“With a missionary zeal, one must convert not only the patient’s mind and soul, but also his doctor to the realization that it is worth the effort to control the disease as shown by the sugar-free urine, normal blood sugar and cholesterol”

-Dr. Elliot P. Joslin

**DISCUSSION**

A person once diagnosed with diabetes, enter a kingdom where everyday aspects of life are altered. Increased urbanization and industrialization along with the environmental factors contribute to high incidence rate of diabetes, a major health problem in India. [172] According to current estimates of diabetes by International Diabetes Federation (IDF), globally 415 million people are suffering from it, and the prevalence is growing, particularly in low- and middle-income countries. 78 million diabetic people live in South East Asia; this number is expected to rise to 140 million by the year 2040. The country with highest number of diabetic patients in this region is India, with 69.2(8.7%) million cases, as in 2015.[173] This increasing prevalence of diabetes at national and international levels is imposing a huge economic and social burden on society. The costs involved in the care and management of diabetes are considerable for both the patients and the health care system. [174]

The chronic hyperglycemia, a characteristic of diabetes mellitus, with other risk factors shows long term consequences including microvascular and macrovascular complications which may lead to damage to several organs. The macrovascular complications of diabetes comprise cardiovascular disease (CVD) and stroke, and microvascular complications include diabetic nephropathy, neuropathy and retinopathy. [175]

Uncontrolled hyperglycemia and hypertension are established causal factors for the development of diabetic nephropathy, [176] whose natural history has been changed most as a consequence of the scientific advances over the last 20 years. [177] Diabetic nephropathy is the major single cause of ESRD, [178] a
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Life threatening complication resulting in a poor prognosis for patients as well as high medical costs. [179]

ESRD requires dialysis and is becoming a stumbling challenge to public health care systems due to the unaffordable cost of treatment of renal transplant. About 20 to 40% patients diagnosed with diabetes are more likely to develop kidney damage, and with significant genetic component confers the risk of DN, revealed from family-based studies. [88] DN a clinical condition is manifested by presence of albuminuria, declining GFR, and increased risk for CVDs. [88, 180] The identification of microalbuminuria as an early biomarker/predictor for both renal and cardiovascular diseases has allowed patients at high risk to be identified and treated. [177]

An understanding of lipoprotein metabolism and how it influences diabetes and associated diseases is of particular importance. This requires close attention to lipid screening because of the association of lipids & lipoproteins with CVD, which is the most common complication and leading cause of death among people with diabetes. [181] The NCEP and ADA have focused attention on the necessity of managing lipid disorders. [182, 183]

Since, the positive association of glycemic control and lipid levels has been observed, the increased glycemic index play an important role in the altered metabolism of lipids and lipoproteins, thereby increasing the risk of CVDs. Therefore timely diagnosis and treatment of lipid and lipoprotein abnormalities in diabetes mellitus patients should be a clinical priority. [184]

The risk factors for diabetes include age, diet, lifestyle, family history of diabetes, obesity, stress etc. Along with environmental factors, several genes are also known to contribute in the pathogenesis of diabetic nephropathy and are known to be responsible for the differences in clinical progressions. The involvement of genes in diabetic nephropathy is complex and yet not completely understood. Evidence suggests that there are number of genes which play an important role in development of diabetic nephropathy. Therefore, the present study was focused on the alterations in lipid and
lipoprotein metabolism in T2DM patients with and without nephropathy, along with the role of ACE gene insertion/deletion polymorphism in T2DM patients with & without nephropathy and in lipid and lipoprotein metabolism.

**BIOCHEMICAL ANALYSIS AND CORRELATION OF ALBUMIN TO CREATININE RATIO WITH BIOCHEMICAL PARAMETERS:**

In India the burden of diabetes is increasing with the chances of development of complications including diabetic kidney disease. ESRD caused due to DN, increases the CVD events and is a disastrous medical condition with varying magnitude. Several pathways including lipoprotein abnormality leads to causation of hypertension due to aggravation of hyperinsulinemia. Lipid reduction might be helpful to maintain normal GFR, lower protein excretion and reducing the CVD events. [106, 185]

In DM patients, the increased glycation of proteins causes stiffness of arteries, and thereby progression to arteriosclerosis. [87] Dyslipidemia plays a vital part in progression of kidney abnormalities in diabetic patients and ends with CVD complications. Diabetes mellitus leads to dyslipidemia and this aggravated dyslipidemia is seen in diabetic patients with nephropathy. Lipid alterations related to diabetes mellitus and diabetic nephropathy include increased levels of triglycerides, VLDL-C, IDL cholesterol, LDL-C and low level of HDL-C. [186]

Number of observational studies suggested that the glomerular injury might developed or advanced due to abnormal lipids, which are consistent with the results of the present study. Katore et al. [13] assessed atherogenic lipid profile in patients of DM and DN. Values of total cholesterol, triglycerides, LDL-C and TC/HDL, LDL/HDL were significantly higher in DM with nephropathy and DM without nephropathy but values of HDL-C were significantly lower in DM with nephropathy and DM without nephropathy than controls.

