REVIEW OF LITERATURE
2. Review of literature

2.1. Eye Anatomy

Structure of the human Eye
The eye is a highly specialized organ of photoreception, the process by which light energy from the environment produces changes in specialized nerve cells in the retina, the rods and cones. These changes result in nerve action potentials, which are subsequently relayed to the optic nerve and then to the brain, where the information is processed and consciously appreciated as vision.

The eye is made up of three basic layers or coats, often known as tunics. These are the fibrous (corneoscleral) coat, the uvea or uveal tract (composed of choroid, ciliary body, and iris), and the neural layer (retina). The coats surround the contents, namely the lens and the transparent media (aqueous humor and vitreous body). The cornea and sclera together form a tough protective fibrous envelope that protects the ocular tissues.

![Figure 1: Anatomy of the human eye](www.seeweimer.com)
2.1.1. Retina

Retina is a thin transparent membrane, approximately 0.5mm thick and spread over the inside of the back of the eye. It is the innermost of the three coats of the eye. This layer is in the image plane of the eye’s optical system and is responsible for converting relevant information from the image of the external environment into neural impulses that are transmitted to the brain for decoding and analysis. It consists of two primary layers: an inner neurosensory retina and an outer simple epithelium, the retinal pigment epithelium (RPE). These two layers can be traced embryologically to the inner and outer layers of the invaginated optic cup. In the adult they are continuous anteriorly with the epithelial layers over the ciliary processes and posterior iris surface. Between the neural retina and RPE is a potential space, the subretinal space, across which the two layers must adhere. The neural retina is firmly attached only at its anterior termination, the ora serrata, and at the margins of the optic nerve head. The retina is bound externally by Bruch’s membrane and on its internal aspect by the vitreous. It is continuous with the optic nerve posteriorly, the site of exit of ganglion cell axons from the eye.

It contains millions of photoreceptors that capture light rays and convert them into electrical impulses. These impulses travel along the optic nerve to the brain where they are turned into images.

There are two types of photoreceptors in the retina: rods and cones. The retina contains approximately 6 million cones. The cones are contained in the macula, the portion of the retina responsible for central vision. They are most densely packed within the fovea, the very center portion of the macula. Cones function best in bright light and allow us to appreciate color. There are approximately 125 million rods. They are spread throughout the peripheral retina and function best in dim lighting. The rods are responsible for peripheral and night vision.

Each retina possesses about 200 million neurons. Note that light impinges on the retina
from below in the diagram (Figure 2). The layers of the retina can be seen easily in cross-sectional histologic preparations. In youth, the innermost layer, the internal limiting membrane, is contiguous with the most posterior aspect of the vitreous. Progressing from inner to outer retina the layers are

- **Inner limiting membrane (ILM):** is the boundary between the vitreous humor in the posterior chamber and the retina itself.
- **Ganglion cell layer:** comprises the cell bodies and axons of ganglion cells.
- **Inner plexiform layer (IPL):** contains the synapses made between bipolar, amacrine and ganglion cells. The thickness of this layer varies considerably across species, where "simpler" organisms (such as frogs, pigeons and squirrels) possess thicker IPL's than "higher" organisms like primates. The thicker IPL indicates that these retinas perform more peripheral and specialized image processing.
- **Inner nuclear layer (INL):** contains bipolar cells, horizontal and amacrine cells boides.
- **Outer plexiform layer (OPL):** contains bipolar cells, horizontal cells and receptor synapses.
- **Outer nuclear layer (ONL):** contains the nuclei of photoreceptors.
- **Outer limiting membrane (OLM):** a membrane which coincides with the base of inner segments of photoreceptors.
- **Photoreceptor layer:** contains the inner and outer segments of rod and cone photoreceptors.
- **Pigment epithelium (PE):** darkly pigmented cells which absorb light not captured by photoreceptors, thus reducing scattering; also plays a role in "trimming" photoreceptors -- cones are "trimmed" at dusk, and rods are "trimmed" at dawn. Diurnal species (active in bright light environments) typically possess dark PE's; nocturnal species (active in dim light environments) possess an adaptation called a tapetum. The tapetum is a mirrorlike layer behind the photoreceptors which reflects photons not captured by the photoreceptors back out the eye, thus giving
the receptors a "second chance" to capture them. Sensitivity to light in these animals is thus increased by approximately two fold. The dominant wavelength of light reflected by the tapetum is usually close to the absorbance peak of rhodopsin (the photopigment contained by rods). Thus, the "eyeshine" seen in deer, opposums, dogs and the cat below appears greenish to us.

- **Choroid**: highly vascularized layer which supplies nutrients and oxygen to the retina. The eyeshine sometimes observed in flash photographs of humans is caused by light reflected from the choroid layer. The hue is red because oxygenated blood absorbs light of shorter wavelengths.

*Figure 2: Different layers of the Retina*

Courtesy by – [www.iriscameras.com](http://www.iriscameras.com)
2.2. Diabetes Mellitus

2.2.1. Definition
The term ‘diabetes mellitus‘ describes, “a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from the body’s inability to produce insulin or resistance to insulin action or both.” (American Diabetes Association 2006) Diabetes mellitus (DM) may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss.

2.2.2. Types and Pathophysiology of Diabetes Mellitus
The aetiological types designate defects, disorders or processes which often result in DM. It can be subdivided into four clinically distinct types: Type 1 DM, Type 2 DM, Gestational diabetes, Maturity onset diabetes of the young.

Type 1 Diabetes Mellitus
This form of diabetes, formerly called insulin–dependent diabetes, Type 1 diabetes, or juvenile–onset diabetes, results from autoimmune mediated destruction of the insulin producing β-cells of the pancreatic islets of Langerhans, a process that is immunologically mediated and occurs in genetically susceptible individuals. The islet β-cells are destroyed by an autoimmune response mediated by T-lymphocytes (T cells) that react specifically to one or more β-cell proteins (autoantigens) (Almawi et al., 1999). The rate of destruction is quite variable, being rapid in some individuals and slow in others (Zimmet et al., 1994). The rapidly progressive form is commonly observed in children, but also may occur in adults (Humphrey et al., 1998). The slowly progressive form generally occurs in adults and is sometimes referred to as latent autoimmune diabetes in adults (LADA). Individuals with this form of Type 1 diabetes often become dependent on insulin for survival on a lifelong basis eventually and are at risk for ketoacidosis (Willis et al., 1996). At this stage of the disease, there is little or no insulin secretion as manifested by low or undetectable levels of plasma C–peptide (Hother–Nielsen et al., 1988). In adults, type 1 diabetes accounts for 5 to
10 percent of all diagnosed cases of diabetes. Risk factors for type 1 diabetes may be autoimmune, genetic or environmental. Several clinical trials for the prevention of type 1 diabetes are currently in progress or are being planned.

