ASSOCIATION OF VITAMIN D BINDING PROTEIN IN DIABETIC NEPHROPATHY-A CROSS SECTIONAL ANALYTICAL STUDY

THESIS SUBMITTED

TO

SRI DEVARAJ URS ACADEMY OF

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For Awarding the Degree as

DOCTOR OF PHILOSOPHY

IN MEDICAL BIOCHEMISTRY

Under Faculty of Medicine

By

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Under the Supervision of

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DECLARATION BY CANDIDATE

I, **Bhuneshwar Yadav,** declare that thesis titled: **Association of Vitamin D Binding Protein in Diabetic Nephropathy-A Cross Sectional Analytical Study,** is a original research work carried out by me for the award of **Doctor of Philosophy** in Medical Biochemistry.

This study was carried under the supervision of **Dr Shashidhar KN,** Professor and Head, Department of Biochemistry and Co- supervision of **Dr Raveesha A**, Professor and Head Department of General Medicine and **Dr Muninarayana C**, Professor, Department of Community Medicine, Sri Devaraj Urs Medical College, A Constituent of Sri Devaraj Urs Academy of Higher Education and Research.

No part of this has formed the basis for the award of any degree of fellowship previously elsewhere.

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PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Ph. D study entitled **"Association of Vitamin D Binding protein in Diabetic Nephropathy-A Cross Sectional Analytical Study"** being investigated by Mr.Bhuneshwar Yadav, Dr.Shashidhar.K.N, Dr.V.Lakshmaiah[1] & Dr. Muninarayana.C[2] in the Department of Bio-chemistry, General Medicine[1] & Community Medicine[2] at Sri Devaraj Urs Medical College, Tamaka, Kolar. **Permission is granted by the Ethics Committee to start the study**.

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Bhuneshwar Yadav

LIST OF ABBREVATIONS

ACR	Albumin: Creatinine Ratio
AGEs	Advanced Glycation End Products
AIDS	Acquired Immuno Deficiency Syndrome
AKI	Acute Kidney Injury
ALT	Alanine Transaminase
AST	Aspartate Transaminase
AUC	Area Under Curve
BMI	Body Mass Index
BUN	Blood Urea Nitrogen
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CTGF	Connective Tissue Growth Factor
CVD	Cardiovascular Disease
DAG	Diacylglycerol
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DN	Diabetic Nephropathy
DNPH	Dinitrophenyl Hydrazine
EDTA	Ethylene Diamine Tetra Acetic Acid
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme Linked Immuno Sorbent Assay
ESRD	End Stage Renal Disease
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFR	Glomerular Filtration Rate
GPX	Glutathione Peroxidase
GSH	Reduced Glutathione
HbA1c	Glycated Hemoglobin
HHNS	Hyperosmolar Hyperglycemic Non-Ketotic State
HIV	Human Immuno Deficiency Virus
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
IDF	International Diabetes Federation
IL	Interleukin
ISE	Ion Selective Electrode
MAF	Macrophage-Activating Factor
MAPK	Mitogen-Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
MDA	Malondialdehyde
NAD	Nicotinamide Adenine Dinucleotide

NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NF-κβ	Nuclear Factor Kappa β
NO	Nitric Oxide
OGTT	Oral Glucose Tolerance Test
PDGF	Platelet Derived Growth Factor
PEDF	Pigment Epithelium-Derived Growth Factor
PKC	Protein Kinase C
PTC	Proximal Tubular Cells
RAAS	Renin-Angiotensin-Aldosterone System
RAGE	Receptor for AGE
RAS	Renin-Angiotensin System
RBS	Random Blood Sugar
ROC	Receiver Operating Curve
ROS	Reactive Oxygen Species
SNS	Sympathetic Nervous System
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic Acid
TGFβ	Transforming Growth Factor β
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
uACR	Urine Albumin: Creatinine Ratio
UAE	Urinary Albumin Excretion
uVDBP	Urinary Vitamin D Binding Protein
VDBP	Vitamin D Binding Protein
VEGF	Vascular Endothelial Growth Factor

TABLE OF CONTENTS

LIST OF TABLES

PICTORIALS

GRAPHS

Introduction

Diabetes Mellitus (DM) is a metabolic disorder resulting either from deficiency of insulin or resistance. This results in increased in blood glucose level termed as hyperglycemia leading to several complications [1]. Diabetes Mellitus is congenially termed as diabetes.

The classical symptoms of diabetes include polydipsia, polyuria, polyphagia and weight loss. As the disease progress, patients are at high risk for development of complications, such as retinopathy leading to blindness, nephropathy ending with renal failure, neuropathy resulting in nerve damage and atherosclerosis [2].

Prevalence of diabetes is increasing day on day worldwide. International Diabetes Federation (IDF) in year 2019 estimated that around 463 million people had diabetes. Prevalence of diabetes is increasing exponentially and by the end of 2030 it is estimated that the chance of diabetes may be almost doubled [3]. The IDF published data in the year 2007 revealed that India plays a dominant role in the number of diabetics followed by China, United States of America, Russia and Germany [4]. India leads the world with highest number of diabetic patients with 61.3 million in 2011 and the number is likely to rise to 69.9 million by 2025 and 79.4 million by 2030 [5].

Prevalence of diabetes is increasing rapidly in the developing and developed countries. It is linked to transformation of lifestyles, awareness of the diagnostic criteria, improved or enhanced detection modalities, decrease in the mortality, obesity and sedentary lifestyle [6]. People with type 2 diabetes remain unaware of their illness until the complications sets in or often noticed during routine health checkup. Since symptoms of diabetes take years to express, by then the pathological issues might have set in.

Classification Based on Etiology [7]

1. **Type 1 diabetes:** due to autoimmune β-cell destruction, leading to absolute insulin deficiency
2. **Type 2 diabetes:** due to a progressive loss of β-cell insulin secretion with insulin resistance
3. **Gestational Diabetes Mellitus:** diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation
4. **Specific types of diabetes due to other cause**
    a) Monogenic diabetes syndromes
        i. Neonatal diabetes
        ii. Maturity-onset diabetes of the young
    b) Disease of the exocrine pancreas
        i. Cystic fibrosis
        ii. Pancreatitis
    c) Drug/chemical induced diabetes (Example-glucocorticoids use in the treatment of HIV/AIDS or after organ transplantation)

Criteria for the diagnosis of DM [8]

1. Classical symptoms of diabetes and RBS concentration ≥200 mg/dl.
2. Fasting blood sugar ≥126 mg/dl.
3. 2-hour postprandial plasma glucose concentration ≥200 mg/dl during the OGTT.

Complications of DM [9, 10]

People with diabetes are at risk of developing a number of disabling and life-threatening health issues. Consistently high blood glucose may affect the heart and blood vessels, eyes, kidneys, and nerves. People with diabetes are prone to develop infections. Thus, maintenance of blood glucose homeostasis, blood cholesterol and tight control of blood pressure shall help delay or even prevent complications.

Complications of diabetes are classified into acute and chronic complications. Acute complications of diabetes include diabetic ketoacidosis, hyperosmolar hyperglycemic non-ketotic state (HHNS), hypoglycemia, lactic acidosis, and diabetic coma.

Chronic complications lead to multi-organ damage and are further classified into vascular and non-vascular complications.

I. Vascular complications
    1. Micro-vascular complications and
    2. Macro-vascular complications

1. Micro-vascular complications include:
    A. Retinopathy
    B. Neuropathy
    C. Dermopathy
    D. Cataract
    E. Nephropathy
2. Macro-vascular complications include:
    A. Coronary artery disease
    B. Peripheral vascular disease
    C. Cerebro vascular disease etc.

II. Non-vascular complications
    1. Gastroparesis
    2. Sexual dysfunction
    3. Skin changes

The pathogenic processes are multiple and varied for the complications of diabetes. Risk of chronic complications depends on the duration of hyperglycemia.

Diabetic Nephropathy (DN) synonymously termed as Kimmelstiel Wilson syndrome or nodular diabetic glomerulosclerosis or inter-capillary glomerulonephritis, is a clinical syndrome characterized by albuminuria (>300 mg/day or >200 mcg/min) confirmed at least on two occasions 3-6 months apart with permanent and irreversible decrease in glomerular filtration rate (GFR) and arterial hypertension. DN is a leading cause of morbidity and mortality and is a progressive disease caused by glomerular as well as tubular structural and functional alteration. DN is induced by disturbance in glucose homeostasis and accounts for 30-40% of diabetic patients [11]. DN is one of the major causes of end stage renal disease (ESRD) [12]. Diabetic kidney disease (DKD) usually follows a characteristic clinical course. With the onset of diabetes, DKD is first manifested as microalbuminuria, clinical proteinuria, hypertension and finally declining the GFR. Clinical trials have established several effective treatments to slow the development and progression of diabetic kidney disease, including strict glycemic control, angiotensin converting enzyme inhibitors and angiotensin receptor blockers, blood pressure control and perhaps dietary protein restriction.

In India, DN accounts for about 46% of chronic kidney disease (CKD) in elderly people and is associated with increased cardiovascular mortality and morbidity. An exponential increase in prevalence of diabetic nephropathy has been documented in Indian diabetic patients. DN is the most common cause for end-stage renal disease (ESRD) in USA, Europe, Japan and other Asian countries, accounting for 25-45% [12].

Early detection of DN following T2DM helps delay its progression and improve the outcome. However, despite the various diagnostic and treatment modalities, there are challenges. These challenges can be overcome by considering the biomarkers. The advent of biomarkers especially the non-invasive urinary biomarkers shall help early diagnosis even before the onset of albuminuria.

To get a specific and a sensitive marker with a better predictive value, which is simple, accurate, cost effective and affordable needs to be addressed. To overcome these flaws and to meet the present-day requirements of clinicians for better and affordable patient care, as a laboratory personnel and researcher created interest in looking for an early predictive marker and evaluate Vitamin D Binding Protein (VDBP) as a most promising one.

Vitamin D Binding Protein (VDBP) is a low-molecular-weight protein of 58 kDa. The main function of VDBP is to transport the circulating vitamin D and its metabolites to target tissues. In addition, the bioavailability of 1,25-dihydroxyvitamin D, the active form of vitamin D and its precursor 25-hydroxyvitamin D is augmented by VDBP. Furthermore, it has an important role in the biosynthesis of 1,25 dihydroxyvitamin D in renal proximal tubules [13].

The complex formation of VDBP/25-hydroxyvitamin D, its filtration and reabsorption of in the proximal renal tubular cells is critical for the retrieval and activation of vitamin D [14]. The complex is taken up by the megalin receptors in the brush border of proximal tubular cells and is separated into VDBP and 25-hydroxyvitamin D. Inside the tubular cell The VDBP is degraded in lysosome whereas 25-hydroxyvitamin D is converted into 1,25-dihydroxyvitamin D and this active form is secreted into circulation. In normal kidney as the VDBP is catabolized, its urinary excretion is reduced to trace amounts [14]. People with renal damage, like diabetic patients with DKD, have increased urinary VDBP concentrations due to

destruction of megalin/cubilin receptors in the proximal tubular epithelial cells caused by persistent hyperglycemia in diabetic patients [15]. In the recent years, uVDBP is considered to be as a novel biomarker of DN, where it enhances its detection in T2DM patients.

Lacunae of the study

Increased levels of urinary biomarkers can be detected in type 2 diabetic patients before the onset of significant albuminuria. Urinary biomarkers may be used in the early stages to assess the renal injury in DN. Urinary biomarkers would play a significant role in the effective management and treatment modalities in diabetic care. Despite this, there is a lacuna of knowledge with respect to the novel biomarkers in early detection of DN. The clinical need for an ideal diagnostic and prognostic marker is still unmet. This has set in the healthcare providers a drift in their efforts to predict which diabetic patients will progress from incipient nephropathy to overt nephropathy. Estimation of VDBP as a biomarker in urine for early detection of DN would be ideal proposal as collection of urine is a non-invasive procedure and sample can be obtained in large quantities.

However, VDBP has been demonstrated with increased excretion in DN much earlier and before the alteration in Albumin: Creatinine Ratio (ACR). Increased VDBP in urine depict renal tubular dysfunction in both type I and type II diabetes.

Further, the potential role of VDBP as a non-invasive marker for early detection of DN needs to be established. As VDBP is a newer biomarker, very few studies linking its role in diabetes and diabetic nephropathy made us to consider and find its predictive value in the assessment of progressive loss of renal function. Whether urinary VDBP is a more suitable early marker to assess tubulointerstitial damage than the direct tubular biomarkers such as serum cystatin C, creatinine, microalbuminuria needs to be looked into.

Review of Literature

Diabetic nephropathy (DN) also known as Diabetic Kidney Disease (DKD) is the most common microvascular complication of diabetes mellitus, characterized by the presence of urine albumin excretion and/or accompanied by a gradual deterioration in the glomerular filtration rate and raised arterial blood pressure [16].

Natural history of DN varies among type 1 and type 2 diabetics. Around 80% of type 1 diabetic patients will end up with overt albuminuria (urine albumin excretion rate >200 µg/min) over a 15-year period if left untreated. Out of these patients, 50% will develop ESRD over 10 years. On contrary, 20-40% of type 2 diabetics will have overt albuminuria, of which 20% shall end up with ESRD over 20 years. Studies have documented that 40-50% of type 2 diabetic with microalbuminuria may succumb to cardiovascular complications [17].

Diabetic kidney disease is an important cause of chronic kidney disease (CKD). It has been documented that India has a diabetic population of more than 70 million, of these 30-40% develop DKD [18]. The onset of DKD is a leading cause of mortality and morbidity. DKD has a significant impact on the economy of the nation. A recent large-scale study in a rural community in south India found diabetes prevalence rate of 7.8 % [19]. Karnataka with 7.5% prevalence of diabetes, the southern state stands at the sixth position among Indian states. In house study in the year 2010 demonstrated the prevalence of diabetes in local population is 10% in Kolar district [20].

Stages of Diabetic Nephropathy

Diabetic nephropathy, a chronic complication of diabetes is categorized into five different stages based on Glomerular Filtration Rate (GFR) and urine albumin excretion (UAE) (Table 1) [21, 22]:

Table 1: Stages of Diabetic Nephropathy

Stages	Pathophysiology	Description
I	Glomerular Hyperfiltration	Early hyperfunction and hypertrophy
II	Silent Stage	Glomerular lesions without clinical disease
III	Incipient Nephropathy with Microalbuminuria	Urine albumin excretion (UAE) 30-300 mg/day
IV	Overt Nephropathy	Urine albumin excretion (UAE) >300 mg/day
V	End-Stage Renal Disease (ESRD)	Major loss of kidney function, requires dialysis

Risk factors [23]

Numerous risk factors contribute to the development and progression of Diabetic Kidney Disease (DKD) (Table 2) [24]. Risk factors for diabetic nephropathy is classified into:

1. Susceptible factors: age, sex, race/ethinicity and family history
2. Initiation factors: hyperglycemia and Acute Kidney Injury (AKI)
3. Progression factors: hypertension, dietary factors and obesity

Among these notable risk factors, Hyperglycemia and hypertension are the two most established risk factors.

Table 2: Risk factors for Diabetic Nephropathy

Risk Factor	Suceptiblity	Initiation	Progression
Demographic: 1. Older age 2. Sex (men) 3. Race/ethnicity (black, American Indian, Hispanic, Asian/Pacific Islanders)	✓ ✓ ✓		✓
Hereditary 1. Family history of DKD 2. Genetic kidney disease	✓	✓	
Systemic conditions 1. Hyperglycemia 2. Obesity 3. Hypertension	✓ ✓ ✓	✓ ✓	✓ ✓ ✓
Kidney injuries 1. AKI 2. Toxins 3. Smoking	✓	✓ ✓	✓ ✓ ✓
Dietary factors 1. High protein intake	✓		✓

Pathogenesis of Diabetic Nephropathy

Diabetic Nephropathy is associated with a series of morphological changes involving all compartments of the kidney, which affect the function of the organ and associated with clinical manifestations of the disease. The most important factor in the development of diabetic nephropathy is hyperglycemia. Early alterations in diabetic nephropathy include glomerular hyperfiltration, glomerular and tubular

epithelial hypertrophy and the development of microalbuminuria, which is followed by thickening of glomerular basement membrane, accumulation of mesangial matrix and overt proteinuria, eventually leading to glomerulosclerosis and ESRD [25].

The capillary surface area available for filtration reduces with the accumulation of matrix in the mesangial area and contributes to the progressive loss of renal function [25].

Hyperglycemia being a crucial factor in the development of diabetic nephropathy induces hemodynamic and metabolic factors which are thought to be the main mediators of this renal injury.

Hemodynamic factors contributing to DN includes activation of vasoactive systems. The leading vasoactive systems include renin-angiotensin-aldosterone (RAAS) and endothelin system. These vasoactive systems increase secretion of profibrotic cytokines such as transforming growth factor β1 (TGFβ1) [26]. Increased secretion of TGFβ1 leads to further hemodynamic changes ultimately resulting in increased systemic and intraglomerular pressure [27]. RAAS is known to play a crucial role in glomerular hemodynamics regulation and cytokine expression in renal tissues. RAAS is involved in glomerular hyperfiltration, the earliest pathophysiologic features and mesangial expansion in DN. Also TGF-β expression in mesangial cell is stimulated by angiotensin-II and contribute to the development of glomerular sclerosis [28]. Hemodynamic changes play a significant role in the early stage of DKD. These hemodynamic changes exacerbate albumin leakage via glomerular capillaries and contributing to mesangial matrix expansion, podocyte injury, and nephron loss [29].

Metabolic factors may interact with multiple biochemical pathways such as activation of advanced glycation end products (AGEs), protein kinase C (PKC),

acceleration of polyol pathway and overexpression of TGF-β in the progression of diabetic nephropathy [26].

Three mechanisms have been postulated to explain tissue damage following hyperglycemia:

1. Nonenzymatic glycation generating advanced glycation end products
2. Activation of protein kinase C (PKC)
3. Acceleration of the aldose reductase (polyol) pathway [27]

Oxidative stress seems to be a theme common to all the three mechanisms [30].

Nonenzymatic Glycation and Generation of AGEs

Advanced glycation end products (AGEs) are heterogenous group of compounds formed by nonenzymatic glycation of proteins with aldose sugars as a result of hyperglycemia [31]. Early glycation and oxidation processes result in the formation of reversible Schiff bases and Amadori products. These products on later stage is converted to irreversible advanced glycation end products [31]. Accumulation of AGEs in the kidney may contribute to progressive alteration in renal architecture and loss of renal function. These AGEs are known to cross link with proteins e.g., collagen and extracellular matrix proteins that contribute to the associated renal and microvascular complications [32]. Enhanced formation of AGEs and decreased clearance of AGEs by renal dysfunction are believed to be responsible for its accumulation in DN [33].

Mesangial cells occupy a central anatomical position in the glomerulus. These cells maintain structure and function of glomerular capillary tufts providing a structural support for capillary loops and modulate glomerular filtration. AGEs play a key role in glomerular nephropathy as they accumulate in glomerular basement membrane and interact with the mesangial cells, endothelial cells and podocytes.

These factors trigger oxidative stress, inflammatory signaling and apoptosis [34]. Thus AGE- induced mesangial apoptosis and dysfunction may contribute in part to glomerular hyperfiltration, which is an early feature of renal dysfunction in diabetes [35].

AGE accumulation in glomerulus could also be implicated in the initiation of diabetic nephropathy by promoting the secretion of Monocyte chemoattractant protein-1 (MCP-1). Increased MCP-1 expression associated with monocyte infiltration in mesangium has been observed in the early phase of diabetic nephropathy [36].

Activation of Protein Kinase C (PKC)

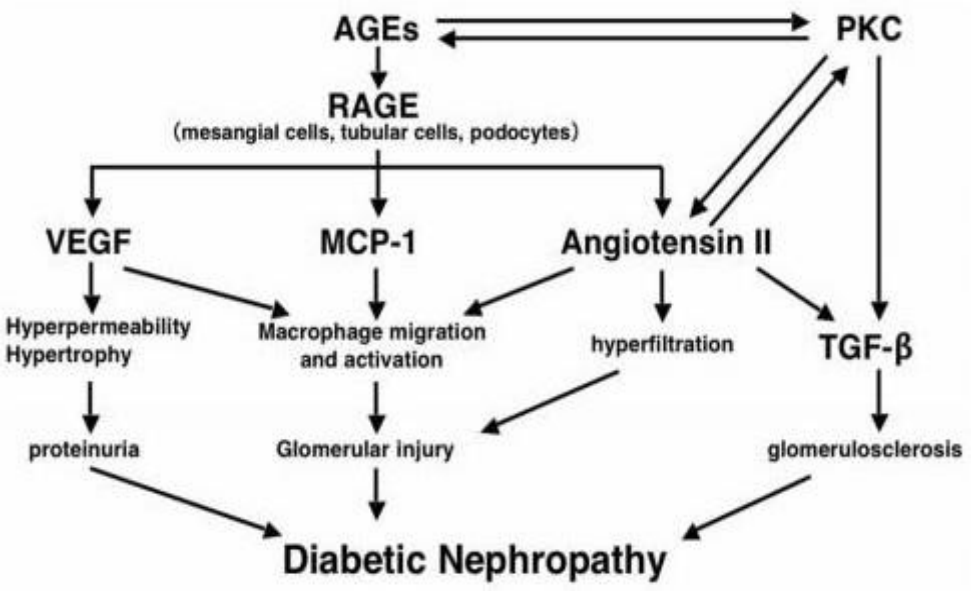
Protein kinase C is a family of protein kinase enzymes involved in controlling the function of other proteins. They play an important role in several signal transduction cascades [37]. Increase in the concentration of diacylglycerol (DAG) or calcium ions (Ca^{2+}) are known to activate PKC enzyme [38].

Elevated blood glucose causes increased de novo synthesis of Diacylglycerol (DAG) by inhibiting Glyceraldehyde-3-phosphate dehydrogenase. This leads to increased flux of dihydroxyacetone phosphate to DAG synthesis [39]. DAG being the regulatory stimulator of PKC, its elevation leads to subsequent activation of PKC.

PKC system is known to be involved in the transcription of growth factors, signal transductions and endothelin production [40]. Activation of PKC increases the expression of TGF-β which is known to regulate extracellular matrix production. This activated PKC increases the extracellular matrix production in mesangial cells via TGF- β resulting in expansion of mesangium and nephromegaly [35]. Further, mesangium expansion due to matrix deposition results in glomerular sclerosis and

diabetic nephropathy [35]. Activated PKC increases the permeability of endothelial cells in diabetes leading to endothelial dysfunction [41].

Figure 1: Possible PKC and AGEs involvement in Diabetic Nephropathy [35]



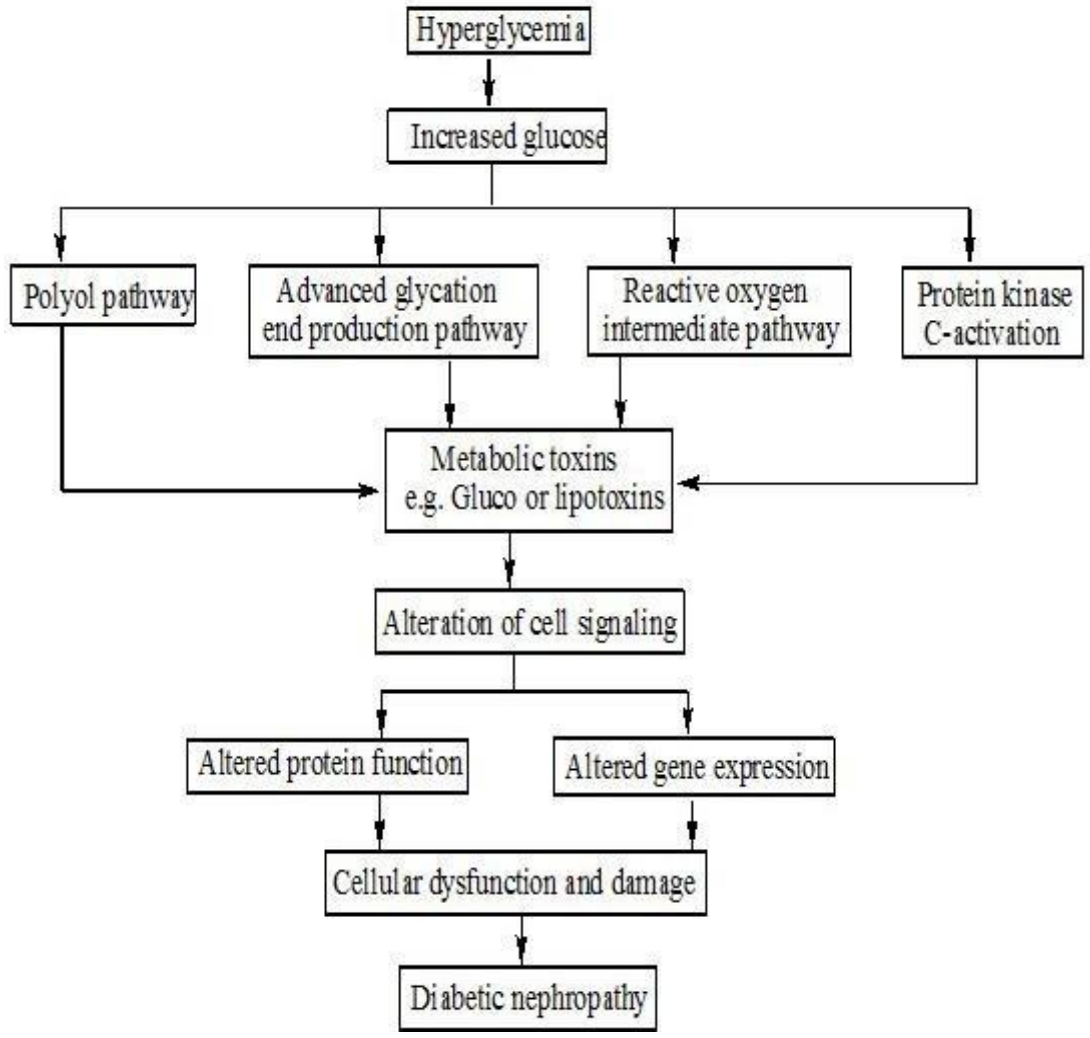
Source: Yamagishi S, Fukami K, Ueda S, Okuda S. Molecular mechanisms of diabetic nephropathy and its therapeutic intervention. Current Drug Targets. 2007;8: 952-959.

Acceleration of the Aldose Reductase (Polyol) Pathway

Hyperglycemia causes upregulation of polyol pathway which involves conversion of glucose to sorbitol via NADPH dependent enzyme aldose reductase. Sorbitol formed through polyol pathway is converted to fructose using NAD^+ as a cofactor [39]. Reduction of glucose to sorbitol via polyol pathway decreases the intracellular NADPH level, which act as a cofactor for reduced glutathione (GSH) synthesis. This reduction in GSH increases susceptibility of intracellular oxidative stress, which in turn causes cell stress and apoptosis [42]. Furthermore, oxidation of sorbitol to fructose results in an elevated intracellular NADH: NAD^+ ratio. This inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and increases triose phosphate. Elevated triose phosphate concentrations could increase

the AGE precursor methylglyoxal and diacylglycerol (DAG) activating PKC, thus contributing to initiation and progression of DN [39].