Suchitra et al. [112] studied atherogenic dyslipidemia in T2DM and DN patients. Atherogenic dyslipidemia with elevated Lp(a), total cholesterol,
triglycerides, VLDL-C, LDL-C, non-HDL-C, lipid ratios, atherogenic index and low HDL-C levels were observed in both T2DM patients with and without nephropathy when compared to controls. Significantly high TG/HDL-C, TC/HDL-C and atherogenic index were observed in DN when compared to T2DM.

Momin et al. [113] found significant increase in lipid and lipoprotein levels, except HDL-C which were significantly decreased in T2DM individuals than controls. BMI, FBG, total cholesterol, triglycerides, LDL-C, VLDL-C, TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, Non HDL-C and cholesterol retention fraction were positively associated with insulin level and HOMA IR, while HDL-C was negatively associated with insulin levels and insulin resistance.

The present study was aimed to find the alterations in the lipids and lipoprotein levels in patients of T2DM with & without nephropathy as compared to controls. ANOVA detected significant difference in BMI, total cholesterol, triglycerides, HDL-C, LDL-C, VLDL-C, blood urea, serum creatinine, urine creatinine, urine microalbumin and albumin to creatinine ratio in T2DM patients with & without nephropathy and healthy controls. Further evaluation disclosed BMI, TC and LDL-C were altered in both T2DM patients with and without nephropathy than healthy controls. The urine creatinine, microalbumin and albumin/creatinine ratio differed significantly between the T2DM patients with nephropathy than healthy controls as well as T2DM patients without nephropathy. Patients having proteinuria had higher BMI, total cholesterol, triglycerides, LDL-C, VLDL-C, blood urea, serum creatinine, urine creatinine, microalbumin, and albumin/creatinine ratio than microalbuminuric patients.

In accordance with the present study, lipid profile parameters viz. total cholesterol, triglycerides, LDL-C and VLDL-C were found to be significantly higher in DN patients compared to controls by Reddy et al.[187] Study by Jha et al. [188], reported significant difference in FBG between normoalbuminuric
and microalbuminuric group. The values of urea, creatinine and triglyceride were significantly different, while cholesterol, HDL-C and LDL-C levels were comparable, in microalbuminuric and overt proteinuric group when compared with T2DM patients without renal pathology.

Similar results were noted by Soher et al. [189], including significant elevations in serum cholesterol, triglycerides and LDL-C, while HDL-C was significantly reduced in diabetics. Our results are similar to that of Al-Jameil et al. [106], who noted that mean values of triglycerides, HDL-C, VLDL-C and TC/HDL-C ratio in diabetics with microalbuminuria group differ significantly from the diabetics with normoalbuminuria but not from the diabetics with overt proteinuria.

Khadkeet al. [114] undertook the study to assess the lipid profile of T2DM and its correlation with FBG. The lower levels of HDL-C and LDL-C were reported in DM patients, and in patients taking statins and had low LDL-C. The significant difference in FBG, lipids and risk ratios was indicated in a correlation analysis. The correlation of alterations in lipid levels with FBG were the key findings in diabetics, along with the increased risk of CVDs.

Samatha et al. [115] investigated the predictive biomarkers of microvascular complications in T2DM patients. The incidence and the progression of microvascular complications was found to be increased with hyperglycemia, a longer duration of DM, dyslipidemia and the presence of microalbuminuria in patients with T2DM suggesting that these factors can predict the microvascular complications in patients with T2DM.

Ozder [116] found association between serum levels of TC, triglycerides, LDL-C and hepatosteatosis and HbA1c. Significantly higher mean serum levels of total cholesterol, triglycerides and LDL-C and significantly lower mean serum levels of HDL-C were noted in patients with DM. FBG showed significant positive correlation with total cholesterol and triglycerides. Sigdel [117] et al. gave the prevalence of microalbuminuria and overt proteinuria to be 45.5% and 11.2%, respectively. Prevalence of microalbuminuria in females was found to
be marginally higher than in males. Also microalbuminuric subjects had significantly higher blood pressure.

