Association Study, Analysis for Genetic and Environmental Interaction of Variants in Candidate Genes for Inflammation (*ICAM-1, CFH*) and Neuroprotection (*PEDF, EPO*) in Type 2 Diabetic Retinopathy Patients from South India

THESIS

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CERTIFICATE

This is to certify that the thesis entitled "Association Study, Analysis for Genetic and Environmental Interaction of Variants in Candidate Genes for Inflammation (*ICAM-1, CFH*) and Neuroprotection (*PEDF, EPO*) in Type 2 Diabetic Retinopathy Patients from South India" submitted by Ms Vinita Kumari, ID No. 2007PHXF427 for the award of Ph.D. Degree of the Institute embodies original work done by her under my supervision.

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LIST OF ABBREVIATIONS/SYMBOLS

AGE	advanced glycation end product
CCL2	chemokine (C-C motif) ligand 2
CFH	complement factor H
CRP	C-reactive protein
DAG	diacylglycerol
DCCT	diabetes control and complications trial
DM	diabetes mellitus
DN	diabetic nephropathy
DR	diabetic retinopathy
DR+	type 2 diabetes subjects with retinopathy
DR-	type 2 diabetes subjects without retinopathy
DRS	diabetic retinopathy study
ECM	extracellular matrix
eNOS	endothelial cell nitric oxide synthase
EPO	erythropoietin
GMDR	generalized multifactor dimensionality reduction
GWAS	genome-wide association study
HbA1c	glycosylated haemoglobin
HDL	high density lipoprotein
ICAM-1	intercellular adhesion molecule-1
IDF	international diabetes federation
IL-6	interleukin-6
IP-10	interferon gamma-induced protein-10
LDL	low density lipoprotein
MCP-1	monocyte chemotactic protein-1
MIP-1ß	macrophage inflammatory protein 1

MODY	maturity onset diabetes of the young
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κß	nuclear factor-kappa-B
NO	nitric oxide
NOS	nitric oxide synthase
NOS3	the gene encoding eNOS
NPDR	non-proliferative diabetic retinopathy
OR	odds ratio
PARP	poly (ADP-ribose) polymerase
PDR	proliferative diabetic retinopathy
PEDF	pigment epithelium derived factor
PKC	protein kinase C
RAAS	renin-angiotensin-aldosterone system
RAGE	receptor for advanced glycation end product
RANTES	regulated on activation, normal T cell expressed and secreted
ROS	reactive oxygen species
SDF-1α	stromal cell-derived factor 1
SNP	single nucleotide polymorphism
STZ	streptozotocin
T2D	type 2 diabetes
T2DR	type 2 diabetic retinopathy
TNF-α	tumor necrosis factor-alpha
UKPDS	United Kingdom prospective diabetes study
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
WHO	world health organization

ABSTRACT

Diabetic retinopathy (DR), a major worldwide cause of permanent visual loss, contributed by numerous risk factors namely increased duration of diabetes mellitus, severity of hypertension, hyperglycemia and exhibits complex inheritance pattern. A substantial variability in the severity of DR, observed in diabetes people, sharing similar environmental and demographic background has been attributed to genetic influences. Pathway centric approaches in the dissection of complex disease etiology, is being explored to identify the potentially interacting factors regulating an individual's risk for the disease. In the current study, single nucleotide polymorphisms (SNPs) in the candidate genes for inflammation (*ICAM-1* and *CFH*) and neurodegeneration (*PEDF* and *EPO*) were selected and tested for interaction along with association in DR.

A total of 401 T2D subjects with ≥15 years of diabetes duration were recruited from Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetic Study (SNDREAMS), an epidemiology project on the prevalence of DR in T2D subjects from south India and the outpatient department of Sankara Nethralaya, a tertiary care hospital, in Chennai, India.

SNPs in *ICAM-1* (rs5498), *CFH* (rs1061170), *EPO* (rs1617640), and *PEDF* (rs12150053, rs12948385) were genotyped by suitable PCR based methods (direct sequencing and RFLP). Multivariate analysis for various clinical covariates was done using SPSS (v. 14). High order gene-gene (GxG) and gene-environment (GxE) interactions were determined using generalized multifactor dimensionality reduction (GMDR) and classification and regression tree (CART) method. Comparative assessment of structural stability of the variants in K469E was assessed using bioinformatics tools like ModellerV97, MuPro, STRIDE etc.

Genotype distributions for all the SNPs were in Hardy-Weinberg equilibrium (>0.05) in both cases and controls. Significant association was observed for the AA genotype of rs5498 of ICAM-1 gene and was frequently

represented in the cases (p = 0.012); OR = 1.94 [95% CI: 1.06-3.55]. The other SNPs however did not show any direct association with DR.

Testing for interaction by GMDR revealed modest interaction between *CFH* and *ICAM-1* genes (p-0.0547). G-E interaction analysis showed significant results for *CFH* and *ICAM-1* variants in 3-locus model with HbA1c levels and insulin user status with a cross-validation consistency (CVC) value of 10/10 and highest testing balance accuracy (TBA) of 60.9% and 60% respectively. CART analysis further confirmed the interaction between *CFH*, *ICAM-1* genes and HbA1c with relative error of 0.638 and ROC of 0.778. HbA1c forms the first split in the CART indicating it to be the strongest risk factor for DR.GMDR and CART analysis for *PEDF* and *EPO* variants indicated insulin user status (IUS), HbA1c levels and blood pressure parameters as potentially interacting with the polymorphisms, specifically for rs12150053 in both single and additive models and blood pressure parameters.

Structural superimposition of *ICAM-1* wild type (K469) and variant (E469) showed 0.943Å of backbone root mean square deviation as calculated by PYMOL software. A difference in the fold rate time was also observed between the wild type (5.4/s) and variant (3.3/s).

Over all the study has attempted a pathway centric analysis of SNPs for the potential role of genetic and environment interaction in the pathogenesis of T2DR using highly recommended high order interaction tools for the first time in literature. HbA1c and insulin user status, among the well known risk predisposing factors for retinopathy in T2D individuals were identified as probable interacting factors with genes involved in inflammatory pathway. The results, however demands replication and also functional validation. The study emphasizes the potential role of interaction analysis in dissecting genetics of complex disorders.

CHAPTER 1

INTRODUCTION: AN OVERVIEW ON DIABETIC RETINOPATHY

Global burden of diabetes and its complications

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia [1, 2]. According to the International Diabetes Federation (IDF), diabetes affects 366 million people worldwide and this number is expected to reach 552 million by the year 2030. This equates to approximately three new cases every ten seconds or almost ten millions per year [1, 3].

Type 1 DM (T1D) earlier referred as insulin dependent diabetes or juvenile-onset diabetes [1], results from a cellular-mediated autoimmune destruction of the ß-cells of the pancreas causing reduction in insulin levels while type 2 DM (T2D) results due to insulin resistance, referred as non-insulin dependent diabetes or adult-onset diabetes [1]. Other forms of DM includes maturity onset diabetes of the young (MODY) and maternally inherited diabetes and deafness (MIDD) [4, 5].

T1D most often occurs in <10 years age and in young adults (<18 years). Its incidence has been reported to be increasing in both rich and poor countries [6]. T2D, a serious global health problem throughout the world, constitutes 85 to 95% of all diabetes [7] due to rapid cultural and social changes, aging populations, increasing urbanization, dietary changes, reduced physical activity and other unhealthy lifestyle and behavioural patterns [7].

Burden of diabetes in India

India ranks first in the list of top ten countries with highest number of diabetics [7-11] that is estimated to increase upto 87.0 million by 2030 (Table 1.1) [7]. There are 97,700 children with T1D in India currently which is more than the relative burden of T2D [12]. Reports from India showed prevalence of diabetes to be higher in urban (5.6%) than rural (2.7%) areas. Moreover the recent transition in socioeconomic status has lead to the increase in prevalence of diabetes in rural areas as well [9, 13].

Table 1.1: Worldwide prevalence of diabetes estimated for the year 2010 and 2030 (reproduced from ref [11])

	2010		2030	
	Country	No. of adults with diabetes (millions)	Country	No. of adults with diabetes (millions)
1	India	50.8	India	87.0
2	China	43.2	China.	62.6
3	USA	26.8	USA	36.0
4	Russian Federation	9.6	Pakistan	13.8
5	Brazil	7.6	Brazil	12.7
6	Germany	7.5	Indonesia	12.0
7	Pakistan	7.1	Mexico	11.9
8	Japan	7.1	Bangladesh	10.4
9	Indonesia	7.0	Russian Federation	10.3
10	Mexico	6.8	Egypt	8.6

The incidence of DM has been shown to increase with age in developed countries compared to the developing nations within the working age group (Figure 1.1) [11].

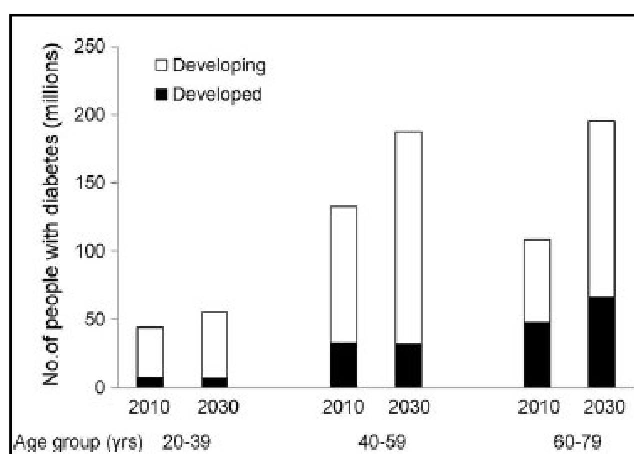


Figure 1.1: Comparison of the distribution of diabetics between developed and developing countries in 2010 and 2030, according to age group [11]

The morbidity and mortality in diabetes arises due to the long-term complications and systemic dysfunction that decrease the life expectancy by an average of 5 to 10 years [10].

Diabetic microvascular complications affect small blood vessels resulting in renal failure (nephropathy), blindness (retinopathy) and distal extremity amputations (neuropathy) mediated by both glycemic and non glycemic factors [10]. The systemic effect of diabetes on large blood vessels (referred as diabetic macrovascular complications) leads to coronary artery disease, peripheral arterial disease and stroke [10].

It has been observed from the Diabetes Control and Complications Trial (DCCT), and United Kingdom Prospective Diabetes Study (UKPDS), that glycemic index, implicated by the level of glycosylated hemoglobin (HbA1c) correlates directly to the severity of diabetic complications in both type of diabetes. Lowering the HbA1c from 8.0% to 7.2% reduced the microvascular complications from 34% to 76% in T1D patients. Additionally the non-glycemic factors including hypertension, smoking and obesity have been shown to contribute to the risk of neuropathy, nephropathy, and cardiovascular diseases [10]. This indicates the cumulative effect of both glycemic and non-glycemic factors in diabetes and its complications and also demands a more holistic approach for disease prevention strategies. [14]

Diabetic retinopathy

Diabetic retinopathy (DR), one of the severe microvascular complications of diabetes, could be sight-threatening when not intervened at the earlier stages of the disease [15]. With the persistent increase in the global prevalence of diabetes, DR pose a major public health concern and has been indicated as the 5th most common cause of blindness in the world, accounting for ~4.8% of global blindness [16] among working aged adults world wide [17].

Retinal physiology underling its vulnerability to diabetes

Structure and function of healthy retina

The human retina constitutes the innermost layer of the eye and functions by processing the light signals to the brain [18]. Retina is composed of 5 major types of cells namely photoreceptor (rods and cones), bipolar, horizontal, amacrine and ganglion cells constituting the ten layers of retina as shown in the figure 1.2 [19].

The retina, partitioned from the systemic circulation by the blood-retinal and blood-aqueous barriers, receives its nutritional supply from the retinal and choroidal circulations and from the ciliary body by diffusion through the vitreous gel [19]. Retina captures the photon, convert the photochemical energy into electrical energy, integrate the action potentials and transmit them to occipital lobe of brain, that further deciphers into recognizable images. Normal vision depends on intact cell-cell communication among the neuronal, glial, microglial, vascular, and pigmented epithelial cells of the retina.

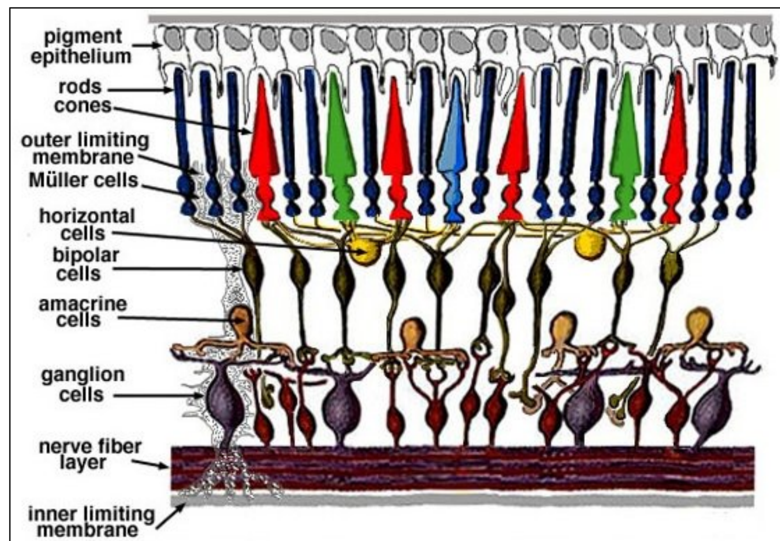


Figure 1.2: Vertical section of the human retina displaying its ten layers (reproduced from ref [20])

In diabetes, the innate retinal physiology remains an important predisposing factor for damages. A sparse retinal vascularity and difference in the oxygen gradient in the retinal layers contrasts the high metabolic demands of the tissue. Also, the density of blood vessels in the inner retina, comparatively lesser than the other tissues, sets a prior hypoxic environment that declines the oxygen gradient. The glial cells of the inner retina possess fewer mitochondria and thus rely on glycolysis which remains as a less efficient machinery for ATP production, when compared to oxidative phosphorylation. ATPs are required for the phototransduction to maintain the ionic gradients across the cell membranes, for neurotransmission at synapses, to replenish the photoreceptor outer segment membranes and to transport proteins and neurotransmitters through axons to the optic nerve. The outer retina however receives its oxygen and nutrients by diffusion from the choroid through the pigment epithelium and is relatively spared from the early insults of diabetes [19,21].

Anatomical lesions in DR

The histological changes of retina in DR include loss of pericytes, basement membrane thickening haemodynamic alterations which eventually lead to the formation of microaneurysms, an earliest visible lesion of DR [22].

Loss of pericytes: Pericytes and endothelial cells resting on the basement membrane (BM) constitute the retinal capillary where the pericytes are shown to provide vascular stability by controlling endothelial proliferation (Figure 1.3). The ratio of pericytes to capillary endothelium (1:1) needs to be maintained in the normal retina to prevent the proliferation of endothelial cells. Loss of pericytes represents an earliest histopathological lesion of DR [22] alters the ratio (~1:10) resulting in acellular capillaries due to the prolonged duration of diabetes [23]. The loss of pericytes have been hypothesized to be mediated by diabetes induced accumulation of toxic products such as sorbitol (polyol pathway flux) or advanced glycation end products (AGEs), oxidative stress and activation of protein kinase C (PKC) [22].

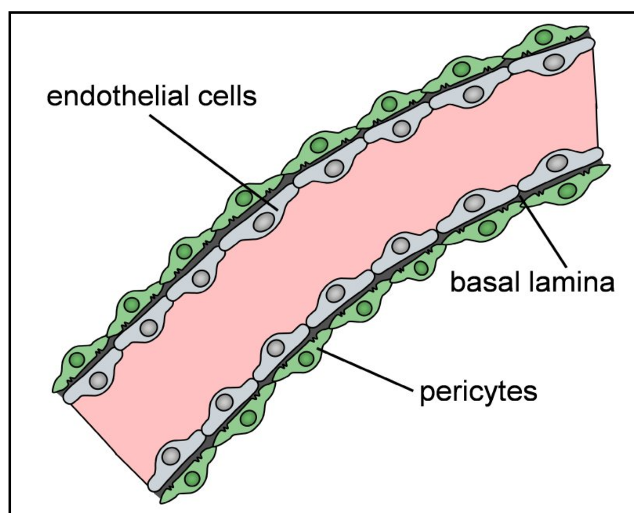


Figure 1.3: Pericytes and endothelial cells shown in the longitudinal section of the blood vessels [24]

Basement membrane (BM) thickening: Vascular BM thickening is a prominent and characteristic lesion in DR, which occurs due to the increased expression and deposition of extracellular matrix (ECM) protein [25]. Fibronectin, an ECM component, that forms the core structure of BM, plays an important role in cell proliferation and migration during wound healing. Its level has been found to be upregulated in the plasma due to the injury of the blood vessels caused by hyperglycemia thus contributing to the structural lesions of DR [26]. Thickening of basement membrane disturbs the retinal hemodynamics.

Microaneurysm: Microaneurysm, an earliest clinical lesion in DR, arises due to dilatation of the retinal capillaries because of the intraretinal hemorrhage (Figure 1.4). It is formed by the weakening and subsequent outpouching of the capillary walls due to fluid leakage. Ophthalmoscopically microaneurysm appears as small, dark red spots referred as “dot hemorrhages”. Though the mechanisms of its formation remains unknown, it is predicted to be formed by vasoproliferative factor or loss of pericytes [25].

Breakdown of blood-retinal barrier (BRB): BRB maintains the homeostasis of retina by preventing the inflow of toxic molecules and outflow

of important ions from the retina. However hyperglycemia activates various cellular mechanisms and molecular pathways causing breakdown of BRB [27]. Increased VEGF expression in diabetes induces ICAM-1 expression that mediates and upregulates leukocyte adhesion to the retinal vasculature, resulting in BRB breakdown.

Diabetic macular edema: This lesion appears because of the abnormal leakage from the retinal capillaries into the retina due to the breakdown of BRB. The lesions, being the most common cause of moderate vision loss in diabetics remain as a greyish area of retinal thickening [25].

Capillary occlusion is another hallmark of early DR presumed to initiate neovascularization. Adherence of leucocytes to the endothelial cells and its entrapment causes capillary occlusion. As a consequence, the migration of leukocytes gets hindered due to the narrow lumen of the capillary channels thus decreasing the blood flow leading to local tissue ischemia. These occlusions appear as cotton wool spots under ophthalmoscope examinations [25]. Retinal capillary occlusion leads to intraretinal microvascular abnormalities and venous calibre irregularities. This leads to the development of new retinal vessels, vitreous haemorrhage, and traction that finally manifests as sight-threatening PDR [25].

Clinical features in DR

Progressive leakage secondary to compromised barrier function of retinal endothelial cells causes macular edema that appears as greyish area of retinal thickening under examination [28]. Clinically significant macular edema (CSME) is defined as retinal thickening with or without hard exudates within the fovea [29]. In addition to this, retinal ischemia due to microvascular occlusion leads to the formation of new fragile blood vessels both at the optic disc called neovascularisation at the disc (NVD) or elsewhere (NVE) [28]. These leaking vessels if not sealed through clinical intervention further progresses to vitreous haemorrhage [28]. Table 1.2 lists the various clinical features observed in DR (28)

Table 1.2: Clinical features in DR [28]

Clinical feature	Description
Microaneurysms	Visible out-pouchings of the fragile blood vessels.
Intraretinal hemorrhage	Results from the ruptured microaneurysms and appear as dot blots if present in the inner nuclear layer of the retina.
Soft exudates	Also known as 'cotton wool spots' formed by the swelling of nerve fiber layers due to impairment of perfusion of the capillaries and ischemia.
Hard exudates	Represent protein and lipid deposits within the retina
Venous beadings	Resemble beads due to alternating thick and thin appearance of the veins
Intraretinal microvascular abnormalities (IRMA)	Distinctive aberrations that affect small blood vessels of the retina
Neovascularization	Growth of new blood vessels to compensate for the ischemia induced oxygen deficit.
Vitreous hemorrhage	Accumulation of blood in the vitreous due to more and more of leakage from the weak newly growing blood vessels

The progressive fibrovascular proliferation leads to blindness due to vitreous haemorrhage and traction retinal detachment. Rubeosis iridis and neovascular glaucoma may occur in rare cases due to the formation of new vessels at the iris and in the anterior chamber drainage angle, leading to a painful blind eye that occasionally requires enucleation [28].

Figure 1.4 shows the fluorescein fundus photos of enrolled study subjects from the SNDREAMS project with typical clinical features of DR including microaneurysm (A), intraretinal hemorrhage (B), hard exudates (C) and neovascularisation.

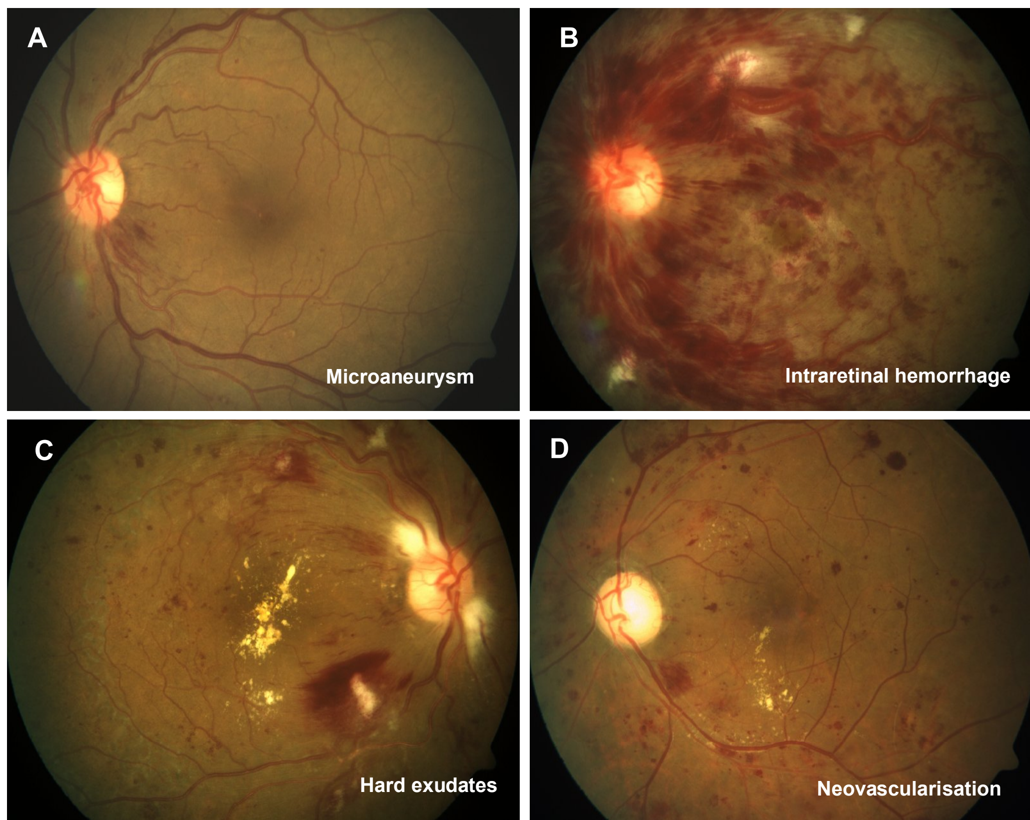


Figure 1.4: Fluorescein fundus angiography (FFA) of retina from DR patient showing (A) microaneurysms, (B) intraretinal hemorrhage, (C) hard exudates and (D) neovascularisation.

Classification of DR

DR has been mainly classified as non-proliferative DR (NPDR) and proliferative DR (PDR) based on the presence or absence of abnormal new vessels. NPDR is being further classified as mild, moderate and severe as per the classification of international American Academy of Ophthalmology (AAO) (Table 1.3) [30].

1. NPDR: The characteristic clinical features include microaneurysm, intraretinal hemorrhages, cotton-wool spots, venous beading and intraretinal microvascular abnormalities (IRMA). These features distinct for the different stages of NPDR (mild moderate and severe) indicate early injuries (Table 1.3) [29].

2. PDR: Refers to neovascularisation of the disc (NVD) or neovascularisation elsewhere (NVE) (Table 1.3) which can cause vitreous hemorrhage, retinal detachment and if left untreated blindness secondary to retinal detachment or neovascular glaucoma [30].

Irreversible vision loss in DR potentially occurs due to macular edema (caused by increased vascular permeability) as seen in CSME and retinal neovascularization (caused by angiogenesis) and seen in PDR [31].

Table 1.3: Classification of DR based on disease severity [30]

DR severity level	Defining features	Clinical implications
No apparent retinopathy	No microvascular lesions	Low risk of progression to sight threatening retinopathy
Mild NPDR	Microaneurysms only	5% microaneurysms seen within a year and 14% within 3 years progress to PDR.
Moderate NPDR	*Microaneurysms* and other microvascular lesions, but not severe NPDR	12-26% (within 1year) and 30-48% (within 3years) progress to PDR.
Severe NPDR	Any of the following: ▪ More than 20 *intraretinal hemorrhages* in each of the 4 quadrants of retina ▪ Definite *venous beading* in 2 or more quadrants. ▪ Prominent *intraretinal microvascular abnormalities* (IRMA) in 1 or more quadrant.	52% (within 1year) and 71% (within 3years) progress to PDR.
PDR	One or more of the following: ▪ Neovacularisation of optic disc (NVD) or elsewhere (NVE). ▪ Vitreous/preretinal hemorrhage. High-risk characteristics include Mild NVD or moderate-to-severe NVD or moderate NVE with or without vitreous haemorrhage	Indication for panretinal photocoagulation; [43] urgent if high-risk characteristics are present
Clinically significant macular oedema (CSME)	Retinal thickening or hard exudates of 500 ìm from centre of macula.	Can develop at any stage of DR; indication for macular laser.

RISK FACTORS FOR DR

Hyperglycemia: The current diagnostic criteria for diabetes by World Health Organisation (WHO) and American Diabetes Association (ADA), based on the glycemic threshold, accurately separates persons at high and low risk of microvascular complications, specifically for the retinopathy signs of diabetes [32]. Poor glycemic control has been shown to cause chronic oxidative stress, a central mechanism for glucose toxicity [33].

Results from two landmark studies DCCT and UKPDS have shown significant benefit of intensive glycemic control (graded based on HbA1C levels) on the development and progression of DR [34]. The change in HbA1c was determined as independent risk factor for the development of DR and nephropathy by the Finnish Diabetic Nephropathy (FinnDiane) Study [34]. Similarly, the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) have shown reduction in incidence and progression of DR. The risk of retinopathy was shown to be lowered by 30–40% with every percent reduction in HbA1c levels (eg, from 9% to 8%) [35] . An increased prevalence of nonproliferative diabetic retinopathy (NPDR) has also been shown in HbA1C category of 6.5–7.0% [36]. However, certain patients with poor glycemic control remain unaffected by the diabetic complications when compared to those with good glycaemic control as observed in the DCCT trial [37].

However in certain patients with poor glycaemic control remain unaffected by the diabetic complications when compared to those with good glycaemic control as observed in the DCCT trial [37]. The level of HbA1c level was strongly related with diabetic complications instead of the type of treatment [38].

Diabetes duration: The diabetes duration, remains as another strongest predictor for DR. The prevalence of DR was found to be directly proportional to the duration of diabetes by many epidemiological studies (WESDR, DCCT); the incidence of developing PDR increased from 0% at 5 years to

27.9% at 14 years and remained stable after 15 years of diabetes. An increased prevalence of DR (87.5%) in patients with greater duration of diabetes (>15 years) when compared with the group which had <15 yr duration (18.9%) was also observed. The Chennai urban rural epidemiology study (CURES) showed 41.8 % of DR patients had > 15 years duration of diabetes and for every five year increase in duration of diabetes the risk for DR increased by 1.89 times [39].

Hypertension: Hypertension exacerbates DR through increased blood flow and mechanical damage of vascular endothelial cells through the release of VEGF. Epidemiological studies WESDR and United Kingdom prospective diabetes study (UKPDS) as well as clinical trials strongly support the role of hypertension as an important modifiable risk factor for DR. According to the UKPDS study, tight blood pressure control reduced the risk of DR progression by about a third, visual loss by half and the need for laser treatment by a third in people with T2D [39].

Dyslipidaemia: An abnormal amount of lipid referred as dyslipidemia has been suggested to play major role in the pathogenesis of DR. The DCCT study has shown a proportional (increased triglyceride) and inverse (HDL cholesterol) correlation of the lipid parameters with the degree of DR severity. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial have shown that fenofibrate, a lipid modifying agent, reduced the need for laser treatment of STDR by 31% in patients with T2D. However this study does not report the effect of the drug on the change in lipid profile and the corresponding mechanism in reducing the risk for DR changes [30].

Genetics: The prevalence of DR among diabetes patients irrespective of the glycemic index suggests the role of genetic factors in regulating DR complications.

The results of few epidemiological studies and clinical trials have shown that the incidences of retinopathy were not altered even after intensive control of glycemia and blood pressure [40-42]. Microaneurysms were also observed

in non-diabetic subjects with normal HbA1c level [43]. Additionally identical twins with diabetes showed same degree of retinopathy [44]. Also a recent study by Hietala et al has shown heritability for the severe manifestation of DR [45]. Thus these observations suggest the role of factors other than hyperglycemia or blood pressure in the development and progression of DR [46]. Population-based studies have also suggested an increased prevalence of DR specific ethnic groups: African Americans, Hispanics, and south Asians in comparison with Caucasians. For example, results from the UK Asian Diabetes Study have shown that after controlling the risk factors of retinopathy people with a south Asian ethnic origin showed high risk for DR than the Caucasians [30]. However no significant difference in the prevalence of DR has been found based on racial or ethnic difference among Chinese, Malays and Indians living in Singapore [47].The Multi-Ethnic Study of Atherosclerosis (MESA) reported moderate differences in the prevalence of DR among different races. The prevalence of DR was higher in African-Americans (36.7%) and Hispanics (37.4%) than in the white Americans (24.8%) [48]. Mexican-Americans and African-Americans have been reported to have higher prevalence and severe retinopathy when compared to non-Hispanic whites [49,50].

GENETICS OF DR

Familial heritability and aggregation in DR: The role of genetics in DR has been demonstrated by familial, twin and transracial studies [51,52]. DCCT study showed 3.1 times increased risk for severe retinopathy in relatives of DR people [52]. A study in south Indian families with two or more siblings with T2D showed three times higher prevalence of DR in siblings of probands with retinopathy [53]. A 3-fold increase in the risk of severe DR for the siblings has been observed.

Genetic association studies in DR: For the past few decades several studies have been undertaken to evaluate genetic links to DR. The search

for genes that might influence the susceptibility to DR has predominantly been undertaken using the candidate gene approach [39].

Candidate gene association study: Genetic variants in candidate genes have been studied for the association with DR in various populations and few of them are represented in table 1.4 [15].

Table 1.4: Candidate genes with the chromosomal location for the different pathways in DR

Gene	OMIM name	Chromosomal location	Role/Pathway
Aldose reductase	*AKR1B1*	7q33	Polyol pathway
Angiotensin I converting enzyme	*ACE*	17q23.3	Renin angiotensin aldosterone system (RAAS)
Erythropoietin	*EPO*	7q22.1	Neovascularisation
Intercellular adhesion molecule-1	*ICAM-1*	19q13.2	Inflammation
Pigment epithelium derived factor	*PEDF*	17p13.3	Anti-angiogenic, neuroprotective etc
Transforming growth factor beta	*TGF-ß*	19q13.2	Neovascularisation
Tumor necrosis factor alpha	*TNF*	6p21.3	Inflammation
Vascular endothelial growth factor	*VEGF*	6p21.1	Neovascularisation

In these studies several polymorphic variants in the promoter, coding, non-coding and other regulatory regions of the genes were analysed for their putative disease associations. Table 1.5 details the list of SNPs identified as susceptible allele for T2DR in various population.

Table 1.5: Genetic variants associated as risk factor for T2DR [54]

Genes	Polymorphism, location	Ethnicity	References
TNFß	Allele 8	Asian Indian	Kumaramanickavel, et al, 2001
ALR2	Z-4 allele for (CA)n repeat, Promoter	Chinese	Lee et al., 2001
TGFß	G (915) C-R 25 P Promoter region/ Signal Peptide sequence	Caucasian	Beranek et al., 2002
VEGF	-634CC, Promoter	Japanese	Awata et al., 2002
iNOS	Pentanucleotide microsatellite or (CCTTT)n, (allele 210bp, 15 repeats)	Asian Indian	Kumaramanickavel et al, 2002
ALR2	Z-2	Asian Indian	Kumaramanickavel et al, 2003
		Caucasians	Petrovic et al, 2005
	C(-106)T	Chilean	.Olmos et al, 2006
VEGF	c.-634CC	Caucasian	Errera et al., 2007
eNOS	Intron 4b/b	West Africa	Chen et al, 2007
eNOS	Haplotype 112 (Glu298/4b/-786C) and 222 (Asp298/4a/-786C)	Tunisian	Ezzidi et al, 2007
VEGF	c.-160 C/C	British	Churchill et al., 2008
	c.-152 A/A		
	c.-116 A/A		
	-160C/-152A/-116A		
	-460C/-417T/-172C/-165C/-160C/-152A/-141A/-116A/+405C haplotype		
	C(-7)T T(-1498)C C(-634)G	Asian Indian	Suganthalakshmi et al, 2006
EPO	Homozygous for T allele of rs1617640, Promoter	Caucasian	Tong et al, 2008
ICAM-1	K469E, EE	Slovenian	Petrovic et al, 2008
Angiotensin convertin enzyme insertion(I) /deletion(D)	DD genotype	Tunisian	Feghhi et al, 2008

Genes	Polymorphism, location	Ethnicity	References
VEGF	-2578 A/A, Promoter	Japanese	Nakamura et al, 2009
PPARG	-2819G	Italian	Costa et al, 2009
VEGF	c.-160CT	British	Churchill et al,2008
	c.-152AG		
	c.-116AG		
VEGF	-160/-152G/-116G	British	
	-460C/-417T/-172C/-165C/-160C/-152A/-141A/-116G/+405G		
EDN1 Lys198Asn	EDN1 Asn/Asn	Chinese	Li et al 2008
eNOS	Haplotype 122 (Glu298/4a/-786C)	Tunisian	Ezzidi et al, 2008
TNF-beta	STR upstream of promoter region : allele 4 (103bp) with (GT)9 repeat	Asian Indians	Kumaramanickavel et al, 2001
Plasminogen activator inhibitor 1	5g/5g	Pima Indians	Nagi et al, 1997
Receptor of AGE	Gly82ser (Ser 82 allele), Exon 3	Asian Indians	Kumaramanickavel et al, 2002

Candidate gene association study in India

In India the genetic preponderance in DR was first described by Rema et al. They found that familial clustering was 3 times higher in sibs of those with DR when compared to those without DR. Subsequently the variants in genes *TNF, iNOS, ALR2, IGF-1, VEGF*, *ICAM-1* etc were studied for their association with DR as given in table 1.6.

Table 1.6: Genetic association studies reported from India [54]

Gene	Polymorphism, location	Risk/Association	References
TNF	(GT_9) and (GT_{13}) promoter microsatellite	Low risk - (GT_9) High risk - (GT_{13})	Kumaramanickavel et al 2001, Rema et al 2002
iNOS	(CCTTT)n	Low risk (CCTTT)13&17 High risk - (CCTTT)13	Kumaramanickavel et al 2002
RAGE	Gly82Ser	Protective	Kumaramanickavel et al 2002
ALR2	Z-2 allele	Risk	Kumaramanickavel et al 2003

Gene	Polymorphism, location	Risk/Association	References
eNOS	VNTR	No association	Suganthalakshmi et al 2006
VEGF	C(-7)T and T(-1498)C	Associated	
eNOS	Intron 4, 27bp, VNTR	No association	Uthra et al 2007
RAGE	-429T/C, -374T/A and 63bp deletion polymorphisms	-374T/A – modestly associated	Ramprasad et al 2007
IGF-1		(CA)18 genotype/Risk	Uthra et al 2007
VEGF	-634C/G 936C/T	Risk No association	Uthra et al 2008
TNF-β	(GT)15 genotype	High risk	Uthra et al 2010
RAGE	Gly82Ser	High risk	
ALR2	(CA)n repeat	High risk	
iNOS	(CCTTT)(n)	High risk	
PRKCB1 (PKC)	-150C/T and -1440G/T	No association	Uthra et al 2010
PEDF	*Exon 4*	Protective	
ICAM-1	*Exon 6*	Risk	Vinita et al 2012

Genome wide association study (GWAS)

GWAS studies in DR have identified many loci associated with DR. Huang et al have identified 5 novel chromosomal regions and *PLXDC2* and *ARHGAP22* genes with susceptibility to DR [55]. These genes are implicated in endothelial cell angiogenesis and increased capillary permeability [55] . Genome-wide meta-analysis by Grassi et al revealed an intergenic polymorphism rs476141 on chromosome 1 between genes, AKT3 and *ZNF238* associated with severe DR in different studies. Of these the *AKT3* regulates cell survival, insulin signaling and angiogenesis after getting activated by both PDGF and IGF-1, both of which have been implicated in PDR. This analysis found an intronic SNP rs10521145 in *CCDC101,* a histone acetyltransferase, gene tagging a copy number region CNVR6685.1 on chromosome 16 by copy number variation (CNV) analysis [56]. However, none of the loci identified in these studies have been previously linked to DR or diabetes, thus suggesting the role of unsuspected pathways in the pathogenesis of DR [55].

Molecular mechanisms in DR

DR shows multifactorial inheritance and involves hyperglycemia mediated changes through polyol pathway, activation of diacylglycerol (DAG)/protein kinase C (PKC) pathway, increased advanced glycation end product (AGE) formation, activation of hexosamine pathway and oxidative stress (Figure 1.5) [57].

