Studies on Vascular Endothelial Growth Factor, Matrix Metalloprotease-2 and Alpha 1-Antitrypsin in Diabetic Retinopathy

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Cell Biology and Molecular Genetics

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by

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I, **Kuksal Ashansha Mohan**, hereby declare that this thesis entitled **"Studies on Vascular Endothelial Growth Factor, Matrix Metalloprotease-2 and Alpha 1-Antitrypsin in Diabetic Retinopathy"** is a bonafide and genuine research work carried out by me in the Department of Cell Biology and Molecular Genetics Sri Devaraj Urs Academy of Higher Education and Research Tamaka, Kolar, under the supervision of **Dr. Mamatha Kunder.,** Assistant Professor, Department of Biochemistry, **Dr. Sharath B.,** Associate Professor, Department of Cell Biology and Molecular Genetics. No part of this thesis has formed the basis for the award of any degree or fellowship previously. Plagiarism check was carried out on the thesis and the report was found to be satisfactory.

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Abbreviations

AATD	α1-AT Deficiency
AGEs	Advanced Glycation End Products
BRB	Blood Retinal Barrier
$CoCl_2$	Cobalt Chloride
DR	Diabetic Retinopathy
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
HbA1c	Glycated Hemoglobin
HIF-1α	Hypoxia-Inducible Factor 1-alpha
HUVECs	Human Umbilical Vein Endothelial Cells
MMPs	Matrix metalloproteases
NPDR	Non-proliferative Diabetic Retinopathy
PDR	Proliferative Diabetic Retinopathy
PKC	Protein Kinase C pathway
RCL	Reactive Center Loop
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus

VEGF	Vascular Endothelial Growth Factor
α1-AT	Alpha 1-antitrypsin

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Abstract

Being one of the leading causes of vision impairment in the developing world, Diabetic Retinopathy (DR) continues to thrive at an exponential rate. Chronic hyperglycemia activates many pathways which result in retinal microvasculopathy, inflammation, and retinal neurodegeneration, all of which lead to the breakdown of the blood-retinal barrier in DR. This increases endothelial cell permeability causing vascular leakage, thickening of the vessel wall, and coagulation further resulting in hypoxia. Vascular endothelial growth factor (VEGF), released in response to hypoxia leads to the formation of new blood vessels ensuing neovascularization. This process is facilitated by the degradation of extracellular matrix (ECM) carried out by Matrix metalloprotease-2 (MMP-2), which allows migration of endothelial cells through degraded matrix. Regulation of MMP-2 activity is required to avoid uncontrolled proteolysis of ECM components. Alpha 1-antitrypsin (α1-AT) has been identified as a potential inhibitor of angiogenesis and also has been observed to inhibit MMP-2. This study intended to assess the levels of VEGF, MMP-2 and α1-AT in DR patients and evaluate their role in pathogenesis of DR.

The sample size calculated for the present study was 186 which were divided into DR, Type 2 Diabetes Mellitus (T2DM), and control with 62 participants in each group. The serum levels of VEGF, MMP-2, and α1-AT were estimated in study groups. An *in vitro* study was carried out using Human Umbilical Vein Endothelial Cells (HUVECs) under different concentrations of glucose and cobalt chloride to assess the effect of α1-AT on the levels of VEGF and MMP-2.

The serum levels of VEGF and MMP-2 were observed to be significantly elevated, contrary to α1-AT which were significantly lower in the DR group as compared to T2DM and control subjects. On further subgroup analysis, it was observed that VEGF and MMP-2 levels were significantly higher in the proliferative diabetic retinopathy group as compared to the non-proliferative diabetic retinopathy group, while, α1-AT was significantly lower. The relationship between the study molecules in DR and T2DM groups were assessed, where VEGF and MMP-2 were positively correlated with each other, and α1-AT was observed to negatively correlate with VEGF and MMP-2. Furthermore, on correlation analysis between the study parameters and HbA1c levels in DR patients, it was observed that VEGF and MMP-2 correlated positively, whereas, α1-AT was observed to correlate negatively with HbA1c levels. The findings of the *in vitro* study showed significantly higher levels of VEGF and MMP-2 in cells cultured under higher glucose concentration, and were observed to decrease significantly post α1-AT treatment.

In conclusion, there is an imbalance in the levels of VEGF, MMP-2, and α1-AT in DR. Also, the *in vitro* study findings demonstrated the inhibitory effect of α1-AT on VEGF and MMP-2 levels. Therefore, targeting the angiogenesis and ECM degradation by decreasing the levels of VEGF and MMP-2 using α1-AT could be exploited as a therapeutic approach toward ameliorating the damaging effect observed during the development and progression of the disease.

Chapter I

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia. Insufficiency in insulin secretion, resistance to the insulin produced, or both results in DM. Type 2 Diabetes Mellitus (T2DM) which usually affects adults is the most prevalent form of DM and occurs because of insulin resistance by the body. For the past three decades, the prevalence of T2DM has raised dramatically in countries with all income levels ranking it as one of the leading causes of morbidity and mortality (1).

Chronic hyperglycemia results in severe diabetic complications categorized into microvascular and macrovascular complications. Diabetic retinopathy (DR), Diabetic nephropathy, and Diabetic neuropathy are attributed to microvascular complications. Whereas, coronary artery disease, peripheral arterial disease, and stroke are classified under macrovascular complications of T2DM (2).

Being one of the major microvascular complications of T2DM, DR has attained the stature of the leading cause of visual loss in working-aged people. A meta-analysis that included data from 59 populations revealed the global prevalence for DM was 22.27%, with a total population of 6.17% affected by DR. In 2020, the number of individuals affected by DR was estimated to be 103.12 million worldwide and are expected to increase by 0.64 fold by 2045. Among the selected population, DR showed the highest prevalence in Africa (35.90%) followed by North American and the Caribbean (33.30%), South and Central America, and the lowest in the Asian population (3). Gadkari SS *et.al.,* reported the overall DR prevalence in the Indian population to be 21.7% (4).

DR is characterized by clinical manifestations of vascular abnormalities such as microaneurysms, hard exudates, hemorrhages, and neovascularization (5) (6). The signs and symptoms of DR onset are not evident until a funduscopy is carried out. Hence, DR causes substantial damage even before it is diagnosed. Based on the evident vascular changes, DR is further categorized into the early stage of non-proliferative diabetic retinopathy (NPDR) and the advanced stage of proliferative diabetic retinopathy (PDR). Progression of DR is observed ranging from mild to severe based on its clinical manifestation. The onset of DR is characterized by the appearance of microaneurysms, loss of retinal pericytes and breakdown of the blood-retinal barrier (BRB), and endothelium damage. This results in blurry vision and marks the initial stage of NPDR. PDR is characterized by increased severity of symptoms which involves proliferation of blood vessels resulting in complete vision loss and requires immediate medical intrusion (7). Diabetic Macular Edema, an important manifestation of DR is observed to occur in severity levels of both NPDR and PDR (8).

Chronic hyperglycemia activates many pathways such as the formation of advanced glycosylation end products and receptors, pro-inflammatory cytokines and chemokines, proliferator-activated receptor-γ disruption, growth factors, oxidative stress, and microRNA that contribute to the progression of DR (9). These pathways when activated result in retinal microvasculopathy, inflammation, and retinal neurodegeneration, all of which leads to the breakdown of the BRB. BRB disruption results in endothelium damage leading to the formation of acellular capillaries and edema in retinal vascular structure

(10). This increases endothelial cell permeability causing vascular leakage, thickening of the vessel wall, and coagulation further resulting in hypoxia. Hyperglycemia-induced hypoxia stimulates angiogenesis in DR by modulating a balance between pro-angiogenic and anti-angiogenic mediators (11). The hypoxic effects are mediated by hypoxia-inducible factor-1alpha (HIF-1α), an oxygen-sensitive transcription factor (12). HIF-1α is involved in the production of vascular endothelial growth factor (VEGF), which is responsible for retinal neovascularization; a classic hallmark of progressive DR (13) (14).

VEGF is a homodimer glycoprotein with a molecular weight of approx. 45kDa (15). Discovered in 1983, VEGF is an angiogenic factor and a potent mitogen for endothelial cells. It is also known as the vasopermeability factor due to its persuasive permeability-inducing properties (16). VEGF is known to be responsible for the proliferation of endothelial cells to create new blood vessels i.e., angiogenesis (17). VEGF activity is initiated by its binding to the receptors for vascular endothelial growth factor (VEGFRs). Predominantly present on vascular endothelial cells, these receptors present three domains: an extracellular domain for VEGF binding, a transmembrane domain, and an intracellular domain with tyrosine kinase activity (18) (19) (20). The binding of VEGF to the extracellular receptor domain promotes the activation of tyrosine kinase enzyme in the intracellular receptor domain. This phosphorylates the tyrosine residues, thus activating several intracellular signaling pathways (18). Extracellular matrix (ECM) degradation has been implicated in pathological angiogenesis during the development and progression of DR (21). VEGF induces the activity of matrix

metalloproteases (MMPs); a zinc-dependent endopeptidase, which facilitates ECM degradation and remodeling (22) (23).

MMPs are a family of 25 protease enzyme which takes part in the degradation of ECM components and regulate many normal and pathological processes. They are involved in tissue remodeling, inflammation, and injury (24). MMPs are considered to possess a dual role in the development of DR: they accelerate apoptosis of retinal capillary cells in early-stage (pre-neovascularization) and assist in the formation of new blood vessels by degrading ECM components in later stages (25). Several experiments have shown elevated levels of MMPs in the retina and vitreous of patients with DR (26) (27) (28). Augmented levels of MMPs result in increased vascular permeability as it carries out proteolytic degradation of tight junction protein occludin and disrupts the overall tight junction complex (29) (30). Out of all the MMPs that have been studied, MMP-2 and MMP-9 have been known to accelerate retinal cell apoptosis (25). Also, MMP-2 has been observed to degrade collagen; one of the largest components of ECM further aiding angiogenesis (23) (26). Thus, proper regulation of MMPs activity is important to hinder the progression of DR. Physiologically, MMPs are regulated by a group of endogenous inhibitors like tissue inhibitors of metalloproteases-1, 2 (TIMPs), α2-macroglobulin, and Alpha 1-antitrypsin (α1-AT) (31) (32).

Alpha-1 antitrypsin is a 52kDa serine protease inhibitor belonging to the SERPINA family and is formed in hepatocytes (33). Existing evidence suggests that α1-AT not only possesses the ability to inhibit proteases but also possesses

anti-inflammatory, anti-apoptotic, and anti-angiogenic properties (34) (35). α1-AT has been linked to the inflammation observed in the pathogenesis of DM. Moreover, it is observed that α1-AT protects pancreatic beta cells against apoptosis (36). Studies have demonstrated the low levels of α1-AT in patients with Type 1 diabetes mellitus (T1DM) and T2DM (37). Multiple clinical trials are currently in progress to assess the safety and effectiveness of α1-AT in patients with T1DM (38) (39). These evidences suggest the protective role of α1-AT against the protection and development of DM. As discussed earlier, ECM degradation is one of the major events that take place in DR development which aids neovascularization (40). Thus, adequate levels of α1-AT are an important requirement for the downregulation of VEGF and MMPs and the subsequent reduction in ECM proteolysis impeding neovascularization.

Chapter II

Rationale

VEGF is an endothelial cell-specific mitogen, responsible for angiogenesis and increased vascular permeability observed during the development and progression of DR. Angiogenesis marks a classic hallmark for the onset of DR development and is mediated by ECM degradation. Endothelial cells migrate through the degraded matrix and form new blood vessels. The proteolysis of ECM is brought about by MMPs. MMP-2 is responsible for the degradation of collagen, one of the major components of ECM. The uncontrolled proteolytic activity of MMPs causes increased neovascularization leading to the DR progression. Therefore, the proteolytic activity of MMPs must be regulated to downregulate this process. The MMP-2 activity is regulated by α1-AT. α1-AT is an anti-proteolytic, anti-inflammatory, and anti-angiogenic molecule which has been known to involve in the angiogenesis process.

From the above explanation, it is evident that VEGF, MMP-2, and α1-AT play an important role in the DR pathogenesis. Studies have been conducted to investigate the levels of these molecules in DR patients. These are however isolated studies. Therefore, this study was intended to investigate the relationship between all three molecules in retinopathy patients. Also, the effect of α1-AT on VEGF and MMP-2 levels in *in vitro* study was carried out to corroborate its protective role against DR development and progression.

Chapter III

Aim and Objectives

Aim: To investigate the relationship between VEGF, MMP-2, and α1-AT in the development and progression of DR.

Objectives:

- To determine the serum levels of VEGF, MMP-2, and α1-AT in study groups.
- To determine the association between VEGF, MMP-2, and α1-AT in study groups.
- To study the effect of α1-AT on VEGF and MMP-2 *in vitro,* in HUVECs cultured under different concentrations of glucose and Cobalt Chloride ($CoCl_2$).

Chapter IV

Review of Literature

Diabetic retinopathy (DR) is one of the leading causes of vision impairment in the developing world and continues to thrive rapidly. Progression of DR is strongly navigated by prolonged hyperglycemia due to the poor glycemic control in diabetic patients (41).

Many risk factors influence the chances of a person being affected by DR.

1. **Long duration of hyperglycemia**

One of the strongest risk factors responsible for the progression of DR is the duration of diabetes (42). According to the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), there is 8% chance for a patient to develop any form of retinopathy in the first 3 years of DM diagnosis. The chance of being affected by DR may gradually rise to 25%, 60%, and 80% at a duration of 5, 10, and 15 years of diabetes respectively (43). However, irrespective of the diabetic control more than 60% of patients affected by DM may develop DR after 20 years of diabetes (44). A study conducted by Raman and co-workers reported a significant association of diabetes duration with DR development in the South Indian population with the prevalence of DR being 37.1% in patients with more than 15 years of diabetes (45). A Nationwide study reported an increase in the prevalence of DR with an increase in the duration of diabetes. As per this survey, 9.23% of subjects with a diabetic duration of fewer than 6 months showed DR development. A significant increase in the DR prevalence was observed with an increase in the duration of diabetes i.e., subjects with the duration period of 6 months -5 years and more than 5 years was 15.12% and 35.12 respectively (4).

2. Poor glycemic regulation and control

Poor glycemic control strengthens the risk of causing DR. Regulated blood sugar levels have been known to reduce the risk of DR (46). According to a survey conducted by Action to Control Cardiovascular Risk in Diabetes (ACCORD), there is an increase in the development of DR by 3.1% in patients with standard glycemic control treatment as compared to those with intensive glycemic control treatment at 4 years of DM (47). Another study reported that with a decrease in the levels of glycated haemoglobin by 10% a decrease in the development of DR by 42% was observed. On the contrary, an increase in the glycated haemoglobin escalated the development of DR by 64% whereas every 10% increase in HbA1c enhanced the progression of retinopathy development to 64% (48).

3. Hypertension

One of the factors that enhances the risk of developing DR is high blood pressure (49). According to the United Kingdom Prospective Diabetes Study (UKPDS) survey conducted in 2002, an association between systolic blood pressure and patients with DR was observed (50). Patients with systolic blood pressure ≥ 140mmHg were likely to be affected 2.8 times more as compared to patients with blood pressure <125mmHg (51). Another meta-analysis conducted by Wat *et.al*. showed a positive correlation between systemic risk factors that included high blood pressure and the development of DR (52).

4. Dyslipidaemia

The formation of hard exudates is one of the classic hallmarks observed during the development of DR. This is caused due to elevated levels of lipids which dysfunctions the endothelial cells and reduces the bioavailability of nitric oxide (53). In a study conducted by Early Treatment of Diabetic Retinopathy Study (ETDRS), patients with elevated levels of serum total cholesterol or elevated serum low-density lipoprotein cholesterol were more likely at the risk of developing retinal hard exudate (54). According to the United Kingdom Prospective Diabetes Study (UKPDS) severity of the retinopathy was associated with higher levels of high-density lipoprotein cholesterol (55). In a report by Diabetes Control and Complications Trial study conducted in 2004, a significant association was observed between elevated serum lipid levels and complications of DR such as hard exudates (56). A systematic review of the meta-analysis showed that the lipid-lowering agents lower the risk of DR (57).

5. Pregnancy

Pregnancy poses a major risk factor in the development of DR and is associated with increased prevalence and severity of retinopathy as compared to pregnant women without diabetes. Women with T1DM are more likely to be at risk of DR (58). The percentage of DR development was found to be higher in pre-gestational diabetic pregnant women (59). A study conducted on the North Indian population of pregnant women revealed deterioration in the proliferative stage of disease with the fibrovascular proliferation during pregnancy (60).

Classification of DR:

Based on the clinical manifestations, DR has been categorized into different categories over the years and has been modified with the improvement in the technologies and more evident characteristics of the disease. The first known classification of DR was carried out in 1968 by the Airlie House Symposium, where the effects of tight metabolic control, pituitary ablation, and photocoagulation were discussed in detail (61). However, the Airlie classification did not cover all the aspects of the disease progression and hence Diabetic retinopathy study classification was generated from the modification of the Airlie classification. This classification was based on the standard photograph of the fundus of different regions of the patient's retina. This was further redefined by the ETDRS. This classification method was based on a variety of diseases, ranging from no retinopathy to severe grades of retinopathy including vitreous haemorrhage and retinal detachment (62). Although this classification was considered the gold standard, at the same time it was difficult to follow due to its intricate categorization of the disease based on fundus photography. To overcome this difficulty, International Clinical Disease Severity Scale for DR was created in 2002. According to this classification, DR was categorized into five categories based on the clinical manifestation of the disease (63). This categorization includes no retinopathy followed by a broad classification of an early stage of NPDR and advanced stage of PDR (64).

Non-proliferative diabetic retinopathy (NPDR)

This is the early stage of DR. Chronic hyperglycemia damages retinal capillaries and gives rise to small bulges which are known as microaneurysms. These microaneurysms with the severity of the disease burst and form dot-blot haemorrhages also known as intra-retinal haemorrhages due to their location in the deep retina. The gradual progression of NPDR leads to the obstruction of the affected vessels and infarction of the retinal nerve fiber layer that results in the development of cotton wool spots in the retina.

NPDR is further categorized into:

i. No retinopathy: No clinical signs of DR progression is observed.

ii. Early or Mild NPDR: With the presence of not less than one microaneurysms, patients with NPDR have a low risk of progressing to PDR.

iii. Moderate NPDR: these patients are categorized based on the presence of multiple microaneurysms, intraretinal haemorrhages, or venous bleeding.

iv. Severe NPDR: This is the advanced stage of DR in which a patient's fundus examination shows all the clinical manifestations of the disease. These include cotton wool spots, venous bleeding, and severe intraretinal microvascular abnormalities (65).

Within 5 years of diagnosis, individuals with moderate NPDR had a 17.6% chance of advancing to severe NPDR or PDR, compared to 5.8% for patients with mild NPDR (66).

Proliferative diabetic retinopathy (PDR)

PDR is the advanced stage of DR and is characterized by the formation and proliferation of new blood vessels in the retina. The formation of new blood vessels is in response to hypoxia-induced vaso proliferative molecules like VEGF. These newly formed vessels are fragile and are leaky which results in haemorrhages (67) (68). They start growing into the vitreous and make fibrovascular proliferations which consist of glial cell types. The retinal traction takes place when these fibrovascular proliferations pull down the retina into the vitreous and cause retinal detachment ultimately resulting in vision loss (69).