Figure 2: Biochemical Pathway Mediating Diabetic Nephropathy [43]



Source: https://www.pharmatutor.org/articles/diabetic-nephropathy-an-review.

Oxidative Stress and Reactive Oxygen Species in DN

Chronic hyperglycemia is the single most important factor in the initiation of early and sustained oxidative stress. Other complications of chronic hyperglycemia that boost oxidative stress include enhanced production of AGEs, reduced nitric oxide production, increased cytokine activation and levels of inflammatory markers [44].

Enhanced ROS formation and reduced bioavailability of nitric oxide is the hallmark of oxidative stress in diabetes mellitus. Oxidative stress coupled with chronic hyperglycemia may have an important role in the pathogenesis of glomerular and tubular functional and structural abnormalities, even before the onset of microalbuminuria. These alterations include deposition of extracellular matrix in the mesangium, promotion of a hypoxic environment by early microvascular damage, induction of cellular oxidant injury and apoptosis and finally promotion of tubulointerstitial fibrosis by activation of TGF-β, which stimulates several pathways of fibrosis [44].

Oxidative stress resulting in the generation of reactive oxygen species (ROS) is recognised as a major factor in the development of diabetic complications [39]. ROS are directly cytotoxic and promote inflammation and fibrosis. The eminent oxidative stress seen in diabetes is the consequences of an disproportion between ROS generation and endogenous anti-oxidant activity, including free radical scavengers and enzyme systems. The oxidative stress is considered as a common and key factor that couples hyperglycemia with vascular complications via metabolic modifications of target tissue molecules and alterations in the renal hemodynamics. The glomerular mesangial cells and tubular cells do not require insulin for glucose uptake and consequently have no control over glucose movement across the cells [44]. In chronic hyperglycemia, glucose and its metabolites such as fructose-1,6-bisphosphate and glyceraldehyde-3-phosphate are shunted to alternate metabolic pathways including polyol pathway, PKC activation through DAG, AGEs formation, thus promotes excessive production of reactive oxygen species (ROS) and are critical in the development of diabetic complications and in pathogenesis of early DN [45,46]. ROS additionally mediate extensive biological injury such as peroxidation of cell

membrane lipids, oxidation of proteins, mutation and cleavage of DNA. ROS furthermore induce transcription factors such as hypoxia-inducible factor alpha and nuclear factor kappa β (NF-κβ), promoting cellular proliferation and hypertrophy and contributing in DN progression [47].

Prominently, there seems to be a tight pathogenic relation between hyperglycemia-induced oxidant stress and other hyperglycemia-dependent mechanisms of vascular damage, namely AGEs formation, PKC activation and increased polyol pathway [48].

Signs and Symptoms of DN [49]

In the early stages of DN, symptoms go unnoticed untill there is a significant damage. During early stage of disease, hyperfiltration, where the glomerular filtration rate (GFR) is significantly higher than normal is observed. Over the years, as kidney disease progresses, small amounts of albumin begin to appear in the urine. This is the first stage of chronic kidney disease and is called moderately increased albuminuria or microalbuminuria where urine albumin excretion is <300 mg/day [50]. However, the kidneys can still filter out the waste. Further progression in the disease results in increased albumin leaking into the urine. This stage is called as severely increased albuminuria or macroalbuminuria with an overt urine albumin excretion of >300 mg/day.

Patients in the advanced stage of diabetic nephropathy complains symptoms of :

1. Uncontrolled blood pressure irrespective of treatment
2. Proteinuria
3. Swelling of eyes, limbs, and abdomen
4. Increased urgency of urination
5. Frequent hypoglycemic attacks

6. Lack of concentration
7. Shortness of breath
8. Loss of appetite
9. Nausea and vomiting
10. Persistent itching
11. Fatigue

Diagnosis of Diabetic Nephropathy

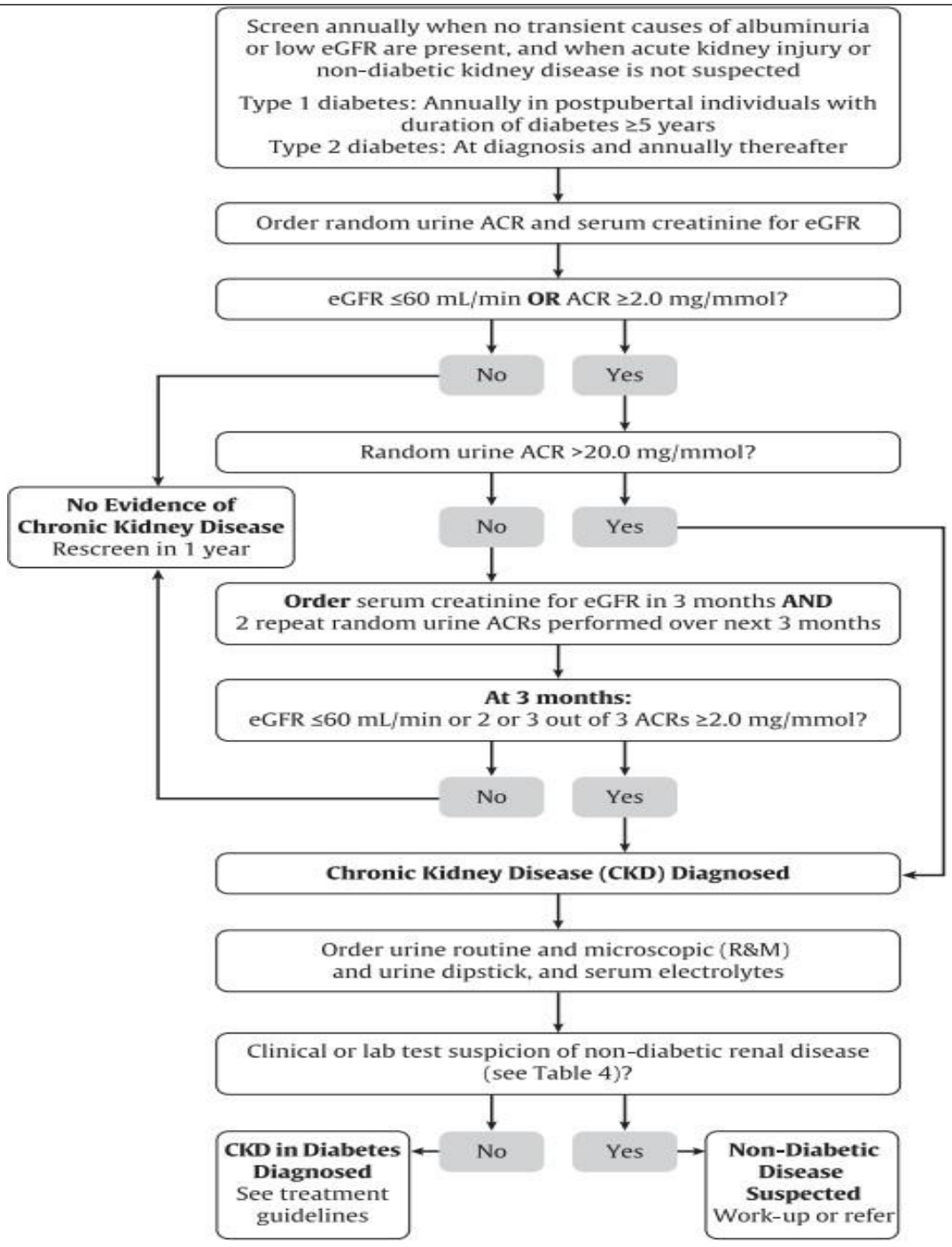
The onset of diabetic nephropathy is often silent and with the early intervention development and progression can be delayed. It is necessary that diabetic patients should be routinely screened for early evidence of renal involvement.

Diagnosis of diabetic nephropathy is based on eGFR measurement and albuminuria with clinical features and duration of diabetes and diabetic retinopathy [51]. Clinically diabetic nephropathy is identified by persistently high urine albumin-to-creatinine ratio of ≥30 mg/g and/or sustained reduction in eGFR below 60 ml/min per 1.73 m^2 [52]. Urine albumin:creatinine ratio performed on spot urine sample preferably in the morning is the preferred test for albuminuria and the eGFR is calculated from the serum creatinine concentration [53]. The presence of diabetic retinopathy in patients with albuminuria is strongly suggestive of diabetic nephropathy. Screening for nephropathy in people with diabetes requires albuminuria or low eGFR confirmed on at least two occasions 3-6 months apart [25].

Individuals with T1DM are not anticipated to have kidney disease at the time of onset of diabetes, thus screening for nephropathy can be postponed till the duration of diabetes exceeds 5 years. On contrary T2DM patients will have a significant renal

disease at the time of diagnosis. Hence, screening shall be initiated immediately at the time of diagnosis of T2DM [54].

Figure 3: Flowchart for screening for CKD in people with diabetes [54]



(ACR, albumin to creatinine ratio; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate)

Source: McFarlane P, Cherney D, Gilbert RE. Senior P. Chronic Kidney Disease in Diabetes. Canadian Journal of Diabetes. 2018;42: 201–209.

Biomarkers and its importance in Diabetic Nephropathy

Biomarker also termed as biological marker. It can be used as an indicator of the presence or severity of any diseased state. It is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention. Biomarker can be possibly useful as a diagnostic tool for the identification of those patients with a disease or abnormal condition, staging of disease, indicator of disease prognosis as well as prediction and monitoring of clinical response to an intervention [55]. Biomarkers offer a dynamic and influential approach to understanding the variety of a disease from the initial manifestation to the terminal stage.

Biomarkers play a prime role in the early detection of DN and may delay its progression with specific intervention. Many biological markers associated with DN were found in recent years, which were important for predicting the occurrence and development of the disease. The identification of novel biomarkers of early stages of DN and progression toward ESRD is thus mandatory to reduce the burden of chronic kidney diseases in the human population. To assess the presence and progression of DN, serum creatinine, estimated glomerular filtration rate (eGFR), blood urea and proteinuria or albuminuria are currently used and considered as standard biomarkers of DN [56].

Serum Creatinine: Creatinine is a by-product obtained from the breakdown of creatine phosphate in muscles and a well-established marker of renal function. It is completely filtered by the glomerulus and excreted from the kidney and is inversely related to the GFR. Creatinine has been found to be a fairly reliable indicator of kidney function because a high creatinine level in the blood is associated with poor

clearance of creatinine by the kidneys. However not very sensitive as creatinine levels do not increase significantly until the GFR is reduced to less than 50% [57]. In addition, concentration of serum creatinine is significantly affected by many factors such as age, gender, muscle mass, dietary intake, changes in tubular secretion and various drugs as well as endogenous substances that interfere with its assay. Furthermore, as a result of direct renal injury the elevated serum creatinine concentration fails to determine location of the renal injury i.e., glomerular versus tubular or proximal versus distal tubular [58].

Blood Urea: Urea is principal metabolite derived from protein metabolism. It has a molecular weight of 60 Da [59]. Urea is released into the blood and is filtered through kidneys and excreted in the urine. Blood urea nitrogen (BUN), the measure of urea nitrogen in blood and serum could be a marker of kidney impairment and dysfunction. If not properly excreted by the kidneys it gets accumulated in the blood. BUN estimation is useful marker for the primary diagnosis of acute or chronic kidney injury. However, BUN-to-creatinine ratio generally provides more precise information about kidney function and its possible underlying cause compared with creatinine level alone [60]. Even though BUN is routinely used and considered as a standard biomarker of kidney function, it is known to be influenced by several factors such as febrile illness, high protein diet, alimentary tube feeding, gastrointestinal bleeding, dehydrated patients and drugs which are known to alter blood volume and renal blood flow. Low levels of urea can be observed in decreased protein intake and chronic liver disease due to reduced synthesis [61].

Estimated glomerular filtration rate (eGFR): GFR measures the rate at which the glomeruli filter the plasma and remove waste products from it. GFR can be used to measure the kidney function using the rate of blood filtration. However, GFR cannot

be measured directly as it is based on renal clearance capacity of the endogenous biomarkers in plasma. eGFR estimation can be done by using either serum creatinine and/or cystatin C values with patients age, gender and weight [62].

eGFR= 135 × min (SCr/K, 1)−α × max(SCr/K, 1)−0601 × min(SCysC/0.8,1) − 0375 × max (SCysC/0.8, 1)− 0.711 × 0.995age [× 0.969 if female] [×1.08 if black] If female: K = 0.7, α =−0.248 If male: K = 0.9, α =−0.207

To measure GFR, exogenous substances can also be used which is continuously infused and analyzed by multiple timed urine collections (inulin, iohexol, Iiothalamate, chromium labelled EDTA). However, these methods are not used in routine clinical practice. GFR is a good marker for the detection of kidney disease by understanding its severity and making decisions on diagnosis, prognosis and treatment [63]. However, eGFR calculation based on serum creatinine concentration are limited by variation in the synthesis of creatinine and on the basis of age, gender, race and body composition [62]. Further eGFR does not predict the initial stage of renal dysfunction.

Albuminuria: Albuminuria is an important marker to predict the progression of diabetic nephropathy. Persistent albuminuria correlates with rapid progression of DN and cardiovascular disease (CVD) among T2DM patients. While evaluating albuminuria the ideal test of choice is the urine albumin: creatinine ratio with a first-morning void spot collection as it correlates with the 24-hour urinary albumin excretion. Twenty four-hour urine collection for albumin remains the gold standard. However, it is difficult to implement on a large scale as it is inconvenient and associated with sample collection bias [66]. Physiologically excreted urine contains approximately 20 mg albumin/L urine. Microalbuminuria and macroalbuminuria are

not only markers of nephropathy but also predicts disease progression [65]. Microalbuminuria has been documented to be the earliest clinical evidence of diabetic nephropathy in T2DM. Microalbuminuria is characterized by the appearance of albumin in urine 30-300 mg/day or 20 μg/min [66]. Persistent microalbuminuria is a strong risk factor for subsequent loss of GFR. This stabilizes the importance of sustained increase in urine albumin excretion in the pathogenesis and diagnosis of diabetic kidney disease. However, patients who lost GFR at a high rate did not have overt albuminuria [67]. This suggest that overt albuminuria does not always precede a significant loss of GFR in the setting of diabetes. Thus, measuring albuminuria alone does not fully capture the scope of early diabetic kidney disease [67]. Furthermore, transient and benign increase in albuminuria can be triggered by a number of factors such as recent major exercise, congestive heart failure, febrile illness, urinary tract infections, menstruation as well as hypertension as these limits use of albuminuria as a marker for DN [65]. At least 2 out of 3 urine samples show elevations in urinary albumin levels over a span of 3 months and are required before it is considered as abnormal [54].

Cystatin C: Cystatin C is a promising marker of renal failure. It is a cysteine protease inhibitor. It is a low molecular mass protein of 13.4 kDa, which is freely filtered at the glomerulus because of its small size and positive charge. Once cystatin C is filtered, it is reabsorbed and completely catabolized. Further, it cannot be secreted by proximal renal tubules even though they absorb it [68]. Cystatin C is constantly produced by all nucleated cells at a stable rate which is unaffected by inflammatory processes, gender, age, diet and nutritional status [69]. Cystatin C is being considered as a potential replacement for serum creatinine as it appears to be less affected by factors known to confound creatinine concentration [70]. These characteristics of cystatin C enables it

as an endogenous marker for GFR assessment and have been suggested as a marker of glomerular and tubular dysfunction in early diagnosis of DN. Urine cystatin C could be an independent factor for identifying renal dysfunction in type 2 DM patients with normoalbuminuria, including patients with GFR [70].

Other Biomarkers of Diabetic Nephropathy:[60]

Table 3: Other Biomarkers of Diabetic Nephropathy

Class	Biomarkers	Sample
Oxidative Stress	1. Pentosidine	Serum/Urine
	2. 8-hydroxy-2′-deoxyguanosine (8-OHdG)	Urine
	3. Uric Acid	Serum
Fibrosis	1. Transforming Growth Factor-β1	Serum/Urine
	2. Connective tissue growth factor (CTGF)	Serum/Urine
	3. Vascular endothelial growth factor (VEGF)	Serum/Urine
Glomerular Damage	1. Transferrin	Urine
	2. Type IV collagen	Urine
Tubular Damage	1. Liver-type fatty acid-binding protein (L-FABP)	Urine
	2. Neutrophil gelatinase-associated lipocalin (NGAL)	Urine
	3. Kidney injury molecule-1 (KIM-1)	Serum/Urine
	4. Angiotensin-converting enzyme-2 (ACE-2)	Serum/Urine
	5. N-acetyl-beta-d-glucosaminidase (NAG)	Urine
	6. Fibroblast growth factor (FGF23)	Serum
	7. α1-microglobulin	Urine
Inflammatory	1. Tumor necrosis factor-α (TNF-α)	Serum/Urine
	2. Monocyte chemoattractant protein-1(MCP-1)	Urine
	3. Osteopontin	Serum
	4. Interleukin (IL)1, 6,8,18	Serum/Urine
Filtration Markers	1. Beta-trace protein	Serum
	2. Beta-2 microglobulin	Serum

Source: Campion CG, Sanchez-Ferras O, Batchu SN. Potential Role of Serum and Urinary Biomarkers in Diagnosis and Prognosis of Diabetic Nephropathy. Canadian Journal of Kidney Health and Disease.2017;4: 1–18.

The pathophysiology in DN affects all the renal cellular elements including the glomerular endothelium, mesangial cells, podocytes as well as tubular epithelium. The links between tubulointerstitial change and functional outcomes of the kidney have been reported. Also it is being suggested that tubular injury contributes in a primary way to the development of DN, rather than in a secondary manner [71].

Owing to limitations of current biomarkers of renal failure and DN, there is a substantial necessity for the discovery of novel early biomarker which should be easily available, cost effective, more of sensitivity, selective, precise, accurate, affordable and predictive.

Vitamin D Binding Protein (VDBP) a multifunctional protein belonging to the albumin superfamily of binding proteins, is a novel promising biomarker for tubular injury in DN and suffice the requirements as an effective early biomarker in assessing tubular damage in patient with DN thus for a better patient care. However, this molecule needs to be studied in the local population as the T2DM in this geographical belt has a varied etiology, multidimensional and multifactorial.

Vitamin D Binding Protein (VDBP)

Vitamin D Binding Protein is a multifunctional circulating α_2-globulin with a molecular weight of 58 kDa [72]. It is identified as a polymorphic protein and known as a group-specific component of serum (Gc-globulin) [73]. VDBP is a serum α_2-globulin primarily responsible for the transport of vitamin D and its metabolites [74]. This multifunctional glycoprotein is a member of the albumin super family of binding proteins (albumin, α-fetoprotein and afamin). It is predominantly synthesized as a single long chain of glycoprotein in the liver [75]. VDBP was isolated from the globulin portion of plasma in the year 1959 and was named as “group-specific component (Gc)”. Later Group-specific component was replaced by VDBP as it was

shown to bind and transport vitamin D analogs. VDBP belongs to albumin superfamily, characterized by unique cysteine residue arrangements, with an adjacent cysteine residue distributed throughout the primary structure [76].

Structure & synthesis of VDBP

Human VDBP is encoded by the VDBP gene located on the long arm of chromosome 4 (4q12-q13). It contains 13 exons and 12 introns and is extended over 35 kb DNA. VDBP is composed of 458 amino acids containing numerous cysteine residues and are arranged in 3 domains in addition to 16 amino acid leader sequence [77].

Three binding domains of VDBP with amino acid residues are [75]:

a. Vitamin D binding domain between 35 and 49

b. An actin-binding domain between residues 373 and 403

c. Membrane binding site between 150-172 and 379-402

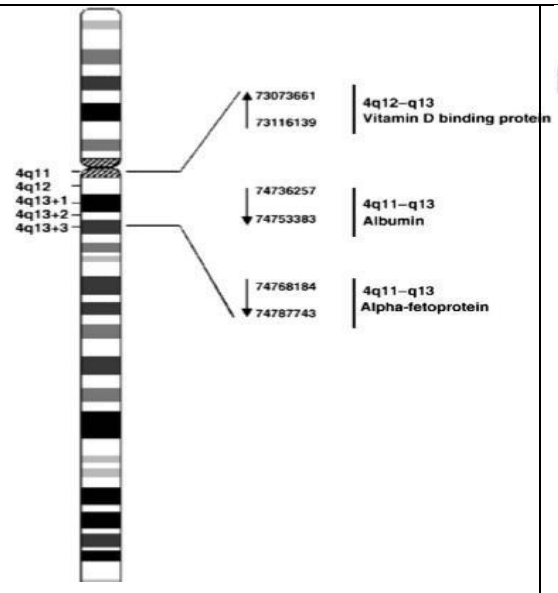


Figure 4: Localization of the vitamin D binding protein gene on the long arm of chromosome 4 (4q12–q13).

Source: Speeckaert MM, Speeckaert R, Van Geel N, Delanghe JR. Vitamin D Binding Protein. Advances in Clinical Chemistry. 2014; 1–57.

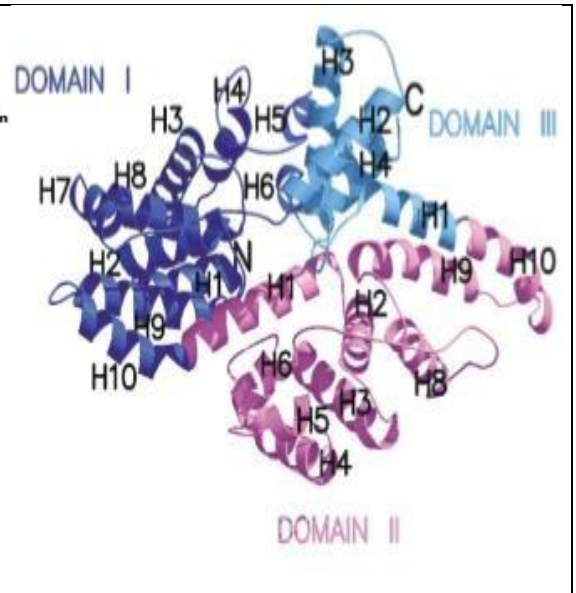


Figure 5: Vitamin D Binding Protein in three dimensions.

Source: Verboven C, Rabijns A, De Maeyer M, Van Baelen H, Bouillon R, De Ranter C. A structural basis for the unique binding features of the human vitamin D-binding protein. Nat Struct Mol Biol. 2002;9: 131–6.

In healthy subjects, the production rate of VDBP varies from 0.69-0.93 g/day, with a mean of 10.1mg/kg body weight/day [78]. VDBP is constitutively synthesized by the liver and circulate in great excess (about 400 mg/L), with less than 5% of vitamin D binding sites normally occupied with a short plasma half-life of 2.5 days [79]. VDBP binds vitamin D and its metabolites particularly 25-hydroxylated metabolites i.e., 25(OH) Vitamin D, $24,25(OH)_2$ Vitamin D and $1,25(OH)_2$ Vitamin D. Hepatic synthesis of VDBP is estrogen dependent and is significantly increased during pregnancy and estrogen therapy. Plasma level of VDBP is low in liver diseases, nephrotic syndrome, malnutrition, septic shock or trauma due to a diminished synthesis or excessive protein loss or consumption [78]. Vitamin D is circulated by binding with VDBP of 85-90% and albumin around 10-15% with ≤1% existing as free form [80].

Functions of VDBP

VDBP has both physiological and pathological functions. The major physiological role of VDBP is transportation of vitamin D metabolites [81]. The three major physiological roles of VDBP in vitamin D metabolism are:

1. Protecting vitamin D from biodegradation
2. Limiting its access to target tissues
3. Reabsorbing vitamin D in the kidney

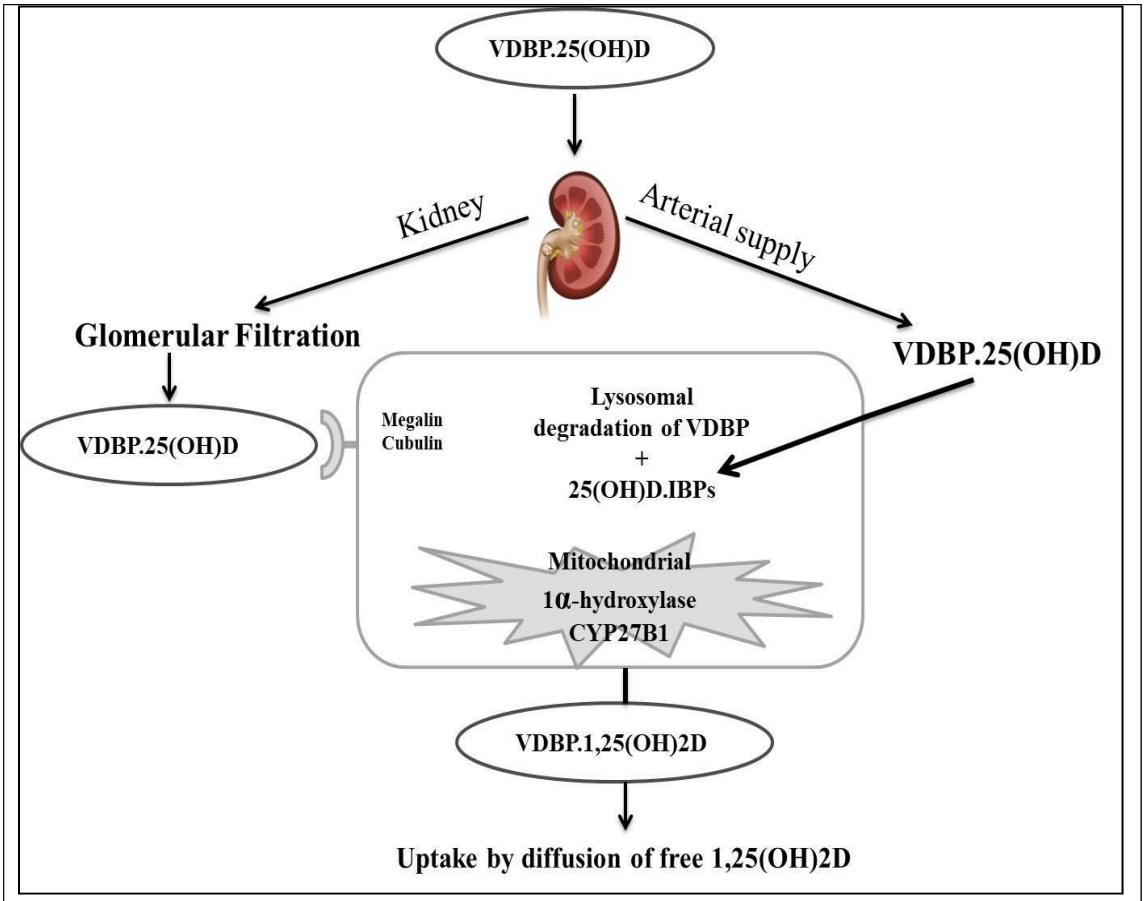
The affinity of VDBP for vitamin D metabolites is quite different being highest for 25-hydroxyvitamin D followed by 24,25-dihydroxyvitamin D. On contrary 1,25-dihydroxy vitamin D has about a 10 to 100 fold lower affinity for VDBP than 25-hydroxyvitamin D [73].

