1.0 Introduction

Diabetes is a group of complex metabolic disorders principally characterized by insulin resistance, pancreatic beta cell dysfunction, and associated hyperglycemia. This disease is increasing to epidemic proportions in many countries throughout the world (Tripathi and Srivastava, 2006). In fact, it was estimated that 171 million people worldwide had diabetes in 2000, and this number is projected to increase to 366 million by 2030 (Wild et al. 2004). In India, currently, there are 40 million people with diabetes. By 2025 this number is estimated to rise to 70 million. This means that every fifth diabetic in the world would be an Indian (Zhaolan et al., 2010).

1.1 Prevalence of Diabetes

It has been estimated that the global burden of type 2 diabetes mellitus (T2DM) for 2010 would be 285 million people (2010) which is projected to increase to 438 million in 2030 as shown in figure 1 (Ramachnadaran, 2009). Similarly, for India this increase is estimated to be 58%, from 51 million people in 2010 to 87 million in 2030 (Snehalatha and Ramachnadaran, 2009). The impacts of T2DM are considerable: as a lifelong disease, it increases morbidity and mortality and decreases the quality of life. (Hoskote and Joshi, 2008). At the same time, the disease and its complications cause a heavy economic burden for diabetic patients themselves, their families and society. A better understanding about the cause of a predisposition of Indians to get T2DM is necessary for future planning of healthcare, policy and delivery in order to ensure that the burdens of disease are addressed (Hoskote and Joshi, 2008).
1.2 Classification of diabetes:

There was several classification systems established for diabetes mellitus by the WHO Expert Committee on Diabetes (1980, 1985). The current WHO classification system has been established in co-operation with the National Diabetes Data Group (USA). It is mainly based on the etiology of diabetes mellitus. (Catchpole B. et al., 2008)

I. Type 1 diabetes

In type 1 diabetes, beta cell destruction, usually leading to absolute insulin deficiency.

A. Immune mediated
B. Idiopathic

II. Type 2 diabetes

It may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance.

III. Other specific types

A. Genetic defects of beta cell function
B. Genetic defects in insulin action
C. Diseases of the exocrine pancreas
D. Endocrinopathies

Figure 1: Estimated number of diabetic subjects in India.
(Source: Ramachnadaran, 2009)
E. Drug induced or chemical induced
F. Infections
G. Uncommon forms of immune-mediated diabetes

IV. Gestational diabetes

1.3 Criteria for the diagnosis of diabetes

1. A1C > 6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.

   OR

2. FPG > 126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.

   OR

3. 2-h plasma glucose > 200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

   OR

4. In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose > 200 mg/dl (11.1 mmol/l).

1.4 Diabetic complications

All forms of diabetes are characterized by chronic hyperglycaemia and the development of diabetes-specific microvascular pathology in the retina, renal glomerulus and peripheral nerve. As a consequence of its microvascular pathology, diabetes is a leading cause of blindness, end stage renal disease and a variety of debilitating neuropathies. Diabetes is also associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply the heart, brain and lower extremities. As a result, patients with diabetes have a much higher risk of myocardial infarction, stroke and limb amputation. Large prospective clinical studies show a strong relationship between glycaemia and diabetic microvascular complications in both type 1 and type 2 diabetes. Hyperglycaemia and insulin resistance both seem to have important roles in the pathogenesis of macrovascular complications (Ebara et al., 2000; Ginsberg, 2000).
1.4.1 Macrovascular complications

Macrovascular complications include coronary artery disease (CAD), Peripheral vascular disease (PVD), and cerebrovascular events (CVA). Diabetes mellitus is an independent risk factor for the development of atherosclerosis. On the other hand, atherosclerotic or macrovascular disease is responsible for more than 50% of all deaths in patients with T2DM. Cardiovascular disease accounts for most cases of diabetic macrovascular complications and the remaining are caused by cerebrovascular events and peripheral vascular disease (Leung et al., 2000). In India, escalating population levels of major coronary risk factors have contributed to the coronary heart disease epidemic.

Mortality among diabetic patients with CAD is higher than non-diabetic subjects. Studies have also shown that myocardial infarction in diabetics was more common than non-diabetics (Haffner et al., 1998). Indian seems to be more predisposed to both diabetes and CAD. Hypertension is the primary preventable cause of the two major causes of mortality: coronary artery disease (CAD), and cerebrovascular disease (CVD). It increases the risk for CAD by two fold, CVD by seven folds and congestive heart failure by four fold. There is ample evidence for a consistent gradient relationship of blood pressure with CVD and CAD. Studies have also shown that an increase of blood pressure of 5 mm Hg is associated with a 34% increase in risk for CVD and a 21% excess risk for CA (Kannel et al., 2003).

1.4.2 Microvascular complications

Microvascular complications include retinopathy, nephropathy, and neuropathy. Diabetic retinopathy (DR) can be defined as damage to microvascular system in the retina due to prolonged hyperglycaemia. Diabetic retinopathy is primarily classified into non proliferative diabetic retinopathy (NPDR), or background retinopathy, and proliferative diabetic retinopathy (PDR). Progression from mild form characterized by increased vascular permeability, to moderate, and then to severe NPDR characterized by vascular closure and an increased risk for the development of PDR distinguished by the growth of new blood
vessels on the retina and posterior surface of the vitreous. Visual impairment in diabetic retinopathy occurs due to diabetic macular edema (DME) and PDR (Rema et al., 2007). Diabetic neuropathies are a family of nerve disorders caused by diabetes. It can be classified as peripheral, autonomic, proximal, and focal. Each affects different parts of the body in different ways. Diabetic foot ulcers may develop, mainly because of the abnormal distribution of pressure. The early detection of diabetic neuropathy results in less hospitalization of patients with foot ulcers and fewer lower-extremity amputations (Leung et al., 2000). Screening for neuropathy can be done reliably by using the 10-g Semmes-Weinstein monofilament over areas of the feet, ankle reflexes and vibration perception over the great toe and ankle. A standard neuropathy disability score (NDS) will be measured and a score of over 6 shows the presence of significant neuropathy is present. One of the most common complications of diabetes in the lower extremity is the diabetic foot ulcer which is often ignored.

Diabetic nephropathy is the most common cause of microvascular chronic complication of type 2 diabetes mellitus which is associated with considerable morbidity and mortality, finally leading to end-stage renal disease (Raine, 1993). Diabetic nephropathy is a progressive disease that takes several years to develop. Glomerular hyperfiltration and increased excretion of urinary albumin (microalbuminuria) are early manifestations of diabetic nephropathy. It also involves various functional clinical abnormalities of the kidney such as elevated creatinine, urea, albuminuria, decline glomerular filtration rate, elevated arterial blood pressure, and fluid retention (Wolf et al., 2007; Balakumar et al., 2009).

1.5 Prevalence of Diabetic Nephropathy

Racial differences in the prevalence of diabetic renal disease have been reported. Asian subjects have significantly (p<0.01) higher prevalence (52.6%) of diabetic end stage renal disease (ESRD) when compared with the Caucasians (36.2%) migrant Asian (Young et al., 2003). Indians have 40 times greater risk of developing ESRD compared with the Caucasians (Chandie et al., 2002).
1.6 Screening for diabetic nephropathy and monitoring kidney function

Detection of diabetic nephropathy as early as possible in the disease process currently offers the best chance of delaying or possibly preventing progression to end-stage disease. Therefore, screening for microalbuminuria and proteinuria in a structured, regular manner is recommended. Most guidelines suggest annual screening, ideally using an early morning urine sample to avoid variable effects of upright posture on albumin excretion. A quantitative, laboratory-based, sensitive assay, specific for albumin, is preferable. The albumin:creatinine ratio should be calculated; albumin concentration on its own is unreliable. If the ratio exceeds the upper limit for microalbuminuria, a less sensitive, conventional assay for total protein should be performed (Table 1).

