" EVALUATION OF SERUM 25 HYDROXY VITAMIN D IN TYPE 2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT NEPHROPATHY "

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BONAFIDE CERTIFICATE

This to certify that this dissertation work entitled "**EVALUATION OF SERUM 25 HYDROXY VITAMIN D IN TYPE 2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT NEPHROPATHY"** is the original bonafide work done by **Dr.P.DEEPA**, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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I, **Dr.P.DEEPA**, solemnly declare that the dissertation titled "**EVALUATION OF SERUM 25 HYDROXY VITAMIN D IN TYPE 2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT NEPHROPATHY"**is the bonafide work done by me at Institute of Biochemistry, Madras Medical College under the expert guidance and supervision of **Prof. Dr.R.Chitraa,** M.D., Professor, Institute of Biochemistry, Madras Medical College. The dissertation is submitted to the Tamil Nadu Dr.M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch III) in Biochemistry.

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ABBREVATIONS

DM – Diabetes Mellitus

DN – Diabetic Nephropathy

WHO – World Health Organisation

ESRD – End Stage Renal Disease

1,25$(OH)_2$D – 1,25 dihydroxy vitamin D

25(OH)D - 25 hydroxy vitamin D

RAS – Renin Angiotensin System

MODY – Maturity Onset Diabetes of Young

GLUT2 – Glucose Transport2

TCA – Tri-Carboxylic Acid

ETC – Electron Transport Chain

ATP – Adenosine Triphosphate

SUR1- sulphonyl urea receptor 1

KIR - potassium Inward Rectifier

ROS - Reactive Oxygen Species

NF^kB – Nuclear Factor kappa B

IL-1- Interleukin-1

TNF-α - Tumour Necrosis Factor alpha

IRS – Insulin Receptor Substrate

PI3K - phospho-inositide 3-kinase

MAP - mitogen-activated protein

HSL - Hormone Sensitive Lipase

FFA - Free Fatty Acid

NEFA - Non Esterified Fatty Acid

IL-6 - Interleukin-6

GFR – Glomerular Filtration Rate

UAER - Urinary Albumin Excretion Rate

NO - Nitric oxide

VEGF - Vascular endothelial growth factor

TGF-β1 - Tumour Growth Factor β1

AGE - Advanced Glycosylation End products

DAG – Diacylglycerol

NAD^+- Nicotinamide Adenine Dinucleotide phosphate

ICAM - Intercellular adhesion molecule

VCAM - Vascular cell adhesion molecule

ECAM - Endothelial cell adhesion molecule

MCP – Monocyte Chemoattractant Protein

UVB – Ultra Violet B

DBP - vitamin D-binding protein

VDR – Vitamin D Receptor

VDRE – Vitamin D Response Element

CoR- co-repressor

CoA - co-activator

POL II – Polymerase II

MARRS - membrane-associated, rapid response steroid-binding

PKC – Protein Kinase C

RANKL - Receptor Activator of Nuclear Factor-κB Ligand

RANK - receptor for RANKL

OPG – osteoproteregin

PTH – Parathormone

cAMP – CyclicAMP

CRE - cAMP response elements

PKA - protein kinase A

BMP -7 - Bone Morphogenetic Protein

ELISA – Enzyme Linked Immunosorbent Assay

eGFR – estimated Glomerular Filtration Rate

IDMS – Isotope Dilution Mass Spectrometry

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disease of carbohydrate metabolism resulting in hyperglycemia mainly due to absolute or relative deficiency of insulin secretion and or its action. As the global effect of Diabetes constantly intensifies, there is a need for intervention to slow down the progression of disease and to achieve metabolic control.

Epidemiology

According to World Health Organisation (WHO), approximately 250 million people currently have diabetes worldwide and this number will reach 380 million by the year 2030.

India is being termed the" Diabetes capital of the world". In India, approximately 40 million people are found to have diabetes and this will reach 70 million by the year 2030[1].

Diabetic Nephropathy (DN) is one of the chronic complication of Diabetes Mellitus(DM). Diabetic Nephropathy is identified clinically at the earliest by micro-albuminuria.

End Stage Renal Disease (ESRD), a devastating disease is mainly due to Diabetic Nephropathy and is the major cause for Diabetes related morbidity and mortality[2]. Around 10 – 20% mortality in diabetes is due to

renal failure. Chronic kidney disease due to Diabetic Nephropathy finally leads onto Renal Replacement Therapy[3].

Diabetics have 17 fold higher mortality rate due to kidney disease than the persons without Diabetes[4]. Vitamin D is the factor having an impact in the differential development of DN, as it posseses anti - proliferative effect in cellular differentiation, immune modulation and inhibition of the renin - angiotensin system (RAS)[5], apart from its role in maintaining bone homeostasis.

Kidney disease might progress depending upon Vitamin D insufficiency versus deficiency[6]. Vitamin D metabolite, i.e., $1,25(OH)_2$ vitamin D inhibited by the RAS has been established in vitro to emphasise its protective role in DN[7].

Rat studies are based on these demonstrations which showed that the administration of $1,25(OH)_2D$ helps in reducing the progression of glomerulo-sclerosis and albuminuria through mechanisms that are not dependent on parathormone functions and by its antiproliferative actions which finally also reduce podocyte hypertrophy and its loss[8,9].These changes might be particularly applicable in individuals with Diabetic Nephropathy .

When adequate vitamin D levels are maintained, there is an increase in the secretion of insulin by the β cells of pancreas and also there is a decrease in insulin resistance and arterial pressure. The potentially

modifiable factor, arterial blood pressure for diabetic nephropathy is said to decrease when there is a decrease in insulin resistance and also by an increase in the secretion of insulin, which are done by adequate vitamin D levels[10].

The role of vitamin D level to decrease insulin resistance and thereby to decrease the formation of overt hyperglycemia needs to be analysed in our population.

Only a very few human have dealt about the relationship between serum vitamin D levels and DN. If the levels of vitamin D are adequately maintained whether it could prevent diabetic nephropathy needs to be studied.

This study is to evaluate the serum 25-hydroxyvitamin D levels in Type 2 Diabetes Mellitus patients with and without nephropathy.

AIM AND OBJECTIVES OF THE STUDY

1. To assess the levels of serum 25-hydroxy vitamin D in patients with Type 2 Diabetes Mellitus with and without Diabetic Nephropathy.

2. To correlate the levels of serum 25- hydroxy vitamin D, with urine microalbumin and serum creatinine in patients with Diabetic Nephropathy.

3. To assess the role of vitamin D insufficiency Versus vitamin D deficiency in facilitating the progression of Diabetic Nephropathy.

REVIEW OF LITERATURE

DIABETES MELLITUS

The most commonly prevalent endocrine disorder is Diabetes Mellitus (DM). More than 170 million people were found to be affected world wide. Around 365 million people would be involved with this disease by the year 2030[11].

Type 2 Diabetes Mellitus is expeditiously blooming as one of the greatest world wide health burden of the 21st century. The complications of diabetes mellitus like coronary artery disease, nephropathy, neuropathy, stroke and retinopathy[12] which are the resultant of micro and macrovascular changes are also said to rapidly increase and thereby causing profound morbidity and mortality in these group of patients. Hence diabetes mellitus can be aptly termed as global endocrine epidemic which needs urgent attention for prevention.

The major devastating complication of Diabetes is Diabetic Nephropathy, which leads on to End Stage Renal Disease (ESRD).

Micro-albuminuria is a characteristic and early marker of DN.

Diabetes Mellitus, the most common metabolic disorder is characterised by chronic hyperglycemia associated with disturbance in the metabolism of carbohydrate, aminoacid and lipid metabolism due to, either absolute or relative deficiency of the secretion /action of insulin[13].

Diabetes Mellitus apart from being a metabolic disorder is also a multisystem disorder involving every vital organ of the body. Most importantly the involvement of heart and kidneys result in life threatening complications.

TYPES OF DIABETES MELLITUS[14]:

1. Type 1 Diabetes Mellitus occurs due to destruction of the β cells of pancreas

Destruction of the β cells of pancreas is mainly due to :

a) Autoimmune cause
b) Idiopathic cause

2. Type 2 Diabetes Mellitus

Causes are :

a) Resistance to insulin in the insulin target tissues.
b) Defects in insulin secretion by the β cells of pancreas.

3. OTHER SPECIFIC TYPES OF DIABETES:

a) Genetic defects in β cell dysfunction eg: MODY 1 to 11
b) Genetic defects in insulin action eg: Type A insulin resistance
c) Pancreatic disease eg : fibrocalculus pancreatopathy
d) Endocrine diseases eg:, Cushings disease, Acromegaly

e) Drugs and chemicals induced eg : steroids

f) Infections due to congenital rubella

g) Rare disease of immune related diabetes eg: stiff man syndrome

h) Other genetic disorders like Down’s syndrome

4. GESTATIONAL DIABETES occurs during pregnancy which may either get corrected after parturition or result in DM (Type 2) accordingly.

PATHOGENESIS OF DIABETES MELLITUS

Characteristic features of Type 2 Diabetes Mellitus is mainly due to the following metabolic defects[15]

1. inadequate insulin secretion due to β-cell dysfunction.
2. decreased ability of peripheral target tissues to respond to insulin (insulin resistance).

Insulin resistance is the initial event in most cases, followed by increasing degrees of dysfunction of the β-cells of pancreas .

Physiology of the insulin secretion

Glucose enters the β-cells of pancreas rapidly, through the glucose transporter 2 (GLUT2). Glucose phosphorylated by glucokinase, is one of the rate limiting steps of glycolysis. Several intermediates are formed and finally, leads to the formation of pyruvate.

In the mitochondria, pyruvate is taken up to be involved in the Tri-Carboxylic Acid (TCA) cycle in which the various intermediates permit

production of energy at substrate level as well as the relevant co-enzymes accepting the reducing equivalents (NADH, $FADH_2$) to access the Electron Transport Chain (ETC), situated on the inner surface of the inner mitochondrial membrane and produce ATP, the currency of energy.

ATP is the essential energy needed for the insulin release as well as the depolarisation of the cell membrane. The adjacent potassium channel (potassium inward rectifier [KIR] 6·2 channel) is closed, when sulphonyl urea receptor 1 (SUR1) protein is stimulated by ADP/ATP ratio. This closure of potassium channels causes a change in the membrane potential and thereby leading to opening of calcium channels and finally there is a release of preformed insulin containing granules[16].

Role of chromium on insulin action

Chromium enhances the number of insulin receptors on the cell membrane and increases the insulin binding to the insulin target cells. It also stimulates insulin receptor kinase, resulting in increased insulin sensitivity[17].

- **Pancreatic dysfunction of the β cell.**

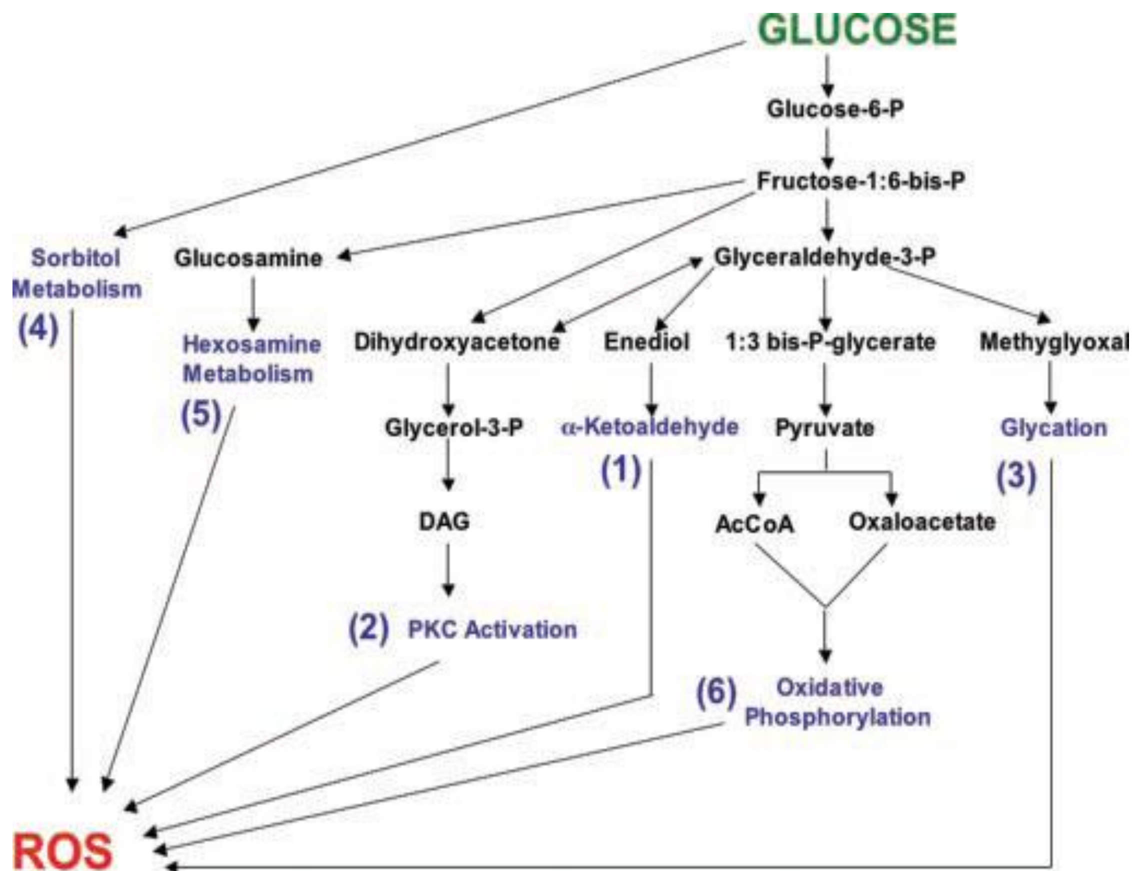
In the initial course of Type 2 Diabetes Mellitus, Insulin secretion appears to be normal. However, normal pattern of insulin secretion is lost and the first rapid phase in the secretion of insulin, stimulated by glucose is decreased. It shows the derangement in the response of the β cells of the

pancreas to hyperglycemia. Finally, irreversible β cell damage occurs due to toxicity of glucose and lipid.

Glucose toxicity

Metabolism of glucose propagates the formation of Reactive Oxygen Species (ROS), in the β cells of pancreas. ROS require superoxide dismutase, catalase, and glutathione peroxidise, for detoxification, which are present in low amounts in the β cells of pancreas[18].

Hyperglycaemia enhances the accumulation of large amounts of ROS in the β cells of pancreas, with consequent damage to the components of the cell by increasing the NFκB activity, which stimulates the apoptosis of the β cell of pancreas.



The above flow chart depicts the formation of Reactive Oxygen species through several metabolic pathways of glucose metabolism.

Lipotoxicity

- Oxidation of fatty acid in the β cells of pancreas, is inhibited in the presence of glucose, resulting in the formation of long chain acyl coenzyme A[19], which in turn decrease the insulin secretory pathway, by opening the K^+ channel of the β cells of pancreas.
- Increased expression of uncoupling protein-2 due to accumulation of acyl coA decreases ATP formation. Insulin secretion will be inadequate in the absence of ATP.

- Higher fatty acyl-CoA levels within the β cell of pancreas enhance the synthesis of ceramide, which increases inducible nitric-oxide synthase[20] and the subsequent rise in nitric oxide stimulates the expression of Interleukin-1(IL-1) and Tumour Necrosis Factor alpha(TNF-α)which are the inflammatory cytokines, that affects the function of the β-cells of pancreas, promoting apoptosis of the β-cells of pancreas[21].

Insulin resistance

Insulin secreted by the β-cells of the pancreas, is the anabolic hormone, its action is necessary for the homeostasis of glucose, fat and protein metabolism[22].

Insulin resistance[23] is defined as the incapability of insulin target tissues, such as skeletal muscle and adipose tissue, to respond effectively to the insulin secretion.

Insulin resistance might be due to reduction in the number of insulin receptors or diminution in the post receptor signalling of insulin receptors with or without deterioration in the number of insulin receptors.

Mechanism of insulin resistance

i) Role of Phosphorylation and De-phosphorylation of Insulin Receptor Substrate (IRS).

The insulin receptor (IR) is a hetero-tetramer has α and β subunits, two each, are linked by disulphide bonds. Insulin binds to the α subunit of the insulin receptor and stimulates the tyrosine kinase in the β subunit.

Once the tyrosine kinase is activated, it favours the auto-phosphorylation of the β subunit, i.e., phosphorylation of three tyrosine residues(Tyr-1163, Tyr-1162, and Tyr-1158)[24]. Two major cascades such as mitogen-activated protein (MAP) kinase pathways and phospho-inositide 3-kinase (PI3K) are triggered, which mediate the growth-promoting and metabolic functions of insulin[25] .

By reducing tyrosine phosphorylation while enhancing serine and threonine phosphorylation of the insulin receptor, insulin resistance is exacerbated that has an blocking effect on insulin signaling[26].

Phosphotyrosine phosphatase1B is expressed widely and has an opposite effect in insulin signaling[27].

ii) Role of adipocyte and inflammation

In normal individuals, insulin impedes lipolysis by inhibiting Hormone Sensitive Lipase(HSL). In Insulin resistance, lipolysis is increased with an increase in circulating Free Fatty Acid (FFA); Hence in obesity, mainly increased adipocity in the visceral area, are of great relevance.

Increased Non Esterified Fatty Acid (NEFA),TNFα and IL-6 released by visceral adipose tissue untowardly affect the insulin signalling cascade[28].

Glucose metabolism in skeletal muscle stimulated by insulin is inhibited by NEFA and gluconeogenesis is triggered by NEFA in liver[29]. NEFA stimulate cellular kinases, such as isoforms of atypical protein

kinase C by enhancing cellular diacylglycerol levels, which in turn can stimulate the c-jun N-terminal kinase and inflammatory kinases inhibitor k B kinase (IKK), enhancing serine/threonine phosphorylation of insulin receptor resulting in decreased insulin signaling[30,31].

TNFα stimulates lipolysis in adipocytes, then stimulate NEFA, and exerts direct inhibitory action on insulin signalling cascade[32].

