Evaluation of serum cystatin-C, Myeloperoxidase and other biochemical markers for the early detection of renal failure in diabetic and non-diabetic patients in Karimnagar, Telangana.

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By

S.SANGEETA

Department of Biochemistry

Registration No: 13PHD004

Department of Biochemistry

BLDE (DU) Shri B. M. Patil Medical College,

Hospital and Research Centre,
Vijayapura, Karnataka, India.

2020

BLDE (Deemed to be University)

Vijayapura, Karnataka, India.

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Evaluation of serum cystatin-C, Myeloperoxidase and other biochemical markers for the early detection of renal failure in diabetic and non-diabetic patients in Karimnagar, Telangana."** is a bonafide and genuine research work carried out by me under the guidance of **Dr. J. G. Ambekar**, Professor of Biochemistry, BLDE (Deemed to be University)'s Shri B. M.Patil Medical college, Hospital and Research Centre, Vijayapura, Karnataka. No part of this thesis has been formed the basis for the award of any degree or fellowship previously. Shall have the rights to preserve, use and disseminate this dissertation/thesis in print or electronic format for academic/research purpose.

Date: Signature of the candidate

Place: Vijayapura.

S. Sangeeta
Registration no: 13PHD004
Department of Biochemistry
BLDE (Deemed to be University')s
Shri B. M. Patil Medical College,
Hospital & Research Centre,
Vijayapura, Karnataka.

BLDE (Deemed to be University)

Vijayapura, Karnataka, India.

CERTIFICATE BY THE GUIDE

This is to certify that the thesis entitled **"Evaluation of serum cystatin-C, Myeloperoxidase and other biochemical markers for the early detection of renal failure in diabetic and non-diabetic patients in Karimnagar, Telangana."**is a bonafide research work done by **S.Sangeeta** under my supervision and guidance in the department of Biochemistry, BLDE (Deemed to be University)'s Shri B. M. Patil Medical college, Hospital and Research Centre, Vijayapura, Karnataka. Inthe fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

Date: **Signature of the Guide**

Place: Vijayapura.

Dr. J. G. Ambekar
Professor of Biochemistry,
BLDE (Deemed to be University)s
Shri B. M. Patil Medical College,
Hospital & Research Centre,
Vijayapura, Karnataka.

BLDE (Deemed to be University)

Vijayapura, Karnataka, India.

CERTIFICATE BY THE CO-GUIDE

This is to certify that the thesis entitled **"Evaluation of serum cystatin-C, Myeloperoxidase and other biochemical markers for the early detection of renal failure in diabetic and non-diabetic patients in Karimnagar, Telangana**." Is a bonafide research work done by **S. Sangeeta** under my supervision and guidance for the award of the degree of the Doctor of Philosophy.

Date:

Place:

Signature of the Co-guide

Dr.T.Sudhakar

Professor of Biochemistry

Chalmeda Ananda Rao institute of medical sciences

Karimnagar, Telangana

BLDE (Deemed to be University)

Vijayapura, Karnataka, India.

ENDORSEMENT BY THE HOD AND PRINCIPAL

This is to certify that the thesis entitled **“Evaluation of serum cystatin-C, Myeloperoxidase and other biochemical markers for the early detection of renal failure in diabetic and non-diabetic patients in Karimnagar, Telangana** is a bonafide research work done by **S.Sangeeta** under the supervision of **Dr. J. G. Ambekar** (Guide), Professor of Biochemistry, BLDE (Deemed to be University)’s Shri B. M. Patil Medical college, Hospital and Research Centre, Vijayapura, Karnataka, **Dr. T.Sudhakar** (Co-guide), Professor of Biochemistry, Chalmeda Ananda Rao institute of Medical sciences ,Karimnagar, Telangana

Signature of the HOD	**Signature of the Principal**
Dr. B. B. Devaranavadagi Professor & Head, Department of Biochemistry BLDE (Deemed to be University)’s Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka.	**Dr. AravindPatil** Principal, BLDE (Deemed to be University)’s Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka.
Date: Place: Vijayapura	Date: Place: Vijayapura

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LIST OF ABBREVIATIONS AND SYMBOLS

ACR	Albumin creatinine ratio
ADA	American Diabetes association
AGES	Advanced glycation end products
AKI	Acute kidney injury
ANOVA	Analysis of variance
ATN	Acute tubular necrosis
AUC	Area under the curve
AGE-RAGE	Advanced glycation end products-Receptor for advanced glycation end products
BUN	Blood urea nitrogen
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration equation
CKD-DM	Chronic kidney disease with Diabetes mellitus
CKD-ND	Chronic kidney disease without Diabetes mellitus
CNO^-	Cyanate
cr	Creatinine
cys	cystatin-C
CSF	Cerebrospinal fluid
DCCT	Diabetes control and complications trial
dl	Deciliter
DN	Diabetic Nephropathy
DM	Diabetes mellitus
DKA	Diabetic ketoacidosis
EDTA	Ethylene diamine tetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme linked immunosorbent assay
EPO	Eosonophil peroxidase
ESRD	End stage renal disease
ESKD	End stage kidney disease
FPG	Fasting plasma glucose
GFR	Glomerular filtration rate

GOD-POD	Glucose oxidase-peroxidase
g/dl	gram per deciliter
HHS	Hyperglycemic hyperosmolar state
HOCl	Hypochlorous acid
HOCN	Cyanic acid
HCNO	Isocyanic acid
H_2O_2	Hydrogen peroxide
HbA_1c	Glycated hemoglobin
IDDM	Insulin dependent Diabetes mellitus
IEC	Institutional Ethical Committee
KDIGO	Kidney disease improving global outcome
KDOQI	Kidney disease outcome quality initiative
Kg	Kilogram
KDa	Kilodalton
LPO	Lactoperoxidase
MA	Microalbuminuria
MD	Mean deviation
MDRD	Modification of diet in renal disease
mGFR	Measured glomerular filtration rate
mg	Milligram
mg/g	milligram per gram
mg/L	milligram per litre
ml	Milliliter
mmol/L	millimoles per litre
MOPS	Morpholinopropanesulphonic acid
MPO	Myeloperoxidase
MPO-H_2O_2	Myeloperoxidase- Hydrogen peroxide
m^2	meter squared
mRNA	Messenger ribonucleic acid
NGSP	National glycohemoglobin standardization programme
NIDDM	Non Insulin dependent Diabetes mellitus
NKF	National Kidney foundation

ng/ml	Nanogram per ml
OGTT	Oral glucose tolerance test
$O_2^{\cdot}$	Singlet oxygen
O_3	Ozone
Ocl^{-}	Hypochlorite ion
OH^{-}	Hydroxyl radical
OPD	Out patient department
OD	Optical density
RAGE	Receptor for glycation end products
REIN	Ramipril Efficacy in Nephrology
ROC	Receiver operating curve
ROS	Reactive oxygen species
SD	Standard deviation
SP	Streptavidin peroxidase Conjugate
scr	Serum creatinine
scys-C	Serum cystatin-C
T2DM	Type 2 Diabetes mellitus
UAC	Urinary albumin concentration
UACR	Urinary Albumin creatinine ratio
UPCR	Urinary protein creatinine ratio
USRDS	U.S.Renal Data system
U/L	Units per litre
WHO	World Health Organisation
α	Alpha
β	Beta
γ	Gamma
µl	Microlitre
-ve	Negative
+ve	Positive
%	Percentage

ABSTRACT

Aim and Objectives:

The present study is undertaken to assess the role of serum cystatin-C, Myeloperoxidase, serum creatinine, urine albumin-creatinine ratio and microalbumin for the early diagnosis of chronic kidney disease in diabetics and non-diabetics in and around Karimnagar, Telangana.

The objective was to evaluate whether combining serum creatinine, cystatin-C and urine albumin to creatinine ratio would improve the identification of risks associated with chronic kidney disease compared to creatinine alone in diabetic and non-diabetic patients and also to establish the possible mechanism of the role of Myeloperoxidase for the early diagnosis of Chronic kidney disease.

Material and Methods:

In the present cross sectional study, a total of 150 participants were included in the study. Group-1 comprises of 50 Normal healthy subjects, Group-2 comprises 50 Chronic Kidney Disease Patients without Diabetes Mellitus (CKD-ND) and Group-3 comprises 50 patients of Chronic Kidney Disease with Diabetes Mellitus (CKD-DM). The biochemical parameters were recorded in the samples collected from all participants of the study using Transasia XL-640 fully automated analyser. Myeloperoxidase was estimated by using ELISA reader. eGFR was calculated by applying the values of serum creatinine and cystatin-C in CKD- EPI creatinine and cystatin-C equation (2012). The results were analyzed with appropriate statistics using SPSS version 20.0 software.

Results:

Serum cystatin-C and serum creatinine were considerably increased in non-diabetic subjects with CKD, and a notable reduction in eGFR levels was observed in Group-2 in comparison to Group-3. A significant negative correlation was observed between cystatin-C and eGFR between the groups. The strength of correlation of serum cystatin-C with eGFR was strong in Group-2 and Group-3 in comparison to controls. The values of Myeloperoxidase were decreased in diabetic patients with chronic kidney disease (Group-3) when compared with non- diabetic patients with chronic kidney disease (Group-2). Myeloperoxidase levels were compared with urea, creatinine, microalbumin and eGFR levels. eGFR levels showed a significant negative correlation with MPO levels.

Conclusion:

Serum cystatin-C can be used as an alternate marker to creatinine in patients with CKD. Diagnostic accuracy is favorable for serum cystatin-C when compared with serum creatinine in patients with decreased renal function. However, it is more sensitive for the estimation of eGFR than serum creatinine. Serum MPO levels can be used as an indicator for CKD patients with diabetes mellitus in assessing the renal impairment and to prevent further complications.

Key words:

Chronic kidney disease (CKD), estimated Glomerular filtration rate (eGFR), Chronic kidney disease epidemiology collaboration (CKD-EPI), creatinine, Albumin creatinine ratio (ACR), cystatin- C, Myeloperoxidase (MPO).

Introduction

Diabetes mellitus (DM) is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use insulin produced by it[1]. According to the study of Wild S et al, it was estimated that there were around 171 million people in the world with diabetes in the year 2000 and this is expected to increase to 366 million by 2030[2].

The metabolic derangements, which are related to DM cause many physiological and pathological changes, thereby affecting various organ systems which may lead to certain complications[3]. If diabetes mellitus is untreated, it may result in acute complications like diabetic ketoacidosis, hyperosmolar hyperglycemic state, or even death[4]. Severe long-term complications include cardiovascular disease, chronic kidney disease, diabetic foot ulcers, diabetic retinopathy and cerebrovascular disease which ultimately results in tissue damage[5]. Although chronic kidney disease (CKD) is considered as a co morbidity of diabetes or hypertension, it has multiple complex causes. The other causes include polycystic kidney disease, glomerulonephritis, acute kidney injury (AKI) and autoimmune disorders like lupus and Ig A Nephropathy[6]

Chronic Kidney Disease (CKD) is a major emerging global health problem due to the increasing prevalence of conditions like diabetes mellitus, hypertension and cardiovascular disease. It is a common and serious complication of diabetes with more than 5 years of duration. Assessment of renal function in individuals with type-2 diabetes is very essential as diabetic nephropathy constitutes the main cause of CKD which ultimately results in end stage renal disease (ESRD). CKD initially is without specific symptoms and is detected by an increase in serum creatinine or protein levels in urine. In developing countries like India, diabetes and hypertension accounts for

40-60% cases of CKD which is associated with significant morbidity, mortality and economic burden[7].

According to Kidney Disease Improving Global Outcomes (KDIGO) guidelines (2013) CKD is defined as either kidney damage or eGFR <60 ml/min/1.73m^2 for ≥3 months with implications for health[8].

The progression of CKD occurs with a very long duration when the disease is not evident clinically and therefore diagnosis, evaluation and treatment is dependent mainly on biochemical markers that evaluate renal function. The biochemical parameter which is being routinely used for the assessment of renal function is serum creatinine.

One of the main diagnostic criteria of CKD is GFR and it can be considered as the best index for assessment of renal function. The "gold standard" for determining GFR was inulin clearance. Inulin is an exogenous substance, has to be administered intravenously. It is a laborious and time consuming process; it is not being practiced for routine monitoring[9]. The eGFR can be calculated based on values of serum creatinine or cystatin-C or a combination of both by using various GFR estimation equations. Routine albumin-creatinine ratio (ACR) is recommended for patients with diabetes for early detection of CKD, but in routine practice it is limited to serum creatinine testing [10].

Serum creatinine is frequently being used as a marker which is affected by factors other than renal function such as muscle mass, age, diet, race etc and hence is less reliable marker for changes in GFR. The values of serum creatinine does not show an increment until GFR levels are moderately decreased (≤40 ml/min/1.73 m^2). Thus, it is insensitive and late marker for changes in GFR[11].Thus, there is a need for identifying an alternative filtration marker for unbiased estimation of GFR. Among

several biomarkers, cystatin-C has been proposed to be a promising marker which can help to detect early kidney injury.

Serum cystatin-C is a low molecular weight (13.3KD), non-glycosylated protein containing 120 amino acids and is encoded by CST3 gene. It is produced at a constant rate by CST3gene, and is expressed in all nucleated cells of the body and being used as a marker of kidney function[12]. Because of its small size and basic pH it is easily removed from the blood stream and freely filtered by the glomerular membrane in the kidneys. After filtration 99% of the filtered cystatin-C is reabsorbed and catabolised by tubular epithelial cells[13]. As it is not secreted into the tubular system, it is not found in urine. The serum levels of cystatin-C are not altered by infections, or neoplastic states, and also by body mass, diet, or drugs[14]. Serum level of cystatin-C is also superior to creatinine as a marker of kidney function and it correlates better with eGFR than serum creatinine. Therefore, on the basis of these key factors cystatin-C has been proposed and attempted to be proved as a superior marker for predicting GFR than creatinine[11].

CKD patients are at higher risk related to oxidative stress, metabolic disorders and other pathology. Oxidative stress is one of the pathophysiological mechanisms in CKD. This could be the reason for an increase in the generation of reactive oxygen species as well as a decrease in antioxidant defence. This oxidative stress further accelerates progression of renal injury.

Myeloperoxidase (MPO) is produced by neutrophilic granulocytes which along with heme is bactericidal and microbicidal by producing HOCl, H_2O_2 and Cl^- ions. In the presence of H_2O_2&halides,MPO catalyses the formation of reactive oxygen intermediates like HOCl (Hypochlorous acid) and respective halides which participate as one of the mediators of oxidative modification of biomolecules/tissues

and contribute to the development of co-morbidities and complications in patients with CKD.

Microalbuminuria & eGFR are independent risk factors in the diagnosis of CKD which are recommended to identify early renal impairment at reversible stage[15]. Proteinuria is the common indication for End Stage Renal Diseases (ESRD) which is more commonly seen in patients with CKD. Microalbuminuria should be corrected at an early stage to delay the renal damage and development of cardiovascular complications in CKD patients. The diagnosis of CKD in diabetics in early detectable stage is important to delay the renal complications.

The need to reduce the complications like CKD in diabetes led the researchers to look for various new markers like cystatin-C and Myeloperoxidase, which help in the early diagnosis of CKD. Hence the present study is undertaken to assess the levels of serum cystatin-C, Myeloperoxidase and their correlation with eGFR and other biochemical parameters in diabetic and non-diabetic patients.

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Aims and Objectives

2.1 Aim of the study:

The present study is undertaken to assess the role of serum cystatin-C, Myeloperoxidase, serum Creatinine, urine albumin-creatinine ratio and microalbumin in the early diagnosis of chronic kidney disease in diabetics and non-diabetics in and around Karimnagar, Telangana.

2.2 Objectives of the study:

1. To evaluate whether cystatin-C along with serum creatinine and ratio of urine albumin to creatinine can be used as markers for early detection of risks associated with chronic kidney disease compared to creatinine alone in diabetic and non-diabetic patients.
2. To evaluate the early detection of chronic kidney disease to prevent the increased risk for the development of diabetic nephropathy by assessing urine microalbumin levels and HbA_1c in diabetic patients.
3. To establish the possible mechanism of the role of Myeloperoxidase for early diagnosis of CKD.

2.3 Hypothesis:

Combination of serum Creatinine, cystatin-C and albumin creatinine ratio is not effective than creatinine alone in the early detection of Chronic kidney disease.

Review of Literature

3.1 Diabetes mellitus and its related complications:

Diabetes mellitus (DM), is more often known as type-2 diabetes, includes a cluster of metabolic disorders marked by elevated blood glucose levels over a lengthy period of time[1].It is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both[2]. The symptoms of diabetes mellitus include polyuria, polydipsia and polyphagia[3].

3.1.1 Epidemiology of Diabetes mellitus:

As per WHO 2016 reports, there were 422 million DM patients worldwide. The overall prevalence of diabetes across the globe in adults over 18 years of age was reported as 4.7% in 1980 which has increased to 8.5% in 2014[4]

In 2016, it was estimated that 1.6 million deaths were caused by diabetes. Prevalence of diabetes has been rising notably in middle and low-income countries[5]. It may be because of their lifestyle, socioeconomic status and dietary habits. Diabetes is the main cause of systemic complications like visual defects, renal failure, cardiovascular disease and diabetic foot.

Prevalence in India:

According to the WHO report published in 2016, the overall prevalence of DM in India was 7.3%. Higher prevalence was seen in urban areas i.e 11.2% compared to rural areas i.e 5.2 %[4]

In India, diabetes is now being given the grade of an epidemic with more than 62 million diabetic patients presently investigated with the disease[6,7]. In 2000, India (31.7 million) topped the world with highest number of cases diagnosed with diabetes mellitus followed by China (20.8 million) and United States (17.7 million) in second and third place respectively. The prevalence of diabetes is anticipated to be more than

double globally from 171 million in 2000 to 366 million in 2030as reported by Wild et al[8], with a maximal increase in India. It is assumed that by 2030 diabetes mellitus may affect around 79.4 million subjects in India, 42.3 million in China and 30.3 million in United States[8,9] .

3.1.2 Classification of diabetes according to American Diabetic association (ADA guidelines):[10]

It can be classified into the following categories:

1. Type 1 diabetes: It occurs either due to autoimmune or β cell destruction, usually resulting in complete insulin deficiency
2. Type 2 diabetes: It is the result of progressive loss of β cell function or insulin secretion commonly on the background of insulin resistance.
3. Gestational diabetes mellitus (GDM): It is usually diagnosed in the second or third trimester of pregnancy.
4. Specific types of diabetes due to other causes, e.g., Monogenic diabetes syndromes such as neonatal diabetes and Maturity-Onset Diabetes of the Young [MODY], diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes.

Type 1 DM: Immune-Mediated Diabetes**:** This form was previously called “insulin- dependent diabetes” or “juvenile-onset diabetes” accounts for 5–10% of diabetes and is due to cellular-mediated auto-immune destruction of the pancreatic β-cells. The rate of β-cell destruction is quite variable, being rapid in some individuals i.e mainly infants and children and slow in others mainly adults.

Type 2 DM: Type 2 diabetes, previously referred to as “noninsulin-dependent diabetes” or “adult-onset diabetes,” accounts for 90– 95% of all diabetes. This form encompasses individuals who have relative insulin deficiency and peripheral insulin

resistance. There are various causes of type 2 diabetes. Most of the patients with type 2 diabetes are overweight or obese. Excess weight itself causes some degree of insulin resistance. Patients who are not obese or overweight by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region. Type 2 DM frequently is undiagnosed for many years because hyperglycemia develops gradually and, at early stages, is often not severe enough for the patient to notice the classic symptoms of diabetes. Nevertheless, even undiagnosed patients are at increased risk of developing macrovascular and microvascular complications.

3. Gestational diabetes mellitus (GDM):

It is defined as any degree of glucose intolerance that is first recognized during pregnancy[11], regardless of whether the condition existed prior to pregnancy or after pregnancy.

4 Specific types of diabetes due to other causes:

a) Monogenic diabetes syndromes such as neonatal diabetes and maturity-onset diabetes of the young [MODY],
b) Diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis),
c) Drug- or chemical-induced diabetes

3.1.3 Signs and symptoms

The classical symptoms of untreated diabetes mellitus are characterized by weight loss, polyuria (increased urination),polydipsia (increased thirst) and polyphagia (increased hunger)[7].Symptoms may develop rapidly within few weeks to months in type-1 diabetes, while they usually develop slowly and may be subtle or absent in type-2 diabetes. The other symptoms of diabetes mellitus include weight loss and tiredness[12]

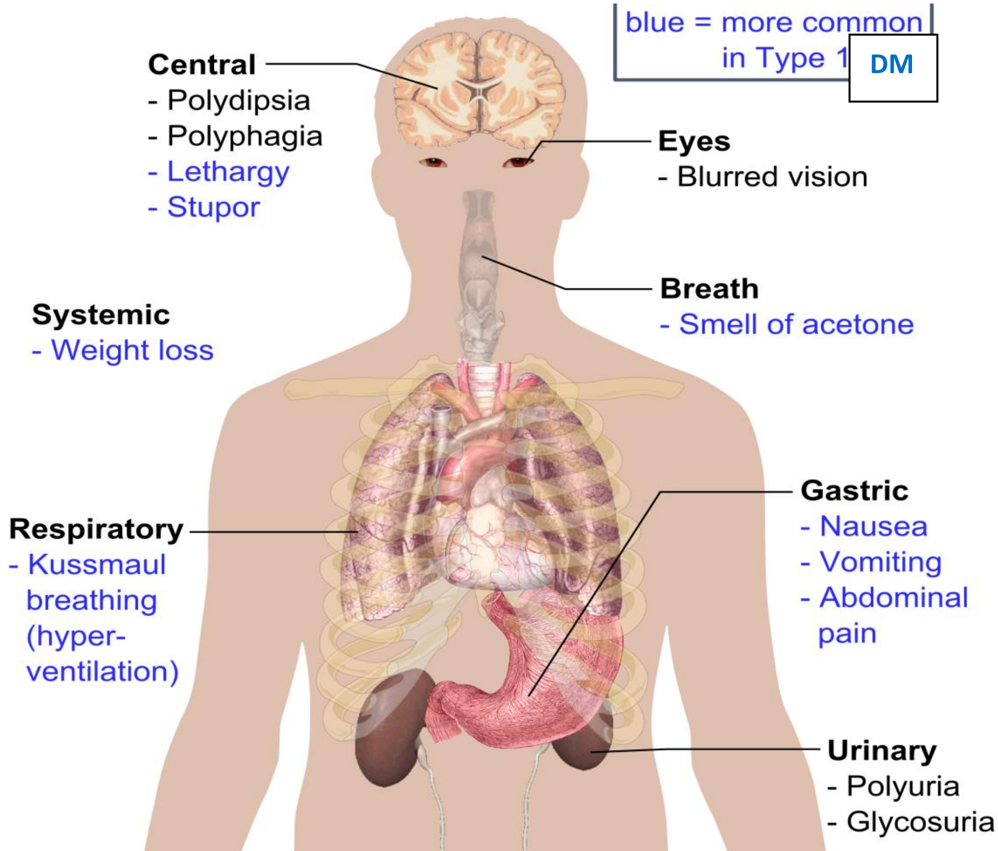

Figure-3.1 Main symptoms of diabetes

Source-www.clinicaltrialsarena.com/projects/bydureon-bcise-treatment-type-2-diabetes.com

3.1.4 Pathogenesis and pathophysiology of diabetes mellitus:

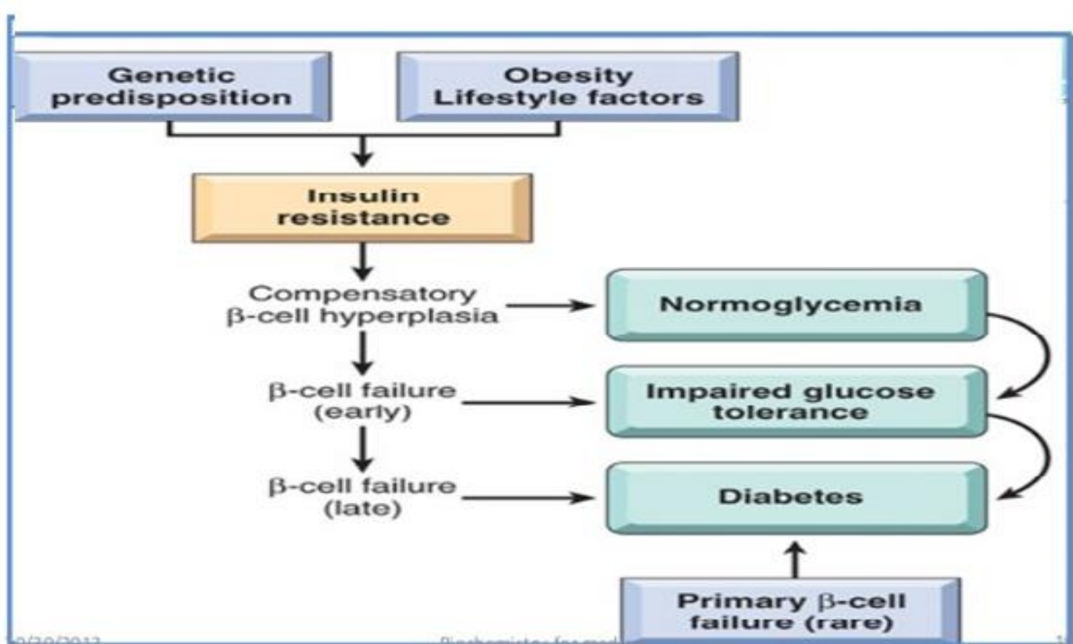

Figure 3.2 Pathophysiology of Diabetes mellitus

Source-Ozougwu JC, Obimba KC, Belonwu CD, Unakalamba CB. The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus.Journal of Physiology and Pathophysiology. 2013 Sep;4(4):46-57.

The two main factors which are important for the pathophysiological conditions of diabetes mellitus are -

1. Impaired insulin secretion: It is a decline in response to glucose, which is noticed before the clinical development of the disease. Insulin secretion is decreased in the early phase which is an essential part of this condition and it is the basic pathophysiological change observed in all ethnic groups at the initial onset of the disease[13].
2. Insulin resistance: It is a condition in which the production of insulin in the body will not exhibit appropriate action as per its concentration in blood in the human body. The common pathophysiological feature of type-2 diabetes is the

decreased action of insulin on the target organs such as liver and muscles. The causes of insulin resistance include excess body weight, too much belly fat, lack of exercise, smoking, and even skimping on sleep. As insulin resistance develops gradually over months and years, it may lead to pre-diabetes or type-2diabetes. Insulin resistance is usually triggered by a combination of factors linked to weight, age, genetics, being sedentary and smoking.

3.1.5 Diagnostic Criteria for the diagnosis of diabetes mellitus

Diabetes can be diagnosed based on either fasting plasma glucose (FPG), 2-hr plasma glucose or HbA_1c (Glycated haemoglobin) criteria. 2hr PG value should be considered for evaluation after administration of 75-g oral glucose .i.e (OGTT)[14, 15]. These tests are being used to screen and diagnose diabetes.