Table 1: Diagnostic criteria used in diabetic nephropathy

<table>
<thead>
<tr>
<th>Albumin creatinine ratio (mg/mmol)</th>
<th>Normal</th>
<th>Microalbuminuria</th>
<th>Proteinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>&lt;2.5</td>
<td>2.5-30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>Women</td>
<td>&lt;3.5</td>
<td>3.5-30</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Albumin excretion rate overnight (ug/min)</th>
<th>Normal</th>
<th>Microalbuminuria</th>
<th>Proteinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>&lt;20</td>
<td>20-200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Women</td>
<td>&lt;30</td>
<td>30-300</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

(Vrhovac et al., 2008)

1.7 Stages of Diabetic Nephropathy

Diabetic nephropathy is a chronic complication of both type 1 DM (beta cell destruction absolute lack of insulin) and type 2 DM (insulin resistance and/or decreased secretion of insulin) (Vrhovac et al., 2008). There are five stages in the development of diabetic nephropathy as shown in table 2.

Stage I: Hypertrophic hyperfiltration. In this stage, GFR is either normal or increased. Stage I lasts approximately five years from the onset of the disease. The size of the kidneys is
increased by approximately 20% and renal plasma flow is increased by 10%-15%, while albuminurias and blood pressure remain within the normal range.

**Stage II**: The quiet stage. This stage starts approximately two years after the onset of the disease and is characterized by kidney damage with basement membrane thickening and mesangial proliferation. There are still no clinical signs of the disease. GFR returns to normal values. Many patients remain in this stage until the end of their life.

**Stage III**: The microalbuminuria stage (albumin 30-300 mg/dU) or initial nephropathy. This is the first clinically detectable sign of glomerular damage. It usually occurs five to ten years after the onset of the disease. Blood pressure may be increased or normal. Approximately 40% of patients reach this stage.

**Stage IV**: Chronic kidney failure (CKF) is the irreversible stage. Proteinuria develops (albumin > 300 mg/dU), GFR decreases below 60 mL/min/1.73 m², and blood pressure increases above normal values.

**Stage V**: Terminal kidney failure (TKF) (GFR < 15 mL/min/1.73 m²). Approximately 50% of the patients with TKF require kidney replacement therapy (peritoneal dialysis, hemodialysis, kidney transplantation) (Mogensen, 1999).

**Table 2: Diabetic nephropathy stages based on urinary albumin excretion**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Urine with marked time (ug/min)*</th>
<th>24-hour urine (mg/24h)*</th>
<th>Random urine sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Albumin concentration (mg/l)**</td>
</tr>
<tr>
<td>Normoalbuminuria</td>
<td>&lt;20</td>
<td>&lt;30</td>
<td>&lt;17</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>20-199</td>
<td>30-299</td>
<td>17-173</td>
</tr>
<tr>
<td>Macroalbuminuria</td>
<td>≥200</td>
<td>≥300</td>
<td>≥174</td>
</tr>
</tbody>
</table>

* Values according to the American Diabetes Association
** Gross et al., Diabetes Care 2005.
1.8 Risk Factors

1.8.1 Hyperglycemia

Hyperglycemia is a significant risk factor for the development of microalbuminuria, both in type 1 and in type 2 DM (Gall et al., 1997; Ravid et al., 1998). A reduction of 1% in HbA1c is associated with a 37% decrease in microvascular endpoints (Stratton et al., 2000). In the presence of micro- and macroalbuminuria the role of metabolic control is less defined, even though some studies showed a deleterious effect of high glucose levels on GFR (Hovind et al., 2003). Moreover, it was demonstrated that pancreas transplantation reversed renal damage in type 1 DM patients with mild to advanced DN lesions. Recently a large trial also reinforced the importance of intensive treatment of DM to decrease the microvascular complications (Patel et al., 2008).

1.8.2 Arterial Hypertension

Arterial hypertension is a main risk factor for the development of DN (Park et al., 1998), and probably the best known relevant factor related to its progression. Analysis of UKPDS showed that every 10 mmHg reduction in systolic BP is associated with a 13% reduction in the risk of microvascular complications, with the smallest risk among those patients with systolic BP <120 mm Hg (Alder et al., 2000).

1.8.3 Smoking

Smoking is a risk factor for DN and might contribute to its progression (Sawicki et al., 1994). It is strongly recommended to quit smoking in any phase of DN, also aiming to reduce the associated cardiovascular and cancer risk.

1.8.4 Dyslipidemia

In type 2 DM, elevated serum cholesterol is a risk factor for the development of DN. In type 1 DM patients increased serum triglycerides, total and LDL-cholesterol were associated with micro- and macroalbuminuria (Chaturvedi et al., 2001; Jenkins et al., 20030. High serum cholesterol also seems to be a risk factor for GFR loss in macroalbuminuric type 1 diabetic subjects.
1.8.5 Proteinuria

Proteinuria itself could lead to progression of DN. Proteinuria >2 g/24 h is associated with a greater risk of ESRD (Ruggenenti et al., 1998). Increased leakage of albumin may induce glomerular damage probably through activation of inflammatory cascades. This would be a reason to target decreased urinary albumin excretion in DN treatment.

1.8.6 Glomerular hyperfiltration

Elevated GFR values are present in about one third of type 2 DM patients and theoretically it could cause DN due to glomerular damage. Studies led to controversial findings regarding its role as a risk factor for the development of DN (Dahlquis et al., 2001). Type 2 DM patients with a single-kidney more often present increased UAE levels (Ficociello et al., 2009). On the other hand, type 1 DM patients with only one kidney do not have a more aggressive disease (Chang et al., 2008). Glomerular hyperfiltration probably plays a small role, if any, in the development of DN.

1.8.7 Dietary factors

Increased dietary protein intake seems to be associated with the presence of higher UAE values, at least in patients with type 1 DM. In patients with type 2 DM this association has not been documented. The source of proteins in the diet also seems to be related to the presence of DN. A higher intake of fish protein is related to a lower risk of microalbuminuria in type 1 DM patients (Mollsten et al., 2001). The mechanisms involved in these findings are unknown but probably related to hemodynamic factors. Regarding the dietary lipid content, an association has been observed between the higher intake of saturated fat and the presence of microalbuminuria in patients with type 1 DM. In patients with type 2 DM, very recently, it was observed that the presence of microalbuminuria was associated with the lower content of polyunsaturated fatty acids, especially those of vegetable origin (Almeida et al., 2008). In a study performed with patients with type 1 and type 2 DM, followed for 6 years, it was also demonstrated that those who evolved with regression of the DN presented a higher intake of polyunsaturated fatty acids and a lower intake of saturated fatty acids (Cardenas et al., 2004).
1.9 The pathogenesis of Diabetic Nephropathy

1.9.1 Hemodynamic Pathways

The early signs of glomerular hyperperfusion and hyperfiltration result from decreased resistance in both the afferent and efferent arterioles of the glomerulus. The afferent arteriole seems to have a greater decrease in resistance than the efferent. Many factors have been reported to be involved in this defective autoregulation, including prostanoids, nitric oxide, vascular endothelial growth factor (VEGF; now formally known as VEGF-A), TGF-β1, and the renin–angiotensin system, specifically angiotensin II. These early hemodynamic changes facilitate albumin leakage from the glomerular capillaries and overproduction of mesangial cell matrix, as well as thickening of the glomerular basement membrane and injury to podocytes (Ziyadeh and Wolf, 2008). In addition, increased mechanical strain resulting from these hemodynamic changes can induce localized release of certain cytokines and growth factors (Wolf and Ziyadeh, 2007). The renal hemodynamic changes are mediated partly by the actions of vasoactive hormones, such as angiotensin II and endothelin. Glomerular hypertension and hyperfiltration contribute to the development of diabetic nephropathy because use of renin–angiotensin blockers preserves kidney function and morphology. Blockade of the renin–angiotensin–aldosterone system antagonizes the profibrotic effects of angiotensin II by reducing its stimulation of TGF-β1 (Hilgers and Veelken, 2005). Support that such profibrotic effects underlie diabetic nephropathy has also been provided by study of an animal model of diabetic nephropathy (Nagai et al., 2005). Transient blockade of the renin–angiotensin system (for 7 weeks) in prediabetic rats reduced proteinuria and improved glomerular structure. Additionally, the administration of an angiotensin-converting-enzyme inhibitor to patients with type 1 diabetes and nephropathy lowered serum concentrations of TGF-β1 (Sharma et al., 1999). A correlation exists between decreased levels of TGF-β1 in serum and urine and renoprotection, as determined by changes in the glomerular filtration rate over time.
Introduction and review of literature

Figure 2: Metabolic and hemodynamic factors contributing to the initiation of DN.

