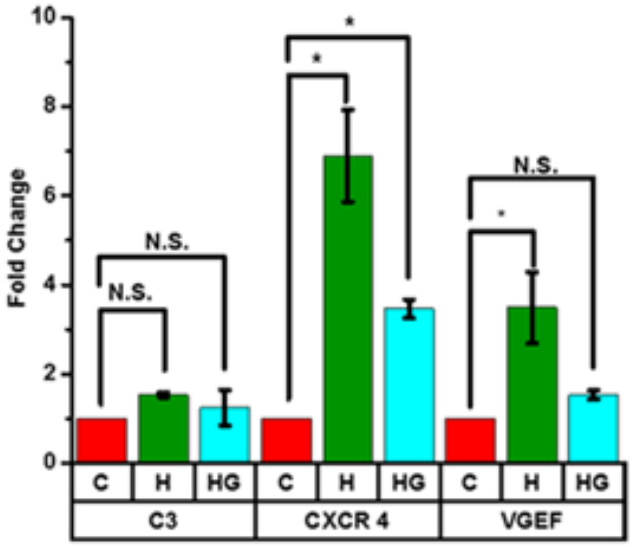


Figure 4.47. Quantitative gene expression analysis of genes involved in inflammation and angiogenesis *p<0.05, N.S: not significant

The apoptotic markers *Caspase*-3 and *BAX* showed an increased expression under hypoxia 2.26±0.63 (p>0.05, N.S) and 1.41±0.3 (*p>0.05, N.S*) and in HG 1.68±0.14; (*p>0.05, N.S*), 1.99±0.36; (*p>0.05, N.S).* The level of proinflammatory cytokines *IL-1β* was found to be upregulated under hypoxia 15.3± 2.5 (p*<0.05) and in HG 2.57 ± 0.5; (*p>0.05*, *N.S*) while the expression of *IL-8* and *IL-6* were unaltered after exposure of both hypoxia (*p>0.05)* and HG (*p>0.05*) (Figure 4. 48).

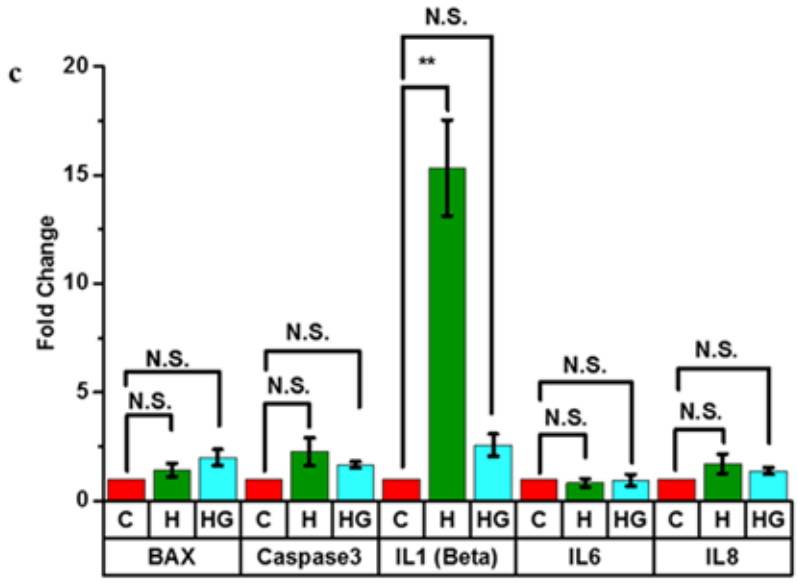


Figure 4. 48. Quantitative gene expression analysis of genes involved in apoptosis and inflammation *p<0.05, N.S: not significant

4.3.7. Quantitative assessment of gliosis (IBA-1 and GFAP) under hypoxic and high glucose condition in primary mixed retina culture

Further, the expression of gliotic markers such as GFAP and IBA-1 was analysed in the MRC population, based on the fact that the increased expression of these two markers indicate glial activation followed by neuronal damage. Proteomic data from the present study also clearly identified activation of glial population in the retina under diabetic stress, especially the microglial population. In order to quantify these proteins under stress conditions, immunofluorescence was performed for the cells of MRC under hypoxic and high glucose conditions and compared them with cells under no stress condition. Due to heterogenous population of cells in culture and also due to spatial heterogeneity observed in various proteins in MRC, a large-scale imaging for protein quantification was performed in these cells using confocal microscopy. A large panorama of Z-stack images were taken and analyzed the

expression of IBA- 1 and GFAP. Representative 3D images of IBA – 1 and GFAP positive cells under control, hypoxia and high glucose conditions are given in the Figure 4.49a and Figure 4.49c. In-order to quantify their expression, 3D surface plots were generated corresponding to spatial profiling of IBA-1 and GFAP under the three different conditions as shown in the Figure 4.49b and Figure 4.49d.

The spatial pattern clearly identified the differential expression of IBA-1 and GFAP under stress compared to control conditions, indicating the activation of microglia and astrogliosis under stress. Further, the quantification of their expression was done by analyzing the protein expression in large number of cells in MRC under three different conditions. This identified a significant 2-fold increase of IBA-1 and 1.7-fold increase of GFAP in hypoxia treated cells (Figure 4.49e), whereas high glucose induced a significant increase of 1.5- fold for IBA-1 and a significant downregulation of GFAP expression (0.7-fold) compared to no stress condition was observed (Figure 4.49f)

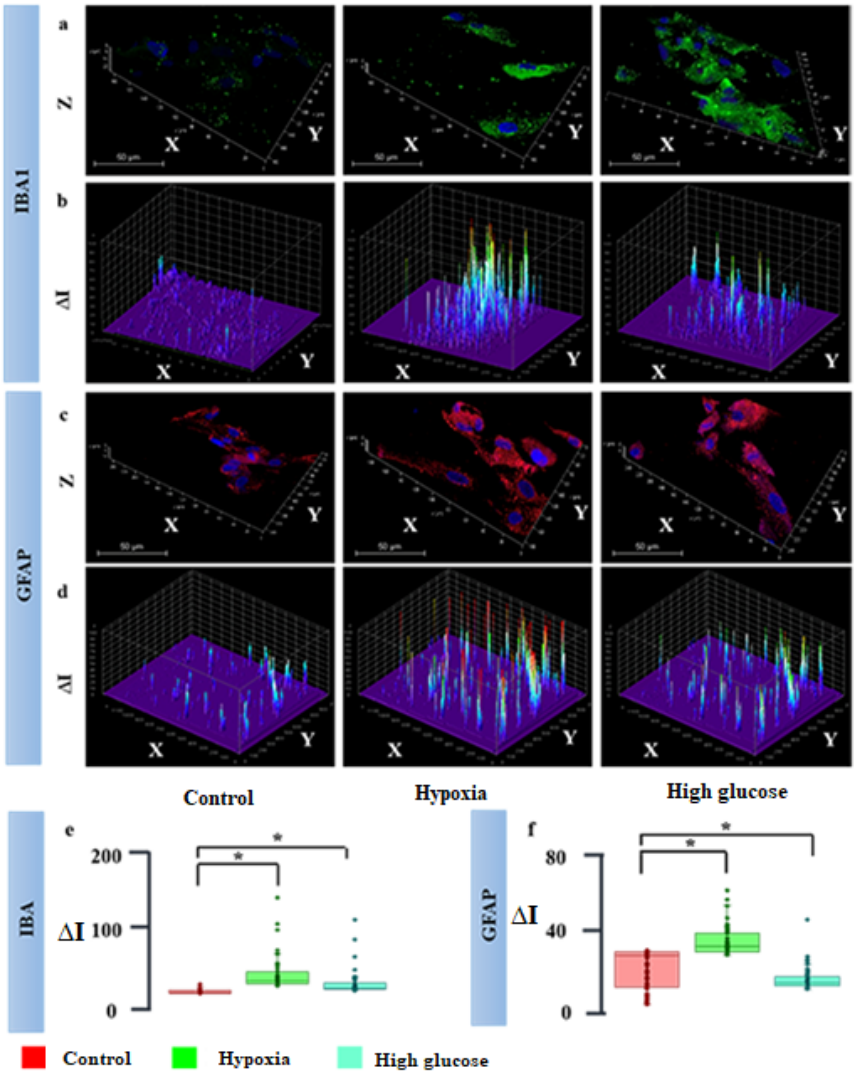


Figure 4.49. IBA1 and GFAP expression in MRC under no-stress, hypoxia and high glucose conditions (a) Representative 3D images showing IBA1 expression under no stress, hypoxia and high glucose; (b) Surface plot showing the spatial profiling of IBA1 expression under no stress, hypoxia and high glucose (ΔI indicating the fluorescent intensity corresponding to protein level). (c) Representative 3D images showing GFAP expression under no stress, hypoxia and high glucose. (d) Surface plot showing the spatial profiling of GFAP expression under no stress, hypoxia and high glucose. (e) Comparison of IBA1 expression between no stress, hypoxia and high glucose (f) Comparison of GFAP expression between no stress, hypoxia and high glucose, *p<0.05.

5. DISCUSSION

Diabetic retinopathy is a complex metabolic disease and causes catastrophic loss of vision, if untreated. A complex interplay between neuro-vasculature, genetic factors, immunological and inflammation related factors are known to be involved in DR progression (Loukovaara, Nurkkala, Tamene *et al.* 2015). However, none of the available treatment modalities can completely arrest the onset and progression of DR and its associated complications. Further an early diagnosis of the disease based on biomarker(s) in relevant biological samples could help in preventing disease development and retarding progression. It is also essential to understand the key mechanisms involved in DR from human samples that can interconnect various other players involved in progression like neurodegeneration and neovascularization. The present study was aimed at identifying the key players of PDR pathogenesis using a combination of proteomics and cell culture-based approaches through global vitreous proteome profiling, targeted investigations of glial mediated complement activation and further assessment of the role of neuro-glia interaction by developing an *in-vitro* culture system.

5.1. Role of proteomics in diabetic retinopathy

Knowledge of protein changes in complex metabolic diseases like DR is very crucial to explore the underlying mechanisms and for providing a better assessment of their relative contributions to disease progression. For the past two decades, several efforts have been undertaken to explore the proteome of DR subjects by characterizing the total proteins involved and their expression levels using various biological samples such as serum, retina, retinal membranes and vitreous humor (George, Chen, Chaudhary *et al.* 2009, Liu, Hu, Wu *et al.* 2011, Sundstrom, Hernandez, Weber *et al.* 2018). However, vitreous humour is the most

reliable and accessible human tissue to explore the mechanism of DR since it is most proximally attached to the retina and can provide a snapshot of all the alterations occurring within this tissue. Thus, studying the vitreous proteome may help to elucidate the pathogenesis of retinal complications, especially ocular angiogenic conditions like DR. Among the vitreous proteome studies undertaken so far, only a few compared the alterations of vitreous proteome in DR compared to diabetes without retinopathy (Gao, Chen, Timothy *et al.* 2008). This is very crucial in order to understand and distinguish the proteome alterations specific to PDR from those to diabetes alone. The present study compared the vitreous proteomes of PDR, NDR and NDM and measured the changes in proteins expression by label free quantification (LFQ) using mass spectrometry. This approach enlisted the proteins differentially expressed during diabetes and those involved in PDR.

5.2. Mass spectrometry-based vitreous protein characterization

MS is one of the sophisticated techniques with high resolution to characterize proteins from a complex mixture like the vitreous humour. But the **extent of protein identification is dependent on multiple factors such as** the choice of sample preparation method, which rely mainly on the complexity of the protein mix, presence of abundant proteins and its depletion, prefractionations strategies and the resolutions of mass spectrometer (Angel, Aryal, Hengel *et al.* 2012). Several groups have tried to catalogue vitreous protein by MS/MS in normal and diseased samples but these studies generated variable data showing significant differences in the identified proteins (Table 5.1), suggesting thereby that no individual technology by itself can cover the entire vitreous proteome.

Table 5.1: Comparison of proteins identified and methods used in different vitreous proteome studies

Method used	Prefractionation method	Abundant protein depletion	Total Proteins identified	Sample Size (n)	References
MALDI-TOF, ESI-MS–MS	2D PAGE	Yes	51	5	(Nakanishi, Koyama, Ikeda et al. 2002)
LC-MS/MS (Q-TOF), MALDI	2D PAGE	No	36	51	(Yamane, Minamoto, Yamashita et al. 2003)
MALDI-TOF	SDS PAGE	No	12	16	(Wu, Sauter, Johnson et al. 2004)
LC MS/MS (tandem)	2D PAGE	No	48	18	(Ouchi, West, Crabb et al. 2005)
MALDI-TOF, MALDI-TOF/TOF	2D PAGE	No	23	30	(Kim, Kim, Park et al. 2006)
MALDI, nano-LC, MALDI MS/MS, nano-LC-ESI-MS/MS	(IS)/2-DE	Yes	531	33	(Kim, Kim, Kim et al. 2007)
MALDI-MS	DIGE	Yes	41	18	(Garcia-Ramirez, Canals, Hernandez et al. 2007)
nano-LC/MS/MS, (LTQ-ion trap)	SDS PAGE	No	252	17	(Gao, Chen, Timothy et al. 2008)
MALDI	DIGE	Yes	25	16	(Hernandez, Garcia-Ramirez, Colome et al. 2013)
LTQ-ORBITRAP XL	SDS-PAGE, Liquid phase IEF and IEF + SDS PAGE	No	1111	3	(Aretz, Krohne, Kammerer et al. 2013)
RP-HPLC coupled with ESI-MS/MS (LTQ)	In-gel digestion	No	96	16	(Wang, Feng, Hu et al. 2013)
LC-MS/MS (LTQ-Orbitrap)	In-gel digestion, SCX and OFFGEL fractionation.	Yes	1205	5	(Murthy, Goel, Subbannayya et al. 2014)
EASY-nLCIIsystem (Orbitrap Elite hybrid mass)	In -solution digestion	No	2482	138	(Loukovaara, Nurkkala, Tamene et al. 2015)
CE-MS (micro-TOF MS)	In -solution digestion	No	101	132	(Nobl, Reich, Dacheva et al. 2016)
CE-MS (micro-TOF MS), LC-MS/MS	In -solution digestion	No	94	68	(Reich, Dacheva, Nobl et al. 2016)
LC-MS/MS (tandem)	In-solution digestion	No	740	17	(Zou, Zhao, Yu et al. 2018)
EASY-nLC-LC-MS/MS (orbitrap), 2018	In-solution digestion	No	610	17	(Li, Lu and Lu 2018)
nano-HPLC- LC-MS (orbitrap) Fourier transformation MS	In-solution digestion	Yes	677	32	(Schori, Trachsel, Grossmann et al. 2018)
LC-MS/MS	In-solution digestion	No	940	25	(Zou, Han, Zhao et al. 2018)
LC-ESI-MS/MS- iTRAQ,	In-solution digestion	Yes	1030	8	(Santos, Gaspar, Ciordia et al. 2018)
Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer	In gel digestion	No	1079	9	Present study

As seen above, four large vitreous proteome databases have utilized either multiple fractionation techniques and/or a larger sample sizes (Table 5.1). Compared to all these vitroeus proteome studies, the present study also identified 1079 proteins from 9818 peptides using 9 vitreous humor samples by a single method of pre-fractionation (SDS-PAGE) followed by LC-MS/MS. The abundant protein depletion was not performed in order to avoid loss of low abundant proteins along with abundant proteins, which may have an important role from the disease perspective. Thus, one of the major advantages achieved in the present study was the identification of a large number of vitreous proteins using a single prefractionation strategy followed by LC-MS/MS without any abundant protein depletion method.

The study identified a greater number of proteins in NDM group, followed by NDR and PDR. Earlier studies reported higher complexity in PDR vitreous based on the disease complexity and increased number of proteins identified (Schori, Trachsel, Grossmann *et al.* 2018, Zou, Han, Zhao *et al.* 2018a). However, compared to the large vitreous proteome studies, the present study identified lesser number of proteins in PDR. This could be due to the masking of low abundant proteins by elevated levels of abundant plasma proteins due to serum infiltration in the advanced stages of the disease as abundant protein depletion was not carried out prior to mass spectrometry. This was further quantitatively validated by evaluating the levels of abundant proteins in the three groups based on the protein intensities and peptide levels, which showed an increase of 1.93% and 3.8 % albumin level in PDR vitreous compared to NDR and NDM groups respectively (Table 4.5).

5.3. Identification of novel vitreous proteins

In order to understand the validity of the data and the methods selected for the present study, the identified proteins were matched with human vitreous proteome database as a part of human eye proteome project published in the year 2013 and 2018 (Ahmad, Zhang, Dufresne *et al.* 2018, Semba, Enghild, Venkatraman *et al.* 2013). Additionally, proteins from the present study were also compared to the vitreous proteome data published between 2018 until date and not listed in the vitreous proteome from the eye proteome database (Li, Lu and Lu 2018, Santos, Gaspar, Ciordia *et al.* 2018, Zou *et al.* 2018a, Zou, Zhao, Yu *et al.* 2018b). Among 1079 proteins identified, 938 proteins were found to be reported in the published vitreous database, which confirmed the validity of the data generated from the present study. Following stringent criteria including identification of proteins in minimum of three vitreous samples with minimum sequence coverage of 2%, 27 novel vitreous proteins were identified in this study. Among these, 16 proteins were reported for the first time (Table 4.3), whereas, the remaining 11 were identified in the eye proteome database (Table 4.2) (Ahmad *et al.* 2018, Semba *et al.* 2013). Eight of these proteins were found to be immunoglobulins and two were HLA proteins such as HLA class I histocompatibility antigen- Cw-12 alpha chain (HLA-C) and HLA class I histocompatibility antigen- Cw-17 alpha chain (HLA-C). Two novel vitreous proteins identified were found to be associated with nervous system including TREM2 and VAMP1. TREM2 is a myeloid cell receptor that is expressed exclusively in microglial cells. Activation of this receptor in microglial cells promote microglial activation and phagocytosis and thus play a critical role in neurodegenerative diseases (Mecca, Giambanco, Donato *et al.* 2018). Even though the role of TREM2 was not explored in DR or diabetes associated complications, its role in Alzheimer's disease in activating microglia and promoting

inflammation has already been reported (Geerlings, de Jong and den Hollander 2017). VAMP1 is nervous system specific protein, which is involved in synaptic functions mainly in the neurotransmitter transport and release (Trimble, Cowan and Scheller 1988). While TREM2 has not been reported in any of the eye proteomes, but VAMP-1 was reported in the retinal proteome database. GTPase IMAP family member 4 (GIMAP4), a protein mainly expressed in the immune cells and involved in thymocyte development and T-helper cells differentiation is another novel protein identified in the present study (Filen and Lahesmaa 2010). ZNF268 and HIST1H3A are the DNA binding proteins, wherein, the former is identified for the first time in the eye proteome while the latter was reported in the proteome of retina, cornea and RPE but not in the vitreous (Dyrlund, Poulsen, Scavenius *et al.* 2012, Funke, Perumal, Beck *et al.* 2016). Stromelysin-1 (MMP3) a metalloproteinases enzyme was also identified for the first time in vitreous proteome in the present study though it was exclusively present only in the NDM and NDR groups but not in the PDR vitreous, suggesting that MMP3 may not have a significant role in the PDR pathogenesis. Two lipid binding proteins SEC14 like lipid binding 1 (SEC14L1) and oxysterol-binding protein-related protein 6 (OSBPL6) were also identified. SEC14L1 is a phospholipid transfer protein and its overexpression was shown to reduce the neurotransmitter choline transporter activity (Ribeiro, Ferreira, Marion *et al.* 2007). More number of peptides of this protein were found in diabetic individuals (PDR and NDR) as compared to NDM group suggesting this to be diabetes specific, while its role in DR pathogenesis needs to be investigated. OSBPL6 is an intracellular lipid receptor and its overexpression is known to enhance the cholesterol transport and efflux in the hepatic cells and macrophages (Ouimet, Hennessy, van Solingen *et al.* 2016). Another Sulfotransferase family cytosolic 2B member 1 (SLUT2B1) protein, which is involved in sulfonation of steroid and has a specific affinity to cholesterol was identified in the present proteome data (Fuda,

Javitt, Mitamura *et al.* 2007). This may suggest that proteins involved in cholesterol transport are likely to have an important role in DR pathology. Other novel proteins identified in the present study included 40S ribosomal protein S15a (RSP15A), a critical protein of eukaryotic protein synthesis (Zhang, Zhang, Li *et al.* 2016), Mediator of RNA polymerase II transcription subunit 30 (MED30), a con-activator of regulated transcription, Eukaryotic translation initiation factor 5A-1 (EIF5A) and Putative neutrophil cytosol factor 1B (NCF1B) but, their role in PDR pathogenesis needs further validation.

5.4. Functional annotation of proteins identified in NDM, NDR and PDR vitreous

GO-annotation for all the identified proteins were done to provide some insights on the possible physiological and pathological alterations involved in the three major categories, such as, biological process, molecular function and cellular components. The percentage of proteins present in each of these sub-categories were computed relative to the total protein content identified in each group. Many of the biological process and molecular functions identified were previously reported in different studies (Gao, Chen, Timothy *et al.* 2008, Wang, Feng, Hu *et al.* 2013a). However, some of the biological process that were defined earlier like muscle contraction, ectoderm development and molecular functions like G-protein modulator etc. could not be validated in the present study (Loukovaara, Nurkkala, Tamene *et al.* 2015). This could be due to the limited number of samples available in the present study compared to the large-scale proteomic analysis. Interestingly, there was an appreciable increase in the biological processes associated with immune system and immune response in the PDR vitreous.

A further analysis of an these increased number of proteins involved in immune system process and immune response in PDR showed a gradual increase in immune proteins in

diabetes to PDR and this corroborates the earlier findings of altered innate immune system activation in DR (Rajab, Baker, Hunt *et al.* 2015, Xu and Chen 2017). Likewise, the GO annotation of molecular functions identified an increased percentage of proteins in receptor bindings in PDR and NDR groups compared to NDM (Figure 4.6a), further suggesting that proteins with increased receptor binding activity as seen in diabetes, may further enhance the risk of various diabetes complications including DR. GO-annotation for cellular components identified increased percentage of ECM proteins in diabetic group (PDR, NDR) including proteins of extracellular region, extracellular region part and extracellular space (Figure 4.7). The ECM matrix provides mechanical support and act as a scaffold for the cells to grow. Vitreous is an ECM matrix, but under altered tissue microenvironment, the retinal cells or inflammatory cells can secrete extracellular proteins, which may further accelerate ECM reorganization and its breakdown (Neve, Cantatore, Maruotti *et al.* 2014).

5.5. Unique proteins present in NDM, NDR and PDR group

The unique proteins to each of the three major categories were further compared and analyzed by KEGG and REACTOME pathway analysis in order to get an overview of their involvement in disease pathogenesis. Collectively 20, 90 and 175 unique proteins were identified in the PDR, NDR and NDM categories in the present study. The unique proteins in PDR cases were found to be associated with complement pathway activation [based on the presence of proteins such as *Mannan Binding Lectin Serine Peptidase 2* (MASP2), C4A, immunoglobulin lambda constant 3 (IGLC3), ECM– interacting and signaling proteins such as thrombospondin 4 (THBS4) and collagen type III–alpha chain (COL3A). While the proteins of the complement pathway were present in all the three groups, presence of some additional complement pathway proteins in PDR supported the previous finding of enhanced

immune activation in the DR vitreous. The presence of THBS4, an ECM remodeling protein and COL3A, an important ECM component (Palao, Medzikovic, Rippe *et al.* 2018) in PDR suggested increased remodeling of ECM as seen in abnormal angiogenesis (Neve *et al.* 2014). The identification of ECM-organization proteins in the NDR group suggested that extensive ECM remodeling might not be involved only in the advanced stages of the disease and it could just be a diabetes induced change. Majority of the pathways identified in the NDR group were found to be associated with cell communication and tissue patterning including Nectin-Necl trans heterodimerization, cell-cell and adherens junction interaction proteins along with various immune pathways such as allograft rejection and endosomal and vacuolar pathways. Nectin signals were previously shown to enhance the angiogenic potential of endothelial cells (Son, Lee, Choi *et al.* 2016). Thus ECM -rearrangement and remodeling was found to be a common phenomenon in subjects with diabetes (PDR and NDR) (Table 4.7 and 4.8).

175 proteins were found to be unique in the NDM group and that could be due to the greater number of proteins identified as compared to NDR and PDR. One of the major pathways identified based on these proteins included glutathione metabolism with the presence of antioxidant proteins such as GPX1, IDH2, and GSR. Glutathione is an important antioxidant, and provides protection against oxidative stress induced neuro-vascular damage (Sakurai, Doci and Gutkind 2012). Glutathione peroxidase-1 (GPX1) protect the release of free Hb from erythrocyte, thus preventing oxidative damage induced by free Hb moiety. A downregulation of GPX1 was shown to induce oxidative damage and retinal neovascularization in a mice model of OIR (Tan, Stefanovic, Tan *et al.* 2013). Isocitrate dehydrogenase 2 (IDH2) is required for the generation of NADPH from oxidative decarboxylation of isocitrate-alpha-keto-glutarate. The generated NADH is required for the

production of reduced glutathione, which further prevent ROS induced damage (Yu, Dittenhafer-Reed and Denu 2012). Glutathione S-reductase (GSR) reduces glutathione disulfide into glutathione. The presence of these active anti-oxidant proteins in NDM group suggests its importance in maintaining a homeostatic balance in the retina. In case of diabetes, the levels of these enzyme are reduced, which contributing to oxidative damage (Yildirim, Ucgun, Kilic *et al*. 2007). Thus, the presence of glutathione metabolism in NDM might protect the tissue from oxidative damage and maintenance of homeostatic balance in the retina. Other unique pathways present in the NDM group were found to be Axon guidance and Signaling by ROBO receptors with a large number of proteins (59). Both of these pathways are needed for retinal neuronal function and thus provide good visual acuity (Prieur and Rebsam 2017). Phagosome pathway was found to be shared among the NDR and NDM groups (Table 4.7 and 4.8). Unexpectedly, some of the inflammatory proteins such as MMP9, IL-6 and ITGAM were identified only in the NDM group, but not in PDR and NDR, which could be attributed to the masking due to excess albumin levels in the diabetic samples. To confirm this, the level of MMP9 was assessed in the vitreous by gelatin zymography that revealed an increased expression and gelatinolytic activity of this protein in PDR (Figure 4.33b). Likewise, ITGAM /CD11b is a macrophage receptor-1 molecule and their excess activation was also confirmed in the diabetic retina and vitreous (Figure 4.33a). These experiments further confirmed that the abovementioned inflammatory proteins were masked in the PDR and NDR vitreous by the highly abundant proteins.

5.6. Proteins shared only among diabetic groups (PDR and NDR) and no-retinopathy groups (NDM and NDR)

Methylglyoxal is an important precursor of advanced glycation end products and its degradation is carried out by two important enzymes, Glo-1 and Glo- 2 in humans (Allaman, Belanger and Magistretti 2015). This degradation mechanism for methylglyoxal in the NDR group was evident with the presence of Glo-1, which was not identified in the PDR group (Table 5.2). Further, pyridoxal- 5 phosphate salvage pathway protein, pyridoxal kinase, was found only in the NDR group. Pyridoxal phosphate (vitamin B6) is an important antioxidant that neutralizes AGE (Merigliano, Mascolo, Burla *et al.* 2018). Again their presence in no retinopathy group suggested an active clearance mechanism of AGE but not in PDR. Ten proteins were shared only between the diabetic group (PDR and NDR) (Table 5.3). Neurocan (NCAN), neurotrimin (NTM), Secreted frizzled-related protein 2 (SFRP2), the important neural cell adhesion proteins needed for the cell growth, were some of the prominent ones. Neurocan accumulation in the retina cause retinal vascular alterations (Zhang, Rauch and Perez 2003) (Table 5.3). Most importantly, Wnt signaling pathway proteins, an important angiogenic signaling pathway, were found to be commonly present in diabetic group. The presence of these proteins in PDR and NDR implies their critical role in disease pathogenesis.

Table 5.2: List of proteins found to be shared only between no-retinopathy (NDR, NDM) groups

Proteins shared only between no-retinopathy group	
Proteasome subunit alpha type-2(PSMA2)	Prothymosin alpha; Thymosin alpha-1(PTMA)
Neuroblastoma suppressor of tumorigenicity 1(NBL1)	Histone H2B type 1-J (HIST1H2BJ)
Retinol-binding protein 1(RBP1)	Histone H1.0 (H1F0)
Epsilon-sarcoglycan (SGCE)	Neuroendocrine convertase 2(PCSK2)
GTPase IMAP family member 4(GIMAP4)	Lamin-B1 (LMNB1)
40S ribosomal protein S15a(RPS15A)	Elongation factor 1-gamma (EEF1G)
Cysteine-rich secretory protein 3(CRISP3)	Erythrocyte band 7 integral membrane protein (STOM)
Pyridoxal kinase (PDXK)	Cellular retinoic acid-binding protein 1 (CRABP1)
Alpha-actinin-4(ACTN4)	Beta-arrestin-2; Beta-arrestin-1; Arrestin-C(ARRB2)
Glia maturation factor gamma; Glia maturation factor beta (GMFG)	V-type proton ATPase subunit E 1 (ATP6V1E1)
Non-syndromic hearing impairment protein 5(DFNA5)	Phosphoglucomutase-1(PGM1)
6-phosphogluconolactonase (PGLS)	Nicotinamide phosphor ribosyltransferase (NAMPT)
Aspartate aminotransferase, mitochondrial (GOT2)	F-actin-capping protein subunit alpha-2 (CAPZA2)
Complement C1q subcomponent subunit A (C1QA)	Alpha-mannosidase 2x (MAN2A2)
Aldehyde dehydrogenase, mitochondrial (ALDH2)	Proteasome subunit beta type-3 (PSMB3)
Neuronal growth regulator 1 (NEGR1)	Actin-related protein 2 (ACTR2)
Adipocyte enhancer-binding protein 1 (AEBP1)	Peptidyl-prolyl cis-trans isomerase FKBP1A (FKBP1A)
UPF0556 protein C19orf10 (C19orf10)	Growth factor receptor-bound protein 2 (GRB2)
BTB/POZ domain-containing protein KCTD12 (KCTD12)	Glutathione S-transferase omega-1(GSTO1)
Legumain (LGMN)	Lactoylglutathione lyase (GLO1)
Calcineurin-like phosphoesterase domain-containing protein 1 (CPPED1)	ATP-dependent RNA helicase A (DHX9)
Calsyntenin-2 (CLSTN2)	Filensin (BFSP1)
Golgi-associated plant pathogenesis-related protein 1 (GLIPR2)	Early endosome antigen 1 (EEA1)
WAP four-disulfide core domain protein 1 (WFDC1)	Putative ubiquitin-conjugating enzyme E2 N-like (UBE2NL)
Coronin-1C (CORO1C)	Stabilin-1 (STAB1)
Endothelial protein C receptor (PROCR)	Triggering receptor expressed on myeloid cells 2 (TREM2)

Table 5.3: List of proteins found to be shared only between diabetes (PDR, NDR) groups

Proteins shared only between diabetes group
A0A0B4J2D9
Serpin E3 (SERPINE3)
Neurocan core protein (NCAN)
N(G), N(G)-dimethylarginine dimethylaminohydrolase 1 (DDAH1)
Cathepsin O (CTSO)
Desmocollin-3 (DSC3)
Peptidase inhibitor 16 (PI16)
Secreted frizzled-related protein 2 (SFRP2)
Mannosyl-oligosaccharide 1, 2-alpha-mannosidase IC (MAN1C1)
Neurotrimin (NTM)

5.7 Quantitative comparisons of proteins between the three groups by label free quantitation (LFQ) method

LFQ enables the quantification of proteins based on their spectrometric signal intensity or by spectral counting and allows the absolute or relative abundance of these proteins across all samples for each experimental run (Wang, You, Bemis *et al.* 2008). In the present study, spectrometric intensity based LFQ was done for measuring protein expressions in all the samples, which enabled a quantitative assessment of proteins in all the three groups. This study was also aimed at differentiating the proteome alterations in two specific disease states such as diabetes and retinopathy. A similar approach was undertaken by Gao *et al* 2008, wherein they generated a list of protiens whose expressions were altered due to diabetes based on counts of the number of peptides in NDR and PDR with NDM group (Gao *et al.* 2008), while the remaining studies provided an overall change in the PDR vitreous, which included both diabetes and retinopathy specific changes compared to the NDM group. Studying the diabetes induced changes are essential but more importantly a clear understanding of the key changes that define the risk of PDR is crucial. Hence, the present study evaluated and

identified proteins those were differentially expressed only in diabetic condition and those that were differentially expressed only in DR.

