CHAPTER-1

INTRODUCTION, AIMS & OBJECTIVES AND REVIEW OF LITERATURE
1. INTRODUCTION AND REVIEW OF LITERATURE

The state of a person's health is often directly related to that person's lifestyle. With more and more people aping the Western food culture, which involves stuff high in saturated salt, fat and calories but low on fibre, vitamins and proteins, obesity is on the rise. This, in turn, is leading to a higher incidence of hypertension and diabetes than before. While a third of the population of India is obese, over half the people suffer from hypertension, often leading to ailments of the heart, the kidney and the eye. Diabetes and hypertension are the primary causes of kidney disease and blindness. India is fast becoming world diabetes capital\(^1\). The World Health Organization (WHO) has predicted that going by the current trend India will become the "diabetes capital of the world" by 2025.

A diabetic is two to four times more prone to heart disease and 30 times more prone to kidney disease than others. Seventy per cent of diabetics suffer mild to severe nerve damage and vision impairment. According to the International Diabetes Federation, India has over 33 million diabetics, the largest number for any one country. The figure is expected to double in the next 10 years.\"India cannot afford to manage its rapidly rising chronic kidney disease (CKD) patients. Diabetic nephropathy is one of the leading cause of chronic kidney failure\(^2\). The best long-term option for the country is to resort to preventive and early detection methods.

Diabetes mellitus is still the main cause of kidney disease in adults in the 40-60 age groups. There is a rising incidence of chronic kidney disease that is likely to pose major problems for both healthcare and the economy in future years. In India, it has been recently estimated that the age-adjusted incidence rate of End Stage Renal Disease (ESRD) to be 229 per million population (pmp), and >100,000 new patients enter renal replacement programs annually\(^3\).

It is estimated that 100,000 patients develop End Stage Renal Disease (ESRD) every year in India but 90 per cent of them never see a nephrologist. A mere 9,000 are started on haemodialysis every year. But a whopping 60 per cent of them do not come back for dialysis, unable to afford the programme. Nearly 20 per cent of the remaining dies because of complications or inadequate dialysis. Some 20 per cent of the patients
who consult a nephrologist opt for transplantation from either living related or unrelated donors. Only a small set of patients continues on maintenance dialysis. It is obvious that in India dialysis is not possible for all ESRD patients.

More and more patients are opting for kidney transplantation in India. It is estimated that more than 3,000 transplants are done every year in approximately 100 centres in the country. These are from living unrelated or related donors. Cadaver transplantation is extremely uncommon. Not much of follow-up is available on patients after kidney transplantation. Only a few centres provide post-transplantation information. After kidney transplantation, the patient becomes highly susceptible to infections and the mortality rate varies from 20 per cent to 60 per cent.

Renal replacement therapy, as it is practiced today with dialysis and kidney transplantation, is not an option for the large number of patients who are likely to develop ESRD. Catching them early is a sure way of ensuring the patient does not deteriorate to ESRD, after which, dialysis and transplantation are the only solutions and significantly lengthen the time required to reach the stage of dialysis or renal transplantation. Also, better control of these lifestyle diseases, along with periodic testing, would help keep the disease at bay. Early detection could enable the physician to handle kidney disease with medicines, the costs of which will amount to less than one per cent of the cost of dialysis or transplantation.

### 1.1.1 The rising incidence of kidney diseases in India

Diabetic kidney disease is a worldwide epidemic caused largely by lifestyle changes. People of the Indian subcontinent are more susceptible to diabetes mellitus. Perhaps genetic factors along with lifestyle changes are responsible for the high incidence. Type 2 diabetes mellitus (T2DM) is a non-autoimmune, complex, heterogeneous and polygenic metabolic disease condition characterized by persistent elevated blood glucose levels (hyperglycemia). India as said to be the diabetic capital of the world is likely to experience the largest increase in T2DM and a greater number of diabetic individuals in the world⁴.
1.1.2 The kidney and its function.

The kidneys play key roles in body function, not only by filtering the blood and getting rid of waste products, but also by balancing levels of electrolyte levels in the body, controlling blood pressure, and stimulating the production of red blood cells. The kidneys have the ability to monitor the amount of body fluid, the concentrations of electrolytes like sodium and potassium, and the acid-base balance of the body. They filter waste products of body metabolism, like urea from protein metabolism and uric acid from DNA breakdown. Two waste products in the blood can be measured: blood urea nitrogen (BUN) and creatinine (Cr).

When blood flows to the kidney, sensors within the kidney decide how much water to excrete as urine, along with what concentration of electrolytes. This system is controlled by renin, a hormone produced in the kidney that is part of the fluid and blood pressure regulation systems of the body. Kidneys are also the source of erythropoietin in the body, a hormone that stimulates the bone marrow to make red blood cells. Special cells in the kidney monitor the oxygen concentration in blood. If oxygen levels fall, erythropoietin levels rise and the body starts to manufacture more red blood cells.