(TGF-β: transforming growth factor-β; AGE: advanced glycation end-products; ROS: reactive oxygen species; PKC: protein kinase-c; Glut1: glucose transporter-1. NO: nitric oxide; Ang II: angiotensin II; ECM: extracellular matrix) (Fornoni et al., 2008).

1.9.2 Metabolic factors

1.9.2.1 Hyperglycemia and advanced glycosylation end products

Hyperglycemia is a crucial factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells, but alone it is not causative as shown in figure 2. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity. Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening. In vitro studies have demonstrated that hyperglycemia is associated with increased mesangial cell matrix production and mesangial cell apoptosis (Mishra et al., 2005; Lin et al., 2006). Mesangial cell expansion seems to be mediated in part by an increase in the mesangial cell glucose
concentration, since similar changes in mesangial function can be induced in a normal glucose milieu by overexpression of glucose transporters, such as GLUT1 and GLUT4, thereby increasing glucose entry into the cells. Hyperglycemia might also upregulate VEGF expression in podocytes, which could markedly increases vascular permeability. Hyperglycemia, however, does not account fully for the risk of diabetic nephropathy, as shown by studies in which kidneys from non-diabetic donors were transplanted into patients with diabetes and nephropathy developed irrespective of the glucose control. Hyperglycemia might, therefore, be necessary for but not sufficient to cause renal damage.

Three mechanisms have been postulated that explain how hyperglycemia causes tissue damage: non-enzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway. Oxidative stress seems to be a theme common to all three pathways (Chen et al., 2007; Wolf et al., 2005).

Chronic hyperglycemia leads to accumulation of advanced glycation end products that covalently trapped extravasated serum proteins such as immunoglobulins, albumins and LDL through glucose derived cross linking to the extravascular matrix (Kalia et al., 2004; Mistry K et al., 2009). Circulating levels of advanced glycosylation end products are raised in people with diabetes and diabetic nephropathy, particularly those with renal insufficiency, since they are normally excreted in the urine (Makita et al., 1991; Mistry et al., 2009). The net effect is tissue accumulation of advanced glycosylation end products (in part by cross-linking with collagen) that contributes to the associated renal and microvascular complications (Singh et al., 1998). Moreover, advanced glycosylation end products (AGE) interact with the AGE receptor, and nitric oxide concentrations are reduced in a dose-dependent manner (Hogan et al., 1992).

1.9.2.2 Protein kinase C

Other proposed mechanisms by which hyperglycemia promotes the development of diabetic nephropathy include activation of PKC. Specifically, activation of this enzyme leads to increased secretion of vasodilatory prostanoids, which contributes to glomerular
hyperfiltration. By activation of TGF-β1, PKC might also increase production of extracellular matrix by mesangial cells (Yamagishi et al., 2007). The mechanism by which hyperglycemia leads to PKC activation involves de novo formation of diacylglycerol and oxidative stress (Kunisaki et al., 1994). PKC activation induces the activity of mitogen-activated protein kinases (MAPK) in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. The coactivation of PKC and MAPK in the presence of high glucose concentrations indicates that these two families of enzymes are linked.

1.9.2.3 Aldose reductase pathway
The polyol pathway is implicated in the pathogenesis of diabetic nephropathy. A number of studies have shown a decrease in urinary albumin excretion in animals administered aldose reductase inhibitors (Haneda et al., 1995) but in humans these agents have not been studied widely and the results are inconclusive.

1.10 Role of Oxidative Stress
Chronic hyperglycemia leads to non-enzymatic glycosylation (glycation) of proteins, acceleration of glucose metabolism in polyol pathway, increased production of diacylglycerol (DAG) and protein kinase C (PKC) activation in parallel with increased oxidative stress (Abdelraze et al., 2001; Bartosz et al., 1995). Oxidative stress is extremely important in the pathogenesis of multiple diseases, including DM-2. The oxidative stress is associated with increased generation of reactive oxygen species and the decreased antioxidant defense systems. It is attributed a relevant role in the chronic DM-2 complications (Cai et al., 2001; Opara et al., 2002; Tilton et al., 2002).
Figure 3: Hyperglycemia is the main sign of type 1 and type 2 diabetes (Opara et al., 2002).

In turn, hyperglycemia results in a myriad of metabolic and hemodynamic changes that are intimately associated with vascular complications of diabetes including diabetic nephropathy.

Under normal physiological conditions, there is a balance in the generation of oxygen-free radicals and the antioxidant defense mechanisms used to deactivate free radical toxicity. Impairment in the oxidant/antioxidant equilibrium results in oxidative stress in numerous
pathological conditions including diabetes leading to cellular damage (Anzalone et al., 2009; Zheng et al., 2009; Xiao et al., 2009). Increasing evidence in both experimental and clinical studies suggests that there is a close link between hyperglycemia, oxidative stress, and diabetic complications (Brown, 2008; Wu et al., 2009). Increased oxidative stress in diabetes likely contributes to the pathogenesis of diabetic nephropathy and its progression to end-stage renal disease (Szabo, 2009; Kanwar et al., 2008). Enhanced reactive oxygen species (ROS) production in experimental and clinical diabetes have been linked to vasoconstriction, vascular smooth muscle cell growth and migration, endothelial dysfunction, modification of extracellular matrix (ECM) proteins, and increased renal sodium reabsorption (Asaba et al., 2005; Son et al., 2004; Satoh et al., 2004). The importance of oxidative stress in diabetic nephropathy is underscored by the finding that inhibition of oxidative stress ameliorates the manifestations associated with streptozotocin-induced diabetic nephropathy (Thallas et al., 2008). Streptozotocin selectively targets and kills the beta cells of the pancreas resulting in an experimental model of type 1 diabetes mellitus.

There are huge amount of in vitro and in vivo studies regarding explanation of mechanism of diabetes-mellitus induced nephropathy. All of these mechanisms are a consequence of uncontrolled elevation of blood glucose level. Currently the proposed mechanism is the glomerular hyperfiltration/hypertension hypothesis. According to this hypothesis, diabetes leads to increased glomerular hyperfiltration and a resultant increased glomerular pressure. This increased glomerular pressure leads to damage to glomerular cells and to development of focal and segmental glomerulosclerosis (Anderson et al., 1988). Angiotensin II inhibitors reduce glomerular pressure and prevent albuminuria. Increased angiotensin II level induces OS through activation of NADPH oxidase, stimulating inflammatory cytokines, and so forth (Garrido et al., 2009; Mehta et al., 2007).
The mechanism by which hyperglycemia causes free radical generation thus causes OS to be complex. Increased blood glucose promotes glycosylation of circulator and cellular protein and may initiate a series of autooxidation reactions that culminate in the formation and accumulation of advanced glycosylation end-products (AGEs) in tissues. The AGEs have oxidizing potential and promote tissue damage by oxygen-free radicals (Mansouri et al., 2011).

Figure 4: Oxidative stress is the main driving force for pathogenesis of diabetic nephropathy (Ahmed et al., 2010)
In experimental studies, formation of OS increases because of high level of blood glucose. Sadi et al. showed that in diabetic rat kidney antioxidant enzyme, namely, catalase (CAT) and glutathion peroxidase (GSHPx), activities were found to be reduced; however, α-lipoic acid and vitamin C administration increased these antioxidant enzyme activities. Increased OS is the common finding in tissues effecting from diabetes, including kidney. Reddi et al. showed that transforming growth factor β1 (TGF-β) is pro oxidant and Se (selenium) deficiency increases OS via this growth factor (Reddi et al., 2001). Chen et al. showed that nitrosative stress increases in diabetic rat model (Chen et al., 2008). These results show the induction of oxidative and nitrosative stress in rat kidney. These may have a role in pathophysiology of diabetes-induced morphological and functional changes of kidney.