Marcovecchio et al. [118] explored the prevalence of lipid abnormalities and their relationship with albumin excretion and microalbuminuria in adolescents with T1DM. Total cholesterol, LDL-C, HDL-C, and non-HDL-C were found to be higher in females than in males. A1C was found to be independently related to all parameters except HDL-C. Total cholesterol and non-HDL-C were independently related to longitudinal changes in ACR. The results consistent with present study were also encountered by Al-Jameil et al. [6] where total cholesterol, triglycerides, LDL-C, VLDL-C values were found to be significantly increased in diabetics than normoalbuminuria, microalbuminuria and overt proteinuria and were found to be positively correlated with albumin to creatinine ratio.

The similar pattern of lipid profile was observed by Onovughakpo-Sakpa et al. [190] They reported significant increase in total cholesterol, LDL-C and triglycerides, while decreased HDL-C in diabetics with and without nephropathy, as compared to control subjects. The study by Dwivedi et al.[191] reported significantly decreased levels of serum HDL-C, along with total protein, albumin, plasma vitamin C level and increased serum total cholesterol, triglycerides, LDL-C, malondialdehyde, homocysteine and Lp(a) levels in DN patients as compared to controls. Vinoth et al. [185] reported the significant difference in LDL-C contents in diabetic kidney disease patients than that in diabetics without kidney disease, and no significant difference was noted for HDL-C within these groups.

The precise pathogenesis of diabetic dyslipidemia is still not known [192]. Experimental and clinical evidences suggest that insulin resistance has a central role in development of dyslipidemia. [193] The basic defect in T2DM is the impairment of insulin action or decreased secretion of insulin or combination of two, showing hyperglycemia which adversely affects glucose utilization and also leads to impairment in lipid metabolism. [194]
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Linear relationship between LDL-C levels and the incidence of cardiovascular events was similar in individuals with and without diabetes mellitus in interventional trials on statins, indicative of the role of LDL-C in development and progression of atherogenesis. Low HDL-C and increased triglycerides levels are also the independent risk factors which contribute to the increased risk of CVD in patients with diabetes mellitus. [195]

The basic mechanism behind diabetic hypertriglyceridemia lays in the overproduction of VLDL-TG, which is most likely due to the increased flow of substrates, particularly free fatty acids, to the liver from adipose tissue. [196] DN is the common cause of abnormal lipoprotein metabolism and can be influenced by impairment of renal function and metabolic control of diabetes. [197] The hypertriglyceridemia may be due to higher production of triglyceride rich VLDL by the liver and decreased removal of triglycerides by peripheral tissues—primarily adipose tissue and muscle. [198]

It has been recognized that one of the factor responsible for DN development is lipid nephrotoxicity. (Figure 42) Diabetes in combination with dyslipidemia play a vital role in the development of DN. Macrophage infiltration and excessive extracellular matrix production into the glomeruli are involved in the DN progression, of diabetic mice deficient of LDL receptors. The receptors for triglycerides rich lipoproteins (TGRLs) have been expressed on mesangial cells and podocytes. Inflammatory pathways via the secretion of proinflammatory cytokines are stimulated by TGRLs, resulting in the ROS production, leading to excessive extracellular matrix production. TGF-β-mediated signaling pathways are reported to be potentiated by ROS, this may cause a brutal cycle in the process of excessive ROS and ECM production. In mesangial cells and podocytes oxidized LDL (Ox-LDL) binds to scavenger receptors, thereby increasing ECM as well as chemokine production, such as monocyte chemoattractant protein (MCP)-1 which induces monocyte migration toward the glomeruli and results in macrophage infiltration. With the uptake of Ox-LDL macrophages form foam cells, facilitating the inflammatory
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Pathway. Lipid nephrotoxicity has been shown to be mediated by several factors, including sterol regulatory element binding protein (SREBP)-1 and Toll-like receptor (TLR) 4. [198a]

Figure 42: Role of dyslipidemia in diabetic nephropathy. Dyslipidemia promotes the development of diabetic nephropathy. Abnormal lipoprotein metabolism is accelerated in diabetic nephropathy that causes further renal injury, leading to ESRD as well as cardiovascular (CV) events. [198a]

Insulin resistance has also known to have a central role in the development of diabetic dyslipidemia.[199] Insulin affects many sites of mammalian lipid metabolism and it stimulates synthesis of fatty acids in liver, adipose tissue and intestine. [190]

The increased release of free fatty acids into the liver in the presence of adequate glycogen stores promotes triglyceride production, which in turn
stimulates the secretion of ApoB and VLDL-C. The impaired ability of insulin to inhibit free fatty-acid release leads to enhanced hepatic VLDL-C production, which correlates with the degree of hepatic fat accumulation. Diabetic nephropathy has been reported to have significantly higher plasma concentrations of VLDL-C, LDL-C, triglycerides and lower HDL-C. Dyslipidemia influences on the formation of glomerular lesions, especially focal glomerular sclerosis and it is proposed that dyslipidemia may promote the progression of chronic renal disease and progression of diabetic nephropathy. [199] The sequence of this mechanism is depicted in figure 43.

