Understanding the Role of Adiponectin in Proliferative Diabetic Retinopathy; Design and Development of Therapeutic Molecules.

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CERTIFICATE

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ABSTRACT

Adiponectin (APN) is a protein hormone having anti-diabetic and anti-inflammatory properties. Its presence in ocular tissues has been postulated, to have escaped from circulation due to the dysfunctional blood brain barrier (BRB) in retinal diseases. The purpose of this study is to understand the origin of intraocular APN and its receptors, precisely measure vitreous APN in proliferative diabetic retinopathy (PDR) and macular hole (MH), study its role in disease pathogenesis of PDR and to unravel the therapeutic potential of APN.

All human studies were done with approval from Institutional Review Board (IRB) and consent of the participants. Tissues derived from donor eye balls were used for qPCR, immuno staining, western blot analysis for localisation and expression studies. Vitreous samples were obtained from patients who underwent vitrectomy surgery for PDR and MH and used for measurement of APN by ELISA. Vitreous amino acids were estimated by HPLC. Role of APN in the pathogenesis of PDR was studied in retinal pericytes. Human retinal endothelial cells (HREC), human choroidal endothelial cells (HCEC) and Human umbilical vein endothelial cells (HUVECs) were used for *in vitro* angiogenesis studies. Peptides were designed using bioinformatics tools based on their *in silico* interacting partners and screened for their anti angiogenic property in both microvascular and macrovascular endothelial cells (HREC and HUVECs). Human visceral adipocytes, HCEC and HREC were used for screening small molecules including amino acids and fatty acids, in order to select the one which stimulates APN secretion.

Expression of APN, AdipoR1 and AdipoR2 in retina, choroid, iris more specifically in the neural retina, photoreceptors, and endothelium was unambiguously identified by qPCR, immunostaining and western blot analysis. Moreover, Epiretinal membranes from PDR also stained positive for APN and its receptors. APN level in vitreous of patients with PDR was found to be significantly elevated when compared to MH. Interestingly, APN inhibited tube formation and migration of HREC and HUVEC which suggested a potent anti angiogenic property of APN.

Interacting partners of APN were identified using *in silico* approach and mechanistic pathway derived indicated APN to inhibit VEGF signaling pathway. rAPN also decreased the VEGF

production in RPE cells confirming its potential as therapeutic target in PDR. Peptides designed using bioinformatics approaches were found to have anti angiogenic property suggesting that these molecules can mimic full length protein in this function. Additionally to further understand the role of small molecules in modulating APN expression amino acids were tested. Proline, hydroxy proline, phenylalanine were found to increase APN levels. These data strongly suggest that APN as potential therapeutic target for PDR.

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LIST OF ABBREVIATIONS

1. 1, 4-bis (5-phenyloxazol-2-yl) benzene – POPOP
2. 2, 5-diphenyloxazole – POP
3. Adiponectin – APN
4. Adiponectin receptor 1 – AdipoR1
5. Adiponectin receptor 2 - AdipoR2
6. Advanced glycation end products – AGE
7. Age related macular degeneration – AMDAMPK
8. AMP activated protein kinases -
9. Ammonium bicarbonate buffer – NH4HCO3
10. Ammonium per sulphate – APS
11. Angiopoietin – Ang
12. Blood retinal barrier – BRB
13. Cardiovascular diseases – CVD
14. C – Reactive protein - CRP
15. Diabetic retinopathy – DR
16. Diabetes mellitus – DM
17. Diabetic macular edema - DME
18. Diabetic nephropathy – DN
19. Diabetic sensor motor polyneuropathy – DSPN
20. Diacylglycerol - DAG
21. Diaminobenzidine – DAB
22. Dimethyl sulfoxide – DMSO
23. Endothelial cells – ECs
24. Enzyme linked immune sorbent assay - ELISA
25. Fasting plasma glucose - FPG
26. Fibroblast derived growth factor – FGF
27. Glutamine fructose-6-phosphate amido transferase – GFAT
28. Glycosylated Haemoglobin – HbA1C
29. Heptocyte nuclear factor 4 alpha – HNF4A

30. High molecular weight – HMW
31. High performance liquid chromatography -HPLC
32. Horse radish peroxidase – HRP
33. Human choroidal endothelial cells - HCEC
34. Human retinal endothelial cells – HRECs
35. Human retinal pigment epithelial cells – HRPE
36. Human umbilical vein endothelial cells – HUVECs
37. Hydrogen peroxide – H2O2
38. Hypoxia-inducible factor 1 alpha – HIF-1α
39. Hypoxia – inducible factor 1 beta – HIF 1 β
40. Insulin like growth factor -1 – IGF 1
41. Insulin Receptor – INSR
42. Insulin receptor Substrate 1 – INSR1
43. Interleukins – IL
44. Low molecular weight – LMW
45. Lysyl oxidase – LOX
46. Macular hole - MH
47. Matrix metalloproteases – MMPs
48. Matrix–assisted laser desorption/ionization–time of flight mass spectrometry - MALDI–TOF MS
49. Methylglyoxal – MG
50. Middle molecular weight - MMW
51. Mitogen-activated protein kinase – MAPK
52. Non proliferative diabetic retinopathy - NPDR
53. Nuclear factor kappa B – NFκB
54. O-linked beta-N-acetylglucosamine - O-GlcNAc
55. Oral glucose tolerance test – OGTT
56. Peroxisome proliferator-activated receptor alpha – PPAR α
57. Peroxisome proliferator-activated receptor gamma - PPARγ
58. Phosphatidylinositol-3kinase – PI3K
59. Pigment epithelium-derived factor – PEDF

60. Placental growth factor - PIGF
61. Platelet derived growth factor – PDGF
62. Platelet endothelial cell adhesion molecule 1 – PECAM 1/CD31
63. Proliferative Diabetic retinopathy – (PDR)
64. Protein kinase C – PKC
65. Protein Lynx Global SERVER - PLGS
66. Polyacrylamide gel electrophoresis – PAGE
67. RNA interference – RNAi
68. Reactive oxygen species - ROS
69. Retinol binding protein – RBP
70. Search Tool for the Retrieval of Interacting Genes/Proteins database - STRING
71. Small interfering RNA – siRNA
72. Sodium dodecyl sulphate – SDS
73. Thrombospondin 1 – TSP1
74. Tissue Inhibitor of Matrix metalloproteases – TIMPS
75. Tumor necrosis factor alpha - TNFα
76. Type 1 diabetes – T1D
77. Type 2 daibetes – T2D
78. Vascular cell adhesion molecule – V-CAM
79. Vascular endothelial growth factor – VEGF
80. Vascular endothelial growth factor receptor – VEGFR

CHAPTER 1: INTRODUCTION

Diabetes is a rapidly increasing worldwide problem which is characterized by defective metabolism of glucose that causes long-term dysfunction and failure of various organs. The most common complication of diabetes is diabetic retinopathy (DR), which is one of the primary causes of blindness and visual impairment in adults. Diabetic retinopathy (DR) is a result of damage to the microvasculature in the retina caused by prolonged hyperglycemia, hypertension and other factors in patients with diabetes mellitus. Nowadays, approximately 4 million people worldwide experience blindness or severe vision loss caused by DR and it is expected that this number will significantly rise in the upcoming years because of the increasing global prevalence of diabetes (Wild, Roglic et al. 2004). The increasing incidence of diabetes, the difficulties in early diagnosis and lack of effective treatments make the development of new therapies for DR an urgent issue. DR is a progressive disease that develops from early asymptomatic vascular and neuronal changes (Biallosterski,Vanvelthoven et al. 2007) into a vision-threatening complication.

Clinically DR is classified as non-proliferative diabetic retinopathy (NPDR), proliferative DR (PDR). Fundoscopic findings characteristic for NPDR include micro-aneurysms, intra-retinal hemorrhages, dilation and beading of retinal veins, capillary non-perfusion, exudate deposition and edema (DME), whereas PDR is characterized by intra-ocular neovascularisation, vitreous hemorrhages and fibrosis that may lead to retinal detachment and vision loss. Hyperglycemia is known to induce pericyte death, loss of blood-retinal barrier (BRB) properties, vessel regression and thickening of the basal lamina of retinal capillaries (Hammes , Feng et al . 2011; Yuan SY, Breslin et al. 2007; Klaassen, Van Noorden et al. 2013)

The current therapies for DR are based on multilevel approaches that combine normalization of blood pressure and glycemic control with ocular treatments such as laser photocoagulation, vitrectomy and various pharmacological therapies (Yang, Liu et al. 2009) Laser photocoagulation became the standard care treatment for PDR since 1985. Although laser photocoagulation was shown to reduce the risk of visual acuity loss, it may lead to several complications including loss of central visual acuity, decreased contrast sensitivity, impaired color vision and headaches (Yang, Liu et al. 2009). Despite a tight glucose control and laser therapy, up to 13% of patients with PDR continue to have progressive vision loss (Arevalo.

2013). Pharmacological therapies with corticosteroids have short-term beneficial therapeutic effects on retinal thickness and visual acuity in PDR. However, this type of intervention may lead to side effects that include elevated intraocular pressure, cataract and injection-related complications, such as vitreous hemorrhages and retinal detachment (Arevalo. 2013; Thomas, Shienbaum et al. 2013). Therefore, pharmacological inhibition of VEGF appears to be the most promising treatment strategy for PDR at the moment. Short-term treatment with anti-VEGF in PDR improved visual acuity and reduced central retinal thickness. In PDR, anti-VEGF treatment induced regression of newly formed vessels and prevented vision loss (Nicholson, Schachat et al 2010; Mitchell, Bandello et al. 2011). However, the long term anti-VEGF therapy may have a negative effect on vision acuity.

Angiogenesis in PDR is a tightly-regulated process of new blood vessel formation from existing vasculature. The angiogenic process is orchestrated by pro-angiogenic factors and inhibitors, as well as interactions between pericytes and endothelial cells (le Noble, Klein et al. 2008; Olsson, Dimberg et al. 2006; Gerhardt, Golding et al. 2003). More than 30 known endogenous angiogenic agents and adipokines were involved in maintaining the angiogenic switch.

VEGF is a well-studied molecule in relation to diabetic microvascular complication. One of the important reports by Adamis et al demonstrated elevated levels of VEGF in vitreous of PDR patients (Adamis, Miller et al. 1994). Discovery and isolation of VEGF initiated the development of anti VEGF therapy in the treatment of PDR (Ferrara, Hillan et al. 2004). IGF-1 is also considered as pro angiogenic adipokine which is found to be elevated in PDR. Apart from its proangiogenic activity, it plays a pivotal neuroprotective role in retinal ganglion cells and also improves survival of *in vitro* neuro-retinal cells even under hypoxic condition (Whitmire, Al-Gayyar et al. 2011). Somatostatin anagloues inhibit IGF-1 (Dal Monte, Cammalleri et al. 2007) are also extensively studied in inhibiting retinal neovascularisation.

Apart from VEGF, IGF-1 other pro angiogenic molecules include leptin (Maberley, Cui et al. 2006), FGF (dell'Omo, Semeraro et al. 2013) are also associated with PDR. PEDF is a known anti-angiogenic adipokine, which is reported to have a protective role in the pathogenesis of PDR (Bouck 2002). Adenoviral vector mediated delivery of PEDF (Gehlbach, Demetriades et al.

2003), Peptides derived from PEDF showed potent inhibition of retinal neovascularisation in oxygen induced retinopathy models (Longeras, Farjo et al. 2012).

APN is a protein hormone having anti-diabetic, anti angiogenic and anti-inflammatory properties. APN is a multi functional protein studied widely in association with diabetes and its complication. Recently AdipoRon, small molecule increased insulin sensitivity and glucose tolerance in mice. (Okada-Iwabu, Yamauchi et al. 2013). Recently elevated levels of APN in sub retinal fluid (Ricker, Kijlstra et al. 2012) and aqueous humor (Mao, Peng et al. 2012; Costagliola, Daniele et al. 2013) indicates its association with ocular physiology and pathology. Its presence in ocular tissues has been assumed to seep from circulation due to the dysfunctional BRB. There are no reports on the intraocular expression or localisation of APN. Role of APN in PDR was not yet identified. Identifying the role of APN in relation to PDR would throw light and pave way for better treatment of diabetic retinopathy

This study aimed at highlighting the intraocular expression and localisation of APN and identifying the role of APN in PDR. The purpose of this thesis is to design and develop of therapeutic molecules for PDR. Chapter 2 reviews the current knowledge of diabetes, its complication, and role of APN in particular in the light of recent findings. Scope and objective were mentioned in chapter 3. Detailed methodologies with appropriate references were described in chapter 4. In chapter 5, I have discussed the results and potential functions of this protein, its implication in diseases and possible therapeutic applications of APN in PDR. Part of the work also indicates the designing of anti angiogenic peptide for PDR and emphasized the importance of recognizing small molecules in order to establish the appropriate therapy of diabetes and to prevent the risk of DR. In chapter 6, a general discussion and summary of this thesis are presented. Significant conclusions from this thesis are summarized in chapter 7.

CHAPTER 2: REVIEW OF LITERATURE

2. Diabetes Mellitus:

2.1. Introduction:

Diabetes mellitus (DM), a metabolic disorder has become a common disease with devastating complications. Diabetes is characterized by chronic hyperglycemia due to improper insulin secretion or activity or both. Polyuria, polydipsia, polyphagia, weight loss, lethargy are the striking symptoms in patients with diabetes. The International Diabetes Federation has reported that around 382 million people are diabetic and this number may boost upto 592 million by 2035 worldwide. India ranked second worldwide on having 63 million people affected with diabetes (Nicholson, Hall et al. 2011). Five to ten percent of diabetic patients fit in to type 1 diabetes whereas majority of about 90-95% fall into the type 2 class of diabetes (Konig, Lamos et al. 2013). The worldwide and societal ramifications of the diabetes plague were reported that with changes in human behavior and lifestyle, high fat diet, and obesity increases the incidence of type 2 diabetes around the world (Zimmet, Alberti et al. 2001). Five percent of all death could be attributed to DM (type 1 and 2) in 2000 which increased to 6.8% in 2010 around the world (Roglic, Unwin et al. 2005; Roglic, Unwin et al. 2010).

2.2. Classification of DM:

Classification of DM was first commenced in 1980, later revised in 1985 by World Health Organisation (WHO). The present classification of DM is based on the etiology which was established by WHO along with National diabetes data group (USA). Today DM is broadly classified in to four groups – Type 1 diabetes, Type 2 diabetes, gestational diabetes and diabetes due to other specific diseases as mentioned in table 1(Seino, Nanjo et al. 2010) . Type 1 and Type 2 diabetes are the most common forms whereas the others are rare forms of diabetes.

Table 1: Classification of DM

I.	Type 1 Diabetes- insulin dependent diabetes mellitus (IDDM) – Absolute insulin deficiency
	Immune mediated
	Idiopathic
II.	Type 2 Diabetes – Non insulin dependent diabetes mellitus (NIDDM) – insulin secretory defect/ insulin resistance
III.	Other specific types
	Diabetes due to genetic defects
	Diabetes due to other pathological condition or diseases
IV.	Gestational diabetes

2.2.1. Type 1 diabetes:

Earlier terms such as juvenile diabetes, insulin dependent diabetes mellitus (IDDM) have been replaced by Type 1 diabetes (T1D). T1D develops mostly in young age but it can occur in any age group. T1D is reported to be associated with higher morbidity and mortality (Kesavadev, Sadikot et al. 2014). T1D is caused by absolute deficiency of insulin due to destruction of pancreatic β cells (Holt 2004; Seino, Nanjo et al. 2010). T1D is further classified as autoimmune type 1 diabetes (type1a) and idiopathic type 1 diabetes (type1b). Genetic susceptibility (indicated by specific HLA type) along with environmental triggers, such as viral infection; induce islet autoimmunity which in turn leads to progressive loss of pancreatic β cell mass as well as insulin deficiency. Early phase of type 1a diabetes have detectable amount of islet associated antibodies. T1D with absence of autoantibodies or any genetic abnormality with ketoacidosis is grouped in idiopathic type 1 diabetes. Laboratory diagnosis includes hyperglycemia, ketonuria, insulinopenia, and auto antibodies against islets (Marker, Maclaren et al. 2001). T1D patients completely depend on their insulin injection for their treatment. Proper diet with multiple insulin doses will help them in delaying diabetic complications. Various insulin analogues having short

and long half life, technology aided insulin delivery and continuous glucose monitoring are helpful in the control of blood glucose in patients with type 1 diabetes (Aathira, Jain et al. 2014).

2.2.2. Type 2 diabetes:

Type 2 diabetes mellitus (T2D) is more widespread form of diabetes with increased prevalence than T1D. T2D is also referred to as non-insulin dependent diabetes. This type of diabetes is due to insulin resistance with relative insulin deficiency (Kuzuya, Nakagawa et al. 2002; Seino, Nanjo et al. 2010). T2D have a strong genetic predisposition unlike T1D. Maturity onset diabetes of the young (MODY) occurs in the young adult is often misdiagnosed as T1D. MODY is reported to be inherited in the family which can be due to the mutation in various genes (Fajans, Bell et al. 2001) viz rate limiting enzyme glucokinase mutation also leads to MODY (Polonsky 1995).

2.2.2.1. Genes involved:

T2D is a polygenic disease which involves interaction of defects in multiple genes. Genes involved in pancreatic β cell function, insulin action, glucose metabolism, lipid metabolism, energy intake and expenditure are the most promising candidate genes in T2D. CAPN10 which encodes for calpain 10, HNF4A which encodes for heptocyte nuclear factor 4 alpha, TCF7L2 (Transcription factor 7 like 2), PPARγ have been identified as major genes associated with the risk for T2D. Apart from these identified genes, few more genes are found to be associated with T2D viz ADIPOQ, NOS3, INSR, IRS1, IRS2, FOXO1, RBP4, SIRT1 (Freeman, Cox et al. 2006). ADIPOQ encodes for adiponectin (APN) which is found to be lower in T2D (Snehalatha, Mukesh et al. 2003). APN enhances insulin sensitivity, decreases influx of non-essential fatty acids, increases fatty acid oxidation, and reduces hepatic glucose output in liver. In muscle, APN stimulates glucose use and fatty acid oxidation (Hotta, Funahashi et al. 2001; Yamauchi, Kamon et al. 2003). RBP4 codes for retinol binding protein (RBP) which positively correlates with insulin resistances (Cao 2014). SIRT1 and FOXO 1 also increases the insulin secretion thereby involved in glucose hemostasis. Genes like INSR (insulin receptor), IRS1, and 2 (Insulin receptor substrate 1 and 2) are involved downstream of insulin signaling (Freeman, Cox et al. 2006). Figure 1 depicts overall genes associated with the risk of T2D.

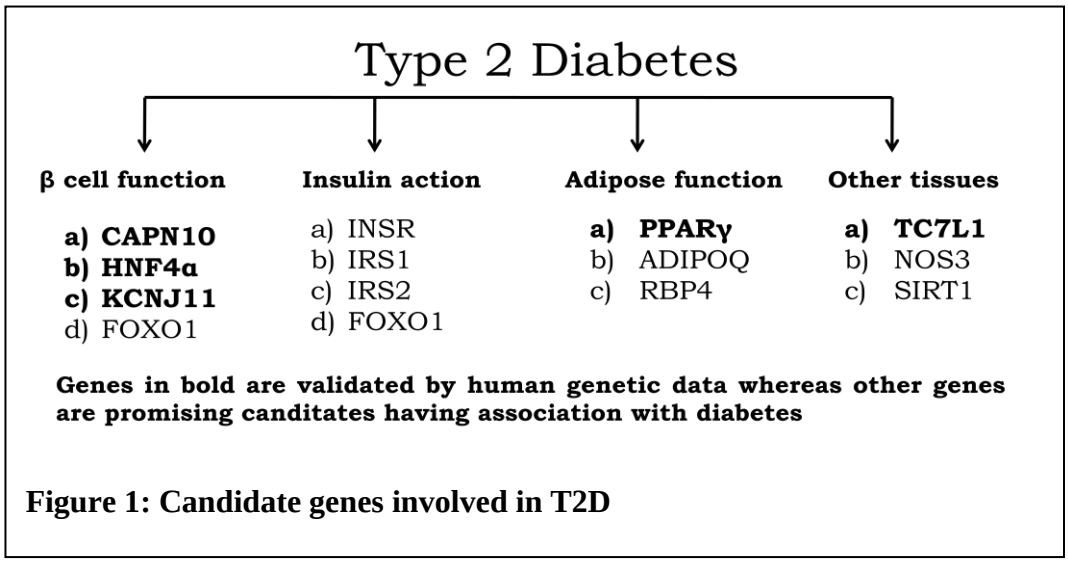


Figure 1: Candidate genes involved in T2D

2.2.2.2. Environmental factors:

Apart from the genetic predisposition various environmental factors act as a trigger for the development of T2D such as obesity and physical inactivity.

2.2.2.2.1. Obesity:

Obesity has become a major burden worldwide. About 1.1 billion people are overweight across the world among which 312 million are obese as reported by WHO and the international obesity task force. Obesity can be measured by calculating body mass index (BMI) and waist to hip ratio (WHR) (Haidar, Cosman et al.2011). About 80% T2D patients are obese. The increased prevalence of obesity not only increase the risk of diabetes it also increases the risk of CVD, dyslipidemia and metabolic syndrome (Hossain, Kawar et al. 2007). Obesity in adulthood is characterized by adipocyte hypertrophy (Berg, Combs et al. 2002). In obesity due to adipocytes hypertrophy, hypoxia occurs resulting in increased secretion of pro inflammatory adipokines as well as decreased production of APN which increase the risk of diabetes. Besides its storage function, adipocytes gain importance as an endocrine organ secreting number of adipocytokines. Adipocyte dysfunction is constantly observed and reported to be associated with development of diabetes (Hajer, van Haeften et al. 2008).

2.3. Complications of diabetes:

Macrovascular complication of diabetes includes cardiovascular diseases stroke and heart attack.

2.3.1. Cardiovascular disease (CVD):

Association of tight glycemic control and macrovascular complication is very minimal. Around 70% of death in T2D is attributed to CVD (Laakso 1999). Obesity, hypertension, microalbuminuria, dyslipidemia increase the risk of CVD in diabetic patients. T2D patients have a higher risk of having CVDs like stroke (Lehto, Ronnemaa et al. 1996), myocardial infarction (Haffner, Lehto et al. 1998), ischemic heart diseases (Almdal, Scharling et al. 2004; Buyken, von Eckardstein et al. 2007).

2.3.1.1. Pathophysiology of CVD:

Hyperglycemia increases advanced glycation end products (AGE) which in turn induce oxidative stress. The production of superoxide in endothelial cells reduces the eNOS activity (Yan, Schmidt et al. 1994; Inoguchi, Li et al. 2000). Diabetic dyslipidemia viz decresed HDL cholesterol and ApoAI also lead to diabetic atherosclerosis (Mazzone, Chait et al. 2008). Furthermore hyperglycemia leads to adipocytes inflammation which reflects in the increased secretion of proinflammatory cytokines, free fatty acids and decreased APN production (Hajer, van Haeften et al. 2008). Diabetic ateriopathy starts with endothelial dysfunction followed by a series of events like inflammation, hypercoagulability, reduced nitric oxide production, vascular wall injury and so on. Tight glycemic control , BP control along with lipid lowering drugs and anti inflammatory drugs may be useful in the prevention of cardiovascular risk (Vinik, Vinik et al. 2003).

2.3.2. Microvascular complications:

Microvascular complications of diabetes include diabetic retinopathy (DR), Diabetic nephropathy (DN) and diabetic sensor polyneuropathy (DSPN).

2.3.2.1. Pathophysiology of microvascular complications in diabetes:

There are multiple factors that contribute to the progression and pathogenesis of microvascular complications. Many causative biochemical pathways have been identified to explain the pathogenesis.

2.3.2.1.1. Polyol pathway:

The surplus glucose is metabolised to sorbitol by aldose reductase and then sorbitol is further metabolised to fructose by sorbitol dehydrogenase (Hotta 1997). During uncontrolled diabetes hyperglycemia increases flux through the polyol pathway via the enzymatic activity of aldose reductase, leading to increase in intracellular sorbitol concentrations. Sorbitol accumulation leads to osmotic imbalance and oxidative stress. They also lead to basement membrane thickening, pericyte loss, and microaneursym formation (Lorenzi 2007).

2.3.2.1.2. Hexoseamine pathway:

Normally a minimal amount of glucose enters the hexoseamine pathway for the synthesis of glycoproteins, proteoglycans, gangliosides and glycolipids. Fructose-6–phosphate is converted to glucosamine-6-phosphate by glutamine fructose-6-phosphate amido transferase (GFAT). Formed glucosamine-6-phosphate is metabolised to UDP-N-acetyl-glucosamine (Werstuck, Khan et al. 2006). O-linked beta-N-acetylglucosamine (O-GlcNAc), glycosylates proteins and also involved in transcription. High concentration of glucose increases UDP-GlcNAc and O-GlcNAc levels which lead to oxidative stress, chronic inflammation and insulin resitance (Tarr, Kaul et al. 2013).

2.3.2.1.3. Protein Kinase C pathway:

Protein kinase C (PKC) is a group of 10 enzymes which are serine/threonine kinases involved in signal transduction. Increase in diacylglycerol (DAG) production due to hyperglycemia in diabetes is an activating factor for PKC isoforms. PKC isoforms and DAG are increased in diabetes and are involved in the pathologic complications associated with hyperglycemia (Tarr, Kaul et al. 2013). Inhibition of PKC- β decreases vascular endothelial growth factors (VEGF), an

important mediator of ocular neovascularisation and also stimulates the induction of apoptosis (Yoshiji, Kuriyama et al. 1999).

2.3.2.1.4. Advanced glycation end products (AGE) pathway:

The nonenzymatic reaction of the amino groups of amino acids, peptides and proteins with reducing sugars, ultimately result in the formation of complex brown pigments and protein-protein crosslinks. Methylglyoxal (MG) is an important glycolytic intermediates and derived from autoxidation of sugars (Phillips, Thornalley et al. 1993). In diabetes (both Type I and Type II) the concentration of MG was reported to be increased in the lens, blood and kidney. As MG major glycating agent, increase in its level inturn increases macromolecule damage, thereby increases ROS production leading to oxidative stress(Chen, Curtis et al. 2013).

2.3.2.1.5. Oxidative stress:

If the balance between oxidants and antioxidants is not protected properly it leads to severe cell damage. Free radicals play an important role in pathogenesis of many diseases like DM, atherosclerosis, aging, etc. ROS increase with auto-oxidation of glucose and glycosylated proteins in DM, and with the activation of the sorbitol pathway during hyperglycemia. Insufficient neutralization of free radical causes the oxidation of cellular lipids, proteins, nucleic acids, glycolipids and glycoproteins. Oxidative stress increases caspases leading to apoptosis. This oxidative effect also causes damage to the vascular endothelial cells (Kowluru, Chan et al. 2007; Eshaq, Wright et al. 2014).

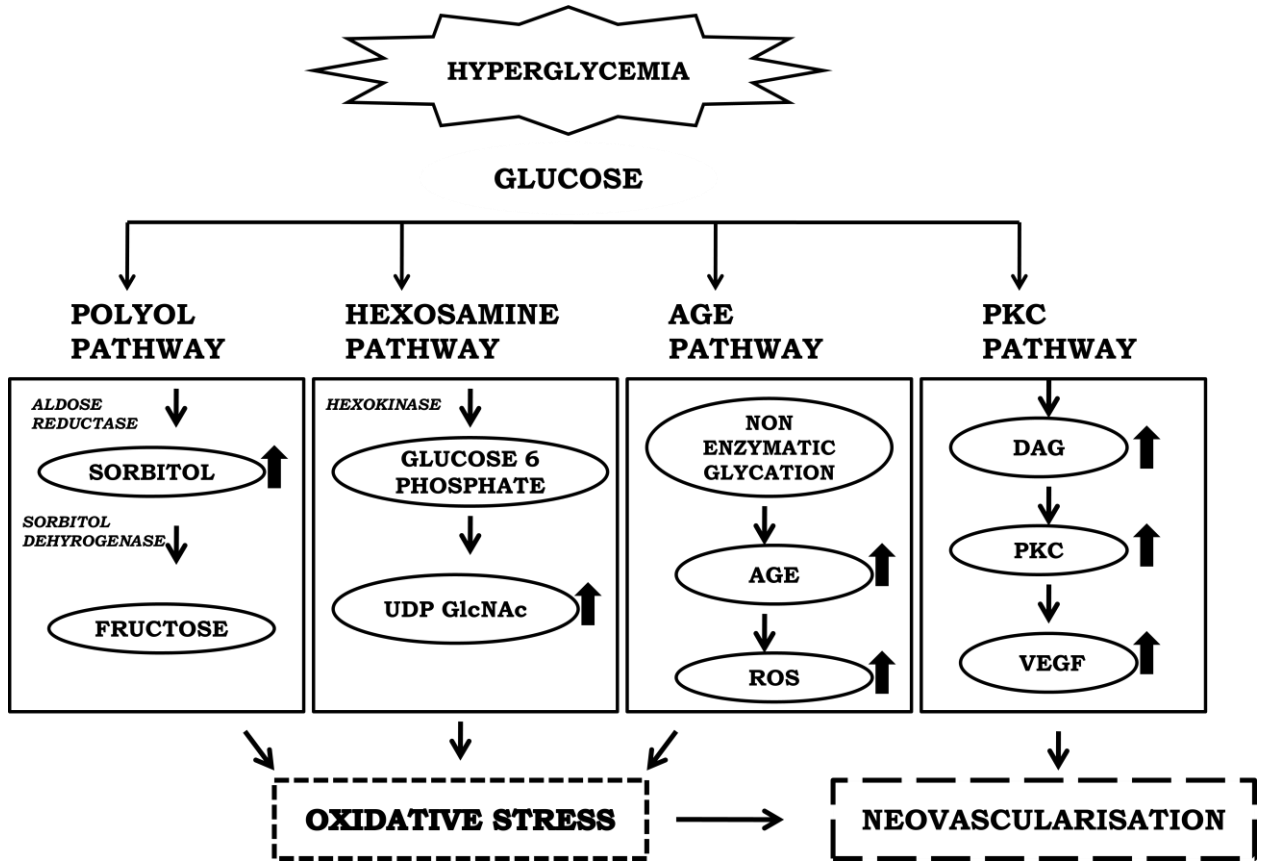


Figure 2: Schematic representation of overall molecular mechanism involved in pathogenesis of microvascular complication.

2.3.2.2. Diabetic Neuropathy:

Sensormotor ployneuropathy is the most common diabetic neuropathy with the symptoms of tingling, loss of sensation, numbness and pain. In diabetic sensor motor polyneuropathy (DSPN), damage to the nerves starts with sensory loss progressing to motor function loss in a glove and stock manner. Around 30% of diabetic patients have diabetic neuropathy (Callaghan, Cheng et al. 2012). More than 80% of amputation are due to diabetic neuropathy (Boulton, Vinik et al. 2005).

Vomiting, diarrhoea, postural hypotension, abnormal heart rate, prolonged QT intervals are referred as autonomic neuropathies. Hyperglycemia, damage the peripheral, motor, and autonomic nerve fibers which leads to foot ulceration. 10-g monofilament is the simple screening

test used for the screening of feet. Electromyography is more sensitive in identifying loss of sensation in nerve fibres compared to 10-g monofilament test. Obesity is considered as major risk factor of DSPN (Dyck, Herrmann et al. 2013). Levels of C peptide play an important role in neuropathies and treatment with C peptide has improved the symptoms of DSPN in T1D. Apart from C peptide polyol accumulation, AGE accumulation, oxidative stress, PKC pathway are involved in the pathogenesis of DSPN (Hosseini, Abdollahi et al. 2013).

2.3.2.3. Diabetic nephropathy:

Diabetic nephropathy (DN) is characterized by distinct proteinuria greater than 500mg/24h or albuminuria more than 300mg/24h (Donaghue, Chiarelli et al. 2009). The prevalence of microvascular complication in diabetic individual is about 30.2% in which diabetic nephropathy, accounts for 10.5% (Raman, Gupta et al. 2012). Hyperglycemia, hypertension, tobacco use, dyslipidemia are the risk factors of DN. Microalbuminuria is the first sign of DN, followed by albuminuria, basement membrane thickening of glomerular, microanuerysm, leading to end stage renal diseases and renal failure. There is positive correlation between serum APN levels and albuminuria. In a nephrectomy model, APN hampered albuminuria and fibrosis playing a pivotal protective role (Ix, Sharma et al. 2010).

2.3.2.4. Diabetic retinopathy (DR):

DR is foremost detected and prevalent microvascular complication in majority of the diabetic patients leading to irreversible blindness in individuals between 20 and 74 years of age. The prevalence of DR in diabetic individual was 34.6% among which proliferative diabetic retinopathy (PDR) was about 6.9% in a worldwide based study (Yau, Rogers et al. 2012). Duration of diabetes is the major risk factor of DR. DR is a pathological retinal angiogenesis due to prolonged hyperglycemia.

DR is broadly classified based on the clinical findings as Non proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) as shown in the table 2 (Wu, FernandezLoaiza et al. 2013)

Table 2: Clinical classification of DR

CLASSIFICATION	CLINICAL FINDINGS
Nonproliferative Diabetic Retinopathy (NPDR)	Presence of microaneursym
Minimal NPDR	Atleast one microaneursym
Mild NPDR	Microaneursym along with any one of the following Retinal Haemorrhages/ Hard exudates
Moderate NPDR	Microaneursym and hemorrhage in atleast one of the quadrant along with any one of the following Nerve fibre infract/ Venous beading/ Intraretinal microvascular abnormalities (IRMA)
Severe NPDR	Microaneursym and hemorrhage in all 4 quadrants. Venous beading in 2 or more quadrants/ IRMA in at least 1 quadrant
Proliferative Diabetic Retinopathy (PDR)	New vessel growth on the retina
High-Risk PDR	New vessels on the disc (NVD) of 1/4 to 1/3 or more within the disc area/ Any neovascularisation or pre retinal vitreous haemorrhage
Advanced PDR	Proliferative retinopathy with tractional retinal detachment or with extensive vitreous haemorrhage

2.4. Screening and diagnosis of T2DM:

Type 2 diabetes remains asymptomatic till its complications are diagnosed. More than 50% of people are still undiagnosed as per WHO reports. The new classification system is now well accepted and strictly followed in most of the organizations, laboratories in the diagnosis of DM. WHO recommends all individual above the age 45 yrs, individuals having family history of diabetes, obese individual, and individual with symptoms of diabetes for diabetic screening. **Fasting plasma glucose (FPG), Oral glucose tolerance test (OGTT), glycosylated haemoglobin levels** (Kuzuya 2000) are the three foremost test practiced in laboratory in the screening and diagnosis of diabetes. American Diabetes Association (ADA) recommends estimation of FPG alone in diagnosing diabetes whereas WHO suggest two step strategy of FPG as well as OGTT for definite diagnosis.

FPG is the first diagnostic test carried out in the laboratory in the screening of diabetes. FPG is easy and less expensive test. FPG more than 126 mg/dL is considered as a diabetic stage.

Impaired FPG will be repeated in another day for the confirmation of DM. The gold standard method OGTT is necessary in identifying diabetic patient with normal fasting glucose (Adam, Tarigan et al. 2004). OGTT is less convenient but more sensitive and accurate than FPG alone. OGTT 2 h glucose level more than 200 mg/dL is considered as DM. Glycosylated haemoglobin is an important diagnostic tool in DM. HbA1c $\geq$ 6.5% is considered to be diabetic stage by ADA and Japanese Diabetes Association (JDA). The optimal cutoff value is reduced to 6.1% and HbA1c $\geq$ 5.5% is considered as pre-diabetes (Tankova, Chakarova et al. 2012; Ma, Gao et al. 2013). Few factors like smoking affect HbA1c values. Smokers have high risk of developing diabetes with elevated HbA1c compared to non-smokers (Vlassopoulos, Lean et al. 2013). Combination of FPG along with HbA1c will be more sensitive and specific in identifying diabetic individuals.

Lack of biomarkers makes diagnosis of DM more difficult however additional test like **lipid profile, protein estimation, albumin, globulin, urea, creatinine, electrolytes, lactate** were also done to evaluate the diabetic complications. **Microalbuminuria,** is a vital laboratory test in the prediction of diabetic nephropathy and also independent risk factor of macrovascular complications (Zitkus 2014). Few biomarkers like plasma free amino acids (Kume, Araki et al. 2014), urinary betaine excretion (Schartum, Ueland et al. 2013) foretell the risk of cardiovascular diseases in diabetic patients. Salomaa et al group has reported 31 novel biomarkers in predicting the risk of incident diabetes which includes adipokines like APN, leptin, c reactive protein and ferritin (Salomaa, Havulinna et al. 2010).

2.5. Medications:

Early diagnosis will help patients in receiving treatment before complications develop. Treatment modalities in T2D aim to attain tight glycaemic control which can prevent the progression of the diseases and also reduce the risk of diabetic complications. Incidence of DM reduced by 30 -51% as a result of weight loss, exercise and lifestyle management (Knowler, Fowler et al. 2009). Reports state that tight glycaemic control can reduce the commencement of microvascular and macrovascular complications in DM (Holman, Paul et al. 2008). Objective of the current medications is to maintain tight glycemic controls in diabetic patients. At present drugs like glucagon-like peptide-1 (GLP-1) receptor agonists, dipeptidyl peptidase-IV (DPP-4)

inhibitors, thiazolidinediones (TZDs; glitazones), insulin analogues, biguanides, sulphonylureas, meglitinides, a-glucosidase inhibitors, and synthetic amylin analogues are available for diabetic patients are listed in the table 3 (Nicholson, Hall et al. 2011).

Table 3: List of medications used for T2D

Current drugs used in T2D	**Mode of action**
GLP agonist	Delay carbohydrate digestion
DPP 4 inhibitors	Delay carbohydrate digestion
Glitazones (Rosiglitazone,pioglitazone)	Improves insulin sensitivity
Metformin	Improve insulin sensitivity
Insulin	Mimics physiological levels of insulin
Sulfonylureas drugs	Stimulates insulin production by pancreas

Use of insulin in T2D having lesser secretion will be helpful in mimicking the physiological level however it may increase insulin resistance, weight gain , obesity and cardiovascular risk (Wyne, Mora et al. 2007). Sulfonylurea and non sulfonylurea drugs decrease plasma glucose by stimulating insulin production by pancreas. They are given in combination with other anti hypoglycemic drugs for the treatment (Whitelaw, Clark et al. 2000). GLP-1 agonist drugs reduce plasma glucose, HbA1c levels and also body weight thus maintain glycaemic control and reduce the risk of obesity related diseases. Increased risk of pancreatitis was reported in clinical trials (Buse, Henry et al. 2004; Amori, Lau et al. 2007). DPP4 inhibitors have similar effects as of GLP-1 agonist drugs but with an advantage of longer half-life (Pratley, Salsali et al. 2007).TZDs and metformin are given as the second line treatment of DM. They are mostly insulin sensitizers which act mainly on adipocytes and muscles. TZDs (Pogiglitazone and Roziglitazone) increase APN levels, increase the efficiency of glucose transporters and thereby decrease plasma glucose levels (Fonseca, Kulkarni et al. 2008).

2.6. Current treatment strategies in DR:

Foremost treatment in management of PDR is by controlling systemic factors like blood glucose levels, lipid levels, blood pressure, proper BMI which remains as a difficult task for most of the diabetic patients. Thus, local treatments for ocular management in PDR patients play a crucial

role. Ocular treatment for PDR includes vitreoretinal surgery, laser photocoagulation, intra vitreal injections of anti VEGF molecules (VEGF inhibitors) and corticosteroids.

2.6.1. Laser Photocoagulation:

Laser photocoagulation is the most widely used standard treatment in PDR. Diabetic retinopathy study confirmed that the treatment of retinal neovascularisation was by applying argon or xenon arc light in scattered pattern on the retina (1976) which has a spot size of 50-500 µm. Retinal photocoagulation reduces diabetic macular edema as well as neovascularisation in diabetic retinopathy. Laser photocoagulation destroys the outer retina thereby increasing the oxygenation of the inner retina which in turn decreases the pro angiogenic growth factors (Stefansson 2006). Hard exudates and retinal thickening caused by micro aneurysms are treated by focal laser treatment whereas diffused area of leakage in the retina are treated by grid treatment (Mohamed, Gillies et al. 2007).

2.6.2. Vitreoretinal surgery:

Blackenship etal demonstrated that retinal neovascularistion in PDR does not progress after successful vitrectomy (Blankenship, Machemer et al. 1978). Vitrectomy was done to improve the visual acuity of the patient having vitreous hemorrhage or in eyes with tractional retinal detachments and in active progressive PDR. The Diabetic Retinopathy Vitrectomy Study (DRVS) demonstrated that patients who underwent early vitreoretinal surgery had better outcome than those treated conservatively. Vitrectomy relives the hypoxia in the ischemic areas. Fluid currents in the vitreous cavity transports oxygen to the ischemic areas. Laser treatment combined with vitrectomy was proven to be more beneficial in treating PDR. Both these treatment modalities increase the oxygenation in the inner retina and also have indirect effect in decreasing VEGF and retinal neovascularisation (Stefansson 2006).

2.6.3. Anti VEGF treatments:

Inhibition of VEGF activity may play a pivotal role in the prevention of DR (Yam, Kwok et al. 2007). Three VEGF antagonists which are currently under investigation are Lucentis (Ranibizumab), Avastin (Bevacizumab), and Macugen (Pegaptanib sodium).

Ranibizumab, a recombinant humanized monoclonal antibody fragment with specificity for all isoforms of human VEGF, has been used for treatment of neovascular age related macular degeneration (AMD) and may also be useful for DR and Diabetic Macular Edema (DME). Intravitreal dosage of ranibizumab is 0.5 mg. Lucentis is designed for better retinal penetration and has greater activity *in vitro* (Bhisitkul 2006).

Bevacizumab is a full-length humanized monoclonal antibody against VEGF which appears to produce similar efficacy for treatment of neovascular AMD, DME and PDR. Typical dose is 1.25 mg, although doses as low as 6.2 μg and as high as 2.5 mg have been used (Simo, Hernandez et al .2008; Yang, Hung et al. 2013).

Pegaptanib sodium is an aptamer that binds the VEGF-A 165 isoform. Aptamers are chemically synthesized and modified oligonucleotides composed of single-stranded nucleic acids. It adopt a specific three-dimensional conformation, allowing them to bind with high specificity and it prevents only VEGF-165 and larger isoforms from attaching to the VEGF receptors (Pai, El Shafei et al. 2010)

VEGF trap eye is the next upcoming drug targeting VEGF which is in phase III clinical trial. It is a recombinant fusion protein consisting of the VEGF binding domains of VEGFR-1 and VEGFR-2 fused to the Fc domain of human immunoglobulin-G having higher binding affinity for all VEGF-A isoforms. In addition, VEGF Trap-eye maintains significant intravitreal VEGF-binding activity for 10–12 weeks after a single injection (Do, Nguyen et al. 2009). Most of the drugs which are in clinical trial targets VEGF and inhibition of VEGF pathway (Sherris 2007).

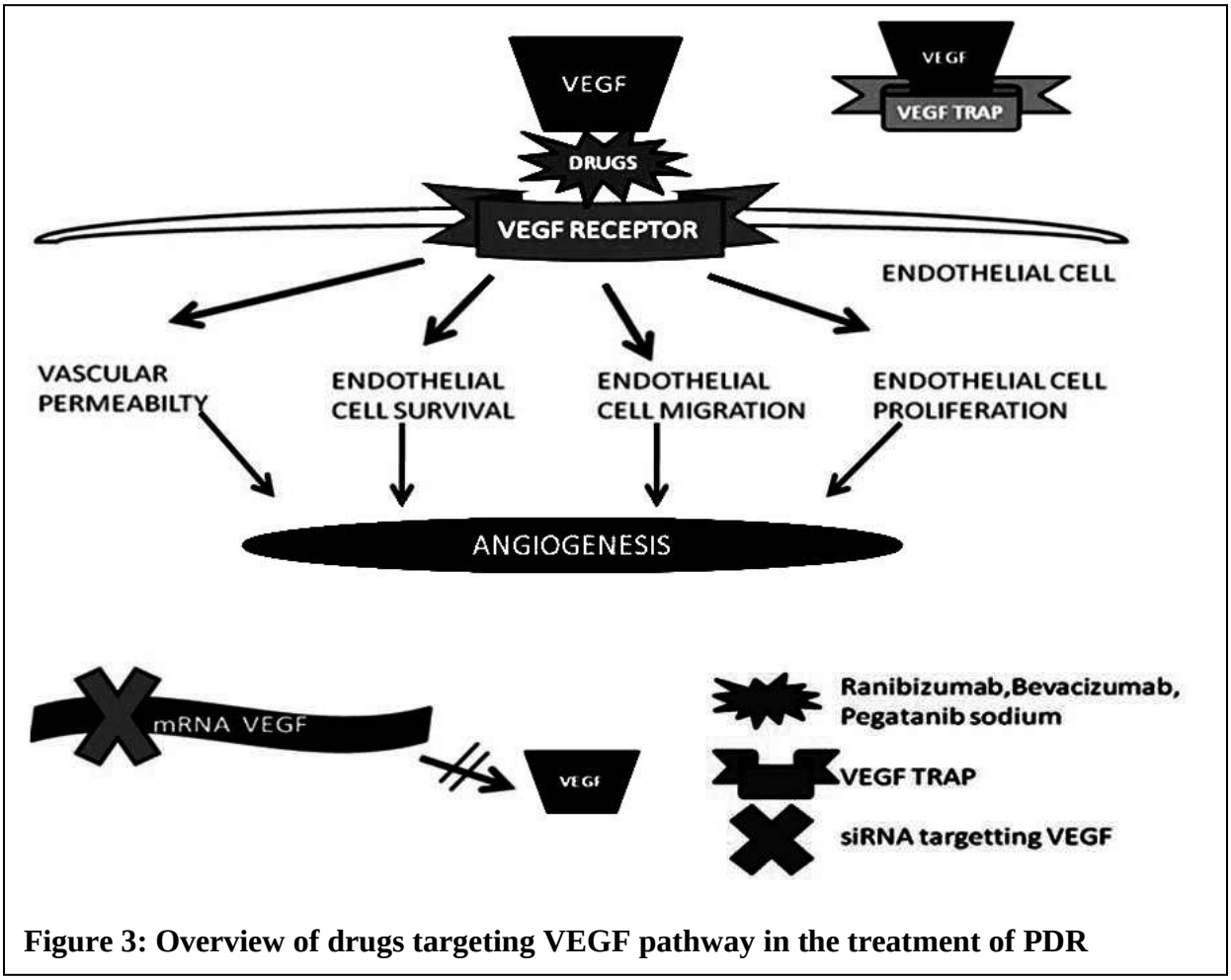


Figure 3: Overview of drugs targeting VEGF pathway in the treatment of PDR

2.6.4. Corticosteroids:

Mechanisms of action of the intraocular corticosteroids are by down regulating VEGF and they are also anti-inflammatory. Intravitreal triamcinolone (IVTA) has shown significant improvements in DME and visual acuity in various random clinical trials (RCTs) (Gillies, Sutter et al. 2006). However, steroid-induced elevation of intra-ocular pressure (IOP) and steroid-induced cataract are commonly reported as adverse effects of IVTA.