ROS include free radicals such as superoxide, hydroxyl and peroxyl, and non radical species such as hydrogen peroxide. It is important to note that there is also a reactive nitrogen species produced from similar pathways, which include the radicals nitric oxide and nitrogen dioxide, as well as the non radical peroxynitrite. There are a number of enzymatic and nonenzymatic sources of ROS in the diabetic kidney, including autoxidation of glucose, advanced glycation, polyol pathway flux, mitochondrial respiratory chain deficiencies, xanthine oxidase activity, peroxidases and NAD(P)H oxidase. Importantly, a direct relationship has been demonstrated between the severity of renal injury and the degree of oxidative stress in DN. Moreover, histological studies have shown the presence of glyco- and lipo-oxidation products in the mesangial matrix and nodular lesions of DN (Vasavada and Agarwal, 2005; Forbes et al., 2008).

Growing evidence indicates that metabolic abnormalities in diabetes lead to mitochondrial superoxide production, which results in the activation of major biochemical harmful pathways (figure 4), including increased AGE formation, activation of protein kinase C, increased flux through the polyol pathway, and overactivity of the hexosamine pathway, each of which, in addition, can initiate and/or perpetuate cellular ROS generation (Vasavada and Agarwal, 2005; Forbes et al., 2008).
The ability of individual cell types to process glucose is the most important factor in the excessive intracellular generation of ROS induced by hyperglycemia. Thus, the control of glucose influx into the cytosol in presence of elevated glucose concentrations is critical in order to maintain an adequate intracellular glucose homeostasis. However, certain renal cell populations, such as endothelial, mesangial, epithelial and tubular cells, are particularly susceptible since they are unable to decrease glucose transport rates adequately. Therefore, intensive glycemic control and interventions that decrease cellular glucose uptake may limit ROS generation in the diabetic kidney (Forbes et al., 2008).

In addition to approaches aimed to reduce ROS production, a critical factor to avoid oxidative damage is the adequate function of endogenous antioxidant systems, including free radical scavengers and enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. A reduction in expression and activity of these antioxidant enzymes have been reported in diabetic micro vascular disease. Importantly, overexpression of SOD or catalase protects against end organ damage in models of DN (Forbes et al., 2008). Finally, the therapeutic use of antioxidants might be a useful approach. However, conventional antioxidants are unlikely to be effective because these compounds neutralize reactive oxygen molecules on a one-for-one basis, whereas hyperglycemia-induced overproduction of superoxide is a continuous process.

1.11 Interaction between oxidative stress and inflammation

There is a close association between oxidative stress and inflammation in diabetes and we hypothesize that an increase in oxidative stress-derived inflammation is a major mechanism in the pathogenesis and progression of diabetic nephropathy. In addition, an increase in inflammatory cytokine levels in diabetes may drive a further increase in oxidative stress as renal injury becomes more pronounced setting up a vicious cycle. However, due to the complex and intimate association between increased oxidative stress and increased inflammation, dissecting the temporal nature of the relationship is a very difficult task. A better understanding of the relationship between oxidative stress and
Inflammatory cytokines in the progression of diabetic nephropathy will facilitate the development of new treatment options and improve current therapeutic strategies (Navarro et al., 2012).

Inflammatory markers, the major mediator of the acute-phase response, along with antioxidant enzyme levels are decreased in the plasma of diabetic nephropathy patients due to hyperglycemia induced oxidative stress in type 2 diabetes mellitus. Different factors prevalent in patients with diabetic nephropathy (ESRD) such as oxidative stress, hypertension, adiposity, insulin resistance, fluid overload and persistent infections which might be associated with elevated inflammatory markers. In addition, reduced renal function, directly or indirectly, seems to be closely related to genetic expressions of various cytokines can cause endothelial dysfunction via oxidative stress might be a probable cause of diabetic nephropathy in type 2 diabetic subjects. The renal risk score for diabetic nephropathy emphasizes the importance of the identification of levels of inflammatory markers, antioxidant levels, albuminuria, hypoalbuminemia as well as increased serum creatinine, and decreased hemoglobin levels to predict the development of ESRD in patients with type 2 diabetes mellitus (Williams et al., 2007).

1.12 Inflammation

In recent years, our knowledge of the pathophysiological processes that lead to diabetic nephropathy has notably improved on a genetic and molecular level. Thus, the classic view of metabolic and hemodynamic alterations as the main causes of renal injury in diabetes has been transformed significantly, with clear evidence indicating that these traditional factors are only a partial aspect of a much more complex picture. One of the most important changes is related to the participation of immune-mediated inflammatory processes in the pathophysiology of diabetes mellitus and its complications (figure 5) (Williams et al., 2007; Navarro et al. 2005).
Although diabetic nephropathy is traditionally considered a nonimmune disease, accumulating evidence now indicates that immunologic and inflammatory mechanisms play a significant role in its development and progression (Tuttle et al., 2005; Mora et al., 2006). Therefore, diverse cells, including leukocytes, monocytes, and macrophages (Galkina et al., 2006; Chow at al., 2004), as well as other molecules, such as chemokines (monocyte chemoattractant protein-1) (Chow at el., 2006), adhesion molecules (intercellular adhesion molecule-1 (ICAM1)), (Okada et al., 2003; Chow at al., 2005) enzymes (cyclooxygenase-2, nitric oxide synthase) (Cheng et al., 2002; Quaggin et al., 2007), growth factors (vascular endothelial growth factor, growth hormone, IGF, TGF-β) (Nakagawa, 2007; Pantsulaia, 2006) and nuclear factors (NF-κ B) (Mezzano et al., 2004; Schmid et al., 2006), are implicated in processes related to diabetic nephropathy. Less is known, however, about the role of inflammatory cytokines in diabetic renal injury.
Figure 5: Schematic overview of the participation of inflammatory mechanisms in the pathophysiology of diabetic nephropathy. MCP: Monocyte chemoattractant protein; CSF: Colony-stimulating factor; ICAM: Intercellular adhesion molecule; VCAM: Vascular cell adhesion molecule. TGF: Transforming growth factor; CTGF: Connective tissue growth factor; VEGF: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor alpha (Navarro et al., 2012).
1.12.1 Inflammatory cytokines in diabetes mellitus

Cytokines are a group of pharmacologically active, low molecular weight polypeptides that possess autocrine, paracrine, and juxtacrine effects with characteristic features (Coppack, 2001). These molecules cluster into several classes (i.e., interleukins, tumor necrosis factors, interferons, colony-stimulating factors, transforming growth factors and chemokines), which are relevant humoral mediators in a highly complex, coordinated network regulating inflammatory immune responses with the participation of different cytokine-associated signaling pathways. In addition, they exert important pleiotropic actions as cardinal effectors of injury (Aldhahi et al., 2003). Cytokines are produced by a wide variety of cells in the body, playing an important role in many physiological responses that have a therapeutic potential.

At the present time it is recognized that chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of diabetes mellitus (Crook, 2004; Pickup, 2004). Increasing evidence suggests that individuals who progress to diabetes mellitus display features of inflammation years before the disease onset (Dandona et al., 2004). Moreover, population-based studies suggest that inflammatory parameters, including inflammatory cytokines, are strong predictors of the development of diabetes (Spranger et al., 2005). The main cytokines involved in the pathogenesis of diabetes are IL-1, TNF-α, and IL 6 (Alexandraki et al., 2006). In addition, studies in recent years have shown that inflammation, and more specifically inflammatory cytokines, are determinant in the development of microvascular diabetic complications, including neuropathy, retinopathy, and nephropathy (Mocan et al., 2006).

1.12.2 Inflammatory cytokines in Diabetic Nephropathy

When considering the role of cytokines in pathophysiological processes underlying disease, it is necessary to take into account the fact that the activities of these molecules are very complex, as reflected by important features, including their pleiotropic actions—and thus a
cytokine may trigger several different cellular responses depending on diverse factors, such as cell type, timing, and context. Cytokines share receptor subunits; act synergistically in many contexts, and, therefore, the association of two cytokines can markedly amplify their effects; cytokines stimulate the cells that produce them, or adjacent cells, or even can intervene through direct cell–cell interaction; and, finally, cytokines induce the expression of other cytokines and cytokine receptors (Vilcek, 2003). In light of the data obtained from experimental and clinical studies, cytokines are often classified according their pro- or anti-inflammatory activities.