Molecular mechanisms in Diabetic retinopathy

Many molecular mechanisms have been put forth that are observed to have been involved in the development and progression of DR. The most studied mechanisms in the progression of DR are polyol pathway, formation of advanced glycation end products (AGEs), increase in the hexosamine pathway flux, activation of protein kinase C pathway (PKC), increased oxidative stress, and inflammation (70).

1. **The polyol pathway**

The polyol pathway follows a two-step mechanism where the excess glucose is reduced to sorbitol by an enzyme present in the retina called aldose reductase. The resulting sorbitol is later converted to fructose. Furthermore, phosphorylation of fructose formed results in the formation of glycating agents like fructose-3-phosphate and 3-deoxyglucosone. This has been observed as a significant contributor towards the progression of DR (71) (72). A study

conducted by Dagher Z *et.al.,* found sorbitol to be increased in the cell culture setup of human retinas which were exposed to high glucose (73). Accumulation of sorbitol has been observed to cause damage to the retinal cells, which includes osmotic damage (72).

2. **Formation of advanced glycation end products (AGEs)**

Accumulation of AGEs has been known to involve in the progression of DR. Some of the most common AGEs are carboxyethyl lysine, carboxymethyl lysine (CML), and pentosidine. CML has been observed to localize in the retinal tissue of diabetic patients and is shown to correlate with the severity of DR (74) (75) (76). Choudhuri S *et.al.,* suggested CML as the key modulator of NPDR amongst patients with poorly regulated T2DM (77). AGEs localized in the retinal blood vessels have shown an association with an increase in the severity of DR (78). AGEs build up in retinal pericytes as a result of diabetes, affecting pericyte survival and function, eventually leading to pericyte loss (79). The binding of AGEs to its receptors in pericytes activates intracellular signaling which leads to the dysfunction of endothelial cells, breakdown of BRB, vascular inflammation, activation of cytokines and growth factors, and angiogenesis (80).

3. **Increased glucose flux through the hexosamine pathway**

Increased flux of glucose through the hexosamine pathway has been observed to involve in insulin resistance, diabetic vascular complications, and activation of growth factors (81). Inside a cell, glucose is metabolized via glycolysis where glucose-6-phosphate (G6P) is converted to fructose-6-phosphate. Nevertheless, at times during the glycolysis process conversion of G6P is diverted to form

glucosamine-6 phosphate. This process is catalyzed by an enzyme called glutamine fructose-6 phosphate amidotransferase (GFAT) (82). The end product obtained is Uridine diphosphate-NAcetylglucosamine (UDP-GlcNAc) through the action of UDP-NAcetylglucosamine synthase, an important metabolic compound for glycosylation of proteins and lipids (83).

Under normal conditions, the activity of GFAT is low, but under hyperglycemic conditions, the GFAT activity is upregulated (84) (85). The upregulated activity of GFAT results in the increased activity of O-Glucosamine-*N*-Acetyl transferase. Hyperactivity of this enzyme in the hexosamine pathway has been linked to changes in gene expression as well as increased expression of transcription factors like TGF- and TGF-ß, which inhibits mesangial cell mitogenesis while activating collagen matrix proliferation and basement membrane thickening (86).

4. Activation of protein kinase C (PKC) pathway

Microvascular changes observed in the DR are believed to be caused due to the activation of PKC which is activated due to chronic hyperglycemia. Several isoforms of PKC are identified to date which are involved in the regulation of other proteins involved in DR. PKC isoforms are divided into classical, novel, and atypical. Few of the identified isomers are activated upon its binding to diacylglycerol (DAG). The DAG-PKC pathway has been observed to involve in the vascular complications of DR (87). DAG levels are found to be upregulated in the vascular retinal tissues in diabetic conditions (88). PKC is a crucial molecule in the control of a variety of physiological functions. As a result, the

activation of this enzyme has a cascade-like impact on multiple other pathways, affecting endothelial permeability, retinal hemodynamics, and VEGF expression in the retinal tissue, as well as recruitment of leukocytes which results in the release of inflammatory molecules such as free oxygen radicals and cytokines (89) (90) (91).

5. Oxidative stress and inflammation in the diabetic retina

With a high amount of polyunsaturated fatty acids, the retinal tissues have the highest oxygen uptake as compared to any other tissue. This makes the retina more vulnerable to oxidative stress. A correlation between hyperglycemia, changes in the redox homeostasis, and oxidative stress have been observed as a crucial event in the progression of DR. Animal studies have shown that oxidative stress has a role in the development of DR as well as the resistance of retinopathy to reverse after resuming excellent glycemic control, a process known as metabolic memory (92). Intracellular reactive oxygen species (ROS) are produced by high glucose levels, either directly through glucose metabolism and auto-oxidation or indirectly through the formation of AGEs and their receptor binding (93). It is observed that the harmful effects of high glucose in the retina may be attributed in part to their capacity to promote the production of ROS and lipid peroxides, resulting in increased oxidative stress and retinal mitochondrial malfunction (94).

The disproportion between formation and/or removal of ROS represents oxidative stress in a cell. The antioxidant defense system of the cell is a significant aspect of the total oxidative stress experienced by a cell. Antioxidant

defense enzymes such as SOD, glutathione reductase, glutathione peroxidase, and catalase which are responsible for detoxifying free radicals are observed to be decreased in the diabetic condition (95). In addition to this, the cell has an internal antioxidant called reduced glutathione (GSH), which is perhaps the most essential protection the cell has. It can scavenge ROS and regulate the intracellular redox state (96) However, this along with other non-enzymic antioxidants namely vitamin C, vitamin E, and β-carotene responsible for maintaining homeostasis are also decreased during oxidative stress induced by hyperglycemia (97) (98). Several evidences point to the increased oxidative stress as a major contributor to diabetic retinal inflammation (99).

DR is associated with a significant increase in the levels of pro-inflammatory cytokines, chemokines, and adhesion molecules in serum, vitreous, and retinas of humans and experimental animals (99). Chronic hyperglycemia-induced cytokines recruit leukocytes at the tissue damage site and form new vasculature by aiding the release of pro-angiogenic factors (100). TNF-α is released by hypoxia-activated macrophages and microglia, the retina's immune cells, which induces the release of IL-8, Monocyte chemoattractant protein-1, and VEGF in vascular cells and retinal microglial cells (101). The accumulation of inflammatory molecules may contribute to neuronal cell death and this has been established with the help of ischemic mouse models (102) (103) (104).

Vascular endothelial growth factor (VEGF)

History of VEGF molecule

Before the discovery and identification of the VEGF molecule in 1983 by Senger, Dvorak, and colleagues, Issac Michaelson proposed the development of pathological angiogenesis and release of an angiogenic factor called "factor X" in 1948 (105). This was followed by the discovery of anti-angiogenic factors by Judah Folkman (106). Nearly forty years later, Senger and co-workers discovered the factor involved in the angiogenesis which was secreted from guinea pig cell lines. It possessed the capability of causing vascular permeability and was initially named vascular permeable factor (VPF) (107). Another molecule was identified by Napoleone Ferrara *et.al.,* that assisted in the process of angiogenesis and was named vascular endothelial growth factor (108). Later, VPF was cloned by Daniel Connelly *et.al.,* where it was discovered that the cloned VPF and molecule identified by Napoleone Ferrara *et.al.,* were the same protein (109). Several experimental studies over time have supported and demonstrated the elevated levels of VEGF in diabetic patients with active retinopathies (110) (111).

Structure of VEGF

VEGF is produced in response to the hypoxic condition which is caused as a result of endothelial damage (10) (13) (14). HIF-1 is a DNA binding protein that is produced in response to tissue hypoxia. HIF-1 is a heterodimeric transcription factor that contains two subunits: constitutively active β subunit and growth factor regulated α subunit (112). HIF-1 activates VEGF transcription by binding

with it. This results in the accumulation of VEGF by upregulation of VEGF mRNA transcription and decreased mRNA degradation (113).

VEGF is a 46kDa glycoprotein secreted by endothelial cells, retinal epithelial cells, pericytes, muller cells, and astrocytes. VEGF is a family of growth factors that has several members including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. VEGF-A is acquired from mRNA splicing of the 8-exon VEGF gene (114). The crystallized form of VEGF represents 8-109 residues out of which 14–106 are characterized in all eight monomers. When present, Glu13 and Asp109 lack density for the side chain and are treated as alanine (115) (Figure 1). Depending upon the number of amino acids present VEGF-A has five different isoforms: 121, 145, 165, 189, and 206. 189 and 206 are the larger isoforms and possess the increased heparin-binding capability which aids them in binding to the cell surface and basement membranes. Whereas, smaller isoforms have limited capability of binding to the basement membrane and are readily diffusible. VEGF 165 is the principal isoform with greater diffusibility and is critical for both physiological and pathological angiogenesis (67).

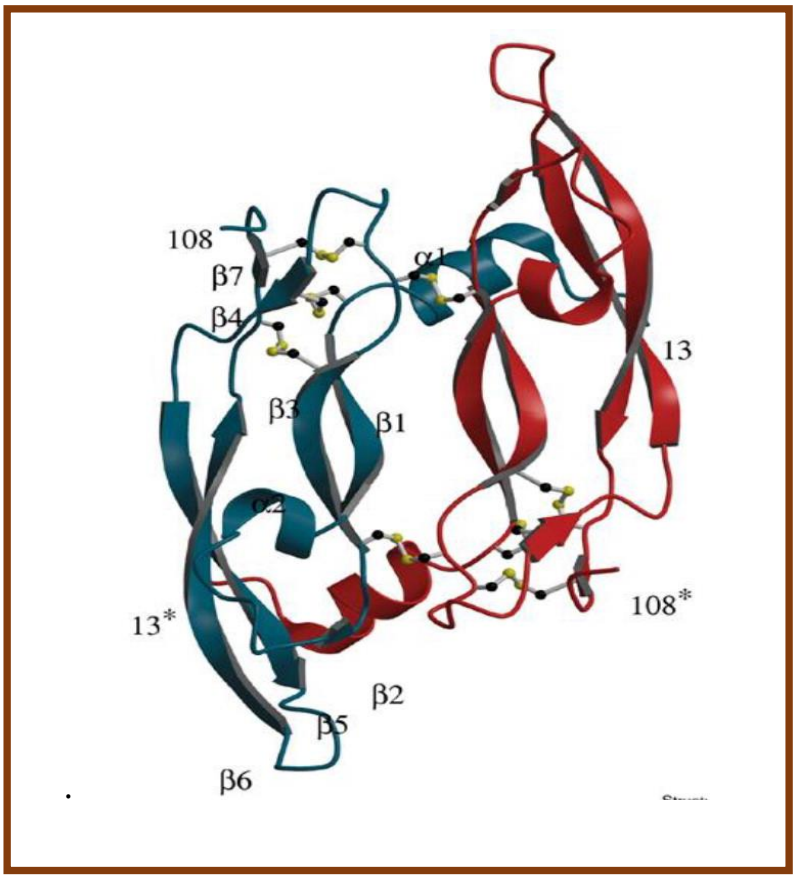

Figure 1. Crystallized structure of VEGF representing monomers with residues
The Sulphur atoms are displayed in yellow while disulphide bonds are represented in white (image source: Muller YA *et.al.*, 1999) (115)

The cellular activities of VEGF are mediated by binding to tyrosine kinase receptors known as VEGFRs. There are 3 types of receptors VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-1 and VEGFR-2 are located primarily on vascular endothelial cells. Although, in some cases, they can also be observed on non-endothelial cells. VEGFR-3 is specifically expressed on endothelial lymphatic cells. Neuropilins are the proteins that increase VEGFR-2 and VEGFR-3 functions and guide endothelial cells toward migration in angiogenesis (20) (116) (117).

These tyrosine kinase receptors have three domains: the extracellular domain, the transmembrane domain, and the intracellular domain. The extracellular domain is for VEGF binding, whereas the intracellular domain carries out the tyrosine kinase activity (118). VEGF binds to the extracellular domain of the receptors and promotes the activation of tyrosine kinase enzymes in the intracellular domain. This in turn phosphorylates tyrosine residues, thereby activating several intracellular signals (Figure 2). VEGFR-2 is phosphorylated in response to VEGF production, which activates downstream ERK1/2 and AKT to increase cell proliferation, motility, and angiogenesis (119).

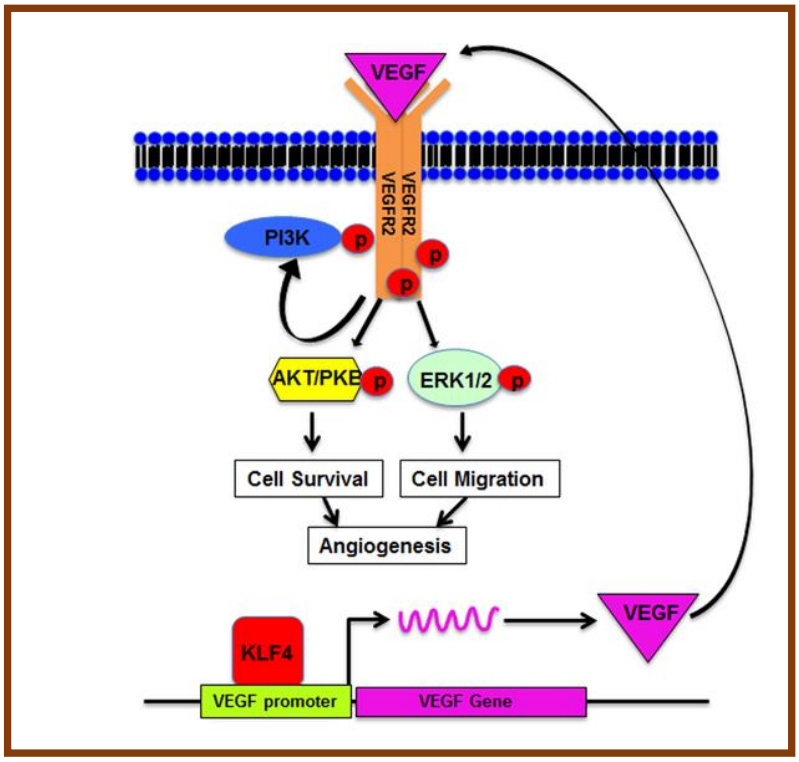

Figure 2. VEGF signaling pathway in retinal microvascular endothelial cells (image source: Melincovici CS *et.al.*, 2018) (119)

Pleiotropic roles of VEGF:

Apart from angiogenesis, VEGF has been observed to play part in many other physiological processes. Post discovery, VEGF was found to regulate hematopoiesis and the behaviour of the white blood cells. VEGF-A is expressed on endothelial cells and promotes homeostasis in adults. This process was observed to maintain across all species including Drosophila (120) (121). Curiosity to learn more about the functions of VEGF apart from its role in angiogenesis has led to the discovery of its paracrine functions on the vasculature. VEGF contains an N-terminal sequence of amino acids that assists in the formation of mature proteins which are linked to each other covalently (122). Apart from its paracrine and autocrine roles, VEGF secreted within a cell i.e., intracellular signaling is emerging as an important aspect in the regulation of cell growth, metabolism, and survival (123). VEGF regulates neuronal migration in the nervous system and has been observed to have neuroprotective and neurotropic effects *in vitro* and *in-vivo* (124).

VEGF in various disease conditions:

According to a meta-analysis conducted by Kut C *et.al.,* normal levels of serum VEGF were observed to be 17–287 pg/ml (125). Angiogenesis mediated by VEGF-A has been implicated in the pathogenesis of many diseases such as DM, cancer, and rheumatoid arthritis (126) (127) (128) (129). VEGF protein expression is upregulated in the pathogenesis of DR. A study conducted by Fu Z *et.al.,* observed the upregulation of VEGF expression by HIF-1α which resulted in the accelerated angiogenesis and thus worsened the disease progression (14)

(130). Elevated levels of VEGF have been observed in several types of cancers like angiosarcoma and breast cancer and have been known to involve in facilitating metastasis (131) (132). It possesses the capability of causing vascular permeability, resulting in the seepage of proteins, RBCs, and other molecules from the blood vessels (133). The physiological importance of permeability remains unclear but the process remains of significance in pathological conditions; for example, brain edema followed by cerebral ischemia (134). Inflammatory conditions like age-related macular edema have been associated with increased levels of VEGF, which results in increased vascular permeability (135). Increased expression of VEGF in glomeruli in the kidney causes glomerular hypertrophy, which is associated with proteinuria (136).

Lymphangioleiomyomatosis is a rare condition that affects the lymphatic system, kidneys, and lungs, where VEGF is observed to be overexpressed. It is currently used as a diagnostic biomarker in the treatment of this disease (137). Lack of VEGF expression is observed in patients with pulmonary emphysema (138). In addition, there is growing interest in the other properties of VEGF that are being studied with the goal of using VEGF to treat ischemic diseased conditions. The aim is to promote blood vessel formation which will act as the biological pass for the affected arteries (139) (140).

Hyperglycemia affects the entire neurovascular system of the retina. Over the course of the disease, there is neuroinflammation, neurodegeneration, BRB damage, retinal endothelium damage, edema, and angiogenesis (141) (142). Endothelial cells are damaged as a result of BRB disruption. The damage caused

results in the formation of acellular capillaries and edema in the retinal vascular structure (10). This leads to increased permeability of the endothelial cells and ultimately results in vascular leakage, thickening of the vessel wall, and coagulation further resulting in hypoxia. HIF-1α is the central regulator of hypoxia in tissues deprived of oxygen (143). Vascular hypoxia in the retina has been proposed as one of the strongest factors in the activation and secretion of growth factors such as VEGF which ultimately results in angiogenesis (144).

Angiogenesis is the physiological process of the formation of blood vessels from pre-existing vessels. Occurring both physiologically and pathologically, it occurs throughout life beginning from a fetus and continuing till old age. The resulting blood capillaries found are required for the transportation of nutrients and metabolites required for the growth of an individual. Unregulated angiogenesis leads to many angiogenic diseases (145). Abnormal blood vessel growth poses an inevitable threat to the development of DR (146). Under physiological conditions, the angiogenesis is in a passive state. The blood vessels are in line with the endothelial cells which are bound by tight-adhesion molecules. These molecules maintain the integrity of the barrier and maintain the blood flow (147). The vessel's stability is maintained by collagen and laminin which are coated with pericytes and also promote endothelial cell survival (148). The degradation of collagen and laminin by MMPs provides the route of development for new blood vessels. The new blood vessels formed are mediated by endothelial cells called tip cells. The tip cells regulate the growth of blood vessels with the help of pro-angiogenic mediators like VEGF (149). The

endothelial cells during angiogenesis develop into stalk cells and elongate in the direction of the tip cells. At the end of the process, the blood vessels return to their passive state, where the basement membrane along with pericytes covers the vessels (147).

During angiogenesis, one of the key events that take place is the proteolysis of ECM. This mechanism is carried out by a group of proteases called Matrix Metalloproteases (MMPs). These MMPs are responsible for the degradation of the basement membrane and ECM components like collagen and laminin which in turn aids the migration of endothelial cells through the degraded matrix (150). In a study conducted by Rodrigues M *et.al.,* VEGF promotes the MMPs activity which is responsible for proteolysis of ECM (23).