5.8. Diabetes induced differential expression of proteins compared with versus No Diabetic Mellitus

Diabetes was found to significantly alter the expression of 15 proteins ($p<0.05$) with a minimum fold change of 1.5- fold (Table 4.10). This included both upregulation (FRZB, AGRN, APLP1, TIMP2, IGFBP6, APLP2, COL1A2 and VCAN) and downregulation (GSTP1, IDH1, LDHA, VASN, LDHB, PRDX1 and C6) of various proteins. The expressions of these significantly altered proteins in DM samples were evaluated in PDR vs NDM group, to understand their relative contribution in PDR. Majority of the upregulated proteins identified in DM were involved in ECM remodeling, rearrangement and degradation. Agrin (AGRN) is an ECM proteoglycan and plays a critical role in neuromuscular junction development during embryogenesis and acetylcholine receptor aggregation during synaptogenesis (Fuerst, Rauch and Burgess 2007). APLP1 (amyloid like protein 1) and APLP2 (amyloid like protein 2) are the member of amyloid precursor proteins (APP) and are involved in functions such as cell viability and neuronal out growth (Sakai and Hohjoh 2006). This family of proteins were also reported to be upregulated as part of the mechanism involving neuronal protection in cases of acute or chronic hypoxic- ischemic conditions (Hefter and Draguhn 2017). Thus, the upregulation of these proteins may suggest a possible survival mechanism of neurons under metabolic stress conditions like diabetes. The level of APLP2 was earlier reported to be downregulated in diabetes vitreous with no identification of APLP2 peptides in PDR vitreous (PDR: 0, NDR: 5.88±2.13, NDM: 6±2.4) (Gao *et al.* 2008), however, in the present study of these proteins showed a significant increase in the DM vitreous (DM

vs NDM: 2.99±0.83, $p<0.04$). Another ECM binding protein that inhibits MMP activity and identified in the present study was TIMP2. The downregulation of TIMP2 was reported in the vitreous proteome of subjects with diabetic macular edema (Hernandez, Garcia-Ramirez, Colome *et al.* 2013). The present study identified significant upregulation of this protein (2.34 ±1.2, $p<0.05$) and a simultaneous downregulation of MMP2 in the diabeteic vitreous (DM vs NDM: 0.95±0.47, $p>0.05$, N.S). However, the downregulation of TIMP2 with no upregulation of MMP2 in PDR (-1.2±0.44, $p>0.05$), suggested that TIMP2 maintained its inhibitory activity towards MMP2 and thereby prevented the MMP2 induced ECM re-arrangement and degradation.

The present study also identified an upregulation of Insulin-like growth factor-binding protein 6 (IGFBP6). The elevated level of this protein was reported in the serum of diabetic patients and also as a marker for proliferative vitreo-retinopathies (Lu, Purohit, Sharma *et al.* 2012, Yu, Peng, Chen *et al.* 2014). Its upregulation promotes alteration in retina and vitreous to induce proliferative changes in diabetic subjects. Further this protein was found to play an important role in the regulation of IGF transport and uptake by IGFBPs in the diabetic subjects along with proteins such as APLP2 and VCAN (Versican). VCAN, a proteoglycan, plays a significant role in intercellular signaling and thereby connects cells with ECM. It also plays a vital role in cell motility, growth and differentiation. VCAN has been shown to target the inflammatory cells and cause retention of those cells and their interactions with ECM. Thus, the presence of increased VCAN in diabetes indicates its role in enhancing inflammation in diabetes complications (Wight and Merrilees 2004). Inflammation is one of the major underlying reason for all diabetes induced complications in the body including DR (Tang and Kern 2011). Another major ECM protein, which was found significantly upregulated in diabetic groups was COL1A2 (collagen type I alpha 2 chain) (DM vs NDM: 3.01±0.11,

p<0.005). It is an important collagen molecule and its integrity is crucial to maintain tissue homeostasis. Increased expression of this protein has been reported previously in various types of cancers (Lin, Goldstein, Nesbit *et al.* 2016, Misawa, Kanazawa, Misawa *et al.* 2011).

The expressions of 7 proteins were downregulated in the diabetic group. These included the proteins involved in detoxification mechanism and metabolic proteins (Table 4.10) glutathione S-transferase pi-1 (GSTP1) and peroxiredoxin 1 (PRDX1) are the enzymes involved in the detoxification of cells from oxidative stress (Mian, Khattab, Hedayati *et al.* 2016). The polymorphism in GSTP1 was associated with diabetes complications (Stoian, Banescu, Balasa *et al.* 2015). The PRDX1 protein has previously been reported in vitreous proteomics and was shown to be upregulated in diabetes (Gao *et al.* 2008). However, the present study identified a significant down regulation of this protein (p=0.04) in both peptide and expression levels in the diabetes group with a fold change -2.04±1.12,- A study by Loukovaara *et al.* in 2015, identified similar proteins in vitreous samples, with no significant change in its expression among the PDR cases (Loukovaara *et al.* 2015). Some studies had shown PRDX1 upregulation as an inflammatory stimuli in various body complications as it enhanced the inflammatory signals like NFkB (Ishii, Warabi and Yanagawa 2012), while others reported that PRDX1 protected cells from oxidative stress induced damage (Kim, Park, Choi *et al.* 2015). The present study identified a significant downregulation of this protein in the DM, suggesting that the downregulation of PRDX1 and GSTP1 enhanced the oxidative stress induced damage in diabetic complications. Vasorin (VASN), a TGF β binding protein was found to be significantly downregulated in diabetes as well as in the retinopathy group (DM vs NDM: -2.66±0.36, p=0.01, PDR vs no retinopathy: -2.61±0.57, p=0.01). It modulates the vascular response to injuiry by inhibiting the level of TGFβ and prevents fibrosis as seen in vascular injury. Thus, the downregulation of Vasorin expression might suggest for its

contribution in fibroproliferative diseases like DR (Ikeda, Imai, Kumagai *et al.* 2004). The present study identified a significant downregulation of this protein in DM as well as PDR, with a simultaneous downregulation of TGFβ observed in both the categories (PDR vs no-retinopathy: -2.11±0.13, p=0.03, DM vs NDM: -1.84±0.63, p>0.05).

The present study also identified a significant downregulation of metabolic protiens such as IDH1 (Isocitrate dehydrogenase), a key enzyme in the Kreb's cycle, lactate dehydrogenase A and B (LDHA and LDHB). LDHA and LDHB are the subunits of lactate dehydrogenase enzyme (LDH). The LDHA has higher affinity towards pyruvate and convert pyruvate to lactate during anerobic respiration, while the LDHB has higher affinity towards lactate and convert lactate to pyruvate under aerobic respiration (Ratter, Rooijackers, Hooiveld *et al.* 2018). These are the key enzymes involved in glucose and pyruvate metabolism in the body. The downregulation of these enzymes in the glycolytic pathway suggests glucose accumulation in the body without efficient breaking down of the glucose moiety in diabetes. A protein of complement pathway C6, was also downregulated in the diabetes group (DM vs NDM: -1.5 ±0.35, p=0.02). Though most of the vitreous proteome studies had shown involvement of complement pathway in diabetes and associated complications like retinopathy, the present study did not identify a clear-cut involvement of proteins of the complement pathway in DM vitreous through the global proteome analysis. This suggested that all proteins in the complement pathway may not contribute to the development of diabetes complications, while some of these complement proteins could have a significant role in disease pathogenesis that needs to be explored further through a targeted proteomic approach.

A total of 10 proteins were found to be differentially expressed in the retinopathy (PDR) vitreous compared with no retinopathy (NDR+NDM) (Table 4.13). These included 4

proteins, which were upregulated and the remaining downregulated. Most importantly the upregulation of angiogenic proteins was observed in the vitreous of PDR samples. LRG1 is a leucine rich glycoproteins present extensively in extracellular matrix and has been shown to promote angiogenesis by modulating TGFβ1 in endothelial cells by activating angiogenic Smad1/5/8 signaling (Wang, Abraham, McKenzie *et al.* 2013b). Elevated levels of LRG1 in PDR vitreous and TGFβ1 in DM retina had been reported earlier in several proteomic studies (Gao *et al.* 2008, Spirin, Saghizadeh, Lewin *et al.* 1999). APCS (Serum amyloid-p-component) and CRP (C-reactive protein) are pentraxin family of proteins and their upregulation was reported in various auto immune and neurodegenerative diseases (Kolstoe, Ridha, Bellotti *et al.* 2009). The present study also identified upregulation of CRP in PDR vitreous (1.26±0.49, p>0.05, N.S). The deposition of CRP in the AMD drusen along with complement proteins, suggested a major role of CRP in excessive complement activation and inflammation in AMD pathogenesis (Molins, Romero-Vazquez, Fuentes-Prior *et al.* 2018). The exact function of APCs is unclear, but it was suggested to be a major protein of humoral innate immune system that had a role in activation of complement- coagulation pathways and inflammation similar to that of the CRP (Behrens, Lipke, Pilling *et al.* 2019, McGeer, Yasojima, Schwab *et al.* 2001). Another important coagulation protein found to be significantly upregulated in the retinopathy vitreous was FGA (2.52 ± 0.86, p=0.04). It is one of the proteins commonly reported in several vitreous proteome studies (Li *et al.* 2018). Its deposition after an injuiry is known to activate systemic inflammatory response along with the complement and coagulation cascade (Levi, van der Poll and Buller 2004).

Oxidative stress plays a key role in the PDR pathogenesis by activating various pathways involved in inflammation and angiogenesis, such as the polyol pathway, AGE pathway and

glucose autooxidation. Oxidative stress in DR is mainly due to the imbalance of ROS generation and its scavenging activity by the antioxidant enzymes (Izuta, Matsunaga, Shimazawa *et al.* 2010). The accumulation of AGE in diabetes was shown to cause the generation of ROS (Nowotny, Jung, Hohn *et al.* 2015). The present study identified a significant downregulation of detoxification enzyme superoxide dismutase- 3 (SOD3). It is a dismutase enzyme and majorly present in the extracellular matrix and is localized within the vitreous cavity of the eye. SOD3, dismutase generates ROS and reduces oxidative stress (Wert, Velez, Cross *et al.* 2018) and thus its downregulation is suggestive of a significant role of elevated oxidative stress in PDR pathogenesis. The present study also identified downregulation of fibronectin binding protein, myocilin (MYOC) in PDR compared to no-retinopathy stage. MYOC is widely studied gene known for its involvement in primary open angle glaucoma, although its exact cellular function is not well known. A study showed that the elevated expression of myocilin increased cellular proliferation rate as well as provided resistance to apoptosis (Joe, Kwon, Cojocaru *et al.* 2014). The elevated expression of this protein cause stress fibre formation in glaucoma and was also shown to competitively interact with proteins of the Wnt signaling pathway (Kwon, Lee, Ji *et al.* 2009). Thus, the downregulation of MYOC could be related to an accelerated apoptosis in PDR, which may increase angiogenesis by not competing with proteins of the Wnt signaling pathway.

PPT1 (palmitoyl-*protein* thioesterase 1) is a protein present in the lysosome, and its function is to remove long chain fatty acids from proteins by their breakdown when they are no longer required. PPT1 is also involved in synapse development and inhibition of this proteins was reported to increase apoptosis in neuronal cells (Cho, Dawson and Dawson 2000). The downregulation of this protein could cause the altered lysosomal function as well as

compromised synaptic activity and neuronal death in PDR group. Thus, downregulation of the critical protein of lysosomal function can cause accumulation of complex macromolecules, that further enhances various inflammatory stimuli and apoptotic signals causing damages to the tissues. Another important protein which was found to be significantly downregulated in the PDR vitreous was ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase 2). It is the major precursor protein of lysophosphatidic acid (Seo, Choi, Shim *et al.* 2012), which is the potent activator of various signaling pathways involved in angiogenesis, inflammation and fibrosis. Based on its elevated level in PDR vitreous, it was thought to serve as a potential biomarker for PDR pathogenesis (Abu El-Asrar, Mohammad, Nawaz *et al.* 2013), however, the present study identified a significant downregulation of LPA precursor ENPP2 in the PDR vitreous, thereby contradicting the previous study. A further validation of this protein in an extended cohort might be useful in further assessment and confirmation of its role in PDR. The present global vitreous proteome study clearly categorized proteins involved in diabetes and retinopathy complication, targeting the key mechanisms and exploring the activation of each of these proteins may lead to a better understanding of DR pathogenesis.

Thus, the overall analysis of the present global protein profiling data provided an understanding of key proteins involved in retinopathy complications and identified various pathways. While ECM organization and downregulation of various metabolic pathways and detoxification mechanisms were predominant in diabetes stage, most of the proteins identified in DR are known to be involved in activating various inflammatory mechanisms in PDR. Additionally, the unique pathway proteins identified in the PDR group had shown the initial trigger for complement pathway with the presence of two of the complement proteins such as C4A, MASP2 as an important immune system pathway. As a logical next step, detailed

investigations were undertaken to understand the role of complement pathway in PDR pathogenesis through a targeted analysis.

5.9. Complement system in retinal homeostasis and pathology

Complement system is the body's innate immune defense mechanism and is a critical for maintaining immune privileged state of the retina by its low-level of activation and is tightly controlled by complement regulators (Sohn, Kaplan, Suk *et al.* 2000). It is comprised of more than 40 complement proteins and regulators, and activated through three different pathways such as classical, alternative and lectin mediated pathway (Nesargikar, Spiller and Chavez 2012). In addition to its role as a defense mechanism of the body, studies had also shown the involvement of complement system in various tissue remodeling process such as liver regeneration, synaptic pruning while development and also in retinal angiogenesis (Langer, Chung, Orlova *et al.* 2010, Stevens, Allen, Vazquez *et al.* 2007, Strey, Markiewski, Mastellos *et al.* 2003). The role of complement in angiogenesis have prime importance, since there are several blinding eye diseases associated with abnormal ocular angiogenesis such as ROP, AMD and PDR (Rajappa, Saxena and Kaur 2010). Diverse mechanism of activation and pathological role of complement system in ocular pathologies of ROP and AMD have been well appreciated through various studies. For instance, the deposition of C3 and MAC complex in neovessels and absence of neovessels formation and angiogenic factors in C3 knockout mice in CNV-models suggested a pro-angiogenic role of complement C3 in AMD (Bora, Sohn, Cruz *et al.* 2005). Additionally, genetic association of polymorphisms in *CFH* gene in complement pathway was identified as a major risk factor for AMD pathogenesis (Klein, Zeiss, Chew *et al.* 2005). The anti and pro-angiogenic role of complement was also

shown in ROP pathogenesis in mice models of OIR and in premature babies, respectively (Langer *et al.* 2010, Rathi, Jalali, Patnaik *et al.* 2017).

In case of DR, the evidence for the involvement of complement in disease pathogenesis was suggested based on the deposition of C3d, MAC complex in the choriocapillaries and decreased levels of inhibitors of complement pathway (CD55 and CD59) in DR eyes (Gerl, Bohl, Pitz *et al.* 2002, Zhang, Gerhardinger and Lorenzi 2002). Later, several proteome studies in DR had also shown the presence of various complement proteins such as such as C3, CFI, C2, C4A, C4B, CFD and CFH in vitreous humor biopsies (Gao *et al.* 2008, Garcia-Ramirez, Canals, Hernandez *et al.* 2007, Gerl *et al.* 2002, Li *et al.* 2018, Wang *et al.* 2013a). The global proteome profiling done in the present study also identified a larger number of complement of the classical and alternative pathways of proteins in all the three groups (PDR, NDR and NDM), though there was no significant differences in their expressions across these groups, except for a significant reduction in C6 levels (1.5 folds, p=0.02) in diabetes compared to NDM.

Even though studies have suggested a strong involvement of complement pathway in PDR pathogenesis based on the presence of complement proteins, none of those studies provided a clear understanding about the specific complement pathway that was involved in PDR, key proteins of complement pathway in PDR progression and the major mediators of complement pathway activation in pathogenesis. Further, it was crucial to assess if complement activation in PDR pathogenesis was a localized phenomenon inside the eye or if it was an additive effect contributed by serum infiltration due to BRB break down, which could occur at any stages of the disease progression.

5.10. Evaluation of complement C3 in PDR pathogenesis:

The activation of complement pathway induces the proteolytic fragmentation of complement component 3 (C3), a large 195kDa protein and generates various activated fragments t with variable functions (Nishida, Walz and Springer 2006). The activation of C3 induces a proteolytic cleavage of C3a of 10kDa size from the 120kDa C3α chain and generate a reactive C3bα' of 110kDa, which is a part of C3b (Clay, Soni, Gunn *et al.* 2008). Thus, the C3bα'together with C3β chain makes 185kDa C3b, which is a pivotal step in the complement pathway activation. The generated C3b with externalized reactive thioester bond can bind with antigen or host tissue and accelerate the generation of C3 convertase. But the regulatory proteins present in the complement pathway mainly CFI with the help of CFH inactivate C3b and generate inactive fragments such as iC3b, C3f and C3c (Law and Dodds 1997). The deposition of these fragment on the pathogen surface enhance opsonization and phagocytosis and results in the elimination of pathogens from the host tissue. If the regulatory mechanism is not intact, the downstream activation of complement continues and leads to the generation of MAC complex which can cause host tissue damage (van Lookeren Campagne, Wiesmann and Brown 2007).

Western blotting of vitreous had shown the presence of various fragments of C3 (Figure 4.16a) such as C3, C3b, C3α, C3bα', C3β, C3α'-1 fragment of iC3b and C3c α' fragment-2. There was no significant difference in the number of activated C3 fragments in PDR vitreous compared to control, suggesting complement activation in the control vitreous as well (Figure 4.17). This could also be attributed to homeostatic changes in retinal microenvironment in conditions of macular hole and retinal detachment cases that served as control for this experiment. The analysis of all identified fragments of C3 found them to be increased in PDR

vitreous compared to no-DM controls, but a significant increase was observed for only total C3 (1.9±0.25a.u ,*p*=0.004*) (Figure 4.16b) and reactive C3bα'–fragment (Figure 4.19) (2.76±0.65a.u, *p*=0.006)* in PDR vitreous. This suggested enhanced complement activation and generation of reactive C3bα' in PDR vitreous to activate the complement pathway. This study demonstrated an enhanced C3bα' level in the PDR vitreous. Further, there were no significant differences in complement activation at the systemic level (serum) in PDR and NPDR compared to no-DM groups. Only four prominent fragments of C3 were identified in the serum (C3, C3b, C3β and an intermediate band of ~90kDa in size) when compared to those observed in the vitreous humor (Figure 4.23). As evident from these findings the extensive complement activation as seen in PDR vitreous could be safely categorized as a localized phenomenon and not contributed by infiltration of complement proteins from systemic circulation. This is unlike AMD where complement activation plays a significant role in disease pathogenesis and was also seen in the systemic circulation in AMD patients (Scholl, Charbel Issa, Walier *et al.* 2008, Sivaprasad, Adewoyin, Bailey *et al.* 2007). Thus, while both DR and AMD are age related conditions and share abnormal angiogenesis as one of the major clinical features, the underlying mechanism for their disease progression is not similar.

5.11. Role of classical vs alternative pathway of complement activation in PDR pathogenesis

The increased level of reactive C3bα' in PDR vitreous suggested an elevated complement activation in PDR but the exact complement pathway that was involved in PDR pathogenesis was not clearly understood. As a next step, major complement proteins in classical (C4b and C1q) and alternative pathways (CFB and CFH) were analyzed by western blotting. There were no significant differences in the levels of classical pathway proteins in vitreous and serum

suggesting that this pathway may not contribute to increased complement activation in PDR (Figure 4.24 and Figure 4.25). Also, none of the previous vitreous proteome studies had assessed the relative contribution of classical complement pathway in PDR pathogenesis.(Balaiya, Zhou and Chalam 2017, Garcia-Ramirez *et al.* 2007).

CFB, a key protein (93kDa) in the alternative pathway of complement, is comprised of two fragments such as BB and Ba. The binding of CFD to CFB cleaves CFB into Ba and Bb , the later fragments binds with C3b and form a stable C3bBb in presence of properdin, that activates the alternative pathway of complement (Noris and Remuzzi 2013). While CFB is known to be involved with neurodegenerative conditions like Alzheimer's, AMD, etc. its role in DR has been not been explored adequately. The present study identified a significant downregulation of Bb in the PDR vitreous unlike the report from Garcia *et al.* (2007), where they showed upregulation of factor B (including Ba and Bb) (Garcia-Ramirez *et al.* 2007). The reduced levels of unbound Bb along with increased levels of reactive C3bα' in the present study suggested the formation of stable C3bBb complex in the PDR vitreous and that explained for a significant reduction in the unbound Bb of factor B (PDR: 0.97±0.15a.u, p^*=0.03) (Figure 4.26).

Likewise, the level of CFH in the PDR vitreous was evaluated. CFH regulates alternative pathway in multiple steps such as preventing C3bBb formation by competing with FB, acting as a co-factor for CFI to degrade C3b to C3bi and thus arrest/slow down the alternative pathway of complement activation (Williams, Stampoulis, Gunter *et al.* 2016). Downregulation of CFH and complement activation was also reported in various other ocular complications (Bora, Kaliappan, Jha *et al.* 2006, Tezel, Yang, Luo *et al.* 2010). Surprisingly there was a significant increase in the level of this protein in the PDR vitreous compared with no-DM

controls (Figure 4.27). A background literature search on the regulatory activity of CFH, revealed that CFH had multiple specific binding regions in C3 to arrest the alternative pathway of complement. As discussed above, the significant increase in reactive C3bα'- chain in the PDR vitreous and CFH could be a feedback mechanism for maintaining C3bα' level in PDR vitreous and thereby regulating the alternate complement pathway. Most importantly the study did not identify increased CFH levels in the serum samples, rather a slight downregulation of this protein was noted (Figure 4.28). This confirmed that upregulation of CFH is a localized phenomenon and could be contributed by the cells of the retina in an altered retinal microenvironment. To further confirm this feedback regulation of alternate complement pathway by CFH, randomly selected vitreous samples of PDR and no-DM controls, were evaluated for CFH and C3bα' levels by western blotting. A clear-cut increase in CFH level in these samples correlated perfectly with higher C3bα' (Figure 5. 1). The novel protein profiling results for CFH in DR vitreous as shown in the present study provided a vital cue for its involvement in disease pathogenesis.

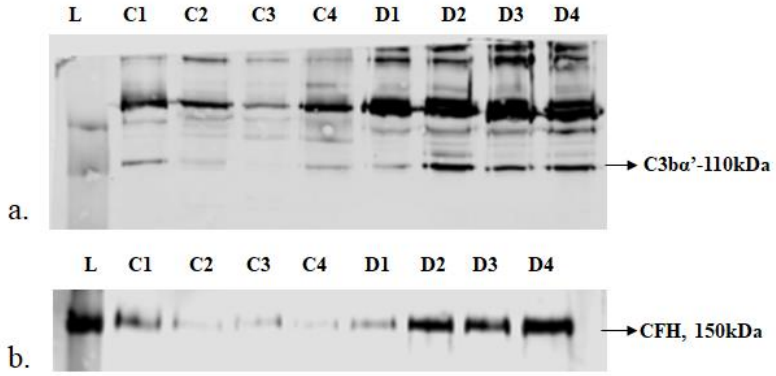


Figure 5.1. Representative Western blot images of C3 and CFH in PDR and no-DM control vitreous. In the western blots 3 of four PDR vitreous samples (D2, D3 and D4) showed higher C3bα' and a concurrent increase in CFH levels.

5.12. Role of microglial activation and complement up regulation in diabetic retina.

Several studies on understanding the DR pathogenesis had suggested that functional alterations of the retina in DR occurs much before the known clinical complications are seen (Jackson and Barber 2010, Lieth, Gardner, Barber *et al.* 2000). These studies had also indicated that these alterations in the retina are mainly contributed by the activated microglia in the retina under overt inflammatory milieu of diabetes (Grigsby, Cardona, Pouw *et al.* 2014, Zeng, Ng and Ling 2000). Microglia are the prime immune cells involved in the maintenance of retinal homeostasis together with complement pathway (Xu and Chen 2017). While a low level of complement activation continues for maintaining retinal integrity as part of aging process (Mukai, Okunuki, Husain *et al.* 2018), the microglial cells remained in ramified morphology under normal physiological conditions (Hristovska and Pascual 2015). The evidence for activation of microglia at different stages of disease progression in DR in human retina was provided based on its morphological changes (Zeng, Green and Tso 2008). While the complement system and retinal microglia are the major innate immune defense mechanism of the retina, it is not clear, if there exists any cross talk between them and if that has any contribution to the disease pathogenesis. Initially the co-staining of complement proteins (C3) with markers of activated microglia (CD11b) performed in diabetic and non- diabetic donor retinas showed an increased deposition of these proteins in the diabetic retina (Figure 4.30). Unlike the previous reports of deposition of complement fragments in choriocapillaries (Gerl *et al.* 2002), the present study identified the deposition of C3 in all the retinal layers. Microglial are known to produce complement proteins both during aging and neurodegenerative disease conditions (Rutar, Valter, Natoli *et al.* 2014). In retinal neurodegenerative conditions like retinitis pigmentosa and AMD, the elevated C3 expression was found localized to the activated

microglial cells and it was thought to be involved in phagocytic clearance mechanism to eliminated apoptotic photoreceptors and prevent further neuro-inflammation (Silverman, Ma, Wang *et al.* 2019). However, an increased staining of C3 throughout the retinal layers and its co-localization with the microglial cells, could suggest an increased activation of microglial cells and phagocytosis of retinal neurons, thereby causing neuronal damage in DR.

The second population of glial cells, which are known to get activated under a retinal injury are the macroglial population comprising of astrocytes and Müller glia, with an increased expression of gliotic marker such as GFAP (Gu, Xu, Zhang *et al.* 2019). Since, the glial population gets activated at an early phase of diabetes, studies were done to understand whether their upregulation also contributed to the elevated level of complement C3 in the PDR pathogenesis. However, the upregulation of C3 was not found to co-localize with GFAP positive cells in the retina (Figure 4.29), suggesting that macroglia were not the prime source of complement C3 in the diabetic retina.

Further, the CFH in the neural retina was shown to have an affinity towards CR3 receptor in the microglial cells. This suggested that CFH upregulation in PDR could also be related to microglial activation. Thus, CFH and CD11b co-staining was done in diabetic and control tissues. Surprisingly, an increased CFH staining was found in the certain cells of the inner nuclear layers in diabetic retina and these cells were found to be $CD11b^{+ve}$ microglial population. In retina, CFH is known to be synthesized mainly by RPE cells (Kim, He, Kase *et al.* 2009), however the present study identified CFH to be produced by activated microglial cells also (Figure 4.31). Since, the increased level of complement activation causes damage to the retinal tissues, it could be speculated that the production of CFH by microglia too is part of its role is to prevent the damage of retinal neurons, which needs to be explored further.

5.13. Role of microglial activation and infiltration into the vitreous of PDR cases.

The microglial activation and infiltration in DR was evaluated by its chemotactic activity. CXCR4, a chemokine receptor, widely expressed in microglial cells as well in astroglial activation (Bezzi, Domercq, Brambilla *et al.* 2001) was found to be increased in the diabetic retina. CXCR4-SDF1-axis has a significant role in sprouting angiogenesis and the antagonism of CXCR4 was shown to reduce neovascular areas with a significant reduction in the number of microglial cells, suggesting its indirect effect on microglial angiogenic activity (Unoki, Murakami, Nishijima *et al.* 2010). The present study clearly showed an infiltration of activated microglial cells ($CD11b^{+ve}$) in PDR but not in the control vitreous (Figure 4.32 and Figure 4.33a). Together these results suggested increased chemotaxis and infiltration of activated microglial cells in the vitreous humor of DR patients. These infiltrating microglia could be the prime source for increased CFH in vitreous and may further enhance pathological neovascularization in the retina. This was further confirmed by evaluating the levels of ECM modulators and angiogenic factors in the vitreous.

Once activated, microglia are known to secrete matrix metalloproteinases, which further contributes to ECM reorganization and thus provide a matrix support for abnormal vessel growth (del Zoppo, Frankowski, Gu *et al.* 2012). An elevated gelatinolytic activity of MMP9 as seen in the PDR vitreous (Figure 4.33b) could be causing ECM degradation and subsequent loss of opticin, an anti-angiogenic ECM-glycoprotein in the PDR vitreous (Figure 5.2). This protein is known to play an important role in preventing pre-retinal neovascularization (Le Goff, Lu, Ugarte *et al.* 2012). This suggested an extensive reorganization of the extracellular matrix contributed to PDR pathogenesis in presence of activated microglial cells.