**Type 2 Diabetes Mellitus**

DM of this type previously known as non–insulin dependent diabetes, or adult–onset diabetes, is a term used for individuals who have relative (rather than absolute) insulin deficiency. It develops when there is an abnormal increased resistance to the action of insulin and the body cannot produce enough insulin to overcome the resistance (DeFronzo et al., 1997 and Lillioja et al., 1993). At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive. Although the specific aetiologies of this form of diabetes are not known, by definition autoimmune destruction of the pancreas does not occur and patients do not have other known specific causes of diabetes. The majority of patients with this form of diabetes are obese, and obesity itself causes or aggravates insulin resistance (Campbell et al., 1993 and Bogardus et al., 1985).

**Maturity Onset Diabetes of the Young (MODY)**

MODY is a monogenic subtype of Type 2 diabetes, characterized by an autosomal dominant inheritance, and an age of onset at 25 yr or younger. Phenotypically, MODY is primarily associated with insulin secretion defects and patients with MODY have impaired insulin secretion with minimal or no defect in insulin action (Byrne et al., 1996 and Clement et al., 1996). It has been estimated that 2-5 % of all patients with Type 2 diabetes may have MODY. Studies by Mohan et al showed a high prevalence of MODY in south Indians (4.8%) besides reporting the insulin responses in them and β-cell response in the offspring of patients with MODY (Mohan et al., 1985).

**Gestational diabetes mellitus**

Gestational diabetes is carbohydrate intolerance resulting in hyperglycemia of variable severity with onset or first recognition during pregnancy. It does not exclude the possibility
that the glucose intolerance may antedate pregnancy but has been previously unrecognized. It is also more common among obese women and in women with a family history of diabetes. The definition applies irrespective of whether or not insulin is used for treatment or the condition persists after pregnancy. Women who become pregnant and who are known to have DM which antedates pregnancy do not have gestational diabetes but have “diabetes mellitus and pregnancy” and should be treated accordingly before, during, and after the pregnancy. Formal systematic testing for gestational diabetes is usually done between 24 and 28 weeks of gestation. During pregnancy, gestational diabetes requires treatment to normalize maternal blood glucose levels to avoid complications in the infant. Immediately after pregnancy, 5 to 10 percent of women with gestational diabetes are found to have diabetes, usually type 2. Women who have had gestational diabetes have a 40 to 60 percent chance of developing diabetes in the next 5 to 10 years.

2.2.3. Prevalence of Diabetes

Global estimates of diabetes

The prevalence of diabetes is rapidly rising all over the globe at an alarming rate in the coming decades. According to the international diabetes federation (IDF), it is estimated that 30 million people worldwide had diabetes in 1985 and in 2000, a little over a decade later; the figure had risen to over 150 million. In 2003, the total was 194 million and in 2007 it was 246 million people with diabetes in the adult population in the seven regions of IDF. By 2025, the figure is expected to rise to 380 million. In 1994, McCarty et al., reported the prevalence of diabetes using the data from population-based epidemiological studies and estimated that the global burden of diabetes was 110 million in 1994 and that it would likely more than double to 239 million by 2010. World Health Organization (WHO) also produced a report using epidemiological information and estimated the global burden as 135 million in 1995, with the number reaching 299 million by the year 2025 (King et al., 1998). In 1997, Amos et al., estimated the global burden of diabetes to be 124 million people, and projected that this would increase to 221 million people by the year 2010. Despite using different methodologies, and at times
showing large differences in country-specific estimates, these reports have arrived at remarkably similar global figures of diabetes.

**Prevalence in India**

India leads the world with largest number of diabetic subjects and crowned as diabetes capital of the world. According to the diabetes atlas 2006 published by the International Diabetes Federation, in India the incidence of diabetes is currently around 40.9 million and is expected to rise to 69.9 million by 2025 unless urgent preventive steps are taken (Sicree *et al.*, 2006). WHO reports show that 32 million people had diabetes in the year 2000 (Wild *et al.*, 2004). The first national study on the prevalence of type 2 diabetes in India was done between 1972 and 1975 by the Indian Council of Medical Research (ICMR, New Delhi). Screening was done in about 35,000 individuals above 14 yr of age, using 50 g glucose load. Capillary blood glucose level >170 mg/dl was used to diagnose diabetes. The prevalence was 2.1 % in urban population and 1.5% in the rural population while in those above 40 yr of age, the prevalence was 5% in urban and 2.8% in rural areas (Ahuja *et al.*, 1979). The overall crude prevalence of diabetes using WHO criteria in CURES (Chennai Urban Rural Epidemiological Study) was 15.5% (age-standardized: 14.3%), while that of IGT was 10.6% (age-standardized: 10.2%).

![Figure 3: Prevalence of Diabetes in different parts of the world](image)

Courtesy by –Wild *et al.*, 2004
2.2.4. Diabetes Related complications

Diabetes can affect many different organ systems in the body and over time, can lead to serious complications. Complications from diabetes can be classified as microvascular and macrovascular. Microvascular complications include nervous system damage (neuropathy), renal system damage (nephropathy) and eye damage (retinopathy). (American Diabetes Association 2006)

![Diabetes Related complications diagram]

Figure 4: Classification of Diabetic complications

2.3. Diabetic Retinopathy

2.3.1. Definition

Diabetic retinopathy is a leading cause of blindness. It is classically defined as a microvasculopathy that affects primarily the small blood vessels of the inner retina due to diabetes mellitus.

2.3.2. Clinical features of diabetic retinopathy

The ophthalmoscopic features include dilated and tortuous retinal vessels, microaneurysms, flame-shaped nerve fiber layer hemorrhages, round deep retinal hemorrhages, cotton-wool
spots and lipoprotein exudates, intraretinal microvascular abnormalities. Microaneurysms are focal outpouchings of capillary walls in the region of vascular occlusions (Gardner et al., 2000). The hemorrhages and exudates result from leaking blood vessels. Increase in retinal blood flow and vasodilation occurs early in the course of the disease, followed by the microvascular leakage and occlusion and histologic data corroborates the clinical evidence (Engerman et al., 1995).

The early stages of DR are characterized by histopathological changes which include loss of pericytes, basement membrane thickening, homodynamic alterations (changes in retinal blood flow and areas of capillary non-perfusion), vascular abnormalities (microaneurysms), venous beading and reduced vascular integrity (Klein et al., 1984 and Patz et al 1980). DR is also characterized by vascular permeability, increased tissue ischemia and angiogenesis, a process of new blood vessel formation arise from the retina and optic disc. These new vessels are fragile and prone to rupture resulting in vitreous hemorrhage and subsequent detachment of the retina.

![Figure 5: Schematic representation of clinical features of DR](www.emeraldeye.com)
2.3.3. Classification

DR is broadly classified as Non proliferative diabetic retinopathy (NPDR) and Proliferative diabetic retinopathy (PDR).