Metabolism of ApoB required in VLDL may also be altered in T2DM patients; the patients may have a fractional decreased catabolic rate for ApoB of VLDL similar to that for VLDL-TG. [201] In addition, the clearance of VLDL-TG decreases in individuals with T2DM. A lipolytic enzyme, LPL, is known for breakdown of triglycerides transported in the form of TGRLs viz. VLDL-C & chylomicrons. LPL activity is found to be decreased in individuals with T2DM, especially those with moderate to severe hyperglycemia who exhibit both insulin deficiency and insulin resistance. [202]

Additionally hypertriglyceridemia, in combination with increased small dense LDL-C and decreased HDL-C levels, are an important contributor to accelerated atherosclerosis in diabetes mellitus and insulin resistance conditions. [203] Further, action of LPL converts the TGRLs to IDL and thereby to LDL-C. LDL-C molecules are then taken up by liver. This binding and uptake of LDL-C is stimulated by insulin. Insulin deficiency and/or insulin resistance is seen in T2DM, leading to impaired LDL clearance. This LDL-C gets modified to become more atherogenic by enhancing atheroma formation. [204]
In T2DM, an increased proportion of TG has been observed in HDL-C molecules than LDL-C. This alignment may be related with the activity of enzyme LPL synthesized by adipose tissue; therefore, LPL deficiency may play a role in altered distribution of HDL-C molecules. [205]

Glycation of apolipoproteins may alter lipoprotein metabolism and may increase susceptibility to oxidation. [206] Due to consistent hyperglycemia, glucose molecules are added to a number of biomolecules like Hb forming glycated Hb i.e. HbA1c. Similarly, hyperglycemia may cause non enzymatic glycation of HDL particles, changing the structural conformation which interferes in binding of HDL with its receptors. Thus, glycation of HDL may be one of the reasons for decreased HDL-C concentration in diabetes. Also, abnormalities in HDL composition have been well-known even in individuals
with optimum glycemic control. All these alterations in HDL composition and modification may alter or lower the normal functioning of HDL i.e. reverse cholesterol transport from extrahepatic tissues to liver. [207]

The hyperglycemia affects the kidneys in several ways. The pathophysiologic mechanisms of DN are attributed primarily to metabolic and hemodynamic derangements including hyperperfusion, hyperfiltration and hyperglycemia induced production of advanced glycation end products (AGEs), and activation of polyol pathway, protein kinase C (PKC) & rennin angiotensin system. Structural changes in DN include thickening of the basement membrane of glomeruli, hypertrophy of glomerulus, glomerulosclerosis, mesangial cell expansion, and injury to podocyte. [106, 208, 209] Figure 44 indicates the outline of pathophysiological mechanisms of diabetic nephropathy.

**Figure 44: Pathophysiological mechanisms of diabetic nephropathy [210]**
The earliest demonstrable abnormalities in diabetic nephropathy include intrarenal hypertension, increased glomerular filtration rate, and microalbuminuria. [211] Elevated levels of lipoproteins and lipids in DM patients cause glomerular and tubulointerstitial injury contributing to DN progression, however, the reduction in albumin excretion has been noted with treatment of dyslipidemia. Increase in the mortality of diabetic patients due to CVD, DN and also other complications of DM is raising because of increased prevalence of DM. [106]
CORRELATION OF ALBUMIN TO CREATININE RATIO WITH BIOCHEMICAL MARKERS AND ROC CURVE ANALYSIS:

About one third of the diabetic patients develop microalbuminuria, almost after 15 years of onset of diabetes. The overt nephropathy is developed in about 50% of microalbuminuric patients with simultaneous increase in risk of cardiovascular disease. [96] The process starts from the early stages of diabetic nephropathy to ESRD; CVDs progressively develop, and are the most important reason of the mortality in DN patients. [212]

Use of the albumin to creatinine ratio (ACR) in urine samples is recommended as the preferred screening strategy for albuminuria in diagnosis of diabetic nephropathy in diabetic patients. [212, 213] Also the relation of increased urine ACR with Apo B containing lipoproteins has been proved. [214]