Diabetic Nephropathy (DN)

The major microvascular complication of Diabetes Mellitus is DN. Diabetic Nephropathy was identified by Clifford Wilson and Paul Kimmelstiel[33] in 1936.

Other names of Diabetic Nephropathy are

- Kimmelstiel -Wilson syndrome or
- Nodular diabetic glomerulo- sclerosis or
- Inter-capillary glomerulo-nephritis,

Diagnostic features of DN are

i. Albuminuria (>300 mg/day or >200 µg/min),
ii. Permanent and irreversible diminution of Glomerular Filtration Rate (GFR)
iii. Elevated blood pressure.

Diabetic Nephropathy is a progressive disorder and is associated with high morbidity and mortality rates. Normally, no albumin is excreted. But, the initial step in Diabetic Nephropathy is the progression from normo- albuminuria to micro- albuminuria, which progresses to macro albuminuria, affecting the renal function by deteriorating the GFR [34].

Albuminuria could be due to structural abnormalities, mainly glomerular in origin[35] presenting with :

i. increased pressure in the glomerulus,
ii. loss of negatively charged glycosaminoglycans in the glomerular basement membrane
iii. the enlargement of the pore size of the basement membrane.

Multi-factorial risk factors for Diabetic Nephropathy are :

i. Hyper-glycemia,
ii. Hypertension,
iii. Genetic factors,
iv. Obesity,
v. Sedentary lifestyle,
vi. Advancing age[36],
vii. Smoking.

Stages of Diabetic Nephropathy [37]

Diabetic Nephropathy can be classified into different clinical stages based on

i. GFR

ii. Systemic Blood Pressure

iii. Urinary albumin excretion rate (UAER).

STAGES OF DIABETIC NEPHROPATHY

STAGES	DESIGNATION	URINE ALBUMIN EXCRETION(UAE)	GFR	BLOOD PRESSURE	STRUCTURAL CHANGE
I	Hyperfunction/ Hypertrophy	May be increased	Maybe increased	Usually normal	Glomerular hypertrophy
II	Normo-albuminuria	Normal albumin loss (UAE excretion <30 mg/24h)	Normal/ Increased	Usually normal	Increasing basement membrane thickening & mesangium expansion
III	Incipient diabetic nephropathy	Microalbuminuria (UAE excretion 30–299 mg/24h)	Normal/ Increased	Increasing	
IV	Overt diabetic nephropathy	Macroalbuminuria (UAE excretion ≥300 mg/24h)	Decreasing	Hypertension	Increasing glomerular occlusion and severe mesangial expansion
V	End stage kidney disease	Macroalbuminuria often decreasing because of glomerular occlusion	<20 ml/min	Frank Hypertension	

PATHOGENESIS

Several mechanisms plays an important role in the development of Diabetic Nephropathy, due to the relationship between

i. Hemodynamic pathways,
ii. Hyperglycemia induced metabolic pathways
iii. Inflammatory pathways.

i. HEMODYNAMIC PATHWAYS

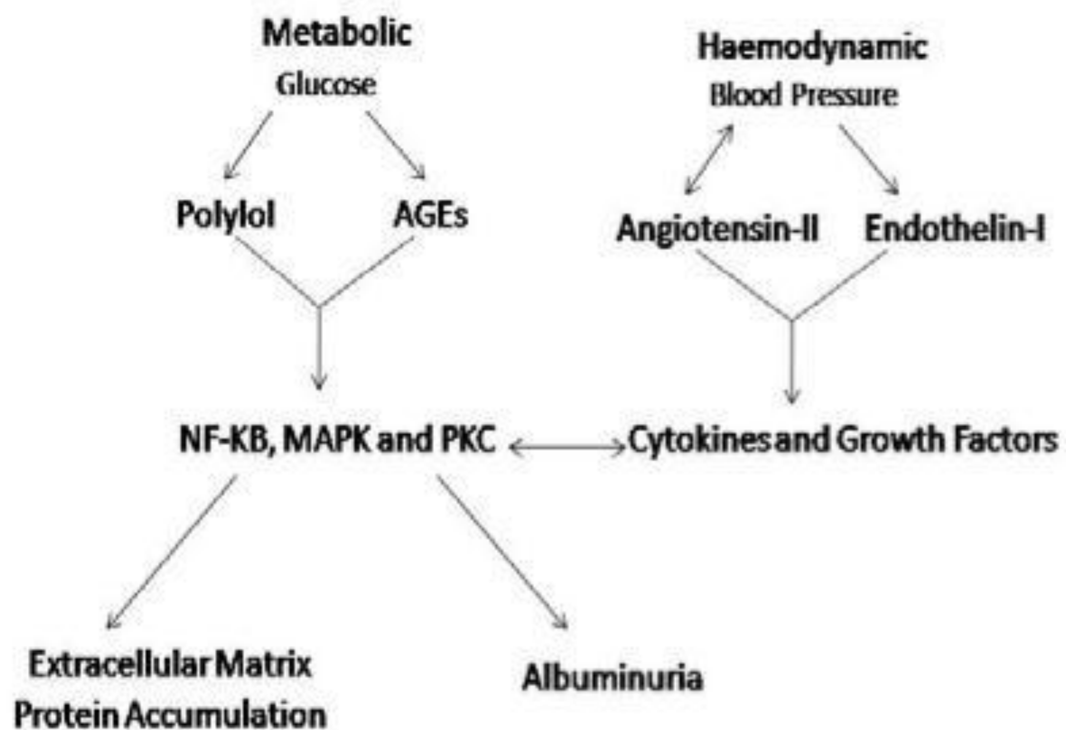
Both the afferent and efferent arterioles of the glomerulus having reduced resistance is the earliest sign. This results in glomerular hyper-filtration and hyper-perfusion.

Many factors are responsible for this defective auto-regulation, including

i. Prostanoids,
ii. Nitric oxide(NO)
iii. Vascular endothelial growth factor (VEGF),
iv. TGF-β1
v. Renin Angiotensin System (RAS).

The early hemodynamic changes contribute

1. Facilitate the oozing of albumin from the glomerular capillaries,
2. Increased production of matrix of mesangial cell,
3. Glomerular basement membrane thickening,
4. Podocytes injury[38],
5. Release of growth factors and certain cytokines [39].



The above flow chart depicts the interaction of hemodynamic and metabolic pathways in Diabetic Nephropathy.

ii. THE EFFECT OF HYPERGLYCEMIA ON METABOLIC PATHWAYS:

Hyperglycemia is a primodial factor in the formation of DN, exerting harmful changes in the glomerular basement membrane and mesangial cells. It initiates a main role in the development of DN by interacting with the metabolic and hemodynamic factors.

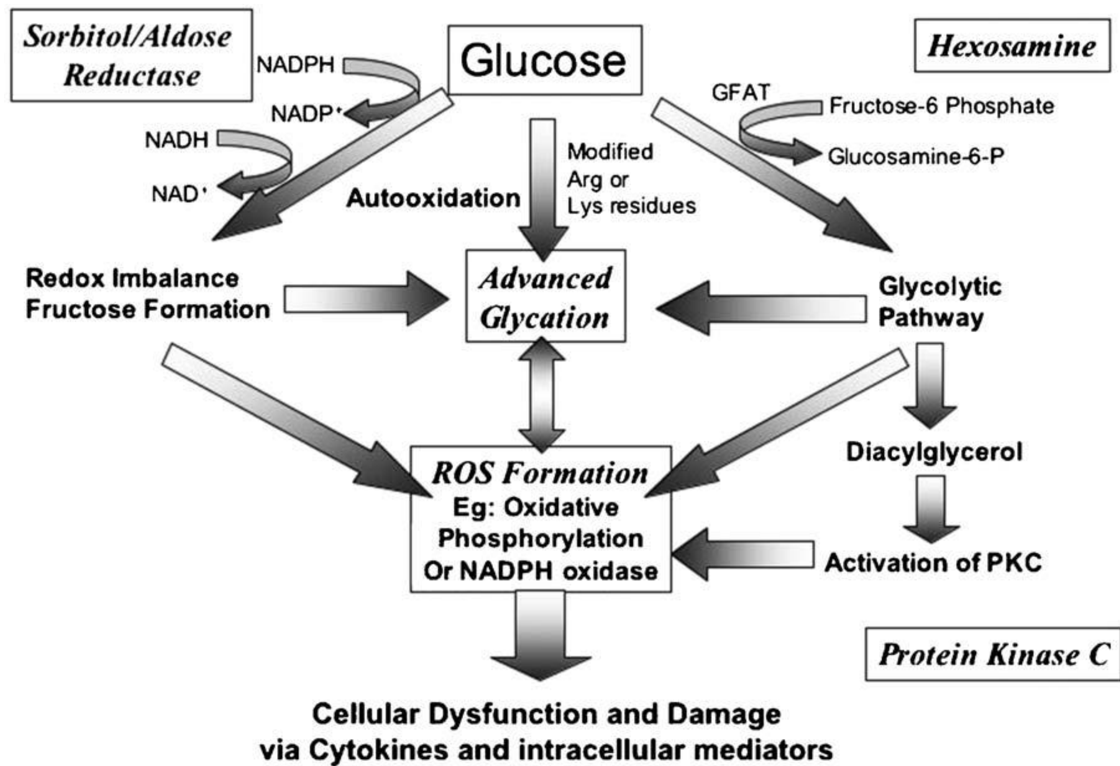
Mesangial cells are responsible for the maintenance of the structure of glomerular capillary, and through smooth-muscle activity,mesangial cells alters the glomerular filtration rate.

Hyperglycemia results in increased proliferation and hypertrophy of mesangial cells[40] in addition, there is increased production of mesangial matrix and thickening of glomerular basement membrane.

Mechanisms causing tissue damage due to hyperglycemia are :

1. Non-enzymatic glycosylation that produces Advanced Glycosylation End products (AGE)
2. Protein Kinase C(PKC) activation
3. Activation of the aldose reductase pathway[41]
4. Increased Hexosamine pathway.

Oxidative stress is the common theme to all these pathways[42].



The above flow chart depicts the various interactions between metabolic pathways in diabetic nephropathy.

a) Increased AGE(Advanced Glycation End products)

Tissue protein glycosylation results in the formation of DN and other microvascular complications. In chronic hyperglycemia, the increased glucose binds non-enzymatically with tissue proteins or free amino acids to form reversible glycated proteins.In the early stage,these reversible glycated products affect the basement membrane of the glomerulus and mesangial matrix components.

By modulating signal transduction through the alteration in the level of signals, such as, free radicals, cytokines and hormones, progressive glycation forms irreversible AGEs, amenable for the pathogenesis of Diabetic Nephropathy,

The accumulation of AGEs in the tissue enhances cross linking with collagen and extracellular matrix proteins, which contribute to renal and micro-vascular complications[43].

AGEs accelerate atherosclerosis, promote glomerular dysfunction, decrease NO synthesis and induce endothelial dysfunction. Finally, there is an alteration in extracellular matrix composition and structure.

b) Activation of Protein kinase C (PKC)

a) De novo formation of Diacylglycerol(DAG) resulting in PKC activation and oxidative stress[44] is mainly due to hyperglycemia.
b) PKC activation is responsible for the increased secretion of prostanoids resulting in glomerular hyperfiltration[45].
c) PKC increases the production of extracellular matrix in mesangial cells[46] by activating TGF-β1.
d) The activation of PKC stimulates the MAPKs activity, through dual phosphorylation at conserved tyrosine and threonine residues,triggered by extracellular stimuli such as hyperglycemia. The co-activation of MAPK and PKC in the presence of

increased glucose concentration suggests the interlink of the two families of enzymes[47].

e) PKC modulates the transcription of gene for collagen Type IV, fibronectin, extracellular matrix proteins and contractile proteins, in neurons and endothelial cells.

C) Increased polyol pathway

.

Hyperglycemia increases the glucose metabolism via sorbitol pathway. Glucose is converted to sorbitol by the enzyme aldose reductase when the intracellular glucose is elevated.

Several mechanisms explain about the polyol pathways.[48]

- Sorbitol induces osmotic stress
- Decreases cytosolic Na^{2+} / K^{+} ATP ase activity
- Reduces cytosolic NADPH, which in turn increases oxidative stress in the cell
- Increase in the cytosolic ratio of $NADH/NAD^{+}$

The surge of the NADH causes inhibition of glyceraldehyde 3-phosphate dehydrogenase which causes an increase in concentration of triose phosphate[49] which could increase the formation of methyl glyoxal, which is a highly active glycating compound and methyl glyoxal is a precursor of AGEs and via α-glycerol -3-phosphate, increase the production of diacyl glycerol which activates protein kinase C. This

pathway is normally inactive and gets activated only when intracellular glucose level starts elevating.

d) Increased hexosamine pathway

Glucosamines formed from excess glucose,in hexosamine pathway could be one of the reason for the development of diabetic complications. All these culminate in the process of DN such as activation of protein kinaseC, increased TGF-β expression and extracellular matrix production[50].Rise in TGF-β expression in the kidney stimulated by glucose,structural alterations in kidney[51] due to glucosamine has been demonstrated in the experimental animals. TGF-β exerts a main role in the development of Diabetic Nephropathy[52] due to the interaction with the RAS and PKC activity.

iii) INFLAMMATORY PATHWAY

Proteinuria, a diagnostic factor in DN, is significantly associated with inflammation, as it induces the renal tubular cell activation, resulting in the expression of pro-inflammatory cytokines, adhesion molecules and numerous chemokines. Consequently, these responses culminate in infiltration of interstitium by lymphocytes, monocytes, macrophages and

neutrophils, which then results in renal cell damage, injury to the tubulo-interstitium and finally fibrosis of kidney[53].

A wide range of pro-inflammatory molecules are involved in the patho- physiological cause of Diabetic Nephropathy.

Pro-inflammatory molecules include :

a) Chemokines with their receptors,
b) Pro-inflammatory cytokines,
c) Adhesion molecules
d) Transcription factors.

Inflammatory molecules in Diabetic Nephropathy:

- Inflammatory cytokines

  Tumor Necrosis Factor (TNF)

  IL-1, IL-6, IL-18.

- Chemokines and their receptors.

  CCL2 (MCP-1) with its receptor CCR2,

  CX3CL1 (fract-alkine) with its receptor CX3CR1,

  CCL5 with its receptor CCR5.

- Adhesion molecules

  Intercellular adhesion molecule (ICAM),

  Vascular cell adhesion molecule (VCAM),

  Endothelial cell adhesion molecule(ECAM),

E-selectin,

α-Actinin 4.

- Transcription factor - Nuclear factor κB (NFκB).

Transcription Factor

Nuclear Factor kappa B(NFκB),a transcription factor, forms a pivotal role in the process of DN.

Metabolic and hemodynamic pathways are the two main pathways in which NFκB plays a significant role in the formation of Diabetic Nephropathy.

NFκB activity is mainly perceived in tubular cells of renal cortex , in some glomerular cells (podocytes), which is related with the amount of proteinuria and the intensity of interstitial cell infiltration[54].

Normally, NFκB dimers are kept in an inactive state by I kappa Bs (inhibitors of NF kappa B). Translocation of NFκB into the nucleus is prevented on binding of I kappa Bs to NFκB and the inactive state of NFκB is maintained in the cytoplasm.In response to extracellular signals, the enzyme I kappa B kinase gets activated.

This causes degradation of I kappa Bs from NFκB and activation of NFκB occurs. NFκB is available in the cytoplasm, which is translocated to the nucleus which in turn activate the NFκB -dependent gene expression.

NFκB is activated by hyperglycemia in:

1. Endothelial cells of the vessels,

2. smooth muscle cells of the blood vessels,

3. Proximal tubular epithelial cells of the kidney [55, 56]

Apart from being activated by hyperglycemia, NFκB is activated by

- Pro inflammatory cytokine molecules
- Oxidant stress
- Protein trafficking in tubular cells of the kidney
- Angiotensin II with its receptors

On activation, NFκB stimulates the various number of gene transcription such as chemokines(i.e; CCL2 and CCL5),nitric oxide synthase, cytokines, adhesion molecules. several proliferative and other inflammatory proteins are also included in the formation of Diabetic Nephropathy [58].

Interestingly, NFκB activation resulting in streptozotocin-induced diabetes[59] in cortical tissue of animals kidney has been demonstrated.

There are many evidences to show that NFκB plays a key role in interrelated pathways which leads to the

i) Structural and functional changes in the kidney,

ii) Stimulation of the RAS,

iii) Accumulation of glycated products,

iv) Increased oxidative stress[57].

Functions of NFκB:

- Proliferation of cell[60]
- Control the cell survival

Hence, NFκB is an integral factor in regulating many chemokines, inflammatory cytokines, cell adhesion proteins and other molecules which are responsible for the pathogenesis of Diabetic Nephropathy.

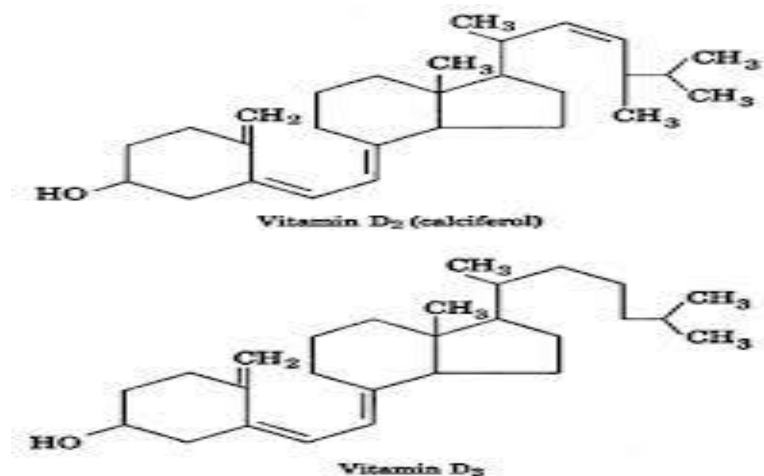
Vitamin D

Vitamin D is a pro-hormone, fat soluble and was identified in the 20th century. Vitamin D, steroid-derivative vitamin, is popularly described as a seco-steroid as, one of the four rings is open[60a]. Vitamin D, well known as "sunshine hormone", was first recognised and framed by Goldblatt and Soames in 1923[61].