HbA_1c (Glycated Haemoglobin): Glycemic control is not evidenced by routine blood glucose estimations due to wide variations. HbA_1c provides the mean value of blood glucose over a period 6-8 weeks. The HbA_1c test should be performed using a method that is certified by the National Glycohemoglobin Standardisation Programme (NGSP) and should be standardized to the Diabetes Control and Complications Trial (DCCT) reference assay.

3.1.5.1 ADA Criteria for the diagnosis of diabetes[14]

The test should be performed in a laboratory using a method that is National Glycohemoglobin standardization programme (NGSP) certified and standardized to the Diabetes control and complications trial (DCCT) assay. FPG should be $\geq$ 126 mg/dl or 2-hr PG should be $\geq$ 200 mg/dl during an OGTT. The test should be performed as illustrated by WHO, using an oral glucose load containing equivalent of 75 g anhydrous glucose dissolved in 250 ml of water or in a patient with classic

symptoms of hyperglycemia, a random plasma glucose ≥200 mg/dl is the diagnostic criteria for diabetes mellitus. Based on HbA_1c, it should be ≥ 6.5%.

3.1.5.2WHO criteria for the diagnosis of DM[16]:

Symptoms of diabetes mellitus and

Random Blood Glucose concentration ≥ 200mg/dl or

Fasting Plasma Glucose (FPG) ≥ 126 mg/dl

or Glycosylated haemoglobin ≥ 6.5%

or 2-hour plasma glucose ≥ 200 mg/dl.

3.1.6 Complications of diabetes mellitus:

1. **Acute complications:** These include hypoglycaemia, hyperglycemic crisis, diabetic ketoacidosis (DKA) and hyperglycaemic hyperosmolar state (HHS)[17].
2. **Chronic complications:** These include micro vascular complications like diabetic retinopathy, diabetic nephropathy, diabetic neuropathy and macro vascular disease.

The other complications and associated conditions:

These include impaired growth and development, associated autoimmune conditions, hypothyroidism, hyperthyroidism, celiac disease, vitiligo, primary adrenal insufficiency (Addison's disease) , non-alcoholic fatty liver disease etc.

The harmful effects of hyperglycemia are divided as

1. Microvascular complications: diabetic nephropathy, neuropathy, and retinopathy
2. Macrovascular complications: coronary artery disease, peripheral arterial disease, and stroke

Microvascular complications:

Diabetic retinopathy:

It is the most common micro vascular complication of diabetes. Retinopathy may begin to develop as early as 7 years before the diagnosis of diabetes in patients with type 2 diabetes.[18]The risk of developing diabetic retinopathy or other microvascular complications of diabetes depends on the duration and the severity of hyperglycemia.

Diabetic Nephropathy:

Microalbuminuria is defined as albumin excretion of 30–299 mg/24 hours. Without timely intervention, diabetic patients having microalbuminuria typically progress to Proteinuria and overt diabetic nephropathy. This progression occurs in both types 1 and type-2 diabetes. The pathological changes that occur in the kidney include thickening of the glomerular basement membrane, micro aneurysms, mesangial nodule formation and certain other changes.

Diabetic neuropathy:

It is the most common complication of both type 1 and type 2 DM. According to ADA guidelines, Diabetic neuropathy is "the presence of signs and/or symptoms of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes."[19] Along with other microvascular complications, the risk of developing diabetic neuropathy is proportional to the severity and duration of hyperglycemia, and in some individuals, it is attributed to genetic factors which affects the predisposition to developing such complications.

Macrovascular complications:

Diabetes increases the risk for development of cardiovascular disease (CVD). Among macrovascular complications of diabetes, coronary heart disease has been associated with diabetes in various studies starting with the Framingham study.[20] Recent studies have shown that the risk of myocardial infarction (MI) in people with diabetes is equivalent to the risk in non-diabetic patients with a history of previous MI.[21]The main pathological mechanism in macro vascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body. Atherosclerosis is the result of chronic inflammation and injury to the arterial walls in peripheral or coronary vascular system.

3.2 Chronic kidney diseases (CKD):

One of the important complications of Diabetes mellitus is Chronic Kidney Disease.

Definition of CKD: According to KDOQI, Chronic kidney disease is defined as:

1. Kidney damage for ≥3 months, as defined by structural or functional abnormalities of the kidney, with or without decreased GFR, that can lead to decreased GFR, manifest by either by a)Pathological abnormalities; or by b)Markers of kidney damage, including abnormalities in the composition of the blood or urine, or abnormalities in imaging tests
2. GFR<60 ml/min/1.73m^2 for ≥ 3 months with or without kidney damage

3.2.1 Types of CKD:

Kidney disease is broadly classified into

1. Acute kidney injury or Acute renal failure and
2. Chronic kidney disease.

3.2.1.1 Acute kidney injury

Acute kidney injury is sudden damage to the kidney. It is usually short term and reversible but in some cases it may lead to long-term chronic kidney disease.

The main causes of AKI are hypotension, shock, over usage of pain relievers, organ failure and allergic reactions. It leads to oedema, obstruction to urinate which results in renal calculi and enlargement of prostate gland.

3.2.1.2 Chronic kidney disease (CKD):

Chronic kidney disease is the result of various conditions affecting the kidney. Kidney function deteriorates gradually over a period of time resulting in chronic kidney disease. Haileamlak A et al (2018) ,described in his study that, in addition to the primary diseases of the kidney various non-communicable diseases like hypertension, diabetes and dyslipidemia are also associated with progressive renal failure[22].

CKD is defined as abnormalities of kidney structure or function, present for more than 3 months, with implications for health[23]. It is a condition which refers to long-term damage of renal function. It is interpreted in the presence of co morbidities like hypertension, diabetes, and cardiovascular disease, and it is also associated with socioeconomic deprivation[24-26]. Effective and timely diagnosis followed by accurate medical management are needed to prevent advancement of the disease, cardiovascular events and also to reduce the hazards associated with acute kidney injury (AKI), and thereby boost up the quality of life.

3.3 Epidemiology of CKD:

Chronic kidney disease is a health crisis globally. In the year 2005, there were approximately 58 million deaths worldwide, out of which35 million were attributed to chronic disease, according to the World Health Organization.[27]In western countries,

diabetes and hypertension accounts for over 2/3rd of the cases of CKD[28]. In India too, diabetes and hypertension accounts for 40–60% cases of CKD[29]

According to a Spanish epidemiological study a higher incidence rate of chronic renal failure was observed in men[30]. A Chinese cross-sectional study proved similar prevalence of CKD among men and women[31]. This might be attributed to geographical variability on the effect of gender on prevalence of CKD. Based on the latest US Renal Data System (2015), 57.8% of the cases were men who reported new onset of ESRD[32].

3.4 Classification of CKD:

CKD is common in the elderly population it is also seen in young population with a gradual deterioration of renal function. About 30% of cases have stable disease with age of more than 65 years[33]. The Kidney Disease Outcomes Quality Initiative (KDOQI) of the National Kidney Foundation (NKF) fixed a definition and classification of CKD in 2002[33].The KDOQI and the international guideline group Kidney Disease Improving Global Outcomes (KDIGO) have finally amended these guidelines[34].

3.5 Staging of CKD:

The staging of CKD is classified according to KDIGO (2012)as follows:

Stage 1: Kidney damage with a normal or increased value of GFR (>90 ml/min/1.73 m^2)

Stage 2: Mild reduction in GFR value (60-89 ml/min/1.73 m^2)

Stage 3a: Moderate reduction in GFR value (45-59 ml/min/1.73 m^2)

Stage 3b: Moderate reduction in GFR value (30-44 ml/min/1.73 m^2)

Stage 4: Severe reduction in GFR value (15-29 ml/min/1.73 m^2)

Stage 5: Kidney failure (GFR value < 15 ml/min/1.73 m^2)

In stage 1 and 2 of CKD, a decrease in GFR will not conclude the interpretation, as GFR value may be normal or near to normal. In such cases, inclusion of other markers of kidney damage like albumin or ACR can clinch the diagnosis[35]. Albuminuria, urinary sediments and impaired electrolytes in serum are due to tubular dysfunction. Histological and anatomical anamolies can be detected by imaging techniques.

STAGES OF CHRONIC KIDNEY DISEASE		GFR*	% OF KIDNEY FUNCTION
Stage 1	Kidney damage with **normal** kidney function	90 or higher	90-100%
Stage 2	Kidney damage with **mild loss** of kidney function	89 to 60	89-60%
Stage 3a	**Mild to moderate** loss of kidney function	59 to 45	59-45%
Stage 3b	**Moderate to severe** loss of kidney function	44 to 30	44-30%
Stage 4	**Severe** loss of kidney function	29 to 15	29-15%
Stage 5	Kidney failure	Less than 15	Less than 15%

Figure-3.3 Staging of CKD

Source-Inker LA, Astor BC, Fox CH, Isakova T, Lash JP, Peralta CA, Tamura MK, Feldman HI. KDOQI US Commentary on the 2012 KDIGO clinical practice guideline for the evaluation and management of CKD.American Journal of Kidney Diseases. 2014 May 1;63(5):713-35.

3.6 Various factors that cause CKD:

The various factors causing CKD are 1. Diabetes Mellitus 2.Hypertensionand 3.Glomerulonephritis in 75% of the cases.

3.6.1 Diabetes:

Diabetes is a metabolic disorder which is represented by very high concentration of glucose in the blood. Increased levels of blood glucose can result in harmful effects on various organs of the body, but in relation to kidneys, it results in the enormous production of chemical intermediates known as Reactive Oxygen Species (ROS) which constitutes peroxides and other related compounds. Chronic exposure of glomeruli to ROS can result in harmful effects. Due to this the larger molecules that are to be filtered can escape and are disposed through urine this leads to excretion of large amounts of protein through urine called as proteinuria, a unique symptom of CKD.

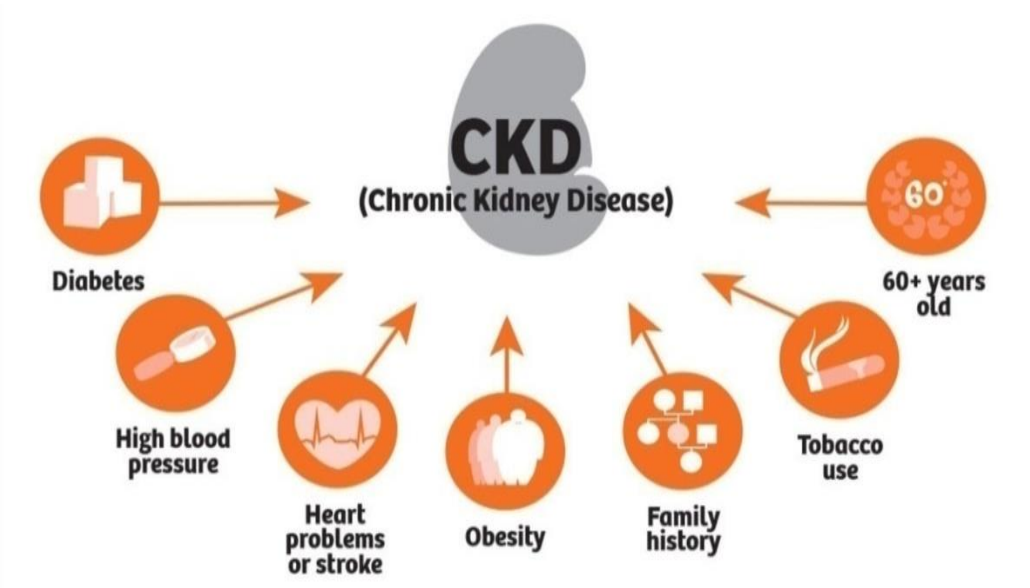

Figure-3.4 Causative factors of CKD

Source-Kazancioğlu R. Risk factors for chronic kidney disease: an update. Kidney international supplements. 2013 Dec 1; 3(4):368-71.

3.6.2.Hypertension

Hypertension accounts for about 20 % of all subjects of CKD. It leads to renal diseases by causing injury to the nephrons of the kidney which results in thickening of microvasculature of the arteries (atherosclerosis), which will further activate the thickening of the minute blood vessels that supply the nephrons thus resulting in reduction in number of functionally active nephrons. Moreover, as the damage proceeds to progression, the functional capacity of the kidneys will gradually decline to produce a hormone called aldosterone, which is involved in regulation of hypertension. This initiates a critical effect in which the cycle of hypertension and renal injury is increased, and more blood vessels are injured and blocked which eventually results in ESRD.

3.6.3 Glomerulonephritis

It is acute or chronic nephritis that involves the inflammation of the capillaries of the glomeruli.

3.6.4 Aging and renal function:

The biological mechanism of aging triggers many anatomical as well as physiological modifications in the kidney[36, 37].

The other, less common causes of CKD include:

Heavy metal poisoning, hemolytic-uremic syndrome, hepatitis B and hepatitis C, interstitial nephritis, pyelonephritis, prolonged urinary tract obstruction, recurrent kidney infections and reflux nephropathy.

3.7 Risk factors for CKD

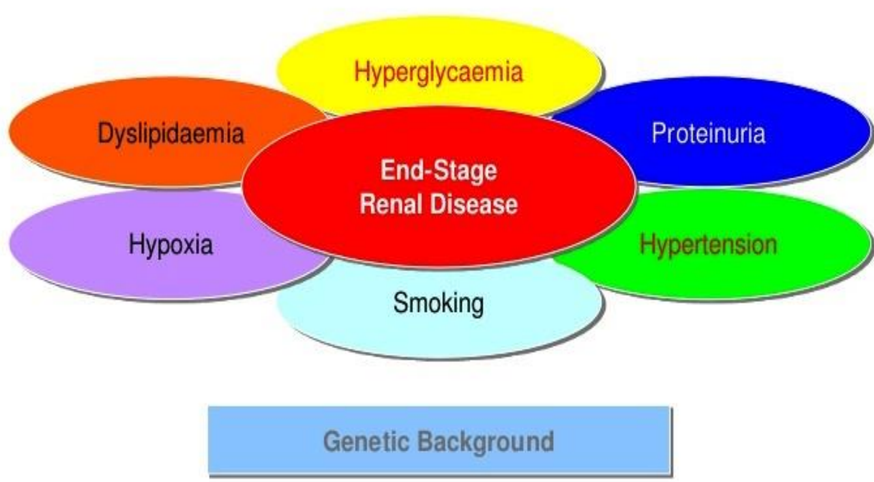

Figure-3.5 Risk factors for CKD

Source- Strippoli GF,Di Paolo S,Cincione R,et al Clinical and therapeutic aspects of diabetic nephropathy.J Nephrol16:487-499,2003

The factors which affect kidney disease are advancing age, malnourishment, obesity, chronic smoking, hypertension, diabetes mellitus, previous history of kidney disease in the family, and being an African-American descent. There are multiple hazards that can promote the risk of developing CKD.

Some of the factors are not modifiable and others are modifiable.

The risk factors which are not modifiable and associated with CKD are:

Heredity: The individual can be susceptible to CKD and the danger of ESRD is much higher if there is a family history of ESRD[38].

Gender: African-Americans are expected to be nearly four times at risk of ESRD as Caucasian-Americans. Whereas Asian-Americans, Hispanic-Americans, and Native Americans are at risk because they are twice as likely to develop diabetes[39].

Age: About 83 percent of ESRD is diagnosed in individuals above the age of 45, according to the data collected from the U.S. Renal Data System (USRDS) [40].

Premature birth: It is related to the developmental anamolies of kidney, which results in decrease in number of nephrons[41].

The modifiable risk factors associated with CKD are:

- Hypertension
- Type-1 diabetes at an early onset of age before 20 years
- Poor glycemic control (both in type 1 and type 2 diabetes population)
- Smoking, which causes constriction of renal arteries
- Excessive weight gain which further leads to Hypertension, and diabetes
- Production of adipokines, the inflammatory molecules that can damage the renal tissue.

3.8 Signs and symptoms of CKD:

The signs and symptoms of chronic kidney disease include:

- Frequent urination, especially at night ;
- Oedema of the legs and puffiness around the eyes ;
- High blood pressure;
- Fatigue and weakness ;
- Loss of appetite, nausea and vomiting;
- Itching, easy bruising, and pale skin ;
- Shortness of breath from fluid accumulation in the lungs;
- Headache, numbness in the feet or hands, disturbed sleep, altered mental status , and restless legs syndrome;

- Chest pain due to pericarditis ;
- hemorrhage
- Bone pain and fractures; and
- Decreased sexual interest and erectile dysfunction[42].

3.9 Pathophysiology of CKD:

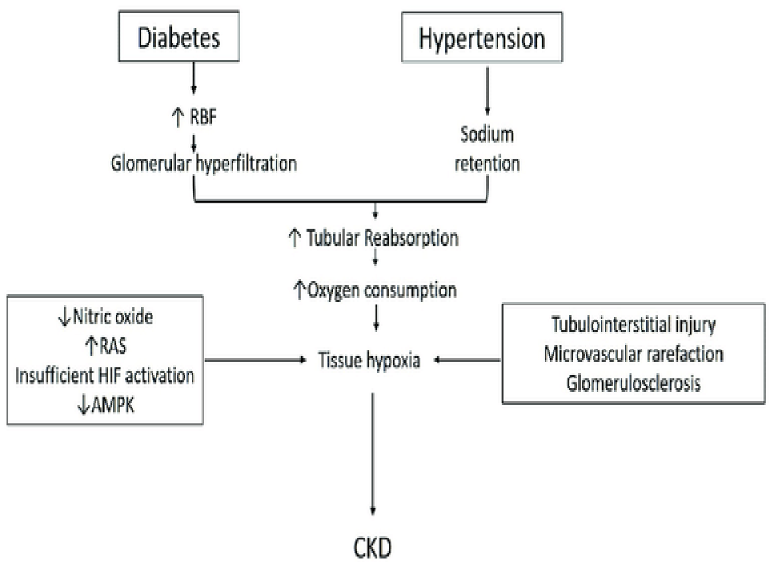

Figure-3.6 Pathophysiology of CKD

Source- Liu ZZ, Bullen A, Li Y, Singh P. Renal oxygenation in the pathophysiology of chronic kidney disease. Frontiers in physiology. 2017 Jun 28;8:385.

A normal kidney consists of about 1 million nephrons; each of it contributes to the total glomerular filtration rate (GFR). During the course of renal injury irrespective of the cause, and progressive functional loss of nephrons, kidney has an innate ability to maintain GFR, as the remaining healthy nephrons signify hyper filtration and compensatory hypertrophy. This mechanism of nephron adaptability allows maintaining normal clearance of solutes. The levels of urea and creatinine will show a notable increase after a significant reduction in total GFR, is observed.(i.e up to 50%)

3.10 Association of Diabetes and CKD:

The main symptom of DM is hyperglycemia which leads to the development of chronic complications of the disease, like Diabetic kidney disease (DKD). It is the result of either dysfunction and apoptosis of pancreatic beta cells caused by an autoimmune abnormality (type 1 DM), or from overstimulation of insulin synthesis and secretion in the presence of insulin resistance (IR), which characterizes type 2 DM[43]. According to recent studies, apart from dysfunction of the pancreas, other organs like liver, adipose tissue, intestine, kidneys, and central nervous system are being involved in the pathogenesis of hyperglycemia in type-2 DM, and may contribute to the hyperglycemic state[44].

Diabetic kidney disease (DKD) is observed due to changes in renal circulation which is caused by hyperglycemia.[45]. The initial renal circulatory events are characterized by glomerular hyper perfusion, hypertension and hyper filtration, which is responsible for functional and structural changes in the glomeruli, resulting in albuminuria followed by decrease in GFR, glomerular hypertrophy, mesangial expansion, glomerulosclerosis, renal fibrosis, and natural history of DKD[46,47]Glucose homeostasis is highly modified in patients with CKD, who are exposed to a increased risk of both hyperglycemia and hypoglycemia. Both high and low glycemic status is associated with increased morbidity and short life span in this group of patients. The factors associated with an increased risk of hypoglycemia in CKD patients include decreased renal gluconeogenesis, deranged metabolic pathways and decreased insulin clearance. On the other hand, decreased glucose filtration, excretion, and inflammation-induced insulin resistance are predisposing factors to hyperglycemic episodes[48].

In a study conducted by Thaker et al, they showed a link between recurrence of acute kidney injury (AKI) and increased danger for the advancement of progressive CKD in patients admitted in a hospital with diabetes mellitus[49].

3.11 Diagnosis of CKD:

As per Kidney Disease Outcome Quality Initiative (KDOQI) guidelines[50,51] all patients should be assessed for the risk factors which cause kidney-disease. Further, screening is performed only in patients with identified risk factors. In randomized controlled trials[52] screening methods for chronic kidney disease have not been evaluated, due to increased occurrence of the disease in at-risk populations, the ease of screening, and the availability of effective treatment at early asymptomatic stages of the disease will provide sufficient rationale for screening[53].

Laboratory tests are used to screen the patients who have risk for chronic kidney disease, wherein some patients may have functional anamolies of kidney which can be identified by the estimation of serum creatinine levels and calculation estimated glomerular filtration rate (eGFR). Screening for proteinuria will identify the presence of chronic kidney disease much before changes in GFR become probable and is an important diagnostic marker for a clinician. As per present KDOQI guidelines measurement of serum creatinine which is useful in estimation of eGFR and estimation of albumin in random urine sample are recommended screening measures for kidney disease[54]. Decrease in eGFR or Proteinuria or combination of both can help in diagnosis of significant kidney disease.

As CKD is usually unclear, its progression in many patients is identified just before the onset of symptomatic kidney failure. At this stage very few opportunities are readily available to prevent and delay the adverse outcomes. Early identification will give more scope for interpretation and treatment. But it requires precise

measurement strategies for individuals without specific symptoms who are at increased risk for the development of CKD. Routinely CKD can be identified by two simple tests:

1) a routine urine examination for detecting the presence of albumin or proteins
2) A blood test i.e estimation of serum creatinine which is used for the calculation of estimated glomerular filtration rate (eGFR).

These tests help in detection of CKD and now there is good information to support a collection of clinical interferences which are beneficial to the patients with CKD after detection[55,56].

CKD is usually not identified with any specific symptoms or deformities; this condition is often masked in 80-90% of cases[57-60]. Initial treatment of CKD however has been proved to be beneficial in order to setback or prevent decline in kidney function[61-63]. Hence early identification and diagnosis of individuals without any specific symptoms will be useful and adequate to reduce the burden of ESRD.

The analysis of creatinine levels for calculation of eGFR and performing simple urine test for the measurement of urine proteins, urine protein/creatinine ratio (UPCR), urine albumin concentration (UAC) or urinary albumin/creatinine ratio (UACR), would be the backbone of CKD screening programs, along with recording of blood pressure. These investigations are simple, affordable and easily available for establishing the presence of CKD. Proteinuria is defined as elevated excretion of urine proteins, predominantly albumin. It is recorded to be most important determinant for likely progression to ESRD[64]. Proteinuria and albuminuria are the early markers of renal impairment in individuals with diabetes, hypertension and glomerular diseases.

3.12 Assessment of renal function:

The estimation of renal function is being commonly done using serum creatinine (scr), blood urea nitrogen (BUN) and simple urine analysis[65].CKD is usually detected by evaluating serum creatinine levels to calculate the GFR and by measuring the urinary albumin/creatinine ratio to detect proteinuria.[66] Recent literature has proved that these parameters are not ideal to identify renal disorders in early stages[67-69]. Special tests may be required to identify rare causes of CKD. Renal ultrasonography is recommended to evaluate kidney size and assess for possible structural abnormalities.[70]

The KDIGO suggests that CKD should be investigated, categorized, and staged on the basis of GFR[73]. In clinical practice GFR plays an important role for interpretation, treatment and prognosis, in addition to its advantage for exploration and public health. GFR is defined as the volume of fluid filtered from the glomerular capillaries into the Bowman's capsule per unit time[72-75]. The levels of GFR are influenced by age, gender, body surface area and its reference range is between 120 to 130 ml/min per 1.73 m^2 in males and females, respectively[76-78]. Routinely, GFR is calculated by using estimation equations which are based on endogenous markers like creatinine or cystatin-C along with the inclusion of demographic variables such as age, gender and race[73, 76,79].

3.13 Endogenous biomarkers of kidney function:

3.13.1 Routine parameters for the assessment of renal function :

3.13.1.1 Blood urea nitrogen:

The values of BUN are increased as GFR decreases and are less advantageous than scr, since the levels of BUN can change irrespective of GFR. The rate of synthesis of urea varies and is raised with intake of diet rich in proteins or tissue

breakdown such as bleeding, muscle damage or by usage of steroids. A low protein diet or in cases of malfunctioning of liver can result in decreased levels of BUN without affecting GFR[80].

3.13.1.2 Creatinine:

Serum creatinine (scr) is derived from creatine degradation and has a molecular mass of 113 Da[81]. It is freely filtered but is neither reabsorbed nor metabolized. However major amount of creatinine in urine is derived from proximal tubular secretion[76, 81]. One of the most important requirements for utilizing estimating equations based on scr is stable kidney function. In addition, non-GFR determinants, such as variation in production of creatinine which is influenced by food intake, or changes in muscle mass, variation in tubular secretion and extra-renal creatinine excretion are to be considered while utilizing creatinine.[82,83] Another relevant factor which checks the precision of equations is the fluctuation in analysis of serum creatinine[84].

3.13.1.3 Microalbumin:

Albuminuria & eGFR have independent additional value in the diagnosis of CKD. Hence they are suggested to diagnose initial risks for renal damage at a reversible stage[85]. However, a large number of glomerular, tubular, and interstitial pathophysiological mechanisms can lead to Proteinuria, and significant structural damage has typically already occurred before proteinuria is measureable[86]. Progressive renal function decline has already commenced at the onset of microalbuminuria[87].

3.13.1.4 Albumin creatinine ratio(ACR):

According to recent guidelines, microalbuminuria (MA) may be defined as urinary excretion of 30–300 mg/D in a timed urine collection in adults. Gerstein et

al(2001) in a systematic review showed microalbuminuria among individuals with type 2 diabetes was found to be associated with a 2.4-fold (95% confidence interval [CI] 1.8 to 3.1) increased risk for cardiovascular death as compared with normo-albuminuria[88]. Volpe M et al (2003), has reported that a similar association existed in hypertensive individuals (without diabetes) and in general population[89]

3.13.2 Special parameters for assessment of renal function :

3.13.2.1cystatin-C:

cystatin-C (cys-C) is a13.3-kDa protein containing 122-amino acids, which belongs to the group of cysteine proteinase inhibitors[90]. It is encoded by 'housekeeping type" CST3 gene, which is formed by nucleated cells at a stable rate[91]. It is freely filtered by the glomeruli and is mostly reabsorbed and degraded in the proximal tubules[92].The estimation of cystatin-C is a good measure of GFR, over established markers like serum creatinine [93].

cys-C is a small molecule easily filtered, readily absorbed and degraded in tubular cells; therefore it is not subjected to tubular secretion when compared to creatinine[94,95]. Itis produced at a stable rate with less intra variability. The levels of cys-C are affected by factors other than GFR determinants, like endocrine disorders (thyroid), usage of corticosteroids, age, gender, race, smoking and obesity[96-99]. In addition, cys-C predicts better outcomes when compared to creatinine. According to Shilpak et al, it was noted thatcys-C has an impact on mortality rate in persons whose GFR range was 60 to 90 ml/min per 1.73 m^2 and were included under the category of "preclinical kidney disease" [100],[101].