Matrix metalloproteases (MMPs)

MMPs are a group of zinc-dependent endopeptidases. To date more than 23 MMPs have been identified in humans whereas, 24 MMPs have been identified in mice. The first MMP was discovered in 1962 by Gross J *et.al.,* which was observed to be responsible for collagen degradation in tadpole tail during metamorphosis and was thus initially termed interstitial collagenase (151). Similarly, another collagenase was identified in the human skin which was later named MMP-1. MMPs have been known to possess the capability of degrading almost all the components of ECM such as collagen, fibronectins, laminins, etc (152). MMPs are categorized into various classes depending upon their functions such as collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs (MT-MMPs).

Structure of MMPs

MMPs contain 4 major regions: a prodomain, a catalytic domain, a hemopexin domain, and a hinge region (153). The propeptide domain is a highly conserved sequenced domain and contains 80 amino acids. The prodomain consists of three α-helices. The enzyme is kept inactive through the cysteine switch, where the unpaired cysteine forms a bond with catalytic zinc and therefore blocking the enzymatic activity (154). The catalytic domain consists of three α-helices and five β-sheets. With a diameter of 40Å, they are spherical. The catalytic domain is a highly conserved sequence in nature and contains a Zn^{+2} ion, three calcium ions, and three histidine residues. The Zn^{+2} and the calcium ion are responsible for maintaining the structural conformation of the protease (155) (Figure 3).

With 170 amino acids, the catalytic domain is a conserved histidine sequence, required for zinc chelation. MMPs and TIMPs interact with each other with the help of hemopexin. The hemopexin domain contains 4β-propeller sheets that are linked to each other with the help of a disulfide bond (156). Having similar scaffolds each sheet is made up of 4 antiparallel β-sheets. The sheets contain one calcium and a chloride ion. The hemopexin domain is important for the degradation of triple-helical collagen degrading activity. This event is also essential for the activation of proMMP-2 which is involved in collagen degradation (157).

There are exceptions where MMP-7, MMP-26, and MMP-23 do not interact with the help of hemopexin or the hinge region. Also, MMP-23 remains

the only one that possesses an immunoglobulin domain and a cystine-rich domain. For their activation, a zinc ion is required at the catalytic site (158).

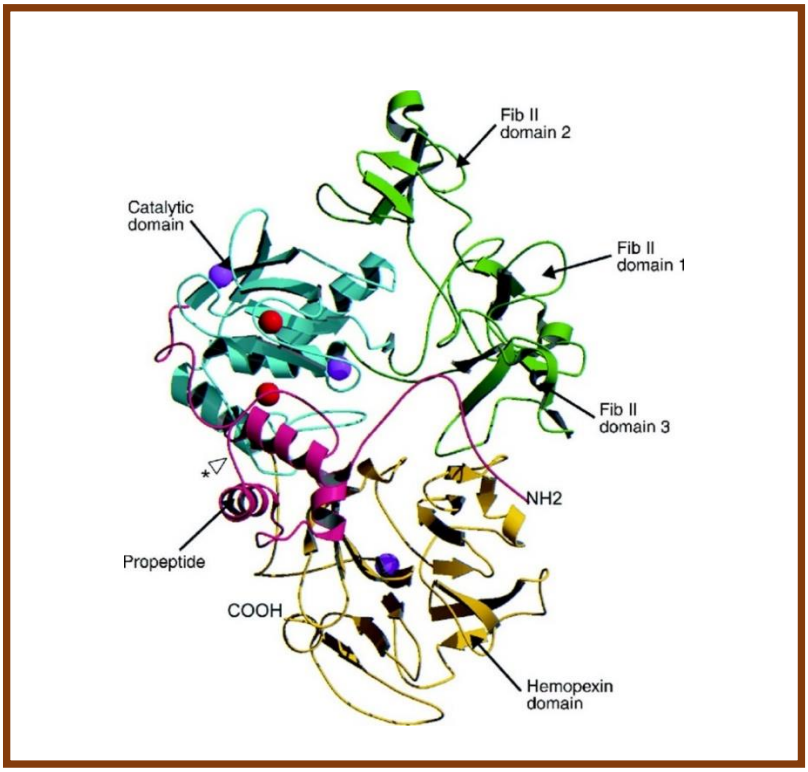

Figure 3. Pro-MMP-2 structure and its domains

Structure of pro-MMP2 with the prodomain, catalytic domain, fibronectin domains, and hemopexin domain. The zinc and calcium ions are represented as red and magenta dots respectively (image source: Morgunova E *et.al.*, 1999) (159)

Table 1. Classifications of MMPs

Family	MMPs	Substrates
Collagenases		
Collagenase-1	MMP-1	Collagen (I, II, III, VII, VIII, X), casein, entactin, laminin, pro-MMP-1, -2, -9, and serpins

Collagenase-2	MMP-8	Collagen (I–III, V, VII, VIII, X), gelatin, aggrecan, fibronectin
Collagenase-3	MMP-13	Collagen (III, IV, XI, X), Aggrecan, fibronectin, laminin, vitronectin, elastin
Gelatinases		
Gelatinase A	MMP-2	Gelatin, collagen (IV–VI, X), elastin, fibronectin
Gelatinase B	MMP-9	Gelatin, collagens (IV, V, VII, X, XIV), elastin, fibrillin, osteonectin
Stromelysins		
Stromelysin-1	MMP-3	Laminin, aggregan, gelatin, fibronectin
Stromelysin-2	MMP-10	Collagens (III–V), gelatin, casein, aggregan, elastin, MMP-1,8
Stromelysin-3	MMP-11	Fibronectin, laminin, aggregan, gelatin
Matrilysins		

Matrilysin	MMP-7	Collagen (IV–X), fibronectin, laminin, gelatin, aggregan, pro-MMP-9
Matrilysin-2	MMP-26	Gelatin, collagen IV, pro-MMP-9
Metalloelastase	MMP-12	Elastin, gelatin, collagen I, IV, fibronectin, laminin, vitronectin, proteoglycan
Membrane-type MMPs (MT-MMP)		
MT-MMP-1	MMP-14	Collagen (I, II, III), gelatin, fibronectin, laminin, aggrecan, tenascin
MT-MMP-2	MMP-15	Fibronectin, laminin, aggrecan, perlecan
MT-MMP-3	MMP-16	Collagen III, gelatin, casein
MT-MMP-4	MMP-17	Fibrinogen, TNF precursor
MT-MMP-5	MMP-24	Proteoglycans

(Table source: Shapiro SD, 1998) (160).

Pleiotropic roles of MMPs

MMPs have been observed to play a direct or indirect role in wound healing and neovascularization *in vivo* as well as *in vitro*. In case of any tissue injury, MMPs are expressed almost in all the cell types which include epithelial, endothelial, mesenchymal, and immune cells. This defines MMPs expression in the process of wound healing and cell migration and has been viewed as a therapeutic target (161).

Genetic knock-out mice have been proven of great use in unraveling the physiological functions of MMPs. As a result, the majority of the knockout models displayed substantial abnormalities in response to external stresses such as injury, infection, and inflammation. MMPs have been observed to play an important role in the process of bone growth and remodeling. To date, 5 MMPs are noticed to be involved in this process namely MMP-2, MMP-9, MMP-13, MT1-MMP, and MT3-MMP. Even though they possess the same capability of influencing the bone remodeling they differ in their specification to the cell type (162). MMP-2 has been implicated in angiogenic development. The knockout model for MMP-2 showed the formation of impaired blood vessels (163). Regeneration of wounded epithelial cells is dependent upon the expression of MMP-7 in mice. Mouse models with MMP-7 mutated genes are observed to be less resistant to intestinal bacterial infection. This is due to their inability to produce an antibiotic called peptide α-defensin as a result of the failure of epithelial regeneration (164).

MMPs in various disease conditions:

MMPs are the primary enzymes responsible for ECM breakdown, with many of them being linked to cancer. MMPs degrade ECM, which not only promotes tumour invasion but also influences tumour cell behavior and contributes to cancer progression (165). Normal serum levels of MMP-2 in adults are observed to be 36.46- 264.4 ng/ml (166). An abnormal increase in MMPs activities results in inducing an incorrect metabolic cascade. Erroneous metabolic cascades are signals that initiate the formation of complex abnormal cell pathways that give rise to tumor/ cancerous phenotype cells. MMP-2 and MMP-9 in particular are involved in the degradation of ECM components during cancer angiogenesis (167). Apart from several types of cancer, MMPs have been involved in neurological disorders like Alzheimer's disease, Parkinson's disease, and Japanese encephalitis. MMPs have been documented to cause inflammation, microglial activation, and disruption of BRB which are all the factors that contribute to Parkinson's disease (168) (169). Neuronal tangles and amyloid plaques are the classic hallmarks of Alzheimer's disease. Accumulation of improperly processed amyloid is the major event in the development of Alzheimer's disease and leads to inflammation. Increased expression of MMPs has been associated with neuronal death in Alzheimer's disease in response to the deposition of plaques (170) (171).

Japanese encephalitis is a neurological disorder that affects the central nervous system and damages neurons in various parts of the brain (172). Even though the exact mechanism for neuronal cell death has not been clear, studies

have implicated MMPs in this mechanism. A study by Shukla *et.al.,* showed elevated levels of MMP-2 in the serum and cerebrospinal fluid of children with Japanese encephalitis as compared to diseased controls. Also, MMP-7 and MMP-9 serum protein levels were elevated in children with Japanese encephalitis as compared to diseased controls but not in cerebrospinal fluid (173).

Several MMPs have been implicated in the progression of DR. MMP-2 has been observed as a salient factor in the activation of retinal cell apoptosis by increasing its membrane permeability (26). Several clinical and experimental studies have observed elevated levels of MMP-2 in patients with DR (30) (174). An experimental study by Mohammad *et.al.,* showed that MMP-2 accelerates retinal cell apoptosis by dysfunctioning the mitochondria (26). MMPs activity is tightly regulated at the transcription levels and its propeptide activation and inhibition is regulated by endogenous inhibitors. These include TIMPs, α2 macroglobulin, and α1-antitrypsin (α1-AT). Poorly regulated MMPs are associated with the progression of various disease conditions (40) (175).

Alpha 1- antitrypsin (α1-AT)

Alpha1-Protease Inhibitor (α1-PI) is an acute phase, 52kDa polymorphic glycoprotein. The isoelectric point of α1-AT ranges from 4.4 to 4.6. α1-AT is synthesized by hepatocytes in the liver and secreted into the bloodstream. Apart from hepatocytes, they are also produced in some amounts by monocytes, macrophages, pulmonary alveolar cells, and the intestinal and corneal epithelium. It was first discovered by Schultz in 1955 and showed anti-

proteolytic activity against trypsin (176). Due to its ability to bind irreversibly with trypsin, it is also known as alpha-1 antitrypsin (α1-AT) (177). The normal circulating concentration of α1-AT is 80-220 mg/dL (178). In response to inflammation, infection, tissue injury, and malignant disease, its concentration is increased by 3-4 folds (177) (179).

Genetics of α1-AT

It belongs to the serine proteinase inhibitor (SERPIN) family, a family of protease inhibitors. The term SERPIN was coined by Carrel and Travis in the year 1983 (180). Encoded by the SERPINA1 gene, α1-AT is located on the protease inhibitor (Pi) locus on chromosome 14q32.1. α1-AT consists of 394 amino acids with an active site of enzyme inhibition at Met358. The SERPINA1 gene is 12.2 kb in length with five exons and six introns (181) (182) (183) (Figure 4). The gene transcription for SERPINA1 is controlled by three non-coding exons (IA, IB, IC) located at the 5' region (184). Through the isoelectric focus technique about 100 genetic variants of α1-AT have been identified (185).

Alleles in α1-AT

Mutation in the SERPINA1 gene results in either low production of α1-AT or its deficiency (AATD). Different versions of alleles produce different amounts of α1-AT. The three alleles responsible for the production of α1-AT are M, S, and Z. The M allele produces an adequate amount of α1-AT required, the S allele produces a moderate amount of α1-AT whereas, the Z allele produces a small amount of α1-AT. Over 70 mutations of the SERPINA1 gene have been identified. During the mutation, there is a switch in the sequence in the position

of amino acids. For example, a mutation in the Z allele results in glutamic acid replacement by lysine at position 342 (Glu342Lys). As a result, there is misfolding of α1-AT protein which in turn gets accumulated in hepatocytes and causes damage to the liver (186).

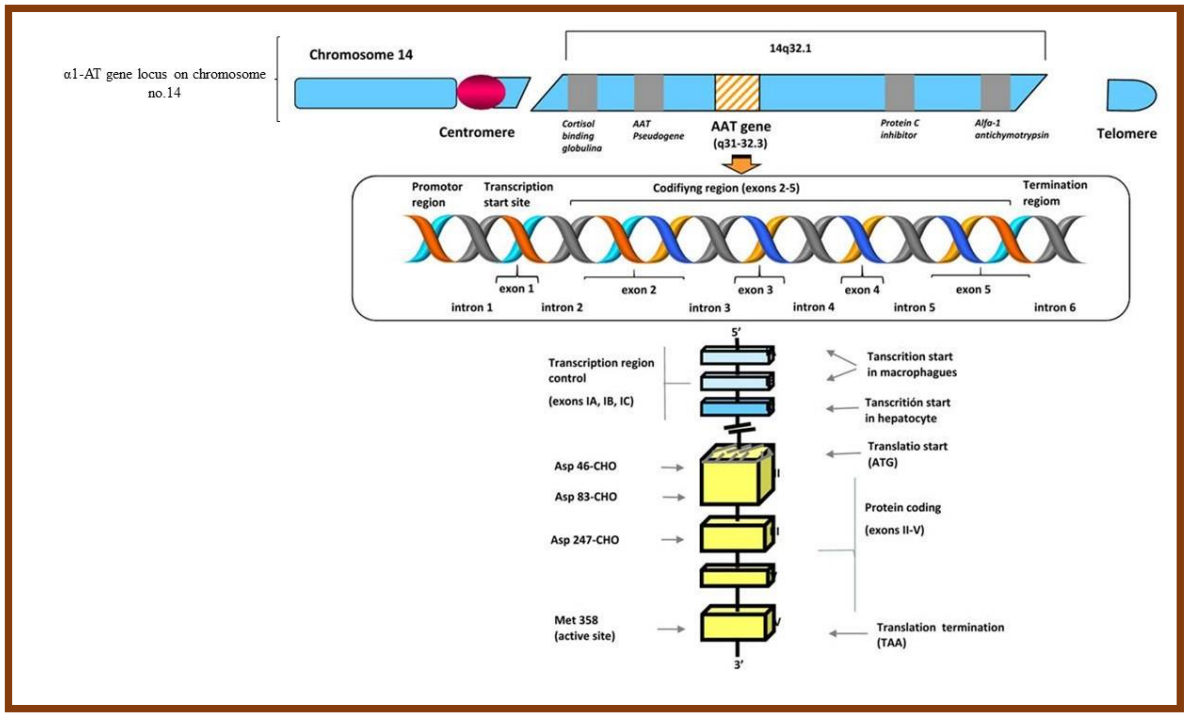

Figure 4. Schematic representation of α1-AT gene

The figure represents α1-AT location on chromosome 14. A schematic illustration of the gene is shown at the bottom, with many locations where diverse activities are carried out (image source: De Serres F *et.al.*, 2014) (184)

α1-AT structure

α1-AT is a single-chain protein composed of 394 amino acids with Met358 residue at the active site. The crystallographic analysis of α1-AT structure showed that it is a globular protein with three N-linked glycosylation sites on the external surface of the one end of the molecule. The side chains consist of N-acetylglucosamine, mannose, galactose, and sialic acid, which are N-linked to amino acids Asn46, Asn83, and Asn247 (187). With a highly ordered internal

structure, α1-AT consists of nine α-helices and three β-sheets that surround the central beta-sheet scaffold. Being an anti-protease α1-AT inhibits the target protein by forming a 1:1 molar α1-AT-enzyme complex which causes conformational changes in the target protein and inhibits its activity (188) (189) (190).

For the past few decades over 80 different structures, representing different conformations have been determined. To attain stability serpins fold into a native, metastable state (192). The reactive central loop (RCL) is a 20-25 functional loop positioned above the body of the molecule and acts as a decoy for target protease and this sequence is responsible for the inhibitory effect on target protease (193). The RCL protrudes from the main body of the molecule and contains the scissile bond (P1 and P1' residues), which mediates α1-AT's inhibitory specificity against the target protease **(**Figure 5**)** (190).

The resulting conformational reorganization is responsible for the overall stability of SERPIN (194). RCL structure plays a decisive role in the ability of the inhibitor to undergo from stress to relax conditions. When activated, α1-AT is in the stressed or metastable form which is essential for protease inhibition. During protease inhibition, α1-AT turns hyper stable (193) (195). There are two possible endings to this reaction. One is protease inactivation, where serpin undergoes stress to relaxed transition, where the distorted protein lies at the base of the molecule. The other possibility is the formation of another β-sheet by RCL, which allows protease to escape the conformational trap leaving active protease and inactive cleaved. *In vivo* α1-AT can exist in three forms: inhibitory

conformation where the RCL is completely exposed, latent conformation where the RCL is exposed partially, and the non-inhibitory conformation. α1-AT adapts to non-inhibitory conformation when the RCL is cleaved by non-target proteinases (196) (197).