Vitamin D, a steroid hormone, synthesised on exposure of skin to sunlight and is also absorbed from foods. Vitamin D is classified as Vitamin D2(ergocalciferol) and vitamin D3 (cholecalciferol) . Cholecalciferol is synthesised naturally in the skin from 7-dehydrocholesterol by UV-B (290-315 nm).Diets rich in vitamin D3 are oily fish such as salmon, sardines, and mackerel[62].

Ergocalciferol is a plant derived vitamin D, produced exogenously by irradiation of ergosterol produced by yeast, and enters the circulation through diet.

Ergocalciferol differs from cholecalciferol by a double bond and a methyl group on carbon 24. As both are equally potent, generally, total vitamin D (vitamin D3 + vitamin D2) and the metabolites are measured.



The vitamin D, plays a pivotal role in homeostasis of calcium,phosphorus & bone metabolism, and is now accepted to sub-serve a entire range of basic functions such as differentiation of cell , cell growth inhibition and modulation of immune system[63].

Synthesis of vitamin D

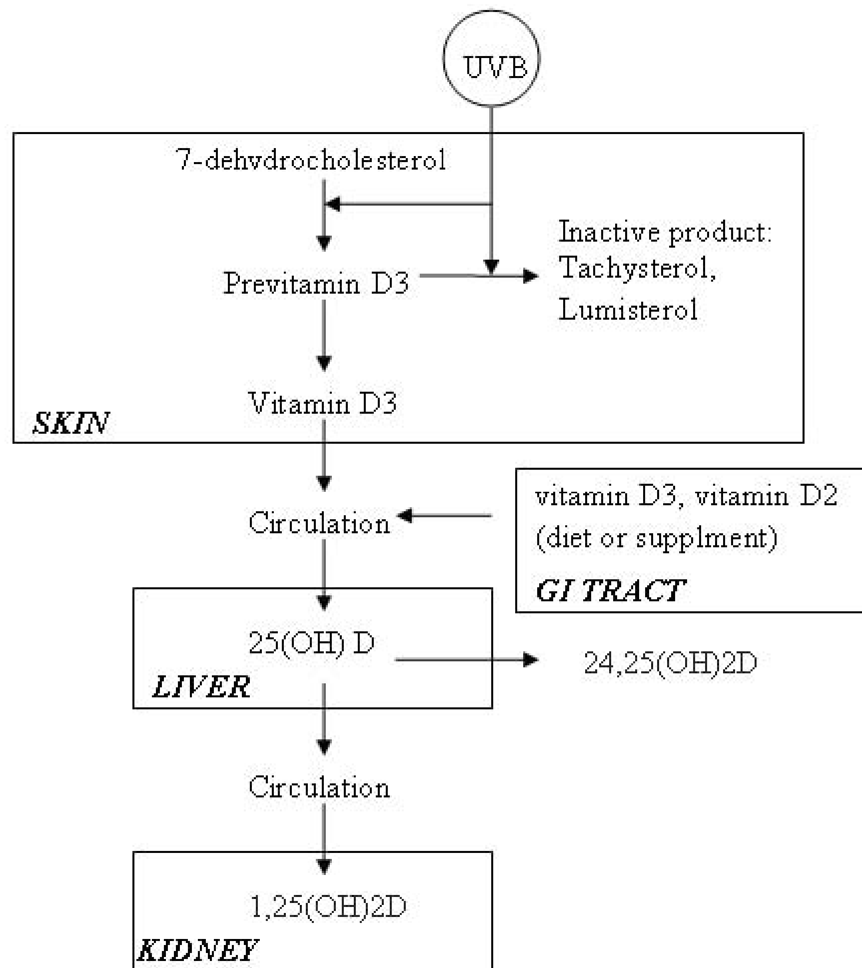
On sunlight exposure, the UV-B photons (290-315 nm) that enter the epidermis causes a photochemical transformation of 7-dehydrocholesterol (pro-vitamin D) to pre-vitamin D3. Depending on heat, pre-vitaminD3 is instantaneously transformed into vitamin D3 (cholecalciferol). Remaining UV-B rays convert pre-vitamin D3 into, tachysterol and lumisterol, which are biologically inactive metabolites [64].

Biologically inactive vitamin D3 are carried in the blood by vitamin D binding proteins(DBP), α_2 globulin. Cholecalciferol, enters the circulation and is delivered to the liver, there it is transformed to form 25-hydroxyvitamin D{25(OH)D } catalyzed by the cytochrome P450

enzymes vitamin D 25-hydroxylases (microsomal CYP2R1 and mitochondrial CYP27A1), 25(OH)D is otherwise known as calcidiol or calciferol, which is the inactive form, it is the major circulating form of vitamin D. Calcitriol is the most relevant index to describe the human vitamin D status.

In the proximal tubules of kidney, cytochrome P450 enzyme 1-α-hydroxylase (CYP27B1) further hydroxylates 25(OH)D to 1, $25(OH)_2D$ (calcitriol), which is the biologically active compound. This is followed by systemic transport of 1, $25(OH)_2D$ to distal target organs, where it binds to a plasma membrane receptor and nuclear receptor or both of the target organs, resulting in the production of specific biological responses[65].

24-hydroxylase (CYP24A1) is responsible to convert 25(OH)D and $1,25(OH)_2D$ to $24,25(OH)_2D$ and 25(OH)D-26,23-lactone,which are the inactive metabolites [66], and through a various pathways, further they are converted to the calcitroic acid (1α-hydroxy-23 carboxy-24,25,26,27-tetranorvitamin D3) which is water soluble, this undergoes urinary and biliary excretion[67] .



The above flow chart summarises the formation of vitamin D metabolites

Regulation of vitamin D- endocrine system

Major regulatory factors are

1. $1,25(OH)_2$ D inhibits the production of itself;
2. Parathyroid hormone, enhances the formation of $1,25(OH)_2$D in kidney,
3. serum concentrations of calcium and phosphorus [68]

$1,25(OH)_2$ D concentration is stringently controlled by itself,parathormone, calcium and phosphorus.

1 α hydroxylase step is inhibited by increased phosphorus level and $1,25(OH)_2D$ directly, but inhibited by hypercalcemia indirectly, as hypercalcemia inhibits parathormone secretion. 25(OH)D stimulates 24 hydroxylase, thereby diverting 25(OH)D to form inactive $24,25\ (OH)_2D$.

Metabolites of vitamin D in the circulation.

The major storage and circulating form of vitamin D is 25(OH) D . It is the biologically inactive form, which is usually measured as an record of vitamin D status and it does not bind to vitamin D receptor.The half-life of 25(OH) D is approximately 10–15 days[69]. The concentration of serum 25(OH) D is usually measured as 20–150 nmol/L or 8–60 ng/mL.

$1,25\ (OH)_2$ D is present in the picomolar range in the blood, about one thousandth that of 25(OH) D, is the biologically active form, which binds and acts through vitamin D receptor .The half-life of $1,25(OH)_2D$ is approximately 4–20 hours[70].

There is a strict regulation of 1α-hydroxylation by parathormone, calcium, phosphate. Moreover, as its half-life, ranges between 4 – 20 hours, the serum concentration of $1,25(OH)_2D$ does not give an access for the exact measurement of total vitamin D status. Assessment of calcitriol, necessitates daily measurement, which is impractical.

Measurement of calcitriol is of much use in altered 1α-hydroxylation states, for example: chronic kidney disease (1,25 $(OH)_2D$ is reduced) or granulomatous disease (1,25$(OH)_2D$ is increased).

Vitamin D metabolite concentrations in serum [71,72]

Vitamin D metabolites	Concentration of vitamin D	Half life
Vitamin D	0.2 – 20 ng/mL	1-2 days
25(OH) D	50–100 nmol/L(20–40 ng/mL)	2 – 3 weeks
24,25$(OH)_2$ D	5–12 nmol/L (2–5 ng/mL)	
1,25$(OH)_2$ D	50–125 pmol/L (20–50 pg/mL)	4-6 hours

Vitamin D-binding Protein

All the vitamin D metabolites are carried in the blood by means of carrier protein known as vitamin D-binding protein (DBP) in circulation. DBP is also known as group-specific component, which is a high affinity transport protein. DBP, with a molecular weight of 51,335 and 458 amino-acid residues, is synthesised in liver.

Vitamin D related molecules, i.e.,25 (OH) vitamin D bind with DBP as well as 24, 25$(OH)_2$; comparatively more than1,25$(OH)_2$ vitamin D[73]. The effect of vitamin D metabolites are regulated by DBP in their target

organs. DBP concentrations are decreased in nephrotic syndrome and increased in pregnancy as well as in subjects on treatment with oestrogen.

Molecular actions of 1, 25-dihydroxyvitamin D

Vitamin D and its analogues, initiate their actions via both genomic and non-genomic cascades.1,25$(OH)_2$D acts occur through plasma membrane-initiated mechanisms and distinct nuclear receptor-mediated.

In the cell nucleus, 1,25$(OH)_2$D binds with the VDR to produce genomic effects or in the caveolae of the plasma membrane to produce non- genomic effects (rapid responses).

These rapid responses can occur within 1–2 min to 15–45 min, but this in contrast with genomic responses, generally occurs within few hours to days to become fully blown.

Vitamin D Receptor (VDR)

Vitamin D intensify its actions,by binding with vitamin D receptor (VDR), is a member of the nuclear receptor family of ligand activated transcription factors. VDR was first identified by Haussler and colleagues in 1969[74] and its structure was further described in 1988[75].

Location of VDR gene is on chromosome 12q13.11 and has 11 exons. VDR is an intracellular receptor present in the nucleus. It is

expressed in more than 30 different human tissues and its stimulation accounts over 60 different genes in different cell lines.

VDR is present in

- Cardiovascular system: cardiomyocytes and smooth muscle cells
- Endocrine system: Thyroid C-cells, Parathyroid glands,
  Islets of Langerhans,
- Epidermis : hair follicles, keratinocytes;
- Gastrointestinal System: liver, stomach, esophagus, intestine,
- Immune System: thymus, bone marrow, T and B lymphocytes,
- Renal system: juxta-glomerular cells, ascending Henle's loop
- Respiratory system: alveolar epithelium,
- Osteomuscular system: striated muscle, chondrocytes, osteoblasts,
- Reproductive system: testis, uterus and ovaries;
- Central nervous system: neurons[76].

Mechanism of genomic actions of 1,25$(OH)_2$ D

1, 25$(OH)_2$ D molecules penetrate the plasma membrane by DBP and their genomic actions are activated by triggering the VDR. After binding to the VDR, it give rise to a change in the conformation of the receptor and further hetero dimerization with RXR. The VDR- RXR

complex linked to the VDRE, which is found in the 5' flanking region of target genes.

From the surface of the VDR, the co-repressor (CoR) proteins are released and it allows the binding with the co-activator (CoA) proteins. These molecules change the structure of chromatin then permits the receptor coordination with the RNA polymerase II transcriptional complex (POL II), resulting in the activation of transcription of the target gene[78].

Non-genomic effects:

Non- genomic effects begins in the caveolae of the plasma membrane. Caveolae are flask-shaped plasma membrane invaginations which are rich in cholesterol and sphingolipids, generally present in variety of cells[77].

Cells having rapid response are

- β cells of pancreas,
- adipocytes,
- vascular smooth muscle,
- sertoli cells,
- intestine,
- monocytes and osteoblast.

It involves a plasma membrane receptor and unique $1,25(OH)_2D$ receptor called 1,25D-MARRS (Membrane-Associated, Rapid Response Steroid-binding)[79,80].

Non-genomic actions of 1,25 $(OH)_2D$ stimulate the rapid translocation of calcium across intestinal mucosal membranes. $1,25(OH)_2D$ binds to the membrane receptor resulting in the stimulation of various second messenger systems, which includes phospho-lipase C, G protein-coupled receptors, PKC, or PI3K.

There are several mechanisms, it includes

a) generation of the second messengers
b) opening of the voltage-gated chloride or calcium channels and

   Second messengers, like RAF/MAPK, may alter the cross-talk with the regulation of nuclear gene expression [81].

Non- genomic actions include

i. In osteoblasts, regulation of voltage-gated calcium and chloride channel
ii. calcium entry into the skeletal muscle cell ,
iii. myogenesis and contractility of the skeletal muscle,
iv. uptake of calcium by the intestinal cells
v. secretion of the insulin by the β cells of pancreas.

Biological actions of 1,25$(OH)_2$D

1,25 $(OH)_2$D, through its actions on bone, kidney, small intestine and parathyroids, helps to maintain calcium and phosphate level in blood.

1) **small intestine**

Vitamin D induce calcium absorption in duodenum and phosphate absorption in jejunum and ileum.

a) Uptake of calcium need epithelial calcium channel CaT1 which is 90% vitamin D dependent,
b) calbindin- D9k which is again vitamin D-dependent, allows diffusion within the cell
c) calcium exit from the cell by Ca ATPase (Na^{+} Ca^{2+} exchanger)[82].

1, 25 $(OH)_2$ D aggravate the transport of phosphate via activation of the Na-P1 cotransporter[83]and alters the enterocyte plasma membrane composition which enhances the fluidity and phosphate uptake.

2) **Skeleton**

It directly stimulates osteoblasts and indirectly, by releasing cytokines, facilitates the activation of osteoclasts. Osteoblasts has 1,25$(OH)_2$ D as its receptor, which expresses the (RANKL) receptor, activator of nuclear factor-κB ligand.

The receptor for RANKL is RANK, present on the preosteoclasts. RANK on interaction with RANKL, enhances preosteoclasts to form mature osteoclasts.

Calcium and phosphorus are reabsorbed from the bone by mature osteoclasts, which maintains calcium(Ca^{2+}) and phosphorus($HPO4^{2-}$) levels in the blood. The mineralization of the skeleton is enhanced by maintaining the adequate levels of blood calcium and phosphorus[84].

1,25 $(OH)_2$D regulates osteoclastogenesis by the reciprocal regulation of RANKL and osteoproteregin(OPG).

On the surface of osteoblasts, 1,25$(OH)_2$D- VDR aggravates the RANKL expression. RANK binds with its receptor RANKL,resulting in osteoclastogenesis. On the other hand, 1,25$(OH)_2$D-VDR complex attenuates the OPG expression, this OPG is a receptor that interacts with RANKL, prevent RANK mediated osteoclastogenesis.

$1,25(OH)_2$ D as well as parathormone and prostaglandins stimulate RANKL expression[85], but $1,25(OH)_2$ D prevents OPG production with corresponding rise in osteoclastogenesis and activity of osteoclast.

Thus, bone remodelling is maintained by the regulation of osteoblasts and osteoclasts

3) **kidney**

Vitamin D stimulates calcium and phosphate reabsorption.

$1,25(OH)_2$ D inhibits its own production in an ultra-short negative feedback loop.

4) **Parathyroid gland**

Vitamin D acts directly on parathyroids to attenuate the synthesis and secretion of parathormone, by inhibiting transcription of parathormone gene and by increasing the calcium sensing receptor in parathyroid gland, sensitizing to inhibit calcium.

5) **Vitamin D is an immuomodulator**, stimulates epithelial differentiation.

Non endocrine actions

Inspite of having endocrine effects, vitamin D has autocrine, paracrine and intracrine actions.

1. VDR has been located in the organs like adrenals, heart, parathyroids, pituitary gland, placenta, skin, ovary, mammary glands, testis, hepatocytes, biliary epithelial cells, thymus, pro-myelocytes, lymphocytes and colon[86].

2. 1α-hydroxylase is also isolated in the pancreas, skin, lymph nodes, brain, adrenal medulla, monocytes, macrophages and colon.These organs have the capacity to synthesis $1,25(OH)_2$ D [87] locally.

Newly recognised biological actions of $1, 25(OH)_2$ D are

- More than 200 genes are controlled by $1,25(OH)_2D$, that includes genes involved in the regulation of differentiation of the cell, cellular proliferation, apoptosis, and angiogenesis. It reduces the multiplication of normal/cancer cells and stimulates their terminal differentiation[88].

- Through VDR mediated gene expression, $1,25(OH)_2D$ induces an antimicrobial peptide,cathelicidin. Toll-like receptor activation of human macrophages de-represses the VDR expression and 25(OH) D-1α hydroxylase genes, resulting in the activation of the

cathelicidin and destruction of intracellular *Mycobacterium tuberculosis*[89]

- In human, concentration of 25(OH)D and insulin secretion/action in target tissues are positively correlated. Deficiency of vitamin D has an opposite effect on the function of β cells of pancreas[90].
- VDR and 1α-hydroxylase are located in human brain favouring a role in central nervous system disease.
- Life-threatening infant heart failure[91] is prevented by vitamin D and calcium.
- 1 α,25$(OH)_2$D has an opposite action on the Renin Angiotensin System and blood pressure[92].
- One clinical application is the therapeutic action of 1, 25$(OH)_2$ D and its active analogues in psoriasis[93].

Circulating concentrations of 25(OH) D associated with vitamin D nutritional status[94,95,96]

Serum 25(OH)D	Nutritional status of Vitamin D
>50 nmol/L (>20 ng/mL)	Sufficient
30–50 nmol/L (12–20 ng/mL)	Insufficient
12–30 nmol/L (5–12 ng/mL)	Deficient
>12 nmol/L (>5 ng/mL)	Severely deficient

In Clinical laboratories, serum 25(OH)D are reported by using international system (SI) units (nmol/L) or conventional units (ng/mL). The conversion factor for converting conventional units to SI units is established based on the assessed value, 1 ng/mL = 2.496 nmol/L[97].

Deficiency of Vitamin D increase the risk of:

- Bipolar disease, multiple sclerosis[98],
- Type 1[99] and 2[100] Diabetes Mellitus,
- Obesity,
- Hypertension[101],
- Muscle weakness[102],
- Heart failure, cardiovascular disease[103];
- Cancers[104] (breast, colorectal, prostate cancer)
- Infectious diseases (i.e., tuberculosis[105], seasonal epidemic influenza virus type A, leprosy);
- Asthma and allergy;
- Polycystic ovary disease, menstrual disorders and infertility;
- Crohn's disease[106] and other inflammatory bowel diseases;
- Bone diorders (osteoporosis[107],rickets, osteomalacia)
- Periodontal disease.