Non proliferative diabetic retinopathy (NPDR)

NPDR indicates progressive ischemia in the retina and an increased risk for the development of PDR and blindness. The prominent clinical features of NPDR include (a) microaneurysms which are the first clinically detectable lesions of DR located in the inner nuclear layer of the retina (b) dot and blot hemorrhages, which are located in the middle retinal layers (c) hard yellow exudates, which are located between the inner plexiform and inner nuclear layer of the retina (d) vascular changes such as beading, looping and sausage like segmentation of the veins (e) cotton wool spots, also called soft exudates or nerve fiber infarcts, result from capillary occlusion of the retinal nerve fiber layer, (f) intraretinal microvascular abnormalities (IRMA), which are dilated capillaries that seem to function as collateral channels, frequently seen adjacent to the areas of capillary closure (g) retinal edema characterized by accumulation of fluid between the outer plexiform layer and inner nuclear layer, which may later involve the entire layers of the retina. ETDRS in 1991 has classified NPDR into four stages. They are Mild NPDR, Moderate NPDR, Severe NPDR, and Very severe NPDR and PDR into early PDR and high-risk PDR. This is as follows:

Mild NPDR

Is marked by at least one retinal microaneurysm, but hemorrhages and microaneurysms are less than Early Treatment Diabetic Retinopathy Stusy (ETDRS) standard photograph 2A in all four retinal quadrants. No other significant retinal lesion or abnormality associated with diabetes is present. Those with mild NPDR have a 5% risk of progression to PDR within 1 year, and a 15% risk of progression to high-risk PDR within 5 years.
Figure 6: Fundus photo shows the clinical features of mild NPDR

Moderate NPDR

It is characterized by hemorrhages and/or microaneurysms greater than those pictured in ETDRS standard photograph 2A in at least one field but less than four retinal quadrants. Cotton-wool spots (soft exudates), venous beading and IRMAs may present to a mild degree (less than standard photograph 8A). The risk of progression to PDR within 1 year is 12-27% and the risk of progression to high-risk PDR within 5 years is 33%.

Figure 7: Fundus photo shows the clinical features of Moderate NPDR
Severe NPDR

Based on the severity of hemorrhages and/or microaneurysms (H/Ma), IRMA, and venous beading (VB), is characterized by any one of the following lesions:

1. H/Ma greater than standard photo 2A in four quadrants or
2. Venous caliber abnormalities (VCAB) in two or more quadrants or
3. IRMAs greater than standard photo 8A in at least one quadrant.

Eyes with severe NPDR have a 52% risk of developing PDR within 1 year, and a 60% risk of developing high-risk PDR within 5 years.

Very severe NPDR

Eyes with very severe NPDR have two or more lesions of severe NPDR, but no frank neovascularization. There is a 75% risk of developing PDR within 1 year. Patients with very severe NPDR may be candidates for scatter (panretinal) laser surgery, and macular edema, if present may require treatment.

Proliferative Diabetic retinopathy

Pre proliferative diabetic retinopathy is the stage before the onset of neovascularization and is characterized by (a) extensive retinal hemorrhages, (b) marked venous beading, (c)
numerous cotton wool spots or retinal infarcts, (d) intra-retinal microvasculature abnormalities (IRMA), and (e) marked retinal ischemia as evidenced by capillary drop outs in the fundus fluorescein angiogram. PDR is characterized by retinal new vessels (neovascularization) in the disc (NVD) or neovascularization elsewhere (NVE), vitreous or pre-retinal hemorrhage, or fibrous tissue, vitreoretinal traction and localized retinal detachment. Early PDR does not meet the definition of high-risk PDR. Eyes with early PDR (less than high risk) have a 75% risk of developing high-risk PDR within a 5 year period.

Figure 9: Fundus photo shows the clinical features of PDR
(a) NVE - Neovascularization elsewhere. (b) NVD – Neovascularization in the disc.
(c) PDR with tractional retinal detachment.
2.3.4. Symptoms of Diabetic Retinopathy
Blurring of vision is the main symptom associated with DR but this usually occurs when the disease is already well established. Many people with DR will have no symptoms at all. DR does not cause pain. People with proliferative retinopathy may experience floating spots in their vision due to bleeding within the eye.

2.3.5. Diagnosis of Diabetic retinopathy
DR is detected during an eye examination that includes:

Visual acuity test: This test uses an eye chart to measure how well a person sees at various distances (i.e., visual acuity).

Pupil dilation: The eye care professional places drops into the eye to widen the pupil. This allows him or her to see more of the retina and look for signs of DR. After the examination, close-up vision may remain blurred for several hours.

Ophthalmoscopy
An ophthalmoscope is an instrument used to examine the retina and vitreous. Ophthalmoscopy requires dilating the pupils with drops to give the doctor the best view inside the eye. There are two types of ophthalmoscopes: direct and indirect. The direct is a hand-held instrument with a battery powered light source. It also has a series of lenses that can be dialed in to focus the doctor’s view of the retina. The direct ophthalmoscope is useful for examining the central retina. Hand-held ophthalmoscopy is insufficient to rule out significant and treatable DR. The indirect ophthalmoscope can be used to examine the entire retina. This instrument is worn on the doctor’s head. While looking through the instrument’s magnifying glasses, a special lens is placed in front of the patient’s eye, allowing the doctor to see the retina clearly.
Ocular Coherence Tomography or OCT
This is a scan similar to an ultrasound which is used to measure the thickness of the retina. It produces a cross sectional image of the retina which is comparable to histological sections and can determine if there is any swelling or leakage. OCT is more sensitive than clinical fundus evaluation in diagnosing clinically significant macularedema.

Fundus fluorescein angiography
Fluorescein angiography (FA) (fluorescein – the type of dye that is used; angiogram – a study of the blood vessels) is an extremely valuable test that provides information about the circulatory system and the condition of the back of the eye. FAs are useful for evaluating many eye diseases that affect the retina. The test is performed by injecting a special dye, called fluorescein, into a vein in the arm. In just seconds, the dye travels to the blood vessels inside the eye. A camera equipped with special filters that highlight the dye is used to photograph the fluorescein as it circulates through the blood vessels in the back of the eye. If there are any circulation problems, swelling, leaking or abnormal blood vessels, the dye and its patterns will reveal these in the photographs. This test is one of the most important tests to diagnose DR.

Tonometry
A standard test that determines the fluid pressure (intraocular pressure) inside the eye. Elevated pressure is a possible sign of glaucoma, another common eye problem in people with diabetes.