3.13.2.2 GFR estimation:

Scr and Scys-C are the frequently used endogenous filtration markers for estimation of eGFR. By using these two parameters various GFR equations were

developed using scr, cys-C along with the inclusion of demographic variables like age, sex, body surface area, etc.

The most commonly used equations include:

1. Cockroft Gault (CG) equation
2. Modification of diet in renal disease (MDRD) equation
3. Chronic kidney disease epidemiology collaboration equation-2009 (CKD-EPI-2009)
4. Chronic kidney disease epidemiology collaboration equation combination of creatinine and cystatin-C-2012(CKD-EPI-2012)

1) **Cockroft Gault (CG) equation**: This formula is used toestimate creatinine clearance which was developed about thirty years ago . Since it was developed in white population the equation does not use the demographic variables. Until recently, CG equation was only utilized for drug dosing

   Cockcroft-Gault : (140-age) x weight in kg x 0.85 if female]/ (0.814 x plasma Cr in umol/L) [102]

2) **MDRD equation**: This equation was developed in 1999 by conducting a study which included mostly white and non-diabetic patients who were at CKD stages 3 and 4 respectively. The original equation comprised of 6-variables which was further abbreviated in year 2000 to a four variable equation that included age, gender, race, and creatinine levels.[103]The four variable equations was equally good in comparison to the six variable equations.

   MDRD equation: 186x (plasma cr in umol/Lx0.033312)-1.154xage-0.203x1.210 if blackx0.742 if female [104]

3) **CKD-epidemiology collaboration equation**: The CKD epidemiology collaboration (CKD-EPI) was developed in 2009 and resulted from a study that included 8250 participants and was validated in similar cohort of 3900 subjects. When Compared to the MDRD cohort, the CKD-EPI equation has used higher GFR (68 ml/min per 1.73 m^2 vs 40 ml/min per 1.73 m^2), young age, included diabetics, blacks and kidney transplant recipients [105-107]. The main purpose for the CKD-EPI was to develop an equation that was advantageous to MDRD equation especially in those cases with GFR > 60 ml/min per 1.73 m^2.

   CKD-EPI equation: 141 x min (Cr/k, 1) α x max (Cr/k, 1) -1.209 x 0.993age x 1.018 if female x 1.159 if black; where k= 0.7 for females, 0.9 for males; α= -0.329 for females, -0.411 for males[108].

4) **CKD-EPI cys-C and combined cys-C and creatinine equations**: To rule out the inaccuracy of creatinine estimating equations, Stevens et al, in the year 2008 developed three eGFR equations for cys-C and compared them with mGFR iothalamate and 51-EDTA in 3418 patients. The combination equation that included cys-C with scr resulted in the most accurate GFR estimates. The equation which is a combination of cys-C with scr yielded the most accurate GFR estimates (P30 of 89%)[109]. Segarra et al (2010) reported that performance of cystatin-C based GFR equations was better than the CKD-EPI equation in a study of 3114 hospitalized patients because the synthesis of creatinine is dependent on muscle mass and malnourishment [110].We have used this equation in our study for the calculation of eGFR

eGFR=135×min (SCr/κ, 1) α×max (Scr/κ, 1)-0.601×min (Scys/0.8, 1)-0.375×max (Scys/0.8, 1)-0.711×0.995Age×0.969 (if female)×1.08 (if black) [111]

3.13.2.3 Myeloperoxidase:

Myeloperoxidase (MPO) is considered as a bactericidal agent[112].Chang KSet al in the year 1986explained the essential role of MPO in persistent, non-microbial inflammatory processes such as neurodegenerative diseases and atherosclerosis [113]. It is a glycosylated, extremely basic protein containing high amount of arginine with an isoelectric pH of 10[114].It is composed of two subunits, fixed within a single mRNA molecule. These subunits combine with heme molecules to produce the functionally active enzyme. It is present in azurophilic granules of leukocytes and the enzyme contributes for about 1% of total cell protein content, in the neutrophils and monocytes, respectively[115].MPO is the main component of the oxygen-dependent microbicidal activity of phagocytes, and is specific with respect to neutrophils. MPO is a constituent of the superfamily of mamalian heme peroxidase, which consists of eosinophil peroxidase (EPO) and lactoperoxidase (LPO).[116,117] This enzyme is bactericidal in nature whose primary function is to produce reactive oxygen species (ROS) which contribute in eliminating the engulfed pathogens.

MPO-derived oxidants:

Based on the local mi- lieu, the MPO-H_2O_2 system is able to generate a broad range of oxidants, like HOCl, chloramines, hydroxyl radicals (HO·), singlet oxygen ($O_2^{\cdot}$), and ozone (O_3) [118,119]. The circumstances in which these oxidants are produced and their reaction with numerous substrates have been reviewed recently in detail[120,121]. HOCl is produced as a result of H_2O_2catalyzed oxidation of chloride. At physiologic pH, HOCl exists as a mixture of the acid (HOCl) and the hypochlorite ion

(OCl^-), whereas under conditions of low pH and high amount of chloride, it can react to form chlorine (Cl_2).

Microbicidal effects of MPO:

Routinely MPO is investigated to add essentially to the microbicidal activity of neutrophils and monocytes through the production of reactive oxidants, predominantly HOCl, and corresponding radical species[122].

MPO and renal diseases

The ability of the MPO-H_2O_2 system to cause injury to the kidney directly was originally established by renal perfusion studies in rats [123-125]. Chaudhary K et al in the year 2010 suggested according to his study that MPO helps in production of HOCl in diabetic nephropathy patients[126.] Diabetic nephropathy (DN) is an advancing disease which is a consequence of hyperglycemia. It is described by a decrease in glomerular filtration rate and proteinuria, which in due course of time results in end-stage renal disease. Reactive oxygen species play a pivotal role in diabetic nephropathy, as illustrated by the findings that the supression of ROS production decreases proliferative and fibrotic changes and enhances renal function as evidenced in experimental studies [127].

The role of MPO in diabetic nephropathy has been implied through the production of advanced glycation end products (AGEs). They are the end products of glycation reactions which can bind and there by stimulate the receptor for AGE (RAGE), leading to the release of profibrogenic mediators and pro-inflammatory cytokines. Stimulation of the AGE-RAGE system is accepted to involve in the prognosis of diabetic nephropathy[128].

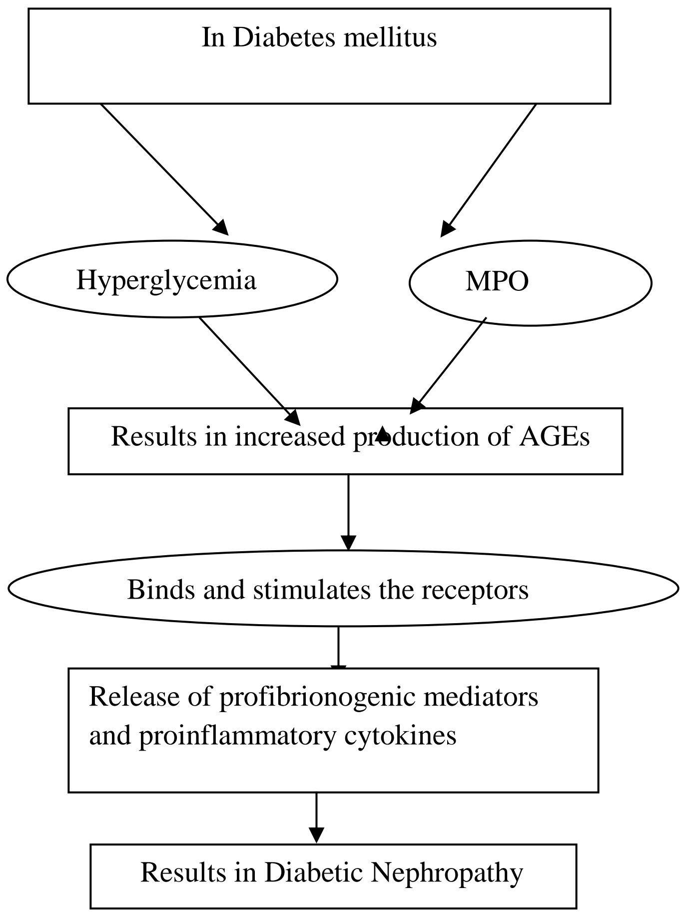

Figure-3.7. Flow chart showing the role of MPO in Diabetic Nephropathy

3.13.2.4 Treatment of CKD:

The main treatment modalities are:

- lifestyle changes – It helps you stay as healthy as possible
- Medicine – to control associated problems, such as high blood pressure and high cholesterol
- Dialysis – treatment to replicate some of the kidney's functions, which may be necessary in advanced (stage 5) CKD
- kidney transplant – this may also be necessary in advanced (stage 5) CKD

More than 60years ago, Addis speculated that the severity of renal disease could be ameliorated by reducing the excretory burden for nitrogen through dietary protein restriction[129].

The Ramipril Efficacy in Nephrology(REIN)study was the first trial to formally test the role of proteinuria in the progression of kidney disease and of proteinuria reduction in renoprotection[130,131,]. Sulodexide, a heterogeneous mixture of sulfated glycosaminoglycans thought to improve glomerular selectivity, was suggested to decrease albuminuria in small studies in diabetic patients[132]:

Estimation of urine microalbumin along with eGFR improves the prognosis of CKD progression. Though albuminuria is a potent biochemical marker, it will appear after the damage has occurred or it may not be detected in other types of renal damage such as tubulointerstitial disease and glomerular kidney disease. The early treatment of patients with CKD would help in reducing the complications of CVD or CKD progression. This has led to the exploration for new biomarkers that are not invasive and that could better associate with the diagnosis of the kidney disease. The inclusion of parameters like serum cystatin-C and myeloperoxidase will help in early diagnosis and delay the complications associated with the disease. The other biomarkers like Beta2-microglobulin, Urinary angiotensinogen, Kidney injury molecule-1, Neutrophil gelatinase-associated lipocalin and Asymmetric dimethyl arginine can also be included.

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Material and Methods

4.1 Study design:

A comparative Cross sectional, hospital based study.

4.2 Study duration:

The study was carried out during January 2015 to September 2016 for a period of two years, during which information was collected over a period of one and half year, collation and analysis was performed over a period of six months.

4.3 Source of data :

A cross sectional study was conducted in the Department of Biochemistry in collaboration with departments of General Medicine and Nephrology at central laboratory of Prathima Institute of Medical Sciences, Karimnagar.

4.4 Sample size : 135-140 cases

4.5 Sample size calculation:

The estimated minimum sample size for this study according to the equation was 45 per group. It was rounded up to 150

The following sample size calculation formula was used

$$n= \frac{(z_{\alpha}+z_{\beta})^2 * 2 * SD^2}{MD^2}$$

Sample size = 45 per group

Anticipated MD of Cystatin C (by table 3 of given article, from stage 4 to normal)= 1.3-0.8= 0.5

Anticipated SD of cystatin-C= 0.7

α=5%

β=10%

Total sample=150.

4.6 Study groups and distribution of participants:

Patients with diabetes mellitus and normal healthy subjects attending the outpatient departments of General Medicine and Nephrology at Prathima Institute of Medical Sciences. The distribution of participants (study groups) who participated in this study is as follows.

Table-4.1Sample size in the studied groups

Group	Subjects	No of participants
Group-I	Normal healthy controls	45
Group-II	Non Diabetics with CKD	45
Group-III	Diabetics with CKD	45

Forty five in each group: 45x3 = 135 which was rounded up to 150

4.7 Sample area:

Patients with Diabetes, Chronic kidney disease and Normal healthy subjects (Visiting the hospital for routine health check up) attending the outpatient departments of General medicine and Nephrology of Prathima Institute of Medical Sciences, Karimnagar.

4.8 Sampling procedure and recruitment:

Systemic random sampling technique is followed**.** The study design and protocol were approved by the Institutional ethics committee. After explaining the purpose of the study and obtaining informed consent, patients attending the Outpatient department for diabetes and CKD follow up and Normal healthy subjects were recruited for the study.

4.9 Inclusion Criteria & Exclusion criteria:

4.9.1 Inclusion Criteria:

Individuals between the age ≥ 40 years, and ≤ 70 years; clinically Confirmed diabetic patients with duration of diabetes > 5 years and <10 years; clinically confirmed patients of CKD.

Age and sex matched healthy controls were recruited for the study.

4.9.2 Exclusion criteria:

Not willing to give consent; Duration of diabetes <5 years and >10 years Patients with hypertension, endocrine disorders, debilitating diseases like tuberculosis, cancer etc., and those on steroid therapy**.**

4.10 Ethical aspects:

- **Informed consent:** All the subjects were explained very clearly about the purpose and outcome of the study in their own local language and a written informed consent was obtained from them.
- **Institutional approval:** An Institutional Ethical committee clearance was obtained from Shri B.M. Patil Medical College, hospital and research centre, BLDE (Deemed to be University),(IEC Ref No: 102/2014-15 dated 15/11/2014) Vijayapura, Karnataka and Prathima Institute of Medical Sciences (IEC/PIMS/2015/01 dated 17/06/2015) Karimnagar, Telangana.

4.11 Collection of blood and urine samples for analysis of biochemical parameters:

4.11.1 Collection of blood samples:

Under aseptic conditions 7 ml of random venous blood sample was collected from each participant, by anti cubital vein puncture. 2 ml was collected in fluoride bulb for the estimation of blood glucose and 2 ml was collected into an EDTA bulb

for the estimation of HbA_1c, the remaining was transferred into a plain bulb for the estimation of other biochemical parameters. After centrifugation, serum was separated and the biochemical parameters were estimated by standard methods.

4.11.2 Collection of urine sample:

A midstream random urine sample of 10 ml was collected in a sterile container for the estimation of Microalbumin and Albumin creatinine ratio.

Random blood glucose, urea, creatinine and cystatin-C were analysed on XL-640 fully automated analyzer. HbA_1c, Urine albumin were estimated on Nycocard reader and Myeloperoxidase was assayed by using Alere AM 2100 ELISA reader.

The following biochemical parameters were estimated in the serum, whole blood and urine samples following the standard methods:

- Random blood glucose
- Blood Urea
- Serum creatinine
- HbA_1c
- Microalbumin
- Serum cystatin-C
- Serum Myeloperoxidase
- Albumin creatinine ratio
- Estimated Glomerular Filtration Rate

4.12 Biochemical analysis:

4.12.1 Estimation of blood glucose[1,2]

Method:

Random blood glucose was estimated by using Glucose Oxidase Peroxidase (GOD-POD) method using commercially available kits of (Erba) Transasia Biomedicals Limited.

Principle:

Glucose is oxidized to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured complex of quinoneimine dye. The intensity of the colour formed is directly proportional to the amount of glucose present in the sample.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2\ H_2O_2 + \text{4- amino antipyrine} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinonemine dye} + 4H_2O$$

Table-4.2 Reagents and reconstitution of glucose kit

Reagents	Concentration
Phosphate buffer (pH 7.40)	100 mmol/l
Phenol	10 mmol/l
Glucose oxidase	>1000 U/L
Peroxidase	>600 U/L
4-Aminoantipyrine	270 mmol/l

Table-4.3 Procedure for the estimation of blood glucose

Reagents	Blank	Standard	Sample
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	-	-
Glucose. Standard (100 mg/ dl)	-	10 µl	-
Sample	-	-	10 µl

After mixing, the standard and sample with the reagent respectively, it was incubated at 37˚C for 10 minutes. The change in absorbance was measured at 505/ 630 nm against reagent blank.

Table-4.4 Programming of Glucose kit

Mode of Reaction	End point
Slope of reaction	Increasing
Wavelength	505 / 630
Temperature	37˚ C
Concentration of standard	100 mg / dl
Blank	Reagent blank
Linearity	500 mg / dl
Incubation Time	10 min
Sample volume	10 µl
Reagent volume	1000 µl
Cuvette	1 cm light path

Calculation:

Concentration of Glucose in (mg/dl) = Absorbance of sample /Absorbance of StandardX100

Reference intervals: Random blood glucose 100-160 mg/dl.

4.12.2 Estimation of blood urea [3, 4]:

Method:

Blood Urea was estimated by Urease Berthelot method by using commercially available kits of (Erba) Transasia Biomedicals Limited.

Principle:

Urease hydrolyses Urea to ammonia and CO_2. The ammonia formed further reacts with a phenolic chromogen and hypochlorite to form a green coloured complex. The intensity of the colour formed is directly proportional to the concentration of urea present in the sample.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

Ammonia + Phenoloic chromogen + Hypochlorite ⟶ Green coloured complex

Table-4.5 Reagents and reconstitution of urea kit

Reagents	Concentration
R1:- Buffer Reagent	120mmol/Lit
R2:- Enzyme Reagent	1g/Lit
R3:- Chromogen Reagent	116mmol/Lit
Urea standard	40 mg/dl

Table-4.6Procedure for the estimation of blood urea

Reagents	Blank	Standard	Sample
R1: Reagent	1000 µl	1000 µl	1000 µl
R2: Reagent	100µl	100µl	100µl
Distilled water	10 µl	-	-
Standard	-	10 µl	-
Sample	-	-	10 µl
After mixing, the standard and sample with the reagent respectively, incubate at 37˚C for 5 minutes and add R3 reagent			
R3: Reagent	200 µl	200 µl	200 µl

Mix well and incubate for 5 minutes at 37˚C. Measure the absorbance of the Standard and Test Sample against the Blank, at 570 nm within 60 min.

Table: 4.7 Programming of blood urea kit

Mode of Reaction	End point assay
Wavelength	570 nm
Zero setting	Reagent blank
Incubation Temperature	37˚ C
Incubation Time	5+5 min
Delay Time	-
Read Time	-
No. of readings	-
Interval	-
Concentration of standard	40 mg / dl
Factor	-
Reaction slope	Increasing
Linearity	250 mg / dl
Sample volume	10 µl
Reagent volume	1.30 ml

Calculation

Concentration of Urea in (mg/dl) = Absorbance of sample / Absorbance of Standard X 40

Reference intervals: 10-40 mg/dl.

4.12.3 Estimation of serum creatinine: [5, 6]

Method:

Serum Creatinine was estimated by Jaffe's Kinetic method by using commercially available kits of (Erba) Transasia Biomedicals Limited.

Principle:

Creatinine reacts with alkaline picrate to produce an orange yellow coloured complex. Specificity of the assay has been improved by the introduction of an initial rate method. The absorbance of orange-yellow colour formed is directly proportional to the concentration of creatinine and is measured photo metrically at 500-520nm.

$$\text{Creatinine + Alkaline picrate} \longrightarrow \text{Orange Coloured Complex}$$

Table-4.8 Reagents and reconstitution of creatinine kit

Reagents	Concentration
R1:-Picric acid	25.8 mmol/Lit
R2:-Sodium hydroxide	95 mmol/Lit
Creatinine standard	2 mg/dl

Table-4.9 Procedure for the estimation of serum creatinine

Reagents	Blank	Standard	Sample
R1: Reagent	500 µl	500 µl	500 µl
R2: Reagent	500 µl	500 µl	500 µl
Distilled water	100 µl	-	-
Standard	-	100 µl	-
Sample	-	-	100 µl

Standard concentration: 2 mg/dl

Mix well and read the initial absorbance A_1, for the Standard and Test after exactly 20 seconds. Read the final absorbance A_2, of the Standard and Test exactly after 80 seconds after mixing. Calculate the change in absorbance ΔA for both the Standard and Test.

Table-4.10 Programming of serum creatinine kit

Mode	Fixed time
Wavelength	505 nm
Sample volume	50/100µl
Reagent volume	500/1000 µl
Lag time	20 sec
Kinetic Interval	60 sec
No. of Readings	1
Reaction temperature	37˚ C
Reaction Direction	Increasing
Normal low	0.6 mg/dl
Normal high	1.4 mg/dl
Linearity low	0 mg/dl
Linearity high	20 mg/dl
Absorbance limit	0.3
Blank	Distilled water
Standard	2 mg/dl

The calculation

Concentration of serum creatinine in (mg/dl) = Δ Abs of Test / Δ Abs of Standard X 2.0

Reference interval: 0.6-1.2 mg/dl

4.12.4 Estimation of HbA_1c[7]:

Method:

HbA_1c is estimated by Nycocard Boronate affinity assay using the test devices based on the technique of immune chromatography.

Principle:

NycoCard HbA_1c is based on boronate affinity assay. The kit has test devices with a porous membrane filter, test tubes prefilled with reagent and wash solution. The reagent contains agents that lyse erythrocytes and precipitate haemoglobin specifically and a blue boronic acid conjugate that binds cis-diols of glycated hemoglobin. When blood is added to the reagent, the RBC lysis occur immediately and haemoglobin is precipitated. Then binding of boronic acid conjugate occurs with the cis-diol configuration of glycated haemoglobin. The aliquot of reaction mixture is added to the test device, and all the precipitated haemoglobin, conjugate-bound and unbound remains on top of the filter. Any excess of coloured conjugate present is removed after washing. The evaluation is done by measuring the blue (glycated haemoglobin) and red (total haemoglobin) colour intensity with the NycoCard reader II, the ratio between them is proportional to the percentage of HbA_1c in the sample.

Reagents:

R1: Glycinamide buffer containing dye-bound boronic acid and detergents.

R2: Wash solution**:** Morpholine buffered NaCl solution and detergents.

Procedure:

- Add 5 µl of whole blood to R_1 reagent. Mix well and incubate at 37°C for 2-3 minutes.
- Mix again and apply 25 µl reaction mixture to the test device, allow it to soak.
- Add 25 µl of R_2/ wash solution and allow it to soak.

- Record the result within 5 minutes using NycoCard Reader.

Reference values:

4-5.6% Normal range

5.7 – 6.5% High risk of developing DM

>6.5% Diabetes mellitus

4.12.5 Estimation of microalbumin[8]:

Method:

Microalbumin is estimated by NycoCard, Boron affinity assay by using the test devices based on the principle of immunochromatographic technique.

Principle:

Urine microalbumin is estimated by solid phase sandwich format, immunometric assay. The test device contains a membrane coated with albumin specific monoclonal antibodies. Then the diluted sample is added to the test device, the sample flows through the membrane and immobilized antibodies on the membrane will capture the albumin molecules.

Reagents:

R1: Dilution liquid

R2: Conjugate

R3: Wash solution

Procedure:

- Add 50 µl urine to the R1/ Dilution liquid and mix well.
- Apply 50 µl diluted urine to the Test Device.
- Apply 50 µl R2/ Conjugate to the test device.
- Apply 50 µl R3/ wash solution to the test device.
- Record the test result within 5 minutes using NycoCard Reader.

Reference range: 0-25mg/L

4.12.6 Estimation of serum cystatin-C[9, 10]:

Method:

cystatin-C is estimated by Latex enhanced ImmunoTurbidimetric Assay by using the commercially available kits from Accurex Biomedicals Limited.

Principle:

cystatin-C assay is based on immunoturbidimetric principle. cystatin-C from the sample reacts with anti-cystatin C antibody and results in immunoparticle aggregation. cystatin-C concentration is determined by measurement of change in absorbance that results from this aggregation.

cystatin-C + Anti-cystatin C antibody ⟶ Immuno complex

Reagents:

R1: MOPS [3-(N- Morpholino)-Propanesulfonic acid] buffered saline and Sodium azide

R2: Polysterene particles coated with Anti-Cystatin C antibody andSodium azide

Table-4.11 Reagents and procedure for serum cystatin-C

Reagents	Test
R1: Reagent	450 µl
Sample	6 µl
Mix well and incubated at 37˚ C for 5 minutes	
R2: Reagent	90 µl

Mix well and read the Test within 60 seconds.

Table-4.12 Programming of cystatin-C

Mode	End point
Wavelength	700/546 nm
Sample volume	6 µl
Reagent volume	540 µl
Linearity	6.5 mg/dl

Reference range: 0.51-1.05 mg/L

4.12.7 Estimation of Myeloperoxidase[11]:

Method:

Myeloperoxidase is estimated by standard protocol by ELISA technique using AssayMax Human Myeloperoxidase kit.

Principle:

The kit is designed for detecting the presence of myeloperoxidase in specimens like human plasma, serum, urine, saliva, milk, CSF, and cell culture samples. This assay involves a quantitative sandwich enzyme immunoassay technique that measures myeloperoxidase in a period of about 4 hours. A polyclonal antibody specific for myeloperoxidase has been pre-coated onto a 96-well micro plate with disposable strips. Myeloperoxidase in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for myeloperoxidase, which is identified by a streptavidin-peroxidase conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The colour development is stopped and the intensity of the colour developed is measured.

All the reagents and microtiter strip plate were supplied as kit components along with MPO ELISA kit. Out of these, few components were ready to use and few reagents require prior reconstitution before use. The reagents were reconstituted as per the instructions of user manual provided along with the kit. All the kit components and reconstitution procedure is described in the following table.

Table-4.13 MPO kit components and reconstitution

Components	Reconstitution
96 wells microtitre plate	Ready to use (pre-coated).
MPO standard	Reconstitution and preparation of serial standards were described below.
Sealing tapes	Sized cut sealing tapes are to be cut according to the requirement.
Biotinylated Human Myeloperoxidase antibody (50X)	Spin down the biotinylated antibody and dilute the desired amount of the antibody in the ratio of 1:50
Mix diluents concentrate (10X)	15 ml made up to 75 ml with deionized water to get working assay mix diluent.
Wash Buffer concentrate (20 X)	25 ml made up to 500 ml with deionized water to get working wash buffer..
Streptavidin peroxidase concentrate	Spin down the SP conjugate and dilute the desired amount of the conjugate in the ratio of 1:100 with mix diluent.
Chromogen substrate solution	Ready to use.
Stop solution (0.5N Hcl)	Ready to use.

Preparation of standards:

600 µl of mix diluent was added to a vial containing powdered form of human MPO (2.4 ng) standard to get stock standard (4 ng/ml). From this, series of standards were prepared as mentioned in the following table.