1.1.3 Renal failure

Renal failure (also kidney failure or renal insufficiency) is a medical condition in which the kidneys fail to adequately filter waste products from the blood. The two main forms are acute kidney injury, which is often reversible with adequate treatment, and chronic kidney disease, which is often not reversible. Acute Renal Failure has now replaced the term Acute Kidney injury (AKI) and a universal definition and staging system has been proposed to allow earlier detection and management of AKI. The new terminology enables healthcare professionals to consider the disease as a spectrum of injury. This spectrum extends from less severe forms of injury to more advanced injury when acute kidney failure may require renal replacement therapy (RRT). Conventional urinary biomarkers such as casts and fractional excretion of sodium have been insensitive and non-specific for the early recognition of AKI.
The cause of Acute Kidney Injury (AKI) is the rapid breakdown of renal function that occurs when high levels of uremic toxins accumulate in the blood. AKI occurs when the kidneys are unable to excrete the daily load of toxins in the urine. The types of Acute Kidney Injury are: Pre-renal AKI, Post- Renal AKI and Intrinsic renal AKI. Acute Kidney Injury does not produce a classic set of symptoms. The most common symptom is decreased urine output, which occurs in 70% of patients. AKI is most easily diagnosed by an increase in blood levels of creatinine and blood urea nitrogen (BUN). The blood level of creatinine typically increases by 0.5 milligrams per tenth of a liter (mg/dL) every day.

### 1.1.4 Acute Kidney Injury (AKI)

The worldwide incidence of acute kidney injury is poorly known because of underreporting, regional disparities, and differences in definition and case mix. New definitions call for revision of the problem with unified criteria. Acute Kidney Injury that is not caused by prerenal or postrenal factors is categorized as intrinsic Acute Kidney Injury. This type involves damage or injury within both kidneys. Intrinsic AKI accounts for approximately 40% of the cases of Acute Kidney Injury. Nearly 90% of intrinsic AKI cases are caused by ischemia or toxins, both of which lead to acute tubular necrosis (ATN). Ischemic AKI is associated with reduced blood flow to the kidneys, which leads to tissue death and irreversible kidney failure. Ischemic AKI occurs most frequently when there is hemorrhage, trauma, or sepsis and in patients undergoing major cardiovascular surgery.

Many types of medication can cause nephrotoxic intrinsic AKI, and the effect seems to be dose related. Most cases occur in the elderly and in patients with chronic renal failure (CRF). Toxins taken into the body that can trigger intrinsic AKI include the following: Radiocontrast dyes (used in imaging procedures), Antibiotics (e.g., acyclovir, roscarnet), Chemotherapeutic drugs (used to treat cancer, e.g., cisplatin, ifosfamide) and Cyclosporine. In AKI serum creatinine can take several days to reach new steady state. Upto 50% of kidney function may be lost before serum creatinine even begins to raise.

Acute Kidney Injury is a common problem, with serious short term and long term consequences. It is present in 5% of all hospitalized patients and upto 30% of
patients in ICUs. The incidence is increasing at an alarming rate. The mortality rate is greater than 50% in dialyzed ICU patients. Levels in serum creatinine are the current gold standard, but this is problematic. Normal serum creatinine varies widely with age, gender, diet, muscle mass, muscle metabolism, medication, and hydration status. In AKI serum creatinine can take several days to reach new steady state. Upto 50% of kidney function may be lost before serum creatinine even begins to rise.

There is a significant development in the field of biomarkers for Acute Myocardial Infarction (Heart disease) but not for Acute Kidney Injury. In the year of 1960, medical research discovered LDH, a biomarker that can detect Acute myocardial infarction, when someone has a heart attack, blood levels of total LDH will rise within 24 to 48 hours, peak in 2 to 3 days, and return to normal in 10 to 14 days. In 1970, CPK and myoglobin were discovered, As a cardiac biomarker, myoglobin is used in conjunction with troponin to help diagnose or rule out a heart attack. Myoglobin levels start to rise within 2-3 hours of a heart attack or other muscle injury, reach their highest levels within 8-12 hours, and generally fall back to normal within one day. Its advantage over other markers is that it turns positive sooner than troponin. In the year of 1980 CK-MB was discovered; CK–MB levels, along with total CK, are tested in persons who have chest pain to diagnose whether they have had a heart attack. Since a high total CK could indicate damage to either the heart or other muscles, CK–MB helps to distinguish between these two sources. Increased CK-MB can usually be detected in heart attack patients about 3-4 hours after onset of chest pain. The concentration of CK-MB peaks in 18-24 hours and then returns to normal within 72 hours. In 1990s Troponin T was discovered. Troponin T, as a result of its high tissue specificity, cardiac troponin is a cardiospecific, highly sensitive marker for myocardial damage. In cases of acute myocardial infarction, Troponin T levels in serum rise about 3-4 hours after the occurrence of cardiac symptoms and can remain elevated for up to 14 days. Troponin T may also be used to identify patients that benefit from antithrombotic therapy and recently in 2000s troponin I was discovered. These biomarkers can detect acute damage to the heart within hours of incidence. Whereas for renal failure, there is no such markers, the only available test is serum creatinine, therefore it is mandatory to see if there is any biomarkers for Acute Kidney Injury that can help to detect renal disease at early stage. Thus, it is important to diagnose kidney disease early. For this to happen, the present investigation such as
blood urea, and creatinine is not sufficient. There is an urgent requirement of new markers which can diagnose Acute Kidney Injury within hours. The subject of early diagnosis of kidney disease in India is very important.