Slit Lamp Biomicroscopy Retinal Screening Programs
Systematic programs for the early detection of DR using slit-lamp biomicroscopy exist either as a standalone scheme or as part of the Digital program (above) where the digital photograph was considered to lack enough clarity for detection and/or diagnosis of any retinal abnormality.
2.3.6. Management

There are three major treatments for DR, which are very effective in reducing vision loss from this disease. In fact, even people with advanced retinopathy have a 90% chance of keeping their vision when they get treatment before the retina is severely damaged. These three treatments are laser surgery, injection of triamcinolone into the eye and vitrectomy. It is important to note that although these treatments are very successful, they do not cure DR. Caution should be exercised in treatment with laser surgery since it causes a loss of retinal tissue. It is often more prudent to inject triamcinolone. In some patients it results in a marked increase of vision, especially if there is an edema of the macula. Avoiding tobacco use and correction of associated hypertension are important therapeutic measures in the management of DR.

Laser photocoagulation

Laser photocoagulation can be used in two scenarios for the treatment of DR.

Pan retinal photocoagulation

Pan retinal photocoagulation, or PRP (also called scatter laser treatment), is used to treat PDR. The goal is to create 1,000 - 2,000 burns in the retina with the hope of reducing the retina's oxygen demand, and hence the possibility of ischemia. In treating advanced DR, the burns are used to destroy the abnormal blood vessels that form in the retina. This has been shown to reduce the risk of severe vision loss for eyes at risk by 50%. Before the laser, the ophthalmologist dilates the pupil and applies anesthetic drops to numb the eye. In some cases, the doctor also may numb the area behind the eye to prevent any discomfort. The patient sits facing the laser machine while the doctor holds a special lens to the eye. The physician can use a single spot laser or a pattern scan laser for two dimensional patterns such as squares, rings and arcs. During the procedure, the patient may see flashes of light. These flashes may eventually create an uncomfortable stinging sensation for the patient. After the laser treatment, patients should be advised not to drive for a few hours while the pupils are still dilated. Vision may remain a little blurry for the rest of the day, though there
should not be much pain in the eye.

**Intravitreal Triamcinolone acetonide**

Triamcinolone is a long acting steroid preparation. When injected in the vitreous cavity, it results in a decrease in the macular edema (thickening of the retina at the macula) caused due to diabetic maculopathy, along with an increase in the visual acuity. The effect of triamcinolone is transient, lasting up to three months, and necessitating repeated injections for maintaining the beneficial effect. Complications of intravitreal injection of triamcinolone include cataract, steroid induced glaucoma and endophthalmitis.

**Vitrectomy**

Instead of laser surgery, some people need an eye operation called a vitrectomy to restore vision. A vitrectomy is performed when there is a lot of blood in the vitreous. It involves removing the cloudy vitreous and replacing it with a saline solution made up of salt and water. Because the vitreous is mostly water, there should be no change between the saline solution and the normal vitreous. Studies show that people who have a vitrectomy soon after a large hemorrhage are more likely to protect their vision than someone who waits to have the operation. Early vitrectomy is especially effective in people with insulin-dependent diabetes, who may be at greater risk of blindness from a hemorrhage into the eye.

Vitrectomy is often done under local anesthesia. The doctor makes a tiny incision in the sclera, or white of the eye. Next, a small instrument is placed into the eye to remove the vitreous and insert the saline solution into the eye. Patients may be able to return home soon after the vitrectomy, or may be asked to stay in the hospital overnight. After the operation, the eye will be red and sensitive, and patients usually need to wear an eye patch for a few days or weeks to protect the eye. Medicated eye drops are also prescribed to protect against infection.
2.3.7. Prevalence of Diabetic Retinopathy

International

The prevalence of DR varies in type 1 and type 2 diabetes. In type 1 diabetes in the EURODIAB IDDM complications study, which included subjects attending 31 European diabetes centres, the prevalence of DR ranged between 25-60% (Abrahamian et al., 1994). The prevalence of DR in Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) was 50.1% (Williams et al., 2004) and 54.2% in the diabetes control and complications trial (DCCT) in IDDM (Malone et al., 2000) and 35-39% in United Kingdom Prospective Diabetes Study (UKPDS) in NIDDM (Kohner et al., 1998). The prevalence of DR was 32.4%, 36.8% and 52% in the Blue Mountains Eye Study, Beaver Dam Eye Study and Melton Study (Mitchell et al., 1998, Klein et al., 1992 and Sparrow et al., 1993).

National

An earlier study done in a clinic-based population reported an overall prevalence of 14 percent. NPDR was observed in 6%, while 4% had macular edema and 4% had PDR (Mohan et al., 1989). Asian Young Diabetes Research (ASDIAB) Study, reported the prevalence of DR in 724 young diabetic subjects of age 12-40 yr with duration of diabetes <12 months in 7 centres of four Asian countries. It is interesting to note that DR prevalence was least among Indians (5.3%) as compared to other ethnic groups like Malays (10%) and Chinese (15.1%) (Rema et al., 2002). The other two studies from South India, the prevalence rate of DR in NIDDM patients were 34.1% and 37% (Rema et al., 1996 and Sharma et al., 1996). In the Andhra Pradesh Eye Disease Study (APEDS) of self-reported diabetes, the prevalence of DR was 22.4% (Dandona et al., 1999). The prevalence of DR in clinic based study showed 30.8% among which 30.8% was NPDR, 3.4% was DR and 6.4%DME (Rema et al., 1996). However, in the Chennai Urban Rural Epidemiology (CURES) Eye study, the overall prevalence of DR was 17.6%. Higher frequency of all the grades of retinopathy (overall, NPDR and PDR) was observed in known diabetic subjects compared to newly detected cases (Rema et al., 2005). The CURES Eye study is the first population-based study, which used four-field stereo retinal
photographs and ETDRS grading to document DR in the Indian population (Deepa et al., 2003).

Two other population-based studies conducted in South India, reported overall prevalence of DR as 22.4% (Dandona et al., 1999) and 26.8% (Narendran et al., 2002) respectively. As per the Chennai Urban Population Study (CUPS), the prevalence of diabetes was higher in the middle income group (12.4%) compared to lower socio-economic strata (6.5%) (Rema et al., 2000), which is contradictory to the western population studies where diabetes is reported to be higher among the lower economic strata (Connolly et al., 2000). In a recent Theni district study estimated the prevalence of DR in the diabetic population was 12.2% (Namperumalsamy et al., 2009).

2.3.8. Etiology and pathogenesis

Various studies have shown that chronic hyperglycemia, hypertension and hyperlipidemia contribute to the pathogenesis of DR. Hyperglycemia damages retinal vasculature in several ways and progression of DR is generally related to the severity and duration of hyperglycemia. The exact mechanism by which raised glucose levels lead to vascular disruption seen in retinopathy is poorly defined. However, various biochemical pathways have been suggested to demonstrate correlation between hyperglycemia and microvascular complications of retinopathy.