2.7. Limitations in the current treatment modalities:

The drawback in laser treatment is that it destroys the peripheral retina to save central retina. Every laser spot leaves a scar on the retina thereby reduce the vision in the area of the treatment. Moreover laser treatment cannot stop the progression of the DR (Petrovic, Bhisitkul et al. 1999). In case of vitrectomy, procedure is invasive and chances of endopthalmitis and retinal

detachment are notable. The anti-VEGF agents to stop VEGF mediated angiogenesis, also have complications like repeated injections to maintain the therapeutic level, cost of the antibody, tractional retinal detachment and endopthalmitis (Ni, Hui et al. 2009).These negative aspects of present treatment drive us forward in identifying newer, effective therapeutic modalities and agents.

2.8. Newer therapeutic approaches and targets in ocular angiogenesis:

Gene therapy, peptide therapy, small molecule which increases anti angiogenic factors are the novel approaches gaining significance in the treatment of ocular angiogenesis.

1. **Gene therapy:** The first gene therapy was initiated for retinal degeneration in humans recently. Gene therapy is the upcoming treatment modality which has emerged over past 20 years. Adenoviral, lentiviral and adeno associated viral vectors are the mostly used viral vectors in ocular gene therapy (Bainbridge, Mistry et al. 2003). Adenoviral vector mediated delivery of PEDF showed betterment in patients with choroidal neovascularisation (Gehlbach, Demetriades et al. 2003) but increased intraocular pressure, intraocular inflammation observed in few patients. Restricted capacity for the insert DNA, oncogenesis, systemic inflammation, adverse immunological response, long term safety are the major concerns in the use of viral mediated gene therapy (Farjo, Ma et al. 2010).
2. **Peptide therapy:** Peptide therapy is advantageous compared to gene therapy because of its easy production by synthesis using automatic synthesizers, low antigenicity, high solubility in water and improved bioavailability with potential oral drug (Sulochana, Ge et al. 2007). Small peptides can mimic protein function as well as its interactive residues. Peptides derived from PEDF showed potent inhibition of retinal neovascularisation in oxygen induced retinopathy models (Longeras, Farjo et al. 2012). Similarly peptide derived from angiopoietin (Palmer, Tiran et al. 2012), ApoE (Bhattacharjee, Huq et al. 2011) displayed anti-angiogenic activity in retinopathy models.
3. **Small molecules** targeting VEGF and its receptor are being widely studied. Small molecules such as 3-(5'- hydroxymethyl-2'furyl)-1-benzyl indazole (YC1) when injected intravitrealy inhibits retinal neovascularisation effectively (DeNiro, Al-Halafi et al.

2010). Alike YC1, Geldanamycin binds to heat shock protein 90 (Kociok, Krohne et al. 2007), carboxyamidotriazole reduces intracellular calcium (Afzal, Caballero et al. 2010), thiazolidinediones upregulate PPARγ (Higuchi, Ohashi et al. 2010), somatostatin anagloues inhibit IGF-1 (Dal Monte, Cammalleri et al. 2007) are also extensively studied in inhibiting retinal neovascularisation. Table 4 gives a picture of current drugs in clinical trial for the treatment of DR.

Table 4: Molecular targets in clinical trial for the treatment of PDR

Agents	**Mechanism of action**	**Route of administration**	**References**
Sorbinil	Aldose reductase inhibitor, phase III clinical trial	Oral	1990
LY333531	PKC*β*-selective inhibitor, phase III clinical trial	Oral	Sheetz, Aiello et al. 2013
Octreotide	Somatostatin analogue; IGF-1 inhibition	Intravitreal/ Subcutaneous	Dal Monte, Cammalleri et al. 2007
Bevasiranib	siRNA for VEGF, phase II clinical trial	Intravitreal	DeNiro, Al-Halafi et al. 2010
rAAV PEDF	PEDF gene therapy, Phase I Clinical trial	Sub retinal	Gehlbach, Demetriades et al. 2003
VEGF trap	Inhibition of VEGF, Phase III clinical trial	Intravitreal	Do, Schmidt-Erfurth et al. 2011

2.9. Role of adipokines in T2D and its complications:

Adipokines provides a link between diabetes and obesity, diabetes and its complication, adipogenesis and angiogenesis. Adipocyte has become an important endocrine organ in secreting numerous adipokines which are listed in table 5.

2.10. Adipokines:

Numbers of adipokines are involved in maintenance of energy homeostasis, glucose metabolism, and lipid metabolism and they are biomarkers / future therapeutic targets for T2D, obesity and metabolic syndrome.

Table 5: Adipocyte derived proteins and their functions

FACTOR	SOURCE	MAJOR ACTION	REFERENCES
ADIPONECTIN	Exclusively from adipose tissue.	Insulin sensitizer, Depicts adipocytes tissue function, Anti diabetic, Anti atherogenic, Anti inflammatory, Anti angiogenic effects	Brakenhielm, Veitonmaki et al. 2004,Mandal, Park et al. 2010,Tishinsky, Robinson et al. 2012,Iwaki, Matsuda et al. 2003
VEGF	Although a Adipokine, can be produced by hypoxic cells.	Vasculogenesis and Angiogenesis, Permeability factor.	Bhisitkul 2006
PEDF	Adipqose tissue , RPE.	Insulin resistance, anti angiogenic factor	Bouck 2002, Chen, Tso et al. 2010
IGF-1	Liver, adipose tissue.	Mimics insulin, proinflammatory.	Laviola, Natalicchio et al. 2007.
Leptin	Adipose tissue,liver , stomach , mammary epithelial cells.	Involved in the regulation of satiety, Marker of body fat mass, pro angiogenic.	Cao 2014; Park, Ahima 2014
Thrombospondin	Adipose tissue, also widely expressed in the Central Nervous system	Endogenous anti angiogenic factor, Involved CNS development.	Scott-Drew, french-Constant et al. 1997
FGF-2	Adipose tissue, also found to be secreted in Pituitary.	Wound healing, angiogenesis and embryo development.	dell'Omo, Semeraro et al. 2013.
TNF-Alpha	Macrophages, Adipose tissue, Mast cells, Endothelial cells, Fibroblasts, Lymphoid cells, Neuronal tissue.	Type 1 immune response, pro inflammatory	Cao 2014
Interleukin-6	Adipose tissue, T-cells, macrophages, muscle cells.	Pro inflammatory	Cohen, Nahari et al. 1996

Chemerin	Adipose tissue, Liver, lung.	Innate immunity, Adipogenesis.	Goralski, McCarthy et al. 2007
Apelin	Vascular Stromal cells, Heart, adipocytes, Brain, adrenal glands.	Improves glucose metabolism, has hypotensive effect.	Lee, Cheng et al. 2000
Visfatin	Adipose tissue , Liver , bone Marrow and Muscle cells.	Mimic Insulin in glucose homeostasis.	Ronti, Lupattelli et al. 2006
Angiopoietin-1	Adipose tissue.	Vascular maturity, Stability, anti – permeability Factor.	Thurston, Rudge et al. 2000
Angiotensin	Adipose Tissue	Modulator of adipocyte lipid metabolism.	Ronti, Lupattelli et al. 2006
Retinol –Binding Protein(RBP)	Liver, adipose tissue.	Lipid metabolism, Insulin resistance	Janke, Engeli et al. 2006,Yang, Graham et al. 2005
Omentin	Adipose tissue.	Predictor of visceral fat mass.	Auguet, Quintero et al. 2011
vaspin.	Adipose tissue	Reduces appetite, glucose lowering effects	Auguet, Quintero et al. 2011
Resistin	Adipocytes, spleen, monocytes, macrophages, lung, kidney, bone marrow, placenta.	Insulin resistance, systemic inflammation. upregulates VEGF;	Calabro, Samudio et al. 2004; Cao 2014

2.10.1. Leptin:

Leptin is an abundantly secreted adipocytokine which is 16 kDa protein hormone encoded in the Ob *(Lep)* gene (Ob for obese, Lep for leptin) on chromosome 7. Leptin plays an important role in the pathogenesis of obesity and eating disorders and is thought to mediate the neuro-endocrine response to food deprivation (Park, Ahima et al. 2014). Leptin stimulates glucose uptake and fatty acid oxidation in muscles through AMPK (Minokoshi, Kim et al. 2002). Recent reports highlight its implication in cell proliferation, angiogenesis and in apoptotic inhibition (Chia, Newcomb et al. 2007). An elevated level of vitreous leptin in PDR (Maberley, Cui et al. 2006), presence of leptin receptor in fibro vascular membrane (Gariano, Nath et al. 2000) and its angiogenic nature (Park, Kwon et al. 2001) indicate its involvement in the pathogenesis of PDR.

2.10.2. Vascular endothelial growth factor (VEGF):

VEGF is a signal protein which belongs to the sub-family of platelet-derived growth factor. VEGF is essential protein involved in vasculogenesis and angiogenesis. VEGF is a adipocytokine which can be secreted by cells which are exposed to hypoxia.VEGF is also produced by several types of cells viz retinal pigment epithelial cells, glial cells, retinal capillary pericytes, endothelial cells, muller cells and ganglion cells.The VEGF family includes VEGF A, VEGF B, VEGF C, VEGF D and placental growth factor (PIGF). VEGF B is more involved in embryonic angiogenesis, PIGF mediates arteiogenesis whereas VEGF C and VEGF D are involved in lymphangiogensis (Tammela, Enholm et al. 2005). The key member of the VEGF family is VEGF A which involved in angiogenesis, vasodilatation and in stimulating recruitment of inflammatory cells. Gene encoding human VEGF A is organized by 8 exons separated by seven introns. Alternative exon splicing results in the generation of 4 major isoforms - $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$ whereas $VEGF_{145}$, $VEGF_{183}$, $VEGF_{162}$, $VEGF_{165b}$ are less frequent spice variant (Zorena, Raczynska et al. 2013).

VEGF mediates its function through binding to its receptors. VEGF 1, VEGF 2 and VEGF 3 are the major receptors for the VEGF family. VEGF R1 (fms like tyrosine kinase 1, flt-1) and VEGF R2 (fetal liver kinase 1, flk-1) have similar structure with single transmembrane region, seven extracellular immunoglobulin homology domain and intracellular tyrosine kinase domain. VEGF A has more affinity to VEGF R1 than VEGF R2. VEGF R3 (fms like tyrosine kinase 4, flt-4) is for VEGF C and VEGF D (Neufeld, Cohen et al. 1999; Tammela, Enholm et al. 2005)

VEGF was first identified as vascular permeability factor. VEGF is a main hypoxia inducible angiogenic factor. Hypoxia blocks the degradation of hypoxia-inducible factor alpha (HIF 1 alpha), promotes the complex formation of HIF 1α and HIF 1β. The HIF1alpha/beta complex binds to promoter sequence of hypoxia inducible gene and provokes the transcription of various genes including VEGF. Circulating VEGF then binds to VEGF receptors on endothelial cells, triggering a tyrosine kinase pathway leading to angiogenesis (Neufeld, Tessler et al. 1994). Platelet derived growth factor (PDGF), Insulin like growth factor 1 (IGF1), BCL, Epidermal Growth Factor (EGF), COX 2 also induce VEGF production.

VEGF is a well-studied molecule in relation to diabetic microvascular complication. One of the important reports by Adamis et al demonstrated elevated levels of VEGF in vitreous of PDR patients (Adamis, Miller et al. 1994). In PDR, VEGF causes the breakdown of blood retinal barrier, increases vascular permeability and stimulates endothelial cell proliferation and migration leading to neovascularisation in ischemic retina (Tarr, Kaul et al. 2013). Discovery and isolation of VEGF initiated the development of anti VEGF therapy in the treatment of PDR (Ferrara, Hillan et al. 2004).

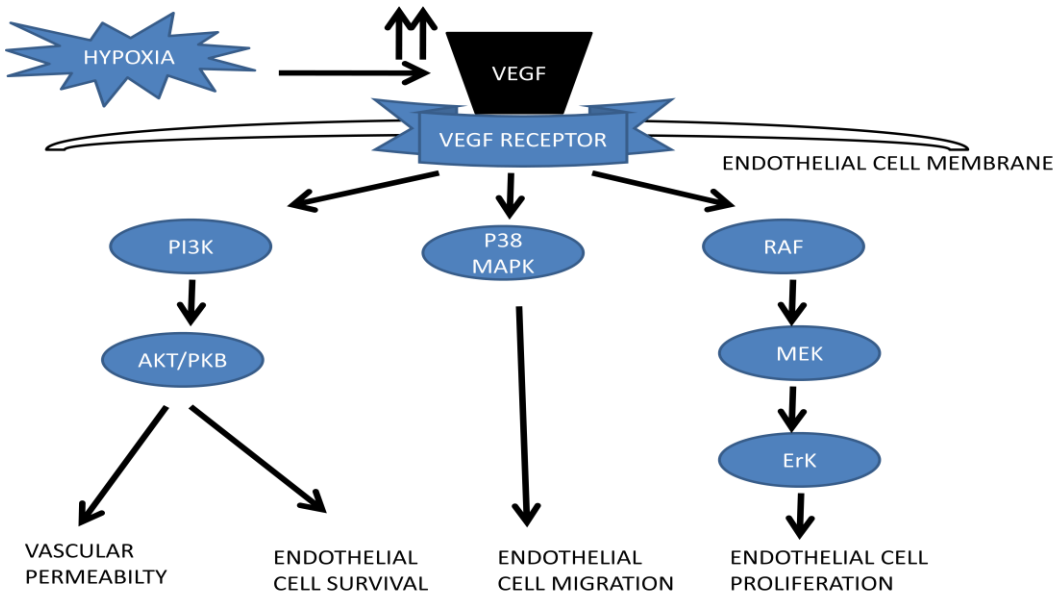


Figure 4: Angiogenic cascade initiated by VEGF

2.10.3. Insulin like growth factor 1 (IGF 1):

IGF 1 is proangiogenic molecule which also has differentiating effect on cells. It is homologous to insulin in structure. IGF receptors have intracellular tyrosine kinase domain alike insulin receptors (Laviola, Natalicchio et al. 2007). Although secreted by adipocytes, it is majorly produced by liver and exist bounded with IGF binding proteins 1-6 in circulation.IGF -1 stimulates cell proliferation (Janssen, Varewijck et al. 2014). IGF plays an important role in pathophysiology of diabetic complication like retinopathy.

Intravitreal injection of IGF 1 initiated retinal neovascularisation and blood retinal barrier breakage in experimental rabbit (Grant, Mames et al. 1993). IGF 1 was also elevated in vitreous of patients with PDR (Meyer-Schwickerath, Pfeiffer et al. 1993). Localisation of IGF-1 and its receptor in the retina (Lambooij, van Wely et al. 2003) indicates its essential role in the pathogenesis of PDR. IGF-1 increases VEGF secretion through HIF1α dependent and independent manner in cultured ARPE 19 cells (Slomiany, Rosenzweig et al. 2006). Apart from its proangiogenic activity, it plays a pivotal neuroprotective role in retinal ganglion cells and also improves survival of *in vitro* neuro-retinal cells even under hypoxic condition (Whitmire, Al-Gayyar et al. 2011).

2.10.4. Fibroblast growth factor (FGF):

FGF family consists of acidic FGF (aFGF) and basic FGF (bFGF) which are involved in wound healing, angiogenesis and in embryonic development. FGF interacts with FGF receptors – FGF R1, R2, R3 and heparin sulphate proteoglycans. FGF receptors possess tyrosine kinase activity similar to VEGF receptors (Simo, Carrasco et al. 2006). FGF induces angiogenesis via MAP kinase pathway and PKC pathway. bFGF is responsible for anti-apoptosis, up regulates VEGF production, increases ECM degradation leading to angiogenesis (dell'Omo, Semeraro et al. 2013).

2.10.5. Interleukins (IL):

Interleukins are the signalling cytokines which play a major role in inflammation as well as neovascularisation. IL 1, IL 10 was reported to be elevated in the vitreous of the patient with PDR. IL 10 positively correlated with VEGF levels. Together with TNF alpha, IL 1 stimulates angiogenesis via collagen synthesis and cell proliferation. Moreover IL1 induces the secretion of IL6 and IL8 in human retinal pigment epithelial cells. IL 6 (Mocan, Kadayifcilar et al. 2006) and IL 8 (Elner, Elner et al. 1995) were also reported to be elevated in aqueous and vitreous humor of the patient with PDR. Their levels correlate with the severity of the disease. IL6 positively correlates with VEGF in gastric carcinoma and upregulates VEGF secretion, increases proliferation of human umbilical vein endothelial cells (HUVEC) (Huang, Wu et al. 2004).

Another study has reported that IL6 promotes cervical tumor by inducing VEGF mediated angiogenesis via STAT3 pathway (Wei, Kuo et al. 2003).

2.10.6. Tumor Necrosis Factor Alpha (TNFα):

TNFα is proinflammatory cytokine secreted by macrophage and monocytes. Apart from its proinflammatory function, it also promotes neovascularisation. TNFα destabilizes the tight junction interrupt blood retinal barrier required for angiogenesis (Zorena, Raczynska et al. 2013). It stimulates the production of IL6 and IL8, extracellular matrix protein, and monocyte chemotaxis. Hypoxia and methylgloyoxal increases the expression of TNFα. Elevated serum levels of TNFα was found in patients with T1D and in NDPR. Elevated intravitreal levels of TNFα, TNF receptors I and II were reported in patients with PDR (Demircan, Safran et al. 2006).

2.10.7. Pigment Epithelium Derived Factor (PEDF):

PEDF is a known anti-angiogenic adipokine, which is reported to have a protective role in the pathogenesis of PDR (Bouck 2002). It is secreted as 50 kDa protein by retinal pigment epithelial cells and was first purified by Tink et al from the conditioned medium of RPE. PEDF belongs to SERPIN family. Gene encoding human PEDF is localised on chromosome 17.

There are reports stating that serum PEDF levels are elevated in diabetic patients (both type1 and type2) and it is positively correlated with body mass index (BMI). PEDF is strongly linked with metabolic syndrome and reported to be predictor of metabolic syndrome (Chen, Tso et al. 2010; Famulla, Lamers et al. 2011). PEDF induces insulin resistance and inflammatory signals in adipocytes. Even though PEDF is said to be proinflammatory in adipocytes and muscle, its anti tumorogenic and anti angiogenic property are well studied in various tumors and ocular angiogenesis.

PEDF binds to PEDF receptor (PEDFR), laminin receptor and low density lipoprotein receptor related protein 6 (LRP6). PEDFR have phospholipase activity which uses phospholipids as a substrate from the lipid bilayer and releases docosahexaenoic acid (DHA) and arachidonic acid (AA). These released DHA makes complex with fatty acid binding protein (FABP7) and these

complexes are translocated into nucleus which in turn increases NFKB transcription, decreases interleukin 8 and increases tumour cell apoptosis (Becerra, Notario et al. 2013).

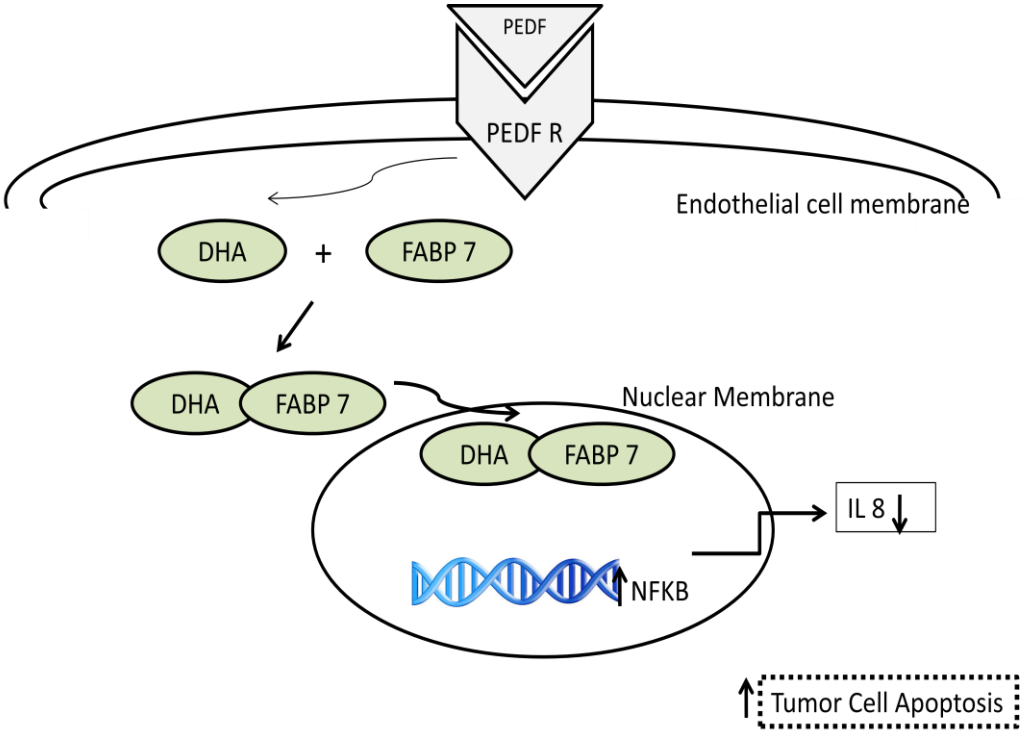


Figure 5: Anti angiogenic mechanism of PEDF.
DHA: Docosahexaenoic acid; FABP7: Fatty acid binding protein; IL 8: Interleukin 8

PEDF levels in the vitreous were significantly lower in patients with PDR and proportional to ocular neovascularisation (Wang, Feng et al. 2012). Besides its anti angiogenic role, PEDF also plays numerous other roles. PEDF protects neural cells and involved in the development of photoreceptors and their survival in retina (Bouck 2002). Recently, Maeda et al, have shown that PEDF could block RAGE-induced APN gene suppression in adipocytes when exposed to AGE through its anti-oxidative properties (Maeda, Matsui et al. 2011). PEDF causes apoptosis in proliferating endothelial cell. It acts as an antagonist to VEGF and counteracts VEGF induced vascular permeability. PEDF is the second most targeted molecule in the treatment for ocular angiogenesis.

2.10.8. ADIPONECTIN (APN):

Among several dozens of adipokines identified so far, APN have attracted attention due to their potential as therapeutic targets for treating obesity and obesity-related vascular disease. Moreover APN exerts pivotal role in the regulation of insulin, vascular homeostasis, anti-inflammatory, anti-angiogenic and anti-atherogenic effects.

APN is a 30 kDa adipocytokine with 244 amino acid encoded by the apM1 gene located on chromosomes 3 (Kawano, Arora et al. 2009). It is also referred to as GBP-28, apM1, AdipoQ and Acrp30. APN belongs to C1q globular domain protein family with structural homology of TNFα. APN has four distinct regions - N terminal short signal sequence (1-18aa), short region that varies between species (19-41aa), 65 amino acid region with similarity to collagenous protein (42-107aa), C terminal globular domain (108-244aa) (Kadowaki, Yamauchi et al. 2005).

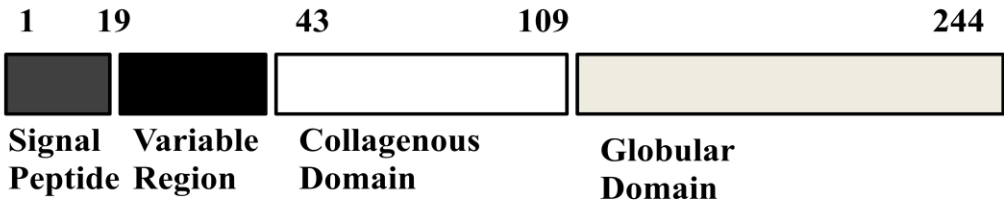


Figure 6: Structure of full length APN

APN can exist as full-length or as globular fragment. Measurable amount of globular APN was detected in plasma (Fruebis, Tsao et al. 2001). Leukocyte elastase secreted from activated monocytes and/or neutrophils cleaves APN to form globular fragment. APN forms a wide range of multimers from trimers (Low molecular weight, LMW), hexamers (Middle molecular weight, MMW) to high molecular weight (HMW) multimers such as dodecamers and 18 mers. HMW forms of APN are the most biological active form with the half-life of around 9 hrs whereas the other forms have less half life (Radin, Sharkey et al. 2009). Different oligomeric forms activate different signaling pathways (Tsao, Tomas et al. 2003).

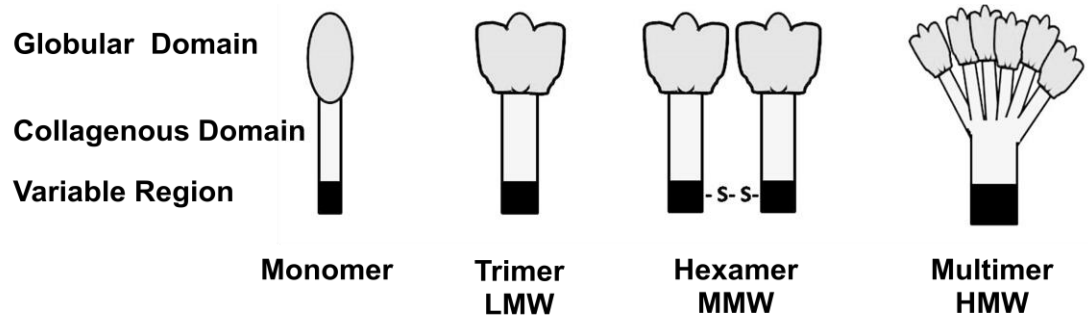


Figure 7: Multimeric form of APN

2.10.8.1. Secretion and localisation:

APN is secreted by mature adipocytes (Radin, Sharkey et al. 2009). There are few reports which demonstrate the presence of APN transcripts in myocytes (Delaigle, Jonas et al. 2004), osteoblasts (Berner, Lyngstadaas et al. 2004), liver endothelial cells (Kaser, Moschen et al. 2005), cardiocytes (Pineiro, Iglesias et al. 2005) and placental tissue (Caminos, Nogueiras et al. 2005). Recently Mori et al localised APN in luminal surface and endocytic vesicles of endothelial cells in normal aorta whereas in atherscelortic lesion APN was also detected in Smooth muscle cells and in monocyte adhering to endothelial cells (Mori, Koyama et al. 2014)

2.10.8.2. APN receptors:

Yamauchi etal reported the cloning of complementary DNAs encoding APN receptors 1 and 2 (AdipoR1 and AdipoR2) by expression cloning. Human AdipoR1 is located on chromosome 1p36.13-q41, whereas AdipoR2 is located on chromosome 12p13.31 (Yamauchi, Kamon et al. 2003).

APN binds to its receptors AdipoR1 and AdipoR2. AdipoR1 found in skeletal muscle and AdipoR2 is found in liver. These two receptors have seven transmembrane domains with homology to G protein-coupled receptors. Both receptors are structurally distinct from most other seven transmembrane proteins, because of their extracellular located C-terminus and cytosolic N terminus. AdipoR1 has high affinity for globular APN and low affinity for full APN

whereas AdipoR2 exhibits intermediate binding affinity for both globular and full APN (Kadowaki, Yamauchi et al. 2006). It has been reported that brain endothelial cells also express both receptors but not APN (Spranger, Verma et al. 2006).

Other than AdipoR1 and AdipoR2, APN binds to T-cadherin. T-cadherin is glycosyl phosphotidylinositol anchored extracellular protein which is expressed in endothelial and smooth muscle cells. Hexameric and high molecular weight APN alone binds to T-cadherin. An APN mutant lacking N-terminal cysteine residue required for formation of trimeric or high molecular weight species did not bind T-cadherin. APN interacts with T.cadherin and restricts the proliferation of the intima (Hug, Wang et al. 2004).

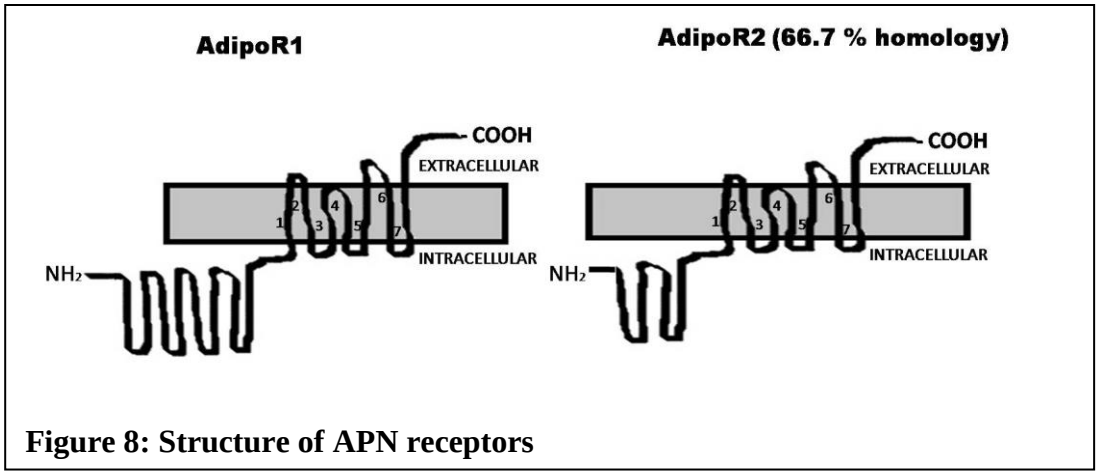


Figure 8: Structure of APN receptors

Plasma APN levels are reduced in patients with obesity (Arita, Kihara et al. 1999), in patients with T2D (Hotta, Funahashi et al. 2001; Weyer, Funahashi et al. 2001) and also in patients with coronary artery disease (Kumada, Kihara et al. 2003). The most portrayed functions of APN are its insulin sensitivity, vascular protective role, anti-inflammatory and anti-angiogenic properties.

2.10.8.3. Role of APN in obesity:

In obese subject, APN levels negatively correlated with body mass index (BMI) and plasma triglycerides concentration but positively correlated with HDL cholesterol (Cnop, Havel et al. 2003). Heterozygous peroxisome proliferator-activated receptor-γ knockout mice were protected from high-fat diet induced obesity, adipocyte hypertrophy and insulin resistance. Systematic gene profiling analysis of these mice revealed that APN was over expressed. Functional analyses of APN transgenic or knockout mice have revealed that APN serves as an insulin sensitizing

adipokine (Kadowaki, Yamauchi et al. 2006). Decreased expression levels of AdipoR1/R2, reduced APN sensitivity leads to insulin resistance, the so called vicious-cycle in obese patients. APN receptor agonists and APN sensitizers should serve as versatile treatment for obesity-linked diseases such as diabetes and metabolic syndrome.

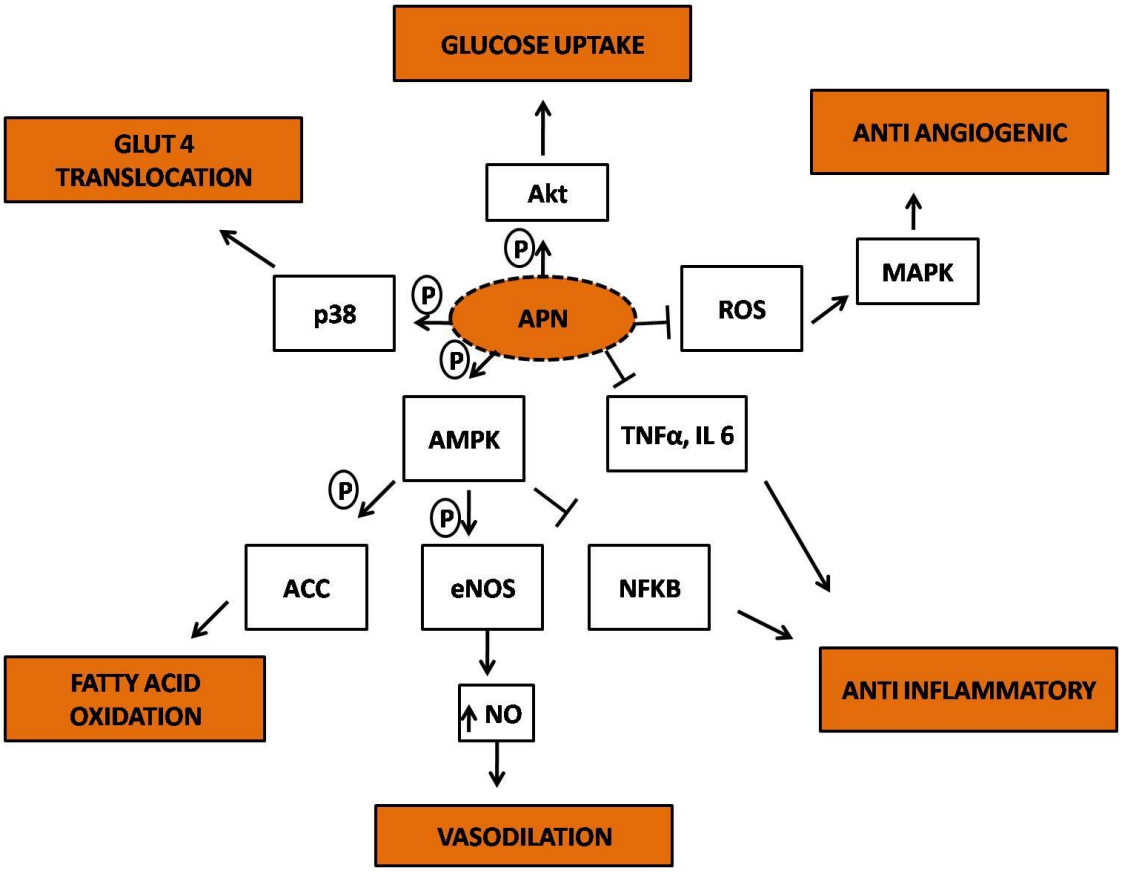


Figure 9: Multifunctional effect of APN.

NO – Nitric oxide, eNOS – Nitric oxide synthase, ROS – Reactive oxygen species, IL 6 – interleukin 6, TNFα – Tumor necrosis factor alpha, MAPK – Mitogen activated protein kinase, ACC – Acetyl CoA carboxylase, AMPK – AMP activated protein kinases

2.10.8.4. Role of APN in diabetes:

Numerous reports showed the association of APN with diabetes. APN knockout mice showed impaired insulin sensitivity indicating the importance of APN as an insulin sensitizer (Bluher 2014). In another study, recombinant full length APN, globular APN reduced hyperglycemia in

alloxan treated rats (Hu, Zhang et al. 2003). Over expression of APN protected mice against diabetes, obesity. APN negatively correlate with glycosylated hemoglobin, plasma glucose (Stejskal, Ruzicka et al. 2003). Recently AdipoRon, small molecule increased insulin sensitivity and glucose tolerance in mice. Moreover AdipoRon improved diabetes and increased the life span in mouse model (Okada-Iwabu, Yamauchi et al. 2013). APN is a key insulin sensitizing adipokine which is considered as a promising drug target in diabetes.

2.10.8.5. Role of APN in cardioprotection:

APN knockout mice show remarkable vascular alteration viz neointimal thickening and increased proliferation of vascular smooth muscle cells in mechanically injured arteries (Kubota, Terauchi et al. 2002). At physiological levels, APN exhibits specific and saturable binding to aortic endothelial cells, but readily binds to the walls of catheter-injured vessels preferentially to intact vascular walls. Studies of vascular reactivity in aortic rings from APN knockout mice showed reduced vasodilation in response to acetylcholine compared with wild-type mice (Okamoto, Arita et al. 2000; Ouchi, Kihara et al. 2003; Yilmaz, Sonmez et al. 2005).

APN is synthesized and secreted by cultured mouse and human cardiomyocytes. In addition, AdipoR1 and AdipoR2 were identified at both mRNA and protein level in these cells. The expression of APN in intact myocardium is low and markedly increases in injured myocardium (Pineiro, Iglesias et al. 2005). Indeed, Takahashi *et al* observed a marked increase in APN content in autopsy specimens obtained from patients with acute myocardial infarction, old myocardial infarction and dilated cardiomyopathy (Takahashi, Saegusa et al. 2005). Apart from circulating blood and local synthesis, APN may reach the myocardium from epicardial adipose tissue. APN exerts typical metabolic effects in cardiomyocytes, including stimulation of AMPK, fatty acid oxidation, and glucose uptake (Ding, Qin et al. 2007).

APN potentiates nitric oxide by activating the AMPK signal pathway favouring vasodilation and inhibiting platelet aggregation, monocyte adhesion and smooth muscle proliferation (Gomez-Ambrosi, Becerril et al. 2004; Anderson, Mehta et al. 2007). APN attenuates ox-LDL and hyperglycemia-induced ROS generation (Morris, Sennello et al. 2006), vascular endothelial growth factor (VEGF)-induced ROS generation (Iwaki, Matsuda et al. 2003). APN inhibits

phorbol ester or N-ormylmethionyl- leucyl-phenylalanine (fMLP)-stimulated superoxide anion radical release by neutrophils (Magalang, Rajappan et al. 2006), superoxide generation in endothelial cells stimulated with oxidized LDL (Tao, Gao et al. 2007).

2.10.8.6. Role of APN in inflammation:

APN attenuates excessive inflammatory responses in the vascular wall by suppressing TNFα stimulated adherence of monocytes to cultured endothelial cells. APN also inhibits the expression of vascular cell adhesion molecule-1, E-selectin and intercellular adhesion molecule-1(Curat, Wegner et al. 2006). The anti-inflammatory properties of APN are mediated partly through activation of receptors AdipoR1 and AdipoR2 in monocytes, macrophages and endothelial cells, and attenuate inflammatory cell accumulation in sites of vascular injury. APN reduces the uptake of oxidized LDL by macrophages, inhibits transformation of macrophages to foam cells (Kamon, Yamauchi et al. 2004). High sensitive (hs) CRP also correlates with several components of metabolic syndrome, since inflammation is believed to play significant role (Lee, Park et al. 2004). Sugiura et al. in a study of 2049 Japanese men aged 35 to 66 reported the negative association of APN with CRP, independently of leptin, proposing a possible inhibitory effect of APN in CRP synthesis through modulations in TNF-α and IL-6 productions (Sugiura, Tamakoshi et al. 2008).

2.10.8.7. Role of APN in angiogenesis:

APN decreased neovascularisation in chick CAM assay and mouse corneal angiogenesis assay via activation of caspase induced apoptosis of endothelial cells (Brakenhielm, Veitonmaki et al. 2004). In addition APN increased tumor cell apoptosis in an *in vivo* study. APN binds to growth factors like bFGF, PDGF, HBEGF and prevents activation of their corresponding receptor thus reducing the effect of these growth factors (Wang, Lam et al. 2005). Yoneda et al reported the presence of AdipoR1 and AdipoR2 in normal colon epithelium as well as in colon cancer tissues. APN plays a major role in the physiological and pathological condition of colon epithelium via its receptors (Yoneda, Tomimoto et al. 2008). APN reduced cell proliferation in breast cancer cells further over expression of APN or recombinant APN also reduced mammary tumourogenesis in mice (Wang, Lam et al. 2006).

On the other hand, APN proangiogenic effect in ischemic limb was also reported in an *in vivo* study (Shibata, Ouchi et al. 2004). Yet another study reported that APN positively correlates with VEGF in rheumatoid arthritis patients , and was able to stimulate the production of VEGF and MMP's in cultured fibroblast like synoviocytes (Choi, Lee et al. 2009). APN plays a pleotropic role in angiogenesis.

2.10.8.7.1. APN in ocular angiogenesis:

Bora et al demonstrated the expression of APN in rat choroidal tissue and also reported that intravitreal or intraperitonal injection of recombinant APN decreased choroidal neovascularisation indicating its anti angiogenic role (Bora, Kaliappan et al. 2007). APN derived peptides decreased choroidal neovascularisation in mouse model (Lyzogubov, Tytarenko et al. 2009; Lyzogubov, Tytarenko et al. 2012). APN decreases retinal neovascularisation by decreasing TNFα mediated inflammatory response in ischemia induced retinopathy in mouse (Higuchi, Ohashi et al. 2009). Same group also reported thiazolidinediones, an anti-hyperglycemic drug reduces retinal neovascularisation via APN (Higuchi, Ohashi et al. 2010). Zietz et al have observed an elevated APN level in the serum of patients with type 2diabetes and PDR. APN gene polymorphism was also observed in PDR patients. They also reported the presence of APN in vitreous from 5 patients who underwent vitreoretinal surgery, as a proof of concept (Zietz, Buechler et al. 2008). Recently elevated levels of APN in sub retinal fluid in patients with PVR (Ricker, Kijlstra et al. 2012) and elevated aqueous level of APN (Mao, Peng et al. 2012; Costagliola, Daniele et al. 2013) in PDR patients have also been reported. Presence of intraocular levels indicates its association with PDR.

2.11. Gap in existing research:

The search for novel, better therapeutic target is still a demanding area to overcome the drawback in conventional treatment modalities. Disturbances in ratio between pro angiogenic and anti angiogenic factors play a major role in the progression of DR. Molecular target which either inhibits proangiogenic factors like VEGF, IGF-1, and TNF α or increase anti angiogenic factors like endostatin, PEDF, angiostatin, APN are encouraged. Understanding the role of APN

in PDR condition can help in understanding the disease pathology and also identify newer drug targets to treat PDR.

So far there are no reports on the localisation of human APN in ocular tissues. Adipokines have been widely studied in PDR in great detail but ocular levels of APN in PDR have not been evaluated. APN has been known to be increased in numerous cancers and also has role as an anti angiogenic molecule. This study aimed in answering key questions such as

1. Is there any presence of APN in ocular tissues?
2. If present what is the role of APN in ocular angiogenesis PDR?
3. Whether modulating APN levels can be beneficial in treating PDR?

In silico based approach will help in predicting mechanistic pathway of APN in PDR. Further, modulating the levels of APN with small molecules viz peptides or amino acid might be useful in ocular angiogenesis. This study focuses on the **role of APN in PDR which is poorly defined.** There is little or no information on the localisation, expression and function of APN and its receptor in human ocular tissues. Unravelling the role of APN will give us more insights on the pathogenesis of disease and paves a way for future therapeutics in ocular angiogenesis.

CHAPTER 3: OUTLINE OF THE WORK

Diabetes mellitus is a major health problem with devastating macrovascular and microvascular complications. DR is foremost detected and prevalent microvascular complication in majority of diabetic patients leads to irreversible blindness. Adipokines plays a major role in the pathogenesis of PDR. Adipokines like PEDF, VEGF, IGF-1, Leptin, APN, TNFα have been studied widely in relation to PDR. APN being a multifunctional adipokine with anti-diabetic, anti atherogenic, anti-inflammatory, vasculo-protective effects is considered as potential therapeutic target for diabetes and its complications.

This study aims to understand the role of APN in PDR which is poorly defined. Various ocular tissues were studied for localisation & expression of APN and its receptors. Vitreous APN levels were measured in patients with PDR. Role of APN in relation to PDR was studied by 1) *invitro* angiogenesis assay in HREC and HUVEC cells, 2) by identifying its interacting partners related to angiogenesis and 3) its ability to alter cytokine production in RPE cells. Peptides were designed using bioinformatic approach based on their *in silico* interacting partners. Further, small molecules like amino acids and fatty acids were screened *in vitro* for modulating the secretion of APN.

Protein localisation and mRNA expression in ocular tissues demonstrated the presence of APN and its receptors AdipoR1 and AdipoR2, confirming that APN is synthesised intraocularly. Vitreous APN levels were found to be increased by 3 fold in LASER treated patients, before vitrectomy. Elevated levels of APN indicate association with PDR. *In vitro* angiogenesis using rAPN in ocular microvascular endothelial cells indicated its anti angiogenic property by reducing VEGF levels. Novel interacting partners of APN were identified using *in silico* approach and mechanistic pathway derived indicated APN to inhibit VEGF signaling pathway. rAPN also decreased the VEGF production in RPE cells confirming its potential as therapeutic target in PDR. Peptides designed using bioinformatics approaches were found to have anti angiogenic property suggesting that these molecules can mimic full length protein in this function. Additionally to further understand the role of small molecules in modulating APN expression amino acids were tested. Proline, hydroxy proline, phenylalanine were found to increase APN levels. These data strongly suggest that APN as potential therapeutic target for PDR.

OBJECTIVES:

1. **To study the intraocular expression and localisation of APN and its receptors**
2. **To study the role of APN in pathogenesis of PDR**
3. **To understand the therapeutic potential of APN in PDR**

CHAPTER 4: METHODS

4.1. Patient recruitment

This study involved the use of clinical specimens for the clinical, biochemical and proteomic analysis. Therefore recruitment of patients played an important role. Study patients were recruited from Medical Research Foundation, Sankara Nethralaya Eye Hospital, Chennai.