Table 4.14 Preparation of MPO standards by serial dilution

Standards	Prepared by adding	Concentration of standards
S1	1 part of standard (4 ng/ml)	4 ng/ml
S2	1 part P1 + 1 part of mix diluent	2 ng/ml
S3	1 part P2 + 1 part of mix diluent	1 ng/ml
S4	1 part P3 + 1 part of mix diluents	0.5ng/ml
S5	1 part P4 + 1 part of mix diluent	0.25 ng/ml
S6	1 part P5 + 1 part of mix diluent	0.125 ng/ml
S7	1 part P6 + 1 part of mix diluent	0.063 ng/ml
S8	Mix diluent	0 ng/ml

Assay procedure:

- Required number of strips were selected from microtitre plate.
- 50 µl of standard and sample were added to appropriate wells.
- Wells were covered and incubated for two hours at room temperature.
- Wash the plate five times using 200 µl of wash buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If auto washer is used, wash six times with 300 µl of wash buffer and then invert the plate, decanting the contents;hit 4-5 times on absorbent material to completely remove the liquid.
- 50 µl of prepared biotinylated antibody was added to each well and incubated for 30 minutes at room temperature.
- Wash the plate five times with 200 µl of wash buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If autowasher is used, wash six times with 300

µl of wash buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.

- 50 µl of prepared streptavidin conjugate solution was added to each well and incubated for thirty minutes at room temperature.
- Wash the plate five times with wash buffer manually.
- 50 µl of chromogen substrate was added to each well and incubated for 12 minutes at room temperature.
- After adding 50 µl of stop solution optical density (OD) was recorded immediately at 450 nanometer (nm) using ELISA reader.
- From the OD values standard graph was obtained and the concentration of serum MPO was calculated from the standard graph.
- **Reference range :** 20-120 ng/ml

4.12.8 Calculation of albumin creatinine ratio[12]:

It is ratio of urinary albumin to urinary creatinine; usually it is expressed as milligram of albumin excreted per gram of urinary creatinine.

$$\text{ACR} = \frac{\text{Urinary albumin in mg/dl}}{\text{Urinary creatinine mg/dl}} \times 1000$$

4.12.9 Estimation of glomerular filtration rate (eGFR)[13]:

From the creatinine and cystatin-C values obtained eGFR was calculated using CKD-EPI Creatinine Cystatin C Equation [2012].

eGFR=135×min (SCr/κ,1) α×max (Scr/κ,1)-0.601×min (Scys/0.8,1)-0.375×max (Scys/0.8,1)-0.711×0.995Age×0.969 (if female)×1.08 (if black)

- **Reference range :** 90-120 ml/min

4.13 Statistical analysis:

- Data was analyzed using SPSS statistical package version 20.0 software
- All characteristics were summarized descriptively using the summary statistics of mean and standard deviation(SD)
- The analysis was performed by one way ANOVA to observe the significant difference between groups.
- Correlation analysis was done using Karl Pearson's correlation coefficient.
- Statistical significance was considered at the level of 5% ('p'- value < 0.05)

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Results

The results of biochemical analysis of the participants are discussed in this chapter. Data of all the participated subjects of the study have been presented in the form of figures and tables. Total 150 subjects participated in the present study. The subjects were divided into three groups as follows:

1. Normal Healthy controls – 50
2. CKD without Diabetes (CKD-ND)–50
3. CKD with Diabetes (CKD-DM)–50

Table: 5.1 Distribution of Subjects Based on Age

Age group	Control N=50	CKD without diabetes (CKD-ND)N=50	CKD with diabetes (CKD-DM)N=50
35-45 years	16 (32 %)	12 (24 %)	10 (20 %)
46-55 years	23 (46%)	17 (34 %)	18 (36 %)
56-65 years	09 (18 %)	13 (26 %)	15 (30 %)

Table -5.1 illustrates the distribution of subjects based on age groups. In this study we found that number of subjects with CKD were higher between the age group of 46-55 years showing (34 %) in CKD without Diabetes and (36 %) in CKD with Diabetes groups respectively.

Table: 5.2 Gender distribution among study groups

Gender	**CKD without diabetes (CKD-ND)**	**CKD with diabetes (CKD-DM)**
Male	33 (33%)	34 (34%)
Female	17 (17%)	16 (16%)

Table-5.2 illustrates the distribution of subjects based on gender among the study groups. The subjects with chronic kidney disease were more in males 67% compared to females 33% .

Table-5.3 ANOVA of the biochemical parameters among the test groups followed by post hoc t test

Parameters	MEAN±SD			p value
	CONTROL (N=50)	CKD-ND (N=50)	CKD-DM (N=50)	
Random Blood Glucose mg/dl	97.54±16.03	101.44±19.21	259.74a,b±80.5	<0.001*
Blood Urea mg/dl	22.9±4.45	84.86a±6.76	81.08a±7.79	<0.001*
Creatinine mg/dl	0.85±0.18	3.92a±1.13	2.86a,b±2.77	<0.001*
HbA1c %	5.09±0.46	5.34±1.49	7.69a,b±1.17	0.041*
eGFR ml/min	102.6±5.52	64.1a±3.69	82.1a,b±4.97	0.004*
Microalbumin mg/L	17.11±8.5	71.94a±8.54	138.78a,b±9.57	<0.001*
cystatin-C mg/L	0.74±0.46	2.62a±1.09	2.21a±1.36	<0.001*
ACR	0.38±0.36	1.82a±0.59	2.11a±0.71	<0.001*
MPO ng/ml	52.95±12.37	9.39a±5.81	7.59a±3.71	<0.001*

Note: * significant among all groups, a is significant with control, b is significant with CKD-ND at 5% level of significance ($p<0.05$)

(CKD-ND Chronic kidney disease without diabetes, CKD-DM Chronic kidney disease with diabetes, ACR –Albumin Creatinine ratio, MPO- Myeloperoxidase ,eGFR-estimated glomerular filtration rate.)

In Analysis of variance all the parameters have shown significant difference among control, CKD-ND and CKD-DM groups. When the parameters of CKD-ND and CKD-DM were compared with controls all the parameters have shown a significant difference. When the parameters of CKD-DM were compared with CKD-ND only Glucose, creatinine, HbA_1c and microalbumin have shown a noted significance. There is no statistically significant difference with respect to blood urea, eGFR, cystatin-C, ACR and Myeloperoxidase between CKD-ND and CKD-DM groups.

The values of mean glucose were found to be significantly different among all the study groups ($p<0.001$*).The mean glucose value in CKD-DM group was (259.74±80.5) which is found to be significantly different from control and CKD-ND group. The mean values of blood urea were found to be significantly different among all the study groups ($p<0.001$*) The mean urea value in CKD-ND was (84.86±6.76) and (81.08±7.79) in CKD-DM groups respectively which was also found significantly different from control .The values of mean creatinine were found to be significantly different among all the study groups ($p<0.001$*) The mean creatinine values in CKD-ND group and CKD-DM group were (3.92±1.13) and (2.86±2.77) respectively which was also found significantly different from control group.

The values of mean HbA_1c levels were found to be significantly different among all the study groups ($p<0.04$*).The mean HbA_1c value in CKD-DM group was (7.69±1.17) which is found to be significantly different from control and CKD-ND group. The mean values of eGFR were found to be significantly different among all the study groups ($p<0.004$*).The mean eGFR value in CKD-ND was (64.1±3.69) and (82.1±4.97) in CKD-DM groups respectively which was also found significantly different from control.

The mean values of cystatin-C were found to be significantly different among all the study groups ($p<0.001$*).The mean cystatin-C in CKD-ND was (2.62±1.09) and (2.21±1.36) in CKD-DM groups respectively which was also found significantly different from control. The mean values of microalbumin were found to be significantly different among all the study groups ($p<0.001$*).The mean in CKD-DM was (138.78±9.57) and (71.94±8.54) in CKD-ND groups respectively which was also found significantly different from control .

The mean values of ACR were found to be significantly different among all the study groups ($p<0.001$*).The mean ACR levels in CKD-DM was (2.11±0.71) and (1.82±0.59) in CKD-ND groups respectively which was also found significantly different from control .The values of mean values of MPO were found to be significantly different among all the study groups ($p<0.001$*).The mean MPO value in CKD-DM group was (7.59±3.71) and (9.39±5.81) in CKD-ND group which is found to be significant. Our study demonstrates that there is a gradual decrease in mean serum MPO levels with progression of renal disease ($p<0.001$) as indicated in table-5.3. However, our results have proved that serum MPO levels are significantly lower in CKD patients with diabetes mellitus in comparison to CKD patients without diabetes mellitus.

Correlation of Biochemical parameters:

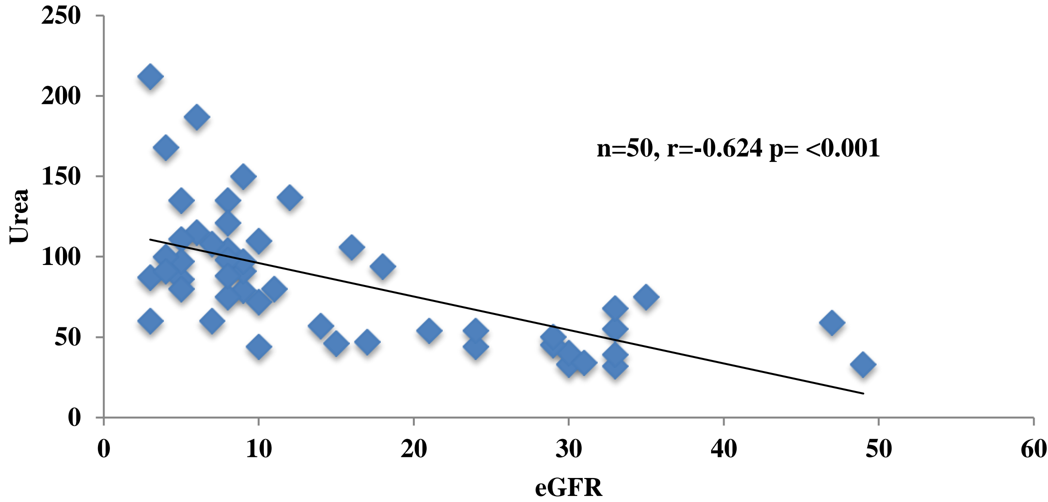


Figure: 5.1 Correlation of Urea and eGFR in non-diabetics with CKD

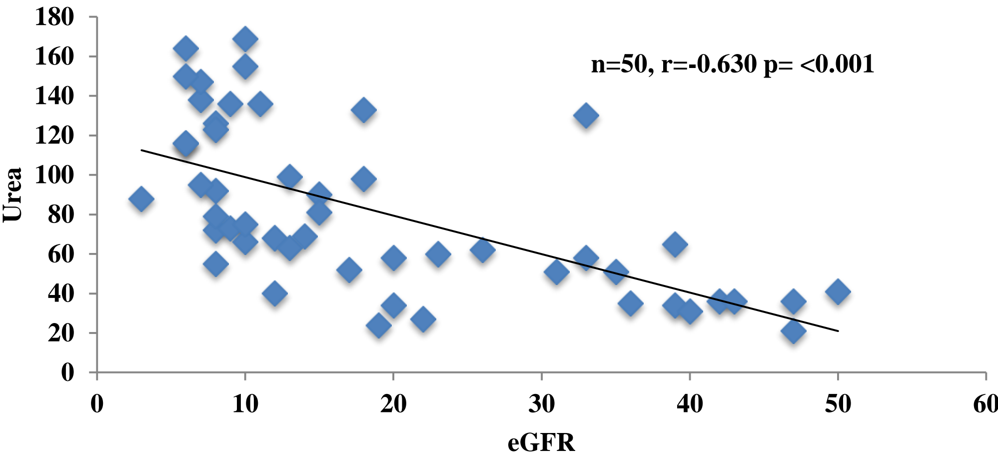


Figure: 5.2 Correlation of Urea and eGFR in diabetics with CKD

In our study we have estimated blood urea and calculated eGFR based on values of creatinine and cystatin-C. We evaluated the correlation among urea and eGFR. Results have shown a negative correlation in CKD-DM ($r = -0.630, p<0.001$) and CKD-ND groups ($r = -0.624$, $p<<0.001$*) as shown in Figure 5.1and 5.2 .

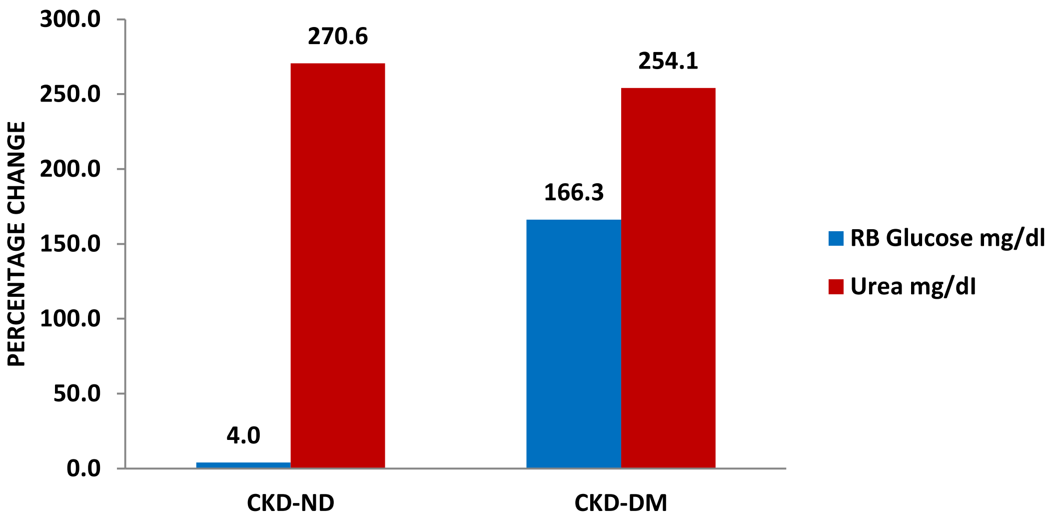
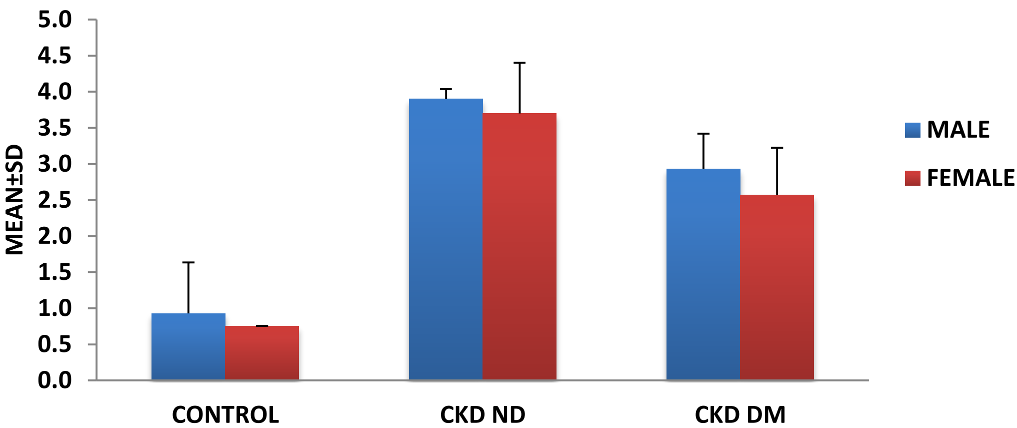


Figure: 5.3 Percentage change of Random blood glucose and Urea among the study groups compared with controls

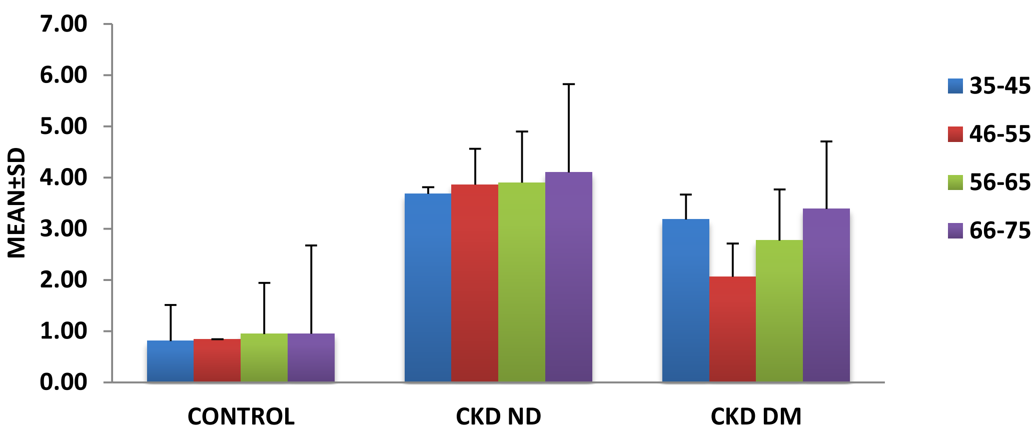
Figure-5.3 shows that there was an increase of 166.3% f Random blood glucose levels in CKD-DM group when compared to healthy controls. It was also noted that there was an increase of 4% in CKD-ND group as compared to control. There was also an increase of 270.6% of blood urea levels in CKD-ND group and an increase of 254.1 % in CKD-DM group when compared with control.



ANOVA p value< 0.001 among males and females is considered as significant

Figure: 5.4 Comparison of mean creatinine based on gender among the study groups

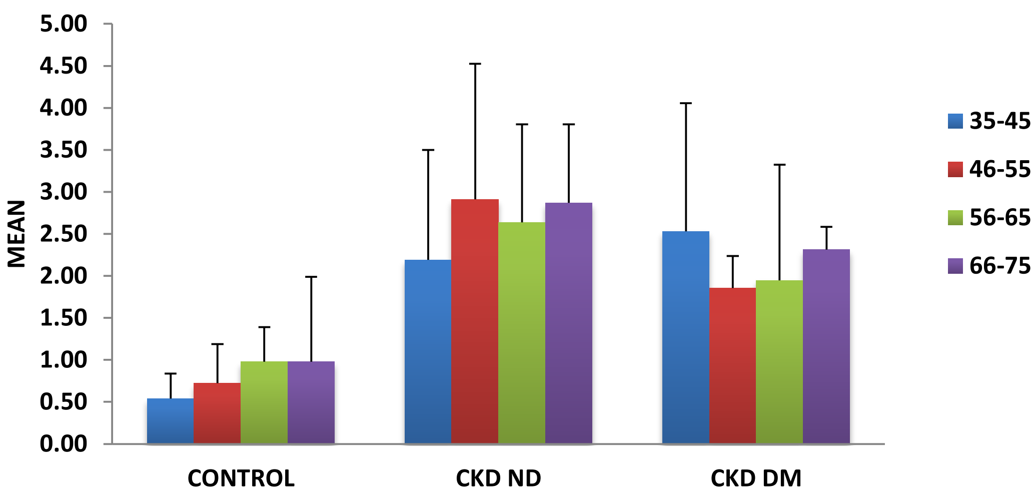
The creatinine levels were significantly increased in CKD-ND group when compared to CKD-DM and control groups as indicated in Figure-5.4. The increment was more in males in all the three groups. It was significant among males and females compared to control ($p<0.001$).



ANOVA p value among 35-45 years age < 0.001(significant), among 46-55 years age < 0.001(significant), among 56-65 years age < 0.001(significant), among 66-75 years age < 0.001(significant)

Figure: 5.5 Comparison of mean creatinine based on age among study groups

Figure -5.5 illustrates that the serum creatinine levels were higher in the age group of 66-75 in both the test groups as compared to controls. The value of mean creatinine obtained from our data in CKD-ND group in the age group 66-75 years was (4.10±1.7) and it was (3.39±1.2) in CKD-DM respectively.The comparison of the test groups in various age groups with control show that it is statistically significant (p<0.001).



ANOVA p value among 35-45 years age < 0.001(significant), among 46-55 years age < 0.001(significant), among 56-65 years age < 0.001(significant), among 66-75 years age < 0.001(significant)

Figure: 5.6 Comparison of mean cystatin-C based on age among study groups

Figure-5.6 shows that cystatin-C levels were higher in the age group of 46-55 years belonging to CKD-ND group. The mean cystatin-C concentration derived from our data was (0.72±0.46) in healthy subjects and (2.91±1.4) in CKD-ND patients and it was higher in the age group of 35-45 years (2.53±1.5) in CKD-DM patients respectively.

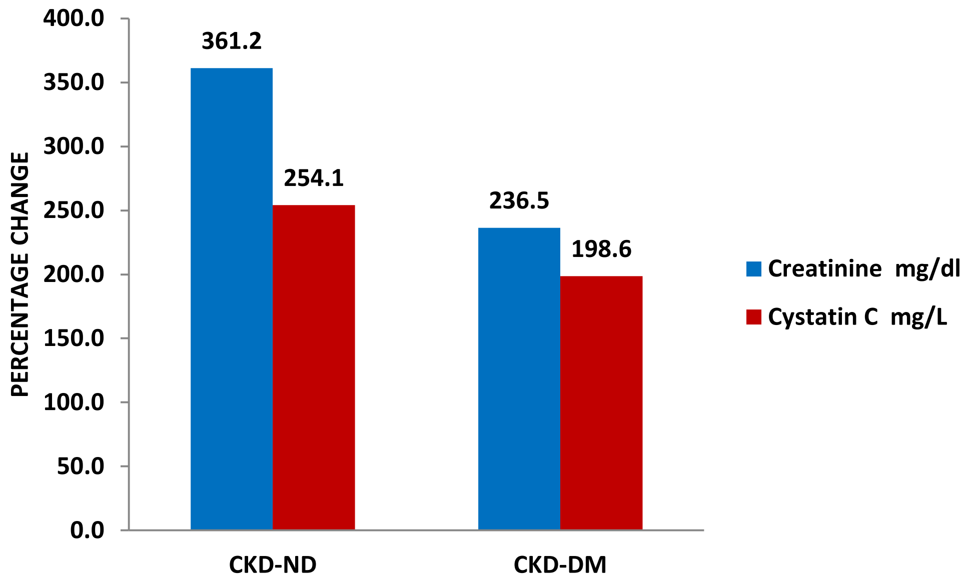


Figure: 5.7 Percentage change of creatinine and cystatin-C among the study groups compared with controls

There was an increase in percentage of serum creatinine levels (361.2%) in CKD ND group compared to control. It was also noted that there was an increase of (236.5%) in CKD DM group as compared to controls indicated in figure-5.7. There was a notable increase in serum cystatin-C levels (254.1%) in CKD-ND group as compared to controls and there was an increase of (198.6%) in CKD-DM group as compared to controls.

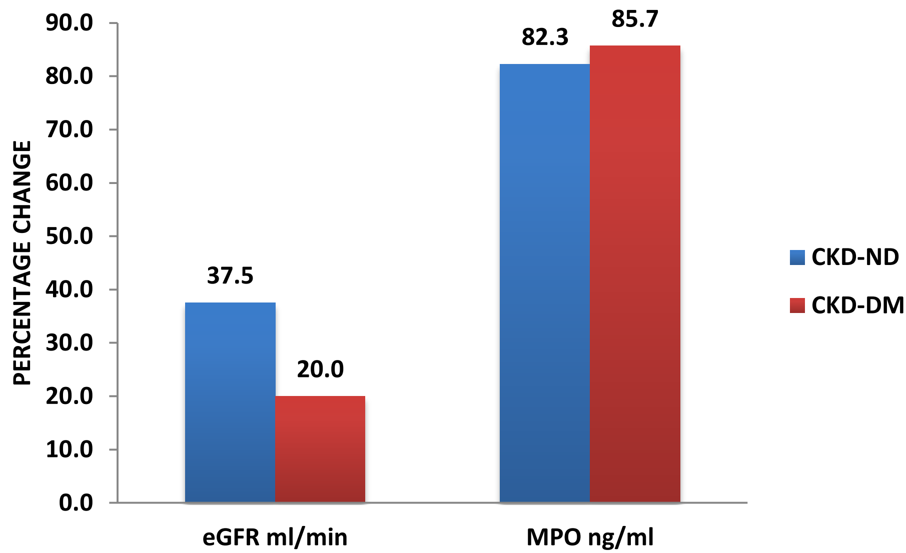


Figure: 5.8 Percentage change of MPO and eGFR among the study groups compared with controls

Figure-5.8 illustrates that there was a percentage decrease of MPO levels in CKD-DM cases was i.e (85.7%) as compared to controls. It was also observed that percentage decrease of MPO levels was (82.3 %) in CKD-ND group as compared to controls. The eGFR levels were decreased in both the groups. There was a percentage decrease of eGFR levels in CKD-ND cases i.e (37.5 %) and it was (20%) in CKD-DM group.

Table:5.4 Receiver operating curve (ROC) analysis of creatinine and cystatin-C with eGFR

Parameters	Area Under the Curve	p value	cut off sensitivity
Creatinine	0.263	0.519	99.9
Cystatin C	0.211	0.404	98.7

Figure: 5.9 Receiver operating curve (ROC) of Creatinine and cystatin-C with eGFR

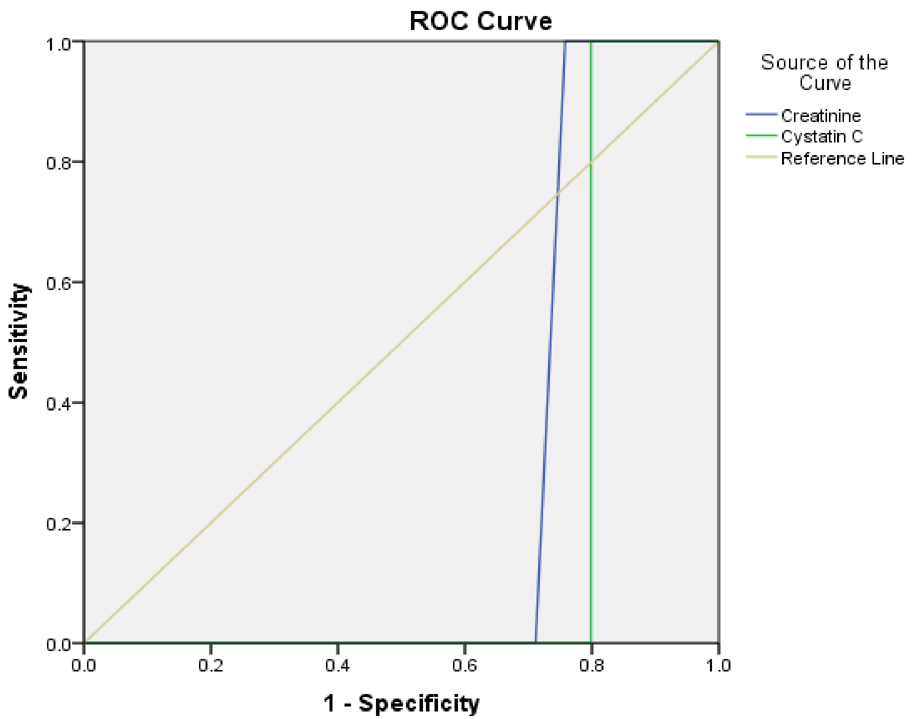


Table-5.4 and figure-5.9 demonstrates that the Area under the curve (AUC) of creatinine is 0.263 with a p value of 0.519 at cut off sensitivity of 99.9 %. Whereas the AUC for cystatin-C is 0.211 with a p value of 0.404 at cut off sensitivity of 98.7%.