ACR has been reported to be significantly positively correlated with total cholesterol, triglycerides, LDL-C, VLDL-C, while HDL-C in diabetics was found to be negatively correlated with microalbuminuria. [106] Zhang et al. [17] found positive association of ACR with LDL-C and log of LDL-C/HDL-C ratio in men, in females none of the lipids or lipid related ratios were associated with ACR. [215] Sun et al. [18] reported association of ACR with the levels of triglycerides, HDL-C, Non-HDL-C/HDL-C ratio, TG/HDL-C ratio, and compared these parameters with other lipids and their ratios. TG/HDL-C ratio exhibited highest rise with increased level of ACR and higher odds of chronic kidney disease. [216]

In the present study attempt was made to find out the association of ACR with biochemical and lipid profile markers in diabetic nephropathy patients. The positive association of ACR was found with BMI, FBG, triglycerides, VLDL-C, urine creatinine & urine microalbumin, while it was negatively associated with HDL-C. Further, we calculated the cut off values of BMI and lipid markers for the prediction of cardiovascular disease in diabetic nephropathy patients by means of ROC curve analysis. With significant area under the ROC curve, we found the cut off values of >24.3, >187.2, >143.1, ≤42.7, >112.12
and >37.2 for BMI, cholesterol, triglycerides, HDL-C, LDL-C and VLDL-C, respectively.

In accordance with our results, a significant positive correlation of urine ACR was observed with plasma creatinine, and urine microalbumin in a study of Karar et al. [217] The increased level of triglycerides was found to be associated with albuminuria in patients with controlled LDL-C, while HDL-C was decreased but not associated with albuminuria. The ROC curve analysis for TG/HDL-C ratio, gave the area under the ROC curve of 0.656 with highest specificity and sensitivity, for differentiating urinary albumin excretion of ≥30 mg/g of creatinine in type 2 diabetic patients. [218]

The significant positive correlation of small dense LDL-C was found with albuminuria, while negative association was found with estimated GFR. The cut off value for small dense LDL, based on microalbuminuria, in the diagnosis of diabetic nephropathy was found to be >55.14 mg/dl. [219]

The study by Bose et al. [220] reported the contrast results as compared to present study. They found association of microalbuminuria and macroalbuminuria with HbA1c levels, and no association was found for other measured lipids. Increase in HDL-C was noted with decreased incidence of microalbuminuria patients, while no significant association of lipid markers was found with urinary ACR.
ASSOCIATION OF ACE I/D POLYMORPHISM IN T2DM PATIENTS WITH AND WITHOUT NEPHROPATHY:

The genetic determinants of complex traits are traceable and that knowledge of genetic variation improves the diagnosis, treatment or prevention of a substantial fraction of cases of the disease that constitute major public health burden of industrialized nations. Much of the enthusiasm is based on the hope that the marginal effects of common allelic variants account for a substantial proportion of the population risk for such diseases in a useful predictive way. [221]

The duration of diabetes or hypertension or the status of glycemic regulation alone cannot explain risk of development and progression of DN. Environmental and genetic factors go hand in hand with these additional factors in the pathogenesis of DN. The genetic evidences can determine the risk of nephropathy in DM patients. Irrespective of strict glycemic control in about 35% of diabetic patients, the development of nephropathy is noted. [88]

Various genes have been mapped on the chromosome where the DN susceptibility is located, that defines the importance of genetic factors in predisposition to DN. [34] Several studies of DN have analyzed the candidate genes that have previously been studied in hypertension, T2DM, and CVD. [122] Polymorphisms in genes encoding PPAR-γ, eNOS, GLUT-1, aldose reductase, MTHFR, ApoE and components of the RAS including angiotensinogen, angiotensin II receptor type 1, and particularly, the ACE gene, have been implicated in the pathogenesis of DN. [121]

One of the important factors in the development of DN is the regulation of blood pressure. The RAS has a predominant role in this process and this system depends on several sequential steps. The enzyme renin is secreted from the juxtaglomerular cells and cleaves angiotensinogen (AGT) to form angiotensin I. Angiotensin converting enzyme (ACE) then turns the inactive angiotensin I into the active octapeptide angiotensin II, which then interacts with specific receptors. When binding to its receptor, angiotensin II increases the
intraglomerular pressure by causing vasoconstriction in the renal vessels via stimulation of different signals in the afferent and efferent glomerular arterioles. Angiotensin II decreases salt and water secretion via the kidneys and this also adds to the rise in blood pressure. ACE not only converts angiotensin I to angiotensin II but also degrades the vasodilator bradykinin. [222] (Figure 45)

![Figure 45: Renin angiotensin system, with role of ACE. [222]](image)

The angiotensin I– converting enzyme (ACE) gene has been tested for its association with DN., High intraglomerular angiotensin II levels increases intraglomerular hydraulic pressure, which favors diabetic glomerulosclerosis, and low ACE concentrations can limit intrarenal angiotensinogen II generation. [125] Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is an exopeptidase which mediates various physiological functions. [122]