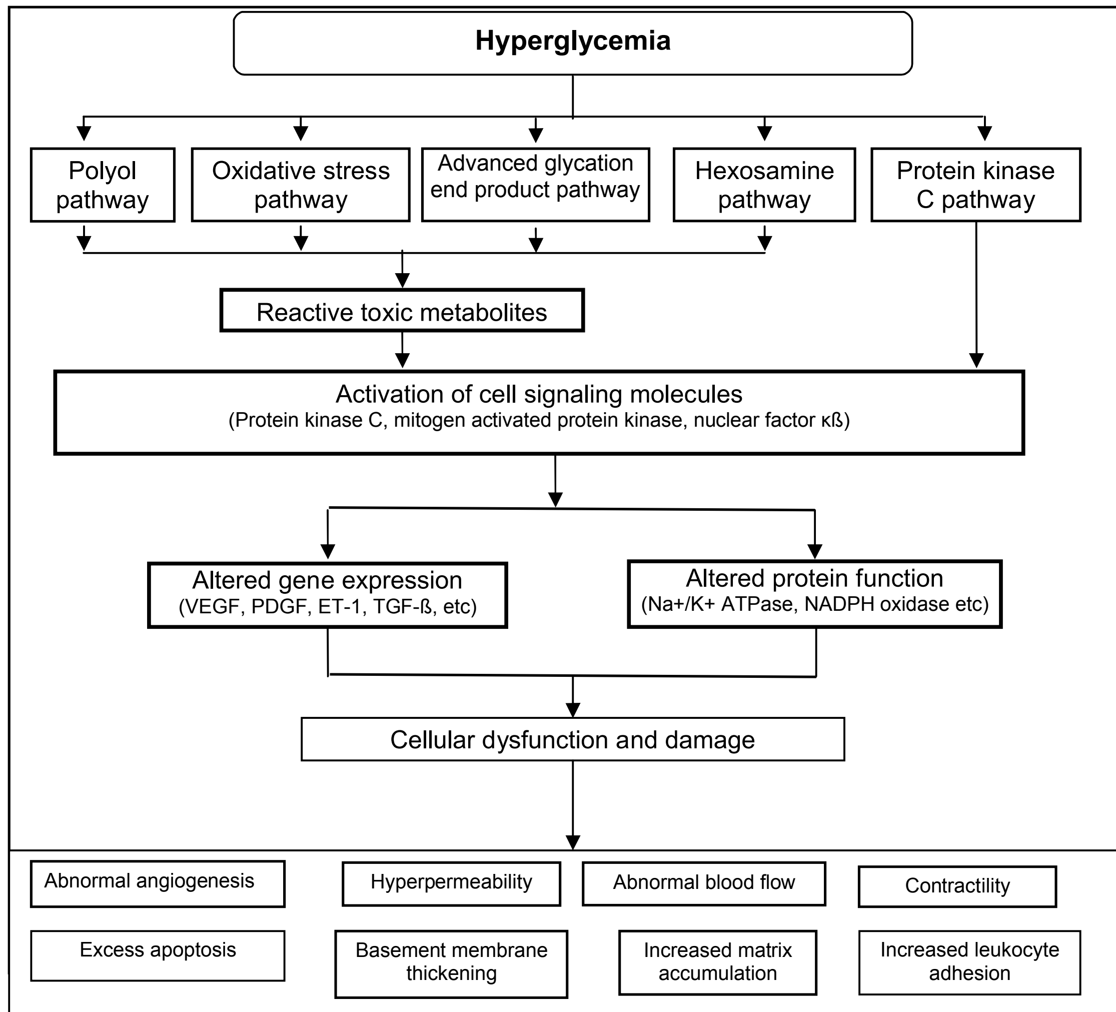


Figure 1.5: Flowchart representation of various mechanisms by which hyperglycemia mediates diabetic vascular complications [57]

Polyol pathway or sorbitol aldose reductase pathway: One of the pathways that metabolizes glucose when its intracellular levels are elevated.

In normoglycemic condition, the glycolytic pathway predominantly metabolizes the cellular glucose and trace amounts of ~3% enters the polyol pathway. In hyperglycemia an increased flux of glucose through polyol pathway accounts for >30% of metabolism. The glucose flux-in through the polyol pathway leads to sorbitol (Figure 1.6) accumulation to produce osmotic stress on cells, decreased Na+/K+ ATPase activity, altered cytosolic NADH/NAD+ ,NADPH ratio, activation of PKC, decreased glutathione and depletion of other antioxidant defences. These metabolic changes cause tissue damage and structural in the retinal vascular changes [58]. However the role of polyol pathway in DR still remains controversial as studies have shown inconsistent results. Animal models of aldose reductase inhibitors (ARI) have shown favorable results by preventing diabetic neuropathy and retinopathy [59,60]. Clinical trials using ARI however has not yielded any successful results on diabetic neuropathy and retinopathy [61].

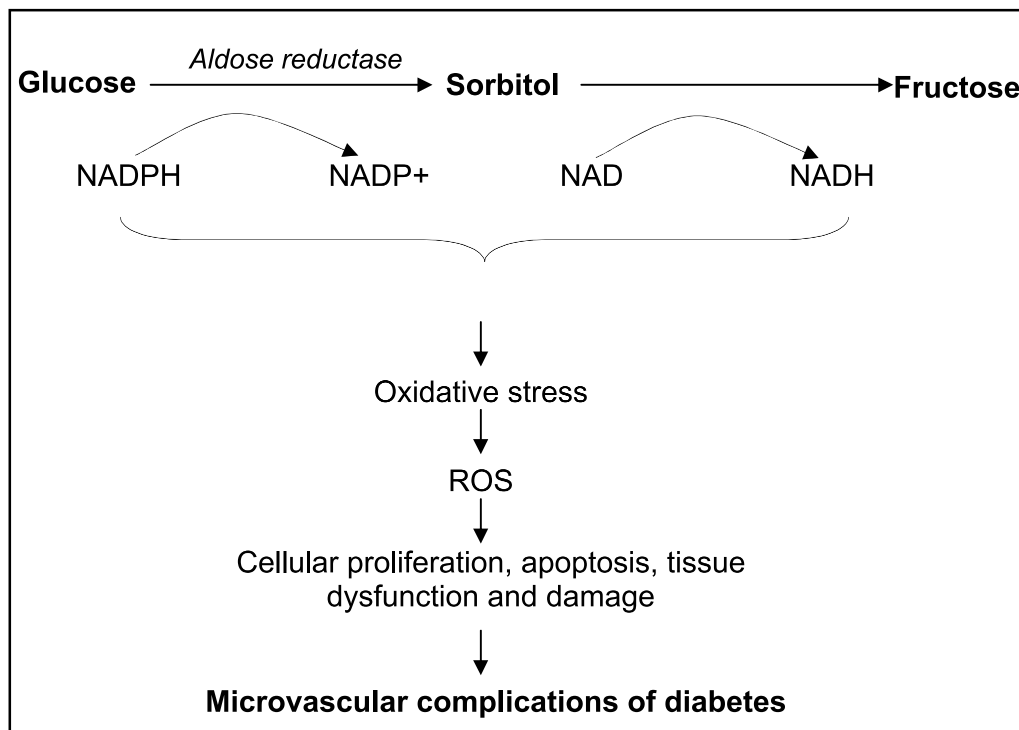


Figure 1.6: Glucose flux through polyol pathway during hyperglycemia [62]

Advanced glycation end product (AGEs) refers to heterogeneous group of molecules generated through the non-enzymatic glycation of proteins, lipids, or nucleic acid (Figure 1.7). AGEs accumulate in the kidney, nerve, retina in a diabetic mileu. They alter the tissue function and mechanical properties through cross linking of the intracellular and extracellular matrix proteins (Figure 1.8). The oxidative stress generated by the interaction between AGEs-RAGE (receptor for AGEs) has also been shown to play important role in DR. Inhibitors for AGE like Aminoguanidine, prevented a range of diabetic vascular complications including retinopathy in animal studies [63] .

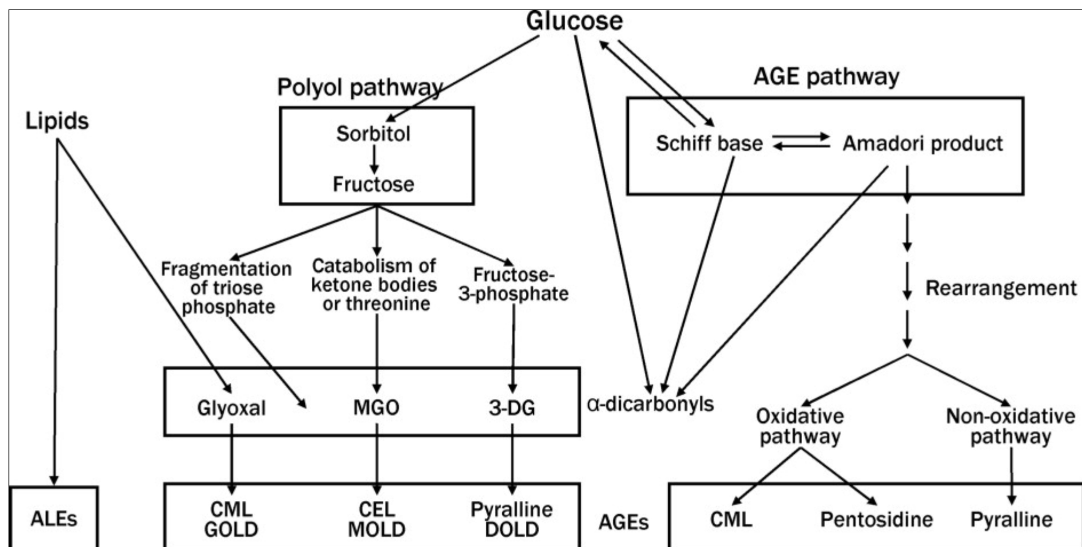


Figure 1.7: Formation of Advance glycation end products mediated by different pathways [64]

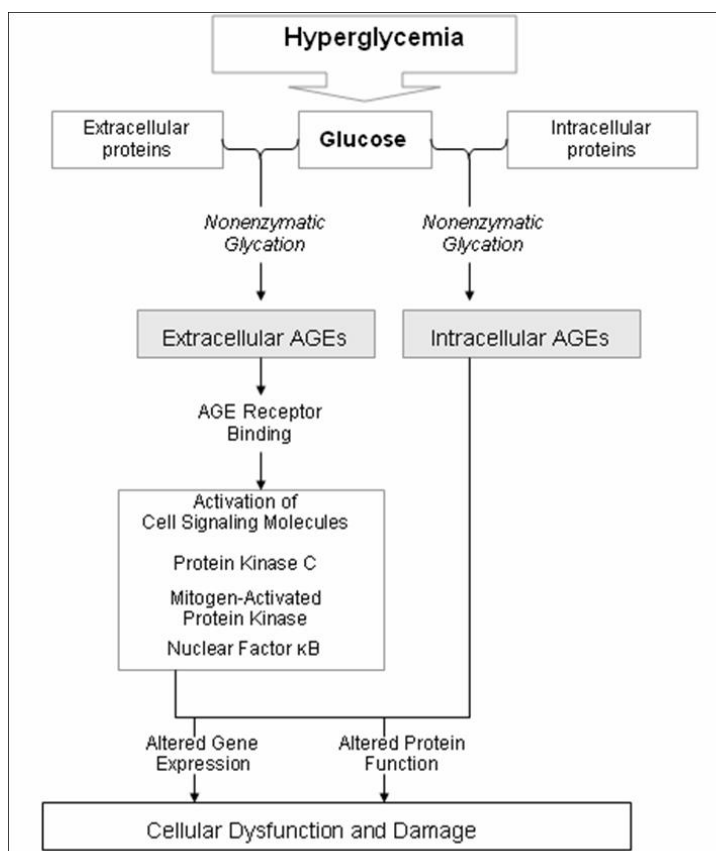


Figure 1.8: Cellular dysfunction due to altered function of AGE [65]

Hexosamine pathway channel the increased glucose flux and has been implicated in chronic hyperglycemia mediated complications The hexosamine pathway merges with glycolysis using fructose D phosphate to form glucosamine-6-phosphate by glutamine fructose-6-phosphate amido transferase (GFAT) enzyme (Figure 1.9) and then rapidly get metabolized to UDP-*N*-acetyl-glucosamine which forms substrates for the synthesis of glycoproteins, proteoglycans, gangliosides and glycolipids. Increased levels of glucosamines due to overexpression of GFAT enzyme causes insulin resistance, an important clinical determinant of DR. The excess glucose flux through the hexosamine pathway also directs the retinal neurons to undergo apoptosis through the perturbation of the neuroprotective effect of insulin mediated by Akt signaling pathway [58].

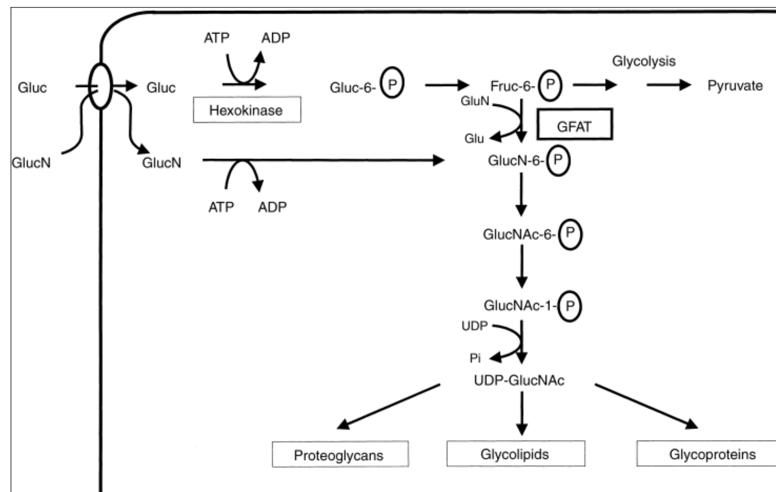


Figure 1.9: The hexosamine pathway [66]

Protein kinase C (PKC) activation

Hyperglycemia stimulates *de novo* synthesis of 1,2-diacyl-glycerol (DAG) in various tissues including vascular cells (Figure 1.10). Increased synthesis of DAG in turn activates PKC (PKC- ß isoforms), implicated in enhanced endothelial permeability, blood flow, retinal leakage, ischemia, neovascularisation, loss of capillary pericytes in DR [42]. Ligation of AGE receptors and increased activity of the polyol pathway also activates the PKC isoforms.

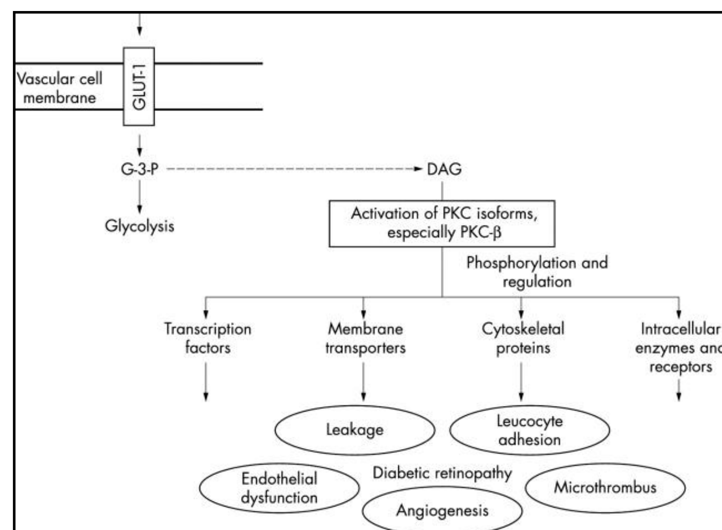


Figure 1.10: Pathway showing protein kinase C activation via diacylglyceral (DAG) and glyceraldehyde-3-phosphate (G-3-P) accumulation [67]

Oxidative stress: Oxidative stress occurs due to the imbalance in reactive oxygen species (ROS) levels and antioxidant potential of the system. The increased production of gluco-oxidants, AGE, by non-enzymatic glycolysis and mitochondrial oxidative phosphorylation, elevated levels of free fatty acid (FFA), signaling cascades, such as PKC mediates the increases ROS production [57] in diabetes condition.

Altered expression of endogenous protective factors: Hyperglycemia mediated metabolic changes causes altered expression of growth factors, mediators of angiogenesis etc like insulin growth factor (IGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) or activated protein C (APC) as well as protective factors (aid in removing the toxic metabolite) like insulin, pigment epithelial derived factor (PEDF), erythropoietin (EPO), somatostatin (SST), neuroprotectin D1 (NPD1), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) etc. These endogenous factors are required for maintaining the vascular homeostasis as well as neutralizing hyperglycemia-induced toxic factors including oxidative stress, AGE or activation of nuclear factor-kB (NF-kB) [57,68] .

Insulin: Insulin aids in maintaining the optimum blood sugar levels in our body via the insulin receptor (IRS) and PI3-kinase signaling pathway. It binds to its receptors and thus translocates Glut-4 transporter to the plasma membrane and causes influx of glucose. It exhibits the vasotropic action on blood vessels through endothelium derived nitric oxide (NO), mediated by the phoshoinositide 3-kinase/protein kinase B (PI3/Akt) signaling. It also provides protection by inhibiting the oxidative stress-induced retinal pericyte apoptosis through the induction of hemeoxygenase-1 (HO-1). The absolute deficiency of insulin defect in its secretion or decreased sensitivity in insulin target tissues causes hyperglycemia in T1D and T2D. Apart from these mechanisms, selective inhibition of PI3/Akt signaling pathway, due to PKC activation has been associated with insulin resistance (IR) mediated vascular dysfunction in diabetes. When given concentration of insulin

provided less-than-expected biological effect leads to IR. Though the exact mechanism causing IR is not fully understood, the dysregulation of fatty acid metabolism, abnormalities of the function and the secretion of adipokines, as well as the increase in stress signaling might contribute to the development of IR [69].

Platelet derived growth factor-B (PDGF-B): PDGF-B is essential for the recruitment of pericytes to the blood vessels and the loss of this factor has been shown to trigger DR development in animal models. DR is characterized by the loss of pericytes that occurs due to persistent hyperglycemia. Hyperglycemia causes activation of PKC to increase Src homology-2 domain containing phosphatase-1 (SHP-1), which in turn causes PDGFR-b dephosphorylation thereby mediating apoptosis of pericytes. Thus PDGF-B plays an important role as retinal vasoprotective factor by maintaining the pericytes survival [57].

Vascular endothelial growth factor (VEFG): VEGF, a major regulator of endothelial proliferation, migration, and survival, also promote angiogenesis. Increased ocular fluid / retinal tissue levels of VEGF have been observed in diabetes probably due to a tissue response to increase survival [57].

Activated protein C (APC): APC, produced when thrombomodulin binds thrombin, is another endogenous protective factor for endothelial cells. This complex catalyses the conversion of protein C to its active form. APC exerts multiple cytoprotective effects including anti-inflammation, anti-apoptotic activities and protection of endothelial barrier function through the endothelial protein C receptor. It has been reported that plasma thrombomodulin levels are elevated in diabetes Patients [57].

The alteration in signal transduction pathways due to hyperglycemia or by the toxic metabolites from glucose metabolism causes multiple vascular and neurological dysfunctions, such as abnormal blood flow, increased rate of apoptosis, hyperpermeability and accumulation of

extracellular matrix (ECM) in vasculature by alteration of gene expression or protein function (Figure 1.5) [57].

It could be thus understood that not single pathway or molecules are solely responsible for the pathophysiology instead cumulatively regulates the physiology in a normoglycemic state [57].

Inflammation and DR

The role of inflammation in diabetes has been hypothesized in 1998 that long-term activation of innate immune system could cause chronic inflammation, which in turn results in T2D. Subsequent studies also showed association of low-grade inflammation with the risk of developing T2D [70,71].

One of the major reasons to elicit the inflammatory response in cell is attributed to the imbalance in the nutritional status of cells which poses metabolic challenges to a cell and makes it susceptible to various inflammatory insults slowly making the cell prone for diabetes related changes [72].

Improvement in the economy and thus the nutrition status in a population have lead to the change from malnutrition status in the past to overnutrition at present, as shown in (Figure 1.11) [73]. The advent of this chronic metabolic overload has lead to the emergence of a new complication called the obesity-linked diabetes known to alter the immune system as well [74].

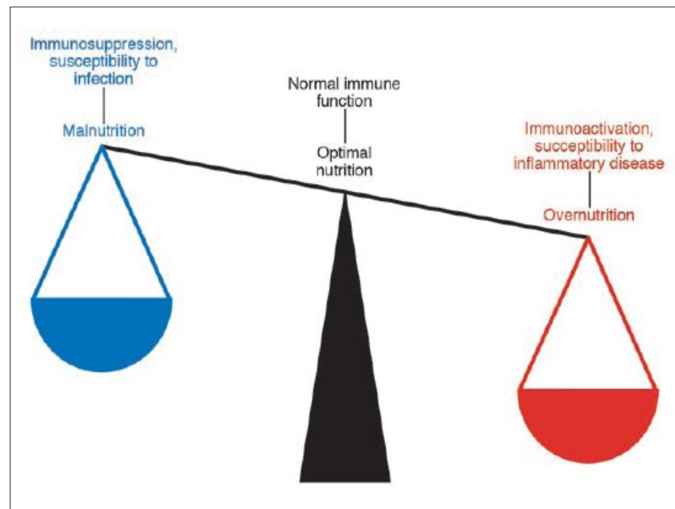


Figure 1.11: Implications of malnutrition and overnutrition on immune system [73]

The pathogenesis of inflammation, involves recruitment and /or activation of leukocytes followed by the action of variety of functional and molecular mediators, typically has beneficial effects under normal physiological conditions; but can have undesirable effects if persisting chronically [75]. There are 3 main stages of inflammation to revive the damaged tissues/cells that includes capillary dilatation to increase the blood flow, structural microvasculature changes that result in the release of plasma proteins, leukocyte recruitment, transmigration and its strong adhesion to the site of injury. The major steps in leukocyte adhesion cascade include capture, slow rolling, firm adhesion and transmigration of the leuckocytes (Figure 1.12) blockage of any of these events can severely reduce the leukocyte accumulation in the tissue.

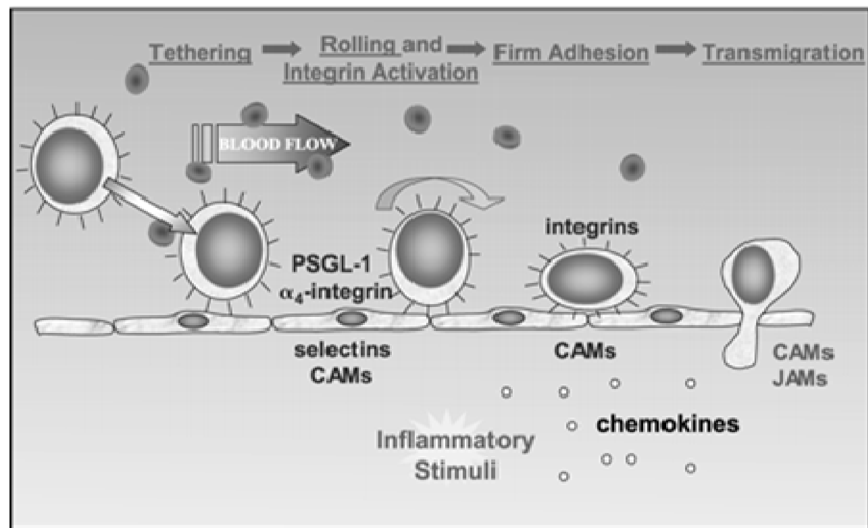


Figure 1.12: Steps involved in adhesion cascade of leukocyte [76]

In addition to these events, increased expression of chemical mediators of inflammation like adhesion molecules, chemokines and others further leads the inflammatory reactions [77].

Adhesion molecules (AM) refer to group of integral membrane proteins (eg; cadherins, Ig superfamily cell adhesion molecules (CAMs), integrins, lectins) located on the cell surface and involved ECM binding. These molecules have cytoplasmic, transmembrane and extracellular domains with the cytoplasmic tail often interacting with cytoskeletal proteins which serve as the actual anchor within the cell (Figure 1.13). The ECM domain of adhesion molecule extends from the cell and bind to other cells or ECM either by homophilic/heterophilic binding or through an intermediary 'linker' which itself binds to other AM [78].

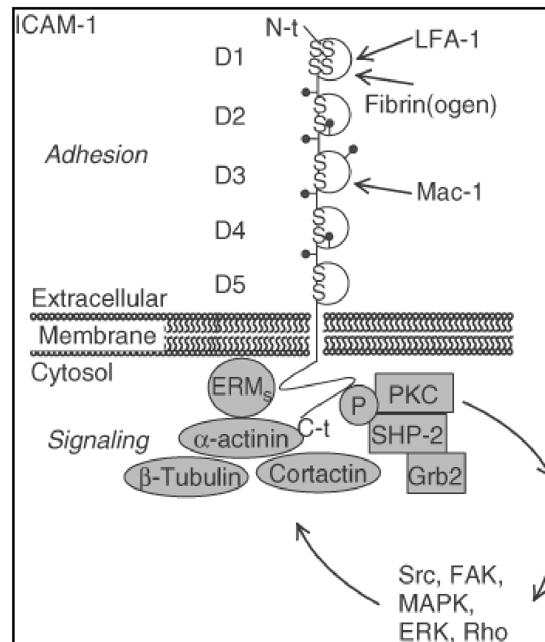


Figure 1.13: A diagram of an ICAM-1 molecule showing binding sites of LFA-1 and Mac-1 [79]

Elevated levels of inflammatory markers including cytokines, chemokines and adhesion molecules have also been observed in the vitreous of DR patients (Table 1.8) [80, 81]. A recent study on diabetic rodents showed an increased level of vascular permeability and caspase-3 activity in the retina [82]. Elevated levels of adhesion molecules were observed in diabetic subjects compared to healthy controls. Funatsu et al (2001) has shown increased levels of interleukin-6 (IL-6) in the aqueous humor of diabetes patients when compared to the non-diabetes controls [83]. Increased levels of leukocyte attractants migration inhibition factor (MIF) and monocyte chemoattractant protein-1 (MCP-1), various inflammatory / proinflammatory mediators, soluble adhesion molecules like sVCAM-1, sICAM-1 etc., have also been observed in the intra ocular fluids of DR patients [84]. Similarly microarray analyses on retinas from diabetic rodents have evidences of inflammatory response [85]. Table 1.7 describes the different clinical features suggesting the role of inflammation in DR.

An inflammatory change in DR has been observed to be mediated through hyperglycemia-induced oxidative stress, dysregulation of NO synthase (NOS), formation of AGEs and production of inflammatory molecules and leukostasis in DR [86]. In addition, recent studies also show that two other risk factors for DR, namely hypertension and dyslipidemia, independently link to inflammation and/or exaggerate the inflammatory reactions induced by hyperglycemia [86].

Table 1.7: Features of inflammation in DR [87]

1	Increased blood flow.
2	Complement activation, FAS ligand upregulation.
3	Microglial cell activation.
4	Increased expression of cytokines and growth factors
5	Increased expression of inflammatory adhesion molecules.
6	Increased leucocyte adhesion.
7	Infiltration of monocytes and neutrophils.
8	Increased vascular permeability.
9	Neovascularization.
10	Accelerated retinal cell death.

Table 1.8 shows common sets of inflammatory biomarkers that are upregulated in both serum (systemic) and vitreous/aqueous (local) samples in subjects with retinopathy.

Table 1.8: Inflammatory biomarkers in diabetic retinopathy

Systemic biomarkers	Local biomarkers
IL-1β, IL-6, IL-8	IL-6, IL-8, IL-10, IL-13, IP-10
TNF-α	TNF-α
VEGF	VEGF
MCP-1, RANTES, SDF-1α	RANTES, MCP-1, MIP-1β
CRP, Selectins, soluble ICAM-1, sVCAM-1	Lipopolysaccharide-binding protein, sCD14

Mechanisms underlying retinal inflammation in DR

The various mechanisms underlying the inflammatory process in retina includes:

(i) Reactive oxygen species (ROS) overproduction: An imbalance in the endogeneous antioxidant system results in oxidative stress to the specific tissues. Increased ROS production by mitrochondria or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, have been demonstrated in diabetes. Hyperglycemia also results in overproduction of superoxide due to enhanced metabolism of glucose-derived pyruvate through the ETC of mitochondria [88].

(ii) Dysregulation of nitric oxide production: The role of nitric oxide (NO) in inflammatory diseases has been indicated depending on the origin (type of nitric oxide synthetase, (NOS) of its expression and the amount produced [89]. Nitric oxide produced by (i) endothelial NOS (eNOS) maintains the blood flow and prevents platelet aggregation and leukostasis; (ii) inducible NOS (iNOS), causes tissue damage and inflammation [89]. In the retina of diabetic mice, iNOS inhibits, ICAM-1, expression and thereafter leukostasis and vascular permeability [90]. In diabetes the NOS pathway is dysregulated thus contributing to the pathogenesis of DR.

(iii) Advanced Glycation End (AGE) product formation: AGEs, byproducts of hyperglycemia have a significant role in retinal inflammation in

DR. Formation of AGEs by non-enzymatic glycation of proteins, lipids and DNA accelerates in diabetes [91] that in turn binds to its receptor (RAGE). RAGE also expressed in retinal cells, when bound by AGE, activates many downstream signaling molecules, including NF-κB and ROS, to induce inflammatory reactions [92]. Activation of NF-κB, increases the expression of other inflammatory genes like IL-*6, iNOS, CCL2, ICAM-1* etc. [93]. Studies have shown enhanced activity of NF-κB is mediated by NADPH oxidase activity in high glucose-treated retinal endothelial cells, pericytes or glial cells and also in the retina of diabetic patient/animal [94]. The NOX2 gene deletions or inhibitions of NADPH oxidase activity prevented diabetes-induced increases in retinal permeability, ICAM-1 expression and leukostasis [95]. Inhibiting AGE formation or blocking AGE with soluble RAGE has shown significant reduction in diabetes-induced ICAM-1 expression, leukostasis and VEGF expression [96].

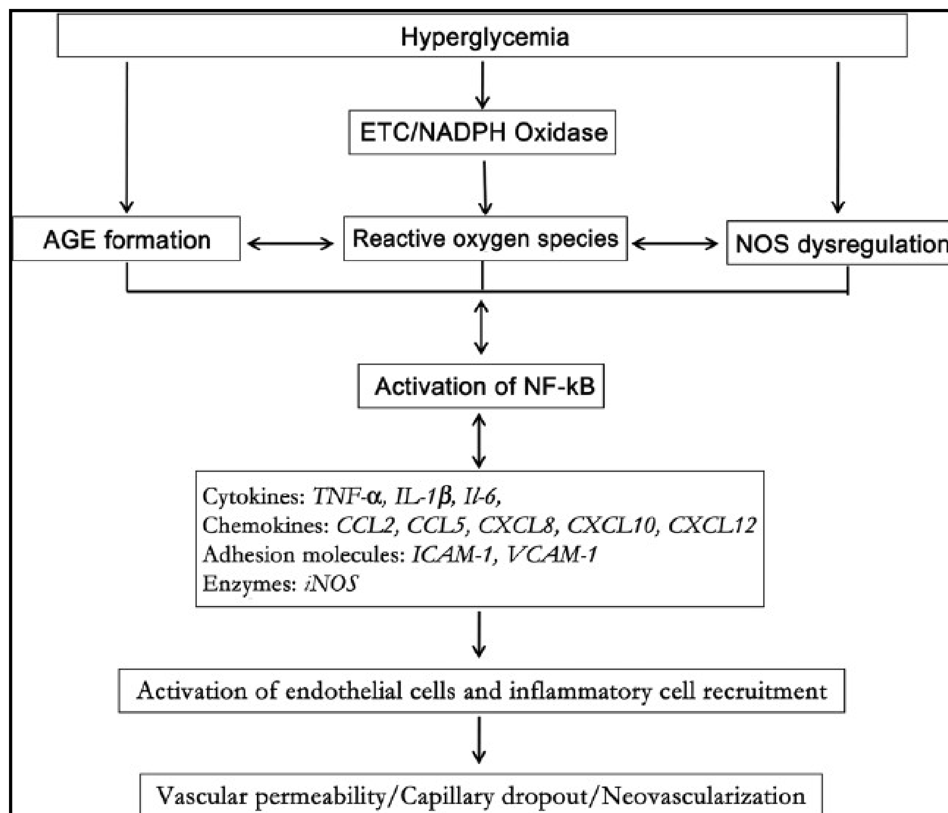


Figure 1.14 Cascade of events induced by hyperglycemia leading to inflammation mediated neovascularization [97]

NF-κB activation, either due to increased ROS production or mitochondria/dysregulation of nitric oxide synthase (NOS) or formation of advanced glycation end products (AGEs) in a hyperglycemic *mileu* (Figure 1.14) increases the expression of proinflammatory molecules that finally activates the endothelial cells which in turn de-arranges the vascular permeability finally leading to neovascularization [97].

Physiological changes in DR mediated by inflammation

Blood-retinal barrier (BRB) breakdown and permeability: Adhesion of leukocytes to the retinal vasculature is one of the earlier events in the diabetic retinal inflammation. Enhanced leukocyte adhesion (leukostasis) has been implicated in the loss of endothelial cells and breakdown of BRB [98]. Breakdown of BRB has been attributed to leukostasis, cytokines and growth factors in the diabetic retinas [99]. These events increase the retinal permeability and results in retinal edema and visual impairment in diabetic patients. Inhibition of leukocyte adhesion has been shown to prevent the loss of pericytes and formation of acellular capillaries, leading to suppression of the BRB breakdown in animal models of DR [100].

Leukostasis: The recruitment of leukocytes from the circulating blood into the tissues is crucial for inflammatory response [101]. Leukocytes role towards the site of microvascular damage in response to the release of cytokines and superoxide thereby causing a local ischemia. They interact and bind to the adhesion molecules (ICAM-1 and VCAM) on the surface of endothelial cells (Figure 1.12) and adhere with the endothelial wall. This interaction (leukostasis) is significantly increased in retinas of diabetic animals is triggered by diabetes-induced oxidative stress, inflammatory molecules, the rennin-angiotensin system etc [101]. An upregulated expression of ICAM-1 in diabetes leads to increase in leukocyte adhesion, thus causing more vascular permeability, BRB breakdown, leukocyte infiltration and finally endothelial cell death [102]. Diabetic mice lacking ICAM-1 and CD18 was found to be protected from developing leukostasis, vascular permeability and degeneration of retinal capillaries, showing the significant role of these molecules in the development of early stages of DR [103].

Intercellular Adhesion Molecule-1 (ICAM-1)

ICAM-1, a biomarker for endothelial cell dysfunction and inflammation, mediates leucocyte influx, leukostasis, vascular leakage, capillary non-perfusion, endothelial injury and cell death subsequently resulting from Fas/FasL-mediated apoptosis in the retina. Its levels are upregulated along with the integrin ligands in patients with DR and retina of animal models [101]. Decrease in the adherent retinal leucocytes have also been observed in *ICAM-1* knock out animal models demonstrating its role in inflammation and DR pathogenesis [103].

ICAM-1 a 90-kD transmembrane glycoprotein located on chromosome 19p13.3 has 7 coding regions (Figure 1.15) and expressed in endothelium, epithelium and fibroblast cells [104, 105]. It belongs to the immunoglobulin superfamily of adhesion molecules and consists of 5 Ig-like domains (D1–D5), a short transmembrane region, and a small carboxyl terminal cytoplasmic domain (Figure 1.16). The extracellular domains mediates interaction with leukocyte β2 integrins, leukocyte function associated antigen (LFA-1, CD11a/CD18), and macrophage-1 antigen (Mac-1, CD11b/CD18). ICAM-1 adheres with (i) LFA-1 at D1 domain (ii) Mac-1 at the D3 domain. The other two molecules ICAM-2 and ICAM-3, have 30% sequence identity with ICAM-1. The cytosolic domain mediates signal transduction upon interaction with the extracellular domains. ICAM-1 mediated signaling includes tyrosine phosphorylation of Src-homology-2 (SH2) domain binds pTyr on (SHP-2) helped by growth-factor-receptor-bound protein-2 (GRB2), PKC activation, phosphorylation of Src kinases, signaling through MAP kinases, Rho-dependent remodeling of the cytoskeleton, activation of cortactin, focal adhesion kinase (FAK), and interaction with α-actinin, ezrin, and moesin, and the microtubule-related protein β-tubulin [79].

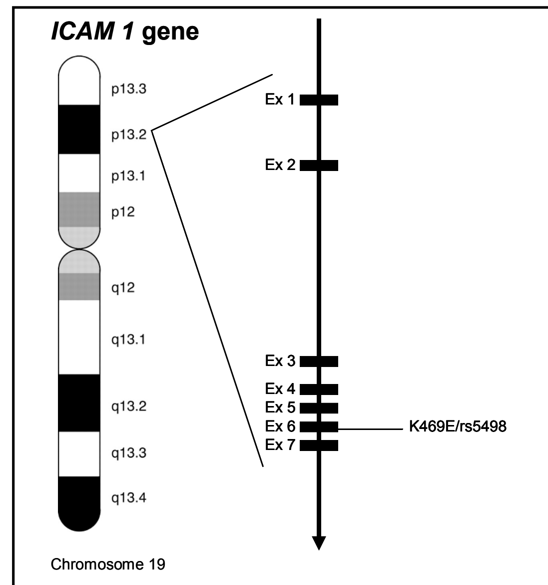


Figure 1.15: Location of K469E polymorphism in *ICAM-1* gene at chromosome locus 19 p13.3

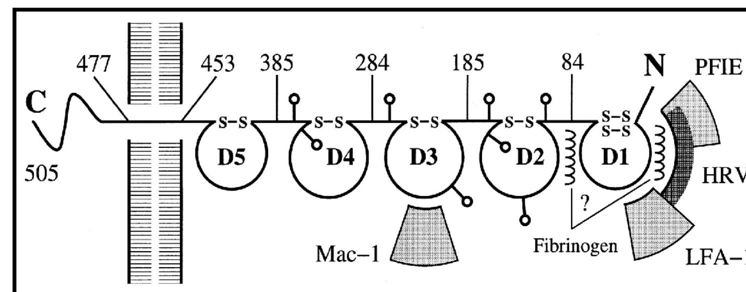


Figure 1.16: A diagramic representation of the various domain of ICAM-1 showing binding sites of LFA-1 and Mac-1 [78]

ICAM-1 and diabetes complications

ICAM-1 proteins in general act as ligands for the integrins and together mediate cell-cell interactions and allow signal transduction. It is targeted by two integrins of the β2 subunit family, i.e. leukocyte adhesion protein-1 (LFA-1) and (Mac-1). The molecule plays a major role in T-lymphocytes activation and leukocyte–endothelial cell interaction. Its role in diabetes has not been completely explored. However recent studies have provided information that ICAM-1 is involved in the pathogenesis of diabetes and complications [106].

