BIOMARKERS AND OXIDATIVE STRESS PARAMETERS IN TYPE 2 DIABETES MELLITUS AND ACUTE KIDNEY INJURY

Thesis Submitted

To

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION AND RESEARCH

For the requirement of degree

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

Under

Faculty of Medicine

By

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Under the Supervision of
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October 2017

DECLARATION BY THE CANDIDATE

I Mrs. Munilakshmi U, hereby declare that this thesis titled: **Biomarkers and Oxidative stress Parameters in Type 2 Diabetes Mellitus and Acute Kidney Injury** is an Original Research Work carried out by me for the award of **Doctor of Philosophy** in the subject Biochemistry.

This study is carried out under the guidance of **Dr. K.N. Shashidhar,** Professor and Head, Department of Biochemistry and Co- guidance of **Dr. Lakshmaiah. V**, Professor of Medicine, **Dr. Muninarayana. C**, Professor of Community Medicine and **Dr. Madhavi Reddy** Clinical Nutritionist, Sri Devaraj Urs Medical College, a Constituent Institute of Sri Devaraj Urs Academy of Higher Education and Research.

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

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Subject: Central Ethics Committee clearance for Ph.D. Research work and Thesis

The Central Ethics Committee of Sri Devaraj Urs Academy of Higher Education and Research, Kolar has examined research proposal for the PhD Thesis titled **"Biomarkers and oxidative stress parameters in type 2 diabetes mellitus and acute kidney injury"** and the detailed work plan of the research work which will be carried under the guidance of Dr. K. N. Shashidhar, Professor & HOD, Biochemistry. The central ethics committee has unanimously decided to approve the Research proposal and grant permission to investigator to start her PhD Thesis. The interim and final report has to be submitted to the ethics committee after completion of the project for the issue of Central Ethics Committee certificate. The principal investigator is hereby advised to maintain the records of the research work details, data and consent form for not less than 5 years from the date of completion or termination of the research work.

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Mrs. Munilakshmi.U

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ABBREVATIONS

ACE	Angiotensin Converting Enzyme
AGEs	Advanced glycation end products
AKI	Acute kidney injury
AKIN	Acute Kidney Injury network
ARF	Acute renal failure
ATN	Acute tubular necrosis
AUC	Area Under the Curve
CKD	Chronic kidney Disease
CCR	Cystatin C to Creatinine Ratio
ClCr	Creatinine clearance
CPB	Cardio Pulmonary Bypass
Cys-C	Cystatin-C
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DN	Diabetic nephropathy
ESRD	End stage renal disease
FDA	Food and drug Administration
GD	Glomerular Dysfunction
GDM	Gestational Diabetes Mellitus
GE	Gastroenteritis
GFR	Glomerular filtration rate
GPx	Glutathione Peroxidase
HNL	Human Neutrophil Lipocalin
HbA1C	Glycated hemoglobin
hs-CRP	High sensitive c-reactive protein
HUS	Hemolytic uremic Syndrome
HTN	Hypertension

ICU	Intensive Care Unit
IL-β	Interleukin
KDIGO	Kidney Disease Improving Global Outcomes
KIM-1	Kidney Injury Molecule
LADA	Latent Autoimmune Diabetes of Adults
L-FABP	Liver type Fatty Acid Binding Protein
MDA	Malondialdehyde
MDRD	Modification of diet in renal disease
MMP	Matrix Metalo Proteinases
NGAL	Neutrophil gelatinase associated lipocalin
NO	Nitric oxide
OP	Organo Phosphate
PCr	Plasma Creatinine Concentration
PKC	Protein kinase
RIFLE	Risk, Injury, Failure, Loss and Endstage
ROC	Receiver Operating Characteristic curve
SCr	Serum Creatinine
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes mellitus
TD	Tubular Dysfunction
TGF-β	Transforming Growth factor-β
Ucr	Creatinine Concentration in Urine
UKPDS	United Kingdom Prospective Diabetes study
UTI	Urinary Tract Infection
V	Urine flow rate
VEGF	Vascular Endothelial Growth Factor
VXDS	Voluntary Exploratory Data Submissions

INTRODUCTION

Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder resulting from a defect in insulin secretion, insulin action or both; characterized by polydypsia, polyuria, polyphagia and loss of weight [1].

Insulin deficiency leads to chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism.

As Diabetes progress; tissue or vascular damage ensues, resulting in complications such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration [2, 3, 4, 5].

Classification:

Type 1 Diabetes Mellitus, Type 2 Diabetes Mellitus, Gestational Diabetes Mellitus and Other Specific Types.

Type 1 Diabetes Mellitus (T2DM): Type 1 diabetes occurs when the body's own immune system destroys the insulin- producing beta cells of the pancreas. Type1 diabetes affects children and adults of young age [5].

Type 2 Diabetes Mellitus (T2DM): Type 2 diabetes mellitus is the most common type of diabetes characterized by insulin resistance, which may be combined with relatively reduced insulin secretion. Defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. Age cannot be used to classify T1DM & T2DM, as Type 1 diabetes can occur at any age. Hence the honus is on measurement of fasting insulin & C- peptide levels in blood which helps to differentiate type of DM [5].

Gestational Diabetes Mellitus (GDM): GDM resembles T2DM in several aspects. GDM occurs in 2- 5% of all pregnancies and may improve or disappear after delivery. About 20- 50% of affected women develop T2DM later in their life [6].

Other types include

a) Maturity- Onset Diabetes of the Young (MODY)

b) Prediabetes [(Impaired Glucose Tolerance (IGT) or Impaired Fasting Glucose (IFG)]

c) Latent Autoimmune Diabetes of Adults (LADA)

Complications of Diabetes Mellitus: Complications of diabetes are observed in uncontrolled diabetes mellitus [7]. Complications are classified into (1) Acute complications and (2) Chronic complications

1. **Acute complications:** includes Diabetic Ketoacidosis, hyperglycemia hyperosmolar state, Hypoglycemia, Diabetic coma, Respiratory infections, periodontal disease, etc.
2. **Chronic complications:** leads to multi-organ damage. Chronic complications of DM are further divided into vascular and non-vascular complications.

   Vascular complications of DM are subdivided into:

   (a) Micro-vascular complications

   (b) Macro-vascular complications

   (a) Micro-vascular complications include: Retinopathy, Neuropathy, and Nephropathy

   (b) Macro-vascular complications include: Coronary Artery Disease, Peripheral Vascular Disease, Cerebro Vascular Disease etc [8, 9].

Non-vascular complications include gastroparesis, sexual dysfunction and skin changes. However, the multiple pathogenic processes are involved in all forms of complications. Risk of chronic complications depends on the duration of hyperglycemia. It is well recorded that T2DM may have a long asymptomatic period of hyperglycemia. Many individuals with T2DM land up with complications at the time of diagnosis. Randomized prospective clinical trials of individuals with Type 1 or Type 2 diabetes have demonstrated a reduction in chronic hyperglycemia which prevents or reduces the micro-vascular complications. However, despite long standing diabetes, some individuals never develop nephropathy or retinopathy. Reasons for control of microvascular complications may be due to well controlled diabetes or genetics. These observations however, create a suspicion that the genetic susceptibility may play a crucial role in the development of micro-vascular complications. However, the genetic locus responsible for these susceptibilities needs to be identified [8, 9].

Causes for Acute Kidney Injury (AKI) and Biochemical Basis with respect to DM

DM is the most frequent cause of end-stage renal disease in our society. Acute kidney injury (AKI) remains a clinical and prognostic problem of fundamental importance since incidences have been increased in recent years while mortality has not substantially been improved. As a matter of fact, not many studies particularly addressed the topic “AKI in diabetes mellitus.”

AKI refers to a syndrome resulting from multiple causative factors and occurs in a variety of clinical settings, with varied clinical manifestations, ranging from a minimal

elevation in serum creatinine to anuric renal failure. AKI is characterized functionally by a rapid decline in the glomerular filtration rate (GFR), and biochemically by the resultant accumulation of nitrogenous wastes such as blood- urea nitrogen and creatinine [10].

There are three major theories that have been proposed to explain how hyperglycemia may lead to chronic complications of DM [8, 9]

1. Increased intracellular glucose may lead to formation of advanced glycation end products (AGEs) example: fructosamine [Glycated albumin], glycated hemoglobin [HbA_1C] via non- enzymatic glycosylation of cellular proteins. Non-enzymatic glycosylation results from interaction of glucose with amino groups on proteins. AGEs have been shown to cross link proteins e.g. Collagen and extracellular matrix proteins which in turn accelerate atherosclerosis, promote glomerular dysfunction, reduce nitric oxide synthesis (NO), induce endothelial dysfunction, alter extracellular matrix composition and structure. The serum levels of AGEs correlates with the level of glycemia, and these products accumulate as GFR declines.
2. Second hypothesis explain how chronic hyperglycemia may lead to complications of DM which is based on the observation that hyperglycemia increase glucose metabolism via sorbital pathway.

Increased glucose *aldose reductase* increased phosphorylation ⟶ increased glycolysis → increased sorbitol → decreased myoinositol → cellular dysfunction. However, this theory with respect to humans using aldose reductase inhibitors has not demonstrated beneficial effects with respect to micro-vascular complications.

3. Third hypothesis proposes: increased glucose → increased diacylglycerol→ activation of certain isoforms of protein kinase C (PKC) → effect cellular events→ micro-vascular complications.

Example of PKC activation by glucose is alteration of transcription of genes for fibronectin, Type- IV collagen, contractile proteins and extracellular matrix proteins in endothelial cells and neurons in vitro.

Growth factors appear to play an important role in DM related complications. Vascular endothelial growth factor (VEGF) is increased locally in diabetic proliferative retinopathy and decreases after laser photo coagulation. Transforming growth factor- β (TGF- β) is increased in diabetic nephropathy and appears to stimulate basement membrane production of collagen and fibronectin by mesangial cells. Other growth factors such as platelet derived growth factor- I, epidermal growth factor, insulin like growth factor- I, growth hormone, basic fibroblast growth factor and even insulin have been suggested to play a role in diabetic complications [8, 9].

Although hyperglycemia serves as a triggering factor for complications of diabetes, it is still unclear whether the same pathophysiological process are operative in all complications or whether certain process predominating certain organs. Oxidative stress and free radical generation as a consequence of hyperglycemia may also promote the development of complications [9].

Diabetes control and complications trial (DCCT) have demonstrated that improvement of glycemic control reduce non-proliferative and proliferative retinopathy by 47%, microalbuminuria 39%, reduction in clinical nephropathy by 54% and neuropathy 60%. DCCT showed that improved glycemic control also slowed the progression of early diabetic complications and 5.8 additional years free from end stage renal disease (ESRD) [11].

Findings of DCCT, United Kingdom Prospective Study (UKPDS) and Kumamoto study support the idea that chronic hyperglycemia plays a causative role in the pathogenesis of diabetic micro-vascular complications [12]. These studies have proved that the value of metabolic control and emphasized the importance of

1. Intensive glycemic control in all forms of DM
2. Early diagnosis and
3. Strict blood pressure control in T2DM.

However such studies have not been documented in Indian population.

Acute Kidney Injury

Diseases of the kidney are diverse, ranging from Acute kidney injury (AKI) to End stage Renal Disease (ESRD). Incidence of kidney disease has been increasing at an

alarming rate across nations [13]**.** The general difficulty of an early diagnosis as well as increased rate of associated multisystem complications makes it a health problem that deserves a great attention worldwide. Treatment of kidney disease poses a major challenge to the health care system and global economy. Hence the detection and management of kidney diseases in the early, reversible and potentially treatable stages is of paramount importance [7]**.**

AKI is defined as rapid deterioration of renal function over hours to weeks, resulting in accumulation of nitrogenous waste products in the body, which may not be due to pre-renal factors. If some of these pre- renal factors are neglected may lead to Acute Tubular Necrosis (ATN). AKI may be reversible with proper diagnosis and treatment [7].

AKI is a syndrome caused by medical, surgical and gynecologic disorders, where the lesions afflicting at different locations of renal system and is mediated through different complex pathogenetic mechanisms. Clinical features and presentations of AKI vary from patient to patient and differs in its prognosis. Multi organ failure associated with many predisposing diseases makes prognosis worse. High risk patients of ARF/AKI include falciparum malaria, obstetrical causes such as septic abortions, IUD, toxemia of pregnancy etc. AKI is also documented following road traffic accidents, crush injuries, polytrauma, hemorrhagic shock, empyema gall bladder, enteric fever, leptospirosis, acute severe pancreatitis, severe gastrointestinal illness, viper snake bite, immune suppressed individual with bacterial or fungal sepsis, post operative sepsis or shock, elderly people, diabetes with sepsis, gangrene and extensive burns [7, 8].

Functional and structural alterations of renal microvasculature are important processes contributing to the pathophysiology of AKI. Persistent deficit in renal blood flow has been put forward to be intrinsically linked to tubular injury, inflammation and vascular alterations. In sepsis, AKI is triggered by both ischemic and inflammatory components, leading to loss of renal epithelial cells and tubular cell dysfunction. Oxidative stress (OS) is the most important factor that is associated with various pathologies during AKI [7].

AKI is largely asymptomatic and establishment of diagnosis relies on functional biomarkers such as serial serum creatinine measurements. Eventhough serum creatinine is a well established marker for kidney injury due to its delayed and unreliability limits its utility in AKI.

Serum creatinine does not accurately depict kidney function until a steady state has been reached which could take several days [14]. Animal studies have identified interventions that can prevent and or treat AKI if identified early in the course of disease, even before the serum creatinine begins to rise [14]. The paucity of early biomarkers has hampered our ability to translate these promising therapies to human AKI. Also lacking are reliable methods to assess the efficacy of protective or therapeutic interventions and early predictive biomarkers of drug toxicity [15].

Pursuit of improved biomarkers for the early diagnosis of AKI and its outcome is an area of intense contemporary research. A troponin like biomarker of AKI that is easily measured, unaffected by other biological variables and capable of both early detection and risk stratification would represent a tremendous advance in the care of hospitalized

patients [15]. Fortunately, understanding the early stress response of the kidney to acute kidney injury has revealed a number of potential biomarkers.

Biomarkers

Biomarkers (Biological markers) are cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells or fluids [16].

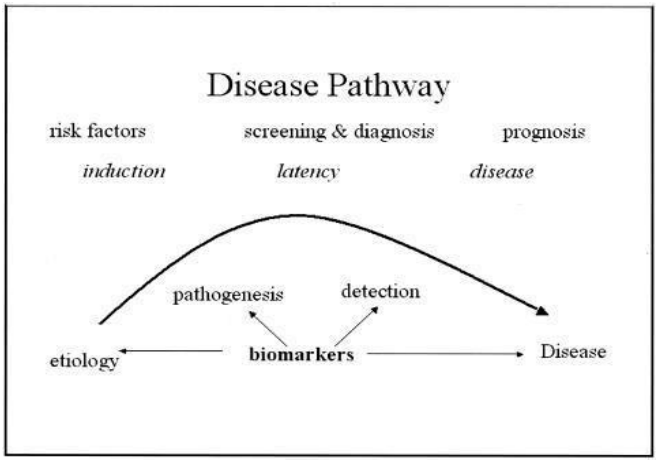
Definition of Biomarker recently has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, or pharmacological responses to a therapeutic intervention [17, 18].

Classification: Based on sequence of events from exposure to disease [19]

Two types:

I) Biomarkers of exposure: used in risk prediction

II) Biomarkers of Disease: used in screening, diagnosis and monitoring of disease progression



Disease pathway and potential impact of biomarkers [20]

Source: Biomarkers, uses and limitations Biomarkers 2000;1:182- 188.

Though biomarkers readily lend themselves to epidemiological investigations, they are also useful in the investigation of the natural history and prognosis of a disease. Schulte has outlined the capabilities of biomarkers to clinical research which include [21, 22]:

1. Delineation of events between exposure and disease
2. Establishment of dose- response
3. Identification of early events in the natural history
4. Identification of mechanisms by which exposure and disease are related
5. Reduction in misclassification of exposure or risk factors and disease
6. Establishment of variability and effect modification
7. Enhanced individual and group risk assessments

Oxidants and Antioxidants

Oxidative stress plays an important role in the development of vascular complications in type 2 diabetes.

Oxidative stress (or oxidant derived tissue injury) occurs when production of oxidants or reactive oxygen species (ROS) exceeds local antioxidant capacity, which includes oxidation of major macromolecules such as proteins, lipids, carbohydrates and DNA. Reactive Nitrogen species (RNS); by- products of nitric oxide (NO) plays a key role in maintaining various physiological functions but can also contribute to several pathological processes at high levels. RNS are comprised of nitrite ($NO2^-$), nitrate ($NO3^-$) and peroxy nitrite ($ONOO^-$). Studies have suggested that oxidative stress is a common pathogenic factor for the dysfunction of beta and endothelial cells of pancreas [23].

Beta cell dysfunction results from prolonged exposure to high glucose, elevated free fatty acid (FFA) levels, or a combination of both. Beta cells are particularly sensitive to ROS as they are low in free-radical quenching (antioxidant) enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) [24]. Thus, the ability of oxidative stress to damage mitochondria and marked blunt insulin secretion is not surprising.

To prevent ROS/ RNS caused cellular damage, the body has a defense system called antioxidant. Antioxidant systems prevent uncontrolled formation of free radicals and activated oxygen species, or inhibit their reactions with biological structures [25]. Antioxidants are any substances which can inhibit the oxidation of oxidative substrates. Antioxidants include molecules play a role in antioxidant defence, including endogenous (initially synthesized) and exogenous (consumed) antioxidants. They can be divided into two types depending on mechanism of action as chain breaking antioxidants or preventive antioxidants. Preventive antioxidants reduce the rate of chain initiation by deactivating metals, quenching singlet oxygen and removing hydroperoxides, including transferrin, ferritin, ethylenediamine tetraacetic acid (EDTA), ceruloplasmin, catalase, SOD and GPx. Chain breaking antioxidants are the molecules that have the ability to receive or donate an electron from a radical with the formation of stable byproducts including α-tocopherol, ascorbic acid, uric acid and β- carotene [25].

Epidemiology

DM and hypertension are the two leading causes of End stage Renal Disease (ESRD) throughout the world. Exponential increase in the prevalence of diabetes is seen

in the last three decades which poses huge clinical and economic challenges in many countries. Around 285 million adults in the age group of 20 years to 79 years were affected by ESRD in 2010 [26]. Epidemiological trends indicate that ESRD prevalence may increase further to 438 million by 2030. This accounts for a global increase of 54% (increase from a prevalence of 6.6% to 7.8% in 20 years). It is documented that around 70% of the people with diabetes live in developing countries; the largest numbers are in the Indian subcontinent and China [26].

India has largest number of diabetic subjects earning the dubious distinction of being termed the "Diabetes Capital of The World". According to the Diabetes Atlas 2006 published by the International Diabetes Federation, number of people with diabetes in India is currently around 40.9 million and expected to rise to 69.9 million by 2025 [1]

"Asian Indian Phenotype" refers to certain unique clinical and biochemical abnormalities in Indians which include increased insulin resistance, greater abdominal adiposity, lower adiponectin and higher highsensitive C-reactive protein levels. This phenotype makes Asian Indians more prone to diabetes and a part of this is due to genetic factors. However, the primary driver of the epidemic of diabetes is the rapid epidemiological transition associated with changes in dietary patterns and decreased physical activity as evinced from the higher prevalence of diabetes in the urban and rural population [27].

Average age of onset of diabetes is 42.5 years, according to World Health Organization; approximately 850,000 patients develop kidney disease every year due to various causes. AKI remains a fundamental problem in hospitalized patients worldwide.

Incidences of AKI have increased steadily in recent years, reaching almost 20% in middle Europe. Studies conducted by Mehta and colleagues evaluated AKI epidemiology in diabetic patients and reported with a total number of 449,524 individuals (total prevalence of DM = 33%) [27].

Lacunae of the study

Incidence of DM is increasing rapidly and has become a global problem that to with developing countries such as India. Studies conducted globally and in Indian population have targeted the urban people. Few studies that have been done to find the microvascular complications such as Diabetic Nephropathy (DN) and AKI in rural population are neither conclusive nor clear. However, studies regarding DN and AKI that are done till date have tried to find the association of kidney injury molecules or biochemical parameters after the occurrence of DN. Among the few studies that have been done to find the cause or consequence of DN with control or uncontrolled diabetes, duration of DM and causes for AKI with and without Diabetes needs to be addressed.

Since, Kolar is a drought hit area and the people here depend on ground water for day today activities. The occupations in rural population are mainly agriculture and silkworm rearing. Agriculturists are chronically exposed to insecticides and pesticides, this is known to affect peptide insulin and insulin resistance, which may alter oxidants, antioxidants, inflammatory molecules and ultimately damage the tubulointerstitium.

Majority of this population are ignorant, illiterate and are not having much awareness of DM cause and/ or consequence of it and also related complications. R L Jalappa Hospital and Research Centre a teaching hospital of Sri Devaraj Urs Medical

College, constituent of Sri Devaraj Urs Academy of Higher Education and Research statistics have documented that Kolar is an endemic area for Leptospirosis, Urinary Tract Infections, Snakebite, Gastroenteritis, OP poisoning, Hypertension and cardiovascular diseases; mainly coronary artery diseases landing with Cardiopulmonary bypass. Majority of diabetes and sequelae of diabetes with predisposing factors have ended up with kidney injury and are addressed by the physicians. These patients have either diabetes or diabetes related complications and/ or associated comorbidties.

As there are very few studies highlighting the importance and usefulness of newer biomarker for diagnosis of AKI/ ARF and associated complications; this made us to take up the study and propose the expediency of NGAL as a biomarker compared with other biomarkers and also estimate oxidants, antioxidants and inflammatory molecules in this population with diabetes and comorbid conditions which in future may help clinicians in early diagnosis and management of AKI and DM.

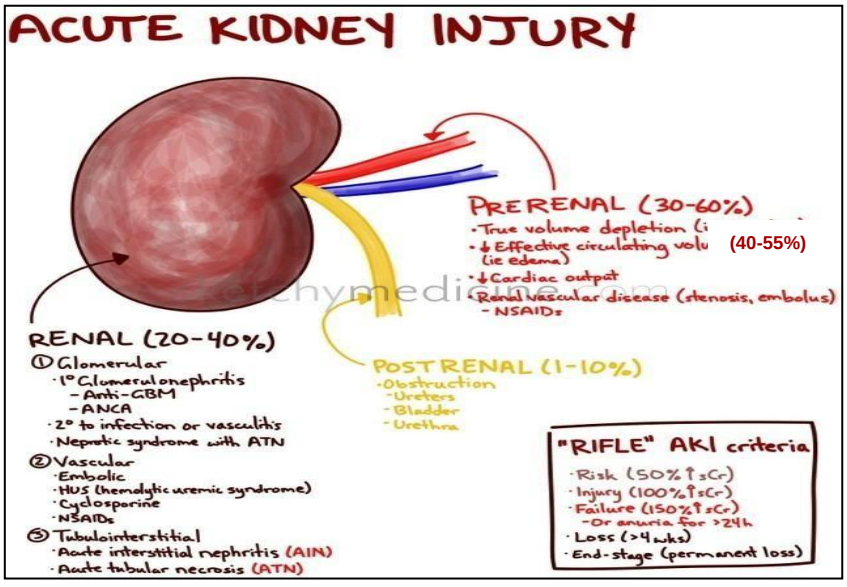
REVIEW OF LITERATURE

Acute Kidney Injury

AKI is a heterogeneous syndrome defined as rapid (hours to days) decline in the glomerular filtration rate (GFR) resulting in the retention of metabolic waste products, such as urea, creatinine and dysregulation of fluid, electrolyte and acid base homeostasis [28].

AKI represent a broad constellation of pathophysiologic process of varied severity and etiology. These include decrease in GFR as a result of hemodynamic disturbances that disrupt normal renal perfusion without causing parenchymal injury, partial or complete obstruction to urine flow and a spectrum of processes with a characteristic pattern of glomerular, interstitial, tubular or vascular parenchymal injury [29].

The term Acute Kidney Injury is largely replaced the older term Acute Renal Failure (ARF). AKI attempts to bring the small acute and transient decrements in kidney function with serious adverse outcomes [29].



Causes of AKI, RIFLE [28]

Source: Google pictures, www.google.co.in

Causes of AKI [30]

1. Prerenal: Diseases characterized by effective hypoperfusion of the kidneys in which there is no parenchymal damage to the kidney such as Gastroenteritis.
2. Intrinsic: Diseases involving the renal parenchyma. Example; Leptospirosis, snakebite, Cardiopulmonary Bypass, etc.
3. Postrenal otherwise obstructive: Diseases associated with acute obstruction of the urinary tract. Example; Urinary tract infection

Prerenal AKI: Prerenal AKI leads to azotemia. Azotemia is the most important cause of AKI accounting for approximately 40% to 55% of all cases of AKI. Azotemia results from kidney hypoperfusion due to reductions in actual or effective arterial blood volume (EABV). Common conditions causing true hypovolemia include hemorrhage secondary to traumatic, gastrointestinal or surgical cases, gastrointestinal loss due to vomiting, diarrhea or nasogastric or suction and renal loss may be due to overdiuresis or diabetes insipidus and third spacing such as pancreatitis or hypoalbuminemia [30].

Note: *Third spacing is defined as movement of fluid from intravascular space example; blood vessels into the interstitial or "third" space, the non functional area between cells.*

This can cause potentially serious problems such as edema, reduced cardiac output and hypotension. Major examples of third space include peritoneal cavity and pleural cavity. In addition, cardiogenic shock, septic shock, cirrhosis, hypoalbuminemia and anaphylaxis or pathophysiologic conditions which decrease EABV and are independent of total body volume status, resulting in reduced renal blood flow [30].

Prerenal azotemia reverses rapidly if renal perfusion is restored because of the integrity of renal parenchyma which has remained intact. However, severe and prolonged hypoperfusion may result in tissue ischemia leading to Acute Tubular Necrosis (ATN). Therefore prerenal azotemia and ischemic ATN are part of a continual spectrum of manifestations of renal hypoperfusion [30].

Intrinsic AKI: Intrinsic or intrarenal acute kidney injury (AKI), occurs when direct damage to kidneys causes a sudden loss in kidney function. Most common causes of intrinsic acute kidney injury are (1) Acute tubular necrosis (ATN) (2) Acute glomerulonephritis (AGN) and (3) Acute interstitial nephritis (AIN) [29].

1. **Acute tubular necrosis (ATN) [30]**

Acute tubular necrosis (ATN) is a condition in which the small filtering tubes in the kidney are injured. ATN is a common cause of intrinsic acute kidney injury often seen in people who are already hospitalized. ATN may occur because of decreased blood flow in the kidneys. Decreased blood flow may be due to:

a) Cardiovascular or abdominal surgery.
b) Direct injury to kidney.
c) Severe burns.
d) Severe muscle injury or extreme physical exertion.
e) Medicines those are toxic to kidneys. Drugs that are non toxic to the kidneys in a healthy person may become toxic in a person who has already existing kidney disease or any other condition that may increase the risk of acute kidney injury. Example, heart failure, diabetes, or multiple myeloma.

2. **Acute glomerulonephritis (AGN) [29]**

Glomerulonephritis is a condition in which the tiny blood vessels in the kidneys become inflamed and damaged. Damaged glomeruli loose the filtering capacity of blood. Causes for Acute glomerulonephritis include:

a) Abnormal immune system response.
b) Lupus (systemic lupus erythematosus).
c) Wegener's granulomatosis, Good pasture's syndrome and other forms of vasculitis.
d) Bacterial or viral infections.

Symptoms of glomerulonephritis include blood and protein in urine, high blood pressure and swelling caused by fluid retention (edema).

3. **Acute interstitial nephritis (AIN) [29]**

Acute interstitial nephritis (AIN) is the inflammation of kidneys, caused by antibiotics or nonsteroidal anti-inflammatory drugs (NSAIDs). Example, naproxen or ibuprofen.

AIN may also be caused by streptococcal, viral or Legionella infection. Symptoms of AIN include a skin rash, fever and abnormal sediment in the urine.

Post- renal AKI: Postrenal azotemia occurs from obstruction of either the ureters of the bladder, outlet or urethra. Most common cause for postrenal azotemia is structural or functional obstruction of the bladder neck [30].

AKI resulting from obstruction accounts less than 5% of cases, in certain settings (eg. transplant), it can be as high as 6% to 10%. Clinically patients can present with pain

and oliguria, though these are neither specific nor sensitive. There is often a degree of overlap between these categories. Renal hypoperfusion may cause a spectrum of renal injury ranging from prerenal azotemia to overt acute tubular necrosis depending on its severity and duration.

As a result, precise categorization of AKI into one of these three groups may not always be possible and transitions between etiologic categories may occur [30].

Staging of AKI

Acute Dialysis Quality Initiative (ADQI) Group in 2002 proposed a new definition for ARF: Risk (R), Injury (I), Failure (F), Loss of kidney function (L) and End-stage kidney disease (E) termed as (RIFLE) criteria, Table 1. Later, the term acute renal failure was replaced by acute kidney injury (AKI), reflecting the fact that structural injury most certainly precedes an acute decline in kidney function [31]. RIFLE criteria define AKI based on three stages of increasing severity (R, I and F) and two outcome criteria based on the duration of renal replacement therapy (L and E). The R, I and F classes are based on either a relative increase in serum creatinine from baseline or an episode of oliguria [31].

Acute Kidney Injury Network (AKIN) Group has proposed a modification of RIFLE classification in September 2007 that includes the Risk, Injury and Failure criteria with the addition of 0.3mg/dL or higher increase in the serum creatinine level to the criteria that define Risk. [32].

The characteristic features of RIFLE criteria versus AKIN criteria are increase in serum creatinine of ≥50% developing over < 7 days compared to An increase in serum

creatinine of ≥0.3mg/dL or ≥ 50% developing over < 48 hours or Urine output of <0.5 mL/kg/hr for 6 hours versus Urine output of <0.5 mL/kg/hr for > 6 hours respectively [32].

Table 1: RIFLE Criteria [31]

Criteria	Serum Creatinine Criteria (Scr)	Urine output (UO) criteria
Risk (R)	Increased SCr of ≥0.3 mg dl^{-1} or increase to ≥150%−200% from baseline	<0.5 mL/kg/h for 6 h
Injury (I)	Increased SCr to >200%−300%	UO <0.5 ml kg^{-1} h^{-1} for >12 h
Failure (F)	Increase SCr to >300% from baseline or (Scr ≥4 mg dl^{-1}) (acute rise ≥0.5 mg/dl)	UO <0.3 ml kg^{-1} h^{-1} for >24 h
Loss (L)	Persistent AKI>4 weeks.	
End stage (E)	Complete loss of kidney function for more than 3 months	

Source: Modified from ADQI work group

Table 2: AKIN Staging of AKI [32]

Stage	Serum Creatinine Criteria (Scr)	Urine output (UO) criteria
1	≥ 50% or ≥ 0.3 mg/dL increase in serum creatinine	<0.5 ml kg^{-1} h^{-1} for >6 h
2	>100% increase in serum creatinine	<0.5 mL/kg/h for >12 h
3*	>200% or 4.0 mg/dL increase in serum creatinine, with an acute rise of ≥5 mg/dL	<0.3 mL/kg/h for > 24 h or Anuria (12 h)

*Stage 3 also includes patients requiring RRT independent of the stage (defined by SCr and/or UO) they are in at the moment they initiate RRT.

Source: Modified from ADQI work group

Kidney Disease Improving Global Outcomes (KDIGO) further in 2012 released their clinical practice guidelines for acute kidney injury (AKI), which build RIFLE criteria and the AKIN criteria [32].

KDIGO definition of AKI should suffice anyone or more of the following [33, 34]:

- Increase in serum creatinine by 0.3mg/dL or more within 48 hours
- Increase in serum creatinine to 1.5 times baseline or more within the last 7 days
- Urine output less than 0.5 mL/kg/h for 6 hours

Table 3: KDIGO Staging of AKI [33]

Stage	**Creatinine criteria**	**Urine output criteria**
1	1.5-1.9 times baseline or ≥0.3 mg/dL increase	< 0.5 mL/kg/h for 6 h
2	2-2.9 times baseline	< 0.5 mL/kg/h for 12 h
3	3 times baseline or Increase in serum creatinine to ≥4 mg/dL or Initiation of renal replacement therapy	< 0.3 mL/kg/h for 24 h or Anuria for ≥12 h

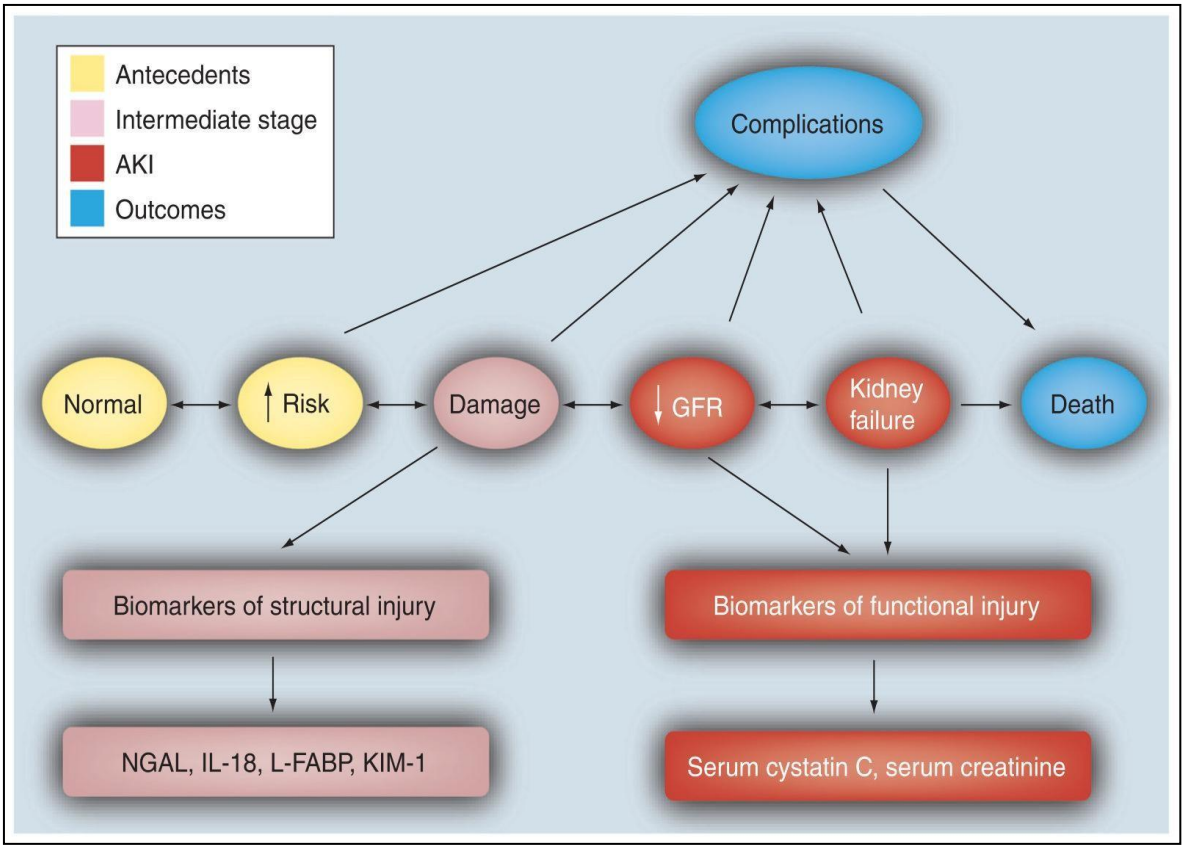
Source: Modified from ADQI work group

Utility of diagnostic criteria such as RIFLE, AKIN and KIDGO definition of AKI is limited by the fact that they rely on serum creatinine concentration, which can increase in cases of pre renal azotemia when there is no tubular injury and can be unchanged under conditions of significant tubular injury, particularly when patients have good underlying kidney function and significant kidney reserve [33]

Pathophysiology of AKI

Acute kidney injury (AKI) refers to a common syndrome that results from multiple causative factors and occurs in a variety of clinical settings, with varied clinical manifestations, ranging from a minimal elevation in serum creatinine to anuric renal failure. AKI is characterized functionally by a rapid decline in the glomerular filtration rate (GFR) and biochemically by the resultant accumulation of nitrogenous wastes such as blood-urea nitrogen and creatinine [14].

Figure 1: Conceptual framework of clinical continuum of AKI



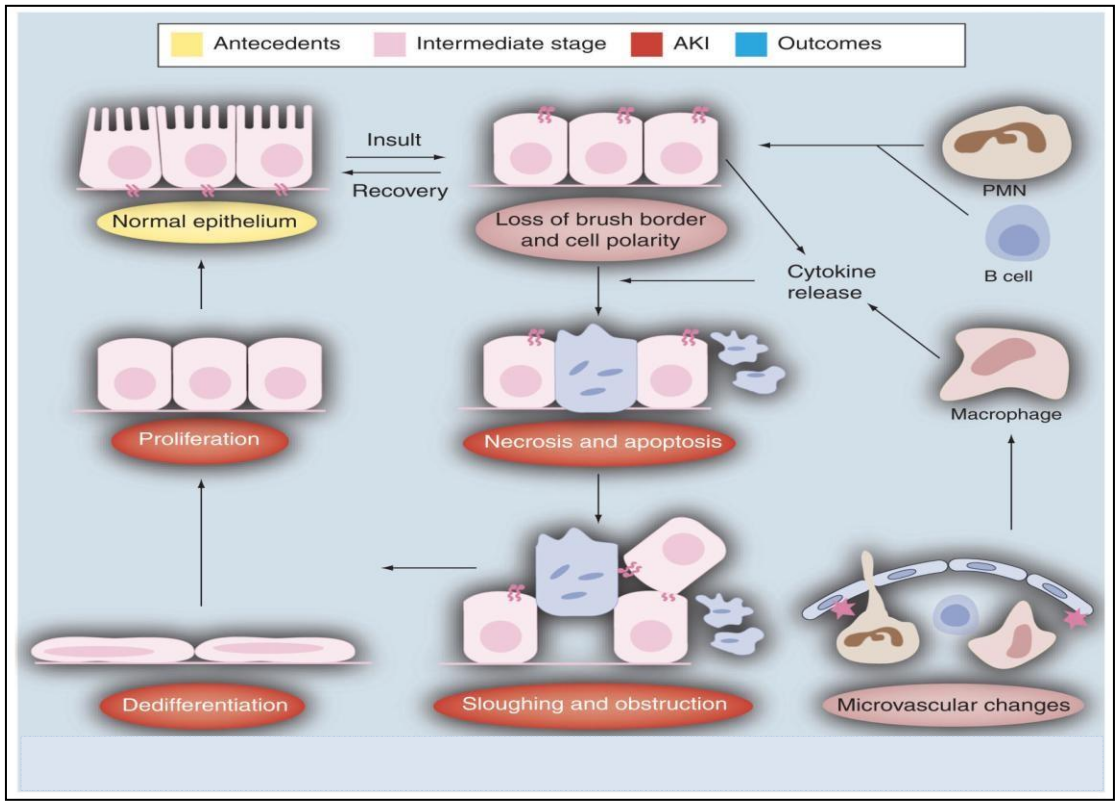
Source: Clinical continuum of acute kidney injury

J Am Soc Nephrol 2006;17:1503- 1520. [17]

AKI: Acute kidney injury; GFR: Glomerular filtration rate; NGAL: Neutrophil gelatinase associated lipocalin.

A conceptual framework of the clinical continuum of AKI has recently been proposed and is illustrated in Figure 1. Color-coded circles depict the various stages in the development of AKI. Based on current diagnostic considerations, AKI (in red) is defined as a reduction in GFR, as reflected by biomarkers of functional injury such as serum creatinine. Antecedents of AKI (in yellow) include risk factors such as increasing age. Nestled between the antecedents and AKI is an intermediate stage of kidney damage (in pink), representing a stage during which structural damage occurs without overt functional injury. Detection of this stage of damage requires emerging structural biomarkers as exemplified by neutrophil gelatinase-associated lipocalin (NGAL) [17].