It is one of the first candidate genes studied in DN for several reasons, like
activation of vasoconstrictor peptide angiotensin to maintain normal blood pressure and on other hand it inhibits the action of bradykinin a vasodilator peptide. By activation of angiotensin II it also regulates microcirculation in the kidney. [33]

The gene of ACE is a 21 kb long situated on the long arm of chromosome 17 (17q23) and it is having 26 exons and 25 introns. Nearly 160 gene polymorphisms in ACE gene are found and many of them are single nucleotide variations. The I/D polymorphism was first reported in 1990 by Rigat et al.[223] for the first time, involving the presence or absence of a 287 bp DNA sequence situated in intron number 16 of the ACE gene. [33]

Since 1990, ACE I/D polymorphism association with DN has been extensively investigated and more than 300 studies have explored genetic associations of this polymorphism in more than 100 conditions including DN. [32] Three genotypes viz. DD, II and ID of this Alu repetitive sequence polymorphism are known. [34, 123]

In a meta-analysis by Staessen et al. [224], it was noted the DD genotype had 1.56 times increased risk of DN than II genotype. Several Japanese studies have found the D allele to be an independent risk factor for DN. [120]

We investigated the ACE I/D polymorphism in type 2 diabetic patients with and without nephropathy, and healthy controls. No significant genotype distribution between any of these three study groups was detected. The ‘D’ allele frequencies did not differ significantly between DM and DN patients, while it is significantly increased in patients of DM and DN than healthy controls.

Logistic regression analysis revealed that the appearance of ‘D’ allele of I/D polymorphism of ACE was positively related to total cholesterol, triglycerides, LDL-C & VLDL-C, while it was negatively associated with HDL-C in type 2 diabetic patients with and without nephropathy. BMI was also positively associated with ACE I/D polymorphism in only type 2 diabetics with
nephropathy.

The levels of total cholesterol, triglycerides, LDL-C, VLDL-C were significantly increased, while HDL-C was decreased in type 2 diabetic patients with and without nephropathy, which were carriers for ‘D’ allele than the homozygotes for ‘I’ allele. BMI was significantly increased in the carriers for ‘D’ allele than ‘I’ homozygotes in type 2 diabetic nephropathy patients.

The role of I/D polymorphism of ACE has been studied in various ethnic groups with inconsistency in results. Ethnicity, interindividual variation determining factor and also defines the action of I/D polymorphism of ACE gene in susceptibility to DN. In Caucasians ACE D allele was not found to be associated with DN. However, the association of D allele of ACE and DN has been reported in Asian and French population. [33]

Several studies have found the D allele to be an independent risk factor for DN and it is used as a marker in population structure analyses. Patel et al. [123] reported that ACE gene polymorphism does not have significant influence on development of DM and non-DN complications, whereas, association of the DD polymorphism has been reported in development of DN in the Western Indian population. Hussein et al. [119] highlighted significant association of dominant model with the risk of DN which rose by three folds. They also showed that the minor allele (D) frequency was significantly higher in DN as compared to controls.

In accordance with the present study, Arfa et al. [135] and Jayapalan et al. [121] showed no association of (I/D) polymorphism within the ACE gene with DN nor with T2DM in the Tunisian and Malaysian population, respectively. Bhaskar et al. [122] reported insignificant differences in genotype frequencies of ACE I/D polymorphisms when DN patients were compared with controls, in south Indian population. Mizuiri et al. [134] investigated a possible association between the ACE I/D genotype and renal ACE mRNA levels in healthy individuals. It is suggested that renal ACE gene expression is associated with the ACE I/D genotype in healthy Japanese people.

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The results of the present study are in accordance with the previous studies from South India by Khan et al. [34]. No significant difference in distribution of genotype frequencies of ACE I/D polymorphism was detected by these authors in type 2 diabetics with nephropathy and controls but significant association of D allele was observed with increased risk of DN. Viswanathan et al. [225] also reported a positive association between the ‘D’ allele (ID and DD genotype) of the Ace polymorphism and diabetic proteinuria is South Indian type 2 diabetic patients.

