## 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.

## 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). 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.

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 (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) 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 also to design and develop of therapeutic molecules for PDR.

## 2. AIM AND OBJECTIVES:

This study focuses on the **role of APN in PDR which is poorly defined.** 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.

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**

## 3. METHODS AND RESULTS:

***In vitro* assay:** Two major microvascular Primary endothelial cells were isolated from human retina, choroid and were maintained in EGM 2 medium. mRNA expression studies by qPCR were done. Protein assays (Western blot, ELISA), immunofluorescence and immunohistochemistry assays were also performed.

Cell culture assays such as cytotoxicity assay, angiogenesis assay, oil red O staining, adipogenesis assay, and Mass spectroscopy was used.

***Clinical samples:*** Vitreous and blood samples were collected from the confirmed diagnosed cases of PDR and MH after getting consent from the patients. Blood samples were also collected from controls I (people without diabetes), control II (people with diabetes and without retinopathy). Routine biochemical assay were performed using a fully automated Dade Behring RxL Max. Vitreous and plasma adipokines level were measured by ELISA.

***In silico* assay:** Bioinformatics approach was employed to design siRNA targeting APN (Ambion web tool) and peptides of APN. Novel interacting partners of APN , Comon interacting partners of AAPN and PEDF were also done.

### 3.1. INTRAOCULAR EXPRESSION AND LOCALISATION OF APN:

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. The two major microvascular endothelial cells - human retinal endothelial cells (HREC), human choroidal endothelial cells (HCEC) were isolated from the donor eye ball. The presence APN mRNA expression and its receptors AdipoR1 and R2 were also observed in primary ocular cells [HRPE, HREC, HCEC].

Western blot analysis revealed the presence of protein expression of APN, AdipoR1 and AdipoR2 in ocular tissues. Increased APN protein expression was observed in vitreous of the patient with PDR. Results obtained from both immunohistochemistry and immunoflourescence method indicates that APN and its receptor AdipoR1, AdipoR2 are localised in the ocular tissue viz ciliary body, retina, choroid layers and in ERM.