In the present study, we performed profiling of new emerging biomarkers of acute kidney injury in combination, similar to troponins, which can increase the sensitivity and specificity of diagnosis of the acute kidney injury earlier than serum creatinine. These include a plasma panel [neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C] and a urinary panel [urinary NGAL, interleukin 18 (IL-18), and kidney injury molecule 1 (KIM)-1].
AIMS AND OBJECTIVES
1.1.5 AIMS AND OBJECTIVES

1.1.5.1 Aims

Serum/urine profiling of new/novel markers for diagnosis of Acute Kidney Injury and evaluating their diagnostic performance associated with contrast administration earlier than serum creatinine.

1.1.5.2 Objective of the study:

This clinical study is designed to investigate whether Serum and Urinary NGAL, Urinary IL-18, Urinary KIM-1 and Serum Cystatin C are early predictive markers for AKI during Contrast induced nephropathy associated with Angiographic procedure. A total of 200 patients scheduled for undergoing angiographic procedure in two groups were included in this study.
1.2. REVIEW OF LITERATURE

Acute kidney injury (AKI) is one of the most challenging problems faced by clinicians in the tropics owing to its fast-changing burden. AKI in the tropics is strikingly different from that in the developed world in terms of etiology and presentation. In addition, there is a stark contrast between well-developed and poor areas in the tropics. Acute Kidney Injury (AKI) is the rapid breakdown of renal (kidney) function that occurs when high levels of uremic toxins (waste products of the body's metabolism) accumulate in the blood. AKI occurs when the kidneys are unable to excrete (discharge) the daily load of toxins in the urine. Acute Kidney Injury (AKI) as well as chronic kidney disease (CKD) are currently categorized according to serum creatinine concentrations. AKI is previously referred to as Acute Kidney Injury (AKI), is significant and devastating problem in clinical medicine. First, serum creatinine concentrations may not change until about 50% of kidney function has already been lost. Second, serum creatinine does not accurately depict kidney function until a steady state has been reached, which may require several days. Serum creatinine, however, has shortcomings because of its low predictive values. The need for novel markers for the early diagnosis and prognosis of renal diseases is imminent, particularly for markers reflecting intrinsic organ injury in stages when glomerular filtration is not impaired.

However, animal studies have shown that while AKI can be prevented and/or treated by several maneuvers, these must be instituted very early after the insult, well before the rise in serum creatinine. The lack of early biomarkers of AKI in humans has hitherto crippled our ability to launch potentially effective therapies in a timely manner. Indeed, human investigations have now clearly established that earlier intervention improves the chance of ameliorating renal dysfunction. The lack of early biomarkers has negatively impacted on a number of landmark clinical trials investigating highly promising therapies for AKI. Thus, it is important to diagnose kidney disease early.

The tools of modern science have provided us with promising novel biomarkers for AKI, with potentially high sensitivity and specificity. These include a plasma panel (NGAL and Cystatin C) and a urine panel (NGAL, KIM-1 and IL-18). Since they represent tandem biomarkers, it is likely that the AKI panels will be useful
for timing the initial insult and assessing the duration of AKI (analogous to the cardiac panel for evaluating chest pain). Based on the differential expression of the biomarkers, it is also likely that the AKI panels will help distinguish between the various types and etiologies of AKI. It will be important in future studies to validate the sensitivity and specificity of these biomarker panels in clinical samples from large cohorts and from multiple clinical situations. Such studies will be markedly facilitated by the availability of commercial tools for the reliable and reproducible measurement of biomarkers across different laboratories.

Therefore, AKI remains an increasingly important clinical condition with a poor outcome. Rapid and significant clinical advances will be possible when diagnostic criteria and "biomarkers" allowing for rapid diagnosis and quantitative staging of the extent of injury are developed, validated and proven useful in the individual patient.

There is no consensus on how best to assess kidney function; namely, what markers best reflect kidney function, and what values of those markers discriminate normal from abnormal kidney function14. To establish a uniform definition for acute kidney injury, the Acute Dialysis Quality Initiative formulated the Risk, Injury, Failure, Loss, and End-stage Kidney (RIFLE) classification15.

RIFLE defines three grades of increasing severity of acute kidney injury – risk (class R), injury (class I) and failure (class F) – and two outcome classes (loss and end-stage kidney disease) RIFLE represents a new classification system issued from a process of formal evidence appraisal and expert opinion16. A unique feature of the RIFLE classification is that it provides three grades of severity for acute kidney injury based on changes in either serum creatinine or urine output from the baseline condition. This allows classification of patients with acute kidney injury into one of the three RIFLE severity classes RIFLE represents a new classification system issued from a process of formal evidence appraisal and expert opinion only. The RIFLE classification is a very sensitive definition of acute kidney
injury: acute kidney injury defined by the RIFLE classification occurred in two thirds of general ICU patients \(^\text{16}\).