Table- 5.5 Relative efficacy of eGFR test equations in detecting CKD

Formula	Group	Sensitivity	Specificity	PPV	NPV	Accuracy
CKD-EPI Creatinine equation (2009)	Control Group	0.0%	98.0%	0.0%	100%	98.0%
	CKD-ND Group	100.0%	0.0%	98.0%	0.0%	98.0%
	CKD-DM Group	100.0%	0.0%	92.0%	0.0%	92.0%
CKD-EPI Creatinine Cystatin-C equation (2012)	Control Group	0.0%	94.0%	0.0%	100%	94.0%
	CKD-ND Group	98.0%	0.0%	98.0%	0.0%	96.0%
	CKD-DM Group	97.8%	25.0%	93.8%	50.0%	92.0%
CKD-EPI Cystatin-C equation (2012)	Control Group	0.0%	88.0%	0.0%	100%	88.0%
	CKD-ND Group	93.9%	0.0%	97.9%	0.0%	92.0%
	CKD-DM Group	91.3%	25.0%	93.3%	20.0%	86.0%

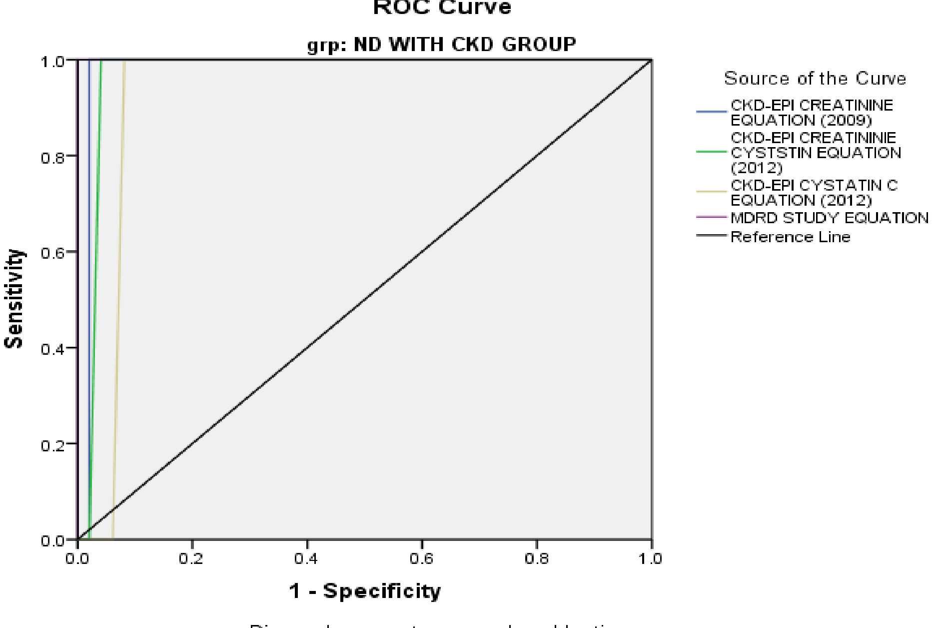
Table-5.5 shows the relative efficacy of eGFR test equations in detecting CKD. Several equations have now been developed to estimate GFR on the basis of cystatin-C, and creatinine including CKD-EPI cystatin-C equation and CKD-EPI creatinine and cystatin- C equation . Table-5.5 also specifies that CKD-EPI creatinine equation (2009) and CKD-EPI creatinine- cystatin-C equation (2012) are more accurate when compared to CKD-EPI cystatin-C equation (2012) CKD EPI creatinine cystatin-C equation (2012) is showing 98% sensitivity and 96% accuracy. We have used this equation in our study for calculation of eGFR .

Table-5.6: Receiver operating curve analysis of test equations among study groups

GROUP	Test Result Variable	Area	Std. Error	p value
CKD-ND Group	CKD-EPI Creatinine equation (2009)	0.98	0.02	0.103
	CKD-EPI Creatinine cystatin C equation (2012)	0.969	0.025	0.111
	CKD-EPI Cystatin C equation (2012)	0.929	0.037	0.146
CKD-DM Group	CKD-EPI Creatinine equation (2009)	0.989	0.014	0.001*
	CKD-EPI Creatinine Cystatin equation (2012)	0.891	0.05	0.01*
	CKD-EPI Cystatin C Equation (2012)	0.745	0.099	0.108

Table-5.6 illustrates ROC Analysis of test equations. The ROC analysis of CKD-EPI creatinine equation (2009) in CKD-ND group shows an AUC value of 0.98 and a p value of 0.103 which is not significant. The CKD-EPI creatinine equation (2009) in CKD-DM group shows an AUC value of 0.989 with a p value of 0.001 which is statistically significant.

Figure: 5.10 Receiver operating curve of Test Equations for CKD-ND Group



As indicated in Figure-5.10 CKD-EPI creatinine cystatin-C equation (2012) in CKD-ND Group, the AUC is 0.969 with a p value 0.11 which is not statistically significant.

Figure: 5.11: Receiver operating curve of test equations for CKD-DM group

Figure-5.11 shows the ROC analysis of test equations it reveals that CKD-EPI creatinine cystatin-C equation (2012) in CKD-DM Group, the AUC is 0.891 with a p value 0.01 which is also statistically significant.

Table:5.7 Sensitivity and specificity

Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
eGFR ml/min	100%	94%	97%	100%	98%
creatinine mg/dl	98%	100%	100%	96%	99%
cystatin-C mg/L	89%	88%	94%	80%	89%

Table-5.7 shows the sensitivity and specificity of eGFR, creatinine and cystatin-C. Sensitivity, specificity and accuracy of cystatin-C is not higher than creatinine and eGFR but it is equally good and can be routinely practiced, since it is not affected by extra renal factors like age, muscle mass and dietary habits .

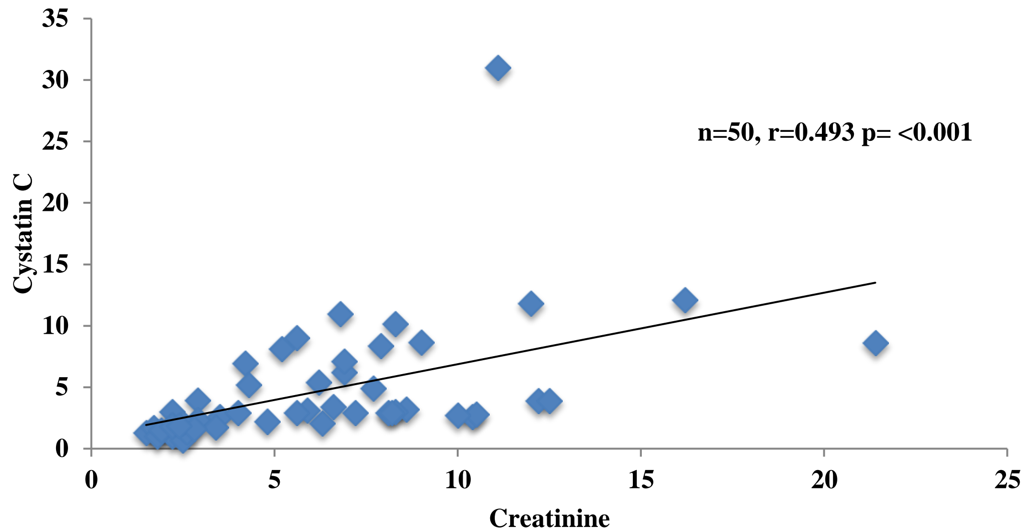


Figure:5. 12 Correlation between cystatin-C and creatinine in non-diabetics with CKD

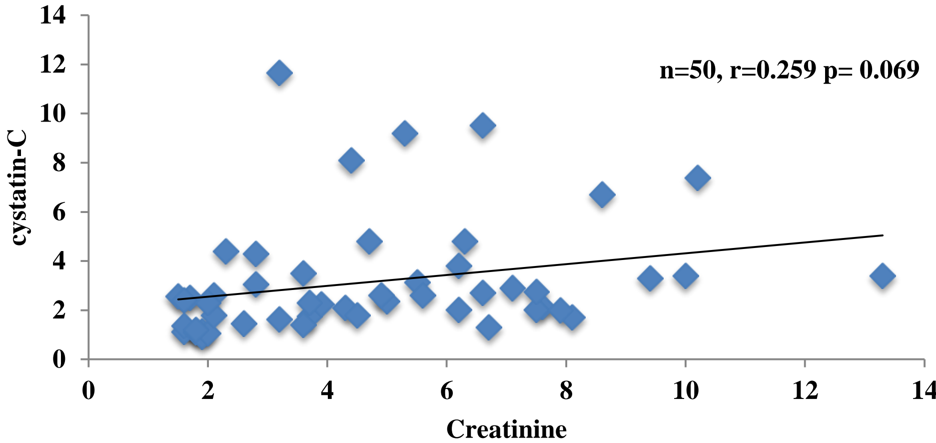


Figure:5. 13 Correlation between cystatin-C and creatinine in diabetics with CKD

In the present study we have evaluated correlation among cystatin-C and creatinine .we applied Pearson's correlation coefficient analysis to evaluate the association between cystatin-C and creatinine in CKD-ND and CKD-DM groups .Figure 5.12 and 5.13 shows that there is a positive correlation between the serum creatinine and cystatin-C levels which is statistically significant ($r=0.493$, $p<0.001$).

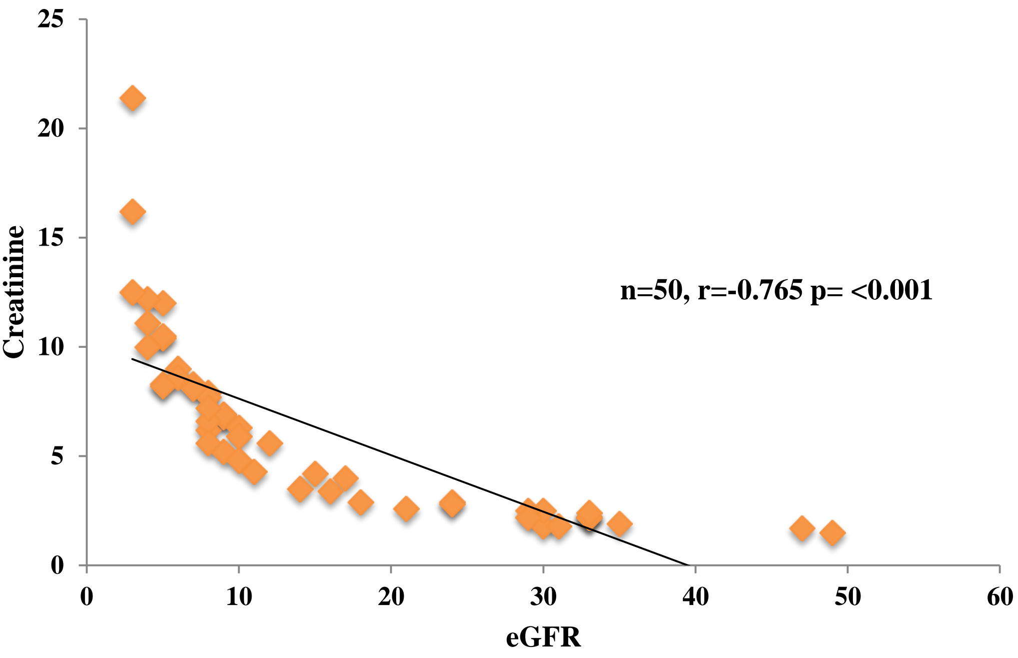


Figure:5.14 Correlation between creatinine and eGFR in non-diabetics with CKD

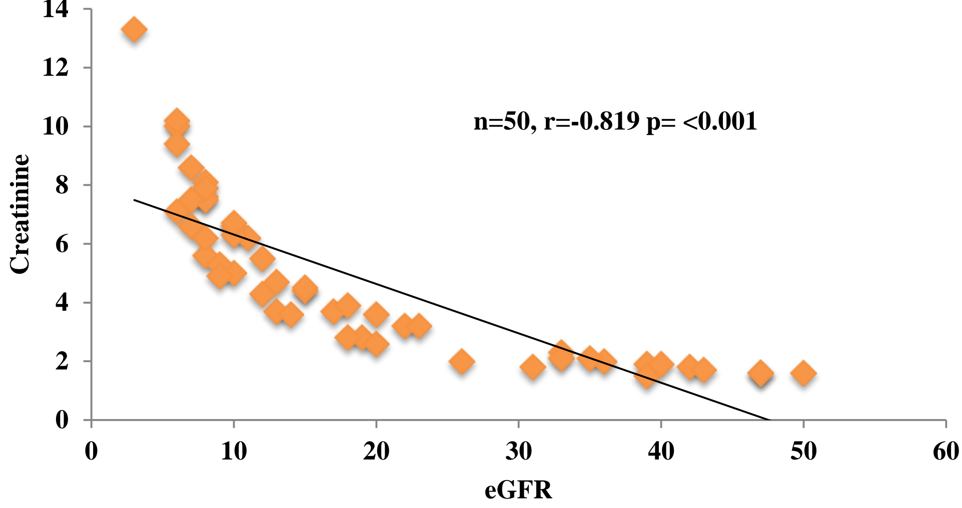


Figure:5. 15 Correlation between creatinine and eGFR in diabetics with CKD

Figure 5.14 and 5.15 shows that there is a negative correlation between creatinine and eGFR in both the test groups. In CKD-ND (r = -0.765,p<0.001) when compared with CKD-DM. (r= -0.819,p<<0.001*) .

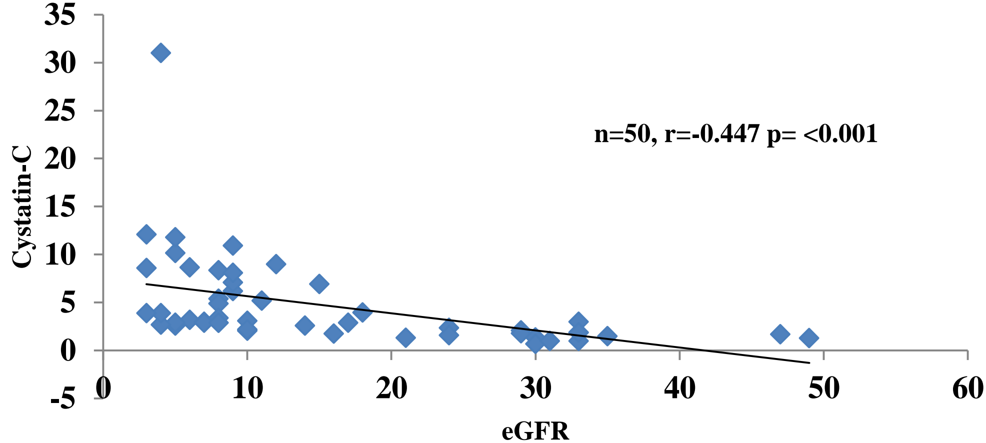


Figure:5.16 Correlation between cystatin c and eGFR in non-diabetics with CKD

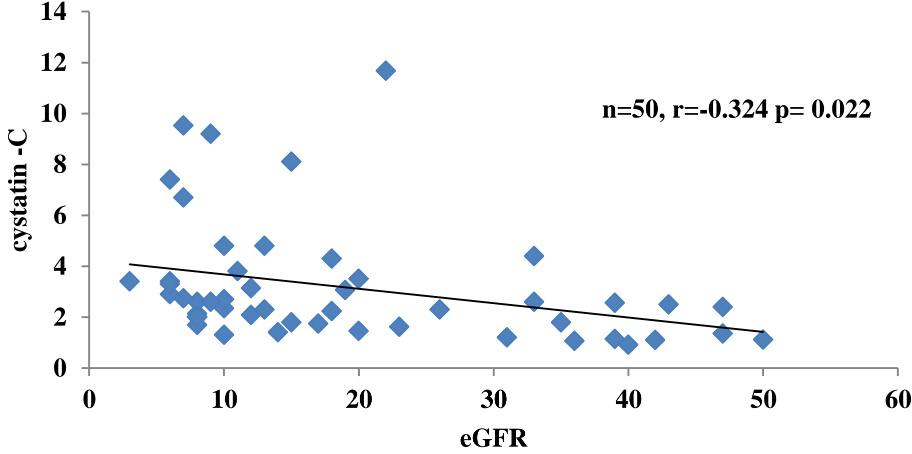


Figure: 5. 17 Correlation between cystatin c and eGFR in Diabetics with CKD

Based on figures 5.16 and 5.17 we found that concentration of serum cystatin-C has negative correlation with eGFR (r= -0.447, p<0.001), which indicates that there is a possibility of renal damage in non-diabetic subjects. The correlation between eGFR and serum cystatin-C was found to be statistically significant among non-diabetic patients with CKD and diabetic patients with CKD as compared to controls. The association between cystatin-C and eGFR was stronger among kidney disease patients than in healthy controls.

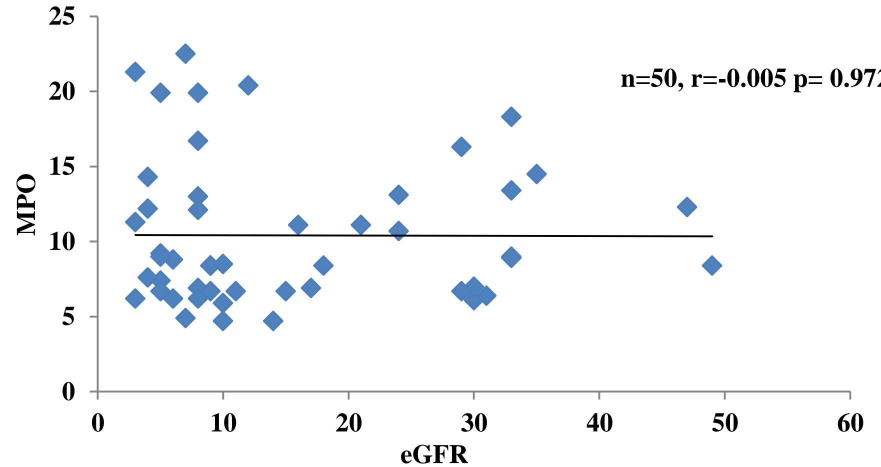


Figure 5.18 Correlation between myeloperoxidase and eGFR nondiabetics with CKD

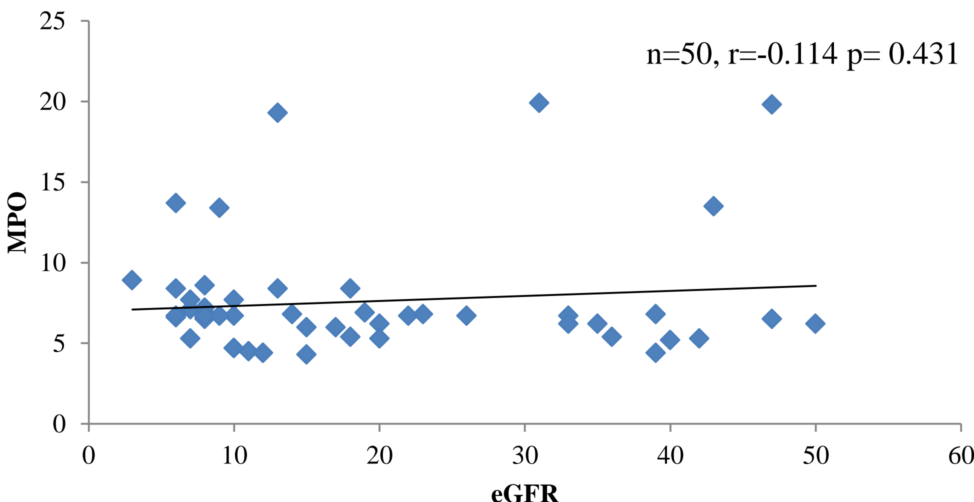


Figure: 5.19 Correlation between myeloperoxidase and eGFR in diabetics with CKD

Figure 5.18 and 5.19 shows that there was a positive correlation between Myeloperoxidase and eGFR in diabetics with CKD when compared non diabetics with CKD (r= 0.114, p=0.431).

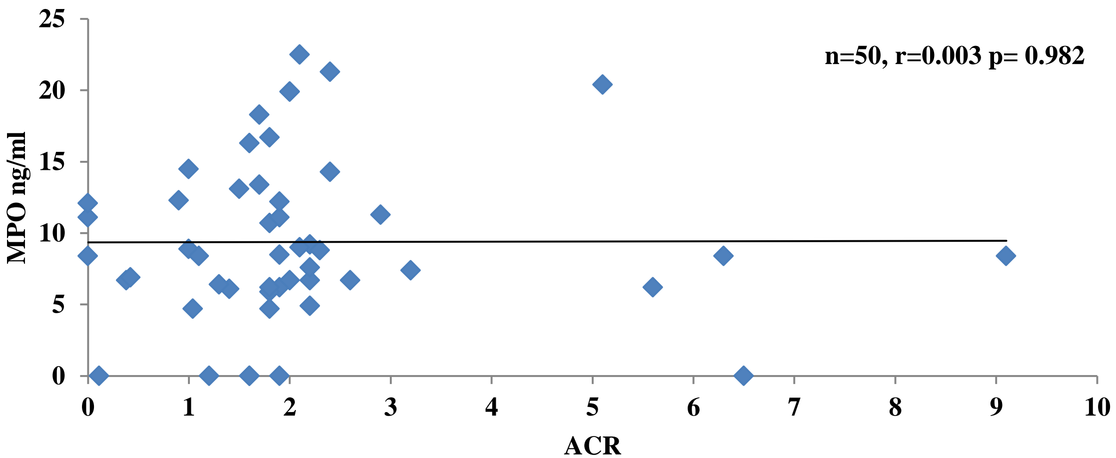


Figure:5. 20 Correlation between MPO and ACR in non diabetics with CKD

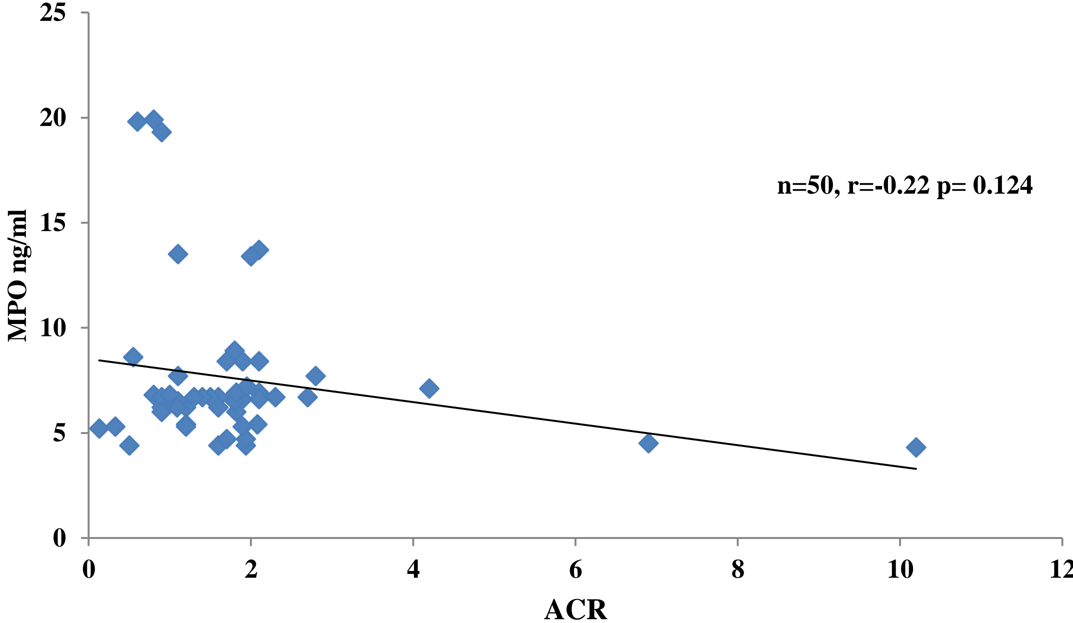


Figure:5. 21 Correlation between MPO and ACR in diabetics with CKD

Figure 5.20 and 5.21 illustrates that a negative correlation was observed between MPO and ACR in diabetics with CKD (r= -0.22, p= 0.124).

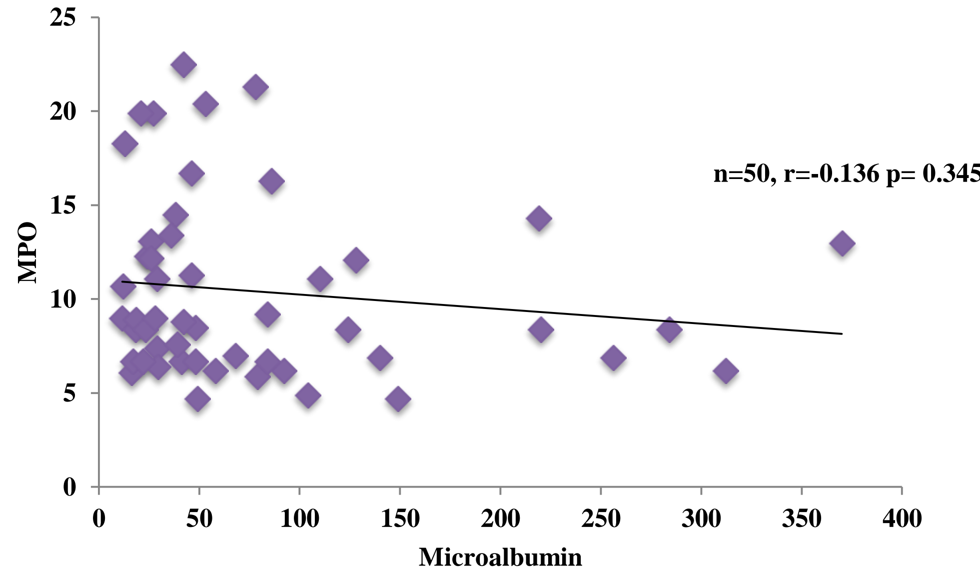


Figure: 5.22 Correlation between MPO and microalbumin in non-diabetics with CKD

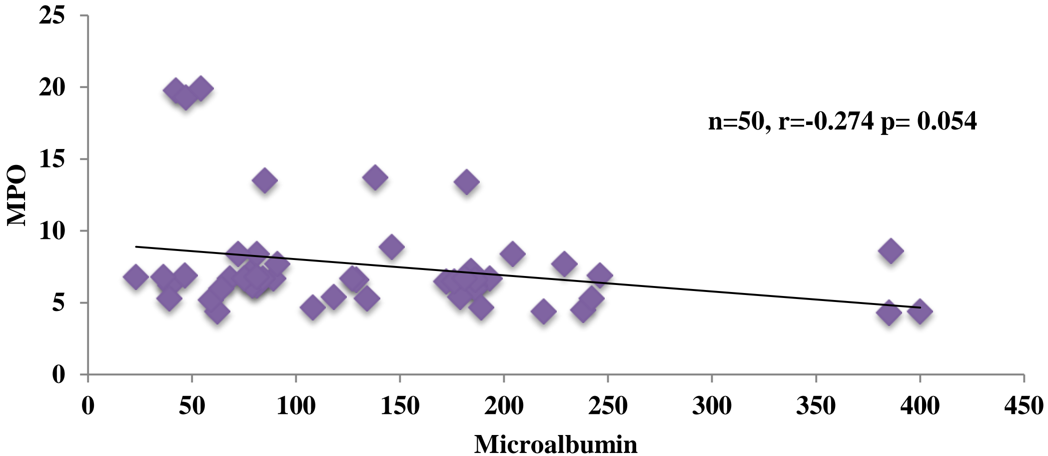


Figure: 5.23 Correlation between MPO and microalbumin in diabetics with CKD

Figure-5.22 and 5.23 shows that there is a negative correlation between MPO and Microalbumin among diabetics with CKD (r= -0.274, p= 0.054). In CKD-ND the correlation was not statistically significant (r = - 0.136 ,p = 0.354)

Table 5. 8 Correlation analysis among the biochemical parameters:

Groups	Correlation Co efficient	r value	p value
Non Diabetics with CKD	eGFR & Urea	-0.624	<0.001
	eGFR & creatinine	-0.765	<0.001
	MPO & Microalbumin	-0.136	0.345
	ACR & cystatin-C	0.505	<0.001
	eGFR & cystatin-C	-0.447	<0.001
	eGFR & MPO	-0.005	0.972
	cystatin-C & creatinine	0.493	<0.001*
	MPO & ACR	0.003	0.982
	cystatin-C & Microalbumin	0.251	0.079
Diabetics with CKD	eGFR & Urea	-0.630	<0.001*
	eGFR & Creatinine	-0.819	<0.001*
	MPO & Microalbumin	-0.274	0.054
	ACR & cystatin-C	0.527	<0.001
	eGFR & cystatin-C	-0.324	0.022
	eGFR & MPO	0.114	0.431
	cystatin C & Creatinine	0.259	0.069
	MPO & ACR	-0.22	0.124
	cystatin-C & Microalbumin	0.249	0.081

The table summarizes the correlation of biochemical parameters among the study groups.