The study was conducted in strict adherence to the guidelines of the Helsinki Declarations and with the approval of the Institutional Ethics Board. The study was conducted for a period of 3 years, and patients who underwent vitrectomy as part of their treatment for PDR and MH were included. In case of diabetes and PDR, patients only on insulin and/or oral anti diabetic drugs were included. Similarly patients who were under any anti-VEGF, statins, piogioglitozone treatment were excluded from the study as these drugs may decrease or increases adipokines (VEGF, APN) level (Ferrara, Hillan et al. 2004; Rasouli, Yao-Borengasser et al. 2006). Patients with complications like coronary heart diseases, renal failure, sepsis and malignancy were excluded from the study. MH patients with diabetes or with macular edema were also excluded.

- Cases: PDR (ocular neovascularisation due to diabetes)
- Disease matched control: MH (without any ocular neovascularisation)
- Control I: age matched control without diabetes
- Control II: age matched control with diabetes and without PDR

Clinical Performa of all the recruited cases was filled by the clinician at the clinic with all the details. All the patients in the study had undergone a complete slit lamp and fundus examination. In case of PDR patients their clinical ocular findings were graded for the presence of vitreous haemorrhage, retinal detachment, presence and absence of neovascularisation in the retina or optic disc prior to vitrectomy. Patients with MH were with an idiopathic full thickness retinal defect of more than 400 μwith posterior vitreous detachment were included.

4.1.1. Sample collection and processing:

4.1.1.1. Vitreous:

Undiluted vitreous samples aspirated during vitrectomy from the confirmed diagnosed cases of PDR and MH after getting consent from the patients. A total of 76 vitreous samples, 53 vitreous from 51 PDR patients and 23 from MH patients was collected in the duration of 3 years. Vitreous samples were transported to laboratory immediately. Vitreous samples were centrifuged, aliquoted and stored at –80^{o}C till the assays were carried out. Assays were performed within one week from the time of collection of samples.

4.1.1.2. Blood samples:

Venous blood samples of 8 mL were collected in fluoride, EDTA and plain vacutainers from the patients. All the routine biochemical assays were done on the day of collection. Routine biochemical assays comprising of plasma glucose, total cholesterol, triglycerides, high density lipoprotein (HDL) and total protein using a fully automated Dade Behring RxL Max (Siemens, USA) were conducted. Glycosylated hemoglobin was estimated by boronate affinity assay (Nycocard reader II, Axis Shied, Norway). EDTA plasma was separated aliquoted and stored at – 80^{o}C till the ELISA were carried out.

Table 6: List of biochemical assay done

Assay	**Assay method**
Plasma glucose	Hexokinase method (Siemens, USA)
Total cholesterol	DEASCIAAP enzymatic method (Siemens, USA)
Triglycerides	LTLGKGTO method (Siemens, USA)
High density lipoprotein	Automated HDL method (Siemens, USA)
Total protein	Biuret method (Siemens, USA)

4.1.1.3. Urine Sample:

Labelled sterile urine containers were also given to the patients to collect urine. Albumin in urine was estimated on the same day of collection by immunometric assay for microalbuminuria (Nycocard reader II, Axis Shied, Norway).

4.1.2. Copy of clinical performa of patients enclosed as appendix I.

4.1.3. Copy of consent letter used to recruit the patients enclosed as appendix II.

4.2. Protein estimation

4.2.1. Bradford method:

Brilliant Blue G-250 dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Amax = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (Amax = 595 nm) which is detected using a spectrophotometer (Beckmann, USA) or microplate reader (Spetramax2e, USA)

4.2.1.1. Materials:

a) Bradford- thermo scientific
b) Sample
c) Normal saline – 0.9 % NaCl in distilled H_2O

4.2.1.2. Procedure:

1. To a 96 well plate, 10 µl of the sample/ lysis buffer /standards were added and 90 µl of normal saline was added.
2. Bradford reagent 100 µl was added and kept for incubation for 10 min at room temperature and read at 595 nm.
3. Standard curve was plotted against known standard concentration (2- 10 µg/ mL).
4. Samples were extrapolated onto the standard curve to calculate the protein concentration.

4.2.2. Bicinchoninic acid assay:

4.2.2.1. Materials:

a) BCA kit- thermo

b) Sample

4.2.2.2. Principle:

This assay is based on the principle reduction of Cu^{2+} ion to Cu^{+} by the peptide bonds in proteins which are dependent on temperature. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Further the two molecules of BCA chelate with each Cu^{+} ion, forming a purple-colored product that absorbs light at a wavelength of 562 nm.

4.2.2.3. Procedure:

1. Solution A 5 mL was mixed with 100 µl of solution B from which 200 µl were added to 96 well plates.
2. Standards (125 – 1000 µg / mL) / samples of 5 µl volume were added to the well. Normal saline 5 µl was added to blank well.
3. The plate was kept for incubation for 30 min at 37°C and read in plate reader at 562 nm.
4. The standard curve was plotted and the unknown samples protein concentrations were calculated by extrapolating the values in the standard graph.

4.3. ELISA

Sensitivity of the ELISA kits used was APN: 0.24 ng/mL, PEDF: 0.09 ng/mL, VEGF: 0.009 ng/mL, IGF- 1: 0.026 ng/mL

4.3.1. Materials required:

a) ELISA kit [APN, VEGF, IGF-1 (R&D, USA), PEDF (Chemicon International, USA)].

b) Samples – Vitreous/Plasma/Cell culture medium

4.3.2. Principle:

Proteins were detected by quantitative sandwich ELISA where specific antibody was pre-coated onto a microtitre plate. Proteins present in the sample binds with the immobilized antibody. Unbound substances are washed away followed by the addition of HRP conjugate. TMB substrate exhibited change in colour which is proportional to the concentration of protein present. The reaction was terminated with sulphuric acid and colour was read at 450 nm.

4.3.3. Procedure for APN/VEGF ELISA:

1. Standards and samples were prepared as per manufacturer's instruction.
2. 100 µl of assay diluent (provided in the kit) was added to each well
3. 50 µl of standard/sample was added to the well and incubated for 2 h at 37°C.
4. Three washes with 350 µl of wash buffer were performed.
5. Then 200 µl of conjugate solution (provided in the kit) was added to each well and incubated for 2 h at 37°C.
6. Three washes with 350 µl of wash buffer were performed.
7. Substrate Solution (provided in the kit) of 200 µl was added to each well. Plate was covered with a sealer and incubated for 30 min at 37°C in dark. Substrate solution colour changed to blue, and then stop solution of 50 µl was added to each well. Yellow colour developed at the end of the reaction.
8. Mixed well and read at 450 nm using an ELISA plate reader (Septramax2e, USA).

4.3.4. Procedure for IGF-1 ELISA:

1. Standards and samples were prepared as per manufacturer's instruction.
2. 150 µl of assay diluent (provided in the kit) was added to each well
3. 50 µl of standard/ samples was added to the well and incubated for 2 h at 2-8°C.
4. Three washes with 400 µl of wash buffer were performed.
5. Then 200 µl of cold conjugate solution (provided in the kit) was added to each well and incubated for 1 h at 2-8°C.
6. Three washes with 400 µl of wash buffer were performed.
7. Substrate Solution (provided in the kit) of 200 µl was added to each well. Plate was covered with a sealer and incubated for 30 min at 37°C in dark. Substrate solution colour changed to blue, and then stop solution of 50 µl was added to each well. Yellow colour developed at the end of the reaction.
8. Mixed well and read at 450 nm using an ELISA plate reader (Septramax2e, USA).

4.3.5. Procedure for PEDF ELISA:

1. Standards and samples were prepared as per manufacturer's instruction.

2. 100 µl of sample/standard was added to the well and incubated for 1 h at 37°C
3. Three washes with 250 µl of wash buffer were performed.
4. Then 100 µl of biotinylated antibody was added to each well and incubated for 1 h at 37°C
5. Step 3 was repeated again
6. 100 µl of streptavidin peroxidase conjugate was added to each well and incubated for 1 h at 37°C.
7. Step 3 was repeated again
8. Substrate solution (provided in the kit) of 100 µl was added to each well. Plate was covered with a sealer and incubated for 5-10 min at 37°C in dark. Substrate solution colour changed to blue, and then stop solution of 100 µl was added to each well. Yellow colour developed at the end of the reaction.
9. Mixed well and read at 450 nm using an ELISA plate reader (Septramax2e, USA).

4.4. Vitreous Amino acid estimation by reverse phase HPLC:

4.4.1. Principle:

Liquid chromatography is a separation technique which takes place in a column containing stationary phase. The stationary phase used is octadodecyl silane. The solvent, which is the mobile phase, is pumped through the particle bed. By this arrangement, sample components dissolve in the mobile phase in continuous manner. A solvent delivery system is connected to the column in order to feed it with fresh solvent. Separated samples are monitored by a VWD detector.

4.4.2. Materials:

a) Vitreous sample
b) 10% TCA
c) Column: C18 column, 5 mm diameter, 150 x 4.6 mm, pH range – 2 to 7.5, stationary phase – silica.
d) Orthophaldedhyde (OPA)
e) Borate buffer (pH - 10.4)

f) Solvent A (pH - 7.2) : sodium Acetate -1.36g, milliQ water – 500mL, triethylamine - 90µl, tetrahydrofuran – 1.5 mL
g) Solvent B (pH - 7.2) : sodium Acetate -1.36g, milliQ water – 100mL, methanol – 200mL, acetonitrile – 200 mL
h) 250 pm amino acid standard

4.4.3. Procedure:

1. Vitreous samples were precipitated with equal volume of 10 % tricholoro acetic acid.
2. Centrifuged at 5000 rpm for 5 min.
3. 10 µL of supernatant was subjected to analysis as per the method of pre column derivatisation with OPA and injected.
4. Prior to injecting samples, the system was calibrated with 250 pm amino acid standard.
5. A flow rate of 0.5 mL/min and column temperature of 40°C was used.
6. Detection wavelength is 338 nm (VWD)

4.5. Western blot

It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electro transferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labelled antibody (HRP) and chemiluminescent substrate.

4.5.1. Materials required:

a) Acrylamide (30 %): Acrylamide - 29.2 g, Bisacrylamide - 0.8 g, H_2O - 100 mL.
b) TRIS-HCl (pH 8.8) - 18 g of Tris dissolved in 50 mL of MQ H_2O and made upto 100 mL (adjust pH with concentrated HCl)
c) TRIS-HCl (pH 6.8) - 6 g of TRIS dissolved in 100 mL of MQ H_2O, pH was adjusted using concentrated HCl.
d) Electrophoresis buffer (pH 8.6) - 3 g of Tris, 15 g of Glycine and 1 g of SDS was added in 1000 mL of MQ H_2O.

e) LaemmLi buffer (2X): 10 % SDS – 4 mL, Glycerol – 2 mL, 1M Tris (pH 6.8) – 1.2 mL, H_2O – 2.8 mL and (0.02 %) bromophenol blue.

f) 10 % Ammonium per sulphate (APS) in distilled H_2O

g) 10 % Sodium dodecyl sulphate (SDS) in distilled H_2O

h) TEMED

i) TBS (Tris buffered saline)

j) Transfer buffer – Same composition as running buffer except addition of 200 mL of methanol made up to 1000 mL using DH_2O. Stored at -20°C.

k) Blocking buffer - 5 % skimmed milk (non fat dry milk) prepared with 1X TBST.

l) Washing buffer-TBST: 0.1 % tween-20 in 1X TBS

m) Secondary antibody

n) Luminescent mixture – Equal volume of HRP substrate peroxide solution and HRP substrate luminol reagent were mixed and added just before use.

o) Nitrocellulose membrane

p) Whatmann filter paper no.1

q) Mini transfer western blot- Bio Rad

4.5.2. Protocol

Table 7: Preparation of 10 % PAGE

S.NO	Reagents	Stacking gel (4 %)	Separating gel (10 %)
1.	Acrylamide 30 % (mL)	1.33	2.5
2.	TRIS HCl (mL)	2.5 (pH 6.8)	3.33 (pH 8.8)
3.	MQ H_20 (mL)	6.1	4.17
4.	10 % APS (µl)	50	50
5.	10 % SDS (µl)	100	100
6.	TEMED (µl)	5	10

4.6. SDS-PAGE

1. Cleaned glass plates and 1.5 mm spacer were clamped together
2. The bottom was sealed with molten agarose gel
3. Separating gel was poured up to 70 % of the plate, overlayed with H_2O and allowed to polymerise.
4. Then stacking gel was poured and comb was placed and allowed to polymerise.
5. After polymerisation comb was removed and washed twice with H_2O.
6. The sample containing 75 µg of protein equal part of 2X SDS sample buffer was added
7. Electrophoresis was performed at 100 V current till the run was over.

4.7. Transfer

1. The gel was cut and incubated in the transfer buffer for 20 min.
2. Nitrocellulose membrane of required size was cut and incubated in the transfer buffer for 20 min.
3. The gel and nitrocellulose membrane were sandwiched in the mini transfer western blot without any air bubble.
4. Transfer was performed under 100 V electric field for 1 h in ice cold buffer.
5. Then the membrane was taken and washed thrice in TBST each for 5 min
6. The membrane was kept in blocking buffer for 1 h at room temperature under mild rocking
7. Washed thrice in TBST.
8. Blot was incubated with primary antibody at 4°C for overnight.
9. Washed in TBST (5 times).
10. Incubated in secondary antibody for 1 h at room temperature.
11. Membrane was washed in TBST thrice and twice with TBS, developed using chemiluminol and documented using fluorchem FC3 chemidoc instrument.

4.8. Immunohistochemistry and immunoflourescence staining:

Donor eye balls were procured from CU shah eye bank (Sankara Nethralaya) after the removal of corneal button. Donor eye balls collected were without any ocular diseases and with the

consent from the donor's relative. Five donor eye balls were used for study. Donor eye ball was stored in 10 % neutral buffered formalin for tissue processing, immediately after collection.

Surgically removed ERMs were collected with proper consent from the patient. The ERMs were transported from the operation theatre to the laboratory in normal saline, and then transferred to 10 % neutral buffered formalin for tissue processing.

4.8.1. TISSUE PROCESSING

Donor eye balls was processed, embedded and sectioned for further immunohistochemistry and immunoflourescence staining.

4.8.1.1. Steps in Processing:

The tissue was removed from 10 % neutral buffered formalin and processed in the following reagents in the order mentioned below.

1. 100 % alcohol – 30 min
2. 100 % alcohol - 30 min
3. 100 % alcohol - 30 min
4. Xylene - 4 min
5. Xylene - 4 min
6. Paraffin wax - 30 min
7. Paraffin wax - 60 min
8. Wax in vacuum - 30min

4.8.1.2. Steps in Embedding

1. Molten paraffin wax 2º or 3ºC above the melting point was dispensed into the mould.
2. It is the process of placing the tissue in a support medium (paraffin wax)
3. Thin film of solid has formed on surface.
4. Gently pressed the tissue into the wax in the oriented plane.

4.8.1.3. Vacuum impregnation

1. Transferred the cleared tissues to a heated, sealed container of molten wax.
2. Degree of vacuum should not exceed 400-500 mm of Hg.

4.8.1.4. Sectioning the tissues

The sections of thin microtome 5 µm was taken on the charged slides (Fisher Scientific) and slides were kept for drying and next day deparffinisation was done

4.8.1.5. Deparaffinisation:

Deparaffinisation of sections was carried in the order mentioned below.

1. xylene 1 - 4 min
2. xylene 2 - 4 min
3. xylene 3 - 4min
4. 100 % Isopropyl alcohol - 4 min
5. 100 % Isopropyl alcohol - 4 min
6. 95 % Isopropyl alcohol - 4 min
7. 80 % Isopropyl alcohol - 4 min
8. 70 %Isopropyl alcohol - 4 min
9. 60 %Isopropyl alcohol - 4 min
10. Distilled water - 4 min

4.8.1.6. H and E staining:

H and E staining was carried out to check the pigmentation in the tissue and the cellular pattern of the tissue. Staining was carried in the order mentioned below.

1. Haematoxylin – 8 min
2. Wash in tap water
3. Acid alcohol – 1 dip
4. Wash in tap water

5. Ammonia water – 2 min
6. Wash in tap water
7. Working eosin solution – 1 min
8. Wash in tap water
9. 95 % alcohol – 1 min
10. 100 % alcohol – 1 min
11. Xylene - 1 min

    Slides were mounted immediately and observed under microscope. After observations tissue sections were taken for immunohistochemistry (IHC).

4.8.2. Immunohistochemistry

4.8.2.1. Principle:

The qualitative identification by light microscopy of antigens in sections of formalin-fixed, paraffin-embedded tissue is studied by immunohistochemistry. The sections were subjected to epitope retrieval prior to staining. Endogenous peroxidase activity is neutralized using the NovocastraTM peroxidase block. This was followed by application of the protein block to reduce non-specific binding of primary and polymer. The sections were subsequently incubated with optimally diluted primary antibody. Post primary block is used to enhance penetration of the subsequent polymer reagent. The polymer recognizes mouse and rabbit immunoglobulins, it detects any tissue-bound primary antibody. Sections were further incubated with the substrate/chromogen, 3,3' - diaminobenzidine (DAB). Reaction with the peroxidase produces a visible brown precipitate at the antigen site (Shanrong, Guo et al. 1999)

4.8.2.2. Reagents

a) Peroxidase Block - 3 % Hydrogen peroxide.
b) Protein Block - 0.4 % Casein in phosphate-buffered saline, with stabilizers, surfactant, and 0.2 % Bronidox L as a preservative.
c) Post Primary Block - Polymer penetration enhancer containing 10 % animal serum in tris-buffered saline/0.09 % ProClin™ 950.

d) NovoLink TM Polymer. Anti-mouse/rabbit IgG -Poly-HRP (each at 8 µg/mL) containing 10 % animal serum in tris-buffered saline/0.09 % ProClin™ 950.
e) DAB Chromogen. 1.74 % 3,3' - diaminobenzidine, in a stabilizer solution.
f) NovoLinkTM DAB Substrate Buffer (Polymer) - Buffered solution containing 0.05 % hydrogen peroxide and preservative.
g) Hematoxylin - 0.02 % Hematoxylin.

4.8.2.3. Procedure:

1. Citrate buffer 0.01M – 294 mg of trisodium citrate and adjusted the pH to 6.0 with citric acid and made to 100 mL with H_2O. This was freshly prepared.
2. Antigen retrieval was performed in microwave oven for 2 min of 4 cycles each lasting for 30 s in citrate buffer.
3. Once antigen retrieval was performed tissues were kept in moist chamber.
4. Neutralized the endogenous peroxidase using peroxidase block for 5 min.
5. Washed in TBS for 2 x 5 min.
6. Incubated with protein block for 5 min.
7. Washed in TBS for 2 x 5 min.
8. Incubated with optimally diluted primary antibody (APN/ADIPOR1/ADIPOR2 - 1:200 dilution) for 2 h.
9. Washed in TBS for 2 x 5 min.
10. Incubated with post primary block for 30 min.
11. Washed in TBS for 2 x 5 min.
12. Incubated with NovoLink TM polymer for 30 min.
13. Washed in TBS for 2 x 5 min with gentle rocking.
14. Developed peroxidase activity with DAB working solution for 5 min.
15. Rinsed the slides in water.
16. Counterstained with Hematoxylin.
17. Rinsed the slides in water for 5 min.
18. Dehydrated, cleared and mounted the sections.

4.8.3. Immunoflourescence staining:

4.8.3. 1. Procedure for immunoflourescence staining:

1. The steps from 1 to 8 were followed as mentioned above for IHC staining.
2. After Primary antibody, slides are washed with TBST.
3. Tissue sections were incubated in fluorescent tagged secondary antibody for 1h in dark.
4. Washed in TBST for 2 x 5 min.
5. Working DAPI solution for 3 min.
6. Rinsed the slides in water for 5 min.
7. Dehydrated, cleared and mounted the sections with anti fade mountant.
8. Slides are sealed with nail polish in all four corners.
9. Unstained sections were used for background elimination for confocal microscopy.

4.9. CELL CULTURE

4.9.1. Isolation of primary cell lines from donor eyeballs:

4.9.2. Materials required:

a) T25 vented flask (Nunc)
b) Fibronectin: Commercially available 1 mg/mL (Sigma) was diluted to 10 µg/mL concentration in PBS and used for coating culture dishes.
c) Collagenase: Collagenase 2 mg of (Sigma) was dissolved in 10 mL of 1X PBS and filter sterilized and 1 mL aliquots were stored at -20 °C. Working collagenase concentration of 0.2 mg/mL was made upto 10 mL and used for eyeball processing.
d) Trypsin (1.5%), EDTA 30 mg, Glucose 500 mg was preparedand filtered using GV membrane filter.
e) Antibiotic solution: Commercially available antibiotic mixture (Gibco) 100 X was diluted to 1X in PBS.
f) Scrappers
g) Drapes
h) Glass petri plate
i) Scissor

j) Tissue homogeniser
k) 10 mL syringes – 2 nos.
l) 21 gauge needle
m) Glass beaker -1000 mL
n) Pair of surgical gloves
o) 15 mL Tarson tubes
p) Phosphate buffered saline: NaCl- 8 g, Na_2HPO_4- 1.15 g, KH_2PO_4- 0.2 g, KCl- 0.2 g. Adjust to pH between 7.2- 7.4.
q) Endothelial growth medium 2 {EGM- 2 medium (Lonza)}
r) Magnetic anti- CD31 coated DYNA beads
s) Magnetic separator

This method was modified from Browning et al. (Browning, Gray et al. 2005)

4.9.3. Procedure for isolation of HCEC:

1. Donor eyeballs were collected in DMEM medium and processed on the same day.
2. Choroid with RPE was isolated from the eye ball.
3. RPE was carefully scrapped gently.
4. Choroidal tissue was minced into small pieces and incubated with 1.5% trypsin for 20 min at 37°C water bath
5. Trypsin activity was arrested by adding FBS and content was transferred to homogenizer.
6. Homogenized sample was aspirated using needle syringe and further treated with 0.2 mg/mL collagenase for 40 min at 37°C
7. The collagenase treatment was terminated by adding medium containing 10 % FBS, followed by anti - CD31 magnetic bead separation at 4°C for 1 h.
8. Then, HCEC was separated using a magnetic separator.
9. The supernatant was discarded and the cell pellets were resuspended in 5 mL of EGM2 with 20 % FBS medium.
10. The cells were seeded in fibronectin coated flask and incubated at 37°C in 5 % CO_2.

4.9.4. Procedure for isolation of HREC:

1. Donor eyeballs were collected in DMEM medium and processed on the same day.
2. Retina was removed after removing the anterior portion of the eye and care is taken that RPE contamination is least.
3. Retinal tissue was minced into small pieces and incubated with 1.5% trypsin for 20 min at 37°C water bath.
4. Steps 5- 10 were same as that of HCEC isolation

4.9.5. Isolation of Human Umbilical Vein endothelial cells (HUVECs)

4.9.5.1. Materials required:

a) T25 vented flask (Nunc)
b) Fibronectin: Commercially available 1 mg / mL (Sigma) was diluted to 10 µg/mL concentration in PBS and used for coating culture dishes.
c) Collagenase: Collagenase 2 mg of (Sigma) was dissolved in 10 mL of 1X PBS and filter sterilized and 1 mL aliquots were stored at -20 °C. Working collagenase concentration of 0.2 mg/mL was made upto 10 mL and used for cord processing.
d) Antibiotic solution: Commercially available antibiotic mixture (Gibco) 100 X was diluted to 1X in PBS.
e) Artery forceps – 2 nos.
f) Drapes
g) Glass petri plate
h) Scissor
i) 10 mL syringes – 2 nos.
j) 21 gauge needle
k) Butterfly needle
l) Glass beaker -1000 mL
m) Pair of surgical gloves
n) 50 mL Tarson tubes

o) Phosphate buffered saline: NaCl- 8 g, Na_2HPO_4- 1.15 g, KH_2PO_4- 0.2 g, KCl- 0.2 g. Adjust to pH between 7.2- 7.4.

p) Endothelial growth medium 2 {EGM- 2 medium (Lonza)}

4.9.5.2. Procedure:

Ethics statement:

All the protocols involving the collection and processing of human samples were strictly adhered to the tenets of Helsinki declarations, and were approved by the Institutional Review Board of Vision Research foundation (IRB – VRF) where the study was conducted. Reference number - 150-2009-P is the IRB-VRF approval number for this work. Written consents were obtained from mothers who volunteered to donate their umbilical cord for research.

This method was modified from Baudin *et al* (Baudin, Bruneel et al. 2007)

1. Cords were procured and processed within 3 h. All the reagents were kept in 37°C water bath before use.
2. To 500 mL of prewarmed PBS, 500 µl of antibiotic solution was added.
3. The cords were washed externally to remove RBCs.
4. A butterfly needle was inserted in one end of the vein and clamped using artery forceps. The cord was internally washed with PBS until the wash appeared to be colorless. The cord was also checked for any clots or leaks while washing. If the cord were very long they were cut into pieces of suitable length and then processed.
5. Collagenase solution was added through the veins after clamping the cord on both sides and then gently massaged.
6. It was incubated for 20 min at 37°C water bath in a beaker containing PBS.
7. After incubation the cord was gently massaged to dislodge the cells from the vein.
8. The clamp was removed from one end, then 10 mL PBS was allowed to flow through the cord gently thereby collecting the dislodged cells. Cells were collected in 5 mL of EGM medium in a 50 mL falcon tube. Centrifuged for 10 min at 1500 rpm.
9. The supernatant was discarded and the cell pellets were resuspended in 5 mL of EGM2 medium.
10. The cells were seeded in fibronectin coated flask and incubated at 37°C in 5 % CO_2.
11. After 2-3 h, change of medium was given to remove unattached cells and incubated till the cells were grown to confluency.

4.9.6. Trypsinisation of HUVECs/HREC/HCEC

4.9.6.1. Materials required: (filter sterilized)

a) Trypsin – 100 mg
b) EDTA- 30 mg
c) Glucose – 500 mg
d) PBS – 100 mL

4.9.6.2. Principle:

Cells were grown to 80-90 % confluency in tissue culture flask containing 5 mL of EGM2 medium. The process of trypsinisation helps in disaggregating the cells into a single cell from a monolayer. Thus, removing cells from the primary culture and transferring them to secondary cultures constitutes a passage, or subculture. Endothelial cells of passages 3- 5 were used for all experiments.

4.9.6.3. Procedure:

1. Medium from the T25 flask was removed and cells were washed with PBS and added 0.5 mL of trypsin and kept in at 37°C incubator for 2-3 min.
2. The monolayer of cells detached to form single cell and this reaction was stopped by addition of EGM medium.
3. The entire solution from the flask was transferred to a 15 mL falcon tube and centrifuged for 5 min at 1500 rpm.
4. The supernatant was removed and the pellet was resuspended with 1 mL of EGM medium.
5. Cells were counted using haemocytometer.
6. The required number of cells were seeded onto 0.1 % gelatin coated dishes for further experiments.

4.9.7. Cryopreservation of endothelial cells:

A cryoprotective agent dimethyl sulfoxide (DMSO) was used in conjunction with complete medium for cryopreserving cells. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage. **Cells grown to confluency were trypsinised and pellet was resuspended in EGM medium with 20 % FBS and 10 % DMSO. Cells were stored kept in one degree step down coolant and**

stored in liquid nitrogen. The number of cells were counted using hemocytometer and diluted with accordingly to get a final concentration of 10^6 - 10^7 cells/mL.

4.9.8. HUVECs/HREC retrieval:

When the cryopreserved cells were needed for study, each vial was taken out, thawed rapidly to prevent formation of ice crystals that can lyse the cells and plated at high density to optimize recovery. **Cells taken from liquid nitrogen were thawed in 37**°C. Fresh 5 mL of EGM medium was added and centrifuged at 1500 rpm for 10 min. The cell **pellet was resuspended in EGM medium and seeded onto gelatin coated culture dishes.**

4.9.9. Cell counting:

4.9.9.1. Materials Required:

1. Trypan Blue (0.4 % in PBS)
2. Haemocytometer

4.9.9.2. Principle:

It is a dye exclusion method where only the membrane of the live cells excludes the trypan blue and the cell remains unstained. Dead cell membrane is permeable to the dye and stains blue.

4.9.9.3. Procedure:

1. Cell suspension of 20 µl was equally mixed with dye and 10 µl was loaded.
2. The total number of cells in the four corners (WBC counting squares) was counted.
3. The total numbers of dead and live cells were counted separately to calculate the % viability.

4.9.9.4. Calculation

No of cells counted = *X*

Therefore cells /mL Y= *X**2 (dilution factor) *10^4

Total no cells = *Y* * volume of the total medium trypsin inactivation.

10^4- 1000*10 (i.e. 1 µl - 1000 µl conversion, depth factor)

4.10. Culturing and maintenance of Human visceral preadipocyte:

4.10.1. Procedure for culturing preadipocytes :

1. Cryopreserved unpassaged human visceral (kidneys and bladder) preadipocytes were purchased from LONZA pvt ltd.
2. Cryovial containing the cells are rapidly thawed and transferred to 10 mL of prewarmed preadipocyte basal medium (PBM-2) in falcon tube.
3. Centrifuged at 1500 RPM for 10 min.
4. The supernatant was discarded and the cell pellets were resuspended in 5 mL of PBM-2 medium.
5. The cells were seeded in T25 flask and incubated at 37°C in 5 % CO_2.
6. Change of medium was given every 3rd day.

4.10.2. Differentiation of preadipocytes to adipocytes:

1. Preadipocyte growth medium (PGM-2) was prepared by adding insulin, dexamethasone, indomethacin, isobutyl-methylxanthine to 100 mL of prewarmed PBM – 2 medium.
2. Preadipocyte cells were seeded on to 6 wells, 12 wells and 24 wells as per experimental requirements.
3. When the cell reached 80 % confluency, PGM- 2 medium was added and left undisturbed for 10 days.
4. Accumulation of lipid droplets was seen from day 7.

4.11. Oil red O staining:

4.11.1. Materials:

a. Stock oil red O

- Oil red o stain – 50 mg
- Isopropanol- 10 mL
- Stain dissolved in isopropanol left at RT for 30 min, filtered.

b. Working stock:

- 3mL of oil red O + 2mL of distilled water
- Prepared freshly. Filtration not required

4.11.2. Procedure for staining:

1. Cells were fixed with 10 % neutral buffered formalin for 30 – 40 min.
2. Discard formalin and add 60 % isopropanol for 5 min.
3. After that working oil red O was added and incubated at room temperature for 30 min.
4. After removal of oil red O four washes with tap water was given.
5. Added haematoxylin for 1 min.
6. Repeat step 4 washing with tap water.
7. Lipids droplets appear red whereas nucleus in blue colour after staining.

4.12. MTT assay

4.12.1. Materials Required:

1. 96 well plate
2. MTT- 5 mg/mL in PBS {(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Invitrogen}
3. DMSO (Merck)

4.12.2. Principle:

The MTT assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by metabolically active cells. After exposure cells were treated with MTT and the formazan crystal formed were dissolved in DMSO and read at 570 nm in spectra max fluorescent plate reader (Molecular devices, USA) (Mosmann 1983).

4.12.3. Procedure:

1. Cells were plated into 96-well plates. Cell densities of 5, 000 to10, 000 cells/ well were seeded and appropriate conditions were exposed.

2. After the exposure, 20 µl solution of MTT with 180 µl of medium was added to wells and incubated for 4 h at 37°C.
3. The medium was removed and 200 µl DMSO was added into each well to dissolve the formazan crystals. Absorbance was measured on plate reader (Spectra max M2^{e}) with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD 570 – OD 630).

4.13. *In vitro* angiogenesis assay

4.13.1. Migration Assay (Wound healing assay) (Liang, Park et al. 2007).

4.13.1.1 Materials required:

a) 0.1 % gelatin
b) 6-well plates
c) HUVECs/HRECs
d) EGM medium
e) 1 % EBM medium
f) 4 % Para formaldehyde – 0.4 g of paraformaldehyde (Merck) dissolved in 10 mL of 1 X PBS at 60°C for 30 min was freshly prepared.
g) PBS

4.13.1.2. Principle:

Cell migration is a process induced by various growth factors and chemokines that are associated with complex signalling. Cell migration occurs in normal condition of wound healing, cell differentiation and embryonic development and pathological condition like tumour metastasis.

4.13.1.3. Procedure:

1. A wound was created in monolayer of cells with a sterile 200 µl tip in a 6-well plate.
2. Cells were exposed to the appropriate conditions and kept at 37°C incubator for overnight.
3. The medium was removed and 500 µl of 4 % paraformaldehyde was added to all wells and kept at room temperature for 30 min for fixation.
4. Cells were washed with PBS twice gently. Photographs were taken from 3 different fields and analysed

4.14. Tube formation assay (Goodwin. 2007)

4.14.1. Materials required:

a) 96-well culture plates- kept at 4 °C
b) ECM matrix- Millipore - kept at 4 °C
c) Microtip - kept at 4 °C
d) EGM medium
e) HUVECs/HRECs

4.14.2. Principle:

The assay measures the ability of endothelial cells to form capillary (tube) like structure in the presence of extracellular matrix support when plated at sub confluent densities. This assay helps in screening pro and antiangiogenic molecules. Upon plating, endothelial cells attach and generate mechanical forces on the surrounding extracellular support matrix to create tracks or guidance pathways that facilitate cellular migration. The resulting cords of cells will eventually form hollow lumens. The comparisons between cells with and without treatment are measured in parameters such as tube length, size and number of junctions.

4.14.3. Procedure:

1. All the plates and consumables were pre cooled at 4°C. The ECM matrix 1 mL was mixed with 100 µl of 10X ECM buffer.
2. ECM gel solution of 50 µl was added to each well and incubated for 30 min at 37°C.
3. Cells were trypsinised and counted. Cells ranging from 15000-30000 cells / well in desired culture medium were incubated with molecules to be tested for 30 min at 37°C.
4. Then 150 µl of the culture medium with cells were added to the well and incubated at 37°C for 8 h. The plates were examined for endothelial tubes under light microscope.
5. The medium was carefully removed without disturbing the tubes and the tubes were fixed with 4 % paraformaldehyde.

4.15. Proliferation assay (Kern, Sondak et al. 1987)

4.15.1. Materials required:

a) 6-well plates

b) Tritiated thymidine – Tritiated thymidine (H^3) was dissolved in 10 mL of serum free medium (EBM) and kept for boiling till 10 min, the mixture was then filter sterilized and appropriate concentration were added to cell culture.

c) EBM medium

d) HUVECs/HRECs

e) Lysis buffer- NaOH (0.2 N) was dissolved in 1 % SDS

f) Scintillant preparation

Toluene- 666 mL (Merck)

Triton X 100- 322 mL (Merck)

POP (2, 5-diphenyloxazole) - 5 g

POPOP (1, 4-bis (5-phenyloxazol-2-yl) benzene) - 0.15 g

4.15.2. Principle:

The process of liquid scintillation counter relies on the transfer of kinetic energy from the beta particle to the solution components through a series of energy transformations. The released ultraviolet light excites fluorchrome molecules in the solvent. The fluorchrome molecules will then release the energy in the UV-Visible range of energy of 300-450 nm. Sometimes, the primary scintillators (POP) are not sensitive in the region of fluorescence emission. Thus, a wavelength shifter (secondary scintillator - POPOP) is introduced which absorbs energy from the primary scintillator and emits light at a wavelength more suitable to the photomultiplier tubes.

Proliferation assay:

Cell proliferation is measured with the amount of radioactive thymidine uptake by the cell which indicates the newly synthesized DNA.

4.15.3. Procedure:

1. Cells were seeded onto 24 well plates (3,000 cells/well). Cells were grown till 60 % confluency and serum starved with 1 % EBM for 4 h.
2. Then cells were exposed to appropriate conditions; H^3 100 nCi was added to the medium and grown in 5 % EBM medium.
3. Cells were kept at 37°C in 5 % CO_2 incubator and covered with aluminum foil.
4. After 48 h cells were washed in PBS twice and 0.2 mL of lysis buffer was added to each well.
5. The lysate was transferred to a vial and heated for 1 h at 60 °C.

6. Scintillation liquid 3 mL each was added to the tubes and 0.1 mL of lysate was added.

4.16. Attachment assay (Goodwin. 2007)

4.16.1. Materials required:

a) 0.1% gelatin
b) 6-well plates
c) HUVECs/HRECs
d) EGM medium
e) 1 % EBM medium
f) Para formaldehyde (4 %) in PBS

4.16.2. Principle:

Cell attachment is a process involved in angiogenesis.

4.16.3. Procedure:

1. Cells were trypsinised and 50,000 cells were taken in each vial.
2. Cells were exposed to the appropriate conditions and kept at 37°C incubator for 30 min.
3. After incubation cells were allowed to attach for 15 min in 0.1 % gelatin coated 24 well plates.
4. Unattached cells were washed carefully with PBS. Attached cells were fixed with 4 % paraformaldehyde and stained with giemsa.
5. Photographs were taken from 3 different fields and analysed using Image J cell counter.

3.17. Apoptosis assay- Cell death ELISA

3.17.1. Materials required:

a) HUVECs – Sample was prepared by adding the lysis buffer to cell grown on 6 well plates. Cells were incubated with 500 µl of lysis buffer for 30 min at room temperature. The sample was centrifuged at 14000 rpm for 10 min. The supernatant was carefully taken and used for the assay.
b) Cell death ELISA kit (Roche)

3.17.2. Principle

A programmed cell death occurs usually by necrosis (osmotic lysis) or apoptosis (zeiosis). Apoptosis is characterized by membrane blebbing, where cytoplasm condenses and activation of

endogenous nucleases occurs. The endogenous nucleases are dependent on Ca^{2+} and Mg^{2+} ions which act upon intracellular DNA to form mono and oligo nucleosomes. Apoptosis assay is based on sandwich ELISA technique using mouse monoclonal antibody towards DNA and histones. This assay allows specific determination of mono and oligo nucleosomes of the cytoplasmic fraction of the cell lysate.

4.17.3. Procedure:

1. Coating solution 100 µl was (anti - histone antibody) added to the 96 well plate. Covered with foil and incubated for overnight at 2-8°C or 2 h at 37 °C.
2. After removing the coating solution 200 µl of incubation buffer was added and kept for 30 min at room temperature.
3. Three washes with 250 - 300 µl of wash buffer were done.
4. Sample solution of 50 µl was added to appropriate wells and incubated for 90 min at room temperature.
5. The washing step was repeated and 100 µl of substrate was added to the wells and incubated for 20 min in dark at room temperature.
6. Colour developed was recorded using a plate reader at 405 nm and 490 nm.

4.18. Adipogenesis Assay:

4.18.1. Materials required:

a) Human visceral adipocytes, Bovine retinal pericytes – sample are prepared as per manufacturer's protocol
b) Adipogenesis assay kit (Biovision, USA)

4.18.2. Principle:

Adipogenesis is the formation of adipocytes or differentiation of cells to mature adipocytes by accumulating triglycerides in the form of lipid droplets. Adipogenesis assay kit solublize the triglyceride accumulated in the cells, followed by hydrolyzing them to glycerol which is further oxidized to convert the probe to generate color (λmax = 570 nm) and fluorescence (Ex/Em = 535/587 nm). Sensitivity of the kit is ranges from 0.2 to 10 nmol.

4.18.3. Procedure:

1. After exposure/ differentiation, 100 µl of lipid extraction solution was added to the cells and incubated at 90-100 C for 30 min.
2. To the each well containing sample and standard 2µl of lipase was added and incubated at room temperature for 10 min.
3. Reaction mix (Adipogenesis assay buffer – 46 µl, probe - 2 µl, Enzyme mix – 2 µl) of 50 µl was added to each well and incubated at 37ºC for 30 min in dark.
4. Colour developed was read at 570 nm.

4.19. Nitric oxide assay:

4.19.1. Materials required:

a) Human visceral adipocytes, Bovine retinal pericytes- conditioned medium after exposure
b) Nitric oxide assay kit (Biovision, USA)

4.19.2. Principle:

Nitric oxide (NO) plays an important role in vasodilation. NO is rapidly oxidized to nitrite and nitrate. This assay kit measures of total nitrate/nitrite in the sample. The first step converts nitrate to nitrite utilizing nitrate reductase. The second step uses Griess reagents to convert nitrite to a deep purple azo compound which is directly proportional to the amount of NO present in the sample.

4.19.3. Procedure:

1. Standards were prepared as per manufacturer's instruction.
2. 85 µl of standard, samples was added to the well and followed by 115 µl assay buffer.
3. Then 5 µl of the nitrate reductase was added to each well.
4. After that 5 µl of the enzyme cofactor was added to each well and incubated at room temperature for 1 h.
5. Then 5 µl of the enhancer was added to each well and incubated at room temperature for 10 min.
6. 50 µl of Griess reagent R1 and 50 µl of Griess reagent R2 were added to each well.

7. Incubated for 10 min at room temperature and colour developed is read at 540 nm using an ELISA plate reader (Septramax2e).

4.20. AMPK activity Assay: (Russell, Kate et al .2005)

4.20.1 Materials required:

a) Human visceral adipocytes cells
b) SAMS peptide assay kit
c) Lysis buffer - 0.1%SDS in 0.2 N NaOH
d) 0.75% orthophosphoric acid
e) P81 phosphocellulose paper
f) P^{32} ATP

4.20.2 Procedure:

1. After exposure, medium were removed and cells were washed twice with PBS.
2. 20 µM of SAMS peptide, 5 µci of p32 ATP was used per assay along with AMPK and incubated at 30ºC for 15 min
3. Then loaded onto p81 phosphocellulose paper.
4. Paper was washed thrice with 0.75 % phosphoric acid and once with acetone.
5. Paper was added to the scintillant and the DPM was read in Liquid scintillation system (LSS-Beckman 6500).

4.21. GLUCOSE UPTAKE (Selvi, Angayarkanni et al. 2010)

4.21.1. Materials required:

a) Human visceral adipocytes cells
b) Lysis buffer - 0.1 % SDS in 0.2 N NaOH
c) ^{14}C-2-deoxyglucose (2DG) (2 µci/mL)

4.21.2. Procedure:

1. Mature adipocyte cells in adipocyte growth medium were serum starved for 2 h and then subjected to the experimental conditions.

2. 33 mM glucose was added along with the ^{14}C-2-deoxyglucose (2DG) (2 µci/mL).
3. After 10 min of exposure at 37ºC, the medium was removed and the reaction arrested by adding ice cold PBS immediately, the cells were washed thrice with PBS
4. Cells are lysed using lysis buffer (0.1%SDS in 0.2 N NaOH) and read in a Liquid Scintillation Counter (Beckman-6500)

4.22. SAMPLE PREPARATION FOR MASS SPECTROSCOPY – *INSOL* TRYPTIC DIGESTION

4.22.1. Procedure:

1. Cells after experimental conditions, were trypsinised and lysed in MPER (Pierce,USA) buffer.
2. Protein estimation was done by BCA assay.
3. 50 µg of protein was digested using rapigest (Waters Incorporation, USA) at 80°C for 15 min followed by addition of 5 µl of 100 mM DTT at 37°C for 20 min followed by 5 µl of 200 mM iodoacetamide at room temperature for 20 min.
4. Lysate was passed through a 10kDa cutoff filter for the removal of salts. After which the protein was kept for tryptic digestion at 37°C overnight.
5. After tryptic digestion the lysate was passed through a cellulose acetate column for concentration.
6. From this 3 µl of tryptic digest protein sample(1µg protein/µl) was taken for mass spectrometry analysis.
7. For label free relative quantification internal standard Alcohol dehydrogenase (ADH) 50 fmol/µL was spiked in the trytic digest samples before subjecting to mass spectrometry
8. The digested peptide mixtures were separated by on-line reversed-phase nanoscale capillary LC and analysed by electrospray MS/MS.

4.22.2. Identification of differential proteins by XEVO G2S-QTof mass spectrometry:

Whole cell proteome analysis for the identification of differential proteins was done using XEVO G2S-QTof mass spectrometry (Waters Incorporation, USA). Binding and chromatographic separation of the peptides took place in a X Bridge BEH 130 C18 (5um)

300X50 µm column (Waters). Peptide mixtures were injected onto the column with a flow of 1500 nl/min and subsequently eluted with a flow of 300 nl/min. A 5 fraction gradient separation from 10 to 40% acetonitrile was done in a 30-min gradient program. Data were acquired in data independent mode using MassLynx 4.1 software. The m/z 50–2000 Da was acquired in the QTof instrument with resolution of 30,000 and the mass accuracy was confirmed with the Lock mass Glu fibrin (m/z). The ESI parameters during analysis were set as scan frequency (sec) 30, Cone Voltage of 40 V and Collision Energy 6.000. The data analysis was done using Protein Lynx Global SERVER (PLGS) 2.5.3 software and the data was analysed using Uniprot database.

4.23. RNA EXTRACTION

4.23.1. Materials required

a) TRIzol (Sigma)
b) DNA RNAase free H_2O, tips and vials
c) Cooling centrifuge
d) Chloroform (Merck)
e) Iso propanol (Merck)
f) Ethanol (Merck) – 70 % made in DEPC treated H_2O

4.23.2. Principle

TRI Reagent has phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate RNAase activity. Biological samples are homogenized or lysed in TRI Reagent; the subsequent addition of chloroform results in the separation of the homogenate into aqueous and organic phase. RNA partitions to the aqueous phase, DNA to the interphase, and protein to the organic phase. The RNA can then be precipitated from the aqueous phase with the addition of iso propanol. The isolated RNA is suitable for any downstream application, including RT-PCR.

4.23.3. Procedure:

1. Cells were trypsinised and centrifuged at 1500 rpm. To the pellet 1 mL of TRIzol reagent was added and incubated at room temperature for 5 min.