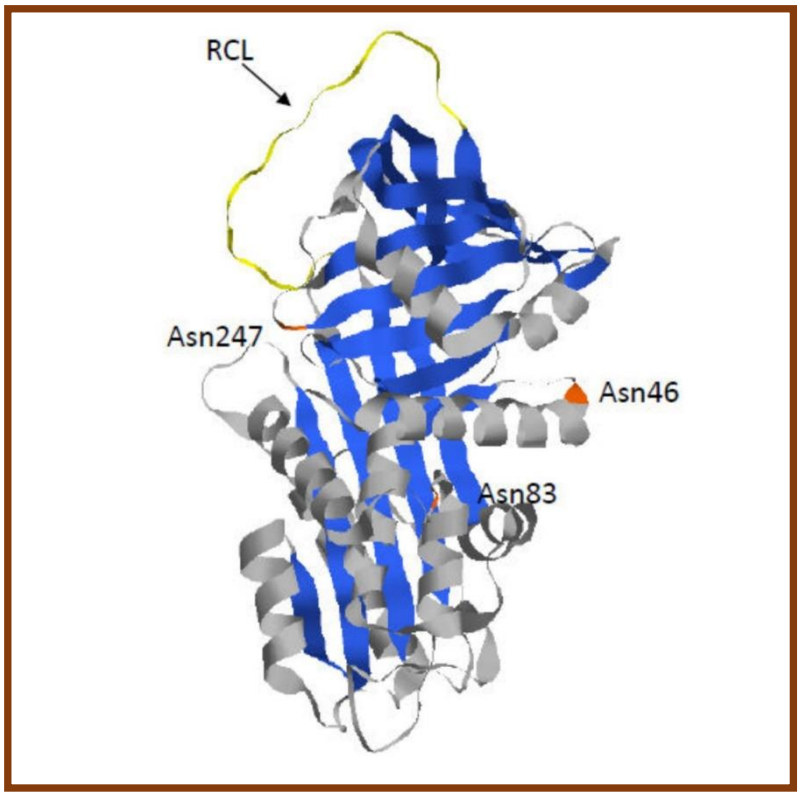

Figure 5. Crystallized structure of α1-AT

It represents asparagine-linked carbohydrates chains at 46, 83, and 247, with three β-sheets and nine α-helices in blue and grey respectively. The reactive center loop is represented in yellow color (image source: Karatas E *et.al.*, 2020) (191)

Pleiotropic role of α1-AT

Previously, α1-AT was thought to primarily inhibit the activity of neutrophil elastase (198). Over time, α1-AT has been known to exhibit other properties as well. α1-AT has been shown to suppress endotoxin-stimulated TNF and increase IL-10 expression in human monocytes *in vitro* via cAMP-dependent protein kinase signaling. A route that is targeted by a variety of Chronic obstructive

pulmonary disease (COPD) anti-inflammatory medications now in use and/or under development (199). Studies have shown the capability of α1-AT in inhibiting caspases. Caspases are apoptosis inducers or initiators. In murine lung endothelial cells and pancreatic beta cells, α1-AT has been shown to inhibit caspase-3 activity (200). Apart from caspase-3, α1-AT have been known to inhibit caspases-6 and -7 in the endothelial cells of lung tissue (201). Apoptosis of retinal endothelial cells is accelerated during DR development and is facilitated by caspases, particularly caspase-3 (202) (203). The ability of α1-AT to suppress caspases could be used to protect microvasculature against DR-induced early damage. Upregulation of α1-AT has been shown to prevent hyperglycemia in non-obese diabetic mice (204). Raising blood levels of α1-AT with augmentation therapy have shown to prevent T1DM development and prolonged islet allograft survival (205) (206).

α1-AT in various disease conditions

C-B Laurell and co-workers of Malmö University Hospital made a significant contribution to protein research in 1952 when he introduced plasma protein electrophoresis as a tool for clinical investigators (207). Using electrophoresis, a lack of bands for α1-AT was observed in the samples of patients with emphysema. This implicated involvement of α1-AT deficiency in emphysema. Further results demonstrated that α1-AT is an effective inhibitor of elastase and therefore indicating its role in the pathogenesis of emphysema (208). In 1978, C. Larsson showed that the age of onset of emphysema in AATD people who were also current or ex-smokers was rapidly lowered (209).

It is widely assumed that ZZ genotypes in patients with AATD account for 1–2% of all COPD cases, and that heterozygous MZ and MS have no chance of developing COPD. However, a study conducted by R. Bals *et.al.,* observed ZZ, MZ, MS, SZ, and 16 other rare genotypes associated with COPD. AATD results in the formation of polymers of the Z variant (210). The unusual accumulation of α1-AT in the endoplasmic reticulum of hepatocytes is linked with liver diseases (211). Children with ZZ variants who do not develop liver disorders in childhood are more vulnerable to liver cirrhosis and hepatocellular carcinoma, later in life (177).

α1-AT is a molecule implicated in various mechanisms observed in DR, including anti-inflammatory activities, apoptosis avoidance, ECM remodeling, and vessel wall and capillary protection (40). Studies on α1-AT have revealed an association between decreased levels of α1-AT and diabetes. Reduced levels of α1-AT have been reported in subjects affected by T1DM (37). Hence, the use of α1-AT may be an effective strategy to prevent or hinder the progression of DR.

Chapter V

Materials and Methods

The present study was an observational study carried out between November 2019 to October 2021. The subjects were enrolled from the Department of Ophthalmology and General Medicine of RL Jalappa hospital and Research Centre, the teaching hospital of Sri Devaraj Urs Medical College, a constituent college of Sri Devaraj University Academy of Higher Education and Research, Tamaka, Kolar, Karnataka. The study was carried out by following the guidelines of the Declaration of Helsinki and the study was approved by Institutional Ethics Committee (SDUMC/KLR/IEC/31/2019-20). Prior to the recruitment in the study, informed consent was obtained from all the study participants.

The study included 186 subjects and were in the age group of 30-70 years. Each group included 62 participants. The subjects were divided into DR, T2DM, and Control groups.

- DR: Included clinically diagnosed patients of DR, confirmed by funduscopy (212).
- T2DM: In accordance with the ADA guidelines 2021 (213), clinically confirmed cases of T2DM with fasting blood sugar (FBS) ≥126mg/dL, Postprandial blood sugar (PPBS) ≥200 mg/dL, and HbA1c levels ≥ 6.5% were included.
- Control: Volunteers without a known history of chronic infections, smoking, alcohol consumption, and with FBS (<100 mg/dL), PPBS (<140 mg/dL), and HbA1c levels (<5%) were included under the control group.

Exclusion criteria: Patients with a known history of smoking, drinking, diabetic nephropathy, neuropathy, and chronic diseases such as cardiac diseases, stroke, COPD, liver disorders, acute renal failures, gestational diabetes mellitus, cancer, and chronic infections were excluded from the study.

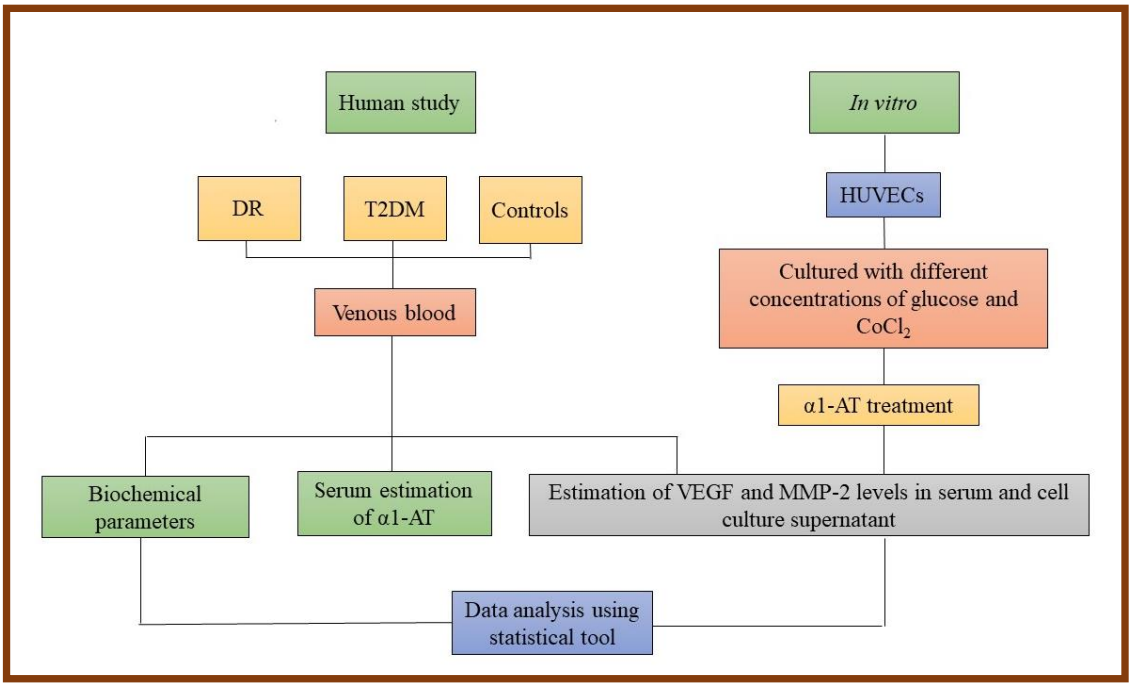

Figure 6. Flow chart for study design

Sample size calculation

The sample size was calculated using an open-source web-based tool viz., OpenEpi version 3.0. with a 95% confidence interval and a power of 80%, the number of study participants required for study in each group was found to be 62 (214).

Sample Collection

Venous blood (6ml) was collected from the study subjects from the antecubital vein in different vacutainers like sodium fluoride (for FBS and PPBS) and EDTA (for HbA1c analysis). For analysis of biochemical parameters

like liver enzymes, urea, creatinine, CRP, and estimation of study molecules VEGF, MMP-2, and α1-AT, the blood was collected in the tube without any anticoagulant. The basic biochemical parameters were analyzed immediately by standard methods using Vitros 5,1 FS autoanalyzer.

Table 2. Measured biochemical parameters

Sr.no	Parameters	Equipment	Reference range	Test method
1.	Fasting Blood Sugar (FBS) (mg/dL)	Vitros 5,1	70-110	Glucose oxidase-peroxidase
2.	Postprandial Blood Sugar (PPBS) (mg/dL)		70-200	
3.	Glycated hemoglobin (HbA1c) (%)	Biorad D10 autoanalyser	<5%	HPLC
4.	Urea (mg/dL)	Vitros 5,1	12-40	Enzymatic method-urease
5.	Creatinine (mg/dL)		0.5-1.4	Enzymatic method-sarcosine oxidase
6.	Gamma-glutamyl Transferase		12-43	L-gamma-glutamyl-p-nitroanilide as

	(GGT) (U/L)			substrate
7.	Alanine transaminase (ALT) (U/L)		<50	Multipoint enzymatic by using LDH
8.	Aspartate aminotransferase (AST) (U/L)		14-36	Oxaloacetate Decarboxylase, Pyruvate Oxidase/Peroxidase
9.	Alkaline phosphatase (ALKP) (U/L)		42-128	P-Nitrophenyl phosphate as substrate
10.	C-reactive protein (CRP) (mg/dL)		<1.0	Anti-CRP monoclonal antibody labelled with horseradish peroxidase

Estimation of VEGF, MMP-2, and α1-AT

For estimation of the study parameters, serum was separated within 2 hours of the sample collection by centrifugation at 3000rpm. The serum was then aliquoted and stored at -80˚C until further analysis. Prior to the analysis, the samples were thawed at room temperature, vortexed, and centrifuged. VEGF, MMP-2, and α1-AT levels were estimated using commercially available Enzyme-linked immunosorbent assay (ELISA) kits (Cloud Clone Corp, USA, #SEA143Hu, #SEA100Hu, and #SEB697Hu, respectively).

The following protocol was followed for quantification of serum VEGF using ELISA:

Samples, reagents, and standards were prepared according to the manufacturer's instructions.100µL of samples or standards were added to the wells. The wells were incubated for an hour at 37°C. The reagents and samples were aspirated and 100µL of detection reagent A was added. The plate was incubated for an hour at 37°C. Wells were aspirated and washed with wash solution. 100µL of detection reagent B was added to the wells. The plate was kept for incubation at 37°C for 30 minutes. The wells were washed again using wash solution. 90µL of substrate solution was added to the well. The plate was incubated for 10-20 minutes at 37°C. 50µL of stop solution was added to the wells. Absorbance was recorded at 450nm.

The following protocol was followed for quantification of serum MMP-2 using ELISA:

Samples, reagents, and standards were prepared according to the manufacturer's instructions.100µL of samples or standards were added to the wells. The wells were incubated for an hour at 37°C. The reagents and samples were aspirated and 100µL of detection reagent A was added. The plate was incubated for an hour at 37°C. Wells were aspirated and washed with wash solution. 100µL of detection reagent B was added to the wells. The plate was kept for incubation at 37°C for 30 minutes. The wells were washed again using wash solution. 90µL of substrate solution was added to the well. The plate was incubated for 10-20

minutes at 37°C. 50µL of stop solution was added to the wells. Absorbance was recorded at 450nm.

The following protocol was followed for quantification of serum α1-AT using ELISA:

Samples, reagents, and standards were prepared according to the manufacturer's instructions.100µL of samples or standards were added to the wells. The wells were incubated for an hour at 37°C. The reagents and samples were aspirated and 100µL of detection reagent A was added. The plate was incubated for an hour at 37°C. Wells were aspirated and washed with wash solution. 100µL of detection reagent B was added to the wells. The plate was kept for incubation at 37°C for 30 minutes. The wells were washed again using wash solution. 90µL of substrate solution was added to the well. The plate was incubated for 10-20 minutes at 37°C. 50µL of stop solution was added to the wells. Absorbance was recorded at 450nm.

***In vitro* culture of Human Umbilical Vein Endothelial Cells (HUVECs)**

HUVECs were procured from ATCC, USA, #CRL-1730. Cells were cultured and maintained in Dulbecco's modified eagle media with 10% foetal bovine serum and 1% antibiotics. Cells were seeded at a density of 0.25x106 in a 24-well cell culture plate. The cells were then cultured for 72 hours with normal (5mM) glucose concentration. To mimic the hyperglycemic conditions cells were cultured under high (15mM and 33mM) glucose concentrations. After 72 hours cells cultured under high glucose concentration (33mM) were treated with different concentrations of $CoCl_2$ i.e., 50µM and 100µM, and incubated for 72

hours to induce hypoxic conditions (35) (215) (216). $CoCl_2$ has been used as an alternative method for inducing hypoxia in experimental settings (217) (218) (219). $CoCl_2$ has been shown to mimic hypoxic conditions by stabilizing HIF1-α by inhibiting its degradation (220) (221).

The cells were then incubated without and with 1mg/mL α1-AT (normal concentration) for 10 hours and were grouped as follows:

Table 3. HUVECs cultured under glucose and $CoCl_2$ concentration without and with α1-AT treatment

Groups	**α1-AT treatment (1mg/mL)**
5mM glucose	Without treatment
	With treatment
15mM glucose	Without treatment
	With treatment
33mM glucose	Without treatment
	With treatment
33mM glucose+50µM $Cocl_2$	Without treatment
	With treatment
33mM glucose+100µM $Cocl_2$	Without treatment
	With treatment

After the incubation period, the cell supernatant was collected from all the groups, and VEGF and MMP-2 levels were estimated by ELISA at 450nm. The samples were run in duplicates.

Statistical Analysis

The data obtained were analyzed statistically using SPSS V20 (International Business Machine Corporation, Armonk, New York) software and Prism GraphPad 9.1. Kolmogorov Smirnov test was performed with Q-Q plots and normality plots to check for the normal distribution of the data. The data were observed to be normally distributed. Results are expressed as mean and standard deviation. Unpaired student t-test was performed to compare means between the two groups and one-way ANOVA was used to compare means between multiple groups. Pearson correlation analysis was carried out to assess the relationship between study parameters. *p*-value <0.05 was considered statistically significant and *p*-value <0.001 highly significant.

Chapter VI

Results

I. Demographic and Biochemical characteristics of the study participants

The demographic information of the study participants is summarized in **Table 4.** The mean age of the study participants was 54.55. Out of the 186 participants, the majority were female. In the DR group, 38% of patients had DR for more than ten years, whereas in the T2DM group, 35% of patients had the condition for more than ten years.

Table 4. Demographic characteristics of the study groups

Parameters	**DR (n=62)**	**T2DM (n=62)**	**Control (n=62)**
Demographic details			
Age (Mean ± SD)	53.24 ± 7.50	55.82 ± 7.72	54.59 ± 7.31
Gender (Male/Female)	33/29	22/40	27/35
Duration of the disease			
<10 years	62%	65%	-
>10 years	38%	35%	-

The biochemical parameters of the study groups have been summarized in **Table 5.** The three groups are comparable with respect to the biochemical parameters except for FBS, PPBS, HbA1c, and CRP.

Table 5. Biochemical characteristics of the study groups

Parameters	Study groups			Comparison between groups (p-value)		
	DR (n=62)	**T2DM (n=62)**	**Control (n=62)**	**DR vs Control**	**T2DM vs Control**	**DR vs T2DM**
FBS	189.63±35.65	186.94±57.49	82.04±7.10	<0.001*	<0.001*	0.04*
PPBS	293.58±52.93	259.47±76.57	110.4±11.65	<0.001*	<0.001*	0.04*
HbA1c	10.01 ± 2.33	9.37 ± 2.44	5 ± 0.54	<0.001*	<0.001*	0.1
Urea	24.41 ± 8.36	26.25 ± 8.19	22.15±7.12	0.2	0.2	0.5
Creatinine	0.75 ± 0.19	0.74 ± 0.46	0.67 ± 0.15	0.1	0.3	0.3
GGT	32.41 ± 9.36	31.27±11.31	28.72±10.37	0.4	0.5	0.3
ALT	28.40 ± 6.60	26.70 ± 6.21	25.74 ± 8.06	0.1	0.4	0.6
AST	26.12 ± 6.14	27.03 ± 7.12	27.24 ± 6.66	0.5	0.6	0.2
ALKP	75.83 ± 20.45	84.67 ± 19.24	77.96±19.60	0.7	0.8	0.6
CRP	5.31 ± 0.5	5.23 ± 0.44	0.1±0.04	<0.001*	<0.001*	0.3

ANOVA test was used. Results represented as mean ± SD.*$p<0.001$ and $p<0.05$ were considered statistically significant.

II. Serum levels of VEGF, MMP-2, and α1-AT in study groups

When serum VEGF levels were compared between study groups, it was observed that VEGF levels were significantly higher in the DR group (468.98±58.14 pg/mL) as compared to the VEGF levels in T2DM (378.37±79.96 pg/mL) and control subjects (238.44±47.63 pg/mL) (Figure 7 (a), p<0.001).

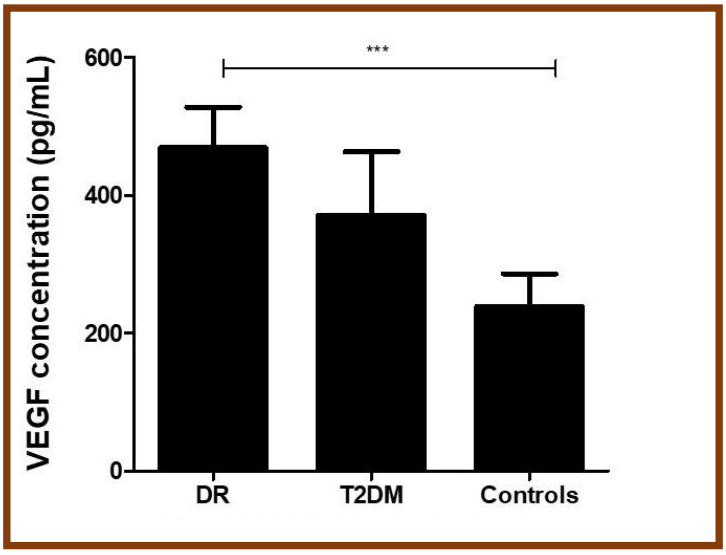

Figure 7 (a). Serum levels of VEGF in the study groups

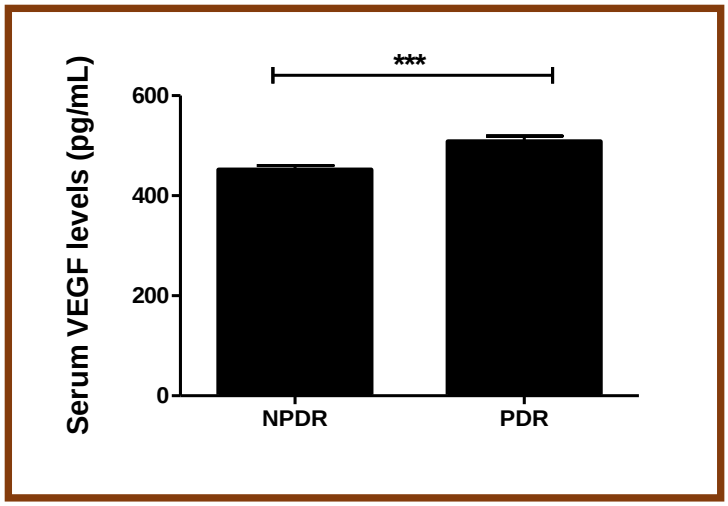

Figure 7 (b). Serum VEGF levels in NPDR and PDR groups

Furthermore, on sub-group analysis of VEGF levels between PDR and NPDR subjects, it was observed that VEGF levels were increased significantly in the PDR group as compared to the NPDR group (Figure 7 (b), $p<0.001$).