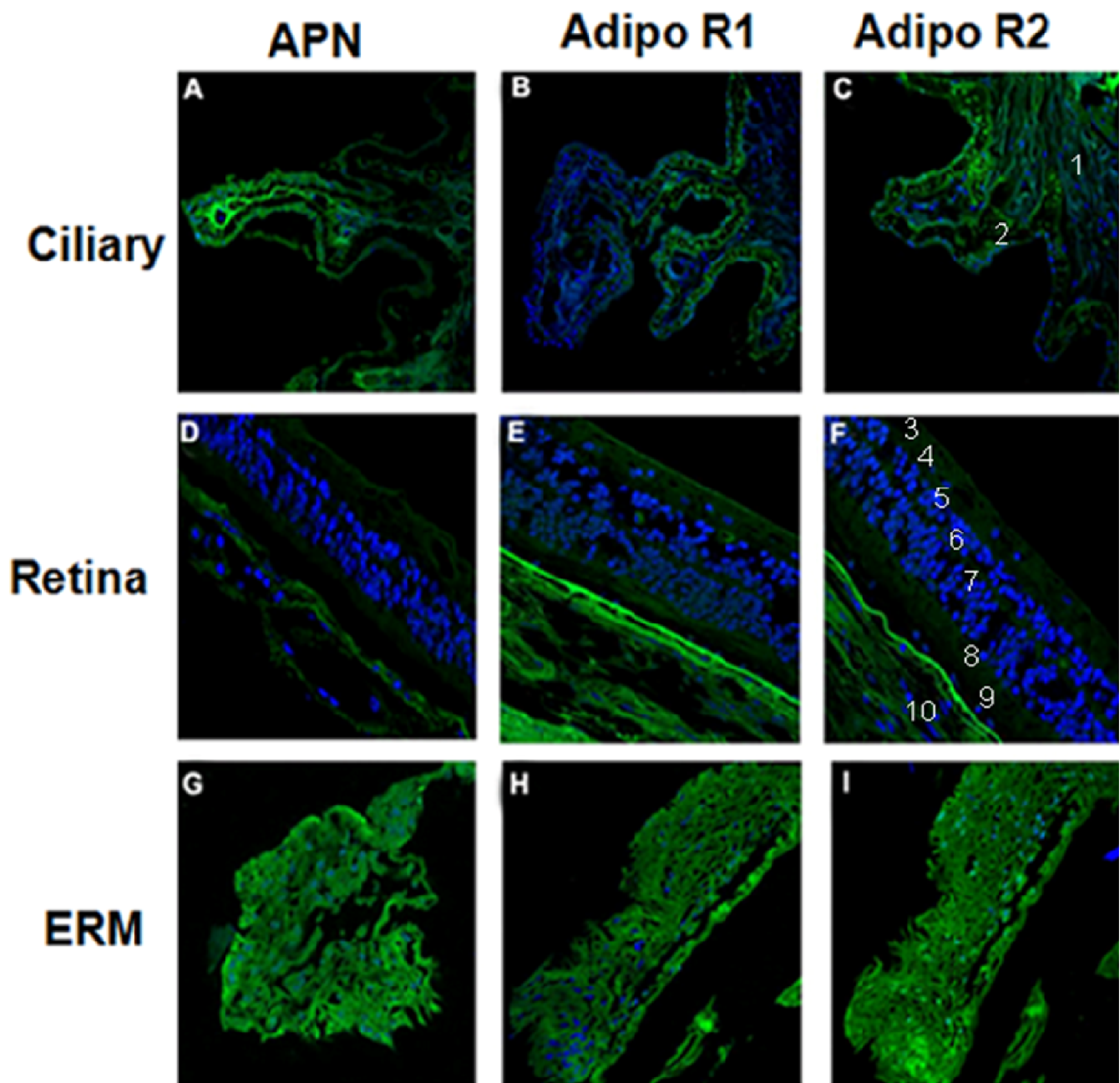


**Figure 1: APN and its receptors localisation by Immunoflourescence staining on 5μm paraffin block sections (n=5).** Confocal micrograph of APN, AdipoR1, AdipoR2 in ocular tissue and surgically removed ERM.

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.

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.

### 3.2. PLASMA AND VITREOUS ADIPOKINES IN PDR:

Plasma APN levels 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 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 and plasma IGF-1 levels didn't show any significance among the groups

Vitreous APN (Figure 2 A), VEGF (Figure 2 B), and IGF 1 (Figure 2 D) were significantly higher in PDR than in MH, whereas, PEDF (Figure 2 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. Vitreous APN levels positively correlated with the PEDF and IGF-1 but negatively correlate VEGF in the PDR and in the PDR patients who had undergone laser prior to vitrectomy.