2. Chloroform 200 µl was added and mixed well for 15 sec and incubated at room temperature for 3 min.
3. Then centrifuged at 12000 rpm for 15 min.
4. The aqueous layer was transferred to a new vial without disturbing the protein layer.
5. Iso-propanol (2- propanol) 500 µl was added and mixed well by inverting and incubated at room temperature for 10 min and step 3 was repeated.
6. The supernatant was discarded and 1 mL of 70 % alcohol was added to the pellet and incubated at room temperature for 2-3 min.
7. Then centrifuged at 14000 rpm for 5 min and after discarding the supernatant it was air dried for 3 min.
8. Finally 20 µl of RNAase free H_2O was added and store at - 80°C (2 µl for quantification and gel electrophoresis)

4.24. Conversion of RNA to cDNA:

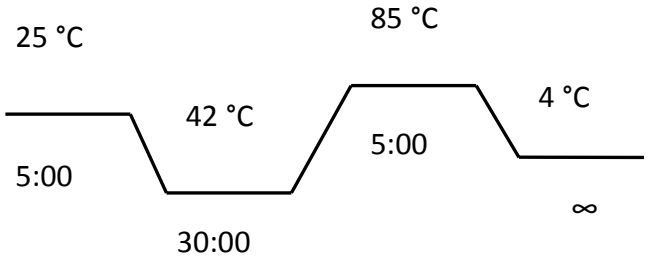
RNA was converted to cDNA using iScript RT-PCR Kit (Bio-Rad) following the manufacturer's protocol using the reagents provided in the kit.

4.24.1. Procedure

Step 1: The following reaction mix was prepared

RNA Template	-	2 µl (1 µg)
Nuclease free H_2O	-	13 µl
5x I script Reaction Mix	-	4 µl
Reverse Transcriptase	-	2 µl
		20 µl

Step 2: The following protocol was followed for cDNA conversion

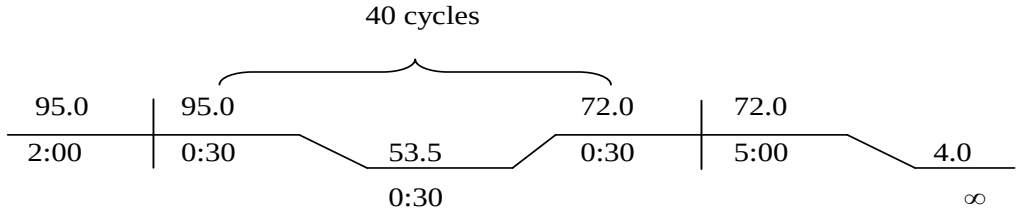


4.25. Polymerase chain reaction

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Primers for APN, Adipo R1, Adipo R2 (Pineiro, Iglesias et al. 2005) , PEDF (Zhang, Xiang et al. 2009) and VEGF are listed in the table 8.

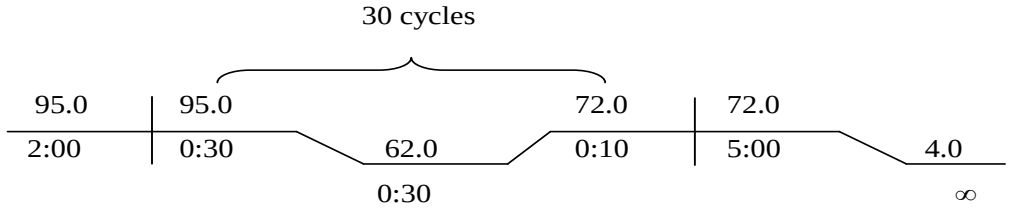
4.25.1. Reverse transcriptase PCR

PCR protocol for APN



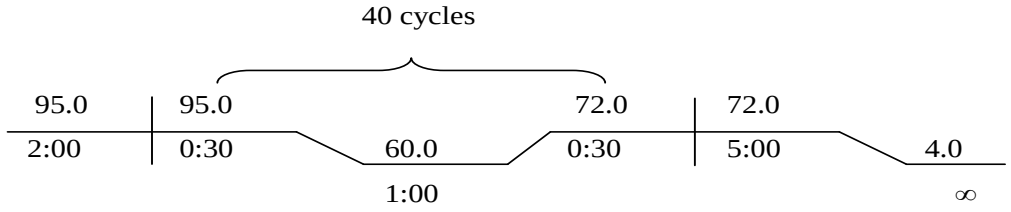
Product size: 220 bp

PCR protocol for AdipoR1, AdipoR2



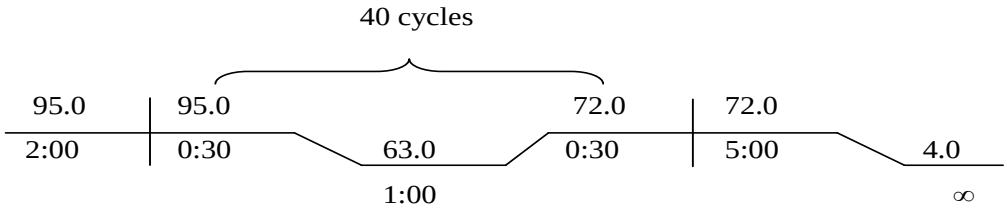
Product size: 71,76 bp respectively

PCR protocol for VEGF



Product size: 180 bp

PCR protocol for PEDF



Product size: 155 bp

4.25.2. Real time PCR

Quantification of RNA was done using nanodrop. Quantitative real-time PCRs were performed using Applied Biosystems 7300 with SYBR Green chemistry (Eurogentech, Belgium). Real-time PCR cycle conditions included the following steps: denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 25 sec. Each sample was run in triplicate and C_t was determined for the target transcripts. Real time calculations were done using $2^{-\Delta\Delta C_T}$ method (Livak, Schmittgen et al 2001).

Table 8: List of primers used

S.No	Target gene	Forward Primer	Reverse primer
1.	Human APN	5’ TGGTGAGAAGGGTGAGAA 3’	5’AGATCTTGGTAAAGCGAATC 3’
2.	Human ADIPOR1	5’TTCTTCCTCATGGCTGTGATGT 3’	5’AGAAGCGCTCAGGAATTCG 3’
3.	Human ADIPOR2	5’ATAGGGCAGATAGGCTGGTTGA 3’	5’GGATCCGGGCAGCATACA3’
4.	VEGF	5’CGGTATAAGTCCTGGAGCGTTC 3’	5’GCCTCGGCTTGTCACATCTG 3’
5.	PEDF	5’ AGGCCCAGAGTCCTGACGGG 3'	5’CTTGAAGTGCGCCACACCG 3'
6.	GAPDH	5’GAACATCATCCCTGCCTCTACTG 3’	5’CGCCTGCTTCACCACCTTC 3’.
7.	Bovine PREF-1	5’ CAACTTCTGCGAGATCGTGA3’	5’ TGCAGGTCTTGTCCATGAAG3’
8.	Bovine PPAR γ	5’TTCTTCCTCATGGCTGTGATGT 3’	5’AAGAAGCGCTCAGGAATTCG 3’
9.	Bovine APN	5’ ATAGGGCAGATAGGCTGGTTGA 3’	5’ GGATCCGGGCAGCATACA 3’
10	Bovine VEGF	5’CATAGCCGCCGCCACCAC3’	5’CGCACAGCCTCCTCTTCCTTC3’
11	Bovine GAPDH	5’- GTTCCAGTATGATTCCA CCC-3’	5’-TCTTCTGGGTG-GCAGTGAT-3’

4.26. Design of siRNA

The siRNAs was designed using Ambion web tools, and the rules described by Reynolds et al for siRNA designing were followed (table 9). Scrambled siRNA (Ssi), was designed using scrambled siRNA software. (Reynolds, Leake et al. 2003)

Rule 1 – GC content (33 – 52 %)

Rule 2 - 15 – 19 (AAA or UUU) residues.

Rule 3 – Melting point (nearest neighbour) less than 60.

Rule 4 – 19th base pair (A)

Rule 5 – 3rd residue (A)

Rule 6 - 10th position "U"

Rule 7 – 19th position (G or C)

Rule 8 – 13th position G

Rule 7 & Rule 8 are negative points G or C residues always absent.

Table 9: List of siRNA for APN using ambion webtool

S.NO	Sense strand	GC%	R1	R2	R3	R4	R5	R6	R7	R8	Score
1	ACCACGACUCAAGGGCCCGUU	61.9	X	X	70.12	X	X	X	√	√	-2
2	GGGCCCGGAGUCCUGCUUCUU	66.7	X	X	63.38	X	X	X	√	X	0
3	GGGGGCCUGCACAGGUUGGUU	66.7	X	X	67.4	X	X	X	√	X	0
4	UGGGGCCCCAGGCCGUGAUUU	66.7	X	X	73.9	X	X	X	X	X	2
5	GGGUGAGAAAGGAGAUCCAUU	47.6	√	X	55.1	√	X	X	X	X	5
6	AGGAGAUCCAGGUCUUAUUUU	38.1	√	X	34.3	X	X	X	X	X	4
7	GGGAGACAUCGGUGAAACCUU	52.4	X	X	58.4	X	X	X	√	X	1
8	GGGAGACAUCGGUGAAACCUU	66.7	X	√	58.4	X	X	X	√	X	2
9	GGUCCCCGAGGCUUUCCGGUU	66.7	X	X	68.7	X	X	X	√	X	0
10	UCCAAGGCAGGAAAGGAGAUU	47.6	√	X	63.5	√	X	X	X	X	4
11	GGCAGGAAAGGAGAACCUGUU	52.4	X	X	60.3	X	X	X	√	√	-2
12	AGGAGAACCUGGAGAAGGUUU	47.6	√	X	54.2	X	X	√	X	X	5

13	CCUGGAGAAGGUGCCUAUGUU	52.4	X	X	46	X	X	X	√	√	-1
14	GGUGCCUAUGUAUACCGCUUU	47.6	√	X	40.6	X	X	X	X	X	4
15	CAUGCCCAUUCGCUUUACCUU	47.6	√	X	52.1	X	X	√	√	X	3
16	GAUCUUCUACAAUCAGCAAUU	33.3	√	X	28.9	√	X	X	X	X	5
17	UCAGCAAAACCACUAUGAUUU	33.3	√	X	45.1	X	√	X	X	X	5
18	AACCACUAUGAUGGCUCCAUU	42.9	√	X	44.7	√	X	X	X	√	3
19	**CCACUAUGAUGGCUCCACUUU**	**47.6**	√	**X**	**40.6**	**X**	√	√	**X**	**X**	**6**
20	AUUCCACUGCAACAUUCCUUU	38.1	√	X	37.8	X	X	X	X	X	4
21	**UCAGCAAAACCACUAUGAUUU**	**33.3**	√	**X**	**43.9**	**X**	√	**X**	**X**	**X**	**6**

Two highlighted sequences of sense strand having score of more than 5 were taken and the antisense strands of the selected sense Si RNA were further given for synthesis.
These two (si 1, si 2) were screened for the silencing effect on adipocytes – APN expression.

The sequence of adiponectin siRNA and scrambled siRNA are as

siRNA 1: 5’AGUGGAGCCAUCAUAGUGGUU 3’

siRNA 2 : 5’AUCAUAGUGGUUUUGCUGAUU 3’

Scrambled siRNA: 5’GUGAUUUACGGGCUAGAGUAU 3’

4.27. PROTEIN - PROTEIN INTERACTION USING *IN SILICO* ANALYSIS:

1. Data mining to Identify existing interacting partners using STRING (Szklarczyk, Franceschini et al. 2011)
2. Multiple sequence alignment of the existing interacting partners
3. Pattern identification through PRATT for the existing interacting partners.
4. Post translational modification analysis through Scan Prosite.
5. Generation of pattern based on the conserved post translational modification analysis.
6. Manual screening of PRATT generated hits by PTM patterns and categorising them based on localisation.

7. Molecular modelling studies for APN and required interacting partners using modeller 9v7/I-TASSER. Refined models will be subjected to energy minimization using GROMACS (Roy, kucukural et al. 2010; Xu, Zhang et al. 2011)
8. Molecular interaction studies for the existing partners and the new partners obtained from sequence based approach using ClusPro and PatchDock.
9. Molecular interaction analysis through PDBsum and HoTPOINT server.
10. Mapping the significant residues involved in all the interactions and predicting putative functions for the new partners.

4.28. Peptide design

Based on bioinformatics work, three peptides were designed. The sequence of these peptides are listed below:

Pep 1: PKGACTGWMAGIP (32-44)

Pep 2: KDKAMLFTYDQYQE (178-191)

Pep 3: DNDNDSTFTGFLLY (226-40)

A scrambled peptide a random sequence was also designed and synthesized along with the adiponectin derived peptides.

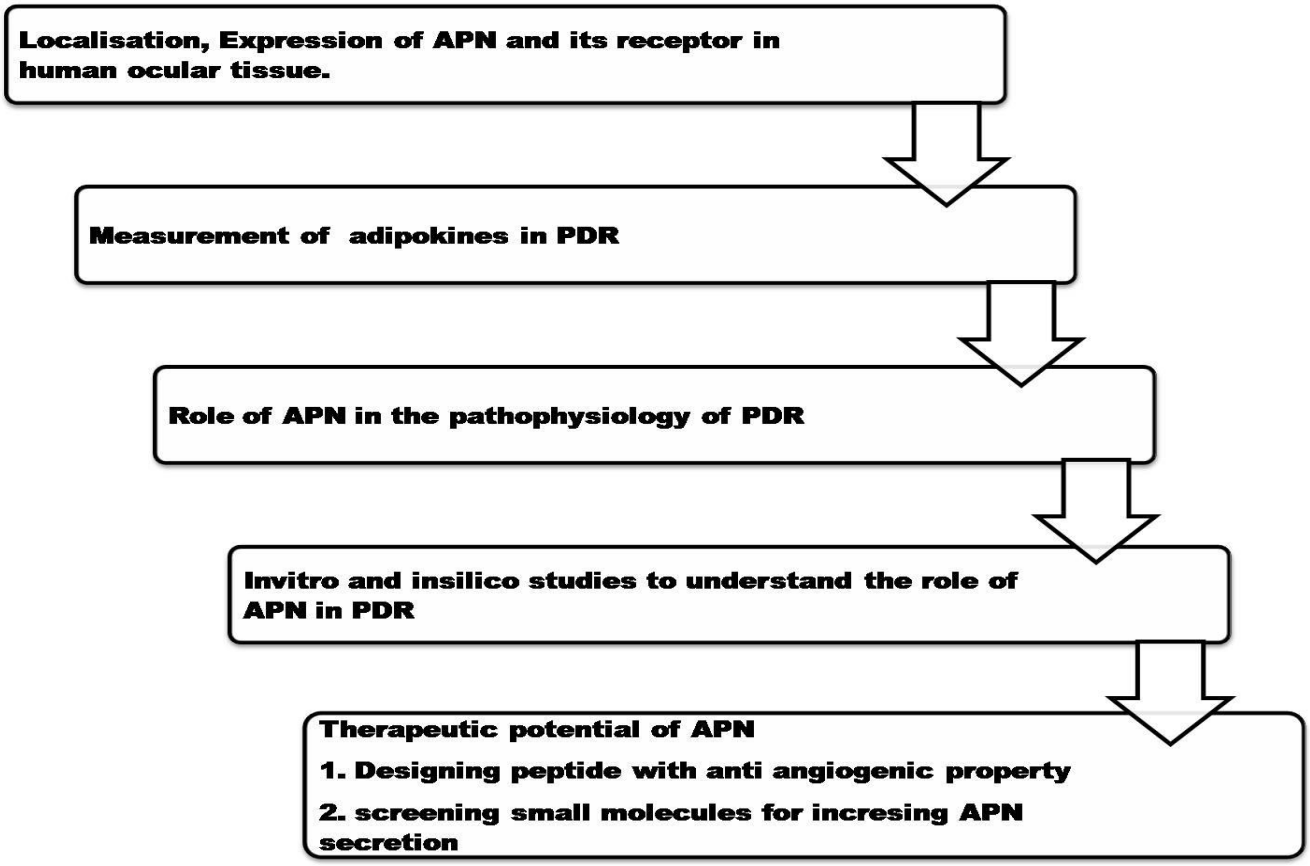
Scrambled peptide (ScP): KAYNDADPP

4.28.1. Purity check using HPLC

Purity check of APN derived peptides:

Peptides were synthetically manufactured. These peptides of 1 mg/ mL concentration were dissolved in H_2O and injected into Agilent HPLC. Buffer A consisting of 0.1 % trifluoroacetic acid in H_2O and buffer B 0.1 % trifluoroacetic acid in acetonitrile were prepared. Buffers were filtered using 0.22 µ filter and degassed by sonicating for 15 min twice. Linear gradient elution from 0 to 90 % was performed. HPLC column (Jupiter) of 5 µm thickness, flow rate of 0.5 mL/ min and detected at 214 nm for 1 h time point.

WORKFLOW:


Localisation, Expression of APN and its receptor in human ocular tissue.
Measurement of adipokines in PDR
Role of APN in the pathophysiology of PDR
Invitro and insilico studies to understand the role of APN in PDR
Therapeutic potential of APN
1. Designing peptide with anti angiogenic property
2. screening small molecules for incresing APN secretion

CHAPTER 5: RESULTS

5.1. Expression and Localisation of APN and its receptors in human ocular tissue

5.1.1. Screening of APN and its receptor mRNA expression in ocular tissues and primary ocular cells

The information about APN expression and localisation is very essential as there are no or few published reports. This will pave way to understand its role in ocular pathology and physiology. Different ocular tissue viz iris, choroid, ciliary, retina, retinal blood vessel (RBV), and optic nerve fibre (ONF) were separated and screened for mRNA expression of APN, AdipoR1 and AdipoR2 by reverse transcriptase followed by real time PCR. Reverse transcriptase PCR for APN was standardized using adipocytes whereas AdipoR1 and AdipoR2 with peripheral blood mononuclear cells (PBMC) as shown in Figure 10.

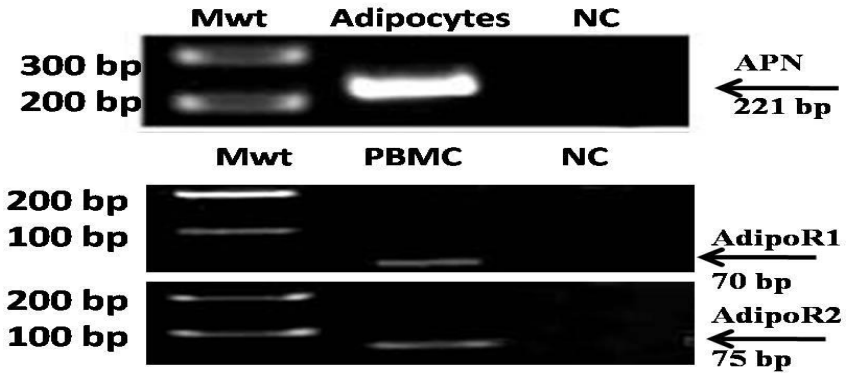


Figure 10: Standaridisation of APN, AdipoR1 and AdipoR2 by reverse transcriptase PCR. The presence of mRNA of APN (221bp), AdipoR1 (70bp), and AdipoR2 (75bp) as visualized in 1.5% agarose gel.

The presence APN mRNA expression and its receptors AdipoR1 and R2 were observed in all the ocular tissue screened viz iris, choroid, ciliary, retina, RBV, and ONF by RT PCR and qRTPCR (Figure 11 & 12). GAPDH was used as a loading control in all PCR.

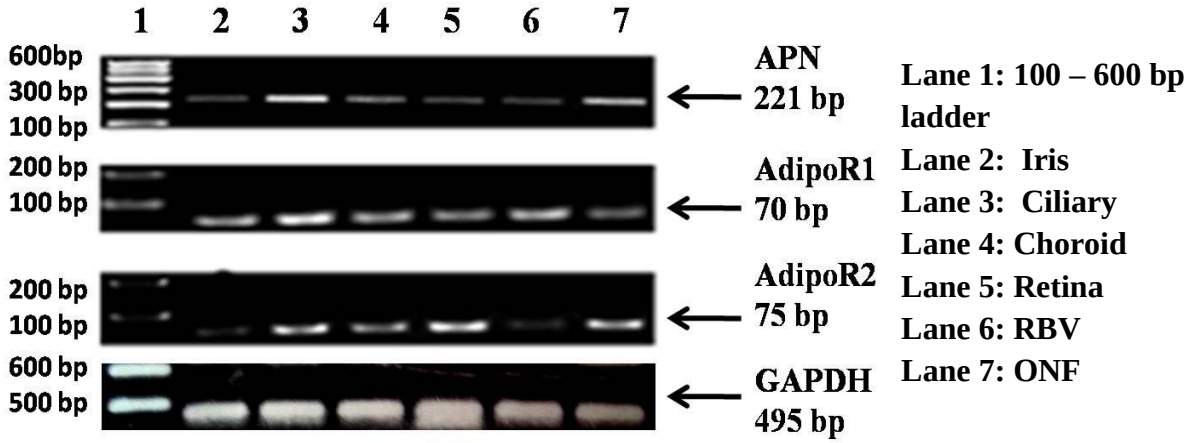


Figure 11: mRNA expression of APN and its receptor in human ocular tissues by RT PCR.

The presence of mRNA of APN (221bp), AdipoR1 (70bp), and AdipoR2 (75bp) in all ocular tissues as visualized in 1.5% agarose gel.

ONF showed higher expression of APN followed by choroid, ciliary, iris retinal blood vessels and retina (Figure 12 A). Both AdipoR1 and AdipoR2 were expressed in all ocular tissues but interestingly AdipoR2 expression was notably higher (Figure 12 B). Both receptors showed maximum expression in choroid and retina.

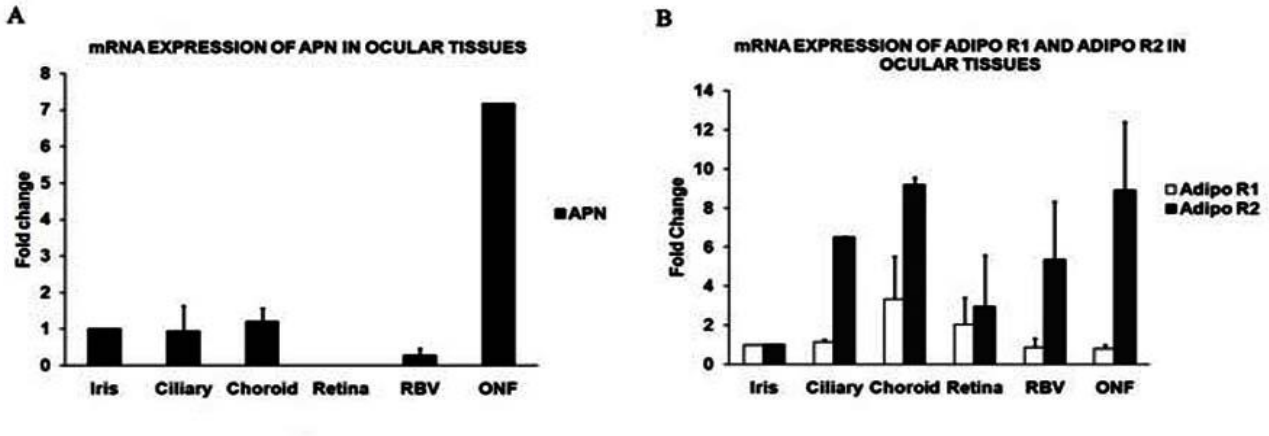


Figure 12: APN and its receptor mRNA expression in human ocular tissues by qRT PCR

A) mRNA expression of APN. B) mRNA expression of AdipoR1 and AdipoR2

The two major microvascular endothelial cells HREC (Figure 13 A), HCEC (Figure 13 C) were isolated from the donor eye ball. Endothelial cell specific marker Von Willebrand factor was used to confirm the primary cultures (Figure 13 C, D) and the cells were used between 2 and 5 passages for further experiments. These microvascular endothelial cells act as an appropriate *in vitro* model for ocular angiogenesis.

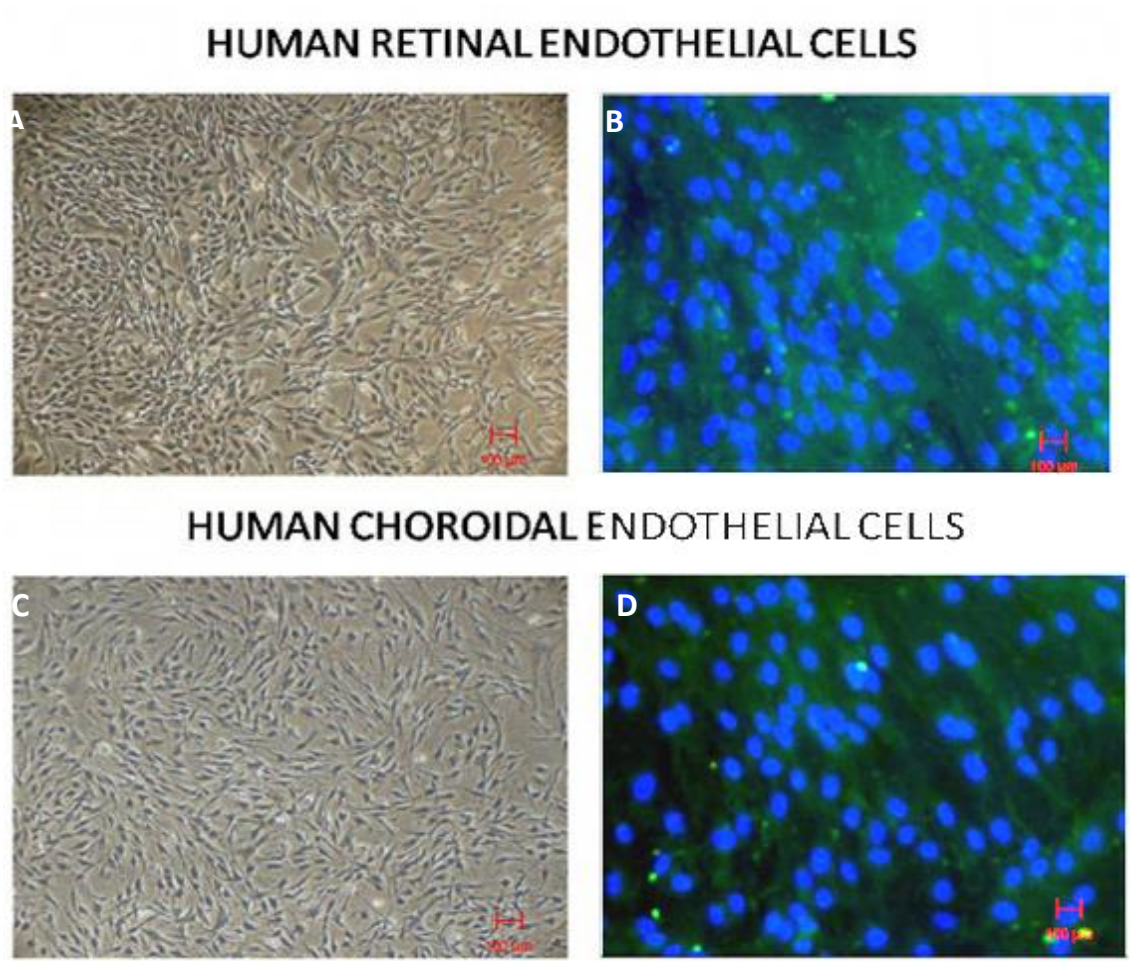


Figure 13: Isolated primary ocular microvascular endothelial cells- HREC & HCEC.

A) Phase contrast image of isolated HREC. B) Phase contrast image of isolated HCEC. C) IF staining for Von Willebrand factor in isolated HREC. D) IF staining for Von Willebrand factor in isolated HCEC. Green florescence shows the positive staining of endothelial cell specific marker

Two primary ocular endothelial cells HREC, HCEC and HRPE were also analysed for the presence of mRNA transcriptome of APN and its receptor. Maximum expression of APN mRNA transcriptome was seen in HREC (Figure 14 A). HRPE and HCEC showed almost similar mRNA expression levels. AdipoR1 and AdipoR2 transcripts were more in HCEC compared to HRPE and HREC (Figure 14 B). Expression of AdipoR2 was significantly more than AdipoR1 in all the three primary cultures.

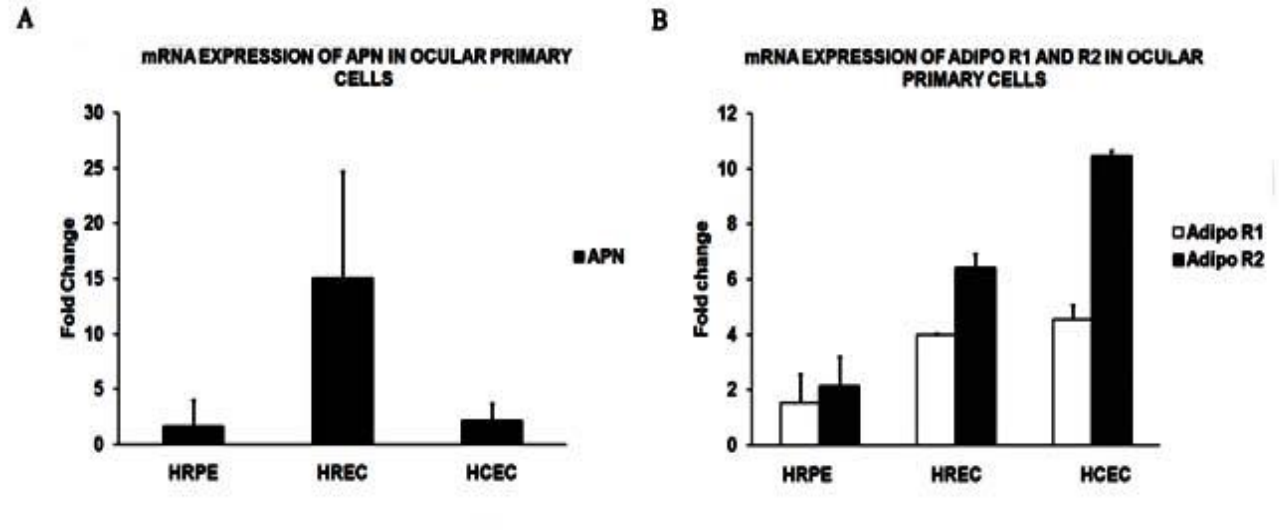


Figure 14: mRNA expression of APN and its receptor in primary ocular cells by qRT PCR.

A) mRNA expression of APN in primary ocular cells. B) mRNA expression of Adipo R1 and R2 in primary ocular cells.

5.1.2. Protein level expression of APN and its receptors in Ocular tissue

Western blot analysis revealed the presence of protein expression of APN AdipoR1 and AdipoR2 in ocular tissues. The protein expression of APN in ocular tissue was also measured quantitatively by ELISA (Figure 15 A). ONF showed maximum expression, followed by ciliary, choroid, retina, RPE and iris, successively in both western blot as well as ELISA. AdipoR1 and AdipoR2 were expressed in all ocular tissue as shown by our western blot analysis (Figure 15 C). Vitreous samples from PDR and MH were also subjected western blot for APN. Increased APN protein expression was observed in vitreous of the patient with PDR.

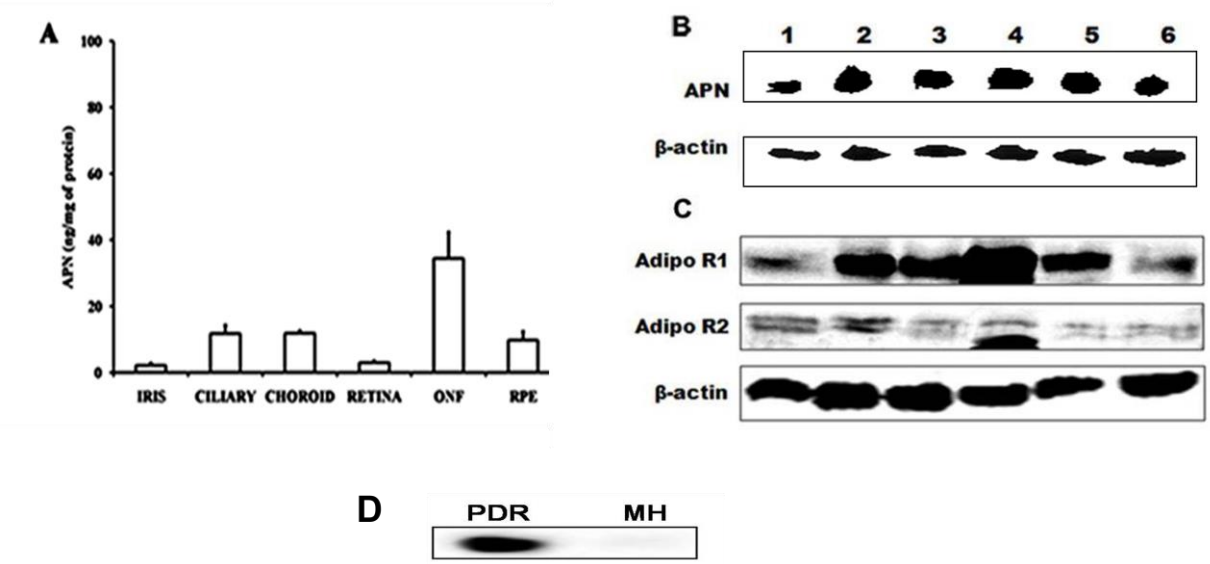


Figure 15: Protein level expression of APN and its receptors in human ocular tissues. Experiments were repeated thrice (n=3)

A) Quantification of APN protein expression by ELISA in ocular tissue.

B) Western blot analysis of APN and beta actin in ocular tissue

C) Western blot analysis of AdipoR1, Adipo R2 and beta actin in ocular tissue. Lane 1: iris, Lane 2: ciliary, Lane 3: choroid, Lane 4: retina, Lane 5: optic nerve fibre, Lane 6: RPE.

D) Western blot analysis of APN in vitreous of the patient with PDR and MH.

5.1.3. Localisation of APN and its receptor in ocular tissue sections

So far APN localisation was studied in rat models as reported by Bora et al, but not in humans. Five donor eyeballs with no diabetic complication and a natural cause of death were used for staining. Immunohistochemistry revealed the presence of APN, AdipoR1 and AdipoR2 in different layers of human donor eye sections. The immunolocalisation of donor eyes showed weak signals of APN, whereas its receptors AdipoR1 and AdipoR2 (Figure 16) showed strong signals in the retinal layers, RPE and choroids.

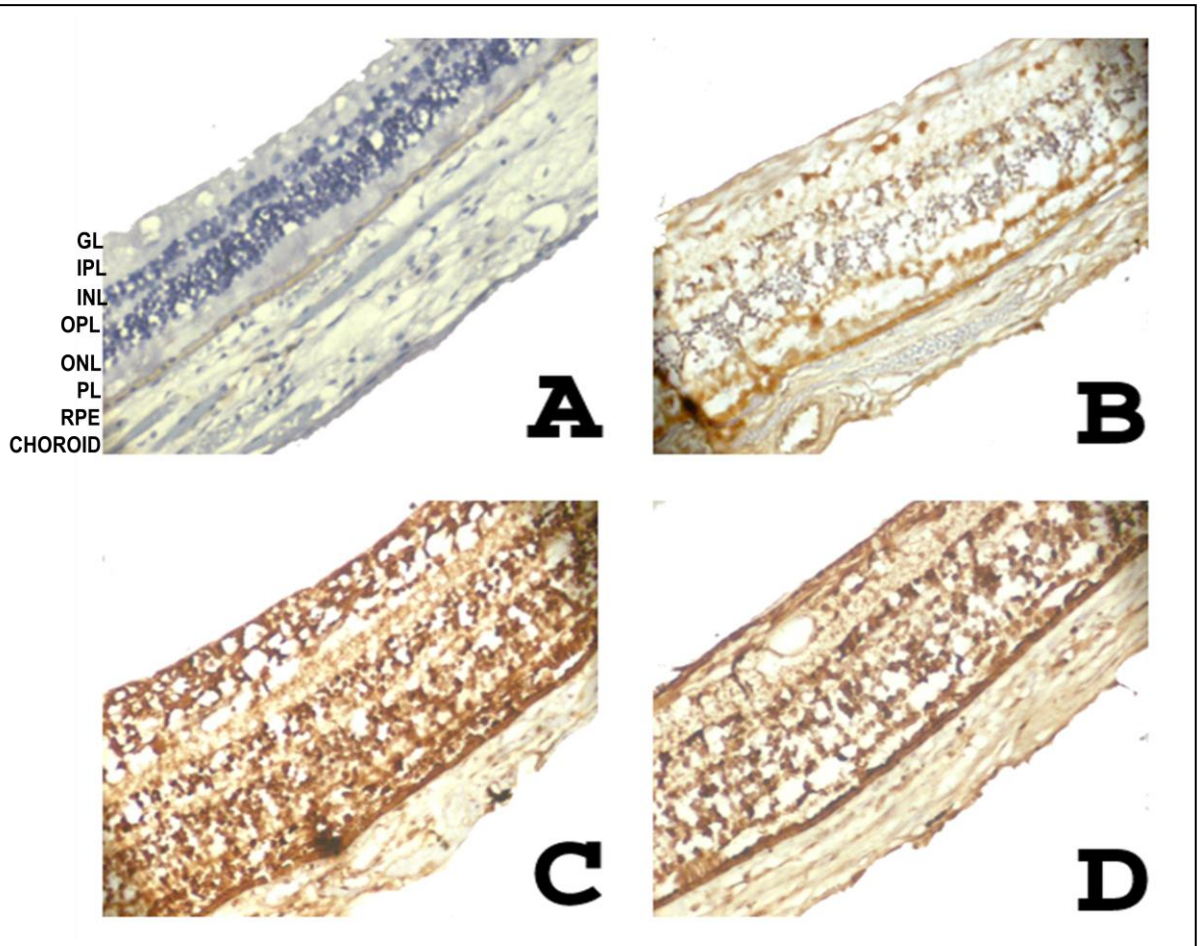


Figure 16: Immunohistochemistry staining for APN, AdipoR1 and AdipoR2 on 5-µm sections of paraffin block of retinal tissue. (n=5)

A) Negative control (without primary antibody). B) APN. C) Adipo R1.
D) Adipo R2.
GL: Ganglion layer, IPL: Inner Plexiform layer, INL: Inner nuclear layer, OPL: Outer Plexiform layer, ONL: Outer nuclear layer, PL: photoreceptors layer. RPE: retinal pigment epithelial cells

Formation of Epiretinal membrane (ERM) is a pathological process. Following vitrectomy, ERM was peeled to improve the visual acuity of the patient. Surgically removed ERM from PDR was also stained by IHC which revealed the presence of APN and its receptors AdipoR1 and AdipoR2 in the membrane (Figure 17).

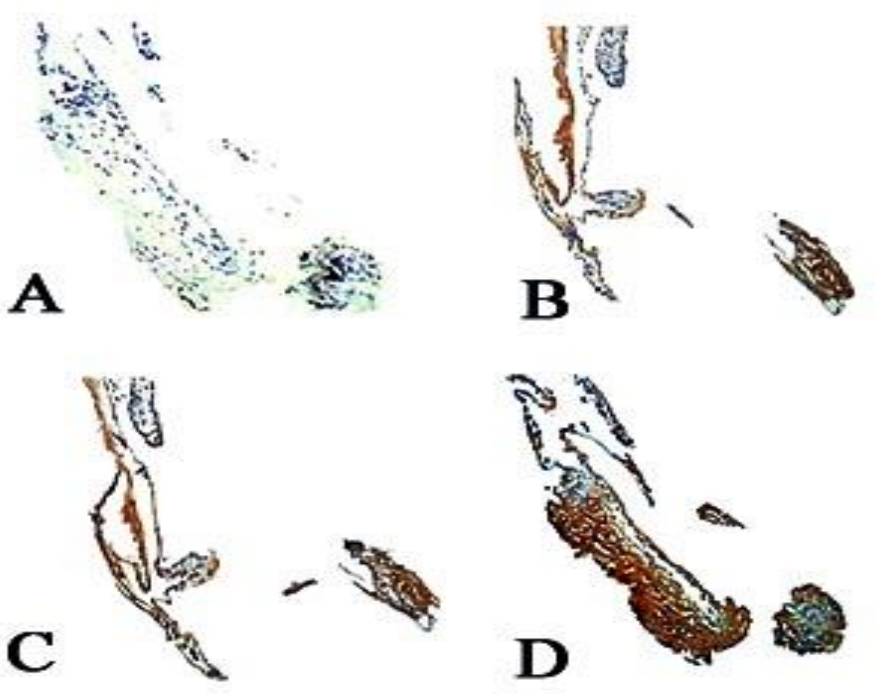


Figure 17: Immunohistochemistry staining performed on 5-µm sections of paraffin block of human ERM tissue (n=5).

A) Negative control (without primary antibody). B) APN.
C) Adipo R1. D) Adipo R2.

Immunoflourescence staining for APN, AdipoR1 and AdipoR2 was done to further confirm immunohistochemistry observation. Similar results were obtained in both immunohistochemistry and immunoflourescence method indicating that APN and its receptor AdipoR1, AdipoR2 are localised in the ocular tissue viz ciliary body, retina, choroid layers and in ERM.

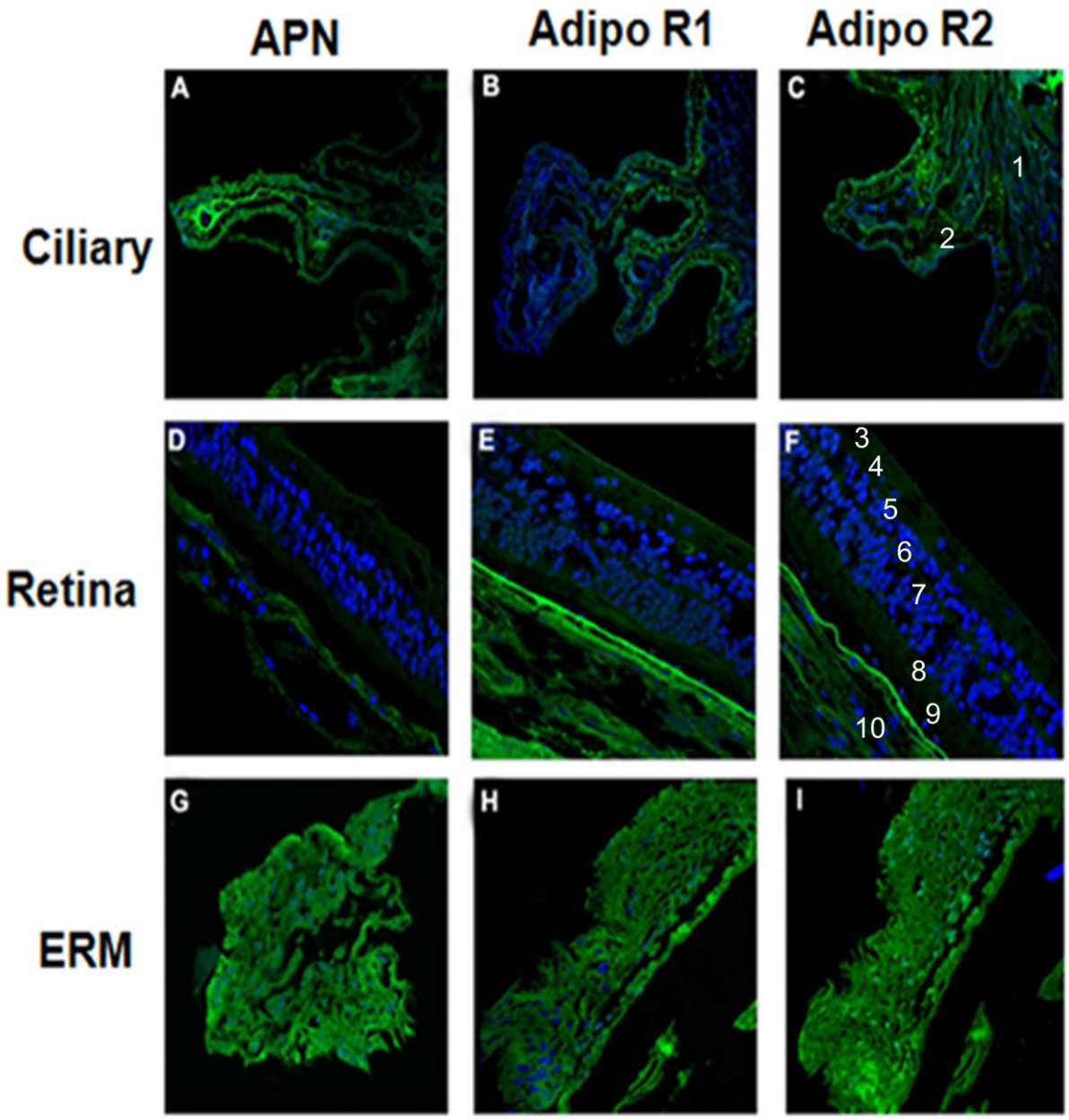


Figure 18: APN and its receptors localisation by Immunoflourescence staining on 5μm paraffin block sections (n=5). Confocal micrograph of APN, AdipoR1, AdipoR2 in ocular tissue and surgically removed ERM. 1: Ciliary muscles, 2: Zonular nodules with pigmented and non pigmented ciliary epithelial layers, 3: Ganglion layer, 4: Inner Plexiform layer, 5: Inner nuclear layer, 6: Outer Plexiform layer, 7: Outer nuclear layer, 8: photoreceptors layer, 9: retinal pigment epithelial cells, 10: Choroidal layer.

5.1.4. Co-localisation of APN with CD31, a retinal endothelial cell marker

APN was localized and expressed in retina APN was found to be more in HREC and to establish the fact that APN is expressed in retinal endothelial cells, colocalisation of APN with retinal endothelial cell markers CD31 was performed. Mild positivity was observed in the retinal capillary indicating the co-localisation of APN with CD31 as in Figure 19.