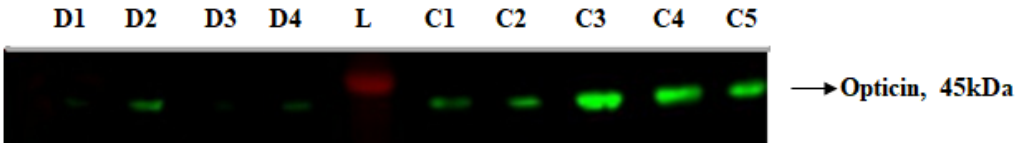


Figure 5.2. Representative western blot image of opticin in PDR (n=4) and no-DM control (n=5) vitreous. D- PDR, C-control, L- protein ladder

Microglia are known to have two different phenotypes upon activation depending on the stimuli (Martinez and Gordon 2014). A significant down regulation of IL-10 and upregulation of IL-8 further confirmed activation of proinflammatory M1 phenotypes in the PDR vitreous. Alongside, an increase in pro-inflammatory markers such as sPECAM ($p<0.05$) analysed by ELISA (Figure 4.35), and CRP analysed by western blotting (Figure 5.3) in the PDR vitreous also indicated excessive inflammatory milieu in PDR pathogenesis. This further suggested the inflammatory milieu in the vitreous of PDR subjects' triggered activation of pro-inflammatory microglial population.

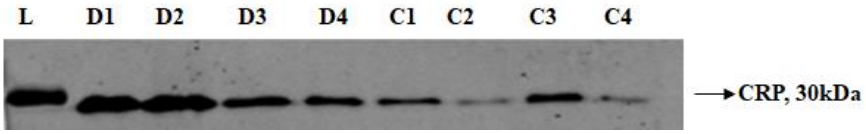


Figure 5.3. Representative western blot image of CRP in PDR (n=4) and no-DM control (n=4) vitreous. D- PDR, C-control, L- protein ladder.

VEGF- VEGFR2 signaling plays a crucial role in neovascular development, and VEGFR2 is the key regulator of angiogenesis (Gampel, Moss, Jones *et al.* 2006). A significant upregulation of these proteins in the PDR compared to no-DM control vitreous suggested an active neovascularization in the PDR vitreous, which is expected since these samples were collected from patients with advanced stage of the disease (Figure 4.34). sVEGFR1 is the truncated version of cell membrane spanning VEGFR1, where it was shown to inhibit VEGF signaling

by trapping of VEGFA and generating non- functional heterodimers (Wu, Stefanini, Mac Gabhann *et al.* 2009). A significant upregulation of sVEGFR1 in the PDR vitreous, could be a part of a feed-back mechanism to suppress VEGF-VEGFR2 signals. Similar findings of upregulation of sVEGFR1 were also reported in previously done PDR studies (Abu El-Asrar, Nawaz, Kangave *et al.* 2013). This suggested a protective mechanism against neovessels formation in DR.

Together, the present study identified a significant involvement of two key complement proteins such as C3bα' and CFH in PDR pathogenesis. While the former was identified as a key player in alternative pathway activation and disease progression in PDR, the later was found as a feedback mechanism to arrest the alternative pathway activation mediated by activated microglial population. Further the activated microglia seemed to synthesize C3, CFH and secrete several inflammatory proteins and ECM re-modelling enzymes that eventually contribute to the underlying pathological changes in PDR.

5.14. Evaluation of neural glial modulations under diabetic condition using a primary human mixed retinal culture system

Functional outcome of the retina depends on the complex interplay between glial, vasculature and neuronal cells by neurovascular coupling (Newman 2015). In this regard, the glial cells in the retina have prime importance, since they are majorly involved in the functional coordination between neurons and vasculature. Thus, it is imperative to understand how the diabetic environment modulates glial cells activity leading to abnormal angiogenesis and neurodegeneration thereby contributing to disease progression.

Ca^{2+} is the secondary messenger, and is one of the major intrinsic signaling systems which interconnects and modulate neuro- glial activation in CNS tissues and thus play a key role in homeostasis regulation. Regulated activity of this signaling mechanism is known to control various key retinal functions such as light adaptation in rod-con photoreceptors, neurotransmitter release, cytoskeletal dynamics, cellular metabolism, gene expression and cell death(Krizaj and Copenhagen 2002). Moreover, it regulates bidirectional communication between homogenous and heterogenous cell population of the retina including signaling between glial cell types, i.e. astrocyte- astrocyte, microglia- macroglial interaction and also critically regulate neuro-glial interaction (Perea and Araque 2005). Previous studies have provided an evidence of regulation of neuronal activity by glial Ca^{2+} wave and vice versa, indicating a strong functional interaction between these heterogenous population of cells mediated by Ca^{2+}signals (Rosa, Bos, Sack *et al.* 2015). Additionally, these cross talks have also been described as a critical mechanism in modulation of retinal response to injury (Vecino, Rodriguez, Ruzafa *et al.* 2016). Since, glial cells evoked Ca^{2+} transient regulate neuronal activity, monitoring the changes in glial Ca^{2+} wave provide much idea about underlying neuronal damage in disease conditions (Metea and Newman 2006). Thus, the present study attempted to understand how the diabetic microenvironment modulates the glial cell activity and its correlation with various degenerative and vascular changes observed in the course of DR by studying Ca^{2+}signaling patterns in these cells under stress. The present study was also intended to understand the effect of two major key factors of DR, such as hypoxia and high glucose in the developed *in vitro* system (Nyengaard, Ido, Kilo *et al.* 2004). The individual effect of these two contributing factors of DR have been studied previously using both *in vivo* and *in-vitro* studies, however, there is no report on a comparative analysis of hypoxia and hyperglycemia

in human mixed retinal cells that can further help to understand the major contributor of disease progression in DR.

5.15. Primary human Mixed retinal culture (MRC) as a model system for understanding the disease pathogenesis.

Most of the existing studies on glial activation and neuro-vascular alteration in DR is based on investigations done in retinal tissues, animal models and also in cell line based studies (Brahmachari, Fung and Pahan 2006, Rosa *et al.* 2015). The studies done on immortalized cell lines are usually derived from tumor cells that suffer from the loss of original tissue specificity and phenotypes with multiple passages (Matteucci, Varano, Mallozzi *et al.* 2015).While there were independent investigations on primary cultures of Müller (Puro 2002), astrocytes (Barber, Antonetti and Gardner 2000), microglia (Ibrahim, El-Remessy, Matragoon *et al.* 2011), only limited investigations have studied them by co-culturing them together and measuring their collective response (Pereira Tde, da Costa, Santiago *et al.* 2010). Most importantly, there are no existing reports on Ca^{2+}modulation in primary human retinal cells of neurons and glia under diabetes stress. The functionality of the retina is interdependent, hence studying the cellular activity in a mixed population of retinal cells may provide better understanding of cellular modulation than cells of homogeneous population.

The cellular characterization in the human mixed retinal cultures done based on immunolabelling and PCR method clearly identified a good proportion of astrocyte, Müller glia, microglia and neurons in the established MRC-culture. Besides neuron and glial cell types, progenitor cell populations (nestin positive cells) that are likely to be Müller glia derived, were also detected, however, due to limited number of cells, no efforts were made to differentiate them into specific cell types or sort them out (Figure 4.37). The cells were making connections

with each other, as was seen clearly through the immunofluorescent labelling of cells using markers for two different populations. A flow cytometry- based counting of each cell types and their relative ratios could not be assessed due to lack of enough cells of each type. However, alternatively relative expression of genes specific to each of the four cell types across different sources of retina tissue was measured by quantitative PCR and found a uniform expression of cell specific genes across different sources implying similar proportion of each cell type in MRC derived from different donor retinas (Figure 4.41).

5.16. Modulation of glial activity measured through Calcium signaling under hypoxia and high glucose stress:

Hypoxic and high glucose stress were given to the cells of MRC using $CoCl_2$ and D-glucose respectively. $CoCl_2$ was used based on its inhibitory activity on prolyl hydroxylases, which leads to the stabilization and accumulation of the HIF-1α protein. Further, being a key protein mediating the responses to hypoxia, it undergoes rapid degradation under normoxic condition by the catalytic activity of prolyl hydroxylases (Cervellati, Cervellati, Romani *et al.* 2014). Before deciding the concentration of drug to induce hypoxia and D-glucose for high glucose, the cells were treated with different concentration of both the molecules. Final concentrations required were selected such that these should create enough stress to the cells and not induce a drastic cell death. Based on this 150μm of $CoCl_2$ and 30mm of D-glucose were selected for the experiments. The viability measurements of the cells clearly identified a significant cell death in both the conditions (hypoxia=63%, $p<0.0001$, high glucose=72%, $p<0.002$, but a greater cell viability was observed in high glucose compared to hypoxic treatment, which suggested hypoxia induce more damage in cells than high glucose (Figure 4.42 and Figure 4.43).

The result of the present study clearly identified a significant increase in Ca^{2+} transient in the cells under hypoxic and high glucose stress compared to cells under no stress condition. Since this was the collective response of different types of cells present in the MRC, a detailed investigation was further done by analysing each cells response in MRC using an algorithm developed at IIT Hyderabad (Swain, Gupta, Ratnayake *et al.* 2018). The clustering study showed that hypoxia induced an increase in percentage of hyper active cells. Since the Ca^{2+} spiking patterns obtained from the mixed culture were found to be highly heterogeneous, the basal level response in control condition was categorized into various types. Calcium imaging along with live markers for each cell type such as neurons and astrocytes may yield better information on cell specific responses in mixed retinal cells though homogenous transfection of primary cells remains a challenge (Guo, Zou, Rensing *et al.* 2017, Peri and Nüsslein-Volhard 2008).

Clustering of Ca^{2+} response in MRC done using an algorithm developed at IIT Hyderabad, helped to understand four different types of response in the culture (Figure 5.4). Most importantly a distinguishable difference in cellular response was identified between hypoxic and high glucose treated cells, suggested further that differences in cellular activity reflects different cellular responses to these two pathological conditions of diabetes. Especially the percentage of hyperactive cells were found to be higher in case of hypoxic cells with a simultaneous lower percentage of silent cells. On the contrary, the percentage of hyperactive cells were lower in high glucose treatment, while there was an increased the percentage of silent population. This clearly indicated that DR pathology represented a coordinated activity of multiple cell populations in MRC system. This was further confirmed based on the

differential gene and protein expression under hypoxic and high glucose conditions compared to cells under no stress.

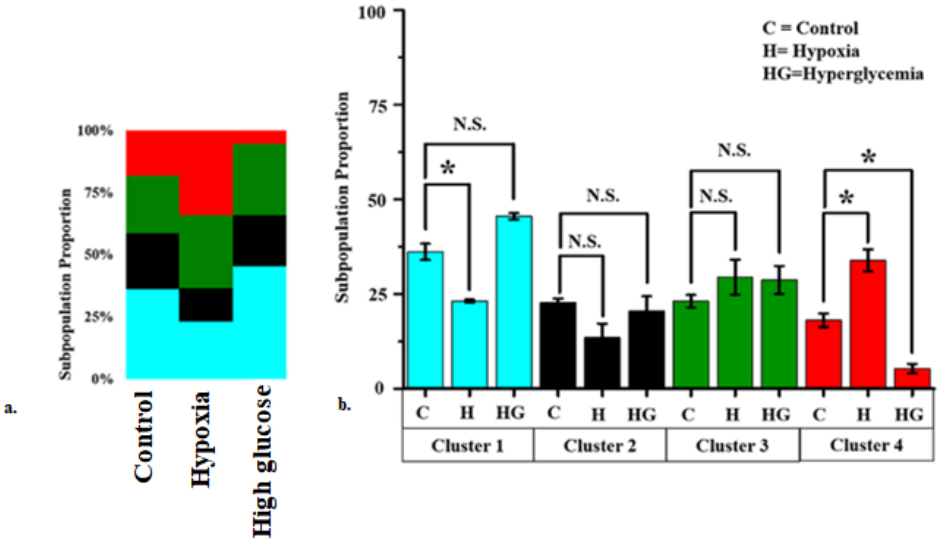


Figure 5.4 a) Stack bars representing the subpopulation profiling of Ca^{2+}spiking corresponding to no stress, hypoxia and high glucose (b) Comparison of relative percentages of four clusters corresponding to no stress, hypoxia and high glucose condition. The clustering was performed based on two features, Ca^{2+} spike count and Ca^{2+}_{max}. N.S: not significant, *p<0.05. **(Courtesy: Dr. Lopamudra Giri, Dr. Sarpras Swain, IIT Hyderabad).**

5.17. Correlation of Ca^{2+} transient with glial activation quantified through gene and protein expression in the cells of MRC

The correlation of Ca^{2+} modulations in glial cells with pathological molecular changes as seen in DR were done for the through gene and protein expression analysis of disease associated genes and markers of gliosis. Similar to the differences in Ca^{2+} modulations as observed for hypoxic and high glucose condition, an increased response of gene and protein expression was seen in hypoxia over high glucose treated cells. An upregulation of genes *HIF-1 a* and *CXCR4* were observed for both the stress conditions (Figure 4.46 and Figure 4.47). Hypoxic

conditions were seen to upregulate the expression of genes such as *NERF2, OXR1, IL1-β* and *VEGF* (Figure 4.46). The expression of *IL1-β* and *VEGF* were known to be upregulated in DR and ischemic retina, which further contribute neuroinflammation and support angiogenesis (Kaur, Sivakumar and Foulds 2006, Liu, Biarnes Costa and Gerhardinger 2012). The viability results from the present study showed increased cell death in hypoxia, and correspondingly, raised caspase 3 gene expression (though not statistically significant). Further this analysis also indicated that while high glucose may not induce more cell death, but it may alter the protein expression or phenotypic changes in glial cells under the oxidative stress. Similar findings were also reported in astrocytes under high glucose conditions (Shin, Huang, Gurel *et al.* 2014).

Further, when the correlation of Ca^{2+} response with glial activation was assessed, by studying protein expression of IBA-1 for microglial cells and GFAP for astrogliosis the changes in the microglial activation and expression of GFAP were more pronounced in the MRC subjected to hypoxia when compared to high glucose (Figure 4.49). Specifically, it was observed that hypoxia lead to a significant increase in IBA1 and GFAP expression whereas significant downregulation of GFAP was found in MRC under high glucose stress. These results were quite similar to the published reports on diabetes, where decrease in GFAP expression in diabetes had been explained as a mechanism of insulin regulatory effect on astrocytes (Coleman, Judd, Hoe *et al.* 2004). It could also be associated with loss of regulatory function of GFAP in BRB maintenance (Barber *et al.* 2000). Some studies have also identified that expression of GFAP in retina occurred in a time dependent manner, based on the findings that downregulation of GFAP in astrocytes in diabetic retina was followed by an upregulation of GFAP in GS positive Müller glial cells (Barber *et al.* 2000). Thus, the GFAP downregulation

could be related to the onset of gliotic change. Interestingly in present study, an increased expression of IBA-1 was observed in both hypoxia and high glucose treatment, thereby indicating proliferation and activation of microglial cells in cells under both hypoxia and high glucose condition. This finding correlated with that of vitreous proteome profiling, where predominance of microglial activation and infiltration was observed in the vitreous samples of PDR patients. Although a precise causal relation between inflammation and neuronal apoptosis has not yet been defined, possible mechanisms could involve microglial activation and cytokine-activated neurodegenerative pathways (Glass, Saijo, Winner *et al.* 2010, Lull and Block 2010) under hypoxic stress. Whereas in case of high glucose, though there are evidences for microglial activation, no other changes were noted further suggesting that high glucose alone may not cause damage to the retina and it is the underlying hypoxia causing detrimental damage. Thus, the present mixed retinal culture system observed significant alterations in cellular functions as assessed through the classification of calcium spiking under stressed condition. The results indicated that a fraction of mixed cell population showed higher activity with higher amplitude and frequency of calcium spiking under hypoxia. However, future studies should include the investigation on the calcium channels and GPCRs involved in this process using channel inhibitor and GPCR targeting drugs to further understand the underlying cellular and molecular changes in DR eyes and for developing newer treatment strategies for this complex disease. Further, measurement of glutamate and ROS may provide insights into whether an excessive stimulation of glutamate receptors results in an uncontrolled intracellular Ca^{2+} flow in neurons as a consequence of oxidative stress.

Thus, the overall strategies used in the present study to understand molecular mechanisms of abnormal ocular angiogenesis in diabetic retinopathy, provided key understanding of few of the basic mechanisms involved in disease pathogenesis. This include identification of key

proteins of retinopathy progression, followed by a targeted analysis of complement pathway that identified its critical involvement in disease pathogenesis with significant involvement of the C3bα' and CFH proteins. Most importantly microglial activation was found to play a significant role in disease progression by causing neovascular and neurodegenerative changes in the retina.

6. CONCLUSIONS

a) Global protein profiling

1. Global protein profiling identified 1079 proteins from the vitreous humor samples, of which 27 were novel.

2. The study segregated proteins that are differentially expressed during diabetes and those involved in retinopathy complications.

3. Fifteen proteins were found to be differentially expressed during diabetes that included upregulation of 8 and down regulation of 7 proteins. The upregulated proteins of DM were found to be play a major role in ECM remodeling and rearrangement, which precedes the onset of neovessels formation in retinopathy complications.

4. Some of the observed proteins could be playing a critical role in detoxification mechanisms (GSTP1 and PRDX1) and glucose metabolism. The significantly downregulated (IDH1, LDHA, LDHB) proteins indicated elevated oxidative stress as well as an inefficient metabolism of glucose in diabetes.

6. Ten proteins were differentially expressed in retinopathy stage with a significant upregulation of 4 and downregulation of 6 proteins. The upregulated proteins in retinopathy were found to be involved in angiogenesis (LRG1) and inflammation (APCS, FGA), suggesting enhanced inflammatory milieu in retinopathy.

8. SOD-3, a vital enzyme required for ROS clearance in retinopathy group suggested an accelerated oxidative stress in retinopathy complication, which may contribute to the activation of various inflammatory pathways.

9. The unique pathway present in the vitreous of retinopathy subjects supported an enhanced inflammatory milieu along with extensive ECM-reorganization in retinopathy with the identification of unique pathways such as initial triggering of complement and ECM- receptor interaction.

b) Role of complement and microglial activation in PDR pathogenesis

1. The present study identified the involvement of alternative pathway of complement in PDR pathogenesis.

2. Two of the proteins of complement pathways (C3bα' and CFH) were found to play a significant role in disease progression.

3. The study clearly demonstrated that activation of complement pathway is exclusive to retina and specific to diabetic retinopathy only and absence of this in serum does not suggest it to be a generalized change due to diabetes

4. An increased level of C3bα' and CFH upregulation in vitreous of PDR and the co-localization of CFH with activated microglia suggested that it could be a feed-back mechanism of microglial cells to arrest complement activation in order to protect complement mediated lysis of retinal neurons.

5. The activation of microglia were found to contribute to enhanced inflammation, ECM organization and neovascularization in PDR pathogenesis

c.) Neuro-glial interaction study by mixed retina culture (MRC) system

1. Increased Ca^{2+} transients were identified in hypoxic and high glucose conditions in cells of MRC and these levels correlated with the activation of various neuroinflammatory and angiogenic genes.
2. Hypoxia was found to be the major contributor of altered Ca^{2+} transients. Gene expression changes suggested hypoxia as a major modulator of diabetes complications
3. Evaluation of glial activation using markers of microglia and macroglia, identified a significant upregulation of microglia under stress, which further suggested the key contribution of microglia in diabetes retinopathy.

7. CONTRIBUTIONS

1. The study highlight the potential involvement of 27 novel vitreous and some key mechanisms in DR complications based on the data from global proteomic profiling.

2. Emphasizes the central role of C3bα' and CFH of complement pathway and microglial activation in the pathogenesis of DR.

3. Demonstrated an optimal retinal culture system to study neuro-glial interactions in DR pathogenesis and this system can be used for further drug screening studies in DR.

8. LIMITATIONS

1. Due to lack of time and resources, all the significantly identified proteins by global proteome profiling could not be validated.
2. Inhibition of calcium channels and GPCRs involved in disease pathogenesis were not performed.

9. FUTURE SCOPE

- In depth functional studies using *in vivo* animal models are required to understand the role of microglial activation and the arrest of complement pathway towards preventing PDR progression

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Informed Consent for Collection of Samples for Research on Diabetic Retinopathy

You are being invited to participate in our ongoing research on genetics of diabetic retinopathy.This consent form describes your role in this study. We will explain the whole procedure and the need for your participation in this study. Please read it carefully and do not hesitate to ask if you have any queries at any point of time.

Diabetic retinopathy (DR) and diabetic macular edema (DME) are common microvascular complications in patients with diabetes and may have a sudden and debilitating impact on visual acuity (VA), eventually leading to blindness. Patients with diabetes for a longer duration, tend to have a higher risk of DR. This study aims at better understanding of the disease development so as to identify individuals at-risk of progressing to DR. This is an experimental study and we will require biological samples (blood) from patients for genetic analysis and surgically removed specimens such as aqueous, vitreous, retina, ILM and ERM from the individuals who are undergoing surgery as part of routine management of the disease for proteomics and cell biology work. Approximately 1200 such individuals would be included in the study.

If you agree to be in this study, we would ask you to do the following things:

1. Complete certain demographic, life style and clinical details in the form of questionnaire for studying the disease pattern
2. Blood (i.e. 4-5ml) would be collected by venipuncture during your routine visit.
3. Vitreous / aqueous/ ILM / ERM/ retina samples removed as part of routine surgery for such patients will be stored for cell culture and immunological tests.

DNA (the genetic material inthe cells) will be extracted from the biological samples and subsequently studied for any change which could be associated with the disease. A portion of your DNA will be stored for any future studies on this disease. We will be using surgically removed tissues for establishing a primary cell culture to identify the key bio-molecules and cellular interactions that regulate ocular angiogenesis and those causing abnormal blood vessel growth and further damage the retina by RNA analysis and proteomic techniques.

There would be no harm to you for providing the sample. The results of study may or may not be of immediate benefit to the patient. However, the results of study would help clinicians to diagnose the disease at an early stage and providing appropriate interventions to check the progression of the disease in future patients. Complete confidentiality will be maintained in the handling and processing of samples and the results of the study. We highly appreciate your cooperation in completing this study. After you join this study, you will also have the liberty to quit at any time without any loss of benefits.

L.V. Prasad Eye Institute has a policy to protect health information that may identify you. By signing this consent form, you agree that health information that identifies you (date of birth, birth place and other details about you and your health or medical condition) may be used as described in this form for the study purposes only.

Your information can be seen by the researcher and the research staff, the institutional review boards and their staff, legal counsel, audit and compliance staff who need to see the information to help this study or make sure it is being done in a proper manner. Governmental bodies that have the duty to protect research participants can also see your information as and when required. Your genetic information may be used and shared to carry out this study and to evaluate the results of this study.Your identity will not be disclosed at any stage when this information is being shared with other organization(s) for academic purposes.

The L.V. Prasad Eye Institute IRB is made up of scientists, non-scientists, doctors, and legal personnel. The IRBs purpose is to review human research studies and to protect the rights and welfare of the

people participating in those studies. You may contact the IRB if you have questions about your rights as a participant or if you think you have not been treated fairly. The IRB office number is **040-30612511.** If you have questions about the study, then you may call the principal investigator, **Dr. Inderjeet Kaur** at phone no.**040-30612508**.

L.V. Prasad Eye Institute is dedicated to finding the causes and cures for ocular diseases. The data, blood/tissue samples from your body collected during this study are important to this study and to future research. If you join this study, L.V. Prasad Eye Institute or its outside partners in this research will own this data, tissue and blood samples. This material will be studied, tested and used by medical scientists. If this material helps lead to the creation of a product or idea, whoever creates that product or idea will own it. You will not receive any financial benefit from the creation, development, use or sale of that product or idea.

By signing this consent form, you are not giving up any legal rights. Your signature means that you understand the information given to you in this form, you accept the provisions in the form, and you agree to join the study.

The above statement has been read out or explained to me, and having understood the same, I voluntarily put my signature or thumb impression.

Patient's Name: Mr. No.:

Signature/left hand thumb impression of patient /guardian

Name (capitals):

Relationship (if guardian):

Witness 1: Signature:

Name (capitals):

Designation: Date:

Ethics Committee
L. V. Prasad Eye Institute
Kallam Anji Reddy Campus
L.V. Prasad Marg, Banjara Hills
Hyderabad-500 034

DR- STUDY PROFORMA
L. V. Prasad Eye Institute

1. Date
2. Study patient no.:

Personal Details:

3. Name:
4. ID no.:
5. Address:
6. Phone no.:
7. Fax no.:
8. Email ID:
9. Age:
10. Date of Birth:
11. Gender: Male \ Female
12. Religion - Hindu \ Muslim \ Jews

13. Educational Background: Literate \ Illiterate

Highest level of Education Primary school \ Higher secondary \ Graduation \ Post-graduation \ Professional

14. Dietary habits:

Staple diet: - Wheat \ Rice \ Maize \ Any other

Green tea/ Black tea / wine/ Chawanprash / any other source of antioxidants

Vegetarian/Non-vegetarian Beef/ Pork/ Chicken/ Fish/Mutton

15. Smoker \ Nonsmoker

In past, then, no of years (smoke): [ ] years

No. of cigarettes/ bidis in a day

16.Pan / Tobacco

In past, then, no of years (chewing): [ ] years

17. Alcoholic \ Non-alcoholic

In past, then, no of years (consumption of alcohol): [ ] years

18. Medical History

a) Height [ ]

b) Weight : [ ]

c) BMI : [ ]

d) Waist [ ] cm e) Hip [ ] cm f. Waist/hip ratio [ ]

g) Resting heart rate : ---------- /min 20. h) BP: Systolic ---------- Diastolic: ---------- (mm/hg)

19. Diabetes mellitus: Yes\No Duration : [ ] HbA1c: [ ]

20. **Control** - well controlled /Fluctuating / Poor control

21. **Treatment** 1.Oral hypoglycemic 2. Insulin 3.Exercise & Diet control 4. No medication

22. **Name & Dose of insulin** ---------- units /day

23. **Duration of insulin**----------

24. **If the patient is a female,** a) history of diabetes during pregnancy: Yes/No

b) history of hypertension during pregnancy: Yes/No

c) Whether the diabetes is present since then tick in the box ☐

25. **Present eye Complaints**: OD ☐ OS ☐

1. Decrease vision 2. Redness 3.Pain 4.Photophobia 5.Haloes 6.Floaters/Flashes 7.Others ---------

---

26.Any other ocular Disease in Past ☐

27. **History of any ocular surgery**:Yes /No If yes, 1) OD 2) OS 3) OU

28. If known type of surgery, specify _____

29. a) **Whether underwent any laser photocoagulation/ Anti VEGF therapy/ IVT A/Steroid:Yes /No**

b) If yes, 1) OD 2) OS 3) OU

b) No. of injections

c) Details of laser treatment OD: ---------- OS: ----------

30. **Systemic Diseases**: 1. Hypertension 2. IHD 3. Asthma 4. Others ----------

31. **Any other significant history related to diabetes**

1.None 2.Nephropathy 3.Neuropathy 4. Others----------

32. **Associated Findings or Disease**:

33. **Comments by Ophthalmologist**:

34. **Allergies,** if yes specify ----------

35. **Ophthalmic examination**

1. Visual acuity with ETDRS chart Distance Near

BCVA a. OD ☐ ☐

b. OS ☐ ☐

1. 4/2 2. 4/2.5 3.4/3 4.4/4 5.4/5 6.4/6 7.4/8 8.4/10 9.4/12 10.4/16 11.4/20 12.4/25 13.4/32 14.4/40 15.3/40 16.2/40 17.1/40 18.CF 19.HM 20. PL 21. No PL	1. N6 2. N8 3.N10 4.N12 5.N18 6. N36 7.< N 36

	Spherical (D)	Cylindrical (D)	Axis	Add
2 a. Subjective OD				
2 b. Subjective OS				

3. Amplitude of Accomodation

FP cms					
NP cms					
Amplitude of Accomodation D					

4. External examination 1.Normal 2. Others (specify) ---------- OD: ☐ OS: ☐

a. Facial asymmetry **b.**Exotropia **c.** Esotropia **d.**Hypertropia **e.** Hypotropia

36.**Fundus Photograph**

a) Fundus Photography 1. 45º fields ☐ 2. 30º fields ☐ 3. both ☐

45ºFields	**OD**	**OS**
1 Temporal	☐	☐
2 Nasal	☐	☐
3 Superior	☐	☐

30ºFields		OD	OS
Disc	1	☐	☐
Macula	2	☐	☐
	3	☐	☐
	4	☐	☐

b). Photographers Notes 1. *Ocular Media* *a. Clear b. Hazy c. Very Hazy OD* ☐ *OS* ☐

2.*Cooperation: a. Good b. fair 3. poor* *3. Fixation: a. Good b. fair c. poor*
4. IOL OD OS 5. Small Pupil OD ☐ OS ☐ 6.Cataract OD ☐ ☐

7. Light Sensitive ☐

37. Clinical diagnosis 1.No DR 2.mild NPDR 3. Moderate NPDR

4. Severe NPDR 5. PDR 6. TRD macula 7. TRD elsewhere 8. Combined RD

38. **Cataract surgery**: Yes/No

39. **Surgery procedure**:----------

40. **Grade of cataract**: I / II / III / IV / V

41. Family History : Familial \ Sporadic

42. Pedigree details:

Father	Age	Affected: Yes/NO
Mother	Age	Affected: Yes/NO

Whether the marriage of parents was within the relation: YES/NO

No of Brothers	Age	Affected: Yes/NO
No of sisters	Age	Affected: Yes/NO
Grandfather	Age	Affected: Yes/NO
Grandmother	Age	Affected: Yes/NO

Whether the marriage of grandparents was within the relation: YES/NO

Spouse	Age	Affected: Yes/NO
No of Sons	Age	Affected: Yes/NO
No of daughters	Age	Affected: Yes/NO

Any other affected members, please specify with age

43. Informed Consent: Yes \ No

44. Permission to contact Family members: Yes \ No

45. Blood sample: Taken \ Not taken

46. Done by: [ ]

47. Signature: [ ] **Date:**

ANNEXURE II

1. Reagents used for PAGE and Zymography

a). 30% acrylamide (acrylamide: bisacrylamide, 29:1)

29 g of acrylamide and 1 g of N,N'-Methylenebisacrylamide was added to autoclaved deionized water to make a stock solution of 100 mL. The solution was made homogenous by mixing on a magnetic stirrer.

b) 0.5M EDTA

186.1 g of disodium EDTA.$2H_2O$ was added to 800 mL of autoclaved deionized water and stirred on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH pellets (approximately 20g of pellets). The solution was sterilized by autoclaving

c)10%APS

0.1g of APS was added to 1mL of autoclaved deionized water in a 1.5mL Eppendorf tube and mixed thoroughly by inverting the tube. The tube was wrapped in aluminum foil and stored at 4°C.

d) Tris-HCl (1.5M –pH 8.8)

For 200 ml of the solution, 36.3 g of Tris base was added to 150 ml of deionized water in a beaker and dissolved on a magnetic stirrer. The pH of the solution was adjusted to 8.8 with HCl

and the volume was adjusted with water and autoclaved.

e) Tris-HCL (1M-pH 6.8)

For 100 ml of the solution, 12.21 g of Tris base was added to 50 ml of deionized water in a

beaker and dissolved on a magnetic stirrer. The pH of the solution was adjusted to 6.8 with HCl

and the volume was adjusted with water and autoclaved.

f) 10% SDS

For 50 ml of the solution, 5 g of SDS was added to 40 ml of autoclaved deionized water in a beaker and dissolved by heating in a water bath. The final volume was adjusted to 50mL with autoclaved deionized water.

g) 5X running buffer

15.1g of Tris base, 5g of SDS and 94g of Glycine were added to 800 mL of autoclaved deionized water. The pH of the solution was adjusted to 8.3 and the final volume of the solution was made to 1000 ml with autoclaved deionized water. The final concentration of the

solution was 5X. The working solution of 1X was prepared by diluting the stock solution with autoclaved deionized water.

h) 1% w/v gelatin

1%Gelatin was freshly prepared by dissolved 50mg of gelatin in 5 mL of dH2O and the solution was heated at 60°C in a water bath (for at least 20 min) until it was dissolved properly.