- Rheumatoid arthritis[108].

Methods for detecting 25 (OH) D:

1. Immunoassays.
2. High Performance Liquid Chromatography (HPLC).
3. Liquid Chromatography- Tandem Mass spectrometry (LC-TMS).

Methods comparison

1. **Immunoassays :**

Some of the technical aspects of ligand binding assays (radioimmunoassay, competitive protein binding assays) present with practical issues.

- Basically difficult, because these assays operate better in a aqueous phase,but 25(OH) D_2 and 25(OH) D_3 are weakly soluble in water.
- Solubility problems can be overwhelmed by manual extraction assays, but suffer from increased imprecision.
- Some assays amplify vitamin D levels significantly, others undervalue them.

- More usually underestimation is noticed. This is especially noticed in patients on treatment with vitamin D_2, some immunoassays cannot detect 25(OH) D_2.
- Not able to differentiate between 25(OH) $D_2$and 25(OH) D_3.Thus it is tough to assess whether the patients are compliant with therapies or presenting with vitamin D malabsorption.

2**. High Performance Liquid Chromatography (HPLC)**[109]**:**

Technical aspects include

- Overcome most of the problems of the ligand binding assays.
- Necessitates a relatively large sample volume (≥ 1.0 mL).

2. **Liquid Chromatography-Tandem Mass spectrometry LC-MS/MS)** [110].

Technical aspects of LC-MS/MS include

- Precise and Accurate, due to internal standard and its physico- chemical methods.
- Gold standard method.
- Uses specified concentrations of standards.
- Rapid than HPLC.

- Distinguish 25(OH) $D_2$and 25(OH) D_3.
- Requires a smaller sample volume than HPLC (0.25 mL).

Role of vitamin D in type 2 DM

The pathogenesis of Type 2 DM is mainly due to insulin resistance and altered insulin secretion by the β-cells of pancreas. Receptors of Vitamin D are present in the β-cells of pancreas and in the immune cells.

1. Vitamin D is the main regulator for calcium absorption,it also enhances the activity of calcium-dependent endopeptidases present in the β-cell and stimulates the transformation of pro-insulin to insulin and there by enhances the insulin secretion by the β cells of pancreas.
2. By regulating calcium pool, vitamin D stimulates the insulin action directly through the induction of insulin receptors expression in target tissues,and stimulates the intracellular processes mediated by insulin.
3. Vitamin D functions as a potent immunosuppressor. It usually de-represses the various proinflammatory cytokine gene transcription. proinflammatory cytokines involved are IL-2, IL-12, and TNF-α, helps in the stimulation of regulatory T-lymphocytes, the synthesis of anti-inflammatory cytokines and preserves the β-cells of pancreas from destruction.

In addition, Vitamin D mediates several other roles. It regulates the cellular proliferation, replication, differentiation, and autoimmune reactions, in various organs and tissues.

Mechanisms of vitamin D action in Type 2 DM

1. Effect of vitamin D on insulin secretion

- Expression of 1,25 hydroxylase enzyme and Vitamin D receptor in the β-cells of pancreas indicate the role of Vitamin D in the secretion of insulin.
- Vitamin D increases the calcium concentration intracellularly through non-selective voltage-dependent calcium channels and thereby act directly to stimulate β-cell to secrete insulin or it may enhance the activation of β-cell calcium-dependent endo-peptidases that converts pro-insulin to insulin.
- 1,25 $(OH)_2D$ is necessary for exocytosis of insulin by up-regulating the calbindin-D28K expression in the β cells of pancreas[112].

Role of Calbindin-D28K in β cell[111]

Calbindin-D28k is a protein, present predominantly in cytosol. This protein is a member of a family of high affinity calcium-binding proteins which includes S100 protein, calmodulin and parvalbumin[113].

Calbindin is also found to be located in many tissues including kidney, bone and in tissues, such as brain and pancreas [114].

- Calbindin-D28K regulates calcium levels intracellularly in the β cells of pancreas, thus enhancing exocytosis of insulin, a calcium-dependent process.
- calbindin- D28K reduces the inflammatory cytokine-stimulated apoptosis of the β cells of pancreas[115].
- Calbindin-D28K attenuates calcium-mediated mitochondrial damage[116], which leads to increased production of reactive oxygen species and subsequent apoptosis of the β cells of pancreas. Activity of caspase-3 is higher in islets of Langerhans of the individuals with Type 2 DM[117].

Vitamin D plays an integral role in preventing Type 2 Diabetes Mellitus by increasing the secretion of insulin, and overall function of the β cells of pancreas.

2. Effect of vitamin D on insulin action

Vitamin D acts directly, by enhancing the insulin receptor expression further aggravates the responsiveness of insulin for glucose transport[118], or vitamin D acts indirectly by regulating calcium extracellularly which controls normal influx of calcium through cell membranes and maintaining enough intracellular ionised calcium pool in the cytosol.

The role of calcium in insulin related action is of paramount importance. Calcium is required for the insulin regulated intracellular processes happening in skeletal muscle and adipose tissue, which are the principal target tissues for insulin.

Vitamin D increases the insulin receptor expression in target tissues and stimulates its action for glucose transport. It also regulates the transcriptional activation of human insulin gene (Vitamin D Responsive Element [VDRE] present in human insulin gene promoter region) .

3. Effect of vitamin D on cytokines

Low grade inflammation which occurs in DM is probably due to the increase in circulating cytokines such as TNFα and IL-6. When these inflammatory markers are significantly increased, there is an increase in insulin resistance in target tissues such as skeletal muscle and adipose tissue[119].

1. Vitamin D may improve insulin sensitivity.It also enhances the survival of beta cells of pancreas by causing an alteration in the function of cytokines.
2. Vitamin D also binds with vitamin D response elements(VDRE),present in the promoter region of cytokine genes. This interference causes a change in the generation and even the action of cytokines.

3. Vitamin D can down regulate the NF-kB activation. NF-kB, a main transcription factor encoding pro-inflammatory cytokines is downregulated by vitamin D and thereby insulin resistance is decreased.
4. Another important action of vitamin D is the upregulation of calbindin which is a calcium binding protein which is present in many tissues especially beta cells of pancreas and hence interfering with the formation of cytokines.

Due to rise in the cytosolic free calcium, calbindin can protect the cytokine stimulated apoptosis of the β cells of the pancreas,

Supplementation of vitamin D in type 2 DM patients and non-diabetic patients with hypo-vitaminosis enhances the secretion of insulin, suggested that vitamin D aggravates the secretion of insulin by the β cells of the islets of Langerhans [120a] and the action of the insulin in the target organs.

The supplementation of vitamin D reduces the cytokines such as IL-6 and TNF-α resulting in decrease in insulin resistance[120] and thus preventing DM

Mechanisms of action of Vitamin D in Diabetic Nephropathy

Hyperglycemia causes the intra-renal production of factors by down regulating VDR and 1α – hydroxylase in kidney, resulting in decrease in

$1,25(OH)_2$ D re-absorption with increased levels of protein urinary excretion[121].

The combination of hyperglycemia and the absence of VDR results in an intra renal increase of Renin Angiotensin System(RAS) activation, and simultaneously there has been suggestion that deficits in the active metabolite of $1,25(OH)_2$ D indirectly stimulate the activation of TGF -β[122].

In experimental animals, 25 (OH) D has a negative effect on the Renin Angiotensin System, and the lower 25 (OH) vitamin D levels are particularly crucial in triggering Renin Angiotensin System activation and hyperfiltration, which are the main characteristic features of Diabetic Nephropathy [123]

Pharmacological use of vitamin D analogues to block Renin Angiotensin System activation exerts a therapeutic effect by increasing the action of Renin Angiotensin System blockers[124].

The mechanisms of action of 1,25 $(OH)_2$ D in the pathogenesis of proteinuria include

1. Haemodynamic and
2. Non -haemodynamic actions that regulate cell proliferation, angiogenesis, apoptosis, and anti -inflammatory action.

1. Hemodynamic action

In Diabetic Nephropathy, the protective action of $1,25(OH)_2$ D is due to its negative regulatory effect on the RAS, by suppressing the

production of renin which is one of the mechanisms responsible for renal injury.

Renin Angiotensin System plays an integral role in the maintanence of blood pressure, homeostasis of fluid and electrolyte[125]. Renin is a protease produced and secreted predominantly by the juxtaglomerular (JG) cells in the kidney. In human, renin is encoded by one gene. Renin formation is the first and rate limiting step in the Renin Angiotensin System.

Cyclic AMP (cAMP) is a main intracellular signal that enhances the synthesis of renin in JG cells. cAMP signals through cAMP response elements (CRE) present in the target gene promoters, interact with the members of the ATF/CREB/CREM bZIP transcription factor family which are in homodimeric or heterodimeric forms.

Adenylate cyclase which are activated by membrane receptors converts ATP to intracellular cAMP . cAMP thus formed, interacts with the regulatory subunit of protein kinase A (PKA) to release the catalytic subunit, which enters the nucleus and phosphorylates CREM at serine 117 or CREB at serine133. This expands the assembly of ubiquitous co-activators CBP/p300 to promote the gene transcription[126].Indeed, a number of CREs have been established in renin gene promoters that play essential roles in transcription of renin gene.

Liganded VDR binds to CREB in the presence of 1,25 $(OH)_2D$ and inhibits the interaction of CREB to the CRE. This step crucially interrupt the formation of CRE-CREBCBP/p300 complex. Finally this leads to decrease in renin gene expression[127].

2. **Non -haemodynamic actions**:

- 1,25$(OH)_2D$ seems to inhibit myofibroblasts proliferation in

the renal interstitium by stimulating hepatocyte growth factors, thus performing a protective effect on the kidneys by suppressing the activation of myofibroblast production in the matrix[128].

- In some experimental models, administration of 1,25$(OH)_2D$ decreased the loss of podocytes and inhibited their hypertrophy[129]. This beneficial effect is due to the direct action in signal modulation, by inhibiting TGF -β and bone morphogenetic protein (BMP -7) expression[130].

1,25$(OH)_2D$ and its analogues reduce proteinuria, a biomarker of kidney involvement. Thus, 1,25$(OH)_2D$ have protective functions by promoting the reduction of proteinuria.

In some animal models, 1,25$(OH)_2D$ and its analogues not only decreased the levels of proteinuria but also preserved the structure of glomerular podocyte apart from reducing TGF-β levels, an enhancer of

renal fibrosis. Apart from these, mesangial cell proliferation[131] which is a marker of renal injury is attenuated.

MATERIALS AND METHODS

The present study on the serum 25(OH) D in patients with Type2 DM with and without Nephropathy is a case-control investigation**,** carried at Diabetology Outpatient clinic in Rajiv Gandhi Government General Hospital, Chennai. This work was conducted after obtaining Institutional Ethical Committee clearance.

The Study group included total number of 90 known diabetics of more than 5 years duration,of which the control group were Type 2 DM patients without nephropathy and the case group were patients with Diabetic Nephropathy.The control group were 45, out of which 22 were males and 23 were females, the case group were 45, out of which 24 were males and 21 were females.

Inclusion criteria:

Diabetics were diagnosed by ADA (American Diabetes Association) criteria

Any one of the following is diagnostic

A. Glucose

- Fasting Plasma Glucose (FPG) ≥ 126mg/dL (7.0mmol/ L) (or)
- Hyperglycemia symptoms and Random Plasma glucose levels ≥200mg/dL(11.1mmol/L) (or)

- 2 hour Plasma glucose ≥200mg/dL(11.1mmol/L)

B. Hemoglobin A1C (HbA1C)

HbA1C ≥6.5%

Exclusion criteria:

- Liver failure patients,
- vitamin D deficiency patients,
- Type 1 diabetes mellitus patients with and without nephropathy,
- Parathyroid disorders,
- Patients with diabetic nephropathy with other coexisting disease like obstructive uropathy, chronic glomerulonephritis
- Patients having malabsorption syndrome,
- Patients on drugs like barbiturates,phenytoin,Rifampicin,calcium, vitamin D
- Pregnant and lactating mothers.

Blood Sample collection

Blood collection was done after an overnight fast for 8-12 hours. About 5ml of blood was drawn from the ante-cubital vein of the study subjects and transferred into red topped serum tubes, from which all the parameters of the study were estimated.

The blood samples were analysed on the same day within 4 hours of blood collection. The biochemical parameters relevant to the study were analysed by the following methodologies.

Urine Sample Collection

Urine samples were collected after giving proper instructions to the patient

- First voided sample was discarded

- Early morning Mid-stream urine specimen was collected

- Sample was collected in plastic sterile containers.

ESTIMATION OF URINE ALBUMIN CREATININE RATIO (UACR)

Step 1 : Estimation of Albumin Concentration in Urine Sample

Methodology : Latex Agglutination method / Immunoturbidimetry

Kit from **Biosystems Reagents & Instruments**

Lot number: **COD 31924(1 x 50 mL)**

Principle:

- Latex particles coated with antihuman albumin are agglutinated by the albumin in the urine sample.
- The agglutination of the latex particles is directly proportional to the concentration of the albumin in the urine and can be measured by turbidimetry.

Composition of reagent:

Reagent A: Borate buffer 0.1mol/L, Sodium azide 0.95g/L,pH 10.0

Reagent B: Suspesion of Latex particles coated with anti-human albumin antibodies,Sodium Azide 0.95g/L

Albumin Standard: 50.8g/L

Reagent preparation:

Working reagent: Prepared the working reagent by mixing Reagent B vial into Reagent A Bottle.

Albumin standard: Standard was reconstituted with 1ml of distilled water

Procedure:

- Working reagent and instrument were brought to 37˚c
- 1mL of working reagent and 7µL of standard/sample were pipetted out
- After mixing, cuvette was inserted in the instrument
- Absorbance was read after 10 seconds (A1) and after 2min(A2) at 540nm.

Calculation

The concentration of the albumin in the sample was calculated using the formula

$$\text{Conc. of Albumin(mg/L)} = \frac{\Delta \text{ A of Test}}{\Delta \text{A of Standard}} \times \text{Concentration of Standard}$$

Reference interval

Urine Albumin = 3.9 to 24.4mg/day

Estimation of the creatinine concentration in the urine sample

Methodology : Modified Jaffe’s method

Principle

Creatinine in the sample combines with alkaline picrate to form creatinine picrate(orange-yellow colour). The intensity of the colour is directly proportional to amount of creatinine present in the sample and is measured spectro-photometrically at 500-520nm.

Composition of reagent

Reagent 1: picric acid reagent – picric acid – 25.8 mmol/L

Reagent 2: sodium hydroxide reagent - sodium hydroxide – 95 mmol/L

Preparation of working reagent

Working reagent was prepared by mixing equal volumes of Reagent 1 and 2. Waited for 15 min before use.

Standard concentration – 2.62mg/dL which is traceable to Isotope Dilution Mass Spectrometry(IDMS).

Sample preparation

Dilute urine samples with 1:10 dilution

Procedure

The analyser was first calibrated using the creatinine standard traceable to IDMS.100µL of diluted sample was added to 1000µL of working reagent. It was mixed well and fed immediately into semi-automatic analyser. The reading obtained was multiplied by the dilution factor to get correct concentration of creatinine in urine sample.

Calculation:

$$\text{Creatinine (mg/dL)} = \frac{\Delta\text{A of Test}}{\Delta\text{A of Standard}} \times \text{Concentration of Standard x dilution factor}$$

Creatinine (g/L) = Creatinine (mg/dL) / 100

Reference interval

Urine creatinine: Adult Male = 14 to 26 mg/kg/day

Adult Female=11 to 20mg/kg/day

Urine Albumin Creatinine Ratio is calculated by the following equation:

$$UACR = \frac{\text{Urine Albumin (mg/L)}}{\text{Urine Creatinine (g/L)}}$$

UACR = mg/g of Creatinine.

Normal UACR = <30 mg of albumin/gm of creatinine,

Microalbuminuria = 30-300 mg of albumin/gm of creatinine,

Macroalbuminuria = > 300mg of albumin/gm of creatinine.

ESTIMATION OF 25 HYDROXY VITAMIN D BY IMMUNOASSAY

Methodology:

Quantitative competitive enzyme immunoassay

Kit from LiLo Diagnostics

Principle:

- 25(OH)D test is a competitive binding assay. It is a direct solid-phase Enzyme-Linked ImmunoAssay (ELISA),
- Calibrators,controls and samples are directly added into a pre-designed anti 25(OH)D, highly specific, monoclonal antibody coated micro-wells.
- A working solution of 200µL biotin labeled 25-OH D reagent is added into each well.
- The reaction mixture is incubated for 90minutes at room temperature, preferably in dark.

- During the first incubation, a biotin-labeled 25(OH) vitamin D binds with the endogenous Vitamin D metabolites in the Calibrators, Controls and sample for a fixed number of binding sites on the anti-Vitamin D metabolites' antibody.

- 100µL Enzyme (horseradish peroxidase) labeled streptavidin, is added and interacts selectively to complexed biotin and, after second wash step,100µL solution of a chromogenic substance, tetramethylbenzidine (TMB) reagent, is added, then incubated at room temperature for 15 minutes, resulting in the development of blue colour.
- Development of the colour is stopped by adding 50µL 1M HCl stop solution. and the intensity of the colour is measured using microtitre plate reader at 450nm,the colour intensity developed being inversely proportional to the 25(OH)D concentration in the sample.

A standard curve was obtained by plotting the absorbance versus the concentration of the standard.

Materials:

- **Standard (STD) 1-6:**

Human serum based buffer containing 25-hydroxyvitamin D, 0.2ml per standard Level. STD 1- 0 ng/mL,STD 2- 2 ng/mL, STD 3- 5 ng/mL,STD 4- 15 ng/mL,STD 5 - 40 ng/mL, STD 6 - 80 ng/mL

- **Microplate - Antibody Coated Plate:**

A 96 well microplate (12stripsx8wells) coated with a highly specific antibody to (OH) vitamin D and its metabolites immobilized on the inner surface of the polystyrene wells.