Variation in VDBP activity can contribute to the pathological progress of osteoporosis by the regulation of calcium metabolism in blood and bone. Study revealed that Low serum VDBP level might be associated with a more severe character of primary hyperparathyroidism with low total 25-hydroxyvitamin D levels [82]. It has been demonstrated that inflammatory modulation effect of 1,25-dihydroxyvitamin D or VDBP-MAF (Macrophage-activating factor) may play an important role in pathogenesis of cancer [83]. Type 1 diabetic patients have been found to have lower serum VDBP levels, which may indicate itself directly or indirectly to the autoimmune destruction of pancreatic β-cells [84]. VDBP affects glucose metabolism by modulating the action of vitamin D metabolites. Low plasma 25-hydroxyvitamin D levels are associated with an increased risk of type 2 diabetes, while 1,25-dihydroxyvitamin D enhances the insulin sensitivity [85,86].

Vitamin D in general circulation is bound with its major carrier protein VDBP and to a lesser extent with albumin. This is then subjected to hydroxylation step by the enzyme 25-hydroxylase in the liver resulting in formation of 25-hydroxy vitamin D [87]. Less than 5% of 25-hydroxyvitamin D is secreted into the bile, whereas bulk of 25-hydroxyvitamin D re-enters the circulation and bound with either VDBP or albumin. VDBP- 25-hydroxyvitamin D complex is later transported to the kidney for final hydroxylation reaction [87]. VDBP and VDBP-bound metabolites are filtered through the glomerulus and reabsorbed by the endocytic receptor megalin into the proximal tubular cells [73]. Further hydroxylation reaction happens in the kidney at 1^{st} & 24^{th} carbon and forming active form of vitamin D i.e., 1,25-dihydroxyvitamin D and non-biologically active form 24,25-dihydroxyvitamin D by the enzymes 1-alpha-hydroxylase and 24- hydroxylase respectively [88]. Megalin-mediated endocytosis of VDBP-bound 25 hydroxyvitamin D appears to be the major pathway

to preserve circulating levels of 25-hydroxyvitamin D and to activate 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D [89].

Figure 6: Renal uptake of 25(OH)D by proximal tubular cells (PTC)



Source: Modified from Bouillon R, Pauwels S. The vitamin D binding protein. In: Davis Feldman editors. Vitamin D. 4th ed. Science Direct: Elsevier; 2018. 97-115.

In addition to transportation of vitamin D, VDBP has varied functions:

- VDBP being a component of "Actin-Scavenger System" plays a vital role in the clearance of actin filaments from the circulation and prevent from harmful effect caused by excess actin filaments in the circulation [90].
- VDBP play a contributory role in fatty acid transport by binding with mainly monounsaturated and saturated fatty acids with low affinity [91].

- VDBP binds with complement component 5a (C5a) during inflammation and enhances the C5a-mediated neutrophil and macrophage chemotaxis [92].
- VDBP can act as a macrophage-activating factor (MAF) and demonstrate a significant tumoricidal activity explained by its role in the immune response to neoplasia as well as stimulate bone resorptive activities of osteoclasts [93, 94].

VDBP in Diabetic Nephropathy

VDBP, a specific globulin is a carrier molecule of vitamin D and helps in transportation of vitamin D through lymphatics. The 25-hydroxyvitamin D-VDBP complex is filtered through glomerulus and reabsorbed in the proximal tubules by the endocytic receptor megalin present in brush border of proximal tubular cells. Carrier VDBP is degraded in lysosomes, while 25-hydroxyvitamin D is converted into active vitamin D; 1,25-dihydroxyvitamin D and resecreted into circulation [74, 89].

Megalin, also known as low-density lipoprotein-related protein 2 (LRP2) is an approximately 600-kDa transmembrane glycoprotein composed of 4655 amino acids. Megalin is expressed in the brush border membrane and endocytic apparatus of the proximal tubule [89]. Megalin drives the reabsorption of nearly all filtered plasma proteins including albumin, vitamin-binding proteins, lipoproteins, hormones, enzymes and drugs in co-operation with the receptor protein cubilin in the proximal tubule [95]. Cubilin is co-expressed with megalin in the apical endocytic compartments of the proximal tubule and also recently demonstrated in rat and human podocytes. In the proximal tubule, it is believed to interact with megalin, forming a two-receptor complex, with megalin directing internalization of the complex and bound ligands [96].

During kidney injury increased urinary excretion of different molecules may be caused by either increased tubular secretion or impaired proximal tubular reabsorption. In normal kidney, VDBP is reabsorbed by megalin mediated endocytosis and catabolized by the proximal tubular epithelial cells reducing urinary excretion of VDBP to trace amount [97]. In DN, hyperglycemia increases ROS and TGF-β production and induces inflammatory cytokines secretion (IL-18) from the podocytes. This causes renal damage with destruction of megalin/cubilin receptors in the proximal tubular epithelial cells. The damaged tubular epithelial cells in areas of tubulointerstitial fibrosis may no longer be capable to deal with VDBP, resulting in its gross loss into the urine [56, 98].

Study conducted by Tian and colleagues in the year 2014 suggested, level of uVDBP is significantly higher in DN with microalbuminuria and macroalbuminuria than in DM patients without albuminuria [99]. They also observed a significant difference between microalbuminuria and macroalbuminuria patients with a strong positive correlation between the expression levels of uVDBP and development of DN. In addition, uVDBP is about 4-fold increase in diabetic patients with normoalbuminuria [99].

Mirkovic K et al. in the year 2013 has shown uVDBP was increased with increasing severity of renal damage and responded well to renoprotective therapy [15]. These facts suggest that tubulointerstitial damage is considered as a final common pathway for end stage renal disease (ESRD) and is present at an early asymptomatic stage of chronic kidney disease [100].

Study conducted by Saleh Sheet MM et al in the year 2018, observed that uVDBP levels were significantly elevated in DN patients and positively associated with higher HbA1c and uACR and concluded that uVDBP can be used as an early predictor for the detection of DN which may help in prevention of the early onset of

DN [101].

Shoukry A et al (2015) reported an increased uVDBP in the early stages of DN in type 2 diabetic patients. VDBP showed more elevation with the progression of DN and were significantly associated with the other risk factors of DN. A strong positive correlation between uVDBP and uACR were observed. uVDBP levels were directly proportional with increased uACR. Results of the study suggest that uVDBP may be considered as a non-invasive diagnostic biomarker for early detection of diabetic nephropathy [102].

Khodeir SA et al. in the year 2016 demonstrated that uVDBP levels were significantly increased in patients with DN. Moreover, a strong positive correlation was observed between the expression level of uVDBP and the development of DN. With their finding they concluded that uVDBP levels were a potential biomarker for the early detection of DN in T2DM patients [103].

However, recent study in the 2019 conducted in Qatar revealed that uVDBP and megalin levels were not significantly elevated in T2DM patients with DN. Moreover, a weak negative correlation was observed between urinary VDBP and megalin levels with eGFR [104].

Study conducted by Ali ANM in the year 2015 in Baghdad observed a significantly elevated uVDBP levels in all the groups in patients with DN. They further reported increased VDBP levels were positively correlated with the development of the DN and concluded that elevation of VDBP level can be considered as a novel predictor for monitoring type 2 diabetes before DN onset since it has a negative correlation with eGFR [105].

In the year 2018, Fawzy MS et al. reported uVDBP levels were highly elevated in Saudi patients with DN and correlated significantly with the severity of DN and also with the degree of albuminuria. Authors further concluded uVDBP could be implicated in combination with other conventional biomarkers for the early prediction of DN patients and help in early diagnosis of DN and prevention of ESRD progression [14]. The urinary excretion of VDBP is increased early after renal injury and is associated with tubulointerstitial inflammation and fibrosisindependently of albuminuria. Urinary excretion of VDBP increases with increased severity of renal damage. This suggests that urinary VDBP could be implicated in a combination with other conventional biomarker for the early prediction of DN and improves early diagnosis and help in prevention of progression of end-stage renal disease.

Despite so much of significance, literature search was futile with respect to Indian population. This made us to take up the study to find out association of VDBP in diabetic nephropathy.

Objectives

RESEARCH QUESTIONS

1. Is Urinary Vitamin D Binding Protein (VDBP) level altered in diabetic nephropathy patients when compared with healthy control individuals?
2. Does Urinary Vitamin D Binding Protein (VDBP) have any role in the early diagnosis of diabetic nephropathy?

PRIMARY OBJECTIVES

1. To estimate and compare Urinary Vitamin D Binding Protein in patients with diabetes, diabetic nephropathy and clinically proven healthy control
2. To assess Urinary Vitamin D Binding Protein as an effective early biomarker in assessing tubular damage in patients with diabetic nephropathy

SECONDARY OBJECTIVES

1. To compare and correlate Urinary Vitamin D Binding Protein with the conventional biomarkers of diabetic nephropathy
2. To find correlation of Urinary Vitamin D Binding Protein with Serum and Urine Fluoride

Materials and Methods

Permission to conduct the study was obtained from the concerned authorities viz.

1. Institutional Ethics Committee on Human Subject's Research of Sri Devaraj Urs Medical College, Kolar **Reference No.: SDUMC/KLR/IEC/17/2019-20; Dated: 24.04.2019**
2. Head of Department, Department of Biochemistry, Sri Devaraj Urs Medical College, Kolar

STUDY DESIGN

This is a Hospital based Cross-sectional Analytical study. All the parameters in methodology section were compared between three groups and within the group.

STUDY PERIOD

3 Years: 2018- 2021

STUDY AREA

Study was conducted in R L Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, affiliated to Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka, India.

Study included three groups;

Group I- Healthy controls

Group II- T2DM without nephropathy and

Group III- Diabetic Nephropathy

Type 2 diabetic patients attending OPD, Department of general medicine and diabetology were recruited for the study. Inclusion and exclusion criteria were confirmed. Clinically healthy subjects were selected from the patient

relatives/attendees and employees of the hospital who were aged between 35-70 years. The purpose of the selection of patient relatives/attendees and employees of the hospital is to prevent selection bias and confounding factors. The entire procedure and purpose of the study were explained to the participants. Informed written consent was taken from all study subjects.

SAMPLE SIZE CALCULATION

For calculation of sample size, we consider the study conducted by Fawzy MS et al [14]. With the minimum mean difference of 65.4 ng/mL for uVDBP between two of the three groups viz Control vs DM without nephropathy, with a standard deviation of ±21.9 and ±141.0 ng/mL in two groups and in comparison, 99% two-sided confidence limits, 90% power was considered.

The minimum sample size derived was 71 in each of the groups. Thus, the overall total sample size was 213. (Calculated using nMaster 2.0).

Formula for comparing means

$$\mathbf{n= [2S_p^2 (Z_{1- \alpha/2}+ Z_{1-\beta}) ^2] /\mu_d^2}$$

$S_p^2 = (S_1^2 + S_2^2)/2$

S_1^2: Standard Deviation (SD) in first group

S_2^2: SD in second group

μ_d^2: Mean differences between samples

α: Level of Significance

$1-\beta$: Power

Group I (n=71): Age and gender matched healthy controls

Group II (n=71): T2DM without Nephropathy

Group III (n=71): T2DM with Nephropathy

INCLUSION CRITERIA

1. Clinically proven Type 2 DM with or without nephropathy

   T2DM subjects were included based on ADA guidelines as follows [8]

   - Fasting Plasma Glucose ≥ 126 mg/ dL with a minimum fasting of 8 hours
   - 2 hours post- load glucose ≥ 200 mg/ dL
   - HbA1c (Glycated Hemoglobin) ≥ 6.5%

   DN is identified by persistently high urine albumin- to-creatinine ratio of ≥30 mg/g and/or sustained reduction in eGFR below 60 ml/min per 1.73 m^2 [52].

2. Non-diabetic age and gender matched healthy control

EXCLUSION CRITERIA

1. Patients with active urinary tract infection, renal disease other than DN
2. Patients with chronic liver or heart diseases
3. Patients on medications known to affect diabetic state and/ or DN
4. Patients on dialysis
5. Patients with hypertension
6. Patients with cancer
7. Patients with Acute Kidney Injury
8. Gestational Diabetes Mellitus
9. Patients with T1DM and Monogenic Diabetic Syndrome
10. Patients on drugs known to alter the parametric values to a major extent

METHOD OF SAMPLE COLLECTION, STORAGE AND DISPOSAL

After explaining the whole procedure to the patient in their understandable language and providing him/her with the patient information sheet, written informed consent complying with the Declaration of Helsinki 2012, was obtained from the study subjects.

Patients who agreed to participate in the study and fulfill inclusion and exclusion criteria were included for the study. Clinical details of diabetes and anthropometric measurements such as age, gender, height, weight of all study subjects was obtained from one-to-one interview. Right arm blood pressure was measured in

orthostatic position using mercury sphygmomanometer. A minimum of 8-10 hours fasting blood sample (3 mL) and 2 hours post- prandial blood sample (3 ml) were collected under aseptic condition and in patient comfortable position. 3 ml fasting sample was split into 1ml each into EDTA tube for HbA1c, Fluoride tube for blood glucose estimation and Plain tube for routine parameters. 3 ml of post prandial blood sample was divided into 1 ml for glucose estimation in fluoride tube and 2 ml into plain tube (Serum sample) for manual parameters as mentioned Table 4 methods characteristics, (Cystatin C, MDA, Vitamin C, GPX and NO). The collected samples were centrifuge at 3000 RPM for 5 minutes to obtain serum and plasma. Serum and plasma were used for routine analysis. Biochemical analysis was carried out in the fresh sample. Samples which require storage and samples for manual parameters were stored appropriately at -80 0C until analysis.

A clean-catch midstream urine sample (around 20 ml) was collected into sterile container and split into two. One part was centrifuged at 3000 rpm x 20 minutes and the supernatant was collected without disturbing and transferred into a sterile fresh container and capped tightly and stored at -20 °C till analysis. The centrifuged sample was used for uVDBP estimation. The uncentrifuged container's urine sample was used for urine albumin/creatinine ratio (uACR), microalbumin and fluoride analysis. All samples were collected with utmost care and precautions. Biomedical waste management guidelines as per the local authorities and present government policies were followed for the disposal of biomedical waste.

METHODOLOGY

All the routine investigations were analyzed using fully automated Vitros 5,1 FS dry chemistry auto analyzer from Ortho Clinical Diagnosis (OCD), United Kingdom, based on the principle of Reflectance Photometry.

Microalbumin was measured by quantitative immuno turbidimetric method on

5,1 FS dry chemistry auto analyzer.

Glycated hemoglobin (HbA1c) was measured by high performance liquid chromatography (HPLC) method on Bio-Rad D10 glycated hemoglobin analyzer at Biochemistry section of Central Diagnostic Laboratory Services facility at RL Jalappa Hospital and Research Centre.

Cystatin C levels were determined using Agappe MISPA i2 based on nephelometry methodology.

Manual methods were carried out at the research lab, Department of Biochemistry of Sri Devaraj Urs Medical College.

Height was measured by manual stadiometer and weight was recorded using digital weighing scale to calculate BMI as kg/m^2.

ELISA kit was supplied by Immunotag Geno Technology Inc. USA- IScience Innovation-Bangalore. **Catalogue Number-ITEH2937.**

Table 4: Method Characteristics

Sl.No	Parameters	Method	Instrumentation	Detectable range	Reference range
Diabetic Profile					
1.	Plasma Glucose (mg/dL)	GOD- POD	Vitros 1,5 FS	20- 625	FBS:70-110 PPBS:70-200
2.	Glycated haemoglobin (%)	HPLC (BioRadD10)	BioRad D10	2- 25%	≥6.5% considered diabetic
Renal Profile					
3.	Blood Urea (mg/dL)	Urease	Vitros 1,5 FS	4- 257	7- 20
4.	Serum Uric Acid (mg/dL)	Uricase	Vitros 1,5 FS	0.5- 17	2.5- 8.5
5.	Serum Creatinine (mg/dL)	Sarcosine oxidase	Vitros 1,5 FS	0.05- 140	0.5- 1.4
Biomarkers					
6.	uVDBP (µg/mL)	Sandwich ELISA	Immuno Tag	10-3800	**To be derived**
7.	Serum CysC (mg/L)	Nephelometry	Mispa i2	0.1- 10	0.5- 1
8.	Urine F (ppm)	ISE	Orion Thermo scientific	1- 100	**To be derived**

9.	Vitamin D (ng/mL)	Chemiluminescence Immunoassay	Vitros ECi/ECiQ	8.0-150	Deficient: <20 Insufficient: 20-30 Sufficient: 30-100
10.	Microalbumin (mg/L)	Quantitative turbidimetric	Vitros 1,5 FS	6-190	<16.7
Oxidative Stress Parameters					
11.	Glutathione Peroxidase (ng/mL)	Sandwich ELISA	Immuno Tag	0.5-180	**To be derived**
12.	Malondialdehyde	Thiobarbituric acid	PerkinElmer UV/VIS Spectro photometer Lambda 35, UK	**To be derived**	
13.	Nitric Oxide	Modified Griess			
14.	Vitamin C	2,4-DNPH			
Lipid Profile					
15.	Serum Triglyceride (mg/dL)	Lipase	Vitros 1,5 FS	10- 525	30–200
16.	Total Cholesterol (mg/dL)	Cholesterol oxidase	Vitros 1,5 FS	50- 325	<200
17.	Direct HDL (mg/dL)	Cholesterol ester hydrolase	Vitros 1,5 FS	5- 110	35- 50
Liver Function Tests					
18.	Total Protein (gm/dL)	Biuret method	Vitros 1,5 FS	2.0-11.0	6.3-8.2
19.	Serum Albumin (gm/dL)	BCG Dye binding	Vitros 1,5 FS	1.0-6.0	3.5- 5.0
20.	ALT (IU/L)	Kinetic	Vitros 1,5 FS	3-750	15-46
21.	AST (IU/L)	Kinetic	Vitros 1,5 FS	6-1000	13-69
Ionic Profile					
22.	Serum Calcium (mg/dL)	Arsenazo III dye method	Vitros 1,5 FS	1.0-14.0	8.4-10.2
23.	Inorganic Phosphate (mg/dL)	Fiske-Subbarow (molybdate)	Vitros 1,5 FS	0.5-13.0	2.5-4.5
24.	Magnesium (mg/dL)	Formazon Azo dye	Vitros 1,5 FS	0.2-10	1.6-2.3

ASSAY PRINCIPLE AND PROCEDURE

Sandwich Enzyme Linked Immuno Sorbent Assay (ELISA)

Principle: Sample containing antigen is added to microtiter well coated with antibody and allowed to react with the antibody attached to the well, forming antigen-antibody complex. A second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. At this stage, the antigen is sandwiched between two antibody molecules, one coated on microtiter well and the other enzyme linked antibody. Unbound secondary antibody is removed by washing. Finally, addition of substrate to the plate is hydrolyzed by enzyme to form colored products. Intensity of the color is directly proportional to the concentration of the analyte in the sample. A standard curve is constructed by plotting absorbance values against concentration of standards and concentrations of unknown samples were determined using the standard curve.

URINARY VDBP (uVDBP): Sandwich ELISA

Kit Components

Item	Specification (96T)
Standard Solution (4000µg/mL)	0.5mL x 1
Pre-coated ELISA Plate	12x8 well strips x 1
Standard Diluent	3mL x 1
Streptavidin-HRP	6mL x 1
Stop Solution	6mL x 1
Substrate Solution A	6mL x 1
Substrate Solution B	6mL x 1
Wash Buffer Concentration (25x)	20mL x 1
Biotinylated human VDBP Antibody	1mL x 1
Plate Sealer	2
Zipper Bag	1

Material Required

- 37 ^{0}C ± 0.5 ^{0}C incubator
- Absorbent paper
- Precision pipettes and disposal pipette tips
- Clean tubes
- Deionized or distilled water
- Microplate reader with 450 ± 10 nm wavelength filter

Reagent Preparation

- All reagents were brought to room temperature before use
- Assay reagents supplied are ready to use
    - Antibody coated microtiter plate
    - Biotinylated human VDBP antibody
    - Streptavidin-HRP
    - Substrate solution
    - Stop solution
    - Standard diluent
- **Standard**
    - Reconstitute 120 µL of standard (4000 µg/mL) with 120 µL of standard diluent to generate a 2000 µg/mL standard stock solution
    - Mix well and keep for 15 minutes
    - Prepare duplicate standard by serially diluting the standard stock solution (2000 µg/mL) 1:2 with standard diluent to produce 1000 µg/mL, 500 µg/mL, 250 µg/mL and 125 µg/mL solutions
    - Standard diluent serves as zero standard (0 µg/mL)

Standards	Reconstitution	Concentration
Standard No. 5	120µL Original Std. + 120µL Std. Diluent	2000 µg/mL
Standard No. 4	120µL Standard No. 5 + 120µL Std. Diluent	1000 µg/mL
Standard No. 3	120µL Standard No. 4 + 120µL Std. Diluent	500 µg/mL
Standard No. 2	120µL Standard No. 3 + 120µL Std. Diluent	250 µg/mL
Standard No. 1	120µL Standard No. 2 + 120µL Std. Diluent	125 µg/mL

- **Wash Buffer:** 20 mL of wash buffer concentrate 25x is diluted into deionized or distilled water to yield 500 mL of 1x wash buffer

Assay Procedure

1. All reagents, standard solutions and samples were prepared as instructed by the kit manufacturer
2. All reagents were brought to room temperature before use
3. Each standard of 50 µL were added to respective standard wells
4. Ensured that antibody was not added to standard wells because the standard solution contains biotinylated antibody
5. Urine sample of 40 µL were added to sample wells
6. Anti-VDBP antibody of 10 µL were added to sample wells
7. Streptavidin-HRP of 50 µL were added to sample wells & standard wells (not blank control well)
8. Mixed well and the microtiter plate is sealed with sealer provided in the kit
9. Incubated for 60 minutes at 37 ^{0}C
10. After incubation, the sealer was removed and the plate was washed 5 times with wash buffer
11. After washing, the plate was blotted onto paper towels

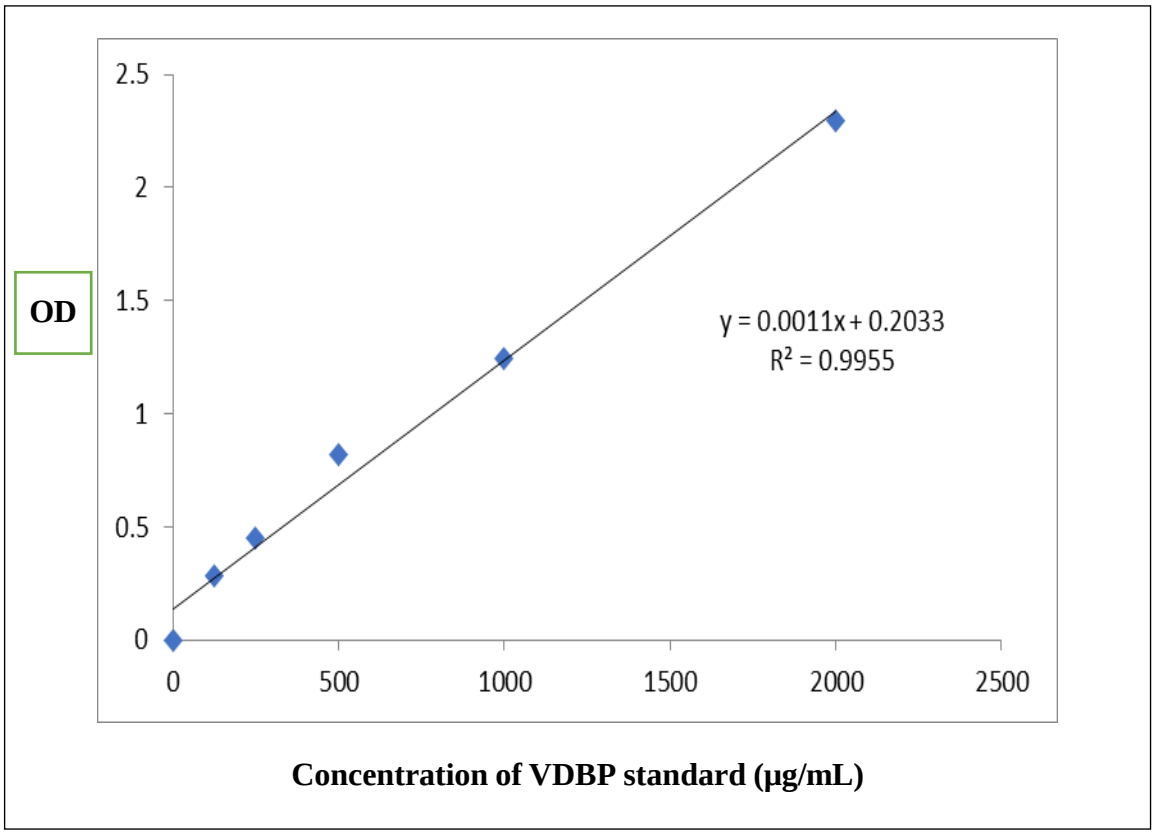
12. Substrate solution A of 50 µL was added to each well
13. Substrate solution B of 50 µL was added to each well
14. Microtiter plate was sealed with a new sealer and incubated for 10 minutes at 37 ^{0}C in the dark
15. Stop solution of 50 µL was added to each well
16. Confirmed change of blue color to yellow
17. Optical density (OD value) of each well was measured immediately using a microplate reader set at 450 nm within 10 minutes after adding the stop solution
18. The values were deduced on the centimeter graph sheets
19. Confirmed the standard graph which followed y=mx+c

Calculation of Result

A standard curve was constructed by plotting the OD for each standard on the vertical (Y) axis against the concentration on horizontal (X) axis and draw a best fit curve through the points on the graph.

The X-axis depicts the concentration of VDBP standard with an incremental increase of 500 µg/mL while Y-axis showed an OD value with an incremental increase of 0.5. The initial concentration of the VDBP standard was 125 µg/mL with an observed OD value of 0.28.

Concentration of Standard (µg/mL)	**Absorbance Observed**	**Concentration Obtained (µg/mL)**
125	0.28	127.81
250	0.453	285.09
500	0.82	618.72
1000	1.244	1004.18
2000	2.294	1958.72



Graph 1: Standard Graph for Vitamin D Binding Protein

Concentration of test samples to be calculated by the equation: **X= [Y-0.2033/0.0011],** where Y is the observed OD of specific sample.

GLUTATHIONE PEROXIDASE (GPX): Sandwich ELISA [106]

Standard curve for GPx was plotted against OD values for each standard on the vertical (Y) axis against the concentration of standard on horizontal (X) axis and draw a best fit curve through the points on the graph. Concentration of test samples to be calculated by the equation: **X= [Y-0.0126/0.0097],** where Y is the observed OD of specific sample.