Similarly, a significant increase in MMP-2 levels was observed in the DR group (552.14±108.80 ng/mL), when compared to T2DM (413.94±82.99 ng/mL) and control group (236.84±78.67 ng/mL) (Figure 8 (a), $p<0.001$).

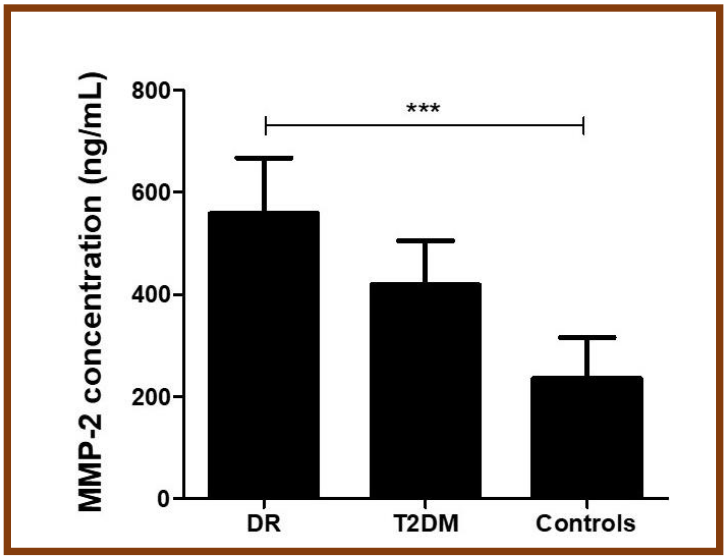

Figure 8 (a). Serum levels of MMP-2 in the study groups

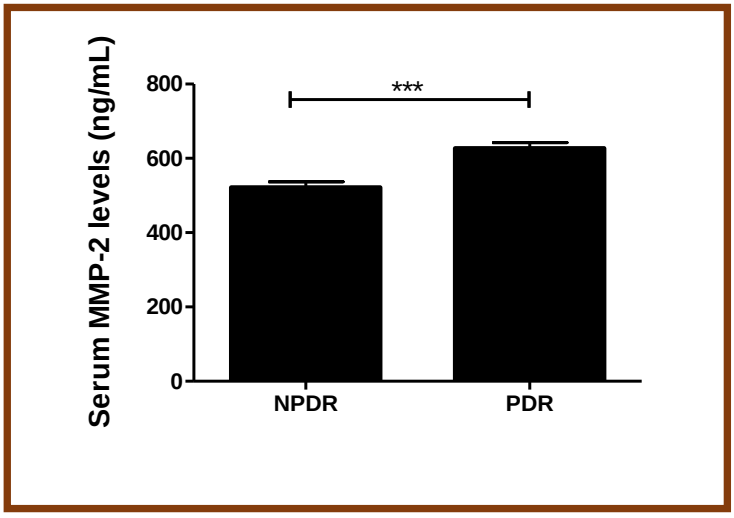

Figure 8 (b). Serum MMP-2 levels in NPDR and PDR groups

The subgroup analysis carried out between NPDR and PDR patients showed significantly elevated levels of MMP-2 in the PDR group as compared to NPDR (Figure 8 (b), $p<0.001$).

However, when serum α1-AT levels were estimated, diabetic patients with (10.04±2.45 mg/dL) and without retinopathy (49.7±7.45 mg/dL) had significantly decreased levels as compared to the control (80.04±4.53 mg/dL). Further, the decrease in the levels of α1-AT was more pronounced in DR patients as compared to T2DM patients without retinopathy (Figure 9 (a), $p<0.001$).

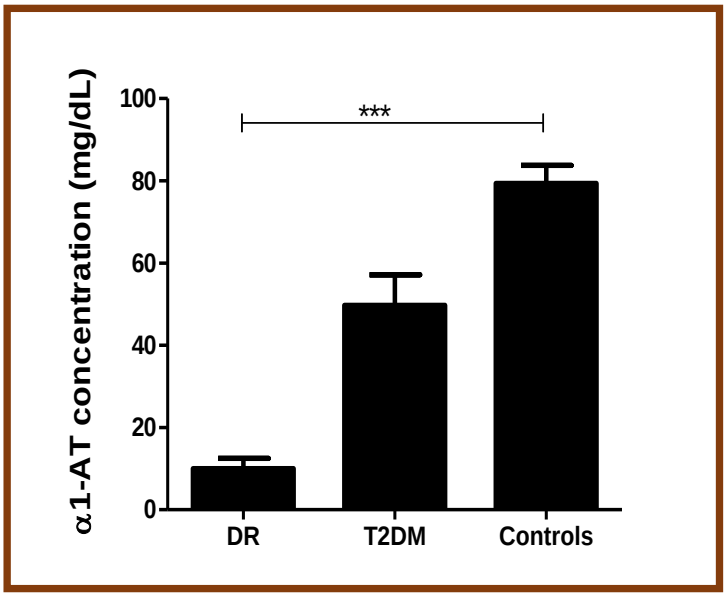

Figure 9(a). Serum levels of α1-AT in the study groups

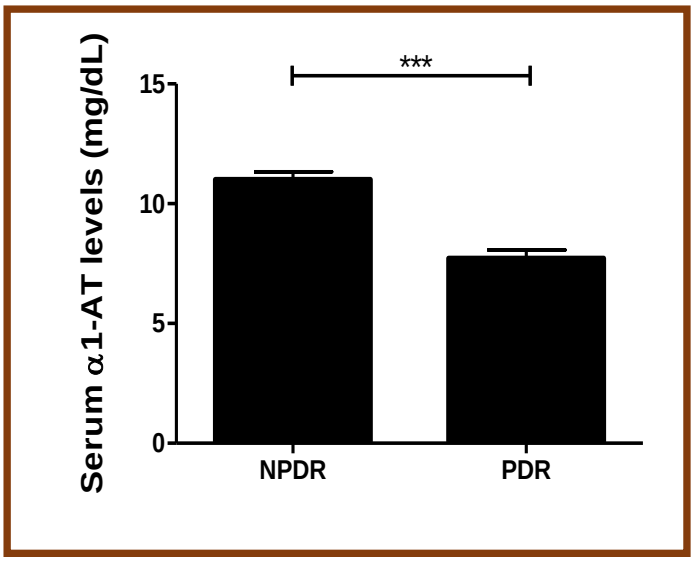

Figure 9(b). Serum α1-AT levels in NPDR and PDR groups

Moreover, on subgroup analysis between NPDR and PDR, a significant decrease in the α1-AT levels was more prominent in the PDR group when compared to NPDR (Figure 9 (b), $p<0.001$).

Ratios of the mean of VEGF vs α1-AT and MMP-2 vs α1-AT were calculated in all the study groups. It was observed that ratios of the mean of VEGF and α1-AT were increased by 6.1 times in DR patients when compared to T2DM. Whereas, the ratios were observed to increase by 16 times in the DR patients as compared to control (Figure 10 (a)). Furthermore, the ratio of the means of MMP-2 and α1-AT was 6.5 times higher in the DR group as compared to the T2DM group and 18 times higher when compared to the control subjects (Figure 10 (b)).

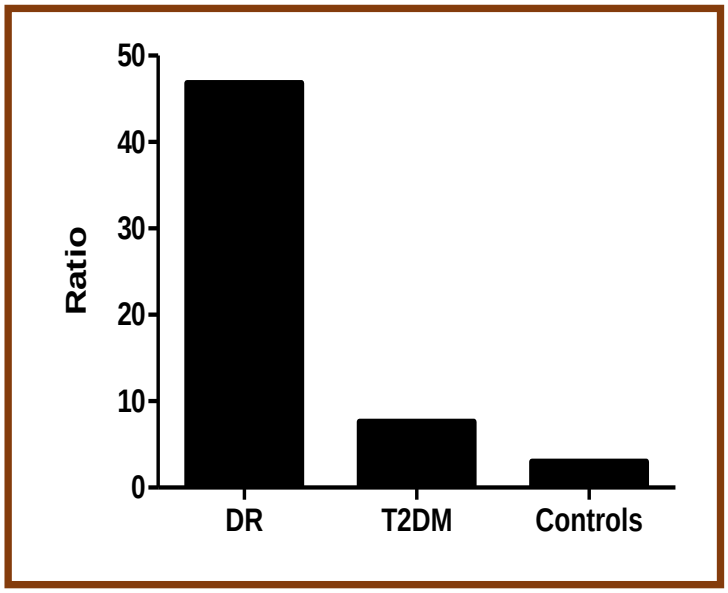

Figure 10(a). The ratio between VEGF and α1-AT in study groups

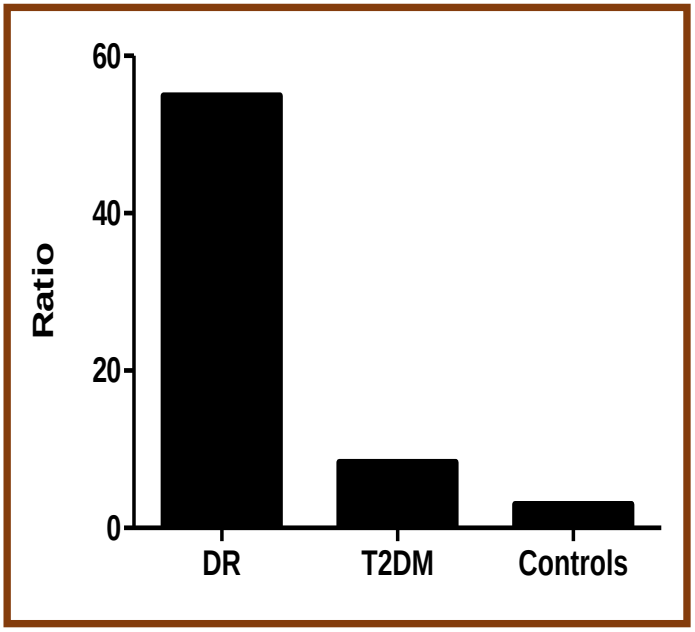

Figure 10(b). The ratio between MMP-2 and α1-AT in study groups

III (a). Correlation analysis between VEGF, MMP-2, and α1-AT in Study groups

Pearson's correlation was carried out to evaluate the association between VEGF, MMP-2, and α1-AT in DR and T2DM groups. A significant positive correlation was observed between VEGF and MMP-2 in DR (r=0.65, p<0.01) as well as T2DM (r=0.22, p=0.08) (Figure 11) patients. On the other hand, a negative correlation was observed between VEGF and α1-AT in DR (r= -0.56, *p*<0.01) and the T2DM group (r=-0.45, *p*<0.01) (Figure 12). Furthermore, when MMP-2 was correlated with α1-AT it was observed that they are negatively correlated in DR (r= -0.72, *p*<0.01) as well as T2DM patients (r= -0.31, *p*<0.01) (Figure 13).

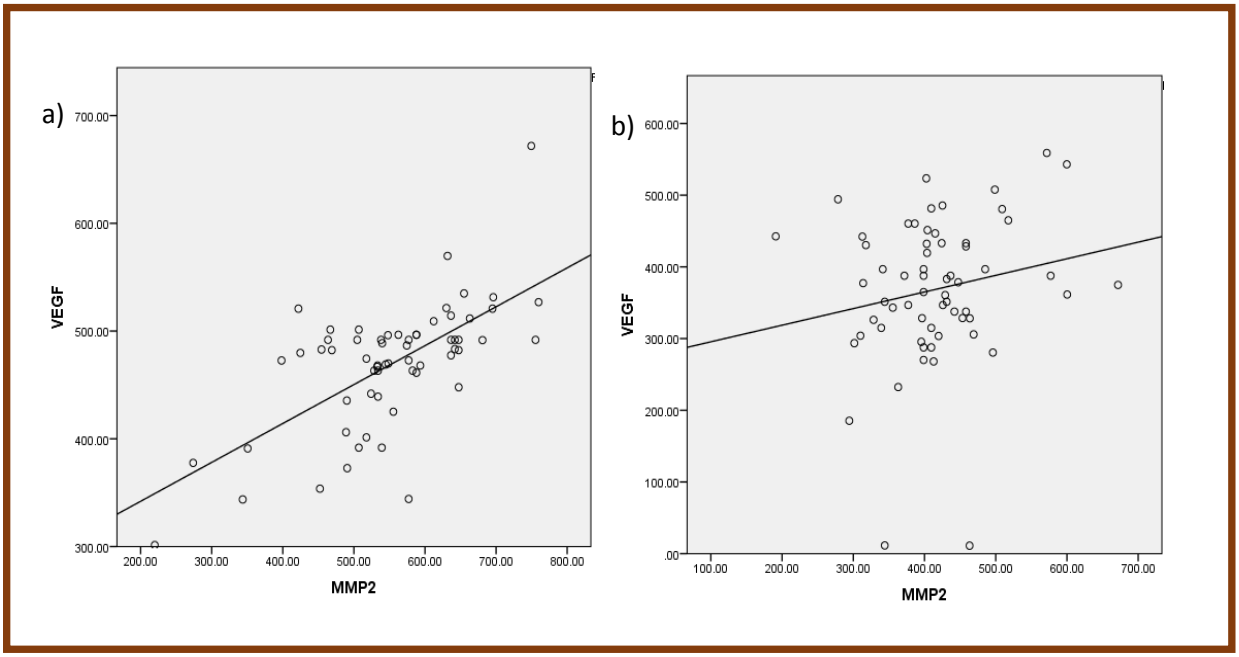

Figure 11. Correlation between VEGF and MMP-2 in DR and T2DM respectively. Pearson's coefficient and p values are as follows: a) r=0.65, p<0.01 and b) r=0.22, p=0.08

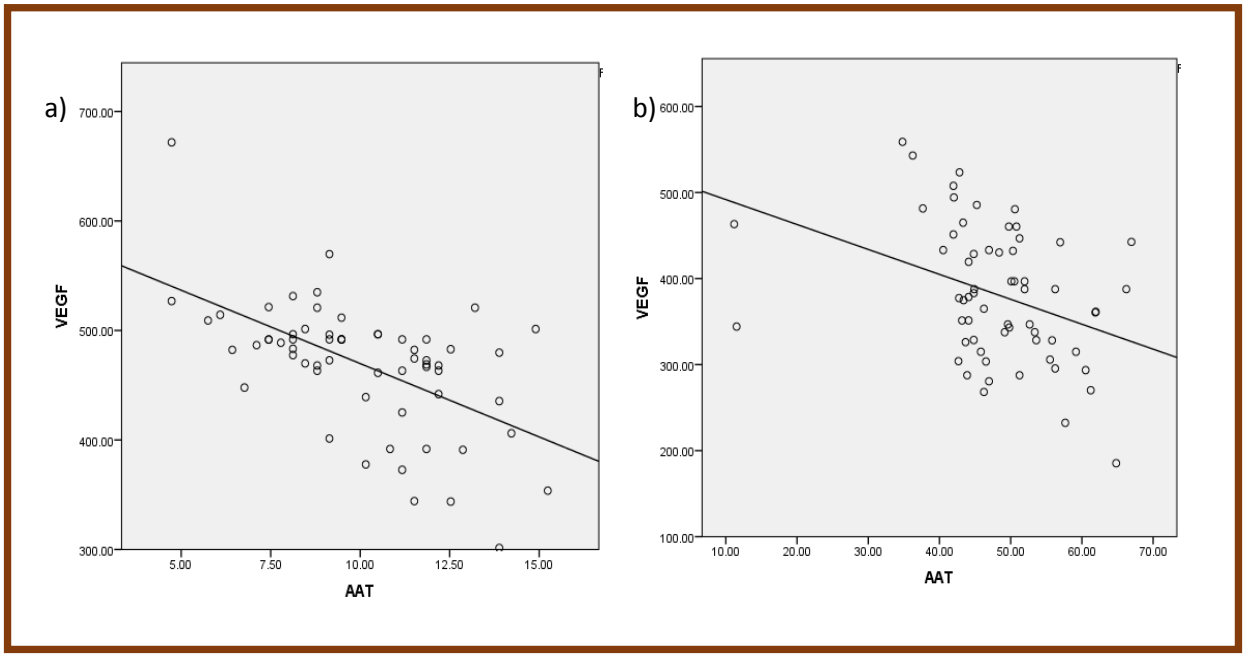

Figure 12. Correlation between VEGF and α1-AT in DR and T2DM respectively. Pearson's coefficient and p values are as follows: a) r= -0.56, p<0.01 and b) r=-0.45, p<0.01

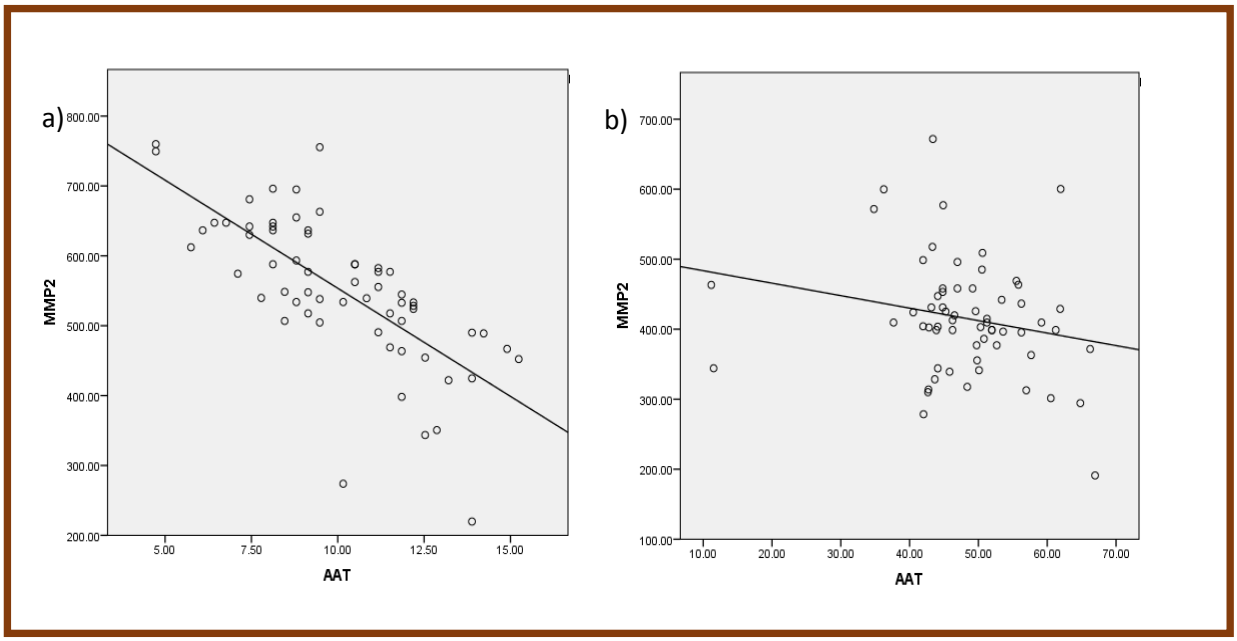

Figure 13. Correlation between MMP-2 and α1-AT in DR and T2DM respectively. Pearson's coefficient and p values are as follows: a) r= -0.72, p<0.01 and b) r= -0.31, p<0.01

III (b). Correlation analysis between study parameters and HbA1c levels in DR patients

On further correlation analysis between study parameters and HbA1c levels in DR patients, it was observed that VEGF and MMP-2 correlated positively with HbA1c levels ($r=0.26$, $p<0.05$ and $r=0.33$, $p<0.01$ respectively) (Figure 14 (a) and (b) respectively). However, α1-AT showed a negative correlation with HbA1c levels ($r=-0.50$, $p<0.01$) (Figure 14 (c)).