Figure 2: Reciprocal cellular changes, clinical AKI continuum

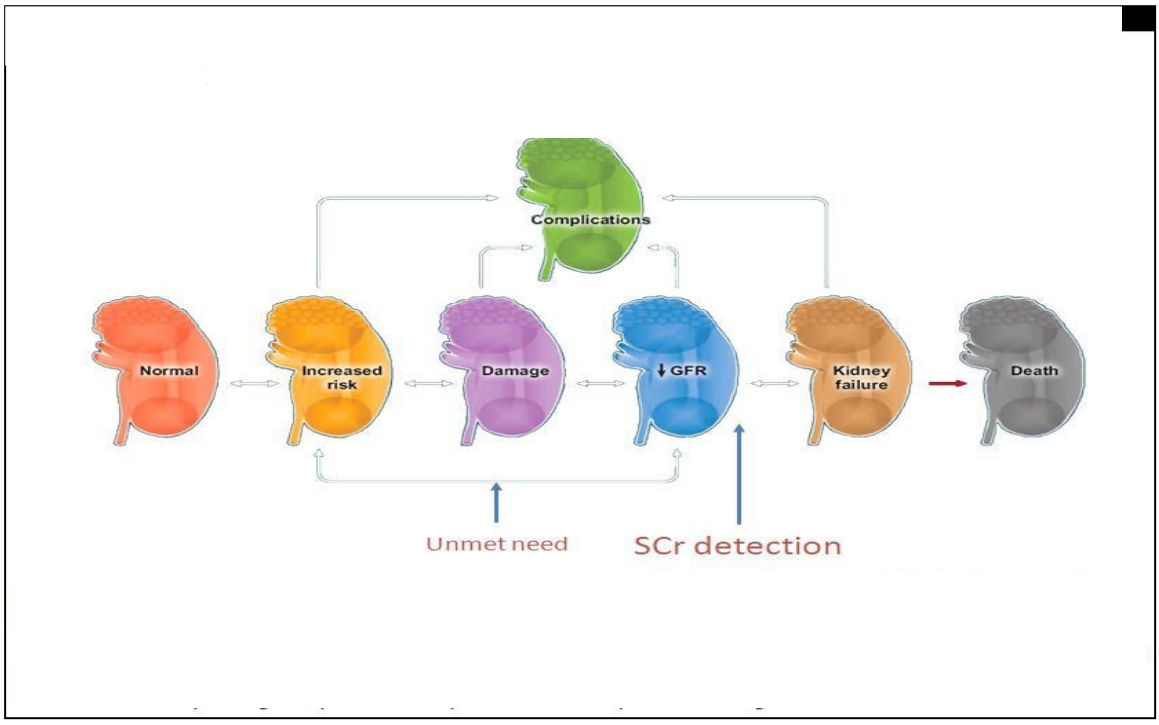


Source: Pathophysiologic continuum of acute kidney injury

J Am Soc Nephrol 2006;17:1503- 1520 [14]

The color-coded ellipses in Figure 2 reflect the reciprocal cellular changes occurring during each stage of the clinical AKI continuum. During the early-damage stage (in pink), only subtle and largely reversible changes, such as alterations in cell polarity and micro-vascular perturbations, are detected. A number of interventions applied at this stage have been successful in preventing and treating AKI in experimental studies. However, once AKI has set in (depicted in red), more severe and irreversible changes, such as cell death, desquamation and intratubular obstruction, become apparent. Although the kidney tubule cells do possess a remarkable ability to regenerate and repair after injury, most therapeutic interventions initiated in the established phase of AKI have been futile [14].

Figure 3: Spectrum of Kidney Injury



Source: Acute Kidney Injury Network group

Crit care 2007;11(2):R31 [17]

Biomarkers need to detect AKI at an earlier time point than the typically delayed rise in serum creatinine (Scr). This helps in rapid diagnosis and interventions that can be applied in a timely manner. A reduction in the GFR causes an increase in Scr. Increase in Scr occurs after possible kidney injury which has already occurred. Creatinine is not an ideal biomarker for diagnosing AKI because it is a marker of GFR and not kidney injury. There can be upto 48 hours delay for Scr levels to raise after kidney injury. This delay may often depict damage to the kidney which has already occurred. This delay in diagnosis prevents timely intervention and treatment of the patient suffering with AKI [17].

Acute Kidney Injury and Cardio Pulmonary Bypass (CPB)

Indicators for CPB such as pulmonary complications, Myocardial Infarction, older age may land up with acute deterioration of renal function, common complication following cardiac surgery. The incidence of AKI range from 7.7% to 42% depending on the criteria used to define AKI [35]. Even small degree of rise in serum creatinine level by only a 0.2-0.3 mg/dL is associated with a significant increase in morbidity and mortality after cardiac surgery. AKI after cardiac surgery is associated with adverse outcomes such as prolonged intensive care and hospital stay, dialysis dependency and increased long term mortality. AKI requiring dialysis occurs in upto 5% of patients after cardiac surgery and represents the strongest independent risk factor death in these patients [36].

Pathogenesis of cardiac surgery associated AKI is complex and multifactorial [37]. Reduced renal perfusion pressure, activation of proinflammatory mediators and direct nephrotoxicity are central in the pathogenesis of AKI- CPB [13].

CPB itself contributes to the pathogenesis via the systemic inflammatory response syndrome, sequelae of alterations in regional blood flow and vasomotor tone in the kidneys and generation of microemboli. CPB associated systemic inflammatory response syndrome is triggered primarily by the contact of blood components with the artificial surface of the bypass circuit.

Normally kidney function is autoregulated that the glomerular filtration rate (GFR) is maintained until the mean arterial pressure falls below 80mmHg. Instituting CPB itself decreases the effective renal perfusion pressure upto 30% altering the vasomotor tone and exposes the renal parenchyma to reduced oxygen tension, contributing to ischemia-reperfusion injury [38]. CPB induced erythrocyte hemolysis and activation of complement proteins further exacerbate CPB systemic inflammatory response syndrome and contribute to ischemia reperfusion injury [38].

Microemboli are formed during CPB and can be composed of combinations of fibrin, platelet aggregates, cellular debris, fat and air. The CPB system can filter emboli larger than 40µm; however, smaller emboli that are not effectively filtered can directly damage renal capillaries[8]. Oxidative stress due to decreased renal perfusion and ischemia in the medulla also contributes to the development of AKI [38].

Acute Kidney Injury and Snakebite

Snakebite is an important health concern in India. It is one of the highest occupational hazard worldwide. The spectrum of presentation may vary from mild local symptoms to rapid envenomation leading to sudden death. Statistical analysis has shown that India as one of the top country with maximum number of snakebite related

envenomations and deaths in a year. Around 19.2% and 55% of all snake bite related envenomations and deaths, respectively occur annually all over the world wide India has leading numbers [39]. AKI is an important consequence of snakebite envenomation seen in 5%- 29% of the hospitalized patients. This variation is due to differences in the type of snakes and degree of envenomation.

Snakebites are commonly reported morbidity in tropical countries, especially during some specific seasons. The complex nature of snake venom is responsible for the wide variety of pathogenic mechanisms involved in tissue and cellular injury which reflect the effects of neurotoxins, myotoxins, hematotoxins, nephrotoxins, and necrotoxins [40].

More than 100 different kinds of peptides or proteins, lipids, amines, carbohydrates etc. have been isolated from snake venoms, not all of which are toxic to humans [41]. Activation or inhibition of various coagulation proteins or platelets and endothelial disruption caused by phospholipases, serine proteases, metalloproteinases, disintegrins and C- type lectins lead to coagulopathy [42]. Hemorrhagins lead to spontaneous bleeding by directly injuring the vascular endothelium. Digestive hydrolases, hyaluronidase and polypeptide cytotoxins contribute to the local tissue necrosis seen after bites of some snakes [43].

Myotoxic phospholipase A_2 seen in venoms of some vipers and sea snakes is responsible for rhabdomyolysis which can later lead to AKI.

Permeability factors that increase extravastion of plasma from intravascular compartment and direct and indirect effects on cardiac muscle and vascular smooth muscle can lead to hypotension without any bleeding manifestations [41]**.**

Oligopeptides potentiating bradykinin action, sarafotoxins bearing homology to endothelin, vascular endothelial growth factor and natriuretic peptides are examples of such toxins seen in snake venom [18, 19]. Neurotoxins impair transmission at neuromuscular junction by acting either pre synaptically or post synaptically [44, 45].

Study conducted by Kanjanabuch T et al. suggests that hemodynamic response to venomous snake bite especially hemotoxic viper bite is similar to sepsis and alter renal hemodynamics (increased renal vascular resistance with decreased renal blood flow and decreased GFR) are probably central to pathogenesis of renal failure [46]

Hemorrhage leading to intravascular volume depletion, hemolysis, rhabdomyolysis and disseminated intravascular coagulation are other factors involved in pathogenesis. There seems to be a good correlation between the degree of renal failure and hemotoxic and myotoxic actions of snake venom [47].

Acute Kidney Injury and Hypertension

Hypertension, one of the most common worldwide diseases afflicting humans and is a major risk factor for stroke, myocardial infarction, vascular disease and kidney disease. Despite extensive research over the past several decades, etiology of most cases of adult hypertension is still unknown, and control of blood pressure is suboptimal in general population. Due to the associated morbidity and mortality and cost to society, preventing and treating hypertension is an important public health challenge. Fortunately,

recent advances and trials in hypertension research are leading to an increased understanding of the pathophysiology of hypertension and the promise for novel pharmacologic and interventional treatments for this widespread disease [48].

According to the American Heart Association (AHA), approximately 86 million adults (34%) in the United States are affected by hypertension, which is defined as a systolic blood pressure (SBP) of 140 mm Hg or more or a diastolic blood pressure (DBP) of 90 mm Hg or more [48]

Acute or severe hypertension can be life-threatening as it can cause end organ damage, particularly in the heart, brain, and kidney. The etiology of hypertension in acute kidney injury patients may be due to fluid overload as evidenced by suppression of the renin-angiotensin-aldosterone system and enhanced release of atrial natriuretic peptide. Although these changes are most prominent with severe disease, the incidence of hypertension is increased even in patients with a normal serum creatinine concentration. Both a familial predisposition to hypertension and subclinical volume expansion are thought to be important in this setting [49].

Acute Kidney Injury and Gastroenteritis (GE)

Gastroenteritis is an infection of the gut (intestines). The severity can range from a mild tummy (abdominal) upset for a day or two with mild diarrhoea, to severe diarrhoea and being sick (vomiting) for several days or longer. Many germs (viruses, bacteria and other microbes) can cause gastroenteritis [50].

Gastroenteritis is an infection of the gut (intestines) with viruses, bacteria or other microbes. It may develop diarrhoea, sickness (vomiting), abdominal pain and other symptoms.

The major gastrointestinal complications associated with AKI include anorexia, nausea and vomiting and upper gastrointestinal bleeding, primarily due to stress ulcers and gastritis.

AKI is recognized as a hypercatabolic state, however the precise mechanism for the hyper catabolic is not known. A variety of factors including the hypercatabolic nature of underlying disorders (eg, sepsis, rhabdomyolysis, trauma); hormonal disturbances including elevated levels of glucogon, catecholamines, growth hormone and cortisol and insulin resistance; and acute uremia perse, which has been associated with accumulation of proteases in the blood [50].

As a result of these factors, protein catabolic rate may exceed 1.5 to 1.7g/kg/day. AKI may also be associated with impaired carbohydrate metabolism with hyperglycemia as a result of insulin resistance and accelerated hepatic Gluconeogenesis and impaired lipid metabolism [24].

Acute Kidney Injury and Urinary Tract Infection (UTI)

UTI is one of the most common bacterial Infections which are common among older people. The prevalence of UTI in the elderly is much higher than younger individuals. At least 20% women and 10% of men aged 65 years or older have bacteriuria [51].

UTI can be either asymptomatic or symptomatic, characterized by a wide spectrum of symptoms ranging from mild irritative voiding to bacteremia, sepsis, shock or even death. In specific patient groups, urosepsis may show high mortality rates of 25% to 60% [51]. Sepsis is one of the most common triggers of acute kidney injury (AKI) and about 60% patients with septic shock developed AKI [52]. Acute UTI may cause sudden deterioration of renal function, especially for urinary tract obstruction [53, 54]. AKI is associated with high morbidity and mortality during acute care [55]. Patients with severe AKI were under increased risk of end stage renal disease and even death after hospital discharge. Since sepsis-related AKI leads to poor outcomes and increased healthcare costs, identification of the risk factors for development of AKI in patients with UTI is critical. However, there were few studies focusing on this important issue [56].

Acute Kidney Injury and Leptospirosis

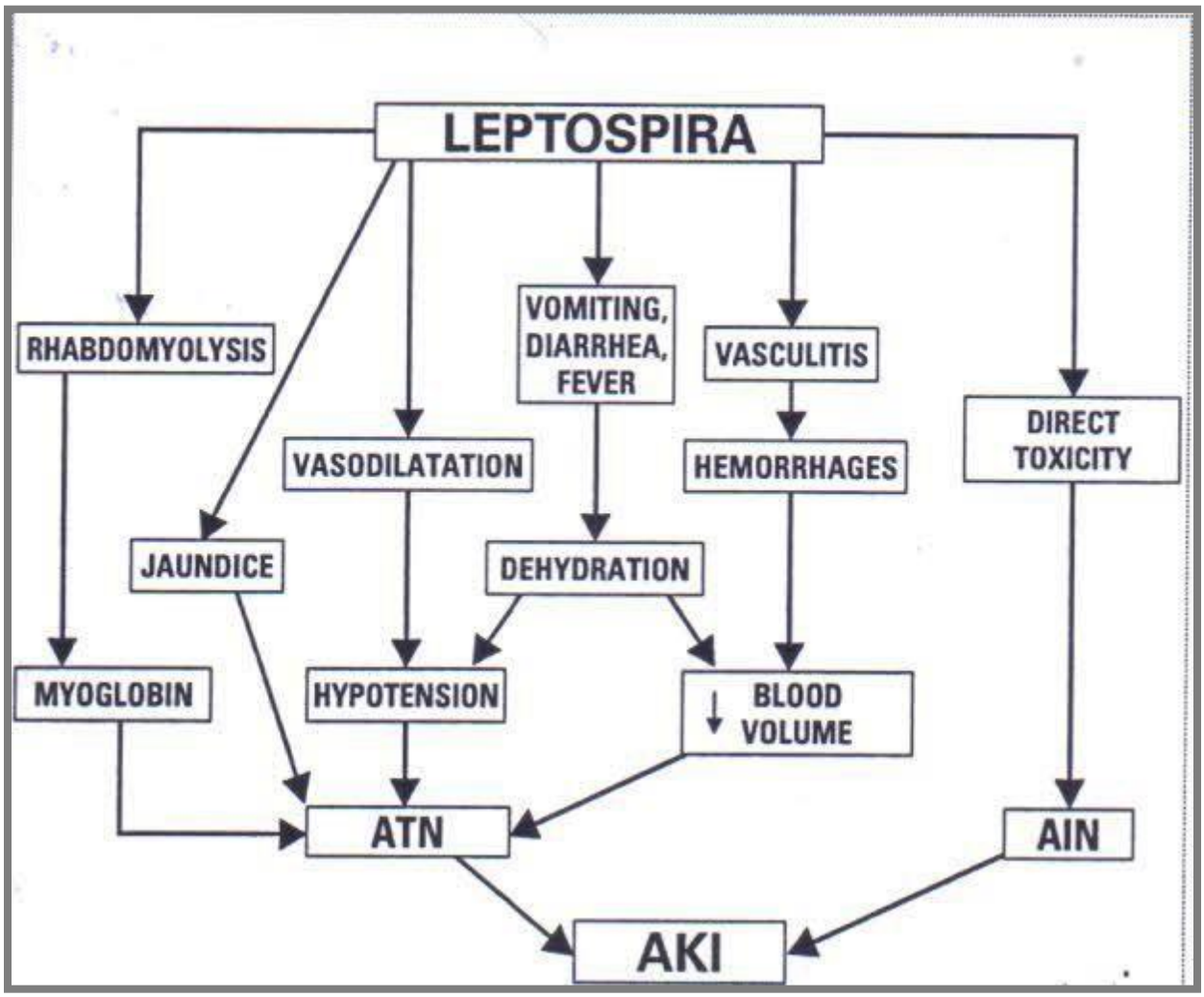
Leptospirosis is a zoonosis caused by a microorganism of the *Leptospira* genus, an obligate aerobic spirochete of worldwide distribution, with two species: *L. interrogans* (pathogenic) and *L. biflexa* (non-pathogenic and saprophytic) [57].

Rat is the major reservoir of leptospira, mainly in urban areas. Transmission to man occurs through direct contact with blood, tissues, organs, or urine of infected animals, or through indirect contact, when injured mucosa or skin is exposed to contaminated water [58, 59]. In tropical countries, leptospirosis is an endemic disease, with outbreaks occurring during the rainy season, coinciding with flooded areas [60].

Leptospirosis is an infectious vasculitis. In the severe form, patients can develop hemodynamic alterations secondary to hypovolemia due to dehydration and the direct effects of the toxins that damage the vascular endothelium and increase permeability [61].

Leptospirosis is an important cause of AKI. The incidence of AKI varies from 10% to 60%, depending on the severity of the disease, age, and definition of AKI [62]. Acute kidney injury due to leptospirosis usually presents in the non-oliguric form with hypokalemia, which can be detected in 41% to 45% of the patients with leptospirosis associated with AKI [63].

Renal impairment is a frequent complication in patients with severe form of leptospirosis, mainly characterized by an association of interstitial and tubular damage [63]. Several factors are involved in AKI in leptospirosis, including direct nephrotoxic action of the leptospira, hyperbilirubinemia, rhabdomyolysis and hypovolemia. Major histological findings are acute interstitial nephritis and acute tubular necrosis. Leptospirosis-induced AKI is usually nonoliguric and hypokalemic. Tubular function abnormalities precede a decline in the glomerular filtration rate, which could explain the high frequency of hypokalemia [63].



J Bras Nefrol 2010;32(4):400- 407

Source: Leptospirosis- associated acute kidney injury [63]

Acute Kidney Injury and OP Poisoning

Organophosphate poisoning occurs most commonly as a suicide attempt in farming areas of the developing world and less commonly by accident [64]. Exposure can be from drinking, breathing in the vapors, or skin exposure [65].

According to the World Health Organization's estimates, there are >3 million cases of OP pesticide poisoning annually; among these, >250,000 deaths are caused by intentional self poisoning, accounting for 30% of suicides worldwide [66, 67].

Mechanism of OP toxicity includes irreversible inhibition of enzyme cholinesterase, leading to massive accumulation of neurotransmitter acetylcholine within the synaptic cleft, and, thus, overstimulation of nicotinic and muscarinic receptors in the central and peripheral nervous system, which form various toxidromes [68, 69]. Patients with OP poisoning have a higher overall incidence and crude risk of developing AKI.

Cavari et al proposed that OP effects on the renal system could be due to direct parenchymal intoxication, secondary to hemodynamic instability, or seizure-induced rhabdomyolysis.

In summary, we hypothesize that bradycardia, dehydration with hypovolemia, and hypotension that induced renal hypoperfusion, direct toxic effects on renal parenchyma and convolutions, endothelial cell damage, activation of immune and inflammatory responses, formation of free radicals, convulsive seizure, and muscular fasciculation–related rhabdomyolysis may all contribute to the decline of renal function after acute poisoning from OP pesticide. In addition, hemodilution induced by massive fluid resuscitation, which impairs the oxygen supply to the kidney may also play a role resulting in AKI [70].

Biomarker

Biomarker is defined as a characteristic which is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to therapeutic intervention [17, 18].

Biomarkers can potentially serve a wide range of functions in drug development, clinical trials and therapeutic management strategies. Biomarkers may be proteins, lipids, genomic or proteomic patterns, imaging determinations, electrical signals and cells present in urine [17].

National institutes of Health further classified biomarkers on the basis of their utility.

1. **Prognostic Biomarker:** A baseline patient or disease characteristic that categorize patients by degree of risk for disease occurrence or progression, informing about the natural history of the disorder in the absence of a therapeutic intervention.
2. **Predictive Biomarker:** A baseline characteristic that categorizes patients by their likelihood of response to a particular treatment, predicting either a favorable or unfavorable response.
3. **Pharmacodynamic Biomarker:** A dynamic assessment that shows that a biologic response has occurred in a patient who has received a therapeutic intervention. Pharmacodynamic biomarker may be treatment specific or broadly informative of disease response, with the specific clinical setting determining how the biomarker is used and interpreted.

4. **Clinical end point Biomarker:** A characteristic or variable that reflects how a patient fares or functions or how long a patient survives.
5. **Surrogate endpoint Biomarker also termed as type 2 biomarker:** A marker that is intended to substitute for clinical endpoint. A surrogate endpoint is expected to predict clinical benefit, harm, lack of benefit or lack of harm on the basis of epidemiologic, therapeutic, pathophysiological or other scientific evidence.

Biomarker Discovery, Assay Validation and Qualification

Biomarker identification and development is arduous and involves several phases. For the purpose of simplicity, this process can be divided into five phases [17]

Phase 1: Discovery of potential biomarkers through unbiased or hypothesis generating exploratory studies

Phase 2: Development and validation of an assay for the measurement or identification of the biomarker in clinical samples

Phase 3: Demonstration of biomarkers potential clinical utility in retrospective studies

Phase 4: Performance of prospective screening studies

Phase 5: Continual assessment of validity of biomarker in routine clinical practice

Phase 1:

Discovery of potential biomarkers through unbiased or hypothesis- Generating Exploratory studies

Primary goal of phase 1 is to identify potential leads using various technologies and to confirm and prioritize the identified leads. Search for biomarkers often begins with

preclinical studies that compare either tissue or biologic fluids in diseased animals eg. Animals with kidney injury compared with healthy animals to identify genes or proteins that appear to be upregulated or down regulated in diseased tissue relative to control tissue.

Once a promising biomarker is discovered, the validation process begins. An assay has to be developed and validated. Validation process is laborious and expensive, requiring access to patient samples with complete clinical annotation and long-term follow-up. In addition, each biomarker must be qualified for specific application [71].

Phase 2:

Development and validation of an assay for the measurement or identification of the biomarker in clinical samples

Primary goal of phase 2 is to develop and validate a clinically useful assay for a biomarker that has the ability to distinguish a person with kidney disease/ injury from persons with healthy kidneys in a high throughput fashion. Phase 2 involves development of an assay, optimization of assay performance and evaluation of the reproducibility of the assay results within and among laboratories. "Defining reference ranges of biomarker values is a crucial step before the biomarker can be used clinically" [48]. It is important to characterize how the levels of these markers vary with patient age, gender race or ethinicity and how biomarker values are related to known risk factors.

Phase 3: Demonstration of biomarkers potential clinical utility in retrospective studies

Primary objectives of phase 3:

1. Evaluate the biomarker potential in samples obtained from a completed clinical study
2. Test the diagnostic potential of the biomarker for early detection and
3. Determine the sensitivity and specificity of the biomarker using defined threshold values of the biomarker for utility in prospective studies.

Phase 3 involves comparing biomarker with several other novel biomarkers or existing "gold standard biomarkers" and defining the biomarkers performance characteristics (sensitivity, specificity) using receiver operating characteristic curve analysis. This process is particularly challenging in kidney disease, given uncertainties in the sensitivity and specificity of the gold standard used [49].

Phase 4: Performance of prospective screening studies

Primary aim of phase 4 studies is to determine the operating characteristics of a biomarker in a relevant population by measuring detection rate and false referral rate. In contrast to phase 1, 2 and 3 studies, which are based primarily on stored specimens, studies in phase 4 involve screening subjects prospectively and demonstrating that clinical care is changed as a result of the information provided by the biomarker analysis.

Biomarker Qualification Process:

The application for FDA qualification of novel biomarkers requires the intended use of the biomarker in non clinical and clinical contexts and collection of evidence supporting qualification. This can be a joint and collaborative effort among regulatory agencies, pharmaceutical companies and academic scientists.

Steps involved in the biomarker qualification pilot process include: [71]

1. Submission to FDA interdisciplinary pharmacogenomic review group with a request to qualify the biomarker for a specific use
2. Recruitment of a biomarker qualification review team (containing both non clinical and clinical members)
3. Assessment of the biomarker context and available data in a voluntary data submission
4. Evaluation of the qualification study strategy
5. Review of the qualification study results
6. Acceptance or rejection of a biomarker for a suggested use.

Data shared between the FDA and pharmaceutical industry or academic laboratories through Voluntary Exploratory Data Submissions (VXDSs) [71, 72].

Submission of exploratory data through VXDSs allows interaction between reviewers at the FDA and researchers in industry or academia regarding study designs, sample collection and storage protocols, technology platforms and data analysis. This pilot process for biomarker qualification allowed the predictive safety testing consortium to apply to both U.S. and European drug authorities simultaneously for qualification of new nephrotoxic biomarkers (kidney injury molecule-1, albumin, total protein, Cystatin C, clusterin, trefoil factor 3 and α_2-microglobulin) as predictors of drug mediated nephrotoxicity [73]. The FDA and the corresponding European Authority (European Medicine Agency or EMA) reviewed the application separately and made decisions as to whether each would allow the new biomarkers to be “fit for purpose” in pre clinical

research [72]. Some of these markers were proposed to be qualified as biomarkers for clinical drug induced nephrotoxicity once further supportive human data are submitted. It is notable that the process described here is specific or the FDA and the United States and that the biomarker validation and approval process varies significantly around the world. USFDA had not approved a new biomarker for the diagnosis or clinical management of acute or chronic renal dysfunction as of June 2014. However, several biomarkers have been approved for clinical use in several European and Asian Countries [73].

Phase 5: Continued assessment of the validity of the biomarker in routine clinical practice

Phase 5 addresses whether measurement of the biomarker alters physician decision making and /or reduces mortality or morbidity associated with the given disease in the population [50].

Analysis of Biomarker

The widely accepted measure of biomarker sensitivity and specificity is the receiver operating characteristic (ROC) curve. ROC curves display the proportion of subjects both with and without disease correctly identified at various cutoff points.

An ROC curve is calculated and represented by the equation:

True positive rate (sensitivity) = False positive rate (1- specificity).

The performance of a biomarker can be quantified by calculating the area under the ROC curve (AUC) and an ideal biomarker have an AUC of 1.0 [11, 73].

Characteristics of an Ideal Biomarker for Acute Kidney Injury [11]

1. Should be organ specific and allow differentiation between Intrarenal, Pre- renal and Post- renal causes of AKI as well as acute glomerular injury
2. Able to detect AKI early in the course and be able to predict the course of AKI and potentially the future implications of AKI
3. Identify the cause of AKI
4. Site specific and able to inform pathologic changes in various segments of renal tubules during AKI as well as correlate with the histologic findings in kidney biopsy specimens.
5. Easily and reliably measured in a non invasive or minimally invasive manner
6. Stable in its matrix
7. Rapid, reliable and measurable at bed side (POCT; Point of Care Testing)
8. Inexpensive to measure

Biomarkers and its importance in Kidney injury

Eventhough AKI and CKD share functional and structural aspects; there is an overlapping of biomarkers with respect to these two conditions.

Among the functional markers, GFR is used as a gold standard marker. Although the true GFR, as determined by agents that are freely filtered and undergo minimal handling by the tubule (iothalamate, iohexol, inulin) represents a sensitive measure for determining changes in kidney function, tests using these agents are invasive and laborious to perform [74].

Moreover, because of renal reserve, changes in GFR may not indicate structural injury until significant injury has occurred. Structural markers of tubular injury are also expressed by tubular cells and subtle changes in epithelial cells which lead to release of these markers excreted through urine. It is becoming increasingly clear that many of these biomarkers serve as signals for both acute and chronic kidney disease and also may be used to monitor progression from AKI to CKD. A challenge is to define at what level of release of these markers the injury is clinically significant in either acute or chronic setting [74].

a. **Biomarkers for AKI:** Biomarkers for AKI help in early detection of AKI and also differential diagnosis of AKI (eg. Distinguishing between volume- mediated AKI [pre-renal] and intrinsic tubular injury [ATN]), predicting outcomes of AKI at the time of clinical diagnosis (need for Renal Replacement Therapy, development of post-AKI CKD, short and long term mortality), predicting recovery from AKI, ascertaining the nephron-specific location and etiology of renal injury and monitoring the effects of an intervention [75].
b. **Biomarkers for CKD:** Early detection and diagnosis of CKD, predicting the progression of CKD (rapid vs slow progression), predicting outcomes of CKD at the time of clinical diagnosis (development of ESRD, short and long term mortality), predicting cardiovascular disease/outcomes among patients with CKD and monitoring the effects of an intervention [75].

Studies have shown that biomarkers of AKI can serve several purposes and are no longer thought of as a replacement for serum creatinine [75].

Creatinine: Product of protein metabolism is a well established marker of renal function and is synthesized in the kidneys, liver and muscle. It is formed by two enzymatically mediated reactions. The most widely used creatinine as a marker of GFR as investigated for the first time by Poul Brandt Rehberg, where he studied renal clearance of orally administered creatinine. Determination of endogenous creatinine clearance was however precluded till 1938 [76, 77].

Creatinine is derived from the conversion of creatine in skeletal muscle. Creatinine concentration is inversely related to GFR. Creatinine is convenient and cost effective to measure, but it is affected by age, gender, exercise, drugs (cimetidine, trimethoprim), muscle mass, nutritional status and protein intake [77].

Plasma creatinine remains unchanged until there is a significant loss of renal function. GFR is a measurement of Glomerular functioning. GFR provides a good estimate for the function of renal tissue in chronic disease state or in acute renal injury. GFR measuring from endogenous creatinine clearance does not offer an advantage over serum creatinine alone. GFR can be estimated by calculating endogenous creatinine clearance (ClCr).

ClCr (ml/min) = Ucr × V/Pcr

Ucr: Creatinine concentration in urine

Pcr: Creatinine concentration in plasma

V: urine flow rate

When GFR falls, Pcr will initially be unchanged and Ucr will increase due to increased tubular secretion, hence Clcr over estimate GFR. Errors in the measurement of urine volume will also limit the accuracy [77].

Limitations of plasma creatinine estimation are: it is independent of renal function and depends on muscle mass. Factors that affect muscle mass include; age, gender, race and physical activity. These factors in turn can affect creatinine values.

NB: women have lower creatinine levels compared to men, vis-a-vis muscle mass.

Eventhough Scr is routinely used and considered as a gold standard biomarker of kidney function, it does not detect dysfunction early and allow prompt therapeutic intervention [76, 77].

AKI is a common and serious condition. Unfortunately creatinine is a delayed and unreliable indicator of AKI for the above reasons.

Creatinine in AKI

The diagnostic criteria for AKI such as RIFLE classification and AKIN definition of AKI largely depend on Serum creatinine concentration [78]. Studies have indicated that creatinine concentration is not ideal marker for diagnosing AKI due to the following reasons [79].

1. Creatinine production and its release into the circulation vary greatly with age, gender and muscle mass, certain disease states and to a lesser extent diet. Example, in rhabdomylosis serum creatinine concentration rise more rapidly due to release of preformed creatinine from muscle. Creatinine production which is

measured by 24 hour urinary excretion is known to decrease with increasing age, largely because of reduction in muscle mass [80].

2. Serum creatinine concentrations are not specific for renal tubular injury. Pre-renal factors such as severe dehydration, loss of blood volume, altered vasomotor tone or age related decrease in renal blood flow and post renal factors like obstruction or extravasation of urine into the peritoneal cavity may falsely elevate serum concentrations in the absence of parenchymal damage. Thus a decrease in eGFR inferred from an increase in serum creatinine level may not distinguish between pre-renal, intrinsic-renal and post- renal causes of impaired kidney function. Even in cases where serum creatinine is elevated as a result of direct renal injury cannot be used to determine the location of the injury i.e., glomerular versus tubular or proximal tubular versus distal tubular [81].
3. A number of acute and chronic kidney conditions exist with no change in serum creatinine owing to the concept of renal reserve. It is estimated that more than 50% of kidney function must be lost before serum creatinine value rises [82].
4. Increase in serum creatinine represents a rate indication of a functional change in GFR that lags behind important structural changes that occurs in the kidney during the early damage stage of AKI [82].
5. Creatinine assay is subject to interferences. Jaffes reaction assay (alkaline picrate method) is routinely used in clinical laboratories to assess creatinine levels but this method overestimates serum creatinine concentrations due to the interference of non-creatinine chromogens like proteins [81].

Urea [83]

Urea is not an accurate filtration marker because it is influenced by various factors such as synthesized in Liver, depends on protein metabolism, drugs etc.

Low urea levels are seen in decreased protein intake and chronic liver disease due to reduced synthesis of urea [83].

Eventhough routinely used and considered as a gold standard biomarker of kidney function, serum creatinine does not detect injury or dysfunction early enough to allow prompt therapeutic intervention [84]. Recently many novel technologies in the field of genomics, proteomics and Metabolomics have made it easier to interrogate potential biomarkers [84]. A renewed interest in discovering novel biomarkers have been reported for AKI. Recent biomarkers include Neutrophil Gelatinase Associated Lipocalin (NGAL), Cystatin C, Interleukin – 18 (IL- 18), Liver type Fatty Acid Binding Protein (L-FABP), Kidney Injury Molecule -1 (KIM- 1) etc. These recent biomarkers have been adequately validated to justify their potential use in making decisions for better patient care [84, 85].

Among the recent biomarkers for AKI, NGAL is a promising biomarker for early diagnosis of AKI. NGAL belongs to Lipocalin family.

Lipocalins are a large and ever expanding group of proteins exhibiting structural and functional diversity. Members of the lipocalin protein family are typically small secreted glycoproteins which have different molecular recognition properties. They are important in the maintenance of health and in combating diseases effectively. The percentage of

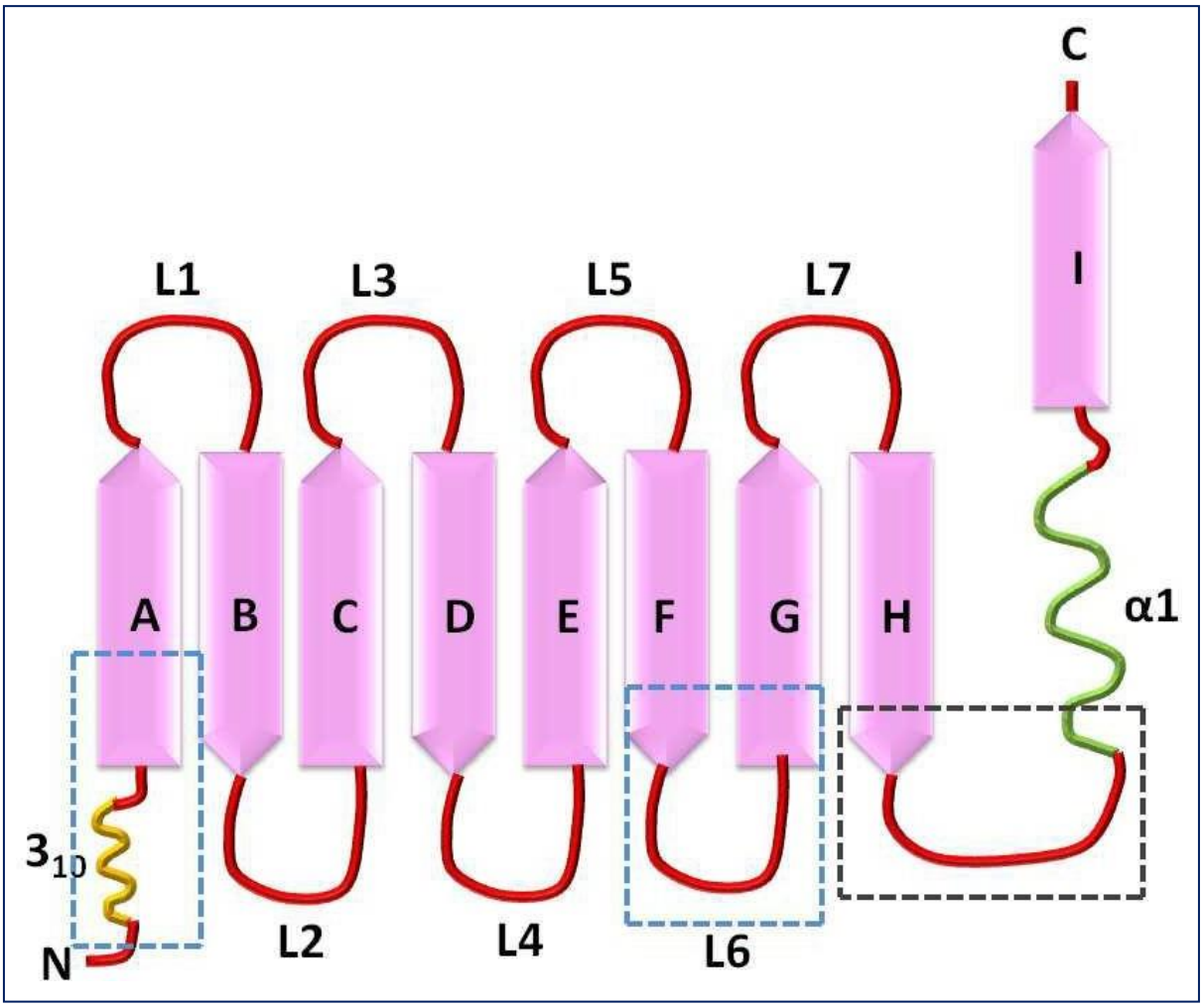
sequence identity among different members of the lipocalin family is even lower than the minimum (20%) identity to call it a reliable alignment [86].

Lipocalins share three short stretches of amino acid sequences or motifs which are structurally conserved regions (SCRs). SCRs classify this large family into two sub families, the kernel and the outlier lipocalins. The kernel lipocalins contain all three motifs while the outlier lipocalins contain only one or two of these SCRs [87]. A list of different lipocalins including both kernel and outlier lipocalins is given in table .Lipocalins are found mainly in vertebrates, but other organisms like butterfly (insecticyanin), cockroach (β1 lactoglobulin), grasshopper (lazarillo) also contain lipocalins [87].

Table 4: List of Lipocalins; Abbreviation with Protein Name

ABBREVATION	PROTEIN NAME
Kernel Lipocalins	
Alpha 1M	Alpha 1-microglobulin
ApoD	Apolipoprotein D
A2U	α2-microglobulin
BBP	Bilin Binding Protein
Blg	β1-Lactoglobulin
CPP	Choroid plexus protein
CRABP2	Cellular Retinoic acid binding protein
ACC	α-crustacyanin
MUP	Major urinary protein
NGAL	Neutrophil Gelatinase Associated Lipocalin
PGDS	Prostaglandin D Synthase
PP14	Pregnancy protein 14
PURP	Purpurin
	Lazarillo
Outlier Lipocalins	
AAG	Alpha 1-acid glycoprotein
	Aphrodisin
OBP	Odorant binding protein
	Probasin
VEGP	von Ebner's gland protein

Figure 4: Structure of Lipocalin



Title: structure and sequence of Lipocalin family [88]
source: Google Pictures

The secondary and tertiary structure of the family members have a characteristic lipocalin fold, which comprises an N-terminal 3- 10 helix followed by eight β pleated sheets arranged in an antiparallel orientation. Eight β sheets are connected to a α helix, which inturn is connected to C-terminal β sheet. These eight antiparallel β sheets are linked to each other by short loops. The N-terminal region forms the ligand binding

cavity. The portion of the lipocalin fold that are structurally conserved between different lipocalins is indicated by the blue boxed regions while the region that shows significant conservation in amino acid sequence is indicated by the black boxed region [88]

The most important three characteristics of this family are

1. Cup shaped cavity of lipocalins enclosed within the β pleated sheets is adapted for binding to hydrophobic ligands.
2. They bind to specific receptors
3. They have a tendency to form complexes with soluble macromolecules.

Some of the well known protein-protein complexes involving lipocalin family are the complex of Retinol binding protein with Transthyretin, NGAL with human neutrophil gelatinase and α_1 macroglobulin with IgA. These associations may either serve to transport proteins and stabilize the interaction or alter the biological activity of the interacting protein [89].

Functions

Lipocalins generally act as transporters (some are enzymes) trafficking small molecules to specific cells. They are proposed to be variously involved in

- Retinol transport
- Invertebrate cryptic coloration
- Olfaction
- Pheromone transport
- Prostaglandin synthesis
- Modulation of cell growth and metabolism

- Regulation of immune response
- Tissue development
- Animal behavior

Of all the lipocalins, NGAL has been extensively studied as a potential marker for diagnosis and prognosis in several human diseases.