Method: Sandwich ELISA, Color change with wavelength λmax = 450nm

Catalogue No.: ITEH03696

Storage

Reagent: 2-8 ^{0}C if unused for ≤ 6 months

Sample: -80 ^{0}C for 6 months

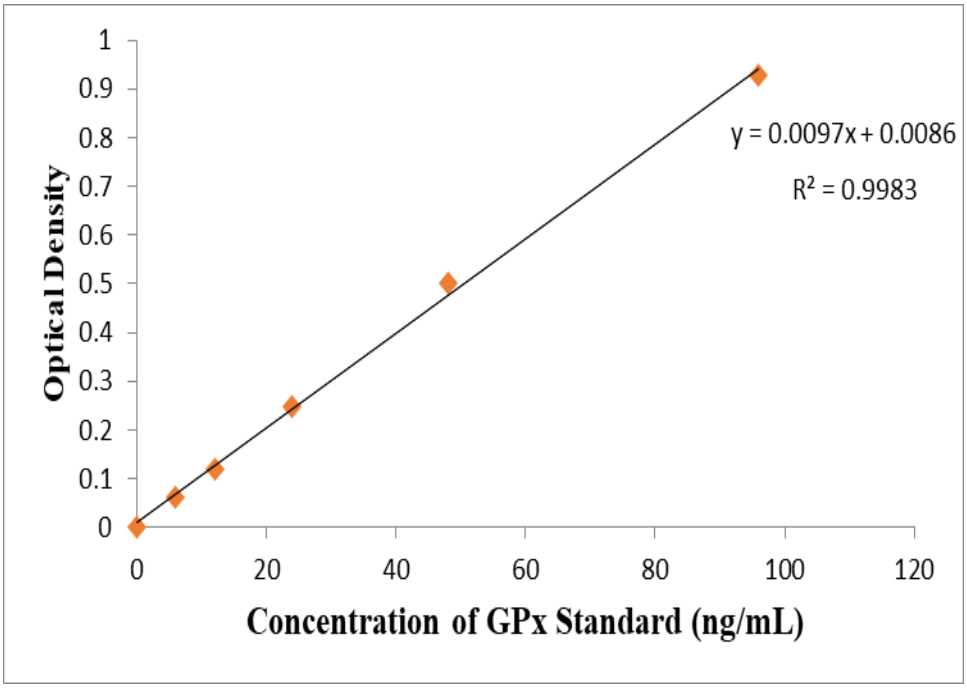
Detection Range (ng/mL): 0.5-180

Sensitivity (ng/mL): 0.27

Intra-Assay precision: <8%

Inter-Assay Precision: <10%

Concentration of Standard (ng/mL)	Absorbance Observed	Concentration Obtained (ng/mL)
6	0.062	5.5
12	0.12	11.48
24	0.248	24.68
48	0.502	50.86
96	0.928	94.78



Graph 2: Standard Graph for Glutathione Peroxidase

CYSTATIN C: Nephelometry (Agappe Mispa i_2) [107]

Principle: Nephelometry is the measure of turbidity of a solution due to immune particles agglutination. Cystatin C in the test sample binds to the specific polyclonal rabbit anti-cystatin C antibody, which has been adsorbed to latex particle and agglutinates. The agglutination is directly proportional to the quantity of cystatin C in the sample.

Kit Components

Reagent	Product Code 12009021	Description
Cystatin-C R1	1 x 3.8 mL	Tris buffer (1.2%) (pH 8.5±0.3, 100 mmol)
Cystatin-C R2	1 x 1.4 mL	Synthesized polystyrene latex particle coated with polyclonal anti cystatin C antibody (rabbit)

Reagent preparation

- Cystatin-C R1 and R2 reagents were ready to use
- The sealed reagents are stable up to the expiry date on the label, when stored at 2-8 ^{0}C
- Once opened the reagents are stable for 60 days
- The validity of the smart card will be up to 60 days from the date of insertion and activation of the card in Mispa i2

Sample: Required sample material is human serum or EDTA/ Heparinized plasma. It is recommended to analyse the sample as fresh as possible

Assay Procedure

1. Insert card to card reader slot and display will prompt to add R1+Sample
2. Pipette 200 µL R1 and 5 µL sample to cuvette and place the cuvette into cuvette holder
3. After incubation display will prompt to add R2
4. Pipette 40 µL R2 using attached sensor pipette to the cuvette
5. The result will show in the display and print out

Agappe MISPA *i*2

Smart Card

URINE FLUORIDE: Fluoride Ion Selective electrode (F^--ISE) method [108]

Principle: F^--ISE, the ion-selective membrane is a single crystal of Lanthanum Fluoride (LaF_3) doped with Europium Fluoride (EuF_2) which produces holes in the crystal lattice through which F ion can pass. When immersed in a fluoride solution and connected via a voltmeter to an AgCl/KCl external reference electrode immersed in the same solution, the negative F ions in the solution pass through the crystal membrane by normal diffusion from high concentration to low concentration until there is an equilibrium between the force of diffusion and the reverse electrostatic

force due to repulsion between particles of similar charge. On the other side of the membrane there is a corresponding build-up of positive ions.

The build-up of negative F ions on the inside of the membrane is compensated for by Cl^- ions in the internal reference solution becoming neutralised by combining with the Ag/AgCl wire, and electrons are thus forced through the external wire to the voltage measuring device (ion meter or computer interface). The other terminal of the voltmeter is connected to the Ag/AgCl wire of the external reference electrode. Here, the influx of electrons causes Ag ions in the filling solution to accept electrons and deposit on the silver wire and, consequently, Cl^- ions to flow out into sample solution.

Reagents Required for Fluoride Analysis

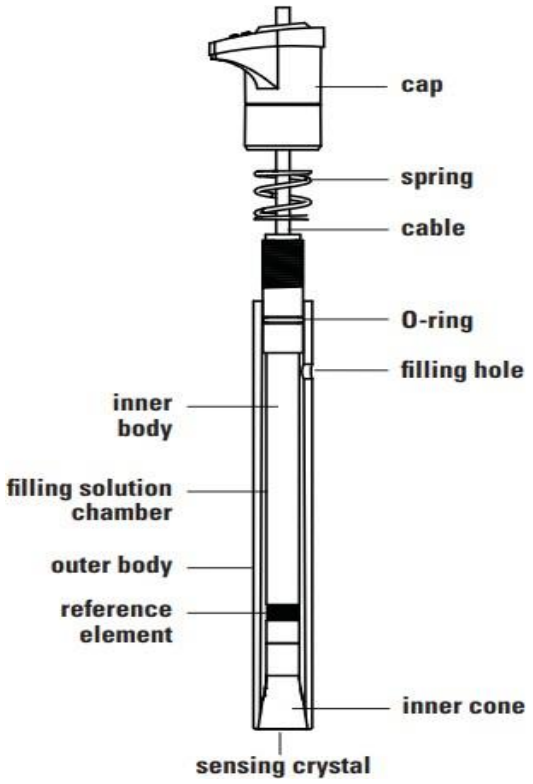
Reagent	Catalogue No.	Qty.
2 ppm std. with TISAB II	Orion 040907	475 mL
10 ppm std. with TISAB II	Orion 040908	475 mL
TISAB II	Orion 940909	3.79 L
Filling solution		50 mL
Measuring cups	Plastic	5-15 mL

Preparation of Fluoride Standard:

1. To prepare 10.0 ppm: Add 9 mL distilled water + 1 mL of Fluoride standard 100 ppm F^- in a plastic beaker, mix well
2. To prepare 1.0 ppm: Add 9 mL distilled water + 1 mL of Fluoride standard 10 ppm F^- in a plastic beaker, mix well
3. To prepare 0.1 ppm: Add 9 mL distilled water + 1 mL of Fluoride standard 1 ppm F^- in a plastic beaker, mix well
4. Add 1 mL of TISAB III in each of the standard

Assay Procedure

1. Measure each standard of fluoride from lower to higher concentration i.e., 0.1 ppm, 1 ppm and then 10 ppm and set the standard in the ion meter (Rinse the electrode with distilled water after each reading of standard solution)
2. Record the result slope value. The slope should be between -54 to 60 Mv
3. Take 9 mL of urine in a plastic beaker of 50 mL and add 1 mL of TISAB III Buffer
4. Mix well and dip the electrode in solution
5. Take the reading and record the fluoride content
6. Rinse the electrode with distilled water after each sample



9609BNWP Fluoride Combination Electrode

Thermo-scientific-stara2147-star-a214-ph-ise-fluoride-meter-kit

VITAMIN D: Chemiluminescence Immunoassay [109]

Principle: A competitive immunoassay technique is used which involves the release of the 25-OH Vitamin D in the sample from the binding protein using a low pH denaturant and the subsequent competition of the free 25-OH Vitamin D with horseradish peroxidase (HRP) labeled 25-OH Vitamin D reagent for monoclonal anti-vitamin D bound to the wells. Unbound materials are removed by washing. The bound HRP conjugate is measured by a luminescent reaction. A reagent containing luminogenic substrate (a luminal derivative and a peracid salt) and an electron transfer agent is added to the wells. The HRP in the bound conjugate catalyzes the oxidation of the luminol derivative, producing light. The electron transfer agent (a substituted acetanilide) increases the level of light produced and prolongs its emission. The light signals are read by the system. The amount of HRP conjugate bound is indirectly proportional to the concentration of 25-OH Vitamin D present.

Detection Range (ng/mL): 8.0-150

Reference Range (ng/mL)

Deficient: <20

Insufficient: 20-<30

Sufficient: 30-100

Potentially toxic: >100

Storage

Reagents: Unopened at 2- 8˚C until expiry,
Onboard ≤ 4 weeks

Samples: ≤ - 20 ˚C for 4 weeks, 2-8 ˚C for 7 days.

From the history elicited from the patients and treating physician it was observed that study subjects were not on either vitamin D or calcium orally or parenterally. However, the dietary sources and synthesis of vitamin D from sun could not be ruled out. This statement was considered with utmost care during the entire study period with a notion of interfering substances and confounding factors.

GLYCOSYLATED HEMOGLOBIN (HbA1c %) [110]

Method: High Pressure Liquid Chromatography (HPLC); Measured at λ_{max}= 340 nm

Instrumentation: Bio Rad D10

Units of Measurements

- **National Glycohemoglobin Standardization Program (NGSP) Unit**

  %A1c= (IFCC x 0.09148) + 2.152

- **International Federation of Clinical Chemistry (IFCC) Units**

  HbA1c (mmol/mol) = {HbA1c [g/dL]/ Hb [g/dL]} × 1000

Detection range (%): 2- 25

Minimum Detection limit (%): 2

Reference Range

- 6.1-6.4% Pre-diabetic
- ≥6.5% are considered diabetic

Storage

Reagent: Onboard ≤ 28 days,

Unopened 2-8 ˚C until expiry

Sample: 2-28 ˚C ≤ 3 days

MICROALBUMIN [111]

Method: Quantitative turbidimetric method; Measured at λ_{max} = 540nm

Reaction: 2-point kinetics

Measurement: Against distilled water

Sample: Urine (7µL)

Linearity: 800 IU/mL

Reaction Direction: Increasing

URIC ACID [112]

Method: Uricase Colorimetric method; Measured at λ_{max} = 670nm

Detection range (mg/dL)

Serum: 0.50-17

Reference range (mg/dL)

- Male: 3.5- 8.5
- Female: 2.5- 6.2

Storage

Reagents: Onboard stability ≤ 2 weeks,

≤ -18˚C for ≤ 6 months

Sample:18- 28 ˚C ≤ 3 days. ≤ -18˚C for ≤ 6 months

GLUCOSE [113]

Method: Glucose Oxidase Peroxidase (GOD-POD); Measured at λ_{max}= 540nm

Detection limit (mg/dL)

Serum: 20- 625

Urine: 20- 650

Reference range (mg/dL)

Serum: Fasting blood sugar: 80- 110

Post- prandial blood sugar: 100-140

Random blood sugar: 90-180

Urine: ≤ 30

Storage

Reagents: ≤ -18 ˚C until expiry,

Onboard: ≤ 1 week

Sample: ≤ -18 ˚C for 1 year

UREA [114]

Method: Urease Colorimetric method; Measured at λ_{max} = 670nm

Detection range (mg/dL)

Serum: 4.29- 257.4

Reference range (mg/dL)

Male: 19- 43

Female: 15- 36

Storage

Reagents: Onboard: ≤ 2weeks,

≤ -18 ˚ C until expiry

Serum: -18 ˚ C ≤ 6 months

CREATININE [115]

Method: Enzymatic Sarcosine Oxidase; Measured at λ_{max} = 670 nm

Detection range (mg/dL)

Serum: 0.05- 14

Urine: 1.2- 346.5 *

* After multiplying with dilution factor x 21

Reference range (mg/dL)

Serum

Female: 0.66- 1.25

Male: 0.52- 1.04

***Urine (mg/day)**

Male: 1000- 2000

Female: 800- 1800

* Creatinine concentration x 24hrs

Storage

Reagent: Onboard: ≤ 2 weeks,

≤ -18 ˚C until expiry

Sample: ≤ -18 ˚C till analysis

CALCIUM [116]

Method: Arsenazo III dye method; Measured at λ_{max}= 680nm

Detection range (mg/dL)

Serum: 1.0-14.0

Urine: 1.0-17.80

Reference range (mg/dL)

Serum: 8.4-10.2

Urine: 100-300 (mg/day)

Storage

Reagent: ≤ -18 ˚C until expiry,

Onboard ≤ 4 weeks

Sample: ≤ -18 ˚C for 1 year

INORGANIC PHOSPHATE [117]

Method: Fiske-Subbarow (molybdate) method; Measured at λ_{max}= 670nm

Detection range (mg/dL)

Serum: 0.5-13.0

Urine: 5.5-143.0

Reference range (mg/dL)

Serum: 2.5-4.5

Urine: 0.4-1.3 (g/day)

Storage

Reagent: ≤ -18 ˚C until expiry,

Onboard ≤ 4 weeks

Sample: ≤ -18 ˚C for 2 months (serum/plasma),

≤ -18 ˚C for 6 months (urine)

TOTAL PROTEIN [118]

Method: Biuret method; Measured at λ_{max}= 540nm

Detection range (gm/dL)

Serum: 2.0-11.0

Reference range (gm/dL)

Serum: 6.3-8.2

Storage

Reagent: ≤ -18 ˚C until expiry,

Onboard ≤ 4 weeks

Sample: ≤ -18 ˚C for 6 months

ALBUMIN [119]

Method: Bromocresol green method; Measured at λ_{max}= 630nm

Detection range (gm/dL)

Serum: 1.0-6.0

Reference range (gm/dL)

Serum: 3.5-5.0

Storage

Reagent: ≤ -18 ˚C until expiry,

Onboard ≤ 2 weeks

Sample: ≤ -18 ˚C until analysis

TRIGLYCERIDE [120]

Method: Lipase hydrolysis colorimetric method; Measured at λ_{max} = 540nm

Detection range (mg/dL): 10- 525

Reference range (mg/dL)

Normal: ≤150

Borderline: 150-199

High: 200-499

Storage

Reagent: Onboard: ≤ 1 week,

≤ -18 ˚C until expiry

Sample: ≤ -18 ˚C for 6 months

TOTAL CHOLESTEROL [121]

Method: Cholesterol oxidase method

Incubation time: 5 min Measured at λ_{max} absorption at 540 nm

Detection range (mg/dL): 50- 325

Reference range (mg/dL)

Desirable: <200

Borderline: 200- 239

High: >240

Storage

Reagent: Onboard: 2 weeks,

≤ -18 ˚C until expiry

Sample: ≤ -18 ˚C for ≤ 3 weeks

DIRECT HDL [122]

Method: Cholesterol ester hydrolase colorimetric method; Detected at λ_{max} = 670nm

Detection range (mg/dL): 5- 110

Reference range (mg/dL)

Low: ≤ 40

High: ≥ 60

Storage

Reagent: ≤ -18 ˚C until expiry,

Onboard ≤ 1 week

Sample: ≤ -20 ˚C for ≤ 3 weeks

MALONDIALDEHYDE (MDA): Thiobarbituric Acid Reactive Substances (TBARS) [123]

Principle: Free MDA, as a measure of lipid peroxidation was measured spectrophotometrically at 530 nm as TBA reactive substances after precipitating the protein with trichloroacetic acid (TCA).

Reagent Preparation

- MDA Standard: 25 µL of TMP (1,1,3,3-Tetra Methoxy Propane) dissolved in 100 mL of distilled water to give a 1 mM stock solution
- Working Standard: 1 mL TMP stock solution dissolved in 50mL of 1% H_2So_4 and incubated at room temperature for 2 hours
- 0.75% TBA Reagent: 75 mg of TBA dissolved in 25% TCA (25 gm TCA in 100 mL distilled water). To this 2.8 mL of N/2 H_2So_4 was added. The final volume was made up to 100 mL with 25% TCA

- N/2 H_2So_4: 3.5 mL of H_2So_4 in 246.5 mL of distilled water (Final volume-250)

Procedure

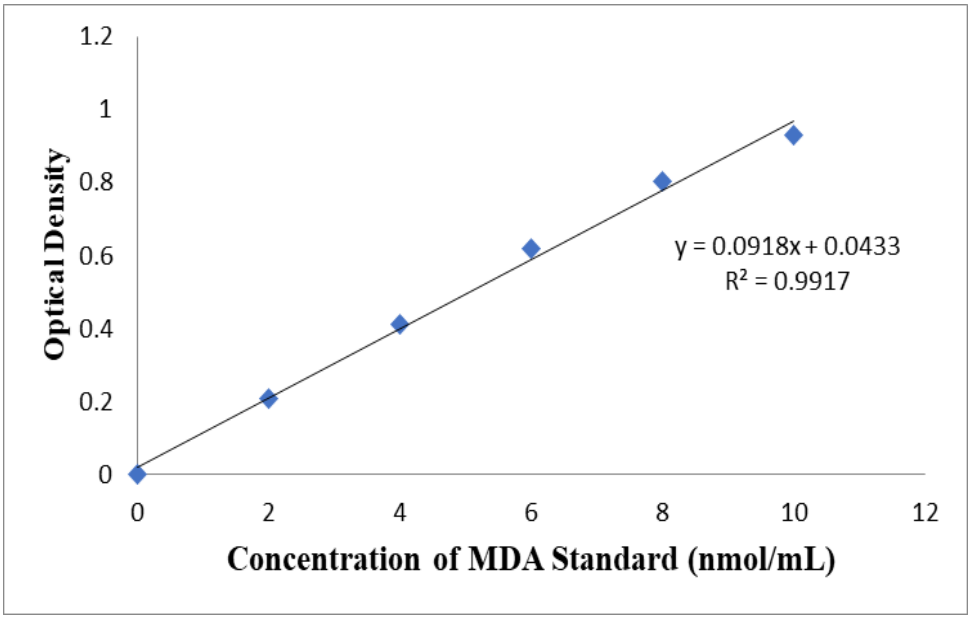
Reagent	Blank	Standards					Test
		S1	S2	S3	S4	S5	
Distilled water (mL)	0.5	-	-	-	-	-	-
Working standard (mL) [20 nmol/L]	-	0.6	1.5	1.8	2.4	3	-
Serum sample (mL)	-	-	-	-	-	-	0.5
TBA (mL) [0.75%]	5.5	5.4	4.5	4.2	3.6	3	5.5

- Mix well and keep in boiling water bath for 15 minutes and centrifuge.
- Take the supernatant and measure absorbance of developed pink color at 530 nm.

Calculation

$$\text{Concentration of MDA (nmol/L)} = \frac{\text{OD of Test-OD of Blank}}{\text{OD of Standard-OD of Blank}} \text{ X Conc. of Standard}$$

Concentration of Standard (nmol/mL)	Absorbance Observed	Absorbance Obtained	Concentration Obtained (nmol/mL)
2	0.207	0.1864	1.97
4	0.411	0.3904	4.12
6	0.619	0.5984	6.31
8	0.804	0.7834	8.26
10	0.928	0.9074	9.57



Graph 3: Standard Graph for Malondialdehyde (MDA)

NITRIC OXIDE: Modified Greiss Method [124]

Principle: Sulphanilic acid- Diazonium salt + N-(1-napthyl) ethylene diaminedihydrochloride – azo dye; formation of the azo dye is detected via its absorbance at 540 nm

Reagent Preparation

- Sodium Nitrite Standard (working): 0.0069 gm $NaNo_2$ dissolve in 100 mL of distilled water
- Reagent A (0.3% N-ethylene diamine dihydrochloride): 300 mg of N-ethylene diamine dihydrochloride dissolved in 100 mL double distilled water
- Reagent B (3% Sulphanilamide): 3 gm of sulphanilamide dissolved in 100 mL of 1N HCl (0.1 mL HCl in 99 ml of distilled water)
- Greiss Reagent: Mix reagent A and reagent B in equal amount. This must be prepared freshly

- 70% Sulphosalicylic Acid: 70 gm of sulphosalicylic acid in 100 mL of distilled water
- 10% NaOH: 10 gm of NaOH in 100 mL of distilled water
- Tris-HCl Buffer (pH-9.0): 18.5 gm of Tris buffer dissolved in 800 mL of distilled water. Set the pH at 9.0 with 6N HCl and make final volume to 1000 mL with distilled water
- 6N HCl: 52 mL of HCl mixed with 48 mL of distilled water (final volume 100 mL)

Procedure

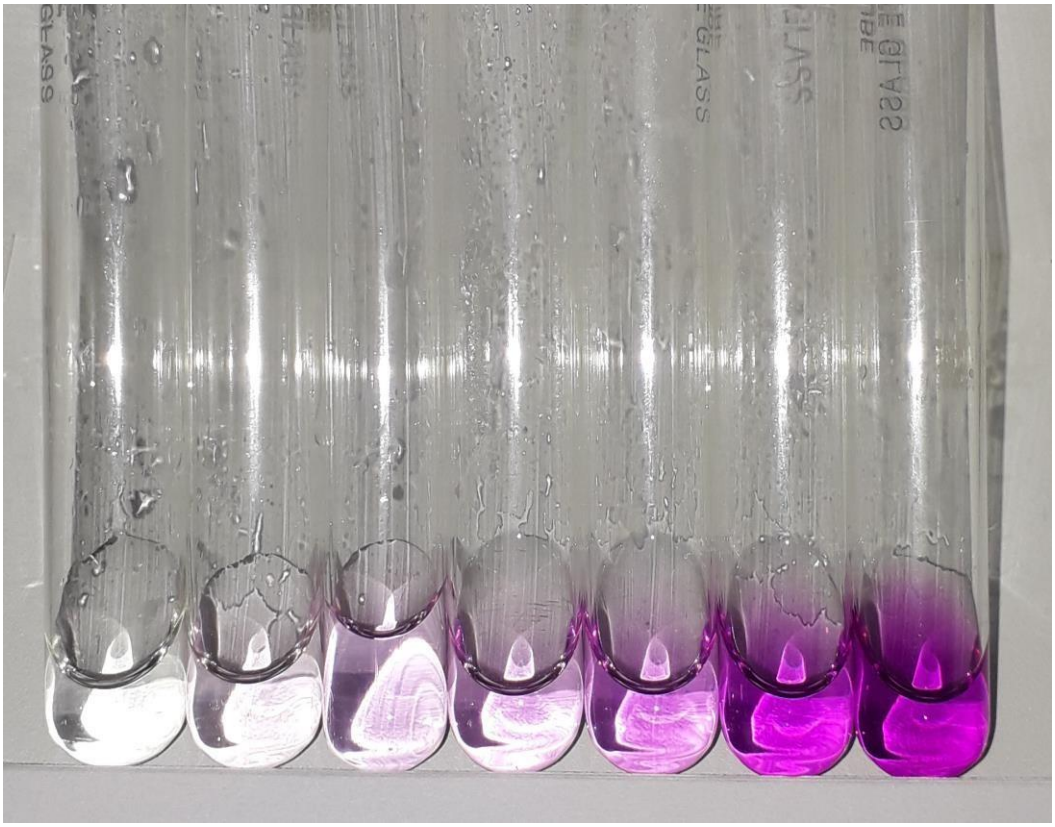
- To 1 mL of serum, 0.1 mL of sulphosalicylic acid is added and mixed every 5 minutes for 30 minutes
- Centrifuge this mixture at 3000 rpm for 20 minutes
- Take 200 µL of supernatant in a separate tube and add 30 µL of 10%NaOH. In this mixture add 300 µL of Tris-HCl buffer
- Now add 530 µL of Greiss reagent
- Keep the tube in dark place for 10 minutes and read the absorbance at 540 nm against blank

Reagent	**Blank**	**Standard**					
		S1	**S2**	**S3**	**S4**	**S5**	**S6**
$NaNo_2$ (µL)	-	50	100	150	250	500	750
Distilled water (µL)	500	-	-	-	-	-	-
Greiss reagent (µL)	500	950	900	850	750	500	250
• Mix and keep the tubes in dark for 10 minutes and read the absorbance at 540 nm.							

