1.1 Background

Diabetic retinopathy (DR) is considered to be one of the most serious complications and a major cause of blindness in diabetic patients (1,2). It is a vascular disorder affecting the microvasculature of the retina. Based on the presence or absence of neovascularization, retinopathy is broadly classified into non-proliferative diabetic retinopathy or NPDR (early stage with no new blood vessels) and proliferative diabetic retinopathy or PDR (severe form with neovascularization in the retina) (1,2).

A systematic review has reported global prevalence of 34.6% for any DR, 6.96% for proliferative diabetic retinopathy (PDR) and 6.81% for diabetic macular edema (DME) (3). CURES Eye Study has reported 16.6%, 1.6% and 0.9% prevalence for any DR, NPDR and PDR respectively among urban south Indian population (4). There was one cross-sectional study among the north Indian population reporting 28.9%, 23.06% and 5.9% prevalence of any DR, NPDR and PDR, respectively (5).

Several factors such as duration of diabetes mellitus, poor glycemic control, hypertension, hyperlipidemia, nephropathy, anaemia, alcohol consumption and pregnancy have been found to influence the development and severity of diabetic retinopathy (1–3). Hyperglycaemia, a key factor in the development of DR, causes increased flux of glucose through several pathways mainly, hexosamine pathway, aldose reductase and protein kinase C (PKC) pathways which lead to their activation (6–12). Activation of hexosamine pathway can lead to the formation of advanced glycated end products (AGEs) resulting in functional alteration of several proteins. AGEs act by binding to the receptors for AGEs (RAGEs) found in different tissues such as lung, kidney, eye, etc. The soluble form of RAGEs called sRAGEs, formed by alternative splicing of RAGE mRNA or cleavage from membrane bound RAGEs, lack transmembrane domain. They freely circulate in the blood stream and are reported to act as decoy receptors, which prevent binding of AGEs to the membrane bound receptors, thus clearing the AGEs from circulation (7). AGEs were also found to be involved in the formation of reactive oxygen species (ROS). Increased formation of sugar alcohols (such as sorbitol) from aldose reductase pathway can deplete NADPH required for the cell to fight against ROS (8–10). Protein kinase C pathway activation can trigger changes in retinal blood flow, basement membrane thickening,
extracellular matrix expansion and increase in vascular permeability (10–12). All these factors finally cause endothelial cell dysfunction, pericyte apoptosis, microvascular leakage and microaneurysms resulting in retinal ischemia. This can trigger angiogenesis in the retina, a hallmark of diabetic retinopathy (9–12).

The pathogenesis of retinopathy starts with the microvascular linkage and/or microvascular occlusion, which reduces blood flow to the retina. Retinal hypoxia induces expression of hypoxia induced factor-1α (HIF-1α). It is a transcriptional factor for several pro-angiogenic factors, such as vascular endothelial factor-A (VEGF-A), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), etc. (13). One of the key pro-angiogenic molecules produced in response to hypoxia is vascular endothelial growth factor-A. It can activate several other angiogenic factors like, matrix metalloproteinases (MMPs), nitric oxide (NO) and inhibit anti-angiogenic factors such as pigment epithelial derived factor (PEDF) (14). MMPs aid in the degradation of matrix proteins and clear the way for migration of endothelial cells towards hypoxic stimuli (15). PEDF is an anti-angiogenic factor found to inhibit the process of angiogenesis in the retina by down regulating VEGF expression in the absence of hypoxia. During hypoxic condition, MMPs cause proteolysis of PEDF thereby, creating a favorable environment for angiogenesis (14). This leads to the formation of a tuft of delicate and leaky blood vessels in the retina. These vessels easily rupture and bleed into the vitreous cavity leading to reduced visual acuity. Nitric oxide can cause hyperpermeability of the blood vessels, which results in macular edema. This is one of the major causes for declined visual acuity seen in retinopathy. The severe untreated PDR leads to fibrovascular growth into the vitreous, which can lead to retinal detachment, rupture and severe loss of vision (1–3).

Phosphodiesterases (PDEs) are a group of enzymes involved in maintaining vascular tone and normal functioning of the photoreceptor cells. They are found to be involved in the angiogenesis of cancerous tissues. But their role in the development of retinopathy is not yet known.