In 1991, Hasegawa et al. reported that glomerular basement membranes from diabetic rats induced significantly greater amounts of TNF-α and IL-1 in cultured peritoneal macrophages than when these cells were incubated with basement membranes from non-diabetic rats. These new findings were the first to suggest that inflammatory cytokines may participate in the pathogenesis of diabetic nephropathy (Hasegawa et al., 1991; Hasegawa et al., 1995). Today, it is known that among inflammatory cytokines, IL-1, IL-6, IL-18 and TNF-α are relevant to the development of diabetic nephropathy, with diverse actions potentially involved in the development of complications (Mocan et al., 2006).

1.13 Predictors of Diabetic Nephropathy

There are various factors which play a role in the development of nephropathy in diabetes. The most well-known amongst them include poor glycemic control, family history of diabetes or hypertension, increased sodium-lithium counter-transport activity in RBCs etc. Previously, it was believed that once albuminuria had become persistent, glycemic control lost its beneficial effect on kidney, but several recent studies documented the importance of glycemic control on progression of nephropathy in patients with Type 1 diabetes. On the other hand, most recent studies have failed to demonstrate any significant impact of glycemic control on progression of nephropathy in Type 2 diabetes. Among the most important putative promoters of progression in kidney disease, blood pressure has been documented to have a close relation with rate of decline of glomerular filtration rate in
both Type 1 and Type 2 diabetes. Serum cholesterol concentration has been shown to be another predictor of progression of nephropathy in both types of diabetes. Dietary protein restriction retards the progression of renal disease in both types of diabetes while smoking has been suggested as a determinant for progression of nephropathy in either types of diabetes. The fact that, a fairly large number of diabetics goes on to develop nephropathy even in the absence of the above-mentioned factors, led scientists to postulate and investigate about genetic factors leading to this dreadful complication (Kalara, 2007).

1.14 Genetic aspects of Diabetic Nephropathy

The etiology of DN is multifactorial, yet clearly has an inherent genetic basis. Evidence of an important genetic component to DN has stimulated extensive efforts to decipher the genetic architecture of disease in multiple populations. In view of the complexity involved, it is not surprising that despite investment of significant resources, there has been limited success in identifying genetic variants that modify the risk of developing diabetic nephropathy. Two strategies have been commonly used to identify DN susceptibility loci: the linkage analysis (i.e. family-based studies) or the association analysis (i.e. case-control studies). Both have led to the discovery of many chromosomal and gene regions that may confer susceptibility to DN (Imperatore et al., 2000). The observation of familial clustering of the disease strongly suggests that genetic factors are involved in the development of DN, whereas segregation analyses point to the existence of susceptibility genes and have established that the onset and progression of DN are influenced genetically (Boright et al., 2005; Jacobsen et al., 2005).

The candidate gene approach involves assessment of genetic variation, typically single nucleotide polymorphisms (SNPs), in one or more genes with plausible physiological roles in DN. The goal is to demonstrate a significant difference in allele frequencies between cases with DN and control subjects (Tabor et al., 2002). In some studies, controls had longstanding diabetes without evidence of nephropathy, while in others, controls lacked diabetes and nephropathy.
The search for genes associated with DN relies on continued innovation. While progress is being made with recent technological advances, genetic variants have not been identified that unambiguously define DN genes. Thus, DN appears typical of common complex diseases. Major research questions remain regarding the underlying genetic architecture of DN. It remains unclear whether shared genetic contributors exist for T1D and T2D associated nephropathy. In addition, as suggested by variable population prevalence rates, the impact of genetic heterogeneity between populations needs to be addressed, i.e. replication across well-powered, ethnically diverse samples to assess impact. It is noteworthy that contemporary studies of DN and other common complex diseases have focused on common genetic variants captured by array technology that inefficiently tag variation in minority populations.

There is now a consensus that genes contribute to risk for DN. Earlier investigations that focused on genetic mapping and analysis of specific candidate genes provided the foundation for current studies that target linkage peaks and specific genes. As knowledge about DN (and other complex disorders) increases, the sophistication of approaches and size and cost of these studies have also increased. These efforts are beginning to identify genes that have increasingly compelling evidence for association (Tong et al., 2008).

1.14.1 Strategies for identification of genes associated with Diabetic Nephropathy

Genes that confer susceptibility to DN can be sought in different ways. A widely established method is the candidate gene approach. The search for candidate genes includes the study of polymorphisms in one or more genes potentially involved in the pathogenesis of the disease. This approach is useful even when the influence of a gene on disease development is small (Adler et al., 2000). Candidate genes are often analyzed in case-control studies by comparing the frequency of polymorphisms/mutations in candidate genes among patients with and without the disease. This is an appropriate study for investigating complex genetic transmission, and it is especially useful in situations where the genetic influence is
relatively low and disease-related alleles are common in a population (Adler et al., 2000). However, this approach is very sensitive to population stratification, which may lead to spurious associations. In the light of that it has been proposed that these studies should include a large sample to obtain very small p-values and be based on well-established a priori assumptions. This approach has allowed to describing many polymorphisms associated with DN (Freedman and Satko, 2000).

Another approach used to analyze candidate genes is the transmission disequilibrium test (TDT). This approach is not influenced by population stratification, but information is required about the individuals studied and their parents and only heterozygote parents are informative. The frequency of transmission of risk allele is compared to the expected 50%. Its main limitation is having access to the individual and his/her parents, especially for type 2 DM that has a late onset in life. More recently candidate genes are being tested in prospective studies. This study design is less prone to survival bias than case control-studies but they are expensive and time-consuming. The limitation of these studies is that they are not specifically designed to address a genetic effect of a specific gene.

1.15 Rationale for study of gene susceptibility in Diabetic Nephropathy
There is enough evidence supporting the concept of genetic susceptibility to nephropathy in patients with diabetes. Discovery of genetic variants that underpin susceptibility to nephropathy could yield important insights into this condition. Firstly, it would permit identification of patients at risk of nephropathy shortly after diagnosis of diabetes rather than much later when persistent microalbuminuria develops, by which time there is already histological evidence of renal injury. This would facilitate targeted therapeutic interventions aimed at primary prevention rather than secondary treatment of established nephropathy. Secondly, and perhaps more importantly, if the susceptibility variants are located in genes that have not previously been implicated in diabetic nephropathy, this may lead to improved understanding of its pathophysiology and development of novel therapies.
The role of genetic factors in the development and progression of diabetic nephropathy, though apparent, has not been conclusively elucidated, as yet. Candidate gene analysis allows us to study both major and minor gene effects, but generally yields conflicting results. The observed discrepancies could be partly explained by differences in the studied populations, particularly dissimilar ethnic backgrounds and genetic heterogeneity, and also by their relatively small sample sizes. The use of single nucleotide polymorphisms in association studies of complex phenotypes has been used most often. At the moment, no single gene with a large effect has been identified and only small effects of a variety of polymorphisms in a number of genes have been reported. Neither linkage analyses nor association studies performed until now support the view of major gene polymorphisms involved in the onset or progression of diabetic nephropathy.

Cytokine genes influence nuclear transcription and cell function with several allelic polymorphisms having demonstrable effects in human disease. Because inflammatory cytokines significantly modulate the pathogenesis of diabetic nephropathy, their genetic variability may affect the susceptibility to renal progression (Lee et al., 2005).

1.16 IL-1

- Chromosome location-2q13
- Size-11.48kb

Figure 6: Cytogenetic location of IL-1 gene on chromosome 2.