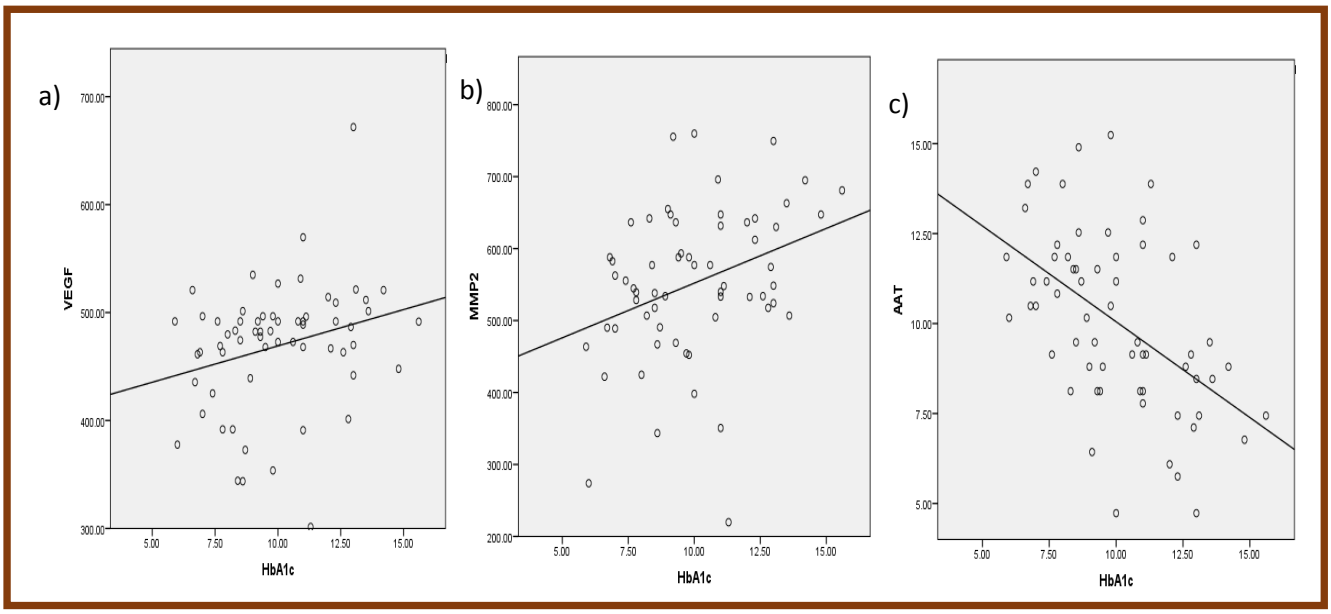

Figure 14. Correlation between the study parameters (VEGF, MMP-2 and α1-AT respectively) and HbA1c in DR. Pearson's coefficient and p values are as follows: a) $r=0.26$, $p<0.05$ and b) $r=0.33$, $p<0.01$ c) $r=-0.50$, $p<0.01$

IV. Effect of α1-AT on the levels of VEGF and MMP-2 in Human umbilical vein endothelial cells: An *in vitro* study

IV (a). Effect of α1-AT on the levels of VEGF and MMP-2 in cells cultured under normal glucose concentration (5mM)

The effect of α1-AT on VEGF and MMP-2 was assessed by evaluating VEGF and MMP-2 levels in HUVEC cells treated without and with α1-AT (1mg/mL)

under normal glucose conditions (5mM). There was no significant difference observed in VEGF and MMP-2 levels between the untreated and treated groups under normal glucose conditions (Figure 15(a) and (b) respectively).

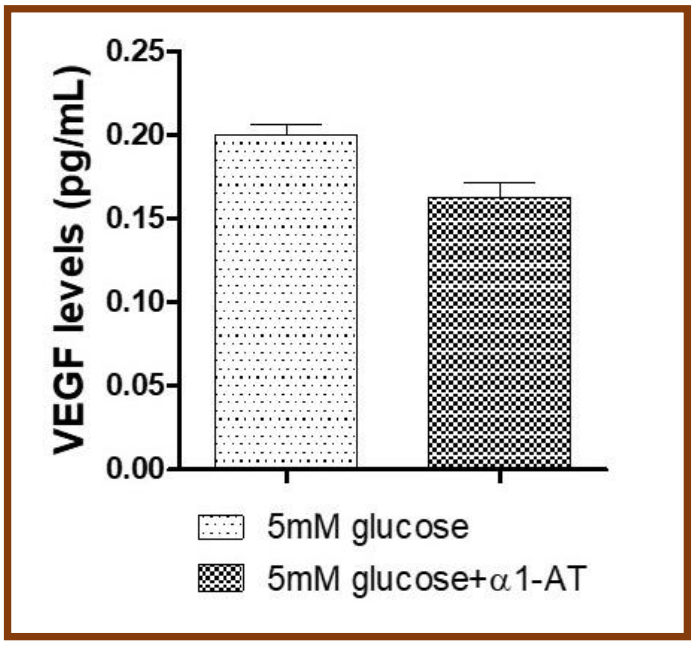

Figure 15(a). VEGF levels in cells cultured under 5mM glucose concentration without and with α1-AT treatment

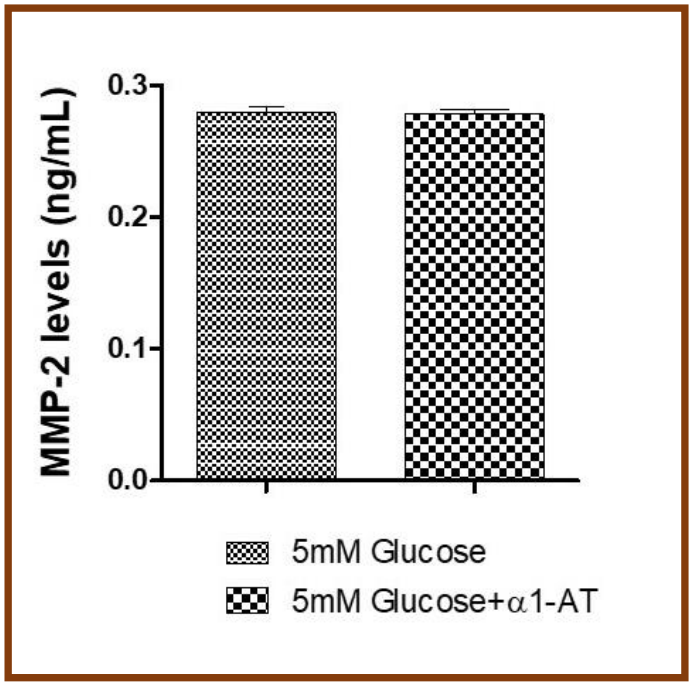

Figure 15(b). MMP-2 levels in cells cultured under 5mM glucose concentration without and with α1-AT treatment

IV (b). Effect of α1-AT on VEGF and MMP-2 levels in cells cultured under high glucose concentrations (15mM and 33mM)

In order to mimic the hyperglycemic conditions observed in diabetes, the cells were cultured with media containing different concentrations of high glucose (15mM and 33mM). Cells cultured with 15mM glucose concentration had significantly higher levels of VEGF and MMP-2 in the untreated group as compared to the treated group ($p<0.001$). Also, with an increase in the glucose concentration (33mM), the VEGF and MMP-2 levels were increased significantly in the untreated group as compared to the treated group ($p<0.001$). The effect of a normal concentration of α1-AT (1mg/mL) on VEGF and MMP-2 levels in cells cultured under high glucose concentrations was investigated. This was done to assess whether the standard concentration of α1-AT was adequate to

decrease the elevated levels of VEGF and MMP-2 in cells cultured with high glucose concentrations. Post-treatment with α1-AT, VEGF and MMP-2 levels were found to decrease significantly (Figure 16 (a) and (b) respectively, $p<0.001$).

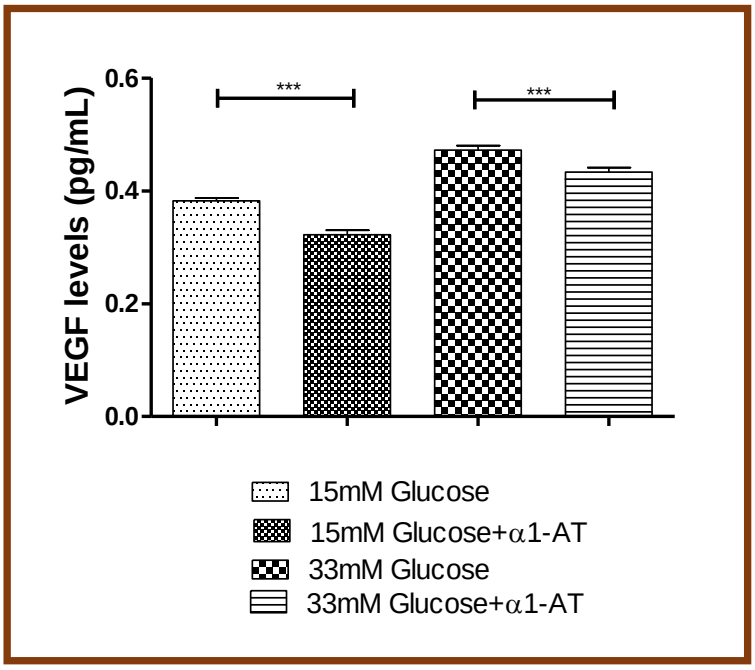

Figure 16(a). VEGF levels in cells cultured under 15mM and 33mM glucose concentrations without and with α1-AT treatment

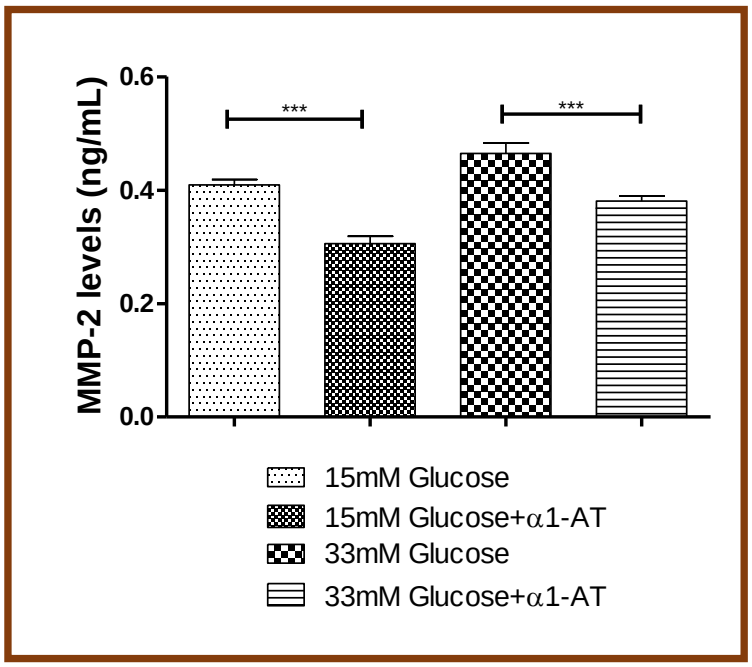

Figure 16(b). MMP-2 levels in cells cultured under 15mM glucose and 33mM glucose concentrations without and with α1-AT treatment

IV (c). Effect of α1-AT on the levels of VEGF and MMP-2 in cells cultured with high glucose concentration (33mM), and hypoxic conditions induced by $CoCl_2$ (50µM and 100µM)

The cells were treated with different concentrations of $CoCl_2$ (50µM and 100µM) after being cultured in a 33mM glucose concentration. This was done to mimic DR conditions. Under high glucose and hypoxic conditions, the effect of α1-AT on VEGF and MMP-2 was assessed. When compared to the treated group, the untreated group had considerably higher levels of VEGF and MMP-2. Furthermore, VEGF and MMP-2 levels were increased in hypoxia-induced cells when compared to VEGF and MMP-2 levels in non-hypoxic cells. We also found that cells treated with 100µM $CoCl_2$ showed significantly higher levels of VEGF and MMP-2 than cells treated with

50µM $CoCl_2$ (Figure 17 (a) and (b) respectively, $p<0.001$). This suggests that an advanced hypoxic situation increases VEGF activity, which in turn increases MMP-2 activity. α1-AT was found to significantly decrease VEGF and MMP-2 at a concentration of 1 mg/mL, indicating a protective effect in DR

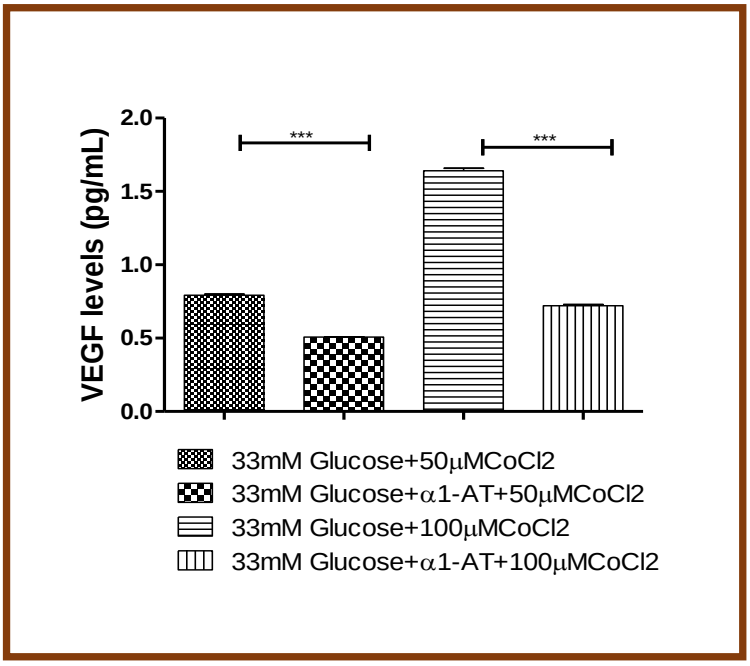

Figure 17(a). VEGF levels in cells cultured under 33mM glucose concentration and different concentrations of $CoCl_2$ without and with α1-AT treatment

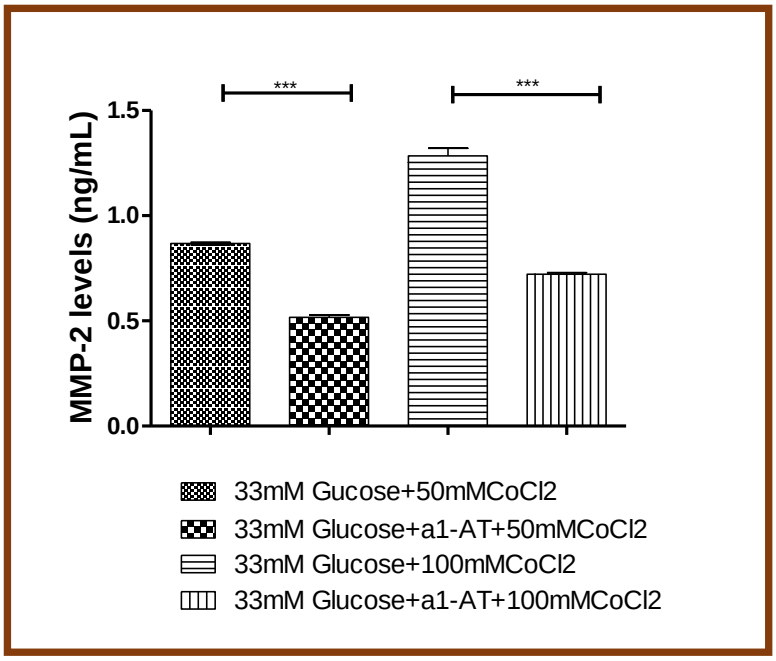

Figure 17(b). MMP-2 levels in cells cultured under 33mM glucose concentration and different concentrations of $CoCl_2$ without and with α1-AT treatment

Chapter VII

Discussion

Long-term exposure to the diabetic environment activates a number of interconnected biochemical pathways that contribute to DR pathology. Dilation of blood vessels and alterations in the blood flow are the first responses of the retinal blood vessels to hyperglycemia. These modifications are thought to represent metabolic autoregulation to boost diabetic retinal metabolism (222). Another feature of the early stages of DR is pericyte loss. Apoptosis of pericytes induced by high glucose levels has been demonstrated in both *in vitro* and *in vivo* studies (223) (224). Because pericytes are in charge of providing structural support for capillaries, their loss results in a localized outpouching of capillary walls. This process is linked to the formation of microaneurysms, which is the first clinical sign of DR (68). In addition, loss of endothelial cells and thickening of basement membrane has been observed to occur during the progression of DR, which disrupts BRB (225). This results in microvascular leakage of the inner retinal capillaries leading to macular edema and lipid exudates (102).

DR is characterized by increased neural cell death, sterile inflammation, and vascular permeability (226). Inflammation plays an important role in the structural and molecular changes associated with DR (227) (228). Inflammation and oxygen levels are linked; inflammation is frequently accompanied by hypoxia, and hypoxia can cause inflammation (229). It has been proposed that metabolic insults to the diabetic retina, such as hypoxia, may cause retinal inflammation (230). Locally, retinal hypoxia causes the release of many molecules, including pro-inflammatory cytokines and chemokines and growth

factors such as VEGF (9) (231). VEGF is the most widely studied growth factor in relation to DR (70).

VEGF causes the breakdown of BRB, stimulates endothelial cell proliferation, causes neovascularization, and increases vascular permeability in the hypoxic retina. Many animal and clinical studies have shown that VEGF, particularly the isoform 165, plays a role in the development and progression of DR (129) (233) (234). VEGF upregulation in DR is regulated by increased levels of HIF-1α as a result of hypoxia, caused due to loss of endothelial cells (14) (235). Furthermore, VEGF increases endothelial cell proliferation by activating mitogen-activated protein (MAP), making it an angiogenic factor (237).