- **25(OH)D biotin concentrate:**

A proprietary stabilizing buffer containing 25-hydroxyvitamin D labeled with biotin, 0.25mL in a vial. To be diluted with Sample Buffer

- **Sample - Buffer :**

A bottle of 22mL proprietary Buffer reagent.

- **Enzyme conjugate :**

A bottle of 11mL proprietary stabilizing buffer containing streptavidin linked to horseradish peroxidase.

- **Controls 1 – 2 :**

Two vial of 0.2mL per level of control in human serum based

Buffer containing25(OH)D. control 1- 10.8 ng/mL, control 2 – 64.7 ng/mL

TMB Substrate :

A bottle of 11mL proprietary aqueous formulation of TMB and hydrogen peroxide.

- **HCL - Stop Solution :**

0.5M Hydrochloric Acid, 7 mL per kit.

- **Wash Buffer Concentrate:**

20ml Tris based buffered saline containing Tween, 20 mL per bottle.

Additional materials used

- Micro-plate reader capable of measuring absorbance at 450nm.
- Precision pipettes.
- Adsorbent paper
- Aluminum foil
- Distilled deionized water

Preparation of Reagents:

- **100X Biotin Reagent:** Reagent was mixed thoroughly before diluting. Prepared 1X working solution at 1:100 with Sample Buffer

- **20X Wash buffer:** Prepared 1X Wash Buffer by adding the contents of the bottle(25mL) to 475 mL of distilled or deionized water.

Assay Procedure:

1. 10µL of standards, controls, and patient specimens were added into pre-designated wells.
2. 200µL working solution (1x) of biotin reagent was added into each well and it was mixed on a plate shaker (400 - 600rpm) for 30 seconds.

3. The plate was covered with an adhesive plate sealer. Incubated at room temperature (18-25°C) for 90minutes.

4. The contents of the wells were decanted by inverting it.

   250 µL of 1x Wash Solution was dispensed to all wells. This was decanted and repeated four times. To remove excess Wash Solution, inverted plate was tapped firmly on absorbent tissue to remove excess wash solution.
5. 100µL of Enzyme Conjugate reagent was added to all wells using a Micro- pipette. Then,incubated at room temperature (18-25°C) for 30 minutes.
6. Repeat the wash step 4.
7. 100µL of TMB Substrate was added to all wells using, a micro pipette. Incubated at room temperature (18-25°C) for 15 to 20 minutes.

8. 50µL of Stop Solution was added to all wells, in the same order the substrate was added, using a micro pipette.
9. The absorbance of each well was measured at 450 nm using microplate reader, within 10 minutes of adding the Stop Solution

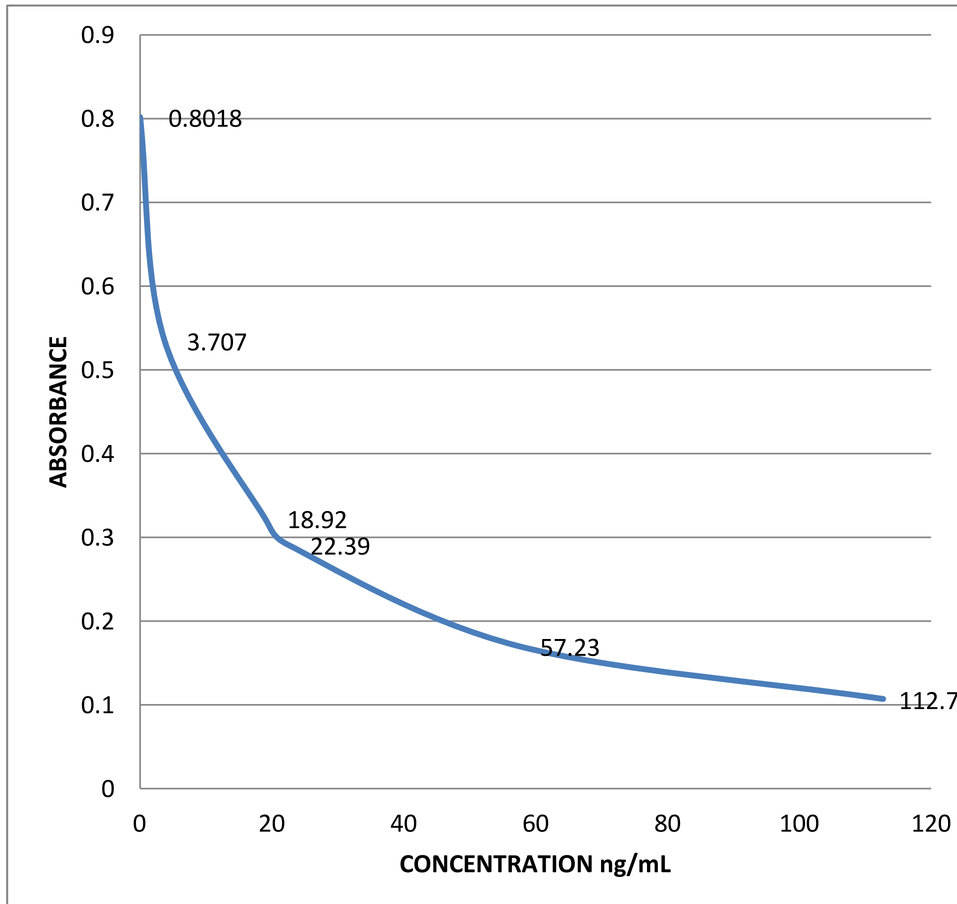
Calculation of results

A standard curve was plotted with standard concentration on x-axis and percentage of absorbance on y-axis. Through the standard points, the best fit straight line was drawn.

The following table shows the absorbance (Optical density) of each standards and the concentration of 25 hydroxy vitamin D in the sample is obtained based on this absorbance.

S.NO	Absorbance (Optical density)	Concentration of Standard (ng/mL)
1	0.1067	112.7
2	0.1702	57.23
3	0.2911	22.39
4	0.3227	18.92
5	0.5348	3.707
6	0.8018	0

Point to point curve



SERUM CREATININE

Methodology : Modified Jaffe's method

Principle:

Creatinine in the sample undergoes chemical reaction with alkaline picrate to form creatinine picrate (orange-yellow colour).The colour intensity is directly proportional to amount of creatinine present in the sample and is measured spectro-photometrically at 500-520nm.

Composition of the reagent:

Reagent 1: picric acid reagent – picric acid – 25.8 mmol/L

Reagent 2: sodium hydroxide reagent - sodium hydroxide – 95 mmol/L

Preparation of working reagent:

Equal volume of Reagent 1 and 2 are mixed to prepare the working reagent.kept for 15 min before use.

Standard concentration – 2.62mg/dl which is traceable to IDMS.

Procedure:

The analyser was first calibrated using the creatinine standard traceable to IDMS.

100µL of diluted sample was added to 1000µL of working reagent. It was mixed well and fed immediately into semi-automatic analyser.

Calculation:

$$\text{Creatinine (mg/dL)} = \frac{\Delta A \text{ of Test}}{\Delta A \text{ of Standard}} \times \text{Concentration of Standard}$$

Reference interval:

Males – 0.7-1.4mg/ dL

Females – 0.6-1.2mg/dl.

ESTIMATED GLOMERULAR FILTRATION RATE (eGFR):

eGFR is calculated using Serum Creatinine by Modification of Diet in Renal Disease(MDRD) formula as follows:

Estimated GFR ($mL/min/1.73\ m^2$) = 175 × ($Sr_{creatinine}$) $^{-1.154}$ × (Age)$^{-0.203}$ × (0.742 if female)× (1.212 if African American)

$Sr_{creatinine}$ = Serum creatinine calibrated using IDMS calibrator.

According to the National Kidney Foundation,

Normal GFR ranges from 90 - 120 $mL/min/1.73\ m^2$.

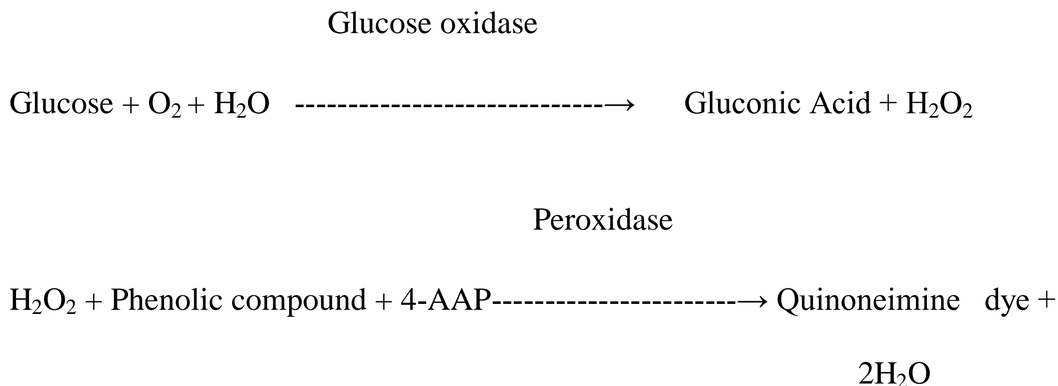
ESTIMATION OF SERUM GLUCOSE

Methodology : Glucose oxidase-peroxidase method(GOD-POD),

endpoint method

Principle:

Glucose present in the sample is oxidised to yield gluconic acid and hydrogenperoxide in the presence of glucose oxidase.The enzyme peroxidase converts the oxidative coupling of 4-aminoantipyrine with phenol to yield a coloured quinoneimine complex with the absorbance proportional to the concentration of glucose in the sample.



Procedure :

To 1mL of working solution,10µL of serum was added and incubated at 37°c for 15 mins and absorbance was measured at 505nm

Calculation:

$$\text{Glucose (mg/dl)} = \frac{\text{Absorbance of test} \times \text{Conc. of standard(100mg/dL)}}{\text{Absorbance of standard}}$$

Reference interval:

Fasting glucose = 70 – 100mg/dL

ESTIMATION OF SERUM CALCIUM:

Methodology : Arsenazo III method, end point

Principle :

At neutral pH, Calcium reacts with arsenazo III, yields a blue coloured complex. The colour intensity is proportional to the concentration of calcium present in the sample.

Procedure :

To 1ml of working solution,25µL of serum was added and incubated at room temperature for 5 mins and absorbance was measured at 650nm.

Standard concentration: 10 mg/dL

Calculation:

$$\text{calcium (mg/dl)} = \frac{\text{Absorbance of Test} \times \text{Conc. Of Standard(10mg/dl)}}{\text{Absorbance of Standard}}$$

Reference interval:

Serum calcium = 8.8-10.2mg/dL

ESTIMATION OF SERUM PHOSPHORUS:

Methodology : ammonium molybdate method, end point.

Principle :

Inorganic phosphorus combines with ammonium molybdate in the presence of strong acids to form phosphomolybdate. The formation of phosphomolybdate is measured at 340 nm and is directly proportional to the inorganic phosphorus concentration in the sample.

Reagent composition: ammonium molybdate, sulphuric acid, surfactant.

Standard concentration: 5 mg/dL

Procedure :

To 1mL of working solution,20µL of serum was added and incubated at 37°C for 5 mins and absorbance was measured at 340nm.

Calculation:

$$\text{Inorganic phosphorus (mg/dl)} = \frac{\text{Absorbance of Test} \times \text{Conc. Of Standard}}{\text{Absorbance of Standard}}$$

Reference interval:

Serum phosphorus = 2.5- 4.5mg/dl.

STATISTICAL ANALYSIS

1. Data were analysed using SPSS statistical software version 16 version and was considered as statistically significant when the p value is less than 0.05.

2. Variables like BMI, serum creatinine, urine albumin creatinine ratio,

eGFR, serum calcium, serum phosphorus, Serum 25 hydroxy vitamin D between cases and controls were compared by unpaired ‘t’ test.

3. Pearson coefficient correlation was done to measure the linear relationship between

UACR and e GFR,

UACR and vitamin D, e GFR and vitamin D,

serum creatinine and 25 (OH)D,

Fasting glucose and 25 (OH)D,

Serum calcium and 25 (OH)D,

Serum Phosphorus and 25 (OH)D.

RESULTS

Age distribution of DN patients and controls

In the present study, in total number of 90 patients, 45 patients with Type 2 DM patients without nephropathy (controls) and 45 patients with diabetic nephropathy (cases) were investigated. The mean and standard deviation of the age of the cases and controls were 58.82 ±5.11 and 56.71±7.11, respectively. There was no statistical difference in the age between cases and controls (p = 0.110). Thus the study included age matched cases and controls as subjects. The details of age distribution among cases and controls were presented in table1,2 and figure 1,2.

Gender distribution of DN patients and controls

In diabetic nephropathy, Males constituted higher percentage than females. in control group, Females constituted higher percentage than males. There was no significant difference in gender among case and controls (p = 0.83). Thus the study included gender matched cases and controls as subjects. The details of age distribution among cases and controls were presented in table 3 and figure 3.

Table 1. Age distribution among controls

Age	Number of individuals	Percentage
41 – 50 years	12	26.66%
51 – 60 years	17	37.77%
61 – 70 years	16	33.33%
>70 years	1	2.22%

Fig 1. Age distribution among controls

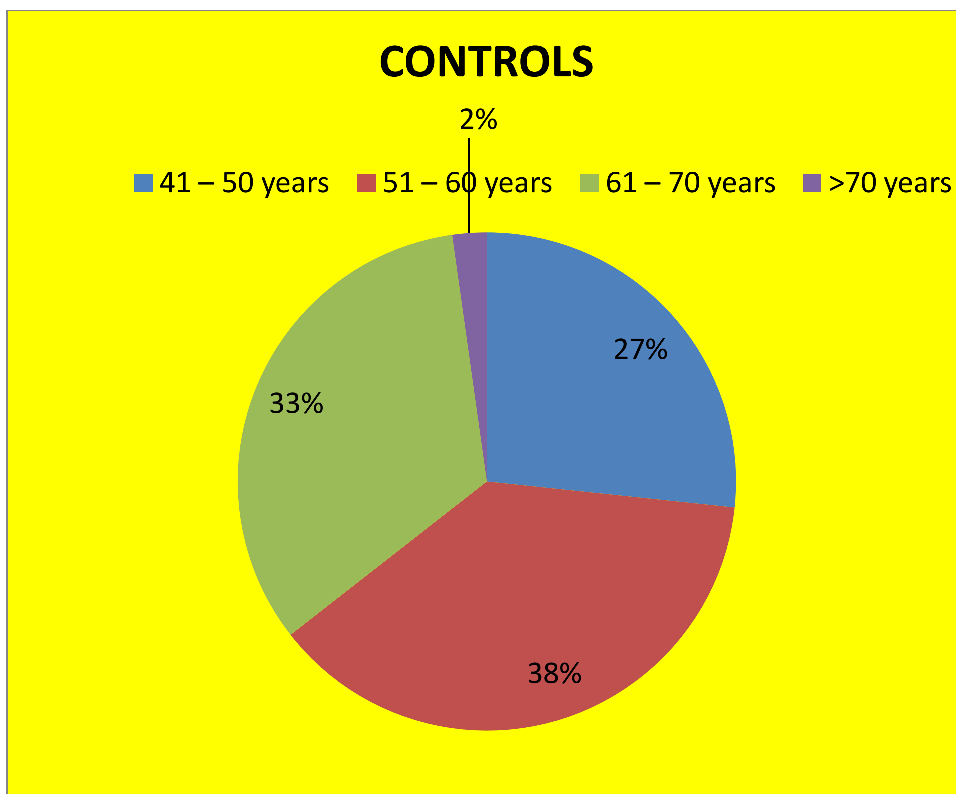


Table2.Age distribution among DN patients

Age	Number of individuals	Percentage
41 – 50 years	3	6.66%
51 – 60 years	28	62.22%
61 – 70 years	13	28.88%
>70 years	1	2.22%

Fig 2. Age distribution among DN patients

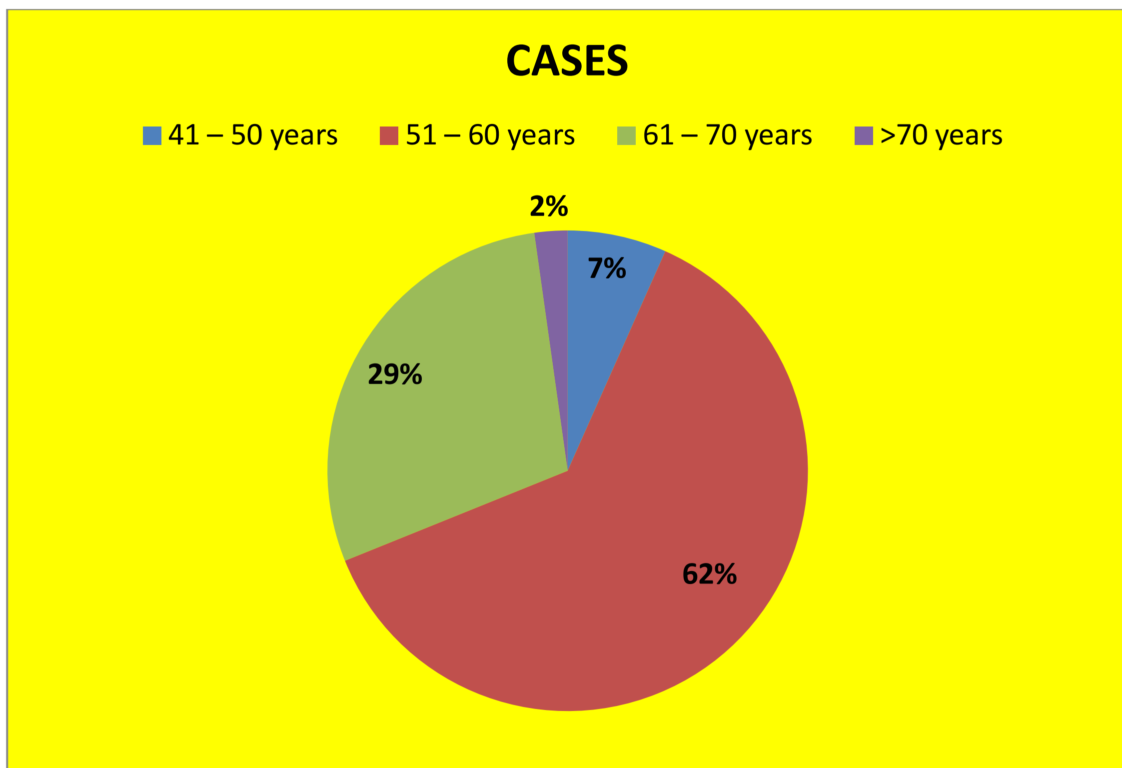


Table 3.Gender distribution among the DN patients and controls

Gender	controls		Cases	
	Number of individuals	percentage	Number of individuals	Percentage
Male	22	49%	24	53.33%
Female	23	51%	21	46.66%