Neutrophil Gelatinase Associated Lipocalin (NGAL)

NGAL is also known as

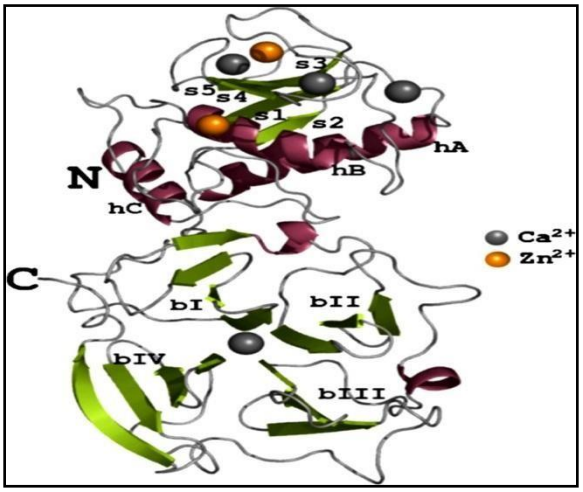
Human Neutrophil Lipocalin (HNL) / Migration Stimulating factor Inhibitor (MSFI) / α_1 Microglobulin related protein / Siderocalin / Uterocalin / 24p3

The human neutrophil contains two major types of granules, peroxidase positive azurophil and the peroxidase negative specific and the gelatinase granules. This lipocalin is associated with gelatinase covalently. NGAL is normally synthesized in the meta myelocyte stage of granulopoiesis. Targeting of proteins to the individual granule subsets are determined by the stage of maturation of cell at which the granule proteins are synthesized rather than by individual sorting information present in proteins [90]. NGAL is a 25 kDa glycoprotein of the lipocalin family. It contains 178 amino acid residues. It is encoded by a gene located on the chromosome locus 3p11. It was first isolated from mouse kidney cells infected with a simian virus (SV- 40) in 1989 [91]. NGAL has a structure that is characteristic of the lipocalin family – the lipocalin domain. Nuclear magnetic resonance studies have revealed that NGAL molecule contains eight antiparallel β strands connected by loops. It contains 20 amino acid signal peptide at the N- terminal end of the protein. In the barrel, three β bulges are

present. One bulge is formed by 1st β strand and the other two bulges are formed by the 6th β strand. These three bulges contribute to the ligand binding site of NGAL. Hydrophobic residues tryptophan, valine and phenylalanine which are present at the base of this barrel like structure is involved in direct binding to the ligand [91].

Another important feature that is binding to the ligands is the patch of positively charged amino acids residues, lysine and Arginine. They are present near the mouth of the barrel projecting into the open end of the molecule. Based on whether NGAL is free to bind to a ligand, it is termed as "apo" or "holo" NGAL respectively. Conformational change between these two forms of the protein is affected by a conformational change occurring at the open end of the NGAL protein. Further a negatively charged pit present at the base of the barrel formed by the amino acid aspartate and glutamate and a nearby unpaired cysteine residue is crucial for binding of NGAL to the gelatinase. Recently data suggest that the ligand binding pocket of NGAL is larger and more polar than a similar pocket in other lipocalin proteins [92].

Figure 5: Structure of NGAL



Source: adapted from google pictures [92]

NGAL is released from neutrophil granules in multiple forms; monomer, disulfide linked homodimer and disulfide linked hetero dimer with gelatinase B (Matrix Metalloproteinase -9). Dimerization does not affect the structure of the protein, it is susceptible to treatment with N-glycanase and N-glycosylation site was identified [93]. A comparison of amino acid sequence between NGAL homologues (different species) reveals that the human NGAL is highly similar to the homologue present in chimpanzees with 98% identity. It shows less identity to the mouse and rat homologues about 62% and 63% identity respectively.

NGAL is packed in specific granules in a high concentration and only liberated from the neutrophil by potent stimuli. Only fully activated neutrophils liberate NGAL along with reactive oxygen species and other bactericidal products. In correspondence with the ability of NGAL to bind N- formylmethionyl- leucyl- phenylalanine (fMLP) in vitro, it could have a potential role of binding to small lipophillic inflammatory mediators like platelet activating factor, leukotriene B_4 and lipopolysaccharide. By this way it seems as a feedback inhibitor of the inflammatory response. It seems to teleologically wise to pack NGAL in granules to prevent early and inappropriate inhibition of the neutrophil response to inflammatory mediators [94].

NGAL Is Induced in Systemic Disease and Renal Injury

Increase of serum NGAL levels have been reported in systemic disease in the absence of overt bacterial infections, most notably during the acute phase response and in renal tubular injury [95]. In the latter setting, human serum NGAL levels are increased in

the order of 7 to 16 fold and urinary NGAL levels increase by 25 to 100 fold which has led to the development of assays for NGAL for the early detection of renal tubular injury [95, 96].

The trafficking of NGAL in renal injury is more complicated. NGAL mRNA in the ischemic kidney is synthesized largely in the loop of Henle and collecting ducts, which are not the primary sites of ischemic renal injury [97, 98]. The loop of Henle responds to renal ischemia inspite of the fact that the site of major damage is the proximal tubule. Measurement of renal vein NGAL indicates that the locally synthesized pool of NGAL is not introduced efficiently into the circulation but seems to be excreted into the urine. Bulk of NGAL protein that is detectable in the post ischemic kidney is localized to the damaged proximal tubule in a lysosomal compartment. In contraindication to NGAL synthesis in distal nephron, it suggests that NGAL is delivered to the proximal tubule from the circulation. This is explained most likely by glomerular filtration of circulating NGAL and subsequent uptake by proximal tubular epithelia via endocytosis [95]. This idea is supported by the observation that tagged NGAL, when injected into the circulation is enriched in the proximal tubule but does not appear in urine in large quantities [99]. A two compartment model of NGAL, trafficking in renal injury has been proposed. Urinary NGAL is delivered from local synthesis in the kidney in distal parts of the nephrons within hours of insult, whereas proximal tubular NGAL derives from circulating NGAL pool which may stem from extra renal sources of NGAL. In this model systemic NGAL that is produced in the setting of sepsis or renal disease may serve to limit proximal tubular damage where as NGAL that is synthesized locally in the kidney may exert bacteriostatic effects in distal urogenital tract [99].

Expression of NGAL in Normal Tissues

Although first discovered as a component of the late granules of neutrophils, NGAL is also strongly expressed in several normal adult human tissues including kidney, liver, trachea, small intestine, bone marrow, thymus and macrophages. Expression of NGAL is very low in normal pancreas, endometrial glands and peripheral blood leucocytes. Studies have shown that NGAL is completely absent from the normal brain, heart, skeletal muscle and spleen [100, 101]. However, only few studies have been done in human fetus. They have shown that NGAL is expressed in the trophoblast cells of placenta, chondrocytes and in epithelial cells present in the developing lung and small intestine. NGAL expression has alo been reported in the epidermis of the fetal skin beginning around the 20^{th} week of gestation [102].

Regulation of NGAL Expression

NGAL is differentially expressed a wide array of benign and malignant diseases. Regulation of transcription of its mRNA has been the focus of many studies. Several cytokines, hormones, vitamins, minerals, drugs and growth factors have been shown to influence the expression of NGAL [89].

NF- κB, a transcription factor and regulator of several key pathways in the cell was found to regulate the NGAL gene expression. NGAL gene has κB elements in its promoter region and has been positively regulated by agents that induce NF- κB like insulin, Interleukin- 1b etc. another pathway that has shown to promote NGAL expression is JNK-MAPK (c- Jun N- terminal kinase -Mitogen-activated protein kinase) pathways has been suggested to act together to upregulate the expression of NGAL [103, 104].

The epidermal growth factor receptor (EGFR) when activated initiates signaling events that promote cell proliferation, survival and enhance migration and invasiveness of a variety of a variety of cancer cells. EGFR treatment decreases the expression of NGAL mRNA. EGF mediated down regulation of NGAL expression occurs by inhibition of the NF-κB pathway [105].

NGAL for the Prediction of AKI

Preclinical transcriptome profiling studies identified NGAL to be one of the most upregulated genes in the kidney very early after acute injury in animal models [106, 107]. Downstream proteomic analyses also reveal NGAL to be one of the most induced proteins in the kidney after ischemic of nephrotoxic AKI in animal models [108]. NGAL protein is easily detected in urine soonafter AKI in animal studies has initiated a number of translational studies to evaluate NGAL as a noninvasive biomarker in human AKI. A marked increase in both urinary and serum NGAL was documented by western blotting in a cross sectional study of adults with established AKI from various etiologies [72]. Urine and serum NGAL correlated with serum creatinine and the kidney biopsies in subjects with AKI demonstrated intense accumulation of immuno reactive NGAL in the cortical tubules. This confirmed NGAL as a sensitive index of established AKI in humans. A number of subsequent studies have now implicated NGAL as an early biomarker for AKI in various clinical settings [82].

NGAL in AKI in Critical Care Setting

In the intensive care unit setting, main problem is to establish the time of renal insult. Often, the baseline kidney function also remains unkown. This patient population

is extremely heterogeneous and the etiology is often unclear [109]. Up to 60% of patients may have already sustained AKI on admission to the intensive care unit [110]. Sepsis accounts for 30-40% of all AKI encountered in critically ill patients and generally portend a poorer prognosis with lower survival [111, 109]. Other etiologies for AKI in this setting include exposure to nephrotoxins, hypotension, kidney ischemia, mechanical ventilation and multiorgan disease. These etiologies are associated with distinct mechanisms of injury that are active at different times with different intensities and may act synergistically [82].

NGAL in the Prognosis of AKI

First study evaluating NGAL as an AKI predictor was conducted on children after cardiac surgery in whom, urinary NGAL raised almost 100- fold and serum NGAL 20-fold up to 48 h before AKI was detected by creatinine [112].

Studies conducted by Haase M et.al, and Awad AS et.al, has expressed variations in NGAL across studies and they ascribed it to a number of factors such as [113, 114]

1. Definition of outcome of NGAL with variable AKI
2. Inclusion of CKD patients may affect the results
3. The time from NGAL measurement to AKI development differs among studies
4. Co-morbid diseases and conditions such as sepsis may affect NGAL levels independently.

Minute amounts of NGAL are produced by different tissues and released into the bloodstream. After free filtration, NGAL is reabsorbed via megalin receptor mediated endocytosis by the proximal tubule.

Bagshaw SM et al, has documented elevated NGAL levels in urine and plasma during early AKI and may lead to [115]:

(1) Impaired reabsorption in the damaged proximal tubule which in turn increases the urinary NGAL

(2) Induced synthesis in different parts of the nephron which has been demonstrated in animal models

(3) Secretion from neutrophils, migrating from capillaries into the tubular lumen and may also be a potential source

(4) Increased NGAL mRNA expression which has been found in distant organs such as lungs in animal AKI models. Thus extra-renal production of NGAL may contribute to elevated plasma levels in AKI.

McIlroy DR et al, and Mori K et al, has observed decline in GFR may amplify plasma levels. Authors have also documented that NGAL is highly expressed and released by liver and circulating neutrophils in response to inflammation. Thus expressed and released NGAL may increase levels of plasma and urinary NGAL irrespective of any potential kidney damage. However, increased urinary NGAL has been observed in patients with urinary tract infection (UTI). [116, 117].

Results from Mishra J et al., has observed that human tubular epithelial cells (HK-2 cells) and neutrophils indicate kidney epithelial cells which mainly secrete monomeric NGAL, whereas neutrophils mainly release the dimeric form detected by Western blot [118].

Flo TH et al and Klausen P et al has documented dimeric NGAL was the predominant form in the urine of patients with UTI and the different forms of NGAL

whether monomeric, dimeric or heterodimeric may expose different epitopes. Choice and configuration of antibodies directed against different epitopes on NGAL molecule may have an impact on the clinical performance of NGAL assay [119, 120].

Cai L et al has shown NGAL as an AKI predictor by use of commercial ELISA supplied by Bioporto (Gentofte, Denmark) and R&D systems (MN, USA). Their NGAL were based on monoclonal anti- NGAL antibodies [121].

Whether NGAL in urine during AKI is derived from both tubular cells and neutrophils, or as a possible contribution from tubular cells or neutrophils may represent different pathophysiological signals during AKI development [121].

Other Biomarkers For AKI

- Cystatin C (Cys C)
- Interleukin -18 (IL-18)
- Kidney Injury molecule – 1 (KIM-1)
- Liver type Fatty Acid Binding Protein (L-FABP)
- N-Acetyl-D-Glucosaminidase (NAG)

Cystatin C (Cys C) and AKI

Cys C is a low- molecular- weight protein of ~13 kDa, produced constantly in all nucleated cells. It is a strong inhibitor of cysteine proteinases. Cys C is freely filtered by the kidney and not secreted. Proximal tubular cells reabsorb and catabolize the filtered Cys C so that minute quantities are normally excreted in the urine [122].

Limitations of estimation of Cys C in biological fluids: It depends on age, gender, obesity, height, cigarette smoking, and higher C - reactive protein [123].

Studies have shown urinary Cys- C to creatinine (CCR) as a good indicator of Cys C reabsorption in the proximal tubules but depend on proximal tubule integrity [124].

Food and Drug Administration (FDA) has approved the use of serum Cys C as a biomarker for renal damage as part of the drug review process (FDA, 2008). However, it is a good complement to serum creatinine and may even be a good replacement for serum creatinine, which means it should be used in combination with other biomarkers [125].

Interleukin - 18 (IL- 18)

IL- 18 is an 18 kDa proinflammatory cytokine produced by renal tubule cells and macrophages. It is a mediator of acute tubular injury, including both neutrophil and monocytes infiltration of renal parenchyma [126]. In the kidney IL- 18 is induced and cleaved mainly in the proximal tubules and released into the urine. IL- 18 has been shown to precipitate in a variety of renal disease processes including ischemia-reperfusion injury, allograft rejection, infection, autoimmune conditions and malignancy. Several studies have demonstrated the usefulness of IL- 18 as biomarker for the detection of AKI. Urinary IL- 18 levels were reported to be significantly higher in patients diagnosed with acute tubular necrosis than in patients with prerenal azotemia or urinary infection or in healthy control subjects with normal renal function. Patients with diabetic kidney disease and proteinuria had higher IL- 18 levels in renal tubular cells compared to patients with non-diabetic proteinuric disease [127, 128].

Clinical utility of IL- 18 as a biomarker to predict or diagnose AKI in other settings such as drug induced kidney injury has not been evaluated. The pathophysiology of IL- 18 is not well elucidated and its role may be a mediator of specific injury subtypes

rather than a marker of injury. Further studies are required to demonstrate the usefulness of IL- 18 as a biomarker in AKI [129, 130].

Kidney Injury Molecule- 1 (KIM- 1)

KIM-1 also reffered as T cell immunoglobulin and mucin domains containing protein (TIM-1) and hepatitis A virus cellular receptor 1 (HAVCR- 1), is a type 1 transmembrane glycoprotein with an ectodomain containing a 6 cysteine immunoglobulin like domain, 2 N glycosylation sites and a mucin domain [131, 132]. KIM- 1 has been shown to significantly expressed in kidneys specifically in proximal tubular cells of humans after ischemia injury, whereas it was virtually absent or present at low levels in healthy kidneys. The ectodomain of KIM- 1 is cleaved by metalloproteinases and sheds from cells both in vitro and vivo into the urine in rodents and humans after proximal tubular injury [133]. The full length form of KIM-1 is 104kDa where as the molecular weight of the shed form of KIM-1 ectodomain is approximately 90kDa. The selective KIM-1 expression by injured proximal tubular cells and the shedding of its ectodomain into urine provided a strong impetus for testing KIM- 1 as a biomarker of kidney damage [134, 135].

Liver type Fatty Acid Binding Protein (L- FABP)

L-FABP was first isolated in the liver as a binding protein for oleic acid and bilirubin. It binds selectively to free fatty acids and transports them to mitochnodria or peroxisomes, where free fatty acids are beta oxidized and participate in intracellular fatty acid homeostasis. There are several different types of FABP which are ubiquitously expressed in a variety of tissues. Different types of FABP's include Liver (L), Intestinal

(I), Muscle and Heart (H), Epidermal (E), Ileal (Il), Myelin (M), Adipocyte (A), Brain (B) and Testes (T) [136].

L-FABP is expressed in proximal tubules of human kidney and localized in the cytoplasm. Increased cytosolic L-FABP in proximal tubular epithelial cells may be derived not only from endogenous expression but also from circulating L- FABP that might be filtered at the glomeruli and reabsorbed by tubular cells [137].

Number of clinical studies has explored the potential utility of urinary L-FABP as biomarker for early diagnosis of AKI. Urinary L- FABP correlated well with the ischemic time of the transplanted kidney and the length of hospital stay in human recipients of living related donor renal transplants [137]. L- FABP predicts the development of AKI in children undergoing cardiac surgery. It was elevated within 4 hours after cardiac surgery and these elevated levels anticipated the subsequent development of AKI with an accuracy of 81% [138]. Urinary L- FABP concentrations reflect the severity of sepsis and response to treatment. Urinary L- FABP levels are predictive of the need for short-term renal replacement therapy, but not hospital mortality [139]. Because L- FABP is also expressed by liver, liver injury can be a potential contributor to increased urinary levels of L-FABP during AKI.

N- Acetyl- D- Glucosaminidase (NAG)

NAG is a lysosomal brush border enzyme that resides in the microvilli of tubular epithelial cells. Damage to these cells results in the shedding of this enzyme into urine. NAG has a high molecular weight of 130kDa and hence plasma NAG is not filtered by the glomeruli. Its excretion into urine correlates with tubular lysosomal activity.

Increased urinary concentrations of NAG have been found in patients with AKI, chronic glomerular disease, diabetic nephropathy, exposure to nephrotoxic drugs, delayed renal allograft function, environmental exposure, contrast medium induced nephropathy and sepsis and following cardiopulmonary bypass [140, 141]. Urinary NAG concentrations were significantly higher in patients with contrast medium- induced nephropathy than in patients without such nephropathy within 24-hours after the administration of a contrast agent [141].

There are some limitations in the use of NAG as a marker of kidney injury. Inhibition of NAG enzyme activity has been reported in the presence of metal ions and at higher urea concentrations in urine. Moreover, increased urinary levels of NAG have been reported in several non-renal diseases, including rheumatoid arthritis and hyperthyroidism as well as in conditions with increased lysosomal activity without cellular damage [142]. Because of concerns about its specificity, the clinical utility of NAG as a biomarker has been limited [143].

OBJECTIVES

1. To estimate and compare NGAL and Cystatin C levels in Acute Kidney Injury in Diabetes Mellitus patients.
2. To compare the levels of NGAL and Cystatin C in diabetes with and without Acute Kidney Injury.
3. Combination assay of biomarkers and its utility in the assessment of Acute Kidney Injury.
4. To test the hypothesis that NGAL is an early biomarker in Acute Kidney Injury compared to traditional markers (Creatinine and Cystatin-C).

MATERIALS and METHODS

Prospective case control study, conducted at RL Jalappa Hospital and Research Centre; teaching hospital of Sri Devaraj Urs Medical College, Constituent college of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India. Study was approved by the Central Ethical Committee. **[Reference: No. SDUAHER/KLR/R&D/18/2014-2015; Dated: 24- 5- 2014]**

To detect a mean difference of NGAL 20 ng/ml with 95% power and 95% confidence interval [144], the sample size was estimated as 134 per group expecting a sample loss or dropout rate of 10% during study, the total sample size calculated was 134+16=150 per group.

Sample size calculation for comparing means:

$N= 2(Z_{\alpha/2}+ Z_{\beta})^2 s^2/(M1- M2)^2=(d)^2$ at 95% confidence interval

d=difference of mean

S^2 = combined variance

$Z_{\alpha/2}$ = 95% confidence interval

Z_{β}= power =95%

Total 475 subjects in the age group 45-75 years of either gender were included and grouped into three categories:

Group I: T2DM without AKI (n=150)

Group II: T2DM with AKI (n=150)

Group III: Age and Gender matched clinically proven healthy controls (n=150)

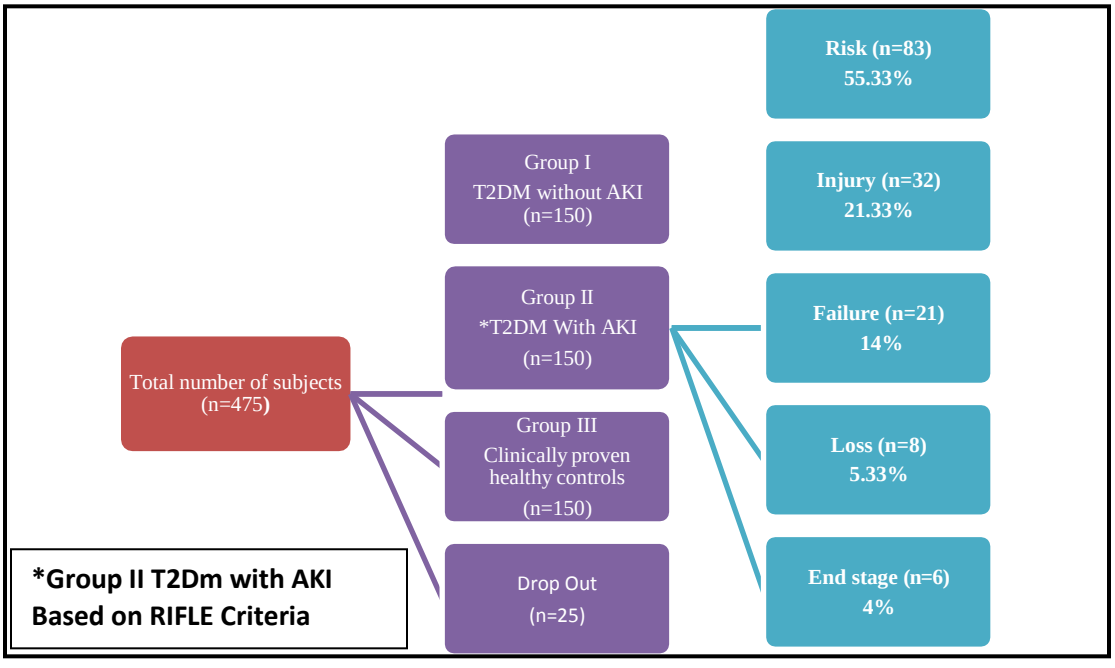
Sampling Method:

- Group I: One in every fifth patient attending to the Medicine outpatient department with history of diabetes was selected
- Group II: Patients admitted in Intensive Care units/ Medical Intensive Care Units with history of T2DM and AKI were categorized into Group II.
- Group III: Patient attendees who are free from diabetes mellitus were investigated and the subjects whose biochemical investigations were within biological reference range and clinically proven healthy were included in the study.

Study was conducted from a period of June 2014 to August 2016. Informed consent was obtained from all the study subjects.

Group II were further sub classified into five groups based on **R**isk (**R**), **I**njury (**I**), **F**ailure (**F**), **L**oss (**L**), and **E**nd-stage (**E**) (RIFLE criteria). Figure 4.

Figure 6: Classification of Subjects



Inclusion criteria:

- Subjects with clinically proven diabetes aged more than 45 years with history of diabetes more than 5 years, irrespective of treatment (oral drugs and/or insulin therapy)
- Subjects without history of diabetes mellitus were included as controls

Exclusion criteria

- Patients with DM predisposed to radio contrast induced nephrotoxicity
- DM with Type- IV renal tubular acidosis (hyperrenin hypoaldosteronism, patients on drugs known to cause proteinuria/albuminuria)
- Patients with hepatobilary disorders leading to proteinuria/ albuminuria
- Gestational diabetes mellitus
- Patients already diagnosed with Diabetic nephropathy

Anthropometric Measurements and Biochemical Estimations

Anthropometric measurements were measured and calculated based on Asian Indian standard guidelines. Right arm blood pressure was measured in comfortable sitting position using mercury sphygmomanometer in all Groups.

Blood sample was collected in plain tubes after overnight fasting of 8-10 hours from clinically proven healthy controls, diabetic subjects and also from DM with AKI.

Group II subject's blood sample was collected from Intensive Care Unit (ICU) and/ or Medical Intensive Care Unit (MICU).

All parameters were estimated by: Table 5

Table 4: Method Characteristics

Sl.No	Parameter (Unit)	Detection limit	Analytical range	Assay Imprecision (%)	Instrumentation/Method
1	Blood Glucose (mg/dL)	20	20-625	1.4-1.7	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom GOD-POD Method
2	HbA1c (%)	3.8	3.8-18.5	1.16-1.22	BIO RAD D10; USA HPLC Method
3	C-Peptide (ng/mL)	0.013	0.013-10	Intra Assay: 2.6-3.6 Inter Assay: 9.8-10.8	Calbiotech-USA; ELISA , Meril Germany
4	NGAL (ng/mL)	0.00	Serum: 0.00-10 Urine: 0.00-2.5	Intra Assay: 7.03-8.38 Inter Assay: 9.73-9.77	Biovendor, USA; ELISA , Meril Germany
5	Cystatin C (mg/L)	0.00	0.00-29	Intra Assay: 6.079-8.149 Inter Assay: 6.388-10.829	Biovendor, USA;ELISA , Meril Germany
6	Serum Creatinine (mg/dL)	0.05	0.05-17	1.6-2.6	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom Jaffes method
7	Urea (mg/dL)	2.00	2.00-120	1.5-1.8	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom Urease method
8	Uric Acid (mg/dL)	0.50	0.50-17	1.0-1.3	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom Uricase method
9	Sodium (mEq/L)	75	75-250	4.2-4.4	Vitros 250; OCD United States (Newyork)
10	Potassium (mEq/L)	1.00	1.00-14	2.5-3.0	Vitros 250; OCD United States (Newyork)
13	Total Cholesterol (mg/dL)	50	50-325	1.5-1.3	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom CHOD/PAP method
14	Triglycerides (mg/dL)	10.00	10.00-525	1.5-1.6	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom GPO/PAP method
15	HDLc (mg/dL)	5.00	5.00-110	3-3.2	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom CHOD/PAP method
16	hs-CRP (mg/dL)	0.010	0.01-1.5	Intra Assay: 6.02-7.28 Inter Assay: 4.27-5.42	PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom Turbidimetric Immunoassay
17	Microalbumin				
18	Malondialdehyde	Thiobarbituric acid Method			PerkinElmer UV/VIS Spectrophotometer Lambda 35, United Kingdom
19	Nitric Oxide	Modified Griess Method			
20	Vitamin C	2,4-Dinitro Phenyl Hydrazine Method			
21	Glutathione Peroxidase	Colorimetric assay; Biovision, USA			

ASSAY PRINCIPLE AND PROCEDURE

NGAL: ELISA [145]

Principle: Human Lipocalin- 2/ NGAL ELISA, standards, quality controls and samples are incubated in microplate wells pre- coated with polyclonal anti-human lipocalin-2 antibody. After one hour incubation and ashing, biotin labelled polyclonal anti-human lipocalin- 2 antibody is added and incubated with captured lipocalin- 2 for one hour. After another washing, streptavidin- HRP conjugate is added. After 30 minutes incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of lipocalin- 2. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using the standard curve.

Reagents Supplied

Kit Components	State	Quantity
Antibody Coated Microtiter Strips	Ready to use	96 wells
Biotin Labelled Antibody Conc. (20x)	Concentrated	0.70 mL
Streptavidin-HRP Conjugate	Ready to use	13 mL
Master Standard	Lyophilized	2 Vials
Quality Control HIGH	Lyophilized	2 Vials
Quality Control LOW	Lyophilized	2 Vials
Dilution Buffer	Ready to use	20 mL
Biotin-Ab Diluent	Ready to use	13 mL
Wash Solution Conc. (10x)	Concentrated	100 mL
Substrate Solution	Ready to use	13 mL
Stop Solution	Ready to use	13 mL
Product Data Sheet + Certificate of Analysis	-	1 pc

Materials Required

- Deionized (distilled) water
- Test tubes for diluting samples
- Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
- Precision pipettes to deliver 5- 1000 ml with disposable tips
- Multichannel pipette to deliver 100 ml with disposable tips
- Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
- Vortex mixer
- Orbital microplate shaker capable of approximately 300 rpm
- Microplate washer (optional). [Manual washing is possible but not preferable.]
- Microplate reader with 450 ± 10 nm filter, preferably with reference wavelength 630 nm (alternatively another one from the interval 550- 650nm)
- Software package facilitating data generation and analysis (optional)

PREPARATION OF REAGENTS

- All reagents need to be brought to room temperature prior to use
- Assay reagents supplied ready to use:
    - Antibody Coated Microtiter Strips
    - Biotin- Ab Diluent
    - Streptavidin- HRP Conjugate
    - Substrate Solution
    - Stop Solution
    - Dilution Buffer

- Assay reagents supplied concentrated or lyophilized:

Human Lipocalin-2 Master Standard

Reconstitute the lyophilized Master Standard with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam). The resulting concentration of the human lipocalin- 2 in the stock solution is 10 ng/ml.

Prepare Set of Standards Using Dilution Buffer As Follows:

Volume of Standard	Dilution Buffer	Concentration
Stock	-	10 ng/ml
300 ml of 10 ng/ml	300 ml	5 ng/ml
300 ml of 5 ng/ml	300 ml	2.5 ng/ml
300 ml of 2.5 ng/ml	300 ml	1.25 ng/ml
300 ml of 1.25 ng/ml	300 ml	0.6 ng/ml
300 ml of 0.6 ng/ml	300 ml	0.3 ng/ml

Quality Controls HIGH, LOW

Reconstitute each Quality Control (HIGH and LOW) with Dilution Buffer just prior to the assay.

Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).

Biotin Labelled Antibody Conc. (20x)

Prepare the working Biotin Labelled Antibody solution by adding 1 part Biotin Labelled Antibody Concentrate (20x) with 19 parts Biotin-Ab Diluent. Example: 50 ml of Biotin Labelled Antibody Concentrate (20x) + 950 ml of Biotin-Ab Diluent for 1 strip (8 wells).

Wash Solution Conc. (10x)

Dilute Wash Solution Concentrate (10x) ten-fold in distilled water to prepare a 1x working solution. Example: 100 ml of Wash Solution Concentrate (10x)+ 900 ml of distilled water for use of all 96 wells.

PREPARATION OF SAMPLE

Dilute serum or plasma samples 30x with Dilution Buffer just prior to the assay, e.g. 5 ml of sample + 145 ml of Dilution Buffer when assaying samples as singlets, or preferably 10 ml of sample + 290 ml of Dilution Buffer for duplicates. Mix well (not to foam). Vortex is recommended.

ASSAY PROCEDURE

1. Pipette 100 µl of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells.
2. Incubate the plate at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital microplate shaker.
3. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add 100 µl of Biotin Labelled Antibody solution into each well.
5. Incubate the plate at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital microplate shaker.
6. Wash the wells 3 times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
7. Add 100 µl of Streptavidin-HRP Conjugate into each well.

8. Incubate the plate at room temperature (25°C) for 30 minutes, shaking at 300 rpm on an orbital microplate shaker.
9. Wash the wells 3 times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
10. Add 100 µl of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
11. Incubate the plate for 10 minutes at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.
12. Stop the colour development by adding 100 ml of Stop Solution.
13. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550 - 650 nm). Subtract readings at 630 nm (550 - 650 nm) from the readings at 450 nm.

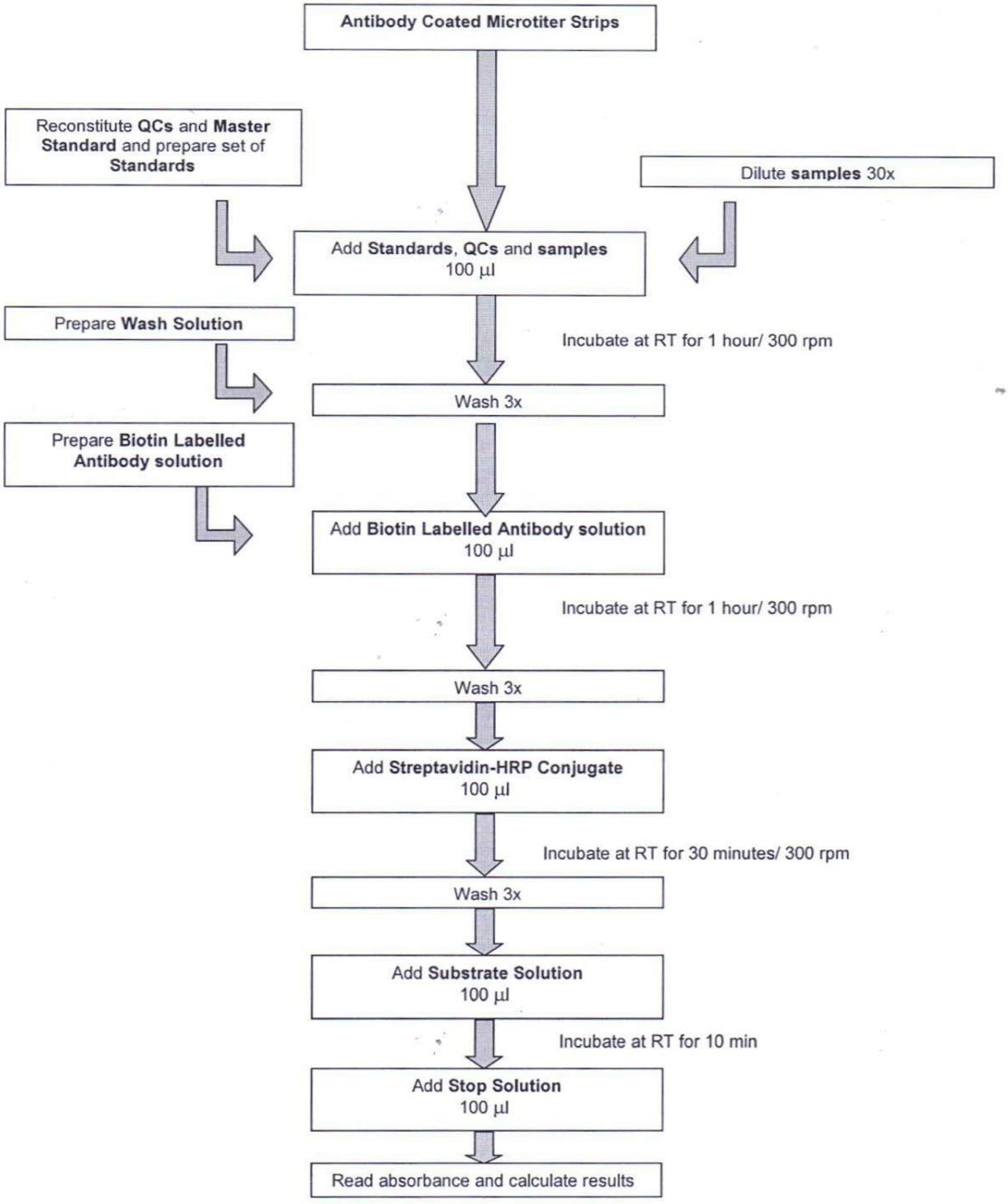
The absorbance should be read within 5 minutes following step 12.

Assay Procedure Cond…

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	Standard 10	Blank	Sample 8	Sample 16	Sample 24	Sample 32
B	Standard 5	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	Standard 2.5	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	Standard 1.25	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	Standard 0.6	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	Standard 0.3	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	QC HIGH	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	QC LOW	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

Standard Operating Procedure for NGAL by ELISA

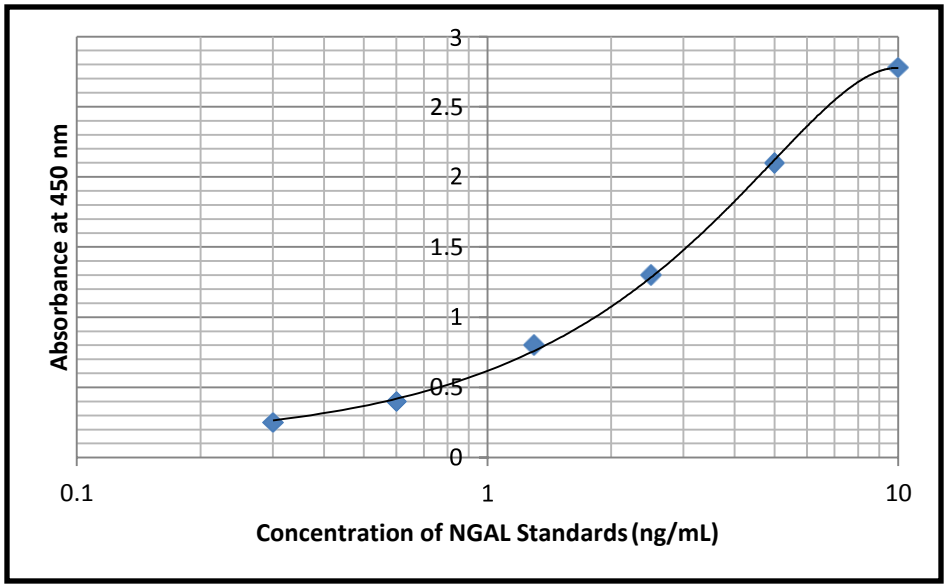
Assay Procedure Summary



CALCULATIONS

The measured concentration of samples and calculated from the standard curve must be multiplied by their respective dilution factor, because samples have been diluted prior to the assay, e.g. 1.75 ng/ml (from standard curve) x 30 (dilution factor) = 52.5 ng/ml.

Concentration of NGAL Standards (ng/mL)	Absorbance at 450 nm
10	2.78
5	2.1
2.5	1.3
1.3	0.8
0.6	0.4
0.3	0.25



Concentration of test samples to be calculated by the equation:

Y= [-0.0273x2+0.5397x+0.106]

CYSTATIN C: ELISA [146]

Principle: The human Cystatin C Kit is a sandwich ELISA. The capture antibody is a polyclonal human Cystatin C antibody pre-coated onto the 96- well strip plates provided in the kit. Human test samples and standards of known Cystatin C concentration are added to these wells and allowed to complex with the bound Cystatin C antibody. A biotinylated human Cystatin C polyclonal antibody is then added. This detection antibody binds to the antigen thus completing the sandwich. After washing, an enzyme Avidin-Biotin- Peroxidase complex (ABC) is added which binds to the second antibody. The peroxidase substrate TMB is added to induce a coloured reaction product. The intensity of this coloured product is directly proportional to the concentration of Cystatin C present in the samples. The purpose of this kit is the in- vitro quantitative determination of human Cystatin C in samples such as serum, plasma, tissue lysates and cell culture supernates.

Reagents Supplied

Kit Components	State	Quantity
Antibody Coated Microtiter Strips	Ready to use	96 wells
Conjugate Solution Concentrate (50x)	Concentrated	0.26 mL
Conjugate Diluent	Ready to use	13 mL
Standard (200-10 000 ng/ml)	Concentrated	6X0.1 mL
Quality Control HIGH	Concentrated	0.1mL
Quality Control LOW	Concentrated	0.1mL
Dilution Buffer Concentrate (10x)	Concentrated	10 mL
Biotin-Ab Diluent	Ready to use	13 mL
Wash Solution Conc. (10x)	Concentrated	10 mL
Wash Solution Concentrate (10x)	Concentrated	100 mL
Substrate Solution	Ready to use	13 mL
Stop Solution	Ready to use	13 mL
Product Data Sheet + Certificate of Analysis	-	1 pc

MATERIALS REQUIRED

- Deionized (distilled) water
- Test tubes for diluting samples
- Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
- Precision pipettes to deliver 10-1000 µl with disposable tips
- Multichannel pipette to deliver 50-100 µl with disposable tips
- Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
- Vortex mixer
- Orbital microplate shaker capable of approximately 300 rpm
- Microplate washer (optional). [Manual washing is possible but not preferable.]
- Microplate reader with 450 ± 10 nm filter, preferably with reference wavelength 630 nm (alternatively another one from the interval 550-650nm)
- Software package facilitating data generation and analysis (optional)

PREPARATION OF REAGENTS

- All reagents need to be brought to room temperature prior to use
- Assay reagents supplied ready to use:
  - Antibody Coated Microtiter Strips
  - Conjugate Diluent
  - Substrate Solution
  - Stop Solution

- Assay reagents supplied concentrated:

Dilution Buffer

Dilute Dilution Buffer Concentrate (10x) ten- fold in 90 ml distilled water to prepare a 1x working solution, e.g. 10 ml of Dilution Buffer Concentrate (10x) + 90 ml of distilled water for use of all 96- wells.

Wash Solution

Dilute Wash Solution Concentrate (10x) ten-fold in 900 ml of distilled water to prepare a 1x working solution, e.g. 100 ml of Wash Solution Concentrate (10x) + 900 ml of distilled water for use of all 96- wells.

Conjugate Solution

Prepare the working Conjugate Solution by adding 1 part concentrated Conjugate Solution Concentrate (50x) with 49 parts Conjugate Diluent.

Example: 0.25 ml of Conjugate Solution Concentrate (50x)+ 12.25 ml of Conjugate Diluent for use of all 96-wells. Prepare only the volume needed for the test. Mix thoroughly and gently.