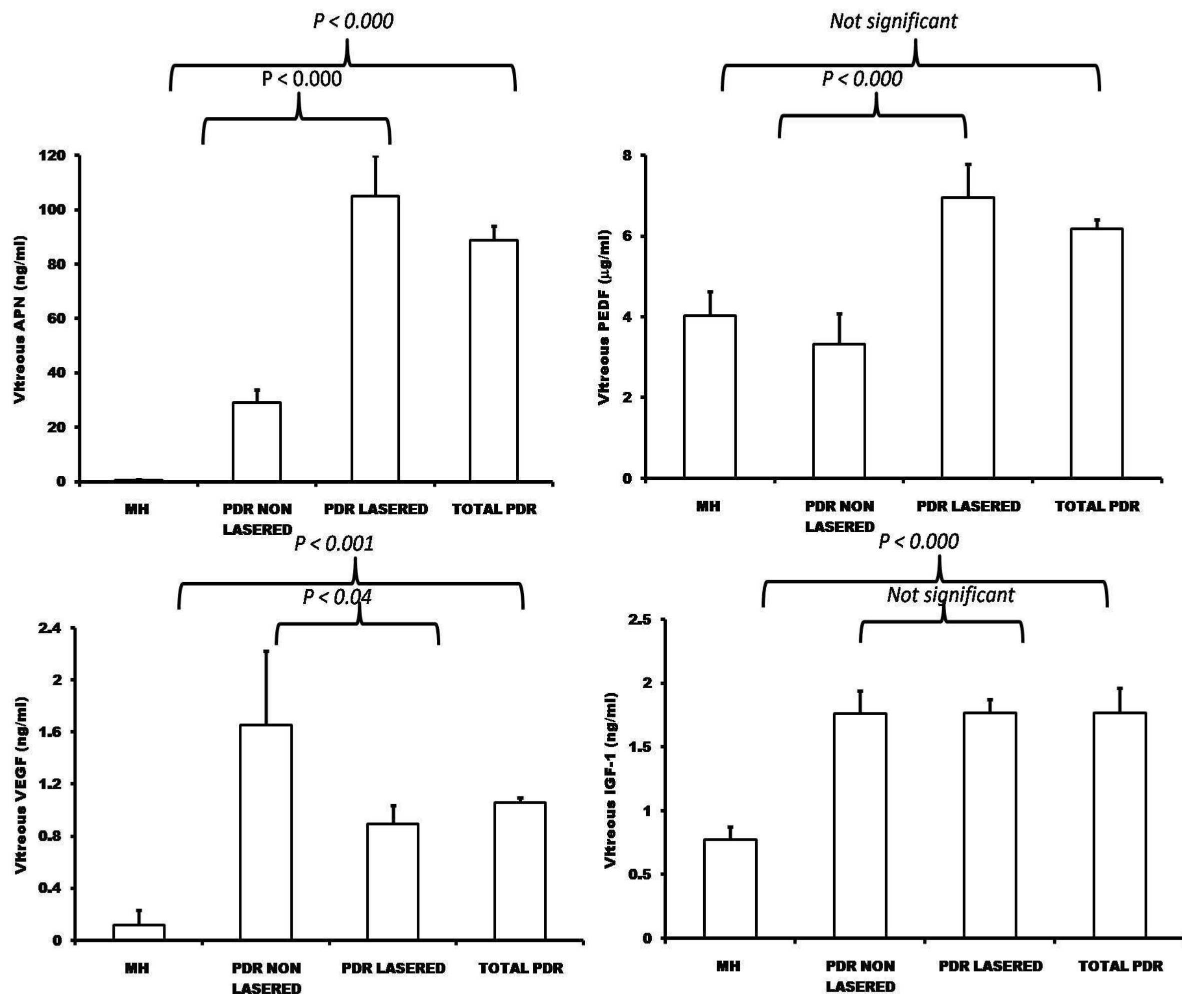


**Figure 2: Vitreous levels of adipokines (Mean ± SEM) in of patients with MH (n =23), patients with PDR (n = 56). Among PDR group, patients with PDR who had not undergone any laser treatment (n=12), patients with PDR who had undergone laser prior to vitrectomy (n=44).**

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.

### 3.3. PATHOPHYSIOLOGY OF PDR:

Elevated vitreous amino acid were also seen in PDR. A total of 12 amino acids were estimated by RPHPLC. 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.

Chronic exposure of amino acid (14 days) in the BRP(bovine retinal p 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 3).

After 14 days of culture, bovine retinal BRP showed a similar morphology of adipocytes with incorporated lipid droplets, increased adipogenecity, expressed adipocyte specific marker PPAR$\gamma$, 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.