Fig 3.Gender distribution among the DN patients and controls

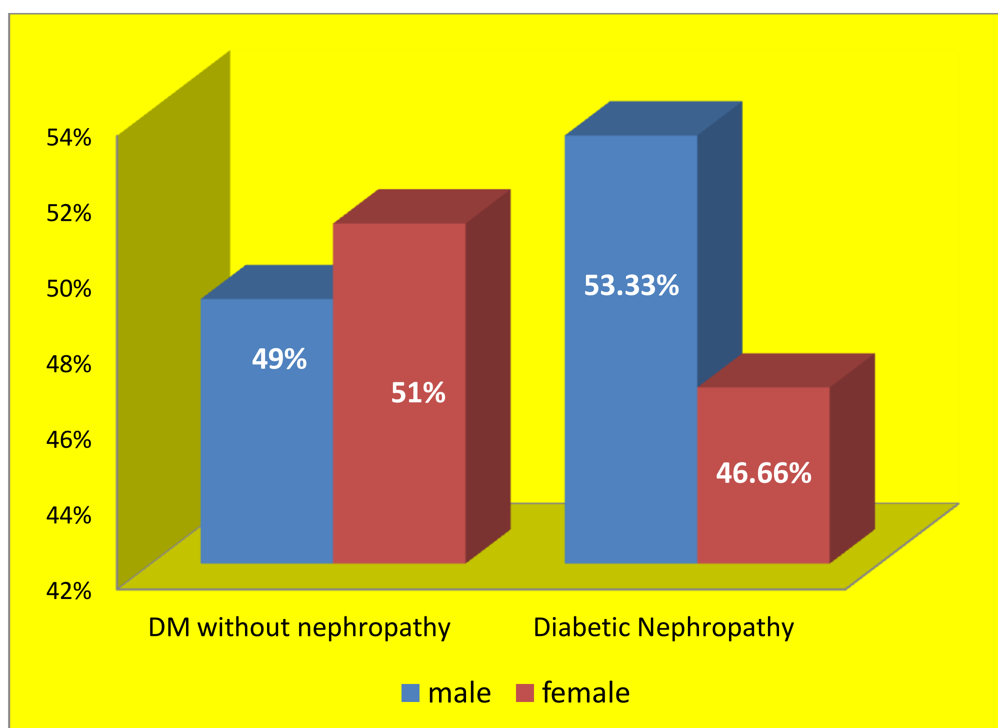


Table 4.Characteristics of patients in the study population

Variables		Control	Case	p value
AGE		56.71± 7.11	58.82± 5.11	0.11 –NS
GENDER	MALE	22(48.88%)	24(53.33%)	0.83 – NS
	FEMALE	23(51.11%)	21(46.66%)	
HYPERTENSION		16(35%)	20(44.44%)	0.51 –NS
SMOKING		5(11.11%)	7(15.55%)	0.75 – NS
ALCOHOLISM		4(8.88%)	4(8.88%)	1.00 – NS
BMI		27.21± 1.78	28.79± 2.47	0.01 – S
UACR		2.56± .64	124.89± 67.29	0.000- HS
e GFR		1.22 ± 7.07	111.51 ± 4.32	0.000- HS
CREATININE		.62 ± .06	.68± .08	0.000- HS
FASTING GLUCOSE		127.78 ± 29.72	188.44± 70.86	0.000- HS
CALCIUM		9.63± .37	9.63± .37	1.000- NS
PHOSPHORUS		3.04 ± .33	3.08± .37	0.540- NS

NS – NOT SIGNIFICANT if p value > 0.05

HS – HIGHLY SIGNIFICANT if p value < 0.001

S - SIGNIFICANT if p value < 0.05

Comparison of patient characteristics

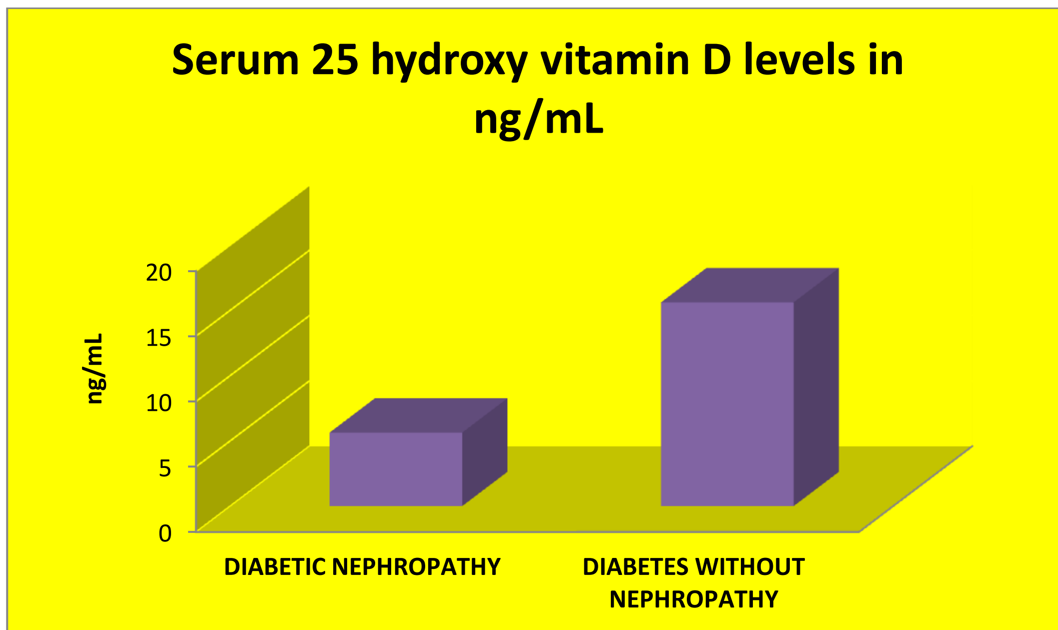
Table 4 compares the mean, standard deviation and p values for cases and controls with respect to variables like smoking, alcohol intake, BMI, presence of hypertension, UACR, eGFR, fasting glucose, serum creatinine, serum calcium, serum phosphorus.

There was highly significant difference between cases and controls with respect to UACR,eGFR, fasting glucose, serum creatinine(p value <0.001). There was significant difference between cases and controls with respect to BMI(p value<0.05). While there was no significant difference among cases and controls with respect to smoking, alcohol intake, the presence of hypertension, serum calcium, serum phosphorus.

Table 5. Serum 25 Hydroxy vitamin D levels among DN patients and controls.

25 Hydroxy vitamin D	Cases		Controls		Student 't' test
	Mean	SD	Mean	SD	.000 – HS
	5.6309	2.34	15.6062	3.46	

Fig 4. Serum 25 Hydroxy vitamin D levels in ng/mL among DN patients and controls (mean± SD)

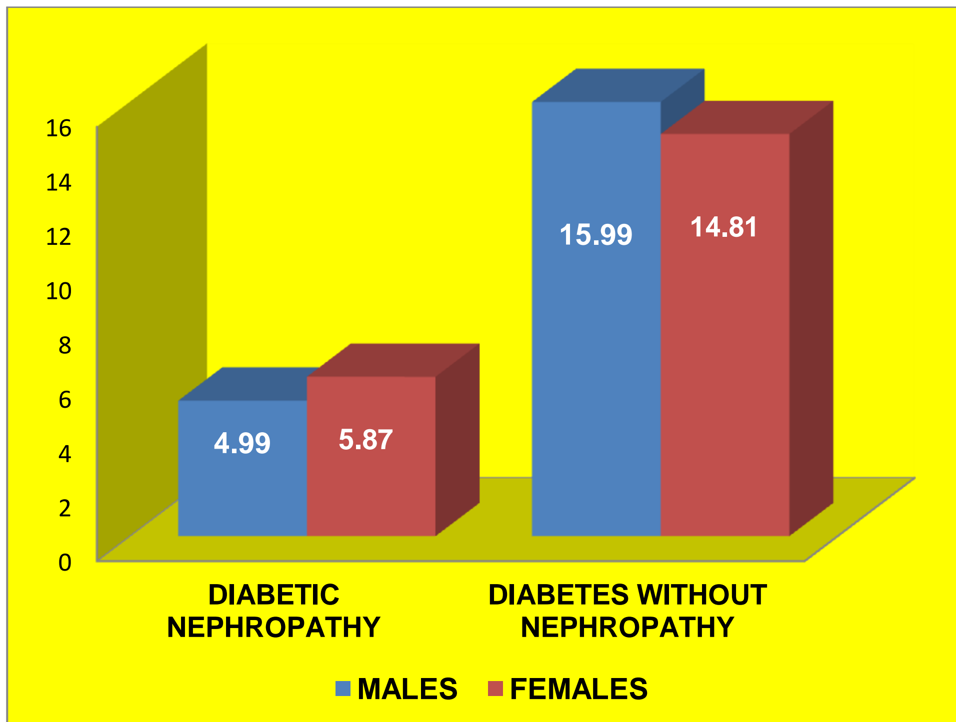


Concentration of serum 25 Hydroxy vitamin D levels among DN patients and controls

The mean and standard deviation of the 25 Hydroxy vitamin D levels among DN patients and controls were presented in Table 5 and figure 4.

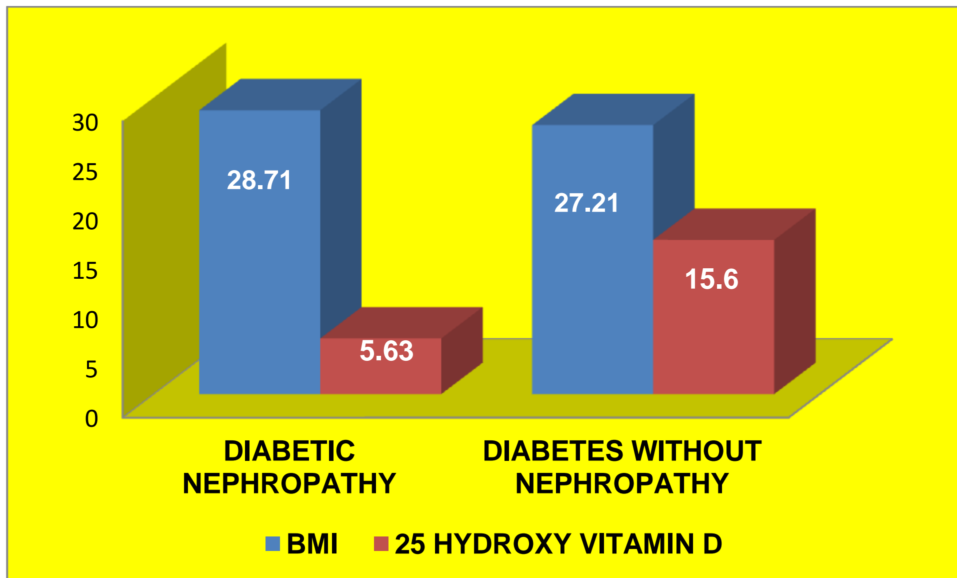
The mean 25 Hydroxy vitamin D concentration in cases (patients with diabetic nephropathy) was 5.63± 2.34 ng/ml, while in controls(diabetic patients without nephropathy) it was 15.60±3.46ng/ml. The difference in 25 Hydroxy vitamin D values between cases and controls were highly significant (p= 0.000).

Fig 5.Serum 25 hydroxy vitamin D levels in ng/mL among males and females in both the groups



In fig 5, male and female distribution in both the groups were depicted. In our study, in Diabetic Nephropathy, 25 hydroxy vitamin D levels were comparatively increased in females than in males. In Type 2 Diabetes Mellitus, 25 hydroxy vitamin D levels were comparatively increased in males than in females.

Fig6. Comparison of BMI and 25 hydroxy vitamin D in DN and in Type 2 DM



This figure 6 shows the relationship between BMI and vitamin D in diabetic nephropathy and in Type 2 Diabetes Mellitus , there was an inverse relationship between BMI and vitamin D in both groups.

Table 6: Pearson correlation co-efficient to measure the relationship between UACR and e GFR

	Cases	**Controls**
correlation	0.017	0.229
p value	0.912	0.136
N	45	45

Table 6 showed Pearson correlation co-efficient to measure the relationship between UACR and e GFR. Pearson coefficient correlation was done on variables like UACR and eGFR in order to find the linear relationship in both the groups. There was weaker positive correlation between UACR and e GFR in both cases & controls.

Table.7 Pearson correlation co-efficient to measure the relationship between e GFR and vitamin D

	Cases	Controls
correlation	-0.145	0.103
p value	0.341	0.501
N	45	45

Table.7 showed Pearson correlation co-efficient to measure the relationship between e GFR and vitamin D

Pearson coefficient correlation was done on variables like eGFR and vitamin D in order to find the linear relationship in both the groups. It was observed that,the concentration of eGFR increases, the concentration of vitamin D decreases with a weaker negative linear relationship with

r=-0.145 in cases.

Fig 7.Comparison of e GFR and 25 hydroxy vitamin D in DN and in Type 2 DM

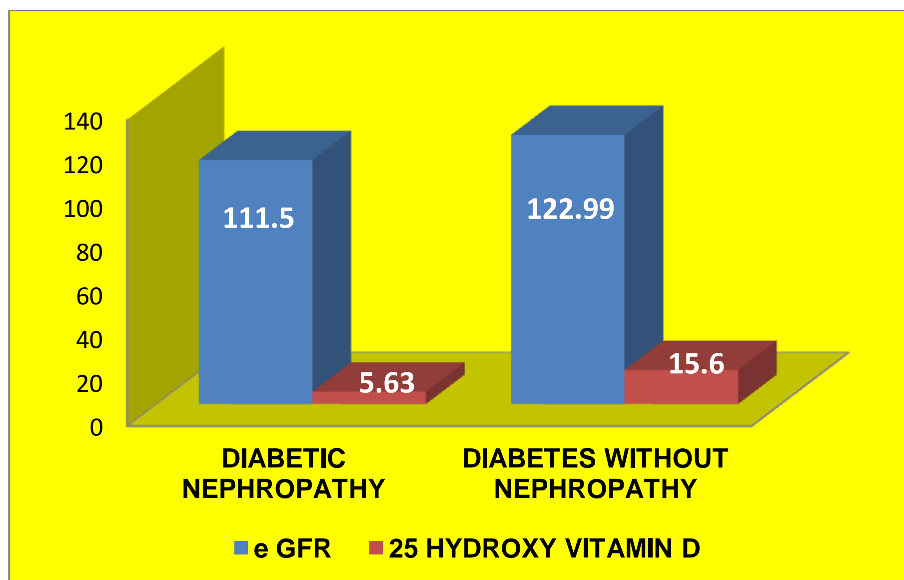


Figure 7 showed in both diabetic nephropathy and in Type 2 Diabetes Mellitus, there was an inverse relationship between e GFR and 25 hydroxy vitamin D .Correlation was significant at the 0.01 level (2-tailed).

Table 8.Pearson correlation co-efficient to measure the relationship between UACR and vitamin D

	Cases	Controls
correlation	-0.115	0.060
p value	0.451	0.699
N	45	45

Table 8 showed Pearson correlation co-efficient to measure the relationship between UACR and vitamin D

Pearson coefficient correlation was done on variables like UACR and vitamin D in order to find the linear relationship in both the groups. It was observed that in cases, the concentration of UACR increases,the concentration of vitamin D decreases with a weaker negative linear relationship with r=-0.115.

Fig.8.Comparison of UACR and 25 hydroxy vitamin D in DN and in Type 2 DM

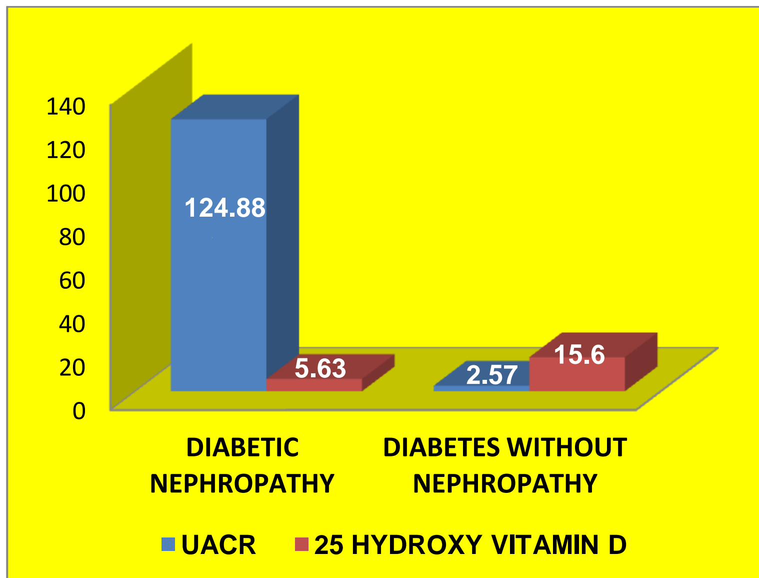


Figure 8 showed, in DN and in Type 2 DM, there was an inverse relationship between UACR and vitamin D, Correlation was significant at the 0.01 level (2-tailed).

In National Health And Nutrition Examination Study (NHANES) III study, there was a relationship between the increase in albuminuria and decrease in plasma 25(OH) D[132].