Human Cystatin C Standards

Dilute each concentration of Standard 400x with the Dilution Buffer prior to the assay in two steps as follows:

Dilution A (10x):

Add 10 µl of Standard into 90 µl of Dilution Buffer. Mix well (not to foam). Vortex is recommended.

Dilution B (40x):

Add 10 µl of Dilution A into 390 µl of Dilution Buffer to prepare final dilution (400x). Mix well (not to foam). Vortex is recommended.

Quality Controls High, Low

Dilute each Quality Control (QC) 400x with the Dilution Buffer prior to the assay in two steps as follows:

Dilution A (10x):

Add 10 µl of QC into 90 µl of Dilution Buffer. Mix well (not to foam). Vortex is recommended.

Dilution B (40x):

Add 10 µl of Dilution A into 390 µl of Dilution Buffer to prepare final dilution (400x). Mix well (not to foam). Vortex is recommended.

PREPARATION OF SAMPLE

Dilute samples 400x with the Dilution Buffer prior to the assay in two steps as follows:

Dilution A (10x):

Add 10 µl of sample into 90 µl of Dilution Buffer. Mix well (not to foam). Vortex is recommended.

Dilution B (40x):

Add 10 µl of Dilution A into 390 µl of Dilution Buffer to prepare final dilution (400x). Mix well (not to foam). Vortex is recommended.

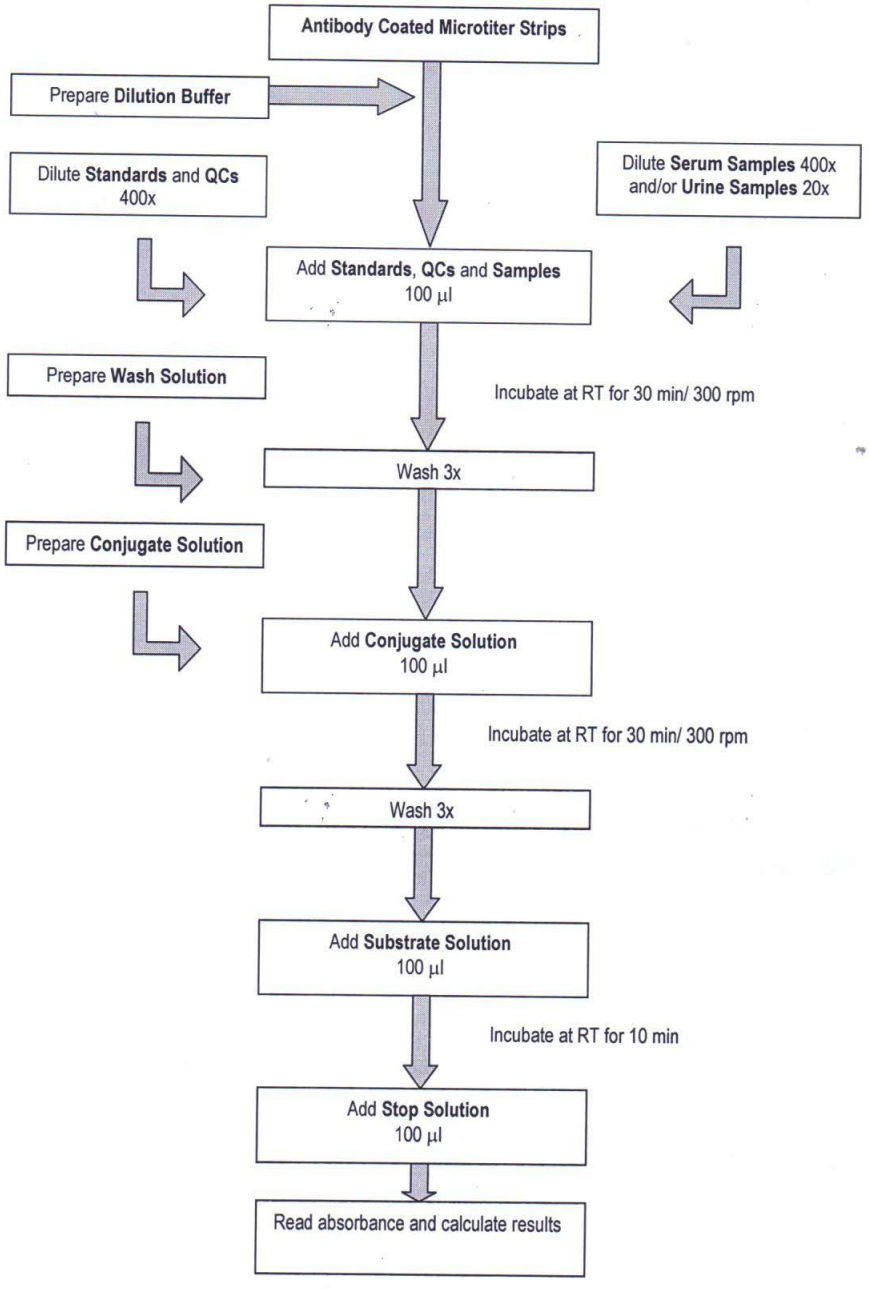
ASSAY PROCEDURE

1. Pipette 100 µl of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells.
2. Incubate the plate at room temperature (25°C) for 30 minutes, shaking at 300 rpm on an orbital microplate shaker.
3. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add 100 µl of Conjugate Solution into each well.
5. Incubate the plate at room temperature (25°C) for 30 minutes, shaking at 300 rpm on an orbital microplate shaker. Incubation without shaking is the alternative that requires to extend incubation with substrate
6. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
7. Add 100 µl of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
8. Incubate the plate for 10 minutes at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below 20°C. Do not shake with the plate during the incubation.
9. Stop the colour development by adding 100 µl of Stop Solution.
10. Determine the absorbance by reading the plate at 450 nm. The absorbance should be read within 5 minutes following step 9.

Assay Procedure Cond…

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	Standard 10 000	Blank	Sample 8	Sample 16	Sample 24	Sample 32
B	Standard 4 000	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	Standard 2 000	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	Standard 1 000	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	Standard 400	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	Standard 200	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	QC High	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	QC Low	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

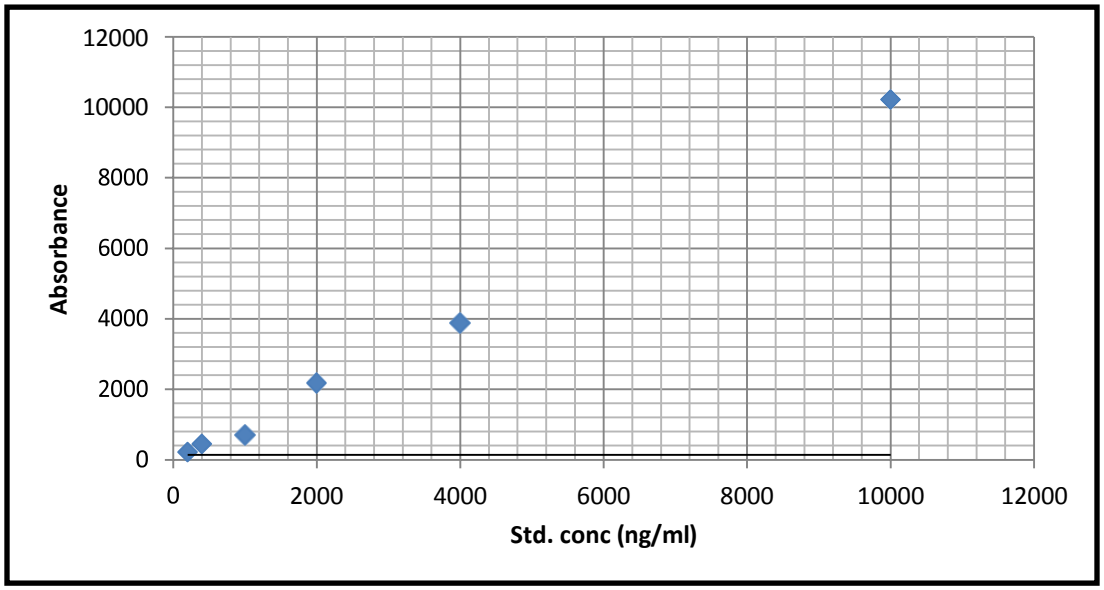
Standard Operating Procedure for Cystatin C by ELISA



CALCULATION

Logit log function can be used to linearize the Standard curve (i.e. logit of the mean absorbance (Y) is plotted against log of the known concentration (X) of Standards. Use values of undiluted standard range: 10 000, 4 000, 2 000, 1 000, 400, 200 ng/ml. Samples, Quality Controls and Standards are all diluted 400x prior to analysis, so there is no need to take this dilution factor into account.

Standard Concentration (ng/ml)	Obtained Concentration (ng/ml)
10000	10218.872
4000	3880.348164
2000	2180.371
1000	707.081294
400	450.772
200	218.092



STANDARD GRAPH FOR CYSTATIN-C
Building a simple linear regression between Absorbance vs Concentration, the relationship is expressed by Linear regression line **(Y=mX + C)**
Concentration (Dependent variable Y)= [-1102.483 (C) + (3109.217 * Absorbance) (mX)]
Note: Precisely for Prediction

C- Peptide: ELISA [147]

Principle: C- Peptide estimation is based on solid phase direct sandwich ELISA method. The standards, samples and controls are added into the selected wells coated with anti C-Peptide monoclonal antibody. C- Peptide in the standards, controls and patients serum binds to anti- C- Peptide Ab on the wells. Unbound protein is washed off by wash buffer. anti C- Peptide HRP conjugated second antibody is added and then binds to C- Peptide. Unbound proteins and HRP conjugate is washed off by wash buffer. Upon the addition of substrate, the enzyme activities measured are proportional to the concentration of C-Peptide in the samples. A standard curve is prepared depending on the color intensity of the concentration of C- Peptide.

REAGENTS SUPPLIED

Kit Components	Quantity
Microwells coated with anti-C-Peptide Ab	12X8X1
Standards (1- 6) 6 vials, Lyophylized	Reconstitute with 2 mL DH_2O
Enzyme Conjugate (Ready to use)	12 mL
TMB Solution	12 mL
Stop Solution	12 mL
Wash Solution 20X	

MATERIALS REQUIRED

- Distilled or deionized water
- Precision pipettes
- Disposable pipette tips
- ELISA reader capable of reading absorbance at 450 nm
- Absorbance paper or paper towel

REAGENT PREPARATION

Standards: Reconstitute the lyophilized standards with 2.0 mL distilled water. Allow them to remain undisturbed until completely dissolved and then mix well by gentle inversion.

Wash Buffer: Prepare 1X Wash Buffer by adding the contents of the bottle (25 mL, 20X) to 475 mL of distilled or deionized water.

Assay Procedure

1. Format the microplate wells for each reference, control and patient specimen to be assayed in duplicate.
2. Pipette 50µl of the appropriate standard, control or specimen into the assigned well
3. Pipette 100 µl Enzyme conjugate into each well
4. Gently mix plate for 15-20 seconds
5. Incubate for 60 minutes at room temperature
6. Remove liquid from all wells. Wash wells three times with 300 µl of 1x wash buffer
7. Add 100 µl of TMB substrate to all wells
8. Incubate for 15 minutes at room temperature
9. Add 50 µl of stop solution to each well and gently mix for 15- 20 seconds
10. Read the absorbance on ELISA reader of each well at 450 nm within 15 minutes after adding the stop solution.

CALCULATION OF RESULTS

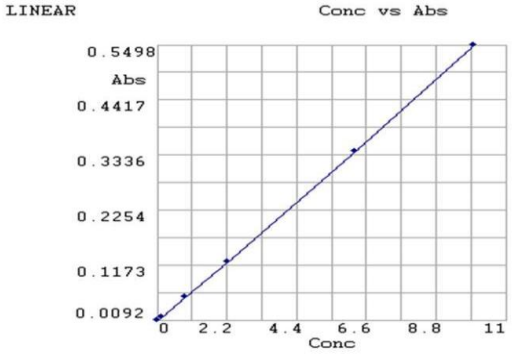
The standard curve is constructed as follows

1. Plot the OD for each C-Peptide standard point (vertical axis) versus the C-Peptide standard concentrations (horizontal axis)
2. Read the concentration (ng/mL) for controls and each unknown sample from the curve.

CALIBRATION REPORT

Print Date: 16/3/2016

Test : CP **Curve Type :** LINEAR **Calibration Date:** 16/03/16 5:09 pm



RESULTS

Sr No.	Lot No	Abs	Conc
1		0.01	0.00
2		0.01	0.15
3		0.05	0.90
4		0.12	2.25
5		0.34	6.25
6		0.55	10.00

PageNo.: 1

hs - CRP Method: Latex Turbidimetry [148]

Principle: CRP- ultrasensitive is a quantitative turbibidimetric test for the measurement of low levels of C- reactive protein in human serum or plasma. Latex particles coated with specific anti- human CRP are agglutinated when mixed with samples containing CRP. The agglutination causes an absorbance change, dependent upon the CRP contents of the patient sample that can be quantified by comparison from a calibrator of known CRP concentration.

TEST PROCEDURE

Bring reagents and sample to room temperature before use

Assay conditions

Wavelength	546nm
Reaction Temperature	$37^{o}C$
Cuvette	1 cm path length

Method for preparation of CRP Calibration Curve

CRP calibrator is reconstituted exactly with 1.0 ml of distilled water, wait for 5 minutes, gently swirl the vial till the solution attains homogeneity. Once reconstituted it is ready to use for preparation of CRP calibration curve.

Dilute reconstituted calibrator serially as mentioned below for preparation for calibration curve.

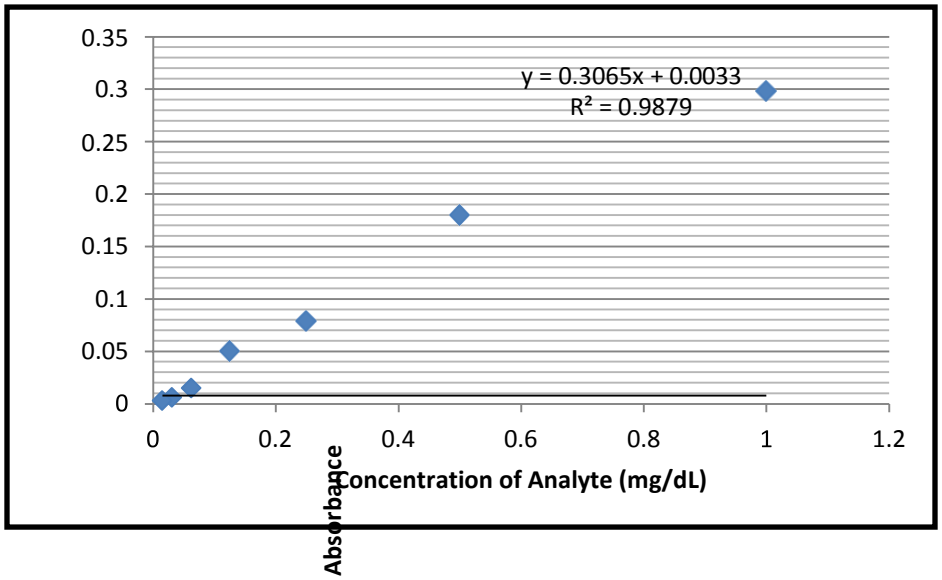
Test Tube No.	1	2	3	4	5	6	7
Calibrator dilution No.	D1	D2	D3	D4	D5	D6	D7
Isotonic Saline	-	100µl	100µl	100µl	100µl	100µl	100µl
Calibrator (S)	100µl	100µl	100µl	100µl	100µl	100µl	100µl
Concentration of CRP in mg/dL	1.0	0.5	0.25	0.125	0.0625	0.031	0.015

Five dilutions of the calibrator including highest concentration 1.0 mg/dL (D1) and lowest concentration 0.015 mg/dL (D7) of measuring range must be used for the preparation calibration curve.

TEST PROCEDURE for PREPARATION of CALIBRATION CURVE

- Zero the instrument with distilled water
- Pipette 400 µl of hs-CRP activation buffer (R1) and 100 µl µl of hs-CRP Latex reagent (R2) in the measuring cuvette. Mix well and incubate for five minutes at 37^{o}C.
- Add 5 µl of calibrator D1, mix gently and start the stopwatch simultaneously
- Read absorbance (A1) exactly at 10 seconds and absorbance (A2) again at the end of exactly our minutes.
- Repeat steps No. 2-4 for each of the diluted calibrator selected for preparing calibration curve
- Calculate ΔA (A2-A1) for each of the diluted calibrator selected for preparing the calibration curve.
- Plot a graph of ΔA versus concentration of CRP

Standard concentration of hS-CRP (mg/dL)	Absorbance	Concentration Obtained
1	0.298	0.091637
0.5	0.18	0.05547
0.25	0.079	0.0245135
0.125	0.05	0.015625
0.0625	0.015	0.0048975
0.031	0.006	0.002139
0.015	0.003	0.0012195



TEST PROCEDURE FOR SPECIMEN

- For determination of hs- CRP concentration in the test specimen;
- Follow steps 2- 4 as mentioned above procedure for calibration curve using the test specimen in place of calibrator
- Calculate ΔA (A2- A1) for test specimen

CALCULATION

1. Interpolate ΔA of the diluted test specimen on the calibration curve and obtain the hs- CRP concentration C of the diluted test specimen.
2. Multiply hs- CRP concentration 'C' with dilution factor (F) of test specimen for obtaining concentration of CRP in test specimen.

Concentration of CRP in test specimen in mg/dL = CxF

(Where 'F' is the dilution factor of test specimen, for e.g. 2 for 1:2 dilution of test specimen)

MALONDIALDEHYDE (MDA): Thiobarbituric Acid Reactive Substances (TBARS) [149]

Principle: Free MDA, as a measure of lipid peroxidation was measured spectrophotometrically at 532 nm as TBA reactive substances after precipitating the proteins with trichloroacetic acid (TCA).

Reagent Preparation

- Normal Saline (0.09%): 900 mg of Nacl dissolved in 100 mL of distilled water
- Thiobarbituric acid (TBA)reagent: TBA with a concentration of 8 mg/mL
- N-Butanol
- 24% Trichloroacetic acid (TCA): 24gm of TCA dissolved in 100 mL of distilled water
- Standard: 10Mmol/L stock standard was prepared (16.5 mg or Tetra methoxy propane) was dissolved in 10 mL distilled water). 1 mL of 10Mmol/TMP stock was dissolved in 100 mL of distilled water to obtain 100µmol/L standard

solution. 1 mL of 100µmol/L standard solution was diluted to 5 mL with distilled water to obtain 20100µmol/L standard solution.

PROCEDURE

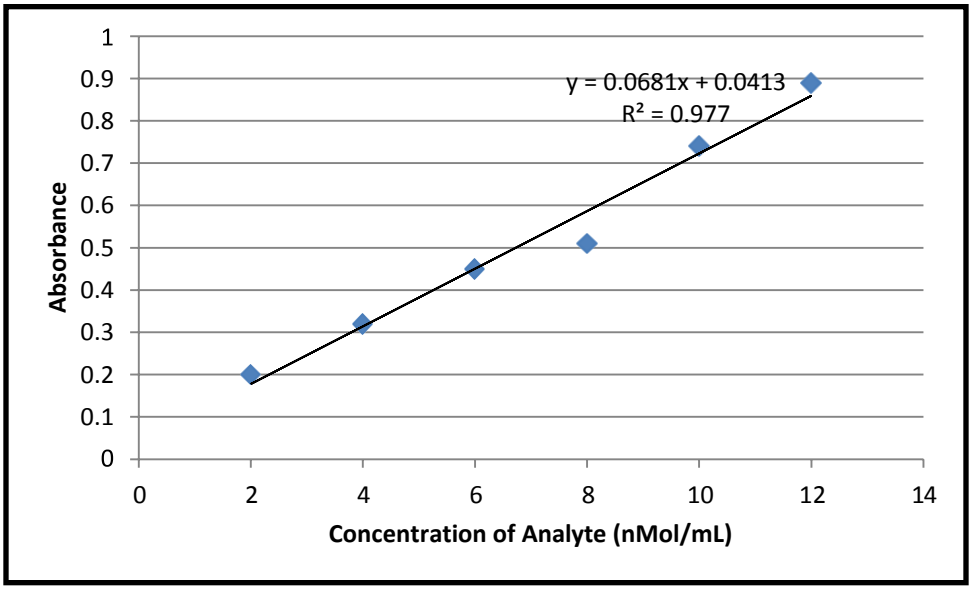
Reagent	Blank	Standard	Test
Saline (µL)	500	-	-
Standard (µL)	-	500	-
Sample (µL)	-	-	500
24% TCA (µL)	1000	1000	1000
Centrifuged at 2000 rpm for 30 minutes			
Supernatant (µL)	1000	1000	1000
TBA Reagent (µL)	250	250	250
Test tubes were covered with rubber cork and kept in boiling water bath for 1 hour. Then removed and cooled under tap water			
N-Butanol (µL)	500	500	500
All the tubes were vortexed for 1 minute Measure OD at 532 nm			

CALCULATION

$$\text{Concentration of MDA (µmol/L)} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{OD of Standard} - \text{OD of Blank}} \times \text{Concentration of Standard}$$

STANDARD GRAPH OF MDA

Concentration of Standards	Absorbance	Concentration Obtained
Blank	0.035	
2	0.2	0.59033
4	0.32	0.62176
6	0.45	0.67115
8	0.51	0.72054
10	0.74	0.76095
12	0.89	0.77891



NITRIC OXIDE: Modified Greiss Method [150]

Principle: Sulfanilicacid → Diazoniun salt + N- (1- napthyl) ethylene diaminedihydrochloride → azo dye; Formation of the azo dye is detected via its absorbance at 548 nm.

REAGENT PREPARATION

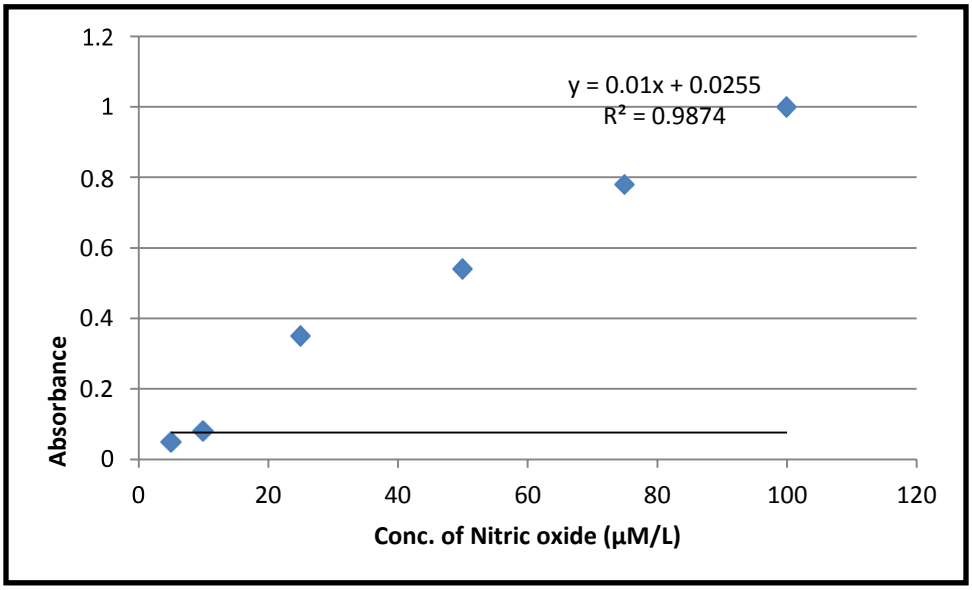
- 0.1mM $NaNO_2$
- 70% Sulphosalicylic acid
- 10% NaOH
- Tris HCL Buffer; pH 9.0
- Reagent A: 0.3% N-ethylene diamine dihydrochloride in doubled distilled water
- Reagent B: 3% Sulphanilamide in 1N HCl
- Greiss reagent: mix reagent A and Reagent B in equal volumes

ASSAY PROCEDURE

- To 1 mL of serum add 0.1 mL of Sulphosalicylic acid and mixed every 5 minutes for 30 minutes
- Centrifuge at 3000 rpm for 20 minutes
- To 200 µl of supernatant add 30 µl of 10% NaOH and 300 µl of tris-Hcl buffer
- Add 530 µl of Greiss reagent
- Keep tubes in dark place for 10 minutes and read absorbance at 540nm against water as blank

Standard Graph of Nitric Oxide

Concentration of Standards(µM/L)	Absorbance	Concentration Obtained (µM/L)
100	1	0.0355
75	0.78	0.033
50	0.54	0.0309
25	0.35	0.029
10	0.08	0.0263
5	0.05	0.026



VITAMIN C: 2, 4- Dinitro Phenyl Hydrazine Method [151]

Principle: Ascorbic acid in colorimetric method is initially converted to dehydroascorbic acid by shaking with bromine. After this conversion, ascorbic acid is coupled with 2,4-dinitrophenyl- hydrazine, the solution is then treated with sulfuric acid and red colour developed at 530 nm is compared with standard.

PREPARATION OF REAGENTS

- Liquid Bromine
- Thiourea solution: 10% Thiourea in 50% alcohol acts as a mild reducing agent
- 2,4-Dinitro Phenyl hydrazine Reagent: 2 % solution in 9N H_2SO_4 (24.4 in 100 mL)
- Ascorbic acid standard: 100 mg ascorbic acid in 100 mL of 4% metaphosphoric acid
- Ascorbic acid working standard: dilute 2.5 mL of stock solution with 17.5 mL of 4% metaphosphoric acid and 2 drops of bromine is added. Keep it in ice chest for 2 hours, excess bromine is removed by aeration. After aeration this content is transferred to 100 mL standard flask and makes it up to 100 mL with 4%

metaphosphoric acid.

- 85% H_2SO_4

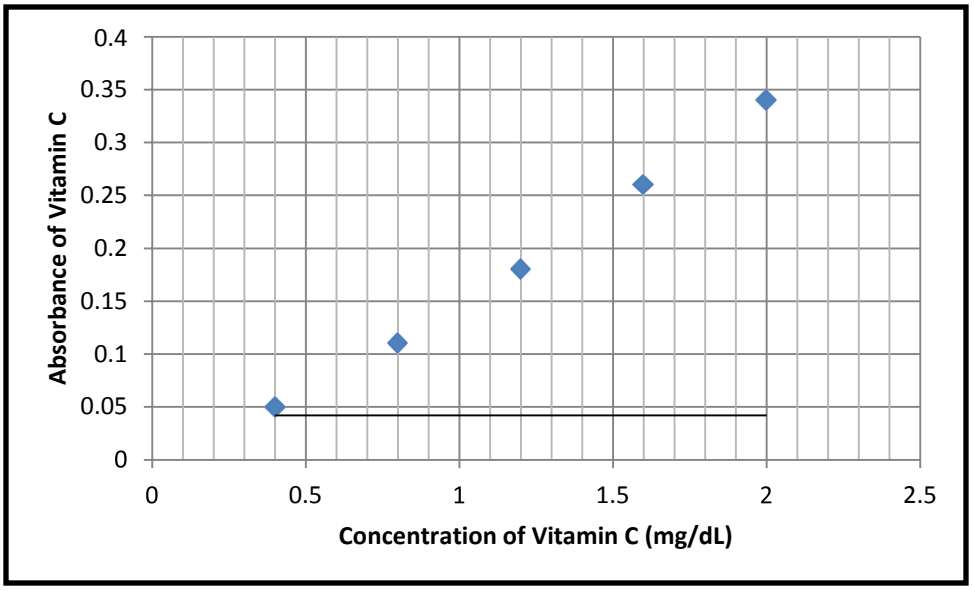
ASSAY PROCEDURE

Reagents	Blank	Standard	Test
Distilled water (mL)	0.4	-	-
Standard (mL)	-	0.4	-
Sample (mL)	-	-	0.4
4% MPA	3	3	3
Thiourea	1 drop		
2, 4- DNPH (mL)	1	1	1
Incubate at room temperature for 3 hours			
85% H_2SO_4	4	4	4
Cool in ice chest for 10 minutes **Absorbance at 530 nm**			

STANDARD GRAPH OF VITAMIN C

Concentration of Standards (mg/dL)	Absorbance	Concentration Obtained (mg/dL)
0.4	0.05	0.1396
0.8	0.11	0.1437
1.2	0.18	0.1472
1.6	0.26	0.1514
2.0	0.34	0.1531

Standard Graph of Vitamin C



$Y=0.59x+0.129$; $R^2 = 0.9856$

MICROALBUMIN- Turbilatex [152]

Principle: Microalbumin turbilatex is a quantitative turbidimetric test for the measurement of microalbumin (mALB) in human urine. Latex particles coated with specific antibodies anti-human albumin are agglutinated when mixed with samples containing mALB. Agglutination causes an absorbance change dependent upon mALB contents of the patient sample that can be quantified by comparison from a calibrator of known mALB concentration.

Automated Parameters	
Wavelength	540 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against distilled water
Reaction	2 point Kinetics
Reaction Direction	Increasing
Sample Volume	7 µl
Reagent Volume	1000 µl
Linearity	800 IU/mL

ASSAY PROCEDURE

Pipette into Test Tubes

	Calibrator	**Sample**
Sample	-	**7** µl
Standard	**7** µl	-
Reagent	1000 µl	1000 µl

Mix well and read the absorbance immediately A1 and after 2 minutes A2 of the sample addition

CALCULATION

$$\text{Micro Alb (mg/L)} = \frac{(A2\text{-}A1)\ \text{Sample}}{(A2\text{-}A1)\ \text{Calibrator}} \times \text{Calibrator}$$

URIC ACID: Uricase Method [152]

Principle: Uric acid is converted by uricase to allantoin and hydrogen peroxide which under the catalytic influence of peroxidase, oxidizes 3, 5- dichloro- 2- hydroxy benzene sulfonic acid and 4-aminophenazone to form a red violet quinoeimine compound. Absorbance is measured at 520 nm.

REAGENT COMPOSITION

Reagent 1: Uric acid reagent; Standard: Uric acid Standard 5 mg/dL (0.3 mmol/L)

Automated Parameters	
Wavelength	505 nm
Reaction Type	End Point
Cuvette	1 cm light path
Room Temperature	37^{o}C
Reaction type	Increasing
Measurement	Against reagent Blank
Sample Volume	25 µl
Reagent Volume	1000 µl
Incubation	10 minutes
Blank Absorbance limit	<0.200
Low Normal	2.4 mg/dL
High Normal	7.2 mg/dL
Linearity	25.0 mg/dL

ASSAY PROCEDURE

Pipette into test tubes

	Blank	Standard	Sample
Sample	-	-	25 µl
Standard	-	25 µl	
Reagent	1000 µl	1000 µl	1000 µl

Mix and incubate for 10 minutes at 37^{o}C. measure absorbance of sample (AT) an Standard (AS) against reagent blank at 505nm. Color produced is stable for 30 minutes at room temperature.

CALCULATION

Uric acid (mg/dl) = AT/AS X Concentration of Standard

UREA: Urease Method [152]

Principle: Urea is hydrolyzed in presence of water and urease to produce ammonia and

carbondioxide. Ammonia produced combines with alfa- oxoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD^+. Wave length at 340 nm.

Automated Parameters	
Wavelength	340 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against distilled water
Reaction	Fixed Time Kinetic
Reaction Direction	Decreasing
Sample Volume	10 µl
Working Reagent Volume	1000 µl
Number of Readings	01
Blank Absorbance Limit	>0.8
Low Normal at 37^{o}C	15 mg/dL
High Normal at 37^{o}C	50 mg/dL
Linearity	300 mg/dL

ASSAY PROCEDURE

	Standard	**Sample**
Sample	-	10 µl
Standard	10 µl	-
Working Reagent	1000 µl	1000 µl

Mix well and read after 30 seconds initial absorbance of sample (A1s) and standard (A1 std) and start timer simultaneously. Read again after 60 seconds.

CALCULATION

$$\text{Urea (mg/dL)} = \frac{(A2s-A1s)}{(A2std-A1std)} \times 50$$

CREATININE: Modified Jaffes Method [152]

Principle: Creatinine in alkaline solution reacts with picrate to form a coloured complex which absorbs at 500-520 nm. The amount of complex formed is directly proportional to the creatinine concentration.

Automated Parameters	
Wavelength	300 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against distilled water
Reaction Type	Fixed Time
Reaction Direction	Increasing
Sample Volume	100 µl
Reagent Volume	1000 µl
Number of Readings	01
Blank Absorbance Limit	>0.8
Low Normal at 37^{o}C	0.8 mg/dL
High Normal at 37^{o}C	1.4 mg/dL
Linearity	25 mg/dL

ASSAY PROCEDURE

Pipette into Test Tubes

	Standard	**Sample**
Sample	-	100 µl
Standard	100 µl	-
Working Reagent	1000 µl	1000 µl

Mix well and read after 30 seconds initial absorbance of sample (A1s) and standard (A1 std) and start timer simultaneously. Read after 1 minute determines Δ A/min of standard (As) and sample (ac) against reagent blank.

CALCULATION

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

GLUCOSE: GOD-POD Method [153]

Principle: Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed, reacts under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoeimine dye as indicator. Absorbance at 550 nm.

Automated Parameters	
Wavelength	505 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against Reagent Blank
Reaction Type	End point
Reaction Direction	Increasing
Sample Volume	10 µl
Reagent Volume	1000 µl
Incubation	05 minutes
Number of Readings	01
Blank Absorbance Limit	< 0.300
Low Normal at 37^{o}C	60 mg/dL
High Normal at 37^{o}C	110 mg/dL
Linearity	400 mg/dL

ASSAY PROCEDURE

Pipette into Test Tubes

	Blank	**Standard**	**Sample**
Sample	-	-	10 µl
Standard	-	10 µl	-
Working Reagent	1000 µl	1000 µl	1000 µl

Mix and incubate for 5 minutes at 37^{o}C. Measure absorbance of sample (AT) and Standard (AS) against reagent blank at 505 nm.

CALCULATION

$$\text{Concentration of Glucose (mg/dL)} = \frac{AT}{AS} \times \text{Concentration of Standard}$$

SODIUM: Spectrophotometric Method [152]

Principle: This method is based on reaction of sodium with a selective chromogen producing a chromophore whose absorbance varies directly as the concentration of sodium in the test specimen

Automated Parameters	
Wavelength	630 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against Reagent Blank

Reaction Type	End point
Reaction Direction	Increasing
Sample Volume	10 µl
Reagent Volume	1000 µl
Incubation	05 minutes
Low Normal at 37^{o}C	135 mmol/L
High Normal at 37^{o}C	155 mmol/L
Linearity	180 mmol/L

ASSAY PROCEDURE

Pipette into Test Tubes

	Blank	**Standard**	**Sample**
Colour Reagent	1000 µl	1000 µl	1000 µl
Standard	-	10 µl	-
Serum	-	-	10 µl

Mix and incubate for 5 minutes at 37^{o}C. Measure absorbance of sample (AT) and Standard (AS) against reagent blank at 630 nm.

CALCULATION

$$\text{Concentration of Sodium (mmol/L)} = \frac{AT}{AS} \times \text{Concentration of Standard}$$

POTASSIUM: Spectrophotometric Method [152]

Principle: potassium ions in a protein – free alkaline medium react with sodium tetraphenylboron to produce a finely dispersed turbid suspension of potassium tetraphenylboron. The turbidity produced is proportional to the potassium concentration and read photometrically.

Automated Parameters	
Wavelength	630 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against Reagent Blank
Reaction Type	End point
Reaction Direction	Increasing
Sample Volume	20 µl

Reagent Volume	1000 µl
Incubation	05 minutes
Low Normal at 37^{o}C	3.6 mEq/L
High Normal at 37^{o}C	5.5 mEq/L
Linearity	7.0 mEq/L

ASSAY PROCEDURE

Pipette into Test Tubes

	Blank	**Standard**	**Sample**
Potassium Reagent	1000 µl	1000 µl	1000 µl
Standard	-	20 µl	-
Serum	-	-	20 µl
Distilled water	20 µl	-	-

Mix and incubate for 5 minutes at 37^{o}C. Measure absorbance of sample (AT) and Standard (AS) against reagent blank at 630 nm.

CALCULATION

$$\text{Concentration of Sodium (mmol/L)} = \frac{AT}{AS} \times \text{Concentration of Standard}$$

CHOLESTEROL: Enzymatic method [148]

Principle: Cholesterol esters are hydrolyzed to produce cholesterol. Hydrogen peroxide is then produced from oxidation of cholesterol by cholesterol oxidase. Indicator quinoneimine is formed from hydrogen peroxide and 4- amino antipyrine in the presence of phenol and peroxide. Absorption (490-550 nm) of red quinoneimine dye is proportional to the concentration of cholesterol in the sample.

Automated Parameters	
Wavelength	505 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against Reagent Blank
Reaction Type	End point
Reaction Direction	Increasing
Sample Volume	10 µl
Reagent Volume	1000 µl
Incubation	05 minutes
Low Normal at 37^{o}C	< 200 mg/dL
High Normal at 37^{o}C	> 200 mg/dL
Linearity	1000 mg/dL

ASSAY PROCEDURE

Pipette into Test Tubes

	Blank	**Standard**	**Sample**
Reagent	1000 µl	1000 µl	1000 µl
Standard	-	10 µl	-
Serum	-	-	10 µl

Mix and incubate for 5 minutes at 37^{o}C. Measure absorbance of sample (AT) and Standard (AS) against reagent blank at 630 nm.

CALCULATION

$$\text{Concentration of Cholesterol (mg/dL)} = \frac{AT}{AS} \times \text{Concentration of Standard}$$

HDL – CHOLESTEROL Enzymatic method [148]

Principle: Direct determination of serum HDL (high density lipoprotein) levels without the need for any pre treatment or centrifugation of the sample. The method depends on the properties of a detergent which solublizes only the HDL so that HDL-cholesterol is released to react with the cholesterol esterase, cholesterol oxidase and chromogens to give color at 600 nm. The non HDL lipoprotein LDL,VLDL and chylomicrons are inhibited from reacting with the enzymes due to absorption of the detergents on their surfaces. The intensity of the colour formed is proportional to the HDL concentration in the sample.

Automated Parameters	
Wavelength	600 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against Reagent Blank
Reaction Type	End point
Reaction Direction	Increasing
Linearity	1300 mg/dL

ASSAY PROCEDURE

Pipette into Test Tubes

	Blank	**Calibrator**	**Sample**
Reagent 1 (µl)	300 µl	300 µl	300 µl
Calibrator (µl)	-	4	10 µl
Sample (µl)	-	-	4

Mix well and incubate for 5 minutes at 37^{o}C.

	Blank	**Calibrator**	**Sample**
Reagent 2 (µl)	100 µl	100 µl	100 µl

Measure absorbance of sample (A1) of samples and calibrator against blank

Read absorbance (A2) of samples and calibrator after 5 minutes against Blank.

Calculate increase of the absorbance $\Delta A = A_2 - A_1$

CALCULATION

$$\text{Concentration of Triglycerides (mg/dL)} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Calibrator}} \times \text{Concentration of Standard}$$

TRIGLYCERIDES: Enzymatic method [148]

Principle: Triglycerides are determined after enzymatic hydrolysis with lipases. Quinoneimine indicator is formed from hydrogen peroxide, 4-aminophenazone, and 4-chlorophenol under the catalytic influence of peroxidase. Absorbance at 520 nm.

Automated Parameters	
Wavelength	520 nm
Cuvette	1 cm light path
Reaction Temperature	37^{o}C
Measurement	Against Reagent Blank
Reaction Type	End point
Reaction Direction	Increasing
Sample Volume	10 µl
Reagent Volume	1000 µl
Incubation	05 minutes
Low Normal at 37^{o}C	40 mg/dL
High Normal at 37^{o}C	165 mg/dL
Linearity	1300 mg/dL

ASSAY PROCEDURE

Pipette into Test Tubes

	Blank	**Standard**	**Sample**
Standard	-	10 µl	-
Serum	-	-	10 µl
Reagent	1000 µl	1000 µl	1000 µl

Mix well and incubate for 5 minutes at 37^{o}C. Measure absorbance of sample (AT) and Standard (AS) against reagent blank at 630 nm.

CALCULATION

$$\text{Concentration of Triglycerides (mg/dL)} = \frac{AT}{AS} \times \text{Concentration of Standard}$$

GLUTATHIONE PEROXIDASE: UV Spectrophotomettric Method [154]

Principle: Glutathione peroxidase (GPX) catalyses the oxidation of glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione reductase (GR) and NADPH the oxidised Glutathione (GSSG) is immediately converted to reduced form with a concomitant oxidation of NADPH to $NADP^{+}$. The decrease in absorbance is measured at 348 nm.