Then the gelatin solution was allowed to cool down at room temperature before use.

i) Renaturing solution stock (10X)

25% v/v Triton X-100 was added in 200 mL of in dH2O.

j) Developing buffer stock (10X)

1 L of developing buffer stock was prepared by adding 500 mM Tris-HCl (pH 7.8), 2 M NaCl,

50 mM CaCl2, and 0.2% v/v Brij 35.

k) Staining solution

1 L of staining solution was prepared by adding 0.5% w/v Coomassie blue R-250, 5% v/v methanol, and 10% v/v acetic acid in dH2O. Store it at room temperature.

l) Destaining solution

Prepare 1 L of 10% v/v methanol, 5% v/v acetic acid in dH2O.

Reagents for western blotting

a) 1X PBS

8 grams of sodium chloride (NaCl), 0.2 grams of potassium chloride (KCl) and 1.44 grams of sodium biphosphate (Na2HPO4) were added to 800 milliliters (ml) of autoclaved deionized water. The pH of the solution was adjusted to 7.4 with 1M hydrochloric acid. The final volume of the solution was made to 1000 ml with autoclaved deionized water. The final concentration of the solution was 10X. The working solution of 1X was prepared by diluting the stock solution with autoclaved deionized water.

b) Transfer buffer

Transfer buffer was made by mixing 50mL of 5X tris glycine with 150mL of deionized water and 50 mL of HPLC grade methanol

3. Reagents for Cell culture

a) Preparation of Trypsin-EDTA Stocks

Prepared a 10X stock solution, dissolving 2.5 g of trypsin (Sigma, Cat No. T4799) and 372 mg of EDTA (Sigma, Cat No. E6758) in 100 ml of 1X PBS solution, filtered sterilize using a 0.22 μm syringe filter, made aliquots and stored frozen at -20°C. To prepare working concentration of 0.25% Trypsin + 1 mM EDTA, 10 ml of stock solution was mixed with 90 ml of sterile 1X PBS solution.

b) Preparation of Penicillin-Streptomycin 100X stock solution

Penicillin G sodium salt (Sigma, Cat No. P3032-10MU) (6 g), Streptomycin sulphate (Sigma, Cat No. S9137-25G), (1x = 100 U/mL or $1X10^5$ U/L Penicillin, 100 μg/mL or 100 mg/L Streptomycin), (100 ml of 100x = 1MU of Penicillin (0.6 g), 1 g of Streptomycin) Weighed out the powders and dissolved in 100 ml of 1X DPBS without calcium and magnesium, filtered and sterilized using a 0.22 μm syringe filter, made aliquots and stored frozen at -30°C.

c) Cobalt chloride:

25mM of stock solution of $CoCl_2$ (Sigma, cat. No. C8661) was prepared by dissolving 0.023g of $CoCl_2$ in 2 mL incomplete DMEM. Filtered and used freshly.

d) D-glucose

1M of D-glucose (Sigma, G7021) was prepared by dissolving 1.8g of D-glucose in 10mL in-complete DMEM, filtered and used freshly

e) Flu4^{Am} dye preparation

Fluo-4^{am} (Cat No. F14201, Life Technologies) of dye was dissolve din 50 μL of DMSO. From this stock 1μL was dissolved in 1Ml of HBSS for 30 min in Hank's Balanced Salt Solution (HBSS) (Invitrogen, Life Technologies, Grand Island, NY). Covered with aluminum foil and used freshy

PRESENTATIONS IN NATIONAL AND INTERNATIONAL CONFERENCES

1. **Shahna Shahulhameed,** Jay Chhablani, Mudit Tyagi, Rajeev Reddy, Subhabrata Chakrabarti, Inderjeet Kaur, Poster- "Role of complement components in proliferative diabetic retinopathy (PDR)", Indian Eye Research Group 21st Annual meeting held on July 29- 30, LV Prasad Eye Institute Hyderabad, India -2014.

2. **Shahna Shahulhameed,** Jay Chhablani, Mudit Tyagi, Rajeev Reddy, Subhabrata Chakrabarti, Inderjeet Kaur, Poster -"Role of Extracellular matrix (ECM) rearrangement in Proliferative Diabetic Retinopathy (PDR)", Indian Eye Research Group 22nd Annual meeting held on July 25-26, LV Prasad Eye Institute Hyderabad, India – 2015
3. **Shahna Shahulhameed,** Lopamudra Giri, Sarpras Swain**,** Jay Chhablani, Mudit Tyagi, Rajeev Reddy, Subhabrata Chakrabarti, Inderjeet Kaur, "Effect of Hyperglycemic and hypoxic stress on the primary cultures of retinal neuron and glial populations: a model system to understand the role of glia in diabetic retinopathy". Association for Research in Vision and Ophthalmology, May 7-11, Baltimore, Maryland, USA. 2017
4. Oral presentation - "Effect of Hyperglycemic and hypoxic stress on the primary cultures of retinal neuron and glial populations: a model system to understand the role of glia in diabetic retinopathy" Indian Eye Research Group 23rd Annual meeting, July 27-29, LV Prasad Eye Institute Hyderabad, India- 2018
5. Inderjeet Kaur, **Shahna Shahulhameed**, Sushma Vishwakarma**,** Jay Chhablani, Mudit Tyagi, Rajeev Reddy, Subhabrata Chakrabarti - Poster "Involvement of complement

activation and its regulation in the pathogenesis of DR", ARVO, April 28- May 02, 2019, ARVO, Vancouver, Canada

Award Received:

DBT international travel grant for attending conference in Association for Research in Vision and Ophthalmology, Baltimore Maryland, USA. 2017

Title: A systematic investigation on the involvement of complement pathway in diabetic retinopathy.

Shahna Shahulhameed[1], Sushma Vishwakarma[1], Jay Chhablani[2], MuditTyagi[2], Rajeev R Pappuru[2],Subhabrata Chakrabarti[1], Inderjeet Kaur[1]

[1]Prof Brien Holden Eye Research Centre, LV Prasad Eye Institute, Hyderabad, India

[2]Smt. Kanuri Santhamma Center for Vitreo Retinal Diseases, LV Prasad Eye Institute, Hyderabad, India

E-mail address of authors:

ShahnaShahulhameed: shahna135@gmail.com

Sushma Vishwakarma:svishwakarma17@gmail.com

Jay Chhablani: jaychhablani@lvpei.org

MuditTyagi: drmudit@lvpei.org

Rajeev R Pappuru: rajeev@lvpei.org

SubhabrataChakrabarti: subho@lvpei.org

Corresponding Author

Inderjeet Kaur

Prof Brien Holden Eye Research Centre, LV Prasad Eye Institute, Hyderabad, India, inderjeet@lvpei.org, Tel. no. +91- 40-30612508, Fax no. +91-40-23548271

Abstract:

Background:

Complement system play a crucial role in retinal homeostasis. Several proteomic studies have shown deposition of complement protein in ocular tissues from diabetic retinopathy, however, their exact involvement in pathogenesis of DR remains unclear.

Methods:

We evaluated major complement pathway proteins in the classical and alternative pathway including C1q, C4b, C3, CFB and CFH in vitreous humor and serum samples from PDR patients and controls by western blotting. Quantitative real time (QRT) PCR was done for PDR, NPDR and no-DM controls for correlating the expression of several key pro and anti -angiogenic genes with their correspondingprotein levels. Inflammation in the vitreous humor samples was assessed by ELISA and metalloproteinase activity measured by gelatin zymography. Glial activation and its association with complement activation in diabetic eyes was assessed by immunohistochemistry.

Results:

A significant increase in C3 proteins, its activated fragment C3bα' (110kDa) along with a concurrent up regulation of CFH was observed for PDR vitreous. QRT identified a significant upregulation of angiogenic genes and downregulation of antiangiogenic genes in PDR and NPDR cases. PDR vitreous had increased MMP9 activity and upregulation of inflammatory markers IL8, sPECAM and down regulation of anti-inflammatory marker IL-10. Increased C3 deposition and CFH upregulation were observed in DM retina. CFH was found co-localizing with CD11b+ve activated microglial cells in inner nuclear layer of DM retina.

Conclusions:

The present study confirms increased activation of alternative complement pathway in PDR. The co-localization of CFH in CD11b +ve cells further suggests microglia as a source of CFH in diabetic retina.Increased CFH levels could be a feedback mechanism for arresting excessive complement activation DR eyes.

Introduction:

Retina being an immune privileged organ, has its own unique immune regulatory mechanisms including retinal neurons and RPE, and immune defense mechanisms comprising microglial population and the complement system. The retinal immune defense mechanism get alerted with any kind of noxious signals and starts a series of inflammatory events as an adaptive response to restore the homeostatic balance[1]. Low- level activation of the innate immune mechanisms, specifically complement system is required for preserving normal eye homeostasis and to maintain the retinal integrity while aging[2]. However, these protective mechanisms can cause detrimental consequences if the insults persist for a longer duration and lead to irreversible functional loss as evident from various neurodegenerative diseases such as Alzheimer's, Parkinson's, Amyotrophic lateral sclerosis, Age related macular degeneration etc[3].

Beyond its role as an immune defense mechanism, studies have shown the involvement of complement system in various tissue remodeling process such as liver regeneration, synaptic pruning while development and also in retinal angiogenesis[4-6]. The role of complement in angiogenesis have prime importance since there are several blinding eye diseases associated with abnormal ocular angiogenesis such as retinopathy of prematurity (ROP), age related macular degeneration (AMD), proliferative diabetic retinopathy (PDR) etc[7]. Both inhibitory as well as promoting role of complement are known in various ocular angiogenic conditions. A study done by Bora *et al*, in 2005, identified C3 and MAC complex deposits in neovessels in mice model of laser induced choroidal neovascularization (CNV), while the C3 knock out (C3-/-) CNV mice showed an absence of neovessels formation with reduced level of

angiogenic factors thereby suggesting complement component C3 as a proangiogenic factor[8]. On the contrary, Langer *et al* in a mice model of ROP, had shown anti-angiogenic property of complement system where C3 and C5aR were required for inhibiting the polarization of macrophage towards its angiogenic potential[6]. Our earlier study (2017) on ROP identified microglia mediated excessive complement activation in vitreous of ROP babies when compared to that of age matched control babies, further suggesting complement as a promoting factor for ocular angiogenesis[9]. In past, genetic studies done in AMD had shown a strong association of *CFB* and *CFH* gene polymorphism with disease pathogenesis and strong deposition of complement components in the RPE-Bruch's layer[10-12]. The balance between angiogenic and anti-angiogenic factors determine the extent of neovascularization[13]. *VEGF* is a potent angiogenic factor, whereas *THBS1* is both angiogenic inhibitor and a potent activator of *TGFβ*. *TGFβ* activation is also required to maintain the RPE mediated immune privilege status of retina[14].

DR is an important ocular angiogenic condition, that displays association with complement component deposits in retina and vitreous. It is one of the most devastating causes of irreversible vision loss with complex pathophysiology and has a global prevalence of 34.6% [15]. Gerl *et al* in 2002, for the first time identified extensive complement C3d and MAC complex (C5b-9) deposits in diabetic retinal choriocapillaries by immunohistochemistry [16]. Later, another study identified the deposition of C5b-9 complex and reduction of glycosylphosphotidyl inositol anchored inhibitors of complements such as CD55 and CD59 in the walls of retinal vessels of diabetic eyes, suggesting for the involvement of alternative complement pathway in diabetic eyes[17]. Additionally, an association of increased systemic level of iC3b/C3 and Bb/Fb was identified with the microvascular abnormalities in diabetic eyes [18]. Several vitreous proteomicsreports have shown the presence of complement proteins such as C3, CFI, CFB, C4A, C4B, C2, C4BPA, CFD, CFH in PDR subjects however the data on the levels of complement components across different studies is highly variable and does not explain clearly how they contribute to DR pathology[19-22]. Likewise, only few studies have observed the associations of polymorphisms in complement pathway genes such as *C5 (rs17611)*, *CFH (rs800292)* and *CFB (rs1048709)* with DR[23, 24].

Thus, while the involvement of complement pathway in the DR pathogenesis was suggested based on the presence of complement proteins in the vitreous, a clear understanding on how complements could contribute to DR pathology is lacking till date. A systematic validation and comparison of alternative and classical complement pathway proteins might address the lacunae and further the knowledge on their role in PDR pathogenesis.The present study, attempted a systematic evaluation and validation of alternative and classical complement pathway proteins in PDR pathogenesis in an extended cohort. In addition, we

have also correlated our findings with the expression of complement proteins in retinal tissues obtained from diabetic cadaveric donors and blood samples of DR patients and controls. Thus, the mRNA expression of pro and anti-angiogenic genes and their correlation with complement C3 and CFH expression was compared among patients and controls. Next, we correlated the level of complement activation with inflammation in PDR vitreous by analyzing inflammatory and proinflammatory cytokines by ELISA. Our study identified, elevation of C3 in PDR vitreous, especially 110kDa C3bα' fragment and a concurrent upregulation of CFH in PDR vitreous.To the best of our knowledge, this is the first report on upregulation of CFH levels in PDR vitreous as revealed through western blotting. Most importantly, increased CFH levels only in the vitreous and not in systemic circulation further, strengthened its major role in the pathogenesis of DR. Additionally, we were also able to show that the increased complement activation correlated with the presence of activated microglia in diabetic retina.

Materials and methods:

Enrolment of study participants and sample preparation:

The study was performed according to the guidelines of Declaration of Helsinki and approved by Institutional Review Board. Vitreous samples (100 µl) were collected from control (n=100) and PDR subjects (n=100) while undergoing pars planar vitrectomy after obtaining written informed consent. Samples were collected in surgery rooms under aseptic conditions and then immediately transferred to the lab in ice cold condition.The samples were then centrifuged at 14,000 rpm for 10 min at 4˚C to remove any cellular debris and then stored at -80 degrees till further use. Proteins were lysed in equal volume of RIPA buffer and precipitated with ice-cold acetone overnight at -80˚C. The precipitated proteins were collected by centrifugation at 14,000 rpm for 1hour at 4˚C and dissolved the protein pellet in 1x PBS containing protease inhibitor. Blood samples were collected in a vacutainer without the anticoagulant from PDR (n=38), NPDR (n=38) and control (n=38) subjects and separated the serum within 1 hour of sample collection by centrifugation at 1500g for 15 minutes and then stored at -80˚C. The total protein concentration was calculated by bicinchoninic acid (BCA) assay. The detailed demographics of the vitreous and serum samples used in this study is given in the supplementary table1 and 2.

Western blotting:

Western blotting was performed in vitreous and serum samples for identifying the role of complement pathway in PDR pathogenesis. Total C3 and its fragmentation pattern in serum samples were compared among PDR, NPDR and no-DM subjects. The quantitative expression of classical complement pathway proteins C1q and C4b was evaluated in vitreous humor of PDR and no-DM subjects. Alternative complement pathway proteins C3, CFB and CFH were evaluated both in serum and in vitreous. Standard

protocol for western blotting were followed[9]. The details of antibodies used and their dilutions are given in supplementary table 3. The blots were developed and protein specific bands were visualized in a LICOR image scanner using LI-COR image studio software and the band intensities were quantified.

Immunohistochemistry (IHC):

Cadaveric control (n=3) and diabetic eyes from type 2 DM with no retinopathy (n=3) were collected in a sterile moist chamber within 24 hours of death from Ramayamma international eye bank, LV Prasad Eye institute, Hyderabad, India, according to the tenets of Declaration of Helsinki. The retinas were removed carefully from the eyes under a dissection microscope and fixed them in 4% formalin and paraffin sections were made. For IHC, antigen retrieval was done for the deparaffinized tissue sections using Tris citrate buffer of pH 6. The sections were permeabilized using methanol for 30 min at -20˚C followed by washing thrice with 1X PBS. Blocking was done with 2% BSA followed by sections were incubated with primary antibodies for overnight at 4˚C (Ms C3- Santacruz, 1:50, sc-28294, Ms CFH- Santacruz, sc-166613, 1: 50, Rb CXCR4, sc- 9036, 1:50, Rb CD11b, CST,49420, 1:200, Rb GFAP, Dako, Z0334). After washing thrice with 1x PBS and the sections were incubated with appropriate fluorescent labelled secondary antibodies (Goat anti Rb594,Life Tech.A-11012, 1:300, Goat anti Ms 594, Life Tech.A-11005, 1:300, Goat anti Rb 488, Life Tech.A-11008, 1:300) for 1hour at room temperature. The sections were counterstained with DAPI and visualized the staining under fluorescent microscope (EVOS) using appropriate filters.

Gelatin zymography:

Gelatin zymography was done to analyze the matrix metalloproteinases activity (MMP2 and MMP9) in the vitreous samples obtained from PDR and control subjectsas per the protocol [25] using 10 µg of vitreous proteins.

Enzyme Linked Immunosorbent Assay:

ELISA was done to evaluate the level of cytokines such as sPECAM, IL-8 and IL-10 in the vitreous samples collected from PDR and control subjects following the standard protocol [9]. Vitreous humor samples were diluted with assay buffer to 1:3 dilution.Quantitative data was obtained using Luminex system with xPONENT® software and the generated results were exported in terms of median fluorescent intensity and calculated the concentration of the analytes in the PDR and controls. The significance was calculated based on the t-test with a p value <0.05.

RNA isolation and quantitative real time PCR:

Blood samples were collected in K3EDTA coated 3mL blood vacutainers from PDR, NPDR and no-DM subjects and RNA was isolated using Trizol-chloroform method. 1µl of RNA was converted into cDNA using iScript cDNA conversion kit (1708891, Bio-Rad) as per manufacture's protocol. Semi-quantitative PCR were performed on 7900 HT system using TaqMan assay chemistry for *C3, TGF*-β and SyBr green chemistry for *VEGF, CFH* and *CXCR4*. β-actin was used as normalization control using standard thermal cycling conditions. Cycle threshold (CT) values for each test gene were obtained for each sample using the *SDS2.3* software and fold change was calculated using $2^{-\Delta\Delta Ct}$. The primer sequence used for qRT are given in the supplementary table 4.

Results:

1. Systematic evaluation of complement pathway activation by analyzing the central complement protein C3.

Complement component C3, is the central complement proteins which converge all the three pathways of complement system. Our vitreous proteomic analysis (manuscript under submission) identified several peptides for complement pathway proteins in the vitreous samples of DR patients (see the supplementary table 5). To study further, western blotting was done for C3 in vitreous samples obtained from PDR and no-DM controls, level of total C3 and its activated fragments were evaluated (Fig1a). A significant increase in total C3 was observed in vitreous PDR (1.9±0.25, $p^{*}<0.004$) (n=38) compared to no-DM controls (0.98±0.18) (n=38) (Fig1b). The activated C3 fragments such as intact C3 consists of 195kDa in size, 110 kDa size C3bα', C3α (120kDa),C3β of 75kDa, α-1 fragment of iC3b (65kDa) and C3c α' fragment-2, (43kDa) were observed in PDR and no-DM controls. A uniform pattern of C3 fragmentation was not observed in vitreous samples, therefore fragments of C3 in each of the samples were analysed and compared. A significant upregulation in C3bα'of 110kDa fragment was seen in PDR vitreous compared to controls (Fig1c). While our western blotting of C3 in PDR, NPDR serum samples identified a slight increase in total C3 compared with no-DM serum however, this increase was not statistically significant (PDR vs no-DM: 1.69±0.58, $p>0.05$, NPDR vs no-DM: 1.38±0.24,$p>0.05$ and PDR vs NPDR 1.19±0.23, $p>0.05$). Additionally, we did not identify significant changes in any of the C3 fragments in serum samples.

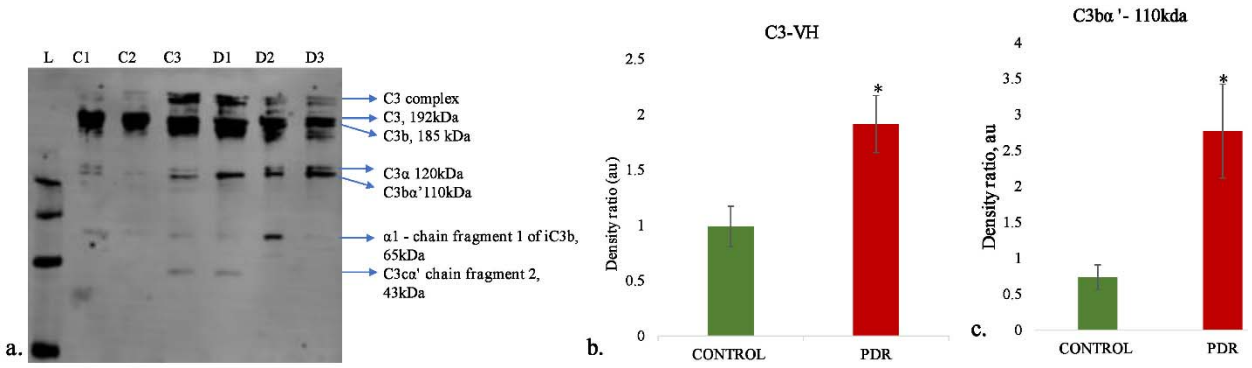

Fig1a. Representative western blot of C3 in PDR no-DM vitreous (b). Quantification of total C3 in PDR and in no-DM control vitreous by densitometry (PDR, n=38 and Control, n=38), Quantification of C3bα' (110 kDa, in PDR (n=13) vitreous compared to control vitreous (n=13) *P*<0.006*, **p<0.05,* Data represented as Mean ±SEM, C- control vitreous, D- PDR vitreous, L- protein ladder, VH-Vitreous Humor.

2. Contribution of classical pathway of complement activation in diabetic retinopathy.

The classical pathway of complement activation was evaluated in vitreous and serum samples by western blotting using classical pathway proteins such as C1q and C4b (Fig 2a and 2c). The levels of C1q and C4b proteins in the vitreous were not significantly different across PDR and controls (C1q- PDR: 1.81±0.44, controls: 1.32±0.38 *p>0.05,* C4b- control: PDR: 1.39±0.4, 1.08±0.2, *p>0.05*) (fig 2c and 2d). Likewise, no significant change of C1q level was observed in serum samples of PDR and NPDR as compared to no-DM controls (PDR: 1.81±0.44, controls: 1.32±0.38 *p>0.05*). Thus, the classical pathway of complement activation was not involved for complement activation in DR.

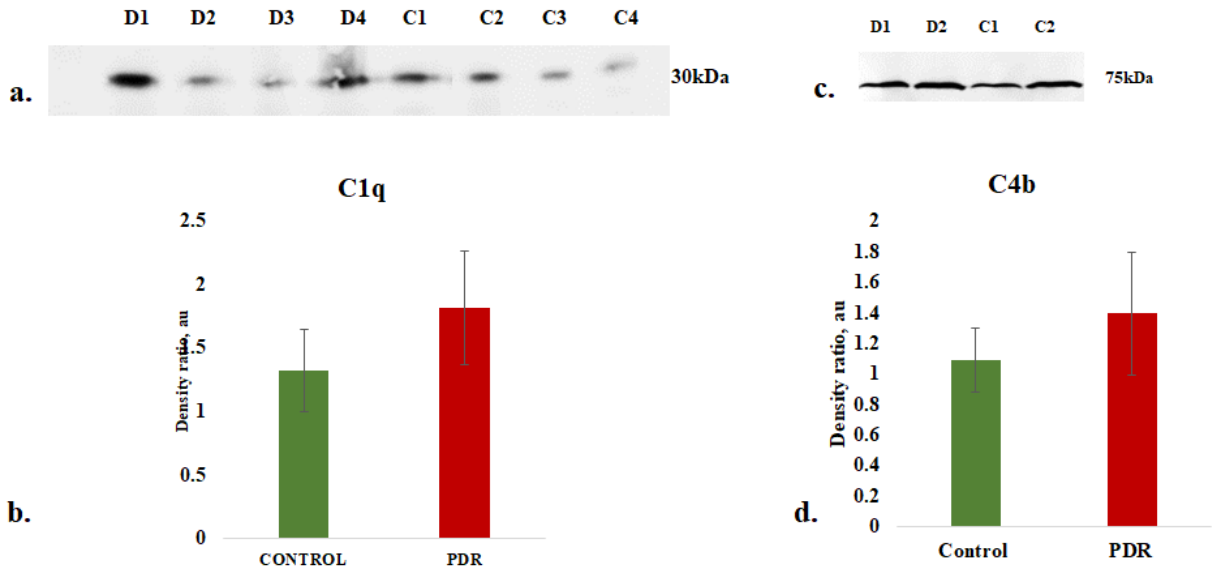

Fig 2. a). Representative western blots of C1q in PDR and No-DM controls, b). Quantification of C1q in PDR (n=17) and no-DM control (n=17) vitreous, *P>0.05*, c).Representative western blots of C1q in PDR and No-DM controls, d).Quantification of C4b in PDR (n=8), no-DM control (n=8) vitreous, *P>0.05* (not significant), Data represented as Mean ±SEM, C- control vitreous, D- PDR vitreous.

3. Contribution of alternative pathway of complement activation in diabetic retinopathy.

Western blotting of CFB in vitreous samples from PDR and controls identified a band corresponding toBb fragment of CFB of molecular weight 48-50kDa (Fig 4a). Densitometry identified a significant downregulation of Bb in the PDR vitreous compared to the controls (PDR: 0.97±0.15, Controls: 1.89±0.38, *p*<0.03*) (fig 4b), indicating more bound form of Bb of factor B in the PDR vitreous for the generation of C3 convertase to activate alternative complement pathway.

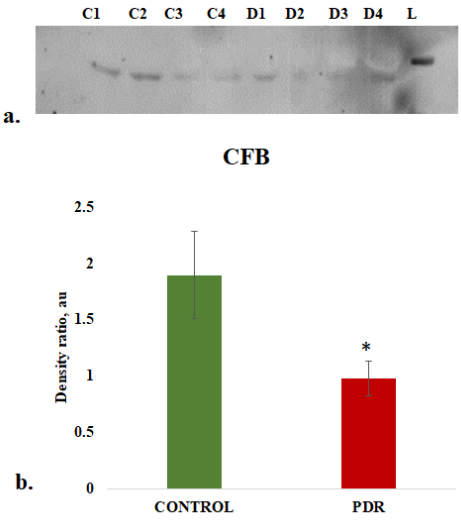

Fig 4. Representative western blot of CFB in PDR and No-DM controls and quantification of Bb in PDR (n=22), no-DM control (n=22) vitreous, *p**<*0.05*,Data represented as Mean ±SEM,D: PDR, C: controls, L- Protein ladder

4. Assessment of regulation of alternative pathway of complement by complement factor H (CFH)

CFH is an important regulator of alternative pathway of complement, western blotting of CFH identified a sharp 150kDa band in vitreous samples and it was found to be significantly upregulated in PDR compared to control vitreous (Control: 0.96±0.172, PDR: 3.68±0.66, *p***<*0.0004*) (fig 5a and 5c). In order to identify whether serum infiltration is contributing to the increased level of CFH in PDR vitreous, CFH levels were measured in serum samples of no-DM, NPDR and PDR (fig 5b) and found to be downregulated for PDR as compared to NPDR and control (PDR: no-DM: 0.78±0.12, *p>0.05*,PDR: NPDR: 0.66±0.07, *p>0.05*, NPDR: no-DM- 1.205±0.2, *p>0.05*) (fig 5d).Thus, the increased level of CFH in PDR vitreous does not to be contributed by the serum infiltration and could represent a localized change in the retina mediated by one of the retinal cell types.

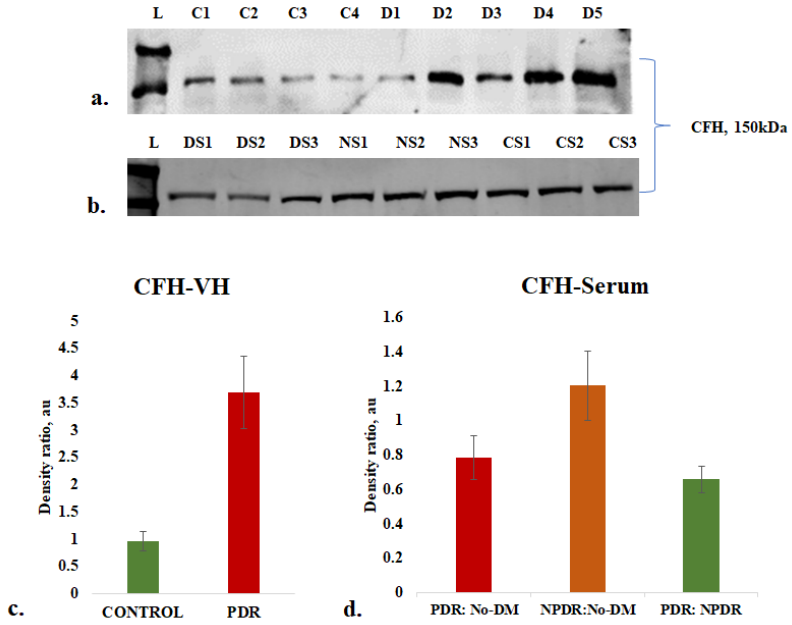

Fig 5. (a). Representative western blot of CFH in PDR and No-DM controls, (b). Representative western blots of CFH (150kDa) in PDR, NPDR and no-DM control serum. (c). Quantification of CFH in PDR (n=31), no-DM control (n=31) vitreous, $p^{**}<0.0004$. (d) Quantification of CFH (150kDa) in PDR (n=12), NPDR (n=12) and no-DM control (n=12) serum, $p>0.05$ (not significant), Data represented as Mean ±SEM, D: PDR vitreous, C: control vitreous , L-Protein ladder, DS: PDR serum, NS: NPDR and CS- Control serum, VH-Vitreous Humor.