Table.9 Pearson correlation co-efficient to measure the relationship between vitamin D and serum creatinine

	Cases	**Controls**
correlation	-0.029	-0.029
p value	0.851	0.849
N	45	45

Table 9 showed Pearson correlation co-efficient to measure the relationship between vitamin D and serum creatinine

Pearson coefficient correlation was done on variables like vitamin D and serum creatinine in order to find the linear relationship in both the groups.

It is observed that in cases, as the concentration of serum creatinine increased,the concentration of vitamin D was decreased with a weaker negative linear relationship with r=-0.029.

Figure.9.Comparison of serum creatinine and 25 hydroxy vitamin D in DN and in Type 2 DM:

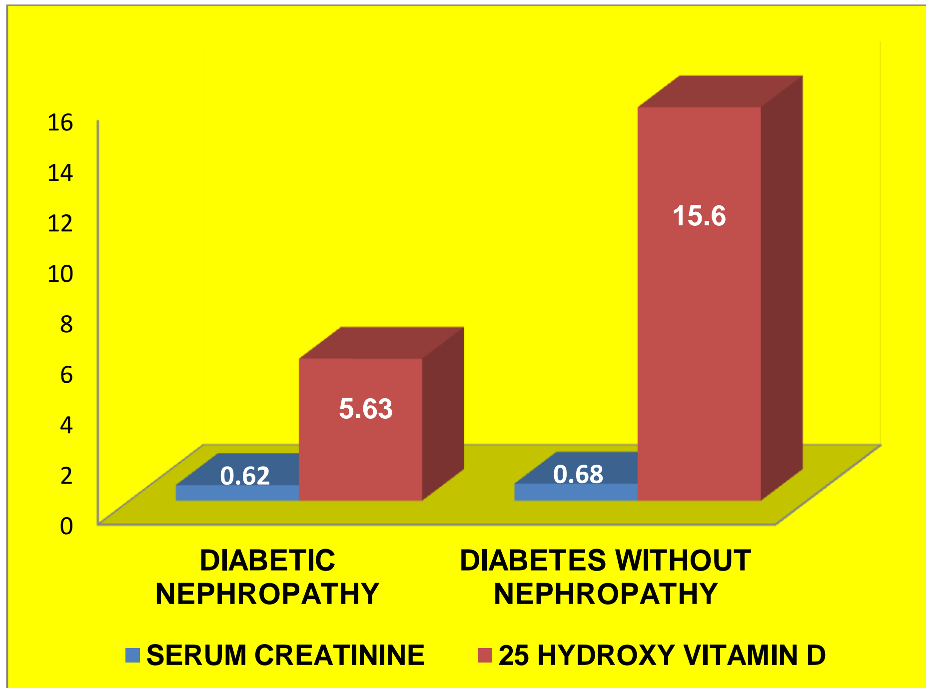


Figure 9 showed in DN and in Type 2 DM, there was inverse relationship between serum creatinine and serum 25 hydroxy vitamin D

Correlation was significant at the 0.01 level (2-tailed).

Table 10.Pearson correlation co-efficient to measure the relationship between vitamin D and fasting glucose

	Cases	**Controls**
correlation	-0.022	0.051
p value	0.884	0.737
N	45	45

Table 10 showed Pearson correlation co-efficient to measure the relationship between vitamin D and fasting glucose

Pearson coefficient correlation was done on variables like vitamin D and fasting glucose in order to find the linear relationship in both the groups. It was observed that in cases, as the concentration of fasting glucose increased,the concentration of vitamin D decreased with a weaker negative linear relationship with r=-0.022

Fig.10.Comparison of fasting glucose and 25 hydroxy vitamin D in DN and in Type 2 DM:

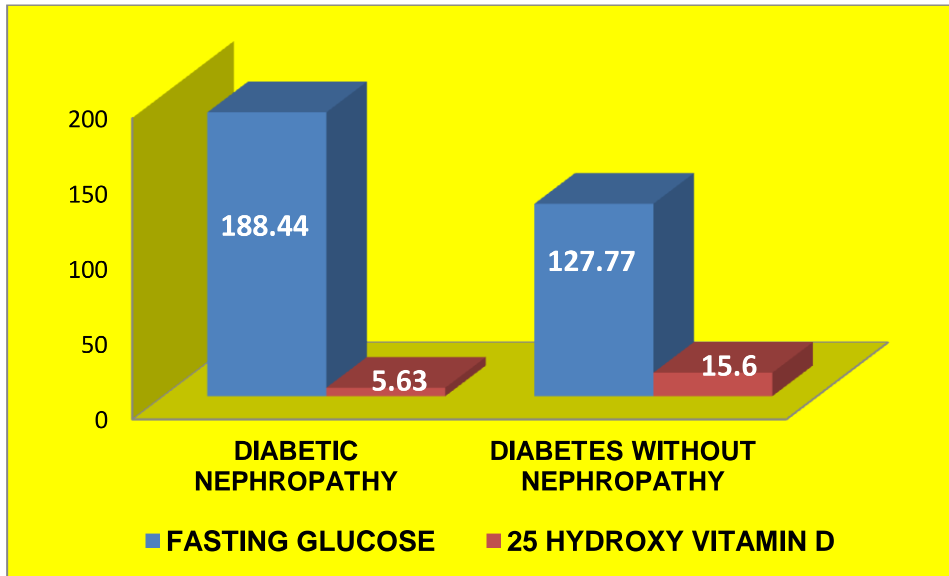


Fig.10 showed the comparison of fasting glucose and 25 hydroxy vitamin D in in DN and in Type 2 DM

Figure 10 showed in DN and in Type 2 DM, there was an inverse relationship between serum creatinine and serum 25 hydroxy vitamin D

Correlation was significant at the 0.01 level (2-tailed).

Table11.Pearson correlation co-efficient to measure the relationship between serum 25 hydroxy vitamin D and calcium

	Cases	**Controls**
correlation	-0.229	-0.228
p value	0.130	0.132
N	45	45

Table11 showed Pearson correlation co-efficient to measure the relationship between serum 25 hydroxy vitamin D and calcium

Pearson coefficient correlation was done on variables like vitamin D and serum calcium in order to find the linear relationship in both the groups. It was observed that as the concentration of serum calcium increases, the concentration of serum vitamin D decreases with a weaker negative linear relationship with r= -0.229 in both cases and controls.

Table12.Pearson correlation co-efficient to measure the relationship between serum 25 hydroxy vitamin D and phosphorus

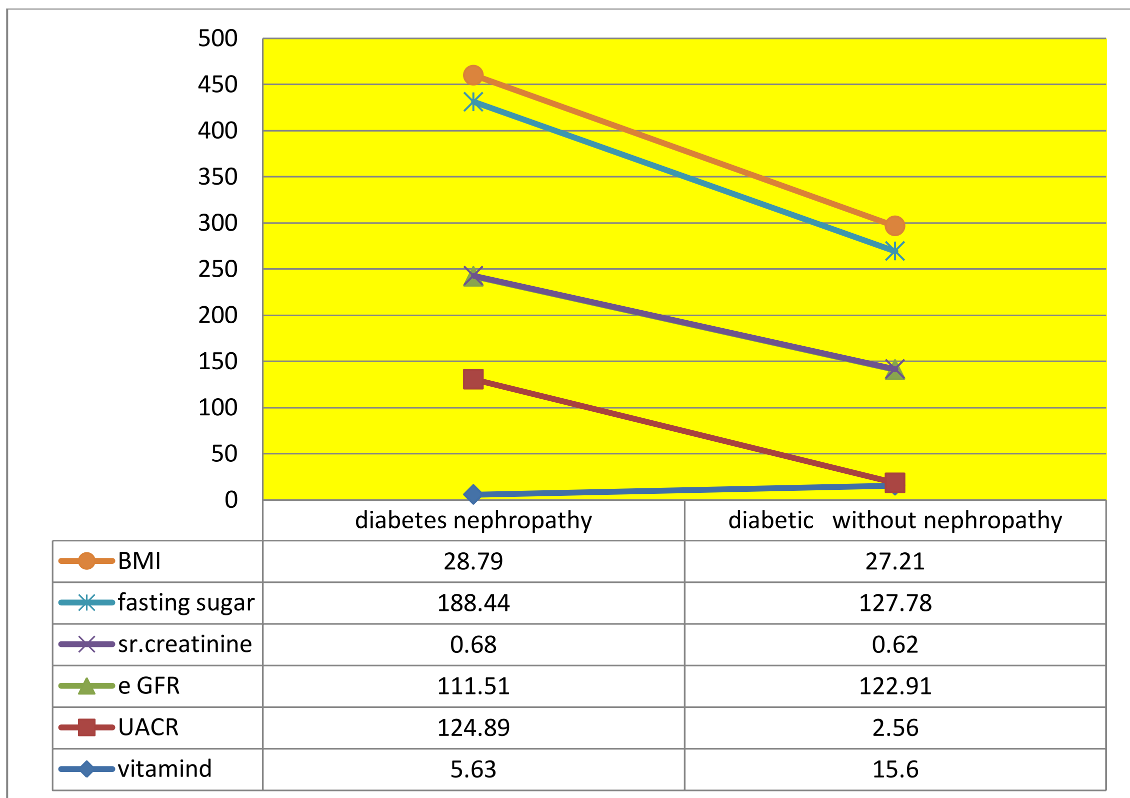
	Cases	**Controls**
correlation	0.043	-0.072
p value	0.777	0.640
N	45	45

Table12 showed Pearson correlation co-efficient to measure the relationship between serum 25 hydroxy vitamin D and phosphorus

Pearson coefficient correlation was done on variables like vitamin D and serum phosphorus in order to find the linear relationship in both the groups. It was observed that there was weaker negative linear relationship with r=-0.072 in controls but weaker positive linear relationship with r=.043 in cases.

Table 13 and 14 showed the master chart showing the details of patient with type 2 DM and DN which included age, gender, BMI, presence or absence of hypertension, smoking,alcohol,duration of diabetes, UACR,eGFR,serum creatinine, serum 25 hydroxy vitamin D, fasting glucose, serum calcium,serum phosphorus

Figure11. change in variables in DN and in type 2 DM



	diabetes nephropathy	diabetic without nephropathy
BMI	28.79	27.21
fasting sugar	188.44	127.78
sr.creatinine	0.68	0.62
e GFR	111.51	122.91
UACR	124.89	2.56
vitamind	5.63	15.6

Figure 11 showed the change in variables like BMI, fasting sugar, serum creatinine, e GFR, UACR, vitamin D in DN(cases) and in Type 2 DM without nephropathy(controls).

In type 2 DM without nephropathy, the mean BMI was 27.21, in DN, the mean BMI was 28.79.

In type 2 DM without nephropathy, the mean fasting sugar was 127.78, in DN, the mean fasting sugar was 188.44

In type 2 DM without nephropathy, the mean serum creatinine was 0.62, in DN, the mean serum creatinine was 0.68

In type 2 DM without nephropathy, the mean e GFR was 122.91, in DN, the mean e GFR was 111.51.

In type 2 DM without nephropathy, the mean UACR was 2.56 , in DN, the mean UACR was 124.89.

In type 2 DM without nephropathy, the mean serum 25 hydroxy vitamin D was 15.6, in DN, the mean serum 25 hydroxy vitamin D was 5.63.

Discussion

Diabetic Nephropathy is the common complication of Diabetes Mellitus, which frequently leads on to ESRD, associated with high mortality rate[133]. DN increases cardiovascular mortality i.e., there is a two to three fold increase when there is associated proteinuria[134].

In the initial stages of DN, there is glomerular and tubular basement membrane thickening, then by hyper-filtration, albuminuria, glomerulo-sclerosis, and tubular-interstitial fibrosis. All these structural and hemodynamic alterations eventually result in ESRD[135]. Microalbuminuria is the early marker of Diabetic Nephropathy.

The pathogenesis of DN is intricate as extracellular glucose acts directly on glomerular, tubular, interstitial and vascular cells,resulting in the release of cytokines and growth factors, such as angiotensin II, TGF-β, and monocyte chemo attractant protein (MCP)-1, which all plays an integral role in the formation of Diabetic Nephropathy [136].

RAS has been known to play a pivotal role in the progression of renal injury in DN.

These findings are demonstrated by clinical studies, on treatment with ACE inhibitors or Angiotensin II type1 receptor blockers can decrease the process of glomerulo-sclerosis, tubulo-interstitial fibrosis, and proteinuria[137].

$1,25(OH)_2D$ is the hormonal form of vitamin D, with multiple physiological functions. Adequate levels of $1,25(OH)_2D$ are necessary to maintain the bone metabolism[140], and there is also evidence that it has anti -proliferative effect in cellular differentiation, immune modulation and inhibition of the RAS[141].

$1,25(OH)_2D$ inhibits renin biosynthesis. Studies involving null-mutant mice lacking the vitamin D receptor (VDR) gene develop hyper-reninemia. The increase in renin results in increased blood pressure and cardiac hypertrophy[139]. In this study, we have demonstrated that $1,25(OH)_2D$ functions as a negative regulator of the RAS[138].

The objective of this article was to summarize the scientific evidence relevant to the role of 25(OH) D in patients with DN and in patients with Type 2 DM without nephropathy.

In this study, we have recruited 90 subjects and divided them into two groups. Cases(45) include DN patients, controls(45) include type 2 DM patients without nephropathy.

We have selected the patients after excluding the patients with liver failure, vitamin D deficiency, type1Diabetes Mellitus, malabsorption syndrome, other co-existing renal disease like chronic glomerulonephritis, obstructive uropathy,pregnant and lactating mothers, patients on drugs like barbiturates,phenytoin, rifampicin.

Insignificant *p* values were obtained for variables viz., age, gender, smoking, alcohol intake,and hypertension while comparing the cases and controls. This implied that the cases and controls were perfectly matched with respect to the confounding variables.

In the present study, the mean of 25(OH) D was 5.6309±2.34 (vitamin D deficiency)and was observed to be very much decreased in DN, the mean of 25(OH) D was 15.6062±3.46(vitamin D insufficiency) and was observed to be reduced in DM without nephropathy.

The difference in 25(OH) D values between cases and controls were highly significant (*p*= 0.000). Similar result was also obtained by *Vanessa A. Diaz et al*[143].

Using t test, the mean difference for vitamin D was *-9.97*(95% CI, -11.21 to - 8.73) and was statistically significant at p =0.000 as supported by Vanessa A. Diaz et al

This study suggested that in DN, the levels of vitamin D could play a significant role and adequate levels of vitamin D are needed to be maintained to decrease the progression of Diabetic Nephropathy.

In the present study, correlation was done to measure the linear relationship between the following variables.

- UACR and e GFR,
- 25(OH) D and UACR,
- 25(OH) D and e GFR,
- 25(OH) D and fasting glucose,
- 25(OH) D and Serum creatinine,
- 25(OH) D and serum calcium,
- 25(OH) D and serum phosphorus.

The study showed

1. UACR and e GFR

There was a weaker positive correlation between UACR and e GFR in both cases & controls, indicated that e GFR increased because of increased UACR.

2. 25(OH) D and UACR

   There was weaker negative correlation between 25(OH) D and UACR, indicated that there was an inverse relationship between 25(OH) D and UACR.

3. 25(OH) D and e GFR

   There was weaker negative correlation between 25(OH) D and e GFR indicated that there was an inverse relationship between

25(OH) D and e GFR

4. 25(OH) D and fasting glucose.

   There was weaker negative correlation between 25(OH) D and fasting glucose indicated that there was an inverse relationship between 25(OH) D and fasting glucose.

5. 25(OH) D and Serum creatinine.

   There was weaker negative correlation between 25(OH) D and Serum creatinine, indicated that there was an inverse relationship between 25(OH) D and Serum creatinine

6. 25(OH) D and serum calcium.

   There was weaker negative correlation between 25(OH) D and Serum calcium, indicated that as the concentration of Serum calcium increases,the concentration of 25(OH) D decreases.

7. 25(OH) D and serum phosphorus

   There was weaker negative correlation between 25(OH) D and Serum phosphorus, indicated that as the concentration of Serum phosphorus increases, the concentration of 25(OH) D decreases.

Studies establishing a benefit in supplementation of vitamin D to prevent the progression of renal disease suggested that this may be a strategy to take into consideration in future studies[144]

These findings were supported by rat studies in which administration of $1,25(OH)_2D$ reduces the formation of glomerulosclerosis and the progression of proteinuria. These actions are brought through parathyroid hormone-independent antiproliferative actions. There is a decrease in podocyte loss and podocyte hypertrophy[8].

According to Kim et al, oral cholecalciferol was given as a treatment for four months for patients with Type 2 DM with low blood levels of 25(OH)D. They found that cholecalciferol reduced albuminuria and urinary TGF -β1. They concluded that dietary $1,25(OH)_2$ D supplemented with cholecalciferol could have a effective action in preventing the progression of Diabetic Nephropathy[122].

In this study, in Type 2 DM without nephropathy, vitamin D levels were reduced significantly(vitamin D insufficiency) and was negatively correlated with fasting glucose.

In DN, vitamin D levels were decreased significantly (vitamin D deficiency) independent of the individuals age, gender and duration of diabetes and was negatively correlated with fasting glucose, UACR and eGFR.

This showed the active role of vitamin D deficiency/vitamin D insufficiency in the progression of diabetes as well as its complication mainly Diabetic nephropathy.

LIMITATIONS OF THE STUDY

1. The sample size is relatively small.
2. We were able to measure only 25-OH-D and not $1,25(OH)_2D$ in this study. As the half life of $1,25(OH)_2D$ is only 4 to 6 hours and is also present in very small quantities we were not able to measure this parameter, which should have to be measured ideally.
3. Parathyroid hormone could have been measured to support the diagnosis, since it is too costly, it has not been done.
4. We might have taken blood samples after giving vitamin D for 6 months for the same groups to highlight the role of vitamin D as a cause, but due to short duration, it has not been adopted.

CONCLUSION

- Serum 25(OH)vitamin D is decreased in diabetic nephropathy(**Vitamin D deficiency**) and in type 2 Diabetes Mellitus without nephropathy patients(**Vitamin D insufficiency**).