Discussion

In the present comparative cross sectional study, there are three groups which comprises of healthy controls and CKD cases with diabetes and without diabetes. A total of 150 subjects were analyzed of which 50 were controls, 50 were CKD with diabetes and 50 were CKD without diabetes.

The distribution of subjects based on age group showed that subjects with CKD were comparatively higher in the age group of 46-55 years showing (34%) in CKD without Diabetes (CKD-ND) and (36%)in CKD with Diabetes (CKD-DM) groups respectively as shown in table-5.1. These findings are similar to the study carried out by Zati Iwani AK et al , in the year 2013 in which the mean age of the subjects was 43.1±14.9 and 53.7±11.0 years.[1]

The gender distribution among study groups shows that the male subjects in CKD-ND group were 33% and females were 17% respectively. The male subjects in CKD-DM group were 34% and females were 16%.The overall total subjects with chronic kidney disease were (67%) males compared to females (33%) as shown in table-5.2.

The current literature suggests that there is a greater incidence of progression and risk related to mortality of CKD in men when compared with women .According to the epidemiological study conducted in Spain by Jungers P et al, in 1990 reported a higher incidence rate of chronic kidney disease in men when compared to women[2]. Our study also revealed similar findings. Whereas a cross-sectional study conducted in China by Zhang L et al, in the year 2008 has demonstrated that the prevalence of chronic kidney disease among men and women were identical[3]. This might be attributed to geographical variability on the effect of gender on prevalence of CKD. Based on the most recent United States Renal Data System (2015) 57.8% of the cases

were men who were diagnosed for the new onset of end stage renal disease[4]. The effect of race on diabetic renal disease is much more uncertain. Studies carried out by Gall MA et al, in 1997, Ravid M et al in 1998, Jacobsen P et al, in the year 1999 and Berg UB in 2006 have shown that the risk factor for diabetic kidney disease was more in men compared to women [5-8]

Routinely blood urea and serum creatinine are being used for the assessment of renal function. Kidney disease results in the decreased excretion of urea which results in increase in blood urea levels .Urea is the end product of protein catabolism. It is a marker of uremic retention in CKD[9].Chronic elevated levels of urea results in the accumulation of harmful waste products which leads to decrease in glomerular filtration rate, and contribute to the increased cardiovascular risk related to CKD. We found blood urea levels were higher in CKD-ND group when compared with the other two groups which is statistically significant ($p<0.001$) table-5.3. The increased levels of urea in CKD-ND group may be due to the accumulation of uremic toxins and also due to secondary causes like hypertension etc, which implies a decline in renal function.

Serum creatinine is commonly used to assess renal function but the value of serum creatinine is affected even by extra renal factors like diet, muscle mass, age etc. An increase in levels of serum creatinine was noted in both the study groups. The levels of serum creatinine were significantly increased in CKD-ND group when compared to CKD-DM and control groups. The increment was more in males in all the three groups which is significant statistically (Figure-5.4).The increase in creatinine levels in CKD-ND group might be due to extra pancreatic factors which might have influenced the renal function. Treating diabetes in CKD-DM patients might be decreasing the nephropathic symptoms compared with CKD-ND group

where several factors are involved in causing CKD[10].. The value of mean creatinine obtained from our data showed that they were higher in the age group 66-75 years (Figure-5.5). The values of serum creatinine gradually increased with age indicating a decrease in renal function.

The detection of reduced renal function can be identified by using the diagnostic tools like cystatin-C, which can help in reducing the complications of CKD and improve the quality of life. At present, cystatin-C is identified as the most authentic endogenous marker to assess GFR (surpasses creatinine[11-16]).Serum cystatin-C synthesized by majority of nucleated cells is a non-glycosylated protein containing 120 amino acid residue polypeptide chains with a molecular weight of 13 kDa[17].

The major diagnostic criteria of CKD is based on GFR. In the present study we calculated eGFR based on serum creatinine and cystatin-C levels. Results have shown a negative correlation between urea and eGFR (Figure-5.1 and 5.2) in both the study groups. A negative correlation was also observed between creatinine and eGFR among CKD-DM and CKD-ND group (Figure-5.14 and 5.15).In the present study, we estimated serum cystatin-C, serum creatinine levels and calculated eGFR using various equations in diabetic and non-diabetic patients.

The mean levels of serum cystatin-C were significantly increased in non-diabetic patients with CKD compared to controls. eGFR levels were decreased in non-diabetic patients with CKD compared to controls. The mean levels of serum cystatin-C were decreased in diabetic patients with CKD compared to non-diabetic patients with CKD (table-5.3). It might be due to the selection of subjects based on duration of diabetes mellitus. In non-diabetics with CKD the increment in serum cystatin-C levels indicate

the severity of renal dysfunction which might be due to the manifestation of glomerular process before the tubular phase[15].

Many individuals with type 2 diabetes mellitus pass through a period of pre-diabetes and may experience renal dysfunction, which may not be diagnosed at an early stage. Diagnosis of CKD at an early stage is important as it can slow down the progressive loss of kidney function, which improves survival rate and lifestyle of CKD patients. The inability of creatinine to detect early fall in GFR levels is due to the fact that serum creatinine levels are evident, due to deterioration of renal function, suggesting that the levels of GFR can change before serum creatinine becomes abnormal[18].

Studies conducted by Rinno H et al (2002) suggested that serum cystatin-C is a more precise parameter for identifying initial changes in GFR in type 2 diabetic patients when compared with creatinine-based measurements[19]. The level of serum cystatin-C rises earlier than serum creatinine since it is filtered easily by the glomeruli and degraded in the proximal tubules without any secretion. Thus, it can be considered as a good marker for estimating glomerular filtration rate.

Serum cystatin-C is not influenced by nutritional status, inflammatory process and neoplastic state. It remains unaffected with age or muscle mass unlike creatinine. Based on the above key factors it can be considered as a potential substitute for creatinine[20-22]. This suggests that serum cystatin-C levels are associated with impairment in tubular function and can be suggested as an early marker of renal diseases. After filtration in the glomeruli, cystatin-C is fully degraded in the proximal renal tubules and small amount of it is excreted through urine. It is a good marker of renal function and correlates better with the direct measurement of GFR. It can be replaced by serum creatinine, due to its constant rate of production and less variability than that of creatinine[16].

Serum creatinine does not rise until there is a reduction in GFR (40 ml/min/1.73 m^2), this insensitivity is associated with minimal to moderate decrease in GFR in creatinine blind GFR area (40-70 ml/min/1.73 m^2) which ultimately results in delay in detection of renal damage. So serum creatinine may not be a reliable indicator for estimation of GFR at reduced levels of glomerular function. Though serum creatinine levels are normal during the initial stage of kidney disease, it does not indicate normal renal function. This is because serum creatinine level begins to rise only after a decline in renal function. The creatinine blind area ranges between 40 and 70 ml/min/1.73 m^2 where the actual decline in GFR occurs initially. cystatin-C does not have any blind area as reported by Hoek et al (2003),Ahlstrom et al (2004) and Peralta et al, in the year 2011 respectively. Initial decrease in GFR cannot be detected with creatinine testing, whereas cystatin-C will show a true positive reduction in GFR[23-25]

Serum cystatin-C helps in diagnosing “preclinical” state of renal dysfunction which can’t be identified with measuring serum creatinine or by estimation of GFR [26]. It offers an advantage to traditional CKD markers with respect to early detection of diabetic nephropathy and its progression which helps in timely intervention preventing further complications. Since creatinine estimation has limited value in CKD prognosis, hence the present study focuses on cystatin-C as it is very sensitive to minor changes in GFR[27]**.** Newman et al in 1995 concluded that, cystatin-C was a more sensitive marker for minor changes in GFR and better estimator of GFR when compared to serum creatinine [22]. According to the studies carried out by Shemesh et al in the year 1985 and Bennet et al in the year 2010 revealed that cystatin-C may be more precise in diagnosing mild decline in kidney function than serum creatinine.[18,28] In our study also we also observed similar findings.

A meta-analysis carried out by Dharnidharka VRet al (2002) was based on 46 studies, which were done using ROC analysis demonstrated that measuring cystatin-C is better when compared to creatinine for detection of decreased GFR[29].Our study also have shown a similar observation. The ROC analysis of creatinine with cystatin-C and eGFR in our study has shown that the area under the curve (AUC) of cystatin-C is 0.211 with a p value is 0.404 showing a cut off sensitivity of 98.7% (table-5.4). Recently Roos et al in the year 2007 reported in a systematic review by correlating the accuracy of cystatin-C with serum creatinine levels have included several studies which estimated efficiency of cystatin-C for all levels of renal function[30].Our study also revealed similar observations.

There was an increase in percentage of serum creatinine levels in CKD ND group compared to control. It was also noted that there was an increase in CKD DM group as compared to controls (Figure-5.7). There was a notable increase in serum cystatin-C levels in CKD-ND group as compared to controls and there was an increase of in CKD-DM group as compared to controls. There was a positive correlation between creatinine and cystatin-C which was statistically significant in CKD-ND group (Figure-5.13).Studies carried out by Dhupper V et al in the year 2015 and Tsai JP et al (2010) reported a positive correlation between scr and scys-C which was statistically significant[31,32] .In our study also a positive correlation existed between the scr and scys-C similar to the findings above.

The cystatin-C levels were higher in the age group of 46-55 years belonging to CKD-ND group (Figure-5.6). In CKD-ND the increment in cystatin-C levels indicate severity of renal dysfunction which is due to the manifestation of glomerular process before the tubular phase[15]. A cohort study carried out by Kittrawee Kritmetapak et al in the year 2007 demonstrated that acute tubular necrosis (ATN) was the most

familiar non-diabetic renal pathology superimposed with diabetic nephropathy in Type 2 DM patients presenting with acute kidney injury/disease[33]. The major causative factor of acute kidney disease is acute tubular necrosis which is characterized by destruction of tubular epithelial cells with acute suppression of renal function. Renal hypo perfusion and ischemia are the most common causes of ATN. Various conditions like acute tubular damage from ischemia, exposure to nephrotoxic drugs or chemicals, tubular obstruction, and sepsis can also result in ATN.

The serum levels of cystatin-C were significantly raised in CKD-ND than CKD-DM (p-value<0.001) table-5.3. This is in agreement with the study carried out by Woo KS et al in 2011[34].It can be explained on the basis that the values of serum creatinine is affected even by extra renal factors like diet, muscle mass, age etc., whereas cystatin-C is not affected by these extra renal factors. The levels of serum cystatin-C rises earlier than creatinine since it is freely filtered by the glomeruli and catabolised in the proximal renal tubules without any secretion, thus it can be considered as a good marker for estimating glomerular filtration rate(eGFR). Though serum levels of creatinine are normal during the initial stages of kidney disease, it does not indicate normal renal function. This is because serum creatinine levels are evident, when 50% of the kidney function is deteriorated, suggesting that GFR can alter even before serum creatinine becomes abnormal[18].

From our findings we observed that there was a decrease in eGFR levels in both CKD-ND and CKD-DM groups as compared to controls which is statistically significant (p<0.001). The eGFR levels were higher in CKD-DM group when compared to CKD-ND group (table-5.3).This might be due to the selection of subjects based on the duration of diabetes mellitus. Based on the values of eGFR the staging of kidney disease can be done which can help in preventing further deterioration of renal

function. From the data obtained in our study we have calculated eGFR (based on values of serum creatinine and cystatin-C) which would be helpful in identifying the stage of kidney disease. By correlating eGFR with the microalbumin and ACR levels and taking into consideration the duration of DM the quality of life of CKD patients can be improved.

Recently several equations are developed to estimate GFR on the basis of cystatin-C, including a CKD-EPI cystatin-C equation[16]. The CKD-EPI creatinine equation (2009) and CKD-EPI creatinine-cystatin-C equation (2012) are more accurate when compared to CKD-EPI cystatin-C equation (2012). CKD-EPI creatinine-cystatin-C equation (2012) is showing 98% sensitivity and 96% accuracy (table-5.5). The following equation was used for calculation of eGFR in our study. CKD-EPI creatinine cystatin-C Equation (2012): The formula for calculation of eGFR is $eGFR = 135 \times \min(SCr/\kappa, 1)\ \alpha \times \max(SCr/\kappa, 1) - 0.601 \times \min(Scys/0.8, 1) - 0.375 \times \max(Scys/0.8, 1) - 0.711 \times 0.995 Age \times 0.969$ (if female) $\times 1.08$ (if black)[35]

The ROC analysis of the test equations revealed that CKD-EPI creatinine-cystatin-C equation (2012) in CKD-ND group has shown that the AUC is 0.969 with a ‘p’- value of 0.11 (Figure-5.10) whereas CKD-EPI creatinine-cystatin-C equation (2012) in CKD-DM group has shown that the AUC is 0.891(Figure-5.11) with a ‘p’- value 0.01 which is statistically significant.Based on the AUC it supports that CKD-EPI creatinine-cystatin-c (2012) equation is the most important in the calculation of eGFR.

The sensitivity, specificity and accuracy of cystatin-C is not higher than creatinine and eGFR but it is equally good and can be routinely practiced, since it is not affected by extra renal factors like age, muscle mass and dietary habits (table-5.7).

In the present study we have observed a negative correlation between creatinine and eGFR in CKD-ND group when compared with CKD-DM group. An identical trend was observed in a more recent study carried out by Dhupper V et al.,(2015) and also in a study done by Kumaresan R and Giri P in the year 2011, a strong correlation was reported between scys-C and eGFR ($r = -0.877$, $p<0.001$) in comparison with SCr and eGFR ($r = -0.777$, $p<0.001$) in CKD subjects[31,36].

We also found that the concentration of serum cystatin-C has negative correlation with eGFR (Figure-5.17, table-5.8) which indicates that there is a possibility of renal damage in non-diabetic subjects. The correlation between eGFR, serum cystatin-C and creatinine was found to be statistically significant among non-diabetic patients with CKD and diabetic patients with CKD as compared to controls.

Dalla Vestra M et al.,[37] in the year 2000 demonstrated that the association between cystatin-C and eGFR was stronger among kidney disease patients than in healthy persons which is in agreement with our study .Type 2 DM patients showed a better correlation with eGFR compared to the normal subjects. This suggests the choice of using cystatin-C for CKD detection in Type 2DM patients over general population. This is in agreement with the study of Xilian Qiu1 et al[38]. Our study also revealed that there is comparatively better correlation between serum cystatin-C and eGFR ($r= -0.447$, $p<0.001$, Figure-5.17) in comparison with creatinine and eGFR. It is in accordance with the studies conducted by Hojs R et al in the year 2006[39].

There was an increase of of Random blood glucose levels in CKD-DM group when compared to healthy controls. (Figure-5.3).The values of mean HbA_1c levels were found to be significantly different among all the study groups ($p<0.04$*).The mean HbA_1c value in CKD-DM group was found to be significantly significant from control and CKD-ND group.

Due to higher levels of HbA_{1C} many changes occur in the glomeruli like thickening of the glomerular basement membrane, leakage of albumin through the glomeruli. The dysfunction of glomerular capillary membrane might lead to increased leakage of albumin at the glomeruli. According to a study conducted by Varghese A et al (2001) in southern India it was observed that in patients with type 2 diabetes, the incidence of microalbuminuria was also closely related to HbA_1c levels[40]. For individuals with higher HbA_1c levels, albuminuria is caused by chronic hyperglycemia, which resulted in loss of charge of selectivity and glomerular hyperperfusion and hyperfiltration accompanied by glycation of basement membrane proteins[41,42].

Myeloperoxidase has a role in initiation of renal damage in type-2 diabetic patients either due to the changes in the glomerular membrane proteins or oxidative damage to lipids. Duration of diabetes has significant role in the development of microalbuminuria by prolonged exposure to hyperglycemia-induced accumulation of advanced glycosylation end products[43].

Hence we made an attempt to study the role of MPO in CKD and its correlation with microalbumin, ACR and eGFR (Figure-5.22 and 5.23, 5.20 and 5.21, table-5.8) in CKD-ND and CKD-DM groups. Our study has shown a significant decrease in MPO levels, and an increase in microalbumin levels in diabetic patients with CKD when compared with non-diabetic patients with CKD (table-5.3).

The microalbumin levels were significantly increased in CKD-DM group when compared to CKD-ND group. Haque et al (2011), Venugopal S and Lyer UM et al,(2010) have reported a significant correlation of HbA_1c with microalbuminuria in type 2 diabetes mellitus patients.[44,45] Studies carried out by Prabhu S et al in the year 2016,Sheik et al (2009) and krolewsi AS et al in 1995 have demonstrated a positive

association between HbA_1c and microalbuminuria[46-48].Microalbumin, eGFR and ACR are considered to be good markers for early renal impairment.

Proteinuria is the common indication for end stage renal diseases (ESRD) which is more commonly seen in patients with CKD. It contributes to the complications associated with the progression of the disease which might influence the mortality in CKD patients. Estimation of urinary microalbumin is the novel marker of reversible nephropathy, by which the initial stages of progressive glomerular disease can be identified. The spot urine ACR is equally good in performance when compared to 24 hours urine ACR for this reason both of them can be suggested to analyse the risk for early renal damage at reversible stage[49]. Patients with DM along with microalbuminuria have insulin resistance and poor glycemic control. In early stages of renal damage, routine parameters like urea & creatinine may be normal, but early changes in the glomeruli like thickening of basement membrane, accumulation of matrix material in the mesangium, subsequent nodular deposits are observed with consequent microalbuminuria. At this stage, pathological changes in the glomeruli can be modified by pharmacological intervention[44]

Urinary ACR should be performed for monitoring the risk in Type 2 DM patients with increased levels of HbA_1c. Microalbuminuria is persistent, increased urinary excretion of albumin[50,51]In study conducted by Coresh J et .al in the year 2000 have also demonstrated there is an excess of excretion of microalbumin through urine among individuals with diabetes and hypertension, the prevalence was increasing with duration of thedisease[51,52].The degree of albuminuria is strongly related to the progression of diabetic renal disease and is also a risk factor for cardiovascular events[53] The condition of microalbuminuria includes an increase in glomerular size, glomerular basement membrane thickening and mesangial expansion[54,55]The increase

in glomerular size might be due both to mesangial expansion and amplification in glomerular capillaries.

Diabetic nephropathy is a chronic microvascular complication of DM. The HbA_1c recorded in CKD-DM group in our study is 7.69%. Due to hyperglycemia many changes occur like thickening of the glomerular basement membrane, leakage of albumin through the glomerulus,. It may gradually progress from hyperfiltration to microalbuminuria followed by macroalbuminuria. There is a decline in eGFR levels which results in suppression of renal function. Hence microalbuminuria should be corrected at an early stage to delay the renal damage and development of cardiovascular complications in CKD patients.

A negative correlation was observed between MPO and microalbumin (Figure -5.23, and table-5.8.) and also between MPO and ACR (Figure-5.21,table-5.8) in CKD-DM group. According to the study carried out by Haque N et al, in the year 2011 reported significant positive correlation of HbA_1c with urinary ACR in type 2 diabetic patients[44] .We also reported a similar observation .Based on our findings there was a decrease in MPO levels with an increase in microalbumin levels and ACR in CKD-DM group indicating early renal damage which can be further prevented by appropriate diagnosis and treatment .Hence it could be speculated that MPO can be considered as earliest marker in CKD-DM cases.

As the levels of HbA_1c increase, in CKD-DM group it causes changes in the metabolic process occurring in the body which results in the production of ROS (AGE s and Cytokines) and damage the glomeruli of the kidneys resulting in leakage of albumin through urine . Microalbuminuria in due course of time leads to Diabetic Nephropathy. By proper monitoring of glycemic control in type 2 DM patients the progression of renal damage can be further prevented.

In the present study we observed percentage decrease of MPO levels in CKD-DM cases and CKD-ND cases when compared to healthy controls as shown in (Figure-5.8) .The eGFR levels were decreased in both the groups. A positive correlation was observed between MPO and eGFR in CKD-DM cases (Figure-5.19, table-5.8). This is in agreement with the study conducted by Rao AM et al (2011)[56]

The decrease in MPO levels implies that MPO and its derived oxidants such as HOCl interferes with various cell functions which may contribute to damage of renal tissues resulting in the accumulation of uremic toxins which indicate a decline in renal function. HOCl and MPO derived oxidants induce damage to the renal tissue there by contributing to the renal complications[57].

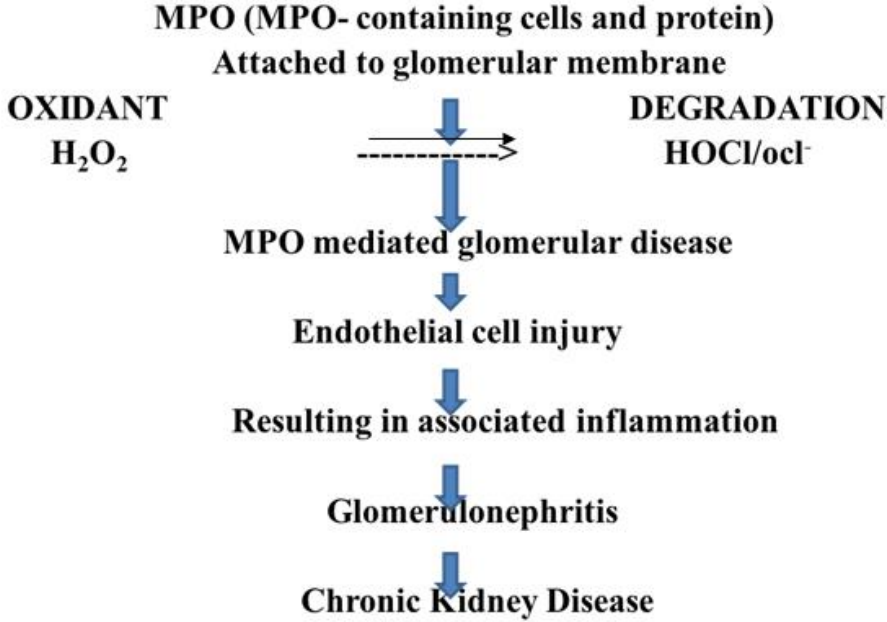


Figure-6.1 flow chart showing the role of MPO in Chronic kidney disease

Lipid peroxidation is induced by extracellular MPO which is capable of catalyzing lipoprotein peroxidation in vivo, thus resulting in atherosclerosis which is common in CKD patients. Due to its cationic character it can bind to the negatively charged structures of endothelial cells and albumin[58].The products of oxidation produced by MPO are microbicidal in nature and play an important role in innate immune response by defending the body against microbes[59].Hypochlorous acid and other MPO-derived oxidants mediate oxidative stress and cause damage to bio-molecules such as proteins, nucleic acids, lipids, and carbohydrates, thus damaging the host tissue. In this manner, MPO derived oxidants may trigger the advancement of atherosclerosis, endothelial dysfunction, cardiovascular, and other complications in patients with CKD[60].HOCl also causes dysfunction of endothelial cells and affect endothelial function by decreasing the adhesiveness of extracellular matrix proteins for endothelial cells and by converting matrix metalloproteinases into their active

form, thus destabilizing the vascular and tissue environment surrounding endothelial cells[61,62]

Our study demonstrated that there is a gradual decrease in values of serum MPO with advancing renal disease ($p<0.001$) table-5.3.This is in agreement with the study carried out by Rao AM et al[56].MPO enzyme has been shown to play a pivotal role in the progression of atherosclerotic disease. Various mechanisms were postulated by which higher levels of MPO can further enhance cardiovascular complications[63].

Therefore the present study was undertaken to determine and understand the activity of MPO in patients with CKD. However, our results have shown that serum MPO levels are significantly less in CKD patients with diabetes mellitus in comparison with CKD patients without diabetes mellitus. Hence, it could be hypothesized that decrease in MPO levels in CKD patients may be due to the inhibitory effect of uremic toxins, particularly CNO^-, on this enzyme. The decrease in MPO levels implies that MPO and its derived oxidants such as HOCl interferes with various cell functions and induces damage to renal tissue thereby contributing to renal complications. This in turn leads to end stage renal disease and cardiovascular complications[64]

Increased levels of blood urea in CKD promote modification of proteins after translation through process of protein carbamylation[65]. It occurs due to non enzymatic chemical modification by isocyanic acid which is derived from urea and also from alternative source, i.e the MPO-catalyzed oxidation of thiocyanate. Urea, in human beings, gradually disintegrates in aqueous solution and forms cyanic acid and its conjugate base cyanate. Cyanic acid (HOCN) is in equilibrium with its active form, isocyanic acid (HNCO).