RIFLE classes injury and failure are independently associated with increased risk for in-hospital dead, but it does not predict or diagnose Acute Kidney Injury at initial stages.

1.2.1 RIFLE and AKIN criteria

In 2002, the Acute Dialysis Quality Initiative (ADQI) group proposed a standard definition and classification system for the syndrome of Acute Kidney Injury through a broad consensus of experts across disciplines and international boundaries. The classification system coins the acronym RIFLE and has three levels: Risk, Injury, and Failure; and two outcomes: persistent Acute Kidney Injury (termed Loss) and End-stage kidney disease.

A unique feature of the RIFLE classification is that it provides retrospectively for three grades of severity of renal dysfunction on the basis of a maximum change in serum creatinine, reflecting changes in GFR or duration and severity of decline in urine output from the baseline. The RIFLE criteria have the potential advantage of providing definitions for the stage at which kidney injury still can be prevented (risk), when the kidney has already been damaged (injury), and when renal failure is established (failure). The RIFLE criteria have been evaluated in clinical practice and seem to be at least coherent with regard to outcomes in patients with AKI \(^\text{16-20}\).

However, the RIFLE classification is not a diagnostic one, but a staging system based, retrospectively, upon the maximum serum creatinine. This has created confusion as the stage (risk, injury or failure) can and does evolve in the same patient from risk to failure depending upon when the diagnosis is completed. For instance, a patient with severe AKI will satisfy the criteria for risk, then injury and finally failure as the serum creatinine rises daily. In epidemiologic studies, this patient would be counted as failure. Therefore, RIFLE does not offer real time quantitative diagnosis regarding severity of injury that can be used to stratify patients for clinical therapeutic studies.
1.2.2 At Risk

According to RIFLE classification, Risk (R) is defined as an increase of baseline serum creatinine 1.5-2.0 folds or decrease of urine output 0.5 ml/kg per h for 6 h. Urine output was included as a diagnostic criterion because in intensive care unit patients it often portends renal dysfunction before the onset of changes in serum creatinine. Recent studies showed that even small changes in serum creatinine were associated with increased morbidity and mortality. Lassnigg et al. 2004, demonstrated a two-fold increase in the risk for death for patients who experienced no change or a small increase (0.5 mg/dl) in SCr 48 h after cardiothoracic surgery compared with patients who experienced a small decline in serum creatinine (SCr). Loef et al found an association between a 25% increase in SCr during the first postoperative week and short- and long-term mortality$^{21}$.

Based on the findings that small alterations of serum creatinine result in adverse outcomes, the Acute Kidney Injury International collaborative Network (AKIN) recently changed the definition of Risk group to include patients with an increase in serum creatinine of 0.3 mg/dl. The proposed diagnostic and staging criteria for AKI are designed to facilitate acquisition of knowledge and to validate the emerging concepts. Serum creatinine is the most widely used parameter for everyday assessment of glomerular filtration rate (GFR), but it has poor sensitivity and specificity in AKI because serum creatinine lags behind both renal injury and renal recovery. Furthermore, creatinine is produced nonenzymatically in skeletal muscle, and the amount of creatinine is directly related to muscle mass. A number of GFR estimating equations have been developed to overcome some of the limitations of estimating GFR from serum creatinine. The Cockroft-Gault equation was developed in 1973 and is used widely. A newer equation, the MDRD Study equation, was developed in 1999 and since then has been validated in a number of populations and is now recommended by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD), the National Kidney Foundation (NKF), and American Society of Nephrology(ASN) for use in clinical practice. These equations assume stable serum creatinine and cannot be used to estimate GRF in patients with AKI and rapidly changing serum creatinine.
Decreased urine output is another important criterion of AKI staging. AKIN proposed documented oliguria of less than 0.5 ml/kg/hour for more than six hours to define stage 1 if AKI (Risk stage by RIFLE classification). The urine output criterion was included based on the predictive importance of this measure but with the awareness that urine output may not be measured routinely in non-intensive care unit settings. Evaluation of urine sediment and urine chemistries helps to differentiate between renal vasoconstriction with intact tubular function and established AKI.

The fraction of filtered sodium that is reabsorbed by intact tubules of the vasoconstricted kidney is greater than 99% resulting in low fractional excretion of sodium (< 1%). FeNa may not be diagnostic in patients with preexisting chronic kidney disease (CKD) or patients taking diuretics because both conditions result in FeNa > 1% even in patients with pre-renal azotemia. On the other hand, contrast induced nephropathy and some cases of myoglobinuria may actually be associated with FeNa less than 1% during the early period post injury.