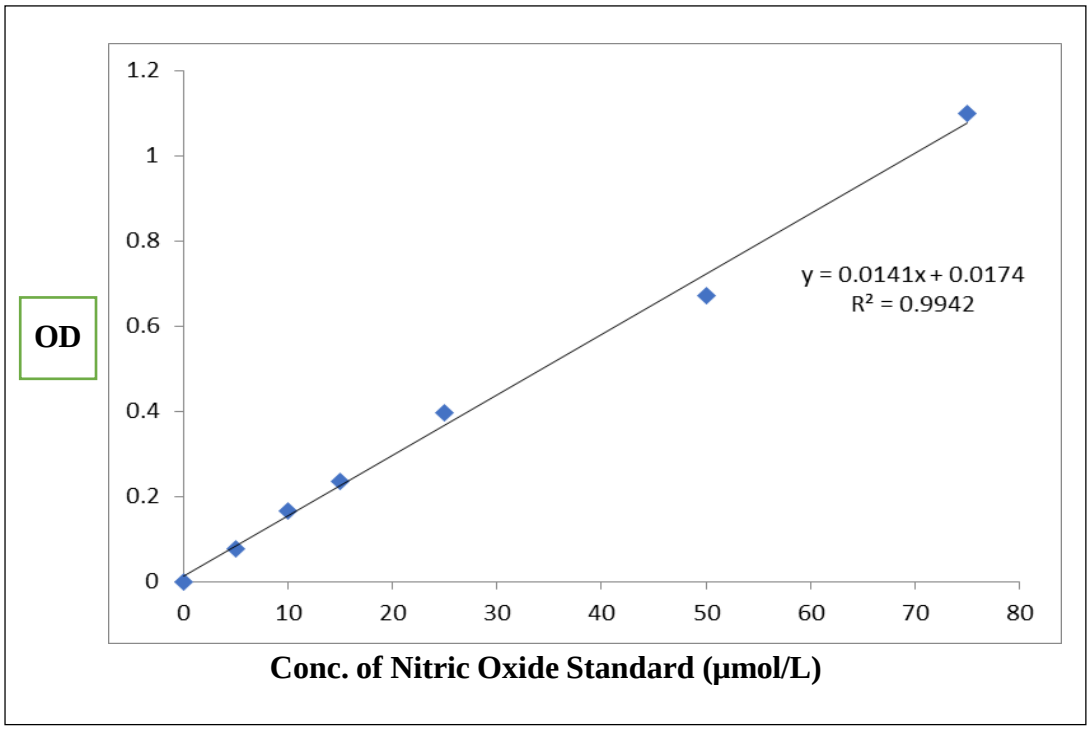
Calculation

$$\text{Concentration of NO (µmol/L)} = \frac{\text{OD of Test-OD of Blank}}{\text{OD of Standard-OD of Blank}} \text{ X Conc. of Standard}$$

Figure 7: Standardization of Nitric Oxide



Concentration of Standard (µmol/L)	Absorbance Observed	Absorbance Obtained	Concentration Obtained (µmol/L)
5	0.077	0.065	4.55
10	0.165	0.153	10.74
15	0.234	0.222	15.60
25	0.397	0.385	27.08
50	0.67	0.658	46.31
75	1.1	1.088	76.59



Graph 4: Standard Graph for Nitric Oxide

VITAMIN C: 2,4-Dinitrophenyl Hydrazine Method [125]

Principle: Ascorbic acid in colorimetric method is initially converted into dehydroascorbate by shaking with liquid bromine. After this conversion, ascorbic acid is coupled with 2,4-dinitrophenyl hydrazine and then treated with sulfuric acid. Absorbance of red color developed is measured at 540 nm

Reagent Preparation

- Liquid Bromine
- Thiourea Solution: 10% thiourea in 50% of alcohol acts as a mild reducing agent
- 2,4-Dinitrophenylhydrazine Reagent: 2 gm of 2,4-DNPH is dissolved in 100 mL of 9N H_2So_4 (24.4 mL of H_2So_4 in 100 mL distilled water)
- Ascorbic Acid Standard (Stock): 100 mg of ascorbic acid dissolved in 100 mL of 4% metaphosphoric acid
- Ascorbic Acid Standard (Working): Dilute 2.5 mL of stock solution with 17.5 mL of 4% metaphosphoric acid and 2 drops of liquid bromine is added. Keep it in ice

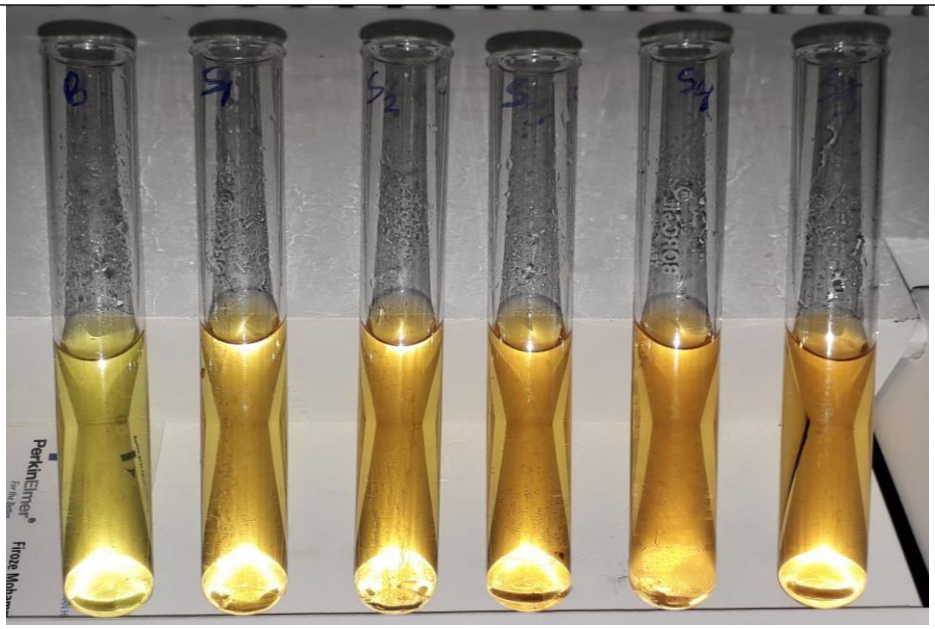
chest for 2 hours, excess bromine is removed by aeration. After aeration this content is transferred to 100 mL standard flask and make it up to 100 mL with 4% metaphosphoric acid

- 85% H_2So_4
- 4% Metaphosphoric Acid: 4 gm in 100 ml of distilled water

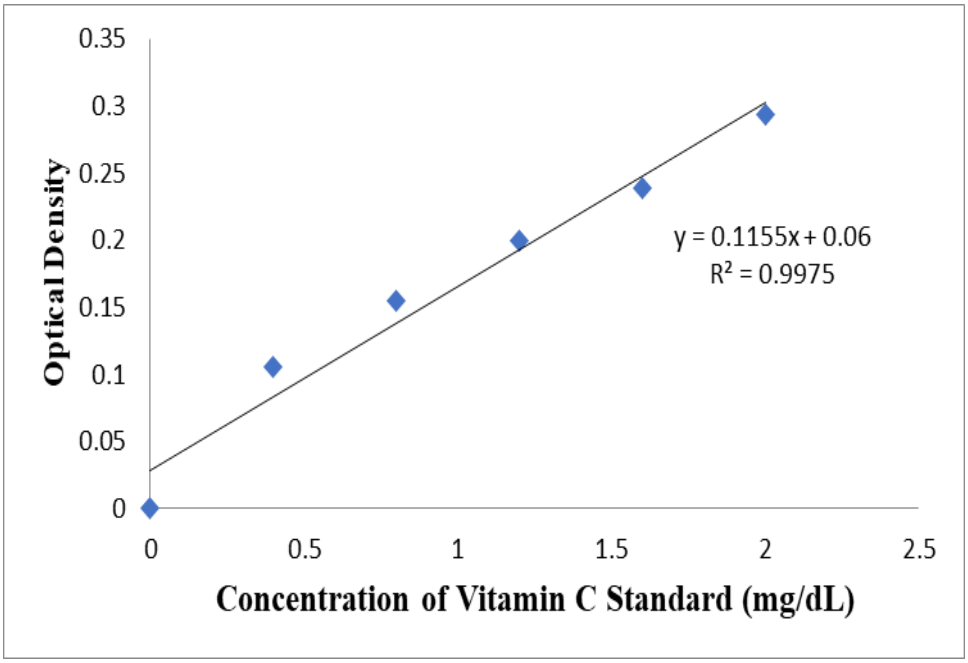
Procedure

Reagents	Blank	Standard	Test
Distilled Water (mL)	0.4	-	-
Standard	-	0.4	-
Serum sample (mL)	-	-	0.4
4% Metaphosphoric acid (mL)	3	3	3
Thiourea	1 Drop		
2,4 DNPH (mL)	1	1	1
• Incubate at room temperature for 3 hours			
85% H_2So_4	4	4	4
• Cool in ice chest for 10 minutes and read the absorbance at 530 nm			

Figure 8: Standardization of Vitamin C



Concentration of Standard (mg/dL)	Absorbance Observed	Absorbance Obtained	Concentration Obtained (mg/dL)
0.4	0.105	0.045	0.39
0.8	0.155	0.095	0.82
1.2	0.2	0.14	1.21
1.6	0.239	0.179	1.55
2.0	0.294	0.234	2.03



Graph 5: Standard Graph for Vitamin C

CALCULATED PARAMETERS

i. eGFR with Cystatin C [126]

eGFR= 135 × min (SCr/K, 1)−α × max(SCr/K, 1)−0601 × min(SCysC/0.8, 1)−0375 × max(SCysC/0.8, 1)− 0.711 × 0.995age [× 0.969 if female] [× 1.08 if black]

If female: K = 0.7, α =−0.248 If male: K = 0.9, α =−0.207

The minimum and maximum are mentioned in the standard formula for eGFR, Reference number 126. In our study we used online eGFR calculator available in the application, online approved by National Kidney Foundation.

Screenshot of NKF eGFR calculator is enclosed for reference.

ii. LDL: **Friedewald Equation** [127]

LDL cholesterol= [Total cholesterol – (HDL cholesterol+Triglyceride/5)]

Considering its limitations

iii. Very Low-density Lipoprotein (VLDL) [128]

VLDL= TG/ 5

iv. Non- HDL

nHDLc = Total Cholesterol- HDL

v. **Albumin Creatinine Ratio (ACR)**

ACR= Urine albumin (mg)/ Urine creatinine (gm)

All the calculations are done considering its limitations.

QUALITY ASSURANCE SCHEME

Regarding quality control (QC) and quality assurance (QA) with respect to uVDBP, fluoride and Cystatin C, we followed the protocol

1. Got the performance verification certificate from the kit manufacturer and confirm the minimum and maximum detectable range and followed the dilution protocol as per the kit insert
2. Specific standards by manufacturer were used to maintain quality for fluoride analysis

We followed level 1 and level 2 of internal quality assurance scheme (IQAS) every day on regular basis for better patient care procured from Bio Rad USA. Level 1 is physiological range and level 2 is pathological range. Since, the chemistry molecules included in my study are routine tests done in Central Diagnostic Laboratory Services, Biochemistry Section; the QC values for patient care analyzed are also considered for my study project. External quality assurance scheme (EQAS) samples from Bio Rad USA were procured and run.

The HbA1c Proficiency Testing provider is Bio Rad USA for both internal and external quality. Three levels of quality testing were run for low range, physiological range and high range values.

STATISTICAL ANALYSIS

The data obtained was tabulated in Microsoft excel and analyzed statistically using IBM SPSS version 20.

- Data were tested for normality by using **Kolmogorov-Smirnov test.**
- **Descriptive statistics:** Calculating Mean ± Standard Deviation (SD) for normally distributed data. Non-parametric variable expressed as median (minimum-maximum)
- **Analysis of Variance (ANOVA):** To find asymptotic significance by calculating probability- value (p- value) between all 3 groups. **Kruskal-walli's** and **Mann-Whitney U** test for were used for non-parametric variables
- **Post- Hoc test:** To further find where exactly the difference in mean lies between two groups and the significance is calculated by Bonferroni Test
- **Pearson's correlation (r):** To find the trend between two variables (either positively correlated or negatively correlated)
- **Receiver Operating Curve:** ROC analysis was done using MedCalc software version 19.8. Area under curve (AUC), sensitivity and specificity were also calculated

All tests were two-tailed, and a p-value of <0.05 was considered as statistically significant.

Results

A total of n=213 study subjects within the age group of 35-70 years of both genders were included in this cross-sectional study. Among the study subjects 65.3% (n=139) were males and 34.7% (n=74) were females. The group wise gender distribution of the study subjects was shown in the following graph 6. The anthropometric measures and physiological variables of study subjects are depicted in table 5.

Graph 6: Group wise gender distribution of study subjects (n=213)

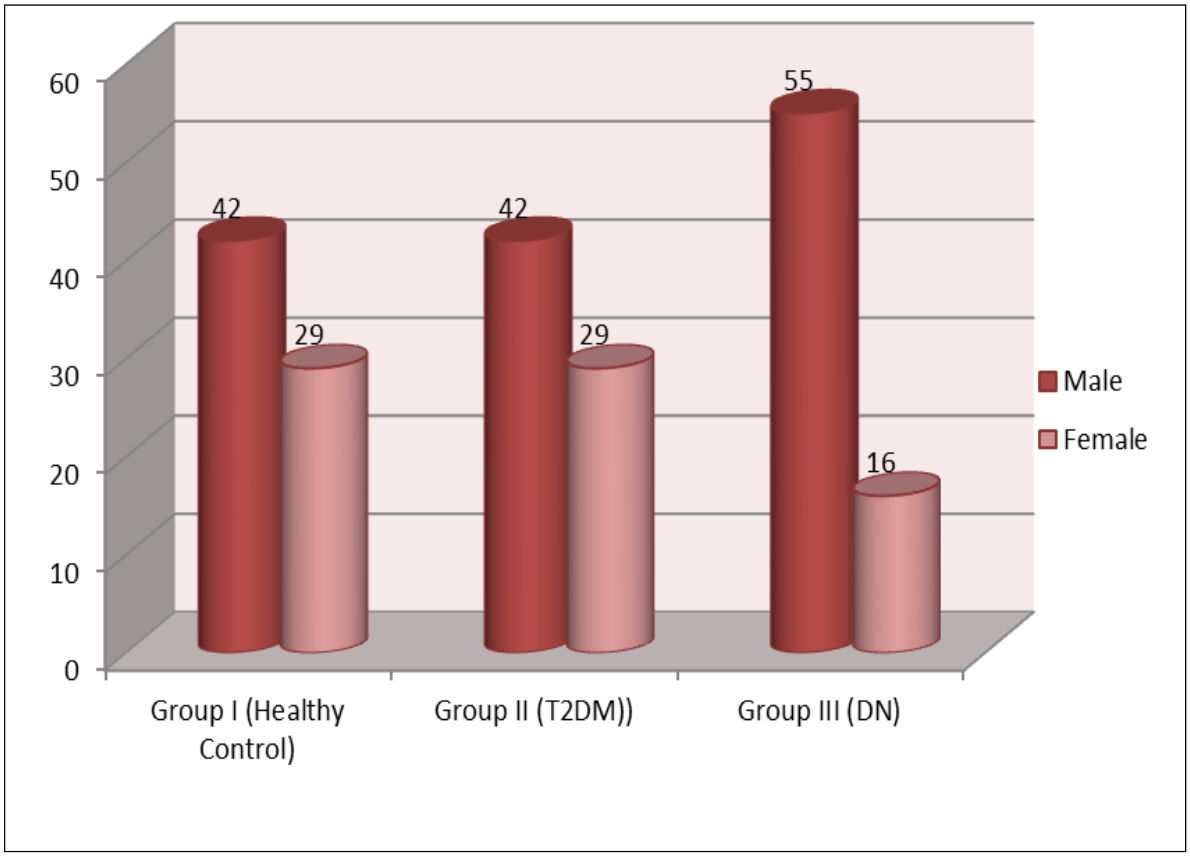


Table 5: Mean ± Standard Deviation (SD) of Anthropometric measures and Physiological Variables of study subjects (n=213)

PARAMETERS	GROUP I Mean ± SD (n=71)	GROUP II Mean ± SD (n=71)	GROUP III Mean ± SD (n=71)	p-value
Age (Years)	55.1 ± 7.4	57.41 ± 8.7	57.06 ± 9.2	0.205
BMI (kg/m^2)	24.08 ± 3.9	24.6 ± 4.02	24.22 ± 4.3	0.767
SBP (mmHg)	120.9 ± 5.2	124.6 ± 15.3	134.2 ± 16.1 [a,b]	0.001*
DBP (mmHg)	73.3 ± 3.44	79.5 ± 8.8	84.4 ± 9.4 [a,b]	0.001*
MAP (mmHg)	93.2 ± 3.7	94.5 ± 10.01	100.9 ± 10.6 [a,b]	0.001*
Duration of Diabetes (Years)	-	6.3 ± 5.46	9.03 ± 7.96 [b]	0.018*

***p<0.05 considered as significant**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

BMI: Body Mass Index; **SBP:** Systolic Blood Pressure; **DBP:** Diastolic Blood Pressure; **MAP:** Mean Arterial Pressure

[a] Compared to Healthy Control (Group I); [b] Compared to T2DM (Group II)

Table 5 shows no significant difference among age and BMI between study groups. With respect to physiological variables, SBP, DBP and MAP were significantly higher in DN compared to healthy control and T2DM group. However, no significant difference was observed between healthy control and T2DM group. Duration of diabetes showed a significant difference between the groups. The duration of diabetes was significantly higher in Group III (9.03 ± 7.96 years) compared to Group II (6.3 ± 5.46 years).

Table 6: Mean ± Standard Deviation (SD) of Basic Diabetic Profile and Renal Parameters of study subjects (n=213)

PARAMETERS	GROUP I Mean ± SD (n=71)	GROUP II Mean ± SD (n=71)	GROUP III Mean ± SD (n=71)	p-value
FBS (mg/dL)	95.85 ± 8.8	212.5 ± 65.6 [a]	152.8 ± 51.7 [a,b]	0.001*
PPBS (mg/dL)	108.1 ± 20.9	284.4 ± 86.7 [a]	243.7 ± 64.7 [a,b]	0.001*
HbA1C (%)	5.52 ± 0.47	10.9 ± 2.57 [a]	8.5 ± 2.23 [a,b]	0.001*
Urea (mg/dL)	19.8 ± 5.8	24.6 ± 13.04	116.5 ± 45 [a,b]	0.001*
SCr (mg/dL)	0.73 ± 0.2	0.69 ± 0.31	5.3 ± 2.8 [a,b]	0.001*
Uric Acid (mg/dL)	4.96 ± 1.3	4.3 ± 1.9	7.8 ± 2.05 [a,b]	0.001*
Uric Acid: Creatinine ratio	6.95 ± 1.65	6.59 ± 2.25	1.8 ± 0.86 [a,b]	0.001*
Total Protein (gm/dL)	7.24 ± 0.44	6.7 ± 0.71 [a]	5.84 ± 0.91 [a,b]	0.001*
Serum Albumin (gm/dL)	4.1 ± 0.27	3.53 ± 0.52 [a]	2.7 ± 0.52 [a,b]	0.001*

***p<0.05 considered as significant.**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

FBS: Fasting Blood Sugar; **PPBS:** Post Prandial Blood Sugar; **HbA1C:** Glycated Haemoglobin**;** **SCr:** Serum Creatinine

[a] Compared to Healthy Control (Group I); [b] Compared to T2DM (Group II)

Mean values of the basic diabetic profile, fasting blood sugar, post-prandial blood sugar and glycated hemoglobin were significantly higher in T2DM (Group II) and DN (Group III) compared to Healthy control (Group I). Significantly lower values for FBS, PPBS & HbA1c were observed in DN patients (Group III) compared to T2DM patients (Group II).

Basic renal parameters were compared between the three groups. Mean value of blood urea, serum creatinine and uric acid were significantly elevated in DN

(Group III) compared to healthy control (Group I) & T2DM (Group II). We observed a reduced serum albumin level in T2DM patients (3.53 ± 0.52 gm/dL) and DN patients (2.7 ± 0.52 gm/dL) compared to Healthy control (4.1 ± 0.27 gm/dL) and is statistically significant. Significantly lower value of uric acid to creatinine ratio was observed in DN subjects (1.8 ± 0.86 mg/dL) compared to healthy control and T2DM patients (Group I and II). The mean value of uric acid to creatinine ratio in Healthy control and T2DM were 6.95 ± 1.65 mg/dL and 6.59 ± 2.25 mg/dL respectively (Table 6).

Table 7: Mean ± Standard Deviation (SD) of Lipid Profile of study subjects (n=213)

PARAMETERS	GROUP I Mean ± SD (n=71)	GROUP II Mean ± SD (n=71)	GROUP III Mean ± SD (n=71)	p-value
TC (mg/dL)	177.6 ± 40.4	166.8 ± 48.8	136.3 ± 43.7 [a,b]	0.001*
TG (mg/dL)	171.7 ± 81.9	232.2 ± 119.8 [a]	170 ± 121.3 [b]	0.001*
HDL-C (mg/dL)	41.7 ± 9.96	31.5 ± 9.45 [a]	30.88 ± 12.76 [a]	0.001*
nHDL-C (mg/dL)	135.8 ± 39.02	135.3 ± 45.7	105.4 ± 47.7 [a,b]	0.001*
LDL-C (mg/dL)	99.32 ± 29.7	87.6 ± 39.8	71.1 ± 34.7 [a,b]	0.001*
VLDL-C (mg/dL)	34.26 ± 16.4	47.6 ± 24.6 [a]	34.15 ± 25	0.001*

***p<0.05 considered as significant.**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

TC: Total Cholesterol; **TG:** Triglyceride; **HDL-C:** High Density Lipoprotein Cholesterol; **nHDL-C:** Non-High Density Lipoprotein Cholesterol **LDL-C:** Low Density Lipoprotein Cholesterol; **VLDL-C:** Very Low-Density Lipoprotein Cholesterol

[a] Compared to Healthy Control (Group I); [b] Compared to T2DM (Group II)

Lipid profile parameters vis-à-vis, total cholesterol and HDL-C is grossly decreased in DN (Group III) compared to healthy control and T2DM (Group I & II) and were statistically significant (Table 7). Triglyceride and LDL cholesterol was decreased

in DN patients compared to T2DM subjects. The level of nHDL-C (105.4 ± 47.7) was observed to be lowered in DN group than healthy control (135.8 ± 39.02 mg/dL) and diabetics (135.3 ± 45.7 mg/dL) with a statistically significant p value. The lipid triads of TC, TG and LDL-C are observed to be lower in DN compared to healthy control and T2DM subjects. The difference between the TC and TG in healthy control versus DN is 42 mg/dL and 28 mg/dL respectively. The TC is 1.5 times higher than the LDL-C between Healthy control and DN. However, the difference between TC and LDL-C in Healthy control versus DN patients is of not great significance. This indicates that the TC needs to be prioritized in DN cases where the population is exposed to high fluoride area. The lipid tetrads considering TC, TG, LDL-C and HDL-C when observed across the three groups, we have noticed the values on higher side in T2DM subjects compared to healthy control and DN patients indicating the glucose homeostasis plays a key role in TG metabolism. The pentad lipid parameters TC, TG, LDL-C, HDL-C and non-HDL-C has shown that in addition to the TG, the non-HDL-C an indicator of HDL devoid of TG is grossly reduced in DN compared to T2DM and healthy controls.

Table 8: Mean ± Standard Deviation (SD) of other Biochemical parameters of study Subjects (n=213)

PARAMETERS	GROUP I Mean ± SD (n=71)	GROUP II Mean ± SD (n=71)	GROUP III Mean ± SD (n=71)	p-value
AST (U/L)	28.31 ± 10.15	27.9 ± 20.6	30.42 ± 18.37	0.639
ALT (U/L)	25.3 ± 13.24	28.63 ± 19.5	26.41 ± 16.5	0.476
Magnesium (mg/dL)	2.01 ± 0.2	1.7 ± 0.26 [a]	2.02 ± 0.46 [b]	0.001*
Phosphate (mg/dL)	3.7 ± 0.53	3.81 ± 3.26	5.31 ± 1.8 [a,b]	0.001*
Calcium (mg/dL)	9.1 ± 0.36	8.87 ± 0.64	7.8 ± 0.85 [a,b]	0.001*
Vitamin D (ng/mL)	25.2 ± 8.07	19.86 ± 8.52 [a]	16.72 ± 8.31 [a]	0.001*
Vitamin C (mg/dL)	1.36 ± 0.26	0.77 ± 0.18	0.48 ± 0.11	0.001*
NO (µmol/L)	32.54 ± 14	13.22 ± 5.52 [a]	11.7 ± 4.83 [a]	0.001*
GPX (ng/mL)	24.23 ± 9.51	18.78 ± 6.87 [a]	12.44 ± 4.84 [a,b]	0.001*
MDA (nmol/L)	2.54 ± 0.92	4.85 ± 1.83 [a]	7.13 ± 1.75 [a,b]	0.001*

***p<0.05 considered as significant.**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

AST: Aspartate transaminases; **ALT:** Alanine transaminases; **NO:** Nitric Oxide; **GPX:** Glutathione Peroxidase; **MDA:** Malondialdehyde

[a] Compared to Healthy Control (Group I); [b] Compared to T2DM (Group II)

Biochemical parameters ALT and AST doesn't show significant difference between the three groups and were within the physiological reference range (ALT <35 IU/L, AST <45 IU/L) indicating the study subject's hepatobiliary systems were physiologically normal.

The magnesium values are within the physiological reference range of 1.6-2.6 mg/dL in all the three groups. To our surprise, we noticed the magnesium values of

DN group correlated with healthy control. In T2DM subjects the magnesium values are towards the lower reference range. Hypothetically indicating that the magnesium might have been utilized by glycolytic enzymes to enhance the conversion of glucose to glucose-6- phosphate, to maintain the homeostasis and prevent entry of glucose into polyol pathway.

Serum phosphate levels in the healthy control and T2DM were within the physiological range (2.5-4.5 mg/dL) compared to DN group where it is grossly elevated with values of 1.61 mg/dL and 1.9 mg/dL respectively compared to healthy control and T2DM. However, there is an elevation of approximately 1 mg/dL of phosphate above the upper limit of reference range in DN group compared to healthy control and T2DM where the phosphate values are nearing the mean values of the physiological reference range.

Serum vitamin D concentration was significantly decreased in T2DM and DN patients compared to healthy control (19.86 ± 8.52 ng/mL, 16.72 ± 8.31 ng/mL, 25.2 ± 8.07 ng/mL respectively). However, it is within the physiological reference range in healthy control and deficient in T2DM and DN patients indicating that the vitamin D may be utilized for other metabolic process or there is laxity in binding of vitamin D with VDBP as much of VDBP is excreted through urine in T2DM and DN patients.

Serum calcium value in healthy control was 9.1 ± 0.36 mg/dL and is within the physiological reference range of 8.6-10.2 mg/dL, compared to DN patients with 7.8 ± 0.85 mg/dL and was statistically significant. The 1.03 mg/dL reduction in the serum calcium in DN group may be contributed to the renal regulation mechanism of this molecule.

Among the oxidative stress parameters Vitamin C, Nitric Oxide and Glutathione peroxidase were showed a significant decline in DN group compared to healthy control and T2DM subjects with a p-value <0.001. However, no significant difference was observed with respect to Nitric oxide in T2DM (13.22 ± 5.52 µmol/L) and DN group (11.7 ± 4.83 µmol/L). The mean value of malondialdehyde in healthy control, T2DM and DN were 2.54 ± 0.92 nmol/L, 4.85 ± 1.83 nmol/L and 7.13 ± 1.75 nmol/L respectively showing a significant (p<0.001) increase in DN and T2DM patients compared to healthy individuals (Table 8).

Table 9: Mean ± Standard Deviation (SD) of Extended Renal Profile and Special Parameters of study subjects (n=213)

PARAMETER	GROUP I Mean ± SD (n=71)	GROUP II Mean ± SD (n=71)	GROUP III Mean ± SD (n=71)	p-value
Cystatin C (mg/L)	0.81 ± 0.2	0.98 ± 0.25 [a]	2.49 ± 0.61 [a,b]	0.001*
eGFR (mL/min)	102.27 ± 19.8	81.77 ± 24.03 [a]	35.5 ± 9.17 [a,b]	0.001*
Serum Fluoride (ppm)	0.7 ± 0.11	0.67 ± 0.18	0.33 ± 0.09 [a,b]	0.001*
Urine Fluoride (ppm)	0.93 ± 0.56	0.98 ± 0.84	0.34 ± 0.25 [a,b]	0.001*
uVDBP (µg/mL)	84.9 ± 50.2	984.9 ± 304.3 [a]	1588.2 ± 277.3 [a,b]	0.001*

***p<0.05 considered as significant**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

eGFR: Estimated Glomerular Filtration Rate; **uVDBP:** Urinary Vitamin D Binding Protein

[a] Compared to Healthy Control (Group I); [b] Compared to T2DM (Group II)

Diabetic nephropathy patients (Group III) showed significantly higher concentration of serum cystatin C (2.49 ± 0.61 mg/L) compared to T2DM and healthy control subjects. However, the eGFR was significantly lowered in DN patients (35.5 ± 9.17 mL/min) compared to healthy control (102.27 ± 19.8 mL/min) and T2DM subjects (81.77 ± 24.03 mL/min).