2.3.8.1. Molecular Mechanisms of DR Pathogenesis

DR is a culmination of numerous biochemical alterations which take place in the vascular tissue of the retina. High levels of glucose affect both the vascular endothelial cells (ECs) and the pericytes. The retinal ECs incorporate glucose via glucose transporter-induced facilitative diffusion (Schalkwijk et al., 2005, Choi et al., 1989 and Khan et al., 2007). Therefore, increased circulating levels of glucose accumulate in the retinal ECs and lead to activation of various biochemical pathways. The main proposed components for induction and progression of DR include: Polyol pathway increased ARactivity (Robison et
Al., 1983); nonenzymatic glycation and glycoxidation with formation of AGEs (Brownlee et al., 1988); increased de-novo synthesis of diacylglycerol from glucose, causing PKC activation (Hammes et al., 2003); oxidative-nitrosative stress with overproduction of reactive oxygen species (ROS) (Kunisaki et al., 1995), rennin-angiotensinogen system (Deinum et al., 1990), VEGF, Growth hormone and IGF-1 (insulin like growth factor) (Ferrara et al., 1995). GLUT1-glucose transporter-1 (Fernandes et al., 2003; Zhang et al., 2003), PPARγ-peroxisome proliferator-activated receptor (Hammes et al., 2002; Miles et al., 1995), TIMP (tissue matrix metalloproteinase) & extracellular matrix homeostasis (Docherty et al., 1992). More recently, it has been established that reactive oxygen and nitrogen species trigger activation of mitogen-activated protein kinases (MAPKs) and poly (ADP-ribose) polymerase (PARP), as well as the inflammatory cascade, and these downstream mechanisms are also involved in the pathogenesis of diabetes complications.

**Aldose Reductase (AR) and the polyol pathway**

Aldose reductase, the first and rate-limiting enzyme in the polyol pathway, reduces glucose to sorbitol using NADPH as a cofactor; sorbitol is then metabolized to fructose by sorbitol dehydrogenase, which uses NAD+ as a cofactor. The polyol (sorbitol) pathway of glucose metabolism is activated in many cell types when intracellular glucose concentrations are very high, and it can generate cellular oxidative stress (Heyningen et al., 1959) through a variety of biochemical abnormalities, including myo-inositol depletion and downregulation of Na/K ATP-ase activity, NAD+/NADH and NADP+/NADPH redox imbalances, changes in fatty acid metabolism, impaired neurotrophic support, and upregulation of VEGF (Dagher et al., 2004). The polyol pathway appears to be both a “dream” and a “dread” target by devising strategies to prevent DR. The pathway is a dream target because its activation is immediately linked to hyperglycemia, generates various types of cellular stress, and occurs prominently in the tissues that develop complications, thus promising returns beyond retinopathy. However, the polyol pathway has become a dread target because AR inhibitors (ARIs) have yielded inconsistent results in the diabetic or diabetic-like retinopathy of experimental animals and only minor benefits in human DR (Dagher et al., 2004). However,
the polyol pathway seems to be really a rational candidate mechanism for the ganglion cell apoptosis and Müller glial cell activation (Dagher et al., 2004 and Oyama et al., 2006). Ganglion and Müller cells are the retinal cells most consistently found to contain AR in all species studied, including humans. Since neuroglial changes may cause vascular changes, and given the general agreement that at least the pericytes of retinal capillaries contain aldose reductase, the inhibition of the polyol pathway could also prevent the vascular abnormalities of DR. In fact, inhibition of AR was also able to prevent the early activation of complement in the retinal vessel wall as well as the apoptosis of vascular pericytes and endothelial cells and the development of acellular capillaries.

Moreover, retinal endothelial cells showed AR immunoreactivity, and human retinas exposed to high glucose in organ culture increased the production of sorbitol. Finally, experimental evidence exists that defects in the polyol pathway may produce thickening of the capillary basement membrane, loss of mural pericytes and microaneurysm formation, and the earliest vascular features of diabetic microangiopathy. In fact, high glucose levels increase flux through the polyol pathway with the enzymatic activity of aldose reductase, thus determining a build-up of intracellular sorbitol concentrations and, consequently, an osmotic damage to the vascular cells. Therefore, it seems possible to conclude that excess of AR activity might be a mechanism in the pathogenesis of DR.

Positive preliminary results of some ARIs (such as sorbinil, zenarestat or fidarestat) in preventing retinal and neural damage in diabetes have been highlighted, thus justifying further clinical trials of specific, potent, and low-toxic ARI (Obrosova et al., 2005).

**AGEs Accumulation**

Advanced protein glycosylation, a process involving the nonenzymatic modification of tissue proteins by physiologic sugars in vivo, appears to play a central role in the pathogenesis of diabetic complications. Chronically increased amounts of glucose amplify the physiological process of nonenzymatic protein glycosylation (glycation). For example, glycated
hemoglobin (HbA1c) is an acknowledged indicator of time-integrated glycemia (Fong et al., 2004). Glucose and other hexoses react with most of the tissue/cellular proteins by combining with free ε-NH2-terminal and side-chain lysine radicals within proteins to form Schiff’s base, which, after a cascade of molecular rearrangements (Maillard reaction), result in molecules of brown color and specific fluorescence (Amadori products), leading to degradation of both structural and functional proteins and accelerated aging (Giusti et al., 2007). While most glycated proteins are eliminated in physiological conditions, they accumulate, on the contrary, in the presence of diabetes and undergo further structural arrangements with the formation of AGEs, which, in turn, are implicated in the development of vascular lesions having a proven effect in determining a significant loss of mural pericytes. Moreover, it is also clear that AGEs formation mechanisms are diverse and complex, encompassing both non-oxidative (glycation) and oxidative (glycoxidation) pathways. These reactions, together with intra- and intermolecular cross-link formation, are able to modify structure and function of target molecules in such a way that they do not respond anymore to biological signals (Wautier et al., 2003 and 2004).

Interaction of AGEs with their receptors (RAGE) has also been implicated in enhanced ROS formation and inflammatory vascular complications. For example, N(carboxymethyl) lysine-protein (CML-protein), macrophage colony stimulating factors (M-CSF) and soluble vascular cell adhesion molecule-1 (sVCAM-1) have been found to be increased in patients with diabetic micro-angiopathy and CML-human serum protein (CML-HSP) levels, which are at variance with the HbA1c index for blood glucose, have been proposed as a good biomarker both for glycoxidation and for the development of microvascular complications in type II diabetes. The use of compounds that inhibit AGE formation (such as pimagedine and aminoguanidine) has been investigated as a possible therapeutic intervention, highlighting promising results in the prevention of DR in animal models. Preliminary results for human diabetic nephropathy have been published and other clinical trials are under way to confirm the efficacy of this new kind of treatment in preventing the onset of diabetic microvascular complications (Bolton et al., 2004 and Yatoh et al., 2006).
AGE formation causes pathological changes via three general mechanisms. First, AGEs alter signal transduction pathways involving ligands on extracellular matrix. Second, AGEs alter the level of soluble signals such as cytokines, hormones, and free radicals through interactions with AGE-specific cellular receptors. Third, intracellular AGE formation by glucose, fructose, and more highly reactive metabolic pathway intermediates can directly alter protein function in target tissues (Brownlee et al., 1995).