Kit Components	**K762-100**	**Cap Code**	**Part Number**
GPx Assay Buffer	25 mL	WM	K762-100-1
NADPH (lyophilized)	1 Vial	Blue	K762-100-2
Glutathione Reductase	1 Vial	Green	K762-100-3
Glutathione (GSH; lyophilized)	1 Vial	Brown	K762-100-4
Cumene Hydroperoxide	1 Vial	Yellow	K762-100-5
GPx Positive Control (lyophilized)	1 Vial	Red	K762-100-6

REAGENT RECONSTITUTION AND GENERAL CONSIDERATION

NADPH: Reconstitute with 0.5 mL distilled water to get a 40mM NAPH solution

GR: Dilute with 0.22 mL Assay Buffer

GSH: Reconstitute with 0.22 mL Assay Buffer

Cumene Hydroperoxide: Dilute with 1.25 mL Assay Buffer. Mix well

GPx Positive Control: Reconstitute with 100µl Assay Buffer

Ensure that the assay buffer is at room temperature before use. Keep samples, GR mix solution and GPx Positive Control on ice during the assay.

Glutathione Reductase Activity Assay:

1. Sample Preparations: Serum can be tested directly. Keep samples at -80 °C for storage. Add 2-50 µl of the samples into a 96-well plate; bring the volume to 50 µl with Assay Buffer.

2. NADPH Standard Curve: Dilute 25 µl of the 40 mM NADPH solution into 975 µl dH2O to generate 1 mM NADPH standard. Add 0, 20, 40, 60, 80, 100 µl of the 1 mM NADPH Standard into 96-well plate in duplicate to generate 0, 20, 40, 60, 80,100 nmol/well standard. Bring the final volume to 100 µl with Assay Buffer. Measure O.D. 340 nm to plot the NADPH Standard Curve.

3. Positive Control (optional) and Reagent Blank:

For Positive Control use 5 - 10 µl of the GPx Positive Control into the desired well(s) and adjust to 50 µl with Assay Buffer. Add 50 µl of Assay Buffer into a well (s) as a Reagent Control (RC).

Reaction Mix: For each well, prepare 40 µl Reaction Mix:

33 µl Assay Buffer

3 µl 40 mM NADPH solution

2 µl GR solution

2 µl GSH solution

Add 40 µl of the Reaction Mix to each test samples, Positive Control (s) and RC(s) mix well, and incubate for 15 minutes to deplete all GSSG in your sample**.** Add 10 µl Cumene Hydroperoxide Solution to start GPx reaction. Mix well. Measure OD 340 nm at T1 to read A1, measure OD 340 nm again at T2 after incubating the reaction at 25 °C for 5 min (or longer if the GPx activity is low) to read A2, protect from light. ΔA340 nm = [(Sample (A1) – Sample (A2)) –(RC(A1) – RC (A2))]

Notes:

A. Measure OD at 340 nm before adding Cumene Hydroperoxide. Add more NADPH if the Sample OD at 340 nm is lower than 1.0 to ensure there is enough NADPH in the reaction system. 1 µl of 40 mM NADPH will give ~ 0.5 OD at 340 nm.

B. If A1 reading is too low (< 0.7), it means either too much GPx or too much GSSG presence in the sample. This may need to dilute the samples, or remove GSSG from sample using methods, such as dialyzing the sample or using spin filters (BioVision Cat.# 1997- 25) to remove GSSG.

C. It is essential to read A1 and A2 in the reaction linear range

5. CALCULATION: Plot NADPH standard Curve. Apply the ΔA340nm to the NADPH standard curve to get NADPH amount B.

$$\text{GPx Activity} = \frac{B}{(T2 - T1) \times V} \text{ X Sample dilution mU/mL}$$

Where: B is the NADPH amount that was decreased between T1 and T2 (in nmol).
T1 is the time of first reading (A1) (in min).

T2 is the time of second reading (A2) (in min).

V is the pretreated sample volume added into the reaction well (in ml).

Unit Definition: One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 μmol of NADPH to NADP+ under the assay kit condition per minute at 25°C.

GLYCATED HEMOGLOBIN: Cation Binding Exchange Resin Method (155)

Principle: Whole blood is mixed with lysing reagent to prepare a hemolysate. This is then mixed with a weakly binding cation-exchange resin. The non glycated hemoglobin binds to the resin leaving GHb free in the supernatant. The GHb percentage is determined by measuring the absorbance of the GHb fraction and of the total Hb.

CALCULATIONS

1. **LDL Is Calculated By Friedewald Equation (156)**

   LDL cholesterol = (Total cholesterol) – (HDL cholesterol) – (Triglyceride/5)

2. **Non-HDL** (156)

   Non-HDL = Total cholesterol - HDL

3. **eGFR Is Calculated By MDRD Formula (157)**

   GFR (ml/min/1.73 m^2) = 186 x $(S.cr)^{-1.154}$ x $(age)^{-0.203}$ x (0.742 if female) x (1.210 if Black)

STATISTICAL ANALYSIS

Comparison of one time data between the three study groups was done by Analysis of Variance (ANOVA).

Bartlett's test was used to identify the skewness for the variables normally distributed or non- normally distributed.

ANOVA and Bonferroni Post hoc test was done within the Group- II (T2DM with AKI).

Correlation among continuous data was performed by the Pearsons correlation coefficients. Comparison between categorical data was done by Fischer's exact test.

Serum NGAL and Cys C concentration was expressed as median and interquartile range (IQR).

All tests were two- tailed, and a $p<0.05$ was considered as statistical significance. ROC analysis was done using MedCalc software. SPSS statistical software program (licensed version 16.0, SPSS, Chicago, IL) was used for all analysis.

RESULTS

Table 6: Demographic Data of the Study Subjects (n=450)

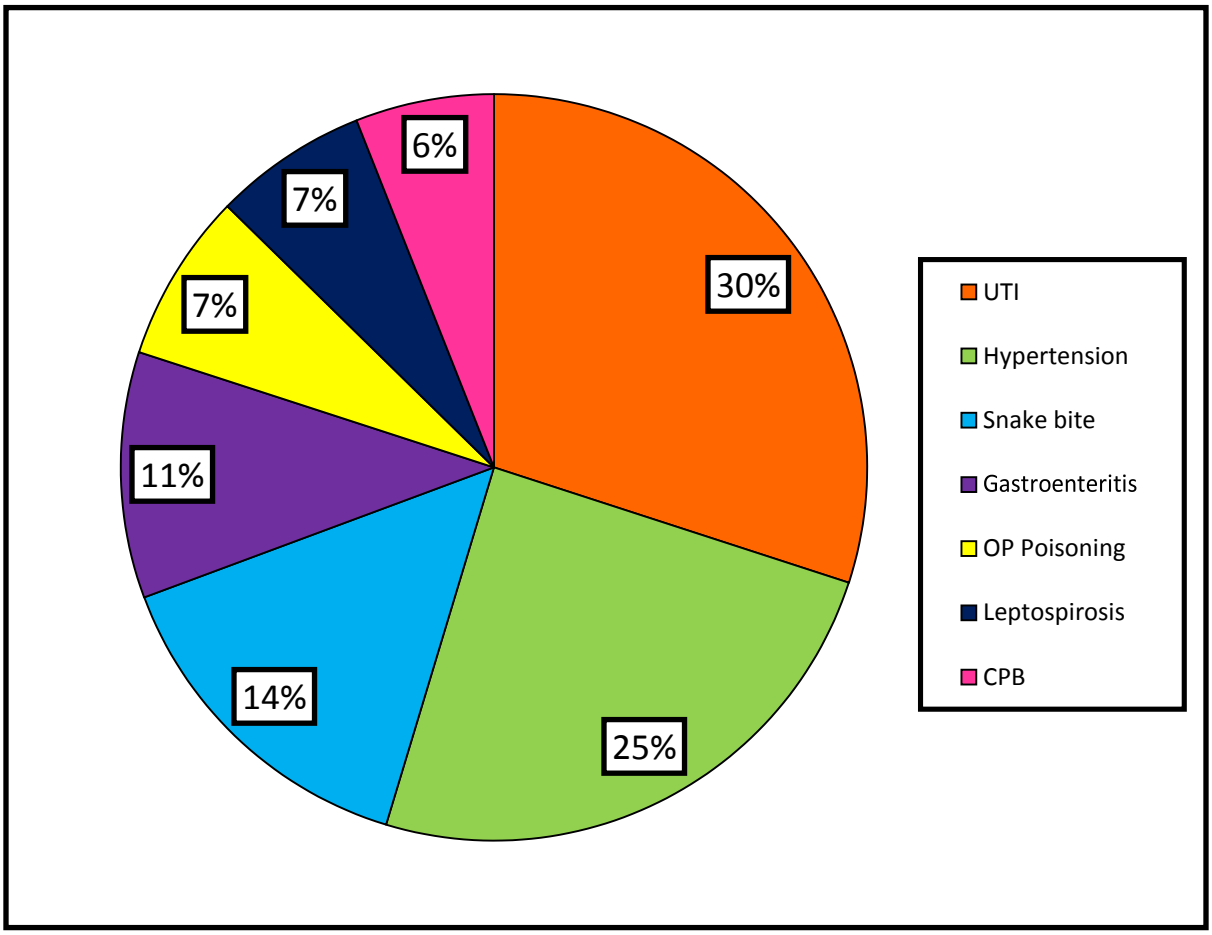
Variables	Group I (n=150)	Group II (n=150)	Group III (n=150)
Gender			
Male	92 (61.33%)	87(58%)	85 (56.66%)
Female	58 (38.66%)	63 (42%)	65 (43.34%)
Region			
Urban	58 (38.66%)	66 (44%)	63 (42%)
Rural	92 (61.34%)	84 (56%)	87 (58%)
Physical activity			
Active/ Moderately active	76 (50.66%)	69 (46%)	65 (43.33%)
Inactive	74 (49.34%)	81 (54%)	85 (56.67%)
Smoking History			
Non-smoker	58 (38.66%)	67 (44.66%)	101 (67.33%)
Daily/Occasional	124 (61.34%)	83 (55.34%)	49 (32.67%)
Diet			
Non-Vegetarians	94 (62.66%)	115 (76%)	85 (56.66%)
Vegetarians	56 (37.34%)	35(24%)	65 (43.34%)
H/o Alcohol			
Former/ non-drinker	61 (40.66%)	72 (48%)	58 (38.66%)
Regular/Occasional	89 (59.34%)	78 (52%)	92 (61.34%)
Life stress			
Not stressful	61 (40.66%)	58 (38.66%)	56 (37.33%)
Stressful	89 (59.34%)	92 (61.34%)	94 (62.34%)

Group I: T2DM without AKI

Group II: T2DM with AKI

Group III: Clinically Proven Healthy controls

Figure 7: Group II (T2DM with AKI; n=150) associated with other complications



Demographic data and characteristics of the study subjects were depicted in Table 6. T2DM with AKI (Group II) are associated with other complications as follows: Among 150 subjects, majority of Group II subjects had Urinary Tract Infection (UTI) n= 45 (30%), Hypertension n=37 (25%), Snake Bite n=22 (14%), Gastroenteritis n= 16 (11%), OP Poisoning n=11 (7%), Leptospirosis n=10 (7%) and cardiopulmonary Bypass Surgery (CPBS) n=9 (6%). Figure 7.

Table 7: Mean and Standard Deviation of Anthropometric & Physiological variables of Groups I, II & III

PARAMETERS	GROUP I Mean ± SD	GROUP II Mean ± SD	GROUP III Mean ± SD	ANOVA F-value	p-value
AGE (years)	64.02±10.50	66.41±9.81	62.22±7.27	13.26	0.081
Height (cm)	162.56±8.48	160.81±6.51	155.77±7.79	14.89	0.001*
Weight (Kg)	69.68±10.16	67.71±8.96	55.05±9.57	48.05	0.001*
BMI (Kg/m^2)	26.13±3.53	24.8±3.45	22.91±3.31	39.21	0.001*
OBI	62.55±8.48	60.81±6.51	55.77±7.79	14.89	0.001*
WC (cm)	97.40±5.01	96.60±0.78	92.38±8.67	10.04	0.001*
HC (cm)	95.47±5.08	96.11±5.09	96.71±5.92	0.62	0.53
WHR	1.02±0.04	1.00±0.06	0.95±0.06	0.85	0.001*
SBP (mmHg)	126±5.04	128±9.85	124±5.33	1.44	0.23
DBP (mmHg)	80±4.21	94±5.14	80±4.62	3.61	0.001*

***p<0.001 highly significant**

Group I: Diabetes without AKI, **Group II:** Diabetes with AKI, **Group III:** Non-Diabetes (clinically proven healthy controls), **BMI:** Body mass index, **OBI:** Obesity index, **WC:** Waist Circumference, **HC: Hip Circumference, WHR:** Waist Hip Ratio, **SBP:** Systolic Blood Pressure, **DBP:** Diastolic Blood Pressure

Among the anthropometric variables, Height 162.56±8.48, 160.81±6.51 and 155.77±7.79; Weight 69.68±10.16, 67.71±8.96 and 55.05±9.57; BMI 26.13±3.53, 24.8±3.45 and 22.91±3.31; OBI 62.55±8.48, 60.81±6.51 and 55.77±7.79; WC 97.40±5.01, 96.60±0.78 and 92.38±8.67 and WHR 1.02±0.04, 1.00±0.06 and 0.95±0.06 were significantly higher in Group I compared to Group II and Group III. With respect to physiological variables, DBP 80±4.21, 94±5.14 and 80±4.62 was significantly higher in Group II when compared to Group I and Group III. **table 6**.

Table 8a: Mean and Standard Deviation of Biochemical parameters of Groups I, II & III

PARAMETERS	GROUP I Mean ± SD	GROUP II Mean ± SD	GROUP III Mean ± SD	ANOVA-F-value	p-value
FBS (mg/dl)	187.99±24.61	249.91±32.18	91.60±14.44	9.96	0.001**
PPBS (mg/dl)	199.84±11.27	387.72±41.72	113.21±9.62	8.45	0.03*
HbA1c (%)	9.06±2.35	10.88±2.08	6.31±1.47	7.33	0.001**
C-Peptide (ng/ml)	2.9±1.33	4.8±1.99	0.62±0.12	5.48	0.001**
NGAL (ng/ml)	160±13.35	210±25.42	90.42±18.36	149.04	0.03*
Cys C (mg/L)	4.73±2.24	5.27±2.53	2.08±1.23	12.10	0.001**
SCr (mg/dl)	0.94±0.31	2.40±0.46	0.6±0.14	29.55	0.001**
Urea (mg/dl)	48.63±4.85	88.26±10.45	30.41±3.42	24.12	0.001**
Uric acid(mg/dl)	5.85±1.17	7.09±1.08	4.92±1.00	5.40	0.001**
Sodium (mEq/L)	145.2±4.9	158.63±8.6	135.61±5.8	98.45	0.03*

***p<0.05 significant, **p<0.001 highly significant**

Group I: Diabetes without AKI, **Group II:** Diabetes with AKI, **Group III:** Non-Diabetes (clinically proven healthy controls), **FBS: Fasting Blood Sugar, PPBS:** Post Prandial Blood Sugar, **HbA1c:** Glycated Hemoglobin, **NGAL:** Neutrophil Gelatinase Associated Lipocalin, **Cys C:** Cystatin C, **SCr:** Serum Creatinine

Table 8b: Mean and Standard Deviation of Biochemical parameters of Groups I, II & III

PARAMETERS	GROUP I Mean ± SD	GROUP II Mean ± SD	GROUP III Mean ± SD	ANOVA-F-value	p-value
TC (mg/dl)	181.81±34.81	175.87±26.13	100.69±15.53	201.03	0.001**
TG (mg/dl)	232.83±52.66	185.03±20.01	94.97±14.15	224.89	0.03*
HDLc (mg/dl)	45.15±6.90	48.65±7.15	39.85±7.39	12.57	0.001**
LDLc (mg/dl)	180.23±37.81	170.22±22.82	79.82±17.76	268.04	0.001**
Non-HDLc (mg/dl)	134.58±6.82	149.85±10.42	94.42±4.36	59.09	0.04*
NO (µM/L)	34.48±7.44	28.78±4.89	54.06±18.59	86.84	0.001**
hs-CRP(mg/L)	1.98±0.45	2.12±0.95	0.94±1.12	0.074	0.022*
MDA (nmol/ml)	8.48±2.50	9.90±2.68	2.25±0.92	12.84	0.031*
Vitamin C (mg/dl)	0.42±0.13	0.35±0.37	1.18±0.56	0.84	0.001**
GPx (mU/mL)	1.91±0.90	0.41±0.12	2.25±1.16	0.97	0.02*
Microalbumin (mg/g)	26.85±4.38	278.15±31.45	12.13±3.36	15.26	0.001**
eGFR(ml/min/1.73m^2)	84.67±16.55	50.78±7.05	120.36±15.46	0.46	0.03*

***p<0.05 significant, **p<0.001 highly significant**

Group I: Diabetes without AKI, **Group II:** Diabetes with AKI, **Group III:** Non-Diabetes (clinically proven healthy controls), **TC:** Total Cholesterol, **TG:** Triglycerides, **HDLc:** High Density Lipoproteins, LD**Lc:** Low Density Lipoproteins, **NO:** Nitric Oxide, **hs-CRP;** High Sensitive C- reactive protein, **MDA:** Malondialdehyde, **GPx:** Glutathione Peroxidase; eGFR: estimated Glomerular Filtration rate.

With respect to biochemical parameters, the mean values of fasting blood sugar 187.99±24.61, 249.91±32.18 and 91.60±14.44; Post Prandial blood sugar 199.84±11.27, 387.72±41.72 and 113.21±9.62; HbA1c 9.06±2.35, 10.88±2.08 and 6.31±1.47 and C- Peptide 2.9±1.33, 4.8±1.99 and 0.62±0.12 were significantly elevated in Group II compared to Groups III and I.

When renal function tests were compared between groups, Mean values of NGAL 160±13.35, 210±25.42 and 90.42±18.36; CysC 4.73±2.24, 5.27±2.53 and 2.08±1.23; Scr 0.94±0.31, 2.40±0.46 and 0.6±0.14; Urea 48.63±4.85, 88.26±10.45 and 30.41±3.42 and UA 5.85±1.17, 7.09±1.08 and 4.92±1.00 was significantly higher in Group II compared to Group I and III.

Concentration of electrolytes such as Sodium 145.2±4.9, 158.63±8.6 and 135.61±5.8 and Potassium 4.2±0.6, 5.8±2.7 and 3.8±1.0 was higher in Group II compared to Groups III & I

With respect to Lipid profile, TC 181.81±34.81, 175.87±26.13 and 100.69±15.53; TG 232.83±52.66, 185.03±20.01 and 94.97±14.15 and LDLc 180.23±37.81, 170.22±22.82 and 79.82±17.76 were significantly elevated in Group I compared with Groups III & II. However, concentration of HDLc 45.15±6.90, 48.65±7.15 and 39.85±7.39 and Non-HDLc 134.58±6.82, 149.85±10.42 and 94.42±4.36 were higher in Group II with a significant p value.

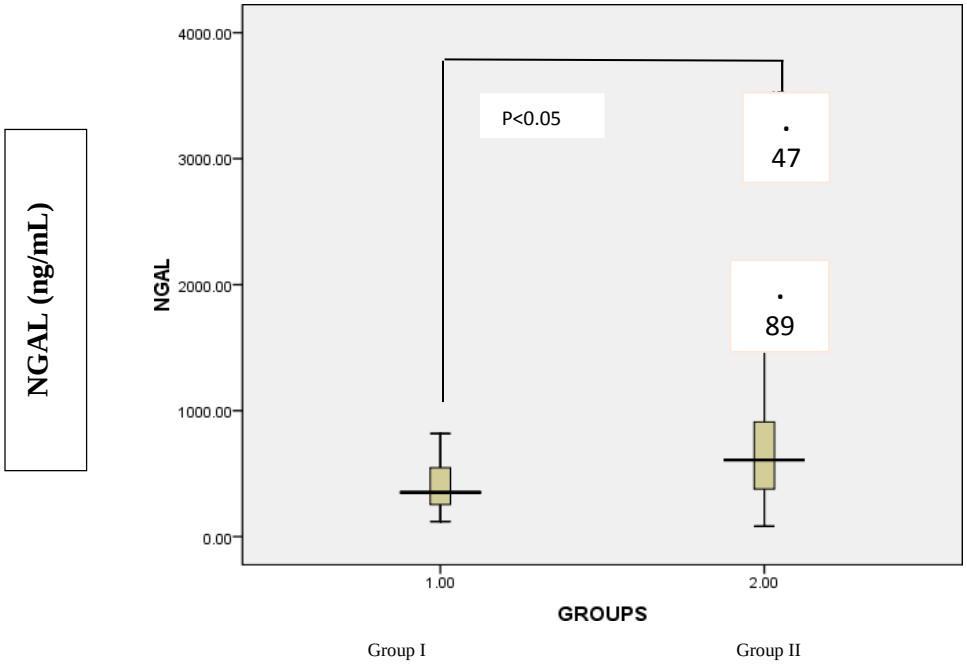
Concentration of inflammatory molecules such as NO 34.48±7.44, 28.78±4.89 and 54.06±18.59 was significantly higher in Group III and hs-CRP 1.98±0.45, 2.12±0.95 and 0.94±1.12 was significantly elevated in Group II.

Mean value of total MDA 8.48±2.50, 9.90±2.68 and 2.25±0.92 was more in Group II compared to Groups I & III with a significant p value.

Antioxidant status such as Vitamin C 0.42±0.13, 0.35±0.37 and 1.18±0.56 and GPx 1.91±0.90, 0.41±0.12 and 2.25±1.16 was significantly higher in Group III compared with Groups I & II

Microalbumin 278.15±31.45, 26.85±4.38 and 12.13±3.36 was significantly increased in Group II when Compared to Group III and Group I. eGFR 120.36±15.46, 84.67±16.55, 50.78±7.05 in Group III, I and II respectively (**table 8).**

Figure 8: Box and whisker plot, serum NGAL in Group I (T2DM without AKI) Vs Group II (T2DM with AKI)

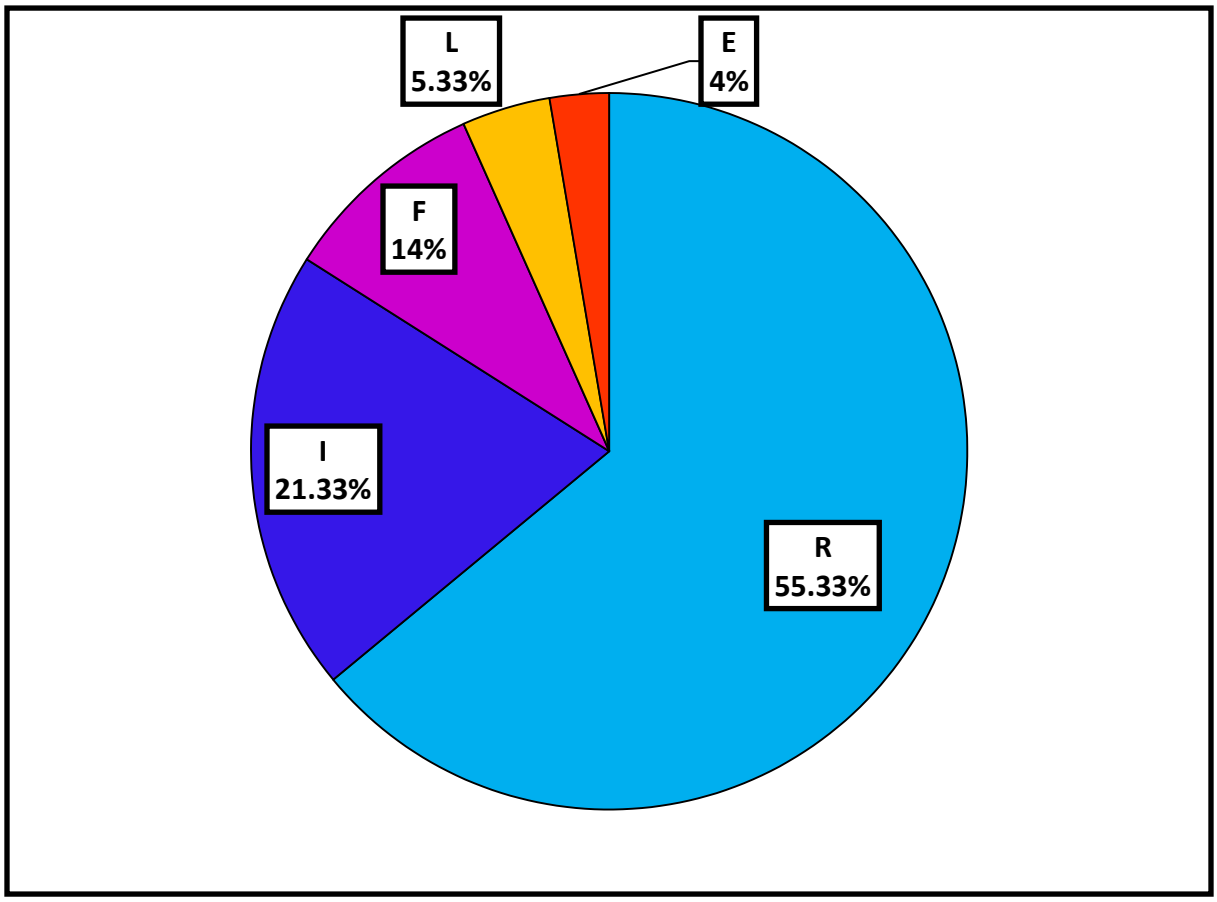


Vertical box represents the 25th percentile (bottom line), median (middle line), and 75th percentile values. Dot (.) represents values outside the 10th and 90th percentile respectively

Note: IQR: Inter Quartile Range, $p<0.05$.

Concentration of serum NGAL was significantly increased in Group II (T2DM with AKI) compared with Group I (T2DM without AKI). Group I (median 359 ng/mL, IQR 236 ng/mL) and Group II (median 612.41 ng/mL, IQR 540.88 ng/mL) reflecting the greater severity of illness in Group II.

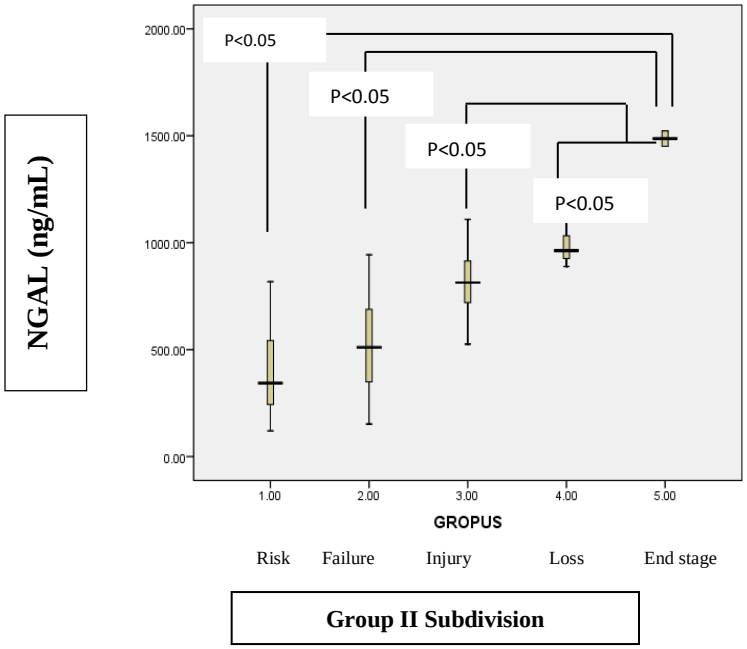
Figure 9: Pie diagram, Group II (T2DM with AKI) classification and Distribution based on RIFLE criteria



Among the 150 Group II subjects (T2DM with AKI), highest distribution was in Risk (R) (n=83; 55.33%) and least in End stage (E) (n=32; 21%).

These data was studied in patients on the day of admission as baseline values and periodical estimation was done daily till the day of discharge (on an average 14 days) in ICU and MICU admissions.

Figure 10: Box and whisker plot, serum NGAL concentration in Group II (T2DM with AKI)



Serum NGAL concentration was significantly elevated between Risk (median 343.69 ng/mL, IQR 314.24ng/mL) Injury (median 510.64ng/mL, IQR 415.94 ng/mL), Failure (median 813.83 ng/mL, IQR 311.00 ng/mL), Loss (median 963.00 ng/mL) and End stage (median 1486.73 ng/mL; $p<0.05$) sub-groups within Group II.

Note: IQR: Inter Quartile Range, $p<0.05$

Table 9: Comparison of biomarkers, oxidants and antioxidants based on RIFLE criteria '0' day and 14th day within Group II (T2DM with AKI)

Criteria Parameters	Days	Risk (R) (55.33%) Mean ± SD	Injury (I) (21.33%) Mean ± SD	Failure (F) (14%) Mean ± SD	Loss (L) (5.33%) Mean ± SD	End stage (E) (4%) Mean ± SD	p value
NGAL (ng/mL)	0	168±12.12	175±19.49	190±23.14	210±24.15	280±25.18	0.003*
	14th	125.02±12.52	142.25±15.10	160.56±20.14	190.96±23.56	250.85±22.15	
Cys C (ng/mL)	0	3.68±2.58	3.47±2.58	2.98±2.14	3.45±2.53	3.57±2.65	0.044*
	14th	2.89±2.23	2.76±2.14	2.66±1.94	2.64±1.93	2.47±1.94	
∞ **SCr (mg/dl)**	0	2.40±0.48	2.9±0.45	3.2±0.49	3.9±0.77	5.4±1.14	0.004*
	14th	1.9±0.21	2.5±1.25	2.8±1.96	3.1±1.99	4.8±2.53	
eGFR	0	51.77±7.35	47.73±4.54	40.28±9.37	35.33±12.7	25.45±15.1	0.02*
(mL/min/1.73m^2)	14th	68.14±5.22	57.44±6.54	55.14±12	50.14±14.52	42.56±13.36	
∞ **MDA (nmol/mL)**	0	9.08±2.47	8.30±2.24	9.60±2.55	10.66±1.75	9.75±2.85	0.03*
	14th	7.52±3.75	6.45±2.89	6.80±4.12	6.45±3.25	7.91±2.42	
NO (µM/mL)	0	58.69±18.80	54.08±9.72	64.07±22.60	56.73±14.16	66.17±21.15	0.02*
	14th	49.10±12.52	51.21±15.15	60.54±11.25	53.02±12.47	58.74±14.85	
hs-CRP (mg/dL)	0	1.99±0.49	1.97±0.38	2.12±0.31	1.66±0.15	2.90±0.95	0.04*
	14th	0.85±0.12	1.32±0.96	1.91±0.19	1.31±1.12	2.35±1.25	
UA (mg/dL)	0	7.28±1.04	5.54±0.86	5.83±0.94	6.13±0.66	5.80±0.14	0.01*
	14th	7.19±2.03	5.48±1.78	5.81±2.45	6.11±1.48	5.80±1.25	
Vitamin C (mg/dL)	0	0.45±0.42	0.38±0.25	0.45±0.21	0.69±0.32	0.55±0.06	0.481
	14th	0.56±1.23	0.51±0.12	0.54±0.99	0.92±0.29	0.56±1.02	
GPx (mU/mL)	0	0.54±0.12	0.51±0.22	0.39±0.21	0.14±0.09	0.06±0.03	0.613
	14th	0.71±1.12	0.55±0.85	0.42±0.14	0.35±0.72	0.23±0.11	

∞: Mann-Whitney U Test, * $p<0.05$ statistical significant

Table 9, show the mean and p values of the data analyzed at the time of admission ('0' day) and day of discharge (on an average 14th day). Concentration of serum NGAL, Scr and NO were significantly elevated in all groups, eGFR was statistically significant between R vs L groups and R vs E groups, UA was significant between R vs L groups. Serum MDA and hs-CRP concentrations were slightly elevated in Loss, Failure and End stage groups respectively compared to Risk and Injury groups.

NGAL data when compared from zero day to fourteenth day (maximum stay in ICU), patients in Group II following varied etiologies, RIFLE criteria on zero day was higher and gradually reduced by 14th day. NGAL compared on '0' day and 14th day for Failure, values are comparable with Group I rather Group III.

Table 10a: Correlation of Scr with oxidants, antioxidants and inflammatory markers in Group I and Group II

	Scr			
Variables	**Group I** T2DM without AKI		**Group II** T2DM with AKI	
	r value	**p value**	**r value**	**p value**
eGFR	0.112	0.012*	0.172	0.05*
Uric acid	0.260	0.015*	0.487	0.001*
NO	-0.130	0.431	0.411	0.002*
hs-CRP	0.062	0.612	0.241	0.004*
MDA	0.005	0.965	0.106	0.021*
Vitamin C	-0.215	0.044*	0.049	0.563
GPx	0.011	0.423	0.038	0.048*

* $p<0.05$ statistical significant

Table 10b: Correlation of NGAL with oxidants, antioxidants and inflammatory markers in Group I and Group II

	NGAL			
Variables	**Group I** T2DM without AKI		**Group II** T2DM with AKI	
	r value	**p value**	**r value**	**p value**
Scr	0.441	0.006*	0.812	0.001*
eGFR	0.217	0.032*	-0.718	0.001*
Uric acid	0.275	0.025*	0.316	0.012*
NO	-0.299	0.041*	-0.424	0.002*
hs-CRP	0.312	0.031*	0.226	0.007*
MDA	-0.055	0.517	0.552	0.002*
Vitamin C	-0.072	0.396	-0.412	0.002*
GPx	0.059	0.441	-0.191	0.037*

* $p<0.05$ statistical significant

Table: 10a make clear that in Group I, Scr was significant and positively correlated with eGFR (r=0.112; p= 0.012) and UA (r=0.260; p=0.015). There is a significant negative correlation with Vitamin C (r=-0.215; p=0.044). In Group II, eGFR (r=0.172; p=0.05), UA (r=0.487; p=0.001), NO (r=0.411; p=0.002), hs-CRP (r=0.241; p=0.004), MDA (r=0.106; p=0.021) and GPx (r=0.038; p=0.048) were positively correlated with Scr with an exception of Vitamin C (r=0.049; p=0.563).

Table: 10b shows NGAL positive correlation with Scr (r=0.441; p=0.006), eGFR (r=0.217; p=0.032), UA (r=0.275; p=0.025), NO (r=-0.299; p=0.041) and hs-CRP (r=0.312; p=0.031) in T2DM without AKI. In Group II NGAL has showed strong positive correlation with Scr (r= 0.812; p=0.001), UA (r=0.316; p=0.012), hs-CRP

(r=0.226; p=0.007) and MDA (r=0.552; p=0.002). However, negative correlation was observed with eGFR (r=-0.718; p=0.001), NO (r=-0.424; p=0.002), vitamin C (r=-0.412; p=0.002) and GPx (r=-0.191; p=0.037) in Group II.

Figures 11, 12 &13: Comparison of NGAL, Cys C and Scr on '0' and 14th day Individually within Group II (T2DM with AKI)

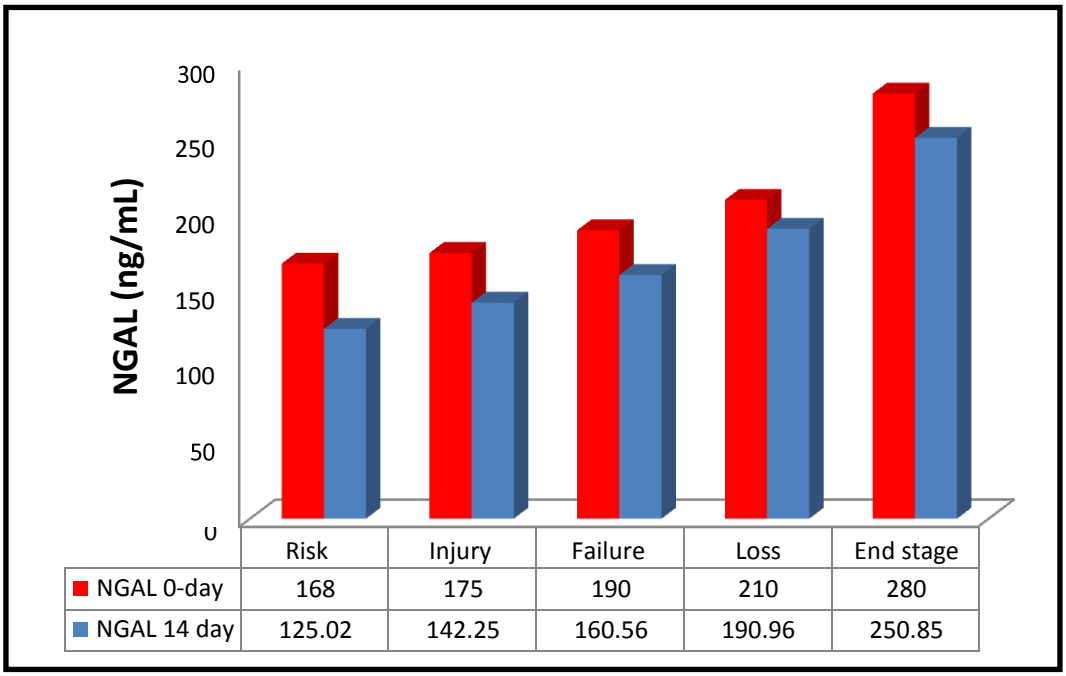


Figure 11: NGAL: '0' Day and 14th– Day

NGAL significantly elevated in all the subgroups in '0' day and exponentially decreased at 14th day in all the groups.