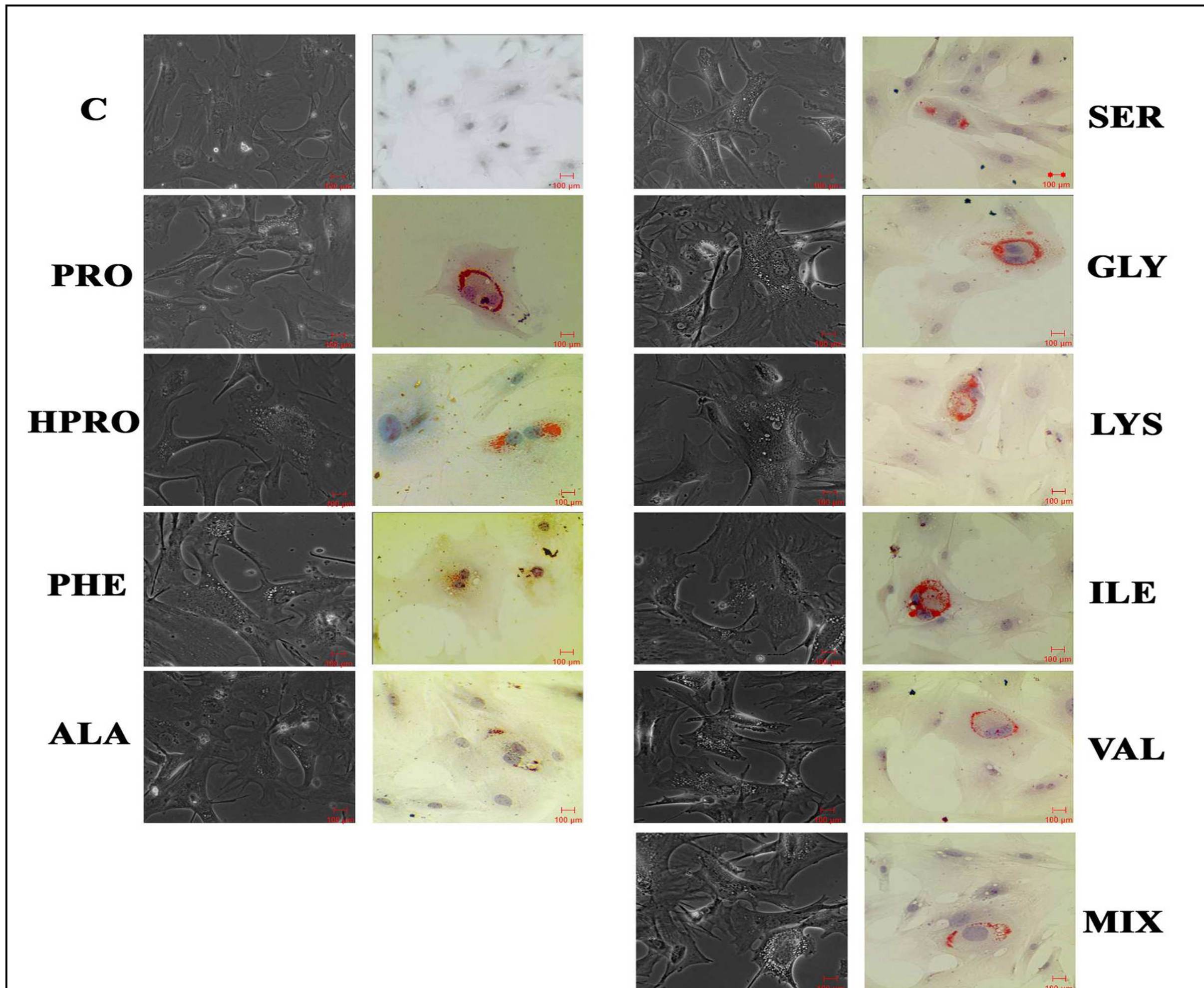


**Figure 3: Oil red O staining of BRP exposed to amino acids.**

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.

### 3.4. Role of APN in ocular angiogenesis:

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

**Figure 4: VEGF protein expression after rAPN treatment.**

b. Id[illegible] atus of APN. Hence, in this study, sequence and structure based approaches were implemented towards predicting of potential APN interacting partners that possibly mediate PDR. 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. It was interesting to observe that novel interacting partners of APN and PEDF identified 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 5).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.