RAGE has been localized in retinal endothelial cells, RPE and pericytes (Sulochana et al., 2001). AGE molecules upon binding to their receptors, exert diverse actions on target cells. Unlike in macrophages, the effect of binding of AGE to RAGE in these cells is different. Binding of AGE to RAGE in pericytes causes the death of the pericytes, while in RPE and endothelial cells, it promotes their migration and proliferation. Retinal microcapillaries are lined by endothelial cells and pericytes (Sulochana et al., 2001). Unlike in pericytes, when AGE binds to endothelial RAGE, it induces a plethora of events including gene expression for a variety of molecules, viz. growth factors such as VEGF, Platelet–derived growth factor (PDGF) (Yamagishi et al., 1997), transcription factors (NFκB, SP1 and STAT 1), activation of proteases such as matrix metalloproteinases (MMP), caspases and calpines, and the synthesis of adhesion molecules like vascular endothelial cell adhesion molecule (VECAM), E-SELECTIN, platelet endothelial cell adhesion molecule (PECAM) and intercellular adhesion molecule (ICAM) (Schmidt et al., 1994). All these events result in the disruption of the cellular homeostasis in DM. The adhesion molecules stimulate cell-cell adhesion and cell extracellular matrix (ECM) interaction. This process recruits macrophages to the local site (vessel wall), setting the stage for diffused and accelerated artherogenesis by elaborating cytokines and growth factors to activate the cascades. These growth factors aid in angiogenesis, thrombogenesis and artherogenesis. The important stages involved in the angiogenic process are invasion, migration and proliferation of microvascular endothelial cells through the capillary basement membrane and their seepage to adjacent ECM, leading to the growth of new microvessels. This invasion is coupled with the production and activation of specific extracellular protease, viz. serine proteinase-urokinase and enzymes of
matrix metalloproteinases (MMPs) family. Blocking the binding of AGEs to RAGE using synthetic chimerical RAGE will be of therapeutic value.

**Protein Kinase C (PKC) activation**

There is increasing evidence that PKC activation is related to hyperglycemia-induced microvascular dysfunction in diabetes (Ways et al., 2000). In this pathway, high glucose activates diacylglycerol (DAG), which then activates PKC. Activation of PKC results which, in turn, is associated with a number of biochemical and metabolic abnormalities, numerous cellular changes, including increased expression of matrix proteins, such as collagen and fibronectin, and increased expression of vasoactive mediators, such as endothelin. The net effect of these changes may be manifested as basement membrane thickening and changes in vessel permeability and/or blood flow. Although the activity of multiple PKC isoforms (α, β1, β2, δ and ε) is increased in vascular diabetic tissues, studies suggest that the PKC-β2 isoform preferentially mediates the pathologic complications associated with hyperglycemia (Shiba et al., 1993 and Inoguchi et al., 1992). Moreover, PKC-β has been shown to be an integral component of cellular signaling by VEGF (Xia et al., 1996), an important mediator of ocular neovascularization, secondary to retinal ischemia and DME (Miller et al., 1997 and Aiello et al., 1994), thus stimulating retinal pericyte proliferation.

A selective inhibitor of PKC-β, ruboxistaurin mesylate (LY333531), was initially reported to prevent the increase in leukostasis and the decrease in blood flow in the retinas of transgenic diabetic rats. However, multicenter clinical trials in humans did not show a same effectiveness on DR progression (Aiello et al., 2004 and Yamagishi et al., 2004). A possible reason of that might be the observation that PKC inhibition augments pro-apoptotic effects of high glucose on cultured pericytes. Therefore, it seem possible to conclude that the potential effectiveness of PKC-β inhibitors on DR progression is at this time still controversial (Wegewitz et al., 2005 and Comer et al., 2004)
Hexosamine pathway

Recent in vitro and in vivo studies suggested that the increased flux of glucose through the hexosamine pathway may contribute to insulin resistance, diabetic vascular complications and to the induction of the synthesis of growth factors (Balasubramaniam et al., 2002). During normal physiology, only ~3% glucose is channeled into the hexosamine pathway. In this pathway, glucose is converted to fructose-6-phosphate. The rate limiting enzyme in this pathway is glutamine: fructose-6-phosphate amidotransferase (GFAT), which catalyses the conversion of fructose-6-phosphate to glucoseamine-6-phosphate which is further metabolized to uridine diphosphate (UDP)-N-acetyl-glucosamine, including the synthesis of proteoglycans, gangliosides, glycolipids and O-linked glycoproteins. In parallel, this pathway activates transcription factors such as tumor-related growth factor-β and plasminogen activator inhibitor-1, both of which cause macrovascular and microvascular occlusion and ischemia.

Therefore, the hexosamine pathway not only potentiates the damaging effects of high glucose, it also activates growth factors that cause vascular ischemia, which in turn triggers free radical formation and more oxidative stress.

Figure 10: Pathways involved in DR

Hyperglycemia-driven biochemical alterations precipitated by mitochondria-driven oxidative stress leading to diabetic complications. (Modified from Brownlee 2002). Balasubramaniam et al., 2002
Oxidative Damage

Diabetes and hyperglycemia are associated with increase in oxidative stress, and overproduction of ROS (free radicals) are thought to be responsible for microvascular damage, being consistent with increased malonyldialdehyde, isoprostanes, nitrotyrosine or 8-hydroxy-2’deoxyguanosine levels as well as an overall decreased antioxidant status (Matteucci et al., 2001, Yokoi et al., 2005 and Amano et al., 2005). Production of ROS may result from various mechanisms, including glucose auto-oxidation, protein glycation, increased flux through the polyol pathway, and prostanoid production (Giugliano et al., 1996). These high ROS levels are thought to determine structural and functional changes in all cellular components, leading to DNA and protein modification and lipid peroxidation. In particular, pericytes are highly sensitive to the oxidative stress not only directly but also indirectly, due to significantly decreased levels of scavenging enzymes and increased rate of apoptosis. Pericyte loss or functional deficiency has been found to reduce the inhibition of endothelial proliferation in vivo. As damage progresses, the blood vessel wall becomes more porous, letting proteins and other materials leak out abnormally, thus determining the typical features of nonproliferative DR (e.g. hard exudates and clinically significant DME). Furthermore, animal studies suggest that antioxidants such as vitamin E may prevent some of the vascular dysfunction associated with diabetes by means of several different mechanisms: reduced retinal DAG levels; normalized PKC-β activation; normalized retinal blood flow; restored nitric oxide-mediated endothelium-dependent relaxation. The use of anti-oxidants is promising, but further studies are needed to determine appropriate doses, and/or whether this approach will translate into long-term benefits of reduced DR and DME (Porta et al., 2004).