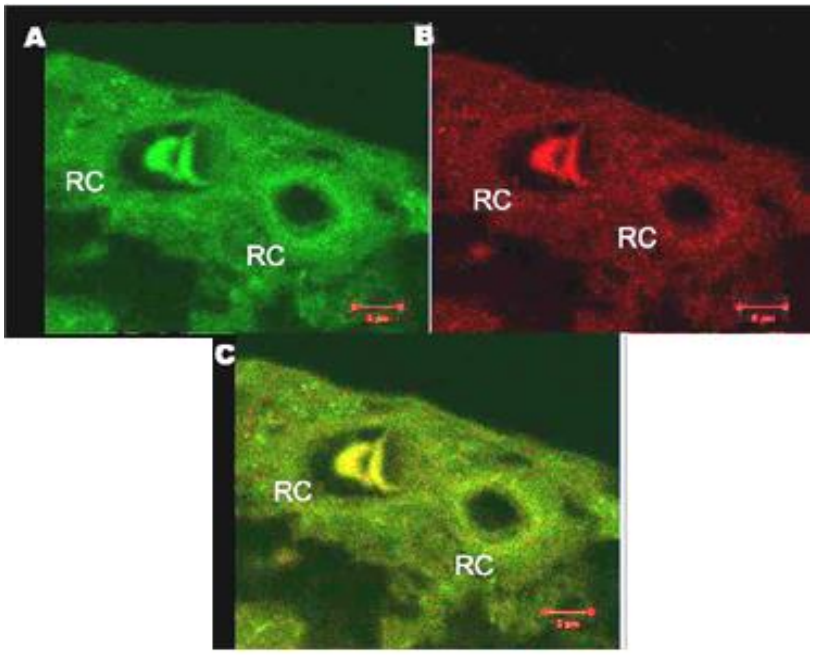


Figure 19: Colocalisation of APN and CD31 in retinal layer.

A) Green fluorescence of APN. B) Red fluorescence of CD31. C) Merged image of A, B.
RC: Retinal capillary

This is the first report on the expression and localisation of APN and its receptor in human ocular tissues. Presence of mRNA transcripts of APN and its receptors, immunolocalisation, protein expression in ocular tissues indicates possible role of APN in ocular physiology and pathology.

5.2. Measurement of Adipokines in PDR

From the ERM results it is clear that APN is over expressed in PDR and thus to understand its role in PDR we also measured several other adipokines in this disease. Earlier studies reported the vitreous levels of various adipokines like VEGF (Adamis, Miller et al. 1994; Bouck 2002), PEDF (Bouck 2002), IGF-1(Meyer-Schwickerath, Pfeiffer et al. 1993) in patients with PDR. Additionally Zeitz *et al* has reported the presence of vitreous APN in just five samples as proof of concept (Zietz, Buechler et al. 2008). The objective was to measure APN levels in vitreous from PDR and MH (disease control) patients and to compare with already known players of retinopathy namely VEGF, PEDF and IGF-1 in vitreous and plasma of patients with PDR.

Table 10: Physical and biochemical parameters of the study group

PARAMETERS	CONTROL I	CONTROL II	PDR	MH
Number of cases	N = 14	N = 11	N = 53	N = 23
			Laser: N = 42	
			Non Laser : N= 11	
Age (Years)	44 ± 10	47 ± 12	54 ± 7	61 ± 7
[Mean ± SD]				
Body Mass Index (kg/m^2)	24.0 ± 3	30 ± 4.8	26.7 ± 5.1	25 ± 5
BP Systolic (mm Hg)	116 ± 11	127 ± 7	148 ± 20	132 ± 17
BP Diastolic (mm Hg)	73 ± 10	83 ± 7	84 ± 8	82 ± 7
Random blood Glucose (mg/dL)	95 ± 2	150 ± 14	192.3 ± 12.7	122 ± 22
[Mean ± SEM]		[p< 0.000]	[p< 0.000]	[p< 0.000]
HbA1c (%)	5.3 ± 0.1	6.5 ± 0.3	7.4 ± 0.2	6 ± 0.2
		[p< 0.001]	[p< 0.000]	[p< 0.009]
Serum Total Cholesterol (mg/dL)	169 ± 8	198 ± 17	177 ± 10	177 ± 9
Serum Triglycerides (mg/dL)	100 ± 10	190 ± 15	147 ± 14	130 ± 18
		[p< 0.000]	[p< 0.03]	
Serum HDL Cholesterol (mg/dL)	47 ± 2	37± 3	43 ± 2	45 ± 3
Serum protein (gm/ dL)	7.5 ± 0.1	7.5 ± 0.2	7.3 ± 0.1	7.5 ± 0.2
Microalbumin in urine (mg/ L)	< 20	< 20	> 20	< 20

Table 10 shows the levels of physical and biochemical risk factors for PDR in the pool of study participants. The control group was age, and BMI matched to the PDR group. Biochemical analysis of the known risk factors showed increased plasma glucose, triglycerides, and glycosylated hemoglobin in the control II and PDR group with statistical significance compared to control I. Patients with PDR had > 20 mg/L of microalbuminuria while, in all control subjects, it was < 20 mg/L.

Table 11: Plasma Adipokines levels in the study group

[*Significance compared to control I]

Plasma Adipokines	**CONTROL I**	**CONTROL II**	**PDR**	**MH**
APN (μg/mL)	6.05 ± 0.9	1.6 ± 0.4 (**p*< 0.000)	6.2 ± 0.8	5.4 ± 0.8
PEDF (μg/mL)	0.54 ± 0.1	35.5 ± 5.5 (**p*< 0.000)	4.6 ± 0.9 (**p*< 0.000)	1.9 ± 0.4
VEGF (pg/mL)	50.5 ± 5.6	83.3 ± 2.6	28.3 ± 7.7	65.7 ± 4.0
IGF- 1 (ng/mL)	60.7 ± 3.8	70.3 ± 4.2	75.7 ± 7.8	80.1 ± 12.1

5.2.1. Plasma adipokines level

Plasma adipokines viz APN, VEGF, PEDF and IGF-1 levels were quantified among the groups which are listed in the Table 11. Plasma APN levels (Figure 20 A) didn't show much difference between Control I, PDR and MH but the plasma APN level were significantly decreased in control II (patients with diabetes) compared with control I ($p < 0.000$) indicating decreased insulin sensitivity of the group. Plasma PEDF levels (Figure 20 B) were significantly elevated in control II ($p < 0.000$) and in PDR group ($p < 0.000$) compared to control I indicating increased insulin resistance in these group. Plasma VEGF (Figure 20 C) and plasma IGF-1 (Figure 20 D) levels didn't show any significance among the groups.

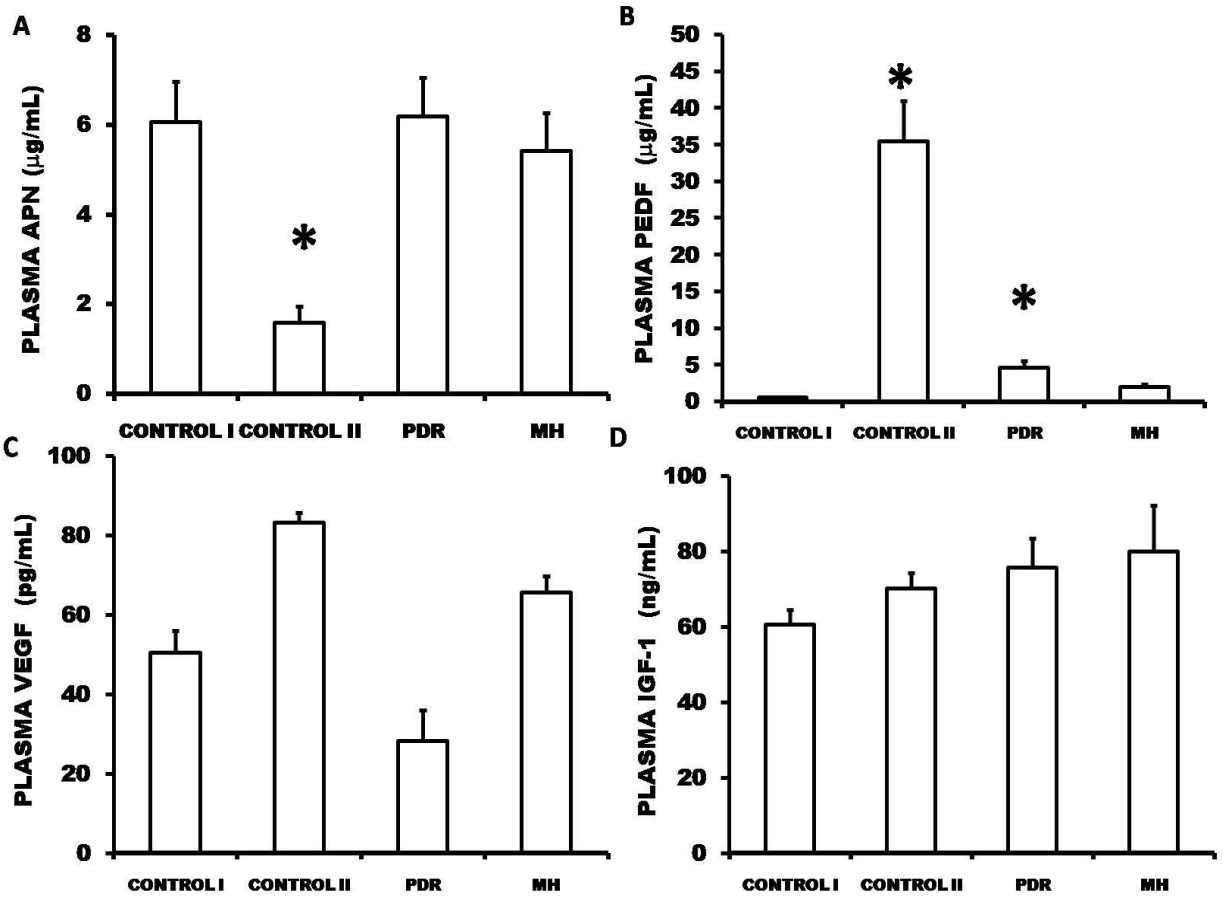


Figure 20: Plasma levels of adipokines (Mean ± SEM) in control I (n = 14), Control II (n = 11), patients with MH (n =23), patients with PDR (n = 53).

A. Levels of plasma APN. B) Levels of plasma VEGF.C) Levels of plasma PEDF. D) Levels of plasma IGF-1

5.2.1.1. Plasma APN positively correlated with HDL

Plasma APN was positively correlated with HDL in control II ($p < 0.001$, Figure 21 A), patients with PDR ($p < 0.000$, Figure 21 B) and also in the whole study group ($p < 0.000$, Figure 21 C) signify its vasculo-protective role.

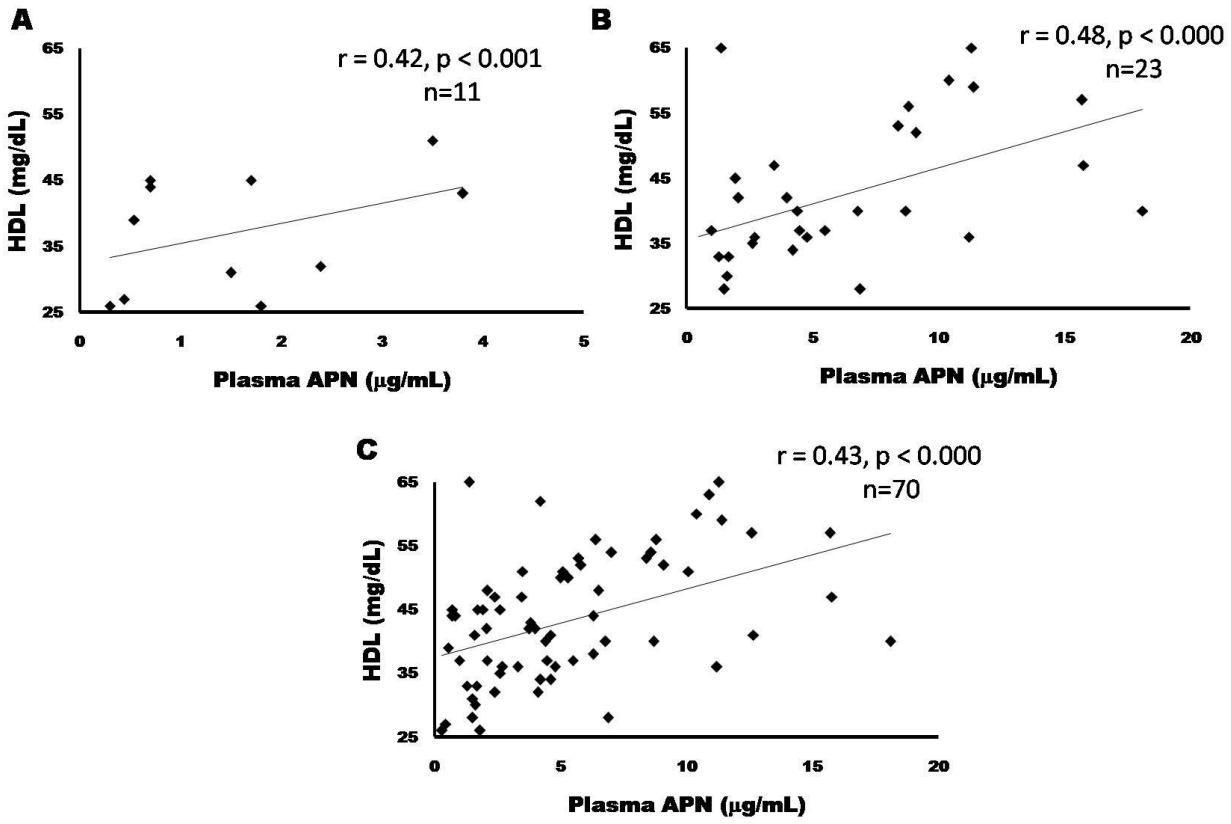


Figure 21: Plasma APN positively correlates with HDL

A) Correlation between plasma APN and HDL cholesterol among control II group
B) Correlation between plasma APN and HDL cholesterol among PDR group
C) Correlation between plasma APN and HDL cholesterol in total study group

5.2.2. Elevated vitreous adipokines in PDR

Vitreous APN (Figure 22 A), VEGF (Figure 22 B), and IGF 1 (Figure 22 D) were significantly higher in PDR than in MH, whereas, PEDF (Figure 22 C) does not show any significant difference in comparison to MH The levels of adipokines are as follows, APN: 88.7 ± 5.2 versus 0.54 ± 0.33 ng/mL, ($p < 0.000$); VEGF: 1.05 ± 0.03 ng/mL versus, not detectable levels, ($p < 0.0001$), IGF - 1: 1.76 ± 0.19 versus 0.77 ± 0.10 ng/mL, ($p < 0.0001$) and PEDF: 6.16 ± 0.24 versus 4.09 ± 0.60 ng/mL. Further within the PDR group, patients who underwent LASER treatment prior to surgery showed a significant increase in vitreous APN and PEDF levels, decreased VEGF than those who had not undergone any LASER treatment (APN: 105.1 ± 14.8 versus 28.9 ± 4.88 ($p < 0.01$), PEDF: 6.95 ± 0.8 versus 3.32 ± 0.75 ($p < 0.01$), VEGF: 0.89 ± 0.1 versus 1.65 ± 0.5,($p < 0.04$). In

addition, IGF -1 level was not altered much in the PDR patients who had undergone LASER treatment.

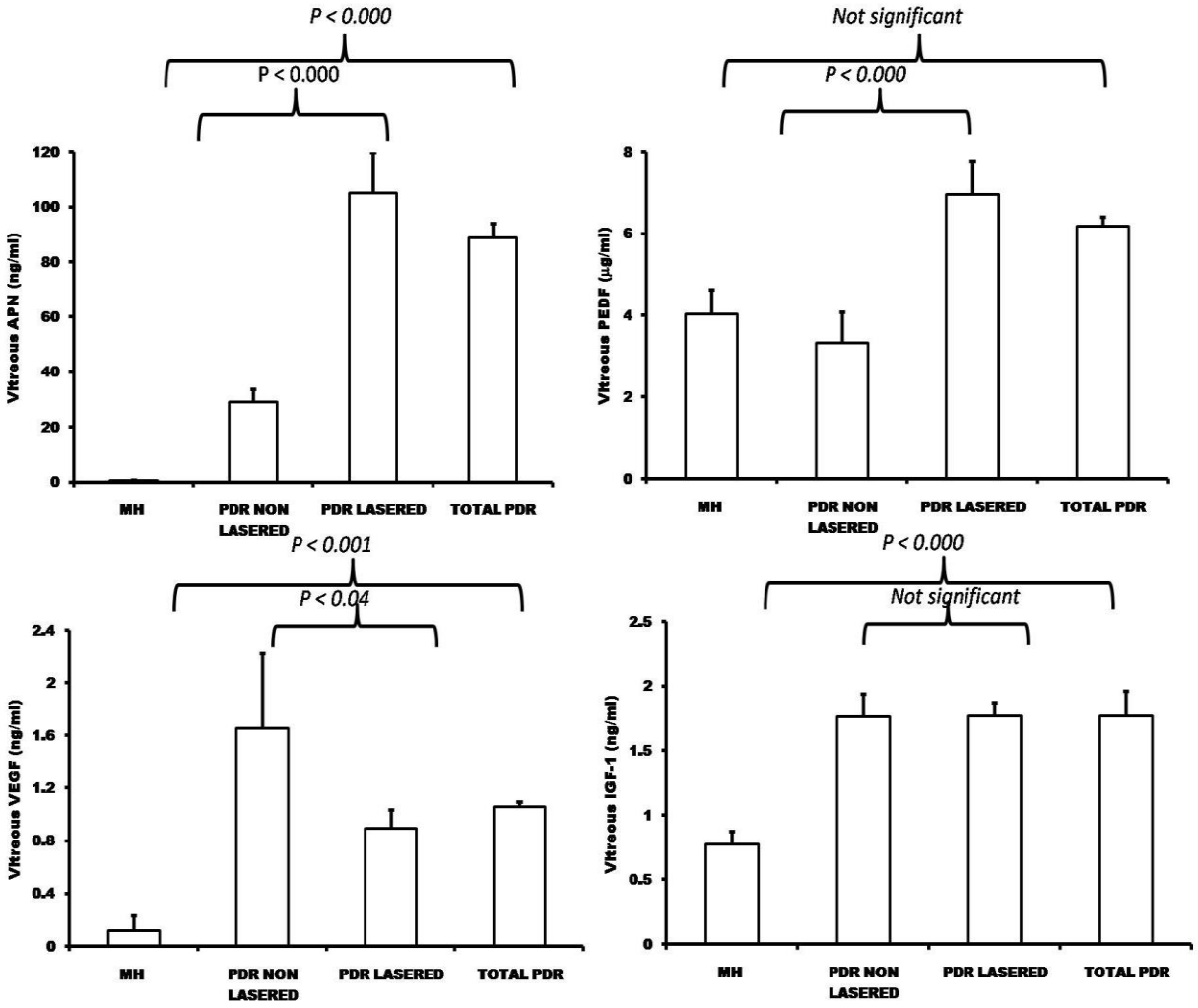


Figure 22: Vitreous levels of adipokines (Mean ± SEM) in of patients with MH (n =23), patients with PDR (n = 56). Among PDR group, patients with PDR who had not undergone any laser treatment (n=12), patients with PDR who had undergone laser prior to vitrectomy (n=44).

A) Levels of vitreous APN in the study group. B) Level of vitreous VEGF in the study group. C) Level of vitreous PEDF in the study group. D) Level of vitreous IGF-1 in the study group.

5.2.2.1. Vitreous APN positively correlated with PEDF and IGF-1, negatively correlated with VEGF

Vitreous APN levels were correlated with other growth factors PEDF, VEGF and IGF in PDR group and in the PDR patients who had undergone laser prior to vitrectomy. Vitreous APN levels positively correlated with anti angiogenic molecule PEDF in both PDR (Figure 23 A) and in PDR patients who underwent laser (Figure 23 B). In the same way APN positively correlated with the IGF-1 in the both the groups (Figure 23 E, F) but negatively correlates VEGF in the both the groups (Figure 23 C, D).

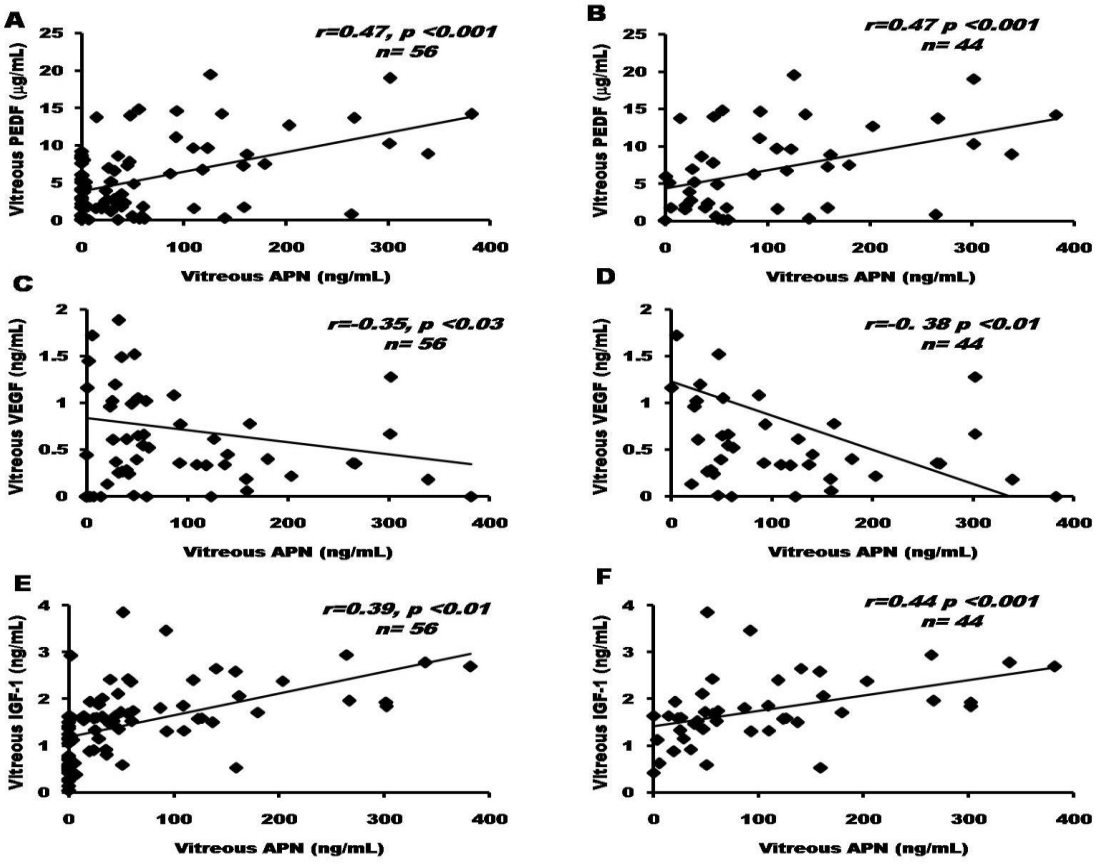


Figure 23: Results of correlation studies between adipokines in Vitreous

A) Correlation between vitreous APN and PEDF in PDR group (n = 56).B) Correlation between vitreous APN and PEDF in PDR group who underwent laser treatment prior to vitrectomy (n = 44). C) Correlation between vitreous APN and VEGF in PDR group (n = 56).D) Correlation between vitreous APN and VEGF in PDR group who underwent laser treatment prior to vitrectomy (n = 44).E) Correlation between vitreous APN and IGF-1 in PDR group (n = 56).F) Correlation between vitreous APN and IGF-1 in PDR group who underwent laser treatment prior to vitrectomy (n = 44).

Adipokines viz APN, VEGF, IGF-1 and PEDF are elevated in vitreous of PDR patients compared to MH. There was a 3 fold increase in APN level in patients, who had undergone LASER treatment, before vitrectomy. This elevated APN levels negatively correlated with VEGF and positively correlated with PEDF, IGF-1 indicating it may have an anti angiogenic role.

5.3. Pathophysiology of PDR:

In vitro reports state that amino acid mixture can stimulate the APN production intracellularly and glycine has already been shown to induce the mRNA of APN. Hence intraocular levels of free amino acids were estimated first followed by studying its effect on pericytes.

5.3.1. Elevated levels of amino acids in the vitreous of PDR:

A total of 12 amino acids viz glutamic acid, serine, histidine, glycine, alanine, tyrosine, methonine, valine, phenylalanine, isoleucine, leucine and lysine were estimated using RP HPLC in the vitreous obtained from patients with PDR (n = 30) and MH (n = 20). Vitreous from MH served as control. The analysis showed that out of the 12 amino acids 8 of them namely serine ($p \leq 0.000$), glycine ($p \leq 0.001$), alanine ($p \leq 0.017$), phenylalanine ($p \leq 0.013$), valine ($p \leq 0.004$), isoleucine ($p \leq 0.000$), leucine ($p \leq 0.022$), and lysine ($p \leq 0.001$) were significantly elevated in PDR patients when compared to MH. Other amino acids like glutamic acid, histidine, tyrosine, methionine did not attain statistical significance (Table 12). There was a significant positive correlation between serine ($p \leq 0.021$), glycine ($p \leq 0.045$), alanine ($p \leq 0.000$), phenylalanine ($p \leq 0.04$), isoleucine ($p \leq 0.023$), leucine ($p \leq 0.043$), and lysine ($p \leq 0.026$) with APN level in the vitreous. All these amino acids were further taken for *in vitro* analysis at 0.5mM concentration.

Table 12: Levels of Amino acids in PDR Vitreous

[*Comparison between PDR vitreous and MH vitreous]

Aminoacid	**PDR (µm/L) (n = 30)** **Mean ± SEM**	**MH (µm/L) (n = 20)** **Mean ± SEM**	***p* value***	**Correlation with Vitreous APN**
Glutamic acid	36.41 ± 6.7	37.2 ± 6.2	0.921	r = 0.038, *p* = 0.798
Serine	55.9 ± 6.6	29.9 ± 4.0	0.000	**r = 0.328, *p* = 0.021**
Histidine	22.0 ± 3.5	26.6 ± 6.7	0.874	r = 0.075, *p* = 0.610
Glycine	51.3 ± 6.8	41.3 ± 16.4	0.001	**r = 0.316, *p* = 0.045**
Alanine	39.3 ± 3.7	26.0 ± 3.1	0.017	**r = 0.509, *p* = 0.000**
Tyrosine	8.7 ± 1.2	5.5 ± 0.8	0.118	r = 0.273, *p* = 0.058
Methionine	25.7 ± 5.4	20.1 ± 5.2	0.060	r = 0.015, *p* = 0.917
Valine	72.0 ± 7.1	48.0 ± 6.7	0.004	r = 0.266, *p* = 0.065
Phenylalanine	33.8 ± 2.6	24.3 ± 2.8	0.013	**r = 0.289, *p* = 0.044**
Isoleucine	28.3 ± 2.3	16.4 ± 2.4	0.000	**r = 0.325,p = 0.023**
Leucine	50.9 ± 4.4	34.7 ± 3.9	0.022	**r = 0.291, *p* = 0.043**
Lysine	54.7 ± 3.2	35.5 ± 3.8	0.001	**r = 0.317, *p* = 0.026**

Pericytes plays a vital role in maintaining the vasculature and loss of pericytes is considered as hallmark of PDR, the effect of these elevated amino acids was studied in Bovine Retinal Pericytes (BRP). For the experiment BRP were incubated in preadipocyte basal medium with 0.5mM individual amino acids (proline, hydroxyproline, phenylalanine, alanine, serine, glycine, lysine, isoleucine, valine) and also with mixture of the mentioned amino acid along with 100 nM of insulin and 8 mM glucose for 14 days with addition of 0.5ml of medium every 3 days.

5.3.2. Effect of amino acid on BRP cells:

MTT assay was performed to check the cytotoxicity of amino acid exposure. There was no cytotoxicity of these amino acids at 0.5 mM concentration on the BRP cells till 48h. Cells unexposed to amino acid served as control (Figure 24 A). In addition to cytotoxicity assay,

apoptosis assay was also performed using cell death detection kit. Cell death detection assay also revealed no apoptotic changes as the apoptotic index was almost similar to control (Figure 24 B).

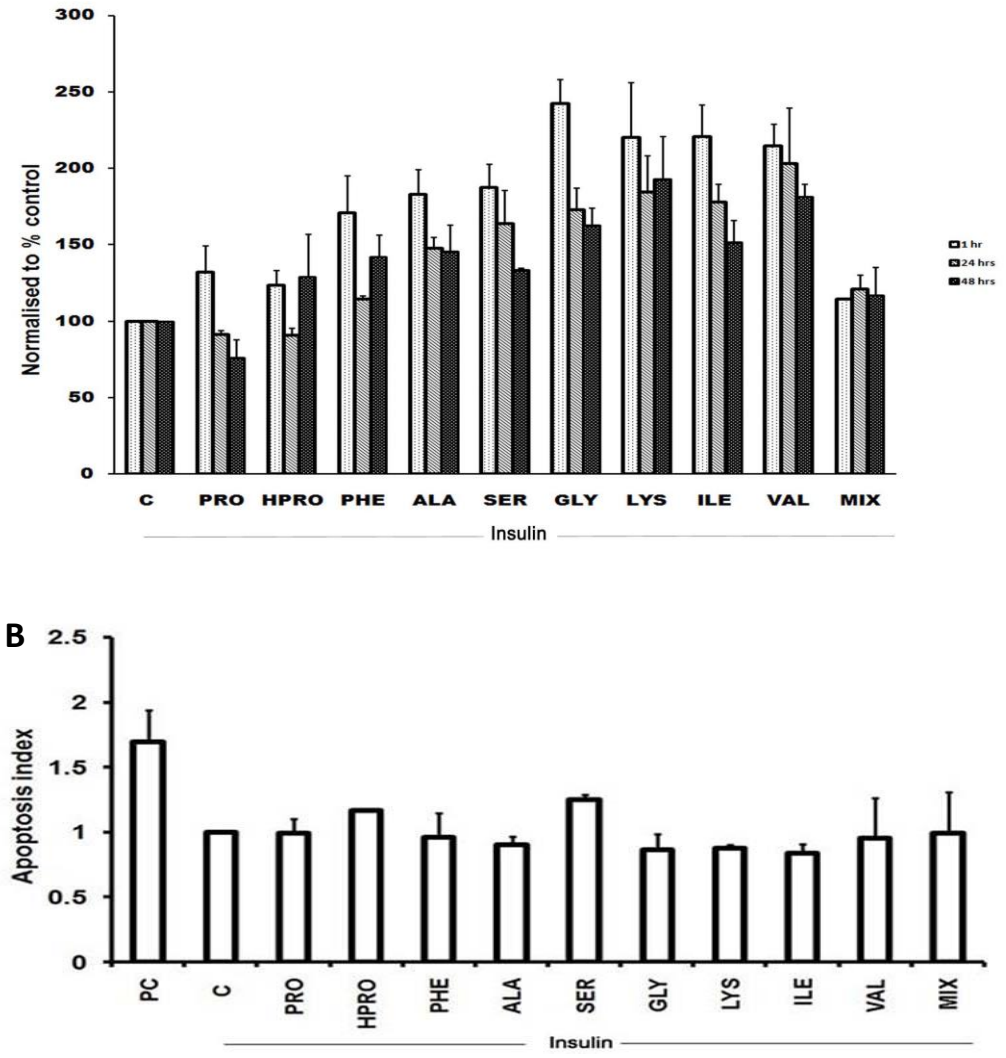


Figure 24: Cytotoxicity assay performed on BRP cells.

A). MTT Assay shows that amino acids are non toxic to BRP cells. B) Apoptotic assay studied using cell death detection kit revealed that amino acids exposure did not induce apoptosis.

All experiments were done in triplicates (n=3)

5.3.3. Adipogenic changes in the BRP exposed to amino acids:

Chronic exposure of amino acid in the BRP showed morphological changes in the cell cytoplasm, nucleus movement towards periphery and refractile bodies resembling oil droplets under phase contrast microscope but cells without amino acid did not show any effect. This is an indication of BRP differentiation into adipocyte like cells. Thus to confirm the adipogenic changes in BRP, presence of lipid accumulation was tested by standard oil red O staining method. These differentiated BRP showed a positive staining for lipid droplets (Figure 25).

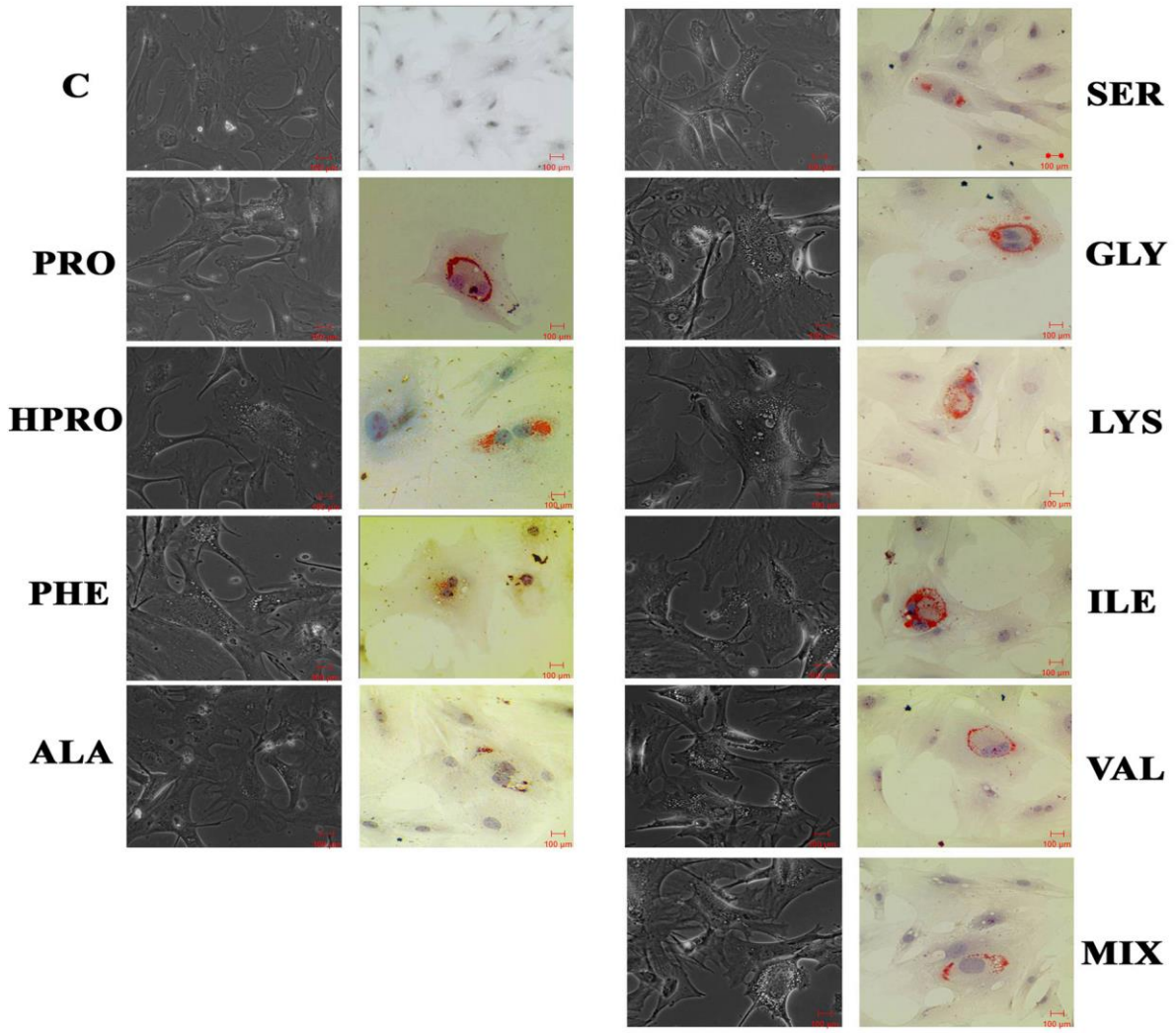


Figure 25: Oil red O staining of BRP exposed to amino acids.

First panel is phase contrast image and the second panel is oil red staining. Maximum staining was observed in proline, hydroxyl proline, glycine, lysine and isoleucine.

In order to confirm the oil drop as triglyceride, adipogenesis assay was also done in BRP cells (Figure 26). The amino acids hydroxyproline, serine, glycine, lysine and valine showed a statistically significant increase in triglyceride accumulation on exposure to BRP cells.

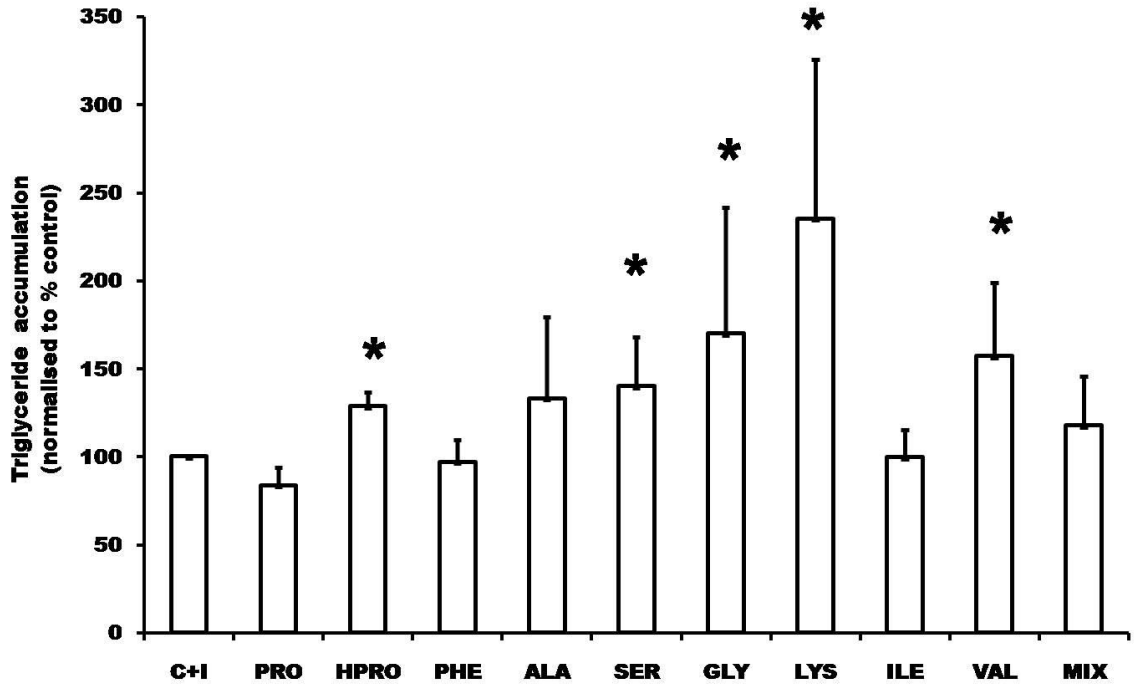


Figure 26: Adipogenesis assay performed in amino acid treated BRP.

BRP treated with hydroxyproline, serine, glycine, lysine and valine showed a significant increase in triglyceride accumulation
(* - statistically significant compared to control; $p \leq 0.05$).

All experiments were done in triplicates

5.3.4. Amino acid exposure displayed markers for adipocytes in BRP:

In addition to the Oil red O staining and triglyceride measurement the gene expression of the specific transcription factors like Pref-1, PPAR γ, APN and VEGF which are crucial for adipogenesis were analysed in the BRP cells treated with amino acids.

Pref-1 which is a marker for preadipocyte was found in all cells treated with amino acids. It was observed that in certain amino acids like proline, hydroxyproline and glycine which showed

higher triglyceride accumulation had significantly down regulated Pref-1 expression. This shows that the cells are beginning to differentiate. The rest of the six amino acids and the mixture did not show much variation in Pref-1 expression (Figure 27 A). PPAR γ which is the marker of adipocyte was present in all amino acid treated cells and control where as amino acids hydroxyproline, glycine, lysine and isoleucine showed a significant up regulation of the PPAR γ (Figure 27 B).

Adipocyte secretory protein APN and VEGF gene expression was also measured. Proline, hydroxyproline, glycine, lysine, isoleucine, and alanine showed a significant increase in the mRNA expression of adipocyte secretary protein, APN (Figure 27 C). Interestingly VEGF gene expression was decreased in all amino acid treated cells and significant decrease was observed in proline, hydroxyproline, glycine, lysine and isoleucine treatment (Figure 27 D).

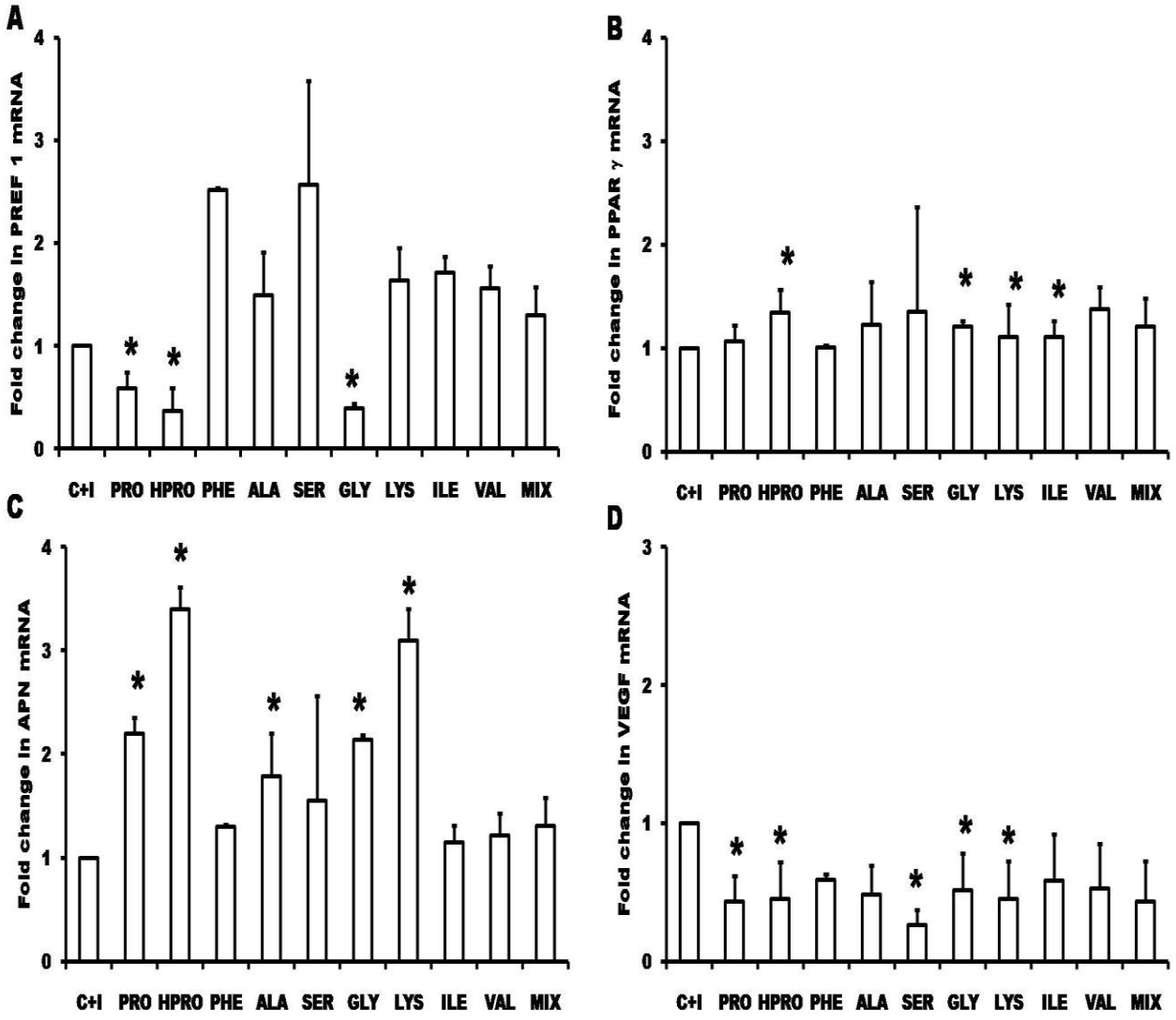


Figure 27: Real time PCR for the mRNA expression of Pref-1, PPAR γ, APN and VEGF in BRP cells exposed to amino acids. (* - statistically significant compared to control; $p \leq 0.05$)

A) Proline, hydroxyproline and glycine showed a significant decrease in Pref-1. B). Hydroxyproline, glycine, lysine and isoleucine showed a significant increase in PPAR γ. C) Proline, hydroxyproline, alanine, glycine, lysine and isoleucine showed a significant increase in *APN*. D) Proline, hydroxyproline, serine, glycine and lysine showed a significant increase in *APN*.

All experiments were done in triplicates

5.3.5. Proteomic analysis of differentiated BRP cells treated with hydroxyproline:

Adipogenic changes in the BRP after chronic exposure to amino acids showed lipid accumulation which was confirmed by Oil red O and adipogenesis assay. Further adipocytes markers were confirmed by real time. Few amino acids like hydroxy proline, glycine, lysine showed intense Oil red O staining, increased triglyceride accumulation, decreased Pref-1 expression, increased PPARγ and APN expression and decreased VEGF expression. Among these hydroxyproline treated cells were taken for proteomic analysis for the further confirmation of adipogenic changes.