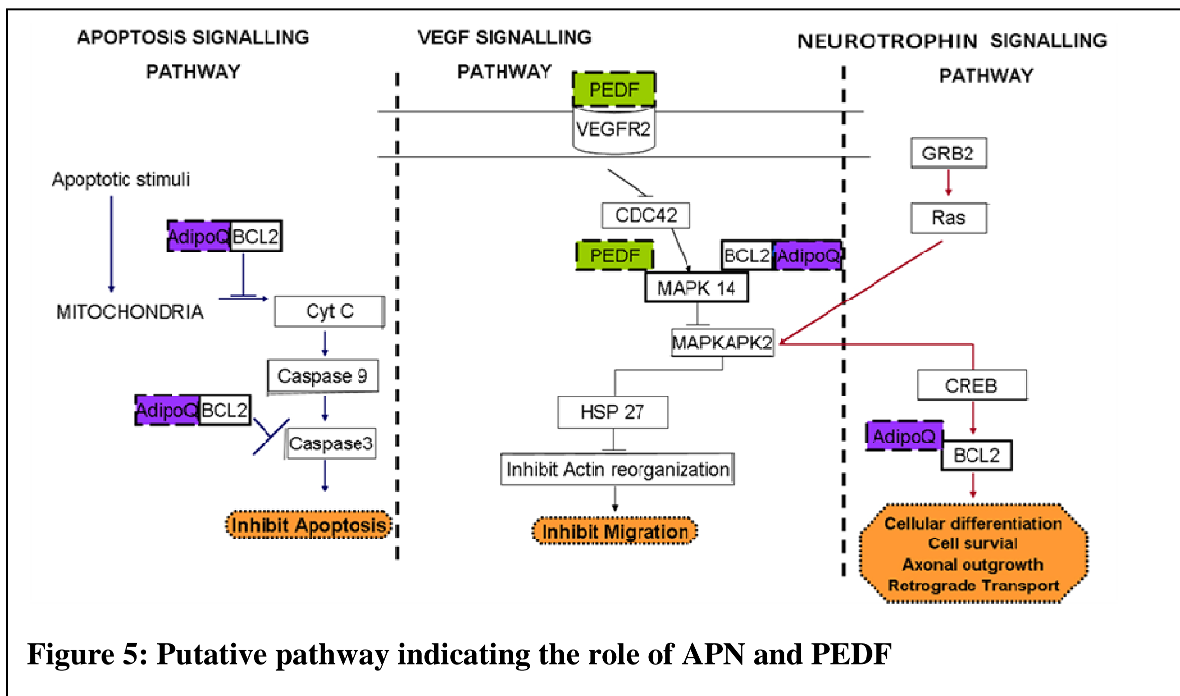


**Figure 5: Putative pathway indicating the role of APN and PEDF**

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

c. The ability of APN in modulating the secretion of VEGF and PEDF at a lower concentration was studied in cultured RPE. 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.

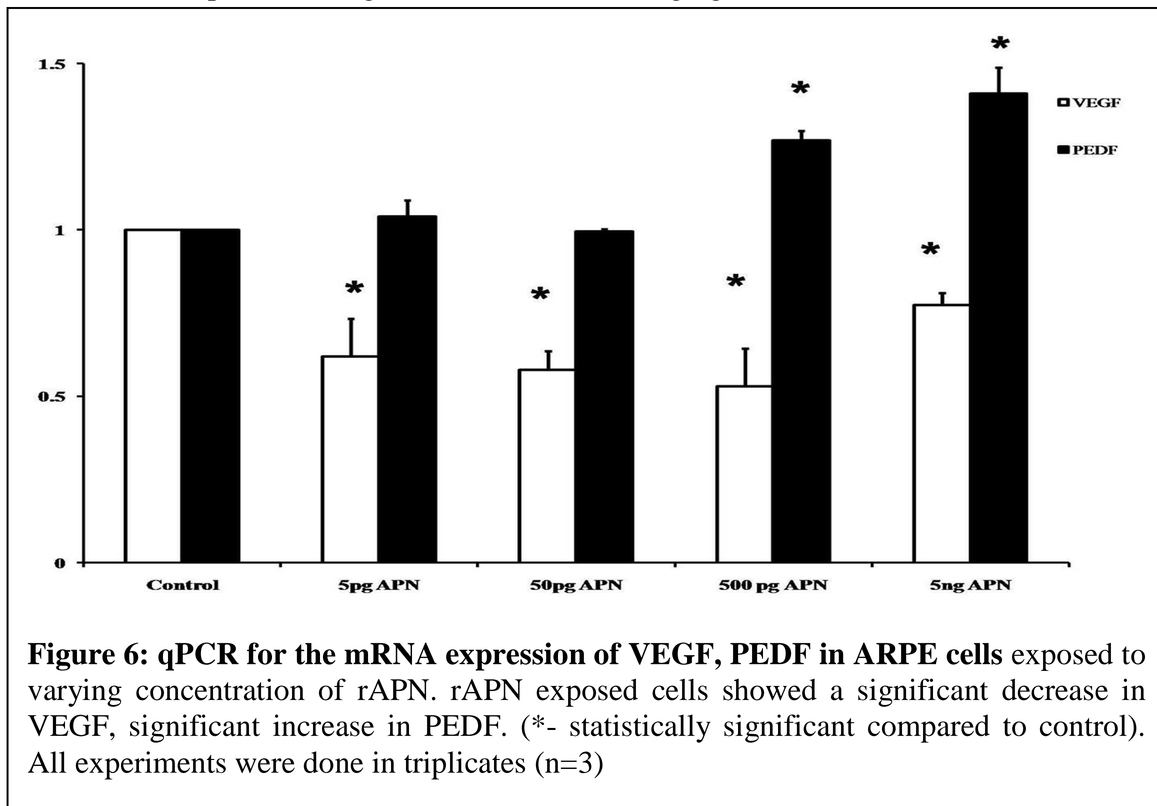


**Figure 6: qPCR for the mRNA expression of VEGF, PEDF in ARPE cells** exposed to varying concentration of rAPN. rAPN exposed cells showed a significant decrease in VEGF, significant increase in PEDF. (*- statistically significant compared to control). All experiments were done in triplicates (n=3)

## 3.5. THERAPEUTIC POTENTIAL OF APN:

### a. Peptides derived from APN having anti angiogenic property:

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**MAGI**P** (32-44)

Pep 2: KDKAMLFTYDQ**YQE** (178-191)

Pep 3: **DNDNDSTFTGFLLY** (226-40)

The designed peptides were meticulously screened in two endothelial systems and were found to be potent in inhibiting angiogenesis. Peptide treatment results were very much comparable with the effect of rAPN in inhibiting cell proliferation, migration and tube formation in HREC cells.