Role of Growth factors

Understanding of the biochemical pathways underlying DR have clearly demonstrated the important role of a number of growth factors VEGF, growth hormone, insulin-like growth factor-1, PKC, transforming growth factor-β and pigment epithelium derived factor) in the
development of structural changes in the retinal vasculature (increased retinal vascular permeability, retinal ischemia, neovascularisation) and progression of DR. Many growth factors having potential angiogenic property have been identified. Only five of them have so far been implicated in DR. They are basic fibroblast growth factor (bFGF), insulin like growth factor (IGF), VEGF, platelet-derived growth factor (PDGF) and hepatocyte growth factor (HGF).

**bFGF**

bFGF a heparin binding protein is associated with extracellular matrix. It stimulates endothelial cell proliferation, migration, and neovascularization in chick chorioallantoic membrane and cornea models. It is present in the retina.

**IGF**

IGF stimulates migration and proliferation of retinal cells. While, IGF-1 has been found elevated in vitreous and serum of patients with proliferative DR, IGF-2 is not elevated (Grant et al., 1986). Levels of IGF-1 and IGF-2 in the vitreous of patients with proliferative DR have been reportedly elevated in a large population–based study (Dills et al., 1991). IGF-1 levels and neovascularization by demonstrating that IGF-1 is a vascular endothelial mitogen and migration factor *invitro* and an angiogenic factor in vivo (Grant et al., 1986). It is also present in the retinal cells. IGF-1 is the predominant mediator of intraocular neovascularization in Diabetes mellitus.

**PDGF and HGF**

PDGF have been reported to be elevated in cases of proliferative DR and it is also up regulated by AGE in cultured retinal pigment epithelial cells. HGF had found to be expressed in retinal pigment epithelial cells. HGF might have a potential role in retinopathy.

**VEGF**

VEGF, a major mediator of vascular permeability and angiogenesis, may play a pivotal role
in mediating the development and progression of DR (Awata et al 2002) VEGF a 45-kDa-homodimeric glycoprotein (Duh et al., 1999), as an important mediator of retinal ischemia associated intraocular neovascularization (Ferrara et al., 1997). VEGF levels are markedly elevated in vitreous and aqueous fluids in the eyes of individuals with PDR (Aiello et al., 1994, Murugeswari et al., 2008). In addition, the observation of increased retinal VEGF expression was early in DR.

2.3.9. Risk Factors of Diabetic Retinopathy:

DR is a multifactorial disease. Epidemiological surveys have shown that various risk factors known to be associated with DR, tend to accelerate its course and increase its severity

Systemic factors
Gender, Duration of diabetes, Glycaemic control, Hypertension, Renal disease, Elevated serum lipids, Pregnancy, Alcohol, Anaemia and Obesity

Ocular factors
Posterior vitreous detachment, Old chorioretinopathy and Cataract surgery

Other factors
Environmental, Biochemical growth factors and Genetic factors

2.3.10. Genetics of Diabetic Retinopathy

DR is a multifactorial disease with a complex inheritance pattern. Genetic as well as environmental factors play a crucial role in understanding the differential susceptibility to DR. It is believed that, genetics may predispose to the development of DR among the diabetic patients despite their good glycemic control. Familial aggregation as well as racial and ethnic differences in incidence also suggested that genetic components play a role in the development of DR. Identifying the genes that contribute to the disease pathogenesis has been a challenging task for geneticists worldwide due to the innate complexity of the disease in terms of the number of possible genes involved in the disease mechanism, unlike single gene disorders. Molecular genetics have taken several approaches to identify genes
contributing to the development of retinopathy, ranging from relatively simply designed analysis of specific candidate genes in case-control studies to systematic evaluations of the human genome using genome scans and linkage analysis in large collections of families.

**Linkage Analysis**

Gene mapping studies using linkage analysis are very difficult to perform in complex diseases like DR due to inherent barriers such as late-onset, non-availability of parents of the affected individuals, etc. However other family based approaches like sib-pair analysis have been performed to map the genes responsible for DR in a few studies. Imperatore et al., (1998) conducted a study on Pima Indians with T2DM, by performing linkage analysis on 103 sib-pairs. They found suggestive linkage on chromosomes 3 and 9 (LOD=1.36, 1.46 respectively). Recently a genome wide linkage analysis on 211 sibships has revealed an evidence of linkage on chromosome 1p yielding LOD scores of 3.1 and 2.58 by single and multipoint analyses respectively (Looker et al., 2007). A similar study on Mexican Americans with 282 sibpairs showed linkage to chromosomes 3 (LOD=2.41) and 12 (LOD=2.47) (Hallman et al., 2007). However these studies have only suggested critical genomic regions on certain chromosomes and the possible susceptibility genes are yet to be identified in these loci.

**Genetic Association Studies**

Researchers have long been concentrating on association studies. The simplicity of such a study design is intriguing. The prevalence of a sequence variant is simply compared between a case and a control group without need of collecting large pedigrees; only a large number of unrelated affected individuals are required. Although this approach is widely used in investigating candidate genes, the first generation of whole-genome association maps, which will be available shortly, will enable to move from candidate gene approach towards systematic screening of genomes for association of genetic markers (SNPs – single nucleotide polymorphisms) with inherited diseases (Anonymous et al., 2003; Carlson et al., 2004). Substantial amount of research has been done in identifying genetic markers
associated with risk for developing DR using indirect approach like case-control association studies in different ethnic populations. Most of the genes that have been screened for variations are implicated in the pathogenic pathways of DR such as polyol pathway, formation of AGEs, activation of PKC and hypoxia induced angiogenesis. However, only a fraction of them have shown consistent associations with occurrence of DR or its severity in different studies. Some of them have been inconsistent in various ethnic groups and different study populations. Discrepancies among these studies are likely due to variations in case definition, sample sizes, and medical conditions of control subjects.

Candidate genes and Diabetic Retinopathy

Over 20 candidate genes involved in different metabolic mechanisms and functional pathways have been reported to be associated with DR. The candidate genes which show major contribution to the development of DR are the following:

- Aldose Reductase (ALR2, now it is known as AKR1B1) gene
- Vascular Endothelial Growth Factor (VEGF) gene
- Receptor for Advanced Glycation End products (RAGE) gene
- Hemochromatosis HFE gene
- Endothelial Nitric Oxide synthase (eNOS) gene
- Glucose transporter (GLUT1) gene
- Paraxonase 1 (PON1) gene
- Angiotensin Converting enzyme (ACE) gene
- Plasminogen activator inhibitor 1 (PAI) gene and
- β3-Adrenoreceptor (β3-AR) gene.
Table 1 summarizes the polymorphisms of candidate genes identified to date associated with DR and Table 2 the loci for which the causative genes are yet to be identified.