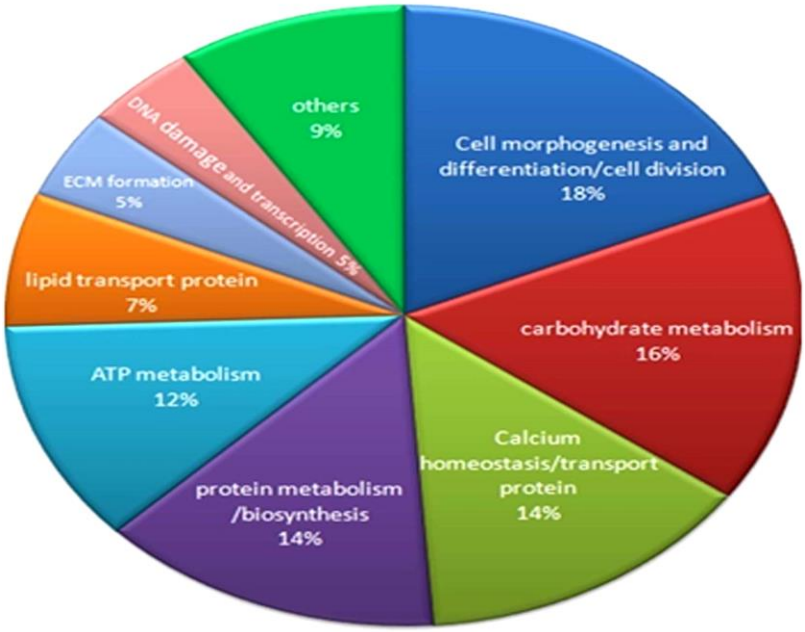


Figure 28: Pie chart showing the functional distribution of uniquely expressed proteins (43 proteins) in BRP cells treated with hydroxy proline.

Proteomic analysis of the BRP cells treated with hydroxyproline was done in Xevo G2S QTof to identify the differential proteins. A total of 322 protein hits were seen in hydroxyproline treated condition and 350 protein hits in control cell lysate. 43 proteins were unique hits in hydroxyproline treated cells which was differentially expressed when compared to control (Figure 28). Out of these 43, twenty five proteins (58 %) showed more than 10 fold increase in the amino acid treated condition compared to control (Table 13). When these 25 proteins were analysed by PANTHER, twelve proteins were already reported to be helping in the

differentiation of preadipocyte into adipocytes. CD151 (Adipocyte marker), Retinol binding protein (transport protein in adipocyte), Apolipoprotein O, non specific lipid transfer protein (lipid transport protein), nuclear migration protein (Cell morphogenesis and differentiation) are few important unique proteins found in the hydroxyproline treated cells.

Table 13: Unique proteins which were more than 10 fold elevated in the differentiating BRP treated with amino acid Hydroxyproline

S. No	PROTEIN DESCRIPTION	PLGS SCORE	MASS NUMBER OF THE PROTEIN	FOLD CHANGE COMPARED TO CONTROL (fmol)	PROTEIN SEQUENCE COVERAGE (%)	PROTEIN MATCHED PEPTIDES
1	ATP synthase subunit e mitochondrial OS Bos taurus GN ATP5I PE 1 SV 2	1023	8320.7	21.7	16.9	2
2	**Beta galactosidase OS Bos taurus GN GLB1 PE 2 SV 1**	**863.4**	**73812.5**	**15.2**	**8.3**	**7**
3	Beta hexosaminidase subunit alpha OS Bos taurus GN HEXA PE 2 SV 1	554.7	60752.1	15.4	9.3	10
4	**CD151 antigen OS Bos taurus GN CD151 PE 2 SV 1**	**994.2**	**28842.2**	**12.0**	**20.9**	**6**
5	**Dolichyl diphosphooligosaccharide protein glycosyltransferase subunit 2 OS Bos taurus GN RPN2 PE 2**	**1092.4**	**69328.2**	**10.0**	**11.4**	**6**
6	Elongation factor Tu mitochondrial OS Bos taurus GN TUFM PE 1 SV 1	582.9	49740.5	10.2	5.9	9
7	**Glutamate dehydrogenase 1 mitochondrial OS Bos taurus GN GLUD1 PE 1 SV 2**	**587.9**	**61854.2**	**23.8**	**26.3**	**20**
8	**Heat shock 70 kDa protein 1A OS Bos taurus GN HSPA1A PE 2 SV 2**	**3398.4**	**70543.7**	**42.9**	**26.4**	**15**
9	Hemoglobin subunit alpha OS Bos taurus GN HBA PE 1 SV 2	1285.5	15184.4	34.8	26.8	7
10	**Histone H2A V OS Bos taurus GN H2AFV PE 2 SV 3**	**3127.1**	**13508.7**	**55.7**	**20.3**	**3**
11	**Isocitrate dehydrogenase NAD subunit alpha mitochondrial OS Bos taurus GN IDH3A PE 1 SV 1**	**817.9**	**40124.2**	**14.3**	**24.9**	**11**
12	Myosin light chain 3 OS Bos taurus GN MYL3 PE 1 SV 1	2855.6	22110.1	50.1	13.6	4
13	**NADH dehydrogenase ubiquinone 1 alpha subcomplex subunit 4 OS Bos taurus GN NDUFA4 PE 1 SV 1**	**1694.0**	**9324.7**	**16.4**	**12.2**	**1**
14	Non specific lipid transfer protein OS Bos taurus GN SCP2 PE 1 SV 2	542.2	59204.9	14.4	21.9	13
15	Phosphoglucomutase 1 OS Bos taurus GN PGM1 PE 2 SV 1	597.7	61874.6	11.9	14.1	13

16	Polyubiquitin C OS Bos taurus GN UBC PE 1 SV 1	1577.6	77570.3	27.7	3.5	4
17	Proactivator polypeptide OS Bos taurus GN PSAP PE 1 SV 3	1462.7	59876.3	66.9	14.3	8
18	**Protein S100 A16 OS Bos taurus GN S100A16 PE 3 SV 1**	**857.5**	**11776.4**	**11.9**	**21.4**	**2**
19	Regucalcin OS Bos taurus GN RGN PE 2 SV 1	532.1	33878.0	110.9	7.4	7
20	Retinol binding protein 1 OS Bos taurus GN RBP1 PE 1 SV 4	620.5	15994.2	10.4	30.4	9
21	**Septin 11 OS Bos taurus GN SEPT11 PE 2 SV 1**	**1160.9**	**49277.0**	**20.5**	**22.4**	**9**
22	Tyrosine aminotransferase OS Bos taurus GN TAT PE 2 SV 1	552.9	50603.9	13.3	7.6	4
23	**Ubiquitin conjugating enzyme E2 N OS Bos taurus GN UBE2N PE 2 SV 1**	**2272.5**	**17194.8**	**43.8**	**42.1**	**5**
24	V type proton ATPase subunit B brain isoform OS Bos taurus GN ATP6V1B2 PE 1 SV 3	531.1	56919.0	10.6	14.8	9
25	**Voltage dependent anion selective channel protein 2 OS Bos taurus GN VDAC2 PE 2 SV 2**	**1112.2**	**32132.8**	**30.4**	**14.3**	**6**

Proteins which were already reported in mature adipocytes are given in bold. PLGS score was calculated by Protein Lynx Global SERVER (PLGS) 2.5.3 software.

After 14 days of culture, bovine retinal BRP showed a similar morphology of adipocytes with incorporated lipid droplets, increased adipogenecity, expressed adipocyte specific marker PPARγ, 2-3 fold increase in APN mRNA but decresed VEGF mRNA. Additionally proteomics analysis revealed adipocytes specific markers and proteins involved in cell morphogenesis and differentiation in BRP exposed to amino acids confirming adipogenic changes. Amino acids act as a trigger for the differentiation of retinal BRP to adipocytes, increasing APN and decreasing VEGF during the pathogenesis of PDR *in vivo*. Extrapolating these *invitro* results it is hypothesized that pericyte loss reported in PDR could be contributed by its adipogenic change which might be defence mechanism for increasing APN and decreasing VEGF.

5.4. Role of APN in ocular angiogenesis:

Most of the characteristic steps of the angiogenic process can be mimicked *in vitro*. Induction or inhibition of angiogenesis can be easily monitored by *in vitro* angiogenesis assay. Recombinant APN (rAPN) 10, 50, 100 ng/ mL were tested for anti angiogenic ability by *in vitro* angiogenesis

assay viz cell migration (*in vitro* wounding assay), proliferation (thymidine incorporation assay), cell adhesion assay and tube formation assay in three-dimensional matrigel in human retinal microvascular endothelial cells (HREC).

5.4.1. In vitro angiogenesis assay:

Cell proliferation assay by triated thymidine showed that rAPN decreased proliferation of HREC compared to cells without treatment. All the three concentration of rAPN (10 ng/mL, 50 ng/mL, 100 ng/mL) decreased proliferation by 30% compared to control (Figure 29).

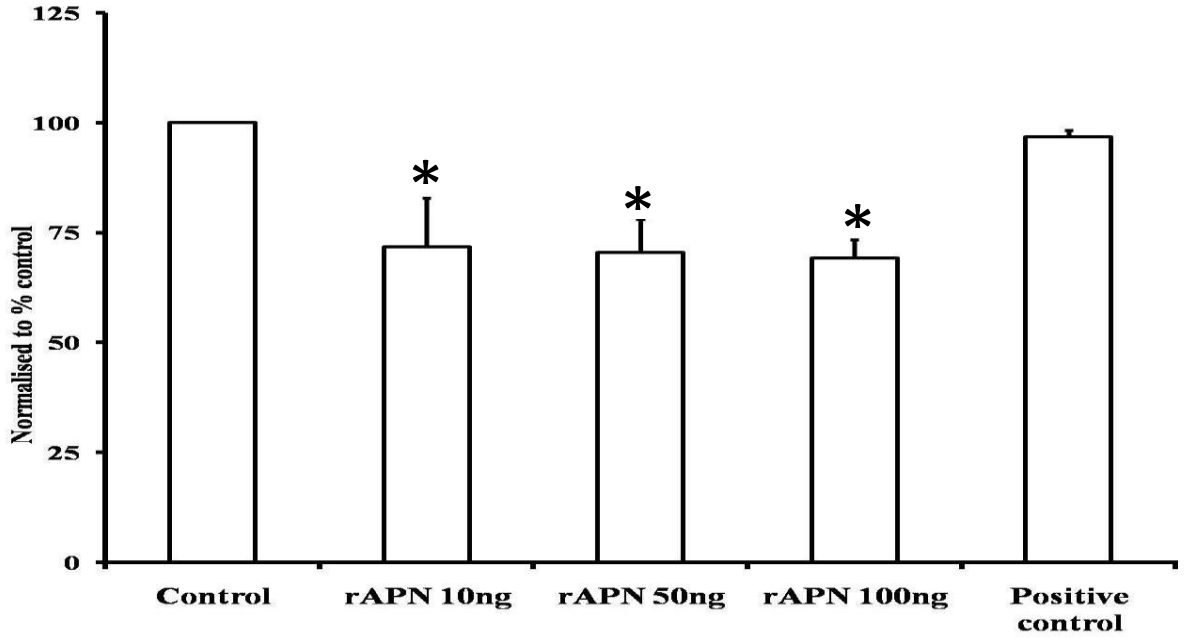


Figure 29: Proliferation assay using tritiated thymidine showed significant decreased proliferation at the end of 48 h of rAPN treatment. Positive control were the cells treated with 10% FBS. Comparison between control and rAPN treatment is represented as *. All experiments were done in triplicates (n=3)

Scratch assay was performed to determine the influence in migration at 16 h time point in HREC. All three concentration of rAPN (10 ng/mL, 50 ng/mL, 100 ng/mL) inhibited migration whereas the cells induced by growth medium (10% FBS) increased migration of HREC (Figure 30).

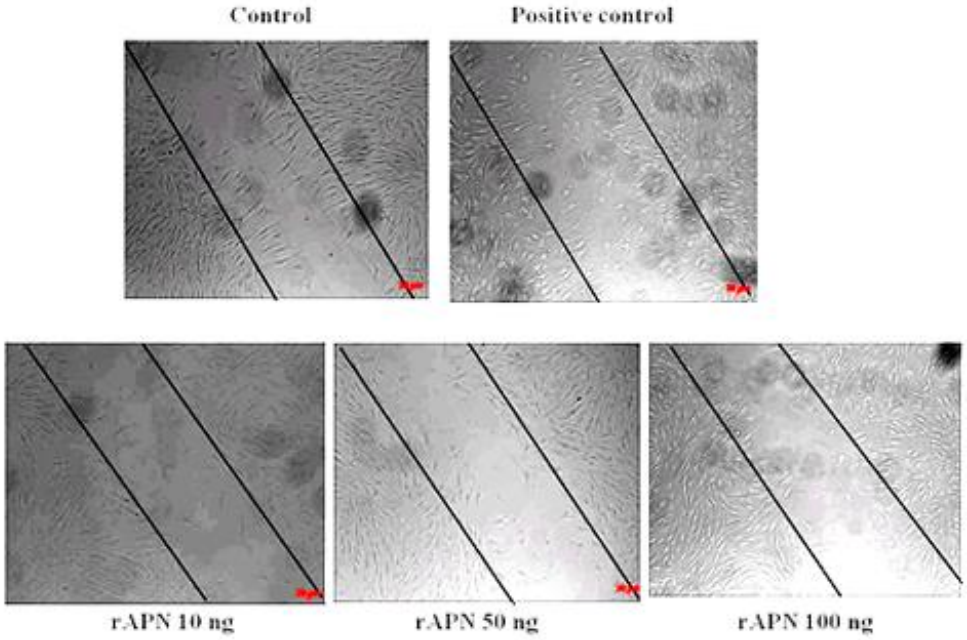


Figure 30: Scratch assay showed significant decreased migration in rAPN treatment compared to cells without treatment. Positive control were the cells treated with 10% FBS

All experiments were done in triplicates (n=3)

Similarly, in tube formation assay, HREC treated with rAPN (10 ng/mL, 50 ng/mL, 100 ng/mL) showed decreased tube formation while cells treated with growth medium showed increased tube formation (Figure 31).

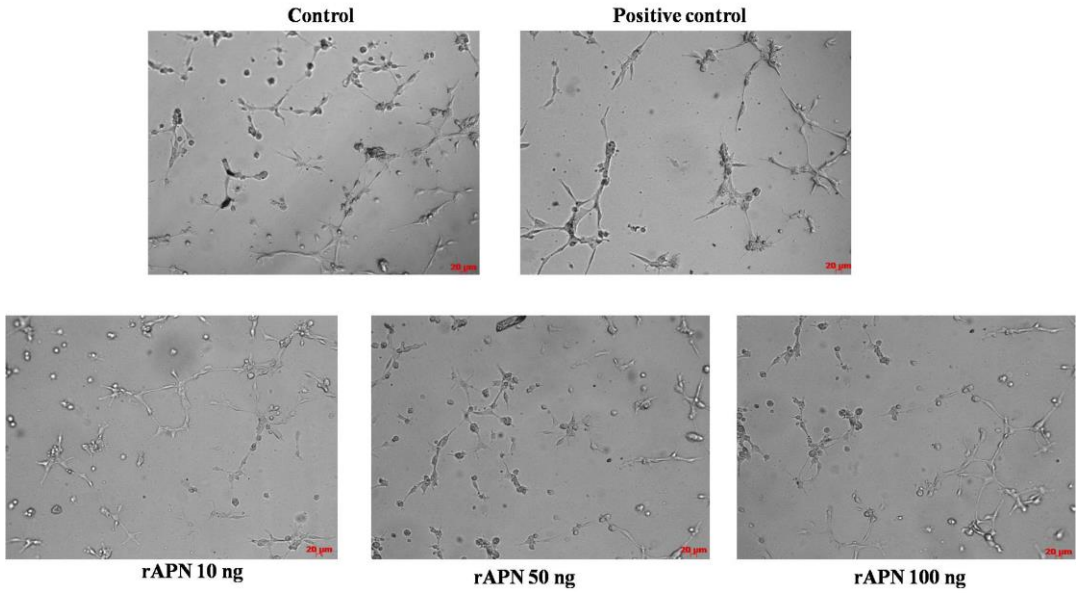


Figure 31: Matrigel assay showed significant decreased tube formation in rAPN treatment compared to cells without treatment. Positive control were the cells treated with 10% FBS. All experiments were done in triplicates (n=3)

VEGF is a pro angiogenic protein which is increased in PDR. On the addition of APN, VEGF levels decreased. This emulates the negative correlation of APN and VEGF levels after laser therapy where there is increased vitreous APN and decreased VEGF. WB analysis of VEGF levels confirmed that rAPN decreased VEGF levels. (Figure 32).

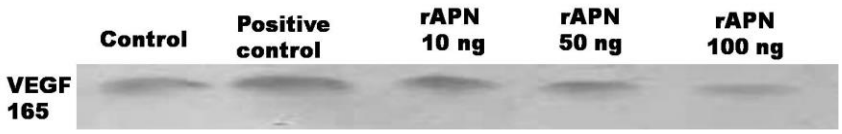


Figure 32: VEGF protein expression after rAPN treatment.

The rAPN added exogenously to microvascular endothelial cells i.e., HREC reduced migration, proliferation and tube formation, thus inhibited angiogenesis by decreasing VEGF levels.

5.4.2. Identification of interacting partners of APN - an insilico approach:

Multiple functions of APN are mediated by interacting with its transmembrane receptors and other growth factors. Identification of APN interacting partners aids in better understanding of functional status of APN. Hence, in this study, sequence and structure based approaches were implemented towards predicting of potential APN interacting partners that possibly mediate PDR.

5.4.2.1. Sequence based approach:

Documented and predicted interacting partners of APN were elucidated based on Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING). This database predicts the interacting based on Literature mining, Experimental evidence and also assigns confidence score for each prediction. The predictions with confidence value higher than 0.900 is considered as highly significant and the hits falling in this range were preceded with further analysis (Figure 33).

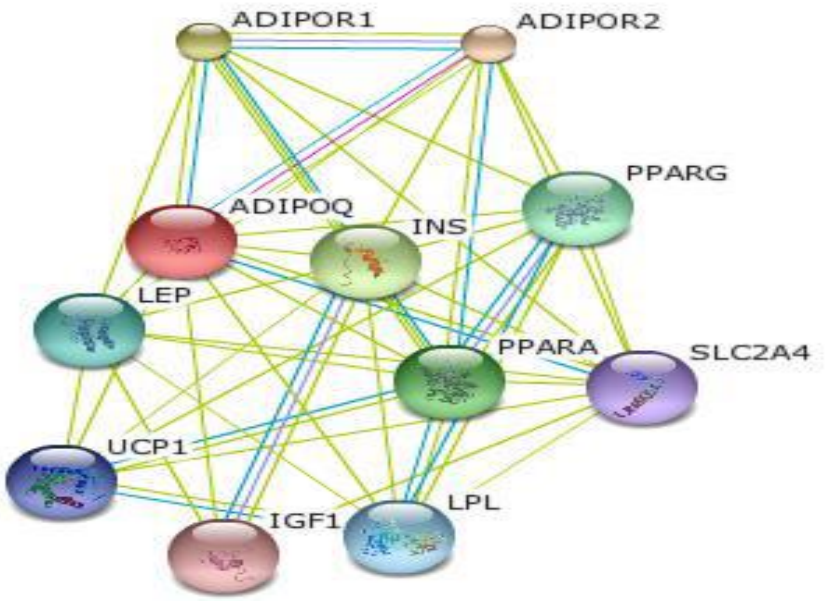


Figure 33: Predicted functional partners of APN from STRING.

List of direct interacting partners of APN with the highest confidence level of 0.998. ADIPOR2 represents Adiponectin receptor protein 2, ADIPOR1 represents Adiponectin receptor protein 1, INS represents insulin precursor, PPARA represents Peroxisome proliferator-activated receptor alpha, PPARG represents peroxisome proilferator-activated receptor gamma, LEP represents leptin precursor, LPL represents lipoprotein lipase precursor, UCP1 represents mitochondrial brown fat uncoupling protein, SLC2A4 represents solute carrier family 2, facilitated glucose transporter member 4, IGF1 represents insulin-like growth factor IA precursor.

PRATT software was used to identify the conserved pattern among the interacting partners form its unaligned sequences. PRATT predicted **S-X(3,4)-L-X(0,1)-L-[AIV]-X-[AGV]-[EGQS]** as the conserved regular expression pattern. This pattern was furthers scanned against PROSITE which resulted in 314 hits from Homo sapiens. These hits were further narrowed down based on the conserved post translational modifications. This analysis was performed in the existing interacting partners using PROSITE where in N-myristoylation site and Protein kinase C phosphorylation site were found to be the key conserved PTMs. Protein kinase C phosphorylation site at serine was also found to be conserved in all the interacting partners.

Based on the manual analysis of sequence conservation, **-X-[KR]-X(0,146)-G-[CMV]-X-X-[NSG]-[RHLF]** was derived as the conserved pattern. When this pattern was scanned against

the sequences which used for PRATT pattern generation, 40 hits were identified as significant with few false positives. All these hits were further screened for the organelle localisation and its association with APN. Proteins with very large sequence length were omitted as there were more chances of them being a false positive. After final screening, five proteins were found to be most potential interacting partners of APN which includes ATP binding cassette subfamily A member, Apoptosis regulator bcl2, Scavenger receptor containing cysteine rich domain, solute carrier family 12 member 9, and serine/threonine protein kinase.

5.4.2.2. Modelled structure of APN and its partner:

Molecular modelling was performed for the interacting partners for which the experimental structure is unavailable. Suitable structural templates were chosen based on homology for comparative modelling of the interacting partners using MODELLER9v7 and for other proteins which doesn't have structural template; a fold recognition based structure prediction was carried out using ITASSER server.

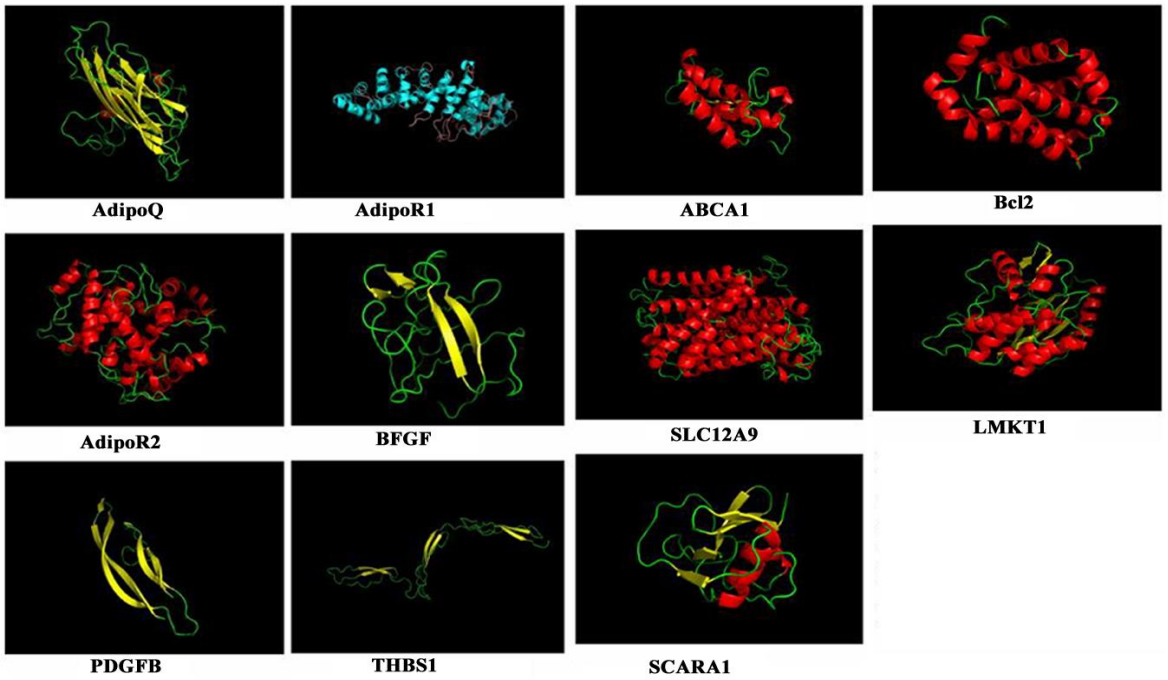


Figure 34: Modelled structure of APN and its interacting partners. AdipoQ stands APN, AdipoR1 – APN receptor 1, ABCA1- ATP partners binding cassette subfamily A member, BCL2 - Apoptosis regulator bcl2, AdipoR2 – APN receptor 2, BFGF- basic fibroblast growth factor, SLC12A9- solute carrier family 12 member 9, LMKT1 - serine/threonine protein kinase, PDGFB – Platelet derived growth factor, THBS1- Thrombospondins, SCAR1- Scavenger receptor containing cysteine rich domain.

The modelled proteins were submitted to WHATIF server and atomic bumps were removed. The structures are then energy minimized with GROMACS using OPLS force field and the stereo chemical quality was checked using PROCHECK server. All the structures had a fairly good quality (Figure 34). These structures were further used for docking studies. Templates used, energy minimization value and quality of structure were listed in the Table 14.

Table 14: Templates and quality of the proteins modelled.

PROTEIN	TEMPLATE	RAMACHANDRAN PLOT	ENERGY MINIMISATION VALUES KJ/MOL
APN	Collagen like domain modeled using ITASSER C1Q domain- 1C3H [acrp-30 calcium complex] as template using modeller 9v7	Most favoured :88.0% Disallowed :0.0%	-2.6011648e+04
AdipoR1	Template identity less than 20%, hence modelled using ITASSER	Most favoured:86.1% Disallowed:0.0%	-4.5189449e+04
AdipoR2	Template identity less than 20%, hence modelled using ITASSER	Most favoured:86.1% Disallowed:0.0%	-4.9370957e+04
TSP-1	1VEX and 1LSL used as a template and modeled using MODELLER9v7	Most favoured:89.5% Disallowed:0.0%	-2.0149645e+04
FGF	Modeled using ITASSER	Most favoured:86.6% Disallowed:0.0%	-9.3889980+e03
Solute carrier family 12 member 9	Modeled using ITASSER	Most favoured:82.6% Disallowed:0.0%	-4.5613203e+04
Serine threonine protein kinase	Modeled using ITASSER	Most favoured:86.8% Disallowed:0.0%	-5.3325633e+04
Scavenger receptor cysteine rich domain	1BY2 used as a template and modeled using MODELLER9v7	Most favoured:88.0% Disallowed:0.0%	-1.2923983e+04
ABCA1	2PCL used as a template and modeled using MODELLER9v7	Most favoured:89.7% Disallowed:0.0%	-1.6268828e+04

5.4.2.3. Docking studies of APN and its interacting partners

Docking studies were carried out between APN and its interacting partners. The energy minimized structures of modelled structures were taken for the analysis. Best docked complexes (Figure 35) were chosen based on the binding energy and molecular interactions as reported by Cluspro and Ligplot respectively.

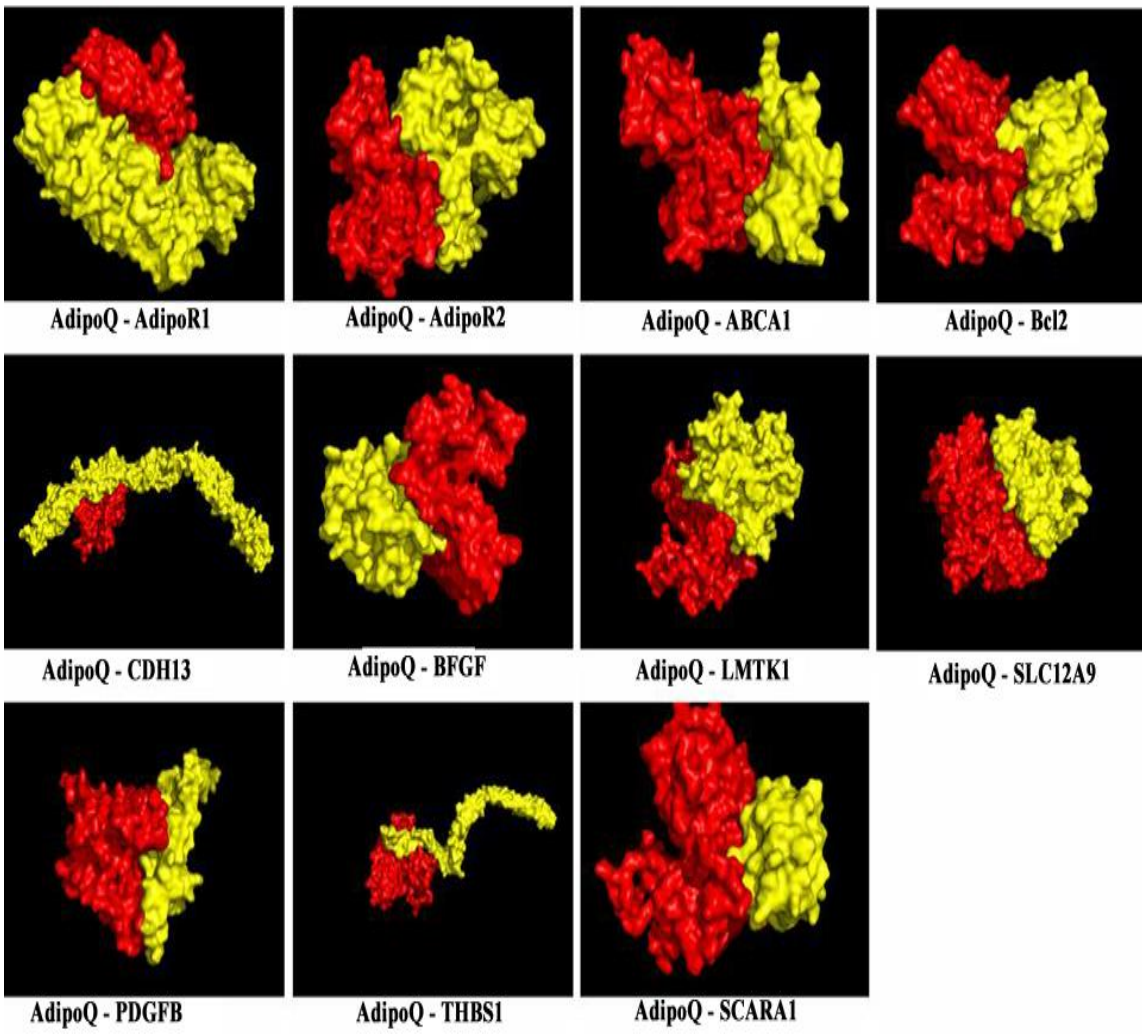


Figure 35: Docked complex of APN with its interacting partners. Red – APN; yellow – interacting partners

On analysis of interactions, important hydrogen bonds among APN and its interacting partners are listed in Table 15.

Table 15: Interacting residues between APN and its partners.

Complex	Hydrogen bonds (APN–Interacting partners)	Binding energy (Kcal/mol)
AdipoR1	Arg112-phe345 Tyr159-tyr321 Leu238-ser347 Tyr240-313 Lys33-glu209 Gly24-arg278	-1821.5
AdipoR2	Arg112-gln11 Gly24-lys39 Thr37-trp88 Ser116-ser185 Tyr137-ser187 Tyr240-leu27 Ser200-phe148	-1447.8
T Cadherin	His46-arg63 Glu106-his111 His241-his111 Tyr240-arg124 Arg112-asp212 Val110-asp213 Thr37-lys163	-1100.3
Thrombospondin	Gln23-glu5 Thr37-arg20 His46-gln44 Tyr137-leu27 Tyr159-ser1 Tyr159-pro2	-1003.2
Platelet derived growth factor	Tyr189-arg4 Tyr159-arg12 Tyr137-arg37 Ser198-cys28 Ala35-arg53 Lys33-gln32 Pro32-gln32	-1081.0
Fibroblast growth factor Basic	Arg112-ile94 His163-arg104 His204-arg14 Glu103-arg16 Tyr137-asp108 Leu239-ile94 Gln196-lys86	-1045.9
ABAC1	Pro25-his33 Cys36-thr15 His46-tyr41 Lys101-gly66	-1102.5

BCL2	His79-his46 Glu138-tyr240 His143-leu238 Arg142-glu106 Glu138-tyr240 Arg88-gly24 Arg86-gly105	-1077.7
SLC12A9	Asp231-gly1 Thr233-gly1 Gly34-cys50 Glu120-arg150 Tyr240-gly196 Glu191-lys487 Lys33-asn405	-1841.2
LMKT1	Lys33-ser26 Lys33-gln28 Arg112-thr242 Tyr159-arg108 Tyr189-gly23 Ser200-pro103 Tyr240-gln240	-1032.7
SCARA1	Thr37-asn91 Thr37-leu50 Gly38-pro51 Arg112-cys7 Tyr137-arg10 Tyr240-glu12	-943.4

The new interacting partners identified from the PTM approach was validated using docking studies and the significant residues involved in interaction were mapped: l**eu31, thr37, trp39, ile43, pro46, arg112, tyr137, leu157, ala161, ser200, leu202, thr235, phe237, leu238, leu239, tyr240,** these residues might contribute towards significant functional regulation of APN. In our earlier studies, we have identified interacting partners of PEDF in a similar way. It was interesting to observe that novel interacting partners of APN and PEDF identified (Table 16) might converge together and shall participate in a common pathway. Thus, the predicted interacting partners in both of these cases were further analysed and a common putative pathway is proposed in this study (Figure 36).

Table 16: Novel interacting partners of APN and PEDF

NOVEL INTERACTING PARTNERS OF APN	NOVEL INTERACTING PARTNERS OF PEDF
ATP binding cassette subfamilyA member[ABAC1]	VEGF Receptor 3
Apoptosis regulator bcl2 [Bcl2]	CABL
Scavenger receptor containing cysteine rich domain [SCARA1]	MAPK 14
Solute carrier family 12 member 9 [SLC12A9]	MAP 4
Serine / threonine protein kinase [LMKT1]	Serine threonine protein kinase [OSR 1]
	Serine threonine protein kinase [MST4]
	Serine threonine protein kinase 24 [MST 24]
	TRAF 2 and NCK interacting protein kinase
	LIM domain kinase 1
	Nuclear receptor subfamily1 group D
	DNA dependent protein kinase catalytic unit
	STE20/SPS1 related proline alanine rich protein kinase

Apoptosis regulator BCL2, is an interacting partner of APN which interacts with MAPK 14 (interacting partner of PEDF). These interactions modulate in VEGF signalling pathway, Apoptosis signalling pathway, and Neurotrophin signalling pathway, thereby, modulating cell migration, apoptosis and also helps in cell survival, respectively.

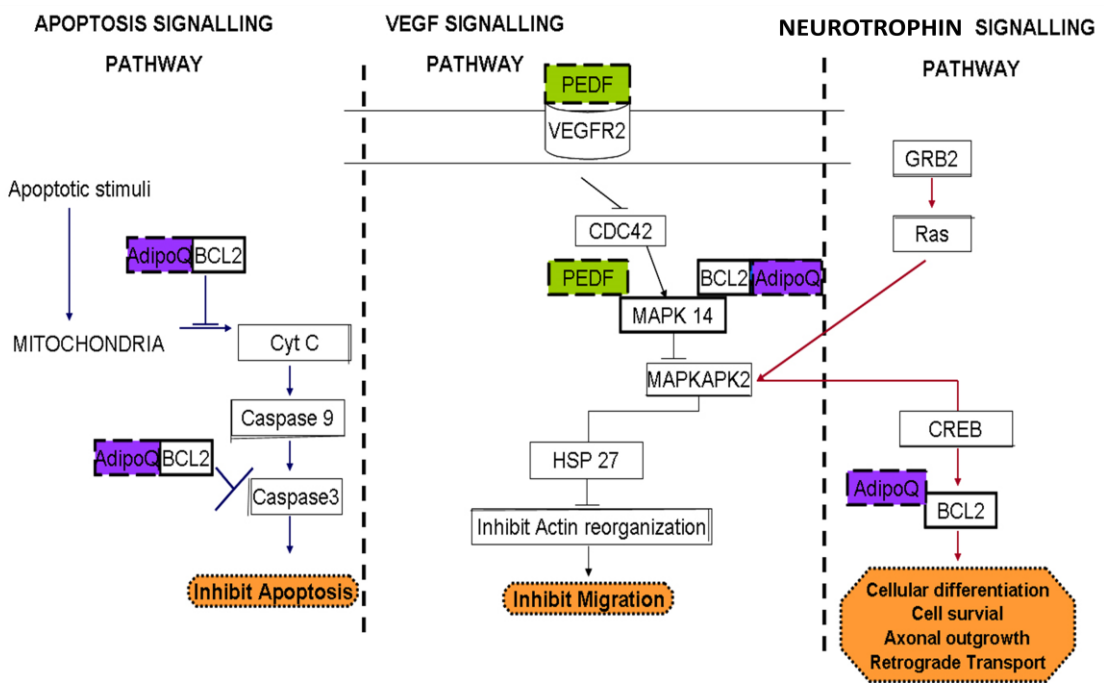


Figure 36: Putative pathway indicating the role of APN and PEDF

Novel interacting partners of APN and the putative pathway identified also indicates anti angiogenic role of APN by inhibiting VEGF signalling.

5.4.3. APN modulates the expression of VEGF and PEDF in cultured RPE cells:

The ability of APN in modulating the secretion of VEGF and PEDF at a lower concentration was studied in cultured RPE. RPE is a single layer of epithelial cells which runs between Bruch's membrane and photoreceptors layer of retina. RPE maintains the outer retinal barrier and it both pro angiogenic VEGF, anti angiogenic cytokine PEDF. Thus VEGF and PEDF are the two widely studied molecules in relation to ocular angiogenesis. Both HRPE and ARPE 19 were exposed to varying concentration (5 pg – 5000 pg /ml) of exogenously added rAPN for 1 h.

5.4.3.1. APN influence VEGF and PEDF expression:

HRPE and ARPE 19 exposed to rAPN showed decreased VEGF mRNA and increased PEDF mRNA compared to cells not exposed to rAPN (Figure 37) by reverse transcriptase PCR. GAPDH was run as an internal control.

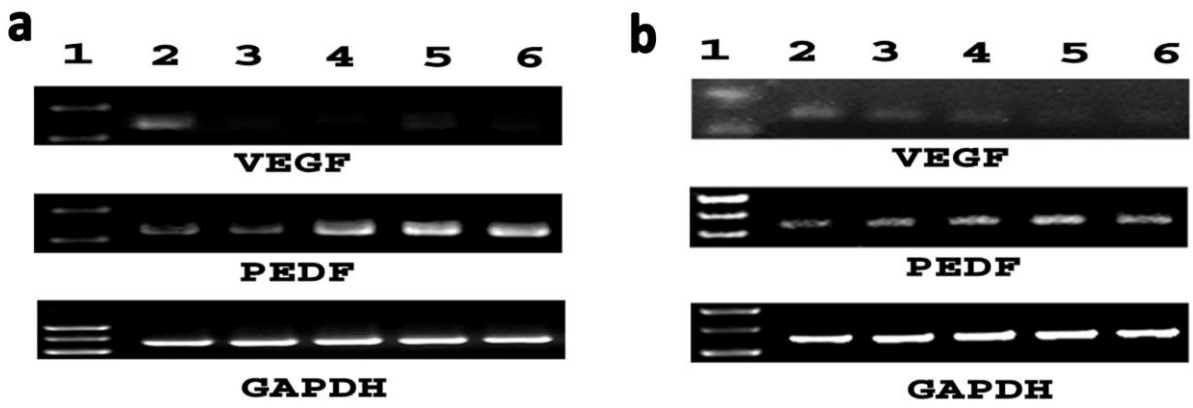


Figure 37: RT PCR for VEGF, PEDF in RPE cells.

a) RT PCR for VEGF, PEDF in ARPE 19 cells after exposing with various concentration of rAPN.
b) RT PCR for VEGF, PEDF in HRPE cells after exposing with various concentration of rAPN.

Lane 1: 100 – 600 bp ladder, Lane 2: 5 pg of APN, Lane 3: 50 pg of APN , Lane 4: 500 pg of APN, Lane 5: 5000 pg of APN, Lane 6: 50 ng of APN

All experiments were done in triplicates (n=3)

ARPE 19 cells functionally bear a resemblance to pathological RPE *in vivo*. Experiments were further continued in ARPE 19 confirming and quantifying mRNA expression by qPCR, protein expression was quantified by ELISA. ARPE 19 cells exposed to rAPN showed 38% decrease ($p < 0.01$) in VEGF expression in the cells treated with 5 pg rAPN, 42 % decrease ($p < 0.01$) in cells treated with 50 pg rAPN, 47% decrease ($p < 0.01$) in cells treated with 500 pg rAPN, 22% decrease ($p < 0.01$) in cells treated with 5000 pg rAPN compared to control cells. Whereas,

PEDF expression showed 27% increase ($p < 0.01$) in cells treated with 500 pg rAPN and 41% increase ($p < 0.03$) in 5 ng rAPN treated cells compared to control (Figure 38).

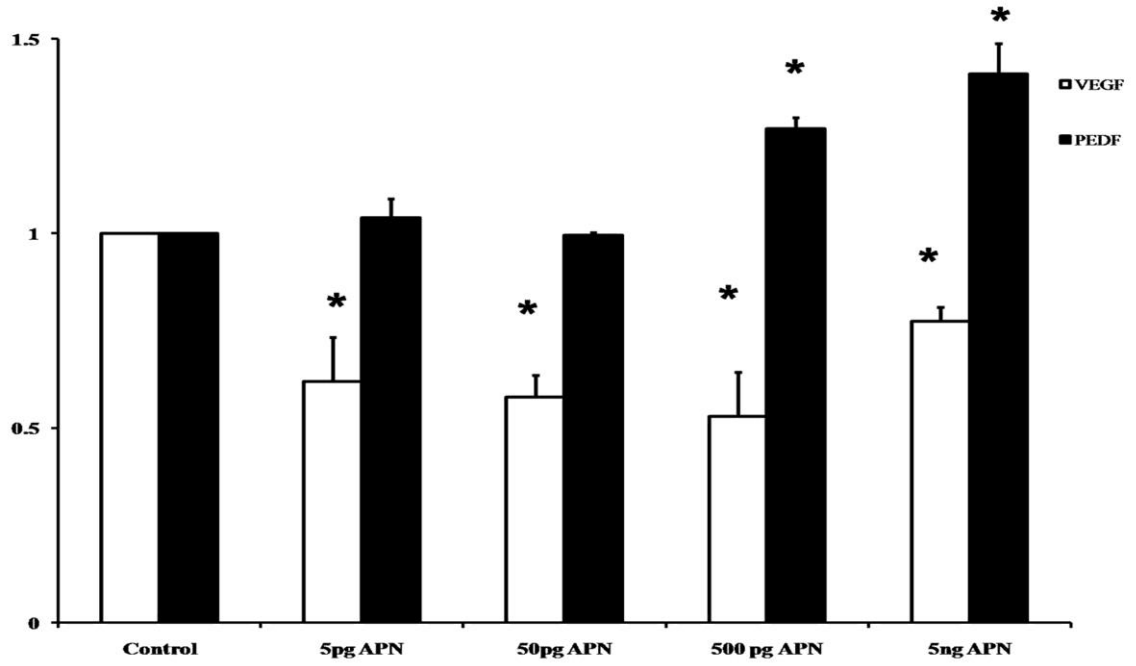


Figure 38: qPCR for the mRNA expression of VEGF, PEDF in ARPE cells exposed to varying concentration of rAPN. rAPN exposed cells showed a significant decrease in VEGF, significant increase in PEDF. (*- statistically significant compared to control). All experiments were done in triplicates (n=3)

5.4.3.2. APN exposure to RPE decreased VEGF protein Expression:

Conditioned medium of cells after exposure of rAPN along with the control were used to quantify VEGF and PEDF protein expression. VEGF protein expression was decreased significantly in cells treated with 5 pg rAPN ($p < 0.007$), 50 pg rAPN ($p < 0.01$), 500 pg rAPN ($p < 0.02$), 5000 pg rAPN ($p < 0.01$) treated cells compared to control (Figure 39 A) whereas PEDF protein didn't show much variation nor were statistically significant (Figure 39 B).

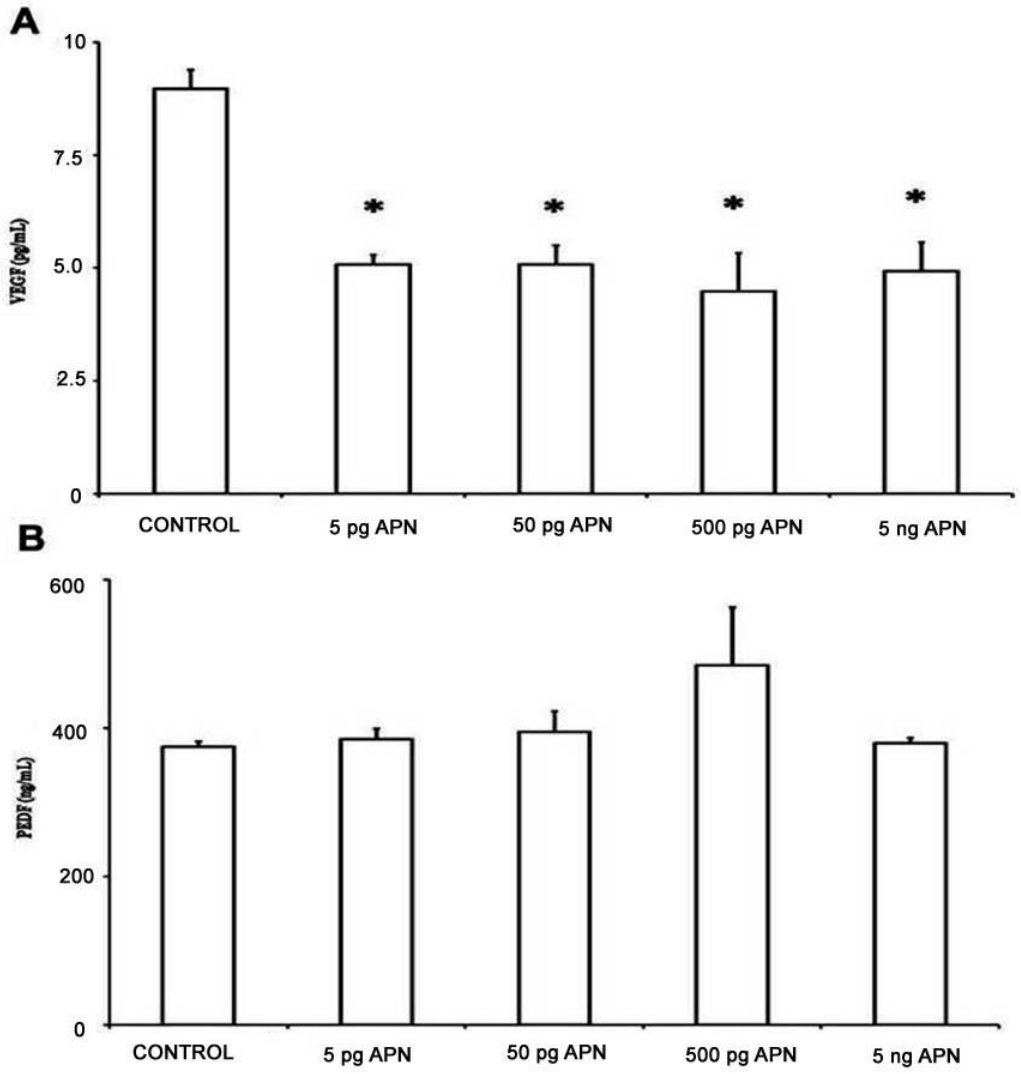


Figure 39: Quantification of VEGF and PEDF protein expression by ELISA.