5. Validation of complement activation and CFH upregulation by immunohistochemistry using diabetic and non-diabetic cadaveric retinal tissues.

Diabetic and non- diabetic donor retinas were collected from cadaveric donors and performed immunohistochemistry to validate complement activation and Factor H upregulation in the retina. The retinal tissues were also stained with markers of glial activation such as CD11b for activated microglia and glial fibrillary acidic protein (GFAP) for macroglial population of the retina. Level of CXCR4 were also evaluated in the retinal tissues. The IHC results clearly demonstrated an intense staining of C3 in all the retinal layers of DM retina. GFAP was found to be present in the inner retinal layers in both DM and control retinas, however, the expression of GFAP in diabetic retina was found to be slightly higher than that of control retinas (fig 6a and 6b), suggesting the onset of gliosis in DM retinas. Increased expression of C3 and CD11b were observed in DM retina, while control retina had a relatively lower expression of these proteins (Fig.7a and 7b).

Further similar to the C3, an intense staining of CFH and its co-localized expression with CD11b positive cells in the retinal layers were observed in DM retina(Fig. 8a and 8b). This suggests that upregulation of CFH in the diabetic retina could be a feedback mechanism of excess complement activation by microglial cells. Further, the IHC results identified distribution of CXCR4+ co-localizing with CD11b positive cells in DM retina, whereas in control retinas, the CXCR4 staining was negligibly low (Fig. 9), suggestive of microglial activation and its enhanced chemotaxis under the diabetic stress.

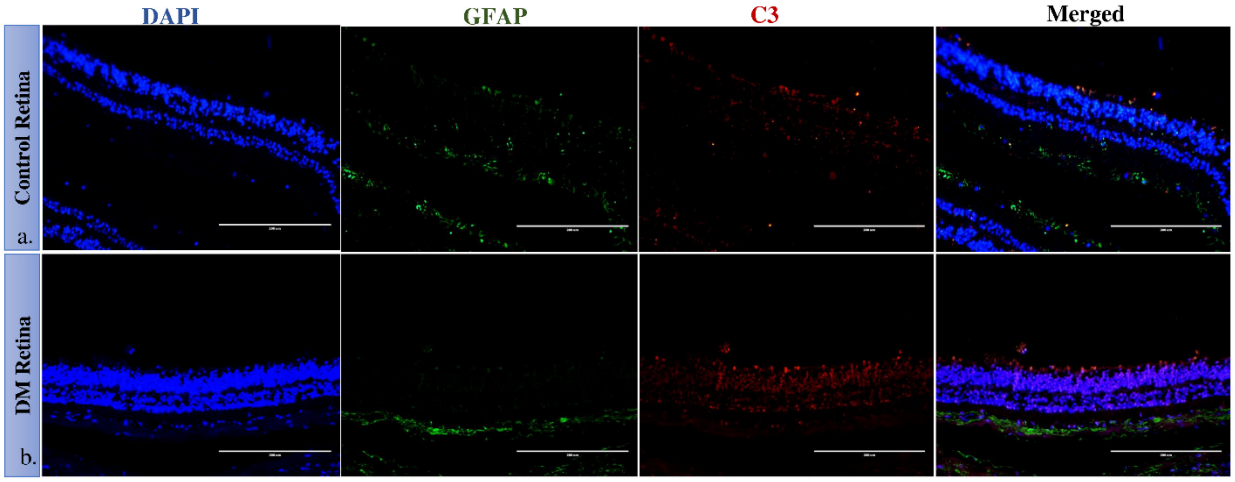

Fig 6. Representative immunofluorescence and localization of C3 and GFAP in retinal tissues obtained from control and diabetic retina, magnification 20X. GFAP was found to be expressed in the inner layers of the retina. C3 expression was observed in all retinal layers and intense in DM retina.

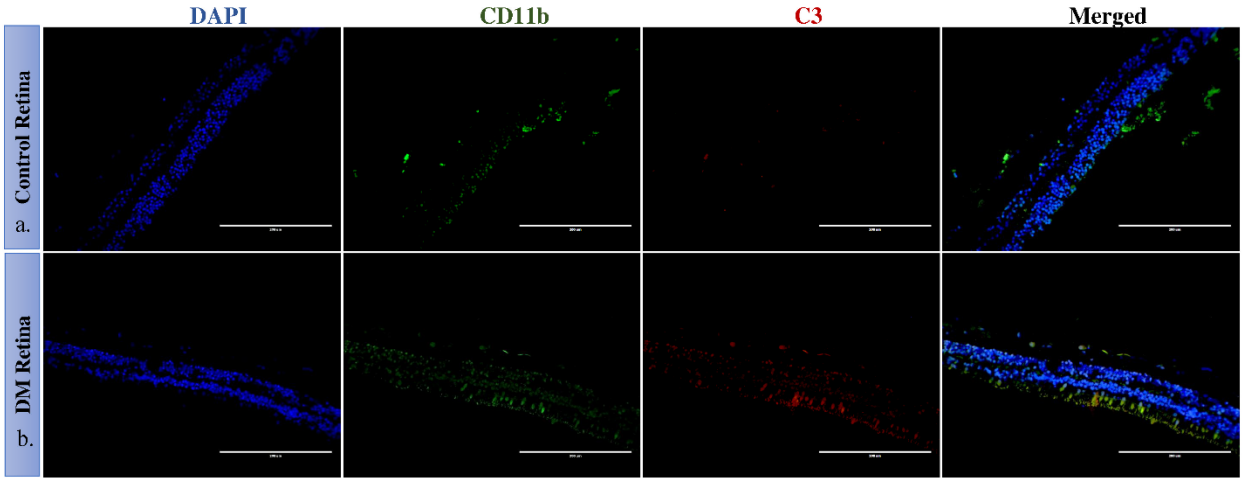

Fig 7. Representative immunofluorescence of C3 and CD11b in retinal tissues obtained from control and diabetic retina, magnification 20X. CD11b cells were found to be present in the inner nuclear layers in control and DM retina. In DM retina, an obvious increase in C3 expression and CD11b was observed.

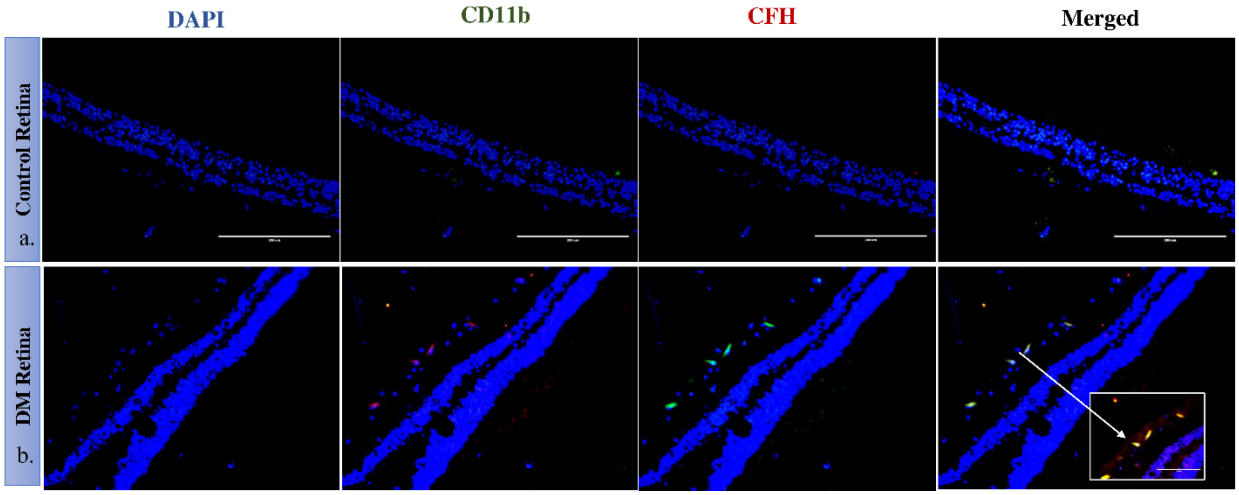

Fig 8. Representative immunofluorescence of and localization of CFH and CD11b in retinal tissues obtained from control and diabetic retina, magnification 20X. Co-localized expression of CFH and CD11b is highlighted in 40X magnification in the panel B

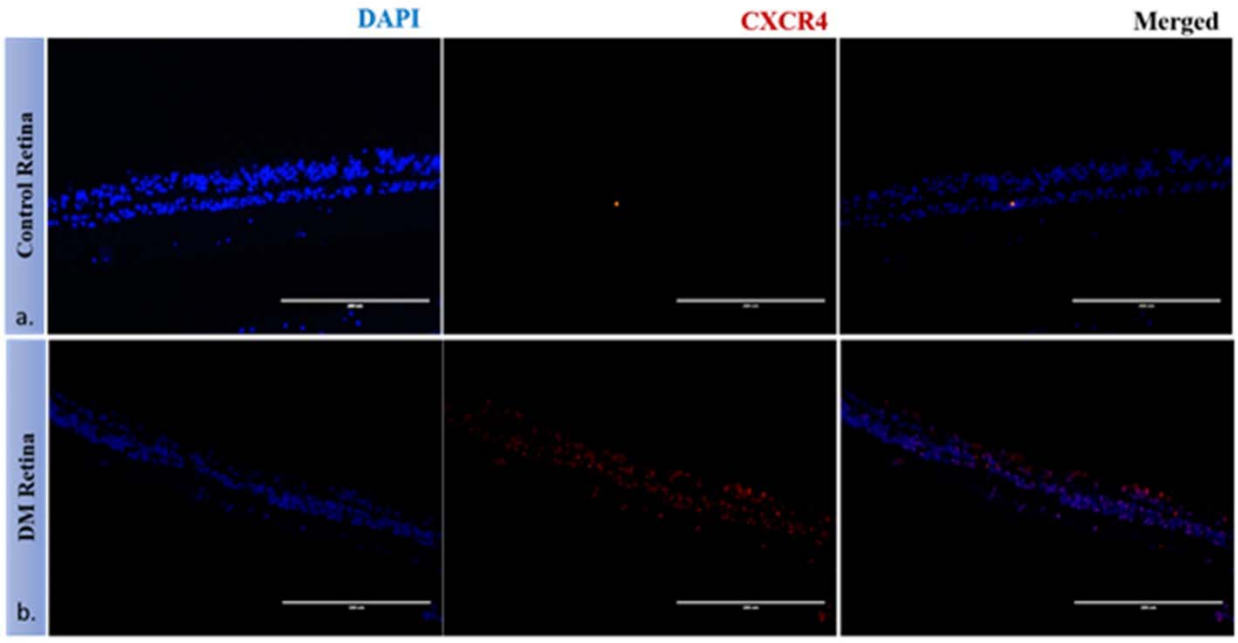

Fig9: Representative immunofluorescence of CXCR4 in retinal tissues obtained from control and diabetic retina, magnification 20X. A clear invasion of CXCR4 cells is seen

6. **Assessment of microglial activation by analysing the level of matrix metalloproteinases (MMPs)**

Microglia are the major source of gelatinolytic MMPs such as MMP9 and MMP2 in the retina. In order to evaluate the enzymatic activity of MMPs in the PDR vitreous gelatin zymography was done and compared MMP activity with vitreous samples from controls. The zymography results identified a clear gelatinolytic band in the PDR and control vitreous at 82-85kDa molecular weight, which corresponds to active MMP9. This band was found to be more pronounced in PDRs than control vitreous, indicating more gelatinolytic activity and active MMP9 in the PDR cases as compared to controls (fig 10a).

7. Analysis of inflammation and microglial activation in vitreous samples by analyzing the level of sPECAM, IL-8 and IL-10.

The level of inflammation in vitreous samples were analysed by the quantitative estimation of pro-inflammatory markers such as sPECAM, IL-8 and also the estimation of anti-inflammatory marker IL-10. The level of sPECAM and IL-8 were found to be higher in PDR vitreous as compared to the control vitreous. (sPECAM- Control (n=8): 49.54±4.76, PDR:105.45±16.69, $p^{*}<0.01$, IL-8: Control (n=8), 12.79±3.13, PDR (n=8): 29.47±14.14, $p>0.05$, non-significant). In contrast to this, the level of anti-inflammatory cytokine IL-10 was found to be significantly downregulated in PDR vitreous as compared to control (Control (n=8): 2.24±0.42, PDR (n=8): 0.57±0.09 $p^{**}<0.001$) (Fig 10 b and 10c).

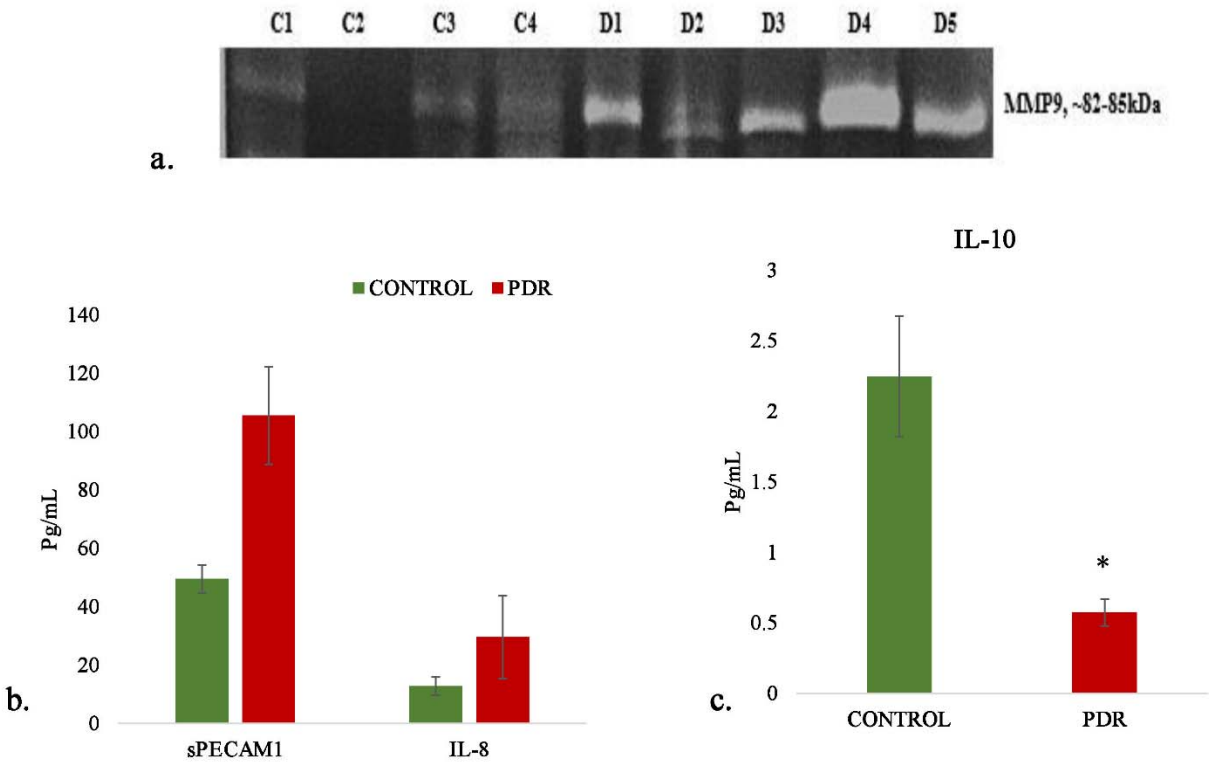

Fig 10: a. Representative Gelatin zymography of vitreous samples from PDR (n=10) as compared to controls (n=10) (C-control, D- PDR),(b). Quantitative estimation of sPECAMIL-8and (c) IL-10 in PDR vs Control vitreous by multiplex ELISA, *$p<0.05$, PDR=8 and Control=8

8. Analysis of complement and angiogenic genes by quantitative real time PCR.

Blood samples were collected and RNA was isolated from PDR, NPDR and control subjects and quantitative real time PCR was performed for the genes such as *TGF β1*, *THSB1*, *CXCR4*, *VEGF*, *C3* and *CFH*. The gene expression analysis identified the expression level of these genes as , *TGF β*- NPDR (n=20): 0.92 ± 0.22 ($p>0.05$, *N.S*), PDR (n=20) 0.73 ±0.08 ($p*<0.05$), *THSB1*- NPDR (n=20): 0.499±0.15 ($p**<0.004$), PDR (n=20): 0.495 ± 0.06 ($p**<0.008$), *VEGF*- NPDR (n=20): 1.43± 0.21 ($p>0.05$, *N.S*), PDR (n=20): 1.53 ± 0.07 ($p*<0.02$), *C3*- NPDR (n=20): 1.17 ±0.173 ($p>0.05$, *N.S*), PDR (n=20): 1.45±0.14 ($p*<0.01$), CFH – NPDR (n=20): 0.628 ± 0.13 ($p*<0.0001$), PDR (n=20), 0.45± 0.13 ($p*<0.0003$) and CXCR4- NPDR (n=20), 1.03± 0.18 ($p>0.05$, *N.S*), PDR (n=20): 1.21±0.22 ($p>0.05$, *N.S*) (Fig 11).

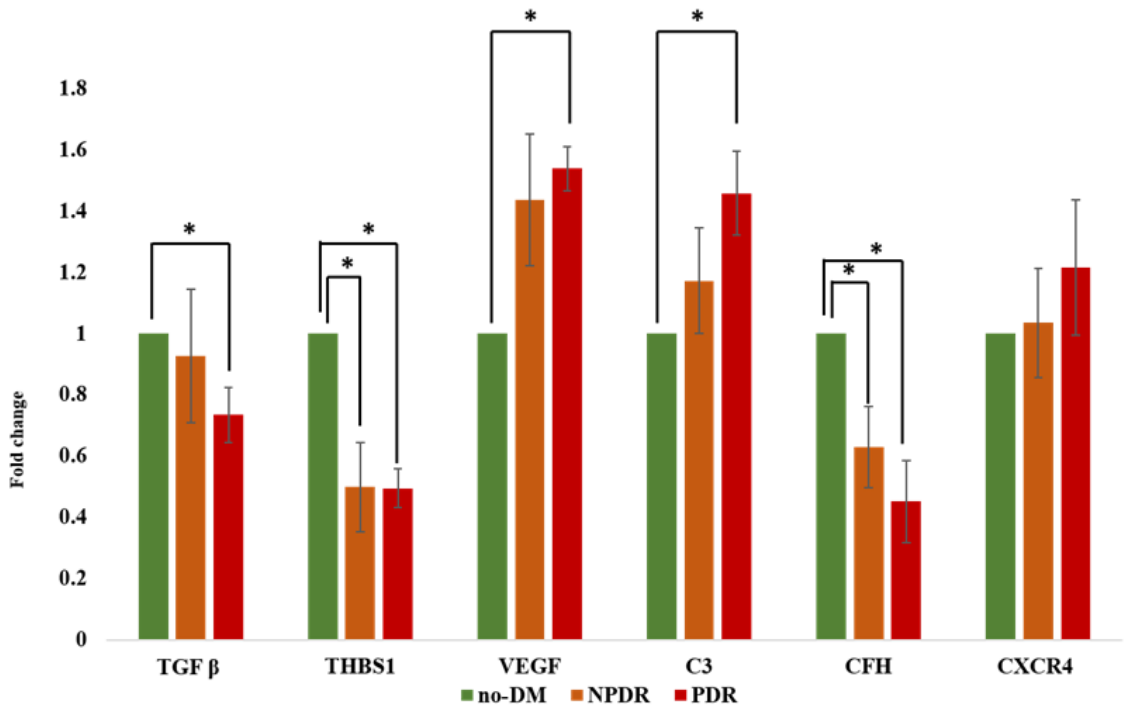

Fig 11: Quantitative real time PCR of complement and angiogenic genes in PDR (n=20), NPDR (n=20) compared to no-DM controls (n=20), *p<0.05, Date represented as Mean ± SEM

Discussion:

Diabetic retinopathy is a serious neuro-vascular complications of retina and involvement of complement pathway in DR progression is paid more attention after the identification of complement deposits in choriocapillaries of DR retina and reduced levels of complement pathway inhibitors in diabetic retina[16, 17]. A comprehensive study done by Garcia *et al*, identified a significant increase in 42kDa fragment of C3 and CFB in PDR vitreous and found it to correlate with the mRNA expression in diabetic retina [22]. Additionally, various vitreous proteome studies in DR also identified the predominant deposition of complement proteins in PDR vitreous [20, 21, 26, 27] though exactly how complements are involved in PDR pathogenesis was not explored. Thus, here for the first time, we attempted a systematic evaluation of both alternative and classical pathwayof complement activation in PDR pathogenesis by analysing proteins of these two pathways in serum as well as in vitreous humor.The major rationale behind this comparison is that, if these proteins are contributed by blood retinal barrier breakdown in advance stages of the disease. Hence, we also checked for the systemic complement activation in serum from PDR,

NPDR and no-DM subjects to make sure if complement dysregulation as seen in vitreous proteome in PDR is a localized effect and not an additive effect due to serum infiltration.

C3 is the central complement protein, where all the three complement pathways converge. The activation of complement pathway causes proteolytic fragmentation of C3 and these fragments can bind to the nearby tissues and enhance inflammatory process [28]. In our study cohort, no significant increase in total C3 or any of its activated fragments was seen among PDR and NPDR serum compared to control serum, while a significant increase in total C3 was observed in PDR vitreous. Though, there was no significant differences in number of activated C3 fragments in PDR vitreous compared to control, suggesting significant complement activation in control vitreous as well. This could also be attributed to homeostatic changes in retinal microenvironment in conditions of macular hole and retinal detachment cases that serve as controls for this experiment. However, a significant upregulation of C3bα'(110kDa) fragment was noted in the PDR vitreous. The reactive C3bα' is generated from 120kDa α-chain of C3 after the proteolytic removal of 10kDa C3a fragment and is the part of active C3b [29]. A significant increase in the level of C3bα'indicated for complement pathway activation in PDR vitreous. C1q and C4b are the proteins specific for classical pathway of complement activation and both these proteins identified an insignificant increase of C1q in PDR and NPDR serum and C1q and C4b in PDR vitreous. This suggested that classical complement pathway might not be playing asignificant role in PDR pathogenesis.

Further, we have looked into the alternative complement pathway by selecting CFB, a protein specific for alternative pathway that is required for the formation of C3 convertase (C3bBb) and activates the alternative pathway of complement[30]. Fragment Bb of factor B was found to be significantly downregulated in PDR vitreous which could be due to the formation of more C3bBb in PDR vitreous. Next, we tested the levels of CFH, one of the major regulators of alternative complement pathway. CFH regulate alternative complement pathway in multiple steps such as competing with FB for C3b binding, also act as a co-factor for factor I to degrade C3b to C3bi [31]. We have expected a low level of CFH in PDR vitreous after observing the downregulation of free Bb in the PDR vitreous. But surprisingly, we found a significant upregulation of *CFH* gene expression in PDR vitreous. Several studies have shown that one of the binding sites of CFH for C3b is located in the C3bα' region[32], and thus quite possibly the significant upregulation of CFH could be a feed- back mechanism for maintaining the level of C3bα' in the PDR vitreous. Further the serum CFH levels on the other hand, showed a down-regulation of this protein in PDR cases compared to NPDR and control, suggesting thereby that the upregulation of CFH as seen in vitreous is a localized phenomenon.

Further immunohistochemistry experiments confirmed the CFH and C3 upregulation as seen in PDR vitreous, in the retinal tissues collected from diabetic donor eyes as compared to the control non

diabeticdonor'sretina. Similar to the vitreous data, we found a concurrent deposition of C3 and CFH in the diabetic retina.To test further if this CFH and C3 overexpression as seen in retinal tissues from diabetic donor by microglial cells as seen in AMD and ROP conditions, we performed co-localization of CFH and C3 proteins with activated microglial specific marker CD11b. A significant amount of CFH expression was seen to localize with microglial cell that were present in the inner nuclear layers. We also noticed significant gliosis in the diabetic tissues as evident from the upregulation of GFAP protein in diabetic retina. However, it was microglial cells and not the macroglia that seemed to be the major source of complement proteins in diabetic retina. Further confirmation of the microglial activation as seen in PDR cases, was evident from the significant down regulation of IL-10 in the PDR vitreous samples while IL-8 was upregulated (though not statistically significant). IL-8 is a proinflammatory and IL-10 is an anti-inflammatory cytokine secreted mainly by M1 and M2 microglia [33]. The down regulation of IL-10 and upregulation of IL-8 further confirmed activation of proinflammatory M1 phenotypes in PDR vitreous. Microglial once activated are known to secrete the matrix metalloproteinases and this too was confirmed using. zymography that showed significantly increased level of MMP9 secretion in PDR vitreous[34]. This further validated microglial activation in PDR vitreous. Alongside an increase in proinflammatory markers such as Il-8 and MMP9, a significant upregulation of sPECAM in the PDR vitreous, also indicated excessive inflammation in the PDR vitreous. This also suggests for an inflammatory environment in the PDR retina that could be a driving force for microglial activation. Microglial cells being the resident cells gets activated and move up from deep RGC layers towards photoreceptors. Microglial activation was further evaluated in the diabetic retina using CXCR4, which is a chemokine receptorand known to be involved in astroglial activation and microglial signaling. Our IHC results identified an increased expression of these receptors in the inner retinal layers, suggesting microglial activation and its migration in diabetic retina.

Next, we correlated the increase in microglial cells with the mRNA expression of C3, CFH, THSB1, VEGF, TGFβ and CXCR4 in PBMNC isolated from PDR, NPDR and no-DM cases. Our gene expression data showed upregulation of C3 in PDR and NPDR cases and a concurrent downregulation of CFH. This was found to be consistent with the serum level C3 and CFH expression, where we found insignificant upregulation of C3 and downregulation of CFH in PDR and NPDR cases. Further the level of *THSB1*, a potent anti-angiogenic gene and also the activator of *TGFβ1*[35] were evaluated and we found a significant downregulation of THSB1 in PDR and NPDR cases, simultaneously pro-inflammatory gene *TGF β*1 also found to be downregulated. Our study also confirmed that THSB1 is a negative fluid phase regulator of alternative pathway of complement activation[36].and therefore the downregulation of THSB1 further correlated with the complement pathway activation in DR.

In conclusion, our study provided a systematic analysis of classical and alternative complement pathway activation in PDR pathogenesis. We for the first time showed a significant upregulation of 110kDa C3bα'and concurrent increase of CFH in PDR vitreous, and that this upregulation of complement cascade was localized to retina and not contributed by the blood retinal barrier breakdown which is common sight in advance PDR cases. Further, a correlation of increased Complement factor H expression and downregulation of Complement factor B with genetic changes in these genes could underscore the contribution of the alternate complement pathway in DR pathogenesis Lastly, our study suggested that activated microglia are the main source of alternative pathway of complement activation in PDR. In future, targeting microglial mediated complement activation could pave way for an effective therapeutic management of DR by the reducing underlying inflammation and abnormal angiogenesis.

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CHAPTER 4

Understanding the Pathogenesis of Neurodegeneration in Diabetic Retinopathy (DR)

Shahna Shahulhameed[1], Subhabrata Chakrabarti[1], Jay K. Chhablani[2] and **Inderjeet Kaur[1,*]**

[1] *Brien Holden Eye Research Centre, LV Prasad Eye Institute Hyderabad, India*

[2] *Smt. Kannuri Santhamma Centre for Vitreo Retinal diseases, LV Prasad Eye Institute Hyderabad, India*

Abstract: Diabetic Retinopathy (DR) is the leading cause of irreversible global vision loss. It affects the entire neurovascular unit of the retina, along with gradual neuro-degeneration and neuroinflammation. DR has primarily been considered a microvasculature complication of diabetes, a well-known metabolic disorder. However, recent studies have reported the presence of neurodegenerative changes in the retina of DR patients prior to clinical manifestations. In this review, we have compiled clinical, histopathological, biochemical and genetic evidences that suggest a role of neurodegeneration in DR progression and pathogenesis. These studies indicated neural changes in the retina that have lead to microvascular alterations. Furthermore, the mechanisms underlying the neural changes can help identify drug targets for effective management of the disease, which in turn will help reduce the burden of visual impairments caused by DR.

Keywords: Degenerative disease, Diabetes, Inflammation, Neurons, Retina.

NEURODEGENERATION

Neurodegeneration can be defined as the degenerative changes (both structural and functional) in neurons that lead to progressive loss of neuronal function whilst promoting their death through apoptosis or other mechanisms like autophagy and necrosis [1]. Neuronal damages are irreversible and show detrimental effects on the human body. Neurodegenerative changes include an increased rate of cell death and proliferation of macroglial population (known as reactive gliosis and recognized by the increased expression of glial fibrillary acidic protein (GFAP) and microglial activation [2, 3]. Neurodegeneration has been implicated in the pathogenesis of central nervous system diseases like Parkinson's, Alzheimer's

* **Corresponding author Inderjeet Kaur:** Scientist, Brien Holden Eye Research Centre, KAR Campus, L.V. Prasad Eye Institute, Road No. 2, Banjara Hills, Hyderabad- 500034, India; Tel: +91-40-30612508; E-mails: inderjeet@lvpei.org; ikaurs@gmail.com

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and Huntington's disease [4, 5]. The eye is also vulnerable to neurodegenerative changes, as found in the pathogenesis of vision threatening diseases such as glaucoma, retinitis pigmentosa and age related macular degeneration [6 - 8]. However, neuronal damage has not been documented in diabetic retinopathy (DR) [9].

DIABETIC RETINOPATHY

Diabetes is one of the major causes of socio-economic burdens in the developing world. It is comprised of a group of metabolic diseases that affect multiple organs. The retina can be severely affected by the diabetic changes, leading to catastrophic loss of vision (termed Diabetic Retinopathy). DR manifests as damaged vascular as well as neuronal networks causing vitreous hemorrhage, microaneurysms, lipid exudates, cotton wool spots, macular edema and abnormal neovascularization [10].

A recent population-based study (2012) estimated the overall global prevalence of DR to be 34.6%. This number is increasing exponentially [11]. The duration of diabetes and glucose levels are major risk factors that determine the prevalence of DR [12]. The Wisconsin Epidemiologic Study of DR observed that 80% of patients with diabetes developed retinopathy within 15 years of its onset. The study also noted that the prevalence of advanced stage of DR was 67% in people with a longer duration of diabetes (>35 years), while it occurred in only 1.2% of people with shorter disease duration (<10 years) [13]. According to a WHO report, the occurrence of diabetes in India reached 31.1 million people. The WHO report also claims that this number will double each year [14]. The 2007 Andhra Pradesh Eye Disease Study (APEDS) in Southern India suggested an estimate of around 2.77 million people with DR and nearly 0.07 million people with severe DR [15].