IL-1 is known to be pro inflammatory cytokine involved in many inflammatory disease. IL-1 gene is present on chromosome 2 and its cytogenetic location is 2q13 as shown in figure 6.
In experimental models of diabetic nephropathy, renal expression of IL-1 increases (Sassy et al., 2000; Navarro et al., 2006) which is related to subsequent expression of chemotactic factors and adhesion molecules. IL-1 enhances the synthesis of ICAM-1 and vascular cellular adhesion molecule-1 by glomerular endothelial cells, and induces de novo synthesis and expression of ICAM-1 by glomerular mesangial cells and renal tubular epithelia. In addition, this cytokine induce transient expression of E-selectin by endothelial cells (Park et al., 2000). IL-1 is also involved in the development of abnormalities in intraglomerular hemodynamics related to prostaglandin synthesis by mesangial cells. Treatment of glomerular mesangial cells with recombinant human IL-1 induces prostaglandin E2 synthesis and the release of a phospholipase A2 activity. In addition, pretreatment of resting mesangial cells with this cytokine results in an amplified secretory prostaglandin E2 response to angiotensin II. Furthermore, in vitro studies demonstrate that IL-1 directly increases vascular endothelial cell permeability (Pfeilschifter and Muhl, 1990). Primary cultures of human proximal tubular epithelial cells stimulated with IL-1 leads to a significant increase in hyaluronan concentrations in the culture supernatant. Increased production of glomerular hyaluronan initiates glomerular hypercellularity in experimental model of diabetes (Jones et al., 2001).

1.17 IL-6

- **Chromosome location**: 7p15
- **Size**: 4.88 kb

![Figure 7](image)

**Figure 7: Cytogenetic location of IL-6 gene on chromosome 7.**

Interleukin-6 (IL-6) is a pleiotropic cytokine expressed in many tissues. It is located on chromosome 7 as indicated in figure 7.
In 1991, Sekizuka et al. (Sekizuka et al., 1994) reported that serum levels of IL-6 were significantly higher in patients with type 2 diabetic nephropathy than the levels observed in diabetic patients without nephropathy, which suggests that this cytokine may play a role in the pathogenesis of diabetic nephropathy. Early after that report, Suzuki et al. (Suzuki et al., 1994) analyzed kidney biopsies in patients with diabetic nephropathy by high-resolution in situ hybridization. These authors observed that cells infiltrating the mesangium, interstitium, and tubules were positive for mRNA encoding IL-6. Furthermore, they found a relationship between the severity of diabetic glomerulopathy (mesangial expansion) and expression of IL-6 mRNA in glomerular cells (mesangial cells and podocytes), which indicated that IL-6 may affect the dynamics of extracellular matrix surrounding those cells.

More recent studies in type 2 diabetic patients demonstrate a significant association between IL-6 and glomerular basement membrane thickening, a crucial lesion of diabetic nephropathy and a strong predictor of renal progression (Nosadini et al., 2000; Dalla Vestra et al., 2005). Studies by Navarro et al., 2008 (Navarro et al., 2008) also show a significant overexpression of IL-6 in the diabetic rat kidneys, with an increase in the levels of mRNA encoding IL-6 in the renal cortex being directly associated with an elevation in its urinary excretion. Importantly, wet kidney weight, an accurate index of renal hypertrophy and one of the earliest renal changes during diabetes, (Thomson et al., 2001) is increased in diabetic rats and associated with renal mRNA expression of IL-6 and urinary excretion of this cytokine. Moreover, a direct correlation was observed between urinary levels and renal expression of IL-6 with urinary albumin excretion. These results support previous findings on the development of renal injury mediated by IL-6, which has been related to alterations in endothelial permeability, induction of mesangial cell proliferation, and increased fibronectin expression (Ruef et al., 1990; Coleman et al., 1992).
1.18 IL-18

- Chromosome location-11q23
- Size-20.87kb

**Figure 8: Cytogenetic location of IL-18 gene on chromosome 11.**

IL-18 is a potent inflammatory cytokine that induces IFN-γ (Okamura et al., 1995), which in turn induces functional chemokine receptor expression in human mesangial cells (Schwartz et al., 2002). Location of IL-18 is depicted in figure 8.

In addition, IL-18 leads to production of other inflammatory cytokines (including IL-1 and TNF-α), upregulation of ICAM-1, as well as apoptosis of endothelial cells (Dai et al., 2004). IL-18 is constitutively expressed in renal tubular epithelia, and recent studies demonstrate that infiltrating monocytes, macrophages, and T cells, along with proximal tubular cells, are potential sources of this cytokine (Melnikov et al., 2002).

Serum and urinary levels of IL-18 have been reported increased in patients with diabetic nephropathy, with an independent relationship between these parameters and urinary albumin excretion (Moriwaki et al., 2003; Nakamura et al., 2005). In addition, urinary excretion of β-2 microglobulin, a marker of tubulointerstitial injury, is also positively associated with serum IL-18. Moreover, in a longitudinal study, serum and urinary concentrations of this cytokine were directly correlated with albumin excretion rate, as well as with changes in albuminuria during the follow-up period (Nakamura et al., 2005).
1.19 TNF-α

- Chromosome location-6p21.3
- Size-2.77kb

Figure 9: Cytogenetic location of TNF-α gene on chromosome 6.

TNF-α is a pleiotropic inflammatory cytokine that is mainly produced by monocytes, macrophages, and T cells. It is present on chromosome 6 as shown in figure 9. In addition, and similar to other inflammatory cytokines, the expression and synthesis of TNF-α is not limited to hematopoietic cells. Thus, intrinsic renal cells, including mesangial, glomerular, endothelial, dendritic, and renal tubular cells are able to produce this cytokine (Sugimoto et al., 1999; Dong et al., 2007; Zhang et al., 2007). Furthermore, studies done by Wang and colleagues show that TNF-α can be stored within cells in a proactive form, and the TNF-α converting enzyme can rapidly increase levels of the active cytokine (Wang et al., 2007). Reported actions of TNF-α on renal cells include the activation of second messenger systems, transcription factors, synthesis of cytokines, growth factors, receptors, cell adhesion molecules, enzymes involved in the synthesis of other inflammatory mediators, acute phase proteins, and MHC proteins. This variety of biologic activities results in diverse effects with a significant role in the development of renal damage in diabetes. TNF-α is cytotoxic to renal cells and able to induce direct renal injury.

In addition, other relevant effects of TNF-α have been reported, such as induction of apoptosis and necrotic cell death (Boyle et al., 2003), alterations of intraglomerular blood flow and GFR as a result of the hemodynamic imbalance between vasoconstrictive and vasodilatory mediators (Baud et al., 1998), as well as alterations of endothelial permeability. TNF-α alters the distribution of adhesion receptors involved in cell–cell adhesion (i.e., vascular endothelial-cadherin catenin complexes) and prevents the
formation of F-actin stress fibers. This results in restructuring of the intercellular junction leading to loss of endothelial permeability. On the other hand, TNF-α directly induces reactive oxygen species (ROS) in diverse cells, including mesangial cells.

Recent experimental studies using isolated rat glomeruli demonstrate this cytokine activates NADPH oxidase through protein kinase C/phosphatidylinositol-3 kinase and mitogen activated protein kinase pathways (Koike et al., 2007). Therefore, TNF-α, independent of hemodynamic factors, prompts the local generation of ROS, resulting in alterations of the barrier function of the glomerular capillary wall and leading to enhanced albumin permeability (MaCarthy et al., 1998). Experimental studies have consistently reported that mRNA encoding TNF-α and protein levels increase in glomerular and proximal tubule cells from diabetic rats (Navarro et al., 2005; DiPetrillo et al., 2004). These investigations demonstrated a significant role of TNF-α in the development of renal hypertrophy and hyperfunction, two main alterations during the initial stage of diabetic nephropathy. TNF-α has a stimulatory effect on sodium-dependent solute uptake in cultured mouse proximal tubular cells (Schreiner et al., 1990), and in those studies diabetic rats exhibited enhanced urinary TNF-α excretion, sodium retention, and renal hypertrophy, which were prevented by administration of the anti–TNF-α agent TNFR:Fc, a soluble TNF-α receptor fusion protein. In addition to these data on renal hypertrophy and hyperfunction, mRNA levels and urinary TNF-α concentrations in the renal cortex directly and independently correlate with urinary albumin excretion.