1.2.3 Renal Injury

Injury stage is defined as a doubling of serum creatinine or urine output below 0.5 ml/kg/h during 12 hours of longer by RIFLE classification. AKIN proposed no changes of this stage of AKI. In one retrospective cohort study 5,383 ICU patients were evaluated. Patients with Injury stage of AKI (26.7% of total) had in-hospital mortality rate 11.4% compared with 5.5% for patients without acute kidney injury. More than 50% of the patients with RIFLE class R progressed to RIFLE class I within the next day.

As the severity of AKI progresses, the urine-concentrating capacity is abolished. At this stage of AKI, kidney concentrating capacity, assessed by urinary osmolality may complement the use of fractional excretion of sodium in the differential diagnosis of renal vasoconstriction from established ATN. Urine osmolality is usually higher in patients with pre-renal azotemia (> 500 mOsm/kg) and lower in those with ATN (<400 mOsm/kg) This diagnostic parameter may be less
sensitive than fractional excretion of sodium in patients with advanced age or with low protein intake\textsuperscript{20}.

### 1.2.4 Failure

Failure stage of AKI in RIFLE classification is defined as a 3- of higher fold increased serum creatinine or higher than 4 mg/dl. Failure stage also is confirmed by urine output criteria: urine output below 0.3 mg/kg/h for 24 hours or anuria for 12 hours. AKIN proposed to use the same criteria for defining stage 3 of AKI. Additionally, the AKIN classification considers patients receiving renal replacement therapy to have met criteria for stage 3\textsuperscript{20}.

The role and time of initiation of RRT in AKI is not well defined nor supported by evidence-based studies. Hyperkalemia, severe metabolic acidosis, diuretic unresponsive pulmonary edema, and uremic symptoms are universally accepted indications for RRT in patients with AKI. Since the consequences of these complications are likely to be more severe for critically ill patients with AKI, ADQI recommends initiation of renal replacement therapy prior to their development. Renal replacement therapy, as it is practiced today with dialysis and kidney transplantation, is not an option for the large number of patients who are likely to develop ESRD\textsuperscript{20}.

Outstanding advances in basic research have illuminated the pathogenesis of AKI and have paved the way for successful therapeutic approaches in animal models. However, translational research efforts in humans have yielded extremely disappointing results. A major reason for this is the lack of early markers for AKI, AKIN to troponins in acute myocardial disease, and hence an unacceptable delay in initiating therapy\textsuperscript{24-26}.

In addition to aiding in the early diagnosis and prediction, biomarkers may serve several additional purposes in AKI. Thus, biomarkers are also needed for: (a) discerning AKI subtypes (pre-renal, intrinsic renal, or post-renal); (b) identifying AKI etiologies (ischemia, toxins, sepsis, or a combination); (c) differentiating AKI from other forms of acute kidney disease (urinary tract infection, glomerulonephritis, interstitial nephritis); (d) predicting the AKI severity (risk stratification for
prognostication as well as to guide therapy); (e) monitoring the course of AKI, and (f) monitoring the response to AKI interventions. Furthermore, AKI biomarkers may play a critical role in expediting the drug development process.

1.2.5 Desirable characteristics of clinically applicable AKI biomarkers include:

(a) An ideal biomarker should be noninvasive and easy to perform at the bedside or in a standard clinical laboratory, using easily accessible samples such as blood or urine;

(b) It should be rapidly and reliably measurable using a standardized assay platform;

(c) It should be highly sensitive to facilitate early detection, and with a wide dynamic range and cutoff values that allow risk stratification.

(d) It should be highly specific for AKI, and allow the identification of AKI subtypes and etiologies. This will almost certainly involve a combination of a panel of biomarkers, along with clinical information.

The quest for AKI biomarkers is an area of intense contemporary research. Fortunately, the application of innovative technologies such as cDNA microarrays and proteomics to human and animal models of AKI has uncovered several novel genes and gene products that are emerging as biomarkers.

As noted in the previous section, small increases in serum creatinine may reflect significant renal insult and be associated with significant morbidity in patients with AKI. Extensive preclinical investigation has led to the identification of several potential biomarkers that may herald AKI prior to a rise in serum creatinine. This may be important because early intervention in the course of AKI may lower the extent of injury, the need for renal replacement therapy and may possibly decrease morbidity and mortality. Numerous potential biomarkers have been identified in pre-clinical AKI studies and are now beginning to be tested in clinical validation studies. However, very few prospective studies have validated initial findings in diverse patients in multiple institutions, or done so using well-established standardization of all steps in the biomarker development process. The extreme nature of human
biologic heterogeneity, coupled with the uncertainties in many disease processes, contributes to the variability in clinical outcomes. To be clinically useful, a biomarker must be relevant to the individual patient, not just to a population of patients. While a rapid and convenient method to determine the patients' actual GFR would be preferable; no such diagnostic tool exists.

1.2.6 Angiographic procedure

Angiographic procedures are an important treatment option for patients with coronary artery disease and are performed in large numbers around the developed world. Interventional cardiologists are being asked more frequently to perform an Angiographic procedure in increasing numbers of patients; contrast induced nephropathy (CIN) is a potentially serious complication.