Complications of Diabetic Retinopathy

DR is classified in two categories: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). These categories are based on the detectable changes in the retinal microvasculature. NPDR represents the earliest stage of DR. In patients with poor diabetic control, NPDR slowly progresses to the severe proliferative PDR stage. In mild NPDR, patients show one microaneurysm or dot blot hemorrhage in fundus quadrants. In severe NPDR, hemorrhage, venous bleeding and abnormalities in intra-retinal microvasculature are commonly observed [16].

In PDR, the ischemic retina releases growth factors like the vascular endothelial growth factor (VEGF) that induce the proliferation of abnormal vessels in the

retina [17]. The newly formed vessels known as neovessels are fragile and tend to bleed at any time, causing vitreous hemorrhage. These neovessels create tractions in the retina as well as the detachment of the retina from the choroid. Neovascular glaucoma is also a vision-threatening complication of PDR, which is caused by the formation of new vessels, which can block the normal aqueous humor flow in the anterior chamber of the eye [18]. Another major factor of vision loss in DR is diabetic macular edema (DME), which is characterized by the accumulation of leaked fluids from the retinal capillaries in the macula, area of central vision [19].

Therapeutic approaches for DR depend upon the severity of the complications in patients. The most widely used approach during early disease stage is retinal laser photocoagulation [20]. This treatment seals leaked vessels, which redirects the blood supply and reduces overall oxidative damage.

Lately, anti-VEGF therapy has become a preferred strategy in the management of DME. VEGF is essential for many physiological functions in the retina. It plays a major role in vasculogenesis and neurogenesis. It is also an important neuroprotective agent in the retina. However, a side effect of anti-VEGF therapy is neurodegeneration [21].

NEURODEGENERATION IN DIABETIC RETINOPATHY

The retina is a highly metabolically active tissue in the eye. It is a well-organized laminated structure of multiple cell types (Fig. **1**). The vertebrate retina contains two synaptic layers intercalated between three nuclear layers: outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL). The ONL contains rod and cone photoreceptor nuclei, whereas the INL is composed of horizontal, bipolar and amacrine cell nuclei. The GCL contains the nuclei of retinal ganglion cells (RGC) and displaced amacrine cells [22]. The retinal nerve fiber layer (RNFL) forms the innermost layer and is composed of axons of the ganglion cells. The nourishment to neuronal cells in the retina is provided through the blood vessels which are organized in a specific pattern in the retina. The communication between the blood vessels and neuronal cells in the retina is maintained by two types of glial cells: microglia and macroglia [23]. This entire cellular network in the retina gets compromised in DR. A majority of the studies on DR is primarily focused on the changes in the microvasculature of the retina. However, the neuronal damage in DR pathogenesis has largely been overlooked. Various preclinical and clinical studies have also provided plenty of evidence for neuronal damage in diabetic eyes, which is discussed in the next section.

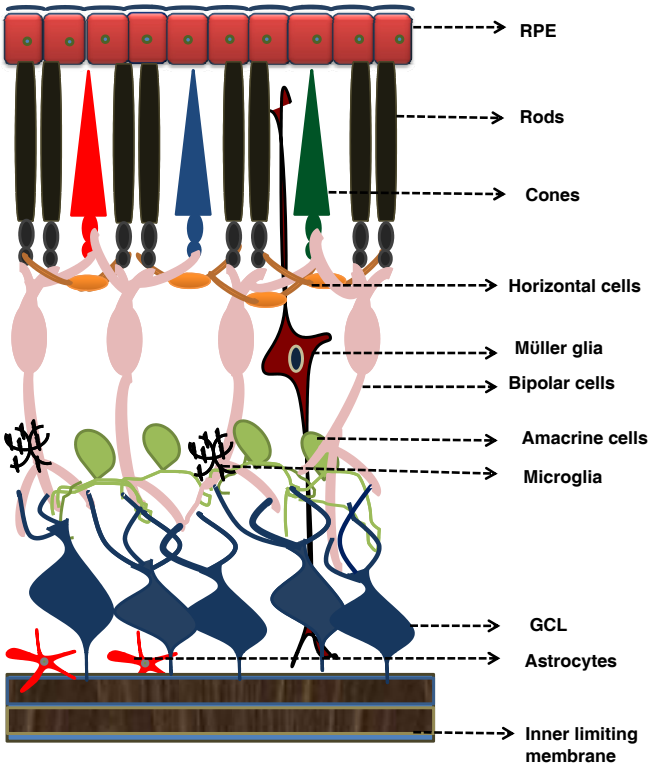

Fig. (1). Schematic representation of the retina: The vertebrate retina contains ten layers with 5 different types of neurons such as photoreceptors, horizontal cells, bipolar cells, amacrine cells and ganglion cell. The RPE provides nutrients to the photoreceptors. The glial cells are positioned critically between the cells of the vasculature and neurons, which maintain retinal homeostasis. The ganglion cells (GCs) receive the information from photoreceptors through bipolar and amacrine cells.

Clinical Evidences of Neurodegeneration in Diabetic Retinopathy

Several clinical studies have been conducted to understand the involvement of neuronal loss in DR pathogenesis. Evidences for RGC loss and reduced color and contrast sensitivity in diabetic eyes prior to the onset of retinopathy points towards neuronal damage in the retina [24 - 27]. Retinal imaging using spectral-domain optical coherence tomography (SD-OCT) has depicted the loss of RGCs in diabetic patients who have not yet developed any clinical symptoms of DR. Progressive loss of RGCs was also noted in moderate and severe DR [28]. A reduction in RNFL thickness was also reported in patients with diabetes and no DR [29]. A strong correlation between thinning of the ganglion cell-inner plexiform layer (GCIPL) and poor visual acuity was also seen in diabetic maculopathy patients. In these patients, the region in the retina responsible for central vision (called macula) is damaged due to the accumulation of fluid from

the leaky vessels in the retina [30]. Likewise, the assessment of the GCIPL and RNFL in eyes of patients with different stages of DR identified a generalized thinning of the GCL and RNFL in all stages of DR as well as in the diabetic patients with no DR [31]. The thinning of GCL and RNFL seen in patients with minimal DR and vascular damage could be due to apoptosis of RGCs and further suggests that chronic neuronal damage precedes vascular damage in DR [32].

Animal Models of Neuronal Damage in Diabetic Retinopathy

Several studies employed animal models of DR to examine the early neuronal damage. Significant neural apoptosis within one month of the onset of diabetes and reduction of RGCs after long-term exposure to diabetes were noted in a rat model [33]. In $Ins2^{Akita}$ mice, the thinning of the INL was observed due to the loss of bipolar, amacrine and horizontal cell bodies. Reduction in IPL thickness [34] and the loss of cholinergic and dopaminergic signaling in the retina were also associated with severe vision loss [35, 36]. The reduction of tyrosine hydroxylase immunoreactive amacrine cells, responsible for dopaminergic neurotransmission were seen in the immunohistochemical analysis in streptozotocin-induced male Sprague-Dawley rats [37].

The functional and pathological alterations of the neurosensory retina of the db/db (BKS/DB−/−) diabetic mice models were studied by pattern electroretinography (ERG), OCT, fundus fluorescein angiography (FFA) and immunohistochemistry using neuroinflammatory markers expressed by activated microglia. Significant alterations were seen in the ERG of diabetic compared to the non-diabetic mice. OCT revealed the thinning of the dorsal and temporal retina in the diabetic mice. These mice displayed significantly higher apoptosis of the RGCs along with the presence of neuroinflammatory markers. However, no vascular damages were observed on FFA, confirming that neural changes precede the vascular damage, and neuroinflammation is one of the earlier events in DR pathology [38].

Evidence from Pathophysiology of Affected Tissue

The immunohistopathology of retinal tissues from diabetic individuals revealed a higher glial reactivity, as suggested by the higher expression of GFAP compared to the control and noticeable growth of Müller glia into the occluded retinal vessels [39]. Müller cells are the major glia in the retina. They span the entire retina with long extensions. These cells play a major role in maintaining retinal homeostasis mainly by regulating the levels of neurotransmitters like glutamate. The effect of diabetes on Müller cells was studied by staining the major proteins in the Müller glia, such as glutamine synthetase (GS), GFAP and anti-apoptotic protein Bcl2 in human post-mortem retinal eyes of diabetics and of controls. This study could not find major changes in the expression of GS and Bcl2 in the Müller

glia, however, significant upregulation of GFAP was observed throughout the Müller glia extensions [40]. Further, a significant drop in RGC count along with the presence of abnormal dendritic structures in the retina were noticed in rat models [41].

MECHANISMS OF NEURONAL DAMAGE IN DIABETIC RETINA

The structural and functional alterations of the neurons are the major manifestations of any neurodegenerative condition. A study by Barber *et al.,* used a rat model of DR and evaluated relative retinal layers thickness of diabetic rats *versus* the age-matched control. Significant reduction of the thickness of the INL and IPL was observed in the diabetic rats compared to controls. Additionally, a 10% reduction in the ganglion cell density in the retina was seen in diabetic rats. The diabetic retina displayed increased terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positivity as compared to controls at all time periods. Further, minimal co-localization of TUNEL positive cells and Von Willebrand factor (vWF) stained endothelial cells indicated that endothelial cells were not apoptotic in the early stages of diabetes. Most of the TUNEL positive cells were seen in the GCL. Similar data were obtained with human tissue samples [33]. Martin *et al.,* also used a similar approach in diabetic mice and found 20-25% reduction in ganglion cell count after 14 weeks of diabetes. A higher number of TUNEL positive cells along with the active caspase-3, which plays a central role in apoptotic DNA fragmentation was seen in diabetic retina, further emphasized that neuronal death in DR is mediated by apoptosis [42].

The presence of apoptotic molecules in the diabetic retina provided the evidence for apoptosis to be one of the major mechanisms of neuronal damage under the hyperglycemic state. But the trigger for apoptosis has not been well established. Several studies attempted to identify the major apoptotic molecules in the diabetic retina by IHC using various known markers of apoptosis. Upon comparing the retinas of diabetic mice *versus* non-diabetic controls, a weak GFAP, Bcl2 and extracellular-signal-regulated *kinases 1/2*(ERK1/2) expression was noted in the controls, whereas a higher expression of these markers was seen in the RNFL and GCL along with the accumulation of apoptotic molecules like caspase-3, Fas, Fas L and Bax in the diabetic retinas. The strong expression of ERK1/2 in glial cell nuclei in diabetic retina indicated the protection of glial cells in diabetic induced damage. The presence of Fas in the glial cells suggested the glial induced apoptosis of ganglion cells [43].

In another study, the key molecules involved in the apoptosis and survival of the neuroretina were studied using human retinal diabetic and non-diabetic tissue samples. This study also identified FasL, a pro-apoptotic molecule in the

neuroretina of the diabetic subjects. FasL is also known to induce the formation of death-inducing signaling complex (DISC) through the death receptor pathway and by recruiting caspase-8. Additionally, molecules such as Bim and Caspase-3 that are involved in the apoptosis *via* the mitochondrial pathways had higher expression in the diabetic neuroretina. This study clearly proved the involvement of death receptor pathway in neural apoptosis in DR [44].

Electron microscopy studies of diabetic rat retinas revealed the loss of photoreceptors in the ONL after 24 weeks of diabetes, but the degenerative changes such as myelinated and multi-vesicular mitochondrial features appeared within 1 week of diabetes [45]. Somatostatin, a neuromodulator as well as an antiangiogenic factor produced by neural cells of the retina, was downregulated in diabetic retinas [46]. This suggested that reduction of somatostatin could be an early event in DR that damaged the neural retina [47].

Piano *et al.,* demonstrated a role of autophagy in DR by examining the progression of retinal dysfunction in streptozotocin-induced diabetic C57BL/6J mice at an interval of 4, 8 and 12 weeks. There was a significant reduction in the amplitudes of scotopic “a” and “b” waves due to rod cell damage in diabetic mice compared to the controls. Synaptic degeneration was seen mainly after 8 and 12 weeks of diabetes and a significant reduction in deep plexus vessel complexity was observed [48].

Inflammation and Neuronal Damage

DR being a metabolic disorder has also been considered an inflammatory disorder. Support for this hypothesis comes from previous studies demonstrating significant correlation with the symptoms of inflammation such as hemorrhage, edema, leukocyte adhesion. Moreover, inflammation plays a major role in the progression of DR [49]. The analyses of vitreous samples in various studies identified proinflammatory and inflammatory cytokines such as *Tumor necrosis factor* alpha (TNFα), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Monocyte chemoattractant protein-1(MCP-1) *etc.* [50 - 52].

Another important component of inflammation is the complement pathway. Retinal microglial cells, RPE and the complement system comprise the retinal innate immune system. Excessive complement activation has been implicated in the pathogenesis of various ocular conditions, such as corneal neovascularization, age-related macular degeneration and other retinopathies [53]. Accumulation of complement components was seen in the choriocapillaris of diabetic patients. The inflammatory reactions in the retina further activate other glial cells in the retina and damage the neurosensory retina. Inflammation and associated gliosis are involved in the pathogenesis of neurodegenerative, diseases like Parkinson’s and

Alzheimer's [54]. Thus, inflammation, glial cell activation and functional alteration of the retina neurons, especially thinning of the RNFL by ganglion cell loss point to a chronic neurodegeneration in DR.

Glial Activation, A Cellular Mechanism of Neuronal Damage

The macroglial and microglial populations in the retina are involved in retinal homeostasis. Several studies have shown that gliosis and increased expression of GFAP are indicators of neuronal damage [44, 55]. The inflammatory environment and the presence of markers of glial activation indicate a role of glial cells in neuroinflammation and vascular damage in DR. Under ischemic stress or altered microenvironment, both the macroglia and microglia play independent roles in maintaining retinal homeostasis and causing damage.

Astrocytes and Müller glia are the major macroglial population in the retina. Unlike other cells types, Müller glia span the retina with its long stem like extensions, *i.e*. they contact all retinal neuronal cell types. The Müller cells contain neurotransmitter receptors, and their interaction with neurons plays a crucial role in retinal development. Calcium plays a significant role in bidirectional signaling between the neuroglial cells. In response to various neurotransmitters, Müller cells show highest calcium transients (rise of calcium from intracellular spaces) in the developing retina. The calcium transients was reduced upon neuronal development due to changes in neurotransmitter release, indicating the role of neuroglial crosstalk during development and homeostasis of the retina [56]. Furthermore, there is an increase in intracellular calcium in mechanically stimulated astrocytes with the simultaneous release of glutamate [57].

Gene expression analysis of Müller glia in streptozotocin-induced rats revealed an increase in the expression of the major proteins associated with gliosis, such as GFAP and Ceruloplasmin [58]. Inflammation can also alter the function of Müller glia by changing its function of neuroprotection. High glucose stress to Müller glia caused glucose toxicity by inducing the production of IL-1β and IL-6. IL-1β further caused apoptosis of the Müller glia by caspase-1 dependent mechanism [59]. The Müller glial cells in the retina also play a major role in maintaining glutamate levels [60]. Glutamate is a major excitatory neurotransmitter and is released from the presynaptic neuronal terminal. The released glutamate is taken up by the glutamate transporter in the Müller glia, where it is converted to glutamine and transported to the retinal neurons. However, if not cleared from the extracellular space, glutamine acts as a neurotoxin [61]. Various animal model and patient studies have revealed elevated glutamate levels in diabetic eyes [62]. The elevated levels of glutamate have deleterious effects on the RGCs. Higher

levels of glutamate were also found in vitreous of patients with DR [63].

Glial reactivity and elevation of glutamate levels have been shown to cause neural apoptosis. Lieth *et al.,* used streptozotocin-induced diabetic rats to study the glial reactivity in the early course of diabetes. A remarkable increase in GFAP was noted after 3 months of diabetes in rats compared to their age matched control by two-site ELISA, indicating the macroglial activation in diabetic retina [63].

The ability of the glial cells to metabolize glutamate is dependent on the receptors which are assigned for glutamate uptake. L-glutamate/L-aspartate transporter (GLAST) is a major glutamate transporter present in the macroglial population of the retina. Its major role is to remove the glutamate from the extracellular space and thus, to protect the neurons, especially RGCs [64]. The activity of GLAST in Müller glia under hyperglycemic condition was analyzed by electrophysiological methods. A significant decrease in GLAST activity was found in Müller glia isolated from diabetic rats, indicating hyperglycemia-induced oxidative stress affected the ability of glutamate uptake in Müller glia [61].

Glutamine synthetase, an important enzyme specifically present in Müller cells is involved in the conversion of glutamate to glutamine. In the streptozotocin-induced diabetic rat model, reduced activity of this enzyme was noted in the Müller glia [65]. Thus, the altered receptor function, gliosis, and reduction in the enzyme activity cause glutamate excitotoxicity and neuronal damage.

Microglia

Microglia represents the resident immune cells of the central nervous system (CNS), which are distributed throughout the inner retinal layers. The resting microglia maintains a ramified shape, but in the case of any homeostatic disturbance in the retina like diabetes or ischemia, these cells sense the signals and attain an amoeboid shape (activated microglia). Microglial activation is an invariable factor of neuronal damage and chronic neurodegeneration, indicating its detrimental role in DR [66]. Upon activation, these cells secreted various cytokines such as such as TNF-α and IL-1β [67]. Inflammatory conditions and hyperglycemia activate microglia and stimulate inflammation, and cause vascular breakdown leading to glial dysfunction and neuronal death [68].

The comparison of microglial morphology in patients with pre-proliferative and proliferative retinopathies compared with normal subjects using a specific cluster of differentiation (CD) markers such as CD68, CD45, and human leukocyte antigen-D related (HLA-DR) antigens revealed markedly increased and hypertrophic microglia during disease. In exudative retinopathy cases, increased cell number and labeling intensity of microglia were reported whereas, in pre-

proliferative DR patients, the microglia became hypertrophic and clustered around the peripheral region of cotton wool spots and the dilated venules. Likewise, in proliferative retinopathy, the microglial clusters were observed around the new vessels in the nerve fiber layer of the retina. The new vessels were heavily surrounded by labeled microglial cells in the region of retinal vascularization breaking through the internal limiting membrane and further grown into the vitreous cavity [69].

Apart from microglial activation, microglial trafficking is another feature in diabetic eyes. Omri *et al.,* compared the distribution pattern of Iba1 positive cells in diabetic rat *versus* control. They reported numerous positive cells in the retina and the subretinal space. Interestingly, RPE breakdown was observed in diabetic rats with the formation of an intracellular pore [70]. Various factors and mechanisms can activate microglia during the early phase of DR. The accumulation of advanced glycation end (AGE) products affects multiple organs. A study by Ibrahim *et al.,* correlated the AGE product such as amygdala glycated protein and microglial activation in diabetic eyes. The deposition of AGE was associated with increased microglial activation and TNFα level in diabetic rat retinas. The activation of microglia by AGE was mediated by phosphorylation of Mitogen-activated protein kinases (MAPK) [71].

The cytokine released from the microglial cells further caused a neuronal damage. A study conducted by Cardona *et al.,* tried to understand the microglial activation and cytokine release and its association with the neuronal damage in DR. A significant reduction of Fractalkine in diabetic mice retina and subsequent increase of microglial activation was observed. Fractalkine is a neuronal membrane molecule and controls microglial activation by the CX3C chemokine receptor 1 (CX3CR1) present on microglia. Fractalkine also acts as an inhibitory signal on microglia, whereby its reduced expression leads to the production of neurotoxic cytokine IL-1β that further induces the RGC death [72].

Role of Oxidative Stress in Neuroinflammation and Degeneration

Ischemia due to hyperglycemia is one of the major factors involved in DR. Ischemia causes oxidative stress and leads to the generation of reactive oxygen species (ROS), which cause mitochondrial dysfunction by superoxide production [73, 74]. The ROS induces DNA damage, membrane permeability and autophagy [75]. The RGCs contain numerous mitochondria because of its higher energy requirement, hence are susceptible to the oxidative stress and autophagy [76]. The oxidative stress also causes activation of various inflammatory pathways like polyol pathway, PKC pathway, AGE pathways, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation [77].

Another important feature of hypoxic stress is the production of VEGF. Müller cells are the major source of VEGF in the retina. Conditional ablation of VEGF in mice led to reduced inflammation, leukocytosis and decreased vascular leakage [78]. These observations indicated the dual role of Müller glia in vascular proliferation by providing VEGF and neuronal damage by secreting inflammatory molecules (Fig. **2**).

Anti-VEGF drugs can be used as a treatment strategy. However, VEGF being a neuroprotectant, its normal levels are required for neuronal survival [21, 79].

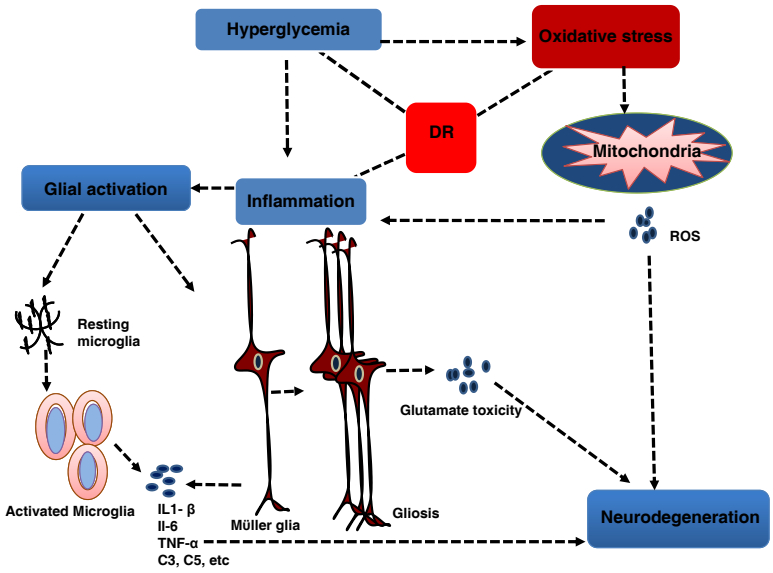

Fig. (2). Cellular Mechanism of neurodegeneration in Diabetic retinopathy: The altered microenvironment of diabetes induces multiple effects on the retina by various mechanisms. High glucose induces mitochondrial damage and ROS generation. These changes induce the activation of the glial population and lead to the secretion of various inflammatory molecules. Accumulation of glutamate due to Müller glia dysfunction, inflammatory cytokines and other various unfavorable factors in the retina affect the normal neuronal function and lead to degeneration of neurons.

Genetic Factors in Diabetic Retinopathy

Along with the altered cellular mechanisms, genetic predisposition also contributes to DR. However, only a few candidate genes have been associated with DR. The gene that codes for aldose reductase, Aldo-Keto Reductase Family 1, Member B1 (*AKR1B1),* a major regulator of the polyol pathway, is one of the important genes in diabetes-associated complications like nephropathy [80]. The polyol pathway plays a role in the apoptosis of retinal neurons in DR during high threshold hyperglycemic condition [81].

The Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) genetics study identified multiple variants in *VEGF* that were associated with severe diabetic retinopathy [82]. The genes involved in apoptosis and retinal permeability at 1 and 3 months of diabetes were studied using diabetic rats by microarray analysis. These studies revealed significant alteration in the expression of 32 genes in diabetic animals. These included Doublecortin-Like Kinase 1(*DCAMKL1*) and peptide transporter *PEPT2* [83]. *DCAMKL1* encoded protein Doublecortin is required for the regulation of microtubule polymerization and is highly expressed in the GCL [84]. *PEPT2* is involved in peptide transport across the membrane and is expressed in the Müller cells and astrocytes [85]. Another important finding was increased expression of proinflammatory genes, such as complement component 1 inhibitor (*C1-INH*), CC motif receptor 5 (*CCR5*), *CD44* and chitinase 3-like 1 (*CHI3L1*) [83].

Evidence from Proteomic Analysis of Vitreous Humor in Diabetic Retinopathy

Several vitreous proteomic studies were conducted to identify differentially expressed proteins under hyperglycemic condition. Interestingly, the majority of proteins that were dysregulated in the vitreous of PDR patients are responsible for neurodegeneration. Some of these proteins are summarized in Table **1**. Notable among these were Crystallins, Vimentin, GFAP, C3, C1, MCP-1, and PEDF [86 - 89]. Retinal expression of β and γ crystallins pertains to vascular remodeling during the development [90]. Upregulation of β and γ crystallins was also seen in neurons and cells involved in ganglion cell axon regrowth. Upregulation of α crystallin with an increase in the vimentin and GFAP in the early disease stages suggested a role of Müller glia cells in disease pathogenesis [86]. Complements are shown to be synthesized by RPE, Müller glia and microglia in the retina and in turn activate the microglia further leading to the upregulation of angiogenic proteins and downregulation of antiangiogenic proteins [91, 92].

Table 1. Proteins associated with the development of diabetic retinopathy.

Sl. No	Protein	Functions	Findings in DR	Inference	Ref.
1	Crystallin	Neuronal protectant	Increased expression of Crystallin isoform in DR, especially γ-Crystallin in RGC and β in other retinal neurons	To protect neurons from inflammation associated damage in DR	[86]

(Table 1) contd.....

Sl. No	Protein	Functions	Findings in DR	Inference	Ref.
3	Pigment epithelium derived factor (PEDF)	Maintenance of vascular permeability and angiogenesis; required for the stability of neurons	Decreased expression of PEDF along with increased expression of VEGF	Reciprocal regulation of PEDF and VEGF is essential to maintain retinal homeostasis	[89]
4	C3, C1	Plays a role in maintain innate immune system and activation of microglia in the retina	Increased expression	Activation of complement pathway	[87]
5	Cadherin-5	Maintenance of endothelial cell (EC)barrier	Reduced level in DR eye	Reduction of cadherin expression cause increased vascular leakage	[93]
6	MCP1	Regulate infiltration and migration of monocytes; activation of microglia	Increased expression after 4 weeks of diabetes	Neuronal MCP 1 induced microglial activation.	[88]
7	Synaptophysin	Synaptic vesicle protein	Down regulation of synaptophysin	Neuronal dysfunction	[94, 95]
8	Carbonic anhydrase	For the conversion of CO_2 and water in to carbonic acid	Increased expression in the vitreous of DR subjects	Promote retinal vessel leakage by activating intrinsic coagulation pathway, neurovascular edema	[87, 96]

CONCLUDING REMARKS

The current therapeutic strategies like laser photocoagulation, anti-VEGF therapy, and vitreous surgery are mainly focused on the microvasculature complications of the retina. However, neurodegeneration is one of the major causes of vision impairment in DR. Despite being considered a metabolic disorder, DR is an inflammatory disorder of the CNS. Retinal homeostasis is maintained by the glial cells. Many of the reasons for neuronal damage and vascular changes in the DR are associated with glial activation, including gliosis, altered metabolism, VEGF production as well as glutamate excitotoxicity. The major point of concern in DR is the glial activation and associated release of various cytokines, which induce the progression of the disease. The increased secretion of inflammatory cytokines in the retina and the altered metabolism cause severe damage to the neurons. These neurodegenerative changes precede the vascular damage in DR. Identification of the neural changes might help in early prediction and effective prevention of the disease. Targeting glial activation and associated changes like inflammation in the DR may give a better understanding and management

strategies for DR.

CONFLICT OF INTEREST

The author (editor) declares no conflict of interest, financial or otherwise.

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Abnormal Complement Activation and Inflammation in the Pathogenesis of Retinopathy of Prematurity

*Sonika Rathi[1], Subhadra Jalali[2], Satish Patnaik[1], Shahna Shahulhameed[1], Ganeswara R. Musada[1], Divya Balakrishnan[2], Padmaja K. Rani[2], Ramesh Kekunnaya[3], Preeti Patil Chhablani[3], Sarpras Swain[4], Lopamudra Giri[4], Subhabrata Chakrabarti[1] and Inderjeet Kaur[1]**

[1] Prof Brien Holden Eye Research Centre, Hyderabad, India, [2] Smt. Kanuri Santhamma Centre for Vitreo Retinal Diseases, Hyderabad, India, [3] Jasti V Ramanamma Children's Eye Care Centre, L V Prasad Eye Institute, Hyderabad, India, [4] Indian Institute of Technology, Hyderabad, India

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Uday Kishore, Brunel University London, United Kingdom

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***Correspondence:**
Inderjeet Kaur
inderjeet@lvpei.org, ikaurs@gmail.com

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Retinopathy of prematurity (ROP) is a neurovascular complication in preterm babies, leading to severe visual impairment, but the underlying mechanisms are yet unclear. The present study aimed at unraveling the molecular mechanisms underlying the pathogenesis of ROP. A comprehensive screening of candidate genes in preterms with ROP ($n = 189$) and no-ROP ($n = 167$) was undertaken to identify variants conferring disease susceptibility. Allele and genotype frequencies, linkage disequilibrium and haplotypes were analyzed to identify the ROP-associated variants. Variants in *CFH* ($p = 2.94 \times 10^{-7}$), *CFB* ($p = 1.71 \times 10^{-5}$), *FBLN5* ($p = 9.2 \times 10^{-4}$), *CETP* ($p = 2.99 \times 10^{-5}$), and *CXCR4* ($p = 1.32 \times 10^{-8}$) genes exhibited significant associations with ROP. Further, a quantitative assessment of 27 candidate proteins and cytokines in the vitreous and tear samples of babies with severe ROP ($n = 30$) and congenital cataract ($n = 30$) was undertaken by multiplex bead arrays and further validated by western blotting and zymography. Significant elevation and activation of MMP9 ($p = 0.038$), CFH ($p = 2.24 \times 10^{-5}$), C3 ($p = 0.05$), C4 ($p = 0.001$), IL-1ra ($p = 0.0019$), vascular endothelial growth factor (VEGF) ($p = 0.0027$), and G-CSF ($p = 0.0099$) proteins were observed in the vitreous of ROP babies suggesting an increased inflammation under hypoxic condition. Along with inflammatory markers, activated macrophage/microglia were also detected in the vitreous of ROP babies that secreted complement component C3, VEGF, IL-1ra, and MMP-9 under hypoxic stress in a cell culture model. Increased expression of the inflammatory markers like the IL-1ra ($p = 0.014$), MMP2 ($p = 0.0085$), and MMP-9 ($p = 0.03$) in the tears of babies at different stages of ROP further demonstrated their potential role in disease progression. Based on these findings, we conclude that increased complement activation in the retina/vitreous in turn activated microglia leading to increased inflammation. A quantitative assessment of inflammatory markers in tears could help in early prediction of ROP progression and facilitate effective management of the disease, thereby preventing visual impairment.