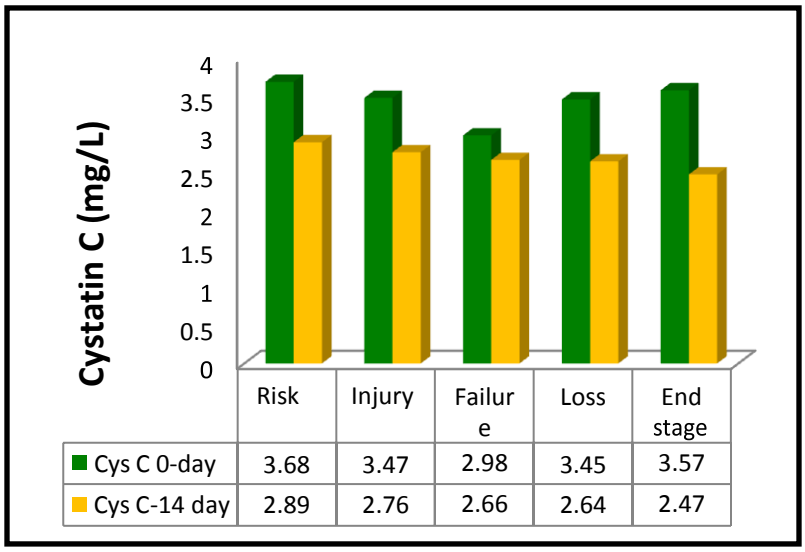


Figure 12: Cys C: '0'Day and 14th – Day

Concentration of Cystatin C higher in all the subgroups in Group II at '0' day and gradually decreased at 14th day

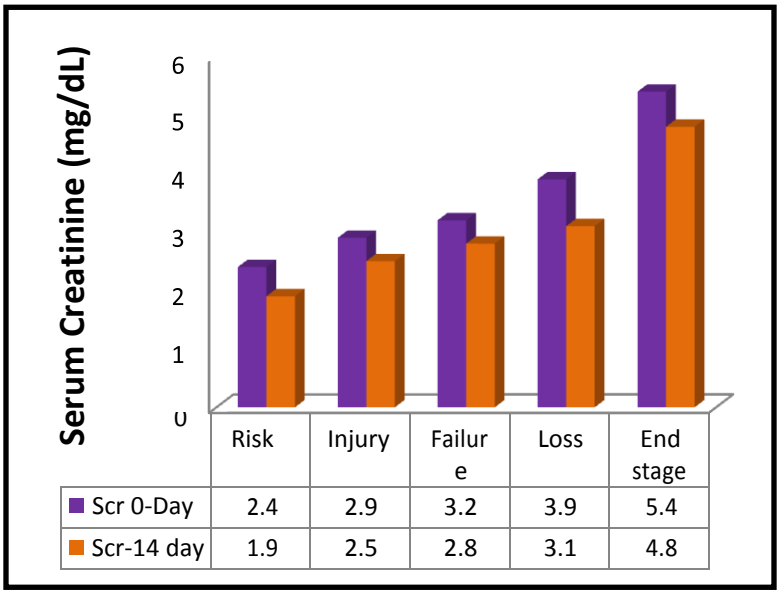
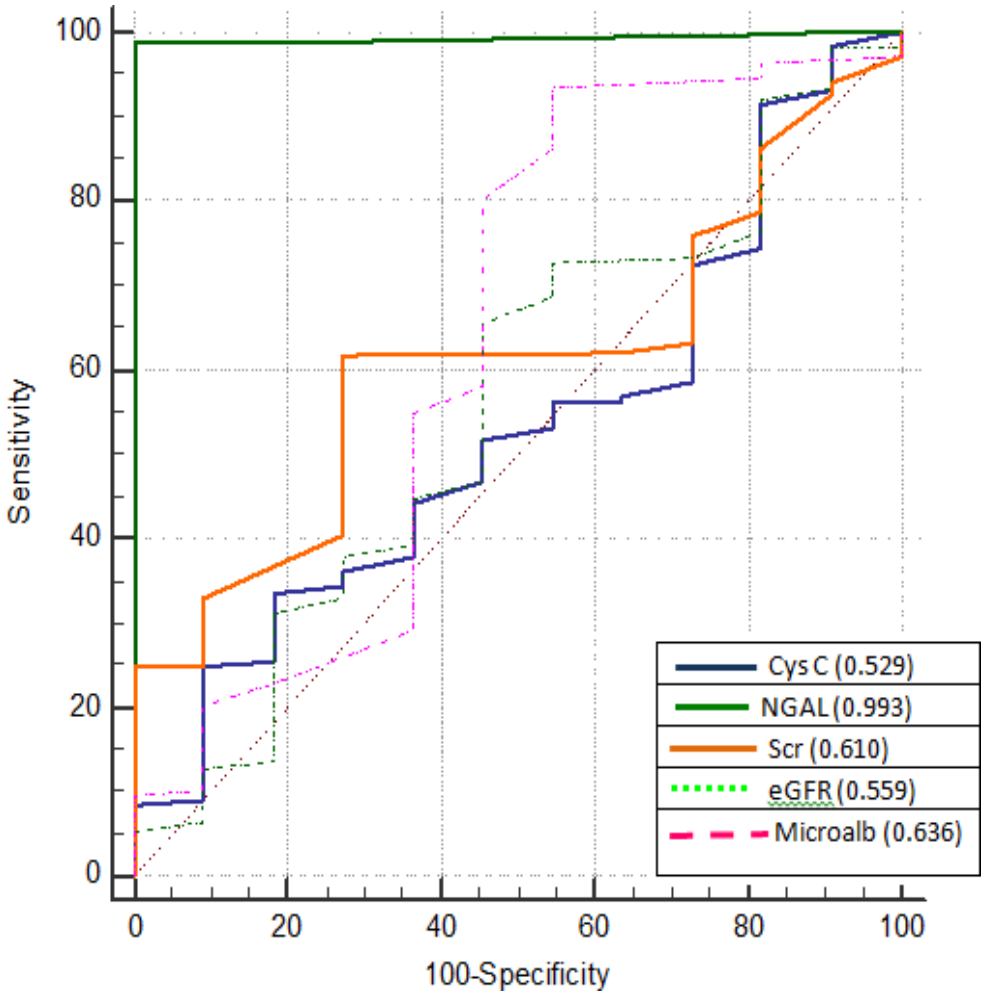


Figure 13: Serum Creatinine: '0' Day and 14th Day

Serum creatinine significantly elevated uniformly with a difference of 0.3 mg/dL in each group in '0' day and decreased gradually at '14th' day.

Figure 14: Receiver Operating Characteristic curve (ROC) of Serum NGAL, Cys C and Scr and Urine eGFR and Microalbumin



ROC analysis to define the diagnostic profile of Serum NGAL, Cystatin C Scr, eGFR and Microalbumin in Group I and Group II, serum NGAL showed an AUC of 0.993 (95% CI, 0.969-1.00) with a cutoff value of 1.06 (sensitivity, 81.0%; specificity, 87.1%), Serum Cystatin C with an AUC of 0.529 (95% CI, 0.457- 0.600), Scr AUC of 0.610 (95% CI, 0.539- 0.679), AUC of eGFR 0.559 (95% CI, 0.487- 0.629) and microalbumin AUC of 0.636 (95% CI, 0.565- 0.703).

Table 11, shows the pair wise comparison of biomarkers with existing gold standard parameters Scr, Microalbumin and eGFR at 95% confidence interval and with p-value < 0.05.

Table 11: Pair wise comparison of ROC curves

CYS ~ NGAL	
Difference between areas	0.464
Standard Error [a]	0.0864
95% Confidence Interval	0.295 to 0.634
z statistic	5.370
Significance level	P < 0.0001
CYS ~ Scr	
Difference between areas	0.0815
Standard Error [a]	0.115
95% Confidence Interval	-0.144 to 0.307
z statistic	0.709
Significance level	P = 0.4783
CYS ~ eGFR	
Difference between areas	0.0302
Standard Error [a]	0.101
95% Confidence Interval	-0.168 to 0.228
z statistic	0.299
Significance level	P = 0.7647
CYS ~ Microalb	
Difference between areas	0.107
Standard Error [a]	0.169
95% Confidence Interval	-0.225 to 0.439
z statistic	0.629
Significance level	P = 0.5291
NGAL ~ Scr	
Difference between areas	0.383
Standard Error [a]	0.0704
95% Confidence Interval	0.245 to 0.521
z statistic	5.435
Significance level	P < 0.0001

NGAL ~ eGFR	
Difference between areas	0.434
Standard Error [a]	0.0958
95% Confidence Interval	0.246 to 0.622
z statistic	4.531
Significance level	P < 0.0001
NGAL ~ Microalb	
Difference between areas	0.358
Standard Error [a]	0.110
95% Confidence Interval	0.143 to 0.572
z statistic	3.265
Significance level	P = 0.0011
Scr ~ eGFR	
Difference between areas	0.0513
Standard Error [a]	0.137
95% Confidence Interval	-0.218 to 0.320
z statistic	0.374
Significance level	P = 0.7087
Scr ~ Microalb	
Difference between areas	0.0251
Standard Error [a]	0.109
95% Confidence Interval	-0.188 to 0.238
z statistic	0.232
Significance level	P = 0.8168
eGFR ~ Microalb	
Difference between areas	0.0764
Standard Error [a]	0.147
95% Confidence Interval	-0.213 to 0.365
z statistic	0.518
Significance level	P = 0.6044

Table 12a & 12b: Calculation of Biomarker Sensitivity and Specificity Compared with Gold Standard Parameter (Scr)

Formula for Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value [157]

1. **Sensitivity: (a/a+c) X 100**
2. **Specificity: (d/b+d) X 100**
3. **Positive predictive value (PPV): (a/a+b) X 100**
4. **Negative Predictive Value (NPV): (d/c+d) X 100**

12(a). NGAL vs Scr

	Scr (+)	Scr (-)	
NGAL (+)	182 a	96 b	278 a+b
NGAL (-)	13 c	159 d	172 c+d
	195	255	450

12(b). Cys C vs Scr

	Scr (+)	Scr (-)	
Cys C (+)	158 a	89 b	247 a+b
Cys C (-)	37 c	166 d	203 c+d
	195	255	450

Table 12c: Calculation of Biomarker Sensitivity and Specificity Compared with Cys C

12(c). NGAL vs Cys C

	Cys C (+)	Cys C (-)	
NGAL (+)	301 a	43 b	344 a+b
NGAL (-)	24 c	82 d	203 c+d
	325	125	450

On comparing with serum creatinine as gold standard parameter, NGAL has sensitivity of 93%, specificity 62%, positive predictive value 65% and negative predictive value of 92%.

With respect to Cys C, sensitivity 81% was observed, specificity 65%, positive predictive value 63% and 81% negative predictive value. However with Cys C; NGAL showed sensitivity 92%, specificity 65%, positive predictive value 87% and negative predictive value 77%.

Table 13: Comparison of biomarkers, oxidants and antioxidants within Group II (T2DM with AKI) secondary to Urinary Tract Infection

No. of Days / **Variables**	**0-4**	**5-9**	**10-14**	**p-value**
NGAL (ng/mL)	184±18.51	210±25.16	174±19.18	0.012*
Cys C (ng/mL)	5.27±2.53	6.23±2.89	5.12±2.11	0.032*
Scr (mg/dL)	5.8±1.16	5.2±1.14	5.1±1.12	0.021*
NO (µM/mL)	51.21±15.15	56.73±14.15	58.54±14.41	0.044*
MDA (nmol/mL)	9.58±2.66	10.68±1.75	9.62±2.55	0.891
hs-CRP (mg/dL)	2.91±0.95	2.35±1.24	1.91±0.19	0.042*
Uric acid (mg/dL)	5.91±1.45	5.62±1.62	5.41±0.92	0.196
Vitamin C (mg/dL)	0.55±0.08	0.62±0.31	0.91±0.29	0.037*
GPx (mU/mL)	0.44±0.14	0.52±0.85	0.71±1.12	0.025*

***p<0.05 considered as significant**

Table 13 shows oxidants and antioxidants status within Group II subjects associated with UTI, observed from baseline '0'day to 14th day, the mean values of NGAL 184±18.51, 210±25.16 & 174±19.18 and Cys C 5.27±2.53, 6.23±2.89 &

5.12±2.11 were significantly higher in frequency distribution 5-9 days compared to 0-4 days and 10-14 days. Scr 5.8±1.16, 5.2±1.14 & 5.1±1.12 and hs-CRP 2.91±0.95, 2.35±1.24 & 1.91±0.19 were significantly higher in 0- 4 days. Concentration of NO 51.21±15.15, 56.73±14.15 & 58.54±14.41 Vitamin C 0.55±0.08, 0.62±0.31 & 0.91±0.29 and GPx 0.44±0.14, 0.52±0.85 & 0.71±1.12 were significantly increased in 10-14 days.

Table 14a and 14b: Correlation of Scr and NGAL with oxidants antioxidants and inflammatory markers in Group II (T2DM patients with AKI) secondary to UTI

14a: Correlation with Scr

	Scr	
Variables	**Group II T2DM with AKI (UTI)**	
	r value	**p value**
NGAL	0.613	0.001
eGFR	0.174	0.021*
Uric acid	-0.417	0.004*
NO	0.315	0.002*
hs-CRP	0.221	0.04*
MDA	0.117	0.03*
Vitamin C	-0.025	0.325
GPx	-0.042	0.411

***p<0.05 considered as significant**

14b: Correlation with NGAL

	NGAL	
Variables	**Group II T2DM with AKI (UTI)**	
	r value	**p value**
Scr	0.613	0.001*
eGFR	0.571	0.001*
Uric acid	0.326	0.031*
NO	0.413	0.003*
hs-CRP	0.525	0.001*
MDA	-0.424	0.001*
Vitamin C	-0.672	0.001*
GPx	-0.055	0.372

***p<0.05 considered as significant**

Table 14a and 14b depicts correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers in UTI. Scr shows significant positive correlation

with NGAL (r=0.613; p= 0.001), eGFR (r=0.174; p=0.021), NO (r=0.315; p=0.002), hs-CRP (r=0.221; p=0.04) and MDA (r=0.117; p=0.03), with an exception of UA (r=-0.417; p=0.004) significant negative correlation was observed. Concentration of NGAL was significant and positively correlated with Scr (r=0.613; p=0.001), eGFR (r=0.571; p=0.001), Uric acid (r=0.326; p=0.031), NO (r=0.413; p=0.003), hs-CRP (r=0.525; p=0.001) and significant negative correlation with MDA (r=-0.424; p=0.001).

Table 15: Comparison of biomarkers, oxidants and antioxidants within Group II (T2DM with AKI) secondary to Hypertension (HTN)

No. of Days / **Variables**	**0-4**	**5-9**	**10-14**	**p-value**
NGAL (ng/mL)	105±18.14	125±11.16	115±10.14	0.042*
Cys C (mg/L)	1.85±0.90	2.85±1.04	2.11±2.04	0.032*
Scr (mg/dL)	1.7±0.46	2.3±0.42	1.9±0.41	0.024*
NO (µM/mL)	52.07±19.71	46.17±18.16	49.18±18.32	0.043*
MDA (nmol/mL)	8.41±2.25	7.62±2.17	7.21±2.04	0.128
hs-CRP (mg/dL)	0.58±0.41	0.45±0.46	0.52±0.38	0.023*
Uric acid (mg/dL)	6.4±1.03	6.4±0.91	6.0±0.94	0.512
Vitamin C (mg/dL)	0.09±0.35	0.32±1.15	0.46±0.29	0.041*
GPx (mU/mL)	0.51±0.25	0.55±0.27	0.69±0.21	0.055*

***p<0.05 considered as significant**

Table 15 makes clear that the concentration of Serum NGAL 105±18.14, 125±11.16 & 115±10.14 and Cys C 1.85±0.90, 2.85±1.04 & 2.11±2.04 is significantly increased in 5-9 days compared to 0-4 days and 10-14 days.

Concentration of inflammatory markers such as NO 52.07±19.71, 46.17±18.16 & 49.18±18.32 and hs-CRP 0.58±0.41, 0.45±0.46 & 0.52±0.38 were significantly elevated in 0-4 days compared to 5-9 days and 10-14 days, however Vitamin C 0.09±0.35, 0.32±1.15 & 0.46±0.29 was more in 10-14 days with a significant p value.

Table 16a and 16b: Correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers in Group II (T2DM patients with AKI) secondary to Hypertension (HTN)

16 a: Correlation with Scr

	Scr	
Variables	**Group II T2DM with AKI (HTN)**	
	r value	**p value**
NGAL	0.418	0.001*
eGFR	0.517	0.001*
Uric acid	-0.428	0.011*
NO	-0.032	0.412
hs-CRP	-0.017	0.816
MDA	-0.092	0.05
Vitamin C	-0.045	0.496
GPx	-0.039	0.667

***p<0.05 considered as significant**

16b: Correlation with NGAL

	NGAL	
Variables	**Group II T2DM with AKI (HTN)**	
	r value	**p value**
Scr	0.418	0.001*
eGFR	0.782	0.001*
Uric acid	0.457	0.001*
NO	-0.392	0.001*
hs-CRP	-0.426	0.001*
MDA	0.629	0.001*
Vitamin C	-0.475	0.032*
GPx	-0.182	0.044*

***p<0.05 considered as significant**

Table 16a emerges; Scr as a strong positively correlation with NGAL (r=0.418; p=0.001), eGFR (r=0.517; p=0.001) and negative correlation with UA (r=-0.428; p=0.11). However, (**table 16b**) concentration of Serum NGAL showed significant positive correlation with Scr (r=0.418; p=0.001), eGFR (r=0.782; p=0.001), UA (r=0.457; p=0.001) and MDA (r=0.629; p=0.001) as well as negative correlation with inflammatory and antioxidant molecules such as NO (r=-0.392; p=0.001), hs-CRP (r=-0.426; p=0.001), Vitamin C (r=-0.475; p=0.032) and GPx (r=-0.182; p=0.044) respectively.

Table 17: Comparison of biomarkers, oxidants and antioxidants within Group II (T2DM with AKI) following Snakebite (SB)

No. of Days / Variables	0-4	5-9	10-14	p-value
NGAL (ng/mL)	190±23.16	216±24.16	185±22.62	0.032*
Cys C (mg/L)	4.73±2.24	4.61±2.14	4.60±2.01	0.137
Scr (mg/dL)	4.8±0.77	3.9±1.12	3.2±0.48	0.762
NO (µM/mL)	55.09±19.72	59.12±21.17	51.72±18.12	0.041*
MDA (nmol/mL)	9.72±2.57	8.45±2.27	7.67±2.41	0.044*
hs-CRP (mg/dL)	1.97±0.41	2.14±0.52	1.72±0.37	0.612
Uric acid (mg/dL)	6.41±1.43	6.01±1.12	5.9±1.11	0.154
Vitamin C (mg/dL)	0.45±0.21	0.55±0.32	0.62±1.12	0.021*
GPx (mU/mL)	0.52±0.21	0.55±0.87	0.61±1.01	0.011*

***p<0.05 considered as significant**

Table 17 depicts, T2DM with AKI subjects co-morbid with snakebite observed the mean values from base line to 14th day. Concentration of NGAL 190±23.16, 216±24.16 & 185±22.62 NO 55.09±19.72, 59.12±21.17 & 51.72±18.12 and MDA 9.72±2.57, 8.45±2.27 & 7.67±2.41 were significantly elevated in 5-9 days. However, Antioxidant molecules such as Vitamin C 0.45±0.21, 0.55±0.32 & 0.62±1.12 and GPx 0.52±0.21, 0.55±0.87 & 0.61±1.01 were significantly increased during 10-14 days compared to 0-4 days.

Table 18a and 18b: Correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers in Group II (T2DM patients with AKI) following Snakebite (SB)

17a: Correlation with Scr

	Scr	
Variables	**Group II T2DM with AKI (SB)**	
	r value	**p value**
NGAL	0.942	0.001*
eGFR	0.415	0.023*
Uric acid	0.029	0.496
NO	0.058	0.397
hs-CRP	0.088	0.06
MDA	0.242	0.034*
Vitamin C	-0.049	0.382
GPx	-0.039	0.581

***p<0.05 considered as significant**

17b: Correlation with NGAL

	NGAL	
Variables	**Group II T2DM with AKI (SB)**	
	r value	**p value**
Scr	0.942	0.001*
eGFR	0.647	0.001*
Uric acid	0.173	0.033*
NO	0.478	0.001*
hs-CRP	0.317	0.025*
MDA	0.526	0.001*
Vitamin C	-0.471	0.001*
GPx	-0.382	0.001*

***p<0.05 considered as significant**

Table 18a & 18b shows correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers. Concentration of Scr show significant positive correlation with NGAL (r=0.942; p=0.001), eGFR (r=0.415; p=0.023) and MDA (r=0.242; p=0.034). Serum NGAL was significant and positively associated with Scr (0.942; p=0.001), eGFR (r=0.647; p=0.001), UA (r=0.173; p=0.033), NO (r=0.478; p=0.001), hs-CRP (r=0.317; p=0.025), MDA (r=0.526; p=0.001). However, NGAL show significant negative correlation with Vitamin C (r=-0.471; p=0.001) and GPx (r=-0.382; p=0.001).

Table 19: Comparison of biomarkers, oxidants and antioxidants within Group II (T2DM with AKI) following Gastroenteritis (GE)

No. of Days	**0-4**	**5-9**	**10-14**	**p-value**
Variables				
NGAL (ng/mL)	169±13.14	154±11.16	145±10.14	0.013*
Cys C (ng/mL)	2.63±2.10	2.61±2.01	2.51±2.00	0.411
Scr (mg/dL)	2.9±0.46	2.8±0.42	2.5±0.41	0.452
NO (µM/mL)	54.07±19.71	58.17±18.16	54.18±18.32	0.613
MDA (nmol/mL)	8.41±2.25	9.62±2.17	9.21±2.04	0.025*
hs-CRP (mg/dL)	1.97±0.41	2.21±0.46	1.96±0.38	0.031*
Uric acid (mg/dL)	5.4±1.03	5.2±0.91	5.2±0.94	0.312
Vitamin C (mg/dL)	0.69±0.35	0.72±1.15	0.76±0.29	0.041*
GPx (mU/mL)	0.51±0.25	0.55±0.27	0.61±0.21	0.06

***p<0.05 considered as significant**

Table 20a and 20b: Correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers in Group II (T2DM patients with AKI) following Gastroenteritis (GE)

20a: Correlation with Scr

	Scr	
Variables	**Group II T2DM with AKI (GE)**	
	r value	**p value**
NGAL	0.417	0.001*
eGFR	0.582	0.001*
Uric acid	0.065	0.496
NO	0.032	0.381
hs-CRP	0.075	0.398
MDA	0.086	0.576
Vitamin C	-0.035	0.811
GPx	-0.066	0.427

***p<0.05 considered as significant**

20b: Correlation with NGAL

	NGAL	
Variables	**Group II T2DM with AKI (GE)**	
	r value	**p value**
Scr	0.417	0.001*
eGFR	0.897	0.001*
Uric acid	0.097	0.063
NO	-0.125	0.047*
hs-CRP	-0.278	0.011*
MDA	0.673	0.001*
Vitamin C	-0.412	0.001*
GPx	-0.098	0.094

***p<0.05 considered as significant**

Table 20a, revealed Scr positively correlated with NGAL (r=0.417; p=0.001), eGFR (r=0.582; p=0.001) and concentration of NGAL was significantly correlated with Scr (r=0.417; p=0.001), eGFR (r=0.897; p=0.001) and MDA (r=0.673; p=0.001). However, significant negative correlation with NO (r=-0.125; p=0.047), hs-CRP (r=-0.278; p=0.011) and Vitamin C (r=-0.412; p=0.001) was documented (**table 20b)**.

Table 21: Comparison of biomarkers, oxidants and antioxidants within Group II (T2DM with AKI) following OP Poisoning

No. of Days / Variables	0-4	5-9	10-14	p-value
NGAL (ng/mL)	90±13.14	164±11.16	145±10.14	0.012*
Cys C (mg/L)	1.05±0.90	2.21±1.04	2.81±2.01	0.032*
Scr (mg/dL)	1.8±0.46	2.6±0.42	2.5±0.41	0.554
NO (µM/mL)	45.07±19.71	46.17±18.16	54.18±18.32	0.043
MDA (nmol/mL)	7.41±2.25	7.62±2.17	8.21±2.04	0.128
hs-CRP (mg/dL)	0.99±0.41	0.95±0.46	0.93±0.38	0.231
Uric acid (mg/dL)	5.8±1.03	5.8±0.91	5.6±0.94	0.312
Vitamin C (mg/dL)	0.29±0.35	0.42±1.15	0.76±0.29	0.041*
GPx (mU/mL)	0.61±0.25	0.65±0.27	0.61±0.21	0.06

***p<0.05 considered as significant**

Mean values of Serum NGAL 90±13.14, 164±11.16 & 145±10.14 was significantly elevated in 5-9 days; however serum Cys C 1.05±0.90, 2.21±1.04 & 2.81±2.01 showed significant increase in 10-14 days compared with 0- 4 days. Concentration of Vitamin C 0.29±0.35, 0.42±1.15 & 0.76±0.29 showed significant increase in 10-14 days compared to 0-4 days and 5-9 days (**Table 21**).

Table 22a and 22b: Correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers in Group II (T2DM patients with AKI) following OP Poisoning (OPP)

21a: Correlation with Scr

	Scr	
Variables	**Group I T2DM without AKI (OPP)**	
	r value	**p value**
NGAL	0.381	0.021*
eGFR	0.179	0.025*
Uric acid	-0.418	0.001*
NO	0.039	0.478
hs-CRP	0.068	0.396
MDA	-0.045	0.375
Vitamin C	-0.017	0.519
GPx	0.035	0.458

21b: Correlation with NGAL

	NGAL	
Variables	**Group II T2DM with AKI (OPP)**	
	r value	**p value**
Scr	0.381	0.021*
eGFR	0.487	0.001*
Uric acid	0.367	0.023*
NO	0.415	0.011*
hs-CRP	0.179	0.035*
MDA	-0.286	0.024*
Vitamin C	-0.125	0.044*
GPx	-0.036	0.397

p<0.05 considered as significant

In T2DM subjects with AKI and OP poisoning, Scr showed a weak positive correlation with NGAL (r=0.381; p=0.021), eGFR (r=0.179; p=0.025) and significant negative correlation with UA (r=-0.418; 0.001). However, Serum NGAL showed significant positive correlation with Scr (0.381; p=0.021), eGFR (r=0.487; p=0.001), UA (r=0.367; p=0.023), NO (r=0.415; p=0.011) and hs-CRP (r=0.179; p=0.035) and significant weak negative correlation with MDA (r=-0.286; 0.024) and Vitamin C (r=-0.125; p=0.044) (**Table 22a & 22b).**

Table 23: Comparison of biomarkers, oxidants and antioxidants within Group II (T2DM with AKI) following Leptospirosis

No. of Days / Variables	0-4	5-9	10-14	p-value
NGAL (ng/mL)	196±24.14	220±24.15	210±20.62	0.015*
Cys C (ng/mL)	5.81±2.64	5.81±2.51	5.72±2.41	0.044*
Scr (mg/dL)	4.2±0.44	4.8±0.49	3.9±0.77	0.032*
NO (µM/mL)	54.06±9.64	64.05±22.52	58.73±20.19	0.025*
MDA (nmol/mL)	9.68±2.91	10.14±1.75	9.08±2.52	0.371
hs-CRP (mg/dL)	1.91±0.19	2.66±0.16	1.96±1.01	0.035*
Uric acid (mg/dL)	6.19±2.03	6.04±1.92	6.0±1.85	0.198
Vitamin C (mg/dL)	0.66±0.35	0.69±0.41	0.72±0.42	0.011*
GPx (mU/mL)	0.55±0.86	0.62±1.16	0.69±1.18	0.036*

***p<0.05 considered as significant**

Table 23 shows Group II subjects (T2DM with AKI) with Leptospirosis; concentration of NGAL 196±24.14, 220±24.15 & 210±20.62 Scr 4.2±0.44, 4.8±0.49 & 3.9±0.77, NO 54.06±9.64, 64.05±22.52 & 58.73±20.19 and hs-CRP 1.91±0.19, 2.66±0.16 & 1.96±1.01 were significantly increased in 5-9 days. Mean values of antioxidants such as Vitamin C 0.66±0.35, 0.69±0.41 & 0.72±0.42 and GPx 0.55±0.86, 0.62±1.16 & 0.69±1.18 were increased in 10-14 days. However, Cys C concentration 5.81±2.64, 5.81±2.51 & 5.72±2.41 was significantly higher in 0-4 days compared with 5-9 days and 10-14 days frequency distribution.

Table 24a and 24b: Correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers in Group II (T2DM patients with AKI) following Leptospirosis (Lepto)

24a: Correlation with Scr

	Scr	
Variables	**Group I T2DM without AKI (Lepto)**	
	r value	**p value**
NGAL	0.815	0.001*
eGFR	0.325	0.021*
Uric acid	0.049	0.352
NO	-0.112	0.024*
hs-CRP	-0.027	0.412
MDA	-0.135	0.04*
Vitamin C	-0.038	0.321
GPx	-0.029	0.451

24b: Correlation with NGAL

	NGAL	
Variables	**Group II T2DM with AKI (Lepto)**	
	r value	**p value**
Scr	0.815	0.001*
eGFR	0.629	0.001*
Uric acid	0.089	0.211
NO	-0.535	0.001*
hs-CRP	-0.255	0.032*
MDA	0.475	0.001*
Vitamin C	-0.562	0.001*
GPx	-0.157	0.04*

***p<0.05 considered as significant**

Table 24a depicts, Scr was positively correlated with NGAL (r=0.815; p=0.001), eGFR (r=0.325; p=0.021) and significant negative correlation with NO (r= -0.112; p=0.024) and MDA (r=-0.135; p=0.04). **Table 24b** shows concentration of NGAL with significant positive correlation for Scr (r=0.815; p=0.001), eGFR (r=0.629; p=0.001) and MDA (r=0.475; p=0.001) and significant negative correlation with NO (r=-0.535; p=0.001), hs-CRP (r=-0.255; p=0.032), Vitamin C (r=-0.562; p=0.001) and GPx (r=-0.157; p=0.04).

Table 25: Comparison of biomarkers, oxidants and antioxidants within Group II (T2DM with AKI) following Cardio Pulmonary Bypass (CPB) Surgery

No. of Days Variables	0-4	5-9	10-14	p-value
NGAL (ng/mL)	215±24.15	205±20.12	210±23.17	0.023*
Cys C (ng/mL)	3.01±1.27	3.12±1.37	3.11±1.28	0.022*
Scr (mg/dL)	3.9±0.75	3.8±0.71	3.6±0.69	0.07
NO (µM/mL)	54.08±19.63	58.69±17.15	56.27±20.31	0.144
MDA (nmol/mL)	6.52±2.71	6.71±2.32	6.35±2.19	0.496
hs-CRP (mg/dL)	1.95±0.19	1.97±0.35	1.63±0.16	0.321
Uric acid (mg/dL)	6.11±1.56	6.04±1.48	6.00±1.39	0.152
Vitamin C (mg/dL)	0.54±0.21	0.56±1.02	0.58±1.29	0.011*
GPx (mU/mL)	0.54±0.15	0.55±0.45	0.71±1.12	0.032*

***p<0.05 considered as significant**

Table 25 shows, concentration of NGAL 215±24.15, 205±20.12 & 210±23.17 significantly increased in 0-4 days compared to 5-9 days and 10-14 days. However, Cys C 3.01±1.27, 3.12±1.37 & 3.11±1.28 showed significant elevation in 5-9 days. Mean values of antioxidant markers such as, Vitamin C 0.54±0.21, 0.56±1.02 & 0.58±1.29 and GPx 0.54±0.15, 0.55±0.45 & 0.71±1.12 were significantly increased in 10-14 days compared to 0-4 days and 5-9 days.

Table 26a and 26 b: Correlation of Scr and NGAL with oxidants, antioxidants and inflammatory markers in Group II (T2DM patients with AKI) following CPB Surgery

26a: Correlation with Scr

	Scr	
Variables	**Group II T2DM with AKI (CPB)**	
	r value	**p value**
NGAL	0.435	0.001*
eGFR	0.387	0.013*
Uric acid	0.127	0.042*
NO	-0.158	0.035*
hs-CRP	-0.039	0.445
MDA	-0.094	0.061
Vitamin C	-0.064	0.091
GPx	-0.041	0.396

26b: Correlation with NGAL

	NGAL	
Variables	**Group II T2DM with AKI (CPB)**	
	r value	**p value**
Scr	0.435	0.001*
eGFR	0.687	0.001*
Uric acid	0.258	0.022*
NO	-0.456	0.015*
hs-CRP	-0.397	0.001*
MDA	-0.492	0.021*
Vitamin C	-0.366	0.014*
GPx	-0.217	0.023*

***p<0.05 considered as significant**

Scr was positively correlated with NGAL (r=0.435; 0.001), eGFR (r=0.387; p=0.013) and UA (r=0.127; p=0.042) and weak negative correlation with NO (r=-0.158; 0.035). However, serum NGAL was positively correlated with Scr (r=0.435; p=0.001), eGFR (r=0.687; p=0.001) and UA (r=0.258; p=0.022) and significant negative correlation with NO (r=-0.456; p=0.015), hs-CRP (r=-0.397; p=0.001), MDA (r=-0.492; p=0.021), Vitamin C (r=-0.366; p=0.014) and GPx (r=-0.217; p=0.023) (**Table 26a & 26b).**

Table 27: Comparative results of Parameters Group I (‘0’ day), Group II (‘0’ day and 14th day) and Group III (‘0’ day)

Variables / Groups		Days	NGAL (ng/mL)	Cys C (mg/mL)	SCr (mg/dl)	NO (µM/mL)	MDA (nmol/mL)	hs-CRP (mg/dL)	Uric acid (mg/dL)	Vitamin C (mg/dL)	GPx (mU/mL)
Group I		0	160±13.35	4.73±2.24	0.94±0.31	34.48±7.44	8.48±2.50	1.98±0.45	5.85±1.17	0.42±0.13	1.91±0.90
GROUP II	UTI	0	184±18.51	5.27±2.53	5.8±1.16	51.21±15.15	9.58±2.66	2.91±0.95	5.91±1.45	0.55±0.08	0.44±0.14
		14th	174±19.18	5.12±2.11	5.1±1.12	58.54±14.41	9.62±2.55	1.91±0.19	5.41±0.92	0.91±0.29	0.71±1.12
	HTN	0	105±18.14	1.85±0.90	1.7±0.46	52.07±19.71	8.41±2.25	0.58±0.41	6.4±1.03	0.09±0.35	0.51±0.25
		14th	115±10.14	2.11±2.04	1.9±0.41	49.18±18.32	7.21±2.04	0.52±0.38	6.0±0.94	0.46±0.29	0.69±0.21
	SB	0	190±23.16	4.73±2.24	4.8±0.77	55.09±19.72	9.72±2.57	1.97±0.41	6.41±1.43	0.45±0.21	0.52±0.21
		14th	185±22.62	4.60±2.01	3.2±0.48	51.72±18.12	7.67±2.41	1.72±0.37	5.9±1.11	0.62±1.12	0.61±1.01
	GE	0	169±13.14	2.63±2.10	2.9±0.46	54.07±19.71	8.41±2.25	1.97±0.41	5.4±1.03	0.69±0.35	0.51±0.25
		14th	145±10.14	2.51±2.00	2.5±0.41	54.18±18.32	9.21±2.04	1.96±0.38	5.2±0.94	0.76±0.29	0.61±0.21
	OP	0	90±13.14	1.05±0.90	1.8±0.46	45.07±19.71	7.41±2.25	0.99±0.41	5.8±1.03	0.29±0.35	0.61±0.25
		14th	145±10.14	2.81±2.01	2.5±0.41	54.18±18.32	8.21±2.04	0.93±0.38	5.6±0.94	0.76±0.29	0.61±0.21
	LEPTO	0	196±24.14	5.81±2.64	4.2±0.44	54.06±9.64	9.68±2.91	1.91±0.19	6.19±2.03	0.66±0.35	0.55±0.86
		14th	210±20.62	5.72±2.41	3.9±0.77	58.73±20.19	9.08±2.52	1.96±1.01	6.0±1.85	0.72±0.42	0.69±1.18
	CPBS	0	215±24.15	3.01±1.27	3.9±0.75	54.08±19.63	6.52±2.71	1.95±0.19	6.11±1.56	0.54±0.21	0.54±0.15
		14th	210±23.17	3.11±1.28	3.6±0.69	56.27±20.31	6.35±2.19	1.63±0.16	6.00±1.39	0.58±1.29	0.71±1.12
Group III		0	90.42±18.36	2.08±1.23	0.6±0.14	54.06±18.59	2.25±0.92	0.94±1.12	4.92±1.00	1.18±0.56	2.25±1.16

UTI: Urinary tract infection; HTN: Hypertension; SB: Snakebite; GE: Gastroenteritis; OP: Organophosphate poisoning; LEPTO: Leptospirosis; CPB: Cardio Pulmonary Bypass Surgery

DISCUSSION

Acute Kidney Injury (AKI), previously known as acute renal failure (ARF), is characterized by sudden impairment of kidney function resulting in the retention of nitrogenous and other waste products normally cleared by kidneys. AKI is not a single disease but, rather a designation for heterogeneous group of conditions that share common diagnostic features: specifically, an increase in the blood urea nitrogen (BUN) concentration and / or an increase in plasma or serum creatinine concentration often associated with a reduction in urine volume. It is important to recognize that AKI is a clinical diagnosis and not a structural one. A patient may have AKI without injury to kidney parenchyma [158].

AKI may be community acquired or hospital acquired or region specific. Common causes of community acquired AKI include volume depletion, adverse effects of medications and obstruction of the urinary tract. Most common clinical settings for hospital acquired AKI are sepsis, major surgical procedures, critical illness involving heart or liver failure, intravenous iodinated contrast administration and nephrotoxic medication administration and region specific issues such as envenomations from snakes, spiders, caterpillars and bees [158].

AKI complicates 5-7% of acute care hospital admissions and up to 30% of admissions in the intensive care unit, particularly of diarrheal illness, infectious diseases such as malaria and Leptospirosis and natural disasters example earth quakes [158].

Presence of AKI is usually inferred by an elevation of Scr concentration from baseline of minimum 0.3mg/dl within 48 hours or 50% higher than baseline within 1 week, or a reduction in urine output to less than 0.5ml/kg/hr for more than 6 hours.

Unfortunately, BUN and Scr are functional markers of glomerular filtration rather than tissue injury. Thus, these markers may be suboptimal for the diagnosis of actual parenchymal kidney damage and these markers are relatively slow to rise after kidney injury. Several novel kidney injury biomarkers have been investigated and show promise for earlier and accurate diagnosis of AKI [158].

As there are very few studies in India highlighting the importance and the usefulness of newer biomarker for diagnosis of AKI and associated complications; this created an interest in studying biomarkers in early diagnosis and management, renal parameters, diabetic profile, in addition to oxidative stress markers and other variables.

Kolar district is an endemic area for Leptospirosis. Our hospital statistics has documented an increased incidence of Urinary Tract Infections, Snakebite, Gastroenteritis, OP poisoning, Hypertension and cardiovascular diseases. It is observed that most of the patients with coronary artery disease who have undergone Cardiopulmonary bypass were diabetic and had Acute Kidney Injury. In addition to this AKI was also documented as sequelae to Leptospirosis, UTI, hypertension, GE, OP poisoning and snake bite.

In this study we observed serum NGAL was significantly elevated in Group II compared to Group III and Group I. In Group II sub classification, concentration of NGAL was significantly and exponentially elevated from Risk group to End stage groups.

Group II subjects had associated UTI, hypertension, snakebite, GE, OP Poisoning and Leptospirosis. NGAL concentration was increased at 5- 9 days phase in UTI, HTN,

Snakebite, OP Poisoning and Leptospirosis subjects and then gradually decreased between10- 14 days. Whereas, subjects with GE and CPB conditions concentration of NGAL was decreased at 5- 9 and 10- 14 days frequency distribution compared to 0- 4 days.

In this study we have observed patients with UTI, serum NGAL was significantly higher at 5- 9 days and decreased at 10- 14 days phase after antibiotic treatment compared to levels before treatment.

UTI is a type of infection which is common among older people. The prevalence of UTI in the elderly is much higher than younger individuals. At least 20% women and 10% of men aged 66 years or older have bacteriuria [159]. In our study, 30% (45/150) of the cases are over 66 years old. Multiple age-related changes including cell-mediated immunity recession, bladder defenses alteration due to obstructive uropathy, neurogenic dysfunction, bacterial receptivity intensification of uroepithelial cells, contamination due to fecal and urinary incontinence, uretheral instrumentation and catheterization, and antibacterial factors resulting in reduction in prostate and vagina associated with changes in zinc levels, PH and hormones contribute to the risk associated with UTI in elderly [160].

DM has been reported as a risk factor for the development of both upper and lower UTIs [161]. Patients with diabetes had a higher risk of AKI (OR 2.23, 95% CI 1.35–3.68, p = 0.002). UTI is one of the most common sources of bacteremia in diabetic patients and patients with DM are at a greater risk of developing various complications of UTI including sepsis [162, 163]. Severe sepsis may induce vital organ dysfunction, including AKI. Robbins et al. showed that UTI induced acute kidney injury in

approximately 40% of diabetic patients with bacteremia [164]. Our results also suggested that DM was an independent risk factor for AKI in UTI patients.

Studies indicated that hypovolemia, hypotension, sepsis, use of nephrotoxic drugs, contrast media and urinary obstruction were AKI risk factors in UTI patients [165]. Our study showed that UTI patients with DM, upper UTI, afebrile or septic shock during hospitalization and impaired baseline renal function were at higher risk for development of AKI.

Upper UTI is not a well-recognized cause of AKI. Acute pyelonephritis can involve entire lobules of the medulla and cortex [166]. Interstitial infiltration of neutrophils and phagocytes and extensive destruction of the parenchymal by the acute inflammatory process were found in AKI patients with acute pyelonephritis [167, 168]. These reports demonstrated that severe upper UTI might cause serious damage to the kidney and resulted in AKI. Our study found that patients with upper UTI had higher risk of AKI than those with lower UTI (OR 2.63, 95% CI 1.53– 4.56, p = 0.001).

NGAL is a 25 kDa protein originally purified from human neutrophils. It is considered a specific marker of neutrophil activity and a strong bacteriostatic agent, as it involves the antibacterial iron-depletion strategy of the innate immune system. NGAL is expressed at low levels in normal organs and increases in injured epithelia, including in the lung, colon and especially in the kidney [169].

Nature of NGAL molecule is complex and related to its cellular origin. NGAL exists in three different molecular forms in blood and urine; (1) 25- KDa monomer (2) 45-KDa disulphide- linked homodimer and (3) 135 KDa heterodimer, covalently

conjugated with gelatinase. Results of studies conducted in vitro indicate activated neutrophils mainly release homodimeric NGAL and to a lesser extent the monomeric form. In contrast, stressed kidney epithelial cells predominantly secrete monomeric NGAL apparently unable to form dimers. This is supported by elevated urinary homodimeric levels seen in patients with UTI and a relative abundance of monomeric NGAL in AKI patients [169].

Results of our study are consistent with the study conducted by de Geus et al., He reported that, the expression pattern of NGAL prior to rise in Scr is early and its predictive power also increased closer to AKI presentation time and also suggested that the time to injury relationship is important and should be obtained for a correct interpretation of its AKI predictive value [170].