A decrease in MPO levels is observed which is due to a rise in the urea levels. The uremic plasma interferes with many biological functions. When urea is present in high concentrations in the body fluids, it dissociates to release ammonia and cyanate. It is a potential toxin which combines irreversibly with normal proteins in cells there by affecting their normal function [66]. It has an inhibitory effect on both peroxidative and halogenating activities of MPO and inhibits the enzyme within intact neutrophils. The cyanate also inhibits Cl^-/H_2O_2/MPO mediated bacterial phagocytosis[67].

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Summary

7.1 SUMMARY:

All the objectives were evaluated with a comparative cross sectional study among diabetic and non-diabetic subjects with chronic kidney disease in the age group of 40-70 years. 150 subjects were included in the study and divided into three groups with 50 subjects in each group.

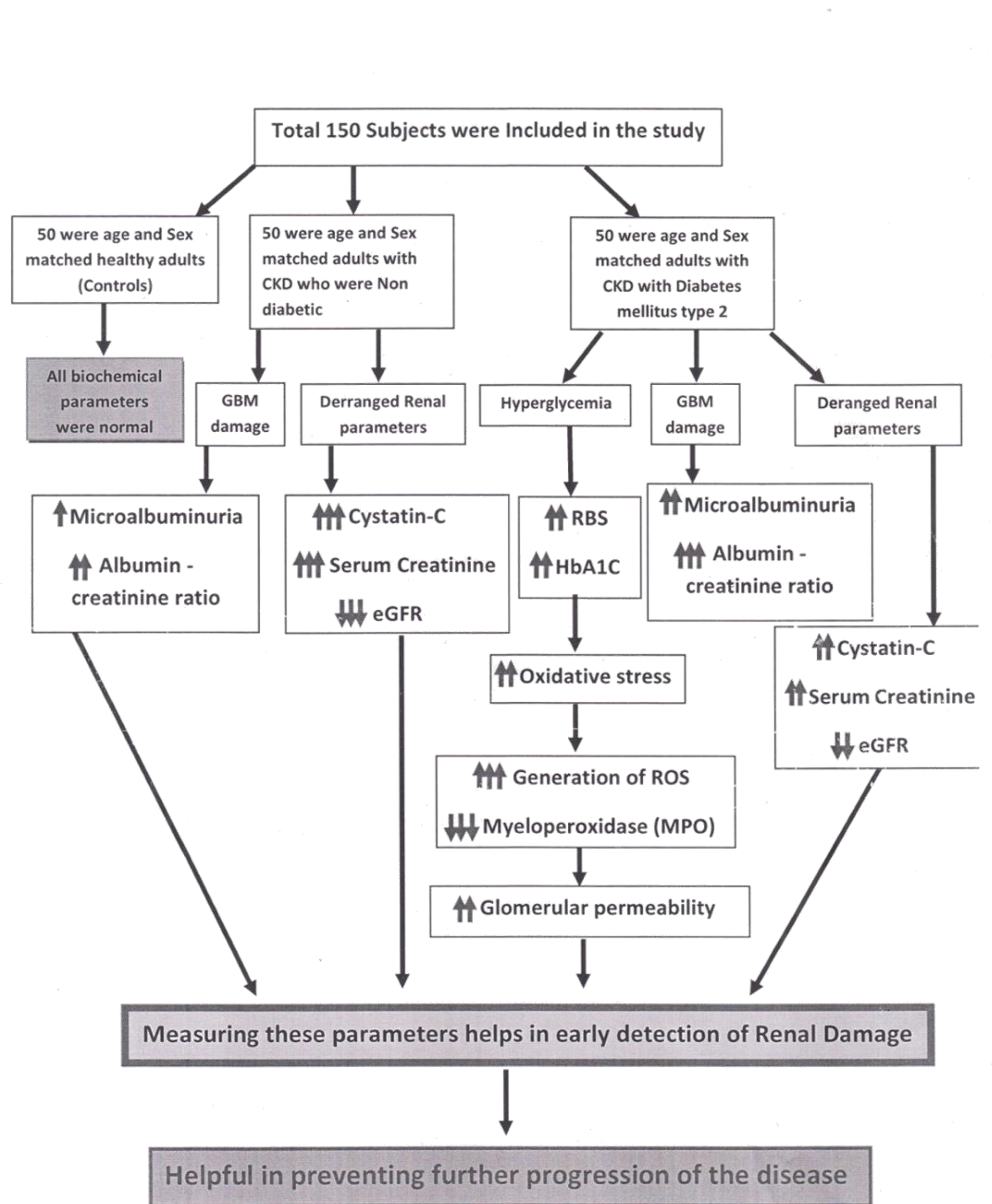
The following biochemical parameters i.e serum cystatin-C, Creatinine, Myeloperoxidase, Microalbumin, ACR and HbA_1c were analyzed following standard procedure. eGFR was calculated using CKD-EPI cystatin-c and creatinine equation (2012).

- Results showed that the subjects with chronic kidney disease were more in males 67% compared to females 33%.
- The values of Random blood glucose in CKD-DM were higher (259.7±.80.5) when compared to CKD-ND and controls.
- The blood urea levels were increased in both CKD-ND (84.86±6.76) and (81.08±7.79) in CKD-DM groups respectively
- The values of creatinine were increased in CKD-ND (3.92±.1.13) and (2.86±.2.77) in CKD-DM groups compared to controls.
- The values of HbA_1c were (7.69± 1.17 %) in CKD-DM group and (5.34±1.49 %) in CKD-ND group compared to controls.
- The eGFR values lower in CKD-ND group (64.1±3.69) and (82.1±4.97) in CKD-DM group compared to controls
- The values of microalbumin were higher in CKD-DM group (138.78±9.57) and (71.94±8.54) in CKD-ND group compared to controls.

- The ACR levels obtained in our study were higher in CKD-DM (2.11±0.71) and it was (1.82±0.59) in CKD-ND group.
- The values of cystatin-C were higher in CKD-ND (2.62±1.09) and (2.21±.36) in CKD-DM group when compared to controls.
- The levels of Myeloperoxidase were notably decreased in Diabetics with CKD when compared with Non Diabetics with CKD. The MPO levels were lower in CKD-DM group (7.59±3.71) and (9.39±5.81) CKD-ND group when compared to controls.
- There was a negative correlation between urea and eGFR, creatinine and eGFR, cystatin-C and eGFR among the study groups.
- There was a positive correlation between MPO and eGFR among the groups, whereas a negative correlation was observed between MPO &ACR and also between MPO and microalbumin among the study groups.
- In this study we found that there was an increase in serum creatinine levels in CKD-ND group and in CKD-DM group as compared to controls. There was a notable increase in serum cystatin C levels in CKD-ND group and in CKD-DM group as compared to controls.
- Serum cystatin-C and creatinine levels were found to be statistically significant (p-value<0.001*) among CKD-ND and CKD-DM groups as compared to controls.
- The sensitivity, specificity and accuracy of cystatin-C is not higher than creatinine and eGFR but it is equally good and can be routinely practiced, since it is not affected by extra renal factors like age, muscle mass and dietary habits.

- The ROC analysis of the test equations revealed that CKD-EPI creatinine-cystatin-C equation (2012) in CKD-ND group has shown that the AUC is 0.969 with a ‘p’- value of 0.11, whereas CKD-EPI creatinine-cystatin-C equation (2012) in CKD-DM group has shown that the AUC is 0.89 with a ‘p’-value 0.01 which is also statistically significant.
- The microalbumin and ACR levels were increased in CKD-DM group compared to CKD-ND group. Based on our findings there was a decrease in MPO levels with an increase in microalbumin levels and ACR in CKD-DM group indicating early renal damage which can be further prevented by appropriate diagnosis and treatment .Hence it could be speculated that MPO can be considered as earliest marker in CKD-DM cases.
- Serum MPO levels were significantly decreased ($p < 0.001$*) in CKD-DM and CKD-ND as compared to controls which can be used an indicator for CKD patients with Diabetes mellitus in assessing the renal impairment which can prevent further complications.

Graphical abstract of the study



Conclusion

7.2 CONCLUSION:

- Serum cystatin-C is a specific and better biochemical parameter to know renal status in non-diabetics with CKD as it is not affected by extra renal factors like age, gender, muscle mass, nutritional status. Based on its sensitivity among the previous established CKD parameters like eGFR and Creatinine it can be a potential substitute to creatinine.
- Diagnostic accuracy is favourable for serum cystatin-C when compared with serum creatinine in patients with decreased renal function in nondiabetic patients with CKD.
- It offers an advantage to traditional CKD markers with respect to early detection of diabetic nephropathy and its progression which helps in timely intervention preventing further complications. Since creatinine estimation has limited value in CKD prognosis, hence the present study focuses on cystatin-C as it is very sensitive to minor changes in GFR.
- CKD-EPI creatinine-cystatin-C equation (2012) is showing 98% sensitivity and 96% accuracy .Hence this equation was used for calculation of eGFR in our study.
- Based on the values of eGFR the staging of kidney disease can be done which can help in preventing further deterioration of renal function and improve the quality of life in CKD cases.
- Urinary microalbumin and ACR should be performed for monitoring the risk in Type 2 DM patients with increased levels of HbA_1c.
- Microalbuminuria should be corrected at an early stage to delay the renal damage and development of cardiovascular complications in CKD patients

- Based on our findings there was a decrease in MPO levels with an increase in microalbumin levels and ACR in CKD-DM group indicating early renal damage which can be further prevented by appropriate diagnosis and treatment .Hence it could be speculated that MPO can be considered as earliest marker in CKD-DM cases.
- MPO is the earliest marker in diagnosing kidney damage in CKD-DM at early stage i.e., more than 5 years and less than 10 years of onset of DM.
- Although cystatin-C and Myeloperoxidase are economically higher in price compared to creatinine it is accurate, better and beneficial to the patients.

7.3 STUDY LIMITATIONS:

- Since there was constraint regarding budget as this study was self funded;
- We did not do any molecular study
- Further molecular studies pertaining to gene expression can be carried out to confirm expression of genes

B.L.D.E. UNIVERSITY
(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act,1956)
The Constituent College
SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE

IEC Ref No-102/2014-15 November 15, 2014.

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 22nd September 2014 at 11 AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.

Title ""Evaluation of serum cystatin C, Myeloperoxidase and other Biochemical markers for the early detection of renal failure in diabetic and non diabetic patients in Karimnagar, Telangana(India)."

Name of Ph.D./ P. G. / U. G. Student / Faculty member. Mrs.S.Sangeeta. Department of Biochemistry.

Name of Guide :.Dr.J.G.Ambekar Professor Department of Biochemsitry.

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPUR – 586 103

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
VIJAYAPUR – 586 103.

Member Secretary,
Institutional Ethical Committee,
BLDE University, BIJAPUR.

Following documents were placed before Ethical Committee for Scrutinization:

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant documents.

Smt. Bangaramma Sajjan Campus, Sholapur Road, Vijayapur – 586103, Karnataka, India.
University: Phone: +918352-262770, Fax: +918352-263303, Website: www.bldeuniversity.ac.in, E-mail: office@bldeuniversity.ac.in
College: Phone: +918352-262770, Fax: +918352-263019, Website: www.bldeuniversity.ac.in, Emaibmpmc.principal@bldeuniversity.ac.in

17-06-2015

PRATHIMA INSTITUTE OF MEDICAL SCIENCES

INSTITUTIONAL ETHICS COMMITTEE

Reference Number: IEC/PIMS/2015/01

Dear, S. Sangeeta,

RE: Approval for ethical clearance for a study entitled "Evaluation of serum Cystatin C, Myeloperoxidase and other biochemical markers for the early detection of renal failure in diabetic and non diabetic patients in Karimnagar, Telangana."

Reference is made to the above heading.

I am pleased to inform you that the institutional ethics committee has approved ethical clearance of the above mentioned study based on the recommendations of the committee members.

The validity of this ethical clearance is three years effective from 17th June 2015. You will be required to apply for renewal of ethical clearance if the study is not completed at the end of this clearance. You will be expected to provide six monthly progress reports and a final report upon completion of your study.

17th June 2015

Chairman
Institutional Ethics Committee,
Prathima Institute of Medical Sciences

Chairman,

Institutional Ethics Committee (for human research)

Prathima Institute of Medical Sciences,

Karimnagar, India.

INFORMED CONSENT FORM

Patient identification number for this study: ………………………………………
Title of the project:……………………………………………………………………

Name of the principal investigator ………………………………………………Tel.No……………… The contents of the information sheet dated …………………… that was provided has been read carefully by me/explained in detail to me ,in a language that I comprehend, and I have fully understood the contents. I confirm that I have had the opportunity to ask questions.

The nature and purpose of the study and its potential risks/benefits and expected duration of the study, and other relevant details of the study have been explained to me in detail. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without giving any reason,without my medical care or legal right being affected.

I understand that the information collected about me from my participation in this research and sections of any of my medical notes may be looked at by responsible individuals from Prathima institute of medical sciences or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

I agree to take part in the above study.
…………………………………

Date:

Signature/thumb impression   Place:

Name of the participant:

Son/daughter of/spouse of:

Complete address:

This is to certify that the above consent has been obtained in my presence.

……………………….
Date:
Signature of principal investigator   Place:

Witness I   Witness II

………………………………
…………………………
Signature   Signature

Name: Name:

విశదీకరించిన ఒప్పంద పత్రము

పరిశోధన కొరకు రోగి యొక్క గుర్తింపు సంఖ్య :

పరిశోధనా శీర్షిక :

ముఖ్య పరిశోధకుడి పేరు : దూరవాణి సంఖ్య:

నాకు అర్థమగు బాషలో ఈ తేది నందు ______________ విశదీకరించిన ఒప్పంద పత్రమునందున్న సమాచారమును నాచే విపులంగా చదవబడినది/వివరింపబడినది. నాకు ప్రశ్నలడుగుటకు స్వేచ్ఛ వున్నదని స్పష్టమైనది.

పరిశోధన యొక్క రీతి, కారణము, ఉపద్రవములు, లాభములు, పరిశోధనాకాలము, మరియు ఇతర పరిశోధన వివరములు నాకు విపులముగా తెలియజేయబడింది.

నేను ఈ పరిశోధనలో స్వచ్ఛందంగా పాల్గొనుచు మరియు ఏ సమయంలోనైనా ఇందుండి అకారణముగా నా హక్కులు, వైద్యసహాయాలకు ఉల్లంఘన జరగకుండా వైదొలగుటకు స్వేచ్ఛవున్నదని స్పష్టమైనది.

నేను పరిశోధనలో పాలుపంచుకొన్న సమయంలో నా నుంచి సేకరించిన వివరములు, నా ఆరోగ్య స్థితి వివరములకు ప్రతిమ వైద్య సంస్థలోని/సంబంధిత బాధ్యయుత అధికారులచే చూడబడులకు నా సమ్మతిని తెలియజేయచున్నాను

ఈ పరిశోధనలో పాల్గొనుటకు సమ్మతించుచున్నాను.

తేది:

స్థలము:

నంతకము:

ఎడమచేతి వేలి ముద్ర:

పాల్గొను వ్యక్తి యొక్క పేరు:

యొక్క పుత్రుడు/పుత్రిక/భార్య/భర్త:

పూర్తి తపాలా చిరునామా:

ఈ ఆమోదము నా యొక్క హాజరీలో గైకొనబడినది.

ముఖ్య పరిశోధకుడి నంతకం:

తేది:

స్థలము:

సాక్షి-1

నంతకము

సాక్షి-2

నంతకము

Proforma

Name of the project: Evaluation of serum cystatin C, Myeloperoxidase and other Biochemical markers for early detection of renal failure in diabetic and nondiabetic patients in Karimnagar, Telangana(India).

Name of the research scholar : Mrs.S.Sangeeta

Name of the guide : Dr. J.G. Ambekar

<u>Patient information sheet:</u>

Name of patient :

OP/IP No :

Age :

Height :

Sex :

Weight :

Occupation :

BMI :

Address :

Provisional Diagnosis :

Investigations :

1) Serum Cystatin C
2) Myeloperoxidase
3) Urine albumin creatinine ratio
4) Serum creatinine
5) RBS
6) HbA_1C
7) Microalbumin
8) eGFR

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGARISM VERIFICATION CERTIFICATE

1. **Name of the Student:** S. SANGEETA **Reg No.** 13PhD004
2. **Title of the Thesis:** "Evaluation of serum cystatin-C, Myeloperoxidase and other biochemical markers for the early detection of renal failure in diabetic and non diabetic patients in Karimnagar, Telangana
3. **Department:** BIOCHEMISTRY
4. **Name of the Guide & Designation:** Dr. JEEVAN G. AMBEKAR, PROFESSOR
5. **Name of the Co Guide & Designation:** Dr. T. SUDHAKAR, PROFESSOR

The above thesis was verified for similarity detection. The report is as follows:

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Signature of the Guide
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J G Ambekar
Professor

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Name & Designation

Signature of Student

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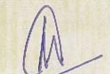

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Contemporary Developments in Laboratory Medicine & Laboratory Management

Certificate of Participation

Dr /Ms /Mr .... Samudrala Sangeeta .... has

presented a **Poster** titled "Evaluation of Serum cystatin C and Serum creatinine in the for early detection of Renal injury in Diabetic + Non diabetic patients of North Telangana region"

DIAGNOCON 2018 National Conference held from **23rd – 25th February 2018**

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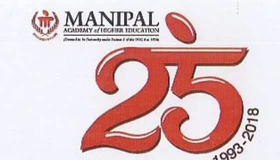

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participated as a Speaker/Chairperson/**Delegate** in the **DIAGNOCON 2018** National

Conference held from **23rd – 25th February 2018**

Dr. Chiranjay Mukhopadhyay Organising Secretary Kasturba Medical College Manipal	**Dr. Pragna Rao** Dean Kasturba Medical College Manipal

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Department of Biochemistry
King George's Medical University, Lucknow

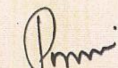
44TH NATIONAL CONFERENCE OF ASSOCIATION OF CLINICAL BIOCHEMISTS OF INDIA

Theme: Emerging Trends in Clinical Biochemistry: From Evidence Based Medicine to Molecular Medicine

3rd - 6th December, 2017 | Scientific Convention Centre, KGMU, Lucknow

CERTIFICATE *of* Participation

This is to certify that Prof/Dr./Mr./Ms. SANGEETA SAMUDRALA has participated as Delegate/Faculty/Chairperson held as a part of 44th Annual Conference of the Association of Clinical Biochemists of India - ACBICON 2017.



Dr. Poornima A Manjrekar	**Dr. Abbas Ali Mahdi**	**Dr. Rajiv R Sinha**
President, ACBI	Organizing Secretary, ACBICON 2017	Secretary, ACBI

Uttar Pradesh Medical Council has granted 12 Credit Hours

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Participation

Presented to Prof./ Dr./Mr./Ms. SANGEETA SAMUDRALA for his/her participation as delegate in Paper/Poster on 04-12-2017 "ASSOCIATION OF BIOCHEMICAL PARAMETERS AND MPO IN KIDNEY DISEASE PATIENTS WITH AND WITHOUT DIABETES" held as a part of 44th Annual Conference of the Association of Clinical Biochemists of India - ACBICON 2017.

Dr. Poornima A Manjrekar President, ACBI	**Dr. Abbas Ali Mahdi** Organizing Secretary, ACBICON 2017	**Dr. Rajiv R Sinha** Secretary, ACBI

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Assessment of eGFR, using Cystatin-C and Creatinine Based Equations for the Early Detection of Renal Injury in Diabetic and Non Diabetic Patients

Biochemistry Section

S. SANGEETA[1], JEEVAN AMBEKAR[2], TUNGUTHURTHI SUDHAKAR[3], MOHD SHANNAWAZ[4], NILIMA DONGRE[5]

ABSTRACT

Introduction: Assessment of renal function in individuals with Type-2 diabetes is very important as diabetic nephropathy is the major cause of Chronic Kidney Disease (CKD) which leads to the most frequent cause of End Stage Renal Disease (ESRD) in diabetic patients. Glomerular Filtration Rate (GFR) can be considered as best index for assessment of renal function.

Aim: To assess the eGFR using Serum Cystatin-C and compare with Serum creatinine based equations for the early detection of renal injury in Diabetic and Non Diabetic patients.

Materials and Methods: The present cross-sectional study was carried out after getting approval by institutional human ethical committee. A total of 150 participants were part of the study after obtaining the informed consent. Group-I included 50 Normal healthy controls, Group-II included 50 Chronic Kidney Disease Patients without Diabetes Mellitus (CKD-ND) and Group-III included 50 patients of Chronic Kidney Disease with Diabetes Mellitus (CKD-DM). Serum Cystatin-C, Creatinine, Urea and Glucose were estimated in the serum sample. eGFR was calculated by using Creatinine and Cystatin C based CKD-EPI equation. Data was analysed by SPSS 20.0. Correlation analysis was done using Karl Pearson's correlation coefficient. A p-value less than 0.05 were considered as significant.

Results: Serum Cystatin-C and serum creatinine were significantly increased in Non diabetic patients with CKD, a considerable decrease in eGFR was observed in Group-II compared to Group-III. Serum Cystatin-C showed a significant negative correlation with eGFR among the groups. There was a strong correlation of serum Cystatin-C with eGFR in Group-II and Group-III compared to Controls.

Conclusion: Serum Cystatin-C can be used as an alternative marker to creatinine in CKD patients without diabetes.

Keywords: Chronic kidney disease, Chronic kidney disease epidemiology collaboration, Diabetic nephropathy

INTRODUCTION

Chronic Kidney Disease (CKD) is an emerging global health problem due to the increasing prevalence of conditions like diabetes mellitus, hypertension and cardiovascular diseases. It is a global threat to health in general and for developing countries like India, because of the dietary habits, socio economic status and life style. CKD initially is without specific symptoms and is detected by an increase in serum creatinine or protein levels in urine. In developing countries like India, diabetes and hypertension accounts for 40-60% cases of CKD [1] which is associated with significant morbidity, mortality and economic burden. It is expected by 2025 that nearly 380 million adults will become diabetic patients worldwide. Indian statistics reveal that there were 41 million diabetics in 2012 and this number is expected to rise to 70 million by 2025 [2]. The metabolic derangements, which are related to diabetes mellitus causes many physiological and pathological changes, thereby affecting various organ systems which may lead to certain complications [3]. The diagnosis of CKD at an early detectable stage is important to delay the renal complications. The "gold standard" for determining GFR was inulin clearance. Since it is an exogenous marker, which has to be administered intravenously, this is a laborious process and hence it is not being practiced for routine monitoring [4]. Serum creatinine is the most routinely used marker for the assessment of renal function. The values of serum creatinine does not show an increase until GFR levels are moderately decreased (40 mL/min/1.73 m^2). The levels of serum creatinine are affected by factors such as age, diet, muscle mass etc. Hence, it is insensitive and late marker for changes in GFR [5]. Hence, there is a need for identifying an alternative filtration marker for unbiased estimation of GFR. Among several biomarkers, Cystatin-C has been proposed to be a promising marker which can help to detect early kidney injury. Cystatin-C is a low molecular weight non glycosylated protein, which is produced by all nucleated cells in the body. It is removed from the blood stream and freely filtered by the glomerular membrane in the kidneys. It serves as an index of renal function. The serum levels of Cystatin-C are not influenced by infections, inflammation or neoplastic states, and also by body mass, diet, or drugs [6]. Therefore, on the basis of these key factors Cystatin-C has been proposed and attempted to be proved as a superior marker for predicting GFR than Creatinine [7]. Cystatin-C is also superior to creatinine as a marker of kidney function and it correlates better with eGFR than serum creatinine. Hence, the present study was undertaken to assess and compare the eGFR using Serum Cystatin-C and Serum creatinine based equations for the early detection of renal injury in Diabetic and Non Diabetic patients which will help in early identification of renal impairment there by reducing the risk of further complications.

MATERIALS AND METHODS

Laboratory setting: The present cross-sectional study was conducted in the Department of Biochemistry in collaboration with Medicine and Nephrology at Central laboratory of Prathima Institute of Medical Sciences during the period of June 2015 to September 2016. Random blood glucose, HbA1c, Urea, Creatinine and Cystatin-C were estimated in selection of patients for CKD without DM.

The sample size (45 per group) was calculated using type one error (α) as 5%, power of the test as 90% with anticipated mean difference of Cystatin-C between groups was 0.5 and anticipated SD 0.7.

A total of 50 patients with CKD without Diabetes (CKD-ND), 50 CKD with Diabetes patients (CKD-DM) and 50 age and sex matched healthy controls were a part for this research. The study was approved by Institutional Ethics committee of Prathima Institute of Medical Sciences (IEC/PIMS: 2015/01). After obtaining written informed consent from all the participants, clinical history and demographic details of the patients were collected using structured questionnaire. The following criteria was used for recruiting the participants.

Inclusion Criteria

- Age ≥40 years, and ≤70 years
- Clinically confirmed diabetic patients with duration of diabetes >5 years and <10 years
- Clinically confirmed patients of CKD.

Exclusion Criteria

- Not willing to give consent
- Duration of diabetes <5 years and >10 years
- The patients with thyroid disease and those who are on treatment for thyroid disorders were excluded. Because thyroid function could affect the levels of Cystatin-C [8].
- Age and sex matched healthy controls were selected from the staff of college and hospital.

Sample Collection

Under aseptic conditions 5 mL random venous blood sample was collected from each participant, by anticubital vein puncture in a sterile disposable syringe of which 2 mL was collected in fluoride bulb and remaining was transferred in a plain bulb. After centrifugation, serum was separated and used for the estimation of biochemical parameters like Urea, Creatinine, Cystatin-C (renal profile).

From the Creatinine and Cystatin-C values obtained eGFR was calculated using CKD-EPI equation [9]. Serum Cystatin-C levels were measured by immuno-turbidimetric method using Accurex kits [10]. The eGFR level can be calculated using the Modification of Diet in Renal Disease (MDRD) formula, and CKD-EPI equation.

MDRD=186×{serum creatinine (mg/Dl)}$^{-1.154}$×age$^{-0.203}$×(0.742 if female)×(1.212 if black) [11].

A correction factor of 0.742 was used for women. (Based on body surface area)

CKD-EPI creatinine equation (2009) eGFR=141×min $(S_{Cr}/\kappa,1)^{\alpha}$×max $(S_{Cr}/\kappa,1)^{-1.209}$×$0.993^{Age}$×1.018 (if female)×1.159 (if Black) [12];

The $eGFR_{cys}$ level was calculated by the Chronic Kidney Disease Epidemiology (CKD-EPI)

CKD-EPI equation: eGFR=127.7×(cystatin C in mg/L)$^{-1.17}$×(age in years)$^{-0.13}$×(0.91 if female) [9].

CKD-EPI Creatinine Cystatin Equation [2012] eGFR=135×min $(S_{Cr}/\kappa,1)^{\alpha}$×max $(S_{Cr}/\kappa,1)^{-0.601}$×min $(S_{cys}/0.8,1)^{-0.375}$×max $(S_{cys}/0.8,1)^{-0.711}$×0.995^{Age}×0.969 (if female)×1.08 (if black) [13].