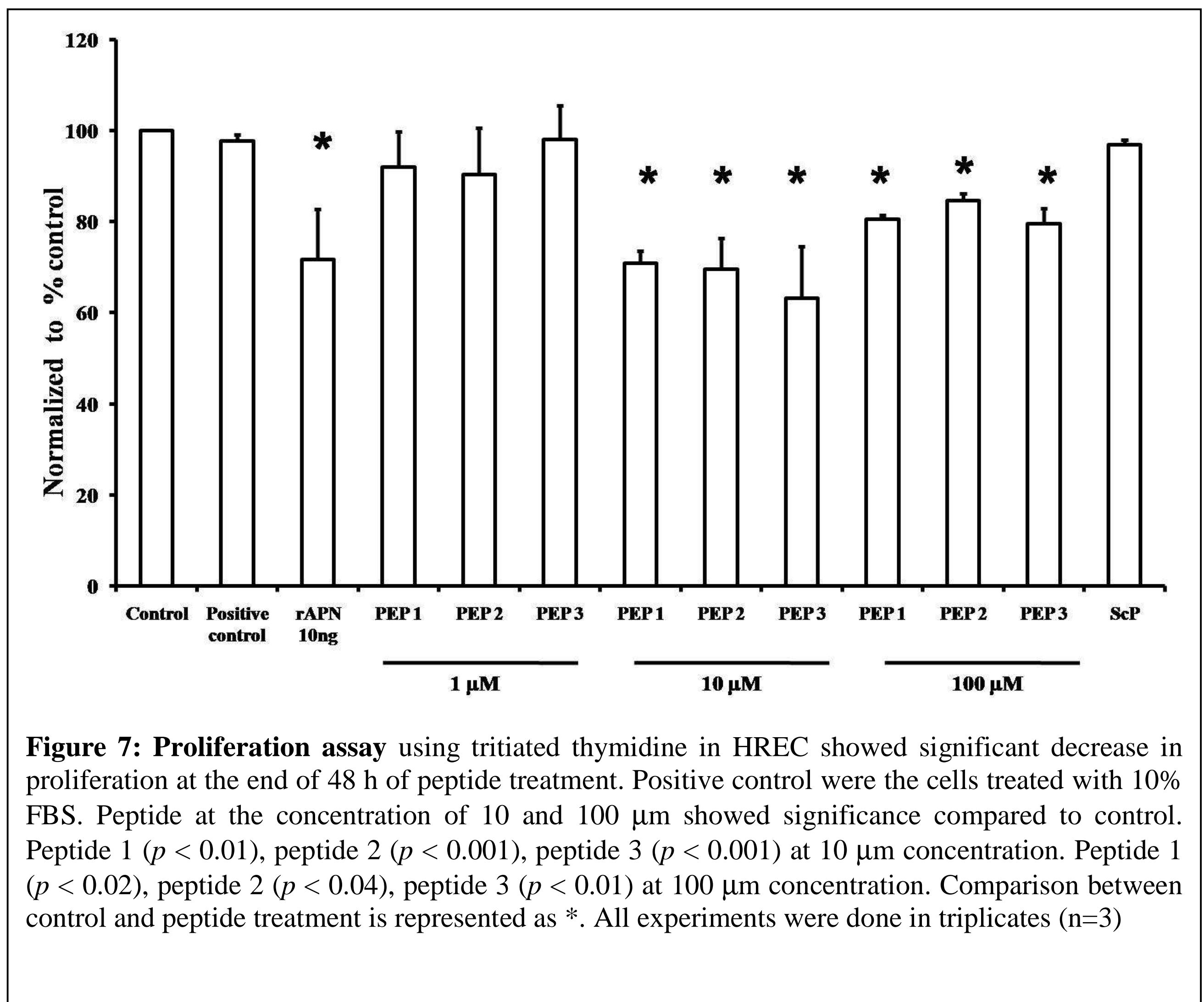


**Figure 7: Proliferation assay** using tritiated thymidine in HREC showed significant decrease in proliferation at the end of 48 h of peptide treatment. Positive control were the cells treated with 10% FBS. Peptide at the concentration of 10 and 100 µm showed significance compared to control. Peptide 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)

Peptide treatment decresed migration, inhibited tube formation, decresed cell adhesion and decresed VEGF levels in HUVEC cells.