Adherence of leukocytes in the retinal vasculature (leukostasis) could be attributed to diabetes-induced increase of ICAM-1/integrins in endothelial cells and leukocytes [103, 107]. Retina from diabetic mice lacking ICAM-1 and CD18 were protected from the development of diabetes induced increase in leukostasis, vascular permeability, and degeneration of retinal capillaries, showing these proteins to be important in the development of early stages of DR [103].

Immunohistochemical studies have demonstrated high expression of ICAM-1 on cell surface in human and animal models [108]. Experiments have also shown prevention of leukostasis after treatment with the monoclonal antibody against ICAM-1. *ICAM-1* knockout diabetic mice did not develop the expected retinal vascular changes [108], strongly indicating the role of *ICAM-1* in diabetes and its complications.

Genetic association of *ICAM-1* polymorphisms in DR

Genetic determinants for diabetic complications have been studied in various candidate genes postulated in the cellular pathways elicited due to hyperglycemia [109].The two coding polymorphisms of *ICAM-1* [Gene ID: 3383] G241R (Exon 4, A>G, rs1799969) K469E (Exon 6, A>G, rs5498) have been extensively studied for the association with various inflammatory disorders including Graves disease, Behcet’s disease, inflammatory bowel disease (IBD), coronary artery disease (CAD) and T1D and complications of diabetes including DR [110-119].

Genetics variant in *ICAM-1* gene have been shown to regulate expression level and have been widely studied for possible genetic association with range of degenerative and inflammatory diseases including diabetic retinopathy (table 1.9). Strong heritability and genetic control on ICAM-1 expression has been observed by genome wide association studies.

The K469E (rs5498) polymorphism in exon 6 of *ICAM-1* gene has been shown to influence the binding of ICAM-1 on endothelial cells and LFA-1 and Mac-1 on leukocytes, mediating leukostasis and its migration in an

inflammatory environment (Figure: 1.15). This domain is essential for the structure and function of ICAM-1. Recent GWAS studies have demonstrated a strong correlation between rs5498 and sICAM-1 levels [120]. In the present study, we have investigated the association of the K469E polymorphism with retinopathy in type 2 diabetes (T2D) subjects from south India.

Table 1.9: Details of genetic study of *ICAM-1* (K469E) associated with inflammatory diseases and cancer in various populations

Diagnosis	Year	Population	Association	Genotype/ Allele associated/ p value
Diabetic Retinopathy	2002, Kamiuchi et al	Japanese	Risk	KK/0.035
	2006, Liu et al	Chinese	Risk	KK
	2008, Petrovic et al	Caucasian	Risk	EE/0.013
Coronary heart disease	2011, Ji et al	Meta-analysis Asian and Caucasians	Risk	K allele
Chronic obstructive pulmonary disease (COPD)	2012, Huang et al	Chinese	Risk	E allele
Gastric cancer	2012, Tian et al	Chinese	Risk	KK
Peripheral arterial occlusive disease (PAOD)	2010, Shaker	Eygptians	Risk	EE
Colorectal Cancer	2009, Wang et al	Chinese	Risk	KK
IgA nephropathy (IgAN)	2009, Yamamoto		Risk	EE
Inflammatory bowel disease (IBD)	2003, Matsuzawa	Japanese	Risk	KK
Graves disease	2003, Kretowski et al.	Polish	Risk	EE

Complement system and complement factor H: In health and diseases

Complement system, a part of innate immune machinery, plays an important role in the inflammatory process [121].

Such inflammatory responses mediated by macrophage and leukocytes include dilatation and leakage of blood vessels that results in redness, warmth, swelling and pain at the site of pathology. Complement system constitutes of multiple plasma and membrane bound proteins produced by the hepatocytes, macrophages and intestinal epithelial cells. These proteins exist as inactive molecules in the circulation that gets converted to proteases upon activation. Complement proteins work in a cascade where the binding of one protein promotes the binding of the other. During activation, the inactive complement components like C3 and C5 are split into two parts. The larger part of the molecule called "b" while the smaller fragment called "a" may diffuse away. In most cases it is the "b" fragment which binds to the surface of the cell to be lysed except for the fragment C2, for which C2a binds to the membrane while C2b is released in the serum or tissue spaces [122].

Complement system gets activated through 3 distinct pathways: classical (CP), alternative (AP) and lectin (LP) (Figure 1.17) initiated by complement 1q (C1q), mannose binding lectin (MBL) and complement 3 (C3) respectively; the endpoint of all the 3 pathways being cleavage of C3 to C3a and C3b, mediated by C3 convertase. C3b leads to the opsonisation of pathogens and forms C5 convertase by associating with C3 convertase which in turn cleaves C5 to C5a and C5b (Figure 1.17). The C3a and C5a mediates the migration of phagocytes to the site of infection, induce the release of histamine and TNF-α, which further augment the inflammatory responses [123].

The activation of the complement system are being regulated by a group of proteins called the complement regulatory proteins (CRegs) [123] and complement factor H (CFH) is one such protein. CFH act as a cofactor

for C3b and regulate the formation of C3 and C5 convertases (Figure 1.17) [123].

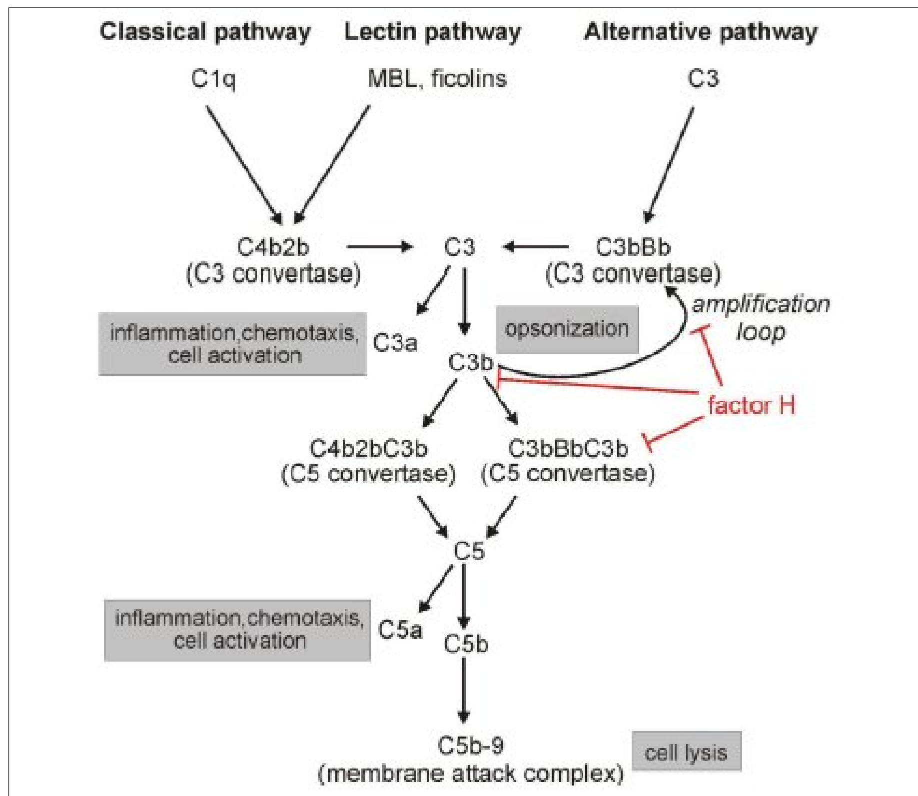


Figure 1.17: The activation of complement pathway depicting the regulatory role of CFH [123]

Inappropriate and excess activation of complement system have been shown to damage multiple organs thus leading to various autoimmune diseases in humans including Alzheimer’s, Huntington’s, Crohn's diseases, schizophrenia, rheumatoid arthritis, atypical hemolytic-uremic syndrome, atherosclerosis, angioedema, macular degeneration, complement-mediated hemolytic anemia, and infertility in both males and females [124]. Additionally it has been implicated in the pathogenesis of the vascular complications of diabetes [125].

Complement system and ocular disorders:

The complement system is being implicated in various ocular diseases including AMD, glaucoma, anterior uveitis and others. There are spontaneous and continuous deposits of complement activation product on self-tissue in small amounts under normal conditions while in larger quantities during inflammatory reactions [126]. Human eye expresses several complement components in various ocular tissues (cornea, uvea, retina) and fluids (tears, aqueous, vitreous humor) [126]. Differential expression of membrane-bound complement regulatory proteins such as membrane cofactor protein (MCP), decay-acceleration factor (DAF) and membrane inhibitor of reactive lysis (MIRL) have been reported in normal human eye [126]. High levels of C1, C2, C3, C4, C5, C6, C7, properdin and factor B observed in the cornea, facilitates its function in protecting the tissues from infections and inflammation [127].

The classical and alternative pathways observed in the RPE, choroid and retinal microglia from mice and humans [128], when activated uncontrollably, damages the photoreceptors and neurons in the retina [129]. Experiment in mice deficient in complement C1q and C3 have provided evidence on the possible role of CP in neurodegeneration [130]. Increased immunolabeling for C1q, C3b, MAC composed of components C5b, C6, C7, C8, and multiple C9 have also been observed in human glaucomatous retina [130]. Study by Tezel et al suggests the role of oxidative stress-related epigenetic factors in the regulation of CFH expression in glaucoma [130]. The activation products of complement system, like C3a, C5a, MAC and regulatory proteins (CFH, CD46, vitronectin, MCP) are also observed in the drusens of AMD [127]. Collectively, these observations provide the supporting evidence for the implication of complement system in various ocular diseases.

Complement pathway in diabetes and its complications

The local immune reactions intervened by complement activation has also been implicated in diabetes and its complication [131]. Some of the complement components have shown altered expression in diabetes and related complications as discussed below.

C4 level are higher in T2D complications like retinopathy and neuropathy thus strongly suggesting, the role of inflammation due to complement activation. Membrane attack complex (MAC) formed due to antibodies mediated complement activation are also implicated in the loss of retinal pericytes in DR [129]. The choriocapillaries and vitreous of DR patients exhibits enhanced C3d and complement factor B. The retinal tissues from diabetic donors also showed reduced level of CD55 and CD59 [127, 132]. CFH, regulated by interleukin-27 (IL-27) levels, plays a critical role in the long term functioning of retina. In mice, IL-27 has been shown to inhibit hyperglycemia and pancreatic islet inflammation [133].

Genetic variants in *CFH* gene (i) regulate its expression like SNPs, in promoter, coding regions rs1061170 (exon 9), rs2274700 (exon 11) and introns (rs1410996 and rs7535263) (ii) mediates surface binding and alter its interaction with cell surface for example the variant Y402H lies within the cluster of positively charged amino acids implicated in the binding of heparin, C-reactive protein (CRP) and M protein [134].

Y402H and eye diseases

CFH gene [Gene ID: 3075] encoding a 155kDa glycoprotein is located in chromosome 1q32 region (Figure 1.18). Various SNP has been identified in *CFH* gene; however the coding variant Y402H (rs1061170) has been exclusively studied as it is located within the binding site for C-reactive protein (CRP) and heparin. CFH has three binding sites for C3b, additional binding site for heparin and C-reactive protein (CRP). It has been postulated that the Y402H variant may lead to decreased binding of CRP and heparin to *CFH* and thus less inhibition of the complement pathway, causing overactivity and deposition of the complement pathway proteins as shown by drusen formation in AMD [127, 135, 136]. Defective function of CFH due to Y402H polymorphism leads to unregulated complement activation at the cell surface [137]. The tyrosine residue at 402 position of the protein is considered as protective while the histidine as risk variant for AMD [137].

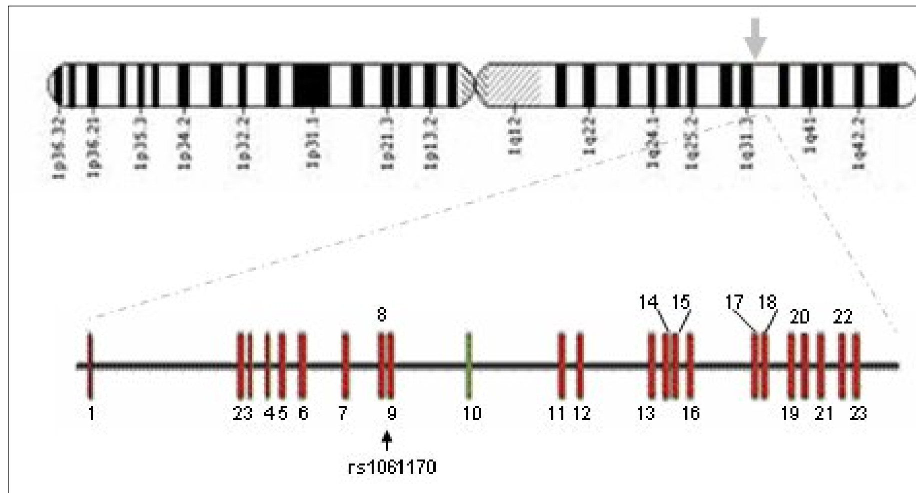


Figure 1.18: Chromosomal location and organization of *CFH* gene on chromosome 1q31.2 with the position of rs1061170 (Y402H) polymorphism

CFH variants have been widely studied for its possible association with retinal diseases including AMD, geographic atrophy (GA), choroidal neovascularization (CNV), polypoidal choroidal vasculopathy (PCV), proliferative DR etc. as shown in table 1.10 [138-141].

Table 1.10: Genetic association studies of polymorphisms with various complex diseases [138-140, 142-144]

Diagnosis	Year/ Population	SNP
Cardiovascular disease (CVD)	2005, Koeijvoets et al	Y402H *(rs1061170)*
Exudative AMD	2005, Souied et al, French	Y402H
AMD	2006, Li et al, Western European	20 SNP showed stronger association than Y402H
Geographic atrophy (GA) and choroidal neovascularization (CNV),	2006, Sepp et al, UK population	Y402H
Polypoidal choroidal vasculopathy (PCV) in patient with AMD	2008, Lee et al, Chinese	rs3753394, rs800292 (exon 2), rs1061170 Y402H
Coronary artery disease (CAD)	2009, Qian et al/Chinese	
PDR (type 1 diabetes)	2012, Toni et al	rs1410996
Coronary artery disease (CAD) Polypoidal choroidal vasculopathy (PCV)	2011, Sakurada et al	rs800292 (I62V)
DR	2010, Balasuubbu et al, Indian	*rs1061170, rs3753394*
AMD (Exudative)	2008, Xu et al/ Chinese	*rs1061170*

The Y402H polymorphism has been identified as a potential biomarker for AMD due to its strong association with the disease in various populations [127, 145]. Additionally, other variants namely rs1410996 (*CFH*), rs2230199 (R102G) and rs1047286 (P314L) of complement component 3 (*C3*) gene, have also been associated with AMD in Caucasians [146].

Smoking, a potential risk factor for many age related eye diseases like AMD has been shown to interact with Y402H of *CFH* in numerous studies [147]. Oxidative stress decreases the complement regulatory proteins in RPE cells, thus indicating the role of environmental factors in the reduced expression of the protein [123]. Though few studies have shown the association of smoking with DR, the exact role of smoking however has not been established for DR [148].

Since CFH, one of the complement regulatory proteins, is required for the long term functioning of retina, which undergoes inflammatory changes in DR, the gene is selected for its possible association and interaction mediating DR.

Neurodegenerative changes in DR

Diabetic retinopathy (DR), earlier thought to be exclusively a microvascular disease of the retina, is now being considered as a neurodegenerative disease [149]. Multiple mechanisms including hyperglycemia, hyperlipidemia, oxidative stress, growth factors etc. are being implicated in DR. The corresponding physiological retinal changes observed in the earliest stages of DR include neural apoptosis, loss of ganglion cell bodies, glial cell reactivity, microglial activation, altered glutamate metabolism and reduction in thickness of the inner retinal layers [150]. Retinal neurodegeneration occurs soon after the onset of diabetes and progress at a steady rate over a long period, resulting in gradual loss of neurons accounting for the chronic vision loss [151]. Impaired vision in DR begins prior to the vascular lesions as demonstrated by ERG recordings of oscillatory potentials, thinning of the inner retinal layer (detected by OCT) in

diabetes patients without clinically detectable retinopathy changes. Experiments using animal models have indicated the role of oxidative stress and inflammation in retinal neuronal tissue in DR pathology [152]. These observations suggests that onset of ND changes precedes microvascular abnormalities in DR [149].

Thus developing strategies to ensure neuroprotection for ocular diseases involving ND remains as a promising treatment modality and many trials are being initiated to address the same [153].

Retina as an extension of central nervous system (CNS)

Retina displays many similarities to CNS in its anatomical structure, functions, response to injury or microbial invasion and immunological responses [154]. Anatomy of the retina indicates a rich inner neural layer referred as the sensory or neural retina that forms the major component of retina as discussed below:

1. Retinal neurons: comprised of photoreceptors, bipolar, horizontal, amacrine, and ganglion cells (Figure 1.19); mediates phototransduction and nerve impulses transmission to the brain [155].

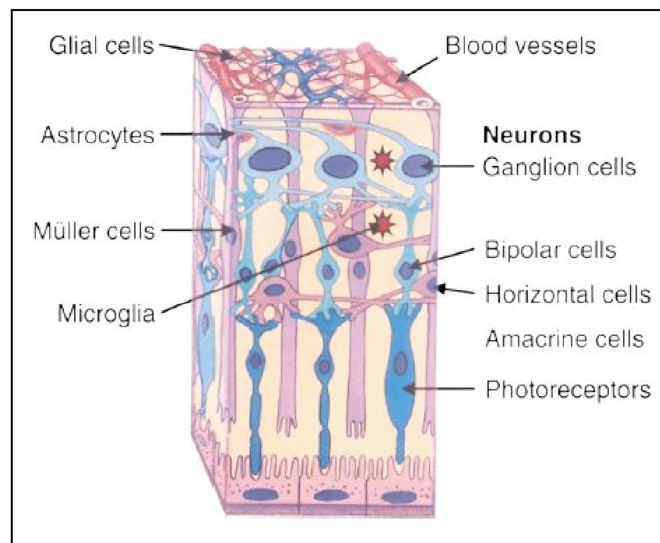


Figure 1.19: Schematic of major types of retinal cells [155]

2. Glial cells include Muller cells and astrocytes which interconnect the neurons with its vasculature, provide nutrition and precursors of neurotransmitters to the neuronal cells (Figure 1.20). Muller cells participate along with the neurons in the glutamate/glutamine cycle to control neurotransmission and protect the neurons from glutamate excitotoxicity [87].

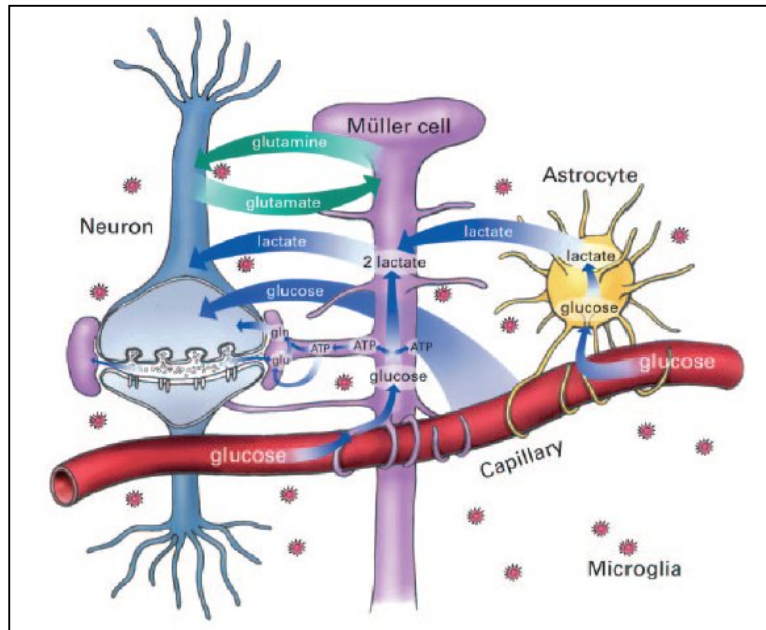


Figure 1.20: Retinal anatomy displaying metabolic interactions between blood vessels, astrocytes, Muller cells, and neurons [87]

3. Microglia consists of macrophages that form the active immune defence by releasing proinflammatory cytokines in response to stress, phagocytosing the necrotic or apoptotic cells, interaction with neurons, glia, and endothelium [87].

Physiology of neural retina in diabetes condition

The unique anatomic and physiologic specialization of retina serves both bidirectionally: facilitates its function on one side and also makes the tissue more vulnerable for diabetes-induced tissue damages [87].

Under normal physiology, the glia and neurons closely interact with retinal vasculature to maintain the homeostasis in the retina. The condition of hyperglycemia disturbs this interaction and compromises the functioning of RGCs and glial cells during early stages of the disease progression as identified by the retinal function tests. Diabetes causes a chronic loss of retinal neurons by increasing the frequency of apoptosis and activating the glial cells [87]. Microglial activation further leads to increased expression of inflammatory cytokines TNF-α which initiates neuronal loss and BRB breakdown in DR [156]. These ND changes mediate the early microvascular changes in DR. Electroretinograms (ERGs), detecting electrical response from ganglion, amacrine and Muller cells, have revealed dysfunction of neuronal cells early in diabetes [157]. The ability of Muller cells to convert glutamate to glutamine reduces due to diabetes as observed in the diabetic retina/ vitreous in rat and human [157].

Astrocytes, another type of glial cells in the eye that wraps the blood vessels of the inner retina maintains the homeostasis with neuronal cells by supplying energy metabolites, mediating neurovascular coupling and neurotransmitter re-cycling. These functions of astrocytes, mediated by gap junctions composed of connexin proteins, which decreases due to hyperglycemia as demonstrated in the retina of 4 weeks old diabetes rats [158]. The quiescent microglial cells found in the retina, activated in hyperglycemic condition, releases pro-inflammatory cytokines and chemokines that activates angiogenic factors. VEGF and tumor necrosis factor, which further exacerbates retinal vascular permeability in diabetes [157].

In addition to these changes, the innate retinal physiology succumbs to diabetes related changes. The oxygen gradient declines from the outer to the inner layer, making it relatively hypoxic due to lower density of blood vessels. In spite of this sparse vascularity and low pO2, the retina has one of the highest metabolic demands than any tissue. ATP is required for phototransduction to maintain the ionic gradients across cell membranes, for

neurotransmission at synapses, to replenish photoreceptor outer segment membranes, and to transport proteins neurotransmitters through the optic nerve. The unmyelinated axons of the retinal neurons require more energy to maintain the membrane potentials. The inner retina however, relies on glycolysis, a less efficient process of generating ATP than oxidative phosphorylation to meet the high energy requirement. The combination of high metabolic demand and minimal vascular supply thus limit the inner retina's ability to adapt to the metabolic stress of diabetes. In addition to this, the supply of oxygen and nutrients to the outer retina, through diffusion from the choroid through the pigmented epithelium is also being affected by the damages to these tissues induced during the early stages of diabetes [87].

The inner neural layer constructed by the 4 types of cells as listed below mediates the process of neuroconduction in the retina [154].

[i] Sensory receptors: First layer near the choroid composed of photoreceptors cells [rods and cones], and various other neurons [99].

[ii] Layer of bipolar neurons: The nerve cells that receive impulses generated by the rod and cone cells and connect them with the ganglion cells.

[iii] Third or inner layer consists of ganglion cells attached directly to the optic nerve.

[iv] The muller cells functions as the supporting tissues in the neuroretina [154].

Pathogenic mechanism in retinal ND

Neurodegenerative changes in the early stages of DR include neural apoptosis, loss of ganglion cells, glial cell reactivity, and activation of microglial cells and reduction of the inner retinal layer thickness [150]. Mechanisms supporting the neuroretinal homeostasis like the blood-retinal barrier, metabolite delivery, and the effect of neurotrophins on the retina are shown to be impaired in DR [159]. The probable mechanisms mediating the above mentioned retinal changes include excitotoxicity due to glutamate accumulation, downregulation of neuroprotective factors in retina, increase

in oxidative stress, neuro-inflammation, loss of insulin-mediated trophic factors, increased vascular permeability due to elevated glucose, injury due to accumulation of excess hexosamines, tumor necrosis factor-α, etc. as discussed below [160].

Glutamate excitotoxicity: Glutamate, an important neurotransmitter is elevated in the retina of diabetes rats as well as in the vitreous of PDR patients. The excessive accumulation of glutamate in diabetes have been shown to be mediated by (i) activated glial cells, (ii) loss of the Muller cell-specific enzyme glutamine synthetase which catalyses the conversion of glutamate to non-toxic amino acid glutamine, (iii) reduction in the retinal ability to oxidize glutamate to α-ketoglutarate, (iv) impairment of glutamate uptake by the glial cells. Thus elevated concentrations of extracellular and synaptic glutamate in the retina lead to 'excitotoxicity' which causes an uncontrolled intracellular calcium response in postsynaptic neurons thus leading to neuro-vascular damage in DR (Figure 1.21) [161]. Higher levels of glutamate due to the impairment of Muller cells (glutamate transporter) by diabetes-induced oxidative stress have been reported in DR [151].

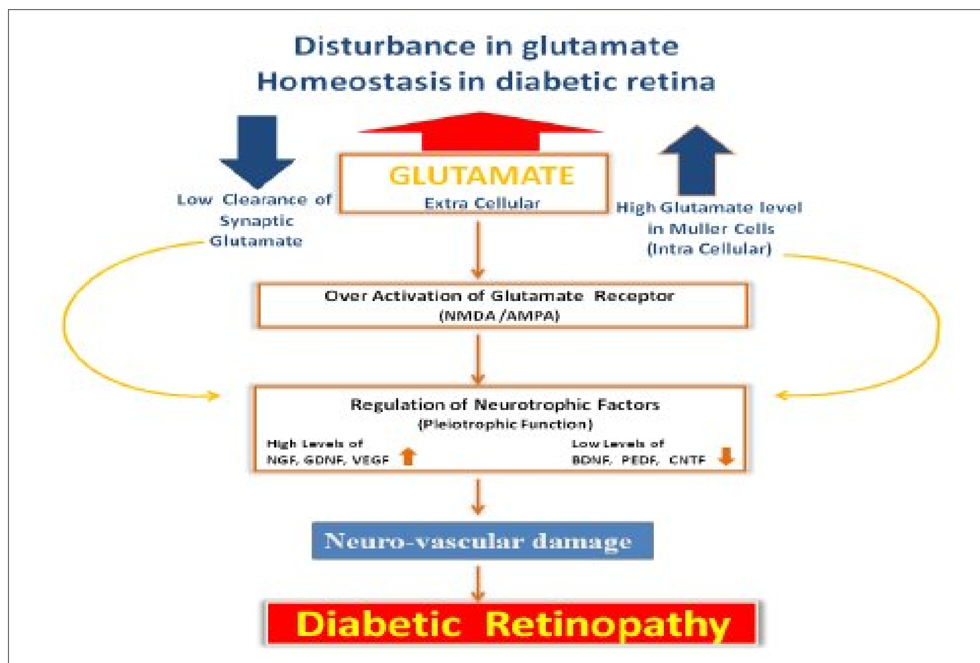


Figure 1.21: Pathway of neurovascular damage in DR due to glutamate exitotoxicity [162]

Loss of neuroprotective factors synthesized by the retina: The neurotrophic or neuroprotective factor promotes neuronal survival, regeneration and protects retina from neurotoxic factors. Therefore a balance between the levels of neurotoxic and neurotrophic factors is necessary to protect the neurons against degeneration [151]. These factors include insulin, pigment epithelial derived factor (PEDF), somatostatin (SST) and erythropoietin (EPO), neuroprotectin D1 (NPD1), brain-derived neurotrophic factor (BDNF), glial cell-line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and adrenomedullin (AM) [162]. Neuronal retina also produces other neurotrophic factors such as nerve growth factor (NGF) and neurotrophin-3 and 4 (NT-3 and NT-4) [162]. Their level gets altered in the retina due to diabetes. The vitreous level of EPO and GDNF are increased in DR patients while the level of PEDF and BDNF are reduced in the vitreous and serum respectively. Diabetes-induced oxidative stress has been suggested to contribute to the neuronal degeneration via reduced formation of neurotrophic factors [163-165].

Elevated oxidative stress: Retina, composed of polyunsaturated fatty acids has the highest oxygen uptake and glucose oxidation relative to any other tissue. Thus making retina more susceptible to oxidative stress which occurs due to an imbalance between excess formation and removal of ROS Hyperglycemia, and impaired antioxidant defense induces ROS formation, which eventually leads to oxidative damage of these biomolecules (lipid, proteins and DNA) as observed in diabetic retina [166]. A reduction in the oxidative stress has also been observed in ND model of experimental DR [161]. Long-term administration of lipoic acid, an anti-oxidant has also shown to prevent the development of DR in rats [167].

Neuro-inflammation refers to the response of neural tissue to injury. It has been often observed in inflammatory and neurodegenerative diseases and can exert beneficial and detrimental effect for the survival of neurons. The systemic increase in oxidative stress indicated by increased accumulation of ROS levels in the retinal neurons, mediates the proinflammatory pathways.

Thus activated retinal neuron produces abundant levels of inflammatory cytokines namely CCL2, ICAM-1, STAT3, CCR, CD44 [161].

Increased vascular permeability due to elevated glucose: Hyperglycemia increases the permeability of retinal vasculature through enhanced expression of various growth factors. VEGF, one of the well studied factors upregulated in the vitreous of diabetic rats as well as in PDR patients. VEGF has been implicated as a survival factor and anti-apoptotic agent for the vascular endothelial cells and neurons. Other angiogenic factors that act as neuronal survival factors include platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-1). Enhanced expression of these factors due to diabetes might have dual role of inducing survival mechanisms in neurons while increasing the vascular permeability and angiogenesis [160].

Neural apoptosis: Diabetes increases the apoptosis of neurons in the inner retina as revealed by histological analysis of retina from streptozotocin induced diabetes rats. The neural apoptosis has been indicated as an earlier event than vascular apoptosis and also remains constant throughout. These changes lead to chronic neuronal degeneration. Studies in diabetic mice model has shown increased TUNEL positive, active caspase 3 immunoreactive cells in the RGC that showed cummulative loss along with amacrine cells. Data from human retina showed increased apoptotic markers in the inner retina. Glutamate excitoxicity, oxidative stress, inflammation, reduced growth/trophic factors were identified as the cause of neural apoptosis in DR [150, 162].

Injury due to accumulation of excess hexosamines and tumor necrosis factor-α: Excessive glucose flux through the hexosamine pathway has been implicated to cause insulin resistance and may also direct apoptosis in the retinal neurons via perturbation of the neuroprotective effect of insulin mediated by Akt and via induction of apoptosis possibly by altered glycosylation of proteins [168].

Genetic studies on neuroprotection and neurodegeneration

Genetic variants in neuroprotection and neurodegeneration genes like *PEDF*, *EPO* have been studied for their putative association with DR in various populations including Japanese, Austrian, European, Australian [169-171]. ATP-binding cassette transporter A1 (ABCA1), *Clusterin* (rs11136000) and *APOEε4* gene have been associated with Alzheimer's disease in Chinese population [172, 173]. However the polymorphism in the *Notch4* gene was not associated with Alzheimer's disease [174].

The polymorphism in angiotensin II receptor, component of renin-angiotensin system, that has neuroprotective role and has been studied with glaucoma and cardiovascular disease. The polymorphism AGTR2/3123C>A in angiotensin II receptor gene has been significantly associated with the risk for normal tension glaucoma [175]. While AT1R-A1166C (rs5186) polymorphism of angiotensin II type 1 receptor (AT1R) has been increased risk of coronary heart disease (CHD) [176].

Role of variants in pigment epithelium derived factor (*PEDF*) and erythropoietin *(EPO*) gene involved in neuroprotection in DR.

Physiological role of PEDF in normal and disease

PEDF or SERPINF1, a multifunctional glycoprotein, exerts multifunctional role namely neurotrophic, anti-angiogenic, anti-tumorigenic, anti-inflammatory, antioxidative effect in cells and regulate their normal functioning. This gene is expressed in the CNS, retina and in systemic tissues and biological fluids of the body including vitreous and aqueous humor. PEDF, exhibiting protective effects on various neuronal cells, have been found in abundance in the retinal pigmented epithelium (RPE) and Muller glial cells [177]. PEDF, a 50kD glycoprotein (418 amino acids) binds to the glycosaminoglycans of the interphotoreceptor matrix to affect the underlying neural retina after getting released from RPE. PEDF acts as a

survival factor for cultured cerebellar granule cells, spinal motor neurons and hippocampal neurons [177].

Altered PEDF levels were reported in various ocular diseases; upregulated in uveitis [178, 179] and reduced in AMD patients with CNV, PDR [180] and early phase of experimental DR in mice models [181, 182].

PEDF protect neurons from glutamate mediated excitotoxicity. Diabetes induced retinal vascular hyperpermeability were prevented by PEDF administration which inhibited the NADPH oxidase-driven oxidative stress in diabetic rats [183]. PEDF knock out models also demonstrated an increased microvessel density in the retina [177]. Human PEDF (hPEDF) gene has also shown to be effective in preventing retinal ganglion cell death for diseases like glaucoma by intraocular gene transfer [184]. These evidences support the neurotrophic role of PEDF in DR.

Altered PEDF levels in DR

Comparison of the vitreous humour protein profiles between PDR and normal human eyes have also shown reduced PEDF levels [185]. This reduced level has been shown to induce neuroglial cell toxicity. Ogata et al. (2001) reported increased levels of PEDF in the vitreous of patients with rhegmatogenous retinal detachment, thus signifying the neuroprotectant role of PEDF for the detached retina. Clinical evidence has shown that lower vitreous concentration of PEDF is related to higher retinal vascular hyperpermeability and aggravation of DME [183].

Genetic association study of DR with *PEDF* gene polymorphisms

The gene spans 15.6kb length on chromosome 17p13.3 with 8 exons (Figure 1.22) [186]. Genetic variations of *PEDF* gene have been studied for their association with diabetic microangiopathy/retinopathy and age related macular degeneration (AMD), polypoidal choroidal vasculopathy (PCV) in various populations [187-189].

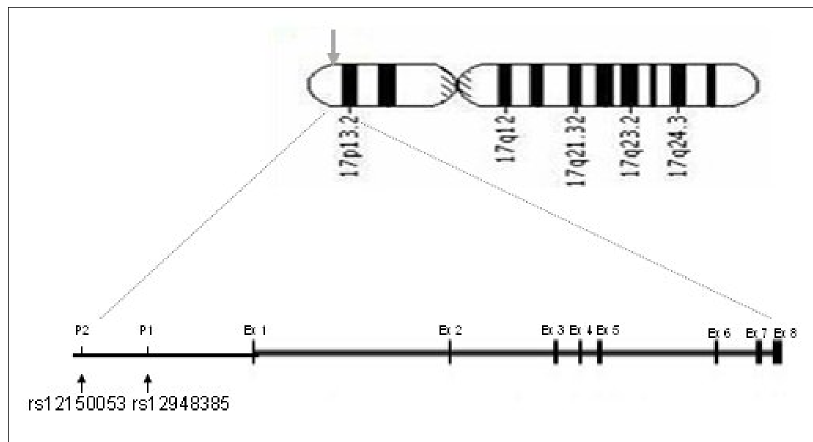


Figure 1.22: Organization of *PEDF* gene in 17p13.3 region

PEDF promoter polymorphisms -5736T>C (rs12150053) and -5304G>A (rs12948385) (Figure 1.22) studied in Japanese population showed the TC/CC genotypes of rs12150053 and GA/AA genotypes of rs12948385 to be a significant risk factor for DR [169]. SNP Met72Thr (rs1136287) in exon 3 (T/C change) of *PEDF* gene has been extensively studied for its possible association with DR, AMD, diabetic microangiopathy in various population [188]. The ‘T’ allele of rs1136287 has been identified as a risk allele for wet AMD in the Taiwan Chinese population while heterozygous genotype (C/T) exerted a protective effect against exudative AMD in the Chinese cohort. Yet another coding region polymorphism T130T in exon 4 showed moderate protective association with DR in the Indian population [190]. Recently it has been shown that functional genetic variants in the gene contribute to adipose tissue-related prediabetic phenotypes in humans. SNP rs12603825, associated with increase in fat mass, was also linked with elevated PEDF and leptin concentration [191].

Physiological role of EPO in normal and disease condition

Erythropoietin (EPO) a 30.4 kDa glycoprotein shown to play major role in stimulation of bone marrow stem cells and erythropoiesis. However it is also implicated in the development, maintenance, protection and repair of the nervous system [192].

Though the underlying mechanism of EPO neuroprotection in diabetes is not fully understood, it has been observed that EPO reduces glutamate toxicity, nitric oxide-mediated injury, inflammation and antioxidant effects [193, 194]. Various experiments in the preclinical models of neuronal, retinal, cardiac and renal ischemic injury support its neurotrophic effect [195]. EPO also induces the generation of neuronal anti-apoptotic factors that prevents destruction of viable tissue surrounding the site of an injury [196]. Diabetic rats exhibiting the neuroglial and vascular degenerative pathology were shown to be protected after being treated with EPO-derived peptide without altering hematocrit or exacerbating neovascularization [197].