More importantly, studies that used the microdialysis technique demonstrate a significant increase in levels of TNF-α in renal interstitial fluid and urine (without evidence of cellular infiltrates in cortex or medulla), which precedes the development of albuminuria, suggesting a direct correlation between urine and renal interstitial fluid concentrations of TNF-α and urinary albumin excretion. Furthermore, shortly after the rise in albuminuria, urinary TNF-α concentration increases significantly, indicating a stimulatory effect for albuminuria on the production of renal TNF-α (Kalantarinia et al., 2003). Finally, important
data about the potential implications of TNF-α in diabetic nephropathy also derive from clinical investigations.

Studies by Navarro et al. (Navarro et al., 2007) found that diabetic patients with nephropathy have higher serum and urinary concentrations of TNF-α than non-diabetic subjects or diabetic patients without renal involvement. These studies suggest a direct and independent association between the levels of this cytokine and clinical markers of glomerular and tubulointerstitial damage, with a significant rise in serum and urinary TNF-α as diabetic nephropathy progresses (Navarro et al., 2003; Navarro et al., 2006).

1.20 IL 4

- **Chromosome location-5q31**
- **Size-8.69kb**

![Figure 10: Cytogenetic location of IL-4 gene on chromosome 5.](image)

Interleukin 4 is secreted by T helper 2 (Th2) cells. Important roles have been identified for IL-4 in the context of the immune response (Feve et al., 2009). IL-4 gene is found on chromosome 5 and its cytogenetic location is 5q31 (figure 10). It stimulates the development of Th2 lymphocytes by acting upon the undifferentiated T cells after exposure to antigens, it induces the shift from IgM and IgG towards IgE production by plasmocytes, and it determines the secretion of the whole Th2 cytokine spectrum. In addition, it has an inhibitory effect upon interferon (IFN) secretion and the differentiation of Th2 lymphocytes (Dinarello et al, 2010; Enriquez et al, 2010). The association of IL-4 with immunological disorders such as multiple sclerosis, systemic lupus erythematosus (SLE), nephrotic syndrome, graft rejection, asthma, and type-1 and 2 DM is well established (Colin, 2003). The key roles of IL-4 as an inhibitory cytokine of autoimmunity and inflammations raise
questions concerning the impacts of this cytokine on the pathogenesis of some diseases including nephropathic type 2 DM (Elbe-Burger, 2002; Arabadadi et al, 2010). In hypercholesterolemia, the accumulated low density lipoproteins (LDL) in the arterial wall would be oxidized to release oxidation products that lead to activation of inflammatory responses (Cornicelli et al, 2000). In mice model, severe hypercholesterolemia is associated with a switch to Th2 immune response, with increased IL-4 expression in the atherosclerotic lesions (Feve et al, 2009; Yuxia et al, 2011). IL-4 mRNA can also be detected in atherosclerotic lesions in human body. The micro-environmental IL-4 in the atherosclerotic lesions has multiple effects on atherogenesis, such as augmentation of LDL cholesterol esterification by a concentration- and time-dependent manner. In addition, IL-4 can regulate the expression of 15-lipoxygenase (15LO), a key enzyme in LDL oxidation (Jingfang et al, 2010). It had been demonstrated that the adipocyte layer in the dermis is reduced in IL-4 transgenic mice. Accordingly, local micro-environmental expression of IL-4 is suggested to be involved in the atherogenic process (Hyun et al, 2009).

Although the initiation and etiology of T2DM still await identification, accumulating evidences have proved the hypothesis that DM type 2 is a state of chronic inflammation, with increased acute phase proteins and various cytokines (Ming-Yuh et al, 2009). Genetic studies exploring susceptible or resistant genes for T2DM could provide clues for understanding the mystery of diabetic pathogenesis and for future design of diabetic treatment (Brown, 2010). It seems likely that the risk for diabetes associated kidney disease is magnified by inheriting risk alleles at several susceptibility loci (Hyun et al, 2009). Genome-wide linkage studies have recently identified several chromosomal regions that likely contain diabetic nephropathy susceptibility genes. Previous studies had documented two polymorphisms affected gene for IL-4. One of these polymorphisms is a single nucleotide polymorphism (SNP) at region -590 in the promoter region (Mohammad, 2010).
1.21 IL 10

- Chromosome location- 1q32
- Size- 4.89kb

![Cytogenetic location of IL-10 gene on chromosome 1.](image)

IL-10 has been shown to limit the cascade of proinflammatory cytokines activation and to downregulate T cell-mediated immune responses and its cytogenetic location is depicted in figure 11 (Korholz et al., 1997). In accordance with its anti-inflammatory activity, high concentrations of IL-10 in critically ill, septic patients protected them from death (Lowe et al., 2003), while low levels were found in non-survivors (Yeh et al., 2002). The TH2 cytokines have been documented to be dominating in nephropathies with a relatively long pre-dialysis or pre-transplantation course such as idiopathic membranous nephropathy (Hirayama et al., 2002), primary IgA nephropathy (De Fijter et al., 1998) and HBV associated membranous disease (Lin et al., 1997), all of which are characterised by an increased production of IL-10 in different in vitro experimental settings. The role of the TH1 system in nephropathies is the reverse, as a high expression of TH1 cytokine transcripts predicts severe glomerular lesions and poor clinical outcome (Lim et al., 2001).

The studies on experimentally induced, passive, antilglomerular basement membrane antibody-induced model of glomerulonephritis in rats showed that systemic treatment with IL-10 significantly reduced the degree of proteinuria and systemic inflammation, and attenuated renal injury (Huang et al., 2000). Those rats with mesangial, proliferative glomerulonephritis that received treatment with IL-10, displayed a lesser degree of histological lesions, limited cellular proliferation and a lower expression of inflammatory mediators (Kitching et al., 2002). When IL-10 was delivered by means of gene therapy, to mice with naturally occurring renal failure, it effectively reduced the level of proteinuria.
and progression to glomerulosclerosis (Choi et al., 2003). DN has been regarded as a slowly evolving disease with a long pre-dialysis period, which suggests that the course of the disease may be shaped by IL-10.

Exel and colleagues discovered that low IL-10 production capacity is associated with the metabolic syndrome and T2DM (Van exel et al., 2002). The capacity for IL-10 production in individuals has been shown to be correlated with genetic composition of the IL-10 locus (Chang et al., 2005). Thus, examination of genetic polymorphisms of IL-10 may explain individual differences in T2DM risk. Several molecular epidemiological studies were conducted in recent years to evaluate the risk of T2DM associated with the polymorphisms of IL-10 (Ezzidi et al., 2009; Tsiavou et al., 2004).

1.22 VEGF

- Chromosome location-6p21.1
- Size-16.3kb

The primary function of the VEGF is to maintain the integrity and viability of the endothelium throughout diverse actions, including promotion of endothelial cell proliferation, differentiation and survival, participation in interstitial matrix remodeling, and mediation of endothelium-dependent vasodilatation. Figure 12 indicates the cytogenetic location of VEGF gene. In the kidney, VEGF expression is most prominent in podocytes and tubular epithelial cells, while VEGF receptors are mainly found on endothelial cells. It has recently been proved that VEGF participates in processes of neovascularization and glomerulosclerosis (Nakagawa, 2007; Schrijvers et al., 2003) and
Growing evidence highlights the relevance of VEGF in the pathogenesis of DN. Different studies suggest that podocyte-derived VEGF is directly involved in the glomerular capillary hyperpermeability of macromolecules (Wolf et al., 2005). VEGF expression is significantly increased in the diabetic state and stimulation of VEGF secretion by podocytes can affect blood flow, glomerular endothelial cell function, and also have an autocrine effect, altering podocyte synthesis of glomerular basement membrane constituents and foot processes (Cruz et al., 2002). From a therapeutical perspective, animal studies using inhibition of VEGF activity by neutralizing antibodies or small molecule inhibitors of VEGF receptor kinase signaling have demonstrated a marked amelioration of albuminuria in the diabetes setting (Flyvbjerg et al., 2002; Sung et al., 2006).