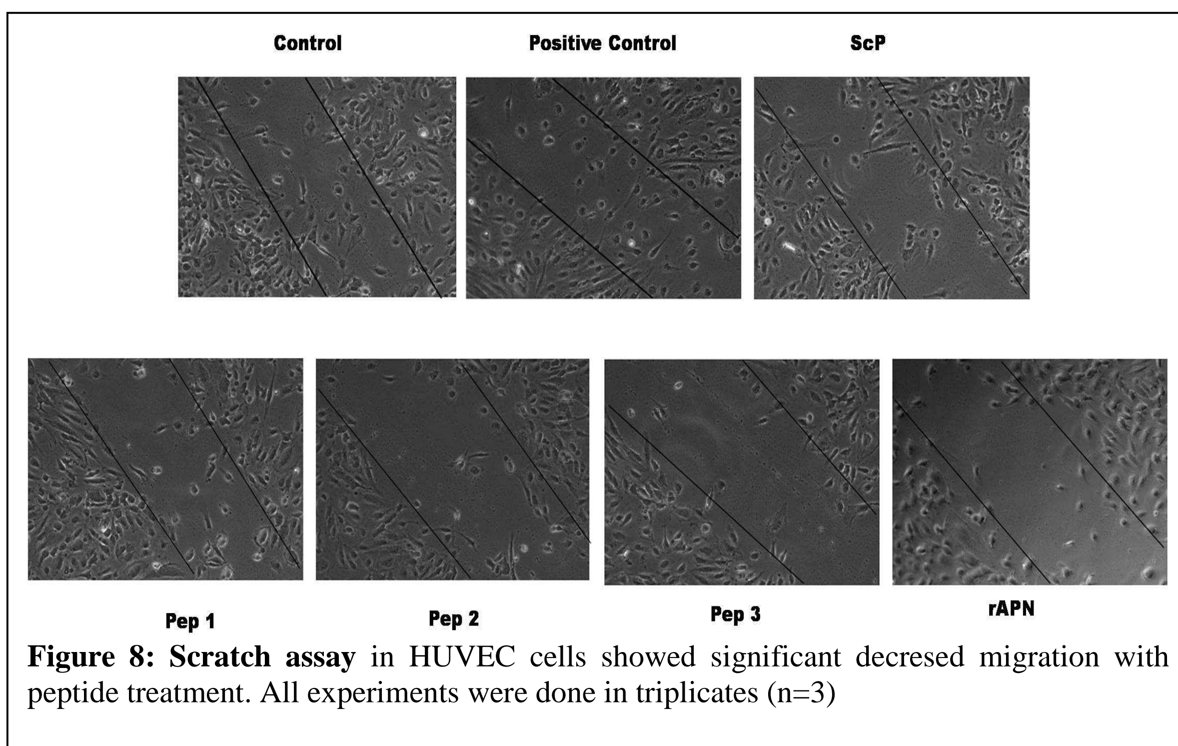


**Figure 8: Scratch assay** in HUVEC cells showed significant decresed migration with peptide treatment. All experiments were done in triplicates (n=3)

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.