EPO and its receptor (EPOR) have been reported in human retina, brain liver, kidney, heart, lung, intestine and uterus [154]. Elevated levels of EPO have been demonstrated in the vitreous of patients with diabetes and DR when compared to controls without diabetes. Animal studies have also shown increased concentration of EPO in ischemic retinas while its inhibition was shown to prevent neovascularisation. Thus confirming the role of EPO in the development of DR as well as an endogenous neuroprotectant against ischemia [170]. Its expression has also been shown to be under genetic control.

EPO observed to be upregulated in RPE, neuroretina and vitreous of donor eyes from diabetic, PDR patients when compared to the non diabetic controls [198]. Systemic administration of EPO or intravitreal injection of rhEPO delivered at the onset of hyperglycaemia prevented subsequent neuronal dropout, as well as retinal edema in rat models [192].

It has also been observed that diabetic rats treated with EPO-derived peptide [197] were protected from neuroglial and vascular degenerative pathology thus suggesting its therapeutic implications in DR. However the exact role of this protein in mediating neuroprotection remains unclear.

Genetic association studies in *EPO* gene

EPO gene localized to 7q22.1 that has been linked to diabetes by sib-par analysis in Pima Indians [199]. The gene encodes a 30.4kDa glycoprotein (Figure 1.23) with 165 amino acids [200].

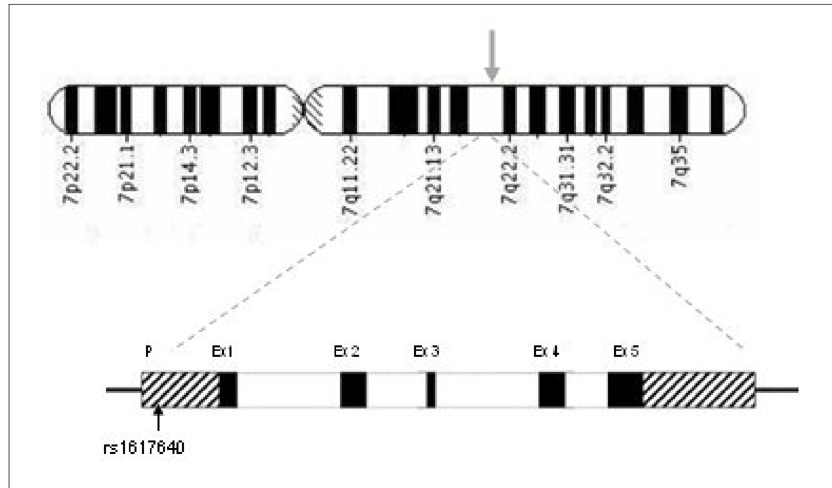


Figure 1.23: Organization of *EPO* gene in 7q22.1 region

Polymorphisms in *EPO* gene (rs1617640, rs551238, and rs507392) have been studied for their possible association with DR, end-stage renal disease in patients with diabetes, breast cancer, myelodysplastic syndrome [170, 171, 201, 202].

The polymorphism rs1617640 located -1125bp upstream of transcription site in the promoter region of *EPO* gene (Figure 1.23) has been correlated with increased levels of the protein. Study by Tong et al has identified a significant association of TT genotypes of rs1617640 with the risk for PDR and end-stage renal disease in patients. The 'T' allele was also reported to have a major effect on its transcription levels as shown in the in-vitro model [170]. However the GG genotype of rs1617640 has been associated towards the risk of myelodysplastic syndrome (MDS) [201].

The present study determines the genetic association of promoter variants of *PEDF* and *EPO* in T2DR patients from south India.

Pathway-based approaches to complex disease

Multiple SNP analysis by GWAS has revealed novel genes for complex diseases like T2D, inflammatory bowel disease, osteoporosis etc. These genes interact as complex molecular networks or pathways and thus involved in disease susceptibility and progression. Therefore by taking into account prior biological knowledge about pathways we may have better chance to identify the genes and mechanisms that are involved in disease pathogenesis. Thus the pathway-based approach typically examines a group of related genes from a common pathway or condition [204,205].

The pathway-based approach has been used in genetic studies of amyotrophic lateral sclerosis (ALS), multiple sclerosis, rheumatoid arthritis, Crohn's disease, age-related macular degeneration, Parkinson's disease, etc. Study by Xu et al has explored the joint effect of multiple genes in the inflammation pathway on prostate cancer risk by multiple dimensionality reduction (MDR) method and has revealed interactions between IL-10 (rs1800896), IL-1RN (rs878972), TIRAP (14115), and TLR5 (IIPGA-5187) genes in four-locus model and thus predicting prostate cancer risk [206].

Gene-gene and gene-environment interaction studies

Epistasis or interactions between genes and environmental factors are vital in understanding the intricacy of the genetic pathways involved in the pathogenesis of complex diseases. In recent years interaction between different genes and/or with the environment are being exclusively dissected in the pathology of many genetic diseases, including single gene disorders. The phenotypes of even single-gene diseases such as cystic fibrosis and phenylketonuria are caused by multiple gene-gene (G X G) and gene-environment (G X E) interactions [207].

Till date G x G interactions have been reported in various diseases including systemic lupus erythematosus, multiple sclerosis, alcoholism, obesity and T2D [208-212]. The polygenic nature of diabetes and its complications demands the need of such interaction studies between genes and environment. Study by Wiltshire et al showed epistasis interaction

between loci on chromosomes 1q21-25 and 10q23-25 in T2D [212]. At the same time Zuniga et al found Killer cell immunoglobulin-like receptors (KIR) genes and G1M immunoglobulin allotypes to be possibly interacting and thus conferring susceptibility to T2M [213]. Also epistatic interaction among RAS related genes have been directly correlated with the susceptibility of T2D using multifactor reduction dimensionality (MDR) analysis [214].

G x E interaction studies, important for risk prediction and evaluating the benefit of modifiable environmental exposures, thus sheds light on the fundamental biological mechanisms of complex diseases. G x E interactions could improve the power for discovering genes involved in the etiology of disease primarily through an interaction effect with no marginal effect; conversely, power for discovering environmental effects that act primarily in genetically susceptible individuals can be improved by searching for interactions [215]. Major environmental risk factors for development of proliferative DR includes hyperglycemia, duration of diabetes, age at diagnosis of DR, blood pressure, glycosylated hemoglobin (HbA1c), microalbuminuria [17, 58, 216]. One of the best established G×E interactions is between smoking and *NAT2* for bladder cancer [215]. *The -677TT* genotype in methylenetetrahydrofolate reductase *(MTHFR)* gene has been found to be associated with NPDR in patient with higher HbA1c (≥6.5%) [217].

Moreover the advent of genome-wide association studies (GWAS) has shown considerable progress in understanding the role of G X G and G X E interactions in the development of complex disease [218].

Methods for analyzing GxG/GxE interactions

Potential interaction occurs between genes as shown in figure 1.24 (i) Synthetic interactions where the 2 genes exert the effect independently to result in a common phenotype (ii) epistatic, interaction in which a gene exerts its effect on a phenotype by interacting and modifying another gene (s) and can result in the same or different phenotype and such interactions often correlated with varied degree of phenotype expression (iii) suppressive-interaction [219].

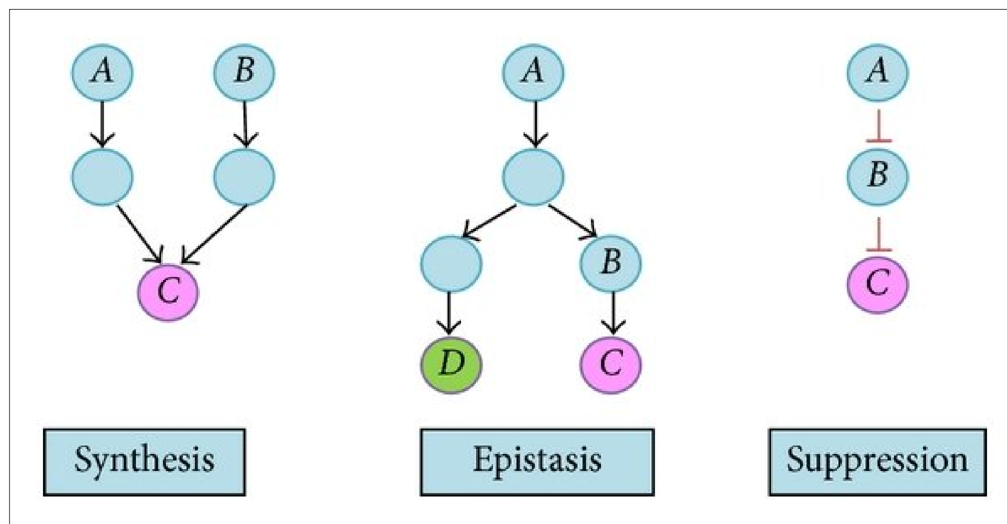


Figure 1.24: Methods of gene gene interactions [219]

(ii) Statistical methods

Detection of genetic interactions can involve statistical modeling, data mining and computational methods [220]. Simple regression based approach forms the first line approach for modeling and testing the interactions [221]. These traditional regression-based methods were however unable to deal with non-linear models for high dimensional data containing numerous potentially interacting predictor variables, leading to sparse contingency tables with many empty cells [222]. Therefore alternative advanced methods were applied which proved to be powerful in handling the

challenges in statistical analysis of genetic interactions by performing high-dimensional data analysis [221].

High order interactions: Exhaustive search however did not scale up to consideration of higher-order interactions as the number of tests increases exponentially with the order of interaction considered. Thus high order interactions was performed in two-stage procedures, when subsets of loci that passes single-locus were chosen, and exhaustive search of all two-locus interactions were carried out on this 'filtered' subset [222].

(iii) Data-mining/machine learning approaches:

High-dimensional data containing many potentially interacting variables often fail to provide better results by the traditional regression-based methods leading to sparse contingency tables with many empty cells.

(a) Neural network: Neural networks handles large quantities of data with reasonable computation time as the scalability of data increases exponentially. Moreover, neural networks are able to approximate any type of genetic etiology that underlies phenotypic values because neural networks are universal approximators. This property is particularly important when mining the high dimensional data [219]. It was used by Tomita et al to predict the development of childhood allergic asthma. It analysed 25 SNPs of 17 genes and select 10 susceptible SNPs among the Japanese people [219].

(b) Support vector machine (SVM): It is a machine learning algorithm which utilizes hyperplanes in high dimensional plane and often used in classification and regression task [219].

(c) Recursive partitioning approaches: The recursive partitioning approaches includes single classification tree based on classification and regression trees (CART) and random forest [222].

In CART analysis, the splitting rule applied at each node in such a way to maximize the reduction in a quantity known as the Gini impurity. As shown in figure 1.25. SNP 3 maximizes the reduction in at the first node and

thus splits the original data set of 1000 cases and 1000 controls into two smaller data sets. Once a node gets split, the same logic was applied to each child node. This defines the recursive nature of the procedure. The splitting stops when no further gain can be made (e.g. when all terminal nodes contain only cases or only controls or all possible SNPs have been included in a branch). At this stage it is usual to prune the tree back (i.e. remove some of the later splits or branches) according to certain rules to avoid over-fitting and to produce a final, more parsimonious model [222].

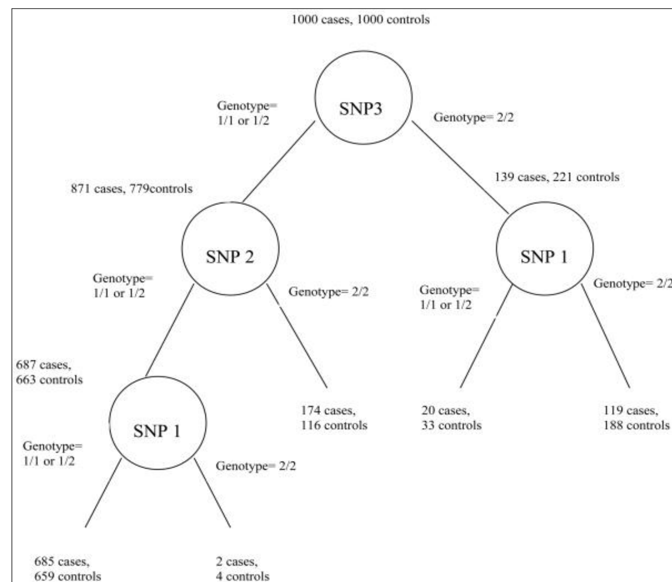


Figure 1.25: Structure of the classification and regression trees (CART) [222]

Multifactor dimensionality reduction method (MDR)

MDR has been a powerful approach to detect epistasis and ideally discriminates between discrete clinical endpoints when using multilocus genotypes. MDR analysis identifies combinations of loci influencing a disease outcome, possibly through interactions. MDR achieves dimension reduction by converting a high-dimensional (multi-locus) model to a one-dimensional model, thus avoiding the issues of sparse data cells. The

method classifies the genotypes either as ’high risk’ or ’low risk’ according to the ratio of cases and controls [222].

For investigation of higher-order interactions, MDR has been best suited for use with small numbers of loci (up to a few hundred), from a candidate gene study or selected from a larger set of potential predictors via a prior filtering step [222].

GMDR statistical framework is applicable to both dichotomous and quantitative phenotype that allows adjustment for covariates in population based study designs. So far GMDR statistical approach has identified many interacting genetic variants underlying various complex human diseases such as Alzheimer disease, asthma, atrial fibrillation, autism, bladder cancer, hypertension, nicotine dependency, prostate cancer, schizophrenia, sporadic breast cancer, thrombotic stroke, and T2D [223].

Examples of studies involving GxG and GxE interaction

GxG and GxE interaction studies have been used in the filed of cancer genetics. A recent Su et al study has suggested the possible GxG interaction between GSTP1, INSIG2 and IL4Ra genes towards susceptibility to asthma in school children from Taiwanese population. Similarly the N-acetyl transferase (*NAT2*) gene has been shown to be interacting with aryl amines, present in tobacco smoking, hair dyes, and various occupational exposures, towards the risk for bladder cancer [224]. Another strong interactions between polymorphisms in genes *GSTM1*, *iNOS* with smoking and asbestos exposure has been implicated in the development of asbestosis [225]. Genetic interaction between the variants of genes *ALOX5AP* and *CYP3A5* were shown to increase the risk for cerebral infarction in Chinese population [226]. Epistatic interaction between *KIR* genes and *G1M* immunoglobulin allotypes as well as RAS related genes have been shown to confer susceptibility to T2DM [227, 228].

There are only few reports on interaction studies in DR. Interactions between HIF1α (hypoxia-inducible factor alpha) and VEGF via their common miRNAs have been implicated in DR [229]. Additionally reduced activity of manganese superoxide dismutase (*sod2*) gene due to increased trimethyl histone H4 lysine 20 (H4K20me3) has also been shown to contribute to the

development of DR [230]. These studies have used the simple regression analysis and not employed the high order interaction studies.

Analyzing and identifying the interacting genes and gene-environment interactions would shed light on the fundamental biological mechanisms involved in the disease process. It would also improve disease risk prediction and facilitate prevention by modifying the environmental exposures [224].

Genetic studies in DR from Indian population have identified many susceptible and risk predisposing genetic factors of DR [67]. Genome wide/candidate gene association studies have been extensively performed in DR in various populations; but the potential effect of genes/environment interaction in DR pathology is not addressed adequately in the literature. Pathway based approaches, investigating variants in a cluster of genes that may be involved in specific pathways are often addressed as a significant area of research in complex disorders. In the current study, we attempt to perform such interaction studies between the genes involved in 2 major pathways; Inflammation and neuroprotection; determine the effect of SNPs in *PEDF*, *EPO* and *CFH*, *ICAM-1* genes. Additionally, high order interaction analysis using generalized multifactor dimensionality reduction (GMDR) and classification and regression tree (CART) analysis are used to assess the possible gene-gene and gene-environment interactions in DR.

Aim:

To study the genetic association and test the potential role of gene-gene and gene-environment interaction in the pathology of T2DR in Indian population.

Objectives:

1. To genotype and analyse the statistical association of SNPs in genes for inflammation (a) *ICAM-1* (rs5498), (b) *CFH* (rs1061170) with T2DR.
2. To genotype and analyse the statistical association of SNPs in genes for neurodegeneration *(a) PEDF* (rs12150053, rs12948385) and (b) *EPO* (rs1617640) with T2DR.
3. To perform high order genetic interaction analysis between the variants of a) *PEDF* and *EPO* genes b) *ICAM-1* and *CFH* genes and with the clinical covariates of DR.

CHAPTER 2

MATERIALS AND METHODS

Recruitment of patients

The study participants were enrolled from the SNDREAMS project, (Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Biology Study), an epidemiology study to understand the prevalence of DR in south Indian population [231] and from the vitreo retinal clinic of Sankara Nethralaya, a tertiary eye care hospital in Chennai between years 2003-2006 and 2007-2010. The study protocol adhered to the Declaration of Helsinki [232] and approved by institutional review board. After an informed consent all the subjects underwent detailed history, physical examination and pedigree analysis. Ocular examination included 45^0 fundus photograph using 4-field stereoscopic digital photography that were graded by 2 independent observers in a masked fashion; the grading agreement was high (κ =0.83) [231]. The diagnosis of DR was based on the modified Klein classification of the Early Treatment Diabetic Retinopathy Study scale. The methodology for sample selection was as described earlier by Agarwal et al (2005) and Pal et al (2011) [233, 234].The inclusion criteria for the study participants were T2D, south Indian origin and ≥40 years of age. The duration of diabetes varied between cases (≥ 10 years) and controls (≥ 15 years). Subjects with sight threatening diabetic retinopathy [STDR, inclusive of severe non-proliferative diabetic retinopathy (NPDR), proliferative diabetic retinopathy (PDR) or clinically significant macular edema (CSME)] constitute the case group (DR+) and those without any signs of DR were included as the controls (DR-). Age related macular degeneration (AMD), other hereditary retinal disease and non south Indian origin were the exclusion criteria.

A total of 401 T2D subjects with retinopathy [cases (DR+): 201] and without retinopathy [controls (DR-): 200] were recruited and genotyping

performed after stringent inclusion and exclusion criteria and clinical evaluations.

Collection of patient details and blood sample

Baseline characteristics and clinical details including age, sex, duration and treatment of diabetes, user of insulin, age at onset of diabetes, history of hypertension, body mass index (BMI), smoking status and blood pressure were documented. Biochemical parameters such as glucose, HbA1c, total cholesterol, HDL cholesterol, triglycerides were estimated in blood whereas microalbuminuria in the urine. HbA1c and microalbuminuria were measured using NycoCard READER II (Axis-Shield, Norway). Blood glucose, total cholesterol, HDL cholesterol, triglycerides were measured using semi automated clinical chemistry analyzer Stat Fax 3300 (Awareness Technology. Inc. USA).

DNA extraction

a) Manual method: Phenol chloroform method [235]

Genomic DNA was extracted from whole blood by phenol chloroform method.

Reagents

- TE buffer pH 8.0
  - 100mM tris - 2mL
  - 50mM EDTA - 200μL
  - MilliQ water - 7.8mL
- Digestion buffer
  - 1M Tris - 200μL
  - Triton - 50μL
  - MilliQ water - 740μL
  - Proteinase K - 10μL (15mg/ml)
- 5M NaCl

- Phenol: chloroform: isoamylalchohol – 25:24:1
- 100% Ethanol
- 70% Ethanol

Equilibration of phenol

Phenol crystals stored at -20^0 C were equilibrated to room temperature and then liquefied at 68°C. Then quinolone was added to a final concentration of 0.1%. Equal volume of 0.5M Tris Chloride (pH 8.0) was added, stirred with magnetic stirrer at room temperature for 15 minutes. When the two phases were separated, the upper aqueous phase was aspirated. Then equal volume of 0.1M Tris Chloride (pH8.0) was added to the organic layer and stirred for 15 minutes at room temperature. The aqueous phase was removed and the above step repeated until the pH of the phenol was > 7.8. The pH of phenol should remains as one of the rate limiting step in DNA extraction, because, at an acidic pH (PH<7.0) DNA separates in organic phase. After the phenol was equilibrated and the final aqueous phase removed, 0.1 volume of 0.1M Tris Chloride (pH 8.0) containing 0.2% β-mercaptoethanol was added [235].

Procedure [5]:

1. 4.8ml of heparinised blood was mixed well with 2.0 ml of TE buffer and 800μL of digestion buffer and incubated at 60^0C for one hour.

2. Then 7.6 ml of equilibrated phenol was added, mixed in a vortex mixer and incubated at 37^0C shaker water bath (100 rpm) for 10 minutes.

3. Then it was taken out and centrifuged at 14,000 rpm for 10 minutes.

4. The aqueous layer was transferred to a fresh tube.

5. To this equal volume of equilibrated phenol-chloroform-isoamylalcohol mixture (25: 24:1) was added, mixed and centrifuged at 2500 rpm for 10 minutes.

6. The aqueous phase was washed with equal volume of phenol: chloroform: isoamlyalchohol until the interphase was minimum.

7. To the final aqueous layer equal volumes of chloroform: isoamyl alcohol mixture (24:1) were added and centrifuged for 10 minutes at 2500 rpm. This is to remove the residual phenol.

8. Then the aqueous layer (upper layer) was transferred to a fresh tube.

9. DNA was precipitated out by the addition of 1/10 volume of 5M NaCl and two volumes of chilled absolute ethanol.

10. This was incubated at -20°C for 16 hours.

11. At the end of 16 hours, it was brought to room temperature, centrifuged at 2500 rpm for 10 minutes.

12. The supernatant was discarded by gently inverting the tube.100% ethanol was added till three-fourth of the tube mixed well and centrifuged at 2500 rpm for 10 minutes.

13. The supernatant was discarded and the pellet was washed twice with 70%alcohol.

14. Then the tube was blotted on a blotting paper and the mouth of the tube covered with paraffin film. Holes were made on the paraffin film and tubes were kept at 37^0C till ethanol dried.

15. Then 500ul of TE buffer was added to dissolve the DNA and kept at 37°C water bath overnight.

b) NucleoSpin® Blood XL column [236]

Genomic DNA was extracted from the sodium heparinsed whole blood of patients included as cases with retinopathy (the STDR group) and control without retinopathy by NucleoSpin blood XL kit method. For the better yield the DNA was extracted from the blood collected on the same

day. The sample could be stored at 4^0C for short storage and -80^0 C for long storage, however this would result in lower yield of DNA [236].

Materials and Reagents [236]:

- Lysis buffer BQ1
- Wash buffer BQ2
- Elution buffer BE
- Proteinase K (lyophilized)
- Proteinase buffer PB
- Phosphate-buffered saline (PBS)
- NucleoSpin blood XL columns
- Collections tubes
- 1.5ml vials
- 50ml tarson tube
- 96–100 % ethanol

Reagent preparation [236]**:**

Required temperature was set in the water bath before starting the extraction procedure followed by preparation of reagents:

1. Wash buffer BQ2: 200ml of absolute ethanol (96–100 %) was added to the BQ2 buffer concentrate and stored at room temperature (18–25 °C) for up to one year.
2. Proteinase K: 5.75ml of proteinase buffer was added to dissolve 126mg of lyophilized proteinase K in a vial and stored at -20 °C for up to 6 months.
3. Setting up of water bath at to 56 °C.
4. Elution buffer prewarmed at 70 °C.

For centrifugation, a centrifuge with a swing-out rotor and appropriate buckets capable of reaching 4,000–4,500 rpm is required.

Procedure [236]:

1. 500μl proteinase K was added to 10ml heparinsed blood sample (equilibrated to room temperature) in a 50ml tarson tube. [Note: In case of blood sample ≤ 5ml, PBS is added to adjust the volume to 10ml]
2. To this 10ml of lysis buffer BQ1 is added and mixed vigorously for 10 s. [Note: Vigorous mixing is important to obtain high yield and purity of DNA]
3. It was then incubated at 56 °C for 15 min.
4. Lysate was then brought to room temperature before proceeding with addition of ethanol. [Note: The lysate should become brownish during incubation with Buffer BQ1 and should have cooled down to room temperature before loading it onto the columns. Loading of hot lysate may lead to diminished yields]
5. 10ml of ethanol (96–100 %) was added to each sample and mixed by inverting the tube 10 times.
6. Column (with silica membrane) was placed in a collection tube and 15 mL of lysate was load in it without moistening the rim. The tubes are closed with screw caps and centrifuged at 4,000 rpm for 3 min. Discard the flow-through and load 15 mL of the remaining lysate to the respective column and centrifuge again at 4,000 rpm for 3 min. Discard the flow-through and place the column back into the collection tube.
7. 7.5 mL wash buffer BQ2 was then added to each column and centrifuged at 4,000 rpm for 2 min. [It is not necessary to discard the flow-through after the first washing step]
8. 7.5 mL wash buffer BQ2 was again added to each column and centrifuge at 4,000 rpm for 10 min. The column was carefully taken from the rotor to avoid that flow-through gets in contact with the column outlet. [By prolonged centrifugation during this second

washing step, residual ethanolic buffer BQ2 is removed from the silica membrane of the NucleoSpin Blood XL Column]

9. Column was then dried by performing prolonged centrifugation for 10 min.

10. The column was then placed in a new collection tube (50 mL) and 1000 µL of preheated elution buffer BE (70 °C) directly to the center of the silica membrane and incubated at room temperature for overnight/2 min. Centrifuged at 4,000 rpm for 2 min.

11. The eluted DNA was then collected in 1.5 ml vials and proceeds further with DNA quantification.

Quantification of DNA

NanoDrop (ND-1000 Spectrophotometer) [237]

Direct 2µl of extracted genomic DNA was taken for quantification to check the concentration and purity of nucleic acid in NanoDrop ND-1000 (220-750nm) spectrophotometer. It is more reliable, accurate and requires less volume of the sample in comparison to the regular spectrophotometer method [237].

The quantification was done spectrophotometrically at 260nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as “pure” for DNA. If the ratio is appreciably lower then it indicates the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Ratio of absorbance at 260 and 230 nm is a secondary measure of nucleic acid purity. Ratio in the range of 1.8-2.2 indicates pure DNA however lower ratio indicates the presence of co-purified contaminants [237].

Polymerase chain reaction (PCR)

Optimization of PCR was done on DNA samples from healthy controls followed by genotyping study subjects for regions on candidate genes spanning the polymorphisms under study using the following methodology.

Requirements [238]:

1. DNA: This is diluted from stock DNA with TE buffer.
2. Taq Polymerase.
3. 10X Taq buffer (100mM Tris pH 9.0, 500mM KCl, 15mM $MgCl_2$, 0.1% gelatin)
4. dNTP: Each dNTP 25μmol/100mM (2mM of dNTP per reaction)
5. Primers: Lyophilized forward and reverse primers diluted with TE buffer.

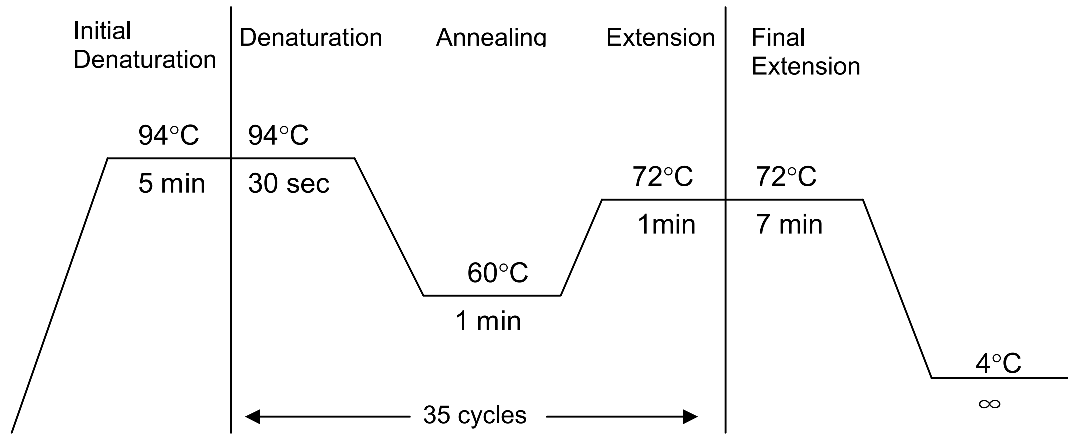
Standard PCR reaction mix [238]:

Reaction Mix	**X1 (µl)**
Forward primer (10pM)	0.2
Reverse primer (10pM)	0.2
dNTPs (2mM)	2.0
Taq buffer (µl)	2.0
Taq polymerase (3u/µl)	0.2
Genomic DNA (50ng/µl)	1.0
Sterile water	13.4

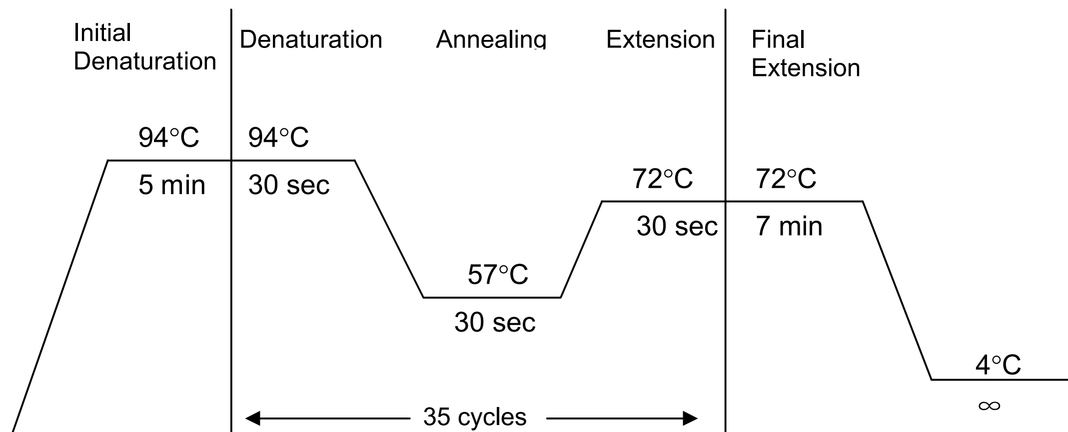
Amplification of DNA was done in thermal cyclers - GeneAmp PCR system 2700, 2720 and 9700 (Applied Biosystem).

PCR reaction was set up using primers provided in Appendix 2. The machine protocol as given below has been used for PCR amplification of each variant in *ICAM-1*, *CFH*, *PEDF* and *EPO* genes.

(a) rs5498 in *ICAM-1* gene:

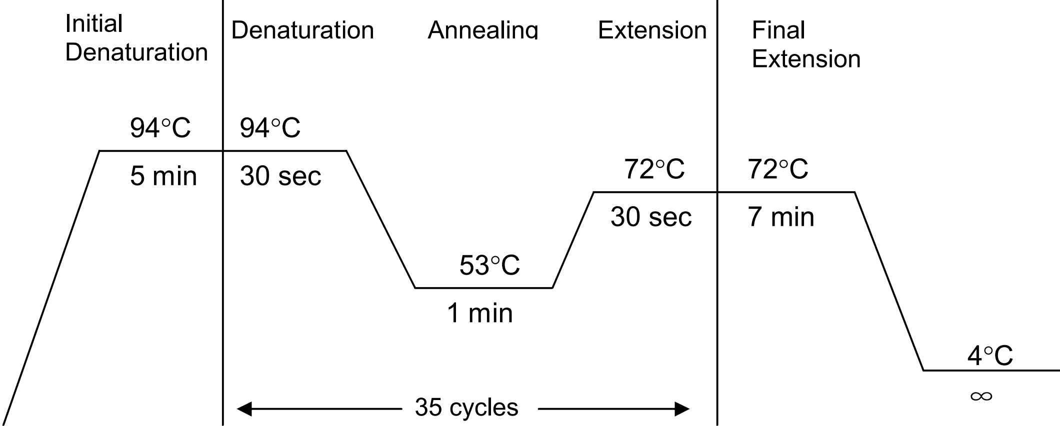


(b) rs1061170 in *CFH* gene

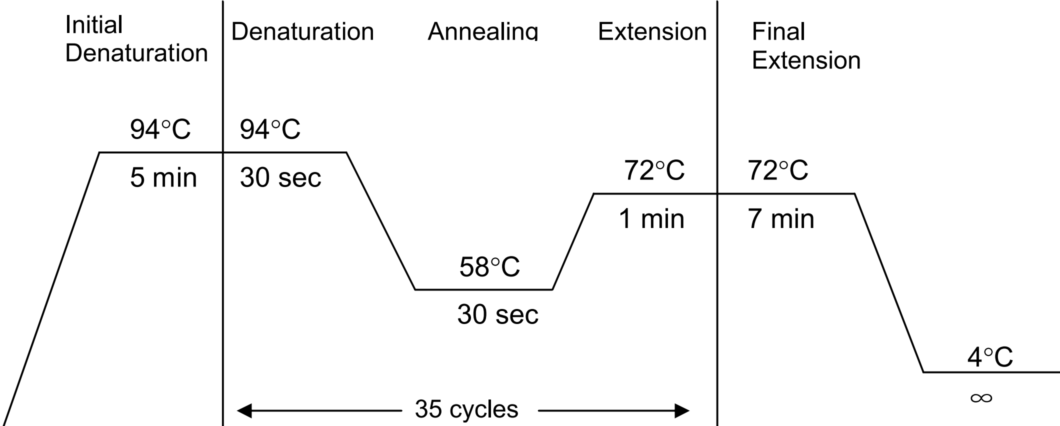


(c) *PEDF* gene

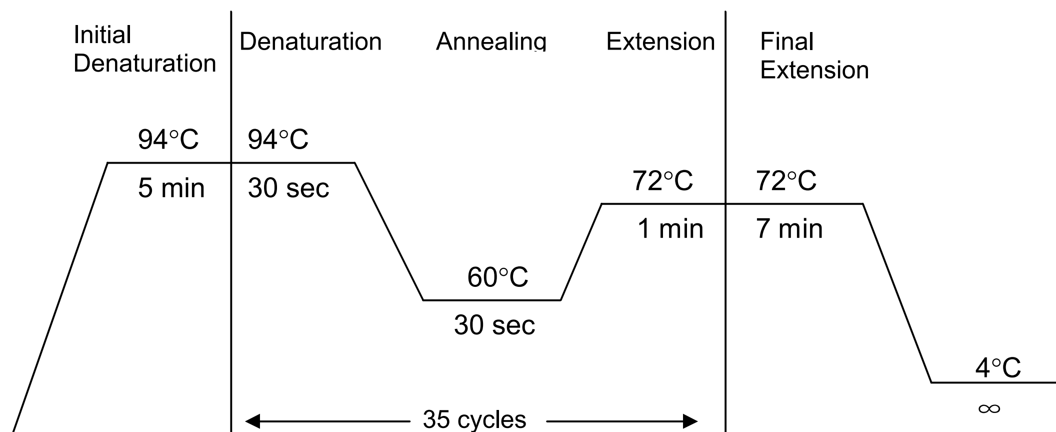
rs12150053



rs12948385



(d) rs1617640 in *EPO* gene



Restriction fragment length polymorphism

PCR based restriction fragment length polymorphism (RFLP), a popular genotyping method for scoring SNPs, has been extensively applied in the present study. This method is based on digestion of the amplified fragment containing the variation (SNP) with an appropriate restriction enzyme. The presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes. Finally the allele or genotype identification can be done by electrophoretic resolvement of the fragments [238].

A digestion condition was optimized to 5U/5μl PCR product (20μl reaction) with restriction enzymes as shown below. Restriction enzymes were diluted to 1U per microlitre when required [238].

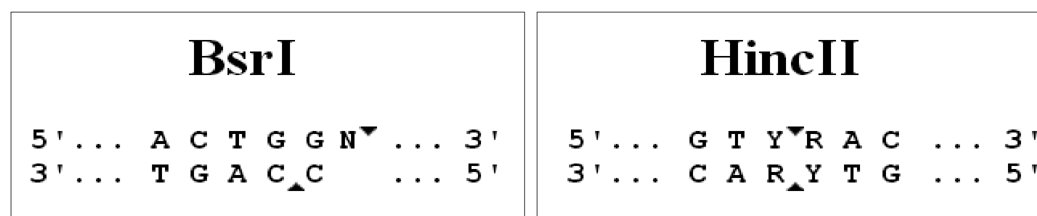
Restriction digestion: Reaction mix

Amplified product	5.0 μl
Restriction enzyme (Diluted)	2.0 μl
Digestion buffer	2.0 μl
Autoclaved milli Q water	12.5 μl

The digestion products were then electrophoresed on 4% agarose gel and genotyped [238].

<u>RFLP protocol for rs1061170:</u> The T>C substitution at position 1277 in exon 9 of *CFH* gene results in gain of recognition site for the enzyme *Tsp*509I [239]. The rs1061170 genotypes were hence scored based on the RE digestion pattern.

<u>RFLP protocol for rs12150053 and rs12948385:</u> Polymorphisms rs12150053 and rs12948385 at -790bp and -358bp position from the transcription start site of the promoter region of *PEDF* gene resulted in generation of restriction site for *BsrI* and *HincII* enzyme [169].



The products subjected for restriction for 10hrs at 65^{0}C (rs12150053) and 12hrs at 37^{0}C (rs12948385) were analysed in 4% agarose gel for genotypes which were scored based on the number of fragments generated by the enzyme (Table 1.11)

Table 1.11: Fragment size of the genotypes for the each polymorphisms rs12150053 and rs12948385 of *PEDF* gene

	rs12150053 Genotype/Fragment size (bp)	***rs12948385*** Genotype/Fragment size (bp)
Homozygous wild	TT: 367	AA: 280, 71
Heterozygous variant	TC: 367, 201,166	GA: 351, 280,71
Homozygous variant	CC: 201,166	GG: 351

Random samples were subjected to sequencing to confirm the genotypes. The sequencing was performed in ABI PRISM 3100 Avant genetic analyzer

and the alleles were differentiated using a reference sequence given in Appendix 1.