Figure 46: Role of ACE I/D polymorphism in pathogenesis of DN [226]

The increased activity of ACE in plasma and in tissues like heart and kidney, is associated with DD genotype than ID and II genotypes. This increased level of ACE leads to the pathogenesis of DN, may be due to increased activation of angiotensin II, increasing GFR and intraglomerular blood pressure, this promotes mesangial cell & matrix proliferation. [123] As the polymorphism is
found in an intron, it has no effect on the structure of the enzyme. [119] The physiological importance of ACE I/D polymorphism is its association with plasma, cellular, and tissue ACE levels (Figure 46). [33, 134]

Plasma levels of ACE enzyme are under genetic control. The ACE I/D polymorphism accounts for over 40% of interindividual variability of serum or tissue ACE activity. [32] It accounts for differences in plasma ACE levels and was thought to influence ACE-mediated physiological functions, and for its genetic susceptibility to glomerular lesions. [121, 122, 125]

Deletion (D) homozygous individuals were found to have the highest levels of plasma ACE, heterozygous individuals were having intermediate levels, while individuals homozygous for the insertion had the lowest levels of plasma ACE. [33] The vasoactive peptide angiotensin II probable be the cause of development of DN, it also increases GFR and intraglomerular pressure, and promotes proliferation of mesangial cells and matrix. [123]

Angiotensin II, via ACE, increases blood pressure and also induces transforming growth factor-β1 (TGF-β1) and thereby stimulates mesangial growth and production of extracellular matrix (ECM), which contributes to DN development. It also induces production of oxygen radicals, at least in vitro. In addition, the angiotensin II molecule stimulates the production of the mineral corticoid hormone aldosterone, which increases the blood pressure by increasing salt re-absorption. [33]
**GENE EXPRESSION ANALYSIS:**

In mammalian tissues, mRNA expression, related to the transcriptional activity of various genes, is in a state of dynamic turnover during different developmental and pathophysiological states. In such a process, various genes are differentially up- or down-regulated depending on a given disease process. The detection of such modulation of gene expression not only would lead to identification of candidate disease susceptible or resistant genes, but also would delineate novel mechanisms and pathways involved in various diseases. [35]

The visceral epithelial cells “Podocytes” are highly specialized, covering the outer side of GBM and important for maintenance of dynamic functional barrier, play a vital role in the regulation of glomerular function. The number of podocytes may be reduced in the glomeruli of both T1DM and T2DM patients. Both foot process effacement and a decreased number of podocytes have been documented to be associated with DN, which is a disease process characterized as a podocytopenia. [88, 227]

Human podocytes (Pods) have been demonstrated to be functionally and structurally injured in the natural history of DN. Furthermore, the number and density of Pods have been reported to be markedly reduced (podocytopenia) in patients with DM. Pods are located outside the GBM. [136]

The understanding of proteinuria and its pathogenesis in DN patients has been increased by analyzing the expression of podocyte proteins. Nephrin and podocin are closely linked to cytoskeletal alpha actinin-4 and synaptopodin, enabling dynamic rearrangements of the podocyte architecture. Podocalyxin, a phenotypic marker of the podocyte at the apical membrane, limits the passage of negatively charged albumin. [39]

The correlation of proteinuria has been evidenced with proteins or genes associated with podocyte. This increases the possibility of studying the podocyte associated proteins in progression of DN. [33] Number of genes expressed by podocytes have been identified and studied their role in the
development of proteinuria and glomerular disease. [138]

Therefore, we investigated the gene expression of proteins associated with podocyte viz. podocalyxin, podocin, and synaptopodin in type 2 diabetic patients with and without nephropathy from PBMCs. The expression of podocalyxin was up-regulated significantly in microalbuminuric and overt nephropathy patients than the T2DM patients without nephropathy and controls. Podocin expression was found to be up-regulated in microalbuminuric patients than T2DM without nephropathy patients, and it was down-regulated in overt diabetic nephropathy patients than T2DM without nephropathy and microalbuminuric patients. The gene expression of synaptopodin was down-regulated in T2DM patients without nephropathy than controls, and it was up-regulated in overt diabetic nephropathy patients than controls and T2DM patients without nephropathy.

Previously, researchers focused on the detection of these podocyte related proteins and expression of their genes in excreted podocytes from urine samples. A sequence of events through epithelial-mesenchymal transition and apoptosis or detachment, and ultimately contributing to glomerulosclerosis and decline of renal function that might trigger the podocyte injury. [227]

Levels of urinary podocalyxin were elevated in patients with various glomerular diseases and patients with diabetes. It was detected by Hara et al. [136] in patients with diabetes. Urinary podocalyxin was higher in 53.8% patients at the normoalbuminuric stage, in 64.7% at the microalbuminuric stage and in 66.7% at the macroalbuminuric stage. Zheng et al. [227] determined the significantly increased mRNA expression of podocyte associated genes in urine viz. synaptopodin, podocalyxin, CD2-AP, α-actinin 4, and podocin from patients with varying stages of DN compared with controls.