MMPs play an important role in VEGF-mediated cell proliferation and the formation of new vasculature in the retina (237) (238). MMPs improve tissue availability of bound VEGF, and plays important role in neovascularization (239). Also, recent evidences have implicated ROS-mediated activation of MMP-2 in the development of DR. The activation of MMP-2 is thought to cause cell death of retinal endothelial cells by causing mitochondrial dysfunction which activates the apoptosis cascade in DR (240). MMP-2 activity has been observed to increase in the epiretinal neovasculature membrane of patients with PDR (25). This advanced stage of DR is followed by thickening of the basement membrane, resulting in loss of pericytes and endothelial cells. Neovascularization begins at this point and the resulting blood vessels are fragile, which if left untreated, results in retinal detachment (241) (242). MMPs being a protease, are controlled by a group of endogenous inhibitors which

regulate their activity by binding to them at their zinc site and thus blocking their activity (243). α1-AT has been known to downregulate MMP-2 (31) (32). In addition to this, α1-AT is known to possess anti-inflammatory, anti-apoptotic, and anti-angiogenic properties, and therefore, has been suggested as a therapeutic approach towards DR (40) (34).

The purpose of this study was to investigate the relationship between VEGF, MMP-2, and α1-AT in the development and progression of DR. To achieve this the present study was designed with the first objective to evaluate the levels of VEGF, MMP-2, and α1-AT in the study groups, followed by *in vitro* study.

I. Serum levels of VEGF, MMP-2, and α1-AT in study groups

VEGF activity has been observed in neovascularization in pathologies of many diseases including DR and has become a topic of interest for researchers. The VEGF levels have been evaluated in the vitreous, plasma, tears, serum, and aqueous humor of DR patients. Our results are in agreement with the previously reported observations of significantly elevated VEGF levels in the serum of DR patients as compared to T2DM and control. Wang J *et.al.*, observed significantly elevated levels of VEGF in PDR patients both in the vitreous as well as plasma (244). In another study conducted, serum VEGF levels in patients with DR were increased significantly (111). VEGF levels in serum and, tears were also observed to be significantly higher amongst diabetic patients with DR compared to those without DR (110) (245). Further, this observation was confirmed at the level of meta-analysis, which showed an increase in the serum levels of VEGF in patients with retinopathy as compared to control (246). Apart from the studies

carried out in humans, VEGF levels were observed to be elevated in the retinas of the diabetic mouse. Also, inhibition of VEGF activity in mice showed the formation of impaired blood vessels and suppressed angiogenesis in a time-dependent manner (247). In the present study, on further subgroup analysis, it was observed that the VEGF levels were increased significantly in the PDR group as compared to the NPDR group, suggesting an increased angiogenic activity in PDR patients which has been supported by the previous observations reported by Ahuja S *et.al.*, (110). Thus, from the above observations, it can be implied that the elevated levels of VEGF may be attributed to the fact that it promotes the proliferation of blood vessels, which is a hallmark of DR progression (248). Our finding of elevated VEGF levels adds to the growing body of evidence that VEGF is a reliable biomarker for the diagnosis and progression of the DR (110) (111) (245).

One of the primary events that occur to mediate angiogenesis is ECM degradation. During DR, under hypoxic conditions, VEGF secreted by hypoxic muller cells induces the neighbouring endothelial cells to produce MMP-2, which aids in the destruction of collagen, one of the key components of the ECM (23). The breakdown of ECM occurs in response to angiogenic stimuli during the early stages of angiogenesis (249). This is facilitated by the migration of endothelial cells through the degraded matrix components (250). The phenomenon of contact inhibition prevents endothelial cells from migrating out of the endothelium lining under physiological conditions. Degradation of the

ECM confiscates contact inhibition, allowing endothelial cells to migrate more freely resulting in neovascularization (251) (252).

In the present study, serum levels of MMP-2 were observed to be elevated significantly in patients with retinopathy as compared to T2DM patients and control. Peeters SA *et.al.,* observed that MMP-2 plasma levels differed significantly across their study groups, with the DR group having the highest levels and was observed to be associated significantly with its severity (253). Elevated levels of MMP-2 have been observed to negatively correlate with the visual functions of the patients (254). Furthermore, in the current study, a significant increase in the MMP-2 levels in PDR patients as compared to NPDR was observed. Thus, the elevated MMP-2 levels observed in DR patients may be attributed to the MMPs' unregulated activity, which leads to uncontrolled proteolysis and ECM degradation resulting in the crucial damage to ECM architecture affecting the cellular functions. Sarray S and co-workers (2022) observed that people with MMP-2 variants are susceptible and are at greater risk of developing DR (255). Moreover, increased levels of MMP-2 have been suggested as a potential biomarker for retinopathy (256). From the above observations, it can be concluded that MMPs play a vital role in the pathogenesis of DR. Also, the observation of an increase in the MMP-2 levels in the PDR group is of relevance and indicates its role in the progression of retinopathy.

MMP-2 promotes an angiogenic phenotype, while its inhibition inhibits angiogenesis (167) (257) (258). MMP-2 activity is controlled *in vivo* by a family of endogenous anti-proteinases that inactivate the enzyme by forming an

irreversible complex with it (259). However, in several disease conditions including DM, the balance between protease and anti-protease is lost due to increased protease activity or decreased anti-protease activity resulting in critical tissue damage (32).

In the present study, a significant decrease in the serum levels of α1-AT were observed in DR patients when compared to T2DM and control. A noticeable reduction in the levels of α1-AT observed in retinopathy patients could point towards its reduced anti-proteolytic activity. This might result in the unregulated activity of MMP-2 and is supported by the present observation regarding significantly increased serum levels of MMP-2 in DR. The increased MMP-2 activity might lead to uncontrolled ECM proteolysis contributing to the DR development and progression. Thus, an adequate amount of α1-AT is thus required to regulate MMP-2 activity and to maintain the protease and anti-protease homeostasis. In addition, studies have demonstrated that α1-AT also possesses anti-angiogenic property (34) from which it can be inferred that lower anti-angiogenic activity of α1-AT may cause unregulated activity of VEGF. This is supported by the current observation of elevated VEGF levels and lower α1-AT levels in DR patients. Therefore, unregulated VEGF and MMP-2 activity observed in DR patients could be attributed to its decreased anti-proteolytic and anti-angiogenic activity.

Low levels of α1-AT have been reported to correlate with an increased risk of developing T2DM (37). α1-AT has been observed to reduce significantly in T1DM patients (261). Also, Hashemi M *et.al.,* observed that T1DM is linked

to a decrease in plasma trypsin inhibitory ability (261). It has been known to protect pancreatic beta cells against apoptosis (36). In the non-obese diabetic mouse model, it was observed that α1-AT gene therapy reduces cell-mediated autoimmunity, changes the T cell receptor repertoire, and effectively averts T1DM (262). Experimental studies have shown that α1-AT obstructs the development of hyperglycemia in diabetic mouse models and the augmentation treatment with α1-AT has been observed to prevent T1DM and extended islet allograft life (263) (264). In addition to these, multiple clinical trials have been carried out to assess the safety and efficacy of α1-AT (39) (265). Based on the evidence presented above, α1-AT may be considered a potential therapeutic agent in DR.

On calculating the ratios of means of VEGF vs α1-AT and MMP-2 vs α1-AT, it was observed that the ratios were higher in the DR group as compared to T2DM and control. This indicates that the angiogenic factors VEGF and MMP-2 are higher in DR patients as compared to patients with T2DM and control subjects suggesting the increased angiogenic activity in DR. With the first objective it can be concluded that there is an imbalance in the levels of VEGF, MMP-2, and α1-AT in DR patients suggesting their role in the development and progression of the disease.

II. To determine the association between VEGF, MMP-2 and α1-AT in Study groups

Correlation analysis was carried out between VEGF, MMP-2, and α1-AT levels in patients with DR and T2DM. The results showed that the VEGF and MMP-2 correlated positively with each other in DR as well as the T2DM group. This suggests VEGF and MMP-2 act synergistically in DM facilitating its progression. Furthermore, we observed α1-AT negatively correlated with VEGF, indicating a disproportion between angiogenic (VEGF) and anti-angiogenic (α1-AT) molecules. Also, a negative correlation was observed between MMP-2 and α1-AT in both DR as well as T2DM. This observation implies that there is an imbalance in the protease and anti-protease activity in diabetic patients. Thus, based on the obtained results, it can be proposed that a balance between VEGF, MMP-2, and α1-AT is important for maintaining the normal vasculature.

On further correlation analysis carried out between HbA1c and study parameters in DR, it was observed that HbA1c levels were positively correlated with VEGF as well as MMP-2 in DR patients, whereas, α1-AT correlated negatively with HbA1c. The positive correlation between VEGF, MMP-2, and HbA1c indicated that chronic hyperglycemia could induce increased production of VEGF and MMP-2 in DR, which could lead to vascular complications of retinopathy, further facilitating the progression of DR. Whereas, the negative correlation between α1-AT and HbA1c could suggest that decreased levels of α1-AT may be associated with an increased risk of developing retinopathy in diabetic patients.

III. Effect of α1-AT on the levels of VEGF and MMP-2 in Human umbilical vein endothelial cells: An *in vitro* study

VEGF, a key regulator of ocular angiogenesis and vascular permeability is produced in response to the hypoxic condition and therefore is linked with the development and progression of DR (9) (266). The released VEGF induces the neighboring endothelial cells to produce MMP-2 (11) (14) (23). α1-AT a major endogenous inhibitor is known to possess anti-proteolytic (31) and anti-angiogenic activities (34) apart from its other biological functions. Therefore, this *in vitro* experiment was planned to study the effect of α1-AT on VEGF and MMP-2 levels in HUVECs, cultured under hyperglycemic and hypoxic conditions.

It was observed that cells cultured under 15mM and 33mM glucose concentrations had significantly higher levels of VEGF and MMP-2 as compared to the cells cultured under 5mM glucose concentration, suggesting high glucose concentration as facilitating factor for the production of VEGF as well as MMP-2. Also, it was noticeable that cells cultured under 33mM glucose concentration showed significantly elevated VEGF and MMP-2 levels with an increase in the hypoxic condition as compared to cells cultured without hypoxic condition. Our study findings are in accordance with the previous studies conducted. Experimental studies have demonstrated that an increase in the VEGF expression under hypoxic conditions promotes *in vitro* angiogenesis (267) (268). VEGF might be one of the long-awaited mediators that connect retinal ischemia to intraocular angiogenesis. Prolonged overproduction of VEGF by ischemic

retinal cells may increase retinal and iris neovascularization in a variety of neovascular eye diseases, regardless of the source of retinal ischemia (269). The impact of hypoxia on the expression of VEGF and angiogenesis in DR in mice was examined by Zhang D *et al.,* (235). Studies have demonstrated that VEGF-activated endothelial cells induce MMP-2 activity which promotes retinal neovascularization (23).

The findings of the present *in vitro* study support hypoxia conditioned with hyperglycemia as the primary inducer of VEGF and MMP-2 activity. MMP-2 could further facilitate the proteolytic degradation of ECM. Thus, the observations of elevated VEGF and MMP-2 under hyperglycemic and hypoxic conditions suggest their potential role in the development and progression of DR.

When cells cultured under normal glucose concentration (5mM) were subjected to α1-AT treatment at a concentration of 1 mg/mL, it was observed that levels of VEGF and MMP-2 did not differ between the untreated and treated groups. Thus, this observation suggests that normal glucose concentration does not have any effect on VEGF and MMP-2 levels. However, cells cultured under 15mM and 33mM glucose concentrations without hypoxia showed significantly reduced levels of VEGF and MMP-2 post-α1-AT treatment. This suggests that α1-AT in physiological levels may have a protective effect against the damaging effects of VEGF and MMP-2 in diabetic patients.

Increased hypoxic conditions resulted in elevated levels of VEGF and MMP-2 in the present study. However, upon treatment with α1-AT, cells cultured with

33mM glucose concentration and $CoCl_2$ showed significantly reduced levels of VEGF and MMP-2, indicating the protective effect of α1-AT in DR. Considering anti-proteolytic and anti-angiogenic properties of α1-AT, it can be suggested that α1-AT is required for the regulated activity of both VEGF as well as MMP-2. Also, in the present study, a significant reduction in the serum levels of α1-AT in patients with DR suggests that an adequate amount of α1-AT is required to hinder the development and progression of DR.

Summary and Conclusion

The current study determined the levels of VEGF, MMP-2, and α1-AT in DR, T2DM, and control subjects. The levels of VEGF and MMP-2 were significantly higher in DR while the levels of α1-AT were lower as compared to T2DM and control subjects. Also, it is noticeable that VEGF and MMP-2 levels were increased with the severity of retinopathy, in contrast to α1-AT, which was observed to decrease significantly with an increase in DR severity. This indicates an imbalance in the homeostasis of VEGF, MMP-2, and α1-AT in DR patients suggesting their potential role in the development and progression of the disease. The *in vitro* study conducted demonstrated that VEGF and MMP-2 levels were increased under hyperglycemic and hypoxic conditions, however, their levels were decreased post-α1-AT treatment, indicating its crucial role against the development and progression of DR. In conclusion this study suggests that an adequate level of α1-AT is required to control the activities of VEGF and MMP-2, which are responsible for two major events that aids the progression of retinopathy; neovascularization and ECM degradation. Targeting these molecules may hinder the progression of DR and therefore, α1-AT can be approached as a therapeutic agent.

New knowledge generated

This is the first study to evaluate the serum levels of VEGF, MMP-2, and α1-AT together in DR patients. Moreover, this is also the first *in vitro* study to demonstrate the inhibitory effect of α1-AT on VEGF and MMP-2. This new insight adds to the wide range of beneficial effects of α1-AT and thus can be used as a therapeutic approach towards DR.

Limitations of study

There are certain limitations to the study. Different concentrations of α1-AT in *in vitro* study could have provided a better understanding of the optimum concentration required for maximum inhibition of VEGF and MMP-2 levels. Furthermore, genetic analysis for α1-AT could have provided a better explanation for the reduced levels of α1-AT in DR patients.

Recommendations

The underlying mechanisms in DR are complex, involving multiple interconnected processes and signaling pathways, where, α1-AT appears to be effective in blocking several of the most significant pathways associated with them. Based on our findings of significantly decreased α1-AT and its inhibitory effects on VEGF and MMP-2, α1-AT could be recommended as a therapeutic agent in DR; to counteract the damaging effects of enhanced angiogenesis and uncontrolled proteolysis.

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List of presentations and Publication

Presentations:

1. Kuksal Ashansha Mohan, Mamatha Kunder, Sharath Balakrishna. Serum levels of MMP-2 and α1-AT in patients with Diabetic Retinopathy. Poster presented at ABGCON 2021, National virtual conference and workshop; 2021 Aug 27 to Aug 28; Sri Balaji Medical College and Hospital, Chennai.
2. Kuksal Ashansha Mohan, Mamatha Kunder, Sharath Balakrishna. Evaluation of serum VEGF, MMP-2, and α1-AT in Diabetic patients with Retinopathy. Oral session presented at Frontiers in Chemistry-CFC2021; 21st to 23rd October 2021; Reva University, Bengaluru.

Publication:

1. Kuksal AM, Kunder M, Balakrishna S. Imbalance between the Serum Levels of VEGF, MMP-2 and α1-AT in Patients with Diabetic Retinopathy. J Krishna Inst Med Sci Univ 2021;10(3):13-20.

ABGCON 2021

CERTIFICATE OF PRESENTATION

SREE BALAJI MEDICAL COLLEGE AND HOSPITAL & BHARATH INSTITUTE OF HIGHER EDUCATION AND RESEARCH

Department Of Biochemistry
National Virtual Conference & Workshop

This certificate is awarded to Kuksal Ashansha Mohan in recognition and appreciation of presentation of e-Poster on the topic Serum levels of MMP-2 and α1-AT in patients with Diabetic Retinopathy in ABGCON 2021, scientific event organised by DEPARTMENT OF BIOCHEMISTRY on 27th and 28th August 2021 at Sree Balaji Medical College and Hospital, Chennai-44

Dr. B. Shanthi
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Professor & HoD
Biochemistry

Dr. J Thanka
Organising
Vice-Chairman
Vice Principal

Dr. WMS Johnson
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DEAN
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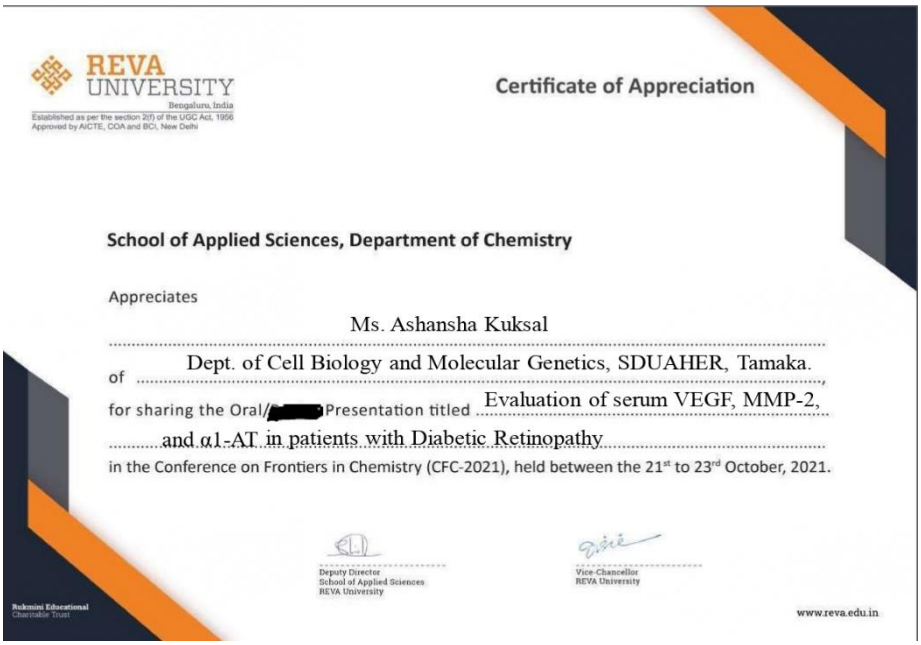

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Approved by AICTE, COA and BCI, New Delhi

Certificate of Appreciation

School of Applied Sciences, Department of Chemistry

Appreciates

Ms. Ashansha Kuksal

of Dept. of Cell Biology and Molecular Genetics, SDUAHER, Tamaka.,

for sharing the Oral/[redacted] Presentation titled Evaluation of serum VEGF, MMP-2, and α1-AT in patients with Diabetic Retinopathy

in the Conference on Frontiers in Chemistry (CFC-2021), held between the 21st to 23rd October, 2021.