DNA sequencing using ABI Prism 3100 AVANT genetic analyzer [240]

The sequence of the PCR amplified DNA were determined with the help of the ABI Prism 3100 AVANT genetic analyzer that works on the principle of Sanger dideoxy sequencing [240].

Protocol for cycle sequencing reaction [240]

Components	**Volume (μl)**
Amplfiied products	2.0
Sequence buffer	2.0
Primer (2pmoles/μl)	2.0
RRMIX	1.0
Water	3.0

PCR conditions for cycle sequencing [240]

PCR step	**Temperature (°C)**	**Time**
Initial denaturation	96	1 min
Denaturation	96	10 sec
Annealing	50	5 sec
Extension	60	4 min

The reaction was carried out for 25 cycles.

Purification of extension products

The extension products were purified to remove the unincorporated dye terminators before subjecting the samples to capillary electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with base calling.

Procedure: 2 μl of 125mM EDTA and 2 μl of 3M sodium acetate (pH 4.8) were mixed to the cycle sequenced products followed by the addition of 50μl of absolute ethanol and incubated at room temperture for 15 minutes followed by centrifugation at 12000rpm for 20 minutes to precipitate the amplified product and remove the unutilized ddNTPs, primer (short length molecules) etc. The pellet were washed twice with 75% ethanol followed by air drying. The purified samples are suspended in fomamide and subjected for capillary electrophoresis in ABI PRISM 3100 genetic analyser. The sequences were then analyzed in Seq scape manager (version 2.1; ABI Prism 3100 AVANT) [240].

Statistical method

Statistical analyses were performed using SPSS software (for Windows version 14.0; SPSS Science, Chicago, Illinois (IL), USA). The results were expressed as mean standard deviation and percentage for continuous and categorical variables. The Student t-test and χ^2-test was performed to compare continuous variables and proportions among groups respectively. Distribution of genotypes and alleles between the case and control groups were compared using χ^2-test. To detect single marker association between cases and controls, two models were tested to compare the allele frequencies in 2X2 contingency tables or genotypes in 3X2 contingency tables. The SNPs were analysed for its association with DR in autosomal dominant and recessive models. To assess the specific effect of the genotypes on the various clinical factors, multivariate analyses were performed in the case group. Odds ratio (OR) and 95% confidence intervals (CI) were estimated with unconditional logistic regression models between cases and controls and p value <0.05 was considered significant. Deviation from Hardy–Weinberg equilibrium for genotypes was analyzed for both cases and controls using online “Simple Hardy-Weinberg Calculator” [241]. Relative risk values between two nodes in CART analysis was carried out using online software [242].

Haplotype analysis

Haplotypes are predictive marker for disease having higher power to detect genetic associations for disease over a single SNP. Haplotype analysis refers to the analysis of a set of linked alleles occurring on the same chromosome.

Genotype data were analyzed using Haploview 4.2 (http://www.broadinstitute.org/mpg/haploview) for haplotype construction [243]. Linkage disequilibrium (LD) between the SNPs used in haplotype analysis were measured by a pairwise D' statistic.

PEDF rs12150053 and rs12948385: Single marker and haplotype block association were estimated and statistical significant results were further confirmed by performing 15000 permutations. [http://www.broad.mit.edu/haploview/haploview]

Bioinformatics analysis

Sequence alignment analysis and effect of polymorphism on structure and function of the protein performed with basic local alignment search tool (BLAST), Polyphen-2 and sorting intolerant from tolerant (SIFT). Using ConSurf 9 conservational analysis performed with Swiss-Prot Accession ID: P05362 as the reference sequence [244]. Three dimensional structural co-ordinates of the ICAM-1 having the natural variant of rs5498, elucidated using X-ray crystallography at resolution of 2.70 Å retrieved from Protein Data bank (PDBID:2OZ4). The structure for K469 variant elucidated using Modeller9V7 [245] and validated for quality by checking the stereo chemical and energetic aspects. The structural stability of the wild and variant analyzed through potential energy of the molecule. The effect of these variants at the cell adhesion studied through protein dimerisation and interaction with integrin alpha-M and Beta-2 using MUpro [246].

Interaction study

High order gene-gene (GxG) and gene-environment (GxE) interactions were determined using generalized multifactor dimensionality reduction (GMDR) and classification and regression tree (CART).

Generalized multifactor dimensionality reduction (GMDR)

A nonparametric and genetic model-free alternative to linear or logistic regression program for the detection of gene-gene and gene-environment interactions [247, 248]. The GMDR software (version 0.9) (http://www.ssg.uab.edu/gmdr/) was developed in 2007 by Lou et al. to implement the GMDR method. GMDR, a score based algorithm based on the MDR framework [249] facilitate the reduction of dimensionality of multilocus information to identify the polymorphisms associated with an increased risk of the disease. This approach takes multilocus genotypes and develops a model for defining the disease risk by pooling high-risk genotype combinations into one group and low-risk combinations into another. The best model, selected based on the combination of marker with maximum cross-validation consistency and minimum prediction error. But unlike the MDR method it permits adjustment for covariates and better handles data with unequal number of cases and controls and can be used to analyze both qualitative (e.g. binary) and quantitative traits.

GMDR software displays the final best 1- to n-locus models along with the training and testing balance accuracy, cross-validation consistency, and sign test p-values as shown in the Figure 2.1 [248].

Best n-locus model determines the overall best epistasis model and selected based on the maximum balanced prediction accuracy and cross-validation consistency (CVC) [248].

Sign tests: The χ^2-test and sign tests determine the factors significantly associated with the genetic disease. A significant sign test suggests that the

best model with one or more SNPs is significantly better than the null model [248].

Prediction accuracy: The prediction accuracy indicates the proportion of subjects that were correctly classified as cases or controls. For example, a prediction accuracy of 0.60 implies that 60% of the subjects were correctly classified as cases or controls [248].

Cross validation consistency: The CVC is measured on a ten point scale for 10-fold cross-validation thus a CVC of eight indicates that the particular model was selected eight times out of ten as the best model [248].

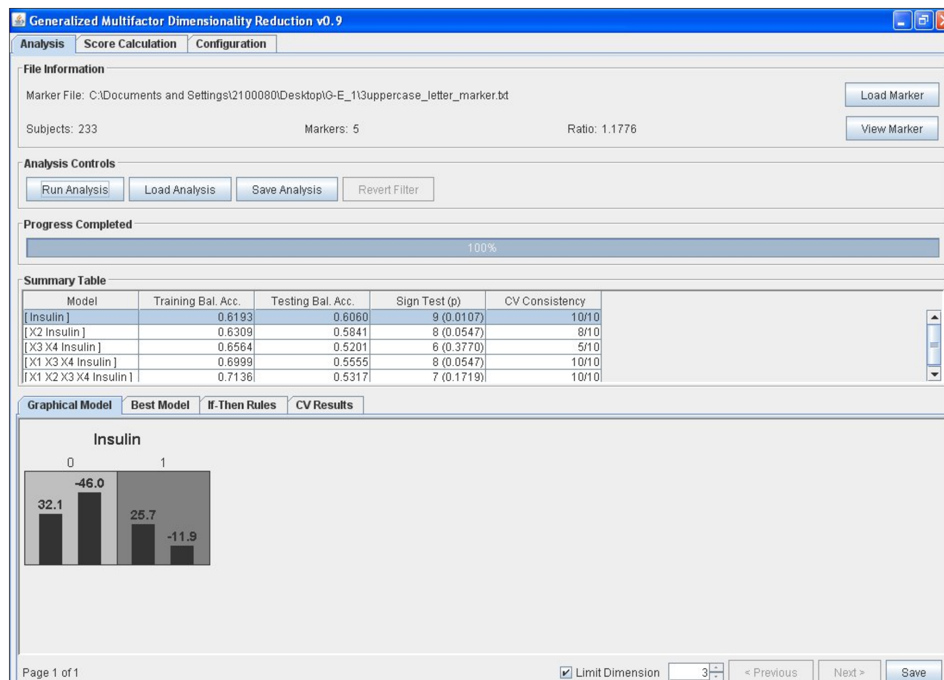
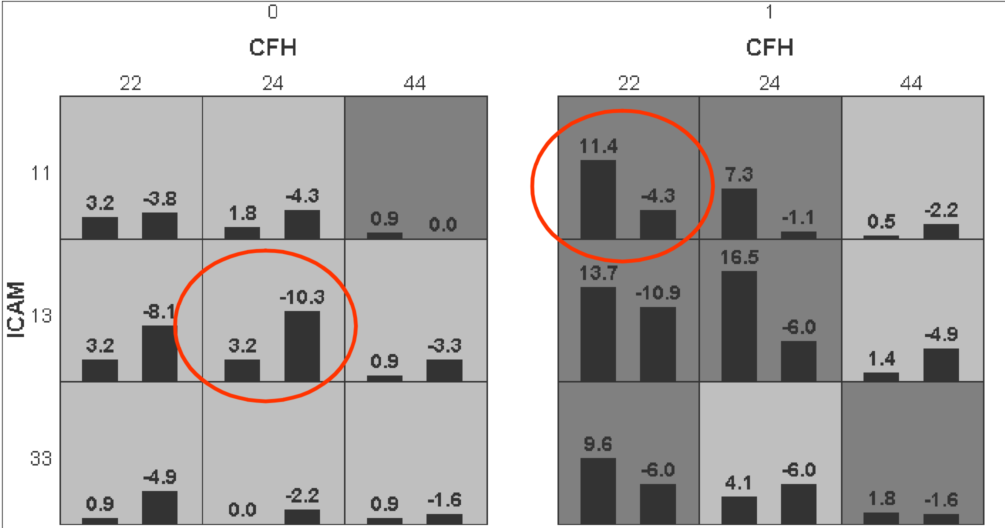


Figure 2.1: Window page showing the results obtained by GMDR software (Version: v0.9)

GMDR method uses the original MDR data reduction method, with the ratio of cases to control being replaced by scores in each cell to discriminate between high risk and low risk followed by determining classification accuracy and prediction error [247]. The threshold equals the ratio of cases to controls in the data set, and a value of zero is equivalent to a one-to-one

case-control ratio [247]. Due to the high dimensionality of the data and the comparatively small sample sizes, some of the cells can be empty in the training set and not in the testing set. The GMDR method identifies these as misclassification cells when adding together the scores of the high-risk and low-risk cells [247].



(A)

Graphical Model | Best Model | If-Then Rules | CV Results

Model Detail:

Combination	Class 1 Score	Class 0 Score	Sum Score	New Class Level
22,11,0	3.201219512195121	3.798780487804879	-0.5976	0
22,11,1	11.432926829268288	4.884146341463416	6.5488	1
22,13,0	3.201219512195121	8.140243902439027	-4.9390	0
22,13,1	13.719512195121945	10.853658536585537	2.8659	1
22,33,0	0.9146341463414633	4.884146341463416	-3.9695	0
22,33,1	9.603658536585362	5.426829268292685	4.1768	1
24,11,0	1.8292682926829267	4.3414634146341475	-2.5122	0
24,11,1	7.317073170731704	1.0853658536585367	6.2317	1
24,13,0	3.201219512195121	10.3109756097561	-7.1098	0
24,13,1	16.463414634146336	5.969512195121953	10.4939	1

(B)

Figure 2.2: Checkerboard model (A) and Score details (B) as derived by GMDR

Checkerboard model representation in figure 2.2 (A) with the highest consistency and testing accuracy in three locus model for a given genetic variant. The two bars within each cell are proportional to the sum of scores in cases and controls, respectively. High-risk cells are indicated by dark shading, while the low-risk cells by light shading, and empty cells by no shading, figure 2.2 (B) shows the scores detail in each cell of the best model for two classes 1 and 2 representing the case and control group respectively. It also shows the new class level '0' and '1' representing the 'low risk' and 'high risk' genotype combinations derived from scores. The circled cells in the checkerboard (A) represent the value from best model (B). Also note that the patterns of high-risk and low-risk cells differ across each of the different multilocus dimensions, presenting evidence of epistasis.

Classification and regression tree (CART)

Classification and regression tree (CART) a robust decision-tree like analysis to identify specific combinations of genetic and environmental factors associated with the disease risk [250].

CART (version 6.6 and 7.0, Salford system): A prediction model where the analysis, represented in the form of a tree constructed by a procedure called "binary recursive partitioning" represents the best interacting parameters. The binary indicates the splitting of a node into two nodes. The term "recursive" refers to the fact that the binary partitioning process can be applied over and over again, while "partitioning" refers to the fact that the data set is split into sections or partitions. The recursive partitioning algorithm starts at the first node (with the entire data) and uses a statistical hypothesis testing method, formal inference-based recursive modeling, to determine the first locally optimal split and each subsequent split of the data set (Figure 2.3). This process continues until the terminal nodes have no subsequent splits or terminal nodes reach a prespecified minimum size of 10 individuals. The variables at each possible split are decided based on the best split values and optimal tree was selected using one standard error and 10 fold cross validation. Subgroups of individuals

with differential risk patterns were identified in the different order of nodes, indicating the presence of gene-gene and gene-environment interactions [251].

Steps in CART

CART analysis consists of four basic steps. The first step consists of tree building, during which a tree is built using recursive splitting of nodes. The overall output of CART is displayed in "Navigator" window as shown in figure 2.3.

1. Tree building: Tree building begins at the root node, which includes all the study subjects in the dataset. Beginning with this node, the CART software finds the best possible variable to split the node into two child nodes. In order to find the best variable, the software checks all possible splitting variables, as well as all possible values of the variable to be used to split the node. A number of clever programming tricks are used to reduce the time required to search through all possible splits.

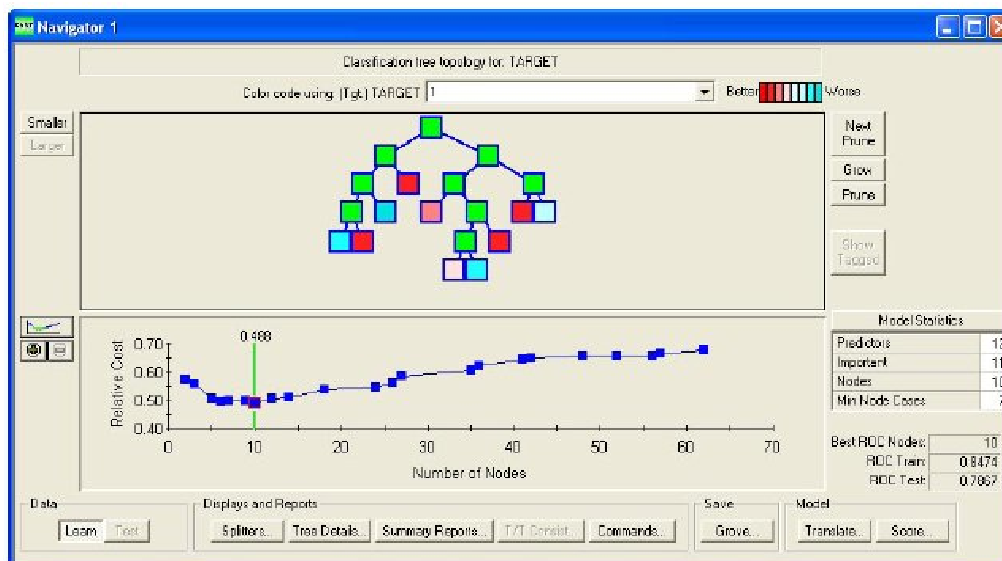


Figure 2.3: CART Navigator showing the best predictive tree model

The initial interpretation of the CART results was based on relative error curve and receiver operating characteristic (ROC) values. The relative error curve is scaled between 0 and 1 where 0 means no error or perfect fit

while 1 represents a random guessing. The best among all is indicated by the green bar marking the low point on the error profile (Figure 2.3). ROC curve, an important metric on the navigator, summarizes the performances of a model. The ROC value ranges between 0 and 1 with higher values indicating better performance. Thus for example CART analysis with an ROC of 0.70 could be considered as suggestive of interaction [251].

2. <u>Assignment of node classes</u>

Each node, even the root node, is assigned a predicted class. This is necessary, as there is no way to know during the tree-building process which nodes will end up being terminal nodes after pruning [251].

3. <u>Optimal tree selection</u>

The maximal tree will always fit the learning dataset with higher accuracy than any other tree [251].

4. <u>Cross Validation</u>

Cross validation is a computationally-intensive method for validating a procedure for model building. In cross validation, the learning dataset is randomly split into N sections, stratified by the outcome variable of interest [251].

CART can deal with missing variables while it is not applicable to GMDR analysis. Thus, CART trees can be generated even when important predictor variables are not known for all study subjects.

Clinical covariates:

Parameters selected for GxE: age, gender, onset of diabetes, history of hypertension, duration of diabetes, insulin user status (IUS), smoking, family history of DM, blood pressure systolic, blood pressure diastolic, HbA1c, BMI

CHAPTER 3

RESULTS, DISCUSSIONS AND CONCLUSIONS

3.1. Genetic association of rs5498 (K469E) polymorphism in *ICAM-1* gene with T2DR.

In the current study we analyzed the association of rs5498 (K469E) polymorphism with DR, in T2D subjects from south Indian population. The demographic details of the study participants given in appendix 3.

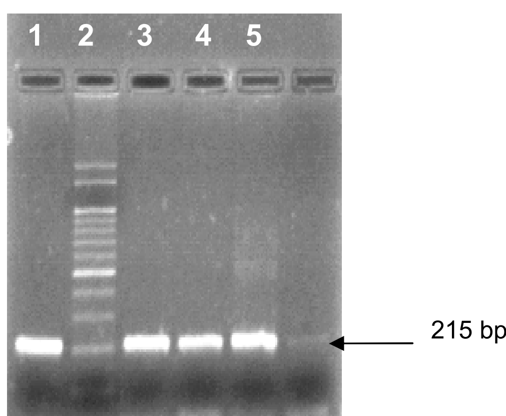


Figure 3.1.1: Ethidium bromide stained, 2% agarose gel showing the PCR amplified product of *ICAM-1* exon 6 flanking K469E polymorphism

Lane 1, 3, 4, 5 – PCR Product (215bp) of *ICAM-1* gene and Lane 2 - Molecular Weight Marker (DNA Ladder 100bp-2000bp)

The total of 356 subjects (199 cases, 157 controls) were genotyped for rs5498 and were in Hardy-Weinberg equilibrium (p>0.5). The AA genotype showed higher frequency of distribution in DR+ group when compared to DR- (p = 0.012); OR = 1.94 [95% CI: 1.06-3.55] (Table 3.1.1). Additionally the AA genotype showed significant association with the risk for DR in recessive (p = 0.01) and additive models (p = 0.03). In the additive models the homozygous genotypes AA showed higher risk OR-1.9 (AA vs. GG, p = 0.03) as shown in table 3.1.1. Also A allele was seen in higher frequency in DR+ group (p = 0.033).

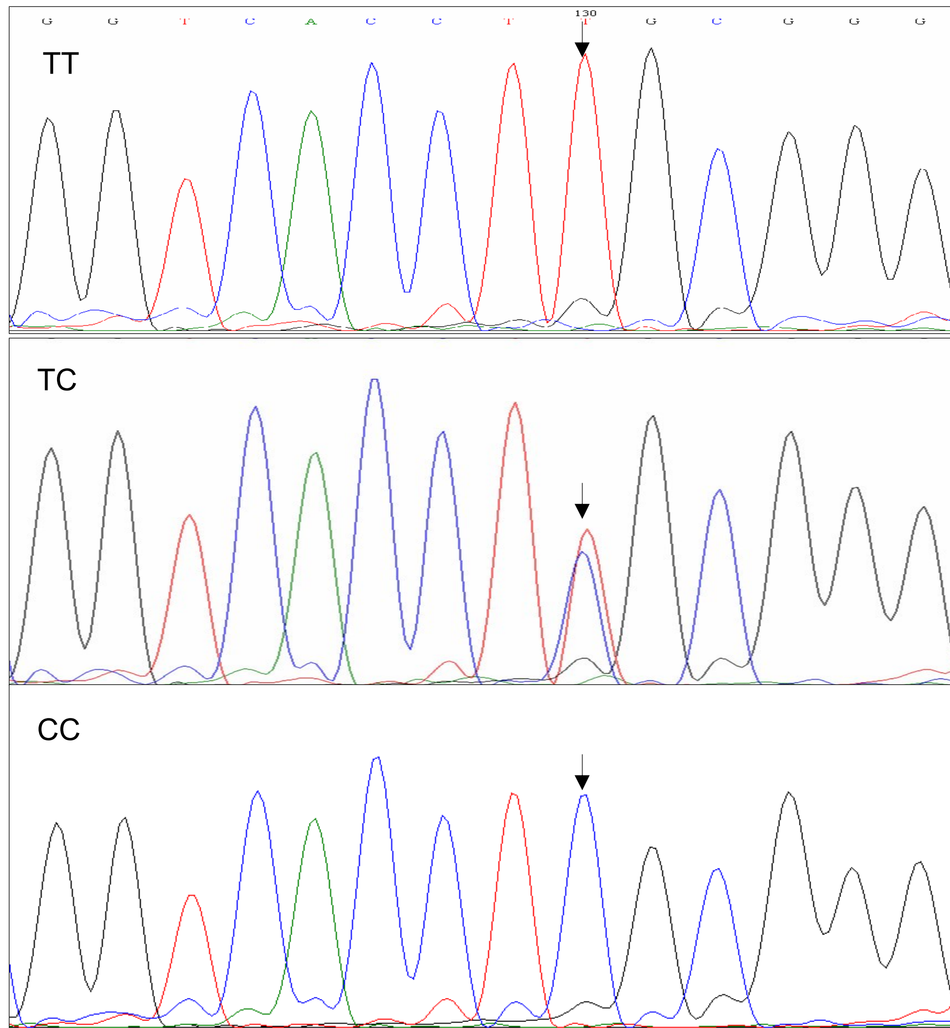


Figure 3.1.2: Electropherogram showing the three genotypes (TT, TC and CC) of the rs5498 polymorphism of *ICAM-1* gene using reverse primer

Multivariate analysis

Multivariate analysis after adjusting for the various clinical risk factors for DR is given in table 3.1.2. A negative association was observed for age/BMI and age/HDL in genotypes GG and AG respectively. A positive association was observed for insulin user status and HBA1c in all the genotypes in addition to microalbuminuria which showed 8.26 times high risk for developing DR in the AG genotype.

Table 3.1.1: Distribution of *ICAM-1* rs5498 genotype and allele frequencies in DR+ and DR- groups

<table>
<tr><th colspan="3">Genotype frequency</th><th colspan="5">P value</th><th>Allele frequency</th><th>P value</th></tr>
<tr><th>Genotype</th><th>Controls DR- (n = 157) Nos (%)</th><th>Cases DR+ (n = 199) Nos (%)</th><th>Direct association P value</th><th>Dominant GG vs. AA+ AG</th><th>Recessive AA vs. AG+ GG</th><th>Codominant AG vs. AA+GG</th><th>Additive AA vs. GG/ AG vs. GG</th><th>G:A DR- /DR+ Nos (%)</th><th>G vs A</th></tr>
<tr><td>GG</td><td>44 (28.0)</td><td>47 (23.6)</td><td>0.344</td><td rowspan="3">0.4</td><td rowspan="3">0.01*</td><td rowspan="3">0.20</td><td rowspan="3">1.9 (0.03)/ 1.0(0.09)</td><td>172(54.8):142(45.2)/ 186(46.7):212(53.3)</td><td>0.033*</td></tr>
<tr><td>AG</td><td>84 (53.5)</td><td>92 (46.2)</td><td>0.173</td><td colspan="2" rowspan="2"></td></tr>
<tr><td>AA</td><td>29 (18.5)</td><td>60 (30.2)</td><td>0.012</td></tr>
</table>

*p<0.05 – significant p value

DR+, T2D subjects with retinopathy; DR-, T2D subjects without retinopathy

Table 3.1.2: Multivariate analysis between DR+ and DR- group for *ICAM-1* rs5498 genotypes and the clinical covariates with the DR status as the dependable variable

Characteristics	**GG**		**AG**		**AA**	
	DR+		**DR+**		**DR+**	
Number	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Age (Years)	*0.91 (0.84-0.98)*	***0.019****	*0.95 (0.91-0.99)*	***0.016****	0.92 (0.84-1.01)	0.077
Male sex	3.28 (0.86-12.44)	0.081	0.94 (0.43-2.05)	0.880	1.82 (0.46-7.10)	0.391
Duration of DM (years)	0.93 (0.82-1.06)	0.277	0.99 (0.94-1.06)	0.880	0.95 (0.82-1.06)	0.404
User of insulin	*4.35 (1.09-17.32)*	***0.037****	*2.66 (1.24-5.69)*	***0.012****	*5.44 (1.03-28.90)*	***0.047****
Age at diabetes onset (years)	0.95 (0.89-1.01)	0.091	0.96 (0.93-1.00)	0.054	0.97 (0.90-1.04)	0.356
HbA1c (DCCT) (%) (IFCC) mmol/mol	*1.47 (1.14-1.89)* *1.04 (1.01-1.06)*	***0.003****	*1.32 (1.11-1.57)* *1.03 (1.01-1.04)*	***0.002****	*1.48 (1.07-2.04)* *1.04 (1.01-1.07)*	***0.017****
Systolic blood pressure(mmHg)	0.99 (0.96-1.03)	0.693	1.01 (0.98-1.02)	0.664	1.03 (0.98-1.07)	0.234
Diastolic blood pressure (mmHg)	1.08 (0.99-1.18)	0.065	*1.04 (1.00-1.08)*	***0.048****	1.08 (0.99-1.17)	0.069
BMI	*0.83 (0.70-0.97)*	***0.020****	0.93 (0.85-1.01)	0.102	0.84 (0.71-1.01)	0.059
History of hypertension	1.70 (0.48-5.97)	0.407	0.69 (0.32-1.46)	0.332	1.26 (0.34-4.74)	0.731
Smokers	1.17 (0.22-6.29)	0.857	1.10 (0.42-2.86)	0.846	3.72 (0.45-31.02)	0.225
Total cholesterol (mmol/l)	0.29 (0.08-1.05)	0.060	0.99 (0.48-2.04)	0.972	0.13 (0.01-1.39)	0.092
HDL cholesterol (mmol/l)	0.55 (0.01-55.94)	0.798	*0.03 (0.00-0.80)*	***0.037****	6.83 (0.04-127.59)	0.471
Triglycerides (mmol/l)	0.40 (0.03-5.75)	0.504	0.41 (0.07-2.49)	0.337	3.34 (0.07-15.72)	0.540
Microalbumin	2.06 (0.21-20.26)	0.537	*8.26 (2.06-33.11)*	***0.003****	3.59 (0.29-43.63)	0.316

*p<0.05 – significant p value

BMI, basal metabolic index; BP, blood pressure; DCCT, Diabetes Control and Complications Trial; DM, diabetes mellitus; DR+, T2D subjects with retinopathy; DR-, T2D subjects without retinopathy; HbA1C, glycosylated hemoglobin; HDL, high-density lipoprotein; ICAM-1, intercellular adhesion molecule-1; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine.

Table 3.1.3 shows the multivariate logistic analysis performed with the genotypes (GG vs. AG, GG vs. AA & AG vs. AA) as the independent variables and DR status as the dependent variables. Unadjusted analysis was performed initially, followed by sequential adjustment of the various clinical factors (covariates) mentioned in table 3.1.3. Significant value of p<0.5 and OR >1.0 was observed for the AA genotype when compared with the other genotypes {unadjusted p = 0.923; OR = 1.02; 95% CI = 0.62–1.70 for GG vs. AG; p = 0.032; OR = 1.94; 95% of CI = 1.06–3.55 for GG vs. AA and p = 0.019; OR = 1.89; 95% of CI = 1.11–3.22 for AG vs. AA}. After covariate adjustment for all risk factors. AA genotype showed significant p value (p = 0.004; OR = 4.82; 95% CI = 1.64–14.14) when compared against AG and GG genotype.

Table 3.1.3: Multivariate logistic analysis in DR+ group with sequential addition of clinical covariates with *ICAM-1* rs5498 genotypes as the dependable variables

Characteristics	**GG vs AG**		**GG vs AA**		**AG vs AA**	
Number	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Unadjusted	1.02 (0.62-1.70)	0.923	1.94 (1.06-3.55)	**0.032***	1.89 (1.11-3.22)	**0.019***
Age	1.02 (0.60-1.74)	0.931	1.97 (1.05-3.69)	**0.035***	1.92 (1.10-3.34)	**0.022***
Age+Gender	1.02 (0.60-1.74)	0.932	2.00 (1.06-3.77)	**0.031***	1.93 (1.10-3.36)	**0.021***
Age+Gender+DD	1.01 (0.59-1.73)	0.958	2.00 (1.06-3.77)	**0.031***	1.94 (1.11-3.39)	**0.020***
Age+Gender+DD+Insulin	1.04 (0.61-1.80)	0.873	2.28 (1.19-4.36)	**0.012***	2.12 (1.20-3.75)	**0.010***
Age+Gender+DD+Insulin+HbA1c	1.31 (0.73-2.34)	0.368	1.83 (1.25-2.69)	**0.002***	2.12 (1.16-3.85)	**0.014***
Age+Gender+DD+Insulin+HbA1c +Systolic BP	1.26 (0.70-2.28)	0.439	1.79 (1.22-2.65)	**0.003***	2.15 (1.17-3.93)	**0.013***
Age+Gender+DD+Insulin+HbA1c+Systolic BP+Diastolic BP	1.28 (0.70-2.32)	0.425	1.81 (1.21-2.70)	**0.004***	2.12 (1.15-3.91)	**0.016***
Age+Gender+DD+Insulin+HbA1c+Systolic BP+Diastolic BP+BMI	1.31 (0.71-2.42)	0.377	1.97 (1.29-3.01)	**0.002**	2.21 (1.18-4.13)	**0.013***
Age+Gender+DD+Insulin+HbA1c +Systolic BP+Diastolic BP+BMI +SMK	1.31 (0.71-2.41)	0.392	1.99 (1.29-3.06)	**0.002***	2.22 (1.18-4.16)	**0.013***
Age+Gender+DD+Insulin+HbA1c +Systolic BP+Diastolic BP+BMI +SMK +Microalbumin	1.25 (0.60-2.59)	0.552	2.04 (1.26-3.32)	**0.004***	3.17 (1.41-7.08)	**0.005***

Characteristics	GG vs AG		GG vs AA		AG vs AA	
Number	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Age+Gender+DD+Insulin+HbA1c +Systolic BP+Diastolic BP+BMI +SMK + Microalbumin +Triglyceride	0.68 (0.27-1.75)	0.430	1.77 (0.98-3.21)	0.059	3.75 (1.28-10.98)	**0.016***
Age+Gender+DD+Insulin+HbA1c +Systolic BP+Diastolic BP+BMI +SMK + Microalbumin +Triglyceride+HDL	0.74 (0.27-1.97)	0.543	1.75 (0.97-3.18)	0.064	4.17 (1.39-12.53)	**0.011***
Age+Gender+DD+Insulin+HbA1c +Systolic BP+Diastolic BP+BMI +SMK + Microalbumin +Triglyceride+HDL +Cholesterol	0.67 (0.25-1.84)	0.439	1.80 (0.98-3.34)	0.060	4.07 (1.34-12.35)	**0.013***
Age+Gender+DD+Insulin+HbA1c+BMI+Cholesterol+HDL+SMK+Triglyceride+ Microalbumin +HOHT	0.63 (0.23-1.72)	0.365	1.72 (0.98-3.02)	0.061	3.56 (1.23-10.03)	**0.020***
Gender+Onset of diabetes +Insulin +HbA1c+ Systolic BP+Diastolic BP +BMI+SMK+Cholesterol+HDL+Triglyceride + Microalbumin	0.67 (0.26-1.76)	0.417	1.89 (1.03-3.49)	**0.041***	4.82 (1.64-14.14)	**0.004***

*p<0.05 – significant p value

BMI, basal metabolic index; BP, blood pressure; DD, duration of diabetes; DM, diabetes mellitus; DR+, T2D subjects with retinopathy; DR-, T2D subjects without retinopathy; HbA1C, glycosylated hemoglobin; HDL, high-density lipoprotein; HOHT, history of hypertension; ICAM-1, intercellular adhesion molecule-1; SMK, smoking

Bioinformatic analysis: Conservational analysis using ConSurf 9 predicted the amino acid at position 469 to be of highly variable nature. SIFT prediction showed the variant as benign and tolerable. Moreover, sequence based stability analysis using MUpro showed an increased in stability for the variant (E469) when compared to the wild (K469) with a confidence score of 0.10666991. Folding rate (using/ Fold rate server) for the wild and variant proteins respectively revealed the values of 5.54/sec and 3.3/sec.

Three dimensional structure model of K469 *ICAM-1* variant was modeled using the natural variant (PDBID: 2OZ4) as a template. The generated structure loop refined using loop.py module of Modeller9v7. The steric clashes and bad contacts removed using What-If server. Structural quality of the protein assessed by checking the Ramachandran plot and ProQ server and tabulated (Table 3.1.4). Moreover, the structure of wild and variant energy optimized using OPLS force filed for 1000 runs of steepest descent. Backbone superimposition of wild and variant forms of ICAM-1 showed RMSD deviation of 0.943 Ǻ. Since structural superimposition studies showed mild deviation (Figure 3.1.3), secondary structural analysis were performed using STRIDE [252] a software tool for secondary structure assignment from atomic resolution protein structures, that however did not show any significant change between the wild and variant .

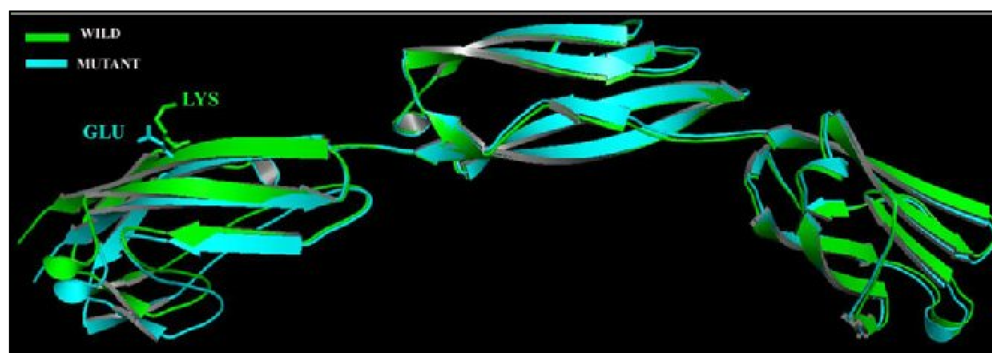


Figure 3.1.3: Structural superimposition of ICAM-1 wild type K469 (green) and variant E469 (blue) with a 0.943 Ǻ of backbone RMSD (Root Mean Square Deviation), as calculated by PYMOL. The variant residues are shown in stick representation.

Table 3.1.4: Comparison of the structural properties of the wild (KK) and variant (EE) proteins for SNP rs5498 of *ICAM-1* gene

	Wild	**Variant**
Experimental type	**Homology Modeling**	**PDBID:** 2OZ4 Resolution:2.70Ǻ
Residues in most favoured regions	95.5%	88.8%
Residues in additional allowed region	4.0%	10.8%
Residues in generously allowed regions	0.4%	0.4%
Residues in disallowed regions	0.0%	0.0%
G-factor	-0.04	0.13
Bond lengths	Main chain:99.8% Within limits:0.2%	Main chain:100% Within limits:0.0%
Bond angles	Main chain:93.9% Within limits:6.1%	Main chain:100% Within limits:0.0%
Planarity	Planar groups:100% Within limits:0.0%	Planar groups:100% Within limits:0.0%
Energy minimization using Gromacs for 1000 runs of Steepest Descent	-2.9554834e+04	-3.7942434e+04

3.1.1 Discussion

In the present study, the frequency distribution of K469E (rs5498) polymorphism in the *ICAM-1* gene has been analyzed for its possible association with T2DR from south India. A higher frequency of AA genotype is being observed in the DR+ when compared to the DR- group [OR = 1.94 (95% CI; 1.06-3.55, p – 0.012)] in the study. The frequency of rs5498 polymorphism observed in the current study simulates the other reports from India and other population like Japanese and Chinese PDR patients [253, 254].

The “A” allele of rs5498 confers disease susceptibility in type 1 diabetes patients with nephropathy of Swedish Caucasian origin 255]. A high heterozygous index was observed for the polymorphism in the current study similar to that observed in GoKinD study [255]. However another similar study from Caucasian cohort shows significant association with GG genotype suggesting the population difference per se [116].

Our results also differ from another similar study in India, by Balasubbu et al. [141]. The possible reasons for the observed differences between our study and the other reports from India could be attributed to the area of sampling and the types of DR included in the study. The present study represents a more homogenous population from the same geographical area of southern India against that of Balasubbu et al which represents a hospital based population. We have included STDR patients with PDR, NPDR or CSME while the ARAVIND study includes only PDR similar to the Caucasian study. One of the major limitations of the study is the smaller sample size that gives a decreased power for the study.

Strong heritability factor has been observed for circulating levels of sICAM-1 by bivariate quantitative genetic analyses [256] and up regulated expressions are being reported in animal models and PDR patients. Such elevated levels are reported to be influenced by glycemic control, disturbances in lipid metabolism, obesity and insulin resistance etc. which are also important clinical determinants of DR. Hence we performed a multivariate logistic regression analysis for the different genotypes between DR+ and DR- groups after adjusting these parameters.

In the current study, we observed an OR of 8.26 [CI, 2.06-33.11] for the heterozygous genotype (AG) in the DR group after adjusting for microalbuminuria (Table 3.1.2) and a genotype dependant risk observed after the sequential addition of gender (Table 3.1.3). Study by Bruno et al also shows increase in the levels of ICAM1, VCAM-1 in the plasma of T2D patients with microalbuminuria thus suggesting a significant correlation between the same [257]. Additionally study by Ma et al conferred decreased risk susceptibility to diabetic nephropathy development with allele G in female T1D patients in GoKinD population [258].