- The association between 25(OH)D and UACR is independent of the individuals age, gender and duration of diabetes.

- When a diabetic patient progresses from Normoalbuminuria to Microalbuminuria, vitamin D levels decrease significantly.

- Renal impairment in diabetics is associated with significant vitamin D deficiency.

FUTURE PROSPECTS OF THE STUDY

1. A prospective cohort study may be performed to obtain vitamin D deficiency as a causative role in the development of nephropathy in Type 2 DM.
2. A similar study may be performed in Type 1 DM to study the role of vitamin D deficiency in the development of nephropathy.
3. The role of VDR gene polymorphism in the development of Type 2 DM may be studied.
4. Studies demonstrating intervention with active vitamin D seem to be associated with improved outcome of renal disease.
5. A similar study may be performed in gestational diabetes to study the role of vitamin D deficiency in the development of diabetes.
6. A study may be done to find the relationship between 25 hydroxy vitamin D and intact parathormone in diabetic Nephropathy.

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PROFORMA

SAMPLE ID:

NAME: HEIGHT:

AGE: WEIGHT:

SEX: BMI:

BP:

DURATION OF DIABETES:

TYPE OF TREATMENT FOR DIABETES:

OHA □ INSULIN □

DRUG INTAKE:

BARBITURATE □ PHENYTOIN □ CALCIUM □ VITAMIN D □

ASSOSCIATED DISEASE WITH DURATION:

LIVER DISEASE □ RENAL DISEASE □ HYPER TENSION □

TYPE1 DM □ MALABSORPTION SYNDROME □

INVESTIGATIONS:

1. FASTING GLUCOSE
2. SERUM CREATININE
3. URINE MICROALBUMIN

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI-3

EC Reg No.ECR/270/Inst./TN/2013
Telephone No : 044 25305301
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CERTIFICATE OF APPROVAL

To
Dr. P. Deepa,
PG in Biochemistry,
Institute of Biochemistry,
Madras Medical College, Chennai-3.

Dear Dr. P. Deepa,

The Institutional Ethics Committee of Madras Medical College, reviewed and discussed your application for approval of the proposal entitled **"Evaluation of 25 Hydroxy Vitamin D3 in patients with Type-2 Diabetes Mellitus with and without Nephropathy"** No.AB05032014

The following members of Ethics Committee were present in the meeting held on 11.03.2014 conducted at Madras Medical College, Chennai-3.

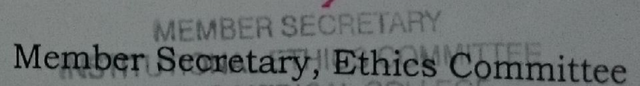
1. Dr. C. Rajendran, M.D. -- Chairperson
2. Prof. Kalaiselvi, MD -- Member Secretary
   Vice-Principal, MMC, Ch-3
3. Prof. Nandhini, M.D. -- Member
   Inst. of Pharmacology, MMC, Ch-3.
4. Prof. Bhavani Shankar, M.S. -- Member
   Prof & HOD of General Surgery, MMC, Ch-3.
5. Prof. V. Padmavathi, M.D. -- Member
   I/c Directory of Pathology, MMC, Ch-3.
6. Thiru. S. Govindasamy, BABL -- Lawyer
7. Tmt. Arnold Saulina, MA MSW -- Social Scientist

We approve the proposal to be conducted in its presented form.

Sd/Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, and SAE occurring in the course of the study, any changes in the protocol and patients information / informed consent and asks to be provided a copy of the final report.

13/3/14



Member Secretary, Ethics Committee

13/3/14

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PATIENT CONSENT FORM

Title of the study : **"EVALUATION OF 25 HYDROXY VITAMIN D3 IN TYPE 2 DIABETES MELLITUS PATIENTS WITH AND WITHOUT NEPHROPATHY"**

Name : Date :

Age : OP No :

Sex : Project Patient No :

Contact No. :

The details of the study have been provided to me in writing and explained to me in my own language.I confirm that I have understood the above study and had the opportunity to ask questions and received the information sheet.

I agree to use my personal clinical history and investigation details for the purpose of the study.I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected.

Having understood ____________________ S/o. ____________________ give my consent to participate in the study conducted by Dr.P.DEEPA, Post Graduate, Institute of Biochemistry, Madras Medical College, Chennai.

Signature of the Investigator : Signature of the Participant/ Thumb Impression

Place :

Date :

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ஆராய்ச்சி ஒப்புதல் கடிதம்

ஆராய்ச்சி தலைப்பு: நீரிழிவு நோயாளிகளில் சிறுநிரக வியாதி உள்ளவர்களுக்கும், இல்லாதவர்களுக்கும் வைட்டமின் D அளவு பற்றி ஆராய்தல்

பெயர் : தேதி :

வயது : புறநோயாளிஎண் :

பால் : ஆராய்ச்சி சேர்க்கை எண் :

இந்த ஆராய்ச்சியின் விவரங்களும் அதன் நோக்கங்களும் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது.

எனக்கு விளக்கப்பட்ட விஷயங்களை புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன்.

எனக்கு இரத்த/ சிறுநீர் பரிசோதனை செய்து கொள்ள சம்மதம்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரில் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் என்பதையும், அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் புரிந்து கொண்டேன்

நான் நீரிழிவு நோயாளிகளில் சிறுநிரக வியாதி உள்ளவர்களுக்கும், இல்லாதவர்களுக்கும் வைட்டமின் D அளவு பற்றிய இந்த ஆராய்ச்சியின் விபரங்களைக் கொண்ட தகவல் தாளைப் பெற்றுக் கொண்டேன்.

இதன் மூலம் எந்த பின்விளைவும் வராது என மருத்துவர் மூலம் தெரிந்து கொண்டு என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள சம்மதிக்கிறேன்

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி :

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s.no	age	sex	YRS	BMI	HT	SMOI	ALC(	S.CRE	U.ALE	U.CR	UACR	e GFR	vit D(	FBG	Ca	P
1	58	M	9	34	YES	NO	NO	0.8	448	2.1	217.33	109	4.89	165	9.3	2.5
2	73	M	8	29	NO	NO	NO	0.7	843	3.1	274.47	122	8.45	275	9.2	2.9
3	59	M	10	29	NO	YES	YES	0.7	796	3	269.95	115	3.76	189	10.1	3.1
4	58	M	7	28	YES	NO	NO	0.8	10.8	0.2	47	110	6.02	342	10.2	2.9
5	60	F	8	29	NO	NO	NO	0.6	58.7	0.6	99.53	111	2.67	187	9.8	2.8
6	60	M	8	27	YES	NO	NO	0.8	376	2.2	169.9	111	5.78	120	10.7	2.8
7	59	F	7	31	NO	NO	NO	0.6	221	1.9	119.69	115	7.89	154	9.8	2.7
8	63	M	10	29	YES	NO	NO	0.8	59.4	0.9	65.26	112	3.85	139	10.3	3.7
9	65	F	9	28	NO	NO	NO	0.6	11.2	0.2	62.06	111	6.97	241	9.4	3.1
10	66	M	9	31	YES	NO	NO	0.6	58.7	0.6	99.53	119	2.74	176	9.3	2.9
11	54	F	10	27	NO	NO	NO	0.6	623	4	157.32	113	3.29	214	9.3	2.8
12	54	M	10	29	YES	YES	YES	0.8	727	2.9	248.08	110	4.13	167	9.8	2.8
13	62	F	7	34	NO	NO	NO	0.6	111	1.1	102.41	112	5.85	450	9.9	3.1
14	65	M	6	31	YES	NO	NO	0.7	125	1.4	87.81	122	1.47	187	9.3	3.2
15	52	F	6	29	NO	NO	NO	0.6	91.7	0.9	104.25	106	2.89	156	9.7	2.9
16	60	F	7	27	YES	NO	NO	0.6	747	3.2	232.82	106	3.78	176	9.9	2.8
17	55	M	7	30	NO	NO	NO	0.8	364	3.4	107.09	116	5.67	271	10.3	2.8
18	65	F	7	27	YES	NO	NO	0.6	59.9	0.6	105.02	111	3.05	355	9.9	3.9
19	56	F	9	31	NO	NO	NO	0.6	125	1.4	87.81	112	9.87	129	9.2	3.8
20	66	M	8	28	YES	YES	YES	0.8	10.7	0.2	46.3	107	8.76	112	9.8	3.8
21	59	F	8	30	NO	NO	NO	0.6	138	0.7	193.94	115	7.68	234	9.4	2.9
22	58	M	9	27	YES	NO	NO	0.7	50.6	1.4	35.64	125	4.89	154	9.3	3.7
23	64	F	8	30	NO	NO	NO	0.6	642	3	213.35	111	2.54	179	9.4	2.5
24	49	M	8	27	YES	YES	YES	0.8	91.7	0.7	125.55	111	6.97	202	9.4	3.2
25	55	F	9	31	YES	NO	NO	0.6	221	1.9	119.69	110	3.12	156	9.8	3.9
26	58	F	7	28	NO	NO	NO	0.6	60.5	0.6	97.65	107	6.97	165	9.2	2.8
27	63	M	10	27	NO	NO	NO	0.8	354	2.9	122.07	110	6.87	187	9.3	2.8
28	54	F	6	30	YES	NO	NO	0.6	756	3	250.33	107	4.26	198	9.2	3.2
29	52	M	7	25	NO	NO	NO	0.8	59.2	1.2	48.15	111	1.32	126	9.7	3.2
30	58	F	8	24	NO	NO	NO	0.6	121	1.4	85.07	107	7.89	214	9.6	3
31	54	F	8	30	YES	NO	NO	0.6	68.6	0.8	90.21	115	6.87	112	9.3	2.8
32	65	M	10	33	NO	NO	NO	0.8	98.9	1	100.89	109	5.46	345	9.4	2.8
33	60	F	10	30	NO	NO	NO	0.6	123	1.5	79.55	106	7.89	167	9.4	3.9
34	62	M	9	26	NO	YES	NO	0.8	325	3.3	97.19	107	8.76	182	9.9	3.2
35	56	F	10	30	YES	NO	NO	0.6	56.8	1.3	42.36	112	9.34	194	9.2	2.9
36	50	F	9	27	NO	NO	NO	0.6	635	3	213.02	110	8.43	146	9.1	2.9
37	60	M	8	26	YES	NO	NO	0.8	59.7	0.8	72.77	106	4.56	178	9.3	2.8
38	56	M	7	28	NO	NO	NO	0.8	119	1.6	76.20	113	7.54	109	9.5	3.1
39	57	M	9	24	YES	NO	NO	0.8	350	3	117.53	108	9.29	154	9.5	3.2
40	62	M	10	28	NO	YES	NO	0.8	221	1.7	132.14	109	6.54	145	9.9	3.2
41	54	M	7	30	YES	NO	NO	0.8	54.4	1.2	46.13	109	4.56	176	9.9	2.9
42	58	M	8	33	NO	NO	NO	0.8	765	3.5	216.17	112	4.79	143	9.7	3.5
43	56	F	7	34	NO	NO	NO	0.6	654	3.5	184.84	112	3.54	110	10.3	3.5
44	67	M	8	29	YES	NO	NO	0.7	76.5	1.2	62.23	114	2.89	143	9.8	2.9
45	50	F	9	25	NO	YES	NO	0.6	321	3.4	93.55	110	8.64	156	9.8	2.8

MEA	59	NA	8	29	NA	NA	NA	0.7	28.8	1.8	124.89	112	5.63	188	9.63	3.09
SD	5	NA	1	2.5	NA	NA	NA	0.1	266	1.1	67.298	4.33	2.34	71	0.37	0.38

s.no	age	sex	YRS	BMI	HT	SMO	ALC(	S.CR	U.AL	U.CREA	UACR(	e GFR	vit D(	FBG	Ca	P
1	48	M	6	26	NO	YES	NO	0.7	3.9	1.13	3.478	132.3	21.8	113	9.3	3.5
2	65	F	6	27	NO	NO	NO	0.6	6.2	2.16	2.884	115.5	13.1	174	9.2	3.2
3	49	F	7	28	YES	NO	NO	0.6	5.5	2.04	2.686	115.1	21	191	10.1	2.8
4	47	F	6	27	NO	NO	NO	0.6	3.5	1.76	1.96	111.7	11.2	145	10.2	2.9
5	55	M	7	26	YES	NO	NO	0.7	3	0.76	3.921	135.6	20.8	93	9.8	3
6	50	F	6	28	NO	NO	NO	0.6	3.7	1.96	1.878	121.8	12.4	123	10.7	3.2
7	48	M	7	28	YES	NO	NO	0.7	2.6	1.07	2.467	130.1	19.4	100	9.8	3.3
8	54	M	7	26	NO	YES	YES	0.7	3.4	0.89	3.775	136.1	11.1	179	10.3	3
9	63	F	8	30	NO	NO	NO	0.5	3.1	1.02	3.069	121.2	13.1	143	9.4	2.9
10	52	F	8	27	YES	NO	NO	0.6	3.3	1.89	1.741	113.8	12.4	98	9.3	2.9
11	59	M	10	27	NO	NO	NO	0.7	3.5	0.98	3.52	120.7	19.6	112	9.3	2.8
12	61	M	6	29	YES	NO	NO	0.7	3.2	1.38	2.29	126	22.8	156	9.8	2.8
13	68	M	7	25	NO	YES	NO	0.7	4.3	1.99	2.171	127.6	14.4	179	9.9	2.8
14	63	F	9	28	YES	NO	NO	0.6	3.9	1.97	2	109.4	16.7	163	9.3	3
15	54	F	10	27	NO	NO	NO	0.6	3.7	0.94	3.926	117.5	14.8	121	9.7	2.5
16	52	F	8	28	NO	NO	NO	0.6	3.3	1.25	2.64	109.5	11.6	99	9.9	2.7
17	67	F	8	26	NO	NO	NO	0.5	4.2	1.57	2.701	119.7	18.9	109	10.3	2.6
18	64	F	7	27	YES	NO	NO	0.6	4.6	1.98	2.303	115.8	16.8	127	9.9	2.5
19	60	M	8	29	NO	NO	NO	0.7	4.1	1.73	2.393	133.2	13.5	110	9.2	2.5
20	50	F	6	28	YES	NO	NO	0.5	3	2	1.505	127	12.5	89	9.8	3.2
21	54	M	7	30	NO	NO	NO	0.7	3.6	1.72	2.105	133.7	14.5	110	9.4	3.1
22	65	F	7	26	NO	NO	NO	0.6	3.2	1.16	2.776	115.5	21	156	9.3	2.9
23	61	M	8	30	NO	YES	YES	0.7	3.2	0.89	3.64	126	12.2	132	9.4	3.8
24	49	M	8	30	YES	NO	NO	0.7	3.4	0.83	4.096	130.6	15.9	120	9.4	3.7
25	54	F	7	28	NO	NO	NO	0.6	3.7	1.82	2.055	122.4	14.3	98	9.8	2.8
26	65	F	9	29	NO	NO	NO	0.5	3.1	1.62	1.938	120.4	19.9	133	9.2	2.8
27	72	M	9	27	NO	NO	NO	0.6	3.2	1.4	2.264	130.7	13.3	174	9.3	3
28	65	M	10	28	NO	YES	NO	0.7	3	1.67	1.766	118.3	15.7	107	9.2	3.2
29	54	F	9	28	YES	NO	NO	0.5	3.1	1.36	2.25	125	14.7	110	9.7	3
30	49	M	9	23	NO	NO	NO	0.7	2.6	1.07	2.43	123.3	11.9	90	9.6	2.8
31	45	F	8	29	NO	NO	NO	0.63	3.4	1.32	2.591	108.6	13.5	89	9.3	2.9
32	54	M	8	28	NO	NO	NO	0.69	4.1	1.5	2.760	127	12.6	171	9.4	2.9
33	67	F	7	28	YES	NO	NO	0.51	4.3	1.81	2.387	127.8	18.7	154	9.4	3.1
34	65	F	8	25	NO	NO	NO	0.54	3.1	0.98	3.184	120.4	12	132	9.9	3.2
35	62	M	7	27	YES	NO	NO	0.68	2.9	1.34	2.142	125.6	20.9	90	9.2	3.2
36	60	M	7	30	NO	NO	YES	0.67	4	1.8	2.244	128.6	19.8	123	9.1	2.9
37	54	F	8	26	YES	NO	NO	0.55	3.3	1.23	2.691	122.4	15.7	156	9.3	2.9
38	46	M	8	25	NO	NO	NO	0.72	3	1.02	2.922	124.9	15	96	9.5	3.9
39	56	F	8	29	YES	NO	NO	0.58	3.2	1.21	2.653	114.3	16.5	89	9.5	3.1
40	54	F	6	26	NO	NO	NO	0.56	2.7	0.99	2.697	119.9	13.6	110	9.9	3.7
41	65	M	6	23	YES	NO	YES	0.65	2.5	1.23	2.065	131	14.6	145	9.9	3.1
42	48	M	9	26	NO	NO	NO	0.72	3.6	1.76	2.068	123.8	11.9	132	9.7	2.8
43	52	M	10	24	NO	NO	NO	0.71	4	1.65	2.442	123.8	12.1	145	10.3	3.8
44	50	F	7	29	YES	NO	NO	0.56	2.4	1.43	1.699	121.8	21.4	166	9.8	3.2
45	57	M	8	23	NO	NO	NO	0.69	3.8	1.32	2.864	125.6	13.2	98	9.8	2.9

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EVALUATION OF SERUM 25 HYDROXY VITAMIN D IN TYPE 2 DIABETES

BY 201223002.MD BIOCHEMISTRY DEEPA P

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INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disease of carbohydrate metabolism resulting in hyperglycemia mainly due to absolute or relative deficiency of insulin secretion and or its action. As the global effect of Diabetes constantly intensifies, there is a need for intervention to slow down the progression of disease and to achieve metabolic control.

Epidemiology

According to World Health Organisation (WHO), approximately 250 million people currently have diabetes worldwide and this number will reach 380 million by the year 2030.

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