Keywords: retina, premature birth, inflammation, genetics, cytokines, abnormal angiogenesis, microglia/macrophage, alternative complement pathway

INTRODUCTION

Retinopathy of prematurity (ROP) is a complex disease of the retina with a multi-factorial etiology and an early intervention has been observed to prevent irreversible vision loss in some of these prematurely born infants (1). Its incidence in developed countries with adequate neonatological facilities (like United States) is 19.88% (2) while it is slightly higher (~30%) for middle-income developing countries (3, 4). In India, approx. two million babies are at risk of developing ROP annually (4) with an overall incidence estimated to be around 45% (5, 6). Hence, ROP is one of the major causes of visual impairment in India. Lower gestational age (GA), lower birth weight (BW), and oxygen supplementation are the primary risk factors associated with ROP (7). It is a self-limiting disease with initial symptoms of avascular retina that progresses to abnormal growth of retinal vessels causing retinal detachment (8). Hypoxia in the avascular retina is considered to be the primary cause for neovascularization in ROP that further activates various cellular pathways such as HIF1α, eNOS/iNOS, and vascular endothelial growth factor (VEGF) signaling leading to abnormal neovascularization (9, 10). However, the detailed molecular mechanisms underlying neovascularization in ROP have not been elucidated yet. So far, few functionally relevant genes (*NDP, FZD4, LRP5, CFH, VEGF, ANGPT2, EPO, BDNF,* and *CETP*) have been associated with ROP in a small fraction of cases. But, many of these variants could not be replicated across different ethnicities (11–13). Further, their roles in risk predictions and disease management are yet to be determined.

The protein profiles in the vitreous have been utilized for studying the underlying pathology of the retina due to its proximity. This has largely been accomplished by analyzing the levels of erythropoietin, VEGF, and cytokines like interleukins (IL-6, IL-7, IL-10, and IL-15), Eotaxin, FGF basic, G-CSF, GM-CSF, IP-10, and RANTES in the vitreous to identify their potential as biomarkers for ROP progression (14–16). Interestingly, interleukin-7 (IL-7), monocyte chemotactic protein-1, and macrophage inflammatory protein 1 (MIP-1α and MIP-1β) levels were also found to be significantly elevated in the cord blood serum of ROP (17). Earlier, low serum levels of IGF-1 and VEGF were reported in preterm babies with severe ROP and low GA (18, 19). Thus, the studies on protein profiling and genetic associations of ROP could explain the susceptibility of some preterm babies progressing to severe ROP.

Our study is an attempt to comprehensively elucidate the genomic basis of ROP and identify the potential biomarkers for progression to severe stages. Since, no mutations were observed in the Norrin signaling genes in ROP in our earlier study (20), we explored genes involved in angiogenesis, growth, and development of the fetal retina, trans-endothelial migration, oxidative stress, inflammation, and neurodegenerative processes, in order to understand their role in ROP pathogenesis. We observed strong associations of ROP with the variants in *CFH*, *CFB, CXCR4, FBLN5,* and *CETP* genes along with increased levels of proteins in the extracellular matrix (ECM) and complement pathways in the vitreous of these babies. We observed the presence of the activated microglia/macrophages in the retina and vitreous. We further demonstrated the activated microglial cells under hypoxia expressed complement C3, VEGF, and IL-1β, thereby resulting in abnormal blood vessel proliferation in the ROP-affected eyes. We also evaluated the inflammatory proteins as potential biomarkers for ROP based on their expressions in the tear samples of the ROP patients.

MATERIALS AND METHODS

Study Subjects

The study protocol adhered to the tenets of declaration of Helsinki and was approved by the Institutional Review Board (LEC02-14-029) of the L V Prasad Eye Institute (LVPEI). Preterm babies referred for further management from the neonatal intensive care units of different hospitals in Hyderabad to the LVPEI between January 2007 and December 2010 were enrolled. Overall, the study cohort comprised 372 preterm babies of GA $\leq$ 35 weeks and/or BW $\leq$ 1,700 g with ROP ($n = 189$) and no-ROP ($n = 167$). A detailed demographic and clinical history (Table S1 in Supplementary Material) of all the preterm babies enrolled were documented and a written informed consent was obtained from their parents. The diagnosis and categorization of ROP cases from mild to severe form was based on severity (stages 1–5), location (zones I, II, III), amount of disease (clock hours), and presence or absence of "plus" disease following ICROP guidelines (20, 21) (Figure S2 in Supplementary Material). Severe ROP includes progressive disease, which requires prompt treatment. It includes any stage (1–5) Zone I with plus and stages 2–3 Zone II with plus. Mild ROP cases include less severe disease, which does not require any treatment. Although until the regression of the disease completely, babies are under regular follow-up for ROP screening.

Sample Collection

Venous blood (0.5–1 mL) was collected from the ROP and no-ROP preterm babies by venipuncture. DNA was extracted from the blood samples using an automated DNA extraction platform (MagNa Pure LC 20, Roche) following the manufacturers guidelines. Likewise, for proteomic studies, the vitreous humor samples (100–500 μL) were collected from preterm babies with stage IV and V ROP ($n = 30$) who had undergone vitrectomy as a part of their routine clinical management. The controls for the proteomic studies included babies with congenital cataract (<6 months of age) who underwent partial vitrectomy as part of the surgical management ($n = 30$). The vitreous samples were immediately centrifuged at relative centrifugal force (rcf) of 10,621 g and the supernatant was stored at −80°C deep freezer until further use.

Additionally, crude tears were collected before instilling any drops or drug for pupil dilation in the eyes of preterm babies with ROP (Stage II–V) ($n = 27$) and no-ROP ($n = 13$) using a capillary tube and without touching the conjunctiva. The tear samples for ROP subjects were collected during the active disease condition either before or after 3 months following medical intervention.

Customized Genotyping of Candidate Variants

A customized panel containing 384 single-nucleotide polymorphisms (SNPs) from 26 chosen genes (Table S2 in Supplementary

Material) involved in growth and development of the fetal retina, angiogenesis, inflammation, neurodegeneration, and oxidative stress processes were genotyped using a microarray platform (Illumina Inc., golden gate assay). Following hybridization, the fluorescent signals were scanned by a bead array reader and the raw signal intensities were imported to the Genome Studio software (version 1.9) for assessing quality scores. The assay and sample reliability were measured by means of the gen call score and the genotypes were called following clustering. The genotypes of a subset of samples for all the genes were validated by resequencing on an automated DNA sequencer (ABI 3130 XL) using the BigDye chemistry.

Quantitative Assessment of Cytokines and Other Proteins in the Vitreous and Tear Samples

The concentrations of 27 cytokines (Bio-Plex Human cytokine 27-Plex, Bio-Rad, Hercules, CA, USA) and 28 different proteins (HMMP1-55K, HMMP2-55K, HNDG1-36K, HNDG2-36K, HTIMP2-54K, TGFB-64K-03, HYCYTOMAG-60K, Merck Millipore, Billerica, MA, USA) involved in ECM remodeling, angiogenesis and inflammatory pathways were screened by multiplex bead immunoassays using the Luminex xMAP technology in vitreous samples that were pre-diluted to concentration 1:3. Similar assay was used for estimating the concentrations of MMPs and cytokines in the tears samples and were quantitated by comparing them with their respective standard curve. All standards and some of the samples (due to less volume of samples) were measured in duplicates.

Validation of the Differentially Regulated Proteins by Western Blotting and Zymography

A part of vitreous sample (50–100 μL) was lysed using a buffer containing 50 mM Tris-HCl (pH = 8), 120 mM NaCl, 0.5% NP40, protease inhibitor cocktail, and precipitated with acetone. The protein pellet was eluted in 50 μL of 1× phosphate buffered saline (PBS) containing the protease inhibitor cocktail and quantified by bicinchoninic acid assay. The normalized vitreous proteins (10 μg) were then subjected to western blotting. Western blotting was done using mouse anti-human C3 antibody (sc-28294, Santa Cruz) and mouse anti-human C5 (MAB2037, R&D Systems) followed by incubation with IRDye® 680RD secondary antibody. The entire procedure was done according to the manufacturer's recommended application protocol (https://www.licor.com).

MMP gelatinase activity was measured in the vitreous and tear of ROP babies and controls by zymography as described earlier (22). An equal volume of crude vitreous and tear samples were electrophoresed under non-reducing conditions in 10% SDS-PAGE gels polymerized with 1 mg/mL gelatin. The gel was washed with 2.5% Triton X-100 for 30 minutes at room temperature with gentle agitation, followed by rinsing with distilled water. The gel was then incubated for 30 min in developing buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM $CaCl_2$, 0.2 M NaCl, 0.02% Brij 35. The gel was incubated with fresh developing buffer at 37°C for 16 h and stained with Coomassie blue (Bio-Rad). This was followed by destaining with 10% v/v methanol, 5% v/v acetic acid in dH_2O. Active MMP (MMP2/MMP9) band was detected in the zymogram in the discovery cohort. The observations of discovery cohort were further validated in tears in an extended cohort of patients at different stages of ROP: no-ROP ($n = 9$), mild ROP regressed ($n = 6$), mild ROP_progressed ($n = 7$), and severe ROP ($n = 12$) by zymography in order to confirm their role as biomarker in ROP pathogenesis. Zymogram band intensities were calculated with ImageJ software.

Immunohistochemistry and Hematoxylin and Eosin (H&E) Staining for Macrophage/Microglia in the Vitreous

Vitreous were subjected to cytospin to separate the cells that were embedded in the parafilm block. Sections were cut, air dried, and stained with H&E (23) for understanding their morphology. Tissue sections were then deparaffinized using xylene and gradually rehydrated with ethanol. Antigen retrieval was done by microwaving the sections at full power for 4–5 min in Tris EDTA buffer (10 mM Tris, 1 mM EDTA, 0.5% tween 20, pH 9.0). Blocking was carried out with 2.5% (w/v) BSA in PBS (10 mmol/L sodium phosphate, pH 7.5, 120 mmol/L sodium chloride) for 30 min at room temperature. Thereafter, the slides were incubated for 60 min with the primary antibody (CD 68 for human 1:100) diluted in 1× PBS, followed by three washings with PBS. Further incubation was carried out with biotinylated anti-mouse immunoglobulin. Sections were then washed in PBS and incubated with avidin DH/biotinylated horseradish peroxidase reagent in PBS for 30 min before final washing. The antigen was localized using 1 mg/mL diaminobenzidene tetrahydrochloride (DAB; Sigma), 0.2% H_2O_2 in 50 mmol/L Tris–HCl, pH 7.6, which appeared as a brown end product. Sections were then counter-stained with DAPI for nuclei staining.

Response to Hypoxia by the Cultured Microglia Cells

The human microglial cell line (CHME3) was cultured in DMEM containing 10% FBS along with antibiotics penicillin and streptomycin. The confluent cells were trypsinized using 0.25% trypsin-EDTA. Hypoxic stress was introduced in the microglia cells by treating them with Cobalt chloride ($CoCl_2$) at various concentrations from 100 to 250 μm. Briefly, around 15,000 cells were seeded on a six well plate and then serum deprived for 6 h, followed by treatment with $CoCl_2$ for 24 h on attaining 70–80% confluency. The serum deprived cells in the same duration that were not treated for hypoxia were used as controls.

Ca^{2+} Staining and Live Cell Imaging

The cells were washed in HBSS (Thermoscientific, Waltham, MA, USA) and then incubated with the calcium binding dye Flu-4 (diluted with HBSS 1:750) for 30 min. After washing the cells with HBSS three times, live cell imaging was performed for 10 min using an EVOS fluorescent microscope (Thermo Fischer

Scientific, Waltham, MA, USA) under 20× magnification. The cytosolic calcium flux was measured using the change in Fluo-4 intensity over time for individual cells (Excitation: 494 nm Emission: 506 nm) (24).

Semi-Quantitative PCR

The RNA from untreated and treated cells was extracted by Trizol method (25). The cDNA was prepared using iScript cDNA synthesis kit (Bio-Rad, CA, USA). Semi quantitative PCR was carried out using the specific primers (Table S3 in Supplementary Material) for *VEGF165*, *C3*, *HIF1*α, *BAX*, and *IL-1*β while β-*actin* was used as an endogenous control.

Statistical and Bioinformatic Analysis

Allele frequencies of all the 384 variants were calculated by gene counting method along with odds ratio and 95% CI. A p value < 0.05 was considered to be significant. The associated allele and haplotype frequencies were further analyzed for statistical correction using Bonferroni and permutations tests ($n = 10{,}000$ permutations). Estimates of Hardy–Weinberg equilibrium ($p > 0.001$), linkage disequilibrium (LD), and haplotype frequencies were calculated using the Haploview software (version 4.2) (26).

Protein and cytokine levels in ROP and control samples were represented as bar plot (the mean ± SE) and box plot (median, interquartile range, and whiskers). Comparison of proteins and cytokines levels between ROP and controls vitreous/tears were calculated using the unpaired Student's t-test. A p-value < 0.05 was considered to be statistically significant. Since the cytosolic calcium level does not follow a normal distribution, we performed the testing of equality in medians for control and hypoxic condition using Wilcoxon rank-sum test.

RESULTS

Involvement of Genes in ROP

Of the 384 variants screened (Table S2 in Supplementary Material), 73 were removed from further analysis as they were either not in Hardy-Weinberg equilibrium in the controls ($n = 16$), were monomorphic ($n = 44$), or had a call rate < 97% ($n = 13$). Thus, 311 SNPs from 26 genes were finally analyzed for association with ROP. Among these 37 SNPs in 14 genes (*AGTR1, ANGPT2, C3, CFH, CFB, CXCR4, FBLN5, H2AFX, IHH, MMP2, TGF*β*1, CETP, VEGF*, and *TSPAN12*) exhibited significant association ($p < 0.05$) with ROP (**Table 1**). Additionally, 5/37 associated SNPs in *CFH, CFB, CXCR4, FBLN5,* and *CETP* genes withstood Bonferroni correction. Intriguingly, only the *CETP* variant (rs891141) conferred significant risk of ROP, while the variants across the other genes were protective (**Table 1**). Strong LD was observed across all the variants (except rs1831821) in *CFH* and rs891141 and rs289716 in *CETP* gene, while moderate LD was observed between rs891141 and rs289713 in *CETP* and rs2268002 and rs2284340 in *FBLN5* (Figure S1 in Supplementary Material).

Likewise, haplotypes generated with the associated and flanking variants of these five genes revealed that only the haplotype C-A-T in *CETP* conferred significant risk of ROP, while those with *CFH* and *FBLN5* were protective. Haplotypes with the *CXCR4* and *CFB* were not informative (**Table 2**). Thus, the present study highlights the potential involvement of novel genes (*CFH, CFB, CETP, FBLN5,* and *CXCR4*) in ROP based on their allelic and haplotype associations.

Quantitative Assessment of Proteins Involved in Complement Cascade and Neurodegeneration in the Vitreous Samples of ROP Subjects

Based on strong associations in the *CFH* and *CFB* genes, a quantitative assessment of a neurodegenerative panel containing CRP, SAP, MIP-4, Complement C4, apolipoprotein AI, apolipoprotein CIII, apolipoprotein E, Complement Factor H, and Complement C3 proteins was carried out by multiplex immuno-bead assay in the vitreous samples of ROP patients ($n = 30$) and controls ($n = 30$). All the complement components and apolipoproteins were detectable in the vitreous samples. Overall, we observed significantly elevated levels of C3 ($p = 0.05$), C4 ($p = 0.001$), CFH ($p = 2.24 \times 10^{-5}$), VEGF ($p = 0.0027$), apolipoprotein AI ($p = 0.0007$), and apolipoprotein CIII ($p = 0.004$) in the vitreous of ROP compared to the control subjects indicating their possible involvement in the disease pathogenesis (**Figure 1A**).

Activation of Complement Pathway in Vitreous Humor of Proliferative ROP

We validated the differential expression of complement component C3 by western blotting. An intense band of 192 kDa corresponding to C3 molecule was observed in ROP cases compared to controls (**Figure 1B**). Additionally, we observed the activated C3 fragments; C3b (182 kDa), C3c (145 kDa), and iC3bα (63 kDa) in the ROP vitreous under non-reducing conditions as confirmed by mass spectrometry (data not shown here), but not in the controls (**Figure 1B**), suggesting a higher activation of C3 in ROP. Likewise, a higher expression of complement component C5 was observed in ROP vitreous compared to the controls (data not shown) suggesting a further activation of the complement pathway.

Hypoxia-Induced Activated Macrophage Secretes Angiogenic Molecules

We further demonstrated that along with increased expression of angiogenic molecules in the vitreous samples of patients, activated macrophages/microglia in turn would also be secreting proinflammatory cytokines that might exacerbate the inflammation, further playing a role in the ROP pathogenesis. We detected activated macrophages/microglia on H&E, further confirmed by immunostaining with CD68 in ROP vitreous but not in the controls (**Figure 1C**). The results of this experiment supported for shift in the proangiogenic state as demonstrated by a significant increase in the levels of cytokines IL8 ($p = 0.0149$), G-CSF ($p = 0.0099$), IL1ra ($p = 0.0019$),

TABLE 1 | Association of gene variants with retinopathy of prematurity (ROP).

Genes screened	Single-nucleotide polymorphism (SNP) ID	Location	Nucleotide change	Amino acid change	RegulomeDB (binding score) (27)	Minor allele	Minor allele frequencies		*p*-Value	Odds ratio [95% CI]
							ROP	Controls		
CFH	**rs374896**	**Intron**	g.71371T>C	–	Minimum binding evidence (6)	**T**	**0.0426**	**0.159**	**2.94×10^{-7}**	**0.241 [0.135–0.431]**
CFB	**rs1048709**	**Exon**	g.19461A>G	p.R150R	Likely to affect binding of POLR2A and linked expression of the HLAC, HLA-DQA1, HLADQB1, HLADRB1, HLADRB5	**G**	**0.15**	**0.269**	**1.71×10^{-5}**	**0.484 [0.035–0.676]**
C3	rs344550	Intron	g.37710G>C	–	Likely to affect binding of GATA2, MYC, NR2F2, STAT5A, SPI1CCNT2 (1F)	C	0.223	0.29	0.0409	0.703 [0.501–0.986]
	rs2287846	Intron	g.24106G>C	–	Minimal binding evidence (5)	G	0.298	0.237	0.0658	1.370 [0.979–1.915]
CXCR4	**rs2228014**	**Exon**	g.2652C>T	p.I142I	Minimum binding evidence (4)	**G**	**0.422**	**0.637**	**1.32×10^{-8}**	**0.416 [0.307–0.565]**
ANGPT2	rs2922889	Intron	g.119194A>T	–	Minimum binding evidence (6)	T	0.497	0.431	0.0776	1.305 [0.971–1.756]
	rs2515464	Intron	g.35092A>C	–	Minimum binding evidence (5)	T	0.173	0.251	0.0102	0.622 [0.432–0.895]
	rs734701	Intron	g.32684C>T	–	Minimum binding evidence (6)	A	0.452	0.527	0.0465	0.741 [0.551–0.996]
	rs2959812	Intron	g.29629T>C	–	Minimum binding evidence (5)	C	0.471	0.548	0.0391	0.732 [0.544–0.985]
VEGF	rs2010963	5′-UTR	–	–	Minimum binding evidence (4)	C	0.302	0.243	0.0084	1.352 [0.969–1.888]
	rs1413711	Intron	g.2758T>C	–	Minimum binding evidence (4)	T	0.39	0.482	0.014	0.688 [0.51–0.928]
	rs1005230	Intergenic	–	–	Minimum binding evidence (5)	A	0.388	0.482	0.011	0.680 [0.505–0.917]
FBLN5	**rs2268002**	**Intron**	g.17582G>C	–	Minimum binding evidence (5)	**G**	**0.32**	**0.423**	**9.2×10^{-4}**	**0.641 [0.492–0.835]**
MMP2	rs2285052	Intron	g.88546A>C	–	Minimum binding evidence (5)	G	0.092	0.036	0.0025	2.732 [1.39–5.368]
TGFb1	rs11466359	Intron	g.22217C>T	–	Minimum binding evidence (5)	T	0.093	0.0482	0.021	2.027 [1.1–3.734]
	rs4803457	Upstream	g.4544T>C	–	Minimum binding evidence (4)	A	0.465	0.536	0.061	0.754 [0.561–1.013]
CETP	**rs891141**	**Intron**	g.7962G>T	–	Minimum binding evidence (5)	**C**	**0.234**	**0.114**	**2.99×10^{-5}**	**2.378 [1.57–3.598]**
H2AFX	rs640603	Intergenic 3′ of a gene	–	–	Likely to affect binding of PLR2A, CHD1, E2F6, MXI1, E2F4, E2F6, MYC (2b)	T	0.161	0.09	0.0049	1.940 [1.215–3.085]
TSPAN12	rs41624	Intron	g.62819A>G	–	Minimum binding evidence (6)	T	0.176	0.243	0.0279	0.665 [0.462–0.958]
	rs41629	Intron	g.59568T>G	–	Minimum binding evidence (6)	A	0.176	0.243	0.0279	0.665 [0.462–0.958]
	rs3735467	Intron	g.47721G>T	–	Minimum binding evidence (5)	C	0.177	0.246	0.031	0.669 [0.465–0.964]
	rs12669167	Intron	g.38926T>G	–	NA (7)	C	0.173	0.24	0.0279	0.664 [0.46–0.958]
	rs10225453	Intron	g.36511C>A	–	Minimum binding evidence (5)	T	0.17	0.236	0.0287	0.663 [0.458–0.959]
	rs6953454	Intron	g.33908G>A	–	NA (7)	T	0.172	0.237	0.03	0.666 [0.461–0.964]
	rs996903	Intron	g.32898A>G	–	Likely to affect binding and linked expression of the FLJ21986 (1F)	C	0.17	0.237	0.0279	0.662 [0.458–1.044]
	rs6959328	Intron	g.32490T>A	–	NA (7)	A	0.17	0.237	0.0279	0.662 [0.458–1.044]
	rs6466759	Intron	g.28767A>T	–	Minimum binding evidence (5)	T	0.17	0.238	0.0251	0.657 [0.454–0.95]
	rs7805211	Intron	g.25107G>A	–	Minimum binding evidence (6)	A	0.168	0.237	0.0218	0.65 [0.449–0.941]
	rs6466760	Intron	g.24403C>G	–	Minimum binding evidence (6)	C	0.168	0.237	0.0218	0.65 [0.449–0.941]
	rs6466762	Intron	g.16639G>A	–	Minimum binding evidence (5)	A	0.169	0.241	0.0167	0.638 [0.441–0.923]
	rs3823859	Intron	–	–	Minimum binding evidence (5)	G	0.17	0.24	0.0219	0.651 [0.451–0.941]
	rs17142995	Intron	g.11660A>G	–	Minimum binding evidence (6)	C	0.173	0.24	0.0279	0.664 [0.46–0.958]
	rs7781985	Intron	g.6475A>C	–	Likely to affect binding and linked expression of the FLJ21986/ monocytes (1F)	C	0.17	0.24	0.0219	0.651 [0.451–0.941]
	rs3757557	5′UTR	g.91G>A	–	Minimum binding evidence (4)	A	0.117	0.171	0.0411	0.644 [0.421–0.985]
	rs4141309	Intergenic, upstream 5′ of gene	–	–	Minimum binding evidence (4)	A	0.112	0.171	0.0236	0.611 [0.398–0.939]
AGTR1	rs2739504	Intron	g.13100A>G	–	Minimum binding evidence (4)	C	0.446	0.371	0.041	1.362 [1.012–1.833]
IHH	rs394452	Exon	g.5153T>C	p.T376T	Minimum binding evidence (5)	T	0.261	0.168	0.0027	1.75 [1.211–2.528]

SNPs in bold withstood Bonferroni correction.

TABLE 2 | Estimated haplotype frequencies of the significantly associated variants in *CETP, CFH*, and *FBLN5* genes in retinopathy of prematurity (ROP) and premature controls.

Genes (single-nucleotide polymorphisms)	Haplotypes	Overall frequencies	ROP frequencies	Controls frequencies	Chi square	*p*-Value	Odds ratios (95% CI)
CETP (rs891141, rs289713, rs289716)	A-A-T	0.327	0.313	0.343	0.734	0.3916	0.871 (0.637–1.193)
	A-A-A	0.3	0.294	0.308	0.156	0.6931	0.937 (0.68–1.292)
	C-A-T	0.149	0.191	0.101	11.358	0.0008	2.1 (1.354–3.256)
	A-T-A	0.143	0.121	0.169	3.357	0.0669	0.674 (0.442–1.029)
	A-T-T	0.053	0.04	0.067	2.488	0.1147	0.586 (0.3–1.144)
CFH (rs3753395, rs374896, rs393955)	T-C-T	0.563	0.578	0.547	0.693	0.4052	1.134 (0.843–1.526)
	A-C-G	0.188	0.233	0.139	10.066	0.0015	1.874 (1.268–2.769)
	A-C-T	0.151	0.149	0.154	0.032	0.8586	0.962 (0.638–1.451)
	A-T-G	0.096	0.04	0.156	27.61	1.48×10^{-7}	0.226 (0.125–0.41)
FBLN5 (rs2268002, rs2284340)	G-C	0.38	0.413	0.343	3.621	0.0571	1.346 (0.992–1.826)
	G-G	0.37	0.311	0.437	12.093	5×10^{-4}	0.58 (0.427–0.789)
	A-C	0.242	0.271	0.21	3.625	0.0569	1.4 (0.989–1.983)

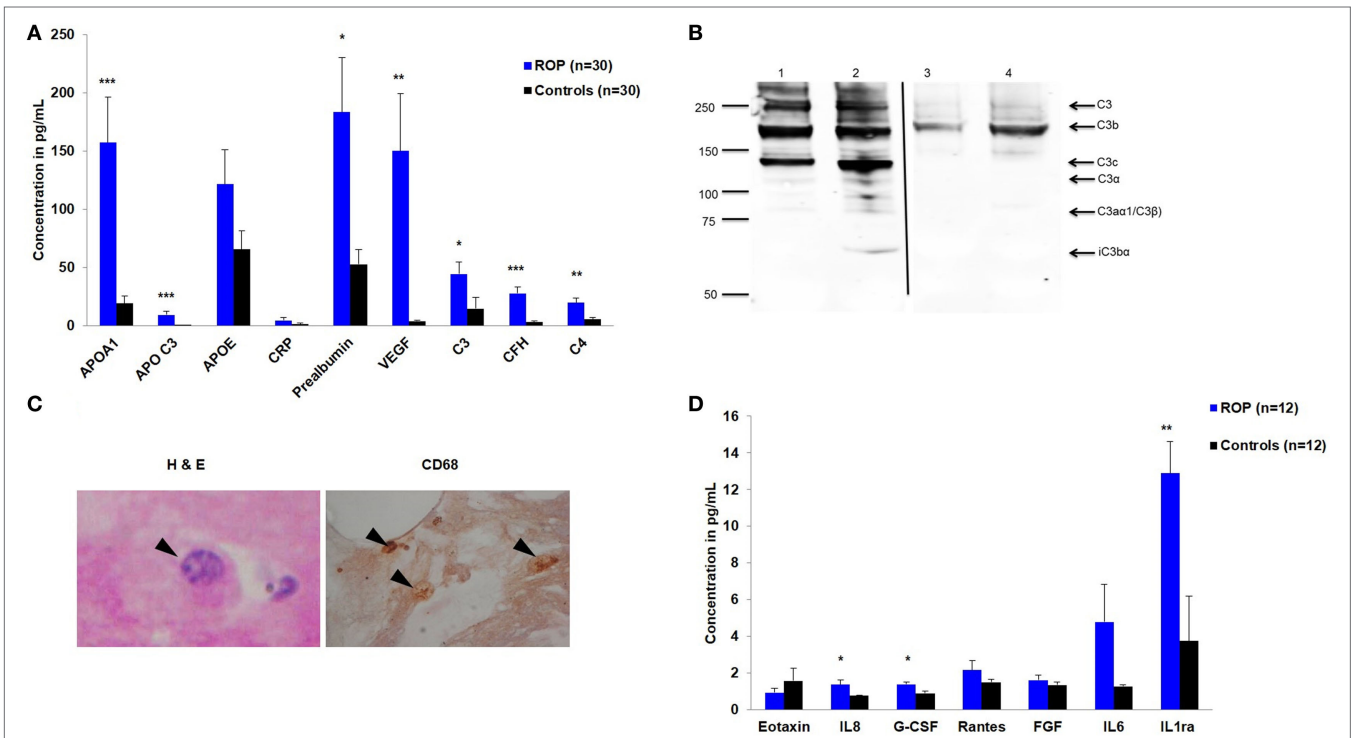

FIGURE 1 | Expression of inflammatory molecules in proliferative retinopathy of prematurity (ROP). **(A)** Differential levels of complement components and apolipoproteins in the vitreous humor of ROP and controls **(B)** Western blotting of C3 done under non-reducing condition, showing its expression in patients (lane 1 and 2) and controls (lane 3 and 4). Arrows indicate the C3 fragments. Thin black line inserted within gel showing that all the lanes were run on the same gel but were not continuous **(C)** Hematoxylin and eosin staining of ROP vitreous. Arrowheads indicate degenerated morphology of macrophages at 40×-magnification (irregular shaped with large vacuole, nucleus with a very prominent nucleolus); Staining of ROP vitreous with CD68 marker. Arrowheads indicate the presence of activated macrophages at 10× magnification. **(D)** Differential levels of inflammatory cytokines in the vitreous humor of ROP and controls. Error bars in **(A)** and **(D)** show SEM, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ (ROP vs. controls).

and VEGF ($p = 0.0027$) (**Figures 1A,D**) along with marginal increase of IL6, IL12, IL7, RANTES, and MCP1 in the ROP vitreous (data not shown).