CKD-EPI Cystatin C equation eGFR=133×min $(S_{cys}/0.8, 1)^{-0.499}$×max $(S_{cys}/0.8, 1)^{-1.328}$×$0.996^{Age}$×0.932 (if female) [14].

Creatinine was estimated by Jaffe's Method [15]. Urea was estimated by Berthelot method [11]. Glucose was estimated by Glucose oxidase-Peroxidase method [16].

STATISTICAL ANALYSIS

Data was analysed by using SPSS 20.0 software. The results of three groups were expressed as Mean±SD. One-way ANOVA was applied to observe the significance of difference between groups. Karl Pearson's correlation coefficient was used to observe correlation. A p-value less than 0.05 were considered as significant.

RESULTS

We have focused on clinical and biochemical parameters and compared among the control and Test groups. The participants included in the study from all the groups were maximum in the age group of 46-55 years. [Table/Fig-1] shows the comparison of Group-I (Normal healthy subjects) with Group-II (CKD-ND) and Group-III (CKD-DM). Serum Cystatin-C, Urea and Creatinine levels were significantly increased (p-value<0.001) in Group-II and Group-III compared to values among controls (Group-I). The eGFR levels were significantly decreased in Group-II and in Group-III compared to Group-I. The correlation between eGFR and serum Cystatin-C and Creatinine was found to be statistically significant among Group II and Group III as compared to controls i.e., Group I [Table/Fig-2].

Parameters	Group-I (Control) n=50	Group-II (CKD-NDM) n=50	Group-III (CKD-DM) n=50	p-value
Cystatin-C (mg/L)	0.74±0.45 (0.05-1.79)	3.59±2.20 (0.7-8.99)	3.17±2.36 (0.9-11.67)	<0.001*
eGFR (mL/ min/1.732 m^2)	92.60±18.85 (48-132)	15.40±12.21 (03-49)	19.14±13.51 (03-47)	<0.001*
Creatinine (mg/dL)	0.85±0.18 (0.6-1.3)	6.24±4.13 (1.5-21.4)	4.78±2.77 (1.5-13.3)	<0.001*
Urea (mg/dL)	22.88±4.48 (17-36)	84.86±40.76 (32-212)	81.08±41.79 (21-169)	<0.001*
Random Blood Glucose (mg/dL)	97.72±16.10 (74-132)	101.44±19.21 (70-138)	259.74±120.50 (158-532)	<0.001*

[Table/Fig-1]: Comparison of Control group with CKD-ND and CKD-DM.
Data was presented as Mean±SD. *significant one-way ANOVA

Groups	Correlation coefficient	r	p-value
Control Group	eGFR and Cystatin-C	-0.317	0.025*
	eGFR and Creatinine	-0.788	<0.001*
	Cystatin C and Creatinine	0.242	0.090
Non-Diabetic With CKD	eGFR and Cystatin-C	-0.447	0.001*
	eGFR and Creatinine	-0.765	<0.001*
	Cystatin C and Creatinine	0.493	<0.001*
Diabetic With CKD	eGFR and Cystatin-C	-0.324	0.022*
	eGFR and Creatinine	-0.819	<0.001*
	Cystatin C and Creatinine	0.259	0.069

[Table/Fig-2]: Correlation analysis between Serum Cystatin-C, Serum Creatinine and eGFR.
*significant with 5% level of significance

There is a strong negative correlation between serum creatinine and eGFR in CKD-ND (Group-II) when compared to CKD-DM (Group-III).

[Table/Fig-3] shows the relative efficacy of eGFR equations in detecting CKD among the three groups. CKD-EPI Creatinine Cystatin Equation (2012) is showing 98% sensitivity and 96% accuracy among the various equations. Correlation analysis showed a negative correlation between Cystatin-C and eGFR among Group-II and Group-III when compared with controls. The correlation co-efficient was -0.447 and p-value was <0.001 for Group-II and r was -0.324 and p-value was 0.022 in Group-III. This indicates that there was a significant negative correlation between Cystatin-C and eGFR in Group-II compared to controls.

Formula	Group	Sensitivity	Specificity	PPV	NPV	Accuracy
CKD-EPI Creatinine Equation (2009)	Control Group	0.0%	98.0%	0.0%	100.0%	98.0%
	ND with CKD Group	100.0%	0.0%	98.0%	0.0%	98.0%
	DM with CKD Group	100.0%	0.0%	92.0%	0.0%	92.0%
CKD-EPI Creatinine Cystatin Equation (2012)	Control Group	0.0%	94.0%	0.0%	100.0%	94.0%
	ND with CKD Group	98.0%	0.0%	98.0%	0.0%	96.0%
	DM with CKD Group	97.8%	25.0%	93.8%	50.0%	92.0%
CKD-EPI Cystatin C Equation (2012)	Control Group	0.0%	88.0%	0.0%	100.0%	88.0%
	ND with CKD Group	93.9%	0.0%	97.9%	0.0%	92.0%
	DM with CKD Group	91.3%	25.0%	93.3%	20.0%	86.0%
MDRD Study Equation	Control Group	0.0%	98.0%	0.0%	100.0%	98.0%
	ND with CKD Group	100.0%	0.0%	98.0%	0.0%	98.0%
	DM with CKD Group	100.0%	0.0%	92.0%	0.0%	92.0%

[Table/Fig-3]: Relative efficacy of eGFR equations in detecting CKD in all three groups.

DISCUSSION

The detection of decline in renal function at an early stage by using the diagnostic tools like Cystatin-C, can reduce and delay the complications there by improving the quality of life. Estimation of GFR is one of the important steps in the diagnosis of CKD. In this study, we aimed at evaluating the serum Cystatin-C, Serum Creatinine levels and calculation of eGFR using various equations in diabetic and non diabetic patients. The levels of serum Cystatin-C were significantly increased in non diabetic patients with CKD compared to controls. eGFR levels were significantly decreased in Non diabetic patients with CKD compared to controls. The levels of serum Cystatin-C were decreased in diabetic patients with CKD compared to Non diabetic patients with CKD. An eGFR levels were increased in diabetic patients with CKD compared to Non diabetic patients with CKD. This might be due to the selection of subjects based on duration of Diabetes mellitus. In Non diabetics with CKD the increment in serum Cystatin-C levels indicate the severity of renal dysfunction which is due to the manifestation of glomerular process before the tubular phase [9].

Many individuals with type 2 diabetes passes through a period of pre-diabetes and may experience renal dysfunction, detection of CKD at early stage is important as early intervention can slow the loss of kidney function, that improves survival and quality of life. The inability of creatinine to detect early decline in GFR is due to the fact that serum creatinine levels only begin to rise above the normal range when approximately 50% of renal function is already lost, suggesting that GFR can change before serum creatinine becomes abnormal [17].

Studies suggested that serum Cystatin-C is a more sensitive marker for detecting early changes in glomerular filtration in type 2 diabetic patients than creatinine-based measurements [18]. Cystatin-C produced by a majority of nuclear cells is a non-glycosylated protein of 120 residue polypeptide chain with a molecular mass of 13 kDa [19]. The level of serum Cystatin-C raises earlier than serum creatinine since it is freely filtered by the glomeruli and catabolized by the proximal tubules without secretion thus, it can be considered as a good marker for estimating glomerular filtration rate. Cystatin-c is not affected by nutritional status and inflammatory processor and dietary factors. It remains unaffected with age or muscle mass unlike creatinine. Based on the above key factors it can be considered as a potential substitute for creatinine [20-22].

This suggests that serum Cystatin-C levels are related to impairment in tubular function and can be suggested as a early marker of renal diseases. After glomerular filtration, it is fully catabolized in the proximal renal tubules and is not returned into blood circulation; only small amounts of Cystatin-C is excreted in urine, which indicates that it is produced at a relatively constant rate irrespective of muscle mass. Cystatin-C provides a better estimate of GFR than estimating equations based on serum creatinine. Cystatin-C is a good marker of renal function and correlates better to direct measurement of GFR. Cystatin-C can be replaced by creatinine, due to its constant production rate and less variability than that of creatinine [9].

In this study we found that concentration of serum Cystatin-C has negative correlation with eGFR which indicates that there is a possibility of renal damage in non-diabetic subjects with CKD. The correlation between eGFR and serum Cystatin-C and Creatinine was found to be statistically significant among Non Diabetic patients with CKD and Diabetic patients with CKD as compared to controls. Serum creatinine does not rise until there is a reduction in GFR (40 mL/min/1.73 m^2) this insensitivity is associated for small to moderate decreases in GFR in creatinine blind GFR area (40-70 mL/min/1.73 m^2) which ultimately results in a delay in detection of renal damage. So serum creatinine may not be a good parameter for estimation of GFR at reduced levels of glomerular function. Though serum creatinine levels are normal during the early stage of kidney disease, it does not necessarily indicate normal renal function. This is because the serum creatinine levels come into picture when 50% of the kidney function is deteriorated. The creatinine blind area is the range between 40 and 70 mL/min/1.73 m^2 where the actual decrease in GFR occurs initially. Cystatin-C does not have any blind area. Early reduction in GFR will not be detected with creatinine testing, whereas cystatin-C will show a true positive reduction in GFR. Cystatin-C helps to identify a "preclinical" state of kidney dysfunction that is not detected with serum creatinine or estimated GFR [23]. It was found that levels of serum creatinine were raised in over 96% of patients that had reduced renal function in comparison with serum Cystatin-C levels. Cystatin-C might offer an advantage to traditional CKD markers with respect to early detection of diabetic nephropathy and its progression which helps in timely intervention preventing further complications. Since creatinine estimation has limited value in CKD prognosis, hence the study focuses on Cystatin-C as it is extremely sensitive to minor changes in GFR.

LIMITATION

The reason for non-significant correlation may be due to non linearity and small sample size. Further studies are recommended by the inclusion of biochemical parameters like NGAL, Beta 2 microglobulin and urinary angiotensinogen for better outcome and quality of life in CKD patients.

CONCLUSION

Though serum Cystatin-C assays are quite expensive compared to conventional serum creatinine assays, estimation of serum cystatin-c can be used as a better diagnostic test to screen patient when serum creatinine level is inconclusive in certain individuals with long duration of diabetes, uncontrolled diabetes and hypertension. Diagnostic accuracy is favourable for serum Cystatin-C when

compared with serum creatinine in patients with decreased renal function. However, it is more sensitive for the estimation of eGFR than serum creatinine.

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PARTICULARS OF CONTRIBUTORS:

1. Research Scholar, Department of Biochemistry, Shri B.M Patil Medical College, BLDE Deemed to be University, Vijayapura, Karnataka, India.
2. Professor, Department of Biochemistry, Shri B.M Patil Medical College, BLDE Deemed to be University, Vijayapura, Karnataka, India.
3. Professor and Head, Department of Biochemistry, Chalmeda Anand Rao Institute of Medical Sciences, Karimnagar, Telangana, India.
4. Lecturer in Statistics, Department of Community Medicine, Shri B.M Patil Medical College, BLDE Deemed to be University, Vijayapura, Karnataka, India.
5. Associate Professor, Department of Biochemistry, Shri B.M Patil Medical College, BLDE Deemed to be University, Vijayapura, Karnataka, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. S. Sangeeta,
Research Scholar, Department of Biochemistry, Prathima Institute of Medical Sciences, Nagunur,
Karimnagar-505417, Telangana, India.
E-mail: samudralasangeeta@gmail.com

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Evaluating the Efficacy of Myeloperoxidase and other Biochemical Parameters in the Diagnosis of Chronic Kidney Disease among Diabetic patients: A cross-sectional Study

Samudrala Sangeeta[1], Jeevan Ambekar[2], Tunguthurthi Sudhakar,[3] Nilima Dongre[4]

[1]Ph.D scholar, Department of Biochemistry, Shri B.M.Patil Medical College, BLDE (Deemed to be University), Vijayapur, [2]Professor, Department of Biochemistry, Shri B.M.Patil Medical College, BLDE (Deemed to be University), Vijayapur, [3]Professor of Biochemistry, CAIMS, Karimnagar, [4]Associate Professor, Department of Biochemistry, Shri B.M.Patil Medical College, BLDE (Deemed to be University), Vijayapur.

ABSTRACT

Introduction: Chronic kidney disease (CKD) is a public health problem across the globe which is characterized by the accumulation of various substances like urea, creatinine, electrolytes, water, in the human body. CKD patients are more prone to increased risk of developing oxidative stress due to metabolic disorders, immunodeficiency and persistent infections and inflammation. Myeloperoxidase may participate as one of the mediators of oxidative modification of biomolecules/tissues and contribute to the development of co-morbidities and complications in patients with CKD.

Aims and objectives: The present study was undertaken to assess the role of Myeloperoxidase, HbA1c, Urea, Creatinine urine microalbumin and eGFR in chronic kidney disease in diabetic and non diabetic patients.

Materials and Method: A cross sectional study consisting of two groups with 50 participants each was carried out. Group-I included 50 Chronic kidney disease patients without Diabetes mellitus (CKD-ND) & Group-II included 50 patients of Chronic kidney disease with Diabetes mellitus (CKD-DM). Myeloperoxidase, HbA1c, Urea, Creatinine and urine microalbumin were estimated in the blood and urine samples by using Erba & ELISA kits on XL-640 clinical chemistry analyzer and ELISA reader.

Results: The values of Myeloperoxidase were statistically decreased in diabetic patients with chronic kidney disease(Group-II)when compared with Non diabetic patients with chronic kidney disease (Group-I). Myeloperoxidase levels were compared with Urea, Creatinine, Microalbumin and eGFR levels. eGFR levels showed a significant negative correlation with MPO levels.

Conclusion: The present study showed that decreased serum MPO can be used as an indicator for chronic kidney disease in diabetic patients which can prevent further complications. MPO levels decline steadily as CKD progresses, which might be due to the inhibitory effect of uraemic toxins on the enzyme.

Keywords: *Myeloperoxidase, Chronic Kidney Disease, Diabetes mellitus, Estimated glomerular filtration rate (eGFR), urine Microalbumin.*

Corresponding author:
Ms. Samudrala Sangeeta,
Ph.D Scholar, Department of Biochemistry,
Shri B.M.Patil Medical College, BLDE (Deemed to be University), Vijayapur.
E-mail id: samudralasangeeta@gmail.com

INTRODUCTION

CKD is a slow and progressive disorder of kidney dysfunction which is reported worldwide affecting 750 million people globally [1]. In India, diabetes and hypertension account for 40–60% cases of CKD [2]. Chronic kidney disease (CKD) is a global threat to

health in general and for developing countries like India, because of the dietary habits, socio economic status and life style.

CKD patients are at higher risk related to oxidative stress, metabolic disorders and other pathologies. Hence CKD is a major cause of morbidity and mortality due to lack of proper diagnosis and treatment. The mammalian heme peroxidase enzymes play a major role in human immune abnormalities. Heme peroxidases are the acceptors which utilize H_2O_2 to catalyze oxidative reactions. MPO is an oxidizing agent whose elevated levels have been associated with CAD, atherosclerotic lesions. Hence this study is first of its kind to correlate the association between MPO levels in CKD with and without diabetes.

MPO is produced by neutrophilic granulocytes which along with heme (as co-factors) is microbicidal/ bactericidal by producing HOCl, H_2O_2 and Cl^- anions. MPO is a basic arginine rich glycosylated protein (isoelectric point >10) [3] which is comprised of two subunits, encoded within a single mRNA molecule. Studies also revealed that apart from being bactericidal its major role also has been associated with non-microbial inflammatory process and neuro degenerative diseases. There are studies which correlate MPO as enzymatic source of bioactive lipids and other products which have emphasized that they adversely affect the cardio protective capacity of high density lipoproteins and as well induce endothelial dysfunction [4,5]. This study is an attempt to understand the role of MPO as a predictor for early detection of CKD in diabetic patients. Hence a cross-sectional study was carried out among CKD patients with and without diabetes. The following are the objectives :

1).Correlation of MPO levels with eGFR

2). Correlation of MPO levels with Microalbumin

MATERIALS AND METHOD

A cross sectional study was conducted in the department of Biochemistry in collaboration with Medicine and Nephrology, at central laboratory of Prathima Institute of Medical Sciences during the period of June 2015 to September 2016. Two comparative groups consisting of 50 study participants each were enrolled in the study. The study was approved by the ethical committee of the institute (IEC/PIMS: 2015/01). Age and sex matched healthy controls were enrolled from the allied hospital. Informed consent was obtained from all the participants in the study. Clinical history and demographic details of the patients were collected using structured questionnaire. In each group, subjects were selected by simple random sampling technique. The groups were divided as follows:

Group-I 50 CKD without Diabetes (CKD-ND)

Group-II 50 CKD with Diabetes (CKD-DM)

Inclusion criteria:

Age ≥ 40 years, and ≤ 70 years

Clinically Confirmed diabetic patients with duration of diabetes >5 years and <10 years

Clinically confirmed patients of CKD

Exclusion criteria:

Not willing to consent,

Duration of diabetes <5 years and > 10 years

Under aseptic conditions, 5ml random venous blood sample was collected from each participant by anti cubital vein puncture in a disposable syringe of which 2 ml was collected into a EDTA vaccutainer for the estimation of HbA1c and the remaining was transferred into a plain bulb. After centrifugation serum was separated and used for the estimation of biochemical parameters like Urea, Creatinine and Myeloperoxidase. A random urine sample of 5 ml was collected in a sterile container for the estimation of Microalbumin. Myeloperoxidase was estimated by ELISA method. Urea and Creatinine were estimated in serum sample and Microalbumin was estimated in the urine sample collected from the above subjects on XL-640fully automated analyser. eGFR was calculated using CKD-EPI formula.

Serum Urea is estimated by Berthelot method..[6]

GFR is calculated by using CKD-EPI creatinine equation 2009.

$GFR = 141 \times \min(S_{cr}/\kappa, 1)^{\alpha} \times \max(S_{cr}/\kappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018$ [if female] $\times 1.159$ [if black][7].

Glycosylated haemoglobin is a boronate affinity which was estimated by immuno chromatographic method using Nycocard reader.

Creatinine was estimated by Jaffe's Method. [8] Microalbumin is estimated by pyrogallol red method [9]. Myeloperoxidase is estimated by standard protocol using ELISA technique [10].

Statistical analysis: The results were expressed as Mean±SD and student't' test was done to compare the mean parameters. Correlation analysis were done using Karl Pearson's correlation coefficient. Statistical significance was considered at the level of 5% (p-value < 0.05). Statistical analysis was performed by SPSS version 20 .

RESULTS

The results of two groups were expressed as Mean±SD. Table1 shows the comparison of Group-I (CKD-ND) with Group-II (CKD-DM). It summarizes the Mean±SD of Serum Myeloperoxidase, Serum Creatinine, Urea, HbA1c,Urine microalbumin and eGFR. The Myeloperoxidase levels of Group-II were (7.59±3.71) which is significantly lower as compared to in Group-I (10.41±4.75). The eGFR levels were significantly decreased in Group-I (15.40±12.21) compared to Group-II (19.14±13.51) respectively. Microalbumin levels were higher in Group-II (138.78±90.47) when compared to Group-I (71.94±64.26). The Creatinine levels of Group-I were (6.24±4.13) as compared to (4.78±2.77) in Group-II. The Urea levels of Group-I were (84.86±40.76) as compared to (81.08±41.79) in Group-II. The values of HbA1c in Group-II (7.69±1.17) were higher when compared to Group-I (5.34±0.49). The results also reveals that the values of serum Myeloperoxidase were significantly decreased in Group-II when compared to Group-I. 'p' value(<0.001)was found statistically significant for Myeloperoxidase and Creatinine in Group-II.

Table 1: Comparison of CKD-ND and CKD-DM

Parameters	Group-I (CKD-ND) n=50 Mean± SD	Group-II (CKD-DM) n=50 Mean± SD	'p'- value
MPO ng/ml	10.41±4.75 (4.7-22.5)	7.59±3.71 (4.3-19.9)	<0.001
eGFR (ml/min/1.732 m^2)	15.40±12.21 (03-49)	19.14±13.51 (03-47)	<0.001
Microalbumin mg/L	71.94±64.26 (11.6-256.1)	138.78±90.47 (23-400)	<0.001
Creatinine(mg/dl)	6.24±4.13 (1.5-21.4)	4.78±2.77 (1.5-13.3)	<0.001
Urea(mg/dl)	84.86±40.76 (32-212)	81.08±41.79 (21-169)	<0.001
HbA1c%	5.34±0.49 (4.3-6.1)	7.69±1.17 (6.4-11.2)	<0.001

Correlation studies revealed a negative correlation between Myeloperoxidase and eGFR in Group-I (CKD-ND) and a positive correlation in Group-II (CKD-DM). The correlation co-efficient value 'r' was -0.005and 'p' value was 0.972 for Group-I (CKD-ND). The value of 'r' was 0.114 and 'p' value was 0.431 in Group-II (CKD-DM). This indicates that there was a significant positive correlation between Myeloperoxidase and eGFR in Group-II when compared with Group-I. The reason for non significant correlation may be due to non linearity and small sample size.

Table 2: Correlation between eGFR, Myeloperoxidase and Microalbumin

Groups	Correlation Coefficent	r	'p' value
Non Diabetic	eGFR & MPO	-0.005	0.972
	eGFR & Microalbumin	-0.348	0.013*
	MPO & Microalbumin	-0.136	0.345
Diabetic	eGFR & MPO	0.114	0.431
	eGFR & Microalbumin	-0.401	0.003*
	MPO & Microalbumin	-0.274	0.054

Note:*significant with 5% level of significance

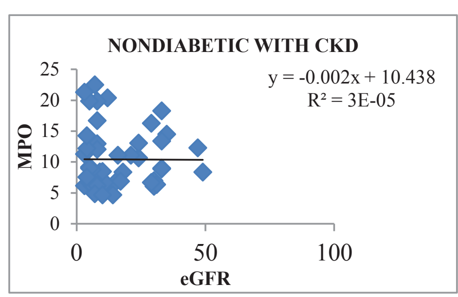

Figure1: CKD-ND (Group-I)

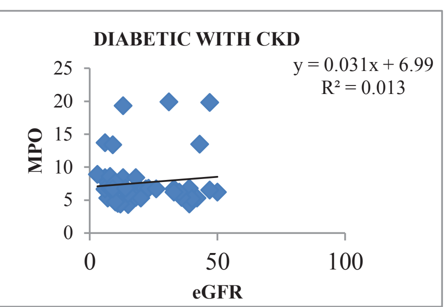

Figure 2: CKD-DM (Group-II)

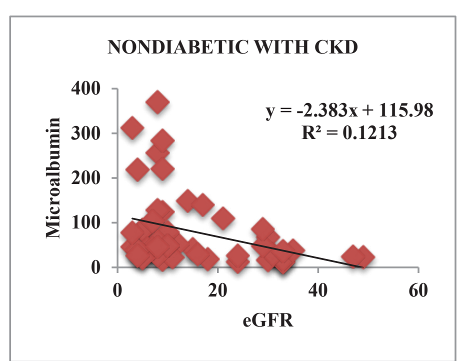

Figure 3: CKD-ND (Group-I)

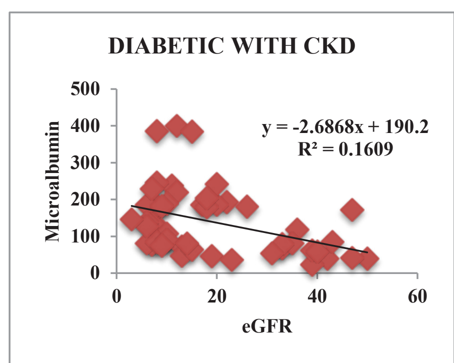

Figure 4: CKD-DM (Group-II)

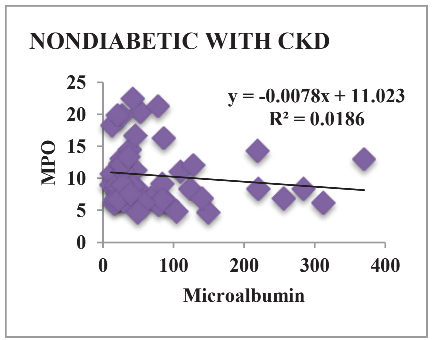

Figure 5: CKD-ND (Group-I)

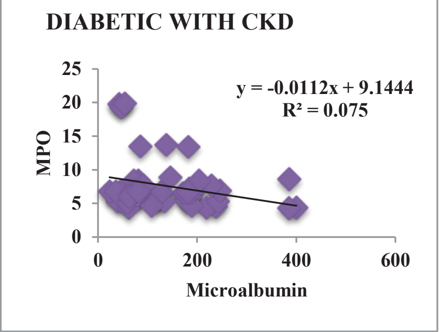

Figure 6: CKD-DM (Group-II)

DISCUSSION

Proteinuria is the common indication for end stage renal diseases (ESRD) which is more commonly seen in patients with CKD. It might contribute to the complications associated with the progression of the disease. It also influences the mortality in CKD patients. HOCl and MPO derived oxidants induces damage to the renal tissue there by contributing to the renal complications [11]. Lipid peroxidation is induced by extracellular MPO which is capable of catalyzing lipoprotein peroxidation in vivo, thus resulting in atherosclerosis which is common in CKD patients. Due to its cationic character it can bind to the negatively charged structures of endothelial cells and albumin [12].

In this study, we aimed at evaluating the levels of Myeloperoxidase, Microalbumin and calculation of eGFR using CKD-EPI equation in diabetic and non diabetic patients. There was a significant decrease in MPO levels, and an increase in microalbumin levels in Diabetics with CKD when compared with non diabetics with CKD.The decrease in MPO levels implies that MPO and its derived oxidants such as HOCl (hypochlorous acid) interferes with various cell functions which may contribute to damage of renal tissues resulting in the accumulation of uremic toxins which indicates decline in renal function.

Our study demonstrates that there is a progressive fall in mean serum MPO levels with advancing renal disease. MPO is an enzyme which has been shown to play an important role in the initiation and progression of atherosclerosis. Several mechanisms by which elevated levels of MPO can promote cardiovascular complications have been described [13]. Therefore this study was undertaken to determine the activity of MPO in patients with CKD. However, our results have shown that serum MPO levels are significantly lower in CKD patients with diabetes mellitus as compared to CKD patients without diabetes mellitus. The present study has also shown a significant negative correlation between Urea and MPO in CKD-DM; while no significant correlation was observed in the CKD-ND subjects. Hence, it could be speculated that the decline in MPO levels in CKD patients might be due to the inhibitory action of uraemic toxins, particularly CNO-, on this enzyme.

CONCLUSION

Estimation of serum MPO in CKD patients can be helpful in better prognosis. This study shows that serum MPO levels can be used an indicator for CKD patients with Diabetes mellitus in assessing the renal impairment and to prevent further complications. Microalbuminuria should be corrected at an early stage to delay the renal damage and development of cardiovascular complications in CKD patients.

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