**b. 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. 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’

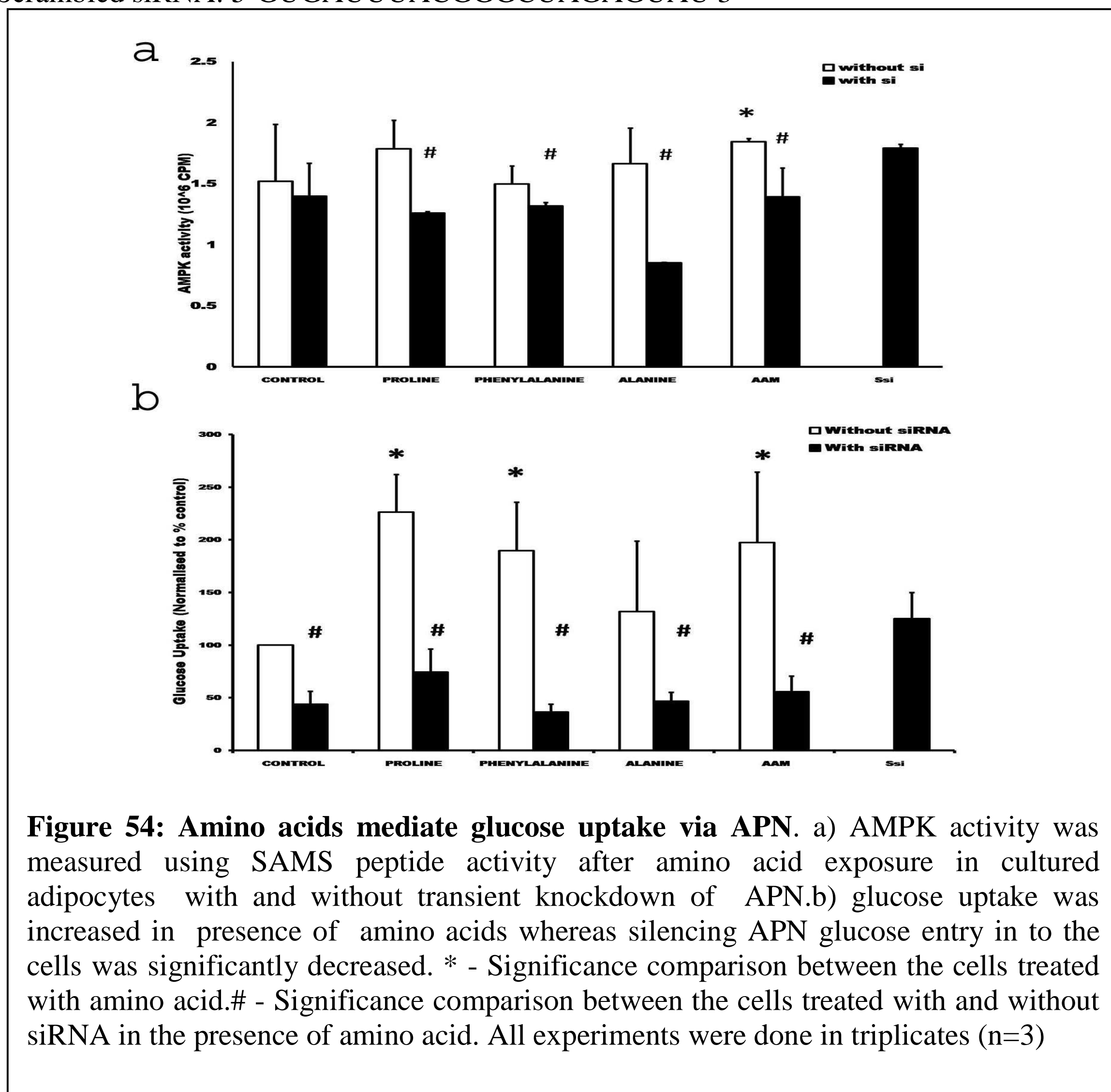


**Figure 54: Amino acids mediate glucose uptake via APN**. a) AMPK activity was measured using SAMS peptide activity after amino acid exposure in cultured adipocytes with and without transient knockdown of APN.b) glucose uptake was increased in presence of amino acids whereas silencing APN glucose entry 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.

## 4. DISCUSSION:

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.

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).

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).

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. *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).

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) 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). 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).

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.

## 5. CONCLUSION:

This is the first report on expression and localisation of APN and its receptors R1 and R2 in human ocular tissues and in isolated ocular cells. Elevated vitreous levels of APN protein in patients with PDR was observed when compared to MH. APN increased by 4 folds in the PDR patients who underwent Laser prior to the vitrectomy surgery. *In vitro* studies in pericytes treated with amino acids increased APN synthesis and decreased VEGF levels which could be beneficial in PDR. *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. 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. 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. Three different peptides were designed and these peptides decreased tube formation, proliferation, migration, adhesion and decreased VEGF levels in both microvascular and macrovascular endothelial cells (HREC & HUVEC) thus inhibiting angiogenesis. 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. 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.

## 6. SCOPE AND LIMITATION OF THE STUDY:

The scope of the current work will be to understand role of APN in PDR and to design and develop of therapeutic molecules for PDR. 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. This work will have translational importance in identifying new drug targets. Inspite of its high efficency of peptide, therapy faces major obstacles with the mode of delivery and stability. Detailed bioavailability, pharmacokinetics and pharmacodynamics are not part of this thesis. Similarly, the designed peptides were not tested *in vivo* models.

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