During the procedure patient are subjected to radio contrast drugs in order to detect the blockage, damage to the heart muscle supplied by the artery being treated. This can be caused by closure of small branch vessels or the release of blood clot or debris.

The contrast dye used to visualise the heart arteries can cause kidney function to deteriorate. Contrast induced nephropathy (CIN) is at present the third leading cause of hospital-acquired Acute Kidney Injury among this branch. Most commonly it is defined as an acute impairment of renal function manifested by an absolute increase in the serum creatinine level of at least 0.5 mg/dl or by a relative increase of at least 25% from the baseline level.

1.3 NGAL as a potential diagnostic marker

Human NGAL was originally identified as a 25-kDa protein covalently bound to gelatinase from neutrophils. Like other lipocalins, NGAL forms a barrel-shaped tertiary structure with a hydrophobic calyx that binds small lipophilic molecules. The major ligands for NGAL are siderophores, small iron-binding molecules. On the one hand, siderophores are synthesized by bacteria to acquire iron, and NGAL exerts a
bacteriostatic effect by depleting siderophores. Although NGAL is expressed only at very low levels in several human tissues, it is markedly induced in injured epithelial cells, including the kidney \(^{34}\).

The promoter region of the NGAL gene contains binding sites for a number of transcription factors, including NF-Alpha B \(^{34}\). NF-Alpha B is known to be rapidly activated in kidney tubule cells after acute injuries and plays a central role in controlling cell survival and proliferation. These findings provide a potential molecular mechanism for the documented role of NGAL in enhancing the epithelial phenotype, both during kidney development and following AKI \(^{35}\).

Preclinical transcriptome profiling studies identified *NGAL* (also known as lipocalin 2 or *lcn2*) to be one of the most upregulated genes in the kidney very early after acute injury in animal models \(^{35}\).

Downstream proteomic analyses also revealed NGAL to be one of the most highly induced proteins in the kidney after ischemic or nephrotoxic AKI in animal models \(^{36}\).

The serendipitous finding that NGAL protein was easily detected in the blood and urine soon after AKI has initiated a number of translational studies to evaluate NGAL as a non-invasive biomarker in human AKI. In a cross-sectional study, adults with established AKI (doubling of serum creatinine) displayed a marked increase in urine and serum NGAL by western blotting when compared to normal controls. Urine and serum NGAL levels correlated with serum creatinine, and kidney biopsies in subjects with AKI showed intense accumulation of immunoreactive NGAL in cortical tubules, confirming NGAL as a sensitive index of established AKI in humans.

NGAL as an AKI biomarker has successfully passed through the pre-clinical, assay development and initial clinical testing stages of the biomarker development process. It has now entered the prospective screening stage, facilitated by the development of commercial tools for the measurement of NGAL in large populations across different laboratories. But will any single biomarker such as NGAL suffice in AKI. In addition to early diagnosis and prediction, it would be desirable to identify biomarkers capable of discerning AKI subtypes, identifying etiologies, predicting
clinical outcomes, allowing for risk stratification and monitoring the response to interventions. In order to obtain all of this desired information, a panel of validated biomarkers may be needed. Clearly, NGAL represents a novel predictive biomarker for AKI. However, the majority of studies published thus far have involved relatively small numbers of subjects from single centers, in which NGAL appears to be most sensitive and specific in homogeneous patient populations with predictable forms of AKI. That's why it is included in this proposal for elucidating with large number of human subjects.

It is apparent that a variety of independent pathologies are associated with raised levels of urinary or plasma NGAL. Therefore the finding of a raised level cannot be independently diagnostic of any one of these pathologies. Other information on the patient must be taken into account in order to assess the significance of the result. The genesis and sources of plasma and urinary NGAL following AKI require further clarification. Although plasma NGAL is freely filtered by the glomerulus, it is largely reabsorbed in the proximal tubules by efficient megalin-dependent endocytosis.

Because of its high predictive properties for AKI, NGAL is also emerging as an early biomarker in interventional trials. Therefore NGAL is considered in this project as a potential marker for Acute Kidney Injury.

1.4 Cystatin C as a potential diagnostic marker

Human cystatin C, a basic low molecular mass protein with 120 amino acid residues, is freely filtered by the glomerulus and almost completely reabsorbed and catabolized by the proximal tubular cells.

The cystatin superfamily encompasses proteins that contain multiple cystatin-like sequences. Some of the members are active cysteine protease inhibitors, while others have lost or perhaps never acquired this inhibitory activity. There are three inhibitory families in the superfamily, including the type 1 cystatins (stefins), type 2 cystatins and the kininogens. The type 2 cystatin proteins are a class of cysteine proteinase inhibitors found in a variety of human fluids and secretions, where they appear to provide protective functions. The cystatin locus on the short arm of
chromosome 20 contains the majority of the type 2 cystatin genes and pseudogenes\textsuperscript{38}. The CST3 gene is located in the cystatin locus and comprises 3 exons (coding regions, as opposed to introns, non-coding regions within a gene), spanning 4.3 kilobase pairs. It encodes the most abundant extracellular inhibitor of cysteine proteases. It is found in high concentrations in biological fluids and is expressed in virtually all organs of the body (CST3 is a housekeeping gene). The highest levels are found in semen, followed by breast milk, tears and saliva. The hydrophobic leader sequence indicates that the protein is normally secreted. There are three polymorphisms in the promoter region of the gene, resulting in two common variants. Several single nucleotide polymorphisms have been associated with altered cystatin C levels\textsuperscript{38}.