Vascular endothelial growth factor (VEGF) is a potent multifunctional cytokine which plays a key role in the pathogenesis of diabetic microvascular complications (Grone 1995; Awata et al., 2002). Endothelial dysfunction and increased blood vessel permeability are observed in both, diabetic retinopathy and diabetic nephropathy (De Vriese et al., 2002). VEGF is a highly conserved homodimeric glycoprotein which promotes angiogenesis and is a potent mediator of microvascular permeability (Ferrarra and Smyth, 1997). The genetic variations in the VEGF gene influence levels of VEGF protein expression. There are several polymorphisms in the VEGF gene and many polymorphisms are associated with the protein production. Among these, four VEGF SNPs, +936C/T in the 39-untranslated region, −634G/C in the 59-untranslated region, and −2578C/A and −1154G/A in the promoter region, were reported to modulate VEGF expression (Stevens et al., 2003; Watson et al., 2000). Uthra et al., 2008 suggests that there is lack of association of VEGF gene polymorphisms with diabetic retinopathy in south Indian population (Uthra et al., 2008). There are very rare studies done so far on role of VEGF gene polymorphism in diabetic nephropathy in Indian population.
1.23 Association between Inflammation and RAS (renin-angiotensin system) system

Intervention trials in adults with CKD have demonstrated that blockade of the RAS slow progression of renal disease via antihypertensive and anti-inflammatory mechanisms (Maschio et al., 1996; Jafar et al., 2003). The RAS generates circulating angiotensin II (AT2), which regulates blood pressure and intravascular volume. In contrast to its endocrine function, tissue RAS produces AT2 that is involved in autocrine and paracrine signaling within all bodily organs, including the heart, blood vessels, and kidneys (Jacoby and Rader, 2003). Tissue RAS exerts a pivotal role in the regulation of cytokine signaling, potentially modulating the inflammatory response associated with renal disease progression. Figure 13 denotes the link between RAS system and inflammation.

Tissue RAS via AT2 regulates the cytokine pathway responsible for progressive injury in the kidney (Ortega et al., 2002; Esteban et al., 2004). Activation of tissue RAS increases the local production of AT2. After AT2 stimulates the AT2 receptor, a number of signaling systems are triggered, including that of nuclear factor kappa B (NFκB), which is responsible for upregulation of proinflammatory cytokines (Ortega et al., 2001). The cytokine signaling modulates endothelial dysfunction, adhesion and migration of circulating immune cells (monocyte, leukocytes, or neutrophils) into the interstitium, and activation of resident fibroblasts. Cytokines are soluble polypeptides that act as important humoral modulators in immunoregulation, hematopoiesis, and inflammation. Cytokines act in a highly complex coordinated network with considerable overlap and redundancy between the function of individual cytokines. Being pleiotropic in their actions, these molecules can induce or repress their own synthesis as well as that of other cytokines and cytokine receptors (Raj et al., 2003). In the kidney, the inflammatory host response leads to renal interstitial fibrosis and progression. These actions within the kidney are mediated by proinflammatory [tumor necrosis factor (TNF)-a, interleukin (IL)-1, IL-6] and profibrotic cytokines [TGF-β and plasminogen activator inhibitor (PAI)-1] (Klahr and Morrissey, 2003). Proteinuria stimulates interstitial inflammation and fibrosis in the kidney; it also is a risk factor for future decline in kidney function (Hogg et al., 2000). NF-Kb activity is stimulated by albumin and is
pathway that links proteinuria and tubulointerstitial inflammation and fibrosis in the kidney (Takase et al., 2005).

Figure 13: Activation of the rennin angiotensin system (RAS) and an increase in the local production of angiotensin II (AT2) triggers the inflammatory host response (Wong et al., 2008).

There is preliminary evidence indicating impaired flow mediated dilation among hypertensive patients with mutations in the promoter region of the NF-κB gene (Park et al., 2007). Hence
the upregulation of RAS-cytokine pathway activity is associated with renal progression, LVH, and atherosclerosis. The magnitude of this response may depend on genetic polymorphisms, which may either increase or decrease expression of these genes (Balakrishnan et al., 2004).

1.24 ACE

- **Chromosome location-17q23.3**
- **Size-44.7kb**

![Figure 14: Cytogenetic location of ACE gene on chromosome 17.](image)

Angiotensin-converting enzyme (ACE) gene has been associated with the pathogenesis and progression of chronic kidney diseases. ACE gene is present on chromosome 17 as indicated in figure 14. Important risk factors for predisposition to DN include hyperglycemia, hypertension and genetic susceptibility. The significance of genetic factors in predisposition to this microvascular complication can be realized from the fact that various genes have been implicated in susceptibility to DN including ACE gene of the RAS (Ergen et al., 2004).

ACE is a key enzyme in the RAS, modulating the synthesis of angiotensin II and inactivation of bradykinin. This gene codes for angiotensin-I converting enzyme (ACE). ACE is a potent vasoconstrictor which catalyzes the conversion of angiotensin-I to angiotensin-II. It also inactivates bradykinin, a vasodilator, by bringing about its proteolysis (Crisan et al., 2000). The ACE gene has 26 exons; of which exons 1-12 code for the amino domain and remaining 13-26 code for the carboxyl domain. An insertion and deletion polymorphism of a 287 bp Alu repetitive sequence in the intron 16 of this gene has been reported (Marre et al., 1994) which results in three genotypes (DD & II homozygotes and ID heterozygotes). The ACE
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gene has an insertion/deletion (I/D) polymorphism, with the D allele being associated with higher ACE levels. The relationship between the ACE I/D polymorphism and insulin sensitivity could therefore provide insight into the role of angiotensin II on glucose metabolism in humans. Quantitative variations in the serum activity of ACE has been reported in individuals with different I/D genotypes, with highest activity in DD homozygotes, intermediate in ID heterozygotes and lowest in II homozygotes (Ng et al., 2005).

Cytokines are important modulators of inflammation. Cytokines are soluble polypeptides acting as important humoral modulators in immunoregulation, hematopoiesis, and the inflammatory cascade. Cytokines act in a highly complex coordinated network with autocrine and paracrine attributes. Many cytokines appear to be pleiotropic in their actions, with considerable overlap and redundancy between the function of individual cytokines. The cytokine network is highly complex, containing interactive cascades of gene activation and suppression. Diabetic Nephropathy is characterized by elevated levels of pro-inflammatory cytokines and markers of inflammation. Cytokines may modulate the risk for progression of DN. Polymorphisms of cytokine genes may influence gene transcription and cytokine secretion and thereby modulate the risk of progression of DN. Although all available evidence indicates up regulation of pro-inflammatory cytokine activity in DN, the etiology of this is largely unknown. Polymorphisms of cytokine genes may influence the expression of gene and their gene products. Those SNPs and their inferred haplotypes that promote increased transcription of pro-inflammatory cytokines may be associated with risk for DN. In this context, cytokine genetics is of special interest to combinatorial polymorphisms among cytokine genes, their functional variations, and general susceptibility to DN. Identification of patients carrying high-risk genotypes may allow early and aggressive interventions to be aimed at appropriate target populations.

In diabetic patients, persistence of hyperglycemia has been reported as a cause of increased production of oxygen free radicals. Hyperglycemia could induce oxidative stress
and become the main factor for predisposing the complications in diabetes. There is a strong belief that renal glomeruli are particularly sensitive to oxidative stress, suggesting the involvement and participation of ROS (reactive oxygen species) in the pathogenesis of diabetic nephropathy. Possible mechanisms for the induction of inflammation in vascular tissues may include activation of PKC pathway and oxidative stress. The stimulus for the increase in inflammation in diabetes is still under investigation; however, reactive oxygen species are a primary candidate. Thus, targeting oxidative stress-inflammatory cytokine signaling could improve therapeutic options for diabetic nephropathy. There is a close association between oxidative stress and inflammation in diabetes and we hypothesize that an increase in oxidative stress-derived inflammation is a major mechanism in the pathogenesis and progression of diabetic nephropathy.

In this context, this study is aimed to examine inflammatory cytokine genes polymorphism, its expression analysis and evaluation of oxidative stress markers in type 2 diabetic and diabetic nephropathy patients of West India.