A) Quantification of VEGF in the conditioned medium showed a significant decrease in rAPN exposed RPE cells when compared to control (* statistically significant compared to control). B) Quantification of PEDF in the conditioned medium in rAPN exposed RPE cells. All experiments were done in triplicates (n=3)

The present study revealed that APN decreased both VEGF mRNA as well as protein expression in cultured RPE cells. Effective decrease in VEGF indicates that APN has therapeutic role against VEGF induced angiogenesis.

5.5. Peptides derived from APN having anti angiogenic property:

Based on the *in silico* analysis, three peptides were designed from the primary sequence of APN. Peptides were designed based on the interactions of APN and its interacting partners. Residues in bold were significant and interacting residues of APN.

Pep1: **PKGACTGW**MAG**I**P (32-44)

Pep 2: KDKAMLFTYDQ**Y**Q**E** (178-191)

Pep 3: **DNDND**S**T**F**T**G**FLLY** (226-40)

APN derived peptides were synthetically produced (USV peptides, India) and were tested for purity using Agilent HPLC system. All the peptides of 10 µg concentrations were injected in HPLC showed a single peak in VWD detector indicating the purity of the synthesized peptides (Figure 40).

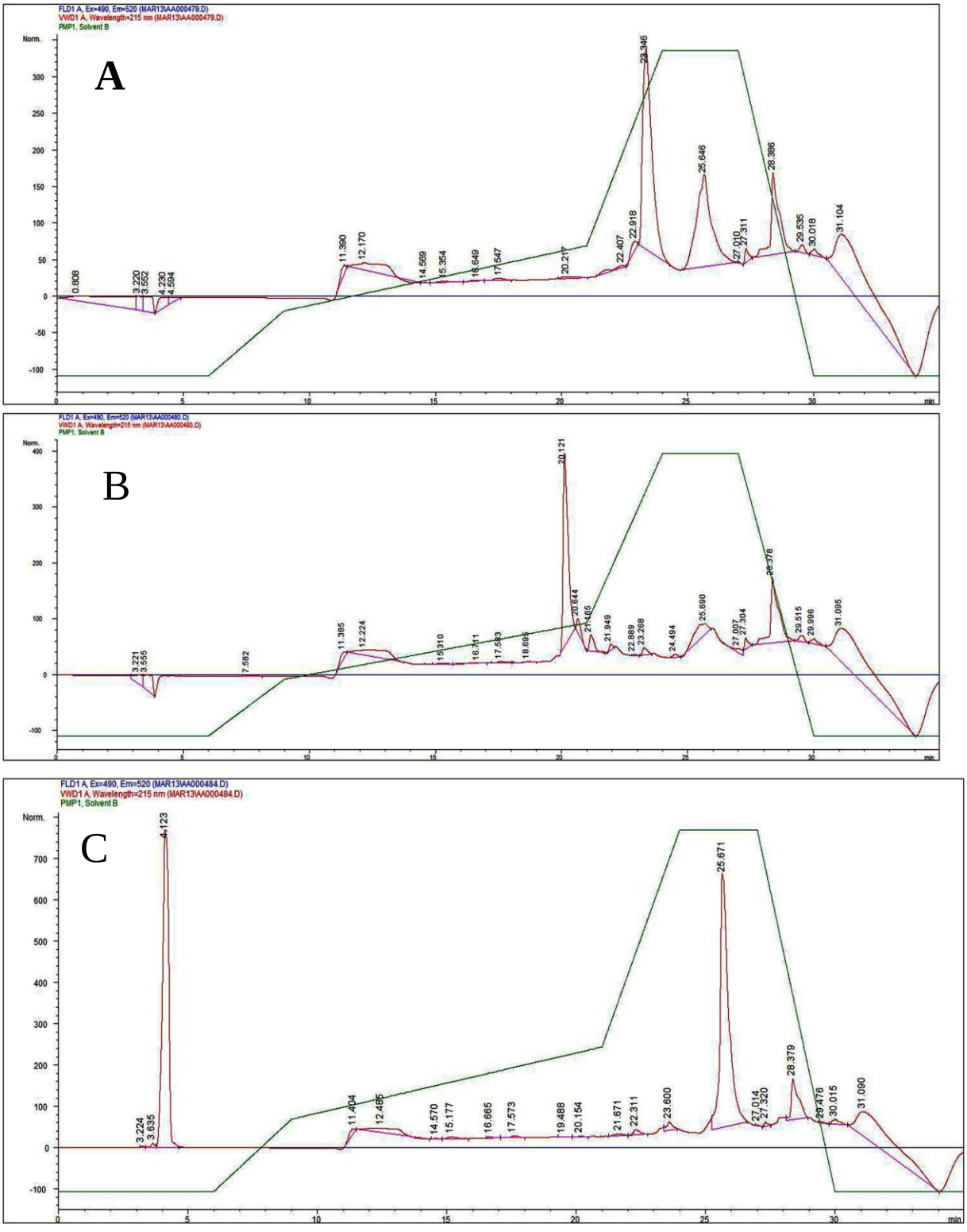


Figure 40: HPLC chromatogram of APN derived peptides. A) Pep1 showed a retention time of 23.3min, B) Pep 2 showed a retention time of 20.2 min and C) Pep 3 showed a retention time of 4.12 min respectively at 215 nm.

5.5.1. In vitro angiogenesis assay in HREC

Concentration ranges from 1 μm – 100 μm of APN derived peptides were tested for its anti angiogenic ability in HREC and HUVEC. Proliferation assay, migration assay, tube formation assay and VEGF western blot were done in HREC.

MTT assay was performed to check the toxicity of peptides. Peptide treatment was found to be non toxic to the cells. (Figure 41)

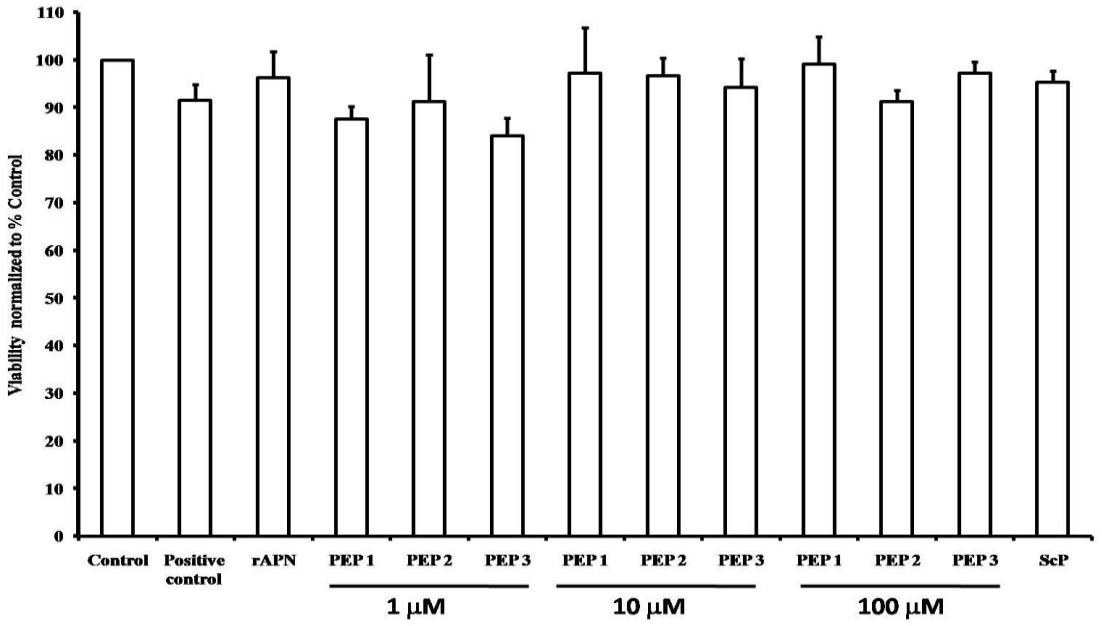


Figure 41: MTT assay with APN derived peptides in HREC. Peptide treatment was not cytotoxic to the cells. All experiments were done in triplicates (n=3)

Cell proliferation assay by triated thymidine showed that APN derived peptides decreased proliferation of HREC compared to cells without treatment. All the three concentration of peptides (1 μm, 10 μm, 100 μm) decreased proliferation compared to control (Figure 42). Peptide 1 decreased proliferation by 8 %, 29 %, and 19 % at 1 μm, 10 μm and 100 μm concentration respectively. Peptide 2 decreased proliferation by 10 %, 31 %, and 15 % at 1 μm, 10 μm and 100 μm concentration respectively. Peptide 3 decreased proliferation by 4 %, 37 %,

and 21 % at 1 μm, 10 μm and 100 μm concentration respectively. Scrambled peptide didn't show any effect.

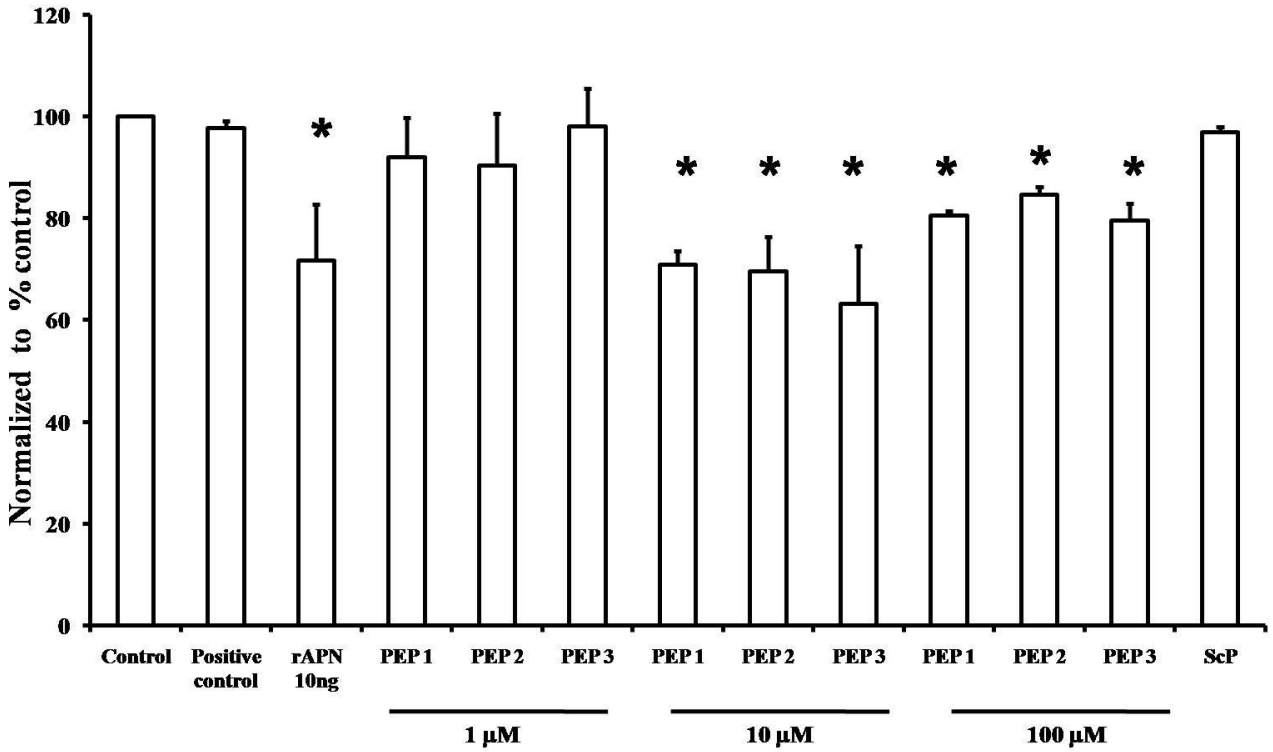


Figure 42: Proliferation assay using tritiated thymidine in HREC showed significant decrease in proliferation at the end of 48 h of peptide treatment. Positive control were the cells treated with 10% FBS. Peptide at the concentration of 10 and 100 μm showed significance compared to control. Peptide 1 ($p < 0.01$), peptide 2 ($p < 0.001$), peptide 3 ($p < 0.001$) at 10 μm concentration. Peptide 1 ($p < 0.02$), peptide 2 ($p < 0.04$), peptide 3 ($p < 0.01$) at 100 μm concentration. Comparison between control and peptide treatment is represented as *. All experiments were done in triplicates (n=3)

Scratch assay was performed for all the three peptide of concentration ranging 10 μM with APN derived peptides. All three peptide inhibited migration whereas the cells induced by growth medium (10% FBS) increased migration of HREC (Figure 43).

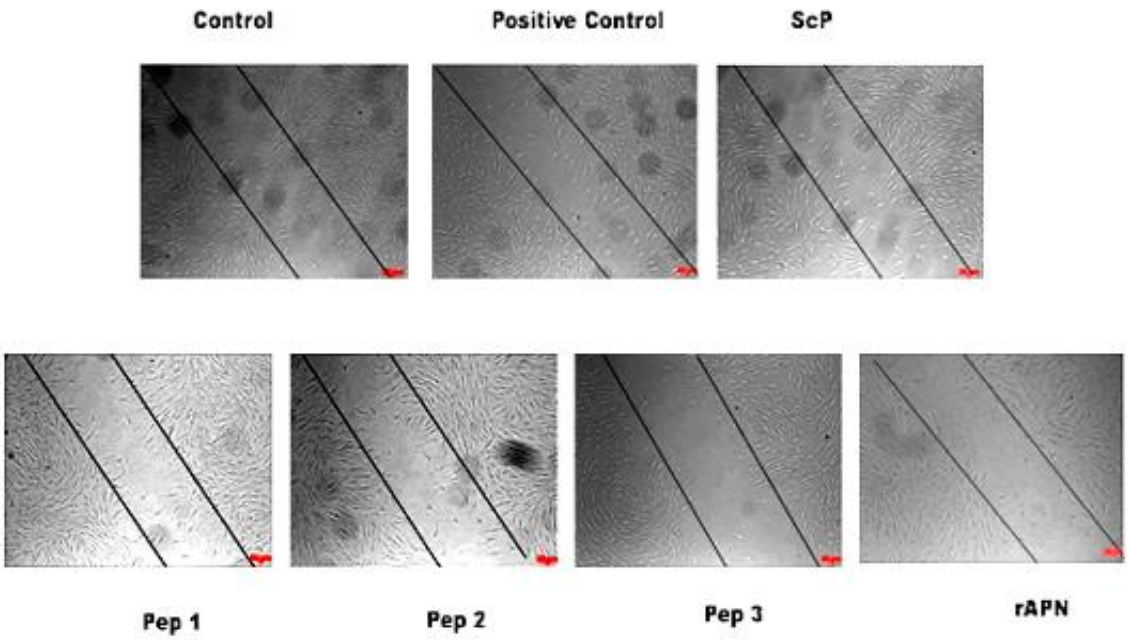


Figure 43: **Scratch assay** in HREC cells showed significantly decreased migration with peptide treatment.

Peptide concentration at 10 μM showed decreased proliferation and inhibited migration compared to 1 and 100 μM concentrations in all the three peptide used. Thus for tube formation 10 μM concentration of all three peptides was used. All the three peptides inhibit tube formation compared to control cells. The cells induced by 10 % FBS showed increased tube formation and scrambled peptide didn't show much effect (Figure 44).

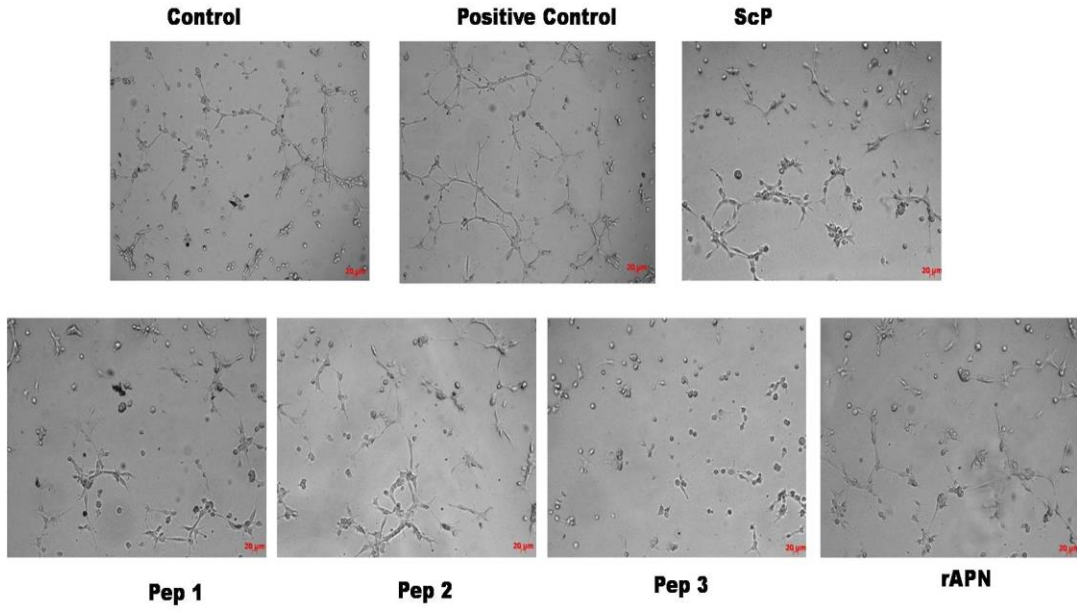


Figure 44: Tube formation assay with APN derived peptides on HREC. Peptide treatment inhibits tube formation compared to control. All experiments were done in triplicates (n=3)

Peptide treatment results were very much comparable with the effect of rAPN in inhibiting cell proliferation, migration and tube formation in HREC cells. The experiments were repeated in macrovascular endothelial cells (HUVEC).

5.5.2. In vitro angiogenesis in HUVEC:

MTT Assay (Figure 45) revealed that peptide treatment were non toxic to the cells.

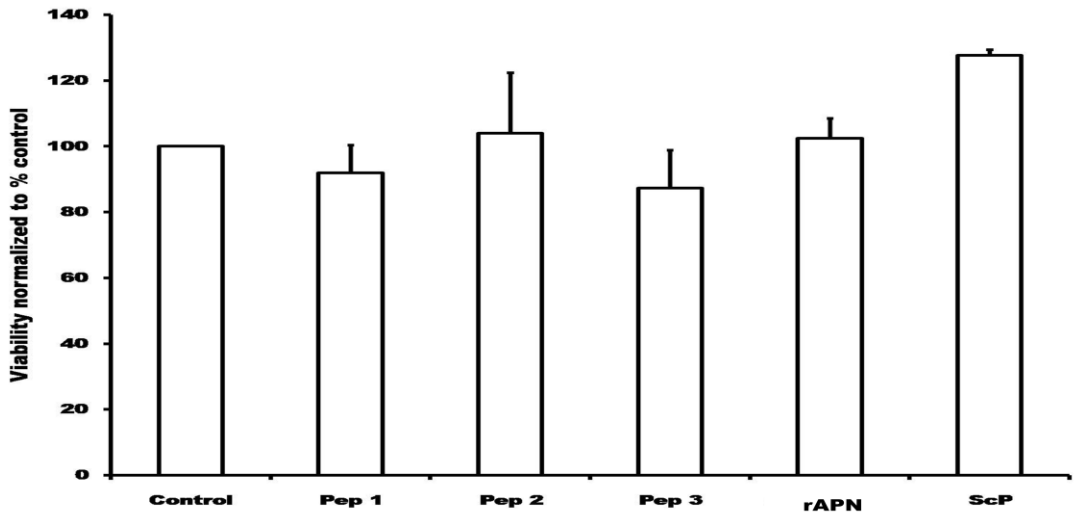


Figure 45: MTT assay with APN derived peptides in HUVEC. Peptide treatmentwas non toxic to cells. All experiments were done in triplicates (n=3)

Cell death detection assay (Figure 46) also showed that peptide treatment was non apoptotic to the cells.

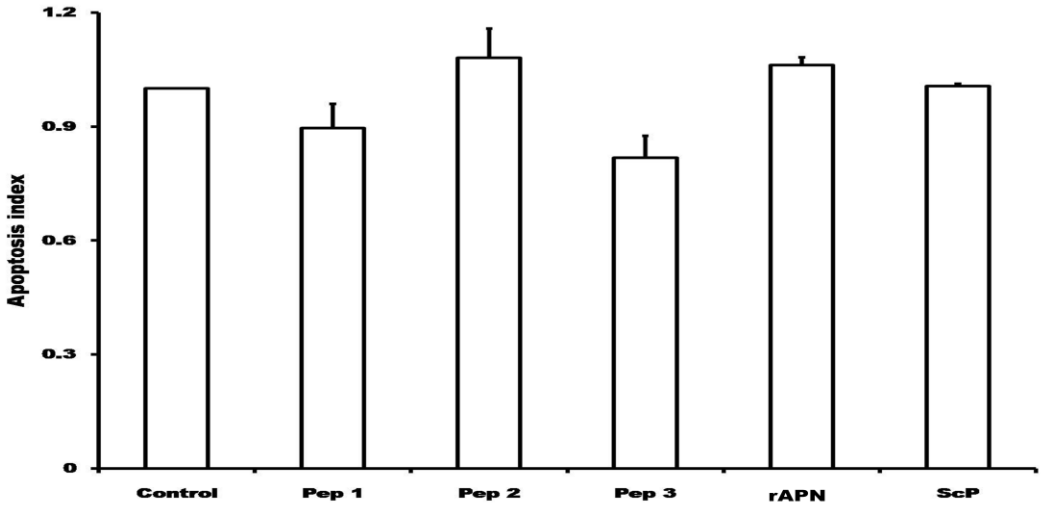


Figure 46:**Cell death detection assay** with APN derived peptides in HUVEC. Peptide treatment was non apoptotic to cells compared to control. All experiments were done in triplicates (n=3)

Alike HREC, Scratch assay in HUVEC cells also showed decreased migration with peptide treatment whereas the cells induced with growth medium increased migration. Scrambled peptide didn't show much effect (Figure 47).

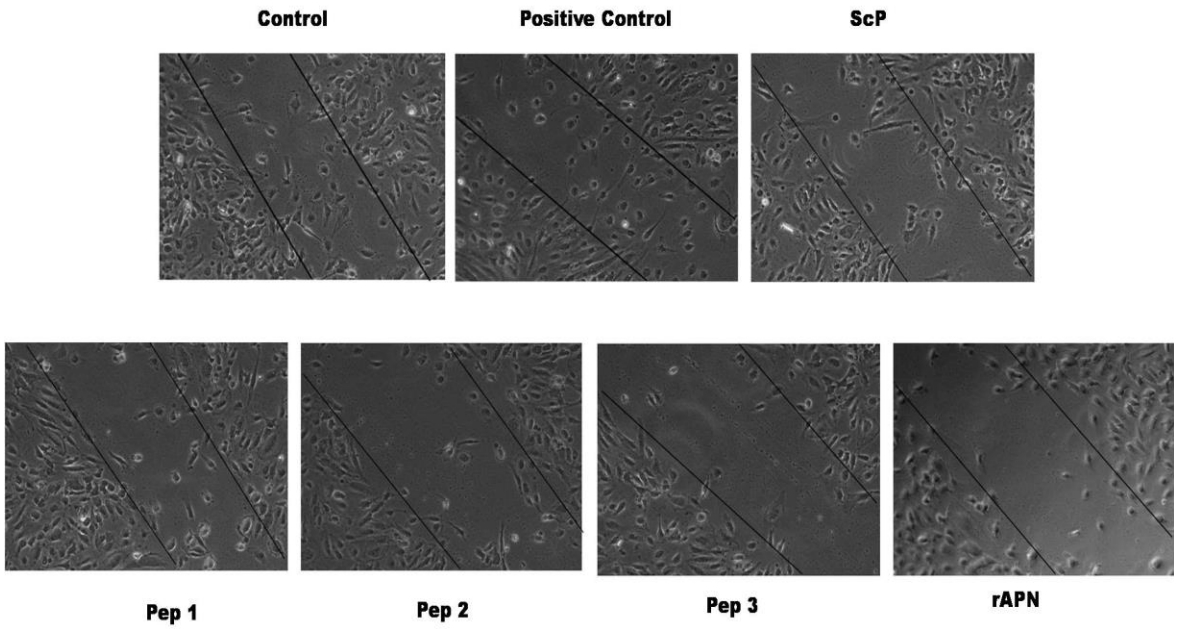


Figure 47: Scratch assay in HUVEC cells showed significant decresed migration with peptide treatment. All experiments were done in triplicates (n=3)

Tube formation assay in HUVEC also showed peptide treatment inhibited tube formation. (Figure 48).

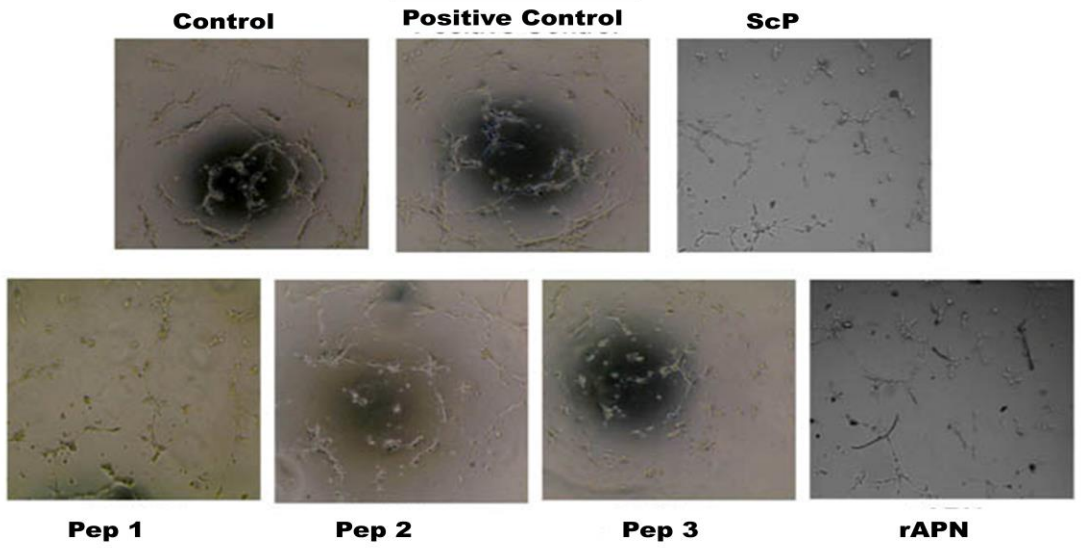


Figure 48: Tube formation assay with APN derived peptides on HUVEC. Peptide treatment inhibits tube formation compared to control. All experiments were done in triplicates (n=3)

Cell attachment assay was also performed in HUVEC. The peptide treatment decreased cell adhesion significantly compared to control. Scrambled peptide didn't show any effect (Figure 49).

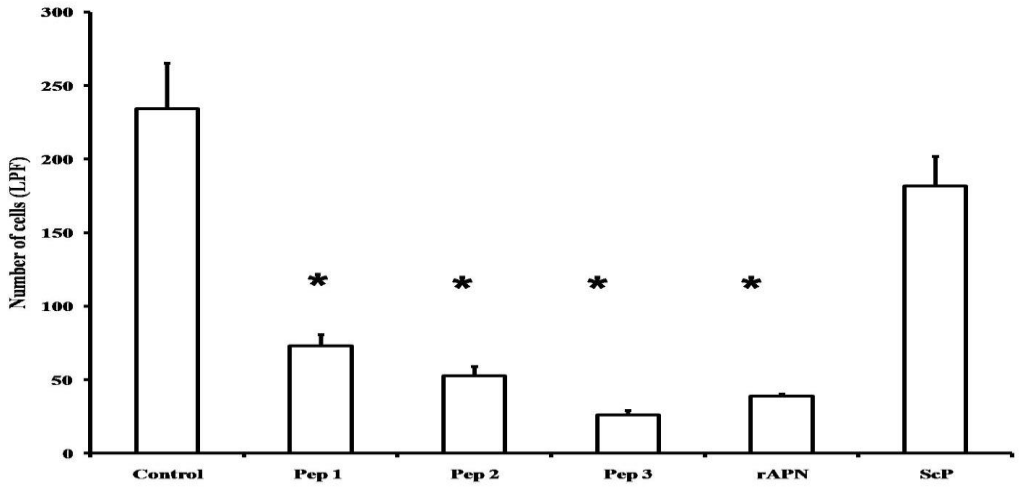


Figure 49: Cell attachment assay in APN derived peptide in HUVEC. Peptide treatment reduced cell adhesion significantly compared to control. All experiments were done in triplicates (n=3)

Western blot analysis showed that VEGF protein expression was reduced by peptide treatment same as rAPN (Figure 50).

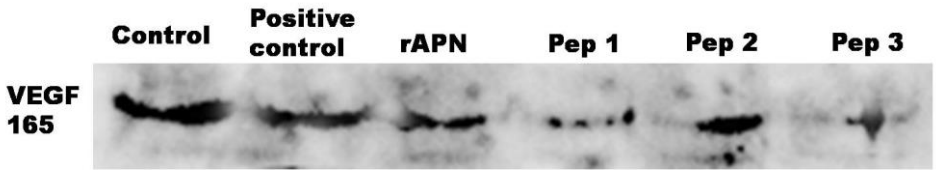


Figure 50: VEGF protein expression after peptide treatment

The designed peptides were meticulously screened in two endothelial systems and were found to be potent in inhibiting angiogenesis. These APN derived peptides decreased migration, proliferation, adhesion, reduced tube formation and decreased VEGF levels. These APN derived peptides will be the lead compound to be further developed for their therapeutic application.

5.6. Small molecule screening for increased secretion of APN and its validation

Non pharmacological agents, endogenous molecules which are general nutrients such as amino acids and fatty acids were screened for increasing the secretion of APN. A panel of 18 amino acids and three essential fatty acids were screened first in human visceral adipocytes which are the primary source of APN *in vivo* (Figure 51).

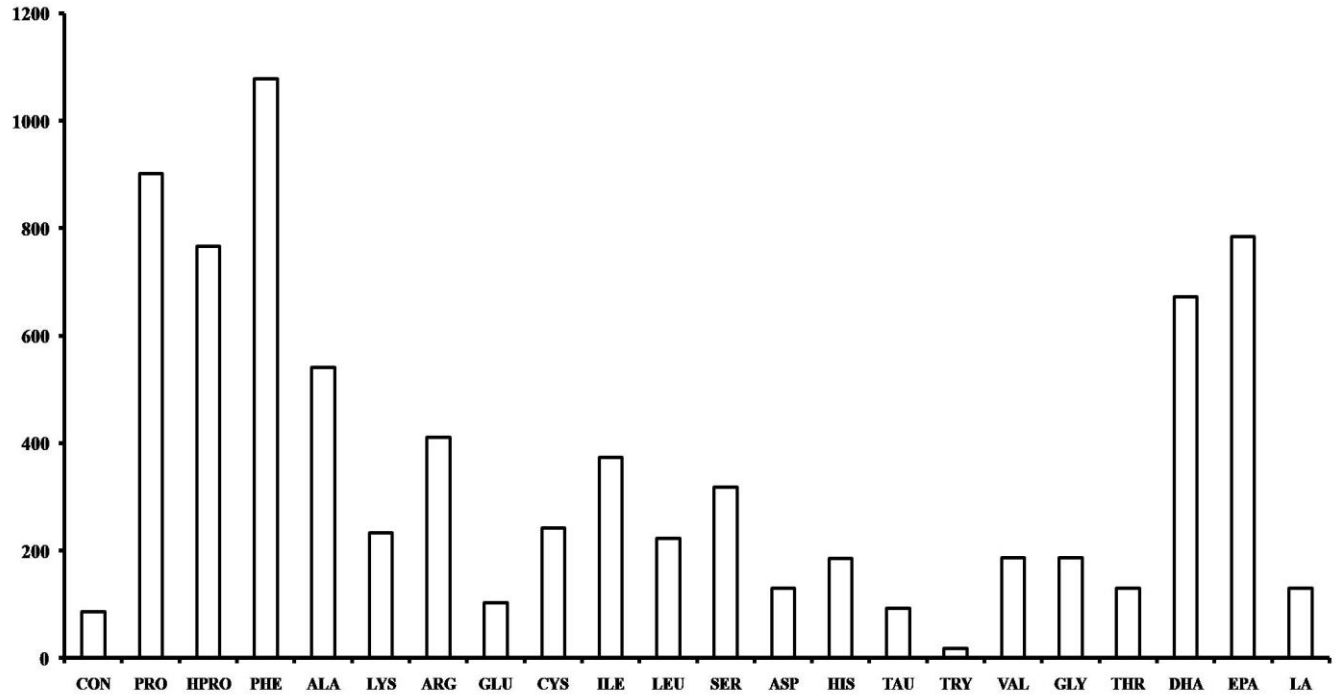


Figure 51: Screening of amino acids and fatty acids for the increased secretion of APN in human visceral adipocytes. Con – control; pro – proline; hpro – hydroxyproline; Ala – alanine; lys – lysine; arg – arginine; glu – glutamic acid; cys – cysteine; ile – isoleucine; leu –leucine; ser – serine; asp – aspartic acid; his – histidine; tau – taurine; try – tryptophan; val – valine; gly – glycine; thr – threonine; DHA – docosahexaenoic acid, EPA – eocosapentaenoic acid, LA – linoleic acid

Free amino acids such as proline, hydroxyproline, alanine, phenylalanine, and fatty acids docosahexaenoic acid, eocosapentaenoic acid increased the secretion of APN. Previous studies from our lab reported amino acids mixture (AAM) increases glucose uptake. Present hypothesis is that amino acids increases glucose uptake via increasing APN levels and was validated using APN siRNA.

Two different siRNA were designed using ambion tool and was purchased from MERCK. The sequence of APN siRNA and Scrambled siRNA are as

APN si1: 5’AGUGGAGCCAUCAUAGUGGUU 3’

APN si2: 5’AUCAUAGUGGUUUUGCUGAUU 3’.

Scrambled siRNA: 5'GUGAUUUACGGGCUAGAGUAU 3'

5.6.1. Optimization of siRNA concentration and time:

APN mRNA silencing effect of the two designed siRNA with varying time period and concentration showed that 10nm of APN si1 at 2hr, 4hr after transfection showed 69% and 56% decrease in APN mRNA, whereas APN si2 didn't show much significant decrease. Scrambled si (Ssi) didn't show any decrease in APN mRNA. GAPDH was used as housekeeping gene to normalize APN expression. Since APN si1 at 10 nm concentration after two hr of transfection showed maximum decrease in APN mRNA it was chosen for the further experiments (Figure 52)

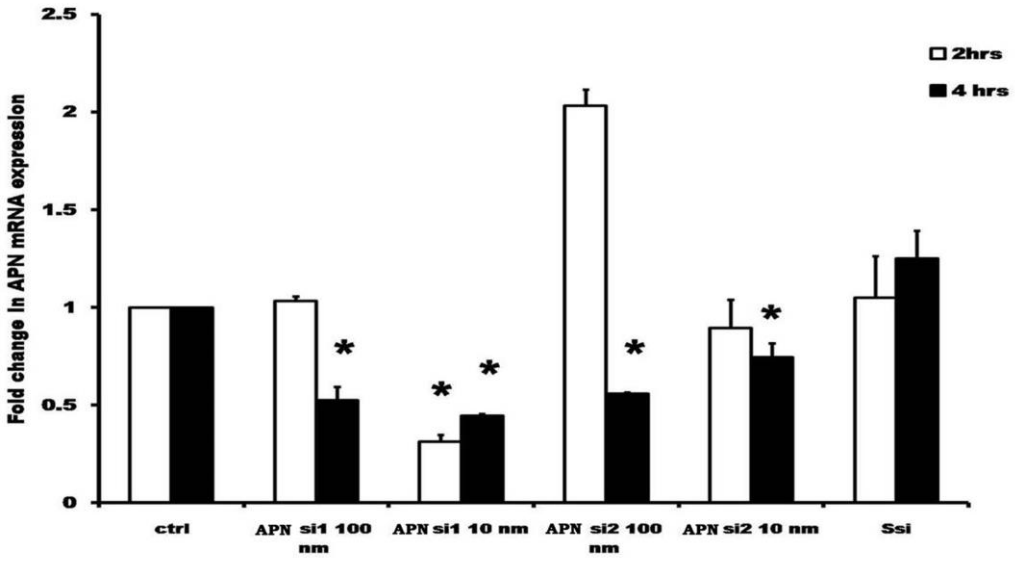


Figure 52: Optimisation of APN siRNA.Quantitative Real time PCR for APN mRNA. APN si1 10 nm at 2 hr ($p \leq 0.001$), 4 hr ($p \leq 0.000$) significantly decreased APN mRNA by 69% and 56%, whereas APN si2 100nm ($p \leq 0.000$) and 10 nm ($p \leq 0.03$) at 4 hr decreased expression by 50% and 30%. The maximum decrease of APN mRNA was observed in APN si1 at 10nm concentration for 2 hr. * Significance compared to control without siRNA. All experiments were done in triplicates (n=3)

5.6.2. Amino acids increase APN mRNA and protein expression:

RNA and Conditioned medium of the mature adipocytes which were exposed to 0.5 mM amino acids (proline, phenylalanine, alanine, AAM) with and without transient knockdown of APN

(APN si1 10 nm) in high glucose environment (33 mM glucose) along with 100 nm Insulin for 2 h were used to quantify the APN mRNA and protein expression.

Amino acid proline, phenylalanine exposure increased APN mRNA by 1.5-2 fold with statistical significance (p <0.007 and 0.009 respectively) when compared to control. Alanine and AAM doesn't show much increase in APN mRNA. APN si1-10 nm decreased the APN mRNA significantly in control ($p < 0.000$) and even in the presence of amino acid proline ($p < 0.001$), phenylalanine ($p < 0.003$) when compared to cells not treated with APN si1. (Figure 53 a)

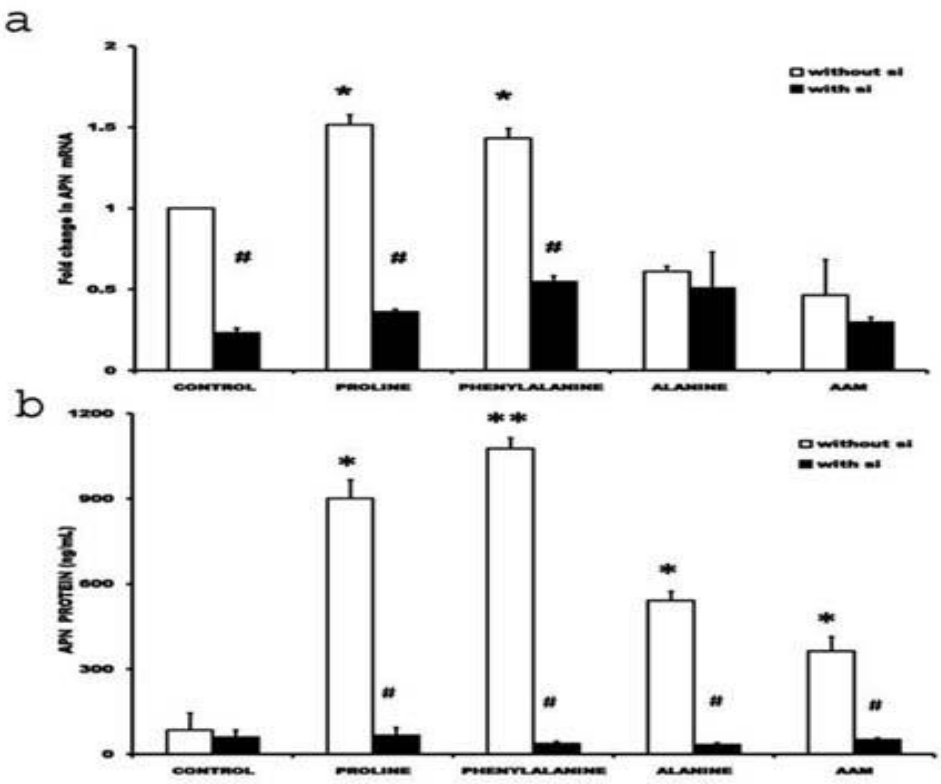


Figure 53: Amino acids increase APN mRNA and protein expression. a) qPCR for APN mRNA after amino acid exposure in cultured adipocytes with and without transient knockdown of APN. B) Quantification of APN by ELISA. Adiponectin levels were increased by 4-13 folds after aminoacid exposure whereas the APN si1 showed a decreased protein expression even in the presence of aminoacid. * Significance comparison between the cells treated with amino acid and without amino acids. # Significance comparison between the cells treated with and without siRNA in the presence of amino acid. All experiments were done in triplicates (n=3)

APN protein expression was increased to 4-13 folds after aminoacid exposure to the adipocytes cells when compared to control. Amino acid proline increased APN secretion by 10.6 fold ($p < 0.01$), phenylalanine increases the APN secretion by 12.7 fold, ($p < 0.000$),Alanine by 6.3 fold ($p < 0.01$), AAM by 4.3 fold ($p < 0.008$) and APN si1- 10 nm decreased APN protein expression in control as well as in the presence of amino acid proline ($p < 0.05$), phenylalanine ($p < 0.000$), alanine ($p < 0.05$) and in AAM ($p < 0.01$) when compared to cells not treated with APN si1 (Figure 53 b).

5.6.3. APN silencing decreased amino acids induced AMPK activity and glucose uptake

Amino acids proline, phenylalanine, alanine were able to increase the AMPK activity but couldn't attain statistical significance where AAM ($p < 0.006$) showed significant increase in AMPK activity. Transient knockdown of APN in the presence of aminoacid proline ($p < 0.03$), phenylalanine ($p < 0.03$), alanine ($p < 0.03$) and in AAM ($p < 0.01$) decreased AMPK activity when compared to cells not treated with APN si1. Transient knockdown of APN by siRNA decreased amino acid induced AMPK activity significantly (Figure 54 a).

Similar results were observed in glucose uptake. Amino acids proline ($p < 0.01$), phenylalanine ($p < 0.006$), AAM ($p < 0.03$) showed a significant increase in glucose uptake where transient knockdown of APN decreased glucose uptake in control ($p < 0.003$) as well as in the presence of amino acid proline ($p < 0.01$), phenylalanine ($p < 0.001$), alanine ($p < 0.05$) and in AAM ($p < 0.04$) when compared to cells not treated with APN si1 (Figure 54 b).

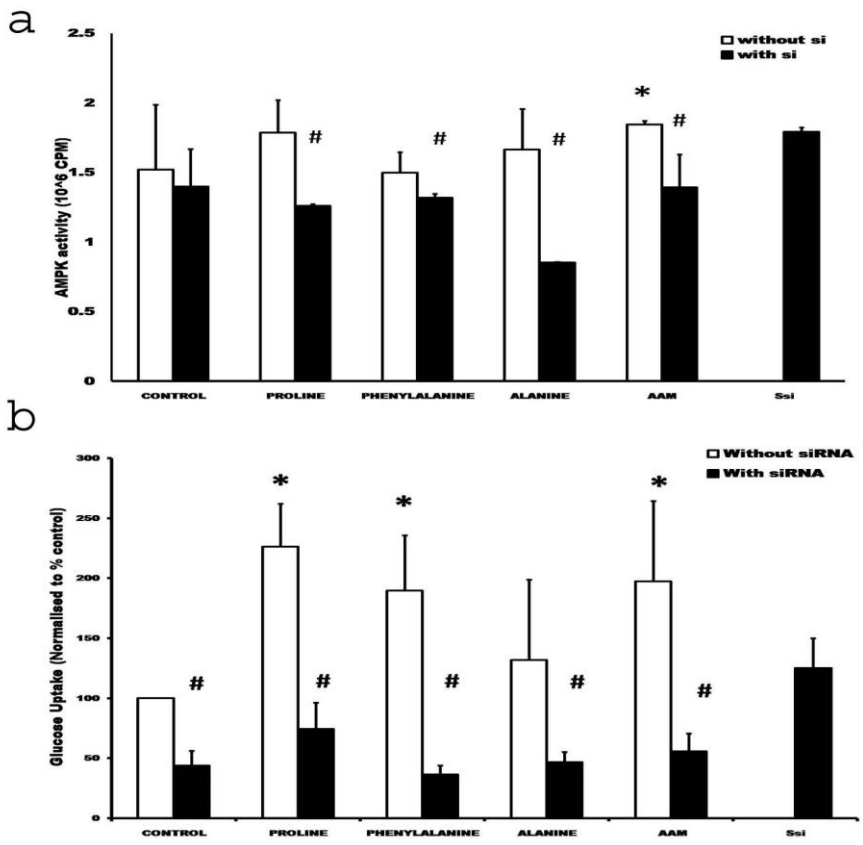


Figure 54: Amino acids mediate glucose uptake via APN. a) AMPK activity was measured using SAMS peptide activity after amino acid exposure in cultured adipocytes with and without transient knockdown of APN.b) glucose uptake was increased in presence of amino acids whereas silencing APN glucose entry in to the cells was significantly decreased. * - Significance comparison between the cells treated with amino acid.# - Significance comparison between the cells treated with and without siRNA in the presence of amino acid. All experiments were done in triplicates (n=3)

Thus free amino acid proline, phenylalanine, alanine and AAM modulate the level of APN thereby increasing AMPK activity and inturn glucose uptake in a high glucose environment. This amino acid induced increase in AMPK activity and glucose uptake was decreased with the transient knockdown of APN indicating that amino acid, by improving the APN levels, increases glucose uptake.