Cystatin C is a non-glycosylated, basic protein (isoelectric point at pH 9.3). The crystal structure of cystatine C is characterized by a short alpha helix and a long alpha helix running across a large antiparallel, five-stranded beta sheet. Like other type 2 cystatines, it has two disulfide bonds. Around 50\% of the molecules carry a hydroxylated proline. Cystatine C forms dimers (molecule pairs) by exchanging subdomains; in the paired state, each half is made up of the long alpha helix and one beta strand of one partner, and four beta strands of the other partner. Cystatin C has been recently proposed as a new sensitive endogenous serum marker for the early assessment of changes in the glomerular filtration rate. Cystatin C is emerging as a superior biomarker for early kidney injury as it is as follows. It is generated at a constant rate by all nucleated cells and is not secreted by the tubules or eliminated by other routes than renal excretion. It does not appear to be affected by body habitus, nutritional state, or comorbid illness. One of its principal advantages is that it identifies kidney injury while creatinine levels remain in the normal range. Cystatin C, a serum protein that is filtered out of the blood by the kidneys and that serves as a measure of kidney function. Cystatin C is produced steadily by all types of nucleated cells in the body. Its low molecular mass allows it to be freely filtered by the glomerular membrane in the kidney. Its concentration in blood correlates with the glomerular filtration rate\textsuperscript{38}.

Cystatin C is a cysteine protease inhibitor that is synthesized and released into the blood at a relatively constant rate by all nucleated cells. It is freely filtered by the glomerulus, completely reabsorbed by the proximal tubule, and not secreted. Since
blood levels of cystatin C are not significantly affected by age, gender, race, or muscle mass, it is a better predictor of glomerular function than serum creatinine in patients with chronic kidney disease\textsuperscript{39}.

The levels of cystatin C are independent of weight and height, muscle mass, age (over a year of age), and sex. Measurements can be made and interpreted from a single random sample. Cystatin C is a better marker of the glomerular filtration rate and hence of kidney function than creatinine which was the most commonly used measure of kidney function\textsuperscript{40}. Therefore, it was included in the study.

\subsection*{1.5 IL-18 as a potential bio-marker}

IL-18 is one of a number of pro-inflammatory cytokines. The activities of IL-18 appear to be species specific. An important function of IL-18 is the regulation of functionally distinct subsets of T-helper cells required for cell mediated immune responses. IL-18 functions as a growth and differentiation factor for Th1 cells. It is produced during the acute immune response by macrophages and immature dendritic cells. IL-18 is expressed by a variety of immune and non-immune cells\textsuperscript{41}. Interleukin-18 (IL-18) is a cytokine produced by macrophages and other cells that belongs to the IL-1 super family. IL-18 works together with IL-12 to induce cell-mediated immunity following infection with microbial products like lipopolysaccharide (LPS). After stimulation with IL-18, natural killer (NK) cells and certain T cells release another important cytokine called interferon-\(\gamma\) (IFN-\(\gamma\)) or type II interferon that plays an important role in activating the macrophages or other cells. Apart from its physiological role, IL-18 is also able to induce severe inflammatory reactions, which suggests its role in certain inflammatory disorders. The protein encoded by this gene is a proinflammatory cytokine. This cytokine can induce the IFN-gamma production of T cells. The combination of this cytokine and IL12 has been shown to inhibit IL4 dependent IgE and IgG1 production, and enhance IgG2a production of B cells. IL-18 binding protein (IL18BP) can specifically interact with this cytokine, and thus negatively regulate its biological activity\textsuperscript{41}.

However IL-18 measurements may be influenced by number of coexisting variables, since renal IL-18 mRNA levels are known to be induced in other disease states such as endotoxaemia, immunological injury and cisplatin toxicity.
Furthermore, plasma IL-18 levels are known to be increased in various systemic inflammatory states and the relationships between plasma and urine IL-18 remain unexplored.

The present work concerns methods of detecting a molecule, interleukin 18, in a sample, and using the detection of IL-18 to predict a condition. In certain embodiments, the method comprise obtaining a sample from a subject such as a urine or blood sample and analyzing the sample for the presence or absence of IL-18 to predict a condition for example Acute Kidney Injury or organ transplant failure. More recently few studies presented urine IL-18 as a highly sensitive indicator for AKI.

Thus, IL-18 may also represent a promising biomarker for AKI. IL-18 is more specific to ischaemic AKI, and levels are largely unaffected by urinary tract infections. Hence IL-18 can be an ideal marker for AKI associated with contract administration followed by angiographic procedure.