Deputy Director
School of Applied Sciences
REVA University

Vice-Chancellor
REVA University

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Annexures

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

1. **Dr. D.E.Gangadhar Rao, (Chairman)** Prof. & HOD of Zoology, Govt. Women's College, Kolar,
2. **Dr. Sujatha.M.P, (Member Secretary),** Assoc. Prof. of Anesthesia, SDUMC,
3. Dr. C.S.Babu Rajendra Prasad, Prof. of Pathology, SDUMC
4. Dr. Srinivasa Reddy.P, Prof. & HoD of Forensic Medicine, SDUMC
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10. Dr. Pavan.K, Asst. Prof of Surgery, SDUMC
11. Dr. Talasila Sruthi, Assoc. Prof. of OBG, SDUMC
12. Dr. Mahendra.M , Asst. Prof. of Community Medicine, SDUMC
13. Dr. Mamata Kale, Asst. Professor of Microbiology, SDUMC

No. SDUMC/KLR/IEC/31/2019-20 **Date:06-06-2019**

PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Ph.D study entitled **"Role of Matris metalloprotease-2 and Alpha1- antitrypsin on Vascular endothelial growth factor levels in Diabetic retinopathy"** being investigated by **Ms. Kuksal Ashansha Mohan,** Dr, Mamatha Kunder1, Dr. sharath.B & Dr. H. Mohankumar2 in the Department of Cell Biology and Molecular Genetics , Bio-chemistry1 & Ophthalmology2 at Sri Devaraj Urs Medical College, Tamaka, Kolar. **Permission is granted by the Ethics Committee to start the study.**

Sujatha .M.P

Member Secretary

Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar.

Chairman

CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar

Note: The same Institutional Ethics Clearance No. can be used for Presentation & Publication. However Presentation to be preceded before Publication.

CASE HISTORY SHEET

Title of the study: Studies on Vascular Endothelial Growth Factor, Matrix Metalloprotease-2 and Alpha 1-Antitrypsin in Diabetic Retinopathy

Case No:	
Patient's Name	
IP/OP Number	
Age	
Gender	M ☐ F ☐
Contact number	
Address	
Phone	
Patient's Name	
IP/OP Number	
Past History	
Hypertension	
Diabetes	
Heart diseases	
Liver diseases	
Renal disorders	
Fundoscopic examination	PDR ☐ NPDR ☐

Biochemical Investigations	
FBS	
PPBS	
HbA1c	
Urea	
Creatinine	
GGT	
ALT	
AST	
ALKP	
CRP	

INFORMATION SHEET

Title of the study: Studies on Vascular Endothelial Growth Factor, Matrix Metalloprotease-2 and Alpha 1-Antitrypsin in Diabetic Retinopathy

Principal Investigator: Ms. Kuksal Ashansha Mohan

Consent for the Interview: Type 2 Diabetes Mellitus is the most common form of diabetes, and is currently one of the major reasons of mortality in the developing world. It is a metabolic disorder caused due to hyperglycemia. Chronic diabetes patients are affected by microvascular and macrovascular complications such as retinopathy, nephropathy, neuropathy, cardiovascular disorder, ischemic heart conditions, etc. The major cause of blindness in patients with the diabetic condition is Diabetic Retinopathy (DR). DR is a disease of the retina and is caused due to damage In the light-sensitive tissue at the back of the eye. Vascular Endothelial Growth Factor (VEGF) is a growth factor whose physiological function is neovascularization and angiogenesis. VEGF also plays a role in abnormal angiogenesis under pathological conditions. Matrix metalloproteinases (MMPs) are the proteases that are responsible for the degeneration and remodeling of Extracellular Matrix (ECM). Alpha 1- antitrypsin (AAT) is the anti-proteases that inhibit MMPs, thus delaying the disease progression. Thus, this study aimed to investigate the relationship between VEGF, MMP-2, and α1-AT in the development and progression of DR.

In this regard, I would like to ask you some questions regarding your present & past health conditions. You do not have to answer any questions that you do not wish to answer & you may end this interview at any time you want to. I will take about half an hour to ask the questions. I would like to take your consent to participate in this study & if you're willing to participate I will be drawing 6ml of blood from you to perform the tests.

Participation in this study does not involve any cost for you. The study is not only beneficial to you but to the community at large. The results gathered from this study will be beneficial in the management of the disease. All information collected from you will be strictly confidential & will not be disclosed to any outsider except if it is required by the law. This information collected will be used only for

research. This information will not reveal your identity. This study has been approved by the local review board & has started only after their formal approval.

There is no compulsion to participate in this study. You will be in no way affected if you do not wish to participate in the study. You are required to sign only if you voluntarily agree to participate in this study. Further, you are at liberty to withdraw from the study at any time. Be assured that your withdrawal will not affect your treatment by the concerned physician in any way. This document will be stored in the safe locker in the Genome Department & a copy given to you for information.

For any further clarification, you are free to contact me.

Name: Miss. Kuksal Ashansha Mohan

Mobile no.: 9860788350

ರೋಗಿಯ ಮಾಹಿತಿ ಹಾಳೆ

ಅಧ್ಯಯನಶೀರ್ಷಿಕೆ: ಡಯಾಬಿಟಿಕ್ ರೆಟಿನೋಪತಿಯಲ್ಲಿ ನಾಳೀಯ ಎಂಡೋಥೆಲಿಯಲ್ ಗ್ರೋತ್ ಫ್ಯಾಕ್ಟರ್, ಮ್ಯಾಟ್ರಿಕ್ಸ್ ಮೆಟಾಲೋಪ್ರೋಟೀಸ್-2 ಮತ್ತು ಆಲ್ಫಾ 1-ಆಂಟಿಟ್ರಿಪ್ಸಿನ್ ಕುರಿತು ಅಧ್ಯಯನಗಳು

ಸಂಶೋಧಕರ ಹೆಸರು: ಮಿಸ್. ಕುಕ್ಸಾಲ್ ಆಶಾನ್ಯಾ ಮೋಹನ್

ಮಧುಮೇಹ ವಿಧ 2, ಒಂದು ಮಧುಮೇಹದ ಸಾಮಾನ್ಯ ಖಾಯಿಲೆಯಾಗಿದೆ, ಮತ್ತು ಪ್ರಸ್ತುತ ಅಭಿವೃದ್ಧಿಶೀಲ ಪ್ರಪಂಚದಲ್ಲಿ ಮರಣದ ಪ್ರಮುಖ ಕಾರಣಗಳಲ್ಲಿ ಒಂದಾಗಿದೆ. ಇದು ಹೈಪರ್ಗ್ಲೈಸೆಮಿಯಾದಿಂದ ಉಂಟಾಗುವ ಚಯಾಪಚಯ ಅಸ್ವಸ್ಥತೆಯಾಗಿದೆ. ದೀರ್ಘಕಾಲದ ಮಧುಮೇಹ ರೋಗಿಗಳು ರೆಟಿನೋಪತಿ, ನೆಫ್ರೋಪಥಿ, ನರರೋಗ, ಹೃದಯರಕ್ತನಾಳದ ಅಸ್ವಸ್ಥತೆ, ರಕ್ತಕೊರತೆಯ ಹೃದಯದ ಪರಿಸ್ಥಿತಿಗಳು, ಮುಂತಾದ ಮೈಕ್ರೊವ್ಯಾಸ್ಕುಲರ್ ಮತ್ತು ಮ್ಯಾಕ್ರೋವಾಸ್ಕ್ಯುಲರ್ ತೊಡಕುಗಳಿಂದ ಪ್ರಭಾವಿತರಾಗಿದ್ದಾರೆ. ಮಧುಮೇಹ ಸ್ಥಿತಿಯಲ್ಲಿರುವ ರೋಗಿಗಳಲ್ಲಿ ಕುರುಡುತನದ ಪ್ರಮುಖ ಕಾರಣವೆಂದರೆ ಮಧುಮೇಹ ರೆಟಿನೋಪತಿ. ಮಧುಮೇಹ ರೆಟಿನೊಪತಿ ಎಂಬುದು ರೆಟಿನಾ ರೋಗವಾಗಿದ್ದು, ಕಣ್ಣಿನ ಅಕ್ಷಿಪಟಲ ಹಿಂಭಾಗದಲ್ಲಿ ಬೆಳಕಿನ ಸೂಕ್ಷ್ಮ ಅಂಗಾಂಶದಲ್ಲಿನ ಹಾನಿ ಕಾರಣದಿಂದ ಉಂಟಾಗುತ್ತದೆ. ಮೆಟ್ರಿಕ್ಸ್ ಮೆಟಾಲೋಪ್ರೋಟೀನೇಸ್ಗಳು (ಎಂಎಂಪಿಗಳು) ಎಕ್ಸ್ಟ್ರಾಸೆಲ್ಯುಲರ್ ಮೆಟ್ರಿಕ್ಸ್ (ಇಸಿಎಂ) ನ ಅವನತಿ ಮತ್ತು ಮರುರೂಪಗೊಳಿಸುವಿಕೆಗೆ ಕಾರಣವಾದ ಪ್ರೋಟಿಯೇಸ್ಗಳಾಗಿವೆ. ಆಲ್ಫಾ-1 ಆಂಟಿಟ್ರಿಪ್ಸಿನ್ ಎಂಎಂಪಿಗಳನ್ನು ಪ್ರತಿಬಂಧಿಸುವ ಪ್ರೋಟೀನ್-ವಿರೋಧಿಗಳಾಗಿದ್ದು, ರೋಗದ ಪ್ರಗತಿಯನ್ನು ವಿಳಂಬಗೊಳಿಸುತ್ತದೆ. ನಾಳೀಯ ಎಂಡೋಥೆಲಿಯಲ್ ಗ್ರೋಥ್ ಫ್ಯಾಕ್ಟರ್ ಒಂದು ಬೆಳವಣಿಗೆಯ ಅಂಶವಾಗಿದೆ, ಇದರ ದೈಹಿಕ ಕ್ರಿಯೆ ನಿಯೋವಾಸ್ಕುಲರೈಸೇಶನ್ ಮತ್ತು ಆಂಜಿಯೋಜೆನೆಸಿಸ್. ರೋಗಶಾಸ್ತ್ರೀಯ ಸ್ಥಿತಿಯಲ್ಲಿ ಅಸಹಜ ಆಂಜಿಯೋಜೆನೆಸಿಸ್ನಲ್ಲಿಯೂ ಸಹ ನಾಳೀಯ ಎಂಡೋಥೆಲಿಯಲ್ ಗ್ರೋಥ್ ಫ್ಯಾಕ್ಟರ್ ಒಂದು ಪಾತ್ರವನ್ನು ವಹಿಸುತ್ತದೆ. ಎಂಎಂಪಿಗಳು ಮೂಲಕ ಆಲ್ಫಾ-1 ಆಂಟಿಟ್ರಿಪ್ಸಿನ್ ಮತ್ತು ನಾಳೀಯ ಎಂಡೋಥೆಲಿಯಲ್ ಗ್ರೋಥ್ ಫ್ಯಾಕ್ಟರ್ ಅನ್ನು ~~ಡಿಆರ್~~ ನ ತೊಡಕುಗಳೊಂದಿಗೆ ಸಂಯೋಜಿಸುವುದು ಈ ಅಧ್ಯಯನದ ಗುರಿಯಾಗಿದೆ. ಈ ನಿಟ್ಟಿನಲ್ಲಿ, ನಿಮ್ಮ ಪ್ರಸ್ತುತ ಮತ್ತು ಹಿಂದಿನ ಆರೋಗ್ಯ ಪರಿಸ್ಥಿತಿಗಳ ಬಗ್ಗೆ ಕೆಲವು ಪ್ರಶ್ನೆಗಳನ್ನು ನಾನು ಕೇಳಲು ಬಯಸುತ್ತೇನೆ. ನೀವು ಉತ್ತರಿಸಲು ಇಚ್ಛಿಸದ ಯಾವುದೇ ಪ್ರಶ್ನೆಗಳಿಗೆ ನೀವು ಉತ್ತರಿಸಬೇಕಾಗಿಲ್ಲ ಮತ್ತು ಈ ಸಂದರ್ಶನವನ್ನು ನೀವು ಯಾವ ಸಮಯದಲ್ಲಾದರೂ ಬಯಸಬೇಕೆಂಬುದನ್ನು ಕೊನೆಗೊಳಿಸಬಹುದು. ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನಾನು ಸುಮಾರು ಅರ್ಧ ಘಂಟೆ ತೆಗೆದುಕೊಳ್ಳುತ್ತೇನೆ. ನಾನು ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನಿಮ್ಮ ಒಪ್ಪಿಗೆಯನ್ನು ತೆಗೆದುಕೊಳ್ಳಲು ಬಯಸುತ್ತೇನೆ ಮತ್ತು ನೀವು ಭಾಗವಹಿಸಲು ಬಯಸಿದರೆ ನಾನು ಪರೀಕ್ಷೆಗಳನ್ನು ನಿರ್ವಹಿಸಲು ನಿಮ್ಮಿಂದ 6 ಮಿಲಿ ರಕ್ತವನ್ನು ತೆಗೆದುಕೊಳ್ಳುತ್ತೇನೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸುವಿಕೆಯು ನಿಮಗೆ ಯಾವುದೇ ವೆಚ್ಚವನ್ನು ಒಳಗೊಂಡಿರುವುದಿಲ್ಲ. ಅಧ್ಯಯನವು ನಿಮಗೆ ಅನುಕೂಲಕರವಲ್ಲ ಆದರೆ ಸಮುದಾಯಕ್ಕೆ ದೊಡ್ಡದಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಸಂಗ್ರಹಿಸಿದ ಫಲಿತಾಂಶಗಳು ರೋಗಗಳ ನಿರ್ವಹಣೆಗೆ ಅನುಕೂಲಕರವಾಗಿರುತ್ತದೆ. ನಿಮ್ಮಿಂದ ಸಂಗ್ರಹಿಸಲಾದ ಎಲ್ಲಾ ಮಾಹಿತಿಗಳು ಕಟ್ಟುನಿಟ್ಟಾಗಿ ಗೋಪ್ಯವಾಗಿರುತ್ತವೆ ಮತ್ತು ಕಾನೂನಿನ ಅಗತ್ಯವಿದ್ದರೆ ಹೊರತುಪಡಿಸಿ ಯಾವುದೇ ಹೊರಗಿನವರಿಗೆ ಬಹಿರಂಗಪಡಿಸುವುದಿಲ್ಲ. ಸಂಗ್ರಹಿಸಿದ ಈ ಮಾಹಿತಿಯನ್ನು ಸಂಶೋಧನೆಗೆ ಮಾತ್ರ ಬಳಸಲಾಗುತ್ತದೆ. ಈ ಮಾಹಿತಿಯು ನಿಮ್ಮ ಗುರುತನ್ನು ಬಹಿರಂಗಪಡಿಸುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನವು ಸ್ಥಳೀಯ ನೈತಿಕ

ಮಂಡಳಿಯಿಂದ ಅಂಗೀಕರಿಸಲ್ಪಟ್ಟಿದೆ ಮತ್ತು ಅವರ ಔಪಚಾರಿಕ ಅನುಮೋದನೆಯ ನಂತರ ಮಾತ್ರ ಪ್ರಾರಂಭವಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಯಾವುದೇ ನಿರ್ಬಂಧವಿಲ್ಲ. ನೀವು ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಬಯಸದಿದ್ದರೆ ನಿಮಗೆ ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನೀವು ಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದ ಸಮ್ಮತಿಸಿದರೆ ಮಾತ್ರ ನೀವು ಸಹಿ ಮಾಡಬೇಕಾಗುತ್ತದೆ. ಮತ್ತಷ್ಟು ನೀವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆಗೆದುಕೊಳ್ಳ ಭಹುದು. ನಿಮ್ಮ ಹಿಂತೆಗೆದುಕೊಳ್ಳುವಿಕೆಯು ನಿಮ್ಮ ವೈದ್ಯರನ್ನು ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಯಾವುದೇ ಚಿಕಿತ್ಸೆಯ ಮೇಲೆ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ ಎಂದು ಖಚಿತಪಡಿಸಿಕೊಳ್ಳಿ. ಈ ಮಾಹಿತಿ ಸುರಕ್ಷಿತ ಲಾಕರ್ನಲ್ಲಿ ತಳಿಶಾಸ್ತ್ರ ಇಲಾಖೆಯಲ್ಲಿ ಮತ್ತು ಮಾಹಿತಿಗಾಗಿ ನಿಮಗೆ ನೀಡಿದ ಪ್ರತಿಯನ್ನು ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ.

ಮತ್ತಷ್ಟು ಸ್ಪಷ್ಟೀಕರಣಕ್ಕಾಗಿ ನೀವು ನನ್ನನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತವಾಗಿರುತ್ತೀರಿ.

ಹೆಸರು: ಮಿಸ್. ಕುಕ್ಸಾಲ್ ಆಶಾನ್ಯಾ ಮೋಹನ್

ಮೊಬೈಲ್ ಸಂಖ್ಯೆ: 9860788350

INFORMED CONSENT

I understand that I remain free to withdraw from this study at any time.

I have read or had read to me & understood the purpose of this study & the confidentiality of the information that will be collected & disclosed during the study.

I have had the opportunity to ask my questions regarding the various aspects of this study & my questions have been answered to my satisfaction.

I agree to participate in this study & authorize the collection & disclosure of my personal information as outlined in this consent form.

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Participant's name & signature/thumb impression Date

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Name and Signature of the witness Date

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Signature of the principal investigator Date:

ಸಮ್ಮತಿಯ ಪ್ರಮಾಣ ಪತ್ರ

ಈ ಅಧ್ಯಯನದಿಂದ ನಾನು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಹಿಂತೆಗೆದುಕೊಳ್ಳಲು ಮುಕ್ತನಾಗಿರುತ್ತೇನೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

ನಾನು ಓದಿದ್ದೇನೆ ಅಥವಾ ನನಗೆ ಓದಿದ್ದಾರೆ ಮತ್ತು ಈ ಅಧ್ಯಯನದ ಉದ್ದೇಶ ಮತ್ತು ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ಸಂಗ್ರಹಿಸಿದ ಮತ್ತು ಬಹಿರಂಗಗೊಳ್ಳುವ ಮಾಹಿತಿಯ ಗೌಪ್ಯತೆಯನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

ಈ ಅಧ್ಯಯನದ ವಿವಿಧ ಅಂಶಗಳ ಬಗ್ಗೆ ನಾನು ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನನಗೆ ಅವಕಾಶವಿತ್ತು ಮತ್ತು ನನ್ನ ಪ್ರಶ್ನೆಗಳಿಗೆ, ನನ್ನ ತೃಪ್ತಿಗೆ ಉತ್ತರ ನೀಡಲಾಗಿದೆ.

ನಾನು, ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಒಪ್ಪುತ್ತೇನೆ ಮತ್ತು ಈ ಸಮ್ಮತಿಯ ರೂಪದಲ್ಲಿ ವಿವರಿಸಿರುವಂತೆ ನನ್ನ ವೈಯಕ್ತಿಕ ಮಾಹಿತಿಯ ಸಂಗ್ರಹಣೆ ಮತ್ತು ಬಹಿರಂಗಪಡಿಸುವಿಕೆಯನ್ನು ದೃಢೀಕರಿಸುತ್ತೇನೆ.

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ಭಾಗವಹಿಸುವವರ ಹೆಸರು ಮತ್ತು ಸಹಿ / ಹೆಬ್ಬೆರಳು ಗುರುತು ದಿನಾಂಕ

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ಸಾಕ್ಷಿಯ ಹೆಸರು ಮತ್ತು ಸಹಿ ದಿನಾಂಕ

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ಪ್ರಧಾನ ತನಿಖಾಧಿಕಾರಿಯ ಸಹಿ: ದಿನಾಂಕ