Wang et al. [147] found that the urinary mRNA expressions of nephrin, podocin, synaptopodin, WT-1 and α-actinin 4 are higher in patients with DN than in normal controls. Urinary nephrin and synaptopodin expressions were correlated with baseline clinical parameters such as proteinuria or renal
function, while WT-1 expression was found to be related to degree of histological damage. In a study by Wang et al. [148], intra-renal expression of podocyte-associated molecules was found to be correlated with glomerular podocyte number, renal function, and tubulointerstitial scarring, and suggested that intra-renal, but not urinary expression of podocyte-associated molecules might be used to assess the degree of podocyte loss in DN.

Aaltonen et al. [144] studied the expression levels of nephrin-specific mRNA in streptozotocin model of rats and non-obese diabetic mouse model and found that the expression levels of nephrin-specific mRNA increased up to two-fold during several weeks of follow-up. They concluded that nephrin is connected to the early changes of DN and thus may contribute to the loss of glomerular filtration function. Toyoda et al. [145] examined the expression of nephrin mRNA in the kidneys of T2DM with DN, and suggested that low expression of nephrin mRNA may be closely linked to development and/or progression of proteinuria in human DN.

Baelde et al. [146] evaluated mRNA expression profiles of glomeruli from DM and healthy individuals. Oligonucleotide microarray analyses on control and diabetic glomeruli were presented and discussed in their relation to vascular damage, mesangial matrix expansion, proliferation, and proteinuria. Their findings suggest that progression of DN might result from diminished tissue repair capability. do Nascimento et al. [39] demonstrated that the stages of DN were found to be associated with excretion rate of nephrin, and nephrinuria also predicts pathological albuminuria. No significant difference was found in urinary mRNA levels of podocyte markers in pre-DM subjects than controls.

Rodrigues et al. [149] evaluated the podocyte-associated mRNA profiles in renal tissue and urine of patients with proliferative or non-proliferative glomerulopathies. Different profiles of mRNA expression were seen, pointing to a higher degree of intra-renal podocytopenia in the non-proliferative and of podocyturia in the proliferative pathologies. The immunosuppressive therapy effectively reduced the urinary levels of podocyte-associated mRNAs.
Inhibition of podocyte-associated mRNAs in kidney tissue suggests that podocyte injury occurs regardless of the severity of lupus nephritis. Increased urinary excretion of podocyte mRNAs, mostly in patients with moderate-to-severe lesions, may reflect a greater burden of glomerular damage with detachment of podocytes into the urine. dos Santos et al. [150]

Numerous factors have been implicated in the pathophysiology of podocyte injury in diabetic nephropathy. Among them, high glucose, angiotensin II, TGF-β, and mechanical stress have widely been studied to explore the precise mechanisms of podocyte injury under diabetic conditions. [37]

![Figure 47: Proposed scheme of glucose-induced activation of a local angiotensin system in the podocyte.](image-url)
In the early stages of DN in T1DM and T2DM patients, the possible podocyte injury mechanisms include foot process effacement, tissue enlargement, detachment, apoptosis, and may be epithelial to mesenchymal transition (EMT). [33, 137]

The involvement of angiotensin II in podocyte injury has been extensively studied in diabetes. In streptozotocin induced diabetic rats, ACE inhibitors were known to prevent loss of podocytes and podocyte injury, in addition, the antagonists of angiotensin II type 1 receptors decreased podocyte foot process broadening in these rats. [88] (Figure 47)

![Figure 47: Mechanism of increase in oxidative stress and resultant podocyte injury due to hyperglycemia. Adapted with modifications from 228](image-url)

![Figure 48: Mechanism of increase in oxidative stress and resultant podocyte injury due to hyperglycemia. Adapted with modifications from 228](image-url)
Hyperglycemia seems to be the centerpiece for podocyte injury and DN as a whole, which is most likely due to a higher degree of oxidative stress. Excess glucose is metabolized through multiple accessory pathways like the polyol pathway that converts glucose into sorbitol, which then depletes the amount of antioxidants like glutathione (GSH), and increases the level of reactive oxygen species (ROS). In addition, increased blood glucose undergoes condensation with free amino acids to form advanced glycation end products (AGEs), which then modulate several important events like the induction of protein kinase C (PKC) and generation of ROS via AGE/a receptor for AGE (RAGE) axis activated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Among kinase signaling pathways, the most commonly activated pathway includes the PKC pathway, which then triggers the production of ROS via NADPH oxidase. All these pathways are interlinked, i.e. AGEs and PKC increase the oxidative stress and oxidative stress in turn exacerbates the generation of AGEs and PKC (Figure 48). [228]