To further confirm these results, we subjected the cultured microglial cells to hypoxic condition and checked for the expression of proinflammatory markers. The effect of hypoxia on the activation of macrophages/microglia was observed with an intense calcium staining in cells exposed to hypoxia compared to the unexposed ones. The result shows that there is increase in cytosolic calcium levels in case of hyperactivated cells subjected to 24 h of hypoxic stress (**Figure 2A**, $n = 50$). Specifically, there is a significant increase ($p < 0.0005$) in cytosolic calcium

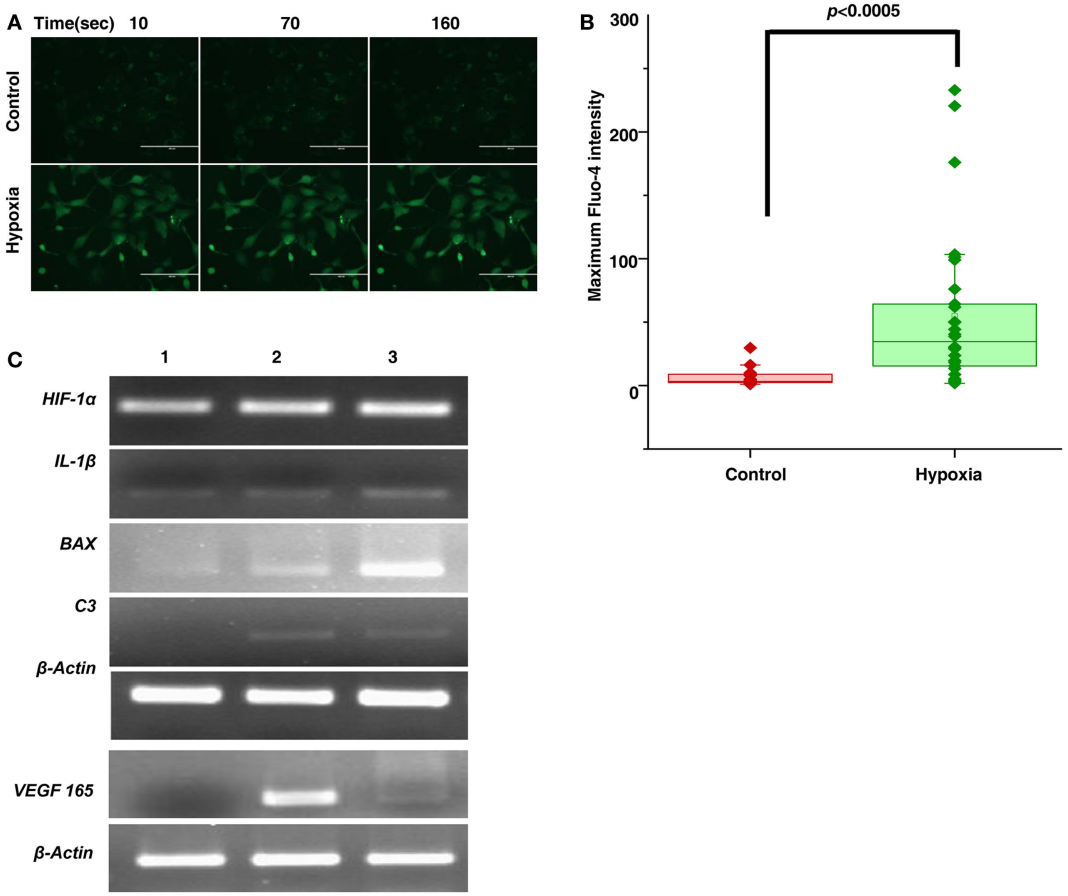

FIGURE 2 | Hypoxia induced microglia secretes inflammatory and angiogenic molecules. **(A)** Time lapse imaging of cytosolic calcium in microglial cells in normal and hypoxic conditions. Scale bar: 200 μm **(B)** Comparison of Ca_{max} (Maximum Fluo-4 intensity) in microglial cells for control and hypoxic condition [Ca^{2+}transients were measured for 50 cells ($n = 50$) for each cases]. Data are presented as box plot and were analyzed using Wilcoxon rank-sum test. **(C)** Semi-quantitative PCR was used to evaluate the expression of secreted product (*HIF-1α, IL-1β, BAX, C3, VEGF*) of hypoxia-induced microglial cells treated with no $CoCl_2$ (lane 1), 100 μM $CoCl_2$ (lane 2), and 150 μM $CoCl_2$ (lane 3) and β-actin as control.

(Ca_{max} = the maximum Fluo-4 intensity) in microglial cells followed by hypoxia exposure (**Figure 2B**). Likewise, a higher expression of complement *C3, VEGF165,* and hypoxia inducing factor-1α (*HIF-1α*) was also observed in exposed cells (**Figure 2C**).

Involvement of Extra-Matrix Metalloproteinases in Pathogenesis of ROP

A strong association of SNPs in *FBLN5* and moderate association of *MMP2, TGF*β gene (**Table 1**) with ROP suggested the role of ECM proteins in ROP pathogenesis. Further, a quantitative assessment of the ECM proteins indicated a significant increase in MMP9 ($p = 0.038$), TIMP1 ($p = 0.004$), and α2 macroglobulin ($p = 0.0018$) in the ROP vitreous (**Figure 3A**). We also assessed the MMP activation in ROP by gelatine zymography. Our results showed higher levels of both pro and activated MMPs (MMP9 and/or MMP2) in the vitreous of patients suggesting its potential role in disease pathogenesis (**Figure 3B**).

Exploring the Potential of Inflammatory Markers in Tear Samples for the Progression of ROP

We explored if increased expression of inflammatory markers (as seen in the vitreous samples of ROP patients) could also be reproducibly detected in tears and further be established as the biomarker for disease progression. A quick multiplex ELISA of tear samples collected from the ROP babies at different stages and no-ROP preterms was performed for some inflammatory markers (interleukins, TNFα, IFNγ, and MMPs). Significantly higher expressions of IL-1ra ($p = 0.014$), MMP2 ($p = 0.0085$), and MMP-9 ($p = 0.03$) were detected in severe ROP cases compared to mild ROP and no-ROP tear samples that was further confirmed by zymography (**Figures 3C–E**). On the zymogram, the tear samples from no-ROP showed very low expression of MMPs as compared to severe ROP (**Figure 3E**). These results were confirmed to be reproducible in the extended cohort of ROP with a significant increased expression of activated MMP2 in severe ROP ($p = 0.0023$) and progressive ROP ($p = 0.007$) as compared

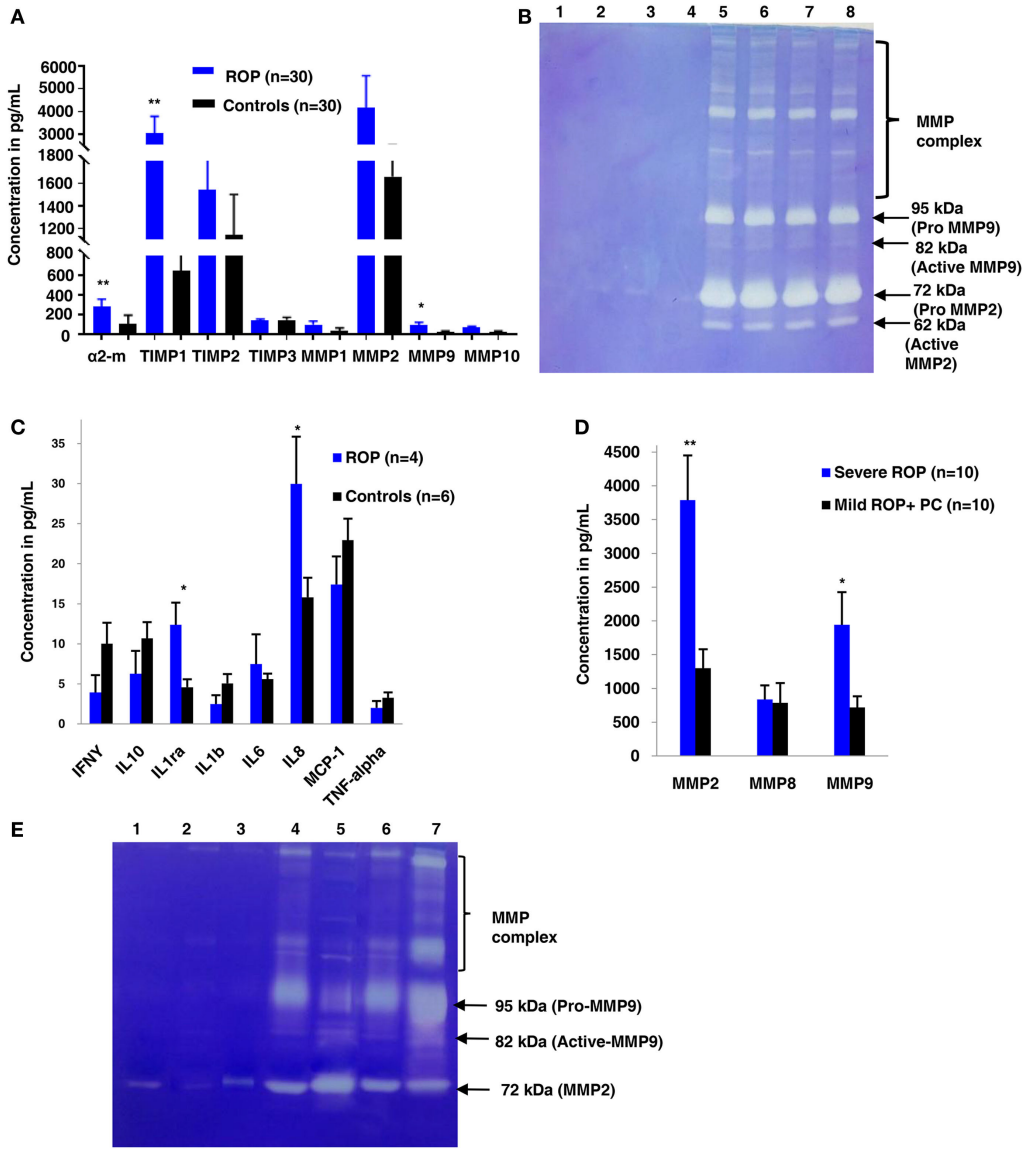

FIGURE 3 | Extracellular matrix (ECM) metalloproteinases and cytokines increases in proliferative retinopathy of prematurity (ROP) in both vitreous and tears samples. **(A)** Differential levels of ECM proteins and its inhibitors in ROP and control vitreous [$^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ (ROP vs. controls)]. **(B)** Zymogram shows activation of MMPs in ROP vitreous (lanes 5, 6, 7, 8) as compared to controls (1, 2, 3, 4). **(C)** Differential levels of cytokines in ROP (5 μL) and control tears (5 μL). **(D)** Differential levels of MMPs in ROP (5 μL) and control tear (5 μL) [$^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ (severe ROP vs. mild ROP + premature controls)]. **(E)** Zymography showing more activation of MMPs in severe ROP (lanes 6, 7) as compared to mild ROP (lanes 4,5) and controls (5 μL tears; lanes 1, 2, 3). Error bars show SEM.

to mild ROP ($p = 0.01$) and premature controls. Similar pattern of gradual increase in MMP9 expression was also observed in mild ROP ($p = 0.02$) to progressive ($p = 0.001$) and severe ROP cases ($p = 1.2 \times 10^{-6}$) with respect to premature controls (**Figure 4**).

DISCUSSION

Retinopathy of prematurity is a biphasic disease that includes an initial phase of hyperoxia leading to blood vessel obliteration followed by hypoxia causing vessel proliferation eventually leading to neovascularization and neurodegeneration. It is a complex disease with multifactorial etiologies. An earlier study on monozygotic and dizygotic twin pairs had also noted the genetic involvement in the development of ROP, in absence of other environmental factors (28). While supplemental oxygen is considered as a major risk factor along with lower GA and BW, studies from India and other Asian countries have reported ROP babies with higher GA and BW (29) and oxygen supplementation does not always predict the risk of ROP (6). Therefore, we hypothesized that genetic predisposition along with environmental/maternal or other risk factors may lead to the development of ROP.

A strong association of gene variants involved in the complement pathway (*CFH, CFB, C3*), ECM remodeling (*FBLN5*, *MMP9*), leukocyte transendothelial migration and activation

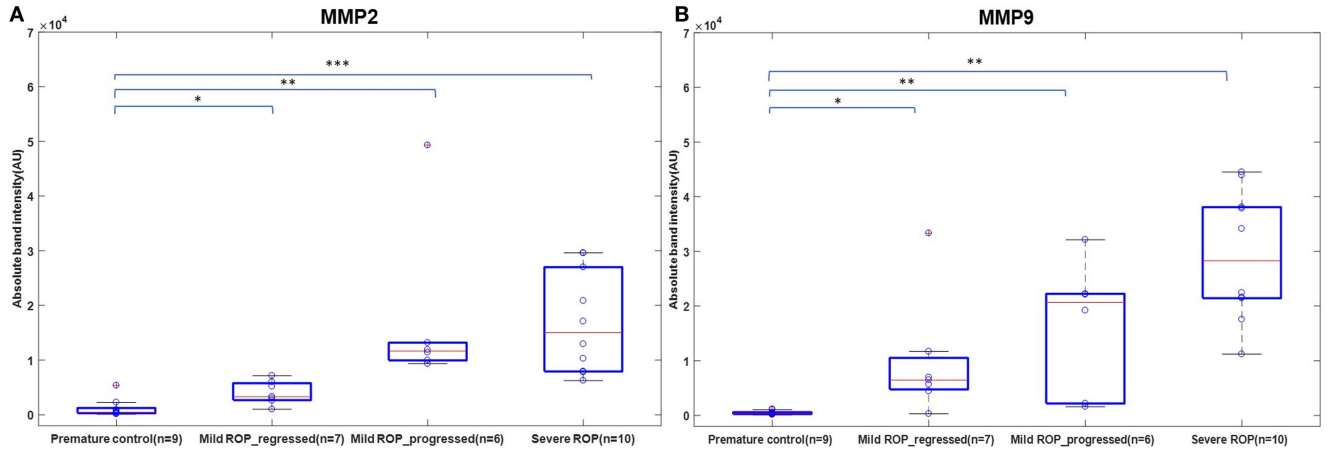

FIGURE 4 | A role of MMPs in tears as biomarker for retinopathy of prematurity (ROP) progression: differential expression of Zymogram band intensities of **(A)** MMP9 and **(B)** MMP2 in extended cohort including severe ROP (n = 10), mild ROP_progressed (n = 6), mild ROP_regressed (n = 7), and no ROP premature controls (n = 9) [*p < 0.05, **p < 0.005, *** p < 0.0005 (ROP vs. controls)], AU, arbitrary unit.

(*CXCR4*), HIF1A signaling and angiogenesis (*ANGPT2, H2AFX,* and *VEGF*), and developmental processes (*TGFb1, IHH*) observed in the present study (**Table 1**), confirms the involvement of genes in ROP pathogenesis. A previous study reported the association of polymorphisms in *IHH, AGTR1, TBX5, CETP, GP1BA, EPAS1, BDNF,* and *CFH* with ROP (11, 13). However, only a few of these associated variants could be replicated in the present cohort, indicating allelic heterogeneity (**Table 3**). Thus, the novel and associated variants identified in the present study (**Tables 1** and **3**) and elsewhere should be screened across multiple populations to understand their implications in ROP.

The strong associations of *CFH*, *CFB*, and *C3* variants in our ROP patients along with elevated levels of C3 and CFH proteins in their vitreous (**Table 1** and **Figure 1A**) indicated a possible involvement of the alternative complement pathway in ROP. CFH and CFB are the regulators of the alternative complement immune pathway (30). Upon activation, CFB is cleaved by complement factor D yielding two subunits, Ba and Bb. The active subunit Bb associates with C3b to form C3 convertase of alternative pathway while CFH regulates the alternative pathway activation by accelerating the decay of C3 convertase (30). It was also noted that there was an increase in the formation of CFB in oxygen induced retinopathy (OIR) mice model (31). Thus, the observed genetic associations of *CFH* and *CFB* complemented with their increased expression of cleaved C3 protein fragments in the vitreous of ROP-affected eyes in our study confirmed their possible involvement in disease pathogenesis (**Figure 5**). Generally, complement factors are known to be downregulated in the normal preterm neonates because of immature development of the immune system (32, 33). On the contrary, we observed an elevation and activation of the complement components and complement factors in the vitreous of ROP patients at infancy (**Figure 1**), suggesting an important role of the complement pathway in ROP pathogenesis.

Interestingly, the genetic variants in *CFH, C3,* and *CFB* genes have also been associated with AMD susceptibility (34, 35).

TABLE 3 | Comparison of commonly associated gene variants in retinopathy of prematurity worldwide.

Associated genes	Associated single-nucleotide polymorphisms (associated allele)	Present study (India), [a]189/167	Study by Mohamed et al. (11) (U.K.), [a]102/228	Study by Hartnett et al. (13) (USA), [a]593/364
CFH	rs529825 (A)	p = 0.7521	p = 0.01	–
	rs800292 (A)	p = 0.3186	p = 0.01	–
	rs379489 (A)	p = 0.4343	–	p = 0.3926
	rs395544 (A)	p = 0.5525	–	p = 0.403
CETP	rs289747 (T)	p = 0.5688	p = 0.004	–
GP1BA	rs2243093 (C)	p = 0.2991	p = 0.005	–
TBX5	rs1895602 (T)	p = 0.352	p = 0.003	–
AGTR1	rs33978228 (G)	p = 0.0196	–	–
	rs427832 (G)	p = 0.2177	p = 0.005	–
IHH	rs3099 (C)	p = 0.1565	p = 0.003	–
EPAS1	rs1867785 (G)	p = 0.958	p = 0.001	–

[a]*Cases/controls.*

A Y402H variant in the *CFH* gene was found to be most strongly associated with AMD patients worldwide. However, the ROP-associated *CFH* variant (rs374896) identified in the present study is located in the intron of the gene (**Table 1**). Further, functional studies on CFH in AMD eyes have shown that chronic low grade intraocular complement activation in patients carrying the risk variants in *CFH* along with exposure to environmental triggers (smoking, oxidative stress, etc.) causes the retinal pigment epithelial damage leading to neurodegeneration and neovascularization and eventually visual loss (36).

Complement components do not mediate neovascularization by itself but *via* the inflammatory cells (37). As was demonstrated in an OIR mouse model, complement factors C3a and C5a activate macrophages by binding to C3aR and C5aR, thereby regulating angiogenesis (38). In the present study, a strong association ($p = 1.32 \times 10^{-8}$) of rs2228014 in *CXCR4* (**Table 1**) along with the presence of activated microglia/macrophages in the vitreous

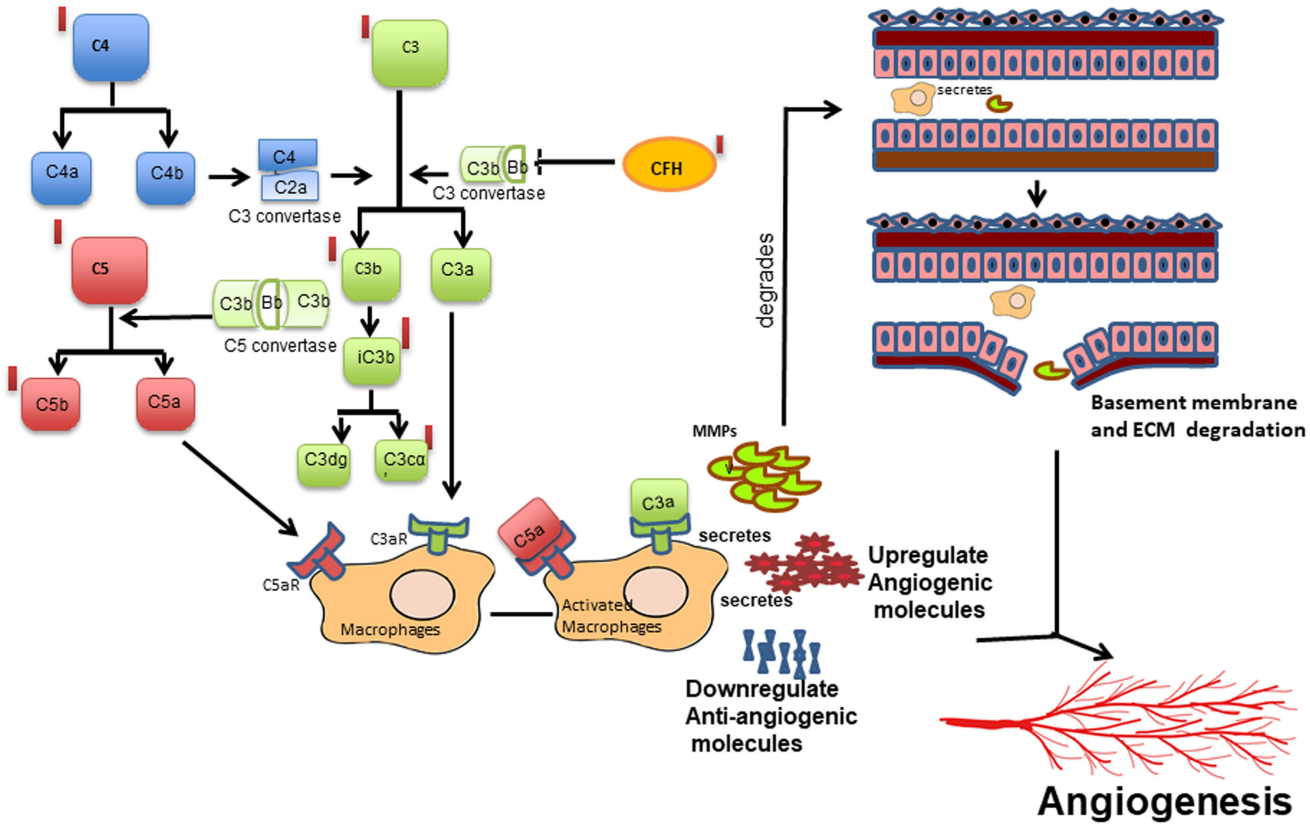

FIGURE 5 | Schematic diagram of the proposed mechanism of complement activation and its potential effect on macrophage-mediated angiogenesis in retinopathy of prematurity. Cleavage of complement components C3 into C3a, C3b and further into iC3b, C3dg and C3cα; C4 into C4a and C4b and C5 into C5a and C5b leading to the activation of complement cascade, which in turn activates macrophage/microglia or *vice versa*. Activated macrophage/microglia secretes MMPs, cytokines, proangiogenic proteins, and reduced angiogenesis inhibitors that may lead to increased vessel proliferation and extracellular matrix (ECM) degradation in turn promoting angiogenesis. Red bars represent upregulation of the complement components observed in the present study.

(**Figure 1C**), implicate their role in ROP angiogenesis via the leukocyte transendothelial migration. CXCR4 is a chemokine receptor for stromal derived factor 1 (CXCL12/SDF-1), which is mainly involved in the extravasation and migration of lymphocytes and monocytes (39). Inhibition of CXCR4 has been shown to result in reduced vascular sprouting following VEGF treatment in retinal explants (40). Our observation of the association of variants in *VEGF, ANGPT2,* and *H2AFX* (**Table 1**) indicate the involvement of HIF1α signaling pathway (hypoxia) in ROP pathogenesis. Furthermore, the secretion of C3 and VEGF by microglial cells under hypoxia (**Figure 2**) validated that hypoxia induced microglia/macrophage along with the complement component, could be contributing to the neovascularization in ROP eyes. The high level of α2-macroglobulin in the ROP vitreous (**Figure 3A**) also indicated the presence of activated macrophages/microglia that further interacts with low-density lipoprotein receptor-related protein 1 (LRP1) which in turn induces MMP9 expression (41, 42).

Based on published studies on macrophages/microglia activation leading to inflammation (37), we proposed that increased expression of the complement components, VEGF, other cytokines, and ECM components (MMPs) in the vitreous of ROP patients are mediated by macrophage/microglia activation by creating an imbalance of angiogenic and anti-angiogenic molecules (**Figure 5**). The proteolytic degradation of ECM is a critical step for the invasion of blood vessels during neovascularization. MMPs are endoproteases that cleaves the protein components of the ECM while TIMPs, α2 macroglobulins, and α1 antitrypsin are the proteinase inhibitors (43). In proliferative diabetic retinopathy, the elevated levels of MMP-2 and MMP-9 were shown to cause ECM remodeling (44, 45) further leading to net collagen IV degradation and vitreous liquefaction (46). The presence of activated MMP-9 in the vitreous along with elevated levels of TIMP1, TIMP2, and α2 macroglobulin in our ROP patients and presence of blood component proteins like apolipoproteins (**Figures 1A** and **3A**) explained its role in the degradation of the basement membrane of blood vessels, seeping out into the vitreous along with the other blood components, thereby causing vitreous hemorrhage and vitreous liquefaction.

Presence of inflammatory markers in the vitreous or other body fluid in young preterm babies might suggest an infectious etiology and inflammatory stimuli contributing to ROP (47). Fetal inflammatory response syndrome (including sepsis, periventricular leukomalacia, intraventricular hemorrhage, necrotizing enterocolitis, and bronchopulmonary dysplasia), chorioamnionitis, and microbial infections are some of the predisposing factors for

inflammation observed in some studies (48). There was no evidence of exposure to infection in our cohort since babies with any microbial infections were excluded. Additionally, we did not find any difference in the complement levels or activation patterns in the serum of these patients and controls unlike in the vitreous samples, further ruling out any systemic infection (data not shown). Based on these evidences, neonatal non-infectious inflammation might be playing a major role in the pathogenesis of ROP.

Based on these findings supplemented with increasing evidences on the role of inflammation in causing neovascularization, we speculated if MMPs could be detected in the tear samples of ROP babies so that it could be used as markers for ROP progression. The tear samples were an obvious choice for this study as it is fairly non-invasive, safe, and convenient, although there were some restrictions of tear volume and sampling in the ROP babies. It was interesting to note that the levels of MMPs in tears were significantly higher in severe ROP compared to no-ROP and mild ROP eyes that underscored its potential use as a biomarker for an early prediction of this condition (**Figures 3E** and **4**). This was further confirmed by zymography, with an increasing trend of the activated MMPs (both 2 and 9) in all the samples of severe stages of ROP along with a case of mild ROP. This mild ROP baby eventually progressed very quickly to a severe stage (plus ROP) in 2 weeks and did not respond to laser therapy (**Figure 3E**). The subsequent validation of these initial findings was done in an extended cohort and the increased levels of MMPs with the increase in severity of disease further established the usefulness of MMPs in tears as potential biomarkers. While our data proved that the levels of MMPs could reliably predict the progression of ROP (**Figure 4**), we could not perform any longitudinal analysis of MMPs levels in the tears due to the difficulties in obtaining samples from preterm babies at regular intervals. Likewise, a direct correlation of genotypes with protein levels or their activities in the corresponding biological material (vitreous/aqueous/tear) and clinical phenotype could not be attempted. Nevertheless, our study provided a proof of concept that tear MMPs levels could be a potential predictor for ROP progression in preterm babies.

In conclusion, the assessment of the activation of alternate complement pathway in ROP based on the novel genetic associations indicated the possible mechanisms of immune activation that could lead to aberrant neovascularization in the retina. However, the detailed underlying mechanisms of immune activation in abnormal blood vessel proliferation and neurodegeneration in the early stages of ROP are yet to be understood. Additionally, our results emphasized the primary role of complement component C3 in abnormal angiogenesis as seen in proliferative ROP. The proteins involved in the alternative complement pathway could be targeted selectively to prevent neovascularization, which might be helpful in preventing vision loss due the progression of ROP. The association of ECM-related genes with ROP along with elevated levels of the corresponding ECM proteins and its activation in the vitreous of ROP patients suggested its possible role in blood-retinal barrier degradation, which could promote neovascularization. Finally, the elevated levels of MMPs in tears of ROP patients established its role as a potential biomarker for the prediction of progression to proliferative stages. However, this needs to be replicated in other extended cohorts worldwide using a longitudinal study design. The present treatment strategies for managing severe ROP are inefficient as they target only the later vasoproliferative phase of ROP. Diagnosing and treating the disease at an earlier stage would definitely help in the timely and efficient management of this disease. The results of this study would aid in finding biomarkers for predictive testing as well as identifying newer drug targets for an efficient management of ROP.

ETHICS STATEMENT

The study protocol adhered to the tenets of declaration of Helsinki and written informed consent was obtained from the parents of all the minor subjects and was approved by the Institutional Review Board (LEC02-14-029) of the L V Prasad Eye Institute (LVPEI).

AUTHOR CONTRIBUTIONS

IK and SJ conceived the idea; IK, SJ, and SC wrote the protocol; IK served as principal investigator; SC, SJ, DB, RK, LG, PR, and PC were co-investigators; SJ, DB, RK, PR, and PC performed clinical examinations, graded the fundus images and did surgeries for the preterm and full term babies; SR, SP, and GM collected blood, vitreous and documented family history in the predesigned questionnaires; SR performed most of the molecular biology based analysis of blood and vitreous; SP performed the tear analysis; SSh performed cell biology work; LG and SS performed analysis for the Ca^{2+} imaging data; SR, IK, and SC analyzed the data and wrote the manuscript; and all authors revised the paper and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01868/full#supplementary-material.

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Effect of Hyperglycemic and hypoxic stresses on the primary cultures of retinal neuron and glial populations: a model system to understand the role of glia in diabetic retinopathy

S Shahna; Lopamudra Giri; Sarpras Swain; Jay Chhablani; Rajeev Pappuru; Mudit Tyagi; Subhabrata Chakrabarti; Inderjeet Kaur

+ Author Affiliations & Notes

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Abstract

Purpose : Retinal gliosis has been shown to be one of the key features in the DR eyes by experimental and immunohistological methods. A human cell culture model system mimicking retinal composition could help in understanding the initial mechanisms of glial activation during disease progression. The aim of present study was to establish a mixed primary culture of retinal glia and neurons from the cadaveric retina for studying their role under the diabetic conditions.

Methods : Primary cultures of retinal neurons and glia were established from human cadaveric retina. The cells from the initial passages (P0-P2) were characterized by immunofluorescence and semi-quantitative PCR. These cells were subjected to a calculated level of hypoxia and hyperglycemia by treating them with $CoCl_2$ and D- glucose. Live cell imaging was done with Fluo-4 AM Ca^{2+} dye to check the activation of cells under stress. RNA and proteins were extracted from the harvested cells after the stress treatment and analyzed for the expression of inflammatory and glial markers by quantitative PCR. Comparisons of GFAP and Iba -1 protein expression (n=60) and Kernel density estimations (n=160) to analyze the spike response of Ca^{2+} were performed in treated versus untreated cells.

Results : Different types of retinal neurons and glial cells in the mixed retinal culture showed positive expression for the specific markers (GFAP, GS, Nestin, Iba-1 and Vimentin). Increased expression of angiogenic and neurodegenerative cytokines were observed in treated cells compared to untreated by real time PCR. Live cell imaging with Flu-4 AM Ca^{2+}dye clearly showed elevated Ca^{2+}signal transduction in the treated cells. Kernel density estimation of Ca^{2+} stained cells further showed higher spiking response and augmented network activity in the treated culture compared to untreated. Compared to untreated cells, 3 and 1.6 fold increase in GFAP protein (n=60) and 4-fold and 2 -fold increase of Iba-1 expression was noted in cells exposed to hypoxia and hyperglycemia respectively.

Conclusions : Hyperactivation of the cultured cells under stress and marked changes in the glial markers confirmed the role of glia in DR progression. The preliminary findings of this study indicate that the proposed culture system can serve as a model for detailed evaluation on the role of glia in DR progression.

This is an abstract that was submitted for the 2017 ARVO Annual Meeting, held in Baltimore, MD, May 7-11, 2017.

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