ICAM-1 expression has been reported to share a common genetic modulation with traits related to obesity, insulin resistance, and HDL3 cholesterol levels [259]. We observed significant p value with OR >1.0 for the AA genotype after the sequential addition of lipid biomarkers (Table 3.1.3). An abnormal endothelial activation after an oral lipid meal, coupled

with an increased oxidative stress is being observed in patients with familial history of T2D [260]. High fat meals are shown to increase ICAM-1 and other adhesion molecules in normal and diabetic subjects [260]. Similarly a recent study on the co-effects of inflammation and endothelial dysfunction and insulin resistance (IR) on hypertension in a large Asian population reports elevated levels of biomarkers for inflammation and endothelial cell dysfunction including sICAM-1 [261]. The *ICAM-1* GG (E469E) genotype reported as an independent contribution factor of plasma fibrinogen level as well as high-density lipoprotein-cholesterol and urinary albumin excretion in atherosclerosis [262]. However we did not identify any statistically significant association for the GG genotype with these 2 parameters in the current study.

Collectively, multivariate analysis after sequential addition of various DR risk factors reveal a high susceptibility for DR (OR>1.0, Table 3.1.3) in the AA genotype of rs5498 for all the DR covariates. Interestingly, these parameters are reported to have a significant effect on ICAM-1 expression [105,108,259]. Estimation of ICAM-1 levels in individuals with E469K polymorphism has been reported to be associated with many inflammatory and infectious diseases. However, population differences are seen [263, 264]. These differences could be due to the dietary influence/epigenetic silencing of ICAM-1 expression by hypermethylation as reported in animal and human models [265,266]. In the study such analysis, functional characterization of K469E SNP and serum correlation weren't performed which otherwise could have validated the functional effect of the polymorphism and thus strengthen the study.

Any disease associated polymorphism can possibly mediate the effect either through altered expression or function of the protein. The rs5498 is located at three-base position upstream of the splice donor site that produces an alternatively spliced short isoform (ICAM-1-S) that has no transmembrane or intracellular domain and speculated to influence the ICAM-1 signal transduction and cell-cell contact including Fas-FasL interaction [267]. Thus correlated decrease in FLIP-L mRNA expression

and apoptosis suggests a putative role of the polymorphism in regulating apoptosis by modifying the inflammatory immune responses [92]. Comparison of the RNA splicing patterns in cells expressing G/G (469E) and A/A (469K) genotypes showed a comparatively higher expression of ICAM-1-S mRNA in A/A cells [267].

The polymorphism rs5498 results in a non-conservative change from lysine to glutamic acid in the fifth immunoglobulin-like domain of ICAM-1 [109] that is essential for dimerization, surface presentation and solubilization of the protein [114]. We therefore performed a bioinformatic analysis to study the putative effect of this SNP on the ICAM-1 structure and thus its influence on the expression. As per the sequence analysis, the variant K469E was shown as benign without any significant secondary structural change. The X-ray structure of ICAM-1 consists of Ig like C2 type domain 3, 4 & 5 that consist of 4 intra disulphide bridges as per Swiss- Prot annotation and it includes 237-290, 332-371, 403-419 and 431-457 (http://www.uniprot.org/uniprot/P05362). It could therefore be inferred that the variant K469E doesn’t affect the disulphide bridges. Interestingly structure superimposition of the 2 variants (Figure 3.1.3), revealed a 0.943 Å deviation of backbone RMSD (Root Mean Square Deviation) as calculated by the software PYMOL thus suggesting a structural effect of the SNP. The difference in the fold rate time observed between the KK (5.4/sec) when compared to the EE (3.3/sec) variant highlights the need of further dimerisation studies.

Our results indicate that the AA genotype of *ICAM-1* (rs5498) gene increases the risk predisposition for retinopathy in T2D patients in south Indian population. Clinical covariates like microalbuminuria, lipid biomarkers etc shows a genotype dependent (AG/AA) increase in the odds of risk for DR among the T2D patients. Bioinformatics analysis of rs5498 showed a deviation in the structure and folding rate of the ICAM-1 protein. These observations emphasize the need for further studies to identify the molecular mechanism connecting the SNP, expression, protein structure and function.

3.2 Genetic association of rs1061170 polymorphism in *CFH* gene with T2DR.

In the current study we have analyzed the association of rs1061170 in *CFH* gene with DR in south Indian population in total of 375 unrelated T2D subjects.

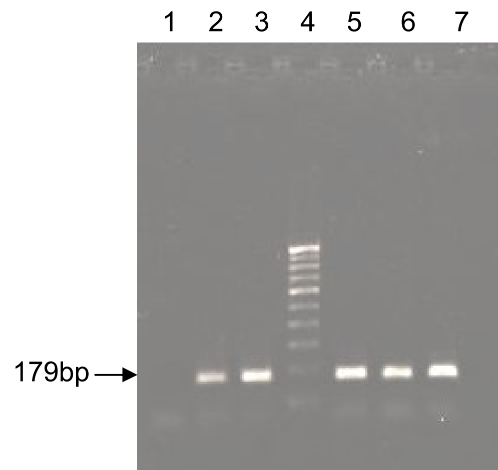


Figure 3.2.1: Ethidium bromide stained, 2% agarose gel showing the PCR product of *CFH* gene. Lane 2, 3, 5, 6, 7: PCR Product (179 bp); Lane 4: Molecular Weight Marker (100bp-2000bp ladder)

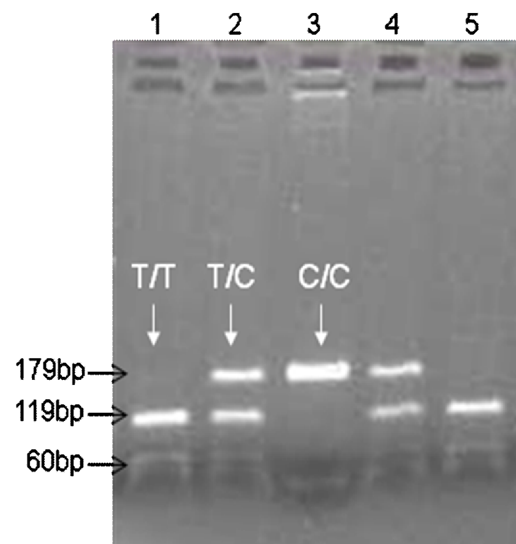


Figure 3.2.2: Ethidium bromide stained, 4% agarose gel showing the RFLP digested products for TT (Lanes 1, 5), (TC) (Lanes 2, 4), CC (Lane 3) genotypes of rs1061170

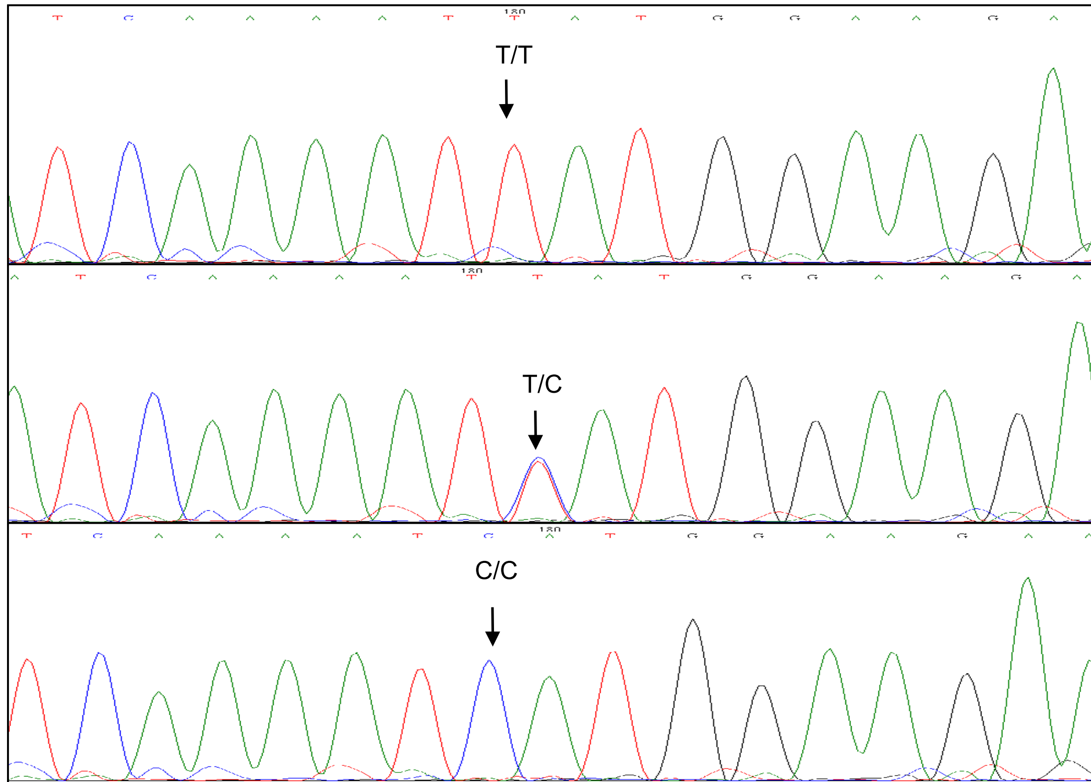


Figure 3.2.3: Electropherogram showing the three genotypes (TT, TC and CC) of the rs1061170 polymorphism of *CFH* gene using forward primer

Genotype distributions were in Hardy-Weinberg equilibrium in both cases (p - 0.896) and controls (p - 0.159) (Table 3.2.1) and there wasn't any significant difference in the distribution of the genotypes and allele frequencies *of* rs1061170 between the two groups (Table 3.2.1).

Table 3.2.1: Distributions of rs1061170 genotypes and alleles between T2D subjects with and without retinopathy

Frequency	**Control/DR-** (n = 185)	**Case/DR+** (n = 190)	**p**	**OR, 95% CI (1)**	**P**
Genotype					
TT	88 (47.6)	97 (51.1)	0.353	Ref.	
TC	73 (39.5)	77 (40.5)		0.96 (0.62, 1.47)	0.84
CC	24 (12.9)	16 (8.4)		0.61 (0.30, 1.21)	0.16
HWE (p value)	0.159	0.896			
Dominant model					
TT	88 (47.6)	97 (51.1)	0.56	0.87 (0.58, 1.30)	0.50
TC+CC	97 (52.4)	93 (48.9)			
Recessive model					
TC+TT	161 (87.0)	174 (91.6)	0.20	1.62 (0.83, 3.16)	0.16
CC	24 (13.0)	16 (8.4)			
Alleles					
T	249 (67.3)	271 (71.3)	0.26	1.21 (0.89, 1.65)	0.23
C	121 (32.7)	109 (26.7)			

Multivariate analysis between DR+ and DR- groups showed significant association for the genotypes when adjusted for age, insulin user status, diastolic blood pressure and HbA1c levels. A higher risk for DR demonstrated by OR>1.0 is being observed for TT and TC genotypes (OR= 2.67 and 6.57) after adjusting for HbA1c levels, in DR group. Similarly TC and CC genotypes showed higher risk (OR = 4.7 and 60.9) after adjusting with insulin user status and CC genotype with diastolic blood pressure (OR = 1.19). Adjusting age as one of the covariates showed less risk for DR in the TT genotype (OR = 0.88).

Table 3.2.2: Multivariate analysis between DR+ and DR- group for *CFH* Y402H genotypes adjusted for the risk factors of DR

Characteristics	TT		TC		CC	
	DR+		DR+		DR+	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Unadjusted	1.134 (0.816 - 1.452)	0.085	1.309 (0.951 - 1.667)	0.133	0.560 (0.0256 - 1.094)	0.082
Gender	0.892 (0.396 - 2.012)	0.78	0.576 (0.201 - 1.649)	0.304	1.092 (0.12 - 9.949)	0.938
Age	0.885 (0.816 - 0.96)	< 0.001*	0.97 (0.889 - 1.059)	0.497	0.954 (0.759 - 1.199)	0.687
Onset of diabetes	1.035 (0.962 - 1.113)	0.355	0.956 (0.88 - 1.04)	0.295	1.13 (0.887 - 1.439)	0.322
History of hypertension	0.81 (0.365 - 1.797)	0.604	0.885 (0.336 - 2.332)	0.805	0.327 (0.046 - 2.307)	0.262
Insulin	2.265 (0.923 - 5.558)	0.074	4.733 (1.627 - 13.768)	0.004*	60.909 (2.147 - 1727.969)	0.016*
Smoking	2.4 (0.715 - 8.052)	0.156	0.958 (0.293 - 3.135)	0.944	0.174 (0.006 - 4.944)	0.306
Family history	1.824 (0.861 - 3.86)	0.116	1.942 (0.846 - 4.458)	0.117	5.628 (0.53 - 59.783)	0.152
Blood pressure systolic	1.015 (0.991 - 1.039)	0.224	1.012 (0.983 - 1.041)	0.431	0.948 (0.889 - 1.011)	0.102
Blood pressure diastolic	1.031 (0.99 - 1.074)	0.142	1.06 (1 - 1.124)	0.051	1.199 (1.033 - 1.392)	0.017*
HbA1c	2.671 (1.115 - 6.395)	0.027	6.571 (2.479 - 17.415)	<0.001	0.567 (0.069 - 4.658)	0.598

*p<0.05 – significant p value

3.2.1 Discussion

The results of the current study did not reveal any association for Y402H (rs1061170) polymorphism with DR in unadjusted model for the various clinical risk factors. The results corroborate a similar report from India by Balasubbu et al in a different cohort of south Indian population [141]. The major alleles in our study consist of T alleles (cases:72%, controls:67%) while the C allele was observed as major allele (cases:70%, controls:72%) by Balasubbu et al. This difference could be attributed to population heterogeneity that emphasizes the need of replication of the study in larger samples across Indian population to arrive at any conclusion. Nevertheless the allele frequency of this polymorphism has been reported to show ethnic variation with lower C allele frequency in Chinese, as similar to the current study, than the North American and European population [268]. The 'C' allele has been associated with risk of AMD in the Chinese and Caucasians [268].

The activation of complement system and its components have been implicated in the pathogenesis of various ocular diseases including uveitis, corneal diseases, AMD and DR [127]. CFH, an important inhibitor of the complement pathway, when activated initiates proteolytic cascade that releases proinflammatory anaphylatoxins and causes formation of a membrane-attack complex ultimately leading to cell lysis. CFH preferentially binds and inactivates complement component C3b, and prevents the production of C3 convertase and progression of the cascade. Histopathological studies by Gerl et al. have shown dense staining for membrane attack complex (MAC) and C3d components in the retinal choriocapillaries of DR subjects [127]. Study by Zhang et al along with Gerl et al. eliminates the involvement classical and lectin pathways in complement activation [127].

Regulation of complement system by CFH is facilitated by its binding with heparin and C-reactive protein at the site where Y402H polymorphism is also located. Y402H polymorphism is postulated to alter the ability of CFH

to suppress excess complement activation, leading to increased complement-dependent damage to the arterial wall and vessel injury [128, 135, 136]. The C allele of Y402H polymorphism has been strongly and consistently associated as risk factor for AMD in several populations worldwide. Apart from AMD, 81% patients with dense deposit disease (membranoproliferative GN type 2) have also been shown to carry at least one copy of the *CFH* His402 [269]. In addition to this genome-wide linkage analysis has identified a locus for DR on chromosome 1 harboring the *CFH* gene [270]. The present study however did not find association of Y402H polymorphism with DR similar to yet another study from India by Balasubbu et al. Further prospective studies on larger sample size with DR from across the population are needed to substantiate this hypothesis.

Inflammatory mediators and glycemic control (HbA1C): Multivariate analysis between DR+ and DR- for various clinical risk factors for DR showed significant $p<0.05$ when adjusted for HbA1c levels. Inflammatory mediators have earlier been correlated with glycemic index in diabetics patients [271].

Complement pathway and insulin resistance (IR): The study results showed that insulin users with homozygous CC genotype, had increased risk for retina complications (OR>1.00). Till data literature does not provide supportive evidence for the direct correlation between CFH and IR except for studies associating CFH with C3 and C3 with IR [272-274].

Elevated levels of C3 and acylation stimulating protein (ASP; cleavage product of C3), along with IR, have been observed in diabetes and proliferative DR [275-277]. Experimental evidence also supports the role of ASP in IR; administration of recombinant ASP to C3 deficient mice on low fat diet increased insulin sensitivity, while on high fat diet caused IR. This study also showed that improvement of IR due to weight loss reduces the concentration of circulating CFH [278]. A recent study has also suggested serum C3 level to be a strong inflammatory marker of IR in women with

polycystic ovary syndrome [279]. Complement anaphylatoxin C5a receptor (C5aR) has also been implicated in the IR mediated by macrophage accumulation and obese adipose tissue dysfunction [280]. These studies show the possible link between the complement system and insulin resistance.

CFH level is shown to be negatively associated with insulin sensitivity. A recent study shows that an increased expression of CFH in subjects with altered glucose tolerance (AGT) reflects elevated AP activation in adipose tissue linked to IR [281].

On the contrary complement proteins have been shown to be reduced in T1D, irrespective of duration or complications thus indicating the role of genetic factors [282]. In the present study, a similar observation is made on genotype specific risk predisposition among insulin users: CC genotypes using insulin renders highest risk for DR (OR: 5.0, p – 0.04) in comparison to TC (OR: 3.4, p – 0.012) and TT (OR: 1.6, p – 0.23) when compared to the non insulin users.

Inflammatory response has shown a direct relationship with age [283]. A complex interaction between the genetic and environmental factors modulating the risk for inflammation has been implicated earlier Genetic factors (SNPs in IL-6 and IL-10) were also shown to influence the susceptibility to inflammation. Polymorphism in the inflammatory markers has been shown to affect its expression due to age [283]. The present study results also showed a genotype specific correlation for Y402H genotypes with age and DR susceptibility. The TT genotypes of Y402H conferred protection for DR (Table 3.2.2) in T2D individual with higher age in comparison to CC and TC genotypes. Inflammation and elevated blood pressure have been associated with each other [284]. The effect of blood pressure in inducing inflammatory reaction in atherosclerotic diseases and association of promoter polymorphism in IL-6 with CHD, angiotensin II receptor type-1 (AGTR1) gene, and hypertension has been earlier observed [285]. Similarly our results also showed increased risk for DR with the CC genotype of Y402H polymorphism of *CFH* [286, 287].

3.3 Genetic association of promoter (rs12150053, rs12948385) polymorphism in *PEDF* gene with T2DR.

In the current study we have analyzed the association of polymorphisms (rs12150053, rs12948385) in *PEDF* gene with T2DR in south Indian population. The genotyping was done in total of 287 unrelated T2D subjects. The demographic and clinical characteristic of the study subjects is given in appendix 2.

The genotypes of *PEDF* rs12150053 and rs12948385 variant were determined by running the RFLP digested product in 4% agarose gel electrophoresis. Figure 3.3.1 represents the 2% agarose gel showing the PCR product while, figure 3.3.2 represents the RFLP digested product. The genotypes were also confirmed by random sequencing (Figure 3.3.3 and 3.3.4).

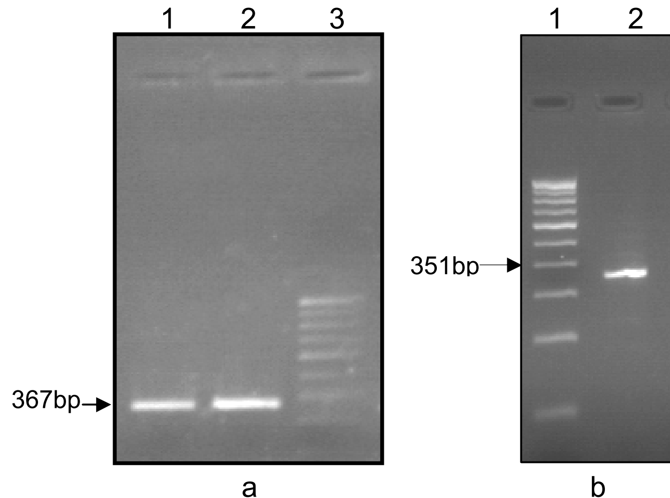


Figure 3.3.1: Ethidium bromide stained 2% agarose gel showing the PCR amplified product of rs12150053 (Fig a; lane 1,2), **rs12948385** (Fig. b; lane 2) Lanes 3 and 1 of figure a and b shows Molecular weight marker (DNA Ladder 100bp-2000bp)

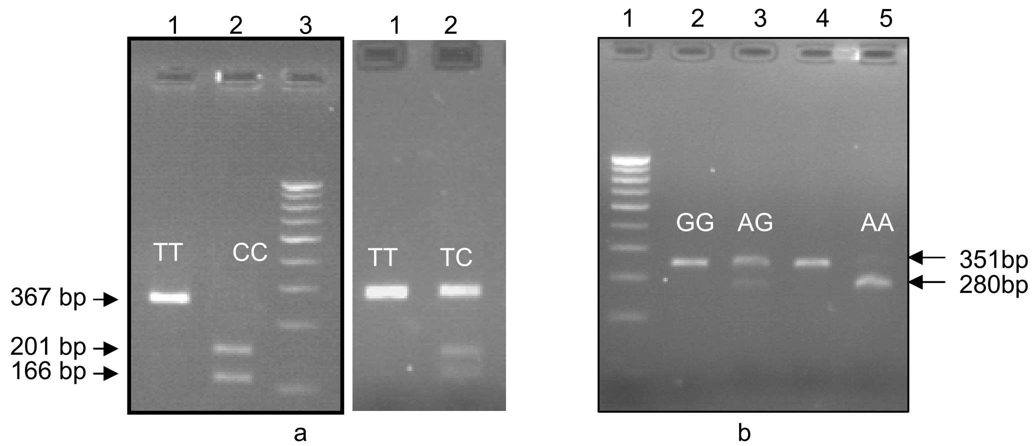


Figure 3.3.2: Ethidium bromide stained 4% agarose gel showing RFLP digested pattern of rs12150053 in Fig 2a (lanes 1,2) and rs12948385 in Fig. 2b (lanes, 2, 3, 4, 5). Lane 3 and 1 of figure a and b shows Molecular weight marker (DNA Ladder 100bp-2000bp)

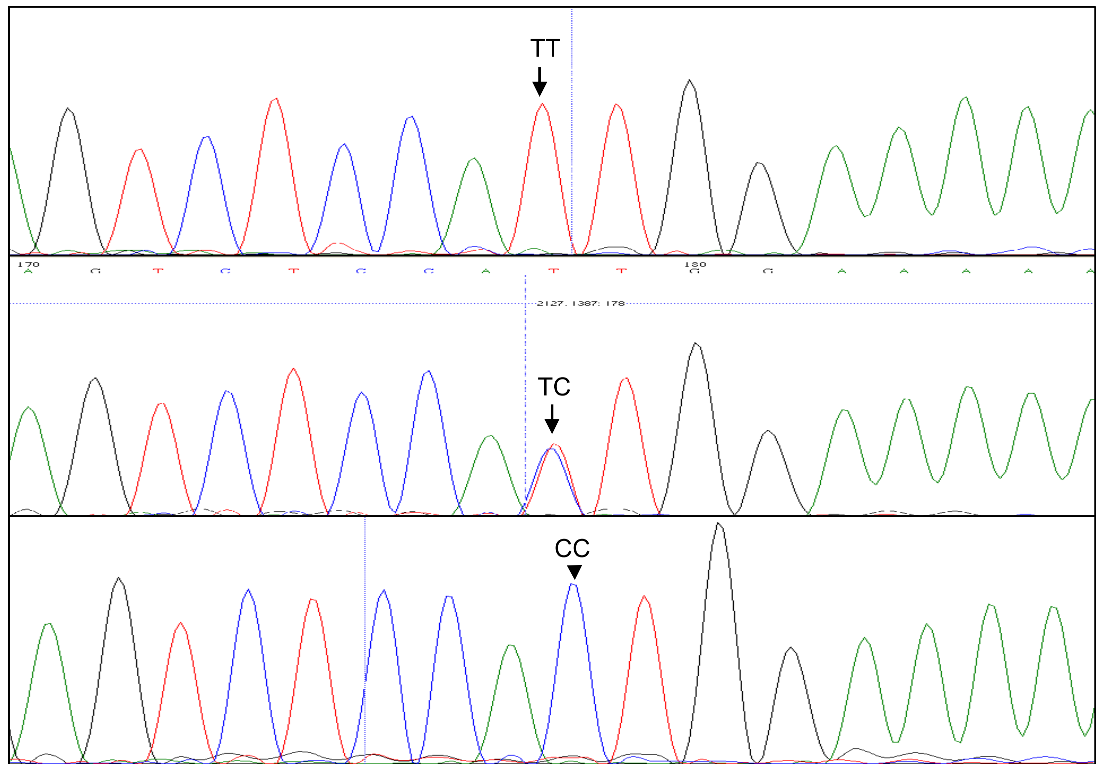


Figure 3.3.3: Electropherogram of TT, TC, CC genotypes of rs12150053 using forward primer

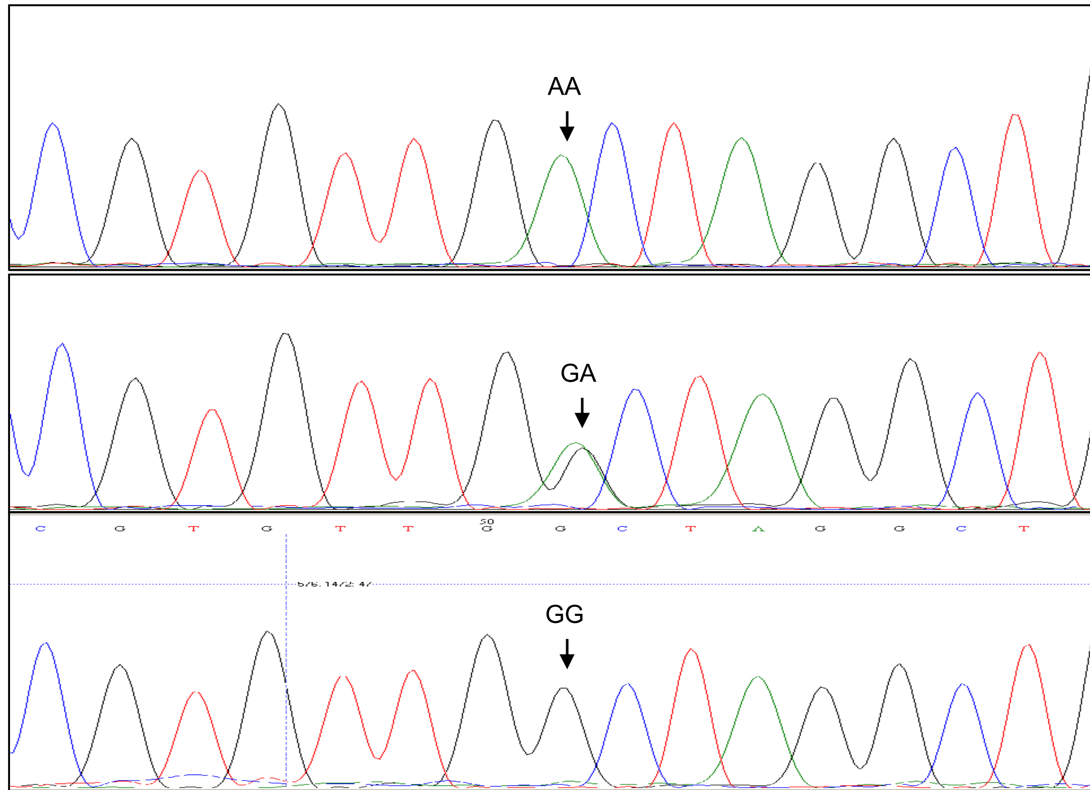


Figure 3.3.4: Electropherogram of AA, GA, GG genotypes of rs12948385 using forward primer

The genotypes and alleles of rs12150053, rs12948385 were in HWE ($p>0.05$). We did not find any significant difference in the distribution of the SNPs between cases and controls (Table 3.3.1). The genotypes of rs12150053, rs12948385 of *PEDF* were obtained for 287 T2D subjects.

Haplotype analysis: Single marker association test (Table 3.3.2) showed T and A alleles of rs12150053 and rs12948385 *PEDF* genes to be highly represented in the cases (DR+) though not statistically significant (p=0.0765, 0.1515). The promoter SNPs (rs12150053 and rs12948385) showed D' value of 0.93 and r^2=78 (D' and r^2 – measure of LD) suggesting strong LD between the 2 SNPs. Four haplotypes identified by the haploview analysis (Figure 3.3.5) did not differ in their distribution between cases and controls (Table 3.3.3).

Table 3.3.1: Distribution and χ^2 analysis for genotypes and allele frequencies for rs12150053 and rs12948385 of *PEDF* gene between DR+ and DR- T2D subjects.

Frequency					p value#			
Gene/SNP	**Allele(1/2) / Genotype (11/12/22)**	**HWE p- value Cases/ Controls**	**DR+ (N = 165)**	**DR- (N = 122)**	**Allele (1/2)**	**Genotype (11/12/22)**	**Genotype (11+12/22)**	**Genotype (11/12+22)**
rs12150053	C/T		76/254	43/201	0.114	0.210	0.235	0.254
	TT/TC/CC	0.74/0.26	97/60/08	81/39/02				
rs12948385	A/G		82/248	48/196	0.143	0.285	0.257	0.806
	GG/GA/AA	0.62/0.32	92/64/09	77/42/03				

#p value by χ^2-square test

Table 3.3.2: Distribution of single marker of PEDF variants among the DR+ and DR-

SNP	Association allele	Case, control ratio	χ^2	p
rs12150053	T	61:181, 38:170	3.137	0.0765
rs12948385	A	64:178, 43:165	2.057	0.1515

Table 3.3.3: Frequency distribution and statistical analysis of haplotypes in the study population

Markers in Block 1	Haplotype	Frequency	Case, Control Ratios	χ^2	p
rs12150053, rs12948385,	CG	0.751	174.9 : 67.1, 162.9 : 45.1	2.193	0.1387
	TA	0.209	57.9 : 184.1, 35.9 : 172.1	3.001	0.0832
	CA	0.029	6.1, 235.9, 7.1 : 200.9	0.306	0.5803
	TG	0.011	3.1 : 238.9, 2.1 : 205.9	0.079	0.7784

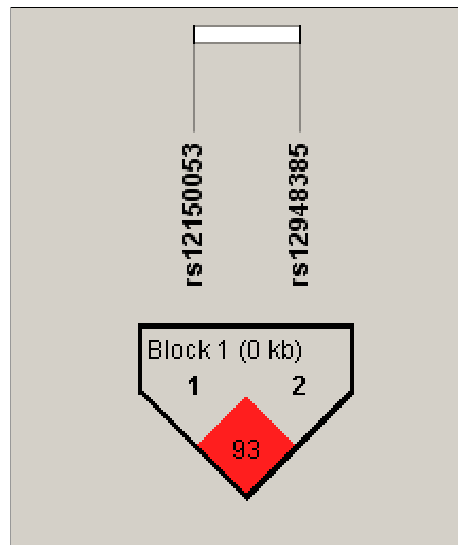


Figure 3.3.5: Software Haploview version 4.0 were used to estimate haplotype blocks

3.3.1 Discussion

The present study on genetic association of SNPs in *PEDF* gene with the risk of T2DR in south Indian population did not show any significant association with DR. PEDF, a multifunctional protein, possess potential neuroprotective properties. Its intraocular levels have been observed to be lower in PDR [180] and early phase of experimental DR in mice models [288]. Thus emphasizing the need to study the promoter SNP with the risk for DR. Promoter polymorphisms (rs12150053 and rs12948385) have been earlier studied for their putative association with DR and microangiopathy in various populations [170, 191, 289]. The present study however did not find significant distribution of genotypes for promoter SNPs. This consistency was also observed in yet another study on DR in Indian cohort as well as with AMD in white Europeans [141, 188]. The observed differences among these studies could be attributed to population difference and disease phenotypes. Study subjects of Izuka et al consist of hospital based patients whereas subjects enrolled in the current study represents a more homogenous population recruited prospectively from an epidemiology study SNDREAMS.

The function of the *PEDF* polymorphisms investigated in the present study is currently unclear. The promoter region is shown to possess a variety of putative binding sites for transcription factors and two Alu repetitive sequences [290] however two polymorphisms investigated in our study do not affect any of these binding sites. The properly folded PEDF protein has been shown to protect the neurons from glutamate mediated injury in rats. Moreover its neurotrophic and neuroprotective activity is attributed to a 44 mer peptide, spanning amino acid positions 78–121, derived from the N-terminal region of the human PEDF molecule (418aa) [291]. Our haplotype analysis result could not detect any difference in the haplotypes frequencies between the cases and controls similar to the finding by Mattes et al. [188].

3.4 Genetic association promoter (rs1617640) polymorphism in *EPO* gene with T2DR.

In the current study we have analyzed the association of polymorphism rs1617640 in *EPO* gene with DR in a total of 369 unrelated T2D subjects from south Indian population. The demographic and clinical characteristic of the study subjects is given in appendix 3.

The genotypes for the *EPO* variant rs1617640 was determined by direct DNA sequencing as shown in figure 3.4.2.

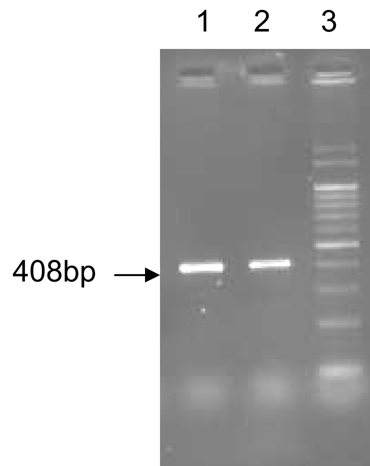


Figure 3.4.1: Ethidium bromide 2% agarose gel showing amplified product of rs1617640 polymorphism in *EPO* gene Lane 1 – Molecular weight marker (100bp – 2000bp DNA ladder), lane 2 & 3: amplified product.

The genotypes and alleles of SNP (rs1617640) were in HWE ($p>0.05$). We did not find any significant difference in the distribution of the SNPs between cases and controls (Table 3.4.2). The genotypes were obtained for 369 T2D subjects.

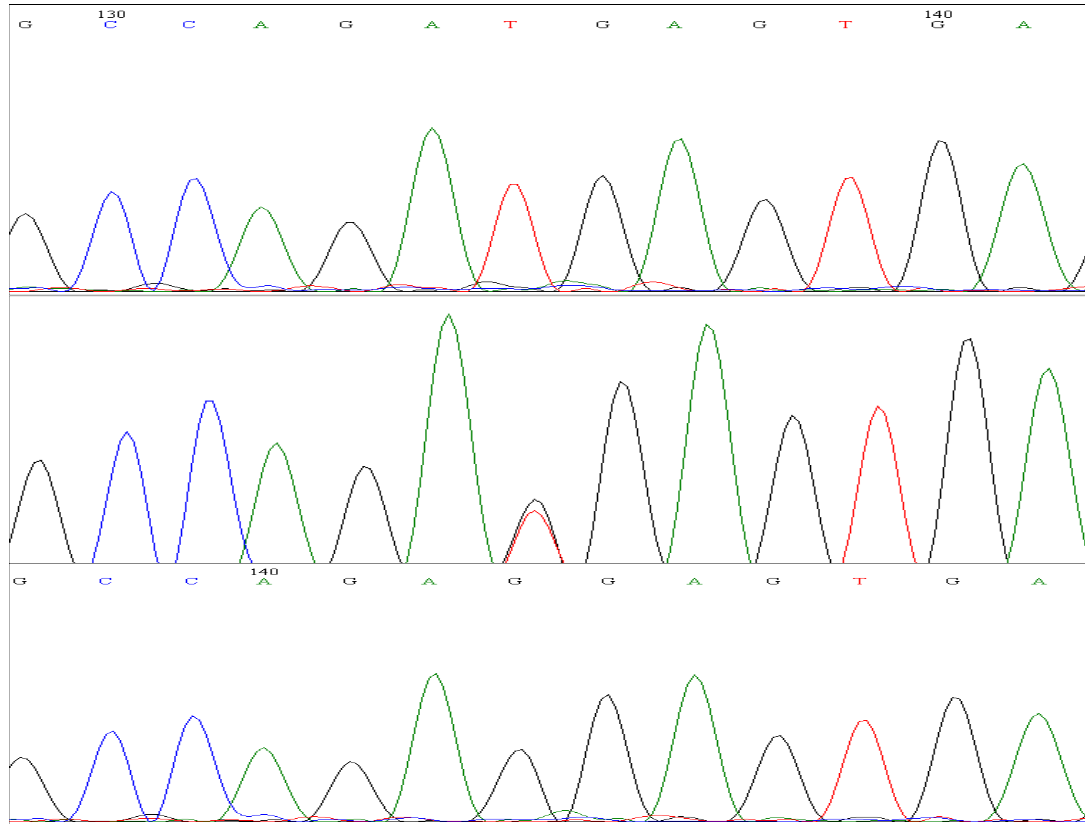


Figure 3.4.2: Electropherogram of TT, TG and GG genotypes of rs1617640 using forward primer

Table 3.4.2: Comparison of genotypes and allele frequencies for rs1617640 *EPO* gene between DR+ and DR-

Frequency					p value#			
Gene/SNP	**Allele(1/2) / Genotype (11/12/22)**	**HWE p- value Cases/ Controls**	**DR+ (N = 201)**	**DR- (N = 168)**	**Allele (1/2)**	**Genotype (11/12/22)**	**Genotype (11+12/22)**	**Genotype (11/12+22)**
***EPO*/rs1617640**	G/T		139/263	104/232	0.297	0.580	0.2506	0.5657
	TT/TG/GG	0.82/0.97	86/91/24	80/72/16				

#p value by χ^2-square test

3.4.1 Discussion

The present study evaluates genetic association of rs1617640 of *EPO* gene with the risk of T2DR in south Indian population. We did not find any significant difference in the distribution of the alleles / genotypes of rs1617640 (*EPO*) between cases and controls (Table 3.4.2) that simulates the results of another study from south India [141].