The urinary fluoride as well as serum fluoride level was lowered in DN group (0.34 ± 0.25, 0.33 ± 0.09 ppm) compared to healthy control and T2DM subjects. However, we did not observe statistically significant difference of serum and urine fluoride between healthy control and T2DM subjects.

Urinary VDBP levels were observed to be grossly elevated in diabetic patients irrespective of nephropathy than in healthy control (Table 9). Mean uVDBP values were 84.9 ± 50.2 µg/mL, 984.9 ± 304.3 µg/mL and 1588.2 ± 277.3 µg/mL in healthy control, T2DM and DN group respectively. The elevation is statistically significant among the groups.

Microalbumin and ACR were not normally distributed and we considered these variables as non-parametric which are represented as median (minimum-maximum).

Table 10: Non-parametric data of study subjects (n=213)

PARAMETERS	GROUP I Median (Min-Max) (n=71)	GROUP II Median (Min-Max) (n=71)	GROUP III Median (Min-Max) (n=71)	p-value
Microalbumin (mg/L)	6 (6-43)	15 (6-430) [a]	700 (70-3800) [a,b]	0.001*
ACR (mg/gm)	11.3 (2.36-84.4)	43.6 (4.9-4294.5) [a]	1884.9 (145.8-9959.8) [a,b]	0.001*

***p<0.05 considered as significant**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

ACR: Albumin Creatinine Ratio

[a] Compared to Healthy Control (Group I); [b] Compared to T2DM (Group II)

Elevated microalbumin and ACR were observed in T2DM and DN group compared to healthy control and was statistically significant (Table 10).

The significant increase in uVDBP, cystatin C, microalbumin, ACR and decrease in eGFR in DN group compared to T2DM reflects the greater severity of illness in nephropathy group (Table 9 & 10).

Table 11: Correlation of uVDBP with oxidants and antioxidants markers

Variables	uVDBP			
	Group I (Healthy Control)		**Group II & III** (T2DM & DN)	
	r value	**p value**	**r value**	**p value**
Uric Acid	-0.120	0.319	0.477	0.001*
Vitamin C	0.029	0.813	-0.510	0.001*
Nitric Oxide	-0.088	0.463	-0.219	0.009*
GPx	0.180	0.132	-0.307	0.001*
MDA	0.060	0.617	0.360	0.001*

***p<0.05 considered as significant.**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

GPX: Glutathione Peroxidase; **MDA:** Malondialdehyde; **uVDBP:** Urinary Vitamin D Binding Protein

Table 11 depicts uVDBP is significantly positively correlated with uric acid (r=0.477, p= <0.001) and MDA (r=0.360, p=<0.001) while a significant negative correlation with vitamin C (r=-0.510, p=<0.001), nitric oxide (r=-0.219, p=0.009) & GPx (r=-0.307, p= <0.001) was observed in T2DM and DN group. uVDBP does not showed any significant correlation with oxidant and antioxidant parameters with respect to healthy controls.

Table 12: Correlation of uVDBP with renal profile parameters

Variables	uVDBP			
	Group I (Healthy Control)		**Group II & III** (T2DM & DN)	
	r value	**p value**	**r value**	**p value**
Urea	-0.017	0.888	0.606	0.001*
Serum Creatinine	-0.127	0.290	0.590	0.001*
Albumin	-0.080	0.509	-0.408	0.001*
Cystatin C	0.011	0.929	0.643	0.001*
eGFR	-0.064	0.596	-0.599	0.001*
µALB	-0.070	0.561	0.434	0.001*
ACR	0.230	0.054	0.399	0.001*
Calcium	-0.159	0.186	-0.449	0.001*
Phosphate	0.296	0.013*	0.261	0.002*
Serum Fluoride	-0.019	0.875	-0.527	0.001*
Urine Fluoride	-0.271	0.022*	-0.414	0.001*

***p<0.05 considered as significant**

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

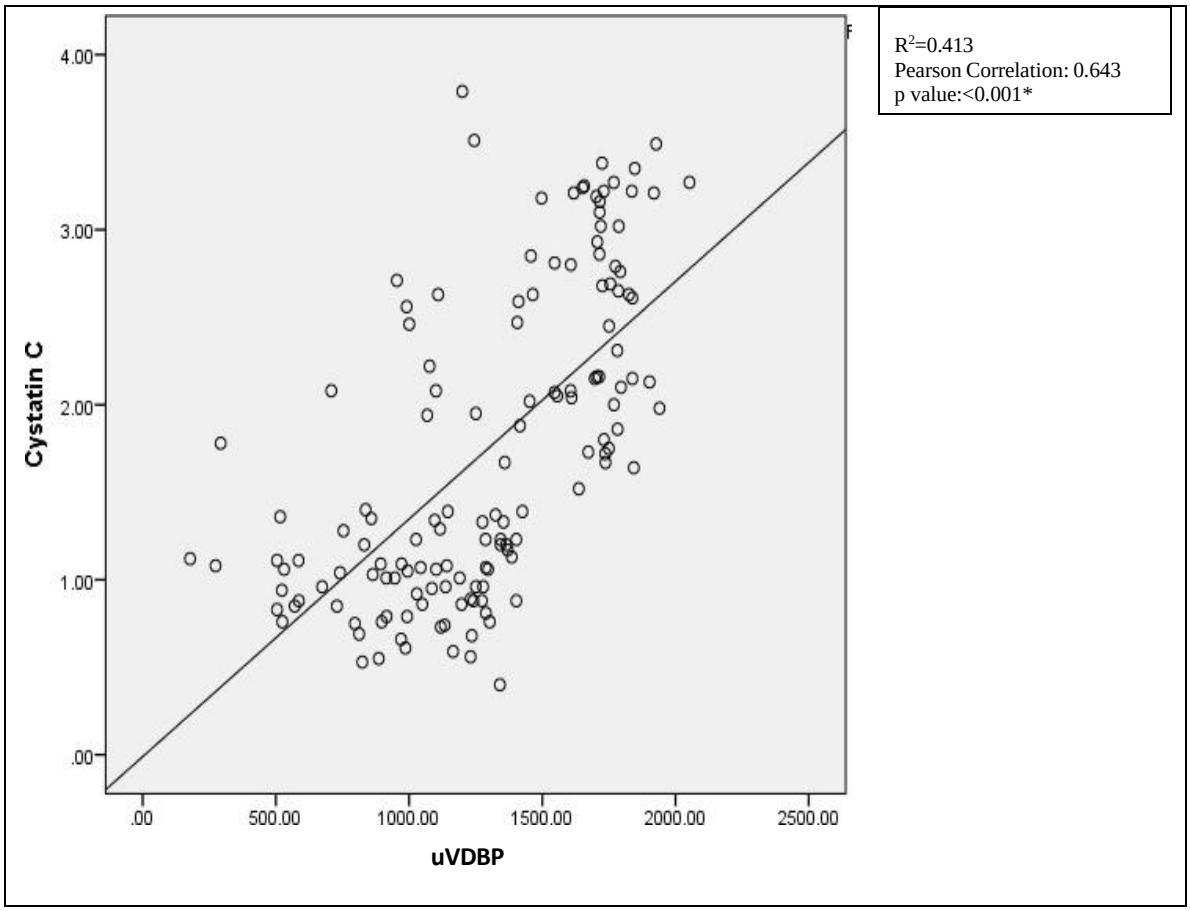
eGFR: Estimated Glomerular Filtration Rate; **ACR:** Albumin Creatinine Ratio; **uVDBP:** Urinary Vitamin D Binding Protein

Table 12 depicts correlation of uVDBP with renal profile parameters. uVDBP shows a significant positive correlation with urea (r=0.606, p=<0.001), serum creatinine (r=0.59, p=<0.001), phosphate (r=0.261, p=0.002), cystatin C (r=0.643, p=<0.001), microalbumin (r=0.434, p=<0.001) and ACR (r=0.399, p=<0.001) while a significant negative correlation with serum calcium (r=-0.449, p=<0.001), albumin

(r=-0.408, p=<0.001) and eGFR (r=-0.599, p=<0.001) in T2DM and DN group. We further observed a significant negative correlation of uVDBP with serum and urine fluoride (r=-0.527, r=-0.414, p=<0.001) in T2DM and DN group. No significant correlation is observed with any of the parameters in healthy controls except phosphate and urine fluoride which shows a weak positive correlation with uVDBP.

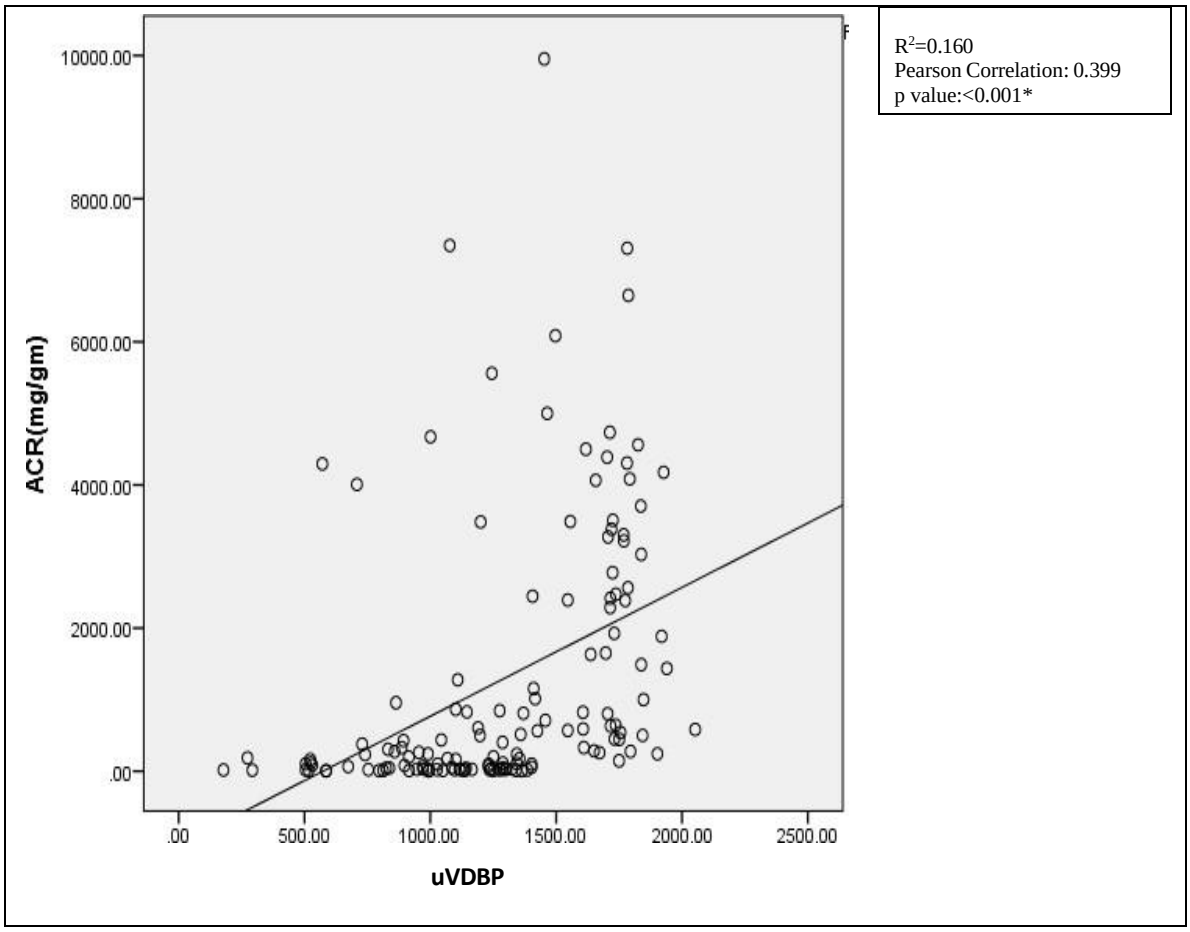
A positive correlation of uVDBP with cystatin C and ACR (Graph 7 and 8), while a negative correlation of uVDBP with eGFR in T2DM and DN group explaining as the renal function declines the urinary level of VDBP grossly elevated.

Graph 7: Correlation of uVDBP with cystatin C in T2DM and DN Subjects (n=142)



***p<0.05 considered as significant,** [uVDBP: Urinary Vitamin D Binding Protein]

Graph 8: Correlation of uVDBP with ACR in T2DM and DN Subjects (n=142)



***p<0.05 considered as significant,** [uVDBP: Urinary Vitamin D Binding Protein; ACR: Albumin Creatinine Ratio]

Table 13: Correlation of uVDBP with Anthropometric, physiological variables and Basic Diabetic profile in study subjects (n=213)

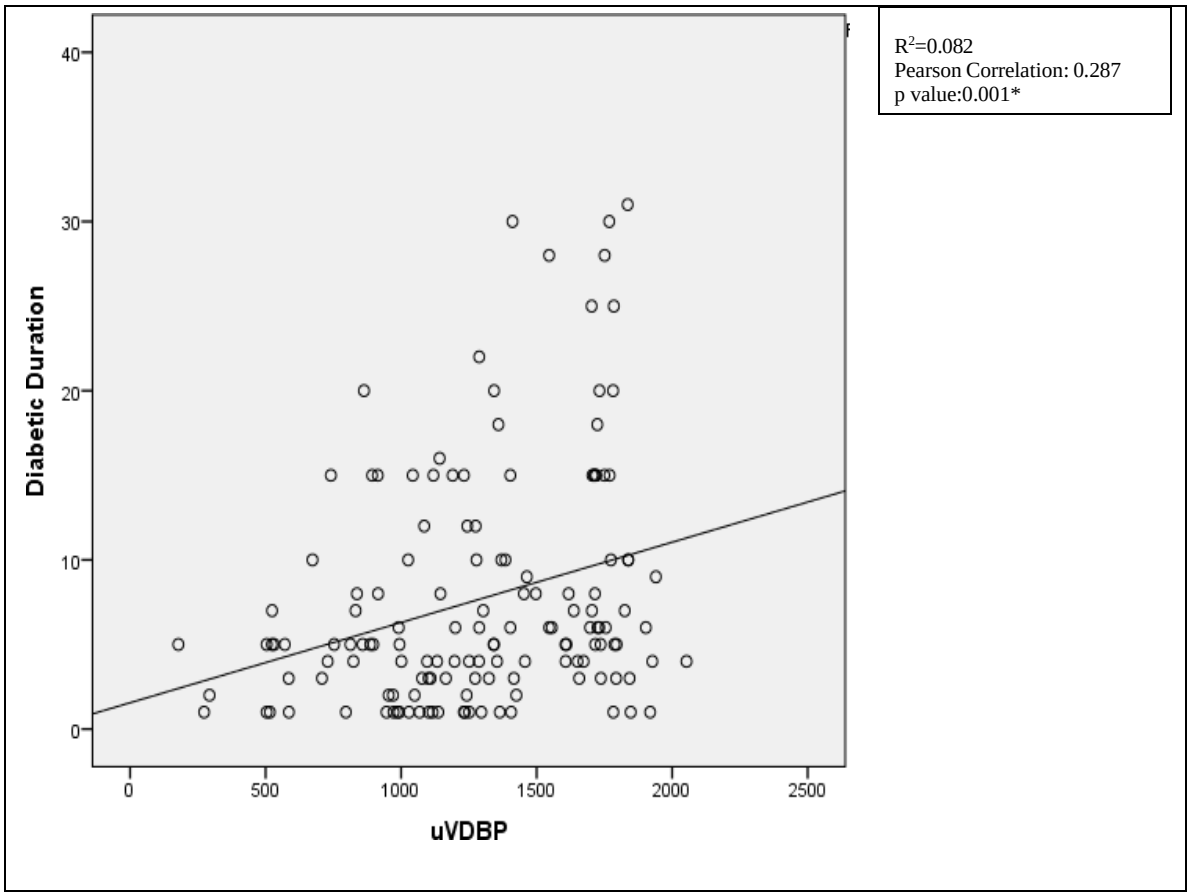
Parameters	uVDBP (r value) Pearson's correlation	p value
Age	0.381	0.001*
BMI	0.024	0.73
SBP	0.334	0.001*
DBP	0.245	0.001*
MAP	0.311	0.001*
FBS	0.352	0.001*
PPBS	0.543	0.001*
HbA1c	0.424	0.001*

***p<0.05 considered as significant.**

BMI: Body Mass Index; **SBP:** Systolic Blood Pressure; **DBP:** Diastolic Blood Pressure; **MAP:** Mean Arterial Pressure; **FBS:** Fasting Blood Sugar; **PPBS:** Post Prandial Blood Sugar; **HbA1c:** Glycated Haemoglobin

Table 13 demonstrates the correlation of uVDBP with anthropometric, physiological and basic diabetic profile parameters in study population. Among these uVDBP showed positive significant correlation with age, SBP, DBP, MAP, FBS, PPBS and HbA1c. There is no significant correlation with respect to BMI (r=0.024, p=0.73) in the study population. A positive significant correlation with basic diabetic profile (FBS, PPBS and HbA1c) showed that chronic hyperglycemic condition aids in tubular damage and a resultant urinary excretion of VDBP.

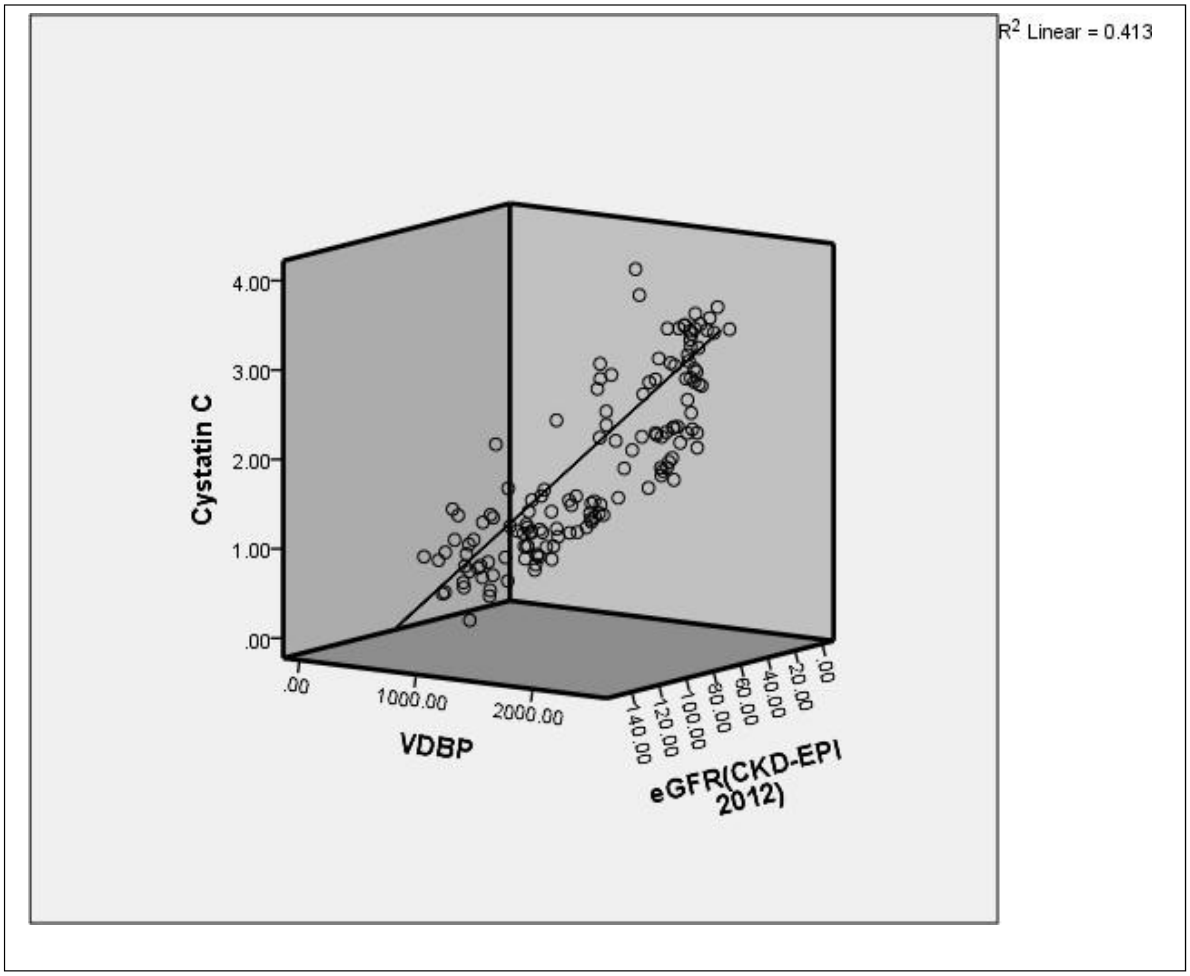
Graph 9: Correlation of uVDBP with Diabetic duration in T2DM and DN Subjects (n=142)



***p<0.05 considered as significant,** [uVDBP: Urinary Vitamin D Binding Protein]

Graph 9 shows correlation between uVDBP and the duration of diabetes in T2DM patients with and without nephropathy. We observe a positive significant correlation between uVDBP and diabetic duration with Pearson's Correlation coefficient (r value) of 0.287 and a significant p value of 0.001 among diabetic population.

Graph 10: Correlation of uVDBP with Cystatin C and eGFR in T2DM and DN Subjects (n=142)

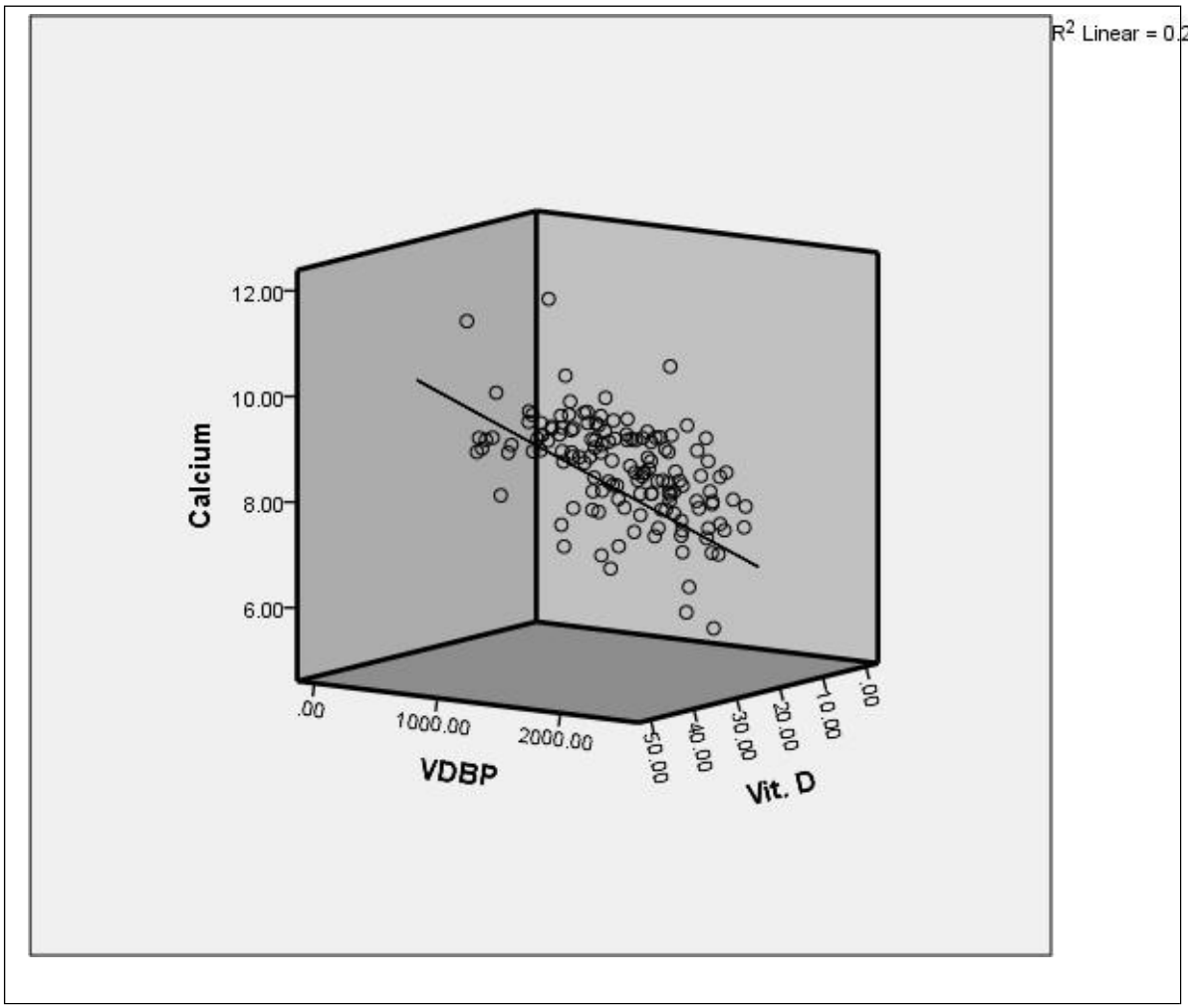


***p<0.05 considered as significant**

[**uVDBP:** Urinary Vitamin D Binding Protein; **eGFR:** Estimated Glomerular Filtration Rate]

Graph 10 demonstrate the 3D scatter plot showing correlation of uVDBP with cystatin C and eGFR together in T2DM and DN patients. With decline in eGFR, the level of serum cystatin C is elevated, and a similar trend is observed with the urinary excretion of VDBP i.e., uVDBP levels are also elevated accordingly.