In our study with respect to GE condition, concentration of NGAL was decreased at 5-9 and 10-14 days frequency distribution compared to 0-4 days.

Gastroenteritis is an infection of the gut (intestines) with viruses, bacteria or other microbes. It may develop diarrhoea, sickness (vomiting), abdominal pain and other symptoms. AKI is recognized with reducing renal blood flow, glomerular filtration rate may decrease and glomerular epithelial cells can be damaged [171]. In this situation, injury of the brush border of proximal tubule cells is the earliest lesion in the pre-renal stage and during this early period minimal acute tubular necrosis cannot be detected using routine laboratory investigations such as serum creatinine levels. GE as a form of pre-renal acute kidney injury may be represented by a tubular enzymuria and a concomitant increase in serum NGAL [160]. Although NGAL is expressed only at very

low levels in several human tissues, it is markedly induced in injured epithelial cells, including the kidney [172].

Depending on these results, we suggested that serum NGAL levels could be a very sensitive biomarker for prerenal kidney dysfunction even in early stage. Similarly, creatinine levels of patients did not different from the healthy controls in our study. On the other hand, serum Cystatin C levels of our patients was slightly higher than the controls.

In this study subjects with T2DM and AKI secondary to snakebite, we observed concentration of NGAL was increased at 5-9 days and then gradually decreased between10-14 days frequency distributions.

Snakebite is an important environmental and occupational hazard. Males are affected more often than females, as they constitute working majority who are actively engaged in farming and other outdoor activities. Involvement of predominantly young working population in poor rural areas compounded by lack of access to health care services in these areas signifies social and economic impact of this problem [42].

Complex nature of snake venom is responsible for the wide variety of effects seen in snakebite patients. Snake venom is a modified salivary secretion and contains more than 100 different kinds of peptides or proteins, lipids, amines, carbohydrates etc. These compounds present in snake venom have been proved that most of them are toxic to humans. Venoms from viper families contain proteins such as disintegrins, C- type lectins etc., which interact with members of coagulation cascade and fibrinolytic pathway.

Enzymes present in snake venom such as phospholipases, serine proteases, metalloproteinases are known to cause endothelial disruption [45].

Hemorrhagins lead to spontaneous bleeding by directly injuring the vascular endothelium. Digestive hydrolases, hyaluronidase and polypeptide cytotoxins contribute to local tissue necrosis seen after bites of some snakes. Myotoxic phospholipase A2 seen in venoms of some vipers and sea snakes are responsible for rhabdomyolysis which leads to acute renal failure [44].

NGAL is gaining momentum as a biomarker for early diagnosis of AKI in different scenarios. Studies have shown NGAL concentrations in both plasma and urine increase soon after the renal insult and AKI could be detected hours or days before creatinine. Concentration of NGAL can be elevated after activation of neutrophils, suggesting influence of systemic inflammation and infections [173]. Off late Plasma NGAL has emerged as a biomarker that predicts the development of AKI in patients 1- 3 days earlier than serum creatinine. NGAL appears to be an early potential 'real time' biomarker for AKI and its expression is proportional to the severity of renal injury.

Leptospirosis is an important zoonosis especially in the tropics. However with the impact of world globalization, there are also reports of this disease as sporadic cases in developed countries. Leptospirosis caused by a microorganism of the *Leptospira* genus, an obligate aerobic spirochete of worldwide distribution, with two species: *L. interrogans* (pathogenic) and *L. biflexa* (non-pathogenic and saprophytic) [57, 58].

Renal impairment is a frequent complication in patients with severe form of leptospirosis, mainly characterized by an association of interstitial and tubular damage [58].

In our study, Group II subjects secondary to Leptospirosis, we have observed concentration of NGAL was increased in 5-9 days frequency distribution and then gradually decreased on 10-14 days.

AKI is one of the most serious complication of Leptospirosis. AKI can be a diagnostic challenge in patients with Leptospirosis because serum creatinine may be interfered by many factors such as volume overload, rhabdomyolysis and jaundice. These confounding factors together make serum creatinine less sensitive and specific in setting of Leptospirosis. It has been documented that most of Leptospirosis patients live in rural areas and cannot afford speciality care due to shortage of nephrologists. Availability of specific novel biomarkers accelerate the timing of AKI detection and triage the Leptospirosis associated AKI patients for further care. Plasma NGAL has been tested as markers for early diagnosis of AKI in various settings such as sepsis, ischemia and post-transplantation condition [174]. Association between NGAL and clinical and laboratory markers of severity such as WBC count, Bilirubin level or lower of platelet count and AKI are common and correspond with the studies [175].

Higher sensitivity of plasma NGAL in diagnosis AKI in Leptospirosis could be due to the fact that Leptospirosis directly invades distal tubule epithelium. This may be because NGAL can be produced in various organs such as liver, colon and lung during the process of inflammation/infection [176]. Moreover, inflammatory cell like neutrophils, monocytes and other immune-competent cells can also synthesize and release NGAL into systemic circulation [177, 178]. The last declining of filtration rate

during AKI may slow down the elimination of systemic NGAL and result in the rising of concentration of plasma NGAL [179, 180].

In our study, Group II subjects secondary to OP Poisoning, had elevated concentration of NGAL on 5- 9 days which gradually decreased at 10- 14 days.

OP compounds are widely used as insecticides in agricultural setting. Since OP compounds are easily available and are handled without proper care. This may result with poisoning and is a common phenomenon in rural setting which presents classically with signs of cholinergic toxicity manifestations [181].

OP compounds inhibit acetylcholinesterase by phosphorylation. Some of phosphorylated cholinesterases dealkylate leading to "aged" enzyme which is a non-reversible state [182]. Further, there will be an uncontrolled stimulation of nicotinic and muscarinic receptors by acetylcholine (Ach). Signs of OP poisoning include diarrhea, excessive salivation, lacrimation, urination, bronchorrhea, bronchospasm, bradycardia, miosis and muscle paralysis [183].

AKI has been seen with OP poisoning. It is seen very rarely and only a few cases have been reported in medical literature. Furthermore, the main mechanism by which OP can cause AKI has been debated. Different mechanisms have been suggested that can cause AKI. Vanden Akker et al proposed that OP effects on the renal system could be due to direct parenchymal intoxication, secondary to hemodynamic instability, or seizure-induced rhabdomyolysis, OP might also cause oxidative stress leads to direct damage to the renal tubules. In addition, hemodilution induced by massive fluid resuscitation, which impairs the oxygen supply to the kidney may also play a role resulting in AKI [184].

Our study findings were consistent with the study done by Lee FY et al., with an observation of patients with OP poisoning who had a 6-17-fold higher risk of AKI [184].

In our study, T2DM with AKI subjects secondary to hypertension, we observed significant increase of NGAL concentration at 5-9 days and then decreased exponentially at 10-14 days frequency distribution. These direct correlation may be a consequence of endothelial dysfunction, on which hypertension and proteinuria probably depend. It has been suggested that an increase in circulating NGAL may be a consequence of leukocyte derived inflammatory activity and endothelial activation, and both these pathophysiological aspects are involved in Hypertensive Disorders [185].

NGAL was found to be significantly correlated with creatinine in cases with the value of correlation coefficient being 0.418. Our study showed that NGAL is a sensitive indicator of AKI and is positively correlated with various parameters.

Group II subjects in our study secondary to CPB, concentration of NGAL were exponentially decreased at 5- 9 and 10- 14 days frequency distribution respectively compared to 0- 4 days.

Kidneys are susceptible to ischemic damage because of their unique blood circulation. The renal medulla is perfused at a low oxygen tension, inducing limitation in its functional reserve [186]. Any decrease in renal perfusion can lead to significant cellular injury, depending on its magnitude and duration. Imbalance between renal oxygen delivery and renal oxygen consumption is generally believed to play a pivotal role in CPB- AKI. Renal oxygen delivery is determined by arterial oxygen content and by

renal blood flow, which varies during CPB with the non-pulsatile pump flow rate and blood pressure.

AKI continues to be a common and important complication of cardiac surgery and is associated with increased mortality, complications and length of hospital stay. Effective clinical protocols for the prevention and optimal management have yet to be defined. Clinical strategies that stress prevention rather than treatment remain the mainstay of effective management of patients at high risk for AKI.

Changes in Scr occur late in the development of CPB- AKI, typically 48 h after the initiating event [187]. Ultimately the diagnosis of CPB- AKI may be delayed when in fact serious tubular injury has occurred and may be ongoing. Thus, one of the important reasons that attempts to treat CPB- AKI have been unsuccessful because interventions are initiated too late and acute tubular necrosis might have already established.

According to RIFLE classification, changes in serum creatinine concentrations and reduced urine output define acute kidney injury. Changes of kidney function are detected with substantial delay in classification and onterpretation. Changes of diuresis and/or serum creatinine levels that appear in the postoperative period can be directly ascribed to CPB, but also to various factors such as hemolysis, blood transfusions, volume deficit, hemodynamic instability, systemic inflammatory response or reduction of renal perfusion in the elderly [188, 189]. Nevertheless, early prediction of the occurrence of AKI after surgery is of crucial importance.

Advent of novel biomarkers of kidney injury has opened a new era of early detection and prognosis prediction for CPB- AKI. Implications are improved monitoring,

early institution of treatment measures, and improved patient counseling [190]. The Assessment, Serial Evaluation, and Subsequent Sequelae of Acute Kidney Injury (ASSESS- AKI) study and the Translational Research Investigating Biomarkers Endpoints in Acute Kidney Injury (TRIBE- AKI) study have evaluated the utility of novel biomarkers to refine the diagnosis and prognosis of AKI [191]. Most frequently studied new promising AKI biomarker to date is neutrophil gelatinase-associated lipocalin (NGAL).

NGAL has been demonstrated to be a highly sensitive and specific predictor of CPB- AKI. Urinary NGAL was demonstrated as an early biomarker of AKI after CPB, increasing 25-fold within 2 h and declining 6 h after surgery. This promoted the use of urine NGAL as an indicator to forecast subclinical CPB- AKI. A multicenter pooled analysis of prospective studies showed that in the absence of diagnostic increases in Scr, NGAL detected patients with likely subclinical AKI who have an increased risk of adverse outcomes. Accordingly, the role of plasma NGAL to classify AKI severity and predict the need for RRT after cardiac surgery has been suggested. Haase et al. reported that plasma NGAL on arrival in the ICU after cardiac surgery correlated with subsequent AKI duration, severity and length of ICU stay [192]. However, studies have found that plasma NGAL was not a useful predictor of AKI within the first 6 h following cardiac surgery. However, urinary NGAL was superior to conventional markers and plasma NGAL in the early diagnosis of CPB- AKI [193].

Studies conducted by Uchino S et al., observed systemic inflammation triggered by conditions such as sepsis or procedures like CPB, where, NGAL was strongly

associated with AKI development. During the process, inflammatory procedures triggers and activate circulating neutrophils to release their granular contents including NGAL [194].

Studies conducted by Flo TH et al., and Grigoryev DN et al., have shown NGAL synthesis increases in non-haematopoietic cells in various tissues such as lung and liver as a part of inflammatory response. The resultant increase in plasma NGAL will increase the filtered load and hence urinary levels irrespective of any potential kidney damage [119, 195]. These findings are supported by studies conducted by Vishal J et al., who also showed elevated plasma and urine NGAL in cardiac surgery patients [196].

Study done by Martensson J et al., observed similar results in the general ICU populations where sepsis is associated with elevated plasma NGAL independent of degree of renal impairment [197].

Under normal conditions, filtered NGAL is almost completely reabsorbed by the proximal tubules via megalin-cubulin receptor- mediated endocytosis, resulting in minimal urinary NGAL levels. Studies by Hvidberg V et al., have shown the total concentration of urinary NGAL in AKI probably representing a mixture of different molecular forms of NGAL with different cellular origins. Monomeric NGAL is known to increase either due to an induced synthesis in the tubular cells or as an effect of impaired reabsorption of the filtered load produced by extrarenal tissues [198]. Based on these findings, NGAL might be a more specific AKI marker in patient populations where the 'noise' from severe systemic inflammation and multi-organ damage is less pronounced.

Studies conducted by Keller CR et al., observed that Cystatin C is related to inflammation and oxidative stress, the key pathogenic components of metabolic

syndrome were elevated in diabetes with or without AKI [199]. We observed serum Cystatin C significantly elevated in Group II compared to Group I and Group III.

Studies have demonstrated that measurement of plasma Cys C concentrations may be a useful tool to diagnose AKI early, but, its superiority over serum creatinine needs to be universally demonstrated [200, 201].

Study done by Zhang and colleagues found that AUC of plasma Cys C to predict AKI was between 0.86 to 0.96 compared to urinary Cys C, where it had only moderate diagnostic value, with a pooled AUC of 0.64 (95% confidence interval) [200].

Royakkers and colleagues measured serial plasma and urinary Cys C prospectively in 151 ICU patients and assessed the performance for AKI prediction on day 1 and day 2 before the RIFLE criteria for AKI were met. On day 2, plasma and urinary Cys C had an AUC for predicting AKI of 0.72 and 0.49 respectively. On day 1, the AUCs were respectively 0.62 and 0.46. Plasma Cys C levels did not rise earlier than serum creatinine [201].

Uric acid the final oxidation product of purine metabolism excreted by kidneys was significantly increased in Group II followed by Group I and Group III in our study.

Within Group II, Risk and Loss groups has shown significant UA increase compared to Injury, Failure and End-stage groups on '0' day and 14^{th} day.

Our findings are consistent with the studies conducted by Kanbay et al., and Shimada MB et al., stating that elevated UA concentration can be a consequence of impaired renal function. They also documented that patients with higher serum uric acid

concentration have associated high systolic blood pressure, increased hs-CRP, decreased eGFR, and low flow-mediated dilatation [202, 203].

An in house study done by Shashidhar KN et.al., observed that elevated UA could induce renin expression from the juxtaglomerular cells and inhibit nitrous oxide system (NOS) expression in the macula densa [204]. They also reported UA is known to impair endothelial function and stimulates the production of cytokines from leukocytes and chemokines from vascular smooth muscle cells [204].

In our study, we observed eGFR was decreased in AKI patients with diabetes when compared with diabetic patients without AKI.

Navaro J et al., has noted that angiotensin II increases efferent arteriolar pressure and plays a key role in the auto-regulation of renal blood flow (RBF) and glomerular filtration rate (GFR). It is also known that prolonged inappropriate increase in angiotensin II lead to decrease in RBF and GFR and also release cytokines and growth factors [205]. In our study around 50% of Group II patients (n=75) were on angiotensin II inhibitors.

DM refers to a group of common metabolic disorders that share the phenotype of hyperglycemia. Several distinct types of DM are caused by a complex interaction of genetics and environmental factors. Depending on the etiology of DM factors contributing to hyperglycemia include reduced insulin secretion, decreased glucose utilization and increased glucose production. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on individuals with diabetes and on the health care system. People

with DM may be at increased risk of developing AKI [1]. Presence of underlying diabetic nephropathy may predispose to AKI resulting from adverse effects such as sepsis, hypotension or exposure to nephrotoxic agents. Increased incidence of cardiovascular disease among diabetic patients may also lead to renal insufficiency as a result of complications of renal artery atherosclerosis or ischaemic heart disease [206].

In our study, we observed significant increase in FBS, PPBS and HbA1c % in Group III vs Group I, Group I vs Group II and Group III vs Group II. Our findings were similar to the studies conducted by Vakrani GP et al, where they pointed out that mesangial expansion is the major lesion of diabetic nephropathy resulting in renal dysfunction in type 2 diabetes [206].

Glucose toxicity is a primary cause of glomerular injury in patients with diabetic nephropathy. Prolonged elevations in blood glucose levels result in the formation of glycation end products which interfere with normal collagen turnover and promote vessel permeability, matrix accumulation, and formation of adhesion molecules. Glucose can also bind reversibly and eventually irreversibly to proteins in the kidneys to form advanced glycosylation end products (AGEs). AGEs can form complex cross-links over years of hyperglycemia and may contribute to renal damage. It has been documented that mediators of proliferation and expansion, including platelet-derived growth factor, TGF-β and vascular endothelial growth factor (VEGF) also gets elevated in diabetic nephropathy [207].

We observed in our study C-Peptide concentration was significantly elevated in Group II compared to Group III and Group I. In T2DM patients, where insulin and C-

Peptide levels are within or above normal range, do not show glomerular hyperfiltration or hypertrophy. Mechanism underlying the beneficial effect of C-Peptide on renal function in diabetes is not known. However, it is possible that C-Peptide may have exerted a direct effect on glomerular handling of albumin, as suggested by the studies of renal function in animals with experimental diabetes [208].

As C-peptide is a contingent upon the presence of insulin, kidney contributes to insulin removal from the circulation. Plasma levels of insulin are slightly to moderately elevated in most uremic patients, in both fasting and postprandial states. Because of diminished renal degradation of insulin, patients on insulin therapy may need progressive reduction in dose as their renal function worsens [208].

Oxidative stress in diabetes is responsible for endothelial dysfunction and release of inflammatory markers such as cytokines from the damaged renal tissue. A number of different inflammatory cells and soluble mediators are shown to be necessary for renal damage and loss of glomerular filtration. Proximal events leading to damage of renal tubular epithelial cells are likely to start in the microvasculature [209].

In our study we observed NO levels were almost half in Group II compared to Group III with a significant p value. Within Group II, NO was highly significant in Risk, Failure, Loss and End stage groups compared to Injury group.

Study done by Saulo K et al., observed similar results of our study and reported increased release of NO into circulation. This increased release of NO may lead to high blood pressure, up-regulation of NO production and affects renal blood flow, shear stress

and other related mechanical stimuli. These factors may account for the increased production of NO and also expression of endothelial Nitric Oxide Synthase (eNOS) [210].

Results of our study are in consistent with the study done by Brodsky et al., where they stated that vascular endothelium undergoes structural and functional changes in early ischemic renal failure and also alter NO production and /or decreased bioavailability of NO which may comprise the endothelial dysfunction in acute renal failure [211].

In our study we included hs- CRP as a marker of inflammation. To our surprise hs-CRP level is almost half in Group II compared to Group III. Reason may be because of the fact that these people were on treatment and constant monitoring. Failure and End stage groups also showed elevated hs-CRP concentration compared to Risk, Injury and Loss groups in Group I.

Similar findings were also observed by Howaida Attia et.al., indicating that acute stressful condition may lead to increased, concentration of hs-CRP rise dramatically during inflammatory processes [212]. Pegues MA et. al., reported that CRP might play an active role in AKI; worsening the damage resulting in renal ischemia and reperfusion injury (IRI) [213]

MDA concentration was four to five times elevated in Group I and Group II respectively compared to Group III. Oxidative stress in diabetes is responsible for endothelial dysfunction releasing inflammatory markers such as cytokines from the damaged renal tissue. Alterations in endothelial dysfunction causes elevated expression and plasma levels of vasoconstrictors such as angiotensin II and endothelin-1 with

increased expression of adhesion molecules and enhanced adhesion of platelets and monocytes to vascular endothelium releasing NO and reduced NO responsiveness causing renal injury [214].

Himmelfarb et.al., has observed Malondialdehyde as a terminal compound of lipid peroxidation, which is commonly used as an index of oxidative stress. In biological matrices, MDA exists as both free (fMDA) and bound (bMDA) bound to -SH and / or -NH_2 groups of proteins, nucleic acids and lipoproteins [215].

Veechi AP et.al., documented that chemically reactive fMDA is an index of recent and potential damage, while bMDA, excreted by the kidney, is a marker of an older injury [215]. However in our study we estimated total MDA which was significantly elevated in Group II and Group I compared to Group III.

Oberley LW et.al., also stated that, under conditions of increased glucose concentration in diabetes, augmented glucose autoxidation may contribute to the enhanced free radical production and facilitate lipid peroxidation [216].

In our study we observed Vitamin C levels were less than 50% in Group II compared to Group III. Within Group II, Vitamin C was gradually decreased from Risk group to End-stage groups. Our study results are consistent with the study done by Yao-Bin Zhu et.al., where he stated that Vitamin C as an antioxidant, plays a major role in the acute phase conditions and as a natural water soluble form and has a potential to intervene in the development of kidney disease by modulating redox steps [217].

In our study we observed Vitamin C concentration was significantly increased in Group II subjects associated with UTI, hypertension, snakebite, GE, OP poisoning, Leptospirosis and CPB.

We observed in our study that concentration of serum GPx was decreased in Group II compared to Group III and Group I. Within Group II concentration of GPx was exponentially decreased from Risk to End Stage group. Our results are on par with the study done by EL-Far MA et.al and Zagrodzki et al., stating that GPx is an important antioxidant and the plasma form of GPx is mainly synthesized in the kidney [218, 219].

Ceballos P et al., have documented utilization of glutathione dependent antioxidant process through generation of ROS which usually accompany AKI. Decreased GPx activity in AKI may be because of increased rate of lipid peroxidation, decrease in functional renal mass, interference of uremic toxins, plasma GPx consumption and inactivation of GPx by biochemical modification and/ or an abnormality in the hexose mono phosphate pathway [220].

Vitamin C is known to influence NO and decrease glutathione concentration. Our study results suggest that the effect of vitamin C might be related to the elevations in NO. Yao BZ et.al., and Ceballos P et.al., also explained generated superoxide is converted into hydrogen peroxide later metabolized to water and oxygen by Catalase and / or GPx. During this reaction a large amount of glutathione reductase (GSH) is released via scavenging superoxide effectively. Free radical $O^{\cdot}_2$ at high concentration is known to react with NO and produce peroxynitrite. This mechanism may possibly restore NO level by reducing $O^{\cdot}_2$. [217, 220].

In our study we also included lipid profile and electrolytes as supportive biochemical parameters. Among these, Triglyceride levels were almost doubled in Group I compared to Group III. This indicates that increased blood glucose in Group I might have contributed to elevated triglycerides. To our surprise we could observe HDL levels were significantly elevated in Group II and Group I compared to Group III, may be because of: a) Life style modification b) Strict diet control c) Statins and d) Awareness of the sequelae. Low Density Lipoprotein cholesterol was significantly elevated and was almost doubled in Group II and Group I compared to Group III.

In our study we documented Na^+ and K^+ levels were increased in Group II compared with Group III and Group I. Except in Group II secondary to Leptospirosis where the K^+ levels were less compared to Group I and III. Most common cause for our observation may be due to drug induced hyperkalemia, triggered either by inhibiting renal potassium excretion or by blocking extrarenal removal. Treatment with Renin angiotensin aldosterone system (RAAS) inhibitors such as Angiotensin Converting enzyme (ACE) inhibitor or angiotensin receptor blockers (ARB) causes blockade of Angiotensin II synthesis with decreased aldosterone secretion, impaired delivery of sodium to the distal nephron and / or competitive binding to the antigen II receptor with decrease of aldosterone synthesis [221].

Tubular impairment plays an important role in the pathogenesis underlying Diabetic Kidney Disease (DKD) [222, 223]. Microalbuminuria activates renal proximal tubular epithelial cells to induce tubulointerstitial inflammation. In contrast, high glucose levels and diabetic substrates, including advanced glycation end-products,

carbonyl intermediates and ultra-filtered growth factors, trigger a number of signaling pathways to promote tubular cell hypertrophy and the interstitial deposition of chemokines, cytokines, growth factors, and adhesion molecules, which are capable of accelerating further inflammation and fibrosis.

The extent of tubulointerstitial injury may ultimately determine the attrition rate of renal function in DKD patients [224]. Therefore, tubular biomarkers may be crucial as glomerular markers for early diagnosis and stratification of DKD or renal impairment in T2DM.

The capability of a biomarker to diagnosis a renal insult early depends on whether or not the alteration in the level of the biomarker detected by current methods precedes the appearance of urinary microalbumin. With respect to being an early biomarker, Table 9a, 9b and Figure 12 NGAL was well correlated with Scr, inflammatory markers, oxidants and antioxidants, ROC curve analyses of serum NGAL with an AUC of 0.993 (95% CI, 0.969-1.00) with a cutoff value of 1.06 (sensitivity, 81.0%; specificity, 87.1%) in T2DM and DKD patients with a normal Urinary Albumin Creatinine Ratio (<30 mg/g).

Thus, the alterations will be detected before the increase in urine microalbumin.

NEW KNOWLEDGE GENERATED

1. Chance of AKI in T2DM were documented with associated comorbid conditions such as UTI, hypertension, Snake bite, Gastroenteritis, OP Poisoning, Leptospirosis and Cardio pulmonary Bypass.
2. Since Glycoprotein biomarker NGAL has completed phases 1,2 and 3 and estimation of this upregulated protein NGAL has positive prediction in assessing development of AKI. This may be an interesting molecule for clinicians to decide in future to include NGAL in diabetic profile as a routine parameter in the management of diabetes mellitus. Our study results with respect to NGAL are true, may be because of larger sample size (total n=450).
3. NGAL as a biomarker in addition may also be used to diagnose superimposed AKI on CKD
4. Cutoff values for NGAL, Cys C, MDA, GPx are proposed in this set of population
5. Comparison of NGAL with established marker and traditional markers were done to find NGAL as an early biomarker and it is proved to be an better option in early diagnosis and management of AKI.
6. We further propose Point of Electronic Care Testing (POECT) validation and approval to reduce turnaround time in estimation of NGAL for early management
7. NGAL is suggested to be added to the existing list of biomarkers for single organ injury particularly kidney injury, as it appear to perform well in specific populations.

8. NGAL has reduced uniformly around 30 ng/mL from'0' day to 14th day in Group II subgroups.
9. Serum creatinine and Serum Cys C levels were also reduced 0.5 and 0.8 respectively on '0' day and 14th day in Group II.
10. NGAL can be considered as an early marker for management of AKI, but needs to be further studied at "omics".

CONCLUSION

- Advent of new biomarkers will help define the kidney at risk rather than relying simply on creatinine.
- Since NGAL have undergone rigorous assessment in validation, our current result with respect to NGAL has lead to success and also improve patient outcome. Thus, it may be used as an early biomarker for AKI.
- Further our observations also suggest; that in addition to clinical examination, estimation of NGAL as a biomarker, base line oxidants and antioxidants may help clinicians in early management of diabetes and associated AKI due to comorbid conditions.

Early management of etiological factors shall prevent the risk of AKI in T2DM, as acute kidney injury has good prognosis with early intervention.

REFERENCES

1. Kumar P, Clark M. Textbook of clinical medicine. In: Saunders, editor. 8th edition. Elsevier; 2002. pp. 1099- 1121.
2. Bearse MA, Han T, Schneck ME. Local multifocal oscillatory potential abnormalities in diabetes and early diabetic retinopathy. Invest Ophthal Vis Sci 2004; 45: 3259- 3265.
3. Seki M, Tanaka T, Nawa H. Involvement of brain- derived neurotrophic factor in early retinal neuropathy of streptozotocin- induced diabetes in rats: therapeutic potential of brain- derived neurotrophic factors for dopaminergic amacrine cells. Diabetes 2004; 53: 2412- 2419.
4. Huang C, Kim Y, Caramori ML. Cellular basis of diabetic nephropathy: The transforming growth factor-beta system and diabetic nephropathy lesions in type 1 diabetes. Diabetes 2002; 51: 3577- 3581.
5. Moran A, Palmas W, Field L. Cardiovascular autonomic neuropathy is associated with microalbuminuria in older patients with type 2 diabetes. Diabetes care 2004; 27: 972- 977.
6. Lawrence JM, Conteras R, Chen W. Trends in the prevalence of preexisting diabetes and gestational diabetes mellitus among a racially/ethnically diverse population of pregnant women, 1999-2005. Diabetes Care 2008; 31 (5): 899- 904.
7. API textbook of medicine vol.1 ed.10 YP Munjal Diabetes mellitus pub. Jaypee brothers medical publishers epidemiology: A Ramachandran and C Snehalatha 2015. Pp.457- 458.

8. Fauci AS, Braunwald E, Kasper DL. Acute renal failure. In: Harrison, editor. Principles of internal medicine, Vol 2, 18th edition. New York: McGraw Hill; 2012. pp.2293- 2306.
9. Suresh CD. Acute renal failure. In: Siddharth NS, editor. API Text Book of Medicine, Vol 2, 10th edition. India: The Association of Physicians of India, Mumbai; 2015. pp.1757- 1761.
10. Sanoff S, Kusa O. Impact of acute kidney injury on chronic kidney disease and its progression. Contrib Nephrol 2011; 171: 213- 217.
11. Chirag RP, Jay LK. Biomarkers in Acute and Chronic Kidney Diseases. In: Brenner & Rector's The Kidney, chapter 30, Vol 1, 10th edition. USA: Elsevier, 2016, pp. 926- 931.
12. UK Prospective Diabetes Study (UKPDS) Group: Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet 1998; 352: 854– 865.
13. Sutton, T.A. (2009). Alteration of microvascular permeability in acute kidney injury. Microvasc. Res., 77: 4- 7.
14. Murray PT, Devarajan P, Level AS. A framework and key research questions in AKI diagnosis and staging in different environments. Clin J Am Soc nephrol 2008; 3: 864- 868.
15. Devarajan. P Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomark Med. 2010; 4(2): 265– 280.

16. Devarajan P. NGAL a promising biomarker for human acute kidney injury. Biomarkers med 2010; 4(2): 265- 280.
17. Devarajan P. update on mechanisms of ischemic acute kidney injury. J Am Soc Nephrol 2006; 17: 1503- 1520.
18. Parikh CR, Devarajan P: New biomarkers of acute kidney injury. Crit Crae Med 2008; 36(Suppl 4):159- 165.
19. Hulka BS, Griffith JD, Wilcosky TC. Overview of biological markers. In: Biological markers in epidemiology. Newyork: Oxford University Press, 1990, pp. 3- 15.
20. Richard M, Gertrude H Sergievesky. Biomarkers: Potential uses and limitations. Journal of American Society for Experimental Neuro Therapeutics 2004; 1: 182–188.
21. Prera FP, Weinstein IB. Molecular Epidemiology: recent advances and future directions. Carcinogenesis 2000; 21: 517- 524.
22. Schulte PA, Perera FP. A conceptual and historical framework for molecular epidemiology. In: Molecular epidemiology: principles and practices San Diego: Academic Press 2014, pp. 3- 44.
23. Evans J, Goldfine ID, Maddux BA, Grodsky GM. Are oxidative stress activated signaling pathways mediators of insulin resistance and beta cell dysfunction? Diabetes 2003; 52 (1): 1- 8.
24. Lu HT, Chin S, Ya WC, Ching YY, Chin CW, Dong ZH. Arsenic induces pancreatic cell apoptosis via the oxidative stress-regulated mitochondria dependent and endoplasmic reticulum stress triggered signaling pathways.

Toxicol Lett 2011; 20: 15- 26.

25. Young IS, Woodside JV. Antioxidants in health and disease. J. Clin. Pathol 2011; 54: 176- 186.
26. Mohan V, Sandeep S, Deepa R, Shah B, Varghese C. Epidemiology of type 2 diabetes mellitus: Indian scenario. Indian J Med Res 2007; 125: 217- 230.
27. Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, Warnock DGl. Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. Crit Care. 2007; 11(2): R31.
28. Lameire N, van Biesen W, Vanholder R. Acute Renal failure. Lancet 2005; 365: 417- 430.
29. Asif AS, Steven DW, Paul MP, Bruce AM. Acute Kidney Injury. In: Brenner & Rector's The Kidney, Vol.1, 10th edition. USA: Elsevier; 2016, pp. 958- 963.
30. Sushrut SW, Joseph VB. Acute Kidney Injury. Cahpter 279, Harrisons principles of Internal Medicine, 18th ed. Vol.1 2012, pp.2293- 2321.
31. Bellamo R, Ronco C, Kellum JA. Palevsky P. Acute renal failure-definition, outcome, measures, animal models, fluid therapy and information technology needs: the second international Consensus Conference of the Acute Dialysis Quality Initative (ADQI) Group. Crit Care 2004; 8: R204- 212.
32. Lopes JA, Jorge S. The RIFLE and AKIN classification for acute kidney injury: a critical and comprehensive review. Clin Kidney J 2013; 6: 8-14.
33. Acute Kidney Injury Work Group. Kidney Disease: Improving Global Outcomes (KDIGO) - Clinical Practice Guideline for Acute Kidney Injury. Kidney Inter. 2012; 2:1-138.
34. Thakar CV, Worley S, Arrigain S. influence of renal dysfunction on mortality

after cardiac surgery: Modifying effect of preoperative renal function. Kidney Int 2000; 67: 1112- 1119.

35. Lassnigg A, Schmidiln D, Mouhieddine M. Minimal changes of serum creatinine predict prognosis in patients after cardiothoracic surgery: A prospective cohort study. J Am Soc Nephrol 2004; 15: 1597- 1605.
36. Loef BG, Epema AH, Smilde TD. Immediate postoperative renal function deterioration in cardiac surgical patients predicts in hospital mortality and long term survival. J Am Soc Nephrol 2005; 16: 195- 200.
37. Fortescue EB, Bates DW, Chertow GM. Predicting acute renal failure after coronary bypass surgery: Cross validation of two risks- stratification algorithms. Kidney Int 2000; 57: 2594- 2602.
38. Bellamo R. Pathophysiology of cardiac surgery associated AKI. Int J Arrif organs 2008; 31(12): 166- 178.
39. Okusa MD. The inflammatory cascade in acute ischemic renal failure. Nephron 2002; 90: 133- 138.
40. Kasturiratne A, Wickremasinghe AR, de Silva N, Gunawardena NK, Pathmeswaran A. the global burden of snakebite: A literature analysis and modeling based on regional estimates of envenomating and deaths. Plos Med 2008; 5: e218- e221.
41. Kohli HS, Sakhuja V. Snake-bites and acute renal failure. Saudi J Kidney Dis Transpl 2003; 14: 165- 176.
42. Mohapatra B, Warrel DA, Suraweera W, Bhatia P, Dhingra N. Snakebite mortality in India: a nationally representative mortality survey. Plos Negl Trop Dis 2011; 5: e1018- e1021.

43. Lu QClemetson JM, Clemetson KJ. Snake venoms and hemostasis J Thromb Haemost 2005; 3: 1791- 1799.
44. Rocha ESM, Beraldo WT, Rosenfeld G. Bradykinin a hypotensive and smooth muscle stimulating factor related from plasma globulin by snake venoms and by trypsin Am J Physiol 1949; 156: 261- 273.
45. Ducancel F, Endothelin like peptides. Cell Mol Life Sci 2005; 62: 2828- 2839.
46. Kanjanabuch T, Sitprija V. Snakebite nephrotoxicity in Asia. Semin Nephrol 2008; 28: 363- 372.
47. Vijeth SR, Dutta TK, Shahapurkar J. Correlation of renal status with hematologic profile in viperbite. Am J Trop Med Hyg 1997; 56: 168- 170.
48. Devarajan P. Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomark Med. 2010; 4: 265– 280.
49. Fiaccadori E, Maggiore U, Clima B Melfa L. Incidence, risk factors and prognosis of gastrointestinal hemorrhage complicating acute renal failure. Kidney Int 2001; 59: 1510- 1519.
50. Rosser CJ, Bare RL, Meredith JW. Urinary tract infections in the critically ill patient with a urinary catheter.Am J Surg. 1999; 177: 287– 290.
51. Mori T, Shimizu T, Tani T. Septic acute renal failure. Contrib Nephrol. 2010; 166: 40– 46.
52. Plataki M, Kashani K, Cabello-Garza J, Maldonado F, Kashyap R, Kor DJ. Predictors of acute kidneyinjury in septic shock patients: an observational cohort study. Clin J Am Soc Nephrol. 2011; 6: 1744– 1751.
53. Baker LR, Cattell WR, Fry IK, Mallinson WJ. Acute renal failure due to bacterial pyelonephritis. Q J Med. 1979; 48: 603– 612.

54. Thakar CV, Christianson A, Freyberg R, Almenoff P, Render ML. Incidence and outcomes of acute kidney injury in intensive care units: A Veterans Administration study. Crit Care Med. 2009; 37: 2552– 2558.

55. Wald R, Quinn RR, Luo J, Li P, Scales DC, Mamdani MM. Chronic dialysis and death among survivorsof acute kidney injury requiring dialysis. JAMA. 2009; 302: 1179–1185.

56. Adler B, de la Peña Moctezuma A. Leptospira and leptospirosis.V. Microbiol 2010; 140: 287- 296.

57. Bharti AR, Nally JE, Ricaldi JN. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003; 3: 757- 771.

58. Ko AI, Galvão Reis M, Ribeiro Dourado CM, Johnson WD Jr, Riley LW. Urban epidemic of severe Leptospirosis in Brazil. Salvador Leptospirosis Study Group. Lancet 1999; 354: 820- 825.

59. Daher EF, Zanetta DMT, Cavalcante M. Risk factors for death and changing patterns in acute renal failure of leptospirosis. Am J Trop Med Hyg 1999; 61: 630- 634.

60. Sitprija V, Losuwanrak K, Kanjanabuch T. Leptospiral nephropathy. Semin Nephrol 2003; 23: 42- 48.

61. Seguro AC, Lomar AV, Rocha AS. Acute renal failure of leptospirosis: Nonoliguric and hypokalemic forms. Nephron 1990; 55:146- 151.

62. Cerqueira TB, Athanazio DA, Spichler AS, Seguro AC. Renal involvement in leptospirosis – new insights into pathophysiology and treatment. Braz J Infect Dis 2008; 12: 248- 252.

63. Elizabeth De FD, Krasnalhia LSD, Geraldo BSJ. Leptospirosis associated acute

kidney injury J Bras Nefrol 2010; 32(4): 400- 407.

64. Carey JL, Dunn C, Gaspari RJ. Central respiratory failure during acute organophosphate poisoning. Respir Physiol Neurobiol. 2013; 189: 403– 410.
65. Iyer R, Iken B, Leon A. Developments in alternative treatments for organophosphate poisoning. Toxicol Lett. 2015; 233: 200– 206.
66. Yang CC, Deng JF. Intermediate syndrome following organophosphate insecticide poisoning. J Chin Med Assoc. 2007; 70: 467– 472.
67. Leibson T, Lifshitz M. Organophosphate and carbamate poisoning:review of the current literature and summary of clinical and laboratory experience in southern Israel. Isr Med Assoc J.2008; 10: 767– 770.
68. Weissmann-Brenner A, Friedman LM, David A. Organophosphate poisoning: a multihospital survey. Isr Med Assoc J. 2002; 4: 573– 576.
69. Cavari BP. Organophosphorus poisoning. JNMA J Nepal Med Assoc. 2008; 47: 251–258.
70. Guidance for Industry and FDA Staff: Qualification Process for Drug Development Tools. Center for Drug Evaluation and Research, US Food and Drug Administration, Department of Health and Human Services; 2014.
71. Chirag R. Parikh, Dennis G. Moledina1, Steven G. Coca, Heather R. Thiessen-hilbrook1 and Amit X. Garg4 Application of new acute kidney injury biomarkers in human randomized controlled trials Kidney Int 2016; 89: 1372– 1379.
72. Kashani K, Al-Khafaji A, Ardiles T. Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury. Crit Care. 2013; 17: R25- R27.
73. Waikar SS, Betensky RA, Emerson SC, Bonventre JV. Imperfect gold standards for kidney injury biomarkers evaluation. J Am soc Nephrol 2012; 23: 13- 21.

74. Levey AS, Bosch JP, Lewis JB, Greene T, Roqers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of diet in renal disease study group. Ann Inter Med 1999; 130 (6): 461- 470.

75. Dieterie F, Sistare F, Goodsaid F, Papluca M, Ozer JS. Renal biomarker qualification submission: a dialog between the FDA-EMEA and Predictive safety testing Consortium. Nat Biotechnol 2010; 28: 455- 462.

76. Van Acker BA, Koomen GC, Koopman MG. Creatinine clearance during cimetidine administration for measurement of glomerular filtration rate. Lancet 1992; 340: 1326- 1329.

77. Rehberg PB. Studies on Kidney Function: The Rate of Filtration and Reabsorption in the Human Kidney. Biochem J 1926; 20: 447- 460.

78. Baum N, Dichoso CC, Carlton CE. Blood urea nitrogen and serum creatinine. Physiology and interpretations. Urology 1975; 5: 583- 588.

79. Soni SS, Ronco C, Katz N. early diagnosis of acute kidney injury: promise if novel biomarker Blood Purif 2009; 28: 165- 175.

80. Kampmann J, Sieraback- Nielson K, Kristensen M. rapid evaluation ofcreatinine clearance. Acta Med Scand 1974; 196: 517- 520.