CHAPTER 6: DISCUSSION

Diabetes is a prevalent metabolic disorder with vascular complications (Nicholson, Hall et al. 2011). Current treatments modalities attempt to keep up tight glycemic control in diabetic patients thereby prevent the onset of the complications (Holman, Paul et al. 2008). Localised treatment becomes unavoidable for the patients with diabetic complications like DR. Laser treatment, vitrectomy and anti VEGF treatments have disadvantages such as repeated injections to maintain the therapeutic level, cost of the antibody, tractional retinal detachment and endopthalmitis (Ni, Hui et al. 2009; Raczynska, Zorena et al. 2014) which still remains challenging part of localized treatment in diabetic complication.

This thesis aimed to study the role of APN, a circulating adipokine in PDR. APN is a multifunctional adipokine reported to have anti diabetic, anti atherogenic, and anti inflammatory effects. APN levels were found to be decreased in obesity (Arita, Kihara et al. 1999), T2D (Hotta, Funahashi et al. 2001; Weyer, Funahashi et al. 2001) and in coronary artery diseases (Kumada, Kihara et al. 2003). APN knockdown showed impaired insulin sensitivity (Bluher 2014), vascular alteration (Kubota, Terauchi et al. 2002) in mouse model indicating its essential role in insulin regulation and cardio-protection. rAPN decreases choroidal neovascularisation (Bora, Kaliappan et al. 2007) and retinal neovascularisation (Higuchi, Ohashi et al. 2009) *in vivo*. Vitreous APN levels from 5 samples (Zietz, Buechler et al. 2008), elevated aqueous level of APN (Mao, Peng et al. 2012; Costagliola, Daniele et al. 2013), in PDR patients were reported, indicating its association with PDR. However there is no information on APN expression, localisation or its function in retina.

Adipocytes were once believed to be the only source of APN expression. It could be possible that, in patients with PDR, the retinal barrier is affected; thus, APN present in the blood could seep into the vitreous humour (Zietz, Buechler et al. 2008; Mao, Peng et al. 2012). However, APN transcripts were also reported in myocytes (Delaigle, Jonas et al. 2004) , osteoblasts (Berner, Lyngstadaas et al. 2004) , liver endothelial cells (Kaser, Moschen et al. 2005), ovarian cancer cell lines (Tiwari, Ocon-Grove et al. 2015) and placental tissue (Caminos, Nogueiras et al. 2005). More interestingly, Bora et al demonstrated the expression of APN in rat choroidal tissue.

Brain endothelial cells express both receptors AdipoR1 and AdipoR2 but not APN (Spranger, Verma et al. 2006).

APN plays a significant role in cancer. Yoneda et al. reported the presence of AdipoR1 and AdipoR2 in normal colon epithelium as well as in colon cancer tissues and that APN plays a major role in the physiological and pathological condition of colon epithelium via its receptors (Yoneda, Tomimoto et al. 2008). APN expression was found to be down regulated in esophageal cancer tissue (Duan, Tang et al. 2014). APN and AdiopR1 expression were found to be significantly lower in haepatocelluar carcinoma (Shin, Yu et al. 2014). These reduced expressions might be associated with the progression of haepatocelluar carcinoma. Expression of AdipoR1 and AdipoR2 might serve as prognostic marker in haepatocelluar carcinoma (Shin, Yu et al. 2014).

This study reports the expression and localisation of APN and its receptors AdipoR1 and AdipoR2 in human ocular tissues. APN and its receptors were found to be localised in ciliary, retinal and choroidal layers as demonstrated by immunohistochemistry and immunoflourescence staining (Figure 16 &18). This study also reports that APN and its receptors mRNA (Figure 12), protein expression (Figure 15), in the retinal layers, iris, ciliary body, optic nerve fibre and choroidal layers.

Two major microvascular endothelial cells HREC and HCEC were isolated in this study. HREC cells showed higher expression of APN compared than HCEC and HRPE. This study also colocalised APN with endothelial cell marker CD31 in HREC which being a sign of APN expression in endothelial cell surface. The coexistence of APN and its receptors points to the possible APN signalling pathway in ocular tissues. Recently, yet another group has also reported the localisation of APN in luminal surface of endothelial cells in normal aorta .The change in the localisation of APN from endothelial cell surface to monocyte adhering to endothelial cell was observed during pathogenesis of atherosclerosis (Mori, Koyama et al. 2014).

Surgically removed ERM and vitreous from PDR patients showed higher expression of APN protein. Further vitreous and plasma APN levels along with other adipokines such as VEGF, PEDF and IGF-1 in the patients with PDR were also measured. Plasma APN levels were

decresed in patients with diabetes (Control II) and found to be elevated in the patients with PDR. Hotta et al also reported decreased plasma levels of APN in patients with T2D (Hotta, Funahashi et al. 2000). Reports also state that blood levels of APN are elevated in patients with diabetic retinopathy, in addition to its positive correlation, with the severity of the disease (Kato, Osawa et al. 2008; Pradeepa, Surendar et al. 2015). Plasma APN levels positively correlated with HDL cholesterol in this study, the latter is considered be beneficial for vascular diseases (Zietz, Herfarth et al. 2003). Another recent study also reported a positive correlation of APN and HDL-2/HDL-3 ratio indicating its associations with insulin resistance and metabolic syndrome (Moriyama, Negami et al. 2014). High levels of PEDF in the plasma of patients with T2D and PDR is not surprising since, it has already been reported that PEDF is one of the most abundant proteins secreted by adipocytes and induces insulin resistance and inflammatory signals in muscles and fat cells (Famulla, Lamers et al. 2011). Increased plasma PEDF correlated with increased glucose in this study which also supports the already known function of PEDF in causing insulin resistance in obese and diabetic patients. Blood levels of VEGF and IGF-1 were not different between the groups.

Vitreous levels of key angiogenic regulators involved in pathophysiology of PDR such as VEGF, PEDF, IGF-1 and APN were analysed in this study. Elevated vitreous levels of APN, VEGF and IGF-1 in patients with PDR when compared to control (MH) were observed. Elevated adipokines were been constantly reported in diabetes and diabetic complications. Imbalance between the pro angiogenic and anti angiogenic molecules lead to pathogenesis of PDR. Elevated levels of VEGF, IGF-1 in vitreous of PDR were reported. (Meyer-Schwickerath, Pfeiffer et al. 1993; Adamis, Miller et al. 1994). VEGF and IGF-1 were two major angiogenic regulators in the pathogenesis of PDR. VEGF is hypoxia dependent (Neufeld, Cohen et al. 1999) whereas IGF-1 is hypoxia independent (Slomiany, Rosenzweig et al. 2006) angiogenic mediators. Earlier Zietz et al reported APN levels ranged from 2.0 to 70.2 ng/ml, (Zietz, Buechler et al. 2008) which is in line, with our finding (2.28 to 301.8 ng/ml) where a larger sample size was used and with 3-4 fold elevation in the patients who had undergone laser treatment before vitrectomy. Yet another study reported elevated APN level in aqueous humour of the patients with PDR (Mao, Peng et al. 2012). Few reports state that elevated level of APN in subretinal fluid could be preventive

mechanism of anti inflammatory response or it could induce the progression of PVR (Ricker, Kijlstra et al. 2012).

There is increased APN, PEDF and decreased VEGF in the patients who underwent laser surgery prior to vitrectomy as observed in this study. Laser treatment decreases the hypoxic environment thereby decreases the VEGF level (Stefansson 2006) and few reports state that laser treatment increases PEDF levels. These adipokines levels were subjected to statistical correlation tests to understand their relationship between each other and with the disease pathogenesis. APN positively correlated with PEDF and IGF-1. APN is reported to have anti tumourgenic and anti angiogenic activity (Brakenhielm, Veitonmaki et al. 2004) and might have similar function in ocular cells as comparable to that PEDF. A positive correlation for APN with IGF-1 was observed in this study. Besides its proangiogenic property IGF-1 is a known neuroprotective agent in retinal cells (Dal Monte, Cammalleri et al. 2007). APN has been shown to have neuroprotective role in brain cells and might have a similar function in ocular tissues as well, where IGF-1 could be a positive regulator in that function. Moreover, there was a negative correlation between APN and VEGF. It has been reported that APN is expressed in choroidal blood vessels, and this expression is increased upon laser treatment in mice (Bora, Kaliappan et al. 2007).

Increased APN levels was observed in the vitreous of patients with PDR who underwent laser treatment prior to surgery, and this increase in APN correlates positively with PEDF and negatively with VEGF, indicating that laser treatment improves APN levels and its convincing role as an anti angiogenic adipokine. Additionally, APN positively correlated with heme oxygenase -1 (HO-1) activity in PDR as well as in study group. HO catalyses the first and rate limiting step of heme degradation resulting in the formation of carbon monoxide, iron and bilirubin. HO exists in three isoforms HO-1, HO-2 and HO-3. HO-1 is a hypoxia inducible enzyme having anti inflammatory and anti apoptotic enzyme in regulation of wound healing (Grochot-Przeczek, Dulak et al. 2010) and found to be elevated in vitreous of the patients with PDR. APN and HO-1 have interdependency in their anti inflammatory function (Bharathselvi Muthuvel, Vidhya Srinivasan et al. 2015).

Interestingly amino acids levels were also found to be elevated in the PDR vitreous when compared to MH control. Panel of 12 amino acids viz glutamic acid, serine, histidine, glycine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine and lysine were estimated using RP HPLC in the vitreous obtained from patients with PDR and MH . The analysis showed that out of the 12 amino acids 8 of them, namely, serine, glycine, alanine, phenylalanine, valine, isoleucine, leucine, and lysine were significantly elevated in PDR patients when compared to MH. Lu et al has shown that the signalling amino acids namely arginine and glutamate are elevated in the vitreous of patients with PDR (Lu, Pulido et al. 2007). Jiang et al also reported increased vitreous level of serine and glutamate in patients with PDR relating it to retinal neural death and toxicity (Jiang, Du et al. 2014). Earlier Coral et al had reported increased proline and hydroxyproline in the vitreous of patients with PDR relating to collagen breakdown (Coral, Angayarkanni et al. 2009).

There was also a significant positive correlation between amino acid level and APN level in vitreous. When retinal pericytes were exposed to these amino acids for a longer period they induced certain morphological changes in the pericytes. That was confirmed as oil drops by oil red O staining. The cells treated with glycine, proline, hydroxyproline, lysine and isoleucine showed the maximal accumulation of oil as well as increased accumulation of triglyceride by adipogenesis assay. Proteomic approach with mass spectrometry revealed 322 proteins hits in hydroxyproline treated condition and 43 proteins were differentially expressed when compared to control and out of which 25 were more than 10 fold elevated. When these proteins were compared with the already reported adipocyte differentiation proteome (Adachi, Kumar et al. 2007; Rajesh, Heo et al. 2010) 12 hits which include β galactosidase, CD151 antigen, glutamate dehydrogenase 1, histone H2A V, septin 11 were also expressed in the amino acid treated condition. Most of the proteins were mitochondrial and the de novo mitochondrial biogenesis is found during 3T3-L1 adipogenesis (De Pauw, Tejerina et al. 2009). Certain proteins which are involved in ubiquitin proteome degradation like Polyubiquitin C and Ubiquitin conjugating enzyme were also observed which may be involved in the β catenin degradation which is an important event for the activation of cEBP alpha and activation of PPAR γ (Gregoire, Smas et al. 1998).

Differentiated pericytes were also subjected to gene expression for adipocyte markers viz Pref-1, PPAR γ and secretory proteins APN and VEGF. The differentiation of preadipocyte to adipocyte requires a sequence of events like the down regulation of Pref-1 which is a preadipocyte factor (Gregoire, Smas et al. 1998). The Pref-1 suppresses the differentiation process by interacting with the transcription factor sox9 which in turn blocks the cEBP alpha expression (Sul 2009). If Pref-1 is suppressed then it leads to the activation of PPAR γ and there is induction of adipogenic genes and proteins. Amino acids proline, hydroxyproline and glycine decreased Pref-1 and increase the PPAR γ expression. The branched chain amino acid isoleucine has also been reported to induce the PPARα expression and also influences the FFA uptake by the cells (Nishimura, Masaki et al. 2010).

Two secretory proteins APN and VEGF in relation to PDR were also analysed. A significant increase in APN expression was observed in cells treated with proline, hydroxy proline, alanine, glycine, lysine and isoleucine There is a single study which states that glycine can induce the mRNA expression of APN in 3T3-L1 adipocytes (Garcia-Macedo, Sanchez-Munoz et al. 2008). Other than the synthesis of APN the amino acids proline, hydroxyl proline and lysine have also been reported to help in the synthesis of the extracellular matrix formation of the adipocytes (Mariman, Wang et al. 2010). However the other secretory protein VEGF expression showed significant decrease in the cells treated with proline, hydroxy proline, glycine, serine and lysine.

NO levels were also found to be elevated in the differentiated pericytes. Vasodilator factor of the endothelium is nitric oxide (NO). It is formed in endothelial cells from the amino acid L-arginine by Nitric oxide synthase (eNOS). Loss of NO leads to vasoconstriction , inflammation, and cellular proliferation (Ganz, Vitaet al. 2003). NO increases mitochondrial biogenesis thereby increasing APN secretion *in vivo* model (Koh, Kim et al. 2010). Increased vitreous amino acid observed in PDR patients could contribute to differentiation of pericytes to some extent which might be mechanism to increase NO levels, APN levels and to decreased VEGF levels.

The precise functions of APN in PDR were studied by *in vitro* angiogenesis assay in microvascular endothelial cells, by *in silico* approach and also studied the effect of APN on VEGF and PEDF levels. Microvascular endothelial cells isolated from retina HREC were used *in vitro* angiogenesis assay. Established HRECs cells expressed endothelial cell marker von wille

brand factor. rAPN commercially purchased were used for the assay. *In vitro* angiogenesis assay indicates APN as an anti angiogenic molecule which decreases tube formation, migration and proliferation of HREC microvascular endothelial cells. Moreover addition of rAPN to HREC cells reduced VEGF protein expression. rAPN inhibits proliferation of pancreatic cancer cells and APN knock down mice showed increased tumor volume compared to wild type indicating anti tumorgenic role of APN (Kato, Watabe et al. 2014). It has been reported that intravitreal and intraperitonal injection of recombinant APN decreased choroidal neovascularisation by 68% and 78% respectively (Bora, Kaliappan et al. 2007). Earlier results also concurs that increased APN in vitreous of the patients with PDR who underwent laser treatment prior to surgery. This increase in APN correlates negatively with VEGF. *In vitro* angiogenesis experiments resulted in decreased VEGF when exposed to APN as reported by Bora et al. APN suppresses VEGF-stimulated human coronary artery endothelial cell migration via cAMP/PKA-dependent signalling which shows implications for APN as a regulator of vascular processes associated with diabetes and atherosclerosis (Mahadev, Wu et al. 2008).

At the sequence level new interacting partners were identified based on the pattern conservation in the existing interacting partners and these proteins were further screened based on the conserved Post Translational Modifications observed in the existing partners. This was further ascertained through proteome docking studies at the structural level. Key interfacial residues mediating the functions of APN were identified through docking studies and it is hypothesized that APN interaction with the newly identified interacting partner's results in anti angiogenic activities and anti apoptotic activities. Further, the common interacting partners between APN and the known anti angiogenic molecule PEDF were analyzed. Results revealed that APN inhibits migration via inhibiting VEGF signalling pathway.

APN also modulates the two major angiogenic regulators in cultured ARPE cells. ARPE 19 was chosen for this experiment for the reason that it maintains the outer retinal barrier and also secretes both pro angiogenic VEGF, anti angiogenic cytokine PEDF (Bouck 2002). *In vitro* studies on RPE cells exposed to various concentration of rAPN showed that APN down regulates VEGF expression and up regulates PEDF gene expression.

APN has been considered as a regulator of vascular remodelling for the following reasons, It is antiangiogenic a) by decreasing interleukin 8 expression and secretion from EC (Kobashi, Urakaze et al. 2005), b) by forming complex with growth factor like platelet derived growth factor (PDGF), Fibroblast growth factors (FGF), and Epidermal growth factor (EGF) reducing their bioavailability (Wang, Lam et al. 2005), c) by inhibiting EC migration and proliferation *in vitro* and *in vivo* assays (Higuchi, Ohashi et al. 2009), d) by attenuating hypoxia induced pathological retinal neovascularisation in mice (Goldstein, Scalia et al. 2004; Kobayashi, Ouchi et al. 2004).

On the other hand, it is also proangiogenic e) by activating AMPK and inhibiting caspase 3 in animal and cell culture experiments and f) by promoting blood flow to ischemic limb (Shibata, Ouchi et al. 2004). APN has been reported to have a pleotropic effect on the maintaining the vascular integrity. At this point we do not know whether APN is pro or anti angiogenic molecule in eye, however, our findings indicate it could inhibit VEGF expression. *In vitro* study on RPE exposed to APN showed that APN alters VEGF levels thus indicating the opportunity for APN in the treatment of PDR.

Administration of physiological dose of APN in diabetic mice and murine type 2 diabetes model reversed insulin resistance (Kadowaki, Yamauchi et al. 2005) indicating the crucial and direct role of APN in insulin resistance. Peptides imitating the function of APN or small molecules for replenishment of APN levels will have therapeutic value in disease like PDR and also in T2D. Functional domains and interacting motifs represent the function of the protein. Peptides can represent complete or partial functional modules as well as possible protein- protein interaction sites. Peptides derived from PEDF (Longeras, Farjo et al. 2012), angiopoietin (Palmer, Tiran et al. 2012) were reported to reduce neovascularisation in retinopathy models. APN was also reported to reduce retinal neovascularisation in mouse model by altering TNFα (Higuchi, Ohashi et al. 2009) APN derived peptides reduced choroidal neovascularisation in mouse model. APN peptides reported to have anti angiogenic property (Bora, Kaliappan et al. 2007; Lyzogubov, Tytarenko et al. 2009; Lyzogubov, Tytarenko et al. 2012) were synthesized from globular domain of APN whereas the peptides used in this study were designed meticulously based on significant interacting residues of APN. Significant residues of APN with its interacting partners were mapped based *in silico* approach. Lyzogubov et al reported APN peptides decresed

proliferation in HUVEC and mouse aorta vein endothelial cells (MAVEC) both being macrovascular endothelial cells (Lyzogubov, Tytarenko et al. 2009). However, this report demonstrates that peptides I,II,III decreased proliferation, migration, tube formation, adhesion and also reduced VEGF expression in both macrovascular endothelial cells (HUVEC) and microvascular endothelial cells (HREC).

Small molecules such as pharmacological drugs like thiazolidinediones (TZD, pioglitazone and rosiglitazone) improve the level of APN thereby increasing insulin sensitivity in diabetic patients.(Yang, Jeng et al. 2002; Yu, Javorschi et al. 2002; Phillips, Ciaraldi et al. 2003; Miyazaki, Mahankali et al. 2004). Pioglitazone increases plasma APN levels by post-transcriptional regulation (Rasouli, Yao-Borengasser et al. 2006). Yet another group reported that thiazolidinediones reduces retinal neovascularisation via APN dependent mechanism in mouse models (Higuchi, Ohashi et al. 2010). GLP-1 was also reported to be beneficial for revascularization via increasing APN level (Ishii, Shibata et al. 2014). Apart from pharmaco agents, nutraceuticals like punicic acids, a compound from pomegranate seed increases APN level and GLUT 4 expression as comparable to rosiglitazone (Anusree, Priyanka et al. 2014). Avocado fruit extract was also found to increases APN level which reduces the risk of obesity and hyperlipidemia *in vivo* models (Padmanabhan, Arumugam et al. 2014).

Amino acids are general nutrients which are non pharmacological and non toxic. Amino acids remove the excess glucose in the blood (Ramakrishnan, Sulochana et al. 1996) and also up regulate IR system (Sulochana, Rajesh et al. 2001). Amino acids are proven to be anti-diabetic and anti-cataractogenic in diabetic rats (Sulochana, Punitham et al. 1998). Oral supplementation of amino acid decreased postprandial blood glucose in a diabetic patients (Natarajan Sulochana, Lakshmi et al. 2002). Additionally beneficial effects of amino acids were studied in CHO-K1 cells in which amino acids mixture increases glucose uptake and GLUT 4 translocation when the cells were exposed to high glucose environment (Selvi, Bhuvanasundar et al. 2012).

Blümer et al have reported that branched chain amino acid mixture can stimulate the APN production and states that amino acids acts as substrates for protein synthesis and thus for APN synthesis (Blumer, van Roomen et al. 2008). Glycine has already been reported to induce the mRNA of APN in cultured 3T3L1 cells. (Garcia-Macedo, Sanchez-Munoz et al. 2008). In this

present study, proline, hydroxy proline, phenylalanine, alanine and amino acids of specific, mixture improve APN mRNA as well as secretion in cultured human adipocytes about 4- 10 fold.

APN stimulates AMPK activation which in turn increases glucose uptake in muscles and decreases gluconeogenesis in liver (Kadowaki, Yamauchi et al. 2006).This increase in APN levels activates AMPK thereby increases glucose uptake in cells as in line with Kadowaki et al. To further confirm this mechanism, APN mRNA was targeted by siRNA. AMPK activity and glucose uptake was measured in the presence of amino acid with and without APN siRNA. Amino acid increased the AMPK activity and glucose uptake but this amino acid induced increase was affected by the transient knockdown of APN. Meng et al reported that 3T3 L1 cells were infected with APN siRNA expression vectors there was a decrease in glucose transport to a great extent (Meng, CB et al. 2008).

Amino acids have been shown to be beneficial in increasing the glucose uptake in the CHO-K1 and clinical studies have shown that they reduce the blood glucose level by potentiating the insulin receptors. This study also stresses that amino acids increases AMPK and glucose uptake via increasing APN. Amino acids can have a therapeutic intervention in insulin resistance, T2D by improving APN levels as in cultured adipocytes cells.

CHAPTER 7: CONCLUSION

Anti diabetic role of APN was well studied and reported in literature. In this work, the role of APN in PDR was studied. Further the approach for modulating angiogenesis by peptides derived from APN and by using small molecules which increase APN levels were evaluated.

1. APN and its receptors R1 and R2 were localized and found to be expressed in human ocular tissues and in isolated ocular cells. Moreover, APN was also colocalised with CD31 in retinal capillaries indicating its presence in endothelial cell surface.
2. Elevated vitreous levels of APN protein in patients with PDR was observed when compared to MH.
3. APN increased by 4 folds in the PDR patients who underwent Laser prior to the vitrectomy surgery and APN levels positively correlated with PEDF and IGF-1. Whereas it negatively correlated with VEGF levels indicating APN could have a possible role as an anti angiogenic molecule.
4. *In vitro* studies in pericytes treated with amino acids increased APN synthesis and decreased VEGF levels which could be beneficial in PDR.
5. *In vitro* angiogenesis assay in microvascular endothelial cells (HREC) revealed that rAPN was anti proliferative, anti angiogenic, anti apoptotic, inhibited migration of cells by decreasing VEGF levels.
6. Unique interacting partners were identified using *insilico* approach, common putative pathway of PEDF and APN also indicates its anti angiogenic property by inhibiting VEGF signalling pathway.
7. Additionally APN was able to reduce the secretion of VEGF in cultured RPE cells confirming the suitability of APN as a therapeutic target for PDR.
8. Three different peptides were designed using bioinformatics tool based on the binding regions of APN and its interacting partners. All the three peptides decreased tube formation, proliferation, migration, adhesion and decreased VEGF levels in both microvascular and macrovascular endothelial cells (HREC & HUVEC) thus inhibiting angiogenesis.

9. Proline, hydroxy proline and phenylalanine increased APN secretions 10 fold. Anti diabetic effect of these amino acids were exhibited by increased glucose uptake via AMPK pathway in an APN dependent mechanism.
10. Newer treatment modalities for diabetes with amino acids, which can enhance glucose uptake and APN secretion, can be developed as drug for treating both diabetes and its complications.

FUTURE SCOPE

- ✓ Role of APN in other ocular pathologies can be studied.
- ✓ Interaction of APN, obtained by *in silico* approach, has to be further validated.
- ✓ The designed peptides will be further validated by *in vivo* experiments. Efficacy and bioavailability of the peptides will be checked.
- ✓ Free amino acids proline, hydroxyproline and phenylalanine can also be tested *in vivo* for their ability to increase APN and its anti diabetic effect.

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APPENDIX I - CLINICAL PERFORMA

ADIPONECTIN AS A "THERAPEUTIC TARGET" FOR OCULAR ANGIOGENESIS

Inclusion criteria	Exclusion criteria
• Age related (controls) • ED,PVR ,PDR • MH (Cases)	• Sepsis and malignancy • Renal failure • Coronary heart diseases.

1. ID No

2. Name:

3. Age & **Sex:**

4. MRD No:

5. Contact Address:

6. Contact number:

7. **Alternate telephone number:**

8. Email id:

9. Date of Examination (dd/mm/yyyy):

10. Occupation: Student/ Employee/ Professinal/Others

11. **Diabetic status**: **1**. Known **2**. Newly detected diabetics **3**. Non-diabetic **4**. IFG **5**. IGT

12. **Present eye Complaints** 1.Yes 2.No

13. **1**. Decrease vision **2**. Redness **3**. Pain **4.** Photophobia **5**. Haloes 6. Floaters/Flashes **7**. Others (Specify)

OD: OS:

14. **Any other ocular Disease in Past Diagnosis** OD: _____ OS: _____

15. a) History of any ocular surgery **1**.Yes **2**.No

16 a) Whether underwent any laser photocoagulation 1.Yes **2**. No

b) Details of laser treatment **1.** Focal **2**. PRP OD OS

c) No of sittings OD : OS:

17. **Other Systemic Diseases: 1**. Hypertension **2**. IHD **3**. Asthma 4.others

18. **Duration of hypertension :** _____(in months)

19. **Treatment 1**.Oral hypoglycemics **2**. Insulin **3**.Exercise & Diet control **4.** No medication **5**. Others specify _____

20. **Any eye examination done after hospital examination**: **1**.Yes 2.No

21. a. If yes when _____ (in months).

b. No of times visited.

c. Last examination date _____

22. **Any other significant history related to diabetes**: **1**.None **2**.Nephropathy **3**.Neuropathy 4**.**Others **(Specify)** _____

23. Smoking status: 1.Non-smoker **2.** Current smoker **3.** Ex smoker

24. Alcohol status: **1**.Never 2. Occasional / social drinker (once /week) **3.** Regular drinker 4**.** Heavy drinker

25. Food Habits: 1. Veg 2. Non-Veg

26 a. Family History of Diabetes: **1**.Yes 2.No. **b) If Yes Relation** : 1.Mother 2.Father 3. Brother 4. Sister 5.Maternal grand Mother 6.Maternal grand Father 7. Paternal Grand Mother 8. Paternal Grand Father 9. Daughter 10. Son 11. Others (**Specify)**

27. **Resting heart rate** /min

28 a. BP Systolic **28 b. Diastolic** (mm/hg)

29 a. Height : _____(cms) **29 b. Weight**:_____(kgs)

30. OPTHALMIC EXAMINATION:

	Distance	Near	Glasses
1. **Visual acuity with ETDRS chart**			
1. PVA a. OD	b. OS		
2. CDVA			
3. Subjective OD			
4. Subjective OS			
5 . Add a. OD	b.OS		

2. EXTERNAL EXAMINATION **1**.Normal 2.Facial asymmetry **3.** Exotropia **4**. Esotropia

5. Hypertropia 6. Hypotropia **7.** Others (specify)

3. Slit lamp examination

Lids: OD: OS:

1.Normal **2**.Blepharitis **3**.Meibomitis **4.** Others (**specify)**_____

Conjunctiva: OD: OS:

1. Normal 2. Others (**specify)** _____

Sclera: OD: OS:

1.Normal **2**.Others (**specify)** _____

Cornea: OD: OS:

1. Normal **2.** Others (**specify)** _____

Fundus details: OD: OS:

1. Seen **2.** Very hazily seen **3.** Not seen

OD:1. Vitreous hemorrhage 2. Cataract 3. Asteroid hyalosis 4**.** Others (**specify)**_____

OS: 1.Vitreous hemorrhage 2. Cataract 3. Asteroid hyalosis 4**.** Others (**specify)**_____

Disc (with 78 D and 20 D): **1.** Normal **2.** Pallor **3.** C: D ratio **4.** Notching of NRR **5**. Disc hemorrhage **6.** NVD 7**.** FVP 8**.** Others

Macula (with 78 D and 20 D): **OD: OS:**

Blood Vessels: OD: OS:

1.Normal 2.AV ratio 3. Venous dilatation 4. Looping of Blood vessels 5. Sclerosed vessel

Periphery: OD: OS:

1. Normal **2**. NVE 3. FVP **4.** Tessellations 5. Hemorrhages **6** laser marks **7**. others **(specify)_____**

Impression :

4. CLINICAL DIAGNOSIS: OD: OS:

1.No DR **2.**Mild NPDR **3**. Moderate NPDR 4. Severe NPDR 5. PDR 6. TRD macula 7. TRD elsewhere 8. Combine RD

Disease Severity Level	Findings Observable With Dilated ophthalmoscopy
No apparent DR	No abnormalities
Mild nonproliferative DR	Microaneurysms only
Moderate nonproliferative DR	More than "mild" but less than "severe"
Severe nonproliferative DR	Any of the following: ▫20 or more intraretinal hemorrhages in 4 quadrants ▫Definite venous beading in 2 or more quadrants ▫Prominent IRMA in 1 or more quadrants and no neovascularization
Proliferative DR	1 or more of the following: ▫Definite neovascularization ▫Preretinal or vitreous hemorrhage

Diabetic Macular Edema: OD: 1. Present **2.** Absent ; **OS: 1.** Present **2.** Absent

If DME present then

OD 1. Mild DME **2**. Moderate DME 3**.** Severe DME

OS:1. Mild DME **2**. Moderate DME 3**.** Severe DME

CSME : **OD: 1**. Present **2.** Absent **; OS: 1**. Present **2.** Absent

Disease Severity Level	Findings on Dilated Ophthalmoscopy
DME absent	No retinal thickening or hard exudates present in posterior pole

DME present	Some retinal thickening or hard exudates present in posterior pole
Disease Severity Level	If DME is present, it can be categorized as follows:
Mild DME	**Findings Observable on Dilated Ophthalmoscopy***
Moderate DME	Some retinal thickening or hard exudates in posterior pole but distant from the center of the macula
Severe DME	Retinal thickening or hard exudates approaching the center of the macula but not involving the center
	Retinal thickening or hard exudates involving the center of the macula

5. **ASSOCIATIONS**: **OS** **OD**

6. **ADVICE:** OD:1. FFA 2. Laser 3. Periodic follow-up 4. Surgery

OS: 1. FFA 2. Laser 3. Periodic follow-up 4. Surgery

7. **OCT OD** 1. Normal 2. Abnormal 3. Not Done

A. **Vitreoretinal Interface :OD: 1**. Normal **2.** Abnormal

OS: 1. Normal **2.** Abnormal

A1. **PVD OD**: 1. No PVD **2**. Incomplete PVD **3**. Complete PVD **4.** Vitreous Schisis

OS:1. No PVD 2. Incomplete PVD 3. Complete PVD 4. Vitreous Schisis

A2. **ERM** : **OD**: 1. Present 2. Absent

OS: 1. Present 2. Absent

B. **Foveal Contour** OD:**1.** Normal **2**. Abnormal

` OS:**1.** Normal **2**. Abnormal

C. **Inner Retinal Layers** OD:1. Normal 2. Abnormal

OS:1. Normal 2. Abnormal

C1 If **(Abnormal) then**

D. **Photoreceptor Layer OD**: **1**. Intact **2**. Altered If 2-quadrant _____

OS: **1**. Intact **2**. Altered If 2-quadrant _____

E. **RPE Layer**: **OD**: **1**. Normal **2**.Abnormal If 2-quadrant _____

OS: **1**. Normal **2**.Abnormal If 2-quadrant _____

8. **FUNDUS PHOTOGRAPHY** **OD** **OS**

1. DONE **2.** NOT DONE Reason (***If-2)*** _____

Others_____

9. **ULTRASOUND: 1.** DONE **2.** NOT DONE **OD** **OS**

9 a. PVD **OD :1**. Complete **2.** Incomplete **3**. Absent

OS :1. Complete **2.** Incomplete **3**. Absent

9 b. If incomplete attached at **OD**:**1**. Disc **2**. Macula **3.** Extra macular

OS:**1**. Disc **2**. Macula **3.** Extra macular

9 c. Impression:

10. **FINAL DIAGNOSIS:** (Decreased Vision due to):

APPENDIX II – CONSENT LETTER

ADIPONECTIN AS A "THERAPEUTIC TARGET" FOR OCULAR ANGIOGENESIS

The doctor / research worker has explained the purpose of this study to me. I understand that vitreous humour will be collected from me, preoperatively, for research purpose that would help in further understanding of the disease mechanism. The vitreous humor is a fluid that fills the back portion of the eye. It is usually removed and replaced with other fluids during vitreoretinal surgery. This fluid which would otherwise be discarded will be used for research. I also understand that 8.0 ml of blood, urine will be collected from me. By giving vitreous humor, blood, and urine I understand that I will not have any side effects or complications.

I consent to participate in the study, having understood its objectives. I was informed about the strict maintenance and confidentiality of the results obtained. I voluntarily give my consent to participate and fully cooperate during the study.

Signature: _____

Date: _____

Name : _____

MRD No: _____

Address : _____

_____

Phone : _____

Email : _____

APPENDIX III

S.No	Product	Company
1.	Vented flask and culture dishes	Nunc
2.	Fibronectin	Sigma
3.	Collagenase	Sigma
4.	Antibiotic solution	Gibco
5.	Endothelial growth medium 2	Lonza
6.	**Gelatin**	Sigma
7.	Dimethyl sulfoxide	Merck
8.	MTT	Sigma
9.	Paraformaldehyde	Merck
10.	ECM matrix	Millipore
11.	Cell death ELISA kit	Roche
12.	TRIzol	Sigma
13.	Chloroform	Merck
14.	Iso propanol	Merck
15.	Ethanol	Merck
16.	iScript RT-PCR Kit	Biorad
17.	SYBR Green	Eurogentech
18.	Bradford	Thermoscientific

19.	BCA	Thermoscientific
20.	Nitrocellulose membrane	Millipore
21.	Horse radish peroxidase tagged antibody	Santacruz
22.	APN ELISA kit	R & D
23.	VEGF ELISA kit	R & D
24.	IGF-1 ELISA kit	R & D
25.	PEDF ELISA kit	Millipore
26.	Oasis column	Waters
27.	DTT	Biorad
28.	Iodaoacetic acid	Biorad
29.	SDS	Sigma
30.	Acrylamide	Sigma
31.	Bisacrylamide	Sigma
32.	TEMED	Biorad
33.	Ammonium per sulphate	Biorad
34.	Rapigest	Waters
35.	APN derived Peptides	USV peptides
36.	SiRNA	Merck
37.	ICA-fectine	Eurogentech
38.	EDTA	Merck
39.	Tris	Sigma
40.	Glycine	Sigma
41.	Trypsin for MS	Proteogen
42.	Trypsin for cell culture	Hi media

43.	Fetal bovine serum	Hi media
44.	Primers	Eurogentech
45.	Adipogenesis assay kit	Biovision
46.	Nitric oxide assay kit	Biovision
47.	AMPK activity kit	Upstate
48.	Novolink min polymer detection kit	Leica
49.	Recombinant Adiponectin	R & D
50.	Bovine serum albumin	Himedia, India

PUBLICATIONS

1. **Vidhya Srinivasan,** karthika palanisamy, Harish vuyuru, Karunakaran Coral Subbulakshmi Chidambaram, Pukhraj Rishi and Sulochana KN. Expression and localisation of adiponectin and its receptors in Human ocular tissues. Int J Pharma Bio Sci. 2014; 5(1): 639-646.
2. **Vidhya Srinivasan,** Subbulakshmi Chidambaram, Karunakaran Coral, Pukhraj Rishi, and Sulochana KN. Measurement of adiponectin in vitreous and plasma of the patients with proliferative diabetic retinopathy and its correlation with VEGF, PEDF, and IGF-1. Int J Pharma Bio Sci. 2013; 4(1), 993-1005.
3. **Vidhya Srinivasan** and Sulochana KN. Effect of adiponectin on VEGF and PEDF – an *in vitro* study. Ind j Pharmacology. 2015; 47, 117-120.
4. Bharath selvi M**, Vidhya Srinivasan,** Pukhraj Rishi, and Sulochana KN. Increased vitreous Heme oxygenase activity is associated with proliferative diabetic retinopathy. Ind j clin biocem. 2015;
5. Radhakrishnan Selvi, Narayanasamy Angayarkanni, Begum Asma, Thiagarajan Seetha, **Vidhya Srinivasan**. Aminoacids influence the glucose uptake through GLUT4 in CHO K1 cells under high glucose conditions. Mol. Cell. Biochem, 2010; 344 (1); 43-53.
6. Saijyothi Aluru Venkata, Angayarkanni Narayanasamy**, Vidhya Srinivasan,** Geetha Krishnan Iyer, Ramakrishnan Sivaramakrishnan, Madhumathi Subramanian, Rajeshwari Mahadevan. Tear ascorbic acid levels and the total antioxidant status in contact lens wearers: A pilot study. Ind j ophth. 2009; 57 (4), 289-92**.**

MANUSCRIPT UNDER PREPARATION:

1. Amino acids proline, phenylalanine, Alanine, AAM increases glucose uptake via adiponectin dependent mechanism.
2. Loss of Pericytes in diabetic retinopathy, are they differentiating into adipocytes?

POSTER PRESENTED:

- Presented poster entitled "Adiponectin, a therapeutic target for Diabetic retinopathy" in **Society of Biological Chemist (India) 2010**. **BS Narasingha Rao Award for the best poster was awarded for the work.**
- Presented poster entitled "Role of Adiponectin in Proliferative Diabetic retinopathy" in CTBPR, **BITS, Pilani (India) 2011. Second Best poster Award was awarded for the work.**
- Presented poster entitled "Expression of Adiponectin in retinal blood vessels and role in ocular angiogenesis**"** in **Society of Biological Chemist (India) 2011.**
- Presented poster entitled"Role of free amino acids in the transformation of retinal pericytes to adipocytes insights into the molecular mechanism of proliferative diabetic retinopathy" in **Society of Biological Chemist (India) 2012.**
- Presented poster entiled"Loss of pericytes in Diabetic retinopathy ,are they transforming into adipocytes" in **ASIA ARVO**,**2013.** Received **National travel grant award** for attending the conference.

AWARD:

1. Received **ASIA –ARVO national travel grant**, held at New Delhi, (India) 2013.
2. **Second Best poster Award** in CTBPR NATIONAL CONFERENCE held at BITS, Pilani (India) 2011.
3. **BS Narasingha Rao Award** for the best poster in 79TH SOCIETY OF BIOLOGICAL CHEMIST (INDIA) 2010 held at IISC BANGLORE.
4. **Wipro Biomed Pvt Ltd Endowment award** for the best performance in Examinations conducted on Biochemistry on successful completion of MS in Medical Laboratory Technology. 2007
5. **Dr.S.Ramaswamy and Dr.S.Narasimhan Endowment award** for the best performance in Examinations conducted on Human Anatomy and Human Physiology on successful completion of MS in Medical Laboratory Technology.2007
6. Secured **First place** in the Major and allied subjects in the university examination II year B.Sc Biochemistry held during the year 2003.

PATENT FILED:

- Identification of Aminoacids inducing high degree of secretion of Adiponectin **[file no. 3219/che/2012]**. Inventor: Dr.K.N.Sulochana. Co- inventor: **Vidhya S** & Dr. R.Selvi
- Method of identifying an aminoacid that enhances the production of nitric oxide **[file no.3220/che/2012].** Inventor: Dr.K.N.Sulochana. Co- inventor: **Vidhya S** & Dr.S RBharthi devi

BRIEF BIODATA OF CANDIDATE

- **Ms. Vidhya Srinivasan** obtained her B.Sc. Biochemistry degree from Sri ram College of arts and Science in 2004. She did her MS in medical laboratory technology in 2007 from Birla institute of Technology & Science – Pilani in collaboration with Medical research foundation. She did MSMLT internship in Biochemistry department on the topics "**Study of Radio labeled Deoxyglucose and Amino acids Uptake by Chinese Hamster Ovary cells in a High Glucose Environment" and "Estimation of Vitamin C by HPLC in Tear specimen of Control subjects and Contact lens Wearer and Standardization of Tear Protein Profile"** which were published in molecular Cell biochemistry and IJO. She joined as **Junior Research Fellow** in SNSC laboratory, Clinical Hematological and pathology department at **Sankara Nethralaya**, Chennai in July 2007. She later joined the department of Biochemistry and Cell biology as a **senior research fellow** in 2010 and registered for PhD under the guidance of Dr. K.N. Sulochana. During which she made 5 poster presentations and got 2 best poster awards and a travel grant award. She has published 4 papers in her PhD topic and other 2 manuscripts are under preparation. She has two patent filed in India and one patent under review. She established HREC and HCEC cell culture in this department and also handled several cell lines like ARPE 19, HRPE, BRP, 3T3, human visceral adipocytes cells. Her keen interest is on diabetes and its related disorders.

BRIEF BIODATA OF SUPERVISOR

Prof. K.N. Sulochana is currently Sr. Professor & Director, R.S. Mehta Jain Department of Biochemistry and Cell Biology, Vision Research Foundation, Chennai. She has been Reader in Biochemistry Research Department, Sankara Nethralaya for nearly 10 yrs, Sr. Research Fellow, National University Singapore, for 2 yrs and as a Sr. Lecturer at Bradford University Singapore campus and Sr. Research Scientist, in pharma industry for 3 yrs. She did her B.Sc. (Chemistry); M.Sc. (Biochemistry) –University of Madras and Ph.D. (Biochemistry-Enzymology) SV. University, India.

She has keen interest in research, teaching and patient care. Her teaching area includes Basic Biochemistry, Biomolecules, Instrumentation, Clinical Biochemistry and Ocular biochemistry. Patient care includes optimization and development of clinical investigations for oxidative stress, analysis of vitamin, amino acids, and enzymes.

Her current interests are Ocular Angiogenesis, Protein- Protein interactions, Molecular mechanisms of metabolic diseases, Drug targets, Design and development of inhibitors of angiogenesis, Pharmacokinetics and Preclinical studies. She has more than 50 Research article published, 5 reviews and 4 Book Chapters.

She has participated in many National and International conferences. She has received Dr. BC. Roy Award, Silver Jubilee Research Award of Medical Council of India, 1996, Swarna Latha Punshi award as "Best Research Worker" Medical Research Foundation, 1997, Women of the Year American Biographical Institute, Inc (2000), The biographical sketch inclusion, 19th edn of Who's Who in the World by Marquis (2001) and Outstanding Woman of the 21st Century", American Biographical Institute, NC, USA, 2001.

She's also a life member of professional bodies in Society of Biological Chemistry (I) (Life Member), Association for the promotion DNA fingerprint DNA technology (Life Member), Active participant in Annual Meeting of IERG for the past 7 yrs, Member in ARVO, Member in Association for clinical biochemist Singapore since 2002 (SAC), Member of the Management development institute of Singapore (MDIS) since Jan-2008.

BRIEF BIODATA OF CO- SUPERVISOR

Prof. Suman kapur is currently Sr. Professor in the centre of Biotechnology, Biological sciences group BITS, Pilani. She has worked in the capacity of unit chief, community welfare and international relations since 1st Jan 2007. From April she has taken charge as Dean, Research and consultancy at the Hyderabad campus of BITS. She did her B.Sc. (Zoology); M.Sc. (Medical Biochemistry) –AIIMS, Delhi and Ph.D. from AIIMS, Delhi (India).

She started Human Genomics works various funds having more than 18 grants awarded since her joining in BITS, 2004. She has ably conducted the day to day activities of research unit with several students. As a mentor she has been able to motivate younger faculty to submit and execute independent grants in DST, research associate and senior research fellows (ICMR & CSIR).

She has more than 80 articles in international peer journals. She is also an editorial board member and Reviewer for international journals. Her research interest is to identify biomarker in disease such as Alzheimer, diabetes, metabolism syndrome, obesity, cataract and depression. Her research group specifically study several genes which include APOE, CAPN, PPAR, TNF-α, ADM, OPRM1, OB, TPH, CRVGA, ICPRSS-1 and several microsatellite markers on chromosome segments of 2, 6 and 10.

She's also a life member of professional bodies in Society of Biological Chemistry (I), Association of Clinical Biochemists of India, Indian immunology Society, society of DNA fingerprinting, Indian academy of Neuro science, Indian Psychiarty society, Research society for Studies in Diabetes in India and also in international society like Research society for Alcoholism, International Society for Biomedical Research in Alcoholism, European Society for Biomedical Research in Alcoholism, society for Neuroscience, Asian Society for Biomedical Research in Alcoholism.