Retinal hypoxia has been shown to upregulate numerous hypoxia inducible genes. One among them is EPO which renders neuroprotection to wide variety of tissues, including the brain, kidney, heart and retina [192].

EPO, a potent neuroprotective factors, has been found to be upregulated in the RPE, neuroretina and vitreous of donor eyes from diabetic, PDR patients when compared to the non diabetic controls [198]. Diabetic rats treated with EPO-derived peptide [198] showed protection from neuroglial and vascular degenerative changes thus suggesting its therapeutic implications in DR. However the exact role of this protein in mediating neuroprotection remains unclear.

This SNP has been associated with DR, end stage renal disease, myelodysplastic syndrome in various populations [141, 170, 171]. Genetic variants in the promoter regions are shown to regulate the expression of EPO genes. SNP rs1617640 (-1125 bp upstream of the transcription start site) has been shown to regulate EPO expression. Elevated levels of EPO observed in vitreous of PDR patients and genetic determinant of the gene expression substantiates the candidature of the promoter SNP rs1617640 for the association for DR in the current study.

SNP rs1617640 has been associated with risk for myelodysplastic syndrome (MDS) while lack of association was observed with early stage breast cancer. Clinical correlation of the genotypes in cardiopulmonary bypass (CPB) showed that the TT genotypes associated more frequently in

patients undergoing cardiac surgery and acute renal replacement therapy [292].

In the current study, we did not find any significant association for EPO SNP (rs1617640) with DR in Indian population in contrast to the other studies worldwide. Our result simulates the finding by Balasubbu et al. from another cohort of south India [141]. However our population showed more frequency of the TT genotypes in comparison to presence of more of GG genotypes in study by Balasubbu et al. This difference in distribution of genotype is attributed to population heterogeneity.In a cohort of European-American descent 'T' allele of rs1617640 polymorphism showed a risk with DR. Similar results associating the GG genotypes with DR is reported in Australian population. Phenotype differences could be one of the possible reasons for the observed difference between the studies. The cohort included in the current study is STDR patients (NPDR, PDR and CSME) while that studied in the European-American descent has PDR and end stage renal disease (ESRD). The ethnic differences also contribute to the observed difference between the other studies.

Conclusion

SNPs in the candidate genes for inflammation and neurodegeneration pathway were studied for the assoicaiton with DR and its clinical covariates. While the association of rs5498 with DR is replicated in Indian population, the other SNPs did not show statistically significant associations; the limitations of sample size to be considered for further conclusions. A gentoype dependent risk was observed for DR for SNPs in inflammatory pathway (i) AG/AA genotype of rs5498 in *ICAM-1* gene with microalbuminuria/lipid biomarkers; (ii) individuals with TT and TC genotypes of Y402H polymorphisms of *CFH* gene with poor control of HbA1c and TC and CC genotypes among insulin users were at higher risk for DR.

3.5 To analyze the putative interactions of candidate genes implicated in inflammatory reaction (*ICAM-1*, *CFH*) and neurodegeneration (*PEDF*, *EPO*) in DR patients.

In the current study the variants of *PEDF* (rs12150053, rs12948385) and *EPO* (rs1617640) genes were analyzed for the potential interactions between the SNPs and also with the clinical covariates such as age, gender, onset/duration of diabetes, history of hypertension, family history, systolic and diastolic blood pressure, smoking, HbA1c and BMI. Permutation was employed to obtain empirical p values of prediction error-based on 5,000 shuffles.

3.5.1 Results: High order interaction between *PEDF* and *EPO* genes

GMDR analysis did not show any significant interaction between the SNPs (Table 3.5.1.1). However, after adjusting for the onset of diabetes and age of the study subjects at the time of enrolment, polymorphisms rs12150053 (*PEDF*) and rs1617640 (*EPO*) showed significant associations ($p<0.05$) that remained unaltered for the rest of the covariates.

Table 3.5.1.1: Best gene-gene interaction models identified by GMDR analysis

Gene (SNP)	CVC		TBA (%)		p value#	
	UAD	AD*	UAD	AD*	UAD	AD*
PEDF (rs12150053)	10/10	6/10	54.82	56.12	0.0547	0.1719
PEDF (rs12150053), *EPO* (rs1617640)	8/10	6/10	46.37	55.10	0.6230	0.1719
PEDF (rs12150053), *PEDF* (rs12948385), *EPO* (rs1617640)	10/10	10/10	44.76	51.15	0.9893	0.6230

Abbreviations CVC: cross validation consistency, TBA: testing balance accuracy, UAD: unadjusted, AD: adjusted

*Adjusted with all the covariates (age, gender, onset of diabetes, history of hypertension, duration of diabetes, insulin user status, smoking, family history of DM, blood pressure systolic, blood pressure diastolic, HbA1c, BMI) #p values based on (Random) 5000 permutations

Analysis for the potential interaction with various clinical covariates showed significance for IUS and rs12150053 at single loci (CVC of 10/10 and TBA >59%) in the unadjusted model. *PEDF* rs121948385 and *EPO* rs1617640 showed best interaction for rs12150053 with IUS in both unadjusted and adjusted models (Table 3.5.1.2). We also tested for the gene interaction with IUS and HbA1c levels with and without adjustment for various clinical factors. While rs121948385 and rs12150053 in *PEDF* gene showed p level of significance that was altered when adjusted for age of onset of diabetes both at the single and multiple loci (Table 3.5.1.2), *EPO* (rs1617640) was never identified to be interacting with these parameters. *PEDF* (rs12150053, rs12948385) and HbA1c interacted significantly both in the adjusted and unadjusted models (Table 3.5.1.2).

Table 3.5.1.2: Best gene-environment interactions identified by GMDR analysis

S. No.	Gene x covariate	Model	p value#	CVC	TBA (%)
Number of locus with insulin user status					
1	IUS	Unadjusted	0.0107	10/10	61.75
		Adjusted*	0.0107	10/10	60.53-62.64
2	*PEDF* (rs12150053), IUS	Unadjusted	0.0107	10/10	62.41
		Adjusted*	<0.02	9/10	61.27-64.61
3	*EPO* (rs1617640), IUS	Unadjusted	0.0107	10/10	60.54
		Adjusted*	<0.01	10/10	60-63
4	*PEDF* (rs12150053, rs12948385), IUS	Unadjusted	0.0107	10/10	61.49
		Adjusted*	<0.05	10/10	58.65-62.08
5	*PEDF* (rs12150053), *EPO* (rs1617640), IUS	Unadjusted	0.0107	10/10	62.61
		Adjusted*	<0.01	9/10	58-63
6	*PEDF* (rs12150053, rs12948385) , *EPO* (rs1617640), IUS	Unadjusted	0.0107	10/10	59.27
		Adjusted*	<0.05	10/10	55-61
		Adjusted with BP_Dys	0.1719	10/10	57.69
Number of locus with HbA1c					
7	*PEDF* (rs12948385), HbA1c	Unadjusted	0.0107	10/10	59.70
		Adjusted**	0.0107	10/10	59.80-60.45
8	*PEDF* (rs12150053, rs12948385), HbA1c	Unadjusted	0.0107	10/10	59.03
		Adjusted**	<0.05	10/10	58-59
Number of locus with insulin user status and HbA1c					
9	*PEDF* (rs12150053), IUS, HbA1c	Unadjusted	0.0010	10/10	63.11
		Adjusted***	<0.01	10/10	59.42-66.67
		Adjusted with onset	0.1719	10/10	59.42
10	*PEDF* (rs12948385), IUS, HbA1c	Unadjusted	0.0107	10/10	61.49
		Adjusted***	0.0107	10/10	59.31-62.21
		Adjusted with onset	0.1719	10/10	>56
11	*PEDF* (rs12150053, rs12948385) , IUS, HbA1c	Unadjusted	0.0547	10/10	59.90
		Adjusted***	<0.05	10/10	57.02-66.09
		Adjusted with onset	0.1719	10/10	57.66

Abbreviations IUS: insulin user status, BP_Dys: blood pressure diastolic.
*Adjusted with covariates (age, gender, onset of diabetes, history of hypertension, duration of diabetes, smoking, family history of DM, blood pressure systolic, blood pressure diastolic, HbA1c, BMI)
** Adjusted with covariates (age, gender, onset of diabetes, history of hypertension, duration of diabetes, insulin user, smoking, family history of DM, blood pressure systolic, blood pressure diastolic, BMI)
*** Adjusted with covariates (age, gender, onset of diabetes, history of hypertension, duration of diabetes, insulin user, smoking, family history of DM, blood pressure systolic, blood pressure diastolic, HbA1c, BMI)
#p values based on (Random) 5000 permutation.

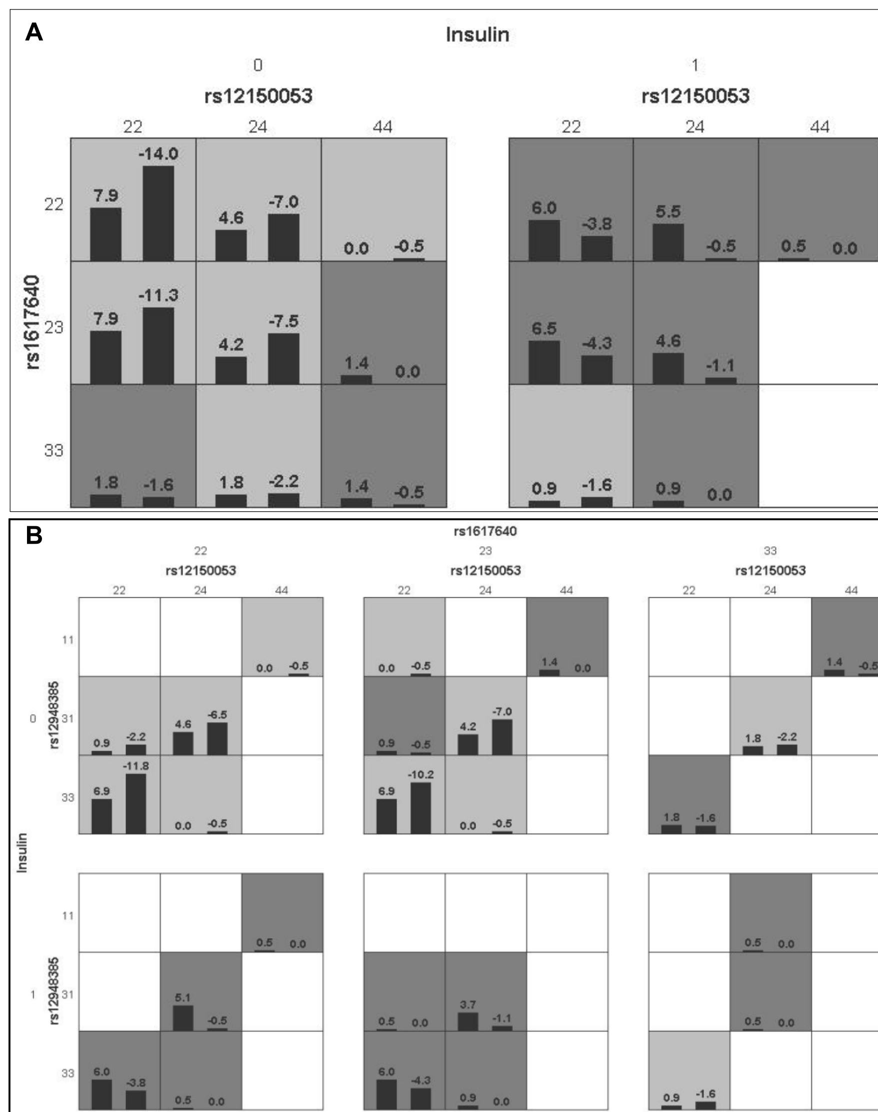


Figure 3.5.1.1: Best predictive model obtained by 3 and 4-locus interaction between the variants of *PEDF* and *EPO* gene and IUS without adjustment with clinical covariates. Numbers 22, 24, 44 corresponds to TT, TC, CC genotypes of rs12150053 while 11, 31, 33 to AA, AG, GG genotypes of rs12948385 of PEDF gene and 22, 23, 33 to TT, TG, GG genotypes of rs1617640 of EPO gene, respectively.

Figure 3.5.1.1 depicts the checkerboard model with the highest CVC (10/10) and TBA of >61% in 3 and 4-locus model (Table 3.5.1.2) (p-0.0107) unadjusted to clinical covariates. In each cell, the left bar represents a positive score, and the right bar a negative score. The two bars within each cell are proportional to the sum of scores in cases (DR+) and controls (DR-),

respectively. The heights of the bars are proportional to the sum of scores in each group. High-risk cells are indicated by dark shading, while the low-risk cells by light shading, and empty cells by no shading. Note that the patterns of high-risk and low-risk cells differ across each of the different multilocus dimensions, presenting evidence of epistasis.

CART analysis: We then explored yet another high order interaction by the CART analysis using binary partitioning method. The analysis was performed between all the genetic variants and environmental risk factors. The initial interpretation of the CART results were based on relative error curve and ROC values. The relative error curve is scaled between 0 and 1 where 0 means no error or perfect fit while 1 represents a random guessing. The best among all is indicated by the green bar marking the low point on the error profile (Figure 3.5.1.2). Our gene-environment analysis between PEDF, EPO and insulin user status (IUS) showed the lowest relative error of 0.761, but since it had only two nodes, we therefore selected a tree with five terminal nodes with the relative error of 0.814 (Figure 3.5.1.2). Another important metric on the navigator was ROC value. ROC can range between 0 and 1 with higher values indicating better interaction. ROC for the selected CART model between PEDF, EPO and IUS was 0.589 (Figure 3.5.1.2).

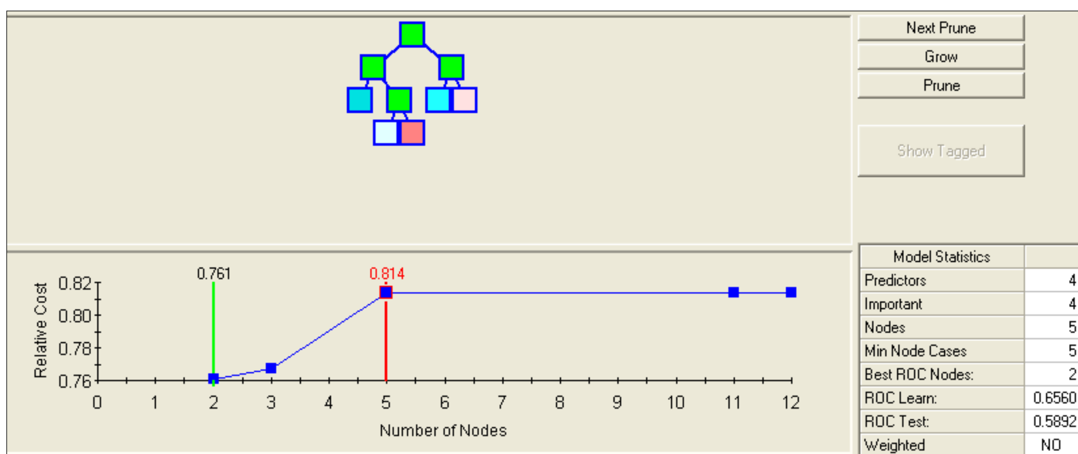


Figure 3.5.1.2: Results of CART between genes (*PEDF* & *EPO*) and IUS as displayed on navigator

The final tree generated by the CART analysis is shown in figure 3.5.1.3. The initial split of the root node was at the IUS predicting the same as the strongest risk factor for DR. Further examination of the tree indicated an interaction between CC/TC genotype of rs12150053 and IUS (89.7%; p=0.003; RR 1.46) with an OR >1.0 (Table 3.5.1.3) for the CC genotype after adjusting for insulin user status/IUS by LR analysis. Insulin users with *PEDF* TT (rs12150053) (Node3) and *EPO* (rs1617640) (TN 2, 3) genotypes were also predisposed to DR. Terminal nodes 4 and 5 were formed by *PEDF* rs12150053 among non insulin users. Additionally, comparison of rs12150053 genotypes between cases and controls among both the groups showed significant Fisher's exact p value of 0.017 among the insulin user group (Table 3.5.1.4).

GMDR and CART results (nonparametric) were further validated by unconditional logistic regression (parametric) analysis (Table 3.13) Since, there was a strong interaction for SNPs (rs12150053, rs12948385, rs1617640) with control of diabetes measured by the HbA1c levels; LR was also done for the same after splitting the HbA1c status as poor (≥7.0%) or good (<7.0%). We did not identify any significance for the distribution of the 3 SNPs when adjusted for HbA1c levels.

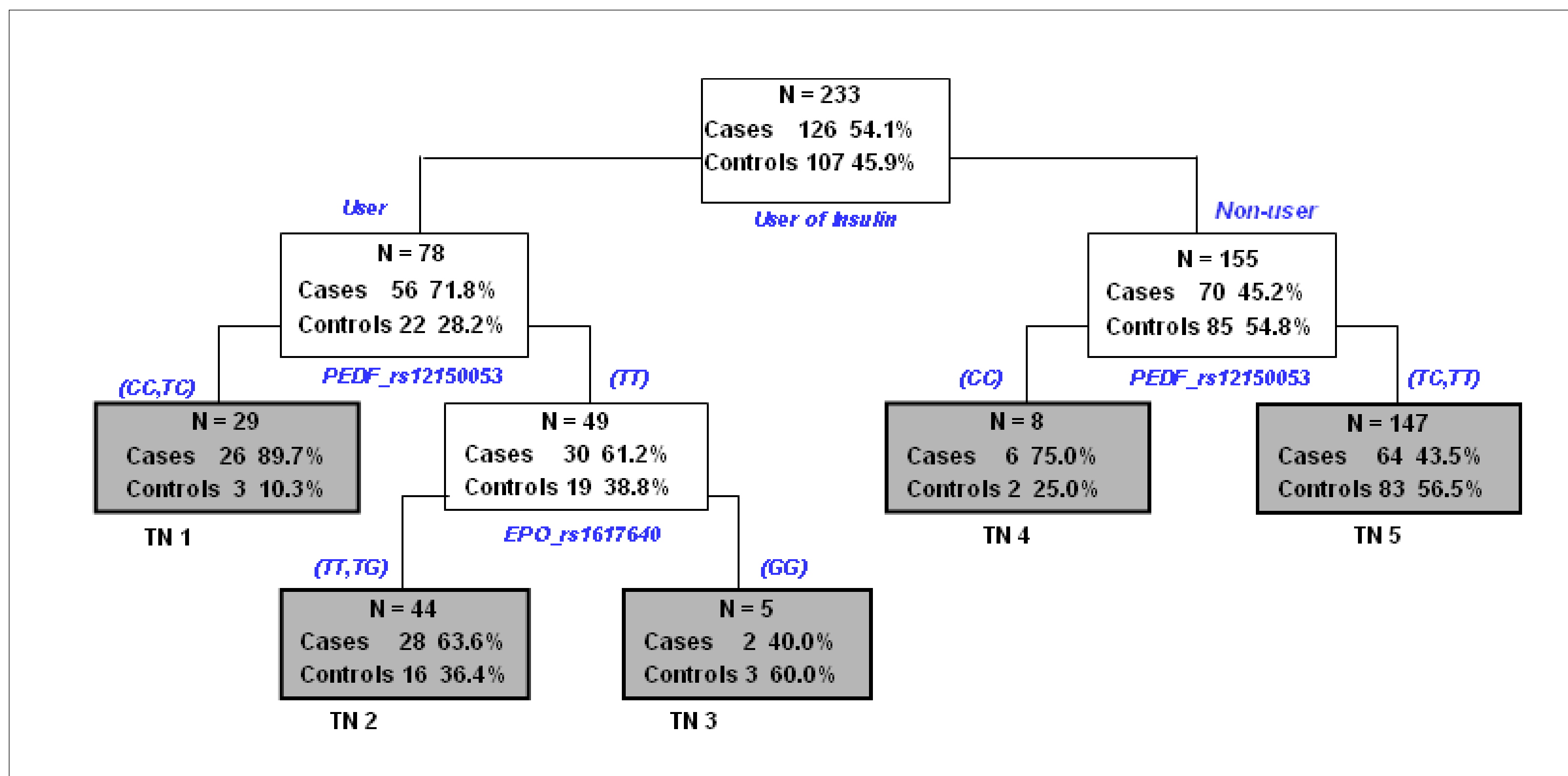


Figure 3.5.1.3: Classification and regression tree model between the variants in *PEDF* and *EPO* genes and IUS risk factor. Terminal nodes (TN) are boxes shaded in gray. Relative Error Curve value: 0.814, ROC value: 0.589 for the above shown CART model

Table 3.5.1.3: Logistic regression analysis for insulin user status and HbA1c levels with the *PEDF* and *EPO* genotypes

Genotypes	Unadjusted		Adjusted with Insulin		Adjusted with HbA1c	
	OR (95% of CI)	**p value**	**OR (95% of CI)**	**p value**	**OR (95% of CI)**	**p value**
PEDF rs12150053						
TT	Ref		Ref		Ref	
TC	1.403 (0.805 -2.445)	0.232	1.470 (0.824 -2.620)	0.192	1.354 (0.786 -2.396)	0.297
CC	3.552 (0.712 – 17.723)	0.122	**4.904 (0.961 – 25.018)**	**0.056**	3.033 (0.593 – 15.506)	0.183
PEDF rs12948385						
GG	Ref		Ref		Ref	
GA	1.28 (0.737-2.215)	0.383	1.476 (0.828-2.630)	0.187	1.194 (0.678-2.102)	0.540
AA	2.63 (0.67-10.34)	0.168	3.515 (0.864-14.296)	0.079	2.135 (0.530-8.599)	0.286
EPO rs1617640						
TT	Ref		Ref		Ref	
TG	1.07 (0.611-1.862)	0.820	1.059 (0.594-1.886)	0.847	0.964 (0.542-1.716)	0.902
GG	1.235 (0.517-2.949)	0.635	1.355 (0.552-3.328)	0.508	1.185 (0.483-2.907)	0.711

#p value by χ^2-square test

Since, there was a strong interaction for rs12150053, rs1617640 and insulin user status, we split the DR groups based on the insulin user status and analyzed. The TC genotype of rs12150053 (*PEDF* gene) demonstrated 5.12 times increased risk for DR [p=0.017; 95% CI (1.35-19.25)], while OR<1.0 was observed for the TT genotype [p= 0.007; OR: 0.18; 95% CI (0.05-0.69] (Table 3.5.1.4). However the distribution of EPO promoter polymorphism (rs1617640) genotypes was not significantly distributed between DR+ and DR-.

Table 3.5.1.4: Association of promoter polymorphisms of PEDF and EPO and the insulin user status with DR susceptibility

	Non insulin user			**Insulin user**		
	(DR-) n=85	**(DR+) n=70**	**p**	**(DR-) n=22**	**(DR+) n=56**	**p**
PEDF rs12150053						
CC	2 (2.4)	6 (8.6)	0.141	0 (0.0)	1 (1.8)	1.000
TT	52 (61.2)	40 (57.1)	0.611	19 (86.4)	30 (53.6)	**0.007***
TC	31 (36.5)	24 (34.3)	0.777	3 (13.6)	25 (44.6)	**0.017****
EPO rs1617640						
GG	8 (9.4)	11 (15.7)	0.234	3 (13.6)	4 (7.1)	0.367
TT	42 (49.4)	29 (41.4)	0.321	8 (36.4)	27 (48.2)	0.344
TG	35 (41.2)	30 (42.9)	0.833	11 (50.0)	25 (44.6)	0.669

'*' OR (95% of CI) between non-insulin user and insulin user = 0.18 (0.05-0.69) (p value = 0.610)

'**' OR (95% of CI) between non-insulin user and insulin user = 5.12 (1.35-19.25) (p value = 0.950)

3.5.1.1 Discussion

Diabetic retinopathy, a complex disease, is shown to be influenced by interactions between genes and environmental factors. However epistatic interactions are a major challenge towards uncovering the gene responsible for DR. Thus the present study evaluates the individual and interactive role of SNPs in *PEDF* and *EPO* genes, the 2 candidate genes earlier studied for their putative association with ocular diseases like DR and AMD [141, 169-171, 190, 293].

In the complex disorders, gene-gene and gene-environment interactions are being employed to identify the role of candidate genes in the etiology of specific conditions. The present study utilized the GMDR and CART program to test these high order interactions between candidate genes in T2DR. These non-parametric methods being preferred for intersecting the genetics of complex diseases as they attempt to identify the combination of loci that influences a disease outcome through interactions [288]. This also addresses the issue of sparse data cells identified in traditional regression-based methods and also the chance of false-positive results even on relatively smaller sample sizes [288]. These methods also overcome inaccurate estimation by using model validation through permutation testing. Additionally GMDR methodology allows adjustment for quantitative traits to analyze qualitative and quantitative phenotypes in a population based study design. GMDR and CART are being extensively used for complex diseases like hypertension, T2D, age related maculopathy, stroke, osteoporosis, obesity, rheumatoid arthritis, coronary artery disease, cancers of breast, prostrate lung, skin, bladder [247, 288, 294-298].

In the current study we did not identify significant score depicting gene-gene interactions between the SNPs. GMDR and CART results indicated insulin user status, glycemic control denoted by HbA1c levels and blood pressure parameters as the clinical factors interacting with the polymorphisms, specifically rs12150053 in both single and additive models.

Insulin user status remained as the best interacting factor with the SNPs even after adjusting for the other covariates except for diastolic blood pressure. A significant correlation of the PEDF levels with the blood pressure parameters and insulin resistance were earlier identified in other studies [299]. A causal role of PEDF levels with obesity induced insulin resistance has also been earlier observed thus correlating PEDF with metabolic syndrome [300]. PEDF concentrations were shown to decrease significantly after weight loss in association with blood pressure thus suggesting a primary or secondary effect of PEDF on these parameters [299]. The changes in circulating PEDF levels were positively associated with the change in systolic and diastolic blood pressure [299].

The GMDR results were simulated by CART analysis which showed the first split of the root as insulin user status, thus indicating insulin as the strongest risk factor for DR. The subsequent CART results picked rs12150053 as a potential predisposing genetic factor for DR that was also confirmed by LR analysis (Table 3.5.1.3). The *EPO* promoter polymorphism (rs1617640) was also observed to interact with IUS in the current study (Table 3.5.1.2). Milutinovic et al have earlier shown a reduction in insulin resistance through iron stores reduction after administration of recombinant human erythropoietin (EPO) [301]. Bohm et al reports that *PEDF* variant contributes to overall body adiposity and obesity-related insulin resistance [191]. We also observed a high risk predisposition for DR in insulin users with rs1617640 and TT genotypes of rs121500053 (Figure 3.5.1.3). Since our CART results did not meet the required criteria for true interaction at a given node (value closer to one and zero of relative error curve and ROC respectively) the putative interaction between the genetic variants and insulin user status detected in the current study remains as a chance observation.

Another covariate that indicated putative interaction with the PEDF variants by GMDR analysis was the HbA1c levels. Interestingly serum PEDF and HbA1c levels have been correlated with T2 diabetic nephropathy by

Chen et al as well as Jenkins et al in the same year [302, 303]. However our GMDR results were not reproduced by CART and LR analysis.

The study subjects enrolled were a part of a large epidemiology study on understanding the prevalence of DR in south India, and hence include a homogenous T2 diabetes samples from similar origin and diabetes duration of ≥15yrs in the control group. This is the first study dissecting the possible gene-gene / gene-environment interactions in DR. This study indicates a biological plausibility of such interactions which explains the complex mechanisms in the disease pathology that however needs to be replicated statistically and functionally validated.

To summarize, the above study suggest a probable interaction between SNPs of *PEDF*, *EPO* genes with insulin user status and index of glycemic controls in the etiology of DR. Our data combined with other reports on clinical correlation between HbA1c, insulin resistance and blood pressure parameters with levels of PEDF and EPO [300, 301] convey a possibility of genetic regulation on PEDF/EPO expression. However we do not have the supportive evidence to prove these observations. A variety of putative binding sites for transcription factors and 2 Alu repetitive sequences have been described in the *PEDF* promoter [177], but the two promoter polymorphisms (rs12150053 and rs12948385) do not affect any of these putative binding sites [169]. The biological plausibility of the associations between *PEDF* (rs12150053, rs12948385), *EPO* (rs1617640) and insulin resistance and that of PEDF levels with glycemic control that we have demonstrated in the current study, demands appropriate functional studies.

3.5.1.2 Conclusion

The study suggests a probable interaction between SNPs of *PEDF*, *EPO* genes with insulin user status. IUS was indicated as the strongest risk factor for DR amongst all the clinical risk factors. However the biological plausibility of the associations between *PEDF* (rs12150053, rs12948385), *EPO* (rs1617640) and IUS demands appropriate functional studies.

3.5.2 Results: High order interaction between *ICAM-1* and *CFH* genes

One hundred and seventy eight cases and 150 controls were analyzed for G-G and G-E interaction and tables 3.5.2.1, 3.5.2.2 & 3.5.2.3 depict the results for the same. The results revealed modest interaction between *CFH* and *ICAM-1* (p - 0.0547) with 10/10 CVC and TBA of 54% without adjustment for the various clinical covariates of (Table 4.5). When adjusted the significance of interaction remained unchanged for duration of diabetes, IUS and smoking. The p value of significance was lost when adjusted for age/onset of diabetes and did not alter for family history, history of hypertension, blood pressure systolic, diastolic and HbA1c in two locus models
(p <0.05)

Table 3.5.2.1: Best gene-gene (*ICAM-1*, *CFH*) interactions models identified by GMDR model

Locus number	Best combination	Cross-validation consistency	Testing accuracy (%)	P-value
Without adjustment				
1	*ICAM-1*	10/10	56.06	0.0547
2	*CFH, ICAM-1*	10/10	54.91	**0.0547**
Adjusted with				
Age	*CFH, ICAM-1*	10/10	53.22	**0.6230**
Onset of Diabetes	*CFH, ICAM-1*	10/10	54.29	**0.6230**
History of hypertension	*CFH, ICAM-1*	10/10	55.91	**0.0107**
Duration of diabetes	*CFH, ICAM-1*	10/10	54.83	0.0547
Insulin	*CFH, ICAM-1*	10/10	56.49	0.0547
Smoking	*CFH, ICAM-1*	10/10	54.85	0.0547
Family history	*CFH, ICAM-1*	10/10	56.50	**0.0107**
Blood pressure systolic	*CFH, ICAM-1*	10/10	56.33	**0.0107**
Blood pressure diastolic	*CFH, ICAM-1*	10/10	56.60	**0.0107**
HbA1c	*CFH, ICAM-1*	10/10	**60.95**	**0.0010**

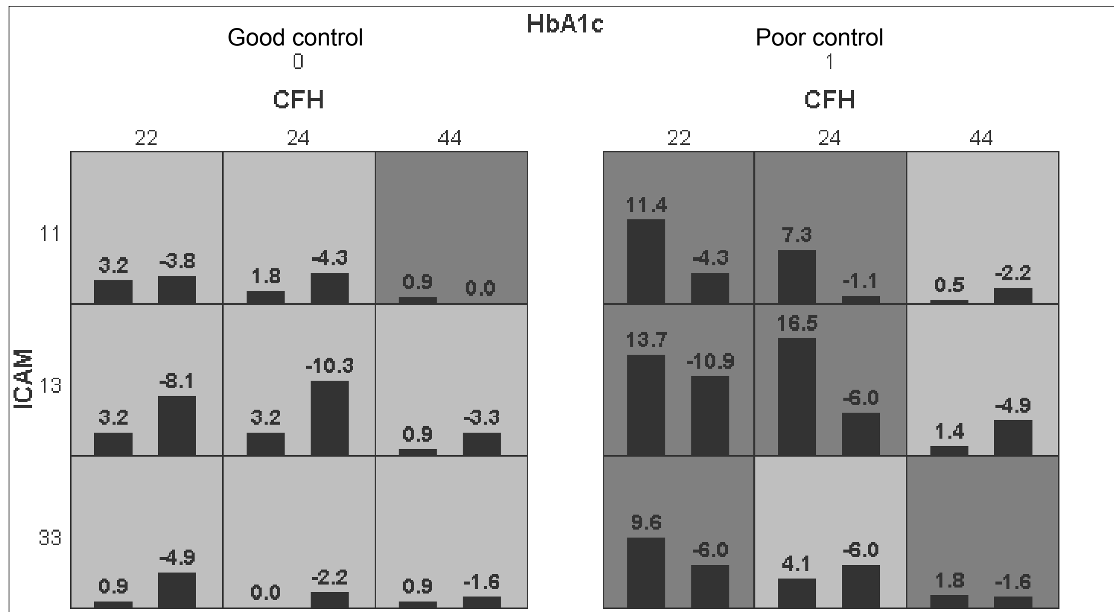
We then studied the gene-environment interaction analysis with the clinical covariates for DR showed significant interactions at 3-locus model (Table 3.5.2.2, 3.5.2.3) interactions of *CFH, ICAM-1* genes with HbA1c, smoking or IUS. The highest testing accuracy was obtained with HbA1c (60.9%) and IUS (60%) while smoking had a testing accuracy of 55.9%.

Table 3.5.2.2: Best gene-environment (*CFH, ICAM-1*, and HbA1c) interactions models identified by GMDR model

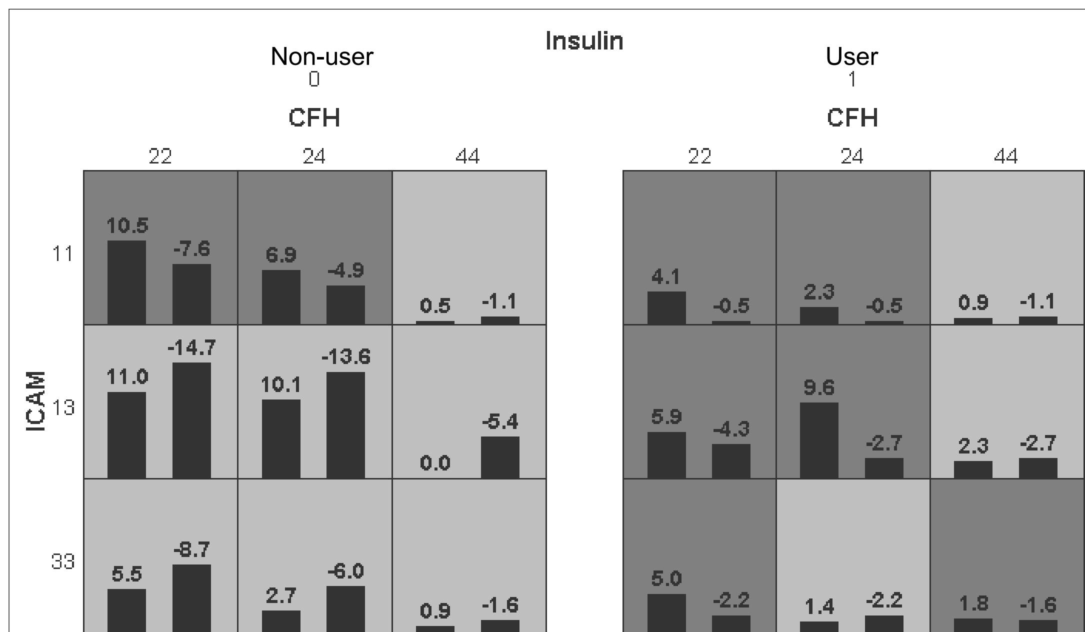
Number of locus	Best combination	CVC	TBA (%)	P-value
Without adjustment				
1	*HbA1c*	10/10	52.41	0.6230
2	*ICAM-1, HbA1c*	10/10	54.68	0.6230
1	*HbA1c*	10/10	52.41	0.6230
2	*CFH, HbA1c*	10/10	58.43	0.1719
1	*HbA1c*	10/10	52.41	0.6230
2	*CFH, HbA1c*	8/10	52.51	0.6230
3	*CFH, ICAM-1, HbA1c*	10/10	**60.99**	**0.0107**
With adjustment				
Gender	*CFH, ICAM-1, HbA1c*	10/10	64.75	**0.0107**
Age	*CFH, ICAM-1, HbA1c*	10/10	64.75	**0.0107**
Onset of diabetes	*CFH, ICAM-1, HbA1c*	10/10	60.79	**0.0107**
History of hypertension	*CFH, ICAM-1, HbA1c*	10/10	62.23	**0.0107**
Duration of diabetes	*CFH, ICAM-1, HbA1c*	10/10	66.96	**0.0107**
Insulin	*CFH, ICAM-1, HbA1c*	10/10	60.89	**0.0107**
Smoking	*CFH, ICAM-1, HbA1c*	10/10	60.89	**0.0107**
Family history	*CFH, ICAM-1, HbA1c*	10/10	63.47	**0.0107**
Systolic BP	*CFH, ICAM-1, HbA1c*	10/10	60.99	**0.0107**
Diastolic BP	*CFH, ICAM-1, HbA1c*	10/10	62.35	**0.0107**

Table 3.5.2.3: Best gene-environment interactions models identified by GMDR model

Number of locus	Best combination	CVC	TBA (%)	P-value
Without adjustment				
3	*CFH, ICAM-1,* Smoking	10/10	**55.93**	**0.0107**
3	*CFH, ICAM-1,* IUS	10/10	**60.01**	**0.0010**



A



B

Figure 3.5.2.1: Best predictive model obtained by 3-locus interaction between the variants of ICAM-1, CFH and HbA1c (A) and IUS (B) without adjustment with clinical covariates age, gender, onset of diabetes, history of hypertension, duration of diabetes, insulin user status, smoking, family history of DM, blood pressure systolic, blood pressure diastolic, HbA1c, BMI

Figure 3.5.2.1 depicts the best-identified checkerboard model of GMDR interactions with the highest consistency (10/10) and TBA (60%) in 3-locus model between the variants of *ICAM-1* (rs5498), *CFH* (rs1061170) and HbA1c levels (Table 3.5.2.2) and IUS (Table 3.5.2.3). The two bars within each cell are proportional to the sum of scores in cases (DR+) and controls (DR-), respectively. High-risk cells are indicated by dark shading, while the low-risk cells by light shading, and empty cells by no shading. Numbers 22, 24, 44 corresponds to TT, TC, CC genotypes of rs1061170 of *CFH* gene and 11, 13, 33 to AA, AG, GG genotypes of rs5498 of *ICAM-1* gene respectively. The patterns of high-risk and low-risk cells differ across each of the different multilocus dimensions, presenting evidence of epistasis.