The mode of treatment for retinopathy is dependent on the severity of the disease and type of lesion. Non-proliferative retinopathy, unless there is a macular edema, is not treated. Tight glycemic control is the goal in such cases to prevent further progression to the proliferative stage (16). Macular edema is treated with either LASER, (when
the lesion is away from the fovea) or anti-VEGF agents (when fovea is involved). For severe NPDR and PDR, only LASER photocoagulation is the available treatment strategy. When retinopathy is complicated with tractional retinal detachment, vitrectomy is done to prevent further loss of vision (17). Pan retinal photocoagulation (PRP) involves inducing small laser burns on the retinal surface, causing death of the peripheral retinal tissue. This destruction of peripheral retina is thought to improve its perfusion by concentrating the available blood flow onto a reduced area of the viable retina (18). The focal/grid laser photocoagulation is used to seal leaky blood vessels and to clear the capillary occlusions in the retina to treat macular edema. Diabetic Retinopathy Study has reported that PRP can decrease the rate of vision loss by more than 50% in patients with proliferative retinopathy (19). Drawbacks of PRP include destruction of peripheral vision, change in colour vision and worsening of retinopathy in few cases (17).

In recent years, several anti-angiogenic molecules such as PKC inhibitors, anti-VEGF agents, tyrosine kinase receptor inhibitors, etc. have been tried and many of them are under clinical trial for treatment of angiogenesis in diabetic retinopathy (18). Anti-VEGF therapy (humanized monoclonal antibody against VEGF) binds and traps VEGF molecules and blocks their action. Bevacizumab is a humanized form of the murine anti-VEGF-A antibody that has been used for the treatment of macular edema (ME). But it is not approved by the United States food and drug administration (USFDA) for treatment of any ocular pathology in humans (18,19). Ranibizumab, an optimized Fab fragment of bevacizumab, can bind and inhibit all isoforms of VEGF-A. It has been approved by USFDA for intraocular use to treat age related macular degeneration (AMD) and macular edema (18,19). Several studies reported complete regression of macular edema in 60-80% of the patients following anti-VEGF therapy (20–22). Various local and systemic side effects such as retinal detachment, increased intra-ocular pressure, endophthalmitis and vitreous haemorrhage had been reported by some of the researchers (22–24).

In case of severe PDR complicated with fibrovascular proliferation and retinal detachment, vitreoretinal surgery or vitrectomy has been recommended. It includes surgical removal of the vitreous fluid from the eye. The Diabetic Retinopathy
Vitrectomy Study has demonstrated a 25% improvement in the visual acuity in the subjects who underwent early vitrectomy versus 15% in the observational group (25).

The outcome measures of treatment in diabetic retinopathy are assessed by fundus examination and visual acuity scores. For focal/grid photocoagulation and anti-VEGF therapy, along with fundus examination and visual acuity scores, retinal thickness is also measured.

There are several studies addressing the vitreous and plasma levels of pro- and anti-angiogenic factors in diabetic retinopathy. Most of these studies have been conducted in developed countries. In India, studies done in Chennai reported increased levels of pro-angiogenic erythropoietin and VEGF-A with decreased anti-angiogenic PEDF in both plasma and vitreous fluid of PDR subjects. Several studies have reported a decrease in the vitreous and plasma levels of VEGF-A and PEDF following anti-VEGF therapy. The therapy also decreased macular thickness (26–29).

With this background, we undertook the study to evaluate the plasma levels of pro- and anti-angiogenic factors, along with oxidative stress in diabetic patients with and without retinopathy. The plasma levels of these factors and oxidative stress were also compared with the outcome of treatment in diabetic patients with retinopathy.

We hypothesized that, Evaluation of these factors could aid in the screening and management of diabetic patients under higher risk of developing diabetic retinopathy. Comparison of these factors before and after the LASER and anti-VEGF therapy could help in predicting therapeutic outcome.
1.2 Aims

- To compare plasma levels of pro- and anti-angiogenic factors in diabetic patients with and without retinopathy
- To compare these factors with treatment outcome in patients with diabetic retinopathy

1.3 Objectives

Primary objectives

1. To compare plasma levels of
   - pro-angiogenic factors (VEGF-A, HIF 1-α, MMP-9) and anti-angiogenic factor (PEDF)
   - Phosphodiesterase (PDE) and nitric oxide (NO)
   - fasting blood glucose and glycated hemoglobin,
   - oxidative stress [sRAGE and malondialdehyde (MDA)] and anti-oxidant (protein thiols) between,

   a. Normal controls
   b. Diabetic patients without retinopathy
   c. Diabetic patients with non-proliferative retinopathy
   d. Diabetic patients with proliferative retinopathy

2. To compare the plasma levels of the above factors with respect to duration of diabetes, type of treatment, glycemic control, body mass index and insulin resistance
Secondary objectives

1. To compare the plasma levels of study parameters before and after LASER and anti-VEGF therapy

2. Correlation of the pre-treatment levels of study parameters with clinical improvement indices in respective treatment groups (LASER and anti-VEGF therapy)

3. Comparison of pre- to post-therapy fluctuations of study parameters in different treatment groups (LASER and anti-VEGF therapy) with respect to treatment outcome