Graph 11: Correlation of uVDBP with Vitamin D and Calcium in T2DM and DN Subjects (n=142)



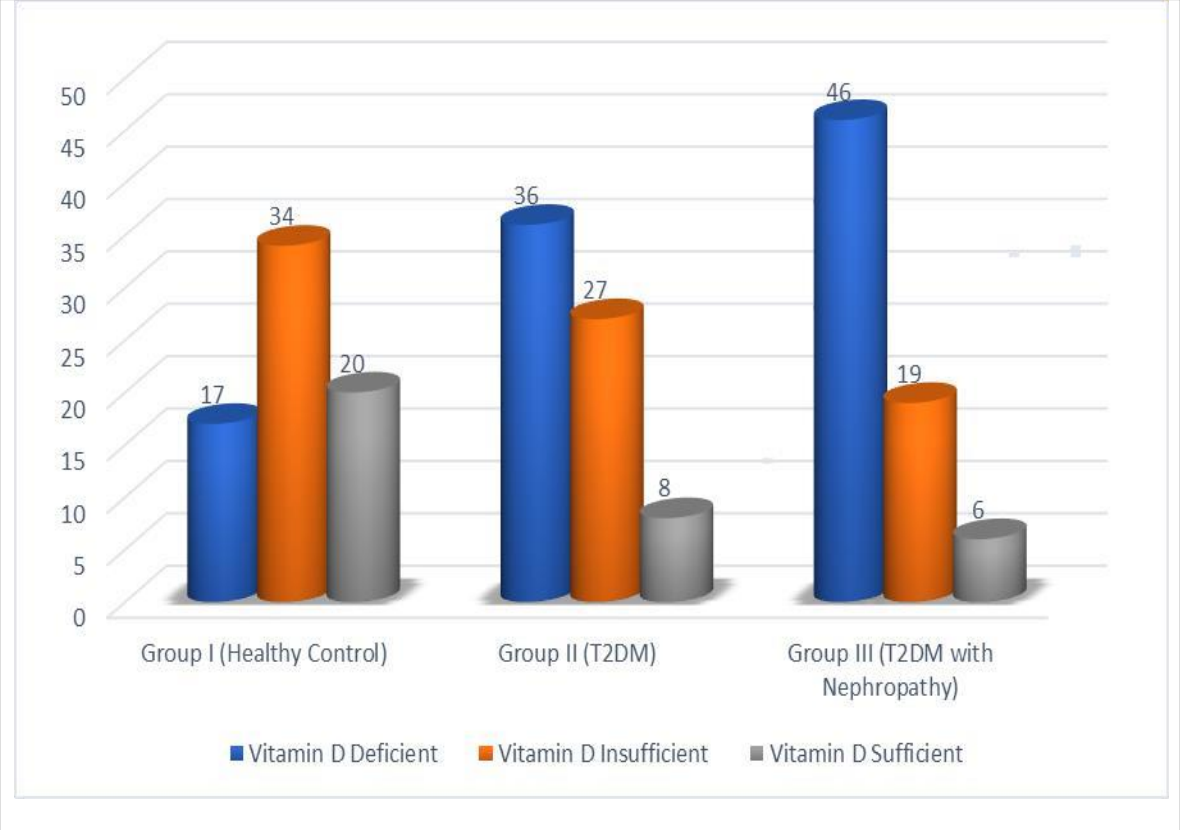
***p<0.05 considered as significant**

[**uVDBP:** Urinary Vitamin D Binding Protein; **Vit D:** Vitamin D]

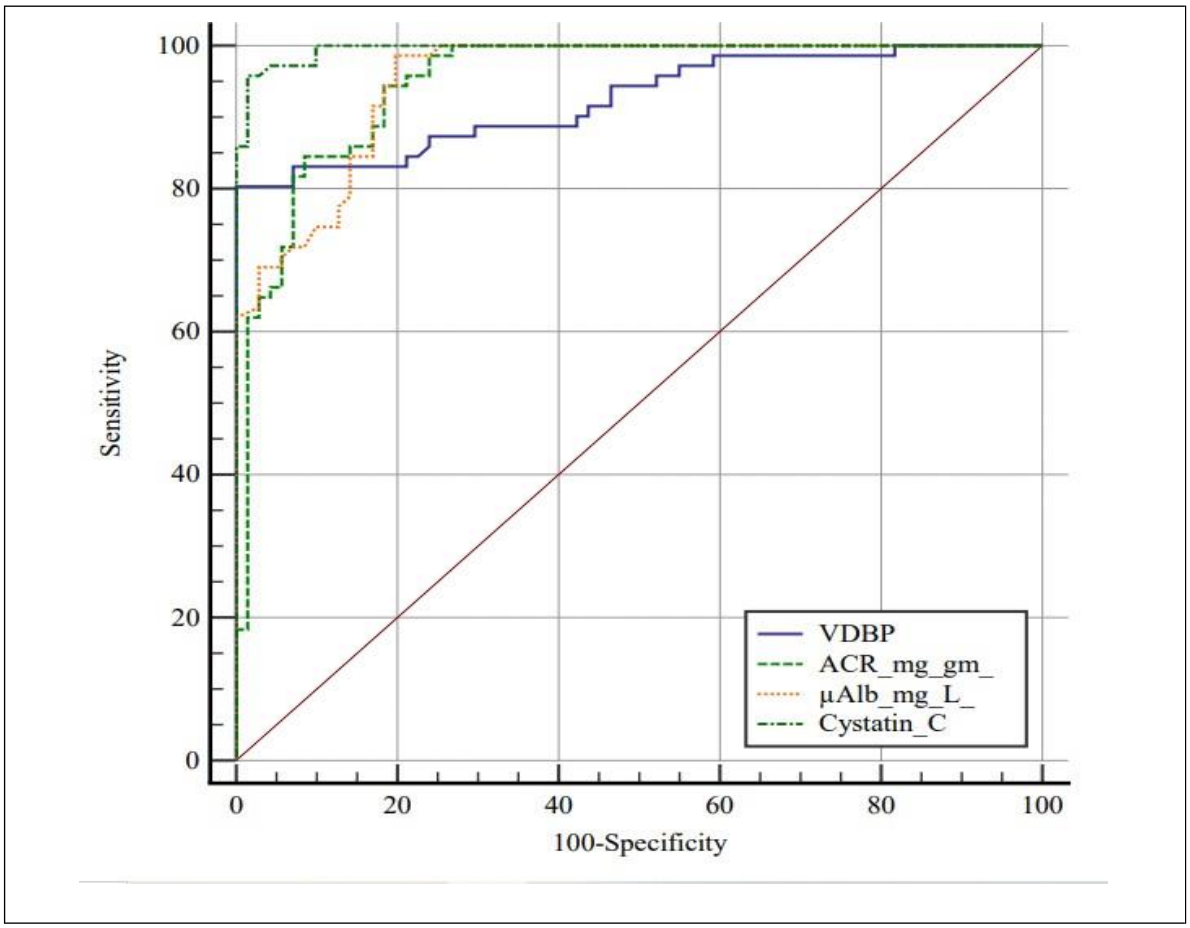
Graph 11 demonstrate the 3D scatter plot showing correlation of uVDBP with Vitamin D and Serum calcium together in T2DM and DN patients. There is an inverse correlation between elevated urinary excretion of VDBP with Vitamin D and serum calcium in diabetic patients.

Based on vitamin D levels, study participants were categorized into subjects having sufficient (>30 ng/mL), insufficient (20-30 ng/mL) and deficient (<20 ng/mL) vitamin D level. Among all the study participants (n=213), 99 study subjects were deficient, 80 with insufficiency and 34 had sufficient vitamin D (Graph 12). Highest number of vitamin D deficient patients were in Group III i.e., nephropathy patients (64.8%), while 50.7% patient with type 2 diabetes and 24% healthy control subjects showed vitamin D deficiency. Numbers of subject with sufficient vitamin D were 20 in control group, 8 in T2DM group and 6 in nephropathy group respectively.

Graph 12: Vitamin D Status in the Study participants



Graph 13: Receiver Operating Characteristic (ROC) Curve of uVDBP, Cystatin C, ACR and Microalbumin



To define the diagnostic performance of uVDBP, Cystatin C, ACR and Microalbumin in T2DM and DN group, Receiver Operating Characteristic (ROC) curve has been derived using MedCalc software version 19.8. The uVDBP showed an Area Under Curve (AUC) of 0.924 (95% CI, 0.868-0.962) and with a cutoff value of 1273 µg/mL, the sensitivity was 85% and specificity was 79% respectively. The AUC of Cystatin C is 0.995 (95% CI, 0.966-1.00) while ACR showed an AUC of 0.949 (95% CI, 0.899 - 0.979) and Microalbumin showed an AUC of 0.952 (95% CI, 0.902 - 0.981) respectively (Graph 13).

All the recruited T2DM study participants with and without nephropathy (n=142) were categorized into three different groups based on the value of urinary albumin: creatinine ratio (ACR) into normoalbuminuric (<30 mg/gm), microalbuminuric (30-300 mg/gm) and macroalbuminuric (>300 mg/gm) group respectively.

Table 14: Comparison of Extended Renal Profile and Special Parameters based on ACR (n=213)

Parameters	Healthy Control (n=71)	T2DM with & without Nephropathy			p-value
		Normo-albuminuria (n=31)	Micro-albuminuria (n=38)	Macro-albuminuria (n=73)	
Urea (mg/dL)	19.8 ± 5.8	22.29 ± 8.01	45.05 ± 41.7[a]	104.3 ± 53.6[a,b,c]	0.001*
SCr (mg/dL)	0.73 ± 0.2	0.62 ± 0.21	1.49 ± 1.6	4.77 ± 3.14[a,b,c]	0.001*
S. Albumin (gm/dL)	4.1 ± 0.27	3.53 ± 0.51[a]	3.26 ± 0.66[a]	2.85 ± 0.61[a,b,c]	0.001*
Uric Acid (mg/dL)	4.96 ± 1.3	4.04 ± 1.68	4.84 ± 2.24	7.54 ± 2.26[a,bc]	0.001*
Vitamin D (ng/mL)	25.2 ± 8.07	20.4 ± 8.6[a]	19.4 ± 7.9[a]	16.8 ± 8.7[a]	0.001*
Cystatin C (mg/L)	0.81 ± 0.2	0.98 ± 0.28	1.3 ± 0.62[a]	2.3 ± 0.8[a,c]	0.001*
eGFR (mL/min)	102.3 ± 19.8	82.4 ± 25.8[a]	68.9 ± 31.1[a]	43.5 ± 23.1[a,b,c]	0.001*
Urine Fluoride (ppm)	0.93±0.56	1.2±0.83	0.67±0.53[b]	0.43±0.58[a,b]	0.001*
uVDBP (µg/mL)	84.9 ± 50.2	981 ± 320.8[a]	1102.1 ± 382.2[a]	1512 ± 340.9[a,b,c]	0.001*

***p<0.05 considered as significant**.

Group I: Healthy Control; **Group II:** Type 2 Diabetes Mellitus; **Group III:** Diabetic Nephropathy

SCr: Serum Creatinine**; eGFR:** Estimated Glomerular Filtration Rate; **uVDBP:** Urinary Vitamin D Binding Protein

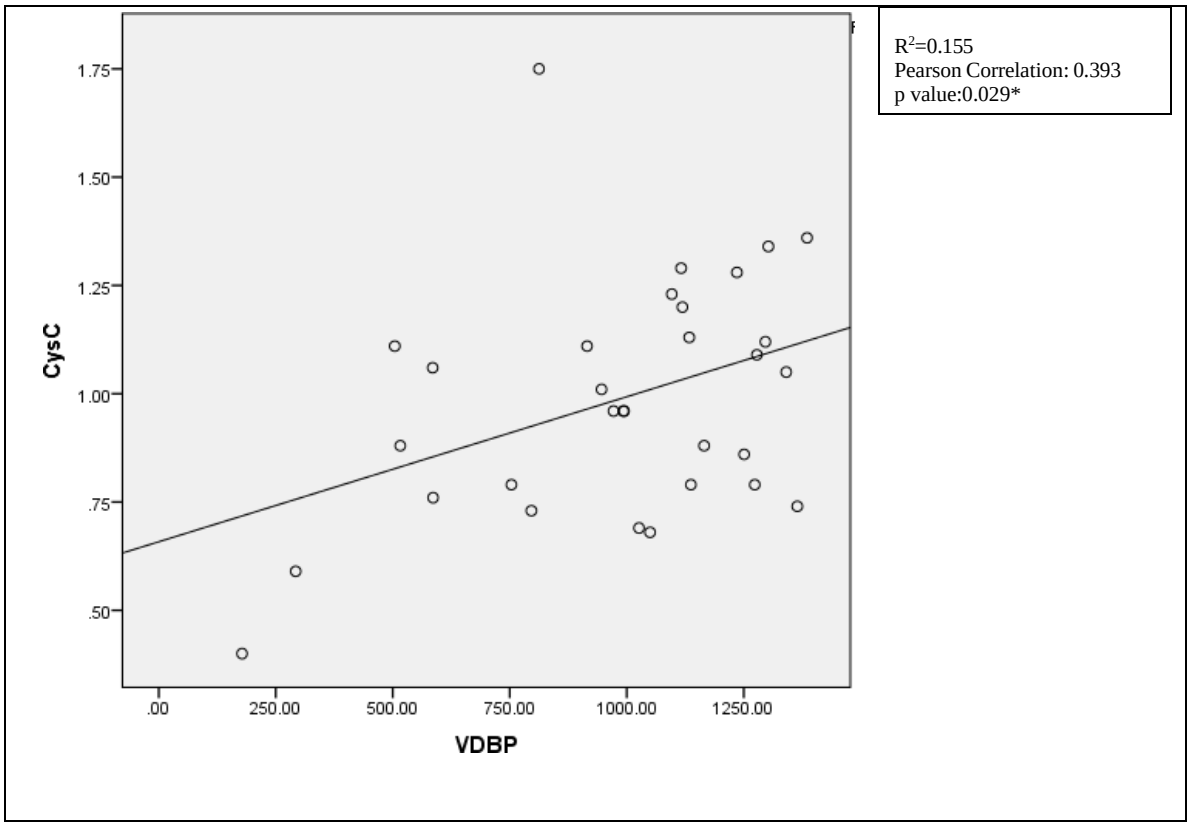
[a] Compared to control group; [b] Compared to diabetic normoalbuminuria group; [c] Compared to diabetic microalbuminuria group.

Renal parameters in the control and normoalbuminuric diabetic groups were in the physiological range compared to diabetic patients with micro and macroalbuminuric group where it is grossly elevated and were statistically significant. Uric acid level was in higher range in macroalbuminuria (7.54±2.26 mg/dL) diabetic subjects than normo and micro albuminuric diabetic group. A significant decline in vitamin D is noted in the three subgroups of T2DM with and without nephropathy categories based on ACR and were compared with healthy subjects. We couldn't observe any significant difference among the diabetic subgroups. Serum Cystatin C showed almost 2.5 times higher values in macroalbuminuric group than control and normoalbuminuric group (Table 14). We also noted that eGFR levels were lowered in T2DM study subjects with progressive albuminuria and was inversely proportional to the microalbumin as well as cystatin C levels in diabetic patients.

An altogether higher mean uVDBP levels were noted in diabetic patients than in control subjects (Table 14). In addition, urinary VDBP levels were observed to be significantly elevated in diabetic subgroups i.e., normoalbuminuria, microalbuminuria and macroalbuminuria group (981 ± 320.8 µg/mL, 1102.1 ± 382.2 µg/mL and 1512 ± 340.9 µg/mL) when compared to control subjects (84.9 ± 50.2 µg/mL). These results showed even in normoalbuminuric diabetic patients where other renal markers were within physiological reference limit, the level of uVDBP showed a marked elevation.

With this finding we further tried to correlate the uVDBP level and cystatin C level exclusively in normoalbuminuric diabetic group. We observed a significant positive correlation with cystatin C in diabetic normoalbuminuric group (Graph 14).

Graph 14: Correlation of uVDBP with Cystatin C in Normoalbuminuric Diabetic subjects (n=31)



***p<0.05 considered as significant,** [uVDBP: Urinary Vitamin D Binding Protein; CysC: Cystatin C]

To conclude the results, uVDBP levels are elevated in DN compared to diabetes and healthy controls and the ratio of increase of uVDBP in DN is 19 times more than healthy controls and 12 times higher in diabetic patients. This correlated inversely with eGFR, urine fluoride and serum fluoride in DN group compared to diabetes and healthy controls. There is a strong positive correlation of uVDBP with cystatin C where cystatin C is elevated approximately 3 times in DN group compared to healthy controls. There is no much significant difference between diabetics and healthy controls with respect to cystatin C.

The serum albumin is directly proportional to vitamin D. serum albumin and vitamin D values combined are inversely proportional to uVDBP in diabetics and diabetic nephropathy patients.

Serum fluoride and urine fluoride values are correlating across all the three groups. However, urine fluoride excretion is inversely proportional to uVDBP in diabetic nephropathy patients. Serum fluoride substantiate the inverse correlation of urine fluoride with uVDBP in DN compared to diabetes and healthy controls.

Discussion

Diabetic nephropathy (DN) is a morbid and extremely dreaded complication of diabetes caused by glomerular and tubular structural and functional alteration because of disturbance in glucose homeostasis. A striking 45% of T1DM and T2DM diabetics are affected by this microvascular complication [129]. As of now, diabetes is the single leading cause of ESRD in the Western world and the prime cause for patients requiring renal replacement therapy worldwide [130]. However, due to the strong link between DN and cardiovascular disease, majority of patients with DN will die even before progression to ESRD, because of cardiovascular related events [130].

The incidence and prevalence of diabetes mellitus have grown significantly throughout the world, primarily due to the increase in type 2 diabetes. This increase in the number of people developing diabetes had a major impact on the development of diabetic kidney disease. Incidence of severe macrovascular disease in diabetic nephropathy is very high leading to an increased mortality in diabetic patients [131]. In India, DN accounts for about 46% of CKD in elderly people and is associated with increased cardiovascular mortality and morbidity. Thus, effective management of the disease must be started at the earliest to prevent from developing ESRD. Therefore, early prediction and detection of DN would play a significant role in the effective management and treatment approaches. To reduce the burden of CKD in diabetic patients and facilitate the monitoring of the condition, identification of novel biomarkers of early stages of DN is mandatory which could improve risk stratification and prediction.

The current most accepted biomarkers of DN are the presence of detectable amounts of albumin in urine i.e., microalbuminuria and eGFR due to their non-invasive

nature and is often linked to established significant glomerular damage [132]. However, recent studies showed that microalbuminuria might be transient and not necessarily reflect permanent renal impairment [133]. Although the albuminuria-based system is used more often than eGFR, it is neither sensitive nor specific as it was found to be normal in the presence of early DN pathological changes and increased in other types of nephropathies [134]. The need for early detection of DN with the deficient performance characteristics of urinary albumin or eGFR raised the question for further evaluation of other biomarkers to be used for early detection of DN.

In recent years, several biomarkers have been broadly studied in serum and urine for early DN detection covering glomerular, tubular, inflammatory and oxidative stress biomarkers with distinct performance features [135]. Data on urinary biomarkers support the view that tubular injury contributes in a primary way, rather than in secondary manner for early DN development in type 2 diabetic individuals [136]. Thus, to explore whether urinary VDBP levels could be a novel non-invasive early biomarker for DN, this study has been conducted and the result of this study demonstrated that uVDBP levels were grossly elevated in DN patients compared to healthy control and T2DM patients without nephropathy.

In the present study, study subjects showed diabetes duration of 9 years in DN compared to 6 years in diabetes mellitus. T2DM patients with advanced age are likely to have vascular and tubulointerstitial changes due to the potential senescence of glomeruli [137]. There was no significant difference observed among groups with respect to BMI in this study and values were within reference range. Our study subject BMI correlated well with study conducted by Al-Hazmi SF et al [138].

Further, the average duration of diabetes was almost 1.5 times more in nephropathy group (9.03 ± 7.96 years) compared with T2DM group (6.3 ± 5.46 years). This finding could be explained by the fact that longer duration with T2DM may lead to poor glycemic control and comorbidities, affecting renal functions through vascular damage [139]. As the diabetic duration increases one year, the chances of developing DN increases by 1.14 times indicating the probabilities of developing DN increased by 3.74 times for ten years of diabetic duration [139].

Result of present study demonstrated that an increase in SBP and DBP in diabetic nephropathy patients compared to control group and T2DM patients and was statistically significant and is concurrent with findings by Grassi G et al [140]. We also observed an elevated mean arterial pressure (MAP) in DN compared to healthy control and T2DM patients. In our study, presence of hypertension among DN patients was associated with renal impairment and agreed with other related studies showing a significant association being hypertensive with renal impairment [141,142]. Study conducted by Noor T et al showed an elevated blood pressure among patients with diabetes developing towards nephropathy [143]. A similar result was also reported in recent study conducted by Al-Rubeaan K et al [144]. The significant reasons for hypertension in diabetes include volume expansion due to elevated renal sodium reabsorption and peripheral vasoconstriction because of dysregulation of factors that regulate peripheral vascular resistance [145]. The mechanism of hypertension in DN is complex and not completely understood which involves excess sodium retention, activation of the sympathetic nervous system (SNS) and the renin-angiotensin-

aldosterone system (RAAS), endothelial cell dysfunction and increased oxidative stress [143].

Our study showed a significant increase in FBS, PPBS and HbA1c in T2DM and DN group versus healthy controls (table 6). These findings are comparable with the finding of study conducted by Patel and Kaila in the year 2019 with Indian population [146]. Comparison between T2DM and T2DM with nephropathy studied groups regarding parameters of glycemic control showed a significant difference with decreased level in nephropathy group and is contradictory to the result of study conducted by Elsayed MS et al [147]. We observed the HbA1c % of 8.5 ± 2.23 in DN group compared to 10.9 ± 2.57 in T2DM group and 5.52 ± 0.47 in healthy control group respectively. Whatever the reduction of the basic diabetic profile parameters we observed in Group III vis patients with DN may be because of the awareness of the clinical consequences that diabetic subjects have landed with and also diet restriction which these patients are subjected to. Hyperglycemia is a primary cause of glomerular injury in patient with DN. Prolonged hyperglycemia results in the formation of glycation end products which interferes with normal collagen turnover and promote blood vessels permeability, matrix accumulation and formation of adhesion molecules [25]. It also causes glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy and the development of microalbuminuria, which is followed by thickening of glomerular basement membrane, accumulation of mesangial matrix and overt proteinuria, eventually leading to glomerulosclerosis and end stage renal disease [25].

Renal dysfunction is a devastating illness that affects a rising number of patients with diabetes. It is very difficult to get a treatment as soon as the disorder has been diagnosed. Thus, to assess the renal function in the study subjects we performed basic renal profile parameters. Among the basic renal parameters estimated (table 6), urea and creatinine values showed an elevated level. Serum albumin lower in DN group compared to T2DM and healthy controls which is a clear sign of renal impairment coinciding with the recommendations of KDIGO 2012 [148]. To our surprise we observed a marginal reduction in total protein in DN group compared to control and T2DM subjects. However, the total protein and albumin values are within physiological reference range in Group I and Group II compared to the lower values documented in Group III. This observation can be hypothetically construed to the fact that albumin is the major protein which contribute to the totality of total protein. Further, the observation of marginal reduction in the total protein compared to the gross reduction of albumin may be contributed to other complimentary proteins which shall give the total protein concentration. Low level of total protein and albumin in nephropathy patients in present study indicates loss of albumin through urine because of damage to the glomerulus and affecting the tubular functions.

A significant elevated uric acid level is observed in DN subjects compared to healthy control and T2DM. This marginal increase in non-protein nitrogenous compound i.e., uric acid level above the physiological reference range of 2.6-7.2 mg/dL indicates that the molecules which are markers of glomerular damage vis urea and creatinine are correlating with uric acid. A similar result was observed in study conducted by

Tanaka K et al [149]. This elevated uric acid level is consequence of renal function decline as renal excretion is the primary mode of its clearance [150]. Uric acid is a product of purine degradation synthesized by enzyme xanthine oxidase. During Uric acid production, oxidants are produced, which can lead to renal dysfunction and cardiovascular disease [151]. It has been documented that uric acid play a significant role in endothelial dysfunction by prompting inflammation with these oxygen-radical products and may lead to DN development [152]. Hyperuricemia-induced endothelial dysfunction has been endorsed to reduce renal perfusion, along with glomerular hypertension and renal hypertrophy by stimulating afferent vascular smooth muscle cell proliferation, which suggests that elevated uric acid levels are an impeding variable in the kidneys [153]. Findings of present study was in concurrent with findings of study conducted by Kocak MZ et al where they observed an elevated serum uric acid level in patients with DN compared to diabetics without nephropathy [154].

Diabetes mellitus is a congenial reason for hyperlipidaemia, especially noticed, if glycaemic control is poor and is a threat for atherosclerosis and coronary heart disease. Role of dyslipidaemia in macrovascular complications of Diabetes have been extensively studied but its role in microvascular complications namely nephropathy is still unclear [155]. Because of the high prevalence of vascular abnormality in patients with diabetes, evaluating for lipid abnormalities is a fundamental part of routine clinical administration. Also, patients with DN are at higher risk of developing CVD mainly because of dyslipidaemia and one of the reasons for morbidity and mortality. We have considered estimation of lipid profile parameters in diabetes as well as in DN and compared them with healthy controls.

Our finding with respect to TG showed a significant increase in T2DM patients compared with controls indicating that increased blood glucose might have been contributed to the elevated triglycerides (table 7). A significant reduction in HDL-C was observed in T2DM and T2DM with nephropathy subjects. This indicates that HDL, a protective molecule against the microvascular complications is lowered. Result of present study correlate with study conducted by Rai S et al in the year 2017 where they observed T2DM and DN are associated with dyslipidemia [156]. Altered lipid profile in T2DM is due to insulin resistance and defective insulin action on lipoprotein metabolism. It will also increase triglyceride synthesis and promote quick breakdown of HDL-C [157]. To our surprise, we observed that total cholesterol and LDL-C was significantly lowered in T2DM and Nephropathy patients compared to control study subjects and it may be because of lifestyle modification, strict diet control and awareness of the sequelae. The unaltered triglyceride levels in DN patients versus healthy control may be a contributory or secondary to the medical management of nephropathy. DN which is considered as an independent risk factor for cardiovascular disease and altered lipid levels is known to enhance the risk [156]. Routine monitoring of lipid profile should be included in the optimal care for diabetic patients. Thus, patients should be managed with lifestyle modifications with or without lipid lowering agents to achieve target lipid values along with adequate glycaemic control to prevent or delay nephropathy in T2DM patients.

Serum phosphate levels in the control study subjects is in the physiological range compared to DN group where it is grossly elevated (5.31 ± 1.8 mg/dL) and significant. We could not observe much difference between healthy control and T2DM subjects. The gross elevation of serum phosphate in DN may be because of low serum calcium. It is a

well-known fact that serum calcium and phosphate are reciprocally regulated in the biological system. Severe hyperphosphatemia may occur when phosphorus cannot be excreted by the malfunctioning kidney either with or without increased cell catabolism, thus resulting in hypocalcaemia. Advanced chronic renal insufficiency may be associated with hypocalcaemia due to accompanying hyperphosphatemia and low levels of vitamin D [158].