We then explored yet another high order interaction (CART analysis) using binary partitioning method. The analysis was performed between all the genetic variants and environmental risk factors. The initial interpretation of the CART results were based on relative error curve and receiver operating characteristic (ROC) values as represented in Figure 3.5.2.2. The relative error curve, scaled between 0 and 1, represents no error or true interactions when its value is close to 0 while the interaction is just a random guess for the values close to 1. However similar range of scaling represented by the ROC value denotes an inverse meaning with value close to 1 indicating true interaction.

The initial output (result) of the CART as shown in figure 3.5.2.2 depicts the best among all the possible interactions in a green bar marking with the lowest relative error curve value (a value close to zero in 0 to 1 scale in Y axis). The GxE interaction between genes (*CFH, ICAM-1*) and HbA1c, smoking and IUS provided relative error curve values of 0.638, 0.904 and 0.778 while ROC values of 0.703, 0566 and 0.627 respectively as shown in figure 3.5.2.2 (A, B and C). Amongst this the interaction between *CFH, ICAM-1* and HbA1c showed the lowest relative error of 0.638 and higher ROC value of 0.703 in comparison to that with smoking and IUS.

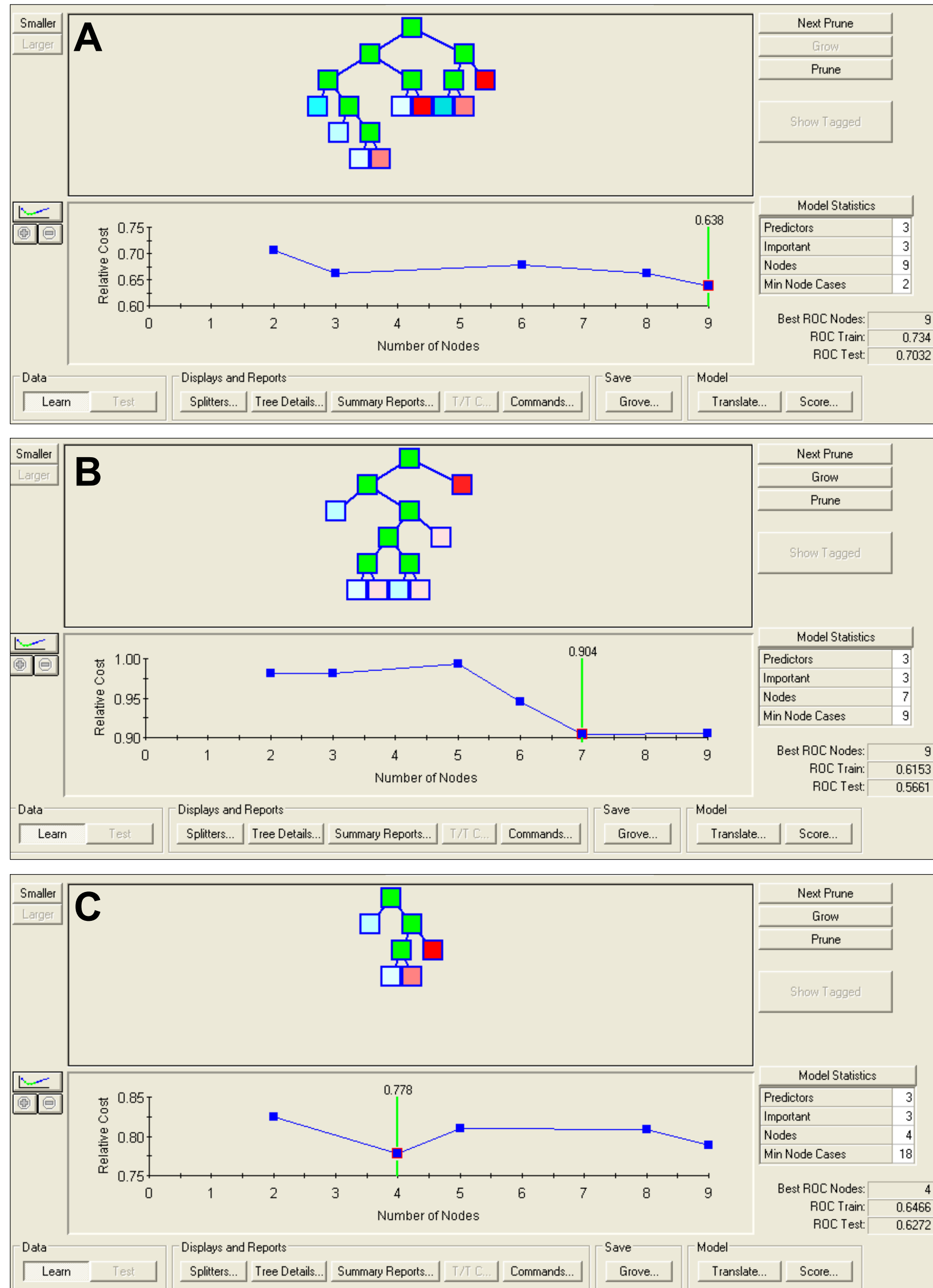


Figure 3.5.2.2: The navigator window displaying the best possible interaction represented by the relative error curve value in the green bar for the GxE interaction between genes *CFH, ICAM-1* with HbA1c (A), smoking (B), insulin user status (C) by CART analysis

The figure 3.5.2.3 depicts the detailed tree model of the interaction between the *CFH*, *ICAM-1* and HbA1c. Nine terminal nodes (TN, shaded in grey) were obtained for the CART analysis. The first split of the root was with HbA1c levels that split the tree based on the glycemic control as bad control and good control of HbA1c. This indicates HbA1c as the strongest risk factor for DR. Among the group with bad control of HbA1c, both *CFH* and *ICAM-1* genotypes were found to be interacting. The first split under bad control of HbA1c was with *CFH* (rs1061170) polymorphism as TC, TT at node 3 and CC at node 6 with a relative risk of 2.05 (p – 0.013) thus indicating TC, TT to be risk genotypes for DR. This was later substantiated by the logistic regression (LR) analysis (table 3.5.2.4) confirming higher risk for DR with an OR of 2.6 and 6.5 for genotypes TT and TC after adjusting for HbA1c levels. Terminal node 1 (TN1) comprised of 80.4% of cases with AA genotype for *ICAM-1* (rs5498) and showing a relative risk of 1.24 (p – 0.017) while TN2 showed 68.4% of cases with AG genotypes thus suggesting the risk genotypes for DR among the bad control of HbA1c as also confirmed by LR (table 3.1.2). The relative risk calculated for other terminal nodes were however not found to be significant.

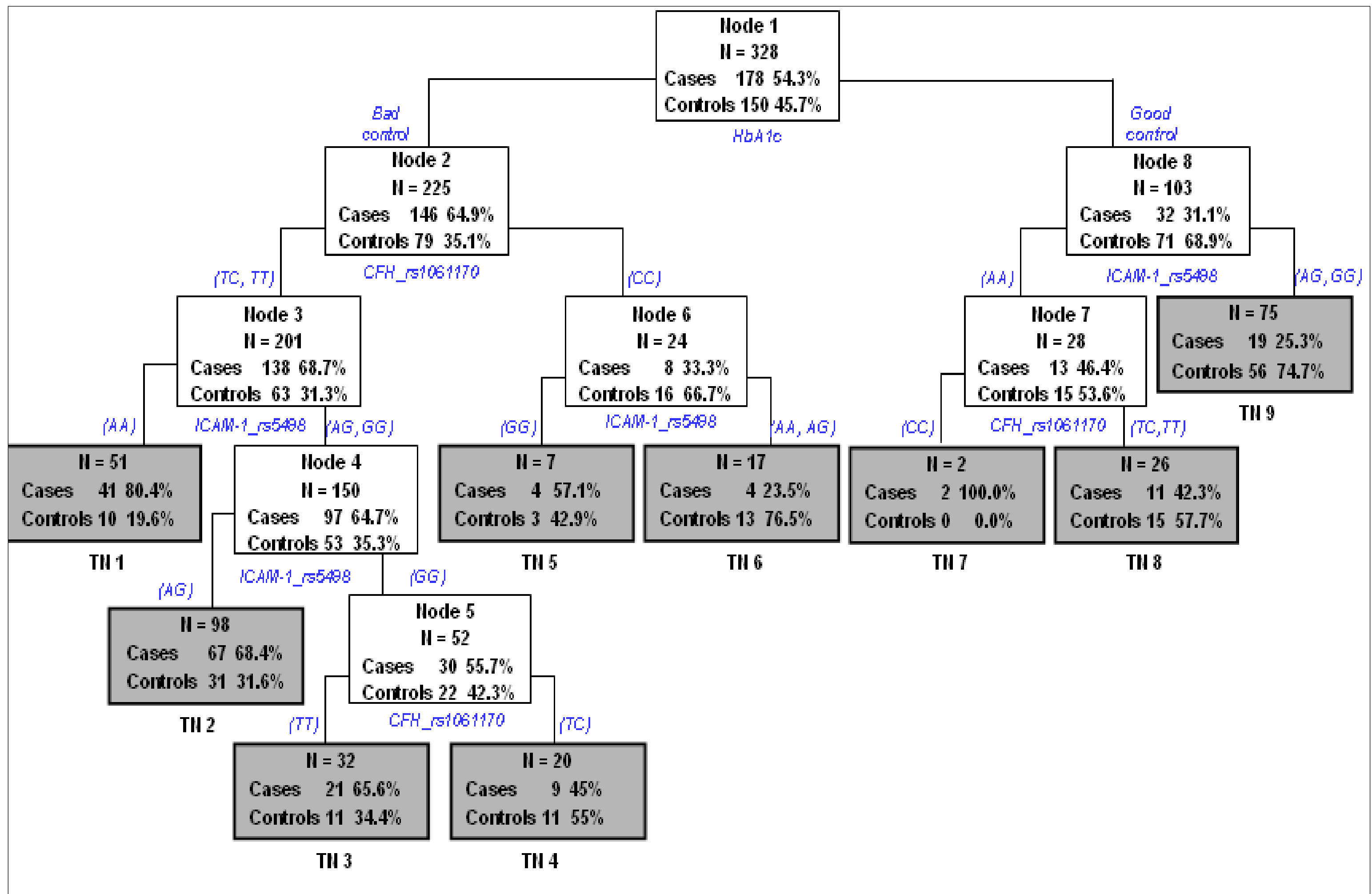


Figure 3.5.2.3: CART model for polymorphisms in *ICAM-1* and *CFH* genes with HbA1c
The unshaded box represents the nodes (8 nodes) and box in grey shade represents the terminal nodes (9 TN)

The results of gene-environment (*ICAM1* or *CFH* vs. HbA1c) interaction by GMDR and CART were further validated by unconditional logistic regression analysis (table 3.5.2.4).

Table 3.5.2.4: Multivariate analysis between DR+ and DR- group for *CFH* Y402H genotypes adjusted for the risk factors of DR

	TT		TC		CC	
Characteristics	DR+		DR+		DR+	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Unadjusted	1.134 (0.816 - 1.452)	0.085	1.309 (0.951 - 1.667)	0.133	0.560 (0.0256 - 1.094)	0.082
Gender	0.892 (0.396 - 2.012)	0.78	0.576 (0.201 - 1.649)	0.304	1.092 (0.12 - 9.949)	0.938
Age	0.885 (0.816 - 0.96)	< 0.001	0.97 (0.889 - 1.059)	0.497	0.954 (0.759 - 1.199)	0.687
Onset of diabetes	1.035 (0.962 - 1.113)	0.355	0.956 (0.88 - 1.04)	0.295	1.13 (0.887 - 1.439)	0.322
History of hypertension	0.81 (0.365 - 1.797)	0.604	0.885 (0.336 - 2.332)	0.805	0.327 (0.046 - 2.307)	0.262
Insulin	2.265 (0.923 - 5.558)	0.074	4.733 (1.627 - 13.768)	0.004	60.909 (2.147 - 1727.969)	0.016
Smoking	2.4 (0.715 - 8.052)	0.156	0.958 (0.293 - 3.135)	0.944	0.174 (0.006 - 4.944)	0.306
Family history	1.824 (0.861 - 3.86)	0.116	1.942 (0.846 - 4.458)	0.117	5.628 (0.53 - 59.783)	0.152
Blood pressure systolic	1.015 (0.991 - 1.039)	0.224	1.012 (0.983 - 1.041)	0.431	0.948 (0.889 - 1.011)	0.102
Blood pressure diastolic	1.031 (0.99 - 1.074)	0.142	1.06 (1 - 1.124)	0.051	1.199 (1.033 - 1.392)	0.017
HbA1c	2.671 (1.115 - 6.395)	0.027	6.571 (2.479 - 17.415)	<0.001	0.567 (0.069 - 4.658)	0.598

Diastolic blood pressure (BP) was shown to confer 1.19 times risk for DR (p – 0.017) while the age of the study subjects was shown to provide protection (OR 0.88, p – 003) with TT genotypes of Y402H polymorphism of *CFH*.

3.5.2.1 Discussion

The pathogenesis of DR involves complex interactions between multiple genes and /or environmental factors. Inflammation has been identified as an important molecular mechanism in the development and progression of DR.

Upregulation of adhesion molecules due to the complement system activation has been implicated in the inflammatory reaction in general and have also been demonstrated in DR pathogenesis [304,305]. The present study explored the possible genetic interactions between *CFH* and *ICAM-1* genes and also with the environmental factors towards the risk for DR by applying data mining approaches namely GMDR and CART analysis and demonstrate a possible interaction between the same.

Our study suggests possible interaction between the variants in *ICAM-1* and *CFH* gene in DR by GMDR analysis. Increased expression of CFH, CFB, C3 and C5 has been observed in the vitreous of DR patients. In addition, genetic variations have also been associated in range of inflammatory diseases. Hence we have taken two inflammatory mediators well studied with the retinal inflammatory disorders (*CFH* and *ICAM-1*) and assessed whether a possible interaction exist between them in DR pathology. A direct link between ICAM-1, CFH and DR however has not been proved by functional studies. Instead another inflammatory mediator C5a, regulated by CFH has been shown to activate ICAM-1 expression in the choroidal endothelial cells for AMD model [306,307]. Increased expression of C5a, a complement activation fragment, has been observed in the vitreous of patient with PDR [308] and the expression of the same remains under the control of CFH. *O*xidative stress-related inflammation has been shown to cause increase ICAM-1 levels in the retina [98] while reduced expression of CFH was observed in the RPE cells in cell culture experiments. These results thus suggest the possible link between the ICAM-1 and CFH through C5a. The reduced expression of CFH due to

oxidative stress leads to higher expression of C5a and ICAM-1 thus causing an inflammatory response.

In the current study high interaction analysis between clinical covariates, *ICAM-1* and *CFH* genotypes has shown possible interaction between poor control of glycosylated hemoglobin (HbA1c), a parameter of glycemic index, with the *CFH* and *ICAM-1* genotypes in a 3-locus model with 10/10 CVC and 60.99 TBA (p-0.010) . The p value was not altered even after adjusting for various other factors thus denoting a strong interaction that was not confounded by other factors. This was further supported by the CART analysis where HbA1c forms the first split showing the lower relative error (0.638) and higher ROC (0.703) values amongst the other covariates. Additionally the analysis by LR also shows risk for DR with the genotypes of *CFH* (rs1061170) and *ICAM-1* (rs5498) after adjusting for HbA1c levels. Glycemic control has been shown to influence the expression of *ICAM-1* gene [309]. A significant correlation between oxidative stress, ICAM-1 and HbA1c levels has been observed in T2D patients. In the current study we have also observed possible interactions between the *ICAM-1, CFH* genetic variants (rs5498, rs1061170) and HbA1c levels; the correlation between CFH levels and HbA1c has not been observed. Instead oxidative stress an important regulator of inflammatory pathway has been linked with HbA1c and ICAM-1 levels.

Insulin user status, another important clinical determinant of DR showed significant interaction with the *CFH* and *ICAM-1* variants in the current study. The anti-inflammatory effect of insulin [310] has been associated with complement system. Study by Bjerre et al suggested the role complement component sC5b-9 in endothelial activation that resulted in the activation of complement system and damage to the heart [311]. Reduced insulin action was observed with an increase activity of complement C3. Insulin resistance (IR) has been observed in subjects with higher C3 levels [281]. The optimum level of C3 has been shown to be maintained by CFH thereby protecting the eye from indiscriminate complement activation and inflammatory insult [312]. Mutations in CFH gene

have been shown to increase the C3 deposition in patients with membranoproliferative glomerulonephritis (MPGN) and atypical hemolytic uremic syndrome (aHUS) [313]. These studies provided information on the observed possible link between IR (indicating insulin user status) and inflammatory markers CFH, and ICAM-1.

Smoking status has also been identified as a potential interacting factor with *CFH* and *ICAM-1*, in the current study. However the results were not confirmed by CART and LR. The impact of *CFH* Y402H genotype and smoking towards the risk for AMD has been known however the role of smoking in DR has not been established.

Diastolic blood pressure (BP), was another factor, that was observed to confer risk for DR with the CC genotypes (OR 1.19, p – 0.017), while age of the study subjects associated with decreased risk (OR 0.88, p – 0.003) for DR with TT genotypes of *CFH*. The role of complement system in hypertension was suggested a century ago. Study by Shkhvatsabaya et al showed higher concentration of C3 and C4 concentration in subjects with essential hypertension [314]. Study by Mansat et al. demonstrated complement inhibition at the C3 convertase level which effectively attenuated inflammatory reactions restoring systemic blood pressure during E coli sepsis in baboons [315]. Additionally the deposition of C3d in the lung section of patients with pulmonary arterial hypertension (PAH) further suggests the role of complement activation in the development of PAH in humans [316].

3.5.2.2 Conclusion

Our study shows for the first time a possible interaction between *CFH* and *ICAM-1* with HbA1c and insulin user status. The results on HbA1C levels were then confirmed by CART analysis indicating HbA1c as risk factor for DR. Overall, HbA1c levels has been indicated as the strongest risk factor for DR. that however, needs to be replicated in larger sample size, in other populations and functionally proven.

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SUMMARY

The present study determines the association of SNPs in candidate genes for inflammation and neuroprotection with type 2 DR in Indian population. The study has identified statistically significant association for SNP rs5498 of *ICAM-1* gene with DR that also showed interaction with clinical covariates like microalbuminuria, lipid biomarkers. In addition, high order genetic interaction between the candidate gene SNPs were also tested that showed a possible interaction for SNPs in *CFH* (rs1061170) and *ICAM-1* (rs5498) genes, *PEDF* (rs12150053, rs12948385) and *EPO* (rs1617640), with each other. HbA1c and insulin user status (IUS) were the 2 clinical covariates that interacted with *CFH* and *ICAM-1* genotypes. The study has shed new light on interaction between the promoter polymorphism (rs12150053) in *PEDF* gene with insulin user status. Over all, these results suggest the possible role of genetic and environment interaction in the complex etiology of type 2 DR. The comprehensive clinical characterization, like longer duration of diabetes (>15years), ethnic homogeneity of cases and controls offers a strong strength to the study. Analysis for a genetic and environment interaction analysis using high order approaches like CART, GMDR is very unique for the study and till date not attempted for understanding the genetics of DR. However, the sample size offers the major limitation in further extrapolating the results to disease pathology. Replication in a larger sample size and functional characterization of the results would possibly aid towards deciphering the implication of the study results. The interaction between genotype and clinical covariates of DR, observed in the current study, has limited ability to measure the genetic and environment components which are also confounded by many other factors namely the genetic and disease heterogeneity. Hence these results demands functional validation for disease correlation (expression studies and serum correlation).

SPECIFIC CONTRIBUTION TO THE FIELD OF RESEARCH

The data generated from the present study has identified the potential role of gene environment interaction in DR pathology for the first time in Indian population. Some of the specific contributions from the study include:

1. Genetic association of AA genotype of SNP rs5498 in *ICAM-1* gene with type 2 DR.
2. A genotype dependent risk predisposition in type 2 diabetes people with AA genotype of *ICAM-1* gene through its potential interaction with the clinical covariates like microalbuminuria and lipid biomarkers.
3. A genotype dependent risk predisposition for the genotypes of rs1061170 (Y402H) in CFH gene is observed with specific clinical determinants namely poor control of HbA1c and insulin user status.
4. Insulin user status and HbA1c levels were indicated as the strongest risk factor for DR in Indian population
5. A pathway based gene environment interaction was analysed using CART and GMDR methods that suggested a genotype dependent risk predisposition with specific genotypes of genes implicated in neurodegeneration and inflammation pathway in DR.
6. High order interaction analysis (GMDR and CART) has identified the probable role of gene-environment interaction with DR in Indian population for the first time in literature that includes interaction between SNPs of *PEDF* (rs12150053, rs12948385), *EPO* (rs1617640) genes with insulin user status and *CFH* (rs1061170) and *ICAM-1* (rs5498) with HbA1c and insulin user status.

FUTURE SCOPE OF WORK

The current study has revealed the scope of (a) pathway centric approach (b) genetic and environment interaction (network) analysis in understanding the genetic etiology of DR. The study has also shown that testing for interaction analysis could identify those genetic variants that otherwise do not show any direct genetic association with DR. One of the alternate approaches that could incorporate both pathway centric and interaction analysis is genome wide association studies (GWAS). However a multicentric consortium based approach is deemed to overcome the major limitation for a successful GWAS results namely, sample size. A multicentric based, GWAS approach is hence being initiated for understanding the genetics of type 2 DR. To overcome the limitation on clinical heterogeneity (parameters like diabetes duration, stages of DR (PDR NPDR), end stage disease, namely, sight threatening diabetes retinopathy are only included as cases for the GWAS. A neural network based analysis is also being suggested to identify the interacting parameters as highlighted by the current study.

APPENDIX 1

Reference sequences

<u>Note:</u> Bold italics font size refers to forward and reverse primers. Bold underlined letters refer to the polymorphism studied.

<u>*PEDF* (rs12150053, -790T/C, PROMOTER)</u>

TTGAGACAAGCGTGACCAATGTGGTGAAACCCTGTCTCTACTAAAAATACAAA
AATTAGCCGGGCATGCTCGTGCACACCTATAGTCCCAACTACTCAGCAGGGTG
AGGCAGGAGAACCTCTTGAACCCGGGAAGCGGAGGTTGCAGTGAGCCGACAT
TGCACCCCTGCACTCCAGCCTGGGTGACAGAGTGAGTCTCCA**<u>T/C</u>**TGGAAAAA
AAAAAAAAAGAACAGTGTGATACATTGACCTAAGGTTTAAGAACATGCAAACTG
ATACTATATATCACTTAGGGACAAAAACTTACATGGTAAAAGTAAAAAGAAATG
TACGAAAATAATAAAAATCAAATTCAAGATGGT***GGTTATGGTGACGGGAA***

<u>*PEDF* (rs12948385, 358G/A, PROMOTER)</u>

CTACAGGTGCGCGCCAACACACCTGGGTAATTTTGTTTGTATTTTTAGTAGAG
ATGGGGTTTCACCGTGTTG**<u>A/G</u>**CTAGGCTGGTCTCGAACTCCTGACCTCAGGT
GATCCCCCGGCCTCGGTCTCCCAAAGTGCTGGGATAACAAGCGTGAGCCACT
GCGCCCAGCTTTGTTTGCATTTTTAGGTGAGATGGGGTTTCACCACGTTGGCC
AGGCTGGTCTTGAACTCCTGACCTCAGGTGATGCACCTGCCTCAGTCTCCCAA
AGTGCTGGATTACAGGCGTTAGCCCCTGCGCCCGGCCCCTGAAGGAAAATCT
AAAGGAAGAGGAAGG***TGTGCAAATGTGTGCGCCTTAG***

<u>EPO (rs1617640, -1125T/G, PROMOTER)</u>

GTCCATTGTGCAGGACACACATGCACCTTGACATCTGTTTATTTGACCTGTGG
TGTAGGTTAGCTAGGCTGCATTGCTGAGTTCTTAAGAACACTGAAATACAGCTA
ACACCAAGAGTGATGGGGGCTGGGATTTACAGCTAAGGTTTTATGGCTCTGGA
AACCCTGAGCCAGA**<u>T/G</u>**GAGTGAGATTCCCAGAGCAGGAGACCAAACTAGCTG
GTCTCTCACACCTTAGAAAGTGGCCTCGTCCTTGAGCCCTCAATGTCCTCATC
TGTCAAATGGGTGTGGTGAAACTACCAGCCTATCCCATTCAGGTTTCATAAAA
GACAGGAAAGGTCCAGGCGCCGCAGCTGACACCTATAATCCCAGCACTTTGG
GAGACCAAGGCAGGAAG***ATCCTTTGAGCCCAGGAGTT***

ICAM-1 (rs5498, K469E, EXON 6)

CTTGAGGGCACCTACCTCTGTCGGGCCAGGAGCACTCAAGGGGAGGTCACC
CGC**A/G**AGGTGACCGTGAATGTGCTCTGTGAGTGAGCCGGCGGGCAGAGCTG
GGTGGGGGCAGGGGCCATGGACCTAATGCAATCCTCACCGCCTGTTGTATCC
TCCCCACAGCCCCCCGGTATGAGATTGTCATCATCACTGTGG***TAGCAGCCGC
AGTCATAATG***

CFH **(rs1061170, Y402H, EXON 1)**

TCATTGTTATGGTCCTTAGGAAAATGTTATTTTCCTTATTTGGAAAATGGATATA
ATCAA*AAT***C/T**ATGGAAGAAAGTTTGTACAGGGTAAATCTATAGACGTTGCCTG
CCATCCTGGCTACGCTCTTCCAAAAGCGCAGACCACAGTTA*CATG*TATGGAG***A
ATGGCTGGTCTCCTACTCC***

APPENDIX 2

Primers used in PCR and DNA sequencing

Gene	SNP	Primer sequence (5' -3')	Annealing temperature (^{0}C)
ICAM-1	rs5498	**F:** CTTGAGGGCACCTACCTCTG **R:** CATTATGACTGCGGCTGCTA	60^{0}C
CFH	rs1061170	**F:** TCATTGTTATGGTCCTTAGGAAA **R:** GGAGTAGGAGACCAGCCATT	57^{0}C
PEDF	rs12150053	**F:** TTGAGACAAGCGTGACCAATGT **R:** GGTTATGGTGACGGGAA	53^{0}C
	rs12948385	**F:** CTACAGGTGCGCGCCAA **R:** TGTGCAAATGTGTGCGCCTTAG	58^{0}C
EPO	rs1617640	**F:** GTCCATTGTGCAGGACACAC **R:** ATCCTTTGAGCCCAGGAGTT	60^{0}C

APPENDIX 3

Demographic and clinical characteristics of study subjects

Characteristics	Control DR-	Case DR+	P
Age (Years)	63.7±9.5	58.9±8.6	**<0.0001**
Male sex	113 (61.1)	155 (64.8)	0.483
Duration of diabetes (years)	18.1±6.19	17.6±5.2	0.577
User of insulin	45 (24.3)	96 (40.1)	**<0.0001**
Age at diabetes onset (years)	45.3±9.4	41.3±8.9	**<0.0001**
HbA1c	59.3±20.9	75.6±28.5	**<0.0001**
Systolic blood pressure (mmHg)	132.6±18.0	135.3±19.4	0.144
Diastolic blood pressure (mmHg)	77.4±9.6	81.3±10.4	**<0.0001**
BMI	24.9±3.9	23.5±4.1	**<0.0001**
History of hypertension	77 (41.6)	91 (36.1)	0.241
Smokers	25 (13.5)	45 (17.9)	0.221
Total cholesterol (mmol/L)	4.3±1.1	4.2±1.2	0.222
HDL cholesterol (mmol/L)	1.0±0.3	1.1±0.9	0.622
Triglycerides (mmol/L)	1.2±0.6	1.2±0.6	0.609
Micro	38 (21.8)	114 (58.8)	**<0.0001**

The subjects in DR- group were older than DR+ group (mean age, 66.7, 58.9, p <0.0001). The mean level of HbA1c (g/dl) were higher in DR+ compared to DR- (mean, 75.6 vs. 59.3, p<0.0001). The age at diabetes onset in DR+ (mean, 41.3) and DR- (mean, 45.3) were statistically significant (p <0.0001). The user of insulin was more in DR+ compared to DR- (40.1% vs. 22.7%, p <0.0001). The subjects with DR were having higher diastolic blood pressure, lower BMI, lower total cholesterol and higher microalbuminuria as compared to the subjects without DR; all variables were statistically significant. No differences between DR+ and DR- with regard to gender, duration of DM, systolic BP, history of hypertension, smokers, HDL cholesterol and triglycerides were observed.

LIST OF PUBLICATIONS

1. **Vinita K**, Sripriya S, Prathiba K, Vaitheeswaran K, Sathyabaarathi R, Rajesh M, Amali J, Umashankar V, Kumaramanickavel G, Pal SS, Raman R, Sharma T; SNDREAMS project. ICAM-1 K469E polymorphism is a genetic determinant for the clinical risk factors of T2D subjects with retinopathy in Indians: a population-based case-control study. *BMJ Open* 2012 Aug 17;2(4). PMID: 22904330 [Impact Factor: 1.58]

2. Raman R, Vaitheeswaran K, **Vinita K,** Sharma T. Is prevalence of retinopathy related to the age of onset of diabetes? Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetic Report No. 5. *Ophthalmic Res.* 2011;45(1):36-41. PMID: 20714189 [Impact Factor. 1.56]

3. Jagadeesan Madhavan, **Kumari Vinita**, Govindasamy Kumaramanickavel. Genetic counselling in Ophthalmology. *Journal of Tamil Nadu Ophthalmic Association.* Vol. 45, Issue – 3, September 2007.

Manuscript submitted/Under review

1. **Vinita K**, Sripriya S, Sharmila FM, Vaitheeswaran K, Raman R, Sharma T. High order interaction analysis of SNPs in PEDF (rs12150053, rs12194385) and EPO (rs1617640) genes with clinical determinants of type 2 diabetic retinopathy patients from south India. [Submitted; under review]

LIST OF PRESENTATIONS

Attended an International Asia ARVO 2013 conference held at Ashok International Convention & Exhibition Centre, New Delhi, and presented my work as poster presentation titled "Genetic interaction between rs5498 (ICAM-1), rs1061170 (CFH) and glycosylated hemoglobin levels among type 2 diabetic retinopathy patients from India" **on 28th-31st January, 2013**.

Presented my thesis work for the Swarnalatha Punshi Award held at Sankara Nethralaya, as paper presentation titled "Association and interaction study of genetic variants in neuroprotective genes (PEDF and EPO) for diabetic retinopathy in south Indian population with type 2 diabetes" **on 10th September, 2012.**

Attended the National Conference on Emerging Trends in Life Sciences Research held at Birla Institute of Technology & Science, Pilani, and presented my work as a poster presentation titled "Genetic association study with diabetic retinopathy – our experience" **on 6th – 7th March, 2009.**

Attended the International conference on vision and ophthalmology Asia ARVO 2009 held at Hyderabad International Convention Centre, Hyderabad, and presented my work as a poster presentation titled "Met72Thr polymorphism of the PEDF Gene is associated with Diabetic Retinopathy" **on 15th-17th January, 2009.**

Attended the International conference on Nano-medicine & Recent Advance in Ophthalmic Research held at Sankara Nethralaya, Chennai and presented my work as a paper presentation titled "Met72Thr polymorphism of the Pigment Epithelium-Derived Factor Gene is associated with Diabetic Retinopathy in the South Indian Population" **on 3rd-5th September, 2008.**

Attended the 17th annual meeting Indian Eye Research Group (IERG) meet held at Aravind Eye Hospital, Madurai, and presented my work as a paper presentation titled "Y402H polymorphism is not associated with Diabetic Retinopathy in the South Indian Population" **on 26th-27th July, 2008.**

AWARDS

1. Received the best poster award for the presentation "Genetic interaction between rs5498 (*ICAM-1*), rs1061170 (*CFH*) and glycosylated hemoglobin levels among type 2 diabetic retinopathy patients from India" at Asia-ARVO 2013 conference at New Delhi on 28th-31st October 2013.

2. Granted "Asia-ARVO National Travel Grant" for attending the Asia-ARVO 2013 conference at New Delhi for poster presentation on "Genetic interaction between rs5498 (*ICAM-1*), rs1061170 (*CFH*) and glycosylated hemoglobin levels among type 2 diabetic retinopathy patients from India" on 28th-31st October 2013.

3. Granted "Travel Fellowship" for attending 17th Indian Eye Research Group (IERG) meeting at Aravind Eye Hospital, Madurai for paper presentation on "*CFH* gene Y402H polymorphism is not associated with diabetic retinopathy in the south Indian population" on 26-27th July, 2008.

4. Awarded the "Best out-going student in Clinical Genetics and Molecular Biology (MS MLT) on 2005.

BRIEF BIOGRAPHY OF THE CANDIDATE

Ms. Vinita Kumari obtained her B.Sc Zoology degree from Jamshedpur Co-operative College, Ranchi University, Jamshedpur in 2000. She obtained her M.S. (Medical Laboratory Technology) degree of Birla Institute of Technology and Science, Pilani in 2005 with course work at Medical Research Foundation, Sankara Nethralaya, Chennai. She did her 1 year internship project in SNONGC Department of Genetics and Molecular Biology, Sankara Nethralaya and studied "Case control study of RAGE gene in diabetic patients with or without retinopathy" and "Mutational screening of Retinoblastoma susceptibility (RB1) gene". She then joined the SNONGC Department of Genetics and Molecular Biology, Sankara Nethralaya as Junior Scientist in July 2005 and worked for 2 years. She then joined as research fellow in Sankara Nethralaya Diabetic Retinopathy project and worked on Genetics of diabetic retinopathy which was a part of this Jamshetji TATA Trust funded project. Her skills include peripheral blood lymphocyte culture, banding, karyotyping, PCR, RFLP, sequencing in fluorescent based techniques, etc. She has made presentations in 3 international and 2 national conferences, comprising of 2 papers and 3 posters presentation. She has attended workshops on "Hands on training in molecular genetic techniques in Diabetic Retinopathy" and "Pre-IERG workshop on Molecular Techniques in Ophthalmic Genetics" conducted at Aravind Eye Hospital, Madurai and "Research Methodology and Biostatistics" organized by Department of Epidemiology, The Tamil Nadu Dr. M.G.R. Medical University, Chennai. She received Bangalore Genie best out-going student in Clinical Genetics and Molecular Biology (MS MLT). 2005. She received travel fellowship grant award to attend the 17th annual IERG meeting at Aravind Eye Hospital, Madurai on 2008 and more recenly Asia-ARVO 2013 conference at New Delhi on 28th-31st October 2013. She also received the best poster award for the presentation "Genetic interaction between rs5498 (ICAM-1), rs1061170 (CFH) and glycosylated hemoglobin levels among type 2 diabetic retinopathy patients from India" at Asia-ARVO 2013 conference at New Delhi on 28th-31st October 2013.She has 3 publications out of which 1 are relevant to her thesis work. Her research interests include ocular genomics in general and molecular genetics of diabetic retinopathy in particular. Currently she is working as Junior Scientist in SNONGC Department of Genetics and Molecular Biology, Sankara Nethralaya.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Saragapani Sripriya is, Lecturer at SN ONGC Department of Genetics & Molecular Biology, Vision Research Foundation, Sankara Nethralaya, Chennai, India. She obtained her Doctorate degree from Birla Institute of Technology and Science, Pilani, in 2006 on Title: "Genetic association studies, genome wide linkage analysis and mutation screening in primary open and closed angle glaucomas". She did MS (Medical Laboratory Technology) Birla Institute of Technology and Science, Pilani, in 1999 with course work at Medical Research Foundation, Sankara Nethralaya, Chennai. She has received the following awards: DST Fast Track fellowship (2012), ICMR fellowship for exchange visit under Indo French collaborative project (2010), Young Scientist at 31st Indian Society of Human Genetics, New Delhi, 2006, Best Poster at 29th Indian Society of Human Genetics, Bangalore, 2004, Chennai Willingdon Corporate Foundation PhD Fellowship, 2001, Young Investigator, 1st Singapore Eye Research Institute International Meeting, Singapore, 2001, Travel fellowship, IX Indian Eye Research Group Meeting, Hyderabad, 1999, Bangalore Genie Best Outgoing Student Award for Clinical genetics, 1999. She works on molecular genetics of inherited ophthalmic diseases and involved in genetic diagnostics of the same. She received funding from ICMR, DST etc for various projects. She has 18 publications in peer reviewed journals including Nature Genetics, BMJ Open, Ophthalmic genetics, Molecular Vision, Clinical Genetics, Molecular diagnostics and therapy, Journal of current glaucoma practice, Ophthalmic Epidemiology, Journal Diabetes and Complications, Diabetes Research and Clinical Practice, In press 3: Clinical Genetics, Meta gene, Current eye research. She has reviewed articles on ocular genetic disorders for journals molecular vision, British Journal of Ophthalmology. She has trained 15 masters' students who worked on specific genetics dissertation topic. She guides 3 PhD students registered under BITS, Pilani and SASTRA, Tanjore, Tamil Nadu. She is also involved in genetic counseling for patients with ocular genetic diseases. Areas of Interest: Homozygosity mapping candidate gene analysis and genetic association studies for oligogenic disorders Bardet Biedl syndrome, ocular developmental anomalies, type 2 diabetic retinopathy, open angle and angle closure glaucomas.