<table>
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<th>Gene</th>
<th>Region</th>
<th>Polymorphism</th>
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<th>p-value or Odds ratio (95% CI)</th>
<th>Ethnicity</th>
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<td>Thomas et al., 2003 and Liao et al., 1999</td>
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<td>PON1</td>
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<td>PEDF</td>
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<td>19p13.3-p13.2</td>
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<td>C(-1214)G, G(-888)C</td>
<td>15pter-q21</td>
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NS – Not significant

Table 2  Chromosome loci associated with Diabetic Retinopathy
2.4. Genetic approach of complex diseases

Genetic complexity
All human traits and diseases that have a heritable component can roughly be divided into two major groups according to the genetic complexity underlying the trait or disease in question.

Monogenic
Monogenic traits and diseases follow the Mendelian patterns of dominant or recessive inheritance. Diseases with Mendelian inheritance in humans have traditionally been identified and studied by finding families with multiple affected members. By examining how the trait or disease is passed on through the generations in families the mode of inheritance can be determined. For a trait or disease to be defined as having Mendelian inheritance it must show either a dominant or recessive pattern in families. Current scientific information about diseases showing Mendelian inheritance is catalogued in the “Online Mendelian Inheritance of Man” (OMIM) database (www.ncbi.nlm.nih.gov/omim/). OMIM also catalogues genes that have been indicated to affect phenotypes and diseases with Mendelian inheritance. The first monogenic disease to have its causative gene identified was cystic fibrosis. The CFTR gene was identified using linkage-based analysis followed by positional cloning and the most common mutation causing cystic fibrosis (ΔF508) was identified (Kerem et al., 1989)

Polygenic
Polygenic traits are most often referred to as complex genetic traits in current literature. The name indicates that in contrast to monogenic traits they are influenced by more than one gene and do not display an obvious pattern of Mendelian inheritance in families. The majority of human traits and common diseases that have a heritable component have a complex genetic makeup (Wang et al., 2005).
2.4.2. Genetic analysis

To find the genetic components of any trait or disease two main methods of genetic analysis have been applied. They are called linkage and association analysis. Both have in common that they utilize known genetic markers like microsatellites or SNPs and share the common purpose to find one or more markers that are correlated to the genetic loci that makes up the heritable component of the trait of interest.

2.4.2.1. Linkage

The process of recombination is where DNA segments are exchanged between paired chromosomes during meiosis. The average number of recombination events is around 38 for females and 24 for males during meiosis (Cheung et al., 2007). Recombination is a key mechanism for generating genetic diversity and gives rise to genetic linkage that can be used to map loci linked to a trait or disease. The frequency of recombination between loci is related to the distance between them. Loci that are closer to each other are inherited together and are said to be in linkage with each other.

To perform linkage analysis is to measure how a known genetic marker is co-inherited with the trait or disease of interest in families. Traditionally in linkage analysis microsatellites have been used as genetic markers. Linkage analysis tracks the recombination in family materials to locate causative loci. The analysis is then limited by the number of meiosis available in the family material which depends on the size of the family material and the number of generations represented. This limitation can result in poor genetic resolution of the markers used finding linkage being detected between a marker and the locus of interest even if they are several mega bases (Mb) apart. Genetic linkage studies in family materials have been very successful in identifying genetic loci linked to traits and diseases with Mendelian inheritance (Jimenez-Sanchez et al., 2001). Using linkage analysis to identify genetic components of common diseases has not been nearly as successful (Hirschhorn et al., 2005)
2.4.2.2. Association

A population cohort sample can be described as a very large pedigree where the family information is unknown, but where it can be assumed that all share common ancestry going back far enough in time. This means that in a pedigree of unknown structure there has been thousands of recombination events that have taken place since the beginning of the common ancestry. In order for this assumption to be valid it is important to ensure that the ethnicity and geographic origin of the samples are matched as far as possible. If not the problem of population stratification arises where population subgroups are present in sample which can cause differences in marker allele frequencies that in turn can result in false positive findings of association (Cardon et al., 2003). Association analysis examines the end result of all the recombination events in the population which provides higher genetic resolution compared to traditional linkage analysis that is limited by the number of generations available in a family material with a known pedigree. The classical set up for studying association is a case-control study using SNP markers. It compares the allele frequencies in a group of “cases” that have a disease of interest for example type 2 diabetes with retinopathy examined in this thesis. This group is then compared to a group of control samples from the same population that does not have the disease type 2 diabetes with no retinopathy. Recent technological advancements and efforts such as the HapMap project have now made genome-wide association studies a reality (Hirschhorn et al., 2005).

Single Nucleotide polymorphisms (SNPS)

Among known types of sequence variation found in the human genome the SNP is the most frequently found. A SNP is most commonly defined as a position in the genome were a single nucleotide has been substituted for another and that this change can be seen at least in 1% of a chosen population. The two different nucleotides at the SNP position are referred to as its two alleles. SNPs can be found throughout the entire genome and to date 11.8 million SNPs have been registered in the dbSNP database (http://ncbi.nih.gov/SNP/, Build 127, September 18, 2007) which is the largest public database for SNPs. SNPs are useful for
finding genes that contribute to disease, in two ways. Some SNP alleles are the actual DNA sequence variants that cause differences in gene function or regulation that directly contribute to disease processes. Most SNP alleles, however, probably contribute little to disease. They are useful as genetic markers that can be used to find the functional SNPs because of associations between the marker SNPs and the functional SNPs. SNPs of various types can change the function or the regulation and expression of a protein. The most obvious type is a nonsynonymous SNP, where the alleles differ in the amino acid of the protein product. Some SNPs are in promoter regions and are reported to affect the regulation and expression of proteins. When SNPs are associated with other SNPs because of linkage disequilibrium, then many SNPs, in introns, exons and other noncoding regions, may all be associated with a disease or phenotype. Because of their abundance and presence throughout the genome, SNPs are well suited for use as biallelic markers in genetic studies. SNPs have been applied to genetic studies ranging in scope from analyzing variants of a single gene to performing genome-wide analyses to investigate the genetics of complex traits and diseases.

**Chi-square test**

A non parametric test of statistical significance appropriate when the data are in the form of frequency counts, it compares frequencies actually observed in a study with expected frequencies to see if they are significantly different.

\[
\text{Chi-square} = \sum \frac{(O-E)^2}{E}
\]

Where

- Chi-square = the test statistic that asymptotically approaches a chi-square Distribution
- \(O\) = an observed frequency
- \(E\) = an expected frequency, asserted by the null hypothesis