81. Ducharme MP, Smythe M, Strohs G. Drug induced alterations in serum creatinine concentrations. Ann Pharmacother 1993; 27: 622- 633

82. Rocci Jr ML, Vlasses PH, Ferguson RK. Creatinine seum concentrations and H2receptor antagonists. Clin Nephrol 1984; 22: 214- 215.

83. McCance K, Huether S. Structure and function of the renal and urologic systems. In Pathophysiology: the biologic basis for disease in adults and children. 2002:

52: 1170- 1190.

84. Zurbig P, Dihazi H, Metzger J.urine proteomics in kidney and urogenital diseases: moving towards clinical applications. Proteomics Clin Appl 2011; 5: 256- 268.

85. Prunotto M, Ghiggery GM, Candiano G. urinary proteomics and drug discovery in chronic kidney disease: A new perspective. J Proteome Res. 2011; 10: 126- 132.

86. Flower DR, North AC, Sansom CE. The lipocalin protein family: structural and sequence overview. Biochem Biophys Acta 2009; 1482: 9- 24.

87. Flower DR. multiple molecular recognition properties of the lipocalin protein family J Mol recognition1996; 8: 185- 195.

88. Darren R F, Anthony CT, Clae ES. The lipocalin protein family: Structural and sequence overview Biochemica et Biophysica Acta Protein structure and molecular enzymology 2000; 1482 (2): 9- 24.

89. Subhankar C, Sukhwinder K, Zhmin T, Sirinder K. neutrophil gelatinase associated lipocalin: structure, function and role in human pathogenesis. Acute Phase proteins- Regulation and functions of Acute phase proteins. 2011; 16: 345-368.

90. Vjeronique le CJ, Cowland JB, Calafatt J, Borregaard N. targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. Cell Biology 1996;93:6454- 6457.

91. Harba Renevy S, Turler H, Kress M, Salomon C. SV40-induced expression of mouse gene 24p3 involves a post transcriptional mechanism. Oncogene 1989; 4: 601- 608.

92. Geotz DH, Willie ST, Armen RS, Bratt T. Ligand preference inferred from the structure of Neutrophill gelatinase associated lipocalin. Biochemistry 2000; 39: 1935- 1941.

93. Coles M, Diercks T, Muehlenweg B. the solution structure and dynamics of human neutrophil gelatinase associated lipocalin. J Mol Biol 1999; 289: 139- 157.

94. Lars Kjeldsen, Dorothy FB, Borregard N. identification of neutrophil gelatinase associated lipocalin as a novel matrix protein of specific granules in Human Neutrophils. Blood 1994; 183(3): 799- 807.

95. Schmiddt KM, Mori K, Kalandadze A, Li JY. Neutrophil Gelatinase Associated Lipocalin mediated iron traffic in kidney epithelia Curr Opin Nephrol Hypertens. 2006; 15: 442- 449.

96. Mishra J, Ma Q, Kelly C, Mitsnefes M. NGAL is a novel early marker of acute kidney injury following transplantation. Pediatr Nephrol 2006; 21: 856- 863.

97. Bonventre JV, sukhatme VP, Bamberger M, Ouellette AJ. Localization of the protein product of the immediate early growth response gene, EGR in the kidney after ischemia and reperfusion. Cell Regul 1991; 2: 251- 260.

98. Megyesi J, Di mari J, Udvarhelyi N, Price PM. DNA synthesis is dissociated from the immediate early gene response in the post –ischemic kidney. Kidney Int 1995; 48: 1451- 1458.

99. Kai M, Schmidt-Ott, Kiyoshi Mori, Jau Yi Li. Action of Neutrophil gelatinase associated lipocalin J Am Soc Nephrol 2007; 18: 407- 413.

100. Le C, Calafat V, Borregard N. Sorting of the specific granule protein, NGAL, during granulocytic maturation of HL-60 cells. Blood 1997; 89: 2113- 2121.

101. Furutani M, Arri S, Mizumoto M, Kato M, Imamura M. identification of

neutrophil gelatinase associated lipocalin mRNA in human pancreatic cancers using a modified signal sequence trap method. Cancer Lett 1998; 122: 209- 214.

102. Mallbris L, Obrien KP, Hulthen A, Cowland JB. Neutrophil gelatinase associated lipocalin is a marker for dysregulated keratinocyte differentiation in human skin. Exp Dermatol 2002; 11: 584- 591.

103. Yang XO, Chang SH, Park H, Nuriveva R. regulation of inflammatory responses by IL-17F.J Exp Med 2008; 205: 1063- 1075.

104. Park H, Li Z, XO, Chang SH. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat immunol 2005; 6: 1133- 1141.

105. Tong Z, Chakraborty S, Sung B, Koolwal P, Kaur S. Epidermal growth factor down regulates the expression of neutrophil gelatinase associated lipocalin through E-cadherin in pancreatic cancer cells. Cancer 2011; 117: 2408- 2418.

106. Supavekin. S, Zhang W, Kuchertapati R. Differential gene expression following early renal ischemia-reperfusion. Kidney Int 2003; 63: 1714- 1724.

107. Devarajan P, Mishra J, Supravekin S, Patterson LT.Gene expression in early ischemic renal injury: clues towards pathogenesis, biomarker discovery and novel therapeutics. Mol Genetics 2003; 80(4): 365- 376.

108. Mishra J, Ma Q, Prada A. identification of nrutrophil gelatinase associated lipocalin as a novel biomarker for ischemic injury. J Am Soc Nephrol 2003; 4: 2534- 2543.

109. Bagshaw SM, George C, Bellomo R. Early acute kidney injury and sepsis: A multicenter evaluation. Crit Care 2008; 12: 47- 49.

110. Joannids M, Metnitz B, Bauer P. Acute Kidney Injury in critically ill patients classified by AKIN versus RIFLE using the SAPS 3 database. Intensive Care Med

2000; 161: 872- 879.

111. Guerin C, Girad R, Selli JM. Initial versus delayed acute renal failure in the intensive care unit. A multicenter prospective epidemiological study. Am J Respir. Crit Care Med 2000; 161: 872- 879.

112. Fjaertoft G, Foucard T, Xu S. Human neutrophil lipocalin (HNL) as a diagnostic tool in children with acute infections: A study of the kinetics. Acta Paediatr 2012; 94: 661- 666.

113. Haase M, Bellomo R, Devarajan P. Accuracy of neutrophil gelatinase associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: A systematic review and meta-analysis. Am J Kidney Dis 2009; 54: 1012- 1024.

114. Awad AS, Huang LM. Compartmentalization of neutrophils in the kidney and lung following acute ischemic kidney injury. Kidney Int. 2009; 75: 689- 698.

115. Bagshaw SM, Bennett M, Haase M. Plasma and urine neutrophil gelatinase associated lipocalin in septic versus non-septic acute kidney injury in critical illness. Intensive Care Med 2010; 36: 452- 461.

116. McIlroy DR, Wagener G, Lee HT. Neutrophil gelatinase-associated lipocalin and acute kidney injury after cardiac surgery: the effect of baseline renal function on diagnostic performance. Clin J Am Soc Nephrol 2010; 5: 211- 219.

117. Mori K, Lee HT, Rapoport D. Endocytic delivery of lipocalin-siderophore iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 2005; 115: 610- 621.

118. Mishra J, Mitsnefes M, Prada A. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. J Am Soc Nephrol. 2003; 14: 2534- 2543.

119. Flo TH, Smith KD, Sato S. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature 2004; 432: 917- 921.

120. Klausen P, Niemann CU, Cowland JB. On mouse and man: Neutrophil gelatinase associated lipocalin is not involved in apoptosis or acute response. Eur J Haematol. 2005; 75: 332- 340.

121. Cai L, Rubin J, Han W. The origin of multiple molecular forms in urine of HNL/NGAL. Clin J Am Soc Nephrol 2010; 5: 2229- 2235.

122. Orlando R, Mussap M, Plebani M. Diagnostic value of plasma Cystatin C as a glomerular filtration marker in decompensated liver cirrhosis. Clin. Chem 2002; 48: 850- 858.

123. Knight EL, Verhave JC, Spiegelman D. Factors influencing serum Cystatin C levels other than renal function and the impact on renal function measurement. Kidney Int 2004; 65: 1416- 1421.

124. Uchida K, Gotoh A. Measurement of Cystatin-C and Creatinine in urine. Clin. Chem. Acta 2002; 323: 121- 128.

125. Conti M, Moutereau S, Zater M. Urinary Cystatin C as a specific marker of tubular dysfunction. Clin. Chem. Lab. Med 2010; 44: 288- 291.

126. Edelstein CL, Hoke TS, Somerest H. Potential tubules from caspase -1-deficient mice are protected against hypoxia-induced membrane injury. Nephrol Dial Transplant. 2007; 22: 1052- 1061.

127. Melinkov VY, Ecder T, Fantuzzi G. Impaired IL-18 processing protects caspase-1 deficient mice ischemic acute renal failure. J Clin Invest 2001; 107: 1145- 1152.

128. Parikh CR, Jani A, Melinkov VY. Urinary IL-18 is a marker of human acute tubular necrosis. Am J Kidney Dis 2004; 43: 405- 414.

129. Parikh CR, Jani A, Mishra J. Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant 2006; 6: 1639- 1645.

130. Miyauchi K, Takiyama Y, Honjyo J. up regulated IL-18 expression in type 2 diabetic subjects with nephropathy: TGF- beta 1 enhanced IL-18 expression in human renal proximal tubular epithelial cells. Diabetes Res Clin Pract 2009; 83: 190- 199.

131. Ichimura T, Bonventre JV, Bailly V. kidney injury molecule-1 (KIM-1) putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J Biol Chem 1998; 273: 4135- 4137.

132. Hubank M, Schatz DG. Identifying differences in mRRNA expression by representational difference analysis of cDNA. Nucl Acid Res. 1994; 22: 5640-5648.

133. Bailly. V, Zhang Z, Meier W. Shedding of Kidney injury molecule-1a putative adhesion protein involved in renal regeneration. J Biol Chem 2002; 277: 39739-39748.

134. Ichimura T, Hung CC, Yang SA. KIM-1 A tissue and urinary biomarker for nephrotoxicant induced renal injury Am J Physiol Renal physiol 2004; 296: 552-563.

135. Vidya VS, Ramirez V, Ichimura T. Urinary KIM-1 a sensitive quantitative biomarker for early detection of kidney tubular injury. Am J Physiol Renal Physol 2006; 290: 17- 29.

136. Yamato T, Noiri E, Ono Y. Renal L-Type fatty acid binding protein in acute ischemic injury. J Am soc Nephrol 2007; 18: 2894- 2902.

137. Portilla D, Dent C, Sugaya T. Liver type fatty acid binding protein in acute

kidney injury after cardiac surgery. Kidney Int 2008; 73: 465- 473.

138. Nakamura T, Sugaya T, Koide H. Urinary Liver type fatty acid binding protein in septic shock: effect of polymyxin B-immobilised fiber hemoperfusion. Shock 2009; 31: 454- 459.

139. Ferguson MA, Vaidya VS, Waikar SS. Urinary Liver type fatty acid binding protein predicts adverse outcomes in AKI. Kidney Int 2010; 77: 708- 714.

140. Bondiou MT, Bourbouze R, Bernard M. inhibition of A and B N-Acetyl D glucosaminidase urinary isoenzymes by urea. Clin Chem Acta 1985; 149: 67- 73.

141. Wiley RA, Choo HY, Triager GJ. The effect of nrphrotoxic furans on urinary NAG levels in mice. Toxicol Lett 1982; 14: 93- 96.

142. Nauta FL, Kakker SJ, Van Oeveren W. Albuminuria, Proteinuria and novel biomarkers as predictors of long term allograft outcomes in kidney transplant receipients. Am J Kidney Dis 2011; 57: 733- 743.

143.Ren L, Ji J, Fong Y. Association of urinary N-actyl Beta D Glucosaminidase as an early marker of contrast induced nephropathy. J Int Med Res 2011; 39: 647- 653.

144. Chou KM, Lee CC, Chen CH, Sun CY. Clinical value of NGAL, L-FABP and Albuminuria in predicting GFR Decline in Type 2 Diabetes Mellitus Patients.www.plosone.org.2013; 8(1): 54863- 54869.

145. Mishra J, Kelly C, Devarajan P. Kidney NGAL is a novel early marker of acute injury following transplantation. PediatrNephrol 2006; 21(6): 856- 863.

146. Adanir T, Aksun M, Alkam TF. The renal effect of replacement fluids in controlled severe hemorrhagic shock: an experimental study Cystatin C. Ulus Travma Acilcerrhai Derg 2009; 5: 423- 432.

147. Blix PM, Willis BC, Landau RL. Urinary C- peptide: an indicator of beta cell

secretion under different metabolic conditions. J Clin Endo Metab 1982; 54(3): 574- 580.

148. Nader R, Warnick RG. Lipids, Lipoproteins, and Apolipoproteins In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and Molecular Diagnostics. 4th edition. India: Elsevier; 2006. pp. 940-963.

149. AHA heart and stroke statistical update: American Heart Association 1999 (Nov): www.americanheart.org.

150. Granger DL, Taintor RR, Bockvar KS. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griessrection. Methods in Enzymology, vol 268. 1996.p.142-151.

151. Roe J, Kuether C A. Determination of Vitamin C. Science1942;96.

152. Lamb E, David JN, Cristopher PP. Renal function and Nitrogen Metabolites. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and Molecular Diagnostics. 4th edition. India: Elsevier; 2006. p. 797-813.

153. David B, Sacks MB, Path FRC. Carbohydrates. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and Molecular Diagnostics. 4th edition. India: Elsevier; 2006. pp. 868- 870.

154. Paglia DE, Valentine WN. Estimation of Glutathione Peroxidase. Methods of Enzymology 1967; 14: 74- 78.

155. Holewijn S, Heijer M, Swinkels DW. Apolipoprotein B, non-HDL cholesterol and LDL cholesterol for identifying individuals at increased cardiovascular risk. J Intern Med. 2010; 268(6): 567- 577.

156. Levy AS, Bosch JP, Lewis JB. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of

diet in renal disease study group. Ann Intern Med 1990;130:461-470

157. Daniel WD. Biostatistics A foundation for analysis in the health sciences edn. 7, Wiley series in probability and statistics, editors: Barnett V, Ralph AB publishers: Wiley, 2009. pp.57- 82.

158. Sushrut SW,Joseph VB. Acute Kidney Injury. In: Harrison, editor. Principles of internal medicine, Vol 2, 19th edition. New York: McGraw Hill; 2012, pp.1799-1874.

159. Kooman JP, Barendregt JN, van der Sande FM, van Suylen RJ. Acute pyelonephritis: a cause of acute renal failure? Neth J Med. 2000; 57: 185–189.

160. Schaeffer AJ. Urinary tract infections in the elderly. Eur Urol. 1991; 19 Suppl 1: 2– 6.

161. Sant GR. Urinary tract infection in the elderly. Semin Urol. 1987; 5: 126– 133.

162. Patterson JE, Andriole VT. Bacterial urinary tract infections in diabetes. Infect Dis Clin North Am. 1997; 11: 735– 750.

163. Scholes D, Hooton TM, Roberts PL, Gupta K, Stapleton AE, Stamm WE. Risk factors associated with acute pyelonephritis in healthy women. Ann Intern Med. 2005; 142: 20– 27.

164. Robbins SL, Tucker AW. The cause of death in diabetes. A report of 307 autopsied cases. N Engl J Med. 1944; 231: 865– 868

165. Boscia JA, Kaye D. Asymptomatic bacteriuria in the elderly. Infect Dis Clin North Am. 1987; 1: 893– 905.

166. Cotran RS, Pennington JE. Urinary tract infection, pyelonephritis, and reflux nephropathy. Philadelphia:W.B. Saunders; 1981: 1571– 1632.

167. Lorentz WB, Iskandar S, Browning MC, Reynolds GD. Acute renal failure due

to pyelonephritis. Nephron. 1990; 54: 256– 258.

168. Soylemezoglu O, Kale G, Saatci U, Akcaoren Z. Acute renal failure due to acute pyelonephritis. Int Urol Nephrol. 1995; 27: 137– 139.

169. Martensson J, Bellomo R. The rise and fall of NGAL in acute kidney injury. Blood purify 2014; 4(37): 304- 310.

170. de Geus HR, Fortrie G, Betjes MG, Vanschaik. Time of injury affects urinary biomarkers predictive values for AKI in critically ill, non-septic, patients. BMC Nephrol 2013; 14: 273- 276.

171. Celik E, Altekin, Rana I, Yasin K, Murat D, Nur A. Evaluation of neutrophil gelatinaseassociated lipocalin in pediatric patients with acute rotavirus gastroenteritis and dehydration. Italian Journal of Pediatrics 2013; 39:52.

172. Dent CL, Ma Q, Dastrala S, Bennett M, Mitsnefes MM, Barasch J, Devarajan P: Plasma neutrophil gelatinase-associated lipocalin predicts acute kidney injury, morbidity and mortality after pediatric cardiac surgery: a prospective uncontrolled cohort study. Crit Care 2007; 11: R127-R129.

173. Akcay A, Ngayen Q, Edeistein CL. Mediators of inflammation in Acute Kidney Injury. Mediattors Inflamm 2009; 2009: 1370- 1372.

174. Srisawat N, Praditpomsilpa A, patarakul K, Techapomrung M, Daraswang T, Sukmark T. Neutrophil gelatinase associated lipocalin in Leptospirosis acute kidney Injury: A Multicenter study in Thailand. Plosone 2016: 10(12); 1- 14.

175. Murugan R, Karajala-S V, Lee M, Yende S, Kong L, Carter M. Genetic and inflammatory Markers of Sepsis (GenIMS) Investigators. Acute kidney injury in

non severe pneumonia is associated with an increased immune response and lower survival. Kidney Int 2010; 77(6): 527- 535.

176.Tuladhar SM, puntmann VO, Soni M, Punjabi PP, Bogle RG. Rapid detection of acute kidney injury by plasma and urinary neutrophil gelatinase associated lipocalin after cardiopulmonary bypass. J Cardiovasc Pharmacol 2009; 53: 261-266.

177. Mehta RL, Pascual MT, Gruta CG, Zhuang S, Chertow GM. Refining predictive models in critically ill patients with acute renal failure. J Am Soc Nephrol 2002; 13: 1350- 1357.

178. Lins RL, Elseviers MM, Daelemans R, Arnouts P, Billiouw JM, Couttenye M. Reevaluation and modification of the stuivenberg Hospital Acute Renal Failure (SHARF) scoring system for the prognosis of acute renal failure: an independent multicenter, prospective study. Nephrol Dial Transplant. 2004; 19: 2282- 2288.

179. Schmid-Ott KM, Mori K, Li JY, Kalandadze A, Cohen DJ, Devarajan P. Dual action of neutrophil gelatinase associated lipocalin. J Am Soc Nephrol 2007; 18: 407- 413.

180. Mori K, Nakao K, neutrophil gelatinase associated lipocalin as the real time indicator of active kidney damage. Kidney Int 2007; 71: 967- 970.

181. Patil G, Murthy N, Nikhil M: Contributing factors for morbidity and mortality in patients with organophosphate poisoning on mechanical ventilation: a retrospective study in a teaching hospital. J Clin Diagn Res. 2016; 10: UC18-UC20.

182. Pohanka M, Novotný L, Misík J. Evaluation of cholinesterase activities during in

vivo intoxication using an electrochemical sensor strip- correlation with intoxication symptoms. Sensors (Basel). 2009; 9: 3627-3634.

183. van den Akker JP, Egal M, Groeneveld AB. Invasive mechanical ventilation as a risk factor for acute kidney injury in the critically ill: a systematic review and meta-analysis. Crit Care. 2013; 17: R98- R101.

184. Lee FY, Chen WK, Lin CL. Organophosphate poisoning and subsequent acute kidney injury risk: a nationwide population-based cohort study. Medicine (Baltimore). 2015; 94: e2107- e2019.

185. Patel ML, Rekha Sachan, Gangwar R, Sachan P, Natu SM. Correlation of serum neutrophil gelatinase-associated lipocalin with acute kidney injury in hypertensive disorders of pregnancy. International Journal of Nephrology and Renovascular Disease 2013: 6: 181- 186.

186. Montan S, Sjoberg NO, Svenningsen N. Hypertension in pregnancy – fetal and infant outcome. A cohort study. Clin Exp Hypertens B. 1987; 6: 337– 348.

187. Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, Ruff SM, Zahedi K, Shao M, Bean J, Mori K, Barasch J, Devarajan P: Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. Lancet 2005; 365: 1231– 1238.

188. Parolari A, Pesce LL, Pacini D, Mazzanti V, Salis S, Sciacovelli C, Rossi F, Alamanni F; Monzino Research Group on Cardiac Surgery Outcomes: Risk factors for perioperative acute kidney injury after adult cardiac surgery: role of perioperative management. Ann Thorac Surg 2012; 93: 584– 591.

189. Kolli H, Rajagopalam S, Patel N, Ranjan R, Venuto R, Lohr J, Arora P: Mild acute kidney injury is associated with increased mortality after cardiac surgery in

patients with EGFR <60 ml/min/1.73 m^2. Ren Fail 2010; 32: 1066– 1072.

190. Han WK, Wagener G, Zhu Y, Wang S, Lee HT: Urinary biomarkers in the early detection of acute kidney injury after cardiac surgery. Clin J Am Soc Nephrol 2009; 4: 873– 882.

191. Go AS, Parikh CR, Ikizler TA, Coca S, Siew ED, Chinchilli VM, Hsu CY, Garg AX, Zappitelli M, Liu KD, Reeves WB, Ghahramani N, Devarajan P, Faulkner GB, Tan TC, Kimmel PL, Eggers P, Stokes JB; Assessment Serial Evaluation, and Subsequent Sequelae of Acute Kidney Injury Study Investigators: The assessment, serial evaluation, and subsequent sequelae of acute kidney injury (ASSESS-AKI) study: design and methods. BMC Nephrol 2010; 11: 22.

192. Haase M, Bellomo R, Devarajan P, Ma Q, Bennett MR, Mockel M, Matalanis G, Dragun D, Haase-Fielitz A: Novel biomarkers early predict the severity of acute kidney injury after cardiac surgery in adults. Ann Thorac Surg 2009; 88: 124– 130.

193. Koyner JL, Bennett MR, Worcester EM, Ma Q, Raman J, Jeevanandam V, Kasza KE, Connor MF, Konczal DJ, Trevino S, Devarajan P, Murray PT: Urinary cystatin C as an early biomarker of acute kidney injury following adult cardiothoracic surgery. Kidney Int 2008; 74: 1059- 1069.

194. Uchino S, Kellum JA, Bellamo R. Acute renal failure in critically ill patients: A Multinational Multicenter study. JAMA 2010; 294: 813- 818.

195. Grigoryer DN, Liumhasson HT, Cheadle C, barnes KC. The local and systemic inflammatory transcriptome after AKI. JAMA Soc Nephrol 2010; 294: 813- 818.

196. Vishal J, Yatin M, Abhinav G, Reetesh S, Arun R, Naresh T. The role of neutrophil gelatinase-associated lipocalin in predicting acute kidney injury in patients undergoing off-pump coronary artery bypass graft: A pilot study Ann

Card Anaesth. 2016; 19 (2): 225– 230.

197. Martensson J, Bellomo A, Xu S, Venge P. NGAL in adult septic patients with and without AKI. Intensive care Med 2010; 36: 1333- 1340.

198. Hvidberg V, Jacobsone, Strong RK, Cowland JB. The endocytic receptor megalin binds the iron transforming NGAL with high affinity and mediates its cellular uptake. FEBS Lett 2011; 579: 773- 777.

199. Keller CR, Iino N, Hosojima M. Megalin-mediated endocytosis of Cystatin C in proximal tubule cells. Biochem Biophys Res Commun 2007; 357: 1130- 1134.

200. Zhang Z, Lu B, Sheng X. Cystatin C in prediction of acute kidney injury: a systemic review and meta analysis Am J Kidney Dis 2011; 58: 356- 365.

201. Royakkers AA, Korevaar JC, Van Suijlen JD. Serum and urine Cystatin C are poor biomarkers for acute kidney injury and renal replacement therapy. Intensive Care Med 2011; 37: 493- 501.

202. Kanbay M, Yilmaz MI, Sonmez A. Serum uric acid level and endothelial dysfunction in patients with nondiabetic chronic kidney disease. Am J Nephrol. 2011; 33 (4): 298– 304.

203. Shimada MB. Dass AA, Ejaz. “Paradigm shift in the role of uric acid in acute kidney injury,” Seminars in Nephrology 2011; 31 (5): 453- 458.

204. Shashidhar KN, Munilakshmi U, Prabhavathi K, Madhavi Reddy, Lakshmaiah V. Correlation of blood uric acid with urinary albumin creatinine ratio in hypertension and diabetic nephropathy. Apollo Medicine 2016; 13: 24- 30.

205. Navarro JF, Milena FJ, Mora C. Renal Pro-inflammatory cytokine gene expression in diabetic nephropathy: effect of angiotensin-converting enzyme inhibition and pentoxifylline administration. Am J Nephrol 2006; 26: 562- 570.

206. Vakrani GP, Ramakrishnan S, Rangarajan D Acute Renal Failure in Diabetes Mellitus (Prospective Study). J Nephrol Ther 2013; 3: 137- 140.

207. Shelley LD, Angela ES, Priscilla AB, Holman, Kenneth J R. The initial noncovalent binding of glucose to human hemoglobin in nonenzymatic glycation. Glycobiology 2013; 23 (11): 1250- 1259.

208. Jones AG, Hattersley AT. The clinical utility of C-peptide measurement in the care of patients with diabetes. Diabet Med 2013; 30: 803- 817.

209. Shestakova MV, Martynov IR, Ivanishina NS, Dedov II: Assessment of vasomotor endothelial function in patients with diabetes mellitus type 1 at different stages of diabetic nephropathy (Russian). Ter Arkh 2003; 75: 17- 21.

210. Saulo K. The role of nitric oxide in hypertension and renal disease progression. Nephrol Dial Transplant. 2001; 16 Suppl 1: 60- 62.

211. Brodsky SV, Yamamoto T, Tada T, Kim B, Chen J, Kijiya F. Endothelial dysfunction in Ischemic acute renal failure: rescue by transplanted endothelial cells. Am J Physiol Renal Physiol 2002; 282 (6): F1140- 1149.

212. Howaida AN, Manal AS, Iman EE. The effect of Nrf2-Keap1pathway on the oxidative stress and inflammations in acute kidney injury patients. International Journal of Advanced Research 2016; 4 (2): 424- 433.

213. Pegues MA, McCrory MA, Zarjou A, Szalai AJ. C-reactive protein exacerbates renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 2013; 304: F1358-1365.

214. Himmelfarb J, Mcmonagle E, Freedman S, Klenzak J, Mcmenamin E. Oxidative stress is increased in critically ill patients with acute renal failure. J Am Soc Nephrol 2004; 15: 2449- 2456.

215. Vecchi. AF, Bamonti F, Novembrino C, Ippolito S, Guerra L. Free and total plasma Malondialdehyde in chronic renal insufficiency and in dialysis patients. Nephrol Dial Transplant 2009; 24: 2524- 2529.

216. Oberley LW. Free radicals and diabetes. Free Radic Biol Med 1988; 5(2): 113- 124.

217. Yao BZ, Ya PZ, Jing Z , Yan BZ. Evaluation of vitamin C supplementation on kidney function and vascular reactivity following renal ischemic injury in mice. Kidney Blood Press Res 2016; 41: 460- 470.

218. El far MA, Bakr MA, Farahat SE, A Fattah EA. Glutathione peroxidase activity in patients with renal disorders. Clin Exp Nephrol 2005; 9: 127- 131.

219. Zagrodzki P, Barton H, Walas S. Selenium status indices, laboratory data, and selected biochemical parameters in end stage renal disease patients. Biol Trace Elem Res 2007; 116: 29- 41.

220. Ceballos P, Witko SV, Merad BM. Glutathione antioxidant system as a marker of oxidative stress in chronic renal failure. Free Radic Biol Med 1996; 21: 845-853.

221. Clinical update on Hyperkalemia. National Kidney foundation 2014.

222. Gilbert RE, Cooper ME. The tubulointerstitium in progressive diabetic kidney disease: more than an aftermath of glomerular injury? Kidney Int. 1999; 56(5): 1627- 1637.

223. Kim SS, Song SH, Kim IJ, Jeon YK, Kim BH, Kwak IS, et al. Urinary cystatin C and tubular proteinuria predict progression of diabetic nephropathy. Diabetes Care. 2013; 36(3): 656- 661.

224. Tang SC, Lai KN. The pathogenic role of the renal proximal tubular cell in diabetic nephropathy. Nephrol Dial Transplant. 2012; 27(8): 3049- 3056.

ANNEXURE- I

Paper Title	Author	Journal	Indexation
Biochemical evaluation, anthropometric Measurements in assessment of Microvascular complications in females With type-2 diabetes mellitus	Munilakshmi U, Shashidhar K.N, Harish R, Madhavi Reddy, Lakshmaiah V.	Asian Journal of Medical Science, Volume-5(2014) (International online Journa-10/2013l)	index Copernicus Google Scholar
Evaluation of HbA1C, Fasting insulin and lipid profile in the assessment of Diabetic Nephropathy in Type 2 diabetes Mellitus In Males- A rural Hospital Study	Munilakshmi. U, Shashidhar K.N, Harish R, Madhavi Reddy, Lakshmaiah. V	International Journal of Biochemistry 2014;195:352-360	index Copernicus Google Scholar
A Gender Based case control study of anthropometric and Renal profile in Rural Diabetic population	Munilakshmi.U Dr.Susanna.T.Y K.N.Shashidhar Dr.MadhaviReddy Dr.Lakshmaiah	International Organization of Scientific Research 2014;4(2):6-11.	index Copernicus Google Scholar Embase
Grain and Milling Quality of Barley and their suitability for preparation of Traditional South Indian Products	Madhavi Reddy. M Raja Reddy.P Raghavendra Prasad. BN Munilakshmi U	IOSR Journal of Pharmacy 2014;4(2):23-27	index Copernicus Google Scholar
Is HOMA IR a determinant in assessing age related insulin sensitivity?	Munilakshmi.U Dr.K.N.Shashidhar Dr.Sumathi ME Dr.MadhaviReddy Dr.Lakshmaiah	AARJMD Asian Academic Research Journal of Multidisciplinary. 2014;1(22):504-516	index Copernicus Google Scholar
Anthropometric and biochemical markers to assess the risk factors for metabolic syndrome in south Indian non diabetic rural population	Dr.MadhaviReddy Dr. Raja Reddy P Munilakshmi.U Dr.Lakshmaiah	International Journal of Medicine 2014;103;179-186	index Copernicus Google Scholar
Association of Serum Uric acid with anthropometric, HbA1c and Lipid profile in Diabetic Retinopathy	Munilakshmi U, Prabhavathi K Shashidhar K.N, Madhavi Reddy, Lakshmaiah V	International Journal of Current research and review 2015:7(4):20-26	index Copernicus Google Scholar Embase

Correlation between fasting insulin and blood pressure in obese and non obese middle aged Indian diabetic adults	Raja Reddy.P Lakshmaiah V Madhavi Reddy. M Karthiyanee Kutty Munilakshmi U	International journal of multidisciplinary research and development 2015;2(5):241-245.	index Copernicus Google Scholar
Potential role of uric acid in correlation with epidemics of hypertension and Albumin Creatinine Ratio in Diabetic Nephropathy	Munilakshmi U, Shashidhar K.N, Prabhavathi K Madhavi Reddy, Lakshmaiah V	Apollo Medicine 2016;13:24-30	index Copernicus Google Scholar Scopus
Expediency of markers for early detection of Acute Kidney Injury Sequelae to type 2 Diabetes Mellitus	Munilakshmi U, Shashidhar K.N, Madhavi Reddy, Lakshmaiah V Muninarayana. C	Asian Journal of Pharmaceutical and Clinical Research2016; 9 (2):113-118	index Copernicus Google Scholar Scopus
Small antioxidant molecules and their importance in Acute Kidney Injuri	Munilakshmi U, Shashidhar K.N, Madhavi Reddy, Lakshmaiah V Muninarayana. C	European Journal of Pharmaceutical and Clinical Research 2017;4(9): 793-800	index Copernicus Google Scholar Scopus pubmed

ANNEXURE- II

Information Sheet

Title of the study: BIOMARKERS AND OXIDATIVE STRESS PARAMETERS IN TYPE 2 DIABETES MELLITUS AND ACUTE KIDNEY INJURY.

Name of the Principal Investigator: Mrs. Munilakshmi. U

Investigator's statement:

Introduction – My name is Mrs. Munilakshmi. U, pursuing PhD in the Department of Biochemistry at Sri Devaraj Urs Medical College, Kolar. We are carrying out a study on "Biomarkers and oxidative stress parameters in type 2 diabetes Mellitus and Acute kidney injury patients". The proposed study is a case control study where the blood sample drawn and analyzed from the patients will be compared with the same age and gender matched clinically healthy controls. The study has been reviewed by the Central Eithics Committee, SDUAHER, Kolarand has been started only after their formal approval.

Purpose for the study – Acute renal failure (ARF) recently termed as Acute kidney injury (AKI) which may be reversible. It is a syndrome caused by medical, surgical and gynecologic disorders, where the lesions affecting at different locations of renal system and is mediated through different complex pathogenetic mechanisms. The clinical features and presentations of AKI vary from patient to patient and also its prognosis. High risk patients of AKI include falciparum malaria, obstetrical causes such as septic abortions, toxemia of pregnancy, road traffic accidents, crush injuries, polytrauma hemorrhagic shock, empyema gall bladder, enteric fever, leptospirosis, acute severe pancreatitis, severe gastrointestinal illness, viper snake bite, burns. Proper management of AKI therefore needs a thorough understanding of pathogenesis and identification of aetiological factors.

Diabetes is a metabolic disorder resulting from a defect in insulin secretion, insulin action or both. Insulin deficiency leads to chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. As the disease progress tissue or vascular damage ensues, leading severe diabetic complications such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration. Thus, diabetes covers a wide range of heterogeneous diseases. Diabetes once is being diagnosed has to be followed up and treated.

In type 2 diabetes mellitus patients (T2DM), diabetic nephropathy may be already present when T2DM is diagnosed for the first time. The microvascular complications progress from an incipient stage to overt nephropathy and later to chronic kidney disease. In these individuals the focus is on detecting kidney injury occurring in early stage of diabetic nephropathy. With this purpose, I will be studying/assessing a new biomarker for AKI. NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN (NGAL) as a
promisingearly predictor of AKI then this NGAL would be a useful and valuable marker in diabetic patients with high sensitivity and specificity. NGAL in conjunction with Cystatin C would prove to be beneficial for predicting early AKI in diabetic nephropathy. Not only in Diabetic Nephropathy but also in AKI due to other etiologies NGAL is an early predictor of ongoing kidney damage.

Recent studies conducted with NGAL as a biomarker of AKI, globally and in India have targeted urban population. Only a few studies have been done to find the microvascular complications such as Diabetic Nephropathy and AKI in rural population. Hence, undertaking this study in the rural population of kolar would contribute significantly towards diagnosis and management of Diabetic

Nephropathy by shedding light on the unexplored teritary of AKI in Diabetic Nephropathy and AKI due to other causes as well as in a rural teritary care hospital/population.

The utility of these biomarkers are best interpreted only by comparing with age and sex matched healthy controls. In this regard I highly appreciate your involvement in this study by providing 6 ml of blood and urine.

In this regard, I will ask you some questions about your personal, past and family history. I also need to collect 6ml of blood and urine for study purpose. You do not have to answer any questions that you do not want to answer and you may end this interview at any time you want to. However, your honest answer to these questions will help us better understand the awareness and practice on prevention of diabetes complications and prevent AKI outcome. We would greatly appreciate your help in responding to this survey. The survey will take about half an hour to ask the questions and examination. Participation in this study doesn't involve any extra cost for you. This study is not only beneficial to you but also to the community in large. All the information collected from you will be strictly confidential and will not be disclosed to any outsider unless compelled by law. This information collected will be used only for research.

I kindly request you to give consent for giving clinical information and for collection of 6ml of blood and urine examination and permit us to preserve the left out sample for any future investigations. There is no compulsion to participate in this study. You will be no way affected if you don't wish to participate in this study. You are required to sign only if you voluntarily agree to participate in this study. You are required to sign only if you voluntarily agree to participate in this study. Further, you are at a liberty to withdraw from the study at any time, if you wish to do so. Be assured that your withdrawal will not affect your treatment by the concerned physician in any way. It is up to you to decide whether to participate or not. This document will be stored in the safe locker in the department of Biochemistry in the college and a copy is given to you for information.

For any further clarification you are free to contact the Principal investigator and Guide, Mrs.

Munilakshmi. U: 08748815373; Dr Shashidhar K.N: 09845248742

INFORMED CONSENT

Sl.no:

Title of the study: BIOMARKERS AND OXIDATIVE STRESS PARAMETERS IN TYPE 2 DIABETES MELLITUS AND ACUTE KIDNEY INJURY.

I do here by give my full consent for the study titled "Biomarkers and Oxidative Stress Parameters in Type 2 Diabetes Mellitus and Acute Kidney Injury". I understand that I remain free to withdraw from this study at any time. I give my consent to give 6ml of blood and urine to the investigator for this study. The procedure and consequence has been explained to me in my own understandable language. I have read or read to me and understand the purpose of this study and the confidential nature of the information that will be collected and preserved throughout the study. The information collected will be used only for research.

I permit you to preserve the left out sample for any future investigations.

I do take the opportunity to ask my questions/doubts regarding the various aspects of this study and my questions have been answered by the investigator to my satisfaction. I the undersigned agree to participate in this study and authorize the collection of samples.

I also understand that there is no risk to my life from this study.Participation in this study does not involve any extra cost to me.

1. Subject's name and signature / thumb impression
2. Name and signature of witness
3. Name and signature of interviewer/Investigator:

PROFORMA

TITLE OF THE STUDY: BIOMARKERS AND OXIDATIVE STRESS PARAMETERS IN TYPE 2 DIABETES MELLITUS AND ACUTE KIDNEY INJURY

Case No:
Name: Mr/Mrs OP No:
Age: IP No:
Gender: Ward:
Date: Occupation:
Weight:
Address:
CHIEF COMPLAINTS:
HISTORY OF PRESENTING ILLNESS:
CLINICAL DIAGNOSIS:

PAST HISTORY:

Hypertension: yes/no if yes, duration:
Diabetes: yes/no if yes, duration:
Heart diseases: yes/no if yes, duration:
Liver diseases: yes/no if yes, duration:
Drug ingestion: yes/no if yes, duration & details:
Others:
Gestational diabetes:

FAMILY HISTORY:

Diabetes: yes/no if yes, duration:
Hypertension: yes/no if yes, duration:

OCCUPATIONAL HISTORY:

PERSONAL HISTORY:

Economic status: Below poverty level/above poverty level Diet:
Vegetarian / mixed/non-vegetarian
Vegan/Non-vegan
Eggitarian/Non eggitarian
Smoking: yes/no if yes, duration:
Alcohol: yes/no if yes, duration:
Menstrual History:

GENERAL PHYSICAL EXAMINATION:

Ht: Wt: BMI:
Waist hip ratio: Abdominal girth:
Built : normal / below normal / well built / obese/athletic
Nourishment: well / poor nourished
Oedema: Icterus:
Pallor: Clubbing:
Cyanosis: Lymphadenopathy
Blood pressure: Pulse rate: Brachial dance:

SYSTEMIC EXAMINATION:

CVS:
RS:
CNS:
PER ABDOMEN:

CLINICAL DIAGNOSIS:

INVESTIGATIONS:

BLOOD:

CYSTATIN C:
NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN:
PLASMA FBS:
PLASMA PPBS :
C- PEPTIDE:
BLOOD UREA:
SERUM CREATININE:
SERUM URIC ACID
GLYCATED ALBUMIN LIPID
PROFILE

→ SERUM TOTAL CHOLESTEROL:
→ SERUM TRIGLYCERIDES:
→ SERUM HDLc:
→ SERUM LDL (calculated):
→ NON HDLc (calculated):
→ eGFR (calculated)

SODIUM
POTASSIUM
hs-CRP
URINE MICROALBUMIN NITRIC
OXIDE (NO) GLUTATHIONE
PEROXIDASE MALONDIALDEHYDE
(MDA) VITAMIN C
URINE ALBUMIN
SUGAR
MICROSCOPE

OTHER PARAMETERS/ INVESTIGATIONS AS AND WHEN REQUIRED FOR THIS STUDY