Calcium has an important role in a wide range of biological functions including insulin secretion [159]. Insulin secretion is a calcium-dependent process requiring the influx of calcium into the beta cell [160]. In the present study we observed a low level of serum calcium in DN (7.8±0.85 mg/dL) and T2DM (8.87±0.64 mg/dL) patients compared to healthy control (9.1±0.36 mg/dL). This reduction in serum calcium levels were most probably due to several factors such as reduction in insulin level which impairs bone formation due to its stimulatory action on osteoblast proliferation and impairment of calcium homeostasis and hyperglycemia which increases calcium and phosphorus excretion in urine being proportional to the degree of glucosuria. Study conducted by Kanchana N et al reported significantly decreased levels of serum calcium in diabetic patients compared with the non-diabetic controls and were negatively correlated with elevated blood glucose levels [161]. Literature review showed that the relationship between total serum calcium and albumin is defined by the simple rule: the serum total calcium concentration falls by 0.8 mg/dL for every 1gm/dL fall in serum albumin concentration [160, 161]. These findings are consistent with our finding as we observed reduction in albumin level in DN group and so also serum calcium.

Vitamin D deficiency is a prominent feature in chronic kidney disease [162].

Result of our study showed prevalence of vitamin D deficiency as well as insufficiency among study subjects. Of the total 213 study participants, 99 were documented to be deficient, 80 with insufficiency and only 34 had sufficient vitamin D (Graph 12). The present study showed that serum vitamin D levels were lower in patients with diabetic nephropathy than in healthy control as well as in T2DM study subjects and a similar trend was documented in studies conducted by Peng Y et al and Xiao X et al [163, 164].

However, there was not much difference observed in T2DM and diabetic nephropathy group. As the renal function declines in patients with DN, serum level of 1, 25 dihydroxyvitamin D decreases progressively leading to active vitamin D deficiency. Low 25 hydroxyvitamin D levels in patients with CKD have been associated with a higher risk of mortality and faster progression of kidney disease [165]. Highest number of vitamin D deficient patients were in Group III i.e., nephropathy patients (64.8%), while 50.7% patient with type 2 diabetes and 24% control subjects showed vitamin D deficiency. Our result is supported by findings of study conducted by Gursoy G et al [166]. A significant inverse correlation between HbA1c and serum vitamin D levels in study population is observed suggesting a possible connection between glycemic control and vitamin D metabolism. Vitamin D is thought to be involved in the pathophysiology of insulin resistance, insulin sensibility and β-cell function [164]. The effect of vitamin D on insulin secretion may be indirect as increasing intracellular calcium which could act as a mediator of insulin secretion and thus improve HbA1c level [167].

Oxidative stress is related to endothelial dysfunction and plays a critical role in CKD progression in T2DM patients. The endothelium secretes NO, which is produced

from arginine by the enzyme NO Synthase and involved in several biological processes, including vasodilatation in smooth muscle cells, inflammation and immune responses [168]. Present study showed significantly reduced level of nitric oxide in diabetic nephropathy when compared with control group. However, there was not much difference in nitric oxide level in T2DM and DN. Glycosylation of proteins and formation of AGE is a consequence of long-term exposure of tissue proteins to high glucose concentrations and may contribute to decreased renal NO synthesis and/or availability through renal endothelial NO Synthase inhibition [169, 170].

Hyperglycaemia is known to enhance free radical generation and facilitate lipid peroxidation resulting in production of large amounts of reactive products, which have been implicated in diabetes and its complications. MDA is a marker of lipid peroxidation. It is formed through lipid peroxidation and also during prostaglandin and thromboxane synthesis [171]. The levels were significantly elevated in both T2DM and diabetic nephropathy compared to control study subjects. The level was almost 3 times higher in nephropathy cases and 2 times higher in T2DM patients compared to the control group. Findings of current study were supported by studies conducted by Pan H et al, Vivian ST et al and Varma M et al [171, 172, 173]. ROS produced in hyperglycemia increases peroxidation of cellular membrane lipids as well as increasing the oxidation of proteins. This mechanism yields protein carbonyl derivatives, producing high level of MDA in diabetic nephropathy. This suggests oxidative stress of long-standing T2DM [173].

Vitamin C act as antioxidant and plays a major role in protecting against oxidative stress damage. We observed in our study the serum level of vitamin C was decreased in diabetes and diabetic nephropathy patients and similar results were observed in previous

studies conducted by Pan H et al and Vivian ST et al [171, 172]. Since vitamin C is one of the major contributors for serum total antioxidant activity our results indicate that diabetic patients have significant defects in antioxidant protection, which may increase the vulnerability to oxidative damage and development of diabetic nephropathy.

The antioxidant enzyme glutathione peroxidase level was found to be decreased in both T2DM and DN patients compared to control study subjects. The current findings are consistent with studies which have documented low level of GPx in patients with DN and T2DM [173,174]. This reduction in glutathione peroxidase may be due to increased level of lipid peroxidation or decrease in functional renal mass as the plasma form of glutathione peroxidase is synthesized in the kidney [175, 176]. Kafle D et al in their study observed a significantly low level of glutathione peroxidase in both T2DM and DN compared to healthy controls. Their findings suggest that this reduction might be due to hyperglycemia induced oxidative stress, glycation of antioxidant enzymes, low hemoglobin concentration and excess utilization of NADPH in renal mesangial cells via polyol pathway in type 2 diabetic nephropathy [177]. Finding of our study is in accordance with finding of Kafle D et al.

Serum creatinine is considered the gold standard marker for diagnosis of renal impairment. However, due to least specificity and other dependent factors there was a need for more specific biomarker, hence emerged Cystatin C. Cystatin C is found high in serum during renal insufficiency and specific to renal function. Cystatin C, a promising marker of renal failure, is a cysteine protease inhibitor. It is a low molecular mass protein with 13.4kDa, is freely filtered at the glomerulus because of its small size and positive charge and then reabsorbed and fully catabolized, but not secreted by proximal renal

tubules although they do absorb it [68]. Cystatin C is being considered as a competency substitute for serum creatinine as it seems to be less affected by muscle mass and protein intake which made it as an endogenous marker for GFR assessment and have been proposed as a marker of tubular as well as glomerular dysfunction for early DN diagnosis [69, 70]. Our study findings observed serum cystatin C levels were high in DN group (2.49 ± 0.61) than other 2 groups. Findings of our study were supported by the studies conducted by Joen YK et al and Takir M et al [70, 178]. However, contrary to our findings, studies showed that cystatin C is not sensitive marker for diagnosis of early diabetic kidney diseases [179,180]. As a result of decreased glomerular filtration rate, accumulation of cystatin C in serum is observed in DN patients because cystatin C is physiologically freely filtered by the glomerulus and then reabsorbed and metabolized in the tubules. This forms the basis for speculation that higher cystatin C concentration in diabetic patients results from a significantly lower eGFR (35.5 ± 9.17 mL/Min) in this group.

CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equations are employed to calculate estimated Glomerular Filtration Rate (eGFR) in this study by using the values of creatinine and Cystatin C. we observed a significantly decline eGFR in DN patients compared with healthy control (102.27 ± 19.8 mL/Min) and T2DM patients (81.77 ± 24.03 mL/Min). This confirms the deterioration of renal function in DN patients. Study conducted by Taderegew MM et al has reported that poor glycemic control and chronic hyperglycemia play a key role in decreasing eGFR and accelerates the annual eGFR decline [137].

The earliest clinical evidence of nephropathy is the appearance of low but abnormal levels of albumin in the urine and the patients with microalbuminuria are referred to as having incipient nephropathy [181]. Microalbuminuria is considered as a marker of endothelial dysfunction and increased risk for cardiovascular morbidity and mortality in diabetics. In our study, the microalbumin levels were elevated significantly in T2DM with nephropathy than healthy control and T2DM. Also, a significant difference between T2DM and healthy control was noted with respect to microalbumin (Table 10). Lim A et al has documented that urinary albumin/creatinine ratio (ACR) is a reflection of albumin excretion rate (AER) [182]. Thus, we estimated ACR as it can be measured in untimed spot urine samples. Findings of current study showed an elevated ACR level in DN group compared to control and T2DM and suggested decline in renal function in nephropathy subjects. Our findings are supported by the findings of Orugbo VP et al, as they also observed significantly higher ACR and lower eGFR in DN patients [181].

DN is documented as a more prevalent disorder in fluoride endemic areas across the world. As this study is conducted in Kolar district which is considered as a fluoride endemic area due to increased water F^- level ranging between 0.6 to 4 ppm in and around the Kolar district [183]. Thus, we tried to measure serum and urinary fluoride level in study subjects. Study conducted by Kido T et al, in the year 2017 stated that the constant exposure of glomeruli to fluoride results in impaired kidney function and decreased renal clearance of fluoride [184]. In the present study, we observed a significant decrease in urinary clearance of fluoride in DN patients compared to T2DM patient and healthy controls (Table 9). There was no much difference observed between serum and urine fluoride level in DN subject. Serum fluoride level is significantly lowered in DN group

compared to healthy control and T2DM subjects. This may be because of reduced water intake by the DN patients. Our findings are in consistent with findings of in-house study conducted by Ram Mohan SD et al [185]. However, in group I and II, serum fluoride levels were high and its urinary excretion is also proportionate compared to DN group. This signifies that fluoride in blood is proportionately cleared in urine in healthy control and T2DM subject than in DN subjects. After metabolism, excretion of Fluoride is mainly through renal tissue, partly through faeces and sweat [186]. Thus, decrease in fluoride clearance indicates decreased filtering capacity of kidneys which is estimated in this study for comparison between healthy controls, T2DM and DN. Though there are not many molecular studies regarding the action of fluoride on renal cells, fluoride levels are to be considered in evaluating renal functioning in fluoride endemic area.

To assess whether vitamin D binding protein could be an effective early biomarker in assessing tubular damage in patients with diabetic nephropathy, the current study results demonstrated that the uVDBP levels were grossly elevated in DN and results were significantly correlated with degree of albuminuria in T2DM with and without nephropathy. Mean uVDBP values were 84.9 ± 50.2, 984.9 ± 304.3 and 1588.2 ± 277.3 µg/mL in healthy control (Group I), T2DM (Group II) and DN (Group III) and the elevation is statistically significant among the groups (Table 9). We further categorized all the recruited T2DM patients based on ACR into normo, micro and marco-albuminuria groups to compare the uVDBP level. An altogether higher mean uVDBP levels were observed in diabetic patients than healthy controls. Further uVDBP levels were observed to be significantly elevated in diabetic subgroups i.e., normoalbuminuria, microalbuminuria and macroalbuminuria (981±320.8, 1102.1±382.2 and 1512±340.9

µg/mL) group compared to control subjects (84.9±50.2 µg/mL) (Table 14). These results showed even in normoalbuminuria diabetic patients where other renal markers were within physiological reference limit, the level of uVDBP showed a marked elevation. Thus, even with normal ACR in diabetic patients, the grossly elevated uVDBP levels may be considered as a promising early biomarker for detecting DN. Findings of current study are in consistent with the results of studies which have been documented a marked increase in uVDBP excretion in normo, micro and macroalbuminuric type 2 diabetics, compared with healthy controls [187, 99]. Study conducted by Tian XQ et al demonstrated uVDBP is significantly higher in DN with microalbuminuria and macroalbuminuria than in DM patients without albuminuria [99]. Mirkovic K et al demonstrated that urinary excretion of VDBP was increased with increasing severity of renal damage and responded well to renoprotective therapy suggesting that uVDBP could be developed into a non-invasive urinary marker to monitor tubulointerstitial inflammation and fibrosis [15].

Correlation of uVDBP with oxidants & antioxidants marker showed a significant positive correlation with oxidant markers (Uric acid and MDA) while a significant negative correlation was observed with antioxidant markers such as vitamin C, NO and glutathione peroxidase. This indicates oxidative damage in T2DM patients which may have a role in damaging renal tubules and a resultant elevated excretion of uVDBP (Table 11).

Further, we analyzed correlation between uVDBP with renal profile parameters in diabetic subjects and results were demonstrated in table 12. A significant positive correlation with urea, creatinine, phosphate, cystatin C (Graph 7), microalbumin and

ACR (Graph 8) were observed in T2DM with and without nephropathy. Our findings are in accordance with the findings of Shoukry A et al where they documented a strong positive correlation ($p<0.001$) between uVDBP and uACR [102]. They observed uVDBP levels were directly proportional with increased uACR levels. Further, we observed a significant negative correlation with serum calcium, albumin and eGFR (Table 12). These findings indicate uVDBP levels were related with renal dysfunction indicated altered levels of renal parameters. Duration of diabetes, a major contributor for kidney damage in T2DM patients showed a significant positive correlation with uVDBP ($r=0.287$, $p<0.001$*) in both T2DM and DN patients (Graph 9).

uVDBP showed a significant negative correlation with urine fluoride in healthy control as well as in T2DM and DN group. However, the correlation between uVDBP and urine fluoride in diabetic group is stronger than to healthy control group with a higher r value of -0.414 compared to Group I ($r=-0.271$). This suggest that lower the fluoride clearance through urine, higher will be the uVDBP as a result of decreased filtering capacity of kidneys due to renal damage in diabetic patients. Further we also observed a significant negative correlation between serum fluoride and uVDBP in T2DM and DN group.

However, study conducted by Sherif E et al revealed that uVDBP and megalin levels were non-significantly elevated in T2DM patients with DN. Moreover, they reported a weak negative correlation between urinary VDBP and megalin levels with eGFR in their study [104]. Findings of our study do not support these findings as we observed significantly elevated level of uVDBP in T2DM and DN group. Further, our study uVDBP showed a significant negative correlation with r-value of -0.599.

Study conducted by Fawzy MS et al demonstrated elevated levels of uVDBP in Saudi patients with DN and their finding correlated well with the degree of albuminuria in DN. Our findings are in accordance with the findings of Fawzy MS et al [14]. Increased urinary excretion of VDBP in the diabetic patients is probably due to hyperglycemia induced ROS and TGF-β production and induces inflammatory cytokines secretion (IL-18) from the podocytes. This causes a direct or indirect renal damage with destruction of megalin/cubilin receptors of epithelial cells in the proximal tubules responsible for VDBP uptake and resultant excretion of VDBP in urine [98]. Megalin, a multiligand endocytic receptors are expressed in the brush border of proximal renal tubular cells and participate in the reuptake of filtered low-molecular weight proteins such as albumin and VDBP from the glomerular filtrate [56].

Study conducted by Ali ANM in the year 2015 in Baghdad observed a significantly elevated uVDBP levels in all the groups in patients with DN. They further reported increased VDBP levels were positively correlated with the development of the DN and concluded that elevation of VDBP level can be considered as a novel predictor for monitoring type 2 diabetes before DN onset since it has a negative correlation with eGFR [105]. Findings of current study are in concurrent with the findings of Ali ANM.

Mirkovic K et al demonstrated that urinary excretion of VDBP was increased with increasing severity of renal damage and responded well to renoprotective therapy suggesting that uVDBP could be developed into a non-invasive urinary marker to monitor tubulointerstitial inflammation and fibrosis [15]. Study done by Chaykovska L et al demonstrated that urinary VDBP is increased by fourfold in diabetic patients with normoalbuminuria and suggested that uVDBP is a predictor of early diagnosis of

asymptomatic chronic kidney disease [100]. It has been postulated that the damaged tubular epithelial cells in area of tubulointerstitial fibrosis may no longer be capable to deal with VDBP, resulting in its gross loss into the urine [15]. Additionally, it has been demonstrated that the major factors involved in the development of glomerulosclerosis and interstitial fibrosis of DN (e.g., TGF-β and angiotensin II) could negatively regulate the receptor-mediated endocytosis, participating in enhanced uVDBP excretion [14].

Graph 10 explain the effect of decline eGFR on uVDBP levels and serum cystatin C together. With this 3D scatter plot, it is crystal clear that with observed exponential decline in eGFR values between T2DM and DN respectively, we observed an increased value of cystatin C and uVDBP. These findings indicate uVDBP could be a better indicator of declined renal function. Additionally, its positive correlation with serum cystatin C suggested that uVDBP could be a promising marker for reduced eGFR.

To assess the effect of uVDBP on serum calcium and vitamin D, correlation was performed between serum calcium and vitamin D with uVDBP in a 3D scatter plot as shown in graph 11. Correlation of uVDBP with vitamin D and serum calcium in T2DM with and without nephropathy showed a negative correlation signifying that urinary loss of VDBP directly affect the serum calcium and vitamin D in T2DM patients. Presence of vitamin D deficiency or insufficiency in patient with diabetes has an important role in the progressive loss of renal function and is independently linked with DN development. In our study we observed low levels of vitamin D among T2DM patients with and without nephropathy compared to healthy controls. Further the reduction in vitamin D is more in DN patients than T2DM. This deficiency of vitamin D in DN may be contributed mechanistically because of excess loss of VDBP through urine, which could be explained by the 3D scatter plot in this current study [188].

To assess the diagnostic performance of uVDBP as a biomarker for DN, we performed Receiver Operating Characteristics (ROC) Curve analysis (Graph 13). The result indicated that AUC for uVDBP is 0.924 (95% CI, 0.868-0.962). With a cutoff value of 1273 µg/mL, uVDBP showed a sensitivity of 85% and a specificity of 79% respectively suggesting uVDBP as an early sensitive and potential marker for diagnosis of nephropathy in diabetic patients.

Thus, renal tubular damage in diabetics can be detected before significant microalbuminuria condition when other renal markers were within physiological reference limit. Findings of this study all together suggested uVDBP as an early non-invasive biomarker for DN and could be implicated with other conventional marker for early diagnosis.

Conclusion

To give conclusive remarks we propose:

- uVDBP could be considered as a marker for early detection of nephropathy in T2DM patients.
- Gross elevation of uVDBP levels in normoalbuminuric T2DM provide an early diagnosis of nephropathy despite other renal markers are within physiological range.
- Elevated uVDBP levels significantly correlate with the severity (degree of albuminuria) of diabetic nephropathy.
- uVDBP is an early non-invasive biomarker for DN and needs to be evaluated with other conventional biomarkers
- uVDBP estimation could predict early diagnosis of nephropathy in T2DM and shall help clinicians and community health care professionals plan to prevent DN progression to ESRD.

RECOMMENDATIONS

- uVDBP estimation needs to be incorporated in the extended renal profile as an early marker for diabetic nephropathy.
- Fluoride estimation needs to be considered as a diagnostic and prognostic molecule in all diabetic patients to assess the prediction of future diabetic nephropathy in fluorosis endemic areas.
- Policy makers needs to consider estimation of fluoride and VDBP in urine in diabetic patients exposed to fluoride and/or staying in fluorosis endemic area.
- Encouragement for startups in estimation of fluoride and VDBP for POCT needs to be recommended and supported.

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Annexure-II

PATIENT INFORMATION SHEET

TITLE OF THE STUDY: Association of vitamin D binding protein in diabetic nephropathy-A Cross-sectional Analytical Study.

Name of the Principal Investigator: **Mr. Bhuneshwar Yadav**

Investigator's statement:

I, Mr. Bhuneshwar Yadav pursuing Ph.D. in the Department of Biochemistry at Sri Devaraj Urs Medical College, constituent of Sri Devaraj Urs Academy of Higher Education and Research Kolar, am carrying out a study titled: Association of Vitamin D binding protein in diabetic nephropathy-A cross-sectional analytical study. The proposed study is a case-control analytical study where the blood sample drawn and analyzed from patients will be compared with the same age and gender matched clinically healthy control.

Diabetes mellitus is a metabolic disorder resulting either from deficiency of insulin or resistance to its action causing increased blood glucose levels (Hyperglycemia) which leads to several systemic complications. Presently India is called as a diabetic capital of the world with the highest number of people with diabetes i.e., 40.9 million and is estimated to be 79.4 million by the year 2030. Diabetic nephropathy is the most common microvascular complication of diabetes mellitus accounting 30-40% of diabetic patients and is one of the major causes of end stage renal disease. In the elderly, diabetic nephropathy accounts for about 46% of chronic kidney disease in India and is associated with increased cardiovascular mortality and morbidity.

Even though persistent microalbuminuria or macroalbuminuria was considered as the best available risk marker for early-stage identification of diabetic nephropathy, certain study has shown it to have inadequate specificity and sensitivity and it doesn't cover all patients with renal impairment. Thus, additional studies for novel noninvasive risk markers and feasible measures are required for the diagnosis of diabetic nephropathy prior to advanced renal dysfunction is of clinical importance with a public health implication. This has created interest in me to do the research on Vitamin D binding protein and to assess its possible role in detecting diabetic nephropathy in early stage with type 2 diabetes mellitus. Finding in this study may help us for the early detection of diabetic nephropathy and preventing its progression to end stage renal disease with suitable and early effective treatment.

Recent studies have demonstrated increased levels of VDBP excretion in patients with DN and the resultant renal tubular dysfunction has been associated with increased level of uVDBP in patient with type 1 and type 2 diabetes mellitus. However, the potential role of VDBP as a non-invasive marker for early detection of DN has not been well established.

The utility of VDBP is best interpreted only by comparing with age and sex matched healthy controls. In this regard I highly appreciate your involvement in this study by providing 3 ml of blood and urine.

In this regard, I will ask you few questions about your personal, past, and family history. I also need to collect 3 mL of blood and 20 mL of spot urine samples for investigations. In case for the present study if 24 hours urine sample is required, I request you to kindly follow the instructions mentioned here with and give 24 hours urine sample.

Procedure for 24 hours urine collection: After getting up in the morning, study subjects must empty the bladder and discard that urine. Note the time. For next 24 hours save all urine voided in the container provided. When 24 hours are over empty your bladder and add this urine to the container.

You are at liberty to ask any questions. An honest answer to my questions shall help us in better understanding the pathological process and may help in quality patient care. I assure you this will not take much of your precious time and the investigations you undergo are not charged.

The information obtained from you shall be maintained strictly confidential unless otherwise compelled by law. The entire information, investigative report and other detail obtained from you is used only for research. However, during study, if any issues need to be addressed and which are found accidentally, they will be intimated to you with a proper guidance for further management with standard patient care and would be referred to higher centers, for those treatment modalities which are not available in our hospital.

If you agree and cooperate with me in carrying out the study, I reassure that you will not be burdened financially, and you are at liberty to withdraw from the study at any point of time. Your withdrawal and/or non-acceptance to participate in this study will not affect the treatment or the rapport with the physician.

About 6 mL of the blood using aseptic precautions will be drawn from you to estimate blood glucose, kidney function tests, lipid profile, vitamin C, vitamin D, Cystatin C, antioxidant markers and fluoride levels. Besides the above, proteins that undergo glycosylation such as, glycosylated hemoglobin is also estimated in your blood. I also request you to give 20 ml of corresponding urine for estimation of Vitamin D binding protein, fluoride, sugar and microalbumin.

Further I also request you that the left-out sample (Secondary sample) shall be stored with proper precautions (labeling, recordings, and anonymization) and used for analysis later if required.

I also assure you that the publications from the present study in the present or future shall be done without disclosing your identity.

Feel free for any clarification pertaining to this study with the principal investigator and Supervisor.

Mr. Bhuneshwar Yadav: 9754590505 (Principal Investigator)

Dr. Shashidhar K.N: 09845248742 (Supervisor)

ANNEXURE - II

WRITTEN CONSENT FORM

Sl.no:

Title of the study: **Association of vitamin D binding protein in diabetic nephropathy –A Cross-sectional Analytical Study**

I do here by give my written consent for the study titled: Association of vitamin D binding protein in diabetic nephropathy –A cross-sectional Analytical Study.

I understand that I remain free to withdraw from this study at any time giving a valid reason. I have accepted to give 3 ml of blood, 20 ml of spot urine and 24 hours urine sample to the principal investigator or any person assigned for this study.

The procedure and consequence have been explained to me in my own understandable language. I have read and understood the purpose of this study and the confidential nature of the information that will be collected and preserved throughout the study as explained to me in the patient information sheet. The information collected will be used only for research.

I permit you to perform the tests as well as preserve the secondary sample for any future investigations.

I have taken the opportunity to ask questions/doubts regarding various aspects of this study and my questions have been answered by the principal investigator to my satisfaction.

I the undersigned agree to participate in this study and authorize the collection of samples. I also understand that there is no risk to my life from this study. Participation in this study does not involve any financial burden to me.

1. Subject's name and signature / thumb impression Date:

2. Name and signature of witness Date:

3. Name and signature of interviewer/Investigator: Date:

ANNEXURE - II

PROFORMA

TITLE OF THE STUDY: Association of vitamin D binding protein in diabetic nephropathy –A Cross-sectional Analytical Study.

Case No:

Name: Mr/Mrs OP No:
Age: IP No:
Gender: Ward:
Date: Occupation:
Weight:
Address:
Phone: e- mail:

CHIEF COMPLAINTS:

HISTORY OF PRESENTING ILLNESS:

PAST HISTORY:

Hypertension: yes/no if yes, duration:
Diabetes: yes/no if yes, duration:
Liver diseases: yes/no if yes, duration:
Others:
Gestational diabetes: Yes/ No

GENERAL PHYSICAL EXAMINATION:

Ht: Wt: BMI: BP: Pulse:
Pedal Oedema:
Waist hip ratio: Abdominal girth:
Built: normal / below normal / well-built / obese/ athletic
Nourishment: Well / poor nourished

CLINICAL DIAGNOSIS:

INVESTIGATIONS:

BLOOD:

Vitamin D:	ng/mL
Vitamin C:	mg/dL
Total Protein and Albumin:	gm/dL
Nitric oxide (NO):	µmol/L
Glutathione peroxidase:	ng/mL
MDA:	nmol/L

Diabetic Profile:

Plasma FBS:	mg/dL
Plasma PPBS:	mg/dL
Glycated hemoglobin (HbA1c):	%

Renal Function Tests:

Blood Urea Nitrogen:	mg/dL
Serum Creatinine:	mg/dL
Serum Uric Acid:	mg/dlL
Serum Calcium:	mEq/L
Serum Phosphate:	mEq/L
Albumin Creatinine Ratio (ACR):	mg/gm
Serum Cystatin C:	mg/dL
eGFR (calculated):	mL/min

Lipid Profile:

Serum Total Cholesterol:	mg/dL
Serum Triglycerides:	mg/dL
Serum HDLc:	mg/dL
Serum nHDLc (calculated):	mg/dL
Serum VLDL (calculated):	mg/dL
Serum LDL (calculated):	mg/dL

URINE:

Fluoride	ppm
Vitamin D binding protein	ng/mL
Microalbumin	mg/L

Other parameters/ Investigations as and when for this study.

New Knowledge Generated

1. uVDBP levels were grossly elevated in DN patients and results were significantly correlated with degree of albuminuria in T2DM patients with and without nephropathy.
2. Comparison of uVDBP with established marker and traditional markers were done to find uVDBP as an early biomarker and it is proved to be a better marker in early diagnosis and management of DN.
3. A positive correlation of uVDBP with renal parameters (urea, creatinine, cystatin C, microalbumin and ACR) were observed in T2DM patients with and without nephropathy.
4. A significant negative correlation with eGFR suggest that uVDBP could be used as marker for decline in renal function.
5. uVDBP is suggested to be added to the existing list of biomarkers for tubular injury in DN patients.
6. uVDBP can be considered as a marker for early diagnosis and management of DN and prevention of ESRD progression in T2DM patients.
7. Fluoride estimation in biological fluids has a critical role in diabetes and its microvascular complication particularly nephropathy in fluorosis endemic areas.