**1.6 Kidney Injury Molecule -1 as a potential biomarker**

Kidney Injury molecule -1 (KIM-1) as a type I membrane glycoprotein, which contain a 6-cystein immunoglobulin-like domain in its extracellular portion, and a Thr/Ser-Pro rich domain characteristic of mucin-like O-glycosylated proteins. KIM-1 presence in the urine is highly specific for kidney injury. No other organs have been shown to express KIM-1 to a degree that would influence kidney excretion. Kidney Injury molecule -1 (KIM-1) expression is induced in a variety of renal diseases, whereas in healthy kidney tissue KIM-1 is virtually undetectable. In the case of kidney damage, KIM-1 is expressed on the apical membrane followed by cleavage of the ectodomain (90 kDa) which is released in the urine in humans. KIM-1 is upregulated in the proximal tubule during dedifferentiation of the kidney epithelium, an early manifestation in response to damage.

Kidney injury molecule (KIM-1) a transmembrane tubular protein, is undetectable in normal kidneys, but it is markedly induced in renal injury including acute kidney injury (AKI) and chronic kidney disease (CKD) damage. Many studies indicate that KIM-1 is a sensitive and specific marker of kidney injury as well as a predictor of prognosis.
There are no studies to date that have examined KIM-1 for detection of AKI associated with contrast administration. There is an urgent need for improved and non-invasive renal biomarkers to permit early detection of AKI associated with contrast induced nephropathy.

Thus KIM-1 may represent a promising AKI biomarker. An advantage of KIM-1 as a urinary biomarker is the fact that its expression seems to be limited to the injured or diseased kidney, and no systemic source of KIM-1 has been described. However, urinary KIM-1 measurements may be influenced by a number of other confounding variables. KIM-1 is induced in the kidney and upregulated in the urine by a large number of nephrotoxins, including cyclosporine, cisplatin, cadmium, gentamicin, mercury and chromium. Similarly, KIM-1 in the kidney and urine is induced in a variety of chronic proteinuric, inflammatory and fibrotic disease states in humans.

Radio-contrast administration is one of the important reasons for Acute Kidney Injury. Acute Kidney Injury is an independent risk factor for mortality in adult and children even with a small increase in serum creatinine. Radiological procedures requiring intravascular administration of iodinated contrast media are becoming a common source of an iatrogenic disease known as contrast induced nephropathy (CIN). Acute Kidney Injury is defined as an acute impairment of the renal function manifested by an absolute increase in the serum creatinine level of at least 0.5 mg/dl or by a relative increase of at least 25% from the baseline level. Now a days Cardiologists are being asked more frequently to perform angiography in increasing number of patients, in which contrast induced nephropathy (CIN) is a potentially serious complication. Contrast-induced nephropathy (CIN) is at present the third leading cause of hospital-acquired acute kidney injury (AKI). Increased serum creatinine values typically occur 3-5 days after contrast administration and return to baseline levels within 1-3 weeks, when patients are discharged from the hospital. Unfortunately, serum creatinine is an unreliable indicator during acute changes in the kidney function. A marked reduction in the glomerular filtration rate (GFR) can be present before it is reflected in a rise in serum creatinine levels. All these reasons contribute to significant delay in the diagnosis of acute Kidney Injury associated with contrast administration. More over the renal function may not return to baseline, leading to an increased risk of chronic Kidney Injury. Therefore new
markers that help to identify Acute Kidney Injury earlier than serum creatinine are required for timely treatment.

1.7 Importance of this research:

India cannot afford to manage its rapidly rising chronic kidney disease (CKD) patients. The best long-term option for the country is to resort to preventive and early detection methods. CKD is a silent epidemic of the 21st century. Its occurrence is universal; not confined to the developed countries. The numbers afflicted with CKD are going to rise sharply because of the rising incidence of diabetes mellitus and hypertension (two of the major causes of CKD). Now, when patients develop kidney disease, they are managed by renal replacement therapy including haemodialysis, peritoneal dialysis and kidney transplantation. These programmes are also increasingly available in the developing world. Unfortunately, they are not sustainable in the long run due to lack of resources. Over a million people are on dialysis worldwide, 90 per cent of them in the developed world, which accounts for less than 20 per cent of the world population. So, it is obvious that not all patients in the world have access to renal replacement therapy. That is why early detection of CKD is important. Despite the effort and expenditure on dialysis, the outcome is not great. The median survival period of patients (aged 55 to 65) on dialysis in the United States today is as low as 2.7 years. This is much less than the outcome of many patients with several kinds of cancer. Kidney transplantation, which is more effective as a renal replacement therapy, is limited by the limited availability of organs. Kidney transplantation is not available in many parts of the world. Hence, it is obvious that early diagnosis and prevention of need for dialysis and transplantation are crucial to prevent CKD fatalities.

Early diagnosis can be possible if better biomarkers were discovered and prevention of kidney failure requiring renal replacement therapy is feasible. For many patients early diagnosis is actually the difference between life and death.
CHAPTER